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Thermal Degradation of the *Fusarium* Mycotoxin Deoxynivalenol

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Deoxynivalenol (DON) is a toxic secondary metabolite produced by molds of the *Fusarium* genus, which are able to infect cereal crops in the field. Concerning its rate of occurrence and mean concentration, DON is one of the most important mycotoxins in cereal commodities. Its toxic effects range from causing diarrhea, vomiting, and gastro-intestinal inflammation to noncompetitive inhibition of the biosynthesis of proteins in eukaryotic cells. To study the stability of DON under food-processing conditions such as cooking or baking, we performed model heating experiments and screened the residue for degradation products. Heating of DON and 3-acetyldeoxynivalenol (3-AcDON), especially under alkaline conditions, gave a mixture of compounds, which were isolated and structurally elucidated by NMR and MS experiments. Three of these compounds were already known (norDON A, norDON B, and norDON C), while four were new and named 9-hydroxymethyl DON lactone, norDON D, norDON E, and norDON F. The significance of the DON degradation products was checked by analyzing commercially available food samples. norDON A, B, and C were detected in 29−66% of the samples in mean concentrations ranging from 3 to 15 μg/kg. Furthermore, cell culture experiments using IHKE cells showed that the compounds that were detected in food samples are less cytotoxic in the formazan dye cytotoxicity assay compared to DON. Whereas DON revealed a median effective concentration (EC_{50}) at 1.1 μmol/L, all other compounds did not show any significant effect up to 100 μmol/L. These findings indicate that the degradation of DON under thermal treatment might reduce the toxicity of DON contaminated food.

KEYWORDS: Deoxynivalenol; mycotoxin; thermal degradation; alkaline degradation; heating; stability; GC-MS; HPLC-MS/MS; thermal treated food; food safety; toxicity

INTRODUCTION

Fusarium molds are a large group of phytopathogenic fungi which are able to infect cereal crops in the field during their growth period. They cause plant diseases, resulting in reduced yields, deterioration of quality, and contamination of the crops with mycotoxins. The *Fusarium* genus consists of more than 100 different species such as *Fusarium culmorum* or *Fusarium graminearum*. These particular species are common on soil and the most important in causing head blight and producing trichothecene mycotoxins in various cereal crops. Trichothecenes are a large group of sesquiterpenes sharing a 12,13-epoxytrichothec-9-ene ring system as the basic chemical structure (*1*). The group is subdivided in type A trichothecenes such as T-2 toxin and type B trichothecenes such as deoxynivalenol **1** (DON, **Figure 1**), the difference being a keto substitution at C-8 in the latter case.

Considering its rate of occurrence and mean concentration, DON, which is produced by *F. graminearum*, *F. culmorum*, and *Fusarium crookwellense*, appears to be one of the most important mycotoxins in cereal commodities (*2*). Its toxicity is attributed to the general ability of trichothecenes to noncompetitively inhibit the biosynthesis of proteins in eukaryotic cells likely by affecting the ribosomal function (*3*). The oral LD_{50} of DON was determined to be 78 mg/kg bw in B6C3F1 mice (*4*), while it was 46 mg/kg bw in DDY mice (*5*). Pigs are especially sensitive to DON: acute intoxications result in vomiting, feed refusal, reduced weight gain, and diarrhea at levels as low as 1−2 mg/kg feed (*1*). Furthermore, inflammation and necrosis of various tissues, mainly the gastrointestinal tract, the bone marrow, and lymphoid tissues, are observed (*3*). So far, DON is not considered to be genotoxic or carcinogenic (*6*). Thus, a tolerable daily intake (TDI) value for DON of 1 μg/kg bw was established in 1999 (*1*). It was derived from the "no observed adverse effect level" (NOAEL) of 0.1 mg/kg bw of a chronic dietary study with mice (*6*). Various summaries on the toxicity of DON can be found in the literature (*1*, *3*, *7*).

In recent years, legislation has adopted the problem of DON in food. Maximum limits were established in the European Union in July 2006. They range from 200 μg/kg for cereal

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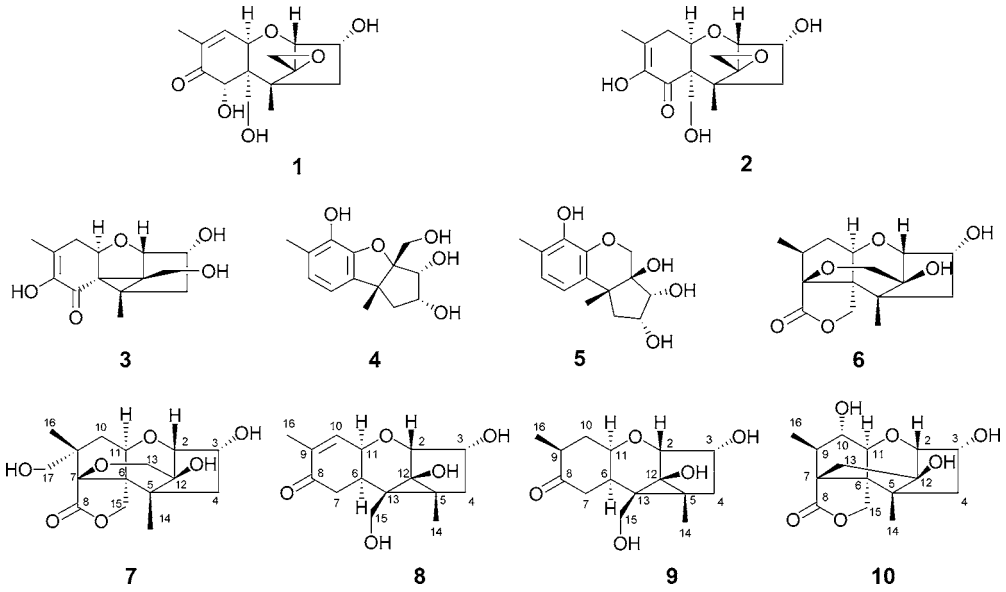

