Simultaneous Determination of 26 Mycotoxins in Sesame Butter Using Modified QuEChERS Coupled with UHPLC-ESI/Triple Quadrupole Mass Spectrometry

Application Note

Food

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Abstract

This application note describes a method for the quantitative determination of 26 mycotoxins in sesame butter based on the work published by Liu *et al*. [1]. The sample was initially extracted and cleaned up using a modified QuEChERS protocol developed in the lab, and the resultant solution was subjected to separation and detection using an Agilent 1290 Infinity LC System coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer. With the matrix-matched external standard calibration, the developed method showed a very good linear dynamic range with correlation coefficients of 0.995 or above. It detected the 26 mycotoxins at limits of quantitation (LOQ) mostly lower than the available maximum residue levels (MRLs) currently regulated by China, EU, and other regulation organizations. Spiking tests demonstrated that the majority of recoveries were within 60–120 %, with RSDs below 15 % for each analyte. The method developed is very sensitive, selective, accurate, and has high throughput. It can be applied to the target screening of mycotoxins in real samples.

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Introduction

A number of agricultural products, including raw materials and processed food products, are susceptible to contamination by mycotoxins, which, under favorable climate conditions, are mainly excreted by *Aspergillus*, *Penicillium*, *Fusarium* and many other fungi species. Many of these mycotoxins are highly toxic, and even carcinogenic [2,3]. Currently, mycotoxins have been regulated by many organizations, especially in main crops such as cereal, corn, milk, and edible oils [4]. Routine monitoring of mycotoxins in food products requires very sensitive and reliable methods.

High performance liquid chromatography coupled with mass spectrometry, especially tandem mass spectrometry (HPLC-MS/MS), has been applied in the determination of mycotoxins over the past decade by combining with immune affinity cleanup (IAC) or solid phase extraction (SPE). The more universal extraction and cleanup method, QuEChERS, has been expanded to the analysis of mycotoxins in some common food matrixes such as wheat, flour, cereals, wines, and so forth [5-7]. However, another more complicated food matrix, sesame butter, has not been as throughly analyzed. Sesame butter is produced mainly from sesame seeds and peanuts, and is susceptible to multiclass mycotoxins contamination, which may lead to an increased risk to human health. This butter is rich in fat, carbohydrates, protein, and natural pigments, therefore, is difficult to clean up. The aim of this study was to develop a reliable method by combing the universal QuEChERS method with ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) for the high-throughput determination of 26 common mycotoxins in sesame butter.

Experimental

Materials and reagents

The mycotoxin standard compounds: neosolaniol (NEO), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), aflatoxin M2 (AFM2), T2 toxin, HT-2 toxin, fumonisin B1 (FB1), sterigmatocystin (ST), verruculogen (VER), ochratoxin A (OTA), and zearalenone (ZEN) were purchased from Alexis Corporation; 4,5-diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), de-epoxy-deoxynivalenol (DOM-1), gliotoxin (GLT), fumagillin (FUM), fumonisin B2 (FB2), fumonisin B3 (FB3), mycophenolic acid (MPA), and paxilline (PAX) were purchased from Romer Lab. Some reagents such as methanol, acetonitrile, formic acid, ammonium acetate, and water were of HPLC grade. Other reagents were all of analytical grade and purchased from local venders. The customized QuEChERS extraction (EN15662 method) and the dispersing cleanup tubes were from Agilent Technologies.

Sample extraction and cleanup

The sample (2.5 g) was weighed into a 50-mL centrifuge tube and sequentially extracted for 30 minutes using two different solutions: 20 mL of 80 % acetonitrile aqueous solution containing 0.1 % formic acid, and 5 mL of 20 % acetonitrile aqueous solution containing 0.1 % formic acid. After each extraction, the vial was centrifuged, and the supernatant solution was collected. The two collected supernatant solutions were then mixed together and salted out using an Agilent QuEChERS extraction kit (p/n 5982-5650CH)with immediate vortexing for 1 minute, followed by centrifugation at 8,000 rpm for 5 minutes. The upper layer was then transferred to a clean vial, and extracted using 20 mL of hexane to remove the lipid. The analytes retained in the lower layer were transferred to an Agilent dSPE cleanup tube containing 150 mg of C18 and 900 mg of magnesium sulfate (p/n 5982-4956CH). The cloudy solution was vortexed for 1 minute and then centrifuged. The resulting supernatant was decanted into a clean dispersive tube, which was sequentially washed twice with 5 mL of acetonitrile. The flow through solution and the washing solution were then combined and dried at 40 °C using a rotary evaporator. Finally, the residue was sequentially dissolved in 1.5 mL of methanol and 1.0 mL of water. The resultant solution was passed through a 0.22-µm membrane for further analysis using UHPLC-MS/MS.

