Optimization and Validation of Multi-class, Multi-residue LC-MS/MS Screening and Confirmation Method for Drug Residues in Milk

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Abstract

This laboratory information bulletin describes the further optimization and validation of a multi-residue veterinary drug screening method for milk. The drug residues of regulatory interest in milk include β-lactams, sulfonamides, tetracyclines, fluoroquinolones, and macrolides. This method is an updated version of LIB #4410 that has been modified to incorporate new compounds and to collect both screening and confirmatory MS information in one acquisition. Milk samples were extracted using the same procedure as before with an equal volume of acetonitrile. The samples were then subjected to clean-up using a bonded solid phase extraction (SPE) cartridge and a molecular weight cut-off filter. The SPE elution protocol was modified to effectively recover a metabolite of flunixin. Established tolerance levels are set for most of these drugs in milk; thus, the screening procedure was semi-quantitative, using positive controls for comparison. Positive controls, consisting of an extracts from milk fortified with the drugs at their tolerance or safe level, were used to set statistically valid minimum response criteria for unknown samples. This updated method was validated with fortified milk, as well as with milk from animals administered veterinary drugs.

Introduction

Recently a multi-class method was developed to monitor for veterinary drug residues in milk (1, 2). In the original procedure, milk samples were first screened for 25 drug residues using one selected reaction monitoring (SRM) precursor to product transition for each compound. If the screen indicated a residue might be present near the level of interest, the extract was reanalyzed using a class-specific method to collect addition SRM transitions. In the current method, the LC-MS/MS parameters have been modified to collect the three SRM transitions for all of the residues in a single analytical run.

The compound list has also been changed to eliminate pirlimycin (standard no longer available) and to include virginiamycin and tilmicosin. In addition, 5- hydroxy flunixin was substituted for the parent compound as a more appropriate marker residue (3). Changing to the hydroxy flunixin metabolite required a modification to the extraction, specifically in the solid phase column elution solvent, to obtain adequate recoveries.

This laboratory information bulletin describes the optimized screening and confirmation method. Complete validation data from fortified samples is presented along with the analysis of incurred milk from cows administered several representative compounds. Variance data from samples fortified at the tolerance for these residues were used to set an appropriate threshold level for presumptive positive samples. The use of automated reporting to obtain information for residue screening and confirmatory status is also discussed. The method was evaluated and validated with an eye towards streamlining the data collection and reporting to meet regulatory requirements, while minimizing instrumental analysis and analyst review time.

The intended purpose of this method is to screen samples to determine if a residue is present at the level of interest and also to confirm the identity of the compound. An exact quantitative determination of any residues is not addressed with this procedure and will need to be obtained using other methodology**.**

Experimental

Equipment

a) *Liquid chromatograph-mass spectrometer*.— Thermo TSQ Quantum triple quadrupole Access mass spectrometer coupled to Thermo Accela LC pump and autosampler. LCQuan *(V 2.5.6)*, Xcalibur Qualbrowser *(V 2.0.7)*, and Xreports *(V 2.0.7)* software was used to obtain and process data (Thermo Corp., San Jose, CA).

b) *LC column*.— YMC ODS-AQ, 120 Å, 2 x 100 mm, 3 micron. (#AQ12S031002WT, Waters Corp., Milford, MA).

c) *Labware*.— 15 mL disposable, conical, graduated, polypropylene tubes with cap (#352097, Becton Dickinson, Franklin Lakes, NJ); Kimax disposable glass tubes, 12 x 75 mm and 16 x 150mm (# 03-341-1 and 03-341-6, respectively, Fisher Scientific, Houston, TX).; polypropylene LC sample vials 2 mL with conical insert (#9301-0978, Agilent, Santa Clara, CA) with pre-scored snap caps (#242775, Wheaton, Millville, NJ).

d) *Centrifuges*.— (1) refrigerated to 5 ºC, capable of accelerating 15 mL tubes to 4,000 rpm (2.730 rcf) and (2) microcentrifuge capable of accelerating 1.5 mL tubes to 13,500 rpm

(17,000 rcf).

e) *SPE cartridge*.— Oasis® HLB 3cc (60 mg) extraction cartridge (#WAT094226, Waters Corp.).

f) *Molecular weight cut-off filters.* — Microcon centrifugal filter device YM-30 (#42410 Millipore Corp., Bedford, MA).

g) *Other laboratory equipment*. — Vortex mixer, SPE manifold, adjustable pipettors, nitrogen evaporator with thermostatted water bath.

Reagents:

a) *Deionized water*.— 18.2 MΩ·cm (Millipore Corp.).

b) *Organic solvents*.— High purity chromatographic and spectrophotometric grade acetonitrile and methanol (EMD Chemicals, Gibbstown, NJ), or equivalent.

c) *Formic acid*.— 96% purity (Sigma Aldrich, St. Louis, MO), or equivalent. Formic acid solution (0.1%) prepared by pipetting 1.0 mL formic acid into a 1000 mL graduated cylinder and diluted to mark with deionized water.

Standard Preparation

The following drug residues are included in this method: ampicillin (AMP), penicillin G (PEN G), cloxacillin (CLOX), cephapirin (CEPH), sulfamethazine (SMZ), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfathiazole (STZ), sulfaquinoxaline (SQX), sulfapyridine (SPD), sulfachloropyridazine (SCP), sulfamerazine (SMR), oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), doxycycline (DC), tylosin (TYL), tilmicosin (TIL), erythromycin (ERY), sarafloxacin (SAR), enrofloxacin (ENR) or ciprofloxacin (CIP), 5-hydroxyflunixin (FLU-OH), bacitracin (BAC), thiabendazole (TBZ), virginiamycin (VIR) and tripelennamine (TRIP). Prepare stock solutions at an approximate concentration of 100 μg mL^{-1} for each residue. This concentration should correspond to the active drug compound, so adjust the amounts weighed to take into account purity and any counter-ions that are present. Standards are obtained from the US Pharma (USP), except for virginiamycin (Sigma Aldrich) and 5-hydroxyflunixin (Schering-Plough, Lafayette, NJ). The solvents used for the stock solutions are listed in Table 1 along with the drug classifications and the tolerance or safe levels for each residue. The stock standard solutions should be prepared every six months, except for BAC, CTC, and the β-lactams, which need to be made every three months. A portion of the β-lactam stock standard solutions can be frozen in separate polypropylene tubes and thawed prior to use. All other stock standard solutions should be stored at 4 ºC.

Prepare two intermediate mixed standard (IMS) solutions. One IMS containing the β -lactam residues at a concentration equivalent to 20 times their tolerance or safe levels should be prepared by adding the amount of stock solution indicated in the second to last column of Table 1 to a 50 mL volumetric flask and bringing to volume with water. Make a second IMS solution with the remaining residues at 20 times tolerance in a similar manner, with the exception that it was brought to the final 50 mL volume with methanol. Store the IMS solutions at 4 ºC. Prepare

the IMS for β-lactams every two weeks. The IMS solution containing the other residues can be made once a month. Prepare solvent standards corresponding to the final concentration of a 1X extract (where X is the concentration at the tolerance or safe level) for instrument system suitability tests by adding aliquots of 50 µL of each IMS standard to 900 µL of 0.1% formic acid.

Table 1. Preparation of Standards

Standard	Class	Tolerance[a] Level (ng mL^{-1})	Stock Std: 100 µg mL^{-1} in	Stock Solution in IMS[b] (µL)	Conc. of Residue in IMS (ng mL^{-1})
Intermediate Mixed Standard #1 (β-lactams)					
AMP	β-lactam	10	water	100	200
CEPH	β-lactam	20	water	200	400
CLOX	β-lactam	10	water	100	200
PEN G	β-lactam	5[c]	water	50	100
Intermediate Mixed Standard #2					
ERY	Macrolide	50[c]	water	500	1000
TYL	Macrolide	50	methanol	500	1000
TIL	Macrolide	None[d]	methanol	1000	2000
ENR or CIP	Fluoroquinolone	None[e]	methanol	50	100
SAR	Fluoroquinolone	None[e]	methanol	50	100
CTC	Tetracycline	300[f]	methanol	1000	2000
OTC	Tetracycline	300[f]	methanol	1000	2000
TC	Tetracycline	300[f]	methanol	1000	2000
DC	Tetracycline	300[f]	methanol	1000	2000
SCP	Sulfonamide[g]	10[c]	methanol	100	200
SDZ	Sulfonamide	10[c]	methanol	100	200
SMR	Sulfonamide	10[c]	methanol	100	200
SDM	Sulfonamide	10	methanol	100	200
SMZ	Sulfonamide	10[c]	methanol	100	200
SPD	Sulfonamide	10[c]	methanol	100	200
SQX	Sulfonamide	10[c]	methanol	100	200
STZ	Sulfonamide	10[c]	methanol	100	200
TRIP	Miscellaneous	20	water	200	400
THBZ	Miscellaneous	50	water	500	1000
FLU-OH	Miscellaneous	2	methanol	20	40
BAC	Miscellaneous	500	methanol/water	5000	10000
VIR	Miscellaneous	None[d]	methanol	1000	2000

[a] Tolerance or safe levels in milk from 9/27/05 FDA/CFSAN Milk Safety Branch memo(4)
[b] IMS = Intermediate Mixed Standard
[c] Amounts listed are "safe level" not a tolerance
[d] No tolerance set for milk; method target level set at 100 ng mL^{-1} (tolerance in muscle)
[e] No tolerance or safe levels have been established; method target levels set at 5 ng mL^{-1}
[f] Tolerance includes sum and individual residues; method target levels set at 100 ng mL^{-1} each
[g] Extra-label use of sulfonamide drugs in lactating dairy cattle (except SDM) is prohibited (4)

Preparation of Milk Samples

Use organic whole milk obtained from a local market as the control matrix. Distribute one (1.0) mL aliquots to 15 mL centrifuge tubes and keep frozen until use. To fortify milk samples with all residues, add 25, 50, or 100 µL of each IMS standard to one (1) mL milk samples to obtain levels of 0.5X, 1X, or 2X the tolerance levels, respectively.