Figure 1. Structures of deoxynivalenol (**1**) and its known degradation products isoDON (**2**), norDON A (**3**), norDON B (**4**), norDON C (**5**), and DON lactone (**6**) and the new compounds 9-hydroxymethyl DON lactone (**7**), norDON D (**8**), norDON E (**9**), and norDON F (**10**).

products for young children to 1750 μg/kg for unprocessed durum wheat and oats (*8*). Germany has implemented more stringent maximum limits for DON in 2004 ranging from 100 μg/kg for infant cereal products to 500 μg/kg for most other cereal products (*9*).

In general, DON is considered to be a relatively stable compound. Most of the studies on its stability are focused on model experiments, observing the degradation depending on time, temperature, or pH in aqueous solutions (*10*−*16*). They showed that DON was especially unstable under alkaline conditions and at high temperatures. In total, five degradation products were isolated after rather harsh thermal or alkaline treatment of DON **1** (**Figure 1**) (*11*−*13*).

The results of studies on the stability of DON in food matrixes under processing conditions such as cooking or baking seem to be contradictory. While some authors report no degradation at all (*17*, *18*), most observe a moderate reduction (5−85%) depending on the respective conditions (*10*, *15*, *19*−*22*). Abbas et al. (*23*) studied the degradation of DON during the production of tortillas from contaminated corn, observing a reduction of up to 82%. This is possibly due to the nixtamalization process, the traditional alkaline treatment of corn kernels in Latin America.

In summary, the available data clearly show that the amount of reduction of DON during thermal food processing is highly variable and depends on the respective processing conditions. However, except for isoDON **2** (**Figure 1**) (*11*), which was detected in bread, little is known about specific degradation products and nothing about their toxicological properties.

In an attempt to investigate the stability of DON under food processing conditions, we used a model heating system, which we have already applied in the investigation of the stability of fumonisins and nivalenol (*24*, *25*). Briefly, the mycotoxins were heated with several model compounds simulating typical food constituents: α-D-glucose was used as sugar model, methyl-α-D-glucopyranoside as starch model, and the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester as protein models (*24*, *25*). The degradation products formed were analyzed by gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS).

MATERIALS AND METHODS

Reagents. DON and 3-acetyldeoxynivalenol (3-AcDON) were prepared according to the procedure described by Altpeter et al. (*26*). Water for HPLC separation was purified with a MilliQ Gradient A10 system (Millipore, Schwalbach, Germany); the other solvents for HPLC and HPLC-MS as well as all other chemicals were purchased from VWR (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) in gradient or reagent grade quality.

Model Experiments. The following compounds were used as food models: α-D-glucose as sugar model, methyl-α-D-glucopyranoside as starch model, and the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester as protein models (*24*). The experiments were performed by heating 100 μg of deoxynivalenol with 1 mg of model compound without solvent. To get a homogeneous mixture, aliquots of stock solutions of the reactants (1−20 mg/mL in methanol) were vortexed in a 1.5 mL glass vial and the solvent was removed under a stream of nitrogen. The mixture was heated in a heating block for various periods (5−20 min) at different temperatures (150−200 °C). The residue was further analyzed by gas chromatography-mass spectrometry (GC-MS). As a control, pure DON was heated under the conditions described above.

GC-MS. Gas chromatography electron impact mass spectrometry (GC-EI-MS) data were acquired on a HP6890 series gas chromatograph and HP5973 mass spectrometer (Hewlett-Packard/Agilent, Böblingen, Germany) after derivatization of the compounds with 200 μL of trimethylsilylimidazole (TMSI, 70 °C, 30 min) and addition of 300 μL of *tert*-butyl methyl ether (t-BME). Data acquisition was performed with the Chemstation software (Agilent). Chromatographic separation was performed on a 60 m × 0.25 mm i.d. fused silica, 0.25 μm methyl silicone J&W Scientific DB-1 column (Agilent) using 1 mL/min helium as carrier gas. The injector temperature was set at 250 °C, and the injection volume was 1 μL with split injection (1:9). The column temperature was held initially at 120 °C for 1 min and then programmed at 4 °C/min to 260 °C and then with 15 °C/min to 320 °C, which was held constant for 10 min. The transfer line was heated at 320 °C. The mass spectrometer was operated in the electron impact mode (EI, 70 eV electron energy) with a source temperature of 230 °C and the quadrupole heated at 150 °C. Mass spectra were acquired in the full scan mode ranging from *m*/*z* 40−800 with a scan rate of 2.0 scans/s.

