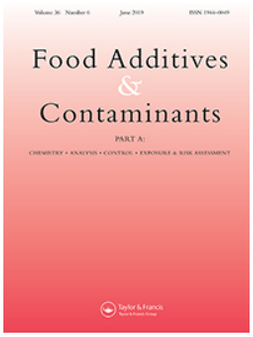

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# Simple validated method for simultaneous determination of deoxynivalenol, nivalenol, and their 3-β-D-glucosides in baby formula and Korean rice wine via HPLC-UV with immunoaffinity cleanup

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**ABSTRACT**

A simple and reliable method for the simultaneous determination of major type B trichothecene mycotoxins, deoxynivalenol (DON) and nivalenol (NIV), along with their 3-β-D-glucosides (DON-3-glucoside (DON3G) and NIV-3-glucoside (NIV3G)) in baby formula and Korean rice wine was validated in the present study. The method was based on immunoaffinity cleanup followed by analysis using an HPLC-UV technique. The method was validated in-house for two matrices as follows: linearity ($R^2 > 0.99$) was established in the range of 20–1000 μg $kg^{-1}$; accuracy (expressed as recovery) ranged from 78.7 to 106.5% for all the analytes; good intermediate precision (relative standard deviation < 12%), and adequate detection and quantitation limits (< 4.4 and < 13.3 μg $kg^{-1}$, respectively) were achieved. Furthermore, the estimated measurement expanded uncertainty was determined to be 4–24%. The validated method was successfully applied to the analysis of 31 baby formulas and Korean rice wines marketed in Korea.

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Baby formula; deoxynivalenol; deoxynivalenol-3-glucoside; nivalenol; nivalenol-3-glucoside; rice wine; simultaneous determination

## Introduction

Trichothecene mycotoxins, produced by *Fusarium* species, constitute the largest group of Fusarium toxins, with over 200 substances isolated to date (Grove 2007). Trichothecenes are classified into groups A, B, C, and D based on their chemical characteristics and origin (McCormick et al. 2011). Among the four subgroups, type B trichothecenes such as deoxynivalenol (DON) and nivalenol (NIV) occur more frequently than other types of trichothecenes (Josephs et al. 2004) and pose a significant food safety problem owed to their prevalence in food crops. In general, the natural occurrence levels of DON are higher than that of NIV (Edwards et al. 2011), although the converse has often been reported in Europe and Asia (Lee et al. 1986; Osborne and Stein 2007) owed to the different geophysical distribution of *Fusarium* species (Desjardins 2006; Nakajima and Yoshida 2007).

Mycotoxins derivatives termed masked (Gareis et al. 1990) or modified (Rychlik et al. 2014) mycotoxins have become an issue. It has been shown that polar groups such as β-D-glucopyranoside or acetate are attached to a hydroxyl group of the parent trichothecenes by plant defence mechanisms. In the case of DON, monoglucosylated DONs that are conjugated with glucose on carbons 3 or 15 of the parent trichothecene have been identified (Berthiller et al. 2005; Yoshinari et al. 2014). Moreover, deoxynivalenol-3-β-D-glucoside (DON3G) and nivalenol-3-β-D-glucoside (NIV3G), major glucose conjugates of DON and NIV, are frequent co-contaminants of the free toxins and are at risk of being hydrolyzed and converted to free toxin during the digestive process of mammals (Nagl et al. 2014). However, at present, these conjugated compounds generally escape routine analysis for monitoring of their parent trichothecenes in food.

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DON and NIV have similar structures, differing only in the oxygen atom at position 4 in the trichothecene core structure. Thus, they share many toxicological aspects in terms of immunotoxicity and hematotoxicity, although NIV is relatively more toxic to humans and domestic animals (Food Safety Commission of Japan, 2010). In contrast, the inherent toxicities of DON3G and NIV3G are considered to be lower than those of their respective native forms (Berthiller et al. 2005; Nagl et al. 2014). Nevertheless, when a human or animal ingests DON3G or NIV3G, the toxic potential could become enhanced because the glucose is hydrolyzed from the parent DON or NIV during digestion, suggesting that DON3G or NIV3G constitute a potential source of dietary DON or NIV exposure (CONTAM 2017a, 2017b).

To avoid the potential health risks caused by the intake of these type B trichothecenes and their modified forms, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a group provisional maximum tolerable daily intake (TDI) of 1 μg $kg^{-1}$ bw $day^{-1}$ for the sum of DON and its acetyl derivatives, such as 3-ADON and 15-ADON (JECFA 2010). Recently, a group TDI of 1 μg $kg^{-1}$ bw $day^{-1}$ for the sum of DON, 3-ADON, 15-ADON, and DON3G was established by the EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel 2017a). In the case of NIV, the TDI of 1.2 μg $kg^{-1}$ bw $day^{-1}$ suggested in 2013 has been retained with NIV3G being included in the group TDI with NIV because it can be hydrolyzed to NIV after ingestion with the same molar potency factor (EFSA CONTAM Panel 2017b).

NIV and DON co-contamination has been reported in a number of studies, along with contamination of the modified form of up to 30% of the free toxin (Dall'Asta et al. 2012; Bryła et al. 2018). Notably, DON3G and NIV3G have a higher polarity than the free toxin and are difficult to detect by general analytical methods. Therefore, rapid and accurate analysis methods that simultaneously detect DON, NIV, and their modified forms are required. Recently, analysis of DON, NIV, and their glucoside conjugates has focused on the method of performing minimal pre-treatment using simultaneous high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) detection to analyze various mycotoxins. MS analysis carries the advantages of simplifying the pre-treatment, obtaining information on the structure of the molecule, and providing high sensitivity and selectivity (Berthiller et al. 2007; Palacios et al. 2017).

