TITLE: 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.

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

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Abstract

A surface plasmon resonance (SPR) biosensor screening assay was developed and validated to detect 11 benzimidazole carbamate (BZT) veterinary drug residues in milk. The polyclonal antibody used was raised in sheep against a methyl 5 (6)-[(carboxypentyl)-thio]-2-benzimidazole carbamate protein conjugate. A sample preparation procedure was developed using a modified QuEChERS method. BZT residues were extracted from milk using liquid extraction/partition with a dispersive solid phase extraction clean-up step. The assay was validated in accordance with the performance criteria described in 2002/657/EC. The limit of detection of the assay was calculated from the analysis of 20 known negative milk samples to be 2.7 µg kg^{-1}. The detection capability (CCβ) of the assay was determined to be 5 µg kg^{-1} for 11 benzimidazole residues and the mean recovery of analytes was in the range 81 to 116%. A comparison was made between the SPR-biosensor and UPLC-MS/MS analyses of milk samples (n = 26) taken from cows treated different benzimidazole products, demonstrating the SPR-biosensor assay to be fit for purpose.

Keywords: SPR Biosensors; benzimidazole carbamates; bovine milk; QuEChERS

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 [1] and weight gain [2, 3]. In the EU, 11 benzimidazoles and pro-benzimidazoles are approved for treatment of food-producing animals giving rise to 20 potential residues. These include the benzimidazole carbamates, albendazole (ABZ), albendazole sulphoxide (ABZ-SO), oxfendazole (FBZ-SO), fenbendazole (FBZ), mebendazole (MBZ), oxibendazole (OXI), flubendazole (FLU), thiabendazole (TBZ) (a thiazole benzimidazole), triclabendazole (TCB) and the pro-benzimidazoles (febantel and netobimin). 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 2377/90/EC [4]. The main concerns over the presence of benzimidazole residues in milk are related to their teratogenic and embryotoxic properties [5, 6]. 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 [7, 8].

Danaher et al. reviewed the analysis of benzimidazole residues in food, highlighting the analytical challenges caused by their extensive metabolism in food producing animals [9]. Recently a number of groups have reported methods for isolating multiple veterinary residues from food using QuEChERS, the so called Quick, Easy, Cheap, Effective, Rugged and Safe method widely used in pesticide residue analysis [10-12]. 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 [12]. HPLC coupled to UV and/or fluorescence detection is the most widely used technique to measure benzimidazole residues in milk [13, 14] However HPLC based methods often require

more intensive sample preparation, particularly when monitoring for low levels of benzimidazole residues [15, 16]. More recently, groups have developed LC-MS/MS methods to detect residues in food that require less complicated clean-up steps [17, 18]. Immunoassay-based detection systems have been developed by other groups as an alternative to chemical assays with improvements in sensitivity, selectivity and require simpler sample preparation in comparison to chemical based assays [19-23]. The SPR-biosensor was first presented as an alternative immunochemical detection system in veterinary drug residue analysis in 1995 [24]. More recently, several SPR-biosensor assays have been developed to detect veterinary drug residues in milk [25-28]. SPR-biosensor assays employ label-free detection and have proven to be versatile, robust and capable of producing rapid and reliable results with minimum sample preparation [25]. Johnsson et al. developed an SPR biosensor assay method capable of detecting benzimidazole residues in bovine serum samples using a simple extraction [29] but no SPR-biosensor method for detecting these substances in food matrices has yet to be 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 combined with a modified QuEChERS method for extraction. The polyclonal antibody does not cross-react to residues of triclabendazole or thiabendazole drugs. The suitability of the assay was demonstrated through its application to samples taken at different withdrawal periods from cows treated with different benzimidazole products and comparing results with UPLC-MS/MS. The method was validated according to the 2002/657/EC guidelines as required for EU monitoring programs [30]. The factors investigated included recovery, repeatability and analytical limits, including the limit of detection and detection capabilities (CCβ) of the method.

2. Materials and methods

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% P20 (v/v) 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 (FBZSO_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 $_{C18}$ were purchased from Biotage (Uppsala, Sweden). Whatman syringe Filter units (polytetrafluoroethylene (PTFE), 0.2 µm) were purchased from Fisher scientific (Dublin, Ireland).

