

GERSTEL AppNote 204

Completely Automated Extraction and Determination of Antibiotics in Eggs using a Robotic Autosampler and LC-MS/MS Platform

Fredrick D. Foster, John R. Stuff, Laurel A. Vernarelli, and Jacqueline A. Whitecavage

GERSTEL, Inc., 701 Digital Drive, Suite J, Linthicum, MD 21090, USA

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Abstract

Antibiotics are a class of antimicrobial veterinary drugs widely used to control disease in food producing animals including egg laying chickens. However, antibiotics can migrate into and accumulate in the egg of the chicken being treated. Eating eggs that contain antibiotic residues could provoke allergic reactions or lead to the development, or dominance of resistant bacterial strains, rendering treatment of human patients with antibiotics ineffective. It is therefore important to develop efficient screening methods for antibiotic residues in eggs intended for human consumption.

Extraction of antibiotic compounds from eggs can be challenging. Eggs are known to contain large amounts of lecithin, which is a mixture of phospholipids, and significant amounts of fats. It is important that the final egg extract be free of these co-extractive substances since they are known to lead to ion suppression and interference during LC/MS/MS analysis. Automating the entire process including liquid-liquid extraction of the raw egg sample, pass-through solid phase extraction to remove ion suppressing phospholipids, and analysis by LC/MS/MS provides the critical high throughput needed for the analysis for antibiotics in eggs.

Introduction

A manual procedure for the extraction of antibiotic compounds in eggs was previously developed and shown to be both simple and effective for the removal of matrix interferences [1]. A variety of sample handling steps are required prior to the analysis of the egg samples to accurately determine the analyte concentrations. Following liquid-liquid extraction, the extract is passed through an Oasis PRiME HLB cartridge which removes significant amounts of potentially interfering phospholipids that are known to be co-extracted with the antibiotic compounds.

In this study, we have shown that the GERSTEL MPS robotic sampler can be used successfully to automate the extraction of a subset of antibiotic compounds in eggs. Using the developed method, antibiotic compounds can be rapidly and reproducibly isolated from egg samples by an automated cleanup procedure coupled to LC/MS/MS analysis using the Agilent Ultivo Triple Quadrupole Mass Spectrometer, allowing their respective required limits of detection to be met. Linear calibration curves resulting in R^2 values 0.99 or greater were achieved based on completely automated extraction of antibiotic compounds. Coupling the liquid-liquid and solid phase extraction to the LC/MS/MS provides high throughput and minimizes matrix interference from these food samples.

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Experimental

Materials

All stock solutions for the compounds listed in Table 1 were purchased from Sigma-Aldrich. Antibiotic stock solutions were prepared using water, except for erythromycin, which was prepared using ethanol. Intermediate analyte stock solutions were prepared by combining the analyte stock solutions with water, resulting in appropriate concentrations for the method evaluation for the different antibiotic compounds.

A deuterated analogue, d_5 -Penicillin G, was purchased from C/D/N Isotopes, Inc. An internal standard stock solution containing d_5 -Penicillin G was prepared in water at a concentration of 1.00 mg/mL. A working internal standard solution was prepared in water at a concentration of 10 μ g/mL. The deuterated internal standard was used for quantitation of penicillin G.

Calibration standard and QC egg samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solutions using homogenized, organic eggs to reach the appropriate concentrations. Calibration standards were prepared using a dilution ratio strategy from the high concentration sample of 1:1.43:1.4:2.5:2.5. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:2:2.5. Table 1 lists the concentrations for the highest calibration standard and the limits of quantitation determined during the analyses.

Oasis PRiME HLB cartridges (Plus Short, 335 mg, p/n. 186008887) were obtained from Waters Corporation. Fresh organic eggs were purchased from a local market. All other reagents and solvents used were reagent grade.

Instrumentation

All automated Prep Sequences were performed using a MPS robotic^{PRO} sampler with the GERSTEL QuickMIX, CF-200 Centrifuge, and Universal Filtration Options as shown in Figure 1. All analyses were performed using an Agilent[®] 1260 HPLC with a Waters[™] Acquity[™] UPLC BEH C18, (2.1 x 100 mm, 1.7 μ m) and an Agilent Ultivo[®] Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Sample injections were made using the GERSTEL LCMS Tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 μ L stainless steel sample loop. The sample extract temperature was controlled at 11 °C while on the autosampler.



Figure 1: MPS robotic^{PRO} Multi-Purpose Sampler with the GERSTEL QuickMix, CF-200 centrifuge, and Universal Filtration option.

Egg Sample Pretreatment

1. Weigh 2.0 grams of homogenized egg sample into a clean 10 mL screw top vial.
2. Pipette 10 μ L of the 10 μ g/mL working internal standard into each sample and cap with a magnetically transportable cap.

