Analysis of *Fusarium* Toxins in Processed Grain Products Using High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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**Abstract**

An analytical method for the simultaneous detection of five types of *Fusarium* toxins (nivalenol (NIV), deoxynivalenol (DON), HT-2 toxin (HT-2), T-2 toxin (T-2), and zearalenone (ZEN)) in processed grains has been developed. Mycotoxins in the sample were extracted using acetonitrile-water (85:15 v/v), purified using MultiSep 226 AflaZon+ multi-functional cartridge columns, and quantified using high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). With wheat flour, the proposed method showed good linearity ($r > 0.999$), and the accuracy was 85 - 97%. The RSD values of the intra-day precision were 1.9 - 13.3% and those of the inter-day precision were 10.2 - 19.6%. In a study of commercial foodstuffs, the limit of quantification was 15 μg/kg for NIV and DON, and 5 μg/kg for HT-2, T-2, and ZEN. Using the validated method, we conducted a contamination study of 55 processed food samples, including a variety of wheat and corn products. DON was detected in 36 samples, with a maximum concentration of 238 μg/kg in corn grits. In addition, HT-2 was detected in six samples, and T-2 was detected in two samples, in products that contained wheat as the raw material, with concentrations of less than 20 μg/kg.

**Keywords:** *Fusarium* toxins; Grain product; Multi-functional cartridge column; LC-MS/MS

1. **Introduction**

Molds from the genus *Fusarium* are called field fungi, and are plant pathogens that propagate by penetrating plant tissues during crop cultivation. These molds are soil fungi and are present around the world [1-3]. The mycotoxins produced by molds of the genus *Fusarium* include trichothecenes, zearalenone (ZEN), and fumonisin. Trichothecenes are over 100 types of compounds that cause toxemia, including nivalenol (NIV), deoxynivalenol (DON), HT-2 toxin (HT-2), and T-2 toxin (T-2) [1-6]. The toxicity of trichothecenes mainly causes digestive system disorders and immune system suppression, and it is known to cause the acute poisoning of vomiting, nausea, dizziness, diarrhea, bleeding, skin inflammation, decreased hematopoietic function, etc to various animals [1-6]. ZEN that does not have a trichothecene skeleton has strong estrogenic activity, which is characterized by female hormone activity in livestock, and it has been reported with trichothecenes in many co-pollution cases [2-5]. Health issues in livestock are currently a worldwide issue. Because of the worldwide presence of *Fusarium* toxins, there is a concern that they could contaminate important grains such as wheat and corn, and cause health issues in humans and livestock [4-7].

In recent years, the calorie-based food self-sufficiency rate of Japan has remained at around 40%, with about 60% of the food supply dependent on imports from overseas. In grains that are staple food, the calorie base rate of self-sufficiency of wheat is only 10% or more, and imports account for about 90% of domestic consumption of wheat [8]. In addition, because of changes in eating habits, the...
importation of processed foods based on wheat and corn as raw materials, such as cereal, pasta, and biscuits, is increasing. Therefore, the mycotoxin contamination of imported grains, which may serve as staple foods, should be carefully monitored.

In relation to the contamination of food by *Fusarium* toxins, Food Sanitation Act of Japan only set a provisional Maximum Residue Limit (MRL) for the presence of DON in wheat (1.1 mg/kg) [9-12]. In the notification method, DON is quantified by HPLC-UV and identified by LC-MS or GC-MS [11]. However, purification of the sample is not sufficient in the notification method. Hence, for a variety of foods, it will not be possible to use HPLC-UV and LC-MS to measure satisfactorily low concentrations of mycotoxins. On the other hand, complicated derivatization is required for a GC-MS analysis. Therefore, we examined LC-MS/MS and attempted a quantitative analysis of DON. Previously, we reported an analytical method for the detection of DON using LC-MS/MS [13]. However, this method was limited to wheat, and the analysis of processed food was difficult.

