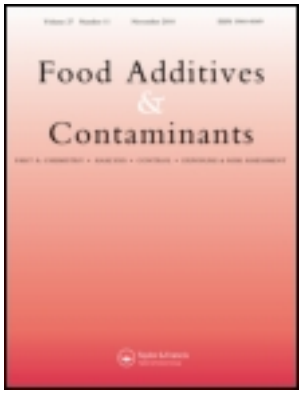

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Determination of aflatoxins in by-products of industrial processing of cocoa beans

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Determination of aflatoxins in by-products of industrial processing of cocoa beans

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This study has examined the occurrence of aflatoxins in 168 samples of different fractions obtained during the processing of cocoa in manufacturing plants (shell, nibs, mass, butter, cake and powder) using an optimised methodology for cocoa by-products. The method validation was based on selectivity, linearity, limit of detection and recovery. The method was shown to be adequate for use in quantifying the contamination of cocoa by aflatoxins B_1, B_2, G_1 and G_2. Furthermore, the method was easier to use than other methods available in the literature. For aflatoxin extraction from cocoa samples, a methanol–water solution was used, and then immunoaffinity columns were employed for clean-up before the determination by high-performance liquid chromatography. A survey demonstrated a widespread occurrence of aflatoxins in cocoa by-products, although in general the levels of aflatoxins present in the fractions from industrial processing of cocoa were low. A maximum aflatoxin contamination of 13.3 ng g^{-1} was found in a nib sample. The lowest contamination levels were found in cocoa butter. Continued monitoring of aflatoxins in cocoa by-products is nevertheless necessary because these toxins have a high toxicity to humans and cocoa is widely consumed by children through cocoa-containing products, like candies.

Keywords: chocolate; mycotoxin; aflatoxin; cocoa processing; methodology; validation

Introduction

Mycotoxins are toxic low-molecular-weight compounds, produced by some fungal species. Their occurrence in foods is regulated in more than 77 countries around the world (FAO 2003), where the maximum level allowed varies among the countries according to the mycotoxin in question and the food matrix. Aflatoxins are the most studied and regulated mycotoxins because they have high toxicity to humans and are widespread in foods. Aflatoxins are hepatotoxic, teratogenic, mutagenic and carcinogenic compounds classified as Group 1 carcinogen by the International Agency of Research on Cancer (IARC 1993).

Because of the diversity of their chemical structure and the wide variety of foods in which mycotoxins can be present, it is not possible to use a single technique for their analysis (Turner et al. 2009). For aflatoxins, the most often employed methods are chromatographic, because the fluorescent properties of these compounds allow for their detection at very low levels. The introduction of immunoaffinity columns with monoclonal antibodies for the clean-up step significantly improved the sensitivity of methods, permitting the isolation and concentration of these mycotoxins from complex matrices without the use of toxic organic solvents such as chloroform (Patel 2004).

Despite the existence of reports on aflatoxin occurrence in levels up to 17 ng g^{-1} in cocoa beans (Campbell 1969) and the description of detection techniques since 1971 (Scott and Przybylski 1971; Hurst et al. 1982), cocoa and cocoa by-products for a long time have been ignored with respect to aflatoxins because of the theory that caffeine inhibits the production of this mycotoxin in cocoa (Buchanan and Lewis 1984). The lack of simple and sensitive detection methods also contributed to the failure to undertake assessment of these contaminants in cocoa.

The increasing restrictions on the presence of aflatoxins in food and recent reports of its occurrence in cocoa and chocolate (Raters and Matissek 2000, 2005; Kumagai et al. 2008; Ulca et al. 2010; Copetti et al. 2011, 2012) demonstrated a need for a more detailed investigation to verify the levels of contamination in cocoa products obtained during the processing of cocoa, the main raw material for producing chocolate products. Thus, the objective of this study was to evaluate the occurrence of aflatoxin-producing fungi

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and aflatoxins in cocoa processed in Brazil after the validation of a methodology for this product.

