OCCURRENCE OF AFLATOXINES IN RAW PEANUTS AND PEANUT PRODUCTS DETERMINED BY FLUORIMETRY AND LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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INTRODUCTION

Aspergillus flavus belongs to the Genus Aspergillus, Subdivision Deutoromycotina (Alexopoulos et al., 1996). *Aspergillus flavus* causes diseases of agronomical important crops, such as corn and peanuts, is second only to *Aspergillus fumigatus* as the cause of human invasive aspergillosis, and is the *Aspergillus* species most frequently reported to infect insects (St. Leger et al., 2000). It was demonstrated that most *A. flavus* strains can cause disease in both plants and animals. Many fungi moved from opportunistic forms to specialized pathogens by gaining the ability to produce host-selective toxins that provided the genetic isolation for evolutionary change. *A. flavus* produces a variety of toxins, including aflatoxins. The occurrence of *Aspergillus flavus* in field maize was first reported 75 years ago (Taubenhaus, 1920). *Aspergillus flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage (Yu et al., 2004). *A. flavus* has been reported to occur in most agricultural soils of the south. The fungus occurs on many types of organic material in various stages of decomposition including forages, cereal grains, food, and feed products. Aflatoxins are harmful or fatal to livestock and are considered carcinogenic (Moreno and Kang, 1999). *A. flavus* is a common contaminant in agriculture. Among the at least 16 structurally related aflatoxins characterized, however, there are only four major aflatoxins, B1, B2, G1, and G2 (AFB1, AFG1, AFB2, and AFG2), that contaminate agricultural commodities and pose a potential risk to livestock and human health. *Aspergillus flavus* produces AFB1 and AFB2. *Aspergillus flavus* causes aspergillosis, a life-threatening human disease, particularly in patients who are immune suppressed or have chronic lung disease. *Aspergillus flavus* is responsible for about 30% of the cases of aspergillosis.

ASPERGILLUS FLAVUS

Peanuts and peanut based products are considered as popular food among all age groups, especially peanut snacks and peanut flips (Dissa nayane *et al*., 2009). Peanut has proved to be a good substrate for the growth of *Aspergillus sp*. and for the production of aflatoxins (Bakhiet et Musa, 2010). Factors responsible for the high incidence of aflatoxin contamination of peanuts include poor agricultural practices during planting, harvesting, drying, transportation and storage of the product Oliveira *et al.,* 2009). There are several reports from all over the world concerning the aflatoxin contamination in peanuts and peanut products. Yentür *et al*., (2009) analyzed 20 samples of peanut butter in Turkey and found that all samples contained aflatoxins ranging from 8.16-75.74 µg/kg. Oliveira *et al*. (2009) reported that 52% of the peanut samples analyzed in Brazil were positive for aflatoxins, ranging from 51-420 µg/kg. Park (2006) analyzed 40 peanut and 30 peanut butter samples. AFB1 was found in 5 peanut butter samples with mean AFB1 concentration of 12 µg/kg, and 10 peanut samples had AFB1 concentration ranging from 19-32 µg/kg. In another study, Mutegi *et al.,* (2009) carried out a survey on a total of 1260 peanut product samples. Thirty eight per cent of all samples tested were noted safe according to the EU regulatory limits. The most contaminated product was peanut flour with 88% of all its products, having aflatoxin levels of more than 10 µg/kg.

MATERIALS AND METHODS

HPLC analysis was performed with Perkin Elmer (PE) chromatographic system equipped with binary pump (PE LC-250), manual injector (PE LC-250), manual injector (PE Rheodyne 7125) and fluorescence detector (PE LC-240). Mycotoxins were separated on Supelco column (250 mm x 4.6 mm, 5mm) at room temperature. The mobile phase was a mixture of water: acetonitrile: methanol (600:50:350, *V/V/V)* with addition of 119 mg KBr and 350 ml 4N HNO3. The mobile phase was degasified in the ultrasonic bath before use. The flow rate was 1 ml/min and the injection volume was 100 ml. The detection was carried out at ex = 360 nm and em = 440 nm.
Fluorometer VICAM, series 4, was used for fluorometric determination, at 360 nm.