Sample Preparation and Extraction

Fortify samples as needed. Allow the samples to sit for approximately 10 minutes. To extract the drug residues and precipitate the milk proteins, add one mL of acetonitrile (ACN) to 1 mL of milk in a polypropylene centrifuge tube; then vortex-mix tube for 10-15 sec. Centrifuge the samples at 4000 rpm (4 ºC) for 10 min. Transfer, using an automatic pipettor, one mL of the resulting supernatant (avoiding any visible fat layer) to a 16 x 150 mm disposable glass tube containing 9 mL of 0.1% formic acid. Vortex-mix the tube at a medium speed for 10 sec. Condition an OASIS® HLB SPE column with 3 mL of ACN followed by 3 mL of a 95:5 0.1% formic:ACN solution using vacuum or gravity flow. Using gravity flow, apply the sample extracts to the SPE cartridges. Wash each glass tube with 2 mL of 0.1% formic acid solution and add this wash to the cartridge after all the sample extract has eluted. Wash the cartridge with an additional 2 mL of the 0.1% formic acid solution. Apply vacuum (or positive air pressure) to the SPE for 30 sec to remove excessive liquid. In preparation for the collecting the SPE eluate, weigh (and mark the weight) 12 x 75 mm disposable glass tubes. Elute (using slight positive air pressure) each SPE cartridge into a weighed glass tube using 2.5 mL of a 70:30 ACN:MeOH solution. Add one mL of 0.1% formic acid to the eluate and briefly vortex-mix the tube. Evaporate the solvent in a water bath set at 50 ºC using nitrogen (10-15 psi) until the volume is slightly less than 0.5 mL. Do not allow the tube to go to dryness. Remove each tube and centrifuge (clinical centrifuge for 10 sec at low speed) to concentrate the extract in the base of the tube. Reweigh each tube using the same balance originally used to weigh the tubes. Add additional 0.1 % formic acid solution until the tube weight reflects an increase of 0.5 g above its original weight. (The 0.5 g weight is equivalent to 0.5 mL volume of the extract.) Briefly vortex-mix prior to the final clean-up step. Final clean-up of the samples was performed by using a 30 Kdalton molecular weight cutoff centrifuge filter. Pipette the extract into the Microcon centrifugal filtration sample reservoir already positioned in a microcentrifuge tube. Centrifuge the samples at 13,000 RPM for 15 min. Transfer the filtered extracts to LC vials for analysis.

Note: Validation data was collected by performing the method as specified above; however, in earlier work (LIB 4410), eluate collection vials were not weighed. Rather, the 0.5 mL extract volume was approximated by consistent measurement of the volume of liquid in the collection tube or of the meniscus against the 0.5 mL gradation on the Microcon reservoir. Analyst demonstration of acceptable precision may permit this time saving approach to be adopted.

Instrumental Parameters

LC: The LC program is the same as the original method. A mobile phase gradient was used, consisting of 0.1% formic acid and ACN, at a flow rate of 250 µL/min. The initial solvent composition is 95% aqueous for 2 min, followed by a linear gradient over 10 min to 50:50 0.1% formic:ACN. Hold the 50:50 ratio for 1 min, then follow by a second linear gradient to 100%

ACN in 3 min. Wash the column with 100% ACN for 2 min, then allow it to return to the initial gradient conditions (95:5 0.1% formic acid:ACN) and re-equilibrate for 3 min prior to subsequent analysis. Set the column oven (35 ºC) and the autosampler tray (10 ºC) temperatures. Use an injection volumes of 10 µL. Divert the LC flow away from the MS for the first four min. Set the syringe flush and needle wash so that each consists of 400 µL of a 50:50 methanol:water solution.

MS-MS Parameters: Use positive ion electrospray ionization for all residues. Tune the instrument by infusing a 10 µg mL^{-1} solution of SMZ (10 µL min^{-1}) into a stream of 50:50 0.1% formic acid:ACN (250 µL min^{-1}) to optimize parameters such as spray voltage and gas flows. Infuse solutions of each residue (at 10 µg mL^{-1}) to determine the optimal tube lens values and collision energies for the SRM transitions. The following general MS parameters should be used: spray voltage, 4 kV; capillary temperature, 220 ºC; nitrogen sheath gas, 50 arbitrary units; nitrogen auxiliary gas, 5 arbitrary units; skimmer offset, 0 V; argon collision gas, 1.5 mTorr; quadrupole peak widths (Q1 and Q3), 0.7 amu. Use a cycle time set at 2.5 sec (~32 msec/SRM transition for 78 transitions). Specific parameters for the SRM transitions are listed in Table 2. LC retention times may vary depending on the instrument, and can be adjusted as needed.

Table 2. MS Acquisition Parameters

Precursor[a]	Product	CE	Start Time	Stop Time	Tube Lens	Name
202.0	92.3	33	6.00	8.00	72	TBZ1
202.0	131.0	31	6.00	8.00	72	TBZ2
202.0	175.2	25	6.00	8.00	72	TBZ3
250.0	92.3	26	6.50	8.00	82	SPD1
250.0	108.3	23	6.50	8.00	82	SPD2
250.0	156.2	16	6.50	8.00	82	SPD3
251.0	92.3	26	5.50	7.00	87	SDZ1
251.0	108.3	22	5.50	7.00	87	SDZ2
251.0	156.2	15	5.50	7.00	87	SDZ3
256.0	92.3	26	6.50	8.00	92	STZ1
256.0	108.3	21	6.50	8.00	92	STZ2
256.0	156.2	13	6.50	8.00	92	STZ3
256.1	91.3	33	7.75	9.25	83	TRIP1
256.1	211.2	14	7.75	9.25	83	TRIP2
256.1	91.3	33	7.75	9.25	83	TRIP3
265.0	92.3	28	7.00	8.50	86	SMR1
265.0	108.3	25	7.00	8.50	86	SMR2
265.0	156.2	16	7.00	8.50	86	SMR3
279.1	92.3	30	7.75	9.25	90	SMZ1
279.1	108.3	25	7.75	9.25	90	SMZ2
279.1	156.2	18	7.75	9.25	90	SMZ3
285.0	92.3	25	9.00	10.50	90	SCP1
285.0	108.3	22	9.00	10.50	90	SCP2
285.0	156.2	13	9.00	10.50	90	SCP3

Precursor[a]	Product	CE	Start Time	Stop Time	Tube Lens	Name
301.0	92.3	29	10.50	12.00	90	SQX1
301.0	108.3	24	10.50	12.00	90	SQX2
301.0	156.3	14	10.50	12.00	90	SQX3
311.1	92.3	30	10.50	12.00	90	SDM1
311.1	108.3	26	10.50	12.00	90	SDM2
311.1	156.2	19	10.50	12.00	90	SDM3
313.0	226.0	32	12.80	15.00	95	FLU-OH1
313.0	252.0	41	12.80	15.00	95	FLU-OH2
313.0	280.0	34	12.80	15.00	95	FLU-OH3
332.1[b]	231.2	36	6.50	8.50	117	CIP1
332.1	245.3	22	6.50	8.50	117	CIP2
332.1	288.4	16	6.50	8.50	117	CIP3
335.1[c]	160.2	23	7.00	9.00	102	PENG1
335.1	176.2	20	7.00	9.00	102	PENG2
335.1	289.2	23	7.00	9.00	102	PENG3
350.1	114.2	29	6.50	8.00	96	AMP1
350.1	160.0	11	6.50	8.00	96	AMP2
350.1	174.2	15	6.50	8.00	96	AMP3
360.1[b]	204.2	26	7.25	8.75	106	ENR1
360.1	245.2	26	7.25	8.75	106	ENR2
360.1	316.3	18	7.25	8.75	106	ENR3
386.1	299.2	28	7.75	9.25	105	SAR1
386.1	322.3	22	7.75	9.25	105	SAR2
386.1	342.3	18	7.75	9.25	105	SAR3
424.0	152.2	21	5.75	7.25	105	CEPH1
424.0	181.2	20	5.75	7.25	105	CEPH2
424.0	292.2	14	5.75	7.25	105	CEPH3
435.3[d]	174.1	24	8.75	10.25	72	TIL1
435.3	522.7	23	8.75	10.25	72	TIL2
435.3	695.5	16	8.75	10.25	72	TIL3
436.0	114.2	31	13.75	15.25	55	CLOX1
436.0	160.2	13	13.75	15.25	55	CLOX2
436.0	277.2	12	13.75	15.25	55	CLOX3
445.1	154.2	26	7.25	8.75	98	TC1
445.1	410.3	19	7.25	8.75	98	TC2
445.1	427.4	12	7.25	8.75	98	TC3
445.105[e]	154.2	31	8.75	10.50	70	DC1
445.105	410.3	25	8.75	10.50	70	DC2
445.105	428.3	19	8.75	10.50	70	DC3
461.1	337.2	29	7.00	8.50	89	OTC1
461.1	426.3	20	7.00	8.50	89	OTC2
461.1	443.4	12	7.00	8.50	89	OTC3
479.1	154.2	27	8.00	9.50	103	CTC1
479.1	444.3	21	8.00	9.50	103	CTC2

