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Mycoflora and Multimycotoxin Detection in Corn Silage: Experimental Study

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Agricultural activities involve the use of crop preservation such as "trench-type" silo, which can sometimes be contaminated by fungi. To investigate the exposure of livestock and farm workers to fungal spores and mycotoxins, a multimycotoxin analysis method has been developed. Six mycotoxins (aflatoxin B_1, citrinin, deoxynivalenol, gliotoxin, ochratoxin A, and zearalenone) were quantified by high-performance liquid chromatography coupled to mass spectrometry after solid-phase extraction. An experimental study of fungal species and mycotoxins was conducted in corn silage (Normandy, France) during 9 months of monitoring. The results indicated the recurrence of around 20 different species, with some of them being potentially toxigenic fungi such as *Aspergillus fumigatus*, *Aspergillus parasiticus*, *Fusarium verticillioides*, and *Monascus ruber*, and the detection of aflatoxin B_1 (4−34 ppb), citrinin (4−25 ppb), zearalenone (23−41 ppb), and deoxynivalenol (100−213 ppb). This suggested a possible chronic exposure to low levels of mycotoxins.

KEYWORDS: Corn silage; mycoflora; mycotoxins; SPE; HPLC-MS

INTRODUCTION

Corn silage consists of grinding corn and represents one of the most important animal feeds in France, particularly in Lower Normandy, where it is used by 56% of cattle-breeding farms (*1*). Because the use of silage is increasing, it is important to assess whether it is a potential threat to human or animal safety.

The production of corn silage entails incorporation of the whole plant. The method of producing silage is essentially based on the principle of preservation under anaerobic conditions together with the growth of lactic acid bacteria, which promote a natural fermentation, lowering the pH to a level at which clostridial growth is inhibited. These conditions (low pH and anaerobiosis) are unfavorable for the growth of most fungi. However, poor storage conditions, caused by insufficient drying, condensation, heating, leakage of rainwater, insect infestation or managerial variation, could lead to undesirable growth of anaerobic and microaerobic acid-tolerant fungi and other microrganisms including yeasts. Among some of these fungi, species of toxigenic fungi, most commonly *Aspergillus flavus*, *Fusarium verticillioides* (syn. *F. moniliforme*), and *Fusarium proliferatum*, are able to grow on corn (*Zea mays* L.) and produce mycotoxins at the preharvest stage. Toxin production in corn has been found to depend on several interacting environmental stress factors (*2*). Other fungal species such as *Aspergillus fumigatus*, *Byssochlamys nivea*, *Monascus* spp., *Penicillium roqueforti*, and *Trichoderma* spp. are adapted to ensiling conditions and were the most frequently determined contaminants of ensilaged feeds (*3*−*5*). Thus, fungal contamination could be present at the preharvest stage (infection in the field) and/or at the postharvest stage (during silage storage).

This fungal growth reduces nutritional value and could result in the production of mycotoxins and allergenic spores (*6*) that constitute a risk factor for human and animal health. Toxic syndromes caused by mycotoxin ingestion by humans and animals are indicated as mycotoxicosis. It has been estimated that at least 300 fungal metabolites are potentially toxic to human and animals (*7*). Surveillance for mycotoxins in cereals and animal feeds has shown that, when mycotoxins are identified, mixtures of these toxins often occur (*8*). These observations showed the need to develop a method to analyze silage for multiple mycotoxins.

Few field studies have explored the fungal contamination of corn silage. During a survey of fungi infecting stored corn silage in France and Italy, Pelhate (*3*) achieved a nonexhaustive account of fungal species: *Geotrichum candidum*, *Monascus ruber* (responsible for a red coloration in silage), *Mucor* spp., *P. roqueforti*, *Trichoderma* spp., *B. nivea*, and *Paecilomyces variotii* were isolated. In an analysis of spoiled corn silage

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carried out in The Netherlands from 1986 to 1990, it was observed that *P. roqueforti* was the predominant fungus found (*4*). A number of strains of *P. roqueforti* isolated from corn silage were identified as producers of roquefortines A, B, C, and D together with festuclavine (*9*). *Alternaria* species were also determined by Muller (*10*). All strains produced tenuazonic acid and alternariol monomethyl ether. Other mycotoxins such as aflatoxin, formed in maize prior to ensilage, or zearalenone, mainly accumulated at the end of the ripening process, have been shown in corn silage (*11*−*13*). The ubiquitous occurrence of *A. fumigatus* means that it can be found on almost any spoiled forage crop and presents a dual hazard from the ingestion of pathogenic spores and from potential mycotoxin production such as fumigaclavine A, fumigaclavine C, and several tremorgens (*14*).

