SPR Biosensor Analysis of β-Lactam Antibiotics in Milk

Development and use of assays based on a β-lactam receptor protein

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Abstract

The aim of this study was to investigate the applicability of an SPR biosensor in combination with a β-lactam receptor protein for generic detection of β-lactam antibiotics in milk.

β-Lactam antibiotics constitute the group of antimicrobials most commonly used for treatment of bacterial infections in dairy cows. Consequently, they are also the most common type of drug residue found in milk and, as such, causing problems to the dairy industry. The advantage of using a receptor protein, instead of antibodies, is that a generic assay, specific for the active form of the β-lactam structure, is obtained. Three assays based on the same receptor protein, a DD-carboxypeptidase from *Actinomadura* R39 (R39) were developed using the SPR biosensor Biacore.

The first assay was based on the binding of the β-lactam receptor to a conjugate that was captured on the sensor surface. The assay showed a low detection limit of 1-2 μg/kg for penicillin G in milk, to be compared with the EU maximum residue limit (MRL), which is set to 4 μg/kg. Furthermore, other β-lactams were detected at or near their respective MRLs. However, there was a high non-specific binding to the sensor surface, varying between different milk samples and batches of conjugate. It was also difficult to obtain a procedure for reproducible synthesis of the conjugate.

As an alternative approach, two assays based on the enzymatic activity of the receptor protein were developed. The carboxypeptidase activity of R39 results in hydrolysis of a 3-peptide (Ac-L-Lys-D-Ala-D-Ala) into a 2-peptide (Ac-L-Lys-D-Ala), a reaction which is inhibited in the presence of β-lactams. Antibodies were developed and used to measure the amount of enzymatic product formed or the amount of remaining enzymatic substrate, respectively. The assays showed low detection limits (1.2 μg/kg and 1.5 μg/kg, respectively) and high precision (CV <5%) for penicillin G in milk and several other β-lactams were detected at or near their respective MRL.

Finally, the two peptide assays were compared with seven commercial tests in screening of producer milk samples. The results showed good agreement and furthermore, the quantitative results obtained were in line with HPLC measurements.

Keywords: penicillin, β-lactams, milk, SPR biosensor analysis, β-lactam receptor, carboxypeptidase.

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Sammanfattning

Mastit (juverinflammation) är den vanligaste sjukdomen hos mjölkkor och cirka 20% av de svenska korna behandlas varje år för detta med antibiotika. β-laktamer, d.v.s. penicilliner och cefalosporiner, är den vanligaste gruppen av antibiotika som används för mastitbehandling och andra bakteriella infektioner hos mjölkkor. β-laktamerna är därför även den vanligast förekommande typen av restsubstanser i mjölk.

För att garantera att animaliska livsmedel är toxikologiskt säkra, finns det inom EU lagstiftning som anger gränsvärden (MRL-värden) för olika restsubstanser, t.ex. vilka halter av penicillin som mjölk maximalt får innehålla. Antibiotikabehandling åtföljs alltid av en karenstid då mjölken inte får levereras till mejeriet, detta för att halten av restsubstans ska sjunka till nivåer under MRL.. Mjölken analyseras regelbundet huvudsakligen av följande anledningar.
1) Den etiska aspekten - att garantera konsumenten att mejeriprodukter är fria från antibiotika. En så kallad avskiljande kontroll har nyligen införts på svenska mejerier, som innebär att all mjölk analyseras innan den används. Om mjölken innehåller β-laktamer kasseras den och därmed kan mejerierna vara säkra på att deras mejeriprodukter ej innehåller denna restsubstans.
2) Hälsoaspekten - vissa personer är överkänsliga mot β-laktamer, vilket kan leda till allergiska reaktioner vid konsumtion av kontaminerad mjölk.
3) Den teknologiska aspekten - tillverkningen av vissa fermenterade mejeriprodukter, t.ex. ost och yoghurt, kan störas om mjölken innehåller förhöjda halter av antibiotika. Vid fermenteringen utnyttjas tillväxten av mjölksyrabakterier, men finns det antibiotika närvarande i mjölken hämmas deras tillväxt och fermenteringen försämras eller uteblir. Ofullständig fermentering leder till försämrad produktkvalitet eller till att mjölken måste kasseras, vilket ger stora ekonomiska förluster för mejerierna.

De vanligaste metoderna för analys av antimikrobiella substanser har under många år varit olika mikrobiella inhiberingstest. Dessa metoder är enkla att utföra, kostnaden per analys är låg och de har ett brett detektionsspektrum. Metodernas största nackdel är den långa inkuberingstiden. Under senare år har antalet receptorbaserade analysmetoder för antibiotikarester i mjölk ökat. Dessa har ofta hög känslighet, kort analystid (5-10 minuter) och är specifika för β-laktamer. Möjligheten till automatiserade analyser är dock begränsad, varför de blir arbetskrävande och kostsamma.

Under det senaste decenniet har en optiska biosensorteknik (Biacore AB, Uppsala) använts för att utveckla automatiserade metoder för detektion av veterinärmedicinska restsubstanser i olika livsmedel. Instrumentet utnyttjar ett optiskt fenomen, SPR (surface plasmon resonance) för att studera interaktioner mellan ligand på en sensoryta och analyt i provet. När exempelvis en tillsatt antikropp binder till sensorytan uppstår en massökning som kan korreleras till hur mycket analyt, t.ex. penicillin, det finns i provet. Metoderna har visat hög känslighet, specificitet och reproducerbarhet. samt kort analystid. Endast små provvolymer behövs (µl) och analystiden är kort (vanligtvis mindre än 10 minuter).

När det gäller mjölk har ingen provupparbetning behövts, utan mjölken har injicerats som den är, vilket är mycket tidssparande.

Syftet med det här projektet var att försöka utveckla en receptorbaserad Biacore-metod för att analysera β-laktamer i mjölk. Fördelen med att använda en β-laktamreceptor i stället för antikroppar, som hittills har varit det vanliga, är att hela gruppen av β-laktamer detekteras – metoden blir generisk. β-laktamerna har en ringstruktur som lätt hydrolyseras, varvid substansen blir inaktiv och förlorar den antibakteriella förmågan. De flesta antikroppar detekterar både den aktiva och inaktiva formen eller enbart den inaktiva, medan en β-laktamreceptor är specifik för den aktiva formen. Det senare är högst önskvärt eftersom gränsvärdena endast avser den aktiva formen.

Tre olika metoder, alla baserade på samma receptorprotein, ett karboxypeptidas från *Actinomadura* R39 (R39), har utvecklats. I den första metoden utnyttjas bindningen av receptorproteinet till β-laktamer. Ett β-laktamkonjugat injiceras och binder till en sensoryta. Beroende på om provet innehöll β-laktamer eller inte, binder receptorproteinet antingen till β-laktamer i provet eller till β-laktamkonjugatet på sensorytan. Metodens detektionsgräns för penicillin G i mjölk var 1-2 μg/kg för, vilket kan jämföras med MRL-värdet som är 4 μg/kg. Dessutom detekterades flera andra β-laktamer vid deras respektive MRL. En nackdel med metoden var att såväl mjölken som konjugatet gav upphov till ospecifik binding till sensorytan, dessutom var konjugatsyntesen svår att göra reproducerbar.

Receptorproteinets enzymatiska aktivitet utnyttjades i två andra metoder. R39 hydrolyserar en 3-peptid (Ac-L-Lys-D-Ala-D-Ala) till en 2-peptid (Ac-L-Lys-D-Ala), men reaktionen inhiberas i närvaro av β-laktamer. Antikroppar producerades och användes för att mäta mängden av bildad produkt (2-peptid) eller den kvarvarande mängden av substrat (3-peptid). Båda metoderna visade låga detektionsgränser (1.2 μg/kg respektive 1.5 μg/kg) och precisionen var hög (CV <5%) för penicillin G i mjölk. Dessutom detekterades flera olika β-laktamer vid deras respektive MRL-värden. Mjölkprover från mjölkproducenter analyserades med de två biosensormetoderna, sju olika kommersiella screeningmetoder och en vätskekromatografisk metod (HPLC) och de erhållna resultaten jämfördes. Resultaten från biosensormetoderna visade hög överensstämmelse med både de kvalitativa (screeningmetoderna) och kvantitativa (HPLC) resultaten.

Contents

Appendix

The present thesis is based on the following papers, referred to by their Roman numerals.

Papers I –IV

I. Eva Gustavsson, Peter Bjurling, Jacques Degelaen and Åse Sternesjö (2002) Analysis of ß-lactam antibiotics using a microbial receptor protein-based biosensor assay. *Food and Agricultural Immunology, 14, 121-131*

II. Eva Gustavsson, Peter Bjurling and Åse Sternesjö (2002) Biosensor analysis of penicillin G in milk based on the inhibition of carboxypeptidase activity. *Analytica Chimica Acta, 468, 153-159*

III. Eva Gustavsson, Jacques Degelaen, Peter Bjurling and Åse Sternesjö. Determination of β-lactams in milk using a surface plasmon resonance-based biosensor. *Submitted for publication*

IV. Eva Gustavsson and Åse Sternesjö. Biosensor analysis of ß-lactams in milk: comparison with microbiological, immunological, receptor-based and HPLC methods. *Submitted for publication*

List of abbreviations

Ab – Antibody
Ac – Acetyl
AcN – Acetonitrile
ADI – Acceptable daily intake
Ala – Alanine
BIA – Biospecific interaction analysis
CV – Coefficient of variation
EDC – N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride
EDTA – Ethylene diamine tetraacetic acid
HPLC – High performance liquid chromatography
IFC – Integrated microfluidic cartridge
LOD – Limit of detection
Lys – Lysine
MRL – Maximum residue limit
MS – Mass spectrometer
MW – Molecular weight
NHS – N-hydroxysuccinimide
NOEL – No-observed effect level
NSB – Non-specific binding
PBP – Penicillin-binding protein
RU – Resonance Unit
SDS – Sodium dodecyl sulphate
SPR – Surface plasmon resonance
$t_{½}$ – Half life

Introduction

Biosensors in food analysis

A biosensor consists of a biological sensing element (ligand) that interacts with the analyte and is in close contact with a physical transducer that senses the physico-chemical change that follows the interaction, *e.g.* change in mass or electrical potential. The transducer converts the biological response into an electrical signal that is amplified, stored and quantified by a processor (Schaertel & Firstenbergeden, 1988; Sethi, 1994; Baird & Myszka, 2001; Patel, 2002; Leonard et al., 2003) (Figure 1). There are many different types of biosensors and they may be classified according to the biological element used, *e.g.* enzymatic or immunological, or to the transducing system applied, *e.g.* optical, amperometric, potentiometric or thermal (Schaertel & Firstenbergeden, 1988).

