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Estimation of Mycotoxin Multiple Contamination in Mexican Hybrid Seed Maize by HPLC-MS/MS

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Abstract

In Mexico, the presence of mycotoxins in chemical treated seed maize by sowing is not well known, despite the need to improve the quality and corn safe for human consumption. It collected twenty-five genotypes maize samples from Morelos State in the spring of 2013, all of them treated with synthetic colors (pink, green, yellow), fungicides and insecticides. Two samples (synthetic seed and hybrid commercial) were selected for analysis of twenty-two mycotoxins by LC-MS/MS and AFB_1 determination by liquid chromatography and fluorescence detection (HPLC-FLD). The results of the 25 samples showed the presence of Aflatoxin B_1 in 25% of samples in a ranged concentration between 2 to 6 μg·kg^{-1}, and average of 4.1^{-1} μg·kg, which were within the allowed limits by national and international legislation. Twenty-two mycotoxins were found in levels ranging between 791.7 and 891.2 μg·kg. The content average in both samples was for total aflatoxins (AFB_1, AFB_2, AFG_1, AG_2) of 16.95 μg·kg, with G aflatoxins the most prevalence. Twelve trichothecenes (Nivalenol, Neosolanol, Fusarenone X, DAS, HT-2, FB_1, FB_2, FB_3, T-2, Zearalenone, ZEA2, ZEA3) were in a level of 292.7 μg·kg^{-1}, Enniantine 8.6 μg·kg^{-1}, Sterigmatocystin 6.5 μg·kg^{-1}, Roquefortine C, 2.9 μg·kg^{-1}. Ochratoxin 8.8 μg·kg^{-1} and Mycophenolic acid at 535 μg·kg^{-1} were the highest content. The synthetic color present in seeds analyzed inhibited a good purification in the extracted mycotoxin by optimizing the step in HPLC-MS/MS quantification system. The information generated in this study would be useful in breeding programs in order to improve the sanitary quality and also to investigate the final contamination of agricultural products with multiple mycotoxin contamination.

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Keywords

Maize Seed, Warehouse, Moulds, Mycotoxins, HPLC, MS/MS

1. Introduction

In Mexico, maize (corn) is the most produced and consumed cereal, like tortilla, an adult may consume 360 g/day [1]. Producer's corn requires protecting seed before planting; using fungicides, insecticides and synthetic dyes as yellow, green and pink to prevent consumption by people [2]. Studies show that a co-occurrence of fungus and mycotoxins in harvest and post-harvest maize is possible around the world [3], but there is not enough information in the chemically treated seed previously sowing. Mycotoxins are a group of secondary metabolites produced by different fungus in maize, one of the most toxic compounds known for humans [4]. The aflatoxin B_1 (AFB_1), is carcinogenic; Ocratoxine A (OTA) and Fumonisins are genotoxic, immunotoxic and recently classified by International Agency for Research on Cancer (IARC) in 2B group of substances possible to human carcinogenic [5] [6]. Esterigmatocistine (ST) is hepatotoxic; in general most of mycotoxins are immunosuppressive [7], indeed it is important to know their exposure since a chronic exposure to low levels can give diseases like cancers [8], additionally it is not well known about the possible interaction between multiple mycotoxins [9]; and on the other hand, fungus and mycotoxins would remain in environment (air, soil, water), due to their stability against degradation [10]-[12]. Among the identified fungus in post-harvest are *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus* which are producers of Sterigmatocystin, Aflatoxin B_1, B2, G_1 and G_2 and Ochratoxin A; *Penicillium spp*, producer of Roquefortine C and Mycophenolic acid, *Fusarium verticilliodes* (Zearalenone, Deoxynivalenol, DAS, Nivalenol, T-2, HT-2, Fusarenona X, Fumonisin B_1, FB_2, FB_3) and *Alternaria alternate*, Alternariol (AL) and Methyl ethyl alternariol (AM) [13]. Various methods have been used to isolate and quantify the levels of mycotoxins, such as liquid chromatography with fluorescense detector (HPLC/FLD), high performance liquid chromatography coupled to system triple-quadruple mass spectrometer (HPLC-MS/MS). In Mexico official tests to determine the presence of AFB_1 and total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1) are HPLC/FLD [14] [15]. The National regulation sets a maximum level of 20 μg/kg and 0.05 μg/kg AFB_1 and AFM_1 in raw maize and milk [16]. In some countries more of 20 mycotoxins are subject to legal guidance and regular monitoring [17]-[19], like in Food Analysis Laboratory in Ghent University at Belgium, which make a survey of 35 mycotoxins and regulated AFB_1 and DON in 5 μg·kg, total Fumonisins at 50 $\mu g \cdot kg^{-1}$, zearalenone at 500 $\mu g \cdot kg^{-1}$ and Ochratoxin A in 5 $\mu g \cdot kg^{-1}$ [20]. This study was designed to analyze AFB_1 as well as twenty-two mycotoxins in sowing maize seed in one synthetic variety and commercial hybrid maize, both adapted to a tropical region in the country.

