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Influence of Fermentation and Drying Practices on the Ochratoxin A Content of Cocoa Beans from the Main Production Areas in Côte d'Ivoire

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Abstract

Côte d'Ivoire has been the world's leading producer of cocoa beans for several decades. Apart from this production performance, the quality of the beans, which are mainly exported to the major chocolate-making countries, presents a quality problem to the point of suffering a discount on the international market. One of these quality problems is the content of ochratoxin A, a mycotoxin produced by fungi. Finally, to verify the level of contamination in beans produced in Côte d'Ivoire, a study was carried out. It consisted of collecting information on fermentation and drying times (The two major post-harvest operations) and collecting beans, which were analyzed by electrophoresis using the High Performance Liquid Chromatography (HPLC) method. The results obtained show ochratoxin A contents of between 0.05 µg/kg and 0.17 µg/kg. The general level of contamination is therefore very low and below the tolerable limit which is 2 µg/kg. In addition, the correlative study between the fermentation and drying times of the beans revealed no significant influence ($p < 0.01$) of the duration of these operations on the level of ochratoxin A contamination. Major contamination can occur after post-harvest activities carried out by producers. This is certainly due to the development of fungi responsible for the production of ochratoxin A during the period of storage and marketing of cocoa beans in conditions of high humidity in storage enclosures. Producers need to be made more aware of the need to ensure that cocoa beans are properly dried and stored in dry areas to avoid moisture build-up, which is a source of mould growth and ochratoxin A production.

Keywords

Ochratoxin A, Cocoa Beans, Fermentation, Drying Practices, Cote d'Ivoire,

Mycotoxins, HPLC Analysis, Fungal Contamination, Post-Harvest Operations, Food Safety

1. Introduction

Cocoa production is a very important economic activity in Côte d'Ivoire. The country is the world's leading producer of cocoa beans, with production estimated at more than 2 million tonnes a year since 1980 [1], contributing 15% of Gross Domestic Product. Most of this production is exported to European and American countries, which are major chocolate manufacturers [2]. Despite this production performance, satisfaction is not total, especially when it comes to the quality of the beans [3]. Indeed, while some countries, such as Ghana and Cameroon, offer quality cocoa on the international market, cocoa from Côte d'Ivoire is said to be of poor quality. One of the problems facing importers of cocoa beans from Côte d'Ivoire is their Ochratoxin A content, which is sometimes considered to be outside tolerable limits. Yet most of this production is intended for export to European and American countries, which are major chocolate manufacturers [4]. The latter often criticise Ivorian cocoa for containing levels of Ochratoxin A, which is a foodborne my-cotoxin found in various agricultural products [5], such as cocoa beans, that are outside tolerable limits [6]. This toxin is produced by different fungi notably by Aspergillus spaces. Studies about Ochatixin A had proven that it is toxic and carcinogenic [7]. Finding high levels of this toxin in products such as cocoa beans is therefore a danger for consumers of cocoa-based products. So, what is the real level of Ochratoxin A in cocoa beans from Côte d'Ivoire? This study was carried out to answer this question. Its general aim is to determine the ochratoxin A content in cocoa beans from the main production areas in Côte d'Ivoire.

2. Material and Methods

2.1. Study Areas

The study was conducted in the main cocoa-growing areas of Côte d'Ivoire. The choice of the East, Centre-West and South-West as the different study areas was motivated by the fact that they represent the major centres of cocoa production in Côte d'Ivoire. Indeed, around 64% of cocoa-growing areas are located in these three zones. What's more, they often provide more than 75% of the country's annual production. Each zone has been represented by an administrative locality. Abengourou represents the East, Oumé the Centre-West and Soubré the South-West.

2.2. Equipment

2.2.1. Plant Material

The plant material used in this study consisted of commercial cocoa beans

obtained after fermentation and drying. They were collected in the three main production areas selected for this study.

2.2.2. Sample Collection Equipment

The samples, each weighing 3 kg, were weighed using a CAMRY with a maximum capacity of 5 kg. Plastic bags were used to package the samples before they were transported to the laboratory.