Enzymes are frequently used as the biological component in biosensors, their high specificity for certain substrates making them very attractive (Brooks & Turner, 1987). The enzymatic product is measured and this can be accomplished by a number of mechanisms, *e.g.* temperature, change in pH, and optical detection, but amperometric detection is by far the most commonly applied transducer (Patel, 2002; Velasco-Garcia & Mottram, 2003). A wide range of enzyme biosensors for applications in food analyses have been described, *e.g.* for detection of glucose, carbohydrates, pesticides, ethanol, starch and phenols (Mello & Kubota, 2002). In the case of antibiotic residues the applications are fewer (Patel, 2002), but systems for detection of penicillin have been described. Setford *et al.* (1999) described a biosensor for detection of β-lactam residues in milk based on glucose oxidase with an amperometric transducer. Despite the high specificity and selectivity achieved by enzymes, enzyme biosensors are sometimes impractical or not possible to use, *e.g.* the enzyme may be unstable, expensive, require a co-factor or be difficult to purify (Brooks & Turner, 1987; D'Souza, 2001).

An alternative to enzyme biosensors is the affinity-based sensor that typically uses the interaction antibody–antigen (immunosensor), receptor–ligand or protein–nucleic acid. Immunosensors are similar to the traditional immunoassays with the advantage of generic applicability, *i.e.* any compound can be detected if there are specific antibodies available. High specificity and selectivity is generally offered based on the antibody-antigen interactions used (Hage, 1999; Mello &

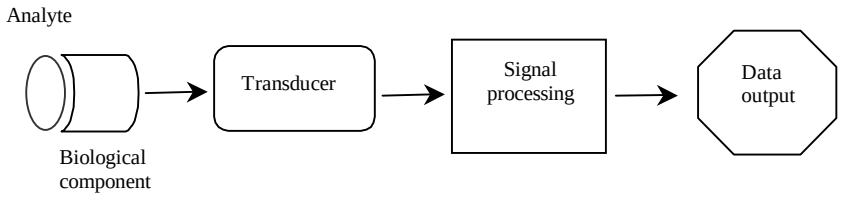


Figure 1. Schematic illustration of the components of a biosensor.

Kubota, 2002). Upon interaction, *e.g.* between an antibody and its antigen, on a transducer surface, the physical properties of the surface will change due to the formation of the antibody-antigen complex, *e.g.* changes in refractive index, mass, density and thickness (Kress-Rogers, 1997). This can easily be followed using acoustic (piezoelectric materials) or optical (*e.g.* surface plasmon resonance (SPR) and resonant mirror) transducers (Bilitewski, 2000) and these are also the most commonly used transducers for detection of chemical or microbial contaminants in food (Patel, 2002). Photometric sensors may, however, also be used and Delwiche *et al.* (2000) described an enzyme immunoassay using a photometric sensor for detection of penicillin residues in food.

Biosensors typically offer automated analysis, resulting in decreased needs for manual operations. The analysis is usually rapid, sensitive and specific with quantification in real time and there is often no need for extensive sample preparation or labelling of reagents (Schaertel & Firstenbergeden, 1988; Bilitewski, 2000; Baird & Myszka, 2001; Patel, 2002; Mello & Kubota, 2002; Velasco-Garcia & Mottram, 2003; Leonard *et al.*, 2003). These features make biosensors interesting as new analytical tools within the food industry. In the literature, there is a steadily increasing number of developed biosensor applications, but many of these are only used for research purposes and the gap between these and the ones being used for routine analysis of biological samples is wide (Luong, Bouvrette & Male, 1997). There are, however, a number of commercially available biosensors, among which Biacore is frequently used for assay developments (Baird & Myszka, 2001; Rich & Myszka, 2002).

Biospecific interaction analysis (BIA) using the Biacore instrument

Biospecific interaction analysis (BIA), as it is applied in the Biacore technique, is based on surface plasmon resonance and enables monitoring of biospecific interactions in real time. The first instrument became commercially available in 1990 and today there are a number of different instruments on the market, all based on the same principle. The Biacore systems comprise of three major components: the optical system, the sensor surface and the microfluidic system.

Optical system

Detection is based on surface plasmon resonance, an optical phenomenon that occurs at the interface of two transparent media of different refractive index (Kretschmann & Raether, 1968; Liedberg, Nylander & Lundström, 1983). A light beam coming from the side of the higher refractive index will be partly reflected and partly refracted, but above a certain critical angle of incidence no light will be refracted and total internal reflection is observed. However, a component of the light, the evanescent wave, will propagate towards the media with the lower refractive index and if the interface between the media is covered with a metal film, *e.g.* gold, the evanescent wave will interact with free electrons in the metal. Light energy will thereby be lost to the metal and the intensity of the reflected light will

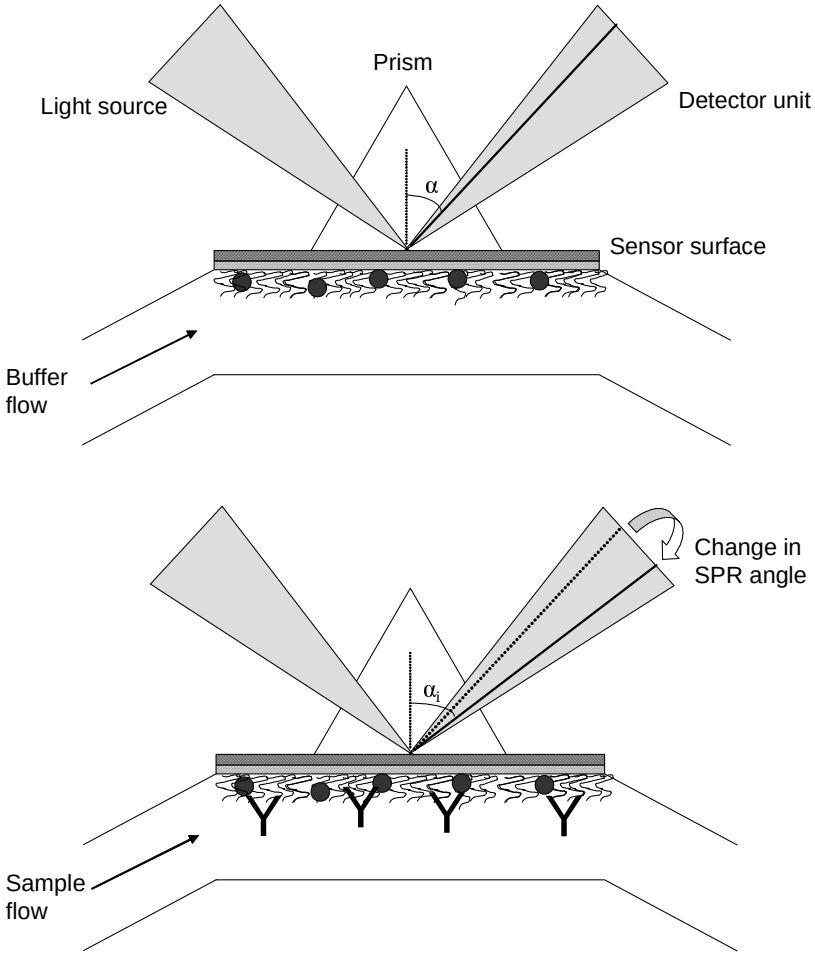


Figure 2. The optical detection system used in the Biacore instrument. Upon binding or dissociation of molecules to the sensor surface the refractive index near the surface changes, resulting in a shift in the SPR angle (α).

decrease. This phenomenon is referred to as surface plasmon resonance (SPR) and only takes place at a sharply defined angle of incidence, the SPR angle.

In the Biacore instrument the interactions between free and immobilised molecules take place at a sensor surface (Löfås & Johnsson, 1990). Upon binding to the sensor surface, the mass at the surface will increase, and thereby also the refractive index (Sjölander & Urbaniczky, 1991), resulting in a shift in the SPR angle that can be measured (Figure 2). The response from the angular change is expressed in resonance units (RU), an arbitrary unit and 1 RU corresponds to a shift in the angle of 0.0001° (Jönsson *et al.*, 1991). By plotting the measured angular shift against time, a sensorgram is obtained illustrating the progress of the interaction at the sensor surface in real time (Figure 3).

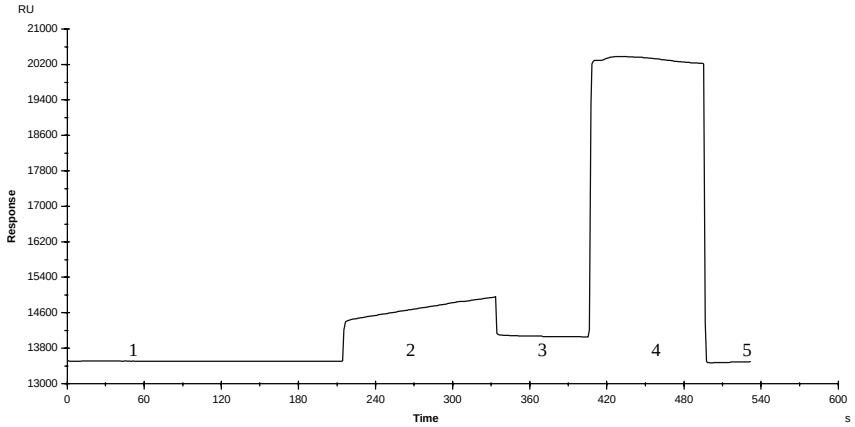


Figure 3. A sensorgram illustrating the interaction between free antibody in the sample and antigen immobilised to the surface. 1) baseline equilibrium (continuous buffer); 2) association of antibody to the sensor surface during injection; 3) response after injection of sample; 4) regeneration of the sensor surface; 5) baseline stabilisation.

Sensor surface

There are different types of sensor surfaces available, but the carboxymethylated dextran surface is the one most commonly used (Baird & Myszka, 2001). The sensor surface consists of a glass support covered by a thin layer of gold to which a coupling matrix, *e.g.* carboxymethylated dextran, is attached via a linker layer (Figure 4). The coupling matrix has an important role in determining the characteristics of the surface. By using this coupling matrix the immobilisation capacity of biomolecules is enhanced, different types of ligands can be immobilised in a general way and a stable covalent binding makes regeneration of the surface possible, thereby enabling repeated analyses to be made (Löfås & Johnsson, 1990; Löfås *et al.*, 1991; Johnsson, Löfås & Lindquist, 1991). Furthermore, the ligand will be easier accessed by the interacting molecule, and the hydrophilic structure of the matrix minimises non-specific adsorption of proteins.

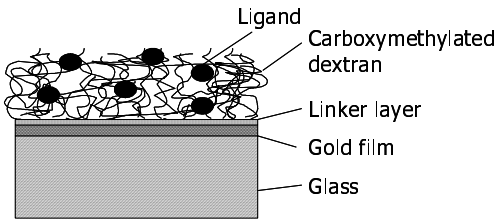


Figure 4. A schematic figure of a Biacore sensor surface in cross-section. The surface consists of a piece of glass covered with a thin gold film. Onto the metal film a linker layer is attached to which a coupling matrix, such as the carboxymethylated dextran layer, is bound.

The solid-phase support, *i.e.* the gold film, would otherwise bind protein in an uncontrollable manner (Löfås & Johnsson, 1990; Löfås *et al.*, 1991; Johnsson, Löfås & Lindquist, 1991; Jönsson *et al.*, 1991).

After ligand immobilisation the sensor surface is stable in most buffers and for short periods of time it can be exposed to high and low pH. Oxidising agents, *e.g.* peroxides, bromine and iodide solutions may, however, destroy the linker layer or the matrix.

