小麦中のデオキシニバレノールおよびニバレノールのLC-UVおよびLC-MSによる同時分析法の試験室間共同試験

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Validation Study

Interlaboratory Study of LC-UV and LC-MS Methods for the Simultaneous Determination of Deoxynivalenol and Nivalenol in Wheat

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To evaluate LC methods with UV or MS detection for simultaneous analysis of deoxynivalenol (DON) and nivalenol (NIV) in wheat, an interlaboratory study was conducted in 11 laboratories. DON and NIV were purified using a multifunctional column, and their concentrations were determined using LC-UV or LC-MS/MS. No internal standards were used. Three fortified wheat samples (0.1, 0.5 and 1 mg/kg), one naturally contaminated wheat sample, and one blank wheat sample were used. The recoveries ranged from 90% to 110% for DON and from 76% to 83% for NIV. For DON, the relative standard deviations for repeatability (RSDr) ranged from 1.1% to 7.6%. The relative standard deviations for reproducibility (RSDR) ranged from 7.2% to 25.2%. For NIV, the RSDr ranged from 2.0% to 10.7%, and the RSDR ranged from 7.0% to 31.4%. Regardless of sample and detector, the HorRat values for DON and NIV ranged from 0.4 to 1.4. Both LC-UV and LC-MS/MS methods were considered to be suitable for application as an official method.

Key words: deoxynivalenol; nivalenol; liquid chromatography (LC); ultraviolet (UV) detector; mass spectrometric (MS) detector; interlaboratory study; wheat

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Introduction

Deoxynivalenol (DON) and nivalenol (NIV), which are trichothecene mycotoxins produced by plant pathogenic Fusarium fungi, cause worldwide health and economic damage. These mycotoxins contaminate grains in temperate and sub-frigid regions and ingestion of contaminated grains can cause growth suppression, immunotoxicity, and hematotoxicity.

JECFA set the provisional maximum tolerable daily intake (PMTDI) of DON at 1.0 μg/kg of body weight per day in 2001. This PMTDI was established based on the "no observed effect level" (NOEL, 100 μg/kg of body weight per day) obtained from a feeding study in mice for 2 years and on a safety factor of 100. The Scientific Committee on Food (SCF) set the temporary TDI (t-TDI) of NIV at 0.7 μg/kg of body weight per day in 2000. This t-TDI was established based on the "lowest observed adverse effect level" (LOAEL, 0.7 mg/kg of body weight per day) obtained from feeding studies in mice for 1 and 2 years and on a safety factor of 1,000. However, the LOAEL of NIV has also been reported to be 0.4 mg/kg of body weight per day based on the results of feeding studies in rats for 90 days.

Both DON and NIV are frequently detected in the same sample, and the naturally occurring level of DON is generally higher than that of NIV. However, cases in which the level of NIV is higher than that of DON are often observed in Japanese wheat. A provisional tolerable level of DON in unpolished wheat has been set at 1.1 mg/kg in Japan. A tolerable level of NIV may also be set in Japan. This is because the LOAEL of NIV is lower than that of DON, and the actual level of NIV is often higher than that of DON in Japan. Therefore, to quantify these mycotoxins reliably and rapidly, an analytical method for simultaneous measurement of the concentrations of DON and NIV is required.

Many methods for simultaneous analysis of DON and NIV have been reported. The analytical instrument most frequently used to determine concentrations of these trichothecenes is GC with electron capture detection (ECD) or with MS detection. However, the GC method requires a derivatization procedure. This procedure is generally time-consuming and leads to poor recovery. On the other hand, LC methods using an UV or MS detector, which requires no derivatization procedure, have also been reported. In recent years, many LC-MS and LC-MS/MS methods have been reported for the simultaneous analysis of trichothecene mycotoxins, including DON and NIV. These methods have been applied to hygiene control and the surveillance of these mycotoxins. However, these LC-MS methods require the use of expensive internal standards such as isotopically substituted compounds. Meanwhile, the LC-UV method offers high precision, although the sensitivity of the method is lower than that of the LC-MS method.

We have previously reported the results of an interlaboratory study of an analytical method for measuring the concentration of DON in wheat by LC-UV coupled with a multifunctional column for cleanup. In the present study, based on the analytical method evaluated in the previous study, we evaluated the LC-UV and LC-MS/MS methods for simultaneous analysis of DON and NIV concentrations in wheat through an interlaboratory study.

Materials and Methods

Standard and reagents

DON and NIV standards were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A 5-mg portion of DON or NIV was accurately weighed and placed in a 50-mL volumetric flask, dissolved in acetonitrile as a stock solution (ca. 100 μg/mL), and stored at −20°C. Portions of the two stock solutions were mixed and diluted with acetonitrile to make a mixed standard solution (20 μg/mL each of DON and NIV) and three concentrations of mixed spiking solutions (5, 25, and 50 μg/mL of DON and NIV). Acetonitrile and methanol for LC were LC grade, and acetonitrile for extraction was reagent grade. The other reagents were of the highest analytical grade available. The Autoprep MP-T 1500 (Showa Denko K. K., Tokyo, Japan) was used as a multifunctional column.

