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Analytical Methods

Analysis of mycotoxin fumonisins in corn products by high-performance liquid chromatography coupled with evaporative light scattering detection

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Abstract

Fumonisins are mycotoxins produced by the fungus *Fusarium verticillioides*, which is a widespread pathogen of corn. The mycotoxins are known to cause fatal diseases in some domestic animals and have been linked to human esophageal cancer in China and South Africa. Here, we describe a simple method for direct and quantitative analysis of the toxins in food products. The method is based on high-performance liquid chromatography coupled with an evaporative laser scattering detector (HPLC–ELSD) without any prior derivatization of the samples. Using this method, we have analyzed corn-based food samples from central markets in eastern China. The results showed that FB_1 was the main contaminant in the samples. The overall level of fumonisin contamination was relatively low, with a range of 0.25–1.80 μg/g (mean 0.74 μg/g) in 66.7% (16 of 24) of corn samples from Middle-eastern Area, 0.21–0.29 μg/g (mean 0.24 μg/g) in 28.6% (6 of 21) of corn samples from Northeastern Area, and 0.30–3.13 μg/g (mean 0.47 μg/g) in 30.0% (6 of 20) of corn samples from Southeastern Area.

Keywords: Fumonisin; *Fusarium verticillioides*; HPLC–ELSD; Corn

1. Introduction

Fumonisins are a group of mycotoxins produced by several agriculturally important fungi, including *Fusarium verticillioides*, which is a common fungal contaminant of corn and maize-derived products worldwide (Wang et al., 2006). Since the first identification of FB_1 by Bezuidenhout et al. (1988), 28 different fumonisin analogs have been characterized (Rheeder, Marasas, & Vismer, 2002). They are classified into four main groups, the A, B, C, and P-series fumonisins. The B-series fumonisins are the most abundant analogs produced by the wild-type strains, with fumonisin B_1 (FB_1) accounting for approximately 70% of the total content (Nelson, Desjardins, & Plattner, 1993). FB_1 is also believed to be the most toxic constituent (Marasas, 2001; Nelson et al., 1993). The chemical structure of the B-series fumonisins is a linear 20-carbon backbone with hydroxyl, methyl, and tricarballylic acid moieties at various positions along the backbone (Fig. 1).

The natural occurrence of fumonisins in maize has become an important concern for animal and human health throughout the world (Wang et al., 2006). Fumonisins have been shown to cause leukoencephalomalacia (ELEM) in horses (Marasas, 2001), pulmonary oedema syndrome (PES) in pigs (Harrison, Colvin, Greene, Newman, & Cole, 1990), and hepatocarcinoma in rats (Gelderblom, Abel, & Smuts, 2001). Although there is no direct evidence of adverse effects of fumonisins on human health (Shephard, Thiel, Stockenstrom, & Sydenham, 1996), studies have shown that these toxins are associated with high incidences of oesophageal cancer in South Africa (Marasas, 2001; Rheeder et al., 1992), China (Chu & Li,

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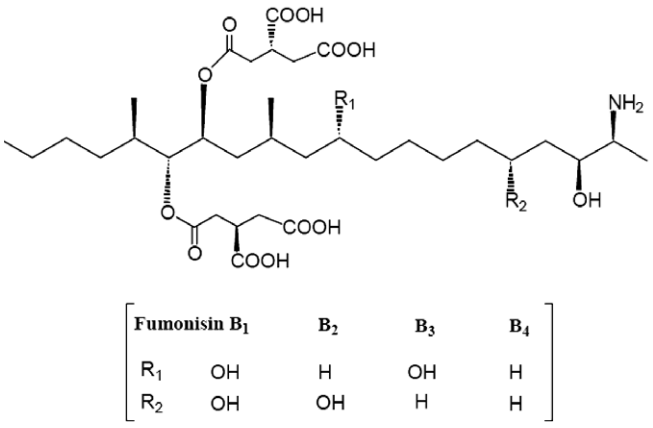

Fumonisin B_1		B_2	B_3	B_4
R_1	OH	H	OH	H
R_2	OH	OH	H	H

Fig. 1. Chemical structure of the B-series fumonisins.

