

QUALITATIVE AND QUANTITATIVE ANALYSIS OF AFLATOXINS IN RAW PEANUTS (*Arachis hypogaea* L.)

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Abstract. Aflatoxins are toxic cancerogenic secondary metabolites, predominantly produced by two fungal species: *Aspergillus flavus* and *Aspergillus parasiticus*. The toxicity of aflatoxins make them primary health hazard because of their occurrence in agricultural food crops. In order to detect presence of contaminants, usually occurring in very low concentrations of several µg/kg, precise analytical methods for detection and quantification are necessary, that have to be simple to carry out and specific, but mainly very sensitive. The subject of this paper is qualitative and quantitative analysis of total aflatoxines (AFB₁ + AFB₂ + AFG₁ + AFG₂) in raw peanuts. The analysis has covered 78 samples of raw peanuts. Preliminary qualitative assessment indicating the presence of contaminants was done using the method of thin layer chromatography, characterised by its simplicity, rapidness, but still sufficient accuracy to separate the contaminated samples. For quantification of aflatoxines, we developed a method of fluorometric spectroscopy. It is an effective procedure for quantification with analytical yield in the range 89.79–104.78%, correlation factor $R^2 = 0.9993$ and sufficiently low value of the limit of quantification LoQ of 2.1 µg/kg. The analysis detected 23% of samples with concentrations of total aflatoxines above the permitted MRL, with average value of 2.6 µg/kg and the maximum of 12.8 µg/kg.

Keywords: aflatoxines, raw peanuts, TLC, fluorometry.

AIMS AND BACKGROUND

The quality of food and its safety have always been important for the human population, but the issue is particularly favoured recently and it grows together with the increase of the number of inhabitants of our planet^{1,2}. Mycotoxins as secondary fungal metabolites are toxic to animals and humans, so their presence in the food chain can cause adverse health effects³. The International Agency for Research on Cancer (IARC) in 1993 announces that aflatoxins in particular aflatoxin B1 (AFB1), are natural substances with the greatest potential for causing cancer. More

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cereal crops such as corn (*Zea mays* L.), peanuts (*Arachis hypogaea*), sorghum (*Sorghum bicolor* L.) and nuts are susceptible to contamination by the fungi that produce aflatoxins *Aspergillus flavus* and *Aspergillus parasiticus*.

The highest prevalence of aflatoxins is observed in South and Southeast Asia, than in Africa, which is partly due to poor hygienic practices in these regions. In Macedonia, and also in Europe, a prominent problem is the trend of increased occurrence of contaminated food, which is especially the case in Eastern European region. In 2011 Biomin published 36% presence of aflatoxins in the food samples at European level, with a mean value of present aflatoxins of 2 µg/kg in positive samples, while in 2012 41% of positive samples are published, with a mean value of 3 µg/kg (Ref. 4). During February-March 2013 several European countries, including Romania⁵, Serbia⁶ and Croatia reported aflatoxin contamination of milk intended for human consumption. The causes for this contamination are not known. In March 2013 feed contamination with Serbian origin that was imported into Netherlands and Germany, was announced.

Due to the cases of increased values of aflatoxin in milk and dairy products in the region discussed above, our country greatly increased controls of the quality of these products. But taking into account the occurrence of aflatoxins in other crops, primarily nuts and peanuts, and the fact that the Macedonian market peanuts are mainly from import origin, in this paper we will focus on this culture.

The main goal of this research is to detect the possible presence and to determine the level of contamination of aflatoxins in peanut samples. The analysis is done in the Laboratory of contaminants and eco-toxicology in the Centre of reference laboratories at the Institute of Public Health of the Republic of Macedonia.

The presence of aflatoxins is determined by two methods:

- Qualitative analysis using thin layer chromatography;
- Quantitative analysis, by fluorometric measurement.

Aflatoxins are group of natural, toxic metabolites, which are probably the most common and prevalent widely known mycotoxins. They create two types of fungi of the genus *Aspergillus* and *Aspergillus flavus* (Fig. 1) and *Aspergillus parasiticus*. The food staff, which is mostly affected, includes rice, maize (Fig. 2), wheat, sorghum, figs and all kinds of nuts (walnuts, peanuts, pistachios and then Brazil nuts, dill, coffee and more).



