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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Material and methods

2.1. Chemicals and reagents

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

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

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

A uniformly ^{13}C-labelled FB1 and FB2 stock standard solution (1200 ng mL^{-1}) was prepared in methanol/water 50/50 (v/v) from U-[$^{13}C_{34}$]-FB1 (25 µg mL^{-1}) and U-[$^{13}C_{34}$]-FB2 (10 µg mL^{-1}) internal standard solutions. A uniformly ^{13}C-labelled ZEA stock standard solution (880 ng mL^{-1}) was prepared in methanol from U-[$^{13}C_{18}$]-ZEA internal standard solution (25 µg mL^{-1}). ^{13}C-labelled stock standard solutions of U-[$^{13}C_{34}$]-FB1 and U-[$^{13}C_{34}$]-FB2 (1200 ng mL^{-1}) and ZEA (880 ng mL^{-1}) were combined to prepare a new mixed ^{13}C-labelled working standard solution in water/methanol/formic acid 75/22/3 (v/v/v) with a concentration of 120 ng mL^{-1} for U-[$^{13}C_{34}$]-FB1 and U-[$^{13}C_{34}$]-FB2, and 88 ng mL^{-1} for U-[$^{13}C_{18}$]-ZEA. This solution was added to test samples, blank samples and calibration curves.

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

138 and U-[$^{13}C_{18}$]-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 ^{13}C-labelled internal standard concentrations were the same for all the levels
141 (3.0 ng mL^{-1} for U-[$^{13}C_{34}$]-FB1 and U-[$^{13}C_{34}$]-FB2 and 2.2 ng mL^{-1} for U-[$^{13}C_{18}$]-ZEA).
142 Because no stable isotope labelled standards are available for ZEA metabolites, the U-[$^{13}C_{18}$]-
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 (N_2,
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

mg ZEA kg^{-1}) and 138 g of corn flour (containing 6800 mg FB1 kg^{-1} and 1600 mg FB2 kg^{-1}) were mixed individually with uncontaminated feed (58 kg; Table SD1). The rice or corn flour were progressively mixed with the feed first in a mixer bowl (Hobart, Croissy Beaubourg, France) containing 10 kg of feed then with a bigger feed mill mixer (Gondard, CTS, Le Pontet, France) able to contain 400 kg feed. This mixture was then pelleted (diameter 2.5 mm) with a feed mill pelletizer without using steam (CPM, CPM Europe B.V., Zaadam, Netherlands). Special precautions had been taken to ensure the homogeneity of the contaminated feed. The introduction of these small quantities of flour into the control feed was not likely to significantly modify the feed value. The levels of contamination of the final feed were measured by liquid chromatography with fluorescence detection (LC-FLD).

For ZEA, the feed level was determined following the NF EN 15792 standard. The ZEA level measured was 0.40 ± 0.12 mg kg^{-1}, which represents 80% of the expected value.

For FBs, levels were determined following the NF EN 16006 standard. The FB1 and FB2 level measured was 12.7 ± 3.3 mg kg^{-1}, i.e. 64% of the expected value. FB1 represented 83% of the sum (10.5 mg kg^{-1}) and FB2, 17% (2.2 mg kg^{-1}).

Control feed was also analysed. ZEA was quantified at a level of 0.027 ± 0.009 mg kg^{-1}. This value is 15 times lower than the level measured in the ZEA contaminated feed. Neither FB1 nor FB2 were detected (LOD: 0.02 mg kg^{-1} for FB1 and 0.03 mg kg^{-1} for FB2).

2.3. Experimental design

The experimental design involved 24 male chickens (genus Gallus – slow growth rate strain JA 657) with a weight of 35 ± 3 g, purchased from Boyé Accouvage (La Boissière-en-Gatîne, France). All the chickens were vaccinated against Marek's disease and infectious bronchitis. The light duration was set to 23 hours daily and the temperature to 30-31 °C up to the age of 3 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 µL 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 ± 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 µm 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

placed in a vacuum manifold. For FBs, the column was washed with 10 mL of PBS and for ZEA and metabolites, the column was washed with 20 mL of PBS. Both columns were then dried by pushing air through them with a syringe. Mycotoxins were finally eluted by applying the backflushing technique with 3.0 mL methanol/acetic acid 98/2 (v/v).

The eluates were evaporated at 50 ± 5 °C under a gentle stream of nitrogen to 0.1 mL and adjusted to 1 mL with water/methanol/formic acid 75/22/3 (v/v/v). The final extracts were mixed using the vortex mixer, filtered (PET, 20 µm), and analysed by UHPLC-MS/MS.

