A Validated Multianalyte LC−MS/MS Method for Quantification of 25 Mycotoxins in Cassava Flour, Peanut Cake and Maize Samples

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Ⓢ Supporting Information

ABSTRACT: This study was designed to develop a sensitive liquid chromatography tandem mass spectrometry (LC−MS/MS) method for the simultaneous detection and quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples with particular focus on the optimization of the sample preparation protocol and method validation. All 25 mycotoxins were extracted in a single step with a mixture of methanol/ethyl acetate/water (70:20:10, v/v/v). The method limits of quantification (LOQ) varied from 0.3 μg/kg to 106 μg/kg. Good precision and linearity were observed for most of the mycotoxins. The method was applied for the analysis of naturally contaminated peanut cake, cassava flour and maize samples from the Republic of Benin. All samples analyzed (fifteen peanut cakes, four maize flour and four cassava flour samples) tested positive for one or more mycotoxins. Aflatoxins (total aflatoxins; 10−346 μg/kg) and ochratoxin A (<LOQ−2 μg/kg) were detected in peanut cake samples while fumonisin B_1 (4−21 μg/kg), aflatoxin B_2 (<LOQ−8 μg/kg), aflatoxin B_1 (<LOQ−9 μg/kg), diacetoxyscirpenol (<LOQ−6 μg/kg) and zearalenone (<LOQ−12 μg/kg) were detected and quantified in cassava flour samples. Fumonisin B_1 (13−836 μg/kg), fumonisin B_2 (5−221 μg/kg), fumonisin B_3 (<LOQ−375 μg/kg) and beauvericin (<LOQ−25 μg/kg) were detected in the maize samples.

KEYWORDS: multimycotoxin, LC−MS/MS, cassava, peanut cake, maize, validation, matrix effect

■ INTRODUCTION

Mycotoxins are toxic secondary metabolites of fungal species, and are common contaminants of food and feed commodities worldwide.[1] Human exposure to mycotoxins can occur through the consumption of food that has been contaminated by toxigenic molds before harvest or during storage and through inhalation of airborne mycotoxins produced by indoor molds.[2] *Fusarium, Penicillium* and *Aspergillus* are the most prevalent toxin-producing genera.[3]

Over 400 mycotoxins are known today, with the aflatoxins, fumonisins, ochratoxin, zearalenone and the trichothecenes being the most widely investigated, due to their frequent occurrence and their severe effects on animal and human health.[4] The trichothecenes form an important group of chemically related *Fusarium* mycotoxins which include deoxynivalenol, 3-acetyl and 15-acetyldeoxynivalenol from *F. graminearum,* T-2 toxin, HT-2 from *F. sporotrichioides*, nivalenol from *F. kyushuense* and diacetoxyscirpenol from *F. scirpi*.[5]

At present, scientific literature on mycotoxin contamination in foods exceeds over 10,000, with just a few reporting on mycotoxin contamination in agricultural commodities from tropical countries, especially from sub-Saharan African countries. Despite the limited number of studies, the results of these studies (surveys) are consistent[6−9] with results from other tropical countries in that fungi and mycotoxins routinely occur at high levels in agricultural commodities from tropical countries especially from sub-Saharan African countries. Mycotoxins cause severe economic loss and impair the health of humans throughout sub-Saharan Africa.[10−12] The largest mycotoxin poisoning was reported in Africa during the past five years[9,13] which resulted in 317 hospitalizations and 125 fatalities.

Cassava, peanuts and maize are the three most widely grown staple foods in most tropical countries.[14] Cassava (*Manihot esculenta* Crantz subspecies *esculenta*) is a starchy root crop and a staple food for half a billion people and the third largest source of carbohydrate for human food in the world, with Africa as its largest center of production.[15] It is highly perishable and thus requires processing to ensure stability during storage. The cassava processing cycle has several steps, which include fermentation, drying and storage. At each of these steps, contamination by fungi may occur.

Analytical methods to screen agricultural commodities for mycotoxins do exist but are scarce in sub-Saharan Africa. Moreover, in previous years, there has been a lack of highly trained personnel, equipped with skills in handling advanced instrumentation such as high-performance liquid chromatography (HPLC) with fluorescence, ultraviolet and tandem mass spectrometry (LC−MS/MS) detections. Therefore, investigations were generally focused on thin layer chromatography (TLC) and enzyme linked immunosorbent assay (ELISA) techniques which are relatively easier to perform, but are however less accurate. Also to simplify analysis, the range of analytes was commonly limited to those mycotoxins which are known to occur in the sample material, for example aflatoxins in peanuts and fumonisins in maize.[16] Thus, most often, only single analyte investigations were performed.

