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# Determination of cephalosporins in raw bovine milk by high-performance liquid chromatography

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**Abstract**

A high-performance liquid chromatographic (HPLC) method based on solid-phase extraction was developed for the determination of cefazolin, cefoperazone, cefquinome and ceftiofur in raw bovine milk. The milk fat was removed by centrifugation and the cephalosporins were extracted in acetonitrile. The extract was cleaned up by solid-phase extraction on an octadecyl sorbent. The compounds were separated by ion-paired gradient HPLC on a phenyl column with ultraviolet detection at 270 nm. The limits of detection estimated by a conservative model were 11 μg/kg for cefazolin and cefoperazone and 7 μg/kg for cequinome and ceftiofur. The mean recoveries were 86–88% for cefazolin, 91–93% for cefoperazone, 69–72% for cefquinome and 84–88% for ceftiofur in the concentration range 20–200 μg/kg. 

*Keywords:* Milk; Food analysis; Cephalosporin; Lactams; Antiobiotics

## 1. Introduction

Cefazolin, cefoperazone, cefquinome and ceftiofur are cephalosporin β-lactam antibiotics which are effective against a broad spectrum of Gram positive and Gram negative bacteria. Cefoperazone and cefquinome are commonly used for treatment of clinical mastitis in lactating cows caused by *Escherichia coli* and *Streptococcus* and *Staphylococcus* species. Some of the cephalosporins, e.g. cefquinome and ceftiofur, show high resistance to β-lactamase activity. Maximum residue limits (MRLs) have been established for some cephalosporins in tissues and milk. In the European Union (EU), MRLs have been established for cefazolin (50 μg/kg) and cefquinome (20 μg/kg) in raw bovine milk [1]. Cephalosporins can readily be detected by microbiological methods and receptor tests. However, these methods generally lack selectivity and only produce qualitative or semi-quantitative results. Chromatographic procedures have been described for determination of single cephalosporins [2–6] or simultaneous determination of two cephalosporins [7] in biological materials. Methods have been reported for determination of ceftiofur [3–5,7] and cephapirin [7] in milk. To date, no single HPLC method has been reported for the simultaneous determination of cefazolin, cefoperazone, cefquinome and ceftiofur in milk.

This paper describes a sensitive multiresidue method for the determination of cefazolin, cefoperazone, cefquinome and ceftiofur in raw bovine milk.

## 2. Experimental procedure

### 2.1. Reagents

Cefquinome sulphate was obtained from Hoechst

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Roussel Vet (Unterschleisheim, Germany), Cefoperazone dihydrate was obtained from Pfizer (Groton, CT, USA), ceftiofur sodium salt was obtained from Upjohn (Vejen, Denmark) and cefazolin sodium salt was purchased from Sigma (St. Louis, MO, USA). Penase (penicillinase) was obtained from Difco (Detroit, MI, USA). Acetonitrile of chromatography gradient grade and octanesulphonic acid for ion-pair chromatography were purchased from Merck (Darmstadt, Germany). Water was purified through a Millipore Milli-Q Plus system (Bedford, MA, USA).

Separate stock solutions of cefazolin, cefquinome and ceftiofur were prepared at a concentration of 1000 μg/ml by dissolving the pure substances in water. Cefoperazone dihydrate was dissolved in one tenth volume acetonitrile and diluted with water. A mixed standard solution containing 2 μg/ml cefazolin, cefoperazone, cefquinome and ceftiofur was prepared by diluting combined aliquots of the stock solutions with water.

Octanesulphonic acid solution pH 2.52 (0.005 *M*) for mobile phase was prepared by dissolving 1.08 g octanesulphonic acid in water followed by dilution to 1000 ml. The pH was adjusted with phosphoric acid.

HPLC eluent A was prepared by diluting 100 ml acetonitrile to 1000 ml with octanesulphone acid solution pH 2.52. HPLC eluent B was prepared by diluting a mixture of 300 ml acetonitrile and 150 ml methanol to 1000 ml with octanesulphonic acid solution pH 2.52.

