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Zearalenone contamination in corn for human consumption in the state of Tlaxcala, Mexico

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Abstract

Corn from various regions of the state of Tlaxcala, Mexico was sampled to determine whether or not corn which is consumed by humans is contaminated with the estrogenic mycotoxin zearalenone (ZEA). In order to quantify this mycotoxin, a method with adequate sensitivity using solid–liquid extraction and high-performance liquid chromatography (HPLC) with UV diode array detection was optimized. ZEA and zearalanol, used as an internal standard, were extracted from 5 g of a finely ground corn sample with an 85:15 v/v methanol:water solution, cleaned by way of a Florisil column, defatted with *n*-hexane, and subsequently re-extracted with chloroform. The separation was performed on a reverse phase analytical column. Detection was confirmed by on-line simultaneous UV spectral scanning during the chromatographic run. The method's limit of detection was 0.7 ng/g and the recovery average was 90%. Analysis of 24 samples indicates that approximately 70% of the monitored samples were contaminated with zearalenone, with levels ranging from 3 to 83 μg/kg of corn kernels.

Keywords: Zearalenone; Zearalanol; Mycotoxins; Corn; Endocrine disruptors; DAD-HPLC

1. Introduction

Zearalenone (ZEA) (Fig. 1), is an estrogenic mycotoxin mainly produced by *Fusarium graminearum*, a species which colonizes a wide variety of grains, including corn, sorghum, wheat, barley oats, and their derived foodstuffs (Bennet & Shotwel, 1979; Caldwell, Tuite, Stob, & Baldwin, 1970). It develops as a consequence of prolonged cold or humidity and of the interaction of multiple factors, such as moisture content of the grains, infections, temperature, microbial interactions, and others (Moss, 1991).

Generically, zearalenone's name derives from *Gibberella zeae*, and chemically speaking, it is the [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)β-resorcylic-acid-lactone]. It is soluble in alkaline solutions, ether, benzene, acetonitrile, ethyl alcohol, and it is virtually insoluble in water. The maximum absorption wavelengths for this mycotoxin are 236, 274 and 316 nm (Shipchandler, 1975; Urry, Wehrmeister, Hodge, & Hidy, 1966).

Of the many derivatives of ZEA which can be produced by metabolism, only trans-α-zearalenol (α-ZOL), [2,4-dihydroxy-6-(6α,10-dihydroxy-*trans*-1-undecenyl)benzoic acid μ-lactone], is naturally found in contaminated grains and cereals (Hagler, Mirocha, Pathre, & Behrens, 1979). In several animal species, including humans, ZEA is mainly metabolized to two stereo-isomeric metabolites, α- and β-ZOL. Another minor possible metabolite is α-zearalanol (ZAL, Zeranol, Ralgro), [2,4,-dihydroxy-6-(6α,10-dihydroxyundecyl)benzoic acid μ-lactone], (Fig. 1), which is the resultant of reduction of both α- and β-ZOL. This metabolic path has been demonstrated in species such as deer, goats, sheep, cattle and horses (Erasmusson, Scahill, & West, 1994; Kennedy et al., 1998; Miles et al., 1996), but

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✠ In memoriam.

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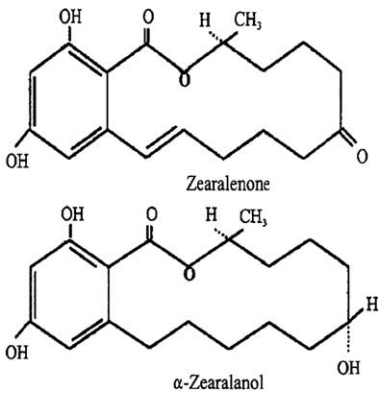

Fig. 1. Chemical structures of zearalenone (ZEA) and the metabolite zearalanol (ZAL).

this compound is mainly synthetically produced from ZEA and is used to stimulate animal growth in the US and other countries, although in the European Union, Zeranol and all hormonal substances used as growth promoters have been banned since 1985 (Council Directive 1985, 1988).

