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# A New Method for Extraction of Aflatoxin B1 from Nuts for Competitive Enzyme-Linked Immuno Assay

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**Abstract:** Nuts are a staple food consumed throughout the world, and their purity is essential to maintaining human health. However, fungal mycotoxins pose one of the real challenges in keeping nuts clean for human consumption. The high cost of equipment, reagents used, standard samples, as well as increased requirements for personnel qualifications limit the use of chromatographic methods in testing laboratories, both manufacturing enterprises and organizations monitoring food safety. The present study aimed to test a new method for extracting aflatoxin B1 using sodium chloride and citric acid instead of methanol present in imported nuts obtained from local markets in Astana (Kazakhstan). The proposed method for extracting aflatoxin B1 from nuts is chemically safe, since the substances used belong to hazard class 3 of the international classification and do not require separate premises in food safety laboratories of the Astana markets and special training of specialists. The technical objective of the method is to replace the reagent (methanol) during the sample preparation process when conducting cocuret enzyme-linked immunosorbent assay. Aflatoxin B1 levels varied depending on the type of nut. Aflatoxin contamination was observed in 35.8% of samples analyzed. These levels of mycotoxin contamination are alarming and raise serious questions about the purity of imported nuts to ensure food safety.

**Keywords:** aflatoxin B1; mycotoxins; various nuts; contamination; food safety; extraction

## 1. Introduction

According to data presented by A. Yarmak, an economist at the FAO Investment Department, world trade in nuts increases annually by an average of 7.8%. At the same time, nut exports are growing annually by $2 billion per year and already reach about $34 billion. In this regard, we can conclude that nuts are the basis of the economy of many developed and developing countries [1].

According to the Rapid Alert System for Food and Feed (RASFF), the majority of notifications in the European Union of non-conformity of agricultural products with subsequent prevention of entry into the internal market or seizure are for nuts. At the same time, nuts and nut products have consistently occupied first place in this ranking over the past four years. Thus, the main reasons for non-compliance with nut requirements were pesticides – 47.7%, mycotoxins – 35.0%, pathogenic microorganisms – 9.6%. Aflatoxins in nuts are the most common cause reported by countries such as Turkey, China, USA, Argentina, Egypt and Iran [2].

Due to unfavorable weather conditions before harvest and poor harvesting, drying, processing, storage and transportation conditions after harvest, the nuts are vulnerable to invasion by various types of toxigenic fungi [3].

Mycotoxins can be found in many foods consumed by humans and animals. These substances are secondary metabolites of some types of mushrooms and are resistant to technological processes (cooking, frying, baking, distillation, fermentation). They most often contaminate products of animal origin (beef, pork, poultry, lamb, fish, game meat, milk) and plant origin (cereals, processed grains,

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vegetables, nuts). These substances damage agricultural crops and can cause various mycotoxicoses. Many mycotoxins can be present in food along with molds, increasing exposure of humans and animals [4].

Aflatoxins are among the most potent mutagenic and carcinogenic substances known (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1999), affecting all vertebrate species, including humans. According to the Food and Agriculture Organization (FAO), a quarter of the world's crops are affected by mycotoxins [5,6].

The health risks of eating nuts contaminated with aflatoxin B1 have been described in many scientific papers [7–11].

Samples of corn, peanuts, rice, walnuts and pine nuts from the provinces and municipalities of Chongqing, Fujian, Guangdong, Guangxi, Hubei, Jiangsu, Shanghai and Zhejiang were randomly selected from markets, with a total of 284 samples. While the peanut content was 3.03% higher than the Chinese and Codex Alimentarius tolerance limits. All samples of rice, walnuts and pine nuts met China's permissible limit for aflatoxins. By conducting a spatial analysis of aflatoxin contamination in peanuts and peanut butter in China, Mei Qin and his team found that AF levels were high in several provinces in East and South China under subtropical temperate monsoon climates [12].

