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

AUTHORS: Jemma Keegan, Richard O'Kennedy, Steven Crooks, Christopher Elliott, David Brandon, Martin Danaher

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

Jemma Keegan[a,b], Richard O'Kennedy[b], Steven Crooks[c], Christopher Elliott[d], David Brandon[e] and Martin Danaher[a*]

[a]*Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland*
[b]*School of Biotechnology and National Centre for Sensor Research, Dublin City University, Dublin 9, Ireland*
[c]*Veterinary Sciences Division, Agri-Food & Biosciences Institute, Belfast, Northern Ireland*
[d]*I nstitute of Agri-Food and Land Use, School of Biological Sciences, Queen's University Belfast, Belfast, Northern Ireland*
[e]*Western RegionalResearch Center, US Department of Agriculture, Agricultural Research Service, Albany, CA, USA*

*Corresponding author
Email: martin.danaher@teagasc.ie
Phone: 353-1-8059552
Fax: 353-1-8059550

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.

Keywords: SPR biosensor, Benzimidazoles, Liver, Screening Assay

1. Introduction

Benzimidazoles are anthelmintic agents with broad spectrum activity against nematodes, cestodes and trematodes. They are widely used for 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 C_2 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 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 that 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 to 0.34% and 1.44 to 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 to 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 H EPES 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 ($MgSO_4$) and 0.5 g $_{C18}$ 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

147 40 µg mL^{-1} by diluting the primary stock in methanol. A FASTH 21 homogenisation unit
148 and sample homogenisation tubes were supplied by Syntec Scientific (Dublin, Ireland), a
149 Mistral 3000i centrifuge (MSE, London, UK), an Elma Transsonic T780/H ultrasonic bath
150 (Bedford, UK) and a Turbovap LV evaporator (Caliper Life Sciences, Runcorn, UK) were
151 used during sample preparation.

152

153 *2.2 Liver samples*

154

155 *2.2.1 Negative control samples*

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

158 *2.2.2 Incurred liver samples*

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

168

169 *2.3 SPR-Biosensor Assays*

170 *2.3.1 Sample preparation*

171 A modified QuEChERS extraction method was used to isolate benzimidazole carbamate
172 residues from liver tissue. Finely chopped liver (2 g) was homogenised in a slurry
173 containing MeCN:$MgSO_4$:NaCl (12:4:1, v/w/w), homogenised (30 sec in a multi-
174 homogenisation unit) and centrifuged (3,000 ×*g*, 10 min, -5°C). The supernatant was
175 transferred to a tube containing $_{C18}$ sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were
176 subsequently shaken (1 min) and centrifuged (3500 ×*g*, 10 min, -5°C). The MeCN layer (6
177 mL) was transferred to polypropylene tubes and DMSO (500 µL) was added. The MeCN
178 was evaporated under nitrogen at 50°C using a Turbovap LV (Caliper Life Sciences,
179 Runcorn, UK). The DMSO extracts were vortexed (2 min) and sonicated (10 min).

180 Amino-benzimidazole residues were extracted using the same procedure as for the
181 carbamate metabolites but did not undergo $_{C18}$ clean-up. Instead, DMSO extracts were
182 defatted with cyclohexane (2 × 2 mL aliquots), and the cyclohexane layer was removed by
183 aspiration. DMSO sample extracts were vortexed (2 min) and sonicated (10 min).

184

185 *2.3.2 SPR-biosensor chip preparation*

186 The preparation of the biosensor chip for benzimidazole carbamate was described in
187 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 immobilized 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 µL 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 µL 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 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}. 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. 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-

234 SO_2) at levels of 0, 50, 100, 250, 500 and 1000 µg kg^{-1} to prepare an extract calibration
235 curve for the benzimidazole carbamate assay. Similarly samples were fortified with
236 albendazole-amino-sulphone (ABZ-NH_2-SO_2) at levels of 0, 25, 50, 75, 125, 250 and 500
237 µg kg^{-1} to prepare an extract calibration curve for the amino-benzimidazole assay.
238 BIAevaluation software was used to construct inhibition assay standard curves based on a
239 4-parameter fit.