Precursor[a]	Product	CE	Start Time	Stop Time	Tube Lens	Name
479.1	462.3	19	8.00	9.50	103	CTC3
526.2	231.0	36	13.00	14.50	105	VIR1
526.2	355.3	15	13.00	14.50	105	VIR2
526.2	508.5	13	13.00	14.50	105	VIR3
712.0[d]	199.2	35	8.75	10.25	100	BAC1
712.0	356.3	29	8.75	10.25	100	BAC2
712.0	669.8	26	8.75	10.25	100	BAC3
716.5[f]	116.2	33	10.5	12.25	107	ERY1
716.5	158.2	28	10.5	12.25	107	ERY2
716.5	540.6	19	10.5	12.25	107	ERY3
916.5	174.2	35	10.25	11.75	106	TYL1
916.5	598.6	33	10.25	11.75	106	TYL2
916.5	772.8	30	10.25	11.75	106	TYL3

[a] Precursor ions = $[MH]^+$ unless otherwise noted
[b] ENR was monitored in validation, but CIP was later substituted as a better marker compound
[c] PEN G is monitored as penicillic acid (isomer at earlier retention time)
[d] Precursor ion for TIL and BAC = $[MH_2]^{2+}$
[e] m/z 445.105 was chosen as precursor for DC to distinguish from TC (isomeric compounds)
[f] Precursor ion for ERY = $[MH- H_20]^+$

Procedure for each batch of samples (QC Controls).

The purpose of the screening method is to rapidly evaluate if a milk sample contained any of the residues of interest at a level that could be considered violative. Because the majority of these residues have established tolerance or safe levels, a minimum response threshold was needed. For that reason, a milk sample fortified at 1X (where X denotes the tolerance or safe level) with all of the drugs should be extracted and analyzed with every set of milk samples. For any residue to be considered presumptive positive, i.e. above the minimum threshold, the measured response or concentration in the unknown sample has to equal or exceed 50% of this "1X milk standard". Additional data analysis is performed to determine if residues meet criteria for confirmation of identity. The individual SRM transitions for each compound are evaluated for adequate signal to noise (>3:1), as well as a retention time (± 5%) and relative abundance (± 20% absolute) matching the 1X milk standard in accordance with established guidelines (5).

Therefore, when analyzing samples using this method, the following procedure and criteria should be followed:

1. Establish system suitability using a solvent standard of all compounds equivalent to a final 1X level milk extract. This standard should be injected (usually twice) until the LC system is equilibrated and all compounds are present in the correct retention time window.
2. Analyze a solvent blank (0.1% formic acid). All compounds should be below the threshold limit.
3. Analyze a reagent blank. All compounds should be below the threshold limit.

4. Matrix control (blank). A blank milk sample should be taken through the extraction procedure. All compounds should be below the threshold limit.
5. Single-point extracted matrix standard (1X Milk Standard). Fortify a milk sample with compounds at the tolerance (1X) level and take it through extraction procedure. The data from this sample will be used to calculate a relative amount of each compound in unknown samples. It can then be determined if residues are present above the threshold level (≥ 50% compared to the 1X Milk Standard) to be considered presumptive positive. This sample is also used to establish parameters (retention time and % relative ion abundances) for the confirmation of identity.
6. Matrix spikes. Fortify samples of milk with compounds at the tolerance (1X) level and take them through the extraction procedure. All compounds should be present above the threshold level. Generally duplicate matrix spikes should be analyzed with each batch.
7. Continuous calibration validation (CCV) standard. The 1X Milk Standard (same as #5) should be reinjected after every ten samples and/or at the end of an analytical sequence. All compounds should be present above the threshold level.
8. Initial calibration validation (ICV) standard. A solvent standard containing three representative compounds from separate (independent) stock solutions at the tolerance level (1X). All compounds should be present above the threshold level.

Data Treatment

In the original method, large numbers of samples were efficiently evaluated for the presence of any of the residues by establishing a data template for the screening SRM transitions with each response normalized to that of the 1X Milk Standard. This approach can still provide a quick, visual evaluation of the results in the Thermo Xcalibur Qualbrowser program. With the data obtained in this modified method, a summation of all three SRMs can be used in the layout. In order for a milk sample to be considered presumptive positive for any residue, the response must have a peak area or height ≥ 50% of the 1X Milk Standard.

Automated reporting with the Thermo LCQuan and Xreports software packages can also be used to rapidly evaluate confirmation data and obtain rough quantitation for large numbers of samples. A data processing method was established using the most abundant SRM transition for each residue. The 1X Milk Standard is used as a one-point "response factor" calibration curve point. Theoretical ion ratios for all of the compounds are also determined from the 1X Milk Standard. An Xreport template can be generated that would calculate the amount of residue present in a sample (based on the quantitative SRM and expressed as a fraction of the 1X Milk Standard) as well as calculate the relative abundances of the confirmatory transitions. If the SRM relative abundances do not meet the required ratio (± 20%), a value for concentration is not calculated automatically using LCQuan. The quantitative SRM peak would then has to be integrated manually and the ion ratio data will be omitted from the report.

Results and Discussion

Changes in the method

In the current method, three SRMs are collected for all of the residues in a single chromatographic run. Previously an initial LC-MS/MS program consisting of 1 SRM for each compound was used as a screen; then if any samples were presumptive positive, a class specific LC-MS/MS acquisition program was selected to reanalyze the extract and collect additional SRMs.(1) With the development of a new software feature allowing for specific SRM start and stop times, it was now possible to collect screening and confirmatory information (3 SRMs) for all residues in one analytical run. A cycle time of 2.5 seconds provided a minimum required number of data points (~10 points) across the chromatographic peaks. The primary advantage of monitoring all three SRMs for each compound in a single chromatographic run is time savings. A validation set of twenty samples can be screened and the identity of any residue present confirmed in just over eight hours (or an overnight run). Using the original method, five separate injections were made from each vial, and the total run time could be a few days. Except for validation samples, it is not likely that all of the residues would need to be confirmed in any one sample. However, some compounds (β-lactams and tetracyclines) are not completely stable in the 0.1% formic acid, so faster confirmation decreases the chance that samples with these residues would decompose before they can be detected.

Modifications were made in the target list to add virginiamycin and tilmicosin. The method was modified to monitor the hydroxy metabolite of flunixin (FLU-OH) instead because this is a more appropriate marker compound (3). As the FLU-OH was tested with this procedure, it was determined that the metabolite did not elute from the Oasis HLB SPE columns using acetonitrile. Changing the elution solvent to a 70:30 acetonitrile:methanol mixture efficiently eluted the hydroxy-flunixin without adversely affecting the recoveries of other compounds.

Validation of Method with Fortified Samples

Sets of milk samples fortified at either 0.5X, 1X, or 2X were analyzed along with a 1X Milk Standard and a control milk. Figure 1 shows the qualitative (screening) data obtained for a 1X spike; Figure 2 consists of a complete Xreport output for a 1X spike with automated residue quantitation and confirmation of identity. Table 3 shows the results for the milks fortified at 1X (n=21). The validation data was used to determine a reasonable cut-off for a presumptive positive sample. This was done using an approach suggested by a European Community Reference Laboratory for the same type of analysis (6). Averages and standard deviations for residue concentrations in the 1X spikes were determined. After evaluating the variance observed in these 21 spikes, a threshold was calculated so that 95% of samples containing residues at or above their tolerance would have a concentration above this level. The calculated threshold values for all residues at the 95% confidence level are listed in Table 3. In order to simplify day-to-day operation, a level of 0.5, corresponding to a response of $\geq$ 50% of the 1X Milk Standard, was selected as the method threshold value. All residues in the 1X spikes were above that level.

The data collected was also used to confirm the identity of the compound. The number of residues that met criteria for confirmation of identity is listed in Table 3. Most drug residues were confirmed at the 1X level. A notable exception is AMP; the ion ratios for AMP varied much more than the allowed ± 20%. This method should be considered only a screen for AMP with additional work being necessary to obtain acceptable confirmatory data. These results are

consistent with what was observed with the original method.(1) A few other residues (CLOX, SMZ, SAR) also fail confirmation criteria occasionally at the 1X level. The final column in Table 3 gives an approximation of the absolute recovery of the method for the twenty-six residues at the 1X level. These numbers were determined by dividing the average concentrations found for 1X spikes by the average calculated amount in the solvent standards.

