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BIOACCUMULATION RISK ASSESSMENT OF AFLATOXIN B1, OCHRATOXIN AND FUMONISIN B1 IN *TENEBRIO MOLITOR* LARVAE

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Mancini A., Dreassi E., Botta M., Tarchi F., Francardi V.- Bioaccumulation risk assessment of aflatoxin B1, ochratoxin A and fumonisin B1 in *T. molitor* larvae.

The industrial farming of edible insects for food and feed generally employs cultivated plants and derivatives as rearing diets. These feed substrates may contain toxins produced by different species of fungi which cause adverse health effects to consumers. Frequently found in cereals and cereal products, mycotoxins aflatoxins, ochratoxins and fumonisins are harmful to human and animal health. In this study the uptake of aflatoxin B1 (AFB1), ochratoxin A (OTA) and fumonisin B1 (FB1) and their elimination rates were investigated in *T. molitor* larvae fed on cereal-based diets contaminated with two concentration levels for each mycotoxin. New analytical methods for extraction and quantification of AFB1, OTA and FB1 residues in larvae were developed and validated.
It has been demonstrated that *T. molitor* larvae did not accumulate in detectable or dangerous concentrations the three tested mycotoxins at the two tested concentration levels and that a fasting period of 24 hours ensured a sufficient elimination rate of AFB1, OTA and FB1 residues. These results represent a preliminary investigation to define an appropriate quality control procedure to assess the safe use of *T. molitor* for food and feed purposes.

KEY WORDS: Edible insects, mycotoxins, diet contamination, *T. molitor*, food and feed.

INTRODUCTION

Currently, there is a rising interest in the use of edible insects as an alternative source for food and/or feed (VAN HUIS *et al*., 2013; YI *et al*., 2013; BELLUCO *et al*., 2013; IPIFF, 2019), as they are recognized to fully meet the requirements for essential nutrients and offer ecological and economic benefits for industrial production. Massive farming of edible insects involves the use of nutrition substrates derived from crop production, for which EU legislation does not foresee specific provisions regarding microbiological or chemical analyses but the responsibility to verify the quality and traceability of the used materials is left to operators. Nevertheless, the uptake of chemical or microbiological contaminants, allergen, and toxins by insects through diet may have consequences for the health of insect consumers [REGULATION (EU) 2015/2283].
The present study focuses on some of the most harmful mycotoxins produced by several fungal species of the genera *Penicillium, Aspergillus*, and *Fusarium*, which may be present in various cultivated plants employed in the mass rearing of insects, among which cereals and their products in storage or field conditions. These mycotoxins are aflatoxin B1 (AFB1), ochratoxin A (OTA), and fumonisin B1 (FB1) and are considered to be responsible for acute toxic, carcinogenic, mutagenic, teratogenic, immunogenic effects in humans and animals. *T. molitor* (Coleoptera, Tenebrionidae) is included in the list of edible insects and larvae are massively reared for food and feed purposes in the EU (EFSA SCIENTIFIC COMMITTEE, 2015; IPIFF, 2019). This study aims to provide data on the capability of *T. molitor* to uptake mycotoxins by feeding on contaminated substrates composed of cereal wheat (mainly grain, oat, and bran) and vegetables intended for human or animal consumption, to evaluate the potential hazard for insect consumers.

MATERIALS AND METHODS

REAGENT AND STANDARDS

All reagents, solvents, and mycotoxin standards (aflatoxin B1, ABF1; ochratoxin A, OTA; fumonisin B1, FB1) were purchased from Sigma-Aldrich S.r.l. (Milano, Italia).

Milli-Q quality water was produced by Millipore Progard 2 MilliQ Water Purification System. (Millipore, Milford, MA, USA).

T. MOLITOR REARING

Yellow mealworms (*T. molitor*) larvae were obtained from laboratory insect mass rearing, carried out in CREA-DC of Florence (Italy), on a diet composed of brewer's yeast (0.5%) (Laboratorio Dottori Piccioni S.r.l., Mi, Italy), wheat flour (49.75%) (Molino F.lli Chiavazza, Casalgrasso, CN, Italy) and minced oat intended for animal use (49.75%) (Michelotti and Zei, Larciano PT, Italy) integrated with carrot pieces as a moisture source. Larvae were maintained in a semi-dark condition in a climate room at 27± 1 °C and RH (Relative Humidity) 40-50%.

