JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of Fumonisin Contamination in Cornflakes on the Belgian Market by "Flow-Through" Assay Screening and LC−MS/MS Analyses

CHARLINE PAEPENS,*,† SARAH DE SAEGER,† LIBERTY SIBANDA,‡ ILDIKÓ BARNA-VETRÓ,§ MARIANNE ANSELME,|| YVAN LARONDELLE,|| AND CARLOS VAN PETEGHEM†

Ghent University, UGent, Faculty of Pharmaceutical Sciences, Laboratory of Food Analysis, Harelbekestraat 72, 9000 Ghent, Belgium, TOXI-TEST NV, Industrielaan 9a, 9990 Maldegem, Belgium, Agricultural Biotechnology Center, Diagnostic Laboratory, Szent-Györgyi Albert u. 4, 2100 Gödöllö, Hungary, and Université catholique de Louvain and Institut des Sciences de la Vie, Unité de biochimie de la nutrition, Croix du Sud 2/8, 1348 Louvain-la-Neuve, Belgium

A total of 205 cornflake samples collected in Belgian retail stores during 2003−2004 were surveyed for the natural occurrence of fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3). These cornflake samples, originating from conventional as well as from organic production, were analyzed using an intralaboratory-validated LC−MS/MS method. Additionally, 90 cornflake samples were subjected to rapid screening using a flow-through enzyme immunoassay method to demonstrate the practicability of a screening test coupled to a validated confirmatory LC−MS/MS method for the management of food safety risks. FB_1 concentrations ranged from not detected (nd) [LOD (FB_1) = 20 μg/kg] to 464 μg/kg with mean and median concentrations of respectively 104 ± 113 and 54 μg/kg. For FB_2 and FB_3, the concentration ranges varied respectively from nd [LOD (FB_2) = 7.5 μg/kg] to 43 μg/kg and from nd [LOD (FB_3) = 12.5 μg/kg] to 90 μg/kg. Mean concentrations for FB_2 and FB_3 were respectively 12 ± 8 and 21 ± 15 μg/kg, while the median concentration was 11 μg/kg for FB_2 and 19 μg/kg for FB_3. From the statistical tests (χ^2 and ANOVA model III), it could be concluded that the agricultural practice did not have any significant effect on the fumonisin concentrations but that the variation between different batches was significant ($p < 0.0001$).

KEYWORDS: Fumonisins, cornflakes, rapid field test, LC−MS/MS, conventional/organic food

INTRODUCTION

Fumonisins are a family of mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*, two of the most prevalent molds associated with corn grown in all regions of the world (*1*). To date, several fumonisins have been isolated and characterized, but FB_1, FB_2, and FB_3 are the major ones found in naturally contaminated foods such as polenta, corn snacks, and cornflakes (*2*−*4*). In animals, these mycotoxins can cause mycotoxicoses, such as equine leukoencephalomalacia (ELEM) and porcine pulmonary oedema (*5*). They are also suspected to be carcinogenic, nephrotoxic, hepatotoxic, and immunosuppressive (*6*−*8*). The minimum doses reported in long-term toxicity/carcinogenicity studies in mouse and rates were 0.25 mg of FB_1 (kg of bodyweight)$^{-1}$ day^{-1} for kidney lesions and 0.7 mg of FB_1 (kg of bodyweight)$^{-1}$ day^{-1} for liver lesions. The lowest dose level at which increased kidney and liver tumor incidences were observed in rats is respectively 2.5 and 7.0 mg (kg of bodyweight)$^{-1}$ day^{-1}. A provisional maximum tolerable daily intake (PMTDI) for nephrotoxicity has been set by the 56th Joint FAO/WHO Expert Committee on Food Additives to 2 μg of total fumonisin kg^{-1} bodyweight (*6*). Their structural similarity with sphingoid bases led to the hypothesis that fumonisins may inhibit ceramide synthase, a key enzyme involved in *de novo* sphingolipid biosynthesis and in the reacylation of free sphingoid bases derived from sphingolipid turnover (*9*). Human exposure to fumonisins is common worldwide and has been statistically associated with the high rate of human oesophageal cancer in certain areas of South Africa (*5*), China (*10*), and Northeastern Italy (*11*).

Fumonisins are water-soluble, and nixtamalization (cooking in alkaline water) lowers the fumonisin content of food products if the cooking liquid is discarded. Baking, frying, and extrusion cooking of corn at high temperatures (≥190 °C) also reduce fumonisin concentrations in foods, with the amount of reduction

* To whom correspondence should be addressed. Telephone: +32-9-264-81-37. Fax: +32-9-264-81-99. E-mail: charline.paepens@ugent.be.
† Ghent University, Laboratory of Food Analysis.
‡ TOXI-TEST NV.
§ Agricultural Biotechnology Center, Diagnostic Laboratory.
|| Université catholique de Louvain and Institut des Sciences de la Vie, Unité de biochimie de la nutrition.

10.1021/jf050858d CCC: $30.25
Published on Web 08/12/2005

achieved depending on the cooking time, temperature, and other factors (*12*−*15*). Data published on fumonisin reduction because of food processing are very inconsistent. However, Meister and Springer reported recently that cooking extrusion (180−220 °C) reduced fumonisin levels to approximately 30−55%, cooking the grits for flaking (30−90 min, 130 °C) to approximately 20−65%, and roasting the flakes (2.5 and 5 min, 250 °C) to approximately 6−35% (*16*). It has not been established whether fumonisin reductions result from their thermal decomposition or from binding of the mycotoxin to food matrix components such as covalent binding to proteins (*17, 18*), saccharides, and polysaccharides (*19*−*21*).

In general, the levels of fumonisin contamination found in cornflakes in different parts of the world are up to 300 μg/kg, although occasional samples with concentrations of FB_1 of more than 1000 μg/kg have been reported (*22*−*23*). Information on the natural occurrence of fumonisins in cornflakes on the Belgian retail market is scarce. A total of 12 cornflake samples originating from the Belgian market were included in the report of the SCOOP project (*22*), aimed to collect data on the occurrence of *Fusarium* toxins in food and to assess dietary intake by the population of European Union member states. In none of these samples was FB_1 detected. This can be due to the high limit of detection (300 FB_1 μg/kg) of the method used.

