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Determination of Mycotoxins Using Hollow Fiber Dispersive Liquid–Liquid–Microextraction (HF-DLLME) Prior to High-Performance Liquid Chromatography – Tandem Mass Spectrometry (HPLC - MS/MS)

Ali Mohamed Ali Alsharif[a,b], Yeun-Mun Choo[a], Guan Huat Tan[a], and Lukman Bola Abdulra'uf[c]

[a]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur , Malaysia; [b]Arab Centre for Desertification and Development of Saharian Societies, Murzuk, Libya; [c]Department of Chemistry, College of Pure and Applied Sciences, Kwara State University, Malete, Nigeria

ABSTRACT

A modified hollow-fiber-supported dispersive liquid-liquid microextraction (HF-DLLME) method was developed for the determination of aflatoxins and ochratoxin A in food samples. The various parameters affecting the efficiency of extraction, such as pH, salt addition, extraction time, stirring rate, desorption time, type and volume of extractant and disperser solvents were carefully studied and optimized using two step strategies. The linearity of the evaluated results was 0.1 to 30 μg L^{-1} for aflatoxins and 0.1 to 20 μg L^{-1} for ochratoxin A, with regression coefficients (R^2) exceeding 0.9990. The precision was satisfactory with relative standard deviation values less than 11%. The method accuracy was within the recommended range from 70% to 120% and analyte accuracy between 83% and 101%. The limits of detection and quantification were in the range from 0.04 to 0.06 μg L^{-1} and 0.08 to 0.13 μg L^{-1}, respectively, for multi-aflatoxins, and 0.02 to 0.04 µg L^{-1} and 0.08 to 0.10 µg L^{-1}, respectively, for ochratoxin A. The developed method was successfully applied for the determination of mycotoxins in food samples.

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Introduction

Aflatoxins and ochratoxins are naturally occurring toxins which cause considerable health problems in humans and animals. Aflatoxins are produced by *Aspergillus flavus and A. parasiticus* and the less common *A. nominus* (Bhat, Rai, and Karim 2010). Ochratoxin A is produced by several types of fungi (*Penicillium Verrucosum* and *Penicillium Nordicum*, *Aspergillus ochraceus* and *Aspergillus Niger*) (Nguyen et al. 2007; Duarte, Pena, and Lino 2010; Skarkova et al. 2013). Aflatoxin is classified as class I human carcinogen (Portier et al. 2014) and ochratoxin A is a group 2B carcinogenic compound (Bui-Klimke and Wu 2015).

CONTACT Yeun-Mun Choo ymchoo@um.edu.my Chemistry Department, University of Malaya, Kuala Lumpur, Malaysia

Supplemental data for this article can be accessed here.

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In view of the potential mycotoxin health risks to human and animal through the consumption of contaminated agricultural products, several organizations such as the European Commission, United State Food and Drug Administration, and Food and Agriculture Organization have set the maximum permissible level to protect the health of consumers from the dangers of continuous exposure to these substances (Food and Agriculture Organization 2004; European Commission 2006; Anater et al. 2016; Leprêtre and Merten-Lentz 2018). There is a need to develop a sensitive and reliable analytical procedure for the determination of mycotoxins in food, in order to ensure compliance with various regulations on the maximum permissible limits, taking into consideration the physicochemical properties of the toxins and food samples (Wong and Lewis 2017).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) provides high sensitivity, selectivity and accuracy with the ability of simultaneous determination of multiple target analytes in a single run (Liao et al. 2011; Beltrán et al. 2013; Li et al. 2018). The determination of mycotoxins in food samples by using liquid chromatography electrospray ionization triple quadruple mass spectrometry (LC-ESI-QQQ-MS) has its challenges due to the presence of high level interfering compounds that suppress the ionization process (Hall et al. 2012). This challenge can be overcome by having a suitable sample preparation procedure (Demirhan, Kara, and Demirhan 2017).

