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Comparative Study Regarding the Application of HPLC and ELISA Methods for Aflatoxins Detection in Peanuts

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Abstract

Aflatoxins are carcinogenic substances produced by fungi and due to the growth of fungi in a wide range of commodities including cereals, nuts, figs and spices, it is necessary to test such products for the presence of aflatoxins prior to their incorporation into other foodstuff or feeds. There is a need for a quick, simple test which can be used to check a diverse range of foodstuff. Two different detection methods of aflatoxins in peanuts were presented and compared (HPLC and ELISA). Both methods allow the determination of aflatoxin content with good recoveries for the levels of interest.

Keywords: mycotoxins, aflatoxins, HPLC, ELISA, peanuts

1. Introduction

In recent years peanuts exportation shows a decrease due to metabolite outcome "Aflatoxins" of *Aspergillus* species. *Aspergillus Fumigatus, Aspergillus Flavus* and *Aspergillus Niger* are the most seen pathogen examples. These species cause a disease named Aspergillosis in humans and animals, pulmonic aspergillosis being a serious and common disease which is seen especially in birds and mammals [1].

The presence of aflatoxins in peanuts and peanut products is caused frequently by the incorrect processing techniques of the raw peanuts, such as drying, and the unsuited storage conditions. Factors as storage duration, humidity in depot, water leaks, insect activity, temperature, faults of air conditioning are the major factors that are causing the production of mycotoxins during the storage period [2,3].

During the time in order to prevent any diseases due to contamination with mycotoxins, maximum tolerated levels of the content of aflatoxins in foodstuff and feedstuff were established by different international organism such as European Commission or the US Food and Drug Administration. Regulations for major mycotoxins in commodities and food exist in at least 100 countries, most of which are for aflatoxins, but maximum tolerated levels differ among countries [4].

In order to control the levels of mycotoxins in food and feeding products different analytical techniques for the detection of mycotoxins have been developed. Conventional analytical methods for mycotoxins determination are involving techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Also, rapid methods for mycotoxin analysis have become increasingly important. Enzyme linked immuno-sorbent assay (ELISA) is one of the rapid methods for determination of aflatoxins in peanuts and peanut products. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin.

The aim of this study is to provide a comparison between two different methods of analysis (HPLC and ELISA) for the detection of Aflatoxin B1 and

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the total content of aflatoxins as sum of aflatoxin B1, B2, G1 and G2 in peanuts.

2. Materials and methods

Thirty peanut samples (groundnuts intended for direct human consumption), 1 kg each, were purchased for analysis from different suppliers. The peanuts were grinded and homogenized and a representative test portion was taken for analysis. The tests have been worked in parallel with two reference materials, peanut and peanut butter, as quality control samples, using the same procedures and reagents. The assigned values for Aflatoxin B1 are 0.44 µg/kg in peanut and 2.12 µg/kg in peanut butter. The assigned values for Total Aflatoxin are 0.98 µg/kg in peanut and 4.58 µg/kg in peanut butter.

The study involves the use of HPLC and ELISA techniques. Both methods require the extraction of aflatoxins from the studied matrix. In order to detect the content of aflatoxins on HPLC, 20 g of test portion were extracted in 100 mL methanol:water (80:20 (v/v)) mixture. Due to the fact that peanut is a fatty matrix, 50 mL of n-hexane were added to the extraction solvent. Also, 2 g of sodium chlorite were added to the extraction mixture. The extraction was performed in a blender jar at high speed for 5 minutes. The extract was filtered through a fluted filter paper and 14 mL of the purified extract were diluted with 86 mL of PBS buffer (pH 7.2). The PBS buffer contains: 8.0 g/L sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L di-sodium hydrogen phosphate, 0.2 g/L potassium dihydrogen hosphate. The diluted extract was passed through a 0.2 µg syringe filter to remove residual turbidity. 40 mL of the diluted extract was applied to an affinity column. A gentle vacuum was used to maintain a flow rate of 2 ml/min. The column was washed with 10 ml of distilled water and carefully the residual water was removed. The immunoaffinity column contains antibodies specific for aflatoxins B_1, B_2, G_1 and G_2. In this way the aflatoxins were isolated, purified and concentrated on the column and then removed from the antibodies with 2 mL of methanol. The aflatoxins are quantified by reversed-phase high-performance liquid chromatography with fluorescence detection and post-column derivatization. Since only the aflatoxins B_2 and G_2 show natural fluorescence, B_1 and G_1 must be derivatized prior to detection. This is done photochemically using the UVE^{TM} high-performance photochemical derivatization system, by irradiation with UV light at 254 nm. The aflatoxins B_1 and G_1 are thus hydroxylated, leading to stable and measurable fluorescence. The chemical or measurement-related properties of the other aflatoxins are not changed by this step.