At the borders of European Union countries, a large number of nuts contaminated with mycotoxins were observed, for example, peanuts from China also contained aflatoxins: France 31 ± 9 μg/kg, Spain 57 ± 18 μg/kg, Croatia 61.5 +/- 23.9 μg/kg, Bulgaria 3.2±1.0 μg/kg, Italy 8.9 μg/kg, etc. [13].

According to our research results, it is clear that nuts are imported from various countries (China) that have excess imports into the European Union. The European Union has one of the highest food safety standards in the world, largely due to robust European Union legislation ensuring food and feed safety.

In turn, countries interested in importing peanuts at the 15th session of the Codex Committee on Contaminants in Foods (CCCF15) propose to set maximum permissible levels of aflatoxins at 10 and 15 μg/kg. The listed countries have repeatedly asked to resume work, since establishing an acceptable maximum permissible levels for aflatoxins is important for export. Kazakhstan cannot support the proposed figure for the amount of aflatoxins in ready-to-eat peanuts. The Chairperson of the Committee decided to set the maximum permissible levels for total aflatoxin at 10 μg/kg. Reservations were expressed by Kazakhstan, the European Union, Singapore and Egypt [14].

Four compounds are commonly produced in plant foods: aflatoxins B1, B2, G1 and G2 (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2002). The three main genera of fungi that produce mycotoxins are *Aspergillus, Fusarium* and *Penicillium* [15]. Among the various types of mycotoxins, aflatoxins (AFs) are highly toxic and are known to contaminate a wide range of foods such as corn, peanuts, dried fruits, meat and milk-based products [16–18]. Aflatoxins are produced by *Aspergillus* species, namely A. *flavus*., A. *nomius* and A. *Parasiticus*, in addition to its production by other *Aspergillus* species such as A. *astellatus* [19]. These mushrooms usually grow in warm and humid conditions in tropical and subtropical regions [12,20].

Although aflatoxins have been found in a variety of foods, the most significant contamination has been found in peanuts, tree nuts and other types of nuts. The Scientific Food Committee noted that aflatoxin B1 is a potent hepatoxic, carcinogen classified as Group 1 by the International Agency for Research on Cancer (IARC) and even at extremely low levels increases the risk of liver cancer [21–23]. Aflatoxin B1 is primarily detoxified in the liver, which is why liver cancer is widespread. It should be taken into account that Mediterranean zones have become susceptible to AFB1 pollution due to shifts in traditional distribution areas due to climate change, such as increases in average temperature, CO2 levels and precipitation patterns. This has led to an increase in fungal infestation of crops worldwide [24,25].

A systematic search of scientific databases was conducted from 2000 to 2020. According to the results, aflatoxin B1 (AFB1) had the highest frequency in the nut samples. The average concentrations of total aflatoxin (AFT) and AFB1 in nuts were as follows: peanuts (37.85, 32.82 μg/kg), pistachios (31.42, 39.44 μg/kg), almonds (3.54, 3.93 μg/kg), walnuts (42.27, 22.23 μg/kg), hazelnuts (17.33, 10.54

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μg/kg), Brazil nuts (4.61, 3.35 μg/kg) and other nuts (26.22, 7.38 μg/kg). By country, the limit of exposure (MOE) for adults was as follows: Argentina (21) > Congo (67) > India (117) > Bangladesh (175) > Cameroon (238) > Iran (357) > Bahrain (438) > Brazil (447) > Ghana (606) > South Africa (1017) > Egypt (1176) > USA (1505) > China (1526) > Cyprus (1588) [26].

K. Huang and A. Zinedine studied the presence of aflatoxins in dried fruits and nuts available in the Rabat-Salé region (Morocco). The results showed that the levels of total aflatoxins (AFT) and aflatoxin B1 (AFB1) in peanuts, walnuts and pistachios were 5%, 30%, 45%, exceeding the maximum permissible limit (2 μg/kg) set for AFB1 by European Union regulations [27–33].

