Solid-Phase Extraction Followed by Liquid Chromatography−Mass Spectrometry for Trace Determination of β-Lactam Antibiotics in Bovine Milk

Federica Bruno, Roberta Curini, Antonio Di Corcia,* Manuela Nazzari, and Roberto Samperi

Dipartimento di Chimica, Università La Sapienza, Piazza Aldo Moro 5, 00185 Roma, Italy

A confirmatory assay able to unambiguously identify and quantify 10 approved-for-use β-lactam antibiotics in milk below stipulated U.S. and EU tolerance levels is presented. β-Lactams are extracted from 10 mL of intact milk by a Carbograph 4 cartridge. After solvent removal, residue reconstitution, and filtration, a completely transparent and uncolored extract is injected into a liquid chromatography −mass spectrometry (LC−MS) instrument equipped with an electrospray (ES) ion source and a single quadrupole. During the chromatographic run, the ES/MS system is operated first in the positive-ion mode (PI) and then in the negative-ion (NI) mode. This is done to circumvent matrix interferences resulting in remarkable signal weakening of the last-eluted analytes, when detecting them as $[M+H]^+$ adduct ions. MS data acquisition is performed by a time-scheduled three-ion selected ion monitoring program. At the 5 ng/mL level, recoveries of the β-lactams are between 70 (nafcillin) and 108% (cephalin), with relative standard deviations ranging between 5 (oxacillin) and 11% (amoxicillin and ceftiofur). The response of the ES/MS detector is linearly related to injected amounts up to 500 ng, irrespective of the chemical characteristics of the β-lactams and the acquisition mode selected (PI or NI modes). Limits of quantification, based on a minimal value of the signal-to-noise ratio of 10, were estimated to be within 0.4 (cephalin) and 3 ng/mL (dicloxacillin). Analyses of milk samples taken after intramammary application of amoxicillin showed that 1.2 ng/mL of this penicillin was still present 6 days after treatment. At this concentration level, the identification power of the method is not weakened, as signals of the three product ions of amoxicillin are still well distinguishable from the background noise.

Keywords: *β-Lactam antibiotics; milk; solid-phase extraction; liquid chromatography−mass spectrometry*

INTRODUCTION

Antibiotic residues in milk, besides inhibiting start of cultures in the production of milk products, can provoke allergic reactions in some hypersensitive individuals. Even more important, low-level doses of antibiotics in foodstuff for long periods has led to the problem of the spread of drug-resistant microorganisms. β-Lactam antibiotics, i.e., penicillins and cephalosporins, are probably the most widely used class of medicines in the treatment of lactating dairy cattle for several infections. To minimize exposure of humans to β-lactam antibiotics, MRL values were established in EEC regulation 2377/90 and subsequent modifications. For milk, these values range between 4 and 125 ng/mL, depending on the specific nature of the β-lactam antibiotic.

For detecting antibiotic residues in milk, bioassay techniques are widely used as screening methods. These methods generally do not distinguish between β-lactams; they provide only semiquantitative measurements of residues detected and sometimes give rise to false positives. Nevertheless, they continue to be used because of their simplicity and economy. But, before samples are condemned for containing levels of β-lactams exceeding the tolerance levels, it is well recognized that these methods need to be supported by highly selective and sufficiently sensitive chemical methods. Carson et al. (*1*) evaluated several proposed gas chromatography (GC) and liquid chromatography (LC) methods using conventional detectors for the analysis of β-lactams in milk and concluded that none of the tested procedures satisfies the requirements for a suitable multi-residue analytical method. The U.S. Food and Drug Administration, as well as public health agencies in other countries, relies on detection by mass spectrometry (MS) for unambiguous confirmation of β-lactam antibiotics in foodstuff. Recent introduction of robust, sensitive, and relatively inexpensive LC−MS benchtop instrumentation with an electrospray (ES) ion source has brought this equipment within the scope of many analytical laboratories. Starting in 1993, several methods based on LC−ES−MS have been proposed for the analysis of β-lactams in bovine milk (*2*−*7*). However, none of these methods satisfies the demand for simultaneous determination of the most commonly used β-lactam antibiotics at levels equal to or below applicable MRLs.

The purpose of this work has been to develop a sufficiently sensitive and selective method for reliable quantification of 10 approved-for-use β-lactams in bovine milk at tolerance levels. This simple method involves analyte extraction from intact milk by solid-phase extraction (SPE) with a Carbograph 4 cartridge followed by LC−ES−MS with a single quadrupole.

* To whom correspondence should be addressed. Fax: +39-06-490631. E-Mail: dicorcia@uniroma1.it.

10.1021/jf010046r CCC: $20.00
Published on Web 06/27/2001

Table 1. Time-Scheduled Three-Ions SIM Conditions for Detecting β-Lactam Antibiotics in Milk

compound	channel mass, *m/z* (relative abundance)	cone voltage, V	retention window, min	ionization mode
amoxicillin	208 (40), 349 (100), 366 (30)	30	0−13	PI[a]
cephapirin	292 (100), 364 (25), 424 (60)	30	-	PI
cephalin	158 (40), 192 (30), 348 (100)	25	13−15.5	PI
ampicillin	160 (30), 192 (30), 350 (100)	30	15.5−20	PI
ceftiofur	241 (80), 285 (30), 524 (100)	35	20−24	PI
penicillin-G	192 (60), 289 (25), 333 (100)	20	24−28.5	NI[b]
penicillin-V (IS)	208 (40), 305 (20), 349 (100)	20	28.5−32	NI
oxacillin	259 (50), 356 (40), 400 (100)	20	-	NI
cloxacillin	293 (40), 390 (40), 434 (100)	20	-	NI
dicloxacillin	327 (25), 424 (30), 468 (100)	20	32−35	NI
nafcillin	272 (60), 369 (20), 413 (100)	20	-	NI

[a] PI, positive ionization. [b] NI, negative ionization.

