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Use of Isotope-Labeled Aflatoxins for LC-MS/MS Stable Isotope Dilution Analysis of Foods

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Aflatoxins are a group of very carcinogenic mycotoxins that can be found on a wide range of food commodities including nuts, cereals, and spices. In this study, the first LC-MS/MS stable isotope dilution assay (SIDA) for the determination of aflatoxins in foods was developed. The development of this method was enabled by easily accessible isotope-labeled (deuterated) aflatoxins B_2 and G_2, which were synthesized by catalytic deuteration of aflatoxin B_1 and G_1, purified, and well-characterized by NMR and MS. All four aflatoxins of interest (B_1, B_2, G_1, and G_2) were quantified in food samples by using these two labeled internal standards. The response factors (RF) of the linear calibrations were revealed to be matrix independent for labeled aflatoxin B_2/aflatoxin B_2 and labeled aflatoxin G_2/aflatoxin G_2. For labeled aflatoxin B_2/aflatoxin B_1 and labeled aflatoxin B_2/aflatoxin G_1 matrix-matched calibration was performed for the model matrices almonds and wheat flour, showing significant differences of the RFs. Limits of detection (LOD) were determined by applying a statistical approach in the presence of the two model matrices, yielding 0.31 μg/kg (aflatoxin B_1), 0.09 μg/kg (aflatoxin B_2), 0.38 μg/kg (aflatoxin G_1), and 0.32 μg/kg (aflatoxin G_2) for almonds (similar LODs were obtained for wheat flour). Recovery rates were between 90 and 105% for all analytes. Coefficients of variation (CV) of 12% (aflatoxin B_1), 3.6% (aflatoxin B_2), 14% (aflatoxin G_1), and 4.8% (aflatoxin G_2) were obtained from interassay studies. For further validation, a NIST standard reference food sample was analyzed for aflatoxins B_1 and B_2. The method was successfully applied to determine trace levels of aflatoxins in diverse food matrices such as peanuts, nuts, grains, and spices. Aflatoxin contents in these samples ranged from about 0.5 to 6 μg/kg.

KEYWORDS: Aflatoxin; mycotoxin; LC-MS/MS; stable isotope dilution assay; internal standard

INTRODUCTION

Aflatoxins are secondary metabolites of fungal species such as *Aspergillus flavus* or *Aspergillus parasiticus* growing on a variety of foods. In particular, peanuts, nuts, spices, and cereals are known to be often contaminated with this class of mycotoxins (*1*). The outstanding prominence of aflatoxins is due to the fact that they are considered to be one of the most potent natural carcinogens. Many mycotoxicoses have been associated with the consumption of aflatoxins, and the incidence of liver cancer in Africa has been proven in epidemiologic studies to be highly correlated with aflatoxin intake (*2*). Besides these chronic effects, acute outbreaks of aflatoxicoses in this millenium are still a current threat in Africa, as reported in Kenya in 2004 with over 100 deaths (*3*). Due to their hepatotoxic and hepatocarcinogenic properties, the content of aflatoxins B_1, B_2, G_1, and G_2 (**Figure 1**) in foods is restricted in many countries (*4*). However, according to a European Union (EU) screening during the years 2000–2006, these mycotoxins were still

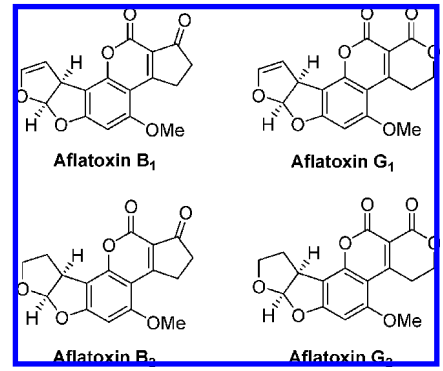

Figure 1. Structures of the four aflatoxins of interest.

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detectable in 26% of all analyzed foods (*1*), which points to the need for an effective food control.

The most frequently used method to detect aflatoxins is HPLC coupled to fluorescence detection after extract cleanup by immunoaffinity chromatography (*5*), but LC coupled to tandem mass spectrometry has gained increasing importance due to its superior specificity (*6–8*). However, both methods are hampered with recoveries as low as 70%, for example, for spices (*9*) or nuts (*10*), as extraction from these matrices may not be complete. Additionally, even the latest LC-MS ionization sources may suffer from matrix-dependent ionization suppression (*11*), for example, in the case of aflatoxins for certain food matrices (*12, 13*). A possible way to overcome both drawbacks is the use of internal standards labeled with stable isotopes. As these analogues show virtually identical chemical and physical properties as the respective analytes, losses and suppressions can effectively be compensated for by applying so-called stable isotope dilution assays (SIDAs). Hence, SIDAs reveal superior validation data compared to commonly used methods, as we reported on the analysis of mycotoxins such as trichothecenes (*14, 15*), ochratoxin A (*16*), and patulin (*17*). Consequently, the objective of the present study was to synthesize novel stable isotope-labeled aflatoxins and to apply them as internal standards in SIDAs for food analysis.

