



Detection of benzimidazole carbamates and amino metabolites in liver by surface plasmon resonance-biosensor

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ABSTRACT

Two surface plasmon resonance (SPR) biosensor screening assays were developed and validated to detect 11 benzimidazole carbamate (BZT) and four amino-benzimidazole veterinary drug residues in liver tissue. The assays used polyclonal antibodies, raised in sheep, to detect BZTs and amino-benzimidazoles. A modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method was developed to isolate benzimidazole carbamate residues. Liver samples were extracted using an acetonitrile extraction method. BZTs were purified by dispersive solid phase extraction (d-SPE) using C₁₈ sorbent. Residues of amino-benzimidazoles were effectively cleaned-up using a simple cyclohexane defatting step. The assays were validated in accordance with the performance criteria described in 2002/657/EC. The BZT assay limit of detection was calculated to be 32 µg kg⁻¹, the detection capability (CCβ) was determined to be 50 µg kg⁻¹ and the mean recovery of analytes was in the range 77–132%. The amino-benzimidazole assay limit of detection was determined to be 41 µg kg⁻¹, the CCβ was determined to be 75 µg kg⁻¹ and analyte recovery was in the range 103–116%. Biosensor assay performance was tested by analysing liver tissue from animals treated with benzimidazole drugs and comparing the results with an ultra high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) confirmatory method. All non-compliant samples were identified using the biosensor assays.

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1. Introduction

Benzimidazoles are anthelmintic agents with broad spectrum activity against nematodes, cestodes and trematodes. They are widely used in the treatment of food producing animals in the European Union (EU). Many benzimidazole drugs have been proven to be safe when product label claims are followed. However, some drugs have shown teratogenic properties and congenital malformations have been reported in gestating ewes after the administration of albendazole and oxfendazole ABZ and OFZ [1]. Hence concern has been raised that high levels of residues may affect developing embryos in pregnant women. Maximum residue limits (MRLs) have been established in the EU for benzimidazole residues in edible tissues to protect public health under Commission Regulation 2010/37/EC. In addition, annual surveillance programmes are

carried out in member states under Council Directive 96/23/EC. 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 [2].

Several assays have been reported for benzimidazoles in liver because residues are known to accumulate in this organ making it an ideal target tissue for residue surveillance purposes. Marti et al. 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 solid phase extraction (SPE) steps [3]. Wilson et al. subsequently developed a simpler method based on ethyl acetate extraction coupled with purification by LLP (acidified ethanol versus hexane) and a further C₂ SPE clean-up step prior to HPLC–UV analysis [4]. This method has been used in many laboratories, but analyte coverage has been limited both by inadequate HPLC resolution and the poor availability of analytical standards [5]. The method has been extended to 12 benzimidazole residues but the throughput of the assay has been limited in our laboratory to 38 samples and associated controls per week for a single analyst [6]. Other groups

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Table 1
Maximum residue limits (MRLs) for benzimidazole residues in liver.

Drug	Marker residue (possible metabolites)	MRL ($\mu\text{g kg}^{-1}$)	Animal species
Albendazole	Sum of albendazole sulphoxide, albendazole sulphone, and albendazole 2-amino sulphone, expressed as albendazole	1000	All ruminants
Albendazole sulphoxide			
Netobimin	Sum of extractable residues which may be oxidised to fenbendazole sulphone	500	All ruminants
Fenbendazole			
Febantel	Sum of thiabendazole and 5-hydroxythiabendazole	400	Caprine
Fenbendazole-sulphoxide			
Thiabendazole	Sum of flubendazole and amino-flubendazole	400	Avian and porcine
Flubendazole			
Oxibendazole	Oxibendazole	200	Porcine
Triclabendazole	Sum of extractable residues that may be oxidised to keto-triclabendazole	250	All ruminants
Mebendazole	Sum of mebendazole, amino-mebendazole and hydroxy-mebendazole, expressed as mebendazole equivalents	400	Ovine, Caprine and Equidae

have increased the analytical scope and throughput of methods using liquid chromatography coupled to mass spectrometry [7]. However, this technology requires a significant amount of consumables, extensive maintenance for effective continued operation and experienced operators to process results. Ideally a method should include a wide range of benzimidazole marker residues as listed in Table 1. The inclusion of metabolites is important because they can be more toxic and persist longer than the parent drug [8–10].

