Universidade do Minho
Escola de Engenharia

Otniel Freitas-Silva

Mycotoxins and mycobiota in Brazil nuts and strategies for their control

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Dissertation for PhD degree in
Chemical and Biological Engineering

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Summary

The Brazil nut (*Bertholethia excelsa*) is an important non timber forest product (NTFP) from the Amazonian forest. Despite their nutritious value, Brazil nuts are susceptible to fungi contamination. The mycobiota associated in nuts is mainly concern due to some species of *Aspergillus* belonging to section *Flavi*, which is composed of a large number of very closely related species, same of which may produce Aflatoxins (AF). AF presence in nuts is not only an increasing concern to the consumer's health but also to economic aspects, causing losses in all phases of global distribution chain, affecting major exporting countries. The purpose of this study was to determine which part of the nut contributes to contamination by aflatoxins and to identify the mycobiota in Brazil nut samples. Unshelled and shell nuts were analyzed by measuring the total count of filamentous fungi (*Aspergillus* sections *Flavi*, *Nigri* and *Circumdati)* in sanitised (1 % sodium hypochlorite for 10 minutes) and non-sanitised treatments. To perform the AF analysis, samples of Brazil nuts were milled separately. The AF from the shell and kernel were extracted with chloroform and analysed by the HPLC-FD system in isocratic mode. The *Aspergillus* section *Flavi* count was 21.67% lower and the production of AF by this section was around 25%. The AFB_1 concentrations of shelled nuts and shell samples were 35.0 and 1.78 μg/kg, respectively. AFB_2 and AFG_2 were detected only in shelled nut samples. The HPLC-FD analysis presented limits of detection (LOD) and quantification (LOQ) of 0.2 and 0.4 μg/kg, respectively.

The occurrence of other fungal metabolites in Brazil nuts was also screened. A total of 201 mycotoxins (including the most prominent ones) were screened by a multi-mycotoxin method based on HPLC-MS/MS. Fifteen mycotoxins were detected and quantified, in at least one sample; namely, AFB_1, AFB_2, AFG_1, and AFM_1, kojic acid, sterigmatocystin, methyl-sterigmatocystin, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin. Aflatoxins were detected in just 1 sample (20%), but above its legal limit in Brazil and EU. Ochratoxin A and *Fusarium* toxins were not detected. Alternariol-methylether (from 0.75 to 3.2 μg/kg) was detected in all five samples. This is the first study dealing with the detection of kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin in Brazil nuts.

One of the agents with great potential to reduce mycotoxins is ozone. In view of this it was assayed the aqueous ozone use as a treatment on the mycotoxin decontamination, since water is a vehicle for washing and decontamination process. Concentrations of 0, 0.1, 1, 10, 20 and in some cases 40 mg/L

were applied with the aim to determine the ideal conditions to degrade AFB_1, AFB_2, AFG_1, and Cyclopiazonic acid (CPA). Aqueous ozone solutions with equal or greater than to 10 mg/L were able to control of AFB_1, AFG1 and CPA. Since ozone first attacks the double bonds of molecules, the less toxic aflatoxins (AFB_2 and AFG_2) were oxidized after the more toxic AFB_1 and AFG_1, being still detected after exposure to the higher ozone concentration tested. The AFB_1, AFB_2, AFG_1, AFG_2 and CPA were degraded by aqueous ozone.

A further experiment with biological samples was realized in order to determine and confirm the aqueous ozone potential ability to destroy fungi on both natural and artificially contaminated Brazil nuts. The former were inoculated with either $1x10^6$ or $1x10^7$ conidia/mL of an *A. flavus* strain. Different aqueous ozone contact time and different ozone concentrations were assayed. A concentrated aqueous ozone solution was assayed on both natural and artificially contaminated nuts. The remaining viable spores in the ozone solution were recorded and the rate of inactivation for each treatment was determined by assessing the ratio between the CFU of each treatment and the control. Ozonized nuts were also plated on MEA to recover the fungi population. Aqueous ozone was effective in reducing the conidia of *Aspergillus flavus* and the natural fungal population associated to Brazil nuts. The optimum ozone concentration depended on the initial viable spores on the nutshell. Also, the effect of the ozonization on the shell nut color was assessed by measuring chromaticity values of the treated fruits in the L*a*b* space coordinates. High concentrations of ozone affected both the luminosity and the hue of the nutshell, affecting a little the external appearance of the shell. Ozone is regarded and generally recognized as safe (GRAS) product and it has been already used in many agricultural products, including the organically labels one like Brazil nuts. Thus, the aqueous ozone solution may be recommended to control mycotoxin producer Aspergilli.

Key words: aflatoxins, *Bertholletia excelsa*, fungi, multimycotoxin method, nontimber forest products, tropical forestry.

Resumo

A castanha do Brasil (*Bertholethia excelsa*) é um genuíno representante de Produtos Florestais não-Madeireiros (PFNM), da floresta amazónica. Apesar de seu valor nutritivo, a castanha do Brasil é muito suscetível à contaminação por fungos. A micobiota associada a esta semente representa uma enorme preocupação à segurança alimentar, principalmente algumas espécies de *Aspergillus* pertencentes a seção *Flavi.* Essa secção é composta por um grande e complexo número de espécies estritamente relacionadas, que podem produzir aflatoxinas (AF). A presença de AF em castanha do Brasil não é apenas uma preocupação crescente para a saúde do consumidor, mas também relacionada com os aspectos sociais e económicos, causando prejuízos em todas as fases da cadeia de distribuição global deste produto, afetando os principais países exportadores.

Um dos objetivos deste estudo foi determinar qual parte da castanha do Brasil contribui mais para a contaminação por AF e identificar a micobiota nestas amostras. Casca e castanha descascada foram analisadas quanto à contagem total de fungos filamentosos (*Aspergillus* seções *Flavi, Nigri* e *Circumdati*) antes e após a sanitização (1 % de hipoclorito de sódio por 10 minutos). Para realização da análise de AF, as amostras de castanha do Brasil foram moídas separadamente. As AF da casca e da castanha descascada foram extraídas com clorofórmio e analisadas por HPLC-FD em modo isocrático. A contagem dos *Aspergillus* da seção *Flavi* foi de 21,67 % e a produção de AF por fungos nessa seção foi de cerca de 25 %. A concentração de AFB_1 na castanha descascada e na casca foi de 35,0 e 1,78 μg/kg, respectivamente. As aflatoxinas AFB_2 e AFG_2 foram detectadas apenas nas amostras descascadas. A análise por HPLC-FD apresentou os limites de detecção (LOD) e os de quantificação (LOQ) de 0,2 e 0,4 μg/kg, respectivamente.

Em outro ensaio também foi investigado a ocorrência de outros metabolitos de fungos em castanha do Brasil. De um total de 201 micotoxinas (incluindo as mais proeminentes) foram analisadas por um método de análise de multi-micotoxinas utilizando um HPLC-MS/MS. Quinze micotoxinas foram detectadas e quantificadas, em pelo menos uma amostra, nomeadamente AFB_1, AFB_2, AFG_1, AFM_1, ácido cójico, esterigmatocistina, metil-esterigmatocistina, citrinina, ciclosporina A, ciclosporina C, ciclosporina D, ciclosporina H, rugulosina, altenariol-metil-éter e emodina. As AF foram detectadas em apenas 1 amostra (20 %), entretanto acima de seu limite legal no Brasil e na UE. A ocratoxina A e as toxinas de *Fusarium* não foram detectadas. Alternariol-metil-éter (0,75-3,2 μg/kg) foi detectado em todas as cinco

amostras. Este é o primeiro relato da detecção de ácido cójico, citrinina, ciclosporina A, ciclosporina C, ciclosporina D, ciclosporina H, rugulosina, altenariol-metil-éter e emodina na castanha do Brasil.

Um dos agentes com grande potencial para o controlo de fungos e de micotoxinas é o ozono. Portanto, no trabalho realizado, aplicou-se o ozono aquoso para a descontaminação de micotoxinas, uma vez que a água é um veículo que pode ser utilizado na lavagem e no processo de descontaminação. Foram analisados os efeitos das concentrações 0; 0,1; 1; 10; 20 e em alguns casos, 40 mg/L para determinar quais as condições ideais de degradação das AFB_1, AFB_2, AFG_1 e do ácido ciclopiazónico (CPA). As soluções aquosas de ozono iguais ou maiores do que 10 mg/L foram capazes de eliminar AFB_1, AFG_1 e CPA. A AFB_1, a AFB_2, a AFG_1, a AFG_2 e o CPA foram degradados pelo ozono aquoso. No entanto, uma vez que o ozono ataca primeiro as duplas ligações das moléculas, as aflatoxinas mais tóxicas (AFB_1 e AFG_1) foram degradadas primeiro do que as menos tóxicas (AFB_2 e AFG_2). Estas últimas foram ainda detetadas após a exposição da maior concentração de ozono testada.

Um outro ensaio com amostra biológica foi realizado para determinar e confirmar o potencial do ozono aquoso na destruição de fungos em amostras de castanha do Brasil artificialmente contaminadas e natural. As primeiras foram inoculadas com 1×10^6 ou 1×10^7 conídios/mL de *A. flavus.* O tempo de contato e as concentrações de ozono foram testados em ambas amostras. Foram registadas a taxa de inativação de conídios através da relação das unidades formadoras de colónias (ufc) de cada tratamento e o controlo. A recuperação de fungos após a ozonização foi realizada pelo cultivo dos filtros em MEA. O ozono aquoso foi eficaz na redução de conídios de *Aspergillus flavus* e da população natural de fungos associados a castanha do Brasil. A concentração de ozono ótima dependeu da concentração inicial de conídios viáveis presentes na castanha. Além disso, o efeito da ozonização na cor da casca também foi mensurado, pela medição dos valores de cromaticidade das coordenadas L* a* b* dos frutos tratados. Em altas concentrações de ozono tanto a luminosidade quanto o matiz das das castanhas foram afetadas, afetando ligeiramente a aparência externa da casca.

O ozono é um produto geralmente reconhecido como produto seguro (GRAS) com seu uso e aplicação amplamente aceitos em diversos produtos agrícolas, podendo ser inclusive aplicado em produtos orgânicos, como é o caso da castanha do Brasil. Desta forma, a solução de ozono aquoso pode ser recomendada para controlar os Aspergilli produtores de micotoxinas.

Palavras chave: Aflatoxinas, *Bertholletia excelsa*, fungos, micotoxinas, método analítico para multimicotoxinas, Produtos florestais não madeireiros, floresta tropical.

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List of Abbreviations

µg - Microgram

AF – Aflatoxins

AFB_1 – Aflatoxin B_1

AFB_2 – Aflatoxin B_2

AFG_1 – Aflatoxin G_1

AFG_1 – Aflatoxin G_1

AFM_1 – Aflatoxin M_1

AFT – Total Aflatoxins

AME - Alternariolmonomethylether

ANOVA - analysis of variance

APCI - atmospheric pressure chemical ionization

a_w - water activity

C18 - *see* ODS

CAC- Codex Alimentarius Commission

C*f* - Correction factor

CV - Coefficient of variation

DFP- Destined for further processing

ELISA - Enzyme linked immunosorbent assay

EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária- (Brazilian Agricultural Research Corporation)

ESI - Electrospray interface/ionization

EU - European Union (

FAO – Food and Agriculture Organization

FD - Fluorescence detection

FDA - U.S. Food and Drug Administration

FVO - Food and Veterinary Office

g - Gram

GAP - Good agricultural practice

GC - Gas chromatography

GMP - Good manufacturing practice

GRAS - Generally recognized as safe

HACCP - Hazard analysis and critical control point

HPLC – High performance liquid chromatography

IAC - Immunoaffinity column

IARC - International Agency for Research on Cancer

IS - Internal standard

IUPAC - International Union for Pure and Applied Chemistry

JEFCA - Joint FAO/WHO Expert Committee on Food Additives (JECFA)

KA - Kojic Acid

Kg - Kilogram

L - Liter

LC - Liquid chromatography

LC-MS/MS - Liquid chromatography mass spectrometry

LOD - Limit of detection

LOQ - Limit of quantification

MAPA- The Ministry of Agriculture, Cattle and Supply (Ministério da Agricultura, Pecuária e Abastecimento - Brasil)

mg - Milligram

MRL - Maximum residue limit

MRM - Multiple reaction monitoring

MS - Mass spectrometry detection

MS/MS - Tandem mass spectrometry

MSMC - Methyl-Sterigmatocystin

MW - Molecular weight

ng - Nanogram

NOAEL - No-observed-adverse-effect level

NTFP - Non-timber forest product

O_3 – Ozone

ODS - Octadecylsilica

p - Probability

PTFE - Polytetrafluoroethylene

Q TRAP - Quadrupole-linear ion trap instrument

R_2 - Coefficient of determination

RASFF - Rapid Alert System for Food and Feed

RP - Reverse phase

RSD - Relative standard deviation

RTE- Ready-to-eat

S - Standard deviation

SAF - agroforestry system

S/N - Signal/noise

SMC - Sterigmatocystin

SRM - Single reaction monitoring

T - Temperature

t tonnes

TLC - Thin layer chromatography

t_R - retention time

UV - Ultraviolet

WHO – World Health Organization

Thesis outputs

The results presented in this thesis are partially published/submitted in:

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Freitas-Silva O, Venâncio A (2011) Brazil nuts: Benefits and risks associated with contamination by fungi and mycotoxins. Food Res Int 44:1434-1440.

Freitas-Silva O, Souza MLM, Venâncio A (2011)Tracing fungi secondary metabolites in Brazil nuts using LC-MS/MS. Drug Metab Lett 5:150-155.

Freitas-Silva O, Teixeira A, Cunha FQ, Godoy RLO, Venâncio A (2011) Predominant mycobiota and aflatoxin content in Brazil nuts. J. Verbraucherschutz Lebensmittelsicherh. DOI: 10.1007/s00003-011-0703-6.

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Freitas-Silva O, Venâncio A (2008) Supressão de *Aspergillus* produtores de aflatoxinas e ácido ciclopiazonico por ozônio. In*:* Encontro Nacional de Micotoxinas (XIII ENM 2008), 6-8/11/2008.Revista Universidade Rural. Série Ciências da Vida, v. 28, p. 128-200.Rio de Janeiro, Brazil. **(Awarded)**.

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Freitas-Silva O, Souza MLM, Venâncio A (2011) A multi-compound LC-MS/MS method for the screening of fungi secondary metabolites in Brazil nuts. 1st International CIGR Workshop on Food Safety: Advances and Trends, Dijon, France.

Freitas-Silva O, Morales-Valle H, Venâncio A (2010) Aqueous ozone controlling fungi in Brazil nuts. In: 4th International Congress on Bioprocesses in Food Industries (ICBF 2010) / X ERSCTA), 2010, Curitiba. Anais do 4th International Congress on Bioprocesses in Food Industries (ICBF 2010) / X ERSCTA). TecArt Editora Ltda, Curitiba, Brazil.

Freitas-Silva O, Teixeira A, Godoy RLO, Venâncio A (2009) Mycobiota predominant and aflatoxins content in shell and shelled Brazil nuts. III BioMicroWorld conference, Lisbon, Portugal.

CHAPTER 1

Outline of the thesis

The Brazil nut tree (*Bertholletia excelsa*) is an important product to the Brazilian Amazon, producing commercially nutritionally harvested edible seeds: the Brazil nuts. This nut is an important product from the Amazon forest, with a 2008 annual world production estimated of 78,000 t, being Brazil responsible for approximately 40 % of it. Although there are beneficial nutritional properties, due to their high content of proteins, carbohydrates, unsaturated lipids, vitamins, essential minerals and mainly by their high level of Selenium, the prevailing mycobiota of Brazil nuts include fungi that are producers of aflatoxins (AF), such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. AF has deleterious effects in consumption considering the global distribution chain, affecting major exporting countries. Currently, its marketing is being affected by the high incidence of AF produced by potentially aflatoxigenic fungi associated with its seeds.

Ozone, a powerful oxidant, may be used for the inactivation of various microorganisms and for the degradation of chemical contaminants. Although there are not many reports on the use of ozone against filamentous fungi or their mycotoxins, promising results have been reported. With a short half-time, at neutral pH and ambient temperature, ozone is able to inactivate microorganisms and to decompose their toxic metabolites leaving no traces of ozone in the treated commodity. This fact makes the use of ozone safe in food applications. There has been relatively limited research in this topic, especially with the use of aqueous ozone. The best management strategy still remains to be developed, but initial studies have indicated that an application of ozone for a short period of exposure is capable of controlling the proliferation of filamentous fungi and of degrading many mycotoxins.

The leitmotiv of this Thesis is therefore to provide possibilities to control fungi and mycotoxins in Brazil nuts, using products compatibles with the appeal of Brazil nut as an organic product. Nowadays, there are no unique technologies to be used as fungi and mycotoxins control.

In these contexts, the aim of this thesis was, at first, to identify the dangerous fungi and mycotoxins in Brazil nut. A second objective was based on the control of the predominant mycobiota

and its mycotoxigenic potential in Brazil nuts. In this respect, chlorine and aqueous ozone were applied directly on the Brazil nuts.

Based on these main objectives, this thesis was organized in two parts and eight chapters. Part 1 is composed of chapters 2 to 4. The chapter 2 provides an overview of the state-of-the-art; chapters 3 to 4 contain the main experimental results with mycotoxins in Brazil nuts. Part 2 is composed of chapters 5 to 7. The chapter 5 presents a detailed review with ozone technology, while chapters 6 and 7 present results by aqueous ozone application on mycotoxins and fungi, respectively. Finally one last chapter (chapter 8) is presented, summarizing the main achievements and conclusions, and presenting perspectives for forecoming work. As the thesis is organized in independent chapters, it should be noted that some subjects could be eventually repeated in some chapters' introduction.

In Chapter 2, the thesis focuses on of the importance of Brazil nuts for the Amazon rainforest and healthy, emphasizing on the social and environmental impact of its production, on the mycobiota contamination of seeds, and on the presence of mycotoxins and related food safety aspects.

Chapter 3 studied the contamination by fungi and AF in shell and shelled nut unshelled and shelled nuts were analyzed by measuring the total count of filamentous fungi (*Aspergillus* sections *Flavi*, *Nigri* and *Circumdati)* in sanitised (1 % sodium hypochlorite for 10 minutes) and non-sanitised treatments. The isolates identified as *Aspergillus* section *Flavi*, the major producers of AF, were plated for determination of their aflatoxigenic potential. To perform the AF analysis, the AF from the shell and kernel were extracted with chloroform and analysed by HPLC-FD.

In Chapter 4 it was done a screening to evaluate quantitatively the occurrence of fungal metabolites in Brazil nuts. A total of 201 fungal metabolites were screened by a multi-mycotoxin method based on HPLC-MS/MS. The recovery was between 56 % and 136 %. Fifteen mycotoxins were detected and quantified, in at least one sample; namely, AFB_1, AFB_2, AFG_1, and AFM_1, kojic acid, sterigmatocystin, methyl-sterigmatocystin, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, alternariol-methylether and emodin. Alternariol-methylether (from 0.75 to 3.2 μg/kg) was detected in all analysed samples. This is the first study dealing with the detection of kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, alternariol-methylether and emodin in Brazil nuts.

In Chapter 5 it was reviewed the potentialities of ozone, as a powerful oxidant, especially against filamentous fungi or their mycotoxins. Ozone is able to inactivate microorganisms and to

decompose their toxic metabolites leaving no traces of ozone in the treated commodity. This fact makes the use of ozone safe in food applications. The best management strategy still remains to be developed, but initial studies have indicated that an application of ozone for a short period of exposure is capable of controlling the proliferation of filamentous fungi and of degrading many mycotoxins.

The use of aqueous ozone to control AF and Cyclopiazonic acid was demonstrated in chapter 6.

Chapter 7 describes the Potential of aqueous ozone to control aflatoxigenic fungi in Brazil nuts**.** All the effects of aqueous ozone on the *Aspergillus flavus* and natural mycobiota from Brazil nuts were studied. Also it was demonstrated that the application of aqueous ozone had a little bit change on its nutshell color change.

Overall conclusions and perspectives are presented in Chapter 8.

PART I

CHAPTER 2

Brazil nuts: Benefits and risks associated with contamination by fungi and mycotoxins

2.1 INTRODUCTION

The Amazon rainforest consists of multiple ecosystems containing a huge biodiversity. The forest has an important role in the global weather balance and it provides shelter and livelihood for many local communities (Vieira et al., 2008). The production and extractivism of the Brazil nut (*Bertholletia excelsa* Humb. & Bompl.) is observed only in the Amazonian biome. This is an important activity for forest people in countries where the trees grow, stimulating the sustainable use of renewable natural resources while combining social development and conservation (Silvertown, 2004). The gathering and commercialization of Brazil nuts by traditional population is an activity that neither destroys the forest nor threats the ecological balance and the environment (Walt et al., 2008). With effect, *B. excelsa* trees are an essential part of the rainforest, as they help maintain the balance in the relationship between flora and fauna (Wadt et al., 2005), since they represent a non-timber forest products (NTFPs), in this way contributing to: i) the household food security and health of local subsistence economies; ii) the local, regional and international trade; and iii) conservation of locally available natural resources, which are compatible with preservation of local forests (FAO, 1995).

Brazil nuts are the main commodity from the Amazon rainforest extractivism. The nuts are destined to national or international trade. Gatherers pick and store the fruits; they are responsible for the initial handling and processing, which is still done in the forest (Codex Alimentarius Commission, 2010). The main stages in Brazil nut production are: production and collection in the forest (cleaning paths between trees, gathering the fruit, opening the fruit and transporting them to the camp), and processing (cleaning, drying and soaking, peeling the nuts, drying the peeled nuts) and commercialization in the packing house (FAO, 2006).

Contamination by aflatoxins (AF) is a major problem for tree nuts, as well as for other stored grains, milk and dry fruits, especially because the causal fungi, *Aspergillus flavus* group, occurs as a natural contamination (Molyneux et al., 2007). Industries and producers have endeavored considerable efforts over the last 15 years to minimize fungal growth and AF production in tree nuts, particularly in the case of Brazil nuts, due to the hot and humid climatic conditions found in the Amazon environment, with an average temperature of 26 °C and relative humidity of 80-95 %, which favors the production of these toxins. In addition, the extractivism characteristics (temperature and relative humidity during gathering and handling) are hard to be controlled, having a direct or indirect effect on toxigenic fungi and on the production of AF. Since contamination is usually associated with shelled nuts, proven processing/treatments that reduce AF levels in Brazil nuts include shelling or sorting by size, specific gravity, color or damage (De Mello-Robert and Scussel, 2009; Pacheco et al., 2010). This review considers the benefits of Brazil nuts and many aspects concerning AF in Brazil nuts, including the occurrence of the fungi, the mycotoxins production and its control.

2.2 ECONOMIC, SOCIAL AND ECOLOGICAL ASPECTS OF BRAZIL NUTS

Despite the importance of the Brazil nut tree, the same is still currently classified as vulnerable to extinction (IUCN, 2010). Native from Amazonian region, the Brazil nut tree is among the highest specimens in the tropical forest, easily reaching 50 m and living up to 1,200 years old. The pod, which weighs about a pound (about 0.45 kg) and may contain 15 to 24 seeds, is known as the fruit of the Brazil nut (Mori and Prance, 1990). Brazil nuts are typical NTFPs and, for that reason, have been increasingly valued in the market, also for its characteristic flavor, high nutritional value and their association with environmental conservation (Yang, 2009). It is the only seed internationally traded that is gathered in the forest (Peres et al., 2003). Its origin comes from the Amazon region, mostly in the north of Brazil and neighbour countries (Peru, Guyana, Venezuela, Suriname and Bolivia), but only Bolivia, Brazil and Peru export this nut. In 2008, Bolivia was responsible for 53 % of in-shell Brazil nut world production, compared with 39.35 % and 0.37 % from Brazil and Peru, respectively (FAOSTAT, 2011).

The majority of attempts to cultivate Brazil nuts for commercial purposes inside and outside the Amazon region failed because no attention was given to pollinator agents, since the Brazil nut tree is a predominantly pollinating species, with a very low autogamy level. The tree produces fruits only with the presence of its natural pollinators, the bees. According to Motta-Maués (2002), the main pollinators

of Brazil nut trees are carpenter bees and bumble bees, from the Apidae and Anthophoridae families, e.g. *Xylocopa frontalis, Epicharis rustica, E. affinis, Epicharis* sp*., Eulaema nigrita, Bombus transversalis* and *B. brevivillus*. This author reinforces the importance of the development of management programs for main pollinators so as to allow the cultivation of Brazil nuts in wide scale. This is to avoid the decrease of the natural population of pollinators in commercial plantations and, consequently, a low fruit production, even in an agroforestry system or managed forestry.

Brazil nuts, although exported since 1800, only had a place in the exports agenda of NTFPs after the beginning of the twentieth century (Emmi, 2003) (Emmi, 2003). After the decline of rubber production (*Hevea brasiliensis*), Brazil nuts became the primary extraction product for export purposes in the Northern Region of Brazil, and its exploitation has a key role in the socio-economic organization of large areas of natural forest.

Sustainable exploitation is widely advocated as a strategy for reconciling economic pressures on natural habitats by means of nature conservation (Silvertown, 2004). The extraction of Brazil nuts has always been seen as an example of sustainable exploitation of the Amazon forest. But this view was shattered by the publication of Peres et al., (2003) showing that there is no regeneration of Brazil nut trees, i.e. they undergo an ageing process and the ageing rate of the population does not follow the occurrence of new plants, which might mean, in the long term, a collapse caused by over gathering. The concern raised by Peres et al., (2003) has been challenged by some studies such as one conducted in Bolivia, assessing the population structure of Brazil nut trees in a forest with and without gathering activities, which was also compared to a secondary (15 years old) forest. This research showed that gathering did not affect regeneration rate, and that the differences observed favoring the secondary forest are due to the positive influence of light on the recruitment of seedlings (Zuidema and Boot, 2002). Such ecological characteristics of Brazil nuts are corroborated also with arguments of Cotta et al. (2008). These authors reinforce the theory of Balée (1989) in which the occurrence and distribution of Brazil nut trees in the forestry is not only the result of natural actions, as it has been thought so far, but rather the result of anthropogenic activities of native Amazon populations in the forest environment. In other words, "*castanhais*" are a sort of cultural forest, and their long-term sustainability will depend on its proper management by humans and appropriate environmental interactions.

Over the last 15 years in Brazil, Brazil nuts have experienced a significant decrease in exports. According to a comparative assessment, one of the explanations for the crisis caused by such decrease in the exports in the Brazilian Amazon was the entry of Bolivia into the international market in 1996

(Coslovsky, 2005), as well as a high incidence of AF in the nut recorded in Brazil. Until 1990, most of Bolivia's production was sent to Brazil, where the nuts were processed, industrialized and exported. From 1990, the exportation of Brazil nuts by Bolivia was fostered mostly by grants from the World Bank and by a low taxation system (Coslovsky, 2005). This caused the product supply to reach prices below those charged by the market, inhibiting the Brazilian exporter, who preferred to wait for the Bolivian stock to end in order to sell their products.

The crash and crisis can still be justified by non-tariff barriers imposed by the European Community since 1998, EC regulation 1525/98 and EC decision 493/2003 (European Communities, 1998; European Union Commission, 2003). Because of those decisions, Brazilian exports of Brazil nuts in shell to Europe fell by almost 90 % between 2000 and 2004. The first regulation reduced the acceptable limit of total aflatoxins (AFT) in Brazil nuts to 4 μg/kg and 2 μg/kg for AFB_1 and rejected contaminated consignments from Brazil (Silva, 2010). While the second one imposed special conditions on the import of Brazil nuts in shell originating in or consigned from Brazil. Moreover, the domestic market absorbs only 10 % of the Brazil nut production, because what is left in Brazil is the lower quality product and no marketing strategy for Brazilians consumers was yet established.

The Brazil nut production is currently recovering in Brazil (Figure 2.1); as of 2009 the national production of it was of 37,467 tonnes, representing an increase of 21.6 % compared to the production of 30,815 tonnes achieved in 2008. The main producer is the State of Amazonas, concentrating 42.7 % of the total gathered, following by the States of Acre (27.5 %), Pará (18.7 %) and Rondônia (6.9 %) (Figure 2.2) (IBGE, 2009).

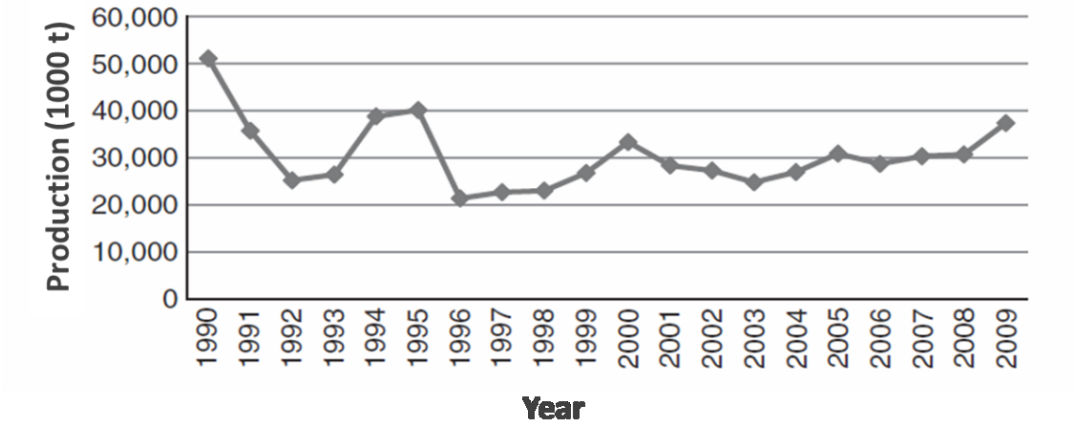

Figure 2.1. Fluctuation in the production of Brazilian Brazil nuts. (IBGE, 2009).

