

## Determination of $\beta$ -Lactams in Milk Using a Surface Plasmon Resonance-Based Biosensor

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Two surface plasmon resonance (SPR)-based biosensor assays for detection of  $\beta$ -lactam antibiotics in milk are reported. The assays are based on the enzymatic activity of a carboxypeptidase converting a 3-peptide into a 2-peptide, a reaction that is inhibited in the presence of  $\beta$ -lactams. Antibodies were used to measure either the amount of formed enzymatic product or the amount of remaining enzymatic substrate. Both assays detected different  $\beta$ -lactams at or below European maximum residue limits (MRLs), and the detection limit for penicillin G was 1.2  $\mu\text{g}/\text{kg}$  and 1.5  $\mu\text{g}/\text{kg}$  for the 2- and 3-peptide assays, respectively. The precision (CV) was < 5%, both within and between assays at the penicillin G MRL (4  $\mu\text{g}/\text{kg}$ ). The biosensor results obtained upon analysis of incurred milk samples were compared with results obtained by liquid chromatography (HPLC), and the method agreements were, in general, good.

**KEYWORDS:**  $\beta$ -lactams; milk;  $\beta$ -lactam receptor protein; SPR biosensor

### INTRODUCTION

The first use of antimicrobials for treatment of infections in veterinary medicine was in the late 1940s, shortly after their development (1). Today, the  $\beta$ -lactam antibiotics (i.e., penicillins and cephalosporins) (Figure 1) are the most frequently used antimicrobials for treatment of mastitis in dairy cows, and consequently, the most commonly found type of residues in milk. Consumer demands for residue-free and in other aspects safe animal-derived food products are high, and to guarantee that food of animal origin is toxicologically safe, withdrawal times and maximum residue limits (MRLs) have been established (2). National monitoring programs to control that veterinary drug residues are below the MRLs in various foods, including milk, are compulsory in all EC countries (3).

In most countries, benzylpenicillin (penicillin G) is the most commonly used substance within the  $\beta$ -lactam group, but other substances are also administered. Information on the type of  $\beta$ -lactam substances that are used for treatments of infections in dairy cows is not easily accessible in many countries. Consumption figures for the overall sales of antimicrobial drugs for veterinary use have been monitored in the Scandinavian countries for some years (4–6); in Denmark, each prescription is also related to animal species, type of medicine, disease to be treated etc. The frequent use of antimicrobials over the years

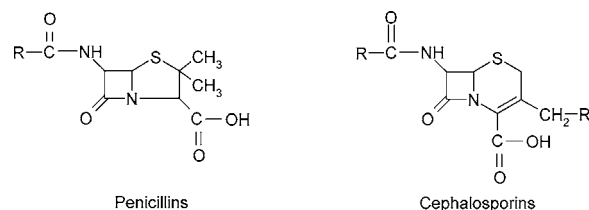


Figure 1. Structures of penicillin and cephalosporin.

has led to a widespread bacterial resistance to  $\beta$ -lactams, in particular to benzylpenicillin. Sweden has applied a successful, restrictive antibiotic policy, and within the  $\beta$ -lactam group, only benzylpenicillin is approved for treatment of mastitis. Recently, however, ceftiofur has been approved for other indications (e.g., foot rot and respiratory diseases). In many other countries, a wider range of  $\beta$ -lactams is approved for use in dairy cows (7–11).

Due to the importance of detecting as many  $\beta$ -lactam substances as possible in milk testing programs, generic assays are to be preferred. Due to the extensive incubation times and insufficient sensitivity for some substances during the last 10 years, the use of the traditional microbial inhibitor tests in certain applications has been replaced by so-called rapid tests (e.g., SNAP) (IDEXX Laboratories, Inc., Westbrook, ME),  $\beta$ -STAR and Penzym (UCB Bioproducts, Braine-l'Alleud, Belgium), and Charm Safe Level  $\beta$ -lactam test (Charm Sciences, Lawrence, MA). These tests are based on receptor proteins that are generic and specific for the active  $\beta$ -lactam compound. This is important because the MRLs are set for the parent compounds (i.e., the active form with closed ring structure). Due to instability of

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the  $\beta$ -lactam ring, the development of antibodies that are specific to the intact  $\beta$ -lactam structure has been achieved with limited success. Immunological assays will, therefore, often detect either both inactivate and active forms, or only the inactive form, of the substances.

