FEASIBILITY STUDY OF OCHRATOXIN A REFERENCE MATERIAL PRODUCTION AND IDENTIFICATION USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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ABSTRACT – *Cocoa is an important agricultural commodity with technical barriers for exportation because of possible contamination with ochratoxin A (OTA), a mycotoxin nephrotoxic and carcinogenic. The maximum limit for OTA in cocoa beans is not stated and can only be found in cocoa powder which is 3.0 µg/kg stated by European Union. This paper describes the development of a candidate reference material (RM) of OTA in cocoa beans. The RM quantitation was based on the extraction method using QuEChRES with slight modification. The obtained recovery at spiking levels of 2 and 5 ng/g ranged between 93.5–110.7 % with relative standard deviations (RSDs) lower than 15.5 %. The method was successfully applied to the feasibility study of the reference material which defined the procedure for the preparation of the cocoa beans with a certain amount of OTA contamination. It was produced by spiking blank cocoa beans with OTA standard, mixing and filling in an amber bottle with 50 g each, and storing at 15 °C. The homogeneity study showed that there were no significant differences between the bottles via ANOVA test and samples were stable for 16 months in the stability study. The results obtained show that the production of the reference material is doable and possible as this material will be an important tool for quality control in laboratories*.

Key words: Reference material, Ochratoxin A, Cocoa beans, Food safety, LC-MS/MS

INTRODUCTION

Cocoa is a vital agricultural commodity and has an important role in the world economy, being produced in West Africa, followed by Asia and central also South America due to its humid and tropical environment (Sánchez-hervás *et al*., 2008). During cocoa processing, the microorganisms could contaminate the bean from the outer surfaces of the pod, through workers' hands and tools, plant leaves, collection baskets, and insects or residual mucilage in equipment used by the workers. Most of these actions may lead to the formation of mycotoxin (Copetti *et al*., 2014).

One of the mycotoxins found in cocoa is Ochratoxin A (Copetti *et al*., 2013) (Figure 1), which is produced by several species of *Aspergillus* and *Penicillium* fungi (Bui- Klimke *et al*., 2015). Ochratoxin A is nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic, and carcinogenic in many species with species and sex-related differences. Ochratoxin A also classified as a possible human carcinogen (group 2B) based on a great amount of evidence of its carcinogenicity discovered in several animal studies. Frequent exposure of animals or humans to OTA may cause a range of health problems. In particular, OTA could be a threat to cancer in humans (Malir et al., 2016).

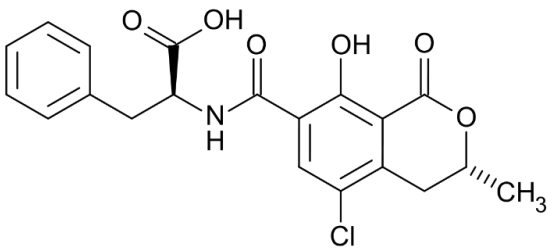

Figure 1: Chemical Structure of Ochratoxin A

To control the intake of OTA in the foodstuff such as cocoa, action should be intensified for implementation of a systematic cocoa harvesting and pod storage, development and validation of analytical methods for OTA analysis, and the production of reference material, which is a fundamental tool for quality control (QC) and reliability of measurements of analytical laboratories. As stated by Guo *et al*., (2019), reference material plays an important role in the analytical measurement of trueness and employed quality control scheme, laboratory accreditations, and the framework of internationally agreed standards of traceability and comparability. However, the reference

material of ochratoxin A in cocoa is lacking which leads in using of spiked samples for quality control purposes. Despite that, the inherent issue with this is that the analyte implemented in this manner is unlikely to be retained as firmly as that which is naturally present in real samples, resulting in an unrealistically high impression of extraction efficiency from the technique (Zainudin *et al*., 2021). Previous studies focused the feasibility and stability study of ochratoxin A in pork meat products and roasted coffee (De Santis *et al*., 2020; Do Rego et al., 2019). However, the study in cocoa beans and cocoa products still lacking. Hence, the purpose of this work is to find a suitable method and process to produce reference material of OTA in cocoa beans.