Conversely, MS equipment is very expensive and requires high proficiency for the analysis. The matrix being analyzed also exerts signal enhancement and suppression effects, requiring optimization of the method in each case. In comparison, a method of analysis of NIV and DON using gas chromatography (GC) with electron capture detection (ECD) or MS detection has also been reported (Ok et al. 2011; MaríaIbáñez-Vea et al. 2011). However, the GC method has some disadvantages such as memory effects from previous sample injections and long-time requirement for derivatization (Lattanzio et al. 2009).

Alternatively, the HPLC method does not require the derivatization process and can provide rapid analyses; moreover, because the device is widely distributed, the HPLC method can be conducted in most laboratories. In addition, when immunoaffinity chromatography (IAC) is used, high selectivity can be ensured because of the use of antigen-antibody reactions; i.e., when cross-reaction of the antibody occurs, it is possible to analyze simultaneously not only target toxins but also modified forms such as sugar conjugates. Simultaneous detection of NIV and DON using HPLC has been carried out by pre-treatment of white rice and rice bran using IAC followed by analysis with an ultraviolet (UV) detector (Ok et al. 2018). Simultaneous analysis of NIV, DON, and DON3G (Trombete et al. 2016) has also been reported by pretreating wheat with IAC and using a photodiode array (PDA detector). The limit of detection (LOD) and limit of quantification (LOQ) are in the range of 10–100ppb, which is comparable to that of the MS/MS detector. However, no commercially available standard is yet available for NIV3G; thus, there have been few reports on simultaneous analysis using HPLC-UV detection based on antibody cross-reactivity.

Baby formula constitutes a solid food matrix that requires sensitive and reliable methods of analysis. Infants are considered a vulnerable group of the population and are more susceptible

to mycotoxin exposure than adults because they have a restricted diet rich in cereals and consume more food on a body weight basis than adults. As a consequence, EU or Korean legal limits for mycotoxins in baby formulas are much lower than those set for all other regulated matrices. In comparison, Korean traditional rice wine (*makgeolli*), a liquid food matrix, is manufactured with grains and a natural fermentation starter termed *Nuruk*. Various types of microbes exist in *Nuruk* because of the coexistence of grain- and environment-derived microorganisms that are acquired during fermentation. Thus, safety issues such as contamination by mycotoxins consequent to the incorporation of pathogenic fungi or bacteria are occasionally also a concern. However, no reports are available regarding the prevalence of co-contamination of DON, DON3G, NIV, and NIV3G in baby formulas and Korean rice wine marketed in Korea.

To address the potential for such contamination in these products, in the present study a method for simultaneous determination of DON, DON3G, NIV, and NIV3G using immunoaffinity column cleanup and HPLC-UV detection was optimized and validated in-house. This method was then applied to a limited survey regarding the presence of these mycotoxins in infant formula and Korean rice wine. To our knowledge, this is the first analytical methodology aimed at the detection and quantification of both free and glycosylated type B trichothecenes by HPLC in processed food matrices such as baby formula and Korean rice.

## Materials and methods

### Chemicals and reagents

DON (100.5 μg $mL^{-1}$) and NIV (101.1 μg $mL^{-1}$) were purchased from Romer Labs (Tulln, Austria) as standard solutions dissolved in acetonitrile (ACN). DON3G (50.3 μg $mL^{-1}$) was purchased from Sigma (St Louis, MO, USA) as a standard solution dissolved in ACN. NIV3G was synthesized and purified at the University of Natural Resources and Life Sciences, Vienna, Austria (BOKU). Its identity and purity (>98%) were verified by nuclear magnetic resonance and HPLC-UV measurements (Li et al. 2017). Working stocks of all trichothecenes were prepared in ACN at a concentration of 10 μg $mL^{-1}$ and stored at – 20 °C. Water (W), methanol (MeOH), ACN, and other solvents used were all HPLC grade from Burdick & Jackson products (Morris Plains, NJ, USA). The IAC column DON-NIV$^{WB}$ used for the pre-treatment was purchased from VICAM (Milford, MA, USA).

### Samples

Different brands of baby formulas (n = 16) and Korean rice wine samples (n = 15) were purchased in the local market in quantities of at least 1 kg. Samples were homogenized, subdivided, and stored in an aluminised zipper bag. Among the baby formulas, porridge products were stored at –20°C and powdered products were stored at room temperature. Korean rice wine samples were stored at 2–5°C. The sample used for validation was confirmed as not being contaminated with the toxin through preliminary analysis.

### Optimization of the HPLC conditions

The HPLC conditions were optimized by modifying an analytical method used for simultaneous analysis of NIV and DON in our previous studies (Ok et al. 2018). To analyze the two glucoside forms simultaneously, six sets of conditions (A-F) considering the sample solvent (or standard solvent) injected, gradient time, mobile phase, and column were compared. Conditions A-C were analyzed using a 4 μm particle size column at 25 °C. Conditions D-F were analyzed using a 2.7 μm particle size column at 30 °C. The sample solvent, gradient time, and mobile phase of the six conditions were as follows. Condition A: 0 min (W/ACN, 95:5, v/v), 8 min (W/ACN, 95:5), 15 min (W/ACN, 95:5), flow rate 1 mL/min, sample solvent W:ACN (90:10, v/v). B: same as A except for sample solvent (W:ACN, 95:5, v/v). C: 0 min (W/ACN, 90:10, v/v), 11 min (W/ACN, 90:10), 12 min (W/ACN, 70:30), flow rate 0.6 mL/min, sample solvent W:ACN (90:10, v/v). D: 0 min (W/ACN/Methanol, 95:2.5:2.5, v/v/v), 5 min (W/ACN/MeOH, 95:2.5:2.5), 20 min (W/ACN/MeOH, 75:12.5:12.5), flow rate 0.8 mL/min, sample solvent W:ACN (90:10, v/v). E: 0 min (W/ACN, 95:5, v/v),

