Advanced systems for the rapid detection of anthelmintic drugs in food

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Declaration

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List of abbreviations

5-AS – 5-aminosalicyclic acid

Ab – Antibody

ABS – Antigen binding site

ABTS – 2,2-azino-di(3-ehtylbezothiazoline-6-sulphonate)

Ag – Antigen

AgAb – Antigen-antibody complex

ABZ – Albendazole

ABZ-SO – Albendazole sulphoxide

ABZ-SO_2 – Albendazole sulphone

ABZ-NH_2-SO_2 – Albendazole amino sulphone

AOZ – 3-amino-2-oxazolidinone

NPAOZ – p-nitrophenyl-3-amino-2-oxazolidinone

ADI – acceptable daily intake

AP – Alkaline phosphatase

b_0 – Maximum response (the biosensor signal in RU for an antibody buffer solution, in absence of analyte, under assay conditions)

BSA – Bovine serum albumin

b.w. – Bodyweight

BIA – Biospecific interaction analysis

C_{18} – Octadecyl bonded silica

CAP – Chloramphenicol

CAP-HMS-BSA – Chloramphenicol-hemisuccinate-bovine serum albumin

CBZ – Carbendazim

CCα – Decision Limit

CCβ – Detection capability

CCD – Charge-couple device

C_H – Constant heavy chain

C_L – Constant light chain

CM – Carboxylated dextran

CMA – Chlormadinone acetate

CMB – (6)-[carboxypentyl)-thio]-2-benzimidazolecarbamate derivative

CMO – Carboxymethyl oxime

CVMP – Committee for Veterinary Medicinal Products

CRL – Community Reference Laboratory

CV – Coefficient of variation

DELFIA – Dissociation-enhanced lanthanide fluoroimmunoassay

DIG-AMPI – Digoxigenin-labeled ampicillin

DMSO – Dimethylsulphoxide

DMZ – Dimetridazole

DMZOH – Hydroxydimetridazole

DNA – Deoxyribonucleic acid

DPV – Differential pulse voltametry

DSC – Di-succinimidyl carbonate

DTR – Discrete test region

EDC – 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EDTA – Ethylenediamine tetra-acetic acid

EIA – Enzyme immunoassay

ELISA – Enzyme-linked immunosorbent assay

EMA – European Medicinal Agency

ESI – Electrspray inoization

FBZ – Fenbendazole

FBZ-SO – Fenbendazole sulphoxide

FBZ-SO_2 – Fenbendazole sulphone

FF – Florefenicol

FLU – Flubendazole

FLUM – Flumequine

FLU-OH – Hydroxy-flubendazole

FLU-NH_2- Amino flubendazole

GC-MS – Gas chromatography mass spectrometry

HEPES – (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HPA – 17α-hydroxyprogesterone acetate

HRP – Horseradish peroxidase

HPLC – High performance liquid chromatography

HSA – Human serum albumin

IAC – Immunoaffinity chromatography

IBIS – Instrument for Biomolecular Interaction Sensing

IC_{50} – Inhibitory concentration 50% (concentration at the midpoint of the calibration curve)

IFC – Integrated microfluidic cartridge

Ig – Immunoglobulin

IgG – Immunoglobulin G

IPZ – Ipronidazole

KLH – Keyhole limpet haemocyanin

LC-MS – Liquid chromatography coupled to mass spectroscopy

LFIA – Lateral flow immunoassay

LOD – Limit of detection

LOQ – Limit of quantitation

mAb – Monoclonal antibody

MBC – Methylbenzimidazole carbamate

MBZ – Mebendazole

MBZ-NH_2 – Amino- mebendazole

MBZ-OH – Hydroxy-mebendazole

MCMS – Mesofluidic system

MeCN – Acetonitrile

MEGA – Megestrol acetate

MIC – Minimum inhibitory concentration

MRL – Maximum residue limit

MRM – Multiple reaction monitoring

MNZ – Metronidazole

MPA – Medroxyprogesterone acetate

MUG – methylumbelliferyl-β-D-galactosidase

MWCNT – Multi-wall carbon nanotubes

NHS – N-hydroxysuccinimide

NOAEL – No-observed-adverse-affect-level

NRL – National Reference Laboratory

NT – Nortestosterone

OPD – Orthophenylene diamine

OVA – Ovalbumin

OWLS – Optical waveguide lightmode spectroscopy

OXI – Oxibendazole

OXI-NH_2 – Amino- oxibendazole

pAb – Polyclonal antibody

PASA – Parallel affinity sensor array

PBS – Phosphate buffered saline

PBP – Penicillin binding protein

PDDA – Poly(diallyldimethylammonium chloride

p-NPP – *para*-nitrophenyl phosphate

QD – Quantum dots

QuECHERS – Quick, Easy,Cheap, Effective, Rugged and Safe

RU – Resonance units

RNZ – Ronidazole

RSD – Relative standard deviation

SAM – Self-assembled monolayers

SANCO – Santé et Consommateurs (Directorate General Health and Consumers; European Commission; Brussels, Belgium)

SAS – Saturated ammonium sulphate

ScFv – Single-chain variable fragment

SEM – Semicarbazide

SMM– Small molecule microarray

SMZ – Sulfmethazine

SPE – Solid phase extraction

SPR – Surface plasmon resonance

iSPR – Surface plasmon resonance imaging

TAP – Thiamphenicol

TC – Tetracycline

TCB – Triclabendazole

TCB-SO – Triclabendazole sulphoxide

TCB-SO_2 – Triclabendazole sulphone

Keto-TCB – Keto-triclabendazole

TBZ – Thiabendazole

TBZ-OH – Hydroxy-thiabendazole

TOF-MS – Time-of-flight mass spectrometry

TR-FIA – Time resolved fluorescent assay

UPLC-MS/MS – Ultra performance liquid chromatography tandem mass spectrometry

UV-HPLC – High performance liqud chromatography with ultraviolet/visible detection

V_H – Variable heavy chain

V_L – Variable light chain

Appendices

Appendix A: Dissemination of Research

Advanced systems for the rapid detection of anthelmintic drugs in food

Abstract

Several surface plasmon resonance (SPR) biosensor assays were developed and validated for the detection of anthelmintic veterinary drugs in liver tissue and milk using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure. The first screening assay was developed to detect 11 benzimidazole carbamates in milk and liver. In bovine milk the assay showed a limit of detection (LOD) of 2.7 μg kg^{-1} and a detection capability (CCβ) of 5 μg kg^{-1}. Analyte recovery was in the range 81 to 116% and the assay was found to be fit for purpose when its performance was compared to UPLC-MS/MS analyses of milk from cows treated with benzimidazole products. In bovine liver the LOD (32 μg kg^{-1}) and the CCβ (50 μg kg^{-1}) were determined and the analyte recovery was in the range 77-132%. All non-compliant samples were identified when the assay performance was tested by analysing liver from animals treated with benzimidazole drugs and comparing the results with a UPLC-MS/MS confirmatory method.

A screening assay was developed for four amino-benzimidazoles in liver. The LOD (41 μg kg^{-1}) and the CCβ (75 μg kg^{-1}) were determined and the analyte recovery was in the range 103-116%. A screening assay for thiabendazole and 5-hydroxy-thiabendazole in ovine liver tissue using a novel recombinant antibody fragment (Fab) was developed. The LOD (12.3 μg kg^{-1}), the CCβ (20 μg kg^{-1}) and analyte recovery (86-107%) satisfied the criteria required for thiabendazole screening in liver tissue.

A biosensor to detect triclabendazole residues in liver tissue was developed through the immobilization of amino-triclabendazole via a glutaraldehyde homo-bifunctional cross-linker. Several experiments were required to reduce non-specific binding in this assay. An LOD of 105 μg kg^{-1} was determined which was close to the maximum residue limit (MRL) in liver matrix (100 μg kg^{-1}).

A biochip array was developed and validated to screen orange juice for fungicide and pesticide residues. The LOD for carbendazim (20 μg kg^{-1}), 2-aminobenzimidazole (4.0 μg kg^{-1}), thiabendazole (4.2 μg kg^{-1}) and ivermectin (10.2 μg kg^{-1}) residues were determined. The CCβ for carbendazim (50 μg kg^{-1}), 2-aminobenzimidazole (10 μg kg^{-1}), thiabendazole (10 μg kg^{-1}) and ivermectin (20 μg kg^{-1}) residues were sufficient for the analysis of orange juice. When orange juice from retail outlets in the greater Dublin area (n = 15) Two samples contained thiabendazole residues above the CCβ (260 and 181 μg kg^{-1}) however these concentrations were below the maximum residue limit.

Research objectives

The overall aims of this research project were to investigate the applicability of SPR biosensors to screen for benzimidazole residues in liver and milk from food-producing animals and to validate a pesticide and fungicide residue screening method for orange juice using a biochip array platform through the development of fast, reliable tests with minimum sample pre-treatment.

The specific objectives of this work were:

- To prepare biosensor chip surfaces for the detection of benzimidazole carbamate, amino-benzimidazole, thiabendazole and triclabendazole residues.
- To develop sample preparation procedures to isolate benzimidazole residues from liver and milk.
- To validate the SPR biosensor screening assays for benzimidazole residues in liver and bovine milk.
- To evaluate the performance of the biosensor assays in "real" samples taken from animals treated with benzimidazole drugs and compare the results to those of mass spectrometry (UPLC-MS/MS) analysis.
- To develop and validate a multiplex biochip array method to detect avermectins pesticides, carbendazim, thiabendazole and 2-aminobenzimidzole fungicidal agents in orange juice.

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"The hardest arithmetic to master is that which enables us to count our blessings".
Eric Hoffer

Chapter 1

Section A: An introduction to veterinary drug monitoring in food and screening methods used for their identification

1.1 Introduction

1.1.1 The role of veterinary drugs in animal health

Veterinary drugs are essential in modern agriculture to maintain the health and yields of food-producing animals. Drugs are used in farm animals for therapeutic and prophylactic purposes and the primary routes of drug administration include oral, intramuscular, subcutaneous and intravenous dosing. Over the last decade, great strides have also been made in using topical 'pour-on' and 'spot-on' applications of pesticide and antiparasitic treatments (Riviere and Papich, 2001). Ruminal boluses are a unique dosage form that provides a prolonged duration of controlled drug release and has particular application in the delivery of anthelmintics (Baggott, 1988). Administration in feed is a convenient approach for simultaneous treatment of large number of animals. However, unlike traditional dosing this may not ensure that a specific dose of the drug reaches each animal because the drug dosage becomes a function of food consumption. Subcutaneous implantable/injectable devices such as ear implants may also be used where prolonged drug release is required in herds (Rothen-Weinhold, Gunry and Dahn, 2000).

1.1.2 Legislation regarding veterinary drugs

The control of veterinary drug residues in live animals and their food is described in Council Directive 96/23/EC (Anonymous, 1996). Drug residues to be monitored in food are listed in Annex I of this document and are divided into two groups, A and B. Group A substances are banned in food-producing animals and Group B substances include veterinary drugs and contaminants. A detailed list of the approved and banned pharmacologically active products is included in the Annexes of Council Regulation 37/2010 (Anonymous, 2010). This regulation describes the procedure to establish maximum residue limits (MRLs) for veterinary products in foodstuffs of animal origin. An MRL is defined as the concentration of residue legally permitted or recognized as acceptable in or on a food that occurs in edible tissues after treatment with a veterinary medicinal product (expressed in mg kg^{-1} or μg kg^{-1} on a fresh weight basis).

Safety assessments of veterinary medicinal products are carried out by the Committee for Veterinary Medicinal Products (CVMP), which is part of the European Medicinal Agency (EMA). Safety assessments take into account assessments by international organizations, in particular the Codex Alimentarius Commission or by other scientific committees established within the European Union. MRLs are established using the acceptable daily intake (ADI) concept, which is based on multiple-dose toxicological studies that represent chronic exposure to drug residues. The ADI is established by applying a safety factor to a minimum inhibitory concentration (MIC) value or a no-observed-adverse-affect-level (NOAEL) value that has been identified in the most sensitive species. In the event that metabolic and pharmacokinetic data identify a species that is more suitable for extrapolation to humans, then the NOAEL is divided by a safety factor to establish an ADI. A safety factor of 100 is usually applied, which is based on the assumption that humans are 10 times more sensitive to the substance than experimental animals and that there is a ten-fold range in sensitivity within the human population (10 x 10).

1.1.3 Food safety

Residues of veterinary drugs can occur in food and may give rise to human health concerns through the direct consumption of meat and milk products. A complex laboratory structure comprising of national reference laboratories (NRLs) and community reference laboratories (CRLs) has been established to provide residue control within the European Union (EU). NRLs are established at a member state level to provide expert monitoring of residues. The role of NRLs is to provide support for residue control including the provision of expert laboratory analysis, input to annual national monitoring plans and to act as a contact point with the CRLs. CRLs are established at an EU level to provide expertise for different substance groups and or foods. The current CRLs and NRLs are listed in Commission Decision 130/2006/EC (Anonymous, 2006a) and Council Regulation 776/2006/EC (Anonymous, 2006b). In those animals where the manufacturers' and legislative directions are followed by the producer, drug residue levels will be within safe limits. In the relatively few cases where residue levels exceed permitted MRLs, the cause is nearly always improper use.

To ensure food is safe to consume, reliable and cost effective analytical methods are increasingly needed to provide rapid and sensitive screening for veterinary drug residues in food.

1.1.4 Analysis of veterinary drug residues

There are two main methodologies used for drug residue analysis in food, namely, screening and confirmatory assays. Screening assays are described in Commission Decision 2002/657/EC as analytical techniques for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of <5 % (β-error). This must be at a level below the minimum required performance level in conformity with Directive 96/23/EC. These are often extremely rapid techniques such as immunochemical methods which permit high sample through-put at low cost. This procedure should be as simple as is possible. Nonetheless, it may be rather complex, due to, e.g. the properties of the drugs of interest or the desired limit of detection, and, in certain cases, will provide (semi)quantitative next to the qualitative information (Aerts, Hoogenboom and Brinkman, 1995). Immunochemical and microbial growth inhibition techniques are two commonly used screening methods.

Confirmatory methods are defined in Commission Decision 2002/657/EC as the analyses of target molecules based on the concept of unequivocal identification and accurate, as well as precise quantification by means of physical-chemical properties unique to the chemical at hand (e.g. molecule characteristic wavelength of emitted or absorbed radiation, atomic mass) at the level of interest. The purpose of a confirmatory method is to definitively confirm the presence and identity of an analyte. Methods used for this purpose must be highly specific and sensitive. Liquid chromatography coupled to mass spectroscopy (LC-MS), gas chromatography coupled to mass spectroscopy (GC-MS) and atomic absorption/emission spectroscopy techniques are commonly used confirmatory methods. The validation of screening and confirmatory methods must demonstrate that the analytical method complies with pre-set performance characteristics which are outlined in the SANCO document 1085/2000 and in Commission Decision 2002/657/EC.

These performance criteria include the determination of the decision limit (CCα) and the detection capability (CCβ). The CCα is the limit at or above which it can be concluded with an error probability of α that a sample is non-compliant (Anonymous, 2002). The CCβ is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which the method is able to detect truly contaminated samples with a statistical certainty of 1 – β. In the case of substances with an established permitted limit, this means that the CCβ is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 – β (Anonymous, 2002).

1.2 Immunoassays

1.2.1 Basic principle

An immunoassay is a molecular recognition-based detection system, which exploits the specific binding of an antibody (Ab) to an antigen (Ag) raised against it. The molecule to which the antibody binds is referred to as an antigen (Ag) and immunoassays can be used to detect or quantify either antigens or antibodies. The classical immunoassay (Yalow and Berson, 1959) is a limited reagent assay whereby there is less binding protein present in the system than antigen, and to quantify the system a labelled form of the analyte is measured. The unlabelled antigen competes with the labelled antigen for the limited number of antibody binding sites, therefore the more unlabelled antigen present, the fewer labelled antigens will be bound by antibody (**Fig. 1.1**).

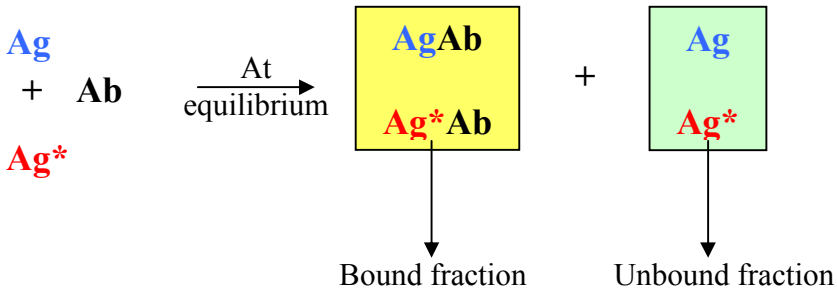


Figure 1.1 Principle of the immunoassay: Ag, unlabelled antigen; Ag*, labelled antigen; Ab, antibody; AgAb, antigen-antibody complex.

1.2.2 A brief history

All developments in immunoassays stem from the first report of an immunoassay by Yalow and Berson (1959) when an assay was applied to detect radiolabelled insulin. This radioimmunoassay (RIA) format was rapidly adapted for the analysis of many other analytes and gained acceptance among clinical scientists. Food scientists were slower to adapt to this technology because of public concern associated with radioactivity in close proximity to food. Subsequently the radiolabel was replaced with an enzyme label (Engvall and Perlman, 1971; van Weeman and Schuurs, 1971) and the enzyme immunoassay (EIA) was established. Shortly after this, the first EIAs for food were developed for the detection of trichinellosis in pigs for slaughter and this work triggered an increase in the type of analysis for which immunoassays were developed (Ljungström *et al.*, 1974). Between the years 1974 and 1978 publications describing food EIAs represented a quarter of food immunoassay publications (Morris and Clifford, 1983).

Since then there have been numerous developments and applications of immunoassay techniques. These include enzyme-linked immunosorbent assays (ELISAs), lateral flow immunoassays (LFIA)/dipstick assays, biosensor assays, time-resolved fluorescent assays (TR-FIAs), biosensors and microarrays.

Many different antigen labels have been applied to the immunoassay including several radioisotopes, enzymes and luminescent molecules. These techniques are now frequently used for the determination of anabolic hormones, bacterial toxins, disease markers, microorganisms, mycotoxins, pesticides and veterinary drugs.

1.2.3 Antibodies

Antibodies / immunoglobulins are proteins that are synthesized by an animal in response to the presence of a foreign substance. Each antibody consists of four polypeptides; two heavy chains and two light chains joined to form a "Y" shaped molecule (**Fig. 1.2**). Antibodies are the recognition elements of the humoral immune response and each antibody has a specific affinity for the foreign material (antigen) that stimulated its synthesis (Stryer, 1988). Effective antigens are proteins, polysaccharides and nucleic acids, which usually result from the presence of bacteria, fungus and viruses.

Antibodies do not have specific affinity for the entire macromolecular antigen, instead they have a particular surface feature on the antigen called the antigenic determinant or epitope. Small foreign molecules (haptens) can elicit the formation of a specific antibody if they are attached to macromolecules (Stryer, 1988). Consequently, low molecular weight haptens and hapten derivatives are conjugated to high molecular weight proteins to form immunogens. Haptens are routinely conjugated to proteins such as human serum albumin (HSA), keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA).

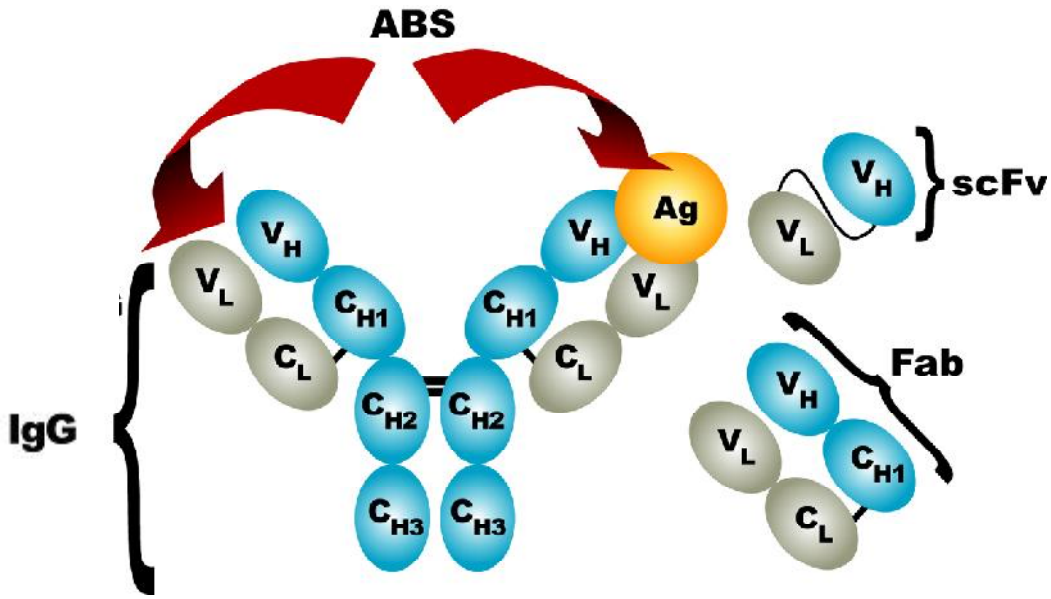


Figure 1.2 Antibody structure: IgG, immunoglobulin G; C_H, constant heavy chain, C_L, constant light chain; V_H, variable heavy chain; V_L, variable light chain; ABS, antigen binding site,; fAb, antibody binging fragment; scFv, single-chain variable fragment.

1.2.3.1 Polyclonal antibodies

Polyclonal antibodies (pABs) are generated from the repeated immunization of an animal with a particular antigen. The animal serum or antiserum will consist of a complex mixture of antibodies produced by many different B cell clones. Each antibody recognizes a different epitope on the antigen and will each differ in their affinity for the antigen. Each antisera preparation differs in specificity, average affinity and cross-reactive specificities and therefore the supply of a single type of pAb is limited.

1.2.3.2 Monoclonal antibodies

Monoclonal antibodies (mAbs) are monospecific antibodies that may be produced in large quantities. This is achieved using hybridoma technology, a technique first introduced by Kohler and Milstein (1975). A hybridoma is formed by fusing a myeloma cell (a bone marrow tumour cell) to an antibody producing lymphocyte, a B cell (from the spleen of a mouse). These hybrid cells have the antibody-producing capability inherited from the lymphocytes and the ability to grow identical cells continuously like malignant cancer cells. This results in the formation of "immortal" cells that can be grown rapidly. These mAbs consist of single type of antigen binding site that recognizes a single epitope produced by a single B cell clone. Therefore, mAbs are consistent, provide a limitless supply of a specific reagent and are more easily tested for cross-reactivity. However, once a mAb is produced its structure can not be easily altered to improve antibody specificity.

1.2.3.3 Recombinant Antibodies

Recombinant antibodies are produced using genetic engineering techniques. Phage display in combination with antibody gene libraries are widely used to select *E. coli* host cells that express desired antibody fragments. The antigen binding site of an antibody is formed by combining the variable gene segments of a heavy chain (V_H) and a light chain (V_L). Genetic engineering is used to create naïve libraries based on one or more of the antibody V_H and V_L gene segments that are diversified by cassette mutagenesis or similar approaches. These libraries are typically unbiased and can be used for any given antigen (Knappik *et al.*, 2000; Soderlind *et al.,* 2000; Hoet *et al.,* 2005). Phage display is used to select desired antibodies from such libraries and is discussed in detail in several reviews (Hoogenboom *et al.*, 1998; Kretzschmar and Von Ruden, 2002). Recombinant antibodies offer many advantages over traditionally generated mAbs because these antibodies can be selected from libraries based on their affinity and avidity for a certain antigen target. The two main types of recombinant antibody fragments expressed in *E. coli* are single-chain variable fragments (scFv) and antibody fragments (Fab) (Albitar, 2007).

A scFv is a fusion of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. A Fab is composed of one constant and one variable domain from each heavy and light chain of the antibody (**Fig. 1.2**).

1.3 Immunoassay detection systems

1.3.1 Introduction

Immunoassay detection systems represent a highly interdisciplinary field of research covering a broad range of research disciplines. This section is intended to present examples from research areas within the scope of this thesis rather than to provide a comprehensive coverage of this topic.

1.3.2 Immunoassay formats

1.3.2.1 Competitive and non-competitive immunoassays

In a competitive immunoassay format the antigen (analyte) in the sample competes with a labelled antigen (e.g. enzyme or fluorescent label) for a limited number of antibody binding sites (Wild, 2005). The bound antigen is separated from the excess analyte, usually with a wash step. The amount of analyte in a sample is inversely proportional to the amount of labelled antigen, which can be measured (e.g. by fluorometry or spectrophotometry).

In non-competitive "sandwich" immunoassays the analyte is sandwiched between two antibodies. Typically the capture antibody is coated onto a solid phase, such as a microplate well and the detection antibody (which should be present in excess) is labelled with an enzyme, radioactive label or fluorophore. As the amount of analyte is increased, the amount of labelled antibody-antigen complex also increases. Thus, the amount of analyte in an unknown sample is directly proportional to the amount of labeled detection antibody measured by the detection system (Kemeny and Challacombe, 1988).

Non-competitive immunoassays may be one-step or two-step methods as with the competitive assay. The two-step assay employs washing steps in which the sandwich-binding complex is isolated and washed to remove excess unbound labelled reagent and any other interfering substances. These steps are omitted in the one-step assay. Examples of competitive and non-competitive immunoassays are outlined in **Figure 1.3**.

1.3.2.2 Heterogeneous and homogeneous immunoassays

Immunoassays that require separation of the bound antibody-labelled antigen complex are referred to as heterogeneous or solid phase immunoassays (Wild, 2005). These assays are performed on a surface that is coated with antibody or antigen (**Fig. 1.3**). The immunological binding takes place on the surface and is followed by one or more washing steps to achieve separation of the antibody-labelled antigen complex from free reactants (Wild, 2005). The solid phase of an immunoassay may be the inner surface of immunoassay plate wells, a tube, the surface of a slide or specialized reagents including magnetic particles or plastic beads. Immunoassays that do not require separation of the bound antibody-antigen complex from labelled antigen are referred to as homogeneous immunoassays. These assays are simpler to perform and are commonly used for the detection of small molecules.

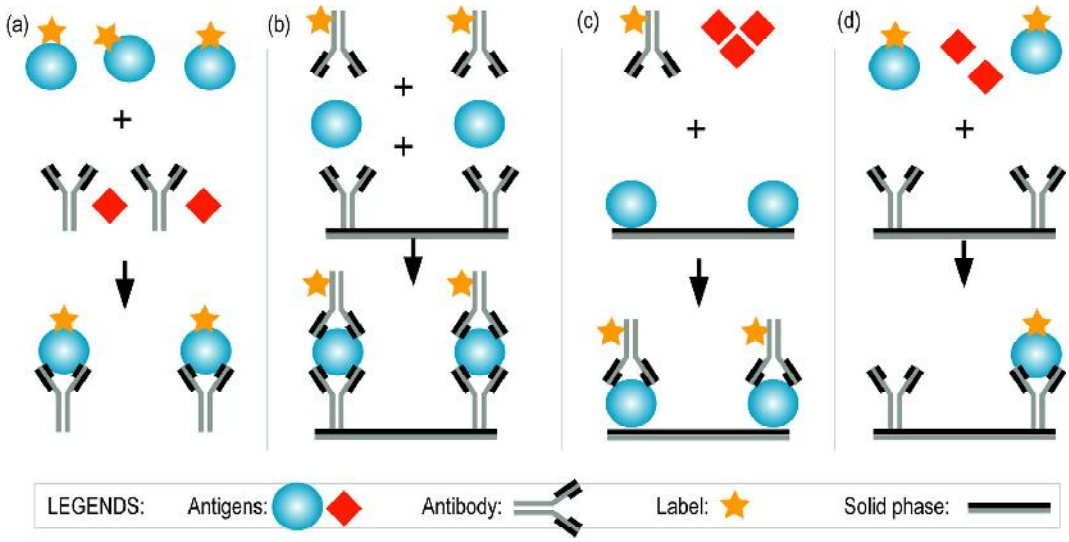


Figure 1.3 Immunoassay formats: (a) a homogeneous competitive immunoassay, (b) a heterogeneous non-competitive immunoassay, (c) a heterogeneous competitive immunoassay and (d) a heterogeneous competitive immunometric assay.

1.3.3 Enzyme-linked immunosorbent assays (ELISAs)

ELISAs are solid phase immunoassays, typically performed in 96-well (or 384-well) polystyrene plates which can passively bind antibodies and proteins. The reactants of the ELISA are immobilized to the microplate surface, which makes it easy to separate bound from non-bound material during the assay. This ability to wash away non-specifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation. Enzyme labels are attached to antibodies or antigens and when the enzyme substrate is added to the reaction it produces a colorimetric readout as the detection signal. Enzyme labels used in ELISAs include horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-galactosidase. Several chromogens may be used with the HRP substrate hydrogen peroxide including orthophenylene diamine (OPD), 2,2-azino-di(3-ehtylbezothiazoline-6-sulphonate) (ABTS), 5-aminosalicyclic acid (5-AS) and 3,3,5,tetramethylbenzidine hydrochloride (Kemeny and Challacombe, 1988). The substrate used in conjunction with AP for spectrophotometric measurement is *para*-nitrophenyl phosphate (*p*-NPP). Chromogenic substrates such as *p*-nitrophenyl-β-D-galactosidase and fluorogenic substrates like 4-methylumbellifeyl-β-D-galactosidase (MUG) may be used with β-galactosidase (Kemeny and Challacombe, 1988). Competitive and sandwich ELISAs formats are generally used in the detection of small molecules such as veterinary drugs. The direct ELISA format is not generally used for the detection of contaminants in food matrices but is more common for immuno-histochemical staining of tissues and cells.

1.3.4 Biosensors

1.3.4.1 Background

A biosensor is an analytical device, incorporating a biological or biomimetric sensing element, either closely connected to, or integrated within, a transducer system (Turner *et al.*, 1987). The principle of detection is the specific binding of the analyte of interest to the complementary biorecognition element or bioreceptor immobilized on a suitable support medium. The biorecognition elements used in biosensors include antibodies, enzymes, nucleic acids, tissue, cells or artificial biomimetic receptors. The specific interaction will result in a change in one or more physico-chemical properties (pH change, electron transfer, mass change, heat transfer, uptake or release of gases or specific ions), which are detected and may be measured by the transducer. Optical, electrochemical, electrical, thermal and piezoelectric transducer types exist for the detection of specific interactions (**Fig. 1.4**). The usual aim is to produce an electronic signal that is proportional in magnitude or frequency to the concentration of a specific analyte or group of analytes, to which the biosensing element binds (Turner *et al.*, 1987). For the detection of veterinary drug residues, the most widely used biological element is the antibody/antigen affinity pair and the most common transducer systems are optical and electrochemical methods. An optical transducer element frequently employed in biosensors for environmental and food safety is the surface plasmon resonance (SPR) device which will be discussed in detail later.

A SPR biosensor assay has been developed to detect microorganisms (Nanduri *et al.*, 2007), antibiotics (Situ *et al.*, 2002), hormones (Gillis *et al.,* 2002), pesticides (Subhash Chand and Gupta, 2007), toxins and antimicrobial drugs (Haasnoot *et al.,* 2001) in food. Electrochemical sensors have been applied to detect microorganisms in food such as *E. coli* 0157 and *Salmonella* (Dill, Stanker and Young, 1999, Ercole *et al*., 2003). In addition, these sensors have been applied to detect hormones (Draisci *et al.,* 2000, Volpe *et al.*, 2006) toxins (Kreuzer *et al.*, 2002) and pesticides (Nunes and Barcelo, 1998) in food.

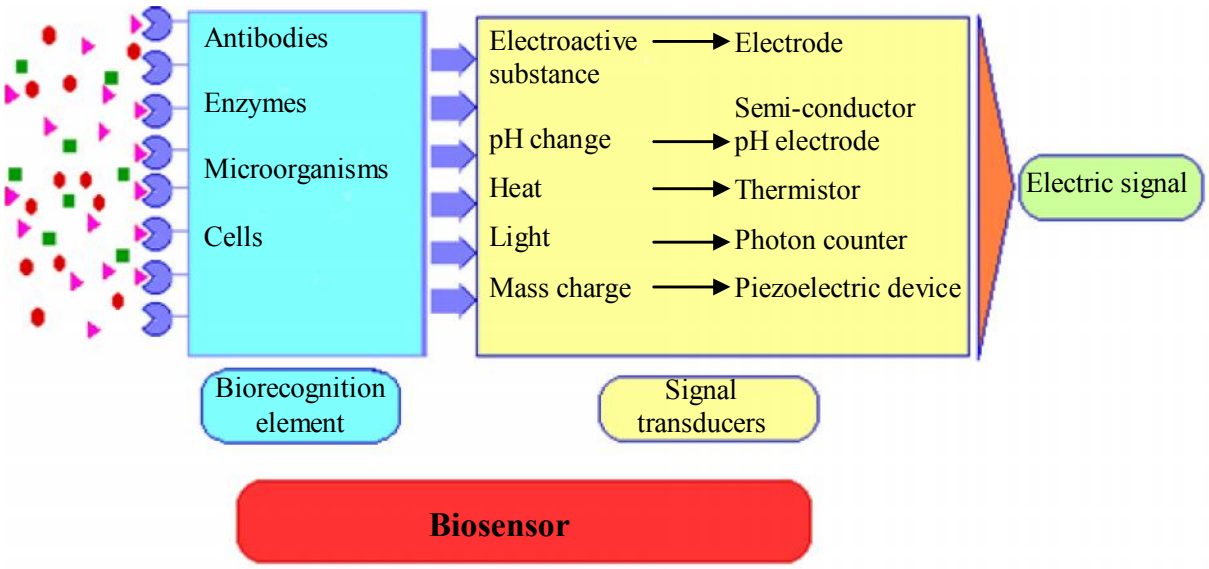


Figure 1.4 Principle of operation of a biosensor showing components: sample matrix, bioreceptor, transducer, electrical amplification system and a data-processing system.

1.3.4.2 Surface plasmon resonance (SPR) biosensors

Surface plasmon resonance (SPR) is a quantum optical-electrical phenomenon that occurs at metal surfaces (typically gold and silver) when an incident beam of plane-polarised light directed through a prism at a given wavelength strikes a surface at a given (incident) angle (**Figure 1.5**). These conditions cause photon-plasmon electromagnetic waves that propagate parallel to the meta-dielectric interface. Changes in the refractive index close to the interface caused by binding between biomolecules and immobilized ligands are detected via changes in the angle of reflection of plane-polarized light (Schasfoort and Tudos, 2008). This SPR instrumentation can be configured in various ways to measure this change in refractive index also known as the SPR dip shift. In general, three different optical systems are used to excite surface plasmons: systems with prisms, gratings and optical waveguides.

The most widespread are instruments with a prism coupler, also called the ‘‘Kretschmann configuration’’ (Schasfoort and Tudos, 2008). In this configuration, a prism couples plane-polarized light into the surface plasmon film and reflects the light onto a light intensity detecting device, e.g. a photodiode.

This configuration can be further divided into three subgroups: fanshaped beam, fixed-angle and angle scanning. In the following section the basic features and characteristics of an optical SPR detection system using a prism coupler, in a fan-shaped beam configuration are discussed.

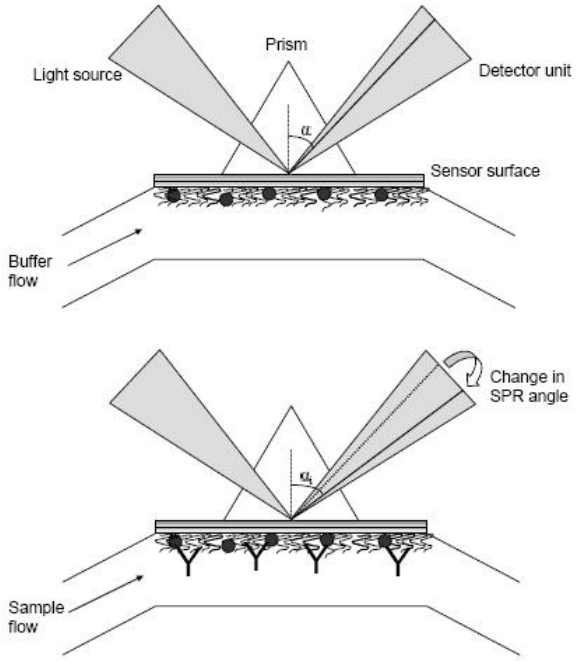


Figure 1.5 The optical detection system used in the Biacore™ instrument. Upon binding or dissociation of molecules to the sensor surface the refractive index near the surface changes, resulting in a shift in the SPR angle (α).

In general, an SPR immunosensor consists of a light source, a detector, a transduction surface (usually gold-film), a prism, biomolecule (antibody or antigen), and a flow system. When a SPR biosensor instrument operates using a fan-shaped beam, a converging beam of plane polarized light is coupled in the higher refractive index medium using a cylindrical or triangular prism. The beam is focused onto an infinitely narrow line on the sensor chip and a photodiode array is used to detect the reflected diverging beam with the SPR dip. Interactions between free and immobilized molecules take place at a sensor surface and these changes are directly related to the amount of sensor surface bound molecules (Löfås and Johnsson, 1990).

The binding events are monitored by a detector (photo-diode array) and time-dependent changes in the refractive index are recorded as a sensorgram (**Figure 1.6**). Resonance units (RU's) are arbitrary units used to monitor binding events where a change of 1000 RU corresponds to a 1° shift in the reflection angle of plane-polarised light (Jönsson *et al.*, 1991).

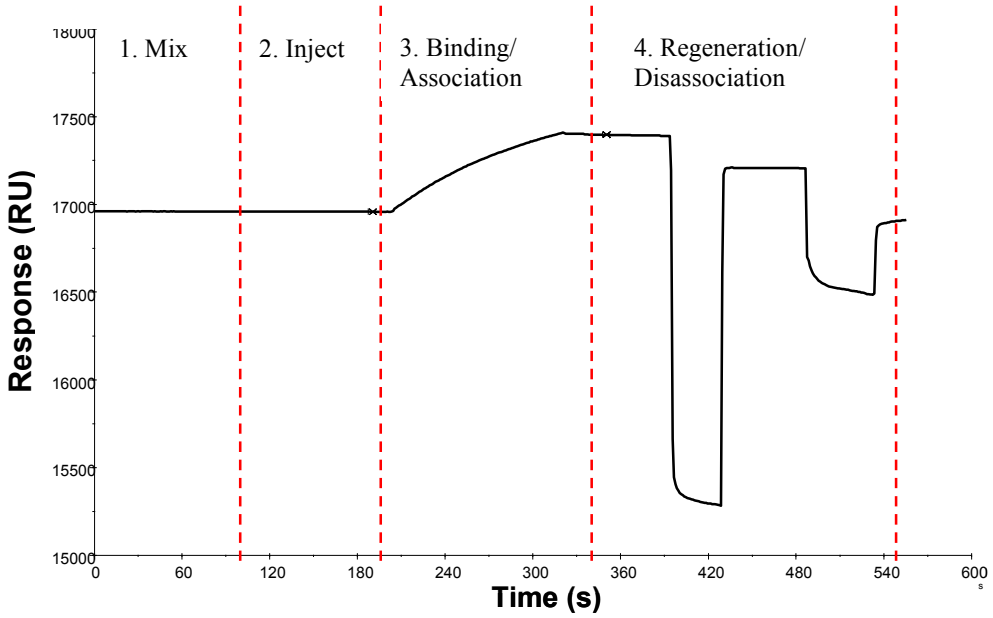


Figure 1.6 A sensorgram illustrating the interaction between free antibody in a sample and antigen immobilized onto the surface. 1) baseline equilibrium (continuous buffer); 2) association of antibody to the sensor surface during injection; 3) response of sample; 4) regeneration of sensor surface.

1.3.4.3 The sensor surface

The immobilisation of an antibody or antigen onto a transducer or a support matrix is a key step in optimizing the analytical performance of an immunosensor in terms of response, sensitivity, stability, and reusability. The immobilisation strategies most generally employed are physical or chemical methods. In general, they fall into following methodologies; physical adsorption, covalent binding or self-assembled mono-layers.

Physical adsorption is generally based on interactions such as van der Waals' forces and electrostatic interactions between the antibody/antigen and the transducer. It is especially common on hydrophobic polymer surfaces (Jiang *et al.*, 2008). The main advantages of this mode of immobilisation are its rapidity and simplicity, while its main drawbacks are random orientation and weak attachment.

Covalent coupling may be used to immobilize antibody or antigen through the formation of a stable covalent bond between functional groups of an antibody and the transducer. The procedure provides increased stability of the antibody but decreases the activity of antibody-antigen and is generally poorly reproducible. Blocking steps are usually necessary to limit the non-specific binding. Self-assembled monolayers (SAMs) may be generated by the spontaneous chemi-sorption of molecules onto a gold surface. SAMS consist of long-chained *n*-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups (Wink *et al.*, 1997).

Sensor chips are commercially available which consist of a glass support covered by a thin layer of gold to which a coupling matrix, e.g. carboxymethylated dextran, is attached via a linker layer (**Figure 1.7**). The coupling matrix determines the surface characteristics and enhances the immobilisation capacity of biomolecules. Different coupling procedures can be used for ligand immobilisation; these include amine coupling, thiol coupling, immobilisation of aldehydes through hydrazide groups and coupling through epoxy groups. Due to its flexibility, relative ease of use, high coupling and robustness, amine coupling via reactive esters is the most frequently employed immobilisation method (Schasfoort and Tudos, 2008).

Carboxymethylated dextran enables ligand immobilisation through amine coupling and has become the most commonly used coupling matrix (Baird and Myszka, 2001). The ligand is more easily accessed by its' interacting partner and the hydrophilic structure of the matrix minimises non-specific adsorption of proteins. Without the matrix the gold film would bind protein in an uncontrollable manner (Löfås *et al.*, 1991).