Microfluidic system

When the sensor surface is inserted into the instrument the surface matrix side, with the immobilised ligand, is docked against an integrated microfluidic cartridge (IFC). Four flow cells are thereby formed, with volumes ranging from 20 to 60 nl depending on instrument model (Baird & Myszka, 2001). The opposite side of the sensor chip, *i.e.* the glass side, is pressed against a glass prism in the optical unit. The IFC, together with an autosampler, controls the continuous flow of buffer or sample over the sensor surface via a number of sample loops (5-50 µl). By using a continuous flow system, the ligand will be exposed to a constant analyte concentration for the time of the interaction measurement (Baird & Myszka, 2001).

Important aspects in Biacore assay development

Immobilisation procedure

The immobilisation procedure will depend on the properties of the ligand to be coupled and the type of surface. The most common immobilisation strategy for proteins and peptides is amine coupling when the ligand is attached to the surface via primary amine groups, but other functional groups, *e.g.* thiol groups or aldehyde groups, can also be used. In amine coupling the carboxyl groups on the dextran are activated by injection of a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Johnsson, Löfås & Lindquist, 1991). Active esters are thereby formed, which may readily react with an amine group of the ligand. After ligand coupling, ethanolamine hydrochloride is added in order to deactivate the remaining non-reacted active esters. Figure 5 shows the different immobilisation steps. In order to obtain a satisfactory degree of ligand immobilisation, several factors are important, *e.g.* ionic strength, pH and concentration of the coupling buffer, ligand concentration and reaction times. These parameters need to be optimised for each ligand used (Johnsson, Löfås & Lindquist, 1991). By injecting the ligand over a non-activated surface (pre-concentration), the suitability of the coupling buffer can be determined without permanently modifying the surface. The non-covalent electrostatic binding of the ligand to the surface may be observed as a response increase providing an indication of whether the coupling conditions are suitable or not.

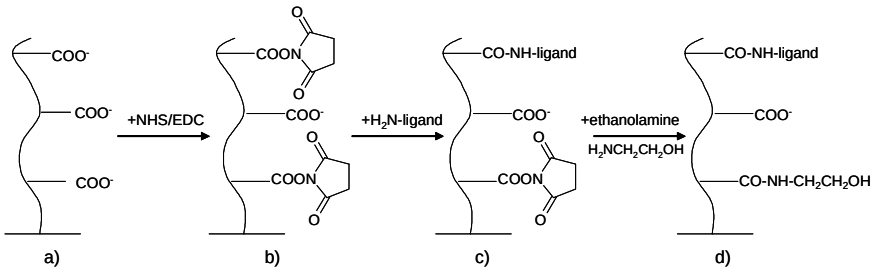


Figure 5. Amine coupling of ligand to a sensor surface: a) Non-activated carboxyl groups on the dextran; b) The carboxyl groups are activated by addition of a mixture of succinimide (NHS) and carbodiimide (EDC); c) The ligand is covalently bound to the sensor surface; d) Remaining esters are deactivated by addition of ethanolamine (Johnsson, Löfås & Lindquist, 1991).

Reduction of non-specific binding to the sensor surface

Matrix effects, *i.e.* interferences with the analytical technique by one or several components in the sample, may have an adverse effect on the detection. The sensitivity and robustness of the assay may decrease and the incidence of false positive and false negative results may increase (Johnsson *et al.*, 2002). Sample components and reagents added to the sample, *e.g.* antibodies, may bind non-specifically to the dextran layer of the surface and thus interfere with the measured specific response. The non-specific binding (NSB) can be hydrophobic and/or electrostatic in nature (Newman, Olabiran & Price, 1997). There are various ways to reduce the extent of this undesired effect, *e.g.* by addition of soluble dextran to the sample (Bergström, 1998), increasing the ionic strength by addition of salt to the sample (Johnsson *et al.*, 2002), or in an extra washing step after the sample injection (Nygren, Sternesjö & Björck, 2003), changing the pH (Johansson & Hellenäs, 2001) and precipitation of proteins with a subsequent cut-off filtration step (Johnsson *et al.*, 2002). Decreased NSB can also be achieved by diluting the sample and reducing the contact time. Additionally, there are sensor surfaces with modifications of the conventional carboxymethylated dextran layer available; *i.e.* shorter dextran chains or reduced degree of carboxylation of the dextran groups.

Surface regeneration

To ensure reproducible measurements, a complete regeneration of the sensor surface between sample injections must be obtained, without affecting the characteristics of the ligand (Andersson, Hämäläinen & Malmqvist, 1999). The regeneration solution should remove all compounds that bind non-covalently to the surface. Ideally, the binding capacity of the surface should not be affected by the regeneration and the baseline should remain at a constant level. The most commonly used regeneration solutions in Biacore applications are agents with high or low pH, *e.g.* NaOH, HCl and glycin (Andersson, Hämäläinen & Malmqvist, 1999). If changes in pH are not sufficient to regenerate the surface, other options may be more successful, *e.g.* high ionic strength, ionic detergents, *e.g.* SDS

(sodium dodecyl sulphate), chaotropic buffers, ethylene glycol, addition of acetonitrile to NaOH (Newman, Olabiran & Price, 1997; Baxter, 1999)

Biacore assays for analysis of food

Methods based on the Biacore technique have frequently been described in the literature during recent years, but only a minor part of the applications are related to food (Myszka, 1999; Rich & Myszka, 2000; Rich & Myszka, 2001; Rich & Myszka, 2002). Bovine milk has been a common matrix for different assays, *e.g.* for detection of non-milk proteins in milk (Haasnoot *et al.*, 2001), determination of progesterone levels to predict a cow's pregnancy (Gillis *et al.*, 2002), determination of folate-binding proteins that are important for supply of folates (Nygren, Sternesjö & Björck, 2003), and for determination of water soluble vitamins, *e.g.* biotin, vitamin B12 and folates, which also can be determined in other matrices like infant formula and cereals (Indyk *et al.*, 2000; Caselunghe & Lindeberg, 2000; Indyk *et al.*, 2002). Many applications developed have been related to the area of food safety; *e.g.* for detection *Salmonella* in chickens (Jongerius-Gortemaker *et al.*, 2002), detection of food pathogens in meat and poultry (Medina, Poole & Anderson, 1998), detection of allergens in different food matrices (Jonsson, Malmheden-Yman & Hellenäs, 2001) and detection of IGF-1 (insulin-like growth factor-1) that increases in milk after treatment with recombinant bovine somatropin to increase milk yield (Guidi *et al.*, 2001). Furthermore, assays for detection of different toxins, *e.g.* aflatoxin B1 (Daly *et al.*, 2000), mycotoxin fumosin B_1 (Mullett, Lai & Yeung, 1998) and staphylococcal enterotoxin B (SEB) (Rasooly, 2001) have been described. Nedelkov, Rasooly & Nelson (2000) also described an assay for detection of SEB together with toxic-shock syndrome toxin-1, where the biosensor was coupled to a mass spectrometer for identification of the toxins. The detection of pesticides (Minunni & Mascini, 1993; Alcocer *et al.*, 2000) and detection of genetically modified organisms (Feriotto *et al.*, 2002; Mariotti, Minunni & Mascini, 2002) have also been addressed. Assays for analysis of veterinary drug residues in different matrices are, however, the major application in food analysis.

Biacore assays for analysis of veterinary drug residues in food

Traditional immunoassays are frequently used for detection of veterinary drug residues in food (Homola, Yee & Gauglitz, 1999) and since the immunosensor assays are similar in their design, these may be a natural choice when developing biosensor assays for residue detection. The first Biacore assay for detection of veterinary drug residues in food, an inhibition assay designed for detection of sulfamethazine in milk, was described by Sternesjö *et al.* (1995). The analyte (sulfamethazine) was immobilised to the sensor surface, antibodies against sulfamethazine were added to the sample and the mixture was injected over the sensor surface. In a contaminated sample containing sulfamethazine the antibodies would be inhibited by free analyte, but if the sample was free from residues, the antibodies would instead bind to the analyte on the sensor surface, giving rise to an increase in response. The results were promising for utilisation of the Biacore

technique in drug residue analysis, *i.e.* high sensitivity, specificity and repeatability, no pre-treatment of the milk before analysis, only requiring small sample volumes and allowing automated analyses. During recent years, the Biacore technique has been used in developing assays for a wide range of veterinary drug residues in food control (Table 1).

All assays, presented in Table 1, besides two, are inhibition assays, based on antibodies for detection and, in most assays, the analyte is immobilised to the surface. The assays for detection of gentamicin (Haasnoot & Verheijen, 2001) and streptomycins (Haasnoot *et al.*, 2002) are direct assays. With a direct assay there is no addition of a detection molecule, *e.g.* antibody, instead this molecule is immobilised to the surface and the amount of analyte is directly measured. Inhibition assays have otherwise become the 'standard' assay format for detection of low molecular weight substances, *e.g.* drug residues, since the increase in mass at the surface upon binding of a small molecule is usually not large enough for concentration determination at low levels. These studies by Haasnoot and colleagues showed, however, that this is possible using a later generation instrument (Biacore 3000) with a more sensitive optical detection system than previous instruments.

The concept of a general capturing sensor surface to facilitate the development of new assays for different analytes was described by Bergström *et al.* (1999). This assay format utilises a conjugate between the analyte and a small synthetic molecule (H1), which will bind to a ligand (H1 antibody) on the sensor surface. By exchanging the analyte part of the conjugate, the assay can be used for typically any substance. Such a design offers several advantages, *e.g.* analyte independent immobilisation and regeneration procedures and, therefore, the development of new assays is simplified. However, one obstacle in this assay is the synthesis of the conjugate needed, which, depending on the analyte, may be problematic.

Penicillins and other β-lactam antibiotics are important substances within veterinary medicine. Therefore, the assay described for detection of penicillins in milk (Gaudin, Fontaine & Maris, 2001) is interesting. An antibody against the hydrolysed form of ampicillin was used for detection, resulting in an assay specific for the inactive form of the penicillins. This is a limitation of the assay, since only the active form is covered by legislation. However, by using antibodies specific for the active form and omitting the hydrolysis of the β-lactam structure, the assay can be improved in this respect.

Table 1. Biacore assays for detection of veterinary drug residues in various matrices

Type of drug	Substance	Matrix	Reference
Antimicrobial substances	Sulfamethazine	Milk, pig bile, pig muscle	(Sternesjö, Mellgren & Björck, 1995; Crooks et al., 1998; Baxter *et al.*, 1999; Bergström *et al.*, 1999; Gaudin & Pavy, 1999; Bjurling *et al.*, 2000).
	Sulfadiazine	Pig bile, pig muscle	(Crooks *et al.*, 1998; Elliott *et al.*, 1999; Bjurling *et al.*, 2000).
	Sulfonamides (8 substances)	Chicken serum	(Haasnoot, Bienenmann-Ploum & Kohen, in press).
	Streptomycin and dihydrostreptomycin	Milk, honey, pig kidney and muscle	(Baxter et al., 2001; Haasnoot *et al.*, 2002; Ferguson *et al.*, 2002).
	Gentamicin	Milk	(Haasnoot & Verheijen, 2001).
	Chloramphenicol	Milk	(Gaudin & Maris, 2001).
	Penicillin	Milk	(Gaudin, Fontaine & Maris, 2001).
	Enrofloxacin and ciprofloxacin	Milk	(Mellgren & Sternesjö, 1998).
β-agonists	Clenbuterol	Bovine urine	(Haughey *et al.*, 2001).
	Salbutamol	Bovine urine	(Elliott *et al.*, 1998).
Antiparasitic agents	Levamisole	Milk, bovine liver	(Crooks *et al.*, in press).
	Ivermectin	Milk, bovine liver	(Samsonova *et al.*, 2002a; Samsonova *et al.*, 2002b).
	Benzimidazole	Bovine serum	(Johnsson *et al.*, 2002).
Coccidiostatic agents	Nicarbazin	Poultry liver and eggs	(McCarney *et al.*, in press).

