Universidade do Minho
Escola de Engenharia

Thalita Cabral Calado

Gamma irradiation in mycotoxins control

janeiro de 2020

Thalita Cabral Calado **Gamma irradiation in mycotoxins control**

Uminho | 2020

Universidade do Minho
Escola de Engenharia

Thalita Cabral Calado

Gamma irradiation in mycotoxins control

Doutoramento em Engenharia Química e Biológica

Trabalho Efetuado sob a orientação do
Doutor Armando Albino Dias Venâncio
Doutor Luís João Abrunhosa Pereira

janeiro de 2020

Agradecimentos

Depois de tanto tempo neste "rebuliço", quando paro para pensar e olho para trás, foi tanta, mas tanta, gente boa no meu caminho... E, se ser feliz é estar grata, estes agradecimentos vão ser longos...

Agradeço em primeiro lugar ao Professor Venâncio pela oportunidade e por acreditar que eu conseguiria. Pelos ensinamentos, pela paciência gigante que sempre teve comigo, com os meus atrasos, inquietações, hesitações e acessos de ansiedade. Por ter sempre uma atitude humilde e humana que tornou a convivência com ele tão agradável que vai deixar saudades. Ao Professor Luís, por todos os ensinamentos e ajuda na concretização deste trabalho. Pelo companheirismo e diversas oportunidades de aprendizagem, pelos ensinamentos, correções e sugestões.

Ao pessoal do INIA, nomeadamente a Doutora María Luisa Fernández-Cruz e Luis Alté por tudo o que me ensinaram em trabalhos com linhas celulares, pelo apoio e pela paciência com o meu "portunhol".

Ao pessoal do C2TN, nomeadamente à Sandra Cabo Verde e à Maria Luisa Botelho por me acolherem desde o início com toda a simpatia e pelas inúmeras irradiações que a Sandra fez durante estes anos.

À FCT pelo financiamento da minha bolsa com a referência SFRH/BD/79364/2011.

Ao Professor Teixeira que me possibilitou uma abertura de horizontes nessa última fase do doutoramento (que eu tanto precisava). Obrigada pela confiança, pelo otimismo e energia!

Aos meus colegas de trabalho no DEB, pela parceria, pelo respeito, pelos empréstimos, por, num centro onde, por vezes, os recursos são poucos para tantos investigadores a trabalhar ao rubro, encontramos sempre forma de nos entreajudar e aprender uns com os outros. Um agradecimento especial aos técnicos que tantas vezes me ajudaram, nomeadamente Eng. Madalena, Sr. Santos, Aline, Vitória, Diana e Maura.

À todos os amigos que angariei no LCTA, pela amizade e pelas partilhas de conhecimentos. Às minhas "amorecas" Ana Sofia "Tola", "Caty" Costa e Ana "Paulete" Santiago por todos os momentos de descontração e grande amizade. À Tina pela amizade, apoio e por todas as conversas e Coca Colas para "arrebentar". Ao Otniel, obrigada pela generosidade e amizade!

Aos melhores colegas de trabalho do mundo: Isa, Nuno, Cláudia, Mey, Aloia, Sara, Ailton, Pedro, por todas as conversas e risadas e por tornar os meus dias muito mais leves e divertidos no meio da correria de sempre.

Ao Filipe, por todo o incentivo e ajuda na altura crítica.

Às minhas Cristiane, Dina, Mónica e Karol, por aturarem todos os meus dramas e fazerem sempre que isso pareça completamente compreensível. Por me lembrarem o verdadeiro conceito do "be a unicorn...", por gostarem de mim porque sim, pelos "puxões de orelha" e por me lembrarem tantas vezes da "fibra de que sou feita". À minha Marisa, vamos tomar aquele café um dia... seja lá onde for...

À Érica pela amizade, ajuda e compreensão e por me "emprestar" o meu Gui (e agora também a minha Pé) que faz sempre o mundo ser um lugar lindo, leve e feliz. Aos meus pais por todo o apoio. À minha mãe por mesmo às vezes sem saber bem como, me dar forças e exemplo de nunca desistir e que temos sempre mais um bocadinho de energia mesmo que não nos pareça...

Ao meu Nuno, pelo amor, por estar sempre, sempre, sempre lá, pelas bachatas, pelas salsas, pelas botijas, pela força, pelas chamadas de atenção para mim, pelo companheirismo, pelos fins de semana a trabalhar, por tudo e muito mais...

A todos que com energia, praticidade, otimismo e amizade ajudaram na concretização deste trabalho, muito obrigada!

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Irradiação gama no controlo de micotoxinas

A minimização da presença e dos efeitos tóxicos das micotoxinas em alimentos representa uma preocupação a nível económico, regulatório e científico. Embora a implementação de boas práticas que evitem o crescimento fúngico e a produção de micotoxinas em produtos agrícolas seja essencial para cumprir os padrões de segurança alimentar, a utilização de novas tecnologias que possam atuar nos processos de pós-colheita e pós-armazenamento é igualmente importante. O uso da radiação gama tem sido estudado como uma alternativa para a detoxificação de alimentos, existindo, contudo, resultados contraditórios em vários estudos. O principal objetivo desta tese foi verificar a eficácia da irradiação gama na detoxificação de algumas das principais micotoxinas – ocratoxina A (OTA), zearalenona (ZEA) e aflatoxinas (AFIs) B1, B2, G1 e G2.

Irradiou-se padrões de micotoxinas com doses de radiação entre 0 e 8,6 kGy e em diferentes condições de hidratação. Verificou-se que a presença de água é fulcral para a eficácia da irradiação gama em todas a micotoxinas em estudo. No caso da AFIs, a irradiação de soluções das quatro AFIs, mostrou-se mais eficaz do que a irradiação de cada uma das AFIs em separado.

A citotoxicidade das micotoxinas foi igualmente avaliada. Para tal, amostras de micotoxinas, irradiadas e não irradiadas, foram inseridas em culturas de células HepG2 de modo a verificar se a degradação das micotoxinas é acompanhada pela diminuição da sua citotoxicidade. Para além disto, no caso da ZEA, a variação da estrogeneicidade também foi avaliada em culturas de células hERα-HeLa-9903. Em todos os casos, verificou-se que existe uma diminuição da toxicidade das micotoxinas depois de submetidas a radiação gama.

O efeito da irradiação de alimentos foi testado para a OTA. Irradiaram-se amostras de trigo, sumo de uva e vinho contaminadas com OTA. Verificou-se que o efeito da radiação em OTA quando esta está numa matriz alimentar é muito distinto do efeito da degradação em suspensão aquosa. Os componentes dos alimentos parecem dificultar a degradação da OTA pela radiação.

Os resultados obtidos mostram que, em alimentos, a radiação poderá ser mais eficaz na eliminação de fungos micotoxigénicos, e consequentemente na eventual acumulação de micotoxinas, do que na destruição de micotoxinas. Ainda assim, ocorre a redução da quantidade de micotoxina com a radiação, sendo esta redução acompanhada da redução da toxicidade. Contudo, a redução dos teores em micotoxinas alcançada com a radiação é reduzida e não contribui por si só para a descontaminação de alimentos.

Palavras-chave: Radiação gama, citotoxicidade, aflatoxinas, zearalenona, ocratoxina A

Abstract

Gamma irradiation in mycotoxins control

The mycotoxin issue requires constant vigilance from economic, regulatory and scientific agents to minimize its toxicological effect on humans and on animals. The implementation of good practices to avoid fungal growth and mycotoxin production in agricultural commodities is essential to achieve most restrictive safety standards; however, the contribution of novel technologies that may act on post-harvest and post-storage situations may be equally important. The gamma irradiation has been studied has an alternative for food detoxification. Nevertheless, the effectiveness of the process reveals contradictory results in literature. The main purpose of this thesis was to test the effectiveness of gamma irradiation in detoxification of the some of the major mycotoxins – ochratoxin A (OTA), zearalenone (ZEA) and aflatoxins (AFIs) B1, B2, G1 and G2.

Mycotoxins standards were irradiated with irradiation doses between 0 and 8.6 kGy and under different moisture conditions. The results show that water plays an important role in gamma irradiation efficiency to all mycotoxins tested. To AFIs, the irradiation of all of the four AFIs together allowed higher detoxification than when AFIs were irradiated separately.

The cytotoxicity of mycotoxins was also evaluated. To this end, irradiated and non-irradiated mycotoxin samples were inserted into HepG2 cell cultures to verify if the degradation of mycotoxins is accompanied by a decrease in their cytotoxicity. In addition, in the case of ZEA, the variation in estrogenicity was also evaluated in hERα-HeLa-9903 cell cultures. In all cases, it was found that there is a decrease in mycotoxin toxicity after gamma radiation.

The effect of food irradiation has been tested for OTA. Samples of OTA contaminated wheat, grape juice and wine were irradiated. The effect of radiation on OTA when found in a food matrix seems to be very distinct from the effect of OTA degradation in aqueous suspension. The presence of food components appears to hinder radiation degradation of OTA.

The results show that, in food, radiation could be more effective in the elimination of mycotoxigenic fungi, and consequently in the eventual accumulation of mycotoxins, than in the destruction of mycotoxins. Even so, there is a reduction in the amount of mycotoxin with radiation, which is accompanied by a reduction in toxicity. The reduction in mycotoxin levels that can be obtained thought irradiation is relatively small, and does not in itself contribute to food decontamination.

Keywords: Gamma radiation, cytotoxicity, aflatoxins, zearalenone, ochratoxin A

Table of contents

Abbreviations

5-CF	5-carboxyfluorescein
AB	Alamar Blue
ADON	3- and 15-acetyldeoxynivalenol
AFB1	Aflatoxin B_1
AFB2	Aflatoxin B_2
AFG1	Aflatoxin G_1
AFG2	Aflatoxin G_2
AFIs	Aflatoxins
AF	Aflatoxin
ATCC	American Type Culture Collection
CFDA-AM	Carboxyfluorescein diacetate acetoxymethyl ester
D_{10}	Dose required to inactivate 90% of a microbial population
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
e^-	Negative free solvated electrons
EMEM	Eagle's Minimum Essential Medium
ERE	Estrogen-Responsive Element
FAO	Food and Agriculture Organization of the United Nations

FBS	Foetal Bovine Serum
H_2O^+	Positively charged water radicals
H_2O_{dd}	Deionized water
HACCP	Hazard Analysis and Critical Control Point
HepG2	Human hepatoma
HPLC	High-performance liquid chromatography
HPLC-FL	High-performance liquid chromatography with fluorescence detection
IAEA	International Atomic Energy Agency
Int-407	Human intestinal epithelial
Kan	Kanamycin
kCi	kilocurie
kGy	Kilogray unit
LOD	Limit of detection
LOQ	Limit of quantification
MEM	Minimum Essential Medium
MEM	Minimum Essential Medium
MeV	Mega electronvolt unit
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue
NEAA	Non-Essential Amino Acids
NIV	Nivalenol

NR	Neutral Red (3-amino-7-dimethylamino-2-methyl phenanzine hydrochloride)
OECD	Organization for Economic Co-operation and Development
OTA	Ochratoxin A
PBS	Phosphate buffer saline
PES	Polyethersulfone
Pk15	Porcine kidney epitelial
RAW 264.7	Macrophage cell line of Mus musculus
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
SH-SY5Y	Human neuroblastoma
TBq	Terabecquerel
TLC	Thin Layer Chromatography
WHO	World Health Organization
ZAN	zearalanone
ZEA	Zearalenone
α-ZAL	α-Zearalanal
α-ZOL	α-Zearalenol
β-ZAL	β-Zearalenal
β-ZOL	β-Zearalenol
λ_{em}	Emission spectra

λ_{exc}	Excitation spectra

List of Figures

List of Tables

Chapter 1. Motivation and outline

1.1 Thesis motivation

Filamentous fungi are naturally present in most crops. However, the presence of these fungi is not innocuous for consumers due to their capacity to spoil food and produce mycotoxins. Mycotoxins are natural fungal contaminants that can be found in a large variety of food commodities, including cereals, pulses, nuts and their products. When these commodities constitute a significant fraction of population's diet, such contamination translates into chronic exposures to high-levels of mycotoxins (Jolly *et al.*, 2009).

Currently there is no available technology to completely eliminate mycotoxins from the food chain (Shapira and Paster, 2004; Wielogorska *et al.*, 2019). Most of the current strategies for mycotoxin reduction are based on prevention, either at pre- or post-harvest stages, or on the segregation of contaminated kernels after harvest (Venâncio & Paterson, 2007; Wielogorska *et al.*, 2019). Other strategies for mycotoxins removal or inactivation are applied on a case by case approach. Methods such as the biological ones (inactivation of patulin by *Saccharomyces* strains or the degradation of ochratoxin A (OTA) by enzymes), physico-chemical ones (adsorption of ochratoxin A by fining agents in wine making), or physical ones (tangential filtration of whey to reduce aflatoxin M_1) are examples of these (Freitas-Silva & Venâncio, 2010). However, these methods have not a broad application. Although the prevention of contamination is better than elimination of contamination, the study of possibilities to detoxification contaminated food and feed was required. The lack of practical solutions to control mycotoxin contamination in the field, at harvest and on processed products leads to the demand of new methods for their partial or total elimination (Wielogorska *et al.*, 2019).

The constant demand of consumers for safer, healthier and processed food drives the development of technologies in food processing to achieve their needs. Food safety is one of the major challenges for technology, although many preservation processes and regulations are already available to control the microbiological and chemical integrity of food. Furthermore, the fact of the climate change is predicted to increase the presence of mycotoxins in agricultural commodities and the increase of world population will lead to food shortage, decontamination technologies are necessary. Food irradiation is one among many of available technologies that contribute to improve the safety of food (Niemira, 2018).

Food irradiation is a physical mean of food processing that involves exposing pre-packaged or bulk foodstuffs to ionizing energy. This process is sometimes called "cold pasteurization" because the inactivation of microorganisms is achieved at low temperatures unlike the traditional heat-pasteurization. Using irradiation, the microbiological safety of food can be improved and its shelf life prolonged without substantially changing, in most cases, its nutritional, chemical and physical properties. The elimination of pests on agricultural commodities can also be achieved, reducing food losses and the use of chemical fumigants and additives. Food irradiation up to an overall dose of 10 kGy has been considered a safe and effective technology since 1981 by several food international organizations (FAO/IAEA/WHO, 1981). Nonetheless, food irradiation is not as widespread as other conventional technologies due to the high costs of irradiation units but, particularly, because of a negative perception of consumers relatively to its safety (Bearth and Siegrist, 2019).

The non-residual feature of ionizing radiation is an important advantage of irradiation (Arvanitoyannis *et al.,* 2009). Irradiation have been studied for the degradation of mycotoxins showing controversial results. Some authors claim promising results (Aziz *et al.*, 1997; Ghanem *et al.*, 2008; Jalili *et al.*, 2010; Simas *et al.*, 2010; Jalili *et al.*, 2012;)and other authors report gamma irradiation was not effective in mycotoxins reduction (Akueche *et al.* 2012; Iqbal et al, 2012; Di Stefano *et al.*, 2014a; Di Stefano *et al.*, 2014b).

Thus, these techniques may be useful not only as a food sanitizing agent to control food decay during storage, but also for its potential in the inactivation of mycotoxigenic fungi and in the degradation of their mycotoxins. Nonetheless, gamma irradiation as a detoxification method needs further investigation to clarify the conditions for irradiation to be effective as well toxicological studies to assess the safety of irradiated products.

1.2 Research aims

This work aims to study the possibility of irradiation gamma to mycotoxins control in food and feed.

Thus, the main objectives to be targeted are:

1. To evaluate the degradation of mycotoxins by the action of gamma radiation using dried and in solution mycotoxins;

2. To evaluate the occurrence of compounds that may result from mycotoxins degradation and their toxicity;

3. To evaluate the degradation of mycotoxins in food (wheat, wine and grape juice).

1.3 Thesis outline

The thesis is organized in seven chapters.

This first chapter describes the motivation, research aims and thesis outline.

Chapter 2 presents an overview on the utilization of gamma radiation to food irradiation with a special enforce on control of fungi and mycotoxins in food and feed.

Chapters 3-5 present a study of irradiation applied to mycotoxins and cytotoxicity studies of irradiation products. Chapter 3, 4 and 5 are dedicated to studies with aflatoxins, zearalenone and ochratoxin A, respectively. In chapter 3 the effect of radiation was tested in the four more widespread types of aflatoxins, separately and in a combination of them. The cytotoxicity of aflatoxins irradiated was evaluated. In chapter 4, in addition to degradation and cytotoxicity assays, other assays to verify the decrease of estrogenicity were performed. In the last chapter, in addition to degradation and cytotoxicity effect of gamma radiation on ochratoxin A standard solutions, the degradation of ochratoxin A in contaminated matrices was developed. Although, some methodology was similar in the work presented in these chapters, in order that the reader can make an easier reading by type of mycotoxin, it was decided to treat the 3 chapters as autonomous works.

In Chapter 6, an integration of all results was made and, finally, Chapter 7 summarizes the main conclusions of this work and highlights perspectives for future work.

1.4 Publications

This thesis is based on the following original research or review articles:

Chapter 2: Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for mold and mycotoxin control: a review. Comprehensive Reviews in Food Science and Food Safety, 13(5), 1049-1061.

Chapter 4: Calado, T., Fernández-Cruz, M. L., Alté, L., Verde, S. C., Abrunhosa, L & Venâncio, A. Degradation of zearalenone by gamma radiation to decrease its cytotoxicity and estrogenicity (manuscript in preparation)

Chapter 5: Calado, T., Fernández-Cruz, M. L., Verde, S. C., Venâncio, A., & Abrunhosa, L. (2018). Gamma irradiation effects on ochratoxin A: Degradation, cytotoxicity and application in food. Food chemistry, 240, 463-471.

References

Akueche, E.C., Anjorin, S.T., Harcourt, B.I., Kana, D., Adeboye, E., Shehu, I., Akande, R., Adeleke, A.T., Shonowo, O.A. & Adesanmi, C.A. (2012). Studies on fungal load, total aflatoxins and ochratoxin A contents of gamma-irradiated and non-irradiated *Sesamum indicum* grains from Abuja markets, Nigeria. Kasetsart J: Nat Sc 46:371-82.

Arvanitoyannis, I., Stratakos, A., Tsarouhas, P. (2009). Irradiation Applications in Vegetables and Fruit: A Review. Critical Reviews in Food Science and Nutrition, 49:427-462.

Aziz, N., Attia, E., Farag, S. (1997). Effect of gamma-irradiation on the natural occurrence of *Fusarium* mycotoxins in wheat, flour and bread. Nahrung, 41:34-37.

Bearth, A., & Siegrist, M. (2019). "As long as it is not irradiated"–Influencing factors of US consumers' acceptance of food irradiation. Food quality and preference, 71:141-148.

Di Stefano, V., R. Pitonzo and G. Avellone, (2014a): Effect of Gamma Irradiation on Aflatoxins and Ochratoxin A Reduction in Almond Samples. Journal of Food Research, 3.4

Di Stefano, V., R. Pitonzo, N. Cicero and M. C. D'Oca, (2014b): Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 31:2034-2039.

FAO/IAEA/WHO. (1981). Wholesomeness of irradiated food (Technical Report Series 659). Joint FAO/IAEA/WHO Expert Committee, Geneva, Switzerland.

Freitas-Silva, O. & Venâncio, A. (2010) Ozone applications to prevent and degrade mycotoxins: a review. Drug Metabolism Reviews, 42 (4):612-620.

Iqbal, Q., Amjad, M., Asi, M.R. & Arino, A. (2012). Mold and aflatoxin reduction by gamma radiation of packed hot peppers and their evolution during storage. J Food Protect 75:1528-31.

Jolly, C., Bayard, B., Vodouhe, S. (2009) Risks of ingestion of aflatoxin contaminated groundnuts in Benin: scale measurements, beliefs and socioeconomic factors. Risk Analysis, 29:1395-1409.

Niemira, B. A. (2018). Safety and Quality of Irradiated Fruits and Vegetables. In Fruit Preservation (pp. 399-416). Springer, New York, NY.

Shapira, R. & Paster, N. (2004) Control of mycotoxins in storage and techniques for their decontamination. In: Magan N, Olsen M. (Eds.) Mycotoxins in Food: Detection and Control. Cambridge, England: Woodhead Publishing Limited and CRC Press, 190-223.

Venâncio, A. & Paterson R (2007) The challenge of mycotoxins. In: McElhatton A., Marshall R. (eds.). Food Safety - A Practical and Case Study Approach. Reykjavík, Iceland: Springer, 26-49.

Wielogorska, E., Ahmed, Y., Meneely, J., Graham, W. G., Elliott, C. T., & Gilmore, B. F. (2019). A holistic study to understand the detoxification of mycotoxins in maize and impact on its molecular integrity using cold atmospheric plasma treatment. Food chemistry, 301:125281.

Chapter 2. General Introduction

2.1 The food irradiation technology

2.1.1 Introduction

Radiation is energy that originates from a source and that travels through some material or through space. Light, heat and sound are types of radiation (Satin, 1996). The forms of radiation are commonly classified according to the wave frequency, for example, radio waves, microwave, infrared, visible light, ultra-violet radiation, X-rays and γ-rays represented in the electromagnetic spectrum (Figure 2.1). The electromagnetic spectrum is also divided in two types of radiation: non-ionizing radiation and ionizing radiation. The radiation discussed in this thesis is of the ionizing type. Ionizing radiation, due to their high energy contents, are used as a source of radiation capable of removing electrons from atoms or breaking molecules chemical bounds. Ionizing radiation is produced by unstable atoms that have an excess of energy or mass or both. To reach stability, these atoms, give off electrons or emitte the excess of energy or mass.

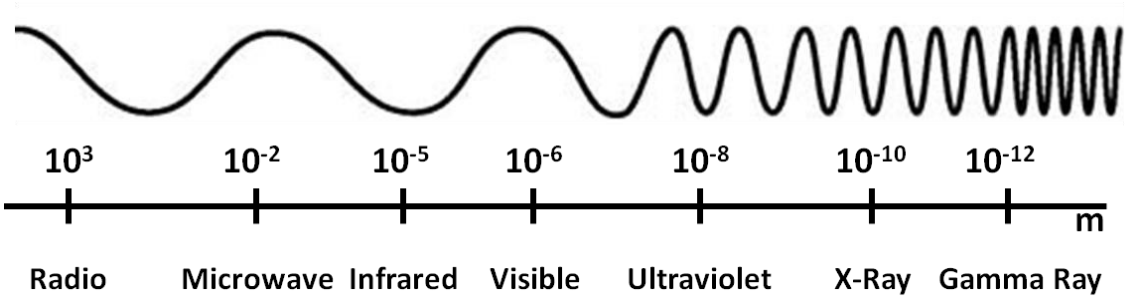

Figure 2.1 - The electromagnetic spectrum (Adapted from Satin (1996)).

The irradiation of food is a process where food is exposed to ionizing energy, such as γ photons emitted by cobalt-60 (^{60}Co) (or much infrequently by cesium-137) radioisotopes, X-rays generated by machines operated below a nominal energy of 5 MeV, and accelerated electrons generated by machines operated below a nominal energy of 10 MeV (Farkas and Mohácsi-Farkas, 2011). Only these sources can be used for food irradiation since the energies emitted by them are too low to induce radioactivity on any exposed material.

Food irradiation using ^{60}Co is currently the preferred method because it has a deeper penetration capacity that enables the treatment of materials with less handling. Nonetheless, irradiators using ^{60}Co must be recalibrated on a monthly basis because of the continuing decay and concomitant loss of radioactive energy of this isotope (Prado, 2005). Gamma radiation can also be sourced by an isotope of cesium (^{137}Cs). In this case, this material is obtained by reprocessing and by extracting spent nuclear fuel from nuclear reactors. This fact has brought much criticism from nuclear opponents who claim that food irradiation was simply invented as a way to eliminate nuclear waste (Satin, 1996). As a result, ^{137}Cs irradiators represent an extremely small proportion of today's irradiators. In contrast, the utilization of X-rays and electron beams involves the use of electrical machine sources of energy. An obvious advantage of such systems is that these systems can be switched on and off similar to a light bulb and are in no way related to the nuclear industry (Satin, 1996). Nonetheless, X-rays have low energetic efficiency, given that only 3-5% of the energy is converted to radiation, and electron beams have a limited penetration ability (Prado, 2005). Typically, the penetration power of electron beams is only suitable for materials with a thickness of no more than 5 to 10 cm (Satin, 1996). However, for certain uses, electron beams have proven to be extremely practical primarily for treating food surfaces, meat and fruits.

