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Co-occurrence of ochratoxin a and aflatoxins in chocolate marketed in Brazil

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ABSTRACT

This study investigated the occurrence of aflatoxin B_1, B_2, G_1 and G_2 and ochratoxin A in chocolate marketed in Brazil. A hundred and twenty-five samples of powdered, bitter, dark, milk and white chocolate were evaluated for mycotoxins. Ochratoxin A was the most common mycotoxin in the evaluated samples, contaminating 98% of the purchased chocolate. A co-occurrence of aflatoxins was observed in 80% of all samples evaluated. The bitter, dark and powdered chocolate samples had the largest presence of aflatoxins. On average, the highest levels of ochratoxin A were found in powdered, dark and bitter chocolate, respectively: 0.39; 0.34 and 0.31 μg/kg. Bitter, powdered and dark chocolate had the highest aflatoxin content; an average of 0.66, 0.53 and 0.43 μg/kg, respectively. This is the first report of co-occurrence of ochratoxin A and aflatoxins in chocolate. The consumption of chocolate with high levels of cocoa in the formulation has been stimulated due to health benefits attributed to some cocoa components but on the other hand, these high cocoa content products tend to have the highest amount of aflatoxins and ochratoxin A. To guarantee a safe consumption of chocolate, there should be a continuous monitoring of both ochratoxin and aflatoxin and more studies attempting to understand the dynamics involving mycotoxin-producing fungi and mycotoxin production in cocoa need to be carried out with the aim of preventing mycotoxin accumulation in this commodity.

1. Introduction

The presence of mycotoxins in products such as chocolate, which has not enough water to support microbial growth and mycotoxin production, occurs due to the proliferation of toxigenic fungi in previous processing steps of the raw material. The amount of toxic metabolites produced during the fungal multiplication in these steps, associated with the stability of the compounds to the manufacturing processes employed, will determine the levels of mycotoxins contaminating the final product.

The processing of cocoa, the main ingredient of chocolate, involves steps of fermentation, drying and storage of cocoa beans on farms located in tropical regions before being marketed and subjected to thermal treatment in the processing industries (Wood, 2001). The development of mycotoxigenic species is frequent during the processing steps on farms, especially during sun drying. Researchers have reported the presence of genus *Aspergillus*, emphasizing ochratoxin and aflatoxin-producing species in cocoa beans, as well as the presence of these mycotoxins (Copetti, Iamanaka, Pereira, Fungaro, & Taniwaki, 2010; Copetti, Pereira, Iamanaka, Pitt, & Taniwaki, 2011; Mounjouenpou et al., 2008; Sanchez-Hervas, Gil, Bisbal, Ramon, & Martinez-Culebras, 2008).

In a following stage, during the industrial processing, the cocoa beans are subjected to roasting steps which are essential to complete the formation of compounds responsible for the flavor of chocolate (Zak, 1988). At the same time this technology contributes to the reduction of contaminants present in the raw material. These treatments are considered sufficient to eliminate vegetative cells of microorganisms (ICMSF, 2005) but on the other hand, it is known that some mycotoxins such as aflatoxins and ochratoxin A maintain a certain stability during most thermal food processing stages (Boudra, Le Bars, & Le Bars, 1995; Ferraz et al., 2010; Kamimura, 1989; Manda et al., 2009). Among the known mycotoxins, aflatoxins and ochratoxin A are of greatest concern due to their frequent occurrence in foods and their severe effects on animal and human health.

Aflatoxin B_1 is the most potent hepatocarcinogen known in mammals classified by the International Agency of Research on Cancer as Group 1 carcinogen and also has toxic, mutagenic and

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teratogenic properties (IARC, 1993). Aflatoxins are produced by members of the genus *Aspergillus*, especially the section *Flavi* (Pitt & Hocking, 2009). Since the aflatoxins are considered genotoxic and carcinogenic, their presence in food should be reduced as low as reasonably achievable (ALARA), with no tolerable daily intake (TDI) indicated.

Ochratoxin A is a mycotoxin with nephrotoxic effects (IARC, 1993) produced mainly by *Aspergillus* sections *Nigri* and *Circumdati* and *Penicillium verrucosum* (Pitt & Hocking, 2009). This mycotoxin has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993). The Joint Committee FAO/WHO of Experts on Food Additives (JECFA) has established the provisional tolerable weekly intake (PTWI) of ochratoxin A at 100 ng/kg of body weight (bw) corresponding to approximately 14 ng/kg bw/day (JECFA, 2002).

