Wageningen Academic Publishers

Evaluation of zearalenone, α-zearalenol, β-zearalenol, zearalenone 4-sulfate and β-zearalenol 4-glucoside levels during the ensiling process.

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Received: 17 September 2013 / Accepted: 15 May 2014

RESEARCH ARTICLE

Abstract

Zearalenone-producing *Fusarium* species can contaminate maize before ensiling and cause reproductive problems in animals. Suspect feeds are only routinely analysed for zearalenone (ZEA), not considering other oestrogenic metabolites or masked derivatives. The aims of the present study were to monitor the levels of ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalenone-4-sulfate (ZEA-4S) and β-zearalenol-4-glucoside (β-ZOL-4G) in artificially contaminated maize silage and determine the effect of the ensiling process on these toxins. A laboratory silo model was designed using polystyrene bags filled with previously contaminated chopped whole-plant maize, stored in a dry and cool room and sampled at days 7, 45, 90, 120 and 127. ZEA, α-ZOL, β-ZOL, ZEA-4S and β-ZOL-4G levels were quantified by liquid chromatography – tandem mass spectrometry. Chemical and physical analysis indicated silage maintained good quality in all stages. pH was reduced favourably ($P<0.05$) from 4.69 to 3.80 during the preservation stage. Dry matter, moisture content and water activity did not vary from day 7 to 127. ZEA, α-ZOL, β-ZOL and ZEA-4S levels also did not change from day 7 to 127, indicating no significant degradation by the ensiling process or silage-native microbiota. This study suggests that ZEA levels remain invariable during the ensiling process, as well as the levels of its derivatives. The presence of highly oestrogenic metabolites, like α-ZOL and the masked ZEA-4S, which are not screened in the routine analyses, increases the overall toxicity of ZEA-contaminated silage.

Keywords: α-zearalenol, β-zearalenol, masked mycotoxins, silage, zearalenone

1. Introduction

Zearalenone (ZEA), chemically described as 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone, is a potent oestrogenic mycotoxin produced by several *Fusarium* species that can colonise grains and forages (Bennett and Klich, 2003). ZEA and its derivatives such as α- and β-zearalenol (α-ZOL and β-ZOL) are frequently found contaminating crops, grains, and other commodities (De Saeger *et al.*, 2003; Golinski *et al.*, 1988; Tiemann *et al.*, 2003). Their toxicity relies in their chemical structure which enables them to couple with the oestrogenic receptor, causing severe effects on the reproductive system of different animal species (D'Mello *et al.*, 1999). In addition to unaltered mycotoxins, there are conjugated and bound mycotoxins (referred to as 'masked mycotoxins') that can be formed by plants as a part of their defence mechanism against xenobiotics. These masked derivatives remain in the plant tissue, but are currently neither routinely screened for in food nor regulated by legislation (Berthiller *et al.*, 2013). Among these masked conjugates, are zearalenone-4-sulfate (ZEA-4S), which is a natural *Fusarium* metabolite produced in fungal cultures and has shown oestrogenic activity in a rat feeding test (Plasencia and Mirocha, 1991), and β-zearalenol-4-glucoside (β-ZOL-4G), that can be formed from ZEA by maize plants (Boutigny *et al.*, 2008).

Reproductive problems, probably related to the presence of ZEA in feed, have often been observed among bovine, porcine and equine livestock in farms located in the central

ISSN 1875-0710 print, ISSN 1875-0796 online, DOI 10.3920/WMJ2013.1638

region of Argentina. In several occasions, our laboratory has received samples of suspect feed (especially silage) for ZEA analysis. However, this toxin, when detected, was not always found in high levels (from 10 to 350 µg/kg) by methods like thin layer chromatography, ELISA or even high performance liquid chromatography (HPLC) coupled with fluorescence or uv detection (HPLC-UV) (González Pereyra *et al.*, 2008 and personal communication). The reproductive problems of these animals could be caused by the presence of masked ZEA derivatives and metabolites, such as α- and β-ZOL that were not screened in the routine analysis. Some of these masked derivatives, when entering the animal's digestive system, can be transformed back to ZEA (Berthiller *et al.*, 2013). In addition, α-ZOL is even more oestrogenic than ZEA. Monitoring only ZEA leads to the underestimation of the overall toxicity of feed.