8 min (W/ACN, 95:5), 20 min (W/ACN, 85:15), flow rate 0.6 mL/min, sample solvent W:ACN (95:5, v/v). F: 0 min (W/ACN/MeOH, 95:4:1, v/v/v), 5 min (W/ACN/MeOH, 95:4:1), 20 min (W/ACN/MeOH, 75:12.5:12.5), flow rate 0.8 mL/min, sample solvent W:MeOH:ACN (95:1:4, v/v/v). The resolution ($R_s$) was calculated using the formula:

$$R_s = \frac{k'}{(k'+1)} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{\sqrt{N}}{4} \quad (1)$$

considering efficiency (N), retention factor (k′), and selectivity (α) of the peak. Peak asymmetry (As) was calculated using the following equation:

$$A_s = b/a \quad (2)$$

where b is the distance from the peak midpoint (perpendicular from the peak highest point) to the trailing edge of the peak measured at 10% of peak height and a is the distance from the leading edge of the peak to the peak midpoint (perpendicular from the peak highest point) measured at 10% of peak height.

The HPLC analysis was conducted using an Agilent 1260 infinity series (Santa Clara, CA, USA): quaternary pump (G1311C), autosampler (G1329B), thermostat (G1330B), thermostatic column compartment (G1316A), and UV detector (G1314F) wavelength of 218 nm. Supelco Ascentis Express C18, 2.7 µm particle size, and 4.6 mm × 150 mm columns (Bellefonte, PA, USA) were used for toxin separation and analysis following 100 µL injection at 30 °C.

### Extraction and purification of samples

For the baby formula samples, 25 ± 0.005 g of sample and 100 mL of 20% ACN were mixed in a 200 mL beaker and homogenized at 6,200 rpm for 5 min (Ultra Turrax; IKA, Staufen, Germany). After homogenization, the mixture was centrifuged at 20,000 *g* for 20 min. The supernatant was diluted fivefold with distilled water. The diluted solution was filtered using a GF/B-grade glass filter. Filtered solution (20 mL) was passed through the IAC column at a rate of 1 drop/s, followed by washing with 20 mL of water. After removing the remaining water using a syringe, the toxin was eluted with 2 mL of MeOH. The eluate was dried evaporated in a heat block at 50 °C with nitrogen gas. A 1 mL aliquot of the mobile phase was added to the residue and vortexed for 20 s before filtering with a 0.2 µm polyvinylidene fluoride syringe filter. For the Korean rice wines, samples (25 ± 0.005 g) were sonicated for 10 min to remove carbonic acid, and 100 mL of water was used as the extraction solvent; the other processes were the same. To determine the levels of DON, NIV, DON3G, and NIV3G in baby formula and Korean rice wine, a triplicate set of samples was prepared as described above and analyzed by HPLC with triplicate injections.

### Validation of the method

Simultaneous analysis of DON, NIV, and their 3-β-D-glucosides in the two matrices of baby formula and Korean rice wine using HPLC-UV was based on single-lab validation (Association of Official Analytical Chemistry 2012). The validation was based on the following parameters: linearity, LOD, LOQ, matrix effect, accuracy, and precision. The calibration curves were prepared by mixing four toxins at five points ranging from 20–1000 µg $kg^{-1}$ in each blank matrix and using the average of 10 measurements. The linearity was evaluated by the coefficient of determination of the calibration curve; it was judged that excellent linearity was obtained when $r^2 > 0.99$. LOD and LOQ were calculated using the formula:

$$\mathrm{LOD} = 3.3 \times \sigma/\mathrm{S}, \mathrm{LOQ} = 10 \times \sigma/\mathrm{S} \quad (3)$$

where σ is standard deviation of the response, and S is the slope of the calibration curve.

The matrix effect was determined at 500 µg $kg^{-1}$, an intermediate concentration within the range from 20–1000 µg $kg^{-1}$, which was confirmed to have linearity, with confirmation using both baby formula and Korean rice wine matrices. Matrix effects are expressed as a ratio of the mean peak area of an analyte in post-extraction spiked samples to the mean peak area of the same analyte in standard solutions, multiplied by 100 (10 measurements).

For the accuracy and precision of the method, intraday and interday recovery tests were carried out at low (2 × LOQ), medium (5 × LOQ), and high (10 × LOQ) concentration. The toxin spike was prepared prior to analysis and left in the fume

hood for 10 min. The intraday analysis was performed in triplicate for each concentration with three independent repeats within a single day (n = 9). The interday analysis was performed in triplicate for each concentration on three different days (n = 9). The accuracy and precision of the method were evaluated using the mean recovery of the triplicate sample and the relative standard deviation (RSD) %.

### Uncertainty of the method

The uncertainty was calculated of the simultaneous HPLC-UV analysis of NIV, DON, and its glucoside conjugates in the two matrices, baby formula and Korean rice wine. Uncertainties of various factors such as pipetting, weighing, standard, and calibration curve were considered. The uncertainty was assessed by the recovery test at low, medium, and high concentration as defined in section 2.5. Calculation of uncertainty was based on the Guide to the Expression of Uncertainty in Measurement and EURACHEM criteria, with the uncertainty factor being selected using the Fishbone diagram (analysis of mass and volume, reference material, calibration curve, instrument). The uncertainty of each factor was calculated according to the type A uncertainty using the standard deviation of the measured value, and the type B uncertainty using the calibration certificate, such as for the balance and pipette. Each calculated standard uncertainty was calculated using the law of propagation of uncertainty, with the expanded uncertainty being calculated by taking the inclusion factor k = 2 (EURACHEM/CITAC directive) at the 95% confidence level into account (Eurachem/CITAC 2000).

