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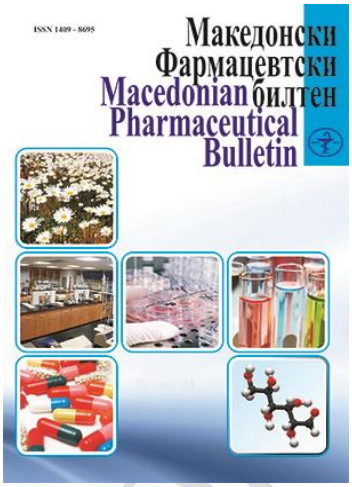

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Analysis of total aflatoxins in commercially available cereal products in the Republic of North Macedonia

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Abstract

Aflatoxins, as naturally occurring toxins, can accumulate in maturing crops or in grain during transportation and represent serious threat for contamination of cereal processed products. Consumption of aflatoxin contaminated foodstuffs can cause acute or chronic toxicity in humans. With an aim to protect public health, maximum residue levels (MRLs) for these toxins are defined by the regulation authorities. To control the compliance of aflatoxin residues in cereal-based products to legislation, a convenient analytical technique that will provide rapid, specific, accurate and costly effective analysis such as ELISA method is required.

In this study, concentration of total aflatoxins in twelve cereal processed products supplied under random selection from several retail outlets in Republic of North Macedonia was determined using commercially available RIDASCREEN Aflatoxin total kit. Analyses have shown that none of the examined samples contained aflatoxins above the MRLs (4 μg/kg). According to the obtained results, the ELISA method can be used for routine detection and quantification of total aflatoxins in cereal products due to simplicity, specificity and sensitivity. In addition, this study may initiate the use of immunochemical methods for analysis of potential aflatoxin contamination in order to establish data collection for the presence of these concerning toxins in cereal-based products available on the national market.

Keywords: aflatoxins, ELISA, immunochemical, cereals

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Introduction

Mycotoxins are toxic secondary metabolites produced by certain molds (fungi) that contaminate cereals, nuts, coffee, cocoa, spices, edible seeds, dried peas and beans, fruit, especially apples as well as dried fruit (Turner et al., 2009). Well-known mycotoxins are aflatoxins (B_1, B_2, G_1 and G_2), citrinin, ergot alkaloids, ochratoxin A, fumonizines, trichothecenes (T-2 toxin and deoxynivalenol), patulin and zearalenone (Bennet and Klinch, 2003). Potentially contaminated products with any of these mycotoxins represent a global food and feed safety concern since mycotoxins are able to penetrate the body of humans and susceptible animals through the digestive system and cause dysfunction of various organs and systems with patterns of both acute and chronic toxicity. In addition, mycotoxins pose a risk for human health when contaminated dust particles are inhaled or entered through the skin. These compounds are rapidly absorbed from the site of exposure to the blood stream and distributed throughout the body. They provoke headaches and various GI symptoms such as abdominal pain, vomiting, and diarrhea, but also serious illnesses leading to death. At particular, the high liposoluble aflatoxins, even at very low concentration levels, have been reported to possess carcinogenic, hepatotoxic, teratogenic, and mutagenic effects on human health (Bbosa et al., 2013; Omotayo et al., 2019).

Aflatoxins are natural mycotoxins produced by many species of the genus *Aspergillus*, fungi of which the most famous are *Aspergillus flavus* and *Aspergillus parasiticus* (Turner et al., 2009). Upon entry into human organism they may be metabolized in the liver to reactive epoxides which can then bind to proteins and cause acute toxicity (aflatoxicosis) or cause DNA mutation via intercalation into DNA and/or alkylation of the bases by epoxide moiety and induce liver cancer (Wild and Montesano, 2009). Aflatoxins are one of the most carcinogenic compounds known. The four primary representatives of this group of structurally related, toxic fungal metabolites, aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are presented in Fig. 1. Of these, AFB_1 has been reported to be the most potent hepatotoxin, the most mutagenic, and the most prevalent worldwide (Benkerroum, 2020).

