# Immunosensor Methods for Drug Residue Control of Food

## Assay design and sample matrix effects

M. Annette Johansson
*Department of Food Science*
*Uppsala*

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# Abstract

The use of drugs to improve animal growth is banned in the European Union. Nevertheless, illegal use has been demonstrated in several countries and Member States have to run extensive control programs to ensure that the ban is respected. The aim of this work was to explore the possibility of using surface plasmon resonance (SPR) biosensors to develop fast and robust screening assays for hormones and ß-agonists.

Modified standard procedures for hapten synthesis were employed to immobilise stable analyte specific sensor chip surfaces used for analysis. Sensor chips for clenbuterol, ethinylestradiol and trenbolone gave assays with $IC_{50}$ values in the region of 0.5 ng $ml^{-1}$. Sample matrix interference from urine and serum with the antibody-antigen interaction and non-specific binding from samples to the sensor surface was systematically studied. Strategies to minimise these effects were investigated and described.

Biosensor assays for clenbuterol in bovine hair and urine, suitable for routine use, were developed and validated. For hair, a sandwich assay format was used. A rapid and simple extraction using 100 mM NaOH was developed, and the assay was validated by analysis of hair samples from animals treated with clenbuterol. The critical factor for assay sensitivity was non-specific binding to the sensor chip surface from hair extracts, which was controlled by ultrafiltration of samples and by the use of a secondary antibody. The LOD of the hair assay was 10 ng $ml^{-1}$.

In the urine assay, the cross-reaction for several ß-agonists, including salbutamol, mabuterol and brombuterol was high, enabling detection of concentrations below 1 ng $ml^{-1}$. Analysis of these low analyte concentrations in urine without sample pre-treatment was limited by high sample-to-sample variation, inhibition of antibody binding to the surface and occasional non-specific binding. A rapid and simple sample clean-up using integrated immunofiltration was developed to minimise these sample matrix effects. With this method, at least 48 samples could be processed and analysed by one person in one working day.

*Keywords:* ß-agonists, hormones, clenbuterol, salbutamol, residue analysis, urine, hair, sample matrix effects, SPR-biosensor, immunobiosensor, immunofiltration

*Author's address:* Annette Johansson, Chemistry Division 1, National Food Administration, P.O. Box 622, S-751 26, Uppsala, Sweden. E-mail address: ajoh@slv.se

# Svensk populärvetenskaplig sammanfattning

Inom EU är det förbjudet att använda hormoner eller andra ämnen med hormonell verkan, såsom beta-agonister, för att stimulera tillväxt hos djur i livsmedelsproduktion. Livsmedelsverket kontrollerar varje år både svenskt och importerat kött efter dessa ämnen. De senaste åren har kontrollen inom den svenska köttproduktionen omfattat drygt 1800 prover/år från slakterier och från levande djur på gård. Kontrollen av djur under uppväxtkedjan är ett komplement till kontrollen på slakterierna i syfte att kunna upptäcka illegal användning på ett så tidigt stadium som möjligt. På levande djur tas oftast urinprov, men det är också möjligt att testa blodprov eller hår. I urin går det i regel att hitta positiva prover under tiden djuret behandlas och under den påföljande veckan efter behandling. I hår lagras flera ämnen in, och det är möjligt att hitta rester av till exempel beta-agonister upp till flera månader efter behandling.

Syftet med mitt doktorandarbete var att undersöka möjligheterna att använda en optisk biosensor för att utveckla snabba och säkra metoder för kontroll av illegal användning av hormoner och beta-agonister speciellt i urin och hår. I biosensorn använder man sig av antikroppar och ett chip med en guldyta, för att kunna mäta de ämnen man söker efter. Först modifieras guldchipets yta kemiskt, så att den blir specifik för ett visst ämne. När väl ytan är modifierad kan man sedan använda samma guldchip till flera hundra analyser.

Under arbetets gång utvecklades specifika chipytor för tre olika hormoner: trenbolon, etinylöstradiol och hexestrol och två olika beta-agonister: klenbuterol och ractopamin. Chipen för klenbuterol, trenbolon och etinylöstradiol fungerade väl och kunde användas till att mäta låga halter, medan chipytorna för hexestrol och ractopamin behöver optimeras ytterligare. Ett av målen var att kunna analysera proverna direkt utan upprening, men det visade sig vara svårt eftersom olika ämnen från provet såsom proteiner och fett störde mätningen. Störningarna undersöktes, och en rad olika metoder att minska dem testades.

Två snabba biosensormetoder för att analysera klenbuterol i hår respektive urin har utvecklats. Hårproverna extraherades först med natriumhydroxid för att lösa ut ämnet. För att bli av med störande ämnen som band in ospecifikt till chipytan krävdes det att hårextrakten filtrerades det genom ett ultrafilter innan analys. Med denna metod kan koncentrationer av klenbuterol i hår ner till 10 mikrogram per kg upptäckas. Antikroppen som användes till urinmetoden kunde förutom klenbuterol också fånga upp flera ämnen, bland annat salbutamol, mabuterol och brombuterol. Halter på 1 mikrogram per liter urin eller lägre av dessa beta-agonister kunde hittas. För att kunna finna så låga halter utvecklades en ny uppreningsmetod, där samma antikroppar används för upprening och analys i biosensorn. Med denna metod kan en person testa minst 48 prover under en arbetsdag. Biosensormetoden för urin används för närvarande i Livsmedelsverkets offentliga kontroll för analys av beta-agonister.

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# Appendix

The present thesis is a synthesis of the following papers, referred to by their Roman numerals.

## Paper I-IV

I. Johansson M. A. & Hellenäs K-E. (2001) Sensor chip preparation and assay construction for immunobiosensor determination of beta-agonists and hormones, *Analyst*, 126, 1721-1727.

II. Johansson M. A. & Hellenäs K-E. (2004) Matrix effects in immunobiosensor determination of clenbuterol in urine and serum, *Analyst*, 129, 438-442.

III. Johansson M. A. & Hellenäs K-E. Immunobiosensor analysis of clenbuterol in bovine hair, *Food and Agricultural Immunology, Accepted*

IV. Johansson M. A. and Hellenäs K-E. Immunobiosensor determination of ß-agonists in urine using integrated immunofiltration clean-up, *International Journal of Food Science and Technology*, *Submitted.*

# List of abbreviations

Ab – Antibody
ACN – Acetonitrile
$b_0$ – Maximum response (the biosensor signal in RU for an antibody buffer solution, in absence of analyte, under assay conditions)
BIA – Biospecific interaction analysis
BRB – Brombuterol
CBL – Clenbuterol
CCα – Decision limit
CCß – Detection capability
CV – Coefficient of variation
EDC – 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA – Ethylene diamine tetraacetic acid
EEO – Ethinylestradiol
GC-MS – Gas chromatography mass spectrometry
HEX – Hexestrol
$IC_{50}$ – Inhibitory concentration 50% (concentration at the midpoint of the calibration curve)
IIF – Integrated immunofiltration
IFC – Integrated microfluidic cartridge
LOD – Limit of detection
MAB – Mabuterol
M-CBL – Monoclonal clenbuterol antibody
MW – Molecular weight
NHS – N-hydroxysuccinimide
NSB – Non specific binding
P-SBL – Polyclonal salbutamol antibody
RCP – Ractopamine
$R_{max}$ – Active antibody concentration
RU – Resonance units
SPE – Solid phase extraction
SBL – Salbutamol
SPR – Surface plasmon resonance
TB – Trenbolone
UF – Ultrafiltration

# Introduction

## Growth promoters in meat production

Anabolic steroids have been used in meat production since the 1950s. Initially the stilbenes, diethylstilbestrol (DES) and hexestrol were widely used but the seventies saw an increase in the use of 19-nortestosterone derivatives: trenbolone, zeranol and the three natural steroids: estradiol-17β, testosterone and progesterone (chemical structures in Figure 1). A hormone scandal in 1980-1981 associated with illegal use of DES in veal calves started a chain of events, which lead to a European ban in 1988. The EU prohibited the use of all substances having a hormonal action for growth promotion in farm animals (Heitzman, 1993). Later ß-agonists were added to the list. This prohibition applies to Member States and imports from third countries alike. The legal instrument in force is Directive 96/22/EC (EC, 1996a), as amended by Directive 2003/74/EC (EC, 2003).

Trenbolone, zeranol, melengestrol acetate and the three natural hormones are legally used in several countries outside the EU, including USA and Canada, and the European ban led to a trade dispute. The Codex Alimentarus Commision Joint FAO/WHO Expert Committee on Food Additives (JECFA) was involved to evaluate the safety risk related to the use of growth promoters. Risk evaluations have been summarised by Orr (1999) and the physiology of anabolic hormones used for meat production was reviewed by Meyer (2001).

Illegal use has been demonstrated in several EU countries and Member States have to run extensive control programs to ensure that the ban is respected. The aim of this work was to explore the possibility to use surface plasmon resonance (SPR) biosensors, to develop fast and robust screening assays for growth promoting hormones and ß-agonists.

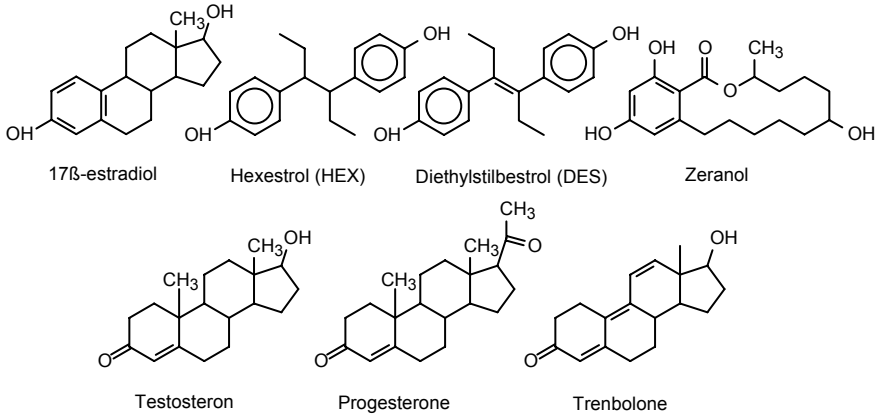


*Figure 1*. The chemical structures of some anabolic steroids. 17ß-estradiol is the major natural estrogen, and hexestrol, diethylstilbestrol and zeranol are synthetic derivatives. Testosterone and progesterone are natural androgens, while trenbolone is derived from 19-nortestosterone.

## The ß-agonists

ß2-agonists are a class of compounds derived from naturally occurring catecholamines (adrenaline, noradrenaline and dopamine). In human medicine they are extensively used for chronic obstructive airway diseases, such as asthma and bronchitis (Witkamp, 1996). For veterinary therapeutics, the use of ß-agonists has only been approved in the case of clenbuterol for bronchodilatation in horses and calves, and for tocolysis in cows (Kuiper *et al*., 1998). For growth promoting purposes, ß-agonists are banned in the European Union under Council Directive 96/22/EC (EC, 1996a). With respect to the therapeutic use, a maximum residue limit (MRL) of 0.5 µg $kg^{-1}$ was established for clenbuterol in bovine and equidae liver (EC, 2000).

ß-agonists act through their binding to ß-receptors located on various cell types, including neuronal and muscle cells, fat cells and blood cells. To date, 3 types of ß-receptors have been classified, ß1, ß2 and ß3 (*e.g.* Witkamp, 1996). The effects of ß2-receptor stimulation include increased heart rate, dilation of coronary vessels, relaxation of bronchial and uterine muscle tone and stimulation of insulin release and glycogenolysis. ß-agonists have proven to be efficient repartitioning agents, promoting reduction in body fat and muscle growth in cattle (Ricks *et al*., 1984), poultry (Dalrymple *et al*., 1984), swine (Jones *et al*., 1985) and sheep (Baker *et al*., 1984; Beermann *et al*., 1987). To achieve these effects, it is necessary to use doses 5-10 times higher than common therapeutic doses (Miller *et al*., 1988). During the last 15 years, illegal use of ß-agonists for growth promoting purposes, have been reported in Europe and USA (Kuiper *et al*., 1998; Mitchell & Dunnavan, 1998). Pharmacological aspects of clenbuterol in growth promotion and methods of analysis were further reviewed by Prezelj, Obreza & Pecar (2003).

The unwanted side effects of ß-agonists are predictable extensions of their pharmacological action: tachycardia, muscle tremor, palpitation, peripheral vasodilatation, nervousness and metabolic effects (Witkamp, 1996). In a series of acute food poisoning outbreaks in the nineties, a large number of patients were diagnosed according to these symptoms after consumption of bovine liver and meat containing high levels of clenbuterol. In Spain, 367 cases in two major outbreaks were reported (Martinez-Navarro, 1990 and Salleras *et al*., 1995). In France, 22 patients developed clinical symptoms associated with consumption of contaminated veal liver (Pulce *et al*., 1991), and in Italy consumption of fillet and rump steaks containing clenbuterol concentrations above 0.5 ppm lead to food poisoning of 16 persons (Maistro *et al*., 1995).

### *Chemical properties and metabolism in food-producing species*

For a ß-agonist to have biological activity it must have a substituted six-membered aromatic ring, hydroxyl group bonded to the ß-carbon in the R configuration, positively charged nitrogen in the ethylamine side chain and bulky substituent (R in Figure 2) on the aliphatic nitrogen. The chemical substitutents on the aromatic ring greatly influence the longevity of the ß-agonist within the mammalian tissue and the compound's efficacy at the receptor (Smith, 1998). Ring hydroxylated ß-agonists, such as salbutamol and ractopamine are rapidly deactivated by

enzymes in the liver and intestine, while halogen substituted ß-agonists withstand these enzymes. The halogens do not however inhibit binding to the receptor. Mabuterol and clenbuterol were designed specifically to resist rapid metabolic degradation by enzymes active toward aromatic hydroxyl groups (Morgan, 1990).

The metabolic pathways of biotransformation for ring hydroxylated ß-agonists such as salbutamol and ractopamine, works exclusively through conjugation with sulphate or glucuronic acid in all species investigated. Conjugation of the aromatic hydroxyl group is the only confirmed site for these types of ß-agonists (Smith, 1998). The major metabolite excreted in bovine urine after clenbuterol treatment was an oxidation product of the primary amine function of CBL, CBL-arylhydroxylamine. It accounted for approximately 40% of the excreted compound, while 40% of the excreted compound was reported to be in the parental form (Zalco, Bories & Tulliez, 1996; Zalco *et al*., 1998).

Clenbuterol accumulates to various degrees in different tissues like blood, urine, muscle, liver and eye (Sauer *et al*., 1995). In blood serum, clenbuterol concentrations peaked at 1.1 ng $ml^{-1}$ within 5 hours after treatment on day 21 of treatment. Within 24 hours of withdrawal, plasma concentrations were below 0.4 ng $ml^{-1}$. In urine, the highest concentration was 193 ng $ml^{-1}$, and concentrations above 0.5 ng $ml^{-1}$ were detected up to 5 days after withdrawal. In liver, concentrations above 0.5 ng $ml^{-1}$ were detected on day 14 after withdrawal, while concentrations in the eye were approximately 30 times higher (Meyer & Rinke, 1991). Similar results were shown in sheep (Elliott, McCaughey & Shortt, 1993).

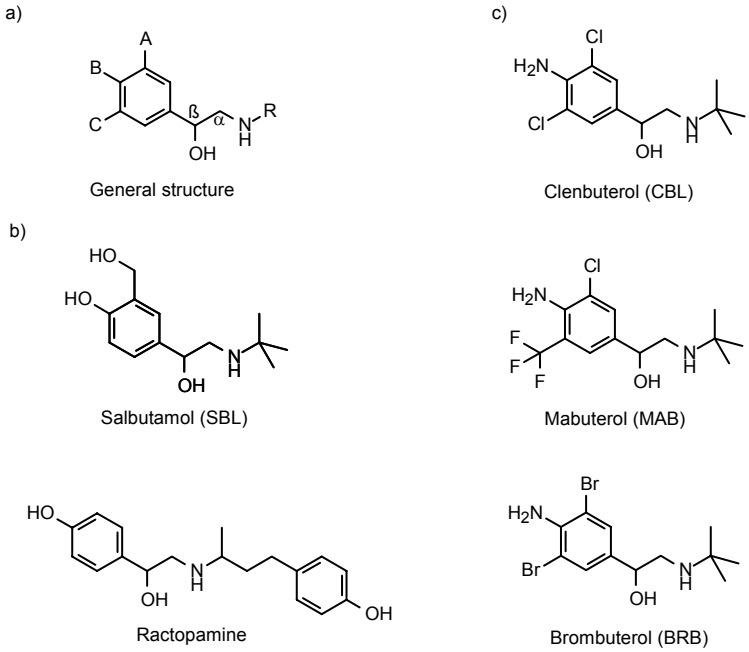


*Figure 2.* Chemical structure of ß-agonists a) the general structure, b) examples of ring-hydroxylated ß-agonists: salbutamol and ractopamine, and c) examples of halogen substituted ß-agonists: clenbuterol, mabuterol and brombuterol.

