Analysis of Peptide Antibiotics in Milk using High Resolution Mass Spectrometry (HRMS)

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ABSTRACT

A liquid chromatography-high resolution mass spectrometry (LC-HRMS) method is described for the quantification and identification of several residual peptide antibiotics in milk. The rapid and effective sample preparation consists of an extraction using dilute formic acid in acetonitrile with a slight amount of trifluoroacetic acid added to improve the peptide recoveries. An aliquot of the extract is evaporated and reconstituted in a formic acid/water-acetonitrile mixture. The reconstituted samples are passed through 0.2 μm PTFE filter to remove the particulates and then analyzed using a Thermo Q-Exactive MS instrument. LC separation is carried out with a higher concentration of formic acid to improve the peak shape and reproducibility of the peptide analytes. Data are collected using a full MS scan followed by all-ion-fragmentation (AIF) acquisition to obtain the exact mass of the precursor and confirmatory product ions. The compounds tested are commonly used peptide antibiotics including colistins (A+B), bacitracin, enramycin A, enramycin B, virginiamycin M1 and virginiamycin S1. Polymyxin B1 is used as internal standard for colistins to correct the process efficiency and matrix interference. The method was validated at four concentration levels ranging from 12.5 to 200 ng/mL in three types of milk (whole milk, raw milk, skim milk). The quantification was performed using a six-point calibration curve of neat solvent standards. The mean recoveries for all analytes at all levels were found within 69 to 138% with relative standard deviations (RSD) less than 24% between days.

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INTRODUCTION

Polypeptide antibiotics, such as colistin, polymyxin, virginiamycin, and bacitracin, have been historically used in food production to improve growth efficiency and for disease prevention.[1-4] Enramycins are approved for use in feed additives as growth promoters in husbandry practices in several countries.[5,6] Virginiamycins are also used to prevent and treat bacterial contamination of commercial fuel ethanol fermentations and may still be present in distiller grains coproducts.[7] These peptide antibiotics are not well incorporated into current regulatory screening because simultaneous determination of multiple peptide antibiotics is relatively difficult due to the differences in their physicochemical properties, for example, the composition of the amino acids, the variety of the functional groups, the helicity (Figure 1), the wide range of molecular weight, and retention similarities (Table 6).

Since the emergence of multi-drug resistant bacteria, many of these antibiotics have been banned for use in animal feed. Colistins, which had been avoided due to high systemic toxicity, are now being re-considered as last-resort for human clinical treatment. Recently, the first mobile resistant gene toward colistins (*mcr*-1) was isolated from swine in China and has since been reported in more than thirty countries.[8] It has been suggested that veterinary use has probably accelerated the dissemination of the bacterial resistance gene in animals and, subsequently humans.[9,10] Therefore, methods to detect and screen these peptide antibiotics in human food or animal feeds should be developed and incorporated into the current surveillance program.

A few methods based on LC-MS/MS have been published for the detection and monitoring of specific peptide antibiotics, such as colistins and bacitracin, in milk or animal tissues.[1-4] These early methods required the use of strong acids or lengthy extraction procedures and resulted in low recoveries of the targeted analytes. Kaufmann *et al* reported a multi-residue method for the detection of bacitracin, colistins, and polymyxins in a variety of food matrices.[11] This method adopted the use of trifluoroacetic acid in the mobile phase to improve the chromatographic separation for the peptide analytes. Boison *et al* also published a method for the detection of seven polypeptide antibiotics simultaneously in chicken tissue without the need of trifluoroacetic acid in the mobile phase, but it required laborious sample extraction.[12] Recently, Tao *et al* developed a method that involved the use of matrix solid-phase dispersion in the sample preparation for the quantification of bacitracin, colistins, and virginiamycins in animal feed.[13] Fu *et al* published a confirmatory method for the determination of colistins in several food commodities.[14] These methods were based on LC coupled with either a triple quadrupole or an ion-trap mass spectrometry instrument, acquiring data in multiple reaction monitoring (MRM) mode. The corresponding transitions and collision energy needed to be optimized individually based on the instrument capability. However, polypeptides have been shown to form multiple charge-states using electrospray. HRMS has been successfully used for contaminants screening in food products.[15,16] Because HRMS has the potential to collect full-scan data rather than preselected ion transitions, different precursor adducts and charge-states of the peptides could be evaluated during method development.

This LIB describes a simple and rapid analysis method with minimum sample preparation to detect and quantify a wide range of residual peptide antibiotics in milk based on LC-HRMS with the capability to monitor several adducts and charge-states of the peptide analytes concurrently.

EXPERIMENTAL

Sources of Milk

Organic whole and skim milk were purchased from local retail establishments. Raw milk was obtained from the FDA Center for Veterinary Medicine/Office of Research.

Chemicals and Reagents

(a) Analyte standards- Standards of bacitracin and colistin sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Enramycin A, enramycin B, virginiamycin M1, and virginiamycin S1 were purchased from LKT Laboratories, Inc. (St. Paul, MN). Internal standard, polymyxin B1 (95%), was purchased from Cayman Chemical (Ann Arbor, MI).

Individual stock solutions- Bacitracin, virginiamycin M1, and virginiamycin S1 were prepared in methanol. Enramycin A and enramycin B were dissolved in 0.1% formic acid (v/v). Colistin and polymyxin B1 were made in 0.1% formic acid in methanol (v/v). All stock standard solutions were made at a concentration of approximately 100 µg/mL (corrected for purity and the counter ion).

Colistin contains two major components, colistin A and colistin B. The percentages of colistin A and B in the reference substance used in this study were estimated by the absolute intensity ratio of their MS signal, and were determined to be 40% A and 60% B.

Working solutions

1) Spiking stock solution contains bacitracin (2000 ng/mL), colistin (A+B) (2000 ng/mL), enramycin A (2000 ng/mL), enramycin B (2000 ng/mL), virginiamycin M1(1000 ng/mL), and virginiamycin S1 (1000 ng/mL). Spiking stock solution was prepared by combining individual stock solutions into a 15 mL polypropylene tube and diluting to the 10 mL mark with 0.2% formic acid in 25 % acetonitrile (v/v).

2) Internal standard (ISTD) spiking stock solution, polymyxin B1 (2000 ng/mL), was prepared by diluting the stock solution into 10 mL 0.2% formic acid in 25 % acetonitrile (v/v).

3) Solvent calibration standards: A set of six calibration solutions were prepared daily by diluting spiking stock solution and ISTD spiking solution into 1 mL 0.2% formic acid in 10 % acetonitrile (v/v).

Table 1. Preparation of solvent calibration standards

Solvent standards	Spiking stock (µL)	ISTD spiking stock (µL)	Final volume (µL)
Level 0	0	25	1000
Level 1	6.25	25	1000
Level 2	12.5	25	1000
Level 3	25	25	1000
Level 4	50	25	1000
Level 5	100	25	1000

Table 2. Concentration of peptide analytes in the individual solvent calibration standard

Analyte	Concentration (ng/mL)					
Calibration standard	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
Bacitracin	0	12.5	25	50	100	200
Colistin (A+ B)	0	12.5	25	50	100	200
Polymyxin B1 (ISTD)	50	50	50	50	50	50
Enramycin A	0	12.5	25	50	100	200
Enramycin B	0	12.5	25	50	100	200
Virginiamycin M1	0	6.25	12.5	25	50	100
Virginiamycin S1	0	6.25	12.5	25	50	100

(b) Solvent, reagents, and mobile phase solutions- LC-MS Optima™ grade acetonitrile, water, methanol, formic acid, and trifluoroacetic acid were purchased from Fisher Scientific (Waltham, MA).

1% Trifluoroacetic acid (TFA)- 1 mL concentrated trifluoroacetic acid diluted in water to a final volume of 100 mL.

Extraction solution- 30 mL 1% TFA and 1.5 mL concentrated formic acid are combined in acetonitrile to a final volume of 500 mL.

0.2% formic acid in 25% acetonitrile- 1 mL concentrated formic acid and 125 mL acetonitrile are combined and diluted to 500 mL with water.