### 2.2. Materials

Solid-phase extraction (SPE) cartridges, tC18 500 mg, were obtained from Waters (Milford, MA, USA). Disposable Acrodisc LC 13 PVDF [poly-(vinylidene diflouride)], 13 mm×0.45 μm, syringe filters were obtained from Gelman Sciences (Ann Arbor, MI, USA). Centrifuge tubes of 15 ml and 50 ml capacity, volumetric flasks and vials were made of polypropylene.

### 2.3. Instrumentation

The instruments used were a Sigma centrifuge model 2-15 (Osterode, Germany), a VF2 test tube shaker (IKA, Staufen, Germany), a vacuum manifold for SPE cartridges (Waters, Milford, MA, USA), a pH meter PHM 240 (Radiometer, Copenhagen, Denmark) and a temperature-controlled heating block with a manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark).

The liquid chromatography system consisted of a Waters pump gradient system 600, a Waters 996 photodiode-array detector and a Waters 717 auto-sampler (Milford, MA, USA). Reversed-phase liquid chromatography was accomplished on a Waters Nova-Pak phenyl column (4 μm, 150 mm×3.9 mm I.D.). The operation of the chromatographic system and acquisition of data were controlled by Waters Millennium 32 software.

The injection volume was 100 μl and the mobile phase flow rate was set at 1.0 ml/min. The HPLC was run isocratic with 90% eluent A and 10% eluent B for 8 min. The relative content of eluent B was then increased linearly to 45% over 15 min. The gradient was further increased linearly to 56% eluent B over 5 min and then to 100% eluent B over 17 min. The system was held constant for 3 min and returned to 90% eluent A over 2 min. The column was conditioned for 10 min before the next injection. The column temperature was kept at 20–24°C. Ultraviolet (UV) detection was performed at 270 nm.

### 2.4. Preparation of samples

Samples of raw milk were obtained from individual farmers and dairies in Denmark. A volume of ca. 20 ml milk was centrifuged at 1500 *g* for 15 min. The skim milk was collected and a volume of 2.00 ml was transferred to a 15 ml polypropylene centrifuge tube. A volume of 7.0 ml acetonitrile was added and the mixture was shaken vigorously for 2 min. The mixture was centrifuged at 1500 *g* for 10 min and the supernatant was evaporated to ca. 2 ml at 45–50°C under a steam of nitrogen. The extract was diluted with 20 ml of water.

An tC18 SPE cartridge was washed with 10 ml methanol followed by 5 ml water. The filtrate was pulled through the cartridge at a flow rate of ca. 2 ml/min. The column was washed with 20 ml water and dried by suction for 1 min. The cephalosporins were eluted with 2.0 ml acetonitrile. The eluate was evaporated to bare dryness at 45–50°C under a

stream of nitrogen. The pellet was redissolved in 1000 μl water and filtered through a 0.45 μm PVDF filter.

Non-matrix calibration standards were prepared by diluting 50, 100, 150 and 200 μl mixed standard containing 2 μg/ml of the individual cephasporins to 1000 μl with water.

### 2.5. Calculation

Linear regression was used for calculation of concentrations in samples.

### 2.6. Penase treatment of samples

Volumes of 20 ml raw milk were mixed with 500 μl penase solution containing 10 000 000 units/ml. The solutions were incubated at 25°C for 1, 2, 4 and 20 h. Extraction with acetonitrile was performed immediately after the incubation period.

### 2.7. Ruggedness

The influence of sample pH on the extraction efficiency was tested on milk samples with pH adjusted to 5.5, 6.0 and 6.7 with acetic acid. Before extraction the samples were spiked to a level of 100 μg/kg with the individual cephalosporins. Extraction was performed with 6.0 and 8.0 ml acetonitrile.