No multiresidue method has been developed for the detection of mycotoxins in corn silage. High-performance liquid chromatography (HPLC) coupled with fluorescence detection has been used for the analysis of citrinin in corn silage (*15*). HPLC coupled with mass spectrometry was performed for the detection of deoxynivalenol and zearalenone (*16*), deoxynivalenol (*17*), and fumonisin B_1 (*18*) in corn.

The aim of our study was to develop a multimycotoxin analysis method based on a description of mycoflora in a corn silage. The developed method was applied to the survey of a silage during its 9 months of use (September 2004−May 2005).

MATERIALS AND METHODS

Corn Silage Selection and sample Collection. Samples of corn silages were collected on a farm located in Calvados (Lower Normandy, France). In this geographical area of 5500 km^2, 80% is devoted to agricultural activities, mainly open field farming and cattle breeding. The selected farm was a dairy farm characterized by 372 acres of cultivated land and a total of 205 cattle fed primarily by corn silage.

Samples were taken through the “trench-type” silo in a transect at three levels (low, middle, and high). For each level, two samples of 800 g were taken from a 15 cm depth. Each sample was homogenized in a blender; 100 g was randomly selected for the analysis of mycoflora, and the other part (700 g) was divided into 100 g aliquots and stored at −20 °C before the multimycotoxins analysis.

Mycological Analysis of the Silage. Preliminary investigations were carried out on a mature corn silage in July 2004 to isolate the potential toxigenic species and select the mycotoxins to be included in the multimycotoxin analysis method. To complete the list of potential *Fusarium* species, the cultivated soil where the corn grew was also studied according to the soil plates method of Warcup (*19*). Some *Fusarium* species are ubiquitous in soil and are actually able to infect growing crops and to produce mycotoxins such as zearalenone or trichothecenes. A second corn silage, monitored during a 9 month period, was studied from harvesting to its final use (September 2004−May 2005).

For each sample, 100 g of silage was suspended in 500 mL of sterile water containing sodium dodecyl sulfate (0.05%, w/v). After 1 h of magnetic shaking, 1 mL of each suspension was sprayed in a Petri dish (90 mm diameter) containing malt extract (1.5%)/agar (1.5%) medium (MEA) complemented with chloramphenicol (0.05%, w/v) following the soil plates method of Warcup (*19*). To limit proliferous fungi such as *Trichoderma* spp., MEA complemented with malachite green (0.0025%, w/v) was also used (*20*).

The plates were incubated at 24 and 37 °C. The identity of each strain, isolated and purified, was achieved through macro- and microscopic examinations (*21*−*27*). For the species belonging to the *Penicillium* genus, growth was also observed on two selective media, Czapek yeast autolysate agar (CYA) and 25% glycerol nitrate agar (G25N), and incubated at 5, 22, and 37 °C (*24*). *Fusarium* species were cultured on MEA and potato dextrose agar medium (PDA).

All of the purified strains were preserved on agar slants (MEA) at 4 °C.

Chemicals and Reagents. Eight mycotoxin standards (aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A, patulin, and zearalenone) were supplied by Sigma-Aldrich (St. Louis, MO). Stock solutions of 250 μg/mL were prepared in LC grade methanol from Chromanorm VWR Prolabo (Fontenay sous Bois, France) and stored at −20 °C in the dark. Diluted solutions were prepared immediately before use by diluting the stock solutions with mobile phase acetonitrile/water (10:90, v/v). The working solution was composed of the eight mycotoxins. It was prepared by combining suitable aliquots of each individual standard stock dilution to obtain each mycotoxin at 1.25 μg/mL.

Milli-Q quality water (Millipore, Bedford, MA) and all other chemicals of LC grade were obtained from Prolabo.

Purification assays used Oasis HLB (6 mL, 200 mg) cartridges purchased from Waters (Milford, MA).

Development of a Multimycotoxin Analysis Method. *Extraction of Mycotoxins from Corn Silage and Solid-Phase Cleanup.* A portion of 5 g of silage was weighed (fresh weight) in an Erlenmeyer flask. Mycotoxins were extracted with 100 mL of a mixture of methanol/water (80:20, v/v) with grinding by an Ultra-Turrax basic T25 homogenizer (IKA-Werke, Staufen, Germany). The slurry was shaken on a rotary shaker for 60 min at 100 rpm and centrifuged at 1500 rpm for 30 min (10 °C).