MATERIALS AND METHODS

Reagents and Solvents

The reagents used were magnesium sulphate ($MgSO_4$) and C18 which were acquired from Agilent Technologies (Palo Alto, USA). Mass spectrometry grade water and acetonitrile were obtained from Fisher Scientific (New Jersey, USA). Formic acid was obtained from Merck (98-100 %; Merck). The solvents used were methanol HPLC grade (99.9 %; Merck) and acetonitrile HPLC grade (99.9 %; Merck).

OTA Standard

The standard solution of Ochratoxin A was obtained from Sigma-Aldrich (98.0 %; Sigma- Aldrich) All weighing was performed in calibrated analytical balances capable of weighing down to 0.0001 g.

Analytical Method

The sample preparation procedure was based on the modified QuEChERS method (Zainudin et al., 2021) and the quantification was realized by LCMS-MS. Five g of cocoa beans samples were weighed in 50 mL screw cap centrifuge tubes. Ten mL of deionised water was added, and the mixtures were homogenised using a vortex mixer for 30 s and left at room temperature for 30 min for matrix hydration. 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. Afterwards, 4 g MgSO4, 1 g NaCl, 1 g sodium citrate dihydrate, and 0.5 g sodium hydrogen citrate sesquihydrate were added and the mixtures were immediately shaken using 1500 ShaQer for 2 min, then centrifuged at 12,000 rpm for 10 min at 4 °C. After centrifugation, an extract of 1 mL organic phase was transferred into 2 mL of microcentrifuge tube contained 50 mg C18 and 150 mg $MgSO_4$. The tube was centrifuged at 13,000 rpm for 5 min. The final solution was then filtered through a 0.2 µm PVDF filter into an autosampler vial to give a 0.5 g sample/mL final extract. Finally, the extract was diluted two times with deionized water.

LC-MS/MS analysis was performed using an Agilent 1260 LC equipped with an Agilent 6420 triple quadrupole mass spectrometry system (Agilent Technologies, Palo Alto, USA). Separation was performed at 40 °C on a Zorbax SB-C18 column (2.1 x 50 mm. 1.8 µm particle size, Agilent Technologies). The mobile phase consisted of water (solvent A: 0.1 % formic acid, 5 mM ammonium formate) and acetonitrile (solvent B: 0.1 % formic acid, 5 mM ammonium formate). The following gradient profile was used: 5 % B for 1 min, linear gradient to 95 % B in 6 min and stayed for 1 min. Finally, the gradient was back to the equilibrium condition of 5 % B for 4 min. The flow rate used was 0.4 mL/min with a sample injection volume of 5 µL. The following MS parameter settings were used: Nitrogen gas temperature 350 °C at a flow rate of 11 mL/min. Nebuliser pressure of 30 psi, capillary voltage of 4 kV, and delta EMV of 200 V. Quantification was performed using MRM in positive ionization mode (404.1 – 220.9 m/z, 404.1 – 238.9 m/z).

Analytical Method Validation and Performance Criteria

The method was validated in the form of linearity, accuracy, precision, and limit of quantitation (LOQ). The LOQ was set at the minimum concentration that can be quantified with acceptable accuracy (70-120 % recovery) and precision (< 20 RSD %). Average recoveries of the spiked blank matrix at 2 and 5 ng/g were used to determine the method's accuracy. On the other hand, the relative standard deviation (RSD %) of within-laboratory reproducibility analyses was used to reflect the method's precision.

Processing Grounded Cocoa Beans

About 0.6 kg of cocoa beans with shells were crushed into smaller pieces using an industrial blender. The smaller pieces of cocoa beans were grounded using a grinding mill (Retsch, ZM 200). Afterwards, the grounded cocoa beans were transferred into the sieve to obtain a particle size of below 1 mm. All grounded cocoa beans were kept in a chiller (4 °C) until ready to be processed in the feasibility study and production of the reference material.