Another site for accumulation of clenbuterol residues is pigmented hair (Dürsch, Meyer & Karg, 1995; Appelgren *et al*. 1996). According to Gaillard *et al*. (1997) hair testing was effective after 7-10 days of treatment, while Gleixner, Sauerwein & Meyer (1996) detected clenbuterol residues in hair samples from day 4 to day 60 of their experiment. Hair analysis has proven to be a good complement to the conventional urine analysis due to the long retention time of the drug and easy sampling compared to other matrices. Since excretion of clenbuterol in urine is faster, but only present for a shorter period, a combination of urine and hair analysis is a possible approach to maximise the chance of detecting illegal use of drugs in meat-producing animals.

## Analytical strategies and methods

Council Directive 96/23/EC (EC, 1996b) requires all European Union (EU) Member States to monitor residues of a range of licensed and illegal compounds in live animals and animal products, to provide assurance to the consumer on the quality and safety of food. Many EU member states have established a two step approach for drug monitoring, which comprises of a screening test followed by, if required, a confirmatory analysis (Elliott *et al*., 1998a; Kuiper *et al*., 1998). Confirmatory analysis of ß-agonists is based on GC-MS (*e.g*. Blanchflower *et al*., 1993; van Ginkel, Stephany & van Rossum, 1992) or LC-MS (*e.g*. De Wasch, De Brabander, & Courtheyn, 1998).

For screening purposes, immunochemical methods are generally used. Yamamoto & Iwata (1982) reported the first competitive enzyme immunoassay (EIA) for clenbuterol. Several methods for urine (*e.g*. Degand, Bernes-Duykaerts & Maghuin-Rogister, 1992; Elliott *et al*., 1993; Haasnoot *et al*., 1996) and hair (*e.g*. Gleixner, Sauerwein & Meyer, 1996; Godfrey *et al*., 1996; Haasnoot *et al*., 1998) have been published, and various EIA-kits are commercially available. Radioimmunoassay procedures (RIA) have also been developed for the detection of clenbuterol in extracts from a range of samples (Delahaut *et al*., 1991; Boyd, O'Keeffe & Smyth, 1994). A more recent detection technique is immunobiosensor analysis. SPR-biosensor methods for liver (Traynor *et al*., 2003) and urine (Haughey *et al*., 2001) have been developed.

Sample clean-up methods for hair generally include an overnight alkaline digestion at elevated temperature, combined with liquid-liquid extraction with tert-butyl methyl ether or solid-phase extraction. In some methods the dissolving agent DTT (1,4-dithiothreitol) was used to break disulfide bonds to release tightly bound clenbuterol (Gleixner, Sauerwein & Meyer, 1996; Godfrey *et al*., 1996; Sauer & Anderson, 1994). The extracts produced were complex and required further purification. Faster sample clean-up was obtained with mild extraction using 200 mM carbonate buffer pH 10 (Elliott Crooks & McCaughey, 1995) and a digestion-extraction using tert-butyl methyl ether (Haasnoot *et al*. 1998).

The optimal screening assay should be fast and should not require any sample clean-up, but direct analysis of ß-agonists in bovine urine has proven to be difficult due to a large sample-to-sample variation. Hahnau and Jülicher (1996) stated that none of the nine commercial ß-agonist-EIA kits on the market was sensitive enough for residue control analysis of urine without a previous clean-up. Today, most immunochemical screening methods are used in combination with liquid-liquid extraction (LLE), solid phase extraction (SPE) or immunoaffinity chromatography (IAC) (Nausch & Galley, 1997). Since salbutamol is predominantly excreted as a sulphate conjugate, enzymatic deconjugation may be required prior to sample clean-up, in order to fully recover the analyte. Sample pre-treatment and sample clean-up methods for ß-agonists in biological matrices were further reviewed by Boyd, O'Keefe & Smyth (1996).

Collins, O'Keeffe & Smyth (1994) used mixed mode SPE columns in combination with a radioimmunoassay (RIA) to analyse four different ß-agonists in urine, achieving a limit of detection (LOD) of 0.13 ng $ml^{-1}$. Iso-butanol was used by Haasnoot *et al*. (1996) to extract several ß-agonists, including both clenbuterol and salbutamol (SBL), to achieve LODs below 1 ng $ml^{-1}$ for their ß-agonist EIA. In the biosensor method by Haughey *et al*. (2001), urine samples were extracted with tert-butyl methyl ether (TBME) and concentrated eightfold, resulting in a LOD of 0.27 ng $ml^{-1}$. This method was, however, not able to extract SBL and other phenolic ß-agonists. The commercial Biacore kit based on the method was reported to be somewhat slower than ELISA, but had a lower false positive rate at the 0.3 ng $ml^{-1}$ level, when tested on-line at a slaughter house (Guggisberg, Widmer & Koch, 2001).

A new approach for sample clean-up is immunofiltration (IF), which was employed in combination with a ß-agonist EIA by Haasnoot *et al*. (2001). The urine sample was mixed with antibodies in an ultra-filtration (UF) device. After centrifugation, the bound CBL was released from the antibodies with a mixture of methanol and acetic acid. The eluate was evaporated, dissolved in PBS-buffer and analysed with EIA, resulting in lower numbers of false positive samples compared to iso-butanol clean-up.

## Biosensors in food analysis

A biosensor is an analytical device that combines the specificity of a biological interaction with a transducer that produces a signal proportional to the target analyte concentration (Mulchandani & Bassi, 1995). The biological recognition element (*e.g.* antibodies, enzymes, receptors and microbial cells) is in close contact with a signal transducer (*e.g.* optical, amperometric, potentiometric and acoustic) coupled to a data acquisition and processing system (Patel, 2002). An overview of the operating principles of a biosensor is shown in Figure 3.

Different types of biosensors and their application in food safety were recently reviewed (Patel, 2002; Velasco-Garcia & Mottram, 2003; Leonard *et al*., 2003). Amperometric devices are the most commonly reported class of biosensors. Detection typically relies on an enzyme system that catalytically converts the analyte to a product, which can be oxidised or reduced at a working electrode maintained at a specific potential. The most commercially important application is the hand-held glucose meter used by diabetics (Velasco-Garcia & Mottram, 2003). A wide range of enzyme biosensors for applications in food analysis have been described, *e.g.* for detection of glucose, carbohydrates, ethanol, starch and phenols (Mello & Kubota, 2002). In contaminant analysis, enzyme biosensors have largely been used for organophosphorus and carbamate pesticide and herbicide analysis. Assays for penicillin G and V have also been reported (Patel, 2002).

Another class of biosensors is affinity-based sensors. They typically use the interaction antibody-antigen, receptor-ligand or protein-nucleic acid. Upon interaction, the physical properties at the sensor surface, *e.g.* refractive index, mass, density or thickness is affected (Kress-Rogers, 1997). The change can be monitored in real time using acoustic (piezoelectric) materials or optical (*e.g.* surface plasmon resonance, SPR or resonant mirror) transducers (Bilitewski, 2000). One of the advantages of optical transduction is that, whatever the biorecognition chemistry, the outcome can be followed in a non-destructive manner (Pearson, Gill & Vadgama, 2000).

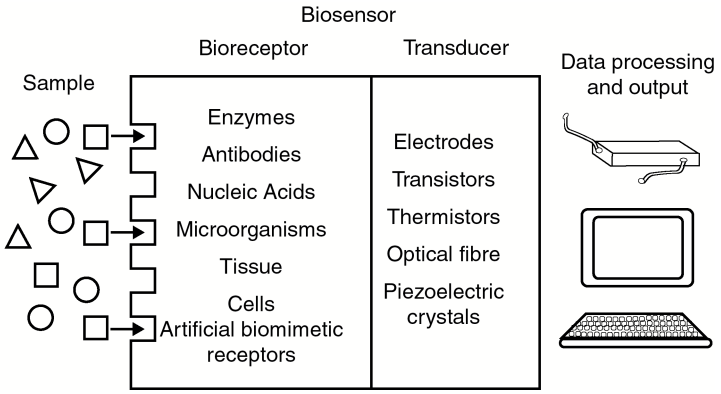


*Figure 3*. Schematic diagram of the main components of a biosensor. The biological event, *e.g.* antibody-antigen interaction elicits a physico-chemical change at the biointerface, *e.g.* change of mass, heat or in electrical potential, which is converted by the transducer to an electrical signal. The output is amplified, processed and displayed as a measurable signal.

Biacore AB, formerly Pharmacia Biosensor AB, was the first company to commercially develop SPR-technology in 1990 (Liedberg Nylander & Lundström, 1995). During the last few years, Biacore technology has been used in approximately 90% of all work published in the optical biosensor field (Myszka, 1999, Rich and Myszka, 2000a; 2001; 2002). Other commercially available SPR-systems include IAsys from Affinity Sensors, Spreeta from Texas instruments and Nippon Laser and Electronics systems (Baird & Myszka, 2001; Mullett, Lai & Yeung, 2000; Leonard *et al*., 2003).

The main applications of Biacore systems are for the determination of affinity and kinetics of interaction between two or more biomolecules (Karlsson & Fält, 1997; Myszka, 1997), receptor-ligand interactions (Cunningham & Wells, 1993) and nucleic acid hybridisation (Persson *et al*. 1997). The systems have also been used for quantitative analysis using antibodies as specific reagents (Mellgren & Sternesjö, 1998) and for the thermodynamic analysis of biomolecular interactions (Roos *et al*., 1998). Only a minor part of the applications published so far is related to food analysis or food safety.

In the current work, two similar models (Biacore 2000 and Biacore Q) of optical SPR-biosensors were used to detect residues of veterinary drugs. Since antibodies were used for biological recognition in these particular applications, the biosensors were referred to as immunosensors or immunobiosensors (Marco, Gee & Hammock, 1995).

## Biacore analysis

The main components of the Biacore system are the interchangeable sensor chip, the microfluidic sample handling system and the surface plasmon resonance (SPR) detector. Aqueous samples are serially injected over the sensor chip surface immobilised with one of the interacting partners. Binding of the interactant is detected and quantified in real-time by the surface sensitive detector (Löfås, 1995).

### *Optical system*

In principle, the SPR-immunosensor measures the mass concentration change caused by binding of an antibody (or analyte) to the corresponding analyte (or antibody) immobilised on the sensor surface (Patel, 2002). The SPR phenomenon occurs when plan polarised light is reflected from a gold film deposited on a glass support (Liedberg, Nylander & Lundström, 1983). Photons react with the free electron cloud in the metal film at a specific angle, the SPR-angle, and cause a drop in the reflected light. Refractive index changes near the surface give rise to a shift of the resonance angle. The shift is directly proportional to the mass increase and mass concentration can thus be measured (Stenberg *et al.*, 1991). The analysis principle is summarised in Figure 4.

The response from angular shift is expressed in resonance units (RU) and 1 RU corresponds to shift of 0.0001° (Jönsson *et al.* 1991). By plotting the angular shifts against time a sensorgram, describing the interaction at the surface in real time, is obtained (Figure 5).

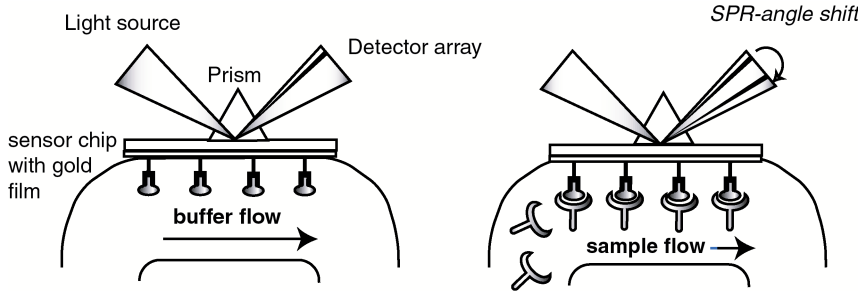


*Figure 4*. Surface plasmon resonance (SPR) biosensor principle. Binding of biomolecules to the surface increase the refractive index, which induces shift of the SPR-angle. The shift is directly proportional to the mass increase.

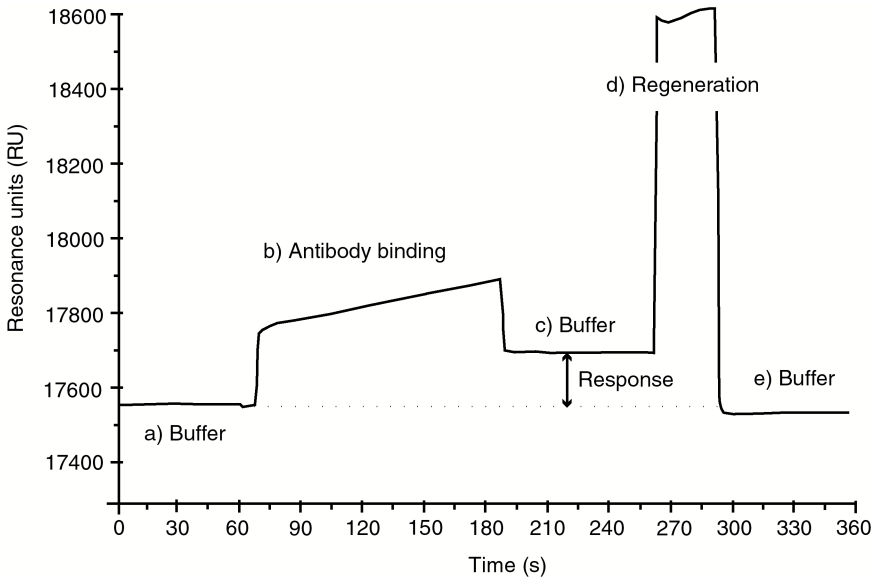


*Figure 5.* A sensorgram illustrating the interaction between free antibody in sample and antigen immobilised on the surface. The different phases are recorded in real time including, (a) baseline equilibrium (continuous buffer flow), (b) binding of antibody to the sensor surface during sample injection, (c) response after sample injection, (d) regeneration of the sensor surface and (e) baseline stabilisation.

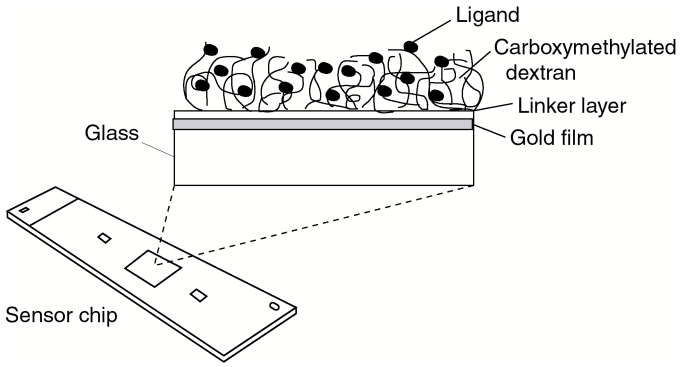


*Figure 6.* The surface of a sensor chip consists of three layers; glass, a thin gold film and a dextran layer, to which biomolecules can be immobilised.

### *Sensor surface*

The sensor chip consists of a gold-coated glass slide embedded in a plastic support platform (Figure 6). The gold film is covered with a covalently bound dextran layer to which biomolecules can be immobilised. Different types of sensor surfaces are available, but the most frequently used surface is carboxymethylated dextran, CM5 (Rich & Myszka, 2000b). The SPR-technique is sensitive to refractive index changes to a distance of approximately 1 µm out into the solution, and the dextran matrix increases the binding capacity of the surface, as it extends out approximately 100 nm in water solutions. In addition, the non-specific binding from proteins compared to a bare gold surface is minimised (Liedberg, Lundström & Stenberg, 1993).

### *Flow system*

When a sensor chip is docked in the instrument, the integrated µ-fluidic cartridge (IFC) is pressed against the chip surface (Sjölander & Urbaniczky, 1991). The IFC forms three flow cell walls, while the sensor chip forms the fourth wall. There are four flow cells that can be fed and monitored separately. Flow cell volumes range from 20-60 nl depending on instrument model (Baird and Myszka, 2001). The sensor surface is exposed to a constant analyte concentration by a continuous flow of buffer sample injections, controlled by an autoinjector in combination with the IFC. Generally, sample volumes of 5-450 µl at flow rates of 1-100 µl $min^{-1}$ can be injected. For some models (*e.g.* Biacore 2000 and 3000), a sample can be passed over the four flow cells in sequence and the response for all flow cells can be monitored in parallel, while for other models (*e.g.* Biacore 1000 and Biacore Q) only one flow cell can be monitored at the time.