Table 4 contains information for the concentration, numbers found above the threshold (≥ 50% of 1X Milk Standard), and numbers meeting confirmation criteria for samples spiked at 0.5X or 2X. Generally, 60-80% of milks fortified at the 0.5X level were presumptive positive, depending on the residue. Most meet confirmation criteria. For the samples spiked at 2X, all had a calculated concentration above the threshold. The residues at the 2X level met confirmation criteria with the exception of AMP (only 2 of 5 confirmed) and SMZ, SMR, and ERY where 4 of the 5 spikes met. No residues were detected in the six matrix controls and two reagent blanks that were also analyzed during method validation.

Application of Method to Incurred Milk Samples

The results from the analysis of milk from cows that were administered selected compounds of interest are shown in Table 5. The zero time points were collected before cows were administered the drugs. Milk was then obtained at different time points (from 8 to up to 144 hours) after dosing. The highest levels of drugs were generally found in the first milking. The residue levels and depletion times varied depending on the drug. For each set of incurred samples, there was a reasonable distribution of residue concentrations above, near, and below the tolerance levels. One exception was OTC where residues were present at almost 5X the tolerance level in milk collected 96 hours post-dosing. In addition, levels of OTC near the tolerance level (0.4X-1.2X) were confirmed in all of the AMP samples, even the zero time point. This is because the cow used for the AMP dosing protocol had previously provided OTC samples, and the OTC residues still persisted. Figures 3 and 4 show the qualitative and confirmatory results from one of these milk samples. A trace of PEN G was detected in the zero time point SDM incurred milk for the same reason (one cow used for both studies). Small amounts of TC were found in some milks from OTC dosed cows, perhaps as a metabolite or break-down product. With the exception of AMP, and the highest level of CLOX, all compounds that were found above the threshold met confirmation criteria. Additional matrix spikes (n=7) and controls (n=4) were analyzed with the incurred milk samples. All the residues in these 1X spikes were above the 50% threshold limit. The confirmatory results for these matrix spikes were similar to what was found in the validation set; AMP did not confirm, SMZ, SAR and CLOX were not confirmed in every sample (data not shown). Residues were not detected in the matrix controls.

The original validation and analysis of incurred milk samples was done with ENR in the analyte list. Subsequent analysis of the milks from cows dosed with ENR using a LC-fluorescence determinative method designed to look for several fluoroquinolones in milk (7) found much higher levels of ciprofloxacin, a known metabolite of ENR (8). From this information, it was determined that CIP would be a better marker for use of ENR. The SRM transitions for CIP were then substituted for ENR in the LC-MS/MS acquisition method. A number of fortified samples with CIP as part of the compound mix (n=12) were analyzed. A threshold of ≥ 50% of the 1X Milk Standard was determined to be reasonable for this compound as well. The incurred ENR samples were reanalyzed and the amount of CIP found is listed in Table 5.

Acknowledgements

The authors would like to thank Dr. Joseph Kawalek of the FDA's Center for Veterinary Medicine (CVM) for providing the incurred milk samples. Also we would like to acknowledge Dr. Philip Kijak, Dr. Randy Arbaugh and Debbie Cera, also of the CVM, for their guidance.

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Table 3. Validation Data for Milk Samples Fortified at 1X[a]

1X	Average Amount Found (* 1X)	Standard Deviation	n	Calculated Threshold[b]	# ≥ 50% of 1X	# confirmed	Average %Recovery
CEPH	1.065	0.247	21	0.640	21	21	31.6
SDZ	1.099	0.219	21	0.721	21	21	61.1
TBZ	1.181	0.301	21	0.663	21	21	84.9
AMP	1.153	0.347	21	0.556	21	7	22.5
STZ	1.153	0.299	21	0.638	21	21	94.4
SPD	1.256	0.376	21	0.609	21	21	92.3
OTC	1.166	0.309	21	0.634	21	21	95.0
SMR	1.238	0.401	21	0.548	21	20	88.9
TC	1.241	0.322	21	0.687	21	21	90.8
ENR	1.205	0.377	21	0.626	21	21	143.3
PENG	1.135	0.289	21	0.637	21	21	86.6
TRIP	1.237	0.392	21	0.562	21	21	75.5
SAR	1.254	0.363	21	0.629	21	19	126.7
SMZ	1.185	0.357	21	0.571	21	17	86.8
CTC	1.258	0.412	21	0.548	21	21	77.1
BAC	1.199	0.372	21	0.558	21	21	135.2
DC	1.256	0.326	21	0.695	21	21	108.4
TIL	1.177	0.340	21	0.591	21	21	99.3
SCP	1.171	0.320	21	0.621	21	21	94.1
TYL	1.127	0.295	21	0.619	21	21	106.7
ERY	1.142	0.340	21	0.558	21	20	87.4
SDM	1.213	0.339	21	0.630	21	21	101.3
SQX	1.229	0.343	21	0.638	21	21	94.1
VIR	1.139	0.443	21	0.628	21	21	142.7
FLUOH	1.225	0.356	21	0.642	21	21	111.3
CLOX	1.179	0.332	21	0.607	21	18	96.1

[a] data collected over four days
[b] Calculated threshold at 95% confidence interval using one-tailed Student T test = [average calculated concentration for 1X spikes – (1.721 * Standard Deviation)]

Table 4. Validation Data for Milk Samples Fortified at 0.5X and 2X

0.5X	Average Amount Found (* 1X)	n	# ≥ 50% of 1X	# confirmed	**2X**	Average Amount Found (* 1X)	n	# ≥ 50% of 1X	# confirmed
CEPH	0.619	5	4	5	CEPH	2.047	5	5	5
SDZ	0.571	5	3	5	SDZ	1.867	5	5	5
TBZ	0.565	5	3	5	TBZ	1.961	5	5	5
AMP	0.609	5	4	2	AMP	2.654	5	5	2
STZ	0.542	5	3	5	STZ	1.824	5	5	5
SPD	0.632	5	3	5	SPD	1.947	5	5	5
OTC	0.638	5	5	5	OTC	1.941	5	5	5
SMR	0.606	5	3	3	SMR	1.927	5	5	4
TC	0.688	5	5	5	TC	2.223	5	5	5
ENR	0.611	5	4	5	ENR	1.885	5	5	5
PENG	0.683	5	4	5	PENG	1.815	5	5	5
TRIP	0.620	5	4	5	TRIP	2.183	5	5	5
SAR	0.663	5	4	5	SAR	2.023	5	5	5
SMZ	0.657	5	3	2	SMZ	2.131	5	5	4
CTC	0.759	5	5	5	CTC	2.248	5	5	5
BAC	0.617	5	5	5	BAC	2.025	5	5	5
DC	0.701	5	5	5	DC	2.175	5	5	5
TIL	0.618	5	4	5	TIL	1.890	5	5	5
SCP	0.573	5	3	5	SCP	1.858	5	5	5
TYL	0.555	5	4	5	TYL	2.04	5	5	5
ERY	0.594	5	4	5	ERY	1.890	5	5	4
SDM	0.573	5	3	5	SDM	1.954	5	5	5
SQX	0.615	5	3	5	SQX	1.916	5	5	5
VIR	0.693	5	3	5	VIR	2.377	5	5	5
FLUOH	0.627	5	4	5	FLUOH	2.175	5	5	5
CLOX	0.651	5	5	3	CLOX	2.178	5	5	5

Table 5. Data for Incurred Milk Samples[a]

Drug	Time point[b]	Calc. Amt (* 1X)	Calc. Amt (ng mL^{-1}) [c]	Other Residue (ng mL^{-1})
SDM	0	ND [d]	ND	PEN G = 3.1
SDM [e]	**8**	**173.15**	**1731.46**	
SDM	**24**	**29.41**	**294.07**	
SDM	**32**	**15.81**	**158.12**	
SDM	**48**	**6.48**	**64.77**	
SDM	**56**	**2.39**	**23.93**	
SDM	**72**	**0.97**	**9.66**	
SDM	**80**	**0.58**	**5.79**	
SMZ	0	ND	ND	
SMZ	**8**	**3182.62**	**31826.2**	
SMZ	**24**	**447.23**	**4472.32**	
SMZ	**32**	**125.74**	**1257.38**	
SMZ	**48**	**11.00**	**110.02**	
SMZ	**56**	**3.47**	**34.66**	
SMZ	**72**	**1.65**	**16.51**	
SMZ	**80**	**1.16**	**11.57**	
SMZ	**96**	**0.61**	**6.08**	
SMZ	104	0.48	4.76	
SMZ	120	0.23	2.27	
SMZ	128	0.18	1.8	
SMZ	144	0.08	0.76	