For this market-orientated survey, cornflakes available in Belgian retail stores situated over the country were collected. The sampling included 11 different brands of cornflakes originating from conventional (*7*) as well as organic production (*4*). Organic agricultural practices do not allow the use of chemical products such as fungicides and growth regulators (EC 2092/91) (*24*). During recent years, organic agricultural practices have grown considerably as an alternative to conventional agriculture and related use of chemical pesticides. Consumers perceive them as pro-environment, healthy, and safe. However, pesticide and chemical residues are only two of the issues related to contamination of the food chain. Another risk for human health is the presence of mycotoxins. Concern has been raised that the organic agricultural practice encourages colonization of fungi in cereal grains with the consequent increase in mycotoxin production. Some studies indicate that agricultural practice had no influence on the mycotoxin concentrations (*25, 26*), and others reported lower mycotoxin concentrations in organic food (*27*−*28*). In contradiction, certain types of organic products, such as cereals, cereal-based products, fresh apples, and apple cider, were found to be more susceptible to fungal contamination than their conventionally grown counterparts (*29, 30*). The inconsistency of these data and the lack of conclusive evidence make it difficult to draw generalized conclusions. At present, there is still not enough information available to provide evidence that organic foods are significantly different from their conventional counterparts, in terms of mycotoxin content (*31, 32*). In this study, it was our objective to evaluate and compare the occurrence of fumonisins in conventional and organic cornflake brands purchased in Belgian retail stores during the years 2003−2004. Therefore, 205 samples of cornflakes were analyzed using an intralaboratory-validated LC−MS/MS method. Additionally, 90 of these samples were subjected to rapid screening using a flow-through enzyme immunoassay method (*33*) to evaluate the usefulness of this field test for the management of food safety risks generated by fumonisins.

MATERIALS AND METHODS

Reagents and Materials for Qualitative Screening with "Flow-Through" Enzyme Immunoassay. Goat anti-horseradish peroxidase (anti-HRP) (number P5774, protein concentration = 52.7 g/L), casein sodium salt (casein), and Tween 20 were purchased from Sigma Chemical Co (Bornem, Belgium). Proclin 300 was purchased from Supelco (Bellefonte, PA). Rabbit anti-mouse immunoglobulin (Ig) G (number Z259, protein concentration = 3.2 g/L) was purchased from DakoCytomation (Heverlee, Belgium). The membrane (Immunodyne ABC; pore size = 0.45 μm) was obtained from Pall France (Saint Germain-en-Laye, France). As the flow-through device, a plastic snap-fit device from Trosley Equipment (Dover, Kent, U.K.) was used. The FB_1−HRP conjugate was prepared by the Diagnostic Laboratory, Agricultural Biotechnology Center, Gödöllö, Hungary. Monoclonal antibodies against FB_1 were produced and characterized by the same institute. The antibody was an IgG1 (protein content = 1 g/L) with κ light chain and an affinity constant of 1.3×10^{10} M^{-1}. Cross-reactivity of the monoclonal FB_1 antibodies against FB_2 and FB_3 was respectively 91.8 and 209% (*34*). For stability reasons, the FB_1−HRP conjugate and anti-FB_1 antibodies were kept in concentrated form. Dilutions in assay buffer were used for 1 week. Phosphate-buffered saline (PBS, 0.01 M) at pH 7.4 was used to prepare the wash solution (PBS−Tween 0.05%), the blocking solution (PBS−casein 2%), and the assay buffer (PBS−casein 0.1%). Proclin 300 was added to the buffers as an antibacterial preservative. A 0.45 μm Chromafil Einmalfilter from Macherey−Nagel (Düren, Germany) was chosen as the membrane filter. The substrate chromogen solution used was ColorBurstBlue obtained from ALerCHEK, Inc. (Portland, ME). A portable colorimeter (Minolta Chroma Meter CR-321) was purchased from Minolta Co. (Osaka, Japan).

Reagents for the LC−MS/MS Analyses. The following reagents were obtained from the firms mentioned: acetonitrile and methanol (BDH, Poole, U.K.), formic acid and hydrochloric acid fuming 37% (Merck, Darmstadt, Germany), and liquid nitrogen (Air Liquide, Belgium). Water was obtained from a Milli-Q Gradient system (Millipore, Brussels, Belgium). Ederol filter paper (24 cm, quality 15) and glass microfiber filters Whatman GF/A (9 cm) were supplied by VWR (Leuven, Belgium). FumoniTest immunoaffinity columns were purchased from Vicam (Watertown, MA).

Preparation of Standard Solutions. Fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) were purchased from Sigma Chemical Co. (Bornem, Belgium). Fumonisin B_3 (FB_3) was obtained from Promec Unit (Tygerberg, South Africa). Stock solutions (1 mg/mL) of FB_1 and FB_2 were prepared in methanol and stored at −20 °C until use. The FB_3 standard (1 mg) was dissolved in 1 mL of ACN/H_2O (1:1, v/v) and stored at 4 °C. For spiking purposes in the flow-through assay, a standard mixture containing FB_1 and FB_2 each at 10 ng/μL was prepared in methanol and stored at −20 °C until use. For spiking purposes in the LC−MS/MS analyses, working solutions of respectively FB_1, FB_2, and FB_3 were prepared in methanol at 10 and 1 ng/μL and stored at 4 °C until use. The sphinganine analogue (2*S*,3*R*)-2-aminododecane-1,3-diol (4.3 mg) served as the internal standard. This component was solved in 1 mL of ACN/H_2O (35:65, v/v) containing 0.3% formic acid, and working solutions (43, 4.3, and 0.43 ng/μL) were prepared by dilution. The stock and working solutions of the internal standard were stored at 4 °C.