The capacity of the tC18 cartridge was checked by connecting to cartridges in series and applying 50% surplus volumes of sample extract spiked to a level of 200 μg/kg with the individual cephalosporins. The cartridge load thus corresponded to extract from 3 g milk. The quantities of cephalosporins adsorbed to each cartridge were determined according to the procedure.

The elution profile of the four cephalosporins from the tC18 sorbent was tested on milk spiked to a level of 100 μg/kg. Volumes of 0.5 ml acetonitrile were used for elution.

The specificity of the method against a range of veterinary drugs (Table 1) was tested on milk samples spiked to a concentration of 200 μg/kg with each of these compounds.

### 2.8. Limits of detection and quantification

The limits of detection (LODs) were determined on milk samples from 20 different livestocks. To obtain realistic LODs, the samples were spiked prior to extraction with cephalosporins to a peak height on chromatograms corresponding to ca. three times the short term baseline variation. The samples were thus spiked with cefazolin and cefoperazone to a level of 7.0 μg/kg and cefquinome and ceftiofur to a level of 3.5 μg/kg. The LODs were determined as the mean results plus three times the standard deviation (SD) of the 20 measurements. The limits of quantification (LOQs) were determined as the mean results plus six times the standard deviation (SD) of the 20 measurements. The limits were corrected for the mean recovery.

### 2.9. Precision and accuracy

The repeatability standard deviation (i.e. variability of independent analytical results obtained by the

Table 1
Veterinary drugs included in the specificity test of the method

| | | |
|---|---|---|
| Benzylpenicillin | Spiramycin | Sulfamethoxazole |
| Penicillin V | Streptomycin | Methylsulfaphenazole |
| Amoxicillin | Dihydrostreptomycin | Albendazole |
| Ampicillin | Trimethoprim | Fenbendazole |
| Oxacillin | Dexamethazone | Febantel |
| Cloxacillin | Thiabendazole | Albendazole sulphoxide |
| Nafcillin | Levamisole | Mebendazole |
| Dicloxacillin | Sulfadiazine | Fenbendazole sulphoxide |
| Oxytetracycline | Sulfathiazole | Fenbendazole sulphone |
| Tetracycline | Sulfamerazine | Oxybendazole |
| Chlortetracycline | Sulfamethazine | |
| Tylosin | Sulfadoxine | |

same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time), intra-laboratory reproducibility standard deviation (i.e. variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different conditions) and recovery of the method were determined on milk samples spiked with the individual cephalosporins to levels of 20, 50 and 200 μg/kg. The spiked samples were equilibrated at 7±2°C for at least 1 h before extraction. Samples were analysed in duplicate on each of 6 different days. Calculation of repeatability was done in accordance with ISO standard 5725-2, 1994 [8]. The intra-laboratory reproducibility was calculated by the same procedure used for determination of reproducibility [8].

## 3. Results and discussion

Precipitation of organic material and extraction of the cephalosporins was achieved by adding acetonitrile to the milk sample. Extraction procedures based on precipitation of proteins at the isoelectric point of casein using acetic acid, acetate buffers, phosphate buffers and a mixture of sulphuric acid and sodium tungstate were less efficient. Different sorbents were tested for clean-up of the extract including a polymeric (divinylbenzene–co-*N*-vinylpyrrolidone) sorbent and trifunctional $C_8$ and $C_{18}$ sorbents. Best overall recovery for the four compounds was obtained on $C_{18}$ sorbents. Chromatographic separation was tested on $C_{18}$ and phenyl HPLC columns. Superior results in minimising interferences were obtained with the phenyl column.

Typical chromatograms of control raw milk and milk spiked to a level of 50 μg/kg with cefazolin, cefoperazone, cefquinome and ceftiofur are shown in Figs. 1 and 2. The retention time ($t_R$) of cefazolin and its separation from interferences were strongly influenced by the pH of the mobile phase. A $t_R$ of 12.5–13.0 min was optimal for separation of cefazolin from interferences. This was achieved when the pH of the octanesulphone acid solution used in mobile phases was adjusted to 2.52.

