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Screening and Mass Spectral Confirmation of β-Lactam Antibiotic Residues in Milk Using LC-MS/MS

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Milk is typically screened for β-lactam antibiotics by nonspecific methods. Although these methods are rapid and sensitive, they are not quantitative and can yield false positive findings. A sensitive and specific method for the quantitation and mass spectral confirmation of five β-lactam and two cephalosporin antibiotics commonly or potentially used in the dairy industry is described using high-performance liquid chromatography with tandem mass spectrometry. The antibiotics studied were ampicillin, amoxicillin, penicillin G, penicillin V, cloxacillin, cephapirin, and ceftiofur. The antibiotics were extracted from milk with acetonitrile, followed by reversed-phase column cleanup. The extract was analyzed by liquid chromatography coupled with a mass spectrometer, using a water/methanol gradient containing 1% acetic acid on a C-18 reversed-phase column. Determination was by positive ion electrospray ionization and ion trap tandem mass spectrometry. Quantitation was based on the most abundant product ions from fragmentation of the protonated ion for amoxicillin, cephapirin, ampicillin, and ceftiofur and on the fragmentation of the sodium adduct for penicillin G, penicillin V, and cloxacillin. The method was validated at the U.S. FDA tolerance or safe level and at 5 or 2.5 ng/mL for these compounds in bovine milk. Theoretical method detection limits in milk based on a 10:1 signal to noise ratio were 0.2 ng/mL (ampicillin), 0.4 ng/mL (ceftiofur), 0.8 ng/mL (cephapirin), 1 ng/mL (amoxicillin and penicillin G), and 2 ng/mL (cloxacillin and penicillin V) using a nominal sample size of 5 mL.

KEYWORDS: β-Lactam antibiotic; HPLC; mass spectrometry; LC-MS

INTRODUCTION

Six β-lactam antibiotics are currently approved for use by the U.S. Food and Drug Administration Center for Veterinary Medicine in lactating dairy cattle. Established tolerances or safe levels for these drugs are as follows: amoxicillin, 10 ng/mL;, ampicillin, 10 ng/mL; ceftiofur, 50 ng/mL; cephapirin, 20 ng/mL; cloxaxillin, 10 ng/mL; and penicillin G, 5 ng/mL. Milk is currently screened for these drugs using rapid, sensitive qualitative screens. These involve microbial growth inhibition, receptor binding, or enzymatic reaction and so are typically class but not compound specific, with the potential to yield many false positive findings (*1*).

The use of chromatographic methods for quantitative multiresidue analysis of β-lactams has recently been reviewed (*2*). Development of quantitative methods is difficult because of the lack of a chromophore in the compounds, the amphoteric nature of ampicillin and amoxicillin, the complex nature of the milk matrix, and the low concentrations of drugs involved. High-performance liquid chromatography (HPLC) with fractionation cleanup (*3*), HPLC with fluorescent detection following derivatization (*4*), and ion-pair liquid chromatography (*5*) have been successfully used as quantitative methods.

Mass spectrometry (MS) provides the structural information needed for confirmation of positive results that HPLC with conventional detectors does not. Thermospray MS (*6*) and positive ion electrospray MS (*7*, *8*) have been used for confirmation methods; however, the method detection limits were well above the established U.S. FDA tolerances. Negative ion electrospray has been used to screen for and confirm several β-lactam residues, although the amphoteric β-lactams were not included (*9*). The feasibility of using ion trap tandem MS has been investigated and was shown to have the sensitivity and specificity to simultaneously confirm the seven β-lactams tested (*10*).

This paper describes a sensitive and specific screening and confirmation method for the extraction, purification, and detection of seven β-lactam antibiotics (Figure 1) using HPLC with positive ion electrospray tandem ion trap mass spectrometry.

