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Analysis of the *Fusarium* Mycotoxin Moniliformin in Cereal Samples Using $^{13}C_2$-Moniliformin and High-Resolution Mass Spectrometry

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ABSTRACT: Moniliformin is a mycotoxin produced by fungi of the *Fusarium* genus and occurs as a contaminant of different cereals worldwide. This study describes the first application of isotopically labeled $^{13}C_2$-moniliformin for the analysis of moniliformin in cereals. Moniliformin is a small and ionic molecule that forms only a single sensitive fragment ion in the collision cell of a tandem mass spectrometer. Therefore, the methods described in the literature for this kind of instrument observe only a single mass transition and show a relatively poor sensitivity. The use of high-resolution mass spectrometry was described to be a suitable alternative technique for the detection of this compound and was therefore applied in this study. The developed method is based on the use of strong anion exchange columns for cleanup prior to HPLC analysis and has a recovery rate of 75.3%, a limit of detection (LOD) of 0.7 μg/kg, and a limit of quantitation (LOQ) of 2.5 μg/kg. Twenty-three different cereal samples were analyzed for their moniliformin content. Twenty of them showed positive results with levels up to 126 ± 12.2 μg/kg.

KEYWORDS: *moniliformin, mycotoxin, Fusarium, high-performance liquid chromatography (HPLC), Fourier transform mass spectrometry (FTMS), high-resolution mass spectrometry (HRMS)*

INTRODUCTION

Moniliformin is a mycotoxin that was first described by Cole et al. in 1973.[1] The structure of moniliformin (salt of 1-hydroxycyclobut-1-ene-3,4-dione) (Figure 1) was characterized

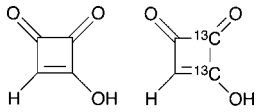

Figure 1. Structures of moniliformin and $^{13}C_2$-moniliformin.[29]

a year after the discovery.[2] The free acid (semisquaric acid) is a strong acid with a pK_a value ranging from 0.0 ± 0.05 to 1.7 as described in the literature.[3−5]

So far, the toxicity of moniliformin has been studied under in vitro and in vivo conditions. In vitro studies indicated an inhibition of multiple enzyme systems such as pyruvate dehydrogenase, transketolase, aldose reductase, glutathione peroxidase, and glutathione reductase.[6−10] Feeding studies with different avian species such as broiler chicks and ducklings using feed contaminated with moniliformin resulted in symptoms such as acidosis and muscular weakness.[11,12] Chicks fed moniliformin levels above 50 mg/kg feed showed increased heart weight, and in those fed levels above 100 mg/kg increased liver weights could be observed.[13] The oral LD_{50} values in ducklings and chickens vary from 3.7 to 5.4 mg/kg body weight, which is comparable to the LD_{50} of T2-toxin as determined in chicks.[12,14,15] Currently, the European Food Safety Authority (EFSA) is working on a scientific opinion on the risk of moniliformin for public health.[16]

The occurrence of moniliformin in cereals and cereal products has been described for different regions worldwide.[17] Levels from 50 to 2000 μg/kg were reported in corn and wheat samples from Austrian fields,[18] and in naturally contaminated corn samples levels of moniliformin between 160 and 1030 μg/kg were detected.[19] Another study screened the moniliformin content in Norwegian grain with levels up to 210 μg/kg in oats, 950 μg/kg in wheat, and 380 μg/kg in barley.[20] Analysis of *Fusarium*-contaminated whole corn plants from Denmark showed positive results of moniliformin contamination in 15 of 28 analyzed samples below the limit of quantitation (LOQ) of 12 μg/kg.[21]

Several analytical methods for the quantitation of moniliformin have been reported so far. An analytical principle common for most procedures is the application of ion-pair reagents to achieve a good chromatographic separation in high-performance liquid chromatography (HPLC) coupled to different detectors such as ultraviolet (UV), diode array detection (DAD), or atmospheric pressure chemical ionization mass spectrometry (APCI-MS).[22−24] For these methods, a LOQ above 24 μg/kg or a limit of detection (LOD) above 5 μg/kg is reported. Techniques such as ion chromatography (LOQ of 120 μg/kg),[25] capillary electrophoresis coupled to DAD (LOD of 50 μg/kg),[26] or GC-MS and HPLC-FLD (LOQ of 20 μg/kg) with previous derivatization[18,27] have also been used for the analysis of moniliformin. A more recent approach to achieve a good chromatographic separation of this highly polar mycotoxin was the use of hydrophilic interaction chromatography (HILIC) coupled to UV (LOQ of 96 μg/kg) or MS (LOQ of 12 μg/kg).[21] Moniliformin has also been analyzed within multimycotoxin methods. For example, one UHPLC-MS/MS method with a LOQ of 8.48 μg/kg based on a single mass transition in the multiple/selected reaction monitoring mode (MRM/SRM) to quantify moniliformin has been published.[28]

