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Detection of five mycotoxins in cocoa bean and its products with QuEChERS Method using Ultra High-Performance Liquid Chromatography Quadrupole-Time-of-Flight Mass Spectrometry

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ABSTRACT

Cocoa beans are a vital raw material for chocolate and various food products; however, they are highly susceptible to contamination by ochratoxin A (OTA) and aflatoxins (B1, B2, G1, and G2), which are toxic mycotoxins of significant food safety concern. OTA is nephrotoxic, immunotoxic, and classified as a possible human carcinogen (Group 2B), while aflatoxin B1 is a Group 1 carcinogen strongly associated with liver cancer. Due to these severe health risks, international authorities such as the European Union and Codex Alimentarius have established strict regulatory limits, and non-compliance often results in trade restrictions. This study developed and validated an analytical method for simultaneous detection of OTA and aflatoxins in cocoa using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) technique coupled with ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) in positive MRM mode. The method demonstrated excellent linearity ($R^2 > 0.999$), with detection limits between 0.11 and 0.99 µg/kg and quantification limits from 0.34 to 2.99 µg/kg. Recovery rates ranged from 73.4% to 103.5%, with RSDs of 8.2–31.5%. The validated method is sensitive, accurate, and reliable, providing a robust tool for monitoring mycotoxins in cocoa to ensure consumer protection and compliance with international trade standards.

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INTRODUCTION

Cocoa beans (*Theobroma cacao*) are an important agricultural commodity, widely used in the production of chocolate, confectionery, biscuits, and beverages [1]. Beyond their economic value, cocoa beans are rich in bioactive compounds such as flavanols, polyphenols, theobromine, and caffeine, which contribute to both their quality and health-promoting properties [2,3]. Flavanols, for example, are known to enhance cardiovascular health by lowering blood pressure and reducing the risk of heart disease [4]. With Malaysia importing over 311,608 tons of cocoa beans mainly from Africa and Indonesia [5,6], ensuring food safety is crucial, as variations in agricultural practices and agrochemical use in exporting countries may impact both safety and quality.

A major food safety concern in cocoa is contamination by mycotoxins, toxic secondary metabolites produced by fungi such as *Aspergillus* and *Fusarium*, which affect about 25% of global crop yield [7]. Among these, ochratoxin A (OTA) and aflatoxins (B1, B2, G1, and G2) are of particular concern. OTA is nephrotoxic, immunosuppressive, teratogenic, and classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (Group 2B) [8,9]. Aflatoxin B1, the most potent of the aflatoxins, is a Group 1 human carcinogen strongly associated with liver cancer, while all four aflatoxins are carcinogenic, mutagenic, and teratogenic [9,10]. Due to these health risks, strict regulatory limits for OTA and aflatoxins have been set by the European Union, Codex Alimentarius, and the U.S. FDA, with non-compliance leading to both trade rejections and public health concerns [10,11,12].

Reliable analytical methods are therefore required to monitor mycotoxin levels in cocoa beans and products. Traditionally, immunoaffinity column (IAC) cleanup and methods such as solid-phase extraction (SPE) or liquid–liquid extraction (LLE) was used, while immunoassays like ELISA and lateral flow devices provide rapid screening, though with lower sensitivity [13,14]. In recent years, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method has gained popularity due to its efficiency, reduced solvent usage, and environmental benefits [15,16,17]. This method uses acetonitrile-based extraction, salting out, and dispersive solid-phase cleanup, making it well-suited for complex matrices such as cocoa. When coupled with advanced instruments like ultra-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS), QuEChERS provides highly sensitive and reliable detection of multiple mycotoxins. This study therefore aims to optimize and validate a UPLC-QTOF-MS method with time-of-flight multiple reaction monitoring (TOF-MRM) for the quantification of OTA and four aflatoxins in cocoa beans and related products, ensuring both consumer safety and compliance with international trade standards.

EXPERIMENTAL

Standards And Reagents

Aflatoxin (B_1, B_2, G_1, G_2) and Ochratoxin A (OTA) standards were obtained from Sigma-Aldrich (St. Louis, USA). Mass spectrometry-grade ammonium acetate, water, and methanol were purchased from Fisher Scientific (New Jersey, USA). C_{18} and magnesium sulfate ($MgSO_4$) were sourced from Agilent Technologies (Palo Alto, USA).