Fig. 1. View of fungi *Aspergillus flavus* under electronic microscopy



Fig. 2. Aflatoxin contamination of maize

There are four main aflatoxins that can be found in food, whose structural formulas are given in Fig. 3. Aflatoxin B1 is the most frequently occurring aflatoxin in food, which also has most adverse cancerogenic and genotoxic effects. Aflatoxin B2 is a dihydroxy derivative of B1. Aflatoxin B1 and B2 are generated in the presence of fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Unlike them, aflatoxin G1 and G2 are generated only from *Aspergillus parasiticus*. Aflatoxin G2 is dihydroxy derivative of G1. Aflatoxins AFB1, AFB2, AFG1 and AFG2 are toxins that glow blue or green, when illuminated under ultraviolet light, and thus get their names and designations AFB-Blue and AFG-Green⁷.

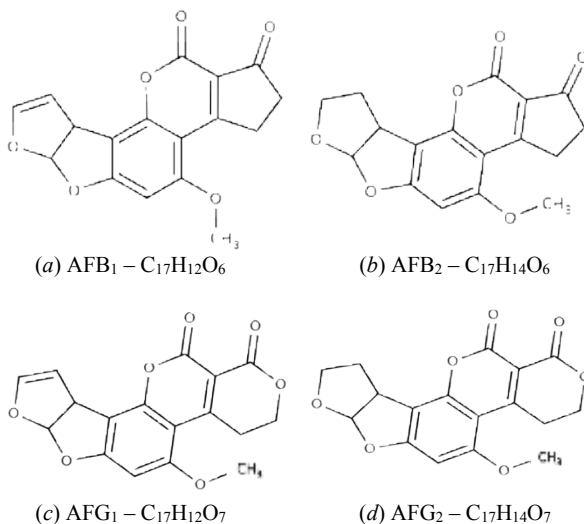


Fig. 3. Structural formulas of aflatoxins

In the European Union, the maximum allowed concentration of aflatoxin in food stuffs is determined by the European Commission Regulation No 1881/2006 from 19 December 2006. The lowest permitted value of AFB₁ in food in EU is 2.0 µg/kg, while the allowed values of AFB₁ in almonds and pistachios are 12.0 µg/kg. The value of total aflatoxins in food for direct consumption is limited to 4.0 µg/kg, and for other food products to 15.0 µg/kg (Ref. 8). Although in 2009 the European Agency for Food Safety EFSA commissioned by the European Commission has made an assessment of the effect of aflatoxins on human health and there are recommendations for raising the limits of allowed aflatoxins from 4 to 10 µg/kg, the European regulations remain the same.

Macedonia has adopted European legislation, defining the maximum allowable limit of total aflatoxins in human foods intended for direct consumption at 4 µg/kg (this applies also to raw peanuts). For foods that are intended for further processing or for ingredients in other foods, the allowed limit of aflatoxin in EU as well as in Macedonia is 15 µg/kg.

Detection of aflatoxins is often at the level of trace (less than µg/l). However, most of analytical instruments can not directly detect the components at this level. Therefore, extraction procedure is imminent because of the need for concentration of target components up to the level which would be measurable. Aflatoxins are generally soluble in organic solvents as methanol, acetone, chloroform and acetonitrile. Thus, for their extraction these solvents or mixtures are used. Extraction varies according to the degree of selectivity, rapidness and convenience, and depends not only on access and conditions at which it is carried out, but also on the configuration of extraction stages.

After extraction next is the process of clean-up of the analyte. A common technique is immunoaffinity clean-up column – IAC (Ref. 9). This technique is based on the specificity and reversibility of the relationship between antibodies and antigens used for separation of aflatoxins extracted from analysed food sample.

Analytical methods for detection and quantification must be simple and specific, but generally sensitive, able to detect the presence of contaminants in very low concentrations of the order of few $\mu\text{g}/\text{kg}$ (Refs 10–12). There are many different analytical methods for analysis of aflatoxins in food samples, which can be divided into three main groups given in Table 1.

Table 1. Overview of standard analytical methods for the detection of aflatoxins in food samples

Type of method	Title of analytical method
Chromatographic methods	thin-layer chromatography – TLC high-performance liquid chromatography – HPLC gas chromatography – GC
Spectroscopic methods	fluorescence spectrophotometry infrared spectroscopy – IR
Immunochemical methods	radioimmunoassay – RIA enzyme-linked immunosorbent assay – ELISA immunoaffinity column assay – ICA

Each of the above methods has its advantages and limitations in terms of sensitivity, ease of use and cost.