2. Materials and Methods

2.1. Collection of Samples

25 genotypes of white and yellow hybrid maize were collected directly (about 1 kg each) in experimental station, Zacatepec Morelos State in the spring of 2013. It is a region geographically defined by the coordinates of 18˚37' and 18˚41' north latitude, 99˚10' and 99˚14' west longitude; with warm humid climate; the annual average rainfall is 892 mm and the minimum and maximum temperatures are 24˚C and 40˚C in an altitude between 900 to 1200 m. The comprised genotypes of maize adapted to temperate and tropical regions, all of them treated with colors (pink, green, yellow), fungicides and insecticides. These two samples were chosen based on their significance, one of two in terms of synthetic variety and the other for production and protein value, both sample were trilinear varieties.

2.2. Chemical, Reagents and Materials

All mycotoxins standards, solvents and material used like methanol, acetonitrile HPLC grade from Merck Company were purchased from Sigma-Aldrich Company, USA.

2.3. Stock Solutions

The mycotoxin standard stock solutions were prepared weighting 1 mg of AFB_1, Sterigmatocistine and Zeara-

lenone, 5 mg each of T-2 toxin Neosolaniol; 10 mg of Deoxynivalenol, Nivalenol and Fusarenone X. Each of them were placed in a 50 mL volumetric flask and dissolved by the addition of acetonitrile.

2.4. AFB_1 Extraction

Aflatoxin B_1 was held at the Laboratory Toxicology at UAM Xochimilco by a liquid chromatography and fluorescence detection (FLD), using Varian Polaris equipment. The method according to the official method [21] was used. In a dried and ground sample (50 g) was mixed with 100 mL of 80% methanol in water (vol/vol) and shaken for 1 h. The aflatoxin B_1 was separated on an analytical reversed phase column C18 (150 mm × 4.6 mm) 5 um particles. The mobile phase was composed of water/methanol/acetonitrile (60:20:20 v/v). Aflatoxin B_1 detection was carried out at excitation λ 360 nm, and emission λ 440 nm wavelengths, in a flow rate was 1 mL min of the mobil phase. Finally the retention time was calculated with five consecutive injections of AFB_1 working solutions.

2.5. Multiple Mycotoxin Extraction

Twenty-two mycotoxins were performed according to the protocol of food analysis laboratory of the Faculty of Pharmacy of the Ghent University, Belgium by HPLC on tandem mass (LC-MS/MS) with ionization detection (Waters UPLC-Quattro Tandem Quadruple MS instrument). Briefly the procedure was taken a 2.5 g of milling sample extracted with acetonitrile/H_2O (95:5 v/v) +10 Mm NH4-acetate in pH 3.0, hexane defatting in a shaker by 60 min, centrifugation 3000 rpm for 15 min, filtration (0.25 µm), clean with tandem immunoassay column, the eluate was evaporated to dryness and redissolution with injection system, so finally made a 10 µL injection to LC/MS/MS.

3. Results

The average of multiple mycotoxins analysis in synthetic and hybrid commercial seed are summarized in **Table 1**. Total mycotoxins mean content was 556.9 µg·kg^{-1}, which is the first time that had been described in chemically treated seeds prior to its sowing in Mexico. Fumonisins concentration (FB_1, FB_2 and FB_3) was 134.3 µg·kg^{-1}, while FB_2 with the highest concentration of 67.4 µg·kg^{-1}, total aflatoxins (AFG_2, AFG_1, AFB_1, AFB_2) in 16.9 µg·kg^{-1}, with AG_2 the most important (8.5 µg·kg^{-1}), Ochratoxin A with 8.80 µg·kg^{-1}, Sterigmatocistine 6.50 µg·kg^{-1}, Roquefortine C 2.9 µg·kg^{-1}, Enniantine 8.65 µg·kg^{-1}, trichothecenes type A and B (NIV, NEO, FUSX, DAS, HT-2, T-2, Zearalenone, ZEA1, ZEA2) with a total levels of 197.4 µg·kg^{-1} Alternariol 17.4 µg·kg^{-1} and Methyl Alternariol 16.7 µg·kg^{-1}.