1994), Italy (Franceschi, Bidoli, Baron, & La Vechhia, 1990) and Iran (Shephard et al., 2000). In 2002, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) released a provisional maximum tolerable daily intake (PMTDI) of 2 mg/kg body weight for fumonisins (World-Health-Organization, 2002). The Food and Drug Administration of USA has announced guidance levels for total fumonisins levels in corn products, 2-4 ppm for human foods and 5–100 ppm for animal feeds depending on the species and the proportion of the contaminated material in the total diets. In 2003, the International Agency for Research on Cancer (IARC, 2002) classified fumonisin B_1 as a Group 2B carcinogen (possible human carcinogen).

Sensitive, accurate and reproducible analytical methods for the detection of fumonisins in foods and feeds are essential to assess exposure. Since the initial discovery and characterization of fumonisins, significant progress has been made in the analytical technology of fumonisins. Current methods used for detection and quantification of fumonisins in foods and feeds include high-performance liquid chromatography (HPLC) with different detectors (Dilkin, Mallmann, de Almeida, & Correa, 2001; Shephard, Sydenham, Thiel, & Gelderblom, 1990; Shephard, 1998), gas chromatography–mass spectrometry (GC–MS) (Plattner, Ross, Reagor, Stedelin, & Rice, 1990a), thin-layer liquid chromatography (TLC) (Karuna & Sashidhar, 1999; Preis & Vargas, 2000), immunochemical methods (Bird et al., 2002), and capillary zone electrophoresis (CZE) (Maragos, Bennett, & Richard, 1997). Each method has its own advantages and limitations. Among them, HPLC methods are the most commonly described procedures for the analysis of fumonisins in foods and feeds. Fumonisins do not fluoresce nor do they contain UV absorbing chromophores. Consequently, most HPLC methods measure fumonisin after derivatizing the free amino group with fluorescent compounds. It is necessary to purify the samples after extraction in most detection methods. However, the accuracy of the methods is affected by selective adsorption of the purification columns or unstable derivatization (Jackson & Jalonski, 2004; Williams, Meredith, & Riley, 2004). Evaporative light scattering detector (ELSD) has been successfully used to quantify underivatized fumonisins in HPLC analysis (Wilkes, Sutherland, Churchwell, & Williams, 1995), but has not been used in detection of fumonisin in corn and corn-based products.

Since Chu and Li (1994) first reported the simultaneous occurrence of FB_1 and other mycotoxins in moldy corns collected from China in regions with high incidences of esophageal cancer, such as Linxian County in Henan Province, the heavy contamination of fumonisin in corn from Linxian County was also reported by some other researchers (Wang, Wei, Ma, & Luo, 2000; Zhang, Nagasshima, & Goto, 1997). However, relatively lower levels of FB_1 and FB_2 in corn were reported in the same regions (Wang & Zhu, 2002; Yoshizawa, Yamashita, & Luo, 1994). In these studies, HPLC with derivatization methods or immunochemical methods were used to detect FB_1 and FB_2 in corn samples from household of limited areas in China. Analysis of FBs content in corn from different areas of China with new developed analytical methods, such as HPLC–ELSD has not been conducted. Thus, the objective of the present study was to develop a HPLC–ELSD detection method of FBs and to analyze the level of fumonisins in corn-based products from the central market of some main corn-producing areas in eastern China.

2. Materials and methods

2.1. Chemicals and culture of fungi

High-performance liquid chromatography (HPLC)-grade acetonitrile was from EM Science (Darmstadt, Germany) or from Fisher Scientific (Pittsburgh, PA). Standard fumonisins and *F. verticillioides* wild-type strain A0149 (FGSC number 7600) were gifts from Liangcheng Du (Department of Chemistry, University of Nebraska, Lincoln, USA.). General fungal growth was in GYP medium (2% glucose, 1% peptone, and 0.3% yeast extract) (Caldas et al., 1998). Conidia were collected from V-8 juice agar plates after 3 weeks growth as described (Bojja, Cerny, Proctor, & Du, 2004; Proctor, Desjardins, Plattner, & Hohn, 1999). CMK (cracked maize kernels) were used for fumonisin production (Bojja et al., 2004).

Approximately 8×10^7 conidia of wild-type strain were used to inoculate CMK (5 g) medium. Mycelia plugs from V8 plates were also used for inoculations in several batches. The cultures were allowed to grow for 3 weeks with occasional shaking to allow even distribution of the cultures (Bojja et al., 2004).

