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Determination of Fumonisin B₁ and B₂ in Corn Products

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ABSTRACT

An analytical method using immunoaffinity column and HPLC was developed for determination fumonisins in corn and corn products. The detection limits for fumonisin B₁ (FB₁) and fumonisin B₂ (FB₂) were 0.03 ppm and 0.07 ppm, respectively. Spiked FB₁ in dried corn (kernel and flour), fresh corn, corn snack and corn flake are at 0.2-3.0 ppm level, with recovery ranged from 79.2-108.8% and relative standard deviation (RSD) of 2.9-21.9%. FB₂ spiked under the same condition as FB₁ had recovery ranges of 70.0-106.0% and RSD of 4.7-20.0%. A total of 76 samples were collected, including 20 dried corn (kernel or flour), 5 fresh corn, 15 corn snacks, 10 corn flakes, 5 corn starch, 5 canned corn from commercial markets and 16 raw corn materials were purchased from corn snack manufacturers. From the total 76 samples, 11 samples (14.5%) were detected with FB₁ and/or FB₂. Seven samples of corn were at 0.05-0.13 ppm level, 1 fresh corn with 0.15 ppm, 2 corn snacks with 0.5 and 0.16 ppm, respectively, where as 1 corn raw material was at the 0.09 ppm level. The highest contamination was only 0.16 ppm. Nevertheless, no fumonisin was detected in corn flake, corn starch and canned corn samples.

Key words: fumonisin B₁, fumonisin B₂, corn products, immunoaffinity column, HPLC

INTRODUCTION

Fumonisin is a mycotoxin which is a structurally related group of long-carbon chain compounds as indicated in Figure 1⁽¹⁾. Fumonisin analogues have been identified and classified into fumonisin A₁, A₂, B₁, B₂, B₃, and B₄ based on their chemical structure. Fumonisin B₁ (FB₁), fumonisin B₂ (FB₂), and fumonisin B₃ (FB₃) are believed to be the most abundant naturally occurring analogues. Since fumonisins are alkaloids and polar compounds, so they can be dissolved in water, methanol, ethanol and acetonitrile. However they are insoluble in nonpolar solvents, such as acetone, chloroform, and hexane⁽²⁾. Because fumonisins are not fluorescent, they should be quantified with *ortho*-phthalaldehyde (OPA) reagent.

Fumonisin is produced mainly by *Fusarium moniliforme* (= *F. verticillioides*), *F. proliferatum*, and several other *Fusarium* species. It was found that water activity between 0.94-0.98 is ideal for growing *F. moniliforme* and *F. proliferatum*. In fact, the optimum pH and temperature for the growing *F. moniliforme* are 5.5 and 25°C, and for *F. proliferatum* are 7.0 and 30°C⁽³⁾. When water activity is below 0.92, growth-inhibited effect will be occurred.

Many kinds of cereals including corn, sorghum, rice and wheat are known to be infected by *Fusarium* species and produce fumonisins, particularly corn crops⁽³⁾. *F. moniliforme* is a soil-borne as well as a seed-borne pathogen of corn that inhabits in the field. Therefore, infection of *F. moniliforme* can infect the roots, stalks, and kernels of corn. Insect invasion on corn kernels can result in the production of fumonisins due to *Fusarium* invasion.

The fumonisin levels found in corn are influenced by various environmental factors such as temperature, humidity, drought stress and the extent of rainfall during the growth and harvesting periods. Post harvest storage of corn kernels under improper moisture conditions can also result in additional accumulation of fumonisins⁽²⁾. Generally, the fumonisin level will increase in corn products during storage as long as proper grain moisture and temperature are maintained⁽⁴⁾. The extent of contamination of corn varies with geographical location, and is found to be highest in the warmer regions of the world⁽⁵⁾. Susceptibility to fungal infection and subsequent contamination of the corn also depend on agricultural practices and genotypes⁽⁶⁾. Corn kernels with insect invasion are easily infected with molds and produce toxins. *Fusarium* species can invade corn kernels by inner route and produce fumonisins. A signifi-