Implementation of biosensors in food control

The large number of assays described during recent years indicates the great potential of the Biacore technique within food analysis. The major advantage is the automated analysis offered by this technique. Immunoassays and similar techniques for detection of drug residues typically offer stability, high sensitivity and specificity and in order to replace these techniques the Biacore instruments and other biosensors need to offer the same, or better, qualities (Patel, 2002). Factors influencing the future use of biosensors by the food industry include cost per analysis, acceptance of the new technology by the food industry, availability of test kits, and adaptation of the instruments to the environment and requirements of food laboratories (Sternesjö, in press). Furthermore, the capacity of the instruments needs to be further improved in order to obtain satisfying sample throughput (Luong, Bouvrette & Male, 1997; Homola, Yee & Gauglitz, 1999; Sternesjö, in press).

In the Biacore instruments a multi-channel sensor chip with four different flow cells is used. These flow cells are serially aligned, but in some instruments the cells are separated, *i.e.* only one flow cell can be used at a time. In a finalised EU-project (Foodsense, FAIR-CT98-3630), a prototype of a high throughput SPR-based biosensor with eight parallel flow cells (one injection needle per flow cell) was developed to evaluate the applicability of SPR-based biosensors for routine monitoring of residues in food. The prototype offered simultaneous detection of either one analyte in up to eight different samples or up to eight different analytes in one sample. The prototype was used in a producer control programme at a pig abattoir to screen for sulphonamide residues in porcine bile and the analyses were also performed in parallel on a commercial Biacore 1000 instrument (Situ *et al.*, 2002). The prototype shortened the analytical time dramatically: from 540 minutes for analysis of a 96-well microtitre plate using Biacore 1000 to 50 minutes for the prototype. To make biosensors competitive in routine food control, an instrument with a capacity comparable to that of this prototype is needed.

Use of β-lactam antibiotics in dairy cows

The β-lactams, *i.e.* penicillins and cephalosporins (Figure 6), constitute the group of antibiotic substances most frequently used within veterinary medicine for treatment of bacterial infections in dairy cows. Mastitis, *i.e.* an inflammation of the udder, is the most common disease affecting dairy cows and in Sweden approximately 20% of the cows are treated for mastitis each year.

The antibacterial effect of β-lactam substances is due to their inhibition of bacterial cell wall synthesis. The cell wall in Gram-positive bacteria consists largely of peptidoglycans, a network of N-acetylmuramyl and N-acetylglucosaminyl residues that are cross-linked by short peptides. The β-lactam antibiotics inhibit the transpeptidases that perform the cross-linking of the peptides (Park & Strominger, 1957; Frère & Joris, 1985) and the resulting structural weakness of the cell wall is followed by activation of autolytic enzymes, causing

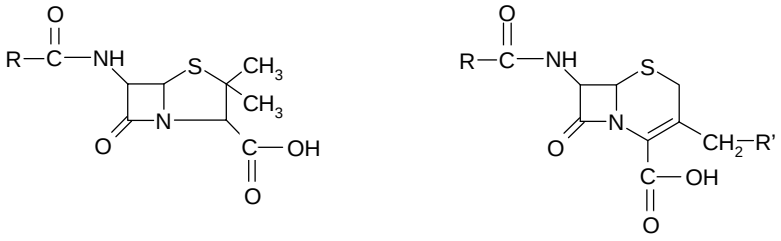
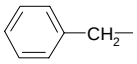
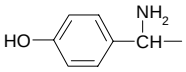
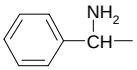
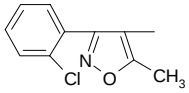
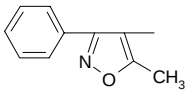
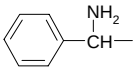
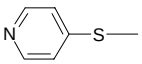
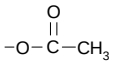
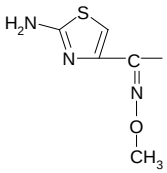
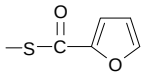


Figure 6. Chemical structures for penicillin and cephalosporin.

lysis of the bacteria (Miller, 2002). Since eucaryotic cells do not have a similar cell wall structure, the β-lactams are not toxic for humans and other eucaryotic species (Greko & Sternberg, 1996).

Some bacteria strains have developed resistance mechanisms against the most common penicillins, which consequently cannot be used in therapy against these bacteria. Chemical modifications of the natural penicillin structure have resulted in β-lactams with broader specificity and improved efficacy (Greko, 1996; Miller, 2002). Table 2 shows the chemical structures of common β-lactams and their respective maximum residue limits (MRL). Despite a wide range of β-lactam substances, benzylpenicillin (penicillin G) is still the most commonly used β-lactam in many countries. Sweden has successfully applied a restrictive policy with respect to the use of antibiotics in veterinary medicine. Antibiotic substances are only available by veterinarian prescription, and since 1986 the administration is limited to therapeutic use, *i.e.* antimicrobial drugs are not allowed for preventative purposes or growth promotion (Greko, 1996; SVARM, 2001). This has most certainly resulted in a low frequency of β-lactam resistance (SVARM, 2001); in Sweden only 5% of the bacteria causing mastitis are resistant to benzylpenicillin (Greko, 1996). Still today, benzylpenicillin is the only β-lactam approved for treatment of mastitis in Sweden (Odensvik, 2002), although recently ceftiofur has also been approved for treatment of dairy cows with the indication foot rot and respiratory disease. In the Scandinavian countries, the use of antibiotic substances has been carefully monitored during recent years (SVARM, 2001; NORM/NORM-VET, 2001; DANMAP, 2001) and annual reports summarise the total administration of antimicrobials and the occurrence of antimicrobial resistance in bacteria. In many other countries these types of figures are not easily accessed.

Table 2. Chemical structures of side chains (compare with Figure 6) for different common β-lactams and their respective EU maximum residue limits (MRLs) (EEC, 1990)

β-lactam	R	R'	MRL (µg/kg)
Penicillin G			4
Amoxilcillin			4
Ampicillin			4
Cloxacillin			30
Oxacillin			30
Cefalexin			100
Cephapirin			60
Ceftiofur			100

Monitoring of β-lactam residues in milk

Control programmes

To ensure that food of animal origin is free from antimicrobial residues, different control programmes are performed. Within all EU countries national control programmes are compulsory and performed by governments (EC, 1996). In addition, food producers and industries carry out self-monitoring programmes to fulfil requirements for export and consumer concerns regarding the safety of food (EEC, 1992). To establish EU MRLs for different substances, the toxicity of contaminants and drug residues is continuously evaluated. Council Directive 2377/90 (EEC, 1990) outlines the establishment of MRLs and acceptable daily intake (ADI), which is the daily amount of substance (mg/day) that can be

consumed without having any toxicological effects. ADI is in turn determined from the no-observed effect level (NOEL) evaluated in animals (Woodward, 1998).

Administration of antimicrobial drugs to lactating cows is always followed by a withdrawal period, during which the milk is not allowed to be delivered to the dairy. This period is necessary for residue levels to decline below MRL and the length varies depending on the drug used. Lack of awareness of withdrawal times or deliberate abuse may lead to elevated levels of drug residues in the milk. In Sweden, the most common causes of occurrence of drug residues in milk are insufficient identification of treated cows, insufficient knowledge about withdrawal periods, and failures due to hired staff (Carlsson, 1991).

Risks associated with drug residues in milk

The control of drug residues in milk is performed for three main reasons:
1) Ethical aspects: consumers should be guaranteed non-contaminated products free from antibiotic substances. The consumers' confidence in milk and dairy products as safe and wholesome food is of utmost importance to the dairy industry. In year 2002, Sweden introduced a new control system for separation of contaminated milk at the dairy. This control is also performed in many other countries, *e.g.* Norway, Germany, France and USA. With this system, all milk is tested for the presence of β-lactam antibiotics before the milk enters the dairy process, *i.e.* the tests are performed on tanker or at silo level. If the milk tests positive, *i.e.* it contains β-lactams, the milk is discarded. In this way the dairies can guarantee that their products are absolutely free from β-lactam contamination.

2) Health aspects: hypersensitive consumers may be subject to allergic reactions. The β-lactam antibiotics are not toxic to humans but there may be adverse effects due to the occurrence of drug residues in food. Allergy and hypersensitivity may lead to severe reactions in humans and up to about 10% of humans are diagnosed to be hypersensitive or allergic to β-lactams (Dayan, 1993; Miller, 2002). However, very few cases of allergic reactions caused by consumption of residue-contaminated food have been reported. According to a review of all published data by Dewdney and Edwards (1984), only 14 cases of allergic responses were related to consumption of contaminated food; all reactions were caused by penicillin residues in milk. This low occurrence can be explained by the fact that if the food is contaminated with antibiotics the drug concentrations are too low to cause any reactions (Dayan, 1993).

3) Technological aspects: many starter cultures used in the manufacture of fermented products, *e.g.* yoghurt and cheese, may be inhibited by β-lactams and other antimicrobial substances. If the fermentation is incomplete, the product quality will be impaired (Mäyrä-Mäkinen, 1995) and occasionally milk may even have to be discarded, in both cases causing the dairy substantial economic losses. The concentration of β-lactams needed for this type of inhibition is, however, rather high. Using penicillin G, total inhibition occurs at approximately 60 µg/kg, but depends on the starter culture (Schiffmann, Shutz & Wiesner, 1992; Grunwald & Petz, in press). However, product quality may be impaired already at

concentrations around 3-5 μg/kg (Mäyrä-Mäkinen, 1993; Grunwald & Petz, in press).

Assays for control of β-lactam residues in milk

In 1952, shortly after the introduction of antimicrobial drugs for treatment of infections within veterinary medicine, the first test for analysis of milk, a microbial inhibition test, was developed (Mitchell *et al.*, 1998). At that time, initiatives for development of milk tests were taken by dairy processors who noticed that starter cultures used for manufacture of fermented dairy products were sometimes inhibited. The assays used for milk screening purposes need to be inexpensive, easy to perform and should preferably detect a broad spectrum of antimicrobials (Suhren & Beukers, 1998). The microbial inhibitor tests have, over the years, proven to be very suitable for screening purposes. The main advantages of microbial inhibition tests are the low cost per test, the simple performance, and their broad detection pattern, *i.e.* not only different antibiotic groups are detected but also abnormalities in the milk composition (Mitchell *et al.*, 1998). The main limitation is the time-consuming incubation, several hours before the result is obtained (Bell *et al.*, 1995).