Feasibility Study for Reference Material Development

About 120 g of ground roasted cocoa was spiked with 12 µg of OTA solution diluted in 200 mL of methanol to obtain a starting material of around 100 ng/g. To

complete the homogenization, the mixture was rotated on a rotary evaporator for 1 h and 15 min at 70 °C and a vacuum of 511 mbar. After that, the residues of solvents and other volatile compounds were removed by spreading the mixture on the tray laid with baking paper and were put into the vacuum oven at 30 °C with a vacuum of 720 mmHg for 21 hours.

Then, the total mass of spiked material with a mass of 98 g was taken and divided gravimetrically into 49 g of two equal parts each. To obtain 500 g of a bulk spiked material, each portion of 49 g was mixed in 20 min blender with one-part blank cocoa beans (uncontaminated) with a mass of 201 g. The two flasks labelled A and B are divided into two equal parts of A1 and A2 and B1 and B2, respectively. Then, a flask of A1 containing cocoa beans was mixed with B2, resulting in the flask of C, and A2 was combined with B1, resulting in flask D. Both flasks of C and D were homogenized separately for a few minutes using a blender to make sure the cocoa beans were mixed uniformly.

A new division was produced, flask C was divided into C1 and C2 while flask D was into D1 and D2. The Flask of C1 was mixed with D2, resulting in flask E, meanwhile, C2 was added to D1, producing flask F. Then, the new flask of E and F were homogenized for 20 min at low speed. Lastly, flasks E and F were packed into 10 bottles (amber glass) with 50 g of cocoa beans. The total blending time was about 2 h without any interruptions.

Homogeneity and Stability Study

The homogeneity study is required to show if the units of a batch are sufficiently homogeneous to each other. For quantitative demonstration, five units were randomly selected from the whole batch of bottles. The samples were prepared in duplicate following the analytical method. The results of the homogeneity were obtained using a one-way analysis of variance (ANOVA), which allows for the calculation of the standard deviation between the units of the material. On the other hand, stability study was conducted for 16 months with four-time intervals with candidate samples stored at 15 °C with 40 % RH. At each interval, samples were randomly picked and analyzed in duplicate.

RESULTS AND DISCUSSIONS

Method Validation

Since the samples revealed matrix-induced response suppression in the cocoa bean matrix when compared with the same standards made in a pure solvent, matrix-matched calibration standards were utilized. Using matrix-matched standards for establishing the calibration curve, good linearity for OTA was attained for the five levels of OTA concentrations used to quantify the samples (1 – 30 ng/g) with correlation coefficients better than 0.990 (Figure 2). On the other hand, recoveries of OTA spiked at 2 and 5 ng/g with five replicates at each concentration ranging between 93.5–110.7 % with RSDs below 15.5 % (Table 1). The recovery and RSD values both met the method performance requirements, demonstrating the proposed technique's high level of precision and accuracy. Since 10 ng/g was the lowest amount of spiking with sufficient accuracy and precision, it was chosen as the method LOQ. Since there is no regulation for OTA maximum limit at the national or international level, it can be concluded that the proposed method is sensitive enough to quantify OTA in cocoa beans.

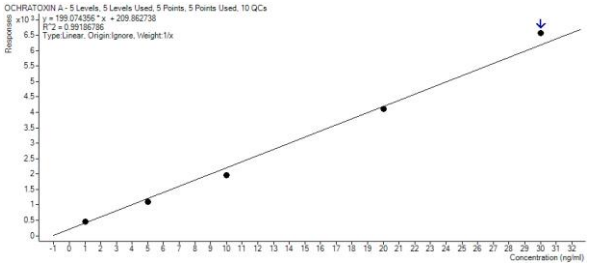

Figure 2: Calibration curve for matrix-matched OTA standard ranged between 1 – 30 ng/g

The separation of the analytes of interest from other components that could obstruct or interfere with the identification and quantification was used to ascertain the selectivity of the analytical method in this work. To do that, 2 and 5 ng/g of spiked matrix solutions and blank matrix solutions were compared (Figure 3). Results indicated that at its retention time of 4.5 min, OTA was free of matrix interferences. Retention time matching and relative ion ratio were also used to identify the OTA, with tolerances of 0.2 min and 30 %, respectively. The spiked OTA gave a relative ion ratio of between 70 – 130 % whereas the blank sample ion ratio deviated more than 30 % compared to the reference standard (Figure 3 A – D). Furthermore, results also show that the retention times of the spiked samples were within ± 0.2 min of the standard.

