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New Approach via Gene Knockout and Single-Step Chemical Reaction for the Synthesis of Isotopically Labeled Fusarin C as an Internal Standard for the Analysis of this *Fusarium* Mycotoxin in Food and Feed Samples

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ABSTRACT: The gold standard for quantitation of contaminants with high-performance liquid chromatography tandem mass spectrometry (HPLC−MS/MS) is the use of isotopically labeled standards. Herein, we report a new strategy for the synthesis of isotopically labeled 21-d_3-fusarin C via a genetically modified *Fusarium* strain, followed by a one-step derivatization reaction. Fusarin C is a *Fusarium* mycotoxin, which is mutagenic after metabolic activation. Its occurrence has been demonstrated recently in corn-based samples, but up to now, little is known about the contamination of other grain samples. To collect further data, the quantitation method was enhanced by application of the 21-d_3-fusarin C and the use of a QTRAP 5500 mass spectrometer. This new method has a limit of detection (LOD) of 1 μg/kg, a limit of quantitation (LOQ) of 4 μg/kg, and a recovery rate of 99%. A total of 21 corn samples and 13 grain samples were analyzed, with resulting fusarin C levels varying from not detectable to 24.7 μg/kg.

KEYWORDS: *Isotopically labeled reference material, 21-d_3-fusarin C, QuEChERS, stable isotope dilution assay, fusarin C, isotope dilution mass spectrometry, Fusarium, mycotoxin*

INTRODUCTION

Fusarin C is a *Fusarium* mycotoxin, which is mutagenic after metabolic activation[1−5] and occurs in food and feed.[1,6−8] Next to fusarin C, several structure analogues, such as fusarin A, fusarin D, and *epi*-fusarin C, are known.[9−11] All fusarins have a 2-pyrrolidone ring in common, which is, in the case of fusarin C and *epi*-fusarin C, substituted with an epoxide group. Under ultraviolet (UV) light, the pentaene chain of fusarin C can easily rearrange to form (10*Z*)-, (8*Z*)-, and (6*Z*)-fusarin C.[12−14] All relevant structures are shown in Figure 1.

Fusarin C is produced by a wide variety of *Fusarium* species, such as for example *Fusarium verticillioides*, *Fusarium acuminatum*, *Fusarium crookwellense*, *Fusarium nygamai*, *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium poae*, *Fusarium sambucinum*, and *Fusarium sporotrichioides*.[15−18] This indicates that fusarin C might contaminate a wide range of grains. However, occurrence data of fusarin C are rather limited because no fusarin C standard is commercially available. Up to now, fusarin C was mainly reported in corn samples of the Transkei region, South Africa,[1] Linxian county, China,[3] Pennsylvania, U.S.A.,[8] and European field and supermarket samples.[6]

Published methods for the quantitation of fusarin C used solvent extraction, followed by fractionation on amino-bonded or silica gel solid-phase columns and high-performance liquid chromatography (HPLC) with UV detection.[1,7,8] The development of a competitive indirect enzyme-linked immunosorbent assay for the detection of fusarins in food has also been reported.[19] In our recent publication, we reported the development of a quantitation method for fusarin C in corn ears and processed corn samples based on a modified QuEChERS protocol with high-performance liquid chromatography tandem mass spectrometry detection (HPLC−MS/MS).[6] QuEChERS is a quick, easy, cheap, effective, rugged, and safe sample preparation, which is used for a broad range of analytes ranging from pesticides to mycotoxins.[20,21] One disadvantage of the former method was the time-consuming preparation of matrix-matched calibration curves to compensate for ion suppression during HPLC−MS/MS measurements. With the use of isotopically labeled fusarin C, those matrix effects can easily be corrected. Besides, the addition of an isotope-labeled internal standard at the beginning of the analysis compensates for any losses during sample preparation. Stable isotope dilution analysis (SIDA) is widely used for the detection of mycotoxins in food and feed. The limiting factor for those analyses is the limited availability of commercially available internal standards and their high price. In the literature, several approaches for the production of those standards have been reported. On the one hand, mycotoxin-producing molds are cultured on completely labeled substrate[22] or labeled biosynthetic precursors[23] to obtain fully or partially labeled reference material. A big disadvantage of these approaches is the efficiency of incorporation, which causes their high price. On the other hand, the isotopically labeled standards can be synthesized by either chemical modification of