0.2% formic acid in 10% acetonitrile- 1 mL concentrated formic acid and 50 mL acetonitrile are combined and diluted to 500 mL with water.

Mobile Phase A- 50 mL acetonitrile and 3 mL concentrated formic acid are diluting into to 1 L with water.

Mobile Phase B- 50 mL water and 3 mL concentrated formic acid are diluting to 1 L with acetonitrile.

Equipment (Equivalent equipment may be substituted)

1. Centrifuge - Thermo Scientific Sorvall RC6+ programmable refrigerated centrifuge capable of speeds of 10,000 rpm or 17,000 RCF(g).

2. Mechanical shaker - multi-tube vortex mixer (Part #02-215-450, Fisher Scientific, Pittsburgh, PA).

3. Sonicator- BRANSON 2000.

4. Vortexer - Vortex Genie 2 (Scientific Industries, Bohemia, NY).

5. Nitrogen evaporator- TurboVap LV heated to 40 °C (Biotage, Charlotte, NC). Evaporation tubes- 15 mL polypropylene tubes.

6. Syringe filters - Acrodisc CR® 13mm, PTFE 0.2 µm (PALL, Port Washington, NY).

Sample Extraction

1. Measure 2 mL of milk into a 50 mL polypropylene tube.

2. Add spiking stock solutions as appropriate. (Spiking stock solution: 2000 ng/mL of Bacitracin, Colistin (A+B), Enramycin A, Enramycin B; 1000 ng/mL of Virginiamycin M1, Virginiamycin S1.).

3. Add 100 µL of 2000 ng/mL Polymyxin B1 for internal standard at final fortified concentration at 100 ng/ mL.
   For method validation, the milk samples were fortified with four levels of analytes.

Table 3. Fortified levels in milk samples

Fortified Level	Milk (mL)	Spiking stock (µL)	ISTD spiking stock (µL)
1	2.0	25	100
2	2.0	50	100
3	2.0	100	100
4	2.0	200	100

Table 4. Concentration of peptide analytes in the individual fortified level

Analyte	Concentration (ng/mL)			
Fortified level	Level 1	Level 2	Level 3	Level 4
Bacitracin	25	50	100	200
Colistin (A+ B)	25	50	100	200
Polymyxin B1 (ISTD)	100	100	100	100
Enramycin A	25	50	100	200
Enramycin B	25	50	100	200
Virginiamycin M1	12.5	25	50	100
Virginiamycin S1	12.5	25	50	100

4. Wait 10 minutes for the spikes to equilibrate before extracting.

5. Add 8 mL of extraction solution. The extraction solution consists of 0.06% trifluoracetic acid (v/v) and 0.3% formic acid (v/v) in acetonitrile (ACN).

6. Sonicate for 20 minutes.

7. Vortex for 30 minutes. (Fisher Multi-tube vortexer, setting speed 2500 rpm.)

8. Centrifuge the tubes for 10 minutes at 4 °C at 10,000 rpm or 17,000 RCF (g).

9. Transfer 2.5 mL of the extract into 15 mL polypropylene tube.

   Note: If the sample is still cloudy, centrifuge again until the supernatant is clear.

10. Dry extract under a nitrogen stream at 40 °C and 15 psi for around 45 minutes. The volume of the remaining portion of the extract is approximate 100 to 200 µL.

    Note 1: If the temperature is too high (> 50 °C), some peptides might degrade, particularly colistins.

    Note 2: If there is large white aggregate, the recovery of enramycin A and virginiamycin M1 might be lower.

11. Add 0.2% formic acid in 25% ACN (v/v) to the tube to make final volume 1 mL. Cap the tube.

12. Sonicate for 3 minutes and vortex for 2 minutes on the multi-tube vortexer.

13. Centrifuge tubes at 10,000 rpm for 10 minutes at 4 °C.

14. Transfer all the sample to the 1mL syringe and pass through 0.2 µm PTFE filter.

    For method validation, matrix-matched calibrants were prepared for each type of milk. Add appropriate spiking stock and ISTD stock solutions for matrix-matched standards after the filtration.

Table 5. Preparation of matrix-matched standards

Matrix-match Level	Spiking stock (µL)	ISTD spiking stock (µL)	Final volume (µL)
0	0	25	1000
1	6.25	25	1000
2	12.5	25	1000
3	25	25	1000
4	50	25	1000

Instrumentation

The instrument used was a Thermo Q-Exactive HF Orbitrap HRMS with a heated electrospray ionization (HESI) source coupled with a Vanquish Flex LC system. Thermo TraceFinder software 4.1 was used for data acquisition and data analysis.

(a) MS Acquisition parameters

The instrument was calibrated for mass accuracy according to the manufacturer's recommendations at least once a week. The optimized tuning method and parameters used for MS acquisition are described below.

Tune parameters

Sheath gas flow rate:	50 units
Aux gas flow rate:	5 units
Sweep gas flow rate:	0 units
Spray Voltage:	4 kV
Capillary temperature:	350 °C
S-Lens RF level:	50
Aux gas heater temperature:	325 °C
Polarity:	Positive

MS acquisition parameters

Acquisition time:	13 min
Lock mass:	OFF
Chrom peak:	15 s
Full MS	
Resolution:	60 K
AGC:	3e6
Max injection time:	200 ms
Scan range:	150 - 1000 m/z
AIF (MS2)	
Resolution:	60 K
AGC:	3e6
Max injection time:	200 ms
Scan range:	80 - 1000 m/z
Normalized collision energy:	10,30,50

(b) Liquid Chromatography

LC separation was performed using a Phenomenex Kinetex Polar C18 reversed-phase column. The mobile phase consisted of 0.3% formic acid in 5% acetonitrile (v/v) (Mobile phase A) and 0.3 % formic acid in 95% acetonitrile (v/v) (Mobile phase B) at a flow rate of 0.3 mL/min. The LC parameters and gradient program are described below, and MS data were collected for 13 minutes. This was followed by a 4 minutes post-run equilibration with the 95% Mobile phase A.

LC parameters

Column:	Kinetex Polar C18, 2.1 x 50 mm, 2.6 µm
Column temperature:	30 °C
Autosampler temperature:	10 °C
Flow rate:	0.3 mL/min
Injection volume:	10 µL
Post-run equilibration:	4 min

LC gradient program

Time (min)	A (%)	B (%)
0.0	95	5
2.0	95	5

2.5	75	25
5.0	75	25
5.5	70	30
6.5	70	30
8.0	50	50
9.0	50	50
9.5	0	100
12.5	0	100
13.0	95	5

Data Analysis

Quantitation was performed using the Thermo TraceFinder "Quantitative Method". The precursor ions listed in Table 6, including protonated molecules $[M+H]^+$, doubly charged $[M+2H]^{2+}$, triply charged $[M+3H]^{3+}$ protonated molecules, and sodiated molecules $[M+Na]^+$ are added together and integrated for quantification.

(a) TraceFinder Quan detection and confirmation of identity

Based on the criteria from FDA guidance using exact mass data,[17] the precursor ions must be present (signal-to-noise >3) and match theoretical exact mass within a 5 ppm mass tolerance. Fragment ion detection is also required with at least 1 product ion; this method used a threshold of 500 count minimum intensity for product ion and the mass accuracy needed to be within 10 ppm. The retention time was set within a time window of 60 s and the isotope match feature was enabled with a 70% fit threshold, 5 ppm mass deviation, and 10 % intensity deviation allowance. The retention times of the test analytes, along with exact masses of precursor and product ions, are listed in Table 6.

