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Sampling of Cocoa Beans and Quantification of Ochratoxin A: Validation of the Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Author AC designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors AD, KB, AT, GHB managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The objectives of this study were compare an alternative method for cocoa beans sampling with the standard method proposed by the European Union (EC 401/2006) and validate a method of Ochratoxin A determination. The alternative method applies to

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samples of 5 kg of cocoa beans while the standard method applies to samples of 10 kg. quality characteristics and validation parameters were determined according to Ivorian Coffee and Cocoa stock exchange and French (NFV03-110-1998) standards. Concerning quality characteristics, no significant difference at 5% risk was revealed in the values of the three parameters considered when assessing marketability quality requirements (moisture, graining and grades). As regards the validation of OTA determination method, the limits of detections and quantifications were 0.05 µg/kg and 0.20 µg/kg. The coefficients of variation for the tests of repeatability and reproducibility were respectively 0.26% and 5.67%. As for the extraction yield, it was equal to 86%. Furthermore, no significant difference (5% risk) was observed between the concentrations of OTA measured by the standard and alternative methods. Hence, although the alternative method goes with a mass reduction of samples analyzed, it did not alter significantly the results of the marketability as well as the concentrations of OTA.

Keywords: Ochratoxin A; cocoa bean; sampling validation method; merchantability.

1. INTRODUCTION

Cote d'Ivoire is the world's largest producer of cocoa, with an average annual production of 1,200,000 tons representing 41% of world supply [1]. The cocoa economy contributes to 10% of the Gross Domestic Product and provides the country with about 40% of its export revenues, which represents 75% of Ivorian exports of foodstuffs [2]. The Ivorian government is particularly responsive to the proper management of strategic issues inherent in sustainable cocoa production, especially in the context of a global economy increasingly liberalized and competitive. After health problems posed by the presence of organochlorine pesticides residues in cocoa and related products, following their use for improving production yields, new threats related to the presence of mycotoxins, particularly ochratoxin A, have arisen [3,4,5]. Ochratoxin A (OTA) is a mycotoxin produced by toxin-producing molds such as *Aspergillus* and *Penicillium*. Studies have shown that OTA can be nephrotoxic, teratogenic, and immunosuppressive [6,7,8,9,10]. OTA was even classified as a carcinogen of the Group 2B [11,12]. It was involved in several human diseases, such as Balkan endemic nephropathy [13], development of tumors of the urinary tract and kidney [14,15].

The European Union, the main partner of Cote d'Ivoire with a volume of 90% of trade in agricultural products, in order to protect its populations, plans to limit the concentration of OTA in cocoa to 2 µg/kg [16]. The implementation of this directive will undoubtedly affect cocoa producing countries. In Cote d'Ivoire, particularly, this will certainly lead to economic and health issues. These concerns involve assessing OTA levels in cocoa beans by means of validated methods. Also, the use of regulation N°401/2006/CE [16] for sampling the beans is disputed by some exporters because the amount of beans used for analysis (10 kg per batch) is considered too important.

This study was initiated to provide an alternative sampling method in which the mass of beans to be taken is reduced to 5 kg and also to validate the method for determining OTA in cocoa beans by high performance liquid chromatography (HPLC).

2. MATERIALS AND METHODS

2.1 Sampling

The plant material used in this study consisted of cocoa beans collected from November 2007 to March 2008 in the port of Abidjan. A total of one hundred and forty (seventy samples of ten kilogram of the beans per batch were collected using 401/2006/CE regulation and seventy samples comprising of five kilogram per batch were collected.