Enzyme linked immunosorbent assays (ELISAs) have been developed by some groups for detecting benzimidazole residues as easier and low cost alternatives to chemical assays [11]. Immunoassays can also offer similar selectivity and sensitivity to those of LC–MS/MS. However, they are slow because of the multiple washing and incubation steps required and have proved difficult to automate in food analysis [12]. In addition, ELISAs with good repeatability can be difficult to develop and results are frequently non-quantitative and susceptible to matrix effects. Alternatively, several surface plasmon resonance (SPR) optical biosensor assays have been developed for detecting low levels of contaminant residues in food [13–15]. SPR is advantageous because labelling of antibodies or enzymes is not required. Furthermore, assay performance is frequently enhanced through automation and real time analysis. Crooks et al. highlighted the advantage of SPR biosensor over ELISA for analysing sulphonamide residues in 2081 pig bile samples over an eight month period [16]. False positive rates of 0.14–0.34% and 1.44–1.54% were observed for SPR-biosensor and ELISA, respectively. No false negative results were observed using SPR biosensor, while ELISA false negative rates were 0.14–0.24%.

The cost of SPR biosensors is higher than ELISA but savings can be seen through automation, which reduces labour costs and improves reliability of results. A further advantage of the technique is the ease of assay transfer between laboratories through reduction of operator effects through the elimination of plate washing and incubation steps. In addition, clear advantages of SPR biosensor assay can be seen over traditional HPLC based detection systems, which are frequently dedicated to specific assays, require laborious sample preparation and take longer to set-up prior to analysis. A typical SPR biosensor system can handle as many as five different assays in a single week because of the speed of changeover. In addition, recent advances in instrumentation have highlighted the ability to multiplex assays and improve sample throughput [17].

Recently a biosensor screening assay for benzimidazole carbamate residues in milk was developed using a modified QuEChERS extraction [18]. Due to limitations in the cross-reactivity of the antibody this assay could not detect a number of key metabolites possessing amino functional groups. The assay was found to be suitable for screening residues in incurred milk samples to below the MRL but extension to amino metabolites is desirable to provide more quantitative results. The aim of this study was to develop and validate multi-residue SPR biosensor assays to

screen liver tissue samples for 11 benzimidazoles and four amino-benzimidazole residues. The suitability of the assays to detect residues in liver tissue was verified through application of each assay to samples that were previously shown to contain benzimidazole residues by UHPLC–MS/MS. The method was validated according to 2002/657/EC [19].

2. Materials and methods

2.1. Chemicals, reagents and apparatus

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 M Ω) 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-NH₂), amino-mebendazole (MBZ-NH₂), hydroxy-mebendazole (MBZ-OH), and hydroxy-flubendazole (FLU-OH) were received as a gift from Janssen pharmaceuticals (Belgium). Albendazole-2-amino-sulphone (ABZ-NH₂-SO₂), albendazole sulphone (ABZ-SO), albendazole sulphoxide (ABZ-SO), fenbendazole sulphone (FBZ-SO₂) and amino-oxibendazole (OXI-NH₂) were purchased from Witega Laboratories (Berlin, Germany). Polypropylene centrifuge tubes with screw caps (50 mL) containing 4 g magnesium sulphate (MgSO₄) and 1 g NaCl were supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50 mL) containing 1.5 g magnesium sulphate (MgSO₄) and 0.5 g C₁₈ were purchased from Biotage (Uppsala, Sweden). The amino-albendazole hapten (Lot No. LK515) stored at –20 °C 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 $\mu\text{g mL}^{-1}$ 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. Liver samples

2.2.1. Negative control samples

Ovine liver samples found to be free of benzimidazole residues by UHPLC–MS/MS, with a limit of detection (LOD) of $<1 \mu\text{g kg}^{-1}$, were used as negative controls.

2.2.2. 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 UHPLC–MS/MS sample preparation, detection conditions and calibration method used in this work were outlined in recent work reported by Kinsella et al. [20].