In synthetic seed, the content of FB_1, FB_2 and FB_3 were 33.3, 66.1, 29.9 µg·kg^{-1} respectively, with a total of 129.3 µg·kg^{-1}, AFG_2, AFG_1, AFB_1 and AFB_2 in 8.5 µg·kg^{-1}, 3.6 µg·kg^{-1}, 1.7 µg·kg^{-1}, 2.4 µg·kg^{-1} respectively with a total of 16.2 µg·kg^{-1}, the Ochratoxin A in 8.70 µg·kg^{-1}, Sterigmatocistine 6.50 µg·kg^{-1}, Roquefortine C, 2.5 µg·kg^{-1}, Enniantine 8.60 µg·kg^{-1}, trichothecenes (Nivalenol, NEO, Fusarenone X, Diacetoxyscirpenol, HT-2, T-2, Zearalenone, ZEA1, ZEA2) with a total content of 123.0 µg·kg^{-1}, Alternariol 14.7µg·kg^{-1} and Methyl Alternariol 15.3 µg·kg^{-1}.

In commercial hybrid seed, the content of FB_1, FB_2 and FB_3 were 42 µg·kg^{-1}, 68 µg·kg^{-1} and 29.3 µg·kg^{-1} respectively with a total of 139.3 µg·kg^{-1}, AFG_2, AFG_1, AFB_1 and AFB_2 in 9.2 µg·kg^{-1}, 3.6 µg·kg^{-1}, 2.4 µg·kg^{-1} and 2.5 µg·kg^{-1} with a total of 17.7 µg·kg, Ochratoxin A 8.90 µg·kg, Sterigmatocistine 6.50 µg·kg, Roquefortine C, 3.3 µg·kg^{-1}, Eniantine 8.70 µg·kg^{-1}, trichothecenes (NIV, NEO, FUSX, DAS, HT-2, T-2, ZEA1, ZEA2, ZEA3) with 171.8 µg·kg^{-1}, Alternariol 20.1 µg·kg^{-1} and Methyl Alternariol 18.1 µg·kg^{-1}. **Figures 1-8** show the chromatograms from LC-MS/MS detected multiple mycotoxins in synthetic and commercial hybrid seed.

Table 1. Mycotoxins contamination in seed samples studies.

Sample	Fumonisin (µg·kg^{-1})	OTA (µg·kg^{-1})	Total AF (µg·kg^{-1})	AB1 (µg·kg^{-1})	Tricothecenes (µg·kg^{-1})	Mycophenolic acid (µg·kg^{-1})	Total ZEA (µg·kg^{-1})	Total Mycotoxins (µg·kg^{-1})
1	129.30	8.90	16.4	1.7	242.90	581.8	30.5	979.3
2	139.30	8.90	17.7	2.4	271.00	479.7		934.6

1, 2 = multiple mycotoxins analysis.

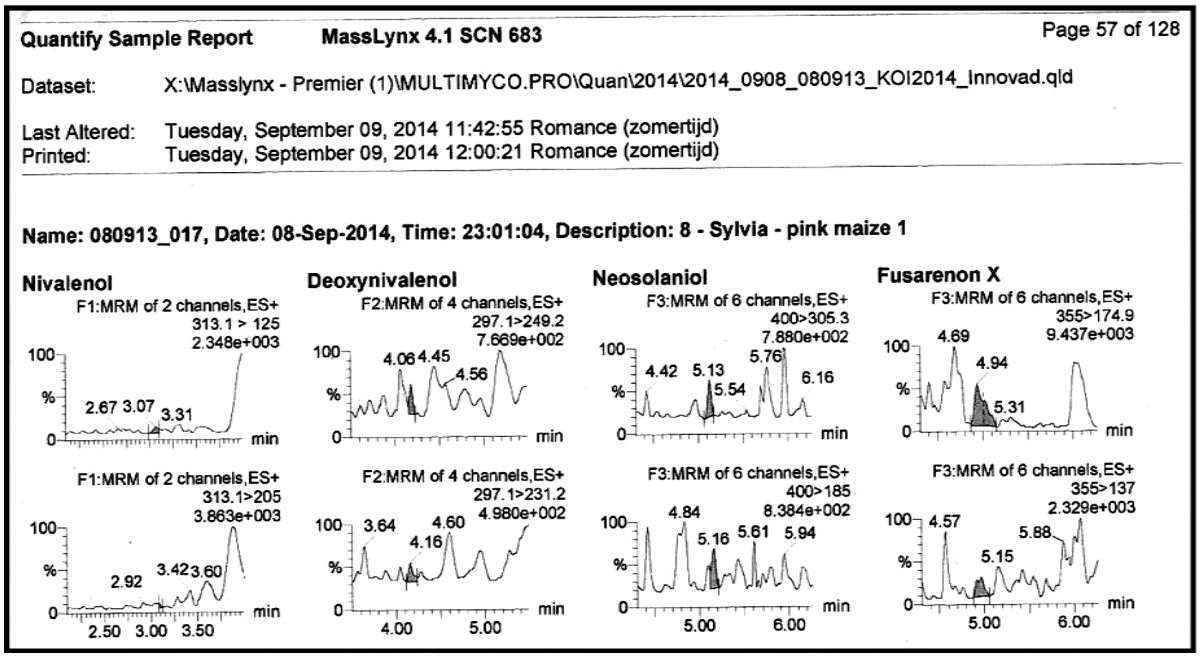

Figure 1. Occurrence of multiple mycotoxin in hybrid seed chemically treated.

