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Article

Confirmation of aflatoxin in fish and shrimp by LC/MS-MS (ZEVO TQD)

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Abstract: Mycotoxins are agricultural contaminants of fungal origin occurring at all latitudes worldwide and being characterized by acute and chronic effects on human health and animal wellness, depending on the species sensitivity. Various types of crops like maize, wheat, soybeans etc are used as raw materials for preparing feed of fish and shrimp. They are particularly susceptible to infection by Aspergillus following prolonged exposure to a high humidity environment. For this reason, the fish and shrimp samples should be tested for identifying and quantifying mycotoxin. The major mycotoxins of food concern are aflatoxin (B_1, B_2, G_1, G_2) for its toxicity. This paper focus on the confirmation of aflatoxin in fish and shrimp by developing method and validated it by LC/MS-MS (ZEVO TQD) which is important for ensuring the safety of fishery product for human consumption. The monitored MRM transitions for B_1, B_2, G_1, G_2 were *m/z* 313→57 and *m/z* 313→71, *m/z* 318→88 and *m/z* 318→256, *m/z* 328.75→242.99 and *m/z* 328.75→199.9, *m/z* 330.34→88 and *m/z* 330.34→106 respectively. Limit of decision (CCα) for B_1, B_2, G_1, G_2 were 0.59 µg/kg, 0.70 µg/kg, 0.68 µg/kg, 0.83 µg/kg respectively and detection capability (CCβ) for B_1, B_2, G_1, G_2 were 1.01µg/kg, 1.19 µg/kg, 1.15 µg/kg, 1.40 µg/kg respectively.

Keywords: aflatoxin; CCα and CCβ; LC-MS/MS; shrimp; fish

1. Introduction

Mycotoxins are toxic secondary metabolites produced by microorganisms of the fungus kingdom, commonly known as molds. The word 'mycotoxin' is commonly set aside for the toxic products produced by some fungi that readily inhabit crops (Turner *et al.,* 2009). A mold species can yield many different mycotoxins, and numerous species could produce similar mycotoxin (Robbins *et al.,* 2000).There are now over 400 recognized mycotoxins that may be found in animal feedings materials and it has been reported that as much as 25% of the worlds cereal grains may be contaminated with mycotoxins (Stead *et al.,* 2014). Aflatoxins are type of mycotoxin produced by *Aspergillus* species of fungi, such as *Aspergillus flavus* and *Aspergillus parasiticus* (Martins *et al.,* 2001). The word aflatoxin denotes to four different types of mycotoxins produced, which are B1, B2, G1, and G2. Aflatoxin B1, the utmost toxic, is a strong cancer-causing agent and has been directly linked to liver cancer in numerous animal species (Martins *et al.,* 2001). The Aflatoxins were first rose to notoriety in 1960 when they caused the deaths of thousands of turkeys on farms in the UK. The bird feed had been made with peanut meal, imported from Brazil, which had been contaminated with the mold *Aspergillus flavus.* The incident highlighted the dangers posed by these compounds, dangers exacerbated by the global nature of modern agricultural trade (de Kok *et al.,* 2007). The appearance of toxigenic fungi and the subsequent production of mycotoxins are more frequently observed in food and feed produced in developing countries. Environmental factors such as high temperature, high humidity and moisture, frequent rainfalls, and poor soil conditions play important roles in growth of fungi and thereby aflatoxin contamination of feed (Cotty and Jaime-Garcia, 2007; Iqbal *et al*., 2015; Shad *et al*., 2019). In addition, improper farm management, which includes poor harvesting techniques and unsuitable storage conditions, can contribute to high occurrence of aflatoxin contamination

