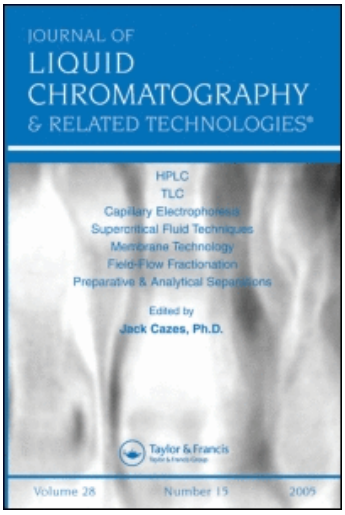

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QUANTITATIVE DETERMINATION OF FUMONISINS B_1 AND B_2 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A high-performance liquid chromatographic method has been developed for the quantitative determination of the recently described mycotoxins, fumonisins B_1 (FB_1) and B_2 (FB_2) utilizing pre-column derivatization with *o*-phthaldialdehyde, isocratic elution, and fluorescence detection. Prior to analysis, sample extracts were purified on strong anion exchange cartridges. The method has been applied to the analysis of naturally contaminated corn and mixed horse feed samples as well as fungal culture material, for the presence of the mycotoxins. Detection limits are approximately 50 ng g^{-1} for FB_1 and 100 ng g^{-1} for FB_2. The method proved to be highly reproducible and recoveries of the toxins from the purification steps were found to be 99.5% and 85.9% for FB_1 and FB_2, respectively.

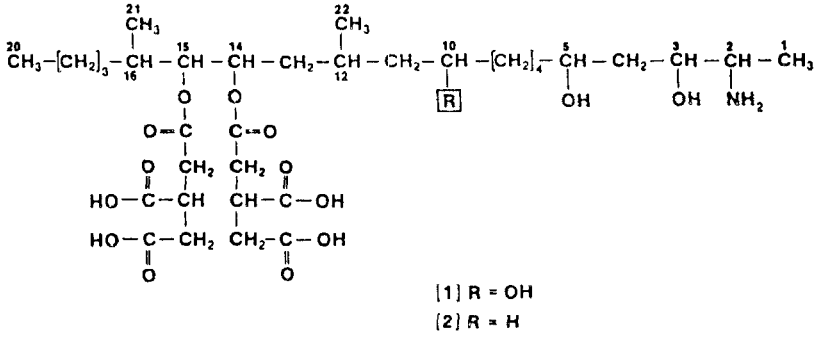

FIGURE 1. Chemical structures of [1] FB_1 and [2] FB_2.

INTRODUCTION

Fumonisins B_1 (FB_1) and B_2 (FB_2) are two recently described structurally similar mycotoxins (1) produced by Fusarium moniliforme Sheldon, a common fungal contaminant of corn throughout the world (2). They are diesters of different polyhydric alcohols and tricarballylic acid, and both contain a primary amine moeity (Fig.1) (1). The fumonisins were isolated from culture material of F. moniliforme (MRC 826) and FB_1 has been shown to be implicated in the disease leukoencephalomalacia (LEM) in horses (4).

Their presence in naturally contaminated corn samples has been qualitatively demonstrated. Two separate corn samples in the USA, which were associated with a field outbreak of LEM, were shown by thin-layer chromatography and liquid secondary ion mass spectrometry to contain both FB_1 and FB_2 (5). Similarly, a moldy corn sample from Transkei, Southern Africa was shown to contain FB_1 by the high-performance liquid chromatograp-

hic (HPLC) analysis of its maleyl derivative (ultraviolet detection) and also of its fluorescamine derivative (fluorescence detection) (6). Supportive evidence was also obtained for the presence of FB_1 or(other fumonisins) in this sample by gas chromatographic - mass spectrometric detection of the tricarballylic acid moeity, following hydrolysis of the sample extracts (6).

Previous quantitative HPLC determination of FB_1 has involved UV-detection at 230 nm of the maleyl derivative (3). Although the latter technique has been used successfully for the analyses of fungal culture material, additional and more sensitive techniques are required for the determination of the fumonisins in naturally contaminated corn samples (6). Attempts to use fluorescamine as a pre-column derivatising agent for fluorescence detection were unsatisfactory as two chromatographic peaks were observed for FB_1 (6).

