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Carcinogens, Aflatoxins and Hydroxylated Metabolites, in Rural Cocoa and Artisanal Chocolate Products

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ABSTRACT

Aflatoxins (AFs) are toxic secondary metabolites for all living creatures, mainly from the fungi Aspergillus flavus, A. parasiticus and A. nomius. They cause mutations, immunosuppression, liver damage, are proven carcinogens and cause death. The 4 basic AFs (AFB1, AFB2, AFG1 and AFG2) and four hydroxylated metabolites (AFM1, AFM2, AFP1 and aflatoxicol) were purified via immunoaffinity columns and quantified by liquid chromatography, in 80 samples of artisanal cocoa and chocolate products which had at least one type of AF. The statistical analysis included Kruskal-Wallis and Wilcoxon tests. AFs contaminated the cocoa (Theobroma cacao L.) in the field and industrialized samples with the following averages percentage and ranges (ng g-1): 63% of AFB1 (0.12-522), 21% of AFB2 (0.2-54), 29% of AFG1 (1.0-665), 33% of AFG2 (0.2-74), 16% of AFM1 (0.2-118), 18% of AFM2 (1.0-33), 36% of AFP1 (0.02-679), and 58% of aflatoxicol (0.3-531.1). The average of total aflatoxins in artisanal chocolate derivatives were 161 ng g-1, concentrations that surpassed the international tolerance levels of the Codex Alimentarius (10 ng g-1) in food for humans so they can be considered a health risk The most contaminated samples were grains, shells, and chocolate, and the least were cocoa butter and beverages.

Keywords

Fungal toxins, Creole cocoa, Derivatives.

Introduction

Theobroma cacao is a native tropical tree from the humid area of Meso-America, and its origin and domestication are considered to have occurred in Mexico in the states of Tabasco, Chiapas and Guerrero [1], although other reports consider its origin in northern South America. The Olmecs, from Mexico, were the first civilization to cultivate cocoa as a beverage at approximately 3500 to 400 BC. The Aztecs (1400 AD) prepared a bitter concentrated beverage from cocoa called techocolatl, prepared in a secret ritual just for the upper classes, emperor and warriors, and the cocoa grain was used as a coin or measurement unit [2].

Ghana by 1951 produced 60% of cocoa world's production. In 2015, Africa continued to contribute with 73% of the world's cocoa production. America produced 18% and Asia with Oceania producing 10% [3]. The six largest producer countries of cocoa are the Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon and Brazil [4]. Cocoa provides an income to 4.5 million families worldwide [2]. In Mexico, cocoa is sown in the southern states with a production of 27,287.25 tons [5]. Cocoa is consumed in "mole", a traditional food ingredient, and beverages in Mexico [1].

Cocoa butter is used to make cosmetics, perfume, soups, creams, emollients, and ointments. The cocoa seeds are 50% oil, containing linalool, aliphatic acid and some esters, that are used as flavoring. Cocoa has alkaloids, theobromine and caffeine, with stimulant properties and are used in candies, jams, ice creams and beverages. Cocoa contains fiber, saturated and unsaturated fatty acids; a low protein/amino acid content, which can be improved by

combining chocolate with milk; carbohydrates; starches, sugars; fats; minerals such as sodium, potassium, calcium, phosphorus, iron, magnesium, zinc, and folic acid; and vitamins A, E, B_1 and B_6 [6].

Aflatoxins

Aflatoxins (AFs), are toxic fungal secondary metabolites, bis-dihydrofuran-coumarins, produced mainly by *Aspergillus flavus, A. parasiticus* and *A. nomius,* and their synthesis is regulated by the AFLR and AFLS genes. Other fungi, such as *A. toxicarius, A. parvisclerotigeneus, A. bombycis, A. pseudotamarii, A. rambelli, A. ochraceoroseus, Emericella astellata* and *E. venezuelensis,* also produce AFs [7].

AFs are proven mutagens and carcinogens in humans [8] and cause various human cancers, such as hepatocarcinoma [9], cervical cancer [10], in lung [11], colorectal [12], pancreatic [13], and kidney cancer [14]. They are teratogens and attack mainly the liver, kidney, and brain, causing liver toxicities such as icteric, hepatitis [15], and cirrhosis [16]. They cause immunosuppression [17], dwarfism [18] miscarriages, protein malnutrition, Reye's syndrome and immunosuppression, vomiting, diarrhea, thymic aplasia [19], and abdominal pain, which affect reproductive functions and cause death [20]. There are 18 different AFs, classified depending on their fluorescence color and relative chromatographic mobility. When mammals eat AF (AFB_1, AFB_2, AFG_1, AFG_2) -contaminated food or feed, the liver produces hydroxylated metabolites (AFM_1, AFM_2, AFP_1, AFL) as a detoxifying product, to make them water soluble and excrete them [21], Figure 1.

Aflatoxin B_1 (AFB_1) is the most toxic and frequently occurring contaminant [22], its toxicological mechanism involves the formation of the epoxide radical, which interacts with DNA nucleosides, generating AFB_1-DNA-FAPY (AFB1-DNA-formamydopirimidine) adducts that are biomarkers of human cancer. These adducts cause genotoxicity and mutation in p53, where the transversion of guanine to thymine occurs at codon 249 [23].

AF determination in dark, white and milk chocolates has been performed [24]. The present research analyses rural cocoa field samples and artisanal chocolate from Mexico in different matrices for the four basic AFs and four hydroxylated metabolites with HPLC-FL for accurate validation of the methodology.