Drug	Time point[b]	Calc. Amt (* 1X)	Calc. Amt (ng mL^{-1}) [c]	Other Residue (ng mL^{-1})
FLU	0	ND	ND	
FLU	**8**	**32.44**[f]	**64.87**	
FLU	**24**	**4.27** [f]	**8.54**	
FLU	**32**	**0.53** [f]	**1.05**	
FLU	48	0.06 [f]	0.24	
FLU	56	ND		
OTC	0	ND	ND	
OTC	**8**	**33.93**	**3393.4**	
OTC	**24**	**45.44**	**4543.5**	TC = 11.4
OTC	**32**	**39.58**	**3958.1**	TC =14.9
OTC	**48**	**78.06**	**7806.0**	TC = 45.0
OTC	**56**	**26.42**	**2641.9**	TC = 14.6
OTC	**72**	**11.94**	**1194.2**	TC = 9.1
OTC	**80**	**8.45**	**845.0**	TC = 5.4
OTC	**96**	**4.85**	**484.90**	TC = 3.4
ENR	0	ND	ND	
ENR	**8**	**166.21**	**831.05**	**CIP =1844.80**[g]
ENR	**24**	**9.65**	**48.27**	**CIP =452.50**[g]
ENR	**32**	**2.04**	**10.20**	**CIP=122.00**
ENR	48	0.40	**1.98**	**CIP=25.30**
ENR	56	0.21	**1.05**	**CIP=12.45**
ENR	72	0.04	0.21	CIP=4.94
ENR	80	0.04	0.22	CIP=2.31
ENR	96	0.04	0.20	CIP=1.36

Drug	Time point[b]	Calc. Amt (* 1X)	Calc. Amt (ng mL^{-1}) [c]	Other Residue (ng mL^{-1})
CEPH	0	ND	ND	
CEPH	**8**	**1071.137**	**21422.70**	
CEPH	**24**	**56.866**	**1137.32**	
CEPH	**32**	**13.547**	**270.94**	
CEPH	48	0.335	6.70	
CEPH	56	0.179	3.58	
CEPH	72	0.08	1.60	
CEPH	80	0.042	0.84	
CEPH	96	0.066	1.32	
AMP	0	ND	ND	**OTC = 107.8**
AMP	**8**	**1.959** [g]	**19.59**	**OTC = 56.4**
AMP	**24**	**1.16**	**11.60**	**OTC = 93.8**
AMP	32	0.396 [g]	3.96	**OTC = 84.4**
AMP	48	0.292 [g]	2.92	**OTC = 120.1**
AMP	56	0.093 [g]	0.93	OTC = 40.5

Drug	Time point[b]	Calc. Amt (* 1X)	Calc. Amt (ng mL^{-1}) [c]	Other Residue (ng mL^{-1})
PEN G	0	ND	ND	
PEN G	**8**	**2.62**	**13.09**	
PEN G	**24**	**2.03**	**10.17**	
PEN G	**32**	**2.05**	**10.25**	
PEN G	**48**	**0.63**	**3.13**	
CLOX	0	ND	ND	
CLOX	**8**	**2745.95** [g]	**27459.50**	
CLOX	**24**	**2.74**	**27.43**	
CLOX	32	0.13 [g]	1.29	
CLOX	48	0.03 [g]	0.31	
CLOX	56	ND		

[a] Cows were dosed with the drugs as follows: SDM 50 mg kg^{-1}, intraveneously (iv); SMZ 100 mg kg^{-1} (iv); FLU 2.2 mg kg^{-1} (iv); OTC 9 mg lb^{-1} subcutaneously (sc); ENR 7.5 mg kg^{-1} (sc); CEPH 200 mg Qtr^{-1} intramammary (imm); AMP 1 mg kg^{-1} intramuscular (im); PEN G 3000U lb^{-1} (im); CLOX 200 mg Qtr^{-1} (imm).
[b] hours after administration that milk sample was collected; zero time point is before dosing
[c] multiplied by method target level (see Table 1) [d] ND = not detected
[e] samples in bold above threshold level [f] detected as FLU-OH [g] not confirmed

Figure 1. Qualitative Data for 1X Spike. The sum of the 3 selected reaction monitoring ion transitions is shown for each compound. The y-axes are normalized to those from a 1X Milk Standard analyzed that day. All residues are present above the 50% threshold.

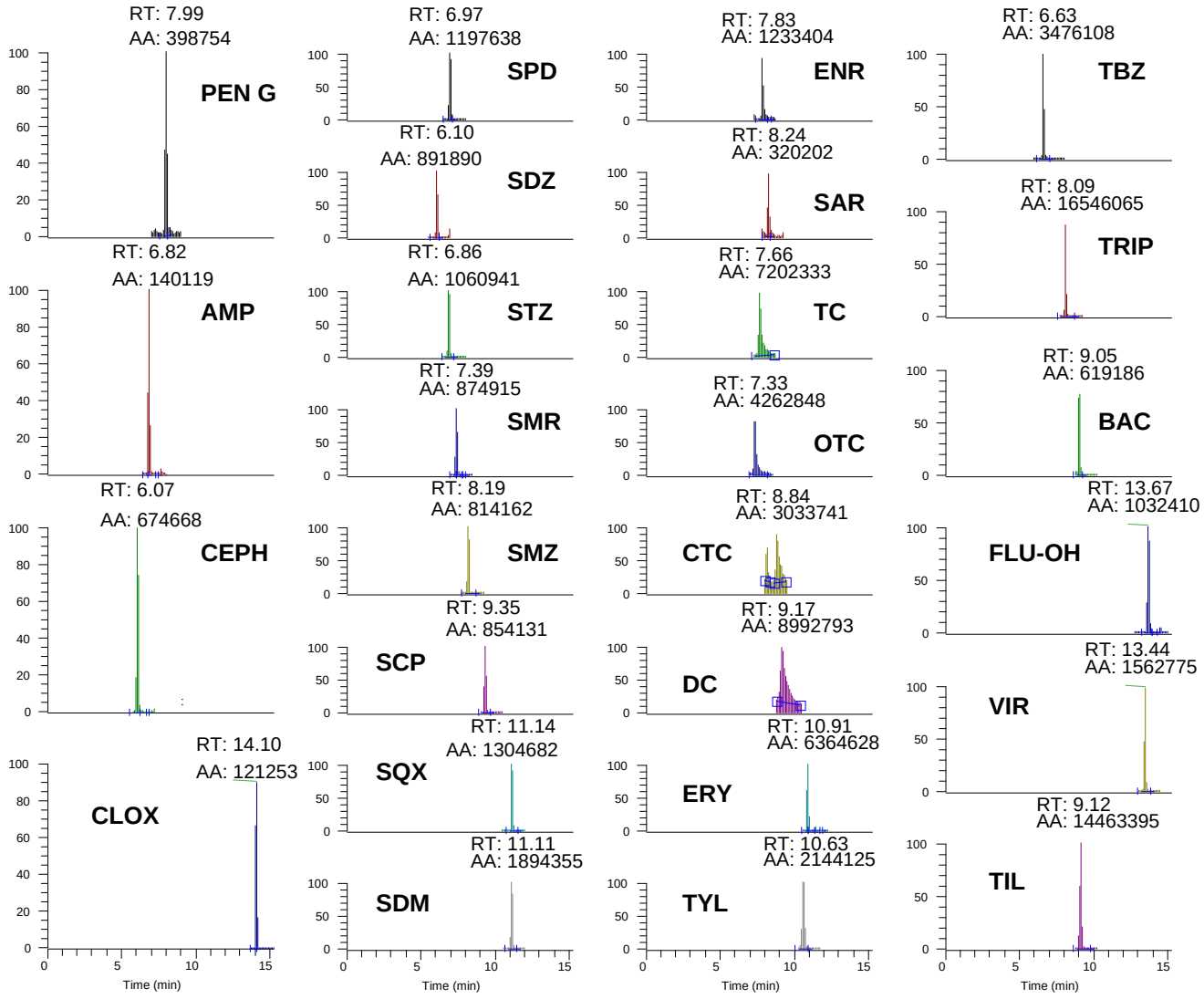


Figure 2. Report for 1X Spike. The most abundant selected monitoring ion transitions is used for quantification. The target ion ratios are set form a 1X Milk Standard analyzed that day.

1x spike

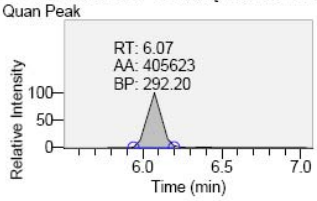
Quan Results Table

Component Name	Sample Type	Area	Calculated Conc	RT	S/N
CEPH	Unknown	405623	1.096	6.07	1000.1
SDZ	Unknown	436683	0.928	6.10	1280.9
TBZ	Unknown	2096652	0.921	6.63	3420.8
AMP	Unknown	30963	0.990	6.82	N/A
STZ	Unknown	514141	1.006	6.86	1905.9
SPD	Unknown	576657	0.945	6.97	3839.6
OTC	Unknown	2535141	0.975	7.38	625.6
SMR	Unknown	339303	0.923	7.39	10762.1
TC	Unknown	3775291	0.992	7.66	619.4
ENR	Unknown	732051	0.791	7.83	374.7
PENG	Unknown	219972	0.961	8.02	290.2
TRIP	Unknown	11506097	0.915	8.09	10516.2
SMZ	Unknown	292158	0.907	8.19	1601.0
SAR	Unknown	128435	0.929	8.24	N/A
CTC	Unknown	1545457	0.839	8.84	190.3
BAC	Unknown	278294	0.824	9.05	1639.6
TIL	Unknown	7903291	0.882	9.12	15515.6
DC	Unknown	7164091	0.963	9.17	306.4
SCP	Unknown	485033	1.125	9.35	2337.5
TYL	Unknown	1504394	0.942	10.63	4927.2
ERY	Unknown	3557130	0.886	10.91	3307.9
SDM	Unknown	1116579	0.916	11.11	2806.9
SQX	Unknown	595093	0.920	11.14	2024.8
VIR	Unknown	1124209	0.893	13.44	1712.8
FLU-OH	Unknown	751728	0.920	13.67	12489.5
CLOX	Unknown	56073	0.807	14.10	2726.3

Figure 2. Report for 1X spike (cont.)

