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Research Article

Investigation of Naturally Occurring Fumonisin B_1 and Glycated Fumonisin B_1 in Korean Feedstuffs

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Abstract

Mycotoxins are potentially dangerous contaminants of livestock feeds. In this study, we measured the levels of fumonisin B_1 and glycated fumonisin B_1 in feedstuffs and then investigated the ability of the extrusion heating regimen to convert the most prevalent mycotoxin contaminant, fumonisin B_1, to a less toxic glycated form. All feed samples were analyzed with fully validated methods. All measured concentrations of fumonisin B_1 were below harmful thresholds, including European Union-recommended levels or US Food and Drug Administration action levels.

Because fumonisin B_1 was highly contaminated mycotoxin in our present investigation and fumonisin B_1 has been shown to be less toxic following Maillard type reaction with reducing sugar, we examined the formation of fumonisin B1 derivatives by Maillard reaction under extrusion process conditions. We employed a variety of tandem mass spectrometric methodologies to selectively detect fumonisin B_1 derivatives and to elucidate their structures partially. We found that compounds of *m/z* 736 were more likely artifacts or side reaction products rather than glycation products. N-(carboxymethyl) fumonisin B1 of *m/z* 780 and other major glycation products of *m/z* 794 and 810 were not detected, and only negligible amounts of methylene fumonisin B1 was found in 10 extruded feed samples. Therefore, either the tested extrusion conditions did not induce fumonisin B_1 glycation or the glycation products simply could not be detected by the method employed in this study.

Introduction

Mycotoxins are secondary fungal metabolites generated in various agricultural commodities during standing crop, harvest, and post-harvest stages. Mycotoxins have adverse health effects on livestock as well as humans. Out of more than 300 mycotoxins, Aflatoxins (AFs), Ochratoxin A (OTA), and Fumonisin Bs (FBs) are considered to present the most serious economic setback as well as risk factors to animals (Figure 1). *Fusarium* genera produce approximately 170 trichothecenes including DON, NIV, T-2, and, fumonisins. Ingestion of FB_1, which is frequently found in corn crops worldwide, has been associated with leucoencephalomalacia in both horses and rabbits, pulmonary edema in pigs, and nephrotoxicity and liver cancer in rats [1-4]. Interestingly, various reports have highlighted the possibility that FB_1 toxicity might be reduced by a Maillard type reaction of the FB_1 amino group with reducing sugar [5-7]. For example, Lu et al. [6] reported that a FB_1-fructose reaction mixture fed to diethyl nitrosamine-initiated Fischer344/N rats resulted in significantly less hepatic cancer promotion than FB_1 alone.

In this study, we had two aims. First, we generated data on contamination of feedstuffs by fumonisin B_1 as of 2013. All samples were analyzed with fully validated methods. Secondly, we studied the ability of a heating regime to reduce FB_1 toxicity, since glycation of FB_1 has been suggested as a decontamination process. We investigated the presence of FB_1 analogues transformed by glycation during extrusion processing from commercial feedstuffs.

The key to the second experimental set was to unambiguously identify glycated FB_1 from a model reaction [6]. Tandem mass spectrometry combined with liquid chromatography (LC-MS/MS) is a powerful technique for studying drug metabolites. In this technique, a parent compound undergoes alteration on a certain moiety, such as oxidation or conjugation, while maintaining its basic structure [7-9]. Likewise, FB_1 was assumed to experience modification of its amino group during the glycation process. Hence, a similar approach to the characterization of drug metabolites was adopted in this study.

Materials and Methods

Samples and Chemicals

Thirty samples of compound feed were collected at manufacturing sites and submitted to our laboratory by field officers. Acetonitrile (HPLC grade) was obtained from Fisher Scientific

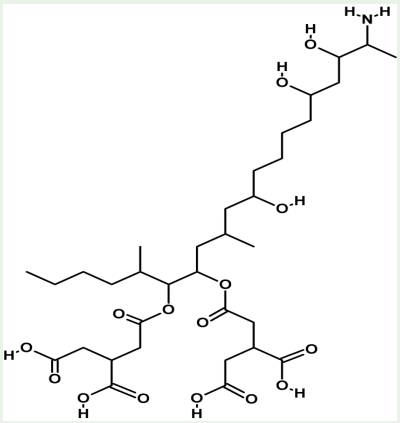

Figure 1: Chemical structure of carcinogenic fumonisin B_1.

