TITLE: A dual validation approach to detect anthelmintic residues in bovine liver over an extended concentration range

AUTHORS: Brian Kinsella, Michelle Whelan, Helen Cantwell, Martin McCormack, Ambrose Furey, Steven J. Lehotay, Martin Danaher

A dual validation approach to detect anthelmintic residues in bovine liver over an extended concentration range

Brian Kinsella[a,b], Michelle Whelan[a,b], Helen Cantwell[a], Martin McCormack[a], Ambrose Furey[b], Steven J. Lehotay[c] and Martin Danaher[a,*]

[a]Teagasc, Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland

[b]Team Elucidate, Department of Chemistry, Cork Institute of Technology, Cork, Ireland

[c]US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA, 19038, USA

*Corresponding author

Email: martin.danaher@teagasc.ie

Phone: 353-1-8059552

Fax: 353-1-8059550

Abstract

This paper describes a method for the detection and quantification of 38 residues of the most widely used anthelmintics (including 26 veterinary drugs belonging to the benzimidazole, macrocyclic lactone and flukicide classes) in bovine liver using two different protocols for M RL and non-MRL levels. A dual validation approach was adopted to reliably quantify anthelmintic residues over an extended concentration range (1-3000 µg kg^{-1}). Sample extraction and purification was carried out using a modified QuEChERS method. A concentration step was included when analysing in the low µg kg^{-1} range. Rapid analysis was carried out by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which was capable of detecting residues to <2 µg kg^{-1}. The method has been single-laboratory validated according to the 2002/657/EC guidelines and met acceptability criteria in all but a few cases. The inclusion of 19 internal standards, including 14 isotopically labelled internal standards, improved accuracy, precision, decision limit (CCα) and detection capability (CCβ).

Keywords

Anthelmintics

Ultra-high performance liquid chromatography-tandem mass spectrometry

QuEChERS

Isotopically labelled internal standards

Ion suppression

Matrix effects

1. Introduction

Anthelmintics are widely used to treat parasitic infections in food-producing animals. They include benzimidazoles, macrocyclic lactones and flukicides, the latter of which are rarely tested in food due to the lack of suitable multi-residue methods. Some of the anthelmintics possess toxicological properties such as teratogenicity and embryotoxicity [1], neurotoxicity [2], hyperplasia [3], goitrogenicity [4] and mutagenicity [5]. The European Union, originally through Council Regulation 1990/2377, established Maximum Residue Limits (MRLs) for a number of these drugs in various animal tissues and species to minimise the risk to human health associated with their consumption [6]. Recently, the EU repealed Council Regulation 1990/2377 and replaced it with Commission Regulation 2010/37 [7]. In bovine liver, MRLs for the chosen anthelmintics range from 10 to 1500 μg kg^{-1}. The validation for benzimidazoles is complicated because their MRLs are expressed in the form of sum-MRLs. This includes marker residues for the benzimidazoles licensed in bovine, namely albendazole, fenbendazole, triclabendazole, thiabendazole. As a result, an infinite number of possibilities exist in terms of the number of concentration level combinations. The SANCO document, published in 2004, aims to clarify how sum-MRLs are validated and gives examples of how the sum-CCα and sum-CCβ values are calculated [8]. Therefore, it is necessary to validate a method over a wide analytical range.

Several groups have published LC-MS methods for the analysis of anthelmintics in liver but few methods have been reported in literature for the flukicide sub-class.

Single residue LC-MS methods have been reported for the determination of closantel [9] and nitroxynil [10]. Multi-residue methods have been reported but are specific to different anthelmintic groups such as benzimidazoles [11], macrocyclic lactones [12, 13] and flukicides [14-16]. Previously our group developed a LC-MS/MS method capable of detecting 38 anthelmintic residues in bovine liver and milk [17]. Sample preparation was carried out using a QuEChERS based method which used $_{C18}$ sorbent for the dispersive solid-phase extraction (d-SPE) step. The limit of detection (LOD) was 5 µg kg^{-1} for all analytes except dichlorvos (10 µg kg^{-1}). Two 15 minute injections were necessary to cover the positively and negatively ionised compounds. Recently, our group demonstrated that UHPLC-MS/MS with fast polarity switching enabled the determination of 38 anthelmintics to 1 µg kg^{-1} in milk [18]. This approach is advantageous because it increases sample throughput while reducing solvent usage in the laboratory.

There is continued demand for more sensitive and reliable methods that will detect MRL violations, identify usage patterns of products and provide more quantitative results for exposure and risk assessment. LC-MS/MS is the most effective means of meeting all of these needs. However, co-eluting matrix components, which often go unseen in LC-MS/MS traces can impact on ionisation efficiency and thus accuracy of methods. This has been highlighted by groups who have reported the need for careful characterisation of matrix effects [19-2 1]. A number of groups have reported on the systematic reduction of matrix effects through the introduction of selective sample preparation [22, 23] or improved chromatographic separation [24]. A drawback of these approaches in multi-residue methods is that they typically increase the time needed to prepare and analyse samples. In addition, more selective sample preparation can result in the loss of certain analytes. Alternatively, the inclusion of isotopically labelled internal standards or structural analogues of analytes can correct for matrix effects [25]. In the area of anthelmintic analysis this has been supported by the recent increase in availability of labelled internal standards. Several internal standards are now available for levamisole and benzimidazole residues.

Unfortunately, there are few isotopically labelled internal standards for other anthelmintic analytes, such as the macrocyclic lactones and flukicides. Such analytes are best covered using structural analogues such as selamectin, ioxynil, salicylanilide and 4-nitro-3 -(trifluoromethyl)phenol until isotopically labelled standards are produced.

In this paper, a sensitive UHPLC-MS/MS method was developed capable of detecting 38 anthelmintic residues to 1 µg kg^{-1} in a 13 min run time. The method uses 14 deuterated and 5 non-deuterated internal standards to improve the precision of the method compared to a previously developed LC-MS/MS method that used two internal standards. A dual validation approach is described to cover both MRL and unapproved/low level substances (2-4 µg kg^{-1}). The sensitive method significantly enhances the detection of anthel mintic drug residues in liver, which is useful for identifying unapproved usage of veterinary medicinal products.

2. Experimental

2. 1 Reagents and apparatus

MS grade ammonium formate, GC grade dimethyl sul phoxide (DMSO) and H PLC grade acetonitrile (MeCN), methanol (MeOH) and 99.5% deuterated MeOH (MeOH-D) were sourced from Sigma-Aldrich (Dublin, Ireland). Analar grade isopropyl alcohol (IPA) and glacial acetic acid (HOAc) were obtained from BDH Chemicals Ltd. (Poole, UK). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore water purification system (Cork, Ireland). Pre-weighed mixtures of 4 g anhydrous (anh.) magnesium sulphate ($MgSO_4$) and 1 g sodium chloride (NaCl) in 50 mL centrifuge tubes; 1.5 g anh. $MgSO_4$ and 0.5 g $_{C18}$ in 50 mL centrifuge tubes; and 2 mL mini-centrifuge tube containing anh. $MgSO_4$ (150 mg) and $_{C18}$ (50 mg) were obtained from UCT, Inc. (Bristol, PA, USA). A Dispensette® lll solvent dispenser (Brand GMBH + Co KG; Wertheim Germany) was used for aliquoting MeCN; an Ultra-Turrax stalk homogenizer (IKA Werke GmbH & Co. KG; Staufen, Germany), Mistral 3000i centrifuge (MSE; London, UK), Eppendorf 5471 R bench top centrifuge (Hamburg, Germany), TopMix multi-vortexer (Fisher Scientific; Dublin, Ireland), Turbovap LV evaporator (Caliper Life Sciences; Runcorn, UK) were used during sample preparation. A Elma Transsonic T780/H ultrasonic bath (Bedford, UK) was used to degas mobile phase and solvent wash solutions. Whatman Rezist® PTFE syringe filters (0.2 µm 13 mm) were obtained from Whatman (Ireland).