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CONTAMINATED DIETS

For each mycotoxin, two levels of diet contamination were selected, to obtain values comparable with the maximum residue limit (MRL) admitted in cereals for human consumption by EU legislation (Commission Regulation (EC) No 1881/2006): 0.02 and 0.20 ppm for AFB1, 0.05 and 0.5 ppm for OTA, 0.4 and 4.0 ppm for FB1.

Contaminated diets were prepared according to methods reported by BOSH *et al*, (2017). The wheat flour used as feed was sprayed with a methanolic solution of AFB1, OTA, and FB1, separately, to obtain the above-mentioned concentration levels. Before using, the flour was kept in a vacuum oven for 24 h to remove any residues of methanol. As a negative control for the toxicity evaluation, the flour was treated only with methanol and then processed as described above.

MYCOTOXINS RESIDUES DETERMINATION

Experimental plan

Four polypropylene food trays (20 × 14 × 10 cm) were prepared for each mycotoxin and concentration added to the diet, each containing 130 larvae of about 1 cm in length. As a negative control, 8 trays were set up with the same number and size of larvae fed on the growth diet. Each diet, contaminated and not, was added *ad libitum* to the larvae and integrated periodically with carrots. Larvae were maintained in the same environmental conditions of *T.molitor* laboratory mass rearing. Tests ended after 50 days and controls were performed as follows to verify detrimental effects on larval growth and mortality. On the 15th and 30th days, 25 larvae per mycotoxin/concentration were collected from each tray/test, washed with drinking water to remove diets components, dried on absorbent paper and weighed. The larvae were then left starving for 24 h and frass collected to evaluate chemical residues and metabolites. Larvae and frass were frozen and stored at −28±8 °C before use. On the 50th day, 25 larvae were collected from each tray/test and treated according to the previous methodology while the remaining ones were frozen directly without fasting to verify the chemical accumulation in the insect body.

During the study, dead larvae were removed twice a week and data was used to estimate *T. molitor* mortality.

Standard solutions

Standard solutions (1 mg/mL) were prepared in methanol and then diluted to make the working solutions. Solutions were prepared weekly and stored at 4°C until used.

Extraction of AFB1, OTA and FB1 residues from larvae

For each group, the extractions were carried out with an IKA Labortechnik homogenizer model T25 basic (Ika Werke Gmb, Staufen, Germany) for 5 min at 13500 rpm.

The supernatant was transferred after centrifugation and another aliquot of methanol (10 mL) was added to the residue and homogenized again. The methanolic phases were dried under vacuum and the residue was redissolved in 500 µL of methanol. The samples were injected into LC-MS/MS after filtration (Nylon filter 0.45µm, Minisart SRP 4, Sartorious, Gottingen, Germany).

LC-MS-MS analyses

LC analyses were performed by Varian apparatus constituted by two 212-LC pumps and a TQ-ESI detector Mod. 320-LC and Varian MS Workstation System control Ver. 6.9 software. Samples, obtained as reported above, were injected after filtration (5µL). For the chromatographic analysis of AFB1, OTA e FB1 column Poroshell 120 EC-C18 (2.7 µm, 3x30 mm) (Agilent Technologies, CA) was used. The separation was performed using linear gradient elution for 7.0 min with a mobile phase of 0.1 % (v/v) formic acid in water and acetonitrile/methanol (50:50 v/v) mixture at the flow rate of 200 µL/min. The gradient started from 100 % water reaching 2 % in 6 min.

The LC-MS/MS detection was performed by operating the MSD in positive ion mode, and the ESI parameters were: detector voltage 1450.000 V, drying gas pressure 18 psi, desolvation temperature 250°C, nebulizer gas 42.0 psi, needle voltage 5850 V, shield voltage 600 V. Nitrogen was used as a nebulizer and drying gas. Collision induced dissociation was performed using argon at a pressure of 1.8 mTorr in the collision cell.

The selected reaction monitoring transitions as well as the capillary voltage and the collision energy are reported in Tab. 1. Two transitions were acquired by using the experimental conditions reported above.