2.2 Determination of Cocoa Beans Marketability

According to Ivorian regulation [17], three parameters are taken into account when it comes to assess the merchantability of cocoa beans: the moisture content, the graining and the classification in grades Table 1. The moisture content of cocoa beans was determined with the help of an oven at 105°C until constant weight [18]. The graining was determined by counting the number of beans in 100 g of whole cocoa beans free of any foreign matter. Finally, the classification in grades was related to the percentage of defective cocoa beans in 100 g of whole cocoa beans. To this end, 100 g of whole cocoa beans were cut lengthwise through the middle with a bean knife brand STANLEY, to expose a maximum cut surface of cotyledons. Visual examination of the inner portions of the two half-beans highlights possible flaws [19].

Table 1. Cocoa beans quality characteristics in Cote d'Ivoire

Moisture content			
Maximum value allowed			8%
Number of beans per 100 g of cocoa beans			
Maximum value			105
Grades			
Grade	Moldy	Slaty	Defective Moth + Germinated + Flat
Grade I (GI)	≤ 3%	≤ 3%	≤ 3%
Grade II (GII)	> 3% and ≤ 4%	3% and ≤ 8%	> 3% and ≤ 6%
Low grade (LG)	> 4%	> 8%	> 6%

2.3 Determination of OTA Levels in Cocoa Beans

2.3.1 Extraction of ochratoxin A

The entire sample was crushed in a hammer mill to obtain a homogeneous fine grind. In a Nalgene jar containing 15 g of homogenate, 150 mL of aqueous methanol-bicarbonate 1% (m/v; 50:50) were added. The mixture was homogenized by Ultra-Turax for 3 minutes and the homogenate was centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was filtered through filter paper into tubes of 25 mL. To 11 mL of filtrate were added 11 mL of saline phosphate buffered (PBS) at pH 7.3. Immunoaffinity columns brand Ochraprep and R-Biopharm were conditioned with 10 mL of PBS. Purification of 20 mL of the mixture was made on immunoaffinity columns and OTA extraction was performed with two volumes of 1.5 mL of solvent (methanol/acetic acid; 98:2; v/v) at a flow rate of 5 mL/minute. The resulting sample was packed in a chromatographic tube and the analysis for OTA was made by HPLC using the European community regulation [16].

2.3.2 Apparatus

A liquid chromatograph HPLC brand Shimadzu coupled to a fluorescence detector was used and the operating conditions are described in Table 2.

Table 2. HPLC analytical conditions

Precolumn	Shim-pack GVP-ODS 10 x 4.6 mm
Column	Shim-pack GVP-ODS 250 mm x 4.6 mm
Detector	Fluorescence, λ excitation: 330 nm, λ emission: 460 nm
Mobile phase	Acetonitrile/Water/Acetic acid (99/99/2)
Injected volume	100 µL
Flow rate	1 mL/minute
Column temperature	40°C
Rinsing solvent	Acetonitrile
Analysis duration	12 minutes

2.4 Validation of the Ochratoxin A Method Analysis

The method validation was conducted using the method of the French Association for Standardization [20]. This procedure includes the study of the linearity of the calibration range, the determination of the limits of detection and quantification, the calculation of the coefficient of variation for the tests of repeatability and reproducibility, and the calculation of the recovery percentage for testing accuracy. The reference material was used to compare the concentration of OTA obtained with the certified value.

2.4.1 Test of linearity

The study on linearity was tested between 0 and 2.0 µg/L using 6 points calibration (0 µg/L, 0.03 µg/L, 0.09 µg/L, 0.20 µg/L, 1.0 µg/L and 2.0 µg/L). Five separate tests were performed for each point.

2.4.2 Limits of detection and quantification

Limit of detection (LD) = mb + 3σ
Limit of quantification (LQ) = mb + 10σ
(mb= Average concentration with the blank ; σ = Standard deviation of blank values)

2.4.3 Tests of repeatability and reproducibility

To test the repeatability, ten trials of extract from a reference sample of cocoa beans were analyzed by HPLC. For reproducibility, five separate trials from a reference sample of cocoa beans were analyzed by HPLC at intervals of each 3 days, or 15 trials in total.

