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Occurrence and simultaneous determination of nivalenol and deoxynivalenol in rice and bran by HPLC-UV detection and immunoaffinity cleanup

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ABSTRACT

A simple and accurate method for simultaneously quantifying two co-occurring *Fusarium* mycotoxins in rice and bran, nivalenol (NIV) and deoxynivalenol (DON), is described. The method involves the use of an immunoaffinity column for cleanup and HPLC-UV detection for quantification. The limits of quantification were $<11.09\ \mu g\ kg^{-1}$ for the two toxins in rice and bran. The mean recoveries from blank samples spiked at levels of 100–1000 $\mu g\ kg^{-1}$ were 86.2–106.6% for NIV and 93.1–106.2% for DON, with relative standard deviations of 6–15% for NIV and 3–11% for DON, respectively. The detection rate of NIV in 482 rice and 239 bran samples was 34–96%, where the level ranging from 5.7 to 2791.4 $\mu g\ kg^{-1}$, whereas that for DON was 10.4–44.8% with levels ranging from 7.1 to 655.6 $\mu g\ kg^{-1}$. The co-occurrence rates of NIV and DON were 9.1%, 14.9%, and 41.5% for white rice, brown rice, and bran, respectively. The estimated dietary intakes of NIV and DON for the Korean population based on the occurrence data were well below the established tolerable daily intake.

1. Introduction

Rice (*Oryza sativa* L.) is the most important food crop worldwide (FAO, 2004). However, if the conditions are favorable, the rice kernels can be contaminated by fungi and mycotoxins during the cultivation and subsequent handling of rice (Ok et al., 2014). Several studies have shown that the main fungi found in rice are the *Fusarium* species, which can produce trichothecene mycotoxins (Pacin et al., 2002; Pitt et al., 1994). In Asian countries, including Korea, rice crops are mostly infected by *F. graminearum* (Lee et al., 2011). Studies have also shown that deoxynivalenol (DON) and nivalenol (NIV), which belong to the type-B trichothecenes, are frequently detected in the same sample, and that the natural occurrence level of DON is generally higher than that of NIV (Edwards et al., 2011). However, cases where the occurrence level of NIV is higher than that of DON have often been reported in Europe and Asia (Lee et al., 1986; Osborne & Stein, 2007). This difference in the occurrence of trichothecene analogs can be explained by the geophysical distribution of *Fusarium* species (Desjardins, 2006; Nakajima & Yoshida, 2007).

DON is associated with feed refusal, vomiting, and suppressed immune functions (FAO/WHO, 2011). NIV is more toxic to humans and domestic animals than DON (Food Safety Commission of Japan, 2010). In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a group provisional maximum tolerable daily intake (TDI) of 1 $\mu g\ kg^{-1}$ body weight (bw) for the sum of DON and its acetyl derivatives, such as 3-acetyldeoxynivalenol (3-ADON) and 15-ADON (JECFA, 2010). The EU Scientific Committee on Food established a tolerable daily intake (TDI) per kilogram of bw per day of 1.2 μg for NIV (CONTAM, 2013). In Japan, the estimates of exposure to DON and NIV are considered to be below the established TDI values (1 $\mu g\ kg^{-1}$bw per day for DON and 0.4 $\mu g\ kg^{-1}$ bw per day for NIV) (Food Safety Commission of Japan, 2010). At least 37 countries, including South Korea, have now set regulatory limits or guidance levels for DON in food, but not for NIV (FAO, 2004).

The confirmatory analytical methods for DON and NIV employ chromatographic methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with different types of detectors. Previously, the presence of type-B trichothecene in cereal-based products was analyzed mainly by GC-electron capture detection (GC-ECD) after derivatization to enhance the sensitivity of the method and solid phase extraction (SPE) cleanup (Ok et al., 2011). The main disadvantages of this

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method are the poor accuracy and precision of the measurements, derived from matrix interferences that induce enhancement of the trichothecene response, as well as the time-consuming derivatization process (Krska, Baumgartner, & Joseph, 2001; Petterson & Langseth, 2002). The use of HPLC methods coupled with ultraviolet (UV) detection for the simultaneous determination of DON and NIV in food and feed using multifunctional column cleanup has also been proposed, but these methods are unreliable due to low sensitivity and matrix interference (Rodrigues, Handl, & Binder, 2011). Recently, liquid chromatography (LC) coupled with mass spectrometry (MS) (LC–MS) has been used for the qualification and quantification of DON and NIV in a multianalyte approach. However, MS-based methods require expensive instrumentation and highly qualified laboratory analysts. Furthermore, complex matrices and signal suppression effects are observed due to matrix effects, particularly in electrospray ionization (ESI)–MS methods, which may require the careful optimization of cleanup process, the use of matrix-matched calibrants, or isotopically labeled internal standards (CONTAM, 2013).

Sample cleanup is an essential step in the analysis of mycotoxins, including DON and NIV, especially when HPLC- or GC-based chromatographic techniques are used for their determination at trace levels. SPE, multifunctional cleanup columns, and immunoaffinity columns (IACs) are frequently used to clean up the extracts of raw cereals, as well as processed cereal products. In particular, IAC cleanup is frequently used for mycotoxin analyses because the specificity of antibodies provides cleaner extracts, compared with other cleanup; good precision, accuracy and sensitivity of the analyticalmethods that include this cleanup procedure have been achieved. However, analytical methods that use IAC cleanup procedures for the determination of DON and NIV in rice and bran have not been reported previously.