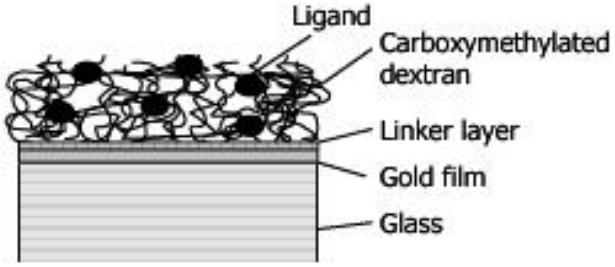


Figure 1.7 A schematic figure of a biosensor chip surface in cross-section

1.3.4.4 Liquid handling unit: The microfluidic system

When a sensor chip is inserted into the Biacore™ instrument, the surface matrix side is docked against an integrated micro-fluidic cartridge (IFC) and four flow cells are formed (**Fig.1.8 (A)**). The opposite side of the chip is pressed against a glass prism in the optical unit. Samples are injected from the autosampler into the IFC, which connects directly with the detector flow cells and controls the continuous flow of buffer or sample over the sensor surface via a number of sample loops. This allows the ligand to be exposed to a constant analyte concentration for the time of the interaction measurement (Baird and Myszka, 2001). This miniaturised system permits the use of low reagent volumes.

1.3.4.5 Biosensor immunoassay formats

The main biosensor assay formats include direct binding, sandwich and inhibition assays. In the direct detection format the target molecules (antigens) bind directly to receptors (antibodies) attached to the surface. This assay requires a biolayer of tens of picometers in thickness and is suited to the detection of medium sized molecules (~20 kDa) and larger sized bacteria (several microns). The concentration of the target molecule that binds to the receptor at the biosensor surface is directly proportional to the biosensor response.

The main limitation of this technique is that the sensitivity depends on the molecular weight of the analyte, implying that low concentrations or small molecules cannot be detected in a direct way.

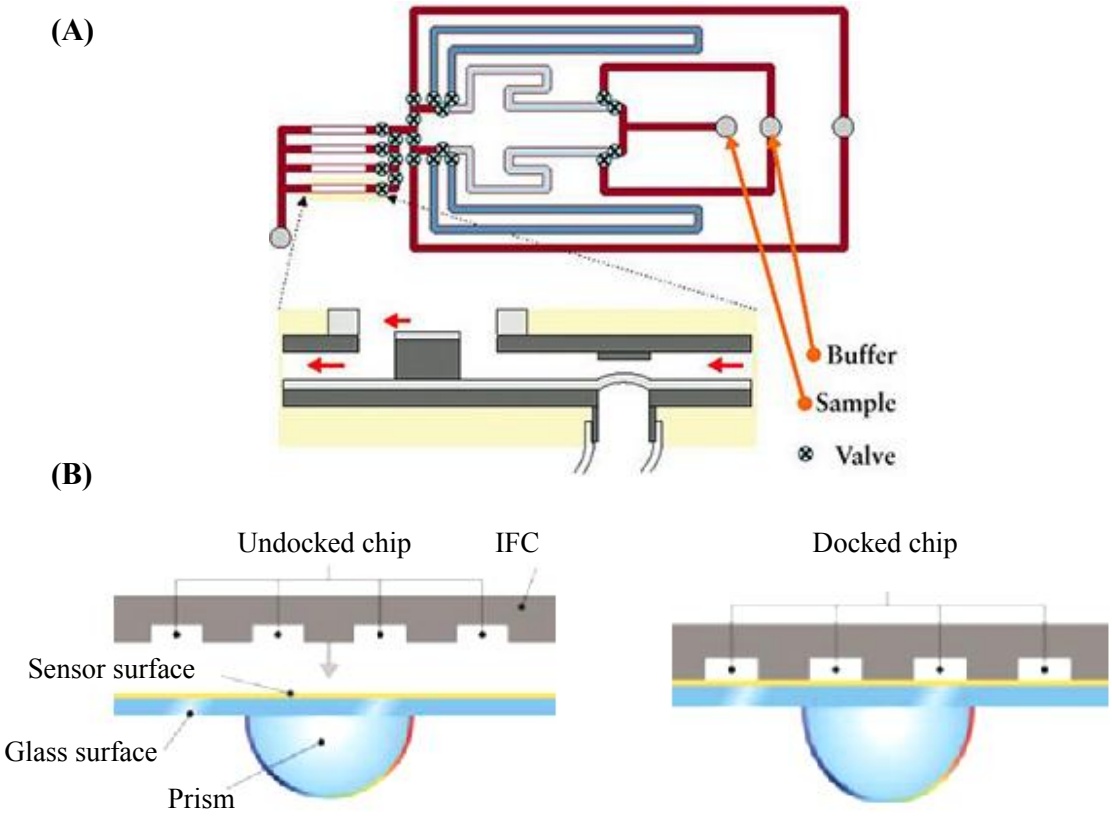


Figure 1.8 **(A)** Integrated micro-fluidic cartridge (IFC) **(B)** Flow cells are formed between the integrated microfluidic cartridge IFC and sensor chip surface.

In these cases a sandwich or competitive assay can be employed. The response of directly captured antigens may be amplified by secondary antibodies (sandwich assay format). Analyte molecules bind to immobilized antibody on the sensor surface, as in the direct format and subsequently a secondary antibody is injected across the surface which binds to the previously captured antigens.

The molecular weight of the antibody (~150 kDa) may be an order of magnitude higher than that of the antigen, a significant amplification of the response and consequently a lower assay detection limit may be achieved.

The inhibition assay is a competitive assay format often used to detect small analytes such as veterinary drug residues. The target analyte or an analyte analogue is immobilized onto the transducer surface (biosensor chip) and receptors (e.g. antibodies) are premixed with the sample to allow binding of the antibodies in a homogenous reaction. The target analyte molecules in the sample bind to the receptors and block their binding sites. The sample is then injected across the sensor surface with immobilized analyte molecules. Depending on the concentration of the target molecules a certain amount of the receptor/antibody is prevented from binding to the sensor surface. The binding of the non-complexed free antibody to the immobilized analyte is monitored. The biosensor response is thus inversely proportional to the analyte concentration in the sample.

1.3.4.6 Commercially available SPR instruments

Commercial SPR instruments typically have the capacity to detect 1 pg mm^{-2} of analyte mass change on the sensor surface (Petz, 2009). Several SPR spectroscopy-based sensors are commercially available, among these the Biacore™ (currently part of GE Healthcare, USA) was the first and provides the highest refractive index resolution measured at approximately 1 x 10^{-7} RU (Xu *et al*., 2010). Texas Instruments have developed portable SPR devices that provide practical application for "real-time" detection with great convenience. The SPR devices in its Spreeta series have been made that are as small as coins. The Spreeta instruments provide a refractive index resolution of approximately 1 x 10^{-6} RU. Windsor Science Instrument for Biomolecular Interaction Sensing (IBIS) Technologies has been focusing on "label-free" analysis and monitoring of biomolecular interactions with array techniques. IBIS has developed a unique label-free surface plasmon resonance imaging (iSPR) sensor device with high accuracy, high dynamic range, and multi-array of real-time imaging (Xu *et al*., 2010). Biosensing Instrument Incorporated uses a unique design, which can detect multiple analytes sensitively and has the largest diversity and flexibility. Some IBIS instruments (BI2000 and BI3000) are equipped with advanced flow injection technique, and can be combined with an electrochemical detector for electrochemical SPR analysis (Xu *et al*, 2010).

1.3.5 Multiplex immunoassay methods

1.3.5.1 Small molecule micro-arrays

Small molecule microarrays are multiplex methods which permit several analyses to be carried out simultaneously resulting in a significant reduction in processing time and the amount of each sample required. An ever-expanding sector in the field of microarray technology is Small Molecule Microarrays (SMMs), whereby small molecules are immobilized on a surface and used as probes for the purpose of screening a single sample for a number of targets (Chiosis and Brodsky, 2005). These SMMs are constructed by printing small molecules onto agarose film-coated modified glass slides. In this way the small molecules retain their ability to interact specifically with corresponding antibodies in solution. Antibodies that are specific towards the immobilized molecules/analytes are combined with each test sample and added to the array plate.

Immobilization methods can be classified based on whether a covalent or non-covalent mode of attachment is employed, and whether the method entails a random or oriented attachment of the molecular probe. Several functional group-based immobilisation procedures have been reported for SMM construction. Thiol-specific immobilisation on malemide-derivatized slides via the Michael reaction, primary alcohol-specific immobilisation on silyl-mediated derivatized slides and diazobenzlidine-mediated immobilisation of functional groups with acidic protons such as phenols and carboxylic acids have been effectively applied (Lee and Park, 2010).

Recently a photo-cross-linking strategy was applied by Kanoh (2010) which depended on the reactivity of carbene species generated from a 4-(3-triflouromethyl)-3H-diazirin-zyl)benzoic acid derivative upon UV irradiation. These photo-generated carbene species are highly reactive towards a variety of chemical bonds including non-activated C-H bonds. Chemical microarrays have also been constructed by selective attachment of hydrazide conjugated substances to epoxide-derivatized glass slides (Park, Lee and Shin, 2010). Flouro-carbon tags have been reported for the non-covalent and homogenous capture of small molecules onto flouro-carbon-coated glass (Vegas and Koehler, 2010).

This is a useful application for applications that require the display of compounds in a specific orientation.

Many SMMs employ Cy5 or Cy3-labelled secondary antibodies to produce a fluorescent signal. Chemiluminescent, radiolabels and colorimetric methods have also been reported. Surface plasmon resonance imaging (iSPR) can also be used for "label-free" SMM detection (Rebe Raz *et al.*, 2008). In the iSPR system the surface is illuminated with incident light at different angles and the images of the surface are captured by a charge-coupled device (CCD) camera. Light reflectivity is determined from the gray values of the pixels and plotted as a function of the scanning angle (Beusink *et al.*, 2008; Lokate *et al.*, 2007).

1.3.5.2 Suspension arrays

A suspension array is simply a transfer of the microarray format from a glass slide (planar and solid microarray) to a microsphere format (Borucki *et al.*, 2005). In this format each array element is prepared in bulk by coupling the appropriate recognition element at the surface of an optically defined microsphere. By optical encoding, micron-sized particles (e.g. polymer particles) can be created to enable highly multiplexed analysis of complex samples (Nolan and Sklar, 2002). Flow cytometry and fibre-optic detection systems are applied for the analysis of suspension arrays.

Multiplexed suspension assays have been commercialised, one example of such a system is the LabMAP^{TM} system made by the Luminex Corporation (Austin, TX, USA) (Fulton *et al.*, 1997; Kettman *et al.*, 1998; Oliver *et al.*, 1998). This system is based on the use of microsphere subclasses, each having a unique combination of two internal identification fluorophore concentrations. The system discriminates among microsphere subclasses on the basis of two longer wavelength fluorescence identification signals (orange and red) leaving the third shorter wavelength fluorescence signal (green) for the determination of the bioaffinity reaction. Currently, the assay steps are manually operated and for each analyte a defined quantity of microspheres is added to the sample.

After mixing and incubation of analytes and microspheres, the detector molecules (e.g. antibodies labelled with streptavidin-R-phycoerythrin) are added. After this incubation period a centrifugation or filtration step is used to separate the unbound components. The washed bead suspension is directly read with the flow cytometer. High-speed digital signal processing classifies the microspheres according to their spectral properties and quantifies the reaction on the surface. Suspension microarrays have not been widely applied to the food safety sector but this emerging technology shows promise for the sensitive and effective detection of drug residues.

1.3.5.3 Biochip arrays

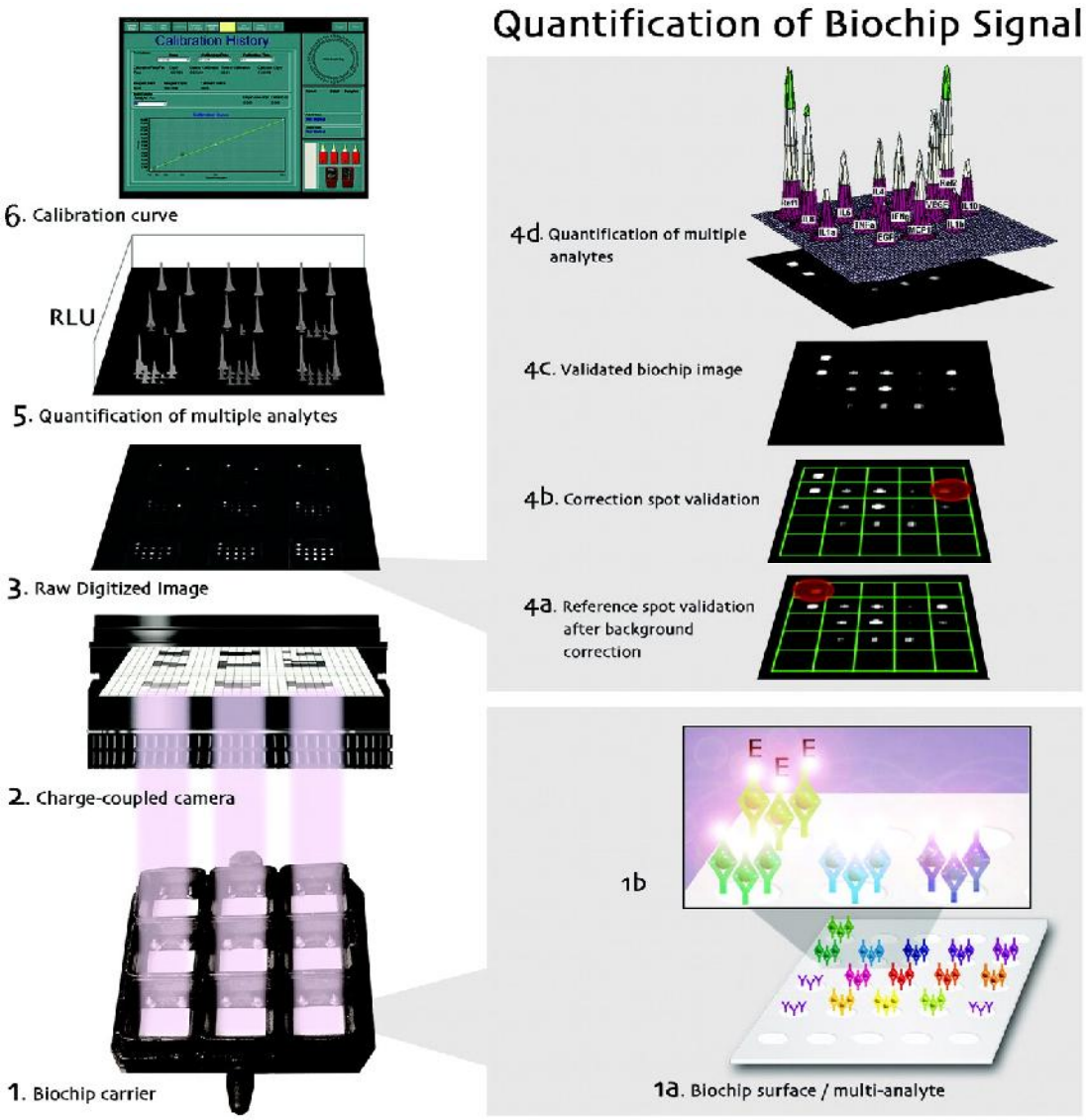
Although biochip array technology is mainly associated with DNA analysis this technology is not limited to DNA analysis. Protein microarrays, antibody microarray, and chemical compound microarrays can also be produced using biochips. In 2003 Randox Laboratories Ltd. launched the Evidence Investigator™, the first protein biochip array analyser. In biochip array technology, the biochip replaces the ELISA plate or cuvette as the reaction platform. Biochip arrays may be fabricated using non-contact piezoelectric nano-dispense techniques for accurate dispensation of capture molecules in picolitre to nanolitre quantities. The silanation method is a contact immobilisation approach which is simple and cost-effective and shows a lower signal-to-noise ratio than other derivatized surfaces.

Photolithography activation methods using light directed through a photo mask to modify the array surface at specified locations has also been reported for ligand attachment. Other array fabrication techniques involve direct array surface contact with solid or split pins.

The biochip array may be used to simultaneously detect several analytes in a single sample using sandwich, competitive and antibody-capture immunoassays. Capture ligands (antibodies) are attached to the surface of the biochip in defined discrete test regions (DTRs), in an ordered array.

Analytes present in the sample are captured by their respective antibodies and on antibody-antigen binding a chemiluminescence reaction produces light which is detected by a charge-coupled device (CCD) camera. The CCD camera is equipped with a sensitive high-resolution sensor which accurately detects and quantifies very low levels of light. The test regions are located using a grid pattern and the chemiluminescence signals are analysed by imaging software to rapidly and simultaneously quantify the individual analytes.

This technology has been used to screen for benzodiazepines, opiates, cocaine, cannabinoids, in haemolysed whole blood (Grassin Delyle *et al.*, 2008), and in clinical and research applications (Licastro *et al.*, 2006; Sachdeva *et al.*, 2007; Fabre *et al.*, 2008; Kavsak *et al.*, 2009; Roh *et al.*, 2009; Zetterberg *et al.*, 2009). In addition, biochip arrays have been developed for the detection of antimicrobial veterinary drugs, synthetic steroids and growth promoters; however the validation of these arrays in food applications has not been widely reported. This multiplex approach to drug residue screening in food may provide an invaluable tool for the rapid screening of veterinary drug residues in food.



Source: http://www.randox.com/Evidence%20Investigator.php

Figure 1.9 Schematic of Randox biochip array assay format

1.3.6 Lateral flow immunoassays

Lateral flow immunoassays (LFIAs) or immunochromatography assays are designed for rapid on-site testing and generally require little or no sample or reagent preparation. LFIAs use membrane-based test devices in either dipstick or flow-through enzyme immunoassay formats (O'Keefe *et al.*, 2003). These tests operate on a purely qualitative basis whereby a positive test is indicated by the presence of a coloured line. The development and combination of specific antibodies, colloidal particles (carbon, silica, gold, latex, etc.) as labels and lateral flow membrane devices have permitted the production of the lateral flow immunoassay (LFIA) (Van Amerongen *et al.*, 1993).

Lateral flow devices operate in both competitive and sandwich assay formats. In the sandwich format the sample is added to the test strip and capillary action draws it towards antibodies labelled with chromagenic particles which are impregnated into the nitrocellulose surface. An antibody-antigen complex is formed and moves further along the surface. This encounters a test strip containing secondary antibodies to the same target but to a different epitope which will produce a coloured line in positive samples. The competitive format is more often used for the detection of small molecules. In this the test sample first encounters antibodies that are already bound to the analyte of interest. As the sample migrate along the surface and reaches the capture zone an excess of unlabelled analyte will bind to the immobilized antibodies and block the capture of the conjugate, so that no visible line is produced. The unbound conjugate will then bind to the antibodies in the control zone producing a visible control line. A single control line on the membrane is a positive result. Two visible lines in the capture and control zones is a negative result. However, if an excess of unlabelled target analyte is not present, a weak line may be produced in the capture zone, indicating an inconclusive result.

1.3.7 Time-resolved flouresence immunoassays (TR-FIAs)

The TR-FIA exploits the fluorescent properties of lanthanide ions such as Eu^{3+}, Sm^{3+}, Dy^{3+} and Tb^{3+}, these tri-valent cations emit light at well-defined wavelengths. The sandwich format of the assay uses lanthanide-labelled antibodies which are added to the sample and an antibody-antigen complex is formed. This complex is added to a solid phase to which a second antibody is attached. The bound lanthanide chelate is then measured with a single-photon-counting fluorometer designed to measure only the specific lanthanide fluorescence with a long decay time. This is achieved when the background fluorescence reaches an insignificant level. The specificity of lanthanide fluorescence is further increased by the large difference between the excitation and emission wavelengths of lanthanide compounds (Stokes shift) and sharp emission profile (Lakowicz, 2006). This fluorescence can be dramatically increased when lanthanide ions are coordinated with the appropriate organic ligand.

Conventional fluorometry using fluorescein has been applied in several routine assays; however there are some drawbacks including insufficient separation of fluorescence from emission and excitation, Rayleigh and Raman scattering, background fluorescence form optics, cuvettes and samples, non-specific binding of reagents and fluorescence quenching (Deshpande, 1996). An assay that utilizes a fluorescent label eliminates the need for an enzyme, thus potentially making this method less susceptible to interferences. In addition, the use of lanthanide chelates and time-resolved technology has been developed to improve the detectability of the label over conventional fluorescence detection methods (Reimer, Gee and Hammock, 1998). TR-FIAs have been widely applied in immunodiagnostics and have become increasingly popular for veterinary drug analysis in food.

Section B

Immunochemical screening assays for veterinary drug monitoring in food

1.4 Anthelmintic and antiprotozoan drug residues

1.4.1 Benzimidazole anthelmintic drugs

Brandon *et al*. (1992) developed a mAb-based ELISA to detect thiabendazole (TBZ) in liver tissue and compared three different TBZ extraction methods for the assay. Homogenized liver samples were added to either 10% (v/v) dimethylsulphoxide (DMSO), phosphate buffered saline (PBS) or water, stirred, centrifuged and the supernatant was assayed directly. The water extraction procedure demonstrated high recovery levels for TBZ and its metabolite 5-hydroxy-TBZ and these results were confirmed by HPLC analysis. An assay LOD of 20 μg kg^{-1} was reported for both TBZ and 5-OH-TBZ residues in liver tissue.

Brandon *et al.* (1994) produced a mAb that cross-reacted to albendazole (ABZ), fenbendazole (FBZ) and several of their metabolites. This mAb was also specific for methyl benzimidazolecarbamate (MBC), a metabolite and breakdown product of the pesticide benomyl. This mAb was used to develop an ELISA to detect multiple benzimidazole drug and pesticide residues in liver tissue. The sulfoxide and sulfone metabolites of ABZ and FBZ were readily extractable and quantifiable by this method. ELISA analysis of liver tissue from cows treated with fenbendazole produced excellent agreement with the results of HPLC analysis. In bovine liver samples fortified with equal amounts of benzimidazole drug and sulfoxide and sulfone metabolites, the limits of detection (LODs) were 58 μg kg^{-1} and 120 μg kg^{-1} for ABZ and the FBZ compounds, respectively. This assay format was later coupled with an ELISA to detect thiazole-containing fungicide compounds (Brandon *et al*., 1992) and a screening assay for a range of benzimidazoles in liver tissue was established (Brandon *et al.*, 1998). Using this ELISA, ABZ residues could be detected below their MRLs, however, the remaining analytes investigated could only be detected at or above their MRLs. Hence this method was not suitable as a screening method for these residues in liver.

In 2002, a competitive ELISA, using a mAb, was developed to detect FBZ residues in bovine milk (Brandon *et al*., 2002). Milk samples were diluted in PBS-Tween containing bovine serum albumin (BSA) prior to analysis. The assay LOD was determined to be 7 μg kg^{-1} and the assay results compared well to chromatographic methods. In addition, mAb-based ELISAs have been produced to detect TBZ residues in the peel of apples and potatoes (Brandon *et al*. 1993). Using an aqueous extraction method a LOD of 200 μg kg^{-1} was reported in both apples and potatoes. The same research group also reported an ELISA for the detection of TBZ in apple, potato, orange, grapefruit and banana peels (Brandon *et al.* 1995). An assay LOD of 100 μg kg^{-1} was reported in peel samples, corresponding to between 10 and 40 μg kg^{-1} in the whole fruit or tuber.

Bushway *et al*. (1995) reported a quantitative ELISA to detect TBZ residues in fruit juice and bulk juice concentrates. Fruit juice samples were prepared by dilution in a phosphate buffer. However, to eliminate matrix effects during analysis, bulk juice concentrate samples required partitioning into methylene chloride and high-speed centrifugation. The average TBZ recovery for juices and concentrates was 93%.
The correlation between samples tested by ELISA and HPLC was reported at an R^2 value of 0.92.

A competitive, indirect ELISA for TBZ was developed and applied to the analysis of fruit juices (Abad *et al*., 2001). Fruit juices were analysed by diluting samples in assay buffer, without extraction or cleanup. The assay detection limits were determined for TBZ in banana (5 μg L^{-1}), apple (20 μg L^{-1}) and pear (20 μg L^{-1}) juices. Polyclonal antibodies have also been developed to detect TBZ residues in vegetables by ELISA (Bushway *et al.,* 1994).
Johnsson *et al*. (2002) developed the first biosensor assay to detect benzimidazole carbamates in bovine serum using a pAb raised in sheep against a carboxyalbendazole derivative. The pAb showed ≥74% cross-reactivity towards ABZ, fenbendazole-sulphone (FBZ-SO_2), mebendazole (MBZ), flubendazole (FLU) and oxibendazole (OXI) residues.

During bovine serum analysis it was observed that matrix effects were caused by non-specific binding of bovine serum globulins and albumins. A sample preparation procedure using saturated ammonium sulphate (SAS) precipitation plus a high speed centrifugation step removed these proteins and minimized the matrix effect. Using this procedure the LOD for the method was detemined to be below 5 μg L^{-1}. This sensitive multiresidue benzimidazole assay demonstrated potential as a screening assay for the detection of benzimidazoles in food.

The screening of food for benzimidazoles residues is, for the most part, performed by ELISA detection. These methods require different sample preparation methods based on the type of benzimidazole drug and the food matrix. In veterinary drug monitoring a single food sample may need to be analysed for several different benzimidazole residues. For this reason a single robust extraction procedure for benzimidazole drugs in a variety of matrices would be extremely beneficial. In this way the same sample extract could be applied to each assay format, and thus both sample preparation time and cost of would be reduced.

1.4.2 Avermectins

A sensitive competitive ELISA method for the detection of ivermectin residues in bovine liver is reported in the literature by Crooks *et al.* (1998a). Liver samples were extracted with MeCN and applied to a competitive ELISA using a rabbit pAb. An assay LOD of 1.6 μg kg^{-1} was reported for the method. Intra- and inter-assay RSDs were determined as 8.8 and 14.6%, respectively. Liver tissue samples from animals dosed with ivermectin (or incurred liver) was analysed by ELISA and the results were confirmed using a HPLC method and a high level of correlation (R^2 = 0.99) was reported.

More recently an ELISA screening test for moxidectin was reported which could detect residues in bovine milk, fat and muscle at 2, 19 and 1 μg kg^{-1}, respectively (Dubois *et al*., 2004). A pAb raised in rabbit towards moxidectin-BSA was applied for moxidectin detection. This effective method was produced as a commercially available ELISA kit.

Subsequently an indirect competitive ELISA to detect three avermectins in liver using a MeOH-based extraction was reported. In this case a pAB raised against an abamectin immunogen in rabbit showed the highest specificity and sensitivity (Shi *et al*., 2006).

A dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) method was reported for ivermectin residues in milk (Crooks *et al*., 2000). Ivermectin was extracted from milk samples into MeCN and the extract washed with hexane. The MeCN layer was evaporated to dryness and the residue resuspended in ethyl acetate. The ethyl acetate was applied to a solid phase extraction (SPE) column, the eluate was evaporated to dryness and the residue was resuspended in assay buffer prior to DELFIA analysis. An LOD of 4.6 μg L^{-1} was reported for this method from the analysis of only 17 milk samples that were negative for ivermectin. Consequently this ELISA does not satisfy any of the current legislative requirements (2002/657/EC) for a qualitative screening assay.

It was a logical progression to further develop ivermectin residue tests using immunosensor technology. Samsonova *et al.* (2002a) described a biosensor method capable of detecting ivermectin at a detection limit of 19.1 μg kg^{-1} in bovine liver. A 5-O-succinoylivermectin-apo-transferrin derivative (Crooks *et al.*, 1998a) was used to produce a mAb (Crooks *et al*., 2000) while a second derivative, ivermectin oxime, was immobilized onto the surface of the sensor chip. The use of the oxime derivative was due to its chemical stability. A MeCN extraction method followed by a C_{18} SPE clean-up was used to prepare bovine liver samples. Although the mAb showed significant cross-reactitvity towards five avermectins the assay was only validated against ivermectin. This was because insufficient extraction efficiency was observed during the analysis of the other four avermectins.

Samsonova *et al.* (2002b) subsequently applied this SPR assay to the detection of ivermectin residues in bovine milk. A detection limit of 16.2 μg L^{-1} was achieved and extraction and analysis of 20 milk samples could be performed within a single working day.

1.4.3 Levamisole

Ovine pAbs were raised to an aminolevamisole-ovalbumin immunogen and incorporated into an ELISA to detect levamisole in muscle and milk by Silverlight and Jackman (1994). Muscle samples were prepared by homogenization in phosphate buffered saline (PBS) pH 7.0 and milk samples were assayed undiluted. The assay LOD was 1 μg kg^{-1} in both muscle and milk. At the time this was acceptable for detection of levamisole in milk. However, in 1998 levamisole was banned in milk for human consumption. Therefore an alternative method of levamisole detection was required.

Crooks *et al.,* (2003) described a SPR biosensor method for levamisole detection in liver and milk. A pAb was raised in rabbit to an aminolevamisole-bovine serum albumin immunogen because levamisole does not lend itself to direct coupling to a carrier protein. Aminolevamisole was immobilized to the sensor surface using amine coupling and levamisole was extracted from liver and milk samples using MeCN. The assay LOD was determined to be 6.8 μg kg^{-1} in liver and 0.5 μg kg^{-1} in milk. When the biosensor assay performance was assessed by direct comparison with an LC-MS procedure a high level of correlation was evident from the results (r^2 = 0.998 and 0.985) for liver and milk analysis, respectively. This correlation demonstrated that the assay was suitable for qualitative analysis and the quantitative information was also reliable.

1.5 Anticoccidial drugs

1.5.1 Ionophores

Several immunochemical assays have been developed to detect ionophore residues. These substances are administered in feed for the treatment of coccidiosis in intensively reared species. As a result of their cardiotoxic properties, ionophore residues need to be continuously monitored in food and feed to protect public health. Substances included in this grouping include monensin, salinomycin, narasin, lasalocid, maduramycin and semduramicin. Several immunoassays have been developed for the remaining molecules. In general, a wide range of antibodies are required to analyse for the complete range of ionophores highlighting the need to multiplex assays.

An ELISA method for the detection and quantification of monensin residues in chicken liver was reported by Crooks *et al.* (1997). The assay was developed using a pAb raised against a monensin–transferrin immunogen raised in rabbitts. Liver samples were extracted using water and MeCN. The pH of extracts was adjusted using sodium hydroxide and samples were extracted using a hexane-diethyl ether mixture. A portion of this extract was evaporated to dryness and extracts were reconstituted in ethanol and sodium acetate prior to analysis. The LOD of this ELISA was 2.91 μg kg^{-1}. The same group subsequently developed a rapid DELFIA assay for detecting monensin residues in avian plasma using an all-in-one dry chemistry concept (Crooks *et al*., 1998b). The assay specific components were pre-dried onto microtitration plate wells and only the addition of the serum sample diluted in assay buffer was required to perform analysis. Results were available one hour after sample addition. The LOD of the assay (14.2 μg L^{-1}) and the intra- and inter-assay RSD were reported to be 15.2 and 7.4%. Hagren *et al*. (2006) developed a method to detect monensin residues in eggs using a competitive time-resolved flouroimmunoassay. Monensin residues were extracted from eggs with MeCN using simple protocol reported by Peippo *et al*. (2004). CCβ was <2 μg kg^{-1} for eggs. Watanabe *et al.* (1998) reported a quantitative ELISA and LFIA for monensin detection in chicken plasma and cattle milk using a mAb.

Monensin was extracted from samples using methanol and chloroform. The LOD of the ELISA was 80 and 16 ng mL^{-1} for bovine plasma and milk, respectively. The LODs of the LFIA were 40, 40 and 160 μg kg^{-1}, in milk, bovine plasma and avian plasma, respectively.

Microbiological bioautographic screening assays developed for salinomycin detection in food (Vander Kop and MacNeil, 1990) did not provide adequate sensitivity and were labour intensive. Elissalde *et al*. (1993) produced a mAb and subsequently employed it to develop a competitive ELISA to detect salinomycin residues in poultry liver. Liver samples were extracted with buffer and the supernatant was directly analysed by ELISA. Muldoon *et al.* (1995) later validated this ELISA and determined the LOQ of assay to be 50 μg kg^{-1}. Kennedy *et al.* (1995) subsequently reported an ELISA for salinomycin detection in avian liver with a LOD of 0.2 μg kg^{-1}. Watanabe *et al.* (2001) reported a mAb-based ELISA for detecting salinomycin in avian plasma. Plasma samples were simply diluted in assay buffer prior to analysis. The assay was also applied to avian liver and muscle, which were extracted in 80% methanol and diluted in assay buffer. The LODs for plasma, liver and chicken muscle were equal to 10 μg kg^{-1}. The current EU MRL for salinomycin is 5 μg kg^{-1} in chicken tissue; therefore this assay did not provide the sensitivity to detect the residues at a suitable level.

A TR-FIA method was reported for screening narasin residues in avian plasma using a pAb raised against monensin (Peippo, Lövgren and Tuomola, 2005). Plasma samples were diluted prior to TR-FIA analysis. The performance of the assay has been confirmed by validation according to Commission Decision 2002/657/EC and a CCα of 1.2 μg L^{-1} and a CCβ of 1.5 μg L^{-1} were reported. A relationship was observed between the concentrations of narasin in plasma and breast muscle ($R^2 = 0.83$) and leg muscle ($R^2 = 0.90$). These results indicated that the analysis of poultry blood samples may be used as a predictor of narasin residues in muscle.

A number of groups have employed antibodies showing cross-reactivity to both salinomycin and narasin. Peippo *et al.*, (2004) developed a TR-FIA using a pAb to simultaneously detect narasin and salinomycin residues in eggs and meat using an MeCN extraction. Muscle samples required additional SPE. Egg and muscle extracts were subsequently concentrated and resuspended in Tris-HCl buffer (pH 7.75) prior to analysis. Mean recovery was 81 to 91% (based on narasin) for muscle and eggs. The LOD of the assay was 0.56 and 0.28 μg kg^{-1} in muscle and eggs, respectively.

Kennedy *et al.* (1998) developed an ELISA for the detection of lasalocid residues in serum, liver and muscle tissues. The assay was capabale of detecting residues as low as 0.15 μg kg^{-1} but depended on the matrix. Watanabe *et al.* (2004) developed ELISAs for detecting lasalocid and semsuramicin in avian muscle and liver. The LODs were 5 and 10 μg kg^{-1} for muscle and liver, respectively. Shen *et al.* (2001) reported an ELISA for the detection maduramicin residues in muscle, liver and fat tissues. Samples were extracted with MeOH and fat was separated by overnight freezing at -20°C and centrifugation. Extracts were concentrated and resuspended in PBS:MeOH (90:10, v/v) and purified by immunoaffinity chromatography (IAC). The collected fraction was diluted in PBS Tween prior to ELISA. LODs for muscle, liver and fat were 1, 2.8 and 1.μg kg^{-1}, respectively.

1.5.2 Chemical coccidiostats

Hagren *et al.* (2005) described a TR-FIA for detecting halofuginone residues in eggs and avian liver. The extracted sample was added to the well and after a 15 min sample incubation period the fluorescence signal is measured directly from the surface of the dry well. LODs were 1.7 and 1.0 μg kg^{-1} for egg and liver, respectively. The assay was validated according to Commission Decision 2002/657/EC and the authors also suggested that the assay sensitivity may be improved further by adjusting the dilution factor of the samples.

M^{c}Carney *et al.* (2003) described an SPR biosensor screening assay for nicarbazin in poultry liver and eggs using a pAb. The pAb was raised in a rabbit to a structural mimic of DNC in an inhibition assay format (Connolly *et al.*, 2002). Another DNC mimic was immobilised onto a CM5 sensor chip. Nicarbazin was extracted from liver and egg samples using MeCN. Liver samples required an additional hexane wash to clean-up samples. The assay LOD for liver (17 μg kg^{-1}) and eggs (19 μg kg^{-1}) were determined and a high correlation between the SPR biosensor and the LC methods was reported when nicarbazin incurred liver was tested. Using this rapid method a single operator can analyse up to 20 livers or 30 egg samples in one working day which can be used in a qualitiative or quantitative mode. Hagren *et al.* (2004) subsequently applied this in a Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) to detect nicarbazin in eggs and liver. LODs were 3.2 and 11.3 μg kg^{-1} for egg and liver, respectively. Huet *et al.* (2005) subsequently developed a competitive ELISA for detecting nicarbazin in eggs and muscle with LODs of 3 and 10 μg kg^{-1}, respectively.

Antibodies have been developed to detect diclazuril (Fodey *et al.,* 2007), robenidine (Fodey *et al.,* 2007) and toltrazuril (Connolly *et al.,* 2003). However, few assays have been reported to detect anticoccidial residues in the literature.

1.5.3 Banned nitroimidazole anticoccidials

Nitroimidazoles are banned within the EU under directives 377/2010 and 2205/201. However, the illegal use of these drugs persists in many countries exporting to the EU (Sanco/3400/2005). As a result, sensitive screening tests have been developed to detect both the parent nitroimidazoles and metabolite forms of the drug. Huet *et al*. (2005) developed a competitive ELISA to detect nitrimidazole residues in egg and muscle that displayed cross-reactivity to dimetridazole (DMZ), ronidazole (RNZ), hydroxydimetridazole (DMZOH), and ipronidazole (IPZ). Residues were extracted from muscle and egg samples with MeCN and were later defatted with hexane. The CCβs for dimetridazole were <1 μg and <2 kg^{-1} for egg and muscle, respectively. CCβs for MNZ, RNZ, MNZ-OH and IPZ in both matrices was <10, <10, <20 and <40 μg kg^{-1}, respectively.

A biosensor assay was developed by Connolly *et al*. (2007) to detect nitroimidazoles in chicken muscle using a pAb raised in sheep. A MNZ-DSC derivative was immobilised onto an EDC/NHS activated CM5 sensor chip via a di-succinimidyl carbonate (DSC) cross-linker (Fodey *et. al*., 2003). Residues were extracted with ethyl acetate and the CCβ was determined as <1 μg kg^{-1} for DMZ, MNZ and RNZ and <2 μg kg^{-1} for MNZ-OH and DMZ-OH. A biosensor method was later reported by the same research group to detect seven nitroimidazoles residues in porcine, bovine and ovine kidney, avian liver, serum and eggs and bovine milk (Thompson *et al*., 2009). Samples were extracted using MeCN and clarified using high speed centrifugation. Egg samples, however, required additional filtration. CCβs of less than 1 μg kg^{-1} were achieved for DMZ in all species and matrices investigated.

1.6 Antimicrobial drugs

1.6.1 Aminoglycosides

Hammer *et al*. (1993) developed a competitive ELISA to detect streptomycin residues in milk. An antibody-capture format was used which could detect streptomycin at 1.6 μg kg^{-1}. Schnappinger *et al.* (1996) applied a nylon membrane support to a rapid enzyme LFIA for streptomycin detection in milk. The LODs for streptomycin and dihydrostreptomycin were 6.0 and 0.8 μg L^{-1}, respectively. An ELISA was subsequently reported for screening honey samples for streptomycin (Heering *et al.,* 1998). A two stage sample preparation procedure was adopted to reduce matrix interference and improve recovery. This assay showed good reproducibility and provided semi-quantitative results. An ELISA was developed by Haasnoot *et al*. (1999) to detect gentamicin, neomycin and streptomycin in milk and honey. LODs for all three aminoglycosides were far below their corresponding MRLs.

Several SPR-biosensor immunoassays have been reported for the direct detection of gentamicin (Haasnoot *et al*., 2001) and streptomycin residues in milk (Haasnoot *et al*., 2002).

In these direct assays, mAbs were immobilised on the biosensor chip surface and binding of aminoglycosides was measured directly. In order to obtain sufficiently high responses, highly purified antibodies and high immobilisation levels were required.

These direct immunoassay formats were difficult to optimise and a competitive biosensor assay was subsequently developed. Gentamicin, streptomycin, kanamycin, and neomycin derivatives were immobilised on the surface of a chip in four flow cells (serially connected), and a mixture of selected specific antibodies was used. The LODs ranged between 15 and 60 ng mL^{-1} (Haasnoot *et al*. 2003).

Ferguson *et al*. (2002) reported a matrix comprehensive biosensor assay for streptomycin and dihydrostreptomycin residues in whole bovine milk, honey, porcine kidney, and porcine muscle. The assay was compared to a commercial ELISA kit and a HPLC assay. A streptomycin derivative was used to prepare a reusable sensor-chip surface, and an antibody showing high cross-reactivity with dihydrostreptomycin was employed (Baxter *et al*., 2001). No extraction was required for the milk assay, and honey samples had only to be diluted. The other matrices were first homogenized in an aqueous buffer and then clarified by centrifugation. Good agreement was found between the biosensor method and both ELISA and HPLC techniques.

1.6.2 β-lactams

The first report of a SPR biosensor assay to screen for penicillin in milk was in 2001 (Gaudin *et al*., 2001). An antibody raised against a hydrolyzed form of ampicillin was used for detection. This antibody was of limited use because it did not provide the specificity required to detect the active forms of penicillin covered by EU legislation (Gaudin *et al*., 2001). Gustavsson, Bjurling and Sternesjo (2002) reported a biosensor to detect penicillin G in milk whereby the β-lactam receptor, protein carboxypeptidase was used as the detection molecule. Using this novel approach a SPR biosensor assay was developed that could detect the active form of the β-lactam ring structure along with penicillin G., amoxicillin, ampicillin, oxacillin, cefalexin, cephapirin, and ceftiofur in milk below their respective MRLs (Gustavsson and Sternesjo, 2004).

The assay performance was compared to several commercial microbial inihibition tests showing good agreement in results (Gustavsson and Sternesjo, 2004)

An SPR-based optical biosensor assay for penicillins and cephalosporins in milk was reported by Cacciatore *et al.* (2004). The assay was based on inhibition of the binding of digoxigenin-labeled ampicillin (DIG-AMPI) to the penicillin-binding protein 2x (PBP2x). This assay could detect four penicillins (penicillin G, ampicillin, amoxicillin, cloxacillin) and two cephalosporins (cephalexin, cefoperazone) in fortified raw milk below their respective MRLs. However the authors reported non-specific binding between matrix components and the sensor surface which affected the reproducibility of the assay (Cacciatore *et al.*, 2004).

