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Determination of isoxazolylpenicillins residues in milk by ion-pair reversed-phase high-performance liquid chromatography after precolumn derivatization

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Abstract

A high-performance liquid chromatographic method has been developed for the determination of isoxazolylpenicillins (oxacillin, cloxacillin and dicloxacillin) residues in milk. This method involves extraction of the penicillins from milk with phosphate buffer pH 8, deproteinization by acidification with sulfuric acid followed by cleanup and concentration on a C_{18} solid-phase extraction column and reaction with 1,2,4-triazole and mercury(II) chloride solution pH 9.0 at 65°C. The derivatized compound is eluted on a C_8 column with a mobile phase containing acetonitrile, methanol and phosphate buffer (pH 6.5, 0.1 mol l^{-1}) loaded with sodium thiosulfate and ion-pairing tetrabutylammonium hydrogenosulphate. The detection limit of the method is 2 μg l^{-1} for oxacillin, 3 μg l^{-1} for cloxacillin and 5 μg l^{-1} for dicloxacillin in milk and the three penicillins have been quantified down to 15 μg l^{-1} in line with the EU criteria of the directive No. 93/256/EEC. © 1998 Elsevier Science B.V.

Keywords: Isoxazolylpenicillins; Oxacillin; Cloxacillin; Dicloxacillin

1. Introduction

Milking cow infections are commonly treated by use of penicillin antibiotics especially with intramammary ointments containing oxacillin, cloxacillin or dicloxacillin active drugs. Residues of these penicillins in milk may cause allergic reactions in people with sensitivity to β-lactam antibiotics and also cause problems in the milk processing industry (yoghurt, cheese and other milk products). To ensure human food safety, maximum residue limits (MRLs) have been fixed to 30 μg l^{-1} for each of the three isoxazolylpenicillins in milk by an EU Council Decision [1] (regulation No. 2701/94, Nov., 1994).

Several high-performance liquid chromatography (HPLC) methods have been described for the assay of penicillin antibiotics in the range of residues concentrations in milk. This paper presents an improved method for the determination of isoxazolylpenicillins residues in milk. It contains a modified extraction procedure based on the methods described by Terada and Sakabe [2] for ampicillin in milk and Boison and coworkers [3,4] for

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penicillin-G in animal tissue and milk. A description of a precolumn derivatization reaction (Fig. 1) formerly proposed by Bundgaard and Ilver [5] for the determination of nine penicillins and later used by Haginaka and Wakaï [6] for ampicillin in serum and urine, by Boison and coworkers [3,4] for penicillin-G in animal tissue and milk and by Verdon and Couëdor [7] for ampicillin in milk is also included. Evaluated as recommended by EEC directive No. 93/256/EEC [8], the results of the validation in terms of selectivity, specificity, resolution, accuracy, precision, recovery and limit of detection and quantification are provided. The effect of an ion-pairing reagent (tetrabutylammonium^{+}) mixed with the phosphate buffer of the mobile phase has also been investigated.

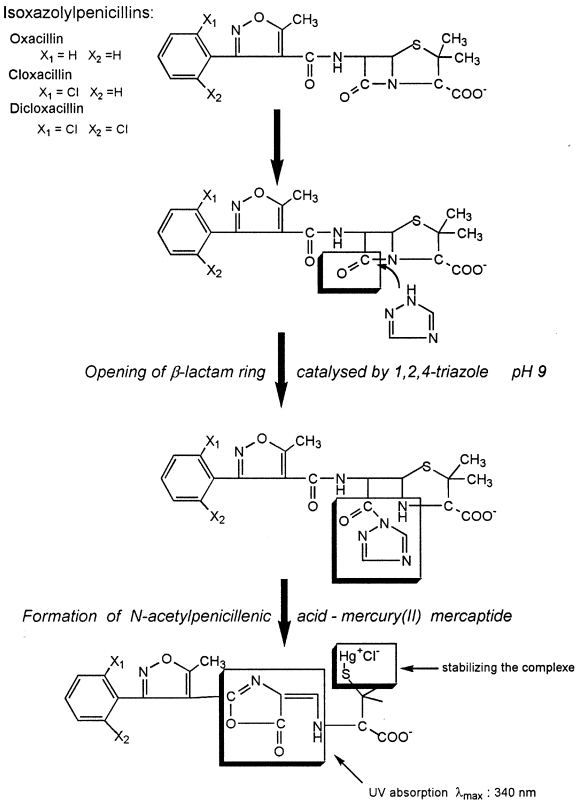

Fig. 1. Isoxazolylpenicillins derivatization – postulated mechanism for the 1,2,4-triazole base-catalyzed reaction of penicillins with mercuric chloride [5,6].