Automated Prep Sequence

1. The MPS adds 8.0 mL of a (80:20) acetonitrile:water solution to the egg sample.
2. The vial is moved to the QuickMix Option where the sample is mixed at room temperature for 2.5 min at 2000 rpm.
3. The vial is moved to the Centrifuge Option where the sample is centrifuged at room temperature for 10 min at 2000 g.
4. The MPS conditions an Oasis PRiME HLB cartridge using 2 mL of (80:20) acetonitrile: water.
5. The MPS filters 2 mL of the resulting supernatant from the extraction through the conditioned Oasis PRiME HLB cartridge into a clean, empty 2 mL autosampler vial.
6. The MPS transfers 250 μ L of the filtered sample into a new, clean, empty 2 mL autosampler vial.
7. The MPS adds 750 μ L of 5 mM ammonium acetate in water to the sample and mixes using the syringe.

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Analysis Conditions LC

Pump	gradient (800 bar), flowrate = 0.4 mL/min
Mobile phase	A - 0.1% formic acid B - 0.1% formic acid in acetonitrile
Gradient	Initial 15% B 2.5 min 40% B 3.9 min 95% B 6.2 min 95% B 6.6 min 15% B 10 min 15% B
Run time	10 minutes
Injection volume	2.0 µL (loop over-fill technique)
Column temperature	30 °C

Analysis Conditions MS

Operation	electrospray positive mode
Gas temperature	350° C
Gas flow (N ₂)	5 L/min
Nebulizer pressure	35 psi
Sheath gas heater	250 °C
Sheath gas flow (N ₂)	11 L/min
Capillary voltage	4000 V
Nozzle voltage	500 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

Table 1: Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]	Fragmentor Voltage [V]	Collision Energy [V]	Ret. Time [min]	High Std Conc. [ng/mL]	MRL [ng/mL]	Limit of Quant. [ng/mL]			
Amprolium	243.2	150.1	94.0	80	80	5	10	0.554	10000	4000	800
Chlortetracycline	479.1	444.1	154.0	90	90	10	20	2.936	1000	400	80.0
Erythromycin	734.5	576.4	158.0	130	130	5	20	3.759	62.5	25	5.00
d ₅ -Penicillin G	339.0	179.9	169.8	90	90	5	5	4.039	-	-	-
Penicillin G ^a	335.1	176.1	160.0	90	90	5	5	4.051	250	(100) ^b	20.0
Tylosin	916.5	174.2	100.8	130	130	30	30	3.931	500	200	40.0

a - d₅-Penicillin used as internal standard
b - No residue allowed

Results and Discussion

Thorough mixing of an egg sample during extraction is important for maximizing the partitioning of the antibiotic residues from the viscous egg matrix into the extraction solvent. To evaluate different technologies for use in the automated mixing step of the method, homogenized egg samples were extracted using either an agitator option at its maximum speed setting of 750 rpm or the GERSTEL QuickMix option at a speed setting of 2000 rpm. Mixing times of both 30 min, to mimic the manual procedure, and 2.5 min were used with the agitator option, and 2.5 min using the QuickMix option. Immediately following the completion of the mixing steps, the resulting mixed samples were removed and photographed. As shown in the comparison pictures in Figures 2 A-C, the most thorough mixing was achieved using the QuickMix option at 2000 rpm. The use of the QuickMix option had the

additional benefit of reducing the amount of time necessary to thoroughly mix the sample by 27.5 min.

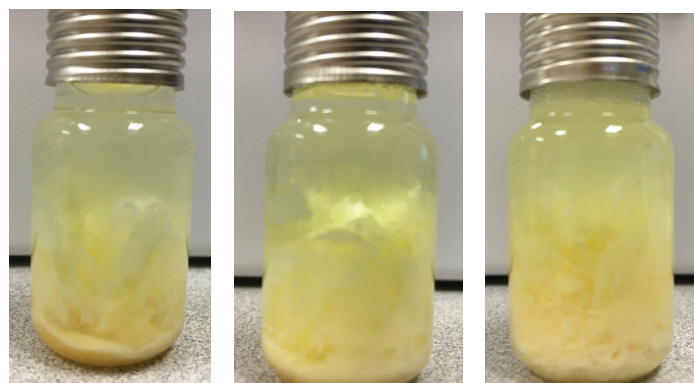


Figure 2: Visual appearance of egg sample extracts following the use of different automated mixing techniques.

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Figure 3 shows representative mass chromatograms for all antibiotics analyzed from an extracted low QC sample. The limits of quantitation for this method were five times lower than industry minimum reporting limits and are shown in table 1. Representative

calibration curves are shown in figures 4 A-C. Regression analysis for all antibiotic compounds analyzed within this method resulted in R^2 values of 0.99 or greater.