Cliquet *et al.* (2005) compared the performances in buffer of two penicillin-specific mAbs and one penicillin-specific pAb in a competitive ELISA and a SPR biosensor assay. One of the mAbs showed a higher level of cross-reactivity to oxacillin, cloxacillin, and dicloxacillin in the biosensor assay system than in the ELISA. In the biosensor assay format ampicillin was detected below its MRL but not in the ELISA. The authors also reported higher sensitivity in both assays using the pAbs as opposed to the mAbs (Cliquet *et al,* 2005).

1.6.3 Sulphonamides

An indirect competitive ELISA was developed to detect sulphonamides using a pAb raised in rabbit towards N^1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]sulphanilamide (Assill, Sheth and Sporns, 1992). The IC_{50} values in buffer were reported for sulphaquinoxaline (0·032 mg L^{-1}), sulphadimethoxine (0·58 mg L^{-1}), sulphachloropyridazine (0·87 mg L^{-1}), sulphathiazole (4·8 mg L^{-1}), sulphamoxole (5·2 mg L^{-1}), sulphamethazine (10 mg L^{-1}) and sulphapyridine (10 mg L^{-1}). The assay performance was not evaluated in a food matrix.

One of the first applications of a SPR biosensor to veterinary drug residue analysis in food was reported to detect sulphamethazine residues in milk and skim milk (Sternesjo *et al*., 1995). Milk samples were simply defatted by centrifugation prior to analysis. The LOD was less than 1 nM.

Akkoyun *et al.* (2000*)* subsequently reported an SPR biosensor assay for sulphamethazine detection in urine using a mAb against sulphamethazine and a corresponding anti-idiotype antibody. Sensor surfaces were prepared by immobilizing the anti-idiotype antibody (Clone 12E12) in the biosensor system using EDC/NHS carbodiimide chemistry. The assay showed a LOD of 5 $\mu g\ kg^{-1}$, which is 0.05% (w/v) of the 100 $\mu g\ kg^{-1}$ MRL for sulfmethazine.

The on-line detection of sulphamethazine and sulphadiazine in porcine bile using a multi-channel high-throughput SPR biosensor was reported by Situ *et al*. (2002). Polyclonal antibodies (Pabs) were raised in sheep and rabbit towards sulphamethazine-bovine thyroglobulin and sulphadiazine-human serum albumin, respectively. Sulphamethazine and sulphadiazine derivatives were immobilised onto the eight lane prototype sensor chip using a standard amine coupling procedure. The performance of this multi-channel biosensor was tested in the laboratory and in an abattoir environment. The instrument was capable of the simultaneous analysis of eight samples for a single analyte or multi-analyte analysis. An automated sample pipetting station was incorporated into the system for the direct analysis of up to 650 bile samples for sulphamethazine and sulphadiazine per day. The assay performance was assessed in a laboratory-based trial by comparing 1751 results of the prototype assay method to a routine Biacore™ procedure. The rate of false positive results were calculated as 0.86% for sulphamethazine and 1.48% for sulphadiazine using the prototype biosensor compared to 0.63% and 0.69% sulphadiazine when using the Biacore™ instrument. During an on-site study at an abattoir 6069 bile samples were analysed and no false negative results were recorded were recorded. This study clearly demonstrated the potential of high-speed SPR biosensor technology for high-throughput veterinary drug detection.

A mAb raised against sulphamethazine was applied in an optical SPR biosensor (Biacore™ Q) to develop a rapid immunoassay for the detection of eight sulfonamides in chicken serum (Haasnoot, Bienenmann-Ploum and Kohen, 2003). The performance of this mAb biosensor assay was compared with two existing pAbs. The mAb-based resulted in improved sensitivity with LODs for eight sulfonamides in diluted chicken serum in the range 7 to 20 μg L^{-1}.

Subsequently, McGrath *et al.* (2005) validated a multi-sulfonamide screening assay for porcine muscle using a commercially available Qflex Kit using a Biacore™ Q instrument. The assay binding protein showed significant cross-reactivity towards 19 sulfonamide drugs but no cross-reactivity was seen towards acetylated sulfonamide metabolites. The LOD was reported to be 16.9 μg kg^{-1} in muscle tissue. Intra-assay and inter-assay precision for sulphamethazine and sulphadiazine fortified was determined to be <10%. The assay LOD could be applied as the CCα for this assay; however no CCβ was reported for the method.

A direct-binding optical waveguide lightmode spectroscopy-based (OWLS) immunosensor was reported to detect sulphamethazine (Kim, Kim and Kim, 2008). The antibody was immobilised in situ by covalent binding of an anti-sulfmethazine antibody over the surface of a glutaraldehyde-activated 3-aminopropyltriethoxysilane-treated sensor chip. The sensor response was directly proportional and specific to antibody–antigen complexation. The authors reported a sensor LOD of 10^{-8} M. This immunosensor has not yet been applied to the detection of sulfamethazine (SMZ) in food.

A LFIA device was reported for the detection of sulfmethazine in bovine and porcine urine using a rat mAb (O'Keefe *et al*., 2003). Apart from sulfmethazine, the mAb showed significant cross-reactivity to N4-acetylsulphamethazine, sulphamerazine and sulphisoxazole. Urine samples from sulfmethazine-treated pigs and fortified bovine and porcine urine were tested in an intra-laboratory evaluation.

In an intra-laboratory evaluation of the LFIAs performance, the device showed an overall sensitivity of 100%, a specificity of 71%, and positive and negative prediction values of 73% and 100%, respectively. This device was fabricated as a test kit for determining SMZ residues in animals produced for slaughter.

1.6.4 Phenicols

Scortichini *et al*. (2005) evaluated a commercial ELISA kit and validated it to Commission Decision 2002/657/EC criteria for detection of chloramphenicol in muscle, eggs, honey and milk (Scortichini *et al*., 2005). Muscle, eggs and honey samples were extracted using an acetone/dichlorometane and purified using alumina (muscle and eggs) or C_{18} (honey) SPE. The milk samples were prepared following the instructions of the ELISA kit producer. CCβ values were <0.3 μg kg^{-1} across matrices and recovery rates ranged between 71 and 106 %.

A sensitive biosensor assay was reported for chloramphenicol and chloramphenicol glucuronide residues in poultry, honey, prawn and milk using a SPR biosensor (Ferguson *et al*., 2005). Milk samples were directly analysed without the need for sample preparation. Avian muscle samples were homogenized in PBS buffer and extracted into ethyl acetate. An aliquot of the organic layer was concentrated and extracts were reconstituted in buffer. Following high speed centrifugation the aqueous layer was carefully removed using a syringe and needle and filtered through 0.22 μm, while avoiding the upper lipid layer. The sample preparation method used for avian tissue was employed during the preparation of prawn and honey samples. During the preparation of prawn samples, the sample was not diluted in PBS buffer and the filtration step was omitted. This filtration step was required during the honey preparation. CCβs of 0.02, 0.02, 0.07 and 0.07 μg kg^{-1} were achieved for poultry, honey, prawn and milk, respectively.

Ashwin *et al*. (2005) reported a SPR biosensor assay for chloramphenicol (CAP) and the metabolite chloramphenicol-glucuronide in honey, porcine kidney, milk powder and prawns. Samples were extracted using MeOH:water and purified by SPE.

Kidney samples required an additional hexane defatting step prior to SPE. CCα and CCβ were reported to be less than 0.1 μg kg^{-1} for all matrices. The assays were also evaluated using incurred tissues with good agreement between this biosensor method and a liquid chromatography–tandem mass spectrometry method.

Zhang, Zuo and Ye (2008) developed a highly selective and sensitive competitive immunoassay based on a mesofluidic system to detect chloramphenicol in milk.
The system used amino-silane modified glass beads covalently immobilized with chloramphenicol succinate which were infused into microchannels. Defatted milk sample extracts were mixed with chloramphenicol antibody and injected across the surface of the glass beads. The chloramphenicol antigen-antibody complex anchored on the beads was probed with Cy5-labelled secondary antibody. The fluorescence intensities of the beads were employed to determine the concentration of chloramphenicol. The assay detection limit was 0.008 μg L^{-1} and recovery ranged between 90 and 108%.

A comprehensive multi-residue SPR biosensor assay was developed for the simultaneous detection of thiamphenicol (TAP), florefenicol (FF), florefenicol amine (FF amine) and chloramphenicol (CAP) residues in shrimps (Dumont *et al.*, 2006)). The assay used a pAb raised in rabbit to two immunogens; chloramphenicol-hemisuccinate-bovine serum albumin (CAP-HMS-BSA) and florefenicol amine-(3-maleimidobenzoyl-N-hydroxysuccinimide ester)-keyhole limpet cyanin (FF amine-MBS-KLH). Stable chip chemistries were produced by immobilising FF amine onto two flow cells and CAP base onto the other two flow cells using EDC/NHS coupling chemistry. Samples were extracted using ethyl acetate, concentrated and resuspended in isooctane/chloroform (40:60, v/v). Residues were subsequently extracted into HBS-EP buffer prior to analysis. The CCβs for CAP, FF, FFA and TAP were 0.1, 0.2 250 and 0.5 μg kg^{-1}, respectively.

1.6.5 Macrolides

Caldow *et al*. (2005) reported the first SPR biosensor screening assay capable of detecting below 10µg kg^{-1} of tylosin residues in honey. The CCβ of the method was determined to be 2.5 µg kg^{-1} and samples examined by both LC-MS/MS and biosensor were in good agreement (R^2 = 0.99, 1.00). No false positive results were observed at ≥2.5 µg kg^{-1}.

1.6.6 Tetracyclines

In sub-therapeutic doses tetracyclines (TCs) show growth promoting effects (Chopra and Roberts, 2001, Michalova *et al*., 2004) and their use for this purpose is banned in the EU (Anonymous, 2003). The UV-HPLC and LC-MS methods reported for the determination of tetracycline residues require extensive sample preparation (Sokol and Matisova, 1994; Stubbings, Tarbin and Shearer, 1996; Cooper *et al*., 1998). Pastor-Navarro *et al.* (2007) synthesized and purified tetracycline haptens based on the formation of their carboxamido and diazo derivatives. The pAbs raised to these haptens were used to develop a sensitive indirect competitive ELISA for tetracycline residues in honey. The pAbs showed significant cross-reactivity to rolitetracycline, oxytetracycline, methacycline and chlortetracycline. Samples were diluted in PBS and filtered through a nitrocellulose membrane prior to analysis. The authors reported an LOD of 0.4 µg L^{-1} and recovery between 79 and 108%.

Zhang *et al.* (2007) developed an indirect heterologous competitive ELISA for TC using pAbs to three different immunogens; TC-o-tolidine-BSA, TC-4-aminobenzoic acid-cationized (c)BSA, and TC-1,1 carbonyldiimidazole-cBSA. The recovery rates from TC-fortified raw milk samples were in the range of 74-116%, while the intra- and inter-assay coefficients of variation were <14.5 and <25.0 %, respectively. The authors reported an LOD of 10 µg L^{-1} however the LOD was estimated as the concentration of TC required to inhibit 10% of antibody binding (IC_{10}). This assay LOD may have been lower had it been calculated as the mean negative response (n = 20) plus three standard deviations above the mean.

A new approach using a biotin-avidin mediated ELISA was developed by Jeon *et al.* (2008) to determine tetracycline residues in milk. Milk samples were added to McIlvaine-EDTA buffer and deproteinised using trichloroacetic acid at pH 4. The fat and protein were separated from the liquid sample by centrifugation and the supernatant was adjusted to pH 7.2. The LOD for TC was 48 ng L^{-1} in raw milk.

1.6.7 Quinolones

The first publication of a biosensor for the detection of quinolones was a SPR assay for the determinatation of enrofloxacin and its main metabolite ciprofloxacin in milk (Mellgren and Sternesjö, 1998). This assay was applied to monitor residues in cows treated for clinical mastitis. Haasnoot *et al*. (2007) developed a SPR biosensor to screen avian muscle and serum for flumequine (FLUM) residues. Polyclonal rabbit anti-sera were raised against two immunogens; FLUM-bovine serum albumin (BSA) and FLUM-keyhole limpet hemocyanin (KLH). Flumequine was immobilised to a CM5 biosensor chip via its carboxyl group using a two-step procedure and an ethylendiamine spacer. Avian muscle samples were extracted using buffer and purified by centrifugation. An IC_{50} of 200 µg kg^{-1} and CCβ of 600 µg kg^{-1} were calculated for this method which showed a high correlation (R^2= 0.99) to an LC-MS/MS confirmatory method.

In 2007 Cao *et al.* demonstrated an SPR biosensor method for the direct detection of enrofloxacin in milk. Denatured deoxyribonucleic acid (dDNA) was deposited layer by layer (LbL) onto a gold sensor surface in the presence a positively charged polymer: poly(diallyldimethylammonium chloride (PDDA). The DNA was used as a broad-selective receptor for flouroquinolones. The binding of enrofloxacin to the DNA coated chip obeyed a Langmuir binding isotherm, being almost linear until 20 µg mL^{-1}. The LOD of the assay was 3 µg mL^{-1}.

An indirect competitive fluorescence-linked immunosorbent assay (cFLISA) using quantum dots (QDs) as the fluorescent marker was developed by Chen *et al.* (2009a) for the detection of enrofloxacin in chicken muscle tissue.

A mAb specific for enrofloxacin was used as the primary antibody and fluorescence detection was carried out using QDs conjugated to goat anti-mouse secondary antibody. This cFLISA demonstrated a linear working range of 1–100 μg L^{-1} for enrofloxacin determination. A 50% inhibition value (IC_{50}) of 8.3 μg L^{-1} and an LOD of 2.5 μg L^{-1} were also reported. The recoveries for chicken muscle samples, fortified with enrofloxacin at levels of 50 – 200 μg kg^{-1}, ranged from 81% to 94% with coefficients of variation (CV) of between 10 and 13%. In tissue taken from enrofloxacin treated chickens, the results of cFLISA were similar to those obtained from an indirect competitive enzyme-linked immunosorbent assay (cELISA) and a HPLC method.

Marchesini *et al*. (2007) described a strategy for coupling a flouroquinolone biosensor screening method with MS for confirmation of analyte identity in chicken muscle by LC-electrospray ionization (ESI) quadrupole time-of-flight (TOF)-MS. A dual SPR-biosensor immunoassay was developed for this purpose, coupling a multi-FQ biosensor immunoassay for the detection of norfloxacin, ciprofloxacin, enrofloxacin, difloxacin, and sarafloxacin with a specific biosensor assay for flumequine. Samples found to be non-compliant in the screen were concentrated and fractionated by gradient LC. The effluent was divided between two identical 96-well plates; one was rescreened with the dual SPR system to generate an immunogram, and the positions of the immunoactive wells were used in the second 96-well plate for identification by high resolution LC-TOF-MS.

It was proposed that the approach could be used to discover unknown chemicals showing activity in the dual biosensor immunoassay. Further developments have led to on-line nanoscale coupling of a SPR biosensor-based screening assay for enrofloxacin and its main metabolite, ciprofloxacin, with nano-LC-ESI-TOF-MS for identification (Marchesini *et al*., 2008).

In 2008 Huet *et al*. developed a rapid SPR biosensor screening assay for 12 flouroquinolones and oxolinic acid in three matrices (egg, fish and poultry meat).
Avian muscle, fish and egg samples were extracted with MeCN, concentrated and resuspended in PBS (pH 7.4). Extracts were defatted prior to analysis. Polyclonal antisera from rabbits injected with the immunogen norfloxacin-BSA effectively bound to a norfloxacin-immobilised chip and significant cross-reactivity was observed for six fluoroquinolones but not for flumequine. Consequently a bi-valent antibody was raised in rabbit against a dual norafloxacin-flumequine immunogen. Several sensor chips were prepared using a combination of hapten derivatives. A fluoroquinolone derivative was immobilized onto a carboxymethyl dextran sensor chip using standard amine coupling. This chip produced the highest sensitivity when used with the bi-active polyclonal antibody. This biosensor assay detected 13 flouroquiniolones below established MRLs. Norafloxacin could be detected in the range 0.1-10 μg kg^{-1} in egg and poultry meat and 0.1-100 μg kg^{-1} in fish. When the SPR biosensor assay was compared to an LC-MS/MS method, using incurred sample material, the data generated were in good agreement (R^2 = 0.96).

1.6.8 Nitrofurans

It is recognised that methods for detecting nitrofuran drugs by measuring the parent species is inappropriate because the drugs are rapidly metabolised in vivo and do not persist in edible tissues (Nouws *et al.,* 1990, McCracken *et al.,* 1995). Nitrofuran metabolites bind to tissue proteins in animal tissues after treatment (McCracken *et al.,* 1997, Hoogenboom *et al.,* 1991, Gottschall *et al.,* 1995, Hoogenboom 1992). A nitrofuran ELISA was developed using a mAb to detect the 3-amino-2-oxazolidinone (AOZ) metabolite in tissues of poultry, shrimp, beef and pork muscle (Diblikova *et al.*, 2005). The sample preparation involved a protease treatment, acid hydrolysis and the derivatization of AOZ to form p-nitrophenyl-3-amino-2-oxazolidinone (NPAOZ). The sensitive ELISA showed a CCβ of 0.4 μg kg^{-1}, close to that of the established confirmatory LC/MS-MS method (0.3 μg kg^{-1}). From the analysis of incurred samples, a high level of correlation ($r^2 = \geq 0.99$) between the ELISA and LC/MS-MS results were seen.

Semicarbazide (SEM) is a nitrofuran metabolite and the marker residue for food contamination. An inexpensive screening method was developed for SEM by Cooper *et al.* (2007) to reduce the need for expensive LC-MS/MS analysis. A polyclonal antibody raised in rabbit against SEM was used to produce a semi-quantitative ELISA for SEM in muscle, validated with a detection capability of 0.25 μg kg^{-1}. This assay satisfies the EU nitrofuran minimum required performance limit of 1 μg kg^{-1}. SEM was isolated from poultry muscle samples by derivatisation to o-nitrobenzaldehyde and simultaneous protease digestion before extraction by cation exchange SPE. This ELISA method is also applicable to egg and chicken liver for SEM detection.

An indirect competitive immunoassay using novel pAbs raised in rabbits was developed to simultaneously detect the seven nitrofurans in swine, poultry and fish feeds (Li *et al.*, 2010). Homogenized feed was added to acetonitrile and incubated at 80 °C. The mixture was filtered through filter paper and the residue on the paper was washed again with acetonitrile. The filtrate was collected and evaporated to dryness and the dry residue was reconstituted in DMF/water (1:1, v/v). The reconstituted extract was filtered again (0.22 μm) and the extract was assayed by ELISA. The assay LOD for furazolidone, nitrofurantoin, nitrofurazone, furaltadone, nifurstyrenate sodium, nitrofuroxazide, nitrofurosol and 5-nitrofurfurol were in the range of 5 to 16 μg kg^{-1}. The recovery levels of nitrofuran residues from fortified feeds were in a range of 82.6 to 108.4% with % CV < 11.4%. The immunoassay performance was compared to a HPLC method and the methods showed good correlation (R^2 = 0.99). Therefore, the proposed immunoassay could be used as a practical method to monitor the illicit use of nitrofurans in animal feeds. Although this ELISA has not yet been applied to screen for nitrofurans in food for human consumption, it may in the future prove to be a useful method for this purpose.

A SPR immunobiosensor assay was reported for the multi-residue screening of a range of nitrofuran compounds in chicken eyes (Thompson *et al.*, 2010). A pAb raised in a rabbit showed significant cross-reactivity to five of the major parent nitrofurans; nitrofurazone, furazolidone, furaltadone, nitrofurantoin, and nifursol. A nitrofuran mimic was immobilised onto a carboxymethylated biosensor chip surface using EDC/NHS amine coupling via a Jeffamine linker. Sample homogenates were extracted into 0.1 M hydrochloric acid and subjected to SPE clean-up and micro-centrifugation prior to biosensor analysis. The authors reported a CCβ of less than 1 ng eye^{-1} for nitrofurazone. Intra-assay variation of 12.9% and 10.1% and inter-assay variation 10.8% and 4.7% were reported for nitrofurazone concentrations of 1 ng eye^{-1} and 2 ng eye^{-1}, respectively. Eye samples from five chickens treated with nitrofurazone were tested using the biosensor method and the corresponding liver samples were analysed by LC-MS/MS. All eye samples showed screening results above the CCβ and the liver samples showed LC–MS/MS confirmatory results of >5 μg kg^{-1} for the semicarbazide metabolite. This work demonstrated a link between the levels of nitrofurans in chicken eyes and metabolite levels in liver samples.

1.6.9 Multiplex screening methods for antibiotic detection in food

1.6.9.1 Small molecule microarrays

A small molecule microarray (SMM) assay was developed for simultaneous detection of chloramphenicol, clenbuterol, and tylosin residues in milk, cheese, chicken and pork by Peng and Bang-Ce (2006). Clenbuterol-OVA, chloramphenicol-OVA and tylosin-BSA conjugates were immobilised onto the surface a modified glass slide. Then the mixture of the drug corresponding antibodies and the samples was added to the microarray surface. The antigen-antibody binding was detected using Cy5-labelled secondary antibody to produce a fluorescent signal. The SMM assay was applied to several food samples, although the sample preparation procedure was not described.

The SMM permitted the detection of tylosin below its MRL and the working range of the SMM for chloramphenical (0.03 – 1.21 μg L^{-1}) and clenbuterol (0.01 – 5.18 μg L^{-1}) indicated adequate sensitivity.

More recently, Rebe Raz *et al.* (2008) described a competitive immunoassay for gentamicin and neomycin developed in a microarray format using SPR imaging. The paper describes the transfer of the assay from a conventional SPR biosensor to the SPR imaging microarray platform and compares the two methods. Microarray sensor chips were prepared by activating the carboxymethylated dextran surface with EDC/NHS and subsequently washing with ice-cold acetic acid. The sensor chip was dried under a stream of nitrogen and immediately spotted with gentamicin and neomycin ligands using a Microgrid II™ contact arrayer (Digilab Incorporated, Hollistin, MA, USA). A competitive immunoassay was developed for parallel detection of gentamicin and neomycin residues in the ng mL^{-1} range. Sensitivity was comparable to that achieved using the Biacore™ assay. However, assay transfer from conventional SPR biosensors to the imaging microarray platform presents new challenges, such as sufficient immobilisation of spots. The authors suggested that this issue must be addressed in future studies if this microarray format is to be adapted for routine analysis of food.

1.6.9.2 Biochip assay

A biochip assay was developed to detect eight antibiotic residues in muscle, and liver tisssue (Chen *et al.*, 2009b). Drug-ovalbumin (OVA) conjugates and OVA negative controls were printed onto activated agarose surface-modified glass slides. Samples were extracted using buffer and incubated at 80°C. Extracts were centrifuged and an aliquot of the middle layer was removed after adjustment to pH 7.0.

The extracts were diluted in PBS prior to analysis. Residues were identified using mAbs in a competitive immunoassay format and secondary Cy5-labelled antibodies provided a fluorescent signal which was detected using a laser confocal scanner.

LODs were in the ranges; sulphamethazine (6.3 - 9.0 μg kg^{-1}), sulphaquinoxaline (4.0 - 9.2 μg kg^{-1}), sulphamethoxazole (4.8 - 8.6 μg kg^{-1}), enrofloxacin (4.9 - 7.4 μg kg^{-1}), streptomycin (80 - 92 μg kg^{-1}).

1.6.9.3. Suspension array

Suspension array technology was recently reported as a novel method for the simultaneous detection of chloramphenicol, clenbuterol and 17-beta-estradiol residues (Liu *et al.*, 2009). Conjugates of chloramphenicol, clenbuterol and 17-beta-estradiol coupled to bovine serum albumin were immobilised via amine onto carboxylated fluorescent polystyrene microspheres/beads and indirectly labelled with streptavidin. Samples and three different biotinylated mAbs were applied to the suspension array in a competitive assay format for analyte detection. A Bio-Plex™ suspension array system based on Luminex xMAP® technology was employed for laser beaming, fluorescent signal capturing, multi-analyzing and data-processing. LODs for chloramphenicol, clenbuterol and 17-beta-estradiol were 0.04, 0.05 and 1 μg L^{-1}, respectively.

Suspension arrays may provide a novel application for analysis and determination of small molecules such as pesticides and veterinary drugs in food. However, the authors suggest that screening food by suspension array for a large numbers of chemicals still merits further investigation and conditional optimization.

1.6.9.4 Protein microarray

Recently, a protein microarray was reported for clenbuterol and sulphamethazine detection in chicken muscle (Zhong *et al.*, 2010). Ovalbumin conjugate chemistries were spotted onto poly-L-lysine microarray slides via amine coupling. The microarray IC_{50s} for clenbuterol and sulphamethazine were 0.0396 μg mL^{-1} and 0.049 μg mL^{-1}, respectively, while a traditional competitive indirect-ELISA showed IC_{50}s of 0.191 μg mL^{-1} and 0.157 μg mL^{-1}, respectively. Both methods were validated with clenbuterol-fortified chicken muscle tissues and a 90% analyte recovery rate was reported for the microarray while the ELISA showed a 76% recovery rate. The authors suggest that this protein microarray is a better approach than this ELISA technique.

1.7 Growth promoters: Steroidal hormones and β-agonist drugs

1.7.1 Steroidal hormones

1.7.1.1 Estrogens

Two competitive electrochemical ELISAs were reported for detecting 17β-estradiol in serum using mAb or pAbs (Draisci *et al.*, 2000). The use of the pAb resulted in a more sensitive assay with a detection limit of 20 ng L^{-1}. Samples were analysed by DELFIA with and without sample pre-treatment. Precision and sensitivity was comparable using both techniques.

A competitive ELISA was developed to detecting hexoestrol residues in porcine muscle and liver tissues using a pAb raised against a hexoestrol-mono-carboxyl-propyl-ethyl-bovine-serum-albumin conjugate (Xu *et al.*, 2006). Samples were extracted with sodium acetate buffer, adjusted to pH 5.2 and incubated overnight. Samples were subsequently diluted with MeOH , centrifuged and defatted with hexane. Extracts were diluted with 1-propanol purified using three different SPE clean-up procedures. CCβ for the ELISA was 0.07 µg L^{-1} and the dynamic range of the assay was between 0.07 and 30.5 ng mL^{-1}. Intra and inter-assay precisions (CV%) were <8 and <15%, respectively. Recovery of hexoestrol determined by ELISA and LC-MS/MS were 102 – 115%, respectively. The ELISA performance compared well to that of the confirmatory method but required a lengthy sample preparation procedure.

A disposable screen-printed electrode immunosensor for the detection of 17β-estradiol in non-extracted bovine serum was reported by Volpe *et al.* (2006). The graphite electrode immunosensor strip was assembled through immobilizing anti-rabbit IgG by passive adsorption, onto the surface of the screen-printed electrode. Serum samples were added directly to the electrode followed by rabbit anti-17β-estradiol pAb and incubated. A 17β-estradiol–alkaline phosphatase conjugate was added next followed by the AP substrate.

The Ag-Ab complex was detected via the current response which was measured by differential pulse voltametry (DPV) using a portable electrochemical detector. Spiked and incurred plasma were analysed and precision values (relative standard deviation, RSD%) ranging from 8.6 to 17.0% and recoveries between 88 to 120% were reported. The assay CCβ was determined to be less than 40 pg mL^{-1} and results obtained for incurred plasma samples were confirmed by LC–MS/MS. One disadvantage of this system is that bovine serum free of 17β-estradiol is required for the preparation of the matrix calibration curve. Hormone-free bovine serum is expensive to purchase and can be expensive to prepare. This requirement significantly increases both the time and cost of this rapid immunosensor assay.

1.7.1.2 Progesterones

The first heterologous multiresidue ELISA was reported to detect acetyl-gestagen residues in animal fat by Peng *et al*. (2008). Previously an ELISA method for medroxyprogesterone (MPA) detection in plasma (Lewis, Elder and Barrel, 1992), a time resolved fluorescence immunoassay (TR-FIA) to detect chlormadinone acetate (CMA) in human serum (Fiet *et al.,* 2002) and a MPA capillary electrophoresis immunoassay (Peng *et al.,* 2007) were available for steroid detection. Four haptens were synthesised (MPA, megestrol acetate (MEGA), 17α-hydroxyprogesterone acetate (HPA) and chlormadinone acetate (CMA)) and conjugated to both BSA. The pAbs used were raised to the 3-CMO-MPA-BSA immunogen because this conjugate showed the highest molar binding ratio of MPA to BSA. Acetylgestagen residues were extracted from fat samples into MeCN. Sample clean-up was carried out using SPE cartridges and the eluate reconstituted in PBS-Tween prior to ELISA analysis. The heterologous ELISA format was shown to be more class-selective than the homologous ELISA format. This phenomenon also occurred during the development of a heterologous multi sulphonomide ELISA (Spinks *et al.,* 2002). The concentration required to inhibit 50% of antibody binding (IC_{50}) was calculated for the four acetylgestagens, CMA, HPA, MEGA and MPA to be 4.5, 2.5, 2.9 and 1.8 μg l^{-1}, respectively. The average recoveries for the assay ranged from 61 to 78% in spiked swine fat.

The sensitivity of the heterologous assay developed in this study was higher than or comparable to that of the methods previously reported by Fiet *et al.* (2002) and Peng *et al.* (2006a, 2007).

A Biacore™ SPR biosensor was also reported to measure progesterone in bovine milk (Gillis *et al.,* 2002). The assay was designed as an inhibition assay with progesterone covalently immobilized to the carboxymethyl dextran matrix of a CM5 sensor chip and a anti-progesterone mAb antibody. The assay concentration range was between 0.5 and 50 μg L^{-1} and the LOD was determined to be 3.56 μg L^{-1}. Reproducibility of the assay showed coefficients of variation of < 5%. The aim of this work was to provide a biosensor assay for progesterone in milk that could be used in-line in the milking parlour and provide an important tool for reproductive management of dairy cattle to predict pregnancy and not as an indication of non-compliant food.

1.7.1.3 Androgens

A solid-phase chemiluminescence immunoassay for 19-nortestosterone (NT) using pAb raised against NT-3-carboxymethyloxime-BSA was reported by Van den Berg *et al.* (1988). This pAb also showed substantial cross-reactivity towards testosterone and trenbolone. The assay was used for the detection of anabolic agents at application sites. Only 250 μg of muscle tissue sample required for the assay and the NT concentrations between 0.4 and 16 000 μg kg^{-1} could be measured. This assay was not applied to monitor for these steroids in food.

A sheep anti-testosterone antibody, a rabbit anti-methyltestosterone antibody (Biogenesis, Poole, UK) and a rabbit anti-nortestosterone antibody (Fitzgerald Inc., MA, USA) were used to develop screening ELISAs for bovine urine (Lu *et al.*, 2006a). Samples were diluted and directly applied to the assays. The LODs for the testosterone, methyltestosterone and 19-nortestosterone assays were found to be 0.074, 0.26 and 0.131 μg L^{-1}, respectively. This method omitted the need for lengthy extraction and hydrolysis of samples necessary for traditional analytical techniques such as HPLC, LC-MS and GC-MS.

The same research group then developed competitive indirect electrochemical immunoassays to screen for boldenone and methylboldenone in bovine urine (Lu *et al.*, 2006b). Fortified urine samples at different levels of boldenone and methylboldenone showed high levels of accuracy and precision. The assay LOD for boldenone was 31 ng L^{-1} and for methylboldenone was 120 ng L^{-1}. Incurred urine samples from heifers treated with boldenone and methylboldenone were analysed using the immunosensors and the results were compared with those obtained in another laboratory using a well-characterised and validated GC–MS method. However, some variation between the two different methods was observed. It was suggested by the authors that this may have been due to the fact that urine samples were analysed directly after a single dilution by immunosensors, while the GC–MS samples were subjected to an hydrolysis step before analysis.

Two indirect competitive electrochemical immunoassays for the detection of testosterone, 19-nortestosterone and methyltestosterone in bovine urine were developed by Conneely *et al.* (2007a, 2007b) using conjugated testosterone–BSA immobilized onto disposable screen-printed electrodes. Undiluted bovine urine, that did not contain hormone residues, was tested and a strong matrix effect was observed; this effect was minimized using a dilution step (1:20 with buffer). The authors claimed satisfactory precision, accuracy and stability. The immunosensor assay was applied to urine from animals treated with testosterone. The results demonstrated the typical metabolic profile of testosterone in bovine urine; however they were not compared to a confirmatory method.

More recently an indirect competitive ELISA was developed for trenbolone residues in food and food products using an anti-trenbolone mAb (Zhang *et al.*, 2010). In buffer the LOD of the method was 0.06 ng.mL^{-1}, which was lower than the MRL (2.0 µg kg^{-1}). Animal tissue, urine and animal feed samples were prepared using MeCN extraction and centrifugation followed by hexane washing.

The recovery rates of the assay in detection of trenbolone-fortified animal tissue, urine and animal feed samples were in the range of 81-90%, while the intra- and inter-assay coefficients of variation were less than 12.0%. The LOD for this ELISA method in sample matrix was not reported

1.7.2 β-agonists

1.7.2.1 Zilpaterol

An ELISA for zilpaterol was developed by Shelver *et al*. (2004) using goat pAbs raised against a zilpaterol-butyrate-BSA immunogen. The average IC_{50} of the assay in buffer was 0.48 ng mL^{-1} (n = 25) and the assay was tolerant up to 10% (v/v) of acetone, ethanol, or methanol, and 15% (v/v) of MeCN or DMSO. This method was not applied to detect zilpaterol in food but the solvent tolerance and low inhibition levels reported indicate that it could provide the basis of a suitable screening method for food.

The same group later developed an SPR biosensor method to detect zilpaterol in sheep urine (Shelver *et al.*, 2005). A carboxy-zilpaterol derivative was coupled to a carboxy-methlated dextran biosensor chip via an ethylenediamine linker. Five mAbs and four pAbs were evaluated for their suitability to detect low levels of zilpaterol. The best sensitivity was achieved using a mAb which resulted in an IC_{50} of 4.47 μg L^{-1}.

Sheep urine was diluted and directly applied to the assay without the need for sample preparation. Both inter- and intra-assay variation were below 10% at concentrations between 2 and 8 μg L^{-1}. When urine samples were analysed using both the biosensor and ELISA methods developed by this group a high level of correlation was reported (r = 0.91).

17.2.2 Clenbuterol

Several ELISAs are described in the literature for the analysis of clenbuterol in animal hair based on overnight digestion and extensive extraction procedures (Gleixner *et al.* 1996; Godfrey *et al.,* 1996). Haasnoot *et al.*, (1998) attempted to reduce this extraction time with a fast digestion-extraction method using tert-butyl methyl ether.

However, this extract proved too complex for application to a sensitive clenbuterol ELISA without further purification. Subsequently, a clenbuterol biosensor assay was reported which dramatically reduced hair sample preparation time (Johansson *et al.*, 2003). Hair samples were washed with water and ethanol prior to incubation with a NaOH extraction buffer (100°C for 30 minutes). An LOD of 10 μg kg^{-1} was reported in hair extract and the method was deemed fit for purpose.

A competitive "label-free" electrochemical immunosensor for the detection of clenbuterol in animal feed was recently reported in the literature (He *et al.*, 2009). Clenbuterol was covalently linked to multi-wall carbon nanotubes (MWCNT). The clenbuterol-MWCNT conjugates were cast on a glassy carbon electrode. Swine feed was ground and added to a phosphate acid–methanol extraction solution, shaken and centrifuged. The supernatant was extracted twice using the extraction solution, an aliquot of the supernatant was evaporated to dryness and reconstituted in PBS for electrochemical analysis. Sample extract was mixed with a mAb and the electrode was immersed in this solution. The electrode was washed and transferred into a cell containing ferricyanate ($K_3[Fe(CN)_6]$). Clenbuterol concentration was determined by monitoring the current response of $K_3[Fe(CN)_6]$ on the electrode. This approach provided a detection limit of 0.32 μg L^{-1}. The immunosensor showed recoveries between 90 and 98% for clenbuterol in swine feed. Accurate detection of clenbuterol in spiked animal feeds was demonstrated by comparison with conventional ELISA assays and LC–MS method. This method may in the future provide an alternative screening method for clenbuterol analysis in food.

A SPR biosensor screening assay was developed and validated, in accordance with Commission Decision 2002/657/EC, to detect a range of β-agonists in liver tissue (Traynor *et al.*, 2003). The mAb used in the assay was raised against a clenbuterol-transferrin immunogen and showed significant cross-reactivity towards 13 β-agonists. Liver samples were prepared by enzymatic digestion with a protease solution (66 mg mL^{-1} in Tris buffer pH8, 15 mL) for two hours at 63°C. Extracts were deconjugated using β-glucuronidase overnight at 37°C or 1 hour at 55°C.

Sample extracts were subsequently purified by SPE on Oasis® HLB cartridges. Purified extracts were evaporated and residues were re-suspended in buffer before biosensor analysis. The LODs were 0.02, 0.11, 0.19 and 1.5 μg kg^{-1} for mabuterol, clenbuterol, salbutamol and remaining β-agonists. The CCβs for clenbuterol, salbutamol and cimbuterol were 0.2, 1.0 and 2.0 μg kg^{-1}, respectively.

1.7.2.3 Ractopamine

An ELISA procedure employing a pAb raised in a goat was developed to detect RCT residues in bovine urine samples (Elliott *et al.*, 1998). Urine samples were simply deconjugated and analysed directly to achieve an LOD of 1.9 μg L^{-1}. A mAb ELISA was later developed to screen for ractopamine in sheep and cattle (Shelver and Smith, 2002). Incurred urine samples analysed by ELISA initially showed elevated concentrations of ractopamine when compared to HPLC analyses. The HPLC method did not detect metabolized ractopamine residues which the ELISA method could detect. In addition, parent ractopamine represented only a small percentage of the total ractopamine (<2%) residue extracted. It was found that the inclusion of an enzymatic hydrolysis step using glucoronidase/arylsulfatase from *P. vulgata* was necessary to improve correlation between ELISA and HPLC results for incurred samples. This process increased the levels of free ractopamine in the sample. This ELISA showed potential as a sensitive, qualitative ractopamine screening assay.

More recently, Zhang *et al.* (2009) reported a direct competitive ELISA to determine ractopamine residues in chicken muscle and in porcine tissue. The matrix effect of the samples was eliminated by one-step extraction with PBS, without any organic solution or clean-up procedures. The ELISA LOD and CCβ were 0.04 μg L^{-1} and 0.2 μg kg^{-1}, respectively.

1.7.2.4 Multiplex methods for B-agonist detection

A novel "label-free" electrochemical immunosensor for the detection of ractopamine in animal feed was reported by Shen and He (2007). The immunosensor was constructed by incorporating a ractopamine–bovine thyroglobulin antigen in agarose hydrogel films modified on a glassy carbon electrode. Swine feed samples were ground and extracted with phosphoric acid–methanol. Sample extracts were mixed with an anti-ractopamine pAb and the electrode was immersed in this solution for analysis. The electrode was washed and transferred into a cell containing ferricyanate ($K_3[Fe(CN)_6]$). Ractopamine concentration was determined by monitoring the current response of $K_3[Fe(CN)_6]$ on the electrode. Ractopamine recovery was in the range 84 and 90%. The dynamic range of the immunosensor calibration curve was reported to be between 1 and 1000 μg L^{-1}. This method shows the potential for fabricating novel immunosensors to detect veterinary drugs in food.

Thompson *et al.* (2008) reported a sensitive SPR biosensor method suitable for screening ractopamine residues in porcine urine and liver. An anti-ractopamine polyclonal antibody was raised in goat against ractopamine-human serum albumin (HSA) immunogen. Liver was found to be unsuitable as a sample matrix because ractopamine was undetectable after five days withdrawal of the drug. In contrast, urine samples allowed ractopamine residues to be detected several weeks after withdrawal. A high level of correlation was reported between the biosensor and LC-MS/MS methods in urine ($R^2 = 0.99$) and liver ($R^2 = 0.97$) samples. An assay LOD of 0.34 and 0.19 μg kg^{-1} and a CCβ of <0.4 and <0.5 μg kg^{-1} were reported for urine and liver, respectively.

Knecht *et al.* (2004) reported a parallel affinity sensor array (PASA) for the rapid automated analysis of ten antibiotics in milk using a multianalyte indirect competitive ELISA format. Disposable microarrays were prepared by immobilizing protein conjugates of the haptens onto modified glass slides. All liquid handling and sample processing was fully automated, and the total sample analysis was five minutes. Each milk sample was mixed with a solution containing ten different mAbs and added to the microarray flow cell.

A HRP-labelled secondary antibody generated a chemiluminescent signal, which was detected using a CCD camera. The assay detection limits ranged from 0.12 to 32 μg L^{-1} for cephapirin and neomycin, respectively. Penicillin G could be detected at the MRL and the detection limits for all other analytes were below the respective MRLs. The multiplex PASA system is ideally suited to on-line monitoring in the dairy industry.

A radio-ligand receptor binding bioassay was also developed to screen for a panel of commonly used β-agonist compounds (Boyd *et al.*, 2009). The assay was developed as a means of detecting low dose cocktails of β-2-agonists in animal feeds. It was also shown that when β-agonists were present as cocktails in samples a pronounced synergistic effect could be measured. The assay proved capable of detecting clenbuterol in animal feed at 250 μg kg^{-1}. Although the sensitivity of the assay was suitable for β2-agonist detection in animal feed it was unsuitable for residue analysis in food of animal origin. The authors' attempts to increase the assay sensitivity showed no marked improvement. Another disadvantage of this method is the need for a radio-isotopic label. This is unpopular due to the safety concerns and cost involved in the disposal of radioactive waste.

