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Simultaneous determination of six penicillins in cows' raw milk by a multiresidue high-performance liquid chromatographic method

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Abstract

A high-performance liquid chromatographic method based on C_{18} solid-phase extraction and ultraviolet detection at 323 nm of analytes derivatized with benzoic anhydride and 1,2,4-triazole mercuric chloride solution was developed for the simultaneous determination of amoxicillin, penicillin G (benzylpenicillin), ampicillin, oxacillin, cloxacillin and dicloxacillin residues in raw milk. The detection limit of the method was 1.3 μg/l for penicillin G; 1.4 μg/l for amoxicillin, oxacillin and cloxacillin, 1.5 μg/l for ampicillin and 2.7 μg/l for dicloxacillin. The mean recovery was 95–102% for amoxicillin, penicillin G and ampicillin, 92–98% for oxacillin and cloxacillin and 87–94% for dicloxacillin, measured by using an internal standard. The relative repeatability standard deviation was 4–9% on level 4–15 μg/l, respectively, 2–7% on level 30–40 μg/l. © 1997 Elsevier Science B.V.

Keywords: Benzylpenicillin; Amoxicillin; Penicillins; Ampicillin; Oxacillin; Cloxacillin; Dicloxacillin

1. Introduction

In the European Union (EU), maximum residue limits (MRLs) have been established for amoxicillin, penicillin G (Pen-G), ampicillin, oxacillin, cloxacillin and dicloxacillin in milk [1]. Several chromatographic procedures have been described for the determination of penicillins in milk [2–6]. However, no sensitive multiresidue high-performance liquid chromatographic (HPLC) method has been reported for the simultaneous determination of the listed penicillins. The simultaneous determination of the penicillins is moreover complicated by the amphoteric properties of amoxicillin and ampicillin.

This paper describes a sensitive multiresidue method for the determination of amoxicillin, Pen-G, ampicillin, oxacillin, cloxacillin and dicloxacillin in milk. The presented method is a further development of the method reported by Boison et al. [2] for the determination of Pen-G in milk.

2. Experimental

2.1. Reagents

Amoxcillin, Pen-G potassium salt, penicillin V (Pen-V) potassium salt, ampicillin sodium salt, oxacillin sodium salt, cloxacillin sodium salt and dicloxacillin sodium salt were purchased from Sigma

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(St. Louis, MO, USA). 1,2,4-Triazole was obtained from Merck-Schuchardt (Hohenbrunn, Germany). Mercuric chloride, sodium tungstate, benzoic anhydride and sodium thiosulphate were obtained from Merck (Darmstadt, Germany). Acetonitrile (gradient grade) and dichloromethane were purchased from Merck. Dimethylchlorosilane (DMCS) was purchased from Fluka (Buchs, Switzerland). Penase was obtained from Leo Pharmaceutical Products (Ballerup, Denmark). Water was purified on a Waters Milli-Q Plus unit. All other reagents were of reagent grade.

Separate stock solutions of each penicillin were prepared at a concentration of 1000 μg/ml by dissolving the pure substances in water. These were stable for at least one month when stored at 5°C. A mixed standard solution containing 2 μg/ml each of amoxicillin, Pen-G, ampicillin, oxacillin, cloxacillin and dicloxacillin was prepared by diluting the stock solution with water. An internal standard solution containing 2 μg/ml of Pen-V was prepared by diluting the corresponding stock solution with phosphate buffer, pH 9.0. The 2 μg/ml standard solutions were stable for at least two weeks when stored at 5°C.

Derivatization reagent I was prepared by dissolving 1.13 g of benzoic anhydride in acetonitrile, followed by dilution to 25 ml. Derivatization reagent II (2 *M* 1,2,4-triazole containing 2.6 m*M* mercuric chloride) was prepared by dissolving 6.905 g of 1,2,4-triazole in 30 ml of water and adding 5 ml of a 26-m*M* aqueous solution of mercuric chloride. The pH was adjusted to 9.0±0.05 with 5 *M* sodium hydroxide and the solution was diluted to 50 ml. The 26 m*M* mercury solution was stable for at least one month when stored at 20–25°C. Derivatization reagents I and II were prepared 1–4 h before use.