2.3. SPR-biosensor assays

2.3.1. 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 $\text{MeCN}:\text{MgSO}_4:\text{NaCl}$ (12:4:1, v/w/w), homogenised (30 s in a multi-homogenisation unit) and centrifuged ($3000 \times g$, 10 min, -5°C). 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 ($3500 \times g$, 10 min, -5°C). The MeCN layer (6 mL) was transferred to polypropylene tubes and DMSO (500 μL) was added. The MeCN was evaporated under nitrogen at 50°C 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 did not undergo C_{18} clean-up. Instead, DMSO extracts were defatted with cyclohexane ($2 \times 2 \text{ mL}$ aliquots), and the cyclohexane layer was removed by aspiration. DMSO sample extracts were vortexed (2 min) and sonicated (10 min).

2.3.2. SPR-biosensor chip preparation

The preparation of the biosensor chip for benzimidazole carbamate was described in previous work by this research group [18].

A new CM5 biosensor chip was prepared for amino-benzimidazoles. 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 (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. 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 immobilised chip was stored in a Sarstedt® tube containing silica crystals (4°C) when not in use.

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 3.0.1 was used for data handling. Studies were conducted at 25°C and all samples and calibrants were analysed in duplicate.

Polyclonal antibody, raised in sheep against 5(6)-[(carboxypentyl)-thio]-2-benzimidazolecarbamate derivative (CMB) coupled to human serum albumin (HSA), was received from the Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, Northern Ireland and was used for the benzimidazole carbamate assay. An antibody dilution of 1/1000 (v/v), was found to give satisfactory results under assay conditions. DMSO extracts were transferred to 96 well microplates and mixed (1:9, v/v) with antibody and passed over the immobilised surface at $10 \mu\text{L min}^{-1}$ (2 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 at $25 \mu\text{L min}^{-1}$.

Polyclonal sheep antibody raised against amino-albendazole coupled to bovine thyroglobulin (BTG) was from Randox Laboratories (Crumlin, Northern Ireland) and was used for amino-benzimidazole detection. The Ig fraction (2.4 mg mL^{-1} in phosphate-buffered saline containing 0.09% 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 $10 \mu\text{L min}^{-1}$ (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 $25 \mu\text{L min}^{-1}$. The binding of the antibody to the chip surface was measured as the change in SPR signal between two report points, 10 s before and 30 s after each injection. A competitive immunoassay format was used to detect inhibition of antibody binding to the chip surface. The SPR signal was expressed in arbitrary resonance units (RU).

2.4. Calibration

Benzimidazole residue-free liver samples were fortified with albendazole-sulphone (ABZ-SO_2) at levels of 0, 50, 100, 250, 500 and $1000 \mu\text{g kg}^{-1}$ to prepare an extract calibration curve for the benzimidazole carbamate assay. Similarly samples were fortified with albendazole-amino-sulphone ($\text{ABZ-NH}_2\text{-SO}_2$) at levels of 0, 25, 50, 75, 125, 250 and $500 \mu\text{g kg}^{-1}$ to prepare an extract calibration curve for the amino-benzimidazole assay. BIAevaluation software was used to construct inhibition assay standard curves based on a 4-parameter fit.

2.5. Method validation

A qualitative approach was used to determine the performance factor $\text{CC}\beta$ (detection capability) as described in 2002/657/EC criteria [19]. 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. $\text{CC}\beta$ is the concentration at which a substance can be identified as positive ($>\text{LOD}$) with a statistical certainty of $(1 - \beta)$, where $\beta = 5\%$. In order to determine $\text{CC}\beta$ for each assay, samples ($n = 20$ for each analyte) were spiked at a concentration above the