Preparation and Isolation of norDON A, norDON B, and norDON C. The compounds were obtained by alkaline degradation of

3-AcDON. Fifty milligrams of 3-AcDON was heated in 2 mL of 0.1 M aqueous NaOH at 75 °C for 60 min (*13*). The mixture was neutralized with 200 μL of 1 M aqueous HCl and 200 μL of MeOH was added to adjust the solution to the HPLC starting conditions. The compounds were separated on an analytical 250 × 4.6 mm i.d., 5 μm Eurospher 100 column (Knauer, Berlin, Germany) using a linear binary gradient delivered by a Knauer Wellchrom Maxi-Star K-1000 HPLC pump with water as solvent A and methanol as solvent B. The HPLC was programmed as follows: isocratic step at 20% solvent B for 1 min followed by a linear gradient to 50% solvent B in 30 min. The flow rate was 700 μL/ min. For injection, a Knauer A-0258 six-port valve with a 250 μL sample loop was used. Three fractions (norDON A, norDON B, and norDON C; yield 1.4−3.3 mg) were collected after peak detection using a Knauer A-0293 single-wavelength detector set at 220 nm. Furthermore, fractions between the UV-detected peaks were collected to isolate compounds without significant UV absorbance.

Isolation of the New Compounds 9-Hydroxymethyl DON Lactone, norDON D, norDON E, and norDON F. The fractions without UV absorbance were lyophilized, redissolved in 2 mL of water−methanol (9:1), and further purified on an analytical 250 × 4.6 mm i.d., 5 μm MicroSorb-MV 100-5 C_{18} column (Varian Inc., Darmstadt, Germany) by isocratic HPLC. The solvent (water−methanol, 9:1) was delivered by a Knauer Wellchrom Maxi-Star K-1000 HPLC pump with a flow rate set at 700 μL/min. Four new compounds were isolated after peak detection using a Type 51.78 Knauer differential refractive index detector. After solvent evaporation under nitrogen streams, all compounds were further analyzed. Yield: 9-hydroxymethyl DON lactone, 1 mg; norDON D, 0.3 mg; norDON E, 0.9 mg; norDON F, 0.8 mg.

Exact Mass Measurements. The exact masses of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer with flow injection and referenced on sodium formiate. The compounds were dissolved in 1 mL of MeOH and 10 μL of a saturated solution of $NaBF_4$ in MeOH was added to measure the exact mass of the sodium adducts. The resolution of the mass spectrometer was $R_{fwhm} = 10000$ (full width at half maximum).

ESI-MS/MS. Electrospray ionization (ESI) mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. For electrospray ionization, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. Nitrogen served as curtain gas (20 psi); the declustering potential, being the accelerating current from atmospheric pressure into high vacuum, was set at −50 V in the negative mode and 50 V in the positive mode. The MS/MS parameters were dependent on the substances, detecting the fragmentation of the $[M - H]^-$ or $[M + H]^+$ molecular ions into specific product ions after collision with nitrogen (4.5×10^{-5} Torr). The collision energies are given at the respective compounds.

NMR Spectroscopy. ^{1}H and 2D NMR data were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany), ^{13}C NMR on a Unity plus (Varian, Palo Alto, CA) NMR spectrometer. Signals are reported in parts per million relative to d_4-MeOH. For structural elucidation and NMR signal assignment 2D NMR experiments such as gradient-selected correlated spectroscopy (gs-COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and 2D nuclear Overhauser enhancement spectroscopy (NOESY) were performed. Pulse programs for the experiments were taken from the Bruker software library.

Spectroscopy Data. For known compounds only the MS data are given, while the NMR data are included for the new ones.

norDON A. ESI-MS: negative mode: *m/z* (%) 265 (100) $[M - H]^-$, MS/MS (−35 V): *m/z* (%) 163 (100), 217 (39), 189 (31), 229 (27), 214 (24), 199 (23), 265 (22), 171 (16); positive mode: *m/z* (%) 267 (100) $[M + H]^+$, MS/MS (10 V): *m/z* (%) 203 (100), 213 (35), 151 (33), 185 (26), 231 (25), 125 (17), 201 (16), 175 (16).

norDON B. ESI-MS: negative mode: *m/z* (%) 265 (100) $[M - H]^-$, MS/MS (−35 V): *m/z* (%) 163 (100), 214 (48), 229 (44), 123 (19), 217 (17), 247 (16), 202 (15), 235 (11), 265 (7); positive mode: *m/z* (%) 267 (100) $[M + H]^+$, MS/MS (35 V): *m/z* (%) 175 (100), 137 (26), 157 (24), 219 (24), 173 (23), 161 (22), 159 (22), 142 (21).

norDON C. ESI-MS: negative mode: *m/z* (%) 265 (100) $[M - H]^-$, MS/MS (−35 V): *m/z* (%) 163 (100), 229 (87), 214 (44), 247 (33), 217 (25), 235 (17), 201 (16), 175 (14), 265 (10); positive mode: *m/z* (%) 267 (100) $[M + H]^+$, MS/MS (35 V): *m/z* (%) 175 (100), 213 (42), 189 (34), 161 (31), 198 (29), 231 (25), 159 (24), 145 (22).

9-Hydroxymethyl DON Lactone. Found: *m/z* 349.1262. Calculated for $C_{16}H_{22}O_7 + Na^+$: 349.1263.

ESI-MS: negative mode: *m/z* (%) 325 (100) $[M - H]^-$, MS/MS (−30 V): *m/z* (%) 325 (100), 247 (73), 235 (39), 123 (34), 295 (31), 219 (29), 207 (28), 165 (28); positive mode: *m/z* (%) 327 (100) $[M + H]^+$, MS/MS (30 V): *m/z* (%) 309 (100), 299 (46), 269 (28), 251 (13), 327 (9), 263 (6), 225 (6), 239 (5).

