

Multi-Class Antibiotic Screening of Honey Using Dual On-Line Extraction Columns in Tandem

Catherine Lafontaine, Yang Shi, Francois Espourteille

Thermo Fisher Scientific, Franklin, MA, USA



Overview

The intent of this study was to develop a broad, generic LC-MS/MS screening method utilizing Thermo Scientific TurboFlow technology for the analysis of multi-class antibiotic residues in honey. The residues were extracted from honey matrix using two TurboFlow™ columns of different chemistries placed in tandem on a Thermo Scientific Aria TLX-1 system. This was followed by transfer to a mixed mode reverse phase (RP) analytical column and gradient elution to a tandem mass spectrometer.

Introduction

Antibiotics are commonly used in bee hives to control bacterial disease in honey bees. Use of these antibiotics requires caution in order to prevent persistent residues from occurring in food-grade honey. If present in sufficient quantities, allergic reactions and bacterial resistance can develop.

Many countries, including those of the European Union and the United States, now monitor antibiotic residues in honey. LC-MS/MS is currently a common analytical method for their quantification in honey. Sample preparation for honey analysis by LC-MS/MS can be time-consuming, involving hydrolysis, pH adjustment, liquid-liquid extraction, evaporation, solid phase extraction, and pre-concentration. A quick, comprehensive on-line screening method by LC-MS/MS has been developed here to monitor several classes of antibiotics (Macrolides, Sulfonamides, Aminoglycosides, and Tetracyclines).

Methods

Sample Preparation

A Mcllvaine/0.1 M Ethylenediaminetetraacetic Acid (EDTA) buffer¹ was used as a 1:1 w/v (gram weight of honey:milliliter volume of buffer) diluent for wildflower honey, the testing matrix in this study. A stock solution was prepared for Sulfapyridine, Sulfathiazole, Tilmicosin, Tylosin, Oxytetracycline, and Erythromycin in 3:1 methanol:water at 0.100 mg/mL. Additionally, one was prepared for Doxycycline, Demeclocycline, Streptomycin, and Dihydrostreptomycin in water at 0.100 mg/mL. These stocks were each spiked into 1:1 honey:buffer matrix and used as a spiking stock to make a set of calibration standards and quality controls (QCs). All blanks, standards, and QCs were prepared and analyzed in polypropylene vials. Injection volumes were 0.050 mL onto a 100uL sample loop using a 100uL syringe.

FIGURE 1. Aria™ TLX-1 TurboFlow Method Parameters

TurboFlow Columns:	Cyclone MAX and Cyclone-P (0.5x50mm), in-tandem
Analytical Column:	Thermo Scientific BETASIL Phenyl-Hexyl, 100x3mm, 3um
Aria TLX-1 System Plumbing:	Focus Mode
Transfer Loop Volume:	0.200 mL
Column and Sample Temperatures:	Ambient
Aria Operating System Software Version:	1.6.2
Loading Pump Mobile Phases	
Mobile Phase A:	1.0 % Formic Acid in Water
Mobile Phase B:	0.1% Formic Acid in Acetonitrile
Mobile Phase C:	10mM Ammonium Acetate in Water, pH 9
Mobile Phase D:	50mM Ammonium Acetate in Methanol with 0.1 % Formic Acid
Eluting Pump Mobile Phase	
Mobile Phase A:	1 mM NFPA [1], 0.5 % Formic Acid, 0.04 % TFA [2] in Water
Mobile Phase B:	0.5 % Formic Acid, 0.04 % TFA in 1:1 Methanol : Acetonitrile

[1] NFPA is nonafluoropentanoic acid.

[2] TFA is trifluoroacetic acid.