2.2 Analytical standards

Abamectin (ABA), albendazole (ABZ), bithionol (BITH), clorsulon (CLOR), closantel (CLOS), coumaphos (COUM), doramectin (DORA), emamectin (EMA), fenbendazole (FBZ), haloxon (HAL), ivermectin (IVER), levamisole (LEVA), morantel (MOR), niclosamide (NICL), nitroxynil (NITR), oxfendazole (OFZ), oxyclozanide (OXY), rafoxanide (RAF), and thiabendazole (TBZ) were purchased from Sigma-Aldrich (Dublin, Ireland). Albendazole-2-amino-sulphone (ABZ-NH_2-SO_2), albendazole-sulphone (ABZ-SO_2), albendazole-sulphoxide (ABZ-SO), 5-hydroxy-thiabendazole (5-OH-TBZ), fenbendazole-sulphone (FBZ-SO_2), triclabendazole (TCB), triclabendazole-sulphone (TCB-SO_2) and triclabendazole-sulphoxide (TCB-SO) were purchased from Witega Laboratories Berlin-Aldershof GmbH (Berlin, Germany). Coumaphos-oxon (COUM-O) was purchased from Greyhound Chromatography and Allied Chemicals, (Merseyside, UK). Cambendazole (CAM) and oxibendazole (OXI) were purchased from QMX Laboratories (Essex, UK). Amino-flubendazole (FLU-NH_2), amino-mebendazole (MBZ-NH_2), hydroxy-flubendazole (FLU-OH), hydroxy-mebendazole (MBZ-OH), flubendazole (FLU) and mebendazole (MBZ) were donated by Janssen Animal Health (Beerse, Belgium). Eprinomectin (EPRI) was donated by Merial Animal Health (Lyon, France). Moxidectin (MOXI) was donated by Fort Dodge Animal Health (Princeton, NJ, USA). Primary stock standard solutions were prepared at a concentration of 2000 µg mL^{-1}, while ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, FBZ, OFZ, FBZ-SO_2, EPRI, CLOS, OXY, NITR, CLOR, BITH and MOR were prepared at a concentration of 4,000 µg mL^{-1}. The macrocyclic lactones were prepared in MeCN; the flukicides, CAM, LEVA, TCB, TCB-SO and TCB-SO_2 were prepared in MeOH; and the remaining analytes were prepared in DMSO.

An intermediate working standard mix solution was prepared for the low level experiments at a concentration of 100 µg mL^{-1} for OXY, CLOR, BITH and MOR, and 50 µg mL^{-1} for the remaining 34 analytes, in MeOH. Three intermediate standard mix solutions were required for the high level experiments, as a single standard solution containing all analytes could not be prepared. The concentration of the high standard was 300 µg mL^{-1} for ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CLOS, MOR and EPRI, and 100 µg mL^{-1} for the remaining 34 analytes.

Deuterated forms of benzimidazoles or structurally similar molecules were used as internal standards. Albendazole-D_3 (ABZ-D_3), albendazole-sulphone-D_3 (ABZ-SO_2-

D_3), albendazole-sulphoxide-D_3 (ABZ-SO-D_3), albendazole-2-amino-sulphone-D_3 (ABZ-NH_2-SO_2-D_3), flubendazole-D_3 (FLU-D_3), levamisole-D_5 (LEVA-D_5), mebendazole-D_3 (MBZ-D_3), hydroxy-mebendazole-D_3 (MBZ-OH-D_3), oxibendazole-D_7 (OXI-D_7), thiabendazole-D_3 (TBZ-D_3), triclabendazole-D_3 (TCB-D_3) and amino-triclabendazole (TCB-NH_2) were purchased from Witega Laboratories. Fenbendazole-D_5 (FBZ-D_5), fenbendazole-sulphone-D_5 (FBZ-SO_2-D_5) and fenbendazole-sulphoxide-D_5 (FBZ-SO-D_5) were from Quchem Ltd. (Belfast,UK). Additional internal standards included selamectin (SELA), which was donated by Pfizer Animal Health (New York, NY; USA), salicylanilide (SALI), 4-nitro-3-(trifluoromethyl)phenol (TFM), and ioxynil (IOX), which were purchased from Sigma-Aldrich. Internal standards were prepared at a concentration of 1000 µg mL^{-1}, except TCB-NH_2 and SELA which were prepared at a concentration of 2000 µg mL^{-1}. IOX, SA LI, SELA, and TFM were prepared in MeCN; TCB-N H_2 was prepared in MeOH; TCB-D_3, TBZ-D_3 and LEVA-D_5 were prepared in MeOH-D (to prevent deuterium exchange in solution); and the remaining internal standards were prepared in DMSO.

An internal standard mix for the low level method (IS1) was prepared in MeOH-D at a concentration of 20 µg mL^{-1} for SELA and TCB-NH_2, 4 µg mL^{-1} for LEVA-D5, TBZ-D_3 and IOX, and 2 µg mL^{-1} for the remaining analytes. A second internal standard mix for the high level method (IS2) was prepared in MeOH-D at a concentration of 200 µg mL^{-1} for SELA and TCB-NH_2, 40 µg mL^{-1} for LEVA-D_5, TBZ-D_3 and IOX, and 20 µg mL^{-1} for the remaining analytes. Primary, intermediate and working standard solutions are stable for at least six months when stored at −20°C.

2.3 Calibration

Two different protocols were found to be necessary to measure anthelmintic residues in the ranges 1 to 100 µg kg^{-1} (low MRL or non-MRL substances) and 10 to 3000 µg kg^{-1} (MRL substances). The majority of positively and negatively ionised analytes fitted a linear calibration line. Exceptions were OXY, TCB-SO and TCB-SO_2, which had a quadratic line fit.

2.3.1 Low level calibration curve

Six-point extracted matrix calibration curves were prepared at concentrations of 1, 2, 5, 10, 25 and 50 µg kg^{-1} (or double for BITH, CLOR, MOR and OXY) to measure low levels of anthelmintic residues. Extracted matrix calibrants were prepared by fortifying negative liver samples with 100 µL volumes of standard solutions containing OXY, CLOR, BITH and MOR at 0.2, 0.4, 1, 2, 5 and 10 µg mL^{-1}, and the remaining 34 analytes at 0.1, 0.2, 0.5, 1, 2.5 and 5 µg mL^{-1}.

2.3.2 High level calibration curve

Seven-point extracted matrix calibration curves were prepared at higher concentrations of 10, 20, 50, 100, 200, 500 and 1000 µg kg^{-1} (or 30, 60, 150, 300, 600, 1500 and 3000 µg kg^{-1} for ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CLOS, EPRI and MOR). Matrix calibrants were prepared by fortifying negative liver samples with 100 µL volumes of standard solutions containing ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CLOS, MOR and EPRI at 3, 6, 15, 30, 60, 150, 300 µg mL^{-1}, and the remaining MRL substances at 1, 2, 5, 10, 20, 50, 100 µg mL^{-1}.

2.4 Quality control samples

Bovine liver samples found to contain no response at the retention time of the analytes were used as negative controls. Quality control (QC) samples (recovery controls) were prepared for the low level method by spiking extracts in duplicate with 50 µL of 0.2 µg mL^{-1} (0.4 µg mL^{-1} OXY, CLOR, BITH and MOR) and 2.5 µg mL^{-1} (5 for OXY, CLOR, BITH and MOR µg mL^{-1}) standards after extraction, and were used to monitor extraction efficiency. QC samples were fortified with 25 µL of the working internal standard (IS1) solution prior to extraction. In the high level method, QC samples were prepared by spiking extracts in duplicate with 5 µL of 2 µg mL^{-1} (6 µg mL^{-1} for ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CLOS, MOR and EPRI) and 20 µg mL^{-1} (60 µg mL^{-1} ABZ, ABZ-SO, ABZ-SO_2, ABZ-NH_2-SO_2, CLOS, MOR and EPRI). QC samples were fortified with 25 µL of the working internal standard (IS2) solution prior to extraction.

2.5 Sample preparation

Liver samples (10 g) were weighed into 50 mL polypropylene centrifuge tubes (Sarstedt, Wexford, Ireland). Samples were fortified with the internal standard solution and let stand for 15 min. Extraction was performed by homogenizing

samples using a stalk homogenizer in the presence of MeCN (10 mL). Phase separation was induced by adding the contents of a tube containing anh. $MgSO_4$ (4 g) and NaCl (1 g) to the sample tube. The remaining salts were transferred using a MeCN (2 mL) wash. Samples were immediately shaken for 1 min (to prevent agglomerates forming during $MgSO_4$ hydration) and centrifuged (2842 x *g*, 12 min).

Two different d-SPE protocols were used to purify extracts depending on the concentration level. In the low level method (2 µg kg^{-1}), the entire supernatant was poured into a centrifuge tube (50 mL) containing anh. $MgSO_4$ (1.5 g) and $_{C18}$ (0.5 g). The sample was vortexed for 1 min and centrifuged (2842 x *g*, 10 min). The purified supernatant (6 mL) was added to a 15 mL polypropylene evaporation tube (Sarstedt) containing DMSO (0.25 mL). MeCN was evaporated under nitrogen at 50°C using a Turbovap.

