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ORIGINAL RESEARCH PAPER

Immunoenzymatic evaluation for aflatoxin, ochratoxin, and zearalenone in grains stored

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Abstract: Mycotoxins cause serious health problems in both humans and animals through ingestion of contaminated processed foods or feed. This study aimed to evaluate the storage of corn, soybean, bean, and chickpea cultivars in grains in High-Density Polyethylene drums for 12 months in the field. Samples of corn (5), soybean (5), bean (3), and chickpea (3) grains were collected, and crushed, and the extract containing mycotoxins was produced. The total quantification of Aflatoxins, Ochratoxin A and Zearalenone were determined by the rapid and direct immunoenzymatic method (ELISA). Four corn samples and two soybean samples showed positive results for the presence of Aflatoxins with results between 37 and 56 µg kg^{-1}. For Ochratoxin A, three samples of corn, two soybeans, and two beans showed positive results between 5 and 9 µg kg^{-1}. Whereas for Zearalenone two samples of corn, three of soybean, and one of bean and chickpea had positive results with means between 108 and 125 µg kg^{-1}. Storage in High-Density Polyethylene drums proved not to be a viable option, and humidity and field storage can be harmful to corn, soybeans, beans, and chickpeas with moisture content between 10 to 17% and temperature between 25-30 °C.
Keywords: *Penicillium*; *Fusarium*; *Aspergillus*; mycotoxins; Immunoenzymatic reaction.

1. Introduction

Grain storage is an important topic for the post-harvest period and is essential for global consumption and processing in the food and feed industries (Matumba et al., 2021). Storage is a science that deals with several ramifications in studies varying from types of storage such as silos, to other variables such as adequate temperatures, moisture content, types of lighting, packaging, production of internal gases, and storage time.

Several species of fungi are capable of reproducing profusely in storage environments where there is no humidity, air circulation, and temperature control, these being factors that influence the successful replication of these beings that each year produce high rates of losses in grains and finished products of plant origin, generating serious problems in the agroindustrial chain (Al-Masoodi et al., 2023).

Some genera are of particular concern for *Aspergillus* and *Penicillium*, although other genera produce mycotoxins. Several authors report more than 50% contamination in grains for *Aspergillus* and *Penicillium* (Brito et al., 2022). A solitary fungal individual in a particular environment conducive to its development proliferates with a high capacity for the production of special metabolites. Mycotoxins are responsible for food poisoning, poor development in animals, cases of immunosuppression, and death (Ricci et al., 2021). Among the mycotoxin research worldwide, we considered Aflatoxins (AFs), Ochratoxin A (OA), and the hormone Zearalenone (ZEA).

Mycotoxins are a serious problem in food safety, as they contaminate around 25% of agricultural food

products (Ricci et al., 2021). Products such as feed, milk (by-products), meat (natura and fermented), and eggs were identified as products with a high potential for contamination by AFs, OA, and ZEA (Lindahl et al., 2018). We know for the AF group the following molecules AFB1, AFB2, AFG1, and AFG2, with AFM1 and AFM2 metabolites of AFB1 and AFB2 respectively (Ismail et al., 2018). For AFB1, AFB2, AFG1, and AFG2 grains, they are related to contamination of agricultural products such as corn grains, soybeans, peanuts, walnuts, cottonseed, and dried fruits, and AFM1 and AFM2 contaminate milk, eggs, and animal urine (Cotty; Jaime-Garcia, 2007).

OA toxin has a high degree of toxicity. This toxin is present in the Penicillium group which infects corn grains, wheat, soybeans, sorghum, chickpeas, beans, oats, peanuts, nuts, coffee, dried fruits, grapes, and animal by-products such as milk, cheese, egg, and other meat products (Malir et al., 2016). ZEA is classified as a non-steroidal estrogenic toxin produced by the *Fusarium* group, *F. graminearum*, and *F. culmorum*. This toxin causes deleterious effects in animals; however, it does not lead to death. Studies have reported cases in cattle, pigs, sheep, and chickens contaminated with ZEA, fed with feed produced from contaminated corn, soy, sorghum, wheat, barley, rye, and oat grains (Rashedi et al., 2011; Ricci et al., 2021).