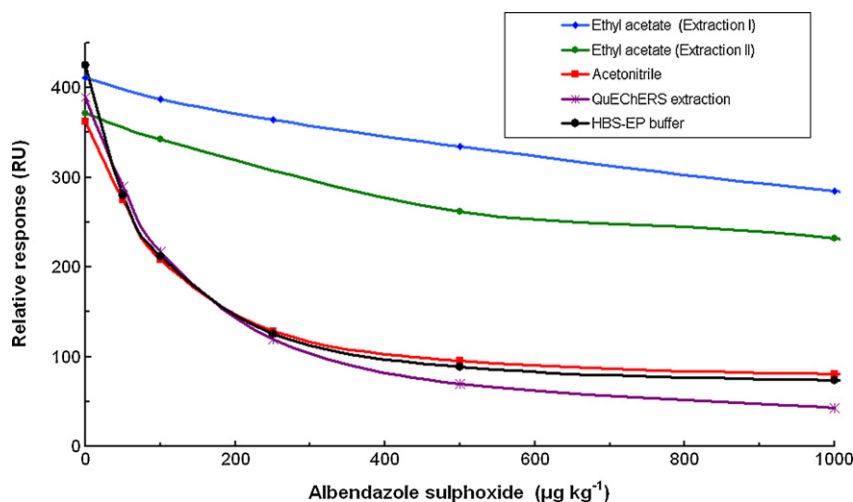


Fig. 1. Comparison of the sensitivity of different extraction methods for ABZ-SO analysis in ovine liver against equivalent curves in HBS-EP buffer.

LOD. If 19 of the 20 fortified samples were identified as positive, CC β 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, CC β was determined to be less than the fortification level and if ≤ 18 samples were identified as positive, CC β was determined to be greater than the fortification level. Liver samples were fortified at arbitrary concentrations above the LOD of each assay and through trial and error CC β levels were determined. Assay repeatability was evaluated by extracting and analysing ovine liver fortified with each analyte on five separate days.

3. Results and discussion

3.1. 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 (extraction procedure I) based on the method reported by Dowling et al. [6] 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 resuspended in MeOH:water (50:50, v/v). This extract was diluted (1/20, v/v) in HBS-EP buffer prior to biosensor analysis. Extracted matrix calibration curves prepared over the range 0–2000 $\mu\text{g kg}^{-1}$ (ABZ-SO equivalents) showed significant lower sensitivity ($\text{IC}_{50} = 770 \mu\text{g kg}^{-1}$) when compared to buffer curves ($\text{IC}_{50} = 88 \mu\text{g kg}^{-1}$) (Fig. 1). Losses in recovery were due to adsorption of analytes onto filter paper containing sodium sulphate. Subsequently the sample preparation procedure was modified by reducing the weights of sample and sodium sulphate (extraction procedure II) but this resulted in only slight improvements in sensitivity ($\text{IC}_{50} = 625 \mu\text{g kg}^{-1}$).

An alternative MeCN extraction was next evaluated for isolating benzimidazoles from liver tissue [6]. MeCN is an attractive solvent for isolating benzimidazole residues from biological samples without pH adjustment, 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 $89 \mu\text{g kg}^{-1}$ (extraction procedure III), not signif-

icantly different from the IC_{50} ($88 \mu\text{g kg}^{-1}$) in buffer. However, the sensitivity required for the recovery for ABZ and FBZ residues was unsatisfactory at <40%.

In earlier work by the present research group, a QuEChERS sample preparation procedure had been successfully applied to the analysis of 11 benzimidazole residues in milk samples. However, we evaluated an alternative clean-up procedure for liver tissue analysis because of the lower sensitivity required. A QuEChERS sample preparation procedure was applied to fortified ovine liver extracts, and the calibration curve showed comparable sensitivity ($\text{IC}_{50} = 86 \mu\text{g kg}^{-1}$) to MeCN and buffer curves. In addition, recoveries of ABZ and FBZ were acceptable, and the assay proceeded to validation. Subsequently, a new antibody became available that showed specificity towards amino-benzimidazole metabolites. Initially, the 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 $_2$, MBZ-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 $\times$ g), and washing with cyclohexane. Liquid–liquid partitioning with cyclohexane showed the highest recovery levels for all amino-metabolites and was selected for further validation.

3.2. Antibody inhibition studies

The cross-reactivity of the benzimidazole carbamate (S48) polyclonal antibody was determined in previous work by analysing inhibition curves obtained for each of 11 analytes in buffer by the SPR-biosensor assay [18]. The cross-reactivity of the S48 antibody towards 11 benzimidazole carbamates was determined by analysing inhibition curves in ovine liver tissue (0–1000 $\mu\text{g kg}^{-1}$) using the QuEChERS method. IC_{50} values in matrix ranged from 78 to 95 $\mu\text{g kg}^{-1}$ for FBZ-SO and FBZ, respectively, and cross-reactivities at 50% inhibition (CR_{50}) were 110 and 91% respectively (Table 2). Matrix calibration curves for 11 benzimidazole carbamates are shown in Fig. 2.