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Generally, due to the broad distribution of moniliformin over a large range of different crops in often relatively low concentrations, a robust and sensitive method is required to achieve a comprehensive overview of the exposure of consumers to this mycotoxin. The aim of the current study was therefore the development of a reliable HPLC-MS quantitation method for moniliformin, which uses $^{13}C_2$-moniliformin (Figure 1), a newly synthesized isotopically labeled standard, to compensate for matrix effects during mass spectrometric analysis.[29] Furthermore, to achieve the selectivity requested by international guidelines[30] and to improve the sensitivity of the moniliformin analysis compared to previously reported methods, an LTQ-Orbitrap-XL high-resolution mass spectrometer (FTMS) was used as detector.

MATERIALS AND METHODS

Reagents. All solvents and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), VWR (Darmstadt, Germany), or Grüssing GmbH (Filsum, Germany) in gradient or analytical grade. Water for extraction and chromatography was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). Moniliformin (purity of 99%, HPLC-UV 260 nm) and $^{13}C_2$-labeled moniliformin (purity ≥ 95%, HPLC-UV 260 nm, NMR, isotope purity of 99%, HPLC-MS) were synthesized in our working group.[29]

Samples. Analyzed samples were all by organic origin purchased in local food stores. The analyzed samples were corn kernels, corn grits, corn flour, wheat kernels, wheat flour, rye kernels, rye flour, oat kernels, and oat flakes.

Standard Solutions. Moniliformin (2.00 mg) and $^{13}C_2$-labeled moniliformin (2.03 mg) were each dissolved in acetonitrile/water (85:15, v/v) to a concentration of 1 mg/mL and stored at −18 °C. These stock solutions were used to prepare the calibration solutions with methanol/water (5:95, v/v).

Sample Preparation. Grain and oat flake samples were ground using an IKA A10 analysis grinder (IKA Labortechnik, Staufen, Germany). Grits and flour samples were directly used for the extraction. All analyses were carried out in duplicate and measured twice, and the arithmetic mean ± standard deviation was calculated.

Ten grams of sample was extracted with 150 mL of acetonitrile/water (85:15, v/v) using an Ultra-Turrax T25 (IKA Labortechnik) at 13000 rpm for 3 min. The extract was filtered through folded filters 3hw (Sartorius Stedim, Göttingen, Germany) and the organic solvent evaporated from an aliquot of 30 mL using a vacuum concentrator at 40 °C (H. Saur Laborbedarf, Reutlingen, Germany). Subsequently, residual water was removed by lyophilization in a Lyovac GT2 (Amsco/Finn Aqua, Hamburg, Germany). The residue obtained was dissolved in 2 mL of methanol using sonification (Bandelin Sonorex, Berlin, Germany) and purified by solid phase extraction using a strong anion exchanger material (SAX) as previously described.[18,21] Briefly, the SAX column (Bond Elut-SAX, 500 mg, 3 mL) (Agilent Technologies, Böblingen, Germany) was activated by adding a sequence of 2 mL of methanol, 2 mL of water, and 2 mL of 0.1 M HCl before the dissolved sample extract was applied. Subsequently, matrix constituents were removed from the column with 2 mL of methanol/water (50:50, v/v) followed by 2 mL 0.1 M HCl. Moniliformin was eluted with 2 mL of 1 M HCl, and the solution was evaporated to dryness at 40 °C under a stream of nitrogen.

The dried residue was reconstituted in 120 μL of methanol/water (5:95, v/v) and spiked with 30 μL of a 300 ng/mL solution of isotopically labeled $^{13}C_2$-moniliformin in methanol/water (5:95, v/v), resulting in a concentration of 4.5 μg/kg isotopically labeled standard. Highly contaminated samples were reanalyzed after dilution to an appropriate concentration within the calibration curve.