Silage is one of the most important feed sources for cattle. In the last 20 years, the use of silage has spread in Argentinean fields, especially in intensive rearing operations (feedlots) and dairy farms. The ensiling process is based on the principle of preservation under anaerobic conditions, where the growth of lactic acid bacteria promotes a natural fermentation that lowers the pH to a level at which clostridia and most moulds are inhibited (Richard *et al.*, 2009). However, silage can be contaminated with mycotoxins before ensiling by toxigenic *Fusarium* species that contaminate plants in the field, or after ensiling by poor storage conditions that can lead to undesirable mould contamination and mycotoxin production (González Pereyra *et al.*, 2008, 2011).

There is a lack of information on whether the ensiling process can positively or negatively affect the levels of ZEA metabolites, and if the masked derivatives can be formed by the ensiled maize fodder. The aims of the present study were to monitor the levels of ZEA, α-ZOL, β-ZOL, ZEA-4S and β-ZOL-4G in artificially contaminated maize silage and to determine the effect of the ensiling process on these toxins.

2. Materials and methods

Production of zearalenone in rice culture

Zearalenone was produced *in vitro* according to Ezekiel *et al.* (2008) with some modifications. 50 g rice was added to 20 ml distilled water in 500 ml Erlenmeyer flasks. Water activity (a_W) was adjusted to 0.95 and flasks were sterilised in an autoclave for 15 min at 1 atm °P. After cooling overnight, 5 mm plugs of a 7-d-old ZEA-producing strain culture (*Fusarium graminearum* Z3639, teleomorph: *Giberella zeae;* deposited in the culture collection of the Department of Plant Pathology, Kansas State University, Manhattan, KS, USA) were inoculated into the flasks. Rice cultures were incubated for 21 days at 30 °C, manually shaking the flasks once per day during the first three days to enable spores to contaminate rice homogeneously. After incubation, cultures were autoclaved and dried in an oven at 60 °C to deactivate the fungal strain after ZEA production. Dry rice was ground in a laboratory mill and ZEA contamination was quantified by HPLC-UV according to Schollemberger *et al.* (2007). Briefly, 5 g of the rice culture were extracted with a mixture of acetonitrile:water, filtered through filter paper followed by an 0.22 µm pore diameter filter (Microclar, Buenos Aires, Argentina). The solvent was evaporated to dryness under a N_2 stream and the residue was dissolved in a volume of 500 µl of the mobile phase (a mixture of methanol:water, 70:30, v/v). Elution was performed at a flow rate of 1 ml/min. The injection volume was 50 µl. Fluorescence was recorded at excitation and emission wavelengths of 235 and 450 nm, respectively. The limit of detection (LOD) for ZEA was 3 µg/kg. Additionally, UV-detection at 235 nm was used with an LOD for ZEA of 2 µg/kg. The HPLC system consisted of a Hewlett Packard 1050 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 3395 integrator. The column was a C18 RP Phenomenex Luna (150×4.60 mm, 5 µm) (Phenomenex Inc., Torrance, CA, USA). *F. graminearum* Z3639 produced 133.7 µg/g ZEA in rice culture. A calibration curve using ZEA standard solutions of different concentrations (1,251, 475 and 299 ng/ml) was constructed and quantification of the samples' ZEA levels was done according to the area under the curve of the peak that eluted at a retention time of 5.3 min.

