# Identification of Cephapirin Metabolites and Degradants in Bovine Milk by Electrospray Ionization−Ion Trap Tandem Mass Spectrometry

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Liquid chromatography−ion trap tandem mass spectrometry (LC-MS/MS) with electrospray ionization was used to identify cephapirin metabolites and degradants in milk from cows dosed with cephapirin. The milk was extracted according to a previously published procedure. Structures for various components were tentatively identified by their molecular weight, product ion mass spectra, and/or correspondence to standard mass spectra. These components may have occurred as metabolites or as degradants that occurred on storage or during extraction. Compounds identified in the milk included cephapirin, desacetylcephapirin, cephapirin lactone, hydrolyzed cephapirin, and a reduced cephapirin lactone that has not previously been reported. Methylcephapirin was also identified, possibly as a trace contaminant in the formulation. Analysis of incurred milk extracts showed that cephapirin and desacetylcephapirin are the major residues in milk. Desacetylcephapirin residues persisted about as long as the parent drug. The detection limit for both residues by LC-MS/MS was ~1 ng/mL in milk. These results have implications for microbiological methods or rapid test kits, if such methods or kits respond to cephapirin metabolites and degradants present in the milk.

**Keywords:** *Cephapirin; desacetylcephapirin; ion trap mass spectrometry; β-lactam antibiotics; metabolism; milk; liquid chromatography−mass spectrometry; electrospray ionization*

## INTRODUCTION

Health concerns require a full characterization of residues that may occur in edible tissues from animals dosed with veterinary drugs. Several important regulatory questions led the Center for Veterinary Medicine (CVM) to apply mass spectrometry in an investigation of cephapirin (CEPH) residues occurring in bovine milk. CVM has approved CEPH for treating dairy cattle but with a tolerance for CEPH residues in milk of 20 ng/mL (ppb; *Federal Register*, 1975; *Code of Federal Regulations*, 1999). Rapid test kits are now in wide use for screening tanker truck loads of milk for CEPH or other β-lactam residues that might exceed the CVM's tolerances or safe levels. Test kits are generally calibrated on the basis of parent alone, but if the kits also respond to metabolites or degradants, there could be positive results even after the parent drug depletes below the CVM's tolerance or safe level. In addition, test kit results would differ from chemical assays, that measure only the parent (Stanker et al., 1998). Test kit cross-reactivity for CEPH metabolites has been suggested as a possibility that warrants further investigation.

Cephalosporins form desacetyl products on acid degradation (Kelani et al., 1998; Simmons, 1981) on standing (Moats, 1993) and when added to milk and allowed to incubate for several hours (Moats, 1993). Desacetylcephapirin (DAC) is a CEPH metabolite that was previously identified by paper chromatography/bioautography (Cabana et al., 1976) and thermospray mass spectrometry (Tyczkowska et al., 1991). The European Union set a maximum residue limit of 10 ppb for the sum of DAC and CEPH (*Off. J. Eur. Communities*, 1999). Assaying both DAC and CEPH levels in bovine milk could address the test kit calibration issue, but the problem has been complicated by the lack of DAC analytical standard in the U.S. regulatory community. For this reason, LC-based methods reported to date for DAC assumed equal molar absorptivity with CEPH (Moats, 1993; Tyczkowska et al., 1991; Moats et al., 2000) On the basis of this assumption, Moats et al. (2000) compared the depletion profiles of DAC and CEPH following intramammary or intramuscular dosing. It was found that relative amounts in milk vary widely and that DAC persisted somewhat after CEPH had depleted below detection limits (Moats et al., 2000).

Desacetylcephalosporins may convert to lactones, which are an inert product of acid degradation (Simmons, 1981). Cabana et al. (1976) reported finding trace amounts of cephapirin lactone (LAC) in the urine of rats, but not mice, dogs, or humans. MacNeil et al. (1986) described an LC procedure for quantitation of CEPH, DAC, and LAC in drug formulations.

The relative biological activities of CEPH, DAC, LAC, and other potential residues are also important in the established withdrawal period for CEPH-dosed animals. The CEPH tolerance of 20 ppb was based on biological assays (microbial sensitivity tests). If the test bacteria were susceptible to other compounds that were present due to CEPH degradation, this may have affected the calculations on which the tolerance was set. Cabana et al. (1976) reported that the potency of DAC versus

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CEPH was ~54%. Also, both the desacetyl and lactone forms of cephalothin, a drug closely related to CEPH, are biologically active (Simmons, 1981).