Aflatoxins are a worldwide problem that affects public health. Research and surveys are fundamental to control their impact on health. The purpose of this research was to identify and quantify eight aflatoxins in creole cocoa samples and processed chocolate derivatives.

Materials and Methods

Sampling

All the samples were bought on September 22th, 2020, in the Cocoa National Exposition in Culhuacán, Iztapalapa Jurisdiction, in Mexico City, where all the products were from the production States of Veracruz, Tabasco, Chiapas, Oaxaca, Puebla, Guerrero and Michoacan, later the samples were stored and refrigerated. There were 2 kinds of samples: creole cocoa and field products

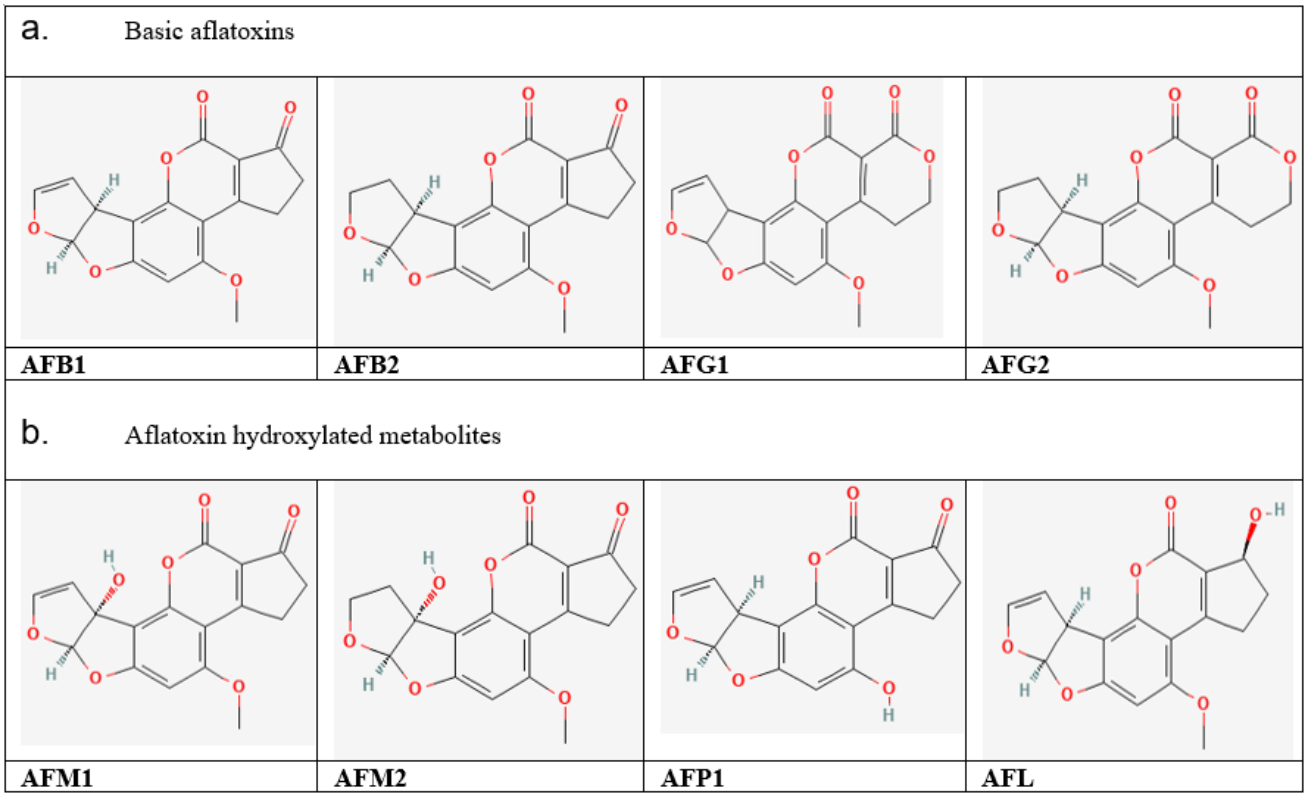


Figure 1: Analyzed basic aflatoxins and hydroxylated metabolites [30].

such as cobs, cocoa grains, hulls and nibs, which are pieces of the cocoa cob, and processed samples from artisanal chocolate derivatives. The shells or hulls were sold in the closed cobs with no grains, the field targets were the immature cocoa grains. The geographical origin of the 80 analyzed cocoa samples is shown in Figure 2, and the kinds of samples in Table 1.

Figure 2: Cocoa and chocolate production and sample origin states in Mexico.

The selection of hydroxylated metabolites were the commercially obtainable standards and the samples from raw materials, no samples had milk, they were chosen as pure cocoa products such as cocoa grains and husks, butter dough, cobs, powder, artisanal chocolate and liquid cocoa with no milk. Chocopinole is a mixture of dry cocoa and maize, with no milk.

Solid artisanal chocolate refers to the chocolate dissolved in water. The external ingredients were sugar, honey, water to wash, sometimes under a process of fermentation, but no milk at all.

Validation of the chemical method

The cocoa and chocolate sample analyses were performed using known parameters. To ensure that the equipment was calibrated and working properly, the method was validated [25]. The spectrophotometer (Genesys 10 UV Thermo Electron Corporation; Madison, WI, USA) was calibrated to measure the absorbance (357 to 360 nm) of the AF standard solutions.

This study demonstrates the real consumption doses, the total AF (AFt) per gram of sample. The parameters used for the validation were as follows:

Linearity (calibration curves) was assessed with 12 concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng mL^{-1}) of the 8 different AFs that were prepared from a 1000 ng stock solution and homogenized in an orbital shaker (Vortex G-560, Bohemia, NY, USA), with calculation of the coefficient of determination (R^2). The purification and concentration of the 8 types of AFs were performed with known methods [26].