Phosphate buffer, pH 9.0, was prepared by dissolution of 0.34 g of KH_2PO_4 in water followed by adjustment of the pH with sodium hydroxide and dilution to 100 ml. Phosphate buffer, pH 2.45, was prepared by dissolution of 2.72 g KH_2PO_4 in water followed by adjustment of the pH using phosphoric acid and dilution to 100 ml. Phosphate buffer (0.1 *M*, pH 6.5) for use in the mobile phase was prepared by dissolution of 9.938 g of Na_2HPO_4, 17.938 g of $NaH_2PO_4 \cdot H_2O$ and 4.964 g of $Na_2S_2O_3$ in water, followed by dilution to 2000 ml.

HPLC-eluent A was prepared by diluting 100 ml of acetonitrile to 1000 ml with phosphate buffer. HPLC-eluent B was prepared by diluting 300 ml of acetonitrile to 1000 ml with phosphate buffer.

2.2. Materials

Solid-phase extraction cartridges, tC_{18}, 500 mg (Waters) were purchased from Waters Chromatography Division (Hedehusene, Denmark). Glass fibre filters, GF/B, were obtained from Whatman (Maidstone, UK). Disposable 0.45 μm Acrodisc LC 13 PVDF filters were purchased from Gelman.

All glassware was silanized with 10% DMCS in toluene.

2.3. Instrumentation

The liquid chromatography system consisted of a Waters pump gradient system 600, a 999 photodiode-array detector and a 717 autosampler (Waters Chromatography Division). Reversed-phase liquid chromatography was accomplished on a Waters Nova-Pak C_{18} column (4 μm, 150×3.9 mm I.D.). Operation of the chromatographic system and acquisition of data were controlled by Waters Millenium 2010.

The injection volume was 150 μl and the mobile phase flow was set at 1.0 ml/min. The gradient was initiated with 0% eluent B followed by a linear increase to 100% eluent B over 30 min and constant 100% eluent B for 13 min. The system was returned to 100% eluent A in 2 min and was re-equilibrated for 5 min before the next injection. The column temperature was kept at 30°C and detection was performed at 323 nm.

2.4. Preparation of samples

Samples of cows' raw milk were obtained from individual farmers in Denmark. A 30-ml volume of milk sample was centrifuged at 1500 *g* for 10 min. A 10-ml volume of the defatted milk was diluted with 20 ml of water and 200 μl of a 2-μg/ml internal standard solution (Pen-V) were added. A corresponding sample, without the addition of internal standard, was prepared. Proteins were removed by addition of 6.0 ml of 0.17 *M* sulphuric acid, immediately followed by 5.6 ml of 5.0% sodium tungstate

solution. The mixture was then immediately shaken vigorously for 1 min and allowed to sit for 5 min. The pH of the mixture was checked, to make sure that it was within the range 4.6–4.8. The mixture was discarded if the pH was outside the indicated range and the precipitation reaction was repeated with an increased or reduced volume of sodium tungstate solution. The mixture was centrifuged at 1500 *g* for 10 min and the pH of the supernatant was adjusted to 8.1–8.2 with 5 and 0.1 *M* sodium hydroxide solutions. The clear liquid phase was vacuum-filtered through a glass fibre filter. The complete precipitation procedure was carried out within 30 min.

A tC_{18} solid phase extraction cartridge was conditioned with 20 ml of methanol followed by 20 ml of water and 10 ml of 2% sodium chloride solution. The filtrate was passed through the cartridge at a flow-rate of approximately 2 ml/min. The column was washed with 2 ml of water and dried by drawing air through the cartridge for 1 min. The penicillins were eluted with 2.0 ml of acetonitrile. The eluate was collected in a 10-ml vial (90×15 mm) with a screw-cap and a Teflon insert.