Figure 2.2. Brazil nut production and occurrence on its states in Brazil (Map adapted from IBGE: http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?c=289&z=p&o=25).

The existence of a market for rainforest products is not enough on its own to prevent the destruction of habitats or overexploitation of the resource. Moreover, a more sophisticated approach to sustainability is required, translated as the development of an ethically-traded Brazil nut market (Silvertown, 2004).

Brazil nut can be processed or unprocessed. The nuts can be sold in their shell, semi dried or dehydrated in bulk (without treatment) (Figure 2.3). Shelled nuts are obtained manually by cracking them to open, and they can be sold with or without their pellicle. The storage and conservation of Brazil nuts are one of the most important issues in their commercialization (Arrus et al., 2005a; Pacheco and Scussel, 2009).

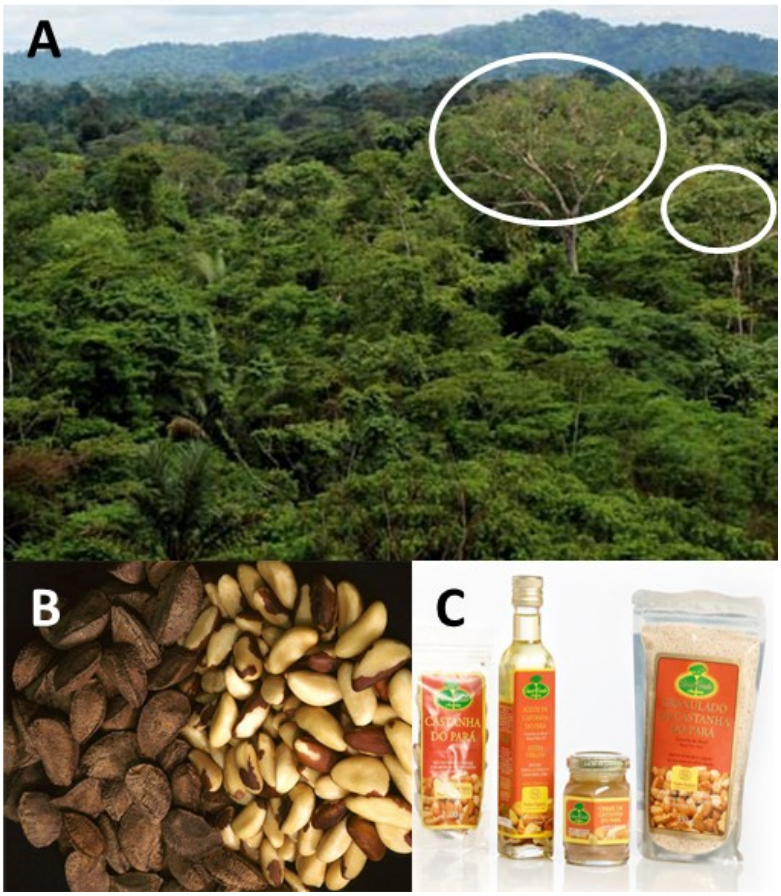

Figure 2.3. Brazil nut trees in the forest (A), seeds (in-shell and shelled) (B) and processed products (C).

In addition to being a foodstuff, the residue of Brazil nuts has several applications. Their pods and shell can be used as fuel and to produce recycled wood and handicraft, and the oil can be used for culinary purposes or in the cosmetic industry (Pacheco and Scussel, 2006). From processed Brazil nut it can be obtained the Brazil nut milk, processed kernels can also be used in variety of food industry products (mix with others nuts, cereals bars, chocolate and the meal to flour preparations) (Souza and Menezes, 2004).

In trade, Brazil nut can be classified in ready-to-eat (RTE) or those destined for further processing (DFP) (Codex Alimentarius Commission, 2010). RTE Brazil nuts are those which are not supposed to undergo any additional processing/treatment before reaching the final consumer. Both, RTE and DFP Brazil nuts can be traded shelled or unshelled and both are susceptible to mycotoxins.

2.3 HEALTH BENEFITS

Among all nuts, Brazil nuts are regarded as one of the most nutritious human foods, due to their high contents of proteins, carbohydrates, unsaturated lipids, vitamins and essential minerals. Brazil nuts have been of interest due to their exceptionally high, by a substantial factor, level of Selenium (Se) (Chunhieng et al., 2004; Chunhieng, et al., 2008; Welna et al., 2008) and other important minerals as calcium, magnesium, phosphorus and potassium (Table 2.1) (USDA, 2008; Yang, 2009; INC, 2010).

Concerning Se, an important antioxidant, it is an essential trace element consumed in sub-milligram amounts, primarily in organically bound forms, in the diet. Gross deficiency leads to a range of diseases or disorders, the most well-known being the cardiomyopathy, Keshan disease (Verkerk, 2010). This antioxidant is required for the function of a number of key selenium-dependent enzymes (selenoproteins) necessary for a wide range of metabolic processes, including thyroid hormone regulation, immune function and reproduction (Pacheco and Scussel, 2007). Studies suggest that proper selenium intake is correlated with a reduced risk of breast, lung and prostate cancer (Pacheco and Scussel, 2007). These findings ask for more research on the correlation between selenium and reduced risk of various cancers (Verkerk, 2010). As a result, Brazil nuts are sometimes recommended as a protective measure.

According to Rodrigues et al. (2005), broken nuts loose 40 % of their commercial value, being used to produce mechanically pressed oil. The byproduct of Brazil nut oil - cake, due to its nutritional characteristics, can be used in the food industry: an extract called “milk” and a protein concentrate and isolate are obtained from the defatted cake, after extraction using hexane or ethanol (Freitas et al., 2007). The vegetable oil is composed of a complex mixture of triglycerides, and the remaining constituents are usually composed of free fatty acids, monoglycerides, diglycerides and other fats at small concentrations: tocopherols, carotenoids and sterols (Rodrigues et al., 2005).

Dry Brazil nuts are about 18 percent protein, 13 percent carbohydrates, and 69 percent fat. The relation among saturated, monounsaturated and polyunsaturated oil in percentile are: 25 : 41 : 34 (USDA, 2008), this represents that content of saturated fat in Brazil nuts is higher than in any other nut. Brazil nuts also are a rich source of vitamin B1 and vitamin E, a good source of niacin and calcium, and a source of iron (USDA, 2008).

Table 2.1. Nutrient profile in Brazil nuts (nutrients/100g)

Nutrient	Units[1]	Value /100g
Proximates		
Water	g	3.48
Energy	kcal	656
Energy	kJ	2743
Protein	g	14.32
Total lipid (fat)	g	66.43
Ash	g	3.51
Carbohydrate, by difference	g	12.27
Fiber, total dietary	g	7.5
Sugars, total	g	2.33
Sucrose	g	2.33
Starch	g	0.25
Minerals		
Calcium, Ca	mg	160
Iron, Fe	mg	2.43
Magnesium, Mg	mg	376
Phosphorus, P	mg	725
Potassium, K	mg	659
Sodium, Na	mg	3
Zinc, Zn	mg	4.06
Copper, Cu	mg	1,743
Manganese, Mn	mg	1,223
Selenium, Se	mcg	1917
Vitamins		
Vitamin C, total ascorbic acid	mg	0.7
Thiamin	mg	0.617
Riboflavin	mg	0.035
Niacin	mg	0.295
Pantothenic acid	mg	0.184
Vitamin B-6	mg	0.101
Folate, total	mcg	22
Folate, food	mcg	22
Folate, DFE[2]	mcg_DFE	22
Choline, total	mg	28.8
Betaine	mg	0.4
Vitamin E (alpha-tocopherol)	mg	5.73
Tocopherol, gamma	mg	7.87
Tocopherol, delta	mg	0.77

Table 2.1. Nutrient profile in Brazil nuts (nutrients/100g) (continued)

Nutrient	Units[1]	Value /100g
Lipids		
Fatty acids, total saturated	g	15,137
Fatty acids, total monounsaturated	g	24,548
Fatty acids, total polyunsaturated	g	20,577
Amino acids		
Tryptophan	g	0.141
Threonine	g	0.362
Isoleucine	g	0.516
Leucine	g	1,155
Lysine	g	0.492
Methionine	g	1,008
Cystine	g	0.367
Phenylalanine	g	0.63
Tyrosine	g	0.42
Valine	g	0.756
Arginine	g	2,148
Histidine	g	0.386
Alanine	g	0.577
Aspartic acid	g	1,346
Glutamic acid	g	3,147
Glycine	g	0.718
Proline	g	0.657
Serine	g	0.683

Data adapted from the International Nut Council / USDA National Nutrient Database for Standard Reference (INC, 2010). 1g = gram, mg = milligram, mcg = micrograms, kcal = kilo calories; 2DFE = dietary folate equivalents).

2.4 CONTAMINATION ROUTES OF AFLATOXINS IN BRAZIL NUT CHAIN

2.4.1.Toxicological aspects of aflatoxins

Mycotoxins are natural contaminants produced by fungi and are commonly found in a wide variety of food products including cereals, legumes, nuts and their products (Paterson and Lima, 2010). Whenever the consumption of these products, including Brazil nuts, is significant in the diet of populations, such infection poses a high risk of chronic exposure (Jolly et al., 2009). Mycotoxins can cause adverse effects to health. Among more than 300 types of mycotoxins, the AF are the most studied ones due to their toxic potential (Klich, 2009). The ingestion of AF may increase the risk of lung and liver cancer and it can also suppress the immune system, increasing the risk of infections. Additionally, it may

cross the placental barrier (Jolly et al., 2009; Meggs, 2009; Partanen et al., 2010). There are no reports on the existence of susceptibility to AF, although there are differences in susceptibility from one species to another.

AFB_1 is responsible for carcinomas in animals, showing a strong relationship with the incidence of cancer in humans (Jolly et al., 2009; Meggs, 2009). AF are a group of related structurally oxygenated heterocyclic compounds produced under favorable conditions by several species of *Aspergillus*. The most known naturally occurring AF are named AFB_1, AFB_2, AFG_1, and AFG_2 (Figure 2.4). AFB_1 occurs in the highest amounts in contaminated commodities; AFB_2, AFG_1, and AFG_2 (Soares et al., 2010) are generally not reported in the absence of AFB_1. Total aflatoxins (AFT) refer to the sum of the four related compounds.

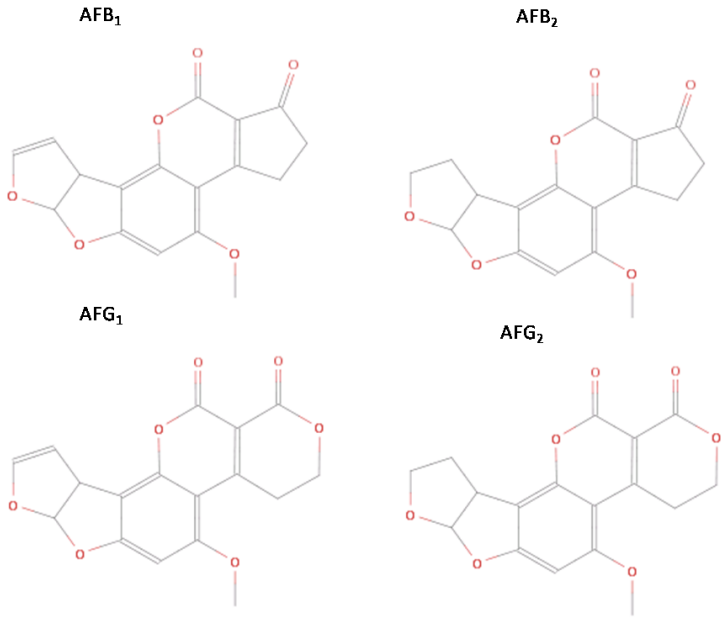

Figure 2.4. Chemical structure of aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2.

In 1998, the joint FAO/WHO Expert Committee on Food Additives (JECFA) considered the carcinogenic potential of AF and the potential risks associated with their intake. No tolerable daily intake was proposed, since these compounds are genotoxic carcinogens. The risk estimative for human liver cancer resulting from exposure to AFB_1 was derived from epidemiological and toxicological studies (FAO/WHO, 2008).

The JECFA reviewed a wide range of studies conducted with both animals and humans that provided qualitative and quantitative information on the hepatocarcinogenicity of AF. The carcinogenic

potency of AFB_1 is substantially higher in carriers of hepatitis B virus (about 0.3 cancers/year/100,000 persons/ng of AFB_1/kg bw/day), as determined by the presence in serum of the hepatitis B virus surface antigen ($HBsAg^+$ individuals), than in $HBsAg^-$ individuals (about 0.01 cancers/year/100,000 persons/ng of AFB_1/kg bw/day). The JECFA also observed that vaccination against hepatitis B virus would reduce the number of carriers of the virus, and thus reduce the potency of the AF in vaccinated populations, leading to a reduction in the risk for liver cancer (FAO/WHO, 2008).

2.4.2.Fungi contamination in Brazil nuts

Fungal infection in Brazil nut kernels has been known since the beginning of last century. One of the first reports described in literature about the loss of quality of Brazil nuts was made by Spencer (1921). This author described a percentage of losses caused by microbiological contamination between 10 and 25 % in Brazil nuts exported from Amazon to the U.S.A. At that time, he found that post-harvest losses of Brazil nuts were predominantly due to fungal contamination. The author, who has described the presence of *Aspergillus flavus* in Brazil nut kernels, also described other fungi infecting the kernels. Among them, *Penicillium* spp., *Pellionella macrospora, Cephalosporium bertholletianum*, *Fusarium* spp. and *Phomopsis bertholletianum* were detected. Other authors, who later studied the fungal mycobiota in Brazil nuts, also highlighted the diversity of species, with the predominance of mitosporic fungi. Since *Aspergillus* is notably the predominant fungus, especially species in section *Flavi*, there is great concern with the production of AF in this product. Subsequent studies also demonstrated the predominance of *A. flavus.* It was reported that among the seventeen fungal species found in Brazil nuts, *A. flavus* was the dominant species, followed by *A. niger*, *Penicillium citrinum* and *Penicillium glabrum* (Freire et al., 2000). According to this study, some species were primarily isolated from Brazil nuts, including *Acremonium curvulum*, *Cunninghamella elegans*, *Exophiala* sp., *Fusarium oxysporum*, *Pseudoallescheria boydii*, *Rhizopus oryzae*, *Scopulariopsis* sp., *Thielavia terrestrial* and *Trichoderma citrinoviride*. The species found by Freire et al. (2000) included fungi associated with deterioration and potentially toxigenic species, including *A. flavus* and *P. citrinum*, producers of AF and citrinin, respectively. The presence of these fungi may be a sign of inadequate drying, processing and storage.

To answer some questions concerning fungal distribution, abundance, specificity and implications of these distribution patterns, and concerning the control of AF contamination in Brazil nuts, Bayman et al. (2002) studied different tree nuts groups. These authors noted that the fungal

incidence in Brazil nuts occurred in the following descending order: *A. flavus*, *Penicillium* spp., *A. tamari, A. niger*, *A. nidulans, A. fumigatus*, and *Rhizopus* spp. These authors stated that the frequencies of *A. flavus, A. tamarii*, *A. niger, Penicillium* and *Rhizopus* in Brazil nuts were the same ones observed in sterilized and unsterilized samples. In this way, they concluded that the internal colonization rate in Brazil nuts was much higher than in other tree nuts they had studied. However, *A. niger* was less common in Brazil nuts than in other nuts. Since Brazil nuts come from wild collection, being stored and transported under conditions that favor fungal growth, those fungi may have more opportunities to colonize the inner tissues of Brazil nuts than of other nuts. This point of view is contrary to the one proposed by Freire et al. (2000), considering that penetration, spore germination and infection takes place during inflorescence or during formation of the seeds, which suggests an invasion and colonization of premature young tissues.

Fungal infections can both occur in harvest and post-harvest stages (Figure 2.5). Fungi may develop during storage and transportation of the product and subsequent kernel contamination is frequent in all stages of a process flow diagram. However, there are bands of moisture and water activity (a_w) which are ideal for the production of AF. The moisture content recommended for in-shell and shelled Brazil nuts to prevent the growth of toxigenic fungi are 5 and 4.5 %, corresponding to a a_w of 0.75 and 0.68, respectively (Arrus et al., 2005a).

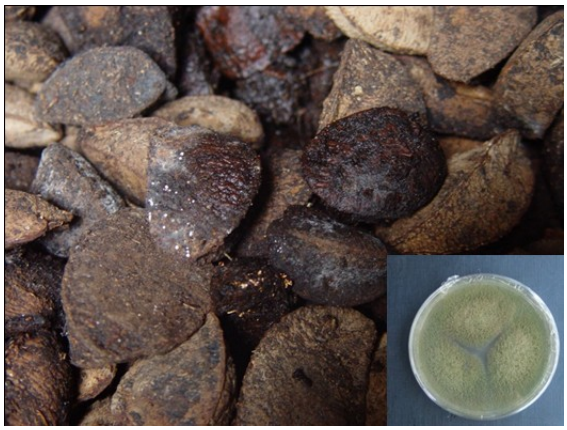

Figure 2.5. Brazil nuts in bad stored conditions infected by *Aspergillus flavus*.

In fact, the most influent parameters in toxin production are temperature and humidity. Storage temperatures between 25 °C and 30 °C and a relative humidity of 97 %, as occur in Amazon environment, favor the production of toxins during storage. Decreasing temperature or relative humidity is not feasible in the Amazon context; so, drying of nuts prior to storage is one possible alternative action (Arrus et al., 2005a). Additionally, these authors recommended in-shell Brazil nuts

storage, since the intact shell acts as a barrier to fungal growth on its surface. This should be considered at appropriate storage conditions, when there is the possibility of cross-contamination with spores from other lots or from the environment. Therefore, if the period during which the product (shelled and in-shell Brazil nuts) is under critical conditions is minimized, the probability of contamination is also reduced (Johnsson et al., 2008). In practice, this usually means reducing the moisture content of Brazil nuts as soon as possible to levels below that required for the activity of fungi.

2.4.3. Aflatoxins in Brazil nuts

Qualitative and quantitative analyses are conducted by a number of available analytical methods to determine AF in nuts. Although most of them have not been specifically developed for the Brazil nut, they are used after in-house validation. In general, the methods include solvent extraction followed by a cleanup step, using liquid-liquid partition, and a solid phase extraction, using silica, florisil, C18, aluminum oxide and immunosorbents. Thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) with UV or fluorescence detector is commonly used for the detection and quantification of AF. More recently, HPLC with mass spectrometry detection (Pacheco and Scussel, 2009) has been used. Its advantage is that there is no need for clean-up and for additional confirmation stages. The limit of quantification (LOQ) for each AF depends on the detection method, and usually ranges from 0.1 to 1 µg/kg (Gilbert and Vargas, 2003; Marklinder et al., 2005; Sobolev, 2007). AF ELISA kits are also available, but are mainly used for screening purposes.

Among mycotoxins, AF are the most commonly observed in Brazil nuts. Additionally, few others have been reported in the literature, such as chaetoglobosin C and spinolosin, found in Brazil nuts with low sanitary quality (Freire et al., 2000). However, one should emphasize that few other fungal metabolites have been screened in Brazil nuts, being the data presented in Chapter 4 the first large surveys about fungal metabolites in Brazil nuts, where a few other mycotoxins have been detected in Brazil nuts.

The production of AF is enhanced by environmental conditions (temperature and relative humidity) and storage conditions. Other factors include agricultural product characteristics, such as water activity, moisture content in food and substrate, in addition to the damage caused by insects (Arrus et al., 2005b).

It should be pointed out that the identification of new species and restriction of the taxon in new groups are the result of a polyphasic taxonomic approach (Ito et al., 2001; Samson et al., 2006; Klich, 2007; Pildain et al., 2008; Godet and Munaut, 2010). Currently, there is a controversy over which species contribute most to AF production in Brazil nuts. It is well known that mycotoxins can exist in products without the fungi associated with the mycotoxin being detected, and vice versa. Therefore, the detection of AFG_1 and AFG_2 in Brazil nuts is often surprising, where only strains of *A. flavus* had been previously detected. In this way, it is assumed that there is a greater contribution by *A. nomius* (Olsen et al., 2008) in the AF contamination of Brazil nuts, since *A. flavus* produces only AFB_1 and AFB_2, while *A. arachidicola, A. bombycis, A. minisclerotigenes, A. nomius, A. parasiticus, A. parvisclerotigenus,* and *A. pseudocaelatus,* additionally produce AFG_1 and AFG_2 (Samson et al., 2006; Rodrigues et al., 2009; Varga et al., 2011). One hypothesis to support this controversy is the erroneous attribution to the taxon, especially after the recent taxonomic guidelines in Section *Flavi* (Samson et al., 2006). Latest, Varga et al. (2011) revisited Section *Flavi* and assigned 22 species belonging to it, of which only 11 are producing AF (*A. arachidicola, A. bombycis, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus, A. parvisclerotigenus, A. pseudocaelatus, A. pseudonomius, A. pseudotamarii,* and *A. togoensis*). All these 11 species produce aflatoxins in group B (AFB) and seven of them produce aflatoxins from group G (AFG) (*A. arachidicola, A. bombycis, A. minisclerotigenes, A. nomius, A. parasiticus, A. parvisclerotigenus*, and *A. pseudocaelatus*). These data are summarized in Table 2.2.

Table 2.2. *Aspergillus* species in Section *Flavi* producers of AFBs, AFGs and CPA

Section *Flavi* species	AFB	AFG	CPA
A. arachidicola	+	+	-
A. bombycis	+	+	-
A. flavus	+	-	+
A. minisclerotigenes	+	+	+
A. nomius	+	+	-
A. parasiticus	+	+	-
A. parvisclerotigenus	+	+	+
A. pseudocaelatus	+	+	+
A. pseudonomius	+ (only AFB_1)	-	-
A. pseudotamarii	+	-	+
A. togoensis	+(only AFB_1)	-	-
A. tamarii	-	-	+
A. oryzae	-	-	+

Data adapted from Varga et al (2011); species assigned to *Aspergillus* section *Flavi* producer (+) and not producer (-) of AFB, AFG and, CPA extrolites.

The incidence and frequency of Brazil nut contamination by AF has been monitored by the Ministry of Agriculture of Brazil since 1998. The data concerning the occurrence of AF in Brazil nut samples, obtained from export batches and from batches rejected by importing countries between 2005 and 2006, analyzing only the edible portion (kernels), demonstrated that about 85 % of 294 samples showed no detectable levels of AFB_1. In AF-positive samples, the lower AFT limit ranged from 0.4 to 2.42 µg/kg and only 13 samples (4.4 %) showed levels above 20 µg/kg (Codex Alimentarius Commission, 2010). These results are favorable, in comparison with previous years. In 2003, Brazil was audited by the European Union with regard to the production and processing of Brazil nuts. Exports of the product were totally restricted since the publication of Decision 2003/493/EC (European Union Commission, 2003). This publication established criteria and conditions for Brazil in order to export in-shell Brazil nuts to the EU. This included the implementation of good manufacturing practices, appropriate sampling plans, favorable analysis conditions and certification of the final product (European Union Commission, 2003). This measure represented the suspension of Brazilian exports, which caused an enormous socioeconomic impact on the entire production chain and led to a stoppage of Brazil nut exports to the EU.

According to the European Commission Directorate General for Health and Consumer Affairs, through the Rapid Alert System for Food and Feed (RASFF), the number of notifications on AF in 2009 (638 notifications) has significantly decreased compared to 2008 (902 notifications). AF findings in nuts, nut products and seeds generated 518 notifications. Brazil received 16 notifications, 4 of which concerned in-shell Brazil nuts (RASFF, 2010).

These findings have resulted in changes in EU legislation. With the adoption of Commission Regulation (EC) No. 1152/2009, of 27 November, imposing special conditions governing the import of certain foodstuffs from certain third countries due to contamination risk by AF and repealing Decision 2006/504/EC, the control frequencies at import were increased, kept or decreased mainly based on the findings reported through the RASFF. The control frequency of imports remained unchanged for in-shell Brazil nuts from Brazil (100 %) (RASFF, 2010).

These numbers reflected a little progress on the production chain of Brazil nuts in Brazil, mainly due to the implementation of good manufacturing practices, appropriate sampling plans, favorable analysis conditions and certification of the final product.

2.5 AFLATOXINS REGULATION

AF are the most regulated mycotoxins and many countries have specific legislation for AF (van Egmond et al., 2007).

Although the limits imposed by the European Union are too restrictive, there was an attempt in recent regulations (European Union Commission, 2010) to abide by the maximum permitted limits of the *Codex Alimentarius* concerning tree nuts. An AFT limit of 10 and 15 μg/kg, for RTE and DFP, respectively, was established for Brazil nuts (Table 2.3). Under these regulations, it is believed that this relaxation in the legislation does not result in increased consumer exposure to AF (EFSA, 2009).

Table 2.3. Current EU Regulations on Aflatoxins in Nuts

Current maximum EU aflatoxin levels*		AFB_1 (μg/kg)	AFT (μg/kg)
Almonds, apricot kernels and pistachios	[1]For further processing	12	15
	[2]Ready-to-eat	8	10
Hazelnuts and Brazil nuts	For further processing	8	15
	Ready-to-eat	5	10
Other nuts	For further processing	5	10
	Ready-to-eat	2	4

Data adapted from European Union Commission, (2010), [(*)]applied as of march 9, 2010. [(1)]For further processing: to be subjected to sorting or other physical treatment, before human consumption or use as an ingredient in foodstuffs; [(2)] Ready-to-eat: intended for direct human consumption or use as an ingredient in foodstuffs.

AFT limit for Brazil nuts in Brazil varies from 10 to 20 μg/kg, to RTE and DFP, respectively (Table 2.4). This new limit was set by the Ministry of Health of Brazil through its regulatory agency (ANVISA) which has recently reviewed and updated the national mycotoxins regulation (BRASIL, 2011). The current limit for AF in Brazil nuts is in agreement with EU levels, in order to meet food safety standards, since the consumption of contaminated Brazil nuts can pose a risk to the consumers' health, both nationally and internationally, in addition to causing serious losses to the economy and agribusinesses due to a rejection of contaminated consignments by the market.

Table 2.4. Current Brazilian Regulation on Aflatoxins in Nuts

Current maximum aflatoxin levels*		AFT (µg/kg)
Almonds, walnuts, pistachios, hazelnuts, cocoa beans and dried fruits	[1]For further processing	10
	[2]Ready-to-eat	10
In-shell Brazil nuts	For further processing	-
	Ready-to-eat	20
Shelled Brazil nuts	For further processing	10
	Ready-to-eat	15

Data adapted from BRASIL, (2011), (*) applied as of February 18, 2011. (1)For further processing: to be subjected to sorting or other physical treatment, before human consumption or use as an ingredient in foodstuffs; (2) Ready-to-eat: intended for direct human consumption or use as an ingredient in foodstuffs.

2.6 POSSIBILITIES OF CONTROLLING THE OCCURRENCE OF AFLATOXINS IN FOODS

Currently there is no available technology to eliminate contamination of food by mycotoxins (Halász et al., 2009; Shapira and Paster, 2004). Most current strategies for reducing mycotoxins are based on prevention, either at the pre or post-harvest stage, e.g. segregation of contaminated grains after harvest (Venâncio and Paterson, 2007). Strategies for the removal or inactivation of mycotoxins are applied on a case by case approach (Sant'Ana et al., 2008). Biological, physical, or chemical methods, for instance, are examples of control methods that do not have a broad application yet (Chapter 5). The lack of practical solutions for controlling mycotoxin contamination in field, at harvest and in processed products leads to the search for alternatives to their partial or total elimination. At the moment, the application of ozone is one of the most promising tools available to ensure food safety (Chapter 5). The application of ozone in low doses can directly protect Brazil nuts from contamination, by reducing the growth of pathogenic microorganisms and decay and, consequently, ensuring product quality.

Compliance with legal limits can also be achieved by investing in inherent natural resistance factors of nuts. This observation was recently evidenced by the addition of natural antioxidants found in tree nuts, such as hydrolysable tannins, flavonoids and phenolic acids (Mahoney et al., 2010). Tannic, caffeic and gallic acids found in almond peel were added to culture media containing *Aspergillus flavus* and the production of AFT was reduced by up to 99.8 %. There is evidence that the oxidative stresses of AF are exacerbated by the fungus and hence compounds that alleviate oxidative stress can limit the accumulation of AF. Therefore, increasing the antioxidant activity of compounds in the almond itself

may restrict the ability of AF biosynthesis by *A. flavus* and, consequently, achieve compliance with regulatory limits (Mahoney et al., 2010). In the same line of research, the use of biological agents to control mycotoxins is possible; it is notorious the action that many lactic acid bacteria have, inhibiting mould growth, and mycotoxin synthesis or degrading mycotoxins (Haskard et al., 2001; Mokoena et al., 2006; Gratz et al., 2006).