To meet the demands of the dairy industry, there has been an increase in the number of rapid tests on the market during the last decade. With these tests, the results are typically obtained within 10 minutes, they are easy to perform, sensitive and specific. One of the first rapid tests that was developed during the early 1980s was the enzyme-based Penzym test (UCB Bioproducts, Braine-l'Alleud, Belgium). Within a few years time also the Charm II test, (Charm Sciences Inc., Lawrence, Massachusetts, USA) was introduced for detection of seven families of antimicrobial drugs. The method is based on the use of entire bacterial cells with intact binding sites for antimicrobials in combination with isotope-labelled drug tracer. More recently, also simpler receptor-binding assays have been developed, *e.g.* SNAP test (IDEXX Laboratories, Inc., Westbrook, ME, USA), β-STAR test (UCB Bioproducts, Braine-l'Alleud, Belgium) and Charm Safe Level test (Charm Sciences Inc., Lawrence, Massachusetts, USA). There are also rapid immunoassays, *e.g.* the Parallux test (IDEXX Laboratories, Inc., Westbrook, ME, USA) using antibodies for detection. By exchanging a disposable cartridge device, also other antibiotic groups than β-lactams can be detected with the Parallux.

The use of penicillin-binding proteins in methods for residue detection

Many assays for analysis of antimicrobial drugs, including Biacore assays, are based on antibodies, usually resulting in sensitive and specific detection. The high specificity also means that the detection spectrum sometimes is limited, since the degree of cross-reactivity within a group may be poor. In the case of β-lactams there is an additional problem; the β-lactam ring structure is easily hydrolysed, whereby the substance becomes inactivated. Since most legislated MRLs are set for the parent compound and sometimes also for active metabolite(s), it is important that only these forms are detected. Many efforts have been made to produce group-specific antibodies against the active β-lactam structure but with limited success,

most often the antibodies will detect both active and inactive forms. However, development and production of generic antibodies against penicillins have been reported, nonetheless showing no cross-reaction with cephalosporins (deLeuw *et al.*, 1997; Usleber, Litz & Märtlbauer, 1998; Dietrich, Usleber & Märtlbauer, 1998; Cliquet *et al.*, 2001).

An alternative approach for group-specific detection of the active form of the β-lactam structure is the use of receptor proteins. β-Lactam specific receptor proteins, or penicillin-binding proteins (PBP), have been successfully applied in some commercially available rapid tests, *e.g.* Penzym, β-STAR, SNAP and Charm Safe Level test. These proteins are found in bacteria and are most often anchored in the membrane of the bacteria (Massova & Mobashery, 1998). Penicillin sensitive bacteria have a number of different PBPs (Ghuysen, 1977) that can be divided into two groups: the high molecular weight and low molecular weight PBPs (Waxman & Strominger, 1983; Granier *et al.*, 1994; Massova & Mobashery, 1998). These, in turn, are divided further into subgroups according to their amino acid sequence similarities (Ghuysen, 1991). The different PBPs have various functions including carboxypeptidase, transpeptidase and transglycosylase activities (Massova & Mobashery, 1998). The low molecular weight PBPs probably control the extent of cross-linking of the peptidoglycan in the cell wall by acting on D-alanyl-D-alanine-terminated peptides, but the high molecular weight PBPs lack this DD-peptidase activity (Granier *et al.*, 1994).

Soluble DD-carboxypeptidases from *Streptomyces* R61 and *Actinomadura* R39 (re-classified from *Streptomyces* R39) have been thoroughly studied by Frère and Ghuysen and co-workers (Frère *et al.*, 1973; Ghuysen *et al.*, 1973; Frère *et al.*, 1974b; Frère *et al.*, 1974a; Frère *et al.*, 1975a; Frère *et al.*, 1976; Frère & Joris, 1985). Upon growth, the bacteria excrete these extra-cellular enzymes (R61 and R39, respectively), which are believed to be soluble forms of membrane-bound transpeptidases participating in the bacteria cell wall synthesis (Leyh-Bouille *et al.*, 1970). The enzymes can act as both carboxypeptidases and transpeptidases: an aqueous environment results in carboxypeptidase activity, whereas a hydrophobic environment, *e.g.* in the membrane, leads to transpeptidase activity (Leyh-Bouille *et al.*, 1970). The natural substrates for these enzymes are peptides ending with D-alanyl-D-alanine. Penicillin is a structural analogue to the dipeptide D-Ala-D-Ala (Ghuysen, 1977; Ghuysen *et al.*, 1996) and therefore the enzymes will also interact with the β-lactam structure. The β-lactam binds covalently to the carboxypeptidase with the formation of a very stable complex, $t_{½}$≈68 h for R39-benzylpenicillin complex at 37° C (Ghuysen, 1977). As a result of the complex formation, the enzymatic activity is inhibited. The interaction is reversible with respect to the enzyme because, being released from the complex, it possesses the same affinity for β-lactams as before the interaction, but the β-lactam is degraded to phenylacetylglycine and *N*-formyl-D-penicillamine, Figure 7 (Frère *et al.*, 1974a; Frère *et al.*, 1975b; Ghuysen, 1977).

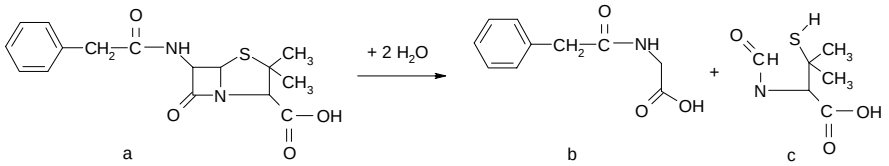


Figure 7. Enzymatic degradation of penicillin G (a) into phenylacetylglycine (b) and *N*-formyl-D-penicillamine (c) (Ghuysen, 1977).

The enzymatic activity of R39, *i.e.* hydrolysis of a 3-peptide into a 2-peptide has the following appearance (Frère, Klein & Ghuysen, 1980):

L-Lys-D-Ala-D-Ala + $H_2O \rightarrow$ L-Lys-D-Ala + D-Ala

and serves as the basis for the commercially available Penzym test (UCB Bioproducts, Braine-l'Alleud, Belgium). The liberated D-Ala will be oxidised, whereupon hydrogen peroxide is formed, which will oxide a chromoforic compound, resulting in a colour change. In presence of β-lactams, however, the reaction is inhibited and, hence, no colour change occurs.

Objectives

The overall aim of this study was to explore the possibilities to use a receptor protein as detection molecule in a surface plasmon resonance (SPR)-based biosensor assay for detection of β-lactam antibiotics in milk. The specific aims of the study were:

- To investigate if the concept of a general capturing sensor surface in combination with a β-lactam binding protein (R39) could be applied in a biosensor assay for detection of β-lactams in milk (paper I).

- To use the enzymatic activity of the β-lactam receptor protein (R39) for detection of β-lactams. The conversion of substrate to enzymatic product was measured by the use of antibodies (papers II and III).

- To compare any developed biosensor assays with commercially available screening tests for β-lactams and liquid chromatography (paper IV).

Materials and Methods

Principles of Biacore assays

All three assays are based on the same receptor protein, a DD-carboxypeptidase from *Actinomadura* R39 (R39). The first assay is based on the binding of the protein to a β-lactam conjugate (paper I) and the two following assays are based on the enzymatic activity of the protein (papers II and III).

In the conjugate assay a small synthetic molecule (H1) is immobilised, creating a general capturing surface (Bergström *et al.*, 1999). A conjugate between an H1-antibody (H1 Ab) and a β-lactam is injected over the surface, whereupon the H1 Ab part interacts with H1 on the surface. The receptor protein, R39, is added to the sample and the mixture is injected over the sensor surface. In a β-lactam free sample R39 will bind to the β-lactam part of the conjugate with a resulting increase in response. In a sample containing β-lactams, however, R39 will react with free β-lactams and its binding to the sensor surface will be inhibited. The response obtained is inversely proportional to the amount of ß-lactam antibiotics in the sample. Figure 8 shows the assay principle and a sensorgram for the conjugate assay.

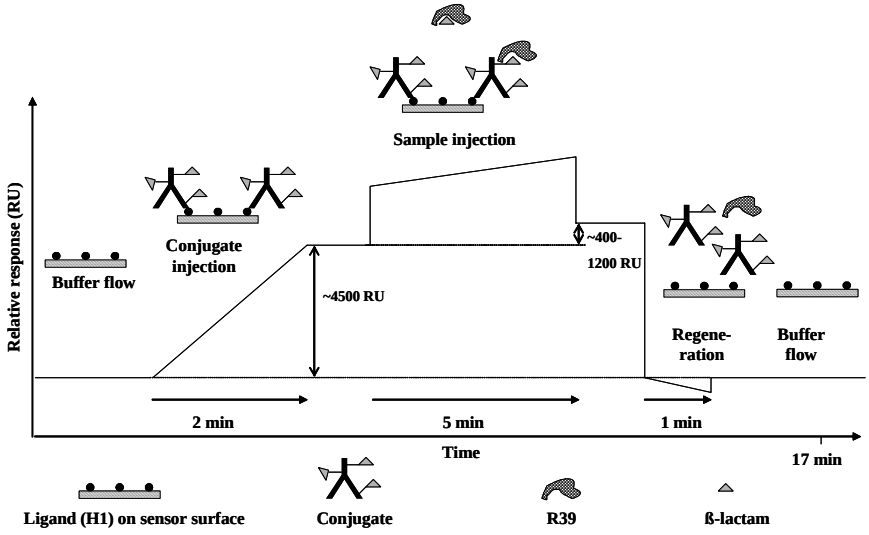


Figure 8. A schematic illustration of a sensorgram and the assay principle of the conjugate assay (paper I). A conjugate between antibody (H1 Ab) and β-lactam is injected over an H1 sensor surface to which the antibody part will bind. The sample, mixed with receptor protein (R39), is injected and R39 will either be inhibited by free β-lactam in the sample, or bind to the β-lactam part of the conjugate, whereby a response increase is obtained. The specific response range between samples with non-inhibited and totally inhibited R39 was approximately 250 RU. Due to non-specific binding to the surface, the response obtained could, however, vary between 400 and 1200 RU.

The assays described in papers II and III, the 2-peptide and the 3-peptide assays, are based on the same enzymatic reaction, *i.e.* hydrolysis of a 3-peptide into a 2-peptide catalysed by R39 according to:

$$\text{Ac-L-Lys-D-Ala-D-Ala} + H_2O \xrightarrow{\text{R39}} \text{Ac-L-Lys-D-Ala} + \text{D-Ala}$$

In both assays, a milk sample is mixed with 3-peptide and R39 and incubated for 5 minutes at 47°C to allow the enzymatic reaction to proceed. If the sample is ß-lactam-free, R39 will hydrolyse 3-peptide into 2-peptide. In the presence of ß-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 (2-peptide Ab) and the mixture is injected over a sensor surface with 2-peptide immobilised. With a ß-lactam-free sample (negative), the 2-peptide Ab will be inhibited by 2-peptide produced in the sample, whereas with a ß-lactam contaminated sample (positive), the 2-peptide Ab will bind to the 2-peptide surface. The response obtained is directly proportional to the amount of ß-lactam antibiotics in the sample.

