JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION AND VALIDATION OF HPLC-FLD METHOD FOR DETERMINATION OF AFLATOXIN B_1 AND PRODUCTION OF THIS MYCOTOXIN BY *ASPERGILLUS* SECTION *FLAVI* ISOLATES

Silvia Jakabová[1], Zuzana Barboráková[2], Dana Tančinová[2], Zuzana Mašková[2], Jozef Golian[1]*

Address(es): PaedDr. Silvia Jakabová, PhD.
[1] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Food Sciences, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, +421376415826.
[2] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Biotechnology, Tr. A. Hlinku 2, 94976 Nitra, Slovakia, +421376414494.

*Corresponding author: silvia.jakabova@uniag.sk

https://doi.org/10.55251/jmbfs.9271

ARTICLE INFO

Received 30. 7. 2022
Revised 26. 11. 2022
Accepted 8. 11. 2022
Published 21. 12. 2022

Regular article

ABSTRACT

A high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) was evaluated for the monitoring of aflatoxin B_1 (AFB_1) production by *Aspergillus* section *Flavi* isolates, which were found in the commodities of Slovak origin (barley and tomatoes). Rapid validation of the method was performed and the method was applied for the determination of the mycotoxin. Based on the calculated validation characteristics, the method showed high sensitivity and reproducibility. The LOD and LOQ determined were 0.03 and 0.10 $\mu g.mL^{-1}$ which is fully acceptable for the determination of the AFB_1 in isolates from plant commodities. The calibration graph was linear in the concentration range of 0.01– 10.0 $\mu g.mL^{-1}$. The optimized method was used for the analysis of the mycotoxin in three *Aspergillus* section *Flavi* isolates. The submerse cultivation was performed at 25±1°C in yeast extract sucrose liquid medium and material was collected on the 7th, 14th and 21st day of cultivation. Simple sample extraction was carried out with use of ethyl acetate, and measurement was done with use of the mobile phase consisting of water and methanol by HPLC-FLD. Production of the AFB_1 by isolates was ranging from <LOD (0.03 $\mu g. mL^{-1}$) to 49.38 $\mu g.mL^{-1}$ with the highest concentration on the 14th day of cultivation. The maximum level of AFB_1 was achieved by the Isolate 1 (isolated from barley, Kolíňany, 2018).

Keywords: aflatoxin B_1, HPLC-FLD, method validation, *Aspergillus* section *Flavi*, isolates

INTRODUCTION

Aflatoxins (AFs) present a major food and feed safety issue. The aflatoxigenic fungi – mainly species of genus *Aspergillus* subgenus *Circumdati* section *Flavi* are responsible for production of various members of the aflatoxin group of chemicals (AFB_1, AFB_2, AFG_1 and AFG_2) and other mycotoxins **(Varga *et al.*, 2011; Coppock *et al.*, 2018)**, such as 3-nitropiazonic acid, tenuazonic acid and cyclopiazonic acid **(Varga *et al.*, 2011)**. Aflatoxins are mainly produced by *Aspergillus flavus*, *A. parasiticus* **(Varga *et al.*, 2011)** and *A. nomius* **(Ayob, 2022)**, which coexist with and grow on almost any crop or food **(Varga *et al.*, 2011)**. AFs of the B type (AFB_1, AFB_2) are produced by *A. flavus* **(Amare *et* Keller, 2014; Varga *et al.*, 2009)**, while as both AFs of group B and G (AFG_1, AFG_2) are produced by *A. parasiticus* and *A. nomius* **(Costa *et al.*, 2019)**. Some reports indicate that *A. flavus* can produce the G type of aflatoxins, as well **(Saldan *et al.*, 2018; Okoth *et al.*, 2018)**. With the help of human metabolism system, AFs are transferred into AFs M1 and Q1. The toxicity levels of AFs are high, while as biological toxicity is contributed measurely from AFB_1 and AFG_1 **(Bacaloni *et al.*, 2008)**.