The toxic effects of ZEA and its metabolites mainly derive from its estrogenic properties, since it can assume a structural shape similar to the naturally occurring estrogens estradiol, estrone and estriol, and interacts with human estrogen receptors in competition with 17β-estradiol (Kuiper et al., 1998; Miksicek, 1994). The estrogenic potency of zearalenone, has been shown to be several orders of magnitude higher than that of other environmental estrogens in various test assays (Kuiper et al., 1998; Leffers, Næsby, Vendelbo, Skakkebæk, & Jorgensen, 2001; Shier, Shier, Xie, & Mirocha, 2001).

In humans, ZEA has been associated with early puberty in youth in Puerto Rico (Ingle & Martin, 1986; Sáenz de Rodríguez, Bongiovanni, & Conde-de-Borrego, 1985) and in Hungary (Szuetz, Mesterhazy, Falkay, & Bartok, 1997). A possible link between ZEA and human cervical cancer and endometric hyperplasia has been suggested (Tomaszewski, Mitursky, Semczuk, Kotarsky, & Jakowicki, 1998). Other authors (Gao & Yoshizawa, 1997; Luo, Yoshizawa, & Katayama, 1990) have also found a possible relationship between ZEA and cancer of the esophagus in China. Although the link between the carcinogenic potential of ZEA and its derivatives in humans is still controversial, positive evidence of carcinogenicity has been demonstrated in laboratory animals (Battershill & Fielder, 1998; IARC, 1993).

Gilts are the most sensitive species to ZEA's effects. When exposed, they may suffer embryonic death in certain stages of gestation, a decrease in fertility, an increase in resorption, smaller litters, changes in thyroid, pituitary and adrenal gland weight, and changes in the serum levels of progesterone and estradiol (Long & Diekman, 1984). The prepubescent female is especially sensitive to ZEA's estrogenic effects, in which tumefaction of the vulva as well as ovarian follicle atresia, apoptoso-like changes in granular cells, and intensified cell proliferation in the uterus and oviduct have been observed (Etienne & Jemmali, 1982; Kurtz, Nairn, Nelson, Christensen, & Mirocha, 1969; Obrenski et al., 2003). A study of ZEA epidemiology in 10 European countries showed that this mycotoxin is present in nearly all grains, corn being the most commonly contaminated, with levels of 1–2900 μg/kg (Kuiper-Goodman, Scott, & Watanabe, 1987).

Tolerance levels for this mycotoxin in food are from 30 to 1000 μg/kg in some countries (Codex Alimentarius, WHO Commission, 1998). European Union (EU) regulation allows between 60 and 200 μg/kg in animal feed, but the presence of the mycotoxin in food, or residual levels in the tissue of animals for human consumption are not allowed (Council Directive, 1985, 1988).

The State of Tlaxcala is located in the center of Mexico, adjacent to Puebla, Hidalgo and the State of Mexico. It has an area of 4016 km^2 and comprises 60 municipalities. Its climate is temperate and semi-humid. The average daily temperature is 25 °C, but there are significant seasonal variations. The rainy season lasts for approximately 7 months, and there is considerable humidity during this period.

Corn is the most important agricultural product in the State of Tlaxcala, followed by barley and wheat. However, corn is the most widely consumed product by humans.

Considering the climate characteristics of the State of Tlaxcala, with prevailing hot and semi-humid conditions for considerable periods of time, there is a strong possibility of corn fungal infestation and subsequent ZEA production in agricultural processes, grain storage and processing.

Reports regarding the extent of zearalenone contamination in corn or other cereals in Mexico are scarce. One study carried out in the State of Nayarit (Robledo, Marín, & Ramos, 2001) found a 15% contamination rate of this mycotoxin in corn fodder, with an average concentration of 1610 μg/g.

Given the high risk involved in consuming corn contaminated with ZEA and due to the lack of information regarding the presence of this type of contamination in the State of Tlaxcala, the goal of this work was to determine its incidence in corn for human consumption in that State.

2. Materials and methods

2.1. Samples

Twenty-four samples of corn kernels for human consumption were collected in the markets of the communities of Nopalucan (1–5), Panotla (6–10), San Jorge (11–15), Xocoyucan (16–20) and Santa Cruz (21–24) in the State of Tlaxcala during the spring–summer 2001 cycle. The samples were stored in refrigeration at +4 °C prior to grinding and analysis. For analysis, the corn kernel samples were first finely ground, and then pulverized in a laboratory mill.