Chromatography. The columns used were a 150 mm × 2.1 mm i.d., 5 μm, Synchronis HILIC with a 10 mm × 2.1 mm i.d. guard column of the same material (Thermo Scientific, Dreieich, Germany), a 150 mm × 2.1 mm i.d., 3.5 μm, ZIC-HILIC with a 20 mm × 2.1 mm i.d. guard column of the same material (Merck, Darmstadt, Germany), and a 150 mm × 2.00 mm i.d., 5 μm, Gemini C6-Phenyl, with a 4 mm × 2 mm i.d. guard column of the same material (Phenomenex, Aschaffenburg, Germany). They were tested on a HPLC-DAD system (Jasco X-LC LC-2000Plus series with a MD-2010Plus diode array detector) (Jasco, Groß-Umstadt, Germany), with a moniliformin standard solution of 10 μg/mL to compare the separation concerning peak shape and retention.

Solvent A was 1% formic acid in methanol, and solvent B was 1% formic acid in water. The detection wavelength for moniliformin was set to 260 nm. The optimized conditions for the chromatographic separation of moniliformin on the different columns were as follows. For the Synchronis HILIC an isocratic run at 20% A was performed for 10 min at a flow rate of 250 μL/min. For the ZIC-HILIC an isocratic run at 95% A was used for 10 min at a flow rate of 150 μL/min. For the Gemini C6-Phenyl column an isocratic run at 5% A for 10 min at a flow rate of 250 μL/min was optimal.

HPLC-FTMS. HPLC-FTMS analysis was carried out on a Thermo HPLC system (Accela LC with Accela Pump 60057-60010 and Accela Autosampler 60057-60020) coupled to a LTQ-Orbitrap-XL Fourier transform mass spectrometer equipped with a heated ESI source (Thermo Scientific, Dreieich, Germany). Xcalibur 2.07 SP1 was used for data acquisition and analysis.

The column used for the separation of moniliformin was the Gemini C6-Phenyl. The flow rate was set to 250 μL/min and the injection volume to 10 μL. Solvent A was 1% formic acid in methanol, and solvent B was 1% formic acid in water. Chromatography was done at a column temperature of 40 °C and an isocratic run at 5% A for 8 min.

Ionization was carried out with heated electrospray in negative mode. Capillary temperature was 225 °C, vaporizer temperature, 350 °C; sheath gas flow, 50 units; auxiliary gas, 10 units; source voltage, 3.5 kV; and tube lens, −110 V.

One scan event was programmed to perform a total ion scan of a mass range from m/z 90.0 to 110.0 at a resolution of 100 000. The base peak traces of the $[M - H]^-$ ions of moniliformin (m/z 96.9931) and $^{13}C_2$-moniliformin (m/z 98.9998) used as internal standard were extracted with a width of ±5 ppm from the mass spectra and used for quantitation. To further confirm the identity of moniliformin, the $[M - H]^-$ ion of the naturally occurring $^{13}C_1$-moniliformin at m/z 97.9964 ± 5 ppm was recorded and the ratio between moniliformin and $^{13}C_1$-moniliformin monitored.

Calibration. For solvent calibration, seven different concentrations of moniliformin in methanol/water (5:95, v/v) between 7.5 and 300 ng/mL correlating with contamination values between 0.6 and 22.6 μg/kg were prepared. They were spiked with the same constant level of 4.5 μg/kg $^{13}C_2$-labeled moniliformin to compensate matrix effects during ionization. Each calibration point was prepared in duplicate, and each of them was analyzed twice, resulting in four data points for each level. Linear regression was used to calculate the regression curve.

Method Evaluation. The LOD and LOQ were calculated according to the method of the German Standard DIN 32645.[31] The calculation was described in detail by Kleigrewe et al.[32] For the matrix calibration, corn grits containing no detectable amount of moniliformin were spiked with unlabeled moniliformin at seven levels ranging between 0.6 and 22.6 μg/kg. To allow an interaction of the standard with the matrix, the flasks were shaken on a Labshaker (GFL, Burgwedel, Germany) at a frequency of 300/min for 3 min before extraction. 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.[33] Additionally, samples of corn grits and rye containing no detectable amounts of moniliformin were spiked with 15.0 μg/kg moniliformin to compare the recovery of different cereals and to check the accuracy of the calculated recovery.

