Multiclass Screening Method Using Automated Solid-Phase Extraction Directly Coupled to a Mass Spectrometer for Drug Residues in Honey

Brian T. Veach, Thilak K. Mudalige, Paula J. Barnes, Bryanna J. Broadaway, Pamela R. Stehle, and Chris A. Baker

Arkansas Laboratory
Food and Drug Administration
Jefferson, AR 72079

ABSTRACT

The use of veterinary drugs in honey bees for the prevention or treatment of infectious diseases is of great concern to the United States, United Kingdom, and many other countries. Traditional analytical methods used for the detection of veterinary drug residues in honey often require labor intensive extraction methods and extended LC-MS analyses. Herein is a rapid, high throughput novel method to screen for 28 different residues, including 6 fluoroquinolones, 13 sulfonamides,4 tetracyclines, 2 macrolides, a lincosamide, an ionophore, and fumagillin. The method utilizes an automated solid-phase extraction system directly coupled to a triple quadrupole mass spectrometer. By using this procedure, samples can be extracted and analyzed in < 30 seconds; reducing analytical time > 85%, when compared to traditional methods. The procedure outlined demonstrated robustness, accuracy, and reproducibility throughout the method validation process.

Correspondence addressed to: Brian T. Veach, Tel: (870)-543-4085; fax (870)-543-4041
email: brian.veach@fda.hhs.gov

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INTRODUCTION

The health of honey bee colonies has a significant economic impact not only in the United States, but many other countries as well. Honey bees are responsible for an estimated $15 billion dollars in increased crop pollination annually in the United States. Furthermore, honey bees produce more than 140 million pounds of honey each year in the United States. This explains why it is estimated that one in every three table spoons in our diet is directly or indirectly associated with honey bees [1-4].

Over the past 10 years, the honey bee population has significantly declined at an alarming rate. A 2013 U.S. Department of Agriculture publication documented high percentages of bee colony losses reported each year by the beekeeping industry. These losses were attributable to four different causes, one of which is pathogens [5]. There are several different bee diseases that have contributed to the declining bee colony populations, including but not limited to American foulbrood, European foulbrood, and Nosema. Most of these pathogens or diseases are mitigated through the use of antimicrobial drugs. The United States Food and Drug Administration's (FDA) Office of Regulatory Affairs (ORA) has had a long-standing interest in the use of antibiotics and veterinary drugs usage for treating honey bees. This is due to the potential adverse health effects of these drugs such as aplastic anemia, bone marrow suppression, carcinogenic affects, and antibiotic resistance.

Traditional analyses of honey for these residues often use liquid-liquid salt assisted extractions, or manual solid-phase extractions (SPE) [6-8]. Although these techniques are effective, they are also time consuming and require a dedicated analyst(s) to be present throughout the entire process. Once the extraction process is completed, the samples are analyzed by liquid chromatography mass spectrometry (LC-MS). The LC-MS analysis can vary from a few minutes to up to an hour in length for each sample. Furthermore, multiple extraction methods and LC-MS analyses are often required to address the vastly different chemical properties of the different classes of drug residues.

In recent years, new advances in technology are accommodating the need for higher sample throughput while maintaining or improving detection levels. This is especially true with respect to automated solid-phase extraction systems directly coupled to mass spectrometers (ASPE/MS). The system in this study conducts high speed solid-phase extraction using multiple pumps running in concert. The system also uses different re-usable SPE cartridge phases. This allows on average 2000+ injections per cartridge; furthermore, the system has a maximum capacity of > 1000 samples.

This study illustrates the method development and validation of a procedure to quickly screen honey samples for 28 different drug residues from multiple different classifications of drugs. The extraction process is automated, and the analysis of a sample is performed in < 30 seconds. Eight different injections were required to address the vastly diverse chemical properties, and the number of multiple reaction monitoring (MRM) transitions for all the drugs

EXPERIMENTAL

Apparatus

a) *Automated Solid-Phase Extraction System (ASE)* – Agilent RapidFire 365 ASE system (Santa Clara, CA)

b) *Solid-Phase Extraction Cartridge* – Agilent Technologies RapidFire reusable C_4 cartridge

c) *Solid-Phase Extraction Cartridge* – Agilent Technologies RapidFire reusable C_8 cartridge

d) *Solid-Phase Extraction Cartridge* – Agilent Technologies RapidFire reusable C_{18} cartridge

e) *Solid-Phase Extraction Cartridge* – Agilent Technologies RapidFire reusable Phenyl cartridge

f) *Solid-Phase Extraction Cartridge* – Agilent Technologies RapidFire reusable Hypercarb cartridge

g) *Mass Spectrometer* (*MS)* – An Agilent 6490 triple quadrupole mass spectrometer equipped with electrospray ionization.

h) *50 mL disposable polypropylene centrifuge tubes with screw tight lids* – (Sarstedt, Newton, NC)

i) *Multi-tube vortex shaker* – capable of holding 50 mL centrifuge tubes

j) *Sodium lamp(s)*– capable of providing enough illumination for extraction of Fumagillin

k) *Centrifuge* – capable of 3700g.

Reagents and Standards

a) *Acetonitrile* – LC-MS grade obtained from Fisher Scientific (Houston, TX)

b) *Methanol*– LC-MS grade obtained from Fisher Scientific

c) *Formic Acid* – LC-MS grade obtained from Fisher Scientific

d) *Water* – Millipore Milli-Q system (Burlington, MA)

e) *Tylosin tartrate (TYL)*– Sigma Aldrich (St. Louis MO)

f) *Erythromycin (ERY)* – Sigma Aldrich

g) *Fumagillin (FUM)* – Sigma Aldrich

h) *Enrofloxacin (ENRO)* – SPEX CertiPrep (Metuchen, NJ)

i) *Sarafloxacin hydrochloride hydrate (SARA)* – SPEX CertiPrep

j) *Ciprofloxacin (CIPRO)* – SPEX CertiPrep

k) *Danofloxacin (DANO)* – SPEX CertiPrep

l) *Difloxacin hydrochloride (DFLX)* – SPEX CertiPrep

m) *Norfloxacin (NOR)* – SPEX CertiPrep

n) *Lincomycin hydrochloride (LIN)* – SPEX CertiPrep

o) *Doxycycline hydrochloride (DC)* – SPEX CertiPrep

p) *Tetracycline (TC)* – SPEX CertiPrep

q) *Oxytetracycline (OTC)* – SPEX CertiPrep

r) *Chlortetracycline (CTC)* – SPEX CertiPrep

s) *Sulfamethazine (SMZ)* – SPEX CertiPrep

t) *Sulfamerazine (SMR)* – SPEX CertiPrep

u) *Sulfadimethoxine (SDM)* – SPEX CertiPrep

v) *Sulfadiazine (SDZ)* – SPEX CertiPrep

w) *Sulfachloropyridazine (SCP)* – SPEX CertiPrep

x) *Sulfaquinoxaline (SQX)* – SPEX CertiPrep

y) *Sulfathiazole (STZ)* – SPEX CertiPrep

z) *Sulfacetamide (SAC)* – SPEX CertiPrep

aa) *Sulfaethoxypyridazine (SEP)* – SPEX CertiPrep

bb) *Sulfamethoxazole (SMX)* – SPEX CertiPrep

cc) *Sulfamethoxypyridazine (SMP)* – SPEX CertiPrep

dd) *Sulfapyridine (SPD)* – SPEX CertiPrep

ee) *Sulfadoxine (SDX)* – SPEX CertiPrep

ff) *Monensin (MON)* – SPEX CertiPrep

gg) *Demeclocycline* – Sigma Aldrich

hh) *Roxithromycin (ROX)* – Sigma Aldrich

ii) *Sulfamethazine $^{13}C_6$* – Sigma Aldrich

jj) *Ciprofloxacin $^{13}C_3$* – Cambridge Isotope Laboratories (Andover, MA)