A competitive microarray assay was reported for the simultaneous detection of clenbuterol, ractopamine and salbutamol (Zuo *et al.*, 2010). Conjugates of these three analytes were immobilized on microarray slides and for detection their corresponding mAbs were added to the samples for indirect competitive immunoassay.
A Cy3-labelled secondary antibody was employed to indicate the antigen–antibody complex. The fluorescence intensity of each spot was imaged and recorded. The microarray method was more sensitive than an ELISA. CCβs were 0.09, 0.5 and 0.01 μg L^{-1} for clenbuterol, ractopamine and salbutamol, respectively. Recovery ranged between 96 and 107% and precision of the assay was <10% for fortified pig urine samples.

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An automated mesofluidic system (MCMS) was developed by Hu, Zuo and Ye (2010) to detect ractopamine, salbutamol, clenbuterol, sulfmethazine and chloramphenicol veterinary residues in meat and milk. The MCMS was based on biorecognitions carried out on meso-scale glass beads in polydimethylsiloxane channels. This integrated MCMS enabled the entire assay to be automated and reduced to a one-step protocol. A competitive immunoassay was carried out on the surface of the glass beads. A Cy3-labelled secondary antibody was introduced to probe the antigen–antibody complex anchored to the beads. The fluorescence intensity of each bead was measured and used to determine the drug residual concentration. CCβs ranged from 0.02 μg L^{-1} (salbutamol) to 3.5 μg L^{-1} (sulphamethazine). The recovery levels of the method in meat and milk samples were in the range of 99 to 109% and the total assay time was 45 mins. This is another example of an automated multiplex approach that could improve the rate at which milk is screened for β-agonist drug residues.

1.8 Conclusions

It has been demonstrated in this chapter that the inherent flexibility of the immunoassay has led to major developments in assay detection systems. It is now a well established technology and has been adapted in an array of applications in veterinary drug residue analysis in food. ELISAs have been widely employed in this field for over 30 years because they are simple to perform and inexpensive. However, they can be slow because of the need for long incubation periods and washing steps. In addition, although they are highly specific they may be prone to false positive or false negative results. Lastly, because these methods provide mostly qualitative or semi-quantitative they cannot be easily automated. In the literature reviewed, many of ELISA screening methods did not meet the performance criteria set out by the regulating bodies because some are dated and have not been developed and validated to meet current regulatory targets laid down for veterinary drug residue analysis in food.

Over the last 15 years technological advances have reduced the immunoassay to a single step procedure. This has been accomplished by the miniaturization and automation of integrated biosensor detection systems. SPR biosensors provide high-throughput "label-free" sample analysis in real-time and can also be left unattended to analyze samples overnight. This system provides more quantitative data, a higher level of sensitivity and reproducibility than many ELISA formats. Recent advances in antibody production and biosensor chip preparation have resulted in several sensitive SPR biosensor assays for veterinary drug screening in food and several other biological matrices.

Whilst compiling this literature review it became apparent that no single effective screening method existed to detect all of the benzimidazole marker residues in both liver tissue and milk. Although some ELISAs demonstrated low levels of sensitivity (Brandon *et al*., 1994; Brandon, *et al.,* 2002) they were usually limited to one or two benzimidazole residues. The extraction techniques used also varied for each benzimidazole drug residue and food matrix. In addition, none of these methods could detect amino-benzimidazole marker metabolites. Hence, these are insufficient screening methods for benzimidazole drugs in food. There have been no reports of anti-triclabendazole antibodies in the literature and as such no immunochemical assay has been reported for triclabendazole (TCB) detection in food. For these reasons the primary focus of this research was to develop SPR biosensor screening methods to detect multiple benzimidazole residues and expand the scope of assays to amino-benzimidazole and TCB marker residues below their MRLs using a single efficient extraction procedure.

Multi-class multiplex methods such as biochip arrays are high-throughput methods which simultaneously detect several analytes in a single sample and as such these methods are ideally suited as screening assays. Currently there are no reports of a validated biochip array screening procedure for the detection of benzimidazole residues in food. The secondary focus of this research will be to produce a validated biochip array to detect benzimidazole fungicides and avermectin pesticides in orange juice.

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Chapter 2

Detection of benzimidazole carbamate residues in liver tissue using a SPR Biosensor

2.1. Introduction

Benzimidazoles are anthelmintic agents with broad spectrum activity against nematodes, cestodes and trematodes. They are widely used for the treatment of food-producing animals in the European Union (EU). Benzimidazole drugs are proven to be safe when product label claims are followed. However, some of these drugs have shown teratogenic properties. Delatour *et al.* (1975) reported congenital malformations in gestating ewes after administration of ABZ and OFZ. As a result, some concern has been raised that high levels of residues may affect developing embryos in pregnant women. This is further highlighted by reported incidences of non-compliant BZT residues in food. Results from this surveillance highlight the need for continued monitoring of benzimidazole residues due to sporadic incidences of non-compliant benzimidazole residues in milk and meat (Danaher, Sherry, O'Mahony, 2009).

Therefore, it is important to develop effective strategies for the control of residues in food and to consider factors such as drug metabolism and toxicity of residues. Several metabolites have been identified in edible tissues formed through hydrolysis, reduction and sulfoxidation routes. In some cases, metabolites are more toxic than the parent form of drug. Hydroxy-mebendazole (MBZ-OH) showed greater embryotoxicity than MBZ in rat (Delatour and Parish, 1989) and oxfendazole (OFZ) is more toxic than its primary chemistry fenbendazole (WHO, 1991a, 1991b).

In the EU, MRLs have been listed for benzimidazoles in edible animal tissues including muscle, liver, kidney and fat. Benzimidazole residues mainly occur in the liver, followed by the kidney, with lower levels of residues detected in other tissues such as fat and muscle. Therefore, it has been concluded that liver is the most suitable matrix in which to monitor benzimidazole residues. Several marker residues have been described for benzimidazole residues in food. The regulatory authorities set maximum residue limits (MRLs) to ensure food is safe for consumers. MRLs in liver range from 100µg kg^{-1} (TCB and TBZ) to 1000 µg kg^{-1} (ABZ) (Table 2.1). Efficient monitoring of benzimidazole residues in liver requires multi-residue methods with the capability to detect residues and their metabolites below their MRL.

There has been extensive research to develop and improve analytical techniques for multi-residue detection of benzimidazoles in liver. Marti *et al.* (1990) developed a HPLC-UV method to detect eight benzimidazole residues in liver tissue using an acetonitrile extraction followed by purification with multiple liquid-liquid partitioning (LLP) and/or solid phase extraction (SPE) clean-up steps using C_{18} sorbent and florisil. Following this work a simpler method was developed to isolate eight benzimidazole residues from liver tissue with HPLC-UV and GC-MS detection (Wilson *et al.,* 1991). This was achieved using ethyl acetate extraction coupled with purification by LLP (acidified ethanol versus hexane) and a subsequent C_2 SPE clean-up step. This method was widely adopted for the isolation of benzimidazole residues from liver tissue and as such has been modified by several researchers e.g. sample size, pH and SPE extraction. More recently it was found that ten benzimidazole residues could be isolated from liver tissue even if SPE clean-up was excluded from the method (Domany and Koviacs, 2000). More recently a HPLC-UV assay was developed that can detect 12 benzimidazole residues in liver tissue using an ethyl acetate extraction and a clean-up with automated SPE on C_{18} cartridges (Dowling *et al.*, 2005). The benzimidazole multi-residue methods described so far involve laborious extraction and clean-up steps that increase analysis time and the risk of errors whereas immunoassays are rapid, simple, selective and low-cost screening techniques. Brandon *et al*. (1994) developed an ELISA screening method for six benzimidazole residues in liver tissue by simply using water as an extraction solvent along with a single centrifugation step. Since a large percentage of benzimidazole parent compounds are metabolised (Delatour and Parish, 1989) most benzimidazoles in tissue are in polar metabolite form. This method detected these more readily extractable oxidized compounds below the permitted MRL for confirmatory analysis by HPLC or LC-MS/MS. Crooks (2003) also described an ELISA method that detected nine benzimidazole residues in liver. Liver samples were defrosted in plastic juice extractors and the neat liver drip extract was analysed.

This chapter describes the development of a novel biosensor screening assay suitable for multi-benzimidazole detection in liver. A multi-residue biosensor screening method for benzimidazoles in liver would increase the sample throughput rate by reducing the sample preparation and clean-up times.

Table 2.1 Maximum residue limits for benzimidazole anthelmintic veterinary drugs

			Maximum residue limit (μg kg^{-1})				
Veterinary Drug	**Marker Residue(s)**	**Species**	**Muscle**	**Liver**	**Egg**	**Fat**	**Milk**
Albendazole Albendazole-sulphoxide Netobimin	Sum of albendazole sulphoxide, albendazole sulphone and albendazole sulphone amine expressed as albendazole	All ruminants	100	1000	NA	100	100
Fenbendazole, Oxfendazole, Febantel	Sum of fenbendazole, fenbendazole sulphoxide and fenbendazole sulphone expressed as fenbendazole sulphone	All ruminants	50	500	NA	50	10
Mebendazole	Sum of mebendazole, amino-mebendazole and hydroxyl mebendazole expressed as mebendazole	Ovine, Caprine and Equidae	60	400	NA	60	N.A
Triclabendazole	Sum of extractable residues that may be oxidised to ketotriclabendazole	All ruminants	225	250	NA	100	N.A
Flubendazole	Sum of flubendazole and amino-flubendazole expressed as flubendazole. flubendazole	Poultry, pigs and chicken	50	400	400	50	N.A.
Oxibendazole	Oxibendazole	Porcine	100	200	NA	500	NA
Thiabendazole	Sum of thiabendazole and 5-hydroxy thiabendazole	Caprine	100	100	NA	100	NA
Cambendazole	Cambendazole	NA	NA	NA	NA	NA	NA

NA= Not approved

2.2 Materials and methods

2.2.1 Chemicals and reagents

CM5 sensor chips (research grade), 96 well polystyrene microplates, NHS (100 mM N-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES, pH 7.4, with 0.05 M NaCl, 3.4 mM EDTA) and 0.005% (v/v) P20 were all obtained from GE Healthcare (Uppsala, Sweden). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore® water purification system (Cork, Ireland). Sodium hydroxide (NaOH), pesticide grade acetonitrile (MeCN), pesticide grade dimethylsulphoxide (DMSO), pesticide grade ethyl acetate, cyclohexane and methanol were supplied by BDH/VWR International Ltd. (Poole, England, UK). Ethylenediamine (99%, v/v), dimethylformamide (DMF), albendazole (ABZ), mebendazole (MBZ) and fenbendazole (FBZ) were supplied by Sigma Aldrich (Steinheim, Germany). Oxibendazole (OXI), fenbendazole-sulphoxide (FBZ-SO) and flubendazole (FLU) were purchased from QMX laboratories (Thaxted, UK). Amino-flubendazole (FLU-NH_2), amino-mebendazole (MBZ-NH_2), hydroxy-mebendazole (MBZ-OH), and hydroxy-flubendazole (FLU-OH) were received as a gift from Janssen Pharmaceuticals (Belgium). Albendazole-2-amino-sulphone (ABZ-NH_2-SO_2), albendazole sulphone (ABZ-SO), albendazole sulphoxide (ABZ-SO), fenbendazole sulphone (FBZ-SO_2) and amino-oxibendazole (OXI-NH_2) were purchased from Witega laboratories (Berlin, Germany). Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g NaCl were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate and 0.5 g C_{18} sorbent were purchased from Biotage (Uppsala, Sweden). The amino-albendazole hapten (Lot no. LK515), stored at - 20ºC, was received from Randox Life Sciences (Antrim, Northern Ireland). Whatman® syringe filter units (polytetrafluoroethylene (PTFE), 0.2 µm) were purchased from Fisher Scientific (Dublin, Ireland). Primary standard stock solutions (1 mg mL^{-1}) for each benzimidazole were prepared in DMSO.

Working standard solutions were then prepared at 40 μg mL^{-1} by diluting the primary stock in methanol. A FASTH 21 homogenisation unit and sample homogenisation tubes were supplied by Syntec Scientific (Dublin, Ireland), a Mistral 3000i centrifuge (MSE, London, UK), an Elma Transsonic T780/H ultrasonic bath (Bedford, UK) and a Turbovap LV evaporator (Caliper Life Sciences, Runcorn, UK) were used during sample preparation.

2.2.2 Negative control samples

Liver samples found to be free of benzimidazole residues by UPLC-MS/MS, with a limit of detection (LOD) of <1 μg kg^{-1}, were used as negative controls.

2.2.3 Incurred liver samples

The suitability of the assay to detect residues was evaluated through application to fortified and naturally positive samples. Liver tissue samples purchased from a supermarket (Samples 1-7) were tested to establish the performance of the assay when low levels of benzimidazole residues are present. To prepare incurred samples, three 16-month old steers were dosed orally with mebendazole (Sample 8), fenbendazole (Sample 9) and albendazole (Sample 10) at 15, 7.5 and 5 mg kg^{-1} body weight, respectively. The animals were humanely euthanized after 24 h and the livers were collected and stored at -20°C until analysis. The UPLC-MS/MS sample preparation, detection conditions and calibration method used in this work were outlined in recent work reported by Kinsella *et al*. (2010).

2.2.4 Biosensor Assay

2.2.4.1 QuEChERS sample preparation

A modified QuEChERS extraction method was used to isolate benzimidazole carbamate residues from liver tissue. Finely chopped liver (2 g) was homogenised in a slurry containing MeCN:$MgSO_4$:NaCl (12:4:1, v/w/w), homogenised (30 sec in a multi-homogenisation unit) and centrifuged (3,000 ×*g*, 10 min, -5ºC). The supernatant was transferred to a tube containing C_{18} sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500 ×*g*, 10 min, -5ºC).

The MeCN layer (6 mL) was transferred to polypropylene tubes and DMSO (500 µL) was added. The MeCN was evaporated under nitrogen at 50ºC using a Turbovap LV (Caliper Life Sciences, Runcorn, UK). The DMSO extracts were vortexed (2 min) and sonicated (10 min). Amino-benzimidazole residues were extracted using the same procedure as for the carbamate metabolites but extracts did not undergo C_{18} clean-up. Instead, DMSO extracts were defatted with cyclohexane (2 × 2 mL aliquots), and the cyclohexane layer was removed by aspiration. DMSO sample extracts were vortexed (2 min) and sonicated (10 min).

2.2.4.2 SPR biosensor chip preparation

A CM5 chip was allowed to equilibrate to room temperature and HBS-EP buffer (50 µL) was added to the chip surface and incubated (10 min). The buffer was removed and 50 mM NHS: 200 mM EDC (1:1, v/v, 40 µL) was added to the chip surface and incubated (20 min) to activate the surface. This solution was removed and 1 M ethylenediamine pH 8.5 (50 µL) was allowed to incubate (1 h) to introduce free amine groups. The remaining unreacted groups on the chip surface were deactivated by addition of 1 M ethanolamine-HCl (50 µL) and allowed to react (20 min). Methyl 5(6)-[(carboxypentyl)-thio]-2-benzimidazolecarbamate (2 mg) was dissolved in DMF (450 µL) and mixed with a solution containing NHS (2 mg) and EDC (5 mg) in 10 mM sodium acetate buffer, pH 4.5 (450 µL) and allowed to react on the chip surface (1 h) at room temperature. The chip was washed with HPLC grade water and dried under a stream of nitrogen gas. The immobilised chip was stored in a desiccated container (4ºC). A schematic representation of the major steps involved in the chip preparation ar shown in **Fig. 2.1.**

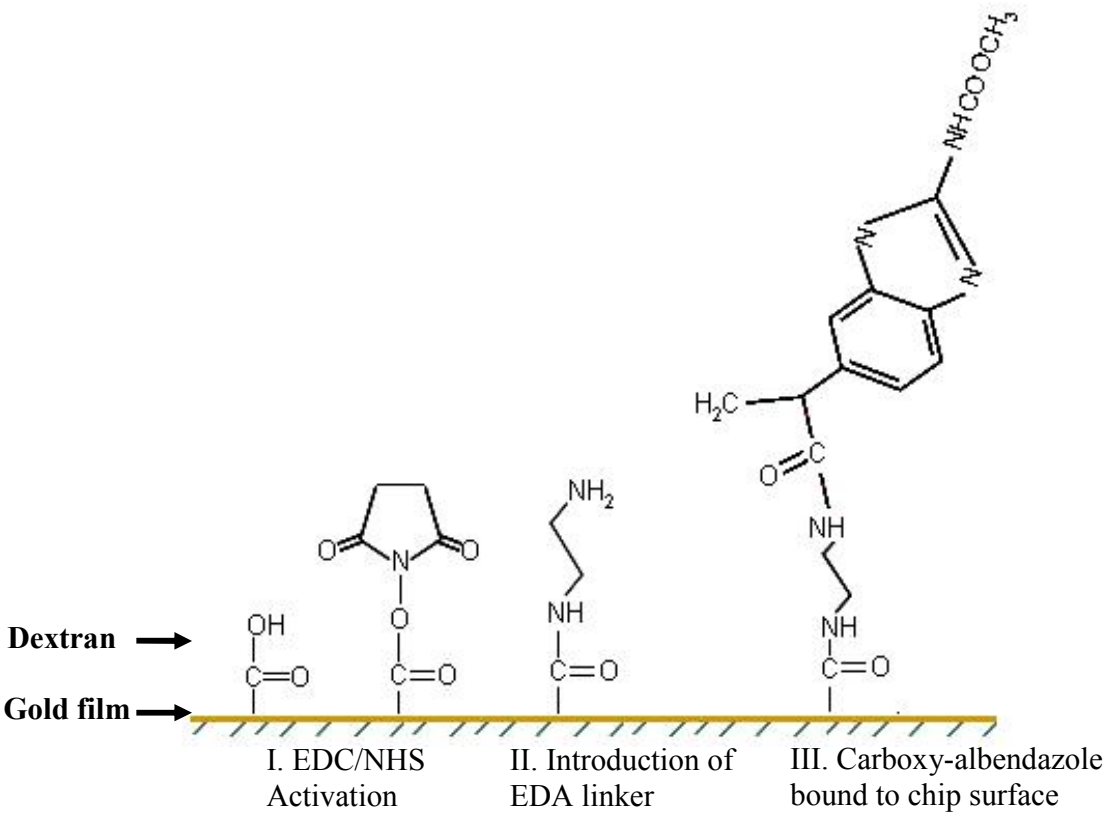

Figure 2.1 Preparation of benzimidazole carbamate biosensor chip surface using an ethylenediamine linker and a carboxy-albendazole derivative

A second CM5 biosensor chip was prepared for amino-benzimidazole detection. Firstly the chip was left to equilibrate to room temperature (20 min). HBS-EP buffer (50 µL) was added to each chip surface and incubated (10 min). The buffer was removed and 50 mM NHS:200 mM EDC (1:1, v/v, 40 µL) was added to the chip and incubated (20 min, room temperature) to activate the surface. This solution was removed from the surface. An amine surface was prepared by adding 1 M ethylenediamine pH 8.5 (50 µL) to the surface (1 h, room temperature). The solution was removed using lint-free tissue paper.

A carboxy-amino-albendazole derivative (2.5 mg) was dissolved in DMF (1 mL), vortexed (2 min) and sonicated (15 min). EDC (1.825 mg) and NHS (1.25 mg) were added to this solution and incubated at room temperature (3 h) to activate the carboxyl groups of the amino-benzimidazole derivative to form o-acylisourea intermediates with a COOH function. This solution was added to the chip surface and incubated (2 h, room temperature). The remaining unreacted groups on the chip surface were deactivated by addition of 1 M ethanolamine-HCl (50 µL) and allowed to react (20 min). Following immobilization, the chip was washed five times with HBS-EP buffer and dried under a nitrogen stream. The amino-albendazole immobilized chip was stored in a Sarstedt® tube containing silica crystals (4ºC) when not in use.

2.2.4.3 Conditions and reagents

Studies were conducted at 25ºC. The optical biosensor used was a Biacore Q (GE Healthcare, Uppsala Sweden) with Biacore® Q control software version 3.0. BIAevaluation software version 3.0.1 was used for data handling. BIAevaluation software was used to construct inhibition assay standard curves based on a 4-parameter fit. The concentration in test samples was read directly from the calibration curve. The polyclonal antibody used in the benzimidazole carbamate assay was raised in a sheep immunised against a methyl 5(6)-[carboxypentyl)-thio]-2-benzimidazolecarbamate derivative (CMB) (Brandon *et al*., 1994). The antibody was obtained from the Veterinary sciences division of the Department of Agriculture and Rural Development for Northern Ireland. An antibody working dilution of 1/1000, (v/v) in HBS-EP buffer, was found to give a satisfactory binding response. Antibody and liver extracts were mixed (1:1, v/v) when ethyl acetate and acetonitrile extraction was applied. During QuEChERS extraction the extracts were mixed (1:9, v/v) with antibody before being passed over the immobilised surface at 10 µL min^{-1} (2 min). Regeneration was carried out by sequential injection of 25 mM HCl (15 µL) followed by 180 mM NaOH (20 µl) across the chip at 25 µL min^{-1}.

Polyclonal sheep antibody raised against amino-albendazole coupled to bovine thyroglobulin (BTG) from Randox Laboratories (Crumlin, Northern Ireland) was used for amino-benzimidazole detection. The Ig fraction (2.4 mg mL^{-1} in phosphate-buffered saline containing 0.09% (w/v) sodium azide) was diluted 1/400 (v/v), to give satisfactory results under assay conditions. DMSO sample extracts were diluted in HBS-EP buffer (1:4, v/v), added to a 96 well microplate and mixed with (1:4, v/v) antibody and passed over the chip surface at 10 µL min^{-1} (3 min). Regeneration of the chip was carried out by sequential injection of 25 mM HCl (15 µL) and 170 mM NaOH (20 µL) at 25 µL min^{-1}. In all studies the binding of the antibody to the chip surface was measured as the change in SPR signal between two report points, 10 sec before and 30 sec after each injection. Competitive immunoassay formats were used to detect inhibition of antibody binding to the chip surface. The SPR signal was expressed in arbitrary resonance units (RU).

2.2.5 Calibration

2.2.5.1 Acetonitrile and ethyl acetate extraction procedures

A stock solution of each benzimidazole carbamate drug was prepared in methanol at a concentration of 40 µg mL^{-1}. Working solutions for calibration curves were prepared by sequential dilutions in methanol. Negative liver samples were fortified at 50, 100, 250, 500, 1000, and 2000 µg kg^{-1} with an ABZ-SO_2 standard prior to extraction.

2.2.5.2 QuEChERS extraction procedure

A stock solution of each benzimidazole carbamate was prepared in methanol at a concentration of 40 µg mL^{-1}. Benzimidazole residue-free liver samples were fortified with albendazole-sulphone (ABZ-SO_2) at levels of 0, 50, 100, 250, 500 and 1000 µg kg^{-1} to prepare an extract calibration curve for the benzimidazole carbamate assay.

Similarly samples were fortified with albendazole-amino-sulphone (ABZ-NH_2-SO_2) at levels of 0, 25, 50, 75, 125, 250 and 500 µg kg^{-1} to prepare an extract calibration curve for the amino-benzimidazole assay.

2.3 Results and discussion

2.3.1 Biosensor assay development

The objective of this research was to develop a multi-residue biosensor assay capable of detecting low levels of benzimidazole residues in animal liver. The BZT biosensor assay was optimised through injecting ABZ-SO standards, diluted in HBS-EP buffer, over the range 0 to 2000 μg kg^{-1}. It was found that by lowering the injection flow rate to 10 μL min^{-1} the antibody could be conserved while maintaining biosensor cycle times of less than eight minutes. The regeneration conditions were based on conditions developed previously by Johnsson *et al*. (2002). Under the optimised conditions, the IC_{50} was determined to be 3.9 ng mL^{-1}, while the dynamic range was found to be between 0.65 ng mL^{-1} (IC_{10}) and 21.5 ng mL^{-1} (IC_{90}) in HBS-EP buffer.

The amino-benzimidazole biosensor assay was optimised through injecting ABZ-NH_2-SO_2 standards diluted in HBS-EP buffer over the range 0 to 125 ng mL^{-1}. The biosensor cycle time was optimised to nine minutes using an antibody injection flow rate of 10 μl min^{-1} for 3 mins followed by 25 mM HCl (15 μL) and 170 mM NaOH (20 μL) at 25 μL min^{-1} for regeneration of the chip surface. The IC_{50} was found to be 5.7 ng mL^{-1} and the dynamic range of the assay was between 0.83 ng mL^{-1} (IC_{10}) and 24.9 ng mL^{-1} (IC_{90}) in HBS-EP buffer.

2.3.2 Development of sample preparation procedures

Several sample preparation procedures have been developed for the isolation of benzimidazole residues from liver tissue based on liquid-liquid extraction with a water immiscible solvent such as ethyl acetate. An ethyl acetate extraction procedure based on the method reported by Dowling *et al*. (2005) was evaluated for the isolation of benzimidazole carbamates from liver tissue.

The automated SPE clean-up step was omitted because it was considered unsuitable for a rapid method. After centrifugation, the ethyl acetate supernatant was reduced to dryness under nitrogen (50°C) and re-suspensed in MeOH:water (50:50, v/v).

This extract was diluted (1/20, v/v) in HBS-EP buffer prior to biosensor analysis (**Fig. 2.2**, Extraction I). Extracted matrix calibration curves prepared over the range 0 to 2000 μg kg^{-1} (ABZ-SO equivalents) showed significantly lower sensitivity (IC_{50} = 770 μg kg^{-1}) when compared to buffer curves (IC_{50} = 88 μg kg^{-1}). Losses in recovery were due to adsorption of analytes onto filter paper containing sodium sulphate. Modifications made to the sample preparation procedure (**Fig. 2.2**, Extraction II) resulted in only slight improvements in sensitivity (IC_{50} = 625 μg kg^{-1}).

An alternative MeCN extraction was next evaluated for isolating benzimidazoles from liver tissue (Domany and Koviacs, 2000). MeCN is an attractive solvent for isolating benzimidazole residues from biological samples without pH adjustment, it also extracts a lower quantity of fat and precipitates protein. Simple liquid-liquid partitioning steps were employed based on cyclohexane and a saturated aqueous NaCl wash to remove non-polar and polar matrix components, respectively. This sample preparation approach resulted in a significant improvement in sensitivity. The calibration curve in liver matrix showed an IC_{50} of 89 μg kg^{-1} (Extraction III), not significantly different from the IC_{50} (88 μg kg^{-1}) in buffer (**Fig 2.2**). However, the sensitivity required for the recovery for ABZ and FBZ residues was unsatisfactory (<40%) for a biosensor screening assay.

In earlier work by the present research group, a QuEChERs sample preparation procedure was successfully applied to the analysis of 11 benzimidazole carbamte residues in milk samples (Keegan *et al*., 2009). However, we evaluated an alternative clean-up procedure for liver tissue analysis because of the level of sensitivity required for benzimidazole detection in milk was much lower (< 10 μg kg^{-1}). When a QuEChERs sample preparation procedure was applied to fortified ovine liver extracts, the calibration curve showed comparable sensitivity (IC_{50} = 86 μg kg^{-1}) to MeCN extracted calibration curves and the buffer curve (**Fig. 2.2**). In addition, recoveries of ABZ and FBZ were acceptable, and the assay proceeded to validation.

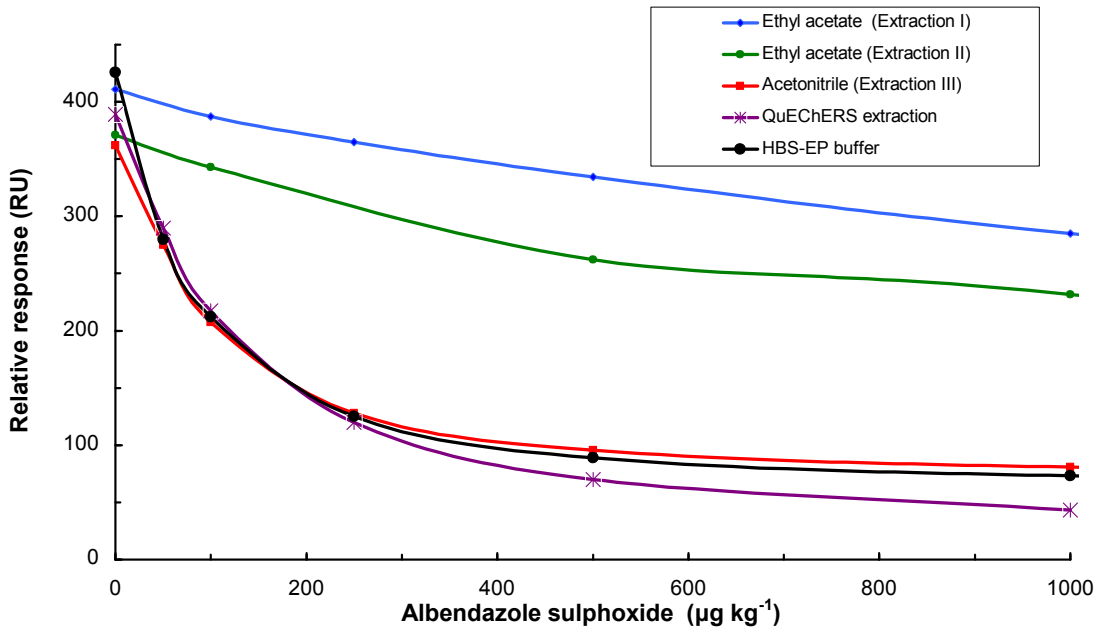

Figure 2.2 Comparison of the sensitivity of different extraction methods for ABZ-SO analysis in ovine liver against equivalent curves in HBS-EP buffer.

Subsequently, a new antibody became available that showed specificity towards amino-benzimidazole metabolites. Initially, the dispersive-solid phase extraction (d-SPE) procedure described in Section 2.3.1 was used for amino-benzimidazole extraction but showed consistently low recovery of <50% for FLU-NH_2, MHZ-NH_2 and OXI-NH_2 residues. Spiking experiments verified that this loss occurred at the clean-up stage. Alternative clean-up methods were investigated using different brands of C_{18} sorbents, high speed centrifugation (18,000 × *g*), and washing with cyclohexane. Liquid-liquid partitioning with cyclohexane showed the highest recovery levels for all amino-metabolites and this clean-up was selected for further validation.

2.3.3 Antibody inhibition studies

The cross-reactivity of the benzimidazole carbamate (S48) polyclonal antibody was determined in previous work by analysing inhibition buffer curves fortified with 11 different analytes by the SPR-biosensor assay (Keegan *et al*., 2009).

The cross-reactivity of the S48 antibody towards 11 benzimidazole carbamates was determined by analysing inhibition curves in ovine liver tissue (0-500 µg kg^{-1}) using the QuEChERS method. IC_{50} values in matrix ranged from 78 to 95 µg kg^{-1} for FBZ-SO and FBZ, respectively, and the cross-reactivities at 50% inhibition (CR_{50}) ranged from 91 to 110% (Table 2.2). Matrix calibration curves for 11 benzimidazole carbamates in ovine liver are shown in **Fig. 2.3.**

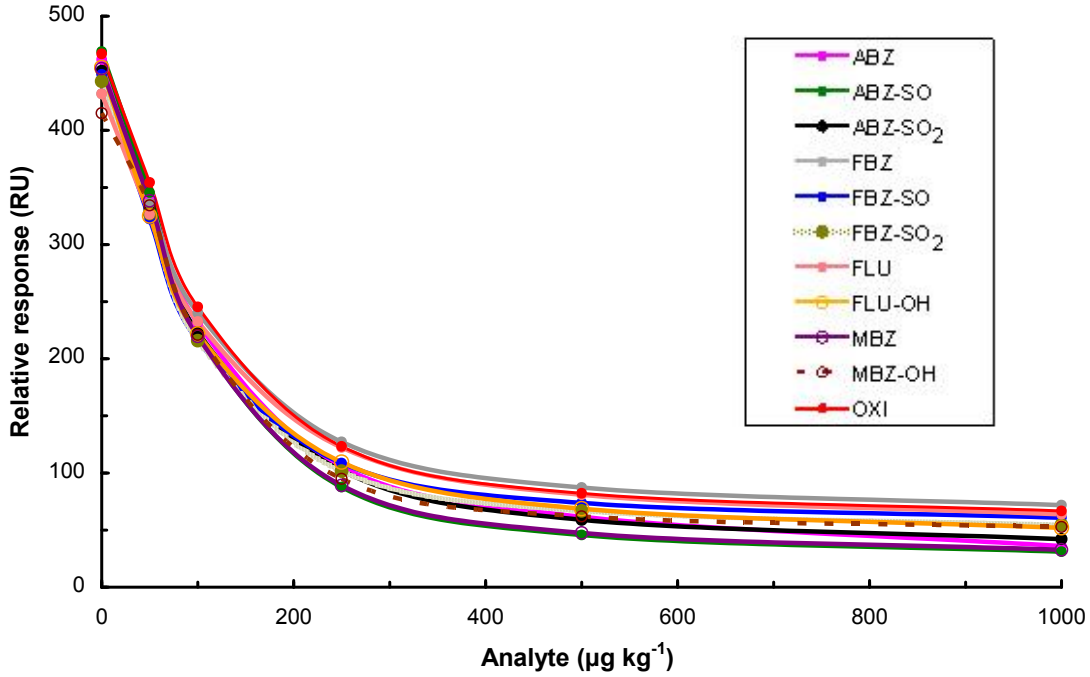

Figure 2.3 Calibration curves for 11 benzimidazole carbamates in ovine liver matrix.

The cross-reactivity of the anti-amino-benzimidazole polyclonal antibody (PAS 9869) was determined by analysing inhibition curves with analyte concentrations from 0 - 125 ng mL^{-1} prepared in HBS-EP buffer and from 0 - 500 µg kg^{-1} in ovine liver tissue. In buffer the antibody showed significant cross-reactivity with four amino-benzimidazoles (80 to 125%) in the following order of affinity OXI-NH_2>MBZ-NH_2>ABZ-NH_2-SO_2>FLU-NH_2 and analyte IC_{50} values were typically less than 7.1 ng mL^{-1} (Table 2.2). IC_{50} values in matrix ranged from 35 to 55 µg kg^{-1} for the four amino analytes. Matrix calibration curves for four amino-benzimidazoles are shown in **Fig. 2.4.**

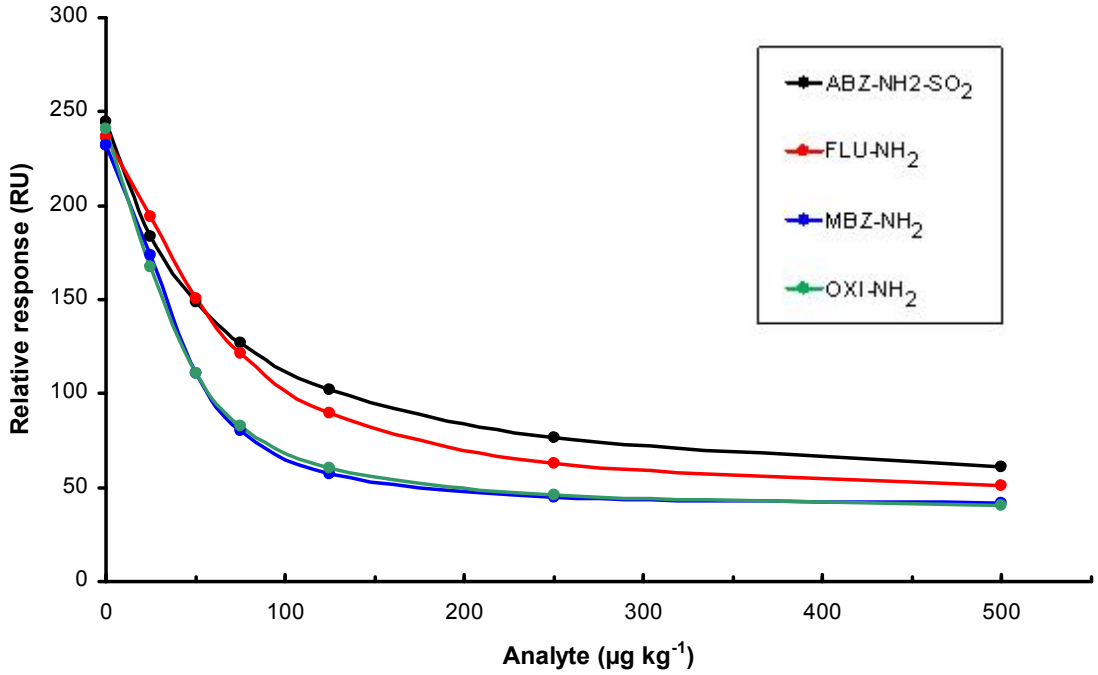

Figure 2.4 Calibration curves for amino-benzimidazole metabolites in ovine liver matrix.

Table 2.2 Cross-reactivity profile of polyclonal amino-benzimidazole antibody (PAS 9869) and polyclonal carboxy-albendazole antibody (S48) in HBS-EP buffer and ovine liver extract.

	Amino-benzimidazole assay			
	Buffer		Liver	
Analyte	[a]IC_{50} (ng mL^{-1})	[b]CR_{50} (%)	[c]IC_{50} (µg kg^{-1})	[d]CR_{50} (%)
ABZ-NH_2-SO_2	5.7	100	44	100
FLU-NH_2	7.1	80	55	80
MBZ-NH_2	5.6	102	39	113
OXI-NH_2	4.5	125	35	126
	Benzimidazole carbamate assay			
	[a]IC_{50} (ng mL^{-1})	[e]CR_{50} (%)	[c]IC_{50} (µg kg^{-1})	[f]CR_{50} (%)
ABZ	4.5	98	90	96
ABZ-SO	4.4	100	86	100
ABZ-SO_2	4.8	93	87	99
FBZ	6.6	67	95	91
FBZ-SO	4.0	110	78	110
FBZ-SO_2	4.0	110	82	105
MBZ	4.5	98	88	98
MBZ-OH	5.0	88	93	92
FLU	5.5	80	90	96
FLU-OH	6.6	67	89	97
OXI	6.2	71	88	98

[a] The concentration of analyte required to reduce the response by 50% in HBS-EP buffer.

[b] Cross-reactivity of antibody towards test amino-benzimidazole at 50% inhibition ((IC_{50} ABZ-NH_2-SO_2/IC_{50} test amino-benzimidazole)×100) in HBS-EP buffer.

[c] The concentration of analyte required to reduce the response by 50% in ovine liver.

[d] Cross-reactivity of antibody towards test amino-benzimidazole at 50% inhibition ((IC_{50} ABZ-NH_2-SO_2/IC_{50} test amino-benzimidazole)×100) in ovine liver.

[e] Cross-reactivity of antibody towards test benzimidazole carbamate at 50% inhibition ((IC_{50} ABZ-SO/IC_{50} test benzimidazole carbamate) ×100) in HBS-EP buffer.

[f]Cross-reactivity of antibody towards test benzimidazole carbamate at 50% inhibition ((IC_{50} ABZ-SO/IC_{50} test BZT) ×100) in ovine liver.

2.3.4 Method Validation

2.3.4.1. Benzimidazole carbamate biosensor assay

The dynamic range of the assay was found to be from 7 μg kg^{-1} (IC_{10}) to 340 μg kg^{-1} (IC_{90}) and the IC_{50} was calculated to be 86 μg kg^{-1}. The LOD was determined to be 32 μg kg^{-1} by measuring the mean response of 20 representative blank ovine liver samples (459 RU) and subtracting three standard deviations (3 × 24 RU). To determine the CCβ a concentration of 50 μg kg^{-1} was selected; this is equivalent to one quarter of the concentration of the analyte with the lowest MRL. The results for the determination of CCβ for each analyte are shown in Table 2.3. The CCβ for ten of the analytes was found to be less than 50 μg kg^{-1}.

The CCβ for MBZ-OH was found to be equal to 50 μg kg^{-1} where one sample was not identified as positive; the false negative sample gave a measured result of 32 μg kg^{-1}. However the method satisfies the false negative rate (5%) as required by 2002/657/EC. The repeatability of the assay was evaluated by analysing fortified ovine liver samples (100 μg kg^{-1}) with the 11 analytes on five separate days (Table 2.3). Results showed acceptable recovery (77-132%) and inter-assay coefficients of variation (11-17%) for the purposes of a screening method. Calibration curves for each day are shown in **Fig. 2.5(A)**.

2.3.4.2 Amino benzimidazole assay

The dynamic range of the assay was found to be from 22 (IC_{10}) to 238 μg kg^{-1} (IC_{90}) and the IC_{50} was 44 μg kg^{-1}. The LOD of the assay using was determined to be 41 μg kg^{-1} by measuring the mean response of 20 representative blank ovine liver samples (236 RU) and subtracting three standard deviations (3 × 21 RU). The CCβ of the assay was determined by fortifying 20 representative blank ovine liver samples at 75 μg kg^{-1} with four different amino-benzimidazoles. The CCβ for three of the four amino analytes was found to be <75 μg kg^{-1} because all 20 fortified samples showed responses above the LOD (Table 2.3). The CCβ for FLU-NH_2 was equal to 75 μg kg^{-1} as one of the samples gave a measured result of 40 μg $kg^{-1}$and was deemed negative.

The repeatability of the assay was evaluated by analysing ovine liver samples fortified (125 μg kg^{-1}) with four analytes on five separate days. Results showed acceptable recovery (103-116%) and inter-assay coefficients of variation (8-16%) for the purposes of a screening method (**Table 2.2**). Calibration curves for each day are shown in **Fig. 2.5(B)**.

Table 2.3 Determination of detection capability (CCβ) and repeatability of biosensor assays: Results from the analysis of fortified ovine liver (n = 20) and the percentage recovery on different days (n = 5).