The results of monitoring aflatoxins B and G in food products of plant origin imported into Southern Italy from countries outside the European Union are presented in the work of G.Pasquale and S.Imbimbo. From 2017 to 2020, they analyzed 1675 samples. It was found that 295 samples (17.6%) were contaminated with aflatoxin B1, 204 with aflatoxin B/G (12.2%), and 75 (4.5%) did not comply with the maximum limits established by European Union legislation [34].

Based on the results of monitoring studies, it was established [35] that in 2021-2022 the largest share of imports of various nuts was from China - 58,294.6 tons (74.6% of total imports). The main types of nuts are: walnuts (43,482.3 tons), almonds (11,688 tons), macadamia (1,596 tons), cashews (344.5 tons), pistachios (119.7 tons), chestnuts (0.2 tons) and pecans (1063.9 tons) . The volume of imports of nuts from the USA amounted to 15,702.9 tons (almonds – 13,712 tons, pistachios – 1985.3 tons, pecans – 4.9 tons), from Russia amounted to 2,126.2 tons (peanuts – 1,693.8). Uzbekistan is one of the leading importers of walnuts – 741.9 tons and almonds – 274.9 tons. Major importers of pistachios are Iran (4,858 tons), Turkey (1,137.8 tons, including almonds – 594.8 tons, pistachios - 454.1 tons), Tajikistan (953.9 tons), Ukraine (438.2 tons), Kyrgyzstan (342.3 tons).

According to official statistics on the import of nuts to the capital, it has been established that the main importers of nuts are Kyrgyzstan (125.8 tons, almonds – 1.3 tons, walnuts – 5 tons) and Russia (with a volume of nuts 64.5 tons), although our monitoring data differs from official statistics.

Currently, there are many methods for determining both qualitative and quantitative content of mycotoxins in food products. An integral part of each method is the preparation of the sample for research. Methanol, which belongs to the 1st class of extremely dangerous substances, is used as an extracting substance; working with it requires an appropriately equipped laboratory and a trained specialist, which makes it impossible to conduct research in ordinary food safety laboratories. When extracting aflatoxins from food and feed, chloroform, which belongs to class 2 hazardous substances, can also be used.

Most often, chromatographic methods are used to determine the content of mycotoxins (gas-liquid chromatography combined with mass spectrometry, high-performance liquid chromatography with UV spectrometric, fluorescent or mass spectrometric detection, thin layer chromatography) with various sample preparation options [36].

The essence of the high-performance liquid chromatography method is that the analyzed sample is extracted with a mixture of methanol and water. The sample extract is filtered, diluted with water and injected into an affinity chromatography column containing antibodies specific for aflatoxins $B_1$, $B_2$, $G_1$, $G_2$. Aflatoxins are quantified using reverse phase high-performance liquid chromatography (HPLC) with fluorescence detection and post-column derivatization [37,38].

The thin layer chromatography method is based on the extraction of aflatoxins B1 and M1 from a product sample, purification of the extract from interfering substances and measurement of the mass concentration of aflatoxins B1 and M1 using thin layer chromatography while visually determining the amount of substance in the stain.

Extraction of aflatoxin B1 from grains, legumes, nuts, confectionery products, baked goods and concentrates for this chromatography method is carried out by adding 25 ml of sodium chloride solution with a concentration of 100 g/dm3 to 25 g of crushed sample. The contents of the flask are thoroughly mixed to ensure uniform wetting of the sample. Using a microsyringe, add 0.0125 ml of aflatoxin B1 stock solution, used as an internal standard, and 100 ml of acetone into the flask using a microsyringe. The contents of the flask are shaken on a shaking apparatus for 30 minutes, after which

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the contents of the flask are filtered through a folded paper filter, and 50 ml of the filtrate is taken from there.

