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1 A dual validation approach to detect anthelmintic residues

2 in bovine liver over an extended concentration range

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17

18 Abstract

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

33

34

35 **Keywords**

36 Anthelmintics

37 Ultra-high performance liquid chromatography-tandem mass spectrometry

38 QuEChERS

39 Isotopically labelled internal standards

40 Ion suppression

41 Matrix effects

42

43

44 **1. Introduction**

45 Anthelmintics are widely used to treat parasitic infections in food-producing animals.
46 They include benzimidazoles, macrocyclic lactones and flukicides, the latter of which
47 are rarely tested in food due to the lack of suitable multi-residue methods. Some of
48 the anthelmintics possess toxicological properties such as teratogenicity and
49 embryotoxicity [1], neurotoxicity [2], hyperplasia [3], goitrogenicity [4] and
50 mutagenicity [5]. The European Union, originally through Council Regulation
51 1990/2377, established Maximum Residue Limits (MRLs) for a number of these
52 drugs in various animal tissues and species to minimise the risk to human health
53 associated with their consumption [6]. Recently, the EU repealed Council Regulation
54 1990/2377 and replaced it with Commission Regulation 2010/37 [7]. In bovine liver,
55 MRLs for the chosen anthelmintics range from 10 to 1500 $\mu\text{g kg}^{-1}$. The validation for
56 benzimidazoles is complicated because their MRLs are expressed in the form of sum-
57 MRLs. This includes marker residues for the benzimidazoles licensed in bovine,
58 namely albendazole, fenbendazole, triclabendazole, thiabendazole. As a result, an
59 infinite number of possibilities exist in terms of the number of concentration level
60 combinations. The SANCO document, published in 2004, aims to clarify how sum-
61 MRLs are validated and gives examples of how the sum-CC α and sum-CC β values
62 are calculated [8]. Therefore, it is necessary to validate a method over a wide
63 analytical range.

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

66 Single residue LC-MS methods have been reported for the determination of closantel
67 [9] and nitroxynil [10]. Multi-residue methods have been reported but are specific to
68 different anthelmintic groups such as benzimidazoles [11], macrocyclic lactones [12,
69 13] and flukicides [14-16]. Previously our group developed a LC-MS/MS method
70 capable of detecting 38 anthelmintic residues in bovine liver and milk [17]. Sample
71 preparation was carried out using a QuEChERS based method which used C_{18} sorbent
72 for the dispersive solid-phase extraction (d-SPE) step. The limit of detection (LOD)
73 was $5 \mu\text{g kg}^{-1}$ for all analytes except dichlorvos ($10 \mu\text{g kg}^{-1}$). Two 15 minute
74 injections were necessary to cover the positively and negatively ionised compounds.
75 Recently, our group demonstrated that UHPLC-MS/MS with fast polarity switching
76 enabled the determination of 38 anthelmintics to $1 \mu\text{g kg}^{-1}$ in milk [18]. This
77 approach is advantageous because it increases sample throughput while reducing
78 solvent usage in the laboratory.

79 There is continued demand for more sensitive and reliable methods that will detect
80 MRL violations, identify usage patterns of products and provide more quantitative
81 results for exposure and risk assessment. LC-MS/MS is the most effective means of
82 meeting all of these needs. However, co-eluting matrix components, which often go
83 unseen in LC-MS/MS traces can impact on ionisation efficiency and thus accuracy of
84 methods. This has been highlighted by groups who have reported the need for careful
85 characterisation of matrix effects [19-21]. A number of groups have reported on the
86 systematic reduction of matrix effects through the introduction of selective sample
87 preparation [22, 23] or improved chromatographic separation [24]. A drawback of
88 these approaches in multi-residue methods is that they typically increase the time
89 needed to prepare and analyse samples. In addition, more selective sample
90 preparation can result in the loss of certain analytes. Alternatively, the inclusion of
91 isotopically labelled internal standards or structural analogues of analytes can correct
92 for matrix effects [25]. In the area of anthelmintic analysis this has been supported by
93 the recent increase in availability of labelled internal standards. Several internal
94 standards are now available for levamisole and benzimidazole residues.

