

Article

The Distribution of Aflatoxins during Corn Dry Milling Process

Mohammad Ramezani

Biotechnology Group, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran;
m_ramezani@modares.ac.ir

Abstract: Background: Aflatoxins (AFs) as toxic compounds are widely spread. They are frequent contaminants of crops that are produced by fungi species. Corn is of crops that widely used in the corn dry milling process; a four-step procedure comprises grain handling and milling, liquefaction and saccharification, fermentation, and co-product recovery that produces ethanol and animal feed. However, it is possible that corn as a feed for dry milling process might be contaminated with AFs. Therefore, monitoring AFs in during widespread this process results having a better understanding of the fate of AFs. **Methods:** In this study, a sample of corn kernel was contaminated with a high level of AFs using *Aspergillus parasiticus*. Then, the corn flour comprising 23400.5 µg/kg of the total level of aflatoxins (AFT), was hydrolyzed by enzymes and acid for ethanol fermentation with *Saccharomyces cerevisiae*. Also, the level of AFs was monitored during ethanol fermentation process. **Results:** The results showed that AFs did not detect in the ethanol produced through fermentation and it was safe. Also, during the experiments, the AFT level reduced by 58.5 and 95.7% for the ethanol fermentation with simultaneous saccharification and fermentation and ethanol production with acidic hydrolysis, respectively. **Conclusion:** The results of this study showed that highly contaminated corn with AFs can be used for ethanol fermentation through dry milling process and the produced ethanol was safe.

Keywords: fermentation; *Saccharomyces cerevisiae*; flour; ethanol

1. Introduction

Mycotoxins as toxic secondary metabolites are included in over 300 chemical compounds. They are frequent contaminants of crops e. g., corn kernel, and tree nuts and fruits (1). Mycotoxins are produced by fungi species, which due to their thermo stability can remain present in food and crop commodities even after removing the fungus (2). Among mycotoxins, aflatoxin is a potent mycotoxin that is naturally produced from a number of fungi species e. g., *Aspergillus* species (3).

The four major aflatoxins (AFs) i. e., aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), are of great importance owing to the pose a considerable risk to human health and widespread occurrence (4). In addition, AFB1 is considered one of the most potent naturally produced carcinogens that are labeled as group 1 of carcinogenic compounds to humans by the International Agency for Research on Cancer (IARC) (5). In a putative pathway of carcinogenicity, AFB1 in cytochrome P450 is metabolized to AFB1-8-9-epoxide, and then covalently reacts with DNA (6). Therefore, the level of aflatoxins in food is regulated in more than 100 countries. For instance, the US Food and Drug Administration (FDA) has set the maximum allowable level of 20 µg/kg of the total concentration of aflatoxins (AFT) for all foods intended for human consumption (7). Also, the Food and Drug Administration of Iran (IFDA) defined a maximum level of 5 µg/kg for AFB1 and 15 µg/kg for an AFT in crops (8).

Numerous studies have reported corn and corn flour contamination with AFs in Iran. For instance, (9) monitored the amount of AFB1 in corn samples from 2006 to 2008 in different regions of Iran i. e., Fars, Khuzestan, and Ardabil province in Iran. The authors

found that the average AFB1 contamination was 0.9, 2.12, and 36.39 $\mu\text{g}/\text{kg}$ for Fars; 14.41, 35.93, and 1.61 $\mu\text{g}/\text{kg}$ for Khuzestan; 15.63, 57.67, and 154.13 $\mu\text{g}/\text{kg}$ for Ardabil province. In another study, (10) collected corn samples, aimed at animal feed and human consumption, from Kermanshah and Mazandaran provinces, Iran, which were analyzed using high-performance liquid chromatography (HPLC) for monitoring AFs contamination. In this regard, the authors reported that AFB1 was detected in 58.3% of the corn samples obtained from Kermanshah province. The maximum detected level of AFB1 and total level of AFs (AFT) contamination were 276.3 $\mu\text{g}/\text{kg}$ and 316.9 $\mu\text{g}/\text{kg}$, respectively. Also, the average level of AFT from contaminated samples was 52.60 $\mu\text{g}/\text{kg}$ which exceeded all the maximum permitted levels regulated for AFT.