Materials and methods

Method validation for analyses of aflatoxins B_1, B_2, G_1 and G_2 in cocoa

The method for aflatoxin B_1, B_2, G_1 and G_2 was reported by Copetti et al. (2011). The validation was based on establishing selectivity, linearity, limit of detection and recovery of the method. Aflatoxins were extracted and detected using immunoaffinity columns specific for this mycotoxin group and HPLC used a fluorescence detector.

For assessment of linearity, 7-point calibration curves were plotted, with a correlation coefficient (r) >0.99. To obtain the limit of detection (LOD), eight artificially contaminated cocoa samples with low total aflatoxin levels ($<0.05\,ng\,g^{-1}$) were analysed and the standard deviation was calculated. These values were multiplied by the corresponding number listed on the Student's t table for 99% significance. The limit of quantification was determined multiplying standard deviation by 10 (Keith et al. 1983; Long and Winefordner 1983; Eurachem Guides 1998). The recovery of aflatoxins was carried out in triplicate after the contamination of three finely ground cocoa beans with 0.40, 0.24, 0.15 and $0.18\,ng\,g^{-1}$, respectively, for aflatoxin B_1, B_2, G_1 and G_2. The recovery was also determined at levels of 4.04, 2.42, 1.48 and $1.80\,ng\,g^{-1}$, respectively, for B_1, B_2, G_1 and G_2.

Clean-up of aflatoxins

Finely ground cocoa beans (20 g), added to 2 g of NaCl, were extracted in 120 mL of methanol–water solution (8:2, v/v). Suspensions were blended (3 min) at high speed (10,000 rpm) using an Ultra-Turrax homogeniser (Polytron, Luzem, Switzerland). Homogenised solutions were filtered through Whatman No. 2 filter paper and Whatman A-H glass microfibre filter (Whatman, Maidstone, England). Filtrate (4 mL) was diluted in phosphate buffered saline (24 mL) and applied to an Aflatest WB immunoaffinity column (Vicam, Nixa, MO, USA) at a flow rate of 2–3 mL min^{-1}. The column was then washed with distilled water (30 mL) and aflatoxins eluted with methanol (4 mL) into an amber vial. After evaporation to dryness at 40°C under a stream of N_2, the dry residue was redissolved in methanol–water (2:3, v/v; 1 mL) and filtered through Millex PTFE 0.45 μm (Millipore, Jaffrey, NH, USA).

HPLC parameters

A Shimadzu LC-10VP HPLC system (Shimadzu, Kioto, Japan) was used with a fluorescence detector set at 362 nm excitation for all aflatoxins and 455 nm emission for aflatoxins G_1 and G_2 and 425 nm emission for aflatoxins B_1 and B_2. A Shimadzu CLC G-ODS (4×10 mm) guard column and Shimadzu Shimpack (4.6×250 mm) column were employed. The mobile phase was water–acetonitrile–methanol (6:2:3, v/v/v) and contained KBr ($119\,mg\,L^{-1}$) and nitric acid (4 M, $350\,\mu L\,L^{-1}$). The flow rate was $1\,mL\,min^{-1}$. A mixture of aflatoxin standards was used for the construction of a 7-point calibration curve of peak areas versus aflatoxin mass (ng). The injection volume was 100 μL for both standard solution and sample extracts. The post-column derivatisation of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhone Ltd, Glasgow, Scotland).

Determination of potentially aflatoxigenic fungi and the occurrence of B_1, B_2, G_1 and G_2 aflatoxins in processed cocoa

Samples

A total of 168 samples of cocoa by-products (19 shell, 29 nibs, 25 mass, 25 cocoa butter, 26 cake, 16 natural powder and 28 alkalised powdered cocoa) were randomly collected from different stages of manufacture at processing plants in Brazil. These samples did not necessarily represent Brazilian cocoa as raw material because it is common to employ cocoa from different origins, especially from African and Asian countries, to make blends. When necessary, the samples were finely ground and all samples were stored at −20°C until analysis. A positive control spiked with 0.40, 0.24, 0.15 and $0.18\,ng\,g^{-1}$, respectively, for aflatoxin B_1, B_2, G_1 and G_2 aflatoxins, was analysed in parallel for cocoa by-products.