2.1.2 Action mechanism

Living cells are inactivated when exposed to factors that substantially change their cellular structure or physiological functions. Lethal structural damages include DNA strand breakage, cell membrane rupture or mechanical damage to cell walls (Lado and Yousef, 2002). During the irradiation of food, DNA is strongly damaged; therefore, primarily by this mechanism, microorganisms, insect gametes, and plant meristems are prevented from reproducing (Farkas, 2006). DNA damage may result from a direct action of the ionizing radiation or from an indirect action of the oxidative radicals that originated from the radiolysis of cellular water (Farkas, 2006).

The radiolysis of water takes approximately 10^{-6} seconds to occur. When water is irradiated by ionizing radiation, water molecules undergo a breakdown sequence that forms several radiolysis products that are extremely reactive with other chemical substances (Figure 2.2). The primary reactions that occur are the ionization and excitation of water molecules. Ionization causes the splitting of water molecules into positively charged water radicals (H_2O^+) and negative free solvated electrons (e^-); excitation causes the formation of excited water molecules ($H_2O^\cdot$) (Le Caër, 2011).

Then, various reactions occur, and hydroxyl radicals (OH·) and hydrogen radical (H·) are predominantly generated. These entities are highly reactive; OH· is a strong oxidant, and H· a strong reducer. At the end of the process, due to various recombination and cross-combination reactions, the following reactive species are present: e^-_{aq}, H·, HO·, HO_2·, OH^-, H_3O^+, H_2 and H_2O_2.

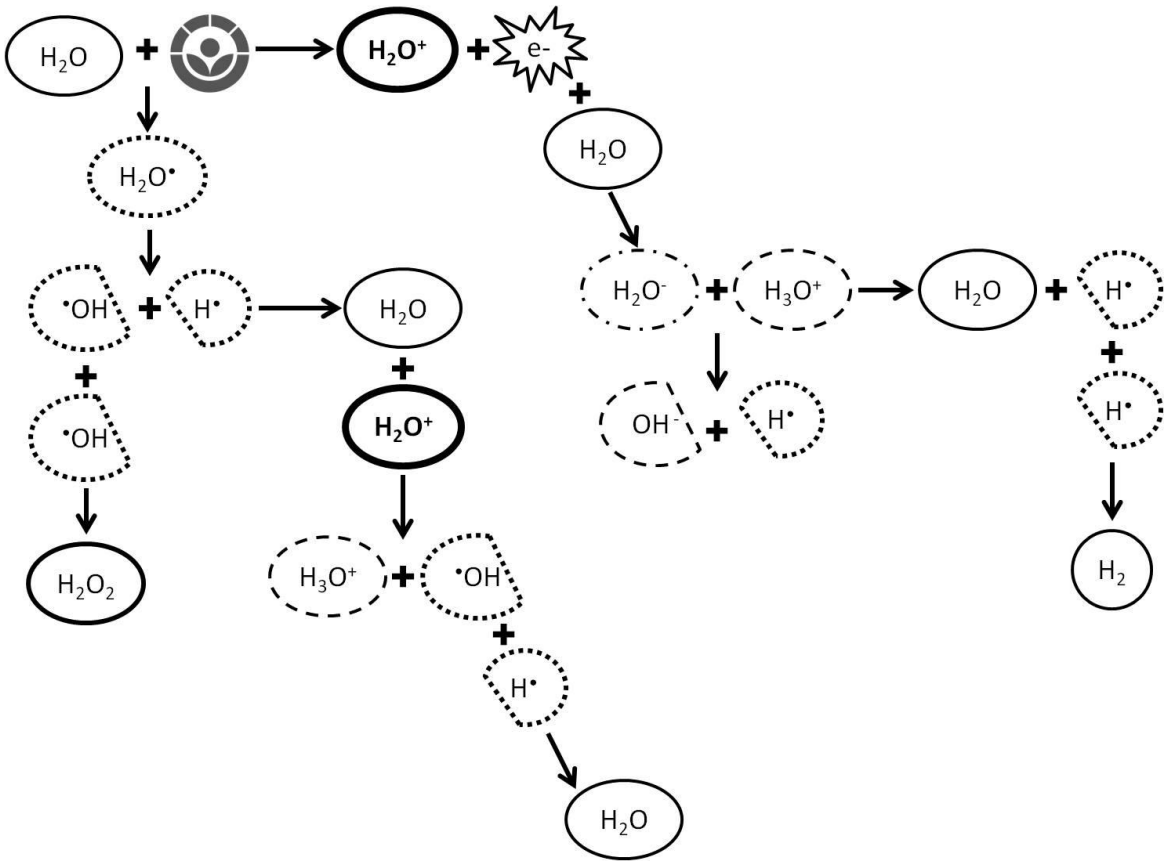

Figure 2.2 - Reactions occurring during water radiolysis (adapted from de Campos and others (2004)).

Thus, these reactive species are free to react with any component present in the cell cytoplasm. The hydroxyl radicals remove hydrogen atoms from sugar and from the bases of DNA strands (Lado and Yousef, 2002). The other free radicals will also attack and break down organic molecules (Stepanik *et al.*, 2007). Potentially lethal DNA lesions are randomly scattered throughout the cell population during ionizing. The cells that are unable to repair their radiation-damaged DNA die (Lado and Yousef, 2002). Differences in radiation sensitivities among microorganisms are related to differences in their chemical and physical structures and in their ability to recover from radiation injury (Farkas, 2006). In general, the sensitivity of organisms to radiation increases with their complexity. Thus, the radiation doses required to achieve effective inactivation usually increase as follows: insects < parasites < molds and yeasts < vegetative (non-spore-forming) bacteria < spore-

forming bacteria < viruses. Therefore, viruses are the most resistant to destruction by irradiation, and insects and parasites are the most sensitive. Moreover, spores (from bacteria and fungi) and cysts (from protozoa and parasites) are quite resistant to the effects of irradiation due to the fact that spores and cysts contain little DNA and are in highly stable resting states (Shea, 2000). Consequently, the irradiation energy required to control microorganisms on food varies according to the type of organism to be eliminated, according to their number and their developmental state. Other factors, such as the composition and moisture content of food, the fresh or frozen state of food, the temperature and levels of oxygen present during irradiation, may also influence the resistance of microorganisms to radiation, particularly in the case of vegetative cells (Farkas, 2006).

Notably, during the irradiation of microorganisms, sublethally injured cells are often subject to mutations, and this occurrence can be dangerous. Mutations can result in greater, less, or similar levels of virulence or pathogenicity from parent organisms. The induction of radiation-resistant microbial populations occurred when cultures were experimentally exposed to repeated cycles of radiation (Shea, 2000). However, mutations in microorganisms develop with any form of food processing (e.g., ultraviolet light, heat and drying). For this reason, it is extremely important to define safe irradiation doses to completely inactivate microorganisms (Shea, 2000). One parameter most often used to compare the susceptibility of microorganisms to irradiation is the D_{10} value, namely, the dose required to inactivate 90% of a microbial population.

2.1.3 Nutritional and organoleptic adequacy

All forms of food processing affect their nutritional organoleptic proprieties, and irradiation is no exception. At doses below 1 kGy, the nutritional losses are considered insignificant. In contrast, the irradiation of food much above 10 kGy degrades nutrients similar to thermal processes, such as cooking, canning, pasteurizing or blanching (Shea, 2000).

Vitamin loss is the largest nutritional concern associated with food irradiation, particularly when synergism between irradiation and heat (cooking) occurs. Additionally, avitaminosis may arise when the irradiated commodity represents a large proportion of the dietary source of an essential vitamin (Shea, 2000; Wood and Bruhn, 2000). Water-soluble vitamins, such as the B vitamins and vitamin C, are the most affected because these vitamins are oxidized during irradiation (Shea,

2000). Nonetheless, the loss of heat-sensitive vitamins with irradiation is considered no greater than that with conventional heat processing, and is often less. In addition, research indicates that vitamin losses can be minimized by irradiating in oxygen-free packaging or at cryogenic temperatures ranging from -20 °C to -40 °C (EC, 2003).

Carbohydrates are not significantly affected during irradiation at less than 10 kGy (Aziz and Mahrous, 2004). In contrast to other preservation methods (*e.g.*, pasteurization), protein denaturation is also not significant (Lado and Yousef, 2002). A change in the bioavailability or quantity of minerals or trace elements has not been identified as a result of irradiation. Fats can be oxidized, leading to rancidity and to odour or colour changes. In contrast, polyunsaturated fatty acids are not generally altered at low to medium irradiation doses (Shea, 2000; Wood and Bruhn, 2000). In addition to nutritional adequacy, organoleptic factors are also extremely important to the feasibility of food irradiation, particularly on fresh fruits and vegetables, which has been reviewed by Arvanitoyannis and colleagues (2009).

2.1.4 Safety and legislative aspects

Food irradiation is not as widespread as other conventional technologies due to the high costs of irradiation units and, in particular, because of its unwarranted association with nuclear radiation, which gives consumers a negative perception of its safety. As a result, the health and safety of irradiated foods have been more exhaustively studied than any other processed food (Satin, 1996). Since 1964, numerous international expert groups, which were jointly set up by the Food and Agriculture Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA), and the World Health Organization (WHO), along with the governments of different countries, collected and reviewed the scientific data produced over the years to consider the question of the wholesomeness of irradiated foods. The first international safety recommendation was presented in 1981, when an expert committee of experts from the joint WHO/FAO/IAEA considered that "... the irradiation of food up to an overall average dose of 10 kGy introduces no special nutritional or microbiological problems" (FAO/IAEA/WHO, 1981). In subsequent years, a number of expert authorities convened their own committees of experts to review and evaluate the data presented and no new concerns were raised. This lead to the FAO/WHO Codex Alimentarius Commission developed the Codex General Standard for Irradiated Foods and the Code of Practice for Radiation Processing of Food (Codex, 2003a; Codex, 2003b). These documents became widely

adopted internationally, and today, specific applications of food irradiation are approved by national legislations in over 55 (IAEA report, 2008) countries worldwide (Farkas and Mohácsi-Farkas, 2011).

Regarding the European Union, the implementation of food irradiation is far less developed when compared with other countries, such as the USA, Brazil or even China. In 2005, the European region represented only 4% of the world production of irradiated food (Kume *et al.*, 2009). The report from European commission in 2015 reveal that the irradiation of food and feeds is done in 26 irradiation facilities approved in the European Union and are located in 14 Member States and Norway.

This situation is primarily caused by the restrictive legislation in use. Directive 1999/2/EC, concerning the "approximation of the laws of the member states concerning food and food ingredients to be treated by ionizing radiation", and Directive 1999/3/EC, concerning "the establishment of a community list of foods and food ingredients treated with ionizing", are the main legislation pieces concerning food irradiation in the European community. In the first document, the European Parliament and the Council adopted a directive framework on the general and technical aspects of food and food ingredients treated with ionizing radiation (EU, 1999a). In the second document, legislators established a list of foodstuffs authorized for irradiation treatment (EU, 1999b). This list of foodstuffs is composed of three items: "dried aromatic herbs, spices and vegetable seasonings", and the permitted maximum overall average absorbed dose is 10 kGy. Since 2009, seven EU Member States have authorization to maintain their national regulation for food products, such as fruits and vegetables, including root vegetables; cereals, cereal flakes, and rice flour; spices and condiments; fish, shellfish; fresh meats, poultry, and frog legs; raw milk camembert; Arabic gum, casein/caseinates, and egg white; and blood products (EU, 2009).

2.1.5 Applications

From a practical point of view, three dose/application categories are typically considered when ionizing radiation is used to treat food: (i) a low-dose of up to 1 kGy, which is used to sprout inhibition and to delay ripening and/or insect disinfestations; (ii) a medium-dose from 1 to 10 kGy, which is used to reduce spoilage microorganisms, non-spore forming pathogens and/or to delay ripening; and (iii) a high-dose from 10 to 50 kGy, which is used to eliminate microorganisms to the point of sterility (Satin, 1996).

Five groups are most often discussed when considering the type of application and of the food irradiated. These five groups include the following: the disinfection of spices and dry vegetables, which represents 46% of all the irradiated products in the world; the sprout inhibition of garlic and potatoes, which represents 22%; disinfestations of grains and fruits, which represents 20%; the disinfection of meat and seafood, which represents 8%; and the treatment of other food items, such as health foods, mushrooms or honey, which represents 4% (Kume *et al.*, 2009). The total quantity of food irradiated in the world in 2005 was approximately 405,000 tons. A report from Kume and Todoriki summarized the quantities of irradiated foods in Asia, the EU, and the US in 2010 were estimated at 285 200, 9 300, and 103 000 tons, respectively. Compared with 2005, the quantity of irradiated foods was 100 000 tons higher in Asia and 10 000 tons higher in the US but 6 000 tons lower in the EU (Kume and Todoriki, 2013).

Irradiation is also used to eliminate or to reduce the presence of pathogenic microorganisms, such as *Aeromonas hydrophila*, *Arcobacter butzleri*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, yeasts, moulds and others, in several food matrices (Farkas, 1998; Stefanova *et al.*, 2010).

2.1.6 Advantages and disadvantages

The primary advantage of food irradiation is most likely the non-residual feature of the process. In contrast to chemical methods, which leave residual components that may have a negative impact on human health, irradiation is free of chemical wastes. An example is the quarantine treatments required to mitigate pests from fruits, vegetables and other plant-derived materials (Ferrier, 2010). Irradiation is an excellent substitute for the conventional fumigation in use. Additional advantages of irradiation technology include the possibility of irradiating packed food at its fresh and frozen state; controlling the hygienic quality of food by eliminating pathogenic and non-pathogenic microorganisms, insects and parasites; extending the shelf-life of foods, thus, increasing its supply and preserving the fresh-like quality of agricultural commodities because irradiation technology is a cold processing method (Stefanova *et al.*, 2010). Furthermore, irradiation may be considered environment-friendly because this method does not consume water and has lower electrical energy demands than other food preservative methods.

Concerning the disadvantages of this method, first, irradiation cannot be applied to all types of foods. Some fruits, such as pears and plums, milk and dairy products are untreatable by irradiation

because these products lose some important sensory and quality properties (Stefanova *et al.*, 2010). In other cases, irradiation can originate minor changes in the nutritional and organoleptic characteristics of treated foods as mentioned above. One of the most relevant characteristic for foods is the reduction of the water- and fat-soluble vitamin contents; the production of off-flavours, lipids oxidation and changes in colour; the creation of oxidation compounds, such as aldehydes, ketones and alcohols; and the formation radiolytic products, particularly 2-alkylcyclobutanones, which are suspected to be toxic (Stefanova *et al.*, 2010). Nonetheless, most of these changes are also induced by traditional food preservative methods, such as cooking, canning, pickling, freezing and drying.

The fact that food irradiation can cause the mutation of pathogenic microorganisms and could create new resistant strains can also be perceived as a disadvantage. However, the risks posed by this occurrence are minimal because safe radiation doses and the required process steps are scientifically settled before any commercial application to avoid this occurrence. A further disadvantage of food irradiation is that this method can be globally more expensive than other preservative methods due to the upfront costs of food irradiation facilities. The poor acceptance of these products by consumers because of unwarranted fears that associate food irradiation to the nuclear technology is also an opposing threat. Nevertheless, consumers trust on this technology can be changed through education, provided that stakeholders disseminate conscious and scientifically rigorous information concerning the subject.

2.2 Irradiation to control mould growth and mycotoxins

2.2.1 The mould and mycotoxin issue

Filamentous fungi are a wide group of eukaryotic microorganisms that are associated with an enormous diversity of habitats, and among which, many are saprophytes, which are responsible for the deterioration of agricultural cultures and food. Saprophytes may be responsible for the decay of commodities at a pre-harvest level; however, this group of organisms may also be responsible for the deterioration of fresh and processed foods, causing their rejection due to the visible development of moulds on these products. In addition, some specific fungal species growing on agricultural commodities may produce mycotoxins. Mycotoxins are a hidden face of food mould

contamination because mycotoxins remain on products well beyond the life cycle of the fungi. Moreover, mycotoxins are extremely stable and moderately heat resistant compounds that remain almost intact after food processing (Bullerman and Bianchini, 2007).

Mycotoxins are fungal secondary metabolites found in many plant foodstuffs, particularly in cereals, fruits, nuts, kernels, seeds and fodder, and are toxic to humans and animals when ingested or inhaled. The most relevant mycotoxins regarding food safety are aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and trichothecenes. These mycotoxins are produced by some species from the genera *Aspergillus*, *Penicillium* and *Fusarium* and have multiple and combined toxic characteristics. These mycotoxins may be carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressive and/or estrogenic (Paterson and Lima, 2010).

Typically, aflatoxins (AFIs) are the most well recognized mycotoxins. AFIs are highly carcinogenic and hepatotoxic (Williams *et al.*, 2004) and are primarily found in peanuts, maize, nuts, spices and in milk (where this mycotoxin occurs as aflatoxin M_1). Ochratoxin A (OTA) is primarily known for its nephrotoxicity; however, OTA is also carcinogenic to experimental animals (Pfohl-Leszkowicz and Manderville, 2007). OTA is mostly found in cereals and in cereal-based products; however, it also occurs in coffee beans, nuts, spices, raisins and in red wine (Jørgensen, 2005). Patulin is primarily associated with fresh fruits and vegetables. Apples, apple juices and purees are the main dietary source of this mycotoxin. Patulin is neurotoxic, immunosuppressive, genotoxic and teratogenic (Moake *et al.*, 2005). Fumonisins, which primarily occur in maize and in maize-based food products, appear to be related to an increased incidence of esophageal and liver cancer in humans and are experimentally associated with leukoencephalomalacia in horses and with pulmonary edema syndrome in pigs (Voss *et al.*, 2007). Zearalenone (ZEA) is estrogenic and interferes with the reproductive system of animals, even if ZEA has a relatively low acute toxicity (Zinedine *et al.*, 2007). Similar to fumonisins, ZEA is primarily associated with maize and with maize-based food products. Trichothecenes are a large group of structurally related compounds. The most relevant trichothecenes for food safety include T-2 toxin, HT-2 toxin, deoxynivalenol (DON), 3- and 15-acetyldeoxynivalenol (ADON) and nivalenol (NIV) (Foroud and Eudes, 2009). These compounds are primarily found in cereal grains and are extremely cytotoxic to mammalian cells, initiating a wide range of toxic effects, such as digestive disorders, followed by diarrhea and vomiting (Foroud and Eudes, 2009).

Therefore, if the presence of mycotoxins in food and feed is not properly controlled, mycotoxins may pose important risks to public health. At low levels, mycotoxins may cause the suppression of immune functions and decrease resistance to infections in individuals. In acute situations, mycotoxins may cause the development of tumours and of chronic diseases in vital organs or high morbidity and premature death among humans and animals (Peraica *et al.*, 1999). Additionally, mycotoxins are also responsible for major economic losses at all levels of the food-production chain. These losses are primarily associated with the rejection and destruction of contaminated materials and with expenses incurred to the implementation of good post-harvested storage conditions, analyses and treatments to guarantee low levels of mycotoxins.

To avoid the introduction of most contaminated products into the food chain, the presence of mycotoxins in certain agricultural commodities and finished food and feeds are regulated by statutory levels in many countries throughout the world (Van Egmond *et al.*, 2007). Additionally, a great diversity of preventive and corrective measures can be applied to control the problem. The preventive measures may include HACCP integrated systems, which involve strategies for prevention at pre- and post-harvested level, good manufacturing practices and quality control. The corrective measures include several physical, chemical and biological decontamination techniques that promote the elimination of the contaminated fraction or that counteract the toxic effects of mycotoxins (Stoev, 2013). The following topics underlines the importance of how food irradiation can contribute to this purpose.

2.2.2 Irradiation to control mould growth

As already mentioned, moulds are one of the main causes of postharvest decay problems. The presence of moulds in food may result in not only a reduction in quality and quantity but also the likely contamination with mycotoxins, that may cause important health problems. Irradiation can be used for the direct purpose of eliminating or reducing the presence of moulds and mould spores in foods and feed, improving their shelf-life and safety. Nonetheless, the application of this technology for other purposes can indirectly aid in the control of contamination with moulds and, subsequently, with mycotoxins. For example, it is well known that grains damaged by insects are more susceptible to mould development and mycotoxin accumulation because insects carry fungal spores and compromise the integrity of grains and plant tissues, facilitating the penetration and access to nutrients of fungal hyphae and, by consequence, fungal development (Jouany, 2007).

Thus, the elimination of insect pests from agricultural commodities through irradiation can indirectly have a positive preventing effect on the reduction of fungal contamination and mycotoxin levels in treated commodities. However, importantly, the irradiation of grains with disinfestations purposes must be combined with good grain handling practices, so that mycotoxin production can also be prevented during storage.

Concerning the direct action of irradiation on moulds associated with foods and feed, there are many studies in the existing literature that evaluate its effect, specifically on spices and on dried vegetables, which are the most irradiated food items worldwide. A study evaluating the effect of gamma-irradiation on the fungal load in red chillies was conducted by Iqbal and colleagues (2013) and concluded that irradiation doses of 6 kGy were sufficient to reduce the fungal load by 5-logs. Another study conducted on hot peppers observed reductions of 90, 99 and 100% of the fungal load with doses of 2, 4 and 6 kGy, respectively (Iqbal *et al.*, 2012). Similarly, Legnani and colleagues (2001) studied the effect of gamma-irradiation on the microbiological qualities of black pepper, red chili, oregano, rosemary and of sage. In this study, radiation doses of 5 kGy were found to be suitable to significantly reduce the load of moulds (between 65 and 80%); however, their complete elimination was only achieved with 10 kGy. *Aspergillus niger*, *Cladosporium* spp., *Penicillium* spp. and *Rhizopus* spp. were the most resistant to irradiation doses of 5 kGy. Similar results were obtained by Farag and colleagues (1995), who studied the effect of irradiation on marjoram, ginger and hot pepper. These authors reported the complete elimination of moulds and, specifically, of *Aspergillus flavus* (a producer of aflatoxins) with a radiation dose of 10 kGy. Coriander, cumin, turmeric and chili were also submitted to irradiation experiments by Alam and colleagues (1992), who obtained D_{10} values for moulds that ranged from 0.71 to 2.14 kGy, depending on the spice studied. In this case, an irradiation dose of 5 kGy was considered sufficient to control fungal contamination because no moulds were detected in samples after 3- and 6-month storage periods.

As evident from the abovementioned studies in the literature, a substantial reduction of the fungal load in spices and in seasonings is most likely achievable with irradiation levels above 5 kGy. Also, high level of irradiation does not seem to affect the quality of the final products since no losses of flavour compounds, changes in volatile oil compositions and antioxidant properties were found at irradiation levels of 10 kGy or even 30 kGy by several researchers, as reviewed by Alam and Abrahem (2010). Thus, the irradiation of spices is widely used as an excellent substitute to fumigation with gases, such as ethylene, propylene oxide or methyl bromide, which leave chemical

residues (e.g., ethylene chlorohydrins and ethylene bromohydrin) that are suspected to be harmful. The dried nature of these products may be the factor that favours their greater resistance to the ionizing energy.