Aflatoxin B_1 (AFB_1) is the most toxic of the many naturally occurring secondary metabolites produced by fungi **(Varga *et al.*, 2011)**. AFB_1, due to its toxic, mutagenic, immunotoxic, teratogenic, and carcinogenic effect on humans and animals, is classified as a group 1 carcinogen in the International Agency for Research on Cancer (IARC) classification of carcinogenic substances **(IARC, 2002; Ostry *et al.*, 2017)**. As potent carcinogen, AFB_1 may affect organs like the liver and kidneys **(Alvarez *et al.*, 2020)**. It is also reported to suppress human's immune systems, rendering them vulnerable to infectious viruses like HIV and subsequent disease AIDS **(Jolly *et al.*, 2013)**. Exposure of human to AF leads to several health-related conditions including acute and chronic aflatoxicosis, immune suppression, liver cancer, liver cirrhosis, stunted growth in children and many others. AF contamination of agricultural commodities poses considerable risk to human and livestock health and economic losses. The effect of AFs on human depends on age, gender, level of exposure, duration of exposure, health condition, strength of their immune system, diet and environmental factors **(Negash, 2018)**.

Although aflatoxin's occurrence is widespread and affects many food crops, certain crops are more susceptible than others **(Jallow *et al.*, 2021)**. AFs are found in the soil as well as in grains, nuts, dairy products, tea, spices and cocoa, as well as animal and fish feeds **(Waliyar *et al.*, 2008)**. Commodities can be contaminated with aflatoxicogenic fungi and AF at any time before harvest and after harvest. The prevention of AF once occurs and treatment of aflatoxicosis is difficult. However, there are some mitigation mechanisms pre- and post-harvest, especially proper storage is essential with proper moisture and temperature. Moreover, awareness creation on AF contamination, its effect and management are essential **(Negash, 2018)**.

Determination of AFB_1 has been object of many studies. Different analytical approaches were applied in this topic. Conventional analytical approaches for identification of aflatoxins present thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) **(Maggira *et al.*, 2022)**. A high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) was applied in the studies of AFs determinations in common food samples by **Blesa *et al.* (2003)**, **Campos *et al.* (2017)**, **Sheijooni-Fumani *et al.* (2011)**, **Golge *et al.* (2016)** etc. HPLC with MS detection was used in the works of **Blesa *et al.* (2003)**, **Stefanovic *et al.* (2015)**, **Fan *et al.* (2015)**, **Pereira *et al.* (2020)** etc. ELISA method is also a way for determination of AFB_1, mentioned in the works of **Stefanovic *et al.* (2015)**, **Pereira *et al.* (2020)**, **Dai *et al.* (2022)**, **Maggira *et al.* (2022)** etc.

The objective of our study was to optimize and validate an HPLC-FLD method for the determination of aflatoxin B_1 and evaluation of the production of this mycotoxin by *Aspergillus* section *Flavi* isolates.

MATERIAL AND METHODS

Chemicals and reagents

The HPLC grade solvents acetonitrile and methanol were purchased from Sigma-Aldrich Chemie GmbH. (Steinheim, Germany), double-distilled deionized water used was prepared in a Millipore Synergy UV water purification system (Millipore, Bedford, USA). Acetonitrile was used for a stock standard solution of AFB_1 (5 mg, 99.7%, Sigma-Aldrich Laborchemikalien GmbH; Seelze, Germany) and preparation of individual standard solutions for calibration in the concentration range 0.01 to 10.0 $\mu g.mL^{-1}$ was done by injecting of appropriate volumes of the stock solution in a mobile phase.

Instrumentation

Measurements were performed on the apparatus Agilent 1260 Infinity (Agilent Technologies, Palo Alto, USA), equipped with the degasser G4225A, binary pump G1312B, auto sampler G1329B, column thermostat G1316A and a fluorescence detector G1321B. A reversed-phase column Eclipse XDB-C18 (3.5 µm, 3.0×150 mm, Agilent, USA) was used for the separation. The column temperature was set at 40 °C and isocratic mode was applied with a composition of the mobile phase water/methanol (60/40, v/v). A flow rate was set at 1 mL.min^{-1} and injected volume of standards and samples was 10µL. Excitation wavelength was set at 362 nm and emission wavelength was 455 nm, while the data acquisition was done within the range 280 – 500 nm. Repeated measurements were done for the purpose of validation of the method, and for determination of individual samples, as well. Instrument control, data acquisition and data analysis were carried out with a ChemStation software package (Agilent Technologies, Palo Alto, USA).