2.2. Sample collection

Corn kernel samples (at least 1 kg) were taken (between September and December 2005) from the largest central markets of the selected main maize-producing areas in eastern China, including Northeastern Area (Heilongjiang,

Liaoning and Jilin Province), Middle-eastern Area (Shandong and Henan Province) and Southeastern Area (Jiangsu and Zhejiang Province). The samples were sent to the laboratory as soon as they were collected, and tested upon arrival or stored at −20 °C to arrest any fumonisin formation up to the time of analysis.

2.3. Extraction and clean-up

After 50 g corn samples were ground to a fine meal in a laboratory mill, a 10-g aliquot of the sample was placed in a flask containing 25 ml acetonitrile/water (1:1, v/v), then was placed in an orbital shaker overnight, and filtered with Whatman No. 1 paper under vacuum. Ten millilitres filtrate was placed on the ice for 15 min in a 50-ml centrifugal tube and centrifuged at 7000 rpm for 10 min at 4 °C, then transferred to a new 50-ml centrifugal tube containing 300 mg Amberlite XAD-4 (37380-42-0, Sigma–Aldrich Co., USA) which had been activated with 2 ml methanol and washed with deionized water, and the tube was stirred for 5 h or overnight in an orbital shaker after adding 40 ml deionized water. The XAD-4 beads were then washed with 200 ml deionized water, then transferred the XAD-4 beads to a Bond Elute column without stuffing by deionized water, and the toxins were eluted with 3 ml 100% methanol. The eluent was dried under vacuum with freezing at −65 °C and dissolved in 200 μl deionized water. The solution was filtered through a 0.2 μm syringe-filter and 20 μl was injected directly into the HPLC–ELSD. All corn samples were analyzed in triplicate.

2.4. HPLC–ELSD analysis

The HPLC system was a ProStar, Model 210 (Varian Walnut Creek, CA) with a column of Alltima C18LL, 5 μm, 250 × 4.6 mm inner diameter (Alltech, Deerfield, IL). The HPLC–ELSD method conditions were performed according to the previously reported procedures with some modifications (Bojja et al., 2004). The mobile phases were (A) water– trifluoroacetic acid (TFA) (100:0.025, v/v) and (B) acetonitrile–TFA (100:0.025, v/v), with a gradient of 0–20% B in A in the first 5 min, 20–40% B from 5 to 10 min, 40–80% B from 10 to 15 min, 80% B from 15 to 20 min, and 80–0% B from 20 to 25 min. The flow rate was 1.0 ml/min. The conditions set for ELSD (ELSD 2000, Alltech, USA) were 45 °C of drift tube temperature, 2.0 l/min nitrogen gas flow, and gain value of 1 in the impactor-on mode. In quantitative analyzes, a volume of 20 μl standard FB_2, FB_3 and FB_4 samples (1 μg/μl) was injected for HPLC–ELSD analysis, and standard FB_1 sample (10 μg/μl) was diluted in four different concentrations of 0.15, 0.3, 0.6, 1.2 μg/μl with the same method above. The peak area from the responding peak was integrated using on-system tools provided by Varian. At least two

