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Liquid Chromatographic Determination of Fumonisins B_1, B_2, and B_3 in Corn Silage

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Corn silage was dried, ground, and then extracted with 0.1 M ethylenediaminetetraacetic acid. The filtrate was applied to a FumoniTest immunoaffinity column. Fumonisins were derivatized with naphthalene-2,3-dicarboxaldehyde, separated on a C_{18} liquid chromatographic column, and detected by fluorescence. The detection limits for fumonisin B_1, fumonisin B_2, and fumonisin B_3 were 50, 25, and 25 ng/g of dried silage, respectively. Recoveries of fumonisin B_1, fumonisin B_2, and fumonisin B_3 from wet and dried corn silage spiked over the range of 100−5000 ng/g averaged 91−106%. The method was applied to corn silage samples collected from the midwestern area of the United States during 2001−2002. Of 89 corn silage samples, fumonisin B_1, fumonisin B_2, and fumonisin B_3 were found in 86 (97%), 64 (72%), and 51 (57%) of the samples. The mean positive levels of fumonisin B_1, fumonisin B_2, and fumonisin B_3 were 615, 93, and 51 ng/g, respectively, in dried silage. This suggests that fumonisins may be frequent low level contaminants in corn silage.

KEYWORDS: Mycotoxin; fumonisin B_1; fumonisin B_2; fumonisin B_3; corn silage

INTRODUCTION

Corn silage is a popular feed source for ruminants and can constitute up to 60% of the dry matter in feedlot diets. Several mycotoxins are known to exist in corn, raising the possibility that they might survive ensiling and constitute a potential hazard to livestock (*1*, *2*). Several studies on the molds and their mycotoxins in silage have been conducted in Europe, especially for roquefortine C, type A trichothecenes, and zearalenone (*3*−*5*).

Even though most *Fusarium* species are unable to grow under anaerobic conditions in silage, these species are prevalent in corn and grass in the field before harvesting. *Fusarium* mycotoxins, in particular the trichothecenes (including deoxynivalenol), zearalenone, and fumonisins, are known to be toxic to animals and potentially could remain intact in corn- and grass-based silage (*6*).

Fumonisins are a structurally related group of water soluble mycotoxins mainly produced by *Fusarium verticillioides*, one of the species formerly known as *Fusarium moniliforme* and *Fusarium proliferatum*, the most prevalent molds associated with corn worldwide (*7*). The chemical structure of fumonisin B_1 is 2*S*-amino-12*S*,16*R*-dimethyl-3*S*,5*R*,10*R*,14*S*,15*R*-pentahydroxyeicosane with the C-14 and C-15 hydroxy groups esterified by a terminal carboxyl group of propane-1,2,3-tricarboxylic acid (tricarballylic acid); fumonisin B_2 is 10-deoxy fumonisin B_1; and fumonisin B_3 is 5-deoxy fumonisin B_1 (**Figure 1**). Among these compounds, fumonisin B_1 is the predominant form found in naturally contaminated corn and usually constitutes about 70% of the total amount of fumonisins present (*8*). These compounds have been implicated as the causative agents in several naturally occurring animal diseases including leukoencephalomalacia in horses and pulmonary edema in pigs (*9*). In controlled experiments, they are also hepatocarcinogenic to rats (*10*). Cardiovascular dysfunction from fumonisin B_1 administered to horses has been recently identified, which may contribute to the development of leukoencephalomalacia (*11*). The toxicity and carcinogenicity of fumonisins are likely due to disruption in sphingolipid methabolism (*12*). Epidemiological studies have also suggested that fumonisins may be partially responsible for the high incidence of esophageal cancer in people from the Transkei region of southern Africa, the Linxian region of China, and the northeast region of Italy (*13*). By contrast, cattle appear