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

100 In this paper, a sensitive UHPLC-MS/MS method was developed capable of
101 detecting 38 anthelmintic residues to $1 \mu\text{g kg}^{-1}$ in a 13 min run time. The method uses
102 14 deuterated and 5 non-deuterated internal standards to improve the precision of the
103 method compared to a previously developed LC-MS/MS method that used two
104 internal standards. A dual validation approach is described to cover both MRL and
105 unapproved/low level substances ($2\text{--}4 \mu\text{g kg}^{-1}$). The sensitive method significantly
106 enhances the detection of anthelmintic drug residues in liver, which is useful for
107 identifying unapproved usage of veterinary medicinal products.

108

109

110 **2. Experimental**

111

112 *2.1 Reagents and apparatus*

113 MS grade ammonium formate, GC grade dimethyl sulfoxide (DMSO) and HPLC
114 grade acetonitrile (MeCN), methanol (MeOH) and 99.5% deuterated MeOH (MeOH-
115 D) were sourced from Sigma-Aldrich (Dublin, Ireland). Analar grade isopropyl
116 alcohol (IPA) and glacial acetic acid (HOAc) were obtained from BDH Chemicals
117 Ltd. (Poole, UK). Ultra-pure water ($18.2 \text{ M}\Omega$) was generated in-house using a
118 Millipore water purification system (Cork, Ireland). Pre-weighed mixtures of 4 g
119 anhydrous (anh.) magnesium sulphate (MgSO_4) and 1 g sodium chloride (NaCl) in 50
120 mL centrifuge tubes; 1.5 g anh. MgSO_4 and 0.5 g C_{18} in 50 mL centrifuge tubes; and 2
121 mL mini-centrifuge tube containing anh. MgSO_4 (150 mg) and C_{18} (50 mg) were
122 obtained from UCT, Inc. (Bristol, PA, USA). A Dispensette® III solvent dispenser
123 (Brand GMBH + Co KG; Wertheim Germany) was used for aliquoting MeCN; an
124 Ultra-Turrax stalk homogenizer (IKA Werke GmbH & Co. KG; Staufen, Germany),
125 Mistral 3000i centrifuge (MSE; London, UK), Eppendorf 5471 R bench top centrifuge
126 (Hamburg, Germany), TopMix multi-vortexer (Fisher Scientific; Dublin, Ireland),
127 Turbovap LV evaporator (Caliper Life Sciences; Runcorn, UK) were used during
128 sample preparation. A Elma Transsonic T780/H ultrasonic bath (Bedford, UK) was
129 used to degas mobile phase and solvent wash solutions. Whatman Resist® PTFE
130 syringe filters ($0.2 \mu\text{m}$ 13 mm) were obtained from Whatman (Ireland).