Contamination of maize

Chopped whole-plant maize ready to ensile was collected from a local farm when a silo was being made. The plant material was taken to the laboratory and fractioned in 8 sections of each 2 kg. These were contaminated with 11.22 g of the ground ZEA-producing *F. graminearum* Z3639 culture described previously (containing 1,500 µg ZEA), obtaining a concentration of approximately 750 ng ZEA/g silage. Three 25 g samples of uncontaminated material were taken to quantify any existing natural contamination with ZEA and/or derivatives.

Laboratory silo model

Eight polystyrene bags (5 kg capacity) were filled with 2 kg of the previously contaminated maize fodder. Plant material was compressed and air was extracted with a vacuum cleaner. Bags were sealed and stored in a dry and cool room and sampled at days seven, 45, 90, 120 and 127.

Sample collection and analysis

Two samples of 250 g were taken from each silo bag, taking material at random from different parts of the bag (top, middle, and bottom) and mixing them. Two silo bags were sampled at each time, obtaining a total of 4 samples (experimental replicates). Sampling days were chosen

in order to obtain representative samples of each of the different phases of the ensiling process. The first two silos were sampled on day 7 representing the start of the fermentation phase. After sampling, the remaining material of the two bags was discarded. The same procedure was followed for the subsequent samplings, except for day 120, where bags were left open after sampling and exposed to air for 24 h, and then sealed and sampled again at day 127. Samples taken at days 45 and 90 represent the stable phase, samples taken at day 120 the opening or cut of the silo for feed-out, and samples taken at day 127 the feed-out and start of the aerobic deterioration phase. a_W was evaluated using an Aqualab CX2 device (Decagon Devices Inc., Pullman, WA, USA) and pH was measured with a pH meter according to Ohyama *et al.* (1975). Samples were then dried in a forced air oven at 60 °C and dry matter (DM%) and moisture content (M%) were evaluated. ZEA, α-ZOL, β-ZOL, ZEA-4S and β-ZOL-4G levels were quantified by liquid chromatography – tandem mass spectrometry (LC-MS/MS) based on the method described by Sulyok *et al.* (2007) with the exception of the use of a more sensitive mass spectrometer (QTrap 5500 instead of QTrap 4000; AB Sciex, Framingham, MA, USA) and a different ratio of solvent to sample (5 g were extracted using 40 instead of 20 ml extraction solvent). Apparent recoveries were determined to be 95.5, 91.0, 80.1, 104.5 and 78.2% for ZEA, α-ZOL, β-ZOL, ZEA-4S and β-ZOL-4G, respectively, by analysing a spiked blank sample. The LODs, defined as 3× signal/noise, were calculated to be 0.4 µg/kg for ZEA, 3 µg/kg for α-ZOL, 4 µg/kg for β-ZOL, 0.3 µg/kg for ZEA-4S and 4 µg/kg for β-ZOL-4G.

Statistical analyses

Data were analysed by the general linear and mixed model (GLMM) using InfoStat (version 2012; University of Cordoba, Cordoba, Argentina) software. Chemical, physical and mycotoxin data were analysed by analysis of variance (ANOVA). Means were given with SE and were compared using the Fisher's protected least significant difference (LSD) test (P<0.05).

3. Results

Chemical and physical analyses of the ensiled maize indicated the laboratory silo model was appropriate and the ensiling process was successful since the silage maintained good quality in all stages. pH was reduced favourably (P<0.05) from 4.69 to 3.80 during the preservation stage (stable phase). DM%, M% and a_W did not vary significantly from day 7 to day 127, indicating there was no dry matter loss (Table 1).

The monitoring of ZEA and its derivatives by LC-MS/MS detected the presence of ZEA, α-ZOL, β-ZOL and ZEA-4S in all samples. β-ZEA-4G was not detected. Phase I metabolites (α-ZOL and β-ZOL) and the masked derivative ZEA-4S were detected after the contamination of silage and were most likely produced in the *F. graminearum* Z3639 rice culture used for contaminating maize. The levels of all ZEA metabolites were stable from day 7 to 127 indicating no significant degradation by the ensiling process or by the indigenous microbiota (Table 2). On the other hand, masked β-ZOL-4G was not formed in the maize fodder.