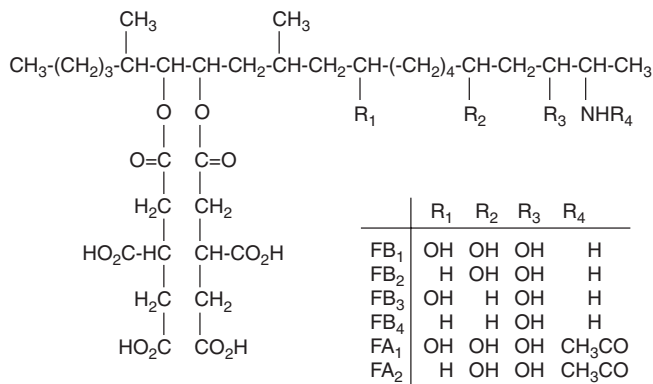


Figure 1. Chemical structure of fumonisins.

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cant percentage of healthy-looking corn kernels contain fumonisin levels of about 1 ppm or higher^(4,7).

FB₁ is believed to be the most toxic fuminin leading to most severely adverse health effects in animals⁽⁸⁾. When plants were contaminated by fumisinis, they could cause physiological damage, growth inhibition, and death in plants⁽⁹⁾. FB₁ can cause acute mycotoxicoses such as equine leukoencephalomalacia^(10,11), pig pulmonary⁽¹²⁾, hepatopathy, liver cancer and nephritic disease in several animal species, including farm animals⁽¹³⁻¹⁵⁾. FB₁ can also influence immunological function, cause liver and kidney damage, laggard growth or even death of poultry. Corn and fumonisin have also been associated with high incidences and increased risk of human esophageal cancer in South Africa and China, where corn and corn products are the main staple food. Investigators in South Africa have noted a correlation between high levels of fumonisin-producing molds on corn and esophageal cancer in human subgroups. The researchers detected high levels of FB₁ were detected in samples that had heavy mold contamination, and these samples also contained high levels of trichothecenes. Epidemiological studies currently available demonstrate only inconclusive associations between fumonisin and human esophageal cancer⁽¹⁶⁻¹⁸⁾.

Since the effect of fumonisin on human healthy has not been proved, worldwide regulations for fumonisin are still in a recommended phase. There are only America and Swiss that have recommended levels for fumonisin. The American advisory level for fumonisin (FB₁ + FB₂ + FB₃) in corn and corn products intended for human consumption range from 2-4 ppm^(19,20), depending on the type of corn products. The Swiss provisional level for fumonisin (FB₁ + FB₂) in corn and corn products intended for human consumption is 1 ppm⁽²⁰⁾.

According to the Agricultural Statistics Yearbook 2001 published by the Council of Agriculture, Executive Yuan, ROC, corn is not a staple cereal among the per capita per year food supply and most parts of corn need to be imported⁽²¹⁾. Because Taiwan is in sub-tropical climate area, *Fusarium* spp. could grow well in warm and humid environment. Tseng *et al.* found only *F. moniliforme* produces FB₁ and FB₂ among *Fusarium* species isolated from the grains collected in Taiwan. Among the 38 strains of *F. moniliforme* isolated, 66% of *F. moniliforme* could produce fumonisin⁽²²⁾. Tseng *et al.* also surveyed corn-based foodstuffs purchased from markets at various districts of Taiwan from 1994 to 1995, 33.9% of samples were found to be contaminated with FB₁ at 0.073-2.375 ppm level. Among the samples contaminated with FB₁, 61.5% were also found to be contaminated with FB₂ at the range of 0.01-0.715 ppm⁽²³⁾. On the other hand, Chung *et al.* surveyed corn products collected in Taiwan in 1995. Twenty-seven of 91 samples were found to be contaminated with fumonisin at the level 0.6-5.6 ppm⁽²⁴⁾.

Cancer is the number one cause of death in Taiwan. Since consumption of mycotoxin contaminated food may be carcinogenic for people, Bureau of Food and Drug

Analysis (BFDA) surveyed alfatoxin contamination in food for years and also examined the contents of other mycotoxins. Purpose of this study was to establish the examination method and apply the method to survey the content of fumonisin in corn and corn products.