Despite the rapid analyses, with results typically available within 5–10 min, the application of the rapid tests is limited by their poor capacity and the frequent attention needed from personnel (12). To increase the applicability of receptor-based assays, it would be an advantage to design an automated assay. By using an SPR-based (surface plasmon resonance) biosensor, a number of assays for detection of veterinary drug residues in milk have been published, for example, sulfamethazin (13), enrofloxacin and ciprofloxacin (14), chloramphenicol (15), penicillins (16), streptomycin (17), and levamisole (18), all of which are based on antibodies for the detection. In contrast, our work has been focused on the possibilities of using a receptor protein to develop assays for  $\beta$ -lactam residues (19, 20).

Our recently published work presented a biosensor assay for analysis of penicillin G in milk, based on the enzymatic activity of a DD-carboxypeptidase from *Actinomadura* R39 (re-classified from *Streptomyces* R39) (20). The enzyme hydrolyses a 3-peptide (Ac-L-Lys-D-Ala-D-Ala) into a 2-peptide (Ac-L-Lys-D-Ala), but in the presence of  $\beta$ -lactam antibiotics, this enzymatic activity is inhibited and less 2-peptide will be formed. The assay described measures the amount of enzymatic product formed by using antibodies against the 2-peptide. Here we describe an optimized version of this assay, together with a related assay based on the same enzymatic reaction, in which the amount of remaining enzymatic substrate (3-peptide) is measured.

## MATERIALS AND METHODS

**Equipment.** The SPR-based biosensor Biacore Q (Biacore AB, Uppsala, Sweden) was used for assay developments. Biacore Q Control Software (version 3.0.1) was used for instrument operation and data handling. A test tube heater (GTF, Gothenburg, Sweden) was used for the incubation step.

**Chemicals and Reagents.** Sensor Chip CM5 (research grade), HBS-EP buffer, pH 7.4 (10 mM 4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid (Hepes), 3.4 mM EDTA, 150 mM NaCl, 0.005% (v/v) surfactant P-20), and amine coupling kit (400 mM *N*-ethyl-*N'*-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), 100 mM *N*-hydroxysuccinimide (NHS), and 1 M ethanolamine hydrochloride) were provided by Biacore AB (Uppsala, Sweden).

The 2-peptide (Ac-L-Lys-D-Ala) was synthesized and purchased from QCB Inc., Biosource International (Hopkinton, MA). The 3-peptide (Ac-L-Lys-D-Ala-D-Ala) was purchased from Bachem AG (Bubendorf, Switzerland).

DD-carboxypeptidase from *Actinomadura* R39 (R39) (21) was provided by UCB Bioproducts (Braine-l'Alleud, Belgium). Polyclonal antibodies directed against the 2-peptide (R513) and the 3-peptide (R499) were developed at the Veterinary Sciences Division, Department of Agriculture and Rural Development, DARDNI (Belfast, United Kingdom); immunogens were prepared by conjugation of 2- or 3-peptide with KLH, and antibodies were raised in rabbits according to a standard procedure.

All  $\beta$ -lactams (penicillin G, amoxicillin, ampicillin, oxacillin, cloxacillin, cefalexin, and cephalirin) were purchased from Sigma-Aldrich Co (St. Louis, MO), except for ceftiofur, which was obtained from Pharmacia Animal Health (Puurs, Belgium).

HNM-buffer, consisting of 0.1 M Hepes, 0.1 M NaCl, and 0.05 M MgCl<sub>2</sub> in Milli-Q water, pH 8.0, was used for dilution of 3-peptide and R39. Borate buffer (50 mM, pH 8.0) was used for dilution of 2-peptide and 3-peptide before immobilization to the sensor chip surface.