LC−MS/MS Equipment. Liquid chromatography analyses were carried out with a Waters Alliance 2695 XE HPLC system coupled to a Micromass Quattro micro triple quadrupole mass spectrometer (Waters, Milford, MA). The analytical column was an Alltima C18, 5 μm, 150 × 3.2 mm (Alltech Associates, Deerfield, IL), while the guard column was an Alltima C18, 5 μm, 7.5 × 3.2 mm (Alltech Associates).

Origin of Samples. A total of 11 brands (7 conventional and 4 organic) were purchased from Belgian retail stores in the Flemish and Walloon parts of Belgium. A total of 205 samples (3 or 4 batches per brand, 5 samples per batch) were collected during the years 2003−2004. From each sample, approximately 100 g was ground using a grinder (Retsch type ZM1, 1 mm sieve, GmbH and Co.KG, Germany), thoroughly mixed, and stored at room temperature prior to analysis.

Qualitative Screening with "Flow-Through" Enzyme Immunoassay. A total of 90 cornflake samples (including 7 conventional and 2 organic brands, 2 different batches/brand, and 5 samples/batch) were screened using the flow-through enzyme immunoassay technique described by Paepens et al. (*33*). This flow-through assay originally developed for the matrix maize was now optimized for the matrix

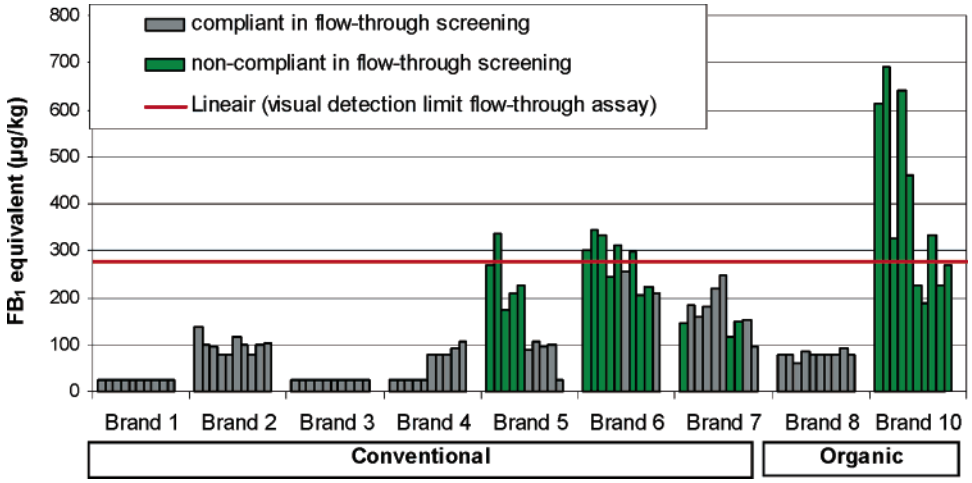

Figure 1. Comparison of the results obtained by flow-through assay and LC–MS/MS analyses. Each bar (=1 sample) presents the sum of FB_1, FB_2, and FB_3 contamination expressed as FB_1 equivalent μg/kg.

cornflakes by lowering the visual detection limit. The samples were subjected to the formerly described sample preparation and the optimized assay procedure. A 5 g portion of ground cornflake sample was mixed with 15 mL of MeOH/H_2O (50:50, v/v) and shaken by hand for 6 min. The supernatant (1 mL) was diluted with 600 μL of PBS and filtered through a 0.45 μm Chromafil membrane filter. This filtrate (600 μL) was used in the flow-through assay. The flow-through assay system (European patent number 0893690) consisted of two layers, with the first being the Immunodyne ABC membrane coated with rabbit anti-mouse antibodies and anti-HRP antibodies. The second layer was an absorbent material (*35*). In the flow-through assay, each reagent was applied in sequence on the membrane. Between each step, it was important to allow the added liquid to flow completely through the membrane. First an aliquot of 100 μL of anti-FB_1 antibodies, diluted 1:150 in assay buffer, was applied on the membrane, followed by a washing step of 300 μL of PBS−Tween 0.05%. Then, the sample extract (600 μL) was added. Analyte, if present, was bound to the anti-FB_1 antibodies. After 300 μL of a wash solution was added, a FB_1−HRP conjugate dilution of 1:500 in the assay buffer was added. The FB_1−HRP conjugate was bound by any remaining free anti-FB_1 antibodies and by anti-HRP antibodies, which acted as the internal control. A last washing step (300 μL) removed residual conjugate solution from the membrane. Finally, 100 μL of color substrate was added. Color development was visually evaluated and also measured with a portable colorimeter, which expressed the color intensity as a single numerical value (ΔE*ab). This assay used monoclonal antibodies against FB_1, with a cross-reactivity against FB_2 and FB_3 of respectively 91.8 and 209%. Results of the visual evaluation corresponded to the concentration of "FB_1 equivalent μg/kg". These were calculated using the corresponding cross-reactivity coefficients for FB_2 and FB_3 (*33*). For samples contaminated with fumonisins equal to or above the above-mentioned visual detection limit, no color appeared on the membrane and they were considered as "positive" or "noncompliant". When a blue-colored spot appeared, even substantially lighter colored than the blank control sample, the sample was considered to be "negative" or "compliant". It should be emphasized that "compliant" and "noncompliant" are only related to the visual detection limit and do not mean containing a violative concentration or not, because the European Regulation (EC) number 466/2001 (*36*) does not include fumonisins. Samples scoring "positive" or "noncompliant" (FB_1 and FB_2 concentration > 275 μg of FB_1 equivalent/kg, see the Results and Discussion) during the screening always require chromatographic confirmation. The cornflake samples were analyzed in a series that consisted of a blank cornflake sample, a compliant spiked cornflake sample (FB_1 and FB_2 concentration < visual detection limit), a noncompliant spiked cornflake sample (FB_1 and FB_2 concentration > visual detection limit), and three cornflake samples. Assay results were valid when a blue internal control spot (consisting of anti-HRP antibodies) developed, and the color intensity of the blank cornflake sample served as a control for immunoreagent stability (i.e., ΔE*ab should be more than 9.50). Visual results were compared with the quantitative LC−MS/MS results to evaluate the accurateness of the assay. Assay precision was expressed as the number of false compliant and false noncompliant results.