The high-performance liquid chromatography method is based on the extraction of mycotoxins from the analyzed sample, identification and quantification of them based on the peak areas of product ions using a calibration characteristic using the HPLC-MS/MS method in the monitoring mode of selected reactions.

Extraction of mycotoxins is carried out by weighing 5 g of the prepared sample, which is placed in a vial or polypropylene tube with a capacity of 50 ml. Add 25 ml of extraction solution, consisting of 790 ml of acetonitrile, 200 ml of deionized water and 10 ml of acetic acid, mix, place for 30 s in a vibrating shaker, then mix for 60 min on a oscillating shaker. Centrifuge at 3500 rpm for 20 min [39].

However, the high cost of equipment, reagents used, standard samples, as well as increased requirements for personnel qualifications limit the use of chromatographic methods in testing laboratories of both production enterprises and organizations monitoring veterinary and sanitary safety.

Screening methods are used to carry out routine measurements, the most common of which is Solid-Phase Enzyme-Linked ImmunoSorbent Assays (SP-ELISA). SP-ELISA methods are based on a highly specific antigen-antibody reaction, detection of which is carried out by introducing an enzymatic label and its subsequent detection using an appropriate substrate that changes its color [40]. The closest technical solution (prototype) is the method of extraction of aflatoxin B1 using a 70% methanol solution, which is the basis for sample preparation of the test system for Enzyme-Linked Immunosorbent Assay "RIDASCREEN FAST", as well as for the determination of aflatoxins by thin-layer chromatography (TLC). The test sample is ground using a mill for grinding bulk products. Weigh 5 g of the ground sample, transfer it to a flask with a ground stopper, add 25 ml of a 70% aqueous solution of methanol. Shake the flask thoroughly for three minutes (it is recommended to use a shaking apparatus). Filter the solution through filter paper. Mix 1 ml of filtrate and 1 ml of distilled water in a test tube.

The disadvantage of this extraction method is the use of a chemically hazardous substance of class 1 – methanol. To do this, the room must be equipped with supply and exhaust ventilation. A specialist with a higher or secondary specialized education must undergo appropriate instruction to obtain a permit on safety measures when working with methanol and master the method during an internship.

The principle of Enzyme-Linked ImmunoSorbent Assays (ELISA) is based on the use of antibodies to detect a target antigen using antibody-antigen interactions. Antibodies are fixed to the appropriate plate or column. When this substrate is exposed to aflatoxin (AF), antibodies recognize AF epitopes to form a complex. Rapid mycotoxin tests combine a simple sample preparation procedure such as methanol:water (or buffer) extraction, filtration, and buffer dilution. This procedure is mandatory because the matrix effect is a significant issue affecting the results.

The purpose of the study was to test a new method for extracting aflatoxin B1 from nuts using a competitive Enzyme-Linked ImmunoSorbent Assays (c-ELISA).

## 2. Materials and Methods

### *2.1. Ethical approval*

The study protocol was discussed and approved (No. 23 of March 31, 2021) at a meeting of the ethical commission of the Saken Seifullin Kazakh AgroTechnical Research University.

The research work was carried out in the food safety laboratory of the department of veterinary sanitation of the Saken Seifullin Kazakh AgroTechnical Research University. The studies were conducted between 2021 and 2022. Samples of various nuts from the China and Uzbekistan were taken from the Astana markets “Artem”, “Asem”, “Alem”, “Eurasia”, “Shapagat” and from a large supplier “Sarvinoz-S” LLP.