2.3. Methods

2.3.1. Sampling of Cocoa Beans

For the determination of ochratoxin A content, samples of merchantable cocoa beans were collected in each production locality. These samples were taken from producers' stocks. A mass of 3 kg of cocoa beans was taken from each farmer immediately after drying, or during storage at the farmers' premises. On arrival at the laboratory, 350 g of beans were collected from each sample and kept in a freezer at a temperature of −22˚C. These samples were used to analyze the ochratoxin A content of the beans.

2.3.2. Collecting Information on the Duration of Fermentation and Drying of Cocoa Beans

Cocoa bean drying and fermentation times were recorded for all samples collected in the production areas. This information was used to establish a correlation between these two practices and the ochratoxin A content of the beans.

2.3.3. Measurement of Ochratoxin A (OTA) Content in Cocoa Beans

1) Characteristics of the HPLC method used

- **Fixed parameters of the method**

The OTA assay protocol was developed by the LARA laboratory in Toulouse. It was developed using the following chromatographic conditions:

- D-star instrument chromatograph, DAS-10 autosampler;
- HPLC column;
- mobile phase: acetonitrile/water/glacial acetic acid mixture (43/55/2);
- injection: 100 µl;
- flow rate: 1 ml/min;
- excitation wavelength: 330 nm;
- emission wavelength: 460 nm;
- OTA retention time between 3 and 4 min;
- detection limit: 0.05 µg/kg;
- chromatographic system thermostated at 25˚C;
- FL 3000 fluorescence detector.

- **Preparation of the ochratoxin A standard range**

A standard range of OTA concentration from 0.05 to 2 ng/ml was prepared from an intermediate solution B of concentration equal to 10 ng/ml, itself obtained from a commercial stock solution of concentration 1 mg/ml. The preparation technique is shown in Table 1.

The calibration curve is defined by the equation y = ax + b.

y = value of the area of the signal obtained.

x = concentration of the corresponding calibration solution (mg/l).

a = slope of the calibration line.

b = intersection of the calibration line with the y-axis.

The correlation coefficient of the calibration curve must be greater than 0.98.

Table 1. Preparation of the ochratoxin A standard range.

Standard solution N°	OTA solution B, concentration 10 ng/ml (µl)	Methanol/Acetic acid/Phosphate Buffer Saline (PBS) (µl)	Concentration of standard solution in OTA (ng/ml)
1	25	4975	0.05
2	100	4900	0.2
3	250	4750	0.5
4	500	4500	1
5	1000	4000	2

2) Determination of ochratoxin A content in samples

Ochratoxin A levels in cocoa beans were determined by High Performance Liquid Chromatography (HPLC) using the method of [8] used by [9].

- **Extraction**

The 350 g of cocoa beans collected and stored in a freezer at −22˚C for toxicological analysis were finely ground cold (frozen) using a propeller grinder. A 150 ml volume of a 3% (v/v) methanol/sodium hydrogen carbonate mixture was added to a 15 g test sample of bean grindings. After stirring for 10 min at 1300 rpm using a magnetic stirrer, the mixture obtained was filtered through Whatman paper. An aliquot volume of 11 ml of the filtrate, to which 11 ml of Phosphate Buffer Saline (PBS) was added, was used to purify the mycotoxin by immunoaffinity chromatography.

- **Purification by immunoaffinity chromatography**

A 20 ml volume of the diluted filtrate was poured at a flow rate of 2.5 ml/min. onto the immunoaffinity column (OchraprepR Rhône Diagnostics) containing a specific antibody that binds ochratoxin A. The column was then rinsed with 20 ml of PBS at a flow rate of 2.5 ml/min. The OTA bound to the immunoaffinity column was eluted with 1.5 ml of a methanol-acetic acid solution (98:2) at a flow rate of one drop/min. The eluate collected was used for the determination of ochratoxin A by HPLC.

- **Dosing**

An injection vial was filled with 1.5 ml of the eluate and then placed in the sample compartment. The ochratoxin A assay was performed on a volume of 100 µl taken automatically and injected into the assay circuit at a flow rate of 1 ml/min.

- **Expression of results**

The OTA concentration was calculated according to the following formula:

$$C(\mu g/kg) = \frac{S_{éch} \times Z_{std}}{S_{std}} \times \frac{V}{m} \times \frac{V_{ext}}{Va} \times \frac{V_1}{V_2}$$

C = OTA concentration in the sample (µg/kg).