In Brazil and several countries, there are regulations regarding the maximum detection levels for these and other mycotoxins. The National Health Surveillance Agency (ANVISA) of Brazil has maximum tolerated limits for mycotoxins in the Resolution of the Collegiate Board (RDC) no. 7. This resolution applies to the control of food for human and animal consumption, products of plant origin, and animal and processed products. However, for products of animal origin, the resolution only addresses maximum limits for AFM1 between 0.5 μg kg^{-1} for fluid milk, 5 μg kg^{-1} for powdered milk, and 2.5 μg kg^{-1} 1 for cheese (Brasil, 2011). Although Brazil has a program to control residues and contaminants in meat and milk products of the National Plan for the Control of Residues and Contaminants (PNCRC), which presents maximum reference limits for ZEA in products such as liver (beef, horse, pork, and poultry) 2 μg kg^{-1} and for AF group AFM1 in milk 0.5 μg kg^{-1} (Brasil, 2010). However, it does not have maximum reference limits for other relevant mycotoxins with high toxic potential or their metabolites such as Ochratoxins A, B, and C.

As noted, mycotoxin contamination problems are directly involved in grain storage. In this sense, this work aimed to evaluate the storage of grains from different cultivars of corn, soybeans, beans, and chickpeas stored in high-density polyethylene (HDPE) drums for twelve months in the field.

2. Material and Methods

2.1. Analytical reagents and equipment

Ethyl acetate P.A – ACS (Synth, Brazil), formic acid P.A – ACS (Neon, Brazil), potassium chloride P.A – ACS (Alphatec, Brazil), methanol P.A – ACS (Neon, Brazil), trifluoroacetic acid P.A – ACS (Sigma Aldrich, USA) and toluene P.A – ACS (Quimex, Brazil).

Analytical balance (Mod. AY220, Shimadzu, Brazil), UV chamber (Mod.SL 204, Solab, Brazil), chromatoplate (DC-Fertigfolien Alugram® Xtra SIL G/UV254, Germany), direct competitive ELISA kits (Veratox®, Neogen, Brazil) for aflatoxin, direct competitive ELISA kits (Veratox®, Neogen, Brazil) for Ochratoxin A and B, direct competitive ELISA kits (Veratox®, Neogen, Brazil) for Zearalenone, digital Thermo-Hygrometer (Icoterm, Cotronic Technology, China), oven with forced air circulation (Nova Ética, Brazil), ELISA microplate reader (Mod. EE CEL 431, Polaris, Brazil), orbital shaker table (Mod. SL-180/DT, Solab, Brazil) and food processor (Mod. L-550-B, Mondial, Brazil).

2.2. Corn, soybean, bean, and chickpea samples

Five samples of corn in grain cultivars ((1) B2801PWU; (2) B2801VYHR; (3) P2719VYH; (4) P3016VYHR, (5) 30F35VYHR, 5 samples of soybean in grain cultivars ((6) M8644 IPRO; (7) BRASMAX VORAZ IPRO; (8) 77A40E; (9) DM74K75CE and (10) NS7901RR), 3 samples in bean cultivars (11) BRS FS307; (12) BRS FC310 and (13) BRS305) and 3 samples in chickpea beans ((14) Cícero); (15) BRS Aleppo and (16) BRS Cristalino)). All grains are early, super-early and conventional hybrids produced in two rural units, where on a high scale (corn and soybean) in the municipality of Rio Verde, Goiás, Brazil, and on a low scale (beans) in the municipality of Campo Grande, Mato Grosso do Sul, Brazil, and (chickpeas) in the municipality of Rondonópolis, Mato Grosso, Brazil.

The different cultivars were kept separately in the same place, kept in stock, packed in drums with a capacity of 240 L made of high-density polyethylene plastic (HDPE) blue in the field for 12 months, harvest (2021/2022) with content of humidity between 10 and 17% (RH%) and temperatures between 25 and 35 °C measured every 30 days using a digital thermo-hygrometer with an external humidity and temperature sensor. The working samples were kept at the Antônio Menezes & Filhos rural property, located in the municipality of Rio Verde, Goiás, Brazil between the years 2021-2023 for the production of feed for family

psychology.

2.3. Collection and processing

250 g of samples were collected for each cultivar of corn, soybean, bean, and chickpea kept in plastic packaging for food and packed in styrofoam packaging containing ice (artificial gel) and temperature between 2-4 °C measured with a thermostat digital. In the laboratory, the samples were crushed in a food processor (one at a time, where between each sample, the "glass" cup of the processor was washed and sanitized with a 70% hydroethanolic solution and dried in an oven with forced air circulation at 80 °C for 3 h, where it was then sterilized in ultraviolet light for 2 h. Each sample processed resulted in a standard fine powder that was stored in plastic food packaging at -12 °C until analysis.