Analyte	Assay Repeatability		Detection Capability (CCβ)	
	Mean recovery (%) ± S (n = 5)	CV (%) (n = 5)	Mean (μg kg^{-1}) ± S (n = 20)	CCβ (μg kg^{-1})
	Fortification = 100 μg kg^{-1}		Fortification = 50 μg kg^{-1}	
ABZ	94 ± 11	11	66 ± 9	<50
ABZ-SO	105 ± 15	15	76 ± 9	<50
ABZ-SO2	122 ± 16	13	71 ± 5	<50
FBZ	132 ± 15	11	79 ± 8	<50
FBZ-SO2	127 ± 15	12	100 ± 15	<50
OFZ	113 ± 18	17	70 ± 7	<50
FLU	95 ± 13	13	55 ± 6	<50
FLU-OH	90 ± 9	10	59 ± 8	<50
MBZ	80 ± 11	13	51 ± 9	<50
MBZ-OH	77 ± 9	11	48 ± 11	50
OXI	106 ± 18	17	67 ± 9	<50
	Fortification = 125 μg kg^{-1}		Fortification = 75 μg kg^{-1}	
ABZ-NH_2-SO_2	109 ± 8	8	121 ± 26	<75
FLU-NH_2	110 ± 18	16	70 ± 18	75
MBZ-NH_2	116 ± 11	10	104 ± 14	<75
OXI-NH_2	103 ± 9	9	93 ± 19	<75

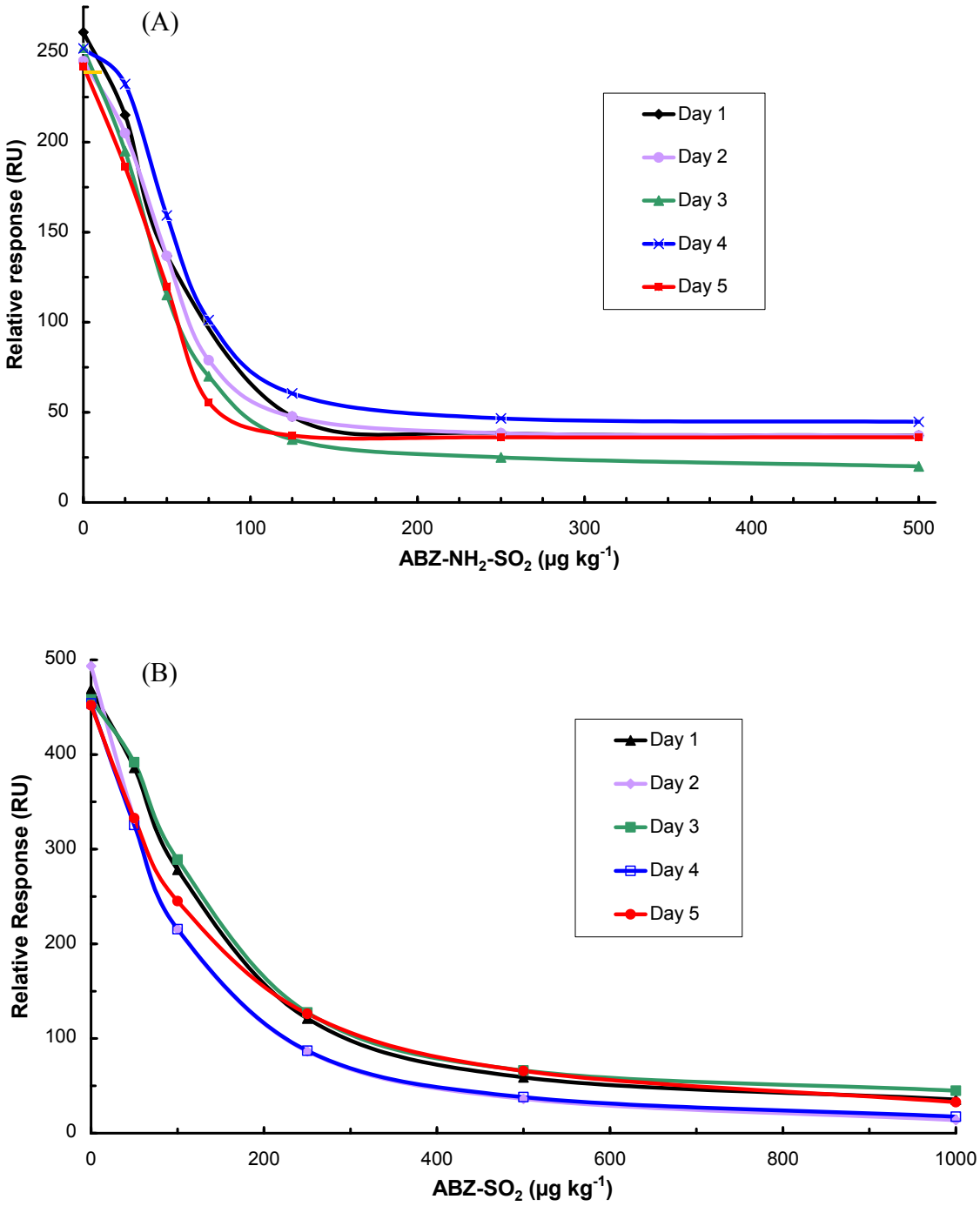

Figure 2.5 SPR biosensor assay calibration curves in fortified ovine liver on different days (n = 5) for (A) albendazole sulphone (ABZ-SO_2) and (B) albendazole-amino-sulphone (ABZ-NH_2-SO_2).

2.3.5 Application of SPR assay to incurred liver tissue

The suitability of the SPR biosensor assays were evaluated by analysing three liver tissue samples from bovine animals treated with albendazole, fenbendazole and mebendazole products and seven supermarket samples found to contain benzimidazole residues. The samples were independently analysed by two different analysts using the SPR-biosensor and UPLC-MS/MS methods. Seven of the ten samples were found to contain benzimidazole residues at concentrations above the LOD, which was 32 and 41 μg kg^{-1} for the benzimidazole carbamate and amino-benzimidazole SPR-biosensor assays, respectively (**Table 2.4**). Samples one to six were determined to be compliant for benzimidazole residues by both the biosensor assay and UPLC-MS/MS. Two of these samples screened above CCβ by the benzimidazole carbamate SPR-biosensor assay (Samples 5 and 6), which indicate that they should be sent for confirmatory analysis. A total of four samples were confirmed to be non-compliant by UPLC-MS/MS (Samples 7 to 10). Three samples contained residues above their respective MRLs (Samples 7, 9 and 10). One sample was categorised as non-compliant because it contained MBZ residues, which are not permitted for use in bovine animals (Sample 8).

The benefits of analysing samples using the amino-benzimidazole biosensor assay can be seen from the results for samples 8 and 10, which gave a screening response >CCβ. UPLC-MS/MS analysis confirmed that these samples contained MBZ-NH_2 and ABZ-NH_2-SO_2 residues at 244 and 228 μg kg^{-1}, respectively. One surprising aspect of this work was that no amino-benzimidazole response was detected in samples confirmed positive for FBZ residues, particularly samples 7 and 9, which were determined by UPLC-MS/MS to contain FBZ marker residues at concentrations above 100 μg kg^{-1}.

Table 2.4 Comparison between biosensor and UPLC-MS/MS analysis of liver samples containing incurred mebendazole, fenbendazole and albendazole residues.

Sample	Species	Biosensor assays			UPLC-MS/MS assay		
		Benzimidazole carbamates (μg kg^{-1})	Amino-benzimidazoles (μg kg^{-1})	[a]Interpretation	[b]Concentration (μg kg^{-1})	Analyte group	[c]Status
1	Bovine	14	ND	Negative	ND	ND	C
2	Ovine	34	ND	Positive	13	FBZ	C
3	Ovine	19	ND	Negative	7	FBZ	C
4	Ovine	12	ND	Negative	5	FBZ	C
5	Ovine	60	ND	Positive	92	FBZ	C
6	Ovine	70	ND	Positive	75	FBZ	C
7	Ovine	>1000	ND	Positive	2659	FBZ	NC
8	Bovine	98	198	Positive	327	MBZ	NC
9	Bovine	>1000	ND	Positive	13096	FBZ	NC
10	Bovine	>1000	211	Positive	1222	ABZ	NC

[a]Negative samples = < LOD and positive samples = > LOD, where benzimidazole carbamate assay LOD = 32 μg kg^{-1} and amino-benzimidazole assay LOD = 41 μg kg^{-1}

[b]UPLC-MS/MS concentrations are expressed as the sum of the FBZ, FBZ-SO and FBZ-SO_2 residues expressed as FBZ-SO_2 , MBZ, MBZ-NH_2 and MBZ-OH residues expressed as MBZ and ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues expressed as ABZ.

[c]C = compliant (< MRL) and NC = non-compliant (> MRL).

2.4 Conclusions

The SPR-biosensor assays presented in this work are suitable for use as rapid screening methods for the detection of 11 benzimidazole carbamate residues and four amino-benzimidazole residues in ovine liver tissue. Both assays were validated according to 2002/657/EC. The benzimidazole carbamate assay can screen for 11 residues at 50 μg kg^{-1}, equivalent to 25% of the concentration of the lowest MRL for benzimidazole carbamates in liver tissue. The amino-benzimidazole assay can screen for four benzimidazole residues at 75 μg kg^{-1}, which is 38% of the lowest MRL for amino-benzimidazoles in liver tissue. No false compliant results occurred during the study and the rate of false non-compliant samples was equal to 5% in both assays. Both screening assays can identify compliant liver tissue samples and thereby reduce the number of samples required to be tested by UPLC/MS-MS. Only suspect non-compliant samples would then require confirmatory analysis by UPLC-MS/MS. Using the methodology presented in this paper it is possible to extract and analyse 25 samples within a single working day.

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Chapter 3

Benzimidazole carbamate residues in milk: Detection by SPR biosensor, using a modified QuEChERS method for extraction

3.1 Introduction

Benzimidazole anthelmintic drugs are widely used in veterinary medicine for the treatment of helminth infections in food-producing animals. These infections result in reductions in milk yields (Vercruysse and Claerebout, 2001) and weight gain (Anderson *et al*., 1965; Eysker and Ploeger, 2000). In the EU, 11 benzimidazoles and pro-benzimidazoles are approved for treatment of food-producing animals giving rise to 20 potential residues. However ABZ and FBZ related drugs are the only ones approved in the treatment of lactating animals and have maximum residue limits (MRLs) in bovine and ovine milk (Table 1) under Commission Regulation 2377/90/EC (Anonymous, 1990). The MRLs for ABZ and FBZ drug residues in milk are 100 and 10 μg kg^{-1}, respectively (**Table 2.1**). The main concerns over the presence of benzimidazole residues in milk are related to their teratogenic and embryotoxic properties (Delatour and Parish, 1989; Mckellar and Scott, 1990). The requirement to monitor benzimidazole residues in milk is supported by pharmacokinetic studies which have shown that benzimidazole residues are excreted in the milk and non-compliant levels of residues may occur if withdrawal periods are not followed (Fletouris *et al*., 1996; Moeller *et al*., 2007).

Danaher *et al*. (2007) reviewed the analysis of benzimidazole residues in food, highlighting the analytical challenges caused by their extensive metabolism in food-producing animals. Researchers have reported methods for isolating multiple veterinary residues from food using QuEChERS, the so called Quick, Easy, Cheap, Effective, Rugged and Safe method. This method is widely used in pesticide residue analysis (Aguilera-Luiz, 2008; Kinsella *et al*., 2009; Stubbings and Bigwood, 2009). QuEChERS offers several advantages over most conventional techniques because it does not require glassware or ancillary equipment (e.g. vacuum manifolds), uses low volumes of solvent, generates little solvent waste and provides high recovery of analytes. The most widely used technique to measure benzimidazole residues in milk is HPLC coupled to UV and/or fluorescence detection is (Constantinou *et al*., 2000; Su *et al*., 2003).

However, HPLC-based methods often require more intensive sample preparation, particularly when monitoring for low levels of benzimidazole residues (Sorenson and Petersen, 1995; Tai, 1990). More recently, groups have developed LC-MS/MS methods to detect residues in food that require less complicated clean-up steps (Radeck and Gowick, 2008; Verdon *et al*., 2008).

Immunoassay-based veterinary drug detection systems have been developed by other groups as an alternative to chemical assays. Lately these assays have shown improvements in sensitivity, selectivity and also require simpler sample preparation in comparison to chemical-based assays (Brandon *et al*., 1994; Bushway *et al*., 1995; 1993). Recently several SPR-biosensor assays have been developed to detect veterinary drug residues in milk (Baxter *et al*., 2001; Crooks, 2003; Gustavsson *et al.,* 2002; Haasnoot *et al*., 2003). The SPR-biosensor assay employs "label-free" detection and have proven to be versatile, robust and capable of producing rapid and reliable results with minimum sample preparation (Baxter *et al*., 2001). A biosensor assay capable of detecting benzimidazole residues in bovine serum samples using a simple extraction was developed in 2002 by Johnsson *et al*. However no SPR-biosensor method for detecting these substances in food matrices is reported in the literature. This research describes the development of a sensitive SPR-biosensor assay to detect trace levels of 11 out of 14 major benzimidazole carbamate residues in milk used in combination with a modified QuEChERS method for extraction. The polyclonal antibody does not cross-react to the residues of triclabendazole, thiabendazole or amino-benzimidazole metabolites. The method was validated according to the 2002/657/EC guidelines as required for EU monitoring programs (Anonymous, 2002). The factors investigated included recovery, repeatability and analytical limits, including the limit of detection (LOD) and detection capabilities (CCβ) of the method.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Sensor chips (CM5, research grade), NHS (100 mM N-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES, pH 7.4, with 0.05 M NaCl, 3.4 mM EDTA and 0.005% (v/v) P20 were all obtained from GE Healthcare (Uppsala, Sweden). Sodium hydroxide (NaOH), HPLC grade water, pesticide grade acetonitrile (ACN), pesticide grade dimethylsulphoxide (DMSO) and methanol were supplied by BDH/VWR international Ltd. (Poole, England, UK). Ethylenediamine (99%, v/v), Jeffamine (4-((4-aminophenyl)methyl)aniline, $C_{13}H_{14}N_2$), dimethylformamide, ABZ, MBZ, TBZ and FBZ were supplied by Sigma Aldrich (Steinheim, Germany). OXI, FBZ-SO and FLU were purchased from QMX laboratories (Thaxted, UK). Amino-mebendazole (MBZ-NH_2), hydroxy-mebendazole (MBZ-OH), amino-flubendazole (FLU-NH_2) and hydroxy-flubendazole (FLU-OH) were received as a gift from Janssen pharmaceuticals (Belgium). ABZ-SO, albendazole sulphone (ABZ-SO_2), albendazole amino sulphone (ABZ-NH_2-SO_2), fenbendazole sulphone (FBZ-SO_2), 5-hydroxy-thiabendazole (5-OH-TBZ), triclabendazole (TCB), triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO_2) and keto-triclabendazole (keto-TCB) were purchased from Witega laboratories (Berlin, Germany). Primary standard stock solutions for each benzimidazole were prepared in DMSO or methanol depending on solubility. Working standard solutions were prepared by diluting the primary standard solutions in methanol. Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g NaCl were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate ($MgSO_4$) and 0.5 g C_{18} sorbent were purchased from Biotage (Uppsala, Sweden). Whatman syringe filter units (polytetrafluoroethylene (PTFE), 0.2 μm) were purchased from Fisher scientific (Dublin, Ireland).

3.2.2 Negative Milk samples

Fresh bovine milk samples were collected from milk tanks on farms and those found to be free of benzimidazole residues by UPLC-MS/MS (limit of quantitation of 1 μg kg^{-1}) were used as negative controls, fortified for validation studies and calibration curve samples.

3.2.3 SPR-Biosensor assay

3.2.3.1 Modified QuEChERS sample preparation

Milk samples (12 g) were extracted using a slurry containing ACN:$MgSO_4$:NaCl (12:4:1, v/w/w) by shaking vigorously by hand (1 min). The samples were centrifuged (3500*g*, 10 min, -5ºC). The centrifuge was set to this temperature to freeze the fat in the sample to avoid the formation of an emulsion when the sample tube was removed from the centrifuge. The supernatant was transferred to a tube containing C_{18} sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500*g*, 10 min, -5ºC). The ACN layer (7.5 mL) was transferred to Pyrex® tubes and evaporated to dryness at 50ºC, under nitrogen. Extracts were reconstituted in DMSO (2.5 mL), vortexed (2 min) and sonicated (10 min). Extracts (2.5 mL) were diluted in water (2.5 mL), vortex mixed (1 min) and filtered (0.22 μm) into Eppendorf® tubes. The sample extract was diluted (1:4, v/v) in HBS-EP buffer and vortex mixed (20 s) prior to biosensor analysis.

3.2.3.2 Biosensor Chip preparation

A CM5 chip was allowed to equilibrate to room temperature and HBS-EP buffer (50 μL) was added to the chip surface and incubated (10 min). The buffer was removed and 50 mM NHS:200 mM EDC (1:1, v/v, 40 μL) was added to the chip surface and incubated (20 min) to activate the surface. This solution was removed and 1 M ethylenediamine pH 8.5 (50 μL) was allowed to incubate (1 h). The remaining unreacted groups on the chip surface were deactivated by addition of 1 M ethanolamine-HCl (50 μL) and allowed to react (20 min).

Methyl 5(6)-((carboxypentyl)-thio)-2-benzimidazolecarbamate (2 mg) (Brandon *et al*., 1994) was dissolved in DMF (450 µL) and mixed with a solution containing NHS (2 mg) and EDC (5 mg) in 10 mM sodium acetate buffer, pH 4.5, (450 µL) and allowed to react on the chip surface (2 h) at room temperature. The chip was washed with HPLC grade water and dried under a stream of nitrogen gas. The immobilised chip was stored in a dessicated container (4ºC).

3.2.3.3 SPR-Biosensor analytical cycle

The optical biosensor used was a Biacore Q (GE Healthcare, Uppsala Sweden) with Biacore® Q control software version 3.0. BIAevaluation software version 3.0.1 was used for data handling. All studies were conducted at 25ºC. The polyclonal antibody (S48) used in this work was raised in sheep against a methyl 5(6)-(carboxypentyl)-thio)-2-benzimidazolecarbamate derivative (CMB)) (Johnsson *et al*., 2002). This antibody was received from the Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland. An antibody dilution of 1/1200, v/v, was found to give satisfactory results under the assay conditions. Antibody and milk extract were mixed (1:3, v/v) and passed over the immobilised surface at a flow rate of 10 µL min^{-1} (1 min). Regeneration of the chip was carried out by sequential injection of 25 mM HCl (15 µL) followed by 180 mM NaOH (20 µL) across the chip surface at 25 µL min^{-1}. The binding of antibody to the chip surface was measured as the change in surface plasmon resonance (SPR) signal between two report points, before (10 s) and after (30 s) each injection. A competitive immunoassay assay format was used to detect inhibition of antibody binding to the chip surface. SPR signal was expressed in arbitrary resonance units (RU). All samples analyses were made in duplicate and the mean response was reported.

3.2.4 Calibration

Calibration curves were prepared in matrix by fortifying negative milk samples at concentrations of 0, 2.5, 5, 10, 15, 25 and 50 μg kg^{-1} with an ABZ-SO_2 standard prior to extraction. BIAevaluation software was used to prepare inhibition assay standard curves based on a four-parametric fit. The concentration in each test sample was read directly from the calibration curve.

3.3. Results and discussion

3.3.1 Antibody inhibition studies

The antibody cross-reactivity was investigated by analysing standards prepared in buffer by SPR-biosensor assay. The antibody showed significant affinity to 11 benzimidazole residues in the following order of affinity FBZ-SO, FBZ-SO_2 > ABZ-SO > ABZ, MBZ > ABZ-SO_2 > MBZ-OH > FLU > OXI > FBZ, FLU-OH (**Table 3.1**). The antibody showed low levels (< 7%) of cross-reactivity towards the amino-benzimidazole metabolites, FLU-NH_2, MBZ-NH_2 and OXI-NH_2. However, it did not show any measurable cross-reactivity towards TCB, keto-TCB, TCB-SO, TCB-SO_2, TBZ, 5-OH-TBZ and ABZ-NH_2-SO_2 when fortified HBS-EP buffer (up to 1000 ng mL^{-1}) curves were analysed. A more detailed investigation of the antibody cross-reactivity was carried out by preparing inhibition curves in buffer at concentrations from 0 to 30 ng mL^{-1} for 11 analytes. The 11 benzimidazole residues studied showed significant cross-reactivity with IC_{50} values of typically <6.6 ng mL^{-1} (**Table 3.1).** A second study was carried out using the modified QuEChERS extraction in milk calibration curves over the range 0 to 50 μg kg^{-1}. The concentration of each analyte required to inhibit 50% of antibody binding (IC_{50}) was calculated using **Formula A**. IC_{50} in matrix typically ranged from 11 to 18 μg kg^{-1} for FBZ-SO to FBZ, respectively (**Table 3.1**). Extracted milk calibration curves for the 11 analytes are shown in **Fig. 3.1**.

Formula A: Response (RU) at $IC_{50} = R_{lo} - ((R_{lo}-R_{hi}) / 2)$

Where Response (RU) at IC_{50} = Relative response at 50% antibody inhibition

R_{lo} = Relative response (RU) in the absence of analyte

R_{hi} = Relative response (RU) at the maximum concentration of analyte

The Response (RU) at IC_{50} was plotted on the inhibition curve to determine the concentration of analyte using the "simulate sample" function in the BiacoreQ Wizard software.

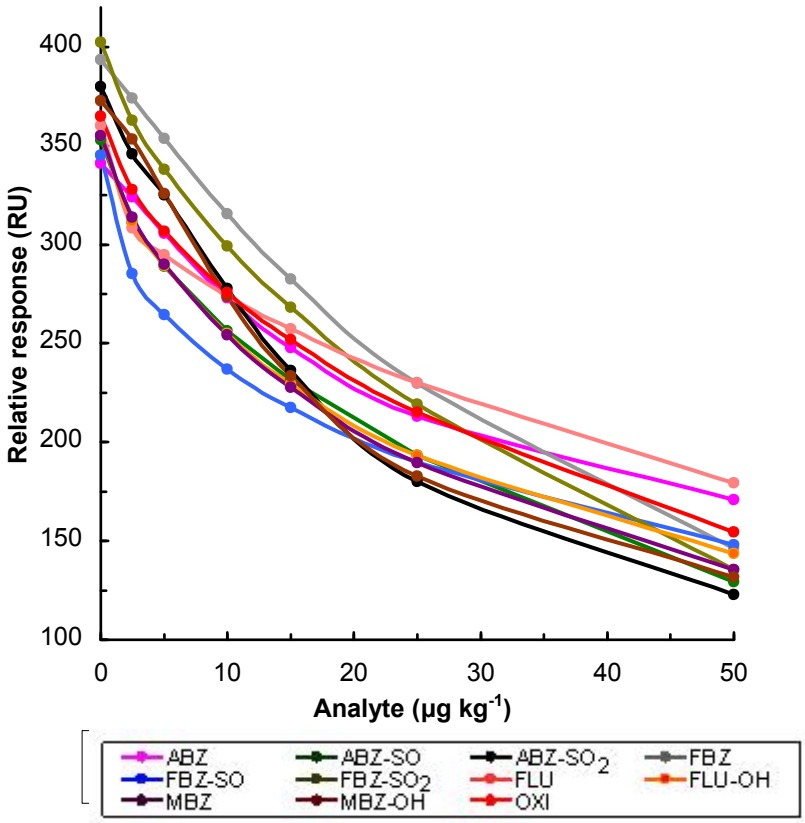

Figure 3.1 Standard curves for 11 benzimidazole carbamates in bovine milk matrix

Table 3.1 Cross-reactivity profile of benzimidazole carbamate drugs to polyclonal antibody (S48) in HBS-EP buffer and in bovine milk.

	Buffer		Milk	
Analyte	[a]IC_{50} (ng mL^{-1})	[b]CR_{50} (%)	[c]IC_{50} (µg kg^{-1})	[d]CR_{50} (%)
ABZ	4.5	98	13.3	95
ABZ-SO	4.4	100	12.7	100
ABZ-SO_2	4.8	93	14.2	90
FBZ	6.6	67	17.3	73
FBZ-SO	4.0	110	11.5	111
FBZ-SO_2	4.0	110	15.3	84
MBZ	4.5	98	12.3	103
MBZ-OH	5.0	88	13.5	94
FLU	5.5	80	15.2	84
FLU-OH	6.6	67	13.6	94
OXI	6.2	71	12.9	98
ABZ-NH_2-SO_2	NA	NA	NA	NA
FLU-NH_2	66	7	NA	NA
MBZ-NH_2	160	3	NA	NA
OXI-NH_2	98	5	NA	NA
5-OH-TBZ	ND[e]	ND[e]	ND[e]	ND[e]
TBZ,	ND[e]	ND[e]	ND[e]	ND[e]
TCB	ND[e]	ND[e]	ND[e]	ND[e]
keto-TCB	ND[e]	ND[e]	ND[e]	ND[e]
TCB-SO	ND[e]	ND[e]	ND[e]	ND[e]
TCB-SO_2	ND[e]	ND[e]	ND[e]	ND[e]

[a] The analyte concentration of inhibitor (analyte) required to reduce the response by 50% in HBS-EP buffer

[b]Cross-reactivity of antibody to test benzimidazole at 50% inhibition ((IC_{50} ABZ-SO / IC_{50} test BZT) x 100) in HBS-EP buffer.

[c] The analyte concentration of inhibitor (analyte) required to reduce the response by 50% in bovine milk

[d]Cross-reactivity of antibody to test benzimidazole at 50% inhibition ((IC_{50} ABZ-SO / IC_{50} test BZT) x 100) in bovine milk

[e]No cross-reactivity detected

3.3.2 Development of sample preparation procedure

The extraction of benzimidazole residues was initially evaluated using conventional solvent extraction with ACN and liquid-liquid extraction with ethyl acetate at different pHs. ACN was found to give the best recovery of benzimidazoles and did not require pH manipulation. However, lower recovery was observed for ABZ and FBZ compared to other benzimidazole metabolites. An extraction method based on QuEChERS, which was recently applied to isolate benzimidazole residues (Constantinou *et al*., 2000) was also investigated but initially gave low recovery. A spiking experiment was performed and the results identified that recovery losses with the QuEChERS method occurred due to the inability to resuspend residues. It was proposed that losses were either due to adsorption of residues onto glassware during evaporation or, more likely, tight binding of residues by milk proteins.

A further QuEChERS experiment was undertaken to evaluate the effect of alternative resuspension solvents such as MeOH:water (50:50, v/v) and various concentrations of DMSO in water on the recovery of ABZ, FBZ, FLU, MBZ and OXI. Recovery was found to be <60% for ABZ, FBZ, FLU, MBZ and OXI residues when reconstituted in MeOH:water (50:50, v/v) (**Fig. 3.2**). The percentage recovery for all 11 benzimidazole residues was found to be acceptable (≥69%) using DMSO:water (50:50, v/v, 5 mL). The recovery of amino-metabolites from fortified milk (100 µg kg^{-1}) was less than 1% and considered insignificant for the purpose of this screening assay. In order to achieve detection of benzimidazoles at less <5 µg kg^{-1} in milk, the sample weight was increased to 12 g and extracts were diluted (1:4, v/v) with HBS-EP buffer. A working antibody dilution (1/1200, v/v), flow rate (10 µL min^{-1}), contact time (1 min) and antibody:extract mix ratio (1:3, v/v) were optimised to give a response approximately equal to 380 RU (b_0) for benzimidazole-negative milk samples. The SPR-biosensor assay regeneration conditions were based on conditions developed by Johnsson *et al*. (2002).

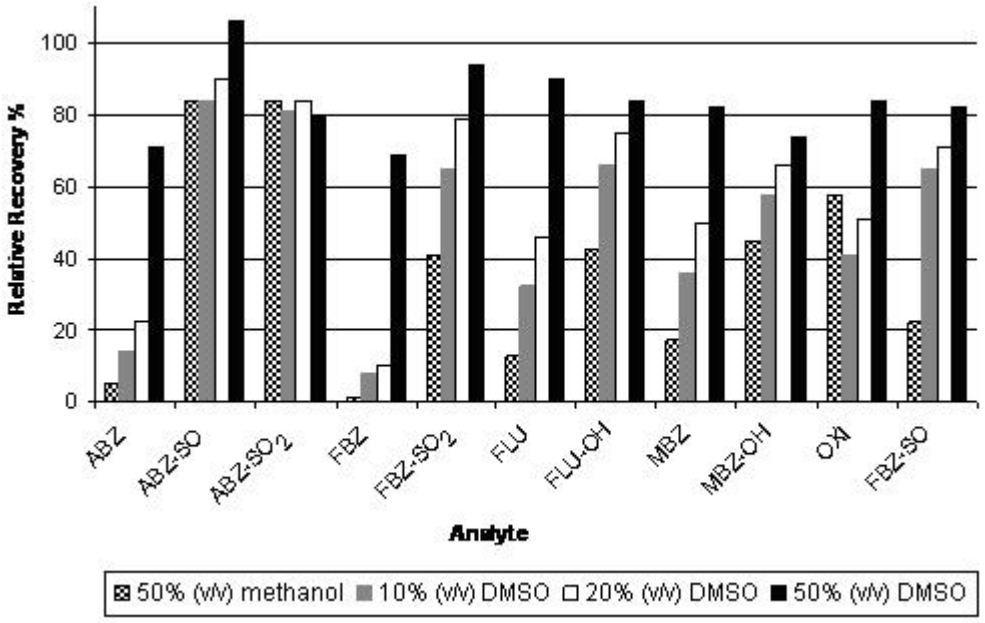

Figure 3.2 Effect of methanol and dimethylsulphoxide reconstitution on the recovery of benzimidazole residues in milk using a modified QuEChERS extraction method.

3.3.3 Method validation

A qualitative approach was used to determine the performance factor CCβ (the detection capability) as described in 2002/657/EC (EC, 2002). Firstly, the limit of detection (LOD) of the assay was determined to be 2.7 μg kg^{-1} by measuring the mean response for 20 different negative bovine milk samples (371.4 RU) and subtracting three standard deviations (3 x 12.5 RU). Secondly, in order to determine CCβ values, samples (n = 20 for each analyte) were spiked at a concentration above the LOD. An arbitrary concentration of 5 μg kg^{-1} was selected because this level is equivalent of detection levels that can be achieved by HPLC-based assays and it was considered that the assay under study should routinely measure this concentration level.

In routine applications, where several possible benzimidazole residues may be detected in a naturally positive sample, the assay is able to detect summed metabolites at ≥2.7 μg kg^{-1} (comparable to UPLC-MS/MS). The CCβ is the concentration at which a substance can be identified as positive (>LOD) with a statistical certainty of 1 – β. Samples (n = 20) were fortified at a level of 5 μg kg^{-1} for each analyte and assayed.

If 19 of the 20 fortified samples were identified as positive, CCβ was determined to be 5 μg kg^{-1} (5% probability of a false negative result). If 20 or ≤18 samples were identified as positive, CCβ was determined to be less than or greater than 5 μg kg^{-1}, respectively. The results for the CCβ determination of each analyte are shown in **Table 3.2**

Table 3.2 Determination of assay detection capability (CCβ): The concentration of benzimidazole residues determined by biosensor analysis of milk fortified at 5 μg kg^{-1} with 11 benzimidazole marker residues (n=20).

Analyte	Mean ± SD (μg kg^{-1})	Minimum (μg kg^{-1})	Maximum (μg kg^{-1})	CCβ (μg kg^{-1})
ABZ	5.39 ± 0.87	3.65	6.84	<5.00
ABZ-SO	3.83 ± 0.64	2.90	5.50	<5.00
ABZ-SO_2	5.73 ± 1.68	3.39	10.00	<5.00
FBZ	5.15 ±1.56	3.48	8.54	<5.00
FBZ-SO_2	8.93 ± 0.80	7.84	11.10	<5.00
FLU	9.37 ± 2.00	4.90	11.80	<5.00
FLU-OH	3.78 ± 0.76	2.65	5.43	5.00
MBZ	4.06 ± 1.21	2.03	7.01	5.00
MBZ-OH	4.49 ± 1.23	3.00	7.78	<5.00
FBZ-SO	4.45 ± 0.97	3.00	6.08	<5.00
OXI	4.86 ± 2.26	2.76	10.10	<5.00

The CCβ value for nine analytes was found to be <5 μg kg^{-1}. CCβ values for FLU-OH and MBZ were found to be equal to 5 μg kg^{-1}, in each case one sample was not identified as positive. The two false negative samples gave measured results of 2.65 and 2.05 μg kg^{-1}, respectively. However, the method satisfies the false negative rate (≤5%) as required by 2002/657/EC (Anonymous, 2002). The repeatability of the assay was evaluated by analysing fortified milk samples (5 μg kg^{-1}) with the 11 analytes on five separate days (**Table 3.3**).

Results showed that recovery was between 81-116% and that inter-assay coefficients of variation were typically <30%. Calibration curves for each day are presented in **Fig. 3.3**. A calibration curve prepared in HBS-EP buffer is also presented in **Fig. 3.3**, which demonstrates the low rate of non-specific binding and high extraction efficiency of the method.

Table 3.3 Biosensor assay repeatability study: Recovery of 11 benzimidazole marker residues from milk fortified at 5 µg kg^{-1} on five different days.

Analyte	Mean Recovery (%) ± SD (n=5)	[a]CV (%) (n=5)
ABZ	97 ± 34	35
ABZ-SO	111± 27	25
ABZ-SO_2	116 ± 16	13
FBZ	81 ± 16	20
FBZ-SO_2	107 ± 25	23
FLU	111± 37	33
FLU-OH	85 ± 10	11
MBZ	93 ± 25	27
MBZ-OH	81± 22	27
FBZ-SO	101 ± 30	29
OXI	96 ± 25	26

[a] Percentage coefficient of variability: CV % = SD/ Mean x 100

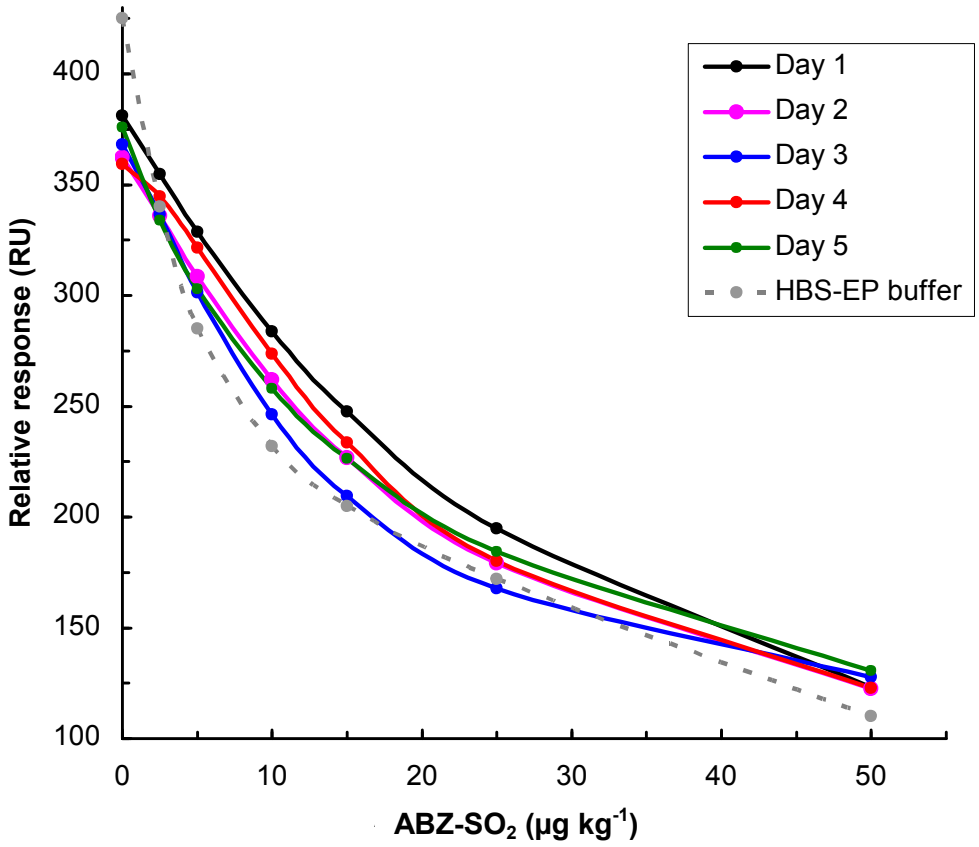

Figure 3.3 Albendazole sulphone (ABZ-SO_2) biosensor calibration curves in fortified bovine milk on different days (n = 5) and in HBS-EP buffer.

3.4. Conclusions

This SPR-biosensor assay is suitable for use as a rapid screening method for the detection of 11 benzimidazole residues in milk. An extensive validation of the assay was carried out for 11 benzimidazole carbamate residues. The LOD and CCβ for benzimidazole residues were determined to be 2.7 µg kg^{-1} and 5 µg kg^{-1}, respectively, which is equivalent to the existing chemical assay. The false negative rate for the assay was ≤5%. This study was performed using artificially fortified / spiked milk samples. The assay performance in "real" incurred milk samples, from animals treated with benzimidazole drugs, will ultimately determine the limitations of this screening assay.

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Chapter 4

Detection of benzimidazole residues in incurred milk samples by SPR biosensor

4.1 Introduction

Benzimidazole residue detection in milk requires sensitive analytical assays (De Ruyck *et al.*, 2002; Su *et al*., 2003; Verdon *et al.*, 2008). These methods can often be described as lengthy and laborious to perform and in order to reduce the number of samples that require confirmatory analysis a two tier testing approach may be used. Using this methodology all samples are screened using a rapid screening technique and subsequently any suspect positive samples are quantified by confirmatory assay. Immunoassay screening techniques have previously been applied to detect benzimidazole residues in both milk and liver. Screening methods, using the ELISA format, have been produced to detect fenbendazole residues in milk (Brandon *et al*., 2002) and the residues of albendazole and fenbendazole in liver (Brandon *et al.*, 1994). Chapter 3 of this work describes the development and validation of the first biosensor screening assay for benzimidazoles in milk using a modified QuEChERS extraction method. This qualitative technique does not identify each individual benzimidazole or metabolites. The assay was developed to generate a positive result (> CCβ) or negative result (<CCβ). The aims of this research were to determine if this assay is applicable to benzimidazole incurred bovine milk samples, to examine the assay performance in incurred ovine milk and to establish if the lack of antibody cross-reactivity to ABZ-NH_2-SO_2 would cause false negative results. A comparison was made between the SPR-biosensor and UPLC-MS/MS analyses of milk samples taken from cows and a goat treated with different benzimidazole products, to demonstrate the SPR-biosensor assay to be fit for purpose.

4.2 Materials and methods

4.2.1 Chemicals and reagents

CM5 sensor chips (research grade), NHS (100 mM N-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES, pH 7.4, with 0.05 M NaCl, 3.4 mM EDTA and 0.005% (v/v) P20 were all obtained from GE Healthcare (Uppsala, Sweden). Sodium hydroxide (NaOH), HPLC grade water, pesticide grade acetonitrile (ACN), pesticide grade dimethylsulphoxide (DMSO) and methanol were supplied by BDH/VWR international Ltd. (Poole, England, UK).

Ethylenediamine (99%, v/v), dimethylformamide, ABZ, MBZ, TBZ and FBZ were supplied by Sigma-Aldrich (Steinheim, Germany). OXI, FBZ-SO and FLU were purchased from QMX laboratories (Thaxted, UK). Amino-mebendazole (MBZ-NH_2), hydroxy-mebendazole (MBZ-OH), amino-flubendazole (FLU-NH_2) and hydroxy-flubendazole (FLU-OH) were received as a gift from Janssen pharmaceuticals (Belgium). ABZ-SO, albendazole sulphone (ABZ-SO_2), albendazole amino sulphone (ABZ-NH_2-SO_2), fenbendazole sulphone (FBZ-SO_2), 5-hydroxy-thiabendazole (5-OH-TBZ), TCB, triclabendazole sulphoxide (TCB-SO), triclabendazole sulphone (TCB-SO_2) and keto-triclabendazole (keto-TCB) were purchased from Witega laboratories (Berlin, Germany). ABZ-D3, ABZ-SO-D3, ABZ-SO_2-D3, FBZ-D5, FBZ-SO-D5, FBZ-SO_2-D5, MBZ-D3, MBZ-OH-D3, FLU-D3 and OXI-D7 were from Witega laboratories (Berlin, Germany). ABZ-NH_2-SO_2-D2 was from Quchem (Belfast, Northern Ireland, UK). Primary standard stock solutions for each benzimidazole were prepared in DMSO or methanol depending on solubility. Working standard solutions were prepared by diluting the primary standard solutions in methanol. Deuterated internal standards were prepared at concentrations of 1 mg mL^{-1} in DMSO or methanol-d. A working standard solution (2 µg mL^{-1}) was prepared by diluting the primary stock internal standard solution in methanol-d.

Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g NaCl were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate ($MgSO_4$) and 0.5 g C_{18} were purchased from Biotage (Uppsala, Sweden). Whatman syringe Filter units (polytetrafluoroethylene (PTFE), 0.2 µm) were purchased from Fisher scientific (Dublin, Ireland).

4.2.2 Milk samples

4.2.2.1 Negative control samples

Fresh bovine milk samples were collected from milk tanks on farms and those found to be free of benzimidazole residues by UPLC-MS/MS (limit of quantitation of 1 µg kg^{-1}) were used as negative controls. The UPLC-MS/MS method was capable of detecting the all of the major metabolites of ABZ, FBZ, MBZ and FLU drugs.

4.2.2.2 Incurred milk samples

Two cows were treated with Panacur SC® 10% (7.5 mg FBZ kg^{-1} b.w. (bodyweight)) and Endospec® 10% (7.5 mg ABZ kg^{-1} b.w.) oral suspension, respectively. Pooled quarter milk samples were taken from each animal immediately prior to dosing and again at subsequent morning and evening milkings for 11 milkings, with a minimum milking interval of 9 hours. The final milk sample was taken 135 hours post-treatment.