Any examination of the relative microbial and test kit sensitivities of the various residues would first require that all metabolites and degradants be characterized and suitable analytical standards obtained. In previous work, we demonstrated the capability of electrospray ionization (ESI) and liquid chromatography−ion trap tandem mass spectrometry (LC-MS/MS) for identifying $\beta$-lactam residues in bovine milk (Heller and Ngoh, 1998). Thermospray LC-MS had been applied to the identification of CEPH residues but with much lower sensitivity (Tyczkowska et al., 1991). ESI-LC-MS/MS with ion trap detection has now been applied to a variety of drug residue identification issues (Josephs, 1996; Carson and Heller, 1998; Heller et al., 2000; Rabbolini et al., 1998; Dear et al., 1999; Wieboldt et al., 1998). Also, metabolites and degradants of other $\beta$-lactam antibiotics have been studied by mass spectrometry, including ampicillin (Suwanrumpha and Freas, 1989) and cloxacillin (Tyczkowska et al., 1992).

ESI-LC-MS/MS was used to identify CEPH metabolites and degradants in milk. The milk was extracted by a determinative procedure for CEPH and ceftiofur (Schermerhorn et al., 1998). Structures for several components were tentatively identified by interpretation of their LC-MS/MS spectra. These components may have occurred as metabolites or as degradants that occurred on storage or during extraction.

## EXPERIMENTAL PROCEDURES

**Apparatus.** Two types of LC cartridge columns were used: (I) ODS-AQ, 4.6 × 50 mm, 3 $\mu$m silica (YMC, Wilmington, NC) and (II) Zorbax SB-C18 rapid resolution, 2.1 × 30 mm, 3.5 $\mu$m silica (Mac-Mod Analytical, Chadds Ford, PA). The liquid chromatograph was a series 1050 quaternary pump with a series 1100 autosampler (Hewlett-Packard, Palo Alto, CA). The mass spectrometer was a model LCQ equipped with ESI and controlled by Navigator 1.2 software (Finnigan MAT, San Jose, CA).

**Reagents.** UV spectrophotometric grade methanol (Burdick & Jackson, Muskegon, MI), formic acid 88% (J. T. Baker, Phillipsburg, NJ), and ammonium formate (Sigma Chemical, St. Louis, MO) were used. All water was prepared with a Milli-Q system to give a resistivity of at least 16 MΩ·cm (Millipore, Bedford, MA). A formic acid solution was prepared in water at 0.1%. A buffer was prepared by dissolving ammonium formate in water at 20 mM and then adjusting the pH to 4 by adding formic acid.

**Standards.** Cephapirin standard was obtained from two sources (U.S. Pharmacopeia, Rockville, MD, and Sigma). Dilutions of standard were prepared in water according to the method of Schermerhorn et al. (1998). Desacetylcephapirin standard was generously provided by by Barbara Evanoff of Bristol-Myers Squibb. Stock solutions were prepared in water at 100 $\mu$g/mL (ppm), after correcting for purity, and were stored at −80 °C until needed. Diluted standard solutions were stored at 4 °C for 2 weeks.

**Dosing.** The dosage formulation was Today Cephapirin Sodium (Franklin Laboratories, Fort Dodge, IA). Control milk samples were taken prior to dosing, and incurred milk samples were collected for up to 120 h after dosing (beyond the regulatory withdrawal period of 96 h). Each 200 mg dose was injected intramammarily twice, at a 12 h interval. Doses were injected just after milking. All milk samples from the animal study had been obtained before the LC-MS/MS study was begun.

**Extraction.** The milk samples were stored at −80 °C, then extracted and analyzed according to a published procedure (Schermerhorn et al., 1998). Thawed milk was deproteinated