RESULTS AND DISCUSSION

The analysis of moniliformin in food and feed to assess the daily exposure of consumers to this toxin requires a robust and sensitive analytical procedure. In previous publications, a good and reliable extraction and purification procedure for moniliformin based on strong anion exchange columns (SAX) has been reported.[18−21,24] However, the reported chromatographic analyses of moniliformin still suffer under high detection limits. As for most analytes, the use of highly sensitive modern mass spectrometers is an approach to improve the LOD and LOQ. Possible types of instruments are either tandem mass spectrometers, which use the fragmentation of the ionized analytes in the MRM mode to improve the sensitivity and selectivity, or high-resolution mass spectrometers. These instruments achieve high sensitivity and selectivity due to the small size of the mass window of a few millidaltons around the accurate mass to charge ratio of the analyte. Thus, coeluting compounds with the same nominal mass but accurate mass and sum formula different from those of the analyte are excluded from detection. For the analysis of moniliformin, the use of tandem mass spectrometers is not recommended as this compound generates only one strong product ion in the collision cell of the instrument. Thus, only one MRM, the fragmentation of m/z 97 to m/z 41 can be programmed,[34] which is not sufficient for an analysis according to international guidelines such as Commission Decision 2002/657/EC of the European Commission.[30] For a reliable qualitative and quantitative analysis with this type of instrument, this institution demands the recording of at least two mass transitions with one transition being regarded as quantifier and the other as qualifier with the intensity ratio between both transitions used as a marker for peak purity. In the case of high-resolution mass spectrometry, no mass transitions are required; instead, two characteristic ions are recorded.[30] In the case of moniliformin, the base peak ($[M - H]^-$) of moniliformin at m/z 96.9931 and the naturally occurring isotopic peak at m/z 97.9964 were recorded, resulting in an observed ratio of $^{13}C_1$-moniliformin relative to all-^{12}C-moniliformin of 2.6%. According to Commission Decision 2002/657/EC of the European Commission, variations of this ratio by up to 50% are accepted.[30]

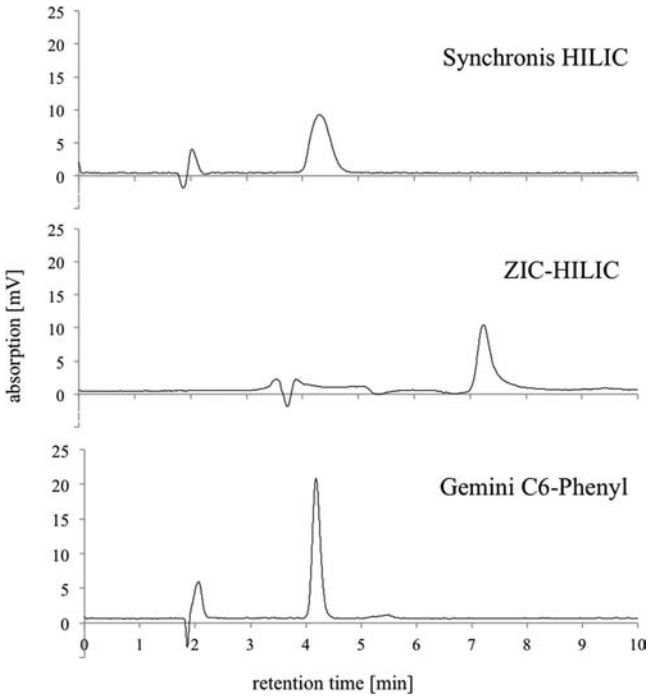

Figure 2. Comparison of three different HPLC columns for their suitability for moniliformin analysis: HPLC-UV chromatograms at 260 nm are shown.