Validation procedure and matrix effect evaluation

The specificity of the methods used was assessed by analysing blank samples extracted from larvae reared on the control diet and blank samples spiked with the three mycotoxins. The absence of interferences in chromatograms indicated the specificity of the methods. To assess the methods' linearity, calibration curves (obtained from 5 replicates) were constructed by analysing pre-extraction-spiked larvae samples. Precision was determined by defining repeatability (intra-day) and intermediate precision (inter-day), assaying added larvae samples during the same day or on different days. The accuracy of the methods was evaluated at two spiked levels and expressed as relative error %. Acceptance criteria were established for precision (residual standard deviation or RSD) and accuracy (percentage error): respectively between ± 20% and ± 15% of the reference values.

The recovery data were determined by spiking blank larvae samples and comparing the results with those obtained from blank samples fortified with the mycotoxins after extraction.

Matrix effect (ME) was calculated by analysing mycotoxin methanol solutions (A) or larvae spiked with mycotoxins after extraction (B) as follows: ME (%) = (A/B) × 100.

Table 1 - Selected reaction monitoring (SRM) parameters used in the analysis (quantification and confirmatory transitions, collision energy and capillary voltage).

Compound	Quantification [a] and confirmatory transition (m/z)	Capillary voltage (V)	Collision energy (eV)
AFB1 $C_{17}H_{12}O_6$ M.W. 312.27	312.9 →240.7 [a]	100	-39.5
	312.9 →212.9		-49.0
OTA $C_{20}H_{18}ClNO_6$ M.W. 403.8	404.2 → 238.8 [a]	10	-27.0
	404.2 → 357.9		-15.0
FB1 $C_{34}H_{59}NO_{15}$ M.W. 721.8	722.5 → 352.4 [a]	150	-39.0
	722.5 → 3334.3		-44.0

ANALYSIS OF MYCOTOXINS RESIDUES AND METABOLITES IN THE LARVAL FRASS

Frass collected from each group of larvae per each mycotoxin/concentration was analysed to evaluate the metabolic potential of *T. molitor* larvae. Two mL of methanol was added to ~30 mg of frass for each mycotoxin/concentration and after 5 min of extraction in an ultrasound bath, the sample was centrifuged and methanol dried under nitrogen flow. Residues were reconstituted with 500 µL of methanol and analysed with an LC-UV-MS method after filtration. An Agilent 1260 Infinity instrument consisted of a binary pump, an autosampler, a UV-DAD, and an ESI-MS detector was used. The chromatographic separation was realized with a Kinetex EVO C18 column (Phenomenex) (4.6 x 150 mm, 5 µm) injecting 20 µL of the sample. The analysis was carried out with linear gradient elution of 0.1 % (v/v) formic acid in water and acetonitrile/methanol mixture (50:50 v/v) at the flow rate of 600 µL/min. The gradient started from 100 % of water reaching 2 % in 12 min. Spectra were acquired over the scan range 50-1500 *m/z* in positive and negative mode using nitrogen as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, and the vaporization temperature were set at 50 psi, 5 Lmin-1, 1000V, and 200°C respectively.

STATISTICAL ANALYSIS

Each analysis was realized in quadruplicate and results expressed as mean ± standard deviation (SD). A T-test was performed to assess significant differences between larvae reared on each diet with respect to the control.

RESULTS AND DISCUSSION

LC-MS/MS DETERMINATION OF THE MYCOTOXINS AFLATOXIN B1 (AFB1), OCHRATOXIN A (OTA) AND FUMONISIN B1 (FB1) RESIDUES IN *T. MOLITOR* LARVAE

For each investigated compound, pseudo-molecular ions $[M+H]^+$ were selected as precursors. The transition reported in the experimental section were used for the quantification of mycotoxin residues in *T. molitor* larvae. For AFB1, OTA and FB1, a good reproducibility of retention times was observed throughout the analysis cycle (4.52± 0.124.94 ± 0.09 and 4.78 ± 0.08 min respectively).

Methods were validated for specificity, linearity, precision and accuracy. Matrix effect was also determined as reported in the experimental section.

