Stable isotope dilution analysis of the *Fusarium* mycotoxins deoxynivalenol and 3-acetyldeoxynivalenol

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Trichothecenes are secondary metabolites produced by several fungi of the *Fusarium* genus during their growth period. They inhibit protein biosynthesis in eukaryotic cells resulting in numerous toxic effects such as diarrhea, vomiting, and gastro-intestinal inflammation. Considering its occurrence in food and feedstuff, deoxynivalenol (DON) is one of the most important trichothecenes. We report the synthesis of stable isotope labeled 15-d_1-deoxynivalenol (15-d_1-DON) from its natural precursor 3-acetyldeoxynivalenol (3-AcDON) as starting material. Furthermore, a method for the analysis of DON and 3-AcDON using HPLC-MS/MS with stable isotope labeled 15-d_1-DON and 3-d_3-AcDON as internal standards has been developed. In total, 18 cereal product samples were analyzed with contamination levels ranging from 10 – 301 µg/kg for DON and 5 – 14 µg/kg for 3-AcDON. This is the first report of an isotope dilution MS method for the analysis of type B-trichothecenes.

Keywords: 3-Acetyldeoxynivalenol / Deoxynivalenol / HPLC-MS/MS / Isotope dilution mass spectrometry / Trichothecenes

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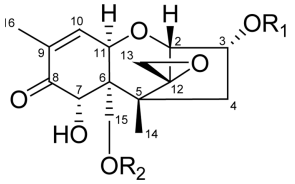
1 Introduction

Mycotoxins are a group of structurally strongly differing compounds produced by moulds during their growth period. Due to their various toxic effects, these fungal secondary metabolites pose a potentially serious health risk to both animals and humans. So far, more than 400 different mycotoxins have been discovered, with aflatoxins, ochratoxin A and the trichothecenes being the most prominent ones. About 100 of these toxins are produced by fungi of the *Fusarium* genus, such as *F. verticillioides*, *F. culmorum* or *F. graminearum*. *Fusarium* moulds are phytopathogenes that mainly infect crops in the field affecting yield and quality of cereal products. The most important *Fusarium* mycotoxins are fumonisins, zearalenone and the trichothecenes [1, 2].

Trichothecenes are a large group of sesquiterpenes with a common 12,13-epoxytrichothec-9-ene ring system as basic chemical structure [1]. The group is subdivided in type A trichothecenes such as diacetoxyscirpenol or T2-toxin and type B trichothecenes such as nivalenol or deoxynivalenol (DON **1**, Fig. 1), the difference being a keto substitution at C-8 in the latter case. They have numerous toxic effects ranging from diarrhea to vomiting and gastro-intestinal inflammation upon oral uptake [1, 2].



1: R_1 = H, R_2 = H

2: R_1 = Ac, R_2 = H

3. R_1 = H, R_2 = Ac

Figure 1. Structures of deoxynivalenol **1**, 3-acetyldeoxynivalenol **2** and 15-acetyldeoxynivalenol **3**.

Considering its occurrence and mean concentration, DON appears to be the most important mycotoxin in cereal commodities: according to the EU scientific cooperation (SCOOP) report on the "Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states" 57% of 11 022 analyzed samples were tested positive for DON [2]. Its biosynthetic precursors are 3-acetyldeoxynivalenol (3-AcDON **2**, Fig. 1) [3] and 15-acetyldeoxynivalenol (15-AcDON **3**, Fig. 1), depending on the particular *Fusarium* chemotype. 15-AcDON occurs mainly in North America and the UK,

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Abbreviations: 3-AcDON, 3-acetyldeoxynivalenol; **DON**, deoxynivalenol; **IDMS**, isotope dilution mass spectrometry; **IS**, internal standard; **MRM**, multiple reaction monitoring

while the 3-AcDON-producing strains are prevalent in Europe and Asia [3, 4]. In the course of mould growth, these acetylated derivatives are hydrolyzed to DON; nevertheless, significant amounts of 3- and 15-AcDON can also occur concomitantly [5].

In recent years, legislation has adopted the problem of trichothecenes in food: in 2005, the European Commission has proposed maximum limits for DON ranging from 200 µg/kg for cereal products for young children to 1750 µg/kg for unprocessed durum wheat and oats [6]. 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 [7].

The legal regulations of maximum limits for DON require the development of accurate methods for its quantitation. Currently, several methods are established [2], yet with inherent limitations: (i) TLC has a general lack of sensitivity and specificity. (ii) HPLC-UV detection at a wavelength of 220 nm is relatively unspecific and can be problematic depending on the respective food matrix. (iii) ELISA tests are useful for sample screening, but produce higher results, which are often not reproducible with chromatographic methods. This is probably due to cross reactions with structural analogues [8, 9]. (iv) GC methods in general require laborious sample preparation and a derivatization step prior to analysis.

HPLC coupled with MS detection has recently become the method of choice, as it offers highest sensitivity, specificity and allows the determination of different mycotoxins within one run. Inherent problems of HPLC-MS analysis are matrix effects, which enhance or more likely suppress the signal intensity of the analyte, depending on the respective sample. To assure, nevertheless, high accuracy and reproducibility of the results, matrix effects have to be compensated either by laborious matrix calibration [10] or by the use of suitable internal standards (IS). In literature, structural analogues and trichothecenes such as neosolaniol [11], verrucarol [12, 13] or deepoxy-deoxynivalenol [14] are described for this purpose. However, the transferability of these IS might be limited, due to differences between analytes and IS in the extraction and ionization efficiency, the recovery, the retention time, etc. In summary, ideal IS would be stable isotope labeled analytes, as only they fully compensate matrix effects, instrument and extraction bias in HPLC-MS analysis. Furthermore, their use reduces total workload as there is no need for recovery determination and the calibration does not have to be repeated with every new analyzed batch.

Here, we report the synthesis of stable isotope labeled DON and its use as IS in the analysis of food samples. Furthermore, 3-AcDON is quantified with 3-d_3-acetyldeoxynivalenol (3-d_3-AcDON **4**, Fig. 2) as isotopically labeled standard, of which the synthesis was recently published [15].