MATERIALS AND METHODS

Reagents and Chemicals. Amoxicillin, cephapirin, cephalin, ampicillin, penicillin-G, oxacillin, cloxacillin, nafcillin, and dicloxacillin were purchased from Sigma, St. Louis, MO. Ceftiofur was a gift from Pharmacia and Upjohn, Kalamazoo, MI. Penicillin V (Sigma) was used as internal standard (IS), as it is not used for veterinary treatment. Individual standard solutions of the analytes and the IS were prepared by dissolving each compound in water to obtain 200 μg/mL concentration. After preparation, these solutions were stored at 4 °C in the dark to minimize analyte degradation. They were freshly prepared every two weeks. A composite working standard solution of the target compounds was prepared by mixing the above solutions and diluting with acetonitrile to obtain analyte concentrations of 10 μg/mL. The same was done with the IS aqueous solution. When unused, these two solutions were stored at 4 °C in the dark and renewed after one week.

For LC, distilled water was further purified by passing it through the Milli-Q Plus apparatus (Millipore, Bedford, MA). Methanol Plus of gradient grade was obtained from Carlo Erba, Milano, Italy. All other solvents and chemicals were of analytical grade (Carlo Erba) and were used as supplied.

Extraction Apparatus. Extraction cartridges filled with 0.5 g of Carbograph 4 and drilled cylindrical Teflon pistons with indented bases and Luer tips for analyte elution in the back-flushing mode (*8*) were supplied by LARA, Rome, Italy. Carbograph 4 is an example of the graphitized carbon blacks sorbent family having a surface area of about 200 m^2/g. It is commercially referred to also as Carboprep (Restek, Bellefonte, PA). The SPE cartridge was fitted into a side−arm filtration flask, and liquids were forced to pass through the cartridge by vacuum (water pump). Before processing milk samples, the cartridge was washed with 20 mL of water acidified with HCl (pH 2) followed by 5 mL of distilled water.

Milk Samples. For recovery studies, pasteurized, homogenized whole milk samples were purchased from retail markets. Preliminary analyses showed they were analyte-free. Raw milk samples were kindly supplied by Dr. R. Condoleo, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana. One sample was antibiotic-free, whereas the other milk samples were taken 3, 4, 5, and 6 days after intramammary application of amoxicillin.

Sample Preparation. For recovery studies, milk samples were spiked with known and varying amounts of the analytes. After 15 min of equilibration, milk was passed through the SPE cartridge at a flow-rate of 1−1.5 mL/min by the aid of a water pump. Thereafter, the cartridge was washed with 5 mL of water that had been used to rinse the milk-containing vial, followed by another 20 mL of fresh water at the flow rate of 1−1.5 mL/min. Most water was eliminated from the cartridge by forcing room air for 1 min. Residual water was eliminated by slowly passing 0.5 mL of methanol. Larger amounts of methanol resulted in some loss of the two amphoteric penicillins, i.e., amoxicillin and ampicillin. Following the passage of methanol, the pressure was again decreased to the minimum for 1 min. Thereafter, the Teflon piston was forced to enter the cartridge until it reached the upper frit. The trap was turned upside down, a 1.4-cm i.d. glass vial with a conical bottom was placed below it, and analytes were back-eluted by passing through the trap 1.5 mL of methanol followed by 6 mL of a methylene chloride/methanol (80:20, v/v) solution acidified with 50 mmol/L formic acid. The flow-rate at which the eluent phase was percolated through the cartridge was ca. 6 mL/min obtained by suitably regulating the vacuum in the extraction apparatus. The last drops of this solvent mixture were collected by a further decrease of the pressure inside the flask. Before removing solvents in a water bath at 40 °C under a nitrogen stream, 25 μL of the solution containing the IS was added to the eluate. This solution was taken to dryness with the precaution of removing the vial from the water bath immediately after complete solvent elimination. The residue was reconstituted with 300 μL of a water/methanol solution (90:10, v/v) basified with 0.1 mol/L sodium acetate, and this solution was passed through a Teflon filter (pore size 0.45 μm, 13 mm d, Alltech). After filtration, a completely uncolored and transparent solution was obtained. A 50-μL portion of it was injected into the LC analytical column.

LC−ES−MS Analysis. Liquid chromatography was carried out with a Thermoquest (Manchester, U. K.) model P2000 equipped with a Rheodyne Model 7125 injector having a 50-μL loop. The analytes were chromatographed on an Alltima 25 cm × 4.6 mm i.d. column filled with 5-μm C-18 reversed-phase packing (Alltech, Sedriano, Italy). For fractionating the analytes, phase A was methanol and phase B was water. Both phases were acidified with 10 mmol/L formic acid. The initial composition of the LC mobile phase was 5% A, which was linearly increased to 30% in 10 min, then to 40% in 3 min, and finally to 70% in 20 min. The flow rate of the LC eluent was 1 mL/min and 250 μL of the column effluent was diverted to the ES source. A Finnigan AQA benchtop mass spectrometer (Thermoquest) consisting of a pneumatically assisted ES interface and a single quadrupole was used for detecting and quantifying target compounds in the LC column effluent. The probe temperature was 230 °C and the capillary voltage was 4 Kv. During the chromatographic run, the ES/MS system was first operated in the positive ionization mode and then in the negative ionization mode. For each analyte, diagnostic fragment ions were obtained by in-source collision-induced dissociation (CID) of the relative quasi-molecular ion by suitably adjusting the voltage of the skimmer cone. Ion signals were acquired by the time-scheduled three-ions selected ion monitoring (SIM) mode (Table 1).

Quantitation. Recovery of each analyte added to milk at any given concentration was assessed by selecting the sum of the ion current profiles for both parent and fragment ions, measuring the peak area relative to that of the IS, and comparing this result with that obtained for a reference solution containing the same nominal analyte quantities and the internal standard. Quantitation of amoxicillin in farm milk was performed by the external standard procedure. Standard solutions at five concentration levels were prepared as reported above. The peak area relative to that of the IS versus injected amount chart was then obtained as described above.