METHOD

Suggested Standard and Reagent Preparation:

Note: Fumagillin is light sensitive. Standard preparation and extractions for the presence of fumagillin should be performed using a low-pressure sodium lamp, or equivalent light source which produces only long wavelength visible light.

a. Stock Internal Standard Solutions (ISTD): Separate sulfamethazine $^{13}C_6$, ciprofloxacin $^{13}C_3$, roxithromycin, and demeclocycline internal standard stock solutions were prepared in methanol at 100 µg/mL, 100 µg/mL, 200 µg/mL, and 500 µg/mL respectively. These solutions are stable for up to 6 months if stored ≤ 5°C).

b. Stock Standard Solutions for Continuing Calibration Verifications (CCVs): Prepare individual stock standards in methanol or other appropriate organic solvent at the following levels:

    i. 250 µg/mL for the sulfonamides (sulfamerazine, sulfadiazine, sulfachloropyridine, sulfathiazole sulfaquinoxaline, sulfamethazine, sulfadimethoxine, sulfadoxine, sulfaethoxypyridazine,

sulfamethoxypyridazine, sulfamethoxazole, sulfapyridine, and sulfacetamide)

ii. 10.0 µg/mL for the ionophore (monensin)
iii. 2500 µg/mL for the tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline)
iv. 2500 µg/mL for the lincosamide (lincomycin)
v. 200 µg/mL for fumagillin
vi. 200 µg/mL for the macrolides (erythromycin, and tylosin)
vii. 100 µg/mL for the fluoroquinolones (ciprofloxacin, enrofloxacin, sarafloxacin, difloxacin, norfloxacin, and danofloxacin)

*Fumagillin, erythromycin, and tylosin are stable for up to six months if stored ≤ 5°C. All other solutions are stable for up to one year if stored ≤ 5°C.

c. Stock Standard Solutions for Initial Calibration Verifications (ICVs): A second set of stock solutions is prepared as the initial calibration verification (ICVs) solutions. .

d. Intermediate Internal Standard Solution: Prepare an intermediate ISTD solution in methanol as described in Table 1.

Table 1:

Internal Standard	Conc. of Stock Solution	Volume Used	Final Volume	Final Conc.
Sulfamethazine $^{13}C_6$	100 µg/mL	500 µL	10.0 mL	5.00 µg/mL
Ciprofloxacin $^{13}C_3$	100 µg/mL	20.0 µL	10.0 mL	0.200 µg/mL
Demeclocycline	500 µg/m	300 µL	10.0 mL	15.0 µg/mL
Roxithromycin	200 µg/mL	40.0 µL	10.0 mL	0.800 µg/mL

These solutions are stable for up to 6 months if stored ≤ -70°C.

e. Intermediate Analytical Standard Solution 1: Prepare an intermediate analytical standard in methanol as described in Table 2.

Table 2:

Analytical Standard	Conc. of Stock Solution	Volume Used	Final Volume	Final Conc.
Sulfamerazine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfadiazine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfachloropyridine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL

Analytical Standard	Conc. of Stock Solution	Volume Used	Final Volume	Final Conc.
Sulfathiazole	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfaquinoxaline	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfamethazine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfadimethoxine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfadoxine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfaethoxypyridazine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfamethoxypridazine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfamethoxazole	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfapyridine	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Sulfacetamide	250 µg/mL	200 µL	25.0 mL	2.00 µg/mL
Ciprofloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Norfloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Sarafloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Enrofloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Difloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Danofloxacin	100 µg/mL	50.0 µL	25.0 mL	0.200 µg/mL
Monensin	10.0 µg/mL	100 µL	25.0 mL	40.0 ng/mL
Oxytetracycline	2500 µg/mL	150 µL	25.0 mL	15.0 µg/mL
Doxycycline	2500 µg/mL	150 µL	25.0 mL	15.0 µg/mL
Chlortetracycline	2500 µg/mL	150 µL	25.0 mL	15.0 µg/mL
Tetracycline	2500 µg/mL	150 µL	25.0 mL	15.0 µg/mL
Lincomycin	2500 µg/mL	150 µL	25.0 mL	15.0 µg/mL

These solutions are stable for up to 6 months if stored ≤ -70°C.

f. Intermediate Analytical Standard Solutions 2: Prepare an intermediate analytical standard in methanol as illustrated in Table 3.

Table 3:

Analytical Standard	Conc. of Stock Solution	Volume Used	Final Volume in methanol	Final Conc.
Tylosin	200 µg/mL	1.25 mL	25.0 mL	10.0 µg/mL
Erythromycin	200 µg/mL	40.0 µL	25.0 mL	320 ng/mL
Fumagillin	200 µg/mL	125 µL	25.0 mL	1.00 µg/mL

These solutions are stable for up to 6 months if stored ≤ -70°C.

g. 0.1% formic acid in water: 4 mL of LC-MS grade formic acid diluted to 4 L with 18 mΩ or equivalent water.

h. 0.1% formic acid in acetonitrile: 4 mL of LC-MS grade formic acid diluted to 4L with LC-MS grade acetonitrile.

Sample Preparation and Extraction:

1. Measure 2.00 grams (+/- 0.03 grams) of honey into a 50 mL disposable polypropylene centrifuge tube.

2. All samples, standards, and quality control are fortified with 50.0 µL of the intermediate internal standard solution. The 1X standard and matrix spikes are fortified with 50.0 µL of the intermediate standard solution #1, and 50.0 µL of intermediate standard solution # 2. This correlates to the following concentrations:
   - Fluoroquinolones = 5.00 ng/g
   - Monensin = 1.00 ng/g
   - Tetracyclines and lincomycin = 375 ng/g
   - Sulfonamides = 50.0 ng/g
   - Fumagillin = 25.0 ng/g
   - Tylosin = 250 ng/g
   - Erythromycin = 8.00 ng/g

3. Add approximately 10 mL of water containing 0.1% (v/v) formic acid to each tube and shake for approximately 5 minutes.

4. Transfer approximately 300 µL of the sample to a clean well plate for fumagillin determination.

5. Add an additional 10 mL of water containing 0.1% (v/v) formic acid to each centrifuge tube and shake for approximately 60 seconds.

6. Transfer approximately 300µL of the sample to a clean well plate for the two separate ASPE/MS analyses of:

a. Erythromycin, and tylosin
b. Tetracyclines, lincomycin and monensin

7. Add approximately 15 mL of heptane to each centrifuge tube and shake for approximately 5 minutes.

8. Centrifuge the sample for 5 minutes, at 4700 RPM, at 5°C.

9. Decant the upper heptane layer, and transfer approximately 300 µL of the sample to a well plate for determination of fluoroquinolones (two separate acquisitions).

10. Add approximately 20 mL of water containing 0.1% (v/v) formic acid to each centrifuge tube.

11. Add 1 mL of glacial acetic acid to each centrifuge tube.

12. Then shake for approximately 60 seconds. Transfer a portion of the sample to a well plate for ASPE/MS analysis for sulfonamides.

Instrumentation:

The mass spectrometer was tuned and calibrated in positive ion detection mode according to the manufacturer's instructions. The instrument was optimized by infusing compounds of interest with (50:50) 0.1% formic acid in water and 0.1% formic acid in acetonitrile, at an HPLC flow rate of 400 µL/min in order to optimize electronic voltages and gas flows. The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source.