The LODs were 11 μg/kg for cefazolin and cefoperazone and 7 μg/kg for cefquinome and ceftiofur (Table 2).

The results for precision and recovery are summa-

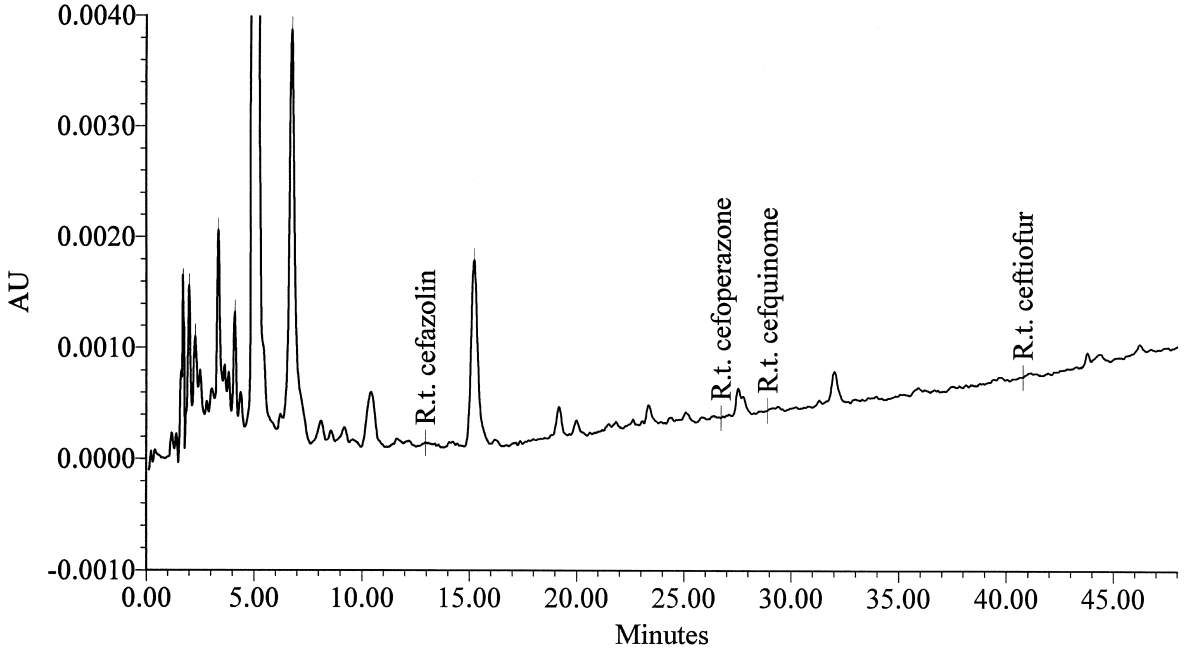


Fig. 1. Chromatogram of a control milk sample.