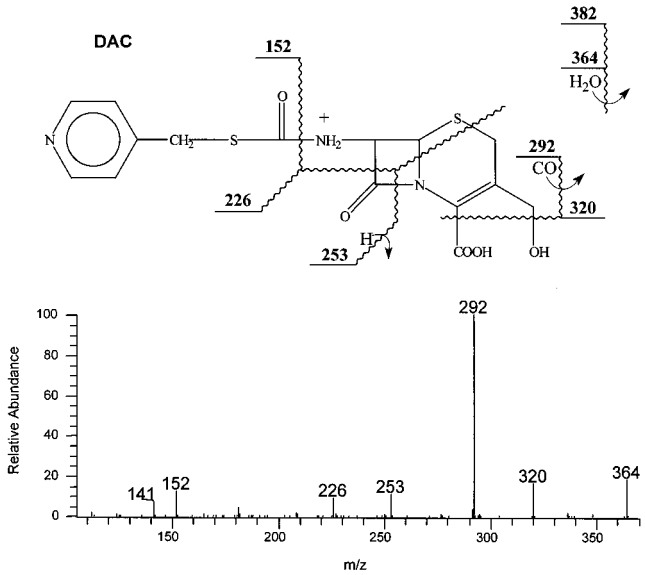


**Figure 1.** Desacetylcephapirin, proposed fragmentation pattern, and MS/MS spectrum from peak appearing in high-level incurred milk sample (LC conditions II).

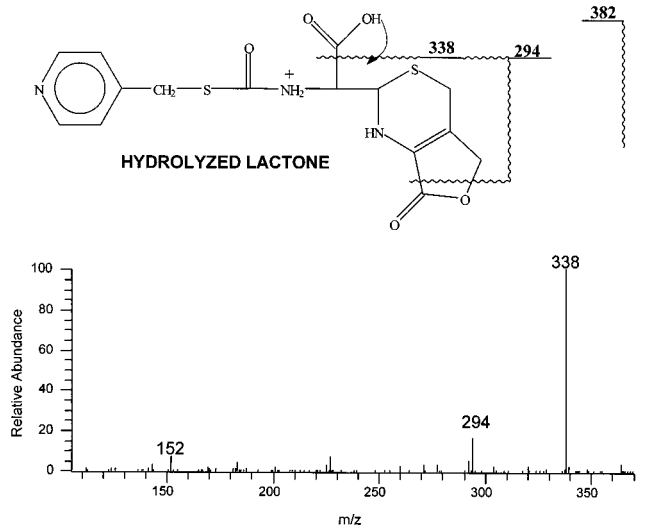


**Figure 2.** Hydrolyzed cephapirin lactone, proposed fragmentation pattern, and MS/MS spectrum from peaks appearing in high-level incurred milk sample and suspected to be from this compound (LC conditions II).

with acetonitrile, and the supernate was mixed with water. Acetonitrile was removed under reduced pressure at 40−50 °C. The resulting aqueous solution was applied to a C-18 solid-phase extraction cartridge. Analytes were eluted with acetonitrile. After acetonitrile had been evaporated under a nitrogen stream, the dried extract was dissolved in water and analyzed by ion-pair LC with ultraviolet (UV) detection. Following LC-UV analysis, the extracts were stored at −20 °C or below prior to LC-MS/MS analysis.

**Liquid Chromatography−Mass Spectrometry.** The ion trap was calibrated according to the manufacturer's specifications. The ESI source was tuned by infusing 10 ppm of CEPH at 10 $\mu$L/min into a mobile phase of 0.1% formic acid/methanol 70:30 at 400 $\mu$L/min. Prior to each day's analyses, source conditions were reoptimized with the LCQ Autotune program while standard was infused. Positive ionization (PI) ESI mode was used. Negative ion ESI and positive ion atmospheric pressure chemical ionization (APCI) were tested, but both gave excessive fragmentation and lower sensitivity than PI-ESI.

The ion trap was operated in various acquisition modes, including full-scan MS, data-dependent MS/MS, and time-scheduled MS/MS. In MS/MS modes, automatic gain control

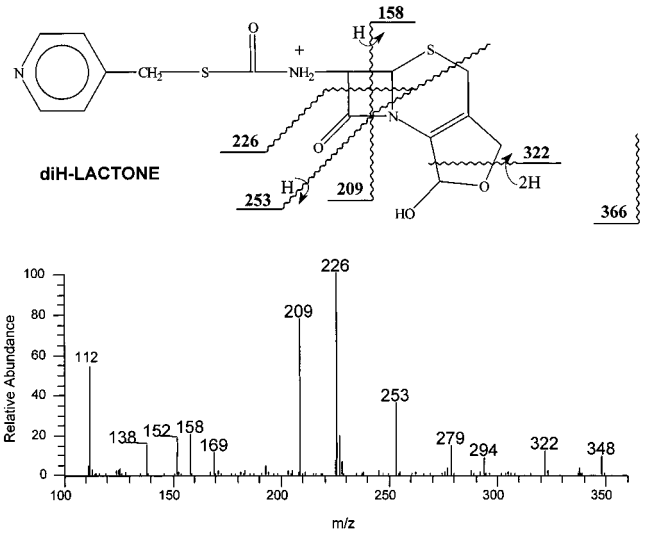


**Figure 3.** Dihydrocephapirin lactone, proposed fragmentation pattern, and MS/MS spectrum from peak appearing in high-level incurred milk sample and suspected to be from this compound (LC conditions II).