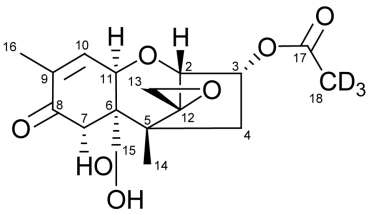


Figure 2. Structure of 3-d_3-acetyldeoxynivalenol **4**.

2 Materials and methods

2.1 General remarks

All solvents and reagents were purchased from VWR (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) in gradient or reagent grade quality. Water for HPLC separation was purified with a MilliQ Gradient A10 (Millipore, Schwalbach, Germany) system. 3-AcDON was prepared according to a procedure previously described by Altpeter *et al*. [16]. Briefly, rice was inoculated in baby food jars with *Fusarium graminearum* (DSMZ 4528) and kept at 28°C for 12 days, followed by extraction with methanol/water (70:30) and further cleanup by column chromatography and repeated crystallization of 3-AcDON.

2.2 GC-MS

Electron impact (EI) GC-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 trimethylsilylimidazol and addition of 300 µL *tert*-butyl methyl ether (*t*-BME). Data acquisition was carried out with the Chemstation software (Agilent). Chromatographic separation was performed on a 60 m × 0.25 mm id fused silica, 0.25 µm Chrompack 5861 CP-SIL 8 CB column (Chrompack, Middelburg, The Netherlands) using 1 mL/min helium as carrier gas. The injector temperature was set at 250°C, injection volume was 1 µL with split injection (1:9). The column temperature was held initially at 100°C for 1 min, then programmed at 4°C/min to 260°C, then with 15°C/min to 320°C, which was held isothermally for 10 min. The transfer line was heated to 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 quadrupol 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.

2.3 Exact mass measurement

Exact masses were measured on a Bruker Micro-TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer with flow injection and referenced on sodium formate clusters. The compounds were dissolved in 1 mL MeOH and 10 µL of a saturated solution of $NaBF_4$ in MeOH were added to measure the exact mass of the sodium adducts and thus avoid the formation of other clusters. The resolution of the mass spectrometer was $R_{FWHM} = 10\,000$.

2.4 NMR spectroscopy

The ^{1}H-, ^{13}C- and 2-D-NMR data were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany) or on a Unity plus (Varian, Palo Alto, USA) NMR spectrometer. Signals are reported in parts per million referenced to $CDCl_3$ or d_4-MeOH, respectively. For structural elucidation and NMR signal assignment 2-D-NMR experiments such as gradient selected correlated spectroscopy (gs-COSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) were carried out. Pulse programs for the experiments were taken from the Bruker software library.

2.5 Synthetic procedures

2.5.1 3-Acetyl-15-oxo-deoxynivalenol (5)

Oxalyl chloride (140 µL, 1.6 mmol) was dissolved in dichloromethane (10 mL) under argon atmosphere and cooled to −70°C in an acetone/dry ice bath. DMSO (230 µL, 3.2 mmol) in 800 µL dichloromethane was added drop wise and the solution stirred for 5 min. Then, solid 3-AcDON (420 mg of 90% purity, equal to 378 mg, 1.12 mmol) was added and stirring was continued for 15 min. Finally, triethylamine (800 µL, 5.7 mmol) was added and after 5 min the solution was warmed to room temperature. $NaHCO_3$ and NaCl saturated solution (10 mL) was added and the mixture was extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over sodium sulfate and evaporated, yielding 405 mg crude product. Purification was achieved with a Kromaton-200 fast centrifugal partition chromatograph (FCPC) (Kromaton Technologies, Angers, France) using the "Extended Arizona approach" solvent system M (H_2O : MeOH : ACN : EtOAc : tBME : n-pentane, 12 : 5 : 5 : 6 : 6 : 10, stationary: upper phase, mobile: lower phase, 800 rpm, descending mode, 10 mL/min) yielding four fractions: 1: 3-acetyl-15-oxo-deoxynivalenol **5** (273 mg, 0.81 mmol, 72% yield). 2: 3-acetyl-7-deoxy-15-oxo-deoxynivalenol **9** (19 mg). 3: mixture of **5** and **9** (31 mg). 4: recovered 3-AcDON **2** (76 mg).

2.5.1.1 3-acetyl-15-oxo-deoxynivalenol (5)

^{1}H-NMR (400 MHz, $CDCl_3$) d 1.21 (3H, s, H-14), 1.87 (3H, s, b, H-16), 2.16 (3H, s, H-18), 2.21 (1H, dd, $J_{4A,3}$ = 11.3 Hz, J_{AB} = 15.3 Hz, H-4A), 2.47 (1H, dd, $J_{4B,3}$ = 4.5 Hz, J_{AB} = 15.3 Hz, H-4B), 3.14 (1H, d, J_{AB} = 4.3 Hz, H-13A), 3.25 (1H, d, J_{AB} = 4.3 Hz, H-13B), 3.89 (1H, d, $J_{7OH,7}$ = 2.1 Hz, OH-7), 3.93 (1H, d, $J_{2,3}$ = 4.5 Hz, H-2), 4.91 (1H, d, $J_{7,7OH}$ = 2.1 Hz, H-7), 4.98 (1H, d, $J_{11,10}$ = 5.8 Hz, H-11), 5.28 (1H, m, $J_{3,4A}$ = 11.3 Hz, $J_{3,2}$ = 4.5 Hz, $J_{3,4B}$ = 4.5 Hz, H-3), 6.57 (1H, dd, $J_{10,16}$ = 1.5 Hz, $J_{10,11}$ = 5.8 Hz, H-10), 9.69 (1H, s, H-15). ^{13}C-NMR (600 MHz, d_4-MeOH) d 15.4 (C-16), 15.6 (C-14), 20.8 (C-18), 41.1 (C-4), 46.9 (C-5), 48.1 (C-13), 62.5 (C-6), 65.7 (C-12), 69.4 (C-11), 72.5 (C-3), 74.4 (C-7), 80.5 (C-2), 138.1 (C-9), 138.3 (C-10), 172.3 (C-17), 200.1 (C-8), 202.7 (C-15).