Limits of detection (LOD), limits of quantification (LOQ), and standard deviation (SD) were calculated using Excel 2003 [26]. The smallest detected AF concentration was considered LOD.

Selectivity

A pair of peaks with less chromatographic resolution, in this case AFM_1 and AFL, were chosen. An acceptable distance of the two compounds is when the resolution is higher to one (Rs > 1).

Percentage of recovery (% PR) [27]

Is the recovered amount of AF from a spiked sample, submitted to the entire analytical method, it allows us to evaluate the efficiency of the entire method. For its accomplishment, subsamples of one gram of dried cocoa were diluted in PBS (1:4 v/v) and individually spiked with 3 different concentrations of 50, 100 and 200 ng $mL^{-1}$of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFP_1 and AFL and the entire method was applied.

A not spiked aliquot was used as a control to provide the basal contamination level. The rest of the chemical extraction method was performed in triplicate using total aflatoxin immunoaffinity extraction columns (Easi-Extract R-Biopharm Rhône LTD, Glasgow, UK). The eluates were derivatized [28]; and quantified by high-performance liquid chromatography with fluorescence detection (HPLC-FL), after which the percentage of recovery was applied for each type of AF.

After obtaining the area and AF concentration, an adjustment to 100% was done to know the AF quantity recovered in the chemical analysis of the samples. This point of view is of upmost importance, because it allows us to adjust the AF concentration in the samples and obtain the real results of them.

AF chemical extraction

The 80 samples of 50 g were individually blended (Waring Commercial Blender, 7010S Mod. WF2211214 Torrington Connecticut, USA), with a 250 mL solution of methanol/ water (80:20 v/v) and 5 g de NaCl, for 2 min at high speed to obtain a homogeneous mixture placed in 50 mL Falcon tubes, that were centrifuged (4235CWS, ALC Cool Working System, Milán, Italy) at 4000 rpm for 15 min to obtain the supernatant.

A sample mixture equivalent to one gram was vortexed and applied to Easi-extract AFt immunoaffinity columns previously activated with 20 mL of phosphate buffer saline (PBS) pH 7.4, and finally washed twice with distilled water [29]. To recover the eluate, 1.5 mL methanol HPLC degree (J.T. Baker MeOH N° Cat. 67-56-1) was applied to the immunoaffinity column and 1.5 mL distilled water with reflux. The 3 mL eluate was received in amber vials and it was dried at 40°C in an oven (Novatech BTC 9100, Guadalajara, Jal. México), and it was derivatized [28], cooled to room temperature. The sample extract quantification method was

Table 1: Geographical origin and artisanal presentation of samples.

Sample N°	State	Municipality	Presentation	
1	Oaxaca	San Felipe Usila	Cob	Unopened creole cob.
7			Cocoa grains	Fermented creole
8				Toasted
9				Dry with shell
67			Powder	Powder
43-55, 62-65		Santa María del Tule	Artisanal chocolate	Solid artisanal chocolate
74-75		Unknown	Beverage	Liquid cocoa
2	Veracruz	Tezonapa	Cobs	Unopened creole cob
10		Paraíso de la Reforma	Cocoa grains	Washed
3		Unknown	Cob	Closed cob
11	Chiapas	Palenque	Cocoa grains	-
13		Tapachula		Toasted and bared
26		Chiapas/Milpa Alta	Chocopinole	Cocoa and maize powder
27		Unknown	Liquor	100% cocoa paste
18		Unknown	Cocoa grains	With chocolate
19		Tapachula, Soconusco	Cocoa husk	Creole
30		Unknown	Butter dough	100% cocoa paste
40-42		Ixcomitan and Pichucalco	Artisanal chocolate	Solid artisanal chocolate
58-59		Unknown		
66		Tapachula, Soconusco	Butter	Cocoa butter
69-70		Soconusco	Powder	Powder to make beverage
79		Unknown	Sweet	Solid with sugar
80		Soconusco		
5	Michoacán	La Mira	Cocoa grains	-
4		Nuevo Urecho		Ivory or porcelaine
6				Coloured
12	Tabasco	La Chontalpa		Toasted
22			Nibs [a]	With honey
23				-
28, 31			Butter dough	100% cocoa paste
29, 32				70% cocoa paste
14		Comalcalco	Cocoa grains	Washed
15				Fermented
16,17				Toasted covered with sugar
20			Cocoa husk	Half bitter bar
24		Comalcalco, Chontalpa	Nibs [a]	-
33		Comalcalco	Butter dough	100% cocoa paste
60,61			Artisanal chocolate	Solid artisanal chocolate
68			Fine powder	Refined powder
71-73			Powder	Powder to make beverages
77			Beverage	Liquid cocoa
21	Guerrero	Unknown	Cocoa	With honey
34		Omeapa	Artisanal chocolate	Solid artisanal chocolate
35		Amusga		
36-39		Unknown		
25		Xochistlahuacan	Nibs	-
56, 57			Artisanal chocolate	Solid artisanal chocolate
78			Sweet	Solid with sugar
76	Puebla	Cholula	Beverage	Liquid cocoa

[a] Nibs = piece of cocoa cob.

High Performance Liquid Chromatography (HPLC), with Series 1200 Agilent Technologies equipment, isocratic pump (G1310A Series DE62957044), fluorescence detector (G1321A Series DE60456380) and autosampler (G1329A Serie DE64761666); with a chromatographic column (Agilent Eclipse XDS-C18), 4.6 × 250 mm, 5 µm of size particle and the computarized program for HPLC was ChemStation 32.