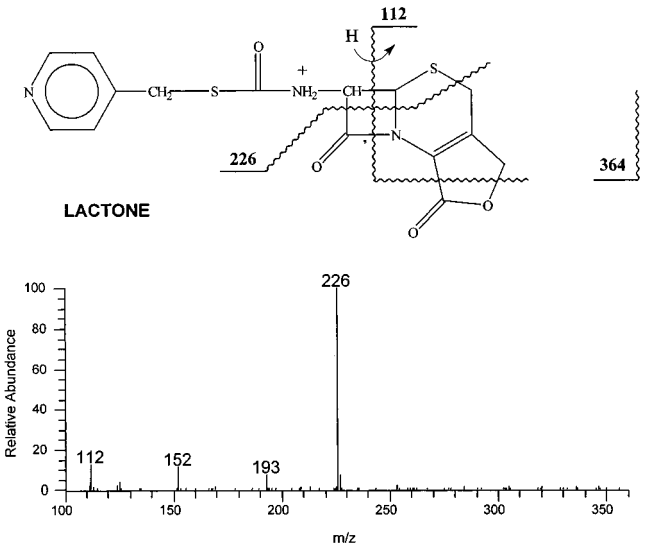


**Figure 4.** Cephapirin lactone, proposed fragmentation pattern, and MS/MS spectrum from peak appearing in high-level incurred milk sample and suspected to be the lactone (LC conditions II).

was on, maximum ion injection time was 500 ms, and one microscan was collected per scan. Time-scheduled MS/MS was carried out by scanning for one or two compounds during each time range (one or two segments per scan event). Isolation width was 2.0 or 4.0 amu. MS/MS collision energy (relative units) was 24%. The full-scan range for MS mode was *m/z* 100−800. For MS/MS mode, product ions were collected over the maximum range allowed (~30% of the precursor ion).

Reconstructed ion chromatograms (RICs) were generated by summing the two or three major ions in each product ion spectrum. Spectra were averaged over the time range for which the RIC peak was 10% of full height or greater.

Initially, CEPH standards and extracts of incurred milk were analyzed by gradient elution in full-scan mode using the YMC cartridge column (LC conditions I). The initial mobile phase was 0.1% formic acid/methanol in a 95:5 ratio; on injection, the mobile phase was ramped to a 40:60 ratio over 10 min. This ratio was held for 4 min, and then the column was reequilibrated at 95:5 for 5 min.

After these initial scouting runs were complete, the shorter cartridge column (Mac-Mod) was used with a different gradient to reduce total run time (LC conditions II). The initial mobile

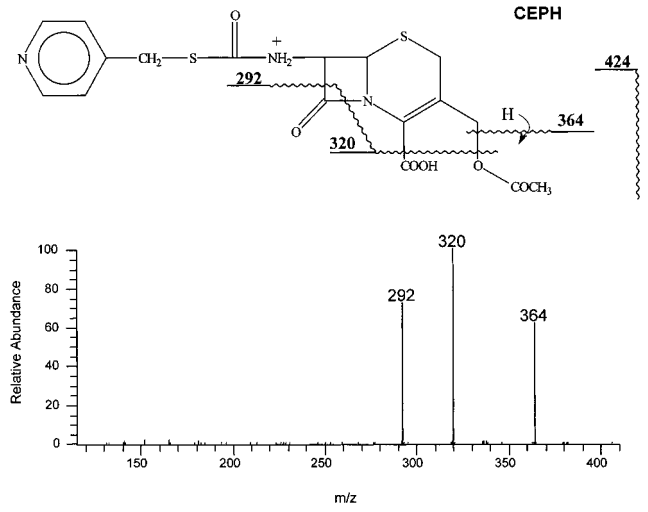


**Figure 5.** Cephapirin, fragmentation pattern, and MS/MS spectrum from peak appearing in high-level incurred milk sample (LC conditions II).