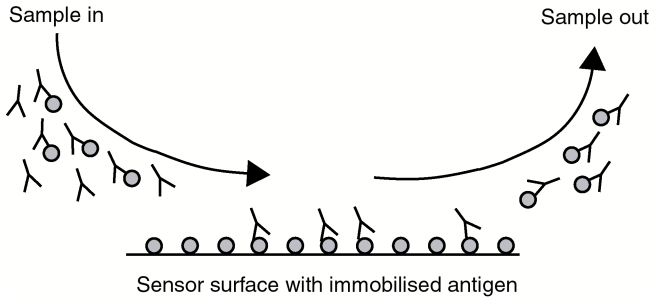


*Figure 7.* In the inhibition assay format the analyte itself is often immobilised to the surface. A mixture of sample and antibody is injected, and the antibody binding is measured.

### *Inhibition assay*

The sensitivity of direct analysis in current instruments is insufficient for determining low levels of low-molecular-weight analytes such as antibiotics, hormones and residues. Instead, an inhibition assay format is used, in which the analyte or analyte conjugate is immobilised on the surface (Bilitewski, 2000). Antibodies are added to the sample prior to injection (Figure 7). If the analyte is present, binding will occur, and some antibodies will be blocked. The mixture is injected over the surface, and the concentration of antibody free to bind to the sensor surface is measured. At a high analyte concentration most antibodies will be inhibited, resulting in a low response, while a blank sample will give a high response. Thus, in the inhibition assay format the SPR change is inversely proportional to the analyte concentration in the sample.

## Assay development

### *Immobilisation of analyte specific chip surface*

The most generally applicable immobilisation strategy is amine coupling, where the ligand is coupled via primary amino groups. Alternative strategies include coupling via thiol or aldehyde groups of the biomolecule, or using streptavidin/biotin interaction (Löfås *et al*., 1995). Amine coupling introduces N-hydroxysuccinimide esters into the surface matrix by modification of the carboxymethyl groups with a mixture of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). These esters then react spontaneously with amines and other nucleophilic groups on the ligand to form covalent links (Johnsson, Löfås & Lindquist, 1991; O'Shannessy, Brigham-Burke & Peck, 1992). Non-reacted active esters are deactivated by addition of ethanolamine hydrochloride. Amine coupling is shown in Figure 8.

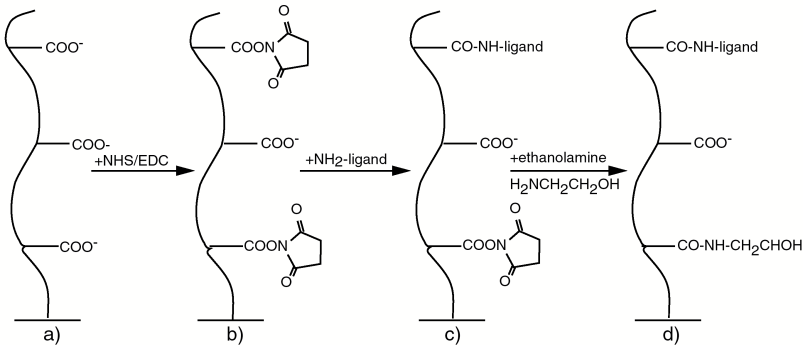


*Figure 8.* Amine coupling of ligand to a sensor surface: a) Non-activated carboxylic groups on the dextran; b) The carboxylic groups are activated by addition of a mixture of succinimide (NHS) and carbodiimide (EDC); c) The ligand is covalently bound to the sensor surface; d) Remaining esters are deactivated by addition of ethanolamine (Johnsson, Löfås & Lindquist, 1991). For some analytes the surface is amino modified by addition of ethylenediamine prior to immobilisation.

The number of ligand molecules that will be immobilised is affected by the number of NHS/EDC activated carboxymethyl groups, the pH, the ionic strength and the concentration of the coupling buffer, and by ligand concentration and reaction times. For proteins, different coupling buffers can be tested prior to immobilisation by injecting ligand over a non-activated surface, without affecting the surface. The process is called pre-concentration and is monitored in a sensorgram. Pre-concentration is however not possible with small ligands due to mass sensitivity limitations of the instrument (Löfås *et al.* 1995).

### *Surface regeneration*

The vast majority of biosensor methods need regeneration of the sensor surface before measurement can be repeated. An ideal regeneration solution should completely remove all non-covalently bound material from the surface, while maintaining the activity of the ligand. A common approach for antibody-antigen regeneration is to use agents with high or low pH, i.e. NaOH, HCl or glycine (Andersson, Hämäläinen & Malmqvist, 1999). A combination of different agents is however more efficient for breaking "stronger" interactions between antibodies and antigens. Dissociation is promoted by a combination of a strong electron-donor organic solvent (*e.g.* dimethylsulfoxide, ethylene glycol or propanol) with an increase or decrease of pH (van Oss, 1994). Addition of chaotropic salts, *e.g.* guanidine hydrochloride, ionic detergents, *e.g.* SDS (sodium dodecyl sulphate) and addition of acetonitrile are other options (Tsang & Wilkins, 1991; Newman, Olabiran & Price, 1997). The binding forces involved in the antibody-antigen interaction and optimal conditions for dissociation are well described elsewhere (Jefferis & Deverill, 1991; van Oss, Good & Chaudhury, 1986). In order to facilitate assay development and enable standardised regeneration, a general capturing surface was used in some Biacore applications (Bergström *et al*., 1999; Gustavsson *et al*., 2002).

*Reduction of sample matrix effects*

In an inhibition assay there are two main types of sample matrix effects present, as shown in Figure 9. First sample matrix components can bind non-specifically to the surface. Secondly, the interaction between antibody and analyte in solution, or analyte liganded to the surface, can be hindered by matrix components. Non-specific binding to the surface (NSB), which is usually caused by larger components such as proteins and fat aggregates, may add to the specific response (SB) and increase the risk of false negative results (c). If the initial interaction between antibody and antigen in the pre-injection mixture is disturbed by matrix components (a), the assay sensitivity in the sample matrix as compared to the buffer is decreased. Hindrance of the interaction between antibody and surface ligand (b) reduces the SB in matrix compared to buffer.

The most frequently discussed matrix effect in biosensor analysis of food is non-specific binding. A common approach to reduce NSB is to use sample dilutions of 10 times or more (Baxter *et al*., 1999, Johnsson *et al*., 2002; Haasnoot, Bienenmann-Ploum & Kohen, 2003). In a folate-binding protein assay, a wash step was introduced after sample injection, to reduce the impact of NSB from rennet-treated milk (Nygren, Sternesjö & Björck, 2003), while a reference flow cell, immobilised with rabbit IgG, was used to correct for NSB in an assay for non-milk proteins in milk powder (Haasnoot *et al*., 2001). Bovine serum proteins were precipitated with saturated ammonium sulphate prior to biosensor analysis of benzimidazole residues (Johnsson *et al*. 2002). Addition of dextran to the assay buffer has also been used (Bergström, 1998).

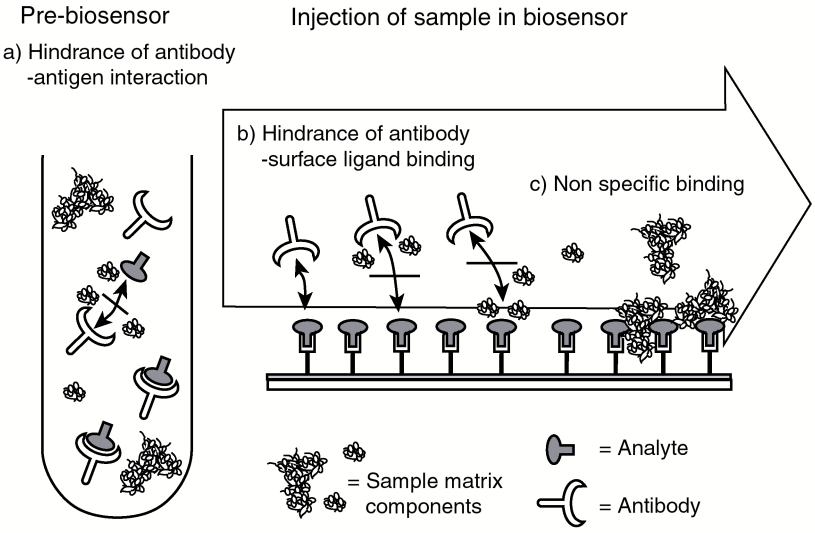


*Figure 9*. Sample matrix effects in a biosensor inhibition assay. Matrix components can interfere with the antibody-antigen interaction in the pre-biosensor mixture (a), adhere to the antibody or the surface ligand and thereby hinder the antibody to bind to the surface (b), and bind non-specifically (NSB) to the surface (c).

Calibration curves spiked in sample matrix (Gaudin & Maris, 2001; Bjurling *et al.,* 2001; Ferguson *et al*., 2002) have been used to compensate for the matrix inhibition effect. Haughey *et al*. (2001) prepared a calibration curve in phosphate buffer with addition of urea and creatinine to mimic the main components in urine. The matrix inhibition effect from liver extracts was substantially reduced by delaying the mixing of antibody and sample until just prior to sample injection (Traynor *et al*., 2003). Standard sample clean-up such as liquid/liquid extraction or solid phase extraction has also been used in several assays (Haughey *et al*., 2001; Traynor *et al.*, 2003; Ferguson *et al*., 2002).

### *Signal enhancement*

The detection sensitivity can be amplified with a second molecule (ligand or antibody) that binds to the analyte in a sandwich assay (Mullett, Lai & Yeung, 2000). Secondary antibodies were used to increase the response and to reduce the impact of non-specific binding (*e.g.* Jonsson, Malmheden-Yman & Hellenäs, 2001). Sub-micron latex particles were shown to improve the sensitivity of an SPR-immunoassay for human chorionic gonadotropin by a factor 30 (Severs & Schasfoort, 1993). In another study, Kubitschko *et al*. (1997) optimised a nanoparticle-enhanced immunoassay, with respect to particle size, coating conditions and non-specific binding of nanoparticles.

## Biacore assays in food analysis

For food borne pathogens and their toxins, a number of assays based on the Biacore technique have been developed over the last decade. Fratamico *et al*. (1998) developed an assay for detection of Escherichia coli O157:H7 and inhibition of binding of the bacteria to extracellular matrix components was studied by Medina (2001 & 2002). Staphylococcal enterotoxin B was detected (Rasooly, 2001; Medina, 2003) and Nedelkov & Nelson (2003) combined a Biacore assay with MALDI-TOF MS to quantify the toxin. Serum antibodies against two types of Salmonella in chicken were detected in parallel with a Biacore 3000 assay developed by Jongerius-Gortemaker *et al*. (2002). Assays for whole cells of Listeria monocytogenes (Leonard *et al*. 2004) and Salmonella cells group B, D and E (Bokken *et al*. 2003) were also developed. Other applications in the food safety area were analysis of the fungal aflatoxin B(1) by Daly, *et al*., (2000) and allergen detection in different food matrices (Jonsson, Malmheden-Yman & Hellenäs, 2001). Insulin-like growth factor 1 (IGF-1) was analysed to detect the use of recombinant bovine somatotropin (rBST), which is used to increase the milk yield from cows (Guidi *et al*., 2001). The detection of pesticides (Minunni, & Mascini, 1993; Alcocer *et al*., 2000) has also been addressed, and genetically modified organisms (GMO) were analysed using DNA hybridisation (Mariotti, Minunni, & Mascini, 2002). Feriotto *et al*. (2002; 2003) developed methods for Roundup Ready soybean gene sequences and quantitative determination of transgenic maize.

A number of assays have been developed for components in bovine milk, *e.g.* detection of non-milk proteins in milk powder (Haasnoot *et al*., 2001), determination of folate binding protein (Nygren, Sternesjö & Björck, 2003) and analysis of progesterone in bovine milk to predict cows' pregnancy (Gillis *et al*., 2002). Casein was measured in milk and ripening cheese (Muller-Renaud, Dupont, & Dulieu, 2004) and immunoglobulin G in bovine colostrum and milk (Indyk & Filonzi, 2003). Biacore assays were also developed for water-soluble vitamins *e.g*. folic acid, biotin, vitamin $B_{12}$ and riboflavin in fortified food, infant formula and milk (Boström Caselunghe & Lindeberg 2000; Indyk *et al*., 2002; Indyk *et al*., 2000; Caelen, Kalman, & Wahlström, 2004).

### *Biacore assays for veterinary drug residues in food*

In drug residue analysis too, the majority of the Biacore applications were developed for milk, *e.g.* sulfamethazine, enrofloxacin/ciprofloxacin, streptomycin and ivermectin (Sternesjö, Mellgren & Björck 1995; 1998; Baxter *et al*., 2001; Samsonova *et al.*, 2002a). An inhibition assay format, with addition of antibodies to the sample prior to injection, was used in most cases (*e.g.* Gaudin & Pavy, 2000; Ferguson *et al*., 2002; Johnsson *et al*., 2002; Samsonova *et al*., 2002b) with a few exceptions. Gentamicin (Haasnoot & Verheijen, 2001) and streptomycins (Haasnoot *et al*. 2002) were detected in direct assays, *i.e*. samples were injected over a sensor surface immobilised with antibodies and binding of analyte was measured directly. Direct assays for these small molecules (MW < 1000 D) were enabled by a higher sensitivity of the optical system in combination with a smaller flow cell and the online data subtraction feature in the Biacore 3000 system compared to earlier models (Rich & Myszka, 2000b).

Most residue applications were developed for veterinary drugs with maximum residue limits (MRL) ranging from 4 ng $ml^{-1}$ for penicillin G in milk (Gaudin, Fontaine & Maris, 2001) to 100 and 200 µg $kg^{-1}$ for levamisole in liver and nicarbazin in chicken respectively (Crooks *et al*., 2003; McCarney *et al*., 2003). Assays for detection of concentrations at or below 1 ng $ml^{-1}$ were developed for clenbuterol in urine (Haughey *et al*., 2001), ß-agonists in liver (Traynor *et al*., 2003) and chloramphenicol in milk (Gaudin & Maris, 2001). These low detection limits all required some sort of sample preparation. Concentrations of ractopamine analysed in urine without sample pre-treatment were somewhat higher (Shelver & Smith, 2003). Two direct assays were discussed earlier (Haasnoot & Verheijen, 2001; Haasnoot *et al*. 2002.) Both assays had $IC_{50}$ values of 20 ng $ml^{-1}$ in buffer and a dynamic range of approximately 100 RU, which indicate that present instrumentation does not enable direct detection of concentrations below 1 ng $ml^{-1}$.

High sample throughput is another feature of the Biacore system, as was demonstrated for sulfamethazine (Elliott *et al*., 1999) and sulfadiazine (Crooks *et al*., 1999) in pigs' bile. Furthermore, the robustness of the Biacore instrumentation was demonstrated by on-site application in a pig abattoir (Baxter *et al*., 1999). Simultaneous analysis of several samples, or for several analytes in one sample, was demonstrated in the EU-project FoodSENSE (FAIR-CT98-3630), using a prototype system with 8 parallel flow cells (Crooks *et al*., 2001). The system was successfully used for on-line detection of sulfamethazine and sulfadiazine in

porcine bile and the analysis time for one 96-well plate was shortened from 540 to 50 minutes using the prototype, compared to analysis using a commercial Biacore 1000 (Situ *et al*., 2002).

Analysis of several groups of drug residues in parallel is the ultimate goal, which require the possibility of immobilising one analyte in each flow cell without contaminating the instrument. For Biacore systems, this has been facilitated by an external immobilisation station (surface preparation unit). Instruments that allow simultaneous detection in four flow cells are available (*i.e*. the Biacore 2000 and 3000 models). Applicability was demonstrated in an inhibition assay with parallel detection of five different aminoglycosides, immobilised in different flow cells, and simultaneously analysed with a mixture of antibodies (Haasnoot *et al*., 2003). Recently, an automated micro array system using multi-analyte immunoassays with an indirect competitive ELISA format for simultaneous detection of 10 different antibiotics in milk was reported (Knecht *et al*., 2004). Another possibility to analyse several compounds, is to use a receptor protein, which was demonstrated in two Biacore sensor assays for ß-lactams in milk (Gustavsson *et al*. 2002; Gustavsson, Bjurling, & Sternesjö, 2002; Gustavsson, 2003). The latter assay was based on the enzymatic activity of the receptor protein, which was inhibited in the presence of ß-lactams.