2.8. LC conditions

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

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

2.11. Quality control

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

2.12. Statistical data analysis

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

3. Results and discussion

3.1. Method validation

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

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

3.2. Carry-over of ZEA to the liver

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

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

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

Carry-over factor (CF) = Toxin level in tissue (µg kg^{-1}) / Toxin level in diet (µg kg^{-1}) (1)

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

3.3. Carry-over of FBs to the liver

FB1 and FB2 levels were assessed in the 15 control samples and in the 9 samples of the FB treated group. These mycotoxins were not detected in the control samples (LOD = 0.3 µg kg^{-1}) (Table 4). FB1 was quantified in all the 9 samples of the FB treated group with a mean level of 30.3 ± 14.8 µg kg^{-1} and a median of 25.8 µg kg^{-1} (minimum and maximum of 17.9 and 65.5 µg kg^{-1}) (Fig. 3). FB2 was also quantified in all the samples (mean: 2.3 ± 1.1 µg kg^{-1}; median 2.1 µg kg^{-1}), with levels ranging from 1.2 to 4.9 µg 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 = 12.8 x + 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 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.

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

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

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

Given that FBs were quantified in all livers in the FB treated group, the chicken muscles belonging to the same animals were also analysed. Results showed that for each sample "liver – muscle" pair, the FB1 and FB2 concentrations were systematically lower in the muscle than 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 metabolisation 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

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

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Declarations of interest

None.

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Figure Captions

Fig. 1: Chromatogram obtained for a chicken liver sample spiked at the quantification limit (1.0 µg kg^{-1} for all mycotoxins): a) quantification transition (Q); b) qualifier transition (q).

Fig. 2: Accuracy profile of FB1 (β = 80%; λ = ± 25%).

