Chromatography
Separation Techniques

Gong et al., J Chromat Separation Techniq 2012, 3:2
DOI: 10.4172/2157-7064.1000125

Research Article Open Access

Determination of 15 Mycotoxins in Foods and Feeds Using High Performance Liquid Chromatography-Tandem Mass Spectrometry with Gel Permeation Chromatography Combined QuEChERS Purification

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Abstract

A reliable, sensitive and selective method was successfully developed to determine 15 different mycotoxins simultaneously in foods and feeds. In this method, the homogenized sample was first treated with gel permeation chromatography (GPC) to eliminate most of coextracts, such as pigments, lipids and waxes. A quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was then carried out as a further cleaned up step, during which the polar interfering compounds such as organic acids and sugars were removed by vortexing with ODS in acetonitrile phase. Using double sample injection method, the analytes were separated by ZORBAX Eclipse plus C18 (1.8 μm, 2.1×100 mm) and detected by a MS/MS system with electrospray ionization (ESI) at multi-reaction monitoring (MRM) mode. The result indicated that the LOD of 15 mycotoxins were ranged from 0.07~5.0 μg/kg. Meanwhile, high correlation coefficients (r^2>0.996) of 15 mycotoxins were obtained within their respective linear ranges. The average recoveries for lower, intermediate, and high spiked levels ranged from 80.1%~95.5% in company with RSD ranged from 10.5%~19.6%. The method not only represents many advantages including simple pre-treatment, good purification and high sensitivity, but also successfully fit the minimum limiting level requests from various countries including EU, USA and Japan.

Keywords: HPLC-MS-MS; GPC; QuEChERS; Mycotoxins; Foods; Feeds

Introduction

Mycotoxins are secondary metabolites produced by molds that are capable of contaminating plant origin products such as crops, foods and feeds. Consumption of mycotoxins-contaminated diet may induce immunosuppression, mutagenicity, and cancer as well as negative effects on various organs and systems of the human body (FAO/IAEA, 1997). Additional symptoms of mycotoxins exposure include dermatitis, sore throat, headache, fatigue, and diarrhea. Hitherto, more than 200 kinds of mycotoxins have been found, and were categorized into *Aspergillus* mycotoxins (e.g. aflatoxin, ochratoxin), *Fusarium* mycotoxins (e.g. deoxynivalenol, zearalenone and T-2 toxin) and *Penicillium* mycotoxins (e.g. verruculogen) [1]. Recently, with the increase of restrictive mycotoxin categories in foods and the decrease of restrictive levels in foods and feeds defined by EU and other developed countries [2], development and validation of more robust methods for the determination of mycotoxins are urgently requested. Current analytical methods for mycotoxins include enzyme linked immunosorbent assay (ELISA) [3], gas chromatography (GC) and LC or GC combined with MS techniques [4-6], high-performance liquid chromatography (HPLC) [7-10]. Although all these methods can successfully determine mycotoxins, issues like false positive results, unfit for quantification of multi-component mycotoxins, and the level of sensitivity still need to overcome. LC/MS/MS has recently attracted increasing attention for the demands of sensitive and selective analyses detection in complex food matrices, biological and environmental sample [11,12].

A number of purification methods such as SPE, IAC were designed to determine mycotoxin residues. However, few if any of these methods can simultaneously achieve high-quality results for a wide range of mycotoxins. QuEChERS was a quick, easy, cheap, effective, rugged, and safe method for pesticide residue analysis [13]. The QuEChERS method has several advantages over most traditional methods of analysis in the following ways: high recoveries (>85%) are achieved for a wide polarity and volatility range of pesticides, including notoriously difficult analytes; high sample throughput of about 10-20 preweighed samples in about 30-40 min is possible; solvent usage and waste are very small, and no chlorinated solvents are used; very little lab ware is used; the method is quite rugged because extract cleanup is done to remove organic acids; the reagent costs in the method are very inexpensive. The weakness of QuEChERS was not very well in removing big molecules. Gel Permeation Chromatography (GPC) was a separation technique based on molecular size, so GPC cleanup could remove specified molecular sizes thoroughly. Especially big molecular QuEChERS used the sorbent to interact with chemicals by hydrogen bonding; to remove the matrix co extractives had no relation to molecular sizes. QuEChERS following GPC cleanup could make up each other and remove most of the matrix coextractives.

The aim of this study is to optimize and establish a reliable and rapid HPLC-MS/MS with gel permeation chromatography combined

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Received February 23, 2012; **Accepted** May 09, 2012; **Published** May 11, 2012

Citation: Gong X, Wang H, Zhang Y, Sun J, Dong J, et al. (2012) Determination of 15 Mycotoxins in Foods and Feeds Using High Performance Liquid Chromatography-Tandem Mass Spectrometry with Gel Permeation Chromatography Combined QuEChERS Purification. J Chromat Separation Techniq 3:125. doi:10.4172/2157-7064.1000125

QuEChERS purification method for the simultaneous analysis of 15 kinds of mycotoxins in foods and feeds.