$S_{éch}$ = peak area of OTA in sample (mm^2).

S_{std} = peak area of OTA in standard solution (mm^2).

Z_{std} = concentration of OTA in standard solution (µg/kg).

m = mass of test sample (15 g).

V = volume of solvent used for extract recovery (2.8 ml).

V_{ext} = volume of extraction solvent (150 ml).

Va = volume of aliquot taken after extraction (11 ml).

V_1 = volume of aliquot after dilution (22 ml).

V_2 = volume of diluted extract actually purified on the column (20 ml).

2.4. Data Analysis

The data collected was processed using Excell 2016 software. An analysis of variance was also performed in order to compare the averages for the quality parameters studied. In the event of a significant difference, the Newman-Keuls test was used to identify the means responsible for the difference observed at the 5% threshold. In addition, a linear regression test using Statistica 7.0 software was used to assess the influence of the duration of fermentation and drying of the beans on the ochratoxin A content.

3. Results

3.1. Fermentation Time

Fermentation time varies according to the cocoa-producing area. It ranged from 2 to 7 days in Abengourou, 3 to 7 days in Oumé and 3 to 8 days in Soubré. Fermentations lasting 4, 5 or 6 days are the most common. In Abengourou, 22% of growers fermented their beans for 4 days, 18% for 5 days and 42% for 6 days. The proportions of growers recorded in Oumé for the same fermentation times were 30%, 36% and 22% respectively. In Soubré, fermentations lasting 4 and 5 days were carried out by 26% of growers. Fermentations lasting 6 days were carried out by 20% of producers. Examination of the duration of fermentation from the point of view of two classes of duration (<6 days and ≥6 days), shows that in Abengourou 54% of growers ferment their beans for at least 6 days. In Oumé and Soubré, less than half (30% and 36% respectively) of farmers practise fermentation during the same period (**Table 2**). The average fermentation time for cocoa beans was 5.30 ± 1.18 days in Abengourou, 5.00 ± 1.01 days in Oumé and 5.04 ± 1.31 days in Soubré. These average times are statistically identical according to the Newman-Keuls test and significantly lower ($p < 0.01$) than the minimum time (6 days) recommended for good fermentation (**Table 3**).

3.2. Drying Time

The duration of cocoa bean drying, which is generally solar, is estimated at

between 7 and 15 days in the farming environment, depending on the drying conditions (duration of sunlight, level of fermentation, quantity of beans, number of stirring operations, etc.). In Abengourou, farmers dried their cocoa beans for 7.00 ± 0.91 days, compared with 5.21 ± 0.98 days in Oumé and 6.69 ± 1.25 days in Soubré. The average drying time in Oumé was statistically lower at the 5% threshold (Newman-Keuls test) than in Abengourou and Soubré, which are identical (Figure 1).

Table 2. Proportion (%) of producers according to the number of days cocoa beans are fermented in the areas studied.

		Number of fermentation days								
	Localities	Less than 6 days					More than 6 days			
		2	**3**	**4**	**5**	**Total**	**6**	**7**	**8**	**Total**
Proportions de Producteurs (%) par localité	Abengourou	2	4	22	18	**46**	42	12	0	**54**
	Oumé	0	4	30	36	**70**	22	8	0	**30**
	Soubré	0	12	26	26	**64**	20	14	2	**36**

Table 3. Average fermentation times for cocoa beans in the areas.

	Fermentation time (days)			
Localities	Abengourou	Oumé	Soubré	Standard
Average	5.30 ± 1.18a	5.00 ± 1.01a	5.04 ± 1.31a	T = 6 days
Comparison of duration	P* < 0.01	P* < 0.01	P* < 0.01	
To the standard				