# Objectives

The overall aim of this study was to investigate the applicability of optical biosensors to screening for low levels of residues of illegal growth promoting drugs in foods and tissues from food producing animals, and to develop fast and reliable screening tests with a minimum of sample pre-treatment.

Specific objectives for this work were:

- To prepare stable analyte specific surfaces for ß-agonists and hormones and to establish optimised assay conditions for binding and regeneration of antibodies.

- To investigate strategies to minimise sample matrix effects through optimisation of assay conditions.

- To develop and validate an immunobiosensor assay for screening of clenbuterol residues in bovine hair.

- To develop and validate a fast immunobiosensor assay for screening of residues of clenbuterol and salbutamol type of ß-agonists, below 1 ng $ml^{-1}$ in urine.

# Materials and methods

## Immobilisation

Immobilisation of analyte-specific chip surfaces with clenbuterol, ractopamine, hexestrol, trenbolone and ethinylestradiol are described in detail in paper I. All immobilisations were performed in a moist chamber on an ELISA plate shaker outside the instrument, by applying 75 µl of each reagent solutions to the chip surface. The chip surface was rinsed with deionised water between each reagent. Reaction times ranged from 1 to 24 hours.

Amine coupling, as described earlier, was used for all immobilisations. The free primary amino groups of ractopamine and hexestrol derivatives were allowed to react directly with the activated chip surface, while the chip surface was covalently modified to carry amino groups, prior to immobilisation of clenbuterol, trenbolone and ethinylestradiol. The remaining unreacted active ester groups on the surface were deactivated by addition of ethanolamine. Chemical structures for the analytes and reagents used for immobilisation are shown in Figure 10.

The immobilisation procedures for ractopamine (RCP) and hexestrol (HEX) were derived from a method for the coupling of fenoterol to a carrier protein (Lommen, Haasnoot & Weseman 1995). One of the phenolic groups of RCP or HEX readily reacted with one of the two epoxy-groups of bisoxirane (BIS) under alkaline conditions, forming stable ether bonds. The remaining epoxy-group of BIS was allowed to react with ammonia to form a primary amino group free to react with the activated carboxylic groups on the sensor chip surface.

Thiophosgene was used to convert the primary aromatic amine group of clenbuterol to the corresponding isothiocyanate. The reaction was performed under neutral conditions and the formed isothiocyanate was allowed to react with an amino-modified surface at pH 10.

Trenbolone was converted to the corresponding succinimidyl carbonate under anhydrous conditions in a DMAP (4-dimethylaminopyridine) catalysed reaction between the hydroxyl group of trenbolone and DSC (N,N-disuccinimidyl carbonate) in a modification of the procedure used by Miron & Wilchek (1993). The product was allowed to react with the amino-modified surface for 1 hour forming a carbamate.

Ethinylestradiol-carboxy-methyloxime (EEO-CMO) in dimethylformamide (DMF) was mixed with EDC and NHS in 10 mM sodium acetate pH 4.6. The product was allowed to react with the amino-modified surface. This chip surface was a gift from Veterinary Science Division, Belfast, UK.

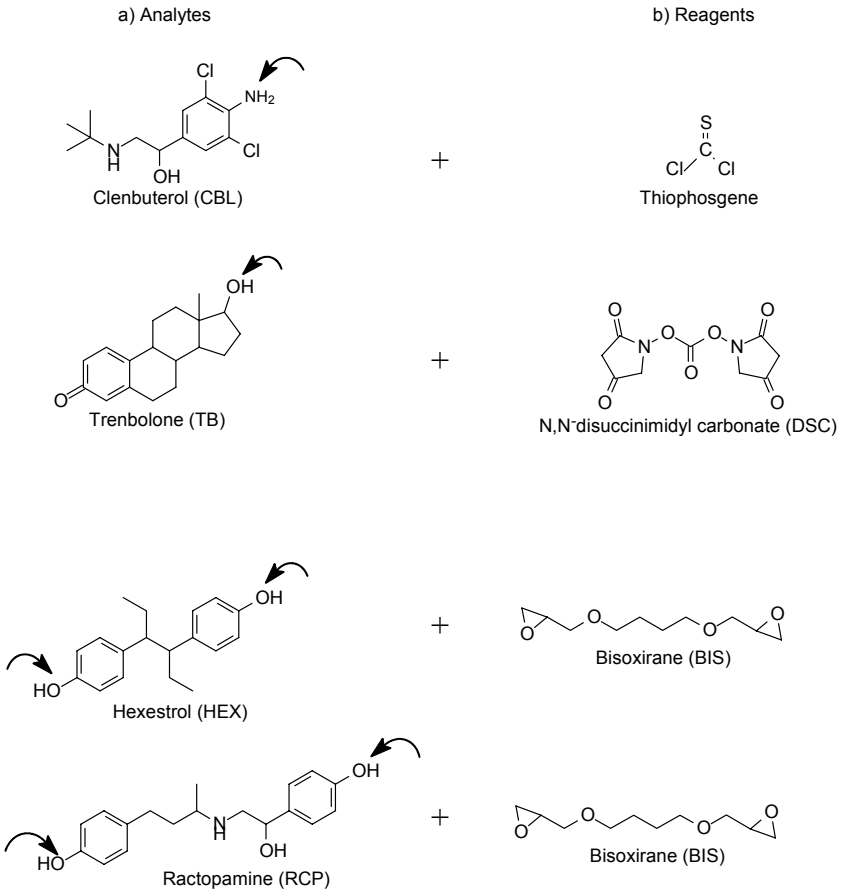


*Figure 10.* Chemical structures of analytes (a) and their corresponding reagents (b) used for immobilisation. Primary reaction sites are indicated by arrows.

## Assay setup and optimisation

Unless otherwise stated, the assay buffer used was 10 mM HEPES pH 8.2 with 0.5 M NaCl, 3.4 mM EDTA and 0.005% P20. All primary antibodies were first diluted in a double strength assay buffer and then mixed (1+1) with samples and standards prior to analysis. Since samples were typically prepared batch by batch, and injected in a sequence, the incubation time could range from minutes to hours with no detectable difference in the result. Assays were typically run at a continuous flow of 30 µl $min^{-1}$, sample injection times were two minutes and regeneration solutions were injected in one or two pulses of 30 seconds each.

### *Antibodies*

The primary antibody preparations used in the present work are summarised in Table 1. All antibody reagents used were dilutions of serum or ascites fluid without further purification. Concentrations of antibodies directed against a specific analyte in serum or ascites were measured as the active antibody concentration ($R_{max}$) according to Karlsson *et al*. (1993) as further discussed in paper I. A secondary antibody (affinity purified goat anti-rabbit) was used for signal enhancement in analysis of clenbuterol in hair (see paper III).

*Table 1*. Overview of primary antibody preparations used in the present work and the coupling chemistry of the immunogen during the production of the antibodies. All these antibodies were gifts from VSD in Belfast (C. T. Elliott), TNO in Zeist (C. Arts) or RIKILT in Wageningen (W. Haasnoot).

| Antibody | Type[a] | Hapten | Immunogen coupling | Host animal |
|---|---|---|---|---|
| P-CBL-1 | PAb | clenbuterol | diazotation | rabbit |
| P-CBL-2 | PAb | clenbuterol | diazotation | rabbit |
| P-SBL | PAb | salbutamol | no information | sheep |
| P-RCP | PAb | ractopamine | bisoxirane | goat |
| P-EEO | PAb | ethinylestradiol | hemisuccinate | goat |
| P-HEX | PAb | hexestrol | carboxy-propyl-ether | rabbit |
| M-CBL | MAb | clenbuterol | diazotisation | mouse |
| M-TB | MAb | trenbolone | hemisuccinate | mouse |

[a]PAb and MAb stand for polyclonal and monoclonal antibody respectively.

### *Maximum binding capacity*

After immobilisation the maximum binding capacity ($R_{max}$) of the different analyte specific chip surfaces was determined by a 20 min long injection of a high antibody concentration. For some of the chip surfaces the maximum binding was monitored over a longer period to observe surface stability during storage and after numerous injections of samples and regeneration solutions (paper I).

### *Calibration curves and optimisation of assay parameters*

Calibration curves in buffer for CBL, EEO, TB, HEX and RCP were prepared by mixing working solutions with concentrations ranging from 0.1 to 100 ng $ml^{-1}$ for each analyte with the respective antibody (1+1). Inhibition assay standard curves were constructed using a 4-parametric fit. To compare the sensitivity of calibration curves, the $IC_{50}$-value, defined as the concentration of the inhibitor (analyte) that is required to reduce the response to 50% of b0, was used. Maximum response (b0) was defined as the signal in biosensor resonance units (RU) for an antibody solution in the absence of analyte under assay conditions. The effect of antibody concentration on assay sensitivity was investigated for antibodies P-CBL-1, P-CBL-2, M-CBL and P-SBL respectively. The relation between response of antibody with a CBL-concentration of 0.5 ng $ml^{-1}$ and in absence of CBL and for six dilutions per antibody was used for evaluation (more details in paper I).

## Bovine urine and hair samples

Twenty lyophilised reference blank bovine urine samples from the EC Reference Laboratory in Bilthoven (RIVM) were used in paper II and IV. These samples were taken from a broad range of animals including veal calves, fattening bulls, heifers, pregnant cows and mature bulls (Sterk *et al*. 1998). Additional samples were collected within the Swedish national control programme. Incurred urine samples (clenbuterol and salbutamol) were obtained from TNO Voeding (Zeist, the Netherlands). Clenbuterol samples were produced according to Haughey *et al.* (2001) and salbutamol incurred samples according to Elliott *et al.* (1998b). All urine samples were stored at –20 °C until analysis. Results by Haasnoot, Kemmers-Voncken & Samson (2002) indicate that urine samples incurred with ß-agonists are stable at –20 °C for several years.

A number of blind coded GC-MS negative and GC-MS positive bovine hair samples (paper III), were obtained from Veterinary Science Division, Queens University of Belfast, UK. Hair samples were stored at room temperature.

## Study of sample matrix effects

Non-specific binding (NSB) from urine and serum to a clenbuterol sensor chip surface was measured through injections of samples mixed with double strength assay buffer (1+1) without antibody present. The effect of dextran addition (1 mg $ml^{-1}$ sample) and ultrafiltration (UF) of samples was tested. UF was done as follows: 500 µl sample (urine or serum) was added to a 10 kD cut-off filter device and spun for 30 min at 14 000 x g (paper II).

The effect of assay buffer composition on non-specific binding (NSB) and matrix interferences with the antibody-antigen interaction were studied in a full factorial design experiment using the clenbuterol assay as a model. In total, 13 different buffer compositions were used as further described in paper II. Three levels of pH (6, 8 and 10), two levels of NaCl (0.02 and 0.5 M) and two levels of HEPES-buffer (10 and 100 mM) were used. For each buffer composition, 8 samples (Table 2) were injected in triplicate, to describe the most important assay parameters.

To represent the maximum sample variation, one dark concentrated urine pool (urine A) and one light urine pool (urine B) were prepared from aliquots of RIVM blank reference standard urine samples. The two urine pools were divided into three aliquots each, pH-adjusted to pH 6, 8 and 10, respectively, and stored frozen in small portions until analysis. The statistical software Minitab 12.22 was used for evaluation of results by response surface regression and ANOVA analysis with a significance level of $p < 0.05$ (see paper II for more details).

*Table 2.* Summary of the primary response variables used in the buffer optimisation experiment. For each buffer composition (n = 13), 8 different samples were injected in triplicate. Reagent (Ab or buffer) was mixed (1+1) with blank or spiked matrix (water or urine). The specified clenbuterol concentration is in matrix.

| Sample name | Sample composition | | | Description |
|---|---|---|---|---|
| | Reagent | Matrix | Clenbuterol (ng $ml^{-1}$) | |
| Max | Ab[1] | H2O | 0 | Max Ab-response in buffer |
| Spike | Ab | H2O | 5 | Spike in buffer |
| NSB A | buffer | urine A[2] | 0 | Non-specific binding for dark urine |
| NSB B | buffer | urine B[3] | 0 | Non-specific binding for light urine |
| MaxA | Ab | urine A | 0 | Max response in dark urine |
| MaxB | Ab | urine B | 0 | Max response in light urine |
| SpikeA | Ab | urine A | 5 | Spike in dark urine |
| SpikeB | Ab | urineB | 5 | Spike in light urine |

[1]Ab is monoclonal clenbuterol antibody (M-CBL) at a final dilution of 1/10 000.
[2]urine A is dark urine pool.
[3]urine B is light urine pool.

Interference from urine with the antigen-antibody interaction at different antibody dilutions was studied through analysis of blank and spiked urine using a monoclonal clenbuterol antibody (M-CBL). The effect of sample dilution (dilution factors from 1 to 32) on background from urine was studied at two antibody dilutions. More details can be found in paper II.

## Sample preparation

In paper III, a biosensor assay for analysis of clenbuterol in bovine hair was presented. Hair samples were washed with deionised water and ethanol, after which they were dried under vacuum at room temperature. The dried hair was cut in 1 cm pieces and 200 mg of hair were extracted with 2.5 ml 100 mM NaOH in water bath at 100 ºC for 30 min. The extracts were purified by microscale centrifuge ultrafiltration with a cut-off of 3 kD (120 min, 14 000 x g) prior to biosensor analysis. See paper III for further details.

Urine, adjusted to pH 8.2 ± 0.2, was pre-filtered through a 10 kD cut-off filter device by centrifugation for 30 minutes. An aliquot of the urine filtrate was mixed (1+1) with antiserum diluted in double strength assay buffer and centrifuged in a 30 kD cut-off filter device for 12 min. After a filter wash, the retained antibodies were collected by placing the filter cup upside down in a clean tube and centrifuged at 1200 g for 3 min. The isolate was diluted to original volume with assay buffer before biosensor analysis. (paper IV). The principle of integrated immunofiltration (IIF) is shown in Figure 11.

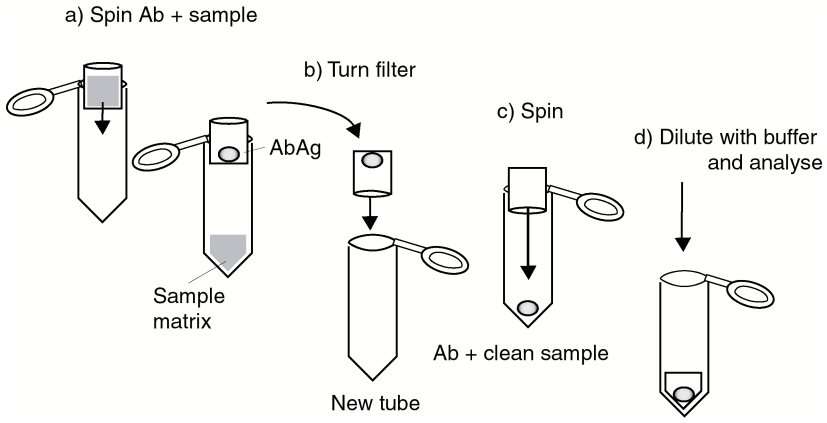


*Figure 11.* The principle of integrated immunofiltration (IIF). The sample is mixed with antibodies and centrifuged in an ultra-filtration device with a molecular cut-off of 30 kD. The filter cup with the retained antibody-antigen complex is turned upside down in a new tube, and after a quick spin followed by addition of assay buffer, the complex is directly analysed in the biosensor.

## Assay validation

According to Council Directive 2002/657/EC (EC, 2002), the only performance criteria specified for screening methods is to have a false negative rate (ß-error) of ≤ 5%. The lowest concentration at which this is fulfilled is termed the detection capability (CCß). For practical reasons, the false positive rate (α-error) also has to be estimated. In the EC Directive, the limit of detection (LOD) has been substituted by the decision limit (CCα), which can be calculated as the mean for 20 blank samples + 2.33 SD. This corresponds to a false positive rate (α-error) of 1%.

## GC-MS analysis

GC-MS analysis of hair samples in paper III was performed at Veterinary Science Division in Belfast, UK (Elliott, 1995) and GC-MS analysis of urine samples in paper IV was performed at the National Food Administration, Uppsala, using a method derived from van Ginkel, Stephany & van Rossum (1992).

# Results and discussion

## Sensor chip preparation and assay construction for immuno-biosensor determination of ß-agonists and hormones (paper I)

Analyte specific chip surfaces for clenbuterol (CBL), ractopamine (RCP), trenbolone (TB), hexestrol (HEX) and ethinylestradiol (EEO) were prepared and surface performance in terms of stability, binding capacity and calibration curve sensitivity was evaluated and optimised with various antibody reagents. In initial immobilisation experiments diazotised CBL was injected over the chip surface in the Biacore instrument, but due to contamination of the liquid flow system, all subsequent immobilisations were done outside the instrument by manually applying the reagents onto the chip.