FIGURE 2. Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer (MS) Parameters

Ion Polarity:	Positive
Ionization Source:	H-ESI
Spray Voltage:	4000 Volts
Vaporizer temperature:	400 °C
Capillary temperature:	370 °C
Sheath Gas pressure (N₂):	30 arb units
Auxiliary Gas pressure (N₂):	60 arb units
Ion Sweep Gas Pressure (N₂):	0.0 arb units
Skimmer Offset:	5 Volts (for Streptomycin), 0 Volts (for all others)
Collision Pressure:	1.2 mTorr
Chrom Filter Peak Width:	8.0 s
Scan type:	SRM
Scan time:	0.020 s
Scan width:	0.100 m/z
Peak Width Q1 Da. (FWHM):	0.700
Peak Width Q3 Da. (FWHM):	0.700

Sample Analysis

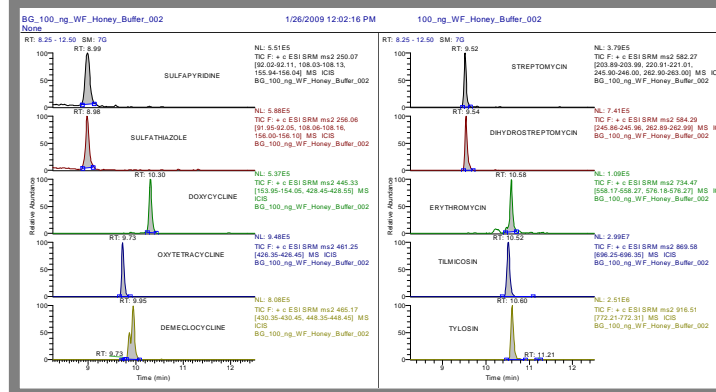
Residues were extracted from wildflower honey using buffer containing EDTA. The extract cleanup was accomplished by on-line passage through a mixed anion exchange column in tandem with a polar polymer-based column. Simple sugars were un-retained and moved to waste during the loading step while the analytes of interest were retained on the extraction column set. This was followed by organic elution to an end-capped silica-based mixed mode RP analytical column and gradient elution to a Heated Electropray Ionization (H-ESI) MS/MS operated in Positive Selective Reaction Monitoring (SRM) Mode. The total LC-MS/MS method run time was less than 18 minutes. Figure 1 shows the TurboFlow method parameters, while Figure 2 highlights the key MS parameters used. Positive SRM transitions and other MS parameters for individual analytes are shown in Table 1.

Table 1. The 10 analytes and their positive SRM transition ions.

STRUCTURAL CLASS	ANALYTE	PRECURSOR ION	PRODUCT IONS
Sulfonamides	Sulfapyridine	250.1	156.0 (Q), 108.1 (C), 92.1 (C)
	Sulfathiazole	256.1	156.1 (Q), 92.0 (C), 108.1 (C)
Tetracyclines	Doxycycline	445.3	154.0 (Q), 428.5 (C)
	Oxytetracycline	461.2	426.4
	Demeclocycline	465.2	448.4 (Q), 430.4 (C)
Aminoglycosides	Streptomycin	582.3	263.0 (Q), 246.0 (C), 203.9 (C), 221.0 (C)
	Dihydrostreptomycin	584.3	262.9 (Q), 245.9 (C)
Macrolides	Erythromycin	734.5	576.2
	Tilmicosin	869.6	696.3
	Tylosin	916.5	772.3

NOTE: (Q)=Quantification Ion ; (C)=Confirmation Ion

FIGURE 3. Example chromatogram of 100 ng/mL calibration standard in 1:1 honey/buffer.



Results

Results were packaged using Thermo Scientific LCQuan 2.5.6 data quantitation software and included subtraction of background due to the presence of a few endogenous analytes in the store-bought honey. Figure 3 shows a representative chromatogram of the 10 analytes at 100 ng/mL in 1:1 honey/buffer. Matrix-matched calibration standards showed linear response of 2 orders of magnitude ($r^2 > 0.99$) for all of the analytes investigated (Table 2). All CVs (n=3) were less than 19% for the lower limit of quantifications (LLOQ) and less than 8% for all other points of the curves. Figure 4 shows an LCQuan™ representative linear regression using Oxytetracycline as an example. QC sample variability was determined by processing and analyzing three replicates of each of four QC samples (2, 50, 100, and 500 ng/mL). All % RSDs were lower than 7% (except for Erythromycin which was below 15%). Data was not used for any QC level which fell below the analyte's determined LLOQ.