In the 3-peptide assay, the sample is mixed with antibodies directed against the 3-peptide (3-peptide Ab) and the mixture is injected over a sensor surface with 3-peptide immobilised. With a positive sample, the 3-peptide Ab will be inhibited by non-hydrolysed 3-peptide, whereas with a negative sample, the 3-peptide Ab will bind to the 3-peptide surface. The response obtained is inversely proportional to the amount of ß-lactam antibiotics in the sample. The principles for the 2- and 3-peptide assays are shown in Figure 9.

Assays used for comparison with the developed assays

In the comparison study (paper IV), a number of screening tests for detection of β-lactams in milk were used.

Microbial inhibitor test

The Delvotest SP (DSM-group, Delft, the Netherlands) is an agar diffusion test that utilises *Bacillus stearothermophilus* var. *calidolactis* in an agar medium containing a pH-indicator. Upon growth of the test organism acid is produced, resulting in a pH-drop and a change in colour of the pH-indicator from purple to yellow. In a β-lactam-containing sample, the growth of the test organism is inhibited and the medium remains purple. This test is not specific for β-lactams, but also reacts upon other antimicrobial substances (sum effect) or abnormal milk composition.

Enzymatic assay

The Penzym S test (UCB Bioproducts, Braine-l'Alleud, Beligum) is based on the hydrolysis of a 3-peptide into a 2-peptide with liberation of D-Ala, *i.e.* the same enzymatic reaction that is used in two of the biosensor assays. The amount of liberated D-Ala is dependent on the amount of active enzyme, which in turn

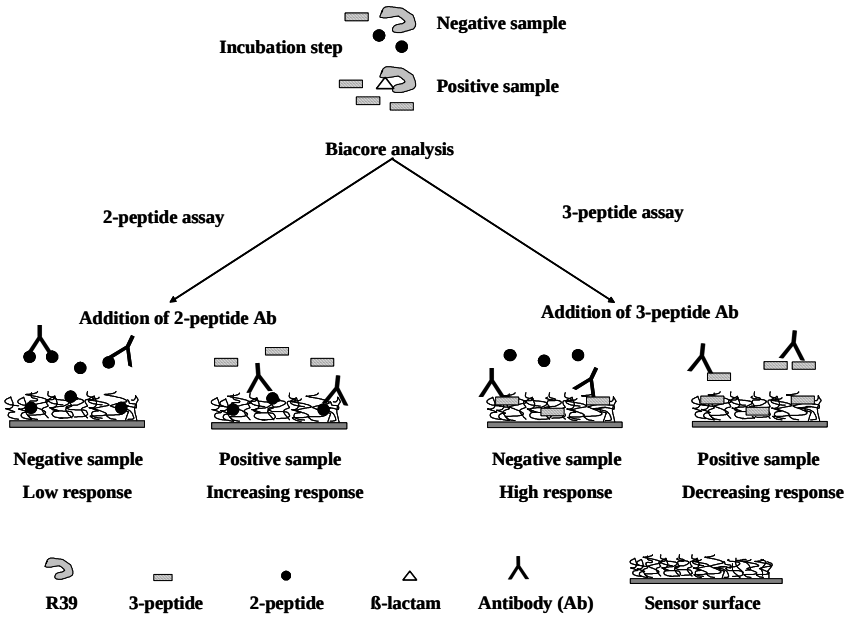


Figure 9. Illustrations of the principles of the 2- and 3-peptide assays (papers II and III), both of which are based on the enzymatic activity of R39. During the incubation step R39 catalyses hydrolysis of 3-peptide into 2-peptide. However, in the presence of β-lactam antibiotics the enzymatic activity of R39 is inhibited. Antibodies are added and the amount of 2-peptide formed, or the amount of remaining 3-peptide, is measured.

depends on the amount of β-lactams present in the sample. Liberated D-Ala will be oxidised to pyruvic acid and H_2O_2, whereupon a colourless redox indicator will change into a pink-orange colour compound. In a β-lactam-containing sample the 3-peptide will not be hydrolysed and, hence, there will be no colour change.

Receptor tests

β-STAR (UCB Bioproducts, Braine-l'Alleud, Beligum) uses a β-lactam-binding receptor protein. The receptor protein is mixed with the milk sample and during a short incubation step the receptor will react with any free β-lactams in the sample. The sample is transferred to a filter paper strip where it migrates towards a test field and any free receptor will be captured by a biomolecule at the test field. Since the receptor protein is linked to gold particles, the protein is directly visualised and can be seen as a pink band. For a sample where the receptor protein has already reacted with free β-lactam molecules, the protein will not be captured at the test field and no band can be observed. The colour intensity of the test band is visually compared with that of a reference band: if the colour intensity of the test band is weaker than that of the reference band, the sample is classified as positive.

The SNAP test (IDEXX Laboratories, Inc., Westbrook, ME, USA) utilises a β-lactam receptor protein conjugated to an enzyme. The conjugate is mixed with the milk sample and after a short incubation step the mixture is transferred onto a filter paper strip, where the sample is allowed to migrate until it passes a test spot. Any free receptor will be captured at this spot, whereas receptor protein that has reacted with β-lactams in the sample will not. A substrate is released, it reacts with enzyme attached to the captured receptor protein and a colour develops at the test spot. The colour intensity of the test spot is compared with that of a reference spot. If the colour of the test spot is weaker than that of the reference spot, the result is interpreted as positive.

Immunological assays

The Parallux (IDEXX Laboratories, Inc., Westbrook, ME, USA) can be used to detect different antimicrobial substances, but in the comparative study in paper IV, only the 'cillin assay' was used. This assay is specific for penicillins, *i.e.* cephalosporins will not be detected. Fluorescent-labelled antibodies are mixed with the sample and will either react with free penicillins in the sample or penicillin immobilised in a capillary tube into which the sample is drawn. The sample fluorescence resulting from antibodies captured in the tubes is compared with that of negative controls and the more fluorescence the less drug in the sample.

The Fluorophos Beta-Screen test (Advanced Instruments, Inc., Norwood, MA, USA) uses a conjugate between β-lactam and alkaline phosphatase. The milk sample is mixed with the conjugate and added to a test tube coated with β-lactam antibodies. Free β-lactams in the sample and the conjugate will compete for the binding sites of the antibodies. After incubation and wash steps, a substrate is added. Conjugated enzyme bound to the antibodies in the test tube will react with the substrate with a subsequent formation of a fluorescent product that is measured and compared with that of a β-lactam standard.

HPLC analysis

The liquid chromatography analysis of β-lactams in paper III and paper IV was performed according to a method described by Suhren & Walte (in press). The method detects seven different β-lactam antibiotics simultaneously, *i.e.* penicillin G, ampicillin, amoxicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin. The milk sample was applied on a solid phase extraction (SPE) column, eluted with acetonitrile (AcN)/water and methanol, and then derivatised with a 1,2,4-triazol and mercury chloride solution. The analysis was performed with a C-18 column and a mobile phase gradient (AcN, acetate buffer and methanol) with UV-VIS detection at 323 nm.

Results and Discussion

The use of a general capturing surface in combination with a β-lactam binding protein in biosensor analysis of β-lactam antibiotics (paper I)

Most previously described Biacore assays for detection of veterinary drug residues in food are inhibitor assays with the analyte immobilised to the surface. This is not, however, possible in the case of β-lactams since the β-lactam ring structure would be hydrolysed in the aqueous environment provided by the continuous buffer flow over the sensor surface. The receptor protein (R39), which only recognises the intact structure, would then no longer interact with the ligand. Furthermore, the interaction between the receptor protein and β-lactams is of covalent character, *i.e.* it would not be possible to regenerate the surface. Therefore, the concept of a general capturing sensor surface, described by Bergström *et al.* (1999), was used. In the present study, instead of using an H1 Ab surface, H1 was immobilised to the surface and the H1 Ab and a β-lactam were combined in a conjugate.

Calibration curves of penicillin G in buffer and milk were constructed. Based on these standard curves, the assay showed a detection limit (LOD) for penicillin G of 1-2 μg/kg, which is below the established EU MRL of 4 μg/kg for penicillin G in milk. Furthermore, the assay detected several other β-lactams, although with different sensitivity (Table 3).

One of the main difficulties associated with this assay was that the synthesis of the conjugate was not easily reproduced. In order to obtain a high sensitivity of the assay, the antibody needed to be highly modified, *i.e.* the number of β-lactam molecules coupled to the antibody should be high. This would then result in a high receptor binding capacity of the conjugate. However, if the degree of modification was too high, the antibody precipitated and, consequently, there was a fine balance between obtaining a high degree of modification and maintaining the antibody in solution.

Non-specific binding (NSB) to the surface was another problem related to this assay. The NSB seemed to be partly due to the nature of the conjugate since a relative response of approximately 200 RU was obtained with R39 in buffer samples also at concentrations where R39 was totally inhibited. It was found that the NSB varied between batches of conjugate. One explanation may be that remaining, non-reacted, active groups on the conjugate would interact non-

Table 3. Penicillin G concentrations corresponding to the response obtained in the analyses of buffer spiked with 15 μg/kg of the respective β-lactam

Substance	Amoxicillin	Ampicillin	Cloxacillin	Oxacillin	Cefalexin	Cephapirin
Conc. (μg/kg)[a]	12	15	5	4	4	20
MRL (μg/kg)	4	4	30	30	100	60

[a] The corresponding penicillin G concentration.

specifically with R39. The NSB also seemed to be affected by the milk composition, since it was clear that it varied greatly between different milk samples. Processed (pasteurised and homogenised) consumer milk resulted in a constant and lower level of NSB than raw milk. To study the effect of heat-treatment, analyses of heat-treated (72ºC for 5 minutes) and non-treated milk from different cows were made. In this experiment, no conjugate or R39 was used, the milk was simply injected over the H1 sensor surface. Heat treatment of the milk samples reduced the NSB by 70% and the standard deviation by more than 80%, *i.e.* 934±213 RU for non-treated and 276±38 RU for heat-treated (n=8). As the whey proteins are the most heat sensitive proteins in milk, they were most probably involved in the NSB.

In order to reduce the NSB, several experiments were performed with varying success. By using molecular weight (MW) cut-off filtration (10,000 Dalton) of the whey, it was possible to reduce the NSB to a constant level. Milk samples from different cows were spiked with 100 µg/kg penicillin G, a concentration where R39 is totally inhibited. Rennet was added to the milk samples in order to precipitate the caseins. The resulting whey was MW cut-off filtered to remove the whey proteins and the filtrates were then analysed by the conjugate assay. Figure 10 illustrates the variation in response between milk samples and the effect of the filtration step in reducing the NSB to a constant level.