For MRL concentrations, 1 mL of supernatant was transferred from the QuEChERS extraction tube into a 2 mL mini-centrifuge tube containing anh. $MgSO_4$ (150 mg) and $_{C18}$ (50 mg). The sample extract was vortexed (1 min) and centrifuged (21913 x *g*, 2 min). An aliquot of the purified supernatant (600 µL) was transferred to a 5 mL glass culture tube containing DMSO (600 µL) and the MeCN was evaporated under nitrogen at 50°C using the Turbovap apparatus. In both cases, extracts were vortexed (1 min) and filtered through a 0.2 µm PTFE 13 mm syringe filter (Whatman Rezist®) into an autosampler vial.

2.6 LC-MS/MS analysis

Separation was conducted on a Waters Acquity UHPLC system (Milford, MA, USA). The analytical column was an Acquity HSS T3 $_{C18}$ (100 × 2.1 mm, 1.8 µm particle size) protected by a 0.2 µm stainless steel in-line filter and maintained at 60°C. Gradient separation was performed using a binary gradient composed of mobile phase A, 0.01% HOAc:MeCN (90:10, v/v) and mobile phase B, 5 mM ammonium formate in MeOH:MeCN (75:25, v/v). The gradient profile was as follows: (a) 0 → 0.5 min, 100% A; (b) 5 min, 50% A; (c) 7 → 8.5 min, 10% A; (d) 8.51 → 10 min, 0% A; (e) 10.01 → 13 min, 100% A. UHPLC weak and strong autosampler needle washes consisted of H_2O:MeOH (80:20, v/v) and MeOH:IPA:H_2O (80:10:10, v/v), respectively. The injection volume was 5 µL. Analytes were detected using a Waters Quattro Premier XE triple quadrupole instrument equipped with an electrospray

ionisation interface (Milford, MA, USA). Nitrogen was used for nebulisation, desolvation (1000 L hr^{-1}) and cone gas (50 L hr^{-1}). Argon was used as collision gas (0.013 L hr^{-1}). The source temperature was set at 150°C and desolvation temperature at 450°C. The ion spray voltage was set at 3000 eV for positive mode and 500 eV for negative mode. The MS conditions were optimised by tuning the analyte-specific parameters, including cone voltage, collision energy and collision cell exit potential for each analyte. This optimisation was carried out by infusion of a 1 µg mL^{-1} standard solution of each analyte and monitoring the two most abundant fragment ions produced from the molecular ion. The SRM windows were time-sectored, and dwell time, inter-scan delay and inter-channel delays were set to get maximum response from the instrument. A summary of the retention times, monitored ions and optimised MS parameters obtained for each analyte is reported in Table 1. The U H PLC-MS/MS system was controlled by MassLynxTM software and data was processed using TargetLynxTM Software (both from Waters).

2.7 Method Validation

Method validation was carried out according to European Commission Decision 2002/657/EC criteria [26]. Parameters investigated included specificity, linearity, recovery, within-laboratory repeatability (WLr) and reproducibility (WLR), Decision Limit (CCα) and Detection Capability (CCβ). LC-MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times, ion recognition (signal-to-noise ratio) and relative ion intensities.

Low level validation was carried out using samples fortified at 1, 1.5 and 2 times the second lowest calibration level, which was 2 µg kg^{-1} for all analytes except OXY, CLOR, BITH and MOR (4 µg kg^{-1}). A second validation study was carried out at 0.5, 1 and 1.5 times the MRL for analytes which had a MRL. Several drugs had low concentration MRLs (NITR, 20 µg kg^{-1}; RAF, 10 µg kg^{-1}; ABA, 20 µg kg^{-1}) and validation was carried out according to the low concentration method (1-50 µg kg^{-1}).

Within-laboratory repeatability (WLr) and reproducibility (WLR) were carried out by fortifying samples with six replicates at each concentration and this was repeated on three separate days by a single analyst (WLr) or by three separate analysts (WLR). Coefficients of variation (CV) were calculated according to the Horwitz Equation. However, concentrations below 100 µg kg^{-1} give unacceptably high values using the Horwitz Equation and according to Commission Decision 2002/657/EC, at

these concentrations the analyst should aim to achieve the lowest possible CV or <23 %.

CCα is the limit from which it can be decided that a sample is truly violative with an error probability of α. In the case of a banned or unauthorised substance the CCα is the lowest concentration level at which a method can discriminate with a statistical certainty of 1-α whether the identified analyte is present. In the case of substances with an established MRL, the CCα is the concentration above which it can be decided with a statistical certainty of 1-α that the identified analyte content is truly above the MRL. CCβ is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of β. The β-error should be less than or equal to 5%. In the case of banned or unauthorised substances, CCβ is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β. In the case of substances with an established MRL, CCβ is the concentration at which the method is able to detect MRL concentrations with a statistical certainty of 1-β.

CCα and CCβ values for unapproved use level were calculated using the intercept (value of the signal, y, where the concentration, x, is equal to zero) and the standard error of the intercept (SEI) for a set of data with 6 replicates at 3 levels (1, 1.5 and 2 x unapproved use level). CCα is the concentration corresponding to the intercept + 2.33 x the SEI, and CCβ is the concentration corresponding to the signal at CCa + 1.64 x the SEI. CCα and CCβ for MRL level were calculated using the calibration procedure for marker residues according to ISO 11843 for a set of data with 6 replicates at 3 levels (0.5, 1.0 and 1.5 MRL). CCα is the concentration calculated from the response at the MRL + 1.64 x the WLR standard deviation (SD), and CCβ is the concentration calculated from the response at CC + 1.64 times the WLR SD.

3. Results and discussion

3.1 Method development

A QuEChERS based method was previously developed by this group which is capable of measuring anthelmintic residues to 5 μg kg^{-1} in liver using LC-MS/MS

technology [17]. Recently, methods have been reported in literature for anthelmintics in milk using UHPLC coupled to single stage [27] and tandem MS [18] analysers. These applications highlight the advantages of UHPLC over LC-based methods in terms of resolution, sensitivity, sample throughput and reduced instrument downtime due to cleaning of the source. In addition, Whelan et al. reported on the advantage of more modern MS instruments with rapid polarity switching capabilities, which allowed the analysis of negatively and positively charged ions in a single injection [18]. A review of published methods has highlighted the scarcity of LC-MS/MS methods for flukicides, particularly in animal tissue [14, 17]. It is proposed that this is likely due to the requirement to monitor negative ions. Jedziniak et al. recently reported difficulties in the analysis of the negative ions of TCB-SO and TCB-SO_2 residues in milk [27]. Our previous method required two injections to cover the positively and negatively ionised compounds, and each injection had a run time of 15 min. Other research groups have reported single class analysis of anthelmintics, including the macrocyclic lactones ($n \leq 6$, <30 min) [12, 13, 28-30], the benzimidazoles ($n \leq 20$, <25 min) [27, 31-33], and the flukicides ($n \leq 5$, <30 min) [14, 16].

Under Council Directive 96/23/EC, it is a requirement for each member state to monitor for the presence of veterinary drug residues in food [34]. Several anthelmintics are licensed for use in cattle and therefore have MRLs. However, it is also important to monitor for drugs for which no MRLs have been established. This includes drugs approved for use in other species, drugs for which MRLs have been established in different matrices (milk, muscle, plasma, kidney, fat) or off-label usage. Therefore, it is vital that an analytical method is capable of not only detecting residues at the M RL level but also at the low µg kg^{-1} region. The objective of this research was to develop a method to detect anthelmintic residues in liver tissue in the range 1 to 3000 µg kg^{-1}. Such a method allows the identification of non-compliant residues caused by the use of unapproved products or failure to adhere to withdrawal periods in approved species.

The development of a single protocol of the method over a broad calibration range proved to be challenging because of the non-linear behaviour of the calibration curves for the negatively ionised compounds (flukicides). Non-linearity problem was observed with and without the inclusion of internal standards. This problem was not

observed for the 29 positively ionised analytes. Experiments were designed to identify the reason for the non-linear behaviour. The potential competition for charge between analytes was investigated by individually injecting calibration curves of the two worst performing analytes (CLOR and TCB-SO_2) but this showed no improvement. A range of other factors were also investigated, including injection volume, desolvation gas flow rate, desolvation temperatures, mobile phase flow rate and detuning the ESI probe position. Reduction of mobile phase flow rate and injection volume improved linearity slightly over the desired range. In an attempt to extend linearity, mobile phase additives used by other groups were evaluated without success, including ammonium acetate [27], ammonium formate [17] and triethylamine [12, 13, 28]. Ultimately, it was decided to use two methods to cover low (1-100 µg kg^{-1}) and high (1 0-3000 µg kg^{-1}) concentration ranges.