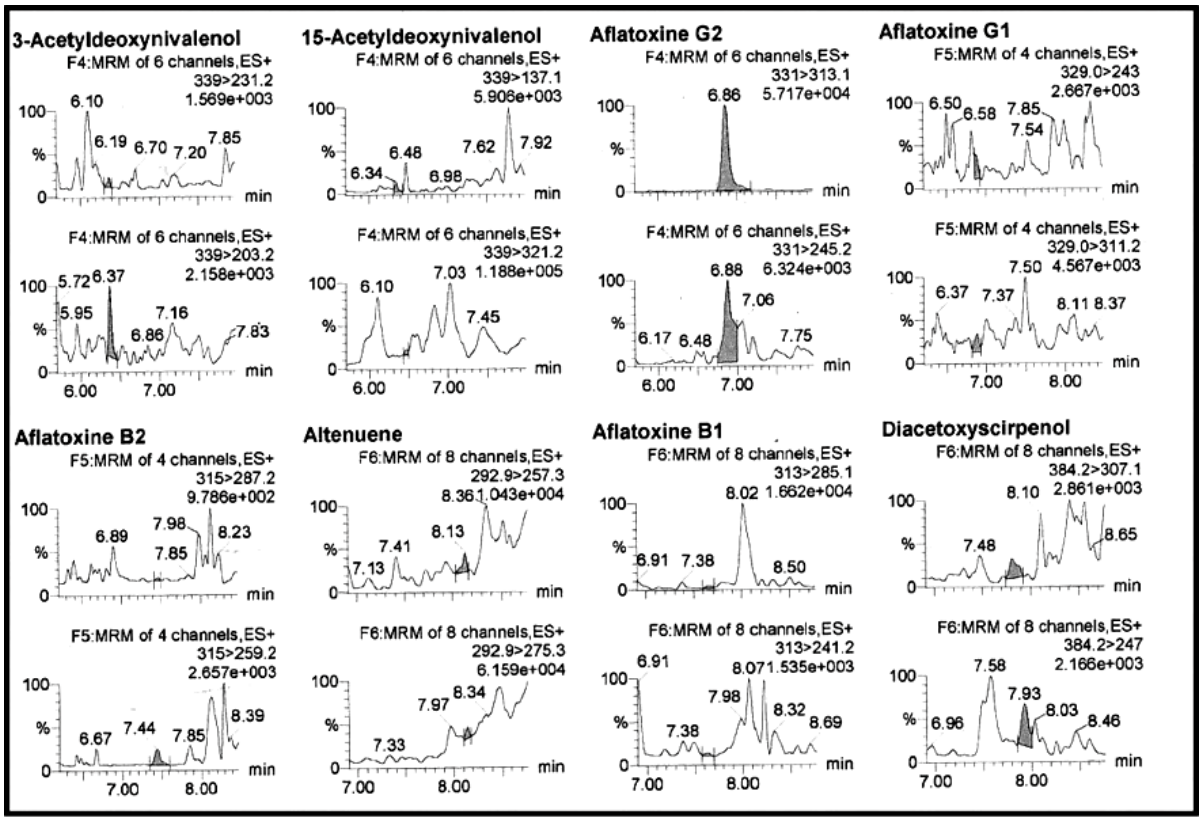

Figure 2. Identification of AFG_2, AFG_1, AFB_2, AFB_1 and derivative mycotoxins.

4. Discussion

The aflatoxins levels detected in eight samples of chemical treated seed maize in our study were lower from