LC/MS conditions

Table 1. Instrument Conditions

LC conditions	
Instrument	Agilent 1290 Infinity LC System with built-in degasser
Autosampler	Agilent 1290 Infinity Autosampler with temperature control
Column temperature	Agilent 1290 Infinity Thermostatted Column Compartment
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Column temperature	40 °C
Mobile phase	A) 0.1 % formic acid (Pos.) and pure water (Neg.) B) 0.1 % formic acid in methanol (Pos.) and pure methanol (Neg.)
Flow rate	0.4 mL/min
Injection volume	3 µL
Post time	2 minutes
Gradient elution profile	0–8 minutes, % B increasing from 30 % to 35 %; 8–8.5 minutes, % B increasing from 35 % to 50 %; 8.5–15 % B increasing from 50 % to 80 % (Pos.); 0–3 minutes, % B maintained at 10 %; 3–3.1 minutes, % B increasing from 10 % to 15 %; 3.1–4.5 minutes, % B increasing from 15 % to 20 %; 4.5–8.0 minutes, % B increasing from 20 % to 30 %; 8.0–8.5 minutes, % B increasing from 30 % to 45 %; 8.5–14.0 minutes, % B increasing from 45 % to 90 % (Neg.)
ESI-MS/MS conditions	
Instrument	Agilent 6460 Triple Quadrupole LC/MS System with Agilent Jet Stream electrospray ionization source
Drying gas temperature	300 °C (+)/350 °C (–)
Drying gas flow rate	6 L/min(+)/10 L/min (–)
Nebulizer gas pressure	45 psi (±)
Sheath gas temperature	350 °C (±)
Sheath gas flow rate	12 L/min(+)/7 L/min (–)
Capillary voltage	3,500 V (±)
Nozzle voltage	0 (+)/2,000 V (–)
Scanning mode	Multiple reactions monitoring

Results and Discussion

Optimization of UHPLC-MS/MS conditions

Standard compounds were initially injected into the mass spectrometer to establish the MS acquisition parameters, see Table 2. The mycotoxins were split into two groups, one group was analyzed under positive ionization, and the other was analyzed in negative mode. As shown in Figure 1, under the optimized UHPLC conditions, both positive and negative groups of mycotoxins can be separated completely by baseline. Such separation avoided cross-analyte interference, and also reduced the interference effects from the matrix itself. Compared to a polarity switching method, in which compromised HPLC conditions (mobile phases, gradient profiles) must be applied, analysis of positive and negative groups separately can achieve higher sensitivity for these 26 mycotoxins.