Brazil nut, being a tropical crop, is so easily subjected to contamination by AF, due to the natural susceptibility of the host. Additionally, Amazon has the ideal climatic conditions for mold growth during flowering and pod collection. Since the Brazil nut tree can up to 50 meters high, the harvest is done when the mature pods falls to the ground in the forest, and therefore it is not possible to schedule an early harvest as it occurs in other perennial crops (e.g., olive and chestnut). Another feature of post-harvest of this nut is that extrativists (*castanheiros*) just start to collect pods when large amount of them is on the forest floor (Pacheco and Scussel 2006). Due to these conditions, the control of the harvest time is an impractical measure. However, it is possible to reduce the time between collection and processing of Brazil nuts, by an adaptation to drying condition standards in order to reduce moisture and water activity of the kernels, thus decreasing the critical cause of contamination. The effects of relative humidity (RH) and temperature on the production of AF have been evaluated after inoculation of Brazil nuts with an aflatoxigenic *Aspergillus* strain (Arrus et al., 2005a). The maximum level of AF occurred in shelled Brazil nuts which were stored at 25-30 °C and at a relative humidity of 97 %, while in-shell Brazil nuts presented lower levels of AF. There was no production of AF in batches stored at 10 °C (97 % RH) or 30 °C (RH 75 %). According to Arrus et al. (2005a), the constraining percentage of the moisture content and a_w that is necessary to control the production of AF (less than 4 µg/kg) at 30 °C for up to 60 days of storage is of 4.57 % (0.70 a_w) for in-shell Brazil nuts, and 4.50 % (0.68 a_w) and 5.05 % (0.75 a_w) for shelled and sliced Brazil nuts, respectively. These authors state that above these values, the production of AF may increase significantly. These results suggest that the growth of fungi after harvest can be minimized by an appropriate control of temperature and water activity, which are important strategies to be adopted to prevent the production of AF in the Brazil nut chain.

2.7 FINAL CONSIDERATIONS

The commercial extraction of NTFPs can be an important component of rainforest conservation strategies. According to this, if the forest becomes economically productive, deforestation will be discouraged. For Amazon areas where *Bertholletia excelsa* occurs, the Brazil nut trade will be particularly positive for the preservation of the forest. Given the profitability of the product and the symbiotic relation of Brazil nut trees with their forest environment, it is argued that this environment will be preserved in order to guarantee a continuous supply of Brazil nuts in Amazon.

A proper management of biodiversity in forestry and agroforestry systems will not allow pests and diseases to reach damage levels characteristically observed in monocultures, because of its equilibrium.

The experience of Mycotoxicology in the last five decades indicates that the current levels of food consumption can be maintained by quantitatively increasing food supply in general. However, in terms of food provision, the task of producing mycotoxin-free products with nutritional quality becomes a challenge, especially in complex production chains of developing countries, such as it is observed in the Brazil nut, where this is more difficult to be achieved.

With regard to AF, the management of the entire Brazil nut chain is still a challenge. This wild commodity needs to be safely offered to consumers, especially for the ones who expect to eat not only a nut but also a functional food, because of its health-related appeal, and mostly because of its oil characteristics and high selenium content. Prevention of contamination by *Aspergillus* spp. through good handling practices is still the best measure to avoid AF in Brazil nuts and to ensure the quality and safety of this product.

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CHAPTER 3

Predominant mycobiota and aflatoxin content in Brazil nuts

3.1 INTRODUCTION

Native to South America and growing only in Amazonian countries, the Brazil nut tree (*Bertholletia excelsa* HBK) is among the tallest specimens in the tropical forest, easily reaching 50 meters and living up to 1,200 years. The pod, which weighs about a pound and can contain 15 to 24 seeds, is known as the Brazil nut fruit (Mori and Prance 1990).

The Brazil nut is one of the most economically important non-timber plant products obtained from the Amazon region (Escobal and Aldana 2003). Because it is sustainably harvested, its exploitation plays an important social and environmental role to the Amazon region and its population, both for forest conservation and poverty alleviation (Newing and Harrop 2000).

Aspergillus is notably the predominant fungus in Brazil nuts, especially section *Flavi* species (Freire et al. 2000; Arrus et al. 2005a), there is great concern with production of aflatoxins (AF) in this product. The presence of these fungi in high counts can be a sign of inadequate drying, processing and storage. The Figure 3.1 illustrates the Brazil nut tree in the forest, as well as its fruits and seeds and fungi isolated from seeds.

The method applied to control fungi and mycotoxins in Brazil nuts must be compatible and consistent with the type of product, namely an organic product from the natural forest, i.e., it has to be generally recognized as safe (GRAS) and authorized for use in organic production systems.

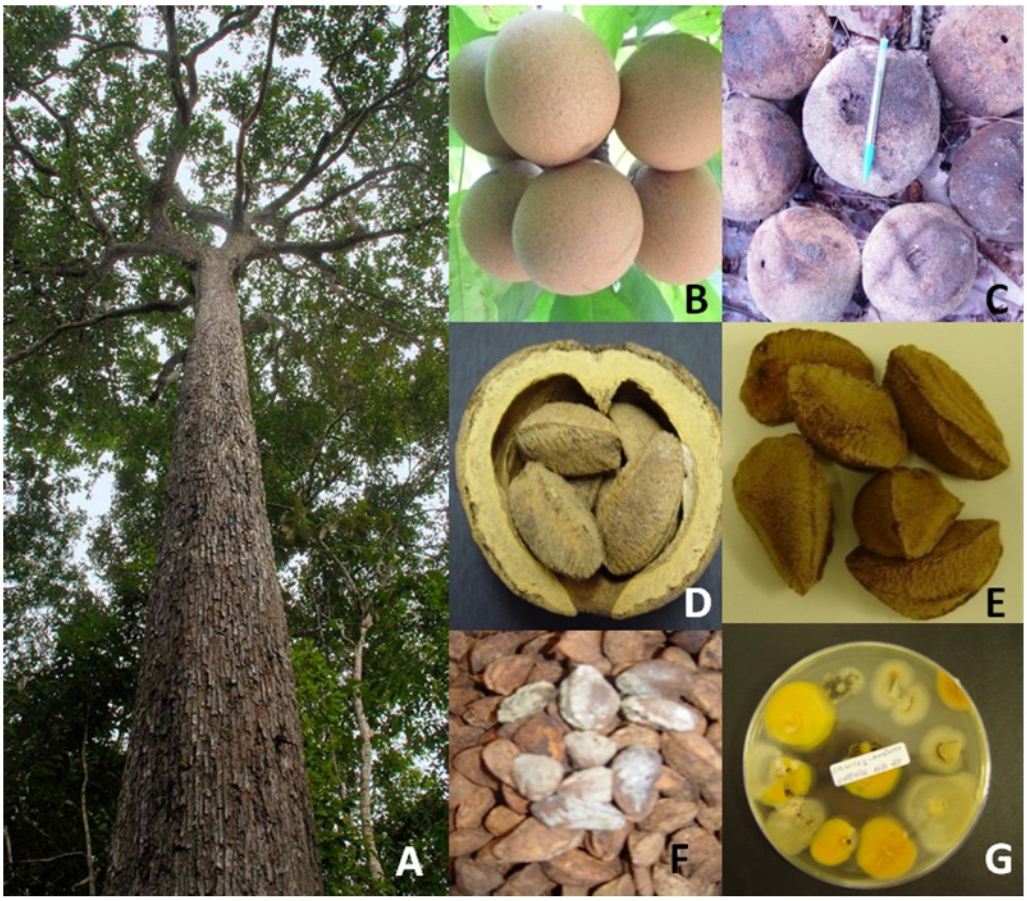

Figure 3.1. Brazil nut tree in the forest (A), immature (B) and mature pods (C, D), seeds (E), fungi on storage nuts (F) and fungi isolated from seeds (G).

The lack of practical solutions for controlling mycotoxin contamination in the field, at harvest and in processed products leads to the search for alternatives to their partial or total elimination. At the moment, the application of ozone is a promising tool available to ensure food safety (Chapter 5) and, consequently, ensuring product quality. Compliance with legal limits can also be achieved by investing in inherent natural resistance factors of nuts. This was recently reported the addition of natural antioxidants found in tree nuts, such as hydrolysable tannins, flavonoids and phenolic acids, to prevent AF accumulation in nuts (Mahoney et al. 2010). Tannic, caffeic and gallic acids found in almond peel were added to culture media containing *Aspergillus flavus* and the production of total aflatoxin (AFT) was reduced by up to 99.8 %. In the same line of research, the use of biological agents such as lactic acid bacteria to control mycotoxins have been shown to inhibit mould growth, mycotoxin synthesis and/or mycotoxin degradation (Mokoena et al. 2006).

AF are a group of structurally related oxygenated heterocyclic compounds produced under favourable conditions by several species of *Aspergillus*, including *A. flavus*, *A. parasiticus, A. nomius, A.*

toxicarius, A. parvisclerotignenus and *A. pseudotamarii* (Ito et al. 2001; Samson et al. 2006). The most common naturally occurring AF are AFB_1, AFB_2, AFG_1, and AFG_2, among which the most toxic is AFB_1 (Soares et al. 2010). Considering the susceptibility to *Aspergillus* section *Flavi* infections, Brazil nuts can contain AF produced mainly by the presence of *A. flavus, A. parasiticus* and *A. nomius* (Olsen et al. 2008).

For public health and export requirements, Brazil nuts must be produced so as to contain AF levels below the limits that could be critical to health (Arrus et al. 2005b). The export market for Brazil nuts to the EU has been greatly reduced because of overly high levels of AF residues (Xavier and Scussel 2008). Currently, the EU has an AF limit of 10 and 15 μg/kg for ready-to-eat (RTE) Brazil nuts and those destined for further processing (DFP), respectively (European Union Commission 2010).

Considering that the EU regulations on maximum residue levels (MRLs) have restricted the export of Brazil nuts, the aim of this work was: (i) to identify the potentially aflatoxigenic mycobiota associated with shelled Brazil nuts (kernels) and the shells and to determine which fraction of the nut contributes most to AF contamination, since many different fungal species have the ability to colonise the external and internal part of the nut to produce AF, and (ii) to detect AF in Brazil nut samples using high performance liquid chromatography with fluorescence detection (HPLC-FD), to provide grounds to the analysis of this complex matrix and to enable making Brazil nuts compliant with the international standards set by *Codex Alimentarius* and the EU.

3.2 MATERIAL AND METHODS

3.2.1.Collection of Brazil nut samples

Samples of Brazil nuts were collected from the agroforestry production area in the municipality of Tomé-Açu in the state of Pará, Brazil (Figure 3.2). In this agroforestry production system (SAF), the Brazil nut tree and Mogno (*Swietenia macrophylla*, Meliaceae) are on top of the agroforestry system, followed by the Assai palm (*Euterpe oleraceae*, Arecaceae), cacao (*Theobroma cacao*, Sterculiaceae), black pepper (*Piper nigrum*, Piperaceae) and other timber and non-timber forest product species cultivated in the Amazon region; such diversity is a way to make sustainably as productive as possible (Figure 3.3).

About 40 kg of Brazil nut samples were collected, labelled, packaged in plastic bags and taken to the laboratory. The samples were divided into: shelled (A), unshelled (B), dried shelled (C), defective (D), dried unshelled (E) and pods (F).

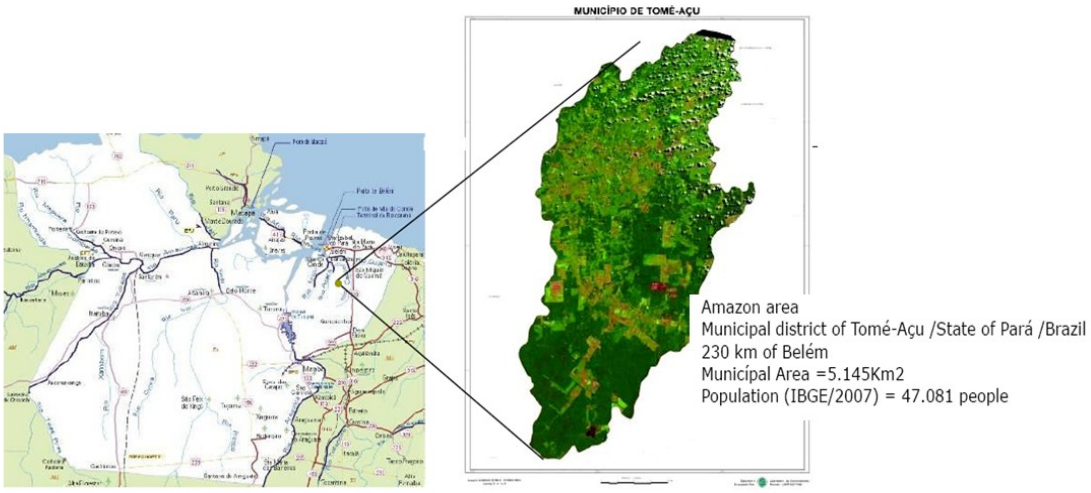

Figure 3.2. Brazil nut colecting area in Tomé açu (Pará State).

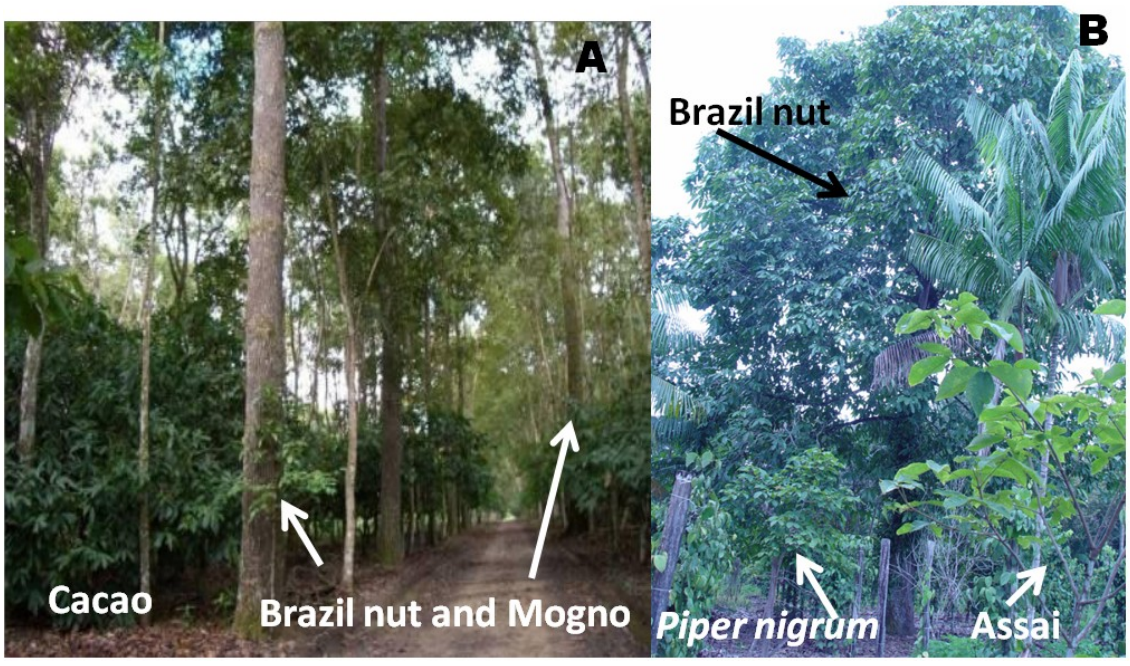

Figure 3.3. Cacao, Brazil nut and Mogno (A); Brazil nut, Assai and *Piper nigrum* trees (B) in agroforestry system production in Tomé-Açu, Pará, Brazil.

In the laboratory, each sample was divided into two aliquots. One aliquot of each sample was immediately used for fungal isolation studies, while the other was stored for later use if needed. In the

case of sample B, the shell was separated from the kernel and each one of these fractions was used for fungal isolation and for AF analysis.

3.2.2. Fungal Isolation and Characterisation

The total count of filamentous fungi was performed in two treatments: with and without surface disinfection. Sanitised samples were prepared by immersion, for 10 minutes, in a solution containing 1 % chlorine. These and the non-sanitised samples were used in a direct plating method. This is considered to be the most effective technique for mycological examination of grains and nuts, since surface disinfection before direct plating is considered essential in most situations to permit the counting of the fungi actually invading the food (Hocking et al. 2006).

For fungal isolation, each sample was sub-sampled and a total of twenty Brazil nuts pieces were plated directly onto four AFPA medium plates, prepared as described below, containing about 15 mL of the medium. Using this medium, it is possible to perform an initial screening for *Aspergillus* section *Flavi* strains, by looking at the reverse colony color.

Composition of AFPA (*Aspergillus flavus* and *parasiticus* differentiation Agar- Fluka®):

Peptic digest of animal tissue	10.0 g/L
Yeast extract	20.0 g/L
Ferric ammonium citrate	0.5 g/L
Dichloran	0.002 g/L
Agar	15.0 g/L
final pH	6.3 ± 0.2 at 25 °C
Sterilization for 15 minutes at 121 °C	

The four inoculated plates per sample were incubated for 5-7 days at 25 °C, inspected for fungal growth and then counted. The overall percent contamination was expressed as the percentage of particles which yielded a visible growth of fungi. All colonies were isolated for further identification, based on their cultural and morphological characteristics (Table 3.1).

Table 3.1. Some major macro and micro morphological characters used in the distinction of some species of *Aspergillus* sections *Flavi*, *Circumdati* and *Nigri* (Ito et al., 2001; Klich, 2002; Doster et al, 2009)

Aspergillus	**Normal colony diameter (mm)[1]**				**Stipe**		**Vesicule**		**Seriation[5]**	**Conidia**		
Sections	CYA 25 °C	MEA	CYA 37 °C	CZ	Length [2]	Surface texture[3]	Diam[2]	Shape[4]		Shape[4]	Length[2]	Surface texture[3]
Section Flavi												
A. flavus	65-70	65-70	55-65	55-65	400-800	rf/fr	20-45	gl/el	u/b	gl/el	3-6	sm/fr
A. parasiticus	60-70	60-70	50-70	45-65	250-500	fr/rf	20-35	gl/el	u/b	gl	3.5-6	rf
A. tamarii	55-70	65-70	40-70	54-70	600-1500	rf	20-45	gl/py	u/b	gl	5.5-8	rf
Section *Circumdati*												
A. melleus	30-50	33-60	25-35	15-35	350-700	rf	20-35	gl/sp or py	B	gl/el	3-3.5	sm/fr
A. ochraceus	39-59	44-55	0-35	22-42	300-1700	rf	25-55	gl/el	B	gl/el	2.5-3.5	sm/fr
Section *Nigri*												
A. awamori	60-70	60-70	65-70	30-60	300-1500	sm	20-40	gl	B	gl	4-5	sm/rf
A. carbonarius	65-70	55-70	10-30	35-45	1000-3500	sm/fr	65-90	gl	B	gl	7-10	rf
A. niger	55-70	50-70	50-70	40-62	400-3000	sm	30-75	gl	B	gl	3.5-4.5	rf/fr

[1] Note: Ranges given are those usually found, outliers have been excluded.

[2] Microscopic characteristics in µm

[3] Surface texture: sm, smooth; fr, finely roughened; rf, rough

[4] Shape: gl, globose; el, ellipsoidal; py, pyriform; sp, spathulate; cl, clavate

[5] Seriation: u, uniseriate; b, biseriate; u/b, both

These features above are based on the growth of fungus on MEA and CYA, by means of a stereomicroscope (Pitt and Hocking 2009).

Malt extract Agar (MEA)

Malt extract	20 g/L
Glucose	20 g/L
Peptone	1 g/L
Agar	20 g/L

Czapeck yeast autolysate agar (CYA)

Saccharose 30 g/L,	30 g/L
Powder Yeast Extract e	5 g/L
K_2HPO_4	1 g/L
$NaNO_3$	2 g/L
KCl	0.5 g/L
$MgSO_4.7H_2O$	0.5 g/L
$FeSO_4.7H_2O$	0.01 g/L
$ZnSO_4.7H_2O$	0.01 g/L
$CuSO_4.5H_2O$	0.005 g/L
Agar	20 g/L

All strains isolated and identified as *Aspergillus* section *Flavi* in the AFPA medium were plated onto yeast extract sucrose agar (YES), for 5 days (25–26 °C), for the determination of their aflatoxigenic potential.

YES (yeast extract sucrose agar) – Difco

Sucrose (15%)	150 g/L
Yeast Extract levedura (Difco) (2%)	20 g/L
$MgSO_4$ (0.5%)	5 g/L
Solution of trace metals ($ZnSO_4.7H_2O$, 1 g; $CuSO_4.5H_2O$, 0.5 g; H_2O 100mL)	1 mL/L
Agar (2%)	20 g/L

The agar plug technique through thin layer chromatography (TLC) (Filtenborg et al., 1983) was used to screening AF metabolites. Discs of mycelium (0.5 cm in diameter) of each isolate from Section *Flavi,* grown in YES, were placed in contact with the silica plate and then removed. AF were separated on thin layer chromatography plates (Merck; Silica Gel 60, 25 mm, 20×20) using ether : methanol : water

(96 : 3 : 1, v/v) as the mobile phase. AF were visualised under ultraviolet light at 365 nm in a chromatovisor. AF producing isolates had a retention factor (Rf) and fluorescent spot similar to the standard sample of AF solution (Figure 3.4).

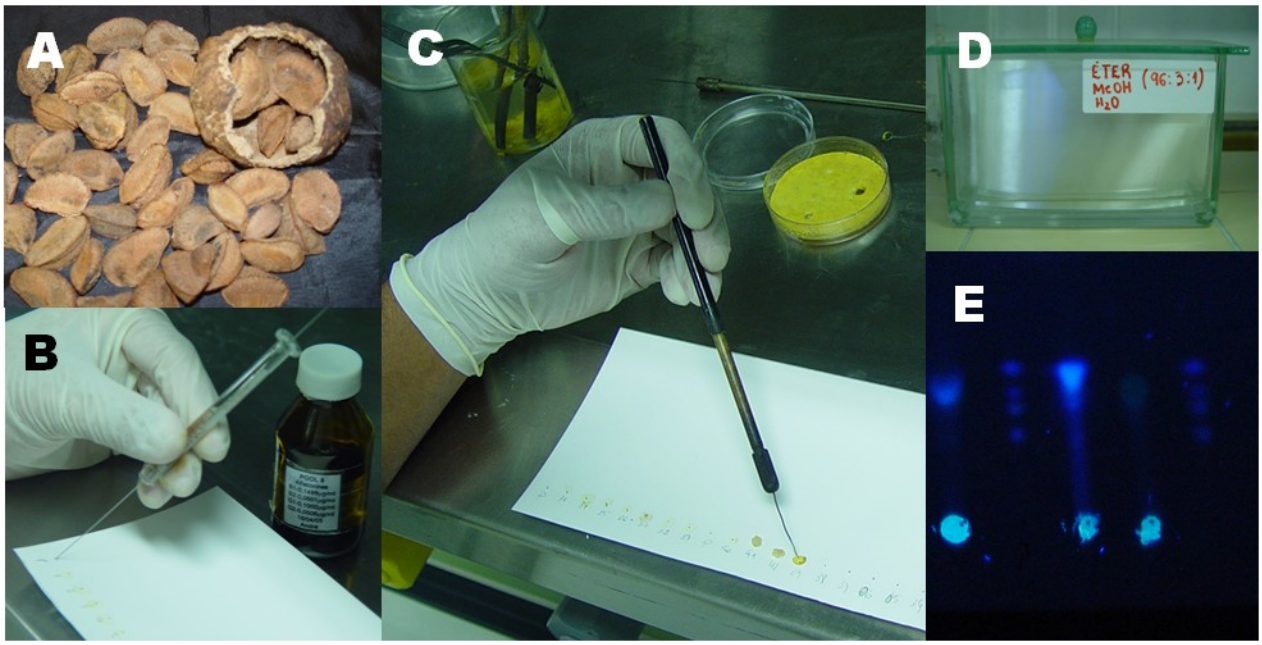

Figure 3.4. Screening of fungal metabolites by TLC using plug agar technique: Brazil nut samples (A), application of an AF pool in TLC plate (B), insertion of plug agar at TLC plate (C), TLC development (D) and results under UV at 365 nm (E).

3.2.3.Aflatoxin Analysis in Brazil nuts

As mentioned before, AF analysis was performed to both fractions of sample B: the shell and the kernel. The shell represents about one-third of the total weight of the unshelled nut. A sub-sample of 500 g of each was ground separately using an industrial mixer to perform the AF analysis.

A standard solution containing all four main AF (AFB_1, AFB_2, AFG_1 and AFG_2), was prepared using individual standards obtained from Sigma-Aldrich (AOAC 2005). From these mother solution, a set of dilutions were prepared and a calibration curve ranged from 0.002 µg/mL to 0.16 µg/mL. (Figure 3.5), was built. The detection (LOD) and quantification limits (LOQ) was calculated according the equations: LOD = 3 *sa/b* and LOQ = 10 *sa/b*, where *sa* is the standard deviation of the intercept of the regression line obtained from the calibration curve and *b* is the slope of the line (Taverniers et al., 2004).

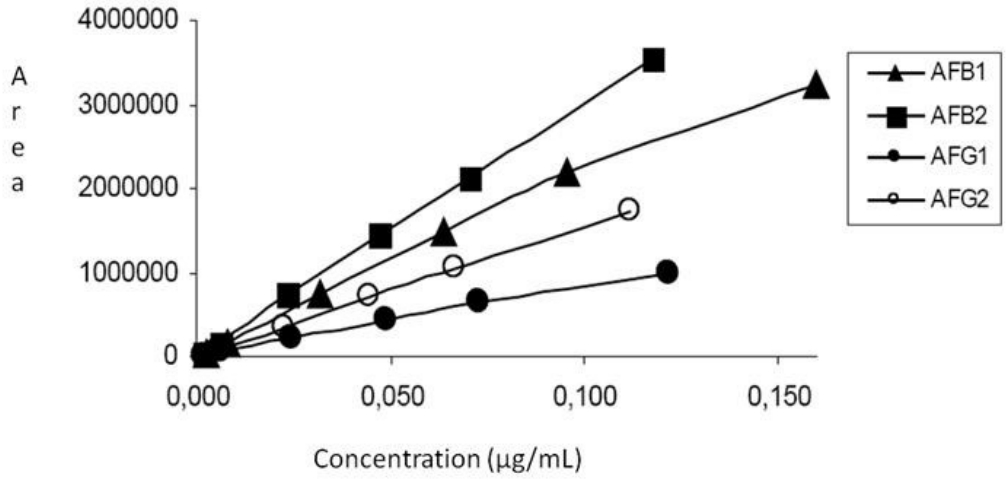

Figure 3.5. AF calibration curves.

The extraction of AF from Brazil nuts was done as follows. In a mixer, 50 g of ground sample was extracted with 270 mL of methanol and 30 mL of potassium chloride aqueous solution (4 %). After filtration through filter paper, 150 mL was collected. At this point, 150 mL of 10 % copper sulphate and 7 g of celite were added. After another filtration, 100 mL of the extract was partitioned with 50 mL of hexane and then with 20 mL of chloroform. The chloroform extract fraction, rich in AF, was dried and resuspended with 300 µL of acetonitrile (Valente-Soares and Rodrigues-Amaya 1989). The method of Valente-Soares and Rodrigues-Amaya (1989) was modified by miniaturization of the extraction step, aiming at the reduction of the amount of solvent used (making it more environmental friendly and economical); and by the change in the AF separation and detection method, from TLC to HPLC-FD (making it more sensitive). With these approaches lower limits of detection was achieved.

After extraction and before chromatography, a derivatization step was performed. For this, to the 300 µL resuspended extract in acetonitrile (the extract rich in aflatoxins), 600 µL of derivatising agent were added water : glacial acetic acid : trifluoroacetic acid (35 : 5 : 10, v/v). The reaction medium was maintained at 65 °C for 9 min in a tightly sealed bottle. After being cooled to room temperature, the solution was analysed by an HPLC-FD system. The AF standard solution was derivatised in the same way.

The chromatographic analysis was performed in isocratic mode in the HPLC-FD system: Waters W600 pump, Waters W717 gun, Waters W2475 detector, with water (milli-Q) : acetonitrile : methanol (600 : 150 : 150, v/v) as the mobile phase. Additionally, the column used was a Waters X-Terra (4.6 x

150 mm and 5 µm - RP18). The injection volume was 5 µL, both for standards and samples. The Figure 3.6 shows an HPLC-FD chromatogram with the separation of aflatoxins, AFG_1, AFB_1, AFG_2 and AFB_2.

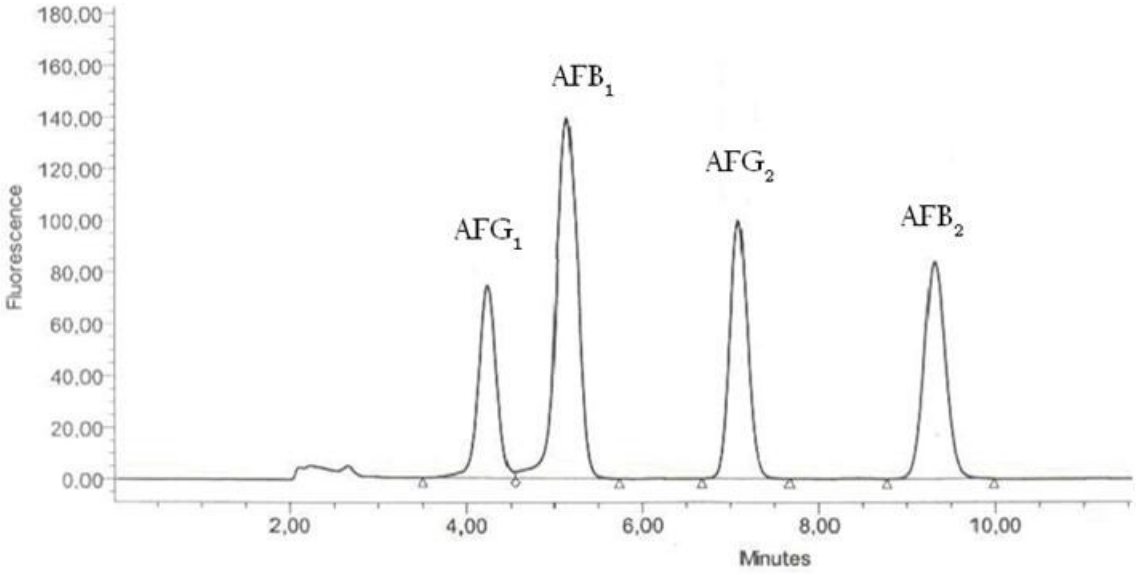

Figure 3.6. HPLC-FD chromatogram showing the separation of AFG_1, AFB_1, AFG_2 and AFB_2.

