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Analytical Methods

Occurrence of ochratoxin A in cocoa by-products and determination of its reduction during chocolate manufacture

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ABSTRACT

This work reports an investigation carried out to assess the natural occurrence of ochratoxin A in 168 samples from different fractions obtained during the technological processing of cocoa (shell, nibs, liquor, butter, cake and cocoa powder) and the reduction of ochratoxin A during chocolate manufacture. Ochratoxin A analyses were performed with immunoaffinity columns and detection by high performance liquid chromatography. Concerning the natural ochratoxin A contamination in cocoa by-products, the highest levels of ochratoxin A were found in the shell, cocoa powder and cocoa cake. The cocoa butter was the least contaminated, showing that ochratoxin A seems to remain in the defatted cocoa solids. Under the technological conditions applied during the manufacture of chocolate in this study and the level of contamination present in the cocoa beans, this experiment demonstrated that 93.6% of ochratoxin A present in the beans was reduced during the chocolate producing.

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1. Introduction

Cocoa beans can become contaminated by fungi during pre-processing at the farm, especially during drying or storage (Copetti, Iamanaka, Pereira, Frisvad, & Taniwaki, 2011a) and some fungal species can produce mycotoxins when growing in foods. The mycotoxins most commonly reported in cocoa are ochratoxin A (Copetti, Pereira, Iamanaka, Pitt, & Taniwaki, 2010; Gilmour & Lindblom, 2008; Raters & Matissek, 2000) and aflatoxins (Copetti, Iamanaka, Pereira, Fungaro, & Taniwaki, 2011b; Copetti et al., 2012b; Raters & Matissek, 2000), stable compounds not completely destroyed during most food processing operations, which may lead to contamination of finished cocoa products (Bonvehi, 2004; Burdaspal & Legarda, 2003; Miraglia & Brera, 2002; Tafuri, Ferracane, & Ritieni, 2004; Brera et al., 2011; Copetti et al., 2012a; Kumagai et al., 2008).

In the last decade concern has increased about human exposure to ochratoxin A, a possible carcinogen to humans (IARC, 1993), and consequently the interest in studies evaluating the sources of this contaminant in the diet. A discussion document was set up in Codex Alimentarius to study the extension and dynamics involving the contamination of cocoa and cocoa products with this toxin, as well as to determine the contribution of these products to daily ochratoxin A consumption and the necessity of establishing a regulation for these products (Codex Alimentarius., 2012).

Studies have shown that in cocoa, ochratoxin A is mainly produced by *Aspergillus carbonarius* and *Aspergillus niger* (Copetti et al., 2010; Mounjouenpou et al., 2008; Sanchez-Hervas, Gil, Bisbal, Ramon, & Martínez-Culebras, 2008). However, the presence of ochratoxigenic isolates of *Aspergillus melleus*, *Aspergillus westerdijkiae* and *Aspergillus ochraceus* have also been reported (Copetti et al., 2010). The contamination of cocoa by ochratoxin-producing fungi can already take place in the fermentation, but a considerable increase in the numbers of these species, as well in ochratoxin A contamination is observed during drying and storage (Copetti et al., 2010). The cocoa beans need to pass through different steps during the industrial processing which bring about a variety of by-products or chocolate, which can contribute to the reduction of ochratoxin A contamination.

One of the first processing steps involves roasting of cocoa that consists in a heat treatment of the beans at 110–140 °C for about 30 min for beans and 12 min for nibs, depending on the equipment. The primary goal of roasting is to complete the development of the chemical reactions responsible for the formation of sensory characteristics of flavour and colour of 'chocolate' (Kamphuis, 2009; Ziegleder, 2009). In addition, there is an important decrease in the water content, volatile acidity (Minifie, 1999) and microbial contamination of cocoa beans (ICMSF, 2005). After roasting the separation of the shell is facilitated, being removed by winnowing. The cotyledon is now breakable, which produces the nibs. The nibs are ground to form a fluid mass of a dark brown colour called liquor (also called cocoa mass when solidified by cooling). The temperature used in this process is 50–70 °C, during a variable time of

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2–72 h, depending on the equipment and cocoa quality and the required chocolate quality (Beckett, 2008).