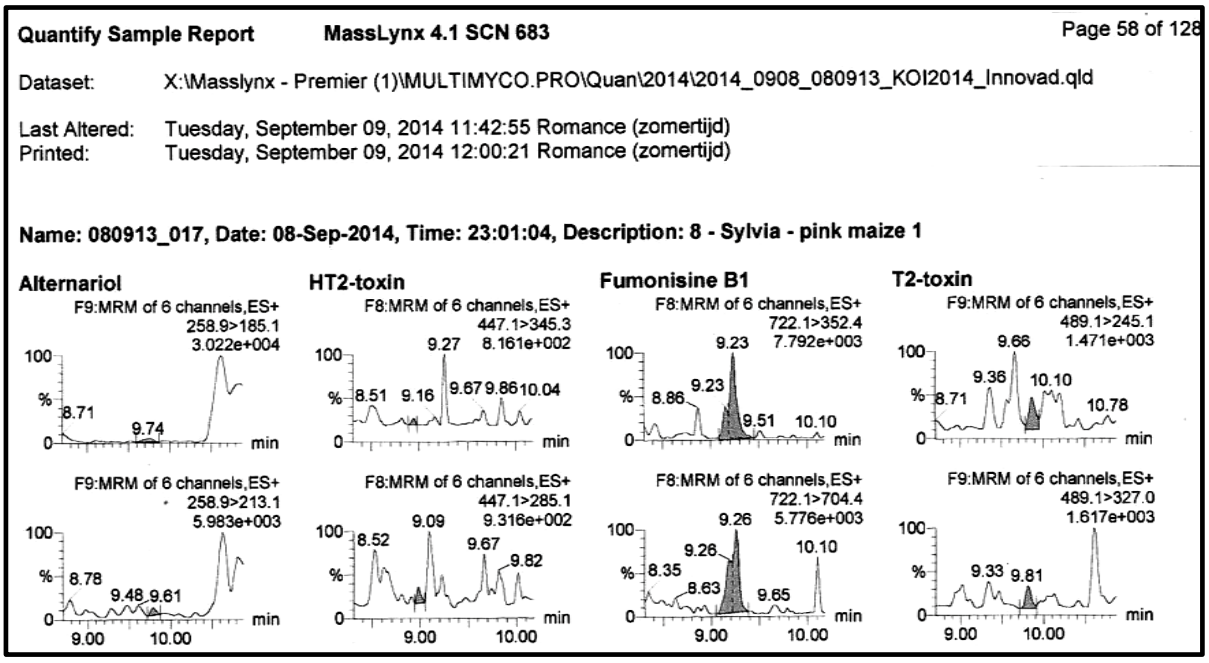

Figure 3. Detection of Alternariol. HT2, Fumonisine B_1, and T2.

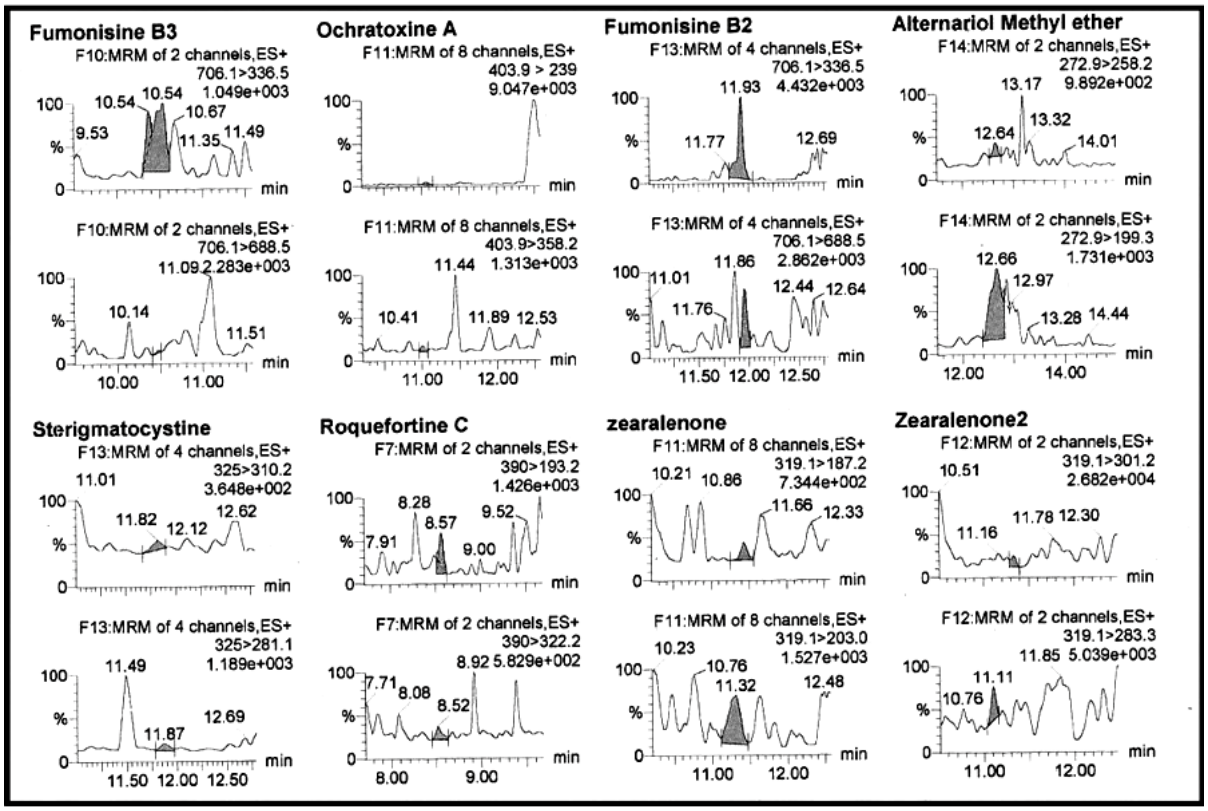

Figure 4. Identification of FB_3, FB_2, Zearalenone, Zearalenone2, Ochratoxin A, Sterigmatocistine and Roquefortine C.