EI-MS: *m/z* (%): 43 (100), 98 (19), 41 (18), 132 (17), 179 (17), 77 (17), 95 (16), 197 (15), 336 (3) [M^+].

2.5.1.2 3-acetyl-7-deoxy-15-oxo-deoxynivalenol (9)

^{1}H-NMR (400 MHz, $CDCl_3$) d 0.85 (3H, s, H-14), 1.79 (3H, s, b, H-16), 2.19 (3H, s, H-18), 2.28 (1H, dd, $J_{4A,3}$ = 11.1 Hz, J_{AB} = 15.0 Hz, H-4A), 2.40 (1H, dd, $J_{4B,3}$ = 4.3 Hz, J_{AB} = 15.0 Hz, H-4B), 2.85 (1H, d, J_{AB} = 16.3 Hz, H-7A), 2.89 (1H, d, J_{AB} = 16.3 Hz, H-7B), 2.93 (1H, d, J_{AB} = 4.6 Hz, H-13A), 3.14 (1H, d, J_{AB} = 4.6 Hz, H-13B), 3.89 (1H, d, $J_{2,3}$ = 4.6 Hz, H-2), 5.03 (1H, d, $J_{11,10}$ = 5.8 Hz, H-11), 5.28 (1H, m, $J_{3,4A}$ = 11.1 Hz, $J_{3,2}$ = 4.6 Hz, $J_{3,4B}$ = 4.3 Hz, H-3), 6.58 (1H, dd, $J_{10,16}$ = 1.4 Hz, $J_{10,11}$ = 5.8 Hz, H-10), 9.64 (1H, s, H-15). ^{13}C-NMR (400 MHz, $CDCl_3$) d 11.4 (C-14), 15.5 (C-16), 20.9 (C-18), 36.5 (C-7), 37.9 (C-4), 44.6 (C-5), 48.0 (C-13), 57.0 (C-6), 64.6 (C-12), 65.9 (C-11), 70.6 (C-3), 78.1 (C-2), 137.2 (C-10), 139.5 (C-9), 170.2 (C-17), 195.7 (C-8), 201.4 (C-15).

EI-MS: *m/z* (%): 43 (100), 79 (21), 108 (20), 77 (20), 278 (19), 41 (19), 109 (17), 91 (17), 320 (4) [M^+].

2.5.2 3-acetyl-15-d_1-deoxynivalenol (6)

The 3-acetyl-15-oxo-deoxynivalenol (273 mg, 0.81 mmol) was dissolved in a mixture of 20 mL CH_2Cl_2 and 1 ml d_4-MeOH under argon and the solution cooled to −70°C in an acetone/dry ice bath. Solid sodium borodeuteride $NaBD_4$ (20 mg, 0.48 mmol, 98% deuterium) was added and the solution stirred for 45 min. Acetone (200 µL) was added to deactivate excess reagent and after 10 min the solution was warmed to room temperature. Saturated NaCl/$NaHCO_3$ solution (30 mL) was added and the mixture was extracted with $CHCl_3$ (3 × 20 mL). The combined organic layers were dried over sodium sulfate and evaporated, yielding 326 mg crude product. Further purification was not necessary, as the byproducts do not interfere in the next synthetic step.

^{1}H-NMR (400 MHz, $CDCl_3$) d 1.15 (3H, s, H-14), 1.89 (3H, s, b, H-16), 2.13 (3H, s, H-18), 2.15 (1H, dd, $J_{4A,3}$ = 11.3 Hz, J_{AB} = 15.1 Hz, H-4A), 2.37 (1H, dd, $J_{4B,3}$ = 4.3 Hz, J_{AB} = 15.1 Hz, H-4B), 3.11 (1H, d, J_{AB} = 4.3 Hz, H-13A), 3.18 (1H, d, J_{AB} = 4.3 Hz, H-13B), 3.83 (1H, b, H-15-AB), 3.90 (1H, d, $J_{2,3}$ = 4.5 Hz, H-2), 4.69 (1H, d, $J_{11,10}$ = 5.8 Hz, H-11), 4.82 (1H, b, H-7), 5.22 (1H, m, $J_{3,4A}$ = 11.3 Hz, $J_{3,2}$ = 4.5 Hz, $J_{3,4B}$ = 4.3 Hz, H-3), 6.60 (1H, dd, $J_{10,16}$ = 1.4 Hz, $J_{10,11}$ = 5.8 Hz, H-10).

Exact mass: *m/z* 362.1342 (calculated for $C_{17}H_{21}D_1O_7$ + Na^+: 362.13205).

EI-MS: *m/z* (%): 43 (100), 98 (29), 108 (21), 136 (20), 80 (20), 41 (18), 137 (18), 164 (18), 339 (2) [M^+]; silylated d_1-AcDON: *m/z* (%): 73 (100), 43 (31), 75 (17),104 (15), 378 (10), 74 (9), 147 (9), 194 (8), 468 (4) [M-15]$^+$, 483 (2) [M^+].

ESI-MS: negative mode: *m/z* 338 [M-H]$^-$, MS/MS (–16 V): *m/z* (%): 307 (100), 173 (64), 338 (40), 247 (21), 217 (21), 229 (11), 191 (7), 278 (6), positive mode: *m/z* 340 [M+H]$^+$, MS/MS (17 V): *m/z* (%): 231 (100), 340 (67), 203 (64), 201 (35), 291 (31), 262 (24), 219 (21), 280 (19).

2.5.3 15-d_1-deoxynivalenol (7)

The crude product obtained from the synthesis of **6** was dissolved in 10 mL 0.1 N NaOH and sonicated for 10 min. The mixture was neutralized with 1 N HCl and 5 mL MeOH were added to adjust the solution to the HPLC starting conditions. The compounds were separated on a semi-preparative Knauer Eurospher 100 column (250 × 16 mm id, 5 µm; Knauer GmbH, Berlin, Germany) using a linear binary gradient delivered by two Varian ProStar 210 HPLC solvent delivery modules (Varian, Palo Alto, USA) with water as solvent A and methanol as solvent B. The following gradient was used: 0 min, 30% solvent B; 1 min, 30% B; 21 min, 52% B; 23 min, 100% B. The flow rate was 4 mL/min. DON was collected after peak detection on a Varian ProStar 325 UV-Vis detector set at 220 nm. After evaporation of the organic solvent, the solution was lyophilized, yielding 129 mg d_1-DON (0.44 mmol, 54% yield, purity >90% (NMR, GC-MS, GC-FID), isotopic purity 97.5 ± 0.1% (HPLC-MS/MS)). The obtained d_1-DON contained one impurity that could not be separated from the target compound by preparative HPLC. However, this impurity does not influence the use of the compound as isotopically labeled IS since its molecular mass (M = 300 g/mol) is different from that of d_1-DON and DON and does not interfere in HPLC-MS/MS analysis.