Fig. 1

Aflatoxin-producing fungi are most commonly found on agricultural crops such as maize, peanuts, cottonseed, and tree nuts (WHO, 2018). Considering the worldwide use of

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cereals and particularly the use of maize in the manufacture of ready-to-eat products for breakfast, analytical confirmation that commercially available foods are safe with regard to aflatoxins is very important. However, the selection of an adequate method for detection and quantitative determination of aflatoxins in food samples is very challenging. Three types of analytical methods have been suggested for analysis of aflatoxins: biological, chemical, and immunochemical methods. Biological methods are based on reactions occurring as a result of an exposure of a biological test organism (chick embryos, day-old ducklings, brine shrimp, *Salmonella* and *Bacillus* spp., and ochra seedlings) to a certain concentration of aflatoxins. However, these methods are qualitative, nonspecific and time-consuming therefore they are only useful for indicating the presence of a toxin in the system. Conventional techniques such as thin layer chromatography, gas or liquid chromatography, spectrofluorometry, spectrophotometry and capillary electrophoresis provide more reliable results, but the cost is significantly higher since specific instrumentation is required (Leszczyńska et al., 2001; Turner et al., 2009). The separation of the analytes (aflatoxins) may be laborious due to the complex nature of food samples. Immunochemical methods are specific, sensitive, simple, and rapid and have become the method of choice for routine analysis of aflatoxins in foods and feeds (Wacoo et al., 2014). Therefore, in this study ELISA technique was applied to examine the concentration of total aflatoxins in twelve randomly selected cereal products available at the market of the Republic of North Macedonia. Concomitantly, the usability of the ELISA method for aflatoxin analysis in cereal-based products was assessed.

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Materials and methods

Samples of commercially available processed cereal products were purchased randomly from local retail outlets. They included rice-, corn-, wheat-, and barley-based snacks and mixed-grain breakfast cereals (Table 1), and were analyzed for quantitative determination of total aflatoxins using an ELISA technique.

Chemicals and tests

The extraction of aflatoxins from food samples was performed using methanol and distilled water mixture (70:30, *v/v*). All the chemicals used were of analytical grade.

Determination of aflatoxins by ELISA was done using Test kit RIDASCREEN Aflatoxin total (R-Biopharm AG, Germany). Referent standard material included in the Aflatoxin total kit for analysis of cereals (TRILOGY TR-A100) was prepared in

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methanol:water mixture (70:30 *v/v*) with concentration of 4.5 µg/L (± 0.5 µg/L). According to the specification of this test kit, total aflatoxin levels lower than 1.7 µg/kg can be detected. The test for determination of total aflatoxins included a 48-well plate coated with capture antibodies; standard solutions of aflatoxin in methanol/water with concentrations 0, 1.7, 5, 15, and 45 µg/L, a conjugate of anti-aflatoxin antibody; a mixture of substrate/chromogen; 0.5 M sulfuric acid as solution for termination of the reaction; and a buffer for dilution (10 mM phosphate buffer that contains 0.05% Tween 20, pH 7.4).

Sample preparation and ELISA procedure

Each sample was disintegrated in homogenizer blender (Iskra, Slovenia). Five grams were weighed and extracted with 25 mL of methanol:water mixture (70:30, *v/v*). The sample was shaken vigorously for three minutes using laboratory shaker (GFL 3006, Germany). Then, the extract was filtered through filter paper (Whatman No. 1, Munktell & Filtrak GmbH, Germany) and 1 mL of filtered extract was diluted with an equal volume of distilled water. After washing the wells as a critical step (microwells were not allowed to dry up totally), substrate was added. Aflatoxins in an aliquot of 50 µL of the samples and standards were allowed to compete with enzyme-labeled aflatoxins (conjugates) for the antibody binding sites. The samples were incubated for 10 min at room temperature and protected from light. The reaction of a substrate with the bound conjugate produces specific color, which intensity is inversely proportional to the concentration of aflatoxins in the sample or in the standard. The absorbance of the examined and standard solutions was measured at 450 nm in a microtiter plate spectrophotometer (Humareader Single Plus, Human GmbH, Germany). Each measurement was performed in triplicate and the concentration of aflatoxins was calculated using a standard curve. The calibration curve was plotted using the above defined five standard solution concentrations. For verification of the obtained analytical data special software adequate for RIDASCREEN® enzyme immunoassays the RIDA®SOFT Win (Art. No Z 9999) was used.