4. Discussion

In the present study, the levels of ZEA, α-ZOL, β-ZOL, ZEA-4S and β-ZOL-4G in properly stored maize silage were monitored and the effect of the ensiling process on these toxins was evaluated. The presence of mycotoxin contamination in silage is a common problem. An improperly made silo will normally produce silage of evident poor quality (bad smell, bad colour, visible mould contamination) that it is expected to contain certain levels of mycotoxins. However, sometimes mycotoxins can be found in properly stored, good quality-looking silage, especially *Fusarium* mycotoxins that come from field contamination and are not formed in the silo. This last case is what constitutes the problem, since the farmer usually does not suspect good quality silage to contain mycotoxins.

Table 1. Moisture (M%), dry matter (DM%), water activity (a_W) and pH mean values found in maize silage during an experiment using a laboratory silo model.

Sampling	M%±SE[1]	LSD[2]	DM%±SE	LSD	a_W±SE	LSD	pH±SE	LSD
Day 7	73.845±0.195	a	26.150±0.200	a	0.980±0.000	a	4.180±0.010	b
Day 45	74.94±0.960	a	25.055±0.965	a	0.980±0.000	a	4.130±0.020	c
Day 90	75.06±0.770	a	24.935±0.775	a	0.980±0.000	a	4.100±0.010	c
Day 120	75.06±0.080	a	24.93±0.080	a	0.975±0.005	a	3.815±0.015	d
Day 127	75.11±0.010	a	24.795±0.095	a	0.980±0.000	a	4.695±0.005	a

[1] SE = standard error.
[2] LSD = least significant difference test; different letters within the same column indicate statistically significant differences (P<0.05).

Table 2. Zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalenone-4-sulfate (ZEA-4S) and β-ZOL-4-glucoside (β-ZOL-4G) mean levels (µg/kg) found in artificially contaminated maize silage during an experiment using a laboratory silo model.

Sampling	ZEA		α-ZOL		β-ZOL		ZEA-4S		β-ZOL-4G
	Mean ± SE[1] µg/kg	LSD[2]	Mean ± SE µg/kg	LSD	Mean ± SE µg/kg	LSD	Mean ± SE µg/kg	LSD	Mean ± SE µg/kg
Natural contamination	2.0±0.00	a	<LOD[3]	a	<LOD[3]	a	<LOD[3]	a	<LOD[3]
Day 7	567.4±124.6	b	28.9±11.5	ab	35.7±3.2	b	17.8±4.3	b	<LOD
Day 45	773.9±162.9	b	18.4±4.4	ab	43.6±7.2	b	25.6±4.8	b	<LOD
Day 90	765.7±72.3	b	15.9±1.0	b	55.3±8.5	b	20.8±3.7	b	<LOD
Day 120	735.2±75.5	b	22.1±4.1	b	52.0±7.4	b	25.2±2.9	b	<LOD
Day 127	737.3±47.4	b	23.5±2.9	b	52.1±2.2	b	21.8±3.7	b	<LOD

[1] SE = standard error.
[2] LSD = least significant difference test; different letters within the same column indicate statistically significant difference ($P<0.05$).
[3] LOD = limit of detection for ZEA = 0.4 µg/kg; α-ZOL = 3.0 µg/kg; β-ZOL = 4.0 µg/kg; ZEA-4S = 0.3 µg/kg; β-ZOL-4G = 4.0 µg/kg.