Concerning the irradiation of grains, pulses and seeds, the inactivation of moulds in rough rice and wheat through gamma-irradiation was reported by Wang and Yu (2010). In wheat, an irradiation dose of 3 kGy was found to be sufficient to reduce the presence of *Alternaria*, *Aspergillus* and *Fusarium* by 10-fold and to completely inactivate *Penicillium* and *Rhizopus* strains. In rice, the irradiation dose required to obtain the same effect was 5 kGy. After irradiation, the detection of moulds did not change significantly during storage periods of 6 and 12 months. Maity and colleagues (2008 ; 2009) also evaluated the effect of gamma-irradiation on the fungal diversity of rice seeds during storage periods up to 12 months. The growth of isolated fungi was found to be completely inhibited during this period with a 3 kGy dose, and no changes in the germination potential was noted with doses ranging from 2 to 4 kGy. Aziz and colleagues (2006b) conducted a research on the effect of gamma-irradiation on wheat, barley, maize and on sorghum and reported that fungi were completely inhibited by a dose of 5 kGy. In this study, aerobic spore-forming bacteria were found to be more resistant to a doses of 5 kGy radiation in comparison to moulds and other aerobic bacteria, leading the authors to conclude that a radiation dose of 10 kGy appears to be sufficient to improve the total hygiene of grains. Furthermore, no significant alteration of their nutritional constitutes was observed. In another study, Aziz and colleagues (2007) studied the control of *Fusarium* species on wheat, barley and maize seeds. *Fusarium* spp. were completely inhibited at 4.0 kGy on barley and at 6.0 kGy on wheat and maize. A 6 kGy dose could also completely inhibit the fungal population in several type of grains; however, 4.0 kGy only reduced the fungal load by 4-logs (Aziz *et al.*, 2006a). Aziz and Moussa (2004) also verified that gamma irradiation at a dose of 5 kGy inactivated the growth of moulds and subsequent mycotoxin formation in maize, chick-peas and in groundnut seeds. In contrast, in ground and whole maize, D´Ovidio and colleagues (2007) found that radiation doses of 30 and 100 kGy, respectively, were required for the complete inactivation of *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp.; and a major reduction in the mould load was also observed with lower doses, at 10 and 30 kGy, respectively. This work does not corroborate with previous studies that also evaluated the effect of irradiation on maize. For instance, Webb and colleagues (1959) found that radiation doses between 2.5 and 10 kGy were sufficient to prevent the growth of moulds in maize during storage and that the required dose increased with maize moisture content. In this study, moulds that required higher

moisture levels to grow were also found to be more resistant to gamma radiation. Similarly, Ferreira-Castro and colleagues (2007) reported that *Fusarium verticillioides* survival percentages on maize irradiated with 2, 5 and 10 kGy were 36, 6 and 0%, respectively; thus, a 5 kGy dose could substantially contribute to the avoidance of maize contamination with this species. A radiation dose of 1.5 kGy was also found to reduce the maize fungal population by 90 and 99%, depending on the maize moisture content, which was 35 and 39%, respectively, in this case (Poisson *et al.*, 1971).

The effect of irradiation on sesame grains was studied by Akueche and colleagues (2012). An irradiation dose of 3 kGy inactivated 75% of the moulds present, and doses above 6 kGy completely eliminated any fungal development. Cowpea bean grains were studied by Lima and colleagues (2011). Irradiation has been shown to be an efficient method to preserve this variety of bean for 6 months. Their results indicated that *A. niger* was eliminated with 1.0 kGy; *Aspergillus ochraceus*, with 2.5 kGy, *A. flavus*, with 5 kGy; and fungi from the genera *Rhizopus*, *Penicillium* and *Fusarium*, with a 10 kGy radiation dose. The effect of irradiation on Lotus seed was studied by Bhat and colleagues (2010). Irradiation with a 7.5 kGy dose substantially reduced fungal contamination, and a 10 kGy dose completely eliminated the presence of fungi. Also, in this study, contaminant yeasts were found to be the most resistant to irradiation because some survived to 10 kGy treatments. Kottapalli and colleagues (2003) used electron beam irradiation to reduce malting barley infection with *Fusarium*, observing that doses higher than 4 kGy effectively reduced the fungal infection without affecting its germination. Zeinab and colleagues (2001) reported that a 6 kGy dose could completely inhibit the fungal population of *Nigella sativa* in black cumin seeds.

Concerning feed, gamma-irradiation was used in order to extend the shelf-life of hydrated feed for fish farming without using preservatives (Kim *et al.*, 2012). A 5 kGy dose was found to be enough to eliminate moulds. Ribeiro and colleagues (2009) studied the effect of gamma radiation on the mycoflora of poultry feed. The total elimination of mould viability was observed at 8 kGy; however, *Aspergillus parasiticus* and *A. flavus* were found to be the most resistant to irradiation, which may pose some safety concerns because these species are aflatoxin producers. Refai and colleagues (1996) studied the elimination of *A. ochraceus* from poultry feed concentrate using gamma radiation and concluded that a dose of 4 kGy could completely inhibit this species and the production of ochratoxin A. Similarly, El-Far and colleagues (1992) studied the inhibition of *A. flavus* in the poultry diet, reporting that no fungal growth and aflatoxin production was observed at a 6 kGy dose. In contrast, Paster and colleagues (1991) reported that irradiation doses of 7 to 10 kGy

delayed fungal development, particularly in feed grains with low moisture content, but did not completely prevent the mouldiness of this product.

Another application of irradiation technology focuses on the treatment of fresh fruits and vegetables to increase their shelf life. Aziz and Moussa (2002) studied the effect of gamma radiation on ten different types of fruits refrigerated at <10 °C for 28 days. The initial viable mould population ranged from 4.8×10^4 to 6.8×10^5 CFU/g (colonies formation units per gram). When fruits were exposed to 1.5 and 3.5 kGy doses, the initial mould population was reduced on average by 2- and 3-logs, respectively. Nonetheless, there is a gap in the literature regarding on studies which focus on the evaluation of the quality and physico-chemical parameters of irradiated fruits. Another study from Mostafavi and colleagues (2012) demonstrated the influence of low irradiation doses on apple preservation. The results demonstrated that the germination of spores from *Penicillium expansum* was completely inhibited with a 0.6 kGy dose and that doses between 0.3 and 0.6 kGy, which were combined with storage at 1 °C, could avoid the development of rot for nine months without significantly changing the phenolic contents, antioxidant activity, firmness, weight loss and total soluble solids of the treated apples. Ben-Arie and Barkai-Golan (1969) showed that the inactivation of *P. expansum* could also be achieved in pears using a treatment with hot water (47 °C during 7 min), followed by gamma irradiation at 0.5 kGy. The same treatment did not prevent the rotting of fruits inoculated with *Botrytis cinerea* and with *Alternaria tenuis*; however, a delay in disease development was observed. Kim and colleagues (2010) studied the effect of gamma radiation on peach. A dose of 1 kGy inactivated *B. cinerea*, *P. expansum*, *Rhizopus stolonifer* var. *stolonifer* and *Monilinia fructicola* in peach pulp, and the calculated D_{10} values for each species were 0.15, 0.23, 0.16 and 0.16 kGy, respectively. El-Samahy and colleagues (2000) studied the microbiological and chemical properties of irradiated mango. An increased reduction in the fungal population on mango fruits was observed with irradiation doses increasing from 0.5 to 1.5 kGy. The ideal treatment reported by the authors involved dipping mangos in hot water (55° C for 5 min) and irradiating with 1 kGy. With these conditions, the ripening of mangos could be delayed for 50 days at 12 °C without significantly changing their nutritive and sensory properties. A similar treatment for tomatoes was found to reduce *B. cinerea*, *R. stolonifer* and *Alternaria alternata* decay (Barkai-Golan *et al.*, 1993). In this case, a hot water dip at 50 °C for 2 min and an irradiation of 1 kGy were required. Nonetheless, the treatment caused a more rapid softening of fruits. The shelf-life of strawberries could also be extended for 2 to 3 days with irradiation doses of 2 to 3 kGy when preserved at 23 °C and for 4 to 8 days when preserved at 8 °C (Shibaba *et al.*, 1967). In this work,

lethal doses for two different strains of *B. cinerea* were found at 9.7 and 5.4 kGy, showing that resistance to irradiation may vary within the same species. Under the same experimental conditions, lethal doses for *Penicillium* sp. and *Aspergillus* sp. were of 2-2.5 and 4 kGy, respectively. Ladaniya and colleagues (2003) studied the influence of gamma radiation on citrus fruits and concluded that radiation treatments could not reduce the decay of these products. Positive effects were only observed in mandarins. In this case, *Penicillium* rot could be delayed with a radiation dose of 1.5 kGy, whereas no significant changes in fruit firmness and in juice content were observed. However, total soluble solids increased, whereas acidity and vitamin C content decreased. In oranges and in limes, radiation treatments considerably changed the texture and appearance of fruits. In fact, in the literature, the irradiation of citrus fruit resulted in injuries that led to the development of black buttons on the skin and, later, to the development of rot (Maxie *et al.*, 1964; O'Mahony *et al.*, 1985). Macfarlane and Roberts (1968) also concluded that irradiation is satisfactory for the disinfection of orange fly because the required doses are extremely low; however, this method is not feasible for mould control because of injury provoked by the high doses required. According to these authors, an irradiation dose of 0.3 kGy should not be exceeded for citrus fruit treatment. A review of the impact of ionizing radiation on fruits and vegetables can be consulted for more information concerning the subject (Arvanitoyannis *et al.*, 2009).

Concerning dried fruits, the irradiation of peanuts was evaluated by De Camargo and colleagues (2012), who concluded that an irradiation level of 5.2 kGy was suitable to prevent the growth of aflatoxigenic fungi without significantly affecting their polyunsaturated fatty acid and polyphenol contents. This observation is in accordance with previous research conducted by Chiou and colleagues (1990), which suggests that radiation levels of 2.5 and 5.0 kGy were effective in retarding the growth of *A. parasiticus* and in reducing the native mold population of peanuts, respectively. Additionally, Hilmy and colleagues (1995) reported that 3.0 and 5.0 kGy could completely inhibit *A. flavus* growth on peanut and on nutmeg meal, respectively. In contrast, Prado and colleagues (2003; 2006) observed only a reduction in fungal infections on peanuts irradiated with 5 kGy and its total elimination only with 10 kGy. The effect of gamma-irradiation on the quality of walnuts was also studied (Wilson-Kakashita *et al.*, 1995). The mould count in walnuts was significantly reduced with irradiation doses above 5 kGy, which were shown to be more effective than propylene oxide treatments. Walnut lipid contents did not change with gamma-radiation treatments; however, a small decrease in iodine contents and an increase in peroxides values were observed. Similarly, Emam and others (1994) compared the irradiation of semidry date fruits with

methyl bromide treatments and concluded that irradiation at 3 kGy was more effective at inhibiting the growth of fungi, despite causing a significant loss in weights of dates.

Concerning the direct effect of ionizing radiation on fungal species, Ribeiro and colleagues (2011) studied the effect of gamma radiation (at 2 kGy) on *A. flavus* and on *A. ochraceus*. Irradiated strains showed different colour and slight differences in the sizes of stipes, metulae and conidia compared with the same non-irradiated strains. The authors also observed that irradiated strains produced two times more mycotoxins than control strains. A similar effect was observed by other researchers. Irradiated strains of *A. flavus*, *A. parasiticus*, *A. niger* and *A. ochraceus* also produced more AFB_1 or OTA then non-irradiated strains (Schindler *et al.*, 1980; Ribeiro *et al.*, 2009). However, this finding is not a consensual observation because other researchers reported that irradiated spores of *A. parasiticus* did not produce more AFB_1 than non-irradiated spores on rice (Sharwia *et al.*, 1990). For these reasons, it is still recommended that appropriate storage practices are implemented after the irradiation process in order to avoid the proliferation of toxigenic fungi and the associated production of mycotoxins.

Maity and colleagues (2011) evaluated the effects of gamma radiation on fungi isolated from rice. The responses of individual cultures of *A. alternata*, *A. flavus*, *Trichoderma viride* and *Curvularia geniculata* submitted to irradiation doses up to 4.2 kGy were evaluated. The inactivation of fungal viability was achieved at 2 and 3 kGy for *T. viride* and for *A. flavus*, respectively, and at 2.5 kGy for *A. alternata* and for *C. geniculata*. Additionally, the following major changes in fungal morphology were observed: a reduction in colony radial growth, a reduction in the germination tube length and diameter, and, in some cases, multi-germ tube formation. Similarly, Saleh and colleagues (1988) reported the gamma radiation doses required to inactivate some fungal species. In this case, dematiaceous fungi with melanized mycelia and conidia, such as *Alternaria*, *Curvularia* and *Cladosporium*, were found to be more resistant to gamma radiation, and the reported inactivation doses were 11.5-13.9, 17-20 and 6.0-6.5 kGy, respectively, for each of these fungi. In contrast, *A. niger*, *A. fumigatus*, *A. parasiticus*, *Fusarium solani* and *Penicillium* sp. were inactivated by doses of 1.7-2.5 kGy, and *A. flavus* was inactivated by doses of 2.5-3.0 kGy. The effect of gamma radiation on *A. flavus* and on *A. parasiticus* was also studied by Kume and colleagues (1989). In humid conditions, the authors obtained D_{10} values of approximately 0.27-0.29 kGy for both species, whereas in dry conditions, the doses required to reduce the load by 1-log were almost double (0.5-0.6 kGy). This study showed that dry spores were more resistant to gamma radiation. The same observation was also reported by other authors (Poisson *et al.*, 1971; Chang and Lee, 1980;

LebaiJuri *et al.*, 1995). Gumus and colleagues (2008) studied two heat-resistant moulds, *Aspergillus fumigatus* and *Paecilomyces variotii*, which were isolated from margarine. The average D_{10} value obtained for *A. fumigatus* was 1.08 kGy, whereas this value was of 0.59 kGy for *P. variotii*. The complete inactivation of *P. variotii* was achieved with 5 kGy, whereas a 7 kGy dose was required for *A. fumigatus*. The radiation sensitivities of *A. flavus*, *A. niger*, *Penicillium* sp., *B. cinerea* and *R. stolonifer* were also evaluated by Chang and Lee (1980). *Aspergillus flavus*, *A. niger* and *Penicillium* sp. presented a similar D_{10} value (0.3-0.35 kGy), whereas *B. cinerea* and *R. stolonifer* showed D_{10} values of 0.55 and 1.0 kGy, respectively. Malla and colleagues (1967) reported that *Penicillium viridicatum* strains were more sensitive to gamma radiation than those strains of *A. flavus*. Their total inhibition was obtained with a dose of 2 kGy. Authors also found that spores of strains with 6-month-old cultures were more susceptible to irradiation than 3-week-old cultures. Aziz and Moussa (2004) reported D_{10} values in saline solutions for *A. alutaceus*, *A. flavus* and for *F. verticillioides* of 0.36, 0.52 and of 0.87 kGy, respectively. Similarly, Geweely and Nawar (2006) evaluated the effect of gamma radiation on *Alternaria tenuissima*, *B. cinerea*, *P. expansum* and on *Stemphylium botryosum*, which are pathogenic to pears. *Botrytis cinerea* and *P. expansum* were found to be more radiosensitive, with complete inactivation by a 1.0 kGy dose, whereas *A. tenuissima and S. botryosum* were only inactivated by a dose of 3.0 kGy. LebaiJuri and colleagues (1995) reported D_{10} values for many species that are pathogenic to plants. For *Fusarium* species, radiation doses required to reduce the load by 1-log were between 0.31-0.71 kGy. The most radioresistant species was found to be *F. moniliforme*, whereas *F. oxysporum* showed the greatest potential for recovery after irradiation. These species are known to produce several mycotoxins, and their resistance to irradiation may raise some concerns for fungal development after treatments if the radiation doses used are not sufficient to completely eliminate them.

As observed, the radiosensitivity of a specific fungal species may be substantially different, depending on the works consulted. These differences may result from innumerous factors whose influence has not been as extensively studied as, for instance, the simple effect of radiation on the fungal load in specific food matrices. Such factors may include moisture contents of spores or commodities, the age of spores, the nature of substrate in which radiation treatments are performed, the existence of periods of refrigeration or of heating before or after treatments, and the combinations of radiation with other technologies. These factors are summarized in Figure 2.3. In general, dried spores are considered more resistant to radiation, as previously discussed; however, commodities with high moisture content may favour fungal recovery after irradiation if

inactivation is not complete. The effectiveness of irradiation also depends on the age of the spores. Spores more than one month old and less than five months old were found to be substantially more resistant to gamma radiation (Poisson *et al.*, 1971). These authors also observed that radiosensitivity increased with moderate heating (40-50 °C) before irradiation and with fungicide treatments (Poisson *et al.*, 1971). When experiments were conducted in inert supports rather than on nutritive media, the radiosensitivity of spores was also higher. Münzner (1969) reported several additional observations, namely, that the recovery of irradiated spores was favoured on optimal nutritive media by optimal incubation temperatures and that actively growing cultures of moulds were more sensitive to radiation than older cultures. A substantial difference in radiosensitivity may also be observed, depending on the strains tested. For example, for two different *B. cinerea* strains, gamma radiation lethal doses were found to be extremely different (9.7 and 5.4 kGy) (Shibaba *et al.*, 1967). Thus, the comparison of the susceptibility of fungal species to irradiation should be performed with care because numerous factors may change their susceptibility, particularly when the irradiation of natural substrates is involved.

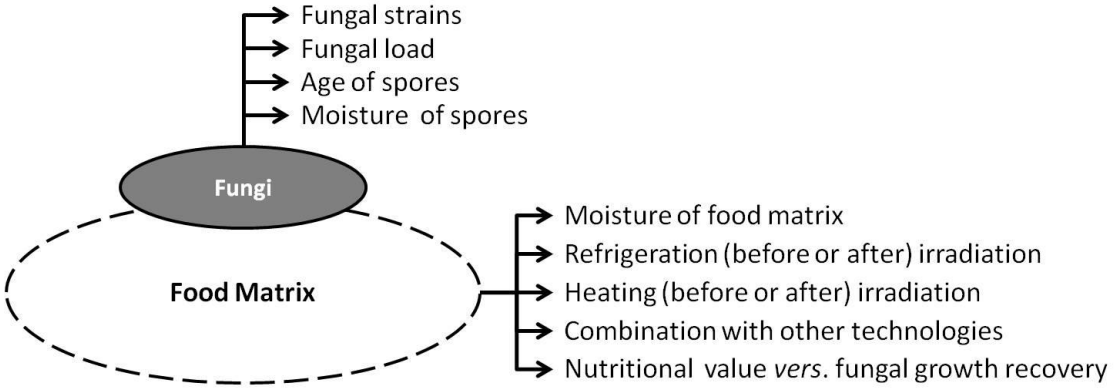

Figure 2.3 - Factors that may influence the effectiveness of the spore irradiation process in food.

2.2.3 Irradiation to control mycotoxins

Due to mycotoxins being highly toxic, it is imperative that their levels in food and feed are reduced as low as technologically feasible. Ionizing radiation is one among many technologies that can contribute to this purpose. As already observed, first, its action on mould viability contributes to the avoidance of fungal development and, consequently, to the production of mycotoxins in commodities. Second, since ionizing radiation can have a direct action on mycotoxins under specific conditions, contributing to their elimination, this subject has been widely investigated,

particularly concerning AFB_1. Nonetheless, the available literature is not always in agreement due to some reports claiming substantial reductions in some mycotoxins through the action of irradiation, whereas others claiming that irradiation is not effective at all. Next, the main achievements reported for this subject will be reviewed.

One of the first reports studied the effect of gamma radiation on dried AFB_1 and on AFG_1 spotted on TLC (thin layer chromatography) silica plates and solubilized in phosphate solutions (Frank and Grunewald, 1970). The authors observed that dried aflatoxins (AFIs) were extremely radioresistant, whereas in solution, AFIs were sensitive to irradiation doses of 1 and 2.5 kGy, with degradation of approximately 90%. Later, Van Dyck and colleagues (1982) studied the radiosensitivity of AFB_1 in water solutions and showed that an identical irradiation dose could eliminate only 34% of the mycotoxin. Furthermore, the authors observed that increasing doses of radiation could destroy increasing amounts of AFB_1 until its total destruction at 20 kGy. Using a test with *Salmonella typhimurium* TA 98, these authors also demonstrated that AFB_1 mutagenicity decreased with increasing doses of gamma radiation. Nonetheless, when the concentration of AFB_1 was increased 50 times, the effect of gamma rays was found to be substantially lower, indicating that the mycotoxin concentration is a determinant factor to achieve satisfactory elimination percentages. Similar observations were also reported by Mutluer and Erkoç (1987), who studied the effect of gamma radiation on AFB_1, AFB_2, AFG_1 and AFG_2 in solutions of water/DMSO. AFB_1 was found to be the most radiosensitive, and AFB_2 was found to be the most resistant. Irradiation doses of 5, 10 and 20 kGy were studied. AFB_1 and AFG_1 were almost completely eliminated at 5 kGy, remaining only 5 and 10% in solution, respectively. In contrast, 90% of AFB_2 and 77% of AFG_2 were resistant to the same radiation dose. With 10 and 20 kGy, AFB_1 could be completely eliminated; however, AFG_1 was only completely eliminated with the 20 kGy dose. Patel and colleagues (1989) also used this approach and investigated the synergetic effects between hydrogen peroxide and gamma radiation on the elimination of AFB_1 in aqueous solutions. In the presence of 5% hydrogen peroxide (H_2O_2), the authors observed that a 1 kGy dose could eliminate 50 μg of AFB_1 and that a 4 kGy dose could eliminate 100 μg. The mycotoxin mutagenicity was also found to be completely lost with 4 kGy in the presence of 5% H_2O_2 using an Ames microsomal test with *S. typhimurium* TA100. Additionally, these authors confirmed that artificially contaminated groundnuts could be detoxified using this strategy due to reductions of AFB_1 from 14 to 3 μg/g and from 6.3 to 1.7 μg/g in treated samples. Despite the observation that AFIs were degraded by gamma radiation, no degradation products were identified, although their presence in samples was observed using TLC in some

cases. Wang and colleagues (2011) approached this subject using gamma irradiated solutions of AFB_1 in methanol/water. Twenty different radiolytic products were obtained; however, only seven products were tentatively identified. Using the quantitative structure–activity relation, six of the seven radiolytic products were considered less toxic than AFB_1 because these products lost the double bond in the terminal furan ring, which is the determinant one for aflatoxin toxicity. Using chicken embryos, the lethality of aflatoxins was observed to decrease with increasing gamma-irradiation doses (Ogbadu and Bassir, 1979).

As observed in various studies, AFB_1 in solution can be effectively degraded and detoxified using gamma radiation. Most likely, this degradation is mediated by the oxidative radicals that originated from water radiolysis because dried AFB_1 is more resistant to radiation than AFB_1 in solution (Frank and Grunewald, 1970). This possibility can be a limiting factor when radiation is applied to food and feed products with the purpose of eliminating mycotoxins. Thus, studies in real matrices are required to evaluate the true effect of irradiation on mycotoxins.

Iqbal and colleagues (2013) evaluated the effect of gamma radiation on the reduction of AFIs in chillies and observed that levels of AFIs decreased with increasing irradiation doses (2, 4 and 6 kGy). The highest reductions obtained ranged from 81 to 91% and were achieved with a 6 kGy dose. In contrast, Akueche and colleagues (2012) did not observe any consistent reduction of AFIs and of OTA on irradiated sesame grains at doses ranging from 3 to 12 kGy; however, these authors obtained the lowest mycotoxin level on grains irradiated with a 15 kGy dose. Jalili and colleagues (2012) also studied the effect of gamma radiation on AFIs and OTA contents on pepper. The tested doses ranged from 5 to 30 kGy. The greatest reductions in mycotoxin levels (35 to 55%, depending on the mycotoxin) were observed in peppers with the highest moisture level (18%) and were irradiated at 30 kGy. Authors also observed that AFB_2 and AFG_2 were more radioresistant than AFB_1 and AFG_1. OTA was the most radiosensitive. In contrast, Hooshmand and Klopfenstein (1995) did not verify any reduction in AFB_1 in soybean, corn and wheat irradiated with doses up to 20 kGy at 9, 13 or 17% moisture content, respectively. Nonetheless, these authors verified significant reductions in DON and ZEA concentrations at doses of 10 and 20 kGy, respectively, and in T-2 toxin with 7.5, 10 and 20 kGy doses. With an irradiation dose of 10 kGy, the maximum allowable for food products, eliminations of 16% for T-2 toxin in wheat, of 33% for DON in soybeans, and of 25% for zearalenone in corn were observed, and with a 20 kGy dose, reductions were 20, 41 and 31%, respectively. The elimination of AFIs from yellow maize and from peanuts using gamma radiation was also studied (Farag *et al.*, 2004). The experiments conducted showed that gamma

radiation at a dose of 20 kGy could eliminate 76% of AFB_1 in yellow maize and 85% of AFB_1 in peanuts. Reductions of 83 to 97% were also observed for the other AFIs at identical conditions. Prado and others (2003) also studied gamma radiation effects on peanuts. Doses of 15 to 30 kGy were found to be enough to eliminate AFB_1 by 55 to 74%. Nonetheless, these authors did not observe any increased effect with increasing irradiation doses. In maize, Aquino and colleagues (2005) observed that an irradiation dose of 10 kGy could completely eliminate the presence of AFB_1 and of AFB_2 in samples. In contaminated feeds, Herzallah and colleagues (2008) found that AFB_1 and total AFL contents decreased by 43% and 40%, respectively, with an irradiation dose of 25 kGy.