This publication reports, for the first time, a quantitative and sensitive HPLC method for the simultaneous determination of FB_1 and FB_2 in naturally contaminated corn and mixed feed. The method is based on methanol/water extraction, anion exchange clean-up and isocratic, reverse-phase chromatography using fluorescence detection of the preformed o-phthaldialdehyde (OPA) derivatives.

EXPERIMENTAL

Methanol, sodium dihydrogen phosphate, orthophosphoric acid, glacial acetic acid, OPA and sodium borate were pro analysi grade from Merck (Darmstadt, FRG). Bond-Elut strong anion exchange (SAX) cartridges

(3cc) were purchased from Analytichem International (Harbor City, CA, USA). Fumonisin B_1 and B_2 were isolated from Fusarium moniliforme MRC 826 fungal cultures as previously described (3, 6).

Commercial samples of whole kernel corn were finely ground in a laboratory mill and sub-samples (25 g) were extracted by blending for 5 min with methanol : water (3:1; 50 ml) in a Sorvall (Newtown, CT, USA) Omni-mixer, followed by centrifugation at 500 x g at 4°C for 10 min. The supernatant was filtered through a Whatman No 4 filter paper and an aliquot (5 ml) was applied to a Bond-Elut SAX cartridge which had been conditioned with methanol (8 ml) followed by methanol : water (3:1; 8 ml). Subsequently, the cartridge was washed successively with methanol : water (3:1; 8 ml) and methanol (3 ml), whereafter the toxins were eluted with 0.5% acetic acid in methanol (14 ml). The eluate was evaporated to dryness under a stream of nitrogen at 60°C. The residue was redissolved in 0.1 M sodium borate (200 µl) and aliquots (50 µl) of this solution were used for derivatization.

The analytical procedure for the determination of the fumonisins in corn was slightly modified to analyse both a commercial mixed feed sample implicated in a recent South African field outbreak of LEM, as well as corn culture samples of F. moniliforme MRC 826. For the analysis of the feed sample, a sub-sample (50 g) was blended in methanol : water (3:1; 500 ml) followed by the same procedure as for corn samples. Due to the high levels of FB_1 and FB_2 in the culture material, only a 5 g sub-sample of this material was extracted by blending with a 50 ml aliquot of the extraction solvent. One ml was applied to the cartridge and the final residue redissolved in 20 ml methanol prior to derivatization.

OPA reagent was prepared by dissolving OPA (40 mg) in methanol (1 ml) and adding 5 ml 0.1 M sodium borate and 50 µl 2-mercaptoethanol. This reagent was stable in the dark at room temperature for at least a week. Derivatives were prepared immediately prior to injection, by the addition of OPA reagent (200 µl) to the sample solution (50 µl). Due to the instability of OPA derivatives, HPLC injections (10 µl) were made between one and two minutes after derivatization (7).

The derivatized samples were analysed by a reverse-phase, isocratic HPLC system consisting of a Waters Associates (Milford, MA, USA) M-45 pump and U6K injector. The analytical column (250 x 4.6 mm) was purchased from Phenomenex (Rancho Palos Verdes, CA, USA) and was prepacked with Ultracarb 7 ODS 30 reverse-phase material. A Waters Guard-Pak pre-column fitted with a Resolve C_{18} cartridge was installed to protect the analytical column. The detector was a model 650S fluorimeter from Perkin Elmer (Norwalk, CT, USA) fitted with an 18 µl flow cell. Excitation and emission wavelengths were 335 nm and 440 nm, respectively. Quantification was achieved by peak area measurement using a Waters 745 data module. The eluent was methanol: 0.1 M sodium dihydrogen phosphate (80:20) adjusted to pH 3.3 with ortho-phosphoric acid. The flow rate was 1 ml min^{-1}.