131

132 *2.2 Analytical standards*

133 Abamectin (ABA), albendazole (ABZ), bithionol (BITH), clorsulon (CLOR),
 134 closantel (CLOS), coumaphos (COUM), doramectin (DORA), emamectin (EMA),
 135 fenbendazole (FBZ), haloxon (HAL), ivermectin (IVER), levamisole (LEVA),
 136 morantel (MOR), niclosamide (NICL), nitroxylin (NITR), oxfendazole (OFZ),
 137 oxyclozanide (OXY), rafoxanide (RAF), and thiabendazole (TBZ) were purchased
 138 from Sigma-Aldrich (Dublin, Ireland). Albendazole-2-amino-sulphone (ABZ-NH₂-
 139 SO₂), albendazole-sulphone (ABZ-SO₂), albendazole-sulphoxide (ABZ-SO), 5-
 140 hydroxy-thiabendazole (5-OH-TBZ), fenbendazole-sulphone (FBZ-SO₂),
 141 triclabendazole (TCB), triclabendazole-sulphone (TCB-SO₂) and triclabendazole-
 142 sulphoxide (TCB-SO) were purchased from Witega Laboratories Berlin-Aldershof
 143 GmbH (Berlin, Germany). Coumaphos-oxon (COUM-O) was purchased from
 144 Greyhound Chromatography and Allied Chemicals, (Merseyside, UK).
 145 Cambendazole (CAM) and oxibendazole (OXI) were purchased from QMX
 146 Laboratories (Essex, UK). Amino-flubendazole (FLU-NH₂), amino-mebendazole
 147 (MBZ-NH₂), hydroxy-flubendazole (FLU-OH), hydroxy-mebendazole (MBZ-OH),
 148 flubendazole (FLU) and mebendazole (MBZ) were donated by Janssen Animal Health
 149 (Beerse, Belgium). Eprinomectin (EPRI) was donated by Merial Animal Health
 150 (Lyon, France). Moxidectin (MOXI) was donated by Fort Dodge Animal Health
 151 (Princeton, NJ, USA). Primary stock standard solutions were prepared at a
 152 concentration of 2000 µg mL⁻¹, while ABZ, ABZ-SO, ABZ-SO₂, ABZ-NH₂-SO₂,
 153 FBZ, OFZ, FBZ-SO₂, EPRI, CLOS, OXY, NITR, CLOR, BITH and MOR were
 154 prepared at a concentration of 4,000 µg mL⁻¹. The macrocyclic lactones were
 155 prepared in MeCN; the flukicides, CAM, LEVA, TCB, TCB-SO and TCB-SO₂ were
 156 prepared in MeOH; and the remaining analytes were prepared in DMSO.
 157 An intermediate working standard mix solution was prepared for the low level
 158 experiments at a concentration of 100 µg mL⁻¹ for OXY, CLOR, BITH and MOR, and
 159 50 µg mL⁻¹ for the remaining 34 analytes, in MeOH. Three intermediate standard mix
 160 solutions were required for the high level experiments, as a single standard solution
 161 containing all analytes could not be prepared. The concentration of the high standard
 162 was 300 µg mL⁻¹ for ABZ, ABZ-SO, ABZ-SO₂, ABZ-NH₂-SO₂, CLOS, MOR and
 163 EPRI, and 100 µg mL⁻¹ for the remaining 34 analytes.
 164
 165 Deuterated forms of benzimidazoles or structurally similar molecules were used as
 166 internal standards. Albendazole-D₃ (ABZ-D₃), albendazole-sulphone-D₃ (ABZ-SO₂-

167 D₃), albendazole-sulphoxide-D₃ (ABZ-SO-D₃), albendazole-2-amino-sulphone-D₃
168 (ABZ-NH₂-SO₂-D₃), flubendazole-D₃ (FLU-D₃), levamisole-D₅ (LEVA-D₅),
169 mebendazole-D₃ (MBZ-D₃), hydroxy-mebendazole-D₃ (MBZ-OH-D₃), oxibendazole-
170 D₇ (OXI-D₇), thiabendazole-D₃ (TBZ-D₃), triclabendazole-D₃ (TCB-D₃) and amino-
171 triclabendazole (TCB-NH₂) were purchased from Witega Laboratories.
172 Fenbendazole-D₅ (FBZ-D₅), fenbendazole-sulphone-D₅ (FBZ-SO₂-D₅) and
173 fenbendazole-sulphoxide-D₅ (FBZ-SO-D₅) were from Quchem Ltd. (Belfast, UK).
174 Additional internal standards included selamectin (SELA), which was donated by
175 Pfizer Animal Health (New York, NY; USA), salicylanilide (SALI), 4-nitro-3-
176 (trifluoromethyl)phenol (TFM), and ioxynil (IOX), which were purchased from
177 Sigma-Aldrich. Internal standards were prepared at a concentration of 1000 µg mL⁻¹,
178 except TCB-NH₂ and SELA which were prepared at a concentration of 2000 µg mL⁻¹.
179 IOX, SALI, SELA, and TFM were prepared in MeCN; TCB-NH₂ was prepared in
180 MeOH; TCB-D₃, TBZ-D₃ and LEVA-D₅ were prepared in MeOH-D (to prevent
181 deuterium exchange in solution); and the remaining internal standards were prepared
182 in DMSO.

183 An internal standard mix for the low level method (IS1) was prepared in
184 MeOH-D at a concentration of 20 µg mL⁻¹ for SELA and TCB-NH₂, 4 µg mL⁻¹ for
185 LEVA-D₅, TBZ-D₃ and IOX, and 2 µg mL⁻¹ for the remaining analytes. A second
186 internal standard mix for the high level method (IS2) was prepared in MeOH-D at a
187 concentration of 200 µg mL⁻¹ for SELA and TCB-NH₂, 40 µg mL⁻¹ for LEVA-D₅,
188 TBZ-D₃ and IOX, and 20 µg mL⁻¹ for the remaining analytes. Primary, intermediate
189 and working standard solutions are stable for at least six months when stored at
190 -20°C.