^{1}H NMR (400 MHz, d_4-MeOH): δ 1.11 (3H, s, H-14), 1.35 (3H, s, H-16), 1.62 (1H, dd, $J_{4A,3} = 4.2$, $J_{AB} = 14.0$, H-4A), 1.72 (1H, d, $J_{AB} = 14.8$, H-10A), 2.15 (1H, dd, $J_{4B,3} = 10.5$, $J_{AB} = 14.0$, H-4B), 2.34 (1H, dd, $J_{10B,11} = 4.2$, J_{AB}=14.8, H-10B), 3.26 (1H, d, $J_{AB} = 10.6$, H-17A), 3.43 (1H, d, $J_{AB} = 10.6$, H-17B), 3.62 (1H, d, $J_{2,3} = 4.6$, H-2), 3.88 (1H, d, $J_{AB} = 12.2$, H-13 A), 3.96 (1H, d, $J_{AB} = 10.2$, H-15A), 4.30 (1H, d, $J_{AB} = 10.2$, H-15B), 4.36 (1H, d, $J_{11,10B} = 4.2$, H-11), 4.53 (1H, d, $J_{AB} = 12.2$, H-13B), 4.59 (1H, m, $J_{3,2} = 4.6$, $J_{3,4A} = 4.2$, $J_{3,4B} = 10.5$, H-3).

^{13}C NMR (150 MHz, d_4-MeOH): δ 15.2 (C-14), 22.6 (C-16), 41.1 (C-10), 43.9 (C-4), 46.0 (C-5), 55.2 (C-9), 62.2 (C-6), 68.7 (C-17), 69.4 (C-15), 71.5 (C-3), 72.8 (C-13), 78.0 (C-11), 78.4 (C-12), 84.2 (C-2), 87.6 (C-7), 179.8 (C-8).

norDON D. Found: *m/z* 303.1202. Calculated for $C_{15}H_{20}O_5 + Na^+$: 303.1208.

ESI-MS: negative mode: *m/z* (%) 279 (100) $[M - H]^-$, MS/MS (−30 V): *m/z* (%) 279 (100), 179 (90), 177 (86), 249 (72), 187 (45), 205 (44), 191 (43), 207 (42); positive mode: *m/z* (%) 281 (100) $[M + H]^+$, MS/MS (25 V): *m/z* (%) 281 (100), 173 (79), 199 (49), 215 (48), 263 (48), 245 (46), 227 (39), 219 (39).

^{1}H NMR (400 MHz, d_4-MeOH): δ 1.07 (3H, s, H-14), 1.80 (3H, d, $J_{16,10} = 1.5$, H-16), 1.99 (1H,dd, $J_{4A,3} = 10.7$, $J_{AB} = 14.5$, H-4A), 2.11 (1H, dd, $J_{7A,6} = 7.3$, $J_{AB} = 13.5$, H-7A), 2.16 (1H, dd, $J = 12.4$, $J_{AB} = 13.5$, H-7B), 2.18 (1H, dd, $J_{4B,3} = 5.2$, $J_{AB} = 14.5$, 4B), 2.63 (1H, m, $J_{6,11} = 1.4$, $J_{6,7A} = 7.3$, $J_{6,7B} = 12.4$, H-6), 3.47 (1H, d, $J_{AB} = 11.9$, H-15A), 3.53 (1H, d, $J_{2,3} = 4.3$, H-2), 3.57 (1H, d, $J_{AB} = 11.9$, H-15B), 4.49 (1H, m, $J_{3,2} = 4.3$, $J_{3,4A} = 10.7$, $J_{3,4B} = 5.2$, H-3), 4.69 (1H, d, $J_{11,10} = 6.4$, $J_{11,6} = 1.4$, H-11), 6.93 (1H, m, $J_{10,16} = 1.5$, $J_{10,11} = 6.4$, H-10).

^{13}C NMR (150 MHz, d_4-MeOH): δ 14.4 (C-14), 15.6 (C-16), 37.0 (C-7), 41.8 (C-4), 47.9 (C-6), 54.6/55.1 (C-13/C-5), 63.7 (C-15), 69.1 (C-11), 72.7 (C-3), 85.8 (C-2), 87.6 (C-12), 139.0 (C-9), 143.7 (C-10), 203.1 (C-8).

norDON E. Found: *m/z* 305.1341. Calculated for $C_{15}H_{22}O_5 + Na^+$: 305.1365.

ESI-MS: negative mode: *m/z* (%) 281 (100) $[M - H]^-$, MS/MS (−25 V): *m/z* (%) 129 (100), 281(57), 251 (54), 121 (32), 179 (12), 233 (11), 151 (10), 205 (8); positive mode: *m/z* (%) 283 (100) $[M + H]^+$, MS/MS (25 V): *m/z* (%) 265 (100), 247 (82), 187 (67), 229 (65), 217 (53), 201 (51), 159 (50), 175 (46).