RESULTS AND DISCUSSION

Derivatization of the primary amine moeity of fumonisins B_1 and B_2 with OPA, yielded highly fluorescent products which were readily separated by reverse-phase isocratic chromatography using methanol : phosphate buffer at a sufficiently acidic pH to ensure

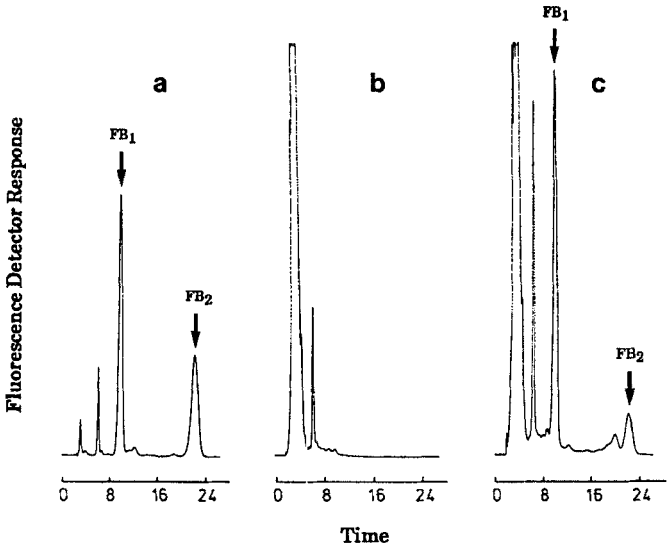

FIGURE 2. Chromatograms of OPA derivatised samples: (A) FB_1 and FB_2 standards; (B) Purified extract from corn free from contamination with F. moniliforme; (C) Purified extract from corn contaminated with F. moniliforme.

suppression of ionization of the tricarballylic acid moeities. Figure 2A shows the chromatographic separation of OPA derivatives of FB_1 and FB_2 standards, with a total run time of approximately 24 min.

Because of the instability of OPA derivatives, it was necessary to prepare the derivatives immediately prior to injection, and to inject within 2 min of mixing the reagents. In this regard, a reaction time of 1 min. between mixing and injection was found to be convenient and to yield reproducible analyses. The reproducibility of analysis of FB_1 and FB_2 standards was 2.5% relative standard deviation based on chromatographic peak area

and 5 replicate injections of 100 ng of each toxin. The detector response was found to be linear in the range 10 to 100 ng toxin per injection.

The application of the OPA derivatization and fluorescence detection to the analysis of corn samples required the establishment of a suitable sample extract clean-up procedure. This was achieved by anion exchange, utilizing a silica sorbent modified with strong anion exchange functional groups. Such a clean-up procedure allowed the direct application of the extract to the cartridge without prior evaporation or solvent manipulation. Subsequent washing of the sorbent cartridge and selective elution of the toxins yielded an eluate suitable for derivatization and chromatographic analysis. The majority of the remaining amine-containing contaminants which react with OPA, eluted from the HPLC column prior to FB_1 as shown in Fig. 2B, which is the chromatogram obtained from a control corn sample free of F. moniliforme contamination as determined by a previously described method (8). Figure 2C shows the chromatogram obtained from a sample of naturally contaminated corn. The peaks representing FB_1 and FB_2 were clearly resolved from the natural background peaks. The reproducibility of the sample clean-up and analysis was 2.8% relative standard deviation for FB_1 and 4.8% for FB_2 based on 6 replicate analyses of an extract of contaminated corn containing 4.4 $\mu g\ g^{-1}$ FB_1 and 1.3 $\mu g\ g^{-1}$ FB_2.

Analytical recoveries of the purification method were assessed by addition of toxin standards to the control corn extract at levels equivalent to 4 $\mu g\ g^{-1}$ and 2 $\mu g\ g^{-1}$ for FB_1 and 8 $\mu g\ g^{-1}$ and 4 $\mu g\ g^{-1}$ for FB_2. Mean recoveries based on triplicate analyses at each

