JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Stable Isotope Dilution Analysis of Small Molecules with Carboxylic Acid Functions Using ^{18}O Labeling for HPLC-ESI-MS/MS: Analysis of Fumonisin B_1

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Supporting Information

ABSTRACT: ^{18}O labeling is a well-known method for the stable isotope labeling of proteins and peptides. This study describes a modified procedure for using ^{18}O labeling on small molecules. Fumonisin B_1, a worldwide occurring mycotoxin, which is routinely analyzed by HPLC-MS/MS, was chosen as model compound. ^{18}O labeling was achieved by acid-catalyzed oxygen exchange from $H_2{}^{18}O$. A mixture of different isotopologues was obtained from the exchange, which, however, could be used as an internal standard for HPLC-MS/MS analysis. The identity of the ^{18}O-labeled fumonisin B_1 was confirmed by NMR and HRMS measurements. The applicability as internal standard has been verified by comparison of results obtained from the method described in this paper to results obtained by reference methods. The presented method is of special interest as the ^{18}O labeling can be generally applied to a large group of small molecules containing carboxylic groups.

KEYWORDS: *fumonisin B_1, ^{18}O labeling, carboxylic groups, Fusarium, mycotoxin, HPLC-MS/MS, stable isotope dilution analysis*

INTRODUCTION

Fumonisins are toxic secondary metabolites produced by a large number of fungi of the genus *Fusarium* and also some fungi of the genus *Aspergillus*.[1,2] The structure of B-type fumonisins (Figure 1) consists of a C_{20} backbone, which bears hydroxy groups at different positions. Fumonisins B_1 (**2**), B_2 (**5**), and B_3 (**6**) are the most abundantly occurring fumonisins in nature. The hydroxy groups at positions C14 and C15 are often esterified by propane-1,2,3-tricarboxylic acid moieties (**1**) but partially (**3a**, **3b**) or fully (**4**) hydrolyzed fumonisins are also known.[1] They can be found worldwide as contaminants of many grains, especially maize, and plant-derived foods.[3,4] Fumonisins are toxic toward many different animals. Known animal diseases caused by fumonisins are hepatocarcinogenic effects on rats and mice, equine leukoencephalomalacia in horses, and pulmonary edema in pigs.[5] Their toxicity toward humans is still unclear. There is some evidence that fumonisins might be involved in the formation of esophageal cancer and might have a negative effect on neural tube development in embryos.[6,7] Maximum levels for fumonisin contamination have been decreed by many countries, for example, the members of the European Union (EU).[8] Therefore, food is routinely analyzed for fumonisins B_1 and B_2.

Liquid chromatography coupled with fluorescence detection after derivatization or with mass spectrometry is the preferred analytical method. Mass spectrometric methods that are used for complex matrices such as food are often limited in their reliability by matrix effects leading to ion suppression. The use of stable isotopically labeled standards can compensate for these matrix effects.[9]

Unfortunately, labeled analytical standards of complex molecules such as fumonisins are not easily available. Often, biochemical production methods are used where fungi or other microorganisms are grown on large amounts of ^{13}C-labeled substrates.[10] A major disadvantage of this production method is the relatively high price for the labeled compounds.

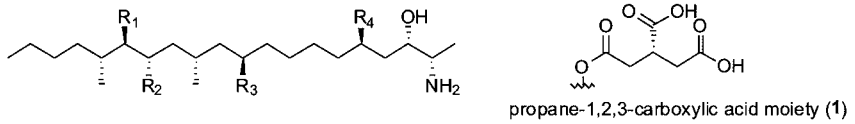
The exchange of oxygen atoms in carboxyl groups by $H_2{}^{18}O$ has been known for a long time. For example, this exchange reaction had already been shown in 1939 by Mears and Sobotka[11] to be suitable for the labeling of amino acids and proteins. The mechanism follows a simple nucleophilic substitution reaction analogous to an ester hydrolysis with a tetrahedral intermediate. The crucial step is the protonation of the carbonyl oxygen. Therefore, the pK_a values are of particular importance.[12] In this study we used fumonisin B_1 as the target compound for ^{18}O labeling. The aim of our work was the synthesis of a stable isotopically labeled standard that can be applied in a simple method to analyze fumonisin B_1 in maize-based food. Therefore, a method described previously for the labeling of proteins was modified to be used for the labeling of fumonisins.[13] The reaction conditions were optimized for high exchange rates of oxygen atoms and low saponification of ester bonds.