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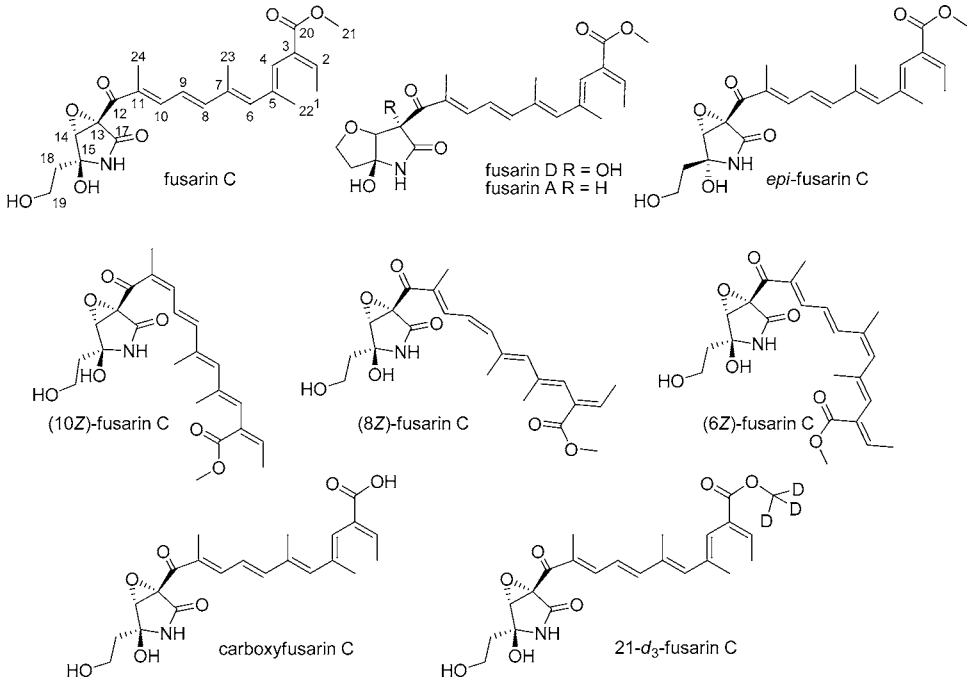

Figure 1. Structures of relevant fusarins.

the original mycotoxin[24−26] or total synthesis of the toxin.[27] For these approaches, often several reaction steps are needed, which consequently reduces the yield of the overall synthesis. In this study, we developed a new strategy for the production of labeled mycotoxins. In the literature, the synthesis of [21-^{14}C]-fusarin C was reported using enzymatic demethylation and remethylation of fusarin C.[28] This enzymatic demethylation was first reported by Gelderblom et al.[10,29] studying the metabolic activation of fusarin C with rat liver microsomes.

In our study, we used a genetically modified strain of *Fusarium fujikuroi*, which produced carboxyfusarin C, the demethylation product of fusarin C (Figure 1). Carboxyfusarin C was then methylated with labeled diazomethane to obtain isotopically labeled 21-d_3-fusarin C in high yield.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals were purchased from VWR International GmbH (Darmstadt, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), or Sigma-Aldrich GmbH (Seelze, Germany). Water for HPLC separation was purified by a Milli-Q gradient A 10 system (Millipore, Schwalbach, Germany). A 30% (w/w) solution of potassium deuteroxide in deuterium oxide (99.8 atomic % D), methanol-d_4, and deuterium oxide were purchased from Armar Chemicals (Döttingen, Switzerland). *N*-Methyl-d_3-*N*-nitroso-*p*-toluenesulfonamide was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bondesil-PSA, 40 μm, was purchased from Agilent Technologies (Böblingen, Germany).