The mass spectrometry data handling system used was the Mass Lab software from Thermoquest.

Table 2. Average Recovery (%) of β-Lactam Antibiotics Added to Milk Samples at Level of 10 ng/mL by Following Two Different Sample Preparation Procedures

	procedure A[a]	procedure B[b]	
compound	pasteurized milk	pasteurized milk	raw milk
amoxicillin	22 (9)[c]	88 (8)	81 (9)
cephapirin	84 (6)	102 (5)	96 (5)
cephalin	62 (6)	105 (8)	96 (7)
ampicillin	27 (6)	100 (7)	94 (8)
ceftiofur	77 (6)	69 (8)	66 (8)
penicillin-G	86 (5)	96 (6)	91 (7)
oxacillin	82 (5)	87 (5)	86 (5)
cloxacillin	94 (6)	92 (4)	85 (5)
dicloxacillin	102 (7)	88 (7)	84 (8)
nafcillin	66 (6)	68 (6)	65 (7)

[a] Procedure A: deproteinization of 10 mL of milk with 30 mL of acetonitrile, solvent reduction to 2 mL, water dilution to 20 mL, and percolation of this solution through the Carbograph 4 cartridge. [b] Procedure B: as described in the Materials and Methods section. [c] Average recovery ($n = 4$), RSD in parentheses.

RESULTS AND DISCUSSION

Recovery Studies. Chromatographic methods developed for β-lactams in milk are generally based on deproteinization with acetonitrile, solvent removal by evaporation, and cleanup treatment by a SPE cartridge. As an alternative to this lengthy procedure, Terada and Sakabe (*9*) proposed a simplified analytical scheme for analyzing three penicillins where intact milk was directly passed through a C-18 extraction cartridge. The extract was then analyzed by LC with UV detection at 210 nm. However, the use of a nonselective instrument made determination of penicillins difficult to be performed at statutory levels due to the presence of endogenous co-extractives which interfered with the analysis. In terms of recovery and speed of analysis, we evaluated comparatively which of the two sample preparation strategies was the more convenient for analyzing β-lactams in milk. In one case (procedure A), a 10-mL portion of whole milk spiked with the analytes at 10 ng/mL individual level was deproteinized with 30 mL of acetonitrile. After centrifugation, the supernatant was first concentrated to about 2 mL, then diluted with water to 20 mL, and this solution was passed through the Carbograph 4 cartridge. From here onward, the sample was carried through the procedure reported in the Materials and Methods section. In the second case (procedure B), a 10-mL aliquot of the same milk sample was directly percolated through the Carbograph 4 cartridge, as reported above. In both cases, four experiments were performed, and the results are reported in Table 2. When milk was submitted to preliminary deproteinization treatment, unacceptable low recoveries of the two amphoteric penicillins, i.e., amoxicillin and ampicillin, were obtained. No additional amount of them was recovered by adding another 15 mL of acetonitrile to the precipitate or by passing the milk effluent through a second Carbograph 4 cartridge. Following a fairly analogous analytical scheme, Heller and Ngoh (*5*) obtained comparable low recovery of the two above penicillins. Probably, the two amphoteric penicillins are so tightly bound to proteins that they remain entrapped in the protein precipitate. Table 2 also shows that the procedure adopted by us is only slightly affected by the type of milk submitted to analysis.

As reported in the Materials and Methods section, re-extraction of the analytes was performed by back-flushing the Carbograph 4 sorbent cartridge with the eluant phase. This expedient is advantageously practiced to quantitatively re-extract, with a moderate eluant phase volume, even those compounds that, having a large affinity for the sorbent surface, are slowly eluted by any eluant system. With low eluate volumes, time for solvent removal is saved and risk of degradation of chemically labile species, such as β-lactams, is reduced. On the other hand, this procedure may appear cumbersome to those who are not familiar with the SPE technique. For the analytes considered, we evaluated whether backward elution of the analytes could be avoided and replaced by conventional forward elution. For this purpose, 10-mL portions of milk fortified with 10 ng/mL of β-lactams were analyzed in duplicate as reported above with the differences that analyte re-extraction was performed without reversing the sorbent cartridge and that 10 mL of the eluant phase was used instead of 6 mL. Compared to the procedure involving back-flush elution, that involving forward elution gave recovery of nafcillin three times lower, but recoveries of the other analytes did not differ significantly. Therefore, if nafcillin is not to be analyzed, a conventional re-extraction procedure could be adopted without affecting analyte recovery.

We varied the flow rate at which the sample and the subsequent 25 mL of distilled water were passed through the extraction cartridge. About 15% of both amoxicillin and ampicillin were unaccounted for in the milk and water effluents when the flow rate was increased from 1−1.5 mL/min to 3−4 mL/min, but recoveries of the other analytes remained unchanged. We believe that a high flow rate partly hinders desorption of the amphoteric penicillins from proteins and subsequent adsorption onto the Carbograph-4 surface.

With a view of developing a method able to determine β-lactam trace levels in milk, increasing milk volumes spiked with 10 ng/mL of each analyte were analyzed as reported in the Materials and Methods section. At each milk volume considered, duplicate measurements were performed, and the results are shown in Figure 1. At sample volumes larger than 10 mL, a remarkable decrease of the recovery of amoxicillin and cephapirin was already observed, while loss of the other analytes occurred on analyzing milk volumes larger than 20 mL. This result appeared rather surprising to us, considering that β-lactams dissolved in 2 L of tap water were quantitatively extracted by the sorbent cartridge (data not shown here). In the past, we observed that a large fraction of proteins pass through a Carbograph cartridge unretained (*10*). To a greater or lesser extent, β-lactams are bound to proteins by various types of interactions. Bearing this in mind, it is conceivable that, when excessive milk volumes are passed through the SPE cartridge, analytes are partially carried away by proteins and lost in the milk effluent. Anyway, processing 10 mL of milk sufficed to quantify most of the β-lactam antibiotics at levels lower than 1 ng/mL (see below).