Table 1: Average recovery (%), RSD (%) and LOQ (ng/g) obtained for OTA in cocoa beans

Mycotoxin	2 ng/g (n=5)		5 ng/g (n=5)		LOQ (ng/g)
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
Ochratoxin A	93.5	15.5	110.7	6.0	10

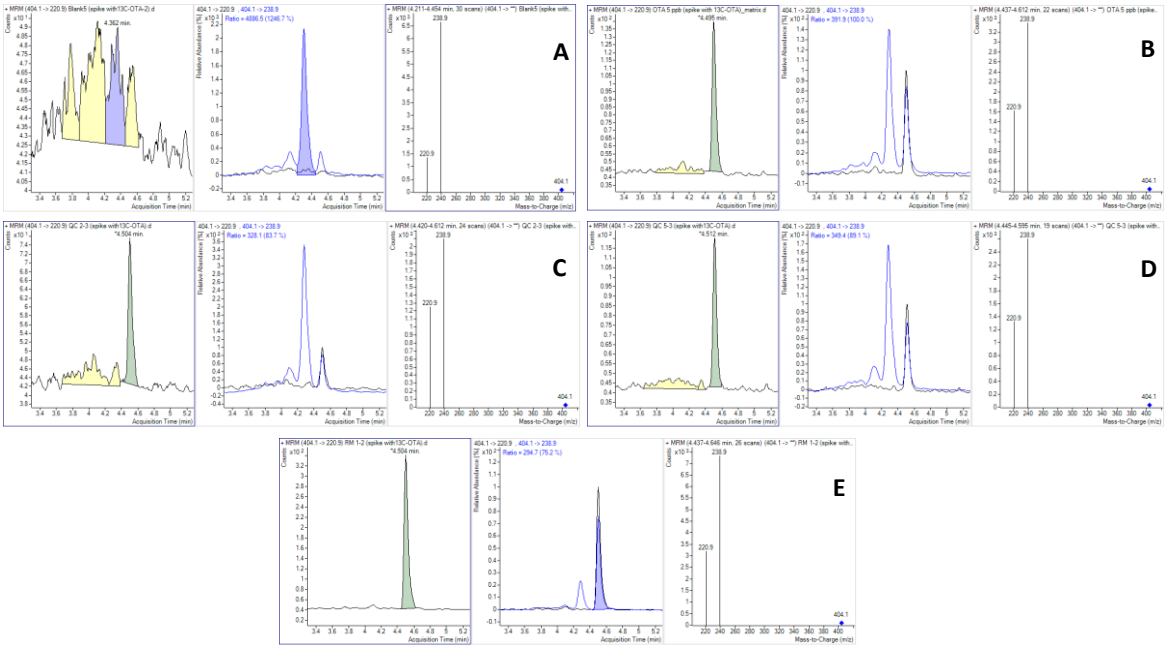

Figure 3: Chromatograms and mass spectra of (A) blank samples, (B) 5 ng/g OTA standard, (C) – (D) spiked samples at 2 and 5 ng/g, (E) Reference material

Ochratoxin A Reference Material Processing

To ensure the complete and uniform fortification of the bulk material, an OTA-free cocoa bean was used for the feasibility study and manufacture of the candidate RM. Samples were subdivided and cross-mixed a couple of times until finally packed in ten amber bottles with 50 g cocoa beans each. Any reference material (RM) must possess both homogeneity and stability. To produce materials that are as homogeneous and stable as possible, the utmost attention must be given throughout preparation. Hence five bottles were randomly picked and analysed for OTA in duplicate. The analysis of candidate reference materials passed the initial identification criteria previously discussed which were the retention time matching and relative ion ratios of the quantifier and qualifier ions (Figure 3E). The results of the homogeneity study are shown in Figure 4. ANOVA test showed the values are statistically equivalent and there is not enough evidence to conclude that there are differences among the means at the 0.05 level of significance with $P > 0.05$ (0.463).

