VALIDATION OF MULTIRESIDUE MYCOTOXIN ANALYSIS IN COCOA SEMI-FINISHED PRODUCTS

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ABSTRACT – *An efficient method based on acidified solvent extraction, dispersive solid phase extraction clean-up (d-SPE) and liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) analysis was validated for the analysis of ochratoxin A and aflatoxins (B_1, B_2, G_1, G_2) in cocoa butter and cocoa liquor. Extraction recoveries were ranged between 91.7 % to 113.8 % with RSDs below 12.3 % for cocoa liquor. On the other hand, cocoa butter gave extraction recoveries in the range of 89.0 – 108.2 % with RSDs below 9.5 %. The method limit of quantification was 2 ng/g for all mycotoxins with good linearity achieved with correlation coefficients better than 0.990, using matrix-matched standards to construct the calibration curve. The method was finally applied to cocoa butter and cocoa liquor samples obtained from the market, which were collected in 2019 – 2020.*

Key words: Mycotoxin, cocoa butter, cocoa liquor, LC-QTOF-MS, multiresidues

INTRODUCTION

Cocoa is known as a main beverage crop, besides tea and coffee (Nair, 2010). Cocoa bean often used as one of the most important ingredients in loads of foods, such as chocolates, biscuits, cakes and sweets, which regularly consumed and become favorite food items among people, especially kids. Cocoa beans had been studied to be very healthy due to its compositions. Recently, cocoa beans recognized as a rich source of polyphenols, which most of them are procyanidins. By consuming cocoa beans and its products, the chances to have a risk for heart disease decreased. Besides, it also has potentially effects on inflammatory activity and act as cancer protective agents (Ruzaidi et al., 2008).

Having said that, one of the threats for cocoa is mycotoxins. Some fungal species produce a group of secondary metabolites which are known as mycotoxins. The presence of the mycotoxins in foods can cause lots of diseases and even death if consumed by man or animals (De Magalhães et al., 2011). Due to their rapid rate of contamination of foods and their widely spread incidence, mycotoxins have become the attention worldwide (Dong et al., 2019). More than 400 mycotoxins had been identified to be presence in agricultural commodities, such as in cereals, fruits, and vegetables. Mycotoxins that presence in products, such as chocolate and cake are occurred due to production of toxigenic fungi in the early stage of processing steps of the raw materials. Mycotoxins are produced in products because there is not enough water presence in products to support the microbial growth which can lead to mycotoxins production (Copetti et al., 2012). Mycotoxins are resistant to disintegration. Once formed in foods or its products, mycotoxins cannot be completely removed by the current food processing techniques.

There are many fungi, such as genera Aspergillus and Penicillium, that able to produce mycotoxins which can cause severe or chronic intoxication to human, and animal once being consumed. Among all those mycotoxins, aflatoxins (AFs) and ochratoxin A (OTA) become the main concern. These are due to their high toxicity and high occurrence in foods (Sánchez-Hervás, Gil, Bisbal, Ramón, & Martínez-Culebras, 2008). Due to the presence of mycotoxins in foodstuff are low (ng/g) and high chances of contamination in matrices, there are variety of extraction techniques have been used for the extraction and purification of mycotoxins (Mbundi et al., 2014).

There are multiple extraction methods for mycotoxins isolation; such as pressurized liquid extraction (PLE) and solid- liquid extraction (SLE) for solid samples, and liquid-liquid extraction (LLE) and solid-phase extraction (SPE) for liquid samples (Miró-Abella et al., 2017). The method that will be used is determined based on the nature of the matrix, its difficulty, and characteristics. However, some of these methods are costly, complex, and/or consume a lot of time and solvent. Until now, there are very few studies on the method and level of mycotoxin residues in cocoa butter and cocoa liquor (Copetti et al., 2013). Therefore, the needs for a fast, robust, and efficient method to determine mycotoxin residues in cocoa semi-finished products is evident. For this reason, the purpose of this work was to validate an

efficient and rapid method for the analysis of different classes of mycotoxin residues in cocoa butter and cocoa liquor. Finally, the validated method was then applied in a real sample monitoring programme carried out on local markets.

MATERIAL AND METHODS

Reagent and materials

Mass spectrometry grade water, methanol, and ammonium acetate were obtained from Fisher Scientific (New Jersey, USA). Magnesium sulphate ($MgSO_4$) and C18 were acquired from Agilent Technologies (Palo Alto, USA). Ochratoxin A (OTA) and aflatoxin (B_1, B_2, G_1, G_2) standards were purchased from Sigma-Aldrich (St. Louis, USA). OTA and aflatoxin stock solutions (1 µg/mL) and intermediate stock solutions (50 ng/mL) were made in acetonitrile and acetone, respectively and kept at -20 °C in the dark.