MATERIALS AND METHODS

Reagents. Sodium sulfate (Fisher Scientific, Pittsburgh, PA) was of ACS reagent grade. Glacial acetic acid, acetonitrile, methanol, and water were of HPLC grade (Fisher Scientific). Phosphate buffer was

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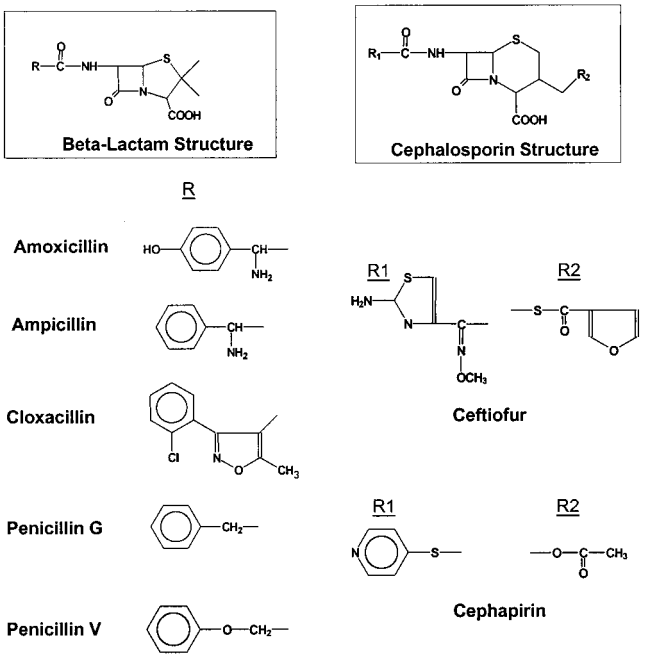

Figure 1. Chemical structures of the seven antibiotics studied.

prepared by dissolving 12 g of anhydrous NaH_2PO_4 (Sigma Ultra) in 1 L of H_2O and adjusted to pH 8.5 with dropwise addition of 10 N NaOH. Ammonium acetate buffer at pH 6.7 was made by adding 4.0 g of ammonium acetate to 1 L of H_2O.

Preparation of Standard Solutions. Amoxicillin, ampicillin, cephapirin sodium salt, penicillin G sodium salt, penicillin V potassium salt, and cloxacillin sodium salt standards were purchased from Sigma Chemical Co. (St. Louis, MO), and ceftiofur was obtained from Pharmacia Upjohn (Kalamazoo, MI). Stock solutions of 1000 μg/mL were made by dissolving standard material after adjusting for salt content and purity in water/acetonitrile (1:1, v/v). Stock standards were stored at 5 °C and were prepared monthly. Mixed and individual daily working standards at 1 μg/mL were made by adding 10 μL of the 1000 μg/mL standards to 10 mL of H_2O.

Fortifications and Calibration Standards. Four-point calibration curves in milk were prepared by adding 12.5, 25, 50, and 100 μL of the 1 μg/mL daily working standard to 5 mL of milk and processing the samples normally. Fortifications at 5 ng/mL were prepared by adding 25 μL of the 1 μg/mL standard to 5 mL of milk.

Procedure. The method used a multiresidue screening method for all seven compounds. Positive samples were re-extracted and quantitatively analyzed for the compounds indicated by the initial screen. Both methods use the same extraction and cleanup methods and the same MS conditions. The quantitative methods used isocratic LC conditions for a rapid analysis. The quantitative analysis was performed with a four-point product standard curve. The screening method used one-point calibration with a 5 ng/mL standard containing all compounds.

Table 2. Summary of Validation Study Results of Fortifications with Seven β-Lactam Antibiotics in Milk, Including Fortification, Average Percent Recovery, and Percent Coefficient of Variation for the Fortifications ($n = 6$)

compound	fortification level (ng/mL)	recovery (%)	CV (%)
amoxicillin	5	108	9.2
	10	96	13.2
ampicillin	5	115	7.5
	10	93	15.6
ceftiofur	5	102	6.1
	50	98	6.2
cephapirin	5	107	13.5
	20	113	14.4
cloxacillin	5	104	6.4
	10	102	5.7
penicillin G	2.5	107	13.2
	5	107	5.9
penicillin V	2.5	95	7.1
	5	85	7.4

(a) Extraction. Well-mixed milk samples (5 mL) were measured into a 50 mL screw-capped glass centrifuge tube (Fisher Scientific), 5 mL of acetonitrile was added, and the sample was mixed on a vortex mixer (Fisher Scientific) for 60 s. Additional acetonitrile (10 mL) was added, followed by mixing for 60 s. The sample was centrifuged at 1800 rpm (260*g*) for 5 min using an IEC Centra-7^R centrifuge (International Equipment Co.), and 10 mL of the clear extract was transferred to a 50 mL glass centrifuge tube. The extract was evaporated to ~0.5 mL using a stream of N_2 at 60 °C, 3 mL of phosphate buffer was added, and the sample was mixed by vortex mixer for 15 s.