The cross-reactivity of the anti-amino-benzimidazole polyclonal antibody (PAS 9869) was determined by analysing inhibition curves with analyte concentrations from 0 to 125 ng mL^{-1} prepared in HBS-EP buffer and from 0 to 500 $\mu\text{g kg}^{-1}$ in ovine liver tissue. In buffer the antibody showed significant cross-reactivity with four amino-benzimidazoles (80–125%) 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). IC_{50}

Table 2

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

Analyte	Amino-benzimidazole assay			
	Buffer		Liver	
	^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

Analyte	Benzimidazole carbamate assay			
	Buffer		Liver	
	^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 ((IC₅₀ ABZ-NH₂-SO₂/IC₅₀ 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 ((IC₅₀ ABZ-NH₂-SO₂/IC₅₀ test amino-benzimidazole) × 100) in ovine liver.^e Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ((IC₅₀ ABZSO/IC₅₀ test benzimidazole) × 100) in HBS-EP buffer.^f Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ((IC₅₀ ABZSO/IC₅₀ test BZT) × 100) in ovine liver.

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. 3.

3.3. Method validation

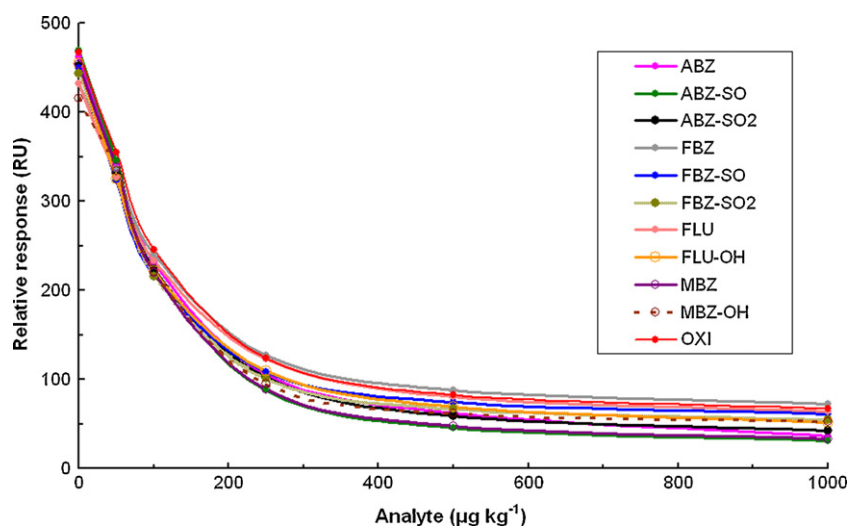
3.3.1. Benzimidazole carbamate biosensor assay

The dynamic range of the assay was found to be from 7 μg kg⁻¹ (IC₁₀) to 340 μg kg⁻¹ (IC₉₀) and the IC₅₀ 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 lowest MRL, which is 200 μg kg⁻¹ for OXI. The results for the determination of CCβ for

each analyte are shown in Table 3. The CCβ for 10 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 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. 4(A).

3.3.2. Amino benzimidazole assay

The dynamic range of the assay was found to be from 22 (IC₁₀) to 238 μg kg⁻¹ (IC₉₀) and the IC₅₀ was 44 μg kg⁻¹. The LOD of the assay

**Fig. 2.** Calibration curves for 11 benzimidazole carbamates in ovine liver matrix.

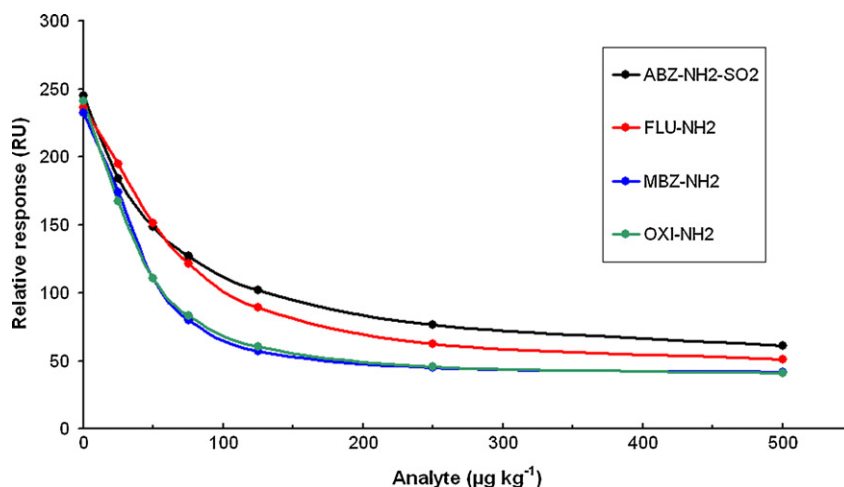


Fig. 3. Calibration curves for amino-benzimidazole metabolites in ovine liver matrix.

