A simple, sensitive, and reliable analytical method is developed for the rapid determination of fumonisin B1 and fumonisin B2 in corn by high-performance liquid chromatography–positive electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS). Fumonisin B1 and fumonisin B2 are extracted from corn with methanol–water (3:1, v/v) by means of ultrasonic extraction, and directly injected into an LC–MS–MS system after centrifugation. Fumonisin B1 and fumonisin B2 are separated on a Zorbax Eclipse XDB-C18 column with a solution of methanol–water–formic acid as the mobile phase. The method is validated with respect to linearity, accuracy, precision, specificity, and stability. Moreover, the method was applied to real samples and demonstrated to be suitable for the determination of fumonisin B1 and fumonisin B2 in corn. The total time required for the analysis of one sample was ≈30 min.

Introduction

Fumonisins are a group of mycotoxins produced principally by *Fusarium moniliforme*, which is a worldwide contaminant of corn and corn products consumed by humans and animals (1–3). There are many naturally produced forms of fumonisins, of which B-series fumonisins are the most abundant analogs (4). Among B-series fumonisins, fumonisin B1 (FB1) and fumonisin B2 (FB2) appear to be biologically significant (5). The chemical structure of FB1 is 2S-amino-1,2S,16R-dimethyl-3S,5R,10R,14S,15R-pentahydroxyecosane with the C-14 and C-15 hydroxy groups esterified by a terminal carboxyl group of propane-1,2,3-tricarboxylic acid; FB2 is 10-deoxy FB1 (Figure 1). Fumonisins are animal carcinogens, and long-term exposure to fumonisins induces liver and kidney tumors in rats (6–7). Human consumption of fumonisin-contaminated corn has been associated with increased risk for esophageal cancer in humans (8–10). The International Agency for Research on Cancer (IARC) classified FB1 as a probable human carcinogen (class 2B) (11). To protect human health, the European Union (EU) has set a maximum level of 1000 μg/kg for the sum of FB1 and FB2 in corn and corn-based foods intended for direct human consumption (12). Therefore, rapid, specific, and sensitive methods for the identification and quantification of FB1 and FB2 in corn are required.

To detect FB1 and FB2 in food and feed samples, many liquid chromatography methods with fluorescence detection (LC–FD) (13–31) and liquid chromatography–mass spectrometry (LC–MS and LC–MS–MS) (23, 32–41) have been developed. However, all of the LC–FD methods require a derivatization step, and false results occur frequently because of retention time shifts or interfering peaks. LC–MS–MS makes a shorter pre-treatment sample preparation possible due to its higher selectivity and sensitivity for the analysis of fumonisins than the LC–MS and LC–FD methods. Recently, several rapid LC–MS–MS methods for the analysis of fumonisins in biological samples have been developed without a solid-phase extraction (SPE) procedure (32, 40, 42). Among them, the method developed by D’Arco et al. was the fastest; the total time for the analysis of one sample is ≈45 min, including 12 min for the sample extraction by means of pressurized liquid extraction (SPE) and 8 min for the LC–MS–MS analysis. However, an expensive accelerated solvent extraction (ASE) system must be used in the method. Moreover, recoveries only ranged from 68% to 83% at fortification levels of 200 μg/kg because the extract must be concentrated to dryness by a complicated concentration procedure. Ultrasonic extraction (USE) is a fast and economic method for extraction. Until recently, there have not been methods for extracting fumonisins in biological samples using USE, with only a 4 min LC–MS–MS analysis. At the same time, a method without concentration procedure will be beneficial for improved recovery.

The purpose of this study was to develop a simple, rapid, and sensitive method for the simultaneous determination of FB1 and FB2 in corn by LC–MS–MS. The pretreatment procedure is very simple without purification, and the total time for the analysis of one sample is ≈30 min.

Experimental

Materials and reagents

Acetonitrile, methanol, and formic acid were of LC grade. FB1 and FB2 were purchased from Sigma (St. Louis, MO). Water was purified with a Milli-Q reverse osmosis system (Millipore, Milford, MA).

Standard solutions

The individual stock solutions of FB1 and FB2 (100 μg/mL) were prepared in acetonitrile–water (50:50, v/v) and stored at 4°C. Three working standard solutions of FB1 and FB2 (10, 20 and 30 μg/mL) and one working standard solution (0.226 μg/mL for FB1 and 0.174 μg/mL for FB2) were prepared by diluting and mixing each stock standard solution with methanol–water (3:1, v/v). Mixed matrix-matched standard solutions (0–400 μg/L) were prepared by diluting and mixing each
stock solution with the extract obtained from a blank corn sample.

Chromatographic conditions
A Waters 2695 LC instrument was used in this study. Separation was carried out on a Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 μm) maintained at 30°C. The LC mobile phase consisted of methanol–water–formic acid (75:25:0.2, v/v/v). The flow rate was 0.2 mL/min. The injection volume was 10 μL, and the runtime was 4 min.