Experiment

Chemical and reagents

The standards of aflatoxins (B1, B2, G1, G2) verruculogen and citrinin were purchased from Sigma-Aldrich (St. Louis, MO, USA). T-2 toxin, zearalenone, 3-Acetyldeoxynivalenol, fusarenone X, deoxynivalenol, nivalenol, 15-Acetyldeoxynivalenol and HT-2 toxin were purchased from Biopure (Tulln, Austria). Methanol (Fischer, Schwerte, Germany), acetonitrile (Tedia, Dayton, OH, USA) were used as mobile phase and extraction solvent. Ammonium acetate, aqueous ammonia and formic acid were of analytical grade. Milli-Q quality water (Millipore, Bedford, MA, USA) was used during the whole analysis.

Standard stock solution

Accurately weigh an appropriate amount of Aflatoxin B1, B2, G1, G2 and dissolve them with methanol to make standard stocks of 0.01 mg/ml. Accurately weigh an appropriate amount of citrinin, verruculogen, T-2 toxin, zearalenone, 3-Acetyldeoxynivalenol, fusarenone X, deoxynivalenol, nivalenol, 15-Acetyldeoxynivalenol and HT-2 toxin and dissolve them with methanol to prepare standard stock solutions of 0.1 mg/ml, stored below 0°C in dark.

Intermediate mixed standard solution

The concentration of the intermediate mixed standard is set up as follows: 0.2 μg/ml of Aflatoxin B1, B2, G1, G2 and 1.0 μg/ml of verruculogen, T-2 toxin, zearalenone, 3-Acetyldeoxynivalenol, fusarenone X, deoxynivalenol, nivalenol, 15-Acetyldeoxynivalenol and HT-2 toxin all stored below 0°C in dark.

Working mixed standard

The working mixed standard was prepared by transferring an appropriate amount of the intermediate mixed standard solution accurately into a mixture of methanol and ammonium acetate solution (50:50, v/v) (Prepare only when required). Aflatoxin B1, B2, G1, G2 were at the concentration of 0.5 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, verruculogen, T-2 toxin, zearalenone, 3-Acetyldeoxynivalenol, fusarenone X, deoxynivalenol, nivalenol, 15-Acetyldeoxynivalenol and HT-2 were at the concentration of 20 ng/ml, 40 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml. The standard curve were established according to these concentrations.

Sample preparation

As for sampling, approximately 10 g of each homogenized sample was stored into a 100 ml centrifuge tube. To perform the extraction process, 40 ml of 84% (v/v) acetonitrile aqueous solution was added and the sample was homogenized 3 min with IKA T25 high speed homogenizer. Samples were centrifuged at 10,000 r/min for ten minutes under 4°C. Then, 16 mL supernatant was evaporated to dryness under a stream of nitrogen at 50°C. The residue was redissolved by 8 ml of mixture of ethyl acetate/cyclohexane (50:50, v/v) and passed through a 0.45 μm nylon filter for GPC injection.

GPC cleanup

The recommended mobile phase for Envirobeads S-X3 column (30 mm×210 mm) was ethyl acetate/cyclohexane (50:50, v/v) and the flow rate was 4.7 mL/min. The sample was injected into the GPC column using 5 mL sample loops. The collection time was begin at 6 min and stop at 15 min. The collected eluent was evaporated to dryness using rotary vacuum evaporation on temperature less than 35°C. The residue was redissolved by 2.5 ml of acetonitrile and wait for further QuEChERS cleanup.

QuEChERS cleanup

To perform QuEChERS process, the extraction was first vortexed with 150 mg of octadecylsilane (ODS) for 1 min. Then, an aliquot of 2.0 ml supernatant was transferred into a test tube and dried by nitrogen at 50°C. The residue was redissolved by 1 ml of mixture of methanol/10 mmol/L ammonium acetate (1:1, v/v). Finally, the solution was passed through a 0.22 μm nylon filter and ready for HPLC injection.

HPLC conditions

The analytes were separated by a HPLC system (consisting of vacuum degasser, autosampler and a binary pump, Agilent Series 1200; Agilent Technologies, Santa Clara, CA, USA) equipped with a ZORBAX Eclipse Plus C18 (1.8 μm, 2.1×100 mm). Column temperature was maintained at 35°C. The injection volume was 20 μl. Gradient elution was used in LC step with a mobile phase containing of solvent A (10 mmol/L ammonium acetate used for the ESI^+ mode and 0.1% (v/v) aqueous ammonia used for the ESI^- mode) and solvent B (methanol) as follow rates: 0~2.0 min, 40% B~60% B; 2.0~7.0 min 60% B~95% B; 7.0~9.0 min, 95% B; 9.0~9.5 min, 95% B~40 % B. A subsequent re-equilibration time (2 min) should be performed before next injection.