Matrix Effect. It is generally accepted that sufficient confirmatory evidence for the presence of a target compound by the LC−MS technique is obtained when, compared to a reference standard analyzed under the same experimental conditions, (i) the LC retention time agrees within 2%, and (ii) the absolute relative abundances of signals of the product ions (the molecular ion plus two diagnostic fragment ions) agree within 20% (*11*). When analyzing milk volumes larger than 10 mL, besides a progressive loss of the analytes in the milk

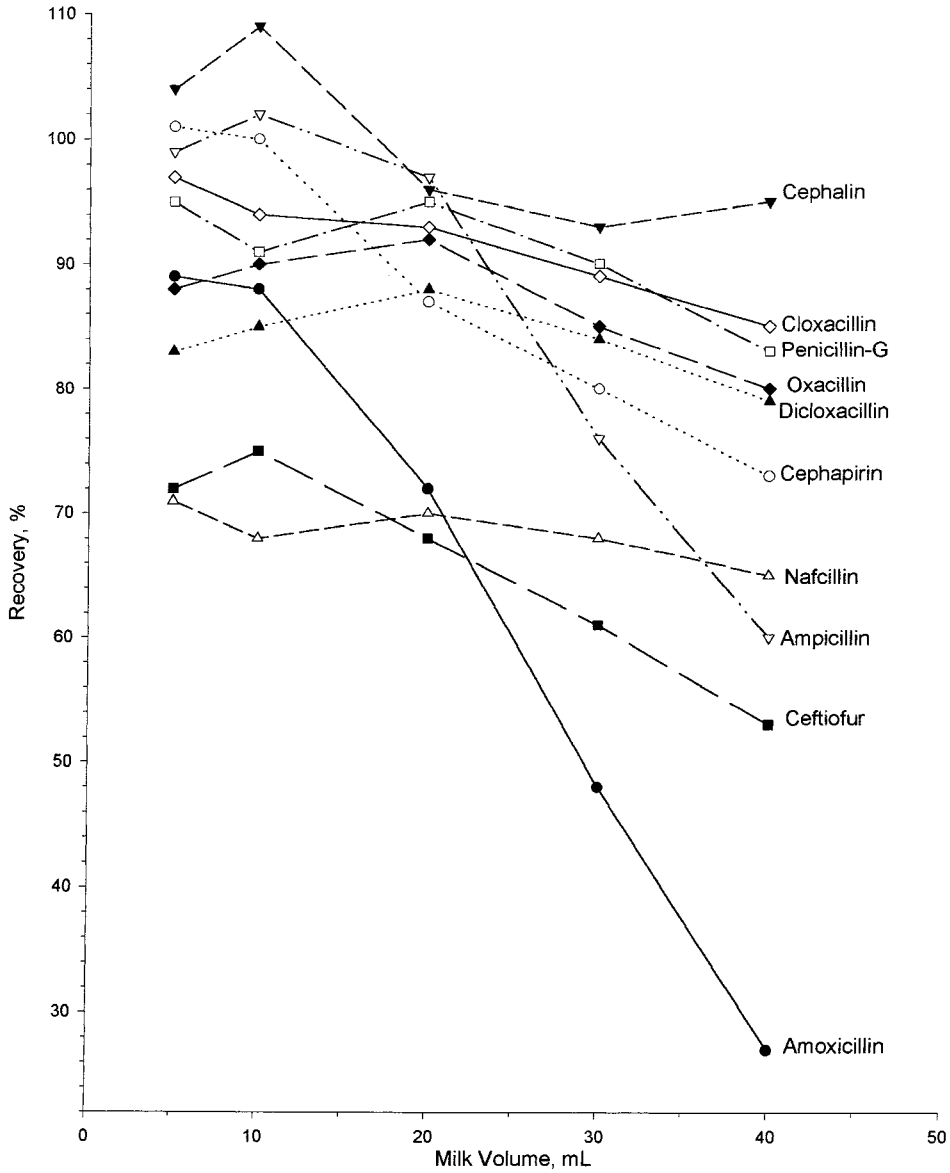

Figure 1. Recovery of 10 ng/mL of β-lactams in milk by varying the sample volume.

effluent, we observed also that the relative abundances of the signals of product ions of cephalin and ceftiofur did not meet the second criterion reported above for unambiguous identification of a targeted compound. For both compounds, abundances of the two fragment ions relative to that of the molecular ion drastically decreased, as compared to spectral data of the reference standard. Analysis of a control milk sample showed that no individual matrix component interfered with the analysis of the above two analytes. We believe this effect could be ascribed to coelution of the two analytes with abundant amounts of some unknown milk matrix components originating ions different from those of the two analytes. In the fragmentation chamber, co-extractive molecular ions could interfere with the in-source CID process by acting as a shield which partially hinders collision of parent ions of the above two analytes with nitrogen molecules.

The ES process is able to produce both negatively and positively charged gas-phase ions of β-lactam antibiotics. According to Straub and Voyksner (*2*), we observed that ion signals of gas-phase $[M+H]^+$ ions of β-lactams were 2-3 times more intense than those of $[M-H]^-$ ions, irrespective of the composition of the LC mobile phase.

Table 3. Recovery of 10 ng/mL of β-Lactam Antibiotics in Milk by Acquiring Ion Signals in Either the Positive-Ion (PI) Mode or the Negative-Ion (NI) Mode

	recovery[a], %	
compound	PI mode	NI mode
amoxicillin	84	82
cephapirin	99	101
cephalin	107	104
ampicillin	95	97
ceftiofur	71	69
penicillin-G	12	97
oxacillin	32	85
cloxacillin	21	88
dicloxacillin	68	90
nafcillin	67	69

[a] Mean values from triplicate measurements.