MATERIALS AND METHODS

Materials and Reagents. Solid standards of aflatoxins were obtained from Sigma-Aldrich (St. Louis, MO). Polyvinylpyrrolidone-stabilized palladium nanoparticles (PVP-Pd) were synthesized by reduction of palladium chloride in alkaline methanolic solution in the presence of PVP (M ~ 10000) (Fluka, Buchs, Switzerland) (*19*). All other chemicals mentioned were of analytical grade and purchased from Sigma. Naturally contaminated peanut butter (NIST standard reference material 2387) was obtained from LGC Promochem (Wesel, Germany).

Synthesis of Labeled Aflatoxins B_2 and G_2. Ten milligrams of aflatoxin B_1 or aflatoxin G_1 was dissolved in 6 mL of dioxane. Four milligrams of PVP-Pd was partly dissolved in 1 mL of dimethylformamide by stirring for 1 h at room temperature. Half a milliliter of the black supernatant was added to the aflatoxin solution, and the mixture was stirred overnight under deuterium atmosphere at normal pressure and room temperature. The solvent was evaporated in vacuo and the residue redissolved in 3 mL of acetonitrile. PVP-Pd was removed by passing this acetonitrile solution through a Thiol MP SPE cartridge (Polymer Laboratories, Shropshire, U.K.). This is a one-way column containing a material that binds heavy metals due to its immobilized thiol functions. However, organic molecules are not adsorbed. For further purification by preparative HPLC, the eluate was added to 80 mL of water and subsequently pumped through a 10 × 10 mm i.d. monolithic C18 cartridge placed in a SecurityGuard cartridge holder (both Phenomenex, Torrance, CA) by suction, followed by washing with 10 mL of water. After this solid phase enrichment, the cartridge holder containing the C18 cartridge was screwed into the six-port injection valve of the HPLC system, replacing the injection loop. This SPE technique allows the purification of about 10 mg of crude substance in one run, which can be difficult in the case of insufficiently water-soluble substances when conventional RP-HPLC injection is applied. Chromatography was carried out on a 150 × 10 mm i.d., 5 μm Gemini semipreparative C18 HPLC column (Phenomenex) using an L-6200A gradient pump (Merck, Darmstadt, Germany) at a flow rate of 4.7 mL/min, variable mixtures of acetonitrile/water as mobile phase, and an L-4250 UV detector (Merck/Hitachi) at 360 nm. The linear gradient started with 10% acetonitrile for 1 min and then increased to 90% acetonitrile within 20 min. The products were collected from 11.5 to 12.5 min (deuterated aflatoxin B_2) and from 10.5 to 11.5 min (deuterated aflatoxin G_2), respectively. After evaporation of the solvent in vacuo, 9.3 mg (93% yield) of colorless needles (deuterated aflatoxin B_2) and 8.2 mg (82% yield) of white powder (deuterated aflatoxin G_2) were obtained.

NMR. The isotope-labeled aflatoxins were characterized by NMR in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C, CH-decoupled) as well as by DEPT and two-dimensional NMR (COSY, NOESY, HMBC, HSQC) on a Varian NMR System 600 MHz (Varian, Darmstadt, Germany). The following data were obtained.

D_2-Aflatoxin B_2: ^{1}H NMR, δ 6.48 (d, 1H, J = 5.6 Hz), 6.34 (s, 1H), 4.16 (d, 1H, J = 5.5 Hz), 3.95 (s, 3H), 3.62 (d, 1H, J = 4.4 Hz), 3.40 (m, 2H), 2.64 (m, 2H), 2.26 (d, 1H, J = 4.5 Hz); ^{13}C NMR, δ 201.5 (s), 177.3 (s), 167.4 (s), 161.9 (s), 155.5 (s), 153.7 (s), 117.3 (s), 114.1 (s), 107.1 (s), 104.0 (s), 90.2 (s), 67.8 (t, J = 23.0 Hz), 56.6 (s), 44.2 (s), 35.3 (s), 31.4 (t, J = 20.3 Hz), 29.2 (s).

D_{2-4}-Aflatoxin G_2: ^{1}H NMR, δ 6.48 (d, 1H J = 5.6 Hz), 6.33 (s, 1H), 4.41 (m, 2H), 4.16 (d, 1H, J = 5.4 Hz), 3.92 (s, 3H), 3.61 (d, 1H, J = 4.4 Hz), 3.38–3.50 (m, ~1.5H), 2.25 (d, 1H, J = 4.4 Hz); ^{13}C NMR, δ 166.8 (s), 162.0 (s), 161.5 (m), 160.7 (s), 155.6 (s), 152.9 (s), 114.2 (s), 107.2 (m), 107.1 (s), 102.6 (s), 90.7 (s), 67.8 (t, J = 23.1 Hz), 64.6 (m), 56.7 (s), 44.2 (s), 31.4 (t, J = 20.3 Hz), 29.2 (m).