^{1}H-NMR (400 MHz, $CDCl_3$) d 1.15 (3H, s, H-14), 1.73 (1H, d, $J_{15\text{-}OH,15}$ = 5.6 Hz, H-15-OH), 1.91 (3H, s, b, H-16), 2.06 (1H, d, $J_{3\text{-}OH,3}$ = 3.7 Hz, H-3-OH), 2.11 (1H, dd, $J_{4A,3}$ = 10.9 Hz, J_{AB} = 14.8 Hz, H-4A), 2.23 (1H, dd, $J_{4B,3}$ = 4.3 Hz, J_{AB} = 14.8 Hz, H-4B), 3.10 (1H, d, J_{AB} = 4.3 Hz, H-13A), 3.17 (1H, d, J_{AB} = 4.3 Hz, H-13B), 3.65 (1H, d, $J_{2,3}$ = 4.5 Hz, H-2), 3.84 (1H, d, $J_{7\text{-}OH,7}$ = 1.5 Hz, H-7-OH), 3.89 (1H, d, b, $J_{15,15\text{-}OH}$ = 5.6 Hz, H-15AB), 4.55 (1H, m, $J_{3,4A}$ = 10.9 Hz, $J_{3,2}$ = 4.5 Hz, $J_{3,4B}$ = 4.3 Hz, $J_{3,3\text{-}OH}$ = 3.7 Hz, H-3), 4.82 (1H, d, $J_{11,10}$ = 5.9 Hz, H-11), 4.85 (1H, d, $J_{7,7\text{-}OH}$ = 1.5 Hz, H-7), 6.63 (1H, dd, $J_{10,16}$ = 1.5 Hz, $J_{10,11}$ = 5.9 Hz, H-10).

Exact mass: *m/z* 320.1313 (calculated for $C_{15}H_{19}D_1O_6$ + Na^+: 320.12149).

EI-MS of silylated d_1-DON: *m/z* (%): 73 (100), 75 (15), 104 (14), 236 (14), 197 (9), 208 (9), 74 (9), 194 (8), 513 (3) [M^+], 498 (2) [M-15]$^+$.

ESI-MS: negative mode: *m/z* 296 [M-H]$^-$, MS/MS (–16 V): *m/z* (%): 265 (100), 296 (66), 247 (29), 138 (18), 278 (8), positive mode: *m/z* 298 [M+H]$^+$, MS/MS (17 V): *m/z* (%): 298 (100), 249 (60), 231 (25), 203 (20), 262 (16), 219 (13), 280 (12), 175 (7).

2.6 Stock solutions

The IS were dissolved in ACN and diluted to a concentration of 25 µg/mL and 10 µg/mL for d_1-DON and 3-d_1-AcDON, respectively. The solutions were stored at 4°C and were stable over several months.

2.7 Sample preparation

Twenty-five grams of commercially available food samples (n = 18; tortilla chips, bread, corn flakes, pretzels) were weighed in an Erlenmeyer flask. Approximately 1 µg 3-d_3-AcDON (40 µg/kg) and 2.5 µg d_1-DON (100 µg/kg) dissolved in an ACN stock solutions were added as IS. ACN/water (80 : 20) (100 mL) was added and the samples homogenized with an Ultra Turrax T25B (Janke & Kunkel IKA, Staufen, Germany) for 3 min at 20 000 rpm.

The extraction solvent was filtered through a Schleicher & Schuell folded filter (595^1/$_2$, 320 mm). Sample cleanup was performed using the method of Klötzel *et al*. [17]. Briefly, 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), the residue was redissolved in 500 µL water/methanol (9+1) and further analyzed by HPLC-MS/MS. Occasionally occurring cloudy solutions were centrifuged at 10 000 rpm prior to injection.

2.8 Recovery determination

The recovery of the method was checked by spiking a corn flakes sample containing 6 µg/kg DON and no detectable

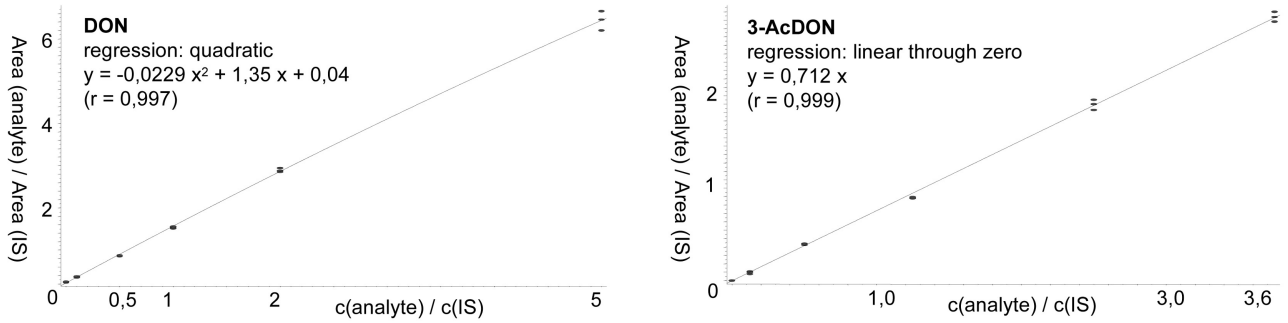


Figure 3. Calibration curves of DON and 3-AcDON using 15-d_1-DON and 3-d_3-AcDON as isotopically labeled IS (for details see Section 2).