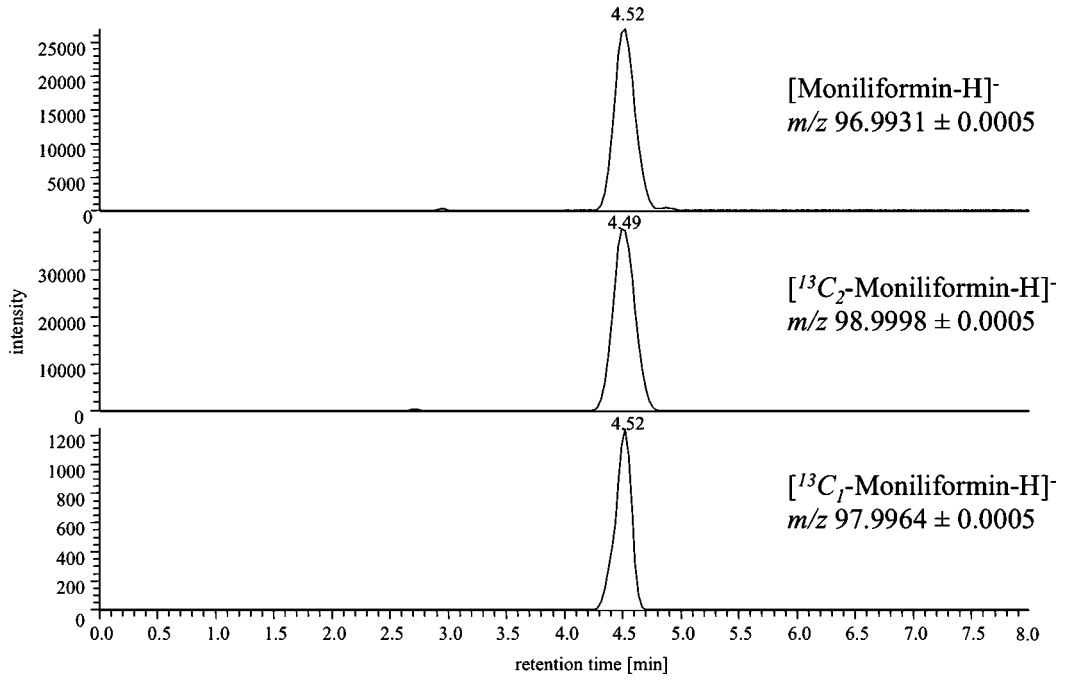

Figure 3. HPLC-FTMS chromatogram of a rye flour sample containing 2.9 ± 0.2 μg/kg moniliformin. $[Moniliformin - H]^-$, extracted ion chromatogram of the base peak of moniliformin m/z 96.9931 (±5 ppm); $[^{13}C_2$-Moniliformin $- H]^-$, extracted ion chromatogram of the base peak of isotope labeled $^{13}C_2$-moniliformin m/z 98.9998 (±5 ppm) used as internal standard; $[^{13}C_1$-Moniliformin $- H]^-$, extracted ion chromatogram of the base peak of the naturally occurring $^{13}C_1$-moniliformin m/z 97.9964 (±5 ppm).

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The analysis of sample extracts by mass spectrometers suffers in almost all cases under matrix effects.[35] They are caused by changes in the ionization efficiency in the ion source due to coeluting matrix compounds and lead to suppression or enhancement of the MS signal. Matrix effects can be compensated by different approaches such as matrix calibration or standard addition.[36] However, the use of stable isotope-labeled standards is the easiest and most efficient approach.[37] Thus, as the synthesis of $^{13}C_2$-labeled moniliformin recently succeeded in our laboratory,[29] we applied this compound as internal standard for the analysis of trace amounts of moniliformin in cereal samples using a HPLC-FTMS system.

Chromatography. Moniliformin is a substance with ionic character that requires special care in the development of a suitable chromatographic separation. Previous studies reported the use of different types of columns for chromatography of moniliformin. In this study two different HILIC columns and one C6-Phenyl column were tested for their suitability. The columns were chosen on the basis of publications of Sørensen et al. and our own observations during the synthesis of $^{13}C_2$-moniliformin.[21,29,38] As depicted in Figure 2, the best peak shape with suitable retention was obtained with the C6-Phenyl column.

Sample Preparation. The sample preparation was performed according to the protocol of Sørensen et al. with slight modifications and is based on the extraction with acetonitrile/water (85:15, v/v) followed by enrichment and purification on a SAX column. In previous studies extraction solvents with different concentrations of acetonitrile have been tested.[18−21,24] In summary, concentrations between 80 and 90% acetonitrile were found to be suitable for an efficient moniliformin extraction. The protocol for the subsequent cleanup uses the high acidity of moniliformin as this compound elutes only with 1 M hydrochloric acid from SAX columns. Due to the good reproducibility of the described method and the limited amount of $^{13}C_2$-moniliformin available, $^{13}C_2$-labeled moniliformin was added after the cleanup prior to HPLC-FTMS analysis to compensate only for matrix effects occurring during the ionization. As an example, Figure 3 shows the HPLC-FTMS chromatogram of a rye flour sample containing 2.9 ± 0.2 μg/kg moniliformin. The base peak traces of the $[M - H]^-$ ions of moniliformin (m/z 96.9931) and $^{13}C_2$-moniliformin (m/z 98.9998) used as internal standard were extracted from the mass spectra. To further confirm the identity of moniliformin, the $[M - H]^-$ ion of the naturally occurring $^{13}C_1$-moniliformin at m/z 97.9964 was also recorded.