However, due to the co-occurrence of multiple toxins in food matrices and their possible synergistic effect in humans, it is thus

absolutely necessary for multianalyte detection to be performed on the different groups of mycotoxins. LC—MS/MS multi-mycotoxin analysis is also expected to be implemented in Africa as more and more countries acknowledge the need to invest in the acquisition of new and modern instrumentation and highly trained personnel. To date, only one multianalyte LC—MS/MS method has been reported for multimycotoxin determination in samples from sub-Saharan Africa. Adejumo et al.[17] reported on determination of trichothecenes in maize from Nigeria. This study was designed to develop and validate a fast and reliable method based on HPLC—MS/MS for the detection of 25 different mycotoxins in cassava flour, peanut cake and maize flour. Furthermore, the validated method was used to analyze samples obtained from markets in the Republic of Benin.

■ MATERIALS AND METHODS

Reagents and Materials. Methanol and *n*-hexane, both HPLC grade, as well as Whatman Glass microfiber filters (GFA, 125 mm) were purchased from VWR International (Zaventem, Belgium). Dichloromethane, *N,N*-dimethylformamide and ethyl acetate were purchased from Acros Organics (Geel, Belgium). Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Acetic acid was supplied by Merck (Darmstadt, Germany). Bakerbond aminopropyl (NH_2) and Grace octadecyl (C18) solid phase extraction (SPE) cartridges were obtained from Grace Discovery Sciences (Lokeren, Belgium). Bond Elut strong anion exchange (SAX) SPE cartridges were obtained from Varian (Sint-Katelijne Waver, Belgium). Ultrafree-MC centrifugal filter devices (0.22 μm) of Millipore (Bredford, MA, USA) were used. Trifluoroacetic acid was obtained from Fluka (Buch, Switzerland). Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium).

Standards. Mycotoxin-reference standards, namely, nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, fusarenon-X, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, HT-2 toxin, alternariol, alternariol methyl ether, altenuene, ochratoxin A, zearalenone, fumonisin B_1, fumonisin B_2, beauvericin, sterigmatocystin, deepoxy-deoxynivalenol and zearalanone, were purchased from Sigma-Aldrich (Bornem, Belgium). Diacetoxyscirpenol and T-2 toxin were purchased from Biopure (Tulln, Austria). Fumonisin B_3 was obtained from Promec Unit (Tygerberg, South Africa). Penicillic acid was purchased from Fermentek (Jerusalem, Israel) while roquefortine C was obtained from Enzo Life Science (Lorrach, Germany). Nivalenol, neosolaniol, deepoxy-deoxynivalenol and diacetoxyscirpenol were obtained as solutions (100 μg/mL) in acetonitrile. Fumonisin B_2 and fumonisin B_3 standards (1 mg) were prepared in 1 mL of acetonitrile/water (50:50, v/v). Stock solutions of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, aflatoxin B1, B_2, G_1, G_2, HT-2 toxin, T-2, altenuene, ochratoxin A, zearalenone, fumonisin B_1, beauvericin, sterigmatocystin, deepoxy-deoxynivalenol, zearalanone, roquefortine C and penicillic acid, were prepared in methanol at a concentration of 1 mg/mL. Alternariol and alternariol methyl ether stock solutions (1 mg/mL) were prepared in methanol/dimethylformamide (60:40, v/v). All stock solutions were stored at −18 °C for 6 months except fumonisin B_2 and B_3, which were stored at 4 °C. From the individual stock standard solutions, a standard mixture was prepared at the following concentrations: nivalenol and alternariol methyl ether (20 ng/μL); fusarenon-X, HT-2, alternariol and neosolaniol (10 ng/μL); deoxynivalenol, 3-acetyldeoxynivalenol and altenuene (5 ng/μL); 15-acetyldeoxynivalenol (2.5 ng/μL); aflatoxin B_2 and zearalenone (2 ng/μL); T-2 (1 ng/μL); diacetoxyscirpenol and sterigmatocystin (0.5 ng/μL); fumonisin B_1, B_2 and B_3 (0.5 ng/μL); aflatoxin B_1, G_1 and G_2 (0.2 ng/μL); roquefortine C, penicillic acid and beauvericin (0.1 ng/μL); ochratoxin

A (0.05 ng/μL). The standard mixtures were prepared in methanol, stored at −18 °C and renewed every 3 months.

Samples. Fifteen peanut cakes, four maize flours and four cassava flour samples were obtained from local markets in Benin. The peanut cakes, locally referred to as "kulikuli", were obtained from different locations, representing three different agroecological zones (the warm arid, warm semiarid and the warm subhumid (forest) regions). Peanut cakes were obtained through peanut processing. The processing of peanut to peanut cake was performed as follows: peanuts were ground and pounded until a smooth paste was formed; water was then added to the paste and mixed thoroughly, and this resulted in a separation of phases. The oily layer (upper phase) was scooped away. Enough water was added to remove most of the oil. The processed peanut was later shaped into balls and fried in oil until a fairly brown color was obtained. Spices could also be added just before frying. These peanut balls (peanut cake) were later kept at room temperature and marketed.

The method used in the processing of cassava roots to cassava flour was based on an early traditional approach mostly practiced in West Africa.[18] Cassava roots were peeled manually, pounded or fermented, sun dried and stored. Upon collection, all samples were sealed in polyethylene bags and dried in desiccators. The samples were later ground in a blender and stored at 4 °C until analysis.