2.2 Milk samples

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.

2.2.2 Incurred milk samples

Milk from cows treated with ABZ and FBZ products

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.

Milk from cows treated with febantel product

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

2.3 SPR-Biosensor assay

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, -5°C) and the supernatant was transferred to a tube containing $_{C18}$ sorbent (500 mg) and $MgSO_4$ (1.5 g). The tubes were subsequently shaken (1 min) and centrifuged (3500*g*, 10 min, -5°C). The ACN layer (7.5 mL) was transferred to Pyrex® tubes and evaporated to dryness at 50°C, 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.

2.3.2 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). 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) [19] 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 desiccated container (4°C).

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. Studies were conducted at 25ºC. The polyclonal antibody (raised in sheep against a methyl 5(6)-[carboxypentyl)-thio]-2-benzimidazolecarbamate derivative (CMB)) [29] 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 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).

2.4 UPLC-MS/MS assay

2.4.1 UPLC-MS/MS *Sample preparation*

Samples were analysed by the method developed by De Ruyck et al [31]. 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 5 0°C. Samples were filtered through 0.2 µm PTFE filters and 5 µL was injected onto the UPLC-MS/MS system.

2.4.2 UPLC-MS/MS 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% 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.

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 2.**

2.5 Calibration

2.5.1 Biosensor 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 test samples was read directly from the calibration curve.

2.5.2 UPLC-MS/MS calibration

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.

3. Results and discussion

3.1 Antibody inhibition studies

The antibody cross-reactivity was investigated by analysing standards prepared in buffer by SPR-biosensor assay. The antibody was shown to have 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). The antibody did not show affinity to TCB, keto-TCB, TCB-SO, TCB-SO_2, TBZ, 5-OH-TBZ, MBZ-NH_2, FLU-NH_2 or ABZ-NH_2-SO_2 residues at a concentration of 100 μg kg^{-1}. 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} (equivalent to 0 to 50 µg kg^{-1}) for the 11 different analytes. The 11 benzimidazole residues studied showed significant cross-reactivity with IC50 values of typically <6.6 ng mL^{-1} (**Table 3).** A second study was carried out using the modified QuEChERS extraction in milk calibration curves over the range 0 to 50 µg kg^{-1}. IC50 values in matrix typically ranged from 11 to 18 µg kg^{-1} for OFZ to FBZ, respectively (**Table 3**). Extracted milk calibration curves for the 11 analytes are shown in **Fig. 1**.

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 was also investigated but initially gave low recovery [14]. 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 or, more likely, tight binding of residues by milk proteins. A further QuEChERS experiment was designed 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. 2**). The percentage recovery for all 11 benzimidazole residues was found to be acceptable (≥69%) using DMSO:water (50:50, v/v, 5 mL). In order to allow the detection of benzimidazoles to 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 for this antibody by Johnnson et al [29].

3.3 Method validation

A qualitative approach was used to determine the performance factor CCβ (detection capability) as described in 2002/657/EC. 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 assay under study could routinely measure this concentration level. In routine application, 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). 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 to be determined to be 5 μg kg^{-1} (5% probability of false negative result). If 20 or ≤1 8 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 validations for each analyte are shown in **Table 4**. 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.

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 5**). Results showed that recovery (81-116%) and inter-assay coefficients of variation (typically <30%). Calibration curves for each day are presented in **Fig. 3**. A calibration curve prepared in HBS-EP buffer is also presented in **Fig. 3**, which demonstrates the low rate of non-specific binding and high extraction efficiency of the method.

3.4 Application of assay to incurred milk samples

The suitability of the SPR-biosensor assay was evaluated by analysing incurred milk samples and comparing 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 from prior to treatment to 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 **(Table 6)** with residues below the MRL at 63 h post-treatment at a level of 7.5 µg kg^{-1}. A typical UPLC-MS/MS trace from incurred milk containing the three major FBZ residues is shown in **Fig. 4a**. 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 also measures other FBZ metabolites, which have no available FBZ standards. 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 high levels for the first three samples but were non-detectable at 168 h post-treatment. The SPR-biosensor results agreed well with the UPLC-MS/MS results for these 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% (active ingredient ABZ) and milk samples were taken from prior to treatment to 135 h post treatment in accordance with the daily milking routine. A typical UPLC-M S/MS trace from an incurred milk found to contain the four major ABZ residues is shown in **Fig. 4b**. 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 7**). 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}.