Stable analyte specific surfaces could be prepared by employing more or less modified standard procedures for hapten synthesis used in antibody production (Table 1). While immunisation conjugates are designed to act in a chemically non-aggressive environment, the immobilised surface has to withstand repeated injections of acids, bases or other disrupting solutions needed for regeneration of the ligand surface. Hemisuccinate conjugates were consequently avoided since the ester-bonds formed are susceptible to both alkaline and acid hydrolysis.

Maximum binding capacities ($R_{max}$), were around 10 000 RU or higher for most of the chip surfaces shown in Table 3, with the exception of the diazo-CBL and the HEX chip. The lower binding capacities of these surfaces were probably due to low recoveries of the non-optimised derivatisation reactions prior to immobilisation.

Typical calibration curves for five analytes in buffer are shown in Figure 12. Sensor chips for CBL, EEO and TB in combination with antibodies developed for conventional immunoassay, such as ELISA and RIA, produced assays with $IC_{50}$ values around 0.5 ng $ml^{-1}$ (Table 3). The sensitivity of these new biosensor inhibition assays compares well with current immunoassays, *e.g.* $IC_{50}$ values between 0.4 and 2.4 ng $ml^{-1}$ were demonstrated for a wide range of commercial ELISA kits for clenbuterol (Hahnau & Jülicher, 1996). Immobilisation of RCP and HEX will however need further optimisation. The RCP calibration curve was sensitive, but levelled out at 60% of b0, while the HEX calibration curve had an $IC_{50}$ value of 12 ng $ml^{-1}$.

The same epoxide reaction and linker (bisoxirane, BIS) was used for both antibody production (Elliott *et al*, 1998b) and surface immobilisation of RCP, while different reactions were used for HEX. During conjugate formation, unwanted epoxide dimers (BIS-RCP-BIS and BIS-HEX-BIS) were formed and immobilised on the chip surface. It is likely that RCP antibodies cross-reacted with these dimers, which affected the calibration curve. No such effect was seen for HEX, which could be taken as an indication of the advantage of using a complementary immobilisation strategy. Optimisation of ligand/BIS ratios during conjugate formation might both improve the calibration curve for the RCP surface and increase the surface capacity for HEX.

*Table 3.* Maximum binding capacity ($_{Rmax}$) and maximum response (b0) during assay conditions for different analyte specific surfaces. $R_{max}$ values are generally averages for the four flow cells of each chip and $IC_{50}$ values were calculated from standard curves with antibodies of dilutions and active antibody concentrations [Ab] indicated in the Table. Antibody dilutions for $R_{max}$ determinations ranged from 1/40 to 1/200.

| | | | | Assay | | | |
|---|---|---|---|---|---|---|---|
| Ligand | Immobilisation reagent | Ab | Max binding[1] $R_{max}$ (RU) | b0[2] (RU) | $IC_{50}$[3] (ng $ml^{-1}$) | [Ab][5] (nM) | Ab dil. |
| CBL | Diazotation | P-CBL-1 | 1 500 | 200 | 2.1 | 3.8 | 1/2000 |
| CBL | Thiophosgene | P-CBl-1 | 13 000 | 205 | 1.8 | 1.5 | 1/5000 |
| CBL | Thiophosgene | M-CBL | 13 000 | 145 | 0.45 | 1.1 | 1/40000 |
| EEO | Carboxy-methyl-oxime | P-EEO | 10 000 | 85 | 0.35 | 0.7 | 1/20000 |
| TB | Succinimidyl-carbonate | M-TB | 9 000 | 72 | 0.25 | 0.7 | 1/40000 |
| RCP | Bisoxirane | P-RCP | 10 000 | 412 | 0.35[4] | 4.0 | 1/400 |
| HEX | Bisoxirane | P-HEX | 1 500 | 347 | 12 | 3.5 | 1/400 |

[1]Maximum binding capacity ($R_{max}$), 20 min injection (flow rate 5 µl $min^{-1}$).
[2]Maximum assay response (b0), 2 min injection (flow rate 30 µl $min^{-1}$).
[3]The $IC_{50}$ was defined as the concentration of the inhibitor (analyte) required to reduce the signal in RU to half the signal measured with no inhibitor present (b0).
[4]The $IC_{50}$ value for RCP was calculated from the dynamic range of the curve (fig. 12)
[5]The concentration [Ab] refers to active antibody concentration as described earlier.

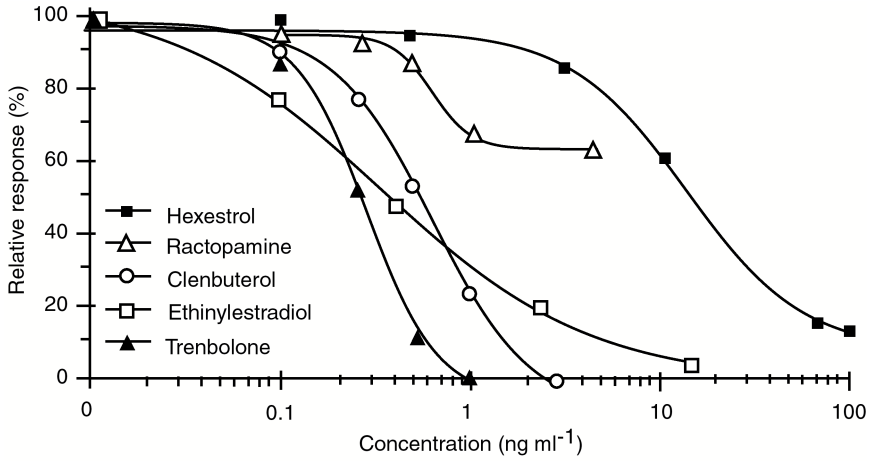


*Figure 12.* Buffer calibration curves from analyte specific surfaces of clenbuterol (CBL), ethinylestradiol (EEO), trenbolone (TB), ractopamine (RCP) and hexestrol (HEX). All calibration curves were normalised against b0 for each substance respectively. Data for assay response, and $IC_{50}$ values for standard curves, are presented in Table 3. For the CBL-curve, the thiophosgene chip was used.

In general, the prepared surfaces were stable during storage and prolonged use. The thiocyanate clenbuterol chip was used for over 5000 sample injections over a three-year period. Despite a response decrease of about 20% for a 1 nM antibody concentration the precision remained constant.

The running buffer used had a pH of 8.2 and a NaCl concentration of 500 mM compared to pH 7.4 and 150 mM NaCl in standard HBS-EP buffer (Biacore AB). The higher NaCl content was used to reduce non-specific binding of proteins to the chip surface, and the pH to operate at the response maximum of the antibody. There was no non-specific binding (NSB) from the two monoclonal antibodies used, while the polyclonal sera gave a constant non-specific binding of 5-10% of the total antibody response over the whole range of dilutions investigated.

Regeneration conditions required for complete regeneration of the chip surface were generally found to be harsher than the conditions for elution of analyte from affinity columns suggested by Tsang & Wilkins (1991). All antibodies tested were totally regenerated with a pulse of 100 mM NaOH with 20% acetonitrile. An alternative with less baseline disturbance was a two step regeneration with a 6 M guanidine-HCl solution at pH 1.5, followed by a pulse of 100 mM NaOH.

In the Biacore system flow rates of 1-100 µl $min^{-1}$ can be used. A flow rate of 30 µl $min^{-1}$ was chosen for the current assays due to maximum sensitivity and acceptable antibody consumption. It was found that an increase of flow rate from 10 to 30 µl $min^{-1}$ resulted in an increase of standard curve sensitivity with approximately a factor 2 or more, while a further increase of the flow up to a 100 µl $min^{-1}$ had no additional effect. In a study by Gaudin & Pavy (1999) an increase in flow rate from 5 to 20 µl $min^{-1}$ decreased the $IC_{50}$ value with a factor of 5 in buffer for their sulfamethazine assay. In milk however no such effect was observed.

Serial dilution of four antibodies spiked with CBL (0.5 ng $ml^{-1}$) indicated that it was not possible to dilute three of them enough to fully benefit from their high avidities due to the response/noise ratio, *i.e.* mass sensitivity limitations of the SPR-instrument, as further discussed in paper I.

One way to increase the response would be to enhance the signal by injecting a secondary antibody after the first sample injection. It was possible to enhance the signal from P-CBL-1 by 4-5 times through a subsequent injection of an affinity purified goat-anti-rabbit IgG antibody. The benefit was however limited by non-specific binding from the secondary antibody to the surface of about 15 RU. At the high concentration end of the standard curve the response from the primary antibody went down to and below the response for non-specific binding from the secondary antibody, causing increased variability between injections. Further work on the control of non-specific binding is needed if secondary antibodies are to be useful for improving assay sensitivity.

## Matrix effects in immunobiosensor determination of clenbuterol in urine and serum (paper II)

The ß-agonist assay was applied as a model to study matrix effects from urine and serum in residue analysis. Interferences with the antigen-antibody interaction, and non-specific binding (NSB) of matrix components to the sensor surface, were systematically studied. A full factorial design experiment was employed for evaluating the effects of assay buffer composition. The experiment showed that it was possible to run the assay at pH 8-10, using NaCl concentrations between 20 and 500 mM, and HEPES buffer concentrations between 10 and 100 mM, while pH 6 was less suitable due to major NSB from both urine samples and antibody.

For blood serum, ultrafiltration (UF) efficiently eliminated an otherwise high NSB of proteins to the sensor chip. NSB was also seen to varying degrees for urine, although to a much lesser extent than for serum. Using an assay buffer at pH 10 in combination with high salt concentration eliminated NSB for the vast majority of urine samples, while ultrafiltration was needed at pH 8. Addition of soluble dextran to the assay buffer reduced the NSB to some extent for serum, but had no effect on urine.

The urine matrix produced a substantial inhibition of the binding of antibodies to the sensor surface immobilised antigen in all the assays for ß-agonists and hormones developed in paper I. The inhibition from serum was smaller compared to urine, as shown for clenbuterol in Figure 13. Furthermore, the variation between individual samples analysed separately was considerably higher for urine (CV values for urine were approximately 35% compared to 1% for serum and buffer). Background correction, *e.g*. by using calibration curves made up in matrix, would therefore not be an efficient way of improving the assay for urine.

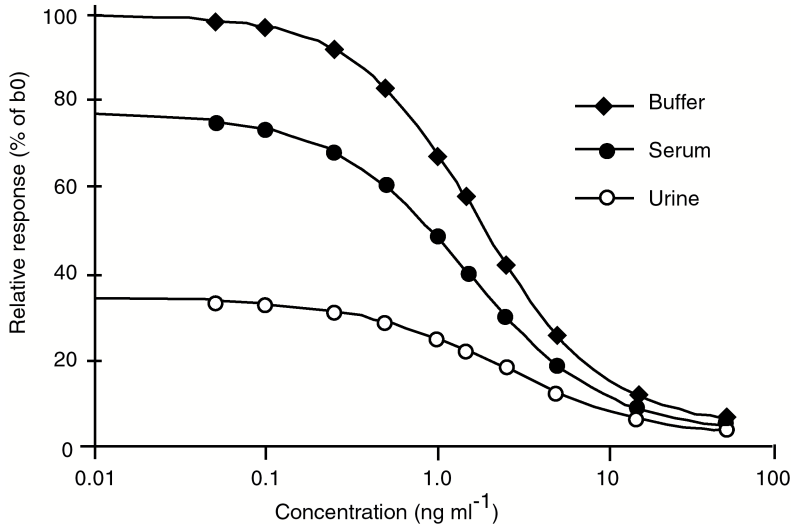


*Figure 13.* Clenbuterol calibration curves in buffer, bovine serum and urine. A polyclonal antibody (P-CBL-1) diluted 1/2000, corresponding to an active antibody concentration of 3.8 nM, was used. All results were normalised against max response in buffer (b0) and expressed as relative response (% of b0).

In addition to NSB, the buffer optimisation experiment was designed to study effects of the running buffer composition on the antigen-antibody interaction. Dark and light urine were used to represent the maximum sample variation, and clenbuterol spikes in both buffer and urine were analysed for each buffer combination according to Table 2. In urine small or no statistically significant systematic effects from the studied buffer composition parameters could be found, although a significant response optimum for the maximum response in buffer ("Max") was obtained at pH 8 with 500 nM NaCl. However, neither the inhibition effect from urine (as illustrated in Figure 13), nor the difference in response between dark and light urine pools, were substantially affected by buffer composition.

As expected, assay sensitivity increased markedly with higher antibody dilution in both buffer and urine, as seen by the steep slopes of the relative response plots for clenbuterol in Figure 14. In comparison, the background from urine matrix was close to constant. Background, expressed as concentration, decreased with higher antibody dilution, e.g. from 3.0 to 1.0 ng $ml^{-1}$, when antibody dilution was increased from 1/10 000 to 1/40 000. Sample dilution is commonly applied in fast assays (Sternesjö, Mellgren & Björck, 1995; Baxter *et al*., 1999) to reduce sample matrix background. In the current assay, background values for urine did instead increase with increased sample dilution as further discussed in paper II. In conclusion, dilution of the antibody increased the sensitivity of the assay and reduced urine background, while sample dilution had the opposite effect on the urine background.

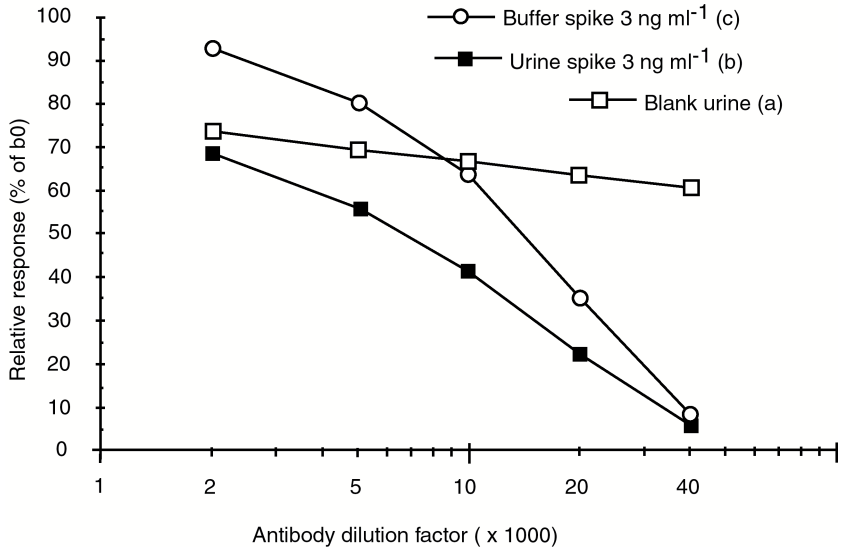


*Figure 14.* The effect of antibody dilution on background from urine. Antibody dilutions from 1/2000 to 1/40 000 corresponding to active antibody concentrations of 16 to 0.8 nM were used. Blank urine diluted 5 times (a), blank urine diluted 5 times and spiked with clenbuterol at 3 ng $ml^{-1}$ (b) and clenbuterol (3 ng $ml^{-1}$) standard in water (c) were mixed (1+1) with the respective antibody dilution. All results were normalised against max response in buffer (b0) for the respective antibody dilution and expressed as relative response (% of b0).

## Analysis of clenbuterol in bovine hair (paper III)

The aim was to explore the possibility of using an SPR-biosensor for fast screening analysis of clenbuterol in hair with a minimum of sample preparation. To validate the assay, a number of hair samples from animals treated with clenbuterol were analysed, and the results were compared with results from GC-MS analysis.

A fast extraction method using 100 mM NaOH was developed. Higher concentrations of NaOH were tested, but dissolved the hair completely, which increased the background for blank sample extracts markedly. Compared to the carbonate buffer extraction developed by Elliott, Crooks & McCaughey (1995), the recovery of clenbuterol from incurred samples was higher with the new extraction method. Non-specific binding (NSB) of about 400 RU from the hair extracts was reduced down to 5-50 RU by ultrafiltration.

Results were evaluated with and without enhancement as indicated in Figure 15. The sample was mixed with primary antibody (1+1) and injected, followed by a subsequent injection of a secondary antibody, enhancing the signal from the primary antibody about three times both in buffer and hair extract. Buffer and hair extract curves were of similar shape, but the maximum response (b0) in hair extract was approximately 10 % lower than in buffer. The maximum response (b0) of the standard curve was about 500 RU, and the dynamic range of the standard curve was about 0.4 to 14 ng $ml^{-1}$, corresponding to 10 and 350 ng $g^{-1}$ hair. With a higher antibody dilution lower concentrations could be detected, but the lower antibody-specific biosensor response would increase the risk for false negative results due to NSB from samples.