EXPERIMENTAL

In this work we made a qualitative and quantitative analysis of aflatoxins in raw peanuts. The analysis is performed using a chromatographic method (thin layer chromatography) for qualitative identification of the presence of aflatoxins and spectroscopic method for quantitative analysis. The analysis includes around 80 samples of raw peanuts, collected from trade network in Macedonia in the period from January 2014 to September 2015. The analysis of the presence of aflatoxins using chromatographic methods is conducted through the multistage process presented with the following sequence (Fig. 4).

The process of purification is performed by passing of the sample solution through the column with chloroform, and using glass wool at the bottom. The chloroform extract is then collected and dissolved into a mixture of organic solvents, 30 ml of chloroform-hexane (1:1) and 20 ml of chloroform-methanol (9:1). Then, eventual aflatoxins are eluted with a mixture of 30 ml of distilled water-acetone (99:1) from the column. This purified sample is subjected to chromatographic testing, by applying spots of the extract onto the TL chromatographic plate by the spots working standard of aflatoxins. The standard mixture of aflatoxins has a concentration of

0.1 µg/ml (B1, B2, G1, G2). The possible presence and identification of aflatoxins is registered by the presence of coloured spots under UV light (wavelength of 365 nm). We used CAMAG UV lamp.

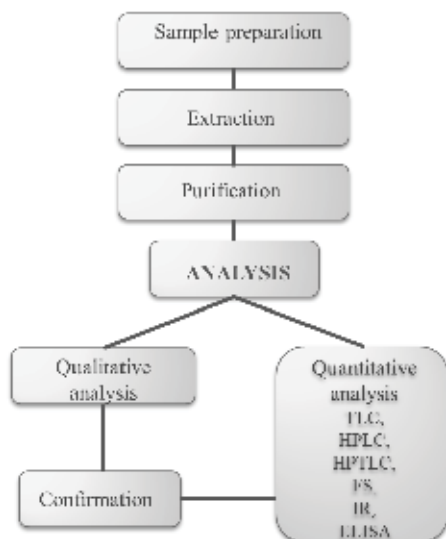


Fig. 4. Sequence for aflatoxin analysis

Fluorometry is a technique by which we measure fluorescence parameters, intensity and wavelength distribution of emission spectra with stimulating light at certain wavelength. Fluorescence analysis technique is much more sensitive than other techniques and it is widely used in medicine, pharmacy, biochemistry, chemistry. In this work is used fluorometer VICAM.

At the beginning the instrument is calibrated using standard aflatoxin solutions, with concentration of 1–22 µg/l of total aflatoxins, in order to avoid readings of uncorrect values. For the measurement, 1 ml of sample eluate is filled into the clean fluorometric cuvette, by adding 1 ml of diluted developer.

RESULTS AND DISCUSSION

In this paper, the presence of aflatoxins in the examined samples is identified by using of simple quantitative TLC method, and quantitative analysis of the samples is done with fluorometric measurement. The analysis covered about 80 samples of raw peanuts which are collected from trade network in Macedonia in the period from January 2014 until September 2015.

Part of the tested samples are first subjected to a qualitative TLC analysis. The plates were developed according to the standard procedure and observed under a

UV lamp. Figure 5 presents developed TLC plate, containing spots from the sample, observed under UV lamp.

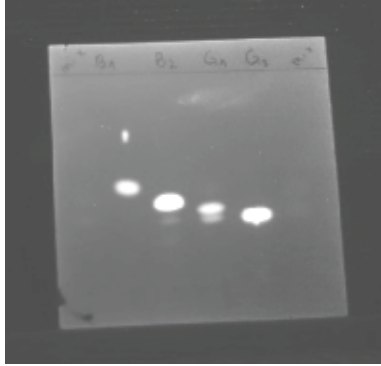


Fig. 5. Developed TLC plate under UV lamp

Fluorometry is an efficient method for quantification of aflatoxins in samples of raw peanuts, showing correlation factor of $R^2 = 0.9993$ and value of the analytical input in the range from 89.79 to 104.78%. The lower limit of quantification of the proposed method is 2.1 $\mu\text{g}/\text{kg}$.

The results for the tested 78 samples of raw peanuts, using fluorometry as a method, are presented in Fig. 6.