The homogeneous combination of cocoa materials (liquor and butter) with milk products, sugars and/or sweeteners, and other additives, produces the chocolate (Codex Alimentarius., 2003). The process occurs at temperatures between 45 and 100 °C (Minifie, 1999) and at this stage a reduction in acidity and moisture content is observed, and the Maillard reaction is enhanced.

Some steps of cocoa processing involve heat treatment or segregation of fractions, which can play an important role in the reduction of contamination of cocoa by ochratoxin A. The purpose of this study is to determine the natural contamination present in cocoa by-products and to evaluate the effect of the chocolate manufacturing process on the reduction of ochratoxin A contamination in chocolate.

2. Material and methods

2.1. Analyses of ochratoxin A in cocoa by-products

2.1.1. Samples

A total of 168 samples of cocoa by-products (19 shells, 29 nibs, 25 mass, 25 cocoa butter, 26 cake, 16 cocoa powders and 28 alkalized cocoa powders) were randomly collected from different steps of manufacture at processing plants in the region of Ilhéus/BA and São Paulo/SP, Brazil. They did not necessarily represent Brazilian cocoa as raw material since it is common to make blends with cocoa from different sources, especially imported from African and Asian countries.

When necessary, the samples were finely ground (<1 mm) and all samples were stored at −20° C until the analyses were performed.

2.1.2. Analyses of ochratoxin A

Ochratoxin A was determined according to the methodology described by Copetti et al. (2010).

For assessment of linearity, five-point calibration curves were plotted, with a correlation coefficient (r) >0.99.

To obtain the limit of detection (LOD) eight contaminated cocoa samples with low ochratoxin A levels (<0.02 μg/kg) were analyzed and the standard deviation was calculated. These values were multiplied by the corresponding number listed on the t-Student table for 99% significance (Keith et al., 1983; Long & Winefordner, 1983).

The recovery of ochratoxin A was carried out in triplicate after the contamination of three finely ground cocoa beans with 0.49, 1.96 and 9.8 μg/kg.

A positive control spiked with ochratoxin A in cocoa by-products was analyzed in parallel with the samples for analytical control.

2.1.2.1. Clean-up. Ten grams of finely ground cocoa were extracted using $NaHCO_3$ (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 Ochraprep immunoaffinity column (R-Biopharm Rhône Ltd., Scotland) 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).

2.1.2.2. 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 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. Evaluation of the stability of ochratoxin A during cocoa processing

2.2.1. Production of beans contaminated with ochratoxin A

Fermentation was carried out at the Comissão Executiva para o Plano da Lavoura Cacaueira (CEPLAC) in Itabuna-BA, Brazil. Mature and healthy cocoa pods were harvested and cut by hand with machetes. The beans were manually removed from the pods, separated from the placenta and 20% of the pulp was removed by a mechanical pulp extractor.

Cocoa beans were inoculated with a suspension of *A. carbonarius* spores (1.67 mL/kg), containing 10^5 conidia/mL, before the curing process. The inoculum was prepared with two ochratoxigenic strains of *A. carbonarius* (ITAL 792 cc and ITAL 1375 cc) isolated from cocoa. The inoculum was aseptically prepared after growing the isolates for 7 days in CYA at 25 °C and suspending the colonies in 0.1% peptone–water.

A combined fermentation-drying, whereby the beans were placed on sun drying wood platforms to reduce their humidity up to 6–7%, was carried out for curing the beans.

2.2.2. Processing of cocoa beans to chocolate

The chocolate was processed at the Cereal and Chocolate Technology Center of the Instituto de Tecnologia de Alimentos (ITAL) in Campinas, São Paulo, Brazil.

The cured beans were roasted in an electric oven (Probat, TP2, Germany) for 40 min with the heating jacket temperature set at 140 °C, according to the conditions optimized by (Gilabert-Escriva, Pezoa-Garcia & Marsaioli, 1998). Then the beans were manually shelled and the obtained nibs were ground in a blender (Walita, Brazil) and refined in a roll mill refiner (Draiswerke GMBH, Germany) made up of three steel jacketed horizontal cylinders and cooled internally with water at 15 °C until obtaining a maximum particle size of 25 μm for the cocoa mass. The measurement of the maximum particle size was performed in a digital micrometer (Mitutoyo, Japan). The refined mass was conched at 70 °C for 3 h in a laboratory conche (Hans Sröter, Dusseldorf KG BEGE BV, Germany).