3.2.4.Statistical Analysis

Analysis of variance in a completely randomised design was performed, and the Tukey test was applied to compare means. Binomial testing was used for sanitized and non-sanitised nuts, which compares the two treatments (sanitized and non-sanitised) in relation to the proportions of a dichotomous variable. The alpha level was 0.05 for the null hypothesis. The BioEstat 5.0 program (Ayres et al., 2007) was used in the study at 5 % significance.

3.3 RESULTS AND DISCUSSION

Fungal Contamination

The presence of *Aspergillus* section *Flavi* (Table 3.2) was detected in all the samples studied (sanitised and non-sanitised), with the exception of sanitised sample F (pod), in which the chlorine treatment was able to eliminate the low occurrence of *Aspergillus* section *Flavi* in the pod samples. This indicates that pod treatment can reduce the population of potentially aflatoxigenic fungi, reducing the

initial inoculum. Table 3.3 shows all possible comparisons between the incidence of filamentous fungi in the non-sanitised and in the sanitized samples, according to each part of the nut.

Table 3.2. Incidence of Aspergilli and other fungi in Brazil nuts from agroforestry orchards

Brazil nut samples	Percentage of Brazil nuts with fungi									
	Aspergillus Section *Flavi*		*Aspergillus* Section *Circumdati*		*Aspergillus* Section *Nigri*		*Fusarium* spp.		Others	
	S	NS	S	NS	S	NS	S	NS	S	NS
A	95	90	0	0	0	0	0	30	0	15
B	80	100	0	0	0	0	0	0	0	100
C	15	100	0	5	15	0	0	0	0	70
D	5	10	0	0	15	0	5	0	30	95
E	30	40	5	0	65	0	0	0	0	80
F	0	15	0	0	0	0	15	0	0	60

The first number in each column corresponds to the percentage of infection on sanitised (S) and the second number corresponds to the % of non-sanitised (NS) treatments. The analysed Brazil nut samples were: shelled nuts (A), in-shell nuts (B), dried shelled nuts (C), defective nuts (D), dried in-shell nuts (E) and pods (F).

As can be observed in Table 3.2 and Table 3.3, *Aspergillus* was the predominant fungus in all the Brazil nut fruits, especially species in the *Flavi* section. For this reason, there is great concern over the production of AF in the product. Previous studies have also demonstrated the predominance of *A. flavus.* Freire et al. (2000) reported that among the 17 species found in Brazil nuts, *A. flavus* was the dominant one, followed by *A. niger*, *Penicillium citrinum* and *Penicillium glabrum*. The species found by these authors included fungi associated with deterioration and potentially toxigenic species, including *A. flavus* and *P. citrinum*, producers of AF and citrinin, respectively.

Our study showed little difference between sanitised and non-sanitised samples. This result is in accordance with a previous study concerning fungal distribution, abundance, specificity and control of AF contamination in Brazil nuts (Bayman et al. 2002). These authors noted that the fungal incidence in Brazil nuts occurred in the following descending order: *A. flavus*, *Penicillium* spp., *A. tamarii, A. niger*, *A. nidulans, A. fumigatus*, and *Rhizopus* spp. The same authors stated that the frequencies of *A. flavus, A. tamarii, A. niger, Penicillium* and *Rhizopus* in Brazil nuts were the same as observed in sterilised and non-sterilised samples. Therefore, they concluded that the internal colonisation rate in Brazil nuts was much higher than in other tree nuts they had studied. However, *A. niger* was less common in Brazil nuts than in the other studied nuts. Since Brazil nuts come from collection in the wild, being stored and transported under conditions that favour fungal growth, those fungi may have more opportunities to

colonise the inner tissues of Brazil nuts than those of other nuts. Freire et al. (2000) considered that penetration, spore germination and infection take place during inflorescence or during formation of the seeds, suggesting invasion and colonisation of premature tissues.

Fungal infections also can occur in the harvest and post-harvest stages. Fungi can develop during storage and transportation of the product and subsequent kernel contamination is frequent in all stages of the process flow. However, there are ranges of moisture and water activity (a_w) which are ideal for the production of AF. The moisture percent values recommended for unshelled and shelled Brazil nuts to prevent the growth of toxigenic fungi are 5 and 4.5 %, respectively (Arrus et al. 2005b).

The average incidence of infection by *Aspergillus* spp. was higher in the *Flavi* section, decreasing in the *Nigri* and *Circumdati* sections (Table 3.3). The sanitisation treatment reduced the incidence of *Aspergillus* section *Flavi*, but not the other ones (Figure 3.7).

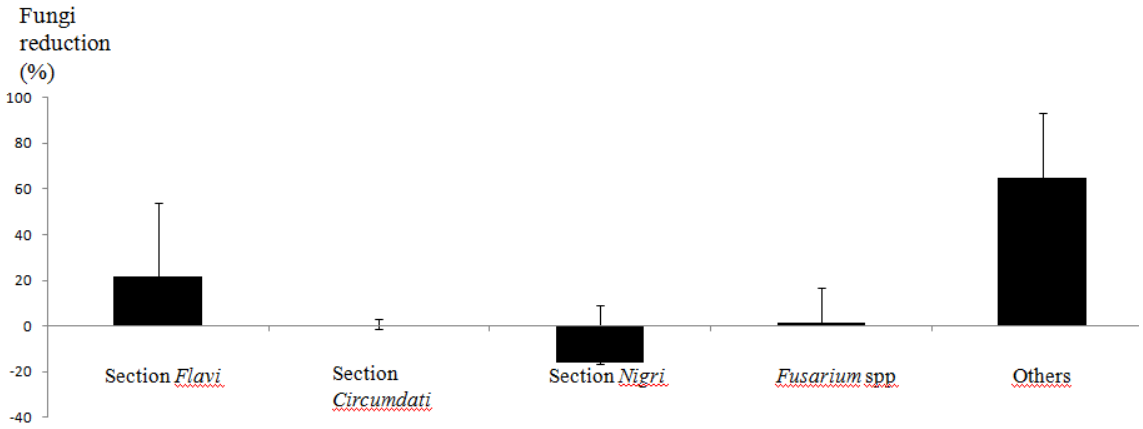

Figure 3.7. Reduction of total average percentage of fungi by chlorine in Brazil nuts.

Table 3.3. Comparative analysis of fungal control by chlorine (sanitised x non-sanitised) on different fractions of Brazil nuts

			Non-sanitised				
			Section *Flavi*	Section *Circumdati*	Section *Nigri*	*Fusarium* spp.	Others
Shelled			(18/20)	(0/20)	(0/20)	(6/20)	(3/20)
Sanitised	Section *Flavi*	(19/20)	0.5488	**< 0.0001***	**< 0.0001***	**< 0.0001***	**< 0.0001***
	Section *Circumdati*	(0/20)	**<0.0001***	1.0	1.0	**0.0007***	0.0717
	Section *Nigri*	(0/20)	**<0.0001***	1.0	1.0	**0.0007***	0.0717
	Fusarium spp.	(0/20)	**<0.0001***	1.0	1.0	**0.0007***	0.0717
	others	(0/20)	**<0.0001***	1.0	1.0	**0.0079***	0.0717
Shell			(20/20)	(0/20)	(0/20)	(0/20)	(20/20)
Sanitised	Section *Flavi*	(16/20)	**0.0350***	**< 0.0001***	**< 0.0001***	**< 0.0001***	**0.0350***
	Sec. *Circumdati*	(0/20)	**<0.0001***	1.0	1.0	1.0	**< 0.0001***
	Section *Nigri*	(0/20)	**<0.0001***	1.0	1.0	1.0	**< 0.0001***
	Fusarium spp.	(0/20)	**<0.0001***	1.0	1.0	1.0	**< 0.0001***
	others	(0/20)	**<0.0001***	1.0	1.0	1.0	**< 0.0001***
Dried shelled nuts			(20/20)	(1/20)	(0/20)	(0/20)	(14/20)
Sanitised	Section *Flavi*	(3/20)	**< 0.0001***	0.2918	0.0717	0.0717	**0.0004***
	Section *Circumdati*	(0/20)	**<0.0001***	0.3112	1.0	1.0	**<0.0001***
	Section *Nigri*	(3/20)	**<0.0001***	0.0717	0.0717	0.0717	**0.0004***
	Fusarium spp.	(0/20)	**<0.0001***	0.3112	1.0	1.0	**< 0.0001***
	others	(0/20)	**<0.0001***	0.3112	1.0	1.0	**< 0.0001***
Defective nuts			(2/20)	(0/20)	(0/20)	(0/20)	(19/20)
Sanitised	Section *Flavi*	(1/20)	0.5483	0.3113	0.3113	0.3113	**<0.0001***
	Section *Circumdati*	(0/20)	0.1468	1.0	1.0	1.0	**<0.0001***
	Section *Nigri*	(3/20)	0.6236	0.0717	0.0717	0.0717	**<0.0001***
	Fusarium spp.	(1/20)	0.5483	0.3112	0.3112	0.3113	**<0.0001***
	others	(3/20)	0.6326	0.0717	0.0717	0.0717	**< 0.0001***
Dried in-shell nuts			(8/20)	(0/20)	(0/20)	(0/20)	(16/20)
Sanitised	Section *Flavi*	(6/20)	0.5073	**0.0079***	**0.0079***	**0.0079***	**0.0015***
	Section *Circumdati*	(1/20)	**0.0080***	0.3112	0.3112	0.3112	**<0.0001***
	Section *Nigri*	(13/20)	0.1134	**<0.0001***	**< 0.0001***	**<0.0001***	0.2881
	Fusarium spp.	(0/20)	**0.0016***	1.0	1.0	1.0	**<0.0001***
	others	(0/20)	**0.0016***	1.0	1.0	1.0	**< 0.0001***
Pods			(3/20)	(0/20)	(0/20)	(0/20)	(12/20)
Sanitised	Section Flavi	(0/20)	0.0717	1.0	1.0	1.0	**<0.0001***
	Section *Circumdati*	(0/20)	0.0717	1.0	1.0	1.0	**<0.0001***
	Section *Nigri*	(0/20)	0.0718	1.0	1.0	1.0	**<0.0001***
	Fusarium spp.	(3/20)	1.0	0.0718	0.0718	0.0717	**0.0033***
	others	(0/20)	0.0717	1.0	1.0		**<0.0001***

* Binomial test for two independent samples at 5 %.

Aspergillus section *Flavi* strains isolated from both types of samples produced AF (Figure 3.8), with 30 % of the 20 isolates from the non-sanitised samples being aflatoxigenic, being detected all four main aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in at least one isolate. In the sanitised samples, 23.8 % of the 21 isolates produced AFB_1, AFB_2, and AFG_1 (Figure 3.8). As expected, AFB_1 was the main mycotoxin produced by these strains. In only two cases, AFB_2 was detected without AFB_1 being detected. This was already reported by other authors (Dutton et al., 1985; Reddy et al., 2005; Liu et al., 2006). Since most studies have assumed that AFB_1 is the biosynthetic precursor of the other AF, Dutton et al. (1985), working on AF biosynthesis with an *Aspergillus flavus* strain which accumulates only AFB_2 had proposed a different pathway for production of AFB_2 directly by versicolorin C instead of versicolorin A (precursor of AFB_1), which is probably blocked.

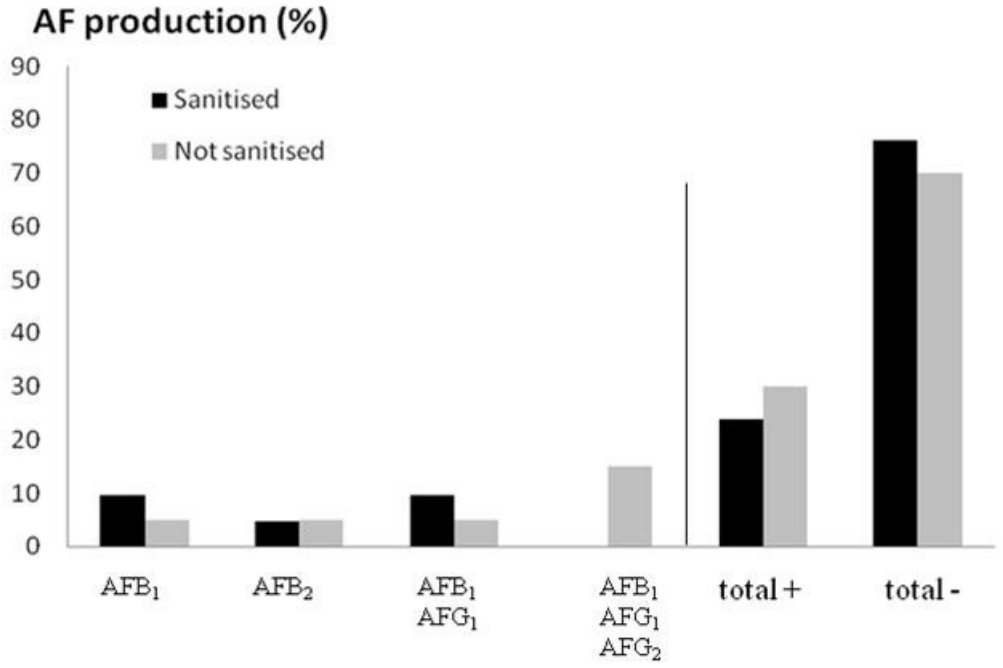

Figure 3.8. Production of aflatoxins by *Aspergillus* strains section *Flavi* in sanitised and non-sanitised samples (%). (+) aflatoxigenic, (-) non aflatoxigenic strains.

Aflatoxin Analysis in Brazil nuts

In the present work, the HPLC-FD method showed good separation of the four metabolites (AFG_1, AFB_1, AFG_2 and AFB_2). AF analysis was performed on the kernel and shell of the Brazil nut samples B. Initially, each array was analysed in quadruplicate and the average results for each AF can be seen in Table 3.4 The concentration of AFB_1 and AFG_1 obtained from the kernel was higher than the concentrations found in the shell. The AFB_1 contents were 35.281 and 1.782 μg/kg in shelled nuts and

shells, respectively. The presence of AFB_2 and AFG_2 was detected only in shelled samples (Table 3.4). According to Vargas et al. (2011), AFT is distributed in all parts of the nut, not just in the kernel (the edible part of Brazil nut). Those authors found AFT in the shell fractions (both good and rotten) and considered this situation unique among tree nuts.

The experimental results reported above may be associated with different combinations of matrices. When the kernel is separated from the shell, two different arrays are obtained, thus allowing a better AF assessment in both kernel and shell. When working with combined arrays, i.e., analysing unshelled nuts, the effect of the matrix is enhanced, reducing the efficiency of the analytical method.

Table 3.4. Aflatoxin contents in shelled Brazil nuts and their shells

Aflatoxins	Shelled Brazil nuts (μg/kg)	Shell (μg/kg)	Limit of Detection (LOD) (μg/kg)	Limit of Quantification (LQ) (μg/kg)	Recovery (%)
AFB_1	35.281 ± 0.051	1.782 ± 0.023	0.263	0.790	100
AFB_2	3.330 ± 0.031	nd	0.345	1.036	93
AFG_1	21.457 ± 0.015	1.656 ± 0.016	0.241	0.725	102
AFG_2	1.728 ± 0.025	nd	0.254	0.763	87

nd: not detected

The matrix-induced chromatographic response enhancement, or matrix effect, is a factor of selectivity of the analytical method and the magnitude of this effect can affect the sample concentrations measured (Mansilha et al., 2010). The approach used to analyse parts of Brazil nuts separately (shell and kernel) proved to be superior to the official method, which uses the nut with shell; although the shell is not edible, it can contribute to the total AF contamination reading of the lot being tested. According to Häubl et al. (2005), to circumvent the matrix effects, internal (stable) standards can be used. An internal standard is an ideal analyte molecule labelled with an isotope, usually prepared by organic synthesis to replace some ^{12}C atoms with ^{13}C atoms (Han et al., 2010).

Table 3.4 also shows that the current HPLC-FD method for AF has LOD and LOQ suitable to meet all international regulations on AF levels.

The incidence and frequency of contamination by AF in Brazil nuts have been monitored by the Brazilian Ministry of Agriculture since 1998. The data concerning the occurrence of AF in samples of Brazil nuts obtained from exported lots and lots rejected by importing countries between 2005 and 2006

showed that about 85 % of 294 samples presented no detectable levels of AFB_1. In AF-positive samples, the lower limit ranged from 0.4 to 2.2 μg/kg and only 13 samples (4.4 %) showed a value higher than 20 μg/kg (Codex Alimentarius Commission, 2010).

These results are favourable when compared with those from previous years. In 2003, Brazil was audited by the European Union in relation to production processes and the export of Brazil nuts was totally restricted as of publication of Decision 2003/493/EC (European Union Commission 2003). This decision by the EU commission established criteria and conditions for Brazil to export unshelled Brazil nuts to the EU. These criteria included the implementation of good manufacturing practices, appropriate sampling plans, analysis conditions and certification of the final product. This measure caused suspension of Brazilian exports to the EU, which in turn caused an enormous socioeconomic impact on the entire production chain.

AF is the most regulated mycotoxin and many countries have specific legislation for AF (van Egmond et al. 2007). Although the limits imposed by the European Union are very restrictive, there was an attempt in the final regulations (European Union Commission 2003; European Union Commission 2010) to abide by the maximum permitted limits of the Codex Alimentarius concerning tree nuts. Total AF limits of 10 and 15 μg/kg, for RTE and DFP, respectively, were established for Brazil nuts (European Union Commission 2010). This relaxation of the rules does not result in increased exposure of consumers to AF.

The AF limit for Brazil nuts in Brazil recently was adjusted to similar levels as in the EU (BRASIL 2011), in order to meet food safety standards, since the consumption of contaminated Brazil nuts can pose a risk to the consumers' health, both nationally and internationally, in addition to causing serious losses to the economy and agribusinesses due to a rejection of contaminated lots by the market.

3.4 CONCLUSIONS

The average incidence of infection by *Aspergillus* spp. in Brazil nuts was, respectively, from the *Flavi, Nigri* and *Circumdati* sections. Shelled nuts, in-shell nuts and dried shelled nuts showed the highest levels of contamination by *Aspergillus* section *Flavi*, while dried in-shell nuts, pods and defective nuts samples, in this order, had lowest levels of contaminated by these AF producing fungi. In general, the sanitisation treatment reduced the fungal counts. Samples sanitized with chlorine have decreased the

population of *Aspergillus* from *Flavi* section, but allowed an increase of the section *Nigri* ones, showing the characteristic not superficial of the fungal colonization.

Aspects concerning identification of fungi in the fractions of Brazil nut fruits can help efforts to decontaminate the product and can be incorporated in a flow diagram of good handling practices for Brazil nuts. AF were produced by fungi isolated and recorded from both types of samples shelled Brazil nut and its shell, being the contribution of the shell to the overall contamination with AF calculated as less than 5 %.

The HPLC-FD used in this work presented limits of detection (LOD) and quantification (LOQ) suitable to satisfy all international regulations and quality conformity standards.

The food safety of Brazil nut will enable its competitiveness in the international market, avoiding trade barriers and reinforcing the importance of Brazil nut safety as a non-timber tropical forest product in the world.

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CHAPTER 4

Tracing fungi secondary metabolites in Brazil nuts using LC-MS/MS

4.1 INTRODUCTION

Brazil nut tree (*Bertholletia excelsa* Humb. & Bompl), specie from Lecythidaceae family, originated from South America, is produced only in Amazonian countries. The total world production of Brazil nuts in 2008 was 78,000 tonnes, being Brazil responsible by 39.35 % of it, Bolivia by 53 % and 0.37 % from Peru (FAOSTAT, 2011).

The Brazil nuts extractivism represents an important activity for the forest people in countries where the tree grows, stimulating a sustainable use of renewable natural resources, while bringing together social development and conservation. It is an activity that neither destroys the forest nor threats the ecological balance and the environment (Wadt et al., 2005). Additionally, the Brazil nut trees are essential in the rain forest, as they help to maintain the equilibrium in the relationship between flora and fauna (Escobal and Aldana, 2003).

Industries and producers have been making considerable efforts in the last 15 years to minimize fungal growth and aflatoxin production in tree nuts. Particularly in the case of Brazil nuts, the climatic conditions in the Amazonian environment and the characteristics of the extractivism activity (collecting and primarily handling) cannot be controlled, exerting direct or indirect effects on the toxigenic fungi and on aflatoxins production. Processing that has been proven to reduce aflatoxin levels in Brazil nuts include shelling and sorting by size, specific gravity, colour or damage (Pacheco and Scussel, 2006; De Mello and Scussel, 2009).

Research efforts to establish the magnitude of the aflatoxins occurrence in Brazil nuts started in the late 1990s, when European Regulation 1525-98 EC decreased the acceptable levels of aflatoxins in Brazil nuts from 20 μg/kg to 4 μg/kg, invoking the precautionary principle (Newing and Harrop, 2000). In this way, nuts consignments moving within commercial channels were often found to be contaminated

with mycotoxins, primarily aflatoxin B_1 (AFB_1). Economically, Brazil nut export market to the European Union (EU) was greatly reduced because of its high frequency of contamination with AF (Xavier and Scussel, 2008). Recently, the EU revised its limit for AFB_1 and total aflatoxins (AFT) (AFB_1+AFB_2+AFG_1+AFG_2) in Brazil nuts. In ready-to-eat (RTE) nuts the limit for AFB_1 and AFT are 5 and 10 μg/kg; while in nuts destined for further processing (DFP), these limits are 8 and 15 μg/kg, respectively (European Union Commission, 2010). At this moment, Brazil also restructured its legislation on mycotoxins (BRASIL, 2011). The new limits on Brazil nuts is harmonized with the *Codex Alimentarius* and the European Union, in order to meet food safety standards, since the consumption of contaminated Brazil nuts may represent a risk to the health of consumers both internally and externally.

Considering the worldwide increasing demand for food safety (Codex Alimentarius Commission, 2010), there is a need for a highly sensitive, self-confirmatory and faster method for measuring mycotoxins, especially aflatoxins, to comply with recent regulation; however, most screening methods for mycotoxins addressed by legislation are based on thin layer chromatography (TLC), high performance liquid chromatography with fluorescence detector (HPLC-FD) and immunoassay (AOAC, 2005), with ambiguous identification of analytes. Unambiguous analytes confirmation can be easily achieved with tandem mass spectrometric methods, such as gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). During the last few years, this progress had also an increasing impact on the expanding field of mycotoxin analysis (Zöllner and Mayer-Helm, 2006). The development of multi-mycotoxin methods (Sulyok et al. 2007; Şenyuva et al. 2008) enables simultaneous analysis of a larger fraction of the 300 to 400 fungal metabolites which are currently recognized as mycotoxins. A methodology by LC-MS/MS able to detect and quantify AFB_1, AFB_2, AFG_1 and AFG_2 (Xavier and Scussel, 2008) at lower levels than the current recommended methodology (solid phase extraction and quantification by HPLC-FD) (AOAC, 2005) for Brazil nuts, was already developed. Now it is opportune to evaluate other fungal metabolites in Brazil nuts, since many different fungi species have ability to produced others mycotoxins than AF. In view of this, the present work aimed to investigate mycotoxin contamination in a Brazil nut matrix by using a HPLC-MS/MS multi-mycotoxin method.

4.2 MATERIAL AND METHODS

Chemicals and reagents: It was used methanol and acetonitrile (both LC gradient grade); ammonium acetate (MS grade) and glacial acetic acid (p.a.). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Details concerning standards of the investigated mycotoxins (which include aflatoxins, ochratoxins and some other metabolites produced by *Aspergillus*, *Penicillium* and *Fusarium* species) were previously described (Sulyok, et al. 2007; Vishwanath et al. 2009).

Collection of samples: A total of five samples of Brazil nuts were collected in different years; initially stored in the field for about three months (in a not confined environment), and then transferred to the lab until analysis. Samples 1 to 3 were collected in 2009 and stored in the field and in the laboratory for 3 months in each place. Sample 4 was collected in 2008 and stored in the field and in the laboratory for 3 and 15 months, respectively. Sample 5 was collected in 2007 and stored in the field and in the laboratory for 3 and 27 months, respectively. Collection and initial storage took place in the rainy months with average temperatures around 26-33 °C and very high relative humidity. Samples 1 to 4 were obtained from an agroforestry production area in Pará state, Brazil; while sample 5 was collected from the natural forest in Acre state. Better conditions for storage were observed in the agroforestry area. The samples size (40 kg) was according to in shell Brazil nuts sampling plan for the ready-to-eat and for further processing nuts. Each sample was thoroughly mixed, and a working sample of 300 g was withdrawn.

After storage, no visible mould development was detected in any one of the samples. Working samples were milled, homogenized during 15 min, packed under vacuum and frozen stored until being analyzed.

Sample preparation and LC-MS/MS determination: To 5 g of milled sample, 20 mL of extraction solvent (acetonitrile : water : acetic acid, 79 : 20 : 1, v/v) was added. Extraction, dilution and analysis were performed as described by Vishwanath et al. (2009).

Detection and quantification (Sulyok et al., 2007) was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionisation (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C_{18} column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4×3-mm-i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). Both

eluents contained 5 mM ammonium acetate and were composed of methanol : water : acetic acid, 10 : 89 : 1, v/v (eluent A) or 97 : 2 : 1, v/v (eluent B), respectively. After an initial time of 2 min at 100 % A, the proportion of B was increased linearly to 100 % within 12 min, followed by a hold-time of 3 min at 100 % B and 4 min column reequilibration at 100 % A. The flow rate was 1 mL/min. ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. In this work, 235 metabolites were analyzed by the LC-MS/MS, this protocol was published with 186 metabolites (Vishwanath et al., 2009) and was in the meantime extended to 234 metabolites: 201 fungal metabolites (6 aflatoxins, 30 other *Aspergillus* toxins, 11 *Alternaria* toxins, 39 *Penicillium* toxins, 3 ochratoxins, 19 trichothecenes; 4 fumonisins, 7 zearalenone-derivatives, 11 other *Fusarium* metabolites, 7 depsipeptides, 25 ergot alkaloids, 7 cytochalasins, 4 cyclosporins, 4 *Chaetomium* toxins, and 24 exotic metabolites) and 33 bacterial ones (Table 4.1).

Table 4.1. Fungal and bacterial metabolites analysed

Fungal metabolites	**Analised metabolites by LC-MS/MS**	**Total 201**
Trichothecenes	T2-Tetraol , Verrucarol, 3-Acetyldeoxynivalenol, 15-Acetyldeoxynivalenol, Neosolaniol, Monoacetoxyscirpenol, Diacetoxyscirpenol, T2-Triol, HT-2 Toxin, T2-Toxin, Verrucarin A, Roridin A, Nivalenol, Deoxynivalenol, DON-3-Glucosid, Fusarenon-X, Deepoxy-DON, Satratoxin G, and Satratoxin H	19
Fumonisins	hydrolysed Fumonisin B_1, Fumonisin B_1, Fumonisin B_2, and Fumonisin B_3	4
Zearalenone-Derivatives	beta-Zearalenol Glucosid, Zearalenon-Glucosid, alpha-Zearalenol Glucosid, beta Zearalenol, alpha Zearalenol, Zearalenon-Sulfat, and Zearalenon	7
Aflatoxins	Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Aflatoxin M_1, and Aflatoxin M_2	6
Ochratoxins	Ochratoxin A, Ochratoxin B, and Ochratoxin alpha	3
Depsipeptides	Enniatin A, Enniatin A_1, Enniatin B, Enniatin B_1, Enniatin B_2, Enniatin B_3, and Beauvericin	7
Fusarium metabolites	Aminodimethyloctadecanol, Moniliformin, Antibiotic Y, Gibberellic acid, Fusaric acid, Apicidin, Aurofusarin, Equisetin, Fusarielin, Clamydosporol, and Fusaproliferin	11
Alternaria Toxins	Altenuene, AAL TA-Toxin, Altenusin, Tentoxin, Tenuazonic acid, Alternariol, Alternariolmethylether, Altertoxin-I, Macrosporin, and Altersolaniol	11
Cytochalasins	Cytochalasin A, Cytochalasin B, Cytochalasin C, Cytochalasin D, Cytochalasin E, Cytochalasin H, and Cytochalasin J	7
Cyclosporins	Cyclosporin A, Cyclosporin C, Cyclosporin D, Cyclosporin H	4
Ergot alkaloids	Ox-Elymoclavine, Ergine, Ox-Luol, Elymoclavine-Fructoside, Elymoclavin, Lysergol, Dihydrolysergol, Chanoclavin, Ergometrin, Ergometrinin, Agroclavin, Festuclavin, Methysergide, Ergosinin, Ergosin, Dihydroergosine, Ergocornin, Ergotamine, Ergotaminine, Dihydroergotamine, Ergocorninine, Ergocryptine, Ergocristine,Ergocryptinine, and Ergocristinine	25

Table 4.1. Fungal and bacterial metabolites analysed (continued)

Fungal metabolites	Analised metabolites by LC-MS/MS	Total 201
Exotic metabolites	HC Toxin, Pseurotin A, Alamethicin, Wortmannin, Stachybortylactam, Calphostin, Cochliodinol, Rubellin D, Oxaspirodion, Paspalitrem A, Paspalitrem B, Paspalin, Paspalinin, Aureobasidin, Ustiloxin A, Ustiloxin B, Ustiloxin D, Ophiobolin A, Ophiobolin B, Fusidic acid, Phomopsin A, Phomopsin B, Lolitrem B, and Citromycetin	24
Chaetomium	Chaetoglobosin A, Chetomin, O-Methlysterigmatocystin, and Chaetocin	4
Aspergillus Toxins	3-Nitropropionic acid, Asterric acid, Austocystin A, Austdiol, Cyclopiazonic acid, Fumagillin, Fumigaclavin, Gliotoxin, Sulochrin, Fumitremorgin C, Kojic acid, Mevinolin, Physcion, Sterigmatocystin, Viomellein, Tryprostatin, Pyripyropene A, Aspyrone, Asperlactone, Territrem B, Aspinonene, Asperloxine A, Nornidulin, Nidulin, Aspercolorin, Cycloechinulin, Setusosin, and Terphenyllin	30
Penicillium Toxins	Patulin, Penicillic acid, Emodin, Meleagrin, Roquefortine C, Griseofulvin, Mycophenolic acid, Citrinin, Brefeldin A, Verruculogen, Paxillin, Penitrem A, Secalonic acid, Penicillin G, Penicillin V, Cephalosporin C, Pentoxyfylline, Viridicatin, Atpenin A5, Cycloaspeptide A, Curvularin, Decarestrictin, Citreoviridin, Dechlorogriseofulvin, Brevicompanine B, Cyclopenin, Cyclopeptine, Dexoybrevianamide E, Marcfortine A, Mevastatin, O-Methylviridicatin, Paraherquamide A, Penigequinolone A, Pestalotin, Rugulosin, and Verrucofortine	39
Bacterial metabolites	Actinomycin D, Anisomycin, K252b, K252a, Nonactin, Oligomycin A, Oligomycin B, Ionomycin, FK 506, Staurosporine, Valinomycin, Trichostatin A, Puromycin, Ascomycin, Mithramycin, Bafilomycin, Chromomycin A3, Rapamycin, Geldanamycin, Nigericin, Myriocin, Cerulenin, Radicicol, Cycloheximide, Tetracyclin, Chloramphenicol, Lincomycin, Vancomycin, Thiolutin, A 23187, Amphotericin, Bacitracin, and Erythromycin	33

Recovery of mycotoxins and limits of detection from spiked Brazil nuts samples: The recovery was determined in triplicate by analyzing sample number 2 after spiking with appropriate amounts of a multi-analyte working solution (Vishwanath et al., 2009). Samples were subsequently stored for one day at room temperature to allow solvent evaporation. After this period, 2 mL of extraction solvent (acetonitrile : water : acetic acid, 79 : 20 : 1, v/v) was added and the same analytical procedure used as for the investigated samples was followed. Limits of detection (LOD) were calculated from the signal to noise ratios (LOD = 3 ^{x} S/N) of the respective Multiple Reaction Monitoring (MRM) chromatograms deriving from the analysis of the spiked samples. This work was conducted at Quantas Laboratory, part of Romer Labs®, in Tulln, Austria.