Individual ASPE/MS parameters are as follows:
Pump 1 is used for solvent A
Pump 2 is used for solvent B
Pump 3 is used for solvent C

a. Fumagillin: Solvent A, used for sample loading was water containing 0.1% (v/v) formic acid. Solvent B, used for washing was 20% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 90% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Samples were sequentially analyzed by aspirating 10 µL onto the collection loop under vacuum directly from the well plates. The sample was then loaded onto the C_8 cartridge by pump 1 at a flow rate of 1.50 mL/min for 3000 msec. The C_8 cartridge was then washed by pump 2, using solvent B at a flow rate of 1.25 mL/min for 3000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 3000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 6000 msec.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 290°C, 20 L/min, 400°C, and 12 L/min respectively. Electrical voltages were optimized for the capillary voltage at +3000 volts, nebulizer/nozzle voltage at +500 volts, cell accelerator voltage of +4 volts, and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +75 volts. Both Q1 and Q3 resolution was set to wide. The collision energy and MRM transition information is listed in Table 4.

Table 4:

Name	Precursor m/z	Product m/z	CE (volts)
FUM 1	459.2	233.0	10
FUM 2	459.2	215.3	10
FUM 3	459.2	131.0	10
*ROX	837.1	679.0	25

*Surrogate standard

b. Erythromycin and Tylosin: Solvent A, used for sample loading was water containing 0.1% (v/v) formic acid. Solvent B, used for washing was 5% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 90% acetonitrile (v/v) containing 0.1% (v/v) formic acid. The sample was loaded onto the C_4 cartridge by pump 1 at a flow rate of 1.50 mL/min for 3000 msec. The C_4 cartridge was then washed by pump 2, using solvent B at a flow rate of 1.25 mL/min for 4000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 6000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 6000 msec.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 290°C, 20 L/min, 400°C, and 12 L/min respectively. Electrical voltages were optimized for the capillary voltage at +3000 volts, nebulizer/nozzle voltage at +500 volts, cell accelerator voltage of +4 volts, and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +75 volts. The collision energy and MRM transition information is listed in Table 5.

Table 5:

Name	Precursor m/z	Q1 Resolution	Product m/z	Q2 Resolution	CE (volts)
ERY 1	734.7	Wide	158.2	Wide	30
ERY 2	734.7	Wide	576.5	Wide	20
TYL 1	916.5	Unit	174.0	Unit	40
TYL 2	916.5	Unit	772.8	Unit	35
*ROX	837.1	Wide	679.0	Wide	25

*Internal standard

c. Tetracyclines, Lincomycin, and Monensin: Solvent A, used for sample loading was water. Solvent B, used for washing was 10% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 100% acetonitrile containing 0.1% (v/v) formic acid. The sample was loaded onto the C_{18} cartridge by pump 1 at a flow rate of 1.50 mL/min for 4000 msec. The C_{18} cartridge was then washed by pump 2, using solvent B at a flow rate of 1.25 mL/min for 2000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 4000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 6000 msec.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 290°C, 20 L/min, 400°C, and 12 L/min respectively. Electrical voltages were optimized for the capillary voltage at +2000 volts, nebulizer/nozzle voltage at +500 volts, cell accelerator voltage of +4 volts (+5 volts for monensin), and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +75 volts. The collision energy and MRM transition information is listed in Table 6.

Table 6:

Name	Precursor m/z	Q1 Resolution	Product m/z	Q2 Resolution	CE (volts)
CTC	479.1	Unit	462.3	Unit	23
OXY	461.1	Unit	426.3	Unit	20
TC	445.1	Unit	410.3	Unit	20
DC	445.1	Unit	154	Unit	30
*MON	693.5	Wide	461.4	Wide	45
**DEME	465.1	Unit	448.1	Unit	23
LIN	407.2	Unit	126.2	Unit	20

* Monensin does not use an internal standard
**Internal Standard

d. Fluoroquinolones (two separate acquisitions):
- Danofloxacin and norfloxacin should be analyzed with a C_{18} SPE
- All other fluoroquinolones should be analyzed with a phenyl SPE

Solvent A, used for sample loading was water. Solvent B, used for washing was 100% water containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 100% acetonitrile containing 0.1% (v/v) formic acid. The sample was loaded onto the cartridge by pump 1 at a flow rate of 1.50 mL/min for 4000 msec. The cartridge was then washed by pump 2, using solvent B at a flow rate of 1.25 mL/min for 4000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 4000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 4000 msec.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 290°C, 20 L/min, 400°C, and 12 L/min respectively. Electrical voltages were optimized for the capillary voltage at +4000 volts, nebulizer/nozzle voltage at +1500 volts, cell accelerator voltage of +4 volts (DANO and NOR +5 volts), and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +75 volts. The collision energy and MRM transition information is listed in Table 7.

Table 7:

Name	Precursor m/z	Q1 Resolution	Product m/z	Q2 Resolution	CE (volts)
DANO	358.1	Wide	82.1	Wide	40
*NOR	320.1	Wide	233	Wide	25
Ciprofloxacin $^{13}C_3$	336	Wide	291.1	Wide	20

*Norfloxacin does not use an internal standard

Name	Precursor m/z	Q1 Resolution	Product m/z	Q2 Resolution	CE (volts)
DFLX	400	Wide	299	Wide	35
SARA	386	Wide	299	Wide	30
ENRO	360.1	Wide	245	Wide	20
CIPRO	332.1	Wide	231	Wide	35
Ciprofloxacin $^{13}C_3$	336	Wide	291.1	Wide	20

e. Sulfonamides (excluding sulfacetamide): Solvent A, used for sample loading was water containing 0.1% (v/v) formic acid. Solvent B was 90% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 90% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Samples were sequentially analyzed by aspirating the sample onto the collection loop under vacuum directly from the well plates. The sample was then loaded onto the C_{18} cartridge by pump 1 at a flow rate of 1.50 mL/min for 3000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 6000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 6000 msec. No extra wash was needed for the method. The main function of pump 2 in this method was to wash the sample collection loop with solvent B at a flow of 1.25 mL/min.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 290°C, 20 L/min, 400°C, and 12 L/min respectively. Electrical voltages were optimized for the capillary voltage at +2000 volts, nebulizer/nozzle voltage at +500 volts, cell accelerator voltage of +4 volts, and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +75 volts. The Q1 resolution was set to unit, and Q3 resolution was set to wide. The collision energy and MRM transition information is listed in Table 8.

Table 8:

Name	Precursor m/z	Product m/z	CE (volts)
SDZ 1	251.0	156	12
SDZ 2	251.0	92.1	25
STZ 1	256.0	156.0	15
STZ 2	256.0	92.1	30
SPD 1	250.0	108.0	25
SPD 2	250.0	92.1	30
SMR 1	265.0	156.1	15
SMR 2	265.0	92.1	30
SMZ 1	279.1	156.1	15
SMZ 2	279.1	92.1	30
SMP 1	281.0	156.0	15
SMP 2	281.0	108.0	25
SCP 1	285.0	156.0	12
SCP 2	285.0	92.1	30
SDX 1	310.9	156.1	15
SDX 2	310.9	92.1	30
SEP 1	295.0	156.0	15
SEP 2	295.0	92.1	30
SMX 1	254.0	156.0	15
SMX 2	254.0	92.1	30
SQX 1	301.1	156.0	15
SQX 2	301.1	92.1	32
SDM 1	311.1	156.0	20
SDM 2	311.1	108.0	25
Sulfamethazine $^{13}C_6$	285.0	124.1	25

*It should be noted, that the number of MRM transitions in the sulfonamides analyses exceeds the scan speed capability of most triple quadrupole mass spectrometers. Therefore, it is advisable to use two separate ASPE/MS injections to acquire all the MRM transitions.

f. Sulfacetamide: Solvent A, used for sample loading was water. Solvent B, used for washing was 10% acetonitrile (v/v) containing 0.1% (v/v) formic acid. Solvent C, used for sample elution, was 100% acetonitrile containing 0.1% (v/v) formic acid. The sample was loaded onto the hypercarb cartridge by pump 1, using solvent A at a flow rate of 1.50 mL/min for 4000 msec. The hypercarb cartridge was then washed by pump 2, using solvent B at a flow rate of 1.25 mL/min for 2000 msec. The retained analytes were then eluted to the mass spectrometer by pump 3, using solvent C at a flow rate of 1.00 mL/min for 4000 msec. The system was re-equilibrated by pump 1 using solvent A at a flow rate of 1.5 mL/min for 6000 msec.