MATERIALS AND METHODS

I. Materials

(I) Sample Collection

Sixty samples of corn product including dried corn (kernel or flour), fresh corn, corn snack, corn flake, corn starch, canned corn from hyper-market, supermarkets, and convenience stores in north Taiwan. Sixteen samples of corn raw materials from 9 corn snack manufacturing plants were collected during May to October, 2002. Each sample was blended and mixed, packed in sealed bag, stored in freezer (-20 ± 2°C) and analyzed as soon as possible.

(II) Reagents and Apparatus

1. FB₁ and FB₂ standards were purchased from SigmaAldrich Chemie GmbH (Germany). Immunoaffinity columns FumoniTest™ were purchased from VICAM (Watertown, MA, USA). *O*-phthaldialdehyde (OPA), 2-mercaptoethanol (MCE), sodium dihydrogenphosphate dihydrate, sodium tetraborate, hydrochloric acid, phosphoric acid, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and potassium chloride were of reagent grade. Acetonitrile and methanol were of LC grade. Distilled, deionized water was used throughout the procedure.

2. Prepared Reagents

- A. Extraction solvent: acetonitrile/methanol/water (25/25/50, v/v/v).
- B. Phosphate buffer saline (PBS): Dissolve 8.0 g of NaCl, 1.2 g of Na₂HPO₄, 0.2 g of KH₂PO₄ and 0.2 g of KCl in 990 mL of water. Adjust pH to 7.0 with 2 M HCl, and dilute to 1 L.
- C. OPA reagent: Dissolve 40 mg of OPA in 1 mL of methanol, and dilute with 5 mL of Na₂B₄O₇ solution. Add 50 μL of MCE and mix. Store the mix in the dark for up to 1 week at room temperature in a capped amber vial.
- D. LC mobile phase: methanol/0.1 M NaH₂PO₄ (77/23, v/v), adjusted to pH 3.35 with H₃PO₄.

(III) Instruments and Analytical Condition

A Hitachi (Japan) HPLC system equipped with Hitachi L-7100 pump, a L-7480 fluorescence detector and a L-7200 autosampler were used. The column (150 × 4.6 mm,

Cosmosil 5C18-AR, 5 μm , Nacalai, Japan), and the fluorescence detector wavelength settings were 335 nm (excitation) and 440 nm (emission). The mobile phase, methanol/0.1 M NaH_2PO_4 (77/23, v/v) adjusted to pH 3.35 with H_3PO_4 was pumped at a constant flow rate of 1.0 mL/min. Injection volume was 20 μL .

II. Method

(I) Preparation of Standard Solution

Dissolve FB_1 and FB_2 standard with acetonitrile/ H_2O (50/50, v/v) to make 100 ppm stock solution, then dilute the solution with acetonitrile/ H_2O (50/50, v/v) to prepare a series of standard solution.

(II) Extraction

Using a modification of the method by Visconti *et al.*⁽²⁶⁾. Weigh 20 g of test portion of mixed sample into blender, add 50 mL of extraction solvent, and then homogenize for 2 min. Afterwards, centrifuge for 10 min at 2500 \times g and filter supernatant through filter paper (Whatman No.4, 12 cm). Again extract remaining solid material by adding 50 mL of extraction solvent and repeat above procedure. Collect and combine the 2 filtrates and pipet 10 mL of filtrate into 50-mL centrifuge bottle. Add 40 mL of PBS and mix well. Filter diluted extract through microfiber filter (Whatman GF/A, 9 cm) and collect 10 mL of filtrate for cleanup through immunoaffinity column.

(III) Immunoaffinity Column Clean Up

Follow manufacturer's instruction, connect a 10-mL syringe reservoir with a FumoniTestTM immunoaffinity column, pipet 10 mL of filtrate into syringe reservoir, let filtrate flow through column at ca 1-2 drops/sec and discard elute. Then wash the loaded immunoaffinity column with 10 mL of PBS at 1-2 drops/sec until air comes through column. Place 4-mL vial under column, elute fumonisins with 1.5 mL of LC grade methanol at 1 drop/sec. Evaporate methanol eluate to dryness under a gentle stream of nitrogen. Retain dried residue at 4°C for derivation and HPLC analysis.