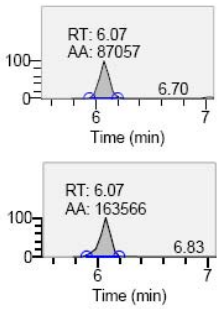
1x spike CEPH

081009a07 - m/z= 292.20-292.20 RT:
F: + c ESI SRM ms2 424.000 [152.199-152.2 ...
Quan Peak



Calculated Amount: 1.096

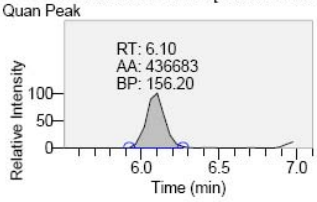
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
181.20	21.46	23.00	Yes
152.20	40.32	46.00	Yes

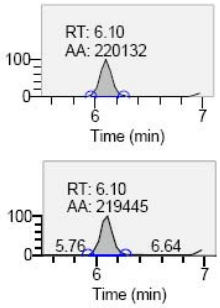
1x spike SDZ

081009a07 - m/z= 156.20-156.20 RT:
F: + c ESI SRM ms2 251.000 [92.299-92.301, ...
Quan Peak



Calculated Amount: 0.928

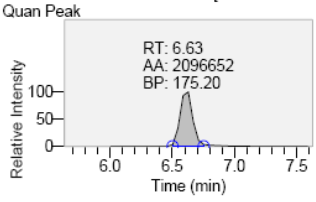
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	50.41	54.00	Yes
108.30	50.25	51.00	Yes

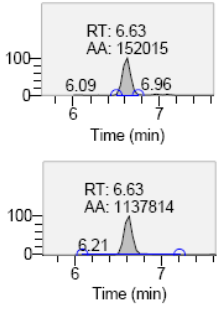
1x spike TBZ

081009a07 - m/z= 175.20-175.20 RT:
F: + c ESI SRM ms2 202.000 [92.299-92.301, ...
Quan Peak



Calculated Amount: 0.921

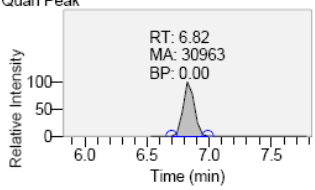
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	7.25	7.00	Yes
131.00	54.27	60.00	Yes

1x spike AMP

081009a07 - m/z= 160.00-160.00 RT:
F: + c ESI SRM ms2 350.100 [114.199-114.2 ...
Quan Peak



Calculated Amount: 0.990

Confirmatory Ions

There's no data available to display this graphic object.

There's no data available to display this graphic object.

There is no Ion Ratio Confirmation data to

Figure 2. Report for 1X spike (cont.)

1x spike STZ

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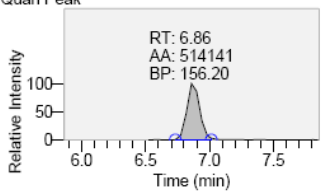
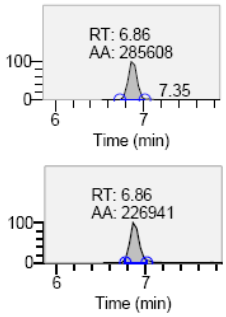
F: + c ESI SRM ms2 256.000 [92.299-92.301, ...
Quan Peak



Calculated Amount: 1.006

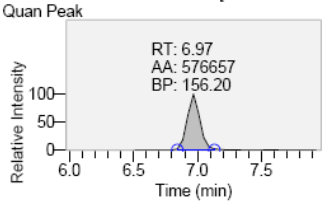
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	55.55	52.00	Yes
108.30	44.14	43.00	Yes

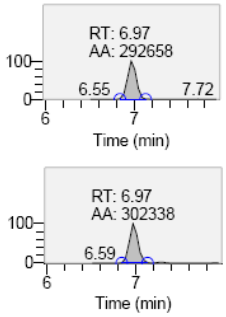
1x spike SPD

081009a07 - m/z= 156.20-156.20 RT:
F: + c ESI SRM ms2 250.000 [92.299-92.301, ...
Quan Peak



Calculated Amount: 0.945

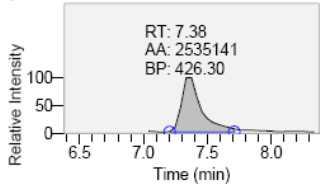
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
108.30	50.75	48.00	Yes
92.30	52.43	47.00	Yes

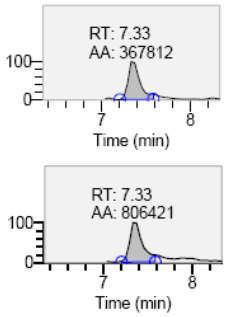
1x spike OTC

081009a07 - m/z= 426.30-426.30 RT:
F: + c ESI SRM ms2 461.100 [337.199-337.2 ...
Quan Peak



Calculated Amount: 0.975

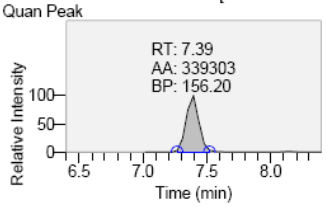
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
337.20	14.51	14.00	Yes
443.40	31.81	32.00	Yes

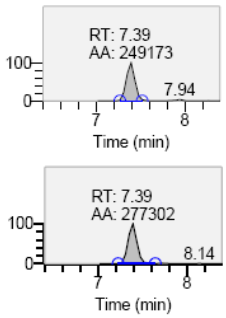
1x spike SMR

081009a07 - m/z= 156.20-156.20 RT:
F: + c ESI SRM ms2 265.000 [92.299-92.301, ...
Quan Peak



Calculated Amount: 0.923

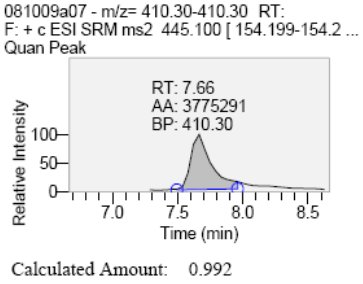
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	73.44	62.00	Yes
108.30	81.73	70.00	Yes

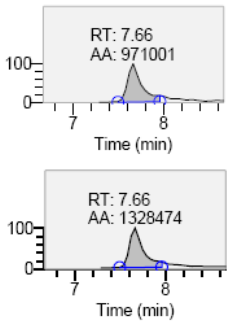
Figure 2. Report for 1X spike (cont.)

1x spike TC



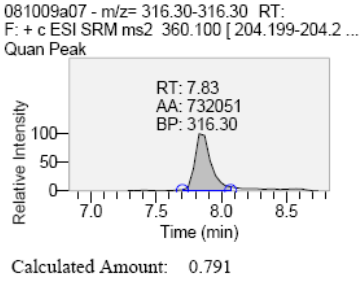
Calculated Amount: 0.992

Confirmatory Ions



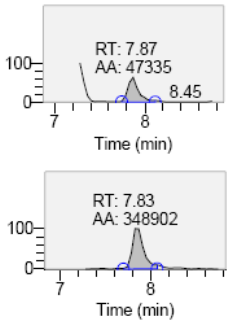
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
154.20	25.72	27.00	Yes
427.40	35.19	34.00	Yes

1x spike ENR



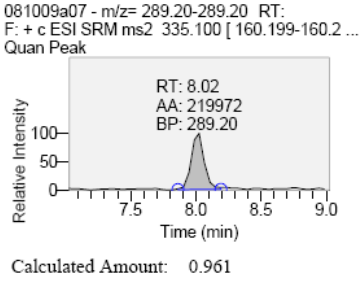
Calculated Amount: 0.791

Confirmatory Ions



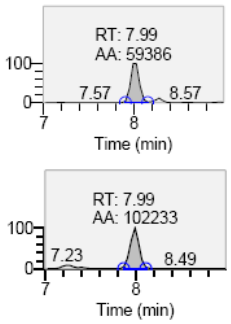
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
204.20	6.47	6.00	Yes
245.20	47.66	54.00	Yes

1x spike PENG



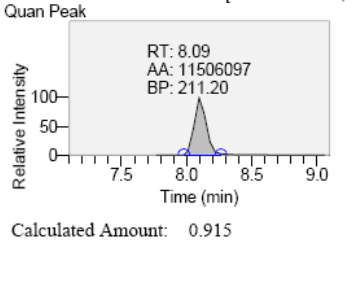
Calculated Amount: 0.961

Confirmatory Ions



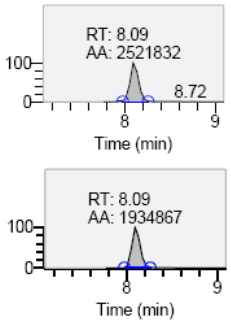
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
160.20	27.00	37.00	Yes
176.20	46.48	33.00	Yes

1x spike TRIP



Calculated Amount: 0.915

Confirmatory Ions

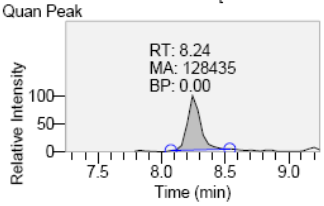


Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
91.30	21.92	24.00	Yes
119.30	16.82	18.00	Yes

Figure 2. Report for 1X spike (cont.)