On the other hand, corn is widely used in the dry milling process to produce products for human consumption and animal feed. Corn dry milling is a four-step procedure that comprises grain handling and milling, liquefaction and saccharification, fermentation, and co-product recovery. In this regard, Ground corn is cooked and liquefied to dextrins, then dextrins are hydrolyzed into fermentable sugars using the saccharification process. During fermentation, the fermentable sugars by means of microorganisms such as *Saccharomyces cerevisiae* are converted to ethanol and carbon dioxide (11). Also, it should be noted that the fermentation and hydrolysis process can take place separately, separate hydrolysis and fermentation (SHF), or simultaneously, known as simultaneous saccharification and fermentation (SSF) (7,12). Also, distiller's dried grains with solubles (DDGS) are a co-product of dry-milled ethanol production and can be used as animal feed (13). Moreover, as we know, AFs are transferred and accumulated into different body parts after consumption and pose a major threat to human health as these toxins play a major role in a number of health problems e. g., cancer, blood, and nerve defects (14). Therefore, it is necessary to monitor and determine the distribution of AFs in the product of the corn dry milling.

Therefore, this study aimed to contaminate a sample of corn with a high level of AFs using *Aspergillus parasiticus* and evaluate the fate of AFs during fermentation of corn using *Saccharomyces cerevisiae* under both enzymatic and acidic hydrolyses process of starch, based on corn dry milling process.

2. Material and Methods

2.1. Microorganism, growth medium, and chemical

Aspergillus parasiticus PTCC 5286, capable of producing four major AFs from Iranian Research Organization for Science and Technology (IROST), and *Saccharomyces cerevisiae* from **Razavi Yeast Company, Iran, were prepared**. Potato dextrose agar (PDA) (Merk, Germany) was used to maintain cultures. Also, corn was purchased from a local store in Tehran, Iran. In this study the commercial thermo-stable α -amylase, Termamyl® 120L (Novo Nordisk A/S, Bagsvæ rd, Denmark) obtained from *Bacillus licheniformis* was used for liquefaction. The activity of this enzyme was 120 KNU g^{-1} . 1 KNU is the amount of enzyme responsible for 5.26 g of starch degradation per hour (Merck, Amylum soluble Erg B 6, Batch 9947275). Moreover, for the saccharification, the commercial enzyme mixture, SAN Super 240L (Novo Nordisk) was applied. This enzyme consists of the α -amylase Fungamy® (1,4-aD-glucanohydrolase EC 3.2.1.1) from *Aspergillus oryzae*, and the amyloglucosidase, AMG (Exo-1,4-a-D-glucan, glucanohydrolase, EC 3.2.1.3) from *Aspergillus niger*. The activity of SAN Super 240L was 240 AGU mL^{-1} where 1 AGU is equal to the amount of enzyme hydrolyzing 1 μmol maltose min^{-1} .

2.2. Aflatoxin production

After the cultivation of *A. parasiticus*, it was transferred into a sprayer containing 0.5 L of autoclaved water, then sprayed on 1000 g corn kernels that were placed in a chamber (10 L). The chamber was placed in a dark place for 20 days, and temperature was set at 24 °C.

2.3. Corn Grinding

To produce ethanol through fermentation, firstly, corn kernels were ground. Then, the powder was sifted by two successive sieves (with mesh sizes of 250 and 53 μm). As a result, corn flour and corn barn obtained that their AFs content was evaluated. Also, the collected powder after sieving, corn flour, was used to prepare a 20% *v/w* solution that was used as the stock solution for further processes.

2.4. Hydrolysis

For carrying out enzymatic hydrolysis, first, α -amylase Termamyl (200 mm^{-3} enzyme/kg corn flour) was added to the stock solution, then, the solution was heated up to 105 $^{\circ}\text{C}$ for 5 minutes, and subsequently, the temperature decreased to 80 $^{\circ}\text{C}$ for 120 minutes. Afterward, SAN Super 240L, (1 mm^{-3} SAN Super/g total starch), was added to the solution. Finally, at room temperature, the solution was cooled. Also, it should be noted that in the SSF the enzymatic hydrolysis can take place in the next 24 to 48 h.