2.4.4 Test of accuracy

The extraction yield was determined from a reference material containing 4.5 µg OTA/kg, obtained from the European Community. Ten separate extractions were performed. Each extraction was assayed three times. The average and standard deviation were calculated from these 30 results.

2.5 Statistical Analysis

The averages were calculated with their standard deviations to assess OTA levels, moisture content, graining and grades of cocoa beans. The squared coefficient of Bravais-Pearson was calculated to assess the correlation between OTA and the criteria for marketability. The homogeneity of means (OTA, moisture content and graining) was determined by a one or two-way analysis of variance (sampling location or period and/or grade) through Fisher test, using SPSS 12 software at 5% risk.

3. RESULTS AND DISCUSSION

3.1 Merchantability of Cocoa Beans

The results of the marketability of cocoa beans are shown in Table 3. The average moisture contents were respectively 6.6% and 6.7% for samples of 10 kg and 5 kg. No sample analyzed had moisture content above the limit value of 8% set by the Ivorian regulation [16]. Results for graining indicated 94 and 95 beans per 100 g of cocoa beans for samples of 10 kg and 5 kg. The study on the homogeneity of means between moisture contents and graining values performed by a one-way analysis of variance (mass of sample), through Fischer-test revealed no significant difference at 5% risk. The classification in grades revealed that respectively 87% and 89% of samples of 10 kg and 5 kg could be marketed.

Regardless of the number of samples collected for analysis (10 kg or 5 kg) no significant influence on the marketability of cocoa was noticed. Indeed, no significant differences were revealed between the parameters taken into account to assess marketability (moisture content, number of beans per 100 g of cocoa beans, classification in grades). The classification in grades shows that 84% of lots are exportable for samples of 10 kg against 89% for samples of 5 kg. To say it more exactly, 5% of lots classified by the standard method as low-grade and therefore not exportable are exportable when we apply the alternative method. Thus the two sampling methods are adequate for the determination of the marketability of beans. But the standard method seems more stringent.

Ivorian cocoa beans marketability seems decreasing in comparison with the results of a previous study by Laine [20], whereas it seems to have been improved compared to the results of Dembele et al. [5]. Indeed, Laine [21] obtained an average moisture content of 7.6%, an average of 88 beans for graining and 93% of exportable beans, while Dembele et al. [5] pointed out 66% and 61% respectively of exportable beans for the ports of Abidjan and San Pedro.

3.2 Validation of Ochratoxin A Determination Method

Fig. 1 shows the pic of OTA with good resolution and baseline correct. The results of validation tests are presented in Table 4. The limits of detection and quantification were 0.05 µg/kg and 0.20 µg/kg. The coefficients of variation calculated for tests of repeatability and reproducibility are respectively 0.26% and 5.67%. The extraction yield is estimated at 86%.

The validation tests have substantiated the reliability of the alternative method proposed for the determination of OTA in samples of cocoa beans. Indeed, the linearity test showed normal distribution across the calibration range [0-2.0 µg/L] Fig. 2. The detection limit 0.05 µg/kg showed a high sensitivity of the technique used for the determination of OTA. The

coefficients of variation for repeatability tests showed the stability and reliability of the HPLC chromatograph and its accuracy as well. The reproducibility tests confirmed the results of repeatability. Moreover, they also showed the reliability of the method of extraction of OTA. The extraction yields also showed the accuracy of the dosage, for no significant difference was obtained with the compliance test. Therefore, the results of the validation tests are consistent with values showing the acceptability of an analytical technique as stated by experts of the Joint FAO/IAEA [22] documented in the collection of standard methods of American Public Health Association [23].