Nevertheless, for the purpose of evaluation, preliminary recovery studies were conducted with the ES/MS system operated simultaneously in both positive ionization (PI) and negative ionization (NI) modes. Recoveries of the analytes were estimated by analyzing a reference standard under the same instrumental conditions. Data reported in Table 3 show that, with the exception of

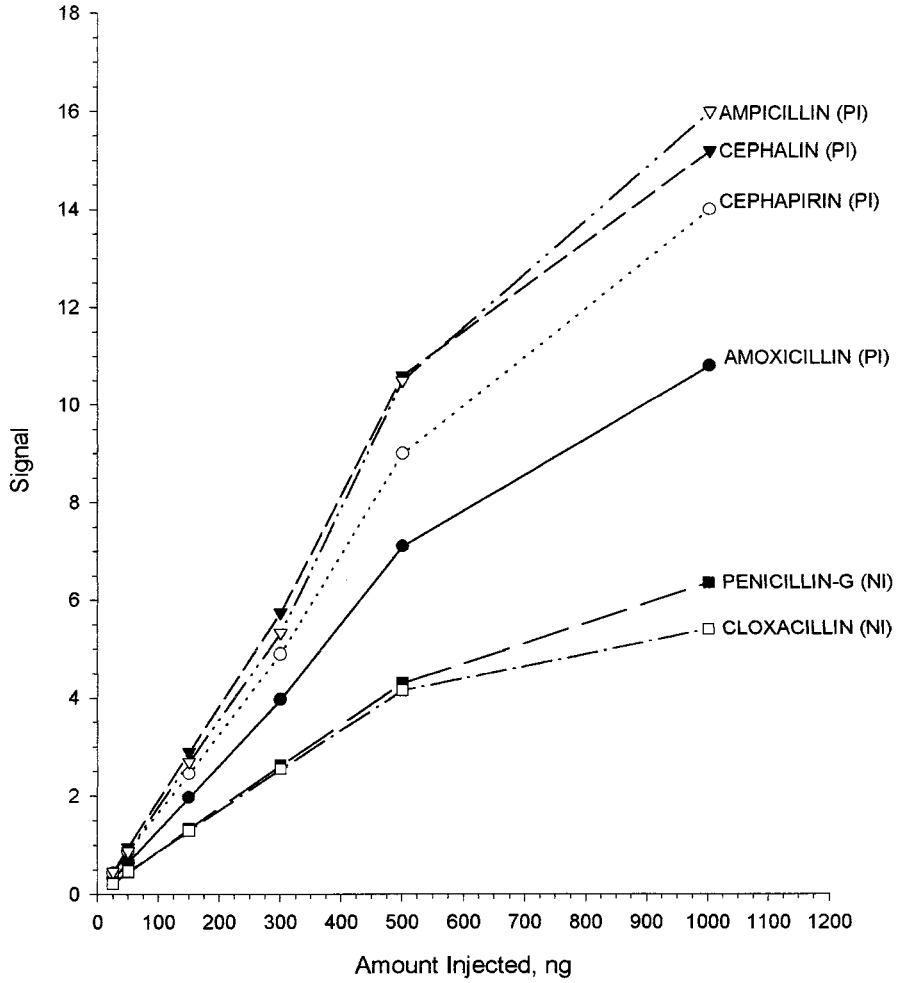

Figure 2. Signal vs amounts of some selected β-lactams injected into the LC column. PI (positive ion) and NI (negative ion) refer to the acquisition mode selected.

nafcillin, the apparent recoveries of the last-eluted analytes were strongly dependent on which of the two signals was considered. It was shown (*12, 13*) that generation of gas-phase $[M+H]^+$ adduct ions by the ES process is a critical step, as competition effects between two or more compounds can affect production of molecular ions of that species being least abundant or having the least affinity for the proton. Probably, coelution of natural constituents of milk with penicillin G, oxacillin, cloxacillin, and dicloxacillin was responsible for signal weakening of these β-lactams. This problem was circumvented by setting the ES/MS detector in the NI mode during the second part of the chromatographic run. Under this condition, matrix components did not interfere with the analysis, probably because they were unable to give $[M-H]^-$ ions. Or, the process of formation of negatively charged gas-phase ions from preformed ions is not so critical as that leading to gas-phase protonated molecules.

Linear Dynamic Range. Under the instrumental conditions reported in the Materials and Methods section, the linear dynamic range of the ES/MS detector was estimated for all the analytes. Varying amounts of each compound (from 25 to 1000 ng) and a constant amount of the internal standard (63 ng of Penicillin-V) were injected from suitably prepared standard solutions. At any analyte amount selected, three replicate measurements were performed. Averaging the peak area resulting from the sum of the signals for parent and fragment ions of each analyte, relating this area to that of the internal standard, and plotting the results against the amount injected yielded a graph indicating nearly linear response for β-lactams from 25 to 500 ng. Exemplary curves for some selected analytes are shown in Figure 2. This behavior was not dependent on either the chemical nature of the analyte or the acquisition mode selected (PI or NI). We observed several times in the past that when the ES/MS system was operated in the PI mode for detecting protonated or sodiated adduct ions, the linearity of the response of the detector was already lost by injecting more than 40−70 ng of compounds having a broad range of chemical characteristics

Table 4. Accuracy and Precision of the Method at Varying Concentrations of β-lactam Antibiotics in 10 mL of Pasteurized Whole Milk, Reported as Recovery[a] ± RSD (%)

	concentration, ng/mL			
compound	5	10	50	250
amoxicillin	84 ± 11	89 ± 8	83 ± 6	85 ± 5
cephapirin	98 ± 9	103 ± 5	97 ± 5	95 ± 4
cephalin	108 ± 8	106 ± 6	103 ± 4	99 ± 4
ampicillin	95 ± 10	98 ± 8	94 ± 5	96 ± 7
ceftiofur	71 ± 11	68 ± 8	70 ± 7	67 ± 6
penicillin-G	96 ± 6	98 ± 8	92 ± 4	88 ± 6
oxacillin	89 ± 5	85 ± 5	79 ± 5	83 ± 2
cloxacillin	95 ± 6	92 ± 5	88 ± 4	84 ± 4
dicloxacillin	92 ± 9	88 ± 9	88 ± 4	85 ± 4
nafcillin	70 ± 10	69 ± 8	71 ± 8	68 ± 7

[a] Mean values from six measurements for each analyte concentration considered.