4.3 RESULTS AND DISCUSSION

From the 234 analytes screened, only 15 fungal metabolites and two bacterial metabolites were detected. Table 4.2 reports the detected compounds and the methodology performance data (LOD and average recoveries). Detected fungal metabolites are mainly produced by *Aspergillus* and *Penicillium* species. Also, from the main *Aspergillus* and *Penicillium* mycotoxins, only citrinin and aflatoxins and its precursors were detected. Other relevant mycotoxins, such as ochratoxin A, patulin, and *Fusarium* toxins were not detected. Traces of two bacterial metabolites (valinomycin and chloramphenicol) were identified as well (Figure 4.1).

Overall, the values obtained for the apparent recoveries are in good agreement with the results obtained earlier (Sulyok et al., 2006; Sulyok et al., 2010). Apparent recoveries significantly lower than 100 % occurred for AF, Sterigmatocystin (SMC) and Methyl-Sterigmatocystin (MSMC) (due to matrix effects) and some other polar analytes. Nevertheless, it must be emphasized that matrix effects have to be carefully re-evaluated for every analyte if the method is transferred to a new matrix and differences between the recoveries of individual samples can be related to the precision of the method (van Eeckhaut et al., 2009). During the method development process, significant efforts were made to achieve LODs and LOQs lower than the official regulatory (maximum) limits as set by the EU. The maximum levels of AF were established by the EU (see Table 2.3). 57Table 4.2 gives the LOD values for different analytes presented in Brazil nuts samples. The LODs were in the range of 0.2 to 15 µg/kg (AFB_1, AFB_2, AFM_1, SMC, MSMC, CTN, Cyclosporin A, Cyclosporin C, Cyclosporin D, Cyclosporin H, Rugolusin, AME, Emodin, Valinomycin and, Choramphenicol), except for AFG_1 and KA which were 24 and 60 µg/kg, respectively. Thus, the method proved to be sensitive for almost all analytes presented in this study. Based on the obtained LOQ values, the method allows us to assess, in a single analysis, the compliance of AFB_1, according to EU regulation; however, to make the AFT value, this method still needs improvement, since the LOD found to AFG1 is above the limit of regulation for AFT. Due the co-occurrence of multiple toxins in food matrices and their possible synergistic effect in humans, multianalyte detection on the different groups of mycotoxins is absolutely necessary (Ediage et al., 2011). The search, refinement and validation of analytical methods that meets the law must be a constant search, especially for complex matrices such as the Brazil nut.

Table 4.2. Results of mycotoxin concentrations in 5 Brazil nuts samples analyzed by LC-MS/MS

Analyte	Sample[*] 1 (µg/kg)	Sample 2 (µg/kg)	Sample 3 (µg/kg)	Sample 4 (µg/kg)	Sample 5 (µg/kg)	LOD[+] (µg/kg)	Recovery (%)
Aflatoxin B_1 (AFB_1)	< LOD	< LOD	< LOD	< LOD	180	0.8	56
Aflatoxin B_2 (AFB_2)	< LOD	< LOD	< LOD	< LOD	21	4	56
Aflatoxin G_1 (AFG_1)	< LOD	< LOD	< LOD	< LOD	320	24	60
Aflatoxin M_1 (AFM_1)	< LOD	< LOD	< LOD	< LOD	5.8	4.8	65
Kojic Acid. (KA)	2200	< LOD	< LOD	< LOD	< LOD	60	113
Sterigmatocystin (SMC)	< LOD	< LOD	< LOD	< LOD	5.9	1.5	57
Methyl-Sterigmatocystin (MSMC)	< LOD	< LOD	< LOD	< LOD	84	1.5	69
Citrinin (CTN)	135	< LOD	< LOD	< LOD	< LOD	8	106
Cyclosporin A	< LOD	< LOD	2.3	650	130	1	136
Cyclosporin C	< LOD	< LOD	< LOD	76	< LOD	6	88
Cyclosporin D	< LOD	< LOD	< LOD	180	27	15	82
Cyclosporin H	< LOD	< LOD	< LOD	1100	260	6	72
Rugolusin	< LOD	< LOD	< LOD	830	< LOD	10	90
Alternariolmonomethylether (AME)	0.75	2.7	3.2	1.4	1.6	0.3	91
Emodin	40	< LOD	< LOD	230	< LOD	0.3	80
Valinomycin	0.41	< LOD	< LOD	0.19	0.22	0.2	86
Choramphenicol	< LOD	< LOD	< LOD	< LOD	20	0.3	136

[(*)]Samples 1 to 4 refer to Brazil nuts from agroforestry system areas in Pará State; while sample 5 is from natural forestry in Acre State; [(+)]LOD, limit of detection.

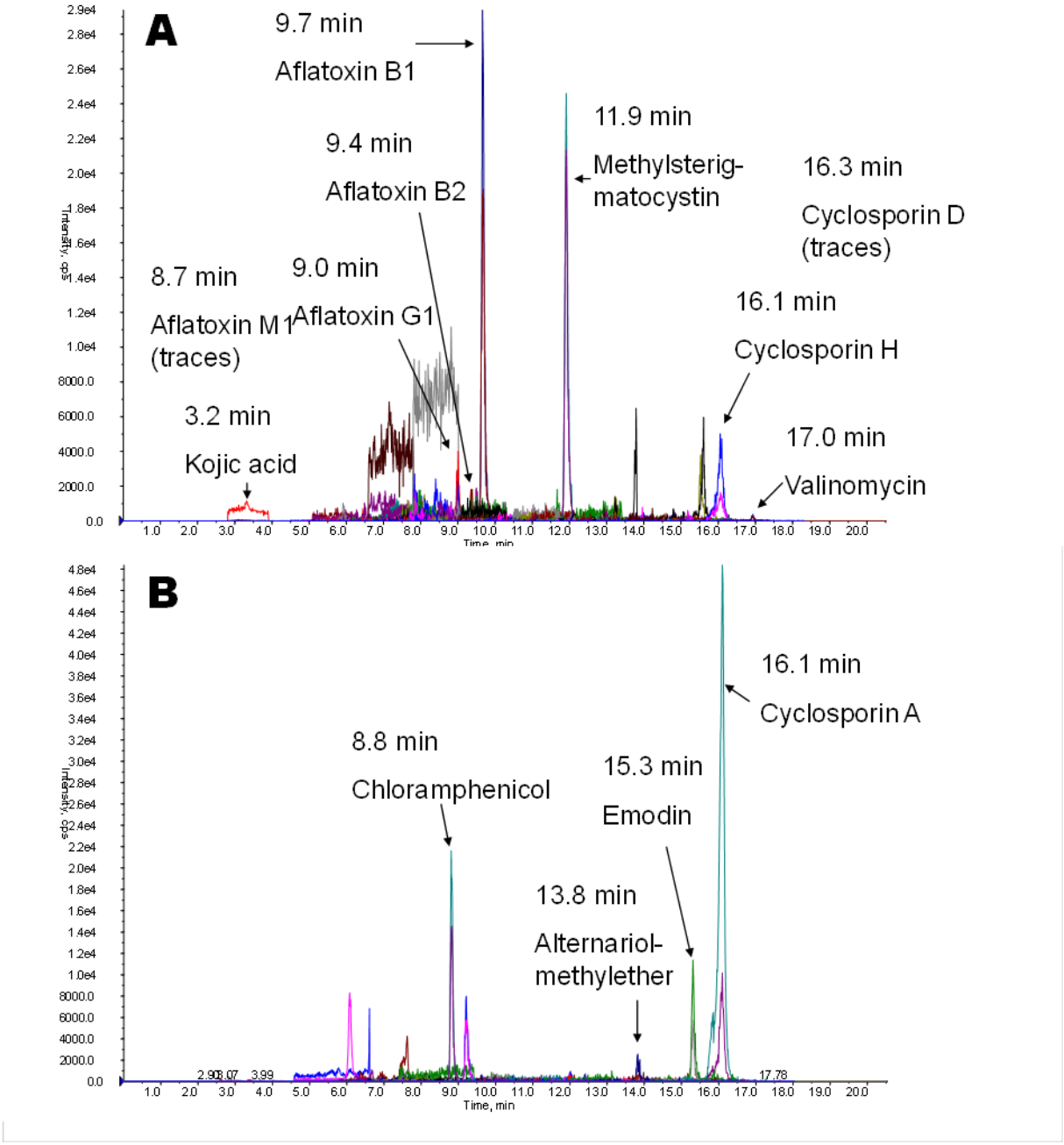

Figure 4.1. Analysis of secondary metabolites in Brazil Nuts of sample 5 by LC-MS/MS. (A) Chromatograms in positive mode. (B) Base-peak chromatogram in negative mode.

Quantitative analysis of Brazil nuts extracts by LC-MS/MS has been mainly focused on AF only (Xavier and Scussel, 2008), where the separation of toxins (AFB_1, AFB_2, AFG_1 and AFG_2) is carried out in a C18 column, with aqueous acetonitrile (80 %) as extraction solvent, and without clean up. This method values for LOD and LOQ for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.040, 0.045, 0.050 and 0.060 µg/kg; and 0.08, 0.09, 0.10 and 0.12 µg/kg, respectively. This method is much more sensitive than the one used in

the current work, since it was optimised for detecting only AF. The current methodology is based on a single extraction step using an acidified aqueous solution (acetonitrile : water : acetic acid, 79 : 20 : 1, v/v) (Vishwanath et al., 2009) which allows the extraction of other metabolites compromising the recovery of aflatoxins and its sensitivity.

Although aflatoxins are the most relevant mycotoxins in Brazil nuts, only one sample (out of five) presented contamination (180.0 µg AFB_1/kg, 21 µg AFB_2/kg, 320.0 µg AFG_1/kg, and 5.8 µg AFM_1/kg) and no AFG_2 was found. It's rare the occurrence of AFM_1 in vegetal matrices, however it was mentioned by Baydar et al. (2007) its presence in commonly consumed types of baby foods, included in cereals based ones; since it is known that AFM_1 is also an *A. flavus* metabolite. These levels are high, considering the maximum limits established in Brazil and in the EU. The high AF value found in this sample could be related to either the condition in the field in the natural forest at the collection site or to the stored period time of more than two years in our facilities. Collection and initial storage occurs in the rainy months with average temperatures among 26-33 °C and very high relative humidity. Collectors tend to pile up the fruit, and are in charge of the handling and primary processing (Peres et al. 2003; Pacheco and Scussel, 2006). The lack of good practices at this stage may be responsible for the accumulation of aflatoxins in sample 5. All other samples are originated from agroforestry production areas, where good practices are implemented. This reinforces the importance of implementing good practices by all actors in the Brazil nuts chain in order to minimize the effects of AF contamination.

As it can be observed in works with Brazil nuts, levels of fungi contamination and AF incidence are usually high, especially in in-shell Brazil nuts (Pacheco and Scussel, 2006). Data obtained from 15 Brazil nuts market samples and analyzed by TLC showed an AFB_1+AFB_2 average of 27 µg/kg and a maximum level of 245 µg/kg (Caldas et al., 2002). Others works, using LC-MS/MS, detected in 7 out of 40 Brazil nuts samples total AF values ranging from 1.2 to 11.5 µg/kg and four samples presented higher levels than the former EU regulation for AF (4 µg/kg) (Xavier and Scussel, 2008). In another study, from a total of 171 samples of Brazil nuts for export, only 8.7 % (14) of samples contained AFT at levels above 4 µg/kg. The data showed that the nuts' AF levels varied with the year of harvest and drying conditions. A good practice at this stage reduces the incidence of AF in dried nuts (Pacheco and Scussel, 2009).

Here, in this work, it was discriminated not only the pool of AF but other secondary metabolites produced by related fungi in the Brazil nuts seeds. In the five samples tested, one to ten fungal metabolites were detected from. The samples with higher number of metabolites detected were sample 5, followed by sample 4 (10 and 7 metabolites, respectively). The most common metabolites were

alternariolmethylether (found in all samples) and cyclosporin A (3 samples). Most of the other metabolites were found in just one sample. Alternariolmethylether occurred from 0.75 to 2.7 μg/kg. It is important to note that it's produced by *Alternaria* spp, which is not as common in Brazil nuts as it is in other nuts (Pryor and Michailides, 2002). Interestingly, kojic acid, a well know *Aspergillus* section *Flavi* extrolite (Pildain et al. 2008), was only found in one sample, but not the one where AFB_1 was also detected.

With multiple metabolites detection in the analyzed samples, it is evident the natural co-occurrence of mycotoxins. However, in just one sample it was found AF contamination, this result enhances the need of good practices to avoid AF contamination, as proposed by Arrus et al. (2005a, 2005b).

The co-occurrence of fungal metabolites (Asan, 2004; Soares et al., 2010) is currently known in many matrices. In Brazil nuts, it was investigated once by Freire et al. (2000) using TLC, where AFG_2, chaetoglobosin C and spinulosin were identified from poor quality Brazil nuts. However, none of these metabolites were detected in our samples.

The performance of the present multimycotoxin method was compared with some of the methods which are already reported in the literature. Compared to conventional methods, its main advantage are the lower sample preparation requirements and the simultaneous detection of all main mycotoxins, while its main disadvantage is the higher limits of detection (lower sensitivity). Improved sensitivity of this methodology is possible, when reducing the range of mycotoxins in the methodology (Xavier and Scussel, 2008).

As concerns AF, they were not detected in the four samples collected in agroforestry areas, while they were detected in the sample collected in the natural forestry. Although the number of samples is not representative, one may speculate that the good practices followed in the agroforestry area are main responsible for this.

The significance of mycotoxin contamination in food gained much attention over the past four decades. The co-occurrence of mycotoxins is more frequently reported and some of them may have synergistic effects or at least additive effects. Moreover, the issue of combined toxicity is very complicated (Speijers and Speijers, 2004; Peraica et al., 2008).

4.4 CONCLUSIONS

An HPLC-MS/MS method constitutes an alternative to conventional techniques for mycotoxin analysis, showing an ultra large mycotoxin spectrum, good sensitivity, rapidness and applicability to complex matrices such as nuts, fat products and processed ones. It could therefore be applied as routine method for complex types of food.

Fifteen mycotoxins were detected and quantified in Brazil nuts, namely, aflatoxins (AFB_1, AFB_2, AFG_1, and AFM_1), sterigmatocystin, methyl-sterigmatocystin, kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin. The last nine are being reported for the first time in Brazil nuts samples. Although some mycotoxins content in Brazil nuts were low, samples presented one to 10 fungal metabolites.

This study suggests that more investigations are needed in this commodity with more samples to be analyzed to understand the complexity fungi metabolites and its ecological role in Brazil nuts, since the fungal metabolites profile may change from year to the other justifying further monitoring and survey studies on emergent mycotoxins in Brazil nuts.

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PART II

CHAPTER 5

Ozone Applications to prevent and degrade Mycotoxins

5.1 INTRODUCTION

The global incidence of foodborne disease is difficult to estimate, but it has been reported that in 2005 alone 1.8 million people died from diarrheal diseases. A great proportion of these cases can be attributed to contamination of food and drinking water (WHO, 2007). Foodborne diseases are a widespread and growing public health problem, both in developed and developing countries. One major example of food safety problems are related with naturally occurring toxins, such as mycotoxins or marine biotoxins.

Mycotoxins are natural fungal contaminants common in a large variety of food commodities, including cereals, pulses, nuts and their products. Where these commodities are significant in the dietary intake of populations, this contamination translates into a high-level chronic exposure (Jolly et al., 2009; Wu and Khlangwiset, 2010). Mycotoxins can have adverse health effects. The most significant mycotoxins are aflatoxins (AF), fumonisins, ochratoxin A, tricothecenes and other *Fusarium* toxins, and patulin (Klich, 2009). AF intake can increase the risk of lung and liver cancer. Also, AF can suppress the immune system (increasing risk of infections), and can cross the placental barrier (entering the blood stream of unborn children) (Jolly et al., 2009; Pasqualotto, 2009; Partanen et al., 2010). Sterigmatocystin can suppress the immune system and also may increase cancer risk (Abramson, 1991). Fumonisins can increase the risk of several forms of cancer and also increase the risk of neural tube defects when unborn children are exposed (Marasas, 1995; Wild and Gong, 2010). Exposure to trichothecenes in food or indoor environments has been associated with asthma, arthritis, neurological problems, mental depression and damage to the immune system (Pestka et al., 2008; Wild and Gong, 2010). Ochratoxins can damage the kidneys and cause kidney and bladder cancer (Bryden, 2007; Wild and Gong, 2010). Patulin exposure in animals has been linked to cancer, birth defects and suppression of the immune system, on humans chronic symptoms is still not clear (Klich, 2009).

Currently there is no available technology to completely eliminate the contamination of food and feed chain by mycotoxins (Shapira and Paster, 2004; Halász et al., 2009). Most of the current strategies for mycotoxin reduction are based on prevention, either pre or post-harvest, or on the segregation of contaminated kernels after harvest (Venâncio and Paterson, 2007). Other strategies for mycotoxins removal or inactivation are applied on a case by case approach. Methods such as biological ones, inactivation of patulin by *Saccharomyces* strains (Scott et al., 1977) or the degradation of ochratoxin A by enzymes (Abrunhosa et al., 2006), physico-chemical ones, adsorption of ochratoxin A by fining agents in wine making (Fernandes et al., 2007), or physical ones, tangential filtration of whey to reduce AFM_1 (Mendonça and Venâncio, 2005) are examples of these. However, these methods have not a broad application. The lack of practical solutions to control mycotoxin contamination in the field, at harvest and of processed products leads to the demand of methods for their partial or total elimination.

From the available tools to ensure food safety, ozone application is one of the most promising ones which come to meet the needs (Graham, 1997; McKenzie et al., 1997; Shapira and Paster, 2004; Naitou and Takahara, 2006). Ozone at low doses can directly protect contaminated food and feed, since it will reduce the growth of spoilage and pathogenic microorganisms, thereby protecting the consumers of vegetal or animal products. Other potential uses of ozone in the food industry include the decontamination of food packaging material and of food contact surfaces, as well as to reduce the presence of undesirable metabolites, such as off-odors or contaminants.

Ozonation is a chemical mean of food processing that involves exposing food or an intermediate product to O_3 (Guzel-Seydim et al., 2004). It can be applied as gaseous ozone or dissolved in as aqueous solution. The first one is a more useful type of application, but both have been successfully used in reducing post harvest diseases (Kim et al., 1999; Nadas et al., 2003; Whangchai et al., 2005), in reducing viability of fungi with potential to produce mycotoxins (Table 5.1) and in reducing mycotoxin accumulation (Table 5.2) in many kinds of food products.

Table 5.1. Potential decaying and mycotoxigenic fungi inactivation by the use of ozone in different food commodities

Fungi	O_3 concentration / application	Matrix	O_3 main effect	Reference
Aspergillus terreus and other fungi	0.0002 to 0.002 g/m^3 of gaseous O_3, for 6 h	Cultivated media	Increase germination at lower O_3 concentration	Hibben and Stotzky, 1969
Aspergillus fumigatus	0.6 to 1.9 g/m^3 of gaseous O_3 (continuously)	Cultivated media	Filamentous fungi are less sensitive than bacteria	Dyas et al., 1983
Total mycobiota	gaseous O_3	Strawberry	Reduction of fungal counts	Perez et al., 1999
Aspergillus parasiticus	0.1 g/m^3 of gaseous O_3 (occasional fumigation)	Maize	Reduction of 63% fungal counts after 3 days	Kells et al., 2001
Penicillium digitatum and *Penicillium italicum*	0.1 to 2 g/m^3 of gaseous O_3, at 4.5 to 10 °C	Oranges and lemons	Increase of radial growth in *P. Italicum,* decrease of radial growth in *P. digitatum,* decrease of sporulation in both	Palou et al., 2001
Penicillium spp	gaseous O_3 at different levels	Cheese	Reduction of fungal counts in the ripening room and on cheese surface	Serra et al., 2003; Pinto et al., 2007; Lanita and Silva, 2008
Total mycobiota	gaseous O_3	Barley	Reduction mycelia grow and spores germination	Allen et al., 2003
Total mycobiota	0.01 to 0.02 g/m^3 of gaseous O_3 for 3 and 4 h	dried figs	Reduction of fungal counts	Öztekin, et al., 2006
Micromycetes *(Fusarium, Geotrichum, Myrothecium, Mucor, Alternaria, Verticillium, Penicillium* and *Aspergillus)*	1.4 g/m^3 of gaseous O_3	Wheat	Reduction of fungal counts	Raila et al., 2006
Fungi from wheat seed	0.33 mg of O_3/g wheat/min of gaseous O_3, for 5 min	Wheat	Reduction of fungal counts (96.9%) and of fungal sporulation	Wu et al., 2006
Aspergillus niger	gaseous O_3	Onion	Reduction in spore germination, change of colony color, change of uniformity of colony and formation of sterile mycelia	Vijayanandraj et al., 2006

Table 5.1. Potential decaying and mycotoxigenic fungi inactivation by the use of ozone in different food commodities (continued)

Fungi	O_3 concentration / application	Matrix	O_3 main effect	Reference
Fusarium, Alternaria, Penicillium and *Aspergillus* species	3.85 g/m^3 of gaseous O_3, for 1, 1.5 and 3 minutes	Seeds of pea, barley and wheat	Reduction of fungal counts	Ciccarese et al., 2007
Total mycobiota	13.8 g/m^3 of gaseous O_3 and 1.7 g/m^3 aqueous O_3, for 7.5, 15 and 30 min.	dried figs	Inactivation of fungi after 15 min Aqueous ozone more effective than gaseous ozone	Zorlugenç et al., 2008
Aspergillus flavus and *A. niger*	1.0 g/m^3 at room temperature	Cultivated media	repeated ozonization presented fungistatic effect	Zotti et al., 2008
Aspergillus nidulans and *A. ochraceus*	0.4 to 0.6 g/m^3 of gaseous O_3, at 18 ºC, for 10 minutes 0.0004 g/m^3 of gaseous O_3, at 18 ºC, for 12 days	Cultivated media	Reduction of growth rates more pronounced at lower ozone levels *A. nidulans* spores is more sensitive than *A. ochraceus*	Antony-Babu and Singleton, 2009
Total mycobiota	0.002, 0.006 and 0.01 g/m^3 of gaseous O_3 for 1 h	date fruits	Reduction of fungal counts	Najafi and Khodaparast, 2009
Aflatoxigenic *Aspergillus* spp	31 g/m^3 of gaseous O_3 for 5 h	Brazil nuts	Inhibit fungal viability No changes on sensory attributes	Giordano et al., 2010
A. flavus, *A. parasiticus* and total mycobiota	10.0 g/m^3 of gaseous O_3 for 90 min	Brazil nuts	Reduction of fungal counts	Scussel et al., 2011
Penicillium digitatum, *P. italicum*, and *Botrytis cinerea*	0.2, 0.25 and 0.30 g/m^3 of gaseous O_3 for 1 h, for conidia 0.8-2.0 g/m^{3}for table grapes.	conidia and table grapes	Inhibition of conidia germination Control of gray mold on table grapes	Ozkan et al., 2011
Total mycobiota including (*A. flavus* and *A. parasiticus*)	13 and 21 g/m^3 of gaseous O_3, at 18 ºC, for 0, 24, 48, 72 and 96 h	peanuts	Reduction of fungal counts Reduction of aflatoxin levels in kernels	Alencar, et al., 2011
Aspergillus brasiliensis	7.0 - 9.0 g/m^3 of gaseous O_3, for 1 min	water	Reduction of fungal counts	Roushdy et al., 2011
Eurotium amstelodami	0.845 to 634 g /m^3 of gaseous O_3, for 21 days and 120min, respectevely	cultivated media	Inhibition of growth, reduction of asexual spores and cleistothecia at low-level O_3 Reduction of spores viability at high-level O_3	Antony-Babu and Singleton, 2011

Table 5.2. Mycotoxins degradation by the use of ozone in different food commodities

O_3 concentration/application	Matrix	O_3 effect	Reference
0.025 g/minute of gaseous O_3	cottonseed meal and peanut meal	Reduction in AFT in cottonseed meal and peanut meal, by 91% (from 214 to 20 ppb) in two hours and by 78% (from 82 to 18 ppb) in one hour, respectively	Dwarakanath et al., 1968
1.1 g/m^3 of gaseous O_3, 5 min	Feed	Inactivation of AF	Maeba et al.,1988
25 g/m^3 gaseous O_3, 15 seconds	corn and rice powder	Rapid degradation of AFB_1 and AFG_1, while AFB_2 and AFG_2 were more resistant to oxidation	McKenzie et al., 1997
18 g/m^3 gaseous O_3, for 92 hours at a flow rate of 200 mg/min	Corn	Reduction of AF by 95%	McKenzie et al., 1998
12-16 g/m^3 of gaseous O_3	corn kernels	Reduction of AF by 92%	Prudente Jr and King, 2002
5 g/m^3 of gaseous O_3, at various temperatures (25, 50, 75 ºC) and exposure times (5, 10, 15 min)	peanut kernels and flour	Reduction of AFB_2 and AFG_2 by 51% in peanut kernels Reduction of AFB_2 and AFG_2 by 20% and 30% for AFB_2 and AFG_2 in peanut flour, respectively	Proctor et al., 2004
0.25 to 25 g/m^3 of aqueous O_3	aqueous solutions	Reduction of toxicity after treatment for most trichotecenes except for fumonisins, where degradation products were still toxic	Young et al., 2006
5.0, 7.0 and 9.0 g/m^3 of gaseous O_3, for 140 and 420 min, at 20 °C and 70 % RH	kernels and ground pistachios	Reduction of AFT and AFB_1 in pistachio kernels by 24% and 23%, respectively Reduction of AFT and AFB_1 in ground pistachio kernels by 5%	Akbas and Ozdemir, 2006
16, 33 and 66 g/m^3 of gaseous O_3at for 7.5, 15, 30, and 60 minutes.	flaked red pepper	Reduction of AFB_1 by 93%, in 60min	Inan et al., 2007
13.8 g/m^3 of gaseous $O_3$and 1.7 g/m^3 aqueous O_3, for 7.5, 15 and 30 min.	dried figs	Reduction of AFB_1 in 15 min Gaseous O_3more effective than aqueous O_3 in AFB_1 reduction	Zorlugenç et al., 2008
15 g/m^3 of gaseous O_3	aqueous solution and diluted apple juice	Reduction of patulin by 92% and 100% in water and in apple juice, respectively	Cataldo, 2008
31 g/m^3 of gaseous O_3 for 5 h.	Brazil nuts	Total degradation of AF Inhibition of fungal viability No changes on sensory	Giordano et al., 2010
10.0 g/m^3 of gaseous O_3 for 90 min	Brazil nuts	Total degradation of AF (from 15 µg/kg to not detection)	Scussel et al., 2011
13 and 21 g/m^3 of gaseous O_3, at 18 ºC, for 0, 24, 48, 72 and 96 h.	peanuts	Reduction of AFB_1 and AFT Reduction of AF levels in kernels	Alencar et al., 2011

Due to the strong desire to reduce the use of chemicals applied in the food and feed chains, and considering the non-residual ozone feature as an important advantage, the application of ozone technology in food has been considered safe and effective by the World Health Organization (WHO), the Food & Agriculture Organization (FAO) and the Food and Drug administration (WHO, 2007; FDA, 2008).