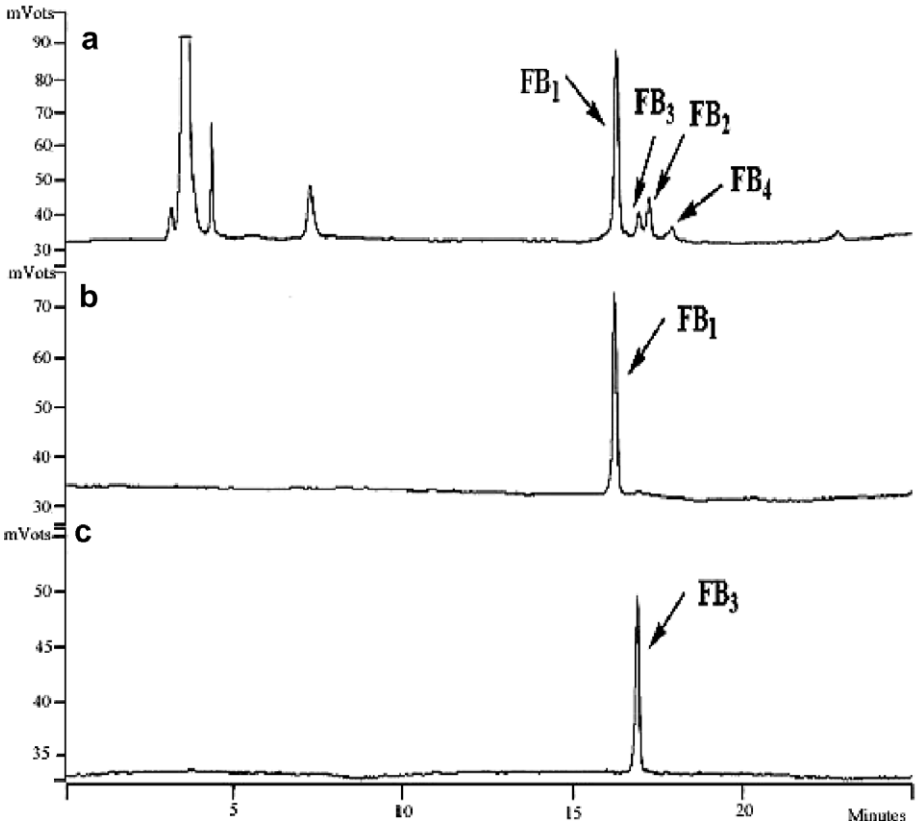

Fig. 2. Separation of fumonisin B series by HPLC–ELSD. (a) An extract from the fermentation broth of 3-week old wild-type strain *Fusarium verticillioides*, (b) standard FB_1 (10 μg) and (c) standard FB_3 (10 μg).

injections were made for each concentration, and the peak areas were then plotted against the absolute amounts of FB_1 used to obtain a standard curve (Fig. 4). In three independent spiking experiments, 3, 30 and 300 μg of FB_1 spiking solution was added into 25 ml original extract (acetonitrile:water, 1:1) of 10 g corn at the beginning of extraction, respectively.

3. Results

3.1. Establishment of the HPLC–ELSD method using standard fumonisins

Under the established conditions, standard FB_1 gave a peak at a retention time of 16.26 min. Retention times for standard FB_3, FB_2 and FB_4 samples were 16.92, 17.22 and 17.90 min, respectively (Figs. 2 and 3). Standard FB_1, ranging from 3 to 24 μg, was tested for its corresponding response as peak areas on the HPLC–ELSD. We found Ln/Ln plot (Fig. 4a) with $R^2 = 0.9934$ was more appropriate than the calibration curve with Peak/Amount approach (Fig. 4b) especially over a wide concentration range. This calibration curve is enough for the detection of FB_1 in corn samples. The accuracy and precision of the method were sufficiently high for tested agricultural commodities spiked within the range of 0.3–30 μg/g, which represents the most critical levels of contamination in terms of FB_1 control. The recoveries of FB_1 in this range varied from 77.27% (0.3 μg/g) to 102.58% (3 μg/g) (Table 1). In the literature, the recovery rate for FB_1 was between 74% and 89% (Jackson & Jalonski, 2004), depending on the quantification method. With HPLC–ELSD analysis, a rate of 93% was described (Wilkes et al., 1995). FBs of various concentrations were injected into the column to determine the limit of detection. The result showed that the established HPLC–ELSD method was sufficiently sensitive to detect 60 ng of FB_1 per injection, and therefore, the limit of detection for components in solution is approximately 3 ng/μl for current system with a N/S ratio of 3.

3.2. Analysis of corn products using HPLC–ELSD

HPLC analysis of extracts from wild-type *F. verticillioides* revealed one major peak with a retention time of