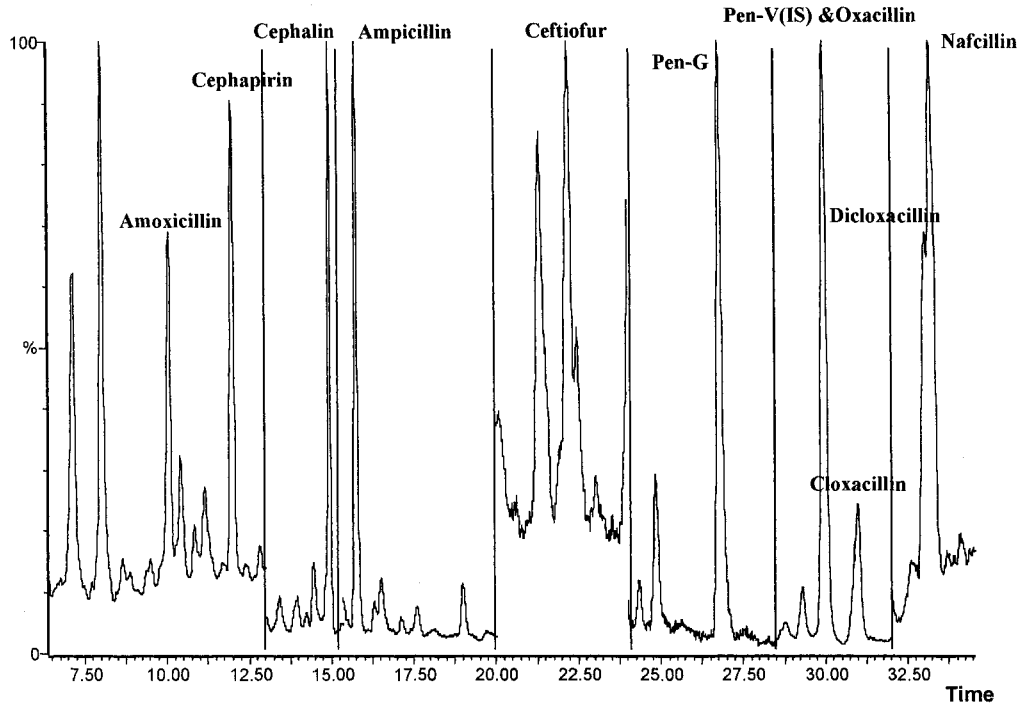

Figure 3. Typical LC−MS SIM chromatogram resulting from analysis of 10 mL of milk spiked with β-lactams at the individual level of 10 ng/mL.

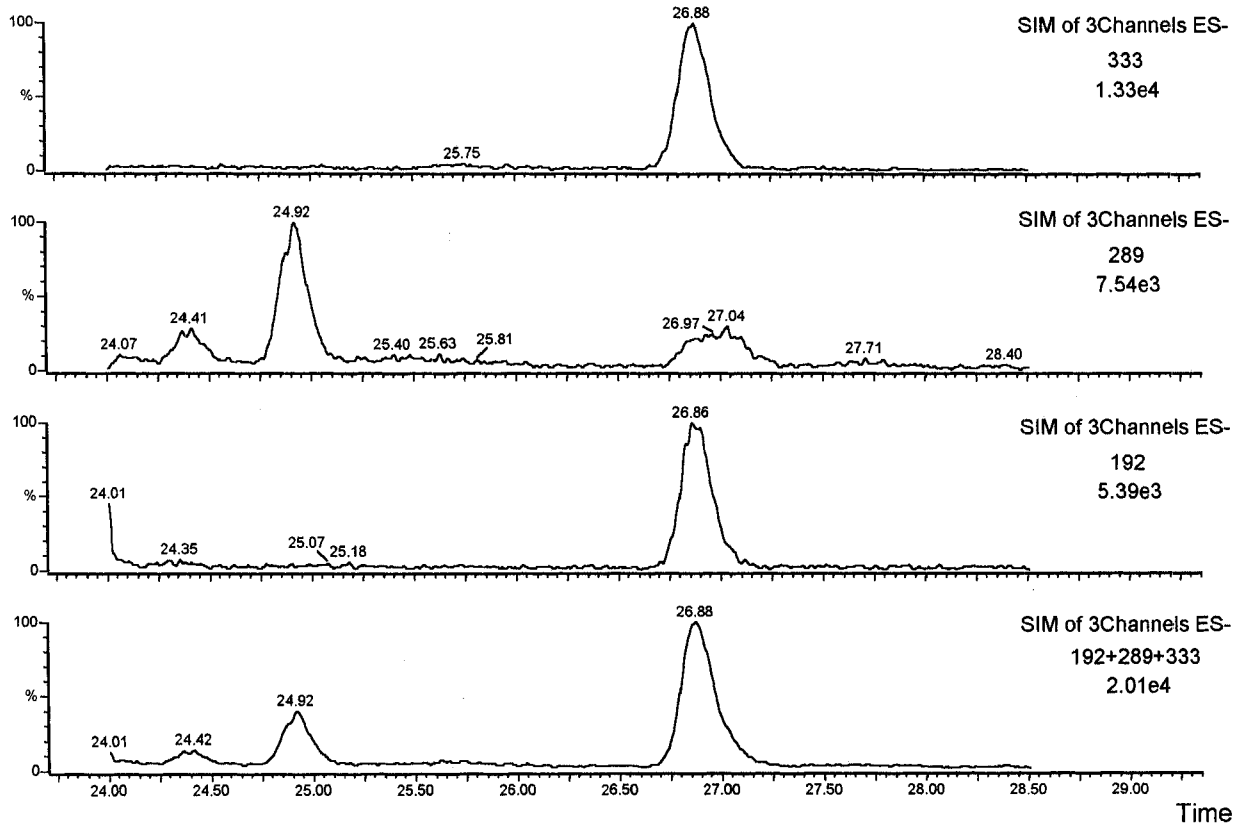

Figure 4. Single and sum of the ion current profiles relative to product ions of penicillin G resulting from analysis of 10 mL of milk spiked with 10 ng/mL of the analyte.