Table 3. Quality characteristics of cocoa beans suitable for the market

Moisture content (%)				
Sample mass	Average	[Min-Max]	Number of sample with> 8%	
10 kg	6.6±0.5a	5.6-8.0	0	
5 kg	6.7±0.6a	5.8-8.0	0	
Graining (number of beans for 100 g of cocoa beans)				
Sample mass	Average	[Min-Max]	Number of samples with> 105	
10 kg	94±4a	80-104	0	
5 kg	95±5a	85-112	3	
Classification in grade (%)				
Sample mass	Grade I (GI)	Grade II (GII)	Low Grade (SG)	Exportables (GI+GII)
10 kg	51%	33%	16%	84%
5 kg	40%	49%	11%	89%

Means in column, for each criterion, followed by the same letter are not significantly different (P=.05)

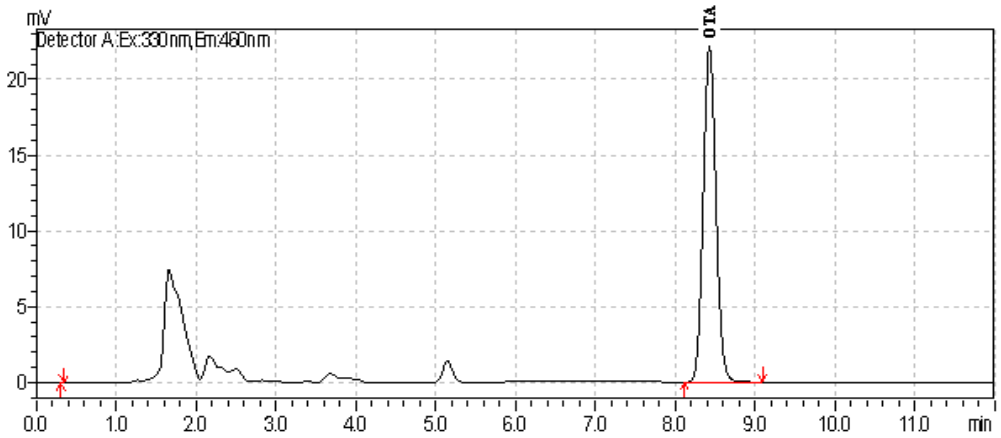

Fig. 1. Chromatogram of OTA reference

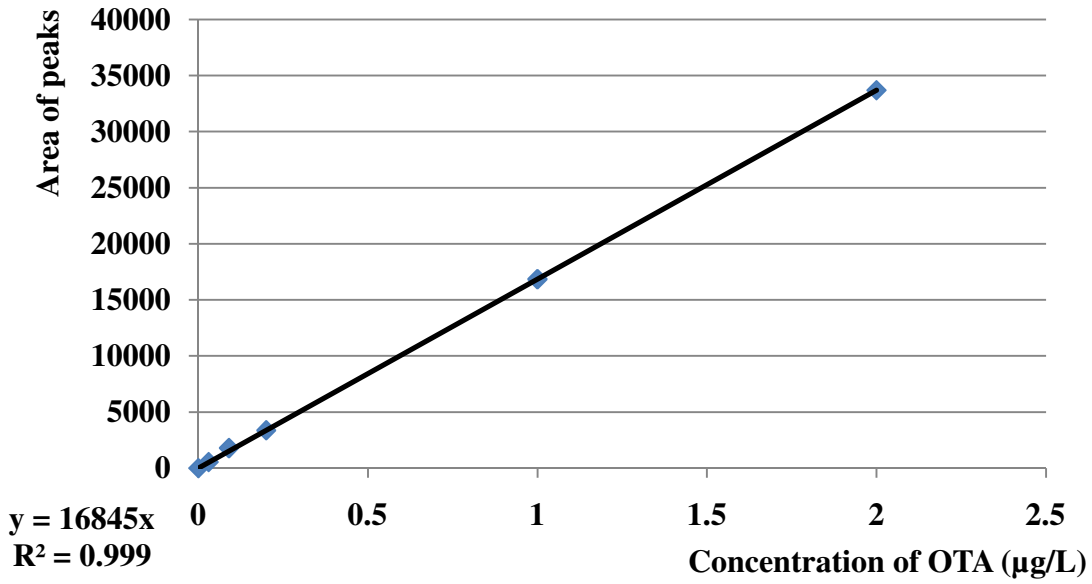

Fig. 2. External calibration curve of ochratoxin A

Table 4. Results of the validation of the method for determination of OTA

Parameters	Results
Linearity (Pearson coefficient (R^2)	0.999
Limit of detection (LOD)	0.05 µg/kg
Limit of quantification (LOQ)	0.20 µg/kg
Extraction yield(EY)	86±0.39%
Repeatability (Coefficient of variation; CV)	0.26%
Reproducibility (Coefficient of variation; CV)	5.67%
Reference material (OTA level in reference material: 4.5 µg/kg)	3.89±0.03 µg/kg

3.3 Concentrations of OTA in Cocoa Beans

Table 5 presents the average and areas of variation of OTA concentrations found in different samples of 10 kg and 5 kg analyzed. The average concentrations are respectively of 0.74 µg/kg and 0.85 µg/kg for samples of 10 kg and 5 kg. The concentrations vary between 0.05 µg/kg and 2.35 µg/kg for sample of 10 kg and between 0.05 µg/kg and 3.85 µg/kg for those of 5 kg. The study on the homogeneity of OTA concentrations by an analysis of variance (mass of sample) performed by Fisher-test did not reveal a significant difference at 5% risk as shown in Table 5.

The average concentrations of OTA were 0.74 µg/kg and 0.85 µg/kg respectively for samples of 10 kg and 5 kg. These averages do not differ significantly at 5% risk and range from 0.05 to 2.35 µg/kg and 0.05 to 3.85 µg/kg respectively for samples of 10 kg and 5 kg. The range of OTA concentrations is larger for samples of 5 kg and it is also observable in samples with OTA content more than 2 µg/kg. We observed 4% of samples having a concentration greater than 2 µg/kg for samples of 10 kg against 10% for the 5 kg. Although