Method Evaluation. The developed method was evaluated before sample analysis, starting with the determination of the LOD and LOQ. However, almost no "noise" signals could be detected due to the high selectivity of the accurate mass traces recorded for quantitation with an HPLC-FTMS system (Figure 3). Thus, a calculation of the LOD and LOQ via the signal-to-noise ratio was not possible. Instead, the dispersion of the signals recorded for the lowest concentrations of a calibration curve of moniliformin was considered as described by the German Standard DIN 32645.[31] A detailed description of the used equations for this method can be found in Kleigrewe et al.[32]

Therefore, a calibration curve in methanol/water (5:95, v/v) with seven different levels of moniliformin (7.5−300 ng/mL corresponding to values of 0.6−22.6 μg/kg) and constant levels of $^{13}C_2$-moniliformin (4.5 μg/kg) and a matrix calibration in corn grits with similar concentrations of moniliformin and the addition of $^{13}C_2$-moniliformin (4.5 μg/kg) just before HPLC-FTMS analysis were recorded. The results obtained with the matrix calibration were used to calculate a LOD of 0.7 μg/kg, a LOQ of 2.5 μg/kg, and a coefficient of variation of 6.5%.

The recovery was determined in corn grits at six concentrations over the whole range of the calibration curve between 2.5 and 22.6 μg/kg. In total, 24 data points were used to calculate the recovery of 75.3%. Additional measurements of another corn grit sample and a rye sample, each spiked with 15.0 μg/kg, confirmed this value, indicating that the calculated recovery of 75.3% is valid for cereals.

Moniliformin in Food Samples. Twenty-three samples from the German market were analyzed for their moniliformin content. As this mycotoxin is reported to occur in wheat, corn, oat, and rye, samples based on these cereals were screened. All samples were prepared in duplicate, each preparation was analyzed twice, and the mean values ± standard deviations were calculated. The relative standard deviation was in all cases below 10%. All results of the screening are summarized in Table 1. Only 3 of 23 analyzed samples were below the LOD.

Table 1. Moniliformin Concentrations of Analyzed Samples from the German Market

food sample	concentration of moniliformin[a] (μg/kg)
corn kernels 1	<LOQ[b]
corn kernels 2	<LOQ
corn kernels 3	18.0 ± 1.1
corn grits 1	31.1 ± 2.1
corn grits 2	<LOD[c]
corn flour	126 ± 12.2
wheat kernels 1	<LOQ
wheat kernels 2	<LOQ
wheat kernels 3	7.0 ± 0.7
wheat flour 1	3.1 ± 0.3
wheat flour 2	6.5 ± 0.4
rye kernels 1	<LOD
rye kernels 2	<LOD
rye kernels 3	<LOQ
rye flour 1	<LOQ
rye flour 2	<LOQ
rye flour 3	2.9 ± 0.2
oat kernels 1	5.4 ± 0.4
oat kernels 2	8.7 ± 0.7
oat kernels 3	<LOQ
oat flakes 1	5.9 ± 0.4
oat flakes 2	5.4 ± 0.4
oat flakes 3	10.0 ± 0.7

[a]Concentrations not corrected for recovery. [b]Limit of quantitation (LOQ) = 2.5 μg/kg. [c]Limit of detection (LOD) = 0.7 μg/kg.

Eight samples showed moniliformin concentrations between 0.7 and 2.5 μg/kg, and in nine samples levels between 2.5 and 12.0 μg/kg were detected. Three samples (all of them corn-based) were above a concentration of 12.0 μg/kg moniliformin, with one corn flour containing 126 ± 12.2 μg/kg.

In summary, a HPLC-FTMS method for the determination of moniliformin in cereal samples using a stable isotope labeled standard was developed for the first time. This method allows a reliable quantitation of moniliformin in food samples down to concentrations of 2.5 μg/kg.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; DIN, Deutsches Institut für Normung (German Institute for Standardization); FLD, fluorescence detection/detector; HILIC, hydrophilic interaction chromatography; MRM, multiple reaction monitoring; SAX, strong anion exchange; SRM, single reaction monitoring; UHPLC, ultrahigh-performance liquid chromatography.

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