Also, for acidic hydrolysis, at first, 0.25 M of sulfuric acid was added to the stock solution, and pH of the solution reduced to about 1. Then, a temperature of 121 $^{\circ}\text{C}$ was applied for 20 minutes. Thereafter, the solution cooled at room temperature, and NaOH solution (2 N) was used to reach a neutral pH of 5-5.5.

2.5. Fermentation and Distillation

For fermentation, the yeast was added to the hydrolyzed solutions, 0.1% *w/v*, and the solutions were placed in a shaker incubator for 2 days, while pH, temperature, and rotation speed were 5-5.5, 32 $^{\circ}\text{C}$, and 150 rpm, respectively. Then, the entire solution was filtered using Whatman filter paper. Also, to separate ethanol from fermented solution, a distillation process was carried out, while temperatures ranging from 80-85 $^{\circ}\text{C}$. As a result, the experiment ended up having three components of distillery spent wash, distillate, and post-fermentation solids, which their AFs content was evaluated (Fig 1).

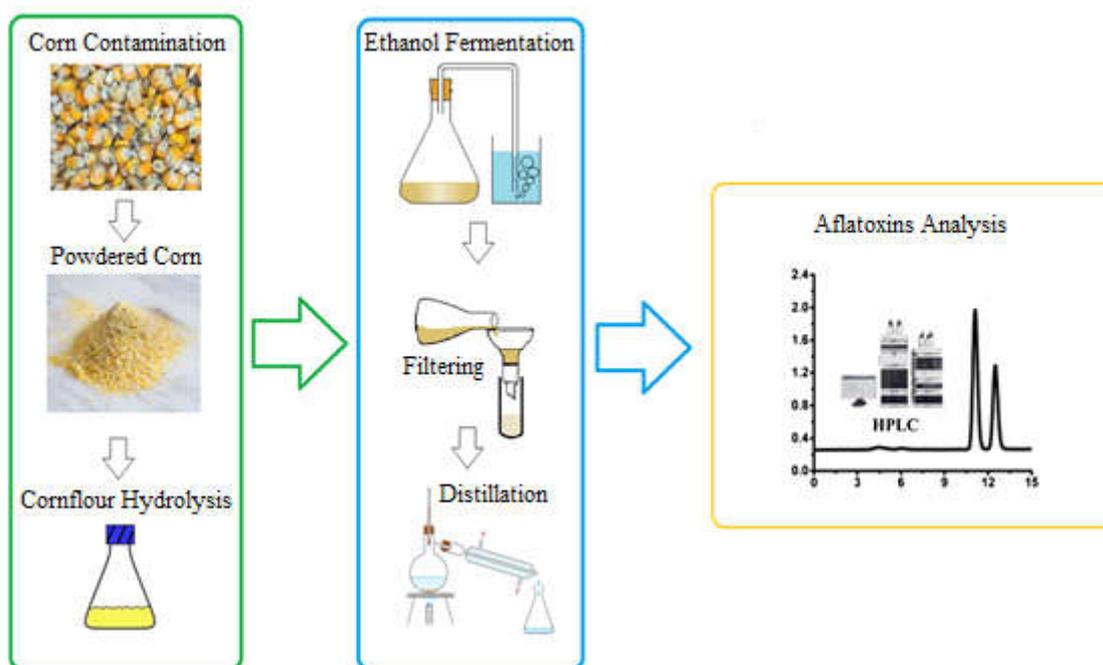


Figure 1. The procedure of assessing the fate of aflatoxins during contaminated corn fermentation.

2.4. Aflatoxins analysis by HPLC method

The level of aflatoxin contamination of samples was measured using the high-performance liquid chromatography (HPLC) method Shimi Darou Kowsar laboratory. For AFs

measurement a protocol (6872) based on the Institute of Standards and Industrial Research of Iran (ISIRI, 6872) was applied. The AFs content of samples was determined by a reversed-phase symmetry C18 column (150 mm of length, 4.6 mm of i.d., and 3 μm particle size) supplied by GL Sciences Inc. (Tokyo, Japan) accompanied by an immunoaffinity column clean-up (ISIRI, 6872). In this study, Waters Alliance blue e2695 separations module (Waters, USA) as the HPLC system accompanied by a fluorescence detector 2475 (Waters, USA) was used to analyze AFs. Also, water: acetonitrile: methanol in a ratio of 6:2:2 (*v/v*) was used as the mobile phase. The flow rate was 1 mL min⁻¹. The concentration of calibrant solutions for AFB1 and AFG1, and AFB2 and AFBG2 were 0.4 to 3.6 and 0.08 to 0.72 ng mL⁻¹. The retention time for AFs was in the range of 10–18 min.