The broad objective of this review is to give support to ozone use, not only as a food sanitizing agent in controlling food decay during storage, but mainly for its potential in the inactivavation of mycotoxigenic fungi and in the degradation of mycotoxins.

5.2 BACKGROUND

In 1982, ozone was declared by the FDA (Food and Drug Administration) as a substance recognized as safe (GRAS - Generally Recognized as Safe), using only as permitted for bottled water disinfection (FDA, 2008). Some years later, its use was extended to food, with ozone still a GRAS substance (Mahapatra et al., 2005). Currently, ozone is gaining prominence in the sanitization of food to replace the use of chlorine as the latter present's problems of occupational and environmental contamination (EPA, 1999). However, studies are still needed so that this substitution may take place in a safe, economic and healthy way.

At room and cold temperatures, ozone is a gas with characteristic odor and can be detected by human olfaction at levels of 0.01 g/m^3 to 0.04 g/m^3, which is ten and six times lower than the limit of exposure defined by the WHO for 1 and 8 hours, respectively (Boccio, 2005). Its high oxidation potential –(E^0 = 2.07 V) makes it 1.5 and 1.3 times stronger than chlorine and hydrogen peroxide against several species of microorganisms (Foegeding, 1985; Kim et al., 1999; Khadre et al., 2001).

The solubility of ozone in water is dependent on factors such as partial pressure, temperature and pH. The purity of water also influences the rate of solubility. Because of its reactivity, the presence of minerals and organic matter will consume ozone (Hoigné and Bader 1985; Khadre et al., 2001, Guzel-Seydim et al., 2004). With short half-life, approximately 20-30 minutes at room temperature and pH 7.0 (Khadre et al., 2001), its stability in aqueous solutions is affected by the pH value. The highest the pH of the solution, the lower the stability of ozone (i.e., shorter half-life).

Modern ozone generators produce high concentrations of ozone, with lower energy consumption and are free from maintenance and from metallic dust generation. These excellent features enable economical ozone generation (Naitou and Takahara, 2006).

5.2.1. Microbial Inactivation by Ozone

The mechanisms involved in microbial inactivation by ozone are complex. Studies conducted to investigate the effectiveness of ozone against various groups of microorganisms (bacteria, virus, algae and fungi) demonstrated that ozone destroys microorganisms by the progressive oxidation of vital cellular components (Karaca and Velioglu, 2007). Cell surface has been suggested as the primary target of ozonation, since ozone acts against unsaturated lipids in the microbial cell envelope, causing leakage of cell contents and eventually microbial lysis (Scott and Lesher, 1963). In addition to this damage, ozone causes widespread oxidation of internal cellular proteins, causing rapid cell death (Karaca and Velioglu, 2007). Also, assays performed with *Salmonella typhimurium* show that ozone may induce mutagenic effects, leading to cell injury or inactivation (Dillon et al., 1992).

The type of microorganism, and the age of culture, the density of the treated population, the presence of compounds in solution with demand for O_3, forms of application of O_3, accuracy of methods for quantification of ozone and procedure to assess the antimicrobial effectiveness are aspects pointed out by some studies that may interfere with the sensitivity of the microorganism to the O_3 and hence its effectiveness for inactivation (Guzel-Seydim et al., 2004). For many pathogenic and spoilage microorganisms a minimum ozonation time and concentration is required to achieve the desired inactivation (Gujer and von Gunten, 2003; Karaca and Velioglu, 2007, Cullen et al., 2009).

5.2.2. Fungi inactivation by ozone

Most of the problems of post-harvest decay are associated to fungi, mainly to *Aspergillus* and *Penicillium* species, development during storage (Filtenborg et al., 1996; Rodrigues et al., 2009). *Aspergillus* and *Penicillium* are together with *Fusarium* the main mycotoxigenic species. Fewer studies have been focused on the use of ozone in filamentous fungi inactivation (Table 5.1). Most of the applications of ozone deal with the direct application of gaseous ozone and evaluate the sporulation, germination or growth of the above mentioned fungi. Aqueous ozone application is restricted to those commodities where a cleaning (washing) processing step is used.

Hibben and Stotzky (1969), after exposing fungal spores to 0.0002 to 0.002 g/m^3 gaseous ozone for 6 hours, found that the sensitivity of fungal species varies within species. An ozone exposition at the lower level was even found to induce germination in certain species (e.g., *Aspergillus terreus*, *Rhizopus*

stolonifer and *Trichoderma viride*). Based on their findings, these authors suggested that membrane integrity was affected, perhaps in a similar way to the increase in membrane permeability reported to occur in other microorganisms. Hibben and Stotzky (1969) also observed the suppression of aerial hyphae growth and the occasional stimulation of sporulation of colonies maintained in sublethal doses of O_3.

Palou and co-workers (2001) studied the development of moulds in decaying citrus fruits. They observed in *in-vitro* tests that gaseous ozone, at a level of 0.00006 g/m^3, neither killed all the spores nor adversely affected their germination ability. However, the sensitivity of species to ozone was different. While *Penicillium italicum* radial growth was inhibit by ozone, *Penicillium digitatum* was resistant. At this ozone dosage, sporulation was not affected. A similar observation was made by Antony-Babu and Singleton (2009), when studying the effect of ozone on *Aspergillus nidulans* and *Aspergillus ochraceus*, where the latter species was found to be more resistant to ozone. Palou and co-workers (2001) concluded that ozone was not able to control fruit decay, but just to delay its occurrence and to reduce its lesions. The disease incidence was not affected.

At higher ozone levels, Vijayanandraj et al. (2006), studying *in-vitro* the effect on *Aspergillus niger* causing black rot disease in onion, found that an exposure to ozone, at a level of 4.8 g/m^3, for 5 minutes, induced the spore germination and few spores show rapid swelling, resulting in the production of 2-3 germ tubes per spore compared to control. However, although inducing germination, the colony morphology was not uniform and the formation of sterile mycelia was induced. When using ozone with mycelia, these authors have also found that aerial mycelia growth was more sensitive to ozone than spores.

Allen and co-workers (2003) studied the use of gaseous ozone during barley storage. These authors verified the relation between contact time, gaseous O_3 concentration, barley water activity and temperature on the fungicidal performance of ozone on barley. At a dosage of 49 mg O_3 per minute (for 50 g barley seeds), ozone was very effective in inactivation of fungi regardless of whether the fungi were in the forms of spores or mycelia. However, mycelia were less resistant to ozone. After 5 minutes exposure to ozone, 96 % of inactivation was achieved for spores, as well as for mixtures of spores. As a side effect, exposure at this dosage for longer periods of time was also found to decrease the germination of barley.

Zorlugenç et al. (2008) compared the effectiveness of gaseous ozone and aqueous ozone on microbial flora and AFB_1 content in dried figs. Figs were exposed to 13.8 g/m^3 gaseous ozone gas or

1.7 g/m^3 aqueous ozone for 7.5, 15 and 30 min. Ozone application for 15 minutes was sufficient to inactivate all fungi. However, aqueous ozone was found to be more effective than gaseous ozone in decreasing microbial counts.

Other authors (Mlikota-Gabler et al., 2010) suggested the fumigation with high concentrations of gaseous ozone (up to 10 g/m^3) on postharvest table grapes, for controlling gray mould (*Botrytis cinerea)* and degradation of pesticides residues. Treatments with 5 to 10 g/m^3 ozone, for 1 hour, were able to reduce postharvest gray mould development by approximately 50 %, after two weeks storage at 15 °C. Also, residues of fenhexamid, cyprodinil, pyrimethanil, and pyraclostrobin were reduced by 68.5, 75.4, 83.7, and 100.0 %, respectively, after a single fumigation of table grapes with 10 g/m^3 ozone for 1 h. Although in some cases the formation of toxic by-products after ozonation may occur.

5.2.3.Mycotoxins Degradation by Ozone

The first studies of ozone application focusing on mycotoxin degradation in contaminated agricultural kernels appeared in the late 1960´s. Dwarakanath et al (1968) reported that gaseous ozone (25 mg/minute) reduced AFT in cottonseed meal and peanut meal by 91 % (from 214 to 20 µg/kg) in two hours and by 78 % (from 82 to 18 µg/kg) in one hour. AFB_1 and AFG_1 were found to be more sensitive to ozone than AFB_2 and AFG_2. By that time, biological experiments with rats have shown that ozone (0.025 g/min) was able to reduce the AF levels and the toxicity (Dollear et al., 1968), indicating that AF degradation residues were less toxic.

Although these preliminary results were promising, only 20 years later the use of ozone in mycotoxin degradation was again tested (Table 5.2). Most of the findings of Dwarakanath et al. (1968) were confirmed in later works. Gaseous ozone was found to (i) be able to degrade AF in many commodities and operation conditions (Maeba et al., 1988; McKenzie et al., 1997 and 1998; Prudente Jr. and King, 2002; Proctor et al., 2004); and (ii) leave less toxic degradation products in the commodity, as tested by the Ames assay (Maeba et al., 1988; Chatterjee and Mukherjee, 1993; McKenzie et al., 1997; Prudente Jr. and King, 2002). Also, the rate of degradation was higher for AFB_1 and AFG_1, compared to AFB_2 and AFG_2 (McKenzie et al., 1997; Proctor et al., 2004).

McKenzie and co-workers (1997) have used radiolabeled AFB_1 to follow the degradation products. Their results suggested the formation of water-soluble compounds, such as organic acids, volatile compounds or mineralization products (i.e. CO_2, O_2 and H_2O); however, they were not able to

detect them. These authors have also tested the action of ozone with other mycotoxins (cyclopiazonic acid, fumonisin B_1, ochratoxin A, patulin, secalonic acid D and zearalenone). With all of these mycotoxins, a reduction in the mycotoxin concentration was observed and degradation products were undetectable by HPLC, except for fumonisin B_1. In this case, degradation compounds were detected. Biological assays to detect toxicity after ozone exposure were performed, and with fumonisins the toxicity remained after treatment.

The degradation of mycotoxins by the action of ozone was found to follow a pseudo first-order rate, as long as a continuous supply of ozone was maintained (McKenzie et al., 1997).

For AF, the higher degradation rate for AFB_1 and AFG_1 was attributed to the presence of an 8,9 double bond forming the vinyl ether at the terminal furan ring, which is not present in AFB_2 and AFG_2 (McKenzie et al., 1997). These latter forms require longer exposure due to a possible second mechanism, when the lactone ring is opened during the ozone exposure (Samarajeewa et al., 1990).

Ozonation efficiency increased with higher temperatures and higher exposition to ozone (higher ozone concentration or longer treatment times); however, regardless of the conditions used, ozonation was more effective in kernels than in flour probably due to the protective effect of flour clumping and the fact that kernel contamination is superficial and easily exposed to gaseous ozone (Proctor et al., 2004).

A study on the use of aqueous ozone to degrade trichothecene mycotoxins was reported by Young et al. (2006). The degradation of ten trichothecene mycotoxins by aqueous ozone was monitored by liquid chromatography–ultraviolet–mass spectrometry (LC–UV–MS). Results of the experiment show that saturated aqueous ozone (about 25 g/m^3) degraded these mycotoxins to products that were not detected by UV or MS. In addition, it was observed that intermediate products were present when treated with lower levels (0.25 g/m^3) of aqueous ozone. Based upon the UV and MS data, it was proposed that an aldehyde was formed with the reaction of ozone and trichothecenes and that degradation began with attack of ozone at the 9,10 double bond with the net addition of two atoms of oxygen with the remainder of the molecule left unaltered. The oxidation state at the allylic carbon 8 position was observed to have a significant effect on the ease of reaction, as determined by moles of ozone required to effect oxidation.

Ozone was used in the detoxification of AFB_1 in red pepper (Inan et al., 2007). Flaked red pepper with moisture content of 12.6 % and containing 20 µg/kg of AFB_1 was treated with gaseous ozone of

various concentrations (16, 33 and 66 g/m^3) for 7.5, 15, 30, and 60 minutes. A significant reduction (over 80 %) in AFB_1 content was observed after ozonation for 60 min, in flaked red pepper and in crushed red pepper.

In the work of Zorlugenç et al. (2008), the use of gaseous ozone and ozonated water was tested for the removal of AFB_1 from dried figs. Artificially contaminated samples with AFB_1 were exposed to 13.8 g/m^3 gaseous ozone or 1.7 g/m^3 aqueous ozone water, for 30, 60 and 180 min. With both ozone application procedures, degradation of AFB_1 increased with increasing exposure time. However, opposite to what was found for the inactivation of fungi, gaseous ozone was more effective than ozonated water for reduction of AFB_1.

Recently, Cataldo (2008) reinforced the potential of ozone application on patulin degradation, a food toxin that can be present in pear juice and, especially, in apple juice. An HPLC-DAD analysis demonstrated that patulin, both in a water solution or in diluted apple juice, was destroyed by the action of ozone. It was found that patulin is the main target of ozone attack even in the presence of sugars from apple juice. This exploratory work suggests that a moderate ozone treatment of the apple juice may become an industrial practice to reduce or eliminate the patulin toxin from the juices, without damaging other juice parameters.

5.2.4.Other effects of ozone

Ozone application may cause other effects besides the microbial and the undesirable compounds degradation. Losses of nutrients or sensory qualities in food may occur (Table 5.3), but these may be negligible when compared to the benefits. Also, gaseous ozone reacts with atmospheric water, decreasing air relative humidity. If the decrease in the air relative humidity is not corrected, drying of products during storage may occur, as observed by Raila et al. (2006).

Table 5.3. Effect of ozone on quality and nutritional parameters

Food product	O_3 application form	Target microbial population	Quality and nutritional attributes	Reference
Blackberries	0.2 to 0.6 g/m^3 of gaseous O_3, for 12 days, at 2°C	Reduction of fungal decay	No effect of Anthocyanin content Reduction in peroxidase activity	Barth et al., 1995
Strawberry	Gaseous O_3	Reduction of fungal decay	Reduction of sucrose Increase of glucose, fructose, vitamin C and aroma quality	Perez et al., 1999
Peaches (cv Elegant Lady)	0.6 g/m^3 of gaseous O_3 for 4 weeks; storage conditions at 5 ºC and 90% relative humidity.	Reduction of mycelia growth and fungi sporulation	Increase of water loss after 5 weeks of storage No changes on respiration and ethylene production rates No phytotoxic injuries	Palou et al., 2002
Orange juice	Gaseous O_3 (pumped into juice)	Reduction of yeast counts (*S. cerevisiae*)	Reduction of ascorbic acid Color changes	Angelino et al., 2003
Apple cider	Gaseous O_3 (pumped into juice)	Reduction of moulds and yeast counts	Increase of sediments Color change	Choi and Nielsen, 2005
Tomatoes	Gaseous O_3	Reduction of microbial counts	Reduction of aroma Increase of texture, ascorbic acid, glucose and fructose No changes on appearance, taste and overall quality	Artes et al., 2006
Kernels and ground pistachios.	5.0, 7.0 and 9.0 g/m^3 of gaseous O_3 for 2.3 and 7 h.	-	No changes on pH, color, moisture content, free fatty acid values, fatty acid compositions, sweetness, rancidity, flavor, appearance and palatability	Akbas, and Ozdemir, 2006
flaked red pepper	16, 33 and 66 g/m^3 of gaseous ozone for 7.5, 15, 30, and 60 min.	-	No changes in color	Inan et al., 2007
Apple juice	15.38 g/m^3 of gaseous O_3	-	No changes on total sugars	Cataldo, 2008.

Table 5.3. Effect of ozone on quality and nutritional parameters (continued)

Food product	O_3 application form	Target microbial population	Quality and nutritional attributes	Reference
Table grapes	2.5, 5 and 10 g/m^3 of gaseous O_3 for 1 h	Reduction of *Botrytis cinerea* contamination	Reduction on residues of fenhexamid, cyprodinil, pyrimethanil, and pyraclostrobin No changes on iprodione and boscalid residues	Milkota-Gabler et al., 2010
Tomatoes	0.01 g/m^3 of gaseous O_3 for 10 min.	-	No changes on color, sugar content, acidity, antioxidant capacity or hemicellulose solubilization Reduction on fruit damage, pectin and weight loss Increase of phenolic accumulation Delay on softening	Rodoni et al., 2010
kiwifruits	0.0003 g/m^3 of aqueous O_3 for 2, 8, 24, 72 and 144 h	Reduction on stem-end rot disease, caused by *Botrytis cinerea*	Negative correlation between disease incidence and antioxidant substances and antioxidant activity	Minas et al., 2010
'Magnum' peaches	3x10^{-5} g/m^3 of gaseous O_3 for 1 h	O_3 reduced growth of lesions caused by *Monilinia fructicola*	Increase of browning on the fruit skin.	Sautter et al., 2011
red bell peppers, strawberries and watercress	6.34x10^{-4} and 0.004 g/m^{3}of aqueous O_3 for 1, 2 and 3 min.	Reduction counts on *Listeria innocua*, total mesophiles and total coliforms.	Combining blanching and ozone did not generate synergistic effects	Alexandre et al., 2011
Lettuce, cherry, tomato and strawberry	0.004 g/m^3 of aqueous microbubble O_3 for 5 and 10 min.	_	Reduction of residual fenitrothion	Ikeura et al., 2011

Akbas and Ozdemir (2006) evaluated the efficiency of ozone for the degradation of AF in pistachio kernels and ground pistachios. Pistachios were contaminated with known concentrations of AFB_1, AFB_2, AFG_1 and AFG_2. Pistachio samples were exposed to gaseous ozone at 5.0, 7.0 and 9.0 g/m^3 ozone concentrations for 140 and 420 min at 20 °C and 70 % RH. Although a significant reduction in AFT and in AFB_1 was achieved for pistachio kernels, no significant change in the fatty acids composition of pistachios was observed. Likewise, no significant changes were found between sweetness, rancidity, flavor, appearance and overall palatability of ozonated and non-ozonated pistachio kernels.

While using ozone in red pepper treatment, Inan et al. (2007) monitored color changes in the product. Treatments with gaseous ozone, with concentration up to 66 g/m^3, for up to 1 hour, did no change significantly the color of red pepper, using the Hunter parameters (L, a and b).

The relative sensitivity of the different vitamins to ozone application depends on the food source, and combination of ozone exposure (combination of concentration with time). Cullen et al. (2009) reports that ozone exposure combinations currently being studied are not expected to produce vitamin losses of notable concern.

Many countries around the world had approved the ozone use in food industry and it has been endorsed or supported by many national and international food and health organizations (USDA, FAO and WHO). Ozone has been already used in many agricultural products, including organically labels ones (Mlikota-Gabler et al., 2010).

5.2.5.Toxicity and Safety

Due the strong oxidizing properties of ozone, ozone is a primary irritant, affecting especially the eyes and respiratory systems and can be hazardous at even low concentrations (OSHA, 2004). The potential symptoms are eye, mucous membrane irritation, headache, fatigue, dizziness, drowsiness, anorexia, nausea, vomiting, cough, sore throat, shortness of breath, pulmonary edema, chronic respiratory disease, conjunctivitis, eye, skin burns, frostbite (on contact with liquid), rapid heart rate (tachycardia), and low blood pressure (hypotension). In the United States, the current permissible level for ozone exposure in the workplace environment is 0.2 g/m^3, as adopted by the Occupational Safety and Health Administration for a breathing period of one hour (OSHA, 2004). According to the World Health Organization, the concentration at which a susceptible individual may be continuously exposed to ozone under normal working conditions for 8 h a day or 40 h a week without adverse effects is 0.12 g/m^3 (Boccio, 2005). The short-term exposure limit is 0.6 g/m^3: Short-term means exposure for less than 15 min not more than 4 times a day, with intervals of at least 1 h between each short-term exposure (Pascual et al., 2007).

5.3 CONCLUSIONS

Accumulation of chemicals in the environment has increased the international focus on the safe use of sanitizers, bleaching agents and other chemicals in industrial processing, food, feed and other areas. The increasing demand for novel products to control infections, intoxications and diseases, without leaving toxic chemical residues in the food and feed chain seems paradoxical.

Ozone in food and feed offers negligible loss of nutrients or sensory qualities in food, as it does not substantially raise the temperature of the food during processing (Cullen et al., 2009). On the other hand, ozone exposure may inhibit grow, germination and sporulation, but its effects are very dependent on species, growth stage, ozone concentration and exposure time.

Ozone is strong oxidizer acting preferentially against unsaturated compounds, by what may be classified by an electrophilic attack mechanism. This is the primary cause for its antimicrobial activity. The reactivity against unsaturated chemical bonds is also responsible for its mycotoxin degradation mechanisms, since molecules with exposed double bonds are found to be more susceptible to degradation. This is the case of AFB_1, AFG_1 and some thricothecenes.

There is an increasing emphasis and trend towards natural food preservation technologies in response to growing consumer demand for 'greener' additives in the food industry. The FDA approval of ozone as a direct additive to food has triggered the use of ozone in fluid food processing applications. Ozone application has given promising results for important problems of food industry, such as mycotoxin and pesticide residues. Degradation products, formed after ozonation of these residues, have not exactly been determined, and this seems to be the most crucial obstacle on this subject. In vivo and in vitro toxicological tests should be conducted to screen the effects of degradation products on human and animal health. Through emerging new techniques, as well as improvements and innovations in ozone generation and application systems, certainly the subject will be evaluated more effectively in the future, facilitating enhanced control of both quality and safety parameters of ozonized foods. For effective and safe use in food processing, optimum ozone concentration, contact time and other treatment conditions should be defined for all products. Pilot test must be conducted at each case, before starting commercial application, since every ozone application is unique.

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CHAPTER 6

Degradation of Aflatoxins and cyclopiazonic acid by aqueous ozone

6.1 INTRODUCTION

Mycotoxins are secondary toxic metabolites, structurally diverse, produced by filamentous fungi. These are natural contaminants in food, feed and many agricultural commodities. The presence of fungi in these commodities, in connection with the right environmental factors and the possibility of producing more than one mycotoxin, makes this a great challenge for quality control and for food safety (CAST, 2003).

Aflatoxins (AF) and Cyclopiazonic Acid (CPA) are examples of these toxic metabolites. They can be produced simultaneously by toxigenic strains of *Aspergillus* in the Section *Flavi* (*Aspergillus flavus, A. minisclerotigenes, A. parvisclerotigenus A. pseudocaelatus* and *A. pseudotamarii*), in this same section *A. tamarii* and *A. oryzae* also have the property to produce only CPA (Varga et al., 2011), as illustrated in the Chapter 2 (Table 2.2, pg 20).

The co-occurrence of AF and CPA contaminants is well documented in cereals and nuts (Rao and Hussain, 1985; Lee and Hagler, 1991; Urano et al., 1992, Resnik et al., 1996; Fernández Pinto et al., 2001; Vaamonde et al., 2003) and together they have been shown to cause health problems in animals and humans, resulting in economic losses (Bamba and Sumbali, 2005; Lee and Hagler 1991; Urano et al., 1992). Although CPA is not as acutely toxic metabolite as AFB_1 (Butler, 1964; Purchase, 1971); the combination of both can produce degenerative changes, inactivity, tremors, necrosis of liver, pancreas, spleen and kidney (Bamba and Sumbali, 2005). Until now, no specific regulations or recommendations exist for CPA. But considering results which indicate that CPA is the mycotoxin who affects muscle tissues, hepatic and renal disorders in animals (Burdock and Flamn, 2000; Malekinejad et al., 2011), the high concentration of this toxin combined with AFB_1 can be a serious threat for human health.

The prevention of mycotoxin contamination in the field is the ideal solution to the health hazards that mycotoxins pose. Many programs, as the GAP, EUREPGAP and the HACCP, focused on the prevention of mycotoxins, seek the safety of product (FAO, 2001; Halkman, 2006). However, despite of all efforts devoted in that direction, a high incidence of mycotoxins in many commodities is still observed. So, decontamination/detoxification procedures are still a challenge for reduction of mycotoxin-contaminated commodities and feeds (Shapira and Paster, 2004; Abrunhosa et al., 2006; Jouany, 2007).

Several strategies have been reported for the management of mycotoxins already present in raw material and food (Goldblatt and Dollear, 1977; Park et al., 1988; Basappa and Shantha, 1996; CAST, 2003). Although certain treatments have been found to reduce the levels of specific mycotoxins, no single method has been developed equally effective against the wide variety of mycotoxins which may occur together in various commodities. According to Shapira and Paster (2004), the ideal decontamination procedure should (i) completely inactivate, destroy, or remove the toxin, or reduce its concentration to acceptable levels; (ii) not produce or leave toxic residues in the food/feed; (iii) preserve the nutritive value of the food/feed; (iv) not alter the acceptability or the technological properties of the product; (v) destroy fungal spores and mycelia so as to prevent revival and toxin production; (vi) be integrated, if possible, into the regular food-processing and preparation steps; (vii) be cost-effective; (viii) be easy to use; (ix) not destroy or damage equipment or pose a health hazard to workers; and, finally, (x) be approved by regulatory agencies.

In an attempt to meet these criteria this work is focused on the use of aqueous ozone, since it fulfills almost all the desirable characteristics reported above, and it is regarded a generally recognized as safe (GRAS) product. The application of ozone (O_3) as a strong anti-microbial agent is well known (Kim et al., 1999; Serra et al., 2003) and its use for mycotoxin destruction has been studied earlier. AF in feeds were reported to be degraded by ozone, without any effect on animals fed with the treated material (McKenzie et al., 1998; Prudente and King, 2002). Ozone was also effective in degrading patulin, ochratoxin, fumonisin, zearalenone, deoxynivalenol, CPA and AF (as seen in Chapter 5). However, most of these studies have used gaseous ozone. Aqueous ozone was successful used for *Fusarium* toxins, AFB_1 and patulin (Young et al., 2006, Zorlugenç, et al., 2008, Cataldo, 2008). In view of this, the objective of this work was to evaluate the control level and degradation of aqueous ozone on AFB_1, AFB_2, AFG_1, AFG_2 and CPA.

6.2 MATERIALS AND METHODS

6.2.1.Aqueous ozone solutions

Water saturated with ozone was prepared by bubbling gas, generated by passing *extra-dry oxygen through* an air-cooled corona discharge generator (Model CD-COM-HF-4) for 15 minutes (with the power generator at 100 %; gas flow of 25 L /h) in a bottle with 1000 mL of Milli-Q water, at 3 °C. The final ozone concentration in water was determined by a colorimetric method in a spectrophotometer (λ_{max} = 258 nm and ε = 2900 M^{-1} cm^{-1}), according to Bader and Hoigne (1981). A concentrated ozone solution with around 40 mg/L was obtained and this was diluted with ozone demand free water as necessary, obtained after ozonation and ozone decay.

6.2.2.Mycotoxins Reagents and Standards solutions for HPLC analysis

Aflatoxins mix and Cyclopiazonic acid standards were provided by Biopure (Austria) and by Sigma (St. Louis, MO), respectively. The mix of aflatoxins contained 2 µg/mL each of AFB_1 and AFG_1, and 0.5 µg/mL each of AFB_2 and AFG_2. The CPA standard presented 5 mg, and a stock solution was prepared by adding 5 mL of methanol.

Mycotoxin standard solutions were prepared by taking an appropriate volume of each concentrated solutions to clean vials. These were dried under a nitrogen stream and solubilized in the corresponding chromatography mobile phase. All solvents (acetonitrile, methanol) used were HPLC grade. All the solutions were stored at +4 °C, according AOAC 2005.

For the assays, two AF and CPA concentrations (I and II) were used, as presented in Table 6.1.

Table 6.1. Final concentration of AF and CPA for ozone assay

Concentration	AFB_1 ng/mL	AFB_2 ng/mL	AFG_1 ng/mL	AFG_2 ng/mL	CPA ng/ml
(I)	0.3278	0.819	0.3327	0.0885	61
(II)	3.278	8.190	3.3327	0.885	264

6.2.3.Samples Preparation

The degradation of mycotoxins by aqueous ozone was tested at 5 defined aqueous ozone levels: 0 (control), 0.1, 1, 10 and 20 mg/L. These concentrations were applied in vials of 2 mL, containing 20 μL solution of the mycotoxin stock.

Each vial was composed by 20 μL mycotoxin stock solution, 200 μL of aqueous ozone solution at each concentration or cold water (control). So, 30 minutes later, 1200 μL of the Mobile phase was added. Five replicates at each condition were made (Figure 6.1).

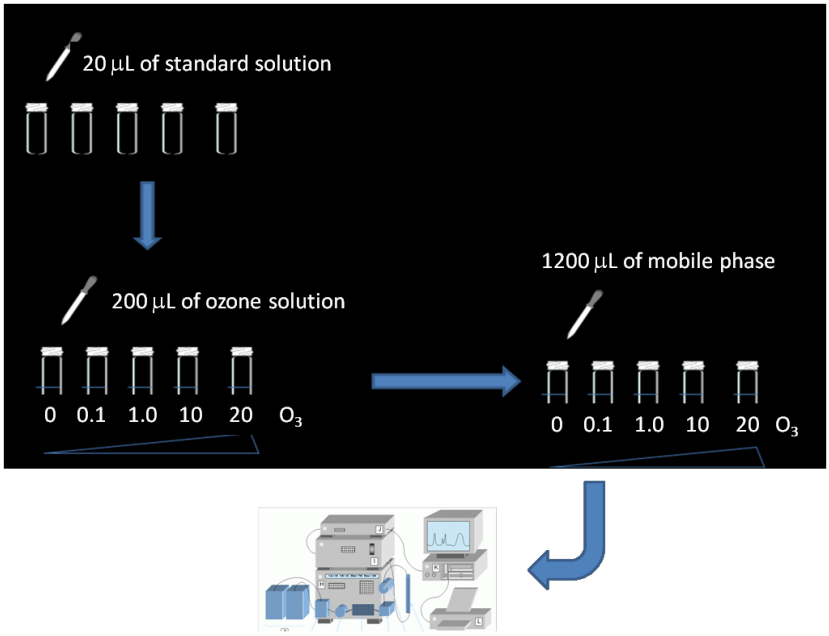

Figure 6.1. Diagram of the preparation of test solutions for the degradation of AF and CPA by aqueous ozone.