A volume of 10 mL of the supernatant obtained from previous centrifugation was diluted in 90 mL of Milli-Q water and was then purified through the OasisHLB cartridge, previously conditioned with 4 mL of methanol and equilibrated with 4 mL of Milli-Q water, at a flow rate of 1 mL/min. The cartridge was washed with 2 mL of Milli-Q water. Citrinin and ochratoxin A were eluted with 5 mL of methanol; aflatoxin B_1, gliotoxin, deoxynivalenol, and zearalenone were eluted with 5 mL of a mixture of methylterbutyl ether (MTBE)/methanol (90:10, v/v).

For recovery studies, silage was artificially fortified at 250 μg/kg in applying 1 mL of the working solution. The spiked sample was incubated for 2 h in the dark at 20 °C. Then it was extracted and purified as described above.

Detection of Mycotoxins by HPLC-MS. The eluted mycotoxins were evaporated to dryness under a stream of nitrogen, and the final residue was dissolved in 0.5 mL of a mixture of acetonitrile/water (10:90, v/v). This was filtered through Millex HV 0.45 μm and injected into the HPLC-MS. Liquid chromatography was performed using an Agilent Technologies series 1100 (Palo Alto, CA) quaternary pump coupled with an autosampler and a model SL mass spectrometric detector. The analytes were chromatographed at 40 °C on a 150 × 2.1 mm i.d., 5 μm, Zorbax SB-C_{18} column (Agilent Technologies) with a 1 mm Optiguard C_{18} precolumn. Mycotoxins were separated using gradient elution with acetonitrile as mobile phase A and water acidified with 0.5% acetic acid (pH 3) as mobile phase B. The gradient program was as follows: at time zero, 5% solvent A; linear gradient to 50% solvent A within 15 min and to 80% at time 25 min. The flow rate was 300 μL/min. The injection volume was 25 μL.

Mass spectrometry was performed on a quadrupole analyzer equipped with an electron spray ionization (ESI) source and operating in positive and negative modes. The parameters used for the mass spectrometer in all experiments were as follows: capillary voltage, 3.0 kV; solvent gas, 720 L/h; evaporation temperature, 350 °C; pressure of nebulization, 35 psi. The ions monitored are presented in **Table 1**. Full scan mass spectra were recorded to select the most abundant m/z value, and then selected ion monitoring (SIM) mode was used for the quantification.

Linearity was established by injecting increasing concentrations (triplicate) of the working solution (5, 10, 20, 50, 100, 200, and 500 μg/kg). Calibration curves were linear in the range studied, showing correlation coefficients of >0.99. Quantification and detection limits (LQ and LD, respectively) were determined by spiked samples based on signal-to-noise ratios of 10:1 for quantification and 3:1 for detection limit.

RESULTS AND DISCUSSION

Mycoflora Associated with Corn Silages. The study of silage and soil in July 2004 was a preliminary report to identify

Table 1. Monitored Ions in HPLC-MS

mycotoxin	RT[a] (min)	monitored ions (*m*/*z*)	quantification ion (*m*/*z*)	mode (SIM[b])
aflatoxin B_1	15.4	313, 335	313	+
citrinin	16.7	251, 273	251	+
deoxynivalenol	6.9	295, 297, 355	297	+
fumonisin B_1	15.1	722, 723, 724	722	+
gliotoxin	14.2	263	263	+
ochratoxin A	20.3	404, 405, 426	404	+
zearalenone	20.4	317, 318, 319	317	–

[a] Retention time. [b] Selected ion monitoring with positive (+) or negative (–) mode of ionization.

the mycotoxins to extract and analyze in the multimycotoxin method. This investigation allowed us to identify 24 fungal species and to select the mycotoxins to be included in the development of the multimycotoxin method. Therefore, the working solution was prepared with eight mycotoxins: aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A, patulin, and zearalenone.