Various mycotoxins extraction methods were reported for food samples such as solid–liquid extraction (SLE) (Rubert et al. 2013), liquid–liquid extraction (LLE) (Jank et al. 2015), solid phase extraction (SPE) (Shar et al. 2015), ultrasonic-assisted extraction (UAE) (Yang et al. 2017), Quick, Easy, Cheap, Effective, Rugged and Safe extraction (QuEChERS) (Annunziata et al. 2017; Zhou et al. 2018) and immunoaffinity columns (IACs) (Wilcox et al. 2015; Iha et al. 2017). As a result of the limitations inherent in some sample preparation methods, it has become necessary to develop more environmentally friendly and efficient extraction methods.

Liquid phase microextraction (LPME) by using a hollow fiber membrane was used in the isolation of different contaminants in liquid samples (Sharifi, Abbasi, and Nosrati 2016; Alsharif et al. 2017). The advantages of this method over other extraction methods include cleaner isolate which resulted in higher detection sensitivity (Alsharif et al. 2017). Hollow fiber liquid phase microextraction (HF-LPME) was successfully applied with a very long extraction time (more than 2 h) for the determination of ochratoxin A in wine (González-Peñas et al. 2004) and ochratoxin A and T-2 toxin in wine and beer (Romero-González et al. 2010), as well as for the detection of aflatoxin M1 in milk (Huang et al. 2015) and multiple aflatoxins in edible oil (Huang et al. 2017).

Even though the extraction time of dispersive liquid-liquid microextraction method (DLLME) was shortened, the traditional DLLME method was not adequate for the isolation of the analytes in complicated food samples (Sarafraz-Yazdi and Amiri 2010; Prosen 2014; Simão et al. 2016). The hollow fiber dispersive liquid–liquid microextraction (HF-DLLME) method presented better results compared to DLLME and/or HF-LPME. Hence, the combination of both methods is expected to introduce advantages and minimize limitation (Simão et al. 2016).

Comparison of the extraction time, precision and recovery for HF-LPME and DLLME methods performed by Xiong and Hu concluded that DLLME had a higher

capacity and faster overall extraction time, while HF-LPME was simpler to employ and more robust especially for complex samples (Xiong and Hu 2008). HF-DLLME was successfully applied for the extraction of pesticides from grape juice (Silva et al. 2015), aflatoxins in soya juice (Simão et al. 2016) and atrazine and triclosan in river water and sewage (Letseka and George 2017).

In the present study, a modified HF-LPME method supported by DLLME was developed and validated for the isolation of mycotoxins. The conditions were optimized by using a mixed strategy of univariate and multivariate methods. The hollow fiber surface contact area with the donor phase was increased and a hollow fiber membrane in a U-shape was used. We observed that adding mixture of extraction and dispersion solvents enhanced the method performance. The modified design boasted better method efficiency with shorter extraction time compared to previously reported HF-LPME mycotoxin protocols. The method was successfully applied for the determination of mycotoxins in commercial fruit juices.

Experimental

Reagents and materials

Aflatoxins were mixed in methanol at 1 mg L^{-1} for aflatoxins B1 and G1 and 0.3 mg L^{-1} for aflatoxins B2 and G2 and ochratoxin A at 10 μg mL^{-1} in acetonitrile from Sigma-Aldrich (Darmstadt, Germany). HPLC grade formic acid and ammonium formate for mass spectrometery (≥99.0%) were employed. The 1-octanol, 1-decanol, dihexyl ether and 2-hexyl-1-decanol were purchased from Sigma–Aldrich (Darmstadt, Germany). Acetonitrile and methanol (LC-MS grade) were used in liquid chromatography, and the dispersive solvent (HPLC grade chloroform) was purchased from Merck (Darmstadt, Germany). Ultra-pure water from a Milli-Q water purification system (MA, USA) was used throughout this study. The working standard solution was prepared by diluting the stock solution in methanol.