(Atungulu *et al*., 2019; Shad and Atungulu, 2019). Many agricultural products such as nuts ,fresh and dried fruits and vegetables , cereals such as like maize, rice, wheat and soybeans, liquids such as wine, grape juice, beer, milk and dairy products, spices and herbs, coffee, cocoa and feed can be contaminated with mycotoxins at all stages of the food and feed chain. Some of these crops like maize, wheat, rice, soybeans, nuts etc are used as raw materials for preparing feed of fish and shrimp. These fish feed are used in fresh water culture. So fish and shrimps are obviously affected with aflatoxin. It is crying need to quantify the amount of aflatoxin in order to consume safe fishery product. According to European Commission (Regulation 1881/2006), the European legislation sets maximum limits for aflatoxin B_1 is 2 ppb and maximum limits for total aflatoxin (B_1, B_2, G_1, G_2) is 4 ppb. Till to date several analytical method has been developed for quantifying Mycotoxin in different cereal based food and animal feedstuffs by using HPLC or LC/MS-MS techniques. But no method developed yet for analysing aflatoxin/Mycotoxin in shrimp and fish sample. This paper reports the development of a quantitative method for determining aflatoxin (B1, B2, G1, G2) of shrimp and fish. This method uses UPLC and ZEVO TQD (LC/MS-MS). Method was validated as per Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive (96/23/EC) establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories.

2. Materials and Methods

2.1. Standards and reagents

Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, M_1 standard ware purchased from Sigma-Aldrich (st. Louis, MO). MS grade formic acid, methanol and acetonitrile were also from Sigma-Aldrich (st. Louis, MO). Beside these HPLC grade chemicals (Ethyl acetate, Acetonitrile, Methanol), Appropriate dispersive sorbent (Z-Sep+) and Deionized water were used.

2.2. Sample preparation procedure

i. Take (1 ± 0.05) g of blend sample in to 50 ml centrifuge tube.
ii. Add 100 µl from 50 ppb M_1
iii. Add 9 ml of ACN
iv. Shake 10 minutes and centrifuge at 8000 rpm for 15 minutes
v. Take 8 ml of supernatant
vi. Add 90 µl formic acid (MS Grade)
vii. Add 500 mg Appropriate dispersive sorbent (Z-Sep+)
viii. Vortex for 5 minutes and centrifuge at 8000 rpm for 15 minutes
ix. Take 7 ml of supernatant in a glass tube
x. Evaporate to Dryness under nitrogen gas Pressure
xi. Reconstitute by 1 ml of 0.1% Formic acid With 10% Acetonitrile
xii. Pass the sample through a 0.45 µm syringe filter and collect in a vial for subsequent LC-MS/MS analysis.

Calibration curve: For calibration curve standards preparation is described in Table 1.

Table 1. Standard preparation for calibration curve.

Sl No.	Volume Mixed Std 10 ng/ml (µl)	Volume M_1 ISTD 50 ng/ml (µl)	Std Equivalent Concentration (ppb)
1	0	100	0.00
2	50	100	0.5
3	100	100	1
4	150	100	1.5
5	200	100	2
6	300	100	3
7	400	100	4
8	500	100	5

2.3. Chromatography conditions

LC/MS-MS Module: Acquity UPLC ZEVO TQD (Waters, USA)
Column: Acquity UPLC BEH C18 1.7 μm, 2.1x100 mm column, Waters, made in Ireland.
Column Temperature: Ambient.

Mobile phase: Pump A: 0.1% FA in water, Pump B: 0.1% FA in Acetonitrile.

Inlet method: Inlet method is created according to Gradient system as described in Table 2.

Table 2. Gradient table.

SL No.	Time	Pump A/0.1% Formic Acid in Water	Pump B/0.1% Formic Acid in Acetonitrile	Flow (ml/min)
1	0.00	90	10	0.4
2	3.0	90	10	0.4
3	10	10	90	0.4
4	10.10	10	90	0.4
5	12	10	90	0.4
6	12.10	50	50	0.4
7	13.10	80	20	0.4
8	14	90	10	0.4
9	15	90	10	0.4

Flow (ml/min) : 0.4
Injection Volume (µl) : 15 (Full loop)

2.4. MS condition
Mass Spectrometer: ZEVO TQD (Waters, USA)

Source (ESP+)
Capillary (kv) : 4
Cone (v) : 42
Extractor (v) : 3
RF Lens : 0.2
Source Temp (ºc) : 135
Dessolvation Temp (ºc) : 400
Desolvation gas flow (L/h) : 900
Cone gas flow (L/h) : 50