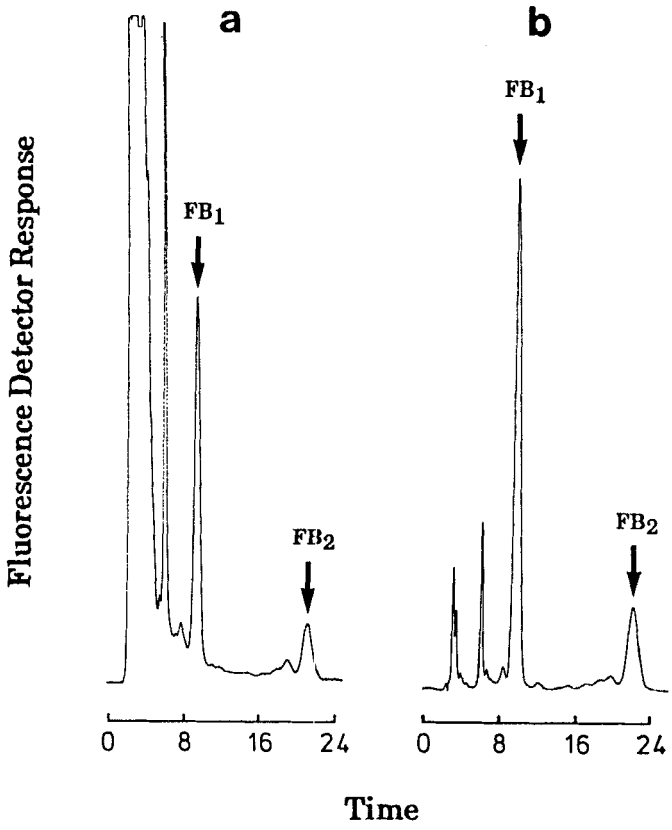

FIGURE 3. Chromatograms of OPA derivatised sample extracts: (A) Mixed feed sample; (B) Fungal culture material (F. moniliforme MRC 826).

level were 99.5% ± 1.9% relative standard deviation for FB_1 and 85.9% ± 4.7% for FB_2.

The minimum detectable amount for the OPA derivatives of FB_1 and FB_2 were 2.5 ng FB_1 (S : N = 7:1) and 5 ng FB_2 (S:N = 4:1) respectively. In terms of the preceding extraction and purification procedure, such

TABLE 1

Levels of FB_1 and FB_2 in Selected Samples

Sample	FB_1 ($\mu g\ g^{-1}$)	FB_2 ($\mu g\ g^{-1}$)
Fungal culture *F. moniliforme* MRC 826	7100	3010
Mixed Feed	8.85	3.00
Commercial corn 1	0.40	0.15
Commercial corn 2	2.90	1.20
Commercial corn 3	4.40	1.30

amounts represent apparent values in the order of 100 ng FB_1 g^{-1} corn and 200 ng FB_2 g^{-1} corn. However, it was found that 10 ml aliquots of extract could be applied to the clean-up cartridge without reducing toxin recovery. Such reserve capacity indicated that these detection limits could be halved, while under normal conditions the cartridges were being operated well within the limit of their ion exchange capacity. These detection limits represent an improvement over the maleyl method where the detection limit for FB_1 is in the order of 10 $\mu g\ g^{-1}$ (6).

Figures 3A and 3B show the chromatograms obtained from the analysis of a mixed feed sample and of *F. moniliforme* MRC 826 culture material, respectively. The

presence of both FB_1 and FB_2 in each sample is clearly shown. Table 1 summarises the analytical results obtained from these samples as well as from 3 separate naturally contaminated corn samples. As previously reported for laboratory culture material (3), FB_1 was present as the major component in all samples analysed (Table 1).

Confirmation of the identity of the peaks assigned as FB_1 and FB_2 was made by spiking sample extracts with authentic standards and observing the chromatographic co-elution of standard and unknown compounds. In addition, the analysis of control corn, free of F. moniliforme contamination, showed the absence of peaks corresponding to the retention times of FB_1 and FB_2.

In conclusion, this isocratic HPLC technique is the first quantitative method to be reported for the determination of FB_1 and FB_2 in corn and feed samples. The high sensitivity of this method will thus enable a survey of the distribution and contamination levels in feed and foodstuffs of these newly identified toxins to be undertaken.

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