Liquid Chromatographic Determination of Fumonisin B₁ and B₂ in Corn by Precolumn Derivatization with 4-(*N,N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F)

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A new, sensitive and rapid method has been developed for the analysis of fumonisin B₁ and fumonisin B₂ in corn. Fumonisin B₁ and fumonisin B₂ which are the major metabolites produced by the fungi *Fusarium moniliforme* and *Fusarium proliferatum*, have been implicated in human esophageal cancer and induced pulmonary edema in pigs and leukoencephalomalacia in horses. Fumonisin B₁ and fumonisin B₂ were extracted from spiked corn with methanol-water (3:1) and then cleaned up by strong anion-exchange (SAX) column chromatography. Fumonisin B₁ and fumonisin B₂ were eluted from the SAX column with 1% acetic acid in methanol and quantitated via gradient elution HPLC using precolumn derivatization with 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) and fluorescence detection. The minimum detectable concentration in corn was 0.01 µg/g. Recovery values for fumonisin B₁ and fumonisin B₂ in spiked corn averaged 92 and 90%, respectively, at the level of 0.5 µg/g.

Key words: fumonisin B₁; fumonisin B₂; 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F); high performance liquid chromatography

Fumonisin are mycotoxins produced by fungi, *Fusarium moniliforme* and *Fusarium proliferatum*, commonly found on corn. It has been suggested that fumonisins isolated from *F. moniliforme* cultures exhibit cancer-promoting activity in rats¹. They have also been found to cause equine leukoencephalomalacia in horses^{2, 3} and pulmonary edema in pigs⁴. The occurrence of *F. moniliforme* has been correlated with human esophageal cancer risk in Southern Africa⁵.

For determination of fumonisins in foods and feeds, several high performance liquid chromatographic (HPLC), gas chromatographic (GC) and thin-layer chromatographic (TLC) methods have been developed⁵⁻⁹. Among these methods, HPLC methods with fluorescence detection after derivatization with *o*-phthalaldehyde (OPA)⁷ and fluorescamine⁶ have been used by many researchers. The OPA derivative formed is,

however, known to be unstable and must be injected immediately into the liquid chromatograph column to get reproducible data. The fluorescamine derivative showed two peaks for fumonisin B₁ (FB₁) on the HPLC chromatogram, which is undesirable for rapid analysis.

Recently, sensitive methods for determination of fumonisins with 4-fluoro-7-nitrobenzofurazan (NBD-F) in corn⁸ and (9-fluorenylmethyl) chloroformate (FMOC) in rodent feed⁹ have been developed. These two fluorescent derivatives were more stable than the OPA derivative, but there are still some problems; that is, the NBD-F derivative was not stable enough for automatic analysis with an autoinjector, while the reaction product with FMOC should be washed with pentane before analysis to remove excess reagent in order to ensure reproducibility of data, which is time-consuming.

We have examined the feasibility of derivati-

zation of FB₁ and fumonisin B₂ (FB₂) with 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) in order to develop a new analytical method for measuring FB₁ and FB₂ in corn by using gradient elution HPLC with fluorescence detection.

Materials and Methods

Apparatus

The gradient HPLC system consisted of two JASCO 880-PU pumps (Tokyo, Japan), a Shimadzu Model SIL-9A autoinjector (Kyoto, Japan) and a Hitachi Model F-1000 fluorescence detector (Tokyo, Japan). A guard column was placed between the autoinjector and the 15 cm × 4.6 mm, 5 μm, TSKgel ODS-80Ts (Tosoh, Tokyo, Japan) column. The integrator used was a Shimadzu Model C-R5A. The Bond-Elut SAX column (Varian, CA90710, U.S.A.) was used for clean-up of corn sample extracts. A coffee mill SCM-40A (Shibata, Tokyo, Japan) was used for grinding corn samples.

Reagents

Organic solvents were of HPLC grade from Merck-Cica (Tokyo, Japan). Water was distilled and deionized before use. The derivatizing reagent was prepared by placing 5 mg of DBD-F (Tokyo Kasei Kogyo, Tokyo, Japan) in 1 ml of acetonitrile.