Sample Preparation. One gram of homogenized sample was spiked with internal standards zearalanone and deepoxy-deoxynivalenol at concentrations of 0.02 μg/g and 0.1 μg/g respectively. The fortified sample was kept in the dark for 15 min, to allow equilibration of the analyte with the matrix. The sample was extracted with 15 mL of extraction solvent, methanol/ethyl acetate/water (70:20:10, v/v/v), during 20 min using an Agitelec horizontal shaker (J. Toulemonde & Cie, Paris, France) and centrifuged for 15 min at 3170g. Using a Pasteur pipet (Novolab, Geraardsbergen, Belgium), the supernatant was transferred into a new extraction tube. Extraction was repeated with fresh solution (10 mL) of the extraction solvent. Both extracts were combined and evaporated at 40 °C under a stream of nitrogen. The residue was reconstituted in 5 mL of methanol/water (85:15, v/v) to which 10 mL of dichloromethane/hexane (30:70, v/v) solution was added. The mixture was shaken for 10 min and centrifuged at 3200g for 10 min. The dichloromethane/hexane phase was discarded while the methanol/water phase was kept for further cleanup. The defatted extract (5 mL) was further split into two parts of 2.5 mL each for sample enrichment (cleanup). One part (of the split-up extract) was cleaned by passing it through a glass fiber filter while the second part was cleaned up using aminopropyl (NH_2) cartridges, mounted on a vacuum elution manifold. The amino cartridge was first conditioned with 5 mL of the reconstitution solvent at a flow rate of one drop per second. After the conditioning step, the sample extract was loaded onto the SPE cartridge and the eluate collected in a test tube. One milliliter of the reconstitution solvent was used to wash the SPE cartridge with the intention of eluting most of the loosely trapped (through weak hydrophobic interactions) mycotoxins with the exception of fumonisin and ochratoxin A, both of which are strongly retained by the SPE absorbent. Both parts (the NH_2 SPE and glass fiber filter) of the cleaned extracts were recombined and evaporated under a gentle stream of nitrogen. The residue was dissolved in 100 μL of mobile phase consisting of methanol/water/acetic acid (57.2:41.8:1, v/v/v) and 5 mM ammonium acetate. Ultrafree MC centrifugal devices were used to further filter the resulting solution prior to injection into the LC—MS/MS system; this was performed for 15 min at 14000g.

The Standard Addition Technique. Considering the fact that the sample matrices used in this study were either processed (ready-to-eat) or semiprocessed products, with possible batch to batch variation, the standard addition technique was chosen and applied for quantification of the analytes. All three sample matrices were subjected to the same analytical protocol. A screening and semiquantitative test was first

performed with external standards to estimate the amount of each analyte present in the samples. Each sample was then split into four portions: three spiked with target analyte at increasing concentrations corresponding to 2-, 3-, and 4-fold the estimated concentration. All four portions were then submitted to the sample preparation procedure as earlier described. Internal standards were added prior to extraction. The ratio of the peak areas of the target analytes to the internal standard was plotted versus the concentration. The absolute value of the intercept of this regression line with the x-axis gave the initial concentration of the analyte in the sample.

Chromatographic Conditions. The column used was a 150 mm × 2.1 mm i.d., 5 μm, Symmetry RP-18, with a 10 mm × 2.1 mm i.d. Sentry guard column of the same material (Waters, Zellik, Belgium). Two solvent mixtures were used as mobile phases, both containing 5 mM ammonium acetate. Solvent A was composed of water/methanol/acetic acid (94:5:1, v/v/v) while methanol/water/acetic acid (97:2:1, v/v/v) was used as solvent B. The sample injection volume was set at 20 μL. A solvent gradient (flow rate of 0.3 mL/min) was adopted for a total run time of 28 min, with all the 25 mycotoxins eluting over 3−14 min while the final 14 min were used for column cleaning and regeneration. The solvent gradient was as follows: 0−7 min, 95−35% A; 7−11 min, 35−25% A, 11−13 min, 25−0% A; 13−14 min, 0% A; 14−16 min, 0−40% A; 16−26 min, 40−60% A; 26−28 min, 60−95% A.

Mass Spectrometry Conditions. Detection and quantification were performed with a Waters Acquity UPLC apparatus coupled to a Micromass Quattro Micro triple quadrupole spectrometer (Waters, Milford, MA, USA). The instrumental control and data processing utilities included the use of the Masslynx 4.1 software. The mass spectrometer analyses were carried out using selected reaction monitoring (SRM) channels in positive electrospray ionization (ESI+) mode. The following were the instrumental settings: source and desolvation temperatures 150 and 350 °C, respectively; capillary voltage 3.2 kV; cone nitrogen and desolvation gas flows 200 and 500 L·h^{-1}, respectively. The MS parameters for each mycotoxin were similar to those optimized and reported by Monbaliu et al.[19] The method was reoptimized, adapted and further extended to include two other toxins: roquefortine C and penicillic acid. Freshly prepared standard solutions (10 ng/μL) were infused at 0.03 mL/min for 30 s, to obtain a diagnostic MS spectrum, from which a precursor ion for each analyte was selected. The precursor ion for each analyte was mass-selected by the first quadrupole and fragmented through a combination of cone voltages and collision energies to obtain the product ion of each analyte. To ensure high specificity and sensitivity, two product ions for each analyte were selected in the final method and their collision energies further optimized. The primary product ion (first transition), which corresponds to the most abundant product ion, was used for quantification while the secondary product ion (second transition) was used for confirmation.