A 150-μl volume of phosphate buffer, pH 9.0, was added to the eluate and the solution was evaporated to approximately 100 μl under nitrogen-flow at 45–50°C. A 400-μl volume of phosphate buffer, pH 9.0, and 75 μl of derivatization reagent I was added to the concentrate. The mixture was vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. The solution was transferred to a 100-ml separatory funnel containing 20 ml of dichloromethane by means of a Pasteur pipette. The tube and pipette were rinsed with 500 μl of water, which was transferred to the funnel. A 5.0-ml volume of phosphate buffer, pH 2.45, was added to the mixture, which was shaken immediately for 60 s. Immediately after the phase separation (max. 5 min of standing), the dichloromethane phase was drained off in a 50-ml pear-shaped flask. The dichloromethane phase was evaporated on a rotatory evaporator at 35–40°C. The evaporation residue was redissolved in 500 μl of phosphate buffer, pH 9.0. The solution was transferred by means of a Pasteur pipette to a screw-capped vial. A 75-μl volume of derivatization reagent I was added, vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. A 450-μl volume of derivatization reagent II was then added. The vial was closed, vortex-mixed for 60 s and allowed to react in a water bath at 55±1°C for 30 min. Hereafter, the solution was cooled down in cold water and filtered through a 0.45-μm Acrodisc.

Calibration standards were prepared by adding 200 μl of internal standard solution to 20, 75, 150 and 225 μl of mixed standard containing 2 μg/ml of the individual penicillins in 10 ml screw-capped vials. The solutions were diluted to 500 μl with phosphate buffer, pH 9.0, and a 75-μl volume of derivatization reagent I was added. The mixtures were vortex-mixed for 30 s and allowed to react at 20–24°C for 10 min. A 450-μl volume of derivatization reagent II was added to each solution, followed by vortex-mixing for 60 s and reaction at 55°C for 30 min. The solutions were cooled down in cold water. The content of the calibration solutions was equivalent to 4, 15, 30 and 45 μg/l, respectively, in milk. Derivatization of calibration standards was done in parallel with test samples.

2.5. Penase treatment of samples

A 600-μl volume of penase solution containing 5000 units/ml was added to 30 ml of milk and allowed to react at 37°C.

2.6. Ruggedness

The optimum pH value for sample extraction on tC_{18} cartridges was determined by analysis of milk spiked to 20 μg/l with the individual penicillins. The pH was adjusted to the selected level after precipitation of proteins.

The stability of penicillins in the acidic environment for dichloromethane extraction was tested on 575 μl of a solution (500 μl phosphate buffer, pH 9.0, and 75 μl of derivatization reagent I) containing 0.15 μg of the individual benzoylated penicillins. The solution was diluted with 5 ml of phosphate buffer, pH 2.45, and, after a scheduled amount of time standing, the solution was extracted with dichloromethane and derivatized with 1,2,4-triazole reagent.

To investigate the reactivity of glassware towards penicillins, approximately 0.15 μg of the individual penicillins was dissolved in a mixture of 2.0 ml of