In table 1 are presented the HPLC operating conditions.

Table 1. HPLC operating conditions.

HPLC column	150 x 4.6 mm with guard column 8 x 4.6 mm; C18; 3 µm
Mobile phase	water/acetonitrile/methanol = 60/15/25 // v/v/v; isocratic
Flow rate	1 mL/minute
Injection volume	10 µL
Column temperature	35 ^{o}C
Fluorescence detection	λ – Excitation: 365 nm
	λ – Emission: 460 nm
Run time	15 minutes

In order to detect the content of aflatoxins using the ELISA method, 50 g of representative test portion were taken for analysis. 10 g of sodium chloride were added and the extraction was performed with 250 mL mixture of methanol:water (70:30 (v/v)) in a blender jar at high speed for 5 minutes. The obtained extract was passed through a Whatman 1 filter paper and the filtrate was collected and used in the ELISA assay (50 µl). The assay is performed in plastic microwells that have been coated with anti-aflatoxins antibody.

100 µl of enzyme conjugate and 50 µl of each sample were added in each premixing well. In the premixing wells the enzyme labelled aflatoxin and the samples are mixed and then 100 µl of the content of each premixing well were transferred into the anti-aflatoxins microtiter plate (anti-aflatoxins antibody coated microwell). Immediately the samples were incubated 10 minutes at room temperature in the dark. During the incubation, free aflatoxins in the sample and enzyme-labelled aflatoxin compete for the anti-aflatoxins antibody binding sites on the solid phase. Any unbound enzyme conjugate and aflatoxin molecule is then removed in a washing step. The bound enzyme activity is determined adding a fixed amount of a chromogenic substrate. 100 µl of development solution was added to each well and then incubated for 5 minutes at room temperature. The enzyme converts the colourless chromogen into a blue product. 50 µl of stop solution was added to each well and mixed thoroughly with rotatory motion for few seconds.

The addition of the stop reagent leads to a colour change from blue to yellow. The absorbance is measured with a microplate reader at 450 nm. The colour development is inversely proportional to the aflatoxin concentration in the sample. [5, 6, 7, 8]

3. Results and Discussion

Both studied methods of aflatoxins detection in peanut samples, HPLC and ELISA, allows the quantitative determination of the content of aflatoxins.

Using HPLC the quantitative determination is performed by external standard method with integration of the peak area which is then related to the corresponding value for the standard substance [9]. The mathematical model is represented by the relationship between the outlet value and the inlet value of the process. Equation (1) represents the mathematical model which describes the dependence between the peak area (Y) expressed in μV·sec (outlet value) and the amount (X) of the standard solution expressed in μg/kg (inlet value). Each standard solution was injected three times.

$$Y = a \cdot X - b \quad (1)$$

In table 2 are presented the coefficients of equation (1) corresponding for each aflatoxin (B1, B2, G1 and G2), calculated by HPLC's Empower2 software. Empower 2 is Waters' chromatography data software package for advanced data acquisition, management, processing, reporting and distribution. Also, in table 2 are presented the accuracy indicators of the mathematical models (R^2 – model accuracy indicator, R – correlation coefficient).

In figure 1, 2, 3 and 4 are presented the dependencies described by equation (1).