Method Validation. For the validation studies, the trueness, linearity and precision of the analytical method were evaluated. For each of the three sample matrices, blank samples were used. Trueness was assessed by estimating the bias. Each sample matrix was split into four portions of 1 g each. Three portions were used to construct a calibration curve (using the method of least-squares), while a fourth sample also spiked to a predetermined concentration was used to estimate the bias of the analytical method. The response (ratio of peak area of analyte to peak area of internal standard) of each analyte was plotted against the spiked concentration levels and the resulting calibration curve used to determine the linearity and bias of the method. In addition to the commonly reported regression coefficients (R^2), which give an indication of the fraction of the variability which can be explained by the model, a lack of fit test was also performed to assess the adequacy of the linear model. For all the analytes, zearalanone

was used as internal standard except for deoxynivalenol, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol, for which deepoxy-deoxynivalenol was used as internal standard. These internal standards correct for volume changes but not for matrix effects as they are not coeluting analytes.

Intraday and interday precision were evaluated by fortifying the different sample matrices at two concentration levels of each mycotoxin (in triplicate), and analyzed in triplicate over 2−4 days. The method limit of detection (LOD) and limit of quantification (LOQ) for each analyte were obtained from the signal-to-noise (S/N) ratio, which have been defined and set as 3 and 10 respectively by the International Union of Pure and Applied Chemistry (IUPAC). For LOD determination, three samples of each matrix were fortified at a level previously determined, analyzed and further confirmed by repeated analysis over three days. The fortification level for each mycotoxin for LOD studies was chosen based on response data collected during the method development. The LOQ was determined based on the knowledge of the LOD, determined by incremental adjustment of the spiked concentration in order to obtain a signal-to-noise of 10.

■ RESULTS AND DISCUSSION

Selection of Extraction Solvent. Owing to the great chemical diversity of mycotoxins and the complexity of plant matrices validated in this study, the solvent composition applied for extraction was an important parameter during the development of the multimycotoxin method. Different mixtures of organic solvents and water with or without organic acids were evaluated. Meanwhile, for selection of the best extraction solvent, it was useful to compare recovery data for the different solvents evaluated. The performances (extraction efficiencies) of the extraction solvents for the different mycotoxins as well as for the different matrices were evaluated as follows. Two sets of three samples each were used; the first set was spiked with known concentrations of analyte prior to extraction (spike begin) while the second set was spiked immediately after sample cleanup (spike end). The ratios of the peak areas of the spike begin to the peak area of the spike end were used to calculate the recovery of the entire sample cleanup procedure.

Mixtures of ethyl acetate/acetic acid (99:1, 97:3 and 95:5, v/v) were the first to be evaluated for their suitability as extraction solvent for all 25 analytes investigated. Use of these solvents resulted in very low recoveries (<50%) for most of the mycotoxins (data not shown). The worst recoveries were observed with the fumonisins (<20%). Mixtures of ethyl acetate with stronger organic acids such as trifluoroacetic acid (TFA), at lower percentages (1, 2 and 5%), were also tested. This solvent mixture led to improved recovery for the fumonisin toxins, sterigmatocystin and beauvericin. Unexpectedly, the recoveries for the other mycotoxins were greatly compromised. These solvent mixtures were not further optimized.

Mixtures of methanol/water/acetic acid (50:49:1 and 80:19:1, v/v/v) were also investigated. Recovery for the aflatoxins (75%), fumonisins (30%) and the type A trichothecenes (>60%) improved significantly when compared to the previously used ethyl acetate/acetic acid mixtures. However, increased amounts of matrix components were also extracted and as a result led to significant signal suppression. This solvent mixture was also not further optimized. Finally, in order not to compromise the recovery of the sample preparation of any of the 25 toxins, a ternary solvent mixture made up of methanol/ethyl acetate/water (70:20:10, v/v/v) was chosen. This solvent mixture