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 metabolites. The CCβ for benzimidazole residues was determined to be 5 μg kg^{-1}, which is equivalent to the existing chemical assay. The false negative rate for the assay was ≤5%. Using the methodology presented in this article, it is possible to extract and analyse 20 samples within a single working day. Any suspect positive samples can be confirmed by UPLC-MS/MS analysis.

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

Table 2 MS/MS parameters for benzimidazole analytes and internal standards

Table 3 Cross-reactivity profile of benzimidazole carbamates drugs to polyclonal carboxy-albendazole antibody (S48) in HBS-EP buffer and in bovine milk

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Fig. 4a LC-MS/MS chromatograms of FBZ, FBZ-SO and FBZ-SO_2 for an incurred sample from Panacur SC 10% study (15 h withdrawal)

Fig. 4b 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)

7. Table 1

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

Drug	Marker residue (possible metabolites)	MRL ($\mu g\ kg^{-1}$)	Animal species
ABZ, ABZ-SO Netobimin	Sum of ABZ-SO, ABZ-SO_2, ABZ-SO and ABZ-NH_2-SO_2, expressed as ABZ	100	Bovine and ovine
FBZ Febantel FBZ-SO	Sum of residues that may be extracted as oxidised to FBZ-SO_2	10	Bovine and ovine
TBZ	TBZ and 5-OH-TBZ	[a]None	Caprine
FLU	FLU, FLU-NH_2 and FLU-OH	[a]None	[b]Not permitted
OXI	OXI	[a]None	[b]Not permitted
TCB	Sum of extractable residues that may be oxidised to ketotriclabendazole	[a]None	[b]Not permitted
MBZ	MBZ, MBZ-OH and MBZ-NH_2 and MBZ	[a]None	[b]Not permitted

[a]No MRL has been set for this drug in milk
[b]Drug is not permitted for use in animals producing milk for human consumption

Table 2 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> 198.1	0.050	40	20
ABZ-NH_2-SO_2-D_3	242> 133	0.050	40	28
MBZ-NH_2	238.1 > 105.09	0.025	50	24
	238.1 > 133.05	0.025	50	34
FLU-NH_2	256.06> 123.05	0.010	45	26
	256.06> 95.1	0.010	45	34
ABZ-SO	282.24> 159.06	0.005	27	35
	282.24> 240.1	0.005	27	15
ABZ-SO-D_3	285.28> 243.02	0.005	41	13
ABZ-SO2	298.1 > 159.08	0.005	42	35
	298.1 > 266.2	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 > 158.95	0.005	40	38
MBZ-OH-D_3	301.15> 160.05	0.005	36	32
OFZ	316.1> 159.05	0.020	35	30
	316.1 > 191.09	0.020	35	24
FBZ-SO_2-D_5	321.04> 158.95	0.020	30	32
OXI	249.9 > 175.9	0.010	35	26
	249.9 > 218	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	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 > 264.1	0.010	35	18
MBZ-D_3	299.15 > 105.05	0.005	39	33
FLU	313.80 > 123	0.005	40	35
	313.80 > 282	0.005	40	24
FLU-OH	316.2 > 125.1	0.05	40	33
	316.2 > 160.05	0.05	40	35
FLU-D_3	317.15 > 123	0.005	40	36
FBZ-SO_2	331.9 > 158.9	0.005	35	36
	331.9 > 300	0.005	35	21
FBZ-SO_2-D_5	337.06> 305.0	0.005	45	23
TCB-NH2	328 > 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

7. Table 3

Table 3 Cross-reactivity profile of benzimidazole carbamates drugs to polyclonal carboxy-albendazole antibody (S48) in HBS-EP buffer and in bovine milk

	Buffer		Milk	
Analyte	[a]IC50 (ng mL^{-1})	[b]CR50 (%)	[c]IC50 (µg kg^{-1})	[d]CR50 (%)
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

[a] The analyte concentration of inhibitor (analyte) required to reduce the response to 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 to 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

7. Table 4

Table 4 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 ± s (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

7. Table 5

Table 5 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 (%) ± S (n=5)	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

7. Table 6

Table 6 Comparison between biosensor and UPLC-MS/MS analysis of milk samples from cows treated with FBZ and febantel.