detected feed corn used in animal production at Mexico [22]. However, we had an Aflatoxin G_2 in the highest content, which was probably due that *Aspergillus flavus* didn't find the environmental conditions for survival or the subspecies flavus were not produced AFB_1 [23] [24]. Nevertheless, we found Sterigmatocistine, which might

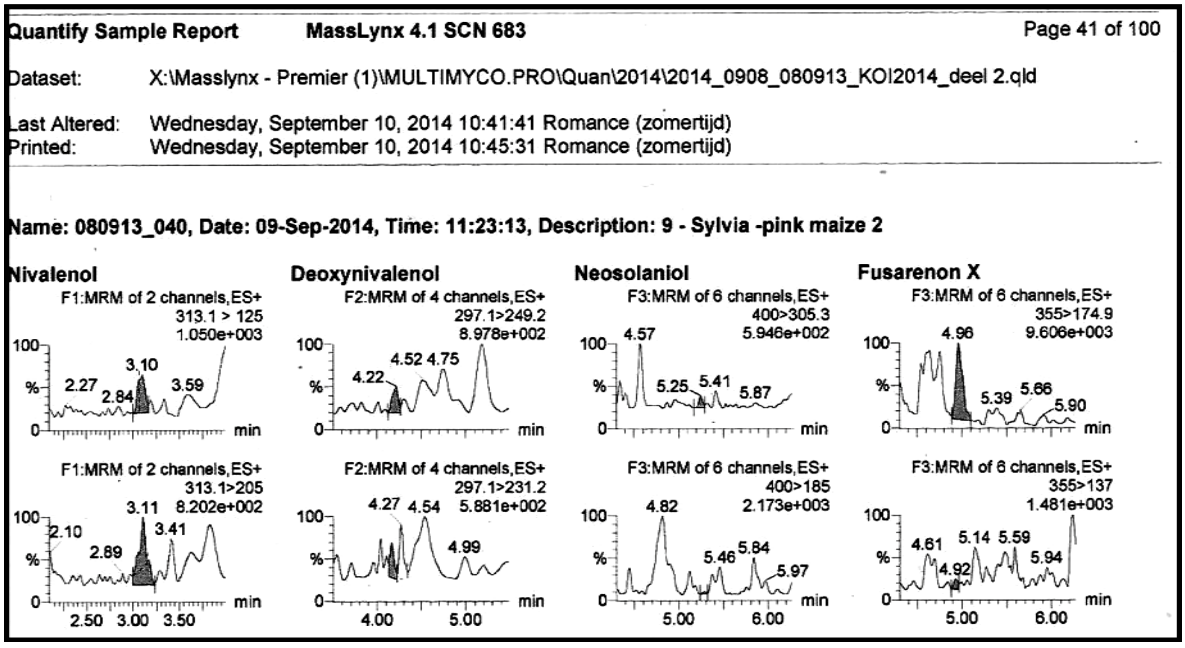

Figure 5. Multiple mycotoxins detection in commercial hybrid seed maize.