The gas temperature, gas flow, sheath gas temperature, and sheath gas flow were set to 200°C, 14 L/min, 250°C, and 10 L/min respectively. Electrical voltages

were optimized for the capillary voltage at +3000 volts, nebulizer/nozzle voltage at +500 volts, cell accelerator voltage of +4 volts, and a fragmentor voltage of 380 volts. The high-pressure RF was set to +150 volts and the low-pressure RF was set to +60 volts. Both Q1 and Q3 resolutions were set to wide. The collision energy and MRM transition information is listed in Table 9.

Table 9:

Name	Precursor m/z	Product m/z	CE (volts)
SAA 1	215.05	156.0	10
SAA 2	215.05	108.0	25
SAA 3	215.05	92.0	25
Sulfamethazine $^{13}C_6$	285.0	124.1	25

Data Interpretation:

Fumagillin was the only compound included in the screening procedure that did not utilize an internal standard. Roxithromycin was used as surrogate standard to demonstrate extraction efficiency. Therefore, the presumptive positive criteria for fumagillin is that all three product ions be present with a response ≥ ½ that of the 1X standard. All three product ions should exhibit a signal to noise ratio > 3:1. Roxithromycin should also be present with a signal to noise ratio > 3:1.

For presumptive positive criteria, the sample concentration should be ≥ ½ the 1X standard. All product ions should be present and have a response ≥ ½ of the 1X standard. The product ion for the internal standard/surrogate standard should also be present. All product ions should have a signal to noise ratio > 3:1.

Semi-quantitation is performed for each drug residue (excluding fumagillin) by selecting the most abundant MRM transition, and/or the MRM transition with the higher signal to noise ratio. All drug residues which utilize an internal standard are calculated by taking the ratio of the chromatographic area of the quantitation ion, to the chromatographic area of the internal standard.

RESULTS AND DISCUSSION

Method Optimization:

The primary focus of this study was to develop an automated, rapid screening method for multiple drug residues in honey. Therefore, efforts were made during the method development process to combine as many residues into a single acquisition as possible. However, multiple ASPE/MS injections were required. This is because of the limited scan speed, and the different chemical properties.

For each of the compounds, the optimization consisted of a two-fold process. The first step was to maximize the performance of the ASPE/MS system with respect to SPE

phases, load times, wash times, elution times, solvent choices, solvent compositions, and flow rates. Subsequently, upon completion of the ASPE/MS optimization, the mass spectrometer needed tuning for each targeted compound with respect to response and peak shape. It should be noted that the ASPE/MS system in conjunction with a triple quadrupole mass spectrometer does have a limitation on the number of MRM transitions which can be acquired during the analysis due to scan speed of the mass spectrometer. Because of this, we were not able to create any single acquisition methods that contained more than approximately 15 different MRM transitions.

The mass spectrometer's parameters initially used to evaluate the automated solid-phase extraction system were obtained from published literature with respects to MRM transitions, temperatures, voltages, and gasses [8]. Although these parameters were not necessarily optimal, they did serve as a basis to optimize the ASPE system. To ensure that all data derived could be directly correlated to method performance and not that of a matrix issue, the initial method development work was done using solvent standards. Two of the most important factors in ASPE optimization was implementing as much automation as possible, and rapidly extracting the samples onto the mass spectrometer. As a result, all of the ASPE methods developed could extract a sample in < 30 seconds, utilizing considerable automation.

Upon completion of ASPE optimization, each tunable mass spectral parameter was optimized through direct injection of a laboratory fortified reagent blank via the ASPE system. Solvent blanks were analyzed immediately following the analysis of the standards to ascertain if any residual carryover was present.

After optimization was completed, laboratory fortified matrix blanks were analyzed in comparison to laboratory fortified reagent blanks. The initial findings indicated that several analytes suffered considerable matrix suppression. Furthermore, the background response in the matrix blanks for some ions could lead to potential false positives. To reduce the matrix effects, multiple sample dilutions were evaluated for each residue.

Method Validation:

All method validation efforts were conducted in accordance to the Guidelines for the Validation of Chemical Methods for the FDA Foods Program and the Center for Veterinary Medicines guidelines for mass spectrometry confirmation criteria [9, 10]. Reference materials were obtained from various commercially available sources and were prepared as described in the sample preparation section. As there are several different types of honey available, it was extremely important to analyze types of honey that are commonly available to consumers. Those chosen for method development and method validation were raw and unprocessed honeys, amber honeys, and wildflower honeys. Additionally, incurred honey samples containing sulfonamides, fluoroquinolones, fumagillin, and macrolides were analyzed both by ASPE mass spectrometry and an approved traditional LC-MS method [8].

The method validation procedure was divided into three separate portions. Sulfonamides were the first set of drug residues to be validated. Once that study was completed, efforts were focused on the validation of fumagillin, tylosin, and erythromycin; which was then followed by validation for fluoroquinolones, tetracyclines, monensin, and lincomycin. Each of the three separate validations was conducted over three days, and used three different honey matrices. The sample sets consisted of laboratory fortified blanks, matrix blanks, reagent blanks, and incurred residues.

Samples were compared against matrix-matched standards that were previously screened and determined to be free of the residues of concern. Each of the three separate validations consisted of ≥ 30 laboratory fortified spikes, and ≥ 18 matrix blanks in accordance with the United States Food and Drug Administration validation guidelines.

Data Analysis:

There are not any chromatographic retention times to use for a means of identification, because the sample was directly injected onto the mass spectrometer via the ASPE system. Therefore, it is impossible to differentiate isomeric or isobaric compounds using this method. Any samples that meet presumptive positive criteria would necessitate further analysis using a confirmatory method. For a sample to meet the presumptive positive criteria, it must have a response for all the monitored product ions at ≥ ½ of the 1X standard. Additionally, the semi-quantitative value should be ≥ ½ of the 1X standard.

Semi-quantitative results were generated for all residues of interest with the exception of fumagillin (see Table 10–13). This was primarily because without an appropriate available stable isotopically labeled standard, we could not obtain accurate quantitative values for this compound. Furthermore, during the method development it was noticed that several of the matrices had a response for one of the product ions at or near that of the standard. To prevent false positive or false negatives, three product ions were monitored for fumagillin. By requiring all three product ions to have a response at ≥½ of the 1X standard, false positives and false negatives were eliminated during the validation procedure.

During the method validation efforts, there were no false positives or false negatives detected for any residue. Furthermore, all of the laboratory fortified matrix spikes and incurred residue samples that were analyzed met the presumptive positive confirmation criteria for all analytes present.

CONCLUSION

The method validation results demonstrate that this rapid screening method is a viable method for analysis of a wide array of drug residues in honey. Through the use of this method analytical times have been reduced by ≥ 85%, with the capability of continuously analyzing > 1000 samples. Furthermore, the method generated precise results with no false positives or false negatives throughout the validation process. Thus, this high-throughput and high-

capacity method is an optimal semi-quantitative screening method for any regulatory laboratory.