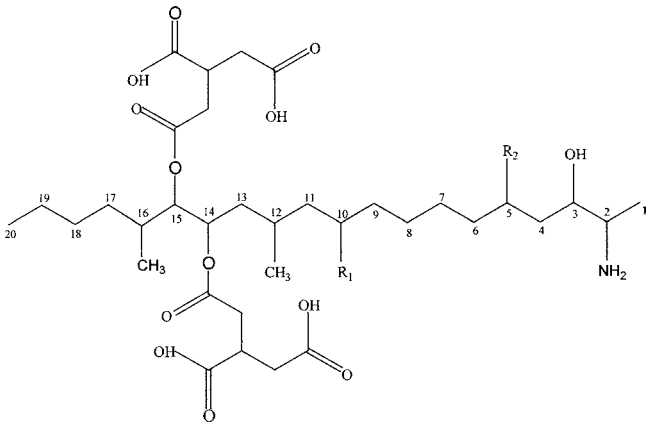

Figure 1. Chemical structures of fumonisin B_1, fumonisin B_2, and fumonisin B_3.

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to be among the more resistant species to fumonisins due to limited absorption and metabolism (*14*, *15*).

Recently, the Food and Drug Administration has announced guidance levels for total fumonisins levels in corn products for foods and feeds ranging between 2 and 4 ppm in human foods and 5 ppm up to 100 ppm in animal feeds depending upon the species and the proportion of the contaminated material in the total diet (*16*).

A number of analytical methods for the determination of fumonisins, mainly using liquid chromatography (LC), have been developed for analysis of corn and other cereals (*17*). Analysis of complex matrixes such as milk, sorghum syrup, forage grass, animal tissues, hair, feces, and urine for the presence of fumonisins has been attempted with some success (*15*, *18*−*23)*. Recently, immunoaffinity column (IAC) cleanup methods have been increasingly applied in surveys for determination of fumonisins in corn and corn-based foods (*24*). Even though significant advances have been made in analytical methodology for the fumonisins and the detection limits of currently available liquid chromatographic methods are below FDA guidelines, analytical problems still exist that hinder accurate and reproducible determination of the fumonisins in complex matrixes or substrates.

Yu et al. (*25*) investigated several mycotoxins such as those from *Alternaria alternata* (AAL TA toxin), deoxynivalenol, cyclopiazonic acid, fumonisin B_1, PR toxin, and zearalenone, respectively, from hay, hay silage, and mixed feed using immunoassay. According to their results, 13 of 25 hay and hay silage samples were contaminated with fumonisin B_1 ranging from 20 to 450 ng/g. However, these amounts were not verified by an established chemical method such as LC.

The aim of this study was to develop a sensitive, reproducible, and reliable analytical method to separate and quantify fumonisins from corn silage samples. High-performance liquid chromatography (HPLC) with fluorescence detection was used, and 89 corn silage samples from the midwestern United States, obtained during 2001−2002, were analyzed. Because no systematic survey of corn silage for fumonisins has yet been undertaken, the results provide useful information about the exposure of livestock to fumonisins from silage.

MATERIALS AND METHODS

Chemicals. Methanol (HPLC grade), acetonitrile (HPLC grade), 2-propanol, and acetic acid, were obtained from Fisher Scientific (Fairlawn, NJ). All reagents were of analytical grade. Ethylenediaminetetraacetic acid tetrasodium salt (Na_4EDTA) was purchased from Sigma (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde was purchased from Molecular Probes (Eugene, OR) and dissolved to a concentration of 0.25 mg/mL in methanol and stored at −20 °C. Deionized water (HPLC grade) was obtained using a Nanopure II Water System (Sybron/Barnstead, Boston, MA). Phosphate-buffered saline (PBS) was prepared as a solution of 1.183 g of disodium hydrogen phosphate, 0.23 g of sodium dihydrogen phosphate, 8.5 g of sodium chloride, and 0.2 g of sodium azide per liter of distilled water, adjusted to pH 7.5 with 1 M hydrochloric acid.

Analytical Standards. All fumonisins standards were graciously provided by Ronald Plattner and Stephen Poling (USDA, Peoria, IL). Fumonisin B_1, fumonisin B_2, and fumonisin B_3 standard solutions were prepared by dissolving 1 mg/mL in acetonitrile/water (1:1) and then keeping them at 4 °C for preparation of working solutions.

Samples. All corn silage samples were collected from the midwestern United States during 2001−2002. The sample size was ca. 1−2 kg. The samples were divided into three sections. Both end parts (ca. 10 cm length), which were exposed to air and were visibly molded for some samples, were separately collected for further analysis. All corn silage samples were dried for 48 h at 45−55 °C in a convection oven.

