Single laboratory validation for a method of analysis for *Fusarium* toxins including deoxynivalenol-3-glucoside in wheat and barley

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**Abstract**
Recent reports have demonstrated that the deoxynivalenol (DON) mycotoxin produced by *Fusarium* sp. is metabolized to deoxynivalenol-3-glucoside (DON-3G) by glycosidation enzymes in infected plants, and subsequently DON-3G accumulates in the plant body. Although DON-3G is less toxic than DON, it is converted back to DON upon release in the intestinal tract of an animal. This reformed DON exhibits the same toxicity as DON initially produced by *Fusarium*. Thus, testing should be extended to DON-3G and other important mycotoxins. This study aimed to evaluate an analytical method based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the detection and quantification of DON, acetyl forms of DON (3-Ac-DON, 15-Ac-DON), a glycoside form of DON (DON-3G), and another *Fusarium* mycotoxins such as nivalenol (NIV), 4-Ac-NIV, T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEA), and diacetoxyscirpenol (DAS) in wheat and barley. The method was validated using wheat and barley samples spiked with each mycotoxin at levels of 0.01 and 0.1 mg/kg. The average recovery of mycotoxins in wheat and barley ranged from 77.4% to 110.3% with a relative standard deviation of 2.2% to 15.7%. These findings demonstrated that this LC-MS/MS method is reliable for the analysis of *Fusarium* mycotoxins, including the acetyl and glycoside forms of DON, in wheat and barley. All parameters corresponding to the trueness, repeatability, and reproducibility as intermediate precision satisfied the criteria values.
ol (VER) and zearalanone (ZAN) were used to correct the matrix effect for the recovery of the mycotoxins. This study is a reliable method for analysis of 10 mycotoxins including DON-3G in wheat and barley. Structures of target mycotoxins were shown in Fig. 1 (type A trichothecene), Fig. 2 (type B trichothecene), and Fig. 3 (ZEA).

Materials

Wheat and barley produced in Japan were used in this study. DON, 3-Ac-DON, 15-Ac-DON, NIV, 4-Ac-NIV, T-2, HT-2, DAS, ZEA (each at 100 mg/L) and DON-3G (50 mg/L) solutions were purchased from Kanto Chemical Co. (Tokyo, Japan). VER and ZAN, as well as acetonitrile (LC-MS grade), distilled water (LC-MS grade), ammonium acetate (HPLC grade), and acetic acid (LC-MS grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Preparation of standard solution

The stock solutions without DON-3G were prepared by diluting in acetonitrile at 20 mg/L. DON-3G were prepared at 5 mg/L. The internal standard VER and ZAN were prepared at 10 mg/L by diluting in acetonitrile.

Working solutions containing each mycotoxin at concentrations between 0.5 – 500 µg/L with fixed concentrations of VER (100 µg/L), ZAN (50 µg/L) were prepared in acetonitrile/water/ acetic acid (5/94/1, v/v/v), and used for calibrations.

Sample preparation

The cereal samples were ground in a grinder mill until all particles passed through a 0.5 µm mesh. Sample preparation was performed using an established procedure. Ground wheat or barley samples without any mycotoxins (10.0 g) was placed in a 100-mL blender cup and the internal standards VER (0.5 mL, 100 µg/L) and ZAN (0.5 mL, 50 µg/L) were added. Subsequently, the samples were spiked with a prepared solution of either DON, 3-Ac-DON, 15-Ac-DON, DON-3G, NIV, 4-Ac-NIV, T-2, HT-2, ZEA, or DAS (0.5 mL, 200 or 2000 µg/L). The samples were stored at –25 °C for 12 h to remove the solvents of the spiked standard solution and internal standards, and held at room temperature for 30 min. An acetonitrile and water mixture (40 mL, 80/20, v/v) and acetic acid (0.4 mL) were added to samples and homogenized at 7000 rpm for 5 min. The extract was transferred to a 50-mL centrifuge tube and centrifuged at 2000×g for 10 min. The supernatant (15 mL) was transferred to a solid phase extraction column (Presep C18, FUJIFILM Wako Pure Chemical Corporation) without pre-conditioning. The eluate was loaded on a Bond Elute Mycotoxin column (Agilent Technologies Japan, Ltd., Tokyo) where the initial 3 mL eluate was discarded and the subsequent 2 mL was collected. The collected eluate (1.6 mL) was dried under nitrogen stream at 40 °C. The residue was dissolved in a mixture of acetonitrile, water, and acetic acid (0.4 mL, 5/94/1, v/v/v) and filtered with a polytetrafluoroethylene (PTFE) disposable syringe filter unit DISMIC-13HP (pore size 0.20 µm, Toyo Roshi Kaisha, Ltd., Tokyo). The filtrate was subjected to LC-MS/MS analysis.