Sample	Withdrawal time (h)	MRL (jig kg^{-1})	Biosensor assay		UPLC-MS/MS	
			Concentration (jig kg^{-1})	Interpretation (LOD = 2.7 jig kg^{-1})	[1]Concentration (jig 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 7 Comparison between biosensor and UPLC-MS/MS analysis of milk samples from a cow treated with albendazole.

Sample	Withdrawal time (h)	MRL (jig kg^{-1})	Biosensor assay Concentration (jig kg^{-1})	Biosensor assay Interpretation (LOD =2.7 jig kg^{-1})	UPLC-MS/MS [1]Concentration (jig kg^{-1})	UPLC-MS/MS [2]Status
			Dairy cow treated at 7.5 mg kg^{-1} b.w. (albendazole)			
1	0	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

[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 =

8. Figure 1

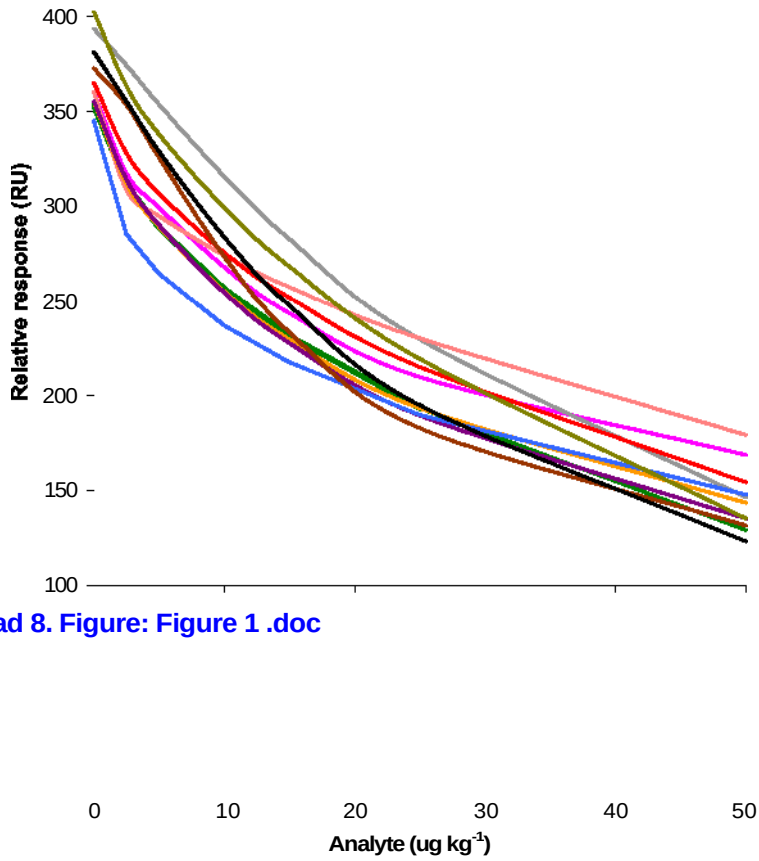