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

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

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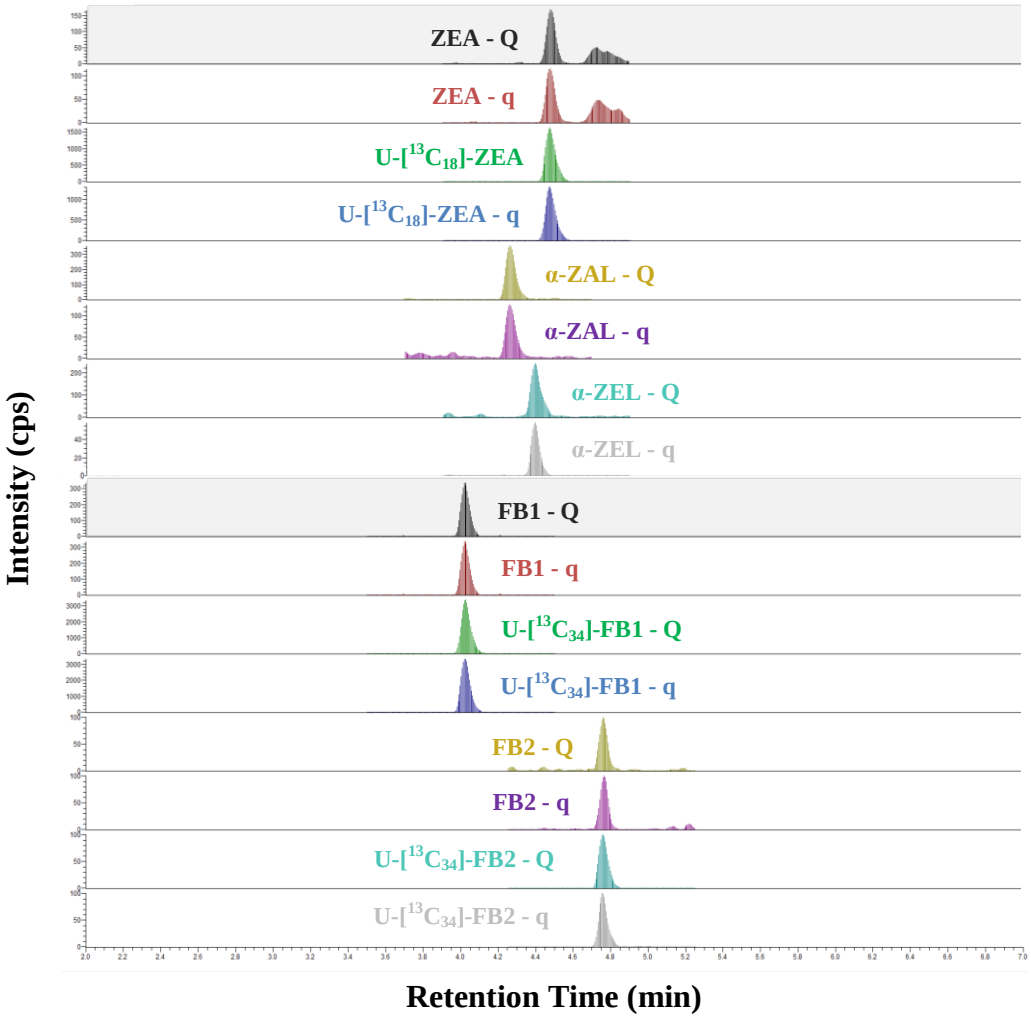
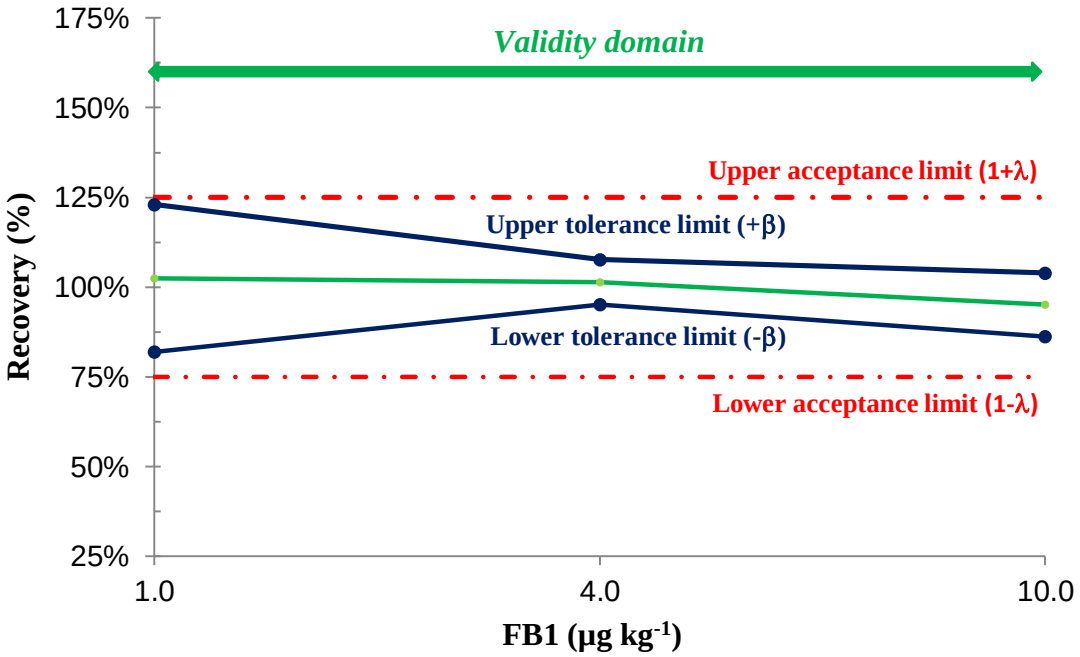
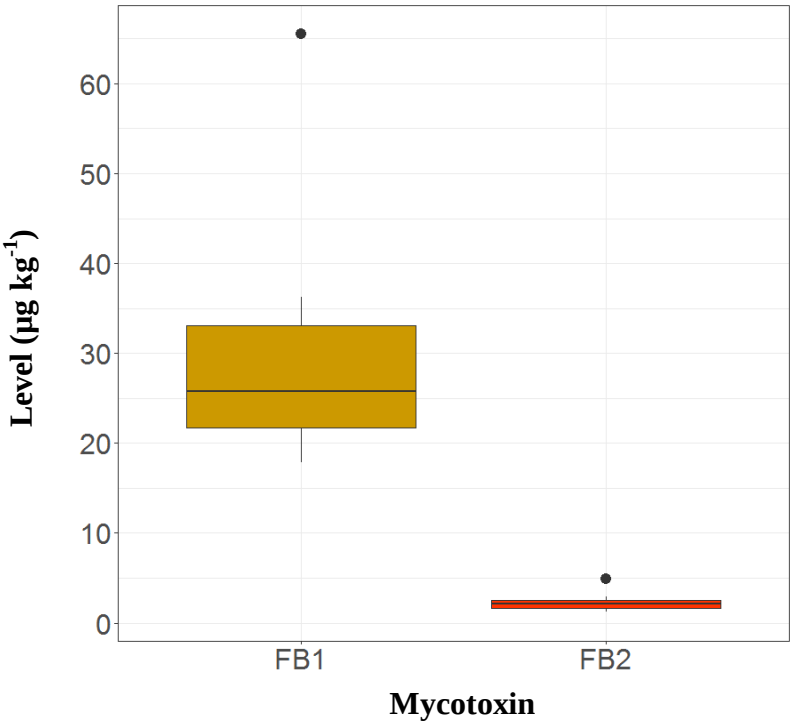