Four milk samples were taken from a cow treated with the FBZ product Rintal® 1.9% (1000 mg Febantel in feed) at 7, 24, 31 and 168 h post-treatment.

Goats (n = 5 each group) were treated with oral suspensions (a) a "normal" dose of Kilan O® 5% (15 mg MBZ kg^{-1} b.w.) and (b) a "high" dose (30 mg MBZ kg^{-1} b.w.). Milk samples were taken from the two groups at 2.5, 4, 6, 10, 14, 18, 26, 32, 38, 48, 72 and 96 h post-treatment. Samples taken at 4 to 10, 18 to 26, and 32 to 38 h were available in low volumes and had to be pooled to allow effective comparison of the biosensor and UPLC-MS/MS methods.

4.2.3 Biosensor assay

4.2.3.1 Modified QuEChERS sample preparation

As per section 3.2.3.

4.2.3.2 Biosensor chip preparation

As per section 3.2.3.

4.2.4 UPLC-MS/MS assay

4.2.4.1 Sample preparation

Samples were analysed by the method developed by De Ruyck *et al.* (2002). Milk samples (5 g) were spiked with internal standard solution and let stand for 30 min. Samples were adjusted to alkaline conditions by addition of 10M NaOH (100 µL). Ethyl acetate (15 mL) was added to samples, which were shaken (60 oscillations min^{-1}, 5 min). Samples were centrifuged (2500*g*, 10 min).

The supernatant layer was transferred to a polypropylene centrifuge tube (15 mL), DMSO (0.25 mL) was added and the ethyl acetate was evaporated under nitrogen at 50°C. Samples were filtered through 0.2 µm PTFE filters and 5 µL was injected onto the UPLC-MS/MS system.

4.2.4.2 Detection conditions

The UPLC-MS/MS system consisted of a Waters Acquity® separations module and a Quattro Premier XE equipped with ESI interface (Waters, Milford, MA, USA). The separation was carried out on a stainless steel Waters Acquity® analytical column (100 x 2.1 mm), packed with HSS T3 C_{18}, 1.8 µm and Waters Acquity UPLC Column In-Line Filter Unit containing a 0.2 µm stainless steel replacement filter (all from Waters). The pump was operated at a flow rate of 0.6 mL min^{-1} and column temperature was maintained at 60^{o}C. The chromatographic separation was achieved using a binary gradient comprised of – Mobile phase A, 0.01% (v/v) acetic acid in water:ACN (900:100 v/v) and Mobile phase B, 5mM Ammonium formate in MeOH:ACN (750:250, v/v) pumped at a flow rate of 0.6 mL min^{-1}. Mobile phase was prepared daily and filtered using 0.2 µm filter membrane and degassed in an ultrasonic bath for 15 min. The gradient profile was as a follows (1) 0 to 0.5 min, 100%A, (2) 5 min, 50%A, (3) 7 min, 10%A, (4) 8.5 min, 10%A, (5) 8.51 min, 0% A, (6) 9.5 min, 0%A, (7) 9.51 min, 100%A, (8) 13 min 100%A. The UPLC-MS/MS system was controlled by Masslynx software and the results were processed by TargetLynx Software. Chromatograms are shown for FBZ, OFZ, ABZ-NH2-SO_2, ABZ-SO_2, ABZ-SO, ABZ, MBZ, MBZ-OH and MBZ-NH_2 (**Fig. 4.1-4.3)**.

MS analyses were performed by atmospheric pressure electrospray ionisation in positive ion mode. The capillary voltage was set at 3 kV. The source and desolvation temperatures were set at 150 and 450°C, respectively. The nitrogen desolvation and cone gases were set at 1000 and 50 L h^{-1}, respectively. The MS/MS conditions were optimised by tuning the cone voltage and collision energy for each analyte by infusing a 1000 ng mL^{-1} standard solution of each analyte individually and monitoring the two most abundant fragment ions produced from the molecular ion. Data were acquired in multiple reaction monitoring (MRM) as outlined in **Table 4.1.**

4.2.5 Calibration

4.2.5.1 Biosensor

Calibration curves were prepared in matrix by fortifying negative milk samples at concentrations of 0, 2.5, 5, 10, 15, 25 and 50 μg kg^{-1} with an ABZ-SO_2 standard prior to extraction. BIAevaluation software was used to prepare inhibition assay standard curves based on a four-parametric fit. The concentration in test samples was read directly from the calibration curve.

4.2.5.2 UPLC-MS/MS

Two approaches were adopted for measurement of benzimidazole residues in samples. In the first approach for measuring low levels of benzimidazoles, calibration curves were prepared by fortifying negative milk samples at concentrations of 1, 2, 5, 10, 25, 50, 100 and 200 μg kg^{-1}, and incubated for 30 min prior to extraction. Samples were also fortified with the internal standard mixture at this time. In the second method for measuring high levels of benzimidazoles, calibration curves were prepared by fortifying negative milk samples at concentrations of 10, 20, 50, 100, 250, 500, 1000, 2000 μg kg^{-1} and incubated for 30 min prior to extraction. A lower volume of ethyl acetate extract (1.5 mL) was carried through to analysis to ensure linearity of curves.

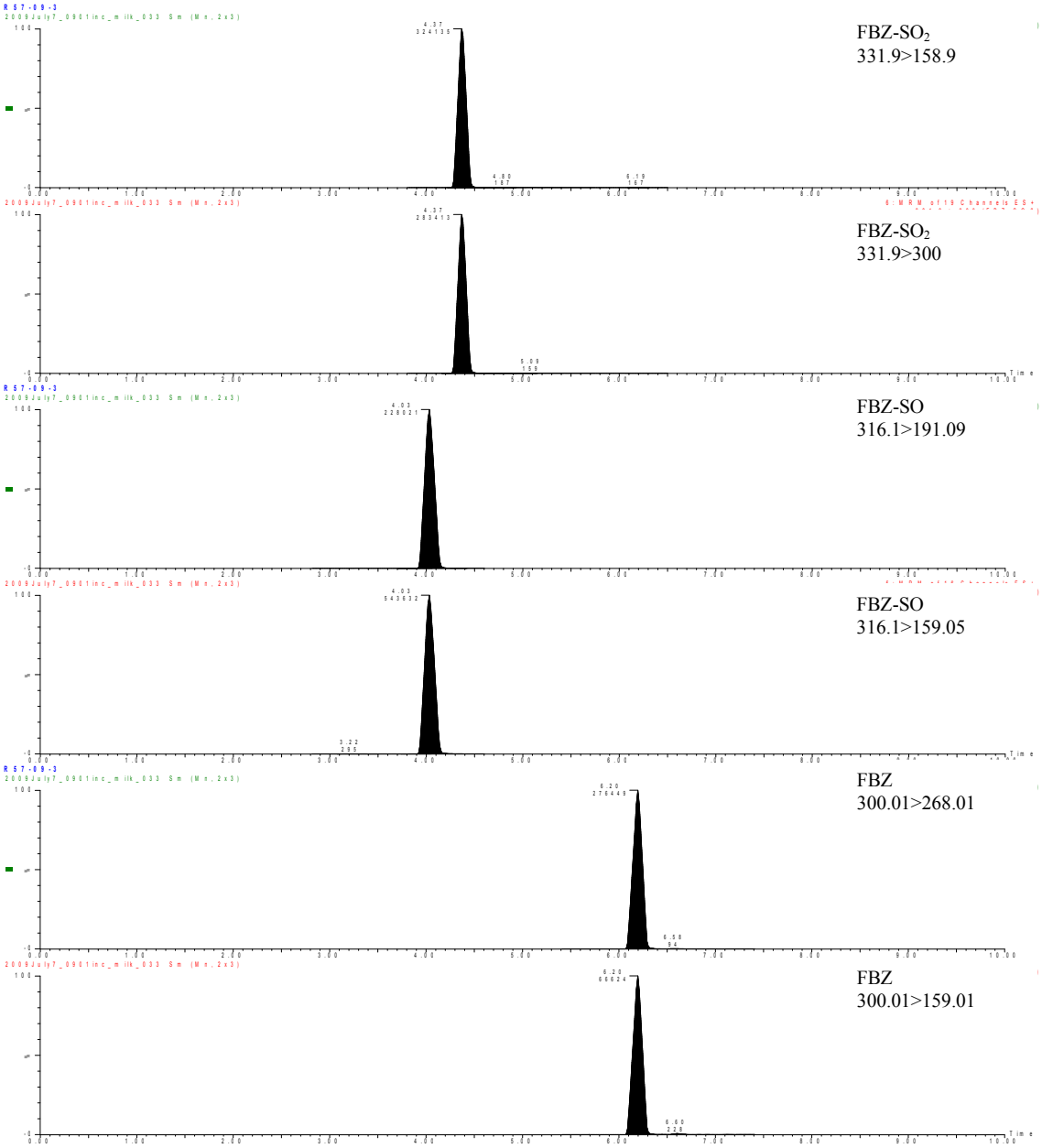

Fig. 4.1 LC-MS/MS chromatograms of FBZ, FBZ-SO and FBZ-SO_2 for an incurred sample from Panacur® SC 10% study (15 h withdrawal). Time in minutes is shown on the x axis and Relative Intensitiy (%) is shown on the y axis.

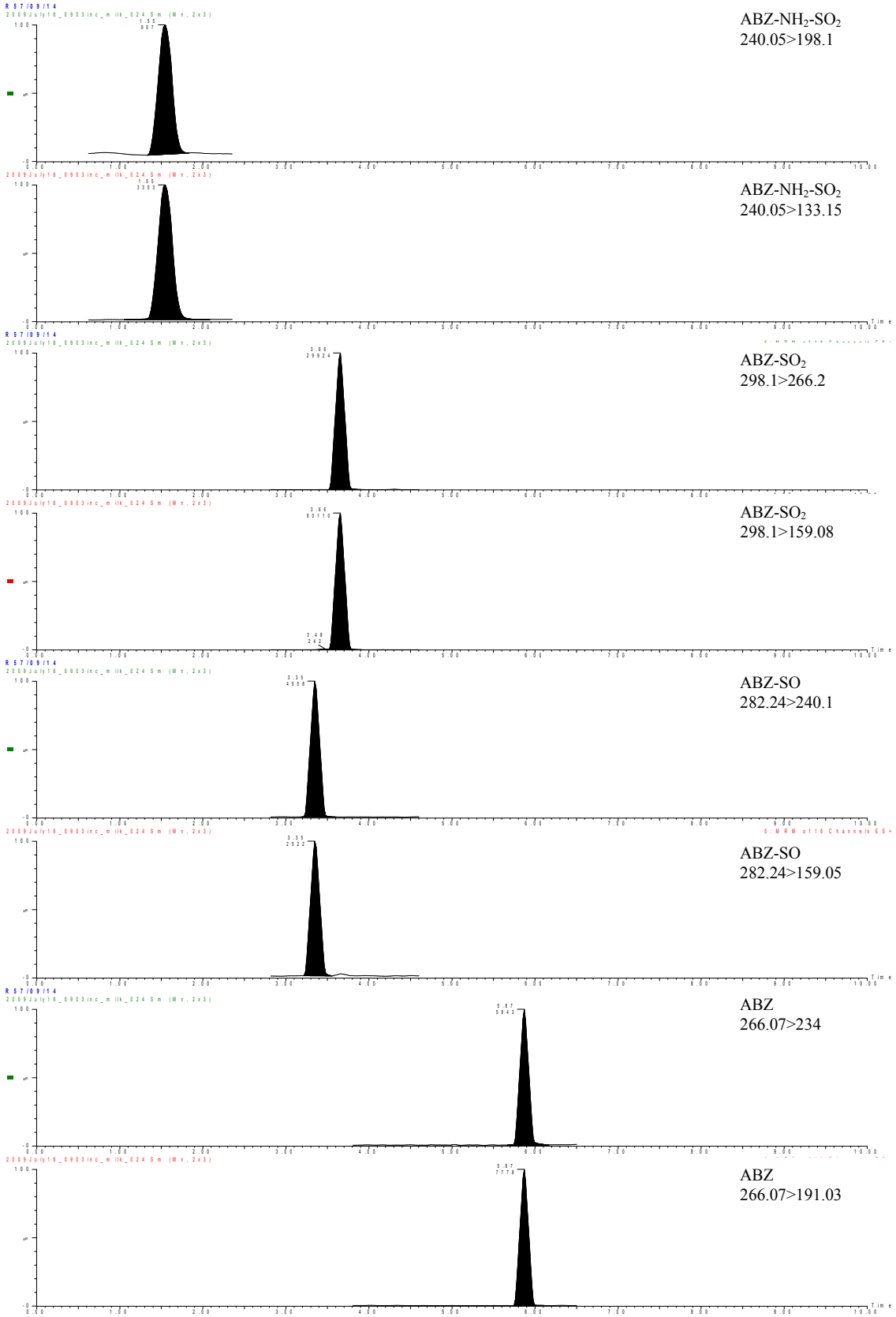

Fig. 4.2 LC-MS/MS chromatograms of ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues detected in milk sample from Endospec® 10% study (15 h withdrawal). Time in minutes is shown on the x axis and Relative Intensitiy (%) is shown on the y axis.

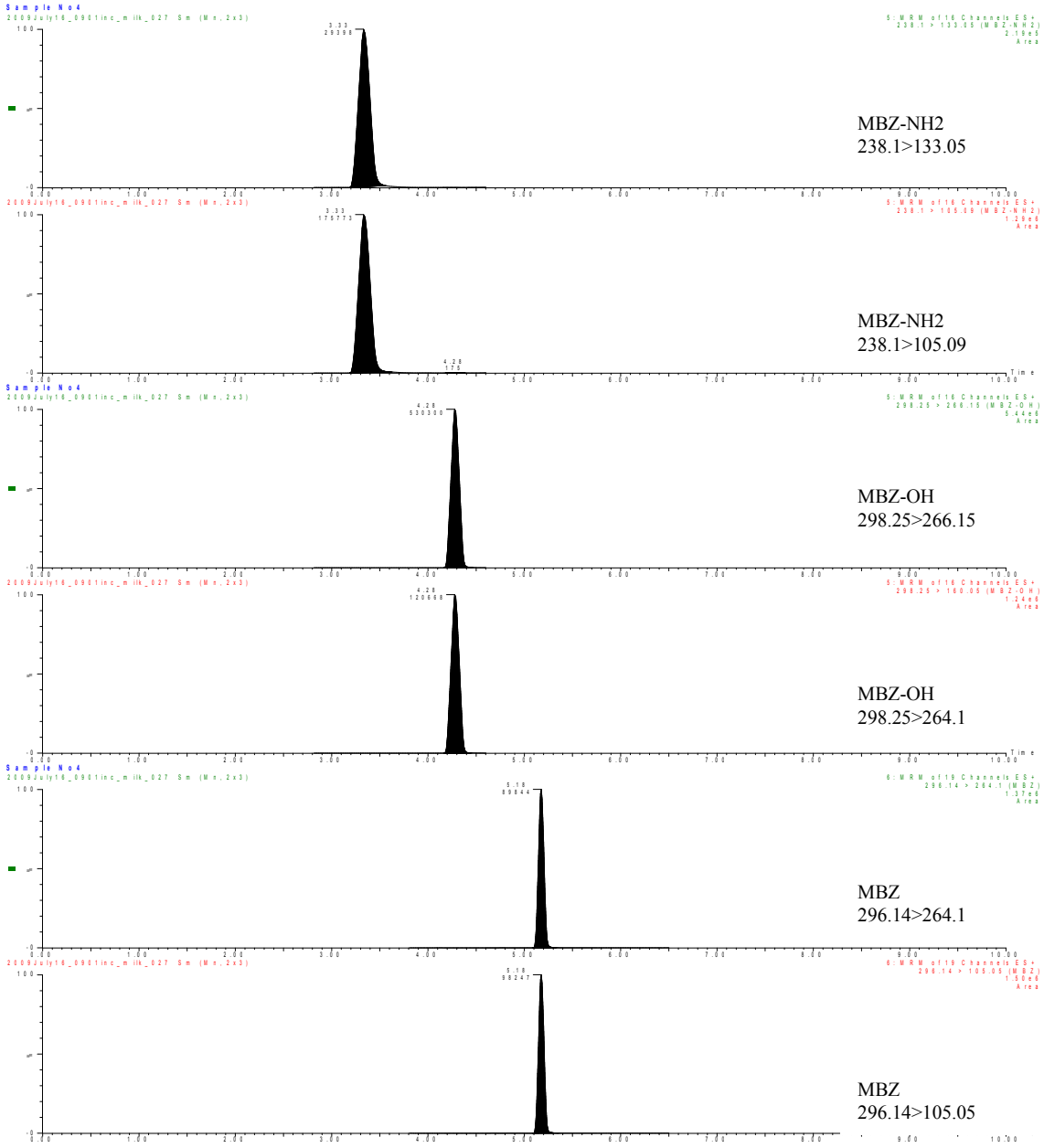

Fig. 4.3 LC-MS/MS chromatograms of MBZ, MBZ-OH and MBZ-NH_2 for an incurred sample from Kilan® O 15% study (15 h withdrawal). Time in minutes is shown on the x axis and Relative Intensitiy (%) is shown on the y axis.

Table 4.1 MS/MS parameters for benzimidazole analytes and internal standards

Compound	Transition (*m/z*)	Dwell time (s)	Cone Energy (V)	Collision energy (eV)
ABZ-NH_2-SO_2	240.08 > 133.15	0.050	40	27
	240.08 > 198.10	0.050	40	20
ABZ-NH_2-SO_2-D_3	242.00 > 133.00	0.050	40	28
MBZ-NH_2	238.10> 105.09	0.025	50	24
	238.10 > 133.05	0.025	50	34
FLU-NH_2	256.06 > 123.05	0.010	45	26
	256.06 > 95.10	0.010	45	34
ABZ-SO	282.24 > 159.06	0.005	27	35
	282.24 > 240.10	0.005	27	15
ABZ-SO-D_3	285.28 > 243.02	0.005	41	13
ABZ-SO2	298.10 > 159.08	0.005	42	35
	298.10 > 266.20	0.005	42	20
MBZ-OH	298.25 > 160.05	0.005	38	33
	298.25 > 266.15	0.005	38	22
ABZ-SO_2-D_3	301.00 > 158.95	0.005	40	38
MBZ-OH-D_3	301.15 > 160.05	0.005	36	32
OFZ	316.10 > 159.05	0.020	35	30
	316.10 > 191.09	0.020	35	24
FBZ-SO_2-D_5	321.04 > 158.95	0.020	30	32
OXI	249.90 > 175.90	0.010	35	26
	249.90 > 218.00	0.010	35	18
OXI-D_7	257.15 > 177.05	0.005	32	28
ABZ	266.07 > 191.03	0.015	33	32
	266.07 > 234.00	0.015	33	13
ABZ-D_3	269.12 > 233.85	0.015	35	19
MBZ	296.14 > 105.05	0.010	35	32
	296.14 > 264.10	0.010	35	18
MBZ-D_3	299.15 > 105.05	0.005	39	33
FLU	313.80 > 123.00	0.005	40	35
	313.80 > 282.00	0.005	40	24
FLU-OH	316.20 > 125.10	0.050	40	33
	316.20 > 160.05	0.050	40	35
FLU-D_3	317.15 > 123.00	0.005	40	36
FBZ-SO_2	331.90 > 158.90	0.005	35	36
	331.90 > 300.00	0.005	35	21
FBZ-SO_2-D_5	337.06> 305.00	0.005	45	23
TCB-NH2	328.00 > 166.95	0.005	48	57
FBZ	300.01 > 159.01	0.005	35	24
	300.01 > 268.01	0.005	35	23
FBZ-D_5	305.01 > 273.01	0.005	28	15

4.3 Results and discussion

4.3.1 FBZ incurred milk samples

The suitability of the SPR-biosensor assay was evaluated by analysing incurred milk samples and comparing the results with UPLC-MS/MS. In the first study, a bovine animal was treated with Panacur® SC 10% (active ingredient FBZ) and milk samples were taken prior to treatment until 135 h post treatment in accordance with the daily milking routine. Milk samples were independently analysed by two different analysts by SPR biosensor and UPLC-MS/MS. FBZ marker residues were detectable in samples by UPLC-MS/MS for 72 h post-treatment with residues below the MRL at 63 h post-treatment at a level of 7.5 µg kg^{-1}**(Table 4.2)**. A typical UPLC-MS/MS trace from incurred milk containing the three major FBZ residues is shown in **Fig. 4.1**. Results from SPR biosensor analysis showed that residues were also detected in milk samples and correctly identified as positive (i.e. >LOD of 2.7 µg kg^{-1}) for 72 h post-treatment. The results of this study showed that the SPR-biosensor results were typically higher than UPLC-MS/MS at the 63 and 72 h sampling periods. It is likely that the antibody used in the assay may also measure other FBZ metabolites for which there are no FBZ standards available.

The method was also applied to milk samples from a cow treated with Rintal® 1.9% (active ingredient FBZ pro-drug – Febantel). The samples in this study were collected at 7, 24, 31 and 168 h post-treatment. FBZ marker residues were detected by UPLC-MS/MS at levels greater than the MRL in the first three samples but were non-detectable at 168 h post-treatment (**Table 4.2**). The SPR-biosensor results agreed well with the UPLC-MS/MS results and no false negative biosensor results were observed in this study.

4.3.2 ABZ incurred milk samples

The ability of the SPR-biosensor assay to detect ABZ residues prior to the study was of concern because of the absence of antibody cross-reactivity to ABZ-NH_2-SO_2. To verify the suitability of the assay a bovine animal was treated with Endospec® 10% (w/v) (active ingredient ABZ) and milk samples were taken prior to treatment until 135 h post treatment in accordance with the daily milking routine.

A typical UPLC-MS/MS trace from incurred milk found to contain the four major ABZ residues is shown in **Fig. 4.2**. The ABZ marker residues were detectable by UPLC-MS/MS for 87 h post-treatment but had depleted to below the MRL of 100 μg kg^{-1} at 39 h post-treatment (**Table 4.3**). The SPR-biosensor assay was capable of detecting ABZ residues in milk samples up to 63 h post-treatment where residues were detected at a level of 4.3 μg kg^{-1}. The discrepancies between biosensor and UPLC results may have been due to the presence of ABZ-NH_2-SO_2 residues not detectable by biosensor nevertheless no false negative results were observed in this study.

4.3.3 MBZ incurred milk samples

Finally, the suitability of the SPR-biosensor assay was evaluated for detecting residues of MBZ residues in goats' milk. Two groups of goats (n = 5 each group) were treated with Kilan® O 5%, (w/v) (active ingredient MBZ) and milk samples were taken from prior to treatment until 96 h post treatment. A typical UPLC-MS/MS trace from incurred milk found to contain the major MBZ residues is shown in **Fig. 4.3**. MBZ marker residues were detectable by UPLC-MS/MS for 48 h post-treatment but had depleted to below the LOQ at 60 h post-treatment (**Table 4.4**). In animals treated at doses of 15 and 30 mg kg^{-1} b.w. MBZ residues could be detected using SPR-biosensor in samples for 48 and 72 h post-treatment, respectively. One false positive was observed at 60 h which was confirmed as compliant. At 72 h residues were detected (3.8 μg kg^{-1}) at a concentration below the detection capability (CCβ) of the biosensor assay (5 μg kg^{-1}) therefore this was not determined to be a false positive. No false negative results were observed in this study. Although the biosensor assay was not validated in goats' milk the results indicated that the assay performed satisfactorily in this species.

Table 4.2 Comparison between biosensor and UPLC-MS/MS analysis of milk samples from cows treated with FBZ and febantel.

Sample	Withdrawal time (h)	MRL (µg kg^{-1})	Biosensor assay		UPLC-MS/MS	
			Concentration (µg kg^{-1})	Interpretation (LOD = 2.7 µg kg^{-1})	[1]Concentration (µg kg^{-1})	[2]Status
			Dairy cow treated at 7.5 mg kg^{-1} b.w. (FBZ)			
1	0	10	ND	Negative	ND	C
2	15	10	>50	Positive	258.9	NC
3	24	10	>50	Positive	263.3	NC
4	39	10	>50	Positive	171.3	NC
5	48	10	>50	Positive	74.2	NC
6	63	10	20.0	Positive	7.5	C
7	72	10	5.7	Positive	2.5	C
8	87	10	ND	Negative	ND	C
9	96	10	ND	Negative	ND	C
10	111	10	ND	Negative	ND	C
11	120	10	ND	Negative	ND	C
12	135	10	ND	Negative	ND	C
			Dairy cow treated at 5 mg kg^{-1} b.w. (febantel)			
13	7	10	>50	Positive	250.5	NC
14	24	10	>50	Positive	336.3	NC
15	31	10	>50	Positive	219.3	NC
16	168	10	ND	Negative	ND	C

[1]UPLC-MS/MS concentrations are expressed as the sum of FBZ, FBZ-SO and FBZ-SO_2 residues expressed as FBZ-SO_2).
[2]C = compliant and NC = non-compliant

Table 4.3 Comparison between biosensor and UPLC-MS/MS analysis of milk samples from a cow treated with albendazole.

Sample	Withdrawal time (h)	MRL (µg kg^{-1})	Biosensor assay		UPLC-MS/MS	
			Concentration (µg kg^{-1})	Interpretation (LOD = 2.7 µg kg^{-1})	[1]Concentration (µg kg^{-1})	[2]Status
			Dairy cow treated at 7.5 mg kg^{-1} b.w. (albendazole)			
1	0 (see note)	100	ND	Negative	ND	C
2	15	100	>50	Positive	507.6	NC
3	24	100	>50	Positive	94.2	C
4	39	100	33.9	Positive	56.1	C
5	48	100	11.9	Positive	38.0	C
6	63	100	4.3	Positive	16.7	C
7	72	100	ND	Negative	10.5	C
8	87	100	ND	Negative	2.3	C
9	96	100	ND	Negative	ND	C
10	111	100	ND	Negative	ND	C
11	120	100	ND	Negative	ND	C
12	135	100	ND	Negative	ND	C
			Dairy cow treated at 1000 mg ABZ			
13	7	100	>50	Positive	238.9	NC
14	24	100	>50	Positive	1479.0	NC
15	31	100	>50	Positive	294.0	NC
16	168	100	ND	Negative	ND	C

[1]UPLC-MS/MS concentrations are expressed as the sum of ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues expressed as ABZ.
[2]C = compliant and NC = non-compliant

Table 4.4 Comparison between biosensor and UPLC-MS/MS analysis of milk samples from goats treated with a mebendazole

Sample	Withdrawal time (h)	MRL (µg kg^{-1})	Biosensor assay		UPLC-MS/MS	
			Concentration (µg kg^{-1})	Interpretation (LOD = 2.7 µg kg^{-1})	Concentration[1] (µg kg^{-1})	Status[2]
			Goat treated at 15 mg kg^{-1} b.w.			
1	2.5	None	8.3	Positive	6.7	NC
2	4	None	>50	Positive	64.9	NC
3	6 – 14	None	>50	Positive	164.1	NC
4	18 – 26	None	>50	Positive	327.7	NC
5	32 – 38	None	>50	Positive	153.7	NC
6	48	None	17.5	Positive	51.0	NC
7	60	None	ND	Negative	ND	C
8	72	None	ND	Negative	ND	C
9	96	None	1.6	Negative	ND	C
			Goat treated at 30 mg kg^{-1} b.w.			
10	2.5	None	10.4	Positive	16.4	NC
11	4	None	>50	Positive	215.3	NC
12	6 – 14	None	>50	Positive	439.3	NC
13	18 – 26	None	>50	Positive	457.2	NC
14	32 – 38	None	>50	Positive	220.8	NC
15	48	None	>50	Positive	68.5	NC
16	60	None	>50	Positive	ND	C
17	72	None	3.8	Negative	ND	C
18	96	None	ND	Negative	ND	C

[1]UPLC-MS/MS concentrations are expressed as the sum of MBZ, MBZ-NH_2 and MBZ-OH residues expressed as MBZ.
[2]C = compliant and NC = non-compliant

4.4 Conclusions

A screening procedure based on optical biosensor technology has been compared with a confirmatory assay based on UPLC-MS/MS. While the confirmatory method is the more sensitive of the two, the screening method was capable of detecting benzimidazole residues below their MRLs in milk. The biosensor identified all of the positive bovine milk samples taken during a 168 h withdrawal period for ABZ and FBZ drugs. The biosensor also correctly identified all of the positive goat milk samples taken during a 96 h withdrawal period of a MBZ-containing drug. The false negative and false positive rates for the assay were <5% and the results obtained compared well with mass spectrometric techniques indicating the reliability and robustness of the method. Advantages of the biosensor compared to UPLC-MS/MS techniques include; shorter sample analysis time (9 mins versus 13 mins), no need for preparation of several mobile phases and no processing of results is required (2 hours processing time per UPLC run). A total of 30 samples may be extracted and analysed in duplicate in 24 h. The SPR biosensor approach was found to be suitable for use as a rapid screening method to detect low levels of benzimidazole residues in milk.

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Chapter 5

Rapid detection of thiabendazole drug residues in liver tissue by a surface plasmon resonance biosensor using a recombinant antibody fragment

5.1. Introduction

Thiabendazole (2-(1,3 thiazole-4-yl) benzimidazole) is a drug used in veterinary medicine for the treatment of helminths such as gastro-intestinal roundworm in cattle, goats, horses and sheep (Campbell, 1990). Parasitic infection disrupts animal feed ingestion and digestibility which leads to reduced live weight, reduced yield and quality of meat and milk products. In severe cases infection may result in the premature death of the animal (Perry, and Randolph, 1999). Benzimidazoles are widely used due to their safety, broad spectrum activity and efficacy against immature and mature helminths (Campbell, 1990). However, the use of benzimidazole drugs has been a cause for concern regarding food safety because studies have shown that thiabendazoles (TBZs) are genotoxic (Sasaki, 1997).

A maximum residue limit (MRL) in ruminants is currently set at 100 μg kg^{-1} under EU Commission Regulation 2010/37/EC (Council regulation 2377/90/EC, 1990). High performance liquid chromatography (HPLC) coupled to both UV and fluorescence; gas chromatography (GC) and liquid chromatography (LC) are used for TBZ detection (Lafuente *et al*., 1987; Arenas and Johnson, 1995; Le Boulaire, 1997; Jedzinik *et al*., 2009; Kinsella, *et al*., 2009; Whelan *et al*., 2010;). Mass spectrometry (MS) has now emerged as a more widespread and sensitive method for the detection of these residues (Danaher *et al*., 2007).

While these methods are sensitive, they are laborious, and expensive when compared to immunoassays which are now routinely used for the rapid, cost-effective identification and detection of agri-food components and contaminants. Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect TBZ residues using polyclonal and monoclonal antibodies. Brandon *et al*. (1992) developed a monoclonal antibody (mAb) ELISA to detect TBZ in liver tissue with a LOD of 20 μg kg^{-1}. This assay was later coupled with an ELISA to detect methyl benzimidazole carbamates to produce a screening assay for a range of benzimidazoles in liver tissue (Brandon *et al*., 1998).

In addition, mAb-based ELISAs have been produced to detect TBZ residues in potatoes and apples (Brandon *et al*., 1993), in the peel of fruits (Brandon *et al*., 1995) and in fruit juice (Bushway *et al*., 1995, Abad *et al*., 2001). Polyclonal antibodies (pAbs) have also been developed to detect TBZ residues in vegetables by ELISA (Bushway *et al*., 1994).

Immunoassay sensitivity and specificity is inherently due to the properties of the assay antibody (Garrett *et al*., 1997) and mAbs provide highly sensitive antibodies (Kohler and Milstein, 1975). However mAb affinity or specificity cannot be readily altered and improved (Conroy *et al*., 2009). Due to their smaller size, recombinant antibodies are easier to manipulate genetically and may be expressed in bacterial systems. Specific recombinant Fab fragments and their coding sequences can be selected simultaneously from a diverse library of displayed antibodies (Yau, Lee and Hall, 1995). Less interference is observed with Fab fragments in biological matrices, Fabs exhibit higher stability than single-chain variable fragments (ScFvs) (Rothlisberger, Honeggar and Plückthun *et al*., 2005), do not suffer from dimerisation and are easier to convert into full length IgG (Bradbury and Marks, 2004). This research describes the development and validation of a SPR biosensor assay to detect TBZ drug residues in ovine liver tissue using a recombinant TBZ Fab.

5.2. Experimental

5.2.1 Chemical, reagents and apparatus

Sodium hydroxide (NaOH), pesticide grade acetonitrile (MeCN), pesticide grade dimethylsulphoxide (DMSO) and methanol were supplied by BDH/VWR international Ltd. (Poole, England). Dimethylformamide (DMF) was supplied by Sigma Aldrich (Dublin, Ireland). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore water purification system (Cork, Ireland). Thiabendazole (99.8% pure) was purchased from Sigma Aldrich (Steinheim, Germany) and 5-hydroxy-thiabendazole (99% pure) was purchased from Witega Laboratories (Berlin, Germany). Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate ($MgSO_4$) and 0.5 g C_{18} were purchased from Biotage (Uppsala, Sweden).

Whatman syringe filter units (polytetrafluoroethylene (PTFE), 0.2 μm) were purchased from AGB scientific (Dublin, Ireland). CM5 sensor chips (research grade), NHS (100 mM N-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES pH 7.4 with 0.05 M NaCl, 3.4 mM EDTA and 0.005% P20 (v/v) were all obtained from GE Healthcare (Uppsala, Sweden). The anti-TBZ recombinant antibody fragment was produced and supplied by our project partners at Dublin City University. This chimeric mouse/human Fab was selected from a phage display library produced from mRNA extracted from hybridoma cells secreting anti-TBZ (Brandon *et al*., 1992).

5.2.2 SPR biosensor assay

5.2.2.1 Biosensor chip preparation

A CM5 chip was allowed to equilibrate to room temperature and HBS-EP buffer (50 μL) was added to the chip surface and incubated (10 min). The buffer was removed and 50 mM NHS:200 mM EDC (1:1, v/v, 40 μL) was added to the chip and incubated (20 min, room temperature) to activate the surface. Amino-thiabendazole (2 mg) was dissolved in dimethyl formamide (100 μL) and added to 10 mM HCl pH 3.0 (900 μL) to give a 9.25 mM amino-thiabendazole solution. This solution (50 μL) was added to the chip surface and incubated at room temperature (3 h). The solution was removed and the chip was washed once with HBS-EP buffer. The remaining unreacted groups on the chip surface were deactivated by addition of 1 M ethanolamine-HCl (50 μL) and allowed to react (20 min). The chip was washed with HPLC grade water and dried under a stream of nitrogen gas. The immobilised chip was stored in a desiccated container (4ºC) when not in use. The orientation of amino-TBZ on the chip surface is shown in **Fig. 5.1**.

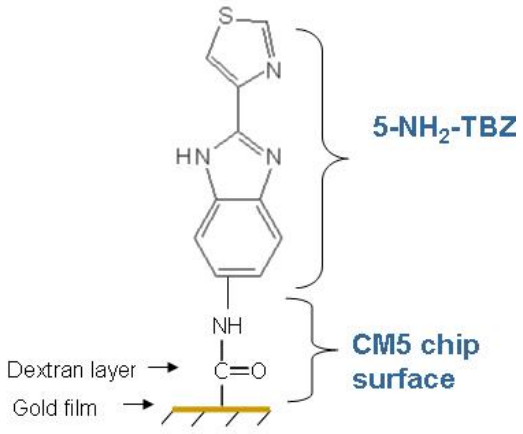

Figure 5.1 Diagram showing the direct amine coupling approach used to prepare the triclabendazole biosensor chip surface.

5.2.2.2 Sample preparation procedure: QuEChERS extraction

Ovine liver samples (2 g) were extracted using a slurry containing MeCN + $MgSO_4$ + NaCl (12 + 4 + 1, v/w/w) by shaking vigorously by hand (1 min). The samples were centrifuged (3,500*g*, 10 min, -5ºC) and the supernatant (6 mL) was transferred to a tube containing C_{18} sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500*g*, 10 min, -5ºC). The MeCN layer (6 mL) was transferred to polypropylene tubes containing DMSO (500 µl). The sample extracts were evaporated (50ºC, under nitrogen) until only the DMSO remained. DMSO Extracts were vortexed (2 min) and sonicated (10 min). The extracts (500 µL) were diluted in HBS-EP buffer (4.5 mL), vortex mixed (30 s) and filtered (0.22 µm) prior to biosensor analysis.

*5.2.2.3 Biosensor assay conditions and reagents*Studies were conducted at 25ºC. The optical biosensor used was a Biacore Q (GE Healthcare, Uppsala Sweden) with Biacore® Q control software version 3.0. BIAevaluation software version 3.0.1 was used for data handling. Antibody production and selection was performed as described by Barbas III et al., 2001 (Barbas III *et al*., 2001). Messenger RNA from hybridoma cells secreting anti-TBZ (Brandon *et al.*, 1992) was extracted and first-strand complementary DNA (cDNA) synthesis performed using a Superscript III™ kit.

Antibody variable and constant regions were amplified and combined by splice by overlap extension PCR using the primer sequences described by Barbas III *et al.* (2001). Amplified genes were then cloned into the pComb3X phage display vector with a hemagglutinin-tag (HA-tag) for detection. Cloned Fab genes were electroporated into *E. coli* XL-1 blue cells generating an antibody library of 4.5 x 10^7 clones. The Fabs were packaged on the surface of M13K07 phage and subjected to one round of panning against immunotubes coated with TBZ-BSA (5 µg mL^{-1}). After panning, eluted phage were re-infected into *E. coli* XL-1 blue cells and single colonies selected for monoclonal ELISA in sterile 96 well culture plates. Positive clones were grown in cultures (20 mL), Fab production was induced (1mM IPTG) and grown overnight (30ºC). Lysates were clarified by centrifugation (10 min, 4000*g*, 4ºC) prior to screening for binding to free TBZ in solution by competitive ELISA.

The TBZ Fab, (1:5, (v/v) in HBS-EP buffer) and liver extract were mixed (1:1) and passed over the amino-thiabendazole immobilised surface at 10 µL min^{-1} (2 min). Regeneration was carried using a single injection of 200 mM NaOH (20 µL) for 1 min at 25 µL min^{-1}. The binding of antibody to the chip surface was measured as the change in surface plasmon resonance (SPR) signal between two report points, before (10 s) and after (30 s) each injection. A competitive immunoassay assay format was used to detect inhibition of antibody binding to the chip surface. SPR signal was expressed in arbitrary resonance units (RU).

5.2.2.4 Calibration

Stock standard solutions of TBZ and 5-OH-TBZ were prepared in methanol at a concentration of 40 µg mL^{-1}. Working standard solutions for calibration curves were prepared by sequential dilutions in methanol. HBS-EP buffer was fortified with TBZ at 0, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 25, 60 and 125 ng mL^{-1} and 5-OH-TBZ at 0, 2.5, 5, 7.5, 10, 25, 60 and 125 ng mL^{-1} for cross-reactivity studies. Negative liver samples were fortified at 0, 10, 25, 50, 125 and 250 µg kg^{-1} with a TBZ standard prior to extraction. BIAevaluation software was used to plot an inhibition assay standard curve based on a four-parametric fit. The concentration in test samples was read directly from the calibration curve.

5.2.3. Biosensor Validation

A qualitative approach was used to determine the performance factor CCβ (detection capability) as described in 2002/657/EC criteria [31]. Firstly, the limit of detection (LOD) of the assay was determined by measuring the mean response for 20 different negative ovine liver tissue samples and subtracting three standard deviations. CCβ is the concentration at which a substance can be identified as positive (>LOD) with a statistical certainty of (1-β), where β = 5%. In order to determine CCβ for each assay, samples (n = 20 for each analyte) were spiked at a concentration above the LOD. If 19 of the 20 fortified samples were identified as positive, CCβ was determined to be equal to the fortification level (5% probability of a false negative result). If 20 samples were identified as positive, CCβ was determined to be less than the fortification level and if ≤18 samples were identified as positive, CCβ was determined to be greater than the fortification level. Liver samples were fortified at arbitrary concentrations above the LOD of the assay and the CCβ level was determined through trial and error. Assay repeatability was evaluated by extracting and analysing ovine liver fortified with each analyte on five separate days.

5.3. Results and Discussion

5.3.1. SPR biosensor assay

5.3.1.1 Antibody inhibition studies

The cross-reactivity profile of the Fab was determined by SPR biosensor assay from the analysis of TBZ and 5-OH-TBZ calibration curves in HBS-EP buffer over the range 0 to 125 ng mL^{-1} **(Fig. 5.2)**. The concentration of analyte required to inhibit 50% of antibody binding (IC_{50}) was calculated to be 2.3 ng mL^{-1} for 5-OH-TBZ and 2.6 ng mL^{-1} for TBZ **(Table 5.1)**. The percentage cross-reactivity of the Fab towards each analyte was calculated at 50% antibody inhibition (%CR_{50}) as a percentage of 5-OH-TBZ, which represented 100% cross-reactivity. Cross reactivity of the TBZ fab was calculated to be 80 and 100% for TBZ and 5-OH-TBZ, respectively. The concentrations of analyte required to inhibit 10%, 50% and 90% of antibody binding ($IC_{10/50/90}$) were calculated for each analyte from their respective inhibition curves **(Table 5.1)**.