(b) SPE Cleanup. Oasis HLB 3 cm^3 500 mg extraction cartridges (Waters Corp., Milford, MA) were prewashed sequentially with 5 mL of methanol, 10 mL of acetonitrile, 5 mL of H_2O, and 3 mL of phosphate buffer. Using no vacuum, the sample extract was applied to the column, and the column was washed with 3.5 mL of phosphate buffer, followed by 2 mL of H_2O and then 2 mL of 3% acetonitrile in water. The antibiotics were eluted at 1−2 mL/min using a vacuum with 6 mL of 40% acetonitrile in water into a 20 mL glass test tube (Fisher Scientific). The eluate was evaporated just to dryness with a stream of N_2 at 60 °C, using 10−20 mL of acetonitrile to wash down the sides of the test tube during the evaporation. Ammonium acetate buffer (0.5 mL) was immediately added, and the extract was mixed by vortexing for 15 s and filtered through a 0.45 μm Millex-HV filter (Millipore, Bedford, MA) for LC-MS/MS analysis.

LC-MS/MS Analysis. A Hewlett-Packard (HP) model 1050 HPLC with a Finnigan LCQ ion trap mass spectrometer was used for all analyses. The analytical column was a Luna C18(2), 25 cm × 4.6 mm × 5 μm particle size (Phenomenex Inc., Torrance, CA). Column temperature was ambient, and injection volume was 40 μL. The HPLC running solvent consisted of a mixture of 1% acetic acid in water (A) and 1% acetic acid in methanol (B).

The screening HPLC method was a gradient method: 80% A/20% B for 3 min, linear gradient to 50% A/50% B at 8 min, linear gradient

Table 1. Summary of Data Acquisition Parameters

analyte	precursor ion (amu)	relative collision energy (%)	isolation window (amu)	scan range (amu)	product ions used for quantitation (amu)
segment 1 (0–13.5 min, tune ion *m/z* 424)					
amoxicillin	365.9	20.0	2.5	100–500	349.0
cephapirin	424.0	22.0	2.5	115–500	292.0, 320.0, 363.9
segment 2 (13.5–16.5 min, tune ion *m/z* 350)					
ampicillin	350.0	22.5	2.5	95–500	160.0, 190.9, 332.9
segment 3 (16.5–22.0 min, tune ion *m/z* 523.9)					
ceftiofur	523.9	28.0	2.5	140–600	197.0, 241.0, 395.9
segment 4 (22.0–33.0 min, tune ion *m/z* 457.9)					
penicillin G	357.0	26	2.0	95–500	181.0, 198.0, 229.4
penicillin V	373.0	25	1.5	100–500	182.0, 214.0, 231.3
cloxacillin	457.9	27	1.5	125–500	182.0, 299.0, 330.3