### LC-MS confirmation

The HPLC-MS analysis was performed on a Velos pro dual-cell two-dimensional linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) hyphenated with an Accela HPLC system (Thermo Fisher Scientific) via a heated-electrospray ionization source. The Accela HPLC system was equipped with a quaternary pump (Accela 600), vacuum degasser, and an open autosampler with a temperature controller. Chromatographic separation of targeted analytes was achieved on a Waters Xbridge C18, 3.4 μm particle size, 2.1 mm × 100 mm column (Dublin, Ireland) with gradient elution. The mobile phase was a mixture of water (A: 0.1% formic acid, v/v) and methanol (B: 0.1% formic acid, v/v). The initial gradient was 90% A and 10% B, which was equilibrated for 3 min. Subsequently, solvent A was changed linearly to 5% at 13 min and then held for 3 min. Solvent B was changed to 95% at 13 min and then equilibrated for 3.1 min. Solvent A was changed directly to 90% and solvent B was changed to 10% at 16.1 min. The total run time was 20 min, the flow rate was set at 0.2 mL $min^{-1}$, the column temperature was at 40 °C, and the sample injection volume was 10 μL. Detection and determination were performed in positive electrospray ionization mode. Optimized MS parameters were as follows: spray voltage, 5.0 kV; source heater temperature, 250 °C; capillary temperature, 275 °C; nitrogen sheath gas, 35 (arbitrary units); nitrogen auxiliary gas, 5 (arbitrary units); nitrogen sweep gas, 5 (arbitrary units); *m*/*z* range, 50–2000, collision-induced dissociation energy; and 35 eV; isolation width, 1.0 *m*/*z*. Helium was employed as a collision gas. The precursor ions $[M + H]^+$ used to identify the four toxins were NIV (313 *m*/*z*), NIV3G (475 *m*/*z*), DON (297 *m*/*z*), and DON3G (459 *m*/*z*).

## Results and discussion

### Optimization of the HPLC condition

For the simultaneous analysis of NIV, DON, and 3-β-D-glucosides using HPLC-UV detection, the retention time, peak area and resolution were compared among seven conditions with different sample solvent, gradient time, column temperature, and column (Table 1). Chromatograms of six different conditions are shown in Figure S1. The NIV–NIV3G resolution was improved from 0.8 (condition A) to 1.7 (condition B) when the sample solvent was the same as the mobile phase by separation on the particle size 4 μm Nova-Pak column (3.9 mm × 300 mm). In the case of decreasing the flow rate to 0.6 mL $min^{-1}$ (condition C), the detection order of NIV and NIV3G was reversed, and the

**Table 1.** Changes in retention time, peak asymmetry, and resolution as affected by six different HPLC conditions for simultaneous determination of DON, NIV, DON3G, and NIV3G.

| Condition | Mobile phase | Sample solvent | Flow rate (mL/min) | Oven temp. (°C) | Retention time (min) | | | | Peak asymmetry | | | | Resolution | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | NIV | NIV3G | DON | DON3G | NIV | NIV3G | DON | DON3G | NIV | NIV3G | DON | DON3G |
| A[1] | W/CAN (95–72:5–28)[3] | W/AC (90:10, v/v) | 1 | 25 | 7.5 | 8.2 | 14.5 | 15.2 | 0.89 | 0.72 | 1.08 | 1.07 | 12.0 | 0.8 | 8.4 | 2.3 |
| B[1] | W/CAN (95–72:5–28)[3] | Same a mobile phase | 1 | 25 | 7.6 | 8.4 | 14.5 | 15.2 | 0.88 | 0.92 | 1.10 | 1.06 | 17.1 | 1.7 | 15.1 | 2.5 |
| C[1] | W/CAN (90–70:10–30)[4] | Same a mobile phase | 0.6 | 25 | 6.1 | 5.7 | 10.6 | 9.3 | 0.80 | 0.81 | 0.97 | 1.09 | 1.2 | 8.2 | 2.3 | 6.9 |
| D[2] | W/ACN/MeOH (95–75:2.5–12.5:2.5–12.5)[5] | W/AC (90:10, v/v) | 0.8 | 30 | 8.2 | 9.9 | 13.4 | 14.4 | 1.76 | 3.24 | 0.93 | 0.86 | 7.9 | 1.6 | 5.3 | 3.5 |
| E[2] | W/CAN (95–80:5–20)[6] | Same a mobile phase | 0.6 | 30 | 8.2 | 9.0 | 15.6 | 16.4 | 0.60 | 0.67 | 0.98 | 0.79 | 10.2 | 2.0 | 21.0 | 3.3 |
| F[2] | W/ACN/MeOH (95–75:1–12.5:4–12.5)[7] | Same as mobile phase | 0.8 | 30 | 7.5 | 8.8 | 12.7 | 13.7 | 0.59 | 0.81 | 1.00 | 0.70 | 10.9 | 3.3 | 13.2 | 4.7 |

[1]Nova-Pak C18, 4 µm particle size, 3.9 mm × 300 mm.
[2]Ascentis Express C18, 2.7 µm particle size, 4.6 mm × 150 mm.
[3]W: water, ACN: acetonitrile, 0 min (W/ACN, 95:5), 8 min (W/ACN, 95:5), 15 min (W/ACN, 85:15), 16 min (W/ACN, 95:5).
[4]0 min (W/ACN, 90:10), 11 min (W/ACN, 90:10), 12 min (W/ACN, 70:30).
[5]MeOH: Methanol, 0 min (W/ACN/MeOH, 95:2.5:2.5), 5 min (W/ACN/MeOH, 95:2.5:2.5), 20 min (W/ACN/MeOH, 75:12.5:12.5).
[6]0 min (W/ACN, 95:5), 8 min (W/ACN, 95:5), 20 min (W/ACN, 80:20).
[7]0 min (W/ACN/MeOH, 95:4:1), 5 min (W/ACN/MeOH, 95:4:1), 20 min (W/ACN/MeOH, 75:12.5:12.5).