Preparation Of Standard Solutions and Reagents

An intermediate OTA standard solution of 1 µg/mL was prepared, by diluting the appropriate amount of the stock solution in acetonitrile and the working standard solution of OTA and aflatoxin mix (50 ng/mL) was prepared by diluting the individual OTA and aflatoxin mix in acetonitrile and both of this solution were

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kept in the dark at a temperature of -20 °C. The working standard solutions for cocoa butter and cocoa liquor fortification were prepared in acetone.

Sample Extraction and Clean-Up

In this study, cocoa bean samples were collected from the Cocoa Research and Development Centre in Tawau. The beans were ground into a fine powder, and a measured portion of 5 ± 0.05 g was mixed in a 50 mL centrifuge tube with 10 mL of ultrapure water. After mixing for 30 seconds, the mixture was allowed to sit for 15 minutes. Then, 10 mL of acetonitrile containing 1% acetic acid was added and shaken for 2 minutes. Following this, a QuEChERS extraction packet containing sodium chloride, magnesium sulfate, sodium citrate, and sodium hydrogencitrate sesquihydrate was added, and the tube was shaken again for another 2 minutes. The mixture was centrifuged at 12,000 rpm for 5 minutes. A 1 mL aliquot of the supernatant was transferred to a microcentrifuge tube containing magnesium sulfate and C18, vortexed for 30 seconds, and centrifuged once more. Finally, 0.5 mL of the extract was combined with 0.5 mL of deionized water and filtered into an autosampler vial for analysis using UPLC-QTOF-MS. The matrix effects were tested by testing the selecting three d-SPE sorbents: PSA, C18 and $MgSO_4$. As for dilution factor, the analysis of peak response was conducted.

UPLC–QTOF-MS Determination

Ultra-performance liquid chromatography (UPLC) was performed on a Waters Acquity UPLC I-Class system with an electrospray ionization (ESI) interface. Samples were separated using an Acquity UPLC BEH C18 column (1.7 µm, 100 mm × 2.1 mm). Mobile phase comprised 5 mM ammonium acetate in water (A) and methanol (B), with a gradient starting at 98% A for 0.1 minutes, transitioning to 1% A from 3 to 4 minutes, and a 3-minute re-equilibration, totaling 7 minutes. Column temperature was set to 45°C, flow rate at 0.45 mL/min, and injection volume at 10 µL. Detection utilized a Vion IMS QTOF Mass Spectrometer in positive ion mode (ESI+), with capillary voltage of 0.45 kV, source temperature of 120°C, and desolvation temperature of 550°C. Multiple reaction monitoring transitions for five target compounds were recorded (Supplementary Data, Table S1).

Method Validation

Method validation for five mycotoxins in cocoa was conducted by assessing specificity, linearity, recovery, precision, and analytical limits. Detailed methods are described in the supplementary data (Methods S1). Specificity was examined by comparing chromatograms of blank cocoa bean samples with those spiked with target analytes to confirm the absence of interfering peaks. Linearity was determined through matrix-matched calibration curves prepared by spiking blank cocoa samples at five concentration levels (0.25–10 µg/kg). Recovery was evaluated by fortifying blank samples at two concentration levels (2 and 5 µg/kg) in five replicates, while precision was assessed as intra-day repeatability using eight replicates at four spiking levels (1, 3, 5, and 10 µg/kg). Analytical limits were established by calculating the limit of detection (LOD) and limit of quantification (LOQ) from spiked samples at six levels (0.25–10 µg/kg) using the formulas,

$$LOD = 3.3 \times Sa/r, \quad (1)$$

$$LOQ = 10 \times Sa/r, \quad (2)$$

where *Sa* is the standard deviation of the response and *r* is the slope of the calibration curve.

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Real Sample Analysis

After optimization and validation, sixty-eight cocoa bean samples, ten cocoa liquor samples, and eleven cocoa butter samples were tested for mycotoxins. Their presence was confirmed by analyzing retention times and detecting quantitative ions, allowing for accurate identification of these harmful substances.