A laboratory silo model was designed to standardise external variables difficult to control in field experiments (temperature, moisture, manipulation of the bags, plastic cover integrity, presence of animals and insects) without affecting conditions inside the silos. The silo model resulted effectively in reproducing the ensiling process with all its characteristics of the different phases, since pH was reduced to proper silo-bag levels (pH values below 4), a_W and M% were maintained and no dry matter was lost. These are all characteristics that are typical of good quality silage. DM%, M%, a_W and pH were monitored during the whole experiment obtaining values typical for each phase according (Oude Elferink *et al.*, 1999). It is well known that a low pH value is the crucial parameter (along with the lack of oxygen) that allows silage preservation. The pH was expected to decrease in the silo, starting from the moment of ensiling, and reaching and maintaining values between 3.8 and 4.2 during the stable phase, in order to allow a correct preservation of the ensiled material. After a silo is cut (in the case of trench-type silos) or the bag is opened for feed-out (in the case of silo-bags), the exposure of the material to oxygen breaks the anaerobiosis allowing spoilage microorganisms to grow and consume the lactic acid, thereby increasing the pH. Therefore, the increase of the pH from day 120 to 127 after the exposure of the silos to oxygen was expected.

The plant material to ensile was contaminated with a deactivated (autoclaved) culture of a ZEA-producing reference strain, to ensure that toxin formation did not continue in the silos. The detection of the phase I metabolites (α-ZOL and β-ZOL) and the masked derivative ZEA-4S after the contamination of silage indicated that they were produced in the *F. graminearum* Z3639 rice culture used to contaminate the maize and moderate levels of these toxins were detected in silage as early as day 7. In the present study, it was observed that the ensiling process conditions, as used in the experimental laboratory silos, did not affect ZEA levels, indicating no degradation or biotransformation to α-ZOL and β-ZOL took place by either the environmental conditions or the indigenous microbiota of this particular ensiled material. The biotransformation of ZEA by different microorganisms has been reported by several authors (Altalhi and El Deeb, 2009; Megharaj *et al.*, 1997; Yi *et al.*, 2011). The levels of all ZEA metabolites detected (α-ZOL, β-ZOL and ZEA-4S) were also stable from day 7 to 127 indicating no significant degradation. On the other hand, the masked β-ZOL-4G was not found in the maize fodder in any of the samples. The detection and quantification of ZEA derivatives, such as α-ZOL, β-ZOL and ZEA-4S is important, since, as observed in the present study, they can be produced in the field and are stable in silage thereby adding up to ZEA's oestrogenic effect. Phase I metabolites α-ZOL and β-ZOL were produced in relation to ZEA in a proportion of 1:32 and 1:15, respectively. The masked ZEA-4S was detected in levels lower than ZEA in a proportion of 1:32. Quantification of ZEA and all its derivatives would give a more realistic assessment of the real overall toxicity of contaminated silage. Currently, there are no regulatory limits established for the presence of ZEA and its derivatives in food or feed in Argentina. However, regulations established by the European Union can be used as a guidance for the acceptable levels (EC, 2006). For feeds, the EU established a limit of 2 µg/g ZEA for cereals and cereal-based feeds, 3 ng/g for maize products, 0.1 µg/g for piglets, 0.25 µg/g for mother and weaning pigs, and 0.5 µg/g for cattle, lamb, sheep and goat complementary feeds. However, none of the existing regulations include α- and β-ZOL or the masked derivatives of ZEA.

To conclude, the present study revealed that ZEA, α-ZOL, β-ZOL and ZEA-4S levels, when produced in the field, are invariable stable in the ensiled material during the whole ensiling process. Microbiota and conditions, such as temperature, a_W, low pH and lack of O_2 did not affect the levels of these toxins in any of the silage phases. A good ensiling process can inactivate mycotoxin-producing fungi. However, if moderate to high levels of ZEA and its derivatives are already present in the vegetal material before ensiling; it is very likely that these will not be reduced in the silo. The presence of highly oestrogenic metabolites like α-ZOL and the masked ZEA-4S, which are not screened in the routine analyses, increase the overall toxicity of ZEA-contaminated silage.

Acknowledgements

This study was supported by grants from CAPES/SPU No. 048/10, FONCyT-PICT No. 2008-02175, PICT-CNPq 35/08 and FONCyT-PICT No. 1607/2011.

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