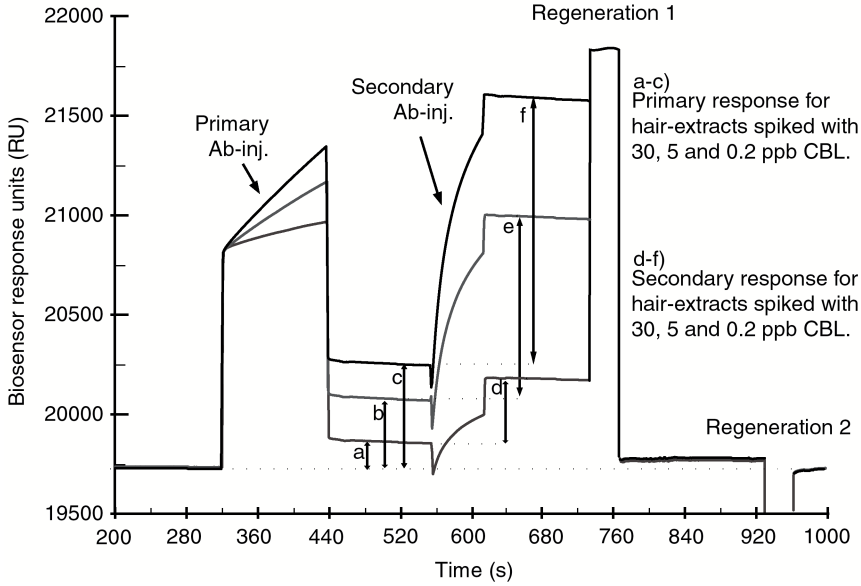


*Figure 15.* Overlay plot of 3 sensorgrams from injections of blank hair extracts spiked with clenbuterol. The concentrations are (a) 30 (b) 5 and (c) 0.2 ng $ml^{-1}$ corresponding to 375, 62.5 and 2.5 ng $g^{-1}$ hair, respectively. Recorded response values (RU) from primary and secondary antibody injections are indicated with arrows (a-c) and (d-f) respectively.

Detection limits (mean + 3SD) for the assay, calculated from seventeen GC-MS negative hair samples, were 10 ng $g^{-1}$, for hair with enhancement, and 15 ng $g^{-1}$, for hair without enhancement. The lower detection limit for enhancement is probably due to the fact that the secondary antibody injection is less influenced by non-specific binding from the hair matrix

Results from biosensor analysis of GC-MS positive hair samples are shown in Figure 16. The correlation between the BIA-method with enhancement and the GC-MS-method was 92.7% ($R^2$). Concentrations found with the biosensor method were generally lower than results from GC-MS analysis, which was probably due to lower extraction recovery for the new simplified extraction procedure developed for the BIA method. It is likely that a more rigorous solubilization of the hair tissue is needed for complete recovery from hair of drug treated animals. In the lower concentration range (up to 100 ng $g^{-1}$ hair), the BIA results were on the average 71 % of those obtained by GC-MS.

The sample capacity of the current method is approximately 48 samples a day. The detection limit of the new assay is comparable to other screening methods, reporting LOD values in the range 0.3 - 22 ng $g^{-1}$ (Gleixner, Sauerwein & Meyer, 1996; Haasnoot *et. al*., 1998; Appelgren *et al*., 1996; Elliott, Crooks & McCaughey, 1995; Godfrey *et al*., 1996). In contrast to most of the published methods in the area, no organic solvent or solid phase extraction step was needed.

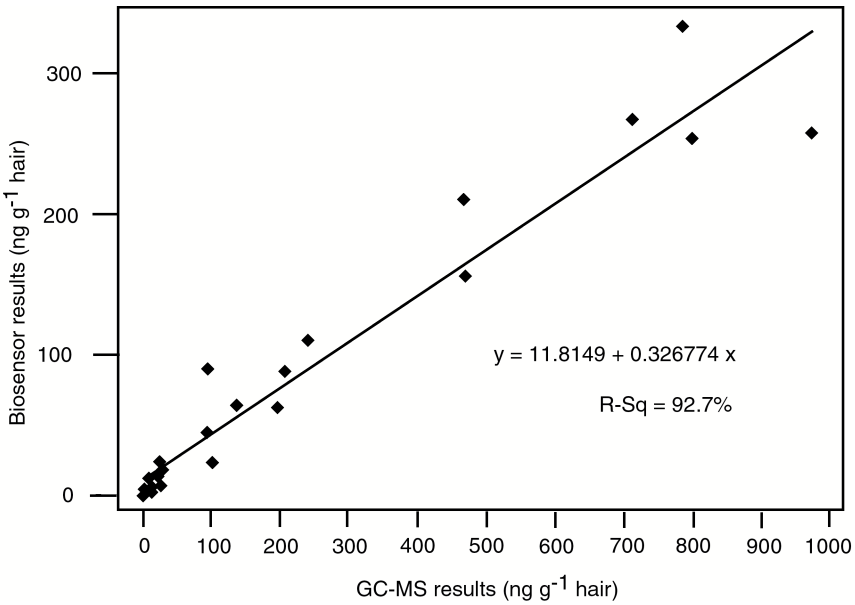


*Figure 16.* Clenbuterol concentrations obtained with the new biosensor method in comparison with results from the GC-MS method in hair from clenbuterol treated animals (n=22).

## Immunobiosensor determination of ß-agonists in urine using integrated immunofiltration clean-up (paper IV)

A fast immunobiosensor method for screening of ß-agonists in urine using integrated immunofiltration (IIF) clean-up is described. The aim was to develop a fast clean-up method suitable for analysis of both CBL and SBL type of ß-agonists without the use of organic solvents. Two antibodies with different cross-reactivities for clenbuterol and salbutamol were used to study optimal conditions for IIF.

In IIF the sample is mixed with antibody in an ultra-filtration device (cut off 30 kD) and after a short incubation and centrifugation the filter is washed with buffer. The antibodies trapped on the filter are recovered and the unliganded fraction is detected directly in the biosensor immunoassay with clenbuterol immobilised on the sensor surface. The principles of IIF clean-up are further described in Figure 12.

A final antibody dilution of 1/40 000 (equivalent to an active antibody concentration of 0.85 and 1.1 nM for M-CBL and P-SBL respectively) was used to obtain a working range of 0.2 to 5 $ng^{-1}$ ml urine. Since the same antibody aliquot is used for clean-up and biosensor analysis, the capturing capacity of analyte in the clean-up step is limited by the degree of antibody dilution required to get a calibration curve with the chosen working range in the biosensor assay. Thus, by increasing the antibody concentration, more analyte can be recovered during IIF but the assay sensitivity is at the same time decreased.

Direct and IIF calibration curves for clenbuterol and salbutamol in the buffer and urine pools for two different antibodies are shown in Figure 17. IIF-curves for both antibodies were somewhat more flat than the corresponding “external” CBL or SBL standard buffer curve, indicating increased loss of analyte at higher concentrations. A higher loss at high analyte concentrations is explained by the limited capturing capacity discussed above. IIF-curves in buffer were generally more sensitive than the corresponding urine curves, indicating additional loss of analyte due to interference from urine on the antibody-antigen interaction.

There were however some differences between the two antibodies during IIF. As seen in Figure 17a, IIF clean-up caused relatively small losses of CBL in buffer and urine when the monoclonal antibody M-CBL was used. Corresponding IIF-curves for SBL in urine and buffer were however much less sensitive, levelling out at 80% and 65% binding respectively. Thus, IIF with this antibody gave a clean-up procedure with a rather high selectivity for clenbuterol.

By comparison, the IIF-curves for the polyclonal P-SBL were much more similar (Figure 17b). There was no or very little difference between the two ß-agonists, although the cross-reactivity rate of the antibody measured direct in buffer in the instrument was 131% for SBL as compared to CBL. The two buffer curves were somewhat more sensitive than the corresponding urine curves, but an analyte concentration of 10 $ng^{-1}$ ml gave an inhibition down to about 50% for both. Since the aim was to develop an assay capable of detecting several ß-agonists, the P-SBL antibody was chosen over the M-CBL for further studies.

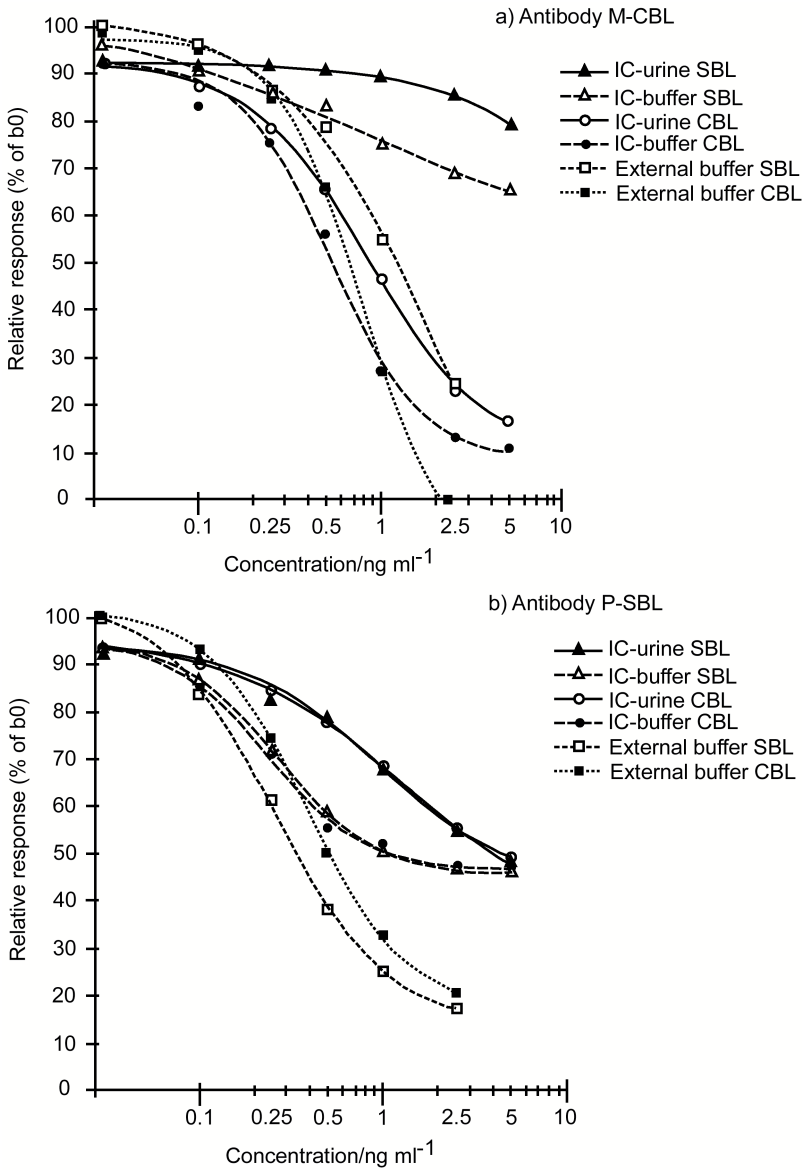


*Figure 17.* Clenbuterol (CBL) and salbutamol (SBL) calibration curves direct in buffer (External buffer) and after sample clean-up with integrated immunofiltration (IIF) in buffer (IIF-buffer) and urine pool (IIF-urine) for a) antibody M-CBL and b) antibody P-SBL, respectively. All concentrations are in final solution (for concentrations in urine multiply by dilution factor 2).

From the results above, it is obvious that quantitative measurements would require the use of an IIF calibration curve in urine. However, if the intended use of the method is screening of high numbers of samples for the presence of banned compounds only, quantitation is not needed. In order to maximise the sample capacity the calibration curve can therefore be excluded or be exchanged for a direct "external" buffer standard curve when the assay is used in routine control.

To validate the assay, 20 reference bovine urine samples were analysed blank and spiked with 1 ng $ml^{-1}$ of clenbuterol. The good result of IIF clean-up on sample-to-sample variation and overall sample matrix background is illustrated in Figure 18. Response values for IIF-treated versus untreated urine increased from 60 ± 17.1% to 91.2 ± 3.5% of b0 resulting in a decision limit CCα (mean of blanks - 2.33 SD) of 83.0 %, corresponding to 0.29 ng $ml^{-1}$ in urine. In conclusion, it was possible to use a decision limit corresponding to a false positive rate as low as 1% (α-error), while at the same time having a false negative rate (ß-error) of < 1% at the 1 ng $ml^{-1}$ level. Since the EU-directive (EC, 2002) requires the ß-error to be ≤ 5% for screening methods, the CCß of the assay is well below 1 ng $ml^{-1}$.

During 2002 and 2003, a total of 348 individual bovine urine samples were screened at the 1 ng $ml^{-1}$ level within the Swedish national residues control programme. None of these samples tested positive. In addition, eight urine samples incurred with clenbuterol, and one with salbutamol were analysed. The concentrations of the CBL-incurred samples determined with GC-MS were 0.17, 0.72, 1.0, 1.2, 1.8, 3.5, 5.2 and 14 ng $ml^{-1}$ respectively, while the SBL sample had a concentration of 1.0 ng $ml^{-1}$. Using CCα as the cut-off gave only one false negative sample (0.17 ng $ml^{-1}$). Results from these incurred samples, and samples spiked at concentrations of 1 and 0.5 ng $ml^{-1}$ respectively, indicate that the method is able to detect salbutamol at 0.5 ng $ml^{-1}$, and clenbuterol, brombuterol and mabuterol at somewhere between 0.5 and 1 ng $ml^{-1}$, as further discussed in paper IV. The sample throughput capacity was at least 48 samples per person and working day.

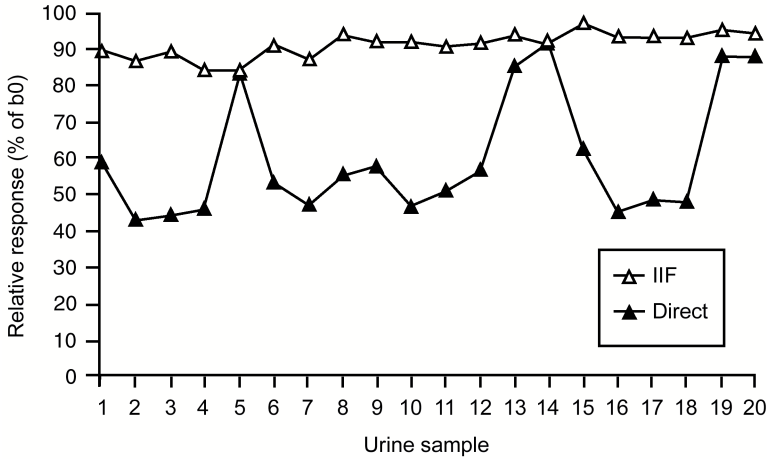


*Figure 18.* Relative response values expressed as % of b0 obtained from 20 reference blank bovine urine samples using the P-SBL antibody for (a) direct BIA analysis of undiluted samples and (b) after integrated immunofiltration (IIF).

# Conclusions and future research

This thesis shows that the immunobiosensor can be applied for fast and sensitive detection screening analysis for various illegal drugs. Sensor chip surfaces, prepared by more or less modified standard procedures for hapten synthesis, resulted in assays with $IC_{50}$ values of around 0.5 ng $ml^{-1}$ for clenbuterol, ethinylestradiol and trenbolone.

Routine assays for clenbuterol in bovine hair and urine were developed and validated. For hair, a sandwich assay format was used. A rapid and simple extraction procedure using 100 mM NaOH was developed, and the assay was validated by analysis of hair samples from animals treated with clenbuterol. The critical factor for assay sensitivity was non-specific binding to the sensor chip surface from hair extracts, which was controlled by ultrafiltration of samples and a secondary antibody. The LOD of the hair assay was 10 ng $ml^{-1}$.

For the urine assay, a novel clean-up procedure using integrated immunofiltration was developed. The clean-up efficiently reduced urine matrix disturbances from non-specific binding to the surface and interference with the antigen-antibody interaction. The new procedure enabled detection of several ß-agonists, including clenbuterol, salbutamol, mabuterol and brombuterol, at concentrations below 1 ng $ml^{-1}$. At least 48 samples could be processed and analysed by one person in one working day.

The major limiting factor for biosensor analysis of residues is interference from the sample matrix. To date, sample clean-up is still needed for most biosensor methods developed for residue analysis. A future goal would be to develop assays with no need for sample clean-up. Improvements of assay sensitivity might also be needed to meet future regulatory requirements. The use of receptors or genetically designed binding biomolecules, possibly in combination with multi-spot or multi-channel instruments, would enable high sample throughput and multi analyte detection. Another intriguing possibility of receptor-based assays is that the response may be directly linked to a specific biological, *i.e.* toxic, effect.