(Schwerte, Germany), water for the HPLC mobile phase was distilled twice, and glucose and formic acid were obtained from Merck (Darmstadt, Germany). Fumonisin B_1 standards and internal standards, $^{13}C_{34}$-FB_1 was purchased from Biopure GmbH (Tulln, Austria). O-Phthaldialdehyde (OPA) 2-mercaptoethanol and a phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (Seoul, Korea). Fumoniprep (Rbiopharm, Glasgow, UK) were used for Immunoaffinity Column (IAC) cleanup. All other solvents and reagents were analytical grade or High Performance Liquid Chromatography (HPLC) grade as appropriate.

Quantification of FB_1 with LC-MS/MS after IAC Enrichment

Each 20-g feed sample with 40 ng ^{13}C-labeled FB_1 in 50 mL of ACN-MeOH-water (25+25+50, V/V/V) was shaken for 20 min on an orbital shaker in a 250-mL centrifuge bottle. The mixture was then centrifuged at 2500 × *g* for 2 min. The supernatant (10 mL) was mixed with PBS (40 mL) in a 100-mL flask and filtered with a microfiber filter. Filtrate (10 mL) was applied to each IAC by gravity and then washed with 10 mL PBS. Adsorbed FB_1 on IAC was eluted with 1.5 mL LC grade methanol by back-flushing at least three times. Sample volume was adjusted to 3 mL by passing 1.5 mL water through the column. Each 10-µL sample was injected into an Agilent HPLC 1200 series and separated over an A 150 × 4.6 mm Hypersil GOLD ODS column (5-µm particle size, Thermo Fisher, Runcorn, UK) at 35°C. The system was programmed to elute with a gradient of 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in ACN (mobile phase B) for a run time of 22 min at a flow rate of 0.9 mL/min according to the following schedule: 2 min, 95% A; 10 min, 50% A; 2 min, 0% A; 4 min, 0% A; 2 min, 95% A; and 4 min, 95% A. The HPLC was coupled through a turbo ion source to a Qtrap tandem mass spectrometer (AB 3200 Qtrap, Toronto, Canada). Infusion of each standard solution was conducted to optimize parameters for Electro Spray Ionization (ESI) acquisition. Parameters were 30 psi curtain gas, medium collision gas, 5.2-kv ion spray voltage, and 600°C turbo gas temperature. Additional parameters are listed in Table 1 and Recovery of fumonisin B_1 in feed are listed in Table 2.

Model Experiment of Fb_1 Incubation for Maillard Reaction

One mL solution containing 139 µM FB_1 and 100 mM d-glucose with 50 mM phosphate buffer (pH 7.4) was prepared in a 30-mL headspace vial and cooked for 48 h at 80°C [6]. The aqueous reaction mixture was lyophilized immediately after being cooled to room temperature. The resultant dry matter was reconstituted in 1 mL of acetonitrile-distilled water (50+50, v/v) and stored at 4°C until analysis.

Table 1: Optimized parameters of LC-MS/MS.

Mycotoxins	Q1	Q3	Declustering potential	Entrance potential	Collision energy
FB_1	722.4	334.5	91	8	53
	722.4	352.4	91	8	43
$^{13}C_{34}$- FB_1[a]	756.4	356.5	91	8	53
	756.4	374.5	91	8	43

$^{13}C_{34}$- FB_1[a]: Internal standard for fumonisin B_1.

Table 2: Recovery of fumonisin B_1 in feed.