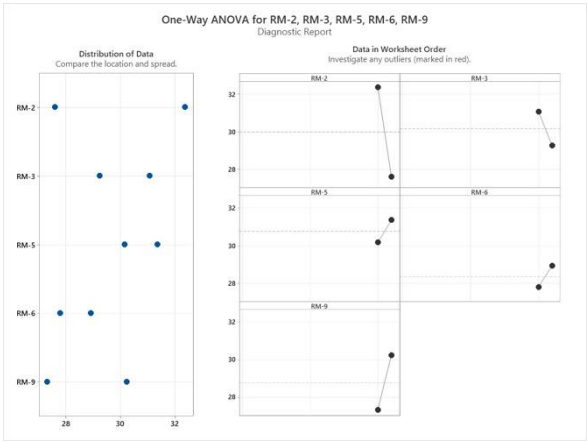

Figure 4: ANOVA distribution chart for homogeneity study for 5 reference materials

On the other hand, the term "stability of RM" refers to the property of the material to retain a particular property value within a specified range for a specified amount of time when stored under specified conditions. In this work, the materials were stored in a humidity chamber at 15 °C with 40 % RH. Assuming that no change in the composition of the reference material happens at this temperature, the stability of the reference material is assessed by examining the values of the OTA in samples of the items held at the specified temperatures. This study looked at the short-term stability throughout the course of 16 months and at four different points in time. Candidate reference material bottles were chosen at random and examined twice. The stability of the candidate reference material was carried out using the ANOVA test (Figure 5). The analysis of the obtained results shows that there is not enough evidence to conclude that there are differences among the means of different time intervals at a 0.05 significance level (P (0.069) > 0.05). Hence, the amount of OTA in cocoa beans is stable at the proposed temperature and relative humidity. Summarizing the findings, it can be said that each material can be utilised for quality control measures because it satisfies the initial prerequisites for homogeneity and stability of reference material with an OTA average concentration of 29.6 ng/g.

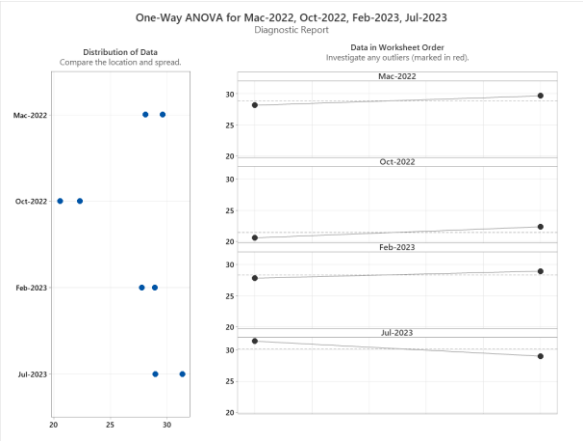

Figure 5: ANOVA of distribution chart for stability study from Mac 2022 to July 2023

CONCLUSIONS

A new reference material for OTA in cocoa beans was produced based on the spiking procedure followed by a multistep subdivision of the mixture of spiked samples with uncontaminated blank samples. A total of ten bottles were prepared with 50 g of each bottle. The preparation procedures gave good homogeneity with acceptable stability for up to 16 months when stored at 15 °C with 40 % RH. The interlaboratory comparison with expert laboratories will be conducted to complete the characterization phase for the value assignment of OTA. The resulting reference materials of OTA in cocoa beans can be used for method validation and quality control purposes, especially for ISO 17025:2017 accredited laboratories as it can help in the reliability of the measurements.

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