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*2.2. Detection of aflatoxin B1 using ELISA*

The RIDASCREEN®FAST Aflatoxin ELISA (Art. No. 5202, R-Biopharm AG, Darmstadt, Germany) was used to determine the aflatoxin B1 content in various nuts. To conduct the study, microwells placed in the plate frame in sufficient quantity for all standards and samples were used. Each well was filled with 50 µl of standards or samples, after which 50 µl of enzyme conjugate and anti-aflatoxin antibody solution were added. The plate was left to incubate at room temperature for 10 minutes. The liquid was then removed from the wells, after which the wells were filled with wash buffer twice. Next, 100 µl of substrate/chromogen solution was added and left to incubate at room temperature for 5 minutes in the dark. The final step was to add 100 µl of stop solution to each well. Optical density was measured at 450 nm in each well within 10 minutes after addition of stop solution. Detection limit:

*2.3. Sample preparation for ELISA*

Reagents: methanol (70%) (preparation of 70% methanol solution: mixing 70 ml of methanol (100%) with 30 ml of demineralized water), NaCl, filter paper: Whatman No. 1.

Weigh 5 g of crushed sample into a 50 ml vial, then add 2.5 g of NaCl to the crushed sample. Then add 25 ml of 70% (v/v) methanol and carry out extraction by stirring for 10 minutes at room temperature. The next step is centrifugation for 15 minutes at 3000 rpm at room temperature. The top fat layer is then removed by aspirating the fat layer using a pipette. Pasteur under vacuum and the extract is filtered through filter paper No. 1 on Whatman paper, if necessary to clarify the remaining supernatant. The clear supernatant is diluted in a ratio of 1:2 (1 + 1) with demineralized water and mixed well.

The performers worked in accordance with safety measures when working with methanol (certificate No. AC 568/1, No. AC 568/2, No. AC 567/1). The following regulatory documents were used in the work: GOST 168832-71, GOST 33303-2015, GOST 16832-71, MVI MN 1364-2000, MVI MN 1363-2000, GOST 10856-96, GOST 28561-90.

*2.4. New sample preparation method for sample extraction for ELISA*

Reagents: 25 ml of 30% sodium chloride and 15 ml of 5% citric acid. Equipment: Filter paper: Whatman paper No. 1; Sodium chloride GOST 4233-77; Citric acid 1-aqueous, Manufacturer: Weifang Ensign Industry CO., LTD. Weigh 5 g of crushed sample into a 50 ml bottle, then add 25 ml of 30% sodium chloride and 15 ml of 5% citric acid kept in a water bath at 45°C for 10 minutes, then actively mix by turning the tube upside down for 10 minutes or use a shaker, centrifuge for 15 minutes at 3000 rpm at room temperature, 1 ml of the resulting filtrate is diluted with distilled water in a 1:1 ratio. Subsequently, filter and dilute 1 ml of the resulting filtrate with distilled water 1:1. If there is a separated fat fraction on the surface of the samples, it is necessary to remove the fraction using a Pasteur pipette. Next, filter through a paper filter.

Statistical processing of the results was carried out using the Student's test. Differences were considered significant at $p < 0.01$. The optical density of each well was measured at 405 nm using a RIDA®ABSORBANCE 96 microplate spectrophotometer with RIDASOFT® Win.NET software (Germany). RIDA® ABSORBANCE 96 is ROHS compliant and tested according to CISPR.

## 3. Results and Discussion

In Astana there are main points of sale of various nuts, the large wholesale market “Sharyn” and the large supplier of nuts, LLP “Sarvinoz-S”. Further, all nuts are sold in large markets: “Alem”, “Eurasia”, “Artem”, “Shapagat”, “Asem”.

According to the results of our research, out of 86 samples of nuts from China and Uzbekistan analyzed for the content of aflatoxin B1, 35.8 (%) exceeded the maximum level allowed by the technical regulation of the Customs Union 021/2011 “On food safety.”

It was found that the compared extractants showed similar results in ±89% of cases, and in ±11% of cases there was a discrepancy between the obtained data. This discrepancy is reflected in an

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increase in aflatoxin concentration when using 25 ml of 30% sodium chloride and 15 ml of 5% citric acid.