In addition, the many co-pollution cases of DON have been reported with ZEN and other *Fusarium* toxins [2-5]. The contamination of grains by trichothecene mycotoxins has frequently been studied and reported for feed, beer, and biscuits with LC/MS and LC-MS/MS [14-22]. However, there are few reports for the processed foods. Therefore, an analytical method corresponding to processed food was needed for the detection of other *Fusarium* toxins that may contaminate grains, in addition to DON.

We examined test methods for routine inspections for *Fusarium* toxins to ensure food safety, and selected the ESI-LC-MS/MS analysis method. In this study, we investigated the simultaneous analysis of four trichothecenes and ZEN (Chemical structures are shown in Fig. 1).

2. Experimental

2.1. Reagents and materials

The standards for NIV, DON, and ZEN, and standard solutions of HT-2 and T-2 at a concentration of 100 μg/mL in acetonitrile were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methanol, acetonitrile (LC-MS grade), and ammonium acetate (reagent grade), along with the acetonitrile used for extraction (pesticide residue and PCB analysis grade), were purchased from Wako Pure Chemical Industries Ltd. Ultrapure water was obtained from a Milli-Q water purification system (Merck Millipore, Darmstadt, Germany), and MultiSep 226 AflaZon+ multi-functional cartridge columns (MultiSep 226) were purchased from Romer Labs (6 mL, Union, MO, USA).

2.2. Standard solution preparation

NIV, DON, and ZEN were dissolved in acetonitrile (100 μg/mL each), and the solutions were stored at -20 °C. Portions of the three stock solutions (NIV, DON, and ZEN) and two purchased standard solutions (HT-2 and T-2) were diluted to 1 μg/mL with methanol-water (1:9 v/v) and stored at 10 °C.

2.3. Preparation of calibration curves of standard samples

Five standard solutions were analyzed by LC-MS/MS. Calibration standards ranged from 1 to 250 ng/mL. Calibration curves were constructed by plotting the peak
area of the mycotoxins from the MS chromatogram against known concentrations. A 10 μL sample of the mixture solution was injected into the LC-MS/MS. The data were subjected to linear regression analysis.

### 2.4. LC-MS/MS conditions

The LC-MS/MS analysis was performed using a Shimadzu HPLC Prominence System (Shimadzu, Kyoto, Japan) equipped with an AB Sciex QTRAP 3200® System (AB Sciex, Framingham, MA, USA). The auto-sampler temperature and injection volume were 6 °C and 10 μL, respectively. An Inertsil ODS-4 (2.1 × 150 mm, 3 μm, GL Sciences, Tokyo, Japan) was used with the flow rate of 0.2 mL/min at 40°C, and a gradient elution system with a mobile phase consisting of solvent A (10 mM ammonium acetate solution) and solvent B (methanol). The linear gradient elution conditions were 0 min (5/95 v/v% B), 12 min (100/0 v/v% B), and 12–20 min (100/0 v/v% B). The system was then returned to the initial conditions (5/95 v/v% B) for 15 min to allow for equilibration prior to the next sample injection. The MS was operated in the multiple reaction monitoring (MRM) mode with the negative ionization mode (DON, NIV) and positive ionization mode (HT-2, T-2, ZEN). The curtain gas, ion source gas 1, ion source gas 2, and collision gas were set at 10, 70, 80, and 5 psi, respectively. The ion source voltages were set at -4200 V (negative) and 5000 V (positive). LC-MS/MS parameters for five Fusarium toxins are listed in Table 1.

### Table 1. LC-MS/MS parameters for five Fusarium toxins.

| Analyte | Polarity | Quantitative ion | Qualitative ion |
|---------|----------|------------------|-----------------|
|         |          | MRM (m/z)        | DP (V) | CE (V) | MRM (m/z) | DP (V) | CE (V) |
|         |          |                  |        |        |          |        |        |
| NIV     | ESI-     | 371>281          | -15    | -18    | 311>281  | -25    | -12    |
| DON     | ESI-     | 355>265          | -15    | -16    | 295>265  | -30    | -14    |
| HT-2    | ESI+     | 442>263          | 11     | 19     | 442>215  | 11     | 19     |
| T-2     | ESI+     | 484>305          | 16     | 19     | 484>215  | 16     | 25     |
| ZEN     | ESI+     | 319>187          | 21     | 23     | 319>283  | 21     | 17     |