The fumonisin B₁ and B₂ standards were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Standard stock solutions of FB₁ and FB₂ with concentrations of 10 μg/ml were prepared in methanol and stored at 4°C. The stock solutions were evaporated to dryness under reduced pressure before use and the residue was taken up in 0.1 M borate buffer containing 1 mM EDTA (pH 9.3) to obtain working standards for precolumn derivatization.

Samples

Whole corn was supplied by Japan Grain Inspection Association, General Research Laboratory.

Extraction and purification of fumonisins

Samples were ground to uniform consistency using a coffee mill, and 10 g was extracted with 50 ml of methanol-water (3:1) by shaking vigor-

ously in a 200 ml glass flask for 15 min. The solution was filtered through Whatman No. 5 filter paper. A 10 ml portion of the filtered extract was passed through a Bond-Elut SAX column at a flow rate of 2 ml/min. A 3-ml capacity Bond-Elut SAX column was previously washed with 5 ml of methanol followed by 5 ml of methanol-water (3:1). The column was then washed with 8 ml of methanol-water (3:1), followed by 3 ml of methanol. The fumonisins were eluted from the SAX column with 10 ml of 1% acetic acid in methanol. The eluate was evaporated to dryness at 50°C and the residue was dissolved in 0.2 ml of 0.1 M borate buffer containing 1 mM EDTA (pH 9.3) and used for precolumn derivatization.

Precolumn derivatization

For the precolumn derivatization, 50 μl of either cleaned up sample extract or fumonisin working standard was added to a 1.5 ml tightly capped Eppendorf tube. Then 50 μl of 0.1 M borate buffer containing 1 mM EDTA (pH 9.3) and 50 μl of DBD-F derivatizing reagent were added to each tube. The tubes were heated at 60°C in an aluminum block for 60 min in the dark and then cooled with ice. A 100 μm portion of each reaction mixture was placed into a 2 ml autosampler vial.

Gradient HPLC

The mobile phases were (A) 0.05 M sodium dihydrogenphosphate-methanol (1:1) and (B) acetonitrile-water (75:25). Each mobile phase was degassed by sonication.

The initial eluate was obtained with 100% A. The gradient program was ramped from 0% B to 15% B in 5 min, then increased to 90% B in the next 13 min, and this was held for the next 7 min. The total analysis time was 25 min. The LC column was TSKgel ODS-80Ts (4.6 mm × 150 mm) and was maintained at 40°C with a flow rate of 0.8 ml/min. The fluorescence derivatives were detected at the excitation and emission wavelengths of 450 nm and 590 nm, respectively. The injection volume was 50 μl.

Results and Discussion

Examination of fluorescent derivatization

DBD-F was developed by Toyo'oka *et al.*¹⁰⁾ as a

Fig. 1. Reaction of DBD-F with fumonisins

Fig. 2. Effects of reaction time on derivatization of FB₁ and FB₂ with DBD-F at 60°C. Each point represents the mean $\pm$ S.E. from triplicate determinations.

fluorescent reagent for the determination of amino acids with primary and secondary amino groups. FB₁ and FB₂ react with DBD-F to yield fluorescent derivatives quantitatively under alkaline conditions (Fig. 1). We used 0.1 M borate buffer containing 1 mM EDTA (pH 9.3) as an alkaline buffer by reference to their report¹⁰. The reaction rate increases with increasing reaction temperature. However, fumonisins are degraded at high temperature and by alkali. Therefore, we employed a reaction temperature of 60°C, which was confirmed not to cause degradation. The effect of reaction time on the derivatization of FB₁ and FB₂ was studied (Fig. 2). Constant fluorescence intensities were observed at reaction times of 60 min or more.

Examination of HPLC conditions

We studied several HPLC conditions to separate the fumonisin DBD-F derivatives (DBD-fumonisin) from impurities. It was found that the most suitable LC column was TSKgel ODS-

80Ts (4.6 mm $\times$ 150 mm). Two mobile phases of (A) 0.05 M sodium dihydrogenphosphatemethanol (1:1) and (B) acetonitrile-water (75:25) were selected and used according to the gradient program described in Materials and Methods. The maximum excitation and emission wavelengths of the fluorescent derivatives were 450 nm and 590 nm, respectively. Therefore, we monitored these wavelengths.