RESULTS AND DISCUSSION

Sample Preparation

A method based on the QuEChERS technique was used in this study to extract five analytes from a complex matrix sample of cocoa beans and their products. The dispersive solid phase extraction (d-SPE) set consisting of 50 mg C_{18}, and 150 mg $MgSO_4$ is chosen due to the good recovery values found in the previous study [Supplementary data, Reference S18]. This combination removes both water residue and non-polar analytes without affecting targeted analytes. On the other hand, primary secondary amine (PSA) and graphitized carbon black (GCB) were not considered in the clean-up procedure because of the adverse effect they brought to the recoveries of mycotoxins [Supplementary data, Reference S18, Reference S19] (Supplementary Data, Table S2).

Among the tested conditions, a dilution factor of 2 with clean-up provided the best compromise between signal intensity and matrix suppression and was therefore selected for routine analysis (Supplementary Data, Table S3). However, since both cocoa liquor (ground cocoa nibs) and cocoa butter (pressed-out fat) contain a high percentage of saturated fat, a visible fat layer was still observed after dilution with deionized water, which risked clogging the instrument. To minimize this issue, the final extracts were re-filtered through a 0.2 µm PVDF membrane prior to UPLC-QTOF-MS analysis.

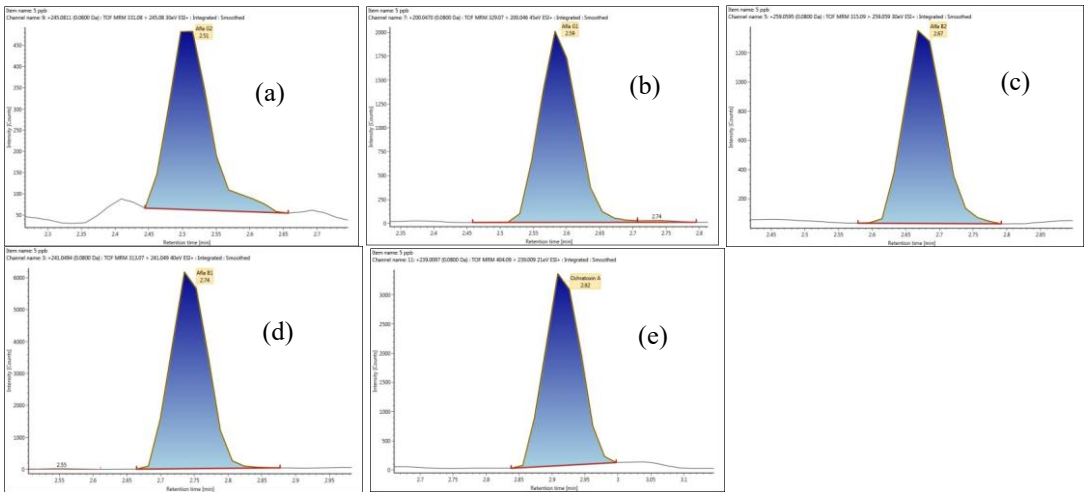

Fig. 1. Chromatogram of each compound spiked at 5 ppb. (a) Afla G2, (b) Afla G1, (c) Afla B2, (d) Afla B1 and (e) OTA

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Method validation

All mycotoxins showed strong linearity ($R^2 > 0.9990$) (Supplementary Data, Table S4). Method selectivity was confirmed by analyzing blank and spiked samples (Fig. 1), with no matrix interferences detected. High-resolution mass spectrometry was used for mycotoxin identification, incorporating retention time and product ion mass accuracy as criteria. A 0.01 Da mass error in TOF-MRM mode improved selectivity and reduced false positives.

Results for spiked samples at 5 µg/kg showed mass errors of less than 0.01 Da for all mycotoxins, while blank samples exhibited errors over 0.01 Da, confirming no mycotoxins were detected. Detection (LOD) and quantification (LOQ) limits varied by cocoa product: for cocoa beans, LOD was 0.11 to 0.62 µg/kg and LOQ ranged from 0.34 to 1.88 µg/kg; for cocoa liquor, LOD was 0.17 to 0.99 µg/kg and LOQ was 0.53 to 2.99 µg/kg; for cocoa butter, LOD ranged from 0.21 to 0.70 µg/kg with LOQ between 0.63 and 2.13 µg/kg (Table 1). Recoveries for all analytes were acceptable at 70% to 120%, indicating the method's accuracy and precision across cocoa beans and their derivatives. Table 2 shows the recovery results for each compound at 2 different levels of concentration. The RSD values for all five analytes were less than 20.2 %. (Supplementary Data, Table S5).

Table 1. LOD and LOQ values for all analytes detected in cocoa bean and its product.