Fungi, media and growth conditions

Three strains of *Aspergillus* section *Flavi* (Isolate 1: KMi-ZB1 isolated from barley from Kolíňany, 2018; Isolate 2: KMi-ZB2 from barley from Oponice, 2019; Isolate 3: KMi- ZB4 from tomatoes originated from Slovakia, 2021, were studied in this work. For each fungal strain, a conidial spore suspension of 2,0x10^8 spores per mL was prepared. Spores were collected by rinsing the colony with physiological saline solution supplemented with Tween 80 (0.5%). 1 mL of spore suspension was inoculated into 40 mL of the liquid medium YES (Yeast extract sucrose; **Samson *et* Frisvad, 2004**) in 50 mL centrifuge tubes (VWR International) in 3 repetitions. Cultivation was carried out on Orbital Shaker PSU-10i (Biosan) and QS-10 Orbital Shaker (Biosan) at 220 RPM. The inoculated media were incubated in darkness at 25±1 °C during the 7, 14 and 21 days.

Preparation of extracts for HPLC analysis

On the 7th, 14th and 21st days of cultivation, 20 ml of the liquid medium were taken into 50 ml centrifuge tubes (VWR International) and centrifuged on a Rotina 420 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) for 3 minutes at a speed of 5,000 × g. 2 mL of the centrifuged liquid medium were filtered through a 25 mm Nylon syringe filter (0.2 µm, Agilent Captiva) into microtubes (1.5 ml, Eppendorf). The filtrate (500 µL) was extracted by adding 500 µL ethyl acetate (Fisher Scientific, Leics, UK) and mixed for 5 minutes using an IKA MS 3 digital vortex (IKA-Werke GmbH & Co., Staufen, Germany). 100 µL of the prepared extract was added into HPLC vials (2 ml, Agilent) and then dried. The extract was reconstituted by adding 500 µL of methanol/water solution (40/60, v/v) before HPLC analysis.

Data processing

Statistical testing was used for data evaluation. A rapid method validation was done in the program Excel (Microsoft, Redmond, WA, USA). Rapid in-house validation of the method for determination of AFB_1 in isolates was performed in terms of intermediate precision and linearity, determination of limit of detection (LOD), and limit of quantification (LOQ). Intermediate precision was tested by determining intraday and interday precision in two parameters - concentration and retention time of analyte. Linearity was assessed by determination of regression analysis, based on repeated measurements of standards in the concentration range from 0.01 to 10.0 µg.mL^{-1}, and coefficient of determination (R^2) was calculated. LOD and LOQ were determined from the calibration curve based on the on the upper limit approach **(Hegedűs *et al.*, 2010)**. Evaluation of the results from determinations of AFB_1 in the isolates was carried out in a statistical program Past4.03. We tested the results by means of kinetics and significant differences between the samples in different periods of cultivation by Tukey HSD test and boxplots were used for the visualisation.

RESULTS AND DISCUSSION

Rapid method validation

The method for determination of AFB_1 was optimized and tested to obtain validation characteristics. The verification the reliability of the method was carried out. Several validation parameters were calculated to evaluate the HPLC method performance for the purpose of routine analysis of AFB_1 in isolates after cultivation and subsequent liquid-liquid extraction from the liquid medium YES. Standard solutions of AFB_1 were used for determination of repeatability and intermediate precision, which were presented as RSD (%) values of repeated measurements performed during the one and within three days. In the table 1 are shown the results for repeatability by means of intermediate precision.

Table 1 Intermediate precision of the retention time and concentration of aflatoxin B_1 in two standard solutions

Compound	Intraday precision					Interday precision			
	Concentration calculated	Retention time		Concentration measured		Retention time		Concentration measured	
	µg. mL^{-1} *	min	RSD %	µg. mL^{-1}	RSD %	min	RSD %	µg. mL^{-1}	RSD %
Aflatoxin B_1	1.00	4.25	0.28	0.97	0.20	4.28	1.33	0.97	1.98
Aflatoxin B_1	2.00	4.26	0.09	1.99	0.13	4.29	1.29	2.00	1.72

Note: *concentration of aflatoxin B_1 in the standard solution,
data presents mean values calculated from 6 repeated measurements within one day for intraday precision and for interday precision data presents mean values calculated from twelve repeated measurements within three days

Variability of retention time expressed by RSD % did not exceeded 0.3% for the both evaluated concentrations of AFB_1 in intraday precision and even lower RSD was determined in interday measurements of retention time (RSDs were 1.98 and 1.72%, respectively). Evaluation of intraday precision of concentration showed RSD % up to 0.20% and for interday variability was up to 1.98%.
We observed a slight delay of retention times of AFB_1 in samples compared to standard solutions. Within one day a mean delay of retention time for samples was 0.033 s. The slight shift was probably due to matrix effect of the extracts of isolates.