Cocoa and its by-products for a long time were not considered a raw material of concern for mycotoxin contamination based on their content of caffeine (Buchanan & Lewis, 1984) and for this reason, and the non-availability of simple and sensitive analytical methods, have not been carefully evaluated. In the last decade, reports on the occurrence of ochratoxin A in cocoa and chocolate have increased (Bonvehi, 2004; Brera et al., 2011; Burdaspal & Legarda, 2003; Copetti et al., 2010; Engel, 2000; Gilmour & Lindblom, 2008; Kumagai et al., 2008; Turcotte & Scott, 2011), although data on the presence of aflatoxins in this product are very limited (Copetti et al., 2011; Kumagai et al., 2008; Raters & Matissek, 2000). The increasing restriction on the presence of both toxins in foods together with the availability of improved equipment and more sensitive analytical techniques allows a better control of public exposure to these contaminants. A major concern related to the occurrence of mycotoxins in chocolate lies in the fact that this product is also present in the diet of children (Brera et al., 2011; Kumagai et al., 2008).

The objective of this study was to evaluate the occurrence of ochratoxin A and aflatoxins in chocolate marketed in Brazil, since monitoring of different classes of mycotoxins in different food is important to evaluate the risk to which a given population is routinely exposed.

2. Material and methods

2.1. Samples

A hundred and twenty-five samples of chocolate from the major chocolate brands marketed in Brazil were acquired from supermarkets in Campinas and Sao Paulo. The samples corresponded to 25 samples of cocoa powder, 25 samples of dark, milk and white chocolate bars and 25 samples of bitter (at least 70% cocoa in composition) chocolate bars.

The bitter chocolate bars corresponded to imported brands while the other samples were produced in Brazil.

The samples were ground and kept at −20 °C until analysis.

2.2. Analyses of mycotoxins in chocolate

The following methods were optimized *in house* for cocoa beans and a positive control inoculated with a known amount of ochratoxin A or aflatoxins was analyzed in parallel for each chocolate matrix evaluated.

2.2.1. Ochratoxin A

2.2.1.1. Clean-up of ochratoxin A. Ten grams of finely ground chocolate were extracted in $NaHCO_3$ (1% aqueous; 200 mL). Suspensions were blended (2 min) at high speed (10,000 rpm) using an Ultra-Turrax homogenizer (Polytron, Switzerland). Homogenized solutions were filtered through Whatman No. 4 filter paper and Whatman A-H glass microfiber filter (Whatman, England). Filtrate (20 mL) was diluted in phosphate buffered saline (20 mL) plus Tween 20 (0.01%) and applied to an Ochratest WB immunoaffinity column (Vicam, USA) at a flow rate of 2–3 mL/min. The column was then washed with distilled water (20 mL) and ochratoxin A eluted with acidified methanol (methanol: acetic acid, 98:2, v/v; 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 mobile phase (0.3 mL).

2.2.1.2. HPLC parameters. A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detection set at 333 nm excitation and 477 nm emission. A Shimadzu CLC G-ODS (4 × 10 mm) guard column and Shimadzu Shimpack (4.6 × 250 mm) column were employed. The mobile phase was acetonitrile: water: acetic acid (51:47:2, v/v/v) and the flow rate was 1 mL/min. An ochratoxin A standard was used for the construction of a five point calibration curve of peak areas versus concentration (μg/L). The injection volume was 100 μL for both standard solution and sample extracts.

2.2.1.3. Chemical confirmation of ochratoxin A. OTA was confirmed by methyl ester formation (Pittet, Tornare, Huggett, & Viani, 1996) in some randomly selected positive samples showing contamination amounts higher than 0.2 μg/kg. Aliquots (about 200 μL) of sample and standard were evaporated to dryness at 40 °C under a stream of N_2 and the residue redissolved in boron trifluoride–methanol complex (20% solution in methanol; 300 μL). The solution was heated at 80 °C for 10 min and allowed to cool to room temperature. The identity of ochratoxin A was confirmed by the formation of methyl ester that gave a retention time of approximately 22 min.