RT: Retention time  DP: Declustering potential  CE: Collision energy

### 2.5. Sample preparation

The samples included flour, corn grits, and imports made from these materials such as biscuits and pasta. Samples were purchased at retail stores in the Kanagawa Prefecture and from the Internet. A 25.0 g of sample was extracted with 100 mL of acetonitrile–water (85:15 v/v), followed by shaking for 30 min. The extract was transferred to a centrifuge tube and centrifuged at 3,000 rpm for 10 min. The supernatant was passed through the MultiSep 226, which did not need conditioning prior to use. The initial 4 mL was discarded. Then, 3 mL aliquots of the remaining fractions were collected, and 2 mL was taken up correctly. The eluate was dried under nitrogen at room temperature, and the residue was dissolved in 1.0 mL of aqueous methanol–water (10:90 v/v). After centrifugation (13,000 rpm, 5 min), the sample was subjected to the LC-MS/MS analysis.

### 2.6. Validation test

The validity of the analytical method was evaluated based on the accuracy, intra- and inter-day precision, linearity, sensitivity (limit of detection and limit of quantification), and selectivity [23]. For the validation test, we used flour that was not contaminated with the target mycotoxins. The flour was spiked with NIV and DON at a concentration of 20 μg/kg, and with T-2, HT-2, and ZEN at a concentration of 5 μg/kg. For the accuracy and intra-day precision, n = 5 tests were conducted on the same day, while for inter-day precision, n = 1 test was conducted over 5 days. The linearity (r) was calculated from the calibration curve obtained using a mixed standard solution prepared with 1, 5, 10, 25, 50, 100, and 250 ng/mL of the mycotoxins in methanol–water (1:9 v/v). The sensitivity was analyzed using a spiked sample standard (NIV and DON at 5 ng/mL, and T-2, HT-2, and ZEN at 2.5 ng/mL) prepared with the wheat flour extract liquid. The spiked sample standard was prepared by mixing equal amounts of the standard solution and sample extract solution. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively. For selectivity, it has confirmed that there is no peak to interfere with the quantification of the target of the mycotoxins.

### 3. Results and discussion

#### 3.1. Optimization of LC-MS/MS conditions

We selected ammonium acetate for the mobile phase additive. Typical chromatograms of the quantitative ions obtained using the MRM mode for each standard solution is shown in Fig. 2.

We examined the analytical conditions using MRM mode, and the ionization conditions were examined using the infusion method. In the negative mode, deprotonated acetic acid adducts [M+CH₃COO]⁻ (NIV m/z 371, DON m/z 355) and deprotonated ions [M-H]⁻ (NIV m/z 311, DON m/z 295) were observed from NIV and DON. In the positive mode, ammonium adduct ions [M+NH₄]⁺ (T-2 m/z 484, HT-2 m/z 442) were observed from HT-2 and T-2, and the protonated ion [M+H]⁺ (m/z 319) was observed from ZEN.

All the adducts were detected at high sensitivities,
therefore these ions were selected as precursor ions. Based on these results, we chose the quantitative and qualitative ions (Table 1) as the product ions. The optimized analytical conditions for each compound are also listed in Table 1.

3.2. Examination of sample preparation methods

The MultiSep 226 is primarily used for the analysis of aflatoxins and ZEN [16]. We thought that it could also be used for other trichothecene mycotoxins. In the present study, we examined the simultaneous analyses of trichothecene mycotoxins and ZEN with MultiSep 226. The MultiSep 226 is a preprocessing cartridge in which an anionic exchange resin and a cation-exchange resin are added to a reversed phase system resin.