2. Experimental

2.1. Reagents and standards

Oxacillin sodium (87.4% as acid form) and dicloxacillin sodium (92.2% as acid form) were obtained from Sigma (St. Louis, MO, USA), cloxacillin sodium (90.9% as acid form) was provided by Smithkline Beecham (Surrey, UK). Methanol, acetonitrile were of analytical-reagent grade (Merck, Darmstadt, Germany). Demineralized ultra-pure water was obtained with Milli-Q ultrafiltration unit from Millipore (Molsheim, France). Monobasic sodium phosphate monohydrate, dibasic sodium phosphate dihydrate, sodium thiosulfate pentahydrate, hydrogenosulfate tetrabutylammonium and mercuric chloride were purchased from Merck. 1,2,4-Triazole was purchased from Sigma.

2.2. Apparatus

Centrifugation was operated with a refrigerated centrifuge (Model GR 4.11, Jouan, France). A vacuum manifold (Model Vac-Elut, Touzart et Matignon, Courtaboeuf, France) and Bond Elut C_{18} cartridges of 6 ml and 500 mg (Varian, Harbor City, CA, USA) were used for the solid-phase extraction (SPE). The liquid chromatography (LC) system was composed of SP8800 pump (Spectra Physics, San Jose, CA, USA); SP8775 autosampler fitted with 100-μl loop and 0.5 ml syringe (Spectra Physics); C_8 analytical column (150×3.9 mm I.D., 5 μm) (Symmetry, Waters, Milford, MA, USA); Kratos 783 variable-wavelength UV detector (Kratos Analytical, Ramsey, NJ, USA); data acquisition was controlled by an SP4290 Integrator and a Winner386 station (Spectra Physics).

2.3. Phosphate buffer

A phosphate buffer (pH 6.5, 0.1 mol l^{-1}) containing 0.015 mol l^{-1} thiosulfate and 0.03 mol l^{-1} tetrabutylammonium hydrogenosulfate was prepared by weighing 4.969 g of anhydrous monobasic sodium phosphate, 10.139 g of dibasic sodium phosphate dihydrate, 3.894 g of sodium thiosulfate pentahydrate and 10.186 g of tetrabutylammonium hydrogenosulfate and dissolving in 800 ml of water by

stirring with a magnetic spinbar. The pH was then adjusted to 6.5 with 5 mol l^{-1} sodium hydroxide. The solution obtained was diluted to volume in a 1 l volumetric flask, mixed thoroughly and filtered under vacuum through a 0.45 μm unit (Millipore, Bedford, MA, USA). It was stored at +4°C and used for a period of one week at the most.

2.4. Mobile phase

Prepared fresh every day, the mobile phase contained 580 ml of the 0.1 mol l^{-1} phosphate buffer (0.015 mol l^{-1} thiosulfate and 0.03 mol l^{-1} tetrabutylammonium hydrogenosulfate), 50 ml of methanol and 370 ml of acetonitrile.

2.5. Extraction solution

A phosphate buffer extraction solution (pH 8, 0.1 mol l^{-1}) was prepared by dissolving 15.6 g of dibasic sodium phosphate dihydrate in 800 ml of water. The solution was adjusted to pH 8 with 10 mol l^{-1} sodium hydroxide and completed to 1 l with water.

2.6. Elution solution

An elution solution ES (60:40) for the SPE step was prepared fresh every day by mixing 60 ml of water with 40 ml of acetonitrile.