using was determined to be $41 \mu\text{g kg}^{-1}$ by measuring the mean response of 20 representative blank ovine liver samples (236 RU) and subtracting three standard deviations (3×21 RU).

The CC β of the assay was determined by fortifying 20 representative blank ovine liver samples at $75 \mu\text{g kg}^{-1}$ with four different amino-benzimidazoles. The CC β for three of the four amino analytes was found to be $<75 \mu\text{g kg}^{-1}$ because all 20 fortified samples showed responses above the LOD (Table 3). The CC β for FLU-NH₂ was equal to $75 \mu\text{g kg}^{-1}$ as one of the samples gave a measured result of $40 \mu\text{g kg}^{-1}$ and was deemed negative. The repeatability of the assay was evaluated by analysing ovine liver samples fortified ($125 \mu\text{g kg}^{-1}$) 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 3). Calibration curves for each day are shown in Fig. 4(B).

3.4. Application of SPR assay to incurred liver tissue

The suitability of the SPR biosensor assays was 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 UHPLC-MS/MS methods. Seven of the nine samples were found to contain benzimidazole residues at concentrations above the LOD, which was 32 and $41 \mu\text{g kg}^{-1}$ for the benzimidazole carbamate and amino-benzimidazole SPR-biosensor assays, respectively (Table 4). Samples one to six were determined to be compliant for benzimidazole residues by both the biosensor assay and UHPLC-MS/MS. Two of these samples (five and six) screened above CC β by the benzimidazole carbamate SPR-biosensor assay, which indicate that they should be sent for confirmatory analysis. A total of four samples (7–10) were confirmed to be non-compliant by UHPLC-MS/MS. Three samples (7, 9 and 10) contained residues above their respective MRLs. The remaining sample, number 8, was categorised as non-compliant because it contained MBZ residues, which are not allowed in bovine animals. 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 $>\text{CC}\beta$.

Table 3
Determination of detection capability (CC β) 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	
	Mean recovery (%) $\pm S$ ($n=5$)	CV (%) ($n=5$)	Mean $\pm S$ ($n=20$) ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)
	Fortification = $100 \mu\text{g kg}^{-1}$		Fortification = $50 \mu\text{g kg}^{-1}$	
ABZ	94 ± 11	11	66 ± 9	<50
ABZ-SO	105 ± 15	15	76 ± 9	<50
ABZ-SO ₂	122 ± 16	13	71 ± 5	<50
FBZ	132 ± 15	11	79 ± 8	<50
FBZ-SO ₂	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
Analyte	Assay repeatability		Detection capability	
	Mean recovery (%) $\pm S$ ($n=5$)	CV (%) ($n=5$)	Mean $\pm S$ ($n=20$) ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)
	Fortification = $125 \mu\text{g kg}^{-1}$		Fortification = $75 \mu\text{g kg}^{-1}$	
ABZ-NH ₂ -SO ₂	109 ± 8	8	121 ± 26	<75
FLU-NH ₂	110 ± 18	16	87 ± 18	75
MBZ-NH ₂	116 ± 11	10	93 ± 14	<75
OXI-NH ₂	103 ± 9	9	105 ± 19	<75