LC−MS/MS Analyses. The presence of FB_1, FB_2, and FB_3 was determined using a LC−MS/MS method as outlined before (*37*). The ground sample (20 g) was extracted with 50 mL of methanol/water (70:30, v/v, adjusted to pH 4 with 0.1 M HCl) by shaking for 30 min using an orbital shaker and then centrifuged for 10 min at 2217*g* and filtered. The remaining solid material was extracted again with 50 mL of extraction solvent, centrifuged, and filtered as above. A total of 10 mL of the combined extracts was mixed with 40 mL of 0.01 M PBS buffer solution at pH 7.4. A 15-mL volume was passed through a FumoniTest immunoaffinity column. After the column was washed with 15 mL of H_2O, fumonisins were eluted with 2 mL of MeOH. The eluted extract was evaporated to dryness under a nitrogen stream at 60 °C and redissolved in 450 μL of mobile phase of acetonitrile/water (60:40, v/v), containing 0.3% formic acid. A volume of 50 μL of (2*S*,3*R*)-2-aminododecane-1,3-diol (0.43 ng/μL) was added after sample cleanup and served as an internal standard. A volume of 20 μL was injected into the Alltima C18 column. The chromatography was performed under isocratic conditions at a flow of 0.3 mL min^{-1} with a mobile phase consisting of acetonitrile/water (60:40, v/v) containing 0.3% formic acid. The mass spectrometer was operated in the positive electrospray ionization (ESI^+) mode using multiple reaction monitoring (MRM). High-purity nitrogen was used as the drying and ESI-nebulizing gas. Argon was used as the collision gas for collision-induced dissociation. For the detection of FB_1, $[MH]^+$ was chosen as the precursor ion at *m*/*z* 722. The product ions selected were at *m*/*z* 352 and 334, with the first one used for quantification. For FB_2, the precursor ion was *m*/*z* 706. The product ions selected for the detection of FB_2 were *m*/*z* 354 and 336 with the first transition used for quantification. For the detection of FB_3, $[MH]^+$ was chosen as the precursor ion at *m*/*z* 706. The product ions *m*/*z* 688 and 354 were selected, and *m*/*z* 354 was used for quantification. For the detection of the internal standard (2*S*,3*R*)-2-aminododecane-1,3-diol, $[MH]^+$ was chosen as the precursor ion at *m*/*z* 218. The product ions selected were at *m*/*z* 170 and 94, with the last transition used for quantification. Quantitative determinations were based on the peak area ratios of the selected product ions of respectively FB_1, FB_2, and FB_3 in comparison to that of the internal standard. Limits of detection (LOD) and limits of quantification (LOQ) were calculated. The corresponding concentration at 3 times the residual standard deviation of the *y* intercept divided through the slope of the regression line equals the LOD. LOQ is equal to 6 times the residual standard deviation of the *y* intercept divided through the slope of the regression line. LOD concentrations for FB_1, FB_2, and FB_3 were 20, 7.5, and 12.5 μg/kg, respectively. LOQ was 40 μg/kg for FB_1, 15 μg/kg for FB_2, and 25 μg/kg for FB_3. Coefficients of variation for analyses carried out under repeatability and under within-laboratory reproducibility conditions were determined at 1, 1.5, and 2 times the LOQ concentration of the respective components (n = 5). The coefficients of variation

under repeatability conditions varied from 7.1 to 13%. Under within-laboratory reproducibility conditions, the coefficients of variation did not exceed 17%. Recoveries were determined using samples fortified at FB_1 levels varying from 50 to 300 μg/kg, while for FB_2 and FB_3, the fortification levels ranged from 25 to 150 μg/kg. Mean recoveries ($n = 6$) for FB_1, FB_2, and FB_3 were respectively 84 ± 10, 78 ± 7, and 87 ± 9%. Results were not corrected for recovery.

Statistical Analyses. The results from fumonisin analyses obtained by the LC−MS/MS method were subjected to statistical analyses (χ^2 and ANOVA model III tests) using SAS Enterprise Guide software (SAS Institute Inc., Cary, NC, version 3.0.0.369). A probability value of 0.05 has been used to determine the statistical significance.

RESULTS AND DISCUSSION

"Flow-Through" Enzyme Immunoassay. On the basis of the fumonisin contamination data for cornflakes collected in the SCOOP project (*22*) and Petersen and Thorup (*23*), it was our goal to situate the visual detection limit of the flow-through assay in the concentration range of 200−300 μg of FB_1 equivalent/kg. The working solutions of the immunoreagents were optimized by trial and error using cornflakes spiked with a standard mixture in methanol containing FB_1 and FB_2 each at 10 ng/μL. A good color intensity (average $\Delta E^*ab = 10.45 \pm 1.23$, $n = 12$) for the blank in combination with complete color inhibition (average $\Delta E^*ab = 6.35 \pm 0.15$, $n = 12$) at 275 μg of FB_1 equivalent/kg was obtained. Therefore, 275 μg of FB_1 equivalent/kg was selected as the visual detection limit. Of the 90 cornflake samples screened with the flow-through enzyme immunoassay, 26 samples were evaluated as "noncompliant"; i.e., a total inhibition of color development was observed indicating that fumonisin contamination was exceeding the 275 μg/kg level. Quantitative LC−MS/MS analyses confirmed that fumonisin concentrations were higher than the visual detection limit for 14 of the 26 "noncompliant" screening results. **Figure 1** shows that, for all of the cornflake samples assessed as "compliant" in the flow-through screening, fumonisin concentrations obtained by LC−MS/MS were lower than 275 μg of FB_1 equivalent/kg. Thus, no false compliant results were observed, and the percentage of false noncompliant results was 18%. This was slightly higher compared to the rates obtained in a previous study for the matrix maize (*33*) and could be due to the complexity of the cornflake matrix. It should be emphasized that for any qualitative screening method working at a specific cutoff level, it is important to avoid false compliant results, because the noncompliant results are confirmed by traditional analytical methods. The presented flow-through assay fulfilled this criterion. Moreover, the method proved its potential as a screening tool, not only with respect to the speed of results but also because of its ease of use and low cost. Additionally, when the immunoreagent concentrations are changed, the visual detection limit can easily be adapted to meet the limits of the upcoming European legislation. Maximum limits for the sum of FB_1 and FB_2 currently considered in the Expert Committee are respectively 2000 μg/kg for unprocessed maize, 1000 μg/kg for maize meal, maize flour, and maize grits, 400 μg/kg for maize for direct human consumption, and 200 μg/kg for maize-based food for infants and young children.