CPA Preparation

The degradation of CPA by aqueous ozone was tested at 5 defined aqueous ozone levels: 0 (control), 1, 10, 20 and 40 mg/L. Two hundred microliters of these ozone solutions were applied in 2 mL vials containing 20 μL CPA solution, to obtain the final CPA concentrations I (61 ng/mL) and II (264 ng/mL) (Table 6.1). Ozone was allowed to react, and samples were analyzed every 5 seconds to determine the kinetics, using a sodium formate solution to stop the reaction.

Sodium Formate (SF) Preparation

To stop ozone reaction, the solution of sodium formate (SF) was used as described elsewhere (Craik, et al., 2003, Biswas et al., 2005). Briefly, at the end of the prescribed contact time, 100 μL of 1 M reagent-grade SF solution was added to the flask to neutralize the remaining dissolved ozone. Before analysis, 980 μL of mobile phase was added. Each experiment was repeated six times.

Aflatoxins detection by HPLC

Samples were analyzed using a HPLC equipped with a Jasco FP-920 fluorescence detector (365 nm excitation wavelength; 435 nm emission wavelength), using photochemical post-column derivatization (PHRED unit - Aura Industries, USA) (Trucksess and Pohland 2000). Chromatographic separations were performed on a reverse phase C18 column (Waters Spherisorb ODS2, 4.6 mm x 250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 mL/min and consisted of an isocratic program as follows: water : acetonitrile : methanol (3 : 1 : 1, v/v). The injection volume was 50 μL.

Samples were taken as positive for if they yielded a peak at a retention time similar to the 4 standards, with a height five times higher than the baseline noise.

Cyclopiazonic acid detection by HPLC

Samples were analyzed using a HPLC equipped with a Jasco UV detector (284 nm), as described by Urano et al (1992) and Losito et al (2002).

Chromatographic separations were performed on a EuroSpher 100 NH_2 column (Knauer, 4.6 mm x 250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 mL/min and consisted of an isocratic program as follows: acetonitrile : 50 mM ammonium acetate (3 : 1, v/v), pH 5. The injection volume was 100 μL. In parallel, a calibration curve was constructed, using six standard CPA solutions, each one analyzed five times.

Samples were taken as positive if they yielded a peak at a retention time similar to the CPA standard, with a height five times higher than the baseline noise.

6.2.4.Simultaneous detection of Cyclopiazonic acid and AFB_1

To test the effect of ozone to degrade simultaneously AFB_1 and CPA mixed in a single chromatographic run was used for the first time with ozone. This method was developed by Soares et al. (2010), to detect at only one run AFB_1 and CPA metabolites from fungal cultures.

The AFB_1 and CPA concentrations used in this assay were 35 and 571 ng/mL, respectively. The mix was analysed by HPLC equipped with a Jasco FP-920 fluorescence detector (372 nm excitation; 462 nm emission), using a photochemical post-column derivatization reactor (PHRED - Aura Industries, USA). Chromatographic separations were performed with a C18 column. The mobile phase was methanol : 4mM zinc sulphate (65 : 35, v/v), pH 5 pumped at 0.8 mL/min (Hayashi and Yoshizawa, 2005; Oliveira et al., 2008). Acetonitrile was replaced by methanol due to a shortage in the availability of this solvent. The injection volume was 50 µL. Samples were taken as positive for each of the toxins when a peak was obtained at a retention time similar to each standard, with a height five times higher than the baseline noise.

6.2.5.Statistical analysis

The statistical analysis was performed with the SPSS 17.0 Microsoft version and significant differences between groups were analyzed by ANOVA test.

6.3 RESULTS AND DISCUSSION

6.3.1.Degradation of Aflatoxins caused by ozone

The aqueous ozone presented effective control to AF. AFB_1 and AFG_1 were more sensitive to ozone treatment than AFB_2 and AFG_2 even in more diluted solutions (Table 6.2 and Table 6.3). A significant degradation of all four mycotoxins was observed, except for the cases of AFB_2 and AFG_2 in solution II (higher concentration), where the decrease in the amount of these two mycotoxins was found to be not significant, by Duncan test ($p \leq 0.05$).

Table 6.2. Aqueous ozone on the AF mix (Solution I) degradation percentage (*)

Ozone concentration (mg/L)	**AFB_1**	**AFB_2I**	**AFG_1**	**AFG_2**
	(mean ± SD)			
0 (control)	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
0.1	6.89 ± 7.62[a]	1.49 ± 3.35[a]	5.70 ± 11.53[a]	13.90 ± 13.41[a]
1	80.00 ± 44.72[b]	34.26 ± 18.87[b]	96.19 ± 4.80[b]	14.80 ± 13.25[a]
10	98.12 ± 1.74[b]	56.38 ± 12.71[c]	100.00 [b]	49.67 ± 21.11[b]
20	100.00[b]	80.50 ± 2.12[d]	99.76 ± 0.53[b]	75.84 ± 26.44[c]

* Means followed by the same letter within a column are not significantly different by Duncan's test at p≤0.05 levels; SD-standard deviation.

Table 6.3. Aqueous ozone on the AF mix (Solution II) degradation percentage (*)

Ozone concentration (mg/L)	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**
	(mean ± SD)			
0 (control)	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
0.1	17.86 ± 11.07[b]	0.97 ± 2.16[a]	13.34 ± 16.94[a]	1.88 ± 2.60[a]
1	37.22 ± 27.38[c]	2.34 ± 5.23[a]	34.53 ± 26.12[b]	2.12 ± 4.74[a]
10	99.81 ± 0.42[d]	16.22 ± 22.23[a]	98.49 ± 3.38[c]	16.75 ± 23.02[a]
20	100.00 [d]	11.61 ± 14.77[a]	100.00 [c]	18.01 ± 20.44[a]

* Means followed by the same letter within a column are not significantly different by Duncan's test at p≤0.05 levels; SD-standard deviation.

As expected, the degradation of mycotoxins increased with increasing ozone concentration, with higher degradation rates observed for AFB_1 and AFG_1 (Table 6.2 and Table 6.3). In conditions where ozone is a limiting reactant (lower ozone concentration and higher AF concentration – Table 6.3), it was observed a significant higher degradation of the latter two mycotoxins (Figure 6.2).

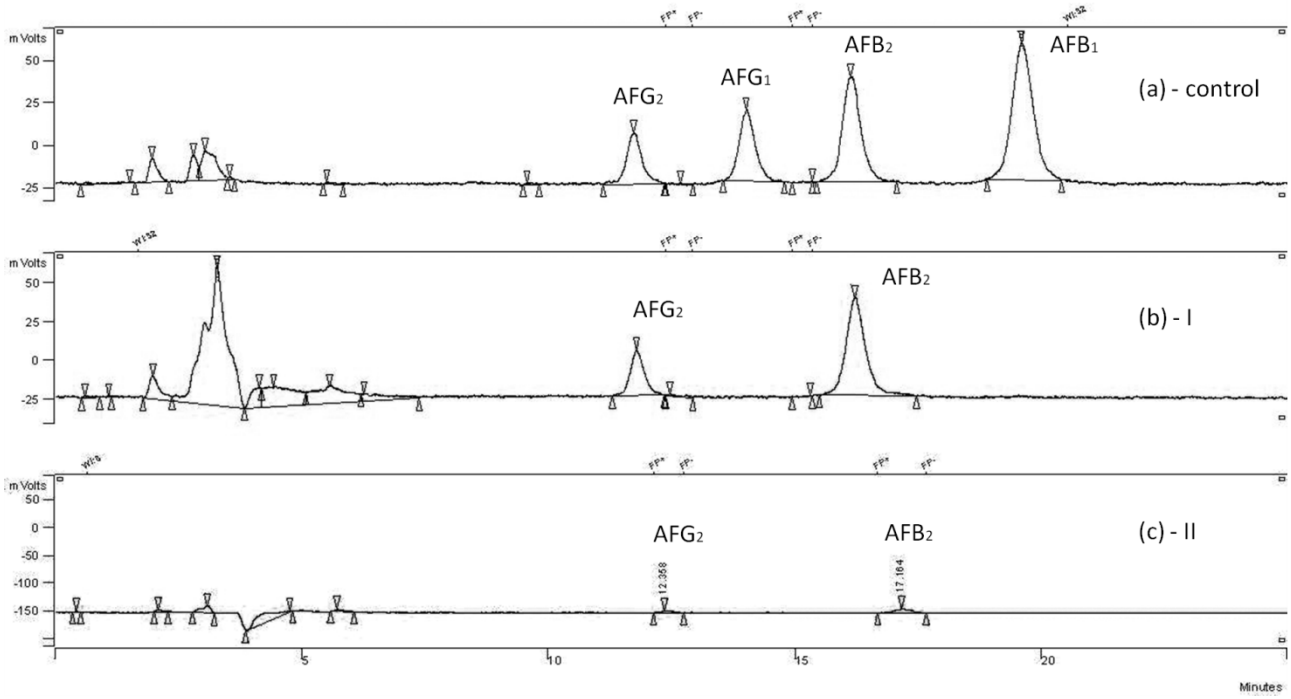

Figure 6.2. AF degradation spectra recorded from HPLC. AF in the non-ozonised sample (a) the total degradation of AFB_1 and AFG1 in solution I (less concentrated) till to almost AF complete degradation (c) in solution II by aqueos ozone at 20 mg/L.

The higher degradation of AFB_1 and AFG_1 is explained by the chain scission of the double bond as proposed by many authors (McKenzie et al., 1997, 1998; Proctor et al., 2004). AFG_2 and AFB_2 showed a higher resistance to ozonation possibly due to the olefin double bond of the terminal furan ring which is the reactive site for ozone (Samarajeewa et al., 1990; McKenzie et al., 1997). This results, reinforces the importance of ozone on aflatoxins degradation, since the AFB_1 and AFG_1 are the more toxicants agent in the AF complex, especially AFB_1, which is the primary source of liver toxicity (IARC, 2002; Proctor et al., 2004; Williams et al., 2004). These results are similar to other results found in the literature on the degradation of aflatoxins by gaseous ozone.

6.3.2. Degradation of Cyclopiazonic acid (CPA) by ozone

Degradation of CPA was conducted at two CPA starting concentrations: 61 and 264 ng/mL, with five distinct ozone concentrations (0, 1, 10, 20 and 40 mg/L). The oxidation of CPA by ozone was found to take place in a very short time, since after 5 seconds (the first sampling point) the reaction had already stopped. Even so, a reaction time of 10 minutes was established and the use of SF was maintained.

Ozone was found to significantly degrade CPA at any concentration used (Table 6.4). For the lower CPA concentration, the amount of ozone used was able to degrade it till not quantifiable levels. A residual amount of CPA (close to its detection limit) was still observed, at all ozone concentration, being the amount of residual CPA not significantly different between experiments.

However, when using a higher concentration of the mycotoxin, quantifiable levels of CPA were observed after the reaction. Significant CPA reduction was observed, increasing this reduction significantly with increasing ozone concentrations (Table 6.4). The reaction of ozone with CPA seem to follow a more than one step reaction, since a degradation product was detected (but not identified), when using the higher CPA and lower ozone concentrations. Increasing the amount of ozone will degrade the by-product as well (Figure 6.3), to not detectable products.

Table 6.4. Effect of Aqueous ozone concentration on the CPA degradation* by 10 minutes in water solution assay

O_3 concentration (mg/L)	% CPA degradation (mean ± SD)			
	I		II	
0	0.00	[a]	0.00	[a]
1	66.66	± 11.66[b]	72.22 b	± 8.39[b]
10	71.43	± 5.07[b]	88.89 c	± 1.92[c]
20	90.47	± 8.25[b]	93.33 c	± 0.23[c]
40	91.47	± 8.07[b]	94.67c	± 1.92[c]

* Means followed by the same letter within a column are not significantly different by Duncan's test at p≤0.05 levels; SD-standard deviation

CPA degradation by ozone was described before by McKenzie et al. (1997), but using gaseous ozone. In the work of McKenzie et al (1997), the degradation of CPA was reported to be complete and no toxic metabolites were formed.

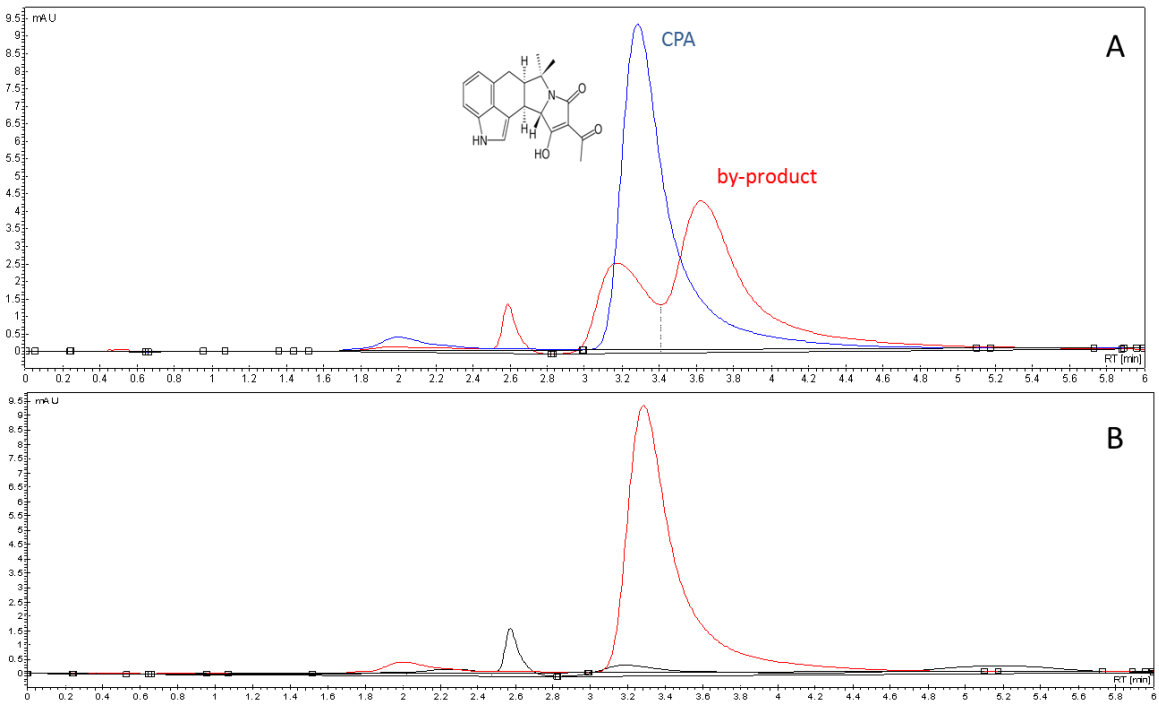

Figure 6.3. CPA by product formation in a lower concentration of aqueous ozone (1 mg/L) and CPA at 264 ng/mL (A); CPA degradation in a higher concentration of aqueous ozone (20 mg/L) (B).

Also with the use of ozone dissolved in water, it is possible to observe a significant reduction in CPA concentration, in any tested ozone to CPA concentrations. This reaction takes places in very short period of time, showing the high reactivity of ozone in aqueous solution, as well as its short half time. After 5 seconds of exposure, it was possible to conclude that the reaction had stopped, due to the absence of ozone in solution.

In situations with a low exposure to ozone, a degradation product is observed by HPLC. It is well known that the mechanism of degradation by ozone starts with an attack to double bounds, which are present in CPA. It is possible, that the exposure to low levels of ozone will induce a partial degradation of the CPA molecule, leaving in solution an intermediate product, which will later be further oxidized.

The mechanism of mycotoxins degradation by ozone has already been proposed by McKenzie et al. (1997, 1998). Here, it was observed the same mechanism. The effect of complete degradation of CPA occurred since the ozone attack the CPA molecule. May be after that there is no more active ozone to degrade other CPA molecules in suspension, according Figure 6.3, especially in a concentrated CPA solution.

The degradation of the chain structure of mycotoxins by O_3 molecule in a less concentrated CPA solution was more efficient than at high CPA concentration. This phenomenon it was related with the total ozone molecules in a solution to oxidise CPA.

The potential of ozone to degrade CPA was studied by McKenzie et al. (1997). They found that O_3 treatment reduced CPA from 242 mg/L to undetectable levels by HPLC. In this work CPA was presented in very lower levels in aqueous solution. Here, it was proposed study two CPA concentrations, both more concentrated than the ones used by McKenzie et al. (1998), and ozone was effective in both. The degradation of CPA is expected to start by the chain scission of the double bonds present in this molecule, as mentioned before.

6.3.3. Simultaneous degradation of Cyclopiazonic acid and aflatoxins by ozone

Simultaneous degradation of CPA and AF was tested in a mixture of CPA and AFB_1. Samples were taken as positive for each of the toxins when a peak was obtained at a retention time similar to each standard, with a height five times higher than the baseline noise. Figure 6.4 and Figure 6.5 shows complete degradation of both AFB_1 and CPA, when using more than 10 mg/L of ozone in solution. Also, the degradation of AFB1 occurs at a higher rate than that of CPA, indicating a higher sensitivite of the aflatoxin to ozone.

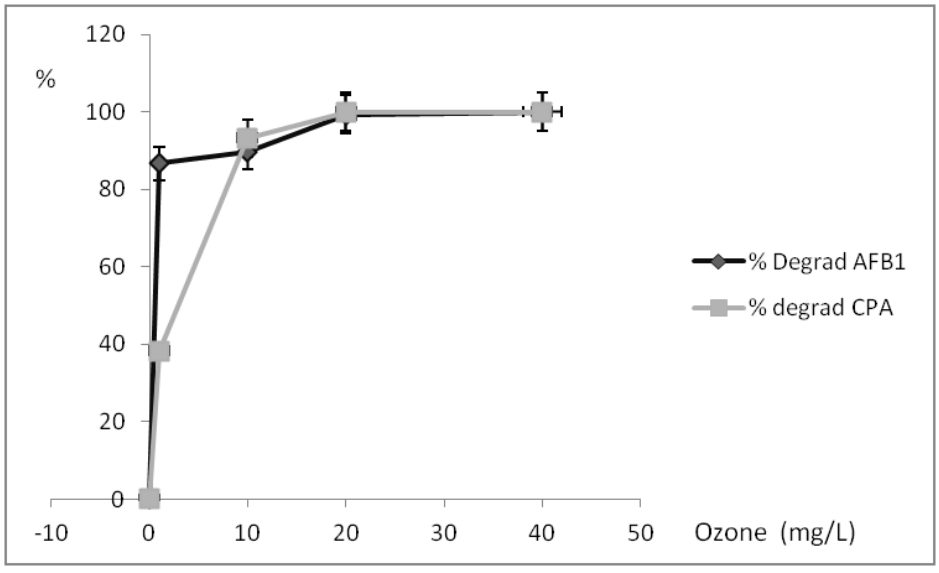

Figure 6.4. Aqueous ozone on the AFB1 and CPA mix degradation percentage.

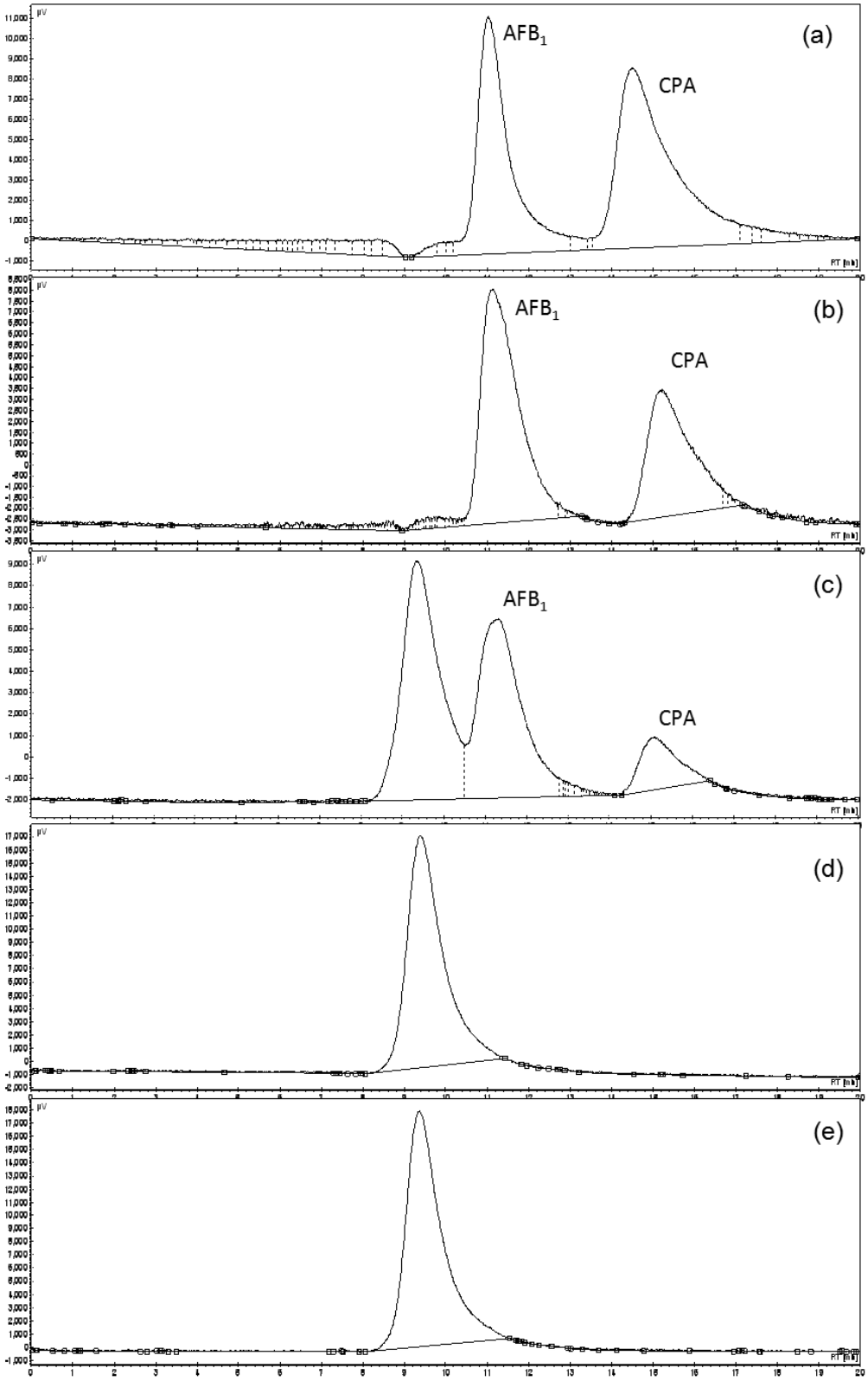

Figure 6.5. Aqueous ozone on simultaneously AFB1 and CPA mix degradation. a) AFB1 and CPA standard solution, b) Ozone at 1 mg/L, c) Ozone at 10 mg/L, d) Ozone at 20 mg/L and, e) Ozone at 40 mg/L .

Results reinforce the importance of ozone on CPA and AFB_1degradation, since CPA and AF are known sometimes to coexist (Bamba and Sumbali, 2005). Especially AFB_1, which is the primary source of liver toxicity (IARC, 2002; Proctor et al., 2004; Williams et al., 2004). In this way only one ozone treatment could be efficient to degrade CPA alone or in combination with AF, as it was proposed.

6.4 CONCLUSIONS

Aqueous Ozone can be used as a treatment on the mycotoxin decontamination. Apart from its foaming ability which permits ozone to be trapped in a better way in water, since water is a vehicle for washing and decontamination process. Five mycotoxins (CPA, AFB_1, AFB_2, AFG_1 and AFG_2) were degraded by aqueous ozone. Samples were well classified by their area from HPLC chromatograms signals. The retention time and peak area detected by HPLC served as useful parameters for classification of potential degradation in contaminated water samples. The method used in this study (AFs, CPA, and a mixture of AFB_1 and CPA together in single run) was a simple and fast way to evaluate toxins degradation to predict its later use in the degradation of mycotoxin during processing of food commodities. However, only a limited volume of tests was performed, with may limit the extrapolation of results. Further experiments with biological samples are recommended in order to determine and confirm the ability of aqueous ozone to destroy mycotoxins. The additional gain of this work was to show that aqueous ozone is a feasible way to decontaminate not only AF and CPA in aqueous solutions but also a way to decontaminated raw materials, as well as laboratory equipment or reagents for disposal.

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CHAPTER 7

Potential of aqueous ozone to control aflatoxigenic fungi in Brazil nuts

7.1 INTRODUCTION

The Brazil nut (*Bertholethia excelsa*) is an important non timber forest product (NTFP) from the Amazonian forest. Brazil participates with approximately 24 % of the total World production of this nut. The Brazil nuts exploitation is an important social and economic activity for the forest population. Besides, it is an important factor in forestry conservation and environmental sustainability (Cotta et al., 2008).

Despite nutrition and health-related aspects of the Brazil nuts, they are susceptible to the colonization by mycotoxin producing fungi, and consequently to contamination with mycotoxins. It has been reported the presence of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, which are the main producers of aflatoxins (AF) (Olsen et al., 2008). EU countries and the United States have been the major importers of in-shell and shelled Brazil nuts, respectively. AF contamination constitutes not only an economic problem for Brazil nut producing countries but also a serious health risk for consumers all over the world (Vargas et al., 2011).

Both industries and producers have been making considerable efforts in the last 15 years to minimize fungal growth and AF production in tree nuts. Particularly in the case of Brazil nuts, the climatic conditions in the Amazonian environment and the characteristics of the exploitation activity (collecting and primarily handling) cannot be controlled, exerting direct or indirect effects on the toxigenic fungi and on AF or multitoxin production (Chapter 4).

Processes and/or treatments that have been proven to reduce AF levels in Brazil nuts include shelling or sorting by size, specific gravity, color or damage (Pacheco and Scussel, 2006). On the other side, several strategies have been reported for the management of fungi and mycotoxins already present in raw material and food. From the available tools to ensure food safety, ozone application is

one of the most promising ones which come to meet the needs. Ozonation is a chemical mean of food processing that involves exposing food or an intermediate product to O_3 (Guzel-Seydim et al., 2004). It can be applied as gaseous ozone or dissolved in as aqueous solution. The first one is a more useful type of application, but both have been successfully used in reducing a) post harvest diseases (Kim et al., 1999), b) viability of potentially mycotoxin producer fungi and c) mycotoxin accumulation in many kinds of food products (Chapter 5).

The fact that ozonation is a non-residual treatment, which is in accordance with the increasing concern related to the use of chemicals in food chains led to the necessity of assay the viability of such treatment in Brazil nuts. The assays performed in the present work are focused on the use of aqueous ozone, since it is well known its application as a strong anti-microbial agent (Khadre et al., 2001) and its use for mycotoxins destruction has been studied earlier (McKenzie et al., 1997). Also, ozone is generally recognized as safe (GRAS) product and it has been already used in many agricultural products, including the organically labelled ones (Mlikota-Gabler et al., 2010), like Brazil nuts. In view of this, the present work aimed to investigate the potential of aqueous ozone controlling the fungi associated with Brazil nuts seeds, focusing on *Aspergillus flavus*.

7.2 MATERIAL AND METHODS

The samples used were collected from 2009 Brazil nuts harvest, obtained from agroforestry production area in Pará state, Brazil. The initial sample weighted about 40 kg and was homogenized to obtain a work sample.

The working samples of Brazil nuts were divided into two groups: (i) natural contaminated nuts (without sterilization), and (ii) sterilized nuts intended for studies with artificially contaminated nuts. For such group, the sterilization of nuts was carried out by autoclaving at 121 °C during 20 min at 1 atm (relative pressure) and the strain MUM 92.01 of *A. flavus* was used. This strain is a well known producer of both AF and Cyclopiazonic acid (CPA) (Rodrigues et al, 2009; Soares et al, 2010).

To obtain the stock solution of ozone, water saturated with ozone was prepared by bubbling gas, generated by the passage of extra dry oxygen through a reactor (Anseros, Model CD-COM-HF-4) for 15 minutes (power generator at 100 %; gas flow of 25 L/h) in a bottle with 1000 mL of water, at 3 °C. The final concentration of aqueous ozone was determined by colorimetric method at JASCO V560

spectrophotometer (λ_{max} = 258 nm and ε = 2900 $M^{-1}\,cm^{-1}$) (Bader and Hoigné, 1981). The ozone concentration in the stock solution ranged from 25 to 40 mg/L.

The different ozone concentrations required for the assays were obtained by the dilution of the stock solution with ozone demand free water as necessary, until the desired ozone concentration is obtained. A nonozonated water was used as control treatments. Only purified water obtained by successive reverse osmosis by Milli-Q plus system from Millipore (Molsheim, France) was used in the whole assay.

MUM 92.01 conidia suspension of $1x10^7$ conidia/mL was prepared in peptone-water (0.1 %). A 10 times diluted conidia (1×10^6 comidia/mL) suspension was prepared, and the number of viable conidia of both suspensions (1 and 0.1 mL) was assessed by inoculation on malt extract agar (MEA), prepared as described in Chapter 3.