The analytical conditions were a mobile phase H_2O/ACN/MeOH (65:15:20 v/v/v), 60 µL inyection, flux 1 mL min^{-1}, for 30 min, 360-362 nm excitation length wave, 425 nm emission length wave for AFB1, AFB2, AFM1 and AFL and 450 nm for AFG1 and AFG2 [21], each sample (60 µL) was injected into the HPLC-FL in triplicate and the percentage of recovery was adjusted to the final quantified amount for each AF.

Statistical analysis

A Kruskal-Wallis test for each of the AFs was performed to determine whether there were significant differences among the samples. Then, the Wilcoxon rank test was used to determine whether there were pairwise differences. There were a number of samples with no AF and a number of samples with AF but no statistically significant difference from zero. We focused on the samples different from zero.

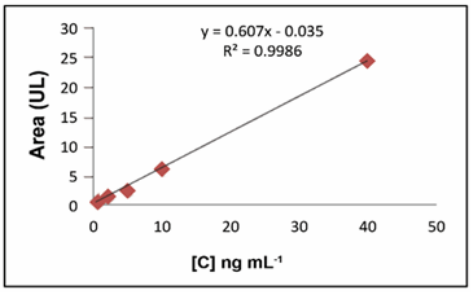
Results and Discussion

Results of the method validation

The validation parameters of the chemical method were demonstrated with the AF standard calibration curves for the basic AFs (AFB_1, AFB_2, AFG_1, and AFG_2) and hydroxylated metabolites (AFM_1, AFM_2, AFP_1 and AFL). Figure 3. The limit of detection (LOD), and the coefficient of determination R^2 are exposed in Figure 3a, 3b; and Table 2.

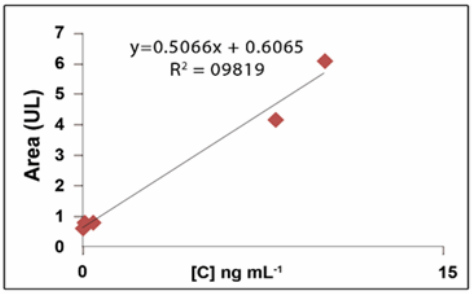
AFB1

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.5	0.341	8.849
1	0.993	8.695
2	1.295	7.877
5	2.357	7.638
10	5.995	7.347
40	24.317	7.085



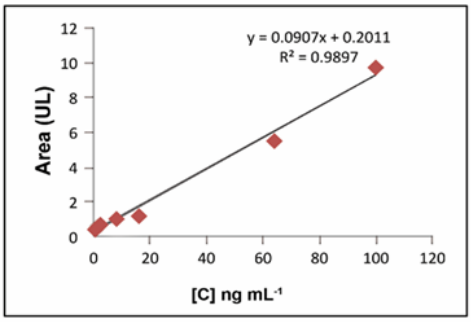
AFB2

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.05	0.63	17.452
0.1	0.8	18.141
0.5	0.82	18.93
8	4.15	20.062
10	6.08	20.228



AFG1

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.5	0.44	8.473
1	0.46	7.681
2	0.58	9.541
8	0.96	9.003
16	1.19	9.166
64	5.46	9.101



AFG2

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.5	0.20	14.177
1	1.05	11.215
2	1.57	12.062
4	5.91	12.002
10	12.78	14.507
16	17.47	14.513
32	35.12	14.476
64	91.13	14.492
100	159.66	14.464

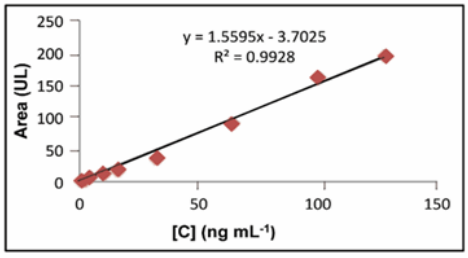
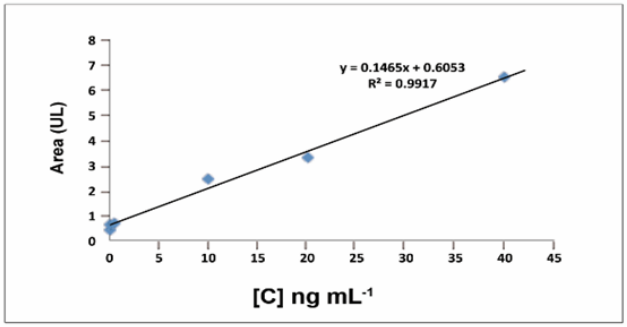


Figure 3a: Calibration curves of the 4 basic aflatoxin standards [30].

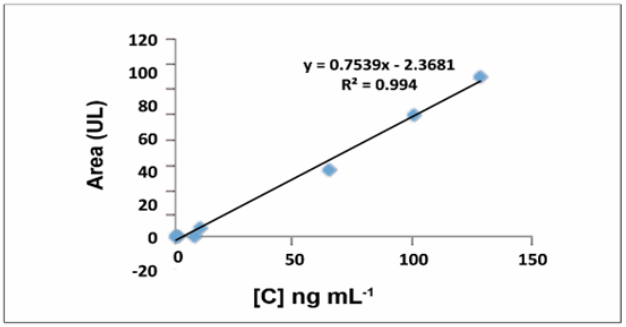
AFM1

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.05	0.425	3.024
0.1	0.636	2.941
0.5	0.696	3.008
10	2.436	2.935
20	3.296	2.971
40	6.495	2.920