Table 1

Results and discussion

Aflatoxins as stable compounds that are not degraded under high temperatures may contaminate cereal derived food products (Kabak, 2012) and enter into the food chain.

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Considering the acute and chronic toxicity of all aflatoxins as well as compelling evidence of the link between exposure to aflatoxins and primary liver cancer (Qamar et al., 2008), determination of aflatoxin levels in commercially available cereal products are of public health importance.

In this paper, the quality of twelve processed cereal products intended to be used as a snack or for breakfast was evaluated in terms of aflatoxin levels. The curve shown in Fig. 2 confirms the accuracy of the measurements of total aflatoxins performed with the commercially available RIDASCREEN Aflatoxin total kit considering the given concentrations of ready-to-use standard solutions of aflatoxins in methanol:water mixture (70:30 *v/v*) and corresponding measured concentrations. The results of the analysis of total aflatoxin levels in different samples of ready-to-eat cereals and cereal products using a competitive ELISA method are presented in Fig. 3. The calculated level for total aflatoxins in each sample examined (< 4 µg/kg) evidently showed that none of them was contaminated with unauthorized level of aflatoxins.

According the Regulation of the Republic of North Macedonia (Rulebook on general food safety requirements for certain contaminants, 2013) which is harmonized with the EU Regulation (EFSA 2013), maximum residue levels (MRLs) of aflatoxins in cereals have to be regularly controlled. MRLs for aflatoxins in foods intended for direct human consumption such as cereal-based products are 4 µg/kg, and 10 µg/kg for both maize and foods intended for further processing.

Fig. 2

Fig. 3

Since presence of aflatoxins in cereal-based products as an important part of a human diet represents a significant health risk, a convenient method for rapid screening and quantification of aflatoxin levels in cereals is required. For routine analysis of aflatoxins, isolation, identification, chemical nature, and immunochemical responses are essential. Although several immunochemical methods have been developed, most of them differ in the solvents used and the measurement of fluorescence. In most cases organic solvents are mixed with a given ratio of a more polar solvent, such as water, so that the aqueous solvents penetrate the hydrophilic tissues and effectively extract aflatoxins. Methanol/water (55 + 45, 80 + 20), acetone/water (85 + 15), acetonitrile/water (90 + 10, 60 + 40), and chloroform/water (250 + 25, 100 + 10) are some of the common solvent mixtures used to

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extract aflatoxins from agricultural commodities (Bertuzzi et al., 2012; Möller and Nyberg, 2004; Sinha, 1999). Since methanol has less negative effect on antibodies compared to other organic solvents such as acetone and acetonitrile, an extraction mixture of methanol-water (80 + 20 *v/v*) is of choice to be used (Lee et al., 2004; Wacoo et al., 2014). Concerning the measurement of fluorescence, group B aflatoxins exhibit blue fluorescence, while group G aflatoxins exhibit yellow-green fluorescence under long-wave ultraviolet light. This difference provides the use of fluorescence for identification and differentiation between different groups of aflatoxins, not only to quantify total aflatoxins, but also to identify and differentiate between the B and G groups. Fluorescence spectrophotometry can rapidly quantify aflatoxins with a limit of detection of 5 μg/kg which is a little above the established legal setting in EU, thus requiring derivatization to improve the fluorescence of these molecules (Wacoo et al., 2014).

