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Do Fumonisin Mycotoxins Occur in Wheat?

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The fumonisin mycotoxins are mainly produced by the fungi *Fusarium verticillioides* and *Fusarium proliferatum*, which are both field pathogens of maize. The natural occurrence of fumonisins has been verified in maize and a large range of maize-based products in many countries of the world. However, occasional reports have emerged of fumonisins being detected in wheat, despite the main producing fungi not being pathogens of this cereal. An investigation was conducted into a recent report of the natural occurrence of fumonisins in the 2003/2004 South African wheat crop at levels up to 1.7 mg/kg, as determined by immunoaffinity column cleanup and direct fluorometric measurement. An AOAC International high-performance liquid chromatographic (HPLC) method for the determination of fumonisins in maize was modified and validated for the determination of fumonisins in spiked wheat samples. HPLC analysis of the wheat samples previously found to be positive for fumonisins revealed no detectable (<5 μg/kg) fumonisins in the 30 samples analyzed. These results, which lay doubt on previous reports of fumonisins in wheat, emphasize the fact that screening methods, especially if used outside their range or matrix of applicability, can produce false positive results despite the use of immunoaffinity cleanup. Such results should be validated and confirmed with a more definitive technique.

KEYWORDS: Fumonisins; maize; wheat; *Fusarium verticillioides*; mycotoxins; HPLC

INTRODUCTION

The fumonisin mycotoxins have been shown to occur worldwide in maize and maize-based products (*1*). They are produced mainly by the maize fungal pathogens *Fusarium verticillioides* (Sacc.) Nirenberg and *Fusarium proliferatum* (Matsushima) Nirenberg (*2*). They are causative factors in a number of animal disease syndromes and have been shown to be hepatotoxic and nephrotoxic in a number of animal species (*3*). They are hepato- and nephrocarcinogenic in rats and mice (*4*, *5*). They have been associated with human esophageal cancer in the former Transkei region of South Africa (*6*) and in Linxian County, Henan Province, and Cixian County, Hebei Province, China (*7*, *8*). The International Agency for Research on Cancer (IARC) has classified fumonisin B_1 (FB_1), the most abundant of the naturally occurring analogues, as possibly carcinogenic in humans (group 2B carcinogen) (*9*). Worldwide concern over their potential impact on human health has led both the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food of the European Commission to evaluate fumonisins and to assign a provisional maximum tolerable daily intake (PMTDI) for FB_1, fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), either alone or in combination, of 2 μg/kg of body weight (*10*, *11*).

The wide geographical distribution of the fumonisin-producing maize pathogen *F. verticillioides* and its endophytic nature in maize have resulted in fumonisins occurring naturally in maize or products containing maize worldwide (*10*). Besides this widespread association with maize, fumonisins have also been detected in a number of other food matrices including rice in Argentina (*10*), China (*10*), Korea (*12*), and the United States (*13*), sorghum in Botswana (*14*), Brazil (*15*), and India (*16*), *Fusarium*-infected moldy navy, adzuki, and mung beans in Canada (*17*), and asparagus in China (*18*), Germany (*19*), and Italy (*20*). The presence of these mycotoxins has also been reported in black tea and medicinal plants in Portugal (*21*) and Turkey (*22*). In addition to these commodities, sporadic reports of the natural occurrence of fumonisins in wheat or wheat products have been published. In a survey of ethnic foods in the United Kingdom, one of four wheat noodle samples was reported to contain 26 μg/kg fumonisins by HPLC (*23*). A survey of fumonisin contamination in cereals conducted in Spain reported FB_1 in 8 of 17 wheat samples in the range of 0.2−8.8 mg/kg (mean = 2.9 mg/kg) and FB_2 in one sample (0.2 mg/kg) (*24*). The fumonisins were determined by HPLC and their presence, but not their levels, was confirmed by LC-MS. The recently published European Commission report on tasks for scientific cooperation contains some occurrence data for fumonisins in wheat and wheat flour from France and Italy (*25*). Analytical methods were either not given or simply reported as HPLC or ELISA. Although 19 Italian wheat, 11 durum wheat paste, and 27 wheat semolina samples were not contaminated, 87 of 91 wheat samples from France were reported to contain fumonisins at levels up to 1044 μg/kg. Of the 42 white wheat

