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1 **Carry-over assessment of fumonisins and zearalenone to poultry tissues after**
2 **exposure of chickens to a contaminated diet – A study implementing stable-**
3 **isotope dilution assay and UHPLC-MS/MS**

4

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15

16 **Abstract**

17 This study assessed the transfer of fumonisins B1 and B2 (FBs), zearalenone (ZEA) and
18 zearalenone metabolites α -zearalanol (α -ZAL) and α -zearalenol (α -ZEL) to poultry tissues.
19 Two experimental groups of 9 male chickens each were exposed for 8 weeks to a
20 contaminated diet (FB group: 12.7 mg FB1 + FB2 kg⁻¹, ZEA group: 0.40 mg ZEA kg⁻¹). To
21 measure the carry-over from feed to animal tissues (liver and muscle), a sensitive and accurate
22 SIDA-UHPLC-MS/MS method was developed and validated. For all mycotoxins, the limit of
23 detection (LOD) was 0.3 μ g kg⁻¹ and the limit of quantification (LOQ) was 1.0 μ g kg⁻¹.
24 Recoveries ranged from 92% to 107% and the intermediate precision coefficients of variation
25 (CV_{IP}) were between 4.3% and 13%. ZEA and α -ZAL were not detected in livers, whereas α -
26 ZEL was detected in five out of eight samples at levels between LOD and LOQ. FBs were
27 detected and quantified in the livers of all animals exposed to the contaminated diet (mean
28 30.3 μ g FB1 kg⁻¹ and 2.3 μ g FB2 kg⁻¹). A significant correlation between the FB1 and the
29 FB2 contents in the liver was demonstrated and FB carry-over factors (CFs) from feed to liver
30 were determined (CF_{FB1}: 0.003 and CF_{FB2}: 0.001). Filet muscles from the same animals were
31 also analysed. FB1 was quantified at trace levels in eight samples out of nine (mean 2.0 μ g
32 kg⁻¹) and was only detected in the remaining sample. FB2 was detected in only one muscle
33 sample.

34

35 **Keywords:** SIDA-UHPLC-MS/MS, fumonisins, zearalenone, poultry tissues, contaminated
36 diet, carry-over

37

38 1. Introduction

39 Fumonisin (FBs) and Zearalenone (ZEA) are mycotoxins produced by *Fusarium* species
40 prior to cereal harvest or during poor storage conditions (Marin, Ramos, Cano-Sancho, &
41 Sanchis, 2013). FBs are hepatotoxic in all animal species studied, nephrotoxic for several of
42 them, and are also considered cytotoxic (Gelderblom et al., 2001; Völkel, Schröer-Merker, &
43 Czerny, 2011). FB1, the most abundant analogue of the fumonisins family, was classified by
44 the International Agency for Research on Cancer (IARC) in group 2B, compounds considered
45 carcinogenic to animals and possibly carcinogenic to humans (IARC, 1993). ZEA is an
46 endocrine disrupter with oestrogenic effects, thought to reduce male fertility in humans and
47 wildlife populations and possibly involved in cancer development (Stopper, Schmitt, &
48 Kobras, 2005).

49 For human consumers, the main sources of exposure to FBs and ZEA are cereals and cereal-
50 based products (AFSSA, 2006; Bailly & Guerre, 2009; Leblanc, Tard, Volatier, & Verger,
51 2005; SCOOP, 2003). Therefore, risk management is mainly based on the control of plant-
52 based food and feed, as demonstrated by the regulations and recommendations adopted by the
53 European Union (EU) (European Union, 2006, 2007). Limits in feed and those in food are not
54 always correlated. Usually, feed limits are defined on the basis of the contamination levels
55 commonly found, applying the principle of “as-low-as-reasonably achievable”, whereas food
56 limits are based on tolerable daily intake levels for human consumption. This approach is
57 adopted mainly because of insufficient data for carry-over from feed to the target organ of the
58 animal or product (van Raamsdonk et al., 2009). Therefore, consumers may be exposed to the
59 toxic compounds indirectly due to the presence of residual contamination in foods from
60 animals that have been fed with contaminated feeds (Maragos, 2010). Although the
61 contribution of animal products is not considered significant, it should be taken into account
62 in risk assessments of compounds presenting chronic toxicity, such as mycotoxins (Meyer,

63 [Mohr, Bauer, Horn, & Kovács, 2003](#)). Moreover, depending on the metabolic pathways
64 involved, the passage through the animal may represent a detoxification process or, on the
65 contrary, lead to the appearance of new and sometimes more toxic compounds for the human
66 consumer ([Bailly & Guerre, 2009](#)).

67 Although FB1 is weakly bioavailable after oral ingestion and is mostly excreted in native
68 form in excreta, the bioavailable amount is extensively distributed and accumulates in tissues
69 ([EFSA, 2018](#); [Norred, Plattner, & Chamberlain, 1993](#); [Prelusky, Trenholm, & Savard, 1994](#)).
70 FB1 toxicokinetic studies showed distribution in animal tissues with most of the absorbed
71 toxin found in the liver and kidneys ([Martinez-Larranaga et al., 1999](#); [Tardieu, Auby, Bluteau,
72 Bailly, & Guerre, 2008a](#); [Tardieu, Bailly, Skiba, Grosjean, & Guerre, 2008b](#)). One study in
73 weaned piglets also highlighted the presence of partially hydrolysed FB1 in tissues, and found
74 that unmetabolised FB1 was the most abundant form ([Fodor et al., 2008](#)). In France, the first
75 total diet study (TDS) revealed high FB1 levels in three poultry liver samples (50% of all the
76 poultry livers analysed), with a content between 90 and 120 $\mu\text{g kg}^{-1}$, suggesting that human
77 exposure to FB1 by the ingestion of animal-derived food products should be considered more
78 closely ([Leblanc et al., 2005](#)).

79 After oral ingestion, ZEA is quickly absorbed and biotransformed in the digestive tract. ZEA
80 and its associated metabolites are then mostly excreted in urine, faeces or bile, depending on
81 the animal species ([Bailly & Guerre, 2009](#); [EFSA, 2011](#)). The principal metabolites known to
82 have affinities for oestrogenic receptors are in the following order: α -zearalanol (α -ZAL) > α -
83 zearalenol (α -ZEL) > β -zearalanol (β -ZAL) > zearalenone (ZEA) > β -zearalenol (β -ZEL).
84 Because some of them have higher affinities for oestrogenic receptors than zearalenone, their
85 appearance during metabolic pathways can be considered as a bioactivation ([Gaumy, Bailly,
86 & Guerre, 2001](#)). However, only a few studies are available on the carry-over of ZEA and
87 metabolites to edible organs of different animal species ([Dänicke et al., 2002](#); [Goyarts,](#)

88 [Dänicke, Valenta, & Ueberschär, 2007; Mirocha, Robison, Pawlosky, & Allen, 1982](#)). The
89 need for studies on the presence of ZEA and metabolites in animal-derived food was pointed
90 out in a ZEA risk assessment conducted by the French Food Safety Agency, to assess their
91 transfer into food products of animal origin ([AFSSA, 2006](#)).