2.2. Reagents

ZEA and ZAL (Fig. 1), which was used as analytical internal standard, and activated magnesium silicate (60/100 mesh PR, Florisil) were obtained from Sigma, St. Louis, MO, USA. Standard solutions of ZEA (1 mg/ml) and ZAL (1 mg/ml) were prepared in an acetonitrile: water (75:25 v/v) solution. Water HPLC grade, was obtained using a Simplicity 185 system (Millipore Corporation, Bedford, Mass, USA). Potassium chloride and acetic acid (reagent grade), and all solvents (HPLC grade) were obtained from J.T. Baker, Xalostoc, Mexico State, Mexico. GHP acrodisc (4 and 13 mm) syringe filters with a pore size of 0.45 μm were obtained from Gelman Sciences (Ann Arbor, MI, USA).

2.3. Equipment and chromatographic conditions

The determinations were carried out using a model 1100 Hewlett–Packard liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic HP 1100 pump, an 1100 HP UV-diode array detector, and a Rheodyne 7125 injection valve with a 50 μl loop. Separation was performed in a C18 reversed-phase column (4.6 mm × 15 cm), with particle size of 5 μm (Beckman Coulter, Fullerton, CA, USA), protected by a guard column packed with the same material.

Elution was performed under isocratic conditions, with a mobile phase of acetonitrile:water:methanol (50:42:8 v/v) solution at a flow rate of 0.8 ml/min. 236, 274 and 316 nm wavelengths were used for detection.

2.4. Procedure

0.5 g of KCl and 1 mg of ZAL (200 μg/g), which was used as an internal standard (IS), were added to a 5 g pulverized corn sample. The mixture was macerated and homogenized using 20 ml of a methanol:water (85:15 v/v) solution. The pH was adjusted to 4 using acetic acid, and the mixture was agitated for 1 h.

The macerate was centrifuged for 20 min at 13,314*g*. The supernatant was collected and eluted through a Florisil column. The column was cleaned, first with 2.5 ml of the previously mentioned methanol:water solution, and then with 2.5 ml of methanol. The fat was removed from the eluate using 10 ml of *n*-hexane, agitated for 10 min, and centrifuged again at 13,314*g*. The *n*-hexane layer was discarded, the water–methanol phase was re-extracted using 10 ml of chloroform and another time centrifuged at 13,314*g* for 10 min. The chloroformic phase was transferred to a concentration tube and evaporated to dryness under a nitrogen stream. The residue was reconstituted with 250 μl of the HPLC mobile phase, filtered through a 0.45 μm syringe filter, and 50 μl were injected into the chromatograph.

Each kernel corn sample was analyzed by duplicate.

2.5. Quantification

The assay linearity of ZEA was verified by linear regression analysis in the 0–400 ng/g range spiked on the 5 g blank corn sample. As internal standard, 1 mg (200 μg/g) of ZAL was added to each sample. The peak-area ratios of ZEA vs. ZAL (ZEA/ZAL) were plotted against the concentration of ZEA. The slope and *y*-axis intercept, as well as the correlation coefficient, were obtained from the calibration curve. The unknown concentrations in samples were obtained for interpolation in the calibration curve.

For recovery analysis, three replicate samples (5 g) spiked with 20, 40, 60 and 80 μg/g of ZEA were prepared on blank corn and processed as it was described previously.

3. Results

3.1. Method validation

Fig. 2(a) shows a DAD chromatogram obtained of a sample of corn previously sterilized by way of microwave, in which, as may be observed, the toxins were undetectable. This sample was used as a matrix for the calibration curve and for the recovery experiments.

Fig. 2(b) and C show DAD chromatograms obtained from samples spiked with 100 and 200 ng/g of ZEA respectively. The retention times of ZAL and ZEA were 5.1 and 8.1 min, respectively. As may be seen, there are no interfering peaks coming from the corn matrix at these retention times. In order to identify the resolved peaks, online spectra were recorded during the chromatographic run. Examples of UV absorption spectra for ZAL and ZEA in accordance with retention times are shown in the insets of Fig. 2(b) and (c). Adequate detection was observed at all wavelengths used (236, 274 and 316 nm). Nonetheless, at 274 nm better resolution was obtained for ZEA and its internal standard (ZAL).