Ms/Ms conditions

MS/MS was performed on a Agilent 6430 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI). Conditions were set up as follows: Scan mode: MRM; drying gas temp: 350°C; drying gas flow: 6 L/min nebulizer pressure: 210 Pa (30 psi); capillary: 4000 V. The parameters of precursor ion, product ion, fragmentor, collision energy, ionization mode of 15 mycotoxins were shown in (Table 1).

Results and Discussion

Optimization of HPLC conditions

Selection of mobile phase besides the optimization of separation efficiency in the chromatographic system, the choice of mobile phase should be concerned based on the consideration of ionization efficiency before the analytes enter the MS/MS system in order to obtain nice resolution and high sensitivity. Results from the MS full scan of 15 kinds of mycotoxins showed that aflatoxin B1, B2, G1, G2, T-2, HT-2, DAS, verruculogen, and ochratoxin A could generate corresponding $[M+H]^+$ ions under the ESI^+ electroscopy mode. Moreover, responses of $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ ions generated from all the mycotoxins were obviously higher than their $[M+H]^+$ ions with the combined mobile phase of water and methanol. However, responses of $[M+H]^+$ parent ions were greatly improved and high sensitivity was subsequently obtained when 10 mmol/L ammonium acetate added. Alternatively, zearalenone, 3-Acetyldeoxynivalenol, fusarenone X, deoxynivalenol, nivalenol, 15-Acetyldeoxynivalenol generated $[M-H]^-$ ions under the ESI^- electroscopy mode. Similarly, when 0.1% (v/v) of

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	Fragmentor (V)	Collision energy (eV)	Ionization mode
aflatoxin B1	313.1	285.1*,241.1	130	20,35	ESI^+
aflatoxin B2	315.1	287.1*,259.1	130	25,25	ESI^+
aflatoxin G1	329.1	243.1*,311.1	130	25,20	ESI^+
aflatoxin G2	331.1	285.1*,245.1	130	25,30	ESI^+
HT-2 Toxin	447.1	345*,285	100	15,25	ESI^+
T-2 Toxin	489.1	245*,387	100	30,20	ESI^+
DAS	384	247*,307	100	18,18	ESI^+
verruculotoxin	534.2	392.0*,191.1	145	13,20	ESI^+
ochratoxin A	404.1	239*,358	100	20,10	ESI^+
deoxynivalenol	295	265*,138	110	5,10	ESI^-
fusarenone-X	353.1	186.9*,263	135	15,10	ESI^-
3-acetyl deoxynivalenol	337	307*,217	90	5,20	ESI^-
15-acetyl deoxynivalenol	337	150*,219	80	10,5	ESI^-
zearalenone	316.9	174.9*,273	160	20,20	ESI^-
nivalenol	310.9	280.8*,205.2	100	5,5	ESI-

quantification ion

Table 1: Qualitative ion pair, quantitative ion pair, cone voltage, collision energy, ionization mode of 15 mycotoxins.

Compounds	Recoveres (%)				
	ODS	NH_2	Alumina-N	GCB	PSA
aflatoxin B1	91.8	54.2	99.1	<10.0	36.8
aflatoxin B2	102.2	60.6	87.3	<10.0	50.1
aflatoxin G1	103.1	68.4	21.8	<10.0	39.3
aflatoxin G2	104.2	89.2	31.3	<10.0	53.1
HT-2 Toxin	91.5	46.5	<10.0	68.8	50.7
T-2 Toxin	102.6	101.31	49.08	75.0	89.5
DAS	98.9	96.4	37.4	74.6	99.4
verruculogen	82.9	60.4	17.5	75.3	70.5
ochratoxin A	80.9	<10.0	<10.0	79.5	<10.0
deoxynivalenol	91.7	68.3	<10.0	60.5	46.2
fusarenone-X	104.3	63.1	<10.0	85.0	70.4
3-acetyl deoxynivalenol	99.4	86.3	<10.0	55.7	80.3
15-acetyl deoxynivalenol	116.4	100.9	<10.0	76.8	40.7
zearalenone	85.9	14.6	21.6	<10.0	<10.0
nivalenol	108.5	<10.0	13.5	<10.0	<10.0

Octadecylsilane (ODS), aminopropyl (NH_2), alumina-neutral (Alumina-N), graphitized carbon black (GCB), and primary secondery amine (PSA)

Table 2: Recoveres of 15 mycotoxins adsorbed by ODS, NH_2, Alumina-N, GCB, PSA.