LC−MS/MS Analyses. A total of 65% of the 205 analyzed samples were contaminated with FB_1, FB_2, and FB_3 concentrations higher than their respective limits of detection. FB_1 concentrations always exceeded FB_2 and FB_3 concentrations. This follows the general pattern of fumonisin contamination in maize and maize-based food. There was an almost perfect correlation between the occurrence of these fumonisins, with a correlation coefficient of respectively 0.98, 0.98, and 1 for FB_1−FB_2, FB_1−FB_3, and FB_2−FB_3. Occurrence data of respectively FB_1, FB_2, and FB_3 in cornflakes collected during the sampling period are shown in **Table 1**. FB_1 concentrations ranged from not detected (nd) to 464 μg/kg with a mean and median concentration of respectively 104 ± 113 and 54 μg/kg. For FB_2 and FB_3, the concentration ranges varied respectively from nd to 43 μg/kg and from nd to 90 μg/kg. Mean concentrations for FB_2 and FB_3 were respectively 12 ± 8 and 21 ± 15 μg/kg, while the median concentration was 11 μg/kg for FB_2 and 19 μg/kg for FB_3. The distributions of the FB_1, FB_2, and FB_3 surveys were right asymmetric (skewness factors = 0.82−1.60), indicating the presence of outliers with high values. Indeed, 9.8% of the analyzed samples had FB_1 concentrations higher than 300 μg/kg. For FB_2, only 1.5% of the samples had a

Table 1. Occurrence and Levels of (a) FB_1, (b) FB_2, and (c) FB_3 in Cornflakes Collected on the Belgian Market (2003–2004)

a. occurrence of FB_1	organic	conventional	total
number of samples	75	130	205
nd[a] (%)	6 (8.0)	64 (49.2)	70 (34.1)
traces[b] (%)	8 (10.7)	8 (6.2)	16 (7.8)
40–100 μg/kg (%)	22 (29.3)	21 (16.1)	43 (20.9)
101–200 μg/kg (%)	19 (25.3)	17 (13.1)	36 (17.6)
201–300 μg/kg (%)	12 (16.0)	8 (6.2)	20 (9.8)
>300 μg/kg (%)	8 (10.7)	12 (9.2)	20 (9.8)
overall incidence (%)	92.0	50.8	65.9
level of contamination (μg/kg)			
mean[c] ± SD	144 ± 114	82 ± 106	104 ± 113
median[c]	116	30	54
range[d]	40–464	40–393	40–464

b. occurrence of FB_2	organic	conventional	total
number of samples	75	130	205
nd[e] (%)	5 (6.7)	66 (50.7)	71 (34.6)
traces[f] (%)	35 (46.7)	23 (17.7)	58 (28.3)
15–30 μg/kg (%)	32 (42.6)	41 (31.5)	73 (35.6)
31–50 μg/kg (%)	3 (4)	0 (0)	3 (1.5)
>50 μg/kg (%)	0 (0)	0 (0)	0 (0)
overall incidence (%)	93.3	49.3	65.4
level of contamination (μg/kg)			
mean[g] ± SD	16 ± 8	10 ± 8	12 ± 8
median[g]	11	4	11
range[h]	15–43	15–27	15–43

c. occurrence of FB_3	organic	conventional	total
number of samples	75	130	205
nd[i] (%)	5 (6.7)	66 (50.8)	71 (34.6)
traces[j] (%)	45 (60.0)	23 (17.7)	68 (33.2)
25–50 μg/kg (%)	18 (24.0)	39 (30.0)	57 (27.8)
51–60 μg/kg (%)	3 (4.0)	2 (1.5)	5 (2.4)
>60 μg/kg (%)	4 (5.3)	0 (0)	4 (2.0)
overall incidence (%)	93.3	49.2	65.4
level of contamination (μg/kg)			
mean[k] ± SD	26 ± 17	18 ± 13	21 ± 15
median[k]	19	19	19
range[l]	25–90	25–50	25–90

[a] nd = not detected (FB_1 concentration <20 μg/kg). [b] Traces (20 μg/kg ≤ FB_1 concentration < 40 μg/kg). [c] Mean and median are computed assuming that nondetected samples contain half of the LOD, while samples with traces of FB_1 contain half of the LOD and LOQ. [d] Range of samples with FB_1 concentrations ≥40 μg/kg. [e] nd = not detected (FB_2 concentration <7.5 μg/kg). [f] Traces (7.5 μg/kg ≤ FB_2 concentration < 15 μg/kg). [g] Mean and median are computed assuming that nondetected samples contain half of the LOD, while samples with traces of FB_2 contain half of the LOD and LOQ. [h] Range of samples with FB_2 concentrations ≥15 μg/kg. [i] nd = not detected (FB_3 concentration <12.5 μg/kg). [j] Traces (12.5 μg/kg ≤ FB_3 concentration < 25 μg/kg). [k] Mean and median are computed assuming that nondetected samples contain half of the LOD, while samples with traces of FB_3 contain half of the LOD and LOQ. [l] Range of samples with FB_3 concentrations ≥25 μg/kg.