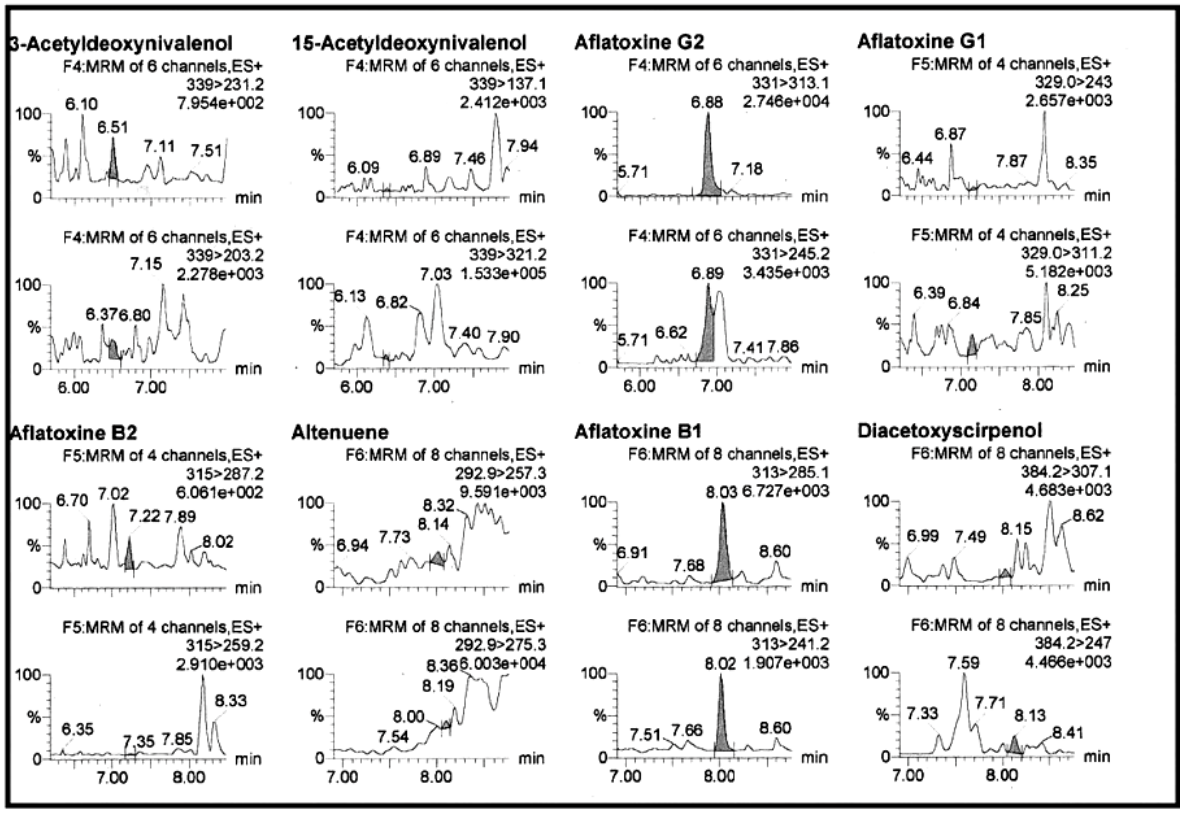

Figure 6. Identification of thricothecene mycotoxins.

be aflatoxins precursor. In Mexico the Fumonisins occurrence in improved corn grain has been noted previously in levels 03 to 64 $\mu g \cdot kg^{-1}$, and commercial hybrid in 32 $\mu g \cdot kg^{-1}$ [25]; similar to the found results in the treated seeds, suggesting that the chemical treatment has not been possible to protect the seed against fungus, according

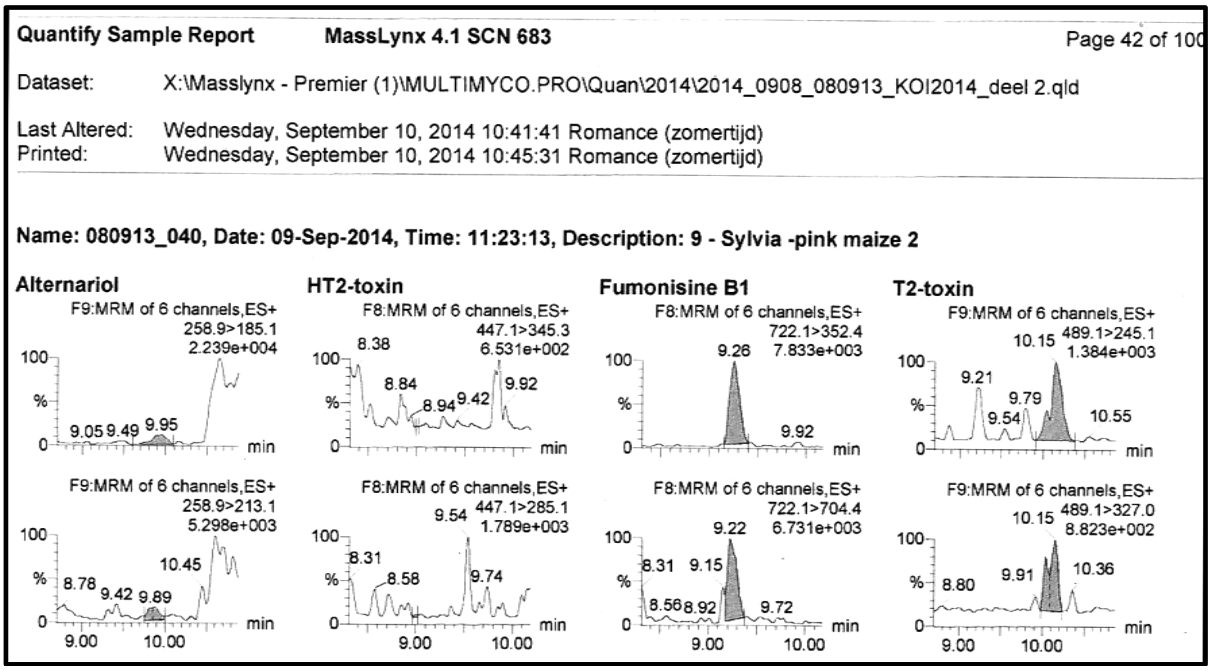

Figure 7. Alternariol, HT2 toxin, FB1, T2 Toxin.