The analysis of larvae reared on the control diet showed the absence of interfering signals at retention times and ion channels. Results obtained in matrix effect determination are reported in Tab. 2 and a signal suppression effect was observed. For this reason, the calibration curves have been realized extracting spiked larvae samples. Satisfactory mean recovery values for the mycotoxins (over 60 %) were obtained (Tab. 3). Calibration curves (n=5 repeated experiments) were constructed and the methods were found linear in the ranges, as reported in Tab. 4, together with intercept, slope, correlation coefficient, the limit of detection (LOD), and limit of quantification (LOQ). As reported in Tab. 5, the developed methods were precise, accurate and reproducible, and then, considered to be suitable for the determination of residues of AFB1, OTA, or FB1 in *T. molitor* larvae.

DETERMINATION OF AFLATOXIN AFB1 RESIDUES AND TOXICITY IN *T. MOLITOR* LARVAE

Fortification levels of 0.02 and 0.2 ppm AFB1 in diets, corresponding to 10 and 100 times higher than the MRLs admitted for cereals intended for human consumption (COMMISSION REGULATION (EC) No 1881 /2006), have been selected according to methods reported by BOSH *et al.*, (2017) and are over the limits admitted for animal feed (DIRECTIVE 2002/32/EC).

To the best of our knowledge, this work is the only literary reference available which examined the toxicity and potential residues of AFB1 in T. *molitor* larvae fed on contaminated feed.

Table 2 – Matrix effect obtained for the AFB1, OTA and FB1 using concentrations reported in Tab. 3.

	Matrix effect % (mean ± SD)
AFB1	444.20 ± 68.25
OTA	327.45 ± 54.87
FB1	190.96 ± 14.03

Table 3– Mean extraction recoveries obtained at two concentration levels (n=5).

Spiked concentration (ng/g)	Recovery % (mean ± SD)	RSD% [a]
AFB1		
0.167 16.7	63.31 ± 6.23 71.09 ± 6.59	9.85 9.27
OTA		
3.0 300	64.32 ± 3.72 61.73 ± 1.82	5.80 2.95
FB1		
8.3 830	64.42 ± 6.24 62.38 ± 5.12	9.69 8.21

[a] relative standard deviation

Table 4– Regression plot parameters for AFB1, OTA and FB1 quantification in *T. molitor* larvae.

	AFB1	OTA	FB1
Slope	241670561	6270802	250460
Intercept	18306	-51030	31210
R^2	0.9911	0.9999	0.9980
Range (ng/g)	0.167-16.7	3.00-300	0.83-830
LOD (ng/g)	0.03	1.00	2.00
LOQ (ng/g)	0.09	3.00	6.00

The validated LC-MS/MS method was applied to the analysis of AFB1 residues in larvae collected after 15, 30 and 50 feeding days and after 24-hours of fasting. The mean results (± SD) obtained from the analysis of larvae fed on the two AFB1 contaminated diets (0.02 and 0.2 ppm) are reported in Tab. 6; after 24-hours of fasting, no AFB1 residues greater than 0.03 ng/g (LOD) were detected in larvae for both the selected contamination levels. These results highlight that larvae fed on these diets do not accumulate AFB1 in detectable concentrations. These results are in accordance with the data reported by BOSH *et al.*, 2017 in larvae fed on substrates added with 0.023 ppm (0.16 ± 0.06 ng/g) and 0.23 ppm (1.29 ± 0.47 ng/g) of AFB1.

During the 50-days trial period, no significant difference in mortality data was observed between larvae fed on contaminated and negative control diets, as already described by BOSH *et al.* (2017), although the reported experiment ended in a shorter period of time (10 days). The solid correspondence of our data with those reported by BOSH *et al.* (2017) pointed out that rearing conditions and the analytical procedures adopted in the present study were correct in supporting the aim of the study. Due to the AFB1 genotoxic and carcinogenic properties (IARC, 2002), it is important to minimize the presence of this toxin in food and feed, and the low levels found in the *T. molitor* larvae after 24-hours of fasting guarantee the acceptability of the presence of AFB1 in low concentrations in insect-food for the safety of consumers.

Table 5– The intra-day and inter-day precision and accuracy of the methods (n=5).