Table 2. Equation's (1) coefficients and the accuracy indicators

Case	Equation's coefficients		Accuracy indicators	
	a	b	R^2	R
Aflatoxin B1	$2.92 \cdot 10^5$	$1.84 \cdot 10^4$	0.998	0.999
Aflatoxin B2	$4.20 \cdot 10^5$	$2.72 \cdot 10^4$	0.999	0.999
Aflatoxin G1	$2.32 \cdot 10^5$	$6.05 \cdot 10^3$	0.999	0.999
Aflatoxin G2	$4.85 \cdot 10^5$	$2.06 \cdot 10^4$	0.999	0.999

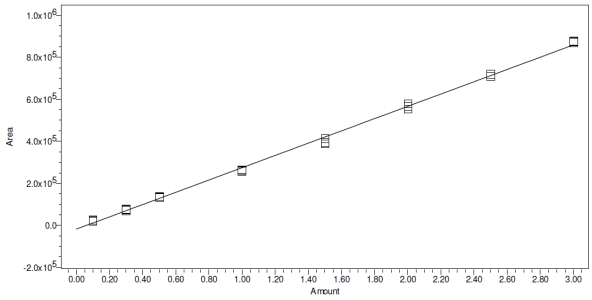

Figure 1. HPLC calibration for Aflatoxin B1

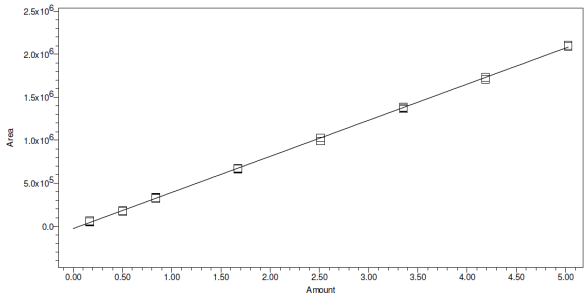

Figure 2. HPLC calibration for Aflatoxin B2

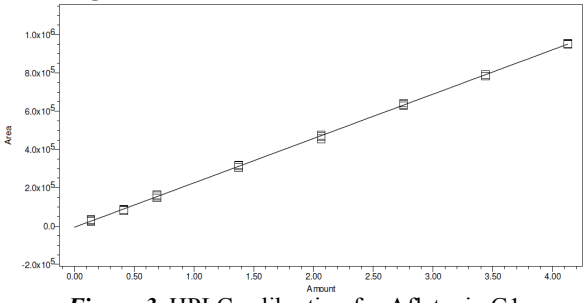

Figure 3. HPLC calibration for Aflatoxin G1

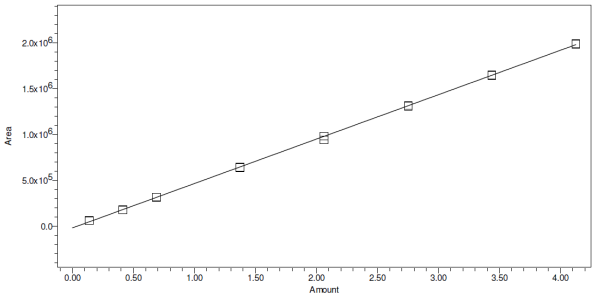

Figure 4. HPLC calibration for Aflatoxin G2

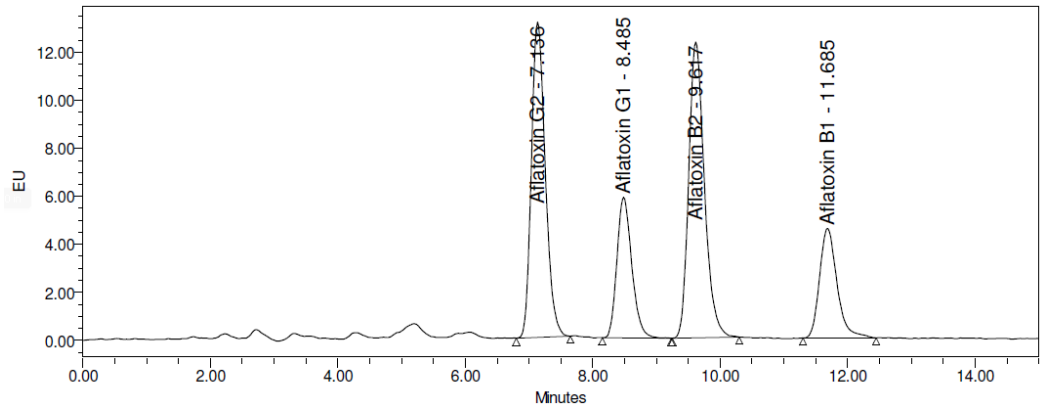

Figure 5. Chromatogram of a standard solution of mixed aflatoxins (G2, G1, B2, B1)