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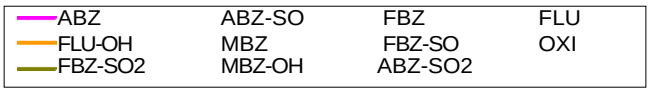
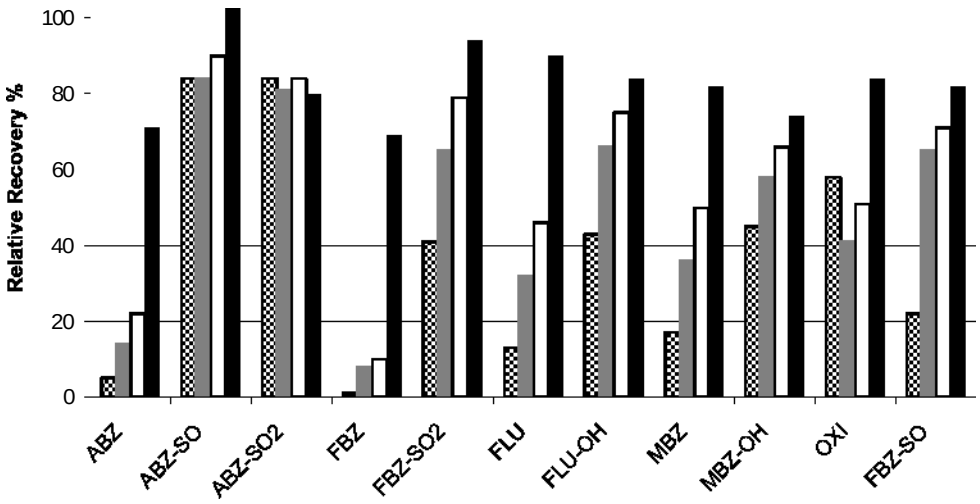
Fig. 1 Standard curves for 11 benzimidazole carbamates in bovine milk matrix



8. Figure 2



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Analyte

Fig. 2 Effect of methanol and dimethylsulphoxide reconstitution on the recovery of benzimidazole residues in milk using a modified QuEChERS extraction method.

8. Figure 3

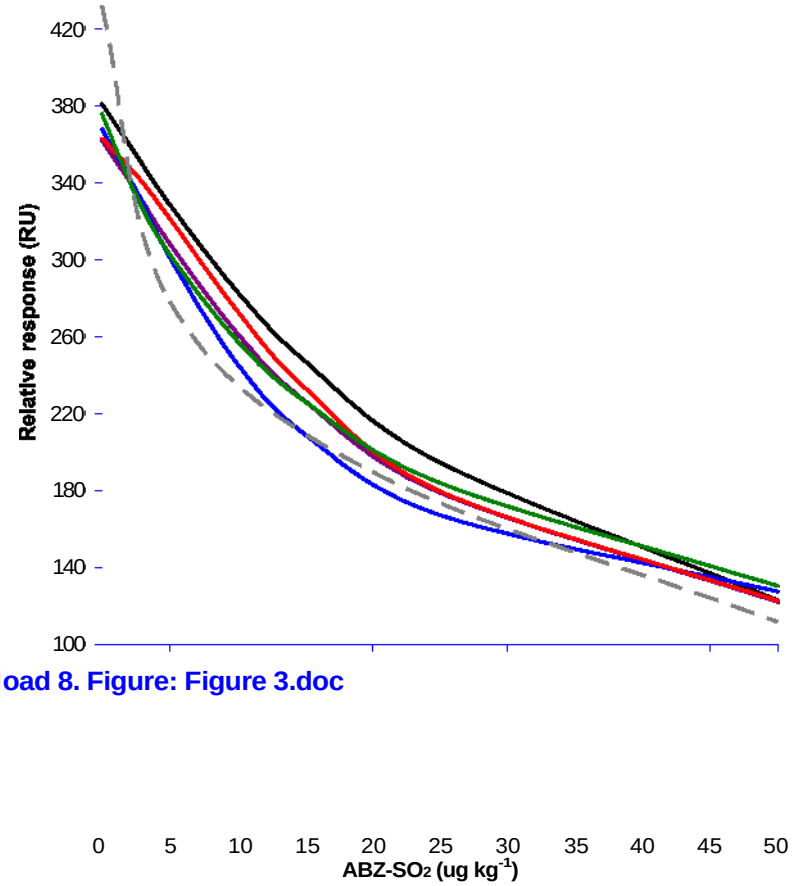

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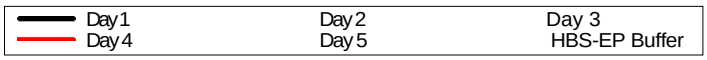
Fig. 3 Albendazole sulphone (ABZ-SO_2) calibration curves in fortified bovine milk on different days (n = 5) and in HBS-EP buffer