Isolation of Carboxyfusarin C. The knockout mutant Δ*fus9* of *F. fujikuroi* IMI58289 was used for the production of carboxyfusarin C. The mutant was preincubated for 72 h in a 300 mL Erlenmeyer flask containing 100 mL of Darken Medium[30] on a rotary shaker at 28 °C and 180 rpm. A 500 μL aliquot of this culture was used to inoculate a battery of 20 Erlenmeyer flasks (300 mL), containing 100 mL of synthetic ICI medium (Imperial Chemical Industries, Ltd., U.K.)[31] with 60 mM glutamine each.

The cultures were incubated for 7 days on a rotary shaker at 28 °C and 180 rpm in the absence of light. The submerge cultures were extracted by solid-phase extraction with C18-SPE cartridges using the same extraction procedure as previously reported for the fusarins.[9] The obtained crude extract was redissolved in CH_2Cl_2/MeOH (92.5:7.5, v/v) and further purified by preparative HPLC.

Purification of Carboxyfusarins with Normal-Phase Preparative HPLC. The isolation was performed according to a previous report,[9] with slight modifications. The crude extract was separated on a 250 × 10 mm inner diameter, 5 μm, LiChrospher Si 60 column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using isocratic conditions. A Jasco PU-2089 low-pressure gradient HPLC pump (Jasco, Gross-Umstadt, Germany) coupled to a Jasco diode array detector (MD-2010 Plus) was used. The solvent composition was 92.5% CH_2Cl_2 and 7.5% MeOH (v/v); the flow rate was set to 5 mL/min; and the run time was 15 min. Data were recorded in a wavelength range of 200−650 nm, and the extracted wavelength of 363 nm was used for identifying the fractions containing carboxyfusarins. Acquisition of data was carried out with Chrompass, Chromatography Data system (version 1.8.6.1, Jasco).

The solvent was removed by a rotary evaporator. One batch of cultivation yielded about 20 mg of carboxyfusarin C (5.5 min), 8 mg of *epi*-carboxyfusarin C (6.0 min), and 3 mg of carboxyfusarin D (6.5 min).

The structure of carboxyfusarin C was confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis and is in accordance with the literature.[10]

Spectroscopic Data of Carboxyfusarin C. ^{1}H NMR (400 MHz, CD_2Cl_2) δ: 7.53 (d, J = 10.9 Hz, 1H), 7.03 (q, J = 7.1 Hz, 1H), 6.82 (d, J = 14.9 Hz, 1H), 6.66 (dd, J = 14.6, 11.2 Hz, 1H), 6.32 (s, 1H), 6.08 (s, 1H), 4.01 (s, 1H), 4.01−3.93 (m, 1H), 3.95−3.81 (m, 1H), 2.09 (s, 3H), 2.07−2.01 (m, 2H), 1.97 (s, 3H), 1.78 (d, J = 7.1 Hz, 3H), 1.75 (s, 3H).

^{13}C NMR (101 MHz, CD_2Cl_2) δ: 191.4, 170.4, 170.0, 149.9, 147.1, 142.0, 141.5, 138.2, 135.6, 134.0, 130.6, 127.0, 124.0, 85.7, 64.1, 62.4, 58.5, 36.5, 19.1, 16.5, 14.4, 11.6.

ESI−MS (positive mode): FTMS m/z 440.1682 (calculated mass for $[C_{22}H_{27}O_7N + Na]^+$: m/z 440.1680); MS/MS (CID 35%; $[M + Na]^+$): m/z (%) 354 (100), 396 (26), 290 (17), 422 (11).