92 Because mycotoxins are present at trace levels in animal products, sensitive and accurate
93 analytical methods are required. For ZEA and metabolites, enzyme-linked immunosorbent
94 assay (ELISA), liquid chromatography with fluorescence detection (LC-FLD) or single mass
95 spectrometry detection (LC-MS) have mostly been implemented ([Gajęcka et al., 2016; Iqbal,
96 Nisar, Asi, & Jinap, 2014; Pleadin et al., 2015; Zielonka et al., 2014, 2015](#)). For analysis of
97 FBs in animal tissues, several liquid chromatography-tandem mass spectrometry (LC-
98 MS/MS) methods have been developed in recent years. The combined implementation of LC-
99 MS/MS methods with stable isotope dilution assays (SIDA) enabled optimal compensation of
100 FB losses at all analytical steps ([Cao et al., 2018; Gazzotti et al., 2011; Schertz et al., 2018;
101 Sørensen, Mogensen, & Nielsen, 2010](#)). To our knowledge, this analytical strategy has not
102 been applied to the determination of ZEA and metabolites in animal products to date.

103 The aim of this work was to assess the carry-over of FBs, ZEA and metabolites from
104 contaminated feed to chicken liver and muscle to meet the needs expressed by risk assessors.
105 This required beforehand the development and validation of a reliable and effective SIDA-
106 LC-MS/MS method able to detect traces of FB1, FB2, ZEA, α -ZAL and α -ZEL in animal
107 tissues.

108

109 **2. Material and methods**

110 *2.1. Chemicals and reagents*

111 All solutions were prepared with analytical reagent-grade chemicals and ultrapure water (18.2
112 M Ω cm) obtained by purifying distilled water with a Milli-Q system associated with an Elix 5

113 pre-system (Millipore S.A., St Quentin-en-Yvelines, France). All standards (solutions, powder
114 and ^{13}C -labelled standards) were purchased from Biopure (Tulln, Austria). A mixed stock
115 solution of FB1 and FB2 (500 ng mL^{-1}) was prepared in methanol/water 50/50 from a $50\text{ }\mu\text{g}$
116 mL^{-1} FB1 standard solution and a $50\text{ }\mu\text{g mL}^{-1}$ FB2 standard solution. A ZEA stock solution
117 ($100\text{ }\mu\text{g mL}^{-1}$) was made by dissolving pure standard in acetonitrile. The ZEA concentration
118 was determined by absorption spectrometry ($\epsilon = 12623\text{ L mol}^{-1}\text{ cm}^{-1}$). A mixed ZEA, α -
119 zearalanol, and α -zearalenol working standard solution (500 ng mL^{-1} for each) was prepared
120 in methanol from the ZEA stock solution ($100\text{ }\mu\text{g mL}^{-1}$), α -ZAL ($10\text{ }\mu\text{g mL}^{-1}$), and α -ZEL (10
121 $\mu\text{g mL}^{-1}$) standard solutions.

122 The FB1 and FB2 (500 ng mL^{-1}) working standard solutions and the ZEA, α -ZAL, and α -
123 ZEL (500 ng mL^{-1}) working standard solution were combined to prepare a new mixed
124 working standard solution in water/methanol/formic acid 75/22/3 (v/v/v) (50 ng mL^{-1} for
125 each). This solution was used for the preparation of calibration standards.

126 A uniformly ^{13}C -labelled FB1 and FB2 stock standard solution (1200 ng mL^{-1}) was prepared
127 in methanol/water 50/50 (v/v) from U- $^{13}\text{C}_{34}$ -FB1 ($25\text{ }\mu\text{g mL}^{-1}$) and U- $^{13}\text{C}_{34}$ -FB2 ($10\text{ }\mu\text{g}$
128 mL^{-1}) internal standard solutions. A uniformly ^{13}C -labelled ZEA stock standard solution (880
129 ng mL^{-1}) was prepared in methanol from U- $^{13}\text{C}_{18}$ -ZEA internal standard solution ($25\text{ }\mu\text{g mL}^{-1}$)
130 ^{13}C -labelled stock standard solutions of U- $^{13}\text{C}_{34}$ -FB1 and U- $^{13}\text{C}_{34}$ -FB2 (1200 ng mL^{-1})
131 and ZEA (880 ng mL^{-1}) were combined to prepare a new mixed ^{13}C -labelled working
132 standard solution in water/methanol/formic acid 75/22/3 (v/v/v) with a concentration of 120
133 ng mL^{-1} for U- $^{13}\text{C}_{34}$ -FB1 and U- $^{13}\text{C}_{34}$ -FB2, and 88 ng mL^{-1} for U- $^{13}\text{C}_{18}$ -ZEA. This
134 solution was added to test samples, blank samples and calibration curves.

135 Calibration standards were prepared by diluting the combined mixed working standard
136 solution of FB1, FB2, ZEA, α -ZAL, and α -ZEL (50 ng mL^{-1} for each) and the mixed ^{13}C -
137 labelled working standard solutions of U- $^{13}\text{C}_{34}$ -FB1, U- $^{13}\text{C}_{34}$ -FB2 (120 ng mL^{-1} for both),

138 and U-[¹³C₁₈]-ZEA (88 ng mL⁻¹) with water/methanol/formic acid 75/22/3 (v/v/v). The
139 following FB1, FB2, ZEA, α-ZAL and α-ZEL concentrations were obtained: 1.0, 2.0, 3.0 and
140 4.0 ng mL⁻¹. The ¹³C-labelled internal standard concentrations were the same for all the levels
141 (3.0 ng mL⁻¹ for U-[¹³C₃₄]-FB1 and U-[¹³C₃₄]-FB2 and 2.2 ng mL⁻¹ for U-[¹³C₁₈]-ZEA).
142 Because no stable isotope labelled standards are available for ZEA metabolites, the U-[¹³C₁₈]-
143 ZEA was used as internal standard for the quantification of α-ZAL and α-ZEL. HPLC grade
144 acetonitrile, methanol and acetic acid were purchased from Fisher Scientific (Loughborough,
145 UK). Formic acid (Fisher Scientific) and ammonium formate (Alfa Aesar, Karlsruhe,
146 Germany) were of analytical grade. Phosphate buffered saline (PBS) tablets were purchased
147 from R-Biopharm AG (Darmstadt, Germany). PBS-buffer (pH 7.4) solution was prepared by
148 dissolving one tablet in 100 ml. In addition to calibration standard preparation,
149 water/methanol/formic acid 75/22/3 (v/v/v) was used to adjust the final volume before LC-
150 MS/MS injection. Ultra-pure grade carrier argon (Ar, 99.9999% pure) and nitrogen (N₂,
151 99.999% pure) were purchased from Linde Gas (Montereau-Fault-Yonne, France).

152

153 *2.2. Preparation of feed*

154 The composition and nutritional characteristics of diets distributed to chickens is indicated in
155 the [Table SD1](#). First, uncontaminated starting feed was given over the period 0-27 days. Then
156 growing-finishing feed contaminated with raw materials provided by the National Veterinary
157 School of Toulouse (France) was given over the period 28-84 days.