Compound		Spiked concentration (ng/g)	Measured concentration (mean ± SD)	RSD% [a]	Accuracy (relative error %)
AFB1	Intra-day	0.167	0.16 ± 0.03	15.87	-5.39
		16.7	16.93 ± 1.11	6.59	+1.35
	Inter-day	0.167	0.17 ± 0.03	18.05	-5.06
		16.7	15.98 ± 1.06	6.61	-5.11
OTA	Intra-day	3.00	3.05 ± 0.48	15.73	+1.67
		300	309.32 ± 18.37	5.94	+3.10
	Inter-day	3.00	3.12 ± 0.62	19.87	+4.00
		300	304.54 ± 21.32	7.00	-1.51
FB1	Intra-day	8.30	7.96 ± 0.68	8.55	-4.14
		830	826.33 ± 19.86	2.40	-0.44
	Inter-day	8.30	8.14 ± 0.84	10.32	-1.89
		830	819.33 ± 23.12	2.82	-1.29

[a] relative standard deviation

Table 6 - AFB1 (ng/g) residues in *T. molitor* larvae fed on the two selected AFB1 contamination levels (n=4).

Rearing days	AFB1 residues in larvae (ng/g)	
	Diet 0.02 ppm	Diet 0.20 ppm
15	<LOD [a]	<LOD
30	<LOD	<LOD
50	<LOD	<LOD
50 without fasting	0.14 ± 0.021	1.69 ±0.098
% AFB1 eliminated within 24 hours	100 %	100 %

[a] LOD = 0.03 ng/g

DETERMINATION OF OCHRATOXIN A (OTA) RESIDUES IN *T. MOLITOR* LARVAE

The two fortification levels of OTA, 0.05 and 0.50 ppm, correspond to 10 and 100-times higher levels with respect to the MRL in unprocessed cereals intended for human consumption **(**Commission Regulation (EC) No 1881/2006). The validated LC-MS/MS method was applied for the determination of OTA residues in larvae collected at 15, 30, and 50 feeding days and after 24-hours of fasting of larva. Tab. 7 shows the mean results (± SD) obtained in the analyse fed for the two levels OTA contaminated substrates. To the best of our knowledge, these results are not comparable with any literary references available but the low levels of residues found after 24-hours of fasting, guarantee the quality of the larvae as they did not exceed the Tolerable Weekly Intake (TWI) of 120 ng/kg body weight reported for OTA by EFSA (EFSA, 2006).

DETERMINATION OF FUMONISIN FB1 RESIDUES IN *T. MOLITOR* LARVAE

FB1 fortification levels of 0.40 and 4.00 ppm in diets have been selected as they include most of the MRLs for maize products (Commission Regulation (EC) N° 1881/2006). Residues determination in larvae has been performed in the same way as for the other two fungal toxins, as previously described.

The validated LC-MS/MS method was applied to the analysis of FB1 residues in larvae collected after 15, 30 and 50 feeding days and 24-hours fasting (n=4). The mean results (± SD) obtained in the analysis of larvae fed on the two FB1 contaminated substrates are shown in Tab. 8.

Results were compared with the only available literary reference published by ABADO-BECOGNEE *et al*. (1998) which reported slightly lower rates of mycotoxin frass excretion than those found in the present study (40%) but in that case, the FB1dose administered to larvae was far greater (450 µg/g). Also in that experiment, no harmful effects were observed in larvae fed on the adopted dietary FB1 fortification concentrations.

The low levels of FB1 residues found in larvae after 24-hour of fasting and the high excretion rate of the mycotoxin guarantee the quality of *T. molitor* larvae bred

Table 7- OTA residues (ng/g) found in larvae fed on OTA contaminated diets at the two selected levels (n=4).

Rearing days	OTA residues (ng/g) in larvae	
	Diet 0.05 ppm	Diet 0.50 ppm
15	5.47 ± 1.97	5.63 ± 2.51
30	32.72 ± 2.36	42.72 ± 7.46
50	70.18 ± 5.32	121.73 ± 13.60
50 without fasting	113.90 ± 9.20	211.91 ± 7.78
% OTA eliminated in 24 hours	61.61 %	57.44 %

Table 8 - FB1 residues (ng/g) in larvae fed on FB1contaminated diets at the two selected levels.

Rearing days	FB1 residues in larvae (ng/g)	
	Diet 0.40 ppm	Diet 4.00 ppm
15	2.12 ± 0.46	4.71 ± 0.35
30	3.96 ± 0.54	12.74 ± 0.87
50	4.17 ± 0.23	27.10 ± 1.80
50 without fasting	18.26 ± 5.22	109.84 ±28.52
% FB1 eliminated within 24 hours	77.16 %	75.33 %

on contaminated substrates at the tested levels, which, in no case, exceeded the human tolerable daily intake of 2 μg per kg body weight (JECFA, 2001).