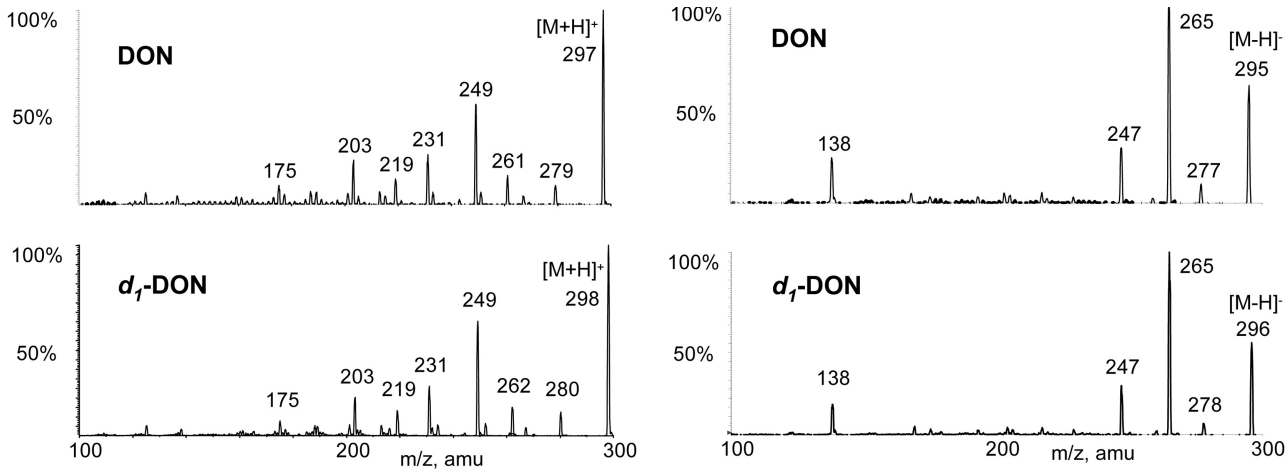


Figure 4. ESI-MS product ion spectra of DON and 15-d_1-DON (collision energy −16 V, respectively 17 V).

3-AcDON, as no blank sample of DON was available. Spiking levels were 50, 250 and 500 µg/kg for DON and 10, 50 and 100 µg/kg for 3-AcDON, respectively. The samples were cleaned up according to the procedure described above and analyzed in duplicate by HPLC-MS/MS.

2.9 Calibration curves

Calibration solutions were prepared as follows. Aliquots of standard solutions of DON and 3-AcDON were mixed in various concentration ratios (DON : d_1-DON 1 : 10 up to 5 : 1; 3-AcDON:3-d_1-AcDON 1 : 8 up to 3.75 : 1) with d_1-DON (100 ng/mL) and 3-d_3-AcDON (40 ng/mL). The mixtures were analyzed in the MRM mode as described in Section 2.10 (each concentration was injected three times). The resulting peak area ratios were plotted against the concentration ratios (see Fig. 3).

2.10 LC-MS

ESI mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. The MS/MS parameters were dependent on the compounds, detecting the fragmentation of the $[M-H]^-$ or $[M+H]^+$ molecular ions into specific product ions after collision with nitrogen (4.0×10^{-5} torr). Both quadrupols were set at unit resolution. The product ion spectra are given at the respective compounds (see Fig. 4).

For HPLC-ESI-MS/MS analysis, an Agilent 1100 series HPLC was linked to the mass spectrometer. Data acquisition was carried out with the Analyst 1.4 software (Applied Biosystems). Chromatographic separation was performed on a Phenomenex SynergiFusion column (150 × 2.1 mm id, 4 µm, Phenomenex, Aschaffenburg, Germany) using a linear binary gradient. The injection volume was 5 µL, the flow rate 200 µL/min, solvent A methanol and solvent B water. The following gradient was used: 0 min, 10% solvent A; 1 min, 10% A; 26 min, 100% A. 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 MRM mode, detecting negative ions. Zero grade air served as nebulizer

gas (30 psi), and, heated at 300°C, as turbo gas for solvent drying (50 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5×10^{-5} torr). 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), d_1-DON: *m/z* 296 → 265 (DP –80 V, CE –16 V, CXP –7 V), 3-AcDON: *m/z* 337(307 (DP – 65 V, CE – 14 V, CXP – 7 V), 3-d_3-AcDON: *m/z* 340 → 310 (DP – 65 V, CE – 14 V, CXP – 7 V). Both quadrupols were set at unit resolution.

3 Results and discussion

The legal regulation of maximum limits for mycotoxins requires the development of accurate methods for their quantitation. In the case of DON, several methods have been established, yet with the inherent limitations mentioned in the introduction. HPLC-MS/MS offers highest sensitivity and specificity, yet, due to matrix effects, especially quantitation is problematic. To assure, nevertheless, high accuracy and reproducibility, the use of a stable isotope labeled analyte as IS is essential.

For the labeling of biomolecules with stable isotopes, a biosynthetical and a chemical approach are possible. DON is biosynthetically built from acetate units [18], so *Fusarium graminearum* cultures would have to be grown on a fully labeled substrate, which we rejected because of the necessary expenses. As neither, the total synthesis of DON has been described, nor an easily accessible precursor is available, we opted for a partial synthesis approach starting from DON or its biosynthetical precursor 3-AcDON. As they are quite expensive, we first produced gram amounts of both, according to the procedure described by Altpeter *et al*. [16].

Chemically speaking, DON proved to be a difficult molecule, with multiple sensitive functional groups, such as three hydroxyls in the presence of an α,β-unsaturated ketone and the 12,13-epoxy moiety (Fig. 1). Furthermore, it is known to be unstable under alkaline conditions [19]. These properties required a careful selection of the reaction conditions.

Approaching from HPLC-MS, we compared the response of DON with ESI in both polarities and found it to be more sensitive in the negative mode, in accordance with literature [10, 12, 14, 20, 21]. In the course of tuning the instrument to develop an MRM method for quantitation purposes, we recorded the product ion spectrum of $[DON\text{-}H]^-$. Figure 4 shows the most intense fragment to be $[265]^-$, corresponding to the loss of CH_2O in the neopentyl moiety at C_{15} [22].