Compound	Linear equation	Coefficent	LOD*/(μg/kg)	Spiked/(μg /kg)	Recovery/%	RSD/%
aflatoxin B1	Y=3349.32*X-1960.02	0.999	0.07	2, 10, 40	94.6, 93.4, 95.5	12.3, 10.5, 10.9
aflatoxin B2	Y=2257.18*X-1418.84	0.999	0.07	2, 10, 40	88.6, 89.5, 91.4	13.5, 12.8, 11.7
aflatoxin G1	Y=1810.28*X-1216.97	0.999	0.07	2, 10, 40	85.5, 85.9, 89.6	16.9, 12.8, 10.5
aflatoxin G2	Y=581.06*X-230.25	0.998	0.1	2, 10, 40	90.2, 91.2, 91.6	17.9, 18.5, 15.8
HT-2 Toxin	Y=71.83*X+357.60	0.999	0.8	10, 50, 200	90.2, 91.5, 91.9	18.4, 15.6, 14.9
T-2 Toxin	Y=297.72*X+2526.65	0.999	0.8	10, 50, 200	85.5, 83.2, 83.5	18.3, 18.5, 16.4
DAS	Y=84.20*X-211.11	0.997	0.8	10, 50, 200	80.5, 81.2, 82.9	15.8, 14.7, 12.8
verruculotoxin	Y=98.14*X+116.68	0.999	1.5	10, 50, 200	82.4, 83.1, 83.8	17.8, 16.5, 14.9
ochratoxin A	Y=56.48*X-0.14	0.998	1.0	10, 50, 200	89.2, 82.1, 83.5	15.4, 14.8, 10.8
deoxynivalenol	Y=241.32*X-67.41	0.999	2.0	10, 50, 200	83.8, 86.6, 87.5	14.8, 12.8, 11.7
fusarenone-X	Y=8.24*X-9.82	0.998	5.0	10, 50, 200	80.1, 84.6, 88.3	19.6, 18.2, 17.5
3-acetyl deoxynivalenol	Y=5.23*X+74.97	0.997	5.0	10, 50, 200	82.8, 86.4, 87.3	18.1, 17.9, 16.8
15-acetyl deoxynivalenol	Y=33.53*X+864.96	0.996	0.2	10, 50, 200	80.9, 82.3, 83.8	16.9, 15.4, 12.8
zearalenone	Y=237.16*X-70.35	0.999	0.3	10, 50, 200	81.8, 84.6, 88.3	18.5, 16.7, 14.9
nivalenol	Y=1097.96*X-5284.32	0.998	0.5	10, 50, 200	83.8, 86.6, 89.5	17.5, 16.2, 14.8

*S/N>3

Table 3: Linear equation, correlation coefficients (*r*2), LOD, mean recoveries and RSD of 15 mycotoxins in peanut butters (n=7).

aqueous ammonia was added into the combined mobile phase of water and methanol, the ESI^- ionization efficiency and sensitivity were also greatly improved. As for the choice of strong elution mobile phase, methanol and acetonitrile were considered as two candidates because most of mycotoxins are easily dissolved in these two solvents. Results indicated that when acetonitrile was chosen as the mobile phase, the ionization of all the selected analytes was significantly mitigated under both $[M+H]^+$ and $[M-H]^-$ electroscopy mode so that the abundance and sensitivity were thus reduced. Conversely, very positive results were observed when using methanol as the mobile phase. Therefore, methanol was selected as the strong elution mobile phase in the present study.

Selection of sample solvent medium before injection

The composition of sample solvent medium before injection directly affects the separation behavior of mycotoxins in the column and their ionization efficiency during MS detection. To select the optimal solvent medium, water, methanol, and different ratios of methanol/water, acetonitrile/water, methanol/ammonium acetate aqueous solution and acetonitrile/formic acid aqueous solution were regarded as the medium candidates. It has been demonstrated that the relative abundance of each analyte obviously increased when choosing the methanol/ammonium acetate aqueous solution (50/50, v/v) compared to other solvent mediums.

Optimization of -Ms/Ms conditions

Selection of parent ions: The stock solutions (1 μg/mL) of 15 mycotoxin standards were prepared with methanol/10 mmol/L ammonium acetate aqueous solution (50/50, v/v) and 0.1% aqueous ammonia/methanol (50/50, v/v). As for the selection of parent ions, the ionization mode (ESI^+/ESI^-) should first be decided according to the chemical ionization characteristics of mycotoxins. The parent ion *m/z* of each mycotoxin was subsequently confirmed by direct injection based on the optimization of MS/MS parameters and solvent medium. It was approved that 9 mycotoxins dissolved in methanol/10 mmol/L ammonium acetate aqueous solution (50/50, v/v) could form generate high responses under the ESI^+ mode while the other 6 analytes dissolved in 0.1% aqueous ammonia/methanol (50/50, v/v) could generate high responses under the ESI^- mode. The final selection of parent ions was summarized in Table 1.