**Milk Samples.** Antibiotic-free bulk tank milk for preparation of standards and spiked milk samples was obtained from the University dairy research farm. Antibiotic-free producer milk samples were obtained from the milk grading laboratory in Jönköping (Steins AB, Sweden). All milk samples were defatted by centrifugation and stored at  $-20^{\circ}\text{C}$  until date of analysis. Lyophilized milk samples from an excretion study (performed in February 2001) with milk from cows treated with penicillin G (Procain-Penicillin G 3 Mio., WDT, Garbsen, Germany) (22) were obtained from the Federal Dairy Research Center (Kiel, Germany).

**Assay Principles.** The two assays are based on inhibition of the enzymatic activity of a carboxypeptidase that hydrolyses a 3-peptide into a 2-peptide. In the presence of  $\beta$ -lactam antibiotics, the enzymatic activity is inhibited. On the basis of the same enzyme, the two assays measure the formation of enzymatic product (2-peptide assay) and the remaining substrate (3-peptide assay), respectively. In both assays, a milk sample is mixed with 3-peptide and R39 and incubated for 5 min at  $47^{\circ}\text{C}$  to allow the enzymatic reaction to proceed. If the sample is  $\beta$ -lactam-free, R39 will hydrolyze the 3-peptide into 2-peptide. In the presence of  $\beta$ -lactams, the enzymatic activity of R39 is inhibited, and less 2-peptide will be formed. Following the incubation, in the 2-peptide assay, the sample is mixed with antibodies directed against the 2-peptide and the mixture is injected over a sensor surface with 2-peptide immobilized. With a  $\beta$ -lactam-free sample (negative), the antibodies will be inhibited by 2-peptide produced in the sample, whereas with a  $\beta$ -lactam-contaminated sample (positive), the antibodies will bind to the 2-peptide surface. The response obtained is directly proportional to the amount of  $\beta$ -lactam antibiotics in the sample.

In the 3-peptide assay, the sample is mixed with antibodies directed against the 3-peptide, and the mixture is injected over a sensor surface with 3-peptide immobilized. With a positive sample, the antibodies will be inhibited by non-hydrolyzed 3-peptide, whereas the antibodies will bind to the 3-peptide surface with a negative sample. The response obtained is inversely proportional to the amount of  $\beta$ -lactam antibiotics in the sample.

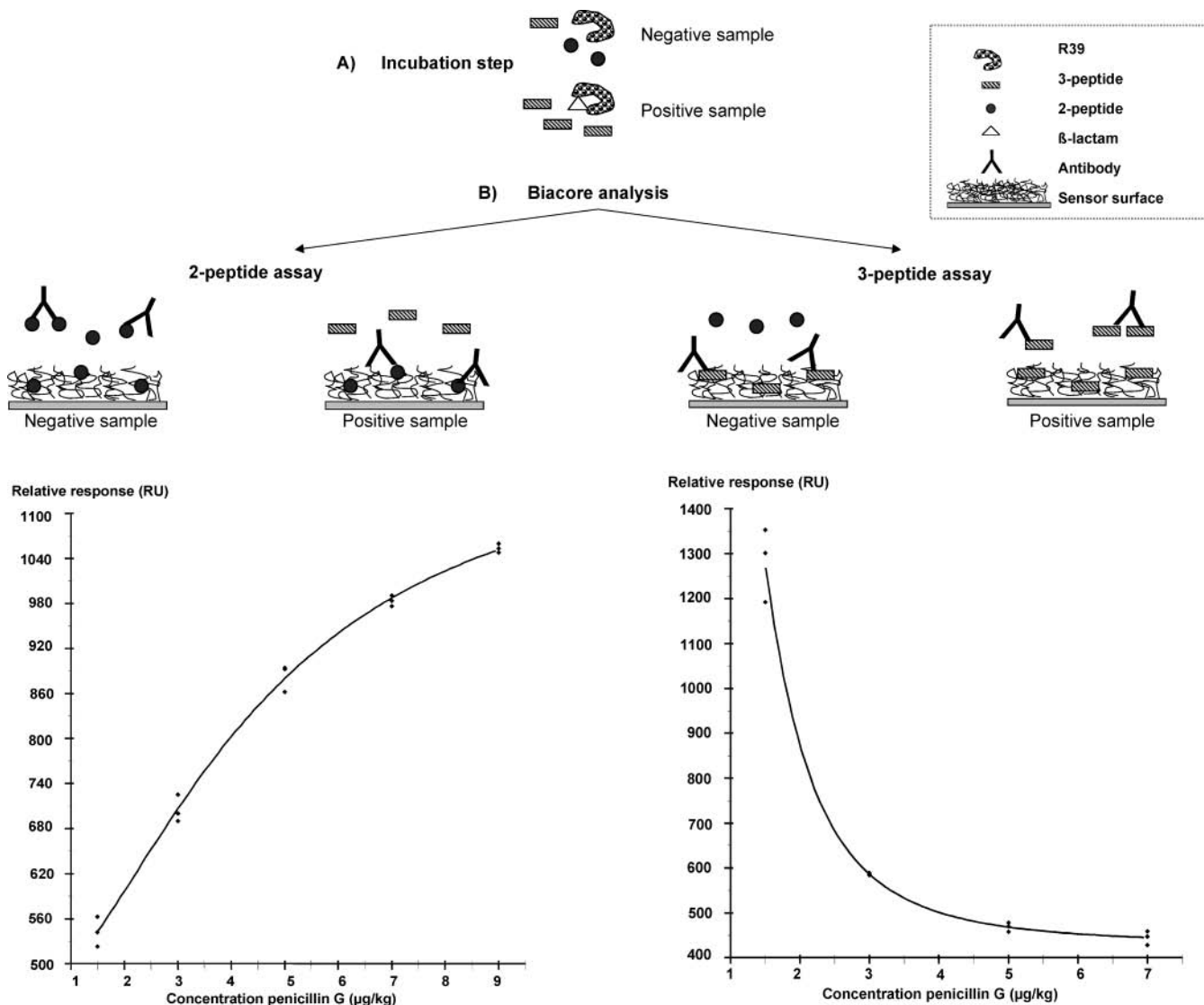
The amount of antibody binding to the surface is measured as a mass-dependent change in refractive index near the sensor surface. For a schematic illustration of the assays, see **Figure 2**.