In order to find a satisfactory regeneration of the surface, different solutions were tested. A solution of 40 mM NaOH containing 20 mM SDS was found to be the best. The surface was not, however, completely regenerated, since the baseline increased approximately 50-100 RU after 30 sample injections. This did not affect the capacity of the surface, *i.e.* the amount of conjugate captured by the surface

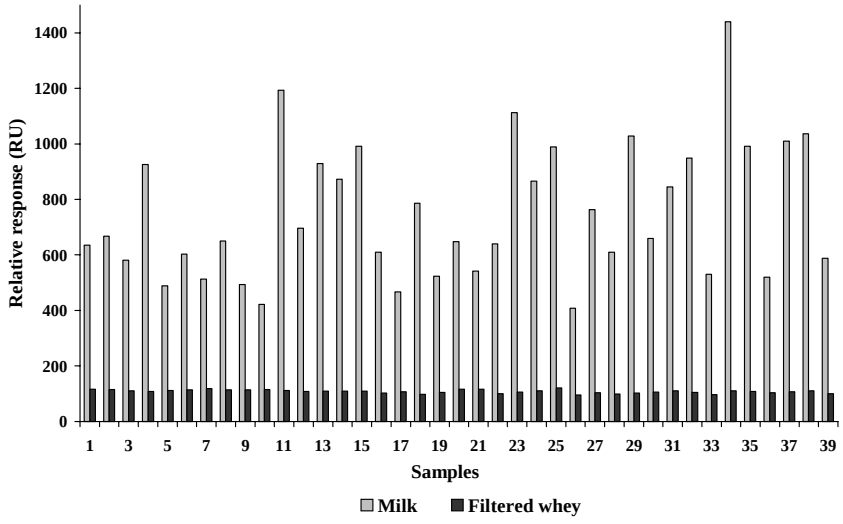


Figure 10. Responses obtained in the analysis of 39 milk samples spiked with 100 µg/kg penicillin G and their corresponding whey filtrates.

remained constant. The increase in baseline response was not observed when samples of filtered whey were analysed, *i.e.* if the NSB was reduced, the sensor surface was completely regenerated.

Unfortunately, due to problems in the production of additional conjugate, further work to optimise the assay could not be performed.

Biosensor assays for β-lactam antibiotics using the enzymatic activity of a receptor protein (papers II and III)

In order to overcome the problems associated with the conjugate assay (paper I), an alternative approach for a β-lactam biosensor assay was developed. The enzymatic activity of the receptor protein, R39, formed the basis for two new assays. Antibodies were used to measure the amount of enzymatic product formed (2-peptide assay) or the amount of remaining enzymatic substrate (3-peptide assay), respectively.

As previously mentioned, existing legislation, *i.e.* MRLs, for β-lactams is only applicable for the active form of the compound. To investigate the specificity of R39, milk samples spiked with penicillin G concentrations of 4 and 8 µg/kg were treated with β-lactamase (Gaudin, Fontaine & Maris, 2001) to hydrolyse the β-lactam structure. These samples, together with non-treated samples, were analysed using the 2-peptide assay as described in paper II. The results showed that there were no detectable residues in the β-lactamase treated samples, whereas the concentrations of the non-treated samples were determined to 4.0 µg/kg and 7.7 µg/kg, respectively. That is, β-lactams with a hydrolysed ring structure did not inhibit the enzymatic activity of R39.

To ensure a secure detection with the respective assay it was crucial that the 2-peptide Ab did not cross-react to a large extent with 3-peptide and vice versa for the 3-peptide Ab. The cross-reactivities of 2- and 3-peptide Ab for 3-peptide and 2-peptide, respectively, were determined to be <0.1% for both antibodies. Considering the high similarity between the two peptides, *i.e.* they differ in only one amino acid, this was better than anticipated.

2-peptide assay

The new approach, using the enzymatic activity of R39 in a biosensor assay, was first described for the 2-peptide assay (paper II). The paper described basic assay performance parameters, *e.g.* detection limit, accuracy and precision determined for penicillin G in milk. An interesting characteristic of this assay format was the positive correlation between the antibody response and β-lactam concentration: the more β-lactam in the sample, the higher the response. The response in inhibition assays is usually inversely proportional to the analyte concentration, but in the 2-peptide assay the amount of formed antigen (2-peptide) will decrease with increasing amount of analyte (β-lactam), resulting in a positive correlation. The validation parameters were promising, but the assay needed to be optimised to be applicable at the required residue level, *i.e.* at, or preferably lower than, the MRL.

The milk fraction in the injection mixture was high (70%) in the first assay version (paper II). It was later reduced to lower the NSB and thereby increase the assay sensitivity. Before injection, the sample fraction (milk, R39 and 3-peptide) was mixed with 2-peptide Ab in the ratio 10:90, which resulted in a reduction of the milk fraction in the injection mixture to 8%. This resulted in less influence by the sample matrix and a reduction in NSB. By increasing the flow rate from 10 µl/min to 30 µl/min a better precision was obtained. Furthermore, the concentration of R39 was reduced from 4.9 µg/ml to 4.6 µg/ml. The lower concentration of R39 resulted in a more sensitive assay, the LOD decreasing from 2.5 µg/kg to 1.2 µg/kg, when using the lower milk fraction and increased flow rate.

The regeneration solution was also changed during the optimisation; 40 mM NaOH with 20 mM SDS was replaced by 0.5 M NaOH with 10% AcN. Use of the alkaline solution containing SDS caused an increase in the baseline response, although the binding capacity of the surface did not seem to be affected. When using the alkaline solution blended with acetonitrile, the baseline was constant and, consequently, this solution was chosen. Table 4 presents the parameters that were modified in order to optimise the 2-peptide assay.

In the first version of the assay, a negative sample gave typically 800 RU and after optimisation approximately 500 RU. Although a low response level would be preferred, it is not a requirement. The main issue is to obtain a response range between a negative and positive sample that is great enough to make secure determinations. However, a lower response level means lower NSB, and is, hence, preferred. The elevated response level observed in this assay partly arose from NSB from the milk and partly from interactions between excess of antibody and surface, *i.e.* in a negative sample all 2-peptide Ab were not inhibited by free 2-peptide but some were still free to bind to the surface. The NSB from the milk was reduced by the lower milk fraction in the injection mix, but the response caused by excess antibodies was not easy to eliminate Calibration curves were constructed using different dilutions of 2-peptide Ab and to obtain a satisfactory response range of the calibration curve, approximately 500 RU, the higher Ab concentration was needed (Figure 11).

Table 4. Parameters that were modified during the optimisation of the 2-peptide assay

Parameter	Version 1 (paper II)	Version 2 (paper III)
Flow rate (µl/min)	10	30
R39 conc.[a] (µg/ml)	4.9	4.6
Mixing ratio sample[b]:Ab (%)	88:12	10:90
Ab conc. in injection mixture (µg/ml)	80	31
Milk fraction in injection mixture (%)	70	8
Regeneration solution	NaOH+ SDS [c]	NaOH+AcN [d]

[a] Before R39 is mixed with sample and 3-peptide.
[b] Sample = milk sample mixed with R39 and 3-peptide.
[c] 40 mM NaOH + 20 mM SDS.
[d] 0.5 M NaOH + 10% acetonitrile (AcN).

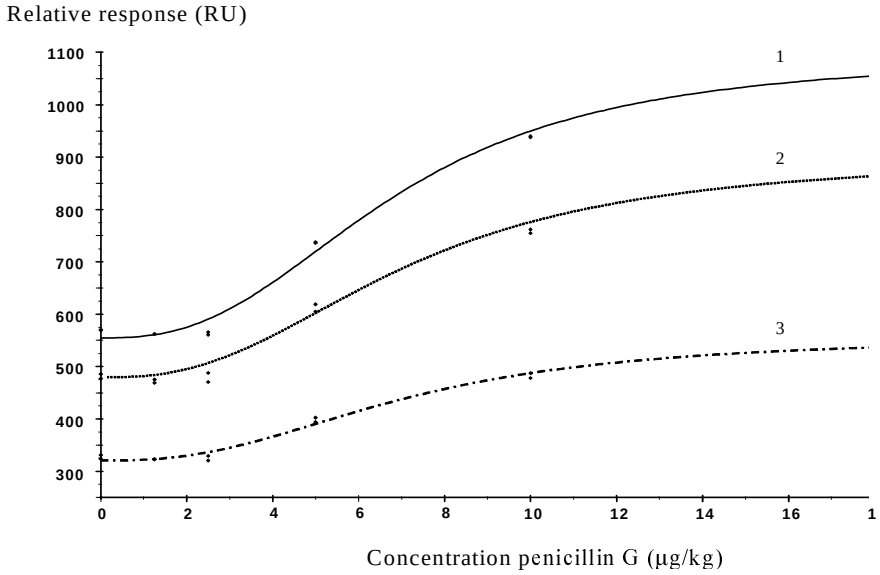


Figure 11. Penicillin G calibration curves constructed in milk using the optimised 2-peptide assay and different concentrations of the 2-peptide Ab in the injection mixture: 1 = 27 µg/ml; 2 = 18 µg/ml; 3 = 14 µg/ml.

By making the described modifications, the assay was improved both with respect to detection limit and precision. The detection limit of the assay for penicillin G in milk decreased from 5.2 µg/kg to 1.2 µg/kg, and the precision was improved from 10-15% to 3.1% within assay at the penicillin G MRL level. The precision between days at MRL was good already in the first version, but was further improved, *i.e.* from 4.7% to 2.2%.

3-peptide assay

Although the 2- and 3-peptide assays are based on the same enzymatic reaction, they differed with respect to performance characteristics. Initially during development of the 3-peptide assay, the response increased with time between replicates, indicating that the enzymatic reaction continued at room temperature after the incubation step at 47ºC. The amount of 3-peptide in the sample would continuously decrease, being hydrolysed into 2-peptide, and resulting in more 3-peptide Ab binding to the surface (Figure 12). This behaviour had not been observed with the 2-peptide assay. Several approaches were investigated to inhibit the enzymatic reaction, *e.g.* heat treatment to denature R39, cooling of the samples to stop the reaction, addition of β-lactams after the incubation step to inhibit R39, and addition of EDTA in order to complex Mg^{2+}, a co-factor of R39. However, all efforts were limited in success. The increase in response between replicates varied with β-lactam concentration: the largest increase was seen at concentrations located within the range of the inclination of the curve. The response was, however, stable

at concentrations where the antibodies were totally inhibited by free 3-peptide in the sample.

The shape of the 3-peptide assay calibration curve was also interesting, with a very sharp decrease in response within a low penicillin G concentration range. This was a positive feature offering a very distinct threshold level for classification of positive and negative samples. However, in order to be of practical use the drop in response needed to be at the appropriate β-lactam concentration, *i.e.* just below the MRL. This was achieved by optimising the reagent concentrations and in the final version the antibodies were totally inhibited at the MRL, resulting in secure determination of positive samples. Figure 12 shows calibration curves constructed in milk using different R39 concentrations, illustrating the sharp decrease in response and the increasing response between replicates.