Apparatus and equipment

Q3/2 Accurel PP hydrophobic polypropylene hollow fiber tubing (200 µm wall thickness, 600 µm i.d., and 0.2 µm pore size) was obtained from Membrana GmbH (Wuppertal, Germany). A hot plate with a magnetic stirrer and a stir bar were from Fisher Scientific. An ultrasonic bath (Branson 3200) was obtained from Branson Ultrasonics and a benchtop pH meter from Mettler Toledo.

Liquid chromatography tandem mass spectrometry

Liquid chromatography was performed on a 1290 series instrument (Agilent Technologies, Waldbronn, Germany). Separation of mycotoxins was achieved on a reversed-phase C18 column (ODS) (15 × 2.1 mm^2; 5 µm) at 30 °C using an injection volume of 4 µL. The mobile phase consisted of (A) 5 mM ammonium formate in water with pH adjustment by formic acid and (B) methanol. The LC gradient program involved 0 min, (A) 40% and (B) 60% at a flow rate of 0.195 mL min^{-1}; 2 min, (A) 75%

and (B) 25% at a flow rate of 0.195 mL min^{-1}; 4–6 min, (A) 0% and (B) 100% at a flow rate of 0.250 mL min^{-1}; and 6–8 min, (A) 10% and (B) 90% at a flow rate of 0.200 mL min^{-1}. The system was re-equilibrated with the initial composition for 5 min prior to the next injection.

Mass spectrometry was performed on a 6490 QqQ system (Agilent Technologies-Singapore) equipped with an Agilent jet-stream electrospray ionization (ESI) interface. Data acquisition, processing, and instrument control were performed using the Mass Hunter Workstation B.06.01 software. The mass spectra were acquired from the mass range of 100 to 1000 *m/z*. The ionization source was operated in positive mode at following conditions: gas flow of 13.65 L min^{-1} at a temperature of 170.20 °C, nebulizer gas pressure of 20 psi, and sheath gas flow of 11 L min^{-1} at a temperature of 250 °C.

Food samples

The food samples used in this study included commercial packaged apple, orange, grape and pomegranate juice which were purchased from Kuala Lumpur markets.

U-shaped hollow fiber dispersive liquid–liquid–microextraction for ochratoxin A

The polypropylene hollow fiber membrane was cut into 6 cm length pieces. To avoid contamination, each hollow fiber was sonicated in acetone (10 min) and dried with an air flow. Each piece was used only once.

The hollow fiber was first soaked in octanol (supported liquid membrane solvent) for 10 s to fill the pores and then washed with ultra-pure water (10 s) to remove the extra octanol. Both ends of the hollow fiber (6 cm) were connected to a microsyringe needle and filled with ~16 µL of octanol (extraction/acceptor phase solvent). The extraction phase was then injected to fill the channel and pores of the hollow fiber, followed by introducing the aqueous sample (12 mL) to the U-shaped configuration in a closed vial. This aqueous sample consisted of 10 µg L^{-1} of ochratoxin A at a pH of 2.66 and contained 7% NaCl.

Prior to introducing the hollow fiber membrane to the donor phase, 200 µL of the extraction/dispersion solvent were added and mixed quickly with the donor phase. Ochratoxin A was extracted for 70 min at a stirring speed of 700 rpm. At the end of extraction period, the hollow fiber membrane was sonicated in 4 mL of acetonitrile to desorb the analyte from membrane and acceptor phase. The extraction phase was subsequently evaporated under vacuum by rotary evaporator to dryness and reconstituted in 1 mL of the mobile phase for LC-MS/MS analysis.

U-shape hollow fiber dispersive liquid–liquid–microextraction for aflatoxins detection

The method is similar to the ochratoxin A method except for changes in the following parameters. The donor phase was prepared at a pH value at 8 and contained 2% NaCl. Prior to introducing the hollow fiber membrane into the donor phase, 150 µL of the

Table 1. Factors and levels used in the central composite design for the optimization of the extraction conditions.