2.7. Derivatizing reagent

The derivatizing reagent (2 mol l^{-1} 1,2,4-triazole and 0.01 mol l^{-1} mercuric chloride) was obtained by weighing 13.78 g 1,2,4-triazole into a 100 ml beaker, adding 60 ml of water and stirring with a magnetic spinbar to dissolve. The solution was mixed with 10 ml 0.1 mol l^{-1} mercuric chloride solution and adjusted to pH 9.0±0.5 with 5 mol l^{-1} sodium hydroxide, and transferred to a 100 ml volumetric flask and diluted to volume with water. Stored at +4°C and protected from sunlight the derivatizing reagent could be used for up to two months.

2.8. Working standard solutions for milk fortification and external calibration curve

Stock solutions of 0.75 g l^{-1} of oxacillin (SSo), cloxacillin (SSc) and dicloxacillin (SSd) were prepared by dissolving the appropriate weight of each pure reference standard in water. An intermediate standard solution (ISSocd) containing the three penicillins was prepared by diluting 2 ml of each isoxazolylpenicillin stock solution to 25 ml with water to obtain a 60 mg l^{-1} solution of oxacillin, cloxacillin and dicloxacillin.

A range of five working standard solutions (WSSs) containing the three penicillins were then prepared by respectively diluting 250 μl, 500 μl, 1 ml, 2 ml and 4 ml of ISSocd to 20 ml with water to obtain 0.75 mg l^{-1}, 1.5 mg l^{-1}, 3 mg l^{-1}, 6 mg l^{-1} and 12 mg l^{-1} solutions. These working standard solutions were stored at +4°C protected from light. Stock solutions, intermediate solutions and working standard solutions were prepared freshly every fortnight.

An external calibration curve was constructed by diluting 100 μl of the corresponding WSSs with 900 μl of the SPS (60:40) into 5 ml glass tubes in order to obtain five levels of concentration: 75, 150, 300, 600 and 1200 μg l^{-1}. The standards preparative solution SPS (60:40) was prepared fresh every day by diluting 60 ml of 0.1 mol l^{-1} phosphate buffer pH 6.5 with 40 ml of acetonitrile. The 5 standards samples were adjusted to pH 8 with 2 mol l^{-1} sodium hydroxide solution and derivatized as described for the fortified milk samples hereafter.

2.9. Milk sample fortification

100-μl volumes of each WSS were used to fortify 5 ml of blank milk samples in order to obtain milk samples spiked at five levels: 15 μg l^{-1} (1/2 MRL), 30 μg l^{-1} (MRL), 60 μg l^{-1} (2 MRL), 120 μg l^{-1} (4 MRL) and 240 μg l^{-1} (8 MRL), respectively.

2.10. Extraction, cleanup and analytical procedure

2.10.1. Acidic extraction

Fortified milk samples or incurred milk samples of 5 ml were placed into 50 ml centrifuge glass tubes.

Thirty ml of phosphate buffer extraction solution pH 8 were added followed by 1.65 ml of 1 mol l^{-1} sulfuric acid to reach pH between 4.0 and 4.5 and the solutions were vortex-mixed for 30 s. The aqueous solutions were centrifuged at 2400 *g* for 10 min (0°C<θ<5°C), and were then transferred to clean glass tubes taking care to avoid cream pieces. 600-μl volumes of 5 mol l^{-1} sodium hydroxide were added to reach pH 8. The aqueous phases were stirred by vortexing and centrifuged one more time at 2400 *g* for 5 min (0°C<θ<5°C).

2.10.2. C_{18} column cleanup

Solvent reservoirs of 50 ml were mounted onto the C_{18} cartridges and placed with adapters on the SPE vacuum manifold. The cartridges were washed with 10 ml methanol followed by 10 ml water, 5 ml 2% sodium chloride solution and finally with 5 ml phosphate buffer extraction solution pH 8. Cartridges were not allowed to run dry at this stage and the aqueous centrifuged supernatants were transferred into the reservoirs and pulled through the C_{18} cartridge with vacuum at a flow-rate of about 3 ml min^{-1}. Vacuum was stopped at this stage, washes were discarded and adapters and reservoirs were removed from the cartridges. 1-ml volumes of the elution solution ES (60:40) were poured into the cartridges and for 1 min were allowed to soak homogeneously the C_{18} phase of the cartridges. Vacuum was set back and the isoxazolylpenicillins were eluted at a flow-rate of about 3 ml min^{-1}.