Synthesis of 21-d_3-Fusarin C. To obtain 21-d_3-fusarin C, carboxyfusarin C was methylated with labeled diazomethane in a single-step reaction. Diazomethane was prepared by hydrolysis of 150 mg of *N*-methyl-d_3-*N*-nitroso-*p*-toluenesulfonamide in 5 mL of dry diethyl ether.[32,33] Because diazomethane can easily exchange hydrogen for deuterium atoms, which decreases the isotopic purity of 21-d_3-

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fusarin C, any source of hydrogen was removed. Therefore, a 30% (w/w) solution of potassium deuteroxide in deuterium oxide, absolute ether, and 2-(2-ethoxyethoxy)ethanol, which was mixed with D_2O and distilled afterward, was used. In addition, carboxyfusarin C was dissolved 2 times in methanol-d_4 and evaporated to dryness to remove any exchangeable hydrogen.

A large excess of diazomethane would yield the 21-d_3-15-O-d_3-dimethoxylated product. Therefore, 10−20 μL of the reaction mixture was diluted in 500 μL of methanol/water (50:50, v/v) and measured via MS with direct infusion. The diazomethane ether solution was added until no carboxyfusarin C was detectable anymore. About 2 mL of the diazomethane ether solution was used for 20 mg of carboxyfusarin C. Afterward, the diazomethane ether solution was removed by vaporization, and the reaction mixture was further purified with preparative HPLC.

Purification of 21-d_3-Fusarin C with Normal-Phase Preparative HPLC. The purification of the reaction products was performed according to our previous report,[9] yielding 5.3 mg of labeled fusarin C.

Spectroscopic Data of 21-d_3-Fusarin C. ^{1}H NMR (400 MHz, CD_2Cl_2) δ: 7.49 (d, J = 11.0 Hz, 1H), 6.96 (qd, J = 7.2, 0.9 Hz, 1H), 6.80 (d, J = 15.0 Hz, 1H), 6.80 (s, 1H), 6.66 (dd, J = 15.0, 10.9 Hz, 1H), 6.30 (s, 1H), 6.07 (s, 1H), 4.11−4.04 (m, 1H), 4.05 (d, J = 1.7 Hz, 1H), 3.97−3.90 (m, 1H), 2.16−2.04 (m, 2H), 2.09 (d, J = 0.8 Hz, 3H), 1.98 (d, J = 0.8 Hz, 3H), 1.77 (dd, J = 7.2, 1.4 Hz, 3H), 1.73 (d, J = 1.1 Hz, 3H).

^{13}C NMR (101 MHz, CD_2Cl_2) δ: 190.5, 170.2, 168.0, 149.4, 146.1, 141.2, 140.6, 138.0, 135.6, 134.1, 130.9, 126.9, 124.0, 86.0, 64.2, 62.3, 59.0, 36.3, 19.1, 16.4, 14.5, 11.7.

ESI−MS (positive mode): FTMS m/z 457.2018 (calculated mass for $[C_{23}H_{26}D_3O_7N + Na]^+$: m/z 457.2024); MS/MS (CID 35%; $[M + Na]^+$): m/z (%) 338 (100), 290 (78), 409 (37), 429 (34), 439 (31), 399 (36), 344 (23), 355 (22), 411 (16), 270 (15), 381 (13), 421 (12), 427 (12).

Isotopic Purity. To determine the isotopic purity, the same HPLC−MS/MS system as described for the food samples was used. Additional multiple reaction monitoring (MRM) transitions were added to the MS method for 21-d_1-fusarin C and 21-d_2-fusarin C: 21-d_1-fusarin C $[M + H]^+$ 433.1−114.9 (CE 129 V, CXP 14 V) and 433.3−141.1 (CE 91, CXP 8) and 21-d_2-fusarin C $[M + H]^+$ 434.1−114.9 (CE 129 V, CXP 14 V) and 434.1−141.1 (CE 91, CXP 8). The first MRM transition was used as a quantifier, and the second MRM transition was used as a qualifier. For determination of isotopic purity, the relative isotope composition of the labeled fusarin C was calculated by dividing the area of each peak of fusarin C, 21-d_1-fusarin C, 21-d_2-fusarin C, and 21-d_3-fusarin C by the sum of all four peak areas. The labeled fusarin C standard solution consists of 0.3% fusarin C, 6% 21-d_1-fusarin C, 32% 21-d_2-fusarin C, and 62% 21-d_3-fusarin C. No exchange of the deuterium atoms was detectable under storage for several months.