In the initial phases of work, samples were extracted and purified using the procedure developed by Kinsella et al. [17]. However, this approach was not suitable for reliably detecting low concentrations of some residues. In order to improve sensitivity for the low level method, the d-SPE step was scaled up by applying the full ≈10 mL of extract to tubes containing $_{C18}$ (0.5 g) + anh. $MgSO_4$ (1.5 g), and subsequent concentration of 6 mL purified extract to a low volume (0.25 mL). The resulting increase in sensitivity (expressed as S/N) is highlighted in Fig. 1, which shows chromatograms for two samples fortified at 10 µg kg^{-1} and prepared according to the low and high level method. An important factor in achieving this improvement was the introduction of DMSO as a keeper solvent to prevent evaporating of samples to dryness. The use of DMSO had several other benefits, including reduction of protein binding, increasing injection volume and sharpening chromatographic peaks [35]. In the high level method, samples were purified with $_{C18}$ (50 mg) + anh. $MgSO_4$ (150 mg). Subsequently, extracts underwent solvent exchange by evaporating the MeCN extract (600 µL) in DMSO (600 µL) to maintain sharp peaks. This dual approach to sample preparation allowed the measurement of residues over a sufficiently wide range for practical application.

To improve the precision of the method by reducing the loss of analyte during sample preparation and reducing matrix effects during MS analysis, 19 internal standards were included in the method. These included 14 deuterated benzimidazoles and 5

non-deuterated compounds. The deuterated benzimidazoles had similar MS conditions and almost identical retention times as their non-deuterated forms. The five non-deuterated internal standards are structural analogues of some of the anthelmintics (Fig. 2). SELA is a macrocyclic lactone used to treat cats and dogs. It is similar in structure to the avermectins (ABA, DORA, EMA, EPRI, IVER) but contains a monosaccharide instead of a disaccharide. It was used as an IS for all the macrocyclic lactones. SALI, a fungicide, is structurally related to several flukicides, including CLOS, NICL, OXY and RAF, for which it was used as an IS. In addition, it was used as an IS for CLOR. TCN-NH_2 was used in both positive and negative ionisation modes. It was used as an IS in ESI^- for TCB-SO and TCB-SO_2, and in ESI^+ for MBZ-NH_2, FLU-NH_2, COUM, COUM-O and HAL. IOX and TFM are herbicides and similar in structure to NITR. IOX was chosen as an IS for NITR as it is structurally more similar and elutes closer to NITR than TFM. TFM was used as the IS for BITH. BITH-SO was also evaluated as an IS but gave unacceptable chromatography and had poor sensitivity. It did not adequately correct for matrix affects and instead the next closest eluting compound (TFM) was chosen as IS for BITH.

3.2 Method validation

3.2.1 Specificity

A specific UHPLC-MS/MS method was developed to separate anthelmintic residues and internal standards. In early experiments, carry-over problems were frequently observed in chromatograms for a number of the most intense substances, namely, CAM, OXI and EMA. Carryover was eliminated through the use of weak (H_2O:MeOH, 80:20 v/v) and strong (MeOH:IPA:H_2O, 80:10:10 v/v) autosampler needle washes. In addition, MeOH was injected after positive controls in routine analytical runs to further ensure no carryover into test samples.

The specificity of the method in terms of potential interferences among analytes was demonstrated by injecting analytes and internal standards individually. After injecting standards and internal standards separately onto the UHPLC-MS/MS, two analyte transitions, ABZ-SO_2 (298.10 > 266.20 *m/z*) and MBZ-OH (298.25 > 266.15 *m/z*), were found to be prone to isobaric interference. However, both peaks were sufficiently separated on the analytical column (3.54 min vs. 4.17 min,

437 respectively), as shown in Fig. 3. MOR was found to contain two peaks, 2.48 and
438 2.87 min (Fig. 4), which correspond to its two stereoisomers. MOR exists as the (*E*)
439 isomer but is known to degrade rapidly under UV light to its (*Z*) isomer [36, 37].
440 Throughout the study, both peaks were integrated and the sum of the areas of both
441 peaks was used to calculate validation parameters. Satisfactory results were achieved
442 for MOR in all validation experiments carried out, as can be seen in Tables 2 and 3.
443
444 *3.2.2 Selectivity*
445 The selectivity of the method was demonstrated by analysing 20 different bovine
446 livers. No interfering matrix peaks were observed in the samples. The selectivity of
447 the method has been since applied to a range of over 1000 liver samples.
448 Selectivity against matrix effects was further demonstrated through a post-
449 column infusion experiment to determine ion suppression/enhancement effects, using
450 the approach described by Bonfiglio et al. [23]. This was achieved by placing a T-
451 junction between the LC system and the MS source. A standard mixture (1000 µg
452 mL^{-1}) was infused, using a 250 µL syringe, at 10 µL min^{-1} into the LC eluent and
453 monitored by MRM. Blank matrix, prepared according to the high and low level
454 methods, was injected (5 µL) via the autosampler into the LC system. The response of
455 the standard mixture was monitored continuously to produce a profile of the matrix
456 effect. Blank matrix samples were then compared to blank DMSO (5 µL) and a 0 µL
457 sample (i.e. only mobile phase). None of the matrix samples analyzed were found to
458 exhibit any major effect on the response, either positive (ion enhancement) or
459 negative (ion suppression), of the anthelmintics that were infused post-column.
460
461 *3.2.3 Within-laboratory repeatability and reproducibility*
462 At MRL level, the majority of analytes gave recovery values within the limits of 80
463 and 110% for WLr and WLR (Table 2). Elevated recovery was observed for TCB-
464 SO, TCB-SO_2, OXY and MOXI in both validation parameters. Additionally, EPRI
465 had elevated recovery for WLR. Precision for all analytes with the exception of
466 CLOR was less than the required limits in both parameters. At low level WLr, the
467 majority of analytes gave satisfactory recovery ranging between 90 and 110% (Table
468 3). Four analytes had elevated recovery, namely FLU, OXI, 5-OH-TBZ and HAL.
469 CVs obtained for the WLr were satisfactory for most analytes. CVs were >23% for
470 BITH, COUM-O, HAL and TCB-SO. At low level WLR, elevated recovery was

obtained for 5-OH-TBZ, BITH, NICL and HAL. CVs were typically <23% except for FLU, OXI, TCB-SO, TCB-SO_2, COUM-O and HAL. The elevated recovery and poor reproducibility obtained for TCB-SO and TCB-SO_2 was attributed to the non-linear behaviour of the calibration curves.

3.2.4 Decision Limit (CCα) and Detection Capability (CCβ)

CCα and CCβ values for MRL and unapproved level are listed in Table 2 and Table 3, respectively. The CCα values ranged from 13-1593 and 0.21-1.69 µg kg^{-1} for MRL and unapproved level, respectively. CCβ values ranged from 15-1765 and 0.28-2.88 µg kg^{-1} for MRL and unapproved level, respectively. The results achieved in this study are better than the results obtained in our previous work [17]. For example, in our previous work, the CCα values obtained for the ABZs ranged from 1077-1383 µg kg^{-1} while in our current method they range from 1062-1115 µg kg^{-1}. The FBZs were reduced from 555-568 µg kg^{-1} to 508-534 µg kg^{-1}. CLOS was reduced from 1228 µg kg^{-1} to 1151 µg kg^{-1}, OXY from 623 to 578 µg kg^{-1} and EPRI from 1721 to 1593 µg kg^{-1}. The improvement in CCα and CCβ values were matched by improvements in recovery and CV. The better results obtained in this study were due to the better reproducibility obtained with the 14 deuterated and five non-deuterated internal standards. In our previous method, only two internal standards were used in the method, namely triphenyl phosphate (ESI^+) and 2,4-dichlorophenoxyacetic acid (ESI^-), which are pesticides and not structurally related to the anthelmintics.