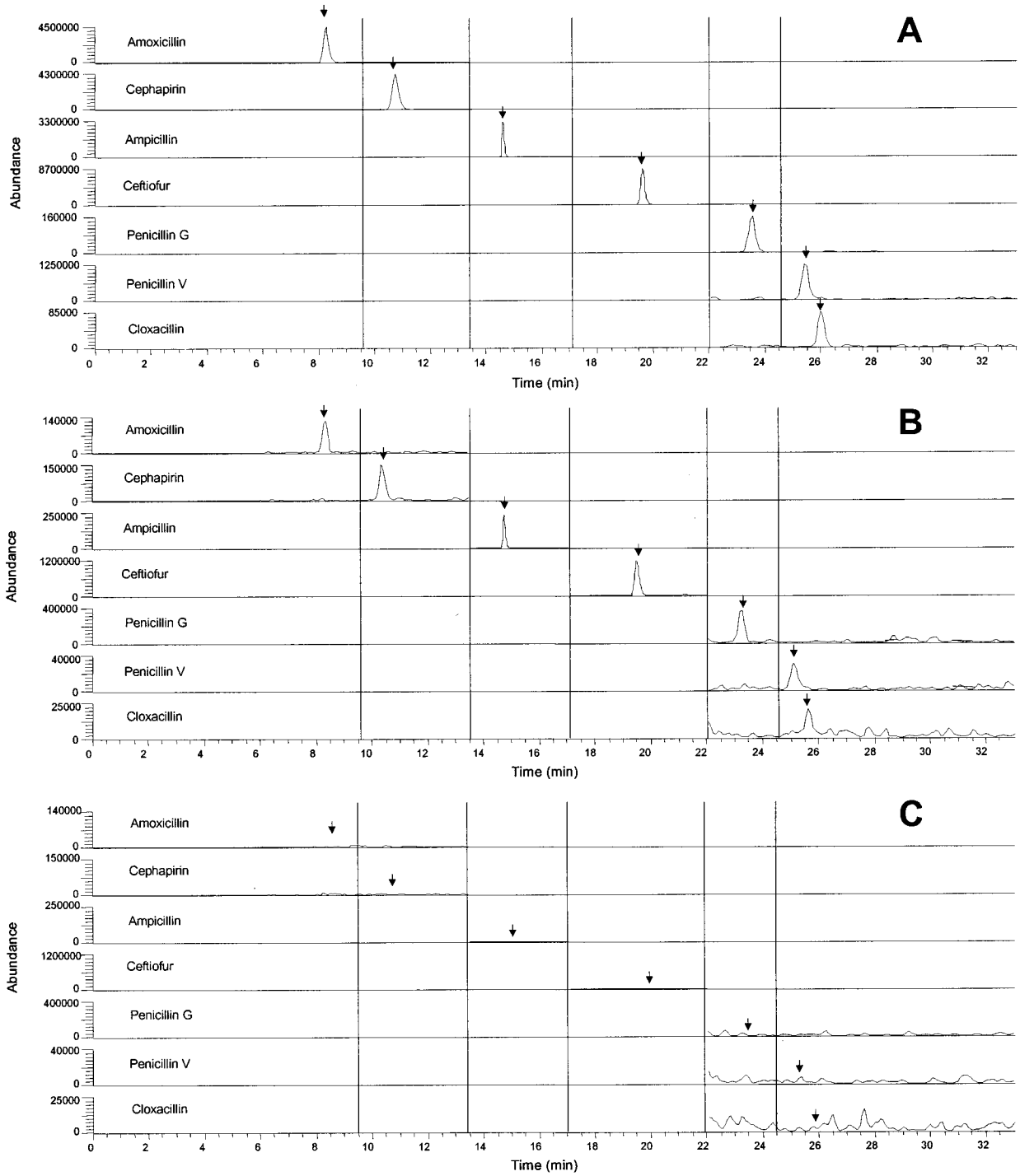

Figure 2. LC-MS/MS chromatograms of β-lactam standards and milk extracts as plots of the ion trap CID transitions of amoxicillin, ampicillin, ceftiofur, cephapirin, cloxacillin, penicillin G, and penicillin V: (A) 40 μL of 0.5 μg/mL mixed standard (equivalent to 100 ng/mL in sample); (B) negative control bovine milk fortified at 5 ng/mL; (C) negative control bovine milk. For mass spectral conditions and ions displayed see Table 1.

to 10% A/90% B at 28 min, hold for 5 min. Flow rate was 0.5 mL/min. The column was equilibrated at starting solvent composition for 12 min between injections. The column effluent was diverted to waste for the first 6.5 min of each run and during equilibration.

The quantitative HPLC methods for ampicillin, ceftiofur, cloxacillin, penicillin G, and penicillin V were isocratic using 30% A/70% B at 0.5 mL/min for 15 min. The amoxicillin and cephapirin methods were isocratic using 65% A/35% B at 0.5 mL/min for 15 min.

The ion trap was tuned according to the manufacturer's specifications. MS data for the screening method were acquired in the positive ion ESI mode using four sequential segments with multiple alternating MS/MS scan events. Table 1 summarizes the precursor ions, collision energies, isolation windows, product ion spectra scan ranges, and product ions used. Scan events used three microscans with 200 ms maximum inject times. The quantitative methods used a single segment with a single scan event, using the MS conditions for that compound (Table 1). Peak areas were from chromatograms generated using the sum of these product ions. Quantification was by comparison with a four-point product calibration curve in milk extract. The calibration curves used nonweighted second-order regression.