Most of the literature that focus on the impact of radiation on mycotoxins address aflatoxins; however, other mycotoxins have also been studied. The effect of gamma radiation on patulin was studied in apple juice concentrate (Zegota *et al.*, 1988). With up to a 2.5 kGy dose, the elimination of patulin was partial and proportional to the irradiation doses. Beyond 2.5 kGy, patulin was completely eliminated. Similar results were obtained in an aqueous solution. At the tested conditions, irradiation did not change the titratable acidity, reducing sugars, carbonyl content or amino acid composition of juice. Yun and colleagues (2008) corroborated these results in apple juice because the researchers observed a reduction of 81% of patulin with a 3 kGy dose and almost total elimination with 5 kGy. These researchers also investigated the effect of irradiation on patulin in water and observed that a 1 kGy dose was sufficient to completely eliminate patulin. Nonetheless, it was also observed that organic acids, such as malic, lactic or ascorbic acid, and amino acids, such as serine, threonine or histidine, conferred a protective action on the radiolytic degradation of patulin.

Pure ochratoxin A, dissolved in methyl alcohol, was also tested and found to be stable, even at 75 kGy (Paster *et al.*, 1985). In contrast, OTA was found to be sensitive to irradiation in water and in other aqueous solution by Kostecki and colleagues (1991), who reported that up to 50% of OTA was decomposed after gamma-irradiation. Similar results were obtained by Deberghes and colleagues (1993), who reported that 50% of OTA in solution was eliminated with 2 and 3 kGy doses and that the elimination increased to 80% when 4 and 5 kGy doses were applied. Kumar and colleagues (2012) irradiated OTA in powder form, in aqueous and in methanolic solutions. In aqueous solutions 30, 79 and 93% of the OTA was eliminated with doses of 1, 2.5 and 5 kGy, respectively. Nonetheless, OTA was found to be more resistant to irradiation when dried or when in methanolic solution. With 10 kGy, only 24% of the OTA was eliminated in methanol, and almost

none disappeared in the powder form. The total elimination of OTA in feedstuffs was achieved with irradiation doses of 15 and 20 kGy in yellow corn and in soybeans, respectively, but not in cotton-seed cake and feed concentrates, for which the elimination reached only a maximum of 47% (Refai *et al.*, 1996). In green coffee beans, at a 10 kGy dose, OTA degradation increased with the moisture content of samples (Kumar *et al.*, 2012). Reductions of 5, 9, 20, 90 and 100% in initial amounts of OTA were observed in coffee beans with moisture contents of 9, 10, 12, 23% and 58%, respectively.

The irradiation effect on DON and on 3-acetyl deoxynivalenol (3-ADON) was tested by O'Neill and colleagues (1993) on maize in aqueous solution and in the dry state. These authors found that both mycotoxins were more sensitive to irradiation when in aqueous solutions than on maize. In aqueous solutions, both mycotoxins were completely destroyed by 50 kGy, and their breakdown began at 1 kGy and at 5 kGy for DON and for 3-ADON, respectively. When irradiated on maize, breakdown only began after 20 kGy. In the dry condition, both mycotoxins were stable to irradiation at 50 kGy. Using electron bean irradiation, Stepanik and colleagues (2007) also demonstrated a dose-dependent reduction of DON contents in wet distiller grains used as feed supplements. Reductions reached 47.5 to 75.5% at the highest doses (approx. 50 kGy). In contrast, the treatment of dry unprocessed wheat produced only a 17.6% reduction in the DON level at the highest dose, and the treatment was ineffective on dried distillers' grains. The effect of irradiation on *Fusarium* mycotoxins in wheat, flour and bread was also studied (Aziz *et al.*, 1997). DON, zearalenone (ZEA) and T-2 toxin concentrations were reduced with increasing doses of irradiation. All mycotoxins were completely eliminated with an 8 kGy dose, and approximately 80% could be eliminated with a 6 kGy dose. T-2 toxin was the most resistant to radiation, and bread prepared from 6 kGy wheat flour was contaminated with levels below 5 μg/kg.

Fumonisin B_1 (FB_1) was also investigated. D'Ovidio and colleagues (2007) studied the effect of irradiation on FB_1 in aqueous solutions and in corn. FB_1 in aqueous solutions was reduced by 99% using only 0.5 kGy; however, irradiation did not significantly reduce levels of this mycotoxin in whole and ground corn using irradiation doses up to 30 kGy. In contrast, Visconti and colleagues (1996) reported that a 15 kGy dose caused a decrease in fumonisin contents of approximately 20% in maize flour. Better elimination of FB_1 was obtained by Aziz and colleagues (2007) in wheat, maize and barley grains, and the application of a radiation dose of 5 kGy inactivated FB_1 by 97%, 87% and

100%, respectively, on these grains. A dose of 7 kGy was also found to be sufficient for the complete destruction of FB_1 in wheat and in maize.

In feedstuffs and feed samples, the influence of irradiation on *Penicillium* mycotoxins was studied by Aziz and Mattar (2007). Ten kGy eliminated citrinin contents up to 97.5% and eliminated ochratoxin A up to 78.5%. Patulin, cyclopiazonic acid and rubratoxin B were not detected after irradiating commodities with a 5.0 kGy dose. Considering the reviewed studies, in Figure 2.4, the factors that must be considered to apply the irradiation process to mycotoxins were summarised.

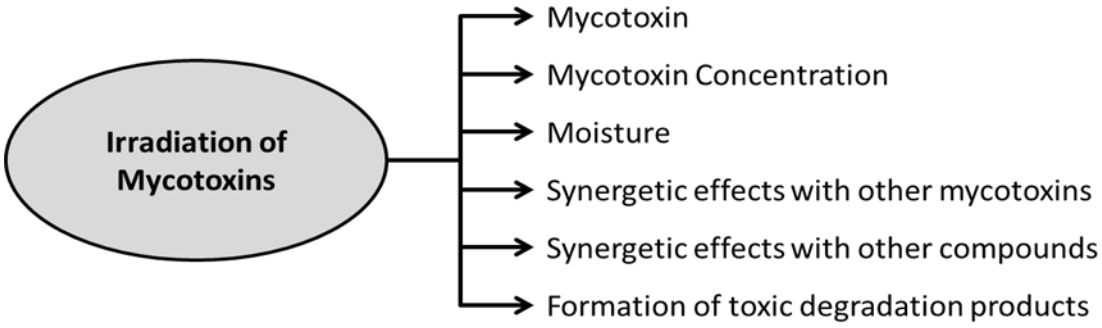

Figure 2.4 - Factors that may influence the effectiveness of the mycotoxin irradiation process.

2.3 Conclusions

Although there are several contrasting reports regarding the effect of gamma rays on fungi and mycotoxins in different foods, gamma irradiation can generally be considered to significantly improve the mycotoxicological safety of food and feed. Indeed, gamma irradiation has an inhibitory effect on mycotoxigenic fungi, inhibiting or delaying their development and, consequently, the production of mycotoxins, and under appropriate conditions, gamma irradiation can directly destroy mycotoxins. Nonetheless, irradiation should only be used in combination with good manufacturing and storage practices to prevent the proliferation of toxigenic fungi and the associated production of mycotoxins.

The following is a brief summary of the reviewed literature:

- The radiolytic process is influenced by many factors, such as absorbed doses, initial mycotoxin concentration or fungal load, the position in the irradiated system, the amount of moisture and/or the presence of other components on matrices.

- Fungi radiosensitivity also depends on strain characteristics, the moisture content of spores or commodities, spore age, commodity characteristics, the existence of periods of refrigeration or of heating before or after treatments, and on the combinations of radiation with other technologies. Fungi with melanized mycelia and spores are also more radioresistant. Commodities with higher moisture content may favour fungal recovery after irradiation if inactivation is not complete.
- The fungal load may be substantially reduced with irradiation levels of 5 kGy and above; however, lower radiation doses can also be effective if products are previously treated with hot water.
- Irradiated fungal strains can occasionally produce more mycotoxins than original strains; however, appropriate storage after irradiation can minimize the development of remaining fungal propagules.
- Dried mycotoxins are extremely radioresistant, whereas in aqueous environment, mycotoxins are more sensitive to irradiation. The oxidative radicals that originate from water radiolysis are responsible for their degradation.
- Combining gamma irradiation with other treatments can improve the breakdown of mycotoxins (e.g., using hydrogen peroxide, ammonium bicarbonate or higher moisture conditions).
- Generally, more than 10 kGy doses are required to eliminate a significant amounts of mycotoxins in food matrices. Patulin is an exception because patulin can be completely destroyed in apple juice by radiation doses between 2.5 and 5 kGy.
- The loss of toxicity after irradiation need further studies to assess the safety of irradiated on food products

References

Akueche, E.C., Anjorin, S.T., Harcourt, B.I., Kana, D., Adeboye, E., Shehu, I., Akande, R., Adeleke, A.T., Shonowo, O.A. & Adesanmi, C.A. (2012). Studies on fungal load, total aflatoxins and ochratoxin A contents of gamma-irradiated and non-irradiated *Sesamum indicum* grains from Abuja markets, Nigeria. Kasetsart J: Nat Sc 46:371-82.

Alam, K.K. & Abrahem, M. (2010). Effect of irradiation on quality of spices. Int Food Res J 17:825-36.

Alam M.K., Choudhury, N., Chowdhury, N.A. & Youssouf, Q.M. (1992). Decontamination of spices by gamma radiation. Lett Appl Microbiol 14:199-202.

Aquino, S., Ferreira, F., Ribeiro, D.H.B., Corrêa, B., Greiner, R. & Villavicencio, A.L.C.H. (2005). Evaluation of viability of *Aspergillus flavus* and aflatoxins degradation in irradiated samples of maize. Braz J Microbiol 36:352-56.

Arvanitoyannis, I.S., Stratakos, A.C. & Tsarouhas, P. (2009). Irradiation applications in vegetables and fruits: A review. Crc Cr Rev Food Sci 49:427-62.

Aziz, N.H. & Mahrous, S.R. (2004). Effect of gamma-irradiation on aflatoxin B1 production by *Aspergillus flavus* and chemical composition of three crop seeds. Nahrung 48:234-38.

Aziz, N.H. & Mattar, Z.A. (2007). Influence of gamma-radiation on the occurrence of toxigenic *Penicillium* strains and mycotoxins production in different feedstuffs. Egyptian J Biotechn 25:130-44.

Aziz, N.H. & Moussa, L.A.A. (2002). Influence of gamma-radiation on mycotoxin producing moulds and mycotoxins in fruits. Food Control 13:281-88.

Aziz, N.H. & Moussa, L.A.A. (2004). Reduction of fungi and mycotoxins formation in seeds by gamma-radiation. J Food Safety 24:109-27.

Aziz, N.H., Attia, E.S. & Farag, S.A. (1997). Effect of gamma-irradiation on the natural occurrence of *Fusarium* mycotoxins in wheat, flour and bread. Nahrung 41:34-37.

Aziz, N.H., El-Far, F.M., Shahin, A.A.M. & Roushy, S.M. (2007). Control of *Fusarium* moulds and fumonisin B1 in seeds by gamma-irradiation. Food Control 18:1337-42.

Aziz, N.H., Mattar, Z.A. & Mahrous, S.R. (2006a). Contamination of grains by mycotoxin-producing molds and mycotoxins and control by gamma irradiation. J Food Safety 26:184-201.

Aziz, N.H., Souzan, R.M. & Azza, A.S. (2006b). Effect of gamma-irradiation on the occurrence of pathogenic microorganisms and nutritive value of four principal cereal grains. Appl Radiat Isotopes 64:1555-62.

Barkai-Golan, R., Padova, R., Ross, I., Lapidot, M., Davidson, H. & Copel, A. (1993). Combined hot water and radiation treatments to control decay of tomato fruits. Sci Hortic 56:101-05.

Ben-Arie, R. & Barkai-Golan, R. (1969). Combined heat-radiation treatments to control storage rots of *Spadona* pears. Int J Appl Radiat Isotopes 20:687-90.

Bhat, R., Sridhar, K.R. & Karim, A.A. (2010). Microbial quality evaluation and effective decontamination of nutraceutically valued lotus seeds by electron beams and gamma irradiation. Radiat Phys Chem 79:976-81.

Bullerman, L.B. & Bianchini, A. (2007). Stability of mycotoxins during food processing. Int J Food Microbiol 119:140-46.

Chang, G.H. & Lee, H.B. (1980). Radiation sensitivity of some food decay fungi. Kor J Microbiol 18:1-6.

Chiou, R.Y., Lin, C.M. & Shyu, S.L. (1990). Property characterization of peanut kernels subjected to gamma irradiation and its effect on the outgrowth and aflatoxin production by *Aspergillus parasiticus*. J Food Sci 55:210-13.

Codex. (2003a). Codex Alimentarius code of practice for radiation processing of food (CAC/RCP 19-1979, Rev.2-2003. Editorial correction 2011). CODEX Alimentarius Commission/FAO/WHO, Rome, Italy.

Codex. (2003b). Codex Alimentarius general standard for irradiated foods (Codex Stan 106-1983, Rev.1-2003). CODEX Alimentarius Commission/FAO/WHO, Rome, Italy.

de Camargo, A.C., Vieira, T.M.F.D., Regitano-d'Arce, M.A.B., de Alencar, S.M., Calori-Domingues, M.A., Spoto, M.H.F. & Canniatti-Brazaca, S.G. (2012). Gamma irradiation of in-shell and blanched peanuts protects against mycotoxic fungi and retains their nutraceutical components during long-term storage. Int J Mol Sci 13:10935-58.

de Campos, S.X., Sanches, S.M., Falone, S.Z. & Vieira, E.M. (2004). Influence of rate dose in the degradation of the 2,4 dichlorophenoxyacetic acid (2,4-D) herbicide in water and methanol by gamma radiation from cobalto-60. Eclética Química 29:41-46.

Deberghes, P., Deffieux, G., Gharbi, A., Betbeder, A.M., Boisard, F., Blanc, R., Delaby, J.F. & Creppy, E.E. (1993). Détoxification de l'ochratoxine a par des moyens physique, chimique et enzymatiques. In: E. E. Creppy, M. Castegnaro and G. Dirheimer editors. Human ochratoxicosis and its pathologies. Paris, France: John Libbey Eurotext Ltd. 231:75-82.

D'Ovidio, K.L., Trucksess, M.W., Devries, J.W. & Bean, G. (2007). Effects of irradiation on fungi and fumonisin B1 in corn, and of microwave-popping on fumonisins in popcorn. Food Addit Contam 24:735-43.

EC. (2003). Revision of the opinion of the Scientific Committee on Food on the irradiation of food (SCF/CS/NF/IRR/24 Final). European Commission Health and Consumer Protection Directorate-General, Brussels, Belgium.

El-Far, F., Aziz, N.H. & Hegazy, S. (1992). Inhibition by gamma-irradiation and antimicrobial food additives of aflatoxin B1 production by *Aspergillus flavus* in poultry diet. Nahrung 36:143-49.

El-Samahy, S.K., Youssef, B.M., Askar, A.A. & Swailam, H.M.M. (2000). Microbiological and chemical properties of irradiated mango. J Food Safety 20:139-56.

Emam, O.A., Farag, S.E.A. & Hammad, A.I. (1994). Comparative-studies between fumigation and irradiation of semidry date fruits. Nahrung 38:612-20.

EU. (1999a). Directive 1999/2/EC of the European Parliament and of the Council of 22 February 1999 on the approximation of the laws of the Member States concerning foods and food ingredients treated with ionising radiation. Official Journal of the European Communities L66/16-L66/22.

EU. (1999b). Directive 1999/3/EC of the European Parliament and of the Council of 22 February 1999 on the establishment of a Community list of foods and food ingredients treated with ionising radiation. Official Journal of the European Communities L66/24-L66/25.

EU. (2009). List of Member States' authorisations of food and food ingredients which may be treated with ionising radiation. Official Journal of the European Communities C283/5.

FAO/IAEA/WHO. (1981). Wholesomeness of irradiated food (Technical Report Series 659). Joint FAO/IAEA/WHO Expert Committee, Geneva, Switzerland.

Farag, R.S., El-Baroty, G.S. & Abo-Hagger, A.A. (2004). Aflatoxin destruction and residual toxicity of contaminated-irradiated yellow corn and peanuts on rats. Adv Food Sci 26:122-29.

Farag, S.E.-D.A., Aziz, N.H. & Attia, E.-S.A. (1995). Effect of irradiation on the microbiological status and flavouring materials of selected spices. Z Lebensm Unters F 201:283-88.

Farkas, J. & Mohácsi-Farkas, C. (2011). History and future of food irradiation. Trends Food Sci Tech 22:121-26.

Farkas, J. (1998). Irradiation as a method for decontaminating food: A review. Int J Food Microbiol 44:189-204.

Farkas, J. (2006). Irradiation for better foods. Trends Food Sci Tech 17:148-52.

Ferreira-Castro, F.L., Aquino, S., Greiner, R., Ribeiro, D.H.B., Reis, T.A. & Corrêa, B., (2007). Effects of gamma radiation on maize samples contaminated with *Fusarium verticillioides*. Appl Radiat Isotopes 65:927-33.

Ferrier, P. (2010). Irradiation as a quarantine treatment. Food Policy 35:548-55.

Foroud, N.A. & Eudes, F. (2009). Trichothecenes in cereal grains. Int J Mol Sci 10:147-73.

Frank, H. K., & Grunewald, T. (1970). Radiation resistance of aflatoxins. Irradiat. Aliments., 11:15-20.

Geweely, N.S.I. & Nawar, L.S. (2006). Sensitivity to gamma irradiation of post-harvest pathogens of pear. Int J Agr Biol 8:710-16.

Gumus, T., Gecgel, U., Demirci, A.S. & Arici, M. (2008). Effects of gamma irradiation on two heat resistant moulds: *Aspergillus fumigatus* and *Paecilomyces variotii* isolated from margarine. Radiat Phys Chem 77:680-83.

Herzallah, S., Alshawabkeh, K. & Al Fataftah, A. (2008). Aflatoxin decontamination of artificially contaminated feeds by sunlight, gamma-radiation, and microwave heating. J Appl Poultry Res 17:515-21.

Hilmy, N., Chosdu, R. & Matsuyama, A. (1995). The effect of humidity after gamma-irradiation on aflatoxin B1 production of *A. flavus* in ground nutmeg and peanut. Radiat Phys Chem 46:705-11.

Hooshmand, H. & Klopfenstein, C.F. (1995). Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat, and soybeans with different moisture contents. Plant Foods Hum Nutr 47:227-38.

IAEA Report. (2008). Retrieved from https://www.iaea.org/sites/default/files/publications/reports/2008/anrep2008_full.pdf Access in 25 July 2019.

Iqbal, Q., Amjad, M., Asi, M.R. & Arino, A. (2012). Mold and aflatoxin reduction by gamma radiation of packed hot peppers and their evolution during storage. J Food Protect 75:1528-31.

Iqbal, S.Z., Bhatti, I.A., Asi, M.R., Zuber, M., Shahid, M. & Parveen, I. (2013). Effect of γ irradiation on fungal load and aflatoxins reduction in red chillies. Radiat Phys Chem 82:80-84.

Jalili, M., Jinap, S. & Noranizan, M.A. (2012). Aflatoxins and ochratoxin A reduction in black and white pepper by gamma radiation. Radiat Phys Chem 81:1786-88.

Jørgensen, K. (2005). Occurrence of ochratoxin A in commodities and processed food: A review of EU occurrence data. Food Addit Contam 22:26-30.

Jouany, J.P. (2007). Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim Feed Sci Tech 137:342-62.

Kim, D., Song, H., Lim, S., Jo, M., Song, D. & Jo, C. (2012). Shelf-life extension of preservative-free hydrated feed using gamma pasteurization and its effect on growth performance of eel. Radiat Phys Chem 81:1095-97.

Kim, K.H., Kim, M.S., Kim, H.G. & Yook, H.S. (2010). Inactivation of contaminated fungi and antioxidant effects of peach (*Prunus persica* L. Batsch cv Dangeumdo) by 0.5-2 kGy gamma irradiation. Radiat Phys Chem 79:495-501.

Kostecki, M., Golinski, P., Uchman, W. & Grabarkiewicz-Szczesna J. (1991). Decomposition of ochratoxin A by heat and gamma-irradiation. IARC Sci Publ 115:109-11.

Kottapalli, B., Wolf-Hall, C.E., Schwarz, P., Schwarz, J. & Gillespie, J. (2003). Evaluation of hot water and electron beam irradiation for reducing *Fusarium* infection in malting barley. J Food Protect 66:1241-46.

Kumar, S., Kunwar, A., Gautam, S. & Sharma, A. (2012). Inactivation of *A. ochraceus* spores and detoxification of ochratoxin A in coffee beans by gamma irradiation. J Food Sci 77:44-51.

Kume, T., Furuta, M., Todoriki, S., Uenoyama, N. & Kobayashi, Y. (2009). Status of food irradiation in the world. Radiat Phys Chem 78:222-26.

Kume, T., Ito, H., Soedarman, H. & Ishigaki, I. (1989). Radiosensitivity of toxigenic *Aspergillus* isolated from spices and destruction of aflatoxins by gamma-irradiation. Radiat Phys Chem 34:973-78.

Kume, T., & Todoriki, S. (2013). Food irradiation in Asia, the European Union, and the United States: A status update. *Radioisotopes*, 62:291-299.

Ladaniya, M.S., Singh, S. & Wadhawan, A.K. (2003). Response of `Nagpur´ mandarin, `Mosambi´ sweet orange and `Kagzi´ acid lime to gamma radiation. Radiat Phys Chem 67:665-75.

Lado, B.H. & Yousef, A.E. (2002). Alternative food-preservation technologies: efficacy and mechanisms. Microb Infect 4:433-40.

Le Caër, S. (2011). Water radiolysis: Influence of oxide surfaces on H_2 production under ionizing radiation. Water 3.1:235-253.

LebaiJuri, M., Omar, M. & Yusof, N. (1995). Sensitivity of the conidia of plant pathogenic fungi to gamma-rays, electron particles and X-ray (Bremsstrahlung) irradiation. World J Microbiol Biotechnol 11:610-14.

Legnani, P.P., Leoni, E., Righi, F. & Zarabini, L.A. (2001). Effect of microwave heating and gamma irradiation on microbiological quality of spices and herbs. Ital J Food Sci 13:337-45.

Lima, K.D.S.C., Souza, L.B., Godoy, R.L.O., França, T.C.C. & Lima A.L. (2011). Effect of gamma irradiation and cooking on cowpea bean grains (*Vigna unguiculata* L. Walp). Radiat Phys Chem 80:983-89.

Macfarlane, J.J. & Roberts, E.A. (1968). Some effects of gamma radiation on Washington Navel and Valencia oranges. Aust J Exp Agr 8:625-29.

Maity, J.P., Chakraborty, A., Chanda, S. & Santra, S.C. (2008). Effect of gamma radiation on growth and survival of common seed-borne fungi in India. Radiat Phys Chem 77:907-12.

Maity, J.P., Kar, S., Banerjee, S., Chakraborty, A. & Santra, S.C. (2009). Effects of gamma irradiation on long-storage seeds of *Oryza sativa* (cv. 2233) and their surface infecting fungal diversity. Radiat Phys Chem 78:1006-10.

Maity, J.P., Kar, S., Banerjee, S., Sudershan, M., Chakraborty, A. & Santra, S.C. (2011). Effects of gamma radiation on fungi infected rice (in vitro). Int J Radiat Biol 87:1097-102.

Malla, D.S., Diehl, J.F.& Salunkhe, D.K. (1967). In vitro susceptibility of strains of *Penicillium viridicatum* and *Aspergillus flavus* to γ irradiation. Experientia 23:492-93.

Maxie, E.C., Eaks, I.L. & Sommer, N.F. (1964). Some physiological effects of gamma irradiation on lemon fruit. Radiat Bot 4:405-11.

Moake, M.M., Padilla-Zakour, O.I. & Worobo, R.W. (2005). Comprehensive review of patulin control methods in foods. Compr Rev Food Sci Food Saf 4:8-21.

Mostafavi, H.A., Mirmajlessi, S.M., Mirjalili, S.M., Fathollahi, H. & Askari, H. (2012). Gamma radiation effects on physico-chemical parameters of apple fruit during commercial post-harvest preservation. Radiat Phys Chem 81:666-71.

Münzner, R. (1969). Some factors affecting the radiosensitivity of moulds. Arch Mikrobiol 64:349-56.

Mutluer, B. & Erkoc, F.U. (1987). Effects of gamma irradiation on aflatoxins. Z Lebensm Unters Forsch 185:398-401.

Ogbadu, G. & Bassir, O. (1979). Toxicological study of γ-irradiated aflatoxins using the chicken embryo. Toxicol Appl Pharmacol 51:379-82.