Finally, the ^{18}O-labeled fumonisin B_1 was employed as an internal standard for the quantitation of samples naturally contaminated with fumonisins. These data were compared to the results that have been quantitated by validated routine methods to examine the suitability of the ^{18}O-labeled standard.

MATERIALS AND METHODS

Chemicals. All solvents and reagents were purchased from Merck (Darmstadt, Germany), VWR (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Grüssing (Filsum, Germany), and Carl Roth (Karlsruhe, Germany) in gradient or analytical grade. $H_2{}^{18}O$ (97

Received: May 23, 2013
Revised: July 24, 2013
Accepted: July 29, 2013
Published: July 29, 2013



2 fumonisin B_1: R_1=**1**, R_2=**1**, R_3=OH, R_4=OH
3a partially hydrolyzed fumonisin B_1: R_1=**1**, R_2=OH, R_3=OH, R_4=OH
3b partially hydrolyzed fumonisin B_1: R_1=OH, R_2=**1**, R_3=OH, R_4=OH
4 hydrolyzed fumonisin B_1: R_1=OH, R_2=OH, R_3=OH, R_4=OH
5 fumonisin B_2: R_1=**1**, R_2=**1**, R_3=H, R_4=OH
6 fumonisin B_3: R_1=**1**, R_2=**1**, R_3=OH, R_4=H

Figure 1. Structures of the most important fumonisins.

atom % ^{18}O) was obtained from Sigma-Aldrich. Water was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

Samples. Ground samples of different maize products from the European Union (EU) were kindly provided by Chemical and Veterinary Laboratory (Chemisches und Veterinäruntersuchungsamt, CVUA, Stuttgart, Germany) and Eurofins Food GmbH (Hamburg, Germany). These samples have been previously analyzed by validated LC-MS/MS methods for the determination of fumonisins in food and were used as reference materials. More detailed information on the analyzed samples can be found in Table 2.

Isolation of Fumonisin B_1. Fumonisin B_1 was isolated according to a procedure previously described by Hübner et al.[14] Briefly, fumonisin B_1 was extracted from solid cultures of *Fusarium verticillioides* MRC 826, which was grown on cracked maize. A liquid–liquid extraction using a solvent system of *tert*-butyl methyl ether/butan-1-ol/ethanol/1% formic acid (5:5:1:10, v/v/v/v) was used for precleaning of the fumonisins that can be found in the organic phase. Afterward, the extract was purified over a strong anion exchange column. The final purification step was a cleanup using centrifugal partition chromatography, a liquid–liquid partition technique.

^{18}O Labeling of Fumonisin B_1. Different reaction conditions have been tested for the exchange reaction that are shown in rows 1–3 of Table 1. The reaction conditions presented in row 3 were deemed best and used for an exchange reaction on a larger scale. For the preparation of larger amounts, 39 mg (0.054 mmol) of dry fumonisin B_1 was dissolved in 190 μL of $H_2{}^{18}O$ (97 atom % ^{18}O) in a 1.5 mL screw cap autosampler vial forming a viscous solution, and 10 μL of trifluoroacetic acid was added. The vial was purged with argon, sealed tightly, and heated for 8 h at 50 °C in an aluminum block. To stop the reaction, water and trifluoroacetic acid were removed in a stream of nitrogen. The obtained crude product contained small amounts of partly hydrolyzed fumonisin B_1 (**3a** and **3b**) or hydrolyzed fumonisin B_1 (**4**). These, however, do not interfere with the use as an internal standard in HPLC-MS/MS. Therefore, no further cleanup was carried out.

Standard Solutions. All standard solutions of ^{18}O-labeled fumonisin B_1 were referred to the $^{18}O_3$-isotopologue. The crude product was dissolved in 10 mL of acetonitrile/water (50:50, v/v), and 2.57 mL of this solution was filled in a volumetric flask to 25 mL with acetonitrile/water (50:50, v/v) to obtain the internal standard stock solution with a concentration of 60 μg/mL of $^{18}O_3$-fumonisin B_1. This solution was diluted with acetonitrile/water (30:70, v/v) containing 1% formic acid 1:40 to a concentration of 1.5 μg/mL of $^{18}O_3$-fumonisin B_1 for the internal standard solution. A standard stock solution of fumonisin B_1 in acetonitrile/water (50:50, v/v) with a concentration of 100 μg/mL was prepared. From this solution a standard solution containing 2.5 μg/mL fumonisin B_1 in acetonitrile/water (30:70, v/v) containing 1% formic acid was prepared.