Linearity of response was observed over the studied ranges of concentration. The mean calibration curve (Fig. 3) may be described by the equation $y = 0.008(x) + 0.027$; $r^2 = 0.98$, and by the estimated standard deviation (S_y) = 0.23 ng. The limit of detection (LOD), defined as mass of the analite which produces a signal three times the standard deviation of baseline noise (Miller & Miller, 1993), was 0.7 ng/g, and the limit of quantification (LOQ) was 1.4 ng/g (twice the value of LOD).

The mean extraction recovery rate of four different concentrations of ZEA was 90.0 ± 5.9%. The mean coefficient of variation (CV) was 9.1 ± 0.7%.

3.2. ZEA in corn kernel samples

Following the optimization and validation of the analytical method, it was used to determine the contamination level in corn samples for human consumption obtained in different communities of the State of Tlaxcala.

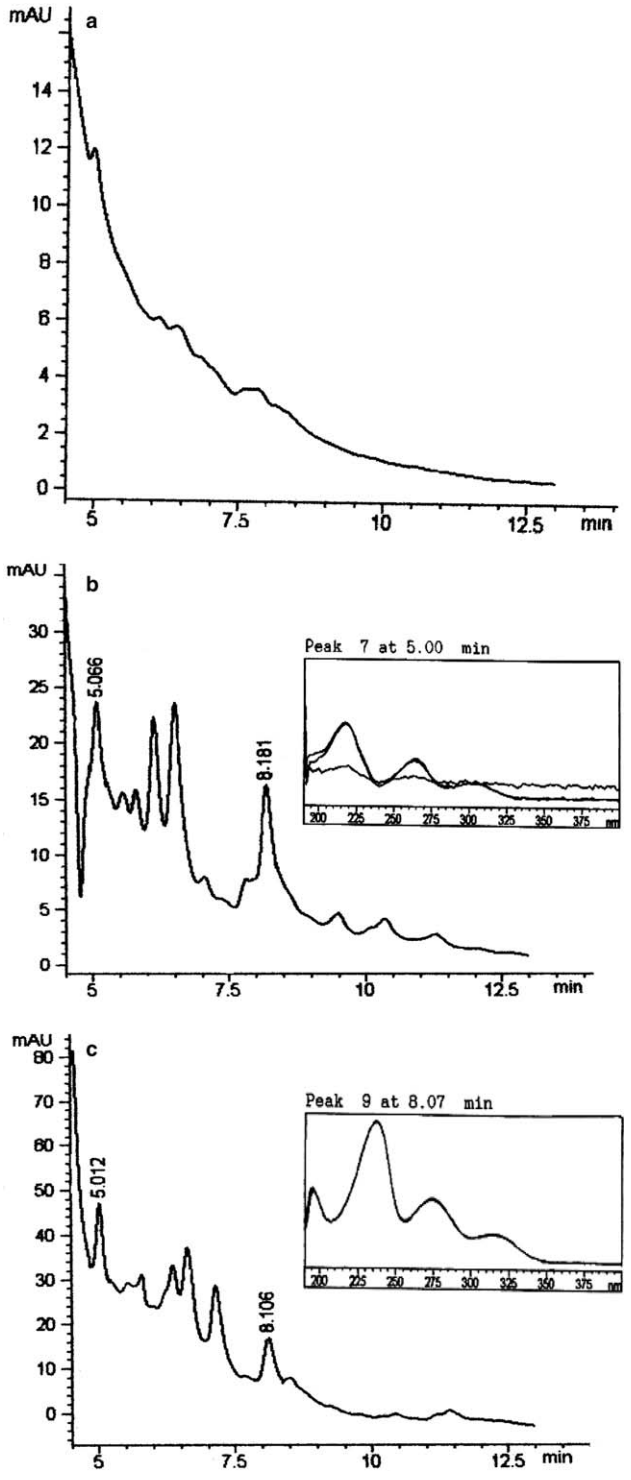

Fig. 2. HPLC-DAD chromatograms for a blank corn sample (a); blank corn sample spiked with 100 ng of zearalenone (b) and blank corn sample spiked with 200 ng of zearalenone (c). All samples were spiked with 200 ng/g of zearalanol as an internal standard. The peak retention time of 5.0 corresponds to zearalanol, and the peak retention time of 8.1 corresponds to zearalenone. An example of the UV spectrum of zearalanol scanned online using DAD during the elution in the chromatographic run is shown at the upper right of b, and the corresponding UV spectrum for zearalenone is shown in c. Chromatographic conditions: C_{18}, 5 μm, reverse phase column; mobile phase acetonitrile:water:methanol (50:42:8, v/v); flow rate 0.8 ml/min. UV detection at 274 nm. Online UV spectral scanning from 0 to 900 nm.