Table 1. Recovery of the Sample Preparation Obtained with Methanol/Ethyl Acetate/Water (Signal Recovery, %)

compounds	signal recovery (%)		
	cassava	maize	peanut
nivalenol	80 (75)	96 (79)	79 (77)
deoxynivalenol	86 (78)	86 (67)	84 (87)
neosolaniol	93 (65)	90 (55)	88 (80)
penicillic acid	96 (65)	90 (55)	88 (80)
fusarenone-X	85 (82)	81 (78)	96 (91)
deepoxy-deoxynivalenol	88 (85)	92 (80)	89 (90)
3-acetyldeoxynivalenol	81 (80)	86 (89)	86 (85)
15-acetyldeoxynivalenol	83 (81)	96 (80)	73 (88)
aflatoxin G_2	72 (66)	76 (54)	107 (80)
aflatoxin G_1	120 (67)	78 (55)	76 (86)
aflatoxin B_2	79 (67)	75 (58)	88 (79)
aflatoxin B_1	82 (76)	75 (75)	94 (79)
diacetoxyscirpenol	82 (89)	84 (91)	82 (80)
altenuene	86 (89)	84 (91)	86 (80)
roquerfortine C	78 (21)	73 (28)	90 (77)
HT-2	87 (75)	82 (68)	90 (77)
fumonisin B_1	92 (81)	113 (84)	92 (81)
alternariol	84 (78)	78 (51)	86 (80)
T-2	87 (79)	88 (66)	86 (82)
fumonisin B_3	89 (78)	104 (78)	86 (76)
ochratoxin A	80 (79)	85 (86)	99 (78)
zearalanone	91 (71)	85 (79)	91 (81)
zearalenone	88 (62)	82 (65)	86 (77)
sterigmatocystin	87 (15)	77 (18)	83 (79)
fumonisin B_2	93 (76)	95 (79)	111 (74)
alternariol methyl ether	87 (15)	77 (18)	83 (79)
beauvericin	75 (79)	73 (84)	105 (80)

showed no separation of phases and also gave very good recoveries for all the mycotoxins. Recovery for the different analytes was between 72% and 120% (Table 1). To the best of our knowledge, this is the first time such a ternary solvent mixture is being used as extraction solvent for multimycotoxin analysis.

The reconstitution solvent prior to sample cleanup was also optimized; different proportions of methanol/water mixtures were evaluated. Use of higher proportions of methanol ($\geq 80\%$) in a methanol/water mixture led to a significant improvement in the recovery of the sample preparation of beauvericin (≥ 10-fold increase). Beauvericin is a fairly big (MW 783 Da) cyclohexadepsipeptide, hardly soluble in water but readily soluble in methanol.[20] As a consequence, with low percentages of methanol in the reconstitution solvent, there is a greater tendency of beauvericin going into the dichloromethane/hexane phase during the defatting step resulting in low recovery of the sample preparation for this analyte. Thus, with a mixture composition of methanol/water (85:15, v/v) as reconstitution solvent, excellent results (recovery of the sample preparation and bias values) were obtained for all the matrices and with all mycotoxins evaluated in this study.

Selection of SPE Cartridge. An efficient SPE sample cleanup protocol should result in enrichment (concentration) of the target analyte while reducing matrix interferences. Four SPE cartridges were evaluated, namely, C_{18}, NH_2, SAX, and Oasis

HLB SPE cartridges. Loading the sample extract onto a preconditioned C_{18} cartridge should result in the retention of a significant amount of matrix components (lipophilic and hydrophobic substances) while the mycotoxins go through unretained. The objective of this protocol was to defat the sample matrices using C18 as adsorbent. Results obtained with the C_{18} cartridges were not satisfactory as it resulted in insufficient matrix retention. However, liquid—liquid extraction (LLE) using a mixture of dichloromethane/hexane (30:70, v/v) gave very good results as a lot more interfering substances (including lipids, proteins) were removed from the sample extract. Based on the above observation, LLE with dichloromethane/hexane was chosen and used for defatting of the sample extract.

Based on the color of the C_{18} eluate, it was decided that further cleanup was necessary. Thus two other SPE cartridges, namely, Oasis HLB and amino (NH_2) SPE cartridges, were also tested. NH_2 SPE cartridges just like the C_{18} SPE cartridges do not retain analytes when sample extract is loaded onto the preconditioned SPE cartridge. However, due to hydrophobic and possibly weak anionic interactions between the NH_2 SPE adsorbent and the analytes (mycotoxins), it was observed that the fumonisins and ochratoxin A were strongly retained onto the NH_2 SPE adsorbent together with significant amount of matrix components.

Sample cleanup with Oasis HLB cartridges resulted in a cleaner extract. Nevertheless, NH_2 SPE cartridges were preferred to Oasis HLB because, unlike the Oasis HLB, NH_2 SPE cartridges did not need a long conditioning protocol and large solvent volumes. However, to be able to analyze both fumonisin and ochratoxin A together with the other mycotoxins in a single analytical run and using NH_2 SPE cartridges as cleanup adsorbents, the sample extract was split up into two parts before sample cleanup. The aim of splitting the sample extract was to use one part of the split extract to further develop an alternative sample cleanup for ochratoxin A and the fumonisins. SAX SPE cartridges were tested as an alternative cleanup procedure for the fumonisins and ochratoxin A since these two analytes bind very strongly onto the SAX adsorbent while matrix components flow through with minimum retention. Improved sample cleanup was observed with the SAX SPE cartridges, but combining this alternative (SAX SPE) sample cleanup protocol with the already established NH_2 SPE cleanup procedure will result in a very tedious and complex sample preparation protocol. For this reason, sample cleanup with SAX cartridges was not further developed. Finally, filtration of one part of the split-up extract using a glass fiber filter was chosen and used as alternative sample cleanup for ochratoxin A and the fumonisins.