Selection of daughter ions: Based on the confirmation of parent ions, more than two daughter ions should be selected when using a low resolution LC–MS analysis in accordance with relevant EU legislation [13]. Therefore, the optimization of daughter ions and their collision energy was performed under the daughter scan mode soon. The final selection of daughter ions and the optimal collision energy was also shown in Table 1.

Optimization of sample pretreatment

Selection of GPC cleanup conditions: Before running samples, the GPC pump flow rate was calibrated. The recommended flow rate for the Envirobeads S-X3 column was 4.7 mL/minute. The primary problem for a GPC method was to find a suitable collection time for interest compounds. Based on the principle of GPC, big molecular elute firstly and small molecular elute lately. We chose the elute time of citrinin whose molecular weight is about 250 as the collect stopping time, the elute time of moxidectin whose molecular weight is about 640 as the collet beginning time. The elute time of moxidectin ranged from 5.0 min to 10.0 min (Figure 1-3). The elute time of citrinin ranged from 7.5 min to 13 min (Figure 4). In order to ensure the precision of collection time, the elution was collected every one minute from 5 min to 20 min and monitored by HPLC-MS/MS. The result observed from HPLC-MS/MS was in accordance with that shown in the GPC detector. The period of 6 to 15 min was chosen as the elution time,

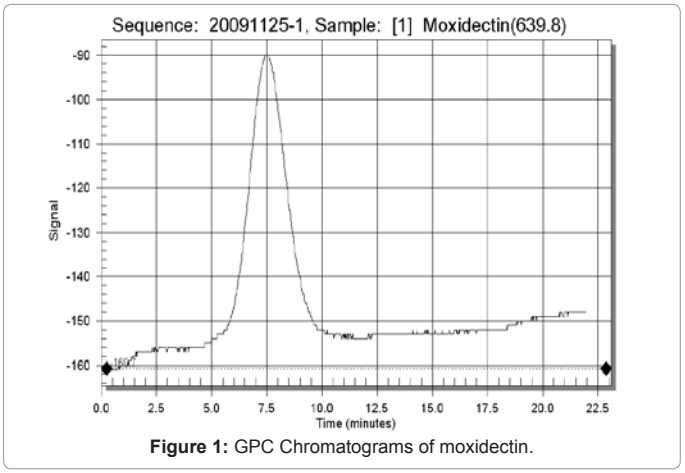

Figure 1: GPC Chromatograms of moxidectin.

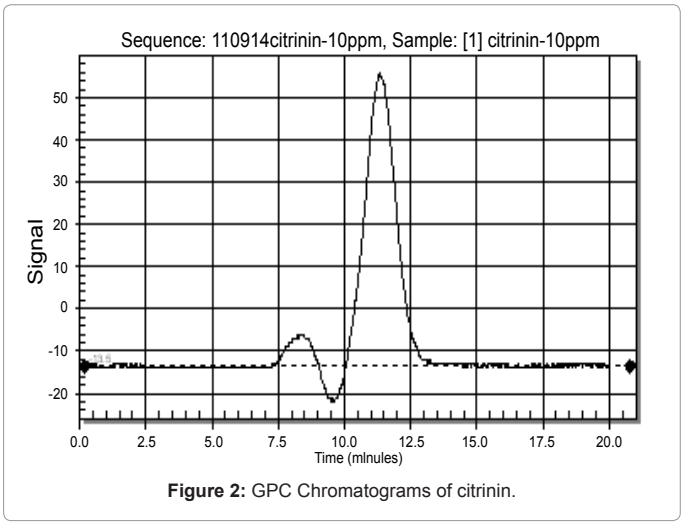

Figure 2: GPC Chromatograms of citrinin.

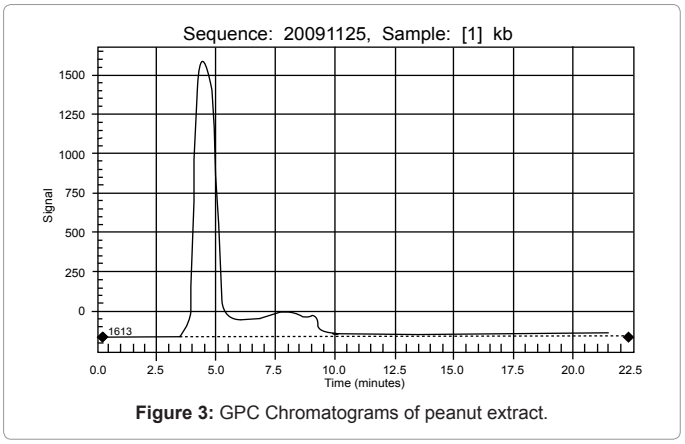

Figure 3: GPC Chromatograms of peanut extract.