1x spike SAR

081009a07 - m/z= 299.20-299.20 RT:
F: + c ESI SRM ms2 386.100 [299.199-299.2 ...
Quan Peak



Calculated Amount: 0.929

Confirmatory Ions

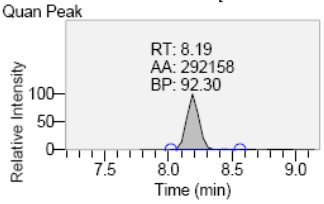
There's no data available to display this graphic object.

There's no data available to display this graphic object.

There is no Ion Ratio Confirmation data to

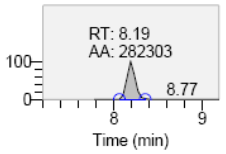
1x spike SMZ

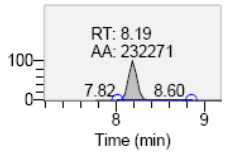
081009a07 - m/z= 156.20-156.20 RT:
F: + c ESI SRM ms2 279.100 [92.299-92.301, ...
Quan Peak



Calculated Amount: 0.907

Confirmatory Ions

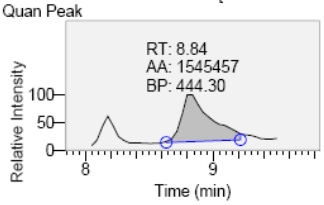




Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	96.63	99.00	Yes
108.30	79.50	89.00	Yes

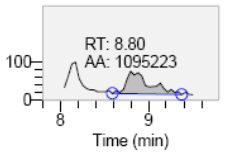
1x spike CTC

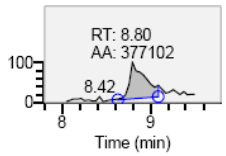
081009a07 - m/z= 444.30-444.30 RT:
F: + c ESI SRM ms2 479.100 [154.199-154.2 ...
Quan Peak



Calculated Amount: 0.839

Confirmatory Ions

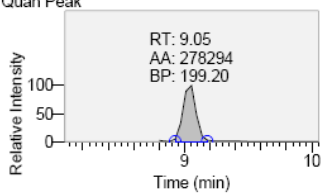




Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
462.30	70.87	60.00	Yes
154.20	24.40	26.00	Yes

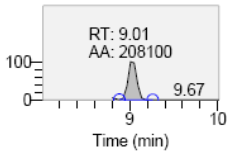
1x spike BAC

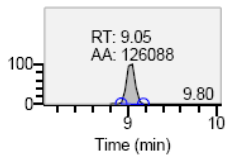
081009a07 - m/z= 199.20-199.20 RT:
F: + c ESI SRM ms2 712.000 [199.199-199.2 ...
Quan Peak



Calculated Amount: 0.824

Confirmatory Ions

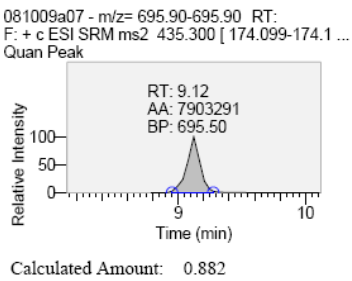




Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
669.80	74.78	57.00	Yes
356.30	45.31	64.00	Yes

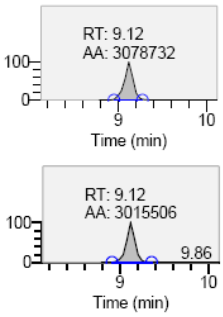
Figure 2. Report for 1X spike (cont.)

1x spike **TIL**



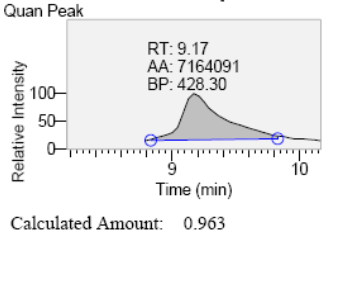
Calculated Amount: 0.882

Confirmatory Ions



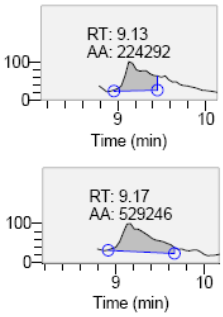
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
174.00	38.96	34.00	Yes
522.70	38.16	26.00	Yes

1x spike **DC**



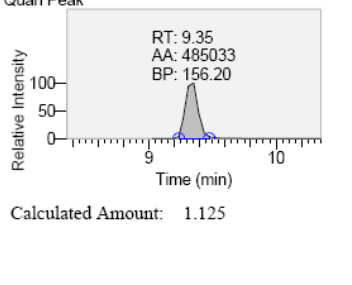
Calculated Amount: 0.963

Confirmatory Ions



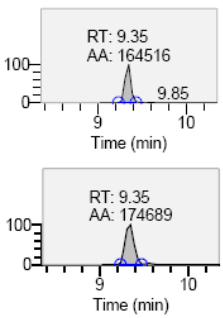
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
154.20	3.13	4.00	Yes
410.30	7.39	6.00	Yes

1x spike **SCP**



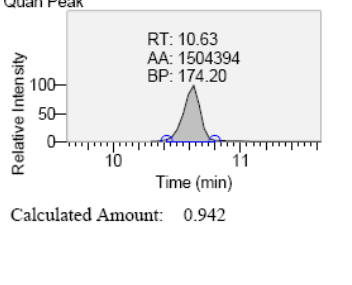
Calculated Amount: 1.125

Confirmatory Ions



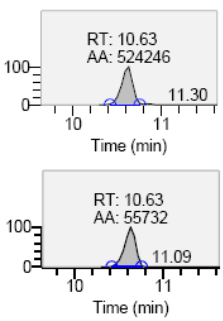
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	33.92	48.00	Yes
108.30	36.02	42.00	Yes

1x spike **TYL**



Calculated Amount: 0.942

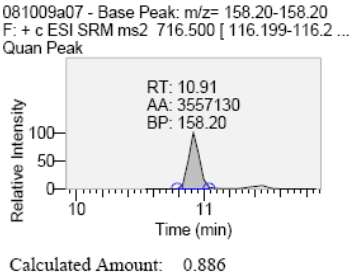
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
772.80	34.85	20.00	Yes
598.30	3.70	4.00	Yes

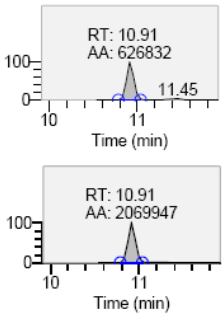
Figure 2. Report for 1X spike (cont.)

1x spike ERY



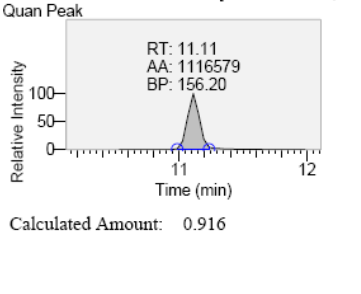
Calculated Amount: 0.886

Confirmatory Ions



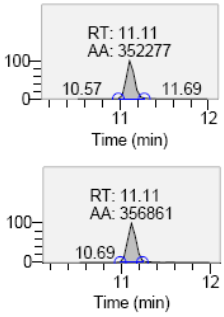
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
116.20	17.62	18.00	Yes
540.60	58.19	53.00	Yes

1x spike SDM



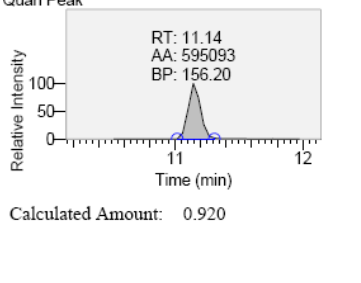
Calculated Amount: 0.916

Confirmatory Ions



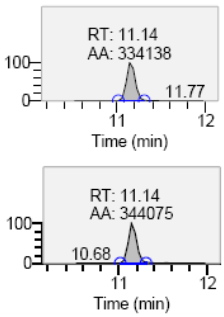
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	31.55	31.00	Yes
108.30	31.96	31.00	Yes

1x spike SQX



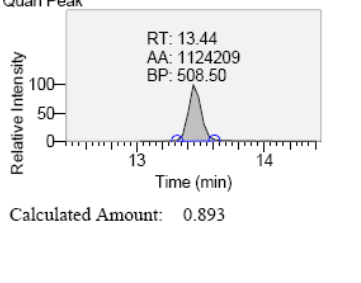
Calculated Amount: 0.920

Confirmatory Ions



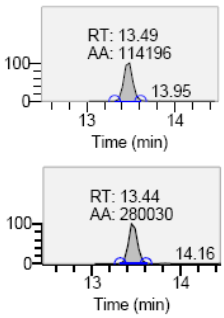
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
92.30	56.15	60.00	Yes
108.30	57.82	56.00	Yes

1x spike VIR



Calculated Amount: 0.893

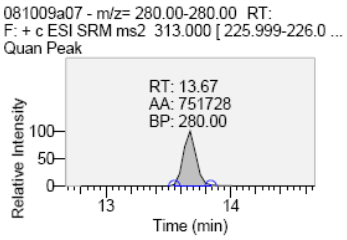
Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
231.00	10.16	11.00	Yes
355.30	24.91	27.00	Yes

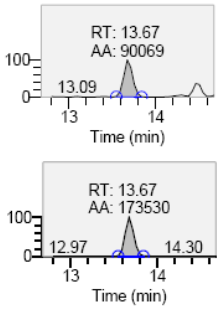
Figure 2. Report for 1X spike (cont.)