	Levels			Star points	
Factors	Low (−1)	Central (0)	High (+1)	−α	+α
pH	3.0	5.5	8.0	0.5	10.5
Time (min)	40	70	100	10	130
Stirring rate (rpm)	400	700	1000	100	1300
NaCl (%)	4	6	8	2	10

extraction/dispersion solvent were added and mixed quickly with the donor phase. The aflatoxins were extracted for 50 min at 700 rpm.

Univariate optimization of U-shape hollow fiber dispersive liquid–liquid–microextraction

The variables assessed by using univariate method included the type of organic solvents (SLM), the length of the membrane, the pH of donor phase, the percentage of NaCl, the extraction time, the agitation speed, the dispersion/extraction solvent on a volume basis, and the desorption time. Excel 365 was used for the statistical analysis. The experiments were carried out using samples which contained 10 μg L^{-1} ochratoxin A, 5 μg L^{-1} of aflatoxins B1 and G1, and 1.5 μg L^{-1} aflatoxins G2 and B2 in pure water

Multivariate optimization of U-shape HF-DLLME

Second-order central composite design (CCD) with response surface methodology (RSM) was used to optimize four extraction variables for ochratoxin A, including the pH of the donor phase, the percentage of NaCl, extraction time, and agitation speed. To minimize the effect of the uncontrolled variables, the central composite design experiments were run in a random manner. The central composite design included 16 cube points, 4 center points in a cube, 8 axial points and 2 center points in axial with α equal to 2 and a total of 30 randomized runs with two blocks. The selected variables involved in the generation of central composite design and levels are shown in Table 1. Design generation and statistical analysis were performed using Minitab Release 17 Statistical Software (State College, PA).

Method validation

Ucontaminated juice samples that were analyzed to ensure that they were free of mycotoxins were extracted by the proposed method without the mycotoxin spike and injected in the liquid chromatography tandem mass spectrometry instrument (Supporting Information Figure S1). To evaluate the linearity of method for each of the analytes, matrix-matched calibration curves were constructed in the matrices of a variety of samples. The limit of detection was estimated to correspond to a factor of three times the signal-to-noise ratio, while the limit of quantitation was estimated to be ten times the signal-to-noise ratio. The relative recovery (RR) was obtained by:

$$\mathbf{RR} = \frac{\mathbf{C}}{\mathbf{B}} \times \mathbf{100} \quad (1)$$

where *B* is the average peak area obtained after the extraction from a spiked standard and *C* is the average peak area obtained from a spiked standard prior to the extraction (Gjelstad and Pedersen-Bjergaard 2013).

The enrichment factor (EF) was determined by (Gjelstad and Pedersen-Bjergaard 2013):

$$\mathrm{EF} = \frac{\mathbf{Vs}*\mathbf{RR}}{\mathbf{100}*\mathbf{Va}} \quad (2)$$

where RR is the method recovery, Vs is the sample volume, and Va is the acceptor solution volume.

The effect of sample matrices (ME) on the ionization efficiency was determined by (Hall et al. 2012; Pizzutti et al. 2014):

$$\mathrm{ME} = \frac{\mathbf{B}-\mathbf{A}}{\mathbf{A}} \times \mathbf{100} \quad (3)$$

where *A* is the average peak area obtained from the neat standard. This equation identifies ion suppression when the value is negative and ion enhancement when the value is positive. An average matrix effect >20% was deemed to be significant.

Results and discussion

Univariate optimization

The influence of the organic solvent, fiber length, stirring rate, extraction time, volume and pH of the donor phase, the salting-out effect and matrix effect were evaluated to determine optimum parameters for the U-Shaped-HF-DLLME method. The HPLC-MS/MS mean peak area was used as the experimental response.