O'Mahony, M., Wong, S.Y. & Odbert, N. (1985). Sensory evaluation of navel oranges treated with low doses of gamma-radiation. J Food Sci 50:639-46.

O'Neill, K., Damoglou, A.P. & Patterson, M.F. (1993). The stability of deoxynivalenol and 3-acetyl deoxynivalenol to gamma irradiation. Food Addit Contam 10:209-15.

Paster, N., Barkaigolan, R. & Padova, R. (1985). Effect of gamma-radiation on ochratoxin production by the fungus *Aspergillus ochraceus*. J Sci Food Agr 36:445-49.

Paster, N., Bartov, I., Menasherov, M., Padova, R. & Ross, I. (1991). Efficacy of gamma-irradiation in preventing moldiness and preserving the nutritional-value of corn grain for broiler chicks. Poultry Sci 70:823-29.

Patel, U.D., Govindarajan, P. & Dave, P.J. (1989). Inactivation of aflatoxin B1 by using the synergistic effect of hydrogen peroxide and gamma radiation. Appl Environ Microbiol 55:465-67.

Paterson, R.R. & Lima, N. (2010). Toxicology of mycotoxins. In: A. Luch editor. Molecular, Clinical and Environmental Toxicology. 100 ed. Berlin, Germany: Birkhauser Verlag Basel. p 31-63.

Peraica, M., Radic, B., Lucic, A. & Pavlovic, M. (1999). Toxic effects of mycotoxins in humans. Bull World Health Organ 77:754-66.

Pfohl-Leszkowicz, A. & Manderville, R.A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res 51:61-99.

Poisson, J., Cahagnier, B. & Guilbot, A. (1971). On the gamma ray sensitivity of the main corn moulds conidies. Incidence on the radurisation of corn. Mycopath Mycol Appl 45:193-209.

Prado, G., de Carvalho, E.P., Madeira, J.E.G.C., Morais, V.A.D., de Oliveira, M.S., Correa, R.F. & Cardoso, V.N. (2006). Gamma-irradiation effect (60Co) in fungi frequency of peanut in natura and after storage. Cienc Agrotec 30:930-36.

Prado, G., de Carvalho, E.P., Oliveira, M.S., Madeira, J.G.C., Morais, V.D., Correa, R.F., Cardoso, V.N., Soares, T.V., da Silva, J.F.M. & Goncalves, R.C.P. (2003). Effect of gamma irradiation on the inactivation of aflatoxin B1 and fungal flora in peanut. Braz J Microbiol 34:138-40.

Prado, G. (2005). Gamma-irradiation effect (60Co) in fungi frequency of peanut in natura and after storage.Lavras, Minas Gerais, Brasil: Universidade Federal de Lavras. 30.5:930-936

Refai, M.K., Aziz, N.H., ElFar, F. & Hassan, A.A. (1996). Detection of ochratoxin produced *by A. ochraceus* in feedstuffs and its control by gamma radiation. Appl Radiat Isotopes 47:617-21.

Ribeiro, J., Cavaglieri, L., Vital, H., Cristofolini, A., Merkis, C., Astoreca, A., Orlando, J., Caru, M., Dalcero, A. & Rosa, C.A.R. (2011). Effect of gamma radiation on *Aspergillus flavus* and *Aspergillus ochraceus* ultrastructure and mycotoxin production. Radiat Phys Chem 80:658-63.

Ribeiro, J.M.M., Cavaglieri, L.R., Vital, H.D., Kruger, C.D. & Rosa, C.A.D. (2009). Gamma radiation on the mycoflora of poultry feed and *Aspergillus* species. Cien Rural 39:1452-58.

Saleh, Y.G., Mayo, M.S. & Ahearn, D.G. (1988). Resistance of some common fungi to gamma irradiation. Appl Environ Microbiol 54:2134-35.

Satin, M. (1996). Food irradiation - a guidebook. 2nd ed. USA: Technomic Publishing Company.

Schindler, A.F., Abadie, A.N. & Simpson, R.E. (1980). Enhanced aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* after gamma-irradiation of the spore inoculum. J Food Protect 43:7-9.

Sharwia, A.R.U.N., Padwal-Desai, S.R. & Nair, P.M. (1990). Aflatoxin-producing ability of spores of *Aspergillus parasiticus* exposed to gamma radiation. J Food Sci 55:275-76.

Shea, K.M. (2000). Technical report: irradiation of food. Pediatrics 106:1505-10.

Shibaba, S., Ito, H. & Iizuka, H. (1967). Effect of gamma irradiation on strawberries as a means of extending its shelf-life and lethal dose of *Botrytis cinerea*. Agr Biol Chem 31:930-34.

Stefanova, R., Vasilev, N.V. & Spassov, S.L. (2010). Irradiation of food, current legislation framework, and detection of irradiated foods. Food Anal Method 3:225-52.

Stepanik, T., Kost, D., Nowicki, T. & Gaba, D. (2007). Effects of electron beam irradiation on deoxynivalenol levels in distillers dried grain and solubles and in production intermediates. Food Addit Contam 24:1001-06.

Stoev, S.D. (2013). Food safety & increasing hazard of mycotoxin occurrence in foods and feeds. Crit Rev Food Sci Nutr 53:887-901.

Van Dyck, P.J., Tobback, P., Feyes, M. & Van de Voorde, H. (1982). Sensitivity of aflatoxin B1 to ionizing radiation. Appl Environ Microbiol 43:1317-19.

Van Egmond, H.P., Schothorst, R.C. & Jonker, M.A. (2007). Regulations relating to mycotoxins in food. Anal Bioanal Chem 389:147-57.

Visconti, A., Solfrizzo, M., Doko, M.B., Boenke, A. & Pascale, M. (1996). Stability of fumonisins at different storage periods and temperatures in gamma-irradiated maize. Food Addit Contam 13:929-38.

Voss, K.A., Smith, G.W. & Haschek, W.M. (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. Anim Feed Sci Tech 137:299-325.

Wang, F., Xie, F., Xue, X.F., Wang, Z.D., Fan, B. & Ha, Y.M. (2011). Structure elucidation and toxicity analyses of the radiolytic products of aflatoxin B1 in methanol-water solution. J Hazard Mater 192:1192-202.

Wang, J. & Yu, Y. (2010). Inactivation of mildew in rough rice and wheat by gamma irradiation. Radiat Phys Chem 79:740-43.

Webb, B.D., Thiers, H.D. & Richardson, L.R. (1959). Studies in feed spoilage: Inhibition of mold growth by gamma radiation. Appl Microbiol 7:329-33.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106-22.

Wilson-Kakashita, G., Gerdes, D.L. & Hall, W.R. (1995). The effect of gamma irradiation on the quality of english walnuts (*Juglans regia*). LWT-Food Sci Technol 28:17-20.

Wood, O.B. & Bruhn, C.M. (2000). Position of the American Dietetic Association: food irradiation. J Am Diet Assoc 100:246-53.

Yun, H., Lim, S., Jo, C., Chung, J., Kim, S., Kwon, J.H. & Kim, D. (2008). Effects of organic acids, amino acids and ethanol on the radio-degradation of patulin in an aqueous model system. Radiat Phys Chem 77:830-34.

Zegota, H., Zegota, A. & Bachman, S. (1988). Effect of irradiation on the patulin content and chemical-composition of apple juice concentrate. Z Lebensm Unters F 187:235-38.

Zeinab, E.M.E.B., Hala, A.F., Mohie, E.D.Z.E.F. & Seham, Y.M.E.T. (2001). Inhibitory effect of gamma radiation and *Nigella sativa* seeds oil on growth, spore germination and toxin production of fungi. Radiat Phys Chem 60:181-89.

Zinedine, A., Soriano, J.M., Moltó, J.C. & Mañes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food Chem Toxicol 45:1-18.

Chapter 3. Gamma Irradiation on Aflatoxins control

3.1 Introduction

Aflatoxins (AFIs) are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus* that are reported to be among the most potent mycotoxins (Bennett and Klich, 2003). Actually, it is known about 20 types of AFIs, being the most common ones Aflatoxin B1 (AFB1) and B2 (AFB2), that present a blue colour under UV light, and Aflatoxin G1 (AFG1) and G2 (AFG2), that present a green colour under UV light (Lee *et al.*, 2015). These mycotoxins were classified by IARC as Group 1 because these agents (or the mixture of them) are carcinogenic to humans (IARC, 2002). Other aflatoxin largely found is Aflatoxin M1 (AFM1) (Bhat *et al.*, 2010). This mycotoxin, despite being produced by some species of *Aspergillus*, is mainly found as a metabolic product of AFB1 in milk and derived products.

Aflatoxins are produced mostly by strains of *Aspergillus flavus* (produces only B aflatoxins) and *Aspergillus parasiticus* (produces both B and G aflatoxins); however, *Aspergillus minisclerotigenes, Aspergillus arachidicola, Aspergillus rambellii, Aspergillus bombycis, Aspergillus ochraceoroseus, Aspergillus nomius*, and *Aspergillus pseudotamari* are also aflatoxin-producing species, but are encountered less frequently (Bbosa *et al.*, 2013, Omar, 2013).

The food products contaminated with aflatoxins include cereals (maize, sorghum, pearl millet, rice and wheat), spices (chillies, black pepper, coriander, turmeric and ginger), oilseeds (groundnut, soybean, sunflower and cotton), tree nuts (almond, pistachio, walnut and coconut), milk (human and animal), and milk products (butter, cheese and yogurt) (Bhat *et al.*, 2010).

Regarding the types of exposures of animal to AFIs, the two most important are ingestion or inhalation routes (Larsson and Tjälve, 2000). The ingestion can occur directly with ingestion of contaminated food or indirectly by consumption of milk products as well as other animal tissues. The inhalation route can occur by inhalation of dust particles with aflatoxins, especially AFB1 in contaminated foods in industries (Bbosa *et al.*, 2013). Since AFIs are highly liposoluble compounds, after entering the body, AFIs are readily absorbed by cells and reach the blood circulation. Once in blood, AFIs are distributed to different tissues and to the liver, the main organ of metabolism of xenobiotics (Bbosa *et al.*, 2013). In liver, AFB1 is metabolized by the microsomal cytochromes P450 (CYPs) in other types of AFIs, namely AFP1, AFM1, AFQ1 and AFB1 -8,9-epoxide (AFBO). This process is a bioactivation of AFB1 that, now, can react with DNA, RNA and proteins (Riley *et al.*, 2011). These new compounds are able to react with covalent bond of biological

macromolecules and can form nucleic acid adducts or undergo conjugation to glutathione. When AFBO binds to DNA, it modifies its structure, and consequently its biological activity. This triggers the mechanisms of mutagenic and carcinogenic effects of AFB1. Additionally, aflatoxins cause hepatotoxicity, nephrotoxicity and genotoxicity in somatic and germ cells, resulting in mitotic and meiotic delay in mice (Omar, 2013).

Gamma irradiation has been shown to be a simple and effective decontamination technique for food preservation. This technique is effective in destroying pathogenic and spoilage microorganisms and in reducing some toxins, such as mycotoxins, without compromising food quality and safety (Domijan *et al.*, 2015). In the field of mycotoxicology, gamma radiation is known to exert a direct action on mycotoxins by degrading them, and an indirect action through the inhibition or delay of fungal development (Calado *et al.*, 2014). Although the application of gamma irradiation to degrade AFIs was studied by several authors, the results of those studies are contradictory (Calado *et al.*, 2014). Some studies have indicated that gamma irradiation can reduce AFIs levels even if applied in low doses (Mohamed *et al.,* 2015; Zhang *et al.*, 2018; Abdel-Rahman *et al.,* 2019), and others have claimed that AFIs reduction can only be achieved at higher irradiation doses (Jalili *et al.,* 2012, Domijan *et al.*, 2015). Furthermore, only a few studies have been carried out on the identification and toxicity of the degradation products of AFIs. The degradation of mycotoxins may form other compounds (radiolytic products) that can be as or more toxic than the original mycotoxin. According to Rychlik *et al.* (2014), the potential exposure to modified mycotoxins is an additional risk to human and animal health. Thus, the study of mycotoxins degradation should be complemented with assays on the safety of the irradiated product. The detection, identification and isolation of each radiolytic product is the ideal option. However, due to the diversity of produced radiolytic products and to their very low concentration, this is not always feasible (Wang *et al.*, 2011). One excellent alternative is to study the toxicity of radiolytic products using *in vitro* assays. Wang *et al.* (2011) identified several degradation products of AFB1 irradiated in methanol:water solution. After identification of radiolytic products, the authors used a quantitative structure-activity relationship analyses to conclude that the toxicity of total radiolytic products compared with that of AFB1 was reduced after the radiation process. Nevertheless, these authors, refer that animal or cytotoxicity experiment must be necessary to confirm the toxicity decrease.

The aims of the current study were:

(i) to study the effect of gamma radiation on aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2

(ii) to verify if the irradiation of aflatoxins separately is different from the irradiation of the four aflatoxins together

(iii) to evaluate the effect of the presence of water on AFIs degradation during the irradiation process; and

(iv) to evaluate the cytotoxicity of radiolytic products formed.

3.2 Material and methods

3.2.1 Chemicals and reagents

Methanol was obtained from Merck (Lisbon, Pt). Standard of AFB1 (A6636-10MG, Sigma), AFB2 (A9887-10MG, Sigma), AFG1 (A0138-10MG, Sigma) and AFG2 (A0263-10MG, Sigma) were purchased from Sigma-Aldrich (Sintra, Pt). Ultraglutamine 1 (200 mM) (L-Gln), fetal bovine serum (FBS), penicillin and streptomycin (P/S) (10000 U/mL / 10 mg/mL), non-essential amino acids (NEAA) 100X, Trypsin EDTA (200 mg/L EDTA, 17000 U trypsin/L), cell culture EMEM (Eagle's Minimum Essential Medium) was sourced by Lonza (Barcelona, ES). Phenol red-free Minimum Essential Medium (MEM) was purchased from PAN-Biotech (Aidenbach, DE). AlamarBlue, 5-carboxyfluorescein diacetate and acetoxy methyl ester (CFDA-AM) were purchased from Life Technologies (Madrid, ES). Neutral red (3-amino-7-dimethylamino-2-methylphenanzine hydrochloride) solution (0.33%), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO) and glacial acetic acid were acquired from Sigma-Aldrich (Madrid, ES). Ethanol was from Panreac (Barcelona, ES). High grade purity water (> 18 MΩ/cm) was obtained from a Milli-Q Element A10 Century (Millipore Iberia, ES).

3.2.2 Preparation of mycotoxins solutions and irradiation process

A stock solution of each mycotoxin at a concentration of 1 mg mL^{-1} was prepared in 10 mL of methanol using commercial standard of each mycotoxin and stored at -20 °C until use. To prepare

the samples of 3 μmol L^{-1} of each mycotoxin and the samples of mix AFIs, the appropriate amount of the stock was pipetted into clean amber 2 mL vials. Samples were then evaporated at 50 °C under a gentle stream of nitrogen. To study the effects of irradiation, three different types of mycotoxin samples were prepared to represent different moisture contents. A set of samples of each mycotoxin was kept dried, another set was resuspended in 1 mL of deionized water (H_2O_{dd}) and the last set was resuspended in 1 mL water:methanol (50/50, v/v). After being prepared, samples were kept in amber vials and stored at -20 °C until irradiated. For the cytotoxicity study, aflatoxins samples at concentrations of 30 μmol L^{-1} in H_2O_{dd} or dried were prepared as described above. The increase in mycotoxins concentrations was necessary to detect its cytotoxicity.

Irradiation was performed at room temperature in a Co-60 experimental equipment Precisa 22 (Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 177 TBq (4.78 kCi; February 2014), located at C2TN. The average dose rate was 1.8 kGy/h and was previously determined by Frick reference dosimeter (ASTM, 1992). To monitor the process during irradiation, two routine dosimeters (Amber Perspex dosimeters, Batch X, from Harwell Company, U.K.) were used to estimate the highest and the lowest dose absorbed by the samples. The absorbance and thickness of Amber Perspex dosimeters were measured in a UV-VIS Spectrophotometer (UV 1800, Shimadzu, USA) at 603 nm and in a micrometer (Mitutoyo America Corporation, USA), respectively, in order to estimate the dose according to a previous calibration curve. Samples of aflatoxins (dried, aqueous and methanolic) were irradiated at 0.4, 0.9, 1.7, 2.5, 5.4 and 8.6 kGy, in triplicates. To evaluate the cytotoxicity after irradiation, only aqueous solutions of all mycotoxins and dried mix of aflatoxins were submitted to radiation doses of 2.4 and 10.3 kGy. During the irradiation process, the vials were closed. Non-irradiated controls were also prepared for each condition.

3.2.3 Determination of Aflatoxins

The HPLC analysis was carried out with a modified version of method described by Rodrigues *et al.* (2009). The HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λexc = 365 nm and λem = 435 nm), a Varian 850-MIB data system interface and a Galaxie chromatography data system. The chromatographic separation was performed with a 30 min isocratic run on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 μm), fitted with a pre-column of the same stationary phase. The mobile phase was a mixture of water/acetonitrile/methanol (3:1:1, v/v/v) that was

filtered and degassed with a 0.2 μm membrane filter (GHP, Gelman). The flow rate was set to 1.0 mL/min and the column temperature to 30 °C. The injection volume was 50 μL. AFIs were identified by retention times (16 min to AFG2, 18 min to AFG1, 25 min to AFB2 and 27 min to AFB1) and quantified by measuring peak areas and comparing them with respective calibration curve, which was prepared by dissolving the required amount of working standard solution in mobile phase. The calibration curve was prepared with AFIs concentrations from 0.02 μmol L^{-1} to 3.2 μmol L^{-1} (0.005 to 1 μg mL^{-1}), used when the detector gain was set to 1000 and to quantify AFIs samples. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated with a signal-to-noise ratio of 3:1 and 10:1, respectively. For working concentration range, LOD = 0.004 μg/mL and LOQ = 0.013 μg/mL.

3.2.4 Cytotoxicity studies

3.2.4.1 Cells culture and exposure

The cell line HepG2 obtained from American Type Culture Collection (ATTC) (Manassas, VA, USA) was cultured in 75 cm^2 Cell Star Cell Culture flasks (Greiner Bio-One GmbH, Frickenhausen, DE) in EMEM supplemented with 1% NEAA, 1% P/S, 1% L-Gln and 10 % FBS (in the following text referred as EMEM+). The flasks were incubated at 37 °C in a humidified 5% CO2 atmosphere and split twice a week using PBS/EDTA and trypsin.

A HepG2 cell suspension (5×10^5 cells/mL) with EMEM+ was seeded into transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, DE) by adding 100 μL of cell suspension to each well. The plates were incubated for 24 h and exposed for 48 h to different concentrations of mycotoxins. Exposure concentrations of irradiated and non-irradiated mycotoxin samples were prepared by drying (if suspended in water) as described in section 3.2.2 and re-suspending in EMEM+ supplemented with 0.5% of DMSO (to improve the solubility) to a final concentration of 30 μmol L^{-1}. Then, those solutions were applied to the cell culture plate in which successive serial dilutions (dilution factor of 2) were performed. As a positive control, a subset of wells was treated with increasing concentrations of SDS (15.6 μmol L^{-1} to 500 μmol L^{-1}, dilution factor 2/3). Cells treated with EMEM+ served as negative control whereas cells treated with 0.5% (v/v) DMSO/EMEM+ were the vehicle control.

3.2.4.2 AB, CFDA-AM and NR Uptake (NRU) assays

The AB, CFDA-AM and NRU assay were performed on the same set of cells. The assays were conducted following a combined version of the protocol described by Lammel *et al.* (2013). Prior to adding the reactants, the exposure medium was removed and the cells rinsed twice with 200 μL of phosphate-buffered saline (PBS). Then, 100 μL phenol red-free MEM containing 1.25% (v/v) AB and 4 μM CFDA-AM was added to each well. The 96-well plates were incubated for 30 min in the dark as described above. The fluorescence intensity was measured at λexc=532 nm and λem=590 nm, for the AB assay, and at λexc=485 nm and λem=535 nm, for CFDA-AM assay, using a microplate reader (Tecan Genios, Tecan Group Ltd., Männedorf, CH). Subsequently, the medium was removed, and the cells were washed once with PBS. One hundred μL of NR solution (0.03 mg/mL in phenol red-free MEM) were added per well and the plates incubated for 1 h in the dark as described above. After the incubation period, the NR solution was removed, the cells were rinsed twice with 200 μL PBS and the retained NR in the cells was extracted with an acidified solution composed of 1% glacial acetic acid/50% ethanol/49% Milli-Q water (150 μL/well). NR fluorescence was measured at λexc=532 nm and λem=680 nm. The fluorescent values were corrected for the cell-free control and normalized against the vehicle control.

3.2.5 Statistical analysis

Statistical analysis was performed using Sigma Plot version 12.0 (Jandel Scientific, San Rafael, CA, USA). To evaluate significant differences between concentrations of each mycotoxin in non-radiated and irradiated samples, means were compared by analysis of variance followed by the Duncan's post-test. Results of cytotoxicity assays represent the means and standard errors (SEM) of at least three independent experiments, in which each treatment was applied in triplicate. Significant differences among treatments were determined by one-way repeated measures analysis of variance (rmANOVA, $p < 0.05$, α 0.05). All data were tested beforehand for normality (Shapiro-Wilk test, $p < 0.05$) and equal variance ($p < 0.05$). Significant differences between treatments and the control were determined by applying a Dunnett's Post hoc test to one-way ANOVA analyses.

3.3 Results and discussion

3.3.1 AFIs concentration after irradiation

The effect of gamma radiation application on aflatoxins detoxification were thoughtfully studied by several authors; however, results are contradictory. These contradictory results can be explained by the influenced of several factors in the effectiveness of gamma irradiation, such as type of mycotoxin and mycotoxin concentration, moisture and synergic effects with other compounds on food matrices (Figure 2.4) (Jalili *et al.*, 2012; Calado *et al.*, 2014). The detoxification efficacy of irradiation can be influenced by the type of food, the moisture content in food, In an attempt to minimize the influence of these factors in this work, the standards of the four aflatoxins were irradiated separately in different solutions (individual assays) and then together to verify if the effect of irradiation was the same (mix assays). On both type of assays, the four AFIs were irradiated in three different conditions (dehydrated, in methanol:water solution, and in water).

The effect of gamma radiation doses on AFB1 samples irradiated separately are presented in Figure 3.1.

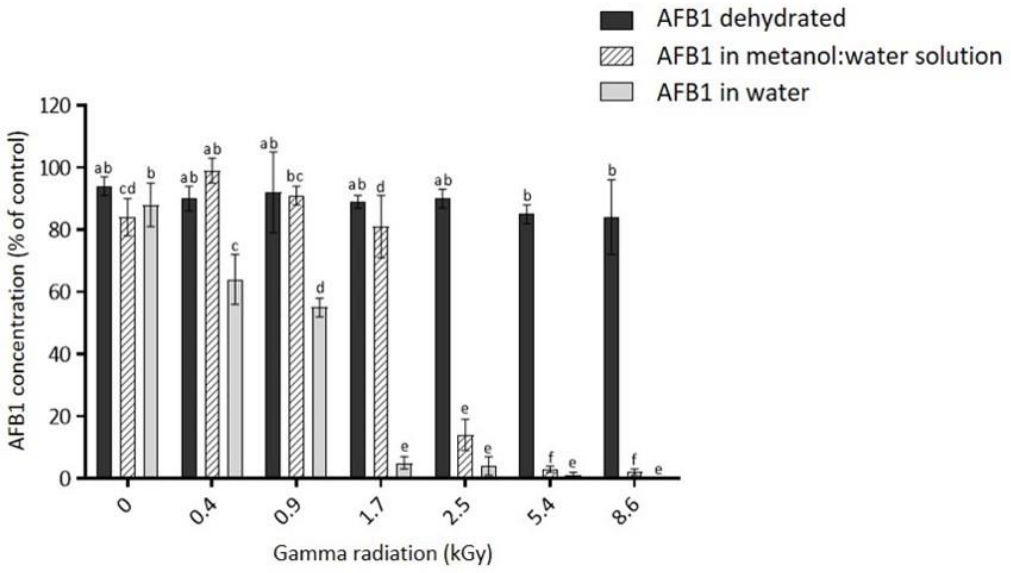

Figure 3.1 - Effects of incremental gamma radiation doses on the AFB1 concentration under different solutions conditions (individual assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

When the effect of gamma irradiation of AFB1 was analysed, it was possible to see that the maximum elimination occurred in samples of AFB1 dissolved in water. In this case, the lowest

radiation dose applied (0.4 kGy) was enough for a significant reduction of AFB1 concentration (36%) and at the highest dose tested (8.6 kGy) no AFB1 was detect in the HPLC analysis. On the opposite side, there was no significant reduction of mycotoxin in dehydrated AFB1 samples, for none of the dose tested. In the assays performed with the mycotoxin dissolved in a water:methanol solution, a significant AFB1 reduction was observed at 2.5 kGy (74%), while a reduction of 98% was observed for the highest dose of radiation. These results clearly reveal that when water is present in samples the degradation of AFB1 increases, and that its reduction is positively correlated to the increase of gamma radiation dose. For dehydrated samples this correlation was not observed.