The results obtained from monitoring of the 24 corn samples are shown in Fig. 4. Zearalenone levels from 3 to 83. 63 μg/kg were observed in 70% of the samples.

4. Discussion

In this work, a method with adequate sensitivity for detecting ZEA in corn kernels was optimized. Although a considerable number of analytical methods for the determination of this mycotoxin have been published, most of them used thin layer chromatography (TLC), immunoassays or immuno-enzymatic techniques (ELISA) when the levels were above 800 ng/g (Bennet, Nelsen, & Miller, 1994). However, when it has been necessary to quantify very small levels, chromatographic methods such as GC or HPLC have been the best option. Since zearalenone is a native fluorescent compound, HPLC with fluorescence detection has been the most widely used HLPC method. Recent advances in mass spectrometry have made this technique the most selective and sensitive for ZEA analysis. However, its high cost does not make it viable for use in routine sample surveys. In this work, an analytical procedure using diode-array detection has been presented as an alternative, because in addition to allowing for the detection of low levels of the mycotoxin in the highly complex corn sample matrix, it allows for the identification and confirmation of analytes of interest by way of the simultaneous combination of UV–VIS detection at different wavelengths and online UV–VIS spectral information in a single chromatographic run. Since zearalanol is not a natural contaminant of cereal samples, it was possible to use this compound as an internal standard when the presence of ZEA is monitored in cereals. The use of an internal standard in this work made it possible to improve the efficiency of the method. Moreover, the simultaneous availability of multiple wavelengths allowed for use of the optimum detection wavelength for each compound.

Another important advantage of the method used was the significant reduction in the quantity of sample used. Only 5 g of corn sample were used, whereas in most of the other reported methods, the minimum sample quantity was 10 g. This allowed us to reduce the clean-up process, with a corresponding reduction in the amount of dangerous solvents and time-consuming steps in liquid–liquid and solid–liquid extractions.

The high correlation coefficient, $r^2 = 0.98$, the low detection limit (≈1 ng/g) and the high recovery level (90 ± 5.9%) show that the optimized method should be used in monitoring of ZEA levels in corn samples.

Monitoring of corn samples which originated from different communities in the State of Tlaxcala showed a 70% degree of ZEA contamination. Although the degree of contamination (3.25–83.63 μg/kg) is considered within the acceptable limits in some countries (Codex Alimentarius, WHO Commission, 1998), the potential risks associated with the cumulative effects of long-term exposure on estrogenic target organs are high when the importance of

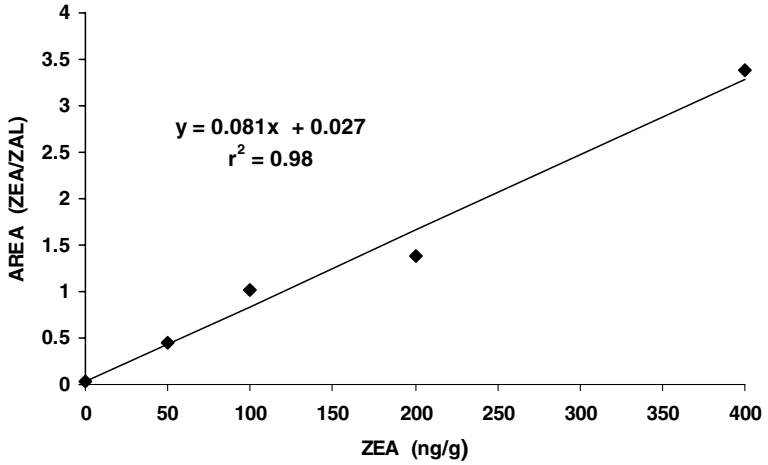

Fig. 3. Calibration plot of the diode array detector (DAD) response at 274 nm. The area ratio of zearalenone and the internal standard zearalanol (ZEA/ZAL) vs. zearalenone concentration was used for the quantification analysis.