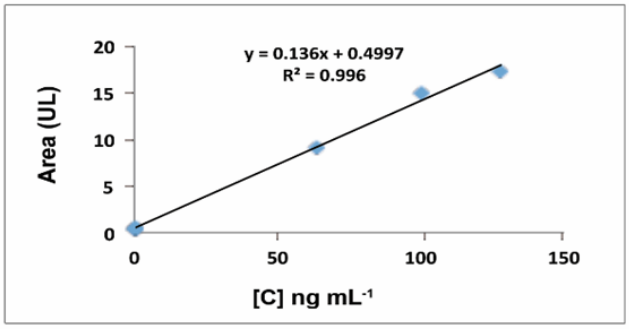
AFM2

[C](ng mL^{-1})	Area (UL)	RT (min)
0.05	0.146	20.208
0.5	0.16	22.447
2	0.5	20.544
64	38.9	20.966
100	73.412	20.905
128	90.17	20.844



AFP$_1$

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.05	0.356861	15.563
0.1	0.432036	16.711
0.5	0.658508	16.817
64	9.10722	19.318
100	14.97025	18.065
128	17.28316	18.213



AFL

[C] (ng mL^{-1})	Area (UL)	RT (min)
0.01	0.39	4.605
0.1	0.55	3.513
1	0.69	4.859
2	1.3	4.16
5	2.9	3.353
15	6.9	4.265
25	10.5	3.032
30	12.6	3.81
40	17.6	5.569

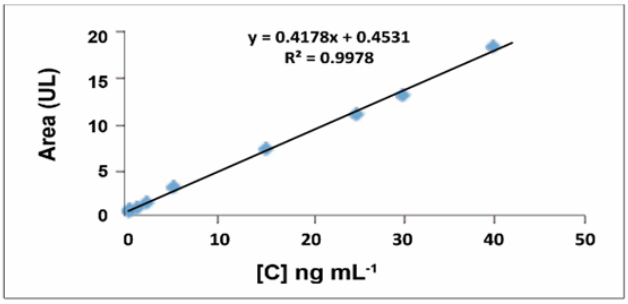


Figure 3b: Calibration curves of 4 hydroxylated metabolites of aflatoxins.

Figure 3a) Calibration curves of the 4 basic aflatoxins. b) Calibration curves of 4 hydroxylated metabolites of aflatoxins. Area µL = Area luminescence units; [C] (ng mL^{-1}) = Concentration (ng mL^{-1}); RT (min)= Retention time (minutes). AFB_1= aflatoxin B_1; AFB_2 = Aflatoxin B_2; AFG_1 = Aflatoxin G_1; AFG_2 = Aflatoxin G_2 [30].

Table 2: Validation parameters of the analyzed aflatoxins (ng g^{-1}).

Aflatoxin	LOD (ng g^{-1})	LOQ (ng g^{-1})	Retention time range (min)	R^2	% of recovery
AFB_1	0.5	5	7.085-8.849	0.9986	100
AFB_2	0.05	0.5	17.452-20.228	0.9817	98
AFG_1	0.5	5	7.681-9.541	0.9898	85
AFG_2	0.5	5	11.215-14.513	0.9946	98
AFM_1	0.05	0.5	2.920-3.024	0.9917	100
AFM_2	0.05	0.5	20.208-22.447	0.9946	98
AFP_1	0.05	0.5	15.563-19.318	0.9960	98
AFL	0.01	0.1	3.032-5.569	0.9978	96

A recovery of 85-100%, good selectivity and chromatographic resolution were obtained, so the measurement of the AFs was considered accurate and reliable.

AF concentrations in cocoa samples

The 80 cocoa samples were contaminated with at least one AF or hydroxylated metabolite per gram. The total AF (AFt) is high because it represents the addition of the 8 AFs and not only the 4 most common basic AFs. AFt represents the real internal consumption ratio of AFs.

Most of the contaminating *Aspergillus* fungi are in the hull, and can be removed in processing. In 80% of the cocoa seeds from New Guinea and Ecuador, one-third contained *A. flavus,* and *A. parasiticus* was not found. *A. parasiticus* has been reported in cocoa substitutes from Africa. The storage conditions of temperature and humidity determine the contamination risk, as do the cocoa seeds for butter [31].

In the fermentation and drying stages, the contaminating fungi diminish [32]. Dry dough has both *A. flavus* and *A. parasiticus*. Chocolate bars, powder and liqueur have mainly *A. flavus* [33]. Creole cocoa and artisanal chocolate derivatives in Mexico had 8 different AFs and both species *A. flavus* and *A. parasiticus* were present. AF contamination in the field cocoa samples are shown in Table 3 and Figures 4a, 4b. The most carcinogenic and frequent AFs were AFB1 and AFL.

The highest health risk and possibility of ingestion of AF types in cocoa were AFB1 and AFL that are the most toxic and dangerous ones. AFB1 and AFL, this fact makes cocoa a food that has mutagens with cancer risk.

Table 3: Concentration of Aflatoxins (ng g^{-1}) and hydroxylated metabolites (ng g^{-1}) in creole cocoa and field products from Mexico.