Calibration dependence was used for calculation of limit of detection (LOD) and limit of quantification (LOQ) based on the upper limit approach **(Hegedűs *et al.*, 2010)**. Coefficient of determination (R^2) was used for determination of linearity of calibration and was determined from three individual measurements of each standard concentration level. Table 2 presents calculated LOD and LOQ values and linearity of calibration.

Table 2 Linearity, limit of detection and limit of quantification for aflatoxin B_1

Compound	Linearity			Limits of the method	
	Concentration range µg. mL^{-1}	Equation*	R^2*	LOD µg. mL^{-1}	LOQ µg. mL^{-1}
Aflatoxin B_1	0.01 – 10.00	$y = 33.003x - 0.3505$	0.99999	0.034	0.102

Note: * mean values calculated from three dependences of peak area and concentrations within three days

Campos *et al.* (2017) reported limits of quantification 0.18 µg.kg^{-1} for AFB_1 for the HPLC-FLD with post-column derivatization. High linearity was obtained over a dynamic range of 0.2–7.0 µg.kg^{-1} for AFB_1. However, fluorescence detection is considered to be high sensitive, for the most of aflatoxin analysis a derivatisation is carried out prior to delivery of the analyte to detector **(Sheijooni-Fumani *et al.*, 2011)**. Derivatisation of extracted aflatoxins increases fluorescence of analytes and thus sensitivity of the method. Pre-column derivatisation is commonly known with use of either trifluoracetic acid or iodine, however they have some significant limitations **(Sheijooni-Fumani *et al.*, 2011)**. Post column derivatisation of aflatoxins can be performed with bromine, using a Kobra Cell system. **Iamanaka *et al.* (2014)** reported optimised method for total aflatoxins with LOD and LOQ of 0.05 and 0.25 µg.kg^{-1}, respectively. **Golge *et al.* (2016)** published data from the AFB_1 determinations in confectionery products containing nuts. Their method had LOQ for AFs ranging from 0.106 to 0.374 µg.kg^{-1} and they applied immunoaffinity column (IAC) clean-up of the samples. For the concentration levels of AFB_1 from isolates cultivated in the liquid medium is the proposed analysis sufficient, however further improvements of sensitivity and selectivity of the method could be applied e.g. the use of IACs for sample clean-up and efficient post-column derivatisation with use of Kobra Cell system.

Determination of AFB1 in the isolates from barley and tomatoes

Aspergillus section *Flavi* (today are known 33 species of this section) contains several species that produce some of the most important mycotoxins known, especially aflatoxins, ochratoxins and cyclopiazonic acid **(Frisvad *et al.*, 2019)**. AFB_1 is the most potent of these compounds and has been well-characterized to lead to the development of hepatocellular carcinoma in humans and animals **(Rushing *et* Selim, 2019)**. In our study, the isolates of *Aspergillus* section *Flavi* from barley and tomatoes samples of the Slovak origin were analysed for the production of AFB_1. Results of description statistics are summarized in the table 3. The highest concentrations were found in isolate 1, while the content in two other isolates contained similar levels of the mycotoxin.

Table 3 Aflatoxin B_1 content (µg. mL^{-1}) in individual isolates of *Aspergillus* section *Flavi* from barley and tomatoes in relation with period of cultivation

	Isolate 1			Isolate 2			Isolate 3		
Days of cultivation	7	14	21	7	14	21	7	14	21
N	6	6	6	6	6	6	6	6	6
Min	3.67	4.81	9.58	< LOD	< LOD	< LOD	< LOD	4.95	6.12
Max	8.05	49.38	33.01	4.62	7.96	4.81	4.87	6.12	7.77
Mean	4.88	25.23	24.77	2.54	5.14	2.09	1.52	5.53	6.85
SD	1.80	20.04	11.43	2.05	3.99	2.16	2.36	0.39	0.68

Note: < LOD the value measured was below the LOD value (LOD: 0.034 µg. mL^{-1}), N – total number of measurements, Min – minimum value, Max – maximum value, SD – standard deviation

Tukey HSD test was used for determination of a difference between the means of all possible pairs using a studentized range distribution (Lee et Lee, 2018). The results from statistical testing of pairs of variants within one isolate are shown in the table 4.