158 The levels of the contamination of mycotoxins in feed were defined to approach the guidance
159 levels set by the European Union (2006/576/EC) relative to a feedingstuff with a moisture
160 content of 12% for calves, dairy cattle, sheep (including lambs) and goats (including kids)
161 (0.5 mg ZEA kg⁻¹) and for poultry, calves (<4 months), lambs and kids (20 mg FBs kg⁻¹)
162 ([European Union, 2006](#)). Contaminated raw materials of 44.62 g of rice flour (containing 650

163 mg ZEA kg⁻¹) and 138 g of corn flour (containing 6800 mg FB1 kg⁻¹ and 1600 mg FB2 kg⁻¹)
164 were mixed individually with uncontaminated feed (58 kg; [Table SD1](#)). The rice or corn flour
165 were progressively mixed with the feed first in a mixer bowl (Hobart, Croissy Beaubourg,
166 France) containing 10 kg of feed then with a bigger feed mill mixer (Gondard, CTS, Le
167 Pontet, France) able to contain 400 kg feed. This mixture was then pelleted (diameter 2.5 mm)
168 with a feed mill pelletizer without using steam (CPM, CPM Europe B.V., Zaadam,
169 Netherlands). Special precautions had been taken to ensure the homogeneity of the
170 contaminated feed. The introduction of these small quantities of flour into the control feed
171 was not likely to significantly modify the feed value. The levels of contamination of the final
172 feed were measured by liquid chromatography with fluorescence detection (LC-FLD).
173 For ZEA, the feed level was determined following the NF EN 15792 standard. The ZEA level
174 measured was 0.40 ± 0.12 mg kg⁻¹, which represents 80% of the expected value.
175 For FBs, levels were determined following the NF EN 16006 standard. The FB1 and FB2
176 level measured was 12.7 ± 3.3 mg kg⁻¹, i.e. 64% of the expected value. FB1 represented 83%
177 of the sum (10.5 mg kg⁻¹) and FB2, 17% (2.2 mg kg⁻¹).
178 Control feed was also analysed. ZEA was quantified at a level of 0.027 ± 0.009 mg kg⁻¹. This
179 value is 15 times lower than the level measured in the ZEA contaminated feed. Neither FB1
180 nor FB2 were detected (LOD: 0.02 mg kg⁻¹ for FB1 and 0.03 mg kg⁻¹ for FB2).

181

182 *2.3. Experimental design*

183 The experimental design involved 24 male chickens (genus *Gallus* – slow growth rate strain
184 JA 657) with a weight of 35 ± 3 g, purchased from Boyé Accoupage (La Boissière-en-Gatîne,
185 France). All the chickens were vaccinated against Marek's disease and infectious bronchitis.
186 The light duration was set to 23 hours daily and the temperature to 30-31 °C up to the age of 3
187 days. These two parameters were then gradually decreased to reach 18 hours light daily and a

188 temperature of 19-20 °C from the age of 21 days. During the whole rearing period, water was
189 always available. Until the age of 28 days, the chickens were reared together in the same
190 room on wood shavings litter. Starter feed was distributed *ad libitum*. During the following 8
191 weeks of the exposure period (28 to 84 days of age), chickens of 28 days of age were
192 distributed into 3 groups presenting the same average body weight after a fasting period of 12
193 hours. There were 15 chickens in the control group and 9 chickens in each mycotoxins treated
194 groups. The first one was treated with ZEA and the second with FBs.

195 The animals were distributed into individual cages allowing monitoring of feed consumption
196 quantities. The grower feed was used until slaughter. It was distributed daily, at 68, 80, 87, 98,
197 106, 111, 126 and 153 g per day, respectively during weeks 5, 6, 7, 8, 9, 10, 11 and 12,
198 according to breeder recommendations. Feed refusals were collected individually and
199 weighed weekly to calculate the feed intake.

200 None of the treatments induced systematic signs of toxicity. However, one chicken died
201 during the 5th week in the ZEA and metabolites treated group, reducing the number of
202 individuals to eight. At the age of 84 days, after a 12 hour fasting period, all chickens were
203 weighed and slaughtered by electronarcosis, followed by bleeding.

204

205 2.4. Sample preparation

206

207 Since previous studies have shown that the liver is more strongly contaminated than muscle
208 (Mirocha et al., 1982; Tardieu, Auby, et al., 2008a; Tardieu, Bailly, et al., 2008b; Völkel et
209 al., 2011), the analytical strategy implemented consisted in first analysing the livers and if
210 their levels were higher than the LOQ, the muscles belonging to the same animals were also
211 analysed.

212

213 *2.5. Extraction*

214 For the extraction process, 250 μ L of ^{13}C -labelled combined working standard solution were
215 added to the test portion of 2.50 ± 0.01 g of muscle or liver sample placed into a 50 mL tube.
216 The tube content was mixed using a vortex mixer. 9.5 mL methanol/water 80/20 (v/v) were
217 added and the samples were homogenised at $10\,000 \pm 500$ rpm for 2 min using a Polytron[®]
218 (Kinematica AG, Luzern, Switzerland). After centrifugation at 9 000 g for 10 min at 3 °C, the
219 supernatant was placed in a volumetric flask of 20 mL. The extraction of mycotoxins from the
220 test portion with 9.5 mL methanol/water 80/20 (v/v), the centrifugation step, and the
221 supernatant transfer were repeated once. The solution was adjusted to 20 mL and filtered
222 using a 0.20 μ m polyester (PET) syringe filter (Chromafil[®], Macherey-Nagel GmbH & Co.
223 KG, Düren, Germany).

224

225 *2.6. Hexane washing of the extract*

226 Before FB clean-up, hexane washing of the extract was necessary. 5 mL of extract were added
227 to a 15 mL falcon tube and 5 mL of hexane were added. The tube was vortex mixed for 30 s.
228 After centrifugation at 3 000 g for 3 min at 20 °C, the upper hexane phase was removed and
229 the hexane washing was repeated once.

230

231 *2.7. Clean-up*

232 Two parallel immunoaffinity clean-up steps were implemented. Fumoniprep[®] immunoaffinity
233 clean-up (R-Biopharm AG, Darmstadt, Germany) was specific for FBs, and Easi-extract[®]
234 Zearalenone immunoaffinity clean-up (R-Biopharm AG, Darmstadt, Germany) was specific
235 for ZEA and metabolites. Both for FBs and for ZEA and metabolites, 8 mL of PBS were
236 added to 2 mL of the hexane washed extract in a 15 mL Falcon tube. These PBS solutions
237 were slowly (drop by drop) passed through their respective immunoaffinity columns, then

238 placed in a vacuum manifold. For FBs, the column was washed with 10 mL of PBS and for
239 ZEA and metabolites, the column was washed with 20 mL of PBS. Both columns were then
240 dried by pushing air through them with a syringe. Mycotoxins were finally eluted by applying
241 the backflushing technique with 3.0 mL methanol/acetic acid 98/2 (v/v).
242 The eluates were evaporated at 50 ± 5 °C under a gentle stream of nitrogen to 0.1 mL and
243 adjusted to 1 mL with water/methanol/formic acid 75/22/3 (v/v/v). The final extracts were
244 mixed using the vortex mixer, filtered (PET, 20 µm), and analysed by UHPLC-MS/MS.