2.2.2. Aflatoxins B_1, B_2, G_1 and G_2

2.2.2.1. Clean-up of aflatoxins. Twenty grams of finely ground cocoa, 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 homogenizer (Polytron, Switzerland). Homogenized solutions were filtered through Whatman No. 2 filter paper and Whatman A-H glass microfiber filter (Whatman, England). Filtrate (4 mL) was diluted in phosphate buffered saline (24 mL) and applied to an Aflatest WB immunoaffinity column (Vicam, USA) at a flow rate of 2–3 mL/min. 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, USA).

2.2.2.2. HPLC parameters. A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detection set at 362 nm excitation 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) and nitric acid (4 M, 350 μL/L). The flow rate was 1 mL/min. A mix of aflatoxin standards was used for construction of a five point calibration curve of peak areas versus concentration (μg/L). The injection volume was 100 μL for both standard solution and sample extracts. The post-column derivatization of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhone Ltd, Scotland).

2.3. Statistical analyses

Correlation analyses were carried out with the software The Unscrambler® 9.2 (Camo Process AS, Norway). Correlation coefficients (r) were calculated to identify possible associations between the occurrence of total aflatoxins and ochratoxin A in the chocolate samples evaluated. Interpretation of values was performed according to Pearson's coefficient (r) which are: very weak $0.000 \leq r \geq 0.200$; weak $0.201 \leq r \geq 0.400$; moderate $0.401 \leq r \geq 0.600$; strong $0.601 \leq r \geq 0.800$ and very strong $0.801 \leq r \geq 1.000$ (Christmann & Badgett, 2009: Chap. 5). When one variable increases and the other variable tends to increase, the relationship shows positive r values. If one variable increases and the other decreases, the correlation coefficient will be negative.

3. Results and discussion

No interferences from matrix components were observed in the same retention time of ochratoxin A or aflatoxins B_1, B_2, G_1 and G_2 peaks (Fig. 1).

The positive control analyses carried out in parallel for each chocolate sample presented recovery values similar to those observed for cocoa beans (Table 1 and Table 2). The Directives 98/53/EC and 2002/26/CE of the European Union (Commission Directives, 1998; 2002) states that analytical methods for control of, respectively, aflatoxins and ochratoxin A in food should provide recovery between 70 and 110% at levels between 1 and 10 μg/kg and 50–120% at levels <1 μg/kg.

Table 1
Ochratoxin A occurrence in chocolate.

Matrix	Samples	Mean concentration ± SD (μg/kg)	Median (μg/kg)	Concentration range (μg/kg)	Positive samples (%)
Powdered chocolate	25	$0.39 \pm 0.23^{a*}$	0.39	0.03–0.92	25 (100%)
Bitter chocolate	25	0.31 ± 0.11^{a}	0.31	0.06–0.60	25 (100%)
Dark chocolate	25	0.34 ± 0.17^{a}	0.31	0.09–0.87	25 (100%)
Milk chocolate	25	0.15 ± 0.08^{b}	0.15	0.08–0.45	25 (100%)
White chocolate	25	0.03 ± 0.01^{c}	0.03	<LOD–0.05	23 (92%)

Method limit of detection (μg/kg): 0.01.
Recovery of method (%): 97.45.
*Lines with the same letter represent not significantly difference ($\rho > 0.05$).

Fig. 2 shows the distribution of total aflatoxins and ochratoxin A contamination according to the chocolate sample evaluated and Tables 1 and 2 present, respectively, the results of ochratoxin A and aflatoxin analyses in samples of cocoa powder and chocolate bars.

Ochratoxin A was the most common mycotoxin present in the evaluated samples. Contamination was noted in all cocoa powder, along with bitter, dark and milk chocolate bars and 92% of white chocolate bars analyzed in this survey, representing 98% of ochratoxin A presence in the purchased products. A co-occurrence of aflatoxins was observed in 80% of all samples evaluated. Bitter, dark