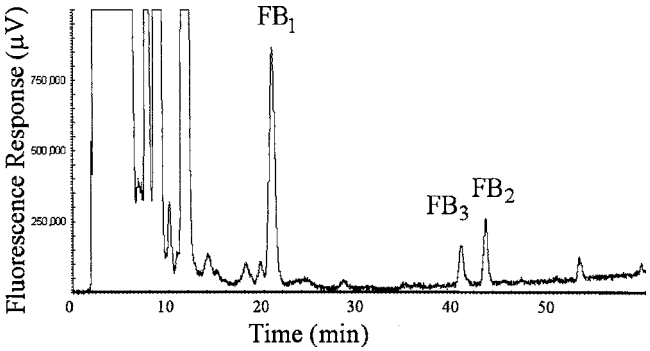

Figure 2. Liquid chromatogram of corn silage (sample no. 26) estimated to contain 354 ng of fumonisin B_1, 54 ng of fumonisin B_2, and 30 ng of fumonisin B_3 per gram of dried silage, respectively, after naphthalene dicarboxaldehyde derivatization.

The dried samples were ground to a fine consistency in a Stein Laboratory Mill (The Stein Corp., Atchison, KS) and stored at 4 °C prior to analysis.

Extraction of Fumonisins and IAC Cleanup. Twenty-five grams of dried silage was extracted with 200 mL of 0.1 M EDTA using a wrist action shaker (Burrell Corp., Pittsburgh, PA) for 1 h. The extract was filtered through a Whatman 2V filter (Whatman International Ltd., Maidstone, U.K.), and 10 mL of the filtrate, equivalent to 1.25 g of dried silage, was added to 10 mL of PBS buffer (pH 7.4) and mixed well. FumoniTest IACs (Vicam, Watertown, MA) were used to isolate fumonisins. Diluted sample extract was pushed through the column using gravity with a flow rate of approximately 1 drop/s. Columns were rinsed with 10 mL of PBS, and fumonisins were eluted with 6 mL of methanol into 8 mL capacity silanized amber vials with Teflon-lined screw caps. The extract was dried under a gentle stream of nitrogen at 60 °C and then derivatized with naphthalene dicarboxaldehyde.

Derivatization of Fumonisins with Naphthalene Dicarboxaldehyde. Fumonisins were derivatized with naphthalene dicarboxaldehyde using a slight modification of the method of Maragos and Richard (*18*). The dried extract was reconstituted with 150 μL of methanol, and 200 μL of 0.05 M borate buffer was added, followed by 300 μL of sodium cyanide (0.1 mg/mL water) and 300 μL of naphthalene-2,3-dicarboxaldehyde (0.25 mg/mL methanol). The solution was mixed well and reacted at 60 °C for 15 min. The derivatized solution was allowed to cool and then diluted with 1050 μL of acetonitrile/water (3:2).

Determination of Fumonisins by LC. The HPLC system consisted of a Spectra-Physics model P4000 pump and Rheodyne 7125 injector with a 20 μL sample loop (Cotati, CA), Alltech Allchrom (version 1.5) chromatography data system (Alltech Associates, Inc., Deerfield, IL), and Spectra-Physics model FL2000 fluorescence detector with excitation at 268 nm and emission at 470 nm. Chromatographic separations were achieved on a 250 mm × 4.0 mm i.d., 5 μm Wakosil II 5C18RS column (SGE Inc., Austin, TX) with a 15 mm × 3 mm i.d., 7 μm NewGuard RP-18 reversed-phase guard column (Applied Biosystems, Inc., Foster City, CA). The mobile phases were A, methanol/acetonitrile/water/2-propanol/acetic acid (100:52:73:20:4, v/v/v/v), and B, 2-propanol, pumped in a gradient of 100% A for 30 min and followed by a ramp to 70% A and 30% B at the end of 60 min. The flow rate was 1 mL/min. Under these conditions, retention times of fumonisins were 20.9 min for fumonisin B_1, 40.7 min for fumonisin B_3, and 43.3 min for fumonisin B_2. Calibration curves for the quantification of fumonisins were derived from peak areas of standard solutions (four concentration levels: 0.75, 1.5, 3, and 7.5 ng, double injection per level).