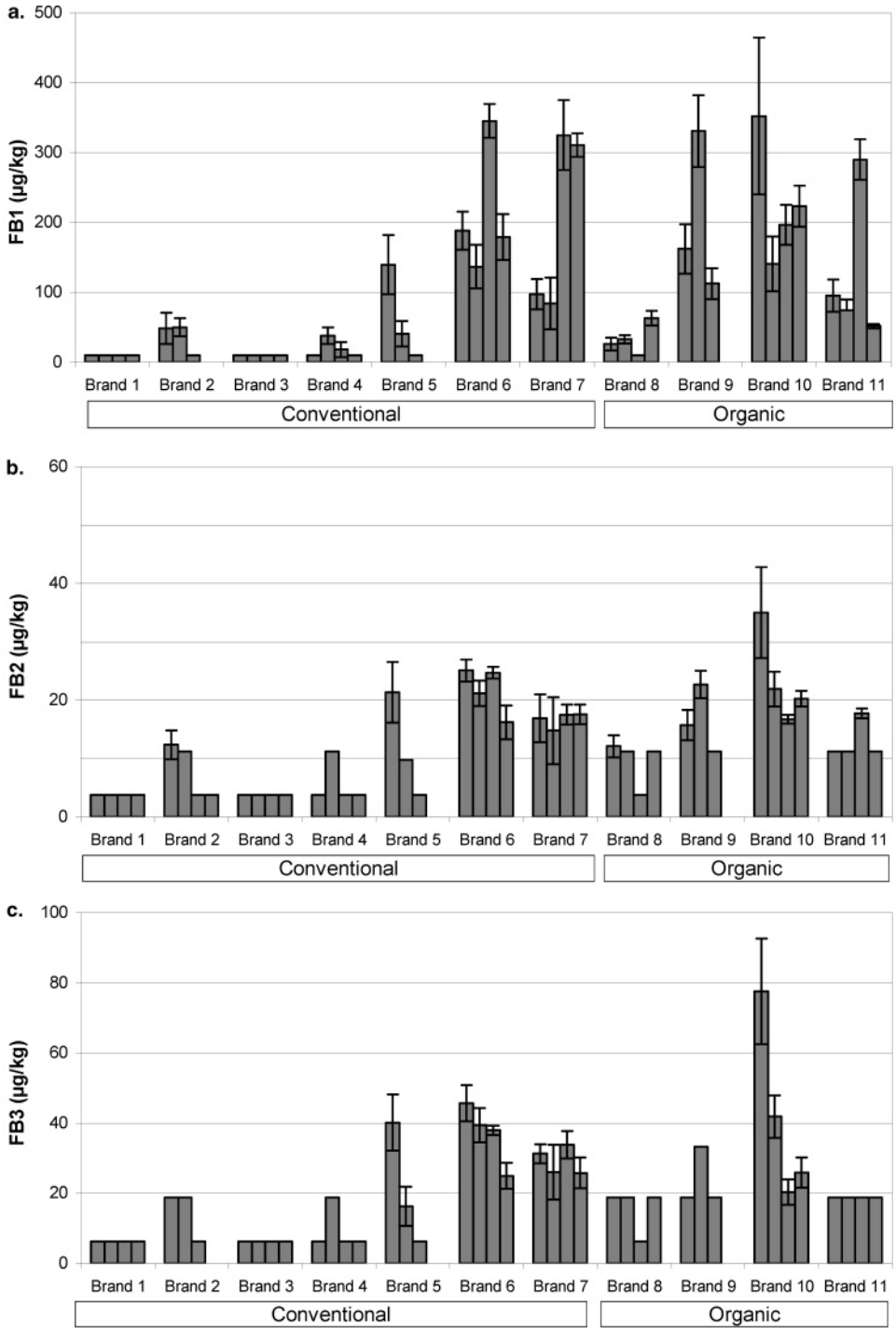

Figure 2. Occurrence data of (a) FB_1 (μg/kg), (b) FB_2 (μg/kg), and (c) FB_3 (μg/kg) in cornflakes collected on the Belgian market. Each brand ($n = 11$) is represented by 3 or 4 batches. Values are the average of five samples for each batch.

contamination level higher than 30 μg/kg, whereas for FB_3, high concentrations (>50 μg/kg) were found for 4.4% of the analyzed samples. Overall, the levels of FB_1 and FB_2 found in the cornflake products in this survey were in accordance with contamination data reported in European studies.

The SCOOP task 3.2.10 reported that 46% of the samples had FB_1 concentrations ranging from 5 to 1092 μg/kg, while the mean was 74 μg/kg. FB_2 concentrations varied from 8 to 235 μg/kg with a mean value of 53 μg/kg (*22*). Petersen and Thorup reported 6 of 10 cornflake samples with detectable FB_1 concentrations (LOD = 5 μg/kg) with a mean value of 110 μg/kg. For FB_2, detectable concentrations ranged from 4 to 243 μg/kg with a mean value of 25 μg/kg (*23*). A comparison of FB_3 results cannot be made because this is the first European survey that includes FB_3 determinations.

Comparison between Conventional and Organic Agricultural Practices. Statistical χ^2 tests were performed to evaluate whether the frequency of samples with contamination levels above the respective LOQs was equal between organic and conventional production. There was an extremely significant difference for FB_1 ($\chi^2_1 = 26.3312$, $p < 0.0001$), with 81.3% organic samples with contamination levels above the LOQ concentration compared to 44.6% samples originating from conventional production. Also for FB_2, a significant difference was observed between the percentage of organic samples with FB_2 concentrations above the LOQ (46.6%) and that for