Preparation of samples

Two samples of wheat, one of which was naturally contaminated with DON and NIV and the other of which was not contaminated, were donated by Dr. T. Nakajima. These wheat samples were ground until all particles could pass through a mesh of 1 mm. After mixing and homogenizing in a V-style mixer (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan), these wheat samples were packed into Stomacher bags containing approximately 30 g each. Six bags each out of 40 bags of naturally contaminated wheat and 200 bags of non-contaminated wheat were chosen at random, and the homogeneity was assayed by the LC-MS/MS method detailed in this report. Each laboratory in the interlaboratory study received the following: (a) six non-contaminated samples for the spike test of wheat found to be free of DON and NIV (<0.005 mg/kg); (b) four random-numbered samples, two of which were naturally contaminated and the others of which were the same as the samples for the spike test; (c) a mixed standard solution; and (d) three mixed spiking solutions, regarding which the laboratory had no knowledge of the DON and NIV concentrations.

Fortification procedure

For evaluating recoveries, 500 μL of mixed spiking solution was added to 25.0 g of non-contaminated sample in a 200-mL flask (final concentration, 0.1, 0.5, or 1 mg/kg) and kept at room temperature in the dark. After 1 h,
DON and NIV were extracted from each spiked sample and quantified according to the protocol.

Protocol used
The method used in this study was based on the method described in our previous report. Briefly, 25.0 g of sample was extracted with 100 mL of acetonitrile–water (85:15) without shaking for 10 min, followed by shaking for 30 min. The extract was transferred to a 50-mL centrifuge tube and centrifuged at 1,500×g for 5 min. Aliquots of 10 to 20 mL of the supernatant solution were applied to a multifunctional cleanup column (Autoprep MF-T 1500) without pre-conditioning. The first 4 mL of eluate was discarded, and the next 4 mL was collected. The collected eluate was divided into two vials; 2.0 mL for LC-UV and 1.0 mL for LC-MS or LC-MS/MS. The divided eluates were dried under nitrogen at approximately 45°C. Each residue was re-dissolved in 1.0 mL of water–methanol–acetonitrile (90:5:5) for LC-UV or 10 mmol/L aqueous ammonium acetate solution–methanol (90:10) for LC-MS or LC-MS/MS. Each solution was filtered through a membrane filter (0.45 μm) and the filtrate was analyzed by LC-UV and LC-MS/MS.

LC-UV conditions
The final solution for LC-UV was loaded onto an octadecylsilane (ODS) column (250 by 4.6 mm i.d., 3–5 μm) at 40°C. The mobile phase was water–methanol–acetonitrile (90:5:5), and the flow rate was set at 0.6–1.0 mL/min. The UV detector was set at a wavelength of 220 nm. The injection volume was set by each laboratory so that the height of the signal peak of 0.05 μg/mL standard solution (equivalent to 0.1 mg/kg in sample) was more than 10 times larger than that of the background noise.

LC-MS or LC-MS/MS conditions
Each laboratory determined the DON and NIV concentrations by LC-MS or LC-MS/MS. Five to twenty microliters of the final solution for LC-MS or LC-MS/MS was loaded onto an ODS column (150 by 2.1 mm i.d., 3–5 μm) at 40°C. The mobile phase was a binary gradient of 10 mmol/L aqueous ammonium acetate solution and methanol, and the flow rate was set at 0.2 mL/min. Electrospray ionization in the negative mode was used for ionization in the MS detector. The selected ion monitoring mode of LC-MS or the selected reaction monitoring mode of LC-MS/MS was selected by each laboratory. All other conditions were set by each laboratory so that the height of the signal peak of 0.025 μg/mL standard solution (equivalent to 0.1 mg/kg in sample) was more than 10 times larger than that of the background noise.

Calibration curve
To prepare standard solutions for calibration, mixed standard solution was diluted with water–methanol–acetonitrile (90:5:5) for LC-UV or with 10 mmol/L aqueous ammonium acetate solution–methanol (90:10) for LC-MS or LC-MS/MS. The concentration of DON or NIV and the peak signal (area or height) were plotted for five standard solutions with different concentrations. The concentration of DON or NIV in the sample solution was calculated from this calibration curve.

Interlaboratory study design
For evaluation of the methods, an interlaboratory study was carried out using pairs of five materials (three spiked samples of wheat, a naturally contaminated sample, and a blank sample). The interlaboratory study involved 10 laboratories in Japan and one laboratory in Korea.