3.2.5 Qualitative Criteria

According to 2002/657/EC [26], three identification points are required to satisfy confirmatory criteria for Group B substances. This was achieved through the selection of one precursor ion and two product ions, which resulted in four identification points and exceeded the minimum requirements. The criteria on relative retention times (RRT), signal-to-noise (S/N) and ion ratios (IR) were examined for all samples and standards used for the validation study. The values for RRT, S/N and IR were in agreement with the EU requirements for all the analytes investigated in the study. In terms of RRT, the analyte peaks in samples were found to be within the ±2.5% tolerance when compared with standards. Furthermore, two transition ions were monitored for each of the 38 analytes, although only the most intense ion was used as the quantification ion. All ion ratios of samples were within

505 the required tolerances as required by EU criteria when compared with standards
506 during the validation study. S/N ratios were found to be greater than 10.
507
508

509 Conclusions

510 A method was developed with two different protocols for the determination of
511 anthelmintic drug residues in the M RL and low μg kg^{-1} regions in bovine liver. Dual
512 validation was necessary because of the need to detect trace levels of some drugs due
513 to low M RLs (NITR and RAF) and also the non-linear nature of calibration curves for
514 negative ion compounds. In addition, a number of drugs are not licensed for use in
515 bovine animals, namely, MBZs, FLUs, OXI, EMA, NICL and CAM. This is further
516 complicated by licensed benzimidazole drugs, which have a sum-MRL marker
517 residue. Therefore, it is necessary to validate analytical methods for anthelmintics at
518 low and high levels.
519 The method has been single-laboratory validated according to the
520 2002/657/EC guidelines and met acceptability criteria in all but a few cases. The
521 method was found to be very sensitive and had LODs of $\leq$2 μg kg^{-1}. The method has
522 since been accredited to ISO1 7025 standard and its robustness has been tested through
523 application to some 1000 liver samples. Typically 36 test samples can be extracted
524 and analysed in a single day.
525 In routine analysis, anthelmintic residues are screened using the more sensitive
526 low level method. If positive samples are found at levels greater than the highest
527 calibration point they are re-extracted and confirmed with the high level method. The
528 same extraction procedure is used for both methods. However, they differ in
529 concentration of internal standards, volume carried through to clean-up and volume of
530 clean-up sorbent used.
531
532

533 Acknowledgements

534 This research was funded by the Irish Department of Agriculture and Food under the
535 Food Institutional Research Measure as part of the National Development Plan
536 (Projects 06RDTAAFRC479) and by EU Sixth Framework programme on Food
537 Quality and Safety, ProSafeBeef (Project FOOD-CT-405 2006-36241). Additionally,

538 we acknowledge Merial Animal Health, Janssen Animal Health, Fort Dodge Animal
539 Health and Pfizer Animal Health for donating standards.

540

References

[1] Q.A. McKellar, E.W. Scott, J. Vet. Pharmacol. Ther., 13 (1990) 223.

[2] F.K. Mohammad, G.A.M. Faris, M.S.H. Rhayma, K. Ahmed, Neurotoxicology, 27 (2006) 153.

[3] G.R. Lankas, C.P. Peter, Food Chem. Toxicol., 30 (1992) 297.

[4] J.D.G. McEvoy, J.P. Ferguson, S.R.H. Crooks, D.G. Kennedy, L.A. van Ginkel, G. Maghuin-Rogister, H.H.D. Meyer, M.W. Pfaffl, W.H.H. Farrington, M. Juhel-Gaugain, Analyst, 123 (1998) 2535.

[5] S.G. Vega, P. Guzman, L. Garcia, J. Espinosa, C.C. Denava, Mutat. Res., 204 (1988) 269.

[6] Off. J. Eur. Union, L224 (1990) 1.

[7] Off. J. Eur. Union, L15 (2010) 1.

[8] SANCO/2004/2726-rev 4-December 2008.

[9] H.W. Sun, F.C. Wang, L.F. Ai, J. Chromatogr. A, 1175 (2007) 227.

[10] W.J. Blanchflower, D.G. Kennedy, Analyst, 114 (1989) 1013.

[11] G. Balizs, J Chromatogr. B, 727 (1999) 167.

[12] H. Noppe, K. Verheyden, J. Vanden Bussche, K. Wille, H. De Brabander, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess., 26 (2009) 1232.

[13] L. Howells, M.J. Sauer, Analyst, 126 (2001) 155.

[14] M. Caldow, M. Sharman, M. Kelly, J. Day, S. Hird, J.A. Tarbin, J Chromatogr. A, 1216 (2009) 8200.

[15] K. Takeba, K. Fujinuma, M. Sakamoto, T. Miyazaki, H. Oka, Y. Itoh, H. Nakazawa, J Chromatogr. A, 882 (2000) 99.

[16] K. Takeba, T. Itoh, M. Matsumoto, H. Nakazawa, S. Tanabe, J. AOAC Int., 79 (1996) 848.

[17] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal. Chim. Acta, 637 (2009) 196.

[18] M. Whelan, B. Kinsella, A. Furey, M. Moloney, H. Cantwell, S.J. Lehotay, M. Danaher, J Chromatogr. A, 1217 (2010) 4612.

[19] J.P. Antignac, K. de Wasch, F. Monteau, H. De Brabander, F. Andre, B. Le Bizec, Anal. Chim. Acta, 529 (2005) 129.

[20] D.N. Heller, Rapid Commun. Mass Spectrom., 21 (2007) 644.

574 [21] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, J Chromatogr. A, 1217
575 (2009) 3929.
576 [22] E. Chambers, D.M. Wagrowski-Diehl, Z.L. Lu, J.R. Mazzeo, J Chromatogr.
577 B, 852 (2007) 22.
578 [23] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass
579 Spectrom., 13 (1999) 1175.
580 [24] B. Le Bizec, G. Pinel, J.P. Antignac, J Chromatogr. A, 1216 (2009) 8016.
581 [25] B.K. Matuszewski, J Chromatogr. B, 830 (2006) 293.
582 [26] Off. J. Eur. Union, L221 (2002) 8.
583 [27] P. Jedziniak, T. Szprengier-Juszkiewicz, M. Olejnik, J Chromatogr. A, 1216
584 (2009) 8165.
585 [28] D.A. Durden, J. Wotske, J. AOAC Int., 92 (2009) 580.
586 [29] S.B. Turnipseed, J.E. Roybal, H.S. Rupp, S.A. Gonzales, A.P. Pfenning, J.A.
587 Hurlbut, Rapid Commun. Mass Spectrom., 13 (1999) 493.
588 [30] Z.G. Wu, J.S. Li, L. Zhu, H.P. Luo, X.J. Xu, J Chromatogr. B, 755 (2001)
589 361.
590 [31] H. De Ruyck, E. Daeseleire, H. De Ridder, R. Van Renterghem, J
591 Chromatogr. A, 976 (2002) 181.
592 [32] T.A.M. Msagati, M.M. Nindi, J. Sep. Sci., 24 (2001) 606.
593 [33] T.A.M. Msagati, M.M. Nindi, Talanta, 69 (2006) 243.
594 [34] Off. J. Eur. Union, L125 (1996) 10.
595 [35] A. Kaufmann, P. Butcher, K. Maden, M. Widmer, J. Chromatogr. A, 1194
596 (2008) 66.
597 [36] J.T. Goras, A.R. Gauthier, J. Assoc. Off. Anal. Chem., 68 (1985) 598.
598 [37] Anon, Environmental Impact Analysis Report, Pfizer Inc., 235 East 42nd
599 Street, New York 10017 (1982) 149.
600
601