**Immobilization of Ligand to the Sensor Chip Surface.** Immobilization of the 2-peptide was performed using the Surface Prep unit (Biacore AB) according to a recently published procedure (20). The 3-peptide surface was prepared in a similar manner.

**Assay Procedures.** The procedures for the two assays are virtually identical, but the concentrations of reagents and the regeneration time differ between the two assays (**Table 1**). Milk was pipetted into a test tube and mixed with 3-peptide and R39, and the mixture (80% milk, 10% 3-peptide, and 10% R39) was incubated for 5 min at  $47^{\circ}\text{C}$ . After incubation, the sample was transferred to a microtiter plate (one replicate/well). In the instrument, the sample was mixed with the respective antibody, and the mixture (10% sample and 90% antibody) was injected for 2 min across the sensor chip surface. The surface was regenerated by injection of 0.5 M NaOH with 10% acetonitrile. HBS-EP was used as running buffer with a constant flow rate of 30  $\mu\text{L}/\text{min}$ .

**Sample Preparation.** Antibiotic-free bulk tank milk was used for preparation of calibration standards. From a stock solution of the  $\beta$ -lactam antibiotic (1 mg/mL in Milli-Q water), working solutions were prepared by dilution in Milli-Q water. These were then further diluted 1:100 in bulk tank milk to the final concentrations 9, 7, 5, 3, and 1.5  $\mu\text{g}/\text{kg}$  of  $\beta$ -lactam in the 2-peptide assay and to 7, 5, 3, and 1.5  $\mu\text{g}/\text{kg}$  of  $\beta$ -lactam in the 3-peptide assay. The same procedure was used to prepare milk samples spiked with 6, 4 and 2  $\mu\text{g}/\text{kg}$  of penicillin G.