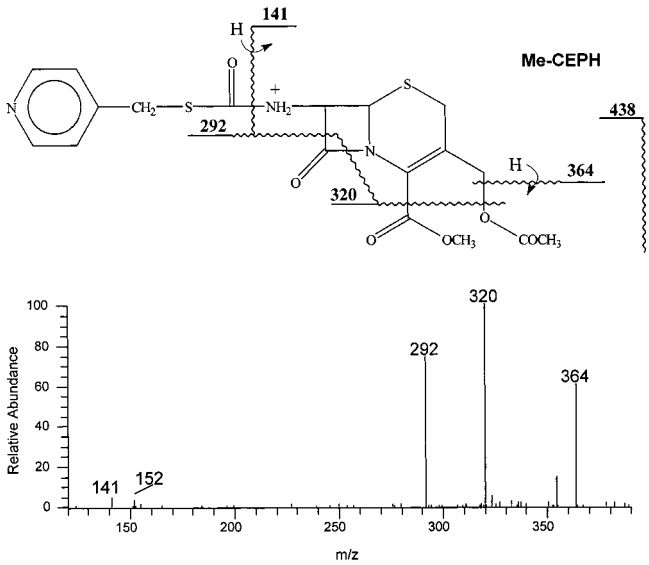


**Figure 6.** Methylcephapirin, proposed fragmentation pattern, and MS/MS spectrum from peak appearing in high-level incurred milk sample (LC conditions II).

phase was ammonium formate buffer/methanol in a 95:5 ratio; on injection, the mobile phase was ramped to a 20:80 ratio over 5 min. This ratio was held for 2two min, and then the column was reequilibrated at 95:5 for 4 min. After using either LC system, the column was flushed with 50:50 MeOH/water and then 95% MeOH for 15 min each at the end of the day.

## RESULTS AND DISCUSSION

We examined the full-scan data for fragment ions indicative of CEPH, DAC, and LAC, as well as peaks that correlated with these ions. Ions indicative of CEPH included *m/z* 364, 320, 292, 253, 226, 209, 152, and 112. Full-scan spectra were obtained after the subtraction of time ranges on each side of suspect peaks, and molecular species ($MH^+$ ions) were tentatively identified.

Data-dependent scanning was used in two modes to acquire MS/MS spectra of potential CEPH residues. First, MS/MS spectra were acquired from any ions giving an above-threshold response in a full mass range prescan. From the full-scan MS data, a list of candidate $MH^+$ ions was created for follow-up data-dependent

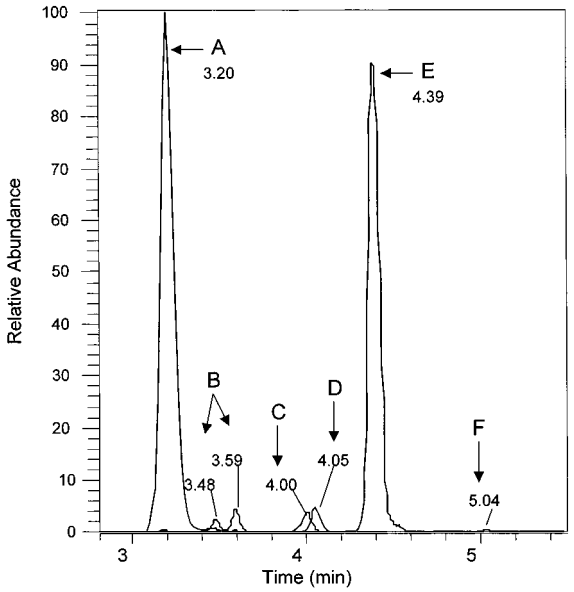


**Figure 7.** Overlay of product ion chromatograms for CEPH residues in an incurred milk extract sample (LC conditions II). The sample contained >1 ppm of CEPH as measured by LC-UV. Chromatograms are normalized to the highest peak. Peaks correspond to summed product ion chromatograms as follows: for DAC (A, *m/z* 292 + 320 + 364), HydroLAC (B, *m/z* 294 + 338), diH-LAC (C, 158 + 209 + 253), LAC (D, *m/z* 152 + 193 + 226), CEPH (E, 292 + 320 + 364), or Me-CEPH (F, *m/z* 292 + 320 + 364).

scanning. When any of these ions gave an above-threshold response in the prescan, MS/MS scanning was triggered.