Determination of fungal species with the potential to produce aflatoxins

The technique of dilution plating was applied for fungal evaluation in the cocoa by-product samples. Under aseptic conditions, 25 g of each sample were weighed and 225 mL of sterile peptone water 0.1% were added. Then, aliquots of the serial dilutions were prepared and inoculated on plates containing dichloran 18% glycerol agar (DG18). The plates were incubated at 25°C for 7 days and the results expressed in colony-forming units per gram of sample (CFU g^{-1}), according to Pitt and Hocking's (2009) methodology.

After incubation, plates were inspected for fungal growth, and all colonies showing characteristics compatible with aflatoxigenic species were isolated on

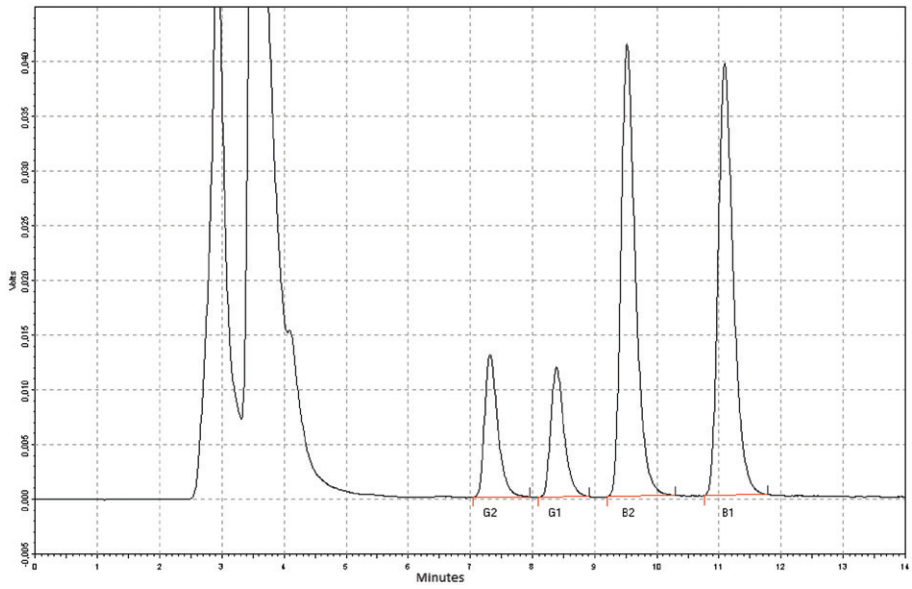

Figure 1. Chromatogram of cocoa beans artificially contaminated with aflatoxins G_2, G_1, B_2 and B_1, in this sequence. Mobile phase = water–acetonitrile–methanol (6:2:3, v/v/v) containing KBr (119 mg L^{-1}) and nitric acid (4M, 350 µL L^{-1}).

Czapek yeast extract agar (CYA) (Pitt and Hocking 2009) for subsequent identification based both on macroscopic (colony diameter, colour, exudate and soluble pigment production) and microscopic features, following appropriate keys (Klich and Pitt 1988).

Fungi identified as potential producers of aflatoxins were inoculated onto yeast extract sucrose agar (YES) (Samson et al. 2002) for 7 days at 25°C, and then the agar plug technique (Filtenborg et al. 1983) was used to evaluate the capability of isolates to produce aflatoxins. Fungal extracts taken as plugs with a cork borer were placed on TLC plates, developed in a toluene–ethyl acetate–formic acid 90%–chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualised under UV light at 365 nm. A mixture of aflatoxins B_1, B_2, G_1 and G_2 standards (Sigma, St. Louis, MO, USA) was used for comparison.