the two sampling methods are adequate for the determination of OTA, the standard method seems more stringent.

The reduction in mass sample does not have a great influence on the results of merchantability and OTA concentrations. All these results show a minor contamination compared to those obtained at the port of Abidjan by Dembele et al. [5] whose average was 1.3 µg/kg in a range of 0.00 to 4.7 µg/kg. However, these averages are in the same range as those obtained at the port of San Pedro by Dembele et al. [5] (0.7 µg/kg in a range of 0.00 to 8.2 mg/kg). These results also indicate a minor contamination than those obtained on cocoa beans from different origins, by Amezqueta et al. [24] whose average was 1.71 µg/kg in a range from 0.00 to 14.8 µg/kg. But they indicate a higher contamination than samples from production areas of Cote-d'Ivoire (average of 0.19 µg/kg in a range from 0.00 to 2 µg/kg) according to the study carried out by Laine [21].

Table 5. Levela of OTA in cocoa bean samples

Sample mass	Ota concentration		Sample percentage	
	Average	[Min-max]	<LD	>2 µg/kg
10 kg	0.74±0.58 a	0.05-2.35	11%	4%
5 kg	0.85±0.79 a	0.05-3.85	10%	10%

(Concentration in µg/kg) Means in a column bearing the same letter do not differ significantly at 5% risk

4. CONCLUSION

The alternative method proposed for the determination of OTA concentrations in cocoa beans was successfully validated. Moreover, the cutback in the amount of sample used for analysis (5 kg rather than 10 kg) does not influence negatively, from a statistical point of view, the results of the merchantability as well as the concentrations of OTA found in cocoa beans. Actually, the method commonly applied remains that of the European Communities, which advocates a sample mass of 10 kg of cocoa beans per batch. However, both methods could be safely used, as shown by the results of the validation, to assess the ever increasing health risk posed by the presence of Ochratoxin A in cocoa beans.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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