This technique for examining samples was applied to CEPH standard, a CEPH standard that had been degraded by exposure to 0.1% formic acid for 2 h, and extracts of milk containing >1 ppm of incurred CEPH, as measured by LC-UV (Schermerhorn et al., 1998). Eight metabolite/degradant peaks were tentatively identified in these solutions. Their relative reponses varied widely. A time-scheduled MS/MS acquisition method was created to focus specifically on the peaks seen in incurred milk extracts.

Figures 1−6 show molecular structures, fragmentation patterns, and product ion spectra for compounds tentatively identified in incurred milk extracts by LC-MS/MS (LC conditions II). All data shown in the figures were obtained with time-scheduled MS/MS. In order of elution, these peaks are identified as desacetylcephapirin (DAC, 1), hydrolyzed cephapirin lactone (Hydro-LAC, 2), dihydrogen cephapirin lactone (diH-LAC, 3), cephapirin lactone (LAC, 4), cephapirin (CEPH, 5), and cephapirin methyl ester (me-CEPH, 6).

The identity of DAC in milk extracts (Figure 1) was confirmed by comparing product ion spectra from extracts with the standard obtained from Bristol-Myers Squibb. Two subsequent peaks at the same molecular weight were probably isomers of hydrolyzed lactone (Hydro-LAC), as each gave the same mass spectrum (Figure 2). Hydrolysis of the $\beta$-lactam ring is a typical degradation pathway of these compounds (Suwanrumpha and Freas, 1989). The proposed structure is supported by the facile loss of $CO_2$ from $MH^+$ in both cases, yielding an ion at *m/z* 338 (Figure 2).

We tentatively identified LAC in milk extracts, on the basis of its $MH^+$ ion and product ion spectrum (Figure 4). We also detected a compound weighing 2 amu more than LAC, which was most likely LAC reduced by the addition of two hydrogens (Figure 3, diH-LAC). On the basis of the product ion mass spectrum, only two sites for this reduction are possible: the carbonyl or double bond of the lactone ring. Reduction of the carbonyl bond may be more likely, as there is a facile loss of water from the precursor ion. The suspect diH-LAC peak was found in CEPH and DAC standards, in fortified milk, and in incurred milk. This form of cephapirin residue has not been reported before, to our knowledge. It was capable of being fully separated from LAC using a slow gradient (not shown).

A compound weighting 14 amu more than CEPH, but giving nearly the same product ion spectrum, was