2.4. Obtaining the extract

Aliquots containing 50 g were homogenized in a 100 mL *Erlenmeyer* flask with methanol and 4% (*w/v*) KCl aqueous solution to extract the mycotoxins. Soon after, filtration was performed on Whatman quantitative blue band paper, porosity (2.0 µm). The supernatant was kept in an amber vails flask in a freezer at -2°C.

2.5. Qualitative determination of aflatoxins by immunoenzymatic method

For quantitative determination, the competitive direct ELISA immunoenzymatic method was used for specific determination of aflatoxins (B1, B2, G1, G2), with a quantification range between 5-50 ppb, 2-25 ppb (Ochratoxin A and B), and (Zearalenone – α and β-zearalenone) 25-500 ppb and immunoresponse time around 5, 20 and 10 min, respectively. A small modification was made to the method where a 96-well microplate reader was used for an Elisa UV-*Vis* spectrophotometer, the reading was performed at 650 nm for all mycotoxins. This technique used the previously produced extract described in the topic (2.3). For this technique, 100 µL was used to carry out the immunoenzymatic analysis according to the manufacturer's guidelines for total aflatoxins, ochratoxin, and Zearalenone, the results were expressed in µg kg-1. Results below the limit of detection on quantitation were reported as < 5 ppb. Each analysis was performed in quadruplicate.

3. Results

3.1. Immunoenzymatic determination of mycotoxins

For maize cultivars, AFs were observed in four of the five samples, OA was detected in three samples, and ZEA in only two samples. For soybean only two cultivars showed contamination by AFs and OA and three showed results for ZEA; beans and chickpeas were negative for AFs, two bean samples were positive for OA and one for ZEA; chickpeas did not show positive results for OA, only one sample was positive for ZEA (Table 1).

Table 1. Determination of mycotoxins in corn, soybean, bean, and chickpea grains stored in HDPE drums for 12 months, using the immunoenzymatic ELISA technique and expressed in (µg kg^{-1}).

Samples	**ELISA***		
	Aflatoxins	**Ochratoxin A**	**Zearalenone**
1	37.83	nd	123.48
2	44.07	5.03	108.04
3	51.06	5.14	nd
4	56.91	nd	nd
5	nd	9.26	nd
6	41.87	nd	131.09
7	nd	nd	110.41
8	50.16	nd	nd
9	nd	7.48	nd
10	nd	8.21	125.90
11	nd	9.56	nd
12	nd	nd	114.15
13	nd	8.31	nd
14	nd	nd	nd
15	nd	nd	nd
16	nd	nd	116.08

Note: nd = not detected. (1) B2801PWU; (2) B2801VYHR; (3) P2719VYH; (4) P3016VYHR, (5) 30F35VYHR; (6) M8644 IPRO; (7) BRASMAX VORAZ IPRO; (8) 77A40E; (9) DM74K75CE; (10) NS7901RR); (11) BRS FS307; (12) BRS FC310; (13) BRS305); (14) Cícero); (15) BRS Aleppo and (16) BRS Cristalino). *Results for the immunoenzymatic method mean followed by ± standard deviation. The standard deviation was below 0.06 for all samples. Source: Authors, 2023.

4. Discussion

AFs are mainly produced by *A. flavus* and *A. parasiticus*, but also by *A. nomiae*, *A. pseudotamarii*, *A. bombycis*, *A toxicarus*, *A. parvisclerotigenus*, *A. minisclerotigenes*, A. arachidicola, A. pseudonomius and

A. pseudocaelatus (Vargas et al., 2011). *Aspergillus* grows mainly on corn kernels, peanuts, walnuts, cottonseeds, and dried fruits. Among animals, the action of AFs is already known, where they can cause serious liver problems, nephrotoxic, immunosuppressive, carcinogenic, and mutagenic, reducing the production of milk and meat in cattle, and eggs in poultry (Ricci et al., 2021).

In Brazil, only the maximum levels of AFs in foods are foreseen in the legislation. The Ministry of Health establishes a maximum limit of 30 μg kg^{-1} of AFB1 + AFG1 in food for human consumption and animals. Total AFs in rations. Our limits established by national law are equivalent to those established by other countries and are recommended by the World Health Organization (WHO) and the Pan American Health Organization (PAHO) (Who/Fao, 1998; Caldas et al., 2002). Our findings are superior to those recommended by national and international legislation, both for corn and soybean grains, however, this study is the first to evaluate storage in 240 L HDPE packages for 12 months stored in the field. In other studies, such as by Milanez et al. (1998), the researchers evaluated the presence of AFs in corn grains using the immunoenzymatic method in two out of seven-grain samples with results of 44.2 and 38.2 μg kg^{-1}. Kawashima & Valente Soares (2006) described in a study with corn that 94.6% of the analyzed samples showed positive results with concentrations ranging between 20 and 8600 μg kg^{-1}, for aflatoxin B1 5 samples showed positive results with a maximum content of 20 μg kg^{-1}, two samples exceeded the limit of 20 μg kg^{-1} in the sum of aflatoxins B1, B2, G1 and G2.