LC-MS. For characterization of the synthesized compounds, mass spectra of the labeled aflatoxins were obtained by LC-MS using an LCT ESI-TOF-MS with a Z-spray source (Micromass/Waters, Milford, MA). For this purpose, 10 μL of a 20 μg/mL solution of each standard in acetonitrile/water (50:50, v/v) was injected into the HPLC system. Except for the MS instrumentation, the HPLC system as well as the chromatographic conditions complied with the LC-MS/MS method described below. The ESI-TOF-MS was operated in the positive mode. The capillary voltage was 3300 V and the cone voltage 45 V at a source temperature of 150 °C. The desolvation temperature was 200 °C at a gas flow of 500 L/h. The following data were obtained.

Labeled aflatoxin B_2: $[M + H]^+$ 316 (1%), 317 (100%), 318 (10%), 319 (1%).

Labeled aflatoxin G_2: $[M + H]^+$ 332 (2%), 333 (100%), 334 (92%), 335 (17%), 336 (2%).

LC-MS/MS. For analysis of food samples, LC-MS/MS was used exclusively. The HPLC system consisted of a Finnigan Surveyor Plus system (Thermo Electron, Waltham, MA) including a Surveyor LC pump and a 150 × 2 mm i.d., 4 μm Synergi Polar-RP column (Phenomenex) at 30 °C. The effluent was monitored by a photodiode array before being introduced into the MS. As mobile phase, acetonitrile and water, both acidified with formic acid (0.1%, v/v), were used. The gradient started with 35% acetonitrile, which was held for 3 min, and then increased to 100% within 12 min at a flow rate of 200 μL/min. The divert valve was used to cut off the first 2 min of every run to the waste. The injection volume was 10 μL.

For MS, a Finnigan TSQ Quantum Discovery (Thermo Electron) was used in positive ESI mode. Spray voltage was set to 4000 V, capillary offset to 35 V, sheath gas pressure to 30 mTorr, and auxiliary gas pressure to 10 mTorr. Capillary temperature was 330 °C. Source collision-induced dissociation (CID) was applied at 10 V. For LC-MS/MS experiments, the collision gas pressure in quadrupole 2 was 1 mTorr, scan time was 0.20 s, and peak width in quadrupoles 1 and 3 was adjusted to ±0.35 amu.

Preparation of Aflatoxin Standard Solutions. Standard solutions of labeled and unlabeled aflatoxins in acetonitrile were prepared in the same manner. Solid aflatoxins were dissolved in acetonitrile to yield a concentration of ~0.1 mg/mL. The stock solutions were further diluted with acetonitrile to yield an aflatoxin concentration of ~10 μg/mL. The exact concentrations were then determined by measurement of the UV absorbance at the maximum around 355 nm in triplicate on a Beckman DU 650 UV spectrometer (Fullerton, CA) using extinction coefficients that have been validated by the Association of Official Analytical Chemists (*18*).

Food Sample Preparation and Cleanup. Food samples were purchased from local stores in quantities of 2 kg, ground (if required), and thoroughly homogenized. Ten grams of sample was weighed and spiked with 40 ng of each of the two labeled standards by addition of the respective amount of standard solution in acetonitrile. The solvent was allowed to evaporate overnight. This particular amount of standard was chosen because it corresponds to a concentration of 4 μg/kg, the European legal limit for the sum of the four aflatoxins for most food matrices. If a higher contamination is intended to be measured, we recommend spiking the sample with a higher amount of standard as

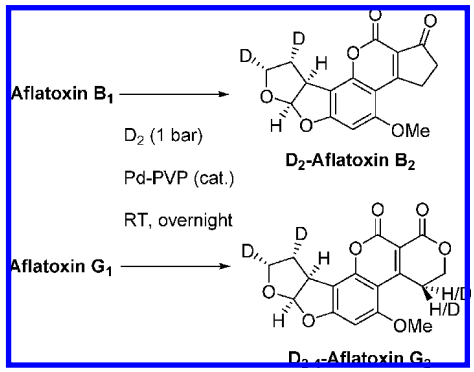

Figure 2. Synthesis of labeled aflatoxins B_2 and G_2.

well, so that the standard/analyte ratio lies within the working range determined by calibration. Extraction was performed by stirring the sample for 2 h at room temperature with 40 mL of methanol/water (80:20, v/v) containing 2.0 g of sodium chloride. After filtration through a folded filter (595.5) (Schleicher & Schüll, Dassel, Germany), 20 mL of the filtrate was added to 180 mL of phosphate-buffered saline (PBS, pH 7.6). We chose to highly dilute the raw extract with buffer, because the affinity of the aflatoxin-selective antibodies that are immobilized on the immunoaffinity column decreases when an extract containing a relatively high content of methanol, which may affect the recovery rate, is applied. Subsequently, the whole amount of this diluted extract was cleaned up by means of a G1024 aflatoxin immunoaffinity column (Vicam, Watertown, MA). After the column had been washed with 5 mL of bidistilled water twice, the analytes were eluted with 3 mL of acetonitrile. The solvent was removed in a stream of nitrogen, and the residue was redissolved in 200 μL of mobile phase for LC-MS/MS analysis and membrane filtered (0.45 μm) (Schleicher & Schüll).