Table 10: Statistical semi-quantitative data generated from screening method. Data is based on 21 matrix spikes analyzed at the target testing limit (1X)

Drug Residue	Mean Recovery (ng/g)	Mean %Recovery	Standard Deviation	%RSD	Threshold Value (ng/g)
Sulfathiazole	46.5	93.0	4.44	9.55	42.3
Sulfaquinoxaline	46.6	93.3	5.22	11.2	41.0
Sulfadimethoxine	48.3	96.6	4.88	10.1	41.6
Sulfamethazine	49.3	98.7	3.92	7.94	43.2
Sulfachloropyridazine	44.0	87.9	4.34	9.88	42.5
Sulfadiazine	50.2	100	8.43	16.8	35.5
Sulfamerazine	48.6	97.3	6.15	12.6	39.4
Sulfamethoxazole	45.0	89.9	7.43	16.5	37.2
Sulfadoxine	52.0	104	5.45	10.5	40.6
Sulfaethoxypyridazine	46.7	93.4	4.60	9.86	42.1
Sulfamethoxypridazine	49.3	98.7	7.96	16.1	36.3
Sulfapyridine	49.5	99.0	4.41	8.91	42.4
Sulfacetamide	52.0	103	11.6	22.4	30.2
*Erythromycin	8.34	104	0.733	8.79	6.92
*Tylosin	263	105	20.8	7.93	222
Chlortetracycline	366	98.4	30.5	8.32	314
Doxycycline	395	105	31.0	7.84	342
Oxytetracycline	380	101	33.6	8.84	322
Tetracycline	363	97.5	35.2	9.71	302
Lincomycin	345	93.3	57.4	16.6	246
Monensin	1.04	104	0.154	14.8	0.772
Ciprofloxacin	5.11	102	0.602	11.8	4.07
Enrofloxacin	4.90	98.0	0.509	10.4	4.02
Difloxacin	5.22	104	0.588	11.3	4.20
Sarafloxacin	5.33	107	0.677	12.7	4.16
Danofloxacin	5.53	111	0.763	13.7	4.23
Norfloxacin	4.74	94.7	1.12	23.7	2.80

* Only 7 matrix spikes were analyzed at the target testing level.

Table 11: Statistical semi-quantitative data generated from screening method. Data is based on 9 matrix spikes analyzed at the target testing limit (2X)

Drug Residue	Mean Recovery (ng/g)	Mean % Recovery	Standard Deviation	%RSD
Sulfathiazole	93.5	93.5	7.79	8.32
Sulfaquinoxaline	97.1	97.1	9.71	9.99
Sulfadimethoxine	101	101	9.70	9.57
Sulfamethazine	102	102	10.3	10.1
Sulfachloropyridazine	90.9	90.9	7.78	8.56
Sulfadiazine	101	101	11.8	11.6
Sulfamerazine	99.0	99.0	13.0	13.2
Sulfamethoxazole	97.3	97.3	16.3	16.7
Sulfadoxine	115	115	14.8	12.5
Sulfaethoxypyridazine	102	102	9.98	9.74
Sulfamethoxypridazine	102	102	14.0	13.7
Sulfapyridine	98.0	98.0	13.9	14.2
Sulfacetamide	124	123	7.16	5.75
Chlortetracycline	732	98.3	60.2	8.23
Doxycycline	767	102	41.9	5.46
Oxytetracycline	749	99.2	50.2	6.70
Tetracycline	699	93.8	38.3	5.47
Lincomycin	696	94.0	101	14.5
Monensin	2.26	113	0.140	6.18
Ciprofloxacin	10.4	104	1.72	16.6
Enrofloxacin	8.71	87.1	0.758	8.70
Difloxacin	11.0	110	1.46	13.2
Sarafloxacin	10.4	104	0.702	6.77
Danofloxacin	12.0	120	1.77	14.8
Norfloxacin	8.81	88.1	3.32	37.7

Table 12: Statistical semi-quantitative data generated from screening method. Data is based on 9 matrix spikes analyzed at the target testing limit (4X)

Drug Residue	Mean Recovery (ng/g)	Mean % Recovery	Standard Deviation	%RSD
Sulfathiazole	196	98.0	22.7	11.6
Sulfaquinoxaline	206	103	14.4	6.95
Sulfadimethoxine	206	103	20.5	9.95
Sulfamethazine	214	107	26.0	12.1
Sulfachloropyridazine	187	93.7	19.7	10.5
Sulfadiazine	210	105	37.1	17.7
Sulfamerazine	207	104	25.0	12.1
Sulfamethoxazole	192	95.9	29.8	15.5
Sulfadoxine	216	108	23.1	10.7
Sulfaethoxypyridazine	210	105	18.3	8.71
Sulfamethoxypridazine	206	103	19.3	9.37
Sulfapyridine	197	98.5	14.1	7.12
Sulfacetamide	246	123	16.1	6.54
Chlortetracycline	1460	97.6	0.103	7.06
Doxycycline	1520	101	0.142	9.33
Oxytetracycline	1360	90.2	0.312	22.9
Tetracycline	1390	93.4	0.112	8.06
Lincomycin	1460	98.4	0.228	15.7
Monensin	4.05	101	0.466	11.5
Ciprofloxacin	21.7	108	4.33	20.0
Enrofloxacin	17.4	86.8	2.38	13.7
Difloxacin	22.4	112	3.12	14.0
Sarafloxacin	21.8	109	2.25	10.3
Danofloxacin	24.4	122	3.28	13.5
Norfloxacin	15.6	78.0	6.28	40.2

Table 13: Incurred Residue Analysis

Drug Residue	RFMS (ng/g)	LC-MS/MS (ng/g)	% Difference
Sulfathiazole	44.8	21.9	68.7
Erythromycin	34.1	28.1	19.3
Enrofloxacin	3.89	3.70	5.00

Figure 1 (*Honey Multi-Residue Analysis Flow Chart*): Due to the broad scope of drug residues and the large number of MRM transitions, this method is divided into sequential dilutions and ASPE/MS analyses.