Cocoa semi-finished products samples for fortification

This study used cocoa butter and cocoa liquor as matrix blanks, spiked samples for recovery purpose, and matrix-matched standards. They were obtained from local cocoa grinders. Five g samples were weighed in 50 mL screw cap centrifuge tubes and fortified with 200 and 500 µL from the 50 ng/mL intermediate standard solution. After that, the samples were left at room temperature before they were analyzed, yielding final spiking concentration levels of 2 and 5 ng/g.

Extraction and clean-up procedure.

The extraction and clean-up method were based on our previous study with some slight modification (Zainudin & Salleh, 2017). In brief, after homogenization, 5 g of samples were weighed in a 50 mL screw cap centrifuge tubes. After the sample was weighed in a centrifuge tube, it was stored in an incubator at 50°C for about 10 to 15 minutes. This was to ensure cocoa liquor and cocoa butter stays in liquid form. Subsequently, 10 mL of acetonitrile (1 % acetic acid) was added to the samples and the centrifuge tubes were shaken using SPEX SamplePrep 1500 ShaQer (New Jersey, USA) for 2 min, then centrifuged at 12000 rpm for 5 min at 4 °C. After centrifugation, an extract of 1 mL organic phase was transferred into 2 mL d-SPE centrifuge (150 mg $MgSO_4$ and 50 mg C18). The tube was vortexed for 1 min and centrifuged at 13000 rpm for 5 min. The final cleaned solution was then filtered through 0.2 µm PVDF filter into autosampler vial to give 0.5 g sample/mL final extract. The extract was diluted two times with deionized water before injected into LC-QTOF-MS. To avoid further dilution factor calculation, standard solutions for quantitation were also diluted with deionized water two times.

Liquid chromatography quadrupole time-of-flight mass spectrometry analysis

LC-QTOF-MS analysis was performed using a Waters I-Class ultra-performance liquid chromatography (UPLC) (Waters Corporation, Manchester, UK). It was equipped with a reversed-phase BEH C18 analytical column of 100 mm x 2.1 mm x 1.7 µm particle size (Waters Corporation, Manchester, UK). The column oven temperature was set to 45 °C and the flow rate was 0.45 ml/min. Mobile phase A and B were water and methanol each containing 5mM ammonium acetate. The linear gradient programme was set as follows: 2% B was maintained for 0.1 min before increased to 99% B from 0.1-3 min, followed by 1 min elution time before re-equilibration back to 2 % B for 3 min. The total run time was 7 min with injection volume of 5 µL. The UPLC system was coupled to a Vion IMS QTOF hybrid mass spectrometer from Waters, equipped with a Lock Spray ion source. The method was optimized for time-of-flight multiple reaction monitoring (TOF-MRM) mode for quantitation purpose. The ion source was operated in positive electrospray ionization (ESI) mode with capillary voltage of 0.45 kV. The reference capillary voltage was set at 3.00 kV. The desolvation gas flow and cone gas flow were set at 800 L/h and 50 L/h, respectively. Nitrogen (>99.5%) was employed as desolvation and cone gas. The source temperature and desolvation gas temperature were set at 120 °C and 550 °C, respectively. Data were obtained in time-of-flight multiple reaction monitoring (TOF-MRM) mode for quantitation purpose (Table 1). Argon (99.999%) was used as collision-induced-dissociation (CID) gas.

Table 1: Time-of-flight multiple reaction monitoring (TOF-MRM) acquisition method parameters.

Mycotoxin	Expected RT (min)	Neutral mass (Da)	Expected m/z	Fragment m/z	Collision energy (eV)
Aflatoxin B_1	2.51	312.0634	313.0707	241.0494	40
	2.51	312.0634	313.0707	285.0750	20
Aflatoxin B_2	2.44	314.0790	315.0863	259.0595	30
	2.44	314.0790	315.0863	287.0900	25
Aflatoxin G_1	2.36	328.0583	329.0656	200.0470	45
	2.36	328.0583	329.0656	243.0640	25
Aflatoxin G_2	2.29	330.0740	331.0812	245.0811	30
	2.29	330.0740	331.0812	313.0700	20
Ochratoxin A	2.72	403.0823	404.0896	239.0097	21
	2.72	403.0823	404.0896	220.9990	41

Analytical method validation and performance criteria

The method was validated in the form of linearity, accuracy, precision, matrix effect and limit of quantitation (LOQ). The matrix effects were evaluated using matrix-matched standards. According to SANTE/12682/2019 document, LOQ is the lowest spike level during validation step which meet acceptable mean recoveries within the range of 70 – 120 % with RSD of less than 20 % (European Commission Directorate General For Health And Food Safety, 2019). Hence, in this study, the LOQ was set according to this document. Average recoveries of spiked blank matrix at 2 and 5 ng/g were used to determine the method's accuracy. On the other hand, relative standard deviation (RSD %) of within-laboratory reproducibility analyses was used to reflect the method's precision. Uncertainty measurement for each mycotoxin was calculated individually based on EURACHEM/CITAC document with some modifications (Ellison & Williams, 2012).