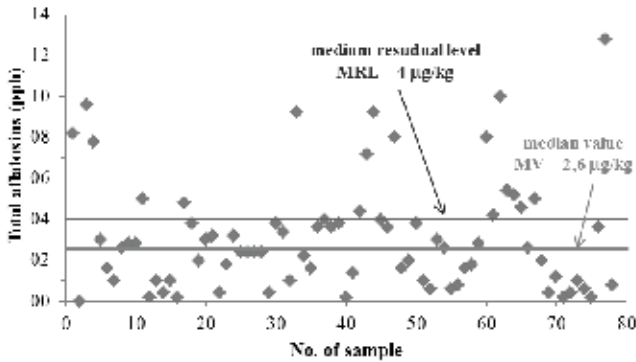


Fig. 6. Results from quantitative analysis of 78 samples of raw peanuts

33 samples of the 78 analysed samples show aflatoxin levels that are below the limit of quantification – LoQ of the method, which is 2.1 $\mu\text{g}/\text{kg}$. The average value of the measured aflatoxins in all samples of raw peanuts (median value MV) is 2.6 $\mu\text{g}/\text{kg}$. From them, 18 samples, i.e. 23.08% of total samples showed levels of aflatoxins above the permitted level according to the European and national regulations (maximum residue level MRL = 4 $\mu\text{g}/\text{kg}$), and the maximum measured value is 12.8 $\mu\text{g}/\text{kg}$ (Fig. 7).

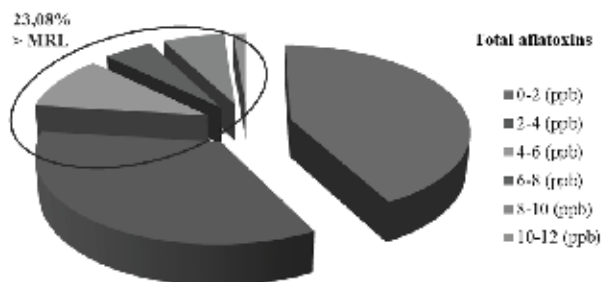


Fig. 7. Percentage of samples with aflatoxin values above MRL

Although only a small part of tested peanut samples confirmed to be contaminated with aflatoxins, this study shows that the occurrence of aflatoxins in food (here raw peanuts) at Macedonian market is not an unknown phenomenon. This observation confirms the need for more serious and more frequent controls of food quality and safety, in order to avoid entry of contaminated food into the food chain. Yet, the highest measured value of aflatoxins is 12.8 $\mu\text{g}/\text{kg}$, is however not too high and even it is below the maximum limit in some countries, such as Brazil, the world major peanuts producer with maximum allowed concentration of 20 $\mu\text{g}/\text{kg}$.

A comparison of the present values of aflatoxin in peanuts on the Macedonian market, obtained in this survey (23% of the tested samples are contaminated), with the values obtained in some other studies in the country and abroad, can essentially only be indicative and informative, mainly due to different values of allowable limits for aflatoxins in different countries and due to usage of different methods for detection. In our previously published results that relate to fluorometric analysis compared to liquid chromatography with fluorescence detection, we had analysed 37 samples of peanuts and peanut products. Even 30 of them showed values below LoD, while 7 products were contaminated with aflatoxin concentrations above MRL, with highest value of 15.8 $\mu\text{g}/\text{kg}$ (Ref. 13). Other authors¹⁴ reported the results of peanuts contamination of 28% of the samples by HPLC.

CONCLUSIONS

In the experimental part of this work, first we done qualitative assessment of the presence of aflatoxins in samples of raw peanuts, using the method of thin layer chromatography, which is distinguished by its simplicity, speed and sufficient precision to separate the contaminated samples.

For the purpose of quantification of aflatoxins, we have developed fluorescence method for analysis of aflatoxins in raw peanuts, which is an efficient method with a value of analytical input in the range from 89.79 to 104.78%, factor of correlation $R^2 = 0.9993$ and sufficiently low value of the lower limit of quantification LoQ of 2.1 $\mu\text{g}/\text{kg}$. This method is applied for the analysis of 78 samples of raw peanuts.

The results show 23% of samples with concentration of total aflatoxins above the permissible level. The average value of total aflatoxins in all measured samples is 2.6 µg/kg and the maximum is 12.8 µg/kg. These observations are somewhat less favourable than the results that have been published in other European countries, but more favourable the results from other countries. Favourable fact is extremely low value of measured maximum concentration of total aflatoxins in the test specimens, which is lower even than the corresponding data in published studies from European countries. This observation should be taken with reasonable reserve, due to the limited number of tested samples, although it is comparable with the number of studied samples published in other similar studies. However, it is only one part of the total quantity of raw peanuts that are entering the domestic market.

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