**Evaluation of Assay Performances.** The following experiments were performed with both assays, using the procedures described above. The antibodies against the 2-peptide (R513) and 3-peptide (R499) were tested for their cross-reactivity with 3-peptide and 2-peptide, respectively. Inhibition curves for R513 and R499 were constructed with both 2-peptide and 3-peptide in HNM-buffer. The relative response corresponding to the 2-peptide concentration resulting in a 50% inhibition of the binding from R513 to the 2-peptide surface ( $\text{IC}_{50}$ ) was determined and compared to the concentration of 3-peptide that inhibited R513 to the same extent. The cross-reactivity of R513 was then determined as



**Figure 2.** Assay principles and typical calibration curves ( $n = 3$ ) for the two biosensor assays. During the incubation step, the DD-carboxypeptidase (i.e., R39) hydrolyses a 3-peptide into a 2-peptide in a negative ( $\beta$ -lactam-free) sample. However, the enzymatic activity is decreased in a positive ( $\beta$ -lactam-containing) sample. In the biosensor analysis, 2- and 3-peptide antibodies respectively, are added, and in the 2-peptide assay the 2-peptide antibodies will only bind to the surface if the sample is positive, while in the 3-peptide assay the 3-peptide antibodies will bind to the surface if the sample is negative.

**Table 1.** Conditions and Reagent Concentrations Used in the 2- and 3-peptide Assays

	2-peptide assay	3-peptide assay
R39 in HNM buffer ( $\mu\text{g/mL}$ )	4.6	4.9
3-peptide in HNM buffer ( $\mu\text{g/mL}$ )	4.7	2.8
Antibodies in HBS-EP buffer <sup>a</sup> ( $\mu\text{g/mL}$ )	34	18
Regeneration time (s)	90	150

<sup>a</sup> Concentration of antibodies determined spectrophotometrically at 280 nm after purification by precipitation via addition of saturated ammonium sulfate.

the ratio of  $\text{IC}_{50}$  for 2-peptide over  $\text{IC}_{50}$  for 3-peptide, expressed in percent. The cross-reactivity of R499 was determined in a similar way, but the cross-reactivity was expressed as a percent of  $\text{IC}_{50}$  relative to 2-peptide.

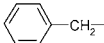
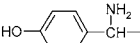
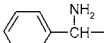
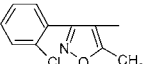
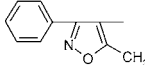
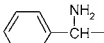
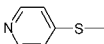

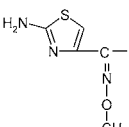
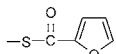
The detection limits (LOD) of the assays were determined by analyzing antibiotic-free bulk milk samples from 20 different producers. The average responses obtained were correlated to a calibration curve with penicillin G constructed at the same occasion (penicillin G concentrations were 0, 1, 2, 4, and 6  $\mu\text{g/kg}$  in the 2-peptide assay and

0, 1, 2, 3, and 5  $\mu\text{g/kg}$  in the 3-peptide assay). The LOD was defined as the concentration corresponding to the response determined to be statistically different (three standard deviations) from the mean response of negative samples.

To determine the precision of the two assays, milk samples spiked with 2, 4, and 6  $\mu\text{g/kg}$  of penicillin G, corresponding to  $0.5 \times \text{MRL}$ ,  $1.0 \times \text{MRL}$ , and  $1.5 \times \text{MRL}$ , respectively, were analyzed. In the 3-peptide assay, only 2  $\mu\text{g/kg}$  and 4  $\mu\text{g/kg}$  of penicillin G were analyzed, because 6  $\mu\text{g/kg}$  would fall outside the linear part of the calibration curve. The precision between days was determined by analyzing three replicates of each concentration on three consecutive days, and the within-assay precision was determined by analyzing 10 replicates of each concentration within the same run. The coefficients of variation (CV) were calculated for each concentration.

To investigate the ability of the assays to detect different  $\beta$ -lactam antibiotics, milk was spiked with different substances (amoxicillin, ampicillin, oxacillin, cloxacillin, cefalexin, cephapirin, and ceftiofur) at their respective  $0.5 \times \text{MRL}$ ,  $1.0 \times \text{MRL}$ ,  $1.5 \times \text{MRL}$ , and  $2.0 \times \text{MRL}$ . The observed responses were related to a penicillin G calibration curve prepared at the same occasion. Each concentration was analyzed twice, and a substance was defined as detected (+) when its concentration was determined to be  $\geq 1.5 \mu\text{g/kg}$ , that is, above LOD for penicillin