Analyser
LM Resolution 1 : 9.7
HM Resolution 1 : 15
Ion Energy 1 : 0.2
Entrance voltage : 50
Exit voltage : 50
LM Resolution 2 : 10.8
HM Resolution 2 : 14.9
Ion Energy 2 : 0.7
Multiplier voltage : 650
Collision gas : Argon @ 3.5 x 10^{-3} mbar
Span (Daltons) : 0.00
Dwell time (Sec) : 0.030
Inter Channel Delay (Sec) : 0.02
Inter-Scan Delay (Sec) : 0.02

3. Results and Discussion

Nowadays, MS/MS is used for accurate mass information (Pascale *et al.*, 2019) and the LC–tandem MS (MS/MS) technique is considered to be the most modern and widely used for mycotoxins analysis at trace levels, as it is more sensitive, specific and reliable compared to HPLC (Woo *et al.*, 2019; Bessaire *et al.*, 2019; Al-Taher *et al.*, 2017). This technique has been successfully used for the simultaneous quantification of mycotoxins with different chemical structures (Zhang *et al.*, 2018) in one single run (Spanjer *et al.*, 2008; Delmulle *et al.*, 2006). The developed MS method (Table 3) for Aflatoxin (B_1, B_2, G_1, G_2) has a good agreement with these previous findings as mentioned above. The separation of each individual aflatoxin from its mixture is very clear as shown in chromatogram (Figure 1 and Figure 2). Aflatoxin (B_1, B_2, G_1, G_2) in Shrimp and Fish matrix was quantified by means of a calibration curve (Figure 3) at seven calibration levels ranging 0.5 ppb to 5.0 ppb. M_1 is used as internal standard. Solvent blank, matrix blank, negative and positive control samples are used each analytical batch as an internal quality control measures.

Table 3. MRM transitions and MS condition.

SL. No.	Prnt(Da)	Dau(Da)	Dwell(s)	Cone(v)	Coll(eV)	Delay(s)	Compound
1	313	57	0.030	40	34	0.02	B_1
2	313	71	0.030	40	34	0.02	B_1
3	318	88	0.030	46	30	0.02	B_2
4	318	256	0.030	46	30	0.02	B_2
5	328.75	242.99	0.030	40	30	0.02	G_1
6	328.75	199.9	0.030	40	30	0.02	G_1
7	330.34	88	0.030	56	28	0.02	G_2
8	330.34	106	0.030	56	28	0.02	G_2
9	328.76	273.03	0.030	32	20	0.02	M_1 (ISTD)

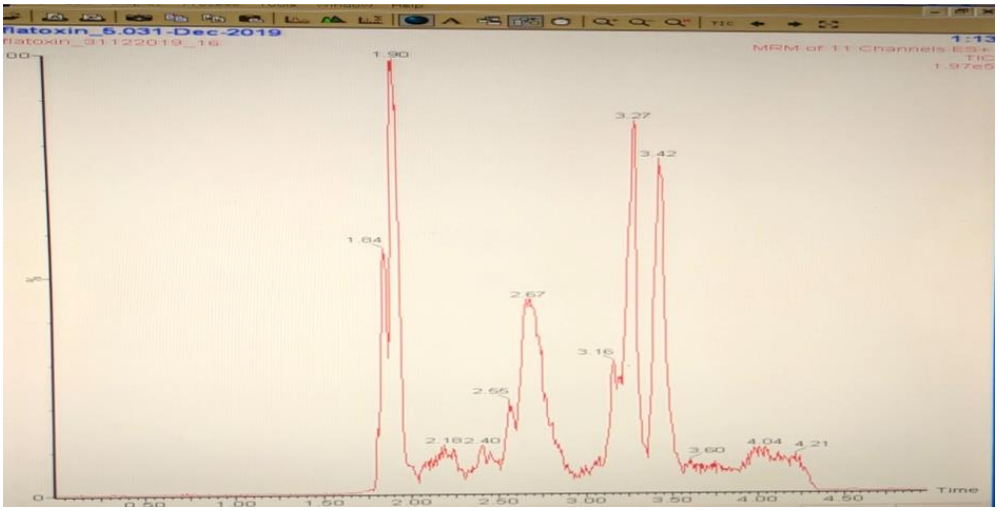

Figure 1. UPLC/MS-MS chromatogram of mixed aflatoxin.