Table 3. Equation's (3) coefficients and the accuracy indicator R^2

Case	Equation's coefficients		Model's accuracy indicator R^2
	a	b	
Aflatoxin B1	-48.828	90.618	0.995
Total Aflatoxin	-48.663	102.597	0.986

Table 4. Limits of quantification

Parameter	HPLC	ELISA
Aflatoxin B1	0.18 µg/kg	1 µg/kg
Total Aflatoxin	0.48 µg/kg	1 µg/kg

Table 5. Recoveries for the quality control samples

Reference Material	Aflatoxin B1		Total Aflatoxin		Recommended value
	HPLC	ELISA	HPLC	ELISA	
Peanut	98 %	50%	94 %	80 %	50 – 120 %
Peanut butter	101 %	93 %	96 %	92 %	70 – 110 %

The identification takes in consideration the following: a) if there is no peak at the characteristic retention time, and the chromatogram is normal in all other aspects, it is assumed that the compound is not present; b) it is assumed that an individual compound is present if the retention time of the compound in the test sample chromatogram coincides with the retention time of a reference compound in the reference chromatogram measured under the same conditions; c) the aflatoxins may be identified by simultaneous injection of the sample test solution and standard solution; d) the disappearance of the aflatoxin B_1 and G_1 peaks if no derivatization is performed is helpful for identification [9].

In figure 5 is presented the chromatogram of a standard solution. The aflatoxins are eluting at the following retention times: Aflatoxin G2 at 7.14 minutes, Aflatoxin G1 at 8.49 minutes, Aflatoxin B2 at 9.62 minutes and Aflatoxin B1 at 11.69 minutes.

The mass fraction of each aflatoxin, m_{AF}, in micrograms per kilogram of sample (µg/Kg), is calculated using the following equation:

$$m_{AF} = \frac{\frac{V_1}{W}\cdot\frac{V_2+V_3}{V_3}}{\frac{V_4}{V_5}} \cdot m_{HPLC} \qquad (2)$$

Where: V_1 is the total volume of the extraction solvent (mL); W is the mass of the test portion (g); V_2 is the volume of the PBS buffer used to dilute the purified extract (mL); V_3 is the volume of the purified extract added to the PBS buffer (mL); V_4 is the volume of extract passed through the immunoaffinity column (mL); V_5 is the final volume of collected and diluted methanol eluate (mL); m_{HPLC} is the mass fraction of each aflatoxin present in the injection volume, corresponding to the measured peak area read off the calibration graph (µg/kg).

The mass fraction of Total Aflatoxins is expressed as sum of the mass fraction of the four aflatoxins

(Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1 and Aflatoxin G_2).

The quantitative determination using ELISA is based on the relationship between the absorbance as outlet value and concentration as inlet value. The absorbance is measured with a microplate reader at 450 nm. The colour development is inversely proportional to the aflatoxin concentration in the sample. Equation (3) represents the mathematical model which describes the dependence between the absorbance (Y) and the concentration (X) of the standard solution expressed in µg/kg.

$$Y = a \cdot \mathrm{Log}(X) + b \quad (3)$$

In table 3 are presented the coefficients of equation (3) corresponding for Aflatoxin B1 and Total Aflatoxin and the model's accuracy indicator (R^2).

From the thirty tested peanut samples only four samples were detected being positive. A proper quantification was possible for all four samples when the HPLC detection was used and only for three samples in the case of ELISA detection (similar results with the HPLC detection were found), the values for the fourth sample being below the limit of quantification. The fourth positive sample was reanalyzed using both detection methods and the same results were obtained in the case of the HPLC test. In table 4 are presented the established limits of quantification for the two methods.

4. Conclusion

There is a need for a quick and simple test which can be used to check a diverse range of foodstuff. Two different detection methods of aflatoxins in peanuts were presented and compared (HPLC and ELISA). The sensitivity of the HPLC and ELISA test kit can provide accurate and reproducible results for the levels of interest stated by the legislation. The HPLC method is suitable, also, for the quantification of the low content of aflatoxins. Both methods are easy to apply in routine laboratory practice.

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