Table 2. MRM Parameters for the Detection of 26 Mycotoxins

CPD	RT (min)	Precursor ion	Frag. volt. (V)	Quant ion (CE/V)	Qual ion (CE/V)	Ionization mode
NEO	1.07	400.2	70	214.8(10)	185(15)	Pos.
AFM2	2.34	331.1	140	285.1(21)	273.1(20)	Pos.
AFG2	3.03	331.2	160	313.1(23)	245.1(30)	Pos.
AFM1	3.33	329.1	140	273.1(23)	259.1(22)	Pos.
AFG1	3.96	329.2	150	311.1(20)	243.1(25)	Pos.
AFB2	5.07	315.2	160	285.1(22)	241.1(38)	Pos.
GLT	6.09	327.1	80	263.0(4)	245.0(12)	Pos.
AFB1	6.45	313.2	160	285.1(22)	241.1(38)	Pos.
DAS	7.17	384.2	75	307.1(5)	228.8(10)	Pos.
HT-2	9.97	447.2	135	345.2(14)	285.0(16)	Pos.
FB1	10.63	722.4	180	352.3(36)	334.3(42)	Pos.
T2	11.07	484.3	125	305.3(8)	215.2(14)	Pos.
FB3	11.58	706.5	120	354.0(30)	336.2(40)	Pos.
ST	11.92	325.0	150	310.0(20)	281.0(36)	Pos.
FB2	12.28	706.5	180	336.3(38)	318.3(40)	Pos.
FUM	13.68	459.2	75	215.0(17)	130.9(23)	Pos.
DON	2.60	295.2	90	265.2(4)	138.1(10)	Neg.
DOM-1	4.93	279.1	70	248.9(1)	230.9(3)	Neg.
FUS-X	5.22	353.2	80	263.1(4)	186.9(20)	Neg.
3-ADON	7.24	337.1	90	217.1(8)	173.1(2)	Neg.
15-ADON	7.43	337.1	100	188.9(8)	150.2(8)	Neg.
MPA	8.75	319.1	150	274.9(3)	242.8(22)	Neg.
ZEN	11.72	317.1	190	175.0(25)	130.8(33)	Neg.
OTA	12.36	402.1	120	358.0(12)	211.0(22)	Neg.
VER	12.54	510.2	110	306.2(10)	167.2(12)	Neg.
PAX	13.31	434.2	90	376.1(8)	358.1(12)	Neg.

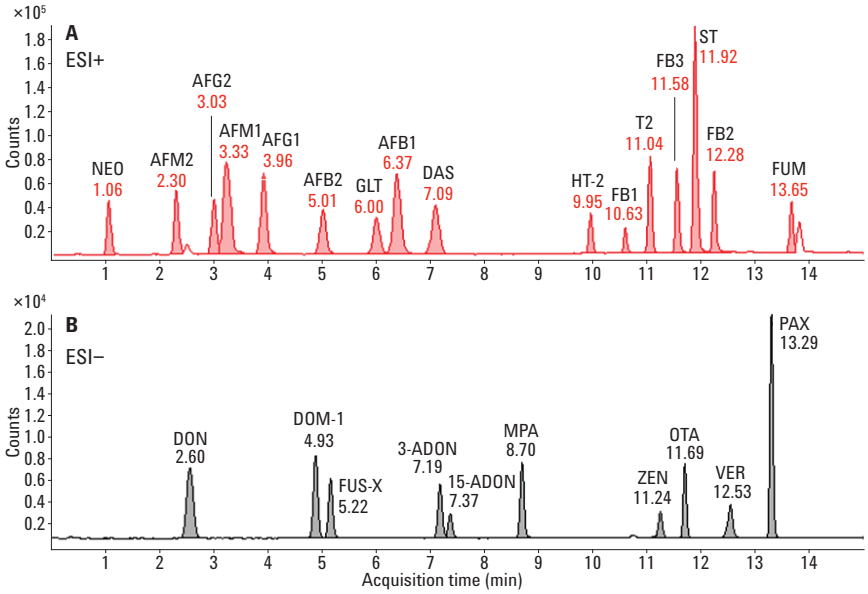

Figure 1. Separation of two groups of mycotoxins under optimized UHPLC-MS/MS conditions. Sixteen compounds were analyzed under positive mode (A), and the other 10 compounds were analyzed under negative ionization (B).

Selection of the QuEChERS extraction solvent

Based on previous reports [8,9], acidified 70 % and 90 % of acetonitrile aqueous solutions (solvents 1 and 2) were initially examined for extraction of mycotoxins from spiked samples. The extractant was directly subjected to UHPLC-MS/MS analysis without cleanup. As shown in Figure 2, the total number of compounds with recovery within 50–120 % were 13 and 14 using solvent 1 and solvent 2, respectively, which covers compounds including aflatoxins, DAS, T2, PAX, and so forth. However, the recoveries for the other compounds are beyond this range, particularly for HT-2, ST, GLT, and DON. This might be due to the wide variation in polarity of these compounds. Hence, to improve the extraction efficiency, a gradient extraction protocol was practiced, with initial extraction using 80 % ACN in water followed by 20 % ACN in water. Using solvents 3, 4, and 5, the number of compounds with recoveries in the range of 50–120 % are 15, 22, and 22, respectively. For solvent 5, more compounds show recoveries within 70–120 %. Therefore, a two-step gradient extraction using solvent 5 was selected for extraction.