**Table 2.** Maximum Residue Limits (MRLs) and Side Chains (compare with Figure 1) for Eight Different  $\beta$ -lactams Used in This Study

$\beta$ -lactam substance	R	R'	MRL ( $\mu\text{g}/\text{kg}$ )
Penicillin G			4
Amoxicillin			4
Ampicillin			4
Cloxacillin			30
Oxacillin			30
Cefalexin		-H	100
Cephapirin			60
Ceftiofur			100

G; however, in the 2-peptide assay, LOD is 1.2  $\mu\text{g}/\text{kg}$ , but the lowest point of the calibration curve (1.5  $\mu\text{g}/\text{kg}$ ) was used for the definition. The chemical structures of the analyzed  $\beta$ -lactams and their respective MRLs are shown in **Table 2**.

Lyophilized milk samples ( $n = 20$ ) from cows treated with a penicillin G-based drug (Procain-Penicillin G 3 Mio., WDT, Garbsen, Germany) were reconstituted and analyzed to investigate if the assays also would detect residues in incurred samples. The samples were prepared as described, and the responses obtained were related to a calibration curve with penicillin G, which was constructed at the same occasion. Samples that gave responses corresponding to penicillin G concentrations higher than the highest calibration point were diluted in antibiotic-free bulk milk to obtain a response in the calibration interval. The results of the two biosensor assays were compared with the results obtained in high performance liquid chromatography (HPLC) analysis performed at the Federal Dairy Research Center (Kiel, Germany) (23).

## RESULTS

Calibration curves for penicillin G in milk for the two assays are shown in **Figure 2**. The cross-reactivity of R513 for 3-peptide and R499 for 2-peptide was determined to be <0.1% for the respective antibody. The limit of detection for penicillin G in milk was determined to be 1.2  $\mu\text{g}/\text{kg}$  in the 2-peptide assay and 1.5  $\mu\text{g}/\text{kg}$  in the 3-peptide assay. The precision (CV) of the 2-peptide assay ranged between 3.1 and 13% within assay and between 2.2 and 6.4% between days. In the 3-peptide assay, the precision within assay ranged between 3.3 and 4.8% and between 1.8 and 9.6% between days (**Table 3**).

The ability of the assays to detect other  $\beta$ -lactams in samples spiked with different concentrations is illustrated in **Table 4**. The results from the biosensor and HPLC analyses of the incurred samples containing penicillin G are shown in **Table 5**.

**Table 3.** Precision (CV) of the 2-peptide and 3-peptide Assays Determined within Assay and between days by Analyzing Milk Spiked with Penicillin G

	2-peptide assay			3-peptide assay	
	2 ( $\mu\text{g}/\text{kg}$ )	4 ( $\mu\text{g}/\text{kg}$ )	6 ( $\mu\text{g}/\text{kg}$ )	2 ( $\mu\text{g}/\text{kg}$ )	4 ( $\mu\text{g}/\text{kg}$ )
within assay					
mean value ( $n = 10$ )	1.9 <sup>a</sup>	4.3	5.8	1.6 <sup>b</sup>	3.7
CV (%)	13 <sup>a</sup>	3.1	6.0	3.3 <sup>b</sup>	4.8
between assay					
mean value ( $n = 3$ )	1.9	4.1	5.6	1.8	3.7
CV (%)	5.0	2.2	6.4	9.6	1.8

<sup>a</sup>  $n = 8$  because two samples were determined to contain penicillin G levels below the lowest concentration of the calibration curve (i.e., <1.5  $\mu\text{g}/\text{kg}$ ). <sup>b</sup>  $n = 7$  because three samples were determined to contain penicillin G levels below the lowest concentration of the calibration curve (i.e., <1.5  $\mu\text{g}/\text{kg}$ ).