Stability of ^{18}O-Labeled Fumonisin B_1. For stability testing a solution of ^{18}O-labeled fumonisin B_1 in water was prepared from the crude product obtained by the reaction conditions presented in Table 1, row 1, containing approximately 2 mg/mL of labeled fumonisin B_1 in total. From this solution four aliquots of 10 μL each were taken. Two were diluted with 190 μL of acetonitrile/water (30:70, v/v) containing 1% formic acid and the other two with 190 μL of acetonitrile/water (60:40, v/v). The first solution was for the simulation of HPLC starting conditions, which might also be used in solutions in the daily laboratory routine; the second solution was for the simulation of long-term storage conditions of stock solutions. One solution of each solvent mixture was kept at room temperature (approximately 21 °C) and the other one in a refrigerator at 2–8 °C. After 0, 1, 2, 3, 28, and 112 days, samples of 10 μL were taken, diluted with 90 μL of water, and analyzed by HPLC-MS/MS.

Identification of ^{18}O-Labeled Fumonisin B_1. High-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopic data (^1H NMR and ^{13}C NMR) were employed to confirm the identity of the obtained ^{18}O-labeled fumonisin B_1.

Determination of the Purity of ^{18}O-Labeled Fumonisin B_1. A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) with an LC-20AT pump, an SIL-20A autosampler, and a low-temperature evaporative light scattering detector (ELSD-LT) was employed for the determination of the purity of the crude product. Data acquisition was performed with LC Solution 1.21 SP1 software (Shimadzu). Chromatographic separation was carried out on a 125 × 4 mm i.d., 7 μm, LiChrosorb RP-select B C8 column with a 4 × 4 mm i.d. guard column (Merck, Darmstadt, Germany) using a binary gradient of 1% aqueous formic acid (eluent A) and 1% formic acid in methanol (eluent B). HPLC parameters were as follows: flow rate, 1 mL/min; injection volume, 20 μL; gradient, 45% eluent A (0 min), 25% A (25 min), 10% A (35 min), 10% A (45 min), followed by returning to starting conditions (46 min) and equilibration of the system (55 min).

Sample Preparation. All ground maize samples were directly used for extraction. Analyses were carried out in duplicate and measured twice. The arithmetic mean ± standard deviation was calculated for each sample. Ten grams of sample was spiked with 100 μL of the internal standard stock solution in a 50 mL conical screw-cap centrifuge tube and extracted with 20 mL of acetonitrile/water (70:30, v/v) containing 1% formic acid. After vortexing (30 s) and sonification (10 min), samples were shaken mechanically for 15 min. Samples were centrifuged (15 min, 8000*g*, 25 °C), and an aliquot of 1 mL of the clear supernatant was diluted with the same volume of 1% aqueous formic acid. Cloudy samples were filtered through a 25 mm diameter, 0.2 μm pore size, polypropylene syringe filter.

HPLC-MS/MS Analysis. An API 3200 mass spectrometer (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1200 series HPLC (Agilent, Böblingen, Germany) was employed for the quantitation of fumonisin B_1 in food samples in the multiple reaction monitoring mode (MRM). Data acquisition was performed with Analyst 1.4.2 software (Applied Biosystems).

A QTRAP 5500 mass spectrometer (AB SCIEX, Darmstadt, Germany) coupled to a VWR Hitachi LaChrom Ultra HPLC system (VWR, Darmstadt, Germany) was employed for the determination of the ^{18}O contents in the ^{18}O-labeled fumonisin B_1 in MRM mode. Data acquisition was performed with Analyst 1.5.2 software (AB SCIEX).

HPLC Parameters. Chromatographic separation was carried out on a 150 × 2 mm i.d., 3 μm, Hyperclone C8 3 μm BDS 130 Å column with a 4 × 3 mm i.d. guard column (Phenomenex, Aschaffenburg, Germany) using a binary gradient of 1% formic acid in acetonitrile (eluent A) and 1% aqueous formic acid (eluent B). HPLC parameters

dx.doi.org/10.1021/jf4022702 | J. Agric. Food Chem. 2013, 61, 7904–7908

were as follows: flow rate, 300 μL/min; injection volume, 20 μL (API 3200) or 10 μL (QTRAP 5500); column temperature, 40 °C; gradient, 35% eluent A (0 min), 62.5% A (4 min), 95% A (4.5 min), 95% A (6.5 min), followed by returning to starting conditions (7 min) and equilibration of the system (11 min).