Type	Sample	Basic Aflatoxins				Hydroxylated metabolites				Total
		AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	AFP_1	AFL	AFt
Cobs	1	0	0	0	0	0	0	412	0	412
	2	164	9	0	0	0	0	0	0	173
	3	56	0	0	28	0	0	0	0	84
Cocoa Grains	4	**24**	0	0	0	0	0	0	0	24
	5	49	0	55	0	0	0	0	53	157
	6	0	0	0	0	0	0	0	148	148
	7	4	0	0	0	0	0	0	100	104
	8	28	0	0	0	0	0	0	0	28
	9	72	0	0	0	0	0	0	0	72
	10	522	0	0	0	0	0	0	0	522
	11	0	0	0	0	0	0	0	387	387
	12	0	6	0	0	0	0	20	531	557
	13	168	0	22	38	0	0	0	0	228
	14	318	0	0	0	0	0	0	293	611
	15	140	0	470	0	3	0	0	137	750
	16	186	0	0	0	0	0	679	10	875
	17	78	0	0	36	0	0	0	0	114
	18	108	0	0	0	0	0	0	81	189
Hull	19	95	0	407	6	0	20	0	0	528
	20	0	5	7	27	0	8	9	0	56
Nibs [a]	21	0	0	0	22	0	0	0	0	22
	22	44	0	0	3	0	0	46	0	94
	23	213	0.2	0	12	0	0	316	46	687
	24	228	0.2	226	0	0	0	0	24	478
	25	0	0	0	<LOD	0	1	0.2	0.5	2
Average [C] (ng g^{-1})		**100**	**1**	**48**	**7**		**1**	**59**	**72**	**288**
Frequency (%)		**72**	**20**	**24**	**32**		**12**	**28**	**48**	**100**

[a] Nibs = Pieces of cocoa cob. [C] = concentration

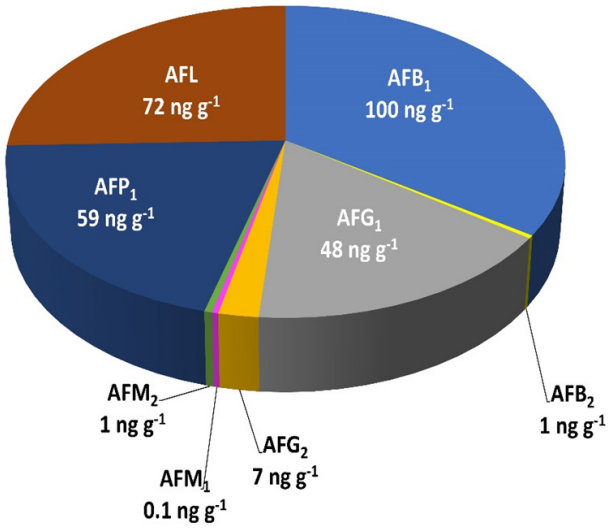


Figure 4a: Average AF concentration (ng g^{-1}), or health risk, of each Aflatoxin type in creole cocoa and in field cocoa products.

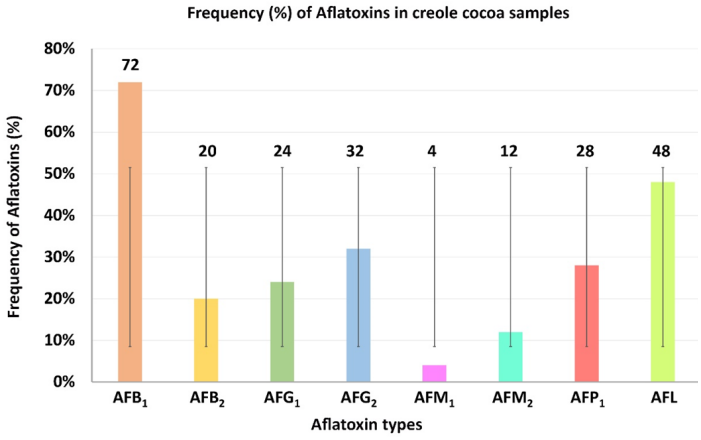
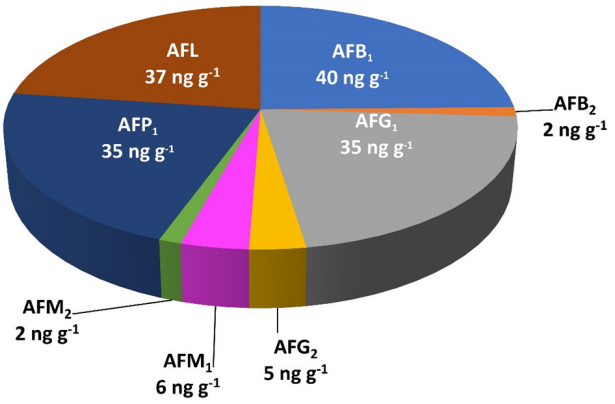


Figure 4b: Frequency (%), or possibility of ingestion, of the different types of AF and their hydroxylated metabolites in creole cocoa.



The rural processed cocoa products are shown in Table 4, Figure 5a, 5b.

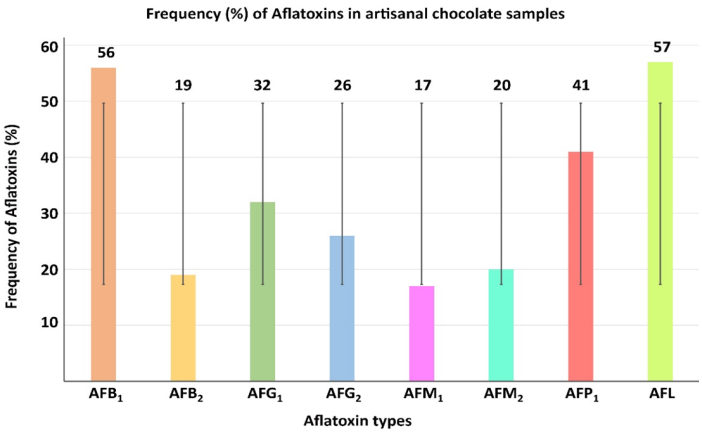


Figure 5a: Average AF concentration (ng g-1), or health risk, of each Aflatoxin type in artisanal chocolate by-products.