Figure 1

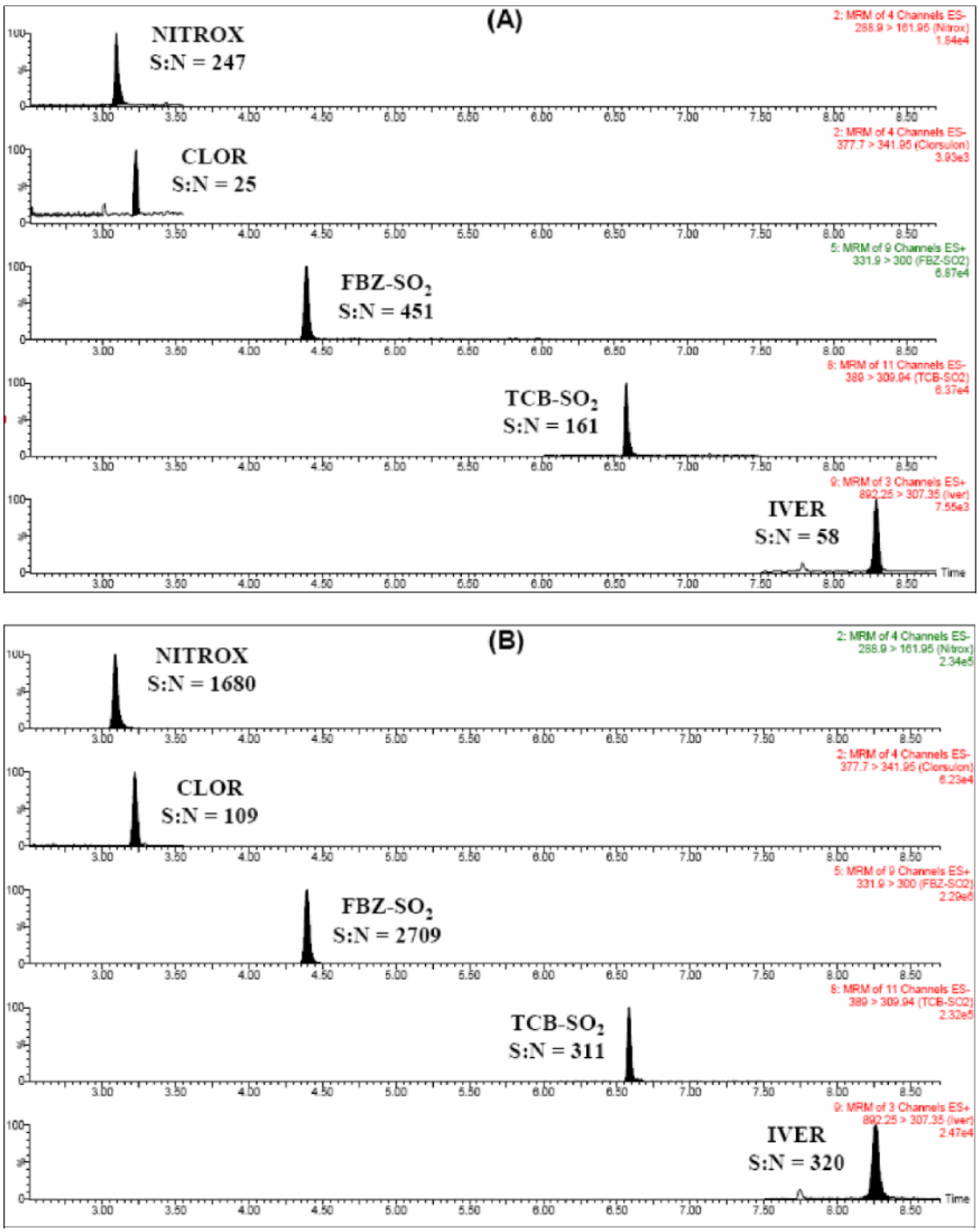

Fig. 1. Chromatogram of samples fortified at 10 µg kg^{-1}, highlighting the increase in S/N between the two methods. (A): high level method, (B): low level method.

Figure 2

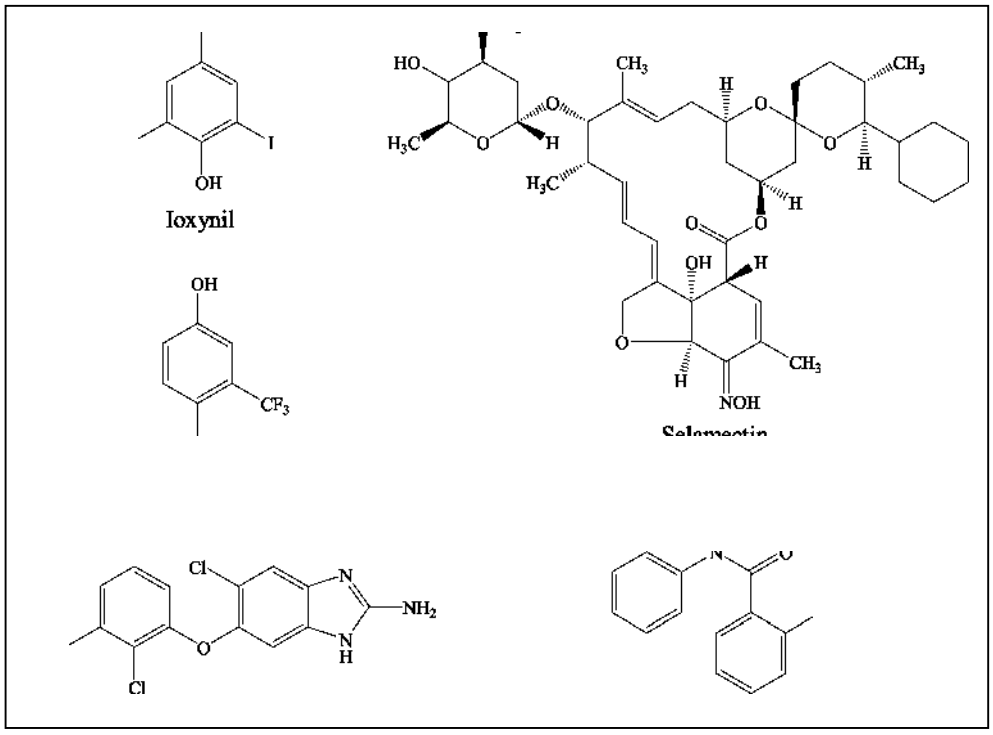

Fig. 2. Structures of the non-deuterated internal standards evaluated during method development.

Figure 3

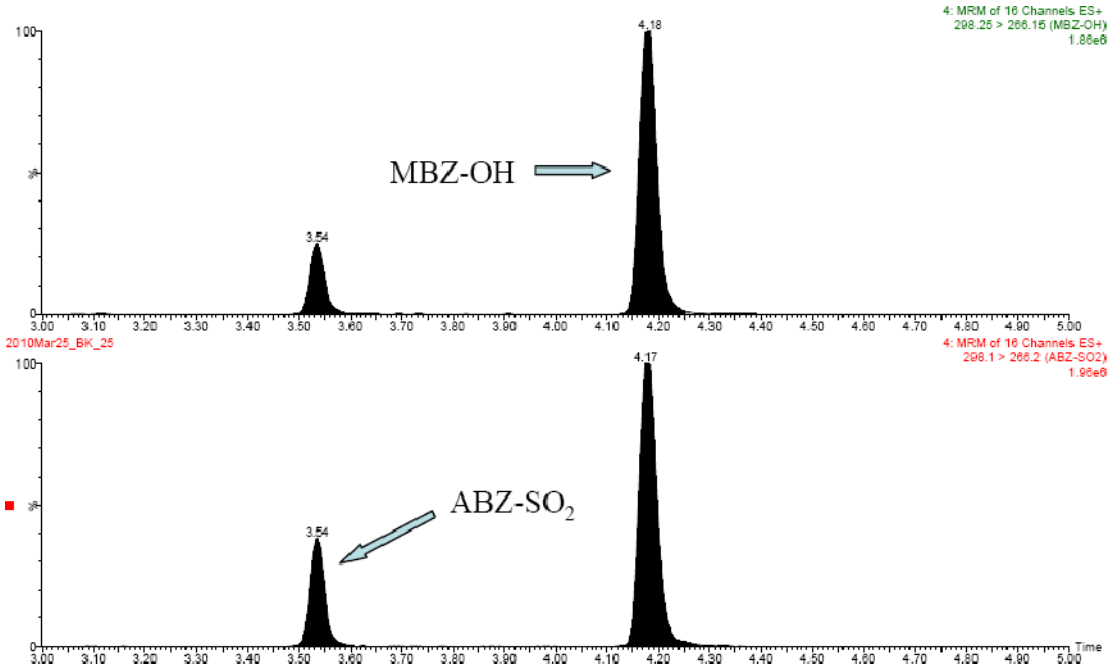

Fig. 3. Chromatogram showing isobaric interference between ABZ-SO_2 (298.10 > 266.20 *m/z*) and MBZ-OH (298.25 > 266.15 *m/z*), and their chromatographic separation