The dark chocolate manufactured was formulated with 56% of cocoa mass (containing 53% cocoa butter), 43.6% of sugar (Glaçucar, União) and 0.4% of soy lecithin (CH Solec, Solae). The chocolates were produced in batches of 700 g with a total content of 29.7% cocoa butter. The mixing step was carried out using 35% of the melted cocoa mass mixed with the sugar in a planetary mixer (Kitchen Aid, K5SS model, USA). The obtained mass was refined to a maximum particle size of 25 μm in a single stage in the same refiner used to obtain the liquor.

Conching was performed in batches of 700 g in the same laboratory conche used for liquor treatment. At the beginning of this step, the remaining liquor (21%) and soybean lecithin were added to the refined mass. This step was performed for 20 h at 60 °C.

2.2.3. Characterization of roasted cocoa beans

The percentage of each fraction related to a whole bean was determined by the manual separation of germen, shell and

cotyledon of a hundred roasted beans, which were weighed separately on a semi-analytical balance (Gehaka, BG 2000, Brazil).

The pH and titratable acidity was measured in a pH meter (Tecnal TE-2, Brazil) according to the methodology of Horwitz (2005).

The total lipid content was determined according to the AOAC International official method 963.15 (Horwitz, 2005) using a Soxhlet extraction battery (Tecnal, model ET-188, Brazil).

2.2.4. Determination of ochratoxin A reduction by chocolate processing

To determine the reduction of ochratoxin A during the chocolate processing, ochratoxin A analyses were carried out according to the methodology of Copetti et al. (2010) with the whole beans before roasting and with roasted nibs and shell, cocoa mass and the formulated chocolate. Five replicates were analyzed.

3. Results and discussion

3.1. Ochratoxin A in cocoa by-products

The mean recovery of ochratoxin A in cocoa beans was 97.45%, 95.02% and 79.91% for lowest, middle and highest level, respectively. The analysis of spikes carried out in parallel for each by-product showed recovery values similar to those observed in cocoa beans, with recovery ranging from 78% to 91%. The Directive 2002/26/CE of the European Union (Commission Directive, 2002) states that analytical methods for control of 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.

The levels of ochratoxin A in cocoa by-products are shown in Table 1. The results found widespread contamination of cocoa by-products by ochratoxin A, but the levels of contamination were generally low.

Out of the 168 samples of cocoa by-products evaluated, 158 (94.05%) showed presence of ochratoxin A (LOD = 0.01 μg/kg). The cocoa butter was the product which had the lowest concentration mean (0.03 μg/kg), contrasting with that found in the cocoa cake (0.97 μg/kg), with both products coming from the cocoa mass (0.34 μg/kg). This observation suggests that during the hydraulic pressing step, most of the toxin remains adhered to the cocoa defatted fraction. The distribution of ochratoxin A in animal tissues is well reported, generally following the order kidney > muscle > liver > fat (Mortensen, Hald, & Madsen, 1983). In vegetal tissues, this toxin appears to show the same non-affinity for fat.

On the other hand, the highest contamination was found in a sample of cocoa powder (5.13 μg/kg) and this product was also, on average (1.42 μg/kg), the most contaminated fraction. The contamination in the alkalized cocoa powder showed on average, a smaller contamination (0.90 μg/kg), suggesting an effect of this process in the reduction of ochratoxin A contamination. The alkalization process is important for obtaining cocoa powders with different shades and this step also influences the dispersibility of the particles in liquids (Minifie, 1999). It is known that treatment with alkali can be applied to reduce contamination of substrates with some mycotoxins such as aflatoxins and fumonisins; however, there are no studies evaluating the effect of alkalization treatment (combination of heat and alkali solution, generally potassium carbonate) on the cocoa content of mycotoxins.

Another interesting point was that the contamination found in shell (1.13 μg/kg) was about 10 times higher than in nibs (0.10 μg/kg). This suggests that the shelling step and the control of the shell content in cocoa nibs after this step have extreme importance in reducing the presence of ochratoxin A.