Organic solvent properties such as volatility and the viscosity affected the extraction efficiency directly. 1-Octanol,1-decanol, dihyxail ether, and 2-hyxail-1-decanol were employed for the extraction of aflatoxins and ochratoxin A. The extractions were carried out in a U-shaped hollow fiber for 30 min from 12 mL aqueous solution containing 2 ppb of aflatoxins at a pH of 7. The stirring speed was maintained at 700 rpm and 1% NaCl was added. Similar procedures were performed for ochratoxin A at 10 µg L^{-1} in pure water. The results indicated that 1-octanol gave the best extraction efficiency for aflatoxins and ochratoxin A. Low water solubility and vapor pressure and the presence of long hydrocarbon chain and hydroxyl group in 1-octanol showed this solvent was the most suitable for the extraction of polar compounds.

Four lengths (4 cm, 5 cm, 6 cm, and 7 cm) of hollow fiber were investigated in this study. The results indicated that the best extraction were achieved using the 6 cm hollow fiber.

Stirring of the sample solution facilitates mass-transfer and improves the extraction efficiency. The effect of stirring rates of 350 rpm, 700 rpm, 1000 rpm, and 1200 rpm were evaluated in this work. The results showed that best stirring speed for extraction aflatoxins was at 700 rpm. Stirring below 700 rpm may be insufficient to reduce the

Table 2. Optimized conditions for U-Shaped-HF-DLLME.

Factors	Univariate optimization		Multivariate optimization
	Aflatoxins	Ochratoxin A	Ochratoxin A
Supported liquid membrane	1-octanol	1-octanol	n.a.
Fiber length (cm)	6	6	n.a.
pH	8	2.6	2.6
Salt (%)	2	7.1	7.1
Extraction time (min)	50	60	70
Agitation speed (rpm)	700	700	700
Extraction/dispersion ratio	1:4	1:4	n.a.
Extraction/dispersion volume (μL)	150	200	n.a.
Desorption time (min)	15	20	n.a.

n.a. – not available.

thickness of diffusion layer effectively, while stirring above 700 rpm leads to vibration of the hollow fiber and resulted in loss of solvent in the hollow fiber pores.

The extraction time profile was evaluated at various intervalsin the range from 30 min to 120 min. The results suggested the optimum extraction time was 50 min with a decrease in extraction efficiency from 60 min onwards.

A donor phase pH range from 3 to 9, adjusted with hydrochloric acid and sodium hydroxide, was investigated in this study. The results indicated that the best donor phase pH was 8.

Seven percentages of NaCl from 0 to 6% were added to the samples to evaluate the influence of salt upon the extraction efficiency. The results indicated that increasing the percentage of salt from 0% to 2% resulted in enhanced efficiency, but the efficiency was reduced when the percentage of salt was higher than 2%.

The experiments also indicated the best extraction/dispersion ratio was 1:4 (chloroform: acetonitrile). The optimum volume was 150 μL for aflatoxins and 200 μL for ochratoxin A. The desorption time was investigated in the range from 1 to 25 min by using an ultrasonic cleaner at 30 °C. The results showed the optimum desorption occurred at 15 and 20 min for aflatoxins and ochratoxin A, respectively. The overall optimized conditions are summarized in Table 2.

Multivariate optimization

The extraction time, agitation speed, salt addition percentage, and pH were further optimized using second-order central composite design with response surface methodology. The number of points in the central composite design were factorial run of 2k, axial runs of 2k and center point runs. The total experimental runs (N) of central composite design was given by $N = 2k + 2k + Co$, where k and Co are the numbers of variables and center points, respectively (Stalikas et al. 2009). The mean peak area of ochratoxin A was used as the experimental relative response. The fit quality of the polynomial model was expressed by the coefficient of determination (R^2) with values exceeding 0.8 (Saguy and Graf 1990; Hair et al. 2016). In the present study, the analysis of variance (ANOVA) results revealed R^2 equal to 0.93 with $p < 0.05$ for the linear and square terms, and no significant interactions between the investigated factors.

The response surface plots of the ochratoxin A relative response with two variables were kept constant and the other two varying within the determined experimental

ranges are showed in Supporting Information Figures S3–S8. The response surface optimization graph shows the best conditions were obtained (0.9725 optimal desirability) using the multivariate method.