2.10.3. Precolumn derivatization

0.5-ml volumes of derivatizing reagent (do not forget to shake the reagent just before using it) were added to the eluate, the glass tubes were capped, stirred by vortexing and allowed to react about 10 min in a 65°C water bath. The tubes were removed from the water bath and quickly cooled to room temperature by immersing them into a beaker of water for 10 min. Protected from light the derivatized penicillins were proven stable for more than 5 h at 30°C.

2.10.4. LC determination

A volume of 100 μl of the derivatized sample was injected into the HPLC system operating in an isocratic mode using a mobile phase of acetonitrile–methanol–0.1 mol l^{-1} phosphate buffer pH 6.5 (37:5:58, v/v/v) at a flow-rate of 1 ml min^{-1}. The peak areas of each of the three isoxazolylpenicillins were detected at 340 nm with retention times of 10 min (oxacillin), 12 min (cloxacillin) and 17 min (dicloxacillin).

2.11. Calculations

2.11.1. Recovery calculation

To determine procedure recoveries, the UV responses for isoxazolylpenicillins in fortified milk samples subjected to extraction, cleanup and LC analysis were compared with those of equivalent external isoxazolylpenicillins standards taking into account the SPE reconcentration (×5) for the milk samples. Recovery was assessed as: R (%)=(S×100)/S_e where R is the recovery of the fortified sample, S is the peak area of the fortified milk sample and S_e the peak area of the corresponding external standard solution. The mean recovery of the method for each of the three penicillins has been measured considering all the values corresponding to the five concentrations obtained during the whole validation study (n=52).

2.11.2. Concentration calculation

The concentration of the fortified sample was calculated as: C (μg l^{-1})=(A×C_e×100)/(A_e×5×R) where A is the peak area of the fortified milk sample, A_e the peak area of the corresponding external standard solution, C_e the concentration of the external standard solution, 5 the SPE reconcentration of the method (1 ml of eluate for 5 ml of milk) and R the percentage of recovery of the method for the designed isoxazolylpenicillin.

2.11.3. Statistical analysis

Linearity of external standards, fortified milk samples, estimated concentrations and recoveries were analyzed by means of regression and analysis of variance (ANOVA). Dose independence of the recovery was also checked by means of a Student test. The limit of detection was defined as LOD=3×C/SB where C is the concentration of the samples fortified at the smallest concentration tested (15 μg l^{-1}) and SB is the signal-to-noise ratio of the response obtained with the same fortified samples.

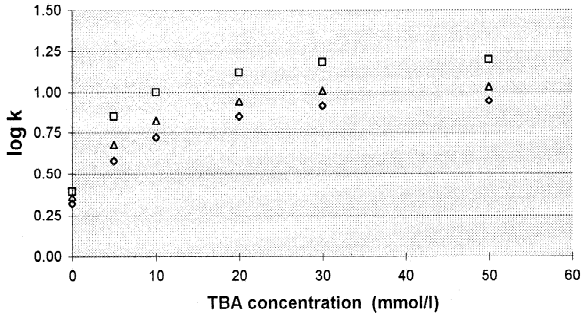

Fig. 2. Effect of the counterion concentration (TBA^+) upon the retention of the derivatized penicillins. Mobile phase, 0.1 mol l^{-1} phosphate buffer–acetonitrile–methanol (58:37:5, v/v/v); flow-rate, 1 ml min^{-1} ; detection wavelength, 340 nm. Analytes: (○) oxacillin; (△) cloxacillin; (□) dicloxacillin.

Precision of the method has been assessed for within-day and between-day variations. The operator carried out four trials each day for both concentrations and during four days. Precision data were determined using the same operator and the same material on milk samples fortified at 15 μg l^{-1} and 30 μg l^{-1}.

3. Results and discussion

The method has been applied to the analysis of oxacillin, cloxacillin and dicloxacillin residues in raw milk and can also be adapted to the determination of the other penicillin antibiotics with only slight modifications in the derivatization and in the chromatographic steps.