Statistics
The homogeneities of DON and NIV in the naturally contaminated material supplied in this study were tested using one-way analysis of variance (ANOVA) and F-test. The results from laboratories have previously been examined for evidence of outliers using statistical Cochrans (between duplicates) and Grubbs single and Grubbs pair value tests (between laboratory means). The relative standard deviations for repeatability (RSDr) and reproducibility (RSDR), and the HorRat values, which is the ratio of the RSDr to the predicted RSDr, were obtained using one-way ANOVA according to the AOAC guidelines. However, the predicted RSDr of the HorRat value was calculated according to the report of Thompson.

Results and Discussion
The F-test at the 95% confidence level showed that each of the naturally contaminated samples could be regarded as homogeneous, because the calculated F-value was less than the critical F-value. No laboratories detected any DON or NIV in the blank. For both LC-UV and LC-MS/MS methods, 11 laboratories reported results, and there were no outliers. Laboratory B reported the result of 0.1 mg/kg spiked wheat as “trace” because the ratio of the signal peak obtained from the spiked test to the background noise was less than 10.

The results of the interlaboratory study are shown in Table 1 (DON) and Table 2 (NIV). The Commission of European Communities has laid down criteria for analytical methods used for the official control of the levels of mycotoxins in foodstuffs. According to the criteria, the recovery, RSDr and RSDr for 0.1–0.5 mg/kg of DON level are required to be in the ranges of 60–110%, ≤ 20% and ≤ 40%, respectively; for >0.5 mg/kg, they are required to be in the ranges of 70–120%, ≤ 20% and ≤ 40%, respectively. All the results for DON in this study were acceptable. Although criteria for NIV were not given, all results for NIV were also acceptable according to the DON criteria. As for the HorRat value, a range of 0.5–1.5 was acceptable according to AOAC International. Although some HorRat values were less than 0.5, no problems were recognized throughout this interlaboratory study. Therefore, the results obtained from both LC-UV and LC-MS/MS methods satisfied...
ly used to correct for purification losses and ionization efficiency. In this study, MS detection gave acceptable results for both DON and NIV even without the use of internal standards.

We have previously reported the results of an interlaboratory study of the determination of DON concentrations in wheat by LC-UV coupled with a multifunctional column\(^\text{[33]}\). In that study, the RSDr ranged from 5.8% to 11.3%, and the RSDs ranged from 12.0% to 20.7%. The HorRat value was below 1.0, and the recovery was

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No of labs: 10
Mean (mg/kg): 0.09
Recovery (%): 90
RSDr (%): 7.6
RSDs (%): 25.2
HorRat: 1.1

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No of labs: 10
Mean (mg/kg): 0.09
Recovery (%): 90
RSDr (%): 7.6
RSDs (%): 25.2
HorRat: 1.1

The ratio of the signal peak obtained from the spiked sample to the background noise was less than 10.
100%. Trucksess et al. have reported the results of an interlaboratory study for the determination of DON concentrations in white flour, wheat flour and bran by LC-UV coupled with a multifunctional column. In their study, the RSDr ranged from 3.1% to 21.7%, and the RSDs ranged from 10.8% to 38.7%. The recovery ranged from 80% to 115%. McDonald et al. have reported the results of an interlaboratory study of the determination of DON concentrations in cereals and cereal products by LC-UV with an immunoaffinity column cleanup step. In that study, the RSDr ranged from 3.1% to 14.1%, and the RSDs ranged from 11.5% to 26.3%. The HorRat value was below 1.3, and the recovery ranged from 74% to 87%. As compared with those results, the results of the UV and MS detection methods presented in this report are similar for both DON and NIV.

Acknowledgements

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小麦中のデオキシジアレノールおよびニバレノールのLC-UVおよびLC-MSによる同時分析法の試験室間共同試験（妥当性評価，英文）

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李 秀樹 小木曾基樹 前田 守 甲斐茂美 田中宏樹
法月廣子 平岡久明 田中敏樹 石黒隆一 伊藤嘉典
永山敏廣 中島正博 内藤成弘 小西良子
食衛誌 53(3), 152~156 (2012)

小麦中のデオキシジアレノール（DON）およびニバレノール（NIV）のLC-UVおよびLC-MS（MS）を用いた同時分析法を評価するために，11試験室で共同試験を実施した。精製には多機能カラムを用い，試料としてDON，NIV添加小麦3試料（0.1, 0.5および1 mg/kg），自然汚染小麦1試料および非汚染小麦1試料を用いた。回収率はDONが90~110%，NIVが76~83%であった。DONにおける室内再現性の相対標準偏差（RSDr）は7.6%以下，室間再現性の相対標準偏差（RSDn）は25.2%以下，NIVにおけるRSDrおよびRSDnはそれぞれ10.7%以下，31.4%以下であった。HorRatは0.4~1.4であり，いずれの方法も公定法として利用可能であることが示唆された。

*（独）農林水産消費者安全技術センター