The results from the monitoring of the silage mycoflora during 9 months are given in **Table 2**. During this monitoring we observed a limited fungal diversity with 20 various species, as well as the co-occurrence and the recurrence of some potential toxigenic strains. Among these fungi four species could be considered as major toxigenic: *A. fumigatus*, *A. parasiticus*, *F. verticillioides*, and *M. ruber*. These fungi, respectively, were considered as possible producers of gliotoxin, aflatoxins, fumonisins, and citrinin. These observations confirmed previous studies (*3*, *14*, *15*, *28*) showing in particular the presence of *A. fumigatus* and *M. ruber*. *P. roqueforti*, observed in the silage from February to April 2005, has been previously reported (*4*, *9*) as a recurrent species in silage. This fungus was able to produce various mycotoxins such as roquefortine, PR toxin, or patulin, which may cause neurologic disorders in ruminants (*29*, *30*). The presence of *P. roqueforti* in silage could be attributed to its growth at low values of pH and high levels of CO_2 (*25*). In our study other species were observed during several months and are known to be toxigenic: *Penicillium brevicompactum* (mycophenolic acid), *Penicillium purpurogenum* (rubratoxins),

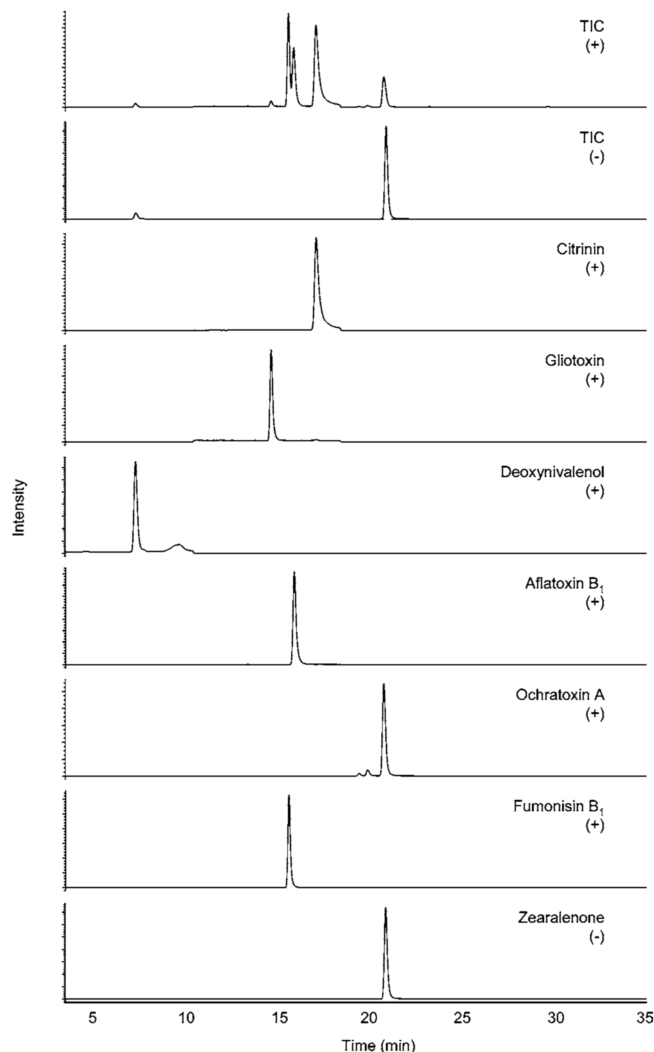

Figure 1. Total (TIC) and extracted ion chromatograms (XICs) of aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A, and zearalenone at 250 μg/L. The signs in parentheses below the mycotoxins correspond to the positive (+) or negative (–) mode used in SIM mode.

Table 2. Monitoring of Mycoflora in Corn Silage

Fungal strains	September	October	November	December	January	February	March	April	May
Arthrinium phaeospermum[a]									■
Aspergillus fumigatus[a]		■	■	■	■				■
Aspergillus parasiticus[a]			■	■	■		■		
Cladosporium cladosporioides						■			
Epicoccum nigrum						■		■	
Fusarium verticillioides[a]		■	■	■					
Geotrichum candidum	■	■	■	■	■	■			■
Monascus ruber[a]			■	■		■		■	
Mucor hiemalis	■	■	■	■		■		■	■
Penicillium aurantiogriseum[a]	■								
Penicillium brevicompactum[a]						■		■	
Penicillium crustosum[a]						■			
Penicillium expansum[a]	■			■					
Penicillium islandicum[a]				■					
Penicillium purpurogenum[a]		■	■		■	■	■	■	
Penicillium roqueforti[a]						■	■	■	
Penicillium viridicatum[a]					■				
Scopulariopsis brevicaulis							■		
Sordaria fimicola		■	■	■					■
Trichoderma viride[a]		■	■			■			

[a] Potentially toxigenic strains. The presence of fungi is indicated by shading.