The NH_2 SPE column load was evaluated for efficient sample cleanup. Relatively small volumes (<3.5 mL) of the sample extract were needed for loading onto the NH_2 cartridges. This further reduced signal suppression because matrix components were better entrapped by the NH_2 cartridges. Further washing of the NH_2 cartridges with 1 mL of the reconstitution solvent improved the recovery for most of the mycotoxins without further elution of matrix components.

Matrix Effect. Matrix effect can be defined by the effect on an analytical method caused by all other components of the sample other than the specific compound of interest.[21] Coeluting matrix components may either enhance or suppress the ionization efficiency of the analytes and as a result could affect the reproducibility and accuracy of the results. Thus, matrix effects must be eliminated or compensated to obtain quantitative accurate results. Matrix effect was assessed by evaluating the

Table 2. LOD[a] and LOQ for the Different Mycotoxins and Matrices

compound	LOD (μg/kg)			LOQ(μg/kg)		
	maize	cassava	peanut cake	maize	cassava	peanut cake
nivalenol	35	31	25	106	93	76
deoxynivalenol	9	15	12	27	46	37
penicillic acid	12	8	8	36	25	24
neosolaniol	5	8	21	16	15	64
fusarenone-X	14	5	23	43	15	70
3-acetyldeoxynivalenol	5	0	3	16	1	9
15-acetyldeoxynivalenol	13	4	2	39	12	8
Aflatoxin G_2	1	2	0	4	6	1
Aflatoxin G_1	2	1	1	5	2	3
aflatoxin B_2	0.5	1	1	2	3	4
altenuene	1	1	1	3	2	2
aflatoxin B_1	4	0.3	2	12	0.8	6
diacetoxyscirpenol	1	0.4	0.1	2	1	0.4
alternariol	3	3	2	10	8	5
HT-2	13	9	11	39	28	34
fumonisin B_1	0.1	0.9	1	0.3	3	3
T-2	0.4	0.1	0.3	1	0.4	1
fumonisin B_3	33	20	13	100	60	40
ochratoxin A	0.1	0.3	0.1	0.3	0.8	0.3
fumonisin B_2	0.3	2	0.3	1	5	1
alternariol methyl ether	2	1	1	6	4	4
beauvericin	5	3	0.2	15	1	0.5
sterigmatocystin	2	3	1	5	9	2
roquefortine C	0.3	0.1	0.2	1	0.3	1
zearalenone	2	1	3	5	3	10
zearalanone	9	12	8	27	36	24
deepoxy-deoxynivalenol	10	15	12	10	45	36

[a] LOD = limit of detection; LOQ = limit of quantitation.

signal recovery for each analyte in each of the three matrices (maize peanut cake and cassava flour). Replicate blank samples and pure solvents spiked at three different concentration levels after cleanup were used to evaluate the signal recovery. Signal recovery was calculated by the formula: signal recovery = $(A_{matrix}/A_{standard}) \times 100$, where A_{matrix} and $A_{standard}$ are the peak areas of an analyte in a spiked sample extract and standard solution, respectively. The closer the value of the signal recovery is to one hundred percent, the lower the matrix effect and as a result the lower the signal suppression. Results are shown in Table 1.

Linearity. The linearity of the MS signal was excellent with p-values greater than 0.05, which demonstrate no lack of fit and thus good adequacy of the model in predicting linearity.

Accuracy. Trueness (expressed as bias) and precision were evaluated to assess accuracy. Bias and precision values (intraday precision (RSDr) and interday precision (RSDR)) for all mycotoxins were within the requirements as specified in the Commission Regulation (EC) No. 401/2006 of 23 February 2006.[22]

Limit of Detection and Quantification (LOD and LOQ). During the method development process, significant efforts were made to achieve LODs and LOQs lower than the official regulatory (maximum) limits as set by the European Commission. The

maximum levels of certain contaminants in some foodstuffs including peanuts and their processed products were established by the European Commission and published in Commission Regulation (EC) No. 1881/2006 of 28 September 2007.[23] Commission Regulation (EC) No. 1126/2007[24] amends Regulation (EC) No. 1881/2006 setting maximum levels for *Fusarium* toxins in maize and maize products. No regulatory limits have been set for cassava or cassava products. Table 2 gives the method LODs and LOQs for the different food matrices. The LODs were in the range of 0.1 to 15 μg/kg except for nivalenol, fusarenone-X and neosolaniol which were greater than 20 μg/kg. Thus, the method proved to be sensitive for all the analytes evaluated in this study. Based on the obtained LOQ values, the method allows us to assess, in a single analysis, the compliance of all the mycotoxins and matrices with the Commission Regulation (EC) No. 1881/2006 of 19 December 2006, except for aflatoxin B_1 in maize and peanut cake with LOQs of 12 and 6 respectively, which are higher than the maximum limits. However, this method is still sensitive enough to detect the presence of this analyte (aflatoxin B_1) at levels close to the maximum limits specified in Commission Regulation (EC) No. 1881/2006 of 19 December 2006 as shown through its LOD values (4 and 2 for maize and peanut cake respectively).