The most desirable option is to have fast, flexible and inexpensive method for analysis of aflatoxins due to their toxicity and hazardous impact on human health. Besides easy to analyze, the method should be robust, reproducible, sensitive and specific. A method that is invariably quicker, cheaper, more specific, more sensitive, and more reproducible than biological and chemical analyses is ELISA technique generally used for the estimation of antibodies present in a specimen. An ELISA method applied in this study is designed specifically to detect aflatoxins with high sensitivity, the result of quantitative ELISA tests can be read visually and a large number of samples can be assayed at the same time. It is based on the competitive interactions between antigen-acting aflatoxins and antibodies labeled with toxin-enzyme conjugate developing a color which intensity depends on the amount of antibody bound toxin-enzyme conjugate. The simplicity of the competitive enzyme immunoassays format, being highly sensitive measure of either antigen or antibody concentration provided a number of commercially available ELISA kits based on a competitive immunoassay to be widely used in analysis of aflatoxins (Kos et al., 2014; Leszczyńska et al., 2001; Ondieki et al., 2014; Thirumala-Devi et al., 2002). The major advantages are: the extraction of tested compounds without or minimal need for purification of sample and low cost. Extraction is a critical step in the analysis of aflatoxins as far as sometimes their detection is required at the trace level of 1 μg/L or even less. In order to improve the extraction efficiency mixtures of organic solvents are used. Besides optimized extraction conditions of the commercially available test kits, one disadvantage is the dependence on a specific matrix and when the kit is used in a matrix not specified by the manufacturer, the results should be confirmed by a chromatographic method (Shephard,

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2016). The second weakness is cross-reactivity to similar compounds that is possible when ELISA technique is used for determination of aflatoxins in foodstuffs. Herein, Kos et al. (2014) have performed screening of aflatoxins in cereals from Serbia and for samples containing higher aflatoxin level than 1 µg/kg high performance liquid chromatography with fluorescence detection (HPLC-FLD) as a confirmatory method was used. However, the comparison of the results obtained with ELISA and HPLC methods revealed only a slight difference. Leszczyńska et al. (2001) have reported that the antibodies contained in the aflatoxin total test may give positive cross-reactions mainly with aflatoxin B_1 and B_2, and at lower level with M_1, G_1 and G_2, but ELISA method remained to be a method of choice for fast analysis of total aflatoxins in considerable number of samples without laborious sample preparation.

HPLC technique coupled with FLD is considered to be advantageous compared to ELISA, providing fast and accurate analysis of aflatoxins with a reported sensitivity (limit) of detection as low as 0.1 ng/kg (Herzallah, 2009). However, a thorough laborious and time consuming sample purification using immune-affinity columns is required. In addition, sometimes natural fluorescence of aflatoxins B_1 and G_1 may not be high enough, thus chemical derivatization may be required to enhance sensitivity and reach the required detection limit (Li et al., 2011). To overcome the challenges associated with pre- and post-column derivatization processes in aflatoxin analysis, HPLC was coupled to mass spectrometry. This technique is up to date using only small amounts of sample and has low detection limits (Miklós et al., 2020), but it is very expensive and without possibility to be adapted to field conditions. Other chromatographic techniques such as TLC and an automated HPTLC have been recognized to be efficient and precise methods for analysis of aflatoxins. Although HPLTC overcome some weaknesses of TLC method such as application of the samples, development and interpretation, the non-transferable equipment that is bulky and expensive and the need of well-trained personnel may be a concern (Wacoo et al., 2014). Gas chromatography is uncommon in analysis of aflatoxins, in particular when cheaper chromatographic techniques are available. Further, a cleanup of the sample is mandatory and sometimes derivatization must be performed because aflatoxins are not volatile compounds (Alshannaq and Yu, 2017). Capillary electrophoresis (CE) combined with fluorescence detection offers the advantage of fast and efficient separation of aflatoxins to be accomplished in aqueous buffer solutions, without use of organic solvents, with small sample size and buffer volumes. Fluorescence interference from the CE buffer and matrix

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components was reported as the main disadvantage of CE, while when coupled with MS the system has become complex and expensive close upon LC-MS (Zhang and Banerjee, 2020).