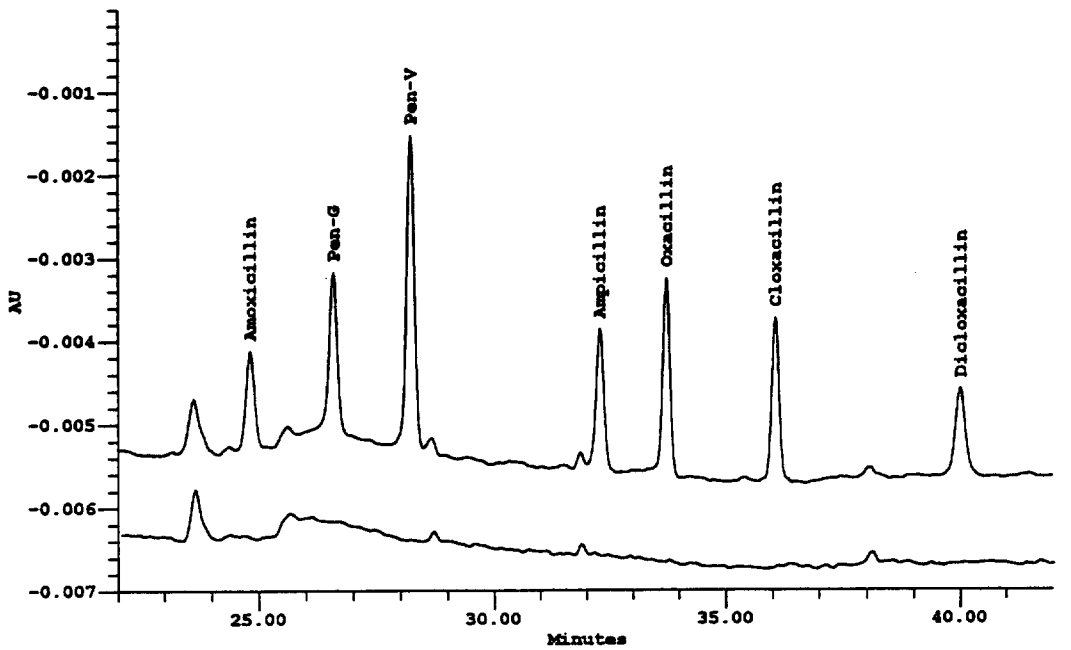

Fig. 1. Chromatograms of a drug-free milk sample (lower chromatogram) and of an identical sample spiked to a level of 15 μg/l with each of the penicillins (upper chromatogram). Pen-V was used as the internal standard.

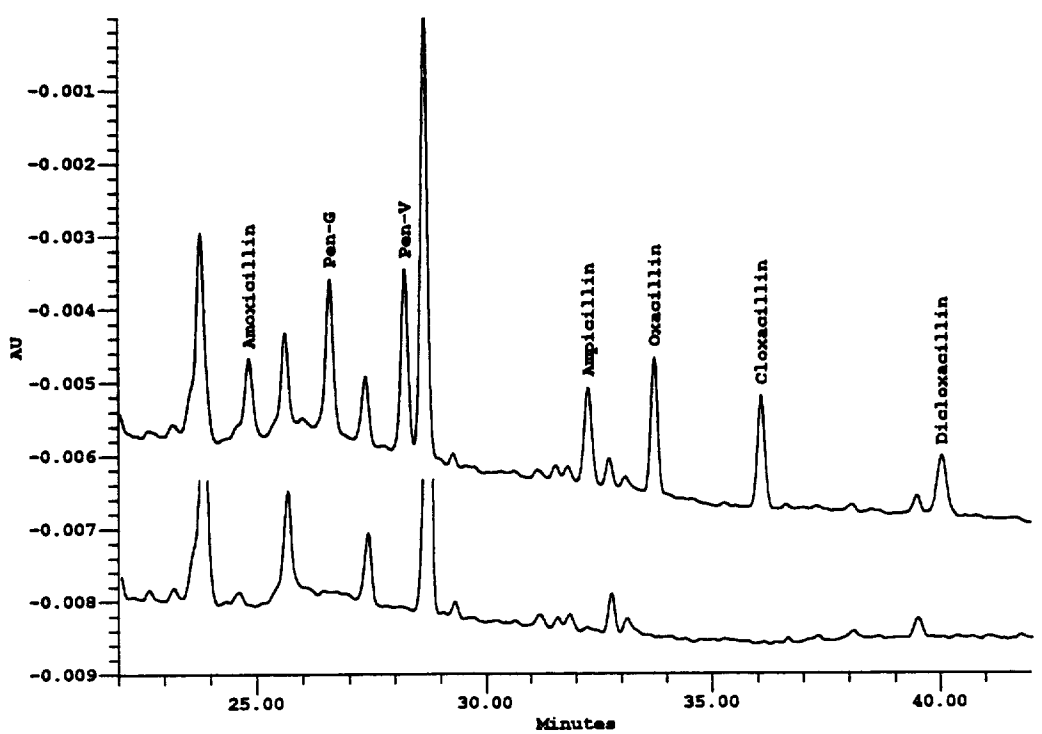

Fig. 2. Chromatograms of a drug-free milk sample (lower chromatogram) and of an identical sample spiked to a level of 15 μg/l with each of the penicillins (upper chromatogram). Liquid–liquid extraction of the SPE extract was omitted. The chromatographic conditions were identical to those in Fig. 1.

acetonitrile and 0.1 ml of phosphate buffer, pH 9.0. The solution was thereafter evaporated, derivatized with benzoic anhydride, shaken with dichloromethane, evaporated and derivatized with triazole reagent. Non-silanized glassware was compared with glassware silanized with 10% DMCS in toluene.

2.7. Limits of detection and quantification

The limits of detection (LOD) were determined on twenty samples of raw milk from different farmers. To obtain realistic LOD, the samples were spiked with penicillins to a quantitative level on chromatograms. Thus, the samples were spiked with amoxicillin, Pen-G, ampicillin, oxacillin and cloxacillin, each to a level of 0.75 μg/l, and with dicloxacillin to a level of 1.5 μg/l. The detection limits were determined as the average results plus three times the standard deviation (SD) of the twenty measurements. The limits of quantification (LOQ) were calculated as the average results plus six times the SD of the twenty measurements.