245

246 *2.8. LC conditions*

247 The LC system was an Accela 1250 (Thermo Fisher Scientific, San Jose, CA, USA). Ultra-
248 high performance liquid chromatographic (UHPLC) separation of mycotoxins was performed
249 using a column with particles composed of a solid core and a porous shell. A Kinetex[®] C18
250 column (100 Å, 2.6 µm particle size, 50 x 2.1 mm) equipped with a Kinetex C18 security
251 guard cartridge (Phenomenex, Torrance, CA, USA) was used. For the FBs analysis, eluent A
252 was composed of water/formic acid 99.9/0.1 (v/v) and eluent B of methanol/water/formic acid
253 94.9/5/0.1 (v/v/v). Both eluents contained 0.5 mmol/L ammonium formate. For the ZEA and
254 metabolites analysis, eluent A was composed of water and eluent B of methanol/water 95/5
255 (v/v).

256 Both gradients were the same and programmed as follows: 25% B (initial), 25-100% B (6.4
257 min), 100% B (hold 1.1 min), 100-25% B (0.5 min), 25% B (hold 1 min). The column
258 effluent was transferred via a divert valve (Rheodyne, USA) either to the mass spectrometer
259 (between 1.5 and 5.5 min) or to waste. The total flow rate was 0.5 mL min^{-1} , while the
260 injection volume was 10 µL. The column temperature was maintained at 30 °C. A
261 chromatogram of a blank matrix spiked at the quantification limit for all mycotoxins (0.1 µg
262 kg^{-1}) is presented in **Fig. 1**.

263

264 *2.9. MS/MS conditions*

265 Detection was performed with a TSQ Vantage triple quadrupole mass spectrometer (Thermo
266 Fisher Scientific), equipped with an ElectroSpray Ionisation (ESI) source (HESI-II probe).
267 The mass spectrometer was operated in Selected Reaction Monitoring (SRM). The spray
268 voltage was -2625 V in negative mode and + 4000 V in positive. The source temperature was
269 set at 500 °C and capillary temperature at 350 °C. Nitrogen was used as the nebulising gas
270 with a sheath gas pressure of 50 (arbitrary unit) and an auxiliary gas pressure of 18 (arbitrary
271 unit). The collision gas was argon, with a gas pressure of 1.5 mTorr. One transition was used
272 for quantification (Q) and another as qualifier transition (q). The optimised SRM parameters
273 by injection of each compound are listed in [Table 1](#). A mass resolution of 0.7 Da full width at
274 half maximum (FWHD) was set on the first (Q1) and the third (Q3) quadrupoles. Instrument
275 control and data were handled by a computer equipped with TSQ Tune Master version 2.3.0,
276 Xcalibur version 2.1.0 and TraceFinder version 1.0.1 (Thermo Fisher Scientific).

277

278 *2.10. Method validation methodology*

279 The linearity of the method was statistically evaluated by the Fisher-Snedecor test with a
280 significance level $\alpha = 0.01$. The other performance characteristics were validated according to
281 the accuracy profile approach. This procedure summarises every validation element on a
282 single plot, giving a graphical representation of the method's performance. The validity
283 domain is defined between the lowest and the highest tested concentrations, with tolerance
284 limits (β -expectation limits) between the acceptance limits (λ). Tolerance limits were
285 calculated at each concentration level and take into account the bias, the repeatability, and the
286 intermediate precision. The limit of quantification (LOQ) was defined as the lowest
287 concentration level validated.

288

289 *2.11. Quality control*

290 To ensure reliable results, samples were analysed in batches including several internal quality
291 controls (IQC). When acceptance criteria were not met, results were discarded and samples
292 were re-analysed. A blank matrix was analysed in the same conditions as for all samples, to
293 check the absence of any contamination. The concentration had to be below the detection
294 limit. A blank matrix spiked at the quantification limit ($1 \mu\text{g kg}^{-1}$ for all the mycotoxins) was
295 prepared and analysed in the same conditions as for all the samples. Recoveries had to be
296 between 70 and 130%. All mycotoxins were quantified using bracketing calibration curves.
297 The determination coefficient (r^2) of the calibration curve had to be ≥ 0.98 and the slope
298 variation between two sets of bracketing calibration curves had to be below 15%. Variation of
299 the retention time in samples had to be below 5% in comparison to the standard retention
300 time. Before use, the capacity of each batch of immunoaffinity columns was checked. For
301 fumonisins, 200 ng of FB1 and 200 ng of FB2 were deposited at the same time on the
302 Fumoniprep[®] immunoaffinity columns, the capacity had to be higher than 100 ng for each
303 mycotoxin. The same quantities of ZEA, α -ZAL and α -ZEL were deposited at the same time
304 on the Easi-extract[®] Zearalenone immunoaffinity column. The capacity also had to be higher
305 than 100 ng for each.

306

307 *2.12. Statistical data analysis*

308 Statistical data analyses were performed using R studio software version 1.0.143 (R Studio,
309 Boston, USA).

310

311 **3. Results and discussion**

312 *3.1. Method validation*

313 For each mycotoxin, an accuracy profile was built following the NF V03-110 Standard
314 (AFNOR, 2010; Mermet & Granier, 2012). The main advantage of this approach is the
315 robustness of the validation methodology. It included a range of 3 concentration levels (1.0,
316 4.0 and 10.0 $\mu\text{g kg}^{-1}$) and 6 series repeated on different days over a period of 1 month for
317 ZEA and metabolites, and 4 months for FBs. For each series, 2 replicates of each of the 3
318 concentration levels were analysed in bracketing with five calibration standards to establish
319 the response function. Spiked samples followed the whole analytical procedure, including
320 extraction. The probability β was set to 80%, meaning that the risk of results falling outside β -
321 expectation tolerance intervals was below 20% on average. The acceptance limits (λ) were set
322 at $\pm 25\%$. For all the concentration levels, β -expectation tolerance intervals were within the
323 acceptability limits. The accuracy profile and the performance criteria obtained for each
324 mycotoxin are presented in Table 2, and as an example, the FB1 accuracy profile is presented
325 in Fig. 2. The method performances in terms of trueness and precision were very satisfactory.
326 Recoveries ranged from 92% to 107% for all toxins. The repeatability coefficient of variation,
327 CV_r , varied from 4.3% to 10.9% on the validity domain, and the intermediate precision
328 coefficient of variation, CV_{IP} , was between 4.3% and 13.1%. Because mycotoxins are present
329 at trace levels in animal matrices, sensitivity was the key parameter of the method. The
330 combined use of immunoaffinity purification, SIDA and UHPLC allowed us to achieve an
331 LOQ of 1.0 $\mu\text{g kg}^{-1}$ for all mycotoxins studied. To the best of our knowledge, the sensitivity
332 of the FB method is the best observed among the existing methods for meat products (Fodor
333 et al., 2008; Gazzotti et al., 2011; Guillamont et al., 2005; Meyer et al., 2003; Sørensen et al.,
334 2010; Szabó-Fodor et al., 2015; Tardieu, Auby, et al., 2008a). For ZEA, method sensitivity is
335 equal to that obtained with several other methods (Fang, Chen, & Guo, 2002; Goyarts et al.,
336 2007; Yan et al., 2018), and is very close to the most sensitive ones (0.4 to 0.5 $\mu\text{g kg}^{-1}$) (Chen
337 et al., 2013; Pleadin et al., 2015; Zöllner et al., 2002). Importantly, in this study, the LOQ was