Mycotoxins	Recovery (%)	Mean ± SD	%RSD	Spiked (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
FB_1 (N=3)	91.33	45.66±3.05	6.69	50	0.07	0.22
	98.26	245.66±26.54	10.80	250		

Detection of Fb_1 Analogues Derived by Maillard Reaction

All samples were filtered through a 4-µm microfiber filter before loading into a column. Precolumn derivatization with OPA was conducted according to a previously described methodology [1]. Chromatographic separation was performed on a Phenomenex ultracarb 5 ODS (150 × 4.6 mm, 5-µm, Torrance, CA, USA). Gradient elution was employed for satisfactory separation as follows: initial elution with solvent A (0.2% formic acid in water), a 5-min isocratic step, a linear increase in solvent B (0.2% in acetonitrile) over 35 min until the column was saturated with solvent B, and a final 10-min step. The flow rate was 300 µL/min.

To identify and measure FB analogues semi-quantitatively, precursor ion scans, neutral loss scans, product ion scans, and multiple reaction monitoring scans were performed with Qtrap combined with chromatographic separation. The optimized parameters for FB_1 were used equally for the detection of FB analogues. The sample preparation method described by Seefelder, et al. [5] was followed to determine FB_1 derivatives in extruded samples.

Results and Discussion

Contamination of Feedstuffs by Mycotoxins

The major ingredients of many livestock feeds are imported and then manufactured into various compound feeds in Korea. When main ingredients were imported, analysis-based certification of their quality is usually required by the trading company. Therefore, legislative framework or guidance implemented by the importing country is very important to ensure that feeds of poor quality are not circulated freely in the market. Regulation limits for AFB_1 and OTA are set to 20 and 50 µg/kg, respectively, in compound feeds in Korea, Table 3.

In the preliminary trial (n=30), we examined levels of FB_1 that remained unregulated as of 2013 in Korea. All compound feeds (n=30) were contaminated predominantly by FB_1. FB_1 was detected in all test samples at concentrations ranging from 52.45 to 1580.20µg/kg. FB_1 was evenly distributed irrespective of sample types such as pig, poultry and cattle feed. Therefore, we designed and carried out a pilot study to detect the glycation of FB_1 in extruded feeds, which has been suggested to be an effective decontamination process [13-15].

Table 3: Contamination of feeds by mycotoxins.

Mycotoxins	Products (sample no)	Positive (%)	Maximum (µg/kg)	Mean±SD (µg/kg)
FB_1	Cattle (n=10)	100.00	1580.20	528.07±298.88
	Pig (n=10)	100.00	1394.45	606.89±557.74
	Chicken (n=10)	100.00	936.62	368.23±406.73

Table 4: CAD fragmentation of reaction products in comparison to that of 722, FB_1

$[M+H]^+$ (m/z)	Retention (min)	$[M+H-nH_2O]^+$ / $[M+H-r-nH_2O]^+$	$[M+H-TCA^a-nH_2O]^+$ / $[M+H-TCA-r^b-nH_2O]^+$	$[M+H-2TCA-nH_2O]^+$ / $[M+H-2TCA-r-nH_2O]^+$	BC[c]
722	25.6	704, 686, 668	564, 546, 528, 510, 492	406, 388, 370, 352, 334, 316	299
736	22.8	718, 700, 682	578, 560, 542, 524, 506	420, 406, 384, 366, 348, 330	313
736	25.9	718, 700, 682	578, 560, 542, 524, 506 564, 546, 528, 510, 492	406, 388, 370, 352, 334, 316	299
780	26.6	762, 744, 726,	622, 604, 586, 568, 550	464, 446, 428, 410, 392, 374	299
794	26.7	776, 758, 740	636, 618, 600, 582, 564	478, 460, 442, 424, 406, 388	299
810	26.7	792, 774, 756	652, 634, 616, 598, 580	494, 476, 458, 440, 422, 404	299

TCA[a]: Tricarballylic acid side chain
r[b]: Mass adduction
BC[c]: Fragment ion representing backbone carbon chain.