(IV) Derivation

Dissolve residue in 200 μL of acetonitrile/ H_2O (50/50, v/v) and then filtered by a 0.45 μm microfilter for HPLC analysis. Transfer 50 μL of sample solution or standard solution to 1-mL test tube, and add 50 μL of OPA reagent. Mix solution for 30 sec with vortex mixer, and inject 20 μL of derived solution into LC system in exactly 3 min after adding OPA reagent.

(V) Identification and Quantification

FB_1 and FB_2 standard were dissolved in acetonitrile/

H_2O (50/50, v/v) to prepare a series of working solutions containing 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 6 and 8 ppm of FB_1 and FB_2 . The standard curves were plotted based on peak area versus concentration. While using equal volume of OPA reagent for derivation, the concentration of FB_1 and FB_2 were injected into HPLC to become 0.025-4 ppm. The sample and standard derived solutions were accurately taken and injected into HPLC according to the analytical condition as described. The retention time and peak area were compared to those in standard curves. The amounts of FB_1 and FB_2 were calculated based on the standard curves.

FB_1 and FB_2 concentration in the test sample was calculated using the following equation:

$$\text{FB}_1 \text{ or } \text{FB}_2 \text{ content (ppm)} = C \times V/M$$

C: concentration of FB_1 or FB_2 in sample derived solution.

V: volume of derived solution (0.1 mL).

M: sample weight (0.1 g) in 0.1 mL of derived solution.

(VI) Recovery Test

Uncontaminated corn, fresh corn, corn snack, corn flake (blank corn products) were spiked with FB_1 and FB_2 standard. The spiked samples were then kept in a hood for 1 hr to evaporate the solvent residue. Corn test sample with 0.5, 1.0, 2.0 and 3.0 ppm FB_1 and FB_2 , the fresh corn with 0.2 ppm FB_1 and FB_2 , and corn snack with 0.5 ppm FB_1 and FB_2 were prepared. The corn flake with 0.5 ppm FB_1 and FB_2 were prepared. Each concentration of spiked samples was prepared in triplicate. The preparation of derivation sample solution was as described. Recoveries for different sample were calculated after HPLC analysis.

(VII) Detection Limit Test

Series of diluted concentrations of FB_1 and FB_2 standard solution were derived as described. The instrument detection limit (IDL) was estimated on the basis of signal to noise (S/N) ratio greater than 3. A suitable amount of FB_1 and FB_2 standard was spiked into blank corn meal and the derived sample solution was prepared as described. The method detection limit (MDL) was estimated on the basis of signal to noise (S/N) ratio greater than 3.

RESULTS AND DISCUSSION

I. Preparation of Derived Sample Solutions

The OPA derivatives of fumonisins, which are not fluorescent, need to be prepared for HPLC fluorescence detector. The fluorescence intensity of the OPA derivative is time dependent (Figure 2). Both FB_1 and FB_2 standards yield stable OPA derivatives after they reacted with OPA

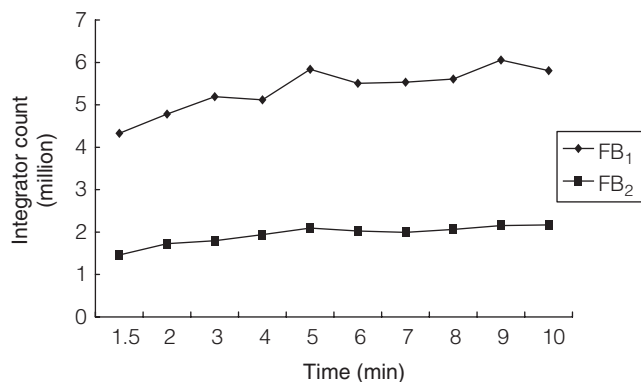


Figure 2. Stability of the FB₁ (1 ppm) and FB₂ (1 ppm) OPA derivatives.