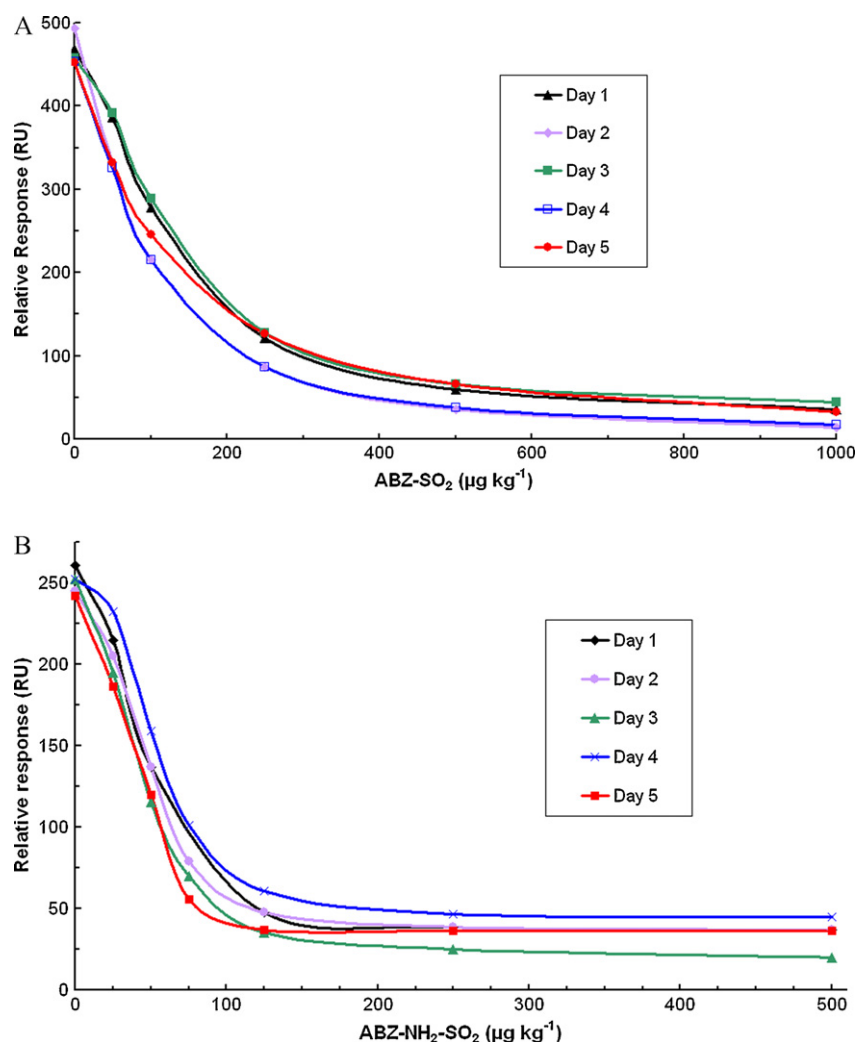


Fig. 4. SPR biosensor assay calibration curves in fortified ovine liver on different days ($n=5$) for (A) albendazole sulphone (ABZ-SO₂) and (B) albendazole-amino-sulphone (ABZ-NH₂-SO₂).

Table 4

Comparison between biosensor and UHPLC-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	13,096	FBZ	NC
10	Bovine	>1000	211	Positive	1222	ABZ	NC

^a Negative samples $\leq$ CC β and positive samples $\geq$ CC β : where benzimidazole carbamate CC β = 50 µg kg⁻¹ and amino-benzimidazole CC β = 75 µg kg⁻¹.

^b UPLC-MS/MS concentrations are expressed as the sum of the FBZ, FBZ-SO and FBZ-SO₂ residues expressed as FBZ-SO₂; MBZ, MBZ-NH₂ and MBZ-OH residues expressed as MBZ; and ABZ, ABZ, ABZ-SO, ABZ-SO₂ and ABZ-NH₂-SO₂ residues expressed as ABZ.

^c C, compliant (<MRL) and NC, non-compliant (>MRL).

UHPLC-MS/MS confirmed these samples to contain MBZ-NH₂ and ABZ-NH₂-SO₂ residues at 244 and 228 µg kg⁻¹, respectively.

One notable 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 UHPLC-MS/MS to contain FBZ marker residues at concentrations above 1000 µg kg⁻¹.

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 $50 \mu\text{g kg}^{-1}$, 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 $75 \mu\text{g kg}^{-1}$, 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 UHPLC/MS–MS. Only suspect non-compliant samples would then require confirmatory analysis by UHPLC–MS/MS. Using the methodology presented in this paper it is possible to extract and analyse 25 samples within a single working day. This is the first reported immunochemical screening assay for amino-benzimidazole residues.

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