Method Validation. The method was validated by analyzing two sets of six replicates of pooled negative control bovine whole milk. One set was fortified at 2.5 or 5 ng/mL and one at the U.S. FDA tolerance or safe level (Table 2).

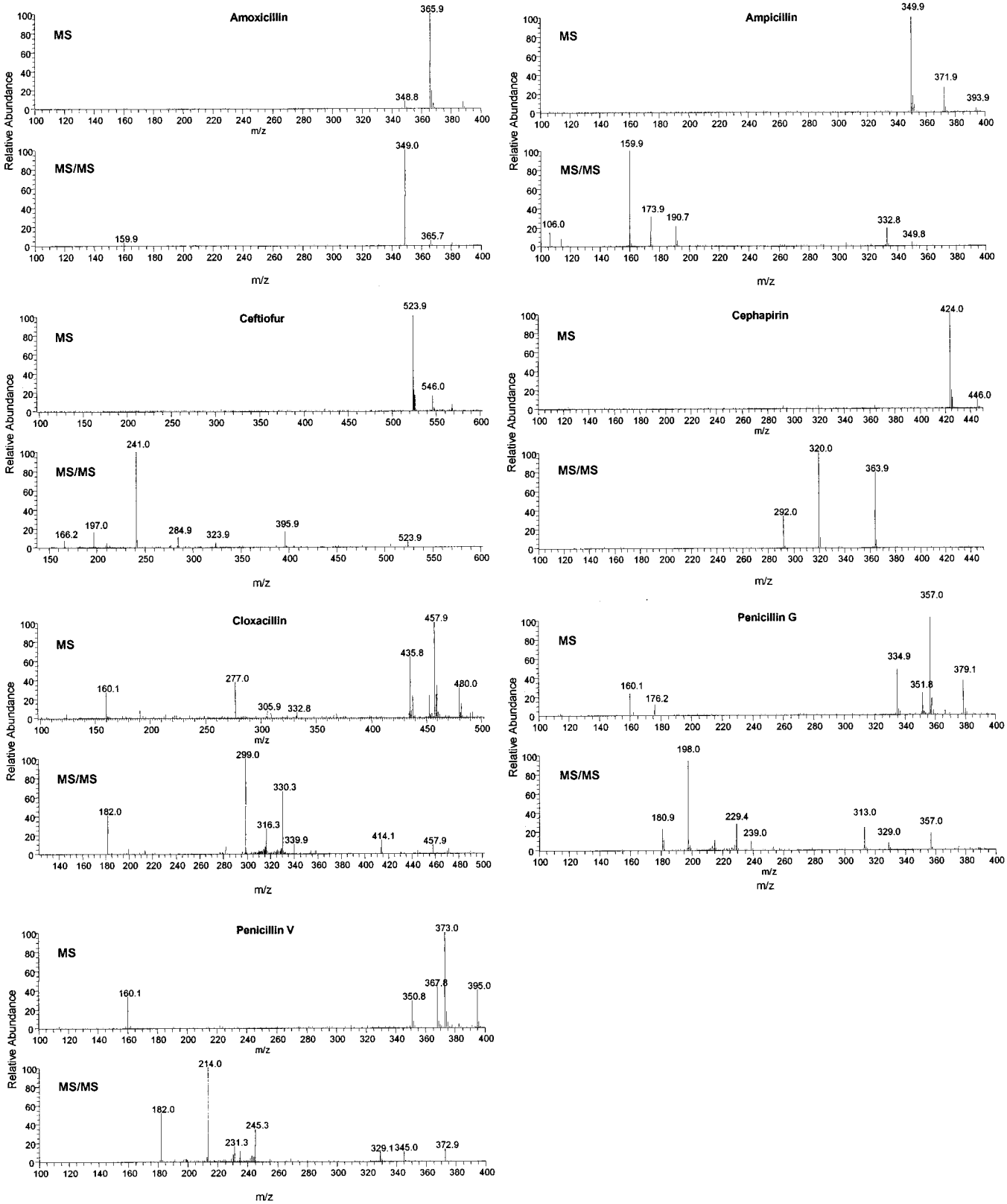

Figure 3. Positive ion ESI mass spectra and MS/MS spectra of the seven β-lactam antibiotics studied. See Table 1 for ion trap CID conditions.