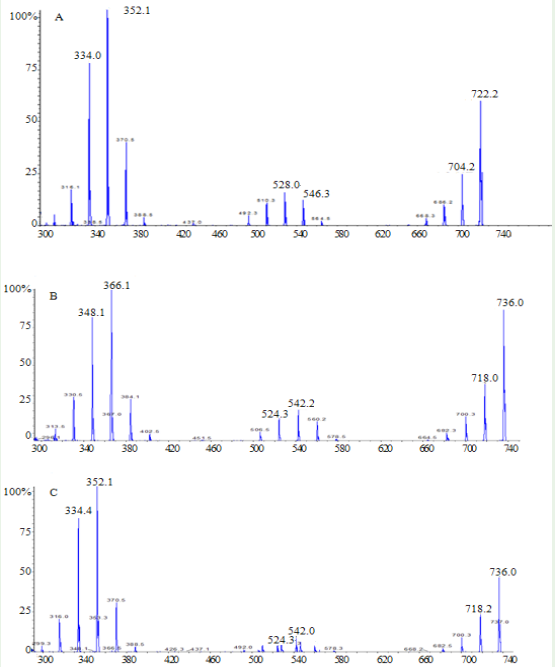

Figure 2: Product ion spectra of 722, fumonisin B1 (A), 736 at 22.9 min (B) and 736 at 25.9min (C).

The measured concentrations of fumonisin B_1 were below all harmful thresholds, such as European Union-recommended levels or U.S. Food and Drug Administration action levels [16,17]. However, as a short-term strategy, manufacturers should use only a restricted ratio of ingredients that represent highly potential hazards, such as corn gluten, and authorities must establish acceptable limits for such ingredients.

Selective Detection of Fb_1 Analogues from Reaction Mixtures Using Precursor Ion and Neutral Loss Scans

FB_1 generates a Schiff base when heated with glucose or fructose as in other glycation processes where a free amino group on an amino acid, peptide, or protein reacts with the carbonyl group of reducing sugars. N-(deoxy-N-fructose-1-yl) FB_1 was reported [6] to be a stable initial product, and presumably this Schiff base undergoes further oxidation during an extended heating time or at a higher temperature.

The mass spectrum of FB_1 shows prominent $[M+H]^+$ ions in comparison to sodium atoms, potassium atoms, or solvent adduct ions in acidic mobile phase. Product ion spectra of protonated FB_1, which are obtained at Q3 after collisionally-activated dissociation of selected mass 722, are depicted in Figure 2A. Fragmentation of protonated FB_1 brought three salient ion bunches to the product ion spectrum. Three consecutive losses of water from the molecule led to *m/z* 704, 686, and 668. Loss of one side chain and sequential dehydrations were represented by *m/z* 564, 546, 528, 510, and 492. In addition, the lowest *m/z* group, including *m/z* 370, 352, 334, and 316, can be explained by two Tricarballylic Acid (TCA) losses and subsequent water deletion (Table 4).

A precursor ion scan of *m/z* 352 representing two side chains losses and a concomitant dehydration was used to screen methyl esterifies FB_1 compounds or FB_1 impurities that have a different side chain from TCA. However, if Maillard type reaction of a free amino group occurs at the backbone, a neutral loss scan of two side chains, *m/z* 370, is more plausible than a precursor ion scan of 352 to determine the molecular weight of the protonated FB_1 glycation products in the incubation mixture. Several ions were detected by the neutral loss scan of 370, although two major peaks were presented in the chromatogram. The retention times of these ions were so similar that they were hardly resolved even with slow gradient elution. Therefore, these ions were selected for the multiple reactions monitoring mode. The chromatographic elution profiles of each ion were compared to avoid a misleading data interpretation that would most likely be due to in-source fragmentation, solvent, or metallic ion adducts added to the compound.

According to these results, *m/z* 736, 780, 794, and 810 were determined tentatively to represent modified FB_1s derived from incubation and have been previously reported as N-methyl FB_1, N-carboxymethyl FB_1, N-(3-hydroxyacetonyl) FB_1, and N-(2-hydroxy, 2-carboxyethyl) FB_1, respectively. However, only N-carboxymethyl FB_1 has been characterized substantially with mass spectral and NMR data. Therefore, we studied these four ions in detail to obtain structural information from the product ion spectra.