The pH of the donor phase played a significant role in the extraction of the analyte. Ochratoxin A is a weak acid with two pK_a values, that is, 4.4 for the carboxyl group of the phenylalanine and 7.05–7.3 for the phenolic hydroxyl group. Because ochratoxin A is a weak acid, the donor phase should be sufficiently acidic to change the analyte to its neutral form and decrease its solubility in this phase at pH values lower than the pK_a of the analyte.

In this study, pH values from 0.5 to 10.5 were evaluated. The results indicated that the highest peak area was obtained with pH of donor phase very close to 2 suggesting that the donor phase has a significant influence on the mass transfer under these conditions. The extraction efficiency was increased with the stirring rate, which was attributed to convection enhancement. However, application of higher stirring speed may cause a decrease in the contact area between the aqueous donor phase and hollow fiber as a result of the formation of fine bubbles on the surface of the hollow fiber (Zargar, Parham, and Hatamie 2015). In the present study, a stirring speed of 700 rpm was found to give the best results.

The extraction efficiency of ochratoxin A is enhanced by the addition of a NaCl salt due to the salting-out effect. It was observed that increasing the NaCl percentage to 7% (w/v) improved the extraction efficiency of ochratoxin A. NaCl levels higher or lower than 7% have been shown to negatively affect the analyte partitioning between the phases (Alsharif et al. 2017).

Lastly, the optimum extraction time was established to be 70 min in this study. The overall optimized conditions are summarized in Table 2.

Method validations

Under the optimized conditions, the performance of U-Shaped hollow fiber dispersive liquid-liquid microextraction (U-Shaped-HF-DLLME) method was evaluated for fruit juice with analysis by HPLC-MS/MS. The matrix-matched calibration curves for aflatoxins B1, B2, G1, and G2 and ochratoxin A were constructed by using seven standard solutions at concentrations from 0.1 to 30 $\mu g\ L^{-1}$ for the former and 0.1 to 20 $\mu g\ L^{-1}$ for the latter. The method accuracy, precision, and matrix effect were investigated using non-contaminated food sample spiked at three concentration levels (Tables 3 and 4).

Linear regression lines were obtained with coefficients of determination higher than 0.9981 in the range between the limit of quantitation and 30 $\mu g\ L^{-1}$ for aflatoxins and 20 $\mu g\ L^{-1}$ for ochratoxin A. Limits of detection and limits of quantitation were calculated to be 3 and 10 times the signal-to-noise ratios, respectively. The limits of detection were between 0.04 and 0.06 $\mu g\ L^{-1}$ were achieved for aflatoxins and 0.02–0.04 $\mu g\ L^{-1}$ for ochratoxin A. The limits of quantification were from 0.08 to 0.13 and 0.08 to 0.10 $\mu g\ L^{-1}$ for aflatoxins and ochratoxin A, respectively. The method revealed suitable enrichment factors from 723.98 to 765.52 for the extraction and purification of multi-aflatoxins and 699.07 to 748.05 for ochratoxin A from liquid food samples.

Table 3. Analytical figures of merit for aflatoxins.