191

192 2.3 Calibration

193 Two different protocols were found to be necessary to measure anthelmintic residues
194 in the ranges 1 to 100 µg kg⁻¹ (low MRL or non-MRL substances) and 10 to 3000 µg
195 kg⁻¹ (MRL substances). The majority of positively and negatively ionised analytes
196 fitted a linear calibration line. Exceptions were OXY, TCB-SO and TCB-SO₂, which
197 had a quadratic line fit.

198

199

200 2.3.1 Low level calibration curve

201 Six-point extracted matrix calibration curves were prepared at concentrations of 1, 2,
202 5, 10, 25 and 50 $\mu\text{g kg}^{-1}$ (or double for BITH, CLOR, MOR and OXY) to measure
203 low levels of anthelmintic residues. Extracted matrix calibrants were prepared by
204 fortifying negative liver samples with 100 μL volumes of standard solutions
205 containing OXY, CLOR, BITH and MOR at 0.2, 0.4, 1, 2, 5 and 10 $\mu\text{g mL}^{-1}$, and the
206 remaining 34 analytes at 0.1, 0.2, 0.5, 1, 2.5 and 5 $\mu\text{g mL}^{-1}$.

207

208 2.3.2 *High level calibration curve*

209 Seven-point extracted matrix calibration curves were prepared at higher
210 concentrations of 10, 20, 50, 100, 200, 500 and 1000 $\mu\text{g kg}^{-1}$ (or 30, 60, 150, 300,
211 600, 1500 and 3000 $\mu\text{g kg}^{-1}$ for ABZ, ABZ-SO, ABZ-SO₂, ABZ-NH₂-SO₂, CLOS,
212 EPRI and MOR). Matrix calibrants were prepared by fortifying negative liver
213 samples with 100 μL volumes of standard solutions containing ABZ, ABZ-SO, ABZ-
214 SO₂, ABZ-NH₂-SO₂, CLOS, MOR and EPRI at 3, 6, 15, 30, 60, 150, 300 $\mu\text{g mL}^{-1}$,
215 and the remaining MRL substances at 1, 2, 5, 10, 20, 50, 100 $\mu\text{g mL}^{-1}$.

216

217 2.4 *Quality control samples*

218 Bovine liver samples found to contain no response at the retention time of the analytes
219 were used as negative controls. Quality control (QC) samples (recovery controls)
220 were prepared for the low level method by spiking extracts in duplicate with 50 μL of
221 0.2 $\mu\text{g mL}^{-1}$ (0.4 $\mu\text{g mL}^{-1}$ OXY, CLOR, BITH and MOR) and 2.5 $\mu\text{g mL}^{-1}$ (5 for
222 OXY, CLOR, BITH and MOR $\mu\text{g mL}^{-1}$) standards after extraction, and were used to
223 monitor extraction efficiency. QC samples were fortified with 25 μL of the working
224 internal standard (IS1) solution prior to extraction. In the high level method, QC
225 samples were prepared by spiking extracts in duplicate with 5 μL of 2 $\mu\text{g mL}^{-1}$ (6 μg
226 mL^{-1} for ABZ, ABZ-SO, ABZ-SO₂, ABZ-NH₂-SO₂, CLOS, MOR and EPRI) and 20
227 $\mu\text{g mL}^{-1}$ (60 $\mu\text{g mL}^{-1}$ ABZ, ABZ-SO, ABZ-SO₂, ABZ-NH₂-SO₂, CLOS, MOR and
228 EPRI). QC samples were fortified with 25 μL of the working internal standard (IS2)
229 solution prior to extraction.

230

231 2.5 *Sample preparation*

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

235 samples using a stalk homogenizer in the presence of MeCN (10 mL). Phase
236 separation was induced by adding the contents of a tube containing anh. MgSO₄ (4 g)
237 and NaCl (1 g) to the sample tube. The remaining salts were transferred using a
238 MeCN (2 mL) wash. Samples were immediately shaken for 1 min (to prevent
239 agglomerates forming during MgSO₄ hydration) and centrifuged (2842 × g, 12 min).