Comparison of the 2- and 3-peptide assays

The 2- and 3-peptide calibration curves were, as previously discussed, different in their appearance. The difference between the penicillin G concentrations, resulting in non-inhibited and totally inhibited antibody binding to the surface, was narrow in the 3-peptide assay and this assay was more sensitive to variations in reagent dilutions and calibrant preparations. In this sense, the 2-peptide assay may, hence, be considered more robust. However, using the reagent combinations described in

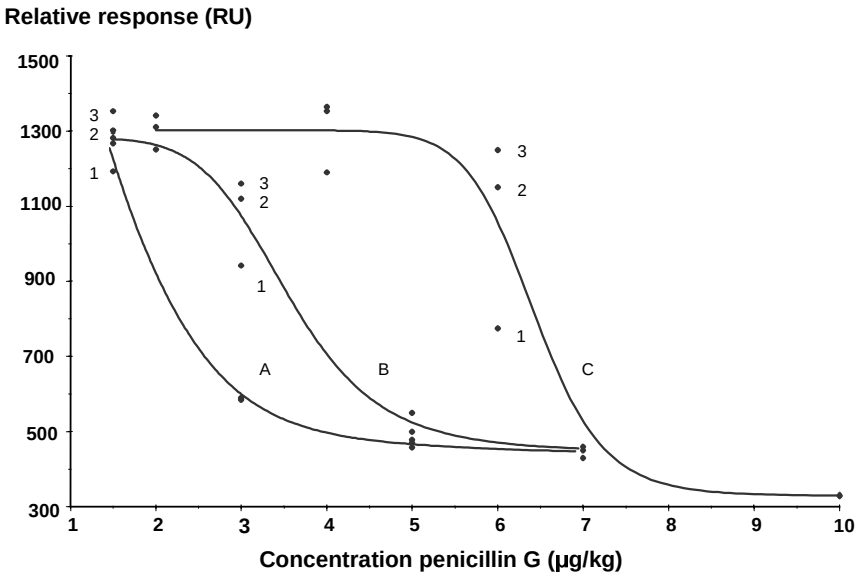


Figure 12. Penicillin G calibration curves in milk using the 3-peptide assay and different concentrations of R39: A: R39=4.9 µg/ml; B: R39=5.0 µg/ml; C: R39=11 µg/ml. The numbers 1-3 are sample replicates at concentrations where the response increases with time.

Table 5. Detection limit and precision (CV) of the 2- and 3-peptide assays at 4 µg/kg penicillin G in milk

	LOD (µg/kg)	Precision within assay (n=10)		Precision between days (n=3)	
		Mean (µg/kg)	CV (%)	Mean (µg/kg)	CV (%)
2-peptide assay	1.2	4.3	3.1	4.1	2.2
3-peptide assay	1.5	3.7	4.8	3.7	1.8

paper III, very reproducible results were obtained and the two biosensor assays were comparable with respect to the LOD and precision (Table 5).

The 2- and 3-peptide assays were tested for their ability to detect different β-lactams at their respective MRL level. Milk samples were spiked with seven different β-lactams at concentrations corresponding to 0.5×MRL, 1.0×MRL and 1.5×MRL of the respective substances and the obtained responses were compared with a penicillin G calibration curve. Both assays detected all investigated β-lactams at their respective MRL, except for cloxacillin, which was not detected by either of the two assays; ceftiofur was detected at MRL by the 2-peptide assay in one of two analyses. These results were compared with the claimed detection limits for the Penzym test (Table 6). Since the Penzym test and the biosensor assays utilise the same enzyme reaction, the similarities in the abilities of the tests to detect different β-lactams were rather expected.

Table 6. The abilities of biosensor assays to detect seven different β-lactams at their respective MRL when comparing the responses against a penicillin G calibration curve and using the LOD of penicillin G (1.5 µg/kg) as threshold limit for positive samples. The LODs of the Penzym test for the β-lactams as claimed by the manufacturer are also presented

β-lactam	MRL (µg/kg)	2-peptide assay	3-peptide assay	LOD Penzym test (µg/kg)
Amoxicillin	4	+[a]	+	4-6
Ampicillin	4	+	+	4-7
Cloxacillin	30	-[b]	-	60-100
Oxacillin	30	+	+	30-50
Cefalexin	100	+	+	20-40
Cephapirin	60	+	+	5-7
Ceftiofur	100	+/-[c]	+	40-70

[a] + means that the sample was positive in duplicate analyses.
[b] - means that the sample was negative in duplicate analyses.
[c] +/- means that the sample was positive in one, negative in one of duplicate analyses.

Comparison of two biosensor assays with commercially available screening tests and liquid chromatography (paper IV)

The two biosensor assays based on the enzymatic activity of R39 (paper III) were used to analyse a total of 195 positive and negative producer milk samples. The samples were also analysed with commercially available screening tests and some of the samples (n=30) were subject to HPLC analysis.

The results obtained by the different screening tests showed good agreement; only 13 samples differed in results. Out of these, eight samples were positive in the first analysis, but negative upon re-analysis with the same method. The differences in results of the remaining five samples may be explained by the different detection limits and principles behind the tests.

The quantitative results obtained by the biosensor assays were also in agreement with those obtained by HPLC (Figure 13). Seven samples with negative results in the biosensor assays were also negative upon HPLC analysis. In both biosensor assays, the concentrations in the upper range of the calibration curve were somewhat underestimated in comparison with the HPLC results. The number of positive samples that could be used in the quantitative comparison was rather low (n=13). Since the biosensor assays were used as screening tools, 10 additional samples, which were determined to contain >7 µg/kg, were not diluted or re-analysed for a more precise quantification of the penicillin G content. It was therefore not possible to reach any conclusion regarding the correlation coefficients between the different analytical methods. To get an indication of the agreement between the methods, the mean difference between the results obtained

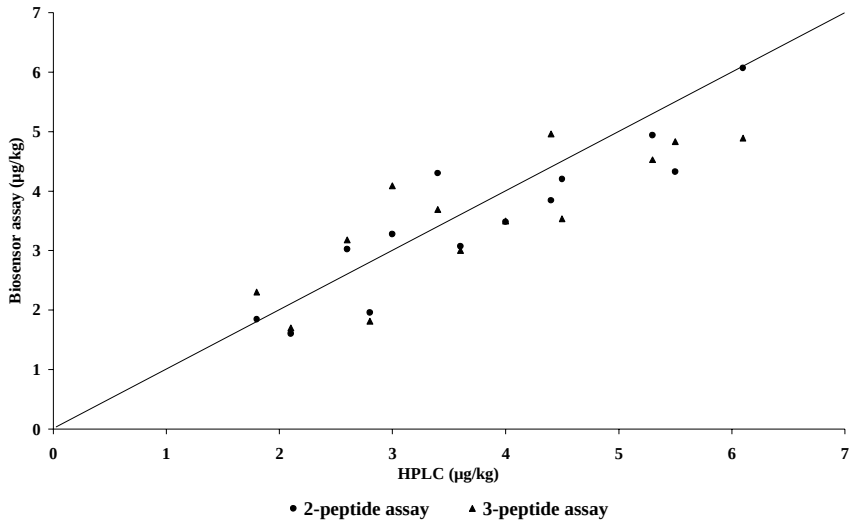


Figure 13. Comparison of penicillin G concentrations obtained by the HPLC and the two biosensor assays.

by the respective biosensor assay and the HPLC was used according to the principle described by Bland & Altman (1986). The concentrations obtained by HPLC were subtracted from the concentrations obtained by the respective biosensor assay. The mean difference was -0.2 μg/kg for both 2- and 3-peptide assays, with standard deviations of 0.5 μg/kg and 0.7 μg/kg, respectively. These figures indicated better method agreements than previously described (paper III), where a mean underestimation of 0.7 μg/kg was observed in both biosensor assays. The results obtained in the present study were probably more reliable since the samples had been frozen and all the analyses were completed within a period of a few weeks. The comparison made in paper III was based on analyses of freeze-dried milk samples with approximately two years between the HPLC and biosensor analyses (February 2001 and December 2002, respectively).

The biosensor assays used a threshold level to classify results as positives or negatives. It was based on the MRL of the analyte and adjusted for the precision and LOD of the respective assay. Ideally, the threshold level should be low enough to detect all contaminated samples, but high enough to avoid too many false-positive results, *i.e.* residue levels below MRL. Accuracy must also be considered before determining the threshold level. The underestimation of 0.2 μg/kg observed in this study will, therefore, not be a problem as long as it is compensated for.

Conclusions and future research

The study showed that the use of a receptor protein in the development of SPR biosensor assays for β-lactam antibiotics in milk was very successful. The β-lactam receptor was specific for the active β-lactam structure and the developed assays detected several β-lactam antibiotics at the respective MRLs, resulting in three generic assays.

The concept of a general capturing sensor surface was applicable, but problems to obtain a procedure for reproducible synthesis of the conjugate could not be overcome. The high variation in NSB between different milk samples made it impossible to use this assay to screen field samples. The assay might probably have been improved by using a lower fraction of milk in the injected sample mixture. Due to problems associated with the conjugate synthesis, further optimisation of this assay was not considered worthwhile, but instead alternative approaches were investigated.

The enzymatic activity of the protein was also shown to be a suitable basis for biosensor detection of β-lactams in milk. High antigen specificity of the respective peptide antibody was a requirement for this concept, resulting in two screening assays with high potential for future use. Both peptide assays showed calibration curves with interesting shapes: the 2-peptide assay with a positive correlation between response and β-lactam concentration, and the 3-peptide assay with a very sharp decline in response within a narrow β-lactam concentration range. The latter may offer a distinct threshold limit for classification of positive or negative samples. Using penicillin G in milk, both assays showed low detection limits and high precision. The linear parts of the calibration curves were quite narrow, making the present assays more suitable for qualitative than quantitative analysis of β-lactams in milk.

Upon screening of producer milk samples the biosensor assays provided results that were comparable to those of commercially available screening tests for β-lactams in milk. Also the quantitative results obtained by the biosensor assays were in good agreement with those obtained by HPLC. The biosensor assays seemed to underestimate the concentrations somewhat compared with HPLC, but by adjusting the threshold level used for screening, this should not be considered a problem.

In this investigation, only one β-lactam receptor protein was used. Other PBPs may have other properties and offer different assay formats. R39 used in this study binds β-lactams covalently, other PBPs may interact differently with β-lactams. The interaction may therefore be used in a different way, *e.g.* in a direct assay with the PBP immobilised to the surface. It may, however, be difficult to reach the high sensitivity obtained in this study with a direct assay.

This study has focused on β-lactams, the most commonly used group of antibiotics. There are, however, many other substances and groups that are important within drug residue detection. To make this biosensor technique an interesting alternative to existing screening techniques, generic assays for a wide range of veterinary drug residues are required. Since this study, based on the use of

a receptor protein, showed very good results it would be interesting to work on receptor assays for detection of other antimicrobial drugs.

In control programmes for residue detection in milk, HPLC has been the traditional technique for confirmation analysis of a sample found positive in the screening. Over the years the requirement for chemical identification of the analyte has increased, as has the number of methods using HPLC in combination with mass spectrometry (MS). Recently, assays where the SPR biosensor is coupled in series with MS have been described. This concept may offer a possible future alternative in confirmation and identification analyses, combining a rapid screening assay and a highly specific identification of the analyte. For such applications of Biacore instrumentation, a direct assay is required.

The future application of the Biacore technique in food analyses will most likely be for screening purposes. In order to replace existing screening techniques, it is necessary to have a biosensor method for β-lactams in milk, being the most common type of drug residues. This application alone will not, however, be enough for the investment in new technology. For other groups of veterinary drugs, such as aminoglycosides and antiparasitic agents, there are no suitable screening methods today. By developing assays also for such substances, the technique will be more interesting, offering screening of residues that are both highly prioritised, *e.g.* β-lactams, and substances that are not easily detected today, *e.g.* aminoglycosides.

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