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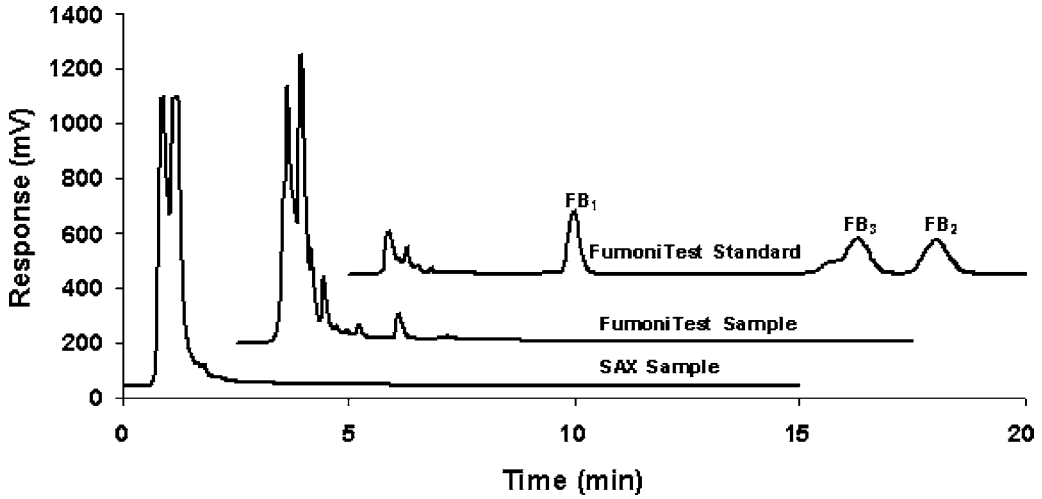

Figure 1. HPLC chromatograms with fluorescence detection of derivatized wheat extracts cleaned up on immunoaffinity (Fumonitest, Vicam) columns and SAX cartridges, as well as a chromatograms of FB_1, FB_2, and FB_3 standards recovered from a Fumonitest immunoaffinity column. The chromatograms represent identical derivatization and injection conditions.

flour samples analyzed in Italy, 5 were contaminated at levels below 100 μg/kg; of the 214 white wheat flour samples from France, 76 were reported to be contaminated, mostly (93%) at levels below 100 μg/kg (*25*).

Other researchers have failed to detect fumonisins in wheat and wheat products. The survey of ethnic foods in the United Kingdom did not detect fumonisins in 4 samples of pita bread and 4 samples of nan bread (*23*), and a further survey of cereals in the United Kingdom failed to detect fumonisins in 5 wheat samples (*26*). Similarly, 2 samples of wheat-based breakfast cereals (*27*), 10 samples of buckwheat, and 22 wheat samples in France did not contain fumonisin (*28*). These surveys were conducted using HPLC and an immunoassay test kit, respectively. In the largest survey of this nature published, 410 wheat samples from two harvesting years (1993 and 1994) in Germany failed to show any fumonisin contamination by HPLC (*29*).

A recent report detailed the occurrence of fumonisins in South African wheat samples collected from the main wheat-producing areas of the country in which 28 of a total of 30 samples showed total fumonisin contamination ranging between 0.29 and 1.7 mg/kg (mean of contaminated samples 0.75 mg/kg), as determined by immunoaffinity column cleanup and total solution fluorometry without HPLC separation (*30*). The method used in this report was based on a published method for fumonisins in maize (Vicam Fumonitest; Vicam, Watertown, MA), but had not been validated for use in wheat. Because of concerns over the human health implications of these high levels of fumonisins in a staple cereal, the samples were sent to the PROMEC Unit, Medical Research Council, Tygerberg, South Africa, for confirmation of the analytical results.