Thus, in the present study, we validated a HPLC method coupled with UV detection and the use of a commercial IAC for the routine and simultaneous determination of DON and NIV in brown rice, white rice, and bran in terms of the linearity, accuracy, precision, and measurement uncertainty. Furthermore, the validated method was employed for monitoring the natural occurrence and estimated dietary intake, based on the analyzed levels of NIV and DON.

2. Materials and methods

2.1. Reagents

Solid crystals of NIV hydrate (purity: 98.6%), DON (purity: 99.4%), 3-ADON (purity: 99.4%), and 15-ADON (purity: 98.8%) were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). Each compound was dissolved in acetonitrile (each 100 μg mL^{-1}) and the solutions were stored at −20 °C. Aliquots of these stock solutions were mixed and diluted with acetonitrile to produce mixed standard solutions (10 μg mL^{-1} each of NIV and DON). DON-NIV™ WB IAC was purchased from VICAM (Milford, MA, USA). All solvents were suitable for LC analysis and they were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Food materials

Freshly harvested paddy rice samples (about 10 kg) were collected from 241 farms in 2010–2012. These farms were located at nine sites with slightly different field cultivation conditions (geographic coordinates: latitude 34.5–37.7°N, longitude 126.6–129.3°E; climatic conditions: annual mean temperature = 12.2–14 °C, relative humidity = 62–74%), i.e., Gangneung (n = 60), Hwaseong (n = 51), Anseong (n = 20), Jeongup (n = 30), Heanam (n = 10), Ulsan (n = 10), Uiseong (n = 20), Hamyang (n = 20), and Boseong (n = 20). The husk was removed from the paddy rice (about 10 kg each sample) using a mill (Model HSMC-4; Hansung Industrial Co., Seoul, Korea). The brown rice was milled to white rice and bran using a commercial abrasive mill (Model VT-21T; Yamamoto Co., Higashine, Japan). All samples were finely ground using a blender until they passed through a No. 20 sieve. The processed samples were then stored in aluminum zipper bags at −18 °C and subsamples were used in the subsequent analyses.

2.3. Extraction and purification of samples

For white rice and brown rice, a ground sample (20 ± 0.002 g) was placed in a 200 mL beaker with 100 mL of distilled water and 1 g of sodium chloride, before homogenizing for 3 min using a high-speed mixer (Ultra Turrax; IKA, Staufen, Germany). After extraction, the sample was centrifuged at 4000 rpm for 20 min and 5 mL of the extract was diluted with 20 mL of phosphate-buffered saline (PBS). If the diluted solution was not clear, it was centrifuged again at 14000 rpm (23700 × g) for 15 min. After filtration through a GF/B filter, 10 mL of the filtrate was passed through an IAC at a flow rate of one drop per second. The IAC was washed with 10 mL of PBS and 10 mL of distilled water, and dried by rapidly passing air through it. The toxins were eluted with 0.5 mL of methanol and 1 mL of acetonitrile. The eluent was evaporated in a water bath at 50 °C. The dried residues were reconstituted with 1 mL of 10% acetonitrile and filtered through a syringe filter (0.2 μm). To analyze the toxins in bran, the extract solution was mixed with 50% acetonitrile. The volume of PBS was 45 mL for diluting the extractant and the volume of the diluted extract solution poured into the IAC was changed to 30 mL. The IAC was washed in 15 mL of PBS and 15 mL distilled water. To determine the levels of NIV and DON in white rice (n = 241), brown rice (n = 241), and bran (n = 239), a duplicate set of samples was prepared as described above and analyzed by HPLC with triplicate injections.

2.4. HPLC conditions

NIV and DON were determined with an Agilent 1200 LC system equipped with an UV detector (model G1314F; Agilent Technologies, Santa Clara, CA, USA), which was controlled by ChemStation software. A Nova-Pak C18 column (300 mm × 3.9 mm i. d., particle size = 4 μm; Waters, Ireland) fitted with a guard column was employed. The mobile phase comprised a mixture of water (A) and acetonitrile (B). The initial gradient was 90% A and 10% B, which was equilibrated for 8 min. Subsequently, solvent A was changed linearly to 70% and then to 90% over 18 min, and then held for 12 min. The flow rate was 0.7 mL min^{-1} and only 0.8 mL min^{-1} after 8–9 min. The detector was set at a wavelength of 218 nm. The injection volume was 100 μL.

2.5. LC/MS confirmation

The chromatographic system comprised an Agilent 1290 infinity series ultra-high performance liquid chromatography system coupled with an ion funnel QTOF mass detector (G6550A) equipped with dual spray electro-spray ionization (ESI) and Agilent jet stream ESI source (Agilent Technologies, USA). LC separation was performed using a ZorbaxEclipe plus C18 column (50 mm × 2.1 mm i. d., 1.8 μm particle size; Agilent Technologies, Santa Clara, CA, USA). The LC mobile phase was a mixture of water (A:0.1% formic acid, v/v) and acetonitrile (B: 0.1% formic acid, v/v). The initial gradient was 95% A and 5% B, which was equilibrated for 0.5 min. Subsequently, solvent A was changed linearly to 70% in 8 min and then held for 1 min. Solvent B was reduced to 5% in 9.5 min and it was then equilibrated for 2.5 min. The total run time