240

241 *2.5 Method validation*

242 A qualitative approach was used to determine the performance factor CCβ (detection
243 capability) as described in 2002/657/EC criteria [19]. Firstly, the limit of detection (LOD)
244 of the assay was determined by measuring the mean response for 20 different negative
245 ovine liver tissue samples and subtracting three standard deviations. CCβ is the
246 concentration at which a substance can be identified as positive (>LOD) with a statistical
247 certainty of (1-β), where β = 5%. In order to determine CCβ for each assay, samples (n =
248 20 for each analyte) were spiked at a concentration above the LOD. If 19 of the 20
249 fortified samples were identified as positive, CCβ was to be determined to be equal to the
250 fortification level (5% probability of a false negative result). If 20 samples were identified
251 as positive, CCβ was determined to be less than the fortification level and if ≤18 samples
252 were identified as positive, CCβ was determined to be greater than the fortification level.
253 Liver samples were fortified at arbitrary concentrations above the LOD of each assay and
254 through trial and error CCβ levels were determined. Assay repeatability was evaluated by
255 extracting and analysing ovine liver fortified with each analyte on five separate days.

256

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. [9] 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 resuspensed 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 to 2000 μg kg^{-1} (ABZ-SO equivalents) showed significant lower sensitivity (IC_{50} = 770 μg kg^{-1}) when compared to buffer curves (IC_{50} = 88 μg 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 (IC_{50} = 625 μg 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 $_{IC50}$ of 89 µg kg^{-1} (extraction procedure III), not significantly different from the $_{IC50}$ (88 µg 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 (IC_{50} = 86 µg 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, 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 (18000 x *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.

303 *3.2 Antibody inhibition studies*

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

312 The cross-reactivity of the anti-amino-benzimidazole polyclonal antibody (PAS 9869) was
313 determined by analysing inhibition curves with analyte concentrations from 0 - 125 ng mL^{-1}
314 prepared in HBS-EP buffer and from 0 - 500 µg kg^{-1} in ovine liver tissue. In buffer the
315 antibody showed significant cross-reactivity with four amino-benzimidazoles (80 to 125%)
316 in the following order of affinity OXI-NH_2>MBZ-NH_2>ABZ-NH_2-SO_2>FLU-NH_2 and
317 analyte IC_{50} values were typically less than 7.1 ng mL^{-1} (Table 2). IC_{50} values in matrix
318 ranged from 35 to 55 µg kg^{-1} for the four amino analytes. Matrix calibration curves for
319 four amino-benzimidazoles are shown in Fig. 3.

320

321 *3.3 Method Validation*

322

323 *3.3.1. 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 $_{IC50}$ 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 x 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 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 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 (IC_{10}) to 238 µg kg^{-1} (IC_{90}) and the $_{IC50}$ 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 x 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 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 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 µg 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 CCβ by the benzimidazole carbamate SPR-biosensor assay, which indicate that they should be sent for confirmatory analysis. A total of four samples (7 to 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 >CCβ. UHPLC-MS/MS confirmed these samples to contain MBZ-NH_2 and ABZ-NH_2-SO_2 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 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 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

392 to extract and analyse 25 samples within a single working day. This is the first reported
393 immunochemical screening assay for amino-benzimidazole residues.

394

395

396 **Acknowledgements**

397 This research was funded by the Irish Department of Agriculture, Fisheries and Food under
398 the Food Institutional Research Measure as part of the National Development Plan (Project
399 05/R&D/TN/355).