resolution was decreased to 1.2. The resolution differ`ence of DON–DON3G in conditions A-C was insignificant at 2.3–2.5. The resolution of NIV–NIV3G and DON–DON3G was improved under all conditions except for NIV–NIV3G in condition D following separation on the particle size 2.7 µm Ascentis column (4.6 mm × 150 mm). Among conditions D-F, the resolution was highest at 3.3 (NIV–NIV3G) and 4.7 (DON–DON3G) under condition F, in which methanol was additionally used in the mobile phase. Among the various chromatographic factors examined in this study, the particle size of the column and the sample solvent were found to have a considerable influence on the resolution, whereas the gradient time, column temperature, and flow rate were less influential.

In HPLC, distorted peaks are causes for integration problems, and very often for poor resolutions. Sample and standard solvent different from that of the eluent represents a possible reason for anomalous peak shapes (Loeser and Drumm 2006). A better peak asymmetry, an indicator of whether a chromatographic system is well-behaved, for NIV and NIV3G, and DON and DON3G, was observed in condition F. However, peak distortion in NIV and NIV3G occurred in condition D, wherein the sample solvent and mobile phase were not identical. This phenomenon appears to be due to the difference in solvent strength between the sample solvent and the mobile phase (Loeser and Drumm 2006). Taken together, our findings indicated that the best condition of separation of DON, NIV, DON3G, and NIV3G was achieved by using the Ascentis Express C18 column (2.7 µm particle size, 4.6 mm × 150 mm) with mobile phase composed of W/ACN/MeOH (0 min, W/ACN/MeOH (95:4:1, v/v/v); 5 min, W/ACN/MeOH (95:4:1. v/v/v); 20 min, W/ACN/MeOH (75:12.5:12.5, v/v/v)), at 0.8 mL/min, volume of injection equal to 100 µL with sample solvent of W:MeOH:ACN (95:1:4, v/v/v), and column oven at 30 °C (condition F).

### *LOD, LOQ, range, linearity, and matrix effect for the two matrices*

Parameters such as LOD, LOQ, range, linearity, and matrix effect were evaluated in optimized conditions (condition F) and are shown in Table 2. The

**Table 2.** LOD, LOQ, linearity, and matrix effects of the method for baby formula and Korean rice wine.

| Matrix | Toxin | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) | $R^2$ | Range (μg kg$^{-1}$) | Matrix effect[2)] (%) |
|---|---|---|---|---|---|---|
| Baby formula | NIV | 4.4 | 13.3 | 0.999 | LOD–1000 | 85 |
| | NIV3G | 2.6 | 7.9 | 0.999 | | 97 |
| | DON | 1.3 | 3.9 | 0.999 | | 91 |
| | DON3G | 1.2 | 3.5 | 0.999 | | 90 |
| Korean rice wine | NIV | 2.5 | 7.7 | 0.999 | LOD–1000 | 89 |
| | NIV3G | 2.0 | 6.1 | 0.999 | | 101 |
| | DON | 1.5 | 4.7 | 0.999 | | 120 |
| | DON3G | 3.2 | 9.8 | 0.999 | | 91 |

[1)]Resolution calculated using formula (1) with 10-fold repeated measures (20 μg kg$^{-1}$)
[2)]Calculated as post-extraction sample peak area mean/standard solution peak area mean (n = 10)

calibration curves of the five points in the range of 20–1000 μg kg$^{-1}$ showed good linearity above 0.999 for all four toxins. LOD and LOQ were calculated to be 1.2–13.3 μg kg$^{-1}$ for all analytes in the infant formula and Korean rice wine matrices (Table 2). A similar result was reported by Trombete et al. (2016), who determined the LOD and LOQ of HPLC-PDA after the IAC pretreatment in wheat as 9.4–31.3 μg kg$^{-1}$ for NIV and DON, and 14.1–47.1 μg kg$^{-1}$ for DON3G. Bryła et al. (2018) reported the LOD, LOQ of HPLC-UV after the IAC pretreatment in wheat as 4.8–16.8 μg kg$^{-1}$ for NIV, 10.1–33.1 μg kg$^{-1}$ for DON, and 15.0–50.0 μg kg$^{-1}$ for DON3G. Matrix effects were negligible for all analytes. The obtained values (%) for all analytes were in range of 85–97% and of 89–120% in baby formula and Korean rice wine, respectively.

The chromatograms of each toxin obtained by applying the optimized HPLC condition F to the two matrices are shown in Figure 1a–c. The retention times were 7.2, 8.3, 12.8, and 13.6 min for NIV, NIV3G, DON, and DON3G, respectively. Interference peaks were not present near the peaks for the toxins. The presence of NIV and DON3G in the naturally contaminated samples was confirmed by LC-MS with electrospray ionization (Figure 1d). The protonated adducts NIV (313 *m*/*z*) and DON3G (459 *m*/*z*) were identified in the toxin-positive samples.