The first detailed data on ochratoxin A contamination in cocoa by-products was reported by Miraglia and Brera (2002). None out of 13 samples of cocoa butter, mass and powder from the Netherlands had ochratoxin A above the limit of quantification (LOQ) (0.25 μg/kg); the contamination of cocoa powder analyzed in Germany and the United Kingdom was, respectively, 0.38 and 1.2 μg/kg, with no distinction between natural or alkalized powder (Miraglia & Brera, 2002). The data from Germany were similar to those observed in our survey.

Another study of ochratoxin A occurrence was carried out by Bonvehi (2004), where 138 samples of cocoa by-products (cake, mass, shell, nibs, butter and powder) from some cocoa producing countries (Indonesia, Ivory Coast, Ghana, Malaysia, Nigeria, Ecuador, Honduras and Peru) were analyzed. A total of 120 (87%) samples had ochratoxin A above the detection limit (0.1 μg/kg). The highest contamination was found in shell (11 μg/kg), followed by cake (2.6 μg/kg) and powder (2.41 μg/kg). The mean values were higher than those reported by us, although the concentration range in both studies were variable. None of the four samples of cocoa butter and two of the nibs had results above the LOD; considering the higher LOD reported by the author (0.1 μg/kg), the contamination present in the samples of butter and most nibs reported in our survey would also not be detected.

3.2. Evaluation of ochratoxin A distribution and stability during chocolate manufacture

An impressive reduction of ochratoxin A was verified during the chocolate manufacturing process (Table 2). For the level of contamination evaluated and under the conditions used in this study, 93.6% of the ochratoxin A was reduced during the chocolate making process.

Considering the results of ochratoxin A in the fractions after roasting and their percentage in the bean composition (Table 3), it is possible to estimate that about 16.6% of the toxin is destroyed by the thermal treatment at 150 °C applied for 40 min, which

Table 1
Ochratoxin A occurrence in cocoa by-products.

Matrix	Samples	Positive samples (%)	Mean concentration ± SD (μg/kg)	Concentration range (μg/kg)
Shell	19	19 (100%)	1.13 ± 1.28	0.13–2.01
Nibs	29	24 (83%)	0.10 ± 0.12	<LOD–0.38
Mass	25	25 (100%)	0.34 ± 0.21	0.03–1.09
Butter	25	20 (80%)	0.03 ± 0.02	<LOD-0.06
Cake	26	26 (100%)	0.97 ± 0.73	0.03–3.18
Cocoa powder	16	16 (100%)	1.42 ± 1.29	0.05–5.13
Alkalized cocoa powder	28	28 (100%)	0.90 ± 0.78	0.14–3.59

SD: standard deviation.
LOD: 0.01 μg/kg.

Table 2
Ochratoxin A contamination in cocoa beans and their fractions (five replicates) before and after roasting and percentage reduction.

Sample	Ochratoxin A ± SD (μg/kg)	Range (μg/kg)	Ochratoxin A reduction (%)[2]
Unroasted beans	9.46 ± 0.80	8.76–10.8	–
Roasted shell[1]	6.53 ± 0.37	6.11–7.12	–
Roasted nibs[1]	1.36 ± 0.21	1.15–1.69	85.7
Cocoa mass	0.96 ± 0.04	0.91–1.03	89.9
Chocolate	0.61 ± 0.03	0.58–0.66	93.6

SD: standard deviation.
[1] Relative to the cocoa bean composition, shell = 12.2% and nibs = 86.8%.
[2] Reduction% = reduction of ochratoxin A contamination when compared with unroasted cocoa beans.

Table 3
Characterization of roasted cocoa beans.

Parameter		
Composition of fractions (%)	Cotyledon	86.8
	Germ	0.95
	Shell	12.2
pH		5.23 ± 0.02
Lipids (%)		53.0 ± 0.93

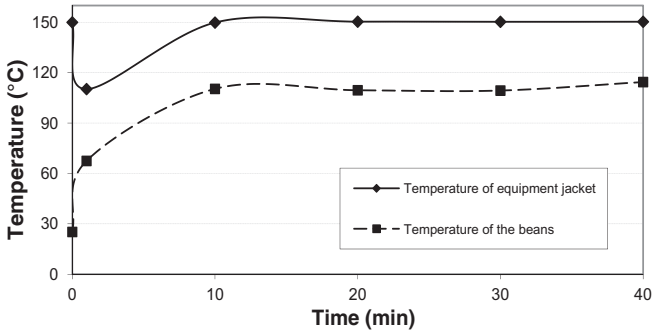

Fig. 1. Evaluation of the temperature in the equipment jacket and of the beans (gases inside the rotating drum) during the roasting of the nibs.