The effect of tetrabutylammonium counterion (TBA^+) on the elution of the three penicillin compounds has been systematically studied (Fig. 2). It has been demonstrated that a concentration of 30 mmol l^{-1} of TBA guaranteed an improved selectivity versus milk coextractives. The derivatization step has also been improved compared to Haginaka and Wakaï [6] and Boison and coworkers [3,4] methods by increasing (from 10^{-3} as stated by Haginaka and

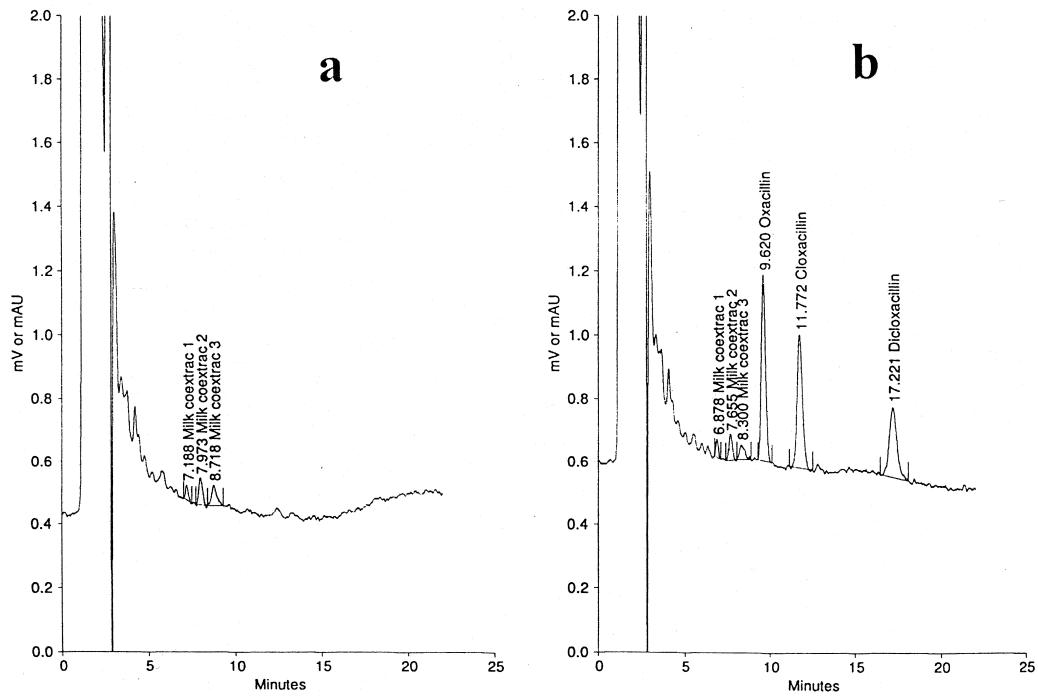

Fig. 3. Chromatograms obtained with a blank milk sample (a) and a milk sample fortified in oxacillin, cloxacillin and dicloxacillin to 15 μg l^{-1} (b).

Wakaï to 10^{-2} mol l^{-1}) the concentration of mercuric chloride in the derivatizing reagent. The saturated reagent obtained, ultrasonicated just before use, consequently insured the stability of the precolumn reaction towards penicillins without increasing the reaction rate.

The method has been validated as follows: concerning the selectivity, the chromatograms corresponding to the extracts of blank milk samples reveal no peak interfering with the isoxazolylpenicillins (Fig. 3a,b). The resolution between the peak of oxacillin and the peak of the milk coextract No. 3 has been calculated to $R_s = 2.27 \pm 0.28$. The other compounds of the penicillin family known to be possibly present in contaminated milk are detected with lower retention times than those of isoxazolylpenicillins (case of benzylpenicillin and phenoxymethylpenicillin) or are not detected at all in our conditions (case of ampicillin and amoxicillin) and thus do not interfere (Fig. 4).