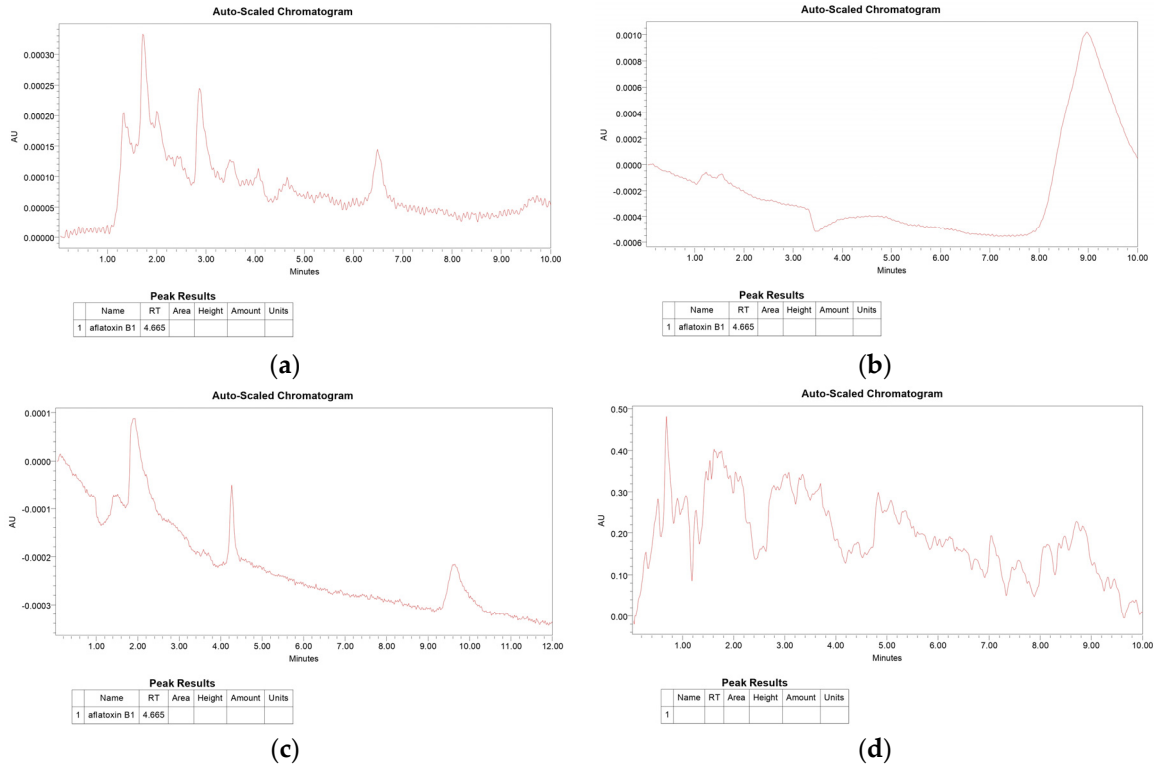
As can be seen from the studies, the difference between the two extractants is small, and the minor fluctuations that exist are within the accuracy of this technique.

Samples that exceeded the permissible values of total aflatoxins were walnuts, peanuts, almonds, badam, hazelnuts, pistachios; the concentration ranged from 0.004 to 0.6 mg/kg (Table 1). There was no significant difference between methanol and the extraction mixture ($p < 0.05$).

**Table 1.** Content of aflatoxin B1 in imported nuts when extracted with various extractants, mg/kg.

| No | Type of nut | Methanol | A mixture of sodium chloride (25 ml 30%) and citric acid (15 ml 5%) |
|---|---|---|---|
| | | China | |
| 1 | Peanuts (n=7) | 0.0017±0.002 | 0.0018±0.002 |
| 2 | Walnut (n=19) | 0.06±0.04 | 0.059±0.04 |
| 3 | Almonds (n=12) | 0.0036±0.004 | 0.0029±0.004 |
| | | Uzbekistan | |
| 1 | Peanuts (n=11) | 0.6±0.004 | 0.6±0.004 |
| 2 | Walnut (n=13) | 0.0016±0.004 | 0.0017±0.004 |
| 3 | Almonds (n=8) | 0.045±0.002 | 0.036±0.002 |
| 4 | Hazelnuts(n=9) | 0.021±0.001 | 0.026±0.001 |
| 5 | Pistachios(n=7) | 0.004±0.004 | 0.007±0.004 |
| | | p <0,05 | |