RESULTS AND DISCUSSION

We tried different extraction solvents and various types of solid phase extraction columns as well as an IAC to optimize the isolation of fumonisins from corn silage. Choosing a suitable extraction solvent and appropriate cleanup column was critical. Solvent combinations of acetonitrile/water (1:1, v/v) and methanol/water (3:1, v/v) in conjunction with C_{18} and strong

Table 1. Recovery of Fumonisins B_1, B_2, and B_3 from Spiked Dried and Wet Silage Samples

samples	spiking level (ng/g)	percentage recovery ± SD: fumonisin B_1	fumonisin B_2	fumonisin B_3
dried silage	100	89 ± 14[a]	108 ± 6	104 ± 5
	1000	107 ± 4	75 ± 4	80 ± 3
	overall average	98 ± 14	92 ± 19	92 ± 14
wet silage	100	133 ± 16	108 ± 6	105 ± 5
	1000	92 ± 4	79 ± 4	83 ± 3
	5000	93 ± 2	88 ± 3	85 ± 2
	overall average	106 ± 22	92 ± 13	91 ± 11

[a] Means and standard deviations were based on triplicate analyses.

anion exchange solid phase extraction columns have been commonly used to isolate fumonisins from corn and corn-based products when tested by HPLC (*26*, *27*). Solfrizzo et al. (*28*) recently reported that methanol/acetonitrile/water (1:1:2, v/v/v) coupled with an IAC gave better recoveries especially from corn-based food products. One tenth molar EDTA has been used to extract fumonisins from feces and white rice flour, which are very difficult matrixes to analyze (*29*, *30*). The use of IACs for cleanup was necessary to obtain clean extracts, particularly for corn flakes, sweet corn, and infant cereals (*28*, *31*, *32*). Thus, several different extraction solvent mixtures and cleanup methods have been evaluated for determination of fumonisins from maize and maize products as well as other matrixes.

Extraction and Cleanup for HPLC Methods Development. When we carried out extraction for fumonisins with either acetonitrile/water (1:1, v/v), methanol/water (3:1, v/v), or methanol/acetonitrile/water (1:1:2, v/v/v), the extracts were