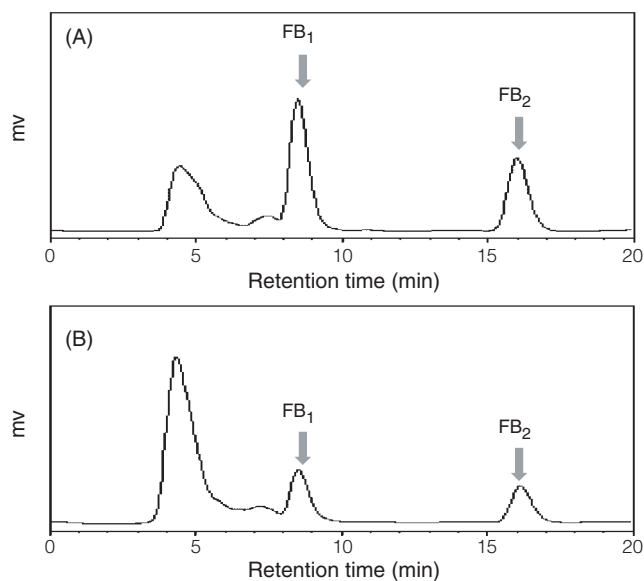


Figure 3. HPLC chromatograms of (A) FB₁ and FB₂ standards, (B) corn sample spiked with 1 ppm FB₁ and FB₂. HPLC condition: column: 150 × 4.6 mm, Cosmosil 5C18-AR, 5 μm; mobile phase: methanol/0.1 M NaH₂PO₄ (77/23, v/v), adjusted to pH 3.35 with H₃PO₄; flow rate: 1.0 mL/min; fluorescence detector: excitation at 335 nm and emission at 440 nm.

reagent for 3 min. Trucksess *et al.*⁽²⁵⁾ found the highest fluorescence intensity of OPA derivatives formed after 0.5 min reaction, but the OPA reagent was freshly prepared by their laboratory. We compared self-prepared OPA reagent and the reagent purchased from VICAM company, and found that VICAM OPA reagent could produce more stable derivatives. In order to prepare the VICAM OPA reagent for this study, we mixed the two solutions. The mixing reagent could be used for 5 days in amber bottle. According to the study of Trucksess *et al.*⁽²⁵⁾, the day-to-day variability of the fluorescence intensity of the OPA derivative was about 10%. Within the same day, the variation of fluorescence intensity of OPA derivative of the same standard solution was about 3%.

The chromatograms of FB₁ and FB₂ standards, blank corn and spiked corn sample are shown in Figure 3. The

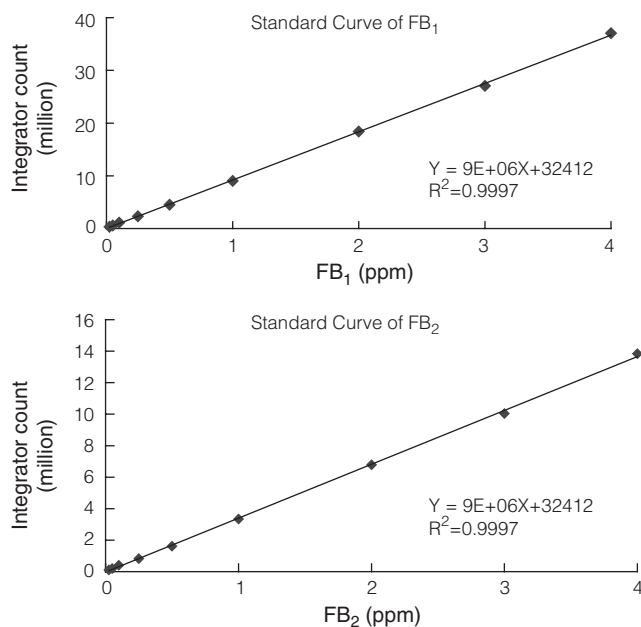


Figure 4. The standard curves for FB₁ and FB₂ derivatives by HPLC.

retention time for FB₁ and FB₂ was 8.4 and 16.1 min, respectively. FB₁ and FB₂ could be separated from other components of the sample solution. The HPLC condition was suitable for analyzing fumonisins in corn.