Mass spectrometry conditions
Detection was carried out by a Waters Quattro Micro™ API triple-quadrupole MS fitted with an electrospray ionization (ESI) probe and operated in the positive ion mode. The following parameters were optimal: capillary voltage, 3500 V; ion source temperature, 120°C; desolvation gas temperature, 350°C; and desolvation gas flow rate, 600 L/h. Detection was carried out in multiple reaction monitoring (MRM) mode. Argon was used as the collision gas, and the collision cell pressure was 3.8 mBar. Other parameters are shown in Table I.

Sample preparation
The fresh corn samples were bought from three local supermarkets. After being dried and grounded to a fine meal in a laboratory mill, the blank corn (5 g) was fortified with FB1 and FB2 by adding 0.25 mL fortifying standard solution in polypropylene tube; it was then vortex-mixed for 15 s and allowed to stand at room temperature for 30 min. The fortified samples contained FB1 and FB2 at a limit of quantitation of (LOQ, 11.7 μg/kg for FB1 and 8.3 μg/kg for FB2) 500, 1000, and 1500 μg/kg.

Ultrasonic extraction
Extraction was performed in an ultrasonic clean bath (300 × 150 mm, Kunshan Ultrasonic Instrument Co. Ltd., Jiangshu, China). Methanol–water (25 mL, 3:1, v/v) was added to 5 g of the fortified corn in polypropylene tube. After the tube was placed in the clean bath, the extraction process was conducted for 10 min at room temperature, and the output powder was set at 120 W. After ultrasonication, the tube was centrifuged at 5000 rpm for 5 min, followed by filtration with a 0.22-μm filter prior to injection into the LC system.

Confirmation
For confirmation of FB in corn, the following three criteria had to be met: (i) the retention time should be within ±2.5% of the external standard solution; (ii) the signal-to-noise ratio (S/N) for each diagnostic ion should be ≥3:1; and (iii) the relative abundance of the two reaction product ions of the samples should be within an acceptable range relative to the average external standards according to the Commission Decision 2002/657/EC for LC–MS methods (43).

Method Validation
An "in house" validation was applied in accordance with an internal procedure as well as the Commission Decision 2002/657/EC (43).

Specificity
To verify the absence of interfering substances around the retention time of analytes, 20 blank corn samples were analyzed.

Linearity
Calibration curves were constructed using matrix-matched standard solutions by plotting the peak area of the quantitative ion of each analyte versus concentrations of 0, 2, 5, 20, 50, 100, and 400 μg/L.

Limit of detection and quantitation
The limits of detection (LODs), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were estimated for those concentrations that provide a signal-to-noise ratio of 3:1. The LOQs were estimated at a signal-to-noise ratio of 10:1.

Table I

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent Ion (m/z)</th>
<th>Daughter Ion (m/z)</th>
<th>Dwell Time (s)</th>
<th>Collision Energy (eV)</th>
<th>Cone Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1</td>
<td>722.4</td>
<td>352.4−(M + H−2TCA+H2O) +</td>
<td>0.5</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>334.4−(M + H−2TCA+2H2O) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FB2</td>
<td>706.4</td>
<td>336.4−(M + H−2TCA+H2O) +</td>
<td>0.5</td>
<td>34</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>318.4−(M + H−2TCA+2H2O) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Ion for quantification, TCA was tricarboxylic acid.
Accuracy and precision
The accuracy and precision (inter-day and intra-day) of the method were determined using a blank corn sample that was fortified at MRL level (1000 µg/kg), at half of the MRL level (500 µg/kg), at one and a half of the MRL level (1500 µg/kg), and at the LOQ (11.7 µg/kg for FB1 and 8.3 µg/kg for FB2). The intra-day precision was assessed by performing six runs of each level during a single day, and the inter-day precision was assessed by performing six runs of each level per day over three different days.

Stability
The stability was determined in two different ways: (A) in solvent (stock solutions), and (B) in a matrix (fortified corn at 1000 µg/kg).

Results and Discussion
Optimization of extraction conditions
Many methods for the determination of FB1 and FB2 use methanol, acetonitrile, or mixtures of both solvents with water as an extraction solution. Among them, methanol–water (3:1 v/v) and acetonitrile–water (1:1, v/v) were used most frequently (44) because they all can provide good extraction efficiency for FB1 and FB2. In this study, the extraction efficiency was studied with methanol–water (3:1, v/v) as an extraction solution. As shown in Figure 2, extraction efficiencies of FB1 and FB2 reached nearly 71.3% and 73.4%, using 25 mL of the extraction solution in the first 5 min. When the extraction lasted for 10 min, ≏ 93.0% of FB1 and 92.6% of FB2 were extracted. A complete extraction of FB1 and FB2 was achieved at 10 min with 25 mL of the extraction solution or 15 min with 10 mL of extraction solution. Thus, the optimized ultrasonication time was 10 min, and 25 mL of the extraction solution was used.