Identification of Fb_1 Analogues from Reaction Mixtures Using Products Ion Scans

The product ion scan of *m/z* 736 resulted in several peaks on the chromatogram with two different types of daughter ion spectra (Figure 2B and C). Peaks between 22 min and 25 min showed identical spectra where all major ions were shifted higher by 14 amu than those of FB_1, indicating that the modification occurs at the backbone. Because we could not speculate on which moiety is different from the original FB_1 with this information, the OPA derivatization method was employed to check whether the free amino group was affected. All these peaks disappeared following OPA derivatization of the sample, and the standard solution itself contained a considerable amount of these substances. However, the solution of FB_1 without glucose remained quantitatively constant, which means that these peaks did not arise from a Maillard reaction and that the substances were vulnerable to a nonenzymatic browning reaction as well. Therefore, these peaks are assumed to be modified FB_1 with an additive methylene group that has been reported previously as an impurity of the FB_1 standard [18].

Another group of peaks between 25 min and 27 min having $[M+H]^+$ 736 gave similar daughter ion spectra to that of FB_1. Ions accounting for the backbone, 388, and sequential dehydration had the same masses as those of FB_1, whereas ion groups that were assumed to have one or two TCA were shifted higher by 14 amu. These could be identified as FB_1 methyl esters that are methylated on one of four carboxyl groups of two TCA side chains. These peaks were phased out by OPA, which mean these compounds still had free amino groups. These peaks might be generated by a side reaction other than glycation.

The peak at 26.6 min with a precursor ion of *m/z* 780 gave a product spectrum identical to that of standard FB_1 except that all the ions were shifted higher by 58 amu, indicating that mass adduction had occurred in the backbone. Howard, et al. [13] identified this as N-(carboxymethyl) FB_1 and suggested that prominent fragmentation at *m/z* 132.1 arose from cleavage between C3 and C4 to yield stable $C_5H_9NO_3$. Two other *m/z* 794 and 810 previously identified by Lu, et al. [6] belong to the same category of mass spectra as those of N-(carboxymethyl) FB_1. Lu, et al. [6] characterized these unknown compounds as N-(3-hydroxyacetonyl) FB_1 and N-(2-hydroxyl, 2-carboxyethyl) FB_1, respectively, according to the general scheme of the Maillard reaction. Now we present supportive additional information on diagnostic product ions, 146.2 of N-(3-hydroxyacetonyl) FB_1 and 162.1 of N-(2-hydroxyl, 2-carboxyethyl) FB_1 corresponding to 132.1 of FB_1.

Detection of Fb_1 Analogues from Real Extruded Samples Using Multi Reaction Monitoring (MRM)

We attempted to measure glycated FB_1 from real extruded feeds using the sample preparation method of Seefelder, et al. [5] and even after only extraction without SPE clean-up. N-(carboxymethyl) FB_1 and other glycation products were not detected in 10 samples. Only negligible amounts of methylene FB_1 were found. These results imply that conventional extrusion conditions, including temperature, pressure, and reactants with reducing groups, were not effective at generating glycated FB_1. However, the possibility that FB_1 underwent glycation successfully but was not extractable in bound forms cannot be excluded, since there is much evidence that thermal activation enables FB_1 to bind to food components such as starch and protein via FB_1's two TCA side chains [15,19].