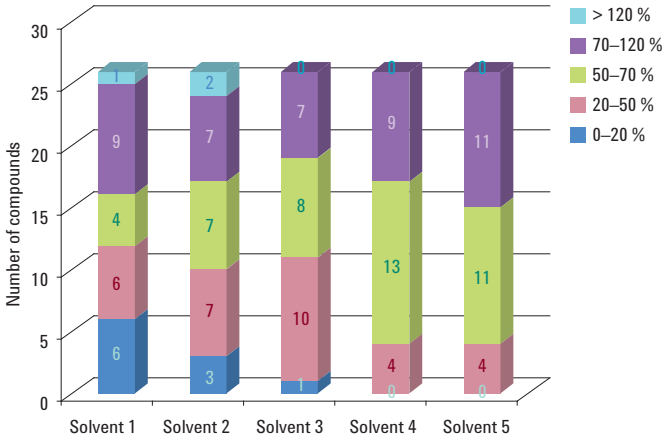

Figure 2. Number of compounds with recoveries in the specified range using five different solvents for extraction:
Solvent 1) 15 mL 70 % ACN-H_2O + 0.1 % FA
Solvent 2) 15 mL 90% ACN-H_2O + 0.1 % FA
Solvent 3) 10 mL 80 % ACN-H_2O+ 5 mL 20 % ACN-H_2O + 0.1 % FA
Solvent 4) 15 mL 80 % ACN-H_2O+ 5 mL 20 % ACN-H_2O + 0.1 % FA
Solvent 5) 20 mL 80 % ACN-H_2O+ 5 mL 20 % ACN-H_2O + 0.1 % FA

Selection of the QuEChERS cleaning sorbent

Based on the lipid-predominant nature of the samples, a degreasing step was applied before further cleanup. It was found that 20 mL of hexane with 10-minute vortexing provided efficient grease removal. Further cleanup was practiced using the Agilent SPE dispersive tube containing 150 mg of C18 and 900 mg of magnesium sulfate, as well as the same SPE dispersive tubes, with the addition of PSA and PSA/graphite carbon black (GCB) combinations. As shown in Figure 3, using the Agilent-customized C18 tube, 24 out of 26 compounds displayed recoveries within 50–120 % (measured values from 61.2 to 115.9 %), except for HT-2 and ST, which showed recoveries below 50 %. In comparison, addition of PSA or PSA/GCB dramatically reduced the recoveries for some compounds such as HT-2, FBs, ST, ZEN, OTA, VER, PAX, and so forth, and reasonable values (50–120 %) were achieved for approximately 45 % of total compounds (Figure 3). Thus, the Agilent-customized C18 SPE dispersive tubes were directly applied for sample cleanup after degreasing.

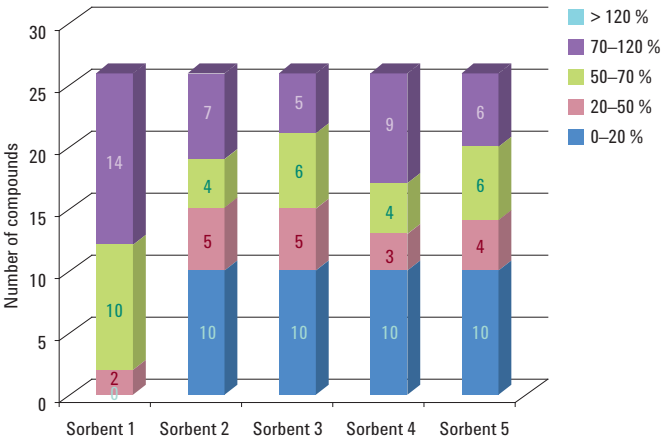

Figure 3. The number of compounds with recoveries in the specified ranges using five different sorbents for cleanup:
Sorbent 1) 150 mg C18
Sorbent 2) 150 mg C18 + 50 mg PSA
Sorbent 3) 150 mg C18 + 200 mg PSA
Sorbent 4) 150 mg C18 + 50 mg PSA + 20 mg GCB
Sorbent 5) 150 mg C18 + 200 mg PSA + 20 mg GCB