From this fragmentation pattern we concluded, that labeling the molecule in this position would be chemically feasible and sufficient to use it as isotope labeled standard. Figure 5a shows the natural isotope distribution of DON, while Fig. 5b takes a closer look at the particular fragmentation patterns of the isotopic forms of DON in the negative ionization mode. The [M+1] isotopic peak (named M′) of organic compounds derives mainly from the natural occurrence of carbon-13 (theoretical value: 1.1%). For DON with 15 carbon atoms, the abundance of this ^{13}C isotopomer [M' = 297] is 16.7% compared to [M = 296] (see Fig. 5a). With one ^{13}C incorporated, the statistical distribution of it would mean a 14/15 (= 93.3%) likeliness, that it is located in the rings and a 1/15 (= 6.7%) likeliness of it being located in the neopentyl moiety at C_{15} (Fig. 5b). This pattern can be monitored by MS/MS in the MRM operation mode: in the negative ionization mode the main transition of $[M'\text{-}H = 296]^-$ is to $[M'\text{-}H\text{-}CH_2O = 266]^-$ (14/15) and the lesser transition is $[M'\text{-}H = 296]^-$ to $[M'\text{-}H\text{-}CH_2O = 265]^-$ (1/15), as shown in Fig. 5b. Summarizing, for natural DON, the total abundance of the 296 → 265 transition (resulting from the ^{13}C isotopomer M′) is only about 1.2% (experimental value) of the 295 → 265 transition (Fig. 6a). This means, that DON and single labeled DON in the neopentyl moiety would generate only minimal spectral overlap under the mentioned analytical conditions (HPLC-MS/MS, negative ESI) and would thus be a suitable isotope labeled IS.

The procedures for the synthesis of mono-deuterated DON are illustrated in Scheme 1. 3-AcDON **2** was used as start-

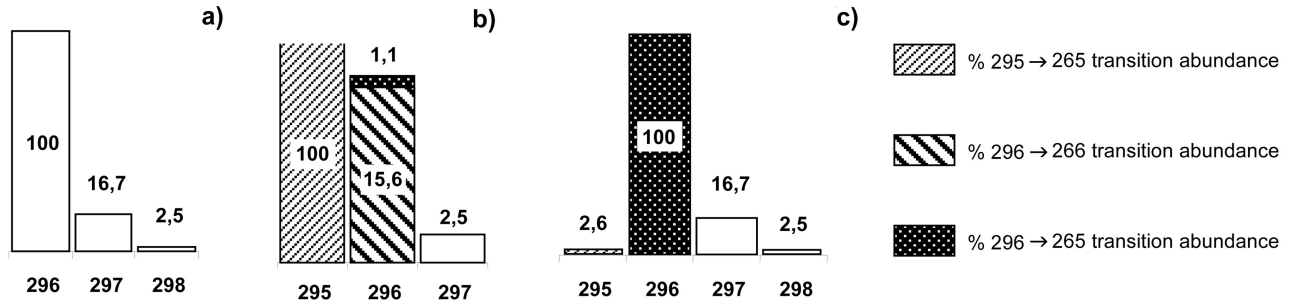


Figure 5. (a) Natural isotopic distribution of DON. (b) Theoretical fragmentation pattern diagram of the respective natural isotopes of DON in the negative mode $[DON\text{-}H]^-$. (c) Fragmentation pattern diagram of 15-d_1-DON in the negative mode $[15\text{-}d_1\text{-}DON\text{-}H]^-$.

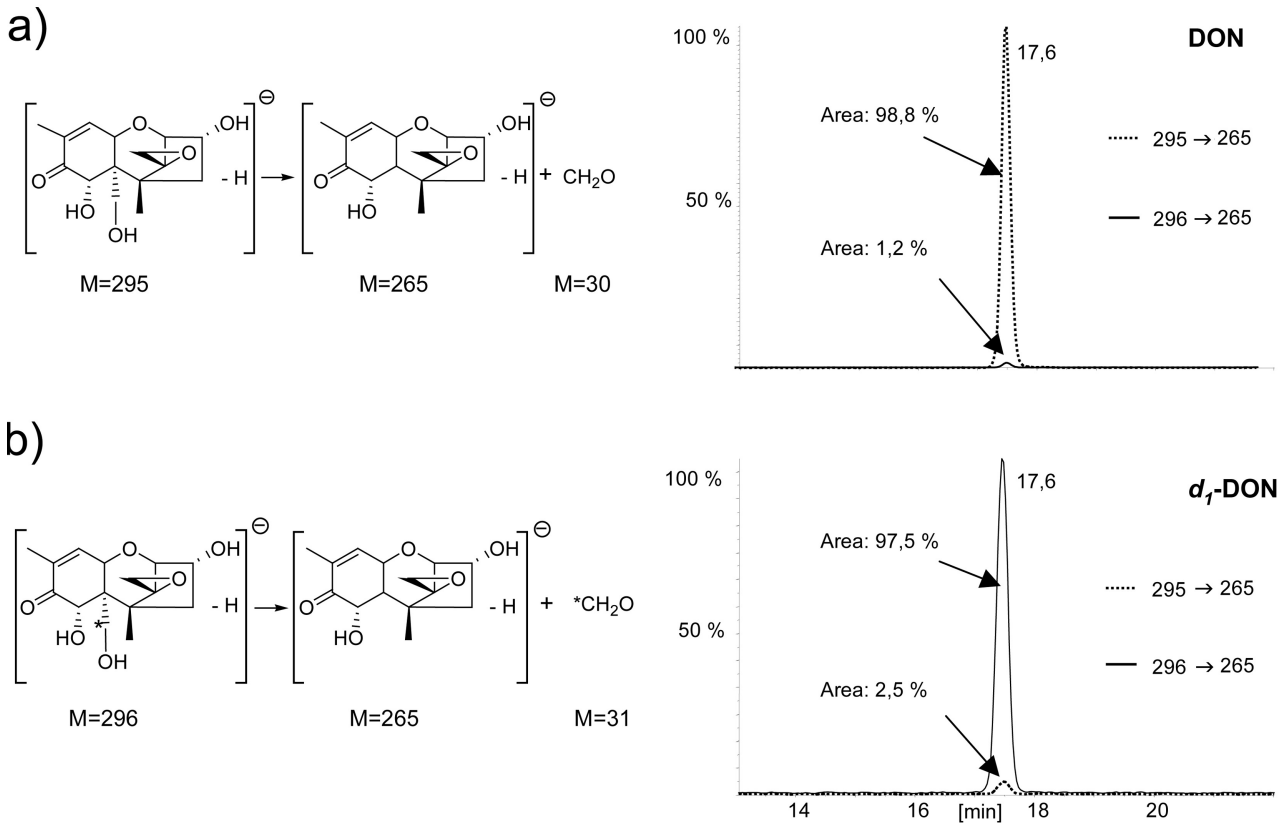
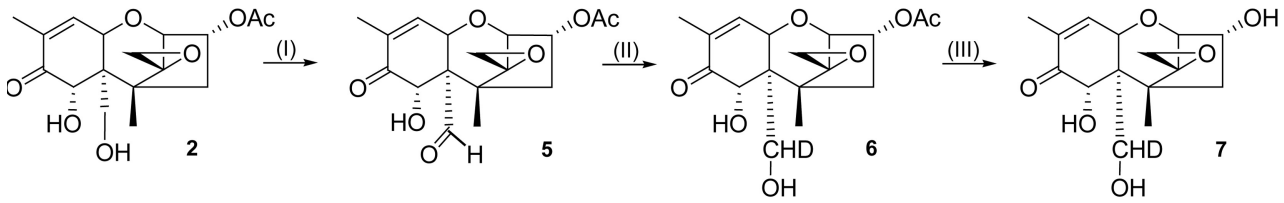