These results point out that the presence of water during the radiation process improves the AFB1 reduction and are in agreement with the conclusion of others published works (Jalili *et al.*, 2012 Di Stefano *et al.*, 2014a; Di Stefano *et al.*, 2014b). Despite most studies reported so far were conducted in food matrices, some of them corroborate our findings. The lower reduction observed in dehydrated samples was in accordance with Hooshmand and Klopenstein (1995) who found that doses of gamma radiation up to 20 kGy did not affect AFB1 concentration in wheat, corn and soybeans having a moisture content around 17%. On the other hand, some authors verified significant reductions of AFB1 concentration when levels of radiation similar to the tested (max 10 kGy) in this present work were used. Recently, Abdel-Rahman and collaborators (2019) reported high reductions on AFB1 levels in roasted peanut samples. They verified that the dose of 5 kGy led to low reduction of AFB1 concentration (<7.8%). But, with higher doses (10 and 20 kGy), a significantly higher decrease on AFB1 in peanut samples was observed at all spiking levels (26.7-59.0% reduction) (Abdel-Rahman *et al.*, 2019).

Mohamed *et al.* (2015) tested the effect of gamma irradiation on AFB1 reduction in samples of maize, wheat and rice. These authors found, with an 8 kGy dose, reductions of about 65%, although the samples had only 13% of moisture content. Zhang *et al.* (2018) verified reductions of 62% and 76% with radiation doses about 10 kGy in soybeans. These authors incubated soybeans, with moisture about 20%, for 30 and 60 days. Zhang and collaborators concluded that the good reduction observed can be explained by the higher moisture content of samples (Zhang *et al.*, 2018). Kanapitsas *et al.* (2015) verified a reduction of 29% of AFB1 in contaminated raisins. In this study the moisture content was not determined. However, the authors refer that samples preparation included a washing step with water, and that this procedure could increase the moisture content of samples (Kanapitsas et a., 2015). Ghanen *et al.* (2008) verified the effect of

gamma irradiation on several samples of food crops and feed. Their work involved the preparation of samples with the addition of 15 mL of water to 100 g of each matrix, autoclave-sterilization, and inoculation with spore suspension of aflatoxigenic fungus. Following a 10-day period of incubation at 27 °C to allow for fungal growth, food and feed samples were irradiated with gamma radiation at the doses 4, 6, and 10 kGy. Their results indicated percentages of AFB1 degradation at 10 kGy of 58.6, 68.8, 84.6, 81.1, 87.8, 90.0, 86.0 and 84.0% for peanuts, peeled pistachios, unpeeled pistachios, corn, rice, barley, bran and corn samples, respectively (Ghanem *et al.*, 2008). The higher degradation observed for these products could be caused by the moisture present in these samples due to the used methodology.

The assays with AFB2 irradiated alone revealed similar results to AFB1 (Figure 3.2). However, in all assays, the degradation of mycotoxin was lower than the one observed for AFB1, suggesting that this mycotoxin in more resistant to gamma irradiation. The maximum elimination was observed for samples dissolved in water. In this assay, a significant degradation was observed for radiation doses from 0.4 kGy. For this radiation dose the reduction of AFB2 was around 16%. For the two higher radiation doses the degradation was up to 99%. As previously observed for AFB1, for the dehydrated samples, no significant reductions were observed at all irradiation doses tested. For the experiment in methanol:water solution, the degradation was only significant for radiation doses of 1.7 kGy or higher.

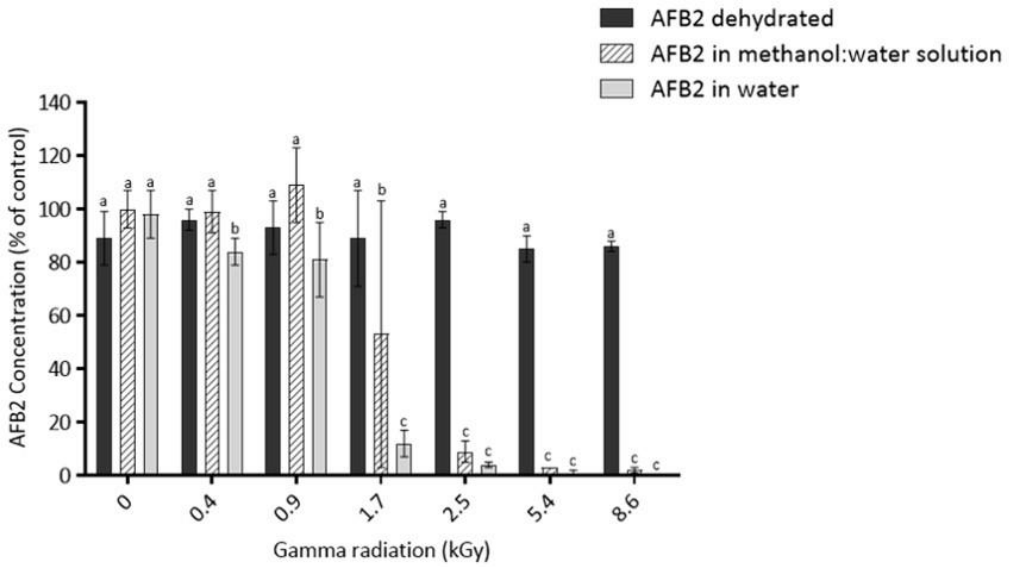

Figure 3.2 - Effects of incremental gamma radiation doses on the AFB2 concentration under different conditions (individual assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

In the same way, AFG1 was submitted to radiation (Figure 3.3). The results point out that this aflatoxin is more sensitive than AFB1 and AFB2 in all conditions tested. When AFG1 was irradiated in water, a degradation of more than 98% was observed to doses superior to 0.4 kGy. In sample irradiated in methanol:water solution, a lower reduction was observed. In this condition, significant reductions were observed for doses greater than 1.7 kGy. For doses >2.5 kGy, reductions above 97 % were observed. Such as observed for other aflatoxins, AFG1 showed more resistance when it was irradiated in dehydrated conditions. However, and contrary to what was observed for both AFB1 and AFB2, for AFG1 it was observed a significant reduction with radiation doses superior to 0.9 kGy. The highest reduction observed was 77% (for 8.6 kGy).

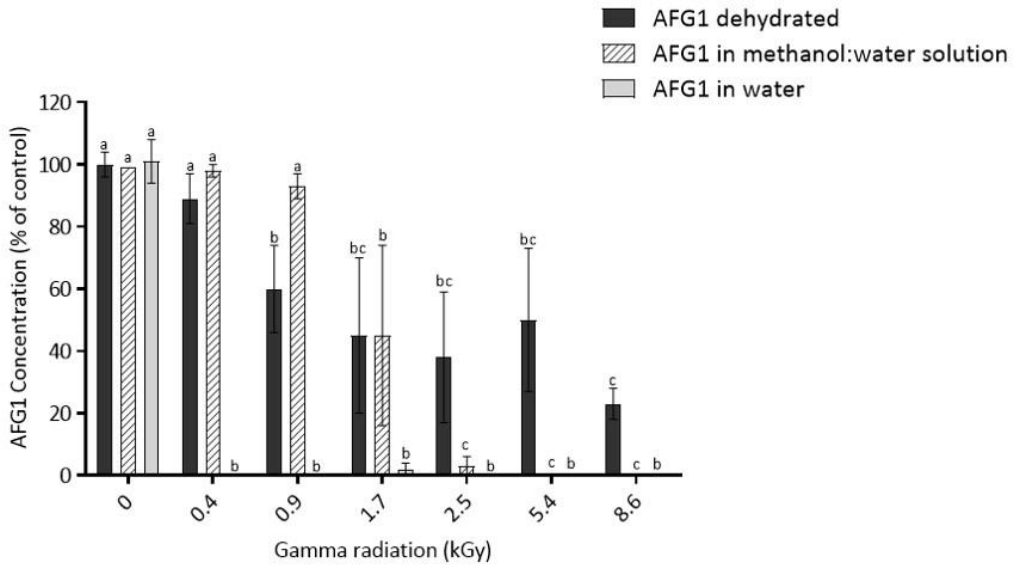

Figure 3.3 - Effects of incremental gamma radiation doses on the AFG1 concentration under different conditions (individual assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

Such as observed for AFG1, the irradiation of AFG2 in dehydrated conditions also revealed a significant reduction (44%) of mycotoxin concentration (Figure 3.4). However, this reduction was only observed at the highest irradiation dose tested. The irradiation of AFG2 in methanol:water solution showed an intermediate trend between the others two conditions, with significant reduction for doses >1.7 kGy. As for the samples irradiated in water, a significant reduction was observed for irradiation doses >0.4 kGy. For doses >5.4 kGy, the total elimination of the mycotoxin was observed.

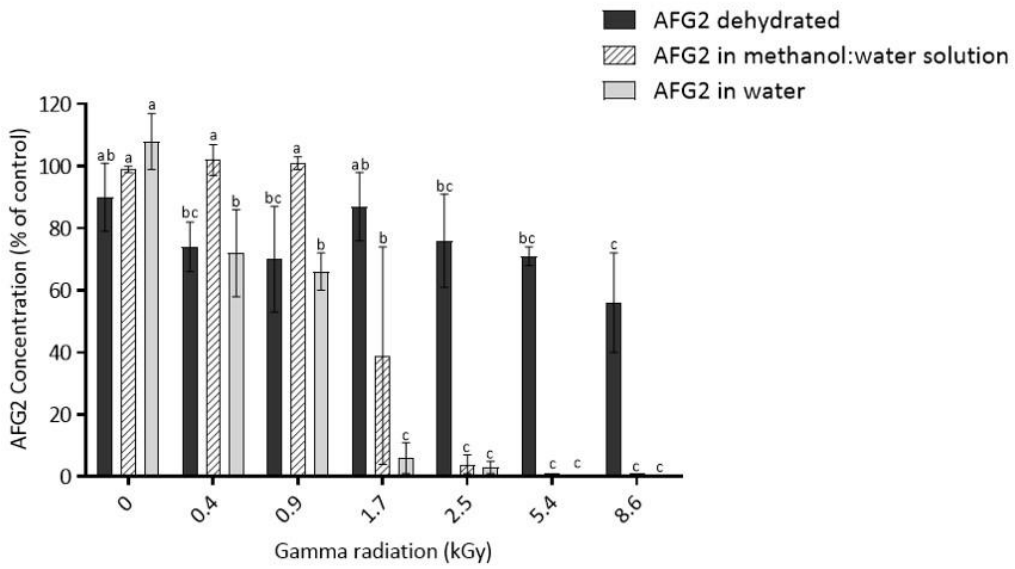

Figure 3.4 - Effects of incremental gamma radiation doses on the AFG2 concentration under different conditions (individual assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

In the reviewed literature, studies may be classified in two groups: some authors irradiated only AFB1, while others irradiated the main 4 aflatoxins together. No studies were found where the irradiation of the four aflatoxins was compared with the irradiation of each aflatoxin alone.

To evaluate if the irradiation of mycotoxins separately or together reveals a different behaviour, a solution containing the four aflatoxins was irradiated under the same three previous tested conditions (mix assays).

Figure 3.5 presents the reduction in AFB1 concentration when this mycotoxin was irradiated with the other AFIs. As it was observed when irradiated alone (Figure 3.1), the most effective condition was when irradiated in water. The less effective condition, in the same way, was when irradiated dehydrated. Additionally, the AFB1 reductions observed were, in all conditions tested, higher than when irradiated separately (compared Figure 3.1 with Figure 3.5). For dehydrated AFB1, the reduction was significant for radiation >0.9 kGy. For the higher irradiation dose, the reduction was 52% (16% when AFB1 was irradiated separately). Regarding the samples irradiated in methanol:water solution, again, higher reductions were observed when all mycotoxins were present - reduction were significant for radiation equal or greater than 0.4 kGy. The irradiation of AFB1 in water showed a reduction above 98% for radiation doses equal or above 0.4 kGy.

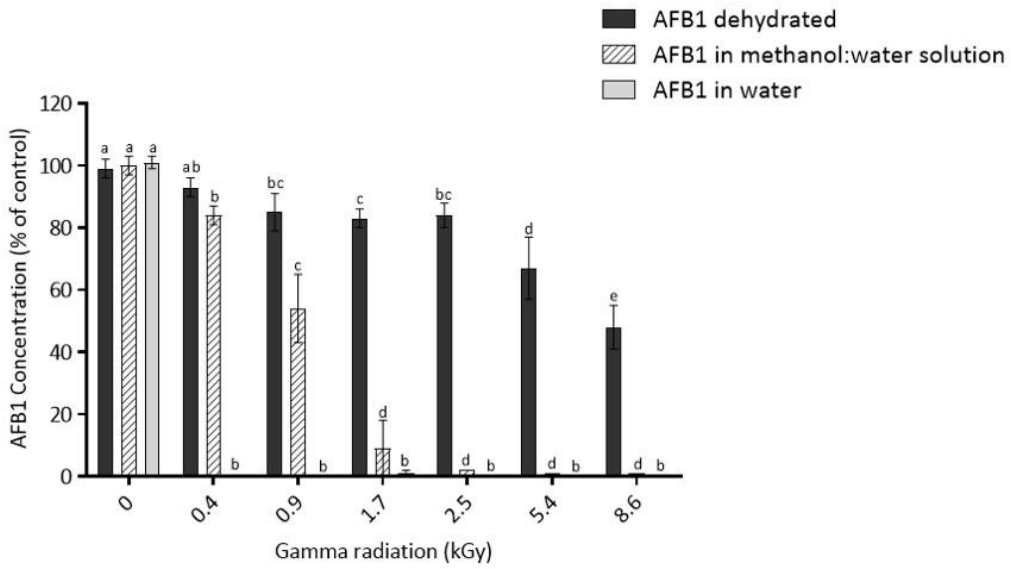

Figure 3.5 - Effects of incremental gamma radiation doses on the AFB1 concentration under different conditions (mix assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

For AFB2 a similar behaviour to AFB1 was observed (Figure 3.6). However, at dehydrated conditions, the increased reduction was not so high as with AFB1. AFB2 reductions were statistically significant for radiation doses equal or above 1.7 kGy, being the reductions for the highest radiation dose of 16%. When water was present, the reduction was again higher. In methanol:water solution, such as it was observed in individual assays, significant reductions were observed for radiation doses equal or greater than 1.7 kGy. However, in mix assays, for radiation dose of 1.7 kGy the reduction increased from 83% (when irradiated only the reduction was 47%). In water the trend was the same - in the two assays reductions were significant for radiation doses above 0.4 kGy, with higher reduction in AFB2 when irradiated together.

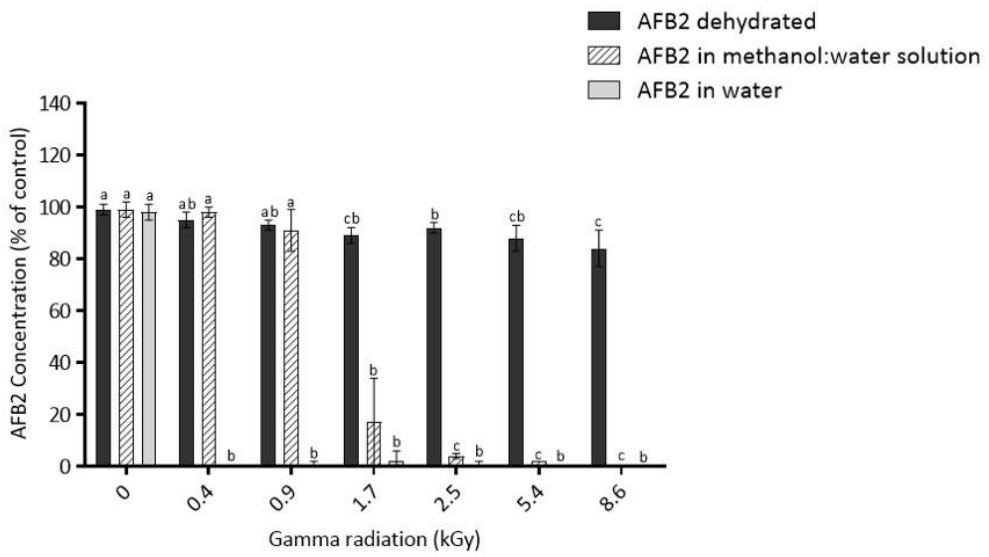

Figure 3.6 - Effects of incremental gamma radiation doses on the AFB2 concentration under different conditions (mix assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

On the other hand, an opposite trend was observed for AFG1 samples irradiated together with other AFIs in dehydrated condition (Figure 3.7). Despite a significant reduction was obtained for a radiation dose of 0.4 kGy, in the mix assays, for the highest irradiation tested, the reduction was lower than the one obtained in the individual assays. In hydrated conditions, the same result was observed in both assays – a total reduction was observed at radiation doses above 0.4 kGy. In methanol:water solution, a little increase was observed in mix assays, and a significant reduction was observed for >0.4 kGy radiation dose.

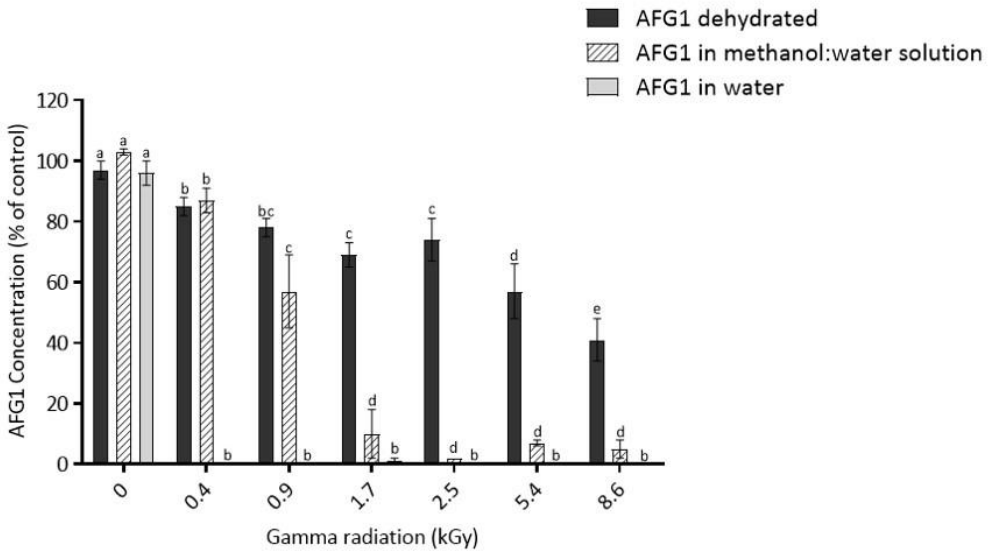

Figure 3.7 - Effects of incremental gamma radiation doses on the AFG1 concentration under different conditions (mix assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

For AFG2, the behaviour was similar to the one for AFB2 (Figure 3.8). In the dehydrated samples, no increase of AFG2 reduction was observed. However, the radiation dose of 0.4 kGy was sufficient to produce a significant reduction. When water is present, although significant reductions were observed for the same radiations doses as observed in the individual assays, an increase of AFG2 destruction was observed. In methanol:water solution, an increase of 23% was observed for radiation doses of 1.7 Gy. In water, a big increase in reduction was observed, with 100 % destruction at 0.4 kGy.

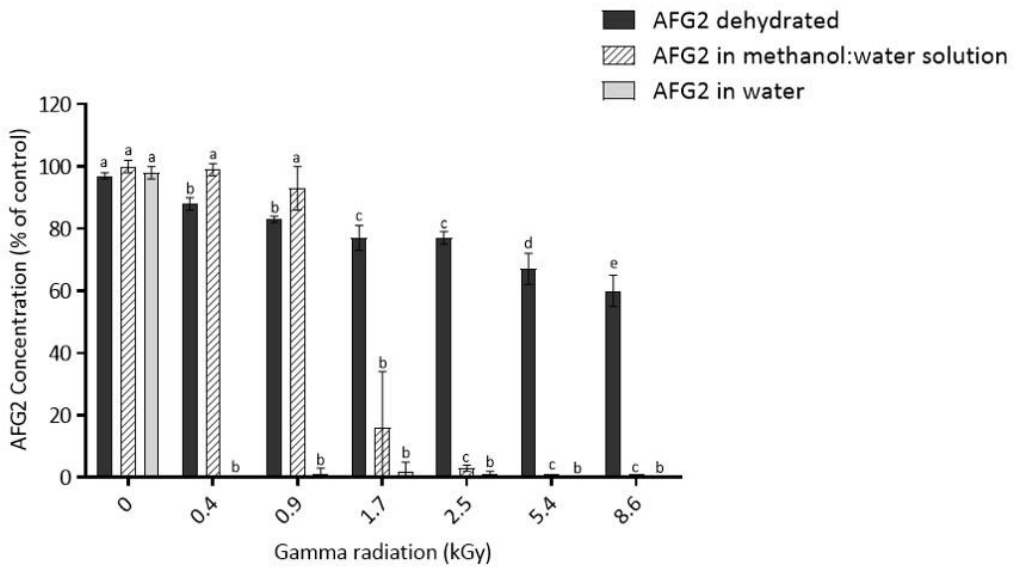

Figure 3.8 - Effects of incremental gamma radiation doses on the AFG2 concentration under different conditions (mix assays). Statistically significant differences for each condition are indicated with different letters, by analysis of variance (two-way rmANOVA and Duncan´s Post-hoc test).

As already mentioned, the majority of published studies performed the irradiation of AFIs together. However, in these studies, the assays were performed in food matrices, which makes difficult to establish comparison with the current study. Nevertheless, as it was observed in the present work, the moisture content seems to be a critical factor for radiation effectiveness.

As explained for AFB1 irradiated separately, the results of published studies with other AFIs and with mixtures of AFIs and/or other mycotoxins are very dissimilar. Jalili *et al.* (2010), in black pepper samples contaminated with AFIs and OTA, with a gamma radiation dose similar to the highest dose tested in this work (9 kGy), observed a reduction of just 10% for all AFIs. These authors say that the low degree of mycotoxin reduction obtained could be explained by the fact that black pepper is a dry product with moisture content of 9 – 12% (Jalili *et al.* 2010). These results are similar to the ones observed in the individual assays for AFB1 and AFB2; however, are about 4-

folds and 6-folds lower than the ones observed for AFG2 and to AFG1, respectively. Another study of the same authors (Jalili et al, 2012), showed no significant reduction to AFIs in black pepper and white pepper for radiation doses of 10 kGy. In addition, in the same study, the authors adjusted the moisture content of samples to test if a higher moisture content (18%) induced a higher mycotoxin degradation by gamma radiation. The results obtained showed significant effects of the gamma dose and moisture content. Moisture content of 18% efficiently reduced the mycotoxin level relatively to the lower moisture content (12%), and authors concluded that water have an important effect on the destruction of aflatoxins by gamma radiation (Jalili et al, 2012). The maximum reduction of mycotoxins was found to be 46% and 55% for moisture contents of 12% and 18%, respectively (Jalili et al, 2012). The study of Pereira *et al.* (2017), that irradiated dehydrated leaves lemon verbena (*Aloysia citrodora*) contaminated with AFB1 and OTA, verified no reduction in AFB1 when samples were irradiated with 10 kGy. These authors proposed, to increase the AFB1 reduction and overcome the limited effect of the treatment generally observed for low moisture products (namely dried herbs), the development of studies on mycotoxin detoxification by gamma radiation with the fresh product, *i.e.*, before drying.

The influence of water activity (A_w) on aflatoxin destruction by gamma radiation was also studied by Aquino *et al.* (2005). These authors tested the effect of gamma radiation in samples of maize inoculated by *A. flavus* on the reduction of AFB1 and AFB2 produced by this fungus. The irradiation of samples with 2, 5 and 10 kGy show a peculiar result - the reduction of aflatoxins was higher in 10 and 2 kGy treatment than in 5 kGy treatment. Before incubation with the fungus, the water activity of samples was adjusted to a_w = 0.91 – 0.94, by sprinkling the samples with distilled water. After incubation, authors verified that a_w of samples irradiated with 5 kGy was inferior to a_w of samples irradiated with 2 and 10 kGy. Thus, author concluded that a_w of treated samples can be a source of the unexpected result (Aquino *et al.*, 2005).