The high concentrate conidia solution (HC) yielded about 400 whereas the low concentrate conidia suspension (LC) yielded about 40 viable conidia. From now on all the assays are based on the number of viable conidia.

7.2.1. Effect of ozonation time on viable conidia

Suspension of *A. flavus (*MUM 92.01) with 0.1 mL of either HC or LC conidia was washed in 20 mL of ozone solution for 2.5 min, 5 min, 10 min, 20 min, 30 min or 60 min. Subsequently, the ozone solution was filtered in a Millipore filtration system. Filters (0.2 μm porosity) with washed conidia were plated directly on malt extract agar (MEA) and incubated at 25 °C for 3 days for colony-forming units (cfu) assessment. Control tests were carried out by filtration of conidia suspension in a solution of tween 80 in Milli-Q water (0.005 % v/v). The duration of the ozone treatment was controlled by the addition of 1 mL of 1 M reagent-grade sodium formate solution to neutralize the remaining dissolved ozone after the desired contact time (Biswas et al., 2003). Three replications of each treatment were carried out and the results underwent full factorial test at $\alpha < 0.05$. In order to assess whether the factor time was affected by the concentration of ozone (interaction between effects), the whole assay was carried out at three different ozone concentrations (1 mg/L; 10 mg/L and 20 mg/L). In summary, the assay consisted of 2 different conidia concentration (HC or LC), 6 submersion times (2.5; 5; 10; 20; 30 or 60 min.), 4 ozone concentrations (0, 1 mg/L; 10 mg/L or 20 mg/L) and 3 replications (2×6×4×3 experiments).

7.2.2.Effect of ozone concentration on recovery of viable conidia from Brazil nuts

On another set of experiments, six sterilized nuts were inoculated with 0.1 mL of either LC or HC conidia solution of *A. flavus* MUM 92.01. These nuts were stored overnight at room temperature. Then, nuts were submerged in 20 mL of ozone solution at different concentrations (1, 5, 10, 15, 20, 25, and 30 mg/L). Control tests were carried out by submerging nuts in a solution of tween 80 in Milli-Q water (0.005 % v/v). After 15 min, 20 mL of the ozone or tween 80 solution were filtered (0.2 μm filter) in a Millipore filtration system and plated directly on malt extract agar (MEA) and incubated at 25 °C for 3 days for the determination of cfu. The whole assay was carried out twice. Three replications per assay were performed. The effect of both factors - ozone concentration and initial conidia concentration - on the viable spores after ozonation was studied by full factorial tests t at $\alpha < 0.05$.

7.2.3.Effect of ozonation on nutshell colour

The effect of the ozonation on the color of the nutshell was tested. For that purpose 20 nuts were submerged in aqueous ozone during 30 min. Five different ozone concentrations were tested (0; 0.1; 1; 10 and 20 mg/L). The control sample consisted on 20 non treated nuts randomly picked. Chromaticity values of the fruits in the L*a*b* space coordinates were recorded (McGuire 1992). The values of L* (lightness or brightness) vary from black (0) to white (100) and the chroma values of a* range from green (-60) to red (+60) and b* range from blue (-60) to yellow (+60). The hue [defined as arctan (b*/a*)] was calculated. The spectrometer Avantes (AvaMouse 2.0, Eerbeek, The Netherlands) was used. The change in nutshell color was evaluated by comparing the parameters hue and L of the control samples with those of the treated ones. The significance of the results was assessed by T tests for independent samples at $\alpha < 0.05$.

7.2.4.Assays with inoculated and natural contaminated nuts

In order to apply the correct ozone concentration during the treatment, it was necessary to know the degree of contamination of the nutshell and the amount of spores recovered after washing. For that purpose, a previous assay with artificially contaminated nuts was carried out to learn the percentage of viable conidia that migrate from the nutshell to an aqueous solution. For that purpose

100 µL of either LC or HC conidia suspension were inoculated onto the sterilized nut surface. After 1 hour at room temperature, the nut was submerged in 20 mL of a solution of tween 80 (0.005 %, v/v) in water. After 1 hour with shake, this solution was filtered (0.2 µm filter) in a Millipore filtration system and the filter was plated directly on MEA and incubated at 25 °C for 3 days for the determination of cfu. The assay was carried out 3 times with 3 replications (3 nuts) each time.

Parallel, in order to know the degree of contamination of natural contaminated nuts, those were submerged in 20 mL of a solution of tween 80 (0.005 % v/v) in water. Homologous to the previous assay, after 1 hour with shake, 20 mL of the aqueous solution were filtered (0.2 µm filter) and the filter plated directly on malt extract agar (MEA) and incubated at 25 °C for 3 days for the determination of cfu. Three assays were performed with 3 nuts per assay. The percentage of migration obtained in the previous assay was used to assess the total initial viable conidia on the nutshell.

Then, three more sterilized nuts were inoculated with 100 µL of either LC or HC conidia solution and left 1 hour at room temperature. Parallel, three natural contaminated nuts were randomly picked. Each sample was submerged in 20 mL of ozone solution (20 mg/L). Each ozone solution was filtered (0.2 µm filter) in a Millipore filtration system and the filter plated directly on MEA. The determination of cfu was carried out after 3 days at 25 °C, The whole assay was carried out 3 times. Those fungi isolated from natural contaminated samples were identified to genus according to Pitt and Hocking (2007) identification key, as already described in chapter 3.

The significance of the efficiency of ozonation was assessed by comparison of viable conidia in aqueous solution to viable conidia in ozone solution by means of T-test for separate samples at $\alpha < 0.05$.

7.3 RESULTS AND DISCUSSION

Neither the duration of aqueous ozone treatment nor the interaction with the ozone concentration affected the efficiency of the treatment. In Table 7.1 it is shown the percentage of viable spores after different treatment duration. However, the factor ozone concentration as a single effect was highly significant ($P < 0.001$) and so it was further suited (see bellow). Therefore, the effect of the ozone against spores is instantaneous at all ozone concentrations. These data may be explained by the short life time of ozone and its high rate of activity. It seems that after a few seconds, the reaction of ozone with the spores stopped, due to the consumption of ozone, and extending the contact time had no effect on the viability of spores. This was corroborated by comparing the results of one assay in

which sodium formate solution was used with the results of another assay in which the treatment duration was determined only by the time of submersion of the nuts in the ozone solution (data not shown), This last results, reinforces the theory that there is no more ozone left in solution of continue the reaction. Thus, the sodium formate solution was no more used and the duration of the treatment (which influence on the nutshell color was studied) was determined by the time the nuts were submerged in the ozone solution.

Table 7.1. Viable conidia of *A. flavus* (MUM 92.01) after different treatment duration and aqueous ozone concentration

	Viable conidia (%) after different time exposure (min) to ozone											
Time (min)	2.5		5		10		20		30		60	
Ozone (mg/L) 0	96	±7	94	±2	92	±0	93	±5	97	±5	94	±3
1	51	±1	69	±2	64	±20	59	±4	58	±7	61	±3
10	7	±4	5	±2	6	±6	5	±3	3	±2	2	±2
20	2	±3	1	±0	2	±1	2	±1	1	±1	3	±1

The effect of the ozone concentration on the efficiency of the treatment was highly significant ($p < 0.001$). In general, whatever the initial charge of spores, the higher the ozone concentrations the lower the final concentration of viable conidia (Figure 7.1). This was an expected result, since it is the reactivity of ozone with surface molecules on the spores that yielded the inactivation. So, the higher the concentration of ozone, the higher the inactivation of spores.

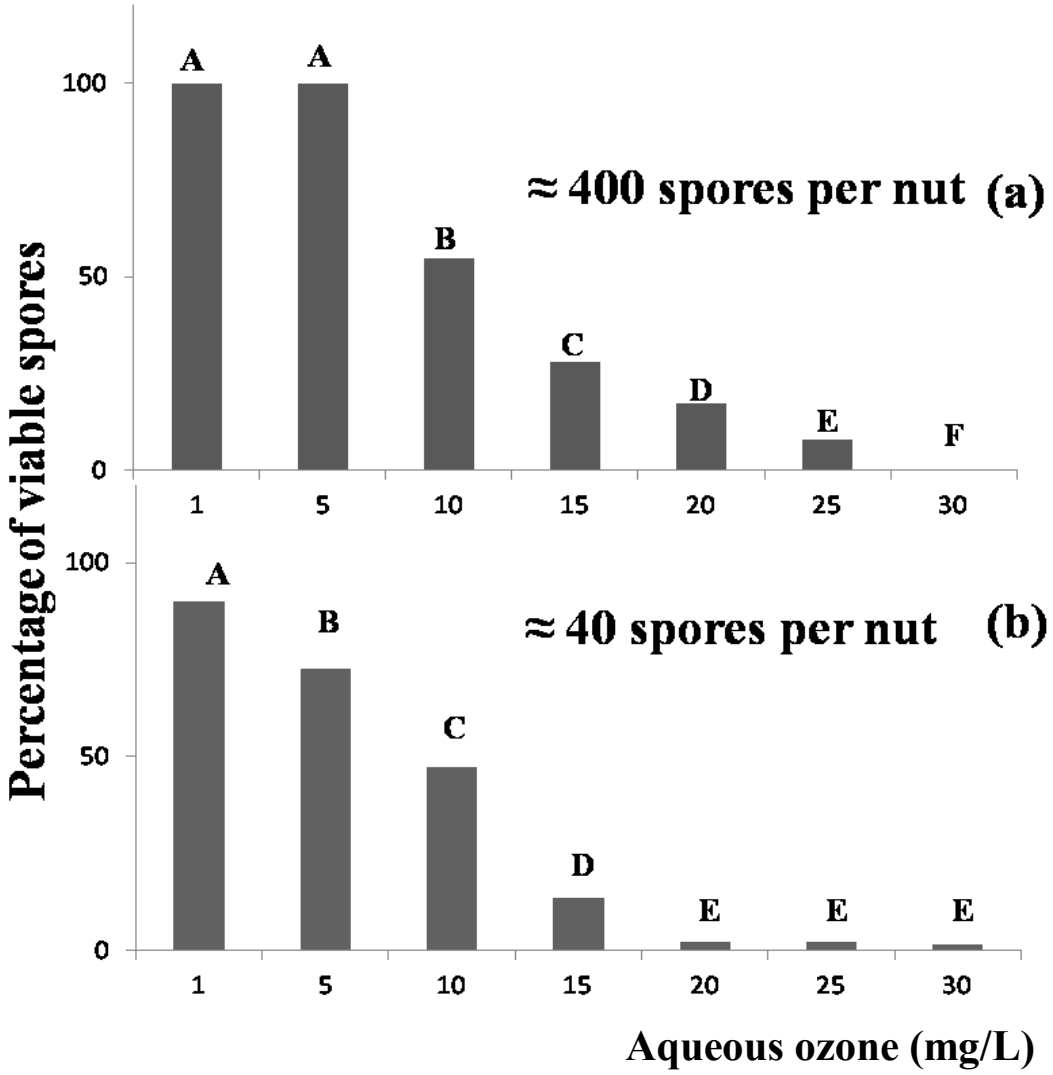

Figure 7.1. Percentiles of *A. flavus* viable conidia recovered after aqueous ozone exposure up to 30 mg/L in high (a) and low (b) concentration of conidia per nut.

However, the interaction of the factor ozone concentration with the factor initial viable spores was also highly significant ($p < 0.001$). Such interaction showed that for the lower initial viable spore charge it was enough to use a concentration of ozone of 20 mg/L, as no significant results were observed with higher ozone concentrations.

The submersion of LC or HC inoculated nuts in solution of tween 80 showed that whatever the initial number of viable spores (HC or LC), about 60 % of them migrate to the water solution. The submersion of natural contaminated nuts in solution of tween 80 yielded 32 ±5 conidia per nut. That means that the initial contamination was 53± 9 conidia per nut. According to the results obtained with

different ozone concentrations (Figure 7.1), a concentration of 20 mg/L of ozone was chosen to assay with HC, LC inoculated nuts and natural contaminated nuts. The most frequently isolated filamentous fungi from natural contaminated nuts were, in descending order, *Aspergillus* section *Nigri*; *Cunnighamella* sp; *Penicillium* spp. and *Aspergillus* section *Flavi* with incidences of 26 %; 21 %; 16 % and 5 %, respectively. The remaining of fungal isolates (33 %) were yeasts.

The results of submersion of a) nuts artificially inoculated with LC solution of *A. flavus* MUM 92.01; b) natural contaminated nuts and c) nuts artificially contaminated with HC solution of *A. flavus* MUM 92.01, in a 20 mg/L ozone solution, showed almost 0 viable spores after treatment, in cases a) and b), and about 3 viable spores per nut in case c) (Figure 7.2). In all cases, the reduction of viable spores was highly significant ($p < 0.001$).

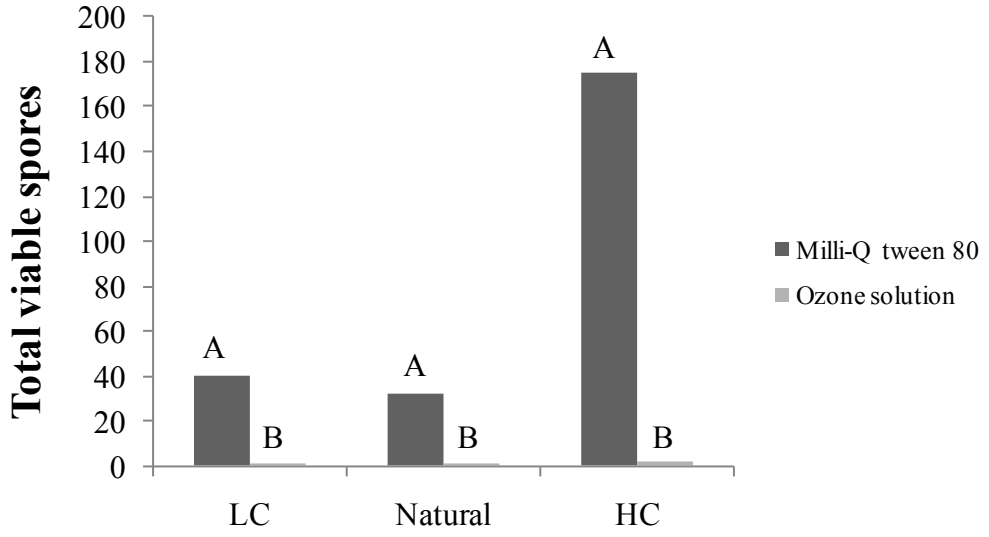

Figure 7.2. Reduction of viable spores on Brazil nuts from natural population and inoculated *A. flavus* (MUM 92.01) after aqueous ozone 20 mg/mL.

The influence of the aqueous ozone on the shell nut color was evident at higher ozone concentrations. The values of the parameter L increased with increasing ozone concentration and significantly differed from the control ones at concentrations higher than 10 mg/L. On the other side, the values of hue decreased with increasing ozone concentration and significantly differed from control ones at concentrations of 10 mg/L and higher (Figure 7.3).

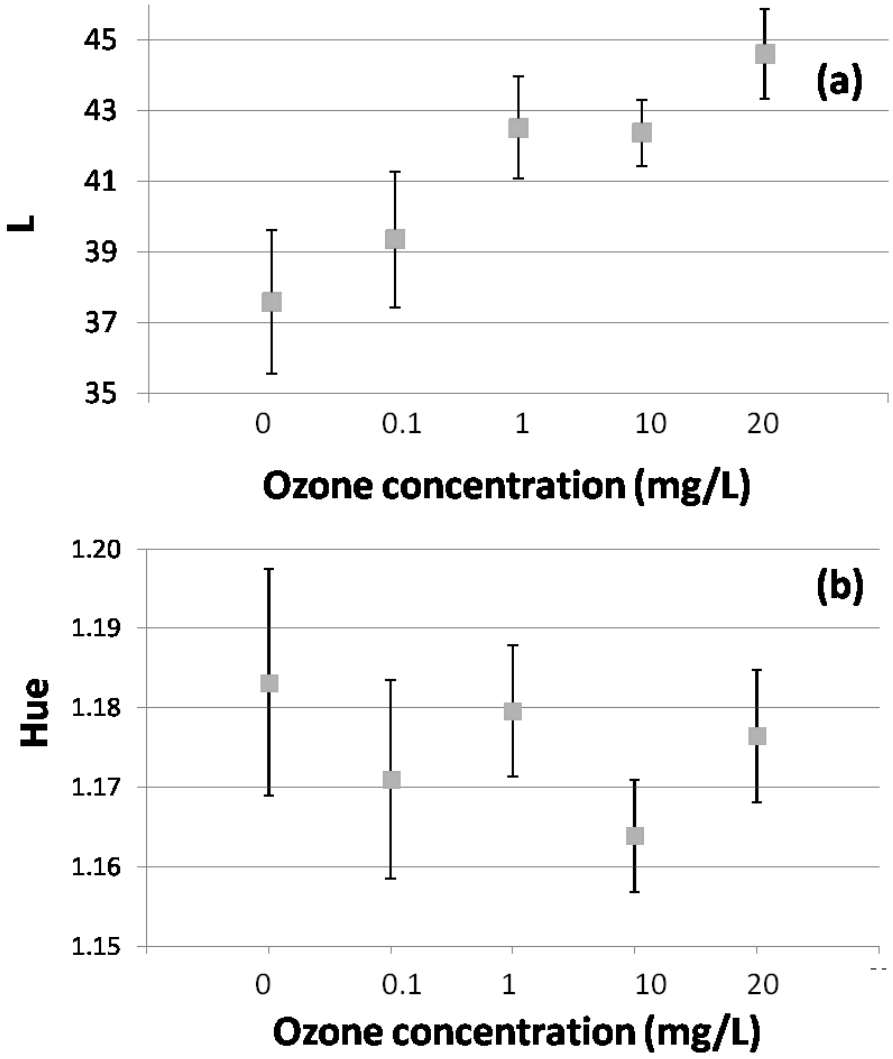

Figure 7.3. Effect of ozone concentration on color parameters a) L and b) Hue.

The genera *Aspergillus* is the main responsible for fungal contamination and subsequent production of AF in the field, at harvest, during post-harvest operations, and storage of nuts (Rodrigues et al., 2009). Due to high potential risk of contamination of nuts, decontamination methods are of great interest from economic, public health, and environmental aspects. Improving post-harvest processing followed by further prevention of fungal growth ozone is one of the best effective ways to restrict AF contamination and would have major effects for the reduction of health related risks and economics losses in production (Basaran et al., 2008).

This work was focused on fungal decontamination of Brazil nuts using different ozone concentrations.The use of ozone in dried fruits and nuts was studied before. The contamination of dried figs using 0.01 to 0.02 g/m^3 of gaseous O_3 for 3 and 4 h resulted in the reduction of total mycobiota counts (Öztekin, et al., 2006). Akbas and Ozdemir (2006) evaluated the efficiency of gaseous ozone for the degradation of AF in pistachio kernels and ground pistachios. Pistachios were contaminated with known concentrations of AFB_1, AFB_2, AFG_1 and AFG_2. Pistachio samples were exposed to gaseous ozone at 5.0, 7.0 and 9.0 g/m^3 ozone concentrations for 140 and 420 min at 20 °C and 70 % RH. Although a significant reduction in AFT and in AFB_1 was achieved for pistachio kernels, no significant change in the fatty acids composition of pistachios was observed. In the same way, no significant changes were found between sweetness, rancidity, flavor, appearance and overall palatability of ozonated and non-ozonated pistachio kernels.

In Brazil nuts, other authors studied the potential of gaseous ozone. Giordano et al. (2010) shown that the O_3 treatment within 5 h at 31 mg/L inhibited the viability of fungi of the nut-contaminating mycobiota, including the toxigenic *Aspergillus*. Scussel et al. (2011) also reinforced the potential of gaseous ozone in Brazil nuts and considered being the best method especially when associated with vacuum. According to these authors, ozone treatment was able to a) degrade AF (15 µg/kg), b) reduce fungi and yeast spores from 1.8×10^4 cfu/g to no growth, c) keep the fatty acid oxidation indicator (i.e. malondialdehyde) stable, and d) even improve the sensory attributes for consumer acceptance. Such features would be of great importance for the gaseous ozone treatment to be considered as a good sanitizer. Unfortunately, the use of gaseous ozone in some cases could cause toxicity. Since, the concentrations of gaseous O_3 necessary to inactivate the conidia of *Penicillium digitatum*, *P. italicum*, and *Botrytis cinerea* from table grapes was relatively high. Such high O_3 concentration make hard to achieve a complete containment of the gas and thus the workers safety may be compromised (Ozkan et al., 2011). Gaseous ozone treatment needs some adjustments in its application in order to accomplish the regulations, since O_3 concentrations cannot exceed 0.075 µL/L during an 8 h workday (USEPA, 2008).

According to our results, the reaction of the ozone is almost instantaneous; the application of aqueous ozone treatments in industries does not seem to be very complicated. It is necessary to learn previously the degree of contamination of the nuts, because if it is not very high, lower concentration of ozone may be used. For the same reason it is necessary to learn which degree of contamination is

acceptable after the treatment, because higher ozone concentrations will be required if lower levels of spores per nut are to be reached.

Although the effect of gaseous ozone in red pepper treated with concentration up to 66 g/m^3, for up to 1 hour, did no change significantly the color of red pepper in the product (Inan et al., 2007), it seems that some changes could happen in the case of Brazil nuts. Such effect has to do with the concentration of ozone but surely longer submersions lead to higher changes in color. Taking into account that the effect of ozone against conidia seems to be immediate it seems feasible to reduce color changes by minimizing as much as possible the submersion time and adjusting ozone concentration to the minimum needed for the initial degree of contamination of the nut. Further assays concerning nutshell color should be carried out as it seems that ozonation do have an effect on it.

Zorlugenç et al. (2008) studied the effectiveness of gaseous ozone and ozonated water on mycobiota and AFB_1 content of dried figs. In both treatments, degradation of AFB_1 increased with increasing ozonation time. Long gaseous ozone treatment, as commented above, are hard to control and may compromise worker safety. Zorlugenç et al. (2008) stated that gaseous ozone was more effective than ozonated water for reduction of AFB_1, whereas ozonated water was affected for decreasing microbial counts.

Our results show that the use of aqueous ozone presented good potential to use for fungi control in Brazil nuts. The aqueous O_3 solution can be recommended to control others fungi and possible bacteria associated to the nuts, being a tool to increase the quality standard of Brazil nuts.

7.4 CONCLUSIONS

The use of aqueous ozone is an actual alternative technique for fungi control in Brazil nuts, showing an optimum control of a large spectrum of fungi and good applicability to complex matrices such as nuts. The aqueous O_3 solution can be recommended to control other mycotoxin producer Aspergilli, since it reduces their counts. However, additional experiments are needed to adjust the method to use in Brazil nuts packing houses.

A food-related application of ozone is currently restricted in packing houses especially on leaves and fruit sterilization (Perry and Yousef, 2011). Food industry constantly searches for new technologies to improve commercial sterilization process of food commodities, especially those which are associated to mycotoxins and other hazards.

As exposed in this assay, the aqueous ozone application with accurate O_3 dosification may be an efficient method for disinfection of dry food surfaces. The O_3 disinfection of food surfaces opens up an alternative and advanced technology for the elimination of fungal contamination, since the ozone application in food is easy to apply and according to the results here exposed, effective at low doses and short durations, yielding no residues on the product or the environment and finally has a GRAS certified (Mlikota-Gabler, et al., 2010).

More research effort must be undertaken to evaluate this techniques to become common in the food industry, as the ideal concentration and projected cost for large quantities of Brazil nuts treatment.

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CHAPTER 8

General Conclusions and Further Developments

The commercial extraction of NTFPs can be an important component of rainforest conservation strategies. According to this, if the forest becomes economically productive, deforestation will be discouraged. For Amazon areas where *Bertholletia excelsa* occurs, the Brazil nut trade will be particularly positive for the preservation of the forest. Given the profitability of the product and the symbiotic relation of Brazil nut trees with their forest environment, it is argued that this environment will be preserved in order to guarantee a continuous supply of Brazil nuts in Amazon.

However, in terms of food provision, the task of producing mycotoxin-free products with nutritional quality becomes a challenge, especially in complex production chains of developing countries, such as it is observed in the Brazil nut, where this is more difficult to be achieved.

With regard to AF, the management of the entire Brazil nut chain is still a challenge. This wild commodity needs to be safely offered to consumers, especially to the ones who expect to eat not only a nut but also a functional food, because of its health-related appeal, and mostly because of its oil characteristics and high selenium content.

Concerning the fungal contamination the average incidence of infection by *Aspergillus* spp. in Brazil nuts was, respectively, from the *Flavi, Nigri* and *Circumdati* sections. The sanitisation treatment reduced the fungal counts, but aflatoxin producing *Aspergillus* species are still isolated from Brazil nuts. Aspects concerning identification of fungi in the fractions of Brazil nut fruits can help efforts to decontaminate the product and can be incorporated in a flow diagram of good handling practices for Brazil nuts.

The AF analysis showed the concentrations of AFB_1 and AFG_1 obtained were higher than of AFB_2 and AFG_2. The HPLC-FD used in this work presented limits of detection (LOD) and quantification (LOQ) suitable to satisfy all international regulations and quality conformity standards.

An HPLC-MS/MS method constitututed an alternative to conventional techniques for mycotoxin analysis, showing an ultra large mycotoxin spectrum, good sensitivity, rapidness and applicability to complex matrices such as nuts, fat products and processed ones. From 235 metabolites (201 fungal metabolites) fifteen mycotoxins were detected and quantified in Brazil nuts, namely, aflatoxins (AFB_1, AFB_2, AFG_1, and AFM_1), sterigmatocystin, methyl-sterigmatocystin, kojic acid, citrinin, cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin H, rugulosin, altenariol-methylether and emodin. The last nine are being reported for the first time in Brazil nuts samples. Although some mycotoxins content in Brazil nuts were low, samples presented one to 10 fungal metabolites. This study suggests that more investigations are needed in this commodity with more samples to be analyzed to understand the complexity fungi metabolites and its ecological role in Brazil nuts, since the fungal metabolites profile may change from year to the other, justifying further monitoring and survey studies on emergent mycotoxins in Brazil nuts. This method could therefore be applied as routine method for complex types of food.

Aqueous ozone can be used as a treatment for mycotoxin decontamination. Apart from its foaming ability which permits ozone to be trapped in a better way in water, since water is a vehicle for washing and decontamination process. The AF and CPA were degraded by aqueous ozone. Samples were well classified by their area from HPLC chromatograms signals. The retention time and peak area detected by HPLC served as useful parameters for classification of potential degradation in contaminated water samples. Sodium formate added to stop ozone reaction had no effects on stopping reaction since the degradation occurs quickly. The method, developed in this study, is a simple and fast way to evaluate the toxins degradation. Measurement of mycotoxins degradation properties by ozone in water is a potential method to predict further content degradation of processed commodities. However, only a limited volume samples was used in this study and this limitation may influenced the result classification, further experiments with biological samples are recommended in order to determine and confirm the aqueous ozone potential ability to destroy mycotoxins. The additional gain of this work was to show that the aqueous ozone use is a way to decontaminate not only CPA standards but also contaminated raw material, as well laboratory equipment/reagent for disposal.

By using aqueous ozone in this study, an alternative technique for fungi control in Brazil nuts packing house was found an ultra large fungi spectra and good applicability to complex matrices such as nuts.

Aqueous ozone was effective in reducing the conidia of *Aspergillus flavus* and natural fungal population associated to Brazil nuts. The process of ozonation on samples of the Brazil nut was efficient in reduction of fungal spores of *A. flavus* and natural mycobiota; however, it had a significant effect on the color of the bark of Brazil nuts after ozonation by *L, *a, *b system, when using aqueous ozone concentration higher than 10 mg/L. The aqueous O_3 solution can be recommended to control to others Aspergilli mycotoxin producers. However, additional experiments are needed to adjust the method to use in packing house of the Brazil nuts.

Prevention of contamination by *Aspergillus* spp. through good handling practices is still the best measure to avoid AF in Brazil nuts and to ensure the quality and safety of this product.

Obviously this subject is not exhausted with this work. It opens the opportunity for some front line research which will be possible to implement over time. Some perspectives of futures actions are listed below:

- Other surveys focusing on different producing areas need to be developed in Brazil nuts, in particular on *Aspergillus* and aflatoxigenic fungi, to identify the predominant species within *Aspergillus* section *Flavi* responsible for AF production in Brazil nuts.

- Given the presence of altenariol-methylether in all analyzed Brazil nuts samples (Chapter 4), which was reported here for the first time, it will be interesting to investigate the occurrence of *Alternaria* spp and the conditions for production of its metabolites. This study suggests that more research is needed in this commodity with more samples to be analyzed to understand the complexity fungi metabolites and its ecological role in Brazil nuts, since the fungal metabolites profile may change from year to the other, justifying further monitoring on other emergent mycotoxins in Brazil nuts.

- In view of monitoring the AF and mycobiota before and after ozonation process, it will be proposed to conduct experiments in a packing house with aqueous ozone applied as a washing step in Brazil nut. This monitoring could be analyzed also after the shipping simulated period in order to understanding the possible AF, mycobiota fluctuation and safety of nuts before reaching marketing channels.

- At present, storage and processing conditions of Brazil nuts needs to be improved for both in-shell and shelled nuts. In view of this, all aspects related to processing need improvement. Monitoring has to be done taken into account all stages of the processing [i.e. cleaning, washing, drying (below to

0.7 a_W)] and other parameters must be carefully checked in order to obtain a quality product, traceable and safe.