3. Result

3.1. Corn grinding and Aflatoxin distribution

With a grinding of the corn, corn flour was obtained. In this regard, the whole level of AFT in the corn flour was 23400.5 $\mu\text{g}/\text{kg}$. This result showed the Corn flour comprised a high level of AFT contamination (23400.5 $\mu\text{g}/\text{kg}$) due to the growth of *A. parasiticus*. Also, for the corn flour, the major part of AFs contamination was contributed to AFB1 and AFG1, concerning 10504.5 and 11848.8 $\mu\text{g}/\text{kg}$, respectively (Table 1).

Table 1. The level of AFs for the products of contaminated corn grinding.

Sample	The total value of AFs ($\mu\text{g}/\text{kg}$)				
	AFB1	AFB2	AFG1	AFG2	AFT
Corn flour	10504.5	508.1	11848.8	539.1	23400.5

3.2. Ethanol Fermentation with SSF

Table 2 shows the distribution of AFs in the fermentation process with enzymatic hydrolysis using *S. crevasse*. In this context, the highest level of AFs, concerning AFB1, AFB2, AFG1, and AFG2 observed in distillery spent wash that was equal to 1960.4, 210, 3890.4, and 329.5 $\mu\text{g}/\text{kg}$, respectively. Also, AFs contamination, concerning AFB1, AFB2, AFG1, and AFG2, was not detected in the distillate.

Moreover, post-fermentation solids with 3328.7 $\mu\text{g}/\text{kg}$ of AFT were the second reservoir of AFs in this experiment. This phenomenon was probably due to the adsorption of AFs to the *S. crevasse* biomass and particles related to corn flour in the post-fermentation solids (15,16).

For the ethanol fermentation with the enzymatic hydrolyzed process, it was observed the contamination level of AFs was slightly lower relative to the initial sample, corn flour. In this respect, the initial sample showed a contamination level of 10504.5, 508.1, 11848.8, 539.1, and 23400.5 $\mu\text{g}/\text{kg}$ for AFB1, AFB2, AFG1, AFG2, and AFT, respectively, while the total value of AFB1, AFB2, AFG1, AFG2, and AFT in three components of fermentation process was equal to 3668.6, 254.4, 5437.9, 358.1, 9719 $\mu\text{g}/\text{kg}$, respectively. Therefore, 58.5% of initial AFT was reduced during this experiment which was probably due to AFs degradation or conversion to other AFs derivatives as a result of hydration (17).

Table 2. The level of AFs for ethanol fermentation with the enzymatically hydrolyzed sample.

Sample	Concentrations of AFs ($\mu\text{g}/\text{kg}$)				
	AFB1	AFB2	AFG1	AFG2	AFT
Distillery Spent Wash	1960.4	210	3890.4	329.5	6390.3
Post-Fermentation Solids	1708.2	44.4	1547.5	28.6	3328.7
Distillate	ND	ND	ND	ND	-

*ND: not detected

3.3. Ethanol Fermentation with Acidic Hydrolysis

Table 3 shows the distribution of AFs for the fermentation process of acidic hydrolysis using *S. crevasse*. In this regard, similar to enzymatic hydrolysis, the maximum level of AFT was contributed to the distillery spent wash, equal to 539.2 µg/kg, while the post-fermentation solids and the distillate comprised an inferior level of levels of AFT. In this regard, post-fermentation solids concerning 459.7 µg/kg, and the distillate did not contain any contamination.

Moreover, for the ethanol fermentation with the acid hydrolysis process, the contamination level of AFs was significantly lower compared to the initial sample, cornflour. Consequently, the total value of AFB1, AFB2, AFG1, AFG2, and AFT in the three components of the fermentation process was equal to 37.4, 461, 52.8, 447.7, 998.9 µg/kg, respectively. However, the initial sample showed a contamination level of 10504.5, 508.1, 11848.8, 539.1, and 23400.5 µg/kg for AFB1, AFB2, AFG1, AFG2, and AFT, respectively. Therefore, ethanol fermentation with the acidic process reduced the level of AFT up to 95.7%.