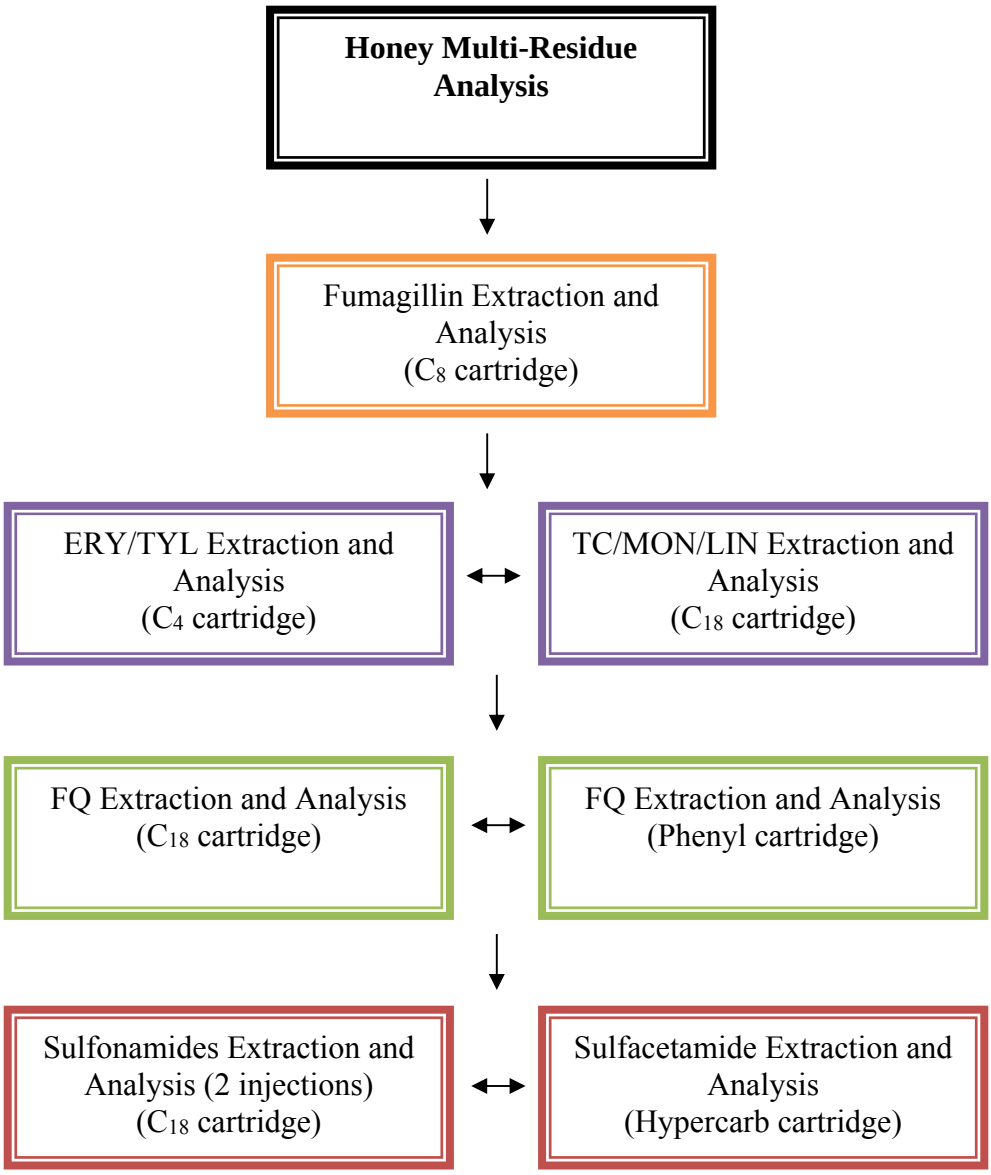


Figure 2. A schematic illustration showing valve configuration and flow path in the aspiration state

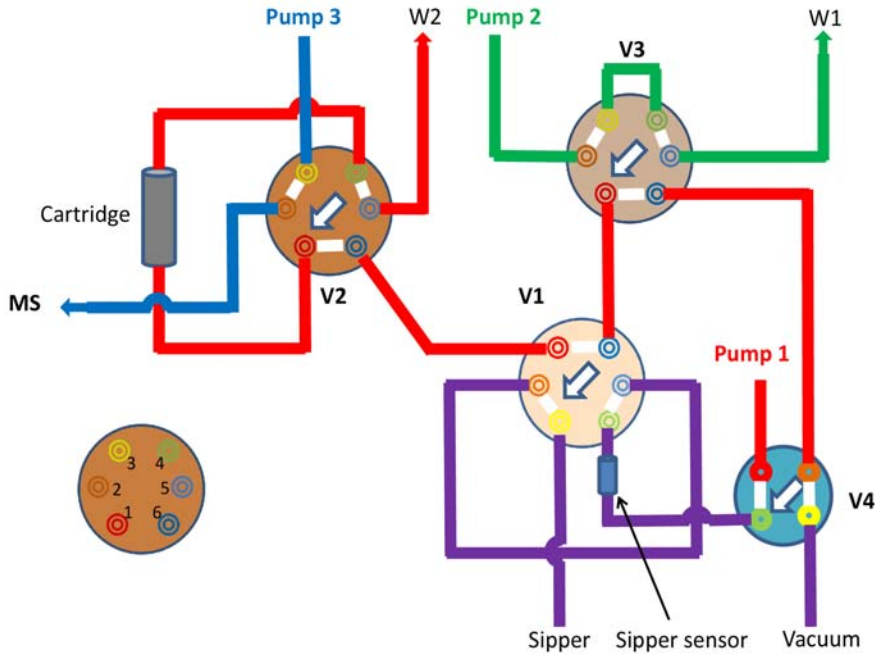


Figure 3. A schematic illustration showing valve configuration and flow path in the load and wash state

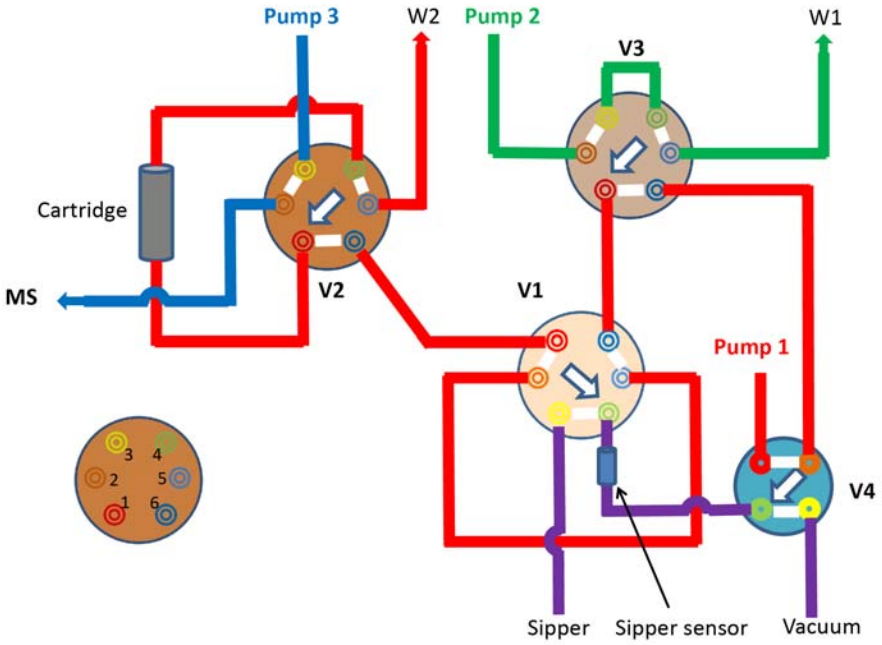


Figure 4. A schematic illustration showing valve configuration and flow path in the extra wash state

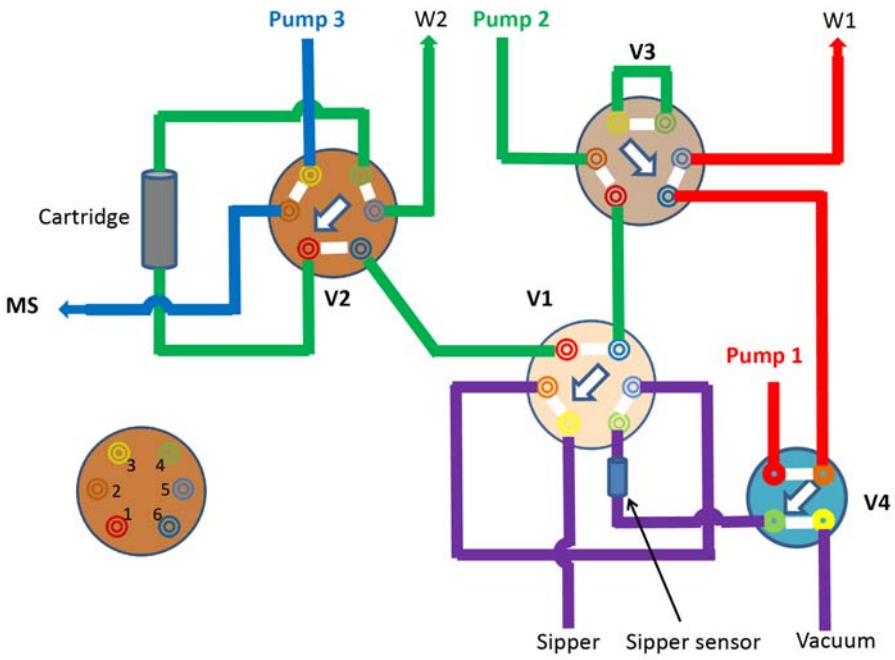


Figure 5. A schematic illustration showing valve configuration and flow path in the elution state