II. Establishment of Standard Curve

Twenty microliter of FB₁ and FB₂ OPA derivatives were injected at concentration of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3 and 4 ppm. The fluorescence responses (peak area) were regressed with injected FB₁ and FB₂ mass and gave a standard curve shown in Figure 4. The linear equation were $y = 9 \times 10^6x + 32412$ and $y = 3 \times 10^6x - 21670$ for FB₁ and FB₂, respectively, where x represents the concentration (ppm) of fumonisins and y represents peak area. Regression coefficients (R^2) are 0.9997 and 0.9995 for FB₁ and FB₂, respectively. Linearity is observed for both FB₁ and FB₂ standard curve at 0.025-4 ppm.

III. Detection Limit Test

Hitachi HPLC was used in this study. The instrument detection limits (IDL) were 0.02 and 0.04 ppm for FB₁ and FB₂, respectively. Converted to mass was 1 and 2 ng for FB₁ and FB₂, respectively. The method detection limits (MDL) for corn products were 0.03 and 0.07 ppm for FB₁ and FB₂, respectively (Table 1). Chu *et al.*⁽¹⁶⁾ found that MDL of fumonisin (not specified FB₁ or FB₂) for corn was 0.05 ppm. On the other hand, Trucksess *et al.*⁽²⁵⁾ detected FB₁ in canned corn and frozen corn, and obtained MDL of 0.025 ppm. Although above-mentioned studies all used immunoaffinity column for extraction and clean up fumonisins, different sample matrix and HPLC instruments supposedly affected the sensitivity.

IV. Recovery and Repeatability Test

Based on consumer's dietary habit, corn products categories surveyed in this study included dried corn (kernel or flour), fresh corn, corn snack, corn flake, corn starch and canned corn, etc. Among them, corn, fresh corn, corn snack and corn flake were chosen for recovery and repeatability study. The detailed recoveries are shown in Tables 2 and 3. Spiked FB₁ at 0.2-3.0 ppm level had recoveries in the range of 79.2-108.8%, and relative standard deviation (RSD) in the range of 2.9-21.9%. Spiked FB₂ in the same condition as FB₁, recoveries of 70.0-106.0% and RSD of 4.7-20.0%.

Trucksess *et al.*⁽²⁵⁾ spiked FB₁ in canned corn and fresh corn at 0.05-0.2 ppm level. They observed recoveries of 76.7-81.3% and 75.8-88.3% and RSD of 4.9-11.9% and 8.2-13.6% for canned corn and fresh corn, respectively. Visconti *et al.*⁽²⁶⁾ had conducted a collaborative study, in which they determined FB₁ and FB₂ in dried corn and corn flakes by liquid chromatography with immunoaffinity column cleanup. Relative standard deviation for the *in vitro* repeatability (RSDr) of the corn analyses ranged from 19-24% for FB₁ and 19-27% for FB₂; for the corn flakes analyses, RSDr ranged from 9-21% for FB₁ and 8-22% for FB₂. Mean recoveries of FB₁ and FB₂ from dried corn spiked with FB₁ at 0.8 ppm and with FB₂ at 0.4 ppm were 76% and 72%, respectively; for corn flakes spiked at the

Table 1. Instrument detection limits (IDL) and method detection limits (MDL) of fumonisins analysis in corn by HPLC

| | IDL | MDL |
|--------------------------|----------|----------|
| Fumonisin B ₁ | 0.02 ppm | 0.03 ppm |
| Fumonisin B ₂ | 0.04 ppm | 0.07 ppm |

Table 2. Recovery of fumonisin B₁ added to corn and corn products

| Product | FB ₁ spiked (ppm) | Recovery ^a (%) | S.D. | RSD (%) |
|------------|------------------------------|---------------------------|------|---------|
| Corn | 0.5 | 91.3 | 11.6 | 12.7 |
| | 1.0 | 90.7 | 6.9 | 7.6 |
| | 2.0 | 79.2 | 2.3 | 2.9 |
| | 3.0 | 79.9 | 11.8 | 14.8 |
| Fresh corn | 0.2 | 81.3 | 17.8 | 21.9 |
| Corn snack | 0.5 | 93.2 | 5.1 | 5.5 |
| Corn flake | 0.5 | 108.8 | 11.4 | 10.5 |

^an = 3.