Matrix effects

Under the optimized QuEChERS procedure, we further examined whether matrix effects were significant and required a particular calibration method for quantitation. We compared the MRM responses of the standard compounds spiked in the blank matrix with those in the clean solvent. For those compounds in the positive ion method, the AFG2 signal was enhanced the most by the matrix, with an enhancement percentage of 50.4 %, followed by the other AFs (Figure 4). In addition, FB1 and FB3 were enhanced 8.5 % and 34.5 %, respectively. The other compounds detected in the positive ion method were suppressed by the matrix by a factor ranging from 3.5 % to 90.7 %. Nine out of ten compounds in the negative ion method were suppressed by 62.6 % or above, and only OTA was slightly suppressed (15.4 %) (Figure 4). Due to the unavailability of isotopically-labeled standard compounds, matrix-matched external standard calibration was applied to avoid quantitation bias.

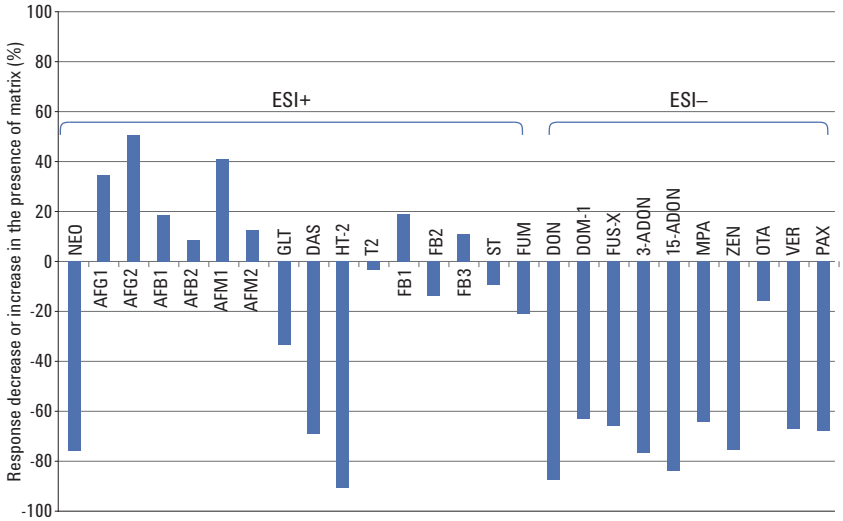

Figure 4. The change for MRM response in the presence of matrix compared to that in the standard solvent. The percentage increase is indicative of matrix enhancement while the decreasing percentage indicative of matrix suppression.

Method validation

Linearity

The calibration standard solutions were prepared in a blank matrix with mycotoxin concentrations at 0.5, 1, 2, 5, 10, 25, 50, 100, 200, 300, 400, and 500 ng/mL. Good linear relationships were achieved with linear regression coefficients (R^2) of 0.995 or higher over the examined concentration range, as shown in Table 3.

Table 3. The Limit of Detection (LOD), LOQ, and Calibration Equations of the Method