Table 4 Significance differences with p-values in relation with period of cultivation within the individual isolates tested by Tukey HSD test

Days of cultivation	Isolate 1			Isolate 2			Isolate 3		
	7	14	21	7	14	21	7	14	21
7		0.046	0.052		0.289	0.961		5.98×10^{-4}	3.22×10^{-5}
14			0.998			0.191			0.282

Note: significant difference ($p < 0.05$) between the concentration of aflatoxin B_1 for the same isolate in different period of cultivation is expressed with one colour

In the Isolate 1 we found significant difference in concentrations of AFB_1 between 7 and 14 days of cultivation, and between 7 and 21 days of cultivation. **Giorni *et al.* (2007)** published, that optimal temperature for growth of *Aspergillus* section *Flavi* is 25–30 °C and for AFB_1 production 25 °C. Isolate 1 produced at 25 °C the highest concentration of AFB_1 (49.38 µg.mL^{-1}) on 14th day of cultivation. **Giorni *et al.* (2008)** mentioned, that the AFB_1 amounts were highest after 7 days of cultivation and then decreased over the subsequent period up to the end of the experiment (21 days). In Isolate 2 we did not observed any significant differences, which could be confirmed by statistical tests. Isolate 2 produced at 25 °C highest concentration of AFB_1 (7.96 µg. mL^{-1}) on 14th day of cultivation, as well. In Isolate 3 significant differences were found in all possible pairs regarding the cultivation period. The highest concentration of AFB_1, which were produced by *Aspergillus* section *Flavi* at 25 °C, was 7.77 µg. mL^{-1}.

Figure 1 shows kinetic behaviour of concentration development of individual isolates. In Isolate 1 and 3 we can observe increasing trend in concentration of AFB_1, however decrease in production of AFB_1 by fungi is visible in the cultivation period between 14th and 21st day. Similar trend of AFB_1 and AFG_1 production by *Aspegillus flavus* in YES medium was observed with the highest AFB_1 and AFG_1 concentrations between 7th and 12th day of cultivation **(Davis *et al.*, 1966)**. Decrease of aflatoxin concentrations was visible after 15th day of cultivation. Concentration of AFB_1 published by the authors was from 1 to 20 µg.mL^{-1} what is in agreement with the results from our experiment achieved in the isolates 2 and 3 isolates, however maximum AFB_1 concentrations in Isolate 1 is higher than those in the published work. **Davis *et al.* (1966)** discussed that YES cultivation medium containing 20% sucrose and 2% yeast extract provided good conditions for production of high concentration of aflatoxins and **Mateo *et al.* (2017)** also confirmed that YES medium is promising for production of aflatoxins in maize. A semisolid YES agar was also used in the study of **Aldars-García *et al.* (2018)** and the concentration of AFB_1 in *A. flavus* isolates from maize grains, chilli and pistachio nuts were achieved at 25°C and 7 days of cultivation in the range between 1.5 to 2114.6 ng.g^{-1}.

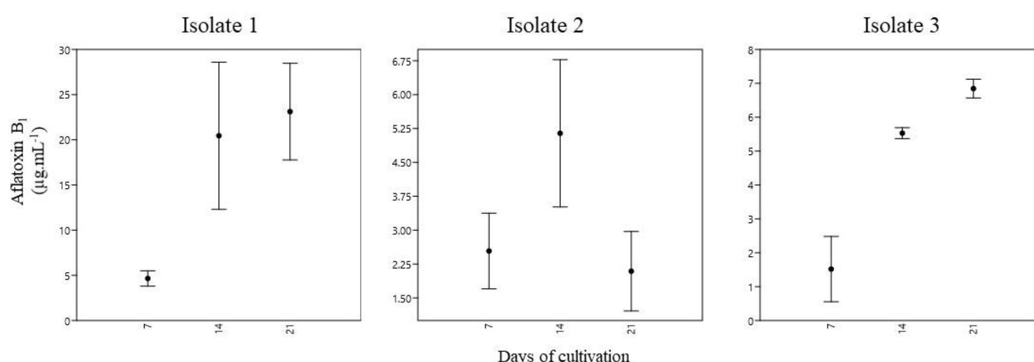

Figure 1 Kinetics of AFB_1 concentration (µg.mL^{-1}) in individual isolates in relation with cultivation period (boxplots of mean content and standard errors)

CONCLUSION

Optimization of the method HPLC-FLD and further rapid validation was carried out to provide suitable method for the determination of AFB_1 applicable on samples cultivated from *Aspergillus* section *Flavi* isolates originated from vegetables and cereals. The testing of the method confirmed high sensitivity suitable for the aflatoxin determination at the sub-µg.mL^{-1} concentration levels. The LOD value was calculated to be 0.03 µg.mL^{-1} for AFB_1. A simple sample preparation was applied for the cultivated isolates containing AFB_1 and could be possibly used also for other types of cultivated isolates with a probable production of aflatoxins. The method showed high sensitivity, reproducibility and accuracy and testing the method on real samples showed its suitability for measurements of AFB_1 in isolates or matrices of similar composition.