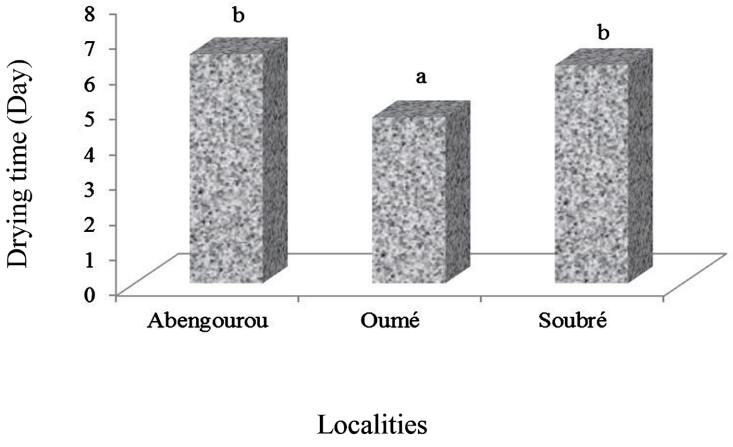

Figure 1. Bean drying time by location.

3.3. Ochratoxin A Content of Beans

Ochratoxin A levels are shown in Table 4. Some cocoa samples collected from producers are free of ochratoxin A. The average level for the Abengourou samples that did contain ochratoxin A was 0.17 ± 0.18 µg/kg. This was statistically different

($p < 0.01$) from the respective levels of 0.05 ± 0.09 and 0.05 ± 0.10 µg/kg obtained in beans from Oumé and Soubré. These averages are significantly below ($p < 0.01$) the tolerance threshold (2 µg/kg) whatever the production locality. In fact, regardless of the production locality, no sample showed an ochratoxin A content above the tolerance limit (2 µg/kg).

Table 4. Ochratoxin A levels in cocoa beans from the various locations studied.

Ochratoxin A levels (µg/kg)				
	Abengourou	Oumé	Soubré	
Mean ± Standard deviation	0.17 ± 0.18b	0.05 ± 0.09a	0.05 ± 0.10a	Quality standard: C < 2 µg/kg
Comparison of average to quality standard	P* < 0.01	P* < 0.01	P* < 0.01	

NB: On the same line, averages followed by the same letter are not significantly different at the 5% level (Newman-Keuls test); C = Ochratoxin A content; *: Mean significantly lower than the standard, at the 5% level (Student's t-test).

3.4. Influence of the Duration of Fermentation and Drying of Cocoa Beans on the Ochratoxin A Content

3.4.1. Influence of Fermentation Time on Ochratoxin A Content

Figure 2 shows the ochratoxin A content of cocoa beans as a function of fermentation time. According to the equation of the regression line ($y = 0.0141x + 0.0218$), there is a linear and positive correlation between the duration of fermentation of the beans and their ochratoxin A content. In fact, an increase in the fermentation time of the beans results in an increase in their ochratoxin A content. However, this correlation is very weak and not significant, as indicated by the correlation coefficient ($r = 0.1224$) and the probability ($p = 0.25$).

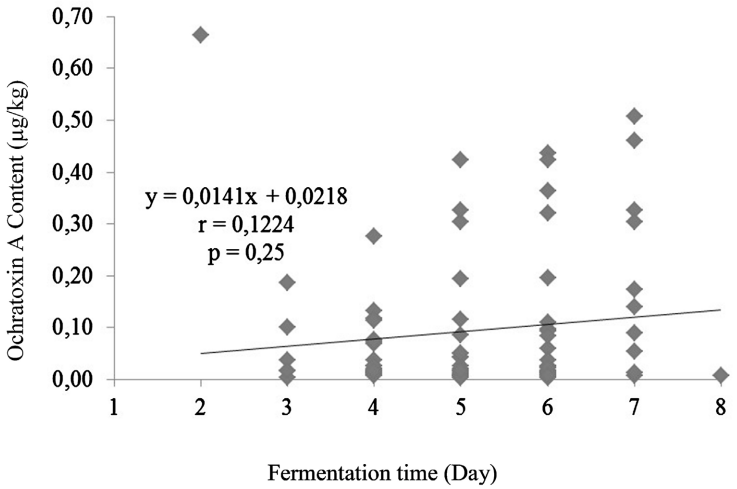

Figure 2. Correlation between bean fermentation time and ochratoxin A content.

3.4.2. Influence of Drying Time on Ochratoxin A Content

Figure 3 shows the influence of the drying time of the beans on their ochratoxin

A content. It shows that the drying time has no major influence on the ochratoxin A content of the beans. The equation of the regression line (y = −0.0018x + 0.0936) does show a negative linear correlation between bean drying time and ochratoxin A content, but this correlation is negligible (r = 0.002) and not significant (p = 0.89).