338 assessed by a very robust method and was defined as the lowest validated level. Finally, the
339 validity domain of the analytical method ranged from 1.0 to 10.0 $\mu\text{g kg}^{-1}$ for ZEA, α -ZAL and
340 α -ZEL and from 1.0 to 16.0 $\mu\text{g kg}^{-1}$ for FB1 and FB2.

341

342 3.2. Carry-over of ZEA to the liver

343 ZEA, α -ZAL and α -ZEL levels were assessed in the 15 control samples and the 8 samples of
344 the ZEA treated group. ZEA and α -ZAL were not detected in any samples from the control or
345 treated groups (Table 3). α -ZEL was not detected in control samples, but levels between the
346 limit of detection (0.3 $\mu\text{g kg}^{-1}$) and the limit of quantification (1.0 $\mu\text{g kg}^{-1}$) were found in 5 out
347 of 8 samples in the ZEA treated group, with concentrations estimated to be in the range 0.4 –
348 0.8 $\mu\text{g kg}^{-1}$. The non-detection of zearalenone could be explained by the fact that ZEA is
349 largely and rapidly eliminated in excreta (Mirocha et al., 1982). Therefore, in this study,
350 excreta were collected during the last two days before animal slaughter, and ZEA was
351 analysed by applying the NF EN 15792 Standard. A level of $0.270 \pm 0.090 \text{ mg kg}^{-1}$ was
352 quantified for the chickens exposed to the contaminated diet, whereas a level 23 times lower
353 was measured for the chickens exposed to the control feed ($0.012 \pm 0.006 \text{ mg kg}^{-1}$). These
354 levels confirm the considerable elimination of ZEA in excreta. This mycotoxin is also rapidly
355 metabolised by three major routes in mammals: hydroxylation resulting, among others, in the
356 formation of α -ZEL; glucuronidation in the small intestine and the liver; and cytochrome
357 P450-mediated oxidation which produces catechol metabolites (EFSA, 2011). The detection
358 of α -ZEL in several samples supports this metabolism process.

359 Dänicke et al. (2002) also studied the carry-over of ZEA to laying hen tissues following hen
360 exposure to ZEA feed with 4 times higher contamination than in the current study (1.6 mg kg^{-1}).
361 Without β -glucuronidase and aryl-sulfatase treatment of the samples, and when no
362 Mycofix[®]Plus was added to the feed, similarly to our results, ZEA was not detected in breast

363 meat or in the liver, whether α -ZEL was quantified in livers. To quantify the extent of carry-
364 over, a carry-over factor (CF) was calculated to measure the degree of release of the
365 mycotoxin from the feed matrix in the digestive tract, and its absorption, distribution
366 metabolism and elimination (**Equation (1)**).

367

$$368 \text{ Carry-over factor (CF)} = \text{Toxin level in tissue } (\mu\text{g kg}^{-1}) / \text{Toxin level in diet } (\mu\text{g kg}^{-1}) \quad (1)$$

369

370 Since our results in tissues were below the limit of quantification, it was not possible to
371 calculate a CF; consequently, we calculated this factor between α -ZEL concentrations in the
372 liver and ZEA concentrations in feed from the [Dänicke et al. \(2002\)](#) study. This CF amounted
373 to 0.0008 (1.3 $\mu\text{g kg}^{-1}$ in liver divided by 1580 $\mu\text{g kg}^{-1}$ in feed). When we applied this carry-
374 over factor to the level of ZEA in the contaminated feed used in this study, we obtained an
375 estimated α -ZEL level in the liver of 0.3 $\mu\text{g kg}^{-1}$ (400 $\mu\text{g kg}^{-1}$ x 0.0008). This value is equal to
376 the LOD (0.3 $\mu\text{g kg}^{-1}$) of the present method. As previously described, among the obtained
377 results, 5 out of 8 were between the LOD and the LOQ (1.0 $\mu\text{g kg}^{-1}$) and the 3 others were
378 less than or equal to the LOD. Therefore, these results are consistent with those reported by
379 [Dänicke et al. \(2002\)](#).

380

381 *3.3. Carry-over of FBs to the liver*

382 FB1 and FB2 levels were assessed in the 15 control samples and in the 9 samples of the FB
383 treated group. These mycotoxins were not detected in the control samples (LOD = 0.3 $\mu\text{g kg}^{-1}$)
384 (Table 4). FB1 was quantified in all the 9 samples of the FB treated group with a mean
385 level of $30.3 \pm 14.8 \mu\text{g kg}^{-1}$ and a median of $25.8 \mu\text{g kg}^{-1}$ (minimum and maximum of 17.9
386 and $65.5 \mu\text{g kg}^{-1}$) (Fig. 3). FB2 was also quantified in all the samples (mean: $2.3 \pm 1.1 \mu\text{g kg}^{-1}$;
387 median $2.1 \mu\text{g kg}^{-1}$), with levels ranging from 1.2 to $4.9 \mu\text{g kg}^{-1}$ (Fig. 3). The mean level

388 observed in the liver, 13 times lower for FB2 compared to FB1, may be partially explained by
389 the 5 times lower concentration in feed. Samples with high levels of FB1 also had high levels
390 of FB2. In order to check whether there is a correlation between the FB1 and FB2 levels
391 measured, a Pearson statistical test with a significance level $\alpha = 5\%$ was applied. A significant
392 correlation was found (p value: < 0.001 ; $y = 12.8x + 0.9$; $R = 0.97$) (Fig. 4). This correlation
393 suggests that even though there was a wide difference in the levels measured between
394 individuals (CV = 46% for FB1 and 43% for FB2), the carry-over of both toxins seems to be
395 very similar between animals.

396 Liver carry-over factors were calculated and compared with previous studies. For FB1, CF
397 was 0.003. This value is very close to the CF of 0.002 reported by [Del Bianchi, Oliveira,](#)
398 [Albuquerque, Guerra, and Correa \(2005\)](#) in broiler chickens, after prolonged FB1 oral
399 administration (10 mg kg^{-1} of feed) from 21 to 41 days of age.