DETERMINATION OF AFLATOXIN B1 (AFB1), OCHRATOXIN A (OTA) AND FUMONISIN B1 (FB1) RESIDUES IN THE FRASS

T. molitor larval frass grouped according to mycotoxins/contamination levels, was extracted with methanol and analysed with the LC-UV-MS method. In all cases, mycotoxins residue values resulted to be proportional to the fortification levels of the diet. In absence of analytical standards of AFB1, OTA, and FB1 potential metabolites, the analysis was performed selecting the masses found for mammalians (GALTIER, 1999; DOHNAL *et al*., 2014; YANG *et al*., 2015).

From the screening, the main AFB1 metabolite observed in *T. molitor* larvae (Fig. I) corresponds to a mass values of 329 *m/z* that could coincide with AFB1 epoxide, AFQ1, or AFM1; the identity of this compound remains to be yet ascertained by comparison with appropriate standards. This is the first study in which the metabolic profile of AFB1 in *T. molitor* larvae was examined; further studies are necessary to clarify the metabolic pathways of this molecule in the mealworm No metabolite was detected for OTA and FB1, only non-metabolised mycotoxins.

TOXICITY OF MYCOTOXINS AFLATOXIN B1 (AFB1), OCHRATOXIN A (OTA) AND FUMONISIN B1 (FB1) AGAINST *T. MOLITOR*

The selected fortification levels were used to verify detrimental effects on larval growth and mortality. Results showed that *T. molitor* mortality was not significantly different among larvae fed on contaminated diets and not as well as the weight values during the 50-day trial period. (Fig. II and Table 9).

CONCLUSIONS

The present study examined the *T. molitor* larvae uptake of aflatoxins (AF), ochratoxins (OT), and fumonisins, some of the more frequent and harmful contaminants in uncontrolled cereals and their products, by feeding on a fortified diet. The concentrations used for each toxin were selected to evaluate larval accumulation, excretion rates, and mortality levels.

New analytical methods for the extraction and quantification of AFB1, OTA, and FB1 residues in larvae were developed and validated for the implementation of the research.

Results showed that *T. molitor* larvae not accumulated AFB1, OTA and FB1 mycotoxins at the tested MRL levels by feeding on a contaminated diet, in amounts exceeding EU legal limits for food and that it is sufficient a fasting period of 24 hours to ensure larvae

detoxification of residues. Furthermore, *T. molitor* larvae showed to be tolerant of AFB1, OTA, and FB1 mycotoxins up to the considered levels. In particular, the metabolic pathways of AFB1 in the mealworm deserve to be investigated in-depth and clarified as potential metabolites have been-highlighted.

The study is a contribution to define appropriate procedures for the safe use of *T. molitor* for food and feed.