8. Figure 4a

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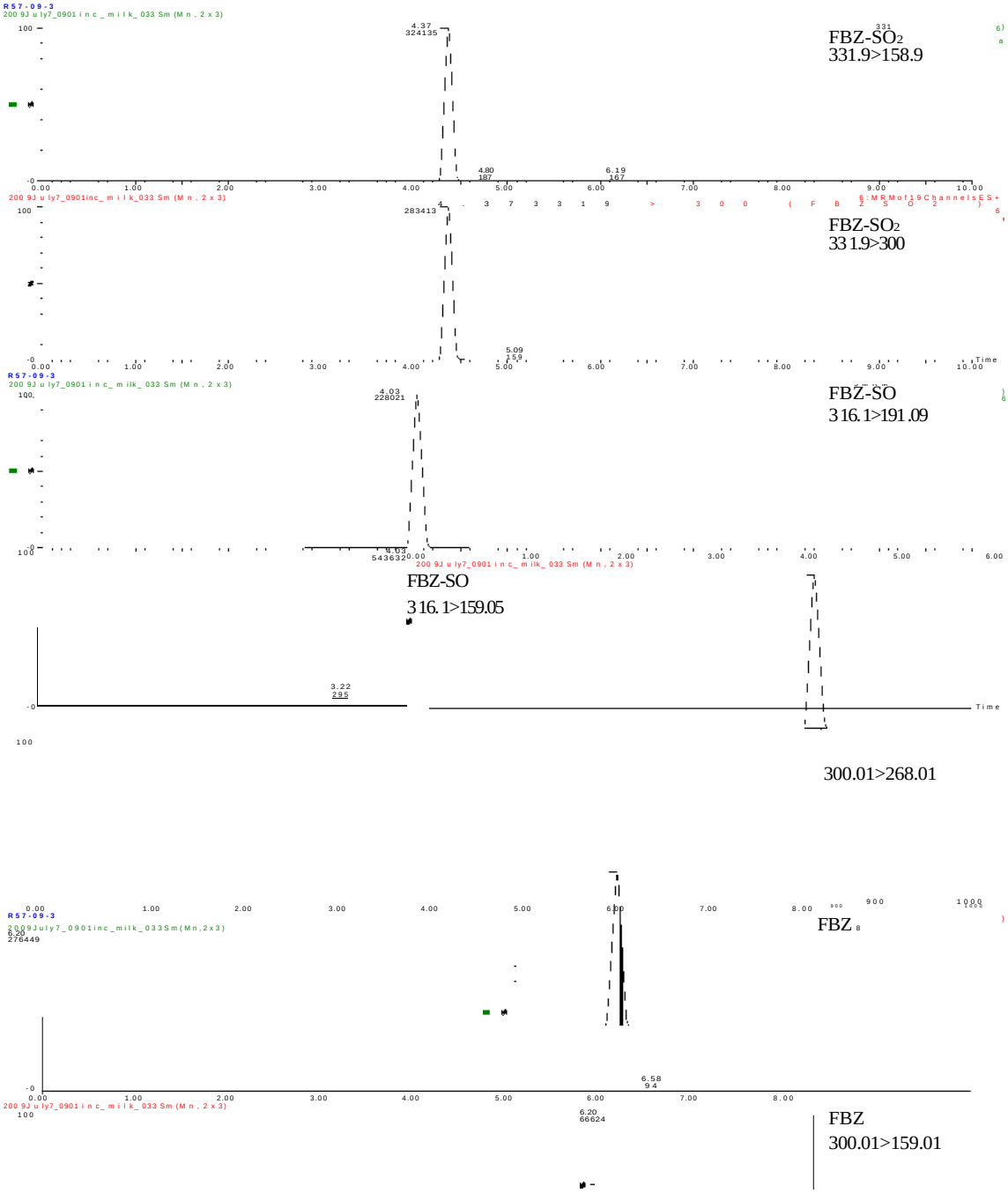

Fig. 4a LC-MS/MS chromatograms of FBZ, FBZ-SO and FBZ-SO_2 for an incurred sample from Panacur SC 10% study (15 h withdrawal)