The chromatograms presented in Figure 2 display the effect of extraction of AFB1-contaminated nut samples. According to the chromatogram results, the AFB1 peak was detected at a retention time of 4.665 min in the reference sample. AFB1 peaks can be seen in sample extraction chromatograms.



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(e)

**Figure 1.** Chromatograms obtained via analysis of HPLC illustrate AFB1 illustrate the extraction of AFB1 from contaminated nuts (a-e).

In many scientific studies, 70% or 60% methanol is mainly used for extraction [16,41–53]. Jubeen and colleagues reported the production of AFD1, a less toxic product, when citric and lactic acids were used to convert AFB1 through hydrolysis of the lactone ring [54]. Citric acid has been found to have better aflatoxin detoxification results compared to traditional methods. The maximum reduction of AFB1 by 99% was observed in walnuts treated with 9% aqueous citric acid solution for 15 min. Similarly, Dhanshetty and colleagues demonstrated that frying in the presence of sodium chloride and citric acid reduced AFB1 content the most [55–57].

The extent to which processing methods reduced B1 content followed the pattern: frying with a combination of NaCl and citric acid > pressure cooking with a combination of NaCl and citric acid > frying. Since the preparation procedures did not involve any complex steps or sophisticated equipment, they could easily be used to decontaminate or reduce B1 levels for safer consumption of peanuts at home without compromising the sensory properties. The extent to which processing methods reduced B1 content followed the pattern: frying with a combination of NaCl and citric acid > pressure cooking with a combination of NaCl and citric acid > frying. Since the preparation procedures did not involve any complex steps or sophisticated equipment, they could easily be used to decontaminate or reduce B1 levels for safer consumption of peanuts at home without compromising the sensory properties. The extent to which processing methods reduced B1 content followed the pattern: frying with a combination of NaCl and citric acid > pressure cooking with a combination of NaCl and citric acid > frying. Since the preparation procedures did not involve any complex steps or sophisticated equipment, they could easily be used to decontaminate or reduce B1 levels for safer consumption of peanuts at home without compromising the sensory properties.

Nuts imported to Kazakhstan are not tested for aflatoxin B1 in the customs zone. When importing nuts, according to the rules for protecting the territory of the Republic of Kazakhstan from quarantine objects and alien species, a quarantine phytosanitary control act is issued for imported nuts, since nuts are regulated products. Sampling for analysis and examination (barn pests) is carried out by state plant quarantine inspectors. Unfortunately, there is no proper control and verification of quality conformity certificates in Kazakhstan.

At the sales sites of the Sharyn wholesale market and large markets of the capital, they are also not tested for aflatoxins, only organoleptic studies (appearance, smell, taste, contamination, etc.) are carried out by a private veterinary and sanitary examination laboratory. To improve the qualifications of specialists in food safety laboratories (crop products) of the Asem, Artem and Alem markets and for large suppliers of nuts in Astana, we conducted a scientific and methodological seminar “Safety and quality of various nuts” [14].

## 4. Conclusion

More than 60 years have passed since the discovery of aflatoxins, and despite scientific progress in the world, consumers still have to eat nuts contaminated with AFB1. It is clear that sample extraction is a key step in aflatoxin determination. As an alternative, to avoid cumbersome and time-

consuming sample preparation, the extraction mixture we tested was as good as methanol in its extraction properties. The current study has shown that the proposed method for extracting aflatoxin B1 is chemically safe, since the substances used belong to hazard class 3 of the international classification and do not require separate premises in food safety laboratories in markets and special training of specialists.

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