(*13, 14*). On the contrary and according to this work, when acquiring signals of preformed anions in the NI mode, the ES/MS detector gave signals linearly related to injected amounts of various species up to 500−1000 ng (*15, 16*). The anomalous behavior of those β-lactams whose signals were acquired in the PI mode is hard to explain. One could speculate that the expanded range of linearity is somehow related to the particular design of the ES ion source used in this work that is substantially different from that used by us in the past (*13, 14*).

Accuracy and Precision. The accuracy and precision of the method was assessed by analyzing 10-mL aliquots of milk spiked with varying concentrations of the analytes. At any analyte concentration six measure-

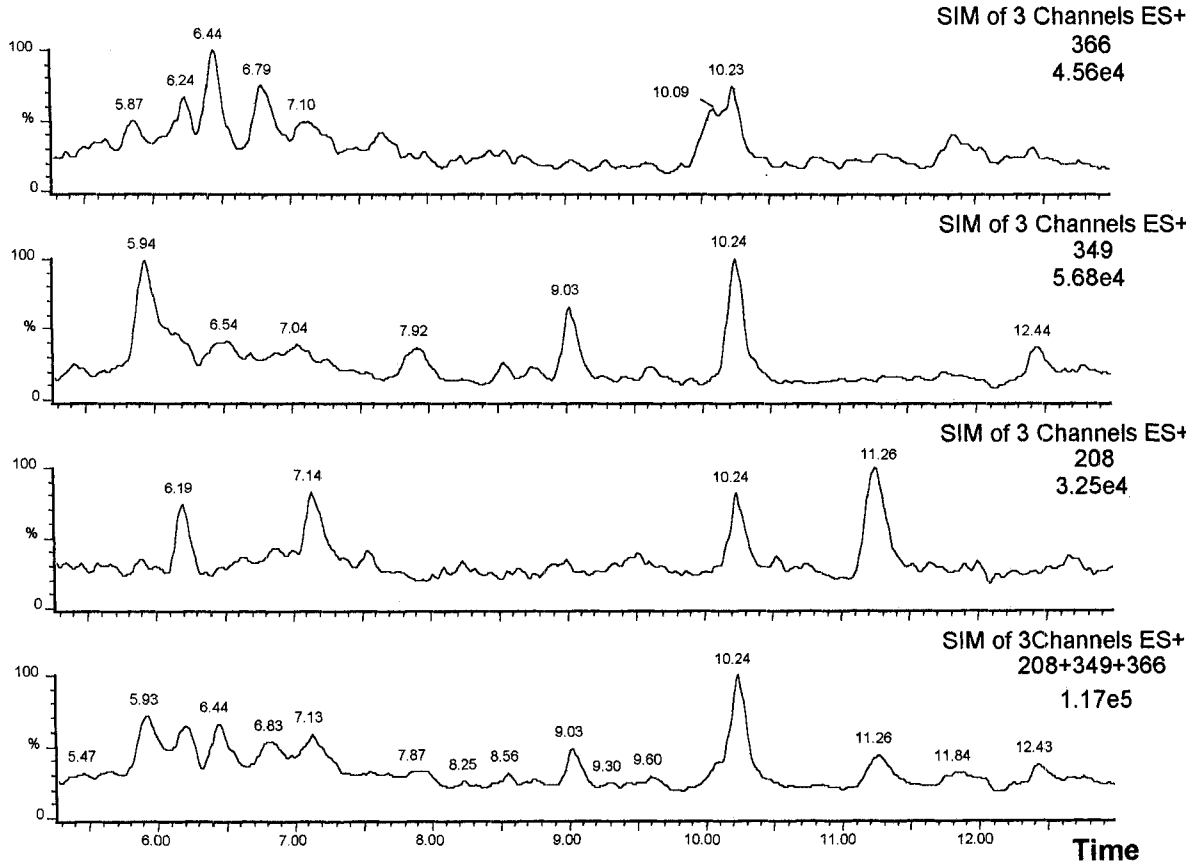

Figure 5. Single and sum of the ion current profiles relative to product ions of amoxicillin resulting from analysis of a raw milk containing 1.2 ng/mL (as measured by us) of the antibiotic.

Table 5. Limits of Quantification (LOQs) of the Method for Ten β-Lactam Antibiotics in Milk and Maximum Residue Limits (MRLs) Established in the U.S. and in the European Union (EU)

compound	LOQ ng/mL	U.S. MRL[a] ng/mL	EU MRL[b] ng/mL
amoxicillin	0.7	10	4
cephapirin	0.6	20	10
cephalin	0.4	N. A.[c]	100
ampicillin	0.5	10	4
ceftiofur	1	50	100
penicillin-G	0.5	5	4
oxacillin	0.7	N. A.	30
cloxacillin	0.6	10	30
dicloxacillin	3	N. A.	30
nafcillin	2	N. A.	30

[a] Source: Code of Federal Regulations, National Archives and Records Administration: Washington, DC, 1997; 21 CFR 556. [b] Source: Commission of the European Communities, *Off. J. Eur. Commun.*, 1992, No. 73, p 8. [c] N. A., not approved for veterinary use.

ments were performed, and the results are reported in Table 4. These data show that the accuracy in determining β-lactams in milk is not significantly affected by their concentration levels. Moreover, the repeatability of the method on measuring levels of β-lactams down to 5 ng/mL ranged between 5 and 11%. Figure 3 shows a typical LC−MS SIM chromatogram resulting from analysis of milk spiked with 10 ng/mL of each β-lactam. More specifically, Figure 4 shows the individual ion current profiles of the (quasi)molecular ion and two selected fragment ions relative to Penicillin G.