MS/MS Parameters. For the API 3200 mass spectrometer, zero grade air was used as nebulizer gas (35 psi) and drying gas (45 psi) and nitrogen as curtain gas (30 psi) and collision gas (5×10^{-5} Torr). Drying gas was heated to 350 °C, and an ionization voltage of 5500 V in positive ionization mode was used. For each analyte of interest, two MRM transitions were measured with a dwell time of 50 ms each (quantifier and qualifier) to ensure correct identification of analytes. MRM transitions were as follows with the according parameters declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) given in volts in parentheses: fumonisin B_1 (**2**) $[M + H]^+$ 722 → 334 (DP 121, EP 10, CEP 35, CE 55, CXP 8) and 722 → 370 (DP 121, EP 10, CEP 35, CE 55, CXP 8); $^{18}O_3$-fumonisin B_1 $[M + H]^+$ 728 → 334 (DP 121, EP 10, CEP 35, CE 55, CXP 8) and 728 → 370 (DP 121, EP 10, CEP 35, CE 55, CXP 8); hydrolyzed fumonisin B_1 (**4**) $[M + H]^+$ 406 → 334 (DP 61, EP 10, CEP 35, CE 35, CXP 8) and 406 → 370 (DP 61, EP 10, CEP 35, CE 27, CXP 8); and fumonisin B_2 (**5**) and fumonisin B_3 (**6**) $[M + H]^+$ 706 → 336 (DP 121, EP 10, CEP 35, CE 55, CXP 8) and 706 → 354 (DP 121, EP 10, CEP 35, CE 55, CXP 8). The first MRM transition listed was used as quantifier and the second as qualifier.

For the QTRAP 5500 system the same conditions were applied except for the following: curtain gas (20 psi), collision gas (medium), and dwell time (15 ms).

Calibration. For solvent calibration, eight different concentrations of fumonisin B_1 in acetonitrile/water (30:70, v/v) containing 1% formic acid between 25 and 500 ng/mL correlating with contamination values between 100 and 2000 μg/kg were prepared from standard solution. They were spiked with the same constant level of $^{18}O_3$-fumonisin B_1 (150 ng/mL) from internal standard solution to compensate for matrix effects during ionization. Each calibration point was analyzed three times. Linear regression was used to calculate the regression curve. For quantitation, the calibration curves measured before and after the samples were averaged.

Method Evaluation. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the method of the German Standard DIN 32645.[15] The calculation was described in detail by Kleigrewe et al.[16]

For the matrix calibration, polenta maize grits containing no detectable amount of fumonisin B_1 were spiked with unlabeled fumonisin B_1 from standard stock solution at eight levels ranging between 100 and 2000 μg/kg. The extraction was carried out as described above. Each spiked sample was worked up in duplicate, measured twice, and used as a calibration point for the matrix calibration. Linear regression was used to calculate the matrix calibration curve and the coefficient of variation. The recovery was determined by comparing the matrix calibration curve and the solvent calibration curve with each other, allowing the calculation of the recovery over the whole calibration range.[17]

RESULTS AND DISCUSSION

Labeling of Fumonisin B_1. On the basis of the recent approach of Kozmin et al.[13] we performed the exchange of oxygen atoms in the side chains of fumonisin B_1 catalyzed by trifluoroacetic acid. The fumonisin B_1 molecule bears 10 oxygen atoms in the two tricarboxylic acid side chains, which could possibly be exchanged with the heavier ^{18}O isotope (Figure 1). However, complete exchange was not the main goal because even a mass increase of 4 Da is sufficient to prevent spectral overlap and because of the undesired ester hydrolysis of the side chains, which occurs during longer exposure to strong acidic conditions. Trifluoroacetic acid was chosen because it can easily be handled and removed after the reaction. Although the use of ^{13}C-labeled fumonisin B_1 as an internal standard has been described by Varga et al.,[18] ^{18}O labeling has a major advantage. As mentioned in the Introduction, ^{13}C labeling is carried out by the use of ^{13}C-labeled substrates; therefore, relatively large amounts of labeled substrate are needed to produce small amounts of labeled secondary metabolites. Thus, ^{13}C labeling is much more expensive when compared to the direct labeling of the desired compound using ^{18}O-labeled water as a reactant.

Different exchange conditions were tested under the premise that the unlabeled fumonisin B_1 should not be detectable; the most intensive isotopologue should bear at least two ^{18}O atoms to prevent spectral overlap with unlabeled fumonisin, and the amount of partially or completely hydrolyzed fumonisin B_1 species should be small. The results are given in Table 1. The best reaction conditions were 5% TFA at 50 °C for 8 h. However, when these reaction conditions were used on a larger scale, the distribution of ^{18}O-fumonisin isotopologues differed considerably from those obtained in the preliminary test. The distribution of fumonisin B_1 isotopologues obtained under the exchange condition at a larger scale is shown in Figure 2.