ZEA - Q
ZEA - q
U-[13C18]-ZEA
U-[13C18]-ZEA - q
α-ZAL - Q
α-ZAL - q
α-ZEL - Q
α-ZEL - q
FB1 - Q
FB1 - q
U-[13C34]-FB1 - Q
U-[13C34]-FB1 - q
FB2 - Q
FB2 - q
U-[13C34]-FB2 - Q
U-[13C34]-FB2 - q
Intensity (cps)
Retention Time (min)


175%
150%
125%
100%
75%
50%
25%
Recovery (%)
Validity domain
Upper acceptance limit (1+λ)
Upper tolerance limit (+β)
Lower tolerance limit (-β)
Lower acceptance limit (1-λ)
1.0
4.0
10.0
FB1 (µg kg-1)


60
50
40
30
20
10
0
Level (µg kg-1)
FB1
FB2
Mycotoxin

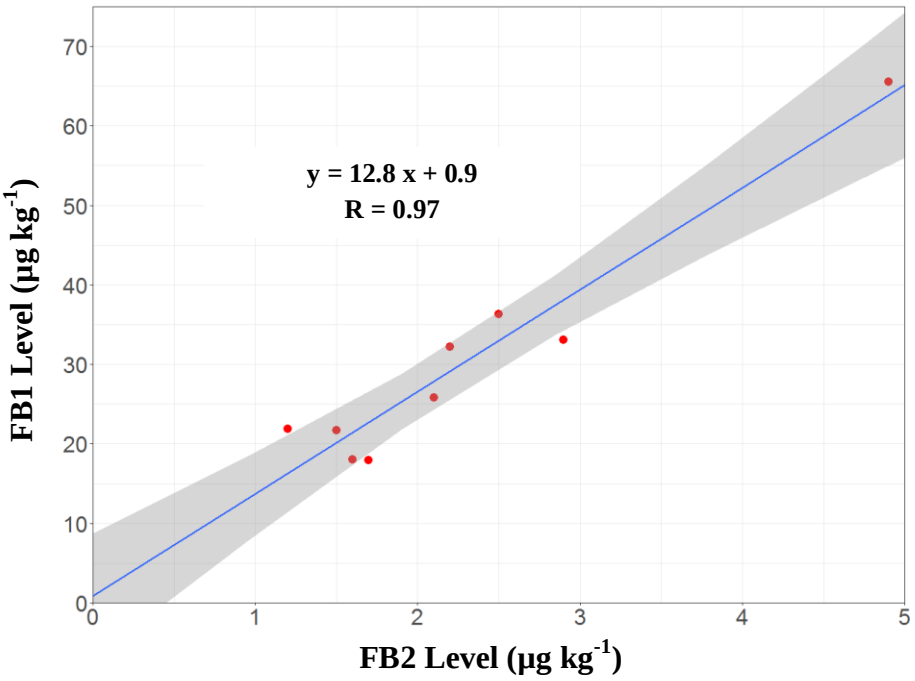

y = 12.8 x + 0.9
R = 0.97
FB1 Level (µg kg-1)
FB2 Level (µg kg-1)
0
10
20
30
40
50
60
70
0
1
2
3
4
5

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)

Table 2: Method performance criteria

	Levels (µg kg^{-1})	LOD (µg kg^{-1})	LOQ (µg 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

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

	Animal Number	Levels in liver (µg kg^{-1})		
		ZEA	**α-ZAL**	**α-ZEL**
ZEA treated group	787	≤ LOD[a]	≤ LOD[a]	≤ LOD[a]
	800	≤ LOD[a]	≤ LOD[a]	≤ LOQ (0.4) [b]
	811	≤ LOD[a]	≤ LOD[a]	≤ LOQ (0.8) [b]
	863	≤ LOD[a]	≤ LOD[a]	≤ LOQ (0.5) [b]
	877	≤ LOD[a]	≤ LOD[a]	≤ LOD[a]
	895	≤ LOD[a]	≤ LOD[a]	≤ LOD[a]
	908	≤ LOD[a]	≤ LOD[a]	≤ LOQ (0.4) [b]
	914	≤ LOD[a]	≤ LOD[a]	≤ LOQ (0.6) [b]

a: LOD = 0.3 µg kg^{-1}

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

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

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

a: LOD = 0.3 µg kg^{-1}

b: LOQ = 1.0 µg kg^{-1}; results comprised between LOD and LOQ were calculated and are presented in brackets