Examination of extraction and purification of fumonisins from corn

The method for clean-up of FB₁ and FB₂ reported by Shephard *et al.*⁷ was modified. We used a first sample volume of 10 g with 50 ml of (CH₃OH-H₂O (3:1, v/v)) as the extraction solvent and 10 ml of 1% acetic acid in methanol for elution from the Bond-Elut SAX cartridge.

Stability and characteristics of DBD-fumonisin

The DBD-F derivative is much more stable than the NBD-F derivative¹⁰. Stability of the DBD-fumonisin was studied at 4°C in the dark (Fig. 3). The DBD-fumonisin were very stable for at least 7 days (168 hr) and were also stable for at least 2 days at room temperature (data not shown). The NBD-F derivatives were stable for 30 hr at 4°C in the dark. The greater stability of DBD-fumonisin made it possible to store the derivative for a few days, if necessary.

Further, the NBD-F derivatives show many unknown peaks derived from the reagent as compared with the DBD-F derivatives, making interpretation more difficult.

Standard curves for analysis of standard fumonisins

The standard curves of FB₁ and FB₂ were linear from 50 ng/ml to 50 μ g/ml. Figure 4 shows the standard chromatograms of FB₁ and

FB₂. The peaks of FB₁ and FB₂ were clearly separated from solvent peaks.

Recovery test from sample corns

The DBD-F derivatization method was applied to the determination of FB₁ and FB₂ in ground corn. Figure 5 shows typical HPLC chromatograms of a cleaned-up extract from a corn sample and a spiked sample. The peaks corresponding to FB₁ and FB₂ were clearly resolved from the background peaks of blank samples.

Fig. 3. Stability of DBD-F derivatives of FB₁ and FB₂ (1 ppm each)

Each point represents the mean $\pm$ S.E. from triplicate determinations.

Analytical recoveries in the present method were assessed by adding fumonisins to corn samples at the levels of 0.25 μ g/g and 0.5 μ g/g for both FB₁ and FB₂. Mean recoveries from the corn samples based on triplicate analyses were $88 \pm 6.3\%$ for FB₁ and $85 \pm 7.2\%$ for FB₂ at the level of 0.25 μ g/g, and $92 \pm 6.9\%$ for FB₁ and

Fig. 4. A typical chromatogram of standard FB₁ and FB₂ (33 ng each) after DBD-F derivatization

Fig. 5. Typical chromatograms of blank corn extract (A) and extract of corn spiked with 0.5 μ g/g of both FB₁ and FB₂ (B) after derivatization with DBD-F

90±5.1% for FB₂ at the level of 0.5 µg/g. The detection limits for FB₁ and FB₂ were about 0.01 µg/g (signal-noise ratio, 3:1).

This method was also used to analyze FB₁ and FB₂ in 10 naturally contaminated corn samples. The levels of fumonisins varied from 0.028 µg/g to 0.56 µg/g for FB₁ (0.034, 0.045, 0.028, 0.090, 0.073, 0.068, 0.034, 0.227, 0.560, 0.089 µg/g) and from 0.013 µg/g to 0.31 µg/g for FB₂ (0.013, 0.018, 0.015, 0.025, 0.018, 0.019, 0.016, 0.080, 0.310, 0.030 µg/g). These levels are less than the FB₁ and FB₂ levels reported in U.S.A. and other countries^{11)~14)}.

Comparison with the OPA method and the DBD-F method

Shephard *et al.* developed a very sensitive HPLC method, using fluorescence detection after derivatization with OPA⁷⁾. This method appeared to work with corn samples, but the fluorescent derivative formed was unstable and had to be applied immediately (within 1 or 2 min) to the liquid chromatography column. Further, in the OPA method, impurities in the corn extract tended to interfere with the peak of FB₁ at low levels (less than 0.25 µg/g).

The DBD-F method is as sensitive as the OPA method, and it is possible to autoanalyze DBD-fumonisin at room temperature because the DBD-fumonisin is highly stable. In addition, impurities in corn extract cause essentially no interference.

Conclusion

This method using DBD-F is a new, simple and reproducible method for the analysis of FB₁ and FB₂ in corn samples. The DBD-F derivative is one of the most stable derivatives among those reported so far. This method makes possible the use of an autosampler for analyzing many samples.

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