Figure 4

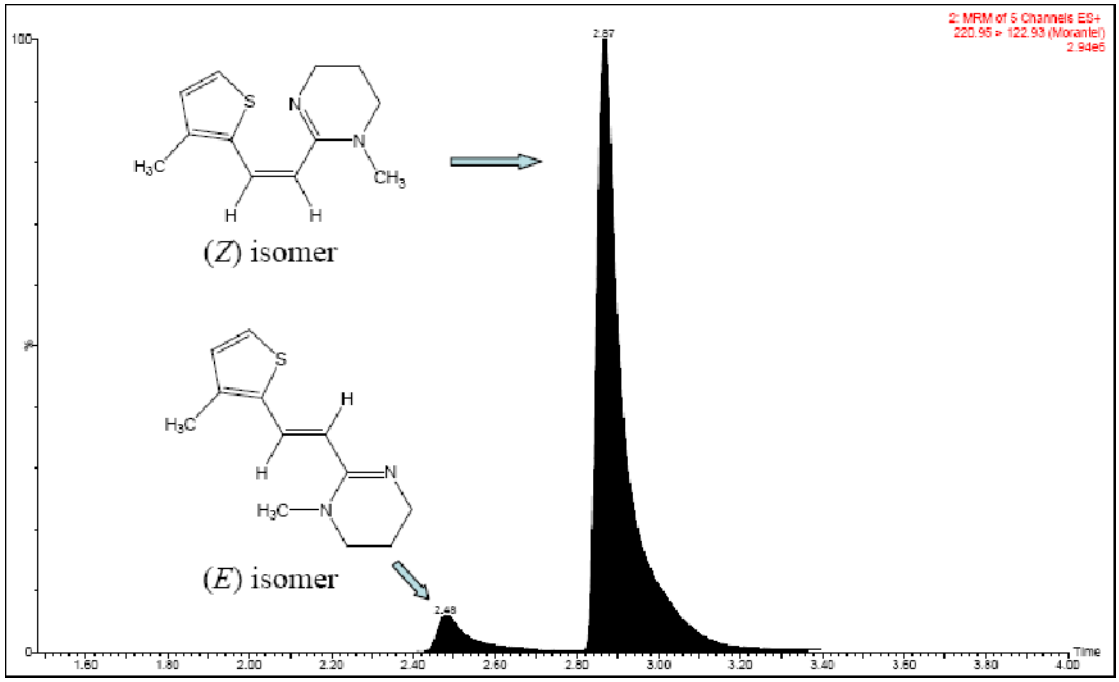

Fig. 4. Chromatogram of MOR showing two peaks corresponding to its isomers.

Table 1

UHPLC-MS/MS conditions.

Analyte	tR (min)	Precursor Ion (m/z)	Product Ion (m/z)	Dwell Time (ms)	Cone (V)	CE (V)	SRM window	IS
ESI$^+$								
LEVA	1.50	204.93	122.89	300	35	27	1	LEVA-D_5
			177.94	300	35	14	1	
ABZ-NH_2-SO_2	1.55	240.08	133.15	5	40	27	1	ABZ-NH_2-SO_2-D_3
			198.10	5	40	20	1	
5-OH-TBZ	1.60	217.87	146.87	8	45	32	1	ABZ-NH_2-SO_2-D_3
			190.85	8	45	24	1	
MOR	2.48 & 2.87	220.95	110.90	50	30	25	2	TBZ-D_4
			122.93	50	30	33	2	
TBZ	3.07	201.90	130.85	5	45	32	2	TBZ-D_4
			174.80	5	45	24	2	
ABZ-SO	3.22	282.24	159.06	5	27	35	4	ABZ-SO-D_3
			240.10	5	27	15	4	
MBZ-NH_2	3.24	238.10	105.09	5	50	24	4	TCB-NH_2 (+)
			133.05	5	50	34	4	
ABZ-SO_2	3.54	298.10	159.08	5	42	35	4	ABZ-SO_2-D_3
			266.20	5	42	20	4	
FLU-NH_2	3.56	256.06	95.10	35	45	34	4	TCB-NH_2 (+)
			123.05	35	45	26	4	
OFZ	4.02	316.10	159.05	5	35	30	4	FBZ-SO-D_5
			191.09	5	35	24	4	
MBZ-OH	4.18	298.25	160.05	5	38	33	4	MBZ-OH-D_3
			266.15	5	38	22	4	
FBZ-SO_2	4.35	331.90	158.90	5	35	36	5	FBZ-SO_2-D_5
			300.00	5	35	21	5	
FLU-OH	4.45	316.20	125.10	8	40	33	5	MBZ-OH-D_3
			160.05	8	40	35	5	
CAM	4.62	302.96	216.85	5	35	26	5	FBZ-D_5
			260.95	5	35	18	5	
OXI	4.93	249.90	175.90	7	35	26	5	OXI-D_7
			218.00	7	35	18	5	
MBZ	5.08	296.14	105.05	5	35	32	5	MBZ-D_3
			264.10	5	35	18	5	
FLU	5.32	313.80	123.00	5	40	35	5	FLU-D_3
			282.00	5	40	24	5	
ABZ	5.77	266.07	191.03	5	33	32	5	ABZ-D_3
			234.00	5	33	13	5	
COUM-O	5.97	347.01	210.99	5	30	29	7	TCB-NH_2 (+)
			291.02	5	30	22	7	
HAL	6.10	414.90	211.00	10	40	35	7	TCB-NH_2 (+)
			272.95	10	40	32	7	
FBZ	6.17	300.01	159.01	5	35	24	7	FBZ-D_5
			268.01	5	35	23	7	
COUM	6.82	363.02	227.05	5	35	25	7	TCB-NH_2 (+)
			307.05	5	35	16	7	
TCB	6.90	359.04	274.07	5	45	36	7	TCB-D_3
			343.97	5	45	27	7	
EMA	7.45	886.54	126.05	5	50	38	9	SELA
			158.01	5	50	37	9	

EPRI	7.66	915.15	144.06	10	19	41	9	SELA
			298.15	10	19	18	9	
ABA	7.77	890.50	305.15	25	14	25	9	SELA
			567.10	25	14	13	9	
MOXI	7.95	640.25	498.30	8	15	12	9	SELA
		640.25	528.40	8	15	8	9	
DORA	7.96	916.60	331.30	18	17	22	9	SELA
		916.60	593.35	18	17	12	9	
IVER	8.26	892.25	307.35	32	15	20	9	SELA
		892.25	569.45	32	15	13	9	
ESI$^+$ int. stds								
LEVA-D_5	1.48	210.10	183.08	300	40	20	1	IS
ABZ-NH_2-SO_2-D_3	1.51	242.00	133.00	5	40	30	1	IS
TBZ-D_4	3.02	205.99	179.00	80	47	24	2	IS
ABZ-SO-D_3	3.20	285.25	243.02	5	41	13	4	IS
ABZ-SO_2-D_3	3.52	301.00	158.95	5	40	38	4	IS
FBZ-SO-D_5	3.99	321.04	158.95	23	30	32	4	IS
MBZ-OH-D_3	4.25	301.15	160.05	5	36	32	4	IS
FBZ-SO_2-D_5	4.16	337.06	305.00	5	45	23	5	IS
OXI-D_7	4.88	257.15	177.05	5	32	28	5	IS
MBZ-D_3	5.07	299.15	105.05	5	39	33	5	IS
FLU-D_3	5.30	317.15	123.00	5	40	36	5	IS
ABZ-D_3	5.75	269.12	233.85	5	35	19	5	IS
FBZ-D_5	6.15	305.01	273.01	5	28	15	7	IS
TCB-NH_2 (+)	6.27	328.00	166.95	5	48	27	7	IS
TCB-D_3	6.90	361.90	343.90	5	43	25	7	IS
SELA	8.19	770.40	333.30	20	40	22	9	IS
ESI$^-$								
NITR	3.02	288.90	126.86	5	37	23	3	IOX
			161.95	5	37	22	3	
CLOR	3.19	377.70	341.95	5	25	12	3	SALI
		379.80	343.95	5	23	12	3	
TCB-SO_2	6.56	389.00	244.16	5	55	28	8	TCB-NH_2 (-)
			309.94	5	55	27	8	
OXY	6.56	397.80	175.75	5	32	26	8	SALI
			201.80	5	32	20	8	
TCB-SO	6.60	375.03	181.00	5	35	40	8	TCB-NH_2 (-)
			212.86	5	35	30	8	
NICL	6.79	324.95	170.91	5	33	26	8	SALI
			288.89	5	33	17	8	
BITH	7.01	352.75	160.70	5	32	23	7	TFM
			191.70	5	32	26	7	
CLOS	7.05	660.85	126.90	5	45	43	8	SALI
			315.10	5	45	35	8	
RAF	7.24	623.79	344.83	10	58	33	8	SALI
			126.90	10	58	36	8	
ESI$^-$ int. stds								
IOX	4.44	369.65	126.80	35	35	33	6	IS
TFM	5.03	205.95	159.95	35	37	24	6	IS
SALI	5.54	212.05	92.00	30	35	28	6	IS

TCB-NH_2 (-)	6.27	325.87	180.90	5	45	26	8	IS

Table 3 **Table 2**

MRL within-laboratory repeatability, reproducibility, Decision Limit (CCα) and Detection Capability (CCβ) results.