Table 1. Recovery results for aflatoxin B_1, B_2, G_1 and G_2 in cocoa beans.

Aflatoxin	Spiking levels (ng g^{-1})	Concentration recovered (ng g^{-1})[a]	Standard deviation	Mean recovery (%)
B_1	0.40	0.40	0.03	99.9
B_2	0.24	0.24	0.01	97.9
G_1	0.15	0.14	0.01	95.6
G_2	0.18	0.16	0.01	92.3
Total[b]	*0.97*	*0.94*	*0.02*	*96.9*
B_1	4.04	3.76	0.25	90.1
B_2	2.42	2.36	0.15	97.4
G_1	1.48	1.34	0.09	90.1
G_2	1.80	1.68	0.15	95.0
Total[b]	*9.74*	*9.14*	*0.16*	*93.8*

Notes: [a]Average of triplicates.
[b]Total = $B_1 + B_2 + G_1 + G_2$.

Results

Method validation

No interferences from matrix components were observed at the same retention time of the aflatoxins B_1, B_2, G_1 and G_2 peaks (Figure 1). When constructing the 7-point dose–response curves for aflatoxin analyses, the coefficient of linearity (r^2) was 0.9983, 0.9992, 0.9994 and 0.996, respectively, for aflatoxins B_1, B_2, G_1 and G_2.

Table 1 shows the recovery data of aflatoxins B_1, B_2, G_1 and G_2 in cocoa beans. The mean recovery of aflatoxin $B_1 + B_2 + G_1 + G_2$ (total aflatoxins) at the lowest level was about 97% and 94% at the highest level. The limit of detection of the method for aflatoxins B_1, B_2, G_1 and G_2 was, respectively, 0.001, 0.003, 0.003 and 0.002 ng g^{-1}.

Aflatoxigenic fungi and aflatoxin contamination in cocoa by-products

Table 2 shows the results for potential aflatoxin-producing fungi, and Table 3 shows the aflatoxins B_1, B_2, G_1 and G_2 contaminating the different cocoa by-products obtained during the industrial processing of cocoa beans. The analysis of spikes carried out in parallel for each by-product showed recovery values

Table 2. Occurrence of potential aflatoxin-producing species in cocoa by-products.

Matrix	Samples	Positive[a] (%)	Species isolated	Range (CFU g^{-1})
Shell	19	11 (58)	*Aspergillus flavus, Aspergillus parasiticus*	ND–10^4 ND–10^4
Nibs	29	8 (28)	*A. flavus*	ND–4
Mass	25	1 (4)	*A. flavus*	ND–1
Butter	25	—	—	ND
Cake	26	—	—	ND
Natural powder	16	3 (19)	*A. flavus*	ND–10
Alkalised powder	28	1 (4)	*A. flavus*	ND–4

Note: [a]Positive: Samples with potential aflatoxin-producing species.

Table 3. Aflatoxin B_1, B_2, G_1 and G_2 occurrence in cocoa by-products.