Standard Solutions. Because fusarin C is not commercially available, reference material was obtained according to our previous report.[9] The purities of the reference material and 21-d_3-fusarin C were checked by HPLC with UV and evaporative light scattering detection (ELSD)[6] and yielded 100% (by ELSD) purity for both standards, taking into account that fusarin C rearranges under reversed-phase conditions, forming *epi*-fusarin C.[9] A total of 21.6 mg of fusarin C and 5.3 mg of synthesized 21-d_3-fusarin C were dissolved in 10 mL of MeOH, each. Because the isotopic purity of 21-d_3-fusarin accounts for 62%, the resulting weight of 5.3 mg was corrected, yielding a total amount of 3.3 mg of 21-d_3-fusarin C. Both solutions were diluted to a concentration of 10 and 1 μg/mL and stored at −80 °C. The stability of these stock solutions under this condition has been previously demonstrated.[6]

Samples. Food and feed samples were from Germany and Poland and were obtained from local food stores or collaborating laboratories. The analyzed samples were corn kernels, corn meal, polenta, wheat bran, wheat whole meal, wheat kernels, oat flakes, rye flour, spelt, and barley.

HPLC−MS/MS. A QTRAP 5500 mass spectrometer (AB SCIEX, Darmstadt, Germany) coupled to a VWR Hitachi LaChrom Ultra HPLC system was used for the detection of fusarin C in the MRM mode. Data acquisition was performed with Analyst 1.5.2 software (AB SCIEX, Darmstadt, Germany).

HPLC Parameters. Chromatographic separation was carried out on a 150 × 2.1 mm inner diameter, 5 μm, Zorbax Eclipse XDB-C18 column (Agilent Technologies, Böblingen, Germany) using a linear binary gradient at a column temperature of 40 °C. The injection volume was 30 μL, and the autosampler was cooled to 7 °C. The flow rate was set to 300 μL/min. Solvent A was MeOH with 5% tetrahydrofuran (v/v), and solvent B was H_2O. HPLC was programmed isocratic for the first 16 min at 55% A. Only for samples, the column was washed afterward with 100% solvent A for 5 min to remove any nonpolar substances and equilibrated at starting conditions. The calibration curves were measured before and after the samples under isocratic conditions without a washing step for the column, which reduced the analysis time.

MS/MS Parameters. For electrospray ionization (ESI), the ion voltage was set to +5500 V in the positive mode and nitrogen was used as the curtain gas (20 psi). Zero-grade air was used as the nebulizer gas (35 psi) and drying gas (45 psi) heated to 350 °C. The declustering potential was set for all transitions to 71 V; the entrance potential was set to 10 V; and the collision gas (CAD) was set to "medium". For quantitation, transition reactions were monitored for a duration of 50 ms each. The proton adduct of fusarin C and 21-d_3-fusarin C was measured with the following collision energy (CE) and collision cell exit potential (CXP): fusarin C $[M + H]^+$ 432.1−114.9 (CE 129 V, CXP 14 V) and 432.3−141.1 (CE 91, CXP 8) and 21-d_3-fusarin C $[M + H]^+$ 435.1−114.9 (CE 129 V, CXP 14 V) and 435.1−141.1 (CE 91, CXP 8). Both quadrapoles were set at unit resolution. The first MRM transition listed was used as a quantifier and the second MRM transition listed was used as a qualifier.