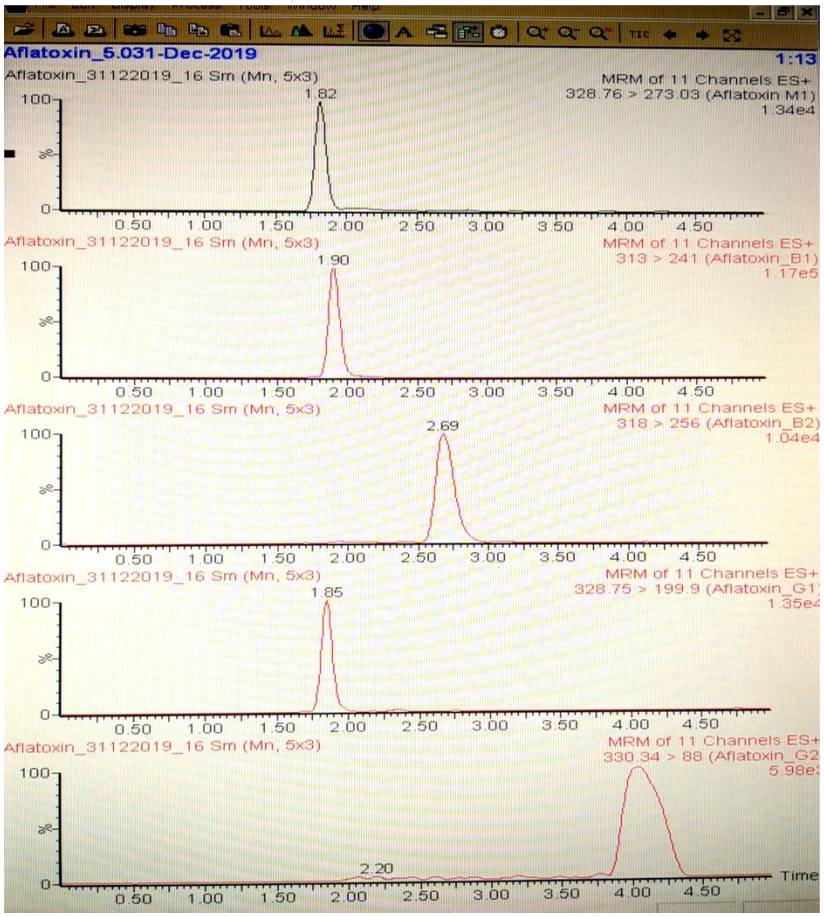

Figure 2. UPLC/MS-MS chromatograms of individual aflatoxin.