Calibration and Quantitation. Calibration was performed both in the absence and in the presence of matrix. For calibration without matrix, solutions of labeled and unlabeled aflatoxins were mixed in seven molar ratios ranging from 0.1 to 10 to give a total aflatoxin concentration of 50 μg/L (i.e., the range of concentrations for the calibration solutions that were directly injected into the LC-MS/MS system was 4.5−45 μg/L for both analyte and standard). Linear calibration curves were constructed from the standard/analyte peak area ratios versus the standard/analyte molar ratios. Additionally, calibration in the presence of matrix was performed by adding seven aflatoxin mixtures with the respective molar ratios of analyte to labeled standard ranging from 0.1 to 10 (40 ng total amount of aflatoxin spiked) to 10 g of ground almonds or type 405 wheat flour (range = 0.4−40 μg/kg). The spiked samples were subsequently analyzed by SIDA, and linear calibration curves were constructed as described above.

Limits of Detection (LODs) and Quantitation (LOQs). LODs and LOQs were determined according to the method of Hädrich and Vogelgesang (*20*), which is related to DIN EN standard 32645 (*21*). It is based on spiking a blank matrix with analyte and deduces LOD from the confidence interval of a calibration line constructed from the concentrations of analyte determined by SIDA versus the spiking concentrations. The LOD is defined as the addition value referring to the upper 95% confidence limit of the calibration line at the zero addition level. The LOQ is defined as the addition level of the lower 95% confidence limit, which meets the upper 95% confidence limit of the addition level at the LOD.

Almonds and type 405 wheat flour were chosen as blank matrices, as no aflatoxin peaks in the corresponding LC-MS/MS chromatograms were obtained in prior analysis. These blank matrices were used for the spiking experiments. For the LOD/LOQ determinations, 10 g of blank sample was spiked with all labeled and unlabeled aflatoxins in absolute amounts of 4, 16, 28, and 40 ng, respectively, as described above. As required by the method, each of the four addition assays was performed in triplicate and analyzed using the SIDA protocol as described above; that is, 12 measurements per model matrix were performed to calculate the corresponding LOD and LOQ values.

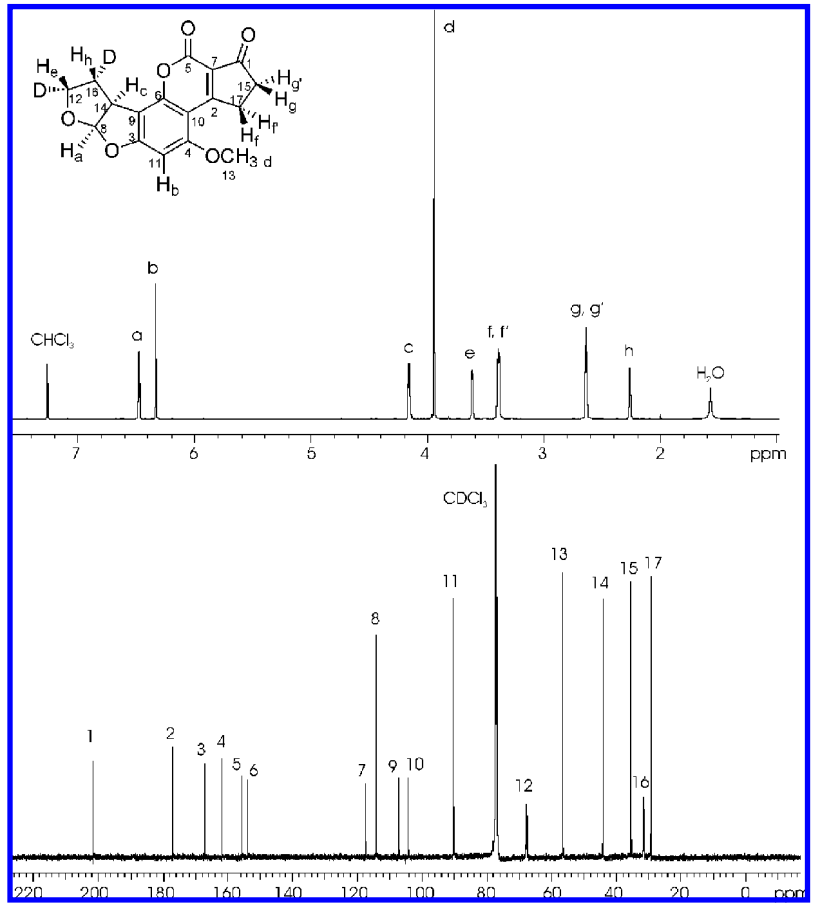

Figure 3. ^{1}H and ^{13}C NMR spectra of D_2-aflatoxin B_2 in $CDCl_3$ with suggested signal assignments.