Specificity of the Analytical Method. Separation of mycotoxins by reversed phase LC followed by detection using an ESI interface and tandem MS, using two SRM (primary and secondary) transitions in the positive ion mode, increased the overall specificity of the analytical method. The ion intensities (ratio of the primary and secondary product transitions) of an analyte spiked in a blank sample extract were compared with those obtained for the same analyte spiked in a pure solvent. The relative ion intensities were within the maximum permitted tolerance limit as specified in the Commission Decision 2002/657/EC.[25]

Application of the Developed Method. The method developed was successfully applied to analyze samples (peanut cake, cassava flour and maize flour) obtained from markets in Benin. All the peanut cake samples tested positive for one or more mycotoxins. Aflatoxin B_1 ranged from <LOQ to 282 μg/kg, aflatoxin B_2 from <LOQ to 31 μg/kg, aflatoxin G_1 from <LOQ to 79 μg/kg and aflatoxin G_2 from 6 to 96 μg/kg (Table 3). <LOQ implies the toxins were detected at concentrations above their respective LODs but lower than the LOQs. Such high levels of aflatoxin contamination are consistent with those already reported in the literature. Awuah et al.[26] reported high levels of aflatoxins (5.7—22168 μg/kg) in market groundnut samples from Ghana, while Barro et al.[5] reported aflatoxin levels of up to 329 mg/kg in raw peanuts from Botswana. There is certainly no doubt that peanuts and their processed products pose a great threat to human health in sub-Saharan Africa.

The presence of such high levels of aflatoxins in these samples however indicates that the raw peanuts used in the processing of the cake were either already moldy or damaged or that contamination occurred after processing (storage under damp conditions). Furthermore, peanut cake samples originating from the warm subhumid (forest) region (samples 1—6) and the warm semiarid (samples 11—15) showed much higher levels of mycotoxin contamination than those obtained from the arid regions (samples 7—10). Five of the 15 peanut cake samples were ochratoxin positive with concentrations ranging from < LOQ to 2 μg/kg. Fumonisin B_1 was detected in one sample (sample 6) at a concentration of 80 μg/kg.

Table 3. Mycotoxin Contamination in Peanut Cake, Cassava Flour and Maize from Benin

| | Mycotoxin Contamination in Peanut Cake from Benin |
| | mycotoxin levels (μg/kg) |

sample	aflatoxin B_1	aflatoxin B_2	aflatoxin G_1	aflatoxin G_2	total aflatoxin	ochratoxin A
1	80	15	52	36	183	0.8
2	183	<LOQ[a]	<LOD	96	279	<LOQ
3	85	<LOQ	79	27	191	0.3
4	<LOQ	<LOQ	4	6	10	<LOD
5	136	<LOQ	40	14	190	<LOQ
6	282	31	15	18	346	2
7	18	<LOD	<LOQ	38	56	<LOD
8	21	<LOD	<LOQ	62	83	<LOD
9	64	<LOQ	<LOQ	58	122	<LOD
10	7	<LOD	<LOQ	51	58	<LOD
11	115	7	13	58	233	<LOD
12	86	<LOQ	17	42	145	0.4
13	152	6	45	58	261	<LOQ
14	62	<LOD	10	65	137	0.7
15	12	<LOD	5	16	33	<LOQ

| | Mycotoxin Contamination in Cassava Flour from Benin |
| | mycotoxin levels (μg/kg) |

sample	aflatoxin B_1	aflatoxin B_2	diacetoxyscirpenol	zearalenone	fumonisin B_1
1	<LOD	4	1	<LOD	4
2	9	<LOD	<LOD	6	8
3	<LOQ	8	9	12	5
4	8	<LOD	5	9	21

| | Mycotoxin Contamination in Maize from Benin |
| | mycotoxin levels (μg/kg) |

sample	fumonisin B_1	fumonisin B_2	fumonisin B_3	beauvericin
1	621	221	375	not detected
2	836	196	250	25
3	13	5	<LOQ	<LOQ
4	51	7	<LOQ	not detected

[a] <LOQ: mycotoxin detected at concentration below LOQ.

On the basis of the liver toxicity of aflatoxin B_1 in animals, the European Commission's Scientific Committee for Food (SCF) has recommended that it would be prudent to reduce exposure of aflatoxin B1 as much as possible, ensuring that exposures are less than 1 ng/kg bodyweight/day.[27] Peanut cake is a delicacy to school age (7−11 yr) African children. The higher exposure of these children to aflatoxins could lead to a greater probability of disease occurrence during a later stage of their lives.