## DISCUSSION

This work presents two related biosensor assays for the detection of  $\beta$ -lactam antibiotics in milk. The assays described are based on the same enzymatic reaction but differ with respect to the substance that is measured: the enzymatic product formed (2-peptide assay) or the remaining substrate (3-peptide assay). An important aspect in the development of these assays was, therefore, the cross-reactivity of the two antibodies for the peptides measured. To ensure a secure detection of 2-peptide in the 2-peptide assay, the 2-peptide antibody (R513) should not cross-react too much with 3-peptide, and vice versa for the 3-peptide antibody (R499) in the 3-peptide assay. The results show that both antibodies were highly specific, because the observed cross-reactivities were below 0.1%. Because the two peptides only differ in one amino acid, and hence, are very similar (Ac-L-Lys-D-Ala-D-Ala and Ac-L-Lys-D-Ala), the results were above expectations.

In previously described SPR-based biosensor inhibition assays for detection of drug residues in food, the response decreases with increasing amounts of analyte. This is also the case with the 3-peptide assay, whereas the 2-peptide assay shows a positive correlation between the concentration of penicillin G and the response. This may, for psychological reasons, be an advantage when used in routine analysis: a positive response = a positive sample. The shapes of the two calibration curves also differ in other aspects; the linear interval of the 3-peptide assay calibration curve is very narrow, ranging from 1.5 to 5  $\mu\text{g}/\text{kg}$ . There is a large steep in response from the level of noninhibited antibody binding to the sensor surface (1.5  $\mu\text{g}/\text{kg}$ ) to a total inhibition of antibody by free 3-peptide in the sample (5  $\mu\text{g}/\text{kg}$ ), see **Figure 2**. This strong decrease in response is interesting, because it provides a very precise cut-off limit between a negative and a positive result.

The detection limits of the two assays, 1.2  $\mu\text{g}/\text{kg}$  and 1.5  $\mu\text{g}/\text{kg}$ , respectively, are low and allow secure detection at the EU MRL of  $\mu\text{g}/\text{kg}$  for penicillin G in milk. Precision (<5%) and accuracy (3.7–4.3  $\mu\text{g}/\text{kg}$ ) were also satisfactory at the MRL for both assays. Because the assays are considered for screening purposes, their ability to quantify detected residues is not as important as the necessity to obtain a true positive result when a sample contains residues above a certain level.

Compared to the previously described 2-peptide assay (20), where the LOD was determined to be 5.2  $\mu\text{g}/\text{kg}$ , and the precision ranged between 4 and 16% at 4  $\mu\text{g}/\text{kg}$  penicillin G, both LOD and precision have been improved in this modified version. The improvements may be explained by the use of a higher buffer flow rate and most certainly also by a reduction

**Table 4.** The Ability of the Assays to Detect Different  $\beta$ -lactams at  $0.5 \times$  MRL,  $1.0 \times$  MRL,  $1.5 \times$  MRL and  $2.0 \times$  MRL When Compared to a Penicillin G Calibration Curve<sup>a</sup>

$\beta$ -lactam	2-peptide assay				3-peptide assay			
	$0.5 \times$ MRL	$1.0 \times$ MRL	$1.5 \times$ MRL	$2.0 \times$ MRL	$0.5 \times$ MRL	$1.0 \times$ MRL	$1.5 \times$ MRL	$2.0 \times$ MRL
Amoxicillin	$\pm^b$	$+^c$	+	+	$-^d$	+	+	+
Ampicillin	-	+	+	+	-	+	+	+
Cloxacillin	-	-	-	-	-	-	$\pm$	+
Oxacillin	$\pm$	+	+	+	-	+	+	+
Cefalexin	+	+	+	+	+	+	+	+
Cephapirin	+	+	+	+	+	+	+	+
Ceftiofur	-	$\pm$	+	+	-	+	+	+

<sup>a</sup> A substance was defined as detected when the determined concentration was higher than the assay LOD for penicillin G. <sup>b</sup>  $\pm$  means that the substance was detected in one of two analyses. <sup>c</sup> + means that the substance was detected in both analyses. <sup>d</sup> - means that the substance was not detected in any of the analyses.