Figure 5b: Frequency (%), or possibility of ingestion, of the different types of AF (ng g^{-1}) and their hydroxylated metabolites in artisanal chocolate by-products.

The storage conditions of temperature and humidity determine the contamination risk, as do the cocoa seeds for butter [31]. Ochratoxin A (OTA) is frequently found together with AFs [34]. The stage of roasting, which occurs between 120 and 150°C, is well supported by AFs [35]. AFB_1 resists 267°C before decomposition, so this stage does not reduce AF contamination [36]. AF hydroxylated metabolites are important to consider because they are detoxification compounds formed by the cocoa plant that are almost never reported in cocoa studies. Samples are naturally contaminated, probably with *A. flavus* [35] or *A. parasiticus*, which produces AFB and AFG [37]. Insects make wounds that facilitate seed and fruit contamination by AFs, which accumulate in the cuticle and tegument. AFs develop in foods rich in carbohydrates, grease and oils and secrete hydrolases, proteases, amylases, lipases and pectinases [38]. Lipases have an important hydrolytic role in metabolism when triglycerides are used as carbon sources. The lipA gene encodes lipase and promotes AF synthesis in environments rich in lipids because it captures carbon nutrients from these sources [39].

The most contaminated samples were grains (522 to 610 ng g^{-1}), hull (508 ng g^{-1}) and chocolate (666 ng g^{-1}). Regarding the hydroxylated metabolites, the most contaminated samples were grains (412 to 688 ng g^{-1}), and chocolate (666 ng g^{-1}). The least contaminated compounds were sweets (35 ng g^{-1}), powder (27 ng g^{-1}), butter (26 ng g^{-1}) and beverages (7 ng g^{-1}). Figure 5 a,b.

The synthesis of polyketidic compounds such as aflatoxins, has similar patterns to lipids where there is a reduction in ketonic groups and the metabolic route of fatty acids is enhanced [40].

Table 4: Aflatoxins and hydroxylated metabolites of artisanal chocolate derivatives.

	Sample	Basic aflatoxins				Hydroxylated metabolites				Total AF
		AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	AFP_1	AFL	
Chocopinole[a]	26	69	0	200	0	0	0	0	42	311
Liquor/paste	27	0	0	0	0	0	0	0	110	110
Paste	28	7	6	4	0	0	0	6	57	80
	29	0	0	0	0	0	0	2	44	46
	30	0	0	0	0	106	0	0	5	111
	31	0	0	0	0	0	0	0	175	175
	32	0	0	0	0	0	0	0	118	118
	33	0	0	0	0	0	0	0	180	180
Chocolate	34	<LOD	0	0	0	0	0	0	14	15
	35	1	0	0	0	0	0	0	17	18
	36	0	0	0	0	0	4	146	0	150
	37	68	0	0	0	0	0	0	0	68
	38	0	0	0	32	0	0	1	0	33
	39	20	0	0	0	0	0	0	20	40
	40	44	0	13	2	0	0	0	34	92
	41	0	< LOD	0	35	0	0	29	0	64
	42	32	0	14	74	0	0	14	0	134
	43	37	0	21	0	0	0	0	0	58
	44	4	0	88	3	0	0	0	0	95
	45	0	0	0	11	0	0	1	0	12
	46	24	5	0	26	0	0	1	0	56
	47	18	0	18	0	0	0	35	0	71
	48	0.5	4	0	0	0	0	1	11	16
	49	0	0	0	0	0	0	15	0	15
	50	0	0	60	22	0	0	<LOD	0	82
	51	70	0	18	3	0	0	0	3	93
	52	111	0	8	0	0.2	0	0	43	162
	53	8	0	0	0	0	0	0	4	12
	54	0	0	665	1	0	0	0	0	666
	55	55	0	6	0	0	0	0	1	62
	56	74	0	0	3	0	0	82	0	159
	57	1	5	306	1	0	0	183	0	495
	58	28	0	0	0	0	0	430	0	458
	59	0	4	0	2	0	3	0.4	82	91
	60	0	0	0	0	118	0	0	0	118
	61	0	0	0	0	117	0	0	1	118
	62	0	0	0	0	50	0	158	0	208
	63	0	0	0	0	45	1	188	0.4	234
	64	0	54	0	0	0	33	0	0	87
	65	0	0	131	0	15	0	0	0	146
Cocoa butter	66	1	0	0	0	0	0	0	25	26
Powder	67	23	0	26	0	0	0	0	3	52
Fine powder	68	8	0	3	0	0	0	0	11	22
Powder for beverage	69	6	0	1	0	0	8	0	8	23
	70	16	0	0	0	0	0	0	37	53
	71	2	0	0	<LOD	0	0	0	0	2
	72	0	0	0	0	18	0	0	15	33
	73	<LOD	0	0	0	0	1	0	4	5
Liquid beverage	74	0	0	0	0	0	0	0	4	4
	75	<LOD	0	0	<LOD	0	0	0	0.4	1
	76	<LOD	0.2	0	1	0	7	0.4	2	11
	77	<LOD	0	0	<LOD	0	11	0	0	11
Sweet chocolate	68	0	4	0	0	0	21	0.3	0	25
	79	<LOD	3	0	0	0	16	22	0.3	41
	80	7	11	0	0	1	21	1	0	39
Average [C]		**40**	**2**	**35**	**5**	**6**	**2**	**35**	**37**	**161**
Frequency (%)		**46**	**19**	**32**	**26**	**17**	**20**	**41**	**57**	**100**

a Chocopinole= Mixture of chocolate with maize powder. [C] = Concentration. <LOD = less than the limit of detection.