Table 6. Peptide analytes, precursor ions for quantitation, retention time, and product ions

Analyte	**Molecular Formula**	**RT (min)**	**Precursor ions [m/z]**	**Product ions [m/z]**
Bacitracin A	$C_{66}H_{103}N_{17}O_{16}S$	5.0	474.9236 $[M+3H]^{3+}$ 711.8817 $[M+2H]^{2+}$	199.0898/227.0850/669.3367
Colistin A	$C_{53}H_{100}N_{16}O_{13}$	4.0	390.5958 $[M+3H]^{3+}$ 585.3901 $[M+2H]^{2+}$	101.0709/241.1911
Colistin B	$C_{52}H_{98}N_{16}O_{13}$	3.9	385.9239 $[M+3H]^{3+}$ 578.3822 $[M+2H]^{2+}$	101.0709/227.1754
Polymyxin B1	$C_{56}H_{98}N_{16}O_{13}$	4.1	401.9239 $[M+3H]^{3+}$ 602.3822 $[M+2H]^{2+}$	101.0709/241.1911
Enramycin A	$C_{107}H_{138}Cl_2N_{26}O_{31}$	4.9	785.3206 $[M+3H]^{3+}$	179.1430
Enramycin B	$C_{108}H_{140}Cl_2N_{26}O_{31}$	5.5	789.9924 $[M+3H]^{3+}$	193.1587
Virginiamycin M1	$C_{28}H_{35}N_3O_7$	8.5	526.2548 $[M+H]^+$ 548.2367 $[M+Na]^+$	337.1170/508.2442 287.0638
Virginiamycin S1	$C_{43}H_{49}N_7O_{10}$	9.8	824.3614 $[M+H]^+$ 846.3433 $[M+Na]^+$	205.0608/290.1135 725.3269

(b) TraceFinder Quan calibration

A set of six-point solvent standards were prepared for calibration and a weighted linear regression (weighted factor 1/x, not forced origin) was used to fit the calibration curve. Concentration of bacitracin, enramycin A, enramycin B, virginiamycin M1, and virginiamycin S1 were calculated with external calibration. Polymyxin B1 was used as internal standard for colistins to compensate for the extraction and separation efficiency of colistins. The composition percentage of colistin A and B in the reference substance colistin (A+B) has been estimated to be 40% and 60% respectively. For example, at the fortified level of 25 ng/mL colistin, there would be 10 ng/mL colistin A and 15 ng/mL colistin B.

The extraction resulted in a 2-fold dilution factor; therefore, a sample fortified at 100 ng/ mL in milk matrices will produce an extract with an equivalent concentration of 50 ng/ mL in the LC vial.

Method Validation

(a) Matrix effect

A set of five-point matrix-matched standards were prepared for each type of milk (whole milk, skim milk, raw milk) to evaluate the matrix effect. The concentration of the matrix-matched standards was estimated using the solvent standard calibration curve and the matrix effect (ME) was calculated by the ratio compared to concentration of solvent standard.

$$\%ME = \frac{Analyte\ concentration\ found_{\text{matrix-matched standard}}}{Analyte\ concentration_{\text{solvent standard}}}\ x\ 100\%$$

The efficiency of the extraction process (RE) for colistins without polymyxin B1 internal standard were calculated based on the method by Matuszewski *et al*.[18]

$$\%RE = \frac{Analyte\ peak\ area\ _{\text{fortified sample}}}{Analyte\ peak\ area\ _{\text{matrix-matched standard}}}\ x\ 100\%$$

(b) Method performance

Three sources of milk (whole milk, skim milk, raw milk) were fortified at four levels with the peptide analytes. Method accuracy and precision were expressed as analyte recovery (%) and relative standard deviation (% RSD). Table 7 shows the number of fortified samples performed at each level for each type of milk meeting FDA OFVM Chemical Method Validation Guidelines[19] for Level Two quantitative validation. Method accuracy was determined by calculating the recoveries of analytes based on the calibration of neat solvent standards. For intra-day analysis the recoveries were determined from n ≥3 replicates, and inter-day recoveries were determined from data collected over at least 3 days (n ≥9). The method detection limit (MDL) was calculated using the standard deviation at the lowest fortification level multiplied by the student's t-value at the 99% confidence level.

Table 7. Number of validation samples

	Whole Milk	Skim Milk	Raw Milk	Total
Controls	4	6	5	15
Fortified Samples				
Level 1	20	20	25	65
Level 2	10	15	14	39
Level 3	10	15	12	37
Level 4	10	15[a]	12	37

Experiments done over 3 or more days

[a] one outlier of virginiamycin M1 was removed from analysis.

RESULTS AND DISCUSSION

Optimization of Liquid Chromatography Separation

Colistins and polymyxins are historically challenging to separate chromatographically due to the presence of terminal amine groups. These analytes exhibit considerable adsorption affinity with the stationary phase or silica surface during LC separation and significant peak tailing to the extent of peak disappearance was observed. Therefore, polymyxin B1 was chosen as internal standard (ISTD) for colistins as it has similar structure and chemical properties to minimize the variation in process efficiency, matrix interference, and chromatographic separation performance. The use of acidified mobile phases, such as a higher percentage of formic acid or the addition of trifluoracetic acid, have been reported to effectively reduce peak-broadening by decreasing the interaction between analytes and the column.[11,13] However, the addition of the trifluoracetic acid as an ion-pairing agent causes significant ion suppression and reduced the signals in the mass spectrometer. To optimize the chromatographic performance, mobile phases with a range of concentrations of formic acid and trifluoroacetic acid were tested. The addition of 0.3% formic acid provided the best balance of peak resolution, peak shape and ionization efficiency. In addition, several types of LC columns from different vendors were investigated for separation performance and consistency. LC columns with conventional fully porous silica-based support showed irreversible interaction with some analytes, such as colistins and polymyxins. A core-shell reversed-phase Kinetex Polar C18 column demonstrated consistency and ruggedness for analytes performance and was selected for this study.

Optimization of Extraction Method

Previous methods reported for peptides extraction from food commodities required the use of strong or highly concentrated acids followed by extensive sample clean-up or pre-concentration steps.[1,3,11,12] In this LIB, the extraction method was optimized to provide an easy and fast procedure for peptides representing several different chemical classes.

1) Extraction solvent- Acetonitrile or methanol with added acids (including formic acid, trichloroacetic acid, or acetic acid) or base (ammonia) were reported in the literature for the extraction of several type of peptide analytes. Acetonitrile was chosen over methanol since it

demonstrated better protein precipitation efficiency in the milk. Various concentrations of acetonitrile in combination with different type of acids or base were carried out for investigation. Higher recoveries of peptide analytes were achieved using an acidic acetonitrile extraction with a higher concentration of formic acid. Instead of using trifluoroacetic acid in the mobile phase, the addition of trifluoracetic acid in the extraction solvent was found to improve the recoveries of the peptide analytes. The addition of a slight amount of trifluoracetic acid helped to achieve the best recoveries of the peptide analytes while not interfering with the MS analysis. The final extraction solvent chosen was acetonitrile with 0.3% formic acid (v/v) and 0.06% trifluoroacetic acid (v/v).

2) Cleanup technique- Several types of solid phase extraction columns were evaluated, including the traditional Waters Oasis® HLBs and the new versions with lipid removal capacities, Oasis PRiME® HLB and Agilent EMR Captiva®. For the traditional HLBs, the structural differences of the various peptide analytes required extensive modification of the conditioning, washing, and elution steps. For the newer one-step pass-through SPE columns, the lipid-like tail or cyclic-shape structure of the peptides caused significant adsorption and resulted in low recoveries. Therefore, other cleanup techniques such as molecular-weight cut-off membranes or simple filtration with different type of membranes were explored prior to MS analysis, and are described below.

3) Extract Evaporation and Reconstitute- The extract contains high organic solvent content and is not suitable for direct LC analysis on reversed-phase columns. An aliquoted amount of the extract was evaporated and reconstituted in different volumes of dissolution solution for comparison. Dissolution solution with a higher percentage of acetonitrile showed higher analyte recoveries but interfered with reversed-phase LC separation. The final dissolution used was 0.2% formic acid in 25% acetonitrile (v/v). There was a 2-fold dilution of concentration of the sample through the extraction and reconstitution procedure that resulted in the best sample recoveries. In addition, it was noted that several of the peptide analytes degraded if higher temperature (>50 °C) were used during evaporation.