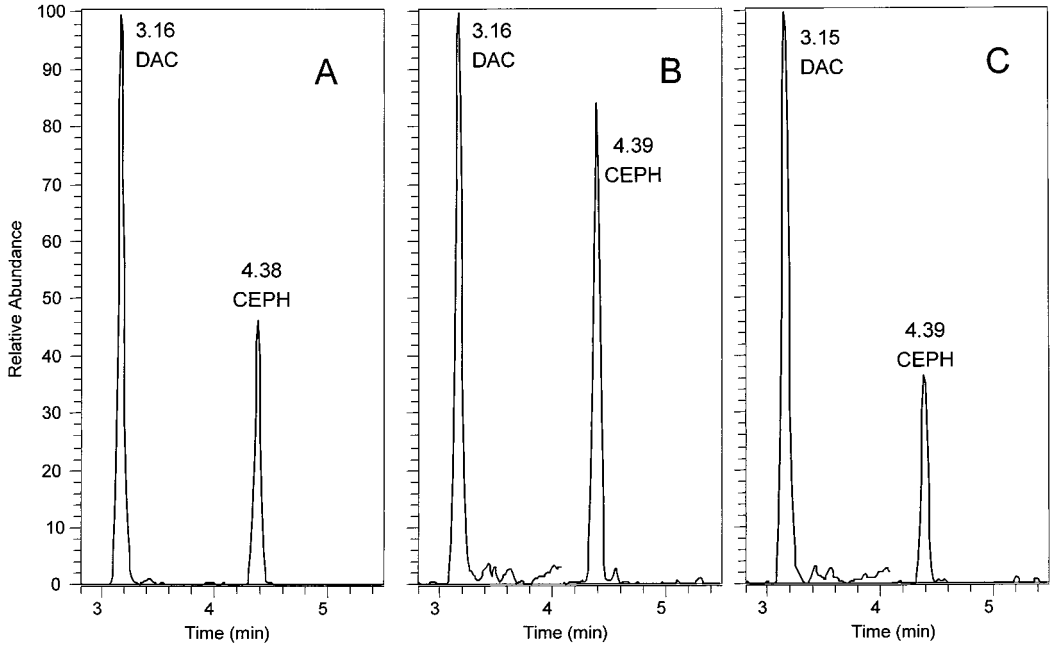


**Figure 8.** Normalized product ion chromatograms from DAC and CEPH in incurred milk extracts (LC conditions I). CEPH levels were measured by LC-UV as follows: A, 14 ppb; B, 3 ppb; C, not detected. Peaks correspond to summed product ion chromatograms as follows: for DAC, *m/z* 292 + 320 + 364, or CEPH, 292 + 320 + 364.

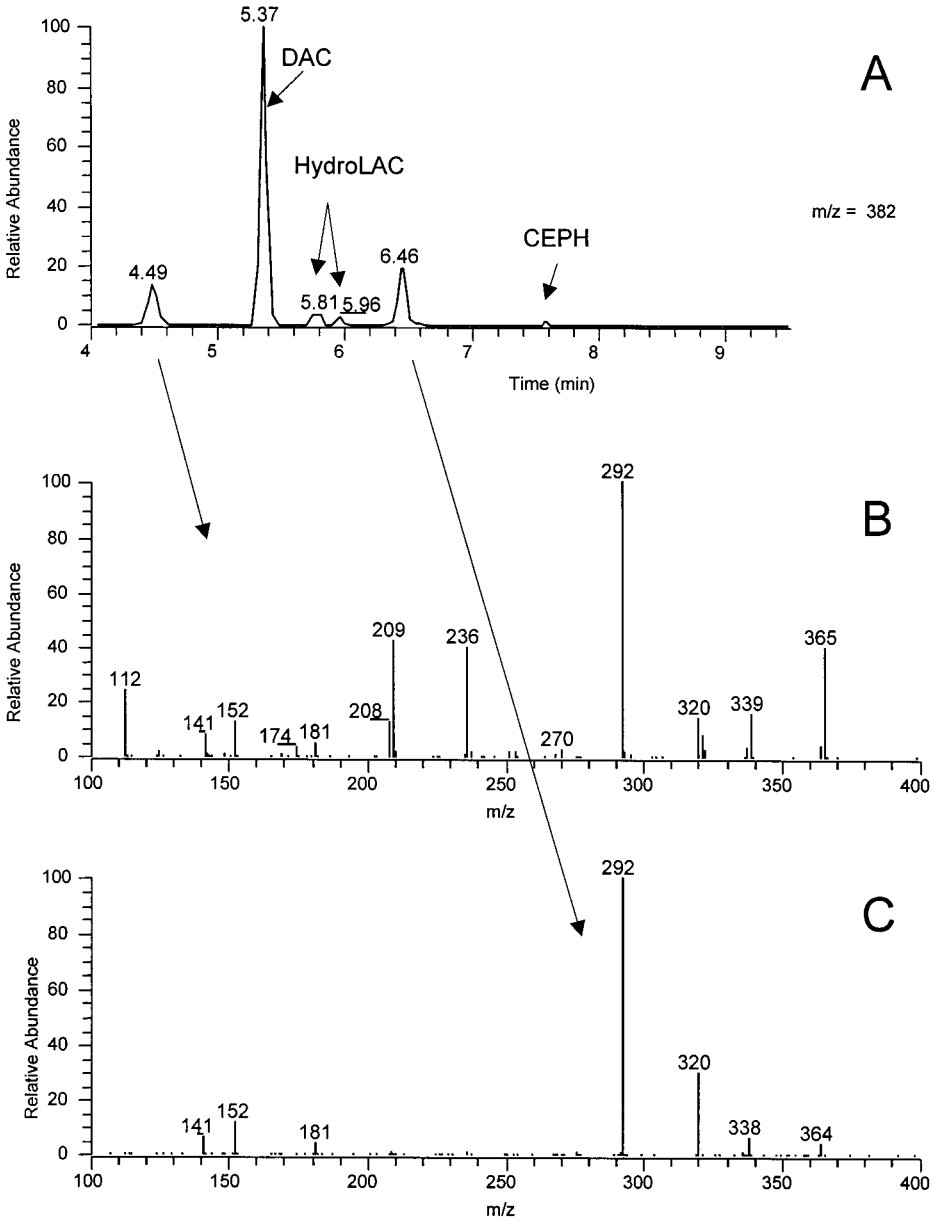


**Figure 9.** Fragment ion chromatograms (A) and MS/MS spectra (B, C) from two metabolite/degradant peaks in acid-degraded CEPH for which structures could not be deduced (LC conditions I). The ion chromatogram in (A) is from *m*/*z* 382, using full-scan MS acquisition. Compound B appeared in acid-degraded and residue-incurred milk. Compound C appeared only in acid-degraded standard.

assumed to be the methyl ester of CEPH (Me-CEPH). This peak was also observed in the drug standard and may be a trace contaminant in the manufactured standard.