Cocoa butter and cocoa powder monitoring study

Cocoa butter and powder samples were obtained from markets from 2019 to 2020 with each sample analyzed in duplicate.

RESULTS AND DISCUSSIONS

Method validation

The purpose of validation on analytical methods is to demonstrate either the methods are fitted for its purposes of establishment or not able to do so (Garcia et al., 2011). Validation process does not only provide estimates of trueness and precision, but it also done to calculate the risks through the measurement of uncertainty (Gustavo González & Ángeles Herrador, 2007). For analytical study, validation process is important to ensure the analysis provide reliable information of the analyte in the sample.

A good linearity was obtained for the six levels of aflatoxins and ochratoxin A concentrations used to quantify the samples (0.5 – 20 ng/g) with correlation coefficients of more than 0.990, using the matrix-matched standards to construct the calibration curve. The limit of quantification (LOQ) value was determined as the lowest concentration of the analyte that has been validated with acceptable accuracy (70 – 20%) and precision (RSD < 20%) by applying the complete analytical method. In this study, the LOQ for all mycotoxins were set at 2 ng/g since it was the lowest spiking level with acceptable accuracy and precision. Currently, there is no known maximum residue limit (MRL) for both mycotoxins in cocoa and cocoa products. Hence the applied LOQ was deemed sensitive enough to quantify the mycotoxins in cocoa butter and cocoa liquor.

As demonstrated in Table 2, the recoveries of the mycotoxins at 2 and 5 ng/g were ranged between 91.7 – 113.8 % for cocoa liquor and 89.0 – 108.2 % for cocoa butter with RSDs below 12.3 % for both matrices. The method specificity was demonstrated using both the ion ratios and retention time matching between mycotoxin in sample extracts and calibration standard. The tolerance was set at ± 30 % and ± 0.1 min for ion ratio and retention time deviation, respectively. Additionally, the method specificity was also demonstrated using mass error of 0.08 Da for product ions from MRM measurement. Finally, the measurement uncertainty was estimated for each mycotoxin using a bottom-up approach, comprised of precision and bias data, which were obtained during method development and validation study. The combined standard uncertainties were then multiplied by a coverage factor of 2 to obtain individual expanded uncertainties for each mycotoxin as shown in Table 2.

Table 2: Average recovery, Rec (%); RSD (%); and measurement uncertainty, $U_{(conc)}$ (%) for cocoa butter and cocoa liquor spiked at 2 and 5 ng/g.

Mycotoxin	Cocoa liquor				Cocoa butter				U (conc), %
	2 ng/g		5 ng/g		2 ng/g		5 ng/g		
	Rec (%)	*RSD (%)*	*Rec (%)*	*RSD (%)*	*Rec (%)*	*RSD (%)*	*Rec (%)*	*RSD (%)*	
Aflatxin G1	111.8	7.2	112.1	2.1	106.8	3.4	101.7	1.5	18
Aflatoxin G2	106.0	12.3	102.0	2.0	102.3	2.9	102.9	1.7	23
Aflatoxin B1	111.5	4.8	113.8	6.0	108.2	4.4	100.6	1.4	23
Aflatoxin B2	109.7	6.8	107.6	5.7	101.0	0.6	100.5	1.5	29
Ochratoxin A	91.7	2.3	91.9	11.1	97.1	4.5	89.0	9.5	31

Cocoa butter and cocoa liquor monitoring

The proposed method was applied to evaluate the status of mycotoxin level in local cocoa butter and cocoa liquor. Since the limit of quantification of the optimized method was set at 2 ng/g, concentration below this value was not reported and only documented for internal purpose. In total, 11 cocoa butter and 11 cocoa liquor samples were analysed using the proposed method and each sample was analyzed in duplicate. Results showed none of these samples contained either ochratoxin A or aflatoxins.

CONCLUSIONS

In conclusion, efficient and rapid method was validated and applied to cocoa butter and cocoa liquor obtained from local markets in 2019 - 2020. The method gave good recoveries with low RSDs and is shown to be satisfactory for quantification of aflatoxins and ochratoxin A in cocoa semi-finished products. Finally, the monitoring of cocoa butter and cocoa liquor from local markets showed that there is no presence of aflatoxins and ochratoxin.

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