4) Final Extract Preparation- PVDF, PES, Nylon, and PTFE membranes were evaluated for use in filtering the final extract before LC injection. Colistins were bound to the PVDF membrane with minimal recovery of these compounds after filtration. PES and Nylon membranes also demonstrated inconsistency with the loss of peptide analytes to a certain degree. PTFE membrane showed the best recoveries and consistency for all peptide analytes.

The use of molecular-weight cut-off filters (3k Da, 10k Da, and 30k Da) was also investigated in this study. Molecular-weight cut-off membranes have been used previously in our lab for the detection of multi-residue antibiotics in milk and demonstrated efficient removal of proteins and lipid interferents.[20] However, the molecular weight of peptide analytes in this study ranged from 500 Da to 3500 Da, and the recoveries after the 3k Da cut-off filtration was extremely low. Most analyte recoveries from the 10k Da filter were around 20 to 50% compared to the PTFE filter (Appendix 1). The recoveries of the 30k Da cut-off filter were about the same level as the PTFE filtration. Therefore, filtration with PTFE membrane was selected due to the simplicity with no need for further centrifugation.

The optimized extraction method involves only one-step extraction followed with a simple syringe filtration clean-up. In previous methods, multiple extractions were performed which could result

in large sample volume (20 ml to 60 ml) for SPE clean-up.[11,12,14] Boison *et al* estimated to analyze thirty samples in a day limited by the size of the vacuum manifold.[12] In this LIB, the sample processing time is reduced to approximate two hours and the sample throughout is significantly improved since the equipment required for the sample preparation is minimized and the steps for SPE columns are skipped.

Data Acquisition

The data were collected using a high-resolution quadrupole-orbitrap instrument which provides accurate mass measurements for precursor and product ions as well as their isotopic ions. The data were acquired in a full-scan MS1 in combination with all-ion-fragmentation (AIF) MS2 mode. Using the HRMS, different adducts and charged-states of the polypeptides, such as $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$ or $[M+Na]^+$ ions, can be monitored simultaneously for enhanced detection. For bacitracin and colistins, the sensitivity was 22% higher when both doubly and triply charged-states were monitored; and for virginiamycins, the intensity was at least doubled if signals from the sodiated and protonated molecules were combined.

Method Evaluation

1) Matrix Effect- Matrix effects were evaluated for each peptide analyte in the individual milk types by comparing the concentration found in matrix-matched standards to the solvent standards (Table 8). Bacitracin, enramycin A, and enramycin B exhibit nearly no matrix effects (86 to 94%) in any of the milk sources tested.

Matrix effects for colistins were initially evaluated without the correction of ISTD and significant matrix enhancement was observed. Colistin A has 137 to 147 % and colistin B has 157 to 172% enhancement in the milk matrices (data not shown). The extraction process efficiency (absolute recovery) for colistin A was 42 to 51 % and 34 to 46% for colistin B (Appendix 2). Polymyxin B1 which is structurally related to colistins also performed similarly in terms of matrix effect (130 to 132%) and extraction efficiency (48 to 61%). Therefore, polymyxin B1 was recommend for use as an ISTD to correct for the variation in matrix interference as well as process efficiency. However, the matrix effect is still relatively high in the raw milk (110 to 139%).

In general, virginiamycins favor the formation of sodiated precursor ions in the milk matrices compared to the dominant protonated molecules in neat solvent standards due to the presence of sodium in milk. The propensity for the formation of protonated or sodiated molecules still varies based on the structures of the individual virginiamycin. For virginiamycin M1, the abundancy of protonated ions is approximate 3-fold higher compared to the sodiated ions in the solvent standards. However, in the matrix-matched standards, the tendency for sodiated ions is preferred. Therefore, if the quantification was based on only protonated precursor ions, the matrix suppression was observed to be around 60% since more virginiamycin M1 ions were in the sodiated form. Using HRMS, the protonated and sodiated precursor ions could be monitored simultaneously to compensate for the different adducts. When the two adducts for virginiamycin M1 were combined, the matrix effect was not significant in the skim milk and whole milk, but was slightly higher in the raw milk (128%). A similar trend was observed for virginiamycin S1, with approximately 1.5-fold more protonated ions in the solvent standards with a preference for sodiated ions in the matrix. While combined, a slight matrix enhancement was observed in the all milk sources (104 to 120%).

Table 8. Matrix effects for peptide antibiotics in milk

Analyte	Skim Milk	Whole Milk	Raw Milk
Analyte	ME (%)	ME (%)	ME (%)
Bacitracin	88	90	93
Colistin A	115	106	110
Colistin B	122	124	139
Enramycin A	92	87	86
Enramycin B	90	87	94
Virginiamycin M1	85	90	128
Virginiamycin S1	110	104	120

If ME (%)=100 no matrix effect is present, if ME(%)>100 there is a signal enhancement and if ME (%)<100 there is a signal suppression.

2) Method validation- Fortified samples were run at four different levels for each type of milk to evaluate the method performance. Figure 2 shows extracted ion chromatograms for combined precursor ions in blank whole milk, solvent standard (50 ng/mL) and whole milk fortified at 100 ng/mL. A six-point solvent standard curve was injected with each analytical batch and used for quantitation. A five-point matrix-matched standards was also injected with each analytical batch for the evaluation of matrix effects and the calculation of matrix-matched recoveries. Accuracy and precision results from the validation are summarized in Table 9 for inter-day and intra-day analysis for each source of milk. Method accuracy (trueness) was determined by calculating the recoveries of analytes in each milk from a solvent standard calibration curve ($R^2 > 0.99$). Method detection limits were evaluated by analyzing twenty to twenty-five replicates fortified at the lowest level. The results demonstrated the method accuracy was satisfactory for most analytes and milk types, according to FDA OFVM guidance.[19] Average recoveries for bacitracin, enramycin A and enramycin B in each milk ranged from 69 to 85 % at the lowest fortified level and 81 to 119 % at higher fortified levels.

The percentage of colistin A to colistin B was estimated at 40:60 in the colistin reference standard used in the study. Therefore, the lowest fortified level of colistin A was 10 ng/mL and colistin B was 15 ng/mL, which produced 5 ng/mL and 7.5 ng/mL after extraction, accounting for the dilution factor. With the correction using ISTD, colistin A performed consistently at all fortified levels in three milk sources (81 to 113%, inter-day RSD 3 to 14%). The recoveries of colistin B were 81 to 107% (RSD 3 to15%) in the skim and whole milk. The recoveries of colistin B in raw milk showed higher variability (RSD 24 to 26%) at lower fortified levels from data obtained over multiple days.

Virginiamycin M1 and virginiamycin S1 exhibited higher signal in the mass spectrometry and thus the fortified concentration was half that for the rest of the analytes; for example, 12.5 ng/ mL for level 1. Virginiamycin M1 and S1 preferably formed sodiated precursor ions in milk and were found to have broader variability in accuracy over multiple days. The variation could be attributed to the preferential formation of the sodiated precursor ions in certain circumstances. For example, the presence of the residual sodium ions on the LC column that accumulated during the sample runs. The mean recoveries of virginiamycin M1 ranged from 74 to 124% (RSD 8 to 24%) in the milk matrices. Virginiamycin S1 gave consistently high recoveries (119-138%, RSD 3-14%) compared to solvent standard in the matrices, indicating ‘matrix signal enhancement’ might occur especially when monitoring sodiated molecules as well.

The matrix-matched recoveries were evaluated to justify if matrix-matched calibration was necessary (Table 10). The data showed that for most of the analytes, the compensation using matrix-matched calibration did not provide significant improvement of accuracies (recoveries). However, for the virginiamycins which generate sodiated precursor ions in the milk, the use of matrix-matched calibration corrected variance of sodium and demonstrated improved accuracy in quantification. When considering the need for fast quantitative applications in a regulatory laboratory, solvent-standard calibration is recommended for use in quantification.