		Within-laboratory repeatability						Within-laboratory reproducibility										
Analyte	**MRL**	**Recovery (%)**			**CV (%)**			**Recovery (%)**			**CV (%)**			**Horwitz CV (%)**			**CCα**	**CCβ**
	(µg kg $^{-1}$)	**0.5 x**	**1 x**	**1.5 x**	**0.5 x**	**1 x**	**1.5 x**	**0.5 x**	**1 x**	**1.5 x**	**0.5 x**	**1 x**	**1.5 x**	**0.5 x**	**1 x**	**1.5 x**	**(µg kg $^{-1}$)**	**(µg kg $^{-1)}$**
ABZ	1000	99	99	98	1.1	1.3	1.2	97	97	96	3.1	3.2	2.8	18	16	15	1061	1115
ABZ-SO	1000	100	101	94	5.3	4.9	13.2	105	103	99	7.1	6.8	5.4	18	16	15	1115	1234
ABZ-SO_2	1000	101	101	100	2.9	2.5	1.4	103	102	101	3.8	3.6	2.6	18	16	15	1062	1118
ABZ-NH2-SO_2	1000	101	101	99	2.1	4.1	3.0	98	100	97	4.5	3.5	4.2	18	16	15	1078	1156
FBZ	500	100	100	100	0.9	0.8	0.6	101	99	99	2.9	1.1	2.2	20	18	17	508	525
OFZ	500	99	100	100	1.4	0.8	0.6	99	97	98	2.9	3.1	3.0	20	18	17	524	551
FBZ-SO_2	500	99	97	100	3.2	3.2	1.7	97	96	97	3.4	5.2	4.6	20	18	17	534	577
TCB	250	100	100	100	1.0	1.5	0.6	103	102	101	5.8	2.9	2.6	22	20	19	263	276
TCB-SO	250	197	152	130	11.0	11.1	7.6	180	145	114	13.6	9.8	10.3	22	20	19	339	436
TCB-SO_2	250	149	115	95	16.6	13.7	16.7	125	104	86	18.5	13.4	13.2	22	20	19	369	495
TBZ	100	98	98	97	2.1	1.6	2.4	98	97	97	3.1	1.8	2.4	23*	23	21	104	108
TBZ-OH	100	105	105	103	6.0	6.9	6.2	101	104	101	5.7	7.4	8.0	23*	23	21	114	131
LEV	100	99	100	100	1.7	1.2	0.9	98	96	96	2.6	6.7	6.0	23*	23	21	111	123
CLOR	100	87	103	111	46.9	38.5	23.9	89	94	99	32.4	17.5	15.1	23*	23	21	124	158
CLOS	1000	112	105	100	12.8	4.9	7.5	98	109	102	4.2	7.2	4.3	18	16	15	1151	1258
MOR	800	101	100	100	1.7	1.5	1.5	100	99	98	3.7	2.0	1.6	18	17	16	832	860
NITR	20	98	101	99	16.1	4.2	6.5	105	100	95	7.3	7.9	10.7	23*	23*	23*	22	27
OXY	500	116	107	97	11.9	13.9	12.0	130	118	106	6.6	3.7	5.7	20	18	17	576	636
RAF	10	93	101	104	9.5	7.6	16.4	100	92	90	7.1	12.2	7.4	23*	23*	23*	13	15
ABA	20	94	92	93	8.2	11.2	8.4	94	90	90	13.7	16.9	18.0	23*	23*	23*	27	39
DORA	100	100	101	101	2.0	3.3	3.6	99	99	101	4.9	5.2	4.7	23*	23	21	108	118
EPRI	1500	110	101	100	4.1	2.2	3.8	112	100	97	3.7	4.0	6.4	17	15	14	1593	1765
IVER	100	107	103	101	6.2	4.3	5.3	102	98	97	4.4	5.6	7.5	23*	23	21	109	123
MOXI	100	119	121	125	6.2	5.8	6.8	119	117	123	5.5	5.9	5.9	23*	23	21	107	118

*Below 100 µg kg^{-1} the Horwitz Equation gives unacceptably high values

Table 3 **Table 3**

Low level within-laboratory repeatability, reproducibility, Decision Limit (CCα) and Detection Capability (CCβ) results.

		Within-laboratory repeatability						Within-laboratory reproducibility							
Analyte	**Validation Level (rig kg^{-1})**	**Recovery (%)**			**CV (%)**			**Recovery (%)**			**CV (%)**			**CCα (rig kg^{-1})**	**CCβ (rig kg^{-1})**
		1 x	**1.5 x**	**2 x**	**1 x**	**1.5 x**	**2 x**	**1 x**	**1.5 x**	**2 x**	**1 x**	**1.5 x**	**2 x**		
ABZ	2	101	100	101	3.4	2.3	3.3	101	100	103	5.3	6.9	5.2	0.21	0.36
ABZ-SO	2	99	99	99	6.3	3.6	4.7	93	92	94	7.4	7.7	7.1	0.27	0.46
ABZ-SO_2	2	99	98	99	3.5	3.6	2.2	100	100	104	9.4	9.9	8.7	0.33	0.56
ABZ-NH_2-SO_2	2	100	100	100	4.3	3.1	1.7	102	104	107	5.5	8.5	7.6	0.27	0.46
CAM	2	99	101	102	3.2	2.7	5.4	102	102	102	5.6	7.6	9.8	0.32	0.55
FBZ	2	99	102	100	3.5	3.0	1.8	100	102	102	6.6	4.1	4.1	0.16	0.28
OFZ	2	100	100	100	2.7	2.2	2.3	100	102	106	5.9	6.8	6.2	0.22	0.38
FBZ-SO_2	2	101	102	103	4.5	4.5	5.3	101	98	104	6.7	11.6	6.3	0.30	0.51
FLU	2	102	111	113	4.7	14.6	17.6	98	99	108	12.9	28.8	23.3	0.79	1.34
FLU-NH_2	2	97	97	96	4.7	4.2	6.4	102	99	96	7.7	11.9	11.0	0.44	0.75
FLU-OH	2	104	104	103	3.3	5.3	2.8	103	102	104	5.5	6.9	6.6	0.24	0.41
MBZ	2	101	100	101	2.4	2.3	1.5	100	99	103	4.5	7.1	4.5	0.20	0.34
MBZ-NH_2	2	97	100	103	5.8	5.2	15.0	103	101	95	10.6	8.3	11.5	0.44	0.74
MBZ-OH	2	100	100	100	2.8	3.5	2.4	100	99	103	5.2	7.5	6.5	0.24	0.41
OXI	2	102	109	112	3.5	13.1	15.3	101	96	108	14.3	29.6	19.7	0.76	1.29
TCB	2	101	100	100	3.9	3.6	1.9	100	98	105	6.3	8.7	7.3	0.27	0.47
TCB-SO	2	94	98	93	26.3	26.8	36.3	105	106	95	22.8	17.2	37.7	1.31	2.24
TCB-SO_2	2	99	96	94	10.0	15.2	22.6	104	100	109	18.2	24.0	51.1	1.45	2.46
TBZ	2	104	103	104	3.4	3.6	4.9	103	96	104	8.6	13.9	6.6	0.36	0.62
5-OH-TBZ	2	125	120	113	15.1	15.0	20.9	111	107	111	14.0	16.3	12.0	0.51	0.86
BITH	4	97	102	102	30.8	19.4	16.2	114	116	120	12.8	9.4	13.8	0.87	1.49
NICL	2	100	100	98	5.6	6.9	11.6	104	108	115	7.3	12.7	20.2	0.56	0.96
COUM	2	106	98	103	8.7	8.2	6.9	100	94	101	11.0	14.6	15.8	0.55	0.94
COUM-O	2	97	97	89	18.9	20.9	36.5	86	79	73	29.2	34.7	38.5	1.69	2.88
HAL	2	117	120	100	27.3	15.3	28.8	115	114	91	32.1	20.8	31.8	1.66	2.84
EMA	2	99	108	101	7.9	11.1	4.2	99	101	99	14.3	22.0	14	0.64	1.09