400

401

402 **References**

403
404 [1] P. Delatour, G. Lorgue, D. Courtot, M. Lampras, Bull. Soc. Sci. Vet. Med. Comp. 77
405 (1975) 197-203.
406 [2] M. Danaher, A.M. Sherry, J. O'Mahony, National Food Residue Database Report 2009.
407 Teagasc, Dublin, 2009.
408 [3] A.M. Marti, A.E. Mooser, H. Koch, J. Chromatogr. A 498 (1990) 145-157.
409 [4] R.T. Wilson, J.M. Groneck, A.C. Henry, L.D. Rowe, J. AOAC Int. 74 (1991) 56-57.
410 [5] G. Domany, L. Koviacs, in: L.A. van Ginkel, A. Ruiter (Eds.), Proceedings of the
411 Euroresidue IV Conference on Residues of Veterinary Drugs in Food, Veldhoven, The
412 Netherlands, May 8-10, National Institute of Public Health and the Environment (RIVM),
413 2000, pp. 361-370.
414 [6] G. Dowling, H. Cantwell, M. O'Keeffe, M.R. Smyth, Anal. Chim. Acta 529 (2005)
415 285-292.
416 [7] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal.
417 Chim. Acta 637 (2009) 196-207.
418 [8] P. Delatour, R. Parish, in: A.G. Rico (Ed.), Drug Residues in Animals, Academic Press
419 Inc., Orlando, FL, 1989, pp. 175–204.
420 [9] P. Delatour, F. Garnier, E. Benoit, C. Longin. J. Vet. Pharmacol. Ther. 7 (1984) 139-
421 145.
422 [10] Q.A. McKellar, E.W. Scott, J. Vet. Pharmacol. Ther. 13 (1990) 223-247.
423 [11] D.L. Brandon, R.G. Binder, A.H. Bates, W.C. Montague Jr., J. Agric. Food Chem. 42
424 (1994) 1588-1594.

425 [12] A-C, Huet, T. Fodey, S.A. Haughey, S. Weigel, C. Elliott, P. Delahaut., TrAC 29
426 (2010) 1281-1294.
427 [13] L. Johnsson, G.A. Baxter, S.R.H. Crooks, D.L. Brandon C.T. Elliott, Food Agric.
428 Immunol. 14 (2002) 209-21 6.
429 [14] J. Samsonova, G.A. Baxter, S.R.H. Crooks, A.E. Small, C.T. Elliott, Biosens.
430 Bioelectron. 17 (2002) 523-520.
431 [15] S.R.H. Crooks, B. McCarney, I.M. Traynor, C.S. Thompson, S. Floyd, C.T. Elliott,
432 Anal. Chim. Acta, 483 (2003) 181-186.
433 [16] S.R.H. Crooks, G.A. Baxter, M.C. O'Connor, C.T. Elliott, Analyst 123 (1998) 2755.
434 [17] C. Situ, M.H. Mooney, C.T. Elliott, J. Buijs. TrAC 29 (2010) 1305-1315.
435 [18] J. Keegan, M. Whelan, M. Danaher, S. Crooks, R. Sayers, A. Anastasio, C. Elliott, D.
436 Brandon, A. Furey, R. O'Kennedy, Anal. Chim. Acta 654 (2009) 111-119.
437 [19] European Commission Decision 2002/657/EC, Off. J. Eur. Comm. L221 (2002) 1-32.
438 [20] B. Kinsella, M. Whelan, H. Cantwell, M. McCormack, A. Furey, S.J. Lehotay, M.
439 Danaher, Talanta 83 (2010) 14-24.
440

441

442

443

444
445

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

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.

Table 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).

Table 4 Comparison between biosensor and UHPLC-MS/MS analysis of liver samples containing incurred mebendazole, fenbendazole and albendazole residues.

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

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

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

Fig. 4. 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).

Drug	Marker residue (possible metabolites)	MRL ($\mu g kg^{-1}$)	Animal species
Albendazole, Albendazole sulphoxide Netobimin	Sum of albendazole sulphoxide, albendazole sulphone, and albendazole 2-amino sulphone, expressed as albendazole	1000	All ruminants
Fenbendazole Febantel Fenbendazole-sulphoxide	Sum of extractable residues which maybe oxidised to fenbendazole sulphone	500	All ruminants
Thiabendazole	Sum of thiabendazole and 5-hydroxythiabendazole	400	Caprine
Flubendazole	Sum of flubendazole and amino flubendazole	400	Avian and porcine
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 hydroxymebendazole, expressed asmebendazole equivalents	400	Ovine, Caprine and Equidae

Table 2

	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)x 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)x 100) in ovine liver.
[e] Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ((IC_{50} ABZSO/IC_{50} test benzimidazole) x100) in HBS-EP buffer.
[f] Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ((IC_{50} ABZSO/IC_{50} test BZT) x 100) in ovine liver.