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**Fig. 2.** Multiple reaction monitoring (MRM) chromatograms of five *Fusarium* toxins using LC-MS/MS. MRM chromatograms were obtained from the monitoring at m/z 371 > m/z 281 (NIV), m/z 355 > m/z 265 (DON), m/z 442 > m/z 263 (HT-2), m/z 484 > m/z 305 (T-2), and m/z 319 > m/z 187 (ZEN). Column: Inertsil ODS-3 (3 μm, 150 × 2.1 mm i.d.); eluent: solvent A (10 mM ammonium acetate solution) and solvent B (methanol), linear gradient 0 min (5v/v% B), 12 min (100v/v% B), 12–20 min (100 v/v % B); injection vol.: 10 μL; flow rate: 0.2 mL/min. (a) Standard solution: NIV and DON (25 ng/mL), HT-2, T-2, and ZEN (5 ng/mL), (b) Sample extracts of the flour fortified with 20 μg/kg NIV and DON, or 5 μg/kg T-2, HT-2, and ZEN.
To examine the loss of five *Fusarium* toxins mainly by the adsorption to MultiSep 226 cartridge, standards of each *Fusarium* toxin (0.05 μg/mL in the wheat flour extract) were passed through the cartridge, and first 8 fractions (1 mL each) were collected. The eluate was dried under nitrogen at room temperature. The residue was dissolved in 1.0 mL of methanol–water (10:90 v/v). After centrifugation (13,000 rpm, 5 min), the sample was analyzed using LC-MS/MS. The elution pattern of five *Fusarium* toxins from MultiSep 226 columns are shown in Fig. 3. In theory, target mycotoxins did not retain on the MultiSep 226, and it eluted earlier. However, the NIV was not completely eluted in the fractions 0–4 mL as shown in Fig. 3. By contrast, NIV was successfully eluted with fractions after the 4 mL. Therefore, to analyze all of the compounds simultaneously, the first 4 mL of the effluent from the MultiSep 226 was discarded, then, the subsequent eluate accurately taken 2 mL, to obtain a sample solution.

### 3.3. Validation test

The data of the validation test are listed in Table 2. The calibration curve showed good linearity \((r > 0.999)\) in the ranges of 5–100 ng/mL for NIV, 5–250 ng/mL for DON, 1–250 ng/mL for HT-2, 1–100 ng/mL for T-2 and ZEN using the quantitative and qualitative ions (Table 1). Furthermore, the accuracy was 85–97%, the RSD values of intra-day precision were 1.9–13.3% and those of the inter-day precision were 10.2–19.6%. The LOD and LOQ values calculated using a spiked sample standard solution prepared with the wheat flour extraction liquid are listed in Table 2.

In the Food Sanitation Law, the validation guidelines show the criterion and calculation method for each parameter, including the selectivity, accuracy, intra- and inter-day precision, and LOQ. For the mycotoxin test, the total aflatoxins test method was specified in the validation guidelines. However, the validation guidelines do not specify a test method for *Fusarium* toxins, therefore we evaluated this test method on the basis of the validation guidelines for the total aflatoxins [23]. The evaluation criteria for validating the aflatoxins test method included an accuracy of 70–110%, intra-day precision of 20% or less, and inter-day precision of 30% or less. Selectivity is defined as follows: the area of the interfering peak is less than 1/10 of the peak area of LOQ equivalent. The examination method fulfilled the evaluation criteria for the accuracy, precision, and selectivity. In the validation guidelines, a LOQ criterion is that the S/N value obtained from the peak of the spiked sample standard should be ten or more. As shown in Table 2, based on these results, this method is believed to be applicable to the analysis of processed food contaminated with *Fusarium* toxins.

### 3.4. Contamination study of commercial foodstuffs

Using the developed method, a contamination study was conducted using 55 samples, including grains such as wheat flour and corn grits purchased at retail stores in the Kanagawa Prefecture or on the Internet. 10 samples were the flours in which the wheat and rye as the raw materials, 19 samples were products based on wheat, such as biscuits, cereals, and pasta, twenty samples were products based on corn such as corn grits, and six samples were snacks composed of corn. In the investigation of contamination, LOQ was a concern that it varies depending on the matrix of the processed food. In the contamination study of commercial foodstuffs, the LOQ was determined to be 15 μg/kg for NIV and DON, and 5 μg/kg for T-2, HT-2, and ZEN.