MATERIALS AND METHODS

Samples. Thirty samples of whole grain wheat from 24 wheat-producing regions of South Africa for the 2003/2004 season were supplied by the Southern African Grain Laboratory. These were exactly the same laboratory samples that had previously been found to be contaminated with fumonisins by solution fluorometry. Samples were stored at 4 °C prior to analysis.

Reagents and Standards. Fumonisin standards were prepared in the PROMEC Unit, Tygerberg, South Africa, according to the method of Cawood et al. (*31*). Fumonisin working standards were prepared at a concentration of 50 μg/mL in acetonitrile/water (1:1). All solvents and chemicals were of analytical grade from Merck (Darmstadt, Germany). Water was purified in a Milli-Q system (Millipore, Bedford, MA).

Determination of Fumonisins. The analytical method for the determination of fumonisins in wheat was adapted from validated methods for maize (*32*, *33*). In brief, milled wheat (20 g) was extracted with acetonitrile/methanol/water (1:1:2, 100 mL) by shaking on a wrist-action shaker for 20 min. The mixture was centrifuged at 1000*g* for 10 min at 4 °C and the supernatant filtered through Macherey-Nagel (Duren, Germany) MN 617 filter paper. The filtrate was adjusted to pH 6−6.5 by the addition of 1 M sodium hydroxide or 1 M hydrochloric acid, and then an aliquot (10 mL) was loaded onto a strong anion exchange (SAX) solid phase extraction cartridge (Bond-Elut, Varian, Harbor City, CA) previously conditioned with methanol (5 mL) and methanol/water (3:1, 5 mL). The cartridge was washed with methanol/water (3:1, 5 mL) and methanol (3 mL) prior to elution of fumonisins with acetic acid/methanol (1:99, 10 mL). The eluate was evaporated to dryness under a stream of nitrogen at 60 °C and the dried residue stored at 4 °C. Sample residues were redissolved in methanol (200 μL) and aliquots derivatized with *o*-phthaldialdehyde (OPA) and determined by reversed-phase HPLC using a Waters (Milford, MA) Breeze system coupled to a Waters 474 fluorescence detector set at excitation and emission wavelengths of 335 and 440 nm, respectively. The fumonisins were separated on a 75 × 4.6 mm i.d., 4 μm, Synergi MAX-RP column (Phenomenex, Torrance, CA) using a mobile phase of methanol/0.1 M sodium phosphate buffer (73:27, pH 3.35) pumped at a flow rate of 1 mL/min. Quantification was by peak area comparison with a similarly derivatized standard in acetonitrile/water (50:50).

The method was validated in-house with respect to precision and recovery. Blank wheat samples were spiked with FB_1, FB_2, and FB_3 at a level of 1000 μg/kg by pipetting an aliquot of standard solution onto the milled wheat and allowing it to dry overnight (16 h). Seven replicate analyses on three different wheat samples gave mean recoveries (± RSD) for FB_1, FB_2, and FB_3 of 63 ± 2, 57 ± 4, and 56 ± 3%, respectively.

RESULTS AND DISCUSSION

As no naturally contaminated wheat samples were available, the analytical method for the determination of FB_1, FB_2, and FB_3 in wheat was validated on spiked samples after the added solvent had been allowed sufficient time to fully evaporate. The extraction method chosen had previously been validated for the determination of fumonisins in corn flakes: shaking for 20 min with acetonitrile/methanol/water (1:1:2) extraction solvent (*33*). For each sample only a single extraction was performed as a second period of shaking with additional extraction solvent did not markedly improve the fumonisin recoveries. Various extract cleanup methods were evaluated. The use of SAX solid phase extraction cartridges gave marginally better recoveries than the

use of immunoaffinity columns (Vicam Fumonitest). Reversed-phase (C_{18}) solid phase extraction was ineffective, yielding a range of chromatographic interferences. On the basis of the method adopted, good analytical precision was achieved, although analytical recoveries were slightly lower than those generally obtained for fumonisins in maize (*32*, *33*).