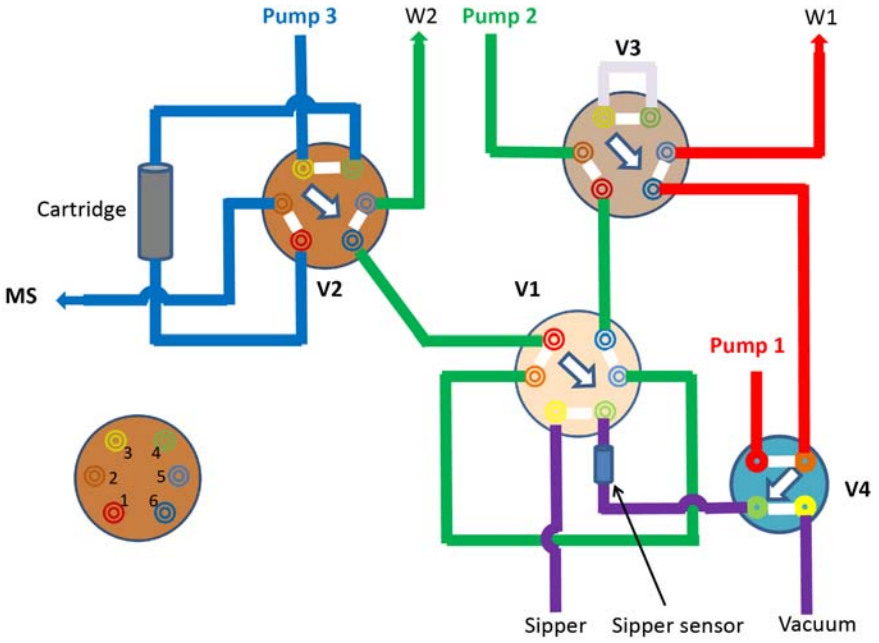
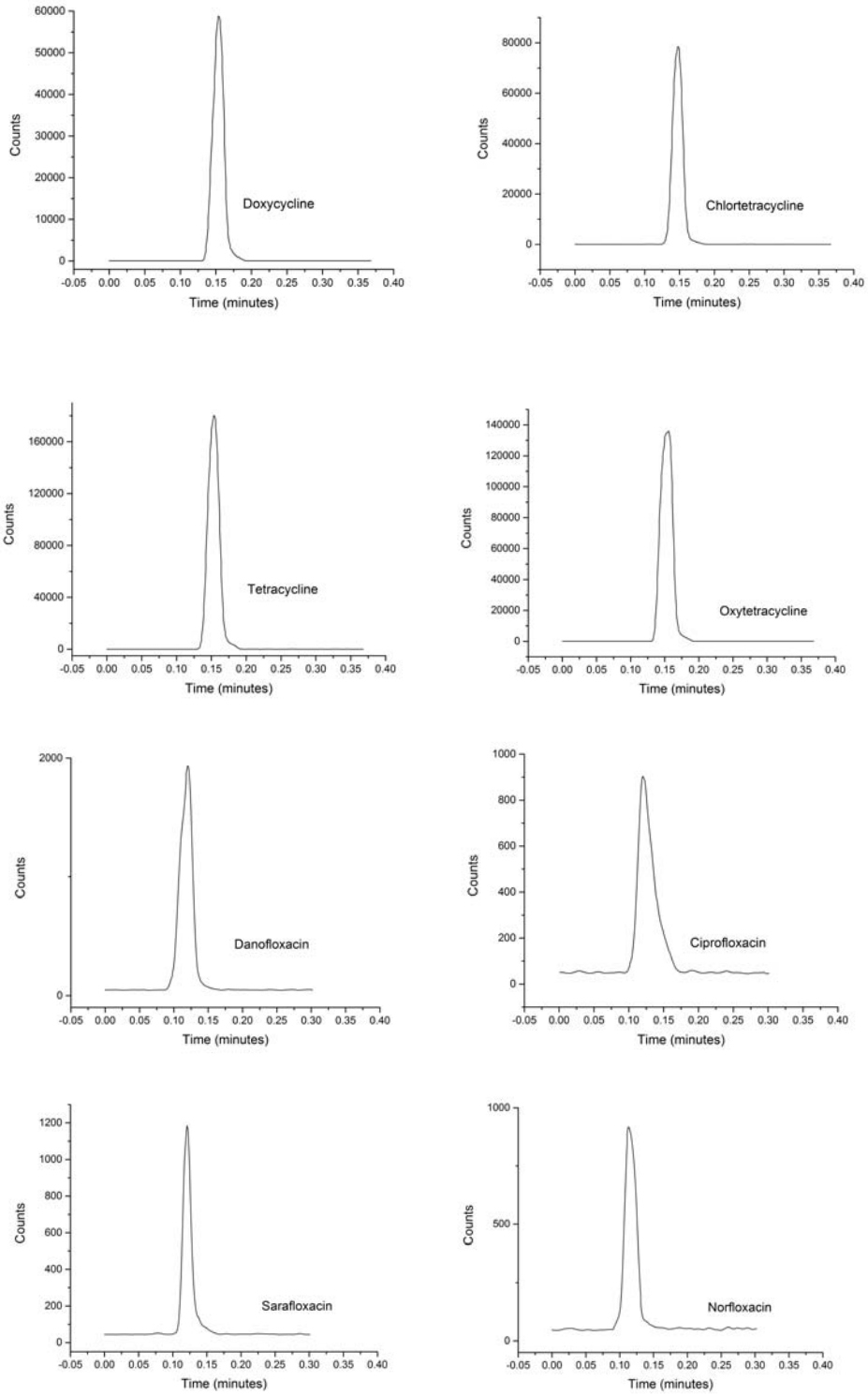
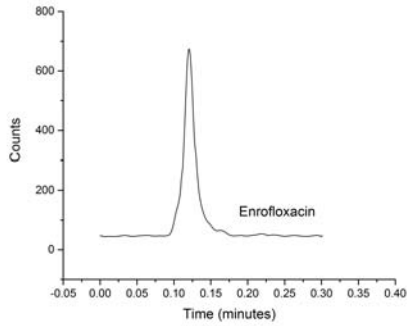
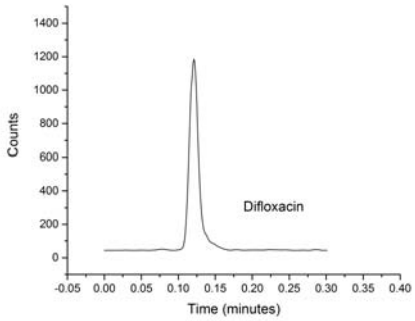
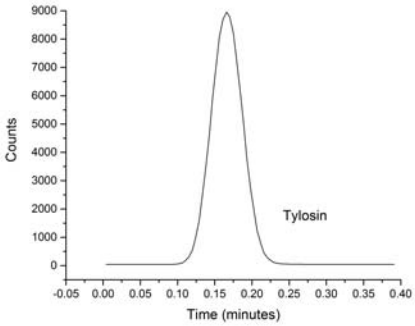


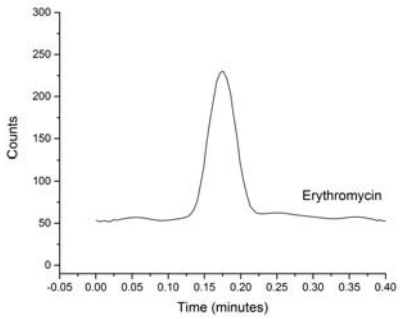
Figure 6. Mass spectra of semi-quantitative ion for drug residues in honey at the target testing limit




Counts
Enrofloxacin
Time (minutes)


Counts
Difloxacin
Time (minutes)


Counts
Tylosin
Time (minutes)