Table 3. The level of AFs for ethanol fermentation with the acidic hydrolyzed sample.

Sample	Concentrations of AFs (µg/kg)				
	AFB1	AFB2	AFG1	AFG2	AFT
Distillery Spent Wash	19.6	216	31.4	272.2	539.2
Post-Fermentation Solids	17.8	245	21.4	175.5	459.7
Distillate	ND	ND	ND	ND	ND

*ND: not detected

4. Discussion

The dry milling is a widespread process that make it possible to obtain products such as ethanol and the DDGS from corn. For such process, it is required to grind corn kernel and convert starch into fermentable sugars for microorganisms. However, it is possible that corn may be contaminated with AFs that are problematic to humans. Therefore, this study, evaluated the distribution of AFs during the ethanol production from contaminated corn using dry milling process. In this regard, not only ethanol, but also post fermentation solids like DDGS, as animal feed, should be investigated.

The result of this study showed that AFs level in ethanol produced with SSF process or acidic hydrolysis was insignificant and the contamination level of distillate was considerably lower than the permitted level determined by IFDA for AFB1 (5 µg/kg) and AFT (15 µg/kg). Also, the post fermentation solid was contaminated with a high level of AFs, higher the level of determined by IFDA, therefore, its consumption as animal feed can pose a great threat. Also, the result showed that during ethanol production with either SSF process or acidic hydrolysis, the level of AFs significantly reduced, concerning 58.8% and 95.7%, respectively. The high level of AFs reduction for ethanol production with acidic hydrolysis was probably due to the combined effect of sulfuric acid (0.25 M) and heat (121 °C for 20 min). This result is comparable with some previous studies. For example, (18) reported that treating rice samples containing AFT concentrations of <30 and >30 µg/kg with citric acid (1 N, 3 ml g⁻¹ of contaminated rice) for 15 min reduced AFT concentration significantly to 100 and 97.22%, respectively. In another study, (19) evaluated the effect of 18 different chemicals including sulfuric acid on AFB1, AFB2, AFG1, and AFG2 in black and white pepper samples (100 g). In this study, the authors did soak samples in 2% concentration of sulfuric acid for 120 min, while AFB1 and AFG1 were at two concentrations of 30 and 60 µg/kg, and AFB2 and AFG2 at two levels of 9 and 18 µg/kg were used. It observed that treatment with sulfuric acid under ambient temperature resulted in

mean reductions of 22.3 ± 6.3 , 17.5 ± 4.8 , 22.8 ± 3.6 , and $22.2 \pm 4.4\%$ for AFB1, AFB2, AFG1, and AFG2, respectively.

5. Conclusion

The results of this study showed that highly contaminated corn with aflatoxins can be used for ethanol fermentation through dry milling process and the produced ethanol was safe. However, post fermentation solids like DDGS are not safe as animal feed. Also, the results showed that corn flour dry milling was capable of degradation of aflatoxins without further aflatoxins detoxification.

Ethics issues: Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the author.

Acknowledgment: The author would like to acknowledge from the Shimi Darou Kowsar laboratory.

Competing interests: The author declare that they have no competing interests.

Authors' contributions: The author have contributed to various sections of this research from guidance and advice to collection of samples, writing and editing the article, and all items related to the article.