240 Two different d-SPE protocols were used to purify extracts depending on the
241 concentration level. In the low level method (2 µg kg⁻¹), the entire supernatant was
242 poured into a centrifuge tube (50 mL) containing anh. MgSO₄ (1.5 g) and C₁₈ (0.5 g).
243 The sample was vortexed for 1 min and centrifuged (2842 × g, 10 min). The purified
244 supernatant (6 mL) was added to a 15 mL polypropylene evaporation tube (Sarstedt)
245 containing DMSO (0.25 mL). MeCN was evaporated under nitrogen at 50°C using a
246 Turbovap.

247 For MRL concentrations, 1 mL of supernatant was transferred from the
248 QuEChERS extraction tube into a 2 mL mini-centrifuge tube containing anh. MgSO₄
249 (150 mg) and C₁₈ (50 mg). The sample extract was vortexed (1 min) and centrifuged
250 (21913 × g, 2 min). An aliquot of the purified supernatant (600 µL) was transferred to
251 a 5 mL glass culture tube containing DMSO (600 µL) and the MeCN was evaporated
252 under nitrogen at 50°C using the Turbovap apparatus. In both cases, extracts were
253 vortexed (1 min) and filtered through a 0.2 µm PTFE 13 mm syringe filter (Whatman
254 Rezist[®]) into an autosampler vial.

255

256 2.6 LC-MS/MS analysis

257 Separation was conducted on a Waters Acquity UHPLC system (Milford, MA, USA).
258 The analytical column was an Acquity HSS T3 C₁₈ (100 × 2.1 mm, 1.8 µm particle
259 size) protected by a 0.2 µm stainless steel in-line filter and maintained at 60°C.
260 Gradient separation was performed using a binary gradient composed of mobile phase
261 A, 0.01% HOAc:MeCN (90:10, v/v) and mobile phase B, 5 mM ammonium formate
262 in MeOH:MeCN (75:25, v/v). The gradient profile was as follows: (a) 0 → 0.5 min,
263 100% A; (b) 5 min, 50% A; (c) 7 → 8.5 min, 10% A; (d) 8.51 → 10 min, 0% A; (e)
264 10.01 → 13 min, 100% A. UHPLC weak and strong autosampler needle washes
265 consisted of H₂O:MeOH (80:20, v/v) and MeOH:IPA:H₂O (80:10:10, v/v),
266 respectively. The injection volume was 5 µL. Analytes were detected using a Waters
267 Quattro Premier XE triple quadrupole instrument equipped with an electrospray

268 ionisation interface (Milford, MA, USA). Nitrogen was used for nebulisation,
269 desolvation (1000 L hr^{-1}) and cone gas (50 L hr^{-1}). Argon was used as collision gas
270 (0.013 L hr^{-1}). The source temperature was set at 150°C and desolvation temperature
271 at 450°C . The ion spray voltage was set at 3000 eV for positive mode and 500 eV for
272 negative mode. The MS conditions were optimised by tuning the analyte-specific
273 parameters, including cone voltage, collision energy and collision cell exit potential
274 for each analyte. This optimisation was carried out by infusion of a $1 \mu\text{g mL}^{-1}$
275 standard solution of each analyte and monitoring the two most abundant fragment
276 ions produced from the molecular ion. The SRM windows were time-sectored, and
277 dwell time, inter-scan delay and inter-channel delays were set to get maximum
278 response from the instrument. A summary of the retention times, monitored ions and
279 optimised MS parameters obtained for each analyte is reported in Table 1. The
280 U H PLC-MS/MS system was controlled by MassLynxTM software and data was
281 processed using TargetLynxTM Software (both from Waters).

282

283 2.7 Method Validation

284 Method validation was carried out according to European Commission
285 Decision 2002/657/EC criteria [26]. Parameters investigated included specificity,
286 linearity, recovery, within-laboratory repeatability (WLr) and reproducibility (WLR),
287 Decision Limit ($\text{CC}\alpha$) and Detection Capability ($\text{CC}\beta$). LC-MS/MS identification
288 criteria were verified throughout the validation study by monitoring relative retention
289 times, ion recognition (signal-to-noise ratio) and relative ion intensities.