Table 2. Occurrence of Fumonisins B_1, B_2, and B_3 in Corn Silages

sample no.	fumonisin B_1 (ng/g)	fumonisin B_2 (ng/g)	fumonisin B_3 (ng/g)	total fumonisins (ng/g)	fumonisin ratio B_2/B_1	fumonisin ratio B_3/B_1	sample no.	fumonisin B_1 (ng/g)	fumonisin B_2 (ng/g)	fumonisin B_3 (ng/g)	total fumonisins (ng/g)	fumonisin ratio B_2/B_1	fumonisin ratio B_3/B_1
1[a]	166 (33)	ND[b]	ND	166			47	921	100	56	1077	0.11	0.06
2[a]	464 (230)	42	16	522	0.09	0.03	48	1004	104	58	1165	0.10	0.06
3[a]	110 (19)	ND	ND	110			49	886	74	34	994	0.08	0.04
4[a]	792 (766)	114	30	936	0.14	0.04	50	579	68	27	673	0.12	0.05
5[a]	227 (22)	ND	ND	227			51	1061	136	65	1262	0.13	0.06
6[a]	216 (10)	ND	ND	216			52	1550	147	62	1759	0.09	0.04
7[a]	354 (39)	25	ND	380	0.07		53	1431	98	41	1569	0.07	0.03
8[a]	136 (9)	ND	ND	136			54	1561	173	71	1804	0.11	0.05
9[a]	606 (555)	73	24	703	0.12	0.04	55	900	66	29	994	0.07	0.03
10[a]	141 (14)	ND	ND	141			56[a]	1151 (770)	116 (210)	83 (90)	1350	0.10	0.07
11	464	26	ND	490	0.06		57	891	111	48	1051	0.12	0.05
12	367	ND	ND	367			58	1201	141	86	1428	0.12	0.07
13	341	26	10	377	0.08	0.03	59	799	109	39	948	0.14	0.05
14	145	ND	ND	145			60	1087	122	75	1283	0.11	0.07
15	150	ND	ND	150			61	834	110	37	981	0.13	0.04
16	141	33	ND	174	0.24		62	ND	ND	ND	ND	_	_
17	91	ND	ND	91			63	458	88	28	574	0.19	0.06
18	108	ND	ND	108			64	899	90	51	1040	0.10	0.06
19	72	26	ND	97	0.36		65[a]	ND (60)	ND (0)	ND (0)	ND	_	_
20	ND	ND	ND	ND			66	184	41	21	246	0.22	0.11
21	228	ND	ND	228			67	706	83	49	838	0.12	0.07
22	202	ND	ND	202			68[a]	354 (500)	66 (160)	24 (60)	444	0.19	0.07
23	215	ND	ND	215			69	1232	147	66	1446	0.12	0.05
24	21	ND	ND	21			70	257	ND	ND	257		
25	208	33	15	256	0.16	0.07	71[a]	183 (130)	ND (0)	ND (0)	183		
26	354	54	30	439	0.15	0.08	72	536	ND	ND	536		
27	474	200	56	730	0.42	0.12	73	734	81	32	847	0.11	0.04
28	153	27	ND	180	0.18		74	218	59	ND	277	0.27	
29	285	ND	ND	285			75	1397	129	174	1701	0.09	0.12
30	218	90	ND	309	0.41		76	68	37	ND	105	0.55	
31	366	21	ND	387	0.06		77	443	62	18	523	0.14	0.04
32	395	39	ND	434	0.10		78	1281	177	71	1529	0.14	0.06
33	351	33	ND	384	0.09		79	369	54	11	435	0.15	0.03
34	149	ND	ND	149			80	211	42	32	286	0.20	0.15
35	403	32	13	448	0.08	0.03	81	313	37	ND	350	0.12	
36	561	48	17	626	0.09	0.03	82	1094	139	64	1297	0.13	0.06
37	182	ND	ND	182			83	1295	165	70	1530	0.13	0.05
38	740	51	26	816	0.07	0.03	84	1582	266	116	1964	0.17	0.07
39	578	63	13	654	0.11	0.02	85	1824	268	112	2204	0.15	0.06
40	273	ND	ND	273			86	1418	73	41	1532	0.05	0.03
41	1009	130	56	1195	0.13	0.06	87	566	47	ND	613	0.08	
42	306	49	21	376	0.16	0.07	88	810	71	32	912	0.09	0.04
43	851	131	57	1039	0.15	0.07	89	1639	276	161	2075	0.17	0.10
44	506	53	28	588	0.10	0.06	no. of positives	86/89 (97%)	64/89 (72%)	51/89 (57%)	86/89		
45	1159	127	76	1358	0.11	0.06	mean	590	66	29	685	0.11	0.05
46	1296	177	100	1573	0.14	0.08	mean positives	615	93	51	707	0.15	0.08

[a] The toxin concentrations as determined by liquid chromatography–mass spectrometry and are indicated in parentheses. [b] Not determined.

highly colored. With these extraction solutions, large interfering peaks were seen on the chromatograms and very low recoveries of fumonisins were observed. IAC cleanup of such extracts was also ineffective due to clogging of the column. The mixture of acetonitrile or methanol and buffer solutions (pH 4.0 and pH 9.0) also gave lower recoveries. When we used pure water for the extraction solvent, the recoveries of fumonisin B_1 were about 60−70% from spiked silage samples. Use of 0.1 M EDTA in water, combined with IAC cleanup, reduced the presence of interfering matrix-related compounds (**Figure 2**).