^{1}H NMR (400 MHz, d_4-MeOH): δ 1.01 (3H, d, $J_{16,9} = 6.7$, H-16), 1.01 (3H,s, H-14), 1.87 (1H, m, $J_{AB} = 14.1$, $J_{10A,11} = 2.0$, $J_{10A,9} = 12.3$, H-10A), 1.90 (1H, dd, $J_{AB} = 13.7$, $J_{7A,6} = 12.7$, H-7A), 1.95 (1H, dd, $J_{4A,3} = 10.7$, $J_{AB} = 14.4$, H-4A), 2.11 (1H, dd, $J_{4B,3} = 5.4$, $J_{AB} = 14.4$, H-4B), 2.18 (1H, dd, $J_{AB} = 13.7$, $J_{7B,6} = 7.3$, H-7B), 2.21 (1H, m, $J_{AB} = 14.1$, $J_{10B,11} = 3.6$, $J_{10B,9} = 6.5$, H-10B), 2.63 (1H, m, $J_{6,11} = 1.7$, $J_{6,7A} = 12.7$, $J_{6,7B} = 7.3$, H-6), 3.10 (1H, m, $J_{9,16} = 6.7$, $J_{9,10B} = 6.5$, $J_{9,10A} = 12.3$, H-9), 3.39 (1H, d, $J_{AB} = 12.0$, H-15A), 3.52 (1H, d, $J_{AB} = 12.0$, H-15B), 3.65 (1H, d, $J_{2,3} = 4.4$,H-2), 4.40 (1H,m, $J_{11,10A} = 2.0$, $J_{11,10B} = 3.6$, $J_{11,6} = 1.7$, H-11), 4.51 (1H, m, $J_{3,2} = 4.4$, $J_{3,4A} = 10.7$, $J_{3,4B} = 5.4$, H-3).

^{13}C NMR (150 MHz, d_4-MeOH): δ 14.3 (C-14/C-16), 35.2 (C-7), 35.6 (C-9), 37.0 (C-10), 42.0 (C-4), 52.7 (C-6), 54.7 (C-5), 57.9 (C-13), 63.6 (C-15), 72.0 (C-11), 73.1 (C-3), 86.2 (C-2), 88.3 (C-12), 217.3 (C-8).

norDON F. Found: *m/z* 319.1152. Calculated for $C_{15}H_{20}O_6 + Na^+$: 319.1158.

ESI-MS: negative mode: *m/z* (%) 295 (100) $[M - H]^-$, MS/MS (−30 V): *m/z* (%) 121 (100), 295 (72), 175 (46), 127 (45), 111 (42), 177 (37), 293 (34), 135 (29); positive mode: *m/z* (%) 297 (100) [M +

H]$^+$, MS/MS (30 V): *m/z* (%) 187 (100), 159 (43), 251 (38), 145 (34), 175 (34), 161 (31), 297 (29), 233 (25).

^{1}H NMR (400 MHz, d_4-MeOH): δ 0.96 (3H, s, H-14), 1.31 (3H, d, $J_{16,9}$ = 7.5, H-16), 1.72 (1H, dd, J_{AB} = 14.1, $J_{4A,3}$ = 5.6, H-4A), 1.82 (1H, d, J_{AB} = 14.2, H-13A), 1.92 (1H, dd, J_{AB} = 14.1, $J_{4B,3}$ = 10.5, H-4B), 2.19 (1H, m, $J_{9,16}$ = 7.5, $J_{9,10}$ = 2.1, H-9), 2.38 (1H, d, J_{AB} = 14.2, H-13B), 3.63 (1H, d, $J_{2,3}$ = 4.1, H-2), 3.84 (1H, d, $J_{10,9}$ = 2.1, H-10), 4.06 (1H, s, H-11), 4.51 (1H, m, $J_{3,2}$ = 4.1, $J_{3,4A}$ = 5.6, $J_{3,4B}$ = 10.5, H-3).

^{13}C NMR (150 MHz, d_4-MeOH): δ 15.4 (C-14), 17.6 (C-16), 33.1 (C-13), 41.9 (C-4), 53.3 (C-5), 53.5 (C-9), 59.2 (C-7), 67.6 (C-15), 70.4 (C-6), 73.2 (C-3), 83.9 (C-11), 86.1 (C-10), 86.3 (C-2), 89.6 (C-12), 184.5 (C-8).

Sample Preparation Method 1. Sixty grams of commercially available food samples (n = 43) were finely ground in a laboratory blender. Twenty-five grams was weighed in an Erlenmeyer flask and 2 μg of zearalanone was added as internal standard. The sample was extracted three times with 100 mL of methanol−water (4:1), and the supernatant was filtered, evaporated to dryness, and redissolved in 10 mL of acetonitrile. For analysis by HPLC-MS/MS, 500 μL of the extract was evaporated to dryness and redissolved in 500 μL of methanol−water (1:9). For quantification, standard solutions of the analytes and the internal standard were mixed to cover concentration ranges equaling 2.5−480 μg/kg for DON, 1−300 μg/kg for norDON A, B, and C, and 80 μg/kg for zearalanone (ZAN). The limits of detection were determined to be 2.5 μg/kg for DON and norDON A and 1 μg/kg for norDON B and C (S/N 10:1).