2.8. Precision and accuracy

The repeatability, day-to-day variation and accuracy were determined on milk spiked with the individual penicillins to levels of 4–45 μg/l. The samples were analysed in duplicate on each of six different days. Calculation of repeatability was accomplished in accordance with International Dairy Federation (IDF) standard 135B, 1991 [7]. The day-to-day variation was calculated by the same principle as used for determination of reproducibility [7].

3. Results and discussion

The amphoteric properties of amoxicillin and ampicillin were removed by derivatization of the $-NH_2$ group with benzoic anhydride. The wavelength of detection was moved to the higher UV-region by forming a complex with the 1,2,4-triazole mercuric reagent in order to reduce the impact of other substances.

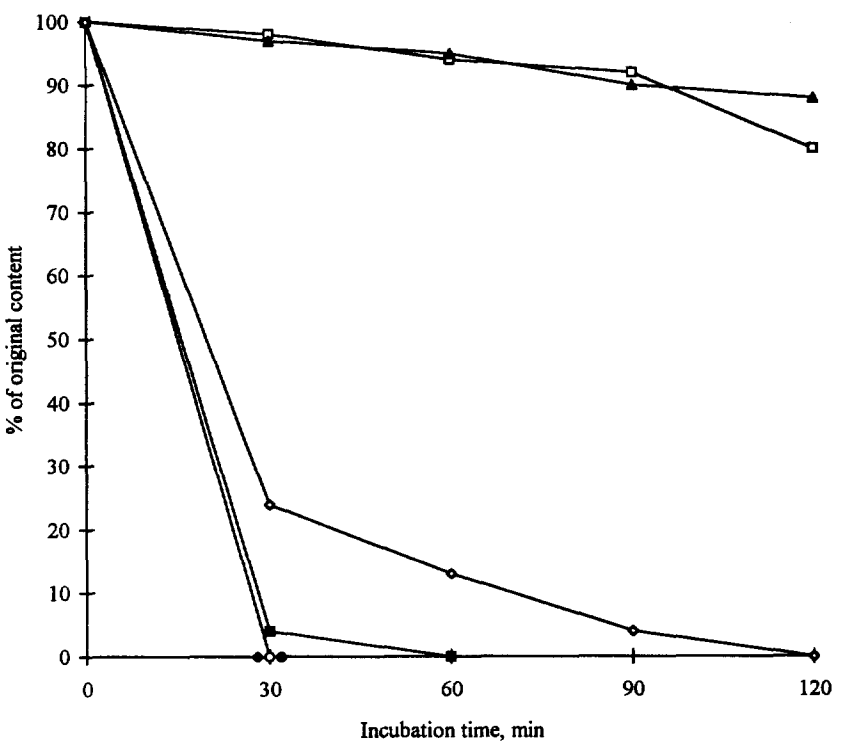

Fig. 3. Degradation of penicillins in milk treated with 100 units of penase per ml at 37°C. The concentration of penicillins was 40 ng/ml. (■) Pen-V, (□) cloxacillin, (▲) dicloxacillin, (●) amoxicillin, (■) ampicillin, (○) Pen-G, (◇) oxacillin.

Typical chromatograms of milk and milk spiked to a level of 15 μg/l each of amoxicillin, Pen-G, ampicillin, oxacillin, cloxacillin and dicloxacillin are shown in Fig. 1. Chromatograms without interferences were obtained.

Accomplishment of the liquid–liquid extraction step was not critical, as seen from Fig. 2. However the liquid–liquid extraction step resulted in cleaner chromatograms.

The LOD was 2.7 μg/l for dicloxacillin and in the range 1.3–1.5 for the other penicillins (Table 1). The LOD and LOQ for oxacillin, cloxacillin and dicloxacillin are far below the EC MRL of 30 μg/kg [1]. The LOD for amoxicillin, Pen-G and ampicillin are a factor of three below the EC MRL of 4 μg/kg [1]. The corresponding LOQ are a factor of two below the EC MRL. It is possible to verify the authenticity of the amoxicillin, Pen-G, ampicillin and oxacillin peaks by prior penase treatment of the milk sample with 100 units/ml at 37°C. The necessary reaction time for complete breakdown of penicillins at levels of 4–40 μg/l was found to be less than 1 h for amoxicillin, Pen-G and ampicillin and 2 h for oxacillin (Fig. 3). Approximately 90% of cloxacillin and dicloxacillin was still intact after a 2-h incubation period. Fig. 4 shows a milk sample from cows treated with Pen-G, subjected to the penase verification procedure. The concentration of Pen-G in the sample was 1.7 μg/l.