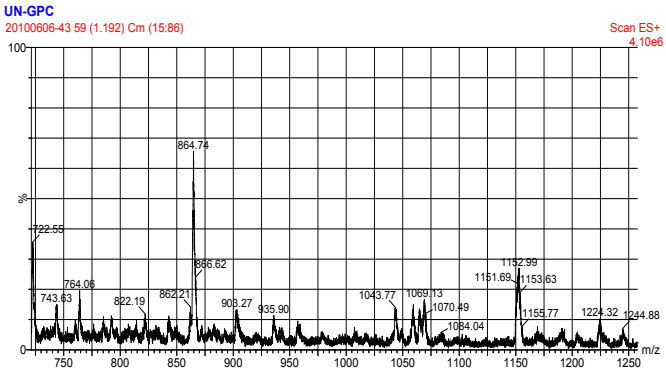

Figure 4: MS scan of peanut extract without GPC purification.

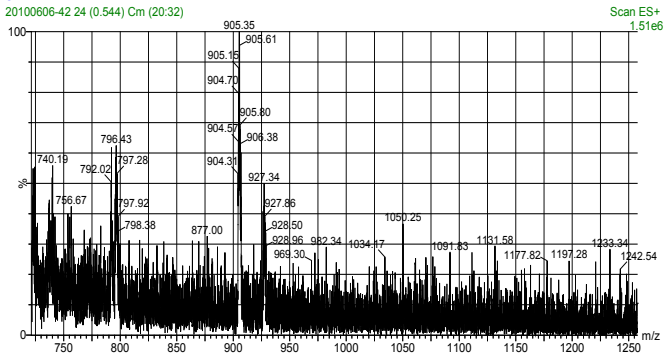

Figure 5: MS scan of peanut extract through GPC purification.

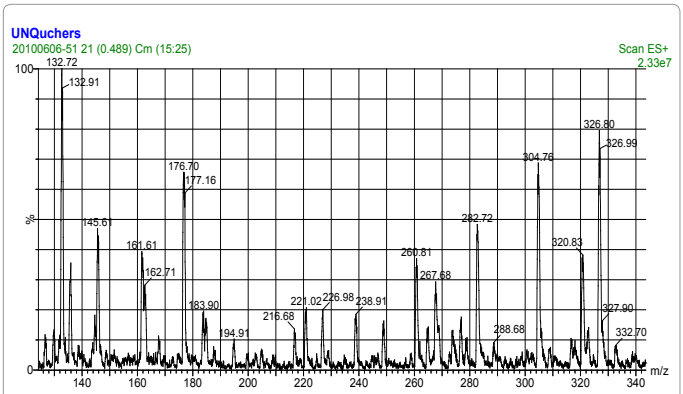

Figure 6: MS scan of peanut extract through GPC purification without QuEChERS cleanup.

which could guarantee all the 15 mycotoxins were recovered and the matrix coextractives were avoided.

Evaluation the efficiency of GPC cleanup: We used MS scan mode to detect ions for evaluating the efficiency of GPC cleanup. The parameter for MS scan was shown in Table 3. The spectrums (Figure 5-7) showed ions (m/z 722.55, 743.63, 764.06, 864.74, 1043.77, 1069.13, 1152.99, 1224.32) were thoroughly removed.

Selection of QuEChERS cleanup conditions: Two aspects need to be considered for the selection of sorbents, one is the removing efficiency of sorbent to matrix co-extractives and the other one is the recovery amount of sorbents to target compounds. Sorbents mainly used for QuEChERS cleanup including octadecyl silane (ODS), aminopropyl (NH_2), alumina-neutral (Alumina-N), graphitized carbon black (GCB), and primary secondary amine (PSA) [14,15]. In order to investigate the recovery amount of different sorbents to 15 mycotoxins, 150 mg different sorbents (such as ODS, NH_2, Alumina-N, GCB, PSA) were added to 2 mL (1 μg/ml) acetonitrile, vortexed for 1 min and held still for 3 min. Then, an aliquot of 1.0 ml supernatant was transferred into a test tube and dried by nitrogen gas at 50°C. The residue was redissolved by 1 ml of mixture of methanol and 10 mmol/L ammonium acetate (1:1, v/v), monitored by HPLC-MS-MS and recovery amount was calculated. The recovery amounts of ODS to 15 mycotoxins ranged from 80.9% to 116.4% (Table 2). So the ODS was chosen as the sorbent for QuEChERS cleanup.

Evaluation the efficiency of QuEChERS cleanup: MS scan mode was used to detect ions for evaluating the efficiency of QuEChERS cleanup. The parameter for MS scan was shown in Table 3. The spectrums shows that the disturbing ions whose molecular weight were close to the target mycotoxins were greatly removed (Figure 8,9).