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

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

302 these concentrations the analyst should aim to achieve the lowest possible CV or <23
303 %.

304 $CC\alpha$ is the limit from which it can be decided that a sample is truly violative
305 with an error probability of α . In the case of a banned or unauthorised substance the
306 $CC\alpha$ is the lowest concentration level at which a method can discriminate with a
307 statistical certainty of $1-\alpha$ whether the identified analyte is present. In the case of
308 substances with an established MRL, the $CC\alpha$ is the concentration above which it can
309 be decided with a statistical certainty of $1-\alpha$ that the identified analyte content is truly
310 above the MRL. $CC\beta$ is the smallest content of the analyte that may be detected,
311 identified and/or quantified in a sample with an error probability of β . The β -error
312 should be less than or equal to 5%. In the case of banned or unauthorised substances,
313 $CC\beta$ is the lowest concentration at which a method is able to detect truly
314 contaminated samples with a statistical certainty of $1-\beta$. In the case of substances
315 with an established MRL, $CC\beta$ is the concentration at which the method is able to
316 detect MRL concentrations with a statistical certainty of $1-\beta$.

317 $CC\alpha$ and $CC\beta$ values for unapproved use level were calculated using the
318 intercept (value of the signal, y , where the concentration, x , is equal to zero) and the
319 standard error of the intercept (SEI) for a set of data with 6 replicates at 3 levels (1,
320 1.5 and 2 x unapproved use level). $CC\alpha$ is the concentration corresponding to the
321 intercept + 2.33 x the SEI, and $CC\beta$ is the concentration corresponding to the signal at
322 $CC\alpha + 1.64$ x the SEI. $CC\alpha$ and $CC\beta$ for MRL level were calculated using the
323 calibration procedure for marker residues according to ISO 11843 for a set of data
324 with 6 replicates at 3 levels (0.5, 1.0 and 1.5 MRL). $CC\alpha$ is the concentration
325 calculated from the response at the MRL + 1.64 x the WLR standard deviation (SD),
326 and $CC\beta$ is the concentration calculated from the response at $CC + 1.64$ times the
327 WLR SD.

328

329

330 **3. Results and discussion**

331

332 *3.1 Method development*

333 A QuEChERS based method was previously developed by this group which is
334 capable of measuring anthelmintic residues to $5 \mu\text{g kg}^{-1}$ in liver using LC-MS/MS

335 technology [17]. Recently, methods have been reported in literature for anthelmintics
336 in milk using UHPLC coupled to single stage [27] and tandem MS [18] analysers.
337 These applications highlight the advantages of UHPLC over LC-based methods in
338 terms of resolution, sensitivity, sample throughput and reduced instrument downtime
339 due to cleaning of the source. In addition, Whelan et al. reported on the advantage of
340 more modern MS instruments with rapid polarity switching capabilities, which
341 allowed the analysis of negatively and positively charged ions in a single injection
342 [18]. A review of published methods has highlighted the scarcity of LC-MS/MS
343 methods for flukicides, particularly in animal tissue [14, 17]. It is proposed that this is
344 likely due to the requirement to monitor negative ions. Jedziniak et al. recently
345 reported difficulties in the analysis of the negative ions of TCB-SO and TCB-SO₂
346 residues in milk [27]. Our previous method required two injections to cover the
347 positively and negatively ionised compounds, and each injection had a run time of 15
348 min. Other research groups have reported single class analysis of anthelmintics,
349 including the macrocyclic lactones ($n \leq 6$, <30 min) [12, 13, 28-30], the
350 benzimidazoles ($n \leq 20$, <25 min) [27, 31-33], and the flukicides ($n \leq 5$, <30 min) [14,
351 16].

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

364

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

369 observed for the 29 positively ionised analytes. Experiments were designed to
370 identify the reason for the non-linear behaviour. The potential competition for charge
371 between analytes was investigated by individually injecting calibration curves of the
372 two worst performing analytes (CLOR and TCB-SO₂) but this showed no
373 improvement. A range of other factors were also investigated, including injection
374 volume, desolvation gas flow rate, desolvation temperatures, mobile phase flow rate
375 and detuning the ESI probe position. Reduction of mobile phase flow rate and
376 injection volume improved linearity slightly over the desired range. In an attempt to
377 extend linearity, mobile phase additives used by other groups were evaluated without
378 success, including ammonium acetate [27], ammonium formate [17] and
379 triethylamine [12, 13, 28]. Ultimately, it was decided to use two methods to cover
380 low (1-100 µg kg⁻¹) and high (1 0-3000 µg kg⁻¹) concentration ranges.