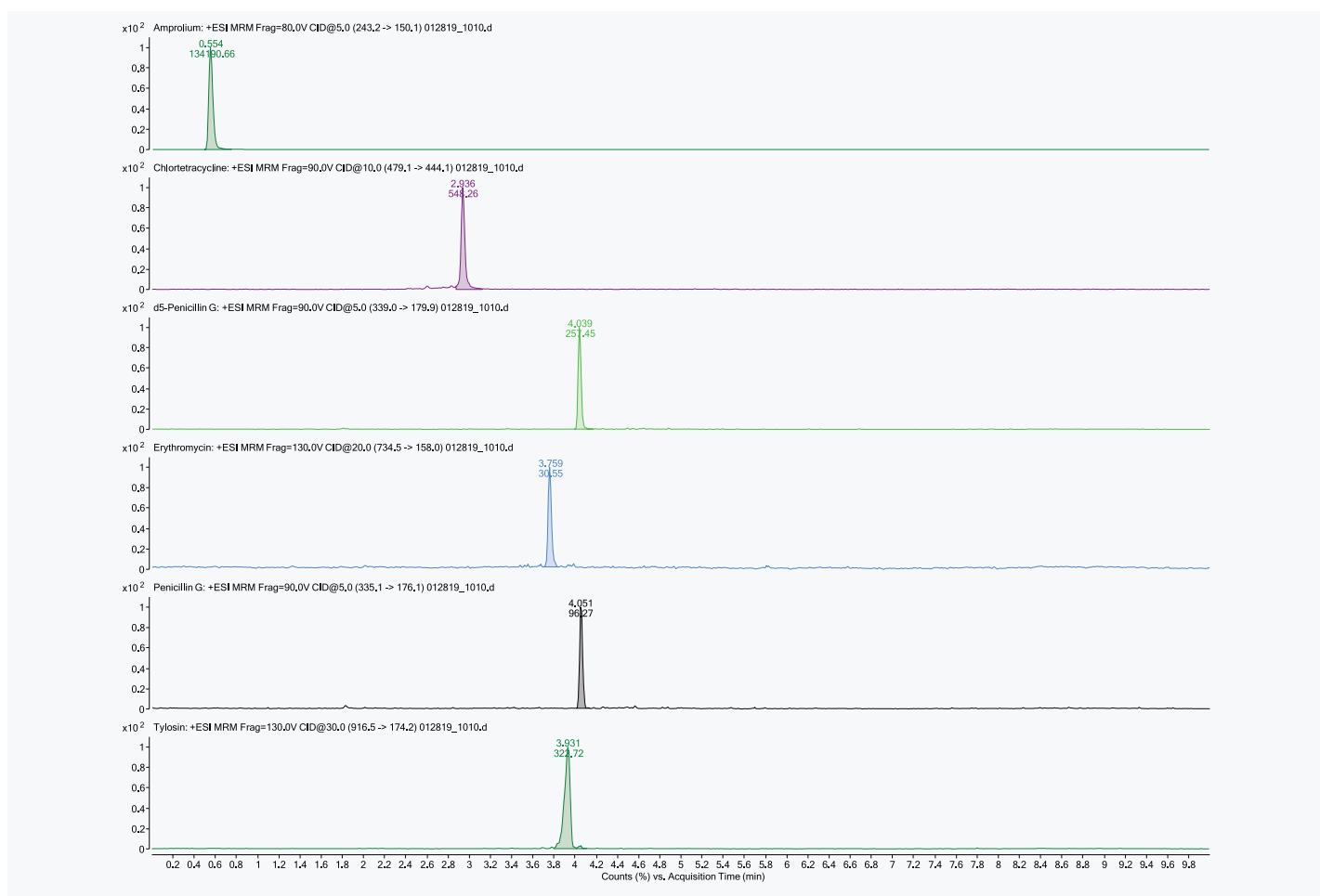
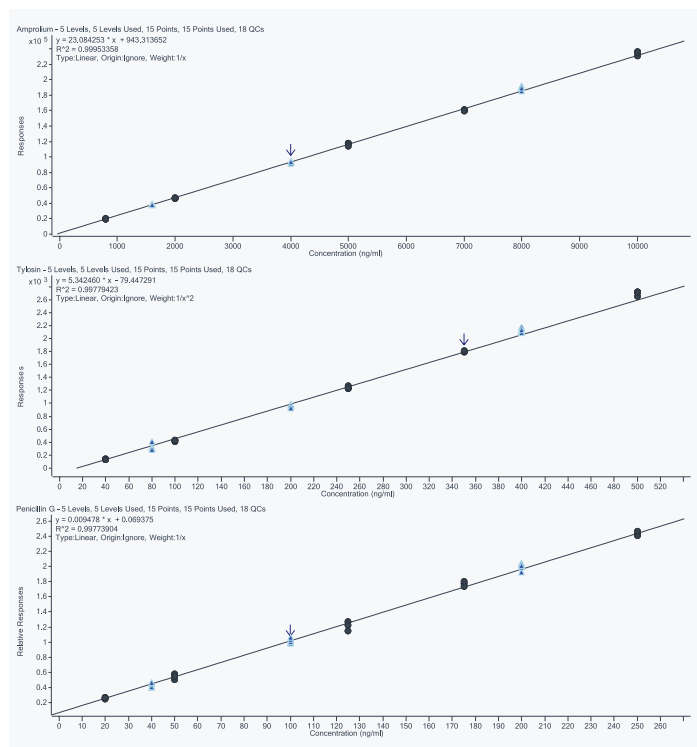