RESULTS AND DISCUSSION

The method quantitatively extracted and recovered the antibiotics at the two levels studied (Table 2). Recoveries ranged from 85 to 115%. The method had variability ranging from 5.7 to 15.6% coefficient of variation (CV). The use of MS/MS produced very clean chromatograms for all compounds with a negligible contribution from the milk background (Figure 2). The method attained method detection limits below the tolerances or safe levels for each compound. Detection limits based on a 10:1 signal to noise ratio (S/N) response of the β-lactams in milk extract were as follows: amoxicillin, 1 ng/mL; ampicillin, 0.2 ng/mL; ceftiofur, 0.4 ng/mL; cephapirin, 0.8 ng/mL; cloxacillin, 2 ng/mL; penicillin G, 1 ng/mL; and penicillin V, 2 ng/mL. Compounds that are present but below tolerance that

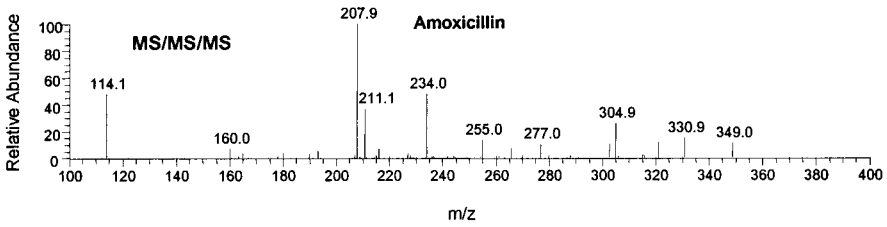

Figure 4. Positive ion ESI MS/MS/MS spectrum of amoxicillin from ion trap CID of the MS/MS ion at *m/z* 349.0.

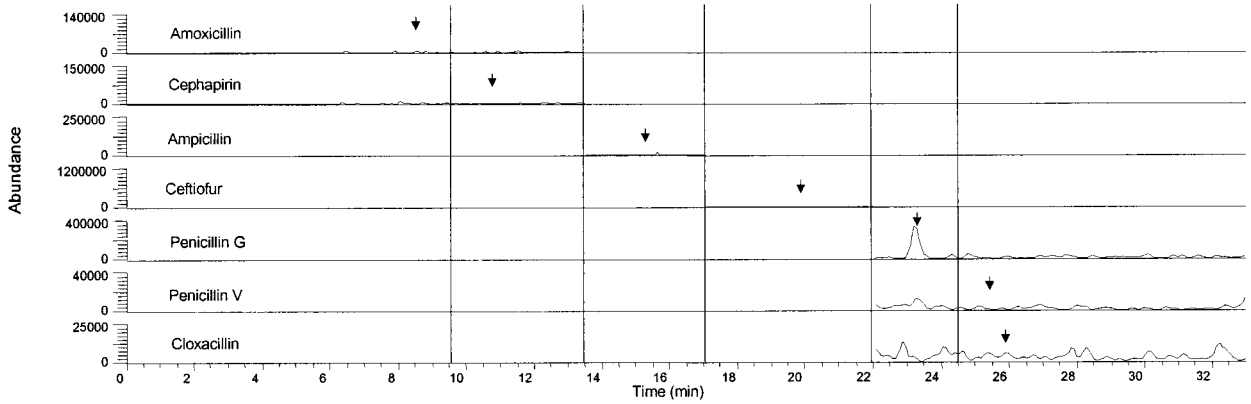

Figure 5. LC-MS/MS chromatogram of an extract of a bovine milk sample positive for penicillin G at 5.2 ng/mL as a plot of the ion trap CID transitions of amoxicillin, cephapirin, ampicillin, ceftiofur, penicillin G, penicillin V, and cloxacillin. For mass spectral conditions and ions displayed see Table 1.