Cassava flour samples were also found to be contaminated with mycotoxins though not at high levels compared to the peanut cake samples (see Table 3). Aflatoxin B_1 (<LOQ− 9 μg/kg), aflatoxin B_2 (<LOQ−8 μg/kg) and fumonisin B_1 (4−21 μg/kg) were detected in the cassava flour samples, a coexistence that is not often reported (to the best of our

knowledge) in the scientific literature. However, Abbas et al.[28] and Spanjer et al.[29] reported the coexistence of aflatoxin B_1 and fumonisin in maize and attributed it to a possible occurrence of heat stress during growth. Other mycotoxins detected in cassava flour were zearalenone (<LOQ−12 μg/kg) and diacetoxyscirpenol (<LOQ−7 μg/kg) and beauvericin (4 μg/kg).

Fumonisins (fumonisin B_1, B_2 and B_3) were detected in all four maize flour samples analyzed (Table 3). Beauvericin was detected in two of the four maize samples at concentrations of <LOQ and 25 μg/kg. Despite the small sample size, the fumonisin levels detected in these samples do agree with the high fumonisin levels which are often reported in maize from tropical and subtropical countries. Fandohan et al.[30] reported fumonisin levels in maize ranging from 8240 to 16690 mg/kg. A much larger survey is planned in order to be able to estimate the actual population exposure to this toxin. The results will be published in other scientific publications.

Comparison with Other Published Methods. The most challenging aspect during the method development was the optimization of extraction solvent. This was due to the diversity in polarity of the different mycotoxins and matrices evaluated in this study and also based on previous reports (by other authors) on the difficulty in obtaining satisfactory recoveries for some of the *Fusarium* toxins (fumonisin B_1, B_2, B_3 and zearalenone). To date, only Cavaliere et al.[31] reported recoveries greater than 80% for the fumonisins, trichothecenes and zearalenone from corn meal by using acetonitrile/water (75:25, v/v/v) as extraction solvent. Attempts to apply the same extraction solvent by other authors[32,21] proved futile as very low recoveries were obtained for *Fusarium* toxins in maize. Though Sulyolk et al.[20] finally found that acetonitrile/water/acetic acid (79:20:1, v/v/v) was a good compensation for the extraction of 39 mycotoxins for wheat and maize, recoveries for the fumonisins remained unsatisfactory (<60%). Lattanzio et al.[33] used a double extraction approach to extract multimycotoxins from maize samples. This technique consisted of phosphate buffer saline (PBS) as first extraction solvent followed by methanol/water (80:20, v/v) as second extraction solvent. The combined extract was further cleaned up using a multimycotoxin immunoaffinity column (IAC). Recoveries obtained with this double extraction protocol were greater than 79% for most of the mycotoxins; however, the presence of salts in the PBS solution makes such an extraction solvent incompatible with the LC−MS/MS method described in this work. The extraction solvent used in this study provided excellent recoveries for all the toxins and for all the matrices evaluated. The extraction yield of the entire sample preparation gave recoveries for maize at 113%, 90% and 104% for fumonisin B_1, B_2, B_3 respectively, thus overcoming the challenges previous authors had earlier reported.

Furthermore, better sensitivity (lower LODs) was obtained for the different mycotoxins when compared to other previously reported multimycotoxin LC−MS/MS methods for maize. Using a dilute and shoot approach Spanjer et al.[29] reported LODs of 100 μg/kg for fumonisin B_1 and fumonisin B_2 in maize; meanwhile for the method reported herein, LODs for fumonisin B_1 and fumonisin B_2 were 0.1 μg/kg and 0.33 μg/kg for fumonisin B_1 and fumonisin B_2 respectively. Furthermore, the method reported by Sulyolk et al.[20] also revealed LODs higher than those reported in this work for most of the mycotoxins evaluated. Immunoaffinity columns (IACs) concentrate sample extracts and minimize matrix effect and as such tend to be more selective than ordinary SPE cleanup cartridges. However, the

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LODs reported by Lattanzio et al.[33] were very similar to those reported in this work except for the aflatoxins. Thus, this present method revealed that sufficiently low LODs can be reached with the use of ordinary SPE cartridges as sample cleanup adsorbent. To date only Lanier et al.[34] have reported on an HPLC—MS method for mycotoxins in oilseed cakes (soya beans, sunflower seeds and rapeseeds). None has been described for peanut cakes.

The validated method reported herein can serve as an important tool for quantitative evaluation of intake (exposure assessment) of multiple mycotoxins in sub-Saharan Africa, a tool which is very scarce in this region. Moreover, during its 56th Meeting, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), however, stressed the importance of using validated analytical methods to ensure that results of surveys provide a reliable assessment of intake.[35]

■ ASSOCIATED CONTENT

ⓢ **Supporting Information.** Tables of optimized ESI+ MS/MS conditions, bias and linearity, and interday precision and intraday reproducibility data and figure depicting sample chromatograms of cassava flour, maize, and peanut cake samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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