Figure 4. LCQuan™ view of Oxytetracycline calibration curve and LLOQ vs. ULOQ chromatograms.

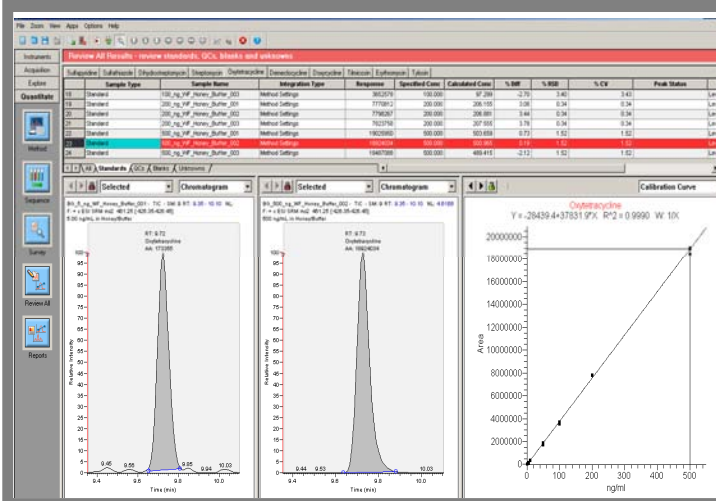


Table 2. Calibration Curve Statistics of the 10 Analytes.

Analyte	R ² (1/x weighting)	Dynamic Range* (ng/mL)**	Limit of Detection (ng/mL)	Percent Carryover (%)
Sulfapyridine	0.9980	50.0-500	10.0	8.95
Sulfathiazole	0.9988	50.0-500	10.0	5.46
Doxycycline	0.9990	10.0-500	5.00	10.8
Oxytetracycline	0.9990	5.00-500	2.00	11.7
Demeclocycline	0.9996	10.0-500	5.00	18.7
Streptomycin	0.9960	50.0-500	10.0	11.6
Dihydrostreptomycin	0.9980	50.0-500	10.0	6.47
Erythromycin	0.9877	50.0-500	10.0	1.16
Tilmicosin	0.9917	2.00-50.0	0.500	16.8
Tylosin	0.9958	10.0-100	5.00	13.7

*Based on analysis using 8 point standard curve (ng/mL): 0.500, 2.00, 5.00, 10.0, 50.0, 100, 200, & 500

**The level of carryover was included in the determination of dynamic range (kept to 20% or less).

Conclusions

During a honey quality monitoring process, it is always an analytical challenge to deal with a large number of antibiotics belonging to different classes. This often requires multiple LC/MS methods. In this study, we introduced a novel application using the TLX-1 system with dual online TurboFlow extraction columns of different chemistries. The results reveal that this design facilitates the separation and quantification of all of the representative compounds in the complex honey matrix. Sample preparation time is minimal, requiring only the addition of a buffer to reduce sample viscosity. These factors enable a broad screening for antibiotic contaminants to be performed quickly for a given sample, thus increasing sample throughput. It is worth noting that multiplexing with an Aria TLX-4 system would further reduce total LC-MS/MS run time four-fold and enable screening of 12 samples per hour. Future work could involve screening a larger range of antibiotic and environmental contaminants and lowering detection limits for all analytes thus combining a screening method with accurate quantification.

References

- United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science, CLG SOP No: CLG-TET2.01, Qualitative Identification of Tetracyclines in Tissues, Rev 01, p. 6, 9/25/03.