In addition to the evaluating quantitative method performance, the results were also examined to determine if the criteria for confirmation of identity using HRMS data[17] were met for the peptide analytes using MS1 and AIF MS2 acquisition. In addition to demonstrating precursor ion mass accuracy (within 5 ppm) and retention time matching (within 0.5 min), at least one fragment ion needed to be detected with a mass accuracy within 10 ppm. Figure 3 shows the theoretical product ions for each analyte and the AIF MS2 spectra in a whole milk sample fortified at 100 ng/mL. Because AIF data acquisition does not isolate precursor ions prior to forming product ions, the resulting spectra in matrix can be quite complex. The TraceFinder software, however, is able to determine if the corresponding product ions are present at an adequate signal (500 counts) and mass accuracy (10 ppm) within the window of the retention time of the precursor ions. Product ions were occasionally detected in the negative control samples, particularly for colistins. However, the peak abundance was very low and none of the precursor ions have matched the isotope patterns. Using these criteria, at least one product ion was detected for each peptide at all levels in the fortified milk samples, indicating that all fortified milk samples met the criteria for peptide identifications.

CONCLUSION

The objective of this study was to develop a sensitive and rapid quantification method for the simultaneous determination of several peptide analytes in milk. The extraction method was optimized to provide a simple procedure with peptides representing several structures. The LC separation was optimized with a higher concentration of formic acid as mobile phase to decrease the undesirable interaction between the additional amine groups of the peptide analytes and the stationary phase of the LC column to improve the peak shape and separation. Polymyxin B1 was chosen as internal standard for colistins to minimize the variation during method performance. A high-resolution mass spectrometer, Q-Exactive Orbitrap, was employed to monitor multiple-charged peptide analytes to achieve enhanced detection. The method was validated for three milk sources (whole, skim, raw milk) with four fortified concentrations to encompass potential targeted testing level and evaluated for fast quantitative and confirmatory applications to support routine regulatory compliance.

Table 9. Method accuracy[a] and detection limits for inter-day[b] (intra-day)[c] in milk

		Trueness (% Recovery) ± % RSD		
Analyte	Fortified level (ng/mL)	Skim Milk	Whole Milk	Raw Milk
Bacitracin	25	80 ± 12 (73 ± 8)	76 ± 10 (81 ± 5)	85 ± 9 (86 ± 2)
	50	87 ± 6 (86 ± 1)	86 ± 7 (89 ± 3)	90 ± 5 (94 ± 4)
	100	91 ± 4 (90 ± 3)	95 ± 2 (94 ± 4)	94 ± 5 (96 ± 4)
	200	93 ± 3 (93 ± 2)	96 ± 4 (96 ± 5)	96 ± 4 (94 ± 4)
	MDL (ng/mL)	2.96	2.41	2.37
Colistin A	10	95 ± 6 (95 ± 6)	81 ± 5 (82 ± 4)	98 ± 14 (124 ± 4)
	20	103 ± 8 (101 ± 3)	88 ± 2 (88 ± 3)	98 ± 10 (113 ± 1)
	40	109 ± 10 (109 ± 1)	93 ± 2 (91 ± 1)	98 ± 9 (109 ± 4)
	80	113 ± 12 (110 ± 2)	97 ± 3 (94 ± 1)	100 ± 7 (109 ± 4)
	MDL (ng/mL)	0.71	0.52	1.73
Colistin B	15	89 ± 10 (88 ± 4)	81 ± 15 (60 ± 8)	112 ± 26 (109 ± 5)
	30	95 ± 12 (91 ± 5)	89 ± 5 (86 ± 2)	100 ± 24 (107 ± 2)
	60	101 ± 13 (96 ± 1)	92 ± 3 (96 ± 1)	107 ± 20 (110 ± 4)
	120	107 ± 14 (98 ± 2)	101 ± 6 (107 ± 1)	100 ± 9 (113 ± 3)
	MDL (ng/mL)	1.78	2.27	5.51
Enramycin A	25	73 ± 21 (65 ± 14)	71 ± 20 (75 ± 8)	69 ± 17 (63 ± 6)
	50	88 ± 13 (83 ± 6)	86 ± 12 (86 ± 5)	81 ± 11 (80 ± 7)
	100	96 ± 9 (96 ± 4)	103 ± 7 (93 ± 3)	87 ± 8 (82 ± 5)
	200	100 ± 5 (100 ± 3)	108 ± 7 (101 ± 5)	90 ± 7 (84 ± 4)
	MDL (ng/mL)	4.78	4.53	3.57
Enramycin B	25	79 ± 11 (67 ± 11)	83 ± 11 (78 ± 4)	79 ± 14 (63 ± 4)
	50	94 ± 11 (83 ± 6)	100 ± 12 (95 ± 4)	87 ± 6 (84 ± 3)
	100	99 ± 10 (90 ± 1)	115 ± 10 (100 ± 4)	96 ± 9 (89 ± 3)
	200	104 ± 9 (99 ± 3)	119 ± 11 (105± 4)	97 ± 6 (91 ± 2)
	MDL (ng/mL)	2.80	3.03	3.57
Virginiamycin M1	12.5	74 ± 20 (78 ± 16)	89 ± 14 (100 ± 8)	121 ± 24 (121 ± 5)
	25	87 ± 10 (95 ± 3)	93 ± 24 (112 ± 12)	122 ± 23 (127 ± 3)
	50	94 ± 8 (100 ± 5)	89 ± 14 (107 ± 7)	124 ± 21 (131 ± 6)
	100	94 ± 20[d] (103 ± 4)	83 ± 21 (101 ± 7)	123 ± 10 (116 ± 2)
	MDL (ng/mL)	2.38	2.00	4.46
Virginiamycin S1	12.5	138 ± 14 (139 ± 12)	132 ± 12 (129 ± 5)	132 ± 10 (120 ± 4)
	25	134 ± 8 (135 ± 1)	126 ± 4 (124 ± 3)	130 ± 10 (121 ± 4)
	50	126 ± 5 (126 ± 3)	124 ± 3 (127 ± 4)	127 ± 9 (126 ± 4)
	100	122 ± 10 (126 ± 3)	119 ± 6 (112 ± 6)	122 ± 8 (113 ± 4)
	MDL (ng/mL)	3.05	2.53	1.95

[a]Method accuracy was based solvent standard calibrations. [b]Inter-day data were collected $n \geq 9$ at each level with experiments conducted more than 3 days. [c]Intra-day data are shown in parentheses, $n \geq 3$ at each level within a day. [d]one outlier was removed from the data set.