Matrix	Aflatoxin	Samples	Mean concentration ± SD (ng g^{-1})	Concentration range (ng g^{-1})	Positive samples (%)
Shell	B_1	19	0.18 ± 0.19	0.01–0.84	19 (100%)
	B_2	19	0.01 ± 0.02	< LOD–0.02	18 (95%)
	G_1	19	0.11 ± 0.12	< LOD–0.44	17 (84%)
	G_2	19	0.02 ± 0.02	< LOD–0.06	0 (0%)
	Total	76	0.34 ± 0.32	0.03–1.37	19 (100%)
Nibs	B_1	29	0.66 ± 1.68	< LOD–11.21	13 (45%)
	B_2	29	0.10 ± 0.26	< LOD–1.66	9 (31%)
	G_1	29	0.28 ± 0.86	< LOD–2.48	6 (17%)
	G_2	29	0.01 ± 0.01	< LOD–0.68	2 (7%)
	Total	116	1.04 ± 2.69	< LOD–13.34	13 (45%)
Mass	B_1	25	0.34 ± 1.19	< LOD–6.14	10 (40%)
	B_2	25	0.05 ± 0.10	< LOD–0.35	7 (28%)
	G_1	25	0.14 ± 0.57	< LOD–2.58	4 (16%)
	G_2	25	0.02 ± 0.10	< LOD–0.73	1 (4%)
	Total	100	0.55 ± 1.93	< LOD–9.90	10 (40%)
Butter	B_1	25	0.03 ± 0.08	< LOD–0.38	7 (28%)
	B_2	25	< LOD	< LOD–0.04	1 (4%)
	G_1	25	< LOD	< LOD	0 (0%)
	G_2	25	< LOD	< LOD	0 (0%)
	Total	100	0.04 ± 0.08	< LOD–0.42	7 (28%)
Cake	B_1	26	0.24 ± 0.18	< LOD–0.63	20 (77%)
	B_2	26	0.08 ± 0.12	< LOD–0.17	17 (69%)
	G_1	26	0.06 ± 0.12	< LOD–0.14	8 (31%)
	G_2	26	< LOD	< LOD	0 (%)
	Total	104	0.38 ± 0.35	< LOD–1.53	20 (77%)
Natural powder	B_1	16	0.42 ± 0.35	< LOD–1.04	12 (75%)
	B_2	16	0.10 ± 0.15	< LOD–0.51	9 (56%)
	G_1	16	0.11 ± 0.26	< LOD–0.91	4 (25%)
	G_2	16	< LOD	< LOD	0 (0%)
	Total	64	0.64 ± 0.59	< LOD–2.09	12 (75%)
Alkalised powder	B_1	28	0.21 ± 0.25	< LOD–0.92	18 (64%)
	B_2	28	0.10 ± 0.13	< LOD–0.43	14 (50%)
	G_1	28	< LOD	< LOD	0 (0%)
	G_2	28	< LOD	< LOD	0 (0%)
	Total	112	0.30 ± 0.36	< LOD–1.36	18 (64%)

Notes: LOD = Method limit of detection in cocoa beans (ng g^{-1}): $B_1 = 0.001$; $B_2 = 0.003$; $G_1 = 0.003$; $G_2 = 0.002$. Recovery of method in cocoa beans (%): $B_1 = 99.93$; $B_2 = 97.93$; $G_1 = 95.56$; $G_2 = 92.32$ (in triplicate).

similar to those observed in cocoa beans, with recovery higher than 90%.

Aspergillus flavus was the most prevalent potentially aflatoxigenic species isolated. This species was found in the shell, nibs, liquor and powder fractions evaluated. Shell and nibs were the most contaminated samples, with *A. flavus* present in, respectively, 59% and 28% of the evaluated samples. *Aspergillus parasiticus* was isolated in only one shell sample.

Nine out of the 18 (50%) *A. flavus* isolates tested were able to produce aflatoxin in culture media. The *A. parasiticus* tested produced aflatoxin B_1, B_2, G_1 and G_2. Considering the results for aflatoxins, out of 168 samples of cocoa by-products evaluated, only 5 had contaminations higher than $2\,ng\,g^{-1}$.

Aflatoxins were present in all shell samples analysed, with levels ranging from 0.03 to $1.37\,ng\,g^{-1}$, with an average of $0.34\,ng\,g^{-1}$. On the other hand, aflatoxins were detected in only 45% of nib samples, which showed a huge variation in the contamination levels, with an average contamination of $1.4\,ng\,g^{-1}$. The highest level of aflatoxin contamination was detected in nibs, reaching $13.3\,ng\,g^{-1}$ in one sample. Two other nib samples also showed high levels ($5.7\,ng\,g^{-1}$ and $4.7\,ng\,g^{-1}$), but in the other positive samples the contamination level was below $2\,ng\,g^{-1}$.