Apart few disadvantages of the ELISA technique such as increase cost of bulk screening related to the single use of the kits and limited detection range due to the narrow sensitivity of the antibodies (Turner et al., 2009), in this study total aflatoxin levels below 1 μg/kg were detected indicating the potential of the described method for routine analysis of cereals in terms of legislation conformity. According to our knowledge, this is the first study to examine the presence of aflatoxins in various cereal-based products available at the market of the Republic of North Macedonia. In 2016 the results of qualitative and quantitative analysis of total aflatoxins in raw peanuts using the TLC method and fluorometric spectroscopy, respectively, have been published, showing concentrations above MRLs in 23% of the total 78 samples analyzed (Georgievski et al., 2016). The concentration of total aflatoxins in feed and aflatoxin M_1 in raw cow's milk in the period 2014-2017 determined mainly by ELISA method, followed by HPLC-FLD analysis of the suspicious samples were in accordance to the Macedonian legal requirements and resulting hazard index below 1 was calculated that means no significant risk to the consumer health, have been reported recently (Santa et al., 2021). Considering the climate changes leading to increased humidity and improper storage of raw foods may significantly support the development of *Aspergillus* molds and scarce investigation of aflatoxins in food commodities as well as processed products in R. N. Macedonia, this study represents a significant contribution in establishing a data collection on presence of these toxins in food.

Conclusion

Results of the analysis of total aflatoxin level in cereal-based products available at the market of the Republic of North Macedonia in this study have shown the presence of aflatoxins to be below the MRLs. The applied ELISA method was capable to detect aflatoxins at a very low level in certain samples, thus emerging as a simple, specific and sensitive alternative and at the same time accurate and portable tool for routine and on-site detection of aflatoxins among various analytical methods employed in analysis of these hazardous and unavoidable toxins in food products. The method is aligned with the criteria set by the regulatory bodies as well as efficient regarding time and expenditure and may be recommended to commercial food testing laboratories to monitor whether aflatoxin levels in final products are within permissible limits.

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Резиме

Анализа на вкупни афлатоксини во комерцијално достапни цереалии на пазарот во Република Северна Македонија

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Клучни зборови: афлатоксини, ELISA, имунохемиски, цереалии

Афлатоксините се природни производи на мувлите *Aspergillus flavus* и *Aspergillus parasiticus*. Овие токсини може да се акумулираат во процесот на зреење на земјоделските култури, како и за време на транспортот на житните растенијата, така што претставуваат ризик за контаминирање на преработените цереалии. Употребата на контаминирана храна со афлатоксини во исхраната на човекот може да предизвика акутна и хронична токсичност. За заштита на здравјето, законската регулатива пропишува максимално дозволени гранични нивоа на афлатоксини во прехранбените производи. Со цел редовна контрола на нивото на афлатоксини во преработените цереалии, односно проверка дали производите го исполнуваат дефинираниот законски критериум, неопходна е примена на аналитичка техника што ќе овозможи брза, специфична, точна и финансиски исплатлива анализа, како што е ензим поврзаната имуносорбентна анализа (ELISA). Во овој труд, со примена на комерцијално достапниот RIDASCREEN кит за определување на концентрација на вкупни афлатоксини беа анализирани комерцијални производи од житарки набавени по случаен избор од малопродажниот пазар во Република Северна Македонија. Направените анализи покажаа дека ниту еден од тестираните дванаесет производи не содржи концентрација на вкупни афлатоксини повисока од максималната дозволена гранична вредност од 4 μg/kg според националната и европската регулатива. Со оглед на тоа што беа детектирани нивоа на афлатоксини пониски од 1.7 μg/kg, применетиот метод може да се користи за рутинска детекција и квантификација на вкупни

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афлатоксини во преработени цереалии поради едноставноста, специфичноста и осетливоста. Овој труд може да служи и како иницијатива за примена на практичните имунохемиски методи за анализа на потенцијална контаминација со афлатоксини на производи добиени од житни растенија со цел основање на национална база на податоци за присуство на афлатоксини во храната.