Limits of Quantification (LOQs). LOQs of the method were estimated from the LC−MS SIM chromatogram (not shown here) resulting from the analysis of 5 ng/mL of each β-lactam in milk. After extracting the sum of the ion currents of both parent and fragment ions relative to each analyte, the resulting trace was two-times smoothed by applying the mean smoothing method (Mass Lab software, Thermoquest). Thereafter, the ratio of peak height to averaged background noise was measured. The background noise estimation was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal value of the signal-to-noise ratio of 10. These data are listed in Table 5 together with the regulatory levels enacted in the U.S. and in the European Union. As can be read, this method enables determination of β-lactam concentrations in milk well below the tolerance levels.

Application to a Real Sample. The effectiveness of this method in measuring trace levels of β-lactams was checked by analyzing raw milk samples after intramammary application of 5 g of amoxicillin. Milk samples were taken 3, 4, 5, and 6 days after treatment with amoxicillin. Results indicated that 2.7 ng/mL of amoxicillin were present at day 3 and that this concentration slowly decreased with time. At day 6, 1.2 ng/mL of the above penicillin still persisted in milk. Figure 5 shows ion current profiles of parent and daughter ions relative to 1.2 ng/mL of amoxicillin in the milk sample taken at day 6 after administration of the antibiotic. As can be seen, even at the concentration reported above, all the three ion signals selected for detection of amoxicillin are well distinguishable from the background noise. This means that the identification power of this method is not weakened even when measuring minute amounts of β-lactams in milk.

LITERATURE CITED

(1) Carson M. C.; Chu, P. S.; Von Bredow, J. Toward a Regulatory Method: Comparison and Validation of Multi-residue Procedures for Determination of β-Lactam Antibiotics in Milk. In *Veterinary Drug Residues: Food Safety; Symposium at the 209th National Meeting of the American Chemical Society, Anaheim, CA, April 2−7, 1995*; Moats, W. A., Medina, M. B., Eds.; ACS Symposium Series 636; American Chemical Society: Washington, DC, 1995.

(2) Straub, R. F.; Voyksner, R. D. Determination of penicillin G, ampicillin amoxicillin, cloxacillin and cephapirin by high-performance liquid chromatography-electrospray mass spectrometry. *J. Chromatogr.* **1993**, *647*, 167.

(3) Straub, R.; Linder, M.; Voyksner, R. D. Determination of β-lactam residues in milk using perfusive-particle liquid chromatography combined with ultrasonic nebulization electrospray mass spectrometry. *Anal. Chem.* **1994**, *66*, 3651.

(4) Blanchflower, W. J.; Hewitt, S. A.; Kennedy, D. G. Confirmatory assay for the simultaneous detection of five penicillins in muscle, kidney and milk using liquid chromatography-electrospray mass spectrometry. *Analyst* **1994**, *119*, 2595.

(5) Heller, D. N.; Ngoh, M. A. Electrospray ionization and tandem ion trap mass spectrometry for the confirmation of seven β-lactam antibiotics in bovine milk. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 2031.

(6) Keever, J.; Voyksner, R. D.; Tyczkpwska, K. L. Quantitative determination of Ceftiofur in milk by liquid chromatography electrospray mass spectrometry. *J. Chromatogr.* **1998**, *794*, 57.

(7) Daeseleire, E.; De Ruyck, H.; Van Renterghem, R. Confirmatory assay for the simultaneous detection of penicillin and cephalosporins in milk using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1404.

(8) Di Corcia, A.; Bellioni, A.; Madbouly, M. D.; Marchese, S. Trace determination of phenols in natural water: extraction by a new graphitized carbon black cartridge followed by liquid chromatography and reanalysis after phenol derivatization. *J. Chromatogr.* **1996**, *733*, 383.

(9) Terada, K.; Sakabe, J. Simultaneous determination of penicillin G, penicillin V and ampicillin in milk by high-performance liquid chromatography. *J. Chromatogr.* **1985**, *348*, 379.

(10) Andreolini, F.; Borra, C.; Di Corcia, A.; Laganà, A.; Raponi, G.; Samperi, R. Improved assay of unconjugated estriol in maternal serum or plasma by adsorption and liquid chromatography with fluorimetric detection. *Clin. Chem.* **1984**, *30*, 742.

(11) Li, L. Y. T.; Campbell, D. A.; Bennet, P. K.; Henion, J. Acceptance criteria for ultratrace HPLC-tandem mass spectrometry: quantitative and qualitative determination of sulfonylurea herbicides in soil. *Anal. Chem.* **1996**, *68*, 3397.

(12) Rule, J. S.; Mordehai, A. V.; Henion, J. Determination of carbofuran by on-line immunoaffinity chromatography with coupled-column liquid chromatography/mass spectrometry. *Anal. Chem.* **1994**, *66*, 230.

(13) Di Corcia, A.; Crescenzi, C.; Laganà, A.; Sebastiani, E. Evaluation of a method based on liquid chromatography/electrospray/mass spectrometry for analyzing carbamate insecticides in fruits and vegetables. *J. Agric. Food Chem.* **1996**, *44*, 1930.

(14) Crescenzi, C.; Di Corcia, A.; Marcomini, A.; Samperi, R. Determination of nonionic polyethoxylate surfactants in environmental waters by liquid chromatography/electrospray mass spectrometry. *Anal. Chem.* **1995**, *67*, 1797.

(15) Crescenzi, C.; Di Corcia, A.; Marchese, S.; Samperi, R. Determination of acidic pesticides by a benchtop electrospray liquid chromatography mass spectrometer. *Environ. Sci. Technol.* **1995**, *67*, 1968.

(16) Di Corcia, A.; Casassa, F.; Crescenzi, C.; Marcomini, A.; Samperi, R. Investigation of the fate of linear alkylbenzenesulfonates and coproducts in a laboratory biodegradation test by using liquid chromatography mass spectrometry. *Environ. Sci. Technol.* **1999**, *33*, 4112.

Received for review January 9, 2001. Revised manuscript received May 14, 2001. Accepted May 14, 2001.

JF010046R