Calibration and method validation: Nice linear relationships and good coefficients of determination (r2 > 0.996) were achieved over the concentration range of 0.5~400 ng/mL. The LOD of 15 mycotoxins ranged from 0.7~5.0 μg/kg. MRM Chromatograms of 15 mycotoxins standards that 4 aflatoxins at 0.02 μg/mL and the other 11 mycotoxins at 0.1 μg/mL and MRM Chromatograms of blank peanut spiked with 4 aflatoxins at 0.01 mg/kg and the other 11 mycotoxins at 0.05 mg/kg (Figure 8,9). The average recoveries at the low, intermediate, high spiked levels (n=7) ranged from 80.1%~95.5% and the relative standard deviations (RSD) ranged from 10.5%~19.6% (Table 3).

Determination of mycotoxins in samples: Twenty peanut butters purchased from different food manufacturers were sampled and analyzed by the validated method in the present study. Four main aflatoxins including aflatoxin (B1, B2, G1,G2) were detected in six samples. The highest level of residues was aflatoxin B1, and it was at a concentration of 15.2 g/kg.

Conclusion

The HPLC–MS/MS method was optimized to yield quickly separation, enhanced sensitivity and specificity for 15 mycotoxins.

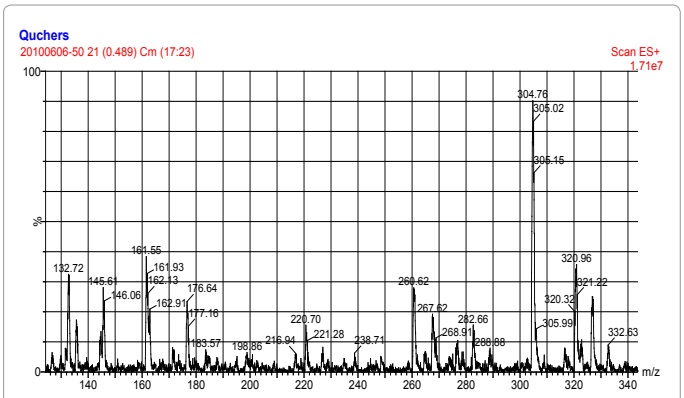

Figure 7: MS scan of peanut extract through GPC purification after QuEChERS cleanup.

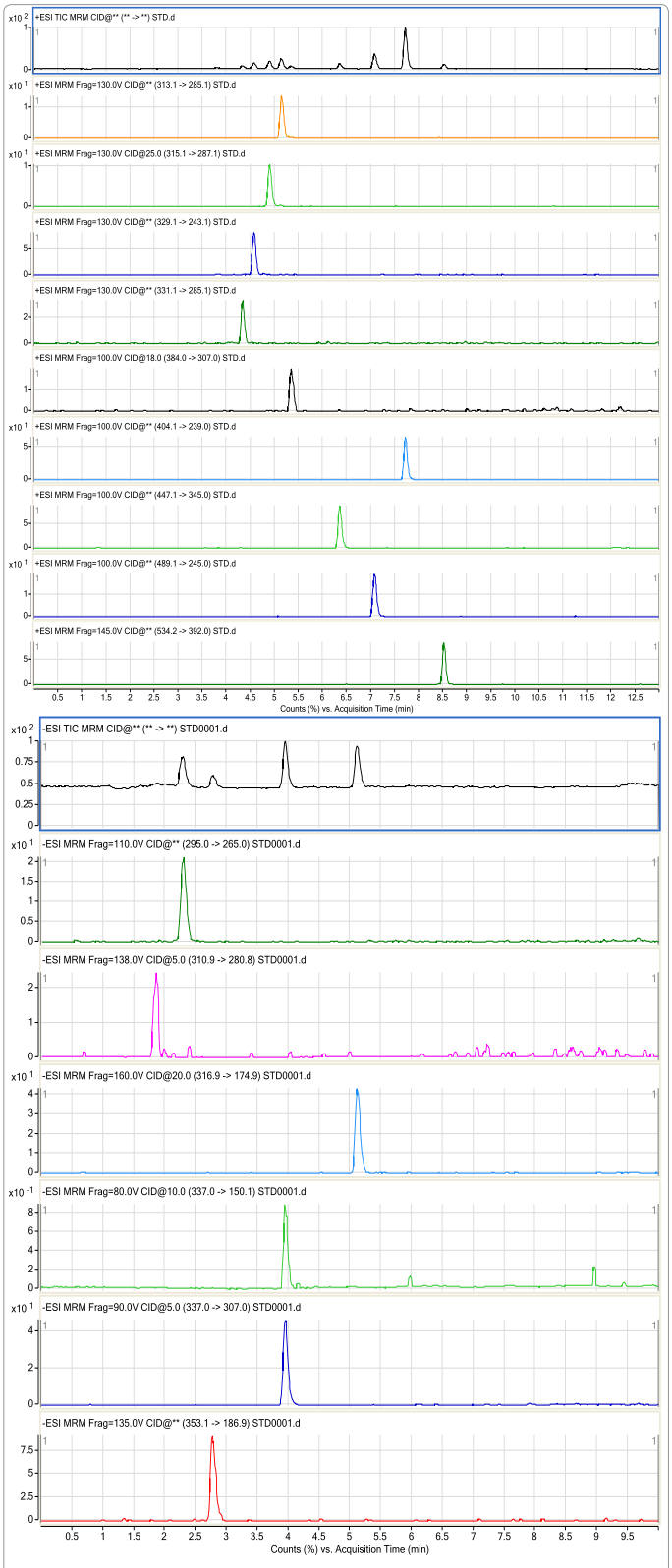

Figure 8: MRM Chromatograms of 15 mycotoxins standards (4 aflatoxins at 0.02 μg/mL and the other 11 mycotoxins at 0.1 μg/mL).