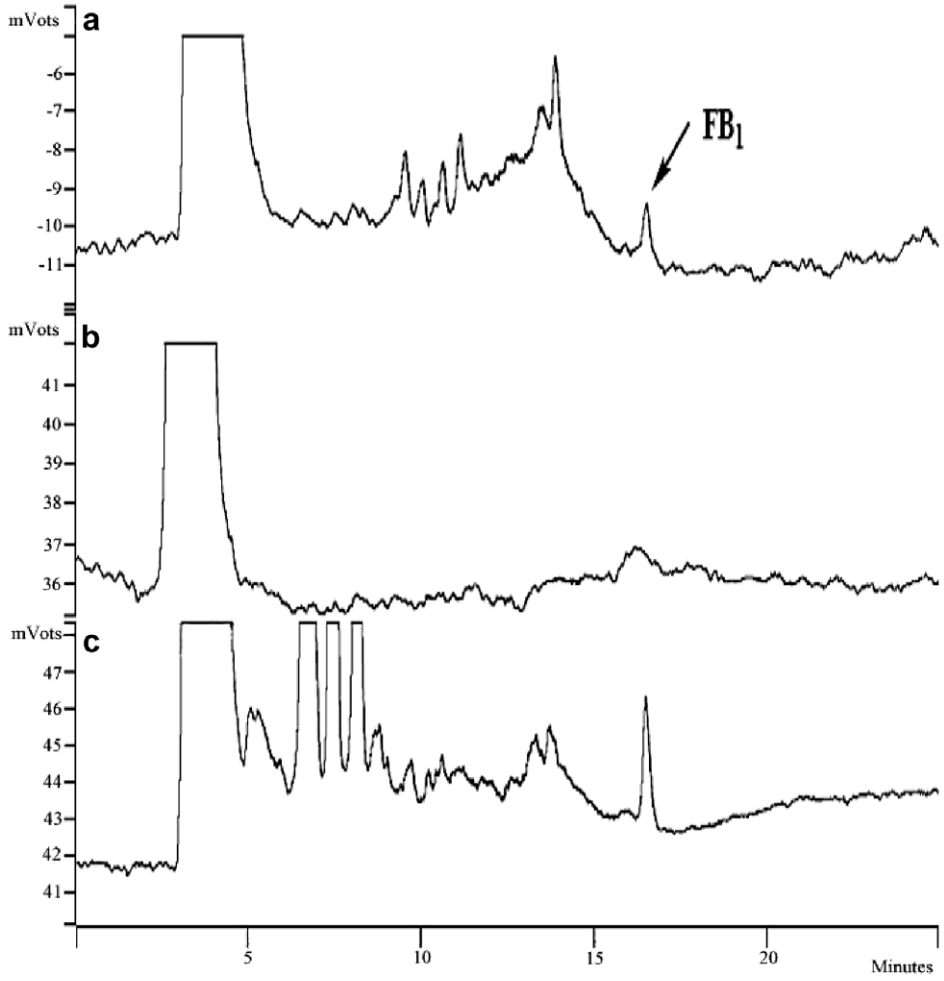

Fig. 3. Analysis of fumonisin contaminations in corn samples using HPLC–ELSD. (a) A representative sample (#8a) containing 0.37 μg/g of FB_1, (b) a representative sample (#20c) without contamination and (c) a representative sample (#22a) containing 0.62 μg/g of FB_1 and many other compounds.

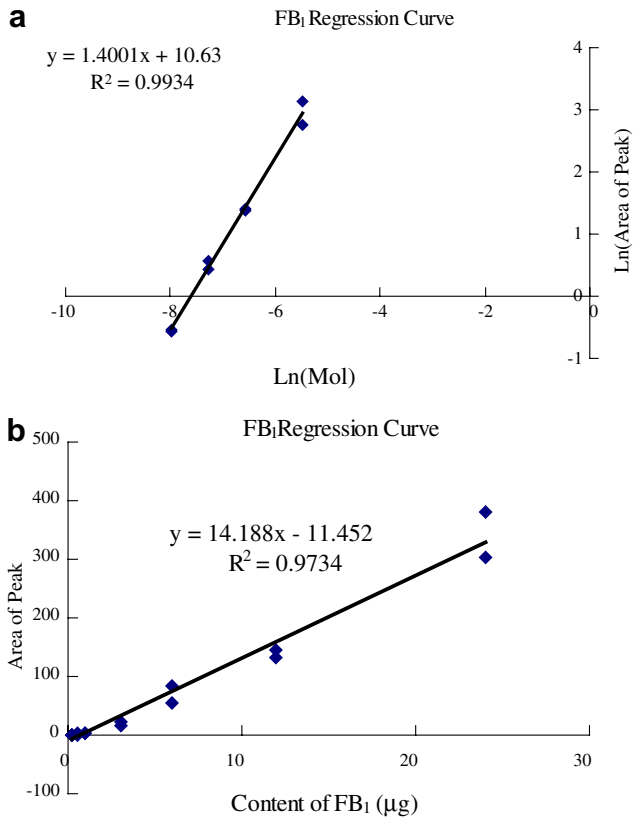

Fig. 4. Regression equation and curve of HPLC–ELSD method for determination of FB_1. (a) Ln/Ln plot and (b) Peak/Amount plot.