CPD	LOD (ng/mL) 3 S/N	LOQ (ng/mL) 10 S/N	Linear equations	R^2	Linear range (ng/mL)
NEO	1.28	3.83		0.9975	5–500
AFM2	0.15	0.46	Y = 380.831470x – 6148.332713	0.9994	0.5–50
AFG2	0.10	0.29	Y = 3770.076774x + 7619.686379	0.9987	0.5–50
AFM1	0.05	0.14	Y = 3389.314337x + 3604.677956	0.9992	0.5–50
AFG1	0.13	0.38	Y = 6783.627614x + 5300.422070	0.9979	0.5–50
AFB2	0.14	0.41	Y = 3327.160443x + 1200.109051	0.9983	0.5–50
GLT	3.95	11.9	Y = 5546.655580x + 4.116544	0.9991	25–500
AFB1	0.07	0.21	Y = 4916.135923x – 242.634875	0.9991	0.5–50
DAS	0.08	0.24	Y = 1108.0438858x – 8130.929299	0.9981	5–500
HT-2	1.66	4.97	Y = 2719.293653x + 41280.602530	0.9985	5–500
FB1	0.23	0.70	Y = 236.852106x – 3123.861876	0.9992	5–500
T2	0.10	0.31	Y = 2411.295594x + 70542.978660	0.9978	5–500
FB3	0.17	0.50	Y = 1174.535695x + 8046.769182	0.9994	5–500
ST	0.05	0.15	Y = 4470.085632x + 85183.182429	0.9984	5–500
FB2	0.17	0.50	Y = 4861.838810x + 80850.952376	0.9983	5–500
FUM	4.32	12.9	Y = 5608.519625x + 105839.123757	0.9995	25–500
DON	6.94	20.8	Y = 52.160185x – 819.847770	0.9992	25–500
DOM-1	1.28	3.85	Y = 373.373396x – 3825.938174	0.9992	25–500
FUS-X	2.80	8.39	Y = 128.820806x – 1564.337340	0.9970	25–500
3-ADON	2.33	7.00	Y = 179.599728x – 3510.676535	0.9948	25–500
15-ADON	7.25	21.7	Y = 30.224230x – 317.701780	0.9992	25–500
MPA	1.27	3.81	Y = 469.797341x – 3923.228345	0.9992	25–500
ZEN	1.03	3.10	Y = 414.474804x – 1603.879798	0.9971	10–500
OTA	0.25	0.74	Y = 1610.057794x + 483.939368	0.9987	1–50
VER	2.92	8.77	Y = 24.953195x – 215.639821	0.9990	25–500
PAX	0.06	0.11	Y = 863.998752x – 6907.946499	0.9991	0.5–50

LOD and LOQ

The LOD was calculated as 3-fold the S/N, based on the MRM chromatograms acquired at the lowest calibration level, and the LOQ was calculated as 10-fold the S/N or more, using the same chromatograms. As shown in Table 3, the LOQs of the samples ranged from 0.11 ng/mL (PAX) to 21.7 ng/mL (15-ADON), which corresponded to 0.11 and 21.7 ng/g in the samples, respectively. The method is sensitive enough for measuring trace amounts of mycotoxins in a food matrix as complex as sesame butter.

Recovery and precision

The matrix-matched external standard calibration method was used throughout this study. As a validation of its performance, three levels of mycotoxins were spiked into blank matrix, and each level contained three replicates. Aflatoxins were set at 2.0, 4.0, and 8.0 ng/g; all other classes of mycotoxins were spiked ranging from 100 to 400 ng/g. The spiked samples were subjected to extraction, degreasing, and cleanup before analysis using the UHPLC-MS/MS method. As shown in Figure 5, 95 % of the recovery values were within 60–120 %, except those of FB2 and PAX at two higher levels, and of ST at the lowest level. All RSDs were below 15 %, with the majority of values (96 % of the total) below 10 %. This suggests that the method is highly reliable.