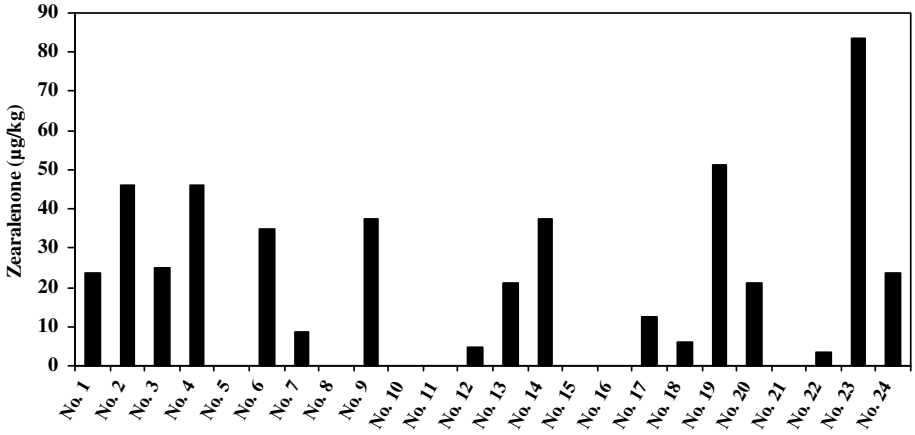

Fig. 4. A representation of the levels of zearalenone found in the analyzed corn samples. Results are the means of two determinations for each sample. Samples were collected in the communities of Nopalucan (1–5), Panotla (6–10), San Jorge (11–15), Xocoyucan (16–20) and Santa Cruz (21–24) in the State of Tlaxcala, Mexico. Spring–summer 2001 period.

corn in the Mexican diet and culture is considered, since corn is the most highly consumed food in Mexico.

This mycotoxin and its metabolites have been shown to be highly dangerous at the parts per billion level. Shier and colleagues (2001) demonstrated that the M50 (the molar concentration (M) resulting in half-maximal effect) of α-zearalenol and ZEA to stimulate cellular proliferation in MCF-7 cell cultures (a breast cancer cellular line) are 0.012 and 1.1 nM, respectively. In this assay ZOL and ZEA are the most potent xenoestrogens. The authors consider that a woman weighing 45 kg, after having consumed 100 g of corn flakes contaminated with 13–20 μg/kg (contamination levels reported by Scott, Panalaks, Kanhere, & Miles, 1978), may have a ZEA plasma concentration of 3.1 nM, a concentration which produces high stimulation in the aforementioned biological assay (Shier and colleagues, 2001). The levels of ZEA found in the present study, in some of the corn samples from Tlaxcala, Mexico are higher than those reported in the aforementioned study. Therefore, although information about the effects related to human exposure to ZEA has not been thoroughly researched, given the hyper-estrogenicity, severe reproductive effects, infertility problems and tumor-promoting properties observed in other species, as well as the early telarche symptoms in children reported in Puerto Rico (Ingle & Martin, 1986; Sáenz de Rodríguez et al., 1985) and in Hungary (Szuetz et al., 1997), the ZEA contamination levels found in this work merit special concern. It is also important to keep in mind the possible additive effect of many estrogenic substances present in the environment (fito-estrogens, pesticides, PCBs, herbicides, alkylphenols, etc.), which could contribute to the estrogenic load in the exposed population.

5. Conclusions

The determination method optimized in this work using DAD detection has the adequate sensitivity and selectivity for the analysis of low levels of ZEA in corn samples, and it is suitable for routine monitoring.

ZEA contamination from 3.25 to 83.63 μg/kg in corn kernel samples for human consumption was found in 70% of the samples analyzed in the State of Tlaxcala, Mexico.

Given the risks people who consume foods contaminated with this mycotoxin are exposed to, the implementation of measures to reduce the probability of contamination in corn and other foods during the harvest, post-harvest, storage and processing periods are recommendable. Better surveillance and monitoring strategies, along with studies to determine the link between the presence of this mycotoxin and health problems, are also highly required.

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