400 [Tardieu et al. \(2008b\)](#) also studied FB1 persistence in poultry tissues after exposure to a diet
401 containing fumonisins at 5, 10 and $20 \text{ mg FB1 + FB2 kg}^{-1}$. After an interval of 8 h between
402 the last ingestion of feed and slaughter of the animals, FB1 concentrations were measured in
403 liver tissues. CFs were slightly higher than the values observed in chickens, with values
404 ranging between 0.004 and 0.007 for the three levels tested. Moreover, with feed less than two
405 times more contaminated, FB levels measured in the liver were about four times higher than
406 the levels measured in the present work. [Tardieu et al. \(2008b\)](#) maintained an interval of 8
407 hours between the last ingestion of feed and slaughter, whereas in the present study, the
408 interval was 12 hours. Therefore, the difference in terms of fasting period duration could
409 explain the difference in the results obtained. The implementation of a fasting period is a
410 practice also carried out in slaughterhouses to improve the safety of animal products by
411 reducing the incidence of carcass contamination due to rupture or laceration of the intestines,
412 intestinal overflow, or faecal discharge from the rectum.

413 To our knowledge, FB2 carry over has so far never been reported in animal tissues. In the
414 present study, we observed a CF three times lower than for FB1 (CF = 0.001 vs. 0.003).
415 Considering the highest FB1 and FB2 concentrations in livers measured in the present study
416 ($70.4 \mu\text{g FB1} + \text{FB2 kg}^{-1}$), a person of 70 kg would have to eat 1.0 kg per day of poultry liver
417 to exceed the FB tolerable daily intake (TDI) of $1.0 \mu\text{g kg}^{-1}$ bw, and 2.3 kg per day of poultry
418 liver for the mean value measured ($32.6 \mu\text{g FB1} + \text{FB2 kg}^{-1}$) (EFSA, 2018). It is important to
419 note that the levels measured in feed during the experiment reached only 64% of the FB
420 guidance level defined for poultry. If consumption data were available for poultry livers, it
421 could be interesting for this feed contamination level to measure the relative contribution of
422 poultry liver consumption to the total exposure of humans to FBs.

423 In the same way as for ZEA, excretion via the excreta is considered the main route of
424 elimination of FBs (Bailly & Guerre, 2009). In the present study, FB excreta analyses were
425 carried out following the NF EN 16006 Standard. An FB excreta level of 15.2 mg kg^{-1} (FB1:
426 $13.0 \pm 3.9 \text{ mg kg}^{-1}$, FB2: $2.2 \pm 0.7 \text{ mg kg}^{-1}$) was measured for chickens exposed to the
427 contaminated diet and a level of 0.065 mg kg^{-1} (FB1: $0.065 \pm 0.033 \text{ mg kg}^{-1}$, FB2 $\leq 0.03 \text{ mg}$
428 kg^{-1}) was found for chickens exposed to the control feed. The comparison of these levels with
429 the levels measured in the contaminated feed points to significant elimination of FBs in
430 excreta.

431

432 *3.4. Determination of FBs in chicken fillets and comparison with the concentration found in* 433 *livers*

434 Given that FBs were quantified in all livers in the FB treated group, the chicken muscles
435 belonging to the same animals were also analysed. Results showed that for each sample “liver
436 – muscle” pair, the FB1 and FB2 concentrations were systematically lower in the muscle than
437 in the liver (Table 4). On average, the FB1 levels measured in meat were 16 times lower than

438 in the liver, and no significant correlation was highlighted between the levels measured for
439 both tissues ($R = 0.08$). In a previous study, [Tardieu et al. \(2008a\)](#) studied the FB1
440 contamination of duck tissues after a single oral dose of 5 and 40 mg kg⁻¹ body weight. Liver
441 levels were between 5 and 18 times higher than muscle levels. Considering the differences in
442 the experimental protocols implemented and in the avian species studied, these results
443 compare well with results presented here. In another study, [Tardieu et al. \(2008b\)](#) did not
444 detect FB1 in muscles of turkeys fed with 20 mg FB1 + FB2 kg⁻¹ feed for 9 weeks, whereas
445 FB1 levels in livers of the same animals reached a mean value of 117 µg kg⁻¹. The absence of
446 FB1 in meat in this former study and its detection in the present work could be explained by
447 the large difference in terms of method sensitivity. The limit of detection of the method
448 presented here is about 33 times lower. Considering the low levels measured in muscles (all <
449 5.0 µg kg⁻¹), high sensitivity was essential to be able to detect and quantify FB1. FB2 was
450 detected but not quantified in one sample and was < LOD for the other eight samples.

451

452 **4. Conclusions**

453 A sensitive and accurate SIDA-UHPLC-MS/MS method using immunoaffinity clean-up was
454 developed, validated and implemented to study the carry-over of FBs and ZEA and
455 metabolites to meat products after exposure of broiler chickens to contaminated feed. The
456 analysis of livers from chickens exposed to a contaminated diet revealed the presence of α -
457 ZEL in several samples. The presence of this compound, more oestrogenic than ZEA, and the
458 absence of ZEA indicate excreta elimination and metabolism processes of ZEA by
459 hydroxylation. FB1 and FB2, which have chronic toxicity in humans, were quantified in livers
460 and muscles. From these results, it was possible to calculate carry-over factors from feed to
461 poultry livers. Finally, FB levels in muscle were also investigated and compared with the
462 levels measured in the livers belonging to the same animals. These new carry-over data will

463 contribute to a better understanding of mycotoxin transfer from feed to meat products.
464 Considering the FB levels measured in poultry tissues, this study could be useful to assess
465 whether the current FB guidance value for poultry is sufficiently protective for consumers.
466 Moreover, given the high excreta elimination of FBs and ZEA, the duration of fasting could
467 be considered an effective parameter for animal product decontamination and deserves further
468 investigation. In future studies, it could also be interesting to adopt this type of approach for
469 the other analogues of FB (FB3 to FB6) and their modified forms, to respond to the recent call
470 for additional data expressed by the European Food Safety Agency (EFSA).

471

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475

476 **Declarations of interest**

477 None.

478

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482

483 **Figure Captions**

484 **Fig. 1:** Chromatogram obtained for a chicken liver sample spiked at the quantification limit
485 ($1.0 \mu\text{g kg}^{-1}$ for all mycotoxins): a) quantification transition (Q); b) qualifier transition (q).

486 **Fig. 2:** Accuracy profile of FB1 ($\beta = 80\%$; $\lambda = \pm 25\%$).

487 **Fig. 3:** Box Plot of FB1 and FB2 obtained from levels measured in chicken livers from the FB
488 treated group.

489 **Fig. 4:** Correlation between FB1 and FB2 levels measured in chicken livers from the FB
490 treated group. The grey shaded area represents the confidence interval.