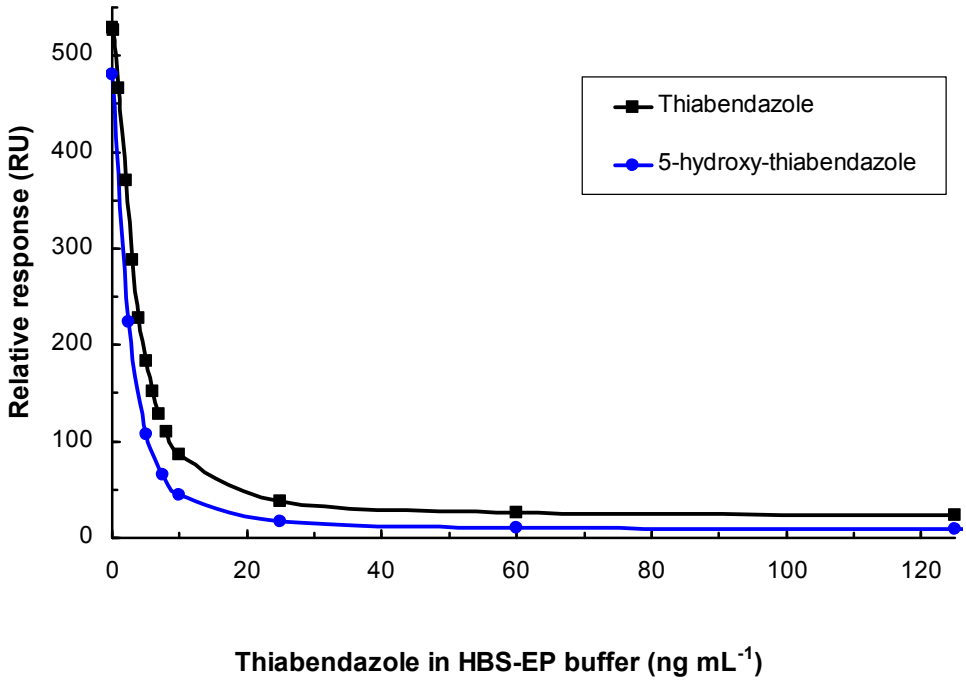

Figure 5.2 Thiabendazole antibody fragment cross-reactivity towards thiabendazole and 5-OH-thiabendazole: Inhibition curves in HBS-EP buffer

5.3.1.2 Calibration curve in ovine liver extract

A TBZ calibration curve (0-250 µg kg^{-1}) was prepared in ovine liver using the QuEChERS extraction method. The concentration at 50% antibody inhibition (IC_{50}) was plotted on this inhibition curve at 16.9 µg kg^{-1} (**Fig. 5.3**). The dynamic range of the TBZ calibration curve was between 2.8 µg kg^{-1} (IC_{10}) and 82.6 µg kg^{-1} (IC_{90}) **(Table 5.1)**. It was concluded from these results that the assay sensitivity was in concentration range required for the determination of TBZ residues in liver tissue below the MRL (100 µg kg^{-1}).

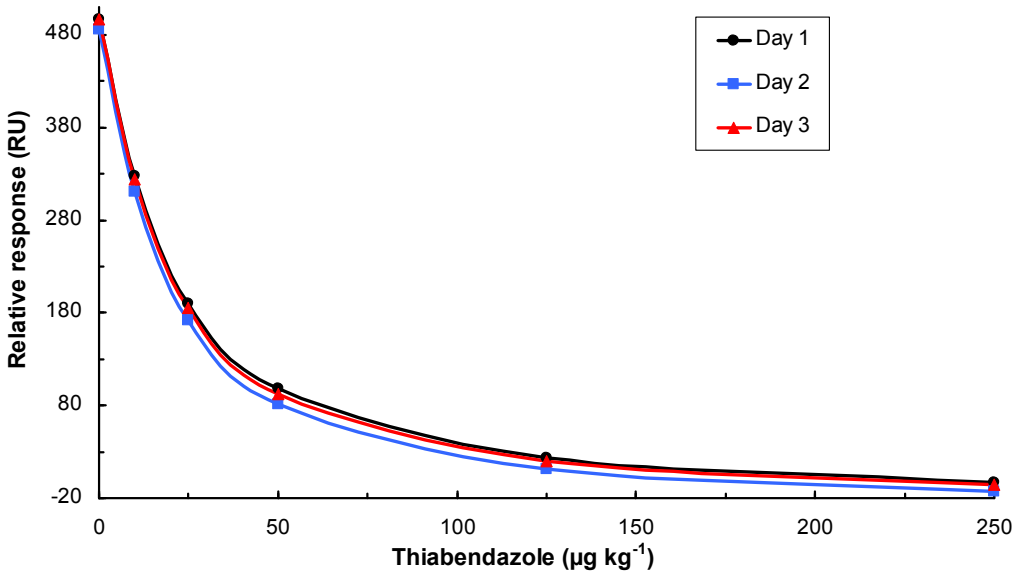

Figure. 5.3 SPR biosensor assay calibration curves in thiabendazole fortified ovine liver on different days (n = 3).

Table 5.1

Cross-reactivity profile of thiabendazole antibody fragment determined by SPR biosensor in HBS-EP buffer and in ovine liver extract.

	HBS-EP Buffer		Ovine liver	
Analyte	[a]IC_{50} (ng mL^{-1})	[b]CR_{50} (%)	[c]IC_{50} (µg kg^{-1})	[d]CR_{50} (%)
Thiabendazole	2.86	80	16.9	86
5-OH-thiabendazole	2.3	100	14.5	100

[a] The concentration of analyte required to reduce the response by 50% in HBS-EP buffer.
[b] Cross-reactivity of antibody fragment towards test analyte at 50% inhibition ((IC_{50} 5-OH-TBZ / IC_{50} test analyte)×100) in HBS-EP buffer.
[c] The concentration of analyte required to reduce the response by 50% in ovine liver.
[d] Cross-reactivity of antibody fragment towards test analyte at 50% inhibition ((IC_{50} 5-OH-TBZ / IC_{50} test analyte) ×100) in ovine liver.

5.3.1.3 Method Validation

The suitability of the assay was evaluated through application to ovine liver samples fortified with TBZ and 5-OH-TBZ residues at 25 and 125 μg kg^{-1}. Three groups of samples were extracted and analysed in duplicate on three different days. Acceptable recovery levels (86-107%) were achieved for both analytes in ovine liver. The repeatability of the assay was determined by calculating the percentage coefficient of variation (CV%) which ranged from 1-10% (Table 5.2).

The assay limit of detection (LOD) was determined from the analysis of 20 different negative ovine livers against a thiabendazole calibration curve (0-250 μg kg^{-1}) prepared in ovine liver. The mean response for 20 negative liver samples was 376 RU and the standard deviation (SD) was 25.7 RU. The LOD was calculated as 299 RU , equivalent to 12.3 μg kg^{-1} when plotted on the thiabendazole calibration curve.

Table 5.2
Determination of detection capability (CCβ) and repeatability of biosensor assays: Results from the analysis of fortified ovine liver (n = 20) and the percentage recovery on different days (n = 3).

Analyte	Assay Repeatability	Detection Capability	
	Mean recovery (%) ± SD (n = 3)	Mean ± SD (n = 20) (μg kg^{-1})	CCβ (μg kg^{-1})
	Fortification = 25 μg kg^{-1}	Fortification = 20 μg kg^{-1}	
TBZ	86 ± 2.1	13.8 ± 1.4	20
5-OH-TBZ	92 ± 5.1	19.6 ± 2.2	<20
	Fortification =125 μg kg^{-1}		
TBZ	101 ± 1.0		
5-OH-TBZ	107 ± 1.5		

The CCβ was determined through the analysis of ovine liver samples (n = 20) fortified with TBZ and 5-OH-TBZ (20 µg kg^{-1}). The assay CCβ for TBZ was determined to be equal to 20 µg kg^{-1} because one fortified sample was not identified as positive; this false negative sample gave a measured result of 9.5 µg kg^{-1} (**Fig. 5.4**). The assay CCβ for 5-OH-TBZ was determined to be less than 20 µg kg^{-1} because all fortified liver samples were identified as positive. The assay CCβ was equal to one fifth of the current MRL permitted for TBZ residues in liver tissue (100 µg kg^{-1}). The mean TBZ and 5-OH-TBZ recoveries from fortified liver samples (20 µg kg^{-1}) were 70 and 98%, respectively. The standard deviations were 1.46 and 2.2 µg kg^{-1} for TBZ and 5-OH-TBZ, respectively.

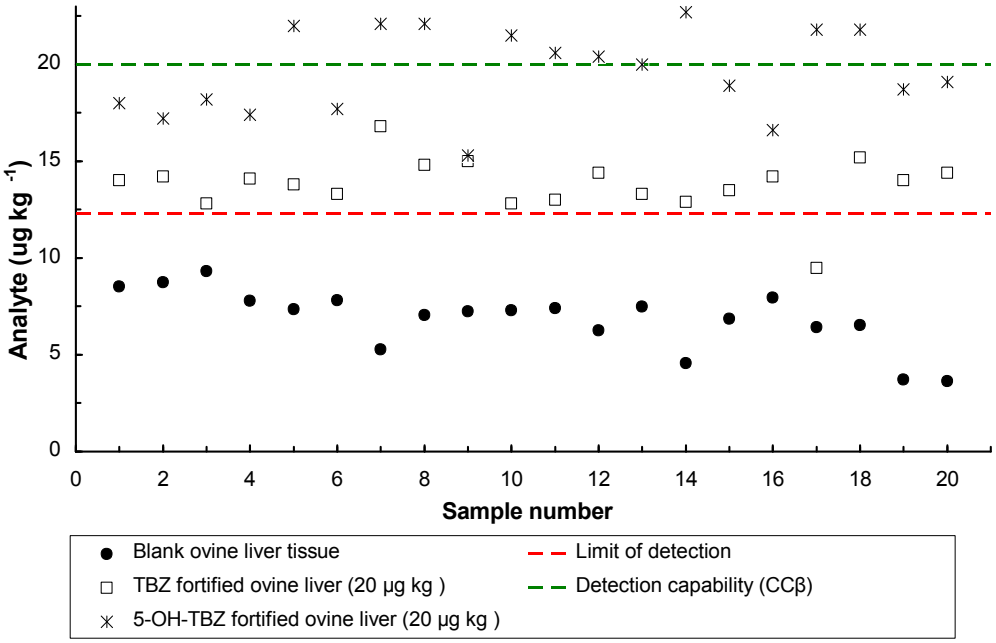

Figure 5.4 Determination of the limit of detection (LOD) and the detection capability (CCβ) of thiabendazole biosensor assay in ovine liver tissue.

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5.4. Conclusions

A sensitive SPR biosensor screening assay was developed and validated for the detection of TBZ and 5-OH-TBZ residues in ovine liver tissue. The assay performance was determined to be acceptable in accordance with 2002/657/EC . The LOD was determined to be 12.3 µg kg^{-1} and the CCβ was calculated to be 20 µg kg^{-1}. The biosensor assay LOD was lower than that of an ELISA screening method reported for TBZ residues in liver using a mAb (Brandon *et al*., 1992).

The QuEChERS method is a versatile extraction technique that has been applied to extract several different pesticide and veterinary drug residues from different matrices (Aguilera-Luiz *et al*., 2008; Kinsella *et al*., 2009; Stubbings and Bigwood, 2009). The scope of this biosensor assay could in the future be expanded to include the detection of thiabendazole residues in a variety of different matrices such as muscle tissue, milk, fruit juices and vegetables.

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Chapter 6

Development of a biosensor assay for the detection of triclabendazole residues in liver tissue

6.1. Introduction

Triclabendazole (6-chloro-5(2-3-dichlorophenoxy)-2-methyl thio-benzimidazole) is a halogenated benzimidazole thiol derivative with specific activity against *Fasciola hepatica* infections in cattle and sheep (Boray *et al*., 1983). Many benzimidazole drugs act by binding to parasite β-tubulin. Immunocytochemical studies showed that tubulin organization was disrupted in the tegument of triclabendazole-susceptible flukes (Robinson *et al.,* 2002). This is a slow mode of action and therefore the efficacy of triclabendazole (TCB) requires prolonged exposure of the parasite to the active forms of the drug (Prichard *et al*., 1978; Hennessy *et al.*, 1987). The rumen acts as a biological slow-release system for TCB towards the posterior digestive tract where these drugs are absorbed (Mestorino *et al*., 2008). Most fasciolicidal compounds have good activity against mature stages of liver fluke but they are not sufficiently effective against immature stages. TCB is one of the most widely used fasciolicides because it shows excellent efficacy against both immature and mature stages of liver flukes (Mottier *et al*., 2004). TCB also requires fewer doses to achieve the same fluke kill as other actives (Boray *et al*., 1983) and is relatively inexpensive in comparison to newer compounds.

The TCB drug is rapidly metabolized and the parent drug is not detected in plasma after oral administration (Hennessy *et al*., 1987). TCB is oxidized to form the sulphoxide (TCB-SO) and sulphone (TCB-SO_2) metabolites. The regulatory authorities set maximum residue limits (MRL's) to ensure food is safe for consumers and the MRL for TCB residues in the liver of all ruminants is set at 250 μg kg^{-1} for extractable residues that may be oxidized to keto-triclabendazole (keto-TCB). Surprisingly few analytical methods have been published for the determination of TCB residues in foodstuffs in comparison to other benzimidazole residues (Danaher *et al*., 2007). This is probably due to the difficulty in obtaining standards for TCB metabolites, which have only become commercially available recently but also due to difficulty in analyzing these molecules because of their tight binding to plasma proteins.

The limited number of published analytical methods for biological and food samples are based on liquid chromatography with UV, fluorescence or mass spectrometry based detection systems (Lehr and Damm, 1986; Alvinerie *et al*., 1986; Cannavan, Haggan and Kennedy, 1998; Takeba *et al*., 2000; De Ruyck *et al*., 2002; Zhou *et al*., 2005; Jedziniak *et al*., 2009; Kaufmann *et al*., 2007; Whelan *et al*., 2010).

The aim of this was to develop a SPR-biosensor screening assay to detect TCB residues in liver. An assay was evaluated using a directly immobilized amino-TCB biosensor chip surface where TCB residues were extracted using a modified QuEChERS procedure. Following this work amino-TCB was immobilized to a biosensor chip via a homobifunctional glutaraldehyde cross-linker. This chip was assessed for TCB detection in liver using a modified QuEChERS extraction procedure. The factors investigated included recovery, repeatability and analytical limits, including the limit of detection (LOD) of the method.

6.2. Experimental

6.2.1 Chemical, reagents and apparatus

Sodium hydroxide (NaOH), ammonium dihydrogen phosphate, pesticide grade acetonitrile (MeCN), pesticide grade dimethylsulphoxide (DMSO) and methanol were supplied by BDH/VWR international Ltd. (Poole, England). Dimethylformamide (DMF) was supplied by Sigma Aldrich (Dublin, Ireland). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore water purification system (Cork, Ireland). Triclabendazole (TCB), triclabendazole-sulphone (TCB-SO_2), triclabendazole-sulphoxide (TCB-SO) and keto-triclabendazole (keto-TCB) were purchased from Witega Laboratories Berlin-Aldershof GmbH (Berlin, Germany). Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate ($MgSO_4$) and 0.5 g C_{18} were purchased from Biotage (Uppsala, Sweden). Whatman syringe filter units (polytetrafluoroethylene (PTFE), 0.2 μm) were purchased from AGB scientific (Dublin, Ireland).

CM5 sensor chips (research grade), NHS (100 mM N-hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM HEPES, pH 7.4, with 0.05 M NaCl, 3.4 mM EDTA and 0.005% P20 (v/v) were all obtained from GE Healthcare (Uppsala, Sweden). The anti-triclabendazole polyclonal antibody (Cat. No. PAS9452) used in this work was raised in sheep towards a triclabendazole-bovine thyroglobulin (BTG) immunogen and was supplied by Randox Laboratories (Co. Antrim, Northern Ireland). A FASTH 21 homogenisation unit and sample homogenisation tubes were supplied by Syntec Scientific (Dublin, Ireland), a Mistral 3000i centrifuge (MSE, London, UK), an Elma Transsonic T780/H ultrasonic bath (Bedford, UK) and a Turbovap LV evaporator (Caliper Life Sciences, Runcorn, UK) were used during sample preparation.

SPR biosensor assay studies were conducted at 25ºC. The optical biosensor used was a Biacore Q (GE Healthcare, Uppsala Sweden) with Biacore® Q control software version 3.0. BIAevaluation software version 3.0.1 was used for data handling. The binding of antibody to the chip surface was measured as the change in surface plasmon resonance (SPR) signal between two report points, before (10 s) and after (30 s) each injection. A competitive immunoassay assay format was used to detect inhibition of antibody binding to the chip surface. SPR signal was expressed in arbitrary resonance units (RU).

6.2.2 Biosensor chip surfaces

6.2.2.1 Surface 1: amino-triclabendazole

A CM5 chip was allowed to equilibrate to room temperature and HBS-EP buffer (50 µL) was added to the chip surface and incubated (10 min). The buffer was removed and 50 mM NHS:200 mM EDC (1:1, v/v, 40 µL) was added to the chip and incubated (20 min, room temperature) to activate the surface. This solution was removed using lint-free tissue paper. Amino-triclabendazole (10 mg) was dissolved in dimethylformamide (500 µL) and this solution was added to 10 mM HCl pH 3.0 (4.5 mL). This solution (50 µL) was added to the chip surface and incubated at room temperature (2 h).

The solution was removed using lint-free tissue paper and the surface was washed once with HBS-EP buffer. The remaining unreacted groups on the chip surface were deactivated by the addition of 1 M ethanolamine-HCl (50 µL) and allowed to react (20 min). The chip surface was washed three times with HBS-EP buffer and once with ultra-pure water. The chip was dried under a stream of nitrogen gas and stored in a desiccated container at +4°C when not in use.

6.2.2.2 Surface 2: amino-triclabendazole with glutaraldehyde linker

The CM5 chip was prepared by adding HBS-EP (50 µL) to the surface (10 min). The surface was activated by the addition of a mixture (1:1) of 50 mM NHS and 0.2 M EDC to the chip surface (50 µL, 20 min). The amine surface was prepared by adding ethylenediamine (1 M, pH 8.5) to the surface (50 µL, 1 h). The surface was capped using ethanolamine-HCl (1 M) to the surface (20 min). A glutaraldehyde homobifunctional cross-linker (10 mM, in borate buffer pH 8.5) was added to the chip surface (20 min). The chip was washed several times using HBS-EP buffer to remove excess gluteraldehyde. The carboxy-amino-triclabendazole derivative (5 mg) was dissolved in DMF and added to an equal volume of sodium borate buffer (pH 8.5) to give a 13.4 mM solution which was added to the chip surface (1 h 20 min). Sodium borohydride (0.1 M) was added to the chip to reduce Schiff bases and form stable secondary amine linkages (20 min). The chip surface was washed three times with HBS-EP buffer and once with ultra-pure water. The chip was dried under a stream of nitrogen gas and stored in a desiccated container at +4°C when not in use. The orientation of the carboxy-amino-TCB derivative on the chip surface using direct and indirect coupling methods is shown in **Fig. 6.1.**

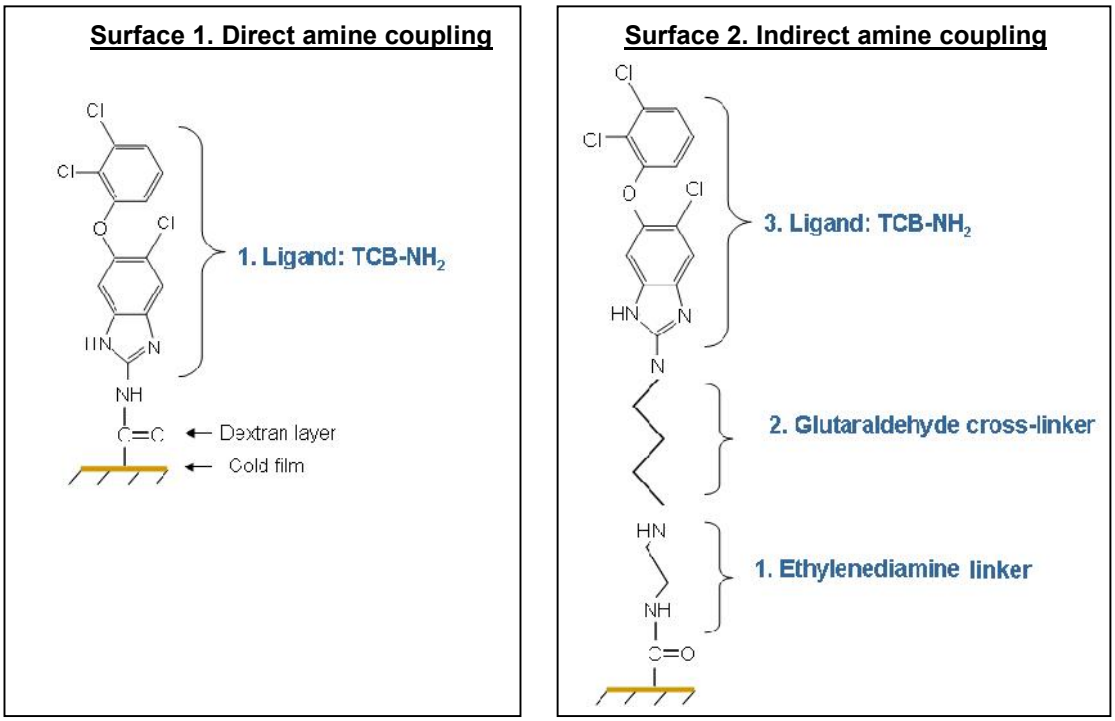


Figure 6.1 Diagram showing direct and indirect amine coupling approaches used to prepare triclabendazole biosensor chip surfaces.

6.2.3 Sample preparation

Finely chopped liver (2 g) was homogenised in a slurry containing MeCN:$MgSO_4$:NaCl (12:4:1, v/w/w) for 30 sec and centrifuged (3500 ×*g*, 10 min, -5ºC). The upper MeCN layer was transferred to a tube containing C_{18} sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500 ×*g*, 10 min, -5ºC). The MeCN layer (10 mL) was transferred to polypropylene tubes and DMSO (500 µL) was added.

The MeCN was evaporated under a stream of nitrogen (50ºC). The DMSO extracts were vortexed (2 min) and sonicated (10 min). Extracts were subsequently diluted in HBS-EP buffer (1:9, v/v) and filtered through 0.45 µm PTFE filters.

6.2.4 Biosensor assay cycles

Two different biosensor assay cycles were developed using the CM5 chip surfaces 1 and 2. In both assay cycles, the polyclonal antibody was diluted with HBS-EP buffer (1:99, v/v), mixed with sample extracts (1:1, v/v) and injected across the chip surface. Extracts were injected across the amino-triclabendazole surface at flow rate of 25 μL min^{-1} (96 s). Regeneration was carried using a single injection of 50 mM NaOH (19 μL) for 45 s at 25 μL min^{-1}. Alternatively, extracts were injected across the amino-triclabendazole-glutaraldehyde surface at 10 μL min^{-1} (360 s). Regeneration was carried using a single injection of 150 mM NaOH (25 μL) at 25 μL min^{-1}.

6.3 Results

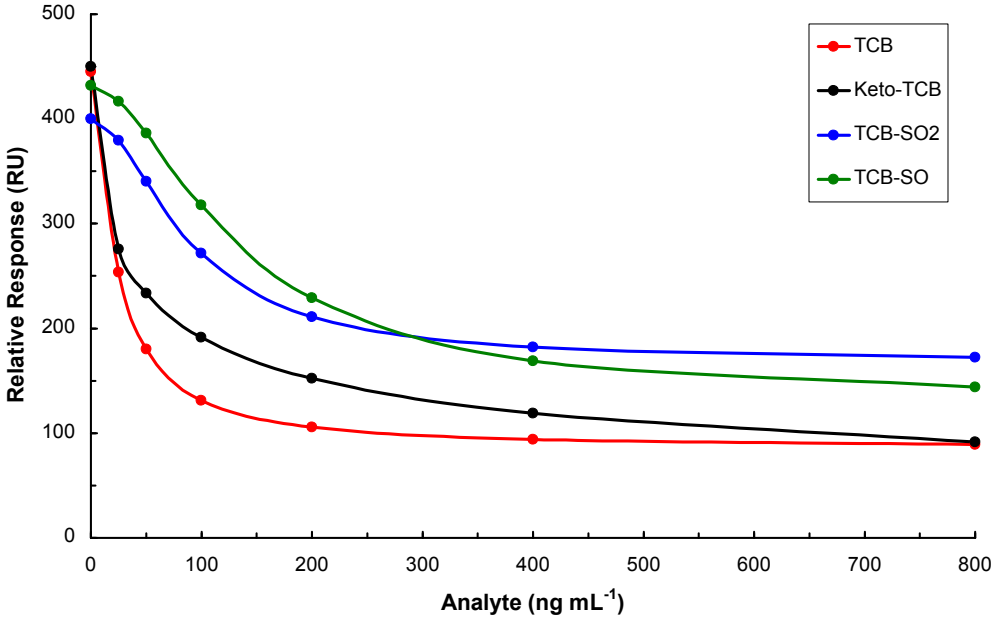
6.3.1 Optimisation of biosensor conditions

The polyclonal antibody cross-reactivity was investigated by analysing standard curves prepared in HBS-EP buffer for analysis using (A) amino-triclabendazole and (B) amino-triclabendazole glutaraldehyde chip surfaces (**Fig. 6.2**). The amino-triclabendazole glutaraldehyde surface was found to be a more suitable chip surface because it demonstrated better stability and lower IC_{50} values for TCB-SO and TCB-SO_2. The cross-reactivity profile of the two chip surfaces to the four benzimidazole residues is shown in **Table 6.1**. The cross-reactivity profile of the antibody on the amino-triclabendazole glutaraldehyde surface was significantly improved towards TCB residues with %CR_{50} values ranging between 56 and 100% in buffer. This level of cross-reactivity was considered adequate for the development of a biosensor assay.

Table 6.1 Cross-reactivity of anti-triclabendazole polyclonal antibody towards triclabendazole residues in HBS-EP buffer using amino-triclabendazole and amino-triclabendazole-glutaraldehyde chip surfaces.

	Amino-triclabendazole				Amino-triclabendazole-glutaraldehyde			
Analyte	IC_{50}	IC_{10}	IC_{90}	$\%CR_{50}$	IC_{50}	IC_{10}	IC_{90}	$\%CR_{50}$
TCB	22	4.1	114	100	30	5.2	161	100
Keto-TCB	28	0.7	330	79	28	5.5	152	106
TCB-SO_2	88	27	265	25	47	3.0	396	64
TCB-SO	122	37	375	18	54	3.2	430	56

(A)



(B)

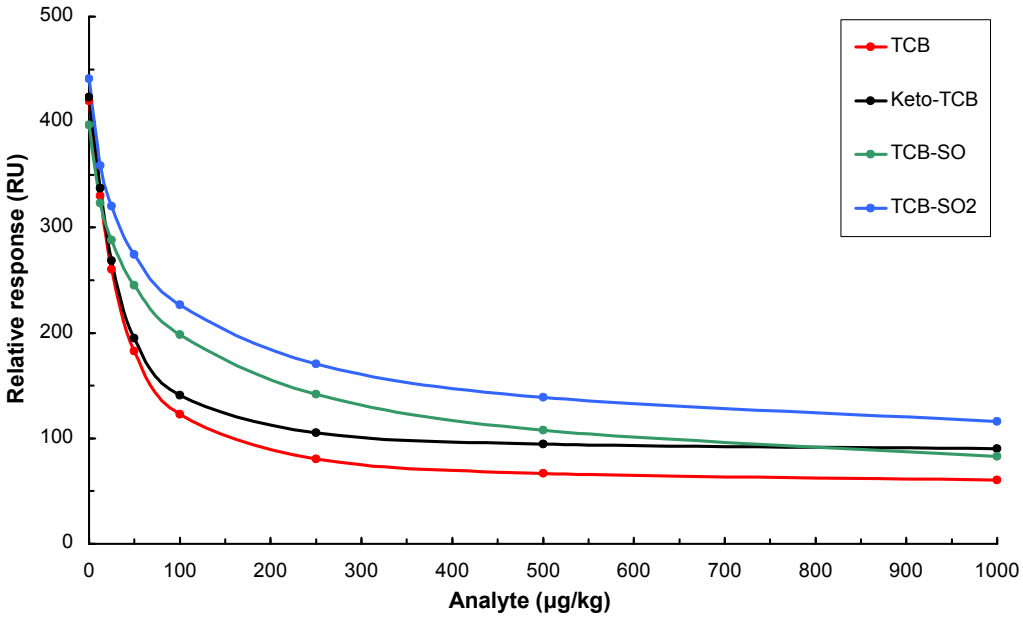


Figure 6.2 Calibration curves for triclabendazole residues in HBS-EP buffer (A) amino-triclabendazole and (B) amino-triclabendazole-glutaraldehyde chip surfaces.

6.3.2. Optimisation of sample preparation

A range of different QuEChERS-based extraction procedures were evaluated using ovine liver samples fortified in the range 0 to 1000 µg triclabendazole kg^{-1} and analysed using the amino-triclabendazole glutaraldehyde chip conditions (**Table 6.2**).

Table 6.2 Conditions, reagents and samples sizes for methods I to VI for the investigation of non-specific binding in a triclabendazole biosensor assay.

Method	Sample size (g)	Antibody: Extract	Dilution in HBS-EP buffer (v/v)	Cyclohexane wash	Filtration
I	2	1:1	1/10	No	No
II	4	1:1	1/10	No	No
III	2	3:1	1/10	No	No
IV	2	1:1	1/5	No	No
V	2	1:1	1/10	No	0.45 µm
VI	2	3:1	1/10	1	0.22 µm

Using method I, the IC_{50} was determined to be 228 µg kg^{-1} and the dynamic range of the assay was between 23 (IC_{10}) and 749 µg kg^{-1} (IC_{90}). It was considered that although the IC_{50} of the assay was greater than the MRL, TCB residues could be detected to below MRL. The LOD of the assay was determined to be 165 µg kg^{-1} by measuring the mean response of 20 representative blank ovine liver samples (272 RU) and subtracting three standard deviations (3 × 4 RU). The sample size was also increased to 4 g to increase the sensitivity of the assay, which lowered the LOD to 122 µg kg^{-1} (**Table 6.3, Fig. 6.3**). Increasing the sample size caused a reduction of 88 RU in the mean response for blank samples. This inhibition implied that non-specific binding had occurred between the sample matrix and the antibody.

Table 6.3 Determination of the impact of altering assay conditions, reagents and sample size on the concentration of triclabendazole required to inhibit 10, 50 and 90% of antibody binding ($IC_{10/50/90}$) and the limit of detection (LOD).

Method	IC_{50}	IC_{10}	IC_{90}	LOD	[a]CV%
	Concentration (µg mL^{-1})				
I	219	23	749	165	1.7
II	239	30	760	122	2.3
III	221	24	735	143	2.1
IV	220	25	730	131	1.3
V	209	23	720	105	0.6
VI	217	28	720	116	1.3

[a] Percentage coefficient of variation = SD / Mean

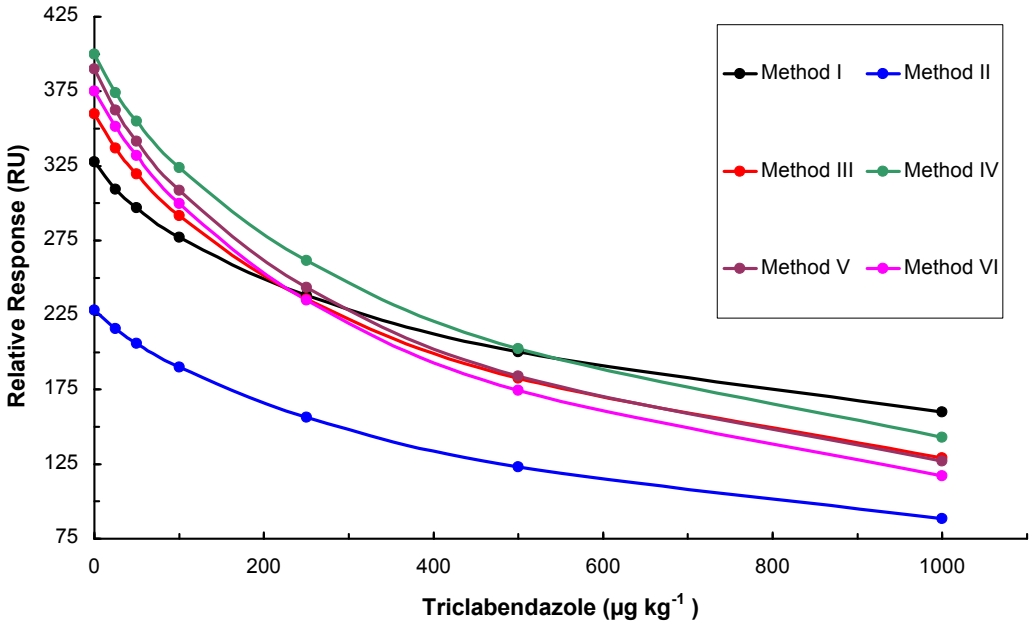


Figure 6.3 Optimisation of biosensor assay conditions, reagents and sample size for the determination of triclabendazole residues in ovine liver tissue using the QuEChERS extraction method.

To further evaluate the cause of non-specific binding the percentage DMSO in the final extract was reduced to 5% and the ratio of antibody to extract was increased from 1:1 to 3:1 (Method III). An increase in the mean response for 20 blank liver samples (291 RU) and a decrease in the LOD (143 μg kg^{-1}) were seen. However, the IC_{50} (221 μg kg^{-1}) was not significantly reduced.

The extract dilution in HBS-EP buffer was reduced from 1 in 10 to 1 in 5, the ratio of antibody to extract was set at 1:1 and the final extract contained 5% (v/v) DMSO (Method IV). A further increase in the mean response for 20 blank liver samples (320 RU) was detected and the LOD (131 μg kg^{-1}) decreased.

To improve the clean-up procedure the final extracts (1:10, v/v, in HBS-EP buffer, 5% DMSO) were filtered (0.45 μm) and mixed in a 1:1 ratio with antibody prior to biosensor analysis (Method V). A slight decrease was seen in the mean response of 20 blank liver samples (311 RU) but there was a significant reduction in the LOD (105 μg kg^{-1}). A cyclohexane wash (2 mL) was introduced after the evaporation step to determine if fat in the final extract was contributing to the cause of non-specific binding. The extract was also filtered using a smaller pore size (0.22 μm) (Method VI). The mean response of 20 blank liver samples (303 RU) was not significantly altered. A marginal increase was seen in the LOD (116 μg kg^{-1}) and it was concluded that fat in the sample extract was not the cause of non-specific binding. Method V was selected as the method for further validation studies.

6.3.3 Method validation

The repeatability of the assay was evaluated by analysing ovine liver samples fortified at 100 μg kg^{-1} and 50 μg kg^{-1} with four different triclabendazole residues on five separate days. The liver samples fortified at 100 μg kg^{-1} did not show acceptable recovery (223-329 %). Only one TCB-SO liver fortified liver showed an acceptable recovery level (146 %) between 80-160 %. However, the TCB antibody showed the lowest cross-reactivity towards this metabolite (%CR_{50} = 56%) and this recovery level was 2.6 times the level of cross-reactivity (**Table 6.4**).

Table 6.4 Repeatability of triclabendazole biosensor assay in ovine liver tissue QuEChERS extraction and assay conditions and reagents outlined in Method V

Analyte	Fortification level (μg kg^{-1})	Mean recovery % ± SD (n = 5)	CV%
TCB	50	186 ± 13.7	14.6
Keto-TCB	50	193 ± 4.3	4.4
TCB-SO	50	146 ± 5.9	8.2
TCB-SO_2	50	167 ± 5.6	6.7
TCB	100	319 ± 32.0	10.0
Keto-TCB	100	321 ± 6.2	1.9
TCB-SO	100	217 ± 8.0	3.7
TCB-SO_2	100	223 ± 9.0	4.0

6.4 Conclusions

A biosensor assay using a QuEChERS extraction procedure was developed for detecting TCB residues in ovine liver. The LOD of the assay was determined to be 105 μg kg^{-1}, which is less than half the MRL for triclabendazole residues. The assay repeatability and recovery were determined indicating good repeatability but with inflated recovery results. Further work is required to optimize this TCB biosensor assay, which showed good potential for the screening of TCB residues in liver tissue. Following this the assay will be validated according to 2002/657/EC criteria.

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Chapter 7

Biochip array for the multi-residue detection of key pesticide and fungicidal residues in orange juice

7.1 Introduction

Macrocyclic lactones (MLs) and benzimidazole are key crop protection agents that are applied as pre- and post-harvest treatments for a broad range of crops. Benzimidazoles are used for the control of fungal spoilage during storage and transportation (Danaher *et al*., 2007; Cacho, Turiel and Perez-Conde, 2009). A range of different benzimidazole actives have been used in the past including carbendazim (methyl 2-benzimidazolecarbamate: MBC), thiabendazole (TBZ) and the probenzimidazoles (Benomyl (BEN), thiophanate-methyl (TPM), thiophanate (-ethyl) (TPE)). Abamectin (ABA) and emamectin (EMA) belong to the family avermectins (AVERs), which are macrocyclic lactones (MLs), produced by the actinomycete, *Streptomyces avemitilis* (Campbell, Fisher and Stapley, 1982). They are used to control mites such as citrus red mites *(Panonychus citri)* and spider mites *(Tetranychidae)* on fruit trees. The compounds are also effective against some insects on fruits and vegetables. These compounds act by stimulating the release of γ-aminobutyric acid thus causing paralysis of the organism (Coccini *et al*., 1993; Cully, *et al*. 1994; Agarwal, 1998).

The drug TBZ is monitored in its parent form in crops. While BEN is very unstable in alkaline media and is converted into MBC in aqueous solutions, organic solvents, field soils and plant tissues (Chiba, 1977; Singh *et al*., 1990; Kiigemagi *et al*., 1991). TPM and TPE can also be partially degraded to MBC and EBC (ethyl benzimidazol-2-yl carbamate), respectively, both of these metabolites can be further decomposed into 2-aminobenzimidazole (2-AB) in very strongly alkaline media or undergo subsequent hydroxylation by the hepatic mono-oxygenase system (Erwin, 1973). The common stable metabolite of BEN and TPM is MBC and this is considered as the major fungal toxic agent of these pro-benzimidazoles. Subsequently, regulatory limits for these fungicides are generally expressed as MBC, the single measurement marker in food safety (Di Muccio, 1995; Danaher *et al*., 2007). Benomyl and MBC have been reported as aneuploidogens (McCarroll *et al*., 2002) and have been attributed to reproductive damage in males when administered at chronic, subchronic, and acute levels (Grey *et al*., 1990).

Abamectin is reported to have neurotoxic effects in mice (Sun *et al*., 2010) and for this reason government authorities set maximum residue limits (MRLs) to regulate their concentration in fruit and vegetables.

The majority of analytical methods reported for benzimidazole fungicides have been based on high performance liquid chromatography (HPLC) coupled to UV and/or fluorescence detection, (Singh, *et al*., 1990, Kiigemagi *et al*., 1991; Di Muccio *et al*., 1995; Zweig and Gao, 1983). In recent, years there has been a move towards liquid chromatography coupled to mass spectrometry, for detecting a wide range of fungicidal agents in crop-based commodities (Liu, Mattern and Rosen, 1990; Fernandez *et al*., 2001; Singh, Foster and Khan, 2007; Wang *et al*., 2007; Economou *et al*., 2009). In the case of the MLs, HPLC-UV (Viuk, 1991, Li and Qian, 1996) is not sensitive enough to ensure compliance with legislation (the MRL set is 0.01 mg/kg). However, HPLC-FLD is sufficiently sensitive but requires derivatization with trifluoroacetic anhydride (Chankasen, Papathakis and Lee; Cobin and Johnson, 1995, 1996; Diserens and Henzelin, 1997). In addition LC-MS can be applied for the analysis of ML residues in crops (Volmer, 1998; Valenzuela *et al*., 2000, 2001; Koesukwiwat *et al*., 2010). Surprisingly, few LC-MS methods have been reported in literature for detecting ML residues in processed fruit juice (Sannino, 2001).

The majority of benzimidazoles and MLs require complex sample preparation procedures that include supercritical fluid extraction (Brooks and Uden, 1995). A new technique known as QuEChERS which involves salting-out, liquid–liquid partitioning/extraction followed by a dispersive solid-phase extraction (DSPE) clean-up, has become increasingly popular for the analysis of multiclass pesticides in a variety of agricultural products (Lambropoulou and Albanis, 2001; Anastassiades *et al*., 2003; Lehotay, Mastovska and Lightfield, 2005).

Due to its inherent advantages such as speed, ease of use, reduced solvent usage with no halogenated waste, and wide applicability with acceptable recovery of an array of analyte–matrix combinations, the QuEChERS procedure is emerging as an alternative official regulatory approach to sample manipulation for multi-residue analysis of fruits and vegetables (Koesukwiwat *et al*., 2010).

Several immunoassay-based screening methods have been reported for the detection of TBZ residues in fruit, vegetables and fruit juices (Newsome and Collins, 1987; Brandon *et al*., 1993, 1995; Abad *et al*., 2001; Blažková, Rauch and Fukal, 2010). Immunoassay detection methods have also been reported to detect avermectin residues in milk (Samsonova *et al*., 2002) and liver (Samsonova *et al*., 2002; Shi *et al*., 2006). However no multi-residue immunoassay screening techniques have been reported for benzimidazole fungicides and avermectin pesticides in fruit juice. No MRLs are set for pesticides in processed orange juice, therefore the convention has been to apply the MRLs for thiabendazole in citrus fruits (7 mg kg^{-1}), carbendazim in oranges (1 mg kg^{-1}) and abamectin in citrus fruits (10 μg kg^{-1}) (Anonymous, 2005).

The Evidence Investigator™ is a bench top semi-automated instrument, which performs the image capture and analysis of biochip arrays and is capable of multiplex analyte analysis. A competitive immunoassay format is applied whereby analytes in a sample are captured by their respective polyclonal antibodies which are immobilized onto the biochip in defined discrete test regions (DTRs). Increased levels of analyte in a sample lead to decreased binding of an enzyme-labelled analyte. The concentration of each analyte in a sample is proportional to the chemiluminescent signal produced at each DTR. The chemiluminescent reactions produced at the DTRs on the surface of the biochip are simultaneously detected and recorded by a cooled charge coupled device (CCD) camera. The light output generated is quantified by the CCD camera and image processing, A European Commission Regulation (No. 1213/2008) was passed in 2008 to establish a three year control programme to monitor pesticides in 30 foodstuffs in food of plant and animal origin (Anonymous, 2008).