### *Accuracy and precision*

Accuracy, expressed as intraday and interday recoveries of DON, NIV, and their 3-β-D-glucosides are shown in Table 3. Intraday accuracy in the matrix of infant food formula artificially spiked with 2×, 5×, and 10× LOQ of each toxin was 83.2–90.8% for NIV, 84.2–89.4% for NIV3G, 82.4–94.3% for DON, and 79.8–82.9% for DON3G. In Korean rice wine, the intraday accuracy was 95.0–100.1% for NIV, 104.7–106.5% for NIV3G, 99.2–104.7% for DON, and 96.2–103.0% for DON3G. Interday accuracies in 2×, 5×, 10× LOQ were 78.7–91.9% for NIV, 83.8–87.2% for NIV3G, 84.0–95.1% for DON, and 81.5–85.4% for DON3G in the baby formula. In Korean rice wine, the interday accuracies were 97.1–105.6% for NIV, 90.1–99.0% for NIV3G, 88.8–97.5% for DON, and 95.4–102.6% for DON3G. The precision of the four toxins was ± 0.2–11.5%, and intermediate precision (day-to-day variation) of the four toxins was ± 0.2–12.4% in the two matrices. Accuracy and precision of the four toxins were acceptable for all of the following criteria for DON of the European Commission regulation 401/2006/EC: repeatability ($RSD_r$) ≤ 20 and reproducibility ($RSD_R$) ≤ 40 (European Commission 2006) in the concentration range of DON > 100 – ≤ 500 μg kg$^{-1}$. Generally, the regulatory limits for DON and NIV in food intended for infants and young children, e.g. baby formula, are set at a lower level than those of other foods. Therefore, in the present study, the accuracy and precision were validated at the lower levels (47–322 μg kg$^{-1}$) than those (100–1000 μg kg$^{-1}$) in previous studies (Bryła et al. 2018; Ok et al. 2018); notably, acceptable accuracy and precision was observed for all four toxins, even at the LOQ level.

As a cleanup method, IAC provides a number of advantages such as the high specificity of the antibody for the analyte, rapid purification process, and effective reduction of toxic solvents (Gonçalves and Stroka 2016). In addition, the cross-reactivity features of IAC can widen the scope of IAC-based methods in terms of number of allowable and/or suitable analytes. Thus, for the simultaneous analysis of DON, NIV, and

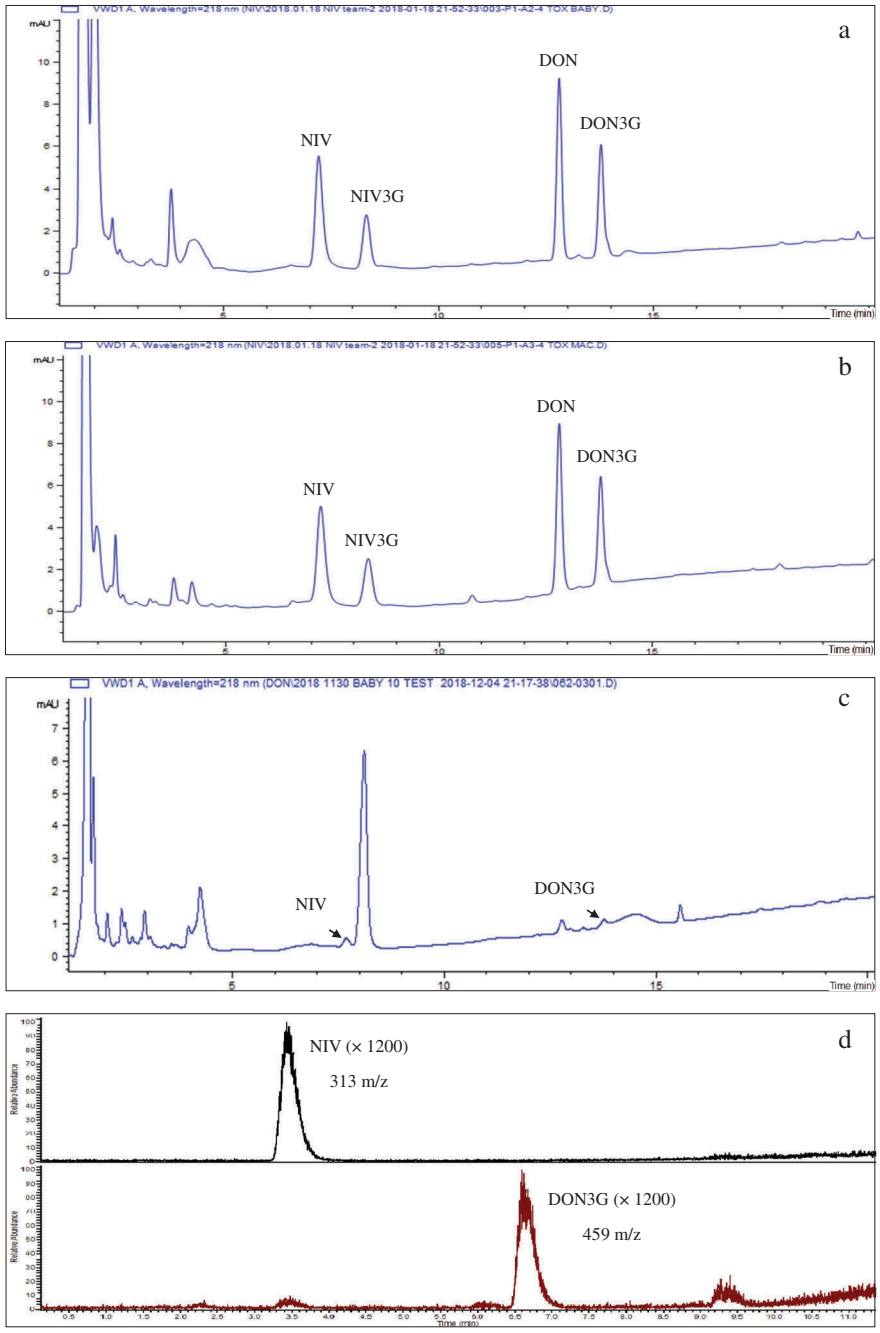


**Figure 1.** HPLC-UV chromatograms of DON, NIV, DON3G, and NIV3G in the spiked baby formula (a) and Korean rice wine (b) at the level of 500 μg $kg^{-1}$, and a naturally contaminated sample of baby formula containing NIV and DON3G (cC). LC-MS chromatogram of the same naturally contaminated sample of baby formula (d). The levels of NIV and DON3G in the sample were estimated as 17.9 and 13.5 μg $kg^{-1}$, respectively.