Aflatoxin P_1 (AFP_1) is a phenolic derivative that results from the O-demethylation of AFB_1, with a reduced toxicity in comparison to AFB_1, and represents more a longer detoxification stage than an activation stage. An average of 59 ng g^{-1} AFP_1 was observed in the field samples versus an average of 35 ng g^{-1} in the processed samples.

Aflatoxins M_1 (AFM_1) and M_2 (AFM_2) are metabolic oxidative products of AFB_1 and AFB_2 produced by the organism that consumes them, with AFM_1 being 2 to 10% less carcinogenic than AFB_1.

The processes of drying, roasting and fermenting cocoa did not diminish the AFs, and the humidity of the washings enhanced them. Although phenols, naphthoquinones, tannins, plumbites, triterpenoids, and sterols have potent effects against AF biosynthesis; they are possibly located only in the seed or in small amounts because they do not show meaningful anti-aflatoxigenic activity. The final edible or cosmetic products will be cocoa cake, butter, pulp, shell, ash of cocoa shell, powder, paste, cream, liquor or chocolate [32].

The most abundant alkaloid in cocoa and chocolate is theobromine, a metabolite of caffeine, followed by antioxidants, such as anthocyanins, flavonoids, and phenolic compounds [41]. In the case of nibs with honey the AFs diminished, in comparison with samples without honey. In the statistical analysis, there was not much significant difference between geographical states and processing methods, such as washing, fermenting, roasting, and drying, but there were significant differences with the AF contaminated added filling.

The hydroxylated metabolite Aflatoxicol (AFL) was the most abundant AF in the cocoa samples, it interconverts with AFB_1 and can increase the mutagenic and carcinogenic risk, although it is 18 times less toxic than AFB_1. AFB_1 is so aggressive that the liver responds by including an OH^- in the molecule, transforming it into AFL, making it more soluble in water than AFB_1, and can be excreted in urine. AFL is frequently present in milk [42], cheese [43], poultry feed and litter [44], breast muscle of laying hens [45], dog food [46], cat food [47], popcorn [30] and many other foods. The average amounts of AFt in cocoa were high (288 ng g^{-1}), especially from the raw field samples, in comparison to the AFt average of other oilseed products, such as almonds (273 ng g^{-1}) [37], walnuts (3 ng g^{-1}), cashew nuts (1.4 ng g^{-1}), pecans (0.4 ng g^{-1}) [48], peanuts (8.56 ng g^{-1}) and pistachios (0.04–8.39 ng g^{-1}), On the other hand, vanilla, mint and anise have been reported to be AFt-negative [49].

Oilseeds as fillings, can increase AFs in traditional chocolate, and honey can diminish them, Table 5.

Cocoa is heavily contaminated with AFt, and lack a legislation to protect human population. The processing of cocoa involves the AF reduction steps of harvesting, cleaning, roasting, shelling, and milling. In cocoa powder, the AFL contamination prevailed, although it has been dismissed in international legislation.

Table 5: Ingredients that contribute with Aflatoxins in the cocoa derivates.

Sample N°	Ingredients	AFt (ng g^{-1}) average
1 to 3	Cocoa cobs	223
4 to 18	Fresh cocoa grains	318
19, 20	Hull	292
34 to 65	Rural chocolate alone	129
21	Nibs with honey	22
22		94
23	Nibs without honey	687
24, 25		478
26	Chocopinole	311
27	Liquor/paste	110
28 to 33	Paste	118
46	Cinnamon	56
63		234
49	Peanuts	15
80		39
51	Hazelnuts	93
52	Almonds	162
61		118
62		208
54	Nuts	666
45	Coconut	12
64		87
47	Coffee	71
50	Mint	82
55	Vanilla	62
60	Tamarind	118
53	Anise	12
48	Flowers	16
66	Cocoa butter	26
67	Powder	52
68	Fine powder	22
69 to 73	Powder for beverage	23
68,79, 80	Sweet chocolate	35
74 to 77	Liquid beverage	7

Statistical Analysis

The values of the Kruskal-Wallis statistical analysis were significant, Table 6.

The Wilcoxon rank test determined that all the samples were significantly different. The AFs AFB_1, AFM_1, and AFL are mutagenic and eventually carcinogenic. Although AFB_2, AFG_2 and AFM_2 were statistically significant, they are not carcinogenic by themselves because they have no double bond in the first furan. AFL is significant because it is mutagenic and a possible reservoir of AFB_1.

Concluding, eight AFs were present in the cocoa products, being more contaminated the samples from the field, creole cobs, fresh

grains, hulls and nips, than the processed chocolate derived products such as chocolate, powder beverage, liquors, etc. The AF contamination in cocoa was high and the amount of AFs might increase in combination with milk, so cocoa can be considered a risk food that contributes to the presence of human cancer.

Table 6: Statistical analysis, chi square and p-value of the eight aflatoxins.

Aflatoxin	Chi squared statistic (79 fd)	p-value
AFB1	131.112	0.0002
AFB2	164.991	<0.0001
AFG1	104.172	0.0305
AFG2	143.122	<0.0001
AFM1	169.546	0.0001
AFM2	172.332	<0.0001
AFP1	111.418	0.0095
AFL	191.214	**<0.0001**
AFt	122.110	0.0013

Fd = freedom degree.

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