**Table 5.** Penicillin G Concentrations in Incurred Milk Samples Determined with the Biosensor Assays and HPLC<sup>a</sup>

sample	HPLC ( $\mu\text{g}/\text{kg}$ )	2-peptide ( $\mu\text{g}/\text{kg}$ )	3-peptide ( $\mu\text{g}/\text{kg}$ )
1	3.2	2.4	3.0
2	3.5	1.8	2.4
3*	8.4	6.0	6.3
4*	5.8	5.1	5.1
5	2.4	2.0	2.9
6	6.1	5.5	5.5
7*	5.3	5.8	5.6
8	4.8	2.5	3.9
9*	2.7	2.6	2.3
10*	4.7	4.3	3.7
11*	4.2	4.8	4.6
12	4.5	3.8	5.2
13	6.4	5.4	5.3
14*	4.4	3.9	3.8
15*	9.7	7.8	6.9
16	6.7	5.7	5.6
17	5.3	5.4	6.2
18*	7.5	5.8	5.5
19	5.7	5.5	4.1
20*	4.0	4.3	4.3

<sup>a</sup> The samples marked with an asterisk (\*) were diluted (1:10) to obtain a response falling in the calibration interval in the biosensor assays.

in matrix interference due to the reduced milk fraction in the injection mixture (8% in this assay compared to 70% in the initially described).

The ability to detect different  $\beta$ -lactam antibiotics was similar for the two assays for the investigated substances. The 3-peptide assay detects both ceftiofur and cloxacillin at lower concentrations than the 2-peptide assay, but the latter has a tendency ( $\pm$ ) to detect amoxicillin and oxacillin at lower concentrations than the former. Both assays detected all substances at their respective MRL except for cloxacillin, which was detected at  $60 \mu\text{g}/\text{kg}$  ( $2.0 \times$  MRL) in the 3-peptide assay but was not detected in the 2-peptide assay. For the other six  $\beta$ -lactams investigated (amoxicillin, ampicillin, oxacillin, cefalexin, cephapirin, and ceftiofur), the results from the two biosensor assays were in agreement with the claimed detection limits of the Penzym test (24).

The results from the analyses of penicillin G in incurred samples with the two biosensor assays agreed well with those obtained by HPLC-analysis. Method agreement can be estimated by calculating the mean differences for two methods (25). By subtracting the concentrations determined by HPLC from the concentrations found by the biosensor assay, the mean difference was calculated to be  $-0.7 \mu\text{g}/\text{kg}$  for both 2- and 3-peptide assays. It is important, however, to consider that prior to this study, there was no experience from analysis of lyophilized

samples and comparison against a calibration curve constructed with normal milk. Furthermore, the HPLC analysis was performed two years ago (in February 2001), and it is therefore difficult to say if the differences between the biosensor and HPLC are due to the circumstances mentioned.

Because the biosensor assays are not considered for quantitative, but rather for qualitative purposes, it is not necessary to obtain a perfectly accurate concentration as long as the sample is correctly determined positive above a certain threshold level. This level must be determined with consideration to assay accuracy, MRLs and the limit of detection of the assay in order to obtain a secure detection of  $\beta$ -lactam-containing samples, without yielding any false negative or too many false positive results.

In conclusion, two receptor/enzymatic-based biosensor assays have been developed that both show promising characteristics. The assays differed mainly in two aspects. The penicillin G calibration curve in the 3-peptide assay showed a very sharp decrease in response between 1.5 and  $5 \mu\text{g}/\text{kg}$ . This is a promising advantage because it allows a clear cut-off limit for classification of the result. On the other hand, the 2-peptide assay had the advantage of showing a positive correlation between response and  $\beta$ -lactam concentration, which must be considered more user-friendly. It has previously been shown that R39 is specific for the active form of  $\beta$ -lactam substances (19), and together with the broad-specificity of R39 shown in this study, where seven  $\beta$ -lactam substances (both penicillins and cephalosporins) were detected at their respective MRLs, the developed assays show high potential to be used for automated screening of  $\beta$ -lactam antibiotics in milk.

#### ACKNOWLEDGMENT

We thank Gertraud Suhren and Heinz Braun at the Federal Dairy Research Center, Kiel, Germany, for kindly supplying us the incurred milk samples and performing the HPLC-analysis.

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Received for review April 25, 2003. Revised manuscript received February 9, 2004. Accepted March 10, 2004. This work was supported by grants from the Swedish Council for Forestry and Agricultural Research.

JF0344284