their 3-β-D-glucosides in food samples, it is necessary to ascertain whether DON-NIV$^{TM}$ IAC exhibits cross-reactivity against the 3-β-D-glucosides of DON and NIV. Trombete et al. (2016) demonstrated the cross-reactivity of DON-NIV$^{TM}$ IAC to DON3G along with a high recovery. However, there have been no reports to date regarding the cross-reactivity or recovery rate for NIV3G. In the present study, NIV3G using DON-NIV$^{TM}$ IAC showed excellent recoveries of 83.8–106.5% in intra- and interday experiments. It was also confirmed that DON-NIV$^{TM}$ IAC enabled the simultaneous analysis of DON, NIV, DON3G, and NIV3G.

**Table 3.** Intraday and interday accuracy and precision of the method for DON, NIV, and their 3-β-D-glucosides (DON3G and NIV3G) in spiked baby formula and Korean rice wine.

| | | Recovery (%) ± RSD (%)[1)] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Intraday (n = 9) | | | Interday (n = 9) | | |
| Matrix | Toxin | 2LOQ | 5LOQ | 10LOQ | 2LOQ | 5LOQ | 10LOQ |
| Baby formula | NIV | 90.8 ± 8.8 | 87.1 ± 7.6 | 83.2 ± 1.2 | 91.9 ± 3.5 | 80.7 ± 4.5 | 78.7 ± 7.0 |
| | NIV3G | 88.2 ± 11.5 | 84.2 ± 1.4 | 89.4 ± 0.4 | 83.8 ± 0.2 | 87.2 ± 5.2 | 84.8 ± 5.5 |
| | DON | 91.6 ± 9.4 | 82.4 ± 1.7 | 94.3 ± 1.9 | 95.1 ± 11.7 | 84.0 ± 2.0 | 89.0 ± 8.0 |
| | DON3G | 82.9 ± 2.4 | 79.8 ± 2.8 | 81.0 ± 0.4 | 85.4 ± 0.3 | 81.5 ± 0.9 | 81.7 ± 1.3 |
| Korean rice wine | NIV | 95.0 ± 3.1 | 100.1 ± 2.9 | 99.7 ± 0.2 | 105.6 ± 6.5 | 97.1 ± 2.5 | 99.4 ± 0.5 |
| | NIV3G | 106.5 ± 3.1 | 105.8 ± 3.7 | 104.7 ± 1.5 | 90.1 ± 12.4 | 97.9 ± 5.7 | 99.0 ± 3.9 |
| | DON | 100.0 ± 4.1 | 104.7 ± 3.7 | 99.2 ± 2.9 | 88.8 ± 11.8 | 95.2 ± 5.4 | 97.5 ± 4.9 |
| | DON3G | 103.0 ± 3.4 | 96.2 ± 2.4 | 98.2 ± 1.1 | 102.6 ± 2.3 | 99.4 ± 1.1 | 95.4 ± 2.6 |

[1)]RSD was calculated using the mean of 10 measurements

### *Measurement of uncertainty for the HPLC method*

To verify the method, uncertainties in the experimental procedure were calculated from the uncertainty of the matrix, instrument, linearity of the calibration curve, final volume of sample, and weight of the sample using a Fishbone diagram. Among the uncertainty factors, the contribution to the overall uncertainty from the uncertainty of the matrix (Rm) and the linearity of the calibration curve (Lc) was the highest (Figure S2). As this result is similar to the previous estimation of measurement uncertainty for the HPLC analysis of DON in wheat in 2009 (Ok et al. 2009), further minimization of the errors that occur in the measurement of the recovery of each matrix and the calibration curve is required to improve the precision of the analysis method.

Calculated uncertainties are shown in Table 4. The uncertainty of 2, 5, 10 LOQ in baby formula was 10–17% for NIV, 15–16% for NIV3G, 17–20% for DON, and 8–9% for DON3G. In Korean rice wine, the uncertainties were 10–20%, 4–11%, 5–21%, and 6–24%, respectively. This result was acceptable according to the following criteria presented by the EU: uncertainty within 32% if the concentration is >100 μg $kg^{-1}$, ≤1000 μg $kg^{-1}$, and within 44% if the concentration is ≤100 μg $kg^{-1}$ (European Commission 2004).