This programme will assess consumer exposure to pesticide residues and to determine their possible aggregate, cumulative and synergistic effects. Abamectin, thiabendazole and carbendazim residue levels in orange juice will be assessed throughout Europe in 2012 and a multi-residue screening method for these pesticide residues would be a valuable asset to large surveys of this kind.

The present study concentrated on the development of a biochip array to simultaneously screen for ivermectin (IVER), thiabendazole (TBZ), carbendazim (MBC) and 2-aminobenzimidazole (2-AB) residues in orange juice (not from concentrate). The method was validated according to the 2002/657/EC guidelines (Anonymous, 2002) to screen for these pesticides below the MRLs for TBZ in citrus fruits, MBC in oranges and (AVER) in citrus fruits.

7.2 Materials and methods

7.2.1 Chemicals, reagents and apparatus

Sodium hydroxide (NaOH), pesticide grade acetonitrile (ACN), pesticide grade dimethylsulphoxide (DMSO) and methanol were supplied by BDH/VWR international Ltd. (Poole, England, UK). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore water purification system (Cork, Ireland). 2-aminobenzimidazole, carbendazim, ivermectin and thiabendazole were supplied by Sigma Aldrich (Berlin, Germany). Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate ($MgSO_4$) and 1 g NaCl were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate ($MgSO_4$) and 0.5 g C_{18} sorbent were purchased from Biotage (Uppsala, Sweden). Whatman syringe filter units (polytetrafluoroethylene (PTFE), 0.45 μm) were purchased from Fisher scientific (Dublin, Ireland). A Mistral 3000i centrifuge (MSE, London, UK), an Elma Transsonic T780/H ultrasonic bath (Bedford, UK) and a Turbovap LV evaporator (Caliper Life Sciences, Runcorn, UK) were used during orange juice sample preparation.

Assay buffer (pH 7.2), an assay-specific multi-conjugate labelled with horse radish peroxidase (HRP), conjugate diluent (pH 7.5), calibrator/sample diluent (pH 7.0), biochips, peroxide, luminol-EV840 and wash buffer (20 mM Tris buffered saline, pH 7.4) were purchased from Randox Laboratories Ltd. (Crumlin, Co. Antrim, Northern Ireland). All antibodies were raised in sheep against haptens coupled to bovine thyroglobulin (BTG) (albendazole-BTG, amino-albendazole-BTG, TBZ-BTG and IVER-BTG), the immunoglobulin fraction of the sheep polyclonal antiserum was used. All antibodies (PAS9618, PAS9847, PAS9900, PAS9283) were sourced from Randox Life Sciences.

7.2.2 Negative control samples

Organic orange juice purchased from a local retailer and found to be free of pesticide residues by UPLC-MS/MS analysis were used as negative controls.

7.2.3 Biochip array

7.2.3.1 Biochip array surface pre-treatment

Aluminum oxide sheets were sonicated in a 50 mL L^{-1} soap solution at an alkaline pH for 1 h, washed extensively under sonication with water and acetone and then dried overnight under reduced pressure (Fitzgerald *et al*., 2005).

7.2.3.2 Surface silanation and characterisation

The biochip surface was functionalised using the 3-glycidoxypropyltrimethyoxysilane (GOPTS) technique described by Fitzgerald *et al*. (2005). The silanised biochip surface was characterised using a contact angle meter (KSV Instruments) equipped with CAM200 software. When twenty-three sessile drops (3.4 µL) were tested over the functionalised surface the coefficient of variation (CV) of the measured contact angle was less than 3%. This confirmed the generation of a hydrophobic surface capable of containing droplets for the fabrication of DTRs. X-Ray Photoelectron Spectroscopy (XPS) measurements were performed with a Kratos Axis Ultra Spectrometer, operating at a base pressure of $3x10^{-9}$ Torr.

The samples were irradiated with monochromatic Al Kα X-rays (1486.6 eV) using an X-ray analysis spot size of 700 μm x 300 μm and ~225 W power. Survey spectra were recorded with pass energy of 160 eV, from which the surface elemental compositions were determined. The standard electron take off angle used for analysis is 90° giving a maximum analysis depth in the range 5 - 8 nm. The elemental analysis profile confirmed uniform silanation on the biochip surface to facilitate reproducible antibody immobilization.

7.2.3.3 Antibody immobilization

The biochip arrays were produced according to previously described methods (Fitzgerald *et al*., 2005). Droplets (330 pL) of antibody in 50 mM sodium carbonate (pH 9.6) were applied sequentially to achieve a volume of 10 nL of ligand solution, without affecting antibody structure and conformation. The ABZ and TBZ antibodies were applied at a concentration of 0.1 mg mL^{-1}. The IVER and 2-AB antibodies were applied at concentrations of 0.75 and 0.3 mg mL^{-1}, respectively. Briefly, diluted antibodies (10 nL) were deposited via a piezoelectric nanodispense technique onto the silanised surface of the biochip at the relevant DTRs. After antibody immobilisation, biochip surfaces were treated with casein (1% in 50mM carbonate buffer, 25°C, 1 h) to eliminate surface reactivity between the DTRs and to reduce non-specific binding.

7.2.3.4 Sample preparation

Orange juice samples (5 g) were adjusted to pH 6 using NaOH (1 M), this step was added because it was reported by Grujic *et al*. (2005) that pH has a decisive influence on the recovery of carbendazim. Samples were mixed gently by inversion (15 sec) and left to stand (15 min). A slurry containing MeCN:MgSO4:NaCl (12:4:1, v/w/w) was added to each sample and shaken vigorously by hand (1 min). After centrifugation (3,000 ×*g*, 10 min, -5ºC) the supernatant was transferred to a tube containing C_{18} sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500 ×*g*, 10 min, -5ºC). The MeCN layer (5 mL) was transferred to polypropylene tubes and the MeCN was evaporated under nitrogen at 50ºC.

Dried extracts were reconstituted in MeOH (500 µL), vortexed (5 min) and sonicated (15 min). An aliquot of this sample (50 µL) was added to an equal volume of DMSO vortexed (2 min) and sonicated (5 min). This sample extract (50 µL) was finally diluted in assay buffer (450 µL) and filtered (0.45 µm) prior to analysis.

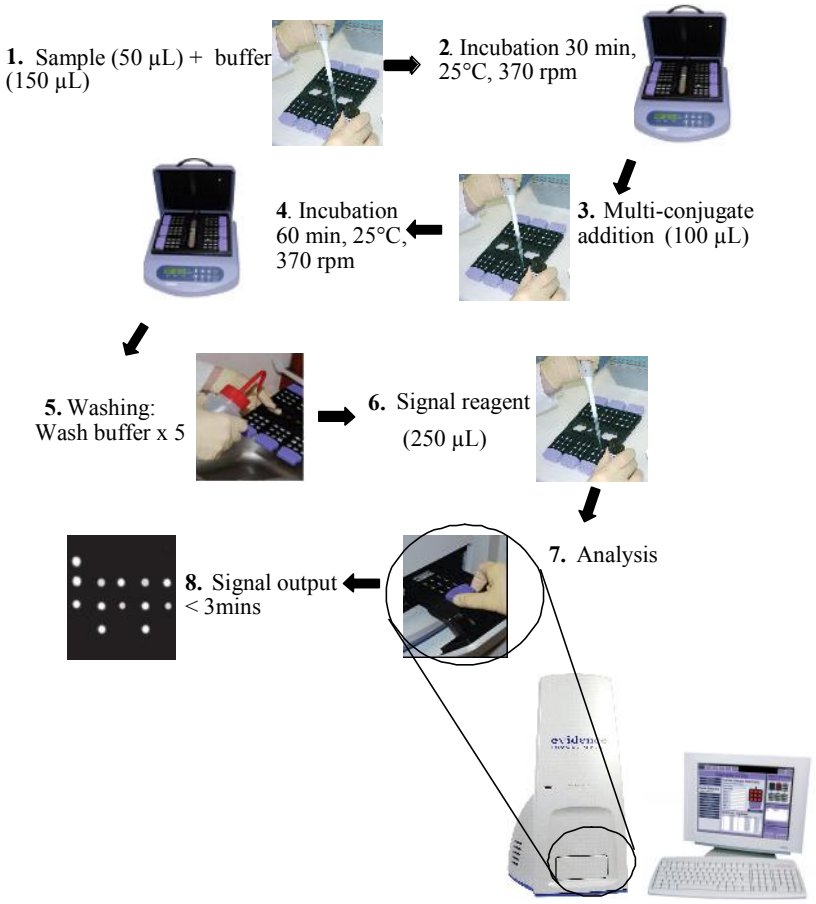
7.2.3.5 Assay procedure

A Randox Evidence Investigator™ was used for the analysis of biochip array (Randox Laboratories Ltd., Crumlin, Co. Antrim). The dimensions of each biochip were 9 x 9 mm, and each carrier held nine biochips in a 3 x 3 format. A competitive immunoassay format was applied whereby increased levels of analyte leads to decreased binding of HRP-labelled conjugates and thus decreases the chemiluminescent signal emitted. The light signal generated at each of the test regions is detected using digital imaging technology. The concentration of analyte present in the sample is then calculated from a calibration curve. The manual steps of the assay procedure are shown in **Fig 7.1**.

7.2.3.6 Calibration

Stock solutions (1 mg mL^{-1}) of 2-aminobenzimidazole, ivermectin, carbendazim and thiabendazole were prepared in DMSO. From these stock solutions a standard mix was prepared containing 2-aminobenzimidazole (20 µg mL^{-1}), ivermectin (40 µg mL^{-1}), carbendazim (100 µg mL^{-1}) and thiabendazole (40 µg mL^{-1}). Working solutions for calibration curves were prepared by sequential dilutions in methanol. Assay buffer was fortified at the concentrations outlined in **Table 7.1**

Negative orange juice samples were fortified at the concentrations outlined in **Table 7.2** prior to extraction and clean-up procedures. Of the six carriers used in a run carrier 1 was used for calibration (9 biochips) and the remaining 45 were used for control / sample analysis.



Source: http://www.randox.com/Evidence%20Investigator.php

Figure 7.1 Schematic diagram showing the manual procedures outlined in the manufacturers assay protocol for the determination of pesticide residues using the Evidence Investigator™ biochip array.

Table 7.1 Calibration curve standards for the determination of pesticide residues in assay buffer.

Calibration standard	2-AB	TBZ	IVER	MBC
	Concentration (ng mL^{-1})			
1	0.01	0.01	0.01	0.5
2	0.08	0.08	0.16	3.5
3	0.16	0.16	0.31	7.8
4	0.31	0.31	0.63	15.6
5	0.63	0.63	1.25	31.3
6	1.25	1.25	2.50	62.5
7	2.50	2.50	5.00	125.0
8	5.00	5.00	10.00	250.0
9	10.00	10.00	20.00	500.0
10	20.00	20.00	[a]N/A	[a]N/A
11	100.00	[a]N/A	[a]N/A	[a]N/A

[a] Not applicable

Table 7.2 Calibration curve standards for the determination of pesticide residues in orange juice.

Calibration Standard	2-AB/TBZ	Ivermectin	Carbendazim
	Concentration (µg kg^{-1})		
1	0.1	0.1	1.0
2	1.5	3.1	7.8
3	3.1	6.3	15.6
4	6.3	12.5	31.3
5	12.5	25.0	62.5
6	25.0	50.0	125.0
7	50.0	100.0	250.0
8	100.0	200.0	500.0
9	200.0	400.0	1000.0

All analyses were run alongside matrix-matched calibrants (a liver sample determined to be free of pesticide residues via UHPLC-MS/MS, fortified at the appropriate levels). Once the response of these calibrants (measured in Relative Light Units, RLUs) was determined, a calibration curve was constructed, applying the 4-parameter logistic model, Equation A below (Findlay and Dillard, 2007).

Equation A $Y= D + ((A-D) / (1+(x/C)^B))$

Where Y is the response generated, x is the concentration of the analyte, A is the response at zero analyte concentration, B is a slope factor, C represents the inflection point of the calibration curve, and D is the response at infinite analyte concentration. An initial estimate was made for each parameter, and this was then optimised by minimising the sum of square residuals via the Microsoft Excel™ component, Solver™. Correlation (R) values of >0.98 were obtained in all cases.

7.2.3.7 Biochip array validation procedure

A qualitative approach was used to determine the performance factor CCβ (detection capability) as described in 2002/657/EC criteria (Anonymous, 2002). Firstly, the limit of detection (LOD) for each of the four analytes in the assay was determined by measuring the mean response for 20 negative organic orange juice samples (not from concentrate) and subtracting three standard deviations. CCβ is the concentration at which a substance can be identified as positive (>LOD) with a statistical certainty of (1-β), where β = 5%. In order to determine CCβ for each assay, samples (n = 20 for each analyte) were spiked at a concentration above the LOD. If 19 of the 20 fortified samples were identified as positive, CCβ was to be determined to be equal to the fortification level (5% probability of a false negative result). If 20 samples were identified as positive, CCβ was determined to be less than the fortification level (0% probability of a false negative result) and if ≤18 samples were identified as positive, CCβ was determined to be greater than the fortification level (≥ 10% probability of a false negative result). Orange juice samples were fortified at arbitrary concentrations above the LOD of each assay and through trial and error CCβ levels were determined.

7.3 Results and discussion

7.3.1 Method development

The calibration range for each curve was optimized through the analysis of negative orange juice samples fortified with each analyte over the range 0 to 1000 µg kg^{-1}. The concentration of 2-AB, TBZ, IVER and MBC required to saturate their respective capture antibody was determined to be 200, 400, 400 and 1000 µg kg^{-1}, respectively. These concentrations were adopted as the maximum concentration levels for each calibration curve.

In an effort to reduce the time required to perform the assay, the volume of acetonitrile supernatant transferred from the initial extraction stage to the C_{18} clean-up stage was optimized. A reduction in analyte recovery and insufficient inhibition levels were seen with 5, 6 and 8 mL aliquots of supernatant and therefore a volume of 10 mL was required. Initially the reconstitution of dried extracts after evaporation was performed in 100% (v/v) DMSO and diluted (1:10, v/v in assay buffer). However this caused a reduced binding response, in negative orange juice matrix, at the MBC and TBZ test regions when compared to the responses of assay buffer. The DMSO may have caused conformational changes in the structure of the capture antibodies which resulted in a lower level of binding of the HRP-labelled conjugate. Sample extracts were reconstituted in methanol: DMSO (50:50, v/v) and diluted as before but in this instance no significant reduction in the negative binding responses were seen.

A filtration step (0.45 µm) was added after the reconstitution and dilution of orange juice samples because increases in the negative binding responses (500-900 RLU) were seen for all four pesticides without a filtration step The increase in binding may have been due to non-specific binding caused by matrix components in the sample which were unidentifiable. The final extracts appeared to be free from particulate matter.

7.3.2 Assay specificity

The concentration of each analyte required to reduce antibody binding by 50% (IC_{50}) was determined from the analysis of calibration curves prepared in assay buffer (**Fig. 7.2**). Each curve displayed a four parameter logistic fit and the IC_{50} values for carbendazim (13 ng mL^{-1}), 2-aminobenzimidazole (0.6 ng mL^{-1}), thiabendazole (0.4 ng mL^{-1}) and ivermectin (0.7 ng mL^{-1}) are shown in **Table 7.3**. From these results (**Fig. 7.3**) it was concluded that the biochip assay format would provide the sensitivity required to detect these analytes below the MRLs set for these pesticides in oranges.

Table 7.3

Determination of the concentration of pesticide analytes required to inhibit 50% of antibody binding (IC_{50}) in assay buffer and in organic orange juice.

Pesticide	IC_{50} in buffer (ng mL^{1})	IC_{50} in orange juice (µg kg^{-1})
Carbendazim	13	90.0
2-aminobenzimidazole	0.6	5.2
Thiabendazole	0.4	5.4
Ivermectin	0.7	14.0

The QuEChERS extraction procedure was optimized for the extraction of pesticides from orange juice. The samples were adjusted to pH 6 prior to extraction, dried extracts were reconstituted in methanol:DMSO 50:50 and diluted (1:10 in assay buffer) prior to biochip array analysis. The assay specificity towards four pesticides was determined in orange juice through the analysis of fortified matrix-matched standard curves (**Fig.7.3**). Carbendazim showed the lowest level of antibody inhibition. This was not unexpected because although carbendazim is a benzimidazole compound, it possesses structural differences from the albendazole hapten to which the antibody was raised. However the assay still provided adequate sensitivity for the purpose of a screening assay. The inhibition of antibody binding shown by 2-AB, TBZ and IVER proved that these assays were also suitable to screen for these pesticides in orange juice.

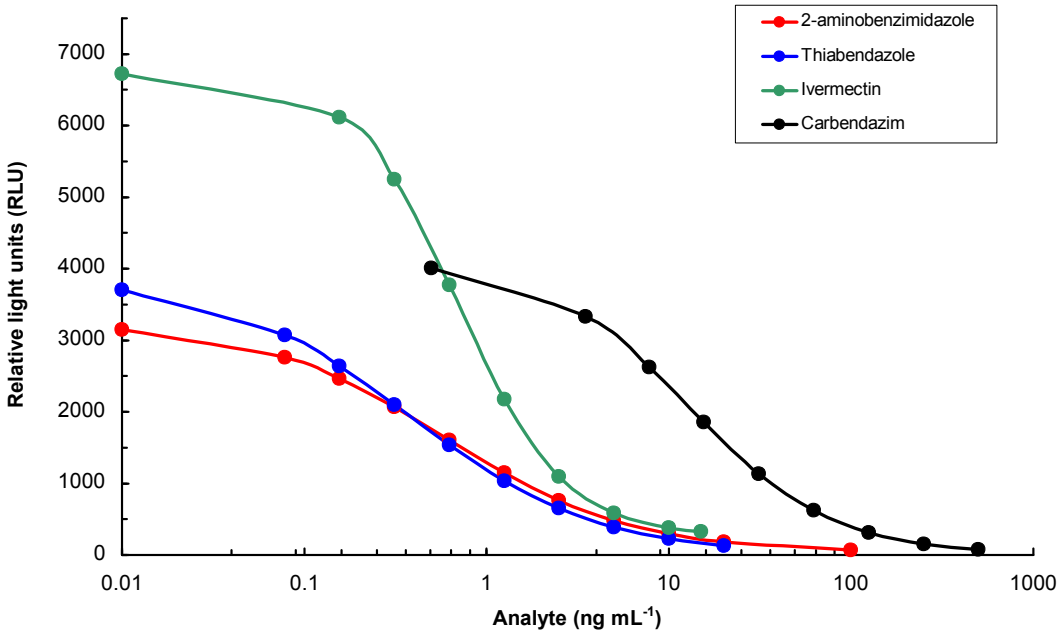


Figure 7.2 Calibration curves for pesticide residues in assay buffer.

7.3.3 Assay validation

A qualitative approach was used to determine the performance factor CCβ (detection capability) as described in 2002/657/EC (EC, 2002). Firstly, the limit of detection (LOD) of the assay was determined for each pesticide by measuring the mean response of 20 different negative organic orange juice samples against each calibration curve and subtracting three standard deviations (**Table 7.4**).

Secondly, in order to determine the assay CCβ values, orange juice samples (n = 20, for each analyte) were fortified with CBZ (50 µg kg^{-1}), 2-AB (10 µg kg^{-1}), TBZ (10 µg kg^{-1}) and IVER (20 µg kg^{-1}). All twenty fortified samples showed responses greater than the LOD of all four analytes and no false positive results were observed. Therefore the CCβ for all four analytes was less than their respective fortification levels (**Table 7.4 and Figs. 7.4-7.7**). The mean recovery of analytes (71 – 148 %) and the percentage coefficient of variation were within the range required for screening assays (CV % = 9-25%).

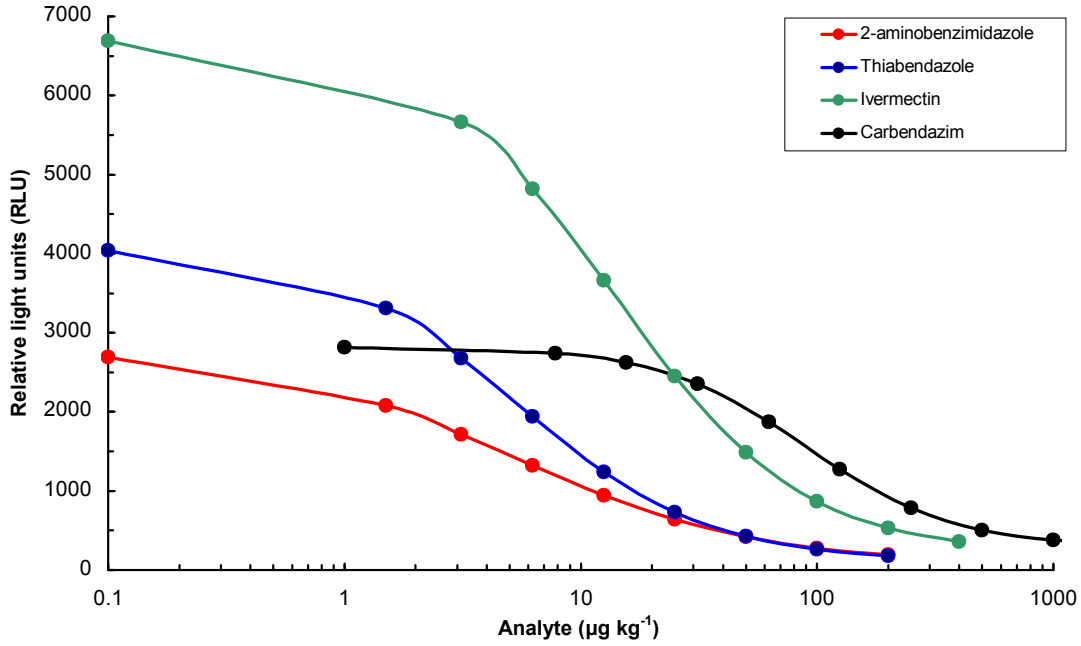


Figure 7.3 Calibration curves for pesticide residues in orange juice (not from concentrate).

Table 7.4

Determination of the limit of detection (LOD) and the capability of detection CCβ of biochip pesticide assay in orange juice (not from concentrate).

Pesticide	LOD ± SD (µg kg^{-1})	CCβ ± SD (µg kg^{-1})	Mean recovery (%)	CV%
Carbendazim	19.6 ± 7.4	< 50 ± 11.0	107	18
2-aminobenzimidazole	4.0 ± 0.7	< 10 ± 2.4	148	16
Thiabendazole	4.2 ± 3.6	< 10 ± 1.8	73	25
Ivermectin	10.2 ± 2.2	< 20 ± 1.2	71	9

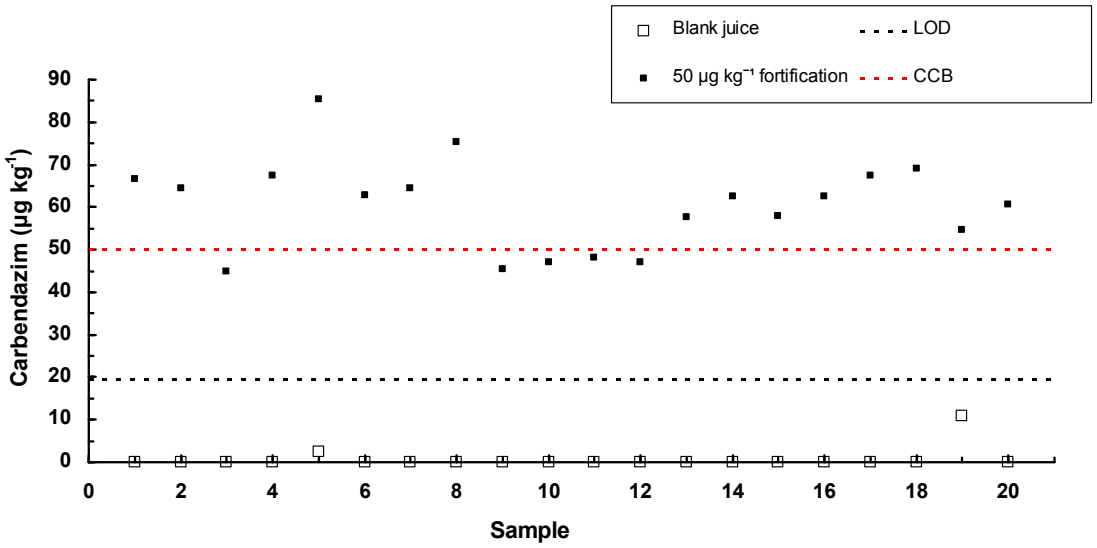


Figure 7.4 Determination of the detection capability (CCβ) of carbendazim (MBC) biochip assay in orange juice (not from concentrate).

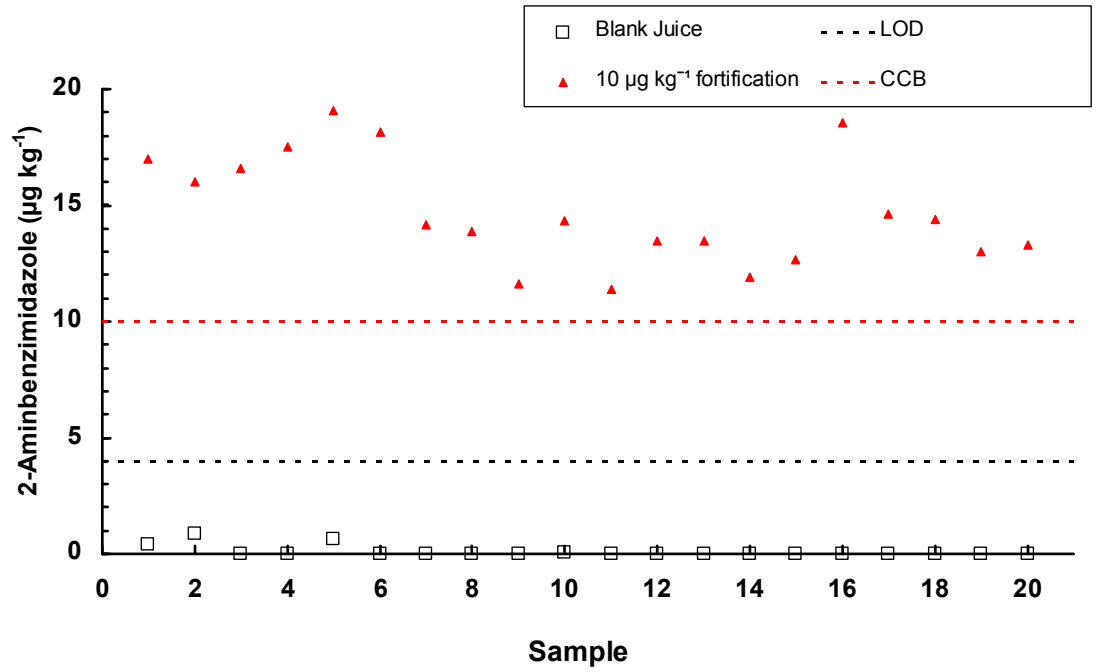


Figure 7.5 Determination of the detection capability (CCβ) of 2-aminobenzimidazole (2-AB) biochip assay in orange juice (not from concentrate)

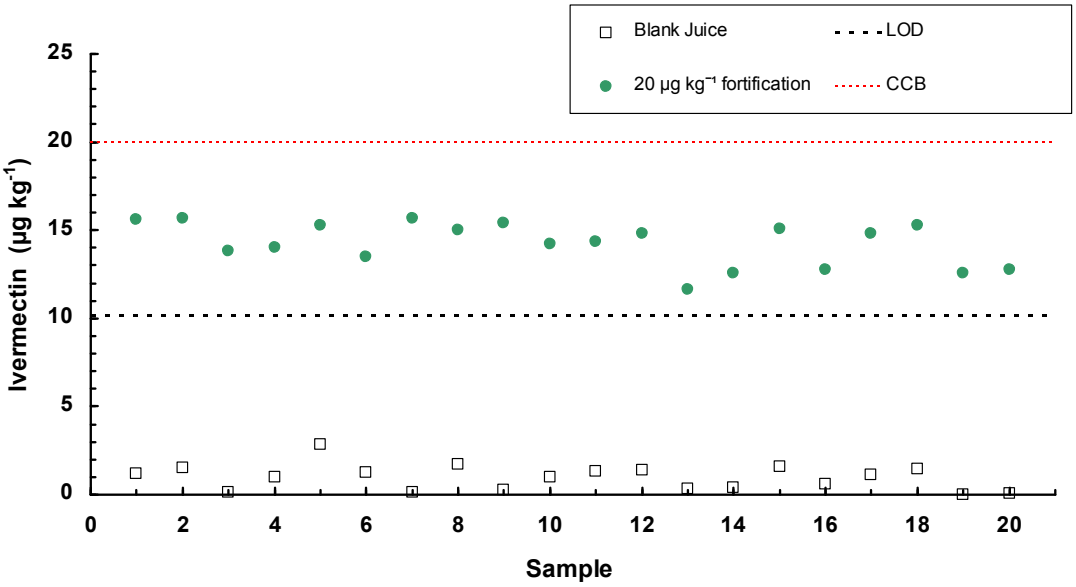


Figure 7.6 Determination of the detection capability (CCβ) of ivermectin biochip assay in orange juice (not from concentrate)

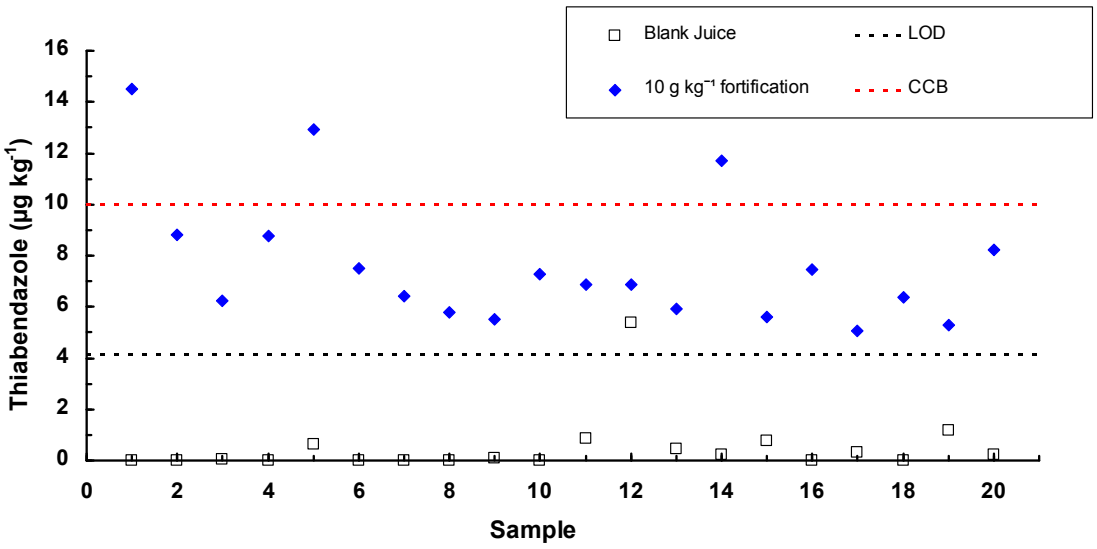


Figure 7.7 Determination of the detection capability (CCβ) of thiabendazole (TBZ) biochip assay in orange juice (not from concentrate)

7.3.4 Application of biochip array to detect pesticides in commercial orange juice

Several non-organic commercial brands of orange juice (not from concentrate) produced by different companies were purchased from retail outlets in the greater Dublin area and analysed using the biochip array assay (n = 15). It was found that two samples contained TBZ residues above the CCβ level. Two samples contained CBZ residues above the LOD however the concentration in both cases was below the CCβ. The concentration of 2-AB and IVER in all samples was below the LOD (**Table 7.5**).

The frequency, identity and concentration of pesticide residues in the samples was also determined. Pesticides were detected in 11 of the 15 samples analysed, but the levels were below the MRLs established by the EU for oranges / citrus fruit. The most commonly detected pesticide was ivermectin, at levels ranging from 2.1 to 7.4 μg kg^{-1}. MBC was found in six samples in the concentration range 5.9 to 41.3 μg kg^{-1}. Five samples contained 2-AB in the concentration range 0.3 to 2.0 μg kg^{-1}. TBZ was the least common pesticide as it was only detected in five of the samples.

On the co-occurance of pesticide residues, two samples contained four pesticide residues, three samples contained three pesticide residues, three samples contained two pesticide residues, thre samples contained one pesticide residue and four samples contained no pesticides.

Table 7.5
Biochip array survey of pesticide and fungicide residues in orange juice samples sourced from local retail outlets.

	Carbendazim	Amino-benzimidazole	Thiabendazole	Ivermectin
Sample	Analyte concentration (µg kg^{-1})			
1	N.D.	N.D.	N.D.	N.D.
2	5.85	0.31	N.D.	2.41
3	8.90	N.D.	N.D.	2.52
4	N.D.	N.D.	N.D.	N.D.
5	41.28	1.35	N.D.	3.28
6	N.D.	N.D.	260.66	2.07
7	N.D.	N.D.	N.D.	5.98
8	N.D.	N.D.	N.D.	N.D.
9	N.D.	N.D.	N.D.	6.17
10	14.62	0.94	0.58	7.35
11	N.D.	N.D.	181.37	N.D.
12	13.22	2.04	1.32	3.29
13	N.D.	0.68	N.D.	2.86
14	N.D.	N.D.	N.D.	N.D.
15	36.32	N.D.	0.55	2.85

7.4 Conclusions

The biochip array described in this study satisfies the performance and validation criteria laid down by Commission Decision 2002/657/EC (Anonymous, 2002). This is a multiplex platform that provides simultaneous analysis of a single sample for two different types of crop protection agents. It is suitable for the qualitative determination of carbendazim, 2-aminobenzimidazole, thiabendazole and avermectin residues in orange juice (not from concentrate) below their EU MRLs. The assay could be incorporated into a two-tiered monitoring system as a screening assay to identify possible non-compliant samples for confirmatory analysis by HPLC or UPLC-MS/MS. This pesticide array is a valuable addition to the regulatory authorities testing laboratories and the food industry in dealing with the issue of pesticide monitoring in processed orange juice. This will in turn improve the chemical safety of orange juice for consumers.

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Final discussion and conclusions

The work presented in this thesis describes the development of surface plasmon resonance (SPR) biosensor assays to screen for several benzimidazole anthelmintic drugs in liver and milk. This thesis also describes the development of a biochip array technique to screen for fungicide and pesticide residues in orange juice. A particular emphasis was placed on developing and validating these techniques according to the performance criteria outlined by EU regulating bodies. This was done to establish if the methods were fit for their intended purpose.

Chapter 2 describes the first application of a SPR-biosensor screening assay to detect a wide range of benzimidazole residues in liver tissue. These included 11 benzimidazole carbamate residues and four amino-benzimidazole residues. Initially, ethyl acetate and acetonitrile extraction procedures were investigated to extract benzimidazole carbamates from liver tissue; however low recoveries were observed for both albendazole and fenbendazole parent drugs. This issue was resolved using the QuEChERS extraction technique. In contrast, amino-benzimidazole residue recoveries were low when this extraction was applied. Surprisingly, a simplified version of this method resulted in higher recovery of amino-benzimidazoles. The detection capabilites (CCβs) of the benzimidazole carbamate assay and the amino-benzimidazole assay were less than 50% of their maximum residue limits (MRLs). Both assays could identify non-compliant liver from animals treated with benzimidazole drugs. It was possible for a single analyst to extract and analyse 25 samples in a single working day. Additionally, this is the first reported immunoassay-based technique to detect the amino-benzimidazole metabolites.

The work in Chapter 3 describes the optimisation of the benzimidazole-carbamate biosensor assay to screen for residues in milk at sub 10 μg kg^{-1} levels using the QuECHERS extraction method. The assay parameters were optimised to develop a sensitive method with a limit of detection (LOD) of 2.7 μg kg^{-1} and a CCβ of 5 μg kg^{-1}, which is equivalent to the existing chemical assay.

In Chapter 4 this assay was compared with a UPLC-MS/MS detection system. Milk from animals treated with benzimidazole drugs was analysed and the biosensor correctly identified all non-compliant samples. Over the course of these studies the biosensor chip surface was regenerated 1214 times and a reduction of just 1% was observed in the baseline response. The SPR biosensor method was found to be ideally suited for use as a rapid screening method to detect low levels of benzimidazole residues in milk.

Chapter 5 describes a sensitive SPR biosensor screening assay for the detection of TBZ and 5-OH-TBZ residues in ovine liver tissue using a novel recombinant antibody fragment (fAb). The assay performance was acceptable in accordance with 2002/657/EC. The LOD was determined to be 12.3 $\mu g\ kg^{-1}$ and the CCβ was calculated to be 20 $\mu g\ kg^{-1}$. The LOD was lower than that of an ELISA screening method reported for TBZ residues in liver using a mAb (Brandon *et al*., 1992). The biosensor assay could in the future be applied to detect TBZ residues in a variety of different matrices such as muscle tissue, milk, fruit juices and vegetables.

In Chapter 6, a biosensor assay was developed to detect triclabendazole (TCB) residues in ovine liver. This is the first immunoassay technique capable of detecting TCB below its MRL in liver. Further work is required to optimise this assay, which shows good potential for the screening of TCB residues in liver tissue. Following this the assay will be validated according to 2002/657/EC criteria.

In Chapter 7 work focussed on the development of a multiplex biochip array platform to qualitatively screen for carbendazim, 2-aminobenzimidazole, thiabendazole and avermectin residues in orange juice below their EU MRLs. This study satisfied the performance and validation criteria laid down by Commission Decision 2002/657/EC. This user-friendly multiplex platform provides simultaneous analysis of a single sample for two different types of crop protection agents and their metabolites. The assay could be incorporated into a two-tiered monitoring system as a screening assay to identify possible non-compliant samples for confirmatory analysis by HPLC or UPLC-MS/MS.

In conclusion, four biosensor assays were developed to screen for a total of 17 different benzimidazole drug residues and for the first time, amino-benzimidazole and TCB residues were detected in an immunoassay format. Three of these methods could be adopted by monitoring laboratories, meat production facilities or large dairies to rapidly screen liver tissue and milk for these residues. The multiplex biochip array could also be used in this way to detect pesticide levels in orange juice during production.

Appendix A

Dissemination of Research

Dissemination activities

Peer-reviewed papers

Keegan, J., Whelan, M., Danaher, M., Crooks, S., Sayers, R., Anastasio, A., Elliot, C., Brandon, D., Furey, A. and O'Kennedy, R. 2009. Benzimidazole carbamate residues in milk: Detection by Surface Plasmon Resonance-biosensor, using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method for extraction, *Analytica Chimica Acta* 654 : 111-119.

Papers currently submitted for peer review

Keegan, J., O'Kennedy, R., Crooks, S. Elliott, C., Brandon, D. and Danaher M.
Detection of benzimidazole carbamates and amino metabolites in liver by surface plasmon resonance-biosensor. (*Analytica Chimica Acta,* Manuscript no. ACA-10-1832).

Papers in preparation for submission to peer review

Keegan, J., Darcy, E., Leonard, P., Fitzgerald, J., Brandon, D. and Danaher, M.
Rapid detection of thiabendazole drug residues in liver tissue by surface plasmon resonance biosensor using a recombinant antibody fragment.
(Prepared for submission to *Analytical Methods: Elsevier)*

Keegan, J., O'Mahoney, J., Danaher, M., and O'Kennedy, R. Biochip array for the multi-residue detection of key pesticide and fungicidal residues in orange juice (not from concentrate). (Prepared for submission to *Analytical and Bioanalytical Chemistry: Springer Journal Series)*

Conferences

Keegan, J., Danaher, M. O'Kennedy, R. 2007. Development of a biosensor method for the detection of benzimidazole residues in food of animal origin. *In: Proceedings of the Berlin Community Reference Laboratory Workshop, Berlin, Germany, 24 - 27th April 2007*. (Invited Speaker)

Keegan, J., Danaher, M. O'Kennedy, R. 2007. Detection of benzimidazole carbamate residues in liver tissue using a surface plasmon resonance biosensor. *In: Proceedings of the IXth ICAFA Annual Conference, Vettre, Norway, 10 -13th September.*
(Poster presentation)

Keegan, J., Danaher, M. O'Kennedy, R. 2007. Detection of benzimidazole carbamate residues in liver tissue using a surface plasmon resonance biosensor. *In: Proceedings of the 121st AOAC Annual meeting and Exposition, California, USA, 16 -20th September*.
(Poster and invited oral presentation)

Keegan, J., Danaher, M. O'Kennedy, R. 2007. Detection of benzimidazole carbamate residues in liver tissue using a surface plasmon resonance biosensor. *In: Proceedings of First Technical Meeting for laboratories involved in the National Residue Programme,* Galway, 22nd November 2007. (Oral presentation).)

Keegan, J., Danaher, M. O'Kennedy, R. 2008. Detection of benzimidazole carbamate residues in liver tissue using a surface plasmon resonance biosensor. *In: Proceedings of EuroResidue VI, Egmond aan Zee, The Netherlands, 19-21th May.* (Poster presentation).

Keegan, J., Danaher, M., Crooks, S., O'Kennedy, R. 2010. SPR biosensor detection of benzimidazoles in ovine liver. *IN: Proceedings of the 6th international Symposium on Hormone and Veterinary Drug Residue Analysis, University of Ghent, Belgium, 1-4th June, 2010*. (Poster presentation).

Keegan, J., Darcy, E., Leonard, P., Fitzgerald, J., Brandon, D., Danaher, M. 2010. Detection of thiabendazole in liver using surface plasmon resonance immunosensor. *IN: Proceedings of the 6th international Symposium on Hormone and Veterinary Drug Residue Analysis, University of Ghent, 1-4th June, 2010*. (Poster presentation).