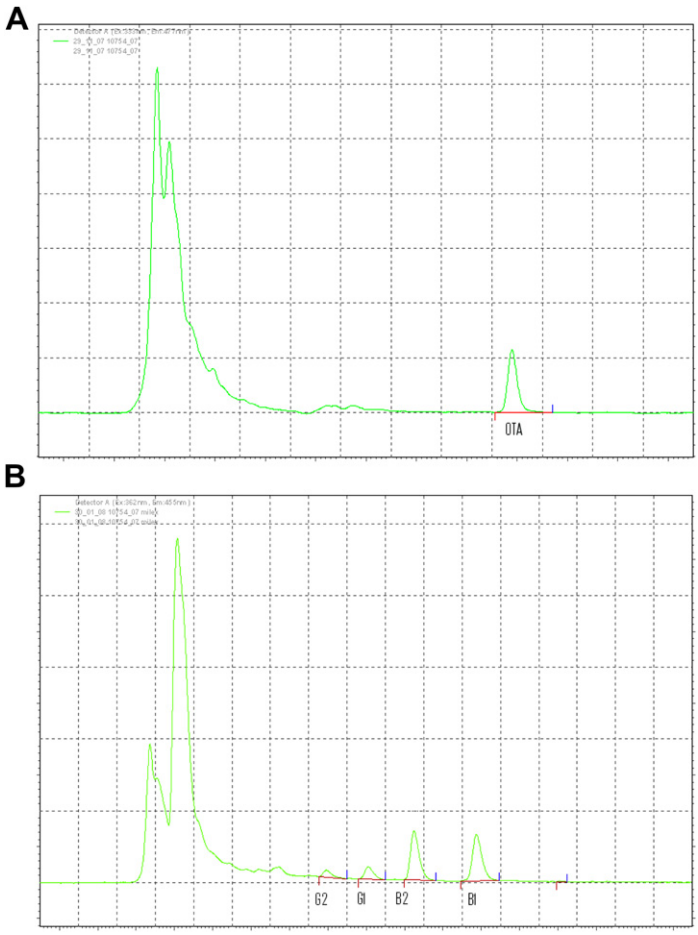

Fig. 1. Chromatograms of cocoa powder naturally contaminated with ochratoxin A (A) and aflatoxins G_2, G_1, B_2 and B_1 (B), in this sequence. 1(A).

Table 2
Aflatoxin B_1, B_2, G_1 and G_2 occurrence in chocolate.

Matrix	Aflatoxin	Samples	Mean concentration ± SD (μg/kg)	Median μg/kg)	Concentration range (μg/kg)	Positive samples (%)
Powder chocolate	B_1	25	0.43 ± 0.16	0.33	<LOD–0.96	24 (96%)
	B_2	25	0.08 ± 0.10	0.07	<LOD–0.60	20(80%)
	G_1	25	0.11 ± 0.16	<LOD	<LOD–0.48	11 (44%)
	G_2	25	0.01 ± 0.03	<LOD	<LOD–0.10	3 (12%)
	Total	25	0.53 ± 0.32[a,b]*	0.45	<LOD-1.70	24 (96%)
Bitter chocolate	B_1	25	0.33 ± 0.20	0.29	0.10–0.87	25 (100%)
	B_2	25	0.07 ± 0.04	0.08	<LOD–0.15	19 (76%)
	G_1	25	0.29 ± 0.18	0.18	<LOD–0.63	24 (96%)
	G_2	25	0.01 ± 0.01	0.02	<LOD–0.03	5 (20%)
	Total	25	0.66 ± 0.36[a]	0.52	0.11–1.65	25(100%)
Dark chocolate	B_1	25	0.43 ± 0.27	0.17	0.04–0.91	25 (100%)
	B_2	25	0.05 ± 0.03	0.05	<LOD–0.11	22 (88%)
	G_1	25	<LOD ± 0.01	<LOD	<LOD–0.06	2 (8%)
	G_2	25	<LOD	<LOD	<LOD	0
	Total	25	0.43 ± 0.27[b]	0.25	0.04–0.91	25 (100%)
Milk chocolate	B_1	25	0.08 ± 0.05	0.05	<LOD–0.27	18 (72%)
	B_2	25	0.02 ± 0.02	0.02	<LOD–0.07	15 (60%)
	G_1	25	0.01 ± 0.02	<LOD	<LOD–0.07	5 (20%)
	G_2	25	<LOD	<LOD	<LOD	0
	Total	25	0.08 ± 0.07[c]	0.09	<LOD–0.32	18 (72%)
White chocolate	B_1	25	<LOD ± 0.02	<LOD	<LOD–0.10	5 (20%)
	B_2	25	<LOD	<LOD	<LOD	0
	G_1	25	<LOD	<LOD	<LOD	0
	G_2	25	<LOD	<LOD	<LOD	0
	Total	25	0.01 ± 0.02[c]	<LOD	<LOD–0.10	5 (20%)