### *Application of the validated method to market samples*

The HPLC-UV and HPLC-MS chromatograms of naturally contaminated samples are shown in Figure 1. Different brands of baby formula (n = 16) and Korean rice wine (n = 15) were used to test the validated method. For baby formula, NIV was detected (> LOD) at a concentration of 17.2 μg $kg^{-1}$ averaged over three samples, NIV3G was detected at 9.8 μg $kg^{-1}$ for one sample, and DON3G was detected at 13.5 μg $kg^{-1}$ for one sample. In the case of Korean rice wine, DON of 7.8 μg $kg^{-1}$ was detected in one sample (Table 5). The toxin that was detected in the HPLC-UV analysis was

**Table 4.** The expanded uncertainty of NIV, DON, and their 3-β-D-glucosides for the baby formula and Korean rice wine matrices analyzed at three spiked concentration levels.

| | Baby formula | | | | Korean rice wine | | | |
|---|---|---|---|---|---|---|---|---|
| Toxin | Spiking conc. (Spiking concg $kg^{-1}$) | Combined uncertainty | Result ± Uncertainty[1)] (Spiking concg $kg^{-1}$) | Uncertainty / Result (%) | Spiking conc. (Spiking concg $kg^{-1}$) | Combined uncertainty | Result ± Uncertainty[1)] (Spiking concg $kg^{-1}$) | Uncertainty / Result (%) |
| NIV | 46.8 | 3.6 | 42.5 ± 7.2 | 17 | 16.0 | 1.6 | 14.9 ± 3.1 | 20 |
| | 117.0 | 4.8 | 90.2 ± 9.7 | 11 | 40.0 | 2.2 | 39.7 ± 4.4 | 11 |
| | 233.9 | 9.7 | 194.6 ± 19.3 | 10 | 80.0 | 3.9 | 79.6 ± 7.7 | 10 |
| NIV3G | 64.4 | 4.5 | 56.8 ± 9.1 | 16 | 22.0 | 1.2 | 23.9 ± 2.4 | 11 |
| | 161.0 | 10.4 | 135.6 ± 20.9 | 15 | 55.1 | 1.3 | 58.6 ± 2.7 | 5 |
| | 322.0 | 22.1 | 287.9 ± 44.2 | 15 | 110.2 | 2.2 | 115.1 ± 4.4 | 4 |
| DON | 42.3 | 3.6 | 35.8 ± 7.2 | 20 | 25.0 | 2.6 | 25.0 ± 5.2 | 21 |
| | 105.7 | 7.5 | 87.0 ± 15.0 | 17 | 62.5 | 2.3 | 65.4 ± 4.6 | 7 |
| | 211.3 | 16.9 | 199.3 ± 33.8 | 17 | 125.0 | 3.3 | 123.9 ± 6.6 | 5 |
| DON3G | 55.2 | 2.0 | 45.8 ± 4.1 | 9 | 13.3 | 1.6 | 14.0 ± 3.2 | 24 |
| | 138.1 | 4.3 | 110.2 ± 8.5 | 8 | 33.1 | 1.3 | 32.4 ± 2.6 | 8 |
| | 276.2 | 8.5 | 223.8 ± 17.0 | 8 | 66.3 | 1.9 | 65.4 ± 3.8 | 6 |

[1)]Expanded uncertainty (k = 2, 95% confidence level)

**Table 5.** Application of the optimized validated method to baby formula and Korean rice wine marketed in Korea.

| Food sample | Toxin | No. of positive (% of positive) | Positive mean (μg kg$^{-1}$) ± Uncertainty[1)] | Total mean (μg kg$^{-1}$) ± Uncertainty[1)] | Range (μg kg$^{-1}$) |
|---|---|---|---|---|---|
| Baby formula (n = 16) | NIV | 3(19) | 17.2 ± 2.9 | 3.2 ± 0.5 | 16.5–17.9 |
| | NIV3G | 1(6) | 9.8 ± 1.6 | 0.6 ± 0.1 | 9.8 |
| | DON | 0 | - | - | - |
| | DON3G | 1(6) | 13.5 ± 1.2 | 0.8 ± 0.1 | 13.5 |
| Korean rice wine (n = 15) | NIV | 0 | - | - | - |
| | NIV3G | 0 | - | - | - |
| | DON | 1(7) | 7.8 ± 1.6 | 0.5 ± 0.1 | 7.8 |
| | DON3G | 0 | - | - | - |

[1)]Expanded uncertainty (k = 2, 95% confidence level)

secondarily confirmed by LC-MS analysis. Therefore, it was confirmed that the established simultaneous analysis method of DON, NIV, and their glucoside conjugates was capable of analyzing a toxin level of tens of μg kg$^{-1}$ in baby formula comprising a solid matrix and Korean rice wine as a liquid matrix, and that this method was applicable to naturally contaminated samples.

Of the four toxins analyzed, only DON is currently controlled in Korea, to 200 μg kg$^{-1}$ or less for baby formula. DON was not detected in the baby formula samples, whereas for NIV, an average contamination level of 17.2 μg kg$^{-1}$ was detected in three samples. The contamination level was 10–100 of μg kg$^{-1}$; although this is not high, it is necessary to keep contamination to the lowest levels possible in food for infants and toddlers. In the case of Korean rice wine, 7.8 μg kg$^{-1}$ of DON was detected in one sample, which is much lower than the standard level. Thus, we found that the overall contamination of domestic circulating baby formula and Korean rice wine was very low. However, because of the nature of mycotoxins, it remains a possibility that they may occur at any time depending on the conditions; therefore, continuous management is required. In addition, as similar results as those identified herein in the case of higher NIV contamination have also been reported in some Asian countries including Korea, monitoring of raw materials for NIV and its glucoside conjugate should be required.

## Conclusions

In this study, a simple method for the simultaneous determination of DON, NIV, and their 3-β-D-glucosides in baby formula and Korean rice wine by HPLC-UV detection with IAC cleanup was optimized and in-house validated. Adequate values of accuracy, intra and intermediate precision, linearity, LOD, and LOQ were acquired. Furthermore, the estimated measurement expanded uncertainty was acceptable. This validated method was successfully applied to the analysis of a total of 31 baby formulas and Korean rice wines marketed in Korea. Thus, the optimized and in-house validated method is suitable for the detection of the conjugated mycotoxins DON3G and NIV3G together with DON and NIV in baby formula and Korean rice wine samples.

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## Conflict of interest statement

The authors have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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