Table 10. Matrix-matched recoveries[a] for inter-day[b] (intra-day)[c] in milk

		Trueness (% Recovery) ± % RSD		
Analyte	Fortified level (ng/mL)	Skim Milk	Whole Milk	Raw Milk
Bacitracin	25	96 ± 12 (92 ± 8)	100 ± 6 (98 ± 5)	98 ± 9 (90 ± 2)
	50	98 ± 8 (92 ± 1)	92 ± 10 (100 ± 3)	98 ± 9 (87 ± 4)
	100	97 ± 5 (92 ± 3)	98 ± 4 (102 ± 4)	97 ± 11 (86 ± 4)
	200	100 ± 7 (96 ± 2)	97 ± 7 (103 ± 5)	96 ± 13 (88 ± 4)
Colistin A	10	91 ± 9 (91 ± 6)	89 ± 6 (84 ± 4)	91 ± 7 (88 ± 4)
	20	94 ± 5 (88 ± 3)	87 ± 8 (87 ± 3)	89 ± 5 (83 ± 1)
	40	98 ± 5 (96 ± 1)	97 ± 5 (91 ± 1)	90 ± 7 (83 ± 4)
	80	98 ± 6 (97 ± 2)	91 ± 3 (93 ± 1)	91 ± 8 (88 ± 4)
Colistin B	15	77 ± 13 (78 ± 4)	73 ± 10 (59 ± 8)	77 ± 14 (70 ± 5)
	30	81 ± 9 (74 ± 5)	72 ± 7 (72 ± 2)	73 ± 13 (67 ± 2)
	60	86 ± 6 (84 ± 1)	73 ± 5 (76 ± 1)	75 ± 8 (69 ± 4)
	120	89 ± 9 (83 ± 2)	79 ± 4 (81 ± 1)	77 ± 10 (73 ± 3)
Enramycin A	25	88 ± 13 (76 ± 14)	105 ± 18 (111 ± 8)	95 ± 16 (82 ± 6)
	50	102 ± 12 (86 ± 6)	100 ± 14 (105 ± 5)	90 ± 19 (85 ± 7)
	100	101 ± 10 (88 ± 4)	109 ± 7 (98 ± 3)	102 ± 24 (78 ± 5)
	200	102 ± 6 (93 ± 3)	114 ± 8 (105 ± 5)	95 ± 20 (82 ± 4)
Enramycin B	25	98 ± 19 (76 ± 11)	112 ± 15 (89 ± 4)	105 ± 18 (80 ± 4)
	50	106 ± 21 (87 ± 6)	112 ± 21 (100 ± 4)	94 ± 8 (94 ± 3)
	100	108 ± 13 (91 ± 1)	130 ± 14 (105 ± 4)	97 ± 16 (86 ± 3)
	200	109 ± 11 (97 ± 3)	126 ± 13 (107± 4)	97 ± 7 (92 ± 2)
Virginiamycin M1	12.5	100 ± 22 (89 ± 16)	109 ± 15 (95 ± 8)	97 ± 19 (94 ± 5)
	25	107 ± 13 (100 ± 3)	103 ± 13 (108 ± 12)	109 ± 28 (94 ± 3)
	50	105 ± 9 (107 ± 5)	100 ± 11 (102 ± 7)	94 ± 23 (91 ± 6)
	100	98 ± 23 (105 ± 4)	91 ± 15 (96 ± 7)	91 ± 15 (85 ± 2)
Virginiamycin S1	12.5	109 ± 10 (113 ± 12)	114 ± 15 (96 ± 5)	105 ± 8 (96 ± 4)
	25	109 ± 5 (112 ± 1)	106 ± 7 (101 ± 3)	96 ± 7 (98 ± 4)
	50	107 ± 5 (110 ± 3)	109 ± 3 (108 ± 4)	107 ± 5 (105 ± 4)
	100	110 ± 4 (116 ± 3)	113 ± 7 (111 ± 6)	107 ± 6 (102 ± 4)

[a]Matrix-matched recoveries were calculated by the concentration determined in the fortified samples compared to the concentration found in the matrix-matched standards (concentration was calculated based on solvent-standard calibration). [b]Inter-day data were collected n ≥ 9 at each level with experiments conducted more than 3 days. [c]Intra-day data are shown in parentheses, n≥3 at each level within a day.

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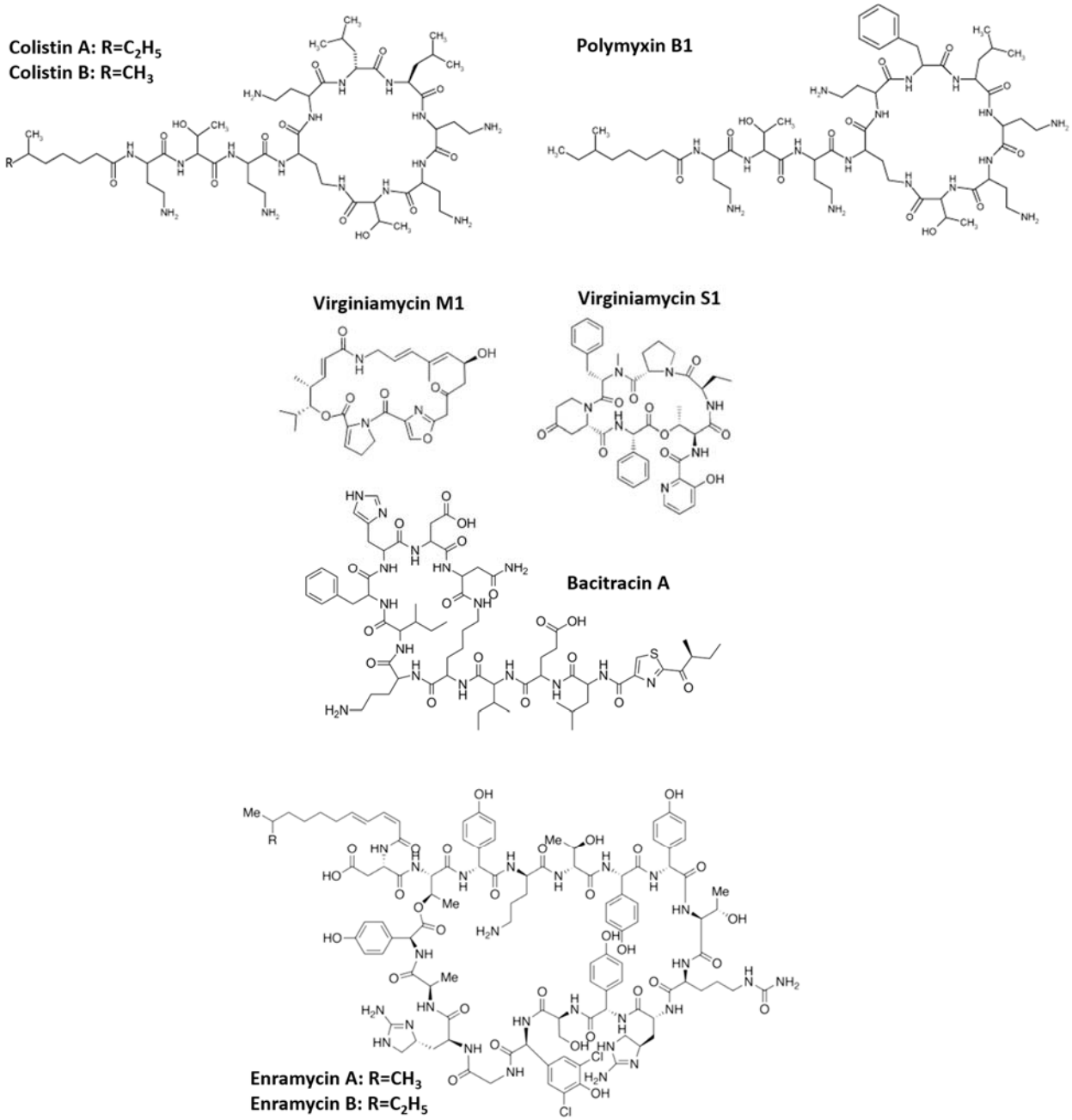


Figure 1. Structures of peptide antibiotics analyzed in this method.