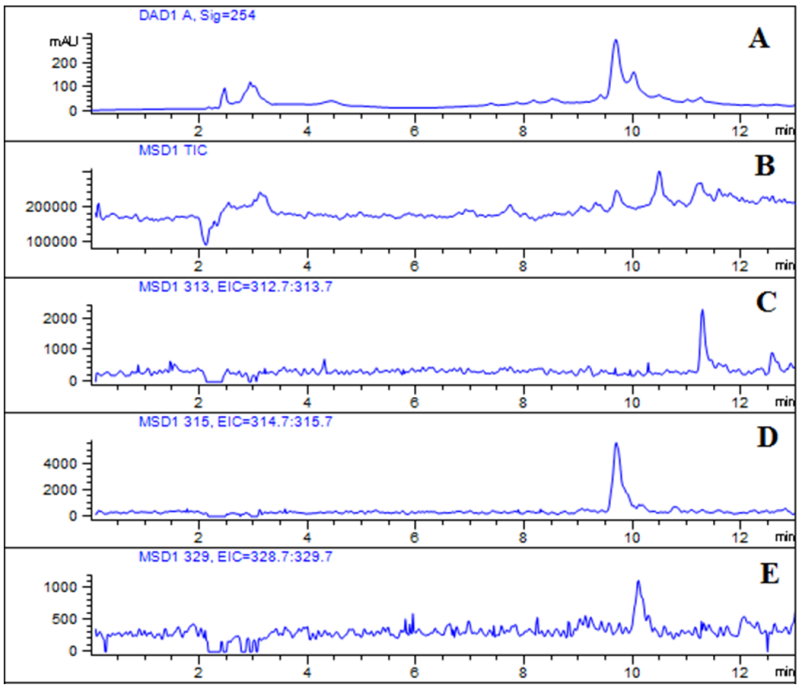

Fig. I– Chromatographic profile obtained in a sample of frass after 50 days on diets contaminated with AFB1 (0.2 ppm). A=UV profile 254nm, B=TIC, C=extract ion 313*m/z* (AFB1), D=extract ion 329*m/z*.

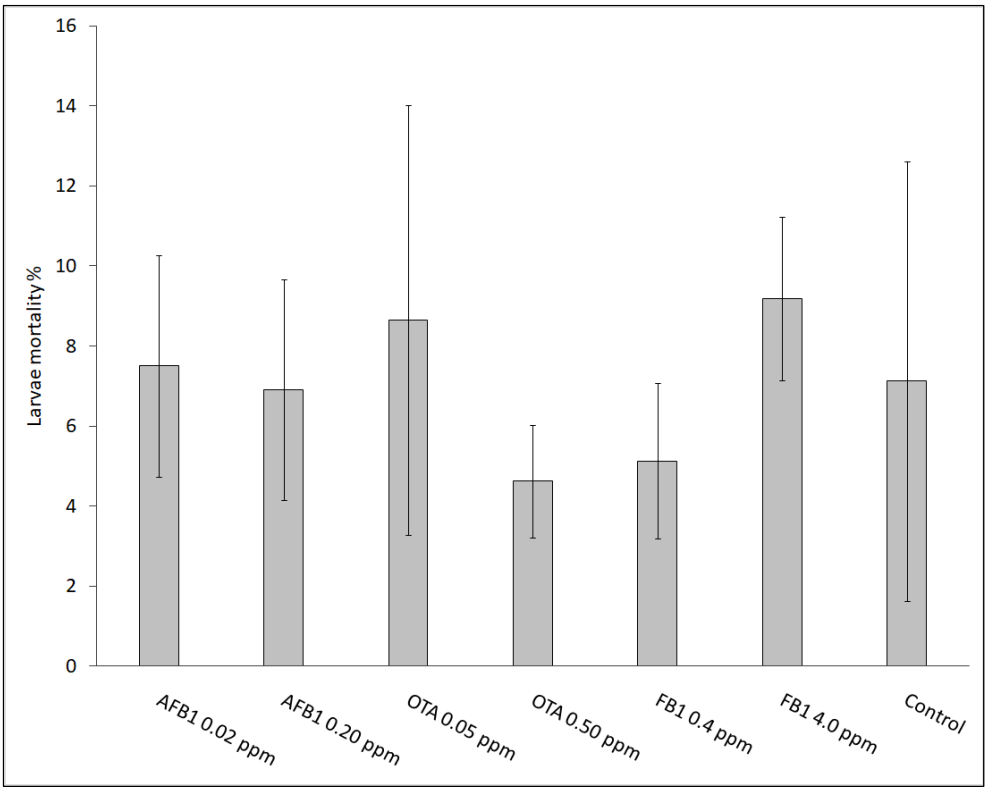

Fig.II – Larvae mortality percentage obtained with diets contaminated with two levels of AFB1, OTA and FB1.

Table 9. Larvae average weight observed in 3 different inspection days.

Larvae diets		Mean weight of larvae ± SD (mg)		
		15 days	30 days	50 days
AFB1	0.02 ppm	0.154±0.008	0.159±0.004	0.189±0.020
	0.20 ppm	0.153±0.007	0.156±0.007	0.181±0.017
OTA	0.05 ppm	0.155±0.004	0.154±0.015	0.190±0.022
	0.50 ppm	0.152±0.005	0.154±0.003	0.181±0.009
FB1	0.4 ppm	0.157±0.011	0.164±0.016	0.201±0.013
	4.0 ppm	0.159±0.014	0.162±0.017	0.202±0.017
Control		0.144±0.014	0.155±0.015	0.185±0.019

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