381

382 In the initial phases of work, samples were extracted and purified using the procedure
383 developed by Kinsella et al. [17]. However, this approach was not suitable for
384 reliably detecting low concentrations of some residues. In order to improve
385 sensitivity for the low level method, the d-SPE step was scaled up by applying the full
386 ≈10 mL of extract to tubes containing C₁₈ (0.5 g) + anh. MgSO₄ (1.5 g), and
387 subsequent concentration of 6 mL purified extract to a low volume (0.25 mL). The
388 resulting increase in sensitivity (expressed as S/N) is highlighted in Fig. 1, which
389 shows chromatograms for two samples fortified at 10 µg kg⁻¹ and prepared according
390 to the low and high level method. An important factor in achieving this improvement
391 was the introduction of DMSO as a keeper solvent to prevent evaporating of samples
392 to dryness. The use of DMSO had several other benefits, including reduction of
393 protein binding, increasing injection volume and sharpening chromatographic peaks
394 [35]. In the high level method, samples were purified with C₁₈ (50 mg) + anh. MgSO₄
395 (150 mg). Subsequently, extracts underwent solvent exchange by evaporating the
396 MeCN extract (600 µL) in DMSO (600 µL) to maintain sharp peaks. This dual
397 approach to sample preparation allowed the measurement of residues over a
398 sufficiently wide range for practical application.

399

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

403 non-deuterated compounds. The deuterated benzimidazoles had similar MS
404 conditions and almost identical retention times as their non-deuterated forms. The
405 five non-deuterated internal standards are structural analogues of some of the
406 anthelmintics (Fig. 2). SELA is a macrocyclic lactone used to treat cats and dogs. It
407 is similar in structure to the avermectins (ABA, DORA, EMA, EPRI, IVER) but
408 contains a monosaccharide instead of a disaccharide. It was used as an IS for all the
409 macrocyclic lactones. SALI, a fungicide, is structurally related to several flukicides,
410 including CLOS, NICL, OXY and RAF, for which it was used as an IS. In addition, it
411 was used as an IS for CLOR. TCN-NH₂ was used in both positive and negative
412 ionisation modes. It was used as an IS in ESI⁻ for TCB-SO and TCB-SO₂, and in
413 ESI⁺ for MBZ-NH₂, FLU-NH₂, COUM, COUM-O and HAL. IOX and TFM are
414 herbicides and similar in structure to NITR. IOX was chosen as an IS for NITR as it
415 is structurally more similar and elutes closer to NITR than TFM. TFM was used as
416 the IS for BITH. BITH-SO was also evaluated as an IS but gave unacceptable
417 chromatography and had poor sensitivity. It did not adequately correct for matrix
418 effects and instead the next closest eluting compound (TFM) was chosen as IS for
419 BITH.

420

421 3.2 Method validation

422

423 3.2.1 Specificity

424 A specific UHPLC-MS/MS method was developed to separate anthelmintic residues
425 and internal standards. In early experiments, carry-over problems were frequently
426 observed in chromatograms for a number of the most intense substances, namely,
427 CAM, OXI and EMA. Carryover was eliminated through the use of weak
428 (H₂O:MeOH, 80:20 v/v) and strong (MeOH:IPA:H₂O, 80:10:10 v/v) autosampler
429 needle washes. In addition, MeOH was injected after positive controls in routine
430 analytical runs to further ensure no carryover into test samples.

431 The specificity of the method in terms of potential interferences among
432 analytes was demonstrated by injecting analytes and internal standards individually.
433 After injecting standards and internal standards separately onto the UHPLC-MS/MS,
434 two analyte transitions, ABZ-SO₂ (298.10 > 266.20 *m/z*) and MBZ-OH (298.25 >
435 266.15 *m/z*), were found to be prone to isobaric interference. However, both peaks
436 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-SO₂, 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

471 obtained for 5-OH-TBZ, BITH, NICL and HAL. CVs were typically <23% except
472 for FLU, OXI, TCB-SO, TCB-SO₂, COUM-O and HAL. The elevated recovery and
473 poor reproducibility obtained for TCB-SO and TCB-SO₂ was attributed to the non-
474 linear behaviour of the calibration curves.