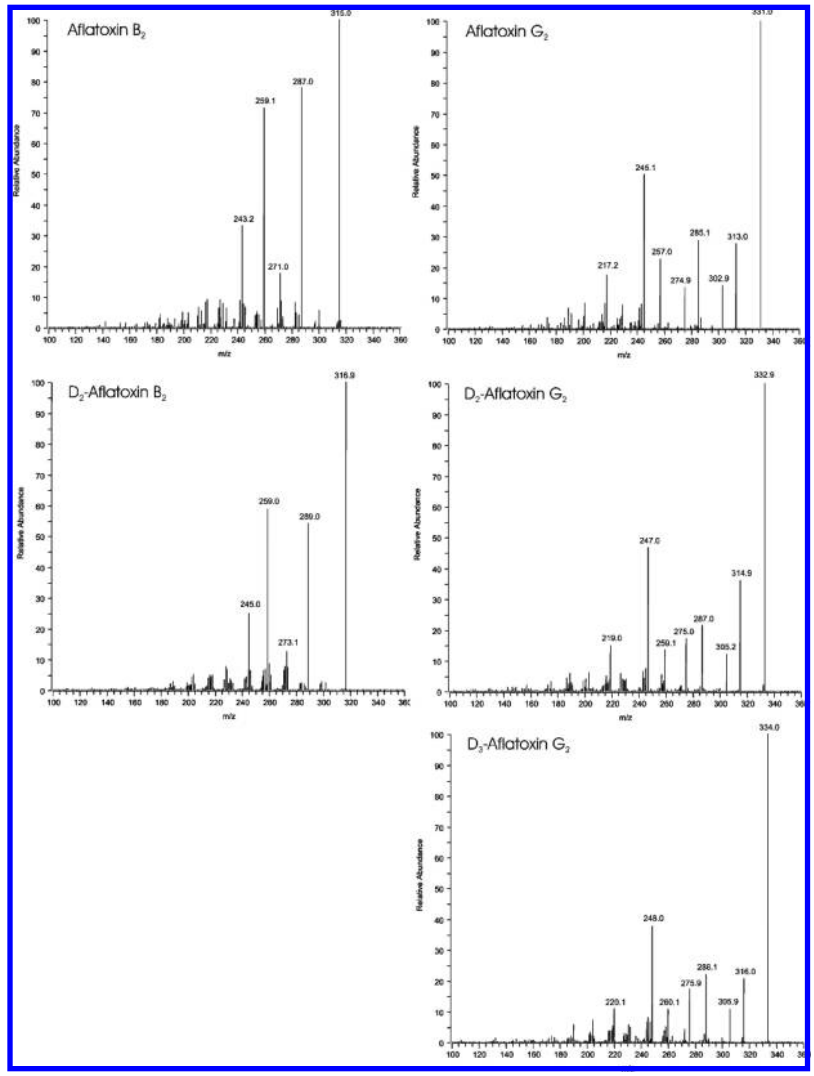

Figure 4. MS/MS spectra of labeled and unlabeled aflatoxins B_2 and G_2 (collision energy for all compounds = 16 V).

Precision and Recovery. Precision was determined by analyzing a naturally contaminated standard reference peanut butter sample for aflatoxins B_1 and B_2 and spiked ground almonds for aflatoxins G_1 and G_2, respectively, three times within 3 weeks in triplicate. Recovery of the analytes was examined at different spiking levels above their LOQs. For this purpose, 10 g samples of wheat flour and ground almonds were spiked with 16, 28, and 40 ng of all labeled and unlabeled aflatoxins and analyzed using SIDA as described above.

RESULTS AND DISCUSSION

Isotope-Labeled Aflatoxins. A SIDA for the determination of aflatoxins in foods is presented here for the first time. For this purpose, novel isotope-labeled aflatoxins (deuterated aflatoxins B_2 and G_2) were chemically synthesized in one step starting from commercially available aflatoxins B_1 and G_1 (**Figure 2**) and purified by preparative HPLC. The synthesis was performed by deuteration of the double bond of the aflatoxin dihydrofuran moiety with catalytically active palladium nanoparticles. The hydrogenation of a similar ring system has been reported previously (*22*). The compounds were extensively characterized by one- and two-dimensional NMR. No impurities were visible in the spectra. The ^{1}H and ^{13}C spectra of deuterated aflatoxin B_2 are shown in **Figure 3**. The isotopic patterns of the labeled aflatoxins were determined by LC-ESI-TOF-MS.

From the NMR and MS data it can be concluded that aflatoxin G_2, in contrast to aflatoxin B_2, was partly further deuterated at its lactone moiety by additional H/D exchange (**Figure 2**), resulting in a mixture of 2-, 3-, and 4-fold deuterated aflatoxin G_2 in a ratio of about 6:5:1. The degrees and positions of deuteration are clearly visible in the mass spectra as well as in the NMR spectra. The ^{1}H NMR spectra of both synthesized compounds show a single set of signals due to the formation of just one of two possible isotopic diastereomers during the synthesis. Comparison with the NMR data of the epoxide of aflatoxin B_1 (*23*) suggests that aflatoxin B_1 was deuterated from the sterically less hindered *exo* face, as a coupling between protons h (δ 2.26) and c (δ 4.16) is absent in the ^{1}H and the COSY spectrum due to a torsion angle of about 90° (**Figure 3**). The presence of the deuterium substituents is also visible in the CH-decoupled ^{13}C NMR spectra, where CD couplings are not eliminated. Further NMR characterization and signal assignments were carried out by means of homo- and heterocorrelated two-dimensional NMR, which further confirm the structural identity of the synthesized isotopologues.