Table 1
Limits of detection and quantification

	Average result (μg/l)	SD (μg/l)	LOD (μg/l)	LOQ (μg/l)
Amoxicillin	0.73	0.21	1.4	2.0
Pen-G	0.72	0.19	1.3	1.9
Ampicillin	0.80	0.23	1.5	2.2
Oxacillin	0.78	0.19	1.4	1.9
Cloxacillin	0.77	0.19	1.4	1.9
Dicloxacillin	1.59	0.35	2.7	3.7

Twenty milk samples were spiked to the minimum level suitable for integration.
LOD and LOQ were calculated from the average result and SD.

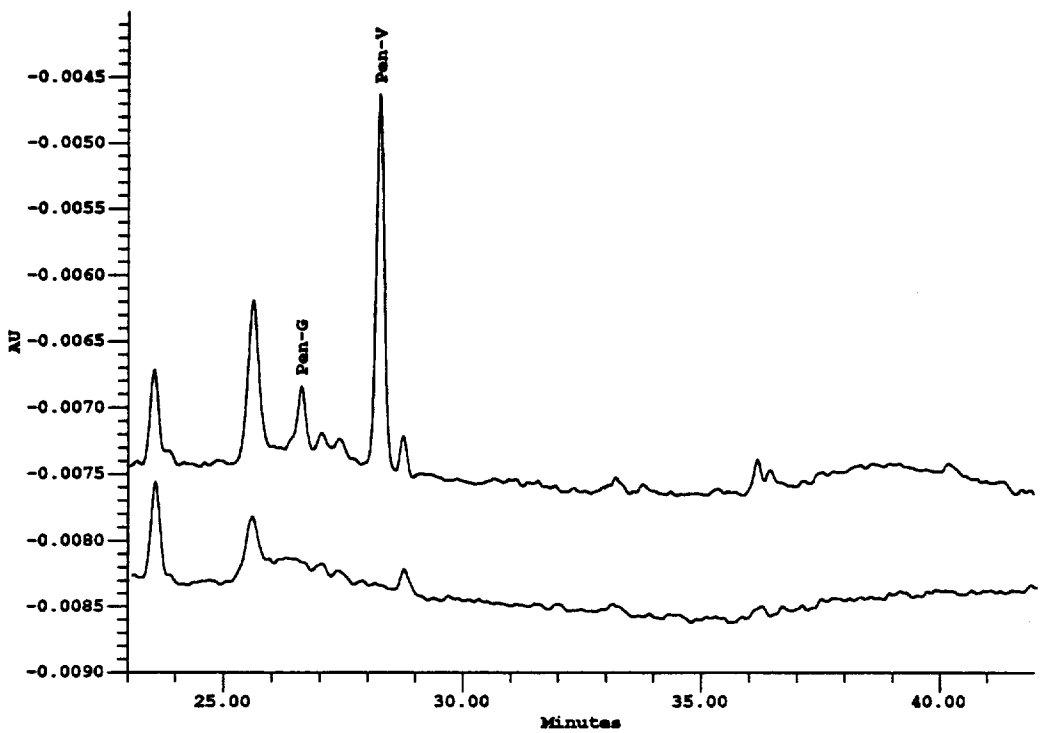

Fig. 4. Chromatograms of a natural contaminated milk sample containing 1.7 μg/l of Pen-G (upper chromatogram) and of an identical sample after treatment with penase (lower chromatogram).

The calibration curves were linear with correlation coefficients (R^2) in the range from 0.995 to 0.999.

The results on precision and recovery are summarised in Table 2. The relative repeatability standard deviation (RSD_r) and the relative day-to-day standard deviation on single measurements were, in all cases, below 10%. The mean recovery calculated for each penicillin and the concentration levels were within the range 87–102%.