Di Stefano *et al.* (2014a) verified the effect of gamma irradiation in almonds artificially contaminated with AFIs and OTA, with radiations ranging from 0 to 15 kGy. The maximum reduction was found at 15 kGy, and achieved 19%, 11%, 21%, and 17% for AFB1, AFB2, AFG1, and AFG2, respectively. These authors concluded that gamma radiation up to 15 kGy was not effective in completely destroying AFIs in almonds (Di Stefano *et al.*, 2014a). In another study, Di Stefano *et al.* (2014b) tested the effect of irradiation, ranging from 0 to 15 kGy, on AFIs and OTA reduction in commercial animal feed. The maximum reductions found at 15 kGy were of 18.2%, 11.0%, 21.1%, and 13.6% for AFB1, AFB2, AFG1, and AFG2, respectively. Results showed that

gamma-rays even at 15 kGy were not effective in the complete destruction of AFIs in the tested feed (Di Stefano *et al.*, 2014b). In these two studies, although the authors did not report the moisture content, authors concluded that the low moisture typically found in these matrices could be the reason for the little reductions observed. These two studies demonstrated that AFG1 is more sensitive to gamma irradiation when compared to the other AFIs, as it was observed in the present work.

Considering all results presented, the mycotoxin that was more sensitive to gamma radiation was AFG1. The aflatoxin that seems more resistant to gamma radiation was not the same in all conditions. In most conditions tested, AFB2 and AFB1 were the most resistant. Jalili *et al.* (2012) reported that AFB1 and AFG1 seem to be more sensitive to gamma radiation, as compared to AFB2 and AFG2. These authors claim that this finding may be related to the 8,9 double bound present in AFB1 and G1 structure, which undergoes a reaction induced by the gamma ray. These results are in agreement with the present work for the mix assays in dehydrated condition and in methanol:water solution and for individual assays in water. In the other conditions tested, this trend was not observed. Different results were observed by other authors. Mutluer and Erkoc (1987) reported that AFB1 was the most radio-sensitive of the four aflatoxins. The radio-sensitivity of the other aflatoxins was in increasing order - G2<B2<G1. In a related study, the B1 and G1 toxins were completely destroyed at irradiation doses of 10 and 20 kGy, respectively (Farag *et al.*, 1995). In experiments on the reduction of mycotoxins in black pepper, Jalili *et al.* (2010) also found that AFB1 and AFG1 were more sensitive to the gamma rays. It seems that more research is needed to investigate the effects of gamma rays on the different mycotoxins in different foods. The sensitive of the four AFIs in study can change according to the matrices.

The results of this work also revealed that the mycotoxin reduction was higher when all mycotoxins were irradiated together. These results may suggest that higher concentrations of AFIs may favour the reduction. In fact, Zhang *et al.* (2018) reported AFB1 reductions of 62% and 76%, with radiation doses around 10 kGy in soybeans, contaminated with 7 and 20 μg/kg respectively. The increase of AFB1 concentration seems to increase the gamma effectiveness in samples. In another study, Abdel-Rahman *et al.* (2019) also demonstrated that the reduction increased by increasing the concentration of AFB1 in spiked samples, notably at the dose 20 kGy. Also, Jalili *et al.* (2010) noticed that the reduction ratios of AFIs in black pepper increased with increasing the AFIs concentration.

Although the present study shows that moisture and concentration are crucial to the effectiveness of gamma irradiation in AFIs destruction, several other components in matrices can affect their degradation. The work of Ghanem and collaborators (2008), that irradiated peanuts, peeled pistachios, unpeeled pistachios, corn, rice, barley, bran and corn samples, observed that AFB1 degradation in food samples correlated negatively with oil content of irradiated samples. In oil-crop food samples (i.e. corn, pistachios, and peanuts), a negative correlation was observed between percentage of oil content and percentage of AFB1 degradation. Thus, in peanuts, which contained the highest oil content, the percentage of AFB1 degradation at 10 kGy was not more than 56.6%, whereas, the corresponding value in corn, which contained the lowest oil content, reached as high as 80% (Ghanem *et al.*, 2008).

3.3.2 Cytotoxicity of irradiated AFIs

Although many studies have focused on the effectiveness of gamma radiation on AFIs reduction on different kind of food and feed, data on toxicity of radiolytic products of mycotoxins (products formed by gamma irradiation of mycotoxins) are lacking (Domijan *et al.*, 2019).

In the present study, a preliminary study about the reduction of cytotoxicity of irradiated AFIs was performed. As mentioned above, the cytotoxicity assays were performed with serial half-dilutions of the 30 μmol L^{-1} irradiated dried and water-dissolved AFIs samples. The cytotoxicity was measured through CFDA-AM, AB and NRU on HepG2 cells after 48 h of incubation.

The three dyes used in these assays measure different cells damages. The CFDA-AM assay is based on the conversion of CFDA-AM to its fluorescent product 5-CF by cytosolic esterases, which are only retained in cells with intact plasma membrane (Dayeh *et al.*, 2005). AlamarBlue is a commercial preparation of the dye resazurin, which is converted to a fluorescent form by viable cells. The diminishment of fluorescence indicates an impairment of cellular metabolism. The NRU assay is based on the accumulation of NR in functional lysosomes (Dayeh *et al.*, 2004).

The result of cytotoxicity effect of AFIs samples irradiated dehydrated is presented in Figure 3.9.

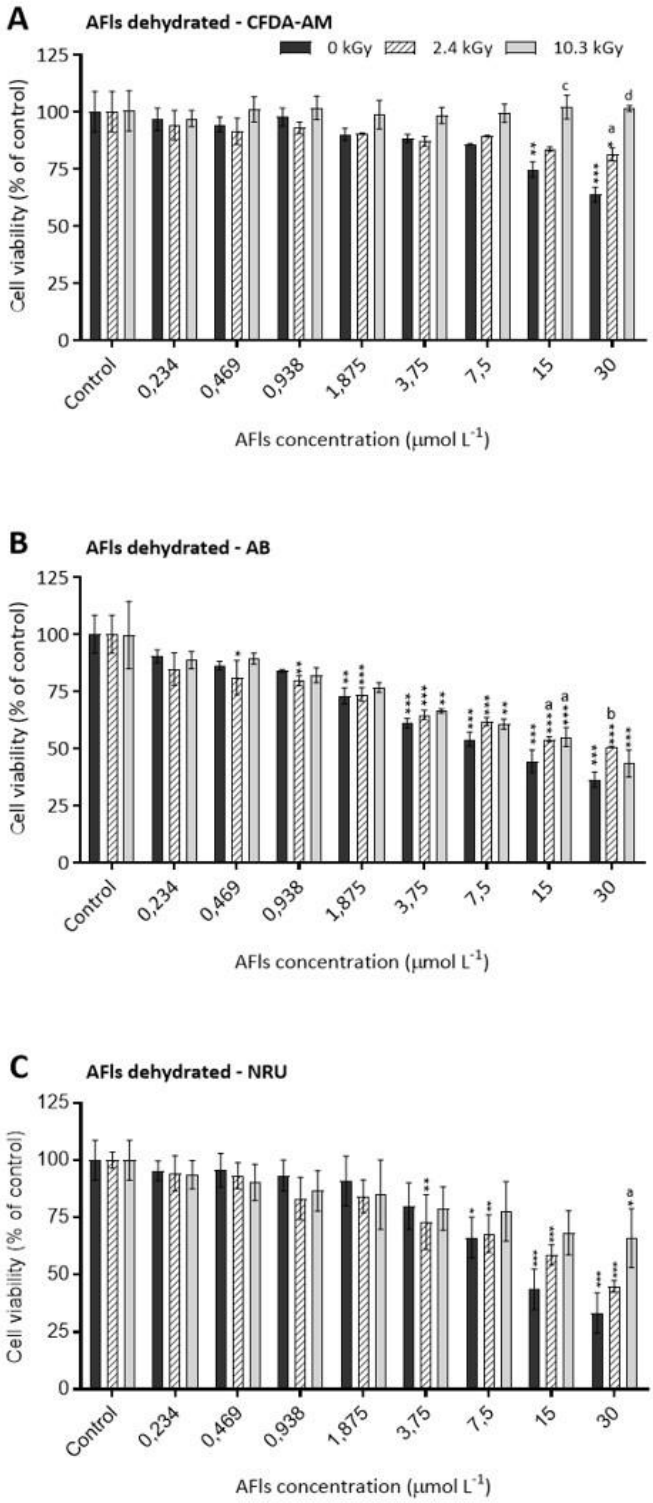

Figure 3.9 - Effects of AFIs dehydrated samples on HepG2 cell viability as assessed by means of (A), CFDA-AM (B) Alamar Blue (AB) and (C) Neutral Red uptake (NRU) assays. The bars represent the means and standard errors of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as follows: ⋆ for $p < 0.05$, ⋆⋆ for $p < 0.01$, ⋆⋆⋆ for $p < 0.001$. Statistically significant differences with respect to the non-irradiated samples (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as follows: a for $p < 0.05$, b for $p < 0.01$, c for $p < 0.001$.

The radiation doses tested were 2.4 kGy and 10.3 kGy. Non-irradiated AFIs samples at concentrations between 0.234 to 30 μmol L^{-1} were also tested causing a significant decrease of HepG2 cells viability that achieved about 37%, 64% and 67% at the highest concentration, to CFDA-AM, AB and NRU, respectively. To all dyes tested it was verified a viability increase of cells to irradiated samples.

To CFDA-AM dyes, the toxicity of samples irradiated with 10.3 kGy is clearly inferior to toxicity of non-irradiated samples and samples irradiated with 2.4 kGy. For the highest concentration, no reduction of viability was verified in cells treated with samples irradiated with 10.3 kGy. For samples irradiated with 2.4 kGy the decrease of viability was 19%.

To AB dyes, the reduction of viability was more evident. The significative reductions, when the sample was compared with controls, were observed for concentrations of 0.469 μmol L^{-1} or higher. The difference between irradiated and non-irradiated samples was less evident than for the other dyes. The samples irradiated with 2.4 kGy and with 10.3 kGy show a similar effect in cells. The AB is a non-toxic dye that can enter the cells and the mitochondria where it is reduced. The reduced dye is released in the medium. The percentage of reduction is dependent on cell viability. The results indicate that AFIs toxic effect was more evident in mitochondrial metabolic activity than in integrity of plasmatic membrane.

The results to NRU assays were similar to CFDA-AM assays. However, in opposition of the verified with CFDA-AM, at higher concentration, the higher radiation dose produced a 34% viability reduction.

AFIs toxicity depends of the dye used in its evaluation, which indicates the damage that was inflicted by the toxin. With irradiation, CFDA-AM and NRU assays indicated a reduction in the cytotoxicity. To CFDA-AM the on two higher concentration it was clearly that sample irradiated (10.3 kGy) is less toxic than non irradiated sample. The plasma membrane cells were less affect by AFIs irradiated than non-irradiated AFIs. However, all results show no increase of toxicity in irradiated samples.

The result of cytotoxicity effect of AFIs samples irradiated in water is presented in Figure 3.10.

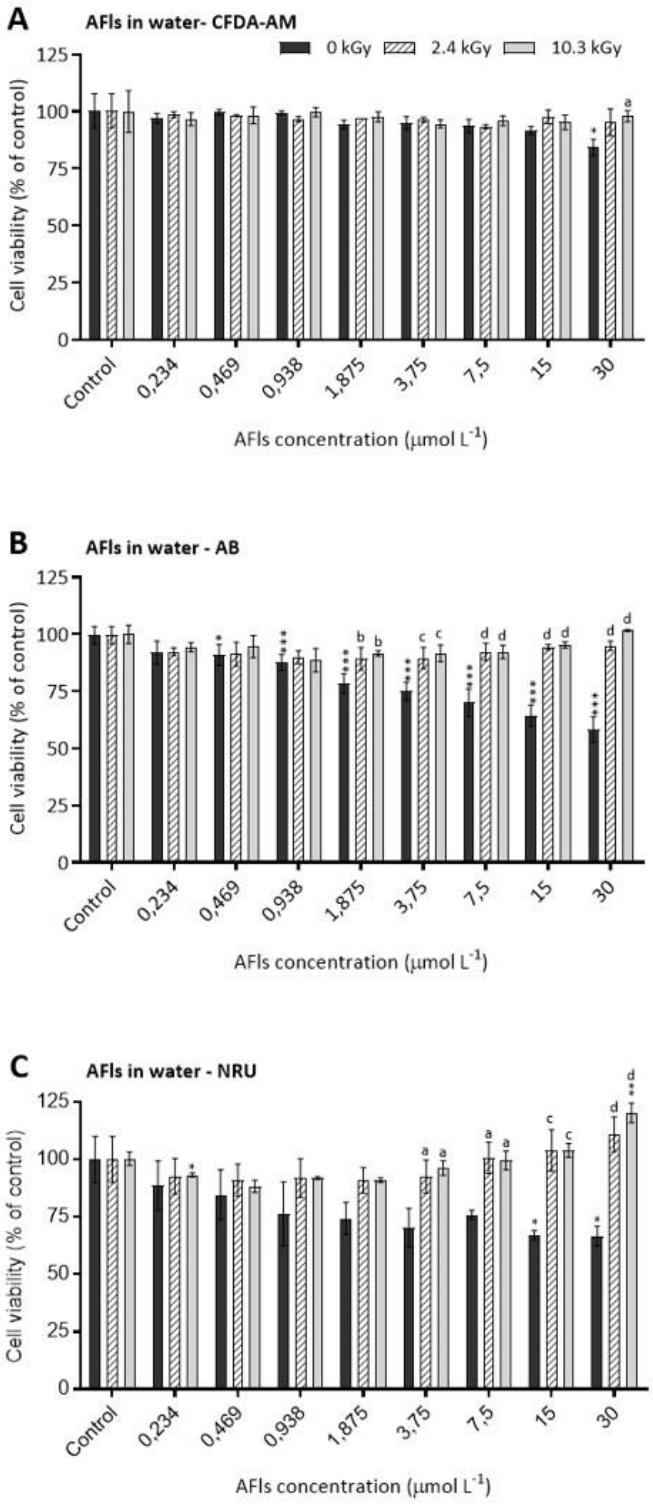

Figure 3.10 - Effects of AFIs water samples on HepG2 cell viability as assessed by means of (A), CFDA-AM (B) Alamar Blue (AB) and (C) Neutral Red uptake (NRU) assays. The bars represent the means and standard errors of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as follows: ⋆ for $p < 0.05$, ⋆⋆ for $p < 0.01$, ⋆⋆⋆ for $p < 0.001$. Statistically significant differences with respect to the non-irradiated samples (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as follows: a for $p < 0.05$, b for $p < 0.01$, c for $p < 0.001$.

As it was observed with dehydrated samples, no increase of toxicity was verified. The results show a clear reduction of toxicity of the irradiated samples. As it was observed for the dehydrated samples, the AB assay is the most sensitive one to the non-irradiated sample. However, in water, after irradiation at both doses, cytotoxicity was no longer detected. This observation can indicate that the degradation of AFIs in water solution was more efficient, as it was explained in the first part of this discussion.

As it is mentioned above, the cytotoxicity effect of gamma irradiated aflatoxins is still poorly studied. Domijan *et al.* (2019) tested cytotoxicity of AFB1 (irradiated and non-irradiated) against three different cell lines – SH-SY5Y, Pk15 and HepG2. For all the cell lines tested, authors verified a higher toxicity of non-irradiated AFB1 comparing to AFB1 irradiated samples, as it was observed in the present work. In 2011, Wang *et al.* conducted a study based on the structure–activity relationship analysis. In this study, the authors verified that the toxicity of radiolytic products was significantly reduced compared with the original AFB1 because the addition reaction that occurred on the double bond in the terminal furan ring. For this reason, authors concluded that gamma irradiation is an effective tool for the detoxification of AFB1 (Wang *et al.*, 2011). In the present study, for the two condition of irradiation, no increase of cytotoxicity was observed. However, the sample irradiated in water showed a more remarkable cytotoxicity reduction. It shows that gamma irradiation can be a promising tool for AFIs detoxification.

3.4 Conclusions

Gamma irradiation was shown capable to degrade AFIs, although the presence of water seems to play a very important role. When no moisture is present the degradation capacity of radiation doses of up to 8.6 kGy had little expression for the majority of AFIs. When individual assays were compared with mix assays the effectiveness of gamma radiation increased when all mycotoxins are present. These behaviours can be caused by increase of concentration of mycotoxins in mix assays. No increase of toxicity was observed when samples were gamma irradiated. The results suggest that gamma radiation is a feasible technology for the detoxification of AFIs. However, more studies are required in foods, to evaluated if AFIs degradation behaves as in standard solutions.

References

Abdel-Rahman, G. N., Sultan, Y. Y., Salem, S. H., & Amer, M. M. (2019). Identify the Natural Levels of Mycotoxins in Egyptian Roasted Peanuts and the Destructive Effect of Gamma Radiation. Journal of Microbiology, Biotechnology and Food Sciences, 9(2): 1174-1177.

ASTM. (1992). Practice for using the Fricke Reference Standard Dosimetry System, ASTM-E 1026 Annual Book of ASTM Standards (Vol. 12.02). Philadelphia, PA: ASTM.

Aquino, S., F. Ferreira, D. H. B. Ribeiro, B. Corrêa, R. Greiner and A. L. C. H. Villavicencio, (2005): Evaluation of viability of *Aspergillus flavus* and aflatoxins degradation in irradiated samples of maize. Brazilian Journal of Microbiology, 36: 352-356.

Bbosa, G. S., Kitya, D., Lubega, A., Ogwal-Okeng, J., Anokbonggo, W. W., & Kyegombe, D. B. (2013). Review of the biological and health effects of aflatoxins on body organs and body systems. Aflatoxins-recent advances and future prospects, 12:239-265.

Bennett, J. W., & Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16(3): 497-516.

Bhat, R., Rai, R. V., & Karim, A. (2010). Mycotoxins in food and feed: present status and future concerns. Comprehensive Reviews in Food Science and Food Safety, 9(1):57-81.

Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for Mold and Mycotoxin Control: A Review. Comprehensive Reviews in Food Science and Food Safety, 13(5):1049-1061.

Dayeh, V. R., Lynn, D. H., & Bols, N. C. (2005). Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicology in vitro, 19(3):399-410.

Dayeh, V. R., S. L. Chow, K. Schirmer, D. H. Lynn and N. C. Bols, (2004): Evaluating the toxicity of Triton X-100 to protozoan, fish, and mammalian cells using fluorescent dyes as indicators of cell viability. Ecotoxicology and Environmental Safety, 57:375-382.

Di Stefano, V., R. Pitonzo and G. Avellone, (2014a): Effect of gamma irradiation on Aflatoxins and Ochratoxin A reduction in almond samples. Journal of Food Research, 3.

Di Stefano, V., R. Pitonzo, N. Cicero and M. C. D'Oca, (2014b): Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of aflatoxins and ochratoxin A concentrations. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 31:2034-2039.

Domijan, A. M., Pleadin, J., Mihaljevic, B., Vahcic, N., Frece, J., & Markov, K. (2015). Reduction of ochratoxin A in dry-cured meat products using gamma-irradiation. Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32(7):1185-1191.

Farag SE-DA, Aziz NH and Attia E-SA. (1995). Effect of irradiation on the microbiological status and flavouring materials of selected spices. Z Lebensm Unters F 201:283-88.

Ghanem, I., M. Orfi and M. Shamma, (2008): Effect of gamma radiation on the inactivation of aflatoxin B1 in food and feed crops. Braz J Microbiol, 39:787-791.

Hooshmand, H., & Klopfenstein, C. F. (1995). Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat, and soybeans with different moisture contents. Plant Foods for Human Nutrition, 47(3):227-238.

IARC. (2002). Evaluation of carcinogenic risks to humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Lyon, France: IARCPress Retrieved from http://monographs.iarc.fr/ENG/Monographs/vol82/mono82.pdf.

Jalili, M., Jinap, S., & Noranizan, A. (2010). Effect of gamma radiation on reduction of mycotoxins in black pepper. Food Control, 21(10):1388-1393.

Jalili, M., Jinap, S., & Noranizan, A. (2012). Aflatoxins and ochratoxin a reduction in black and white pepper by gamma radiation. Radiation Physics and Chemistry, 81(11):1786-1788.

Kanapitsas, A., A. Batrinou, A. Aravantinos and P. Markaki, (2015): Effect of γ-radiation on the production of aflatoxin B1 by *Aspergillus parasiticus* in raisins (Vitis vinifera L.). Radiation Physics and Chemistry, 106:327-332.

Lammel, T., Boisseaux, P., Fernández-Cruz, M. L., & Navas, J. M. (2013). Internalization and cytotoxicity of graphene oxide and carboxyl graphene nanoplatelets in the human hepatocellular carcinoma cell line Hep G2. Particle and Fibre Toxicology, 10:1-27.

Larsson, P., & Tjälve, H. (2000). Intranasal Instillation of Aflatoxin B1 in Rats: Bioactivation in the Nasal Mucosa and Neuronal Transport to the Olfactory Bulb. Toxicological Sciences, 55(2):383-391.

Lee, D., Lyu, J., & Lee, K.-G. (2015). Analysis of aflatoxins in herbal medicine and health functional foods. Food Control, 48:33-36.

Mohamed, N. F., R. S. S. El-Dine, M. A. M. Kotb and A. Saber, (2015): Assessing the Possible Effect of Gamma Irradiation on the Reduction of aflatoxin B1, and on the Moisture Content in Some Cereal Grains. American Journal of Biomedical Sciences,7(1):33-39.

Mutluer, B., & Erkoç, F. (1987). Effects of gamma irradiation on aflatoxins. Zeitschrift für Lebensmittel-Untersuchung und Forschung, 185(5): 398-401.

Omar, H. E. M. (2013). Mycotoxins-induced oxidative stress and disease. Mycotoxin and Food Safety in Developing Countries. Rijeka, Croatia: InTech, 63-92.

Pereira, E., Barros, L., Antonio, A., Cabo Verde, S., Santos-Buelga, C., Ferreira, I., & Rodrigues, P. (2017). Is gamma radiation suitable to preserve phenolic compounds and to

decontaminate mycotoxins in aromatic plants? A case-study with *Aloysia citrodora* Paláu. *Molecules*, *22*(3):347.

Riley, R. T., Voss, K. A., Pestka, J. J., & Williams, D. E. (2011). Developing mechanism-based and exposure biomarkers for mycotoxins in animals. In S. De Saeger (Ed.), Determining Mycotoxins and Mycotoxigenic Fungi in Food and Feed, 262-265.

Rodrigues, P., Venâncio, A., Kozakiewicz, Z., & Lima, N. (2009). A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi isolated from Portuguese almonds. International journal of food microbiology, 129(2):187-193.

Rychlik, M., Humpf, H. U., Marko, D., Danicke, S., Mally, A., Berthiller, Klaffke, H., Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. Mycotoxin Res, 30(4):197-205.

Wang, F., F. Xie, X. F. Xue, Z. D. Wang, B. Fan and Y. M. Ha, (2011). Structure elucidation and toxicity analyses of the radiolytic products of aflatoxin B1 in methanol-water solution. Journal of Hazardous Materials, 192:1192-1202.

Zhang, Z. S., Q. F. Xie and L. M. Che, (2018). Effects of gamma irradiation on aflatoxin B1 levels in soybean and on the properties of soybean and soybean oil. Applied radiation and isotopes, 139:224-230.

Chapter 4. Gamma Irradiation on Zearalenone control

4.1 Introduction

Mycotoxins are secondary metabolites of filamentous fungi commonly found in feed and foodstuffs. Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin classified by IARC as a carcinogen group 3 (IARC, 2002; Ayed-Boussema *et al.*, 2008). This mycotoxin is biosynthesized through a polyketide pathway by several *Fusarium* species, among which *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* and *F. semitectum* are the most relevant ones (Zinedine *et al.*, 2007). Those are common soil fungi found in temperate and warm zones that frequently contaminate worldwide cereal crops, such as maize, barley, wheat, oats, rice and sorghum (Zinedine *et al.*, 2007; Ji *et al.*, 2014).