Table 1
Recovery of HPLC–ELSD resulting from analysis of FBs-spiked blank corn

Spiking level (μg/g)	Recovery (%)	Relative standard deviation (%, $n = 5$)
0.3	77.27	13.56
3	102.58	8.85
30	88.13	5.96

16.26 min, which is the same as that of standard fumonisin B_1 (Fig. 2), and three minor peaks in the same region. These minor peaks had retention times of 16.92, 17.22, and 17.90 min, which comigrated with standard fumonisin B_3 and B_2, and B_4, respectively. The results are consistent with the previously identified fumonisins using LC–MS analysis (Bojja et al., 2004). In the corn samples from the central market of different areas, FB_1 was the only fumonisin detected, while no detectable levels of FB_2, FB_3 and FB_4 were found. Sixteen of 24 samples (66.7%) from Middle-eastern Area were found to be contaminated with FB_1 in concentrations ranging from 0.25 to 1.80 μg/g (mean 0.74 μg/g), FB_1 was also found in concentrations ranging from 0.21 to 0.29 μg/g (mean 0.24 μg/g) in 28.6% (6 of 21) of corn samples from Northeastern Area, and in concentrations ranging from 0.30 to 0.82 μg/g (mean 0.47 μg/g) in 30.0% (6 of 20) of corn samples from Southeastern Area (Table 2).

Table 2
Fumonisin contamination of corn samples from different areas

Area	Samples		Level of FB_1 in positive samples	
	Number	Positive (incidence)	Mean (μg/g)	Range (μg/g)
Northeastern Area	21	6 (28.6%)	0.24 ± 0.04[a]	0.21–0.29
Middle-eastern Area	24	16 (66.7%)	0.74 ± 0.18	0.25–1.80
Southeastern Area	20	6 (30.0%)	0.47 ± 0.10	0.30–0.82

[a] Mean ± standard derivation.

4. Discussion

Our work has demonstrated experimentally that HPLC–ELSD provides a convenient and reliable alternative to common HPLC-fluorescence detection methods for rapid determination of FB_1, FB_2, FB_3, and FB_4 contents without derivatization of the samples. The method ensures accurate quantification with a limit of 3 ng/μl, which is sufficiently sensitive for detection of the fumonisin level in extracts of corn products. The direct analysis of sample extracts reduces not only the cost but also the complexity of the analysis. Clean-up is an important step in analysis of FBs. It is inevitable that there are many interfering matters in detection solution without purification, and therefore, the clean-up is necessary for ideal chromatography figure with litter interfering peaks which might influence the detection results. The use of Amberlite XAD-4 for clean-up in current HPLC–ELSD system proved to be simple, fast, and effective. However, the current HPLC–ELSD method for FBs analysis remains to be further modified, for example, the peaks of standard FB_1, FB_3, FB_2, and FB_4 in current HPLC–ELSD analysis were very close; especially the peaks of FB_3 and FB_2 were almost overlapping. We have conducted LC–MS analysis to further confirm the peaks of FB_1, FB_3, FB_2, and FB_4. Specifically, a fraction corresponding to the range between 15 and 20 min was collected from HPLC–ELSD and subjected to LC–MS analysis. The LC–MS analysis results confirmed the peaks of FB_1, FB_3, FB_2, and FB_4 with the retention time of 16.26, 16.92, 17.22, and 17.90 min, respectively. As only FB_1 was detected in the corn samples we analyzed, we did not further optimize the chromatogram method in this study, which should be improved in our future research.