As shown in Table 3, DON was detected in 36 samples from all of the food groups. The maximum concentration of DON was 238 μg/kg of the corn grits. T-2 and HT-2 were detected in two and six samples, respectively, in products

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**Table 2.** Validation of method used for determining *Fusarium* toxins.

| Analyte | Fortified (μg/kg) | Accuracy (%) | intra-day precision (%) | inter-day precision (%) | Linearity \((r)\) | LOD (μg/kg) | LOQ (μg/kg) |
|---------|------------------|--------------|------------------------|-------------------------|----------------|------------|------------|
| NIV     | 20               | 85           | 12.4                   | 15.8                    | 0.9995         | 3.75       | 12.47      |
| DON     | 20               | 89           | 13.3                   | 19.6                    | 0.9996         | 2.34       | 7.79       |
| HT-2    | 5                | 97           | 7.0                    | 11.0                    | 0.9997         | 1.37       | 4.56       |
| T-2     | 5                | 91           | 1.9                    | 10.2                    | 0.9996         | 0.38       | 1.25       |
| ZEN     | 5                | 86           | 12.8                   | 14.5                    | 0.9996         | 0.75       | 2.50       |

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Table 3. Occurrence of *Fusarium* toxins in 55 processed grain product samples.

| Sample                   | No. of analyzed samples | NIV | DON | HT-2 | T-2 | ZEN |
|--------------------------|-------------------------|-----|-----|------|-----|-----|
| Flour                    | 10                      | 0   | 8   | 1    | 0   | 0   |
|                          |                         | n.d.| 16-177 | 7   | n.d. | n.d.|
| Wheat processed food     | 19                      | 0   | 11  | 5    | 2   | 0   |
|                          |                         | n.d.| 15-68 | 8-19 | 6,18| n.d.|
| Corn grit                | 20                      | 0   | 16  | 0    | 0   | 2   |
|                          |                         | n.d.| 17-238 | n.d. | n.d. | 9,27|
| Snack food made from corn| 6                       | 0   | 1   | 0    | 0   | 0   |
|                          |                         | n.d.| 23  | n.d. | n.d. | n.d.|
|                          |                         | n.d.| 23  | n.d. | n.d. | n.d.|

n.d.: NIV, DON <15 μg/kg, HT-2, T-2, ZEN <5 μg/kg

composed of wheat. Minimum concentration of T-2 and HT-2 in products composed of wheat was less than 20 μg/kg. The provisional MRL for DON in Japan is 1.1 mg/kg in wheat [11]. In addition, although it is unregulated in Japan, the indicative levels for the sum of T-2 and HT-2 is 15–2000 μg/kg for cereals and cereals products in EU [24]. Among the foodstuffs evaluated in the present study, the processed foods were not considered to be staple foods for the Japanese people, and their consumption was not significant. Based on these facts, the quantities of mycotoxins detected in the present study were considered acceptable in relation to the safety of the foodstuffs. However, the occurrence of mycotoxins that may contaminate the grains will depend on the plant growth and climatic conditions. Therefore, the generation of mycotoxin is considered to be changed each year. Based on our results, the mycotoxin contamination of imported processed foodstuffs composed of raw grain materials should be continuously managed and evaluated in a long-term screening study.

4. Conclusions

An investigation of the mycotoxin pollution of imported food is significant to secure safe food. The reported method using LC-MS/MS is thought to be effective for investigating mycotoxins. We inspected 55 food products in this investigation, and detected DON in 36 samples. In the present study, the concentrations of mycotoxins were less than the amounts the causes an immediate health hazards. However, the mycotoxin contamination that occurs in crops such as cereal grains depends on the growth conditions for the plant. Therefore, continuing the investigation of mycotoxins could be considered necessary for contribution to public health.

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