Studies on the stability of the deuterium label were performed with both compounds. The deuterated standards did not show any D/H exchange, neither when stored as stock solutions in acetonitrile within 3 months at 4 °C nor in the presence of water within 1 week at room temperature.

LC-MS/MS was used for the identification and quantitation of aflatoxins in food samples. All aflatoxins showed reproducible MS/MS fragmentation from $[M + H]^+$. The MS/MS spectra of labeled and unlabeled aflatoxins B_2 and G_2 are shown in

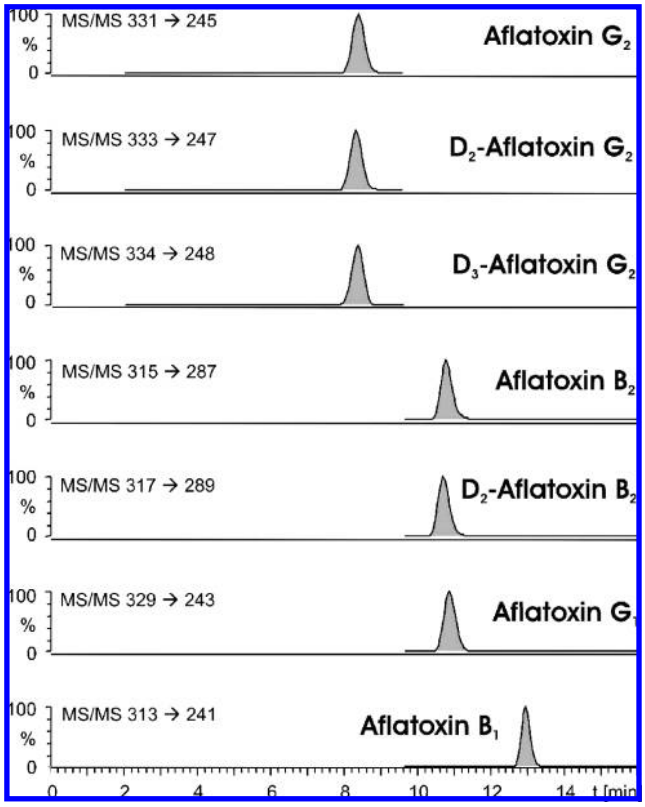

Figure 5. LC-MS/MS selected reaction monitoring (SRM) chromatograms of the labeled and unlabeled aflatoxins.

Figure 4. Selected reaction monitoring (SRM) with mass transitions from $[M + H]^+$ was applied with collision energies as follows: aflatoxin B_1, *m/z* 313.0 → *m/z* 285.0 (22 V) and *m/z* 313.0 → *m/z* 241.0 (38 V); aflatoxin B_2, *m/z* 315.0 → *m/z* 287.0 (26 V) and *m/z* 315.0 → *m/z* 259.0 (31 V); D_2-aflatoxin B_2, *m/z* 317.0 → *m/z* 289.0 (26 V) and *m/z* 317.0 → *m/z* 259 (31 V); aflatoxin G_1, *m/z* 329.0 → *m/z* 243 (28 V) and *m/z* 329.0 → *m/z* 214.0 (36 V); aflatoxin G_2, *m/z* 331.0 → *m/z* 245.0 (30 V) and *m/z* 331.0 → *m/z* 217.0 (36 V); D_2-aflatoxin G_2, *m/z* 333.0 → *m/z* 247.0 (30 V) and *m/z* 333.0 → *m/z* 219 (36 V); D_3-aflatoxin G_2, *m/z* 334.0 → *m/z* 248.0 (30 V) and *m/z* 334.0 → *m/z* 220.0 (36 V). Acquisition during the HPLC run was split into two segments. Labeled and unlabeled aflatoxin G_2 was measured in segment 1 (2–9.5 min), whereas labeled/unlabeled aflatoxin B_2 and aflatoxins G_1 and B_1 were measured in segment two (9.5–16.0 min).

Fragmentation mainly occurs due to loss of CO, CO_2, H_2O, and small organic molecules. The isotope labels were retained during fragmentation for all relevant product ions (except for *m/z* 259 obtained from both labeled and unlabeled aflatoxin B_2). On the basis of a comparison between the MS/MS spectra of labeled and unlabeled aflatoxins, conclusions concerning the structures of the fragment ions can be drawn, which are partly in contrast to hypothetical aflatoxin fragment structures published previously (*24*). Convincing evidence for the structures proposed by us is provided by the MS/MS spectra and their interpretation.