491

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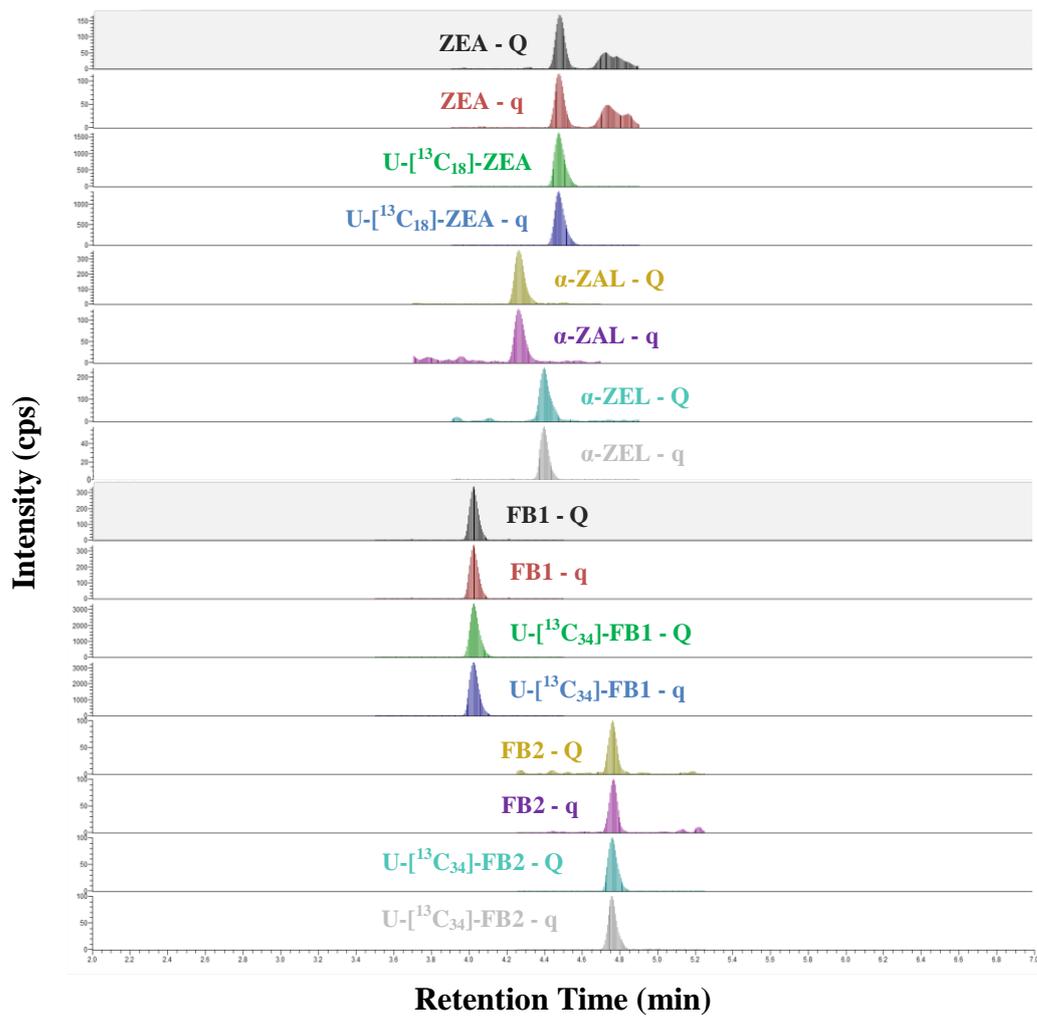
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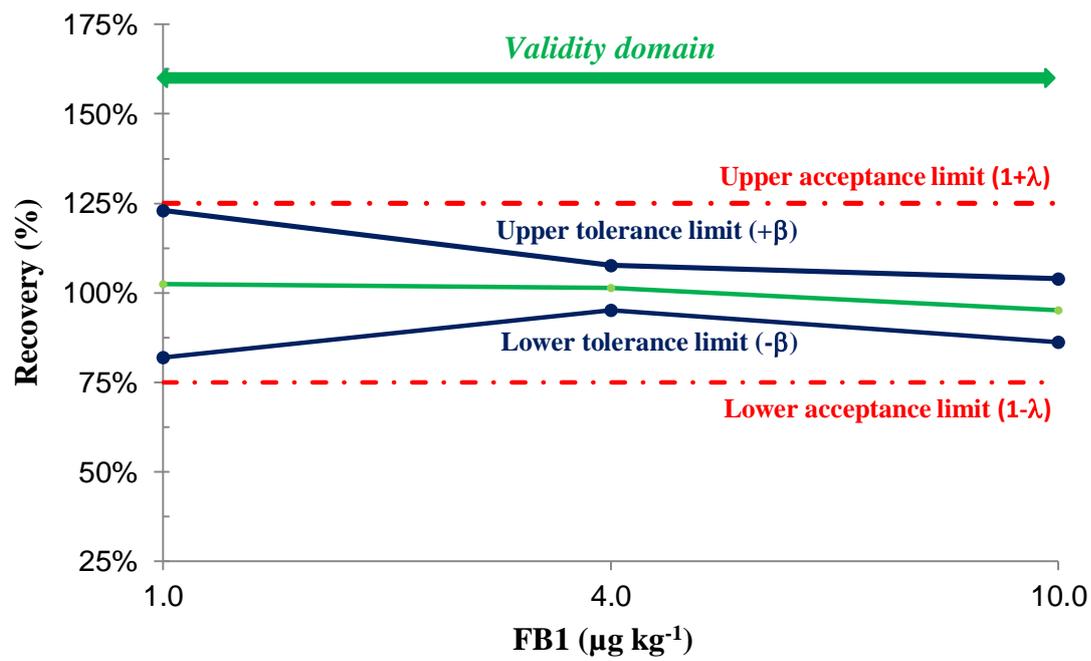
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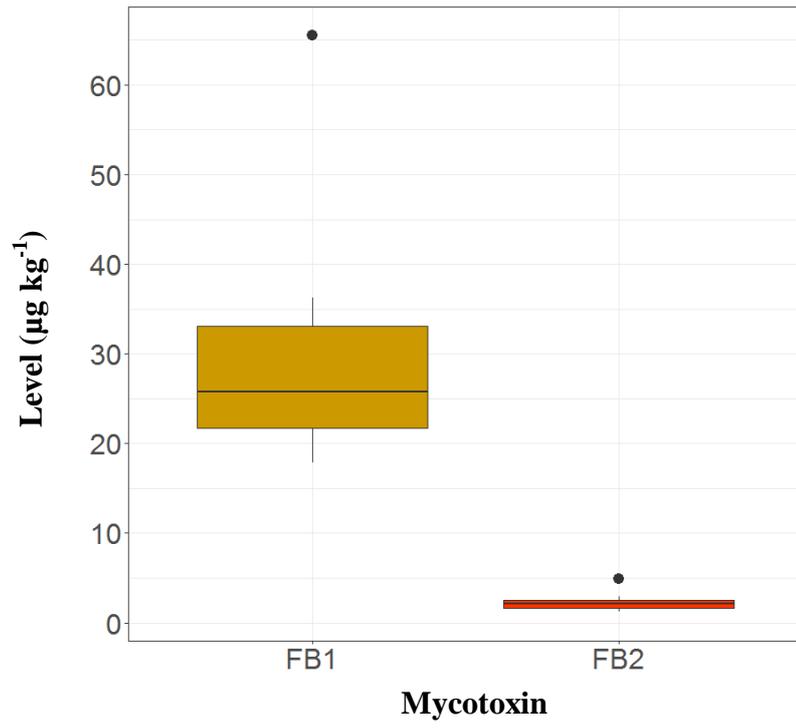
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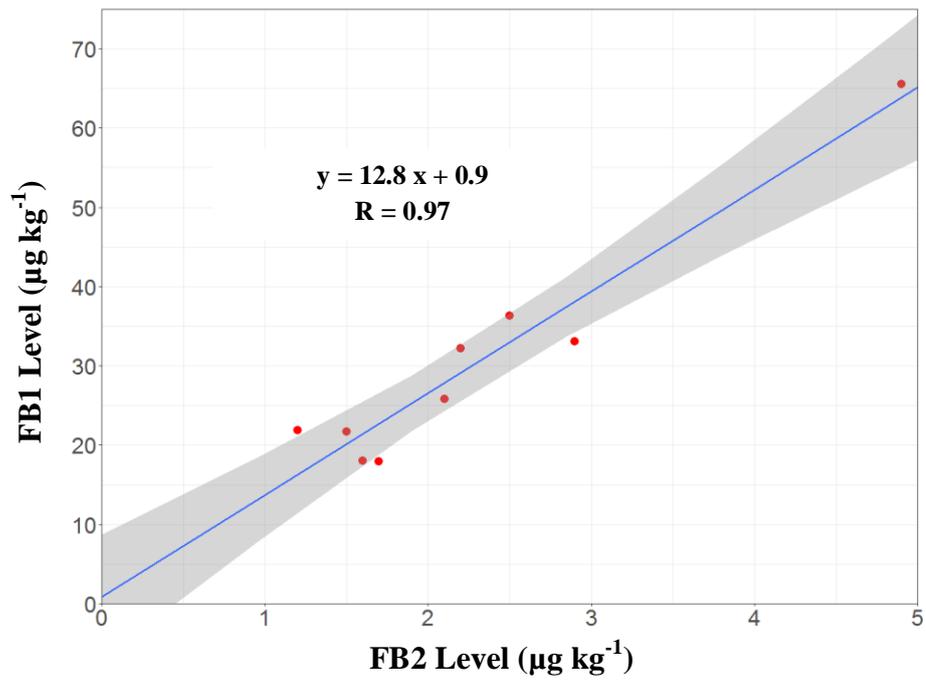
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628