The International Agency for Research on Cancer (Iarc, 1997) presented not encouraging results on the evidence of mixtures of all AFs naturally produced AFB1, AFB2, AFG1, and AFG2 where they demonstrated high carcinogenic power in humans. OA has already been identified in products of animal and plant origin, however, in plants its identification is potentially higher, especially in cereals, coffee, grape juice, cocoa, dried fruits, nuts, wheat grains, oats, corn, and rice (Schrenk et al., 2020). For OA of the sixteen evaluated samples, 7 were positive in our findings for corn, soybeans, and beans. Results reported by Milanez et al. (1998), the researchers did not find positive results for OA in grains of maize cultivars, however, when they evaluated 20 cornmeal samples, only one did not show a positive result. Also in this study, these researchers found a variation in the content between 9.5 and 2.2 μg kg^{-1} of OA, with our results similar to those of Milanez and collaborators for this toxin.

According to Caldas et al. (2002), OA has been reported in up to 50% of corn, rice, wheat, and bean samples analyzed in several Brazilian states, corroborating Schrenk et al. (2020) and in this study. Brazilian human and animal health legislation still does not provide for minimum and maximum limits of this mycotoxin in grains, food, or feed, lacking studies and the need to establish new data on these OA levels so that health problems or even deaths do not occur. in humans and animals by intoxication. This mycotoxin is produced by filamentous fungi of the genera Aspergillus and Penicillium (Cole; Cox, 1981), where its optimal temperature is around 20 °C and water activity of 0.86 (Sweeney; Dobson, 1998). It is possible to say that the average temperature for Goiás is 24.6 °C (Climate-Data, 2023) and the presence of moisture inside the drums provided an ideal micro-climate for the development of this mycotoxin.

OA consists of a dihydroisocoumarin linked by the 7-carboxyl group to an L-β-phenylalanine molecule through an amide bond. A study carried out by Pfohl-Leszkowicz et al. (1998) presents OA as a potent nephrotic mycotoxin capable of causing deleterious effects of hepatotoxicity, teratogenicity, and immunosuppression.

Yet another important ZEA mycotoxin is of non-steroidal estrogenic origin and produced mainly by Fusarium graminearum and F. culmorum species. The effect of this hormone in animals, mainly cattle, sheep, pigs, and poultry, causes early breast development, uterus, and breast enlargement, swelling of the vulva, and infertility, however, with no reported mortality (Rashedi et al., 2011).

In our findings, the highest expressiveness was observed for stored soybeans where three out of five samples showed positive results for ZEA. A study carried out by Sabino et al. (1989) with 328 corn samples from the South and Southwest regions of Brazil, showed positive results in 5% of the total samples. Other researchers reported in studies carried out with corn samples for the States of Minas Gerais and São Paulo, Brazil, positive results in one of 83 samples by Sabino et al. (1986) and one out of 110 samples by Machinski et al. (2001). Again, Milanez et al. (1998) found among five samples of corn grains, one with a positive result containing a total of 166.6 μg kg^{-1} of ZEA. Although it is not a critical storage problem how large grain loads are stored Valente et al. (1989) found negative results for ZEA in 296 samples of corn sold in the city of Campinas, State of São Paulo, Brazil.

5. Conclusion

Note that the storage of grains in 240 L HDPE drums in the field proved not to be a good option for storing corn, soybeans, beans, and chickpeas due to the number of samples in different cultivars that showed contamination fungus producing lethal mycotoxins. Furthermore, studies must be carried out following the humidity, temperature, identification of infesting microorganisms, and how to solve this problem where it is not possible to store grains in silos.

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Author's Contributions

Sebastião Antônio Castro Filho: study design, sample collection, project writing, article writing, publication. *Antonio Carlos Pereira de Menezes Filho*: translation, final corrections, submission, reference checking. *Porshia Sharma*: translation, final corrections, submission, reference checking. *Aurélio Ferreira Melo*: text correction, laboratory analysis. *Matheus Vinícius Abadia Ventura*: advisor, data analysis, laboratory analysis.

Ethics

The authors declare that there are no ethical conflicts.