Previous research works from China have studied the natural occurrence of fumonisin in corn samples collected from households in high-risk areas for human esophageal cancer in eastern China (Linxian County in Henan Province and Cixian County in Hebei Province). In a survey conducted in Cixian and Linxian, Chu and Li (1994) detected high level of FB_1 in concentrations ranging from 18 to 155 μg/g (mean 74 μg/g) in all moldy samples, and relatively lower level of FB_1 in concentrations ranging from

20 to 60 μg/g (mean 35.3 μg/g) in all normal samples from the same households. Similarly, Wang et al. (2000) studied the incidence and levels of FB_1 in corn from Cixian, and found that high levels of FB_1 (88.9 ± 13.1 μg/g) were detected in 100% of 10 moldy samples, while low level (1.40 ± 0.5 μg/g) of FB_1 were detected in 90.9% of 11 healthy corn samples. However, lower incidence and level of FB_1 were also reported. The work by Wang and Zhu (2002) showed that FB_1 at the concentrations ranging from 1.07 to 2.56 μg/g was detected in 50% of moldy corn samples collected from households of Jingtou village in Linxian County, on the other hand, low level (0.21–0.737 μg/g) of FB_1 was detected in 10.5% of 19 normal samples from the same households. In another survey conducted in Henan Province, Yoshizawa et al. (1994) described FB_1 contamination frequencies of 48% and 25% with average FB_1 levels of 0.872 and 0.890 μg/g, respectively, in Linxian and Shangjiu County in Henan Province. Our results showed that the FB_1 was detected at average concentration of 0.74 μg/g in 16 of 24 corn samples from the central market of Middle-eastern Areas (Henan and Shandong Province). These data agree with those from Henan Province (Yoshizawa et al., 1994) and from Linxian County in Henan Province (Wang & Zhu, 2002), but much lower than those from Linxian and Cixian (Chu & Li, 1994) and from Cixian (Wang et al., 2000). No moldy samples were found in all the central markets during our sampling, and all the samples collected were normal apparently healthy corn without any visible mold contamination, which may explain the low incidence and levels of FB_1 in corn in present survey. In previous studies, the co-occurrence of FB_1 and FB_2 in corn (Yoshizawa et al., 1994), corn-based food (Bittencourt, Oliveira, Dilkin, & Corrêa, 2005), and feed (Sanchis et al., 1995), with FB_1 being the predominant fumonisin have been reported. In our test, only FB_1 was detected in corn samples, which agrees with some other surveys in Linxian and Cixian, China (Chu & Li, 1994; Wang et al., 2000; Wang & Zhu, 2002).

Previous surveys in China focused on the occurrence of fumonisin in corn samples from households in high-risk areas for human esophageal cancer (Linxian County in Henan Province and/or Cixian County in Hebei Province). Limited information is available on fumonisin contamination of corn samples in other areas by other source. This is the first report of the natural occurrence of fumonisin in corn samples from central markets of three main maize-producing areas in eastern China. The results showed that fumonisin contamination in corn samples from central markets was not serious and FB_1 was the sole FBs detected. Compared to the maximum level of FDA listed (2–4 μg/g for human food) (FDA, 2002), the result suggests that the corn in central market of eastern China is safe.

In present study, the highest frequency of FB_1 contamination was found in corn from Middle-eastern Area (66.7%), followed by from Southeastern (30.0%) and Northeastern Area (28.6%), while the highest average FB_1 concentration in positive corn samples was detected from Middle-eastern Area, followed by Southeastern Area and Northeastern Area. The corn samples from Northeastern Area are not only with the lowest incidence, but also the lowest FB_1 content (Table 2). Climate conditions, such as relative humidity and temperature influence the invasion of corn by fungi and contamination of fumonisin during pre-harvest and post-harvest periods. The new harvested corn cobs were usually shelled, and dried to reduced moisture content by exposure to the sun on the floor outside. During September to December, the corn kernels traded in the central markets of Northeastern Area and Middle-eastern Area are from local, while the corn kernels traded in the central markets of Southeastern Area are mostly transported from the Northeastern Area. Rich in rain during harvest season in Henan and Shandong Province in 2005 may explain the relatively higher incidence and level of FB_1 contamination in corn form central markets of this area. The lowest incidence and level of FB_1 in corns from central markets of Northeastern Area was due to local climate conditions, the sunny weather with low temperature and relative humidity during harvest season, on the other hand, the relatively higher incidence and level of FB_1 in corns traded in the central markets of Southeastern Area compared to those of Northeastern Area might be due to the unfavorable storage conditions, such as high temperature and relative humidity during the storage and transportation from north to south.

In the future, it will be interesting to further investigate the effect of different ecological factors and storage conditions on accumulation of fumonisin in corn before, during and after harvest.

Acknowledgements

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