Counts
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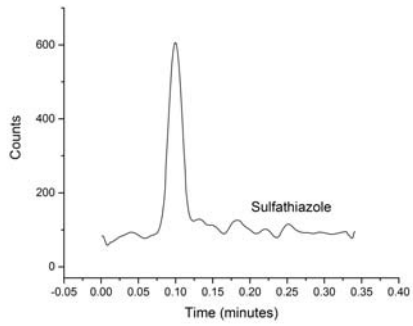
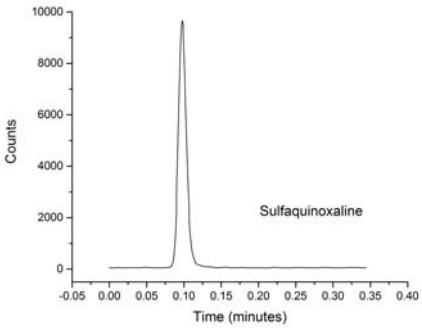
Time (minutes)


Counts
Sulfathiazole
Time (minutes)


Counts
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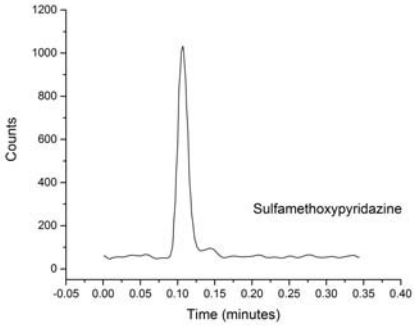
Time (minutes)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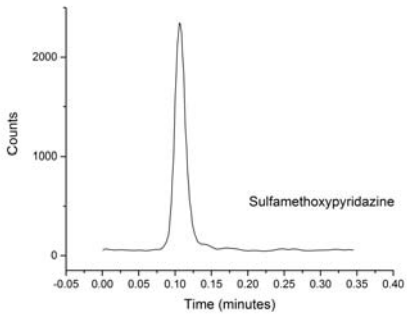
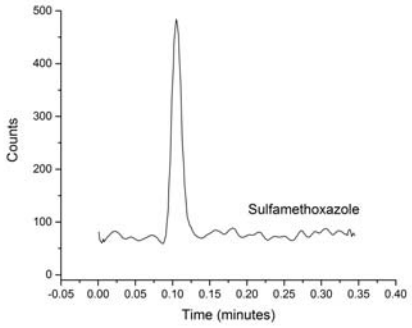
Time (minutes)


Counts
Sulfamethoxypyridazine
Time (minutes)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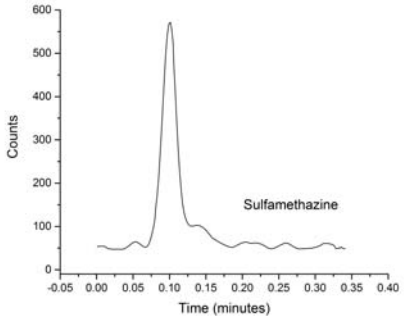
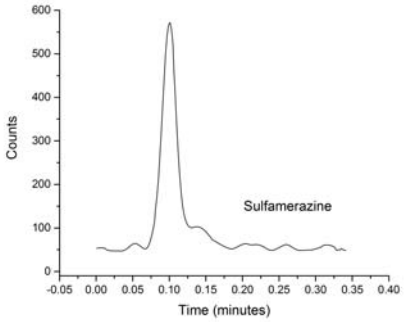
Counts
Time (minutes)


Sulfamethazine
Counts
Time (minutes)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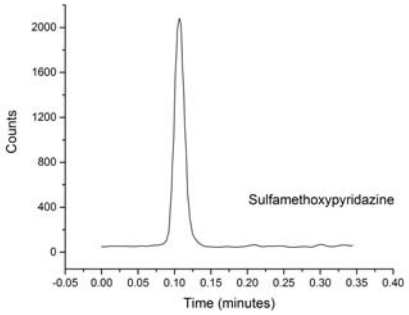
Counts
Time (minutes)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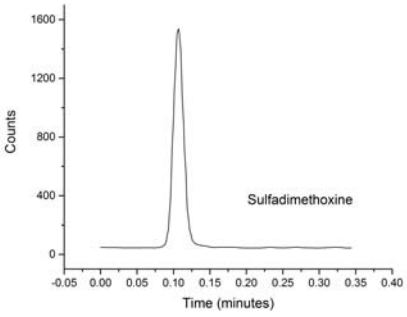
Counts
Time (minutes)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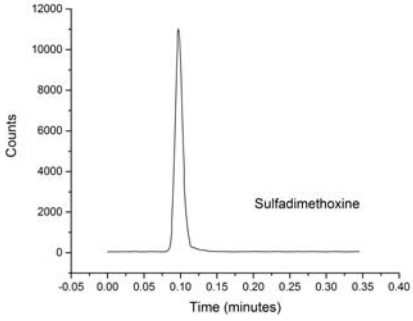
Counts
Time (minutes)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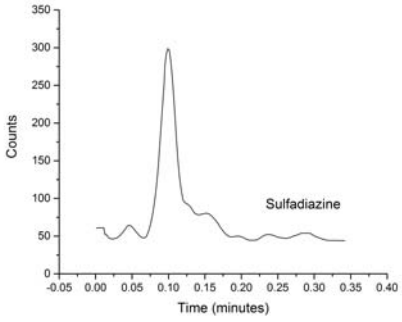
Counts
Time (minutes)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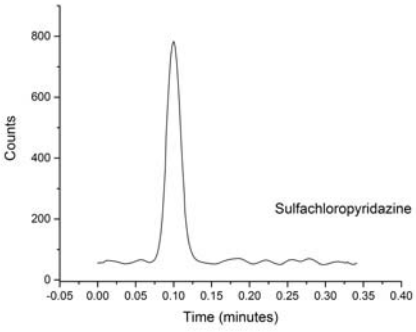
Counts
Time (minutes)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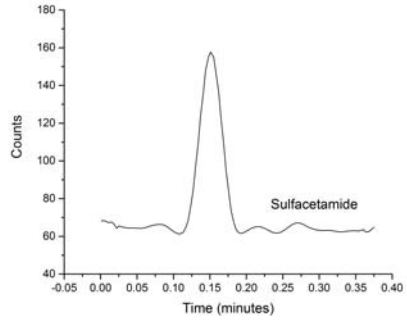
Counts
Time (minutes)


Counts
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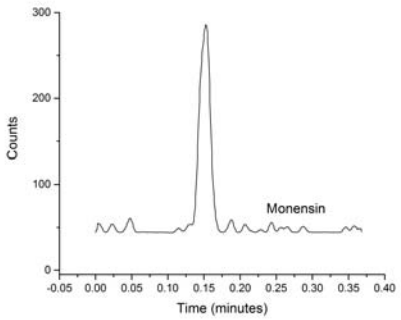
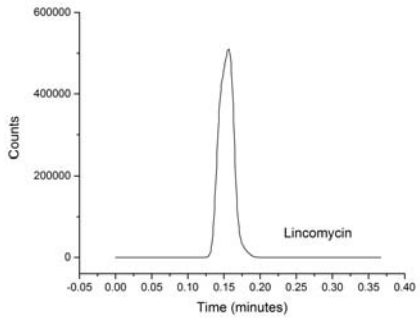
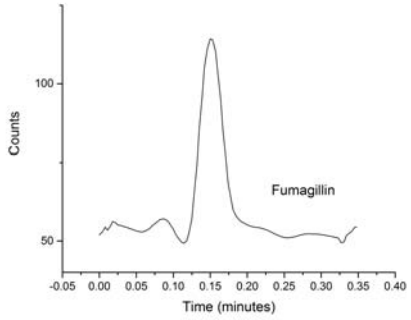
Time (minutes)


Counts
Monensin
Time (minutes)


Counts
Lincomycin
Time (minutes)


Counts
Fumagillin
Time (minutes)

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