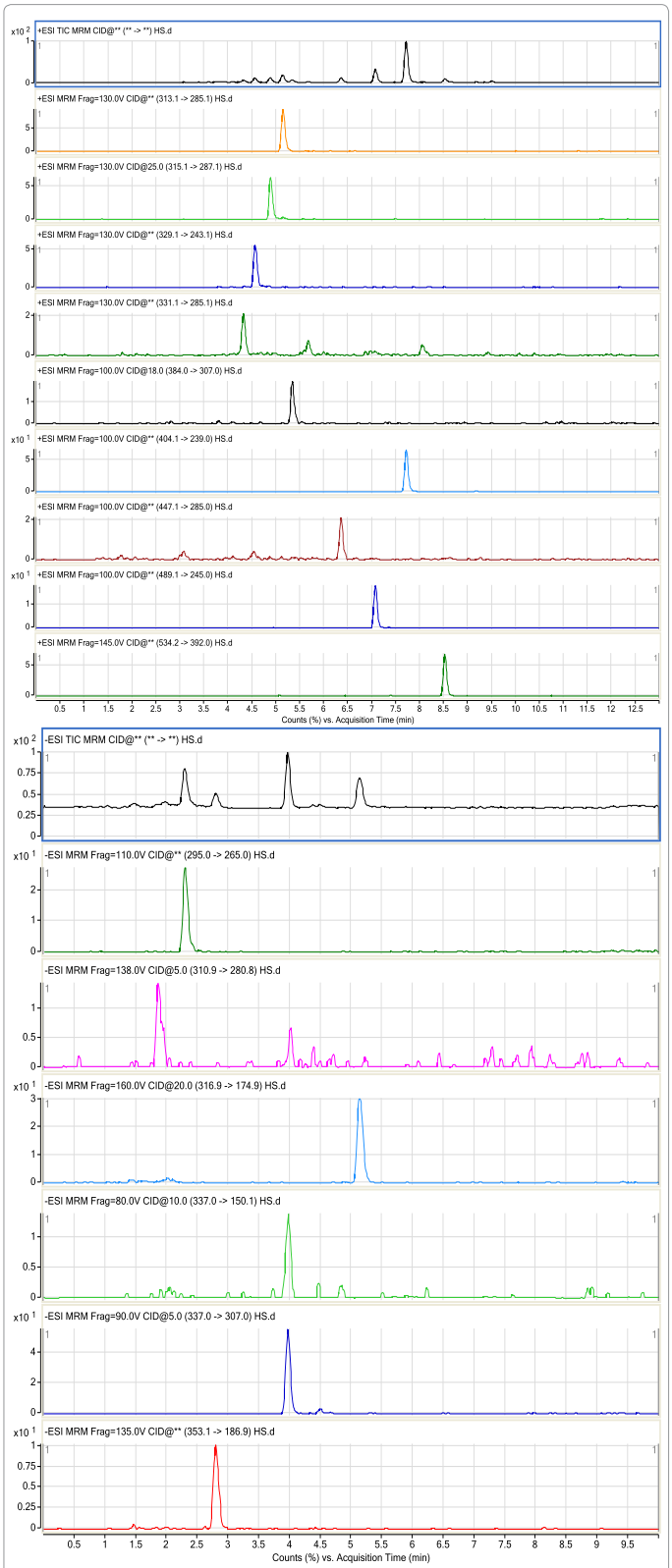

Figure 9: MRM Chromatograms of blank peanut spiked with 4 aflatoxins at 0.01 mg/kg and the other 11 mycotoxins at 0.05 mg/kg.

The use of GPC following QuEChERS cleanup could make up each other and remove most of the matrix coextractives. The method including instrumental method (HPLC-MS/MS) combined with purification methods (GPC and QuEChERS) had built up a platform for simultaneously quantification and determination of mycotoxins. The platform was reliable, sensitive, comprehensive and suitable for

the analysis of multi-component mycotoxin contaminants in complex sample matrixes. With a little modification, more mycotoxins could be easily bought into this platform.

Acknowledgements

We are gratefully for financial support from Shandong Entry-Exit Inspection and Quarantine Bureau.

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