# References


Alcocer, M. J., Dillon, P. P., Manning, B. M., Doyen, C., Lee, H. A., Daly, S. J., O'Kennedy, R. & Morgan, M. R. (2000) Use of phosphonic acid as a generic hapten in the production of broad specificity anti-organophosphate pesticide antibody. *Journal of Agricultural and Food Chemistry*, 48, 2228-2233.

Andersson, K., Hämäläinen, M. & Malmqvist, M. (1999) Identification and optimization of regeneration conditions for affinity-based biosensor assays. A multivariate cocktail approach. *Analytical Chemistry*, 71, 2475-2481.

Appelgren, L. E., Bondesson, U., Fredriksson, E., Ingvast Larsson, C. & Jansson, D. S. (1996) Analysis of hair samples for clenbuterol in calves. *Fleischwirtschaft*, 76, 398-399.

Baird, C. L. & Myszka, D. G. (2001) Current and emerging commercial optical biosensors. *Journal of Molecular Recognition*, 14, 261-268.

Baker, P. K., Dalrymple, R. H., Ingle, D. L. & Ricks, C. A. (1984) Use of a beta-adrenergic agonist to alter muscle and fat deposition in lambs. *Journal of Animal Science*, 59, 1256-1261.

Baxter, G. A., O'Connor, M. C., Haughey, S. A., Crooks, S. R. H. & Elliott, C. T. (1999) Evaluation of an immunobiosensor for the on-site testing of veterinary drug residues at an abbatoir. Screening for sulfametazin in pigs. *Analyst*, 124, 1315-1318.

Baxter, G. A., Ferguson, J. P., O'Connor, M. C. & Elliott, C. T. (2001) Detection of streptomycin residues in whole milk using an optical immunobiosensor. *Journal of Agricultural and Food Chemistry*, 49, 3204-3207.

Beermann, D. H., Butler, W. R., Hogue, D. E., Fishell, V. K., Dalrymple, R. H., Ricks, C. A. & Scanes, C. G. (1987) Cimaterol-induced muscle hypertrophy and altered endocrine status in lambs. *Journal of Animal Science*, 65, 1514-1524.

Bergström, C. (1998) Design and use of optical biosensor assays in analysis of antimicrobial drug residues in milk. Doctoral thesis, Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Bergström, C., Sternesjö, Å., Bjurling, P. & Löfås, S. (1999) Design and use of a general capturing surface in optical biosensor analysis of sulphamethazine in milk. *Food and Agricultural Immunology*, 11, 329-338.

Bilitewski U. (2000) Can affinity sensors be used to detect food contaminants? *Analytical Chemistry*, 1, 692A-701A.

Bjurling, P., Baxter, G. A., Caselunghe, M., Jonson, C., O'Connor, M., Persson, B., & Elliott, C. T. (2000) Biosensor assay of sulfadiazine and sulfametazine residues in pork. *Analyst*, 125, 1771-1774.

Blanchflower, W. J., Hewitt, S. A., Cannavan, A., Elliott, C. T. & Kennedy, D. G. (1993) Detection of clenbuterol residues in bovine liver, muscle, retina and urine using gas chromatography/mass spectrometry. *Biological Mass Spectrometry*, 22, 326-330.

Bokken, G. C., Corbee, R. J., van Knapen, F. & Bergwerff, A. A. (2003) Immunochemical detection of Salmonella group B, D and E using an optical surface plasmon resonance biosensor. *FEMS microbiology letters*, 222, 75-82.

Boström Caselunghe, M. & Lindeberg J. (2000) Biosensor-based determination of folic acid in fortified food. *Food Chemistry*, 70, 523-532.

Boyd, D., O'Keeffe, M. & Smyth, M. R. (1994) Matrix solid-phase dispersion as a multiresidue extraction technique for beta-agonists in bovine liver tissue. *Analyst*, 119, 1467-1470.

Boyd, D., O'Keeffe, M. & Smyth, M. R. (1996) Methods for the determination of beta-agonists in biological matrices. A review. *Analyst*, 121, 1R-10R.

Caelen, I., Kalman, A. & Wahlström, L. (2004) Biosensor-based determination of riboflavin in milk. *Analytical Chemistry*, 76, 137-143.

Collins S., O'Keeffe M.& Smyth M. R. (1994) Multi-residue analysis for beta-agonists in urine and liver samples using mixed phase columns with determination by radioimmunoassay. *Analyst*, 119, 2671-2674.

Crooks, S. R. H., Baxter, G. A., O'Connor, M. C. & Elliot, C. T. (1999) Immunobiosensor – an alternative to enzyme immunoassay screening for residues of two sulfonamides in pigs. *Analyst*, 23, 2755-2757.

Crooks, S. R. H., Stenberg, E., Johansson, M. A., Hellenäs, K-E., & Elliott, C. T. (2001) Optical biosensor for high-throughput detection of veterinary drug residues in foods. *Proceedings of SPIE-The International Society for Optical Engineering, 4206 (Photonic Detection and Intervention Technologies for Safe Food)*, 123-130.

Crooks, S. R. H., McCarney B., Traynor, I. M., Thompson, C. S., Floyd, S. & Elliott C. T. (2003) Detection of levamisole residues in bovine liver and milk by immunobiosensor. *Analytica Chimica Acta*, 483, 181-186.

Cunningham, B. C. & Wells, J. A. (1993) Comparison of a structural and a functional epitope. *Journal of Molecular Biology*, 234, 554-563. Erratum in: *Journal of Molecular Biology*, 1994, 237, 513.

Dalrymple, R. H., Baker, P. K., Gingher, P. E., Ingle, D. L., Pensack, J. M. & Ricks, C. A. (1984) A repartitioning agent to improve performance and carcass composition of broilers. *Poultry Science*, 63, 2376-2383.

Daly, S. J., Keating, G. J., Dillon, P. P., Manning B. M., O'Kennedy, R., Lee, H. A. & Morgan, M. R. (2000) Development of surface plasmon resonance-based immunoassay for aflatoxin B(1). *Journal of Agricultural and Food Chemistry*. 48, 5097-5104.

De Wasch, K., De Brabander, H. & Courtheyn, D. (1998) LC-MS-MS to detect and identify four beta-agonists and quantify clenbuterol in liver. *Analyst*, 123, 2701-2705.

Degand, G., Bernes-Duykaerts, A. & Maghuin-Rogister, G. (1992) Determination of clenbuterol in bovine tissue and urine by enzyme immunoassay. *Journal of Agricultural and Food Chemistry*, 40, 70-75.

Delahaut, P., Dubois, M., Pri-Bar, I., Buchman, O., Degand, G. & Ectors, F. (1991) Development of a specific radioimmunoassay for the detection of clenbuterol residues in treated cattle. *Food Additives & Contaminants*, 8, 43-53.

Dürsch, I., Meyer, H. H. & Karg, H. (1995) Accumulation of the beta-agonist clenbuterol by pigmented tissues in rat eye and hair of veal calves. *Journal of Animal Science*, 73, 2050-2053.

EC (1996a) Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of ß-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC, *Official Journal of the European Communities*, L125, 3-9.

EC (1996b) Council Directive 96/23/EEC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC, *Official Journal of the European Communities*, L125, 10-32.

EC (2000) Commission Regulation (EC) No 2391/2000 of 27 October 2000 amending Annexes I, II and III to Council Regulation (EEC) No 2377/90 laying down Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal of the European Communities*, L276, 5-8.

EC (2002) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Official Journal of the European Communities*, L221, 8-36.

EC (2003) Directive 2003/74/EC of the European Parliament and of the Council of 22 September 2003 amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists, *Official Journal of the European Union*, L262, 17-21.

Elliott, C. T., McCaughey, W. J. & Shortt; H. D. (1993) Residues of the beta-agonist clenbuterol in tissues of medicated farm animals. *Food Additives & Contaminants*, 10, 231-244.

Elliott, C. T. (1995) Detection and pharmacokinetics of beta-agonists. Ph D Thesis, Queens University of Belfast.

Elliott, C. T., Crooks, S. R. H. & McCaughey W. J. (1995) Development of a rapid screening test to detect beta-agonist residues in bovine eye and hair. *Veterinary Record*, 137, 643-644.

Elliott, C. T., Thompson, C. S., Arts, C. J., Crooks, S. R., van Baak, M. J., Verheij, E. R. & Baxter, G. A. (1998a) Screening and confirmatory determination of ractopamine residues in calves treated with growth promoting doses of the beta-agonist. *Analyst*, 123, 1103-1107.

Elliott, C. T., Baxter, G. A., Hewitt, S. A., Arts, C. J. M., van Baak, M., Hellenäs, K-E. & Johannson, A. (1998b) Use of biosensors for rapid drug residue analysis without sample deconjugation or clean-up: a possible way forward. *Analyst*, 123, 2469-2473.

Elliott, C. T., Baxter, G. A., Crooks, S. R. H. & McCaughey, W. J. (1999) The development of a rapid immunobiosensor screening method for the detection of residues of sulphadiazine. *Food and Agricultural Immunology*, 11, 19-27.

Ferguson, J. P., Baxter, G. A., McEvoy, J. D. G., Stead, S., Rawlings, E. & Sharman, M. (2002) Detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using an optical biosensor. *Analyst*, 127, 951-956.

Feriotto, G., Borgatti, M., Mischiati, C., Bianchi, N. & Gambari, R. (2002) Biosensor technology and surface plasmon resonance for real-time detection of genetically modified Roundup Ready soybean gene sequences. *Journal of Agricultural and Food Chemistry*, 50, 955-962.

Feriotto, G., Gardenghi, S., Bianchi, N. & Gambari, R. (2003) Quantitation of Bt-176 maize genomic sequences by surface plasmon resonance-based biospecific interaction. *Journal of Agricultural and Food Chemistry*, 51, 4640-4646.

Fratamico, P. M., Strobaugh, T. P., Medina, M. B. & Gehring, A. G. (1998) Detection of Escherichia coli O157:H7 using a surface plasmon resonance biosensor. *Biotechnology Techniques*, 12, 571-576.

Gaillard, Y., Balland, A., Doucet, F. & Pepin, G. (1997) Detection of illegal clenbuterol use in calves using hair analysis. Application in meat quality control. *Journal of Chromatography. B, Biomedical Sciences and Applications*, 703, 85-95.

Gaudin, V. & Pavy, M. L. (1999) Determination of sulfamethazine in milk by biosensor immunoassay, *Journal of AOAC International*, 82, 1316-1320.

Gaudin, V., Fontaine, J.& Maris, P. (2001) Screening of penicillin residues in milk by surface plasmon resonance based biosensor assay; comparison of chemical and enzymatic sample pre-treatment. *Analytica Chimica Acta*, 436, 191-198.

Gaudin, V. & Maris, P. (2001) Development of a biosensor-based immunoassay for screening of chloramphenicol residues in milk. *Food and Agricultural Immunology*, 13, 77-86.

Gillis, E. H., Gosling, J. P., Sreenan, J. M. & Kane, M. (2002) Development and validation of a biosensor-based immunoassay for progesterone in bovine milk. *Journal of Immunological Methods*, 267, 131-138.

Gleixner, A., Sauerwein, H. & Meyer, H. H. D. (1996) Accumulation of the b2-Adrenoreceptor clenbuterol in calf hair of different pigmentation, *Archiv für Lebensmittelhygiene*, 47, 131-135.

Godfrey, M. A. J., Andersson, P. L., Jülicher, B., Kwasowski, P., & Sauer, M. J. (1996) Detection in cattle hair by IAC/cELISA, *Proceedings of the Euro Residue III conference, Veldhoven May 6-8, 1996*, Haagsma, N. & Ruiter, A. (Eds.), University of Utrecht, Faculty of Veterinary medicin, Utrecht, pp. 421-425.

Guggisberg, D., Widmer, T. & Koch, H. (2001) Detection of clenbuterol in urine of meat stock by biosensor BIACORE Q. *Mitteilungen aus Lebensmittel-untersuchung und Hygiene*, 92, 260-276.

Guidi, A., Laricchia-Robbio, L., Gianfaldoni, D., Revoltella, R. & del Bono, G. (2001) Comparison of a conventional immunoassay (ELISA) with a surface plasmon resonance-based biosensor for IGF-1 detection in cows' milk. *Biosensors and Bioelectronics*, 16, 971-977.

Gustavsson, E. (2003) SPR biosensor analysis of ß-lactam antibiotics in milk. Development and use of assays based on a ß-lactam receptor protein. Doctoral thesis, Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Gustavsson, E., Bjurling, P., Degelaen, J. & Sternesjö, A. (2002) Analysis of ß-lactam antibiotics using microbial receptor protein-based biosensor assay. *Food and Agricultural Immunology*, 14, 121-131.

Gustavsson, E., Bjurling, P. & Sternesjö, A. (2002) Biosensor analysis of penicillin G in milk based on the inhibition of carboxypeptidase activity. *Analytica Chimica Acta*, 468, 153-159.

Haaland, P. D. (1989) Experimental design in biotechnology, Marcel Dekker, New York, p. 91 and p. 197.

Haasnoot, W., Cazemier, G., Stouten, P. & Kemmers-Voncken, A. (1996). In *Immunoassays for residue analysis - food safety*, Immunochemical approaches to the analysis of ß-agonistic drugs. Beier, R. C. & Stanker, L. H. (Eds.), American Chemical Society, Washington DC, pp. 60-73.

Haasnoot, W., Stouten, P., Schilt, R. & Hooijerink, D. (1998) A fast immunoassay for the screening of beta-agonists in hair, *Analyst*, 123, 2707-2710.

Haasnoot, W. & Verheijen, R. (2001) A direct (non-competitive) immunoassay for gentamicin residues with an optical biosensor. *Food and Agricultural Immunology*, 13, 131-134.

Haasnoot, W., Olieman, K., Cazemier, G. & Verheijen, R. (2001) Direct immunobiosensor assays for the detection of nonmilk proteins in milk powder. *Journal of Agricultural and Food Chemistry*, 49, 5201-5206.

Haasnoot, W., Kemmers-Voncken, A. & Samson, D. (2002) Immunofiltration as sample clean up for the immunochemical detection of ß-agonists in urine. *Analyst*, 127, 87-92.

Haasnoot, W., Loomans, E., Cazemier, G., Dietrich, R., Verheijen, R., Bergwerff, A. A. & Stephany, R. W. (2002) Direct versus competitive biosensor immunoassays for the detection of (dihydro)streptomycin residues in milk. *Food and Agricultural Immunology*, 14, 15-27.

Haasnoot, W., Bienenmann-Ploum, M., & Kohen, F. (2003) Biosensor Immunoassay for detection of eight sulfonamides in chicken serum. *Analytica Chimica Acta*, 483, 171-180.

Haasnoot, W., Cazemier, G., Koets, M. & van Amerongen, A. (2003) Single biosensor immunoassay for the detection of five aminoglycosides in reconstituted skimmed milk. *Analytica Chimica Acta*, 488, 53-60.

Hahnau, S. & Jülicher, B. (1996) Evaluation of commercially available ELISA test kits for the detection of clenbuterol and other ß-agonists. *Food Additives and Contaminants*, 13, 259-274.

Haughey, S. A., Baxter, G. A., Elliott, C. T., Persson, B., Jonson, C. & Bjurling P. (2001) Determination of clenbuterol residues in bovine urine by optical immunobiosensor assay. *The Journal of AOAC International*, 84, 4, 1025-1030.

Heitzman, R. J. (1993) Coordination of information on residues of veterinary drugs in the European Community. *Analytica Chimica Acta*, 275, 17-22.

Indyk, H. E., Evans, E. A., Boström Caselunghe, M. C., Persson, B. S., Finglas, P. M., Woollard, D. C. & Filonzi, E. L. (2000) Determination of biotin and folate in infant formula and milk by optical biosensor-based immunoassay. *Journal of AOAC International*, 83, 1141-1148.

Indyk, H. E. & Filonzi, E. L. (2003) Determination of immunoglobulin G in bovine colostrum and milk by direct biosensor SPR-immunoassay. *Journal of AOAC International*, 86, 386-393.

Indyk, H. E., Persson, B. S., Caselunghe, M. C. B., Moberg, A., Filonzi, E. L. & Woollard, D. C. (2002) Determination of vitamin B12 in milk products and selected foods by optical biosensor protein-binding assay: method comparison. *Journal of AOAC International*, 85, 72-81.

Jefferis, R. & Deverill, I. (1991) In *Principles and practice of Immunoassay*, Price, C. P. & Newman, D. J. (Eds.), Stockton Press, New York, pp. 1-18.

Johnsson, B., Löfås, S. & Lindquist, G. (1991) Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Analytical Biochemistry*, 198, 268-277.