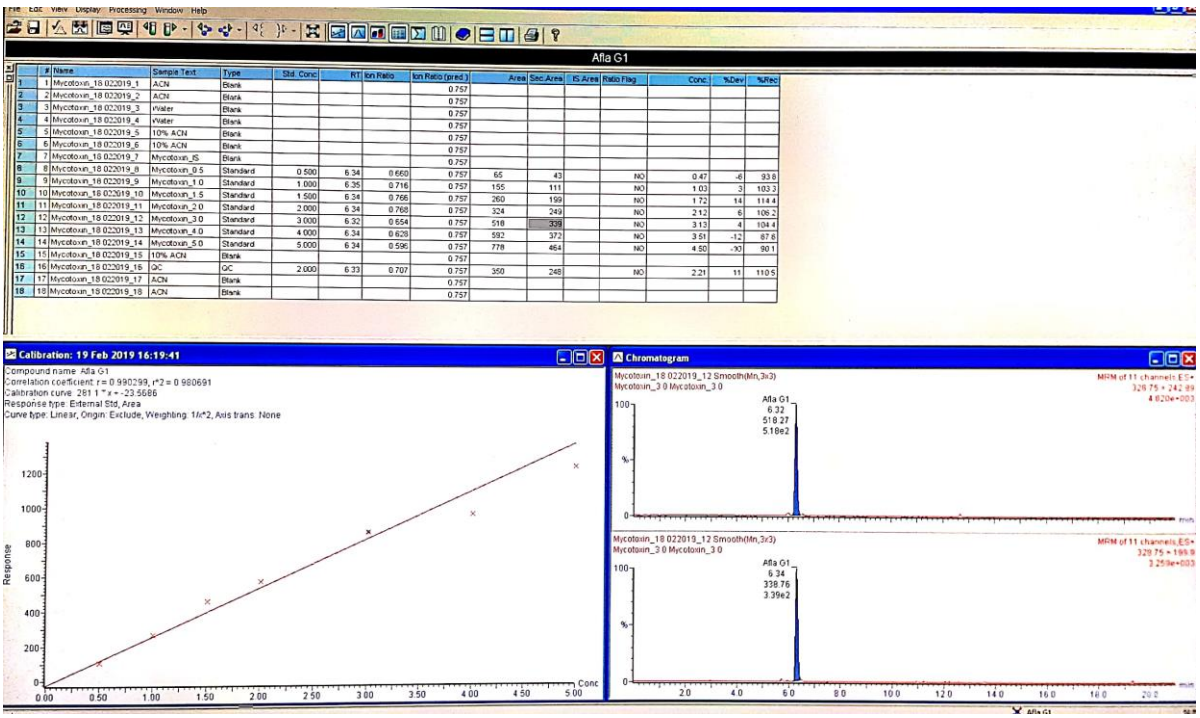

Figure 3. TargetLynx report of calibration curve of aflatoxin (B_1, B_2, G_1, G_2) in shrimp and fish.

3.1. Confirmation criteria

The selectivity of this method is judged by the use of two transitions for each analyte which count for 4 identification points (IPs), as defined by the EU criteria set out in Commission Decision 2002/657/EC. This method fulfills this requirement. The Aflatoxin (B_1, B_2, G_1, G_2) was considered as positively identified in the samples when the peak area ratio of the various transitions was within the tolerance set by Commission Decision 2002/657/EC. In addition, the relative retention time of the analyte must be equal to that of the calibration standard to within ± 2.5%.

3.2. Validation

CCα & CCβ were calculated using the procedure set out in ISO Guide 11843, as described in Commission Decision 2002/657/EC. The validation data were generated in 4 levels of concentrations i.e. 1.0 ppb,2.0 ppb, 3.0 ppb, 4.0 ppb with seven replicates per level for 3different days using fish & shrimp as matrix. The values are shown in the table.

Analyte	CCα(µg/kg)	CCβ(µg/kg)
Aflatoxin B_1	0.59	1.01
Aflatoxin B_2	0.70	1.19
Aflatoxin G_1	0.68	1.15
Aflatoxin G_2	0.83	1.40

Overall summary of the validation data calculation:

B1 in Shrimp and Fish					
Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
1.00	**0.83**	**83**	**7.3**	**3.0**	**7.9**
2.00	**1.67**	**84**	**5.7**	**4.0**	**7.0**
3.00	**3.01**	**100**	**4.4**	**1.9**	**4.8**
4.00	**4.48**	**112**	**2.8**	**3.6**	**4.6**

B2 in Shrimp and Fish					
Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
1.00	**1.12**	**112**	**5.4**	**2.2**	**5.9**
2.00	**2.07**	**104**	**4.6**	**3.2**	**5.7**
3.00	**2.70**	**90**	**4.9**	**2.1**	**5.4**
4.00	**3.26**	**81**	**3.8**	**4.9**	**6.3**

G1 in Shrimp and Fish					
Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
1.00	**1.09**	**109**	**5.6**	**2.3**	**6.0**
2.00	**2.17**	**109**	**4.4**	**3.1**	**5.4**
3.00	**2.86**	**95**	**4.6**	**2.0**	**5.1**
4.00	**3.58**	**89**	**3.5**	**4.5**	**5.7**

G2 in Shrimp and Fish					
Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
1.00	**1.05**	**105**	**5.8**	**2.3**	**6.2**
2.00	**1.82**	**91**	**5.3**	**3.7**	**6.4**
3.00	**2.59**	**86**	**5.1**	**2.2**	**5.6**
4.00	**3.54**	**88**	**3.5**	**4.6**	**5.8**

4. Conclusions

The method was developed and validated as per guideline and commission decision 2002/657/EC. The recovery, linearity and other parameters explains that the developed method is good enough for the confirmatory analysis of Aflatoxin (B_1, B_2, G_1, G_2) by LC/MS-MS in fish & shrimp matrix.

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Conflict of interest

None to declare

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