results in a temperature of about 90 °C in the beans (Fig. 1). This experiment demonstrated that after the roasting processing, 82.9% of the remaining ochratoxin A stayed in the shell fraction, which is mostly removed from the processing line by winnowing (where a maximum of 1–1.5% of shell residues in the nibs is allowed (Minifie, 1999). Thus, among the steps of cocoa processing, the shelling step is the main one responsible for the decrease of ochratoxin A. The amount of toxin physically removed is greater than that destroyed by the heating treatment of cocoa beans, so the winnowing efficiency can be considered critical for reducing the ochratoxin A contamination in chocolate and finished products.

In the next step, the cocoa mass was obtained after the grinding of nibs and a heat treatment of 70 °C for 3 h. After this step the total reduction increased to 89.9%, showing that this step is also important for ochratoxin decrease. The total reduction achieved in the final product, the chocolate was 93.6%.

It has been shown that the ochratoxin A molecule resists the majority of heat treatments used for food production (MAFF (Ministry of Agriculture & Food), 1996). A study conducted by Boudra, Le Bars, and Le Bars (1995) in dry wheat under temperatures and time of exposure similar to those applied in our study demonstrated that the ochratoxin A reduction achieved was 20%, similar to the values found in our study (16.6%). Manda et al. (2009) evaluated the stability of ochratoxin A during the cocoa processing, finding decreases between 23.7% and 40.5% when roasting cocoa at 140 °C for 30 min.

Different studies demonstrate that most ochratoxin A is concentrated in the shell fraction and just a small part of the toxin contaminates the nibs (Amezqueta, Gonzalez-Penas, Murillo, & Lopez de Cerain, 2005; Gilmour & Lindblom, 2008; Manda et al., 2009). About 48% (25–72%) of the toxin is physically removed by industrial shelling (Gilmour & Lindblom, 2008), while hand-made shelling can reduce 50–100% of ochratoxin A contamination (Amezqueta et al., 2005; Manda et al., 2009).

Regarding cocoa mass and chocolate, the ochratoxin A reduction reached 28.9% with the grinding of nibs (70 °C for 3 h). Considering the data from Boudra et al. (1995) in wheat, less than 20% would be expected. Additionally, 36.5% of the decrease was achieved in the chocolate when compared with cocoa mass due to the dilution caused by ingredient addition.

The overall reduction in the ochratoxin A contamination from the unroasted bean to the chocolate manufactured under conditions evaluated in this study was 93.6%, agreeing with the results reported by Manda et al. (2009), which amounted to 91%. In coffee, a fermented product similar to cocoa, the reduction reported by Ferraz et al. (2010) ranged from 8.2% to 98.9%, according to the time and temperature of exposure. In wheat the variation observed was between 2% and 94% (Boudra et al., 1995).

In general, the cocoa processing steps can reduce ochratoxin A contamination by destroying the molecule, as occurs in thermal treatments such as roasting; physically removing the contamination, as observed during shelling; or diluting the concentration, by adding other non-contaminated ingredients. They will therefore influence the contamination found in the finished products. Because the cocoa processing is not capable of eliminating all the ochratoxin A coming with the raw material, the best protection would be to prevent toxin formation in the cocoa producing chain. Consequently, the lower the initial contamination of cocoa beans the safer the manufactured chocolate will be.

4. Conclusion

If the amounts of ochratoxin A found in cocoa by-products is considered and the usually low amounts of these products employed in the formulation of chocolate powders, cakes, biscuits and similar products, it is concluded that cocoa does not represent a major source of ochratoxin A in the diet. However, one concern is the fact that chocolate-containing products are widely consumed by children who are more sensitive to the effects of mycotoxins. Thus, it is important that constant monitoring should be carried out of their occurrence and also to find ways to prevent the contamination in the cocoa production chain.

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