Although 28 of the 30 wheat samples received had previously been found by an immunoaffinty column cleanup and fluorescence method to contain fumonisins in the range 0.29−1.7 mg/kg, HPLC analysis of all 30 samples using the above in-house-validated method failed to detect any fumonisins at a detection limit of 5 μg/kg. Clearly, the fluorescence measured in the original analysis could not have been due to fumonisins, but rather originated in unspecified matrix components retained on the immunoaffinity column and subsequently eluted with methanol. In this regard, it is worth noting the observation that the HPLC chromatogram of a wheat sample extract cleaned up by immunoaffinity column and derivatized with OPA contained significant fluorescent matrix impurities eluting at the front of the chromatogram. **Figure 1** shows a chromatogram of a wheat sample cleaned up on a Fumonitest immunoaffinity column compared to that on a SAX cartridge and that of a set of FB_1, FB_2, and FB_3 standards similarly eluted from an immunoaffinity column and derivatized under the same conditions. The additional matrix-derived impurities eluting early in the chromatogram of both samples, but absent from the standards, are clearly evident.

The fumonisin-producing fungus *F. verticillioides* occurs worldwide across tropical, subtropical, and temperate zones. *F. proliferatum*, although a more recently described species, appears to have a similar geographical distribution (*34*). Although both species are universally associated with maize, they have been isolated from a range of other agricultural products, including wheat (*34*−*39*). Because these fungal species are isolated from wheat only to a limited extent and are not pathogens of wheat, the toxigenicity of these infrequently isolated strains has not been widely investigated. Two strains of *F. verticillioides* isolated from wheat in Poland produced FB_1 levels of 220 and 360 mg/kg on autoclaved rice (*36*), whereas four of five strains of *F. proliferatum* from wheat and buckwheat in Nepal produced FB_1 levels between 863 and 1389 mg/kg on coarsely cracked maize (*37*). Further studies on a total of eight strains of *F. verticillioides* and two of *F. proliferatum* isolated from wheat in Spain and Portugal and cultured on autoclaved maize kernels showed FB_1 production in the range 29.5−2357 mg/kg (*38*, *39*). Whereas all of these studies have cultured isolated *Fusarium* strains on maize or rice, another study has investigated the potential for fumonisin production on irradiated wheat kernels (*40*). Two fumonisin-producing isolates each of *F. verticillioides* and *F. proliferatum* were shown to grow as well on wheat as on maize. Although they produced high levels of FB_1 in maize culture (maximum level of 2019 mg/kg), they failed to produce significant levels of FB_1 in wheat (maximum level of 3.5 mg/kg). The inability of wheat to support fumonisin production, while allowing fungal growth, may lie in the presence of an unknown inhibitory component or may reflect the different protein-to-carbohydrate ratio in the two cereals (*40*, *41*).

In summary, *F. verticillioides* and *F. proliferatum* may occur on wheat and some isolates may produce fumonisins in maize culture, but apparently not in significant amounts in wheat culture. However, the low incidence of *F. verticillioides* and *F. proliferatum* on wheat, the lack of pathogenicity of these strains in the field, and the lack of fumonisin-producing capacity in wheat as opposed to maize culture would indicate that no significant fumonisin contamination of wheat is likely to occur. The results of this study suggest that reports of extensive fumonisin contamination in wheat require careful evaluation of the analytical methods and possible sources of contamination, as well as confirmation by appropriate and validated methods. The finding of fungal toxins in unexpected matrices needs both high-resolution analytical methods and confirmation to prevent the reporting of false positive results from nonchromatographic methods.

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