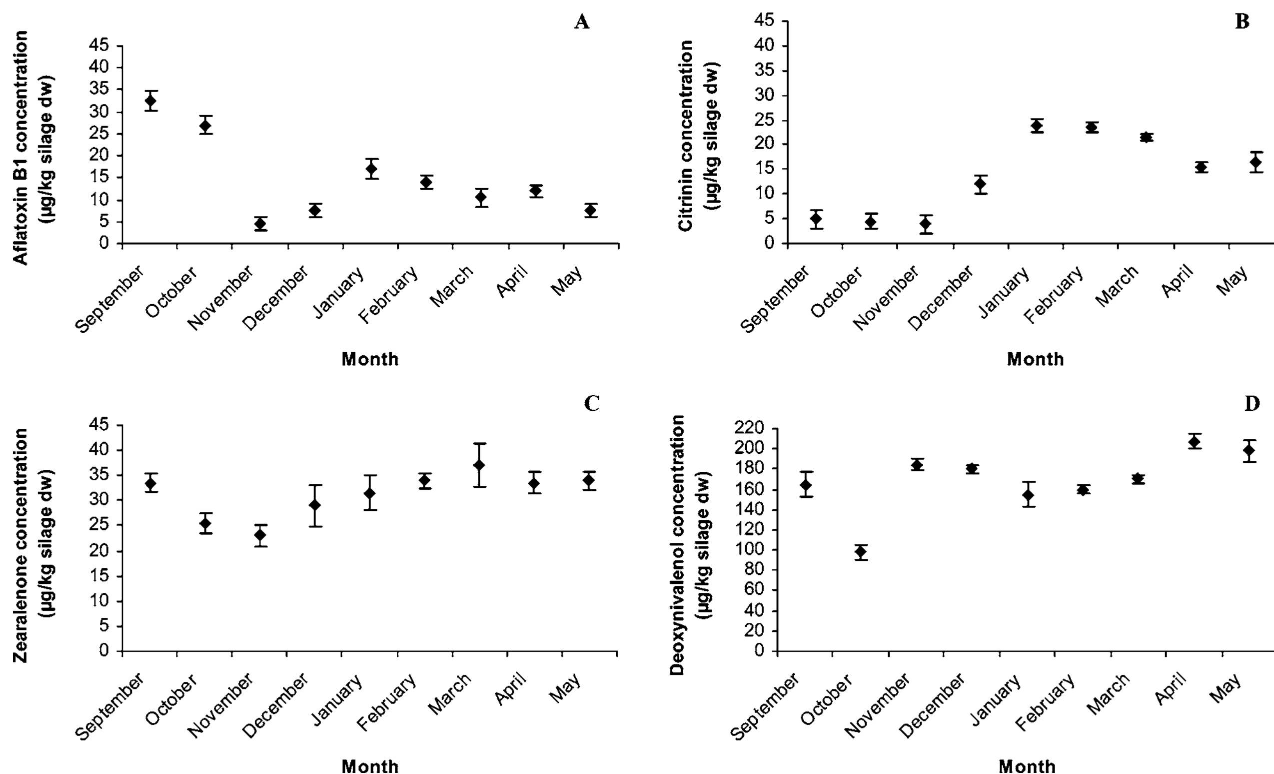

Figure 2. Monthly concentrations of mycotoxins in corn silage: (**A**) aflatoxin B_1; (**B**) citrinin; (**C**) zearalenone; (**D**) deoxynivalenol. For each mycotoxin and month, mean and standard deviation are represented.

and *Trichoderma viride* (trichotoxins) (*31*−*33*). *A. fumigatus* and *P. purpurogenum*, isolated during 5 and 6 months, respectively, could be considered as dominant strains in corn silage. Other fungi were occasionally identified such as *Arthrinium phaeospermum*, *Penicillium aurantiogriseum*, *Penicillium islandicum*, and *Penicillium viridicatum*. However, these fungal species had to be surveyed because of the potential production of nitropropionic acid (*Arthrinium phaeospermum*) (*34*), nephrotoxic glycopeptides and penicillic acid (*P. aurantiogriseum*) (*35*, *36*), tremorgenic mycotoxins such as penitrems (*Penicillium crustosum*), luteoskyrin (*P. islandicum*) (*37*), and citrinin and ochratoxin A (*P. viridicatum*) (*35*, *38*).

Some species such as *Scopulariopsis brevicaulis* and *Epicoccum nigrum* observed in silage contain allergens that could be suspected to play a role in respiratory tract allergy (*39*). Finally, we noted the presence of species distinguished by an optimum growth temperature of 25−30 °C (*22*): *Penicillium purpurogenum* and *P. islandicum* (two biverticillate Penicillia).