[1]-SD: Standard deviation.
LOD = Method limit of detection (μg/kg): $B_1 = 0.01$; $B_2 = 0.01$; $G_1 = 0.01$; $G_2 = 0.01$.
Recovery of method (%): $B_1 = 99.93$; $B_2 = 97.93$; $G_1 = 95.56$; $G_2 = 92.32$.
* Lines with the same letter represent not significantly difference ($\rho > 0.05$).

and powdered chocolate were the samples with a large presence of aflatoxins (100, 100 and 96% contaminated, respectively) and also the ones where the highest contamination levels were observed. Aflatoxin B_1 was the most common aflatoxin isolated.

Considering the samples of chocolate analyzed in this survey, there was a tendency of increasing levels of contamination by mycotoxins parallel to the increased content of fat-free cocoa solids in the products, suggesting an affinity both of aflatoxins and ochratoxin A by the fat-free cocoa fraction. The cocoa powder, which has the highest amount of cocoa solids in its composition, was the commercial product showing, in average, the highest level of contamination by the tested mycotoxins, 1.70 and 0.92 μg/kg respectively for total aflatoxins and ochratoxin A. However the values were not significantly different ($\rho > 0.05$) from the other high content fat-free cocoa solids chocolate (bitter and dark). A total of 4 bitter chocolate samples had aflatoxins at levels superior to 1 μg/kg (Fig. 1) and one sample of powdered chocolate also had contamination at this level. On the other hand, the white chocolate, which contains no added cocoa liquor but only cocoa butter, showed levels close to the limit of detection of the method.

According to the Codex Standard for Chocolate (Codex alimentarius, 2003) the chocolate bars should have in their composition not less than 14 and 2.5% of fat-free cocoa solids for dark chocolate and milk chocolate, respectively. The white chocolate does not present fat-free cocoa solids in its composition and should have at least 20% of cocoa solids. For dark chocolate and milk chocolate the minimum values of total cocoa solids required are, respectively, 35 and 25%.

Table 3 presents the correlation coefficients (r) between the occurrence of total aflatoxins and ochratoxin A in the samples evaluated.

There was a very weak correlation between contamination by aflatoxins and ochratoxin A in powdered, bitter, dark and milk chocolate; this correlation was weak for white chocolate. Once both positive and negative r values were observed according to the chocolate group evaluated, there was no observed pattern of dependency in the co-occurrence of these two mycotoxins. Even if

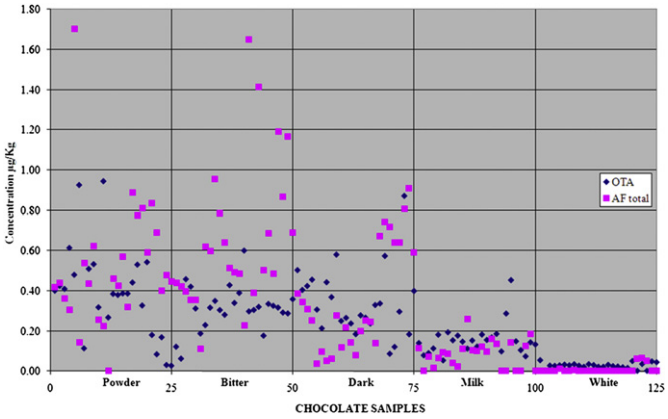

Fig. 2. Distribution of total aflatoxin (AF total) and ochratoxin A (OTA) contamination according to the chocolate samples evaluated (1–25 = powder, 26–50 = bitter 51–75 = dark, 76–100 = milk, 101–125 = white chocolate).

Table 3
Correlation coefficients (r) between the occurrence of total aflatoxins and ochratoxin A in the samples evaluated.

Samples	r
Powdered chocolate	−0.129
Bitter chocolate	−0.048
Dark chocolate	0.172
Milk chocolate	0.119
White chocolate	−0.381

the water and nutrients required for mycotoxin production on raw material by aflatoxigenic and ochratoxigenic fungi are similar (Pitt & Hocking, 2009), many other factors acting together will determine the presence of these mycotoxins in a chocolate sample.