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Table 1. Description of selected cereal processed products available on the market of the Republic of North Macedonia analyzed on presence of total aflatoxin content

Sample number	Product	Origin
1 (RCN)	Rice cakes natural	Republic of Serbia
2 (RCGF)	Rice cakes gluten free	Republic of Serbia
3 (RCWP)	Rice cakes with popcorn	Republic of North Macedonia
4 (CFCP)	Corn flakes classic	Poland
5 (CFCG)	Corn flakes classic	Germany
6 (CFWS)	Corn flakes with no added sugar	Republic of North Macedonia
7 (OFS)	Oat flakes	Republic of Serbia
8 (BF)	Barley flakes	Republic of Serbia
9 (RF)	Rye flakes	Republic of Serbia
10 (MIX5)	Mix 5 flakes: whole grains, oats, barley, spelled and wheat	Republic of Serbia
11 (UGFM)	Universal gluten free flour mixture	Italy
12 (OFG)	Oat flakes	Germany

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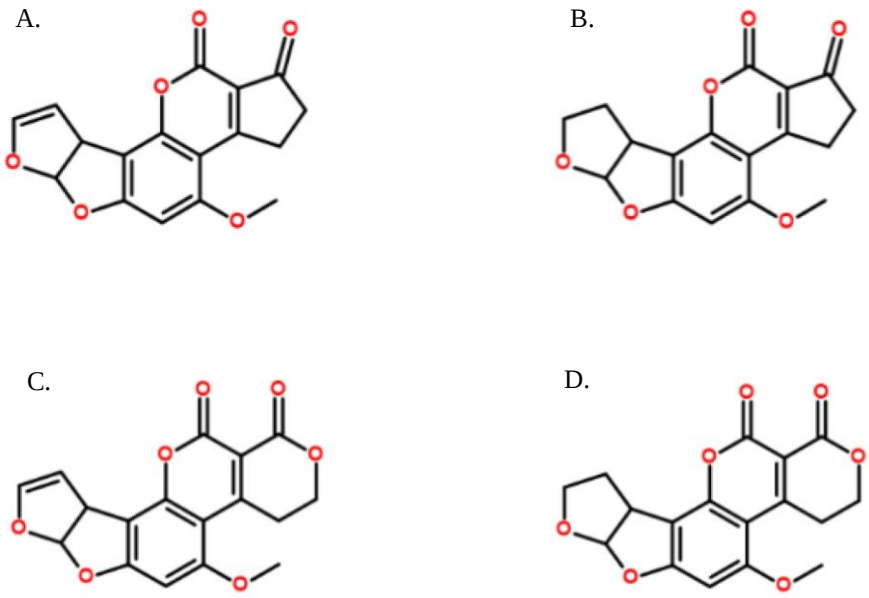

Fig. 1. Chemical structures of aflatoxins: A. Aflatoxin B_1 (AFB_1); B. Aflatoxins B_2 (AFB_2), C. Aflatoxin G_1 (AFG_1), and D. Aflatoxin G_2 (AFG_2).