The concentration of the $aflatoxin_1$ produced by 3 strains of *Aspergillus* section *Flavi*, was ranging between levels below LOD and 49.38 µg.mL^{-1}. The highest concentration of AFB_1 was obtained at 25±1°C after 14 days of experimental cultivation. The highest concentration of AFB_1 (49.38 µg.mL-

[1]) produced Isolate 1 (KMi-ZB1, isolated from barley, Kolíňany, 2018). Comparison of concentration of AFB_1 regarding the cultivation period showed the highest increase in AFB_1 production by *Aspergillus* strains between 7th and 14th day of cultivation.

Acknowledgments: Funding of this work was provided by the VEGA 1/0239/21 and VEGA1/0517/21 projects.

REFERENCES

Aldars-García, L., Marín, S., Sanchis, V., Magan, N., & Medina, A. (2018). Assessment of intraspecies variability in fungal growth initiation of *Aspergillus flavus* and aflatoxin B1 production under static and changing temperature levels using different initial conidial inoculum levels. *International journal of food microbiology*, 272, 1-11. https://doi.org/10.1016/j.ijfoodmicro.2018.02.016

Alvarez, C. S., Hernández, E., Escobar, K., Villagrán, C. I., Kroker-Lobos, M. F., Rivera-Andrade, A., Smith, J. W., Egner, P. A., Lazo, M., Freedman, N. D., Guallar, E., Dean, M., Graubard, B. I., Groopman, J. D., Ramírez-Zea, M., McGlynn, K. A. 2020. Aflatoxin B1 exposure and liver cirrhosis in Guatemala: A case–control study. *BMJ Open Gastroenterology*, 7 (1), 1-7. http://dx.doi.org/10.1136/bmjgast-2020-000380

Amare, M. G., Keller, N. P. 2014. Molecular mechanisms of *Aspergillus flavus* secondary metabolism and development. *Fungal Genetics and Biology*, 66, 11-18. https://doi.org/10.1016/j.fgb.2014.02.0088

Ayob, O., Hussain, P. R., Naqash, F., Riyaz, L., Kausar, T., Joshi, S., Azad, Z. R. A. A. 2022. Aflatoxins: Occurrence in red chilli and control by gamma irradiation. In *International Journal of Food Science and Technology,* 57, 2149-2158. https://doi.org/10.1111/ijfs.15088

Bacaloni, A., Cavaliere, C., Cucci, F., Foglia, P., Samperi, R., Laganà, A. 2008. Determination of aflatoxins in hazelnuts by various sample preparation methods and liquid chromatography- tandem mass spectrometry. *Journal of Chromatography A*, 1179, 182–189. https://doi.org/10.1016/j.chroma.2007.11.081

Blesa, J., Soriano, J. M., Molto, J. C., Marın, R., & Manes, J. (2003). Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography. *Journal of Chromatography A*, 1011(1-2), 49-54. https://doi.org/10.1016/S0021-9673(03)01102-6

Campos, W. E., Rosas, L. B., Neto, A. P., Mello, R. A., & Vasconcelos, A. A. (2017). Extended validation of a senstive and robust method for simultaneous quantification of aflatoxins B1, B2, G1 and G2 in Brazil nuts by HPLC-FLD. Journal of Food Composition and Analysis, 60, 90-96. https://doi.org/10.1016/j.jfca.2017.03.014

Coppock, R. W., Christian, R. G., & Jacobsen, B. J. (2018). Aflatoxins. In *Veterinary toxicology* (pp. 983-994). Academic Press. https://doi.org/10.1016/B978-0-12-811410-0.00069-6

Costa, J., Rodríguez, R., Garcia-Cela, E., Medina, A., Magan, N., Lima, N., Battilani, P., Santos, C. (2019). Overview of fungi and mycotoxin contamination in Capsicum pepper and in its derivatives. *Toxins*, 11(1), 27. https://doi.org/10.3390/toxins11010027