Sample Preparation. Sample preparation was performed according to our previous publication,[6] with slight modifications. A total of 5 g of ground and homogenized grain samples were weighed into a 50 mL centrifuge tube, and a total of 125 ng of 21-d_3-fusarin C (125 μL of a solution of 1 μg/mL 21-d_3-fusarin C in methanol) was added as an internal standard. A total of 5 mL of H_2O and 15 mL of acetonitrile were added. Afterward, the tubes were shaken vigorously for 1 min using a Vortex mixer at maximum speed. Then, 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added, and the samples were again mixed for 1 min on a Vortex mixer to prevent the formation of $MgSO_4$ conglomerates. Before usage, $MgSO_4$ was heated for 10 h to 500 °C in a muffle furnace to remove residual water. Next, the samples were centrifuged for 5 min at 3000 rpm. As a cleanup step, 5 mL of the supernatant was added to 750 mg of $MgSO_4$ and 125 mg of Bondesil-PSA (primary and secondary amines). After 1 min of shaking, the sample was centrifuged for 5 min at 3000 rpm. To concentrate the samples, 3 mL of the dispersive solid-phase extraction cleanup solution was evaporated to dryness and redissolved in 500 μL of MeOH/H_2O (50:50, v/v). Afterward, the samples were centrifuged at 9000 rpm for 10 min to remove insoluble particles. The supernatant was analyzed by HPLC−MS/MS. Every sample was worked up twice, and each sample preparation was analyzed 2 times. The standard deviation (SD) was calculated for all determinations.

All laboratory work was carried out under reduced light conditions because of the instability of fusarin C. Therefore, the centrifuge tubes were wrapped with aluminum foil, and for analysis, amber screw-cap vials were used.

Calibration Curves. For calibration, standard solutions containing 0, 2.5, 5, 10, 30, 50, 70, 90, 110, 130, and 150 ng/mL fusarin C in MeOH/H_2O (50:50, v/v) were prepared. Each calibration point was spiked with 50 ng of 21-d_3-fusarin C (50 μL of a solution of 1 μg/mL 21-d_3-fusarin C in methanol) as an internal standard. For quantitation, the calibration curves measured before and after the samples were averaged. For the calculation of the calibration curves, the peak area ratios of the analyte to the internal standard were plotted against the concentration ratios.

Validation Parameters of the HPLC−MS/MS Method for the Determination of Fusarin C in Corn Products. As previously described, the limit of detection (LOD) and limit of quantitation

(LOQ) were calculated according to the calibration method of the German Standard DIN 32645.[6,34]

A total of 5 g of blank sample was spiked at the beginning of the sample preparation with 12.5, 25, 75, 125, 175, 225, and 275 μL of 1 μg/mL fusarin C stock solution and incubated with the toxin for 1 h. The spiking levels correlate to contamination levels of 2.5, 5, 15, 25, 35, 45, and 55 μg/kg of fusarin C, respectively. The sample preparation was performed as described above. Each calibration point was prepared in duplicate, and each sample was measured twice. Calibration curves were calculated by linear regression. As previously described, the recovery rate over the entire working range was determined from the calibration curve of pure standard in comparison to the calibration curve of the spiked samples by the function of recovery.[6,35]

RESULTS AND DISCUSSION

Stable isotope dilution MS is the gold standard for HPLC−MS/MS analysis to determine mycotoxins as well as other contaminants in food and feed. The stable isotope standard not only compensates for losses during sample cleanup but also corrects the discrimination of signals as a result of ion suppression.[36]

Synthesis of 21-d_3-Fusarin C. According to our approach, isotopically labeled 21-d_3-fusarin C was produced via the methylation of carboxyfusarin C with labeled diazomethane. To obtain higher amounts of carboxyfusarin C as starting material, the knockout mutant Δ*fus9* of *F. fujikuroi* IMI58289 was used. This mutant lacks the gene for the methyltransferase, which is responsible for the methylation of the carboxyl group at position C-20 to obtain fusarin C in the final step of the biosynthesis. Therefore, the knockout mutant Δ*fus9* produces carboxyfusarin C, the demethylation product of fusarin C, and also *epi*-carboxyfusarin C and carboxyfusarin D. Figure 2 shows