8. Figure 4b

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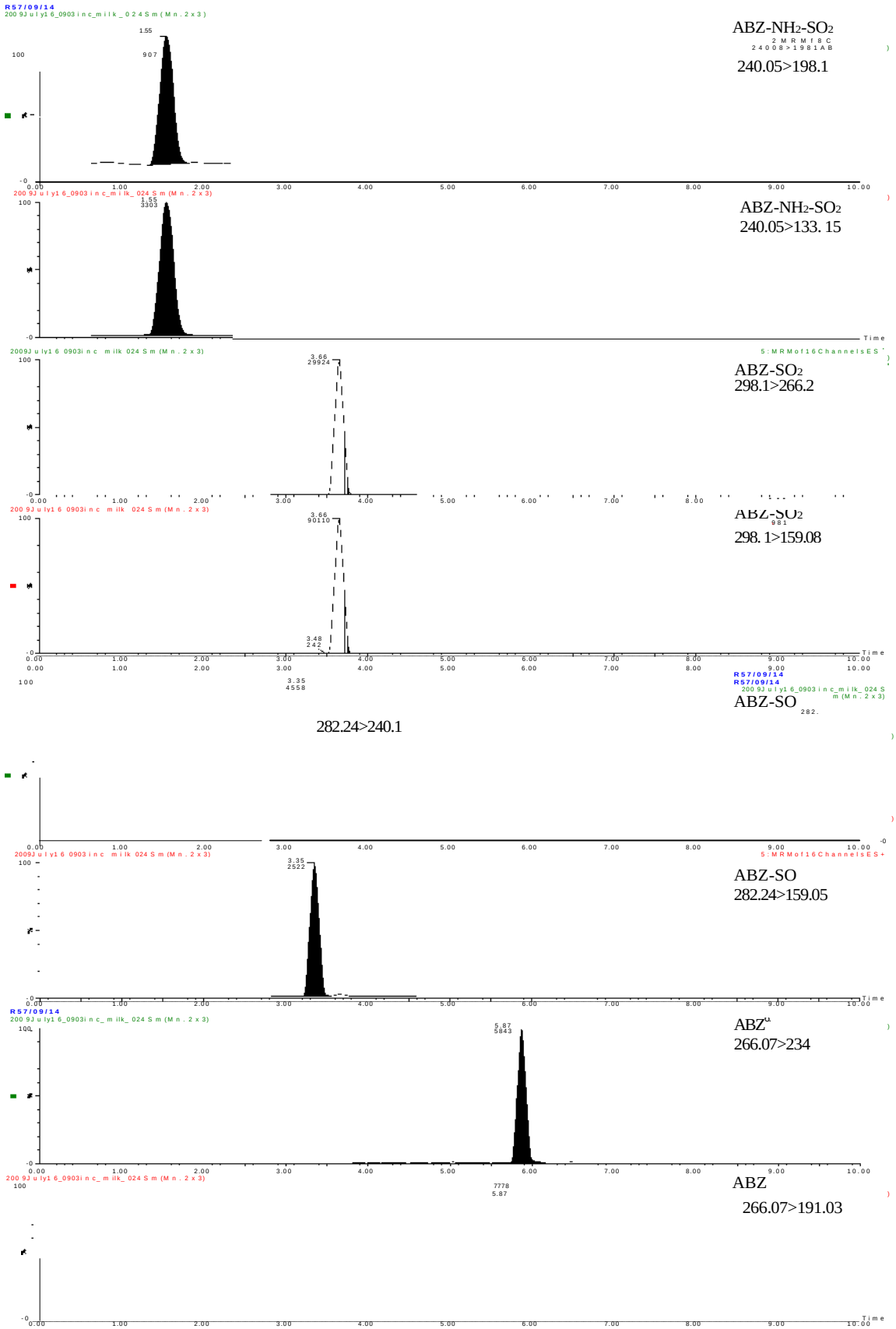

Fig. 4b LC-MS/MS chromatograms of ABZ, ABZ-SO, ABZ-SO_2 and ABZ-NH_2-SO_2 residues detected in milk sample from Endospec 10% study (15 h withdrawal)