Sample Preparation Method 2. Twenty-five grams of commercially available food samples (n = 18, tortilla chips, bread, corn flakes, and pretzels) was weighed in an Erlenmeyer flask and 2.5 μg of 15-d_1-DON (*29*) was added as internal standard. Acetonitrile−water (4:1) (100 mL) was added and the samples were homogenized with an Ultra Turrax T25B (Janke & Kunkel IKA, Staufen, Germany) for 3 min at 20000 rpm. After filtration through a Schleicher & Schuell filter (595^1/$_2$, 320 mm), the extract was cleaned up using the method of Klötzel et al. (*27*): 4 mL of the extract was passed through a Varian Bond Elut Mycotoxin cartridge. Exactly 2 mL of the eluate was evaporated to dryness under a nitrogen stream (42 °C), and the residue was redissolved in 500 μL of water−methanol (9:1) and further analyzed by HPLC-MS/MS. For quantification, five standard solutions containing DON (10−500 ng/mL), norDON A, B, and C (1−75 ng/mL), and the internal standard 15-d_1-DON (100 ng/ mL) were prepared and analyzed by HPLC-MS/MS in the MRM mode. For the calculation of calibration curves, the peak area ratios of the analytes to the internal standard were plotted against the concentration ratios.

HPLC-ESI-MS/MS Analysis. For HPLC-ESI-MS/MS analysis, an Agilent 1100 series HPLC was linked to the API 4000 QTRAP mass spectrometer. Data acquisition was performed with the Analyst 1.4 software (Applied Biosystems). Chromatographic separation was performed on a 150 × 2.1 mm i.d., 4 μm Phenomenex SynergiFusion column (Phenomenex, Aschaffenburg, Germany) using a linear binary gradient. The injection volume was 5 μL, and the flow rate was 200 μL/ min. Solvent A was methanol and solvent B was water. The HPLC was programmed as follows: isocratic step at 10% solvent A for 1 min followed by a linear gradient to 80% solvent A in 26 min. After each HPLC run, the column was washed with 100% solvent A and equilibrated for 15 min at the starting conditions. For HPLC-MS/MS the mass spectrometer was operated in the multiple reaction monitoring mode (MRM), detecting negative ions. Zero-grade air served as nebulizer gas (35 psi) and, heated at 300 °C, as turbo gas for solvent drying (50 psi). The following transition reactions were monitored for a duration of 150 ms each. Declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) are given in brackets: **DON**: *m/z* 295−265 (DP −80 V, CE −16 V, CXP −7 V); **norDON A, B, and C**: *m/z* 265−163 (DP −95 V, CE −28 V, CXP −11 V); **9-hydroxymethyl DON lactone**: *m/z* 325−295 (DP −70 V, CE −25 V, CXP −8 V); **norDON D**: *m/z* 279−249 (DP −70 V, CE −25 V, CXP −8 V); **norDON E**: *m/z* 281−251 (DP −70 V, CE −25 V, CXP −8 V); **norDON F**: *m/z* 295−177 (DP −65 V, CE −30 V, CXP −11 V), ***d*$_1$-DON**: *m/z* 296−265 (DP −80 V, CE −16 V, CXP −7 V); **ZAN**: *m/z* 319−275 (DP −90 V, CE −30 V, CXP −15 V).

Cell Culture. Immortalized human kidney epithelial cells (IHKE cells, passage 164−167) were kindly provided by M. Gekle (Würzburg, Germany) (IHKE cells are originally from S. Mollerup, National Institute of Occupational Health, Norway). They were cultured as described by Tveito (*28*) in DMEM (Dulbecco's modified Eagle's medium)/Ham's-F12 medium (100 μL/cm^2) enriched with 13 mmol/L $NaHCO_3$, 15 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 36 μg/L hydrocortisone, 5 mg/L human apotransferrin, 5 mg/L bovine insulin, 10 μg/L mouse epidermal growth factor, 5 μg/L Na selenite, 10% fetal calf serum, and in addition 1% penicillin/streptomycin under standard cell culture conditions (37 °C, 5% CO_2).

Cytotoxicity Assay. Cytotoxicity was evaluated colorimetrically with the Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan) similar to the manufacturer's instruction. Briefly, cells were grown on a 96-well microplate. One hundred microliters of a cell suspension, containing 3 × 10^3 cells, was added to each well. After 48 h of growth, culture medium was replaced by serum free medium for 24 h. Test compounds were dissolved in methanol and added to serum free medium (the final MeOH concentration was <1%). After 24 h of incubation, the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] solution was added and the cells were incubated for 1 h. WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The absorbance of each well was measured with a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany) at 450 nm. The amount of the formazan generated by the activity of dehydrogenases in cells is directly proportional to the number of viable cells per well. The absorbance of the treated wells was compared with a solvent-treated control.

RESULTS AND DISCUSSION

To study the formation of degradation products of deoxynivalenol under food-processing conditions such as baking, model heating experiments with compounds mimicking typical food constituents were performed. DON was heated at various temperatures (150−200 °C) for different time periods (5−20 min) and with α-D-glucose (sugar model), methyl-α-D-glucopyranoside (starch model), and the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein models). The resulting residues were screened using gas chromatography-mass spectrometry (GC-MS) after trimethylsilylation. Degradation of DON was observed under all conditions, generally accelerating with rising temperatures. As previously reported for nivalenol (*25*), heating with *N*-α-acetyl-L-lysine methyl ester resulted in the fastest degradation: after 10 min at 150 °C, only 20% of DON was left, while it was 50−90% with the other model compounds. Although significant losses of DON were observed in all experiments, only in the heating experiment with the lysine derivative appreciable quantities of three degradation products (up to 40% of the total DON used) were detected. The losses that cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions, as heating DON alone results in a similar rate of decay. The formation of the degradation products in the course of heating of DON with the lysine derivative is most likely due to alkaline catalysis by the ϵ-amino group of the model compound. DON has previously been reported to be unstable under alkaline conditions and degrade into at least four products, namely, norDON A **3**, norDON B **4**, norDON C **5**, and DON lactone **6** (**Figure 1**) (*13*). For the preparation of these compounds, 3-AcDON, which was available and rapidly hydrolyzes to DON under alkaline conditions, was heated with 0.1 M NaOH as described in the literature (*13*). The solution was neutralized and separated by HPLC, collecting fractions after UV detection at 220 nm as well as the fractions between the UV peaks. The compounds

with UV absorption were analyzed by HPLC-MS/MS and ^{1}H NMR and identified as norDON A, norDON B, and norDON C.