Table 3

	Assay Repeatability		Detection Capability	
Analyte	Mean recovery (%) ± S (n = 5)	CV (%) (n = 5)	Mean ± S (n = 20) (µg kg^{-1})	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	87 ± 18	75
MBZ-NH_2	116 ± 11	10	93 ± 14	<75
OXI-NH_2	103 ± 9	9	105 ± 19	<75

Table 4

Sample	Species	Biosensor assays			UPLC-MS/MS assay		
		Benzimidazole carbamates (µg kg^{-1})	Amino-benzimidazoles (µg kg^{-1})	[a]I nterpretation	[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 = < CCβ and positive samples = > CCβ: where benzimidazole carbamate CCβ =50 µg kg^{-1} and amino-benzimidazole CCβ = 75 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, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues expressed as ABZ.

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

Figure 1B

Click here to download high resolution image

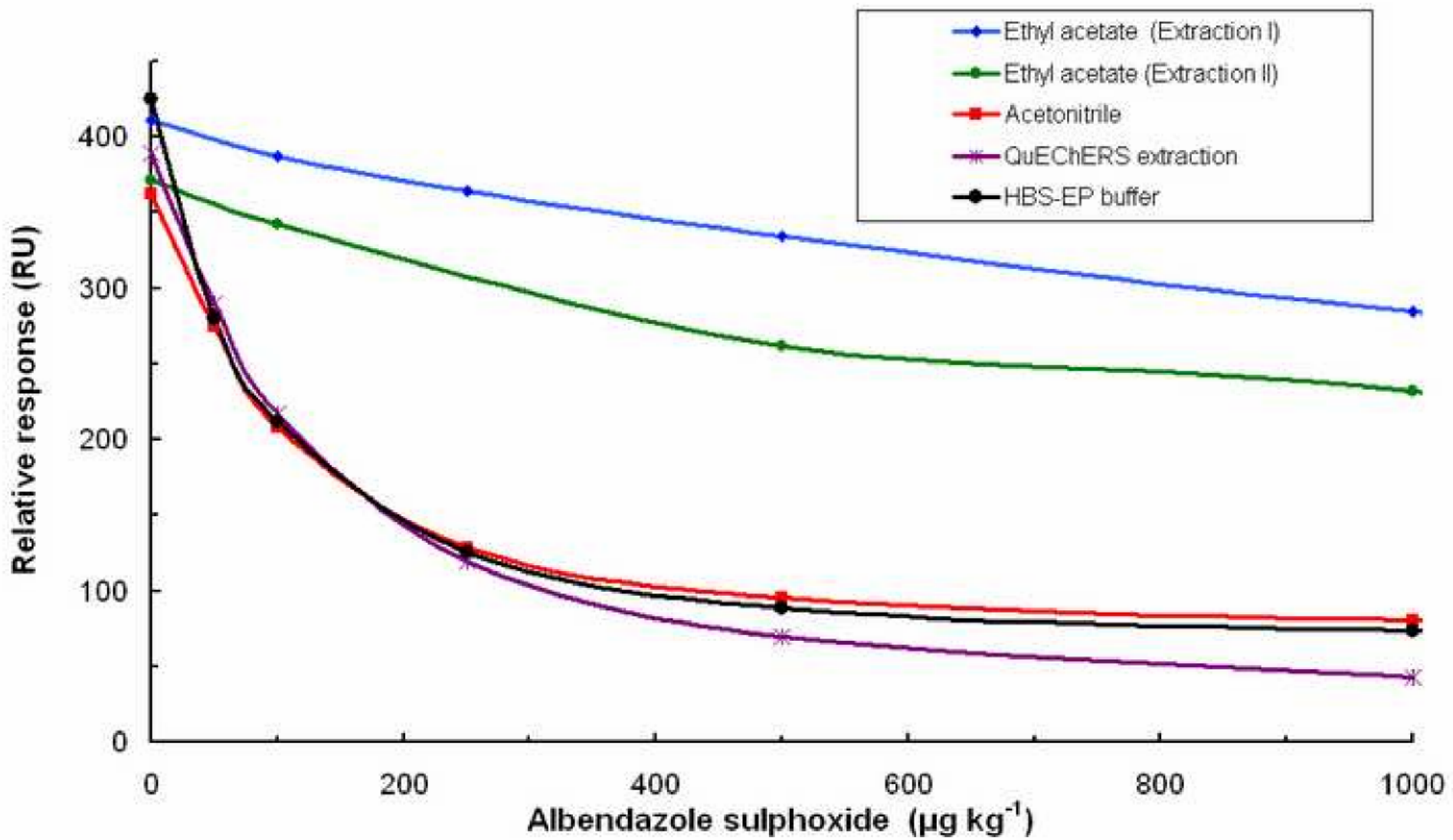

Figure 2B

Click here to download high resolution image