The wheat and barley samples were spiked with the standard solutions at 0.01 and 0.1 mg/kg, and analyzed in duplicate over 5 days. The mycotoxin concentration...
was determined based on the peak area ratio between the mycotoxins and the internal standards (trichothecenes/VER and ZEA/ZAN), where a calibration curve was used.

**LC-MS/MS analysis**

The LC-MS/MS system included an LCMS-8040 mass spectrometer, LC-30AD pump, SIL-30AC auto sampler, CTO-20AC column oven, DGU-20A degasser, CBM-20A communication bus module, and the LabSolution data system (Shimadzu Co., Kyoto, Japan). The analytical column (ZORBAX Eclipse XDB-C18 column, 3.0 × 250 mm, 5 µm particle size, Agilent Technologies Japan, Ltd.) was held at a constant temperature of 40 °C. The mobile phase was water and acetic acid (99.9/0.1, v/v) with 0.5 mmol/L ammonium acetate (eluent A), and acetonitrile/acetic acid (99.9/0.1, v/v) (eluent B). Sample injection volume was 10 µL and the flow rate was 0.3 mL/min. Liner gradient elution was applied using elution A and B, which included constant at 10% eluent B for 1 min, linear increase to 90% over 14 min, and constant at 90% for 4 min, linear decrease to 10% over 1 min, and constant at 10 % for 10 min. Ionization was conducted with electro spray ionization (ESI) to achieve negative polarity for DON, 3-Ac-DON, 15-Ac-DON, DON-3G, NIV, 4-Ac-NIV, ZEA, VER and ZAN, and positive polarity for T-2 and DAS. HT-2 was detected in both polarities. The monitor ions used for detection and retention time were shown in Table 1. The desolvation line (DL) was operated 100 °C, while the heat block was held at 200 °C. The drying gas flow was 15 L/min, nebulizer gas flow was 3 L/min (both gases were nitrogen), and CID gas flow was 230 kPa (argon gas).

| Polarity | Precursor ion (m/z) | Product ion (m/z) | Retention time (min) |
|----------|---------------------|------------------|---------------------|
| DON      | -635                | 59               | 7.5                 |
| 3-Ac-DON | -597                | 59               | 10.1                |
| 15-Ac-DON| -597                | 59               | 9.9                 |
| DON-3G   | -517                | 427              | 7.0                 |
| NIV      | -437                | 281              | 6.2                 |
| 4-Ac-NIV | -413                | 353              | 8.5                 |
| T-2      | +484                | 263              | 14.5                |
| HT-2     | +442                | 263              | 12.4                |
| DAS      | +384                | 247              | 12.0                |
| ZEA      | -317                | 131              | 15.2                |
| VER      | -325                | 59               | 8.7                 |
| ZAN      | -319                | 275              | 15.0                |

The single laboratory validation studies of the ten mycotoxins (DON, 3-Ac-DON, 15-Ac-DON, DON-3G, NIV, 4-Ac-NIV, T-2, HT-2, ZEA, and DAS) were performed according to the Harmonized guideline(6). All of the mycotoxins were well separated under the given conditions in the LC-MS/MS chromatogram of the wheat sample spiked at the lower concentration level of 0.01 mg/kg (Fig. 4). Further, no interfering peaks were detected. The matrix effect was not confirmed in multiple varieties wheat and barley.