conventional samples (31.5%) ($\chi^2_1 = 4.6658$, $p = 0.0308$). Proportions of FB_3 contamination (>LOQ) of conventional and organic origin were statistically equivalent ($\chi^2_1 = 0.2849$, $p = 0.5935$). Analyses of variance were performed on quantifiable concentrations using the GLM procedure of SAS (ANOVA model III) to evaluate the following factors: agricultural practice, brand, and batch. The agricultural practice did not have any statistically significant effect on the FB_1, FB_2, and FB_3 concentrations [$p(FB_1) = 0.6850$, $p(FB_2) = 0.9759$, and $p(FB_3) = 0.6676$], although the incidence of samples with detectable fumonisin concentrations was higher for organic products. These observations illustrate that frequency and actual concentrations do not always coincide. Malmauret et al. reported similar findings for deoxynivalenol in wheat, where the rate of contamination of organic wheat samples with deoxynivalenol was approximately 40% lower than that of conventional ones but the median and maximum levels of this mycotoxin were more than 2-fold higher in organic than in conventional crops (*29*). Cornflake processing may vary considerably from plant to plant depending on the time and temperature of cooking, the kind of additives (salts, iron, vitamins, sugars, etc.) and the quality of the raw material (corn variety, with or without germ and bran layers, etc.). Therefore, the effect of the brand was evaluated, but no significant variations between contamination of different brands [$p(FB_1) = 0.3917$, $p(FB_2) = 0.8178$, and $p(FB_3) = 0.8318$] were observed. However, the variation in fumonisin concentrations between the different batches was significant [$p(FB_1) < 0.0001$, $p(FB_2) < 0.0001$, $p(FB_3) < 0.0001$]. This is visually demonstrated in parts **a**−**c** of **Figure 2** containing the mean concentrations (5 samples of each batch) of respectively FB_1, FB_2, and FB_3 per batch ($n = 3$ or 4) for each brand analyzed ($n = 11$). This could be due to a different level of fumonisin contamination of the raw materials employed in the production of each batch. These observations are in line with the heterogeneous distribution of mycotoxins in bulk commodities and emphasize the importance of a well-defined sampling plan in the process of risk management and food safety control.

In summary, this study demonstrated the practicability of a screening test coupled to an intralaboratory-validated LC−MS/MS method for the management of the food safety risks generated by fumonisins. This survey reflected the situation of cornflake products on the Belgian market during 2003−2004. Fumonisin concentrations found were generally low; however, batches with a considerable contamination (FB_1 concentrated >300 μg/kg) did occur. From the statistical tests (χ^2 and ANOVA model III) performed, we could conclude that in terms of fumonisin occurrence (>LOD), a significant difference was observed between organic and conventional samples. On the other hand, in terms of fumonisin concentrations (>LOQ), the agricultural practice did not have any significant effect but the variation between different batches was significant ($p < 0.0001$).

Safety. Fumonisins have neurotoxic and nephrotoxic properties. On the basis of the available toxicological data, the IARC has classified "toxins produced by *Fusarium moniliforme*" as potential carcinogens for humans (class 2B carcinogens) (*38*). Gloves were worn when working with standards and samples. Standard and sample preparation was done in a fume hood. Glassware and fumonisin waste were decontaminated by keeping them for 24 h in sodium hypochlorite solution (household bleach). Afterward, decontaminated glassware was washed with detergent and rinsed with water.

LITERATURE CITED

(1) Soriano, J. M.; Dragacci, S. Occurrence of fumonisins in foods. *Food Res. Int.* **2004**, *37*, 985−1000.

(2) Shephard, G. S.; Thiel, P. G.; Stockenstrom, S.; Sydenham, E. W. Worldwide survey of fumonisin contamination of corn and corn-based products. *J. AOAC Int.* **1996**, *79*, 671−687.

(3) Trucksess, M. W. Mycotoxins. *J. AOAC Int.* **2001**, *84*, 202−211.

(4) Weidenbörner, M. Food and fumonisins. *Eur. Food Res. Technol.* **2001**, *212*, 262−273.

(5) Marasas, W. F. O. Discovery and occurrence of the fumonisins: A historical perspective. *Environ. Health Perspect.* **2001**, *109*, 239−243.

(6) FAO/WHO, Summary and Conclusions, in 56th joint FAO/WHO expert committee on food additives, Geneva, Switzerland, February 6−15, 2001.

(7) Gelderblom, W. C. A.; Abel, S.; Smuts, C. M.; Marnewick, J.; Marasas, W. F. O.; Lemmer, E. R.; Ramljak, D. Fumonisin-induced hepatocarcinogenesis: Mechanisms related to cancer initiation and promotion. *Environ. Health Perspect.* **2001**, *109*, 291−300.

(8) Voss, K. A.; Riley, R. T.; Norred, W. P.; Bacon, C. W.; Meredith, F. I.; Howard, P. C.; Plattner, R. D.; Collins, T. F. X.; Hansen, D. K.; Porter, J. K. An overview of rodent toxicities: Liver and kidney effects of fumonisins and *Fusarium moniliforme*. *Environ. Health Perspect.* **2001**, *109*, 259−266.

(9) Galvano, F.; Campisi, A.; Russo, A.; Galvano, G.; Palumbo, M.; Renis, M.; Barcellona, M. L.; Perez-Polo, J. R.; Vanella, A. DNA damage in astrocytes exposed to fumonisin B1. *Neurochem. Res.* **2002**, *27*, 345−351.

(10) Zhang, H.; Nagashima, H.; Goto, T. Natural occurrence of mycotoxins in corn, samples from high and low risk areas for human esophageal cancer in China. *Mycotoxins* **1997**, *44*, 29−35.

(11) Franceschi, S.; Bidoli, E.; Baron, A. E.; Lavecchia, C. Maize and risk of cancers of the oral cavity, pharynx, and esophagus in Northeastern Italy. *J. Natl. Cancer Inst.* **1990**, *82*, 1407−1411.

(12) Humpf, H.-U.; Voss, K. Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Mol. Nutr. Food Res.* **2004**, *48*, 255−269.

(13) Castelo, M.; Sumner, S.; Bullerman, L. Stability of fumonisins thermally processed corn products. *J. Food Prot.* **1998**, *61*, 1030−1033.

(14) Saunders, D.; Meredith, F.; Voss, K. Control of fumonisin: Effects of Processing. *Environ. Health Perspect.* **2001**, *109*, 333−336.