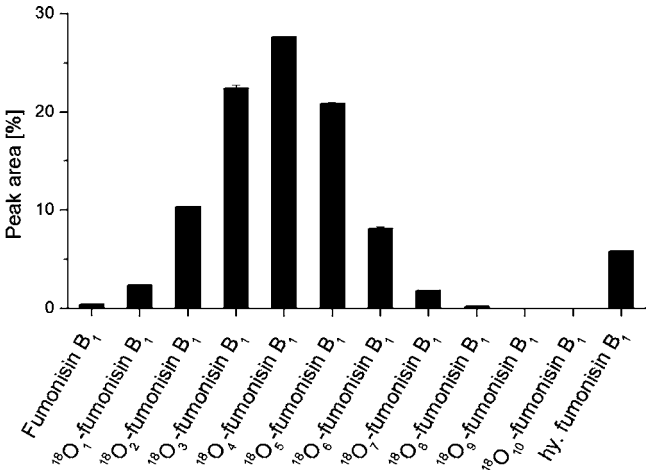


Figure 2. Distribution of the ^{18}O-fumonisin B_1 standard obtained from the large-scale exchange reaction. The column of hydrolyzed fumonisin B_1 shows the percentage of peak areas of the sum of all completely and partially hydrolyzed fumonisin B_1 isotopologues.

Only low amounts of byproducts that do not impair the use as a labeled standard can be found in the crude product. Thus, a further cleanup is not necessary. The purity of the internal standard and occurrence of byproducts was analyzed with HPLC-ELSD, and besides hydrolyzed fumonisins no other impurities were detectable. A purity of 92% was determined for the ^{18}O-labeled standard, which is slightly different from the amount given for the sum of hydrolyzed fumonisins in Table 1. However, the HPLC-ELSD method is much less specific compared to the HPLC-MS/MS measurements, and other impurities such as propane-1,2,3,-tricarboxylic acid from ester hydrolysis of fumonisins will be detected as well.

The identity of the ^{18}O-fumonisin B_1 standard was proven by accurate mass measurements and the characteristic fragmentation patterns of the unlabeled and labeled compounds. Comparison of ^1H NMR spectra showed no change in chemical shifts for the eight methylene protons of the side chains, which were measured between 2.4 and 2.8 ppm. In ^{13}C NMR experiments the signals of the four C atoms of the

dx.doi.org/10.1021/jf4022702 | J. Agric. Food Chem. 2013, 61, 7904−7908

Table 1. Results of Different Reaction Conditions for the ^{18}O Exchange in Fumonisin B_1

educts and reaction conditions					reaction products		
TFA (%)	**2** (mg)	$H_2{}^{18}O$ (μL)	T (°C)	t (h)	**2** (%)	main product[a] (%)	sum of (**3a**, **3b**, **4**)[b] (%)
10	2	100	50	8	nd	24.2 ($^{18}O_6$)	23.6
10	5	90	rt[c]	28	0.5	28.3 ($^{18}O_4$)	6.5
5	4	95	50	8	<0.1	27.1 ($^{18}O_6$)	11.6
5	39	190	50	8	0.4	27.6 ($^{18}O_4$)	5.8

[a]The amount of the most intensive isotopologue is given in percentage of the sum of peak areas of all fumonisin B_1 species. [b]The sum of hydrolyzed fumonisins includes all completely and partially hydrolyzed fumonisin B_1 isotopologues. [c]Room temperature, approximately 21 °C.

carboxylic acid groups were not detectable, which might be caused by different upshifts due to 0−2 ^{18}O atoms directly bound, leading to split signals and severely reducing the intensities.[19] The signals of the carboxylic ester C atoms had intensities comparable to the unlabeled compound with very similar chemical shifts.

The synthesized ^{18}O-fumonisin B_1 is a suitable standard for HPLC-MS/MS measurements due to the specific fragmentation in the mass spectrometer, where both ester bonds of the labeled side chains are cleaved for both most intense MRM transitions. Hence, the ratio of precursor to product ions always remains the same, although oxygen atoms in different positions of the two side chains are labeled.