1x spike **FLU-OH**



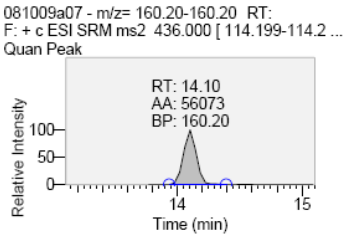
Calculated Amount: 0.920

Confirmatory Ions



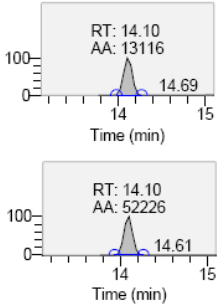
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
226.00	11.98	11.00	Yes
252.00	23.08	24.00	Yes

1x spike **CLOX**



Calculated Amount: 0.807

Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
114.20	23.39	20.00	Yes
277.20	93.14	97.00	Yes

Figure 3. Qualitative Data for Incurred Milk Sample collected 24 hour after dosing with AMP. The sum of the 3 selected reaction monitoring ion transitions is shown for each compound. The y-axes are normalized to those from a 1X Milk Standard analyzed that day. Only residues of AMP and OTC are present above the 50% threshold.

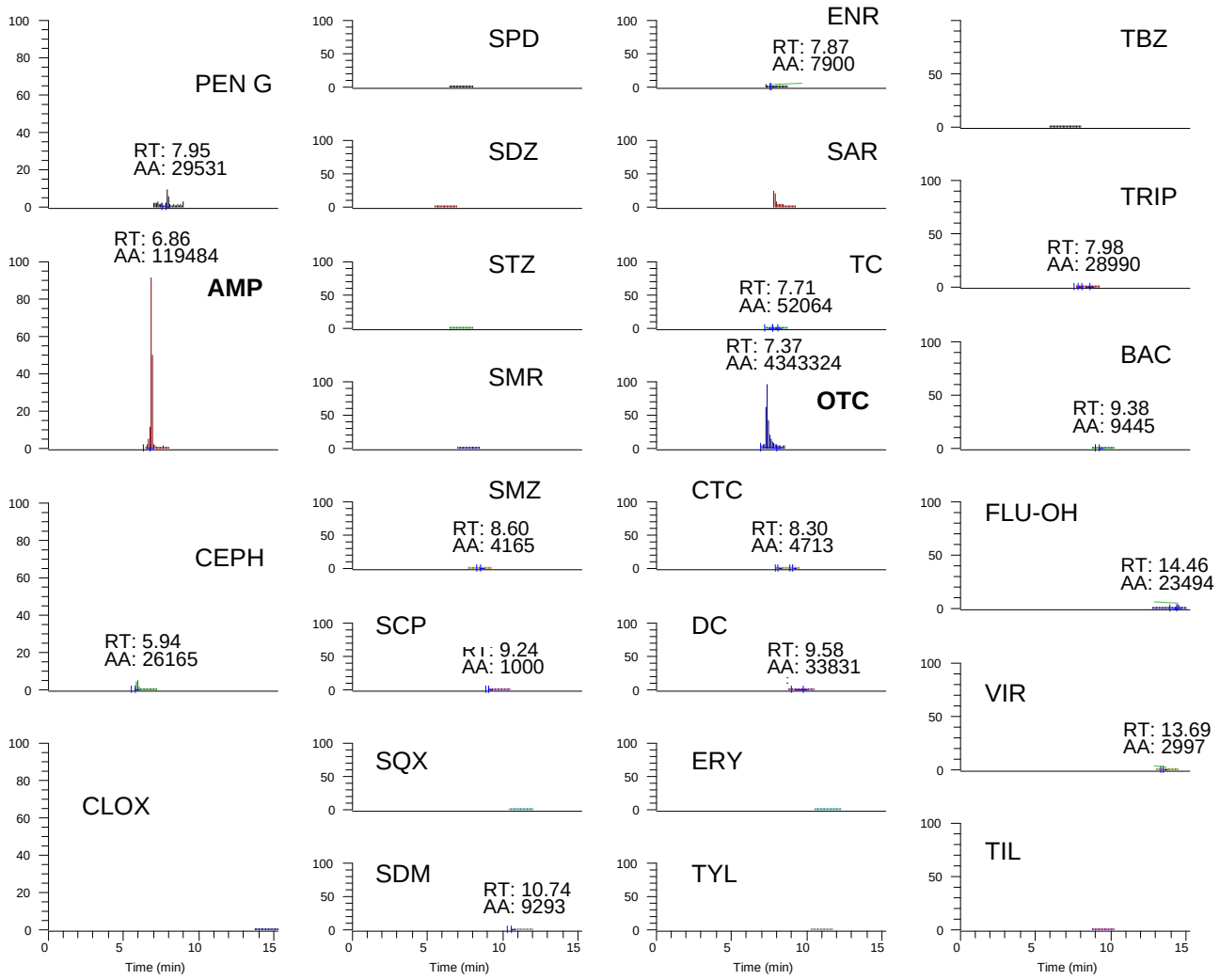
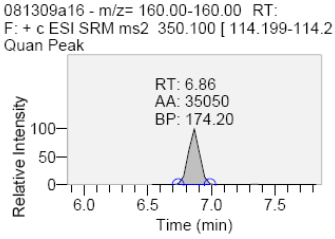


Figure 4. Abbreviated Report for Incurred Milk Sample collected 24 hour after dosing with AMP. The most abundant selected monitoring ion transitions is used for quantification. The target ion ratios are set form a 1X Milk Standard analyzed that day.

Quan Results Table

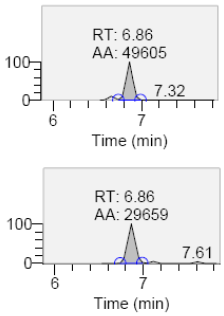
Component Name	Sample Type	Area	Calculated Conc	RT	S/N
CEPH	Unknown	N/A	NC	N/A	N/A
SDZ	Unknown	N/A	NC	N/A	N/A
TBZ	Unknown	N/A	NC	N/A	N/A
FLU-OH	Unknown	N/A	NC	N/A	N/A
STZ	Unknown	N/A	NC	N/A	N/A
SPD	Unknown	N/A	NC	N/A	N/A
VIR	Unknown	N/A	NC	N/A	N/A
SMR	Unknown	N/A	NC	N/A	N/A
TC	Unknown	N/A	NC	N/A	N/A
ENR	Unknown	N/A	NC	N/A	N/A
PENG	Unknown	N/A	NC	N/A	N/A
TRIP	Unknown	N/A	NC	N/A	N/A
SAR	Unknown	N/A	NC	N/A	N/A
SMZ	Unknown	N/A	NC	N/A	N/A
CTC	Unknown	N/A	NC	N/A	N/A
BAC	Unknown	N/A	NC	N/A	N/A
TIL	Unknown	N/A	NC	N/A	N/A
DC	Unknown	N/A	NC	N/A	N/A
SCP	Unknown	N/A	NC	N/A	N/A
TYL	Unknown	N/A	NC	N/A	N/A
ERY	Unknown	N/A	NC	N/A	N/A
SDM	Unknown	N/A	NC	N/A	N/A
SQX	Unknown	N/A	NC	N/A	N/A
CLOX	Unknown	N/A	NC	N/A	N/A
AMP	Unknown	35050	1.160	6.86	1027.6
OTC	Unknown	2468324	0.938	7.37	699.2

amp 24 **AMP**



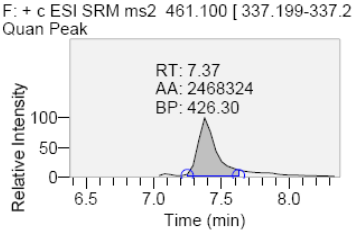
Calculated Amount: 1.160

Confirmatory Ions



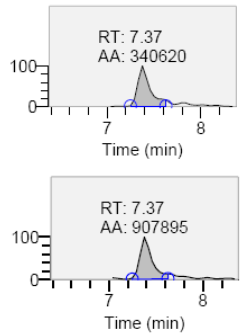
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
174.20	141.53	158.00	Yes
114.20	84.62	88.00	Yes

amp 24 **OTC**



Calculated Amount: 0.938

Confirmatory Ions



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
337.20	13.80	14.00	Yes
443.40	36.78	32.00	Yes