(15) De Girolamo, A.; Solfrizzo, M.; Visconti, A. Effect of processing on fumonisin concentration in corn flakes. *J. Food Prot.* **2001**, *64*, 701−704.

(16) Meister, U.; Springer, M. Mycotoxins in cereals and cereal products—Occurrence and changes during processing. *J. Appl. Bot. Food Qual.* **2004**, *78*, 16−173.

(17) Kim, E.-K.; Scott, P.; Lau, B. Hidden fumonisin in cornflakes. *Food Addit. Contam.* **2003**, *20*, 161−169.

(18) Shier, W. The fumonisin paradox: A review of research on oral bioavailability of fumonisin B1, a mycotoxin produced by *Fusarium moniliforme*. *J. Toxicol., Toxin Rev.* **2000**, *19*, 161−187.

(19) Seefelder, W.; Knecht, A.; Humpf, H.-U. Bound fumonisin B_1: Analysis of fumonisin B_1 glyco and amino acid conjugates by liquid chromatography−electrospray ionization−tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 5567−5573.

(20) Howard, P.; Churchwell, M.; Couch, L.; Marques, M.; Doerge, D. Formation of *N*-(carboxylmethyl)fumonisin B1, following the reaction of fumonisin B1 with reducing sugars. *J. Agric. Food Chem.* **1998**, *46*, 3546−3557.

(21) Kim, E.-Y.; Scott, P.; Lau, B.; Lewis, D. Extraction of fumonisins B_1 and B_2 from white rice flour and their stability in white rice flour, cornstarch, cornmeal, and glucose. *J. Agric. Food Chem.* **2002**, *50*, 3614−3620.

(22) SCOOP Task 3.2.10. Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU Member States, 2003, available: http://europa.eu.int//food/fs/scoop/task3210.pdf.
(23) Petersen, A.; Thorup, I. Preliminary evaluation of fumonisins by the Nordic countries and occurrence of fumonisins (FB1 and FB2) in corn-based foods on the Danish market. *Food Addit. Contam.* **2001**, *18*, 221−226.
(24) European Regulation (EC) number 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs. *Off. J. Eur. Commun*. **1991**, *198*, 1−15.
(25) Biffi, R.; Munari, M.; Dioguardi, L.; Ballabio, C.; Cattaneo, A.; Galli, C. L.; Restani, P. Ochratoxin A in conventional and organic cereal derivatives: A survey of the Italian market, 2001−02. *Food Addit. Contam*. **2004**, *21*, 586−591.
(26) Jestoi, M.; Somma, M.; Kouva, M.; Veijalainen, P.; Rizzo, A.; Ritieni, A.; Peltonen, K. Levels of mycotoxins and sample cytotoxicity of selected organic and conventional grain-based products purchased form Finnish and Italian markets. *Mol. Nutri. Food Res*. **2004**, *48*, 299−307.
(27) Schollenberger, M.; Drochner, W.; Rüfle, M.; Suchy, S.; Terry-Jara, H.; Müller, H.-M. Trichothecene toxins in different groups of conventional and organic bread of the German market. *J. Food Compos. Anal*. **2005**, *18*, 69−78.
(28) Beretta, B.; De Domenico, R.; Garaschi, A.; Ballabia, C.; Galli, C.; Gigliotti, C.; Restani, P. Ochratoxin A in cereal-based baby-foods: Occurrence and safety evaluation. *Food Addit. Contam.* **2002**, *19*, 70−75.
(29) Malmauret, L.; Parent-Massin, D.; Hardy, J.-L.; Verger, P. Contaminants in organic and conventional foodstuffs in France. *Food Addit. Contam*. **2002**, *19*, 524−532.
(30) Marx, H.; Gedek, B.; Kooarczik, B. Comparative investigations of mycotoxological status of alternatively and conventionally grown crops. *Eur. Food Res. Technol.* **1995**, *201*, 83−86.
(31) Magkos, F.; Arvaniti, F.; Zampelas, A. Putting the safety of organic food into perspective. *Nutr. Res. Rev.* **2003**, *16*, 211−222.
(32) Czerwiecki, L.; Czajkowska, D.; Witkowska-Gwiazdowska, A. On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms. Part 2: Occurrence of ochratoxin A and fungi in cereals in 1998. *Food Addit. Contam.* **2002**, *19*, 1051−1057.
(33) Paepens, C.; De Saeger, S.; Sibanda, L.; Barna-Vetró, I.; Léglise, I.; Van Hove, F.; Van Peteghem, C. A flow-through enzyme immunoassay for the screening of fumonisins in maize. *Anal. Chim. Acta* **2004**, *523*, 229−235.
(34) Barna-Vetró, I.; Szabó, E.; Fazakas, B.; L. Solti, L. Development of a sensitive ELISA for the determination of fumonisin B1 in cereals. *J. Agric. Food Chem*. **2000**, *48*, 2821−2825.
(35) De Saeger, S.; Van Peteghem, C. European patent number 0893690: Detection of mycotoxins by flow-through membrane-based enzyme immunoassay. *Bulletin* **2004**, 29.
(36) European Regulation (EC) number 466/2001 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Commun*. **2001**, *77*, 1−13.
(37) Paepens, C.; De Saeger, S.; Van Poucke, C.; Dumoulin, F.; Van Calenbergh, S.; Van Peteghem, C. Development of a LC−MS/MS method for the quantification of fumonisin B_1, B_2, and B_3 in cornflakes. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2021−2019.
(38) World Health Organization−International agency for research on cancer (WHO−IARC), 56 (1993) 445, Toxins derived from *Fusarium moniliforme*: Fumonisin B_1 and B_2 and fusarium C. IARC Monographs on the evaluation of carcinogenic risks to humans.

Received for review April 15, 2005. Revised manuscript received July 13, 2005. Accepted July 15, 2005. This work was carried out within the framework of the SPSDII-CPAA15 project "Mycotoxin contamination of regular and organic foodstuffs", which is supported by the Belgian Federal Science Policy Office and the FWO Flanders Research project number G.0312.03.

JF050858D