	Apple Juice				Grape Juice			
Aflatoxin	B1	B2	G1	G2	B1	B2	G1	G2
R^2	0.9992	0.9991	0.9990	0.9990	0.9993	0.9991	0.9993	0.9991
Limit of detection	0.04	0.05	0.06	0.06	0.04	0.06	0.06	0.05
Limit of quantification	0.08	0.08	0.13	0.09	0.08	0.09	0.1	0.09
Enrichment factor	745.12	737.48	744.6	723.98	744	730.2	765.52	726.38
Matrix effect	0.12	0.03	0.34	−1.23	0.16	−0.13	0.1	0.04
Relative standard deviation	4.74	1.05	2.9	4.77	1.54	4.75	3.89	1.33
Concentration at 1 μg/L								
Relative recovery	98.73 ± 2.34	96.53 ± 3.00	97.33 ± 3.34	91.33 ± 4.30	97.11 ± 2.70	95.12 ± 3.30	98.12 ± 6.76	94.63 ± 6.97
Intra-day precision	2.38	3.11	3.44	4.71	2.79	3.47	6.89	7.37
Inter-day precision	2.42	3.78	4.33	6.74	2.92	4.77	6.38	8.65
Concentration at 10 μg/L								
Relative recovery	100.23 ± 1.17	98.19 ± 0.99	100.87 ± 2.11	98.08 ± 2.63	99.39 ± 3.52	97.92 ± 1.36	107.39 ± 4.06	98.30 ± 5.12
Intra-day precision	1.17	1.01	2.10	2.69	3.55	1.39	3.78	5.21
Inter-day precision	3.55	2.48	3.91	2.76	3.47	3.91	4.20	6.97
Concentration at 30 μg/L								
Relative recovery	99.11 ± 1.55	100.27 ± 1.40	99.66 ± 3.43	100.19 ± 5.82	101.10 ± 1.11	99.05 ± 4.66	100.72 ± 4.18	98.3 ± 5.12
Intra-day precision	1.57	1.39	3.45	5.81	1.10	4.71	4.16	5.21
Inter-day precision	1.92	4.67	2.47	7.13	2.22	4.83	5.33	6.97

Table 4. Analytical figures of merit for ochratoxin A.

	Water	Apple juice	Grape juice	Orange juice	Pomegranate juice
R^2	0.9993	0.9991	0.9990	0.9986	0.9981
Limit of detection	0.02	0.04	0.04	0.04	0.04
Limit of quantification	0.08	0.09	0.09	0.09	0.10
Enrichment factor	748.5	738.6	732.97	711.6	699.07
Matrix effect	0.17	−0.03	−0.07	−0.03	−0.12
Relative standard deviation	2.13	1.05	4.75	5.25	10.35
Concentration at 1 μg/L					
Relative recovery	99.85 ± 1.88	83.18 ± 7.63	89.35 ± 11.41	91.6 ± 6.35	85.58 ± 3.18
Intra-day precision	1.09	9.01	12.78	6.94	3.72
Inter-day precision	2.84	8.52	5.44	4.45	11.87
Concentration at 10 μg/L					
Relative recovery	99.80 ± 0.95	98.48 ± 4.23	97.73 ± 4.30	94.88 ± 1.23	93.21 ± 6.01
Intra-day precision	0.96	4.30	4.40	1.30	6.45
Inter-day precision	1.22	10.93	9.52	5.64	8.95
Concentration at 20 μg/L					
Relative recovery	99.6 ± 2.40	98.44 ± 2.96	96.89 ± 8.08	97.53 ± 4.04	95.54 ± 10.29
Intra-day precision	2.41	3.01	8.34	4.15	10.78
Inter-day precision	1.08	2.80	7.15	14.36	3.54

The method accuracy was determined by comparing the measured concentrations of aflatoxins and ochratoxin A spiked in non-contaminated food samples with the known values. The results shown in Tables 3 and 4 were within the recovery criteria for method development from 80% to 120% (SANTE-11813 2017) with values between 91.33% and 101.10% and 83.18% and 99.80%, for aflatoxins and ochratoxin A, respectively.

The method repeatability values were in the range from 1.01% to 7.37% for intra-day precision and 1.92% to 8.65% for inter-day precision for aflatoxins, and 0.96% to 12.78% for intra-day precision and 1.08 to 14.36% for inter-day precision for ochratoxin A. Supporting Information Figure S2 showed the multireaction monitoring (MRM) chromatogram for the aflatoxins and ochratoxin A using the optimum conditions for spiked apple juice sample.