Stability of ^{18}O-Fumonisin B_1. Under acidic conditions the distribution of ^{18}O labeling remains stable for 3 days at room temperature. These conditions are important for the sample preparation and following analysis. Long-term stability was observed under neutral conditions for up to 112 days at 4 °C. Therefore, storage of prepared standards is possible. Even better stability results can be expected at lower temperatures.

Sample Preparation. The sample preparation was performed according to an in-house method that is based on simple extraction with aqueous acidified acetonitrile solution. The labeled standard was added at the beginning to compensate for losses during sample preparation but also for matrix effects occurring during ionization in the mass spectrometer. Thus, the time needed for sample preparation was reduced because no further sample cleanup such as solid phase extraction or liquid−liquid distribution is necessary. After extraction, the samples were centrifuged, diluted, filtered, and directly analyzed by HPLC-MS/MS.

The results obtained with the matrix-matched calibration were used to calculate a LOD of 53 μg/kg, a LOQ of 188 μg/kg, and a coefficient of variation of 4.5% according to the method of the German Standard DIN 32645.[15,16] Thus, the sensitivity of the used API 3200 mass spectrometer is sufficient to quantitate fumonisin B_1 levels below the lowest maximum level of 200 μg/kg for baby food in the EU.[8] The recovery rate was determined in polenta maize grits at eight concentrations over the whole range of the calibration curve between 100 and 2000 μg/kg. In total, 32 data points were used to calculate the recovery of 94.8%. A further improvement in LOD and LOQ could be achieved when necessary by using a more sensitive mass spectrometer, for example, the QTRAP 5500.

Fumonisin B_1 in Maize-Based Food. To prove the applicability of the developed method, maize-based samples that were previously quantitated by reference methods were analyzed. As can be seen from Table 2, the results are in very good agreement with those obtained by reference methods. Samples containing fumonisin B_1 also showed the MRM transitions of fumonisins B_2 and B_3, which, however, were not quantitated due to the lack of standards.

Table 2. Fumonisin B_1 Levels[a] of Food Samples Analyzed Using $^{18}O_3$-Fumonisin B_1 as Isotope-Labeled Standard in Comparison with Values Determined with Reference Methods

sample	concn (μg/kg ± SD)	concn (ref method) (μg/kg)	rel deviation from ref method (%)
Serbian maize	1280 ± 12	1300	−1.5
whole maize	2263[b] ± 23	2200	2.9
maize kernels	1689 ± 51	1700	−0.7
popcorn maize	1407 ± 11	1383	1.7
tortilla chips nacho cheese	308 ± 8	312	−1.3
maize flour organic	847 ± 26	781	8.5
maize flour common	248 ± 38	244	1.8
ref blank popcorn maize	<LOD[c]		

[a]Four measurements without correction by the recovery rate. [b]Estimated value, concentration outside the calibrated concentration levels. [c]Not detectable (LOD = 53 μg/kg).

In summary, the developed HPLC-MS/MS method for the analysis of fumonisin B_1 using ^{18}O-labeled fumonisin B_1 as internal standard shows very good agreement with the results of reference methods. The stability and suitability of the ^{18}O-labeled fumonisin B_1 were shown for standard conditions. ^{18}O labeling is a convenient method for the labeling of small molecules containing carboxylic acid groups using only small amounts of the labeling reagent $H_2{}^{18}O$. The exchange method can directly be applied to other fumonisins such as fumonisins B_2 and B_3 as well as other compounds with carboxylic acid groups.

The suitability of any labeled compound for stable isotope dilution analysis is affected by a possible "spectral overlap" caused by a too small increase of the m/z value together with an insufficient isotopic purity or by an inappropriate fragmentation behavior in MRM mode. Because the labeling with ^{18}O atoms increases the m/z value by 2 atomic mass units, fewer numbers of exchanged atoms are needed in comparison to ^2H and ^{13}C labeling.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HRMS data of unlabeled and ^{18}O-labeled fumonisin B_1; ^1H NMR spectra of unlabeled and ^{18}O-labeled fumonisin B_1; stability of ^{18}O-labeled fumonisin B_1; and MRM transitions of the QTRAP 5500. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Wentzel C. A. Gelderblom for providing the *Fusarium verticillioides* strain MRC 826; Henning Harrer, Martin Rosenau, and Claudia Tenbrock for the isolation of fumonisin B_1, Uwe Lauber, Stefanie Marschik, and Simone Staiger for supplying samples; and Angela Klusmeier-König for technical assistance. We thank AB SCIEX for supplying us with a QTRAP 5500 mass spectrometer and VWR for supplying us with a VWR Hitachi LaChrom Ultra HPLC system.

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