Reagents and standards

HPLC reagents (methanol, acetonitrile, water) and chemicals (benzene, KBr, NaCl, HNO3) were purchased from Merck (Darmstadt, Germany). For clean-up purification immunoaffinity columns Aflaprep (R-Biopharm Rhône, Glasgow, Scotland) were used. As a standard aflatoxin mix from Supelco, with concentrations of AFB1 982 ng/ml, AFB2 284 ng/ml, AFG1 1034 ng/ml, AFG2 333 ng/ml dissolved in benzene:acetonitrile (98:2), was used. Aflatoxin mix stock solution with concentrations of AFB1 100 ng/ml, AFB2 28.4 ng/ml, AFG1 103.4 ng/ml, AFG2 33.3 ng/ml was prepared from the aflatoxin mix standard, dissolving aliquot (1.01 ml) in a volumetric amber flask of 10 ml with solvent mixture benzene:acetonitrile (98:2). Seven working standard solutions were prepared from the stock solution in volumetric amber flasks of 5 ml. The aliquot of solution needed for preparation of working standards was evaporated under stream of nitrogen and the dry residue was dissolved in methanol:water (1:1). All working standards were kept in a refrigerator at 2-8°C.

Samples

Total of 37 peanuts and peanut based products samples were collected during 2013. All the samples were brought to the laboratory by border health inspectors or food operators. The samples were kept in their original packages in dark, dry and cool place until analysis. For the recovery experiment, aflatoxin-free peanut samples were spiked with known amount of aflatoxin B1 at three concentration levels (1.0, 2.0 and 5.0 mg/kg) and all of them were around maximum residual level (MRL) for peanuts. Those portions (500 ml) of aflatoxin B1 standards were applied to the tested samples and they were kept for app. 15 min before the addition of extraction solvent.

Analytical procedure

The extraction and purification of aflatoxins from peanut samples was done according to AOAC method (AOAC, 2005). HPLC-FLD procedure was performed according to ISO standard (ISO 16050:2003). Twenty five grams of tested sample with addition of 5 g NaCl and 125 ml 70% methanol was mixed in a blender jar for 2 min at high speed. The mixture was filtered through a fluted filter paper. Thirty ml of water was added to a 15 ml of filtrate and filtered again through microfiber filter paper. Fifteen ml of the second filtrate was quantitatively passed through the immunoaffinity column at flow rate of 1ml/min. The column was washed with 10 ml of water. Aflatoxins were eluted with 1 ml of methanol in an amber vial at flow rate of 1 ml/min. The elution step was repeated one more time with 1 ml of water. Then, 100 ml of methanol--water solution was applied to HPLC-FLD system, followed by derivatization with bromine in Kobra cell (R-Biopharm Rhône).

RESULTS AND DISCCUSSION

Seven-point calibration curves were linear in the proposed concentration range for all four aflatoxins and they had satisfactory coefficient of correlation (R^2) in the range of 0.9991-0.9999. The method was appropriate for the tested concentration range, having in mind the European legislation regarding the maximum permitted level of aflatoxins in peanuts (2.0 µg/kg for AFB1 and 4.0 µg/kg for sum of AFB1, AFB2, AFG1 and AFG2 for nuts and processed products thereof, intended for human consumption or use as ingredients in foodstuff). Limits of detection were in the range of 0.004-0.009 µg/kg for all four aflatoxins and limits of quantification were in the range of 0.009-0.024 µg/kg.
The results obtained from repeatability, which was estimated by relative standard deviation (*RSD*), were satisfactory and they are presented in Table 1. The obtained values are in the acceptable range (0.448 % – 2.052 %) at proposed concentration level for all four aflatoxins.

Tab. 1. – Repeatability, estimated by relative standa rd deviation (*RSD*) and recovery of the method

	Spiked concen-tration level (µg/kg)	Concen tration found (µg/kg)	SD n =10	RSD (%)	Reco-very (%)
AFB1	9.867	9.012	0.185	2.052	91.33
AFB2	2.259	2.678	0.012	0.448	118.56
AFG1	10.910	8.765	0.165	1.882	80.32
AFG2	4.710	2.345	0.045	1.919	49.78

Tab. 2. – Results from the analysis of total aflatoxins in peanuts and peanut based products

Type of sample	Number of samples	Number of samples below LOD	Number of samples over MRL	Concentration range for total AF (B1+B2+G1+G2) µg/kg
Peanuts	20	15	5	5.5 – 10.2
Peanut snacks	12	10	2	12.4 – 15.8
Peanut flips	3	3	—	—
Peanut butter	2	2	—	—

CONCLUSION

This study presented the results of application of reliable HPLC-FLD method for the analysis of total aflatoxin. The validation procedure confirmed that the proposed method provides satisfactory aflatoxin recoveries (mean value for total aflatoxins was 84.99%), with acceptable precision values in the range of 0.448–2.052% at proposed concentration levels for all four aflatoxins. The method also showed high level of peak selectivity and low values for limit of detection and limit of quantification. The results from 37 analyzed samples showed that 30 samples (81.1%) were below the LOD. Five peanut samples, and two peanut based products (peanut snack) had positive results over the MRL with total content of aflatoxins of 12.4 µg/kg and 15.8 µg/kg, respectively.