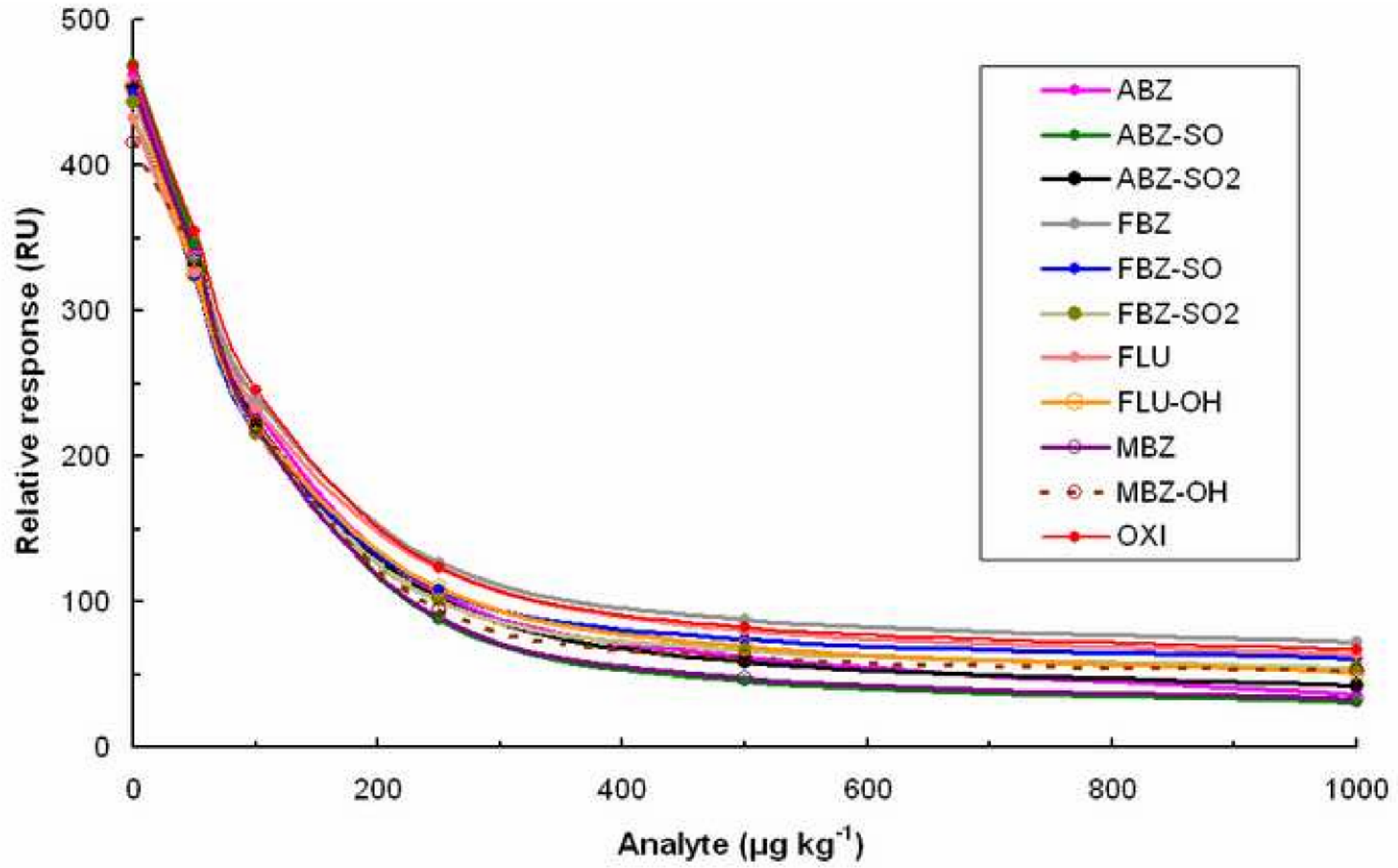

Figure 3B

Click here to download high resolution image

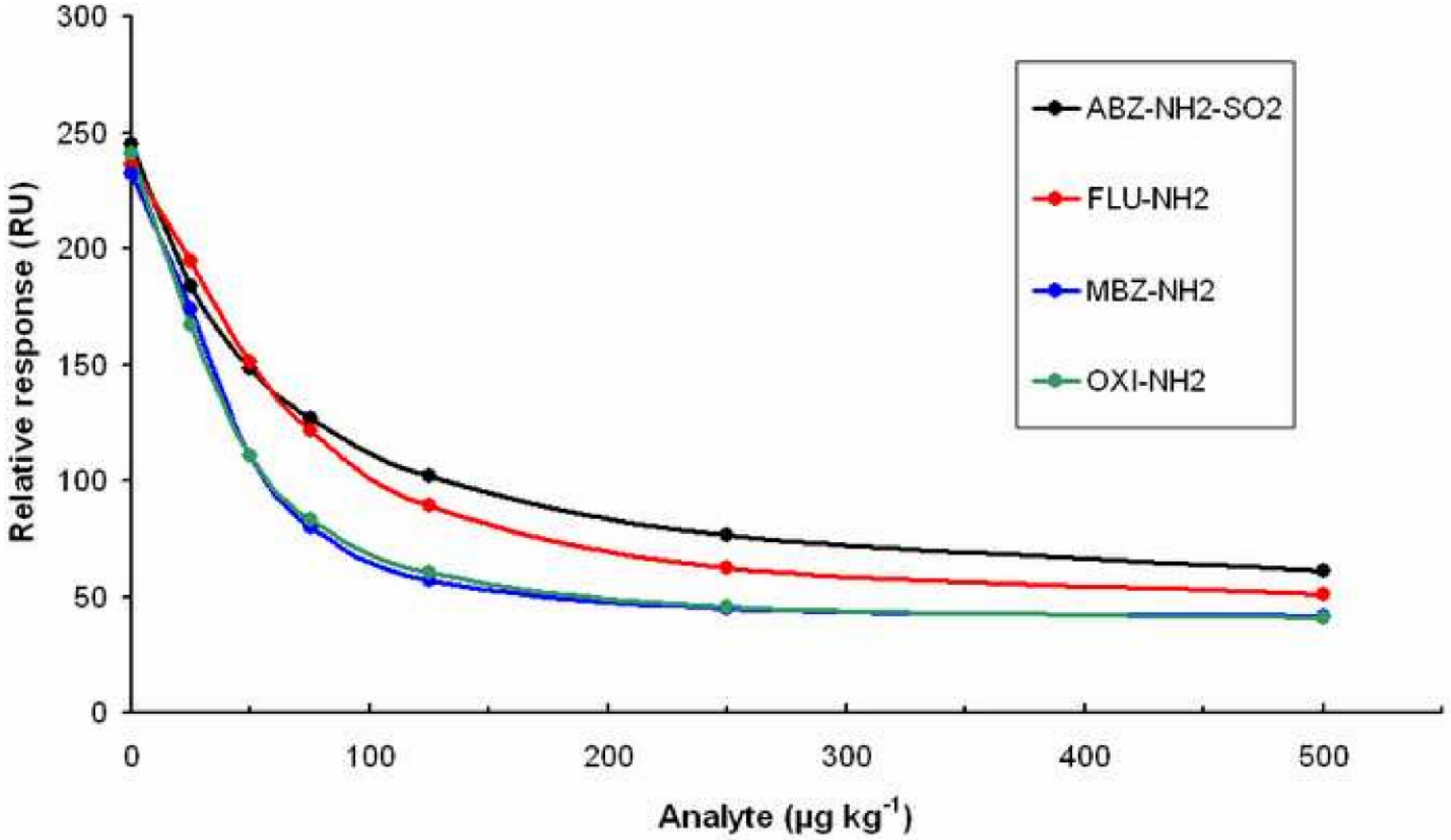

Figure 4B

Click here to download high resolution image

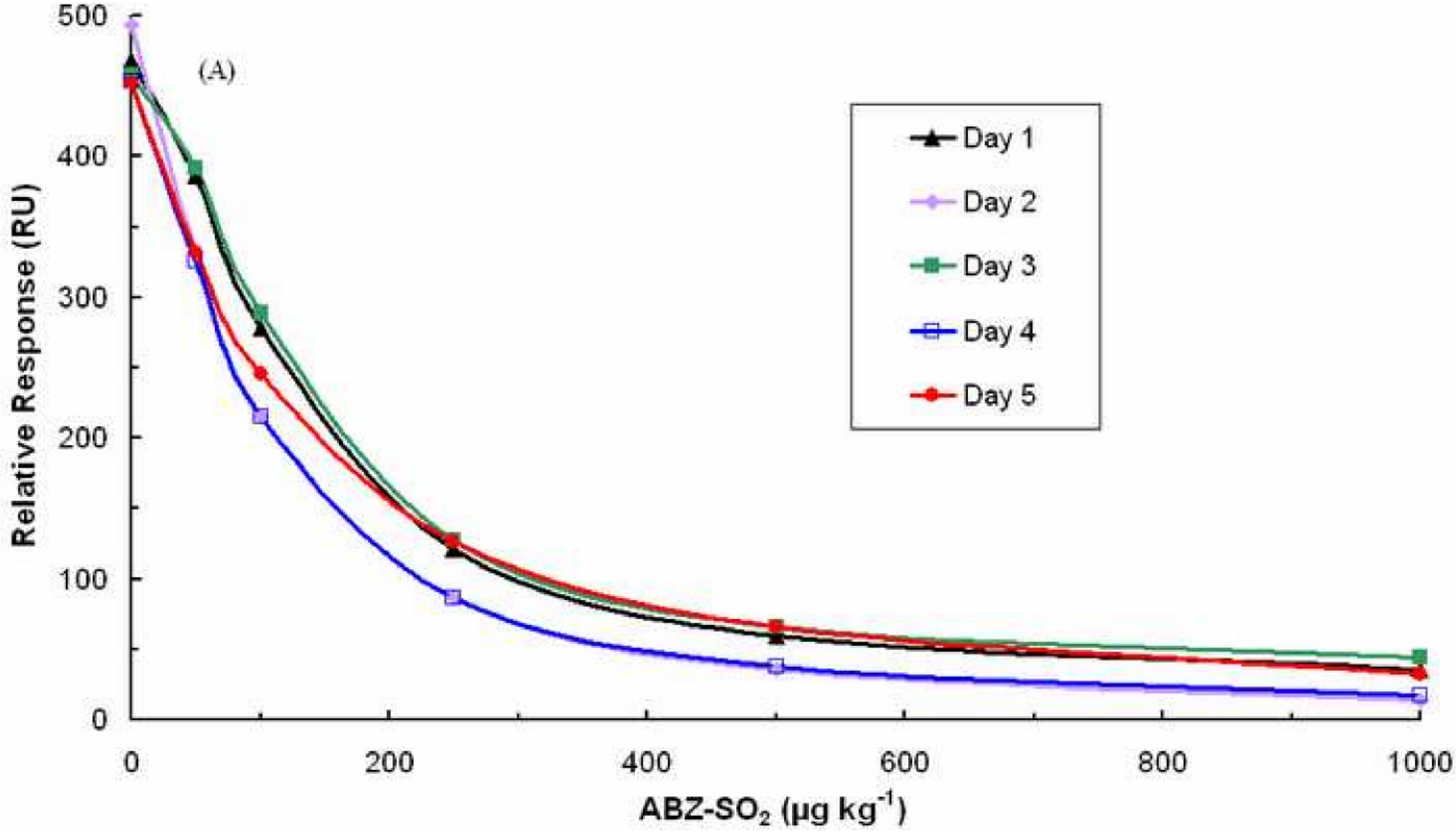

Figure 4B

Click here to download high resolution image