Dai, H., Huang, Z., Liu, X., Bi, J., Shu, Z., Xiao, A., & Wang, J. (2022). Colorimetric ELISA based on urease catalysis curcumin as a ratiometric indicator for the sensitive determination of aflatoxin B1 in grain products. *Talanta*, 246, 123495. https://doi.org/10.1016/j.talanta.2022.123495

Davis, N. D., Diener, U. L., & Eldridge, D. W. (1966). Production of aflatoxins B1 and G1 by Aspergillus flavus in a semisynthetic medium. *Applied microbiology*, 14(3), 378-380. https://doi.org/10.1128/am.14.3.378-380.1966

Fan, S., Li, Q., Sun, L., Du, Y., Xia, J., & Zhang, Y. (2015). Simultaneous determination of aflatoxin B1 and M1 in milk, fresh milk and milk powder by LC-MS/MS utilising online turbulent flow chromatography. *Food Additives & Contaminants: Part A*, 32(7), 1175-1184. https://doi.org/10.1080/19440049.2015.1048311

Frisvad, J. C., Hubka, V., Ezekiel, C. N., Hong, S. B., Nováková, A., Chen, A. J., Aranlou, M., Larsen, T. O., Sklenář, F., Mahakarnchanakul, W., Samson, R. A., Houbraken, J. 2019. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies in Mycology*, 93. 1-63. https://doi.org/10.1016/j.simyco.2018.06.001

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani. 2007. Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *International Journal of Food Microbiology*, vol. 113, issue 3, 330-338. https://doi.org/10.1016/j.ijfoodmicro.2006.09.007

Giorni, P., Battilani, P., Pietri, A., & Magan, N. (2008). Effect of aw and CO2 level on Aspergillus flavus growth and aflatoxin production in high moisture maize post-harvest. International Journal of Food Microbiology, 122(1-2), 109-113. https://doi.org/10.1016/j.ijfoodmicro.2007.11.051

Golge, O., Hepsag, F., & Kabak, B. (2016). Determination of aflatoxins in walnut sujuk and Turkish delight by HPLC-FLD method. *Food Control*, 59, 731-736. https://doi.org/10.1016/j.foodcont.2015.06.035

Hegedűs, O., Hegedűsová, A., Jakabová, S., Vargová, A., Pernyeszi, T., & Boros, B. (2010). Evaluation of an HPIC Method for Determination of Nitrates in Vegetables. *Chromatographia*, 71(1), 93-97. https://doi.org/10.1365/s10337-010-1595-9

Iamanaka, B. T., Nakano, F., Lemes, D. P., Ferranti, L. S., & Taniwaki, M. H. (2014). Aflatoxin evaluation in ready-to-eat Brazil nuts using reversed-phase liquid chromatography and post-column derivatisation. *Food Additives & Contaminants: Part A*, 31(5), 917-923. https://doi.org/10.1080/19440049.2014.895857

IARC (International Agency for Research on Cancer), in IARC. 2002. Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Traditional Herbal Medicines, some Mycotoxins, Naphthalene and Styrene 82, 171 IARC (Ed.), Geneve.

Jallow, A., Xie, H., Tang, X., Qi, Z., & Li, P. (2021). Worldwide aflatoxin contamination of agricultural products and foods: From occurrence to control. *Comprehensive reviews in food science and food safety*, 20(3), 2332-2381. https://doi.org/10.1111/1541-4337.12734

Jolly, P. E., Inusah, S., Lu, B., Ellis, W. O., Nyarko, A., Phillips, T. D., & Williams, J. H. (2013). Association between high aflatoxin B1 levels and high viral load in HIV-positive people. *World mycotoxin journal*, 6(3), 255. https://doi.org/10.3920/WMJ2013.1622

Lee, S., Lee, D. K. (2018). What is the proper way to apply the multiple comparison test? *Korean journal of anesthesiology*, 71(5), 353–360. https://doi.org/10.4097/kja.d.18.00242

Maggira, M., Sakaridis, I., Ioannidou, M., & Samouris, G. (2022). Comparative Evaluation of Three Commercial Elisa Kits Used for the Detection of Aflatoxins B1, B2, G1, and G2 in Feedstuffs and Comparison with an HPLC Method. *Veterinary Sciences*, 9(3), 104. https://doi.org/10.3390/vetsci90301044