1 **Table 1:** Optimised MS/MS parameters

Compounds	Ionisation mode	S-lens voltage (V)	Precursor ion (m/z)	Collision energy (V)	Product ion (m/z)
FB1	ESI+	142	722.4	38	334.3 (Q)
		137		34	352.3 (q)
U- ¹³ C ₃₄]-FB1	ESI+	142	756.4	34	374.3 (Q)
		137		36	356.3 (q)
FB2	ESI+	142	706.4	34	336.3 (Q)
		137		36	318.3 (q)
U- ¹³ C ₃₄]-FB2	ESI+	142	740.4	34	358.3 (Q)
		137		36	340.3 (q)
ZEA	ESI-	117	317.1	24	175.0 (Q)
		117		30	131.1 (q)
U- ¹³ C ₁₈]-ZEA	ESI-	55	335.1	26	185.1 (Q)
		55		32	140.1 (q)
α -ZAL	ESI-	118	321.2	23	277.2 (Q)
		118		22	303.2 (q)
α -ZEL	ESI-	118	319.1	21	277.2 (Q)
		118		26	303.2 (q)

2

3 **Table 2:** Method performance criteria

	Levels ($\mu\text{g kg}^{-1}$)	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	Accuracy		
				Trueness	Precision	
				Recovery (%)	Repeatability (% RSD)	Intermediate precision (% RSD)
ZEA	1.0	0.3	1.0	102	6.1	13.1
	4.0			101	4.3	4.3
	10.0			95	5.8	6.5
α - ZAL	1.0	0.3	1.0	102	5.8	10.1
	4.0			105	6.7	11.7
	10.0			101	3.8	9.6
α - ZEL	1.0	0.3	1.0	107	8.9	9.9
	4.0			96	5.5	7.0
	10.0			92	5.2	5.6
FB1	1.0	0.3	1.0	99	6.5	12.2
	4.0			95	7.8	10.6
	16.0			102	6.2	10.3
FB2	1.0	0.3	1.0	103	7.9	9.4
	4.0			94	10.9	10.9
	16.0			102	10.1	10.1

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6 **Table 3:** Results for ZEA and its associated metabolites in chicken livers in the ZEA treated
 7 group

	Animal Number	Levels in liver ($\mu\text{g kg}^{-1}$)		
		ZEA	α -ZAL	α -ZEL
ZEA treated group	787	\leq LOD ^a	\leq LOD ^a	\leq LOD ^a
	800	\leq LOD ^a	\leq LOD ^a	\leq LOQ (0.4) ^b
	811	\leq LOD ^a	\leq LOD ^a	\leq LOQ (0.8) ^b
	863	\leq LOD ^a	\leq LOD ^a	\leq LOQ (0.5) ^b
	877	\leq LOD ^a	\leq LOD ^a	\leq LOD ^a
	895	\leq LOD ^a	\leq LOD ^a	\leq LOD ^a
	908	\leq LOD ^a	\leq LOD ^a	\leq LOQ (0.4) ^b
	914	\leq LOD ^a	\leq LOD ^a	\leq LOQ (0.6) ^b

a: LOD = 0.3 $\mu\text{g kg}^{-1}$

b: LOQ = 1.0 $\mu\text{g kg}^{-1}$; results comprised between LOD and LOQ were calculated and are presented in brackets.

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12 **Table 4:** Results for FBs in chicken livers and muscle in the FB treated group

	Animal Number	Levels in liver ($\mu\text{g kg}^{-1}$)		Levels in muscle ($\mu\text{g kg}^{-1}$)	
		FB1	FB2	FB1	FB2
FB treated group	821	36.3	2.5	1.3	$\leq \text{LOD}^{\text{a}}$
	846	32.2	2.2	1.3	$\leq \text{LOD}^{\text{a}}$
	855	18.0	1.6	$\leq \text{LOQ (0.5)}^{\text{b}}$	$\leq \text{LOD}^{\text{a}}$
	860	17.9	1.7	1.2	$\leq \text{LOD}^{\text{a}}$
	861	65.5	4.9	1.3	$\leq \text{LOD}^{\text{a}}$
	872	33.1	2.9	2.4	$\leq \text{LOD}^{\text{a}}$
	886	21.9	1.2	1.1	$\leq \text{LOD}^{\text{a}}$
	897	25.8	2.1	4.7	$\leq \text{LOQ (0.5)}^{\text{b}}$
	918	21.7	1.5	2.9	$\leq \text{LOD}^{\text{a}}$

13 a: $\text{LOD} = 0.3 \mu\text{g kg}^{-1}$

14 b: $\text{LOQ} = 1.0 \mu\text{g kg}^{-1}$; results comprised between LOD and LOQ were calculated and are presented
 15 in brackets