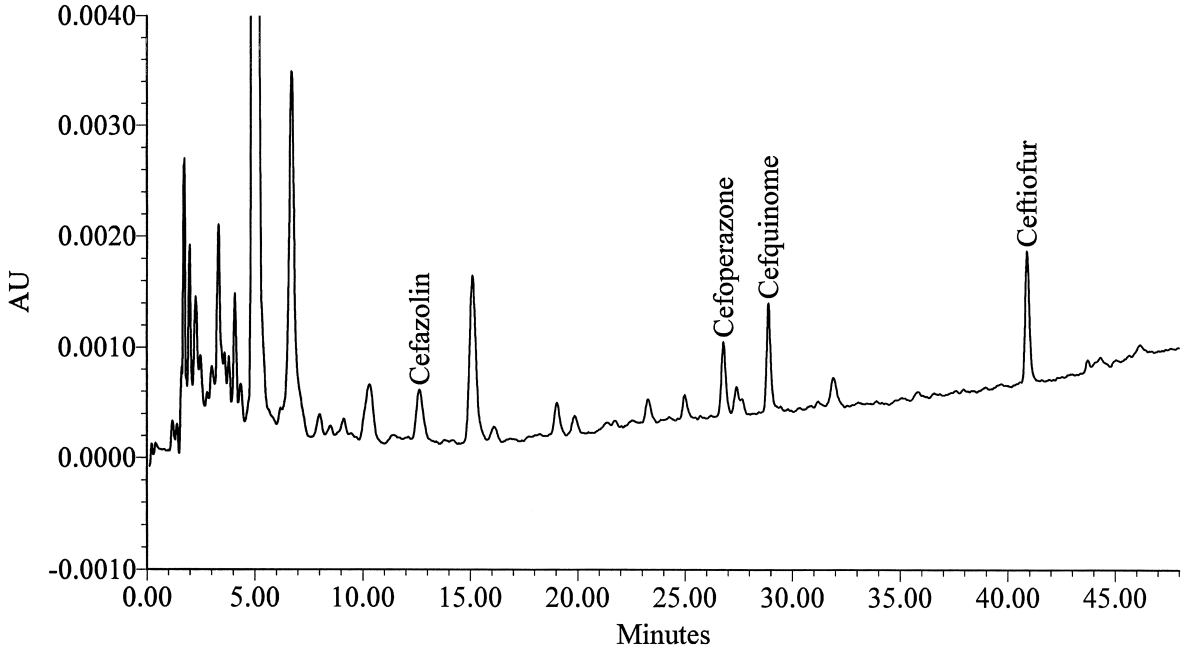


Fig. 2. Chromatogram of a control milk sample spiked to a level of 50 μg/kg with cefazolin, cefoperazone, cefquinome and ceftiofur.

rised in Table 3. The relative repeatability standard deviation ($RSD_r$) was in all cases below 10% for levels 20–200 μg/kg. The mean recoveries were independent of the level in the range 20–200 μg/kg. The mean recoveries were 87% for cefazolin, 92% for cefoperazone, 71% for cefquinome and 87% for ceftiofur.

The calibration curves were linear in the tested range up to 400 ng/ml. The relative standard error of slope converting peak area (μV s) to concentration units (ng/ml) was less than 2.0% for all compounds.

Table 2
Limits of detection and quantification[a]

| | Mean±SD (μg/kg) | LOD (μg/kg) | LOQ (μg/kg) |
|---|---|---|---|
| Cefazolin | 7.13±1.16 | 11 | 14 |
| Cefoperazone | 6.79±1.23 | 11 | 14 |
| Cefquinome | 3.21±1.09 | 7 | 10 |
| Ceftiofur | 4.29±0.71 | 7 | 9 |

[a] Raw milk samples from 20 different livestocks were spiked to a level resulting in a signal equal to ca. three times the short term baseline variation (3.5 μg/kg for cefquinome and ceftiofur and 7.0 μg/kg for cefazolin and cefoperazone). The LODs and the LOQs were calculated from the mean and the SD obtained. The limits were corrected for recovery.

The coefficients of determination ($R^2$s) were better than 0.999.

The retention times (mean±SD) of cephalosporins during the precision study were 12.8±0.23 min (cefazolin), 27.1±0.21 min (cefoperazone), 29.1±0.35 min (cefquinome) and 41.2±0.15 min (ceftiofur).

It was possible to verify the authenticity of cefazolin and cefoperazone peaks by a prior penase treatment of milk sample with 250 000 IU/ml at 25°C. The necessary incubation time for complete breakdown of these cephalosporins at a level of 200 μg/kg was measured to be 2–4 h. The peaks corresponding to cefoquinome and ceftiofur were only reduced to ca. 40% of the original size after 20 h of incubation.

The recovery of the cephalosporins from milk was slightly dependent on the sample pH. Highest recovery was obtained when extraction with acetonitrile was performed at the natural pH level of fresh raw milk. The recovery was significantly lower at pH 5.5 (ca. 12 percentage points). No general difference was observed in using 6.0 and 8.0 ml acetonitrile for extraction of 2.0 ml milk.