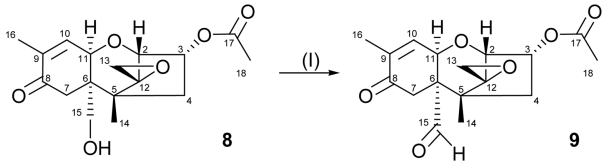
Figure 6. HPLC-MS/MS chromatograms of (a) pure DON and (b) 15-d_1-DON monitoring the respective transitions shown on the left.



Scheme 1. Synthesis of 15-d_1-DON **7**. Reagents and conditions: (I) Swern Oxidation: 1 eq. 3-AcDON, 1.3 eq. $(COCl)_2$, 2.6 eq. DMSO, 4.6 eq. Et_3N, CH_2Cl_2, −70°C. (II) 2.1 eq $NaBD_4$, CH_2Cl_2 + d_4-MeOH, −70°C (20 : 1), 45 min. (III) 0.1 N NaOH, sonication, room temperature, 10 min.

ing material for the synthesis, because the 3-hydroxy-group is already protected. The compound was oxidized under Swern conditions [23] and the crude mixture was purified by fast centrifugal partition chromatography yielding 3-acetyl-15-oxo-DON **5**, 3-acetyl-7-deoxy-15-oxo-DON **9** and unreacted 3-AcDON **2**. Compound **9** is the oxidation product of 3-acetyl-7-deoxy-DON (**8**, Scheme 2), which was an impurity in our 3-AcDON. The hydrolysis product of **8** (7-deoxy-DON) is furthermore described to be a major impurity even of commercially available DON [24].

In the next step, 3-acetyl-15-oxo-DON **5** was selectively reduced with sodium borodeuteride to 3-acetyl-15-d_1-DON **6** following the procedure of Ward *et al.* [25] to avoid reduction of the α,β-unsaturated ketone. The synthesis was completed with the hydrolyzation of 3-acetyl-15-d_1-DON using 0.1 N NaOH. Final purification was achieved by preparative HPLC yielding 129 mg 15-d_1-DON **7** (total yield 39%, purity >90% (NMR, GC-MS, GC-FID), isotopic purity/degree of deuteration 97.5% (LC-MS/MS, see transitions in Figs. 5c and 6b). The ^{1}H NMR data clearly show that deuterium is incorporated at C_{15}, resulting in a broad doublet of 15A and 15B at 3.89 ppm due to the deuterium effect, instead of a split signal with an AB coupling pattern. The product ion spectra and the HPLC-MS/MS transitions of d_1-DON compared to DON are shown in Figs. 4 and 6, respectively.



Scheme 2. Oxidation of 3-acetyl-7-deoxy-DON **8** under Swern Oxidation conditions (see Scheme 1).

The non-complete degree of deuteration and the mass increment of one result in a spectral overlap that is the interference of the IS with the analyte and vice versa [26]. In our case, these interferences are rather small and easily compensated by calibration. To fully comprehend the consequences of spectral overlap, the basic principles of mass spectrometric quantitation are discussed below.

The general procedure for calibration in MS is the preparation of standard solutions containing the same amount of IS and variable amounts of analyte. Calibration curves are recorded by plotting the area ratio of analyte to IS against the concentration ratio of analyte and IS. If no interference occurs, a straight line with the general formula shown in Eq. (1) is calculated through linear regression, with the variable *t* approaching zero.

$$y = mx + t \tag{1}$$

For better understanding, the two possible cases of spectral overlap are discussed separately. First, the non-complete deuteration of d_1-DON leads to the simulation of analyte in the analysis of DON. This means, that an injection of a solution containing only IS would result in peaks for both analyte and IS, as shown in Fig. 6b. This ratio is calculated and describes graphically the intersection of the calibration straight line with the y-axis, mathematically it is variable *t* in Eq. (1). The spectral overlap is thus completely compensated through the calibration curve and is of no further concern to the analyst.

The case of analyte simulating IS is more complicated; however, the solution is quite simple, too. Following the general procedure described above, one would expect the peak area of the IS to stay the same as the IS concentration in the standard solutions is constant. Yet, with a rising analyte concentration, its spectral overlap contributes even more to the peak area of the IS. This effect results in a non-linear calibration curve, with several possibilities for the calculation [26–28]. However, the easiest and most efficient approach is the application of a polynomial fitting routine, generating a general formula as shown in Eq. (2):

$$y = ax^2 + bx + c \tag{2}$$

A few years ago the calculation of non-linear calibration curves required special software [29]. Nowadays it is an incremental part of mass spec as well as spreadsheet software such as Microsoft® Excel™. The analysis of a given sample returns the peak area ratio, which is variable *y*, so the formula can be transformed into Eq. (3).

$$ax^2 + bx + (c - y) = 0 \tag{3}$$

The concentration ratio of analyte to IS, which is variable *x*, is calculated by Eq. (4), yielding two results with one making analytical and the other one only mathematical sense:

$$x_{1/2} = \frac{b \pm \sqrt{b^2 - 4a(c - y)}}{2a} \tag{4}$$

These calculations clearly show that also in this case the spectral overlap is completely compensated through the calibration curve.