Figure 7 shows selected ion chromatograms from the six identifiable compounds observed in an extract of milk containing >1 ppm of CEPH. The chromatograms were generated by summing two or three product ions arising from the selected precurson ion. The primary peaks are DAC and CEPH, which accords with observations of other researchers (Tyczkowska et al., 1991; Moats et al., 2000). Figure 8 shows several low-level incurred samples for which only CEPH and DAC were monitored. The ion chromatogram traces represent milk extracts in which CEPH was measured by LC-UV at 14 ppb, 3 ppb, or not detected, respectively. The persistence of DAC in the milk is evident.

Two other peaks were detected for which unique structures could not be deduced. Analyzed by LC conditions I, these peaks eluted before DAC (Figure 9B) and between hydro-LAC and diH-LAC (Figure 9C), respectively. The first unknown (Figure 9B) was detected at relatively low levels in both incurred milk extracts and acid-degraded standard, but the second unknown (Figure 9C) was detected only in acid-degraded standard. The unknown milk peak of Figure 9B may be due to a rearrangement of DAC. This rearranged product must contain a primary amine and/or amide moiety, which could be readily lost, based on the facile loss of 17 and 43 amu. Rearrangement might involve cleavage of the S−N heterocyclic ring because the product ion at *m*/*z* 236 is interpretable as the entire $\beta$-lactam ring with its amide-linked substituents. However, these clues did not allow for complete structural identification of this peak.

The sensitivity for CEPH using ESI-LC-MS/MS was very good. The limit of confirmation for CEPH was ~1 ppb, and detection limits were <0.5 ppb. This compares well to LC-UV methods for CEPH with limits of detection of 1 ppb (Schermerhorn et al., 1998) or 2 ppb (Tyczkowska et al., 1991). Figure 8C shows that LC-

MS/MS could still detect CEPH when it had depleted below the LC-UV detection limit.

We performed a preliminary evaluation of the LC-MS/MS approach for quantitation of CEPH in milk. Standard curves were prepared equivalent to the range 0.5−40 ppb of CEPH in milk, either by diluting in water (pure standard), spiking into extracts of control milk (matrix standard), or fortifying into milk prior to extraction (processed standard). In addition, a homologous compound with a later elution time, cephalothin (CPTH), was added at 10 pbb in all cases for testing as an internal standard.

First, linearity was assessed in external standard calibration mode. The linearity of pure, matrix, and processed standard curves was good, marginally acceptable, and less than adequate, respectively. Calculations using CPTH as an internal standard calibrant did not much improve the linearity of the matrix or processed standards and made the pure standard case worse. When the matrix standards were compared with pure standards, a matrix effect became apparent: a tendency for coeluting extractants to alter CEPH's ionization efficiency by as much as 20%. Using a matrix standard curve is one way to overcome such a matrix effect. Comparison of matrix versus processed standards revealed a consistent recovery of ~65% CEPH, with an estimated limit of quantitation of 1 ppb. It is possible to use processed standards to compensate for losses on extraction and thus to improve accuracy, but our observations suggest this might be at the cost of lowered precision and linearity.

Our LC-MS work was mainly concerned with qualitative and methodological issues. It was an important result that DAC and CEPH were the major residues observed in milk, even though other minor residues could be detected. Milk from multiple animals was analyzed, but the quantitative data needed for comparing depletion profiles among various residues or animals were not acquired. A quantitative LC-MS/MS procedure for CEPH, DAC, and other residues (if standards are available) could address important questions regarding test kit sensitivity and microbial susceptibility to CEPH residues in milk and could potentially distinguish between metabolism and degradation processes. Advantages of LC-MS/MS for quantitation would include relatively simple cleanup, lower detection limit, higher specificity, shorter run times, and the ability to detect trace metabolites and degradants in the presence of coextracted material.

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