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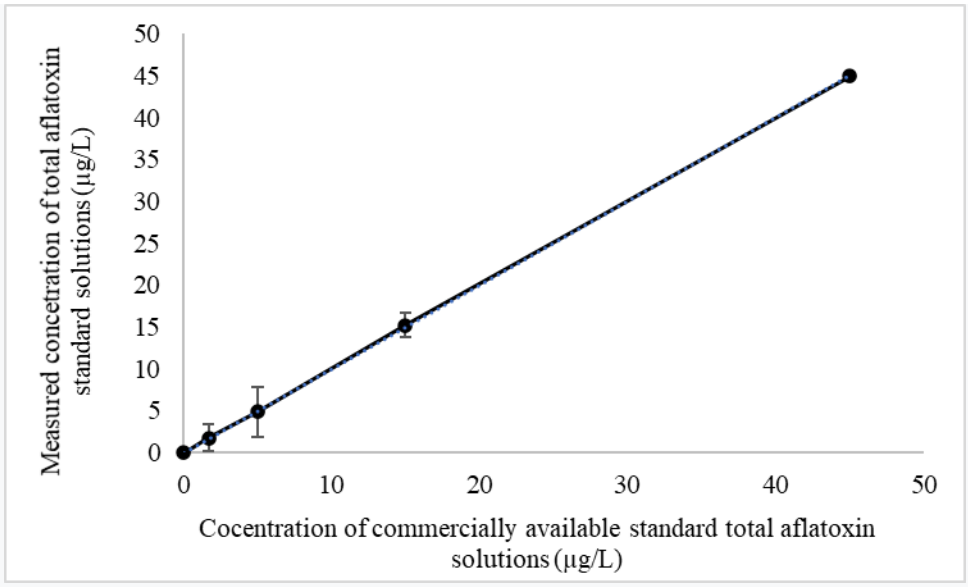

Fig. 2. A calibration curve plotted using five ready-to-use standard solutions of aflatoxins in methanol:water mixture (70:30 *v/v*) at a given concentrations of 0, 1.7, 5, 15, and 45 µg/L and corresponding measured concentrations.

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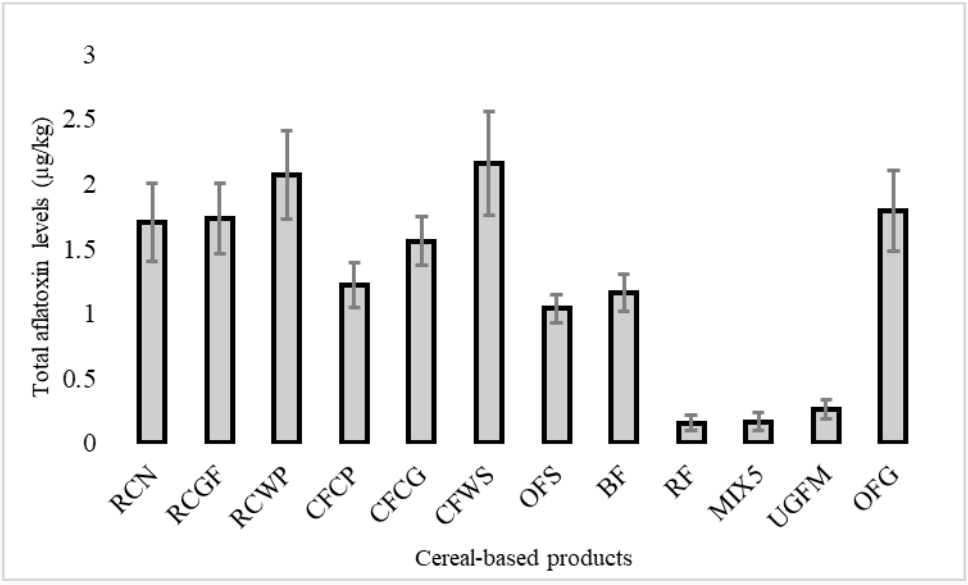

Fig. 3. Total aflatoxin levels (µg/kg) determined in selected cereal-based products available at the market of the Republic of North Macedonia: RCN - Rice cakes natural; RCGF - Rice cakes gluten free; RCWP - Rice cakes with popcorn; CFCP - Corn flakes classic, Poland; CFCG - Corn flakes classic, Germany; CFWS - Corn flakes with no added sugar; OFS - Oat flakes, Serbia; BF - Barley flakes; RF - Rye flakes; MIX5 - Mix 5 flakes: whole grains, oats, barley, spelled and wheat; UGFM - Universal gluten free flour mixture; OFG - Oat flakes, Germany.

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