The same equations can be used, when both types of spectral overlap occur concomitantly as in the case of 15-d_1-DON. While the simulation of IS by the analyte is compensated through quadratic regression, the converse effect is accounted for by variable *c* of Eq. (3), that is the intersection of the curve with the y-axis. As this point is a part of the calibration, the analyst should force the curve through it. The curvature of the calibration functions for DON and 3-AcDON shown in Fig. 3 are in very good accordance with the theoretical predictions. The calibration curve of DON (using d_1-DON as isotopically labeled standard) results in a non-linear regression due to the spectral overlap described above. In the case of 3-AcDON a linear calibration is obtained since the 3-d_3-AcDON used as isotopically labeled standard does not show a spectral overlap with 3-AcDON. It should be noted, that highest measurement precision is usually achieved with the ratio of analyte and standard close to one. However, the increase of the error by using analyte concentrations differing by a certain factor is generally accepted [30]. The CV were calculated from the results obtained by triple injection of the calibration standards. For the calibration curve of DON, they were 6.9 and 5.8% for the analyte-to-standard ratio of 1 : 10 and 5 : 1, respectively, while they were below 2% for the data points in between. For the linear calibration of 3-AcDON the CV were between 0.5 and 5.7%.

Following these considerations, we developed a method for the analysis of DON and 3-AcDON using 15-d_1-DON and 3-d_3-acetyldeoxynivalenol as IS. Briefly, after homogenization and addition of the IS, the samples were extracted with an ACN/water mixture. The extract was filtered and the clean up was performed using a new type of Bond Elut™ column as recently published by Klötzel *et al*. [17]. After a further concentration step, the samples were analyzed by HPLC-MS/MS. The stability of the isotope label during the clean-up procedure was confirmed with standard solutions.

As a general method parameter, we determined the S/N of the compounds in the least concentrated calibration solutions. Injection of 5 µL of a solution containing 10 ng DON/mL (equaling 10 µg/kg sample) gave an S/N of 96, while 5 ng 3-AcDON/mL (equaling 5 µg/kg sample) gave an S/N of 18. These are the lower limits of our calibration, quantitation beyond them would not be in accordance with good laboratory practice. The recovery rates were determined in corn flakes matrix spiked at three concentration levels. Two samples were worked up independently for each concentration level and the results were calculated taking into account the initial DON level of 6 µg/kg. The recovery rates for DON were 101.1 ± 12.1%, 98.8 ± 1.9% and 103.2 ± 5.3% (50, 250 and 500 µg/kg) and 86.6 ± 2.7%, 95.4 ± 6.2% and 96.2 ± 1.6% for 3-AcDON (10, 50 and 100 µg/kg).

The limits of quantitation were not determined, as they are of only little importance in the surveillance of legal limits ranging from 100 to 500 µg/kg. Furthermore, in MS this parameter strongly depends on the day-to-day instrument performance and can easily be lowered by raising the injection volume or adding further concentration steps.

In total, 18 commercially available food samples from the German and US market were analyzed in duplicate. The results including SD are shown in Table 1. 3-AcDON was detected in 17 of the samples, albeit below the quantitation limit given by the calibration curve (5 µg/kg). In 3 samples 3-AcDON was detected in concentrations ranging from 5 to 14 µg/kg. In contrast, DON was detected in all analyzed samples with concentrations ranging from 10 to 301 µg/kg. Concerning the concentrations as well as the occurrence rates, these results are in good accordance with those reported in the EU scientific cooperation (SCOOP) task 3.2.10 report with 11 022 samples analyzed for DON and 3721 analyzed for 3-AcDON [2].

Table 1. Concentrations of DON and 3-AcDON in various food samples

	Concentration, µg/kg ± SD	
Sample	DON	3-AcDON
Whole meal cookies	301 ± 9.4	< 5
Whole meal crackers	134 ± 7.7	n.d.
Pretzels 1	64 ± 1.6	< 5
Pretzels 2	10 ± 1.2	< 5
Pretzel sticks 1	81 ± 1.6	< 5
Pretzel sticks 2	78 ± 11.2	< 5
Tortilla chips 1	208 ± 13.6	10 ± 2.6
Tortilla chips 2	24 ± 3.0	< 5
Tortilla chips 3	38 ± 3.4	< 5
Tortilla chips 4	26 ± 0.8	< 5
Tortilla shells	148 ± 6.1	< 5
Mini-wheats	45 ± 1.6	< 5
Corn flakes 1	216 ± 2.8	14 ± 0.8
Corn flakes 2	10 ± 0.2	< 5
Oat flakes	24 ± 0.4	< 5
Bread	62 ± 0.7	< 5
Maize snack	77 ± 1.1	5 ± 0.1
Maizeca	107 ± 7.6	< 5

4 Concluding remarks

We present a promising approach for the exact quantitation of DON and 3-AcDON in routine food and feed analysis by isotope dilution (ID) HPLC-MS/MS. Stable isotope labeled 15-d_1-deoxynivalenol was synthesized from 3-acetyldeoxynivalenol as starting material in fair yield. Although this compound is only mono-deuterated, we demonstrated its use as IS for ID-HPLC-MS/MS analysis. Furthermore, we developed a method for the analysis of DON and 3-AcDON using 15-d_1-DON and 3-d_3-AcDON as respective isotope labeled standards. In total, 18 samples from the German and US market were analyzed with concentrations ranging from 10 to 301 µg/kg for DON and 5 to 14 µg/kg for 3-AcDON. This is the first report of a stable isotope dilution analysis of type B trichothecenes. The isotopically labeled standards used in this study will be commercially available soon.

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