The results provided in Table 1 underline the specificity of the method in terms of retention time variability. A mean retention time has been calculated for each of the three compounds considering the analysis of the whole fortified milk samples used during the validation ($n = 52$). The good resolution of the peaks of the three compounds ($R_s > 4$) has also been demonstrated in the same conditions (Table 1).

The linearity of the response has been verified for samples of milk fortified from 15 to 240 μg l^{-1}. Recovery is independent of the dose on the range of concentrations studied and the relative standard deviations of the recoveries are always within an acceptable limit of 8% (Table 2).

Accuracy has been measured on milk samples fortified to 15 μg l^{-1}, 30 μg l^{-1}, 60 μg l^{-1}, 120 μg l^{-1} and 240 μg l^{-1} with four replicates for each concentration and taking into account the recovery

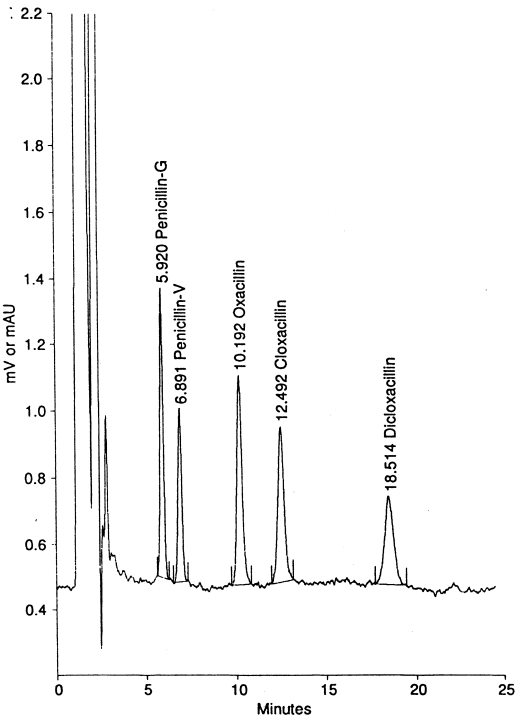

Fig. 4. Chromatogram obtained with a standard solution containing 75 μg l^{-1} of each of seven penicillins (benzylpenicillin, phenoxymethylpenicillin, ampicillin, amoxicillin, oxacillin, cloxacillin and dicloxacillin) and prepared as reported in Section 2.8.

assessed for the whole validation period (Table 2). LODs have been measured at 2 μg l^{-1} for oxacillin, 3 μg l^{-1} for cloxacillin and 5 μg l^{-1} for dicloxacillin. Results for the precision study are shown in Table 3. They correspond to the raw values corrected by the mean recovery of the method. No value was declared doubtful and no sample has been discarded. Considering oxacillin residues analysis and over the four days routine use of the methodology, the mean recoveries for oxacillin residues ranged from 77% to

Table 1
Specificity and resolution of oxacillin, cloxacillin and dicloxacillin obtained from the 52 fortified milk samples of the validation study

Compound	Mean retention time±S.D. (min)	R.S.D. (%)	$R_{s(i/j)}$±S.D.[a]		Number of replicates
Oxacillin	9.70±0.23	2.37	$R_{s(o/c)}$: 4.02±0.27	$R_{s(o/d)}$: 11.54±0.69	52
Cloxacillin	11.88±0.28	2.34	$R_{s(c/o)}$: 4.02±0.27	$R_{s(c/d)}$: 7.66±0.47	52
Dicloxacillin	17.47±0.42	2.38	$R_{s(d/o)}$: 11.54±0.69	$R_{s(d/c)}$: 7.66±0.47	52

[a] $R_{s(i/j)}$: $2 \cdot (t_j - t_i / w_i + w_j)$ where t_i and t_j are the retention times of peaks i and j, respectively, w_i and w_j are the base-widths of peaks i and j, respectively.