Figure 3: Mass chromatograms for extracted low QC sample.

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Figures 4a-c: Representative calibration curves: amprolium, tylosin, and penicillin G.

The Oasis PRiME HLB cartridges had previously been shown to successfully remove lipids from egg extracts that would otherwise lead to matrix interference [1]. The removal of phospholipids from egg extracts was confirmed in our study by monitoring typical transitions of phospholipids during analysis both of an egg extract that had been passed through the Oasis PRiME HLB cartridge and of an extract, which had not gone through additional SPE cleanup. As can be seen in the overlay comparison in figure 5, the Oasis PRiME HLB removes interfering phospholipids from egg extracts.

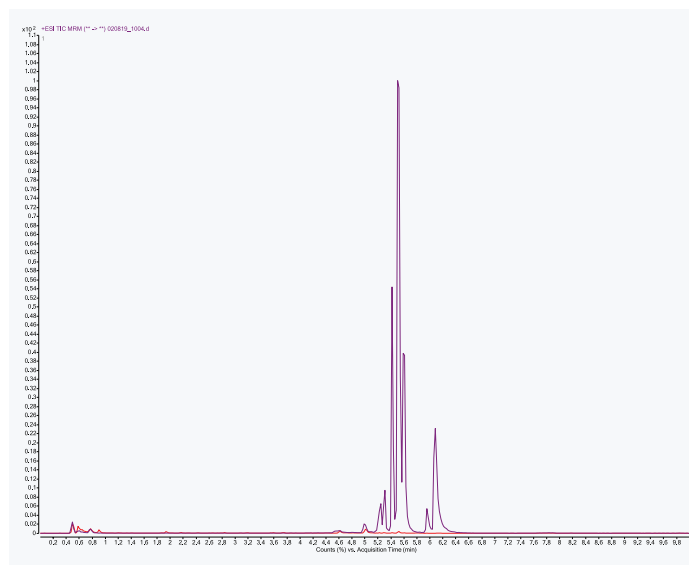


Figure 5: Overlay mass chromatogram results from phospholipid monitoring: PRiME cleanup vs. no PRiME cleanup.

The accuracy and precision of the method were determined for all antibiotics determined in QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for all antibiotic compounds. Accuracy data averaged 102% (range: 94.6% - 115%) and precision data averaged 3.92% RSD (range: 1.23% -11.2%) for all antibiotics analyzed.

Table 2: QC sample accuracy and precision table.

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Prec. [%]	Ave. Acc. [%]
Amprolium	low	1600	1612	1.39	101
	middle	4000	4003	1.28	100
	high	8000	8182	1.23	102
Chlortetracycline	low	160	163	4.37	102
	middle	400	407	2.87	102
	high	800	821	2.75	103
Erythromycin	low	10.0	11.5	11.2	115
	middle	25.0	27.5	3.88	110
	high	50.0	50.0	3.59	100
Tylosin	low	80.0	75.5	10.2	94.6
	middle	200	196	1.94	98.0
	high	400	414	1.58	103
Penicillin G	low	40.0	38.9	7.03	97.3
	middle	100	100	3.14	100
	high	200	204	2.28	102

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Conclusions

As a result of this study, we were able to show:

- Antibiotic compounds in egg samples can be successfully extracted using an automated liquid-liquid extraction procedure with subsequent SPE cleanup and determined using the Agilent Ultivo Triple Quadrupole Mass Spectrometer.
- This method was readily automated using the GERSTEL MPS roboticPRO sampler.
- Evaluation of the method showed the egg extracts to be well mixed within 2.5 min when using the GERSTEL QuickMix Option.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved for the antibiotic compounds analyzed.
- The LLE-SPE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 102% (range: 94.6% - 115%) and precision data averaged 3.92% RSD (range: 1.23% - 11.2%) for all antibiotics analyzed.

References

- [1] *Simple and Effective Cleanup for UPLC-MS/MS Determination of Veterinary Drug Residues in Egg*, Waters Application Note, Retrieved August 2018 from <http://www.waters.com/webassets/cms/library/docs/720005794en.pdf>.

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