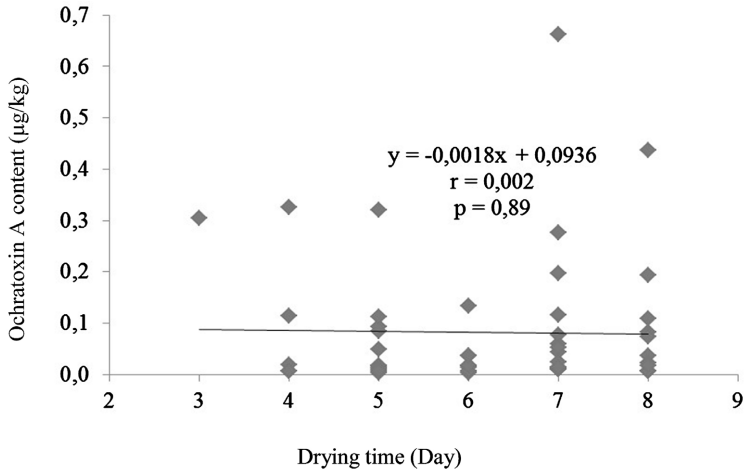

Figure 3. Correlation between bean drying time and ochratoxin A content.

4. Discussion

The analysis carried out to detect the presence of ochratoxin A revealed that in all these localities it was very low. An average level of 0.17 ± 0.18 µg/kg was obtained with the beans collected in Abengourou. This level is significantly higher than those for Oumé (0.05 ± 0.09 µg/kg) and Soubré (0.05 ± 0.10 µg/kg), which are themselves statistically identical. The difference in OTA content between the localities cannot be explained by the post-harvest practices observed in Abengourou, especially as they are better controlled there. Other possible reasons, such as the ochratogenic power of the types of mould found in this locality, could justify this. For example, [6] work showed that in Abengourou, the genus Aspergillus, which is the most pathogenic [10], accounts for 93% of fungi, compared with 88.87% in Oumé and 78.71% in Soubé. In addition, the very low levels of ochratoxin A in the samples (0.66 µg/kg maximum), well below the tolerance threshold given by the quality standards (2 µg/kg), reflect the total acceptability of cocoa beans from the different production areas in terms of the level of contamination by this toxin. This low level of ochatoxin A in cocoa beans from Côte d'Ivoire is comparable to the one obtained in cocoa beans from production areas in Cameroon, where levels vary from 0.20 µg/kg to 0.51 µg/kg [11].

The risks of sample contamination are well avoided by these Ivorian producers during post-harvest practices. Furthermore, this low level of sample contamination is justified by the fact that the cocoa beans were collected at the field edge, just after drying. It is different from the higher level obtained at the end of the internal marketing process, which goes from the end of technological treatments

to the export of the beans. Thus, the work of [12] on the level of contamination of cocoa beans for export places the average ochratoxin A content at 0.67 µg/kg in the warehouses of the port of San-Pédro and at 1.3 µg/kg in those of the port of Abidjan, with respective maximum values of 8.2 ± 0.02 and 4.7 ± 0.02 µg/kg. Indeed, the contamination of producers' cocoa beans is not spontaneous. Between the time when the cocoa is purchased from them and the time when it is delivered to the processors, a period of time may elapse during which contamination may occur. This occurs during storage periods often characterized by moisture regain, conditions favorable to the development of molds [13] and therefore to contaminations by ochratoxin A, if these molds are producers as demonstrated in the work of [14].

5. Conclusion

This study shows that the time taken to ferment cocoa beans does not differ significantly from one location to another. The average values of fermentation times are less than the recommended duration of 6 days for good fermentation of the beans. Cocoa beans from these two operations contain low levels of Ochratoxin. This is due to the fact that the fungi responsible for the production of ochratoxins A develop during the storage and commercial transaction period of cocoa beans, especially if humidity conditions are not respected. Awareness must be raised among producers so that the beans are well dried and stored in dry places to avoid moisture resumption, which is a source of mold development and ochratoxin production.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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