Johnsson, L., Baxter, G. A., Crooks, S. R. H., Brandon, D. L. & Elliott, C. T. (2002) Reduction of sample matrix effects – The analysis of benzimidazole residues in serum by immunobiosensor. *Food and Agricultural Immunology*, 14, 209-216.

Jones, R. W., Easter, R. A., McKeith, F. K., Dalrymple, R. H., Maddock, H. M. & Bechtel, P. J. (1985) Effect of the beta-adrenergic agonist cimaterol (CL 263,780) on the growth and carcass characteristics of finishing swine. *Journal of Animal Science*, 61, 905-913.

Jongerius-Gortemaker, B. G. M., Goverde, R. L. J., van Knapen, F. & Bergwerff, A. A. (2002) Surface plasmon resonance (BIACORE) detection of serum antibodies against Salmonella enteritidis and Salmonella typhimurium. *Journal of Immunological Methods*, 266, 33-44.

Jonsson, H., Malmheden-Yman, I. & Hellenäs, K-E. (2001) Optimizing assay conditions in the detection of food allergens with Biacore's SPR technology. *Biacore Journal*, 1, 16-18.

Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H. & Ronnberg, I. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques*, 11, 620-627.

Karlsson, R., Fägerstam, L., Nilshans, H. & Persson, B. (1993) Analysis of active antibody concentration. Separation of affinity and concentration parameters. *Journal of Immunological Methods*, 166, 75-84.

Karlsson, R & Fält, A. (1997) Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *Journal of Immunological Methods*, 200, 121-133.

Knecht, B. G. Strasser, A. Dietrich, R. Märtlbauer, E. Niessner, R. & Weller, M. G. (2004) Automated microarray system for the simultaneous detection of antibiotics in milk. *Analytical Chemistry*, 76, 646-654.

Kress-Rogers, E. (1997) In *Handbook of biosensors and electronic noses – Medicine, food, and the environment*, Kress-Rogers, E. (Ed), CRC Press Inc., Frankfurt, pp. 3-39.

Kubitschko, S., Spinke, J., Bruckner. T., Pohl, S. & Oranth, N. (1997) Sensitivity enhancement of optical immunosensors with nanoparticles. *Analytical Biochemistry*, 253, 112-122.

Kuiper, H. A., Noordam, M. Y., van Dooren-Flipsen, M. M. H., Schilt, R. & Roos, A. H. (1998). Illegal use of beta-adrenergic agonists: European Community. *Journal of Animal Science*, 76, 195-207.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T. & O'Kennedy, R. (2003) Advances in biosensors for detection of pathogens in food and water. *Enzyme and Microbiological Technology*, 32, 3-13.

Leonard, P., Hearty, S., Quinn, J. & O'Kennedy, R. (2004) A generic approach for the detection of whole Listeria monocytogenes cells in contaminated samples using surface plasmon resonance. *Biosensors & Bioelectronics*, 19, 1331-1335.

Liedberg, B., Nylander, C. & Lundström, I. (1983) Surface plasmon resonance for gas detection and biosensing. *Sensors & Actuators*, 4, 299-304.

Liedberg, B., Lundström, I., & Stenberg, E. (1993) Biosensing with an extended coupling matrix and surface plasmon resonance. *Sensors & Actuators B*, 11, 63-72.

Liedberg, B., Nylander, C. & Lundström, I. (1995) Biosensing with surface plasmon resonance – how it all started. *Biosensors & Bioelectronics*, 10, i-ix.

Lommen, A., Haasnoot, W. & Weseman, J. M. (1995) Nuclear magnetic resonance controlled method for coupling of fenoterol to a carrier and enzyme. *Food and Agricultural Immunology*, 7, 123-129.

Löfås, S. (1995) Dextran modified self-assembled monolayer surfaces for use in biointeraction analysis with surface plasmon resonance. *Pure and applied chemistry*, 67, 829-834.

Löfås, S., Johnsson, B., Edström, Å., Hansson, A., Lindquist, G., Müller Hillgren, R. & Stigh, L. (1995) Methods for site controlled coupling to carboxymethyldextran surfaces in surface plasmon resonance sensors. *Biosensors & Bioelectronics*, 10, 813-822.

Maistro, S., Chiesa, E., Angeletti, R.& Brambilla, G. (1995) Beta blockers to prevent clenbuterol poisoning. *Lancet*, 346, 180.

Marco, M. P., Gee, S. & Hammock, B. D. (1995) Immunochemical techniques for environmental analysis I. Immunosensors. *Trends in Analytical Chemistry*, 14, 341-350.
Mariotti, E., Minunni, M. & Mascini, M. (2002) Surface plasmon resonance biosensor for genetically modified organisms detection. *Analytica Chimica Acta*, 453, 165-172.
Martinez-Navarro, J. F. (1990) Food poisoning related to consumption of illicit beta-agonist in liver. *Lancet*, 336, 1311.
McCarney, B., Traynor, I. M., Fodey, T. L., Crooks, S. R. H. & Elliott, C. T. (2003) Surface plasmon resonance biosensor screening of poultry liver and eggs for nicarbazin residues. *Analytica Chimica Acta*, 483, 165-169.
Medina, M. B. (1997) Hygromycin B antibody production and characterization by a surface plasmon resonance biosensor. *Journal of Agricultural and Food Chemistry*, 45, 389-394.
Medina, M. B. (2001) Binding of collagen I to Escherichia coli O157:H7 and inhibition by carrageenans. *International Journal of Food Microbiology*, 69, 199-208.
Medina, M. B. (2002) Biosensor studies of the binding of extracellular matrix components with immobilized Escherichia coli O157:H7 and inhibition by polysulfated polysaccharides. *Biotechnology letters*, 24, 77-84.
Medina, M. B. (2003) Detection of staphylococcal enterotoxin B (SEB) with surface plasmon resonance biosensor. *Microbiology*, 11, 225-243.
Mellgren, C. & Sternesjö, Å. (1998) Optical immunobiosensor assay for determining enrofloxacin and ciprofloxacin in bovine milk. *Journal of AOAC International*, 81, 394-397.
Mello, L. D. & Kubota, L. T. (2002) Review of the use of biosensors as analytical tools in the food and drink industries. *Food Chemistry*, 77, 237-256.
Meyer, H. H. D. & Rinke, L. M. (1991) The pharmacokinetics and residues of clenbuterol in veal calves. *Journal of Animal Science*, 69, 4538-4544.
Meyer, H. H. D. (2001) Biochemistry and physiology of anabolic hormones used for improvement of meat production. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, 109, 1-8.
Miller, M. F., Garcia, D. K., Coleman, M. E., Ekeren, P. A., Lunt, D. K., Wagner, K. A., Procknor, M., Welsh, T. H. Jr. & Smith, S. B. (1988) Adipose tissue, longissimus muscle and anterior pituitary growth and function in clenbuterol-fed heifers. *Journal of Animal Science*, 66, 12-20.
Minunni, M. & Mascini, M. (1993) Detection of pesticide in drinking water using real-time biospecific interaction analysis (BIA). *Analytical Letters*, 26, 1441-1460.
Miron, T. & Wilchek, M. (1993) A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. *Bioconjugate Chemistry*, 4, 568-569.
Mitchell, G. A. & Dunnavan, G. (1998) Illegal use of beta-adrenergic agonists in the United States. *Journal of Animal Science*, 76, 208-211.
Morgan, D. J. (1990) Clinical pharmacokinetics of beta-agonists. *Clinical Pharmacokinetics*, 18, 270-294.
Mulchandani, A. & Bassi, A. S. (1995) Principles and applications of biosensors for bioprocess monitoring and control. *Critical Reviews in Biotechnology*, 15, 105-124.
Muller-Renaud, S. P., Dupont, D. & Dulieu, P. (2004) Quantification of beta-casein in milk and cheese using an optical immunosensor. *Journal of Agricultural and Food Chemistry*, 52, 659-664.
Mullett, W. M., Lai, E. P. & Yeung, J. M. (2000) Surface plasmon resonance-based immunoassays. *Methods*, 22, 77-91.
Myszka, D. G. (1997) Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Current Opinion in Biotechnology*, 8, 50-57.
Myszka D. G. (1999) Survey of the 1998 optical biosensor literature. *Journal of Molecular Recognition*, 12, 390-408.
Nausch, I. & Galley, K. (1997) Current clean-up procedures for the determination of ß-agonists in bovine urine by enzyme immuno assay (EIA). *Archiv für Lebensmittelhygiene*, 48, 56-58.

Nedelkov, D. & Nelson, R. W. (2003) Detection of staphylococcal enterotoxin B via Biomolecular interaction analysis mass spectrometry. *Applied and Environmental Microbiology*, 69, 5212-5215.
Newman, D. J., Olabiran, Y. & Price, C. P. (1997) In *Handbook of biosensors and electronic noses – Medicine, food, and the environment*, Kress-Rogers, E. (Ed.), CRC Press, Inc., Frankfurt, pp. 59-89.
Nygren, L., Sternesjö A. & Björck, L. (2003) Determination of folate-binding proteins from milk by optical biosensor analysis. *International Dairy Journal*, 13, 283-290.
O'Shannessy, D. J., Brigham-Burke, M. & Peck, K. (1992) Immobilization chemistries suitable for use in the BIAcore surface plasmon resonance detector. *Analytical Biochemistry*, 205, 132-136.
Orr, R. (1999) Growth-promoting hormones in cattle. *Food safety network,* (http://www.foodsafetynetwork.ca/animal/growth-hormones-cattle.html, 07-Apr-2004).
Patel, P. D. (2002) (Bio)sensors for measurements of analytes implicated in food safety: a review. *Trends in Analytical Chemistry*, 21, 96-115.
Pearson, J. E., Gill, A. & Vadgama, P. (2000) Analytical aspects of biosensors. *Annals of Clinical Biochemistry*, 37, 119-145.
Persson, B., Stenhag, K., Nilsson, P., Larsson, A., Uhlen, M. & Nygren, P. (1997) Analysis of oligonucleotide probe affinities using surface plasmon resonance: a means for mutational scanning. *Analytical Biochemistry*, 246, 34-44.
Prezelj A., Obreza, A. & Pecar, S. (2003) Abuse of clenbuterol and its detection. *Current Medicinal Chemistry*, 10, 281-290.
Pulce, C., Lamaison, D., Keck, G., Bostvironnois, C., Nicolas, J. & Descotes, J. (1991) Collective human food poisonings by clenbuterol residues in veal liver. *Veterinary Human Toxicology*, 33, 480-482.
Rasooly, A. (2001) Surface plasmon resonance analysis of staphylococcal enterotoxin B in food. *Journal of Food Protection*, 64, 37-43.
Rich, R. & Myszka, D. G. (2000a) Survey of the 1999 surface plasmon resonance biosensor literature. *Journal of Molecular Recognition*, 13, 1-20
Rich, R. & Myszka, D. G. (2000b) Advances in surface plasmon resonance biosensor analysis. *Current Opinion in Biotechnology*, 11, 54-61.
Rich, R. & Myszka, D. G. (2001) Survey of the year 2000 commercial optical biosensor literature. *Journal of Molecular Recognition*, 14, 273-294.
Rich, R. & Myszka D. G. (2002) Survey of the year 2001 commercial optical biosensor literature. *Journal of Molecular Recognition*, 15, 352-376.
Ricks, C. A., Dalrymple, R. H., Baker, P. K. & Ingle, D. L. (1984) Use of a ß-agonist to alter fat and muscle deposition in steers. *Journal of Animal Science*, 59, 1247-1255.
Roos, H., Karlsson, R., Nilshans, H. & Persson, A. (1998) Thermodynamic analysis of protein interactions with biosensor technology. *Journal of Molecular Recognition*, 11, 204-210.
Salleras, L., Dominguez, A., Mata, E., Taberner, J. L., Moro I., & Salva P. (1995) Epidemiologic study of an outbreak of clenbuterol poisoning in Catalonia, Spain. *Public Health Reports*, 110, 338-342.
Samsonova, J. V., Baxter, G. A., Crooks, S. R. H. & Elliott, C. T. (2002a) Biosensor immunoassay of ivermectin in bovine milk. *Journal of AOAC International*, 85, 879-882.
Samsonova, J. V., Baxter, G. A., Crooks, S. R. H., Small, A. E. & Elliott, C. T. (2002b) Determination of ivermectin in bovine liver by optical immunobiosensor. *Biosensors & Bioelectronics*, 17, 523-529.
Sauer, M. J. & Andersson, S. P. (1994) In vitro and in vivo studies of drug residue accumulation in pigmented tissues. *Analyst*, 119, 2553-2556.
Sauer, M. J., Pickett, R. J., Limer, S. & Dixon, S. N. (1995) Distribution and elimination of clenbuterol in tissues and fluids of calves following prolonged oral administration at a growth-promoting dose. *Journal of Veterinary Pharmacology and Therapeutics*, 18, 81-86.
Severs, A. H. & Schasfoort, R. B. M. (1993) Enhanced surface plasmon resonance inhibition test (ESPRIT) using latex particles. *Biosensors & bioelectronics*, 8, 365-370.

Shelver, W. L. & Smith, D. J. (2003) Determination of ractopamine in cattle and sheep urine samples using an optical biosensor analysis: Comparative study with HPLC and ELISA. *Journal of Agricultural and Food Chemistry*, 51, 3715-3721.

Situ, C., Crooks, S. R. H., Baxter, G. A., Ferguson, J. & Elliott, C. T. (2002) On-line detection of sulfametazine and sulfadiazine in porcine bile using a multi-channel high-throughput SPR biosensor. *Analytica Chimica Acta*, 473, 143-149.

Sjölander, S. & Urbaniczky, C. (1991) Integrated fluid handling system for biomolecular interaction analysis. *Analytical Chemistry*, 63, 2338-2345.

Smith, D. J. (1998) The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock. *Journal of Animal Science*, 76, 173-194.

Stenberg E., Persson, B., Roos, H. & Urbaniczky, C. (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance by using radiolabelled proteins. *Journal of Colloidal Interface Science*, 143, 513-526.

Sterk S. S., van Tricht E. F., Stephany R. W. & van Ginkel L. A. (1998) *RIVM report 3890002047*, RIVM, Bilthoven, the Netherlands.

Sternesjö, A., Mellgren, C. & Björck, L. (1995) Determination of sulfamethazine residues in milk by a surface plasmon resonance-based biosensor assay. *Analytical Biochemistry*, 226, 175-181.

Traynor, I. M., Crooks, S. R. H., Bowens, J. & Elliott, C. T. (2003) Detection of multi-agonist residues in liver matrix by use of a surface plasma resonance biosensor. *Analytica Chimica Acta*, 483, 187-191.

Tsang, V. C. & Wilkins, P. P. (1991) Optimum dissociating condition for immunoaffinity and preferential isolation of antibodies with high specific activity. *Journal of Immunological Methods*, 138, 291-299.

van Ginkel, L. A., Stephany, R. W. & van Rossum, H. J. (1992) Development and validation of a multiresidue method for ß-agonists in biological samples and animal feed. *Journal of AOAC International*, 75, 554-560.

van Oss, C. J., (1994) Nature of specific ligand – receptor bonds, in particular the antigen-antibody bond. In *Immunochemistry*, van Oss, C. J. & van Regenmortel, M. H. V. (Eds.), Marcel Dekker, New York, pp 581-614.

van Oss, C. J., Good, R. J. & Chaudhury, M. K. (1986) Nature of the antigen-antibody interaction. Primary and secondary bonds: optimal conditions for association and dissociation. *Journal of Chromatography*, 376, 111-119.

Velasco-Garcia, M. N. & Mottram, T. (2003) Biosensor technology addressing agricultural problems. *Biosystems Engineering*, 84, 1-12.

Witkamp, R. F. (1996) Pharmacological and toxicological properties of ß-agonists. In *Residues of veterinary drugs and mycotoxins in animal products*, Anonymous (Ed.), Wageningen Pers, Wageningen, pp. 113-123.

Yamamoto, I. & Iwata, K. (1982) Enzyme immunoassay for clenbuterol, an beta 2-adrenergic stimulant, *Journal of Immunoassay*, 3, 155-171.

Zalco, D., Bories, G., & Tulliez, J. (1996) Comparative metabolism of 3H-clenbuterol in the rat and bovine. *Proceedings of the EuroResidue III Conference, 1996*, Haagsma, N. & Ruiter, A. (Eds.), Utrecht, the Netherlands, pp 993-997.

Zalko, D., Perdu-Durand, E., Debrauwer, L., Bec-Ferte, M. P. & Tulliez, J. (1998) Comparative metabolism of clenbuterol by rat and bovine liver microsomes and slices. *Drug metabolism and disposition: the biological fate of chemicals*, 26, 28-35.

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