The ruggedness of the different steps in the procedure was tested by single factor experiments and factorial designs (only data for critical factors are shown). The effect of the pH value of the sample extract on recovery from tC_{18} cartridges is shown in Fig. 5. Amoxicillin was only recovered completely at pH values above 8.0. The procedure describes a step for further clean-up by liquid–liquid extraction with dichloromethane. This was performed at pH 2.5 to obtain complete recovery of the penicillins, although Pen-G is not stable at low pH values. However, the breakdown was not significant within the first 5 min (Fig. 6). Adsorption effects of penicillins, causing less reproducible results, were observed when the surface of the glassware was not deactivated (Table 3). These effects were eliminated by silanization with 10% DMCS in toluene. The pH at the protein precipitation step was not critical within the tested range of 4.5–4.9. However, the efficiency of protein removal was maximal at pH 4.6–4.8.

Using the complete procedure, including the liquid–liquid extraction step, it was possible for a single analyst to obtain 8–10 sample extracts that were ready for HPLC analysis within a working day of 8 h.

Table 2
The relative repeatability standard deviation (RSD_r), day-to-day variation (RSD_d) and recovery from spiked milk samples

Penicillin	Theoretical concentration (μg/l)	RSD_r (μg/l)	RSD_d (μg/l)	Recovery (mean±SD) (%)
Amoxicillin	4	5.1	5.1	95±2.9
	15	6.1	7.4	98±6.0
	30	1.8	4.7	99±4.5
	45	6.5	7.1	99±5.4
Pen-G	4	6.3	7.5	100±6.0
	15	6.6	7.1	97±5.4
	30	5.5	5.5	99±2.9
	45	3.4	5.2	97±4.6
Ampicillin	4	7.3	7.3	99±3.0
	15	7.9	7.9	99±3.1
	30	5.3	5.3	102±3.4
	45	4.7	4.7	101±2.5
Oxacillin	4	6.4	6.4	95±3.9
	15	7.5	7.5	98±2.2
	30	5.4	8.9	97±8.0
	45	3.3	6.6	92±6.1
Cloxacillin	4	4.0	6.6	95±6.0
	15	7.9	7.9	98±1.8
	30	5.4	6.3	93±5.1
	45	4.3	5.5	94±4.6
Dicloxacillin	4	8.8	8.8	94±4.6
	15	5.6	6.6	89±5.2
	30	3.2	6.6	87±6.2
	45	3.7	4.7	87±3.9

Six duplicates were conducted on different days for each concentration.

Table 3
Effect of silanization of glassware. Comparison of results from three experiments using non-silanized glassware and three experiments using glassware treated with 10% DMCS in toluene

Penicillin	Absolute recovery (%)	
	Without silanization (mean±SD)	With silanization (mean±SD)
Amoxicillin	24±13	91±3.6
Pen-G	48±23	96±2.6
Ampicillin	25±15	95±5.3
Oxacillin	58±24	101±4.5
Cloxacillin	57±23	98±2.3
Dicloxacillin	51±22	99±1.0

4. Conclusion

The HPLC method described in this paper provides a sensitive and reliable procedure for the quantitative analysis of amoxicillin, penicillin G, ampicillin, oxacillin, cloxacillin and dicloxacillin in raw milk from cows. The low detection limit and the high selectivity make the method suitable as a confirmation method in residue analyses.

This method is used on a routine basis for the verification of positive results obtained by screening methods in the Danish Milk Quality Recording System.