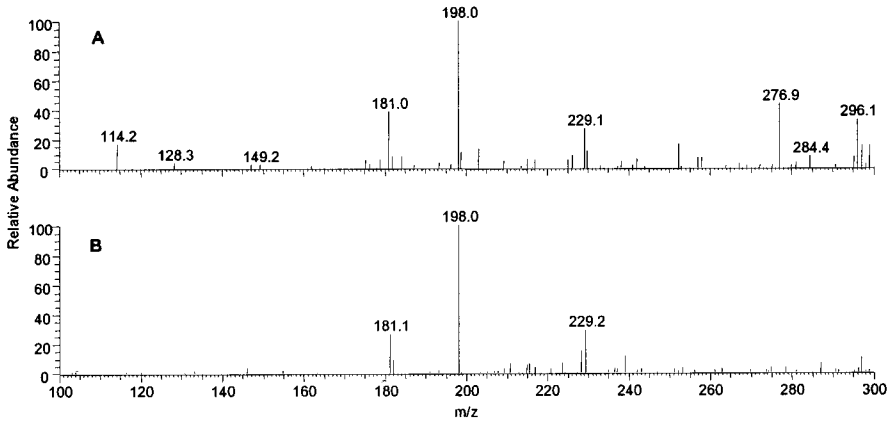

Figure 6. Positive ion ESI MS/MS spectrum of penicillin G from a positive sample for penicillin G at 5.2 ng/mL (A) and a standard equivalent to a sample concentration of 5 ng/mL (B).

might create a positive result by quick screening methods can thus be identified. For example, this laboratory has found cephapirin residues in the concentration range of 1.4−13.2 ng/mL that were submitted because they had been positive by quick screening methods. Milk fortified with cephapirin at 1 ng/mL for these samples averaged 109% recovery with a 3.2% CV ($n = 4$).

Quantitation was based on the most abundant product ions from fragmentation of the protonated ion for amoxicillin, cephapirin, ampicillin, and ceftiofur and on the fragmentation of the sodium adducts for penicillin G, penicillin V, and cloxacillin (Figure 3). Freshly prepared standards of penicillin G, penicillin V, and cloxacillin gave the same proportion of sodium adduct as standards older than 1 month, indicating that the sodium adduct was due to ubiquitous sodium in the HPLC system and not from sodium in the glass of the standard container. Confirmation of a positive result was based on agreement within 20% of the three product ions in MS/MS spectrum (Table 1) of the sample and a standard of equivalent concentration, with the exception of amoxicillin. Amoxicillin produced only one significant product ion at *m/z* 349.0 under the listed MS/MS conditions, which was satisfactory for quantitation but not for definitive identification. For identification MS^3 conditions were developed. The ion at *m/z* 349.0 from the MS/MS experiment was isolated with a window of 3.0 amu and disassociated with a collisional energy of 27%. The resulting spectrum is presented in Figure 4. Confirmation was based on agreement of the ions at *m/z* 207.9 and 234.0 within 20% of the product ions in MS^3 spectrum of the sample and a standard of equivalent concentration.

An important component to this method is the critical need for accuracy, especially in the concentration range of the tolerances or safe levels. Quantitation using electrospray ionization can be seriously affected by ion suppression. The variability and error associated with ion suppression were accounted for by the use of product standard curves in milk extract. No internal standard was found that consistently corrected for this variability. Although use of atmospheric pressure chemical ionization (APCI) may have reduced the effect of matrix on response, it did not provide adequate sensitivity. To attain the best accuracy and for efficiency, it was best to reanalyze positive samples with a product standard curve in the concentration range

determined by the original screen. The product standard curves were nearly linear, but use of nonweighted second-order regression best described the curves.

This method was applied to >100 samples from bulk tankers and storage silos. Positive samples were clearly different from negative even at concentrations near the tolerance (Figure 5), and the identity of the residue was also clearly determined from comparison of the MS/MS spectrum to that of a standard (Figure 6).

β-Lactam degradation was significant at low concentrations in milk extract. The pH of the buffer was optimized at pH 6.7 to provide the most stability. Milk extracts fortified at 7.5 ng/mL and stored at room temperature showed an average decrease in response of 11% for the seven compounds over a 16 h period. Penicillin G and cephapirin decreased the most (18% each), whereas ceftiofur and cloxacillin showed no net change. Thus, for routine analysis of batches greater than four samples, it was more accurate to screen the samples and then re-extract and analyze quantitatively for the compounds found using a rapid isocratic HPLC method.

This method, using ion trap LC-MS/MS, provided a rapid quantitative analysis of milk samples for β-lactam antibiotics at low concentrations. Future work will include validation at lower concentrations, addition of ceftiofur metabolites to the screen, and examination of the use of isotopic internal standards for improved accuracy and precision.

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