The comparison of the response from matrix-matched standards and solvent standards indicated no influence by the sample matrix (Tables 3 and 4), as no ion suppression or enhancement were observed. Moreover, these results reinforce the results from previous reports suggesting the present method is able to minimize the impact of matrix effects (Kataoka 2010; Tan and Abdulra'uf 2012; Alsharif et al. 2017). The validation for developed method demonstrated good precision and accuracy for the determination of aflatoxins and ochratoxin A in liquid food samples. The method performance also satisfies the requirements established by the European Union (EU) legislation (European Commission 2006).

Method efficiency

A comparison was made between the results of this study with a method used for the determination of aflatoxins in soya juice (Simão et al. 2016) (Table 5). The present study had a shorter extraction time (50 min) compared to the reported method (60 min). This improvement may be attributed to the increase in the surface area of the membrane. Also, the present method precision was better than the literature value, with a value less than 10%.

Table 5. Comparison of the reported method with literature protocols.

Method	Mycotoxin	pH	Salt percentage	Extraction time (min)	Limit of quantification	Relative standard deviation (%)	Relative recovery (%)	Reference
U-Shaped-HF- DLLME	Aflatoxins	8	2	50	0.08–0.20 μg L^{-1}	<10	90.13–107.39	This study
U-Shaped-HF- DLLME	Ochratoxin A	2.6	7	60	0.09–0.80 μg L^{-1}	<13	83.18–98.48	This study
Rode-like-HF-DLLME	Aflatoxins	n.a.	2	60	0.03–0.10 μg L^{-1}	12–18	83–117	Simão et al. 2016
Rode-like-HF- LPME	Ochratoxin A	2	10	240	0.02–0.09 μg L^{-1}	12–21	>70	Romero-González et al. 2010
Rode-like-HF-LPME	Ochratoxin A	1.05	n.a.	120	0.25 μg L^{-1}	<10	77	González-Peñas et al. 2004

n.a. – not available.

The improvement in method efficiency may be explained by more favorable extraction kinetics due to the U-shaped design as compared to a rod-like shape (Alsharif et al. 2017). In the case of ochratoxin A analysis, the present method also gave a better extraction time compared to the published method (Table 5). The use of the U-shape with added extraction/dispersion solvent improved the efficiency of the developed method. The overall results of the present method demonstrated an improvement to the previously reported methods (González-Peñas et al. 2004; Romero-González et al. 2010).

Food analysis

The validated U-Shaped-HF-DLLME method for monitoring of aflatoxins and ochratoxin A was successfully applied to commercial apple, grape, orange, and pomegranate juice purchased from Kuala Lumpur markets. Each sample was analyzed in triplicate. The results indicated that all of the juice samples were free of the aflatoxins and ochratoxin A.

Conclusion

A reliable and efficient U-Shaped-HF-DLLME for liquid food samples was developed for the determination of multiple aflatoxins and ochratoxin A in liquid food samples prior to analysis by HPLC-MS/MS. The method provided suitable limits of detection and quantification in the range from 0.04 to 0.06 $\mu g\ L^{-1}$ and 0.08 to 0.13 $\mu g\ L^{-1}$, respectively, for multiple aflatoxins, and from 0.02 to 0.04 $\mu g\ L^{-1}$ and 0.08 to 0.10 $\mu g\ L^{-1}$, respectively, for ochratoxin A. The method accuracy defined by the relative recovery was between 91.33% and 101.10% with relative standard deviations less than 10% for aflatoxins, and 83.18% to 99.80% with relative standard deviations lower than 11% for ochratoxin A. These values meet the standards of the Commission Regulation, which specify relative recovery values from 70 to 120%. The developed method had fulfilled the method performance criteria of the Commission Regulation (EC) No. 401/2006 and SANCO 2013. This method provides an alternative procedure to estimate aflatoxins and ochratoxin A in food.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Ali Mohamed Ali Alsharif http://orcid.org/0000-0001-5214-8494
Yeun-Mun Choo http://orcid.org/0000-0001-9891-5898

Lukman Bola Abdulra'uf http://orcid.org/0000-0002-1702-1049

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