Table 3. Recovery of fumonisin B₂ added to corn and corn products

| Product | FB ₂ spiked (ppm) | Recovery ^a (%) | S.D. | RSD (%) |
|------------|------------------------------|---------------------------|------|---------|
| Corn | 0.5 | 74.8 | 11.9 | 16 |
| | 1.0 | 82.9 | 4.4 | 5.3 |
| | 2.0 | 76.2 | 7.1 | 9.3 |
| | 3.0 | 70.0 | 10.1 | 14.4 |
| Fresh corn | 0.2 | 76.4 | 15.1 | 20.0 |
| Corn snack | 0.5 | 81.0 | 3.8 | 4.7 |
| Corn flake | 0.5 | 106.0 | 11.4 | 10.8 |

^an = 3.

same levels recoveries were 110% and 97% for FB₁ and FB₂, respectively. The recovery and repeatability results of this study are similar with previous studies.

V. Fumonisin Levels in Corn and Corn Products

Seventy-six samples were collected from markets and snack manufacturers, Table 4 shows detected samples of 7 categories of corn and corn products. There are 11 samples (14.5%) among 76 samples were detected FB₁ and/or FB₂, fumonisins were not detected in corn flakes, corn starch and canned corn. Five dried corn samples, 1 corn snack sample and 1 sample of raw material of plant were detected with FB₁. On the other hand, 5 samples were detected with FB₂. Only 1 dried corn sample were contaminated with both FB₁ and FB₂ and the highest contamination 0.16 ppm was found in a corn snack (Table 5).

The limitation levels of fumonisin are not regulated in Taiwan yet, where as the advisory level of fumonisins varies from 1-4 ppm for different corn products in other countries. Based on the data of this survey, FB₁ and FB₂ levels in human foods derived from corn are quite low and corn products are not the staple food in Taiwan. At the present time, fumonisins in corn and corn products for

Table 4. Number of detected samples from corn and corn products

| Product | Number of samples | Number of detected samples | | |
|-------------------|-------------------|----------------------------|-----------------|--------------------|
| | | FB ₁ | FB ₂ | Total ^a |
| Corn | 20 | 5 | 3 | 7 |
| Fresh corn | 5 | 0 | 1 | 1 |
| Corn snack | 15 | 1 | 1 | 2 |
| Corn flake | 10 | 0 | 0 | 0 |
| Corn starch | 5 | 0 | 0 | 0 |
| Canned corn | 5 | 0 | 0 | 0 |
| Corn raw material | 16 | 1 | 0 | 1 |
| Total | 76 | 7 (9.2%) | 5 (6.6%) | 11 (14.5%) |

^aTotal represents the sample number contaminated with fumonisins (FB₁ + FB₂).

Table 5. Fumonisin levels in corn and corn products

| Product | Fumonisin (ppm) | | | |
|-------------------|-----------------|-----------------|-----------------|-----------------------------------|
| | Sample number | FB ₁ | FB ₂ | FB ₁ + FB ₂ |
| Corn | | | | |
| C01 | | 0.06 | 0.07 | 0.13 |
| C02 | | 0.06 | ND ^a | 0.06 |
| C10 | | 0.05 | ND | 0.05 |
| C12 | | 0.07 | ND | 0.07 |
| C15 | | 0.05 | ND | 0.05 |
| C19 | | ND | 0.09 | 0.09 |
| C20 | | ND | 0.08 | 0.08 |
| Fresh corn | | | | |
| R02 | | ND | 0.15 | 0.15 |
| Corn snack | | | | |
| S02 | | ND | 0.16 | 0.16 |
| S03 | | 0.05 | ND | 0.05 |
| Corn raw material | | | | |
| N08 | | 0.09 | ND | 0.09 |

^aNot detected.

human consumption in Taiwan presents negligible public health risk.

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