Calibration. The novel isotope-labeled aflatoxins proved to be valuable as internal standards for the quantitation of all four aflatoxins under study. **Figure 5** shows LC-MS/MS SRM chromatograms of the product ions that were chosen for the quantitation of the analytes. D_2-Aflatoxin B_2 was used for the quantitation of aflatoxins B_1, B_2, and G_1. Whereas the D_2-aflatoxin B_2/aflatoxin B_1 combination was chosen due to structural similarities, the D_{2-4}-aflatoxin G_2/aflatoxin G_1 combination was not used for quantitation. Instead, D_2-aflatoxin B_2 was used as internal standard for aflatoxin G_1 because a similar retention time proved to compensate better for any kind of interference during LC-MS/MS analysis. Additionally, the immunoaffinity columns used for the cleanup of the raw food extracts showed complete recovery for aflatoxins B_1, B_2, and G_1, but a lower recovery (~80%) for aflatoxin G_2. Therefore, D_{2-4}-aflatoxin G_2 appeared to be unsuitable as internal standard for the other three mycotoxins. For the quantitation of aflatoxin G_2, both 2- and 3-fold deuterated aflatoxin G_2 were used because of the distinct isotopic pattern of this internal standard. If only one isotopologue is considered, the response factors (RF) were around 0.5. Using the sum of both transitions, a RF close to 1 was achieved, and hence an excess of internal standard could be avoided. All calibration curves constructed from the peak area ratios versus the molar ratios (labeled/unlabeled) showed excellent linearity with squared correlation coefficients of ≥ 0.995 ($n = 7$). Calibration was performed both with aflatoxin standard solutions and with spiked food samples analyzed by the SIDA method as described above. For these matrix calibrations, aflatoxin-free almonds (as a model matrix for fatty food matrices such as nuts) and wheat flour (as a model matrix for nonfatty food matrices such as grains and spices) were chosen. As expected from SIDA, the slopes of the calibration curves (RF) of D_2-aflatoxin B_2/aflatoxin B_2 ($y = 0.8984x + 0.0238$; $R^2 = 0.9999$; $n = 7$) and (D_2-aflatoxin G_2 + D_3-aflatoxin G_2)/aflatoxin G_2 ($y = 1.1944x + 0.0998$; $R^2 = 0.9982$; $n = 7$) were independent of the presence and the type of matrix. However, for the combinations D_2-aflatoxin B_2/aflatoxin B_1 and D_2-aflatoxin B_2/aflatoxin G_1, RFs were dependent on the type of matrix and differed significantly from the RFs derived from standard solutions.

LODs and LOQs. LODs and LOQs were determined according to the method suggested by Hädrich and Vogelgesang (*20*). The LODs and LOQs for the developed method were determined for the two model matrices almonds and wheat flour. The following LODs were obtained: aflatoxin B_1, 0.31 μg/kg (almonds) and 0.23 μg/kg (wheat); aflatoxin B_2, 0.09 μg/kg (almonds) and 0.10 μg/kg (wheat); aflatoxin G_1, 0.38 μg/kg (almonds) and 0.51 μg/kg (wheat); aflatoxin G_2, 0.32 μg/kg (almonds) and 0.34 μg/kg (wheat). The following LOQs were obtained: aflatoxin B_1, 0.91 μg/kg (almonds) and 0.67 μg/kg (wheat); aflatoxin B_2, 0.27 μg/kg (almonds) and 0.29 μg/kg (wheat); aflatoxin G_1, 1.06 μg/kg (almonds) and 1.50 μg/kg (wheat); aflatoxin G_2, 1.17 μg/kg (almonds) and 1.00 μg/kg (wheat). The values are well below the valid maximum permissible limits in the United States and European Union (*4*).

Validation. Recovery was determined for each analyte as described above. The mean of the recovery rates obtained from the spiking levels 1.6, 2.8, and 4.0 μg/kg was taken, yielding the following data: aflatoxin B_1, 94 ± 5% (almonds) and 100 ± 6% (wheat); aflatoxin B_2, 98 ± 4% (almonds) and 98 ± 3% (wheat); aflatoxin G_1, 90 ± 4% (almonds) and 89 ± 9% (wheat); aflatoxin G_2, 105 ± 9% (almonds) and 106 ± 5% (wheat). For aflatoxins B_2 and G_2 recoveries of about 100% are a consequence of the SIDA technique applied; also for aflatoxins B_1 and G_1 (to which no isotope-labeled standards exist) recoveries of about 90–100% were obtained with the method presented here using matrix-matched calibration with D_2-aflatoxin B_2 as internal standard.

The interassay precision was determined as described above. For aflatoxins B_1 and B_2, naturally contaminated peanut butter

Table 1. Various Food Samples Purchased from Local Food Stores and Their Aflatoxin Content Determined by SIDA

food sample	no. of samples	aflatoxin B_1[a] (μg/kg)	aflatoxin B_2[a] (μg/kg)	aflatoxin G_1[a] (μg/kg)	aflatoxin G_2[a] (μg/kg)
ground hazelnuts	1	0.53[b,d]		1.79[b]	
almonds	2				
green pistachios	1	0.36[b,d]			
roasted pistachios	2				
peanuts	6				
peanut butter 1	1	0.76[b,d]	0.12[d]		
peanut butter 2	1	5.90[b,e]	1.38	0.52[b,d]	
green coffee	2				
cashew nuts	1				
sunflower seeds	1				
maize meal	1	1.60[c]	0.15[d]		
wheat flour	1				
oat	1				
paprika powder	1	1.76[c]		1.56[c]	
black pepper powder	1	1.00[c]			
nutmeg powder	1	1.77[c]	0.40	0.63[c,d]	

[a] All values were corrected with recovery. [b] Determined by applying the calibration for almonds. [c] Determined by applying the calibration for wheat flour. [d] Between LOD and LOQ. [e] Above maximum permissible limit in the EU.

was analyzed three times in triplicate within 3 weeks, resulting in coefficients of variation (CV) of 12% (B_1) and 3.6% (B_2). As there were only a few samples contaminated with aflatoxins G_1 and G_2 above the LOQ of the method, the interassay precision studies for these two analytes had to be performed with spiked material as described above, resulting in CVs of 14% (G_1) and 4.8% (G_2).