Aflatoxins were detected in 40% of the cocoa mass samples analysed. Overall contamination of the mass was low, but in one sample a maximum level of $9.9\,ng\,g^{-1}$ was found, giving an average of $0.55\,ng\,g^{-1}$.

Seventy-seven percent of the cake samples showed some contamination, with an average of $0.38\,ng\,g^{-1}$ total aflatoxins. The average level of cocoa butter contamination was $0.04\,ng\,g^{-1}$. Both the number of positive samples and the mean level of aflatoxins found on alkalised cocoa powder samples were lower than the natural (non-alkalised) ones.

Discussion

Method validation

The solvent used for extraction in the method reported here, methanol–water (8:2, v/v), is less toxic than others described in the literature for aflatoxin determination in cocoa, such as chloroform, ether, hexane and silver nitrate (Scott and Przybylski 1971; Lenovich and Hurst 1979; Hurst et al. 1982). The use of immunoaffinity columns for extraction reduced the use of halogenated solvents such as chloroform that, in addition to its toxicity to those in the laboratory, is problematic as an environmental contaminant.

The best way to assess a method recovery is using reference materials. Because there was no reference material for testing recovery of aflatoxins in cocoa, this work was performed with the contamination of expected blank samples of cocoa beans with a standard mixture of the aflatoxins B_1, B_2, G_1 and G_2, respectively, at levels of 0.40, 0.24, 0.15 and $0.18\,ng\,g^{-1}$, and also 4.04, 2.42, 1.48 and $1.80\,ng\,g^{-1}$.

The recovery values achieved with our methodology (90.1%–99.9%) are in accordance with EU Directive 98/53/EC (1998), which states that analytical methods for control of aflatoxins in food should give recovery between 80% and 110% for levels above $10\,ng\,g^{-1}$, 70%–110% for levels between 1 and $10\,ng\,g^{-1}$ and 50%–120% for levels $<1\,ng\,g^{-1}$.

Aflatoxigenic fungi and aflatoxin contamination in cocoa by-products

No aflatoxin contamination was detected in more than half of the evaluated samples; however, the variation of aflatoxin levels present in some samples was high. At least two cocoa batches would be rejected according to the current Brazilian regulation for aflatoxins in cocoa, which establishes $10\,ng\,g^{-1}$ for raw cocoa beans and $5\,ng\,g^{-1}$ for cocoa products and chocolate (ANVISA 2011).

Despite at least 16 fungal species having their capability to produce aflatoxins reported (Rank et al. 2011; Varga et al. 2011), *A. flavus* and *A. parasiticus* are the most representative aflatoxin producers naturally occurring in agricultural commodities (Cary and Ehrlich 2006). In general, *A. flavus* has a higher prevalence in foods when compared to *A. parasiticus*. However, the opposite is observed in the capability to produce aflatoxins, with the species reaching about 50% and 100%, respectively (Klich and Pitt 1988; Vaamonde et al. 2003). According to Copetti et al. (2011) data, *A. flavus* and *A. parasiticus* were also the most frequent species isolated from cocoa during the early stages of cocoa bean processing on Brazilian farms, and about 64% and 100%, respectively, of the tested isolates were aflatoxin producers. These data are similar to the results found in this study.

A. flavus contamination was higher in the shell fraction. The first processing procedures for the cocoa beans are the pre-roasting and/or roasting. These steps are essential to the formation of chocolate flavour and are also important because they facilitate the separation of the shell and nib fractions during the following winnowing process (Minifie 1989). Once most of the shell is removed from the processing line by winnowing, a reduction of contaminants in the subsequent by-products can be expected. It happens both due to physical removing and thermal inactivation (Minifie 1989).