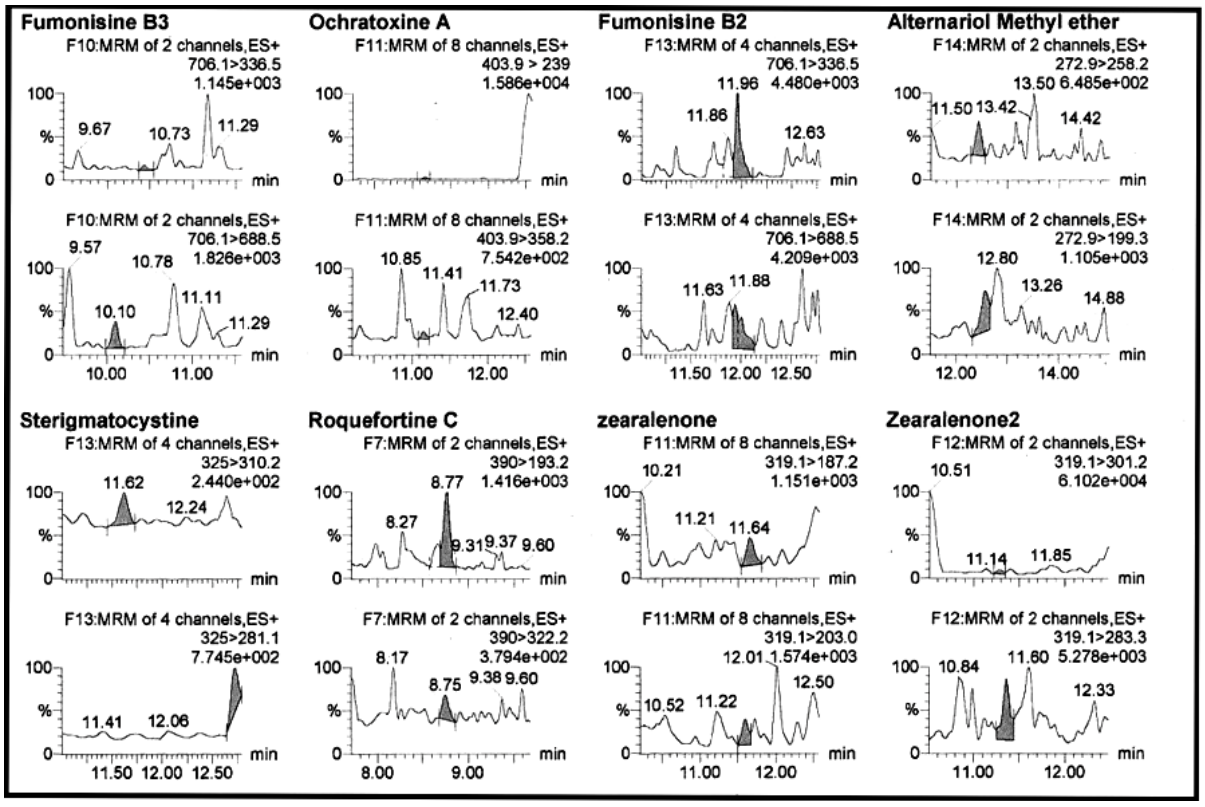

Figure 8. Co-occurrence of eight mycotoxins in commercial seed hybrid maize.

to [26], there is growing evidence that loss of soil biodiversity is a serious issue for arable soils under intensive cultivation. The mycophenolic acid (MPA) [6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-me-thyl-4-hexenoic acid] was the higher mycotoxin incidence in both hybrid seed samples, which is related to *Penicillium*

roqueforti ubiquitous fungus and known to have antibiotic properties but little known about their natural prevalence in feed and foods for human consumption, contrary has been studied in silage corn in the feeding of dairy cows [27]. The alternariol (AOH) and methyl ethyl alternariol (AME) are mycotoxins produced by *Alternaria* sp. It is a common saprophyte pathogen of senescent plant residue and in soil, these mycotoxins are detected in soy, it has been notified the co-occurrence AOH in a minimum level of 25 μg·kg and AME in 62 $\mu g \cdot kg^{-1}$ [28]. All these findings indicate that chemical treated seed maize prior sowing are susceptible to fungus infection and mycotoxins contamination [29]-[31]. The low mycotoxins concentration was due to the extracts purification failure, which could not be removed as interference colorant. It has been mentioned that it is an essential step in the analysis of mycotoxins, especially when chromatographic techniques are used for their determination at trace levels [32].

5. Conclusion

The present study has reported for the first time a survey of multiple mycotoxin contamination in two chemically treated hybrids maize seed for sowing in Mexico, although the median concentrations of the analytes were in a low level allowed in the National Legislation. These data about the presence and content of mycophenolic acid, Ochratoxin A, Fumonisins, Nivalenol, Fusarenone X and Alternariol can be used into National Programs of breeding in order to improve the sanitary quality and to investigate the final contamination of agricultural products with multiple mycotoxin contamination.

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