The presence of aflatoxin and ochratoxin-producing fungi and the related mycotoxins during the processing of cocoa at producing farms was reported in studies carried out in Brazil by Copetti et al. (2010, 2011). The authors suggest the sun drying stage as a critical step for mycotoxin production in cocoa, since during drying, besides the presence of potentially toxigenic species, there is still enough water to sustain fungal growth and mycotoxin production. In the case of ochratoxin A the study (Copetti et al., 2010) demonstrated a strong correlation between the presence of ochratoxigenic fungi and the ochratoxin A content in the samples. On the other hand, besides the intense presence of aflatoxigenic fungi in cocoa samples, the aflatoxin contamination was low and just a weak correlation was observed (Copetti et al., 2011).

Considering the amount of cocoa used for chocolate manufacture (Codex alimentarius, 2003) and the ochratoxin A and aflatoxin levels reported in Brazilian cocoa (Copetti et al., 2010, 2011), a lower contamination both by aflatoxins and ochratoxin A should be expected in the chocolate samples. A possible explanation is that since the 1990s Brazil has changed its status of cocoa bean exporter to cocoa beans importer to meet the internal demand of the cocoa industry (Zugaib, 2008) and it is usual to use cocoa from different origins to make blends and so obtain better sensorial characteristics in the chocolate. A lower contamination of cocoa beans by ochratoxin A has been reported in samples from America, Asia and Australasia and West Africa (excluding Ivory Coast) when compared with Ivory Coast (Gilmour & Lindblom, 2008). Currently Ivory Coast is responsible for about 70% of world cocoa supply (ICCO, 2009). With the exception of the Brazilian survey (Copetti et al., 2011), no data about aflatoxin contamination in cocoa beans has been published in recent years.

A high occurrence of ochratoxin A in chocolate products has been reported in the last decade around the world. Comparing the results obtained from powdered samples in this survey to the literature reports of ochratoxin A presence, they are similar. Data shows presence of ochratoxin A in 92–100% of the products evaluated, with average levels ranging from 0.17 to 2.41 μg/kg (Bonvehi, 2004; Brera et al., 2011; Burdaspal & Legarda, 2003; Engel, 2000; Gilmour & Lindblom, 2008; Miraglia & Brera, 2002). The incidence of ochratoxin A reported for chocolate bars is also high, 60–100% in dark chocolate (0.14–0.38 μg/kg) (Brera et al., 2011; Burdaspal & Legarda, 2003; Engel, 2000; Gilmour & Lindblom, 2008; Kumagai et al., 2008; Turcotte & Scott, 2011), 23–100% in milk chocolate (0.08–0.16 μg/kg) (Brera et al., 2011; Burdaspal & Legarda, 2003; Engel, 2000; Turcotte & Scott, 2011), and 88–100% of contamination in white chocolate (0.03 μg/kg) (Burdaspal & Legarda, 2003).

Regarding the presence of aflatoxins in chocolate, rarely are reports encountered in the literature. The only publication found corresponds to a survey conducted in Japan to assess levels of mycotoxins in products sold in retail outlets where Kumagai et al. (2008) evaluated samples of bitter chocolate for the presence of aflatoxins. The authors found 22 positive for aflatoxins out of 42 samples tested, with an average contamination of positive samples of 0.18 μg/kg, and a maximum of 0.60 μg/kg. Besides the contamination with aflatoxins coming from cocoa products, cocoa butter substitutes can also collaborate in the amount of aflatoxin present in the final product (Kershaw, 1982).

A point to be emphasized in this study is the simultaneous occurrence of ochratoxin A and aflatoxins in the chocolate samples. In the last decade the attention has been focused on occurrence of ochratoxin A in cocoa, by-products and chocolate and this survey demonstrates the necessity to expand this concern also to aflatoxins, regarding the toxicological importance of this food contaminant. This is the first report of co-occurrence of ochratoxin A and aflatoxins in chocolate.

In recent years the consumption of chocolate with increasing levels of cocoa in the formulation has been stimulated due to the beneficial health effects attributed to it, but on the other hand these high cocoa content products tend to have the highest amount of aflatoxins and ochratoxin A, since these mycotoxins appear to be in the free-fat cocoa solids. Because of this, there should be a continuous monitoring of chocolate for ochratoxin A and also aflatoxin and more studies attempting to understand the dynamics involving the mycotoxins production in cocoa need to be carried out with the aim of reducing the accumulation of this toxin in the raw material for chocolate production.

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