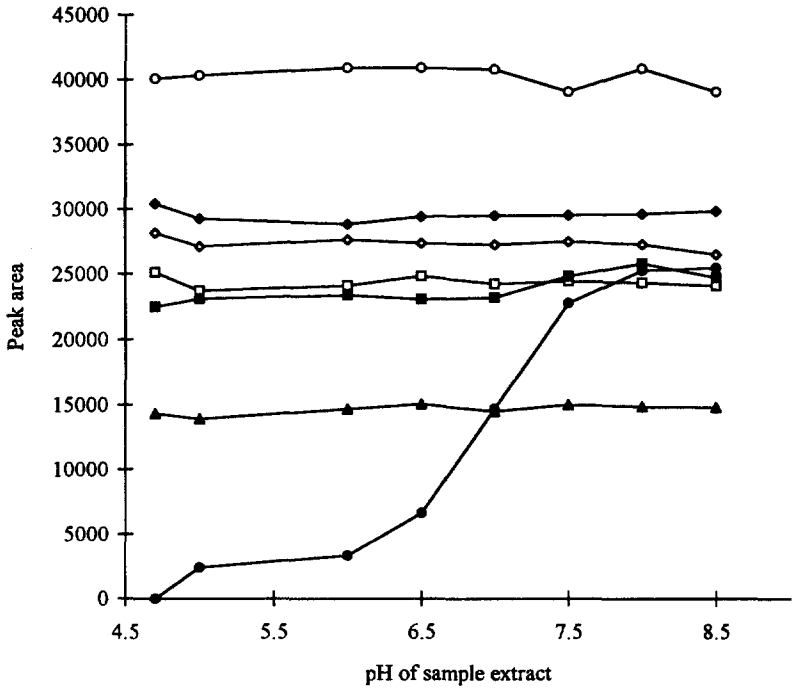

Fig. 5. Recovery from a tC_{18} SPE cartridge at different pH values of the sample extract. (■) Pen-V, (□) cloxacillin, (▲) dicloxacillin, (●) amoxicillin, (■) ampicillin, (○) Pen-G, (◇) oxacillin.

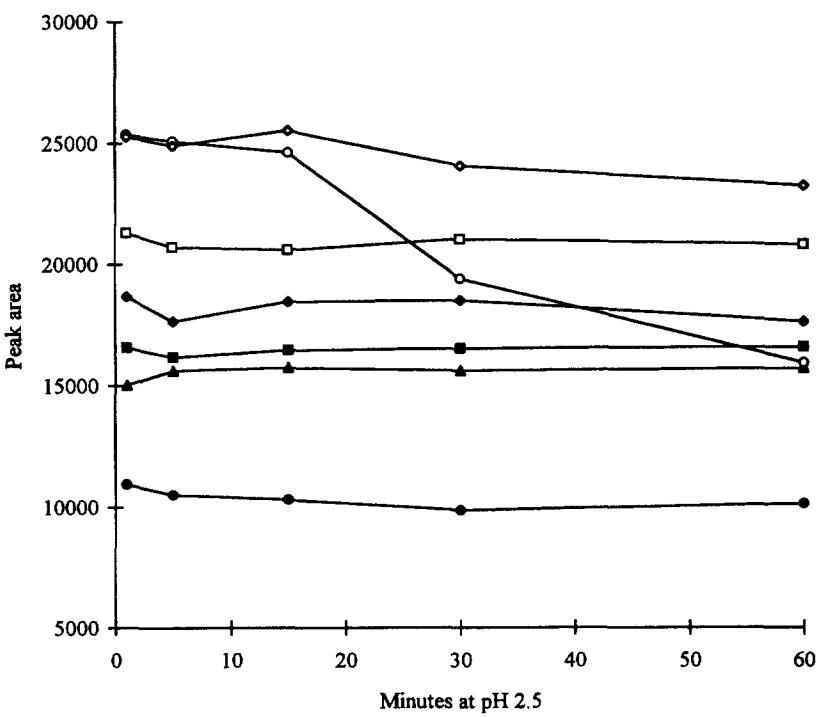

Fig. 6. The stability of penicillins in the buffered mixture (pH 2.5) for liquid–liquid extraction. (■) Pen-V, (□) cloxacillin, (▲) dicloxacillin, (●) amoxicillin, (■) ampicillin, (○) Pen-G, (◇) oxacillin.

References

[1] Commission Regulation (EC) No. 2701/94 of 7 November 1994.
[2] J.O. Boison, L. Keng, J.D. MacNeil, J. Assoc. Off. Anal. Chem. 77 (1994) 565.
[3] D.J. Fletouris, J.E. Psomas, A.J. Mantis, J. Agric. Food Chem. 40 (1992) 617.
[4] W.A. Moats, R. Harik-Khan, J. Assoc. Off. Anal. Chem. 78 (1995) 49.
[5] U. Meetschen, M. Petz, Z. Lebensm. J. Assoc. Off. Anal. Chem. 73 (1990) 373.
[6] W.J. Blanchflower, S.A. Hewitt, G. Kennedy, Analyst 119 (1994) 2595.
[7] International Dairy Federation, IDF standard 135B (1991).