475

476 3.2.4 Decision Limit ($CC\alpha$) and Detection Capability ($CC\beta$)

477 $CC\alpha$ and $CC\beta$ values for MRL and unapproved level are listed in Table 2 and Table 3,
478 respectively. The $CC\alpha$ values ranged from 13-1593 and 0.21-1.69 $\mu\text{g kg}^{-1}$ for MRL
479 and unapproved level, respectively. $CC\beta$ values ranged from 15-1765 and 0.28-2.88
480 $\mu\text{g kg}^{-1}$ for MRL and unapproved level, respectively. The results achieved in this
481 study are better than the results obtained in our previous work [17]. For example, in
482 our previous work, the $CC\alpha$ values obtained for the ABZs ranged from 1077-1383 μg
483 kg^{-1} while in our current method they range from 1062-1115 $\mu\text{g kg}^{-1}$. The FBZs were
484 reduced from 555-568 $\mu\text{g kg}^{-1}$ to 508-534 $\mu\text{g kg}^{-1}$. CLOS was reduced from 1228 μg
485 kg^{-1} to 1151 $\mu\text{g kg}^{-1}$, OXY from 623 to 578 $\mu\text{g kg}^{-1}$ and EPRI from 1721 to 1593 μg
486 kg^{-1} . The improvement in $CC\alpha$ and $CC\beta$ values were matched by improvements in
487 recovery and CV. The better results obtained in this study were due to the better
488 reproducibility obtained with the 14 deuterated and five non-deuterated internal
489 standards. In our previous method, only two internal standards were used in the
490 method, namely triphenyl phosphate (ESI^+) and 2,4-dichlorophenoxyacetic acid (ESI^-
491), which are pesticides and not structurally related to the anthelmintics.

492

493 3.2.5 Qualitative Criteria

494 According to 2002/657/EC [26], three identification points are required to satisfy
495 confirmatory criteria for Group B substances. This was achieved through the
496 selection of one precursor ion and two product ions, which resulted in four
497 identification points and exceeded the minimum requirements. The criteria on
498 relative retention times (RRT), signal-to-noise (S/N) and ion ratios (IR) were
499 examined for all samples and standards used for the validation study. The values for
500 RRT, S/N and IR were in agreement with the EU requirements for all the analytes
501 investigated in the study. In terms of RRT, the analyte peaks in samples were found
502 to be within the $\pm 2.5\%$ tolerance when compared with standards. Furthermore, two
503 transition ions were monitored for each of the 38 analytes, although only the most
504 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 MRL and low $\mu\text{g kg}^{-1}$ regions in bovine liver. Dual
512 validation was necessary because of the need to detect trace levels of some drugs due
513 to low MRLs (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 $\leq 2 \mu\text{g kg}^{-1}$. The method has
522 since been accredited to ISO 17025 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

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540

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600

601

Figure 1

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

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

Figure 3

Fig. 3. Chromatogram showing isobaric interference between ABZ-SO₂ (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

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
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Table 3Table 2

MRL within-laboratory repeatability, reproducibility, Decision Limit (CC α) and Detection Capability (CC β) results.

Analyte	MRL ($\mu\text{g kg}^{-1}$)	Within-laboratory repeatability						Within-laboratory reproducibility									CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)
		Recovery (%)			CV (%)			Recovery (%)			CV (%)			Horwitz CV (%)				
		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		
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 $\mu\text{g kg}^{-1}$ the Horwitz Equation gives unacceptably high values

Table 3

Low level within-laboratory repeatability, reproducibility, Decision Limit (CC α) and Detection Capability (CC β) results.

Analyte	Validation Level (rig kg ⁻¹)	Within-laboratory repeatability						Within-laboratory reproducibility						CCα (rig kg ⁻¹)	CCβ (rig kg ⁻¹)
		Recovery (%)			CV (%)			Recovery (%)			CV (%)				
		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