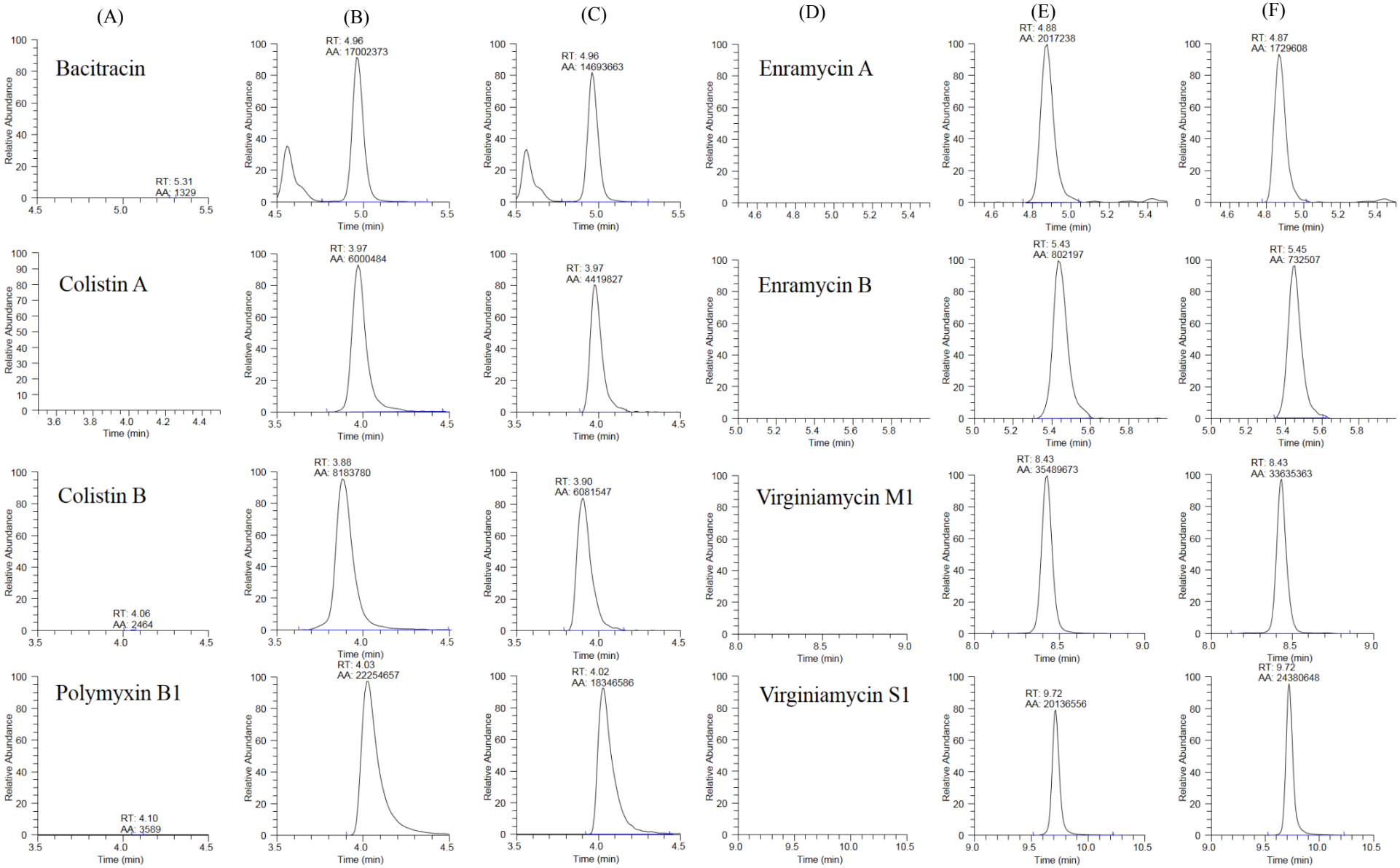
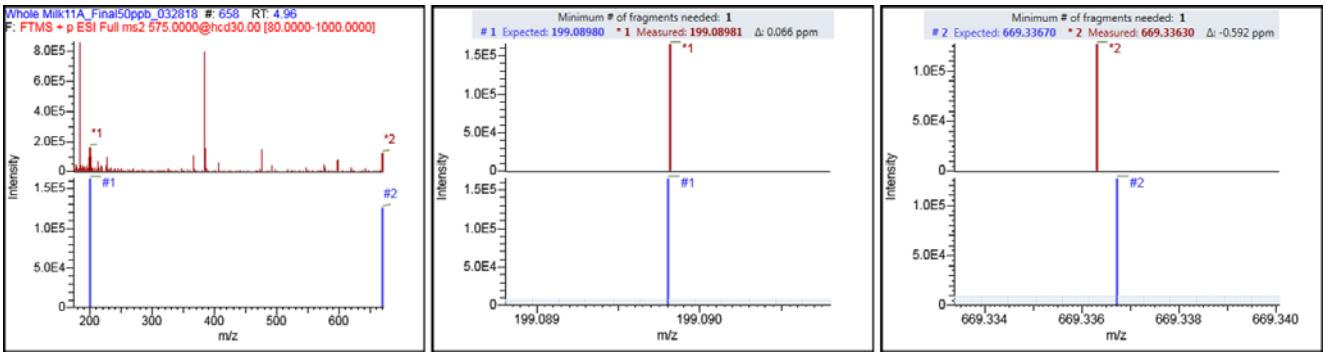
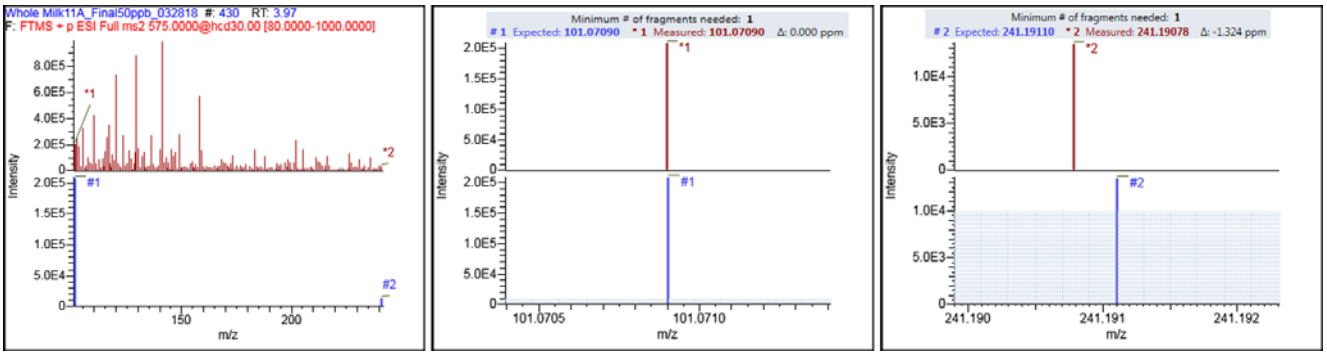


Figure 2. Extract ion chromatogram of combined MS1 ions (5 ppm window) for peptide analytes from whole milk. (A)(D) matrix blank. (B)(E) solvent standard at 50 ng/mL. (C)(F) fortified at 100 ng/mL in milk (final concentration at 50 ng/mL in vial

Bacitracin



Colistin A



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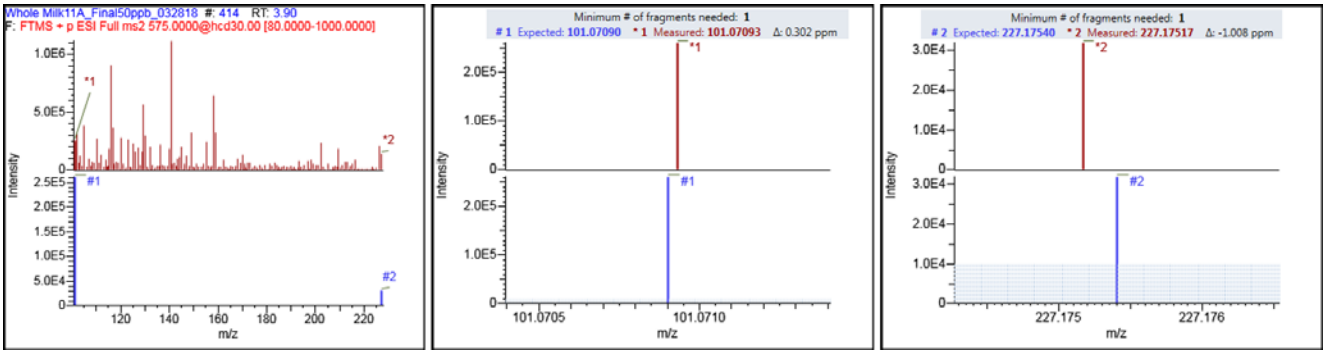