Sample	Cocoa Bean		Cocoa Liquor		Cocoa Butter	
Analyte	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
Aflatoxin G2	0.26	0.78	0.99	2.99	0.66	2.01
Aflatoxin G1	0.11	0.34	0.22	0.67	0.23	0.68
Aflatoxin B2	0.62	1.88	0.67	2.04	0.70	2.13
Aflatoxin B1	0.14	0.43	0.31	0.95	0.25	0.74
Ochratoxin A	0.13	0.41	0.17	0.53	0.21	0.63

Table 2. Recovery results for each compound at two different levels of concentration.

	Level 1[a]		Level 2[b]	
Mycotoxins	**Recovery**	**RSD**	**Recovery**	**RSD**
Aflatoxin G2	101.71	7.91	98.32	16.82
Aflatoxin G1	103.46	10.53	93.82	8.21
Aflatoxin B2	96.55	8.30	89.03	11.20
Aflatoxin B1	102.49	9.79	93.66	18.49
Ochratoxin A	80.20	27.37	73.40	31.50

Note: a is 2 µg/kg for aflatoxin G1, B1 and ochratoxin A, 6 µg/kg for aflatoxin G2 and B2, b is 6 µg/kg for aflatoxin G1, B1 and ochratoxin A, 15 µg/kg for aflatoxin G2 and B2.

Real sample analysis on cocoa bean and their products.

The method was tested on 68 cocoa bean samples, 10 cocoa liquor samples, and 11 cocoa butter samples. Beans were sourced from local farmers and grinding facilities, while liquor and butter came from industry suppliers. Aflatoxins and ochratoxin A were found in 19 cocoa bean samples (Table 3), with only trace amounts in cocoa liquor. No mycotoxins were detected in cocoa butter. This study showed that high-

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resolution instruments are accurate and reduce false positives, helping the cocoa industry manage mycotoxin risks and ensure product safety.

Table 3. Quantification results for targeted mycotoxins in cocoa bean and cocoa products

Type of Cocoa	Origin	G2	G1	B2	B1	OTA
Cocoa bean	Padawan - Sarawak	10.95	12.87	9.18	10.46	
	Padawan - Sarawak					0.66
	I - CI			5.39	5.62	0.15
	Sabah - Misti					3.79
	Sabah - Mohd Mokhyi		33.54	12.36	35.11	9.92
	Kapar - Selangor	10.40				
	Batu Pahat - Johor		1.53	2.23	4.90	
	I - Pasir Gudang - VE			1.69		
	I - Pasir Gudang - NG			1.44		2.85
	I - GH			1.05		
	Jasin - Melaka		1.78			
	Segamat - Johor		0.39			
	Sprai Selatan - Penang			2.79		
	I - T.Pelepas - UG			7.91		
	Jasin - Melaka		1.51			
	Ranau - Sabah			2.04		
	Kota Marudu – Sabah			1.55		
	Tenom - Sabah			1.46		
	Keningau - Sabah			1.85		
Cocoa liquor	Ketua Projek Pembangunan Protokol			1.33		
	Ketua Projek Pembangunan Protokol				0.44	
	Barry Klang	1.46			0.42	

CONCLUSION

A validated method using QTOF-MS for extracting and analyzing mycotoxins in cocoa beans and products was optimized. The technique demonstrated high sensitivity and selectivity through TOF-MRM acquisition, with validation confirming its linearity, precision, and accuracy. Low detection limits enabled effective mycotoxin detection in actual cocoa samples, which showed varying contamination levels. By incorporating mass error of product ions, false positives and negatives were minimized. The QuEChERS method combined with Ultra High-Performance Liquid Chromatography QTOF-MS offers a reliable

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approach for ensuring the safety and quality of cocoa-based foods, supporting regulatory compliance and consumer health.

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AUTHOR'S CONTRIBUTION

The article was written and revised by Amira Syafiqah Harith, Muhammad Isfaiz Iskandar and Muhd Fauzi Safian. The research was done by Muhammad Isfaiz Iskandar. Badrul Hisyam Zainuddin and Muhd Fauzi Safian conceptualized the main idea of the research, theoretical foundation, designed the study. Muhammad Isfaiz Iskandar, Zaidah Zainal Ariffin and Muhd Fauzi Safian support the review, approved corrections, and approved the publication submission.

CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

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