The capacity of the SPE cartridge was checked by

Table 3
The relative repeatability standard deviation ($RSD_r$), intra-laboratory reproducibility standard deviation ($RSD_{R,intra}$) and recovery on spiked milk samples[a]

| Penicillin | Fortification level (μg/kg) | $RSD_r$ (μg/kg) | $RSD_{R,intra}$ (μg/kg) | Recovery (mean±SD) (%) |
|---|---|---|---|---|
| Cefazolin | 20 | 5.7 | 5.8 | 88±3.6 |
| | 50 | 4.8 | 7.1 | 87±5.5 |
| | 200 | 0.96 | 3.2 | 86±2.6 |
| Cefoperazone | 20 | 6.1 | 8.6 | 91±6.7 |
| | 50 | 3.3 | 5.9 | 93±5.0 |
| | 200 | 1.3 | 1.8 | 91±1.4 |
| Cefquinome | 20 | 5.0 | 5.0 | 72±2.7 |
| | 50 | 4.3 | 5.4 | 71±3.1 |
| | 200 | 1.6 | 5.0 | 69±3.3 |
| Ceftiofur | 20 | 4.4 | 6.8 | 88±5.4 |
| | 50 | 2.9 | 3.6 | 84±2.5 |
| | 200 | 1.5 | 3.4 | 86±2.8 |

[a] One duplicate analysis at each level were conducted on each of 6 days.

applying a 50% surplus volume of sample extract to two cartridges connected in series. Analytes were detected in the acetonitrile eluate from the first cartridge but not the second. A volume of 1.5 ml acetonitrile was sufficient for complete elution of cephalosporins from tC18 cartridges. Ca. 80% was eluted in the 0.5–1.0 ml fraction.

The stability at 5±2°C of 100 ng/ml calibration solutions and final sample extracts of raw milk spiked to a level of 100 μg/kg was investigated over a period of 14 days. No significant changes in response were observed for the individual cephalosporins.

The specificity of the method was tested on milk samples spiked to a level of 200 μg/kg with a broad range of veterinary drugs (Table 1). Peaks appeared on the chromatogram from sulfathiazole ($t_R$ 6.20 min), sulfamethazine ($t_R$ 7.50 min), sulfadoxine ($t_R$ 15.14 min), sulfamethoxazole ($t_R$ 16.35 min), albendazole sulphoxide ($t_R$ 23.39 min), thiabendazole ($t_R$ 25.20 min), fenbendazole sulphoxide ($t_R$ 31.82 min), fenbendazole sulphone ($t_R$ 36.61 min), penicillin V ($t_R$ 37.97 min), dexamethazone ($t_R$ 38.84 min) oxybendazole ($t_R$ 40.12 min), mebendazole ($t_R$ 40.24 min), oxacillin ($t_R$ 41.43 min), cloxacillin ($t_R$ 45.98 min) and nafcillin ($t_R$ 47.41 min).

The retention times for cephalosporins measured in the same sample set were 12.97 min (cefazolin), 27.01 min (cefoperazone), 29.05 min (cefquinome) and 41.08 min (ceftiofur). Baseline separation was obtained in all cases except for ceftiofur and oxacillin. The peak resolution for ceftiofur and oxacillin was 0.7. However, the sensitivity of the method was ca. 25 times higher for ceftiofur than oxacillin. Furthermore, the UV spectra of ceftiofur and oxacillin are different and interference from oxacillin could be eliminated completely by moving the detection wavelength to 300 nm. The sensitivity of ceftiofur determination was not decreased by this modification.

For the complete procedure, including the liquid–liquid extraction step, it was possible for a single trained analyst to obtain 8–12 sample extracts ready for HPLC within a working day of 8 h.

## 4. Conclusion

The HPLC method described in this paper provides a simple and reliable procedure for the quantitative analysis of cefazolin, cefoperazone, cefquinome and ceftiofur in raw bovine milk. The method may be used as a determinative method in routine analyses for veterinary drug residues.

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