The degree of cocoa roasting is dependent on the binomial time/temperature applied, where time generally ranges from 5 to 120 min and temperatures between 120°C and 150°C (Wollgast and Anklam 2000). Aflatoxin B_1 is quite stable to dry heating at temperatures below its thermal decomposition temperature of 267°C (Betina 1989), so a large reduction in the aflatoxin content is not expected during cocoa roasting.

There are no data available evaluating the contribution of the shelling process on the levels of aflatoxins in cocoa. No conclusion about the effect of shelling or temperature can be taken from the results observed in

this study because the samples evaluated were provided by manufacturing plants and not necessarily corresponding to the same batches of cocoa beans.

In the case of ochratoxin A in cocoa, studies demonstrate that most of the mycotoxin is concentrated in the shell and just a small fraction of the toxin is contaminating the nibs (Amezqueta et al. 2005; Gilmour and Lindblom 2008; Manda et al. 2009). About 50% of ochratoxin A can be physically removed by industrial shelling (Gilmour and Lindblom 2008), whereas manual shelling reduces 50% to 100% of ochratoxin A contamination (Amezqueta et al. 2005; Manda et al. 2009).

After the removal of shell by winnowing, cocoa nibs are submitted to grinding, forming the liquor (a dark brown fluid). When liquor solidifies with decreasing temperature, it generates the cocoa mass. The cake is obtained after pressing of the liquor, creating, in turn, the cocoa butter. Only *A. flavus* was isolated from these fractions. Minifie (1989) pointed out that the alkalisation process which the cocoa is submitted to (generally carried out with potassium or sodium carbonate) has the aim of making some physicochemical changes and has a strong sterilising action because of the combined effect of water, alkali and heat. It is known that treatment with alkali can be adopted to reduce contamination of substrates with aflatoxins (Piva et al. 1995), but there are no studies evaluating the effect of these treatments on the mycotoxin content of cocoa.

Regarding aflatoxins, their behaviour in relation to migration between the cocoa butter and cocoa cake fractions is similar to that reported for OTA (Manda et al. 2009), with a predominance of toxins in the non-fatty solids fraction. The cocoa powder is obtained after several stages of grinding of the cake and *A. flavus* was isolated from some samples. According to Minifie (1989), the final microbiota is almost exclusively introduced during further processing of the almost sterile alkalised liquor.

The only data concerning the presence of aflatoxins in cocoa by-products are from Germany, showing that 73.5% of the 334 products evaluated contained traces of aflatoxins (Raters and Matissek 2000). No specific data about the contamination of each cocoa fraction processing were found in the literature to be compared with the results observed in this study.

Copetti et al. (2011) reported 5% of aflatoxin positive samples out of 65 cured cocoa beans samples from Brazil. This percentage is lower than that observed in this study if the percentage of contaminated samples of shell (100%) and nibs (45%) is considered. It could be related to the origin of the samples, because in that study the samples were exclusively from Brazilian farms, whereas the samples evaluated here were, in general, a blend of cocoa beans from African and Asian countries and Brazil.

Conclusions

We conclude that the method described here is adequate for use in quantifying the contamination of cocoa by aflatoxins B_1, B_2, G_1 and G_2. *A. flavus* was the main aflatoxin-producing species isolated, with a higher occurrence in samples from the first processing step. A widespread contamination by aflatoxins in cocoa by-products was observed; however, in general, the levels present in the fractions of industrial processing of cocoa were low. No specific data about the contamination of each cocoa fraction processing are available to be compared with the results observed in this study. Usually, only small amounts of cocoa derivatives are employed for the manufacture of chocolate drinks, chocolate powder, cakes, biscuits and similar products, meaning that cocoa contributes only a small proportion of aflatoxin intake in the diet. On the other hand, constant monitoring of aflatoxin occurrence in cocoa and cocoa products is important because of the high toxicity of aflatoxins and the fact that chocolate-containing products are widely consumed by adults and children.

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