Mateo, E. M., Gómez, J. V., Gimeno-Adelantado, J. V., Romera, D., Mateo-Castro, R., & Jiménez, M. (2017). Assessment of azole fungicides as a tool to control growth of Aspergillus flavus and aflatoxin B1 and B2 production in maize. *Food Additives & Contaminants: Part A*, 34(6), 1039–1051. https://doi.org/10.1080/19440049.2017.1310400

Negash, D. (2018). A review of aflatoxin: occurrence, prevention, and gaps in both food and feed safety. *Nutritional Health & Food Engineering*, 8(2),190–197. https://doi.org/10.15406/jnhfe.2018.08.00268

Okoth, S., De Boevre, M., Vidal, A., Di Mavungu, J. D., Landschoot, S., Kyallo, M. Njuguna, J., Harvey, J., De Saeger, S. (2018). Genetic and toxigenic variability within Aspergillus flavus population isolated from maize in two diverse environments in Kenya. *Frontiers in Microbiology*, 9, 57. https://doi.org/10.3389/fmicb.2018.00057

Ostry, V., Malir, F., Toman, J., & Grosse, Y. (2017). Mycotoxins as human carcinogens—the IARC Monographs classification. *Mycotoxin research*, 33(1), 65-73. https://doi.org/10.1007/s12550-016-0265-7

PAST 4.03 for Windows. (2021). Softpedia. https://www.softpedia.com/get/Science-CAD/PAST.shtml

Pereira, C. S., Cunha, S. C., & Fernandes, J. O. (2020). Validation of an enzyme-linked immunosorbent assay (ELISA) test kit for determination of aflatoxin B1 in corn feed and comparison with liquid-chromatography tandem mass spectrometry (LC-MS/MS) method. *Food Analytical Methods,* 13(9), 1806-1816. https://doi.org/10.1007/s12161-020-01805-4

Rushing, B. R., & Selim, M. I. (2019). Aflatoxin B1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. Food and chemical toxicology, 124, 81-100. https://doi.org/10.1016/j.fct.2018.11.047

Samson, R. A., Frisvad, J. C. 2004. *Penicillium* Subgenus *Penicillium*: New Taxonomic Schemes and Mycotoxins and Other Extrolites; Centraalbureau voor Schimmelcultures (CBS): Utrecht, The Netherlands, vol. 449, ISBN 90-70351-53-6.

Saldan, N. C., Almeida, R. T., Avíncola, A., Porto, C., Galuch, M. B., Magon, T.F., Pilau, E.J., Svidzinski, T.I. & Oliveira, C. C. (2018). Development of an analytical method for identification of Aspergillus flavus based on chemical markers using HPLC-MS. *Food chemistry*, 241, 113-121. https://doi.org/10.1016/j.foodchem.2017.08.065

Sheijooni-Fumani, N., Hassan, J., & Yousefi, S. R. (2011). Determination of aflatoxin B1 in cereals by homogeneous liquid–liquid extraction coupled to high performance liquid chromatography-fluorescence detection. *Journal of separation science*, 34(11), 1333-1337. https://doi.org/10.1002/jssc.201000882

Stefanovic, S., Spiric, D., Petronijevic, R., Trailovic, J. N., Milicevic, D., Nikolic, D., & Jankovic, S. (2015). Comparison of two analytical methods (ELISA and LC-MS/MS) for determination of aflatoxin B1 in corn and aflatoxin M1 in milk. *Procedia food science*, 5, 270-273. https://doi.org/10.1016/j.profoo.2015.09.077

Varga, J., Frisvad, J., & Samson, R. (2009). A reappraisal of fungi producing aflatoxins. *World Mycotoxin Journal*, 2(3), 263-277. https://doi.org/10.3920/WMJ2008.1094

Varga, J., Frisvad, J. C., & Samson, R. (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Studies in Mycology*, 69(1), 57-80. https://doi.org/10.3114/sim.2011.69.05

Waliyar, F., Siambi, M., Jones, R., Reddy, S. V., Chibonga, D. et al. 2008. Institutionalizing Mycotoxin Testing in Africa. In Leslie, J. F., Bandyopadhyay, R., Visconti, A. M*ycotoxins: Detection Methods, Management, Public Health and Agricultural* Trade. CABI, 2008, 476 p. ISBN-13: 978 1 84593 082 0