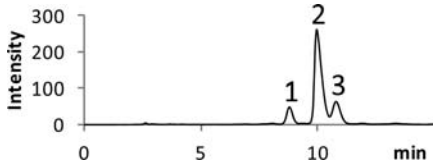

Figure 2. HPLC−DAD chromatogram of a culture filtrate of Δ*fus9* of *F. fujikuroi* IMI58289 (1, *epi*-carboxyfusarin C; 2, carboxyfusarin C; and 3, carboxyfusarin D).

a HPLC−diode array detector (DAD) chromatogram of a culture filtrate of the mutant. The peak pattern is similar to that obtained for fusarin C and its analogues,[9] but carboxyfusarin C, *epi*-carboxyfusarin C, and carboxyfusarin D elute earlier from the column. Carboxyfusarin C was isolated according to the procedure recently published for fusarin C, with slight modifications with regard to the HPLC solvents.[9] The structure of carboxyfusarin C (Figure 1) was confirmed by Fourier transform mass spectrometry (FTMS), NMR, and a comparison to literature data.[10]

In the next step, carboxyfusarin C was methylated with deuterated diazomethane, which was obtained from *N*-methyl-d_3-*N*-nitroso-*p*-toluenesulfonamide according to the commonly used procedure.[32,33] Because diazomethane can easily exchange hydrogen and deuterium, any source of exchangeable hydrogen was avoided during synthesis. With an isotopic purity of 62% 21-d_3-fusarin C (32% 21-d_2-fusarin C, 6% 21-d_1-fusarin C, and 0.3% fusarin C), the labeled fusarin C can be used for HPLC−MS/MS analysis. The structure of 21-d_3-fusarin C was confirmed by NMR and MS analysis and is accordant to literature data for unlabeled fusarin C.[9] In comparison to unlabeled fusarin C, the signal for the d_3-methyl group in position 21 is not detectable. All other signals are in agreement with unlabeled fusarin C.

Sample Preparation. The sample preparation was performed according to a QuEChERS protocol. In our previous publication,[6] we reported the use of QuEChERS for the detection of fusarin C in food and feed. One time-consuming step of this method was the laborious preparation of matrix-matched calibration curves, which had to be performed to compensate for ion suppression during HPLC−MS/MS measurements. This time-consuming step is now eliminated because of the use of 21-d_3-fusarin C. To correct for losses during sample preparation and to correct for matrix effects during measurement, the isotopically labeled 21-d_3-fusarin C was added at the beginning of the sample preparation. A further improvement that yielded lower LOD and lower LOQ was the usage of a QTRAP 5500 mass spectrometer instead of a 4000 QTRAP mass spectrometer. Figure 3 presents HPLC−MS/MS

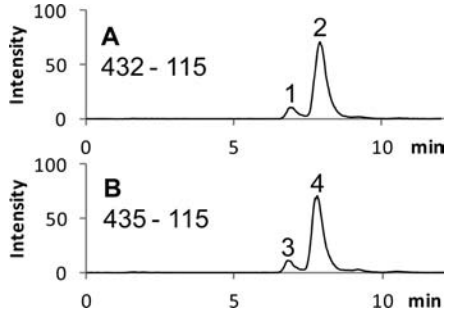

Figure 3. HPLC−MS/MS chromatogram of (A) fusarin C and (B) 21-d_3-fusarin C presenting the quantifier MRM transitions (1, *epi*-fusarin C; 2, fusarin C; 3, 21-d_3-*epi*-fusarin C; and 4, 21-d_3-fusarin C).