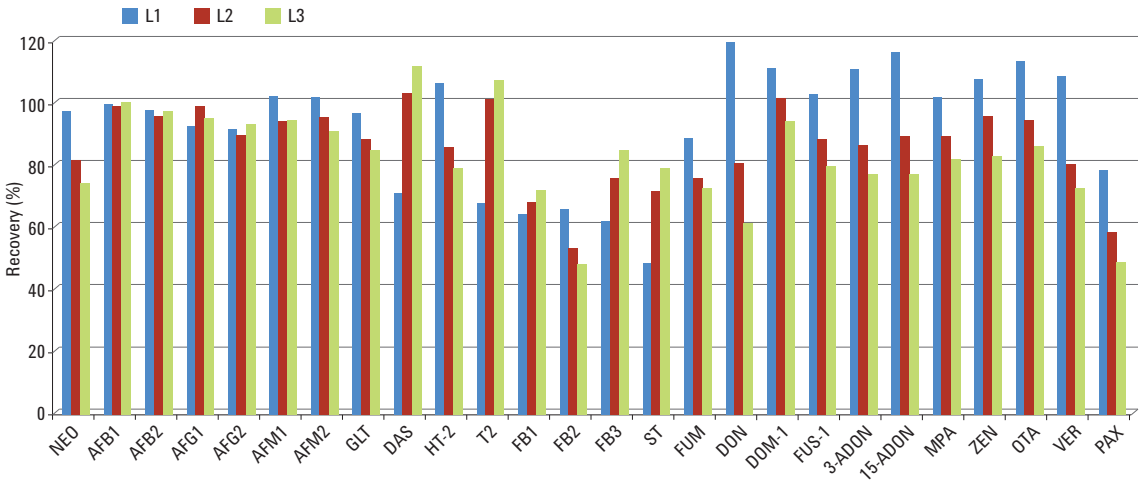

Figure 5. Recovery of mycotoxins spiked in blank matrix at three levels using the matrix-matched external calibration method for quantitation.

Method application

Using the developed method, 30 samples, including 20 sesame butters and 10 peanut butters randomly purchased from local grocery stores, were screened. In total, ten mycotoxins were found, including AFB1, AFB2, AFG1, AFG2, FB1, FB2, FB3, ST, DON, and OTA. The measured ranges for each detected mycotoxin are shown in Figure 6. The medium level and the number of positive samples are also labeled in Figure 6. Among the 20 sesame butters, all of the samples were found to contain FBs (FB1, FB2, and FB3) ranging from 6.8 to 29.3 ng/g; three samples were contaminated with ST ranging from 1.5 to 5.1 ng/g; two samples were found to contain DON and OTA at concentrations of 253.7 and 3.2 ng/g, respectively; four samples were contaminated with AFs (AFB1, AFB2, AFG1, and AFG2) with total concentrations in the range of 0.8 to 12.3 ng/g. Among the AFs, AFB1 showed a level obviously higher than the others (6.6 ng/g), and 25 % of the aflatoxin-contaminated samples showed levels higher than the MRL of EU regulations [7] (B1 < 2.0 ng/g, sum of AFs < 4.0 ng/g).

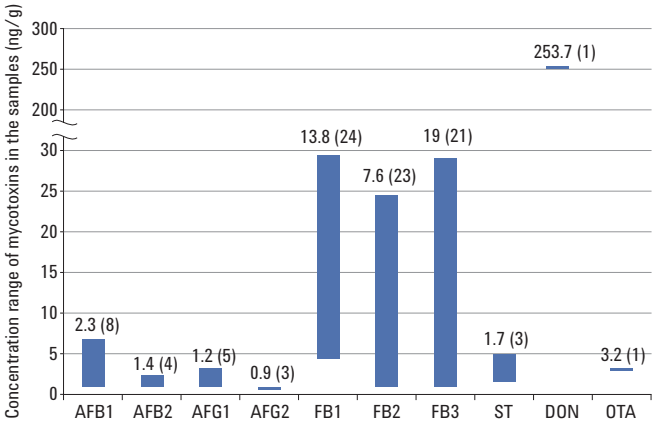

Figure 6. The concentration distribution of detected mycotoxins in the 30 real samples. The number on each bar represents the medium concentration and the number of positive samples among the total 30 samples. Note: samples under the LOD were not included in the calculation.

This method was also extended to peanut butter. It was found that the 10 peanut butter samples were all contaminated with AFs and FBs in the range of 2.4 to 4.6 ng/g and 6.9 to 20.1 ng/g, respectively. Other mycotoxins were under the LOD in peanut butter.

The screening results above demonstrated that AFs and FBs are common contaminants in sesame and peanut butters. However, these mycotoxins cannot be analyzed simultaneously by SPE or IAC pretreatment because of their dramatically different chemical structures and polarities. This method provides a universal extraction and purification method using QuEChERS, thus simplifying the analysis of multiple mycotoxins.

Conclusions

A modified universal QuEChERS method, in combination with an optimized UHPLC-MS/MS method for the quantitation of 26 common mycotoxins in sesame butter, was developed. The developed method showed LOQs mostly lower than the currently available lowest MRLs in most food matrixes. It has the advantages of high sensitivity, selectivity, accuracy, and throughput. It has been successfully applied in mycotoxins screening in sesame butter, and extended to similar food matrixes such as peanut butter.

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