The degradation products without or with only weak UV absorption were separated by isocratic HPLC and fractions were collected after RI detection. For structural elucidation of the isolated compounds, the exact masses were measured and 1D and 2D NMR spectra such as ^{1}H, ^{13}C, H,H-COSY, H,C-HMQC, H,C-HMBC, and H,H-NOESY were recorded. Due to the limited amount of available material (<1 mg), structural elucidation was achieved only for the four compounds **7**, **8**, **9**, and **10** shown in **Figure 1**. DON lactone was not detected; however, substances **7** and **10** are structurally similar to it: **7** has an additional CH_2O moiety at position 9 of the DON lactone skeleton; therefore, it was called 9-hydroxymethyl DON lactone **7** (9-HM DON lactone). The formation of this product can probably be explained by the addition of formaldehyde, which is released in the process of alkaline degradation of DON to norDON A, B, or C.

Compound **10** was named norDON F and is a pentacyclic isomer of DON. In comparison to DON lactone, it is hydroxylated at position 10 of the skeleton and the ring between C-7 and C-13 is linked by a C−C bond instead of an ether bridge. The compounds **8** and **9** were named norDON D and norDON E, respectively, and differ only regarding the type of bond between C-9 and C-10 of the skeleton: while norDON D has a double bond in this position, norDON E is saturated. Due to the complex changes in the molecular structure, in which released formaldehyde was most likely involved, no mechanisms for the formation of the new degradation products are proposed.

To study the relevance of the DON degradation products in food, thermally processed cereal products mainly from the German market were analyzed using a fast screening method without sample cleanup. As the structures of the new compounds had not yet been elucidated, the analysis was focused only on norDON A, B, and C. The samples were homogenized, spiked with zearalanone as internal standard, and extracted with methanol−water (3:1). The extract was filtered, evaporated to dryness, and redissolved in water−methanol (9:1) to adjust the solution to the HPLC starting conditions. No further cleanup was performed prior to analysis by HPLC-MS/MS. The analytes were ionized by electrospray in the negative mode and the mass spectrometer was operated in the multiple reaction monitoring mode (MRM). This method was developed for screening purposes and thus the results discussed below are only semiquantitative.

A total of 43 thermally processed samples were analyzed, three of which were from Mexico. These particular samples had been nixtamalized, that is, the traditional cooking of corn kernels with alkaline calcium hydroxide solution in Central America prior to the preparation of tortillas. Due to strong signal suppression of the internal standard, 9 of the 43 samples were not analyzable. DON was detected in 32 of the samples, norDON A in 21, norDON B in 12, and norDON C in 10. The results of the analyses, which are summarized in **Table 1**, clearly demonstrate the occurrence of DON degradation products in thermally processed food.

To confirm the semiquantitative results, 18 other samples were analyzed in duplicate for degradation products, including the new ones, with a different method based on the determination of DON by isotope dilution mass spectrometry (ID-MS) (*29*). The samples were homogenized, spiked with 15-d_1-DON as internal standard, and extracted with ACN−water (4:1). The extract was filtered and purified using a Bond Elut Mycotoxin cartridge. After a further concentration step, the sample was analyzed by HPLC-MS/MS.

Table 1. Semiquantitative Results of the Analysis of DON and norDON A, B, and C by HPLC-MS/MS in 34 Food Samples

compound	positive samples (>LOQ)	LOQ–10 μg/kg	10–50 μg/kg	>50 μg/kg
DON	32 (94%)	4	10	18
norDON A	21 (62%)	9	11	1
norDON B	12 (35%)	10	2	n.d.[a]
norDON C	10 (29%)	7	1	2

[a] n.d.: not detectable.

Table 2. Concentration of DON and norDON A, B, and C in Representative Food Samples (μg/kg, Mean of Duplicate Analyses)

	concentration, μg/kg			
sample	DON	norDON A	norDON B	norDON C
wholemeal cookies	301	31	<LOQ	<LOQ
wholemeal crackers	134	36	3	4
pretzels 1	64	12	1	2
pretzels 2	10	<LOQ	<LOQ	<LOQ
pretzel sticks 1	81	15	3	5
pretzel sticks 2	78	10	1	3
tortilla chips 1	208	18	<LOQ	<LOQ
tortilla chips 2	24	<LOQ	n.d.[a]	n.d.[a]
tortilla chips 3	39	<LOQ	n.d.[a]	n.d.[a]
tortilla chips 4	26	<LOQ	<LOQ	<LOQ
tortilla shells	148	13	1	2
mini-wheats	45	7	n.d.[a]	n.d.[a]
corn flakes 1	216	16	< LOQ	< LOQ
corn flakes 2	10	n.d.[a]	n.d.[a]	n.d.[a]
oat flakes	24	<LOQ	n.d.[a]	n.d.[a]
bread	62	6	<LOQ	<LOQ
maize snack	77	5	n.d.[a]	n.d.[a]
maize (nixtamalized)	107	14	6	12
positive samples (>LOQ)	100%	66%	33%	33%
mean (positive samples)	92	15	3	5
median	70	14	2	3