The mean recovery (%), relative standard deviation (RSDr, %) within laboratory precision, and HorRat are shown in Table 2 and 37. The mean recovery of mycotoxins spiked at 0.01 mg/kg ranged from 80.4% and 107.7% in wheat and 77.4% to 111.3% in barley, while those at 0.1 mg/kg ranged from 85.9% to 106.2% in wheat and 85.6% to 106.6% in barley. Further, the RSD, at 0.01 mg/kg ranged from 3.0% to 15.7% in wheat and 5.1% to 15.3% in barley, while those at 0.1 mg/kg ranged from 2.2% to 11.4% in wheat and from 5.0% to 12.4% in barley. The HorRat values were within 0.7 for wheat and barley at both mycotoxin concentration levels. Further, all mean recovery and RSD, values were considered acceptable. All parameters corresponding to the trueness, repeatability, and reproducibility as intermediate precision satisfied the criteria values.

These single laboratory validation findings demonstrated that the existing LC-MS/MS method for the quantification of five main mycotoxins (DON, NIV, T-2, HT-2, and ZEA) can be successfully extended to the analysis of additional mycotoxins (3-Ac-DON, 15-Ac-DON, DON-3G, 4-Ac-NIV and DAS) in both wheat and barley. Therefore, this method can be used for the simultaneous analysis of ten Fusarium mycotoxins. This study is the first reliable method for analysis of 10 mycotoxins including DON-3G in wheat and barley.

![Fig. 4 LC-MS/MS chromatogram of various Fusarium mycotoxins at 0.01 mg/kg in wheat sample.](image-url)
Table 2  Recovery and RSD of wheat samples spiked with various *Fusarium* mycotoxins at 0.01 and 0.1 mg/kg.

| Spiked concentration | 0.01 mg/kg | 0.1 mg/kg |
|----------------------|------------|-----------|
|                      | Average recovery (%) | RSD (%) | HorRat | Average recovery (%) | RSD (%) | HorRat |
| DON                  | 98.0       | 9.8       | 0.4     | 100.6     | 4.3       | 0.2     |
| 3-Ac-DON             | 107.7      | 5.0       | 0.2     | 93.4      | 10.1      | 0.5     |
| 15-Ac-DON            | 98.4       | 15.7      | 0.7     | 90.6      | 6.7       | 0.3     |
| DON-3G               | 80.4       | 9.6       | 0.4     | 88.5      | 11.4      | 0.5     |
| NIV                  | 87.9       | 13.2      | 0.6     | 85.9      | 5.8       | 0.3     |
| 4-Ac-NIV             | 86.9       | 15.4      | 0.7     | 90.8      | 11.1      | 0.5     |
| T-2                  | 99.8       | 9.5       | 0.4     | 100.9     | 11.4      | 0.5     |
| HT-2                 | 94.9       | 13.9      | 0.6     | 106.2     | 2.7       | 0.1     |
| DAS                  | 95.1       | 15.0      | 0.7     | 97.2      | 10.9      | 0.5     |
| ZEA                  | 106.0      | 3.0       | 0.1     | 98.8      | 2.2       | 0.1     |

Table 3  Recovery and RSD of barley samples spiked with various *Fusarium* mycotoxins at 0.01 and 0.1 mg/kg.

| Spiked concentration | 0.01 mg/kg | 0.1 mg/kg |
|----------------------|------------|-----------|
|                      | Average recovery (%) | RSD (%) | HorRat | Average recovery (%) | RSD (%) | HorRat |
| DON                  | 111.3      | 7.1       | 0.3     | 101.3     | 6.1       | 0.3     |
| 3-Ac-DON             | 88.7       | 14.3      | 0.7     | 90.2      | 7.3       | 0.3     |
| 15-Ac-DON            | 102.5      | 9.9       | 0.5     | 95.6      | 10.0      | 0.5     |
| DON-3G               | 99.7       | 9.0       | 0.4     | 104.6     | 9.1       | 0.4     |
| NIV                  | 85.0       | 11.1      | 0.5     | 85.6      | 8.5       | 0.4     |
| 4-Ac-NIV             | 87.5       | 15.3      | 0.7     | 87.0      | 8.3       | 0.4     |
| T-2                  | 77.4       | 9.5       | 0.4     | 87.6      | 12.4      | 0.6     |
| HT-2                 | 100.6      | 8.4       | 0.4     | 106.6     | 7.0       | 0.3     |
| DAS                  | 89.6       | 13.0      | 0.6     | 99.8      | 6.6       | 0.3     |
| ZEA                  | 100.8      | 5.1       | 0.2     | 97.3      | 5.0       | 0.2     |

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