References

1. Shephard G, Sydenham E, Thiel P, Gelderblom W. Quantitative determination of fumonisin B1 and B1 by high-performance liquid chromatography with fluorescence detection. J Liq Chromatogr. 1990; 13: 2077-2087.
2. Hopmans E, Murphy P. Detection of fumonisin B1, B2, and B3 and hydrolysed fumonisin B1 in corn-containing foods. J Agric Food Chem. 1993; 41: 1655-1658.
3. Ueno A, Sugiura Y, Wang DS, Lee US, Hirooka EY, Hara S, et al. A limited survey of fumonisins in corn and corn-based products in Asian countries. Mycotoxin Res. 1993; 9: 27-34.
4. Bucci TJ, Hansen DK, Laborde JB. Leukoencephalomalacia and hemorrhage in the brain of rabbits gavaged with fumonisin B1. Nat Toxins. 1996; 4: 51-52.
5. Seefelder W, Hartl M, Humpf HU. Determination of N-(carboxymethyl) fumonisin B1 in corn products by liquid chromatography/electrospray ionization--mass spectrometry. J Agric Food Chem. 2001; 49: 2146-2151.
6. Lu Y, Clifford L, Hauck CC, Hendrich S, Osweiler G, Murphy PA. Characterization of fumonisin B1-glucose reaction kinetics and products. J Agric Food Chem. 2002; 50: 4726-4733
7. Poling SM, Plattner RD, Weisleder D. N-(1-deoxy-d-fructos-1-yl) fumonisin B1, the initial reaction product of fumonisin B1 and d-glucose. J Agric Food Chem. 2002; 50: 1318-1324.
8. Clarke NJ, Rindgen D, Korfmacher WA, Cox KA. Systematic LC/MS metabolite identification in drug discovery. Anal Chem. 2001; 73; 430-439.
9. Hsu FF, Turk J, Thukkani AK, Messner MC, Wildsmith KR, Ford DA. Characterization of alkylacyl, alk-1-enylacyl and lyso subclasses of glycerophosphocholine by tandem quadruple mass spectrometry with electrospray ionization. J Mass Spectrum. 2003; 38: 752-763.
10. Lafaye A, Junot C, Ramounet-Le Gall B, Fritsch P, Tabet JC, Ezan E. Metabolite profiling in rat urine by liquid chromatography/electrospray ion trap mass spectrometry. Application to the study of heavy metal toxicity. Rapid Commun Mass Spectrum. 2003; 17: 2541-2549.
11. Horwitz W. Official methods of analysis of AOAC international. 18th edn. Gaithersburg (MA): Association of official analytical chemist. 2005.
12. Lu Z, Dantzer W, Hopmans E, Prisk V, Cunnick J, Murphy P, Hendrich S. Reaction with fructose detoxifies fumonisin B1 while stimulating liver-associated natural killer cell activity in rats. J Agric Food Chem. 1997; 45: 803-809
13. Howard P, Churchwell M, Couch L, Marques M, Doerge D. Formation of N-(carboxymethyl) fumonisin B1 is following the reaction of fumonisin B1 with reducing sugars. J Agric Food Chem. 1998; 46: 3546-3557.
14. Lemke SL, Ottinger SE, Ake CL, Mayura K, Phillips TD. Deamination of fumonisin B1 and biological assessment of reaction product toxicity. Chem Res Toxicol. 2001; 14: 11-15.
15. Norred WP, Riley RT, Meredith FI, Poling SM, Plattner RD. Instability of N-acetylated fumonisin B1 (FA1) and the impact on inhibition of ceramide synthase in rat liver slices. Food Chem Toxicol. 2001; 39: 1071-1078.
16. Köppen R, Koch M, Siegel D, Merkel S, Maul R, Nehls I. Determination of mycotoxins in foods: current state of analytical methods and limitations. Appl Microbiol Biotechnol. 2010; 86: 1595-1612.
17. Zhang Y, Caupert J, Imerman PM, Richard JL, Shurs GC. The occurrence and concentration of mycotoxins in U.S. distillers dried grains with solubles. J Agric Food Chem. 2009; 57: 9828-9837.
18. Josephs JL. Detection and characterization of fumonisin mycotoxins by liquid chromatography/ electrospray ionization using ion trap and triple quadruple mass spectrometry. Rapid Commun Mass Spectrum. 1996; 10: 1333-1344.
19. Seefelder W, Knecht A, Humpf HU. Bound fumonisin B1: analysis of fumonisin- B1 glyco and amino acid conjugates by liquid chromatography-electro spray ionization-tandem mass spectrometry. J Agric Food Chem. 2003; 51: 5567-5573.