Naphthalene Dicarboxaldehyde Derivatization of Fumonisins. Derivatization of fumonisins is commonly performed with *o*-phthaldialdehyde together with 2-mercaptoethanol. The main advantage of this derivatization mechanism is that the reaction is easy to perform in aqueous solutions under ambient temperature within a short reaction time and the derivative is highly fluorescent. However, 2-mercaptoethanol is toxic and has an unpleasant odor. Therefore, less toxic and odorless alternatives that result in more stable derivatives are highly desirable. The naphthalene dicarboxaldehyde derivatization of fumonisin Bs gave comparable results with those of *ortho*-phthaldialdehyde derivatization of fumonisins after some samples were compared using both derivatization methods. Recently, Lee et al. (*33*) evaluated naphthalene dicarboxaldehyde and *ortho*-phthaldialdehyde methods with sphingoid bases using the criteria of fluorescent intensity, elution profile, and stability. The naphthalene dicarboxaldehyde derivatives could reduce possible error due to differential fluorescence intensities of derivatized sphingoid bases and allowed the determination of sphingoid bases with the same reactivity and with sensitivity at least 10-fold higher than the *ortho*-phthaldialdehyde method.

The standard curve for fumonisin B_1 was linear over the range of 0.75−7.5 ng injected. The detection limit for the fumonisin B_1 in corn silage, defined as a signal-to-noise of 5, was approximately 50 ng/g and slightly lower for fumonisin B_2 and fumonisin B_3.

Recovery of Fumonisins from Wet and Dried Corn Silage. The recoveries of fumonisin B_1, fumonisin B_2, and fumonisin B_3 from dried and wet silage are shown in **Table 1**. A corn silage sample containing no detectable level of fumonisins was spiked at 100 and 1000 ng/g for dried and 100, 1000, and 5000 ng/g for wet corn silage samples. Results from the recovery studies showed that data at 100 ng/g spiked wet silage are questionable because the analytical recoveries were more than 100%, which could be due to the presence of trace fumonisins, below the detection limit 50 ng/g. Nevertheless, good recoveries were obtained at levels above 1000 ng/g from both dried and wet silage.

Survey of Fumonisins in Corn Silage. A total of 89 corn silage samples were collected and analyzed for fumonisin B_1, B_2, and B_3. Fumonisin B_1 was detected in 96.6% of the corn silage samples (**Table 2**) at levels of 21−1824 ng/g. The values in **Table 2** are based on dry weight. Values were much less in the corresponding wet silage when the moisture content (ca. 50−80%) was accounted for. Fumonisin B_2 and fumonisin B_3 were also detected, ranging from 21 to 276 and 10 to 160 ng/g, respectively. Some of the samples were confirmed by LC-MS. The ratios of fumonisin B_2/fumonisin B_1 and fumonisin B_3/fumonisin B_1 were generally around 0.15 and 0.08, respectively, for all corn silage samples. Our results are consistent with the ratios of fumonisin B_1, fumonisin B_2, and fumonisin B_3 observed in a previous report on corn (*34*). Visconti and Doko (*35*) reported the production of fumonisin B_1 and fumonisin B_2 from *Fusarium* strains isolated from corn, sorghum, and wheat. They found that the fumonisin B_2/fumonisin B_1 ratio ranged from 0.1 to 0.37 (average 0.22). Both ends of each silage part, which were visibly molded in some samples and separated from the bulk of the corn silage before testing, were combined and analyzed. Interestingly, the contamination level of this visibly molded material was found to be below 200 ng/g for fumonisin B_1. These data suggest that the presence of visible mold may not be indicative of fumonisin content.

Corn silage is known to be an extremely difficult matrix for analysis, because it contains not only the corn kernels but also other plant parts. In addition, it is fermented by microorganisms during ensilage. Although methods for determining the fumonisins in corn and corn products have been reported, a sensitive method that was suitable for rapid screening or extensive surveys for fumonisins from corn silage was needed. The HPLC method described here combines a 0.1 M EDTA extraction and an IAC cleanup procedure with naphthalene dicarboxaldehyde derivatization for sensitive detection of fumonisins.

Even though cattle do not appear to be extremely sensitive to fumonisin and our results were generally below the U.S. FDA guidance levels, most silage is made from annual crops and there can be variations in quality from year to year. More extended information is needed to assess the risk to livestock from silage containing mycotoxins. The determination of levels of other *Fusarium* mycotoxins such as deoxynivalenol, zearalenone, and T-2 toxin in silage will be important to provide information about the frequency of occurrence and the levels of these important mycotoxins present in the livestock environment, so that the assessments of risk can be made for animal feed.

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