Virginiamycin M1

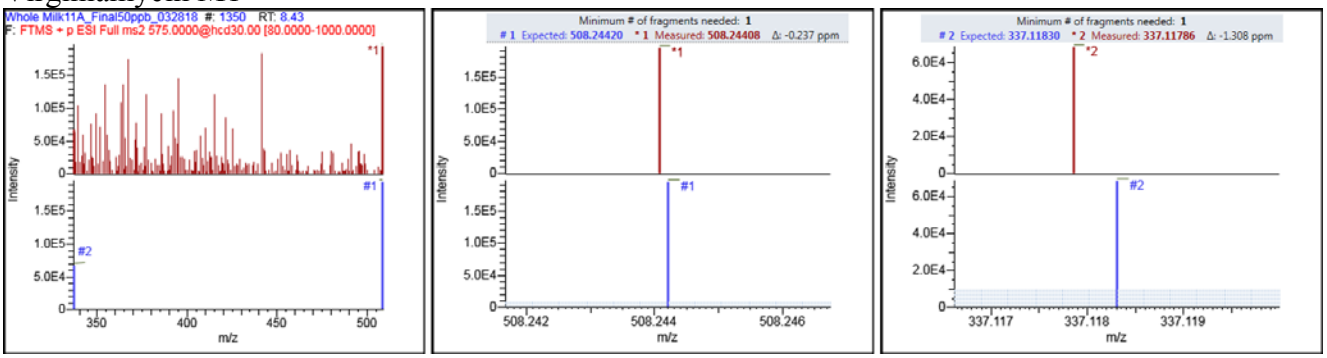
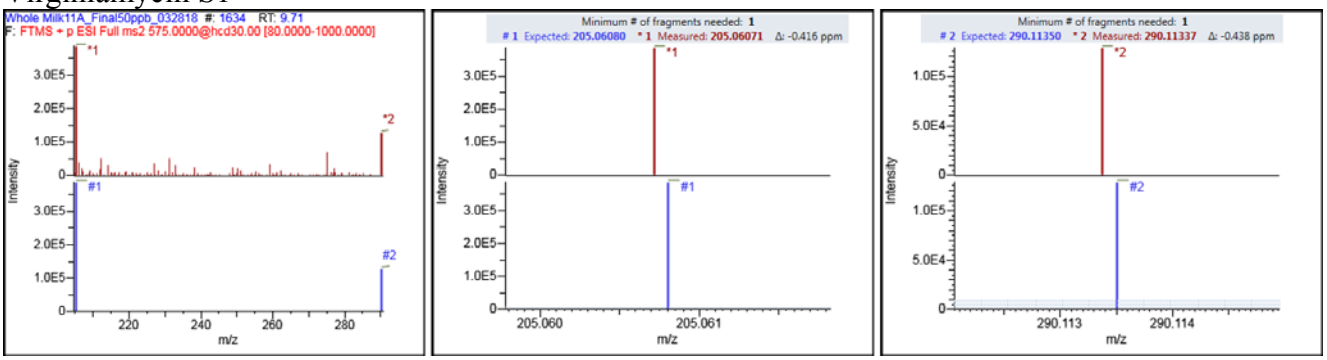
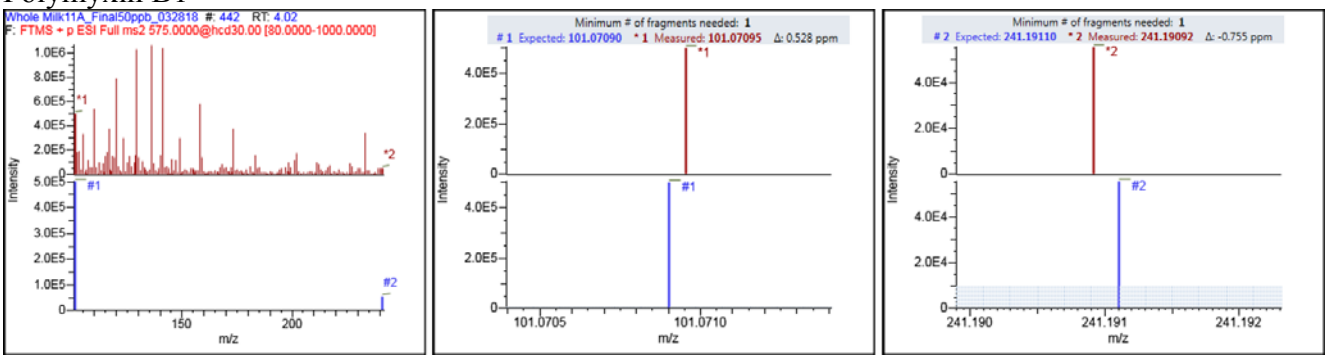


Figure 3. AIF MS2 product ion (top panel, red) compared to theoretical fragment ions in the compound database (bottom panel, blue) for fortified sample at 100 ng/mL in whole milk.

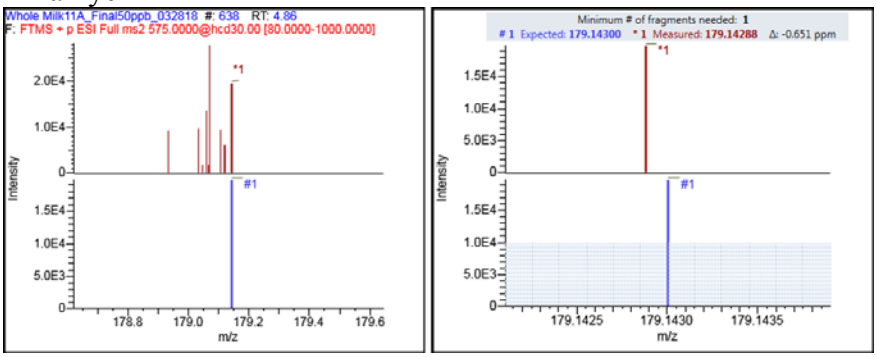
Virginiamycin S1



Polymyxin B1



Enramycin A



Enramycin B

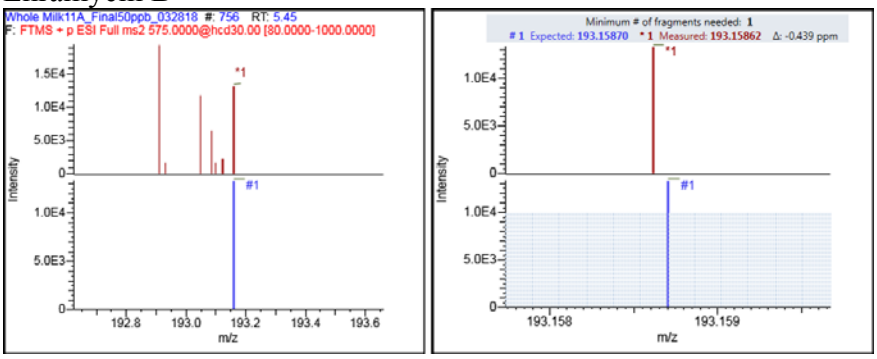


Figure 3. (cont'd) AIF MS2 product ion (top panel, red) compared to theoretical fragment ions in the compound database (bottom panel, blue) for fortified sample at 100 ng/mL in whole milk.

Appendix 1. Peptide analyte recoveries compared to PTFE filtration.[a]

	Recovery compared to PTFE filtration (%)[b]						
Analyte	PTFE	SPE A[c]	SPE B[c]	SPE C[c]	3 kDa	10 kDa	30 kDa
Bacitracin	100	79	75	25	9	50	109
Colistin A	100	89	94	N. D.[d]	11	35	116
Colistin B	100	86	81	N. D.[d]	12	17	114
Polymyxin B1	100	95	97	N. D.[d]	10	39	114
Enramycin A	100	68	53	10	3	21	101
Enramycin B	100	72	50	6	3	20	102
Virginiamycin M1	100	113	72	117	118	145	175
Virginiamycin S1	100	81	56	65	56	101	111

[a]Duplicate of whole milk samples fortified at 100 ng/mL. [b]Recovery from PTFE syringe filtration was set up as 100%. Duplicate of whole milk samples fortified at 100 ng/mL with different clean up procedures were compared to PTFE filtration and displayed as percentage. [c]SPE A: Oasis PRiME® HLB, SPE B: Starta™-X, SPE C: Captiva® EMR-Lipid. [d]Not detected.

Appendix 2. Extraction efficiency of colistins without internal standard.

	Skim milk		Whole milk		Raw milk	
Analyte	Efficiency[a](%)	RSD (%)	Efficiency[a] (%)	RSD (%)	Efficiency [a] (%)	RSD (%)
Colistin A	51	11	55	6	42	8
Colistin B	45	13	46	5	34	9
Polymyxin B1	55	10	61	9	48	5

[a]Extraction efficiency was calculated by the peak areas fortified before extraction to peak areas fortified after extraction into milk extracts. Data were evaluated (n>50) in each type of milk conducted during multiple days.