Instrument conditions
Most of methods used gradient conditions to separate fumonisins (23, 32, 34–35, 37–38, 42). Faberi et al. used an Alltima C18 column to successfully separate fumonisins under isocratic condition at a flow of 0.3 mL/min. The mobile phase was acetonitrile–water (60:40, v/v) containing 0.3% formic acid. The runtime was 15 min. A successful separation of FB1 and FB2 was also obtained using a Zorbax Eclipse XDB-C18 column under isocratic conditions with methanol–water–formic acid (75:25:0.2, v/v/v) as the mobile phase. Moreover, the runtime was only 4 min. The capacity factor (k') was 2.4 and 3.0 for FB1 and FB2, respectively. The chromatogram obtained from corn fortified at LOQ is depicted in Figure 3, demonstrating a good chromatography separation and satisfactory sensitivity.

For the mycotoxins included in Group B of the Annex I of Council Directive 96/23/EC (45), a minimum of three identification points are required. In this experiment, four identification points, one parent (1 point) and two transitions (each 1.5 points), were monitored. Most of the previously developed LC–MS–MS methods of fumonisins are exclusively analyzed with ESI (43). The precursor ion [M+H]+ was chosen because the ion was the most abundant peak in the mass spectra when the mobile phase consisted of methanol–water–formic acid (75:25:0.2, v/v/v).

Confirmation
The retention time of each FB in the fortified samples was within ± 2.5% of the external standards. The signal-to-noise for all the daughter ions listed in Table I was ≥ 3:1. The ratios of the daughter ions of the fortified samples were always within 20% of the ratios of the concurrently analyzed standards.

Method Validation
Linearity
One significant drawback in ESI-MS quantitative analysis is matrix effect. In this study, the matrix effect was evaluated comparing the detector responses from the standard solutions of the FBs in solvent with those from different matrix extracts added of FBs at different concentration levels. From the calculated matrix effect results, it could be concluded that there are ≏ 1.5 times enhancement effect for both FBs, and it did not vary significantly between the individual matrix samples. So, the external calibration using matrix-matched samples was obtained.
by plotting the peak area of the quantitative ion pair of each analyte versus concentrations in 0–400 μg/L. The results of the linearity are reported in Table II. The correlation coefficients (r) of the calibration curves were 0.9970 and 0.9991.

### Specificity

The specificity was evaluated by analyzing 20 blank corn samples. Figure 3 indicate that there were no interfering peaks from endogenous compounds at the retention times of FB1 and FB2.

### Accuracy and precision

The recovery and reproducibility of the method were measured by analyzing seventy-two blank samples fortified with FB1 and FB2 at LOQ (11.7 μg/kg for FB1 and 8.3 μg/kg for FB2), 500, 1000, and 1500 μg/kg on three separate occasions. The results are shown in Table III. The mean recoveries were between 82.6% and 94.4%. The intra-day and inter-day RSDs ranged from 3.08% to 7.91% and 5.28% to 8.62%, respectively.

### LOD and LOQ

The LODs were 3.5 μg/kg and 2.5 μg/kg for FB1 and FB2, respectively. The LOQs were 11.7 μg/kg and 8.3 μg/kg for FB1.
and FB2, respectively. The LOD of this method is better or similar to some of the previously published LC–MS and LC–MS–MS methods for analyzing FB1 and FB2 in corn or corn-based food (23, 36, 39, 41).

**Stability**

The stock standard solutions in acetonitrile/water were stored at 4°C for at least 6 months. The stock solutions were analyzed every month, and the instrumental responses were compared with the peak areas obtained at the moment of solution preparation (t = 0). The acceptance criterion was a response comprised between 95 and 105% of the initial one. Fortified corn samples at 1000 mg/kg were stored at –20°C and were analyzed after 7, 14, and 28 days. It was found that the recoveries of FB1 and FB2 had no significant change.

**Applications of the method**

The applicability of the method for routine analysis was evaluated by analyzing 62 corn samples obtained from three local supermarkets. Positive results were simultaneously quantified and identified by the acquisition of two transitions for each compound and confirmed based on the Council Directive 96/23/EC (45). None of the samples exceeded the limits established by European Union. The highest concentration was 769 mg/kg with 475 mg/kg for FB1 and 294 mg/kg for FB2, respectively. In the other three detected samples, the concentration of FB1 and FB2 were between 37.6 mg/kg (FB1 + FB2) and 133 mg/kg (FB1 + FB2). Figure 4 shows a chromatogram of a positive corn sample.

**Conclusion**

In the present study, a rapid and sensitive method for the simultaneous determination of FB1 and FB2 in corn by LC–MS–MS with USE and without SPE cleanup procedure was developed. This method was validated with fortified corn samples and good recoveries with excellent RSDs were obtained. The LODs were found to be sufficiently low to determine the residues of FB1 and FB2 in corn.

<table>
<thead>
<tr>
<th>Fortified conc. (mg/kg)</th>
<th>Mean recovery (%; n = 6)</th>
<th>RSD (%)</th>
<th>Inter-assay RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1</td>
<td>11.7</td>
<td>82.6, 85.1, 92.0</td>
<td>6.08, 4.27, 5.77</td>
</tr>
<tr>
<td>FB2</td>
<td>8.3</td>
<td>84.5, 87.8, 90.3</td>
<td>5.02, 6.24</td>
</tr>
</tbody>
</table>

**Table III**

Mean Recoveries (MR) and Relative Standard Deviation (RSD) of FB1 and FB2 from Corn by LC–MS–MS

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for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. 


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