References

1. Hajimohammadi B, Ehrampoush MH, Dalvand A, Mohammadzadeh M, Soltani M, Eskandar S. Assessment of aflatoxin exposure using urine biomarker in pregnant and non-pregnant women in Yazd, Center of Iran. *Environ Health Eng Manag* 2021; 8(4):277-286. <https://doi.org/10.34172/EHEM.2021.31>.
2. Inoue T, Nagatomi Y, Uyama A, Mochizuki N. Fate of mycotoxins during beer brewing and fermentation. *Biosci Biotechnol Biochem* 2013; 77(7):1410-5. <https://doi.org/10.1271/bbb.130027>.
3. Tai B, Chang J, Liu Y, Xing F. Recent progress of the effect of environmental factors on *Aspergillus flavus* growth and aflatoxins production on foods. *Food quality and Safety* 2020; 4(1):21-8. <https://doi.org/10.1093/fqsafe/fyz040>
4. Nagatomi Y, Inoue T, Uyama A, Mochizuki N. The fate of mycotoxins during the distillation process of barley shochu, a distilled alcoholic beverage. *Biosci Biotechnol Biochem* 2012; 76(1):202-4. <https://doi.org/10.1271/bbb.110639>.
5. Marchese S, Polo A, Ariano A, Velloso S, Costantini S, Severino L. Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins* 2018; 10(6):214. <https://doi.org/10.3390/toxins10060214>
6. Rushing BR, Selim MI. Aflatoxin B1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem Toxicol* 2019; 124:81-100. <https://doi.org/10.1016/j.fct.2018.11.047>.
7. Yangcheng H, Jiang H, Blanco M, Jane JL. Characterization of normal and waxy corn starch for bioethanol production. *J Agric Food Chem* 2013; 61(2):379-386. <https://doi.org/10.1021/jf305100n>.
8. Mardani M, Rezapour S, Rezapour P. Survey of aflatoxins in Kashkineh: A traditional Iranian food. *Iran J Microbiol* 2011; 3(3):147-151.
9. Karami-Osboo R, Mirabolfathy M, Kamran R, Shetab-Boushehri M, Sarkari S. Aflatoxin B1 in maize harvested over 3 years in Iran. *Food control* 2012; 23(1):271-4. <https://doi.org/10.1016/j.foodcont.2011.06.007>.
10. Ghiasian SA, Shephard GS, Yazdanpanah H. Natural occurrence of aflatoxins from maize in Iran. *Mycopathologia* 2011; 172(2):153-60. <https://doi.org/10.1007/s11046-011-9405-y>.
11. Yu J, Xu Z, He H, Chen S, Wang S, Yu Y, et al. Integration of corn ethanol and corn stover ethanol processes for improving xylose fermentation performance. *Biomass Convers Biorefin* 2021. <https://doi.org/10.1007/s13399-021-01642-3>.
12. Wang P, Singh V, Xue H, Johnston DB, Rausch KD, Tumbleson ME. Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry-grind corn processing. *Cereal chemistry* 2007; 84(1). <https://doi.org/10.1094/CCHEM-84-1-0010>.
13. Peter CM, Faulkner DB, Merchen NR, Parrett DF, Nash TG, Dahlquist JM. The effects of corn milling coproducts on growth performance and diet digestibility by beef cattle. *J Anim Sci* 2000; 78(1):1-6. <https://doi.org/10.2527/2000.7811>.
14. Sipos P, Peles F, Brassó DL, Béri B, Pusztahelyi T, Pócsi I, Gyóri Z. Physical and chemical methods for reduction in aflatoxin content of feed and food. *Toxins* 2021; 13(3):204. <https://doi.org/10.3390/toxins13030204>.
15. Johnston CI, Singletary R, Reid C, Sparks D, Brown A, Baldwin B, Ward SH, Williams WP. The fate of aflatoxin in corn fermentation. *Natural resources* 2012; 3(3):126-136. <https://doi.org/10.4236/nr.2012.33017>.
16. Bueno DJ, Casale CH, Pizzolitto RP, Salvano MA, Oliver G. Physical adsorption of aflatoxin B1 by lactic acid bacteria and *Saccharomyces cerevisiae*: A theoretical model. *J Food Prot* 2007; 70(9):2148-54.
17. Inoue T, Nagatomi Y, Uyama A, Mochizuki N. Degradation of aflatoxin B1 during the fermentation of alcoholic beverages. *Toxins* 2013; 5(7):1219-29. <https://doi.org/10.3390/toxins5071219>.

18. Safara M, Zaini F, Hashemi SJ, Mahmoudi M, Khosravi AR, Shojai-Aliabadi F. Aflatoxin detoxification in rice using citric acid. *Iran J Public Health* 2010; 39(2):24-9.
19. Jalili M, Jinap S, Son R. The effect of chemical treatment on reduction of aflatoxins and ochratoxin A in black and white pepper during washing. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2011; 28(4):485-93. <https://doi.org/10.1080/19440049.2010.551300>.