Multimycotoxin Analysis Method. A first protocol based on extraction and cleanup was tested on the working solution of eight mycotoxins. Because of the low recoveries (<30%), an optimization step was first developed by modifying successively the extraction parameters, such as nature, volume and acidification of the solvent [methanol/water (80:20, v/v), methanol/acetonitrile/water (25:25:50, v/v/v), acetonitrile/water (86:14, v/v), 50 or 100 mL, pH 7 or 3], grinding silage with Ultra-Turrax, filtration through Whatman filter paper (no. 3) or centrifugation, and the purification parameters, such as cartridge load step (acidification at pH 3 with acetic acid or no acidification of the extract), cartridge washing step (no or 2 mL of water), cartridge elution step [pure methanol or MTBE/methanol (90:10, v/v]. These preliminary assays allowed the optimized method, previously described.

Recoveries of the optimized method were, respectively, 65% (aflatoxin B_1, deoxynivalenol, ochratoxin A, and zearalenone), 70% (gliotoxin), and 80% (citrinin). These moderate rates could be explained by the matrix effect due to the presence of some interfering compounds such as pigments or lipids in corn silage. The detection and quantification limits for aflatoxin B_1, citrinin, and ochratoxin A were 1.5 and 5 ppb, respectively. The detection and quantification limits for deoxynivalenol, gliotoxin, and zearalenone were 6.5 and 20 ppb, respectively. This multimycotoxin analysis method based on three steps (extraction, cleanup, and HPLC-MS) allowed the determination of six major mycotoxins in corn silage. **Figure 1** shows chromatograms of the mycotoxins at 250 μg/L. Fumonisin B_1 was detected by HPLC-MS but still required optimization of the purification step. Patulin was neither extracted nor detected in the experimental conditions.

The optimized protocol was applied to the studied silage. **Figure 2** shows the monthly distribution of the four mycotoxins observed in two different samples (three HPLC analyses per sample) originating from the middle of the silage: aflatoxin B_1, citrinin, deoxynivalenol, and zearalenone. Ochratoxin A and gliotoxin were not detected in this experimental study. Aflatoxin B_1 was observed during the period of silaging (September) at a level up to 30 ppb, which could be explained by a contamination in the field and/or during silaging. This concentration decreased and stabilized at ∼10 ppb. A previous study demonstrated that aflatoxin formed in corn prior to ensilage has been shown to break down slowly in stored silage after 2 months (*11*). In our study, aflatoxin B_1 was observed at a low level and *Aspergillus parasiticus*, which also produces aflatoxins G, had been isolated during our survey. These observations showed the necessity to monitor aflatoxin B_1 and other aflatoxins (aflatoxins B_2, G_1, and G_2) in further studies.

Citrinin was detected from September 2004 to May 2005. Its concentration increased from 5 ppb (September) to 25 ppb in January and stabilized at ∼15 ppb in the following months. These data showed that citrinin could be considered to be a

storage mycotoxin. We can assume that its detection could be due to the presence of *M. ruber* isolated from the corn silage. Schneweis (*15*) also observed citrinin in silage samples at similar concentrations.

The toxin zearalenone produced by *Fusarium* species was also detected in the silage at levels between 23 and 41 ppb. The only *Fusarium* species identified during the silage monitoring was *F. verticillioides*, known as a fumonisins producer. The impossibility of identifying *F. graminearum* and *F. culmorum* in the corn silage could be attributed to the presence of some proliferous fungal strains during the mycoflora analysis.

Deoxynivalenol concentrations remained stable from November in corn silage at levels from 150 to 200 ppb. In a previous study (*40*) higher levels (mean of 2000 ppb) of deoxynivalenol were shown in 66% of corn silages analyzed in North Carolina.

In conclusion, this study resulted in the development of a multimycotoxin analysis method applied to corn silage, a complex matrix. It constitutes a tool to explore livestock and human exposure to mycotoxins in corn silage. The experimental study was a first monitoring of a corn silage and allowed the simultaneous determination of aflatoxin B_1, citrinin, deoxynivalenol, and zearalenone. Moreover, the mycoflora analysis showed the recurrence of potential toxigenic species such as *A. parasiticus*, *A. fumigatus*, *F. verticillioides*, *M. ruber*, and *P. purpurogenum*. These analytical and microbiological results suggested a possible chronic exposure to low levels of mycotoxins. Because these compounds are associated with many agricultural commodities, it is clearly possible that the handling of silage and forage crops might present a potential toxic hazard to those involved in these operations.

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