NIST standard reference material 2387 was analyzed for further validation studies. For this naturally contaminated peanut butter, the NIST states reference concentration values for aflatoxins of 4.2 ± 0.9 μg/kg (B_1) and 0.7 ± 0.3 μg/kg (B_2), which had been measured by four different laboratories by HPLC with fluorescence detection. We obtained slightly higher values, that is, 4.9 ± 0.6 μg/kg for aflatoxin B_1 (bias, +16%) and 1.1 ± 0.04 μg/kg for aflatoxin B_2 (bias, +54%). According to a Student's t test at a 95% confidence level, only the value for B_2 may be significantly higher. However, as the NIST peanut butter sample is a noncertified standard reference material for aflatoxin determination and thus may not include all sources of uncertainty, the bias may not be significant.

Determination of Aflatoxins in Food Samples. Twenty-four food samples of different kinds, which are susceptible to aflatoxin contamination, were measured by the SIDA method. **Table 1** summarizes the contents of all samples. All values were corrected with recovery. Both analyzed peanut butter samples were contaminated, one of them above the maximum permissible value in the EU. In contrast, all seven peanut samples examined were free of aflatoxins. Six other food samples were contaminated yet below the EU permissible limit, among them hazelnuts, maize meal, paprika powder, black pepper powder, and nutmeg powder.

Summary. The method presented is an unprecedented LC-MS/MS stable isotope dilution method for aflatoxins, which still pose an actual health hazard since their discovery in the 1960s. The method is based upon the use of novel isotope-labeled aflatoxins B_2 and G_2 for the quantitation of all four aflatoxins of interest. The labeled toxins were readily accessible in good yields and high purity from commercially available starting materials with only minimum synthetical effort. As this is generally the crucial point for the applicability of a SIDA method for complex natural compounds such as mycotoxins, the reported synthetic route may lead to a wider application of these well-characterized standards for both research and routine use. The performance of the developed SIDA method is good also for aflatoxins B_1 and G_1, for which no stable isotope-labeled analogues exist. It needs to be emphasized that only matrix-matched calibration led to reasonable results for these two analytes. The use of model matrices for method validation has been shown to be highly effective, as the aflatoxin B_1 content of NIST peanut butter was quantified with our method showing only a low bias. Limits of detection and quantitation were determined by a statistical approach in the presence of the two model matrices. The values are sufficiently low compared to maximum permissible limits. The method therefore allows measurement of aflatoxin contamination at concentrations low enough to ensure the consumer's health. Altogether, the validation data obtained for the SIDA method are better than for most standard methods used for the determination of aflatoxins (*25*). The new method is furthermore suitable for routine use in laboratories equipped with LC-MS/MS capabilities and, hence, is also suggested as a confirmatory method supplementary to conventional aflatoxin determination. As the labeled material is planned to be made commercially available, the SIDA presented here has the potential to become a reference method for aflatoxin analysis.

ABBREVIATIONS USED

COSY, NMR (homo)correlated proton spectroscopy; CID, source collision-induced dissociation; CV, coefficient of variation; DEPT, NMR distortionless enhancement by polarization transfer; ESI, electrospray ionization; HMBC, NMR heteronuclear multiple bond coherence; HPLC, high-performance liquid chromatography; HSQC, NMR heteronuclear single quantum coherence; LOD, limit of detection; LOQ, limit of quantitation; NOESY, NMR nuclear overhauser effect spectroscopy; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; RF, response factor; SIDA, stable isotope dilution assay; SRM, selected reaction monitoring; TOF, time-of-flight.

SAFETY

Aflatoxins are very carcinogenic substances and should be handled with extreme care. We do not recommend handling milligram amounts of solid aflatoxins in open flasks, because the substance may electrostatically charge and form an aerosol. Instead, we recommend applying the so-called septum technique as follows. Solid aflatoxins are usually delivered in vials covered with a septum. Solvent can be added with a syringe (piercing the septum) to dissolve the toxin. The solution can then be transferred by syringe into another septum-covered vial. Con-

taminated material, for example, syringes, should be treated with an aqueous solution of sodium hypochlorite (5%) before discarding. This technique minimizes health risks due to toxin contamination.

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Supporting Information Available: A table containing the matrix-dependent linear calibration data for the quantitation of aflatoxins B_1 and G_1, additional one- and two-dimensional NMR spectra of the labeled aflatoxins acquired, MS/MS spectra of aflatoxins B_1 and G_1, suggestions for MS/MS fragment structures for all aflatoxins, and SRM chromatograms of a real sample (peanut butter). This material is available free of charge at http://pubs.acs.org.

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