[a] n.d.: not detectable

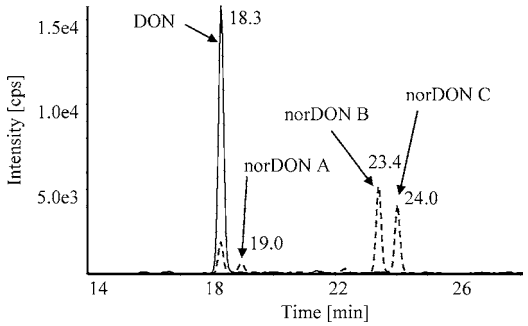

Figure 2. HPLC-ESI-MS/MS analysis of a nixtamalized maize sample (DON, 107 μg/kg; norDON A, 14 μg/kg; norDON B, 6 μg/kg; norDON C, 12 μg/kg).

For the determination of the recovery rates, a blank corn flakes sample was spiked with norDON A, B, and C at three different levels (5, 25, and 50 μg/kg), prepared in duplicate, and analyzed by HPLC-MS/MS. The recovery rates (±SD) were determined to be 113.1 ± 5.6% for norDON A, 42.0 ± 3.7% for norDON B, and 63.5 ± 4.5% for norDON C (n = 6). The low recovery rates for norDON B and C can be explained by losses in the cleanup procedure compared to the internal standard.

The results of the analyzed food samples, which were calculated without correction by the respective recovery rates, are shown in **Table 2**. They confirm the concentration range of

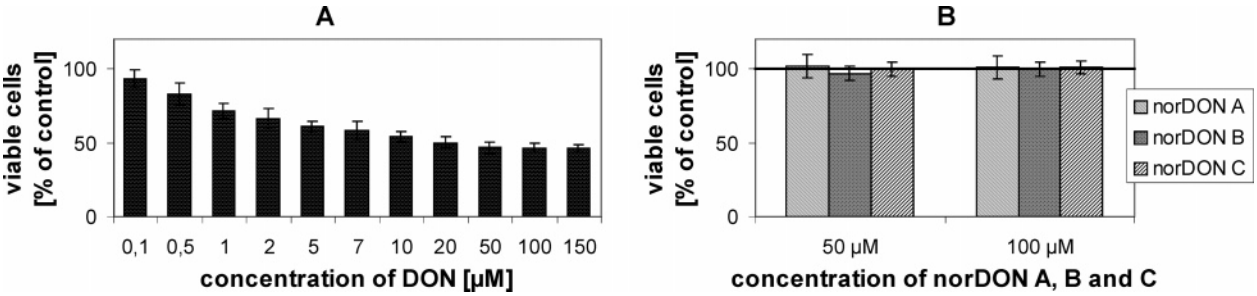

Figure 3. Cytotoxicity of (**A**) DON and (**B**) the norDON compounds in IHKE cells. The cells were incubated with the shown concentrations for 24 h, and then the viable cells were determined colorimetrically.

norDON A, B, and C determined with the first method. norDON A is more abundant than norDON B and C and its concentration is higher in most samples. The new degradation products 9-hydroxymethyl DON lactone and norDON D, E, and F were not detected in any of the samples. As a representative example **Figure 2** shows the HPLC-MS/MS chromatogram of the nixtamalized maize sample (DON, 107 μg/kg; norDON A, 14 μg/kg; norDON B, 6 μg/kg; norDON C, 12 μg/kg). Due to higher ionization efficiency, the signal intensity of norDON B and C is higher than that of DON and norDON A.

To determine the cytotoxicity of the deoxynivalenol degradation products that were detected in food, cell culture experiments were performed using immortalized human kidney epithelial cells (IHKE). As shown in **Figure 3**, DON revealed a median effective concentration (EC_{50}) at approximately 1.1 $\pm$ 0.09 μmol/L, whereas norDON A, B, and C did not exert any significant effect up to 100 μmol. These findings are in agreement with the fact that the epoxy group plays an important role in the toxicity of trichothecenes (*3*, *30*). Cell culture experiments using the 5-bromo-2′-deoxyuridine incorporation assay accessing DNA synthesis showed that the de-epoxides of deoxynivalenol were 24 times less toxic compared to the toxin with an intact epoxide ring (*30*). It was also shown in the brine shrimp bioassay that the de-epoxides are much less acutely toxic than the corresponding trichothecenes (*31*). Only recently, Cetin and Bullerman (*32*) showed that the extrusion of cereal-based products at temperatures from 150 to 200 °C reduced not only DON but also the toxicity of an extract of the extrudate in an MTT bioassay. Furthermore, our own studies showed that the structurally analogous compounds derived from the alkaline degradation of nivalenol are considerably less cytotoxic than nivalenol (*25*).

In summary, heating experiments with DON were performed to study its degradation under thermal food processing conditions. The degradation products were isolated and structural elucidation was achieved by NMR and MS experiments. Subsequently, various cereal-based food samples were analyzed for these compounds. norDON A, B, and C were detected in 29−66% of the samples in mean concentrations ranging from 3 to 15 μg/kg. Since these degradation products are less cytotoxic compared to DON in the cell culture assay used, we conclude that the formation of the degradation products during heating processes might reduce the toxicity of DON-contaminated samples.

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