chromatograms of fusarin C and 21-d_3-fusarin C. As described previously, the LOD and LOQ were determined according to the calibration method of the German Standard DIN 32645.[6] With this new protocol, lower concentrations of fusarin C were detectable with a LOD of 1 μg/kg (2 ng/mL) and a LOQ of 4 μg/kg (8 ng/mL). The recovery rate was determined by the function of recovery calculated from the calibration curves of the standards and from the calibration curves of the spiked samples and yielded a recovery of 99%.[6,35]

Fusarin C in Corn and Other Grains. Food and feed samples based on corn or other grains were analyzed for fusarin C. Table 1 summarizes the contents of fusarin C in polenta, cornmeal, popcorn, and whole corn. Fusarin C was detectable in 7 of 13 samples: in 2 samples of polenta, no fusarin C could be detected, and in cornmeal, 1 sample was below the LOQ and 1 sample contained no fusarin C. The other cornmeal samples had a fusarin C content of 6.1 ± 0.3, 5.2 ± 0.5, and 4.4 ± 0.2 μg/kg, respectively. In corn kernels used for the production of popcorn, 2 of 3 samples contained fusarin C with 24.7 ± 0.1 and 12.7 ± 0.3 μg/kg. All tested whole corn samples contained fusarin C with levels of 14.9 ± 0.1 and 11.5 ± 0.3 μg/kg, and 1 sample was below the LOQ. The 8 tested feed samples contained fusarin C ranging from 7.3 ± 0.2 to 17.0 ± 0.3 μg/kg, and 1 sample was below the LOQ. All data are summarized in Table 2. In summary, the fusarin C levels in corn samples in this study are consistent with the fusarin C content determined previously.[6]

Table 1. Fusarin C Levels in 13 Food Samples Containing Corn

type of food sample	concentration of fusarin C ± SD (μg/kg)
polenta	<1
	<1
	6.1 ± 0.3
	5.2 ± 0.5
cornmeal	4.4 ± 0.2
	<1
	<1
	24.7 ± 0.1
popcorn	12.7 ± 0.1
	<1
	14.9 ± 0.1
whole corn	11.5 ± 0.3
	<4

Table 2. Fusarin C Levels in 8 Feed Samples

type of feed sample	concentration of fusarin C ± SD (μg/kg)
corn kernels	17.0 ± 0.3
	13.5 ± 0.2
	8.7 ± 0.9
	8.3 ± 0.4
	8.2 ± 0.8
	7.3 ± 0.2
	<4
corn mix	11.9 ± 0.2

To evaluate the occurrence of fusarin C in other grains, naturally contaminated samples from the German market containing trichothecenes were analyzed. The recovery of the internal standard was used to evaluate the extraction efficiency, which was acceptable for all tested grain samples. Oat, spelt, rye, wheat, and barley were tested, and Table 3 summarizes the

Table 3. Fusarin C in 13 Different Grain Samples

type of food sample	number of samples	concentration of fusarin C ± SD (μg/kg)
oat	3	the fusarin C content of all samples was below <1
spelt	2	
rye	2	
wheat	5	
barley	1	

results. Fusarin C was not detectable in any of these samples, leading to the preliminary observation that fusarin C does not co-occur with trichothecenes. These results also suggest that fusarin C mainly occurs in corn-based products. Clearly, further studies are needed for a detailed evaluation of the occurrence of fusarin C in food and feed and their co-occurrence with other mycotoxins. Because no tolerable daily intake and no maximum levels have been set, any risk assessment based on the detected fusarin C concentrations is currently not possible.

In summary, we have presented a new method for the production of 21-d_3-fusarin C via gene knockout mutants in combination with a simple chemical derivatization step. With the use of 21-d_3-fusarin C, an improved method for the detection of fusarin C in food and feed samples was developed with a recovery rate of 99%, a LOD of 1 μg/kg, and a LOQ of 4 μg/kg. This method can now be used to improve the limited occurrence data of fusarin C in food and feed.

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Notes

The authors declare no competing financial interest.

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