Since ZEA is heat stable, it is not substantially eliminated from raw materials by processing methods commonly adopted in the food industry, thus being detected in many final-products, like bread or breakfast cereals (Aziz *et al.*, 1997; Bullerman and Bianchini, 2007; Iqbal *et al.*, 2014). This constitutes a serious health problem because ZEA has important toxicological properties. The most relevant one is its estrogenicity since several studies demonstrate that ZEA is implicated in reproductive disorders that result in functional and morphological alterations in reproductive organs in different domestic animal species, particularly in pigs (Gutleb *et al.*, 2002; Cortinovis *et al.*, 2013). After oral administration, ZEA is rapidly absorbed and biotransformed, mainly in the liver, to α-Zearalenol (α-ZOL) and β-Zearalenol (β-ZOL), then reduced to α-Zearalanal (α-ZAL) and β-Zearalenal (β-ZAL), respectively (Tatay *et al.*, 2014). ZEA, α-ZAL and β-ZAL, catalyzed by uridine diphosphate glucuronyl transferases, can be conjugated with glucoronic acid which facilitates their elimination via urine, feces and bile (Zinedine *et al.*, 2007). Nonetheless, conjugated metabolites excreted via the bile are reabsorbed by the intestinal mucosal cells, ultimately entering again in the liver and the systemic circulation via the portal blood supply (Zinedine *et al.*, 2007). This circulation of ZEA and its derivatives extends their biological half-life and increases their total toxicity. Due to the structural similarity of ZEA and its metabolites with endogenous estrogen (17β-estradiol (E2)), these compounds can bind to estrogen receptors (Li *et al.*, 2012), causing reproductive disorders. In addition to estrogenic effects, the toxicity of ZEA includes genotoxicity (Ayed-Boussema *et al.*, 2007), cytotoxicity (Abid-Essefi et al, 2004) immunotoxicity (Marin *et al.*, 2011), reproductive toxicity (Gutleb *et al.*, 2002; Jia *et al.*, 2014), increase of reactive oxygen species in cells (Ferrer *et al.*, 2009;Jia *et al.*, 2014), developmental toxicity (Prouillac *et al.*, 2009), hematotoxicity and

hepatotoxicity (Salah-Abbes *et al.*, 2009). Thus, strategies to reduce or eliminate the toxic effects of ZEA are needed to improve food safety and minimize economic losses in livestock production (Cetin and Bullerman, 2005).

One of the physical methods that can be used to control the presence of mycotoxins in food and feed is irradiation. Presently, due to the power of penetration of gamma rays along with its broad-spectrum efficacy against microorganisms, gamma radiation is the preferred method to irradiate commodities (Fernandes *et al.*, 2012). In the specific subject of mycotoxins, gamma radiation can on the one hand inhibit or delay the development of the mycotoxigenic fungi and, consequently, the production of mycotoxins; and on the other hand, exerts a direct action on mycotoxins, by degrading them (Calado *et al.*, 2014). The elimination of mycotoxins by gamma radiation is a subject that has been widely investigated but the available literature is not always consensual about its efficacy. The irradiation process can be influenced by many factors such as the absorbed dose, the average dose rate, the initial mycotoxin concentration and the moisture content (Wang *et al.*, 2011; Calado *et al.*, 2014), explaining some contradictory results reported in the literature. Moreover, differences in the degradation products formed have also been reported (Wang *et al.*, 2011).

The degradation of mycotoxins may form other compounds (radiolytic products) that can be as or more toxic than the original mycotoxin. According to Rychlik *et al.* (2014), the potential exposure to modified mycotoxins is an additional risk to human and animal health. Thus, the study of mycotoxins degradation should be complemented with assays on the safety of the irradiated product. The detection, identification and isolation of each radiolytic product is the ideal option. However, due to the diversity of the produced radiolytic products and to their very low concentration, make this is not always feasible (Wang *et al.*, 2011). One excellent alternative is to study the toxicity of radiolytic product using *in vitro* assays.

In vitro assays using cells have the advantage of minimizing animal use, allowing the testing of a wide range of chemicals and concentrations (Eisenbrand *et al.*, 2002; Li *et al.*, 2014). However, the use of cells in the toxicological studies requires rapid and sensitive cell viability assays. Viability tests in microwell plates using fluorescent dyes have several desirable features. Many indicator dyes have become commercially available therefore there is a large range of cellular parameters that potentially can be monitored (Dayeh *et al.*, 2004). Examples of indicator dyes are Alamar Blue (AB) for changes in energy metabolism, carboxyfluorescein diacetate acetoxymethyl ester (CFDA-

AM) for evaluating membrane integrity, and neutral red (NR) for evaluating lysosomal function (Dayeh *et al.*, 2005).

According to previous studies, ZEA can induce liver toxicity (Zinedine *et al.*, 2007; Fink-Gremmels and Malekineiad, 2007; Riley *et al.*, 2011). Maaroufi *et al.* (1996) concluded that ZEA exerts liver toxicity in rats and Čonková *et al.* (2001) observed the same toxicity in rabbits. The human hepatoma HepG2 cells were reported to retain many of the properties of primary liver cells and, for this reason, this cells line can be a good approximation to a real situation (Li *et al.*, 2014).

The estrogenicity of ZEA after irradiation should also be studied to prove that the radiolytic products do not increase the estrogenic potency. According to the Organization for Economic Co-operation and Development (OECD), one cell line that can be used to screen and test potential endocrine disrupting chemicals is the HeLa cells transfected to express the human estrogen receptor alpha (hERα) – the hERα-HeLA-9903 (OECD, 2009).

The purposes of the present study were:

(i) to investigate the effect of different doses of gamma radiation on the degradation of ZEA under different moisture conditions;

(ii) to evaluate the cytotoxicity of irradiated ZEA; and

(iii) to evaluate the estrogenicity of irradiated ZEA.

4.2 Material and methods

4.2.1 Chemicals and reagents

Methanol was obtained from Merck (Lisbon, PT). Standard of ZEA (Z2125-10MG, Sigma) was purchased from Sigma-Aldrich (Sintra, PT). Ultraglutamine 1 (200 mM) (L-Gln), fetal bovine serum (FBS), penicillin and streptomycin (P/S) (10000 U/mL / 10 mg/mL), non-essential amino acids (NEAA) 100X, Trypsin EDTA (200 mg/L EDTA, 17000 U trypsin/L) and cell culture EMEM (Eagle's Minimum Essential Medium) were sourced by Lonza (Barcelona, ES). Phenol red-free Minimum Essential Medium (MEM), Charcoal-dextran stripped fetal bovine serum (FBS-charcoal) and kanamycin (Kan) were purchased from PAN-Biotech (Aidenbach, DE). AlamarBlue, 5-carboxyfluorescein diacetate and acetoxy methyl ester (CFDA-AM) were purchased from Life

Technologies (Madrid, ES). Neutral red (3-amino-7-dimethylamino-2-methylphenanzine hydrochloride) solution (0.33%), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), *in vitro* Toxicology Assay Kit Resazurin based, 17β-estradiol (E2) and glacial acetic acid were acquired from Sigma-Aldrich (Madrid, ES). Ethanol was purchased from Panreac (Barcelona, ES). High grade purity water (> 18 MΩ/cm) was obtained from a Milli-Q Element A10 Century (Millipore Iberia, ES).

4.2.2 Preparation of ZEA solutions and irradiation process

A stock solution of commercial standard ZEA at a concentration of 1 mg mL^{-1} was prepared in 10 mL of methanol and it was stored at -20 °C until use. The appropriate amount of the stock was pipetted into clean amber 2 mL vials to prepare the samples of 3 μmol L^{-1} of ZEA. Samples were then evaporated at 50 °C under a gentle stream of nitrogen. To study the effects of irradiation, three types of ZEA samples were prepared to represent different moisture contents. A set of samples was kept dried, another set was resuspended in 1 mL of deonized water (H_2O_{dd}) and the last set was resuspended in 1 mL water/methanol (50/50, v/v). After being prepared, samples were kept in amber vials and stored at -20 °C until irradiated. For the cytotoxicity study, ZEA samples at concentrations of 60 μmol L^{-1} in H_2O_{dd} or dried were prepared as described above. The increase of ZEA concentration was necessary to detect its cytotoxicity.

The irradiations were performed at room temperature in a Co-60 experimental equipment Precisa 22 (Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 177 TBq (4.78 kCi; February 2014), located at C2TN. The average dose rate was 1.8 kGy/h and was previously determined by Frick reference dosimeter (ASTM, 1992). To monitor the process during irradiation, two routine dosimeters (Amber Perspex dosimeters, Batch X, from Harwell Company, U.K.) were used to estimate the highest and the lowest dose absorbed by the samples. The absorbance and thickness of Amber Perspex dosimeters were measured in a UV-VIS Spectrophotometer (UV 1800, Shimadzu, USA) at 603 nm and in a micrometer (Mitutoyo America Corporation, USA), respectively, in order to estimate the dose according to a previous calibration curve. Samples of ZEA (dried, aqueous and methanolic) were irradiated at 0.4, 0.9, 1.7, 2.5, 5.4 and 8.6 kGy, in triplicates. To evaluate the cytotoxicity after irradiation, only dried and aqueous solutions of ZEA at 60 μmol L^{-1} were submitted to radiation doses of 2.4 and 10.3 kGy. During the irradiation process the vials were kept closed. Non-irradiated controls were also prepared for each condition.

4.2.3 Determination of ZEA levels

The HPLC analysis was carried out with a modified version of method described in Keller *et al.* (2015). The HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λexc = 280 nm and λem = 460 nm), a Varian 850-MIB data system interface and a Galaxie chromatography data system. The chromatographic separation, was performed with a 25 min isocratic run on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 μm), fitted with a pre-column of the same stationary phase. The mobile phase was a mixture of methanol/water/acetic acid (65:35:1, v/v/v) that was filtered and degassed with a 0.2 μm membrane filter (GHP, Gelman). The flow rate was set to 1.0 mL/min and the column temperature to 30 °C. The injection volume was 50 μL. ZEA was identified by retention times (21 min) and quantified by measuring peak areas and comparing them with respective calibration curve, which was prepared by dissolving the required amount of working standard solution in mobile phase. Two calibration curves were prepared. The first one, with ZEA concentrations from 0.8 μmol L^{-1} to 3.1 μmol L^{-1}, used with the detector gain set to 1000 to quantify the samples of ZEA with 3 μmol L^{-1}. The second one, with ZEA concentrations from 3.9 μmol L^{-1} to 63 μmol L^{-1} used with gain set to 100 to quantify 60 μmol L^{-1} ZEA samples.

4.2.4 Cytotoxicity studies

4.2.4.1 Cells culture and exposure

As mentioned above, for the cytotoxicity study, ZEA samples at concentrations of 60 μmol L^{-1} in H_2O_{dd} or dried were prepared. The cell line HepG2 obtained from American Type Culture Collection (ATTC) (Manassas, VA, USA) was cultured in 75 cm^2 Cell Star Cell Culture flasks (Greiner Bio-One GmbH, Frickenhausen, DE) in EMEM supplemented with 1% NEAA , 1% P/S, 1% L-Gln and 10% FBS (in the following text referred as EMEM+). The flasks were incubated at 37 °C in a humidified 5% CO_2 atmosphere and split twice a week using PBS/EDTA and trypsin.

A HepG2 cell suspension (5×10^5 cells/mL) with EMEM+ was seeded into transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, DE) by adding 100 μL of cell suspension to each well. The plates were incubated for 24 h and exposed for 48 h to different concentrations of ZEA. Exposure concentrations of irradiated and non-irradiated ZEA samples were prepared by drying (if suspended in water) as described in section 4.2.2 and re-suspending in EMEM+

supplemented with 0.5% of DMSO (to improve the solubility) to a final concentration of 60 µmol L^{-1}. Then, those solutions were applied to the cell culture plate in which successive serial dilutions (dilution factor of 2) were performed. As a positive control, a subset of wells was treated with increasing concentrations of SDS (15.6 µmol L^{-1} – 500 µmol L^{-1}, dilution factor 2/3). Cells treated with EMEM+ served as negative control whereas cells treated with 0.5% (v/v) DMSO/EMEM+ were the vehicle control.

4.2.4.2 AB, CFDA-AM and NR Uptake (NRU) assays

The Alamar Blue (AB), 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester (CFDA-AM) and Neutral Red Uptake (NRU) assay were performed on the same set of cells. The assays were conducted following a combined version of the protocol described by Lammel *et al.* (2013). Prior to adding the reactants, the exposure medium was removed and the cells rinsed twice with 200 µL of phosphate-buffered saline (PBS). Then, 100 µL phenol red-free MEM containing 1.25% (v/v) AB and 4 µmol L^{-1} CFDA-AM was added to each well. The 96-well plates were incubated for 30 min in the dark as described above. Subsequently, the fluorescence intensity was measured at λexc=532 nm and λem=590 nm for the AB assay, and at λexc=485 nm and λem=535 nm for CFDA-AM assay using a microplate reader (Tecan Genios, Tecan Group Ltd., Männedorf, CH). Subsequently, the medium was removed and the cells were washed once with PBS. One hundred µL of NR solution (0.03 mg/mL in phenol red-free MEM) were added per well and the plates were incubated for 1 h in the dark as described above. After the incubation period, the NR solution was removed, the cells were rinsed twice with 200 µL PBS and the retained NR in the cells was extracted with an acidified solution composed of 1% glacial acetic acid/50% ethanol/49% Milli-Q water (150 µL/well). NR fluorescence was measured at λexc=532 nm and λem=680 nm. The fluorescent values were corrected for the cell-free control and normalized against the vehicle control. Fluorescence spectra of ZEA samples (irradiated or not) did not show any significant fluorescence at the excitation/emission wavelengths that were used in the AB, CFDA-AM and NRU.

4.2.5 Estrogenicity studies

4.2.5.1 Cell culture and exposure

To conduct estrogenicity studies, the hERα-HeLa-9903 cell line has been used. This cell line is derived from a human cervical tumor, with two stably inserted constructs: (i) the hER expression

construct (encoding the full-length human receptor), and (ii) a firefly luciferase reporter construct of an Estrogen-Responsive Element (ERE). The cell line hERα-HeLa-9903 was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). It was cultured in 75 cm^2 cell culture flasks treated by vacuum Gas plasma (Becton Dickinson, France) in phenol red-free MEM supplemented with 10% FBS-charcoal, 1% L-Gln, 1% Kan and 1% P/S (in the following text referred to as MEM+). The flasks were incubated at 37 °C in a humidified 5% CO_2 atmosphere and split twice a week using PBS/EDTA and trypsin. A Hela 9903 cell suspension (10×10^5 cells/mL) in MEM+ was prepared. hERα-HeLa-9903 cells were seeded into opaque, flat-bottom 96-well plates (Perkin Elmer, Frickenhausen, DE) by adding 100 µL of cell suspension to each well. The plates were incubated as described above for 3 h.

For the determination of ZEA estrogenicity of irradiated and not irradiated ZEA samples the ZEA solutions of 3 and 60 µmol L^{-1} were diluted into MEM+ to achieve a work solution of 1 µmol L^{-1}. The ZEA working solutions were applied to the cell culture plate in which successive serial dilutions (dilution factor of 2) were performed. As a positive control, a subset of wells was treated with increasing concentrations of E2 (0.004 nmol L^{-1} – 0.125 nmol L^{-1}, dilution factor 2). Cells treated with MEM+ served as negative control and vehicle control. The microwell plates were incubated at 37 °C in a humidified CO_2 atmosphere for 24 h and then subjected to analysis.

4.2.5.2 Transactivation assay

hERα-HeLa-9903 cell line allows conducting the transactivation assay. The assay is used to signal binding of the estrogen receptor with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements (ERE) and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. The assay was conducted following a modified version of the OECD Guideline 455. First, the viability of cells was confirmed by adding 5 µL of resazurin solution per well and by incubating plates for 90 min in the dark at 37 °C in a humidified 5% CO_2 atmosphere. After the incubation period, the fluorescence intensity was measured at λexc=532 nm and λem=590 nm. The results of this assay are not included as no toxic effects were observed at the doses tested indicating that the assay has been conducted adequately. Luciferase activity was measured using a luciferase reporter gene assay kit (Biodetection Systems, Amsterdam, NL) according to the manufacturer's instructions with small

modifications. Briefly, 90 μL of PBS (pH 7.5) and 30 μL of the lysis buffer were added. After 15 min, 80 μL of the luciferase reagent was added and luminescence immediately and was measured using a liquid scintillation counter (1450 MicroBeta Trilux, PerkinElmer, Spain). The luminescence values were normalized against the negative control.

4.2.6 Statistical analysis

Statistical analysis was performed using Sigma Plot version 12.0 (Jandel Scientific, San Rafael, CA, USA). To evaluate significant differences between concentrations of ZEA in non-radiated and irradiated samples, means were compared by analysis of variance followed by the Ducan's post-test. Results of cytotoxicity and estrogenicity assays represent the means and standard errors (SEM) of at least three independent experiments, in which each treatment was applied in triplicate. Significant differences among treatments were determined by one-way repeated measurement analysis of variance (rmANOVA, $p < 0.05$, α 0.05). All data were tested beforehand for normality (Shapiro-Wilk test, $p < 0.05$) and equal variance ($p < 0.05$). Significant differences between treatments and the control were determined by applying a Dunnett's Post hoc test to one-way repeated measurement (RM) ANOVA analyses.

4.3 Results and discussion

4.3.1 ZEA concentration after irradiation

The effect of gamma radiation doses on ZEA samples at the lower concentration tested (3 μmol L^{-1}) is presented in Figure 4.1. The maximum elimination was observed in samples of ZEA dissolved in water. In this case, the lowest radiation dose applied (0.4 kGy) was sufficient to achieve complete degradation of ZEA. On the opposite side, the irradiation of dried ZEA resulted in the lowest reductions in ZEA concentration. In this case, significant reductions of ZEA (between 65% and 87%) were only observed with doses ≥ 1.7 kGy and the complete elimination of ZEA was not achieved even at the highest dose tested (8.6 kGy). In the assays performed with the mycotoxin dissolved in water/methanol solution, a significant ZEA reduction of 24% was observed at 0.9 kGy, while a reduction of 97% was observed for the highest dose of radiation. The HPLC analysis did not revealed any fluorescent degradation products that may have been produced during the irradiation

process of ZEA. Major ZEA derivatives, α-zearalenol (α-ZOL), β-zearalenol, (β-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL), were not detected in chromatograms. As mentioned previously, the effectiveness of irradiation process can be affected by several factors, making a difficult comparison with studies available in the literature. Nevertheless, the observed increase of ZEA degradation with increasing gamma radiation doses (Figure 4.1) corroborates with some published works. For example, Hooshmand and Klopfenstein (1995) demonstrated a 25% reduction of ZEA concentration after irradiation of contaminated corn at 10.0 kGy and Aziz *et al.* (1997) verified a total elimination of ZEA in wheat and flour exposed to a radiation dose of 8.0 kGy.

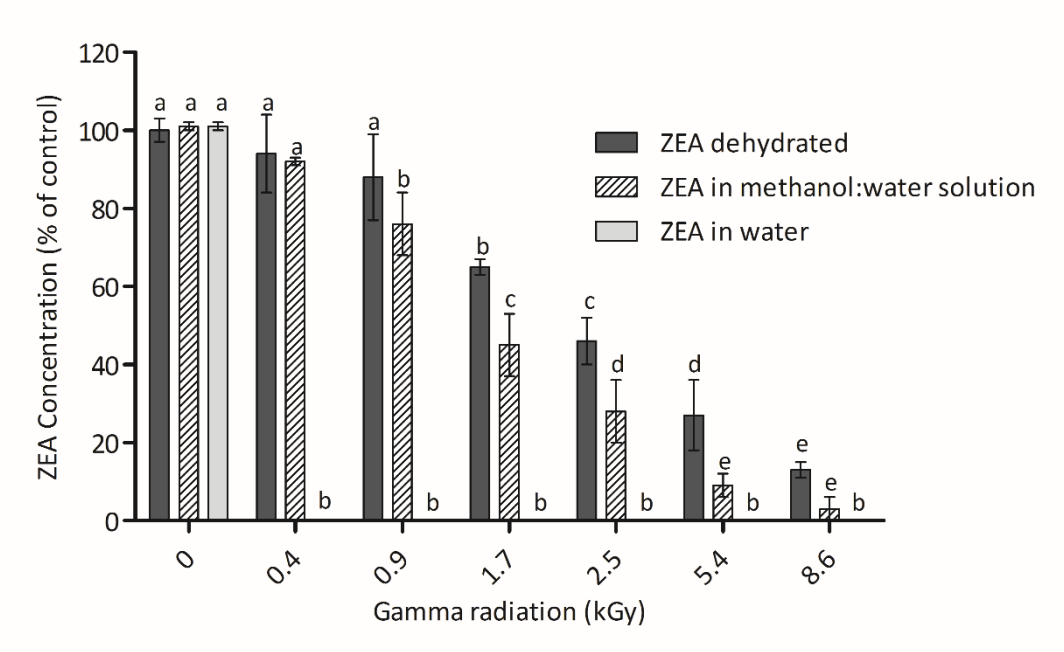

Figure 4.1 - Effect of increasing radiation doses on ZEA (3 μmol L^{-1}) in different conditions. Statistically significant differences in each condition are indicated with different letters, by analysis of variance (two-way rmANOVA, Ducan´s Post-hoc test).

The effect of moisture is overt in our results, particularly when low radiation doses were used. This observation is in agreement with studies using other mycotoxins (Mutluer and Erkoc, 1987; Jalili *et al.*, 2010: Jalili *et al.*, 2012). The higher effect of radiation in the presence of water is justified by the water radiolysis. In this process, the ionization of water occurs, resulting in splitting of water molecules into positively charged water radicals (H_2O^+) and negative free solvated electrons (e–); which after various recombination and cross-combination reactions originates the reactive species e–aq, H•, HO•, HO_2•, OH–, H_3O+, H_2, and H_2O_2. These compounds/radicals are reactive against double bonds, especially to those found in aromatic or heterocyclic rings (Jalili *et al.*, 2012), and

may explain the high reductions observed for ZEA. Eventually, these mechanisms may reduce the mutagenicity and toxicity of mycotoxins.

On the other hand, ZEA elimination in 60 µmol L^{-1} samples was less efficient than previously observed in the 3 µmol L^{-1} ZEA samples. As mentioned above, for 3 µmol L^{-1} aqueous samples, the complete elimination of ZEA was observed at very low radiation doses (≥ 0.4 kGy) and no fluorescent degradation products were observed in samples. However, for 60 µmol L^{-1} aqueous samples, a significant reduction of ZEA was only achieved at higher doses (90% and 96% with 2.4 kGy and 10.3 kGy, respectively). Concerning the irradiation of 60 µmol L^{-1} dried ZEA samples with 2.4 kGy and 10.3 kGy, reductions of only 18% and 21%, were respectively obtained. Calado *et al.* (2014) mentioned that the mycotoxin concentration is one of the aspects involved in effectiveness of mycotoxin irradiation process. Until recently, this effect was not reported for ZEA, it was only studied for others mycotoxins. For example, Van Dyck and others (1982) verified that the effect of gamma radiation was substantially reduced when the concentration of aflatoxin B1 (AFB1) was increased 50 times. Similar results were reported by Mutluer and Erkoc (1987) for aflatoxins (B1, B2, G1 and G2). In spite of this, some authors have verified the opposite effect. Jalili *et al.* (2010), Zhang *et al.* (2018) and Abdel-Rahman *et al.* (2019) described that an increase of AFIs concentration seems to increase the gamma effectiveness in samples. Recently, Kalagatur and collaborators (2018) studied the effect of gamma irradiation on ZEA detoxification and also verified that the increase of ZEA concentration lead to a reducted gamma irradiation effectiveness. Our results are in agreement with these latter authors.

4.3.2 Cytotoxicity of irradiated ZEA

The removal of mycotoxins from food and feed is an area that has gathered research interests and gamma radiation processes has shown promising results. However, the confirmation of the decrease of mycotoxins toxicity after irradiation has been poorly studied.

The cytotoxic effect, measured through AB, of ZEA on HepG2 cells after 48 h of incubation is shown in Figure 4.2A and Figure 4.2B. As mentioned above, the cytotoxicity assays were performed with serial half-dilutions of the 60 µmol L^{-1} irradiated dried and water-dissolved ZEA samples. Non-irradiated ZEA samples at concentrations between 0.5 to 60 µmol L^{-1} were also tested causing a significant decrease of HepG2 cells viability that achieved around 60% at the highest concentration, as shown in Figure 4.2A and Figure 4.2B.