Table 2
Recovery and accuracy of oxacillin, cloxacillin and dicloxacillin from fortified milk samples

Fortification ($\mu g\ l^{-1}$)	Mean concentration found±S.D. ($\mu g\ l^{-1}$)	Recovery (%)	Mean concentration corrected by R[a] (81.9%) ($\mu g\ l^{-1}$)	R.S.D. (%)	Accuracy (%)	Number of replicates
Oxacillin						
15	11.9±1.0	79.3±6.9	14.5±1.3	8.7	−3.2	4
30	24.8±1.7	82.6±5.5	30.2±2.0	6.7	+0.8	4
60	49.1±2.4	81.9±4.0	60.0±2.9	4.9	−0.0	4
120	96.9±2.4	80.7±2.0	118.2±2.9	2.4	−1.5	4
240	200.8±8.3	83.7±3.5	245.1±10.1	4.1	+2.1	4
Cloxacillin						
15	11.5±0.2	76.4±1.4	14.4±0.3	1.9	−4.1	4
30	23.4±0.8	78.2±2.6	29.4±1.0	3.4	−2.0	4
60	47.0±2.6	78.3±4.3	58.9±3.2	5.5	−1.8	4
120	92.3±2.3	76.9±1.9	115.7±2.9	2.5	−3.6	4
240	187.6±11.8	78.2±4.9	235.2±14.8	6.3	−2.0	4
Dicloxacillin						
15	9.7±1.1	64.9±7.6	15.2±1.8	1.6	+1.4	4
30	19.4±1.3	64.6±4.3	30.3±2.0	6.7	+0.9	4
60	40.6±3.0	67.6±5.0	63.3±4.7	7.4	+5.6	4
120	78.5±1.8	65.4±1.5	122.6±2.9	2.3	+2.1	4
240	156.8±11.1	65.3±4.6	244.7±17.4	7.1	+1.9	4

[a] R is the mean recovery determined for the method taking into account all the values corresponding to the five levels of concentration tested during the whole validation of the method (n=52).

89% at the 15 $\mu g\ l^{-1}$ level of concentration and from 75% to 86% at the 30 $\mu g\ l^{-1}$ level of concentration. Thus, on routine use the recoveries range has been estimated as 83±2% at 15 $\mu g\ l^{-1}$ level and 81±3% at 30 $\mu g\ l^{-1}$ level. The mean relative standard deviation of between-day variations was calculated to 4.6% for 15 $\mu g\ l^{-1}$ samples and to 4.2% for 30 $\mu g\ l^{-1}$ samples. They were far within the limit fixed by Directive No. 93/256/EEC concerning within-laboratory variations of reference methods for residues analysis (15% for 15 $\mu g\ l^{-1}$ samples and 13.6% for 30 $\mu g\ l^{-1}$ samples). The same conclusion

Table 3
Precision data—within-day and between-day variations

Fortification ($\mu g\ l^{-1}$)	Mean concentration found ($\mu g\ l^{-1}$)	Recovery (%)	Within-day variation ($\mu g\ l^{-1}$)	Between-day variation ($\mu g\ l^{-1}$)	R.S.D. r (%)	R.S.D. R (%)
Oxacillin						
15	15.1±0.7	82.6±2.4	1.8	2.0	4.1	4.6
30	29.6±1.2	80.9±3.1	1.6	3.5	1.9	4.2
Cloxacillin						
15	15.8±0.8	84.0±3.4	1.6	2.3	3.6	5.1
30	29.1±1.5	77.4±3.7	1.4	4.1	1.8	5.0
Dicloxacillin						
15	15.3±1.5	65.3±5.5	2.5	4.3	5.9	9.9
30	28.6±2.1	61.1±4.0	3.0	5.8	3.7	7.2

can be noted concerning cloxacillin and dicloxacillin precision data shown in Table 3.

4. Conclusions

The analytical method presented in this paper regarding with a selective determination of oxacillin, cloxacillin and dicloxacillin residues in milk allows to quantify these three compounds down to 15 μg l^{-1} (1/2 MRL) in milk according to the criteria of EU Directive No. 93/256/EEC. This multiresidues method is able to detect down to 5 μg l^{-1} of each isoxazolylpenicillin in milk (2.5 ng injected) in the same run. The operator prepared up to 12 milk samples for LC analysis in the same day without any automation. As the method has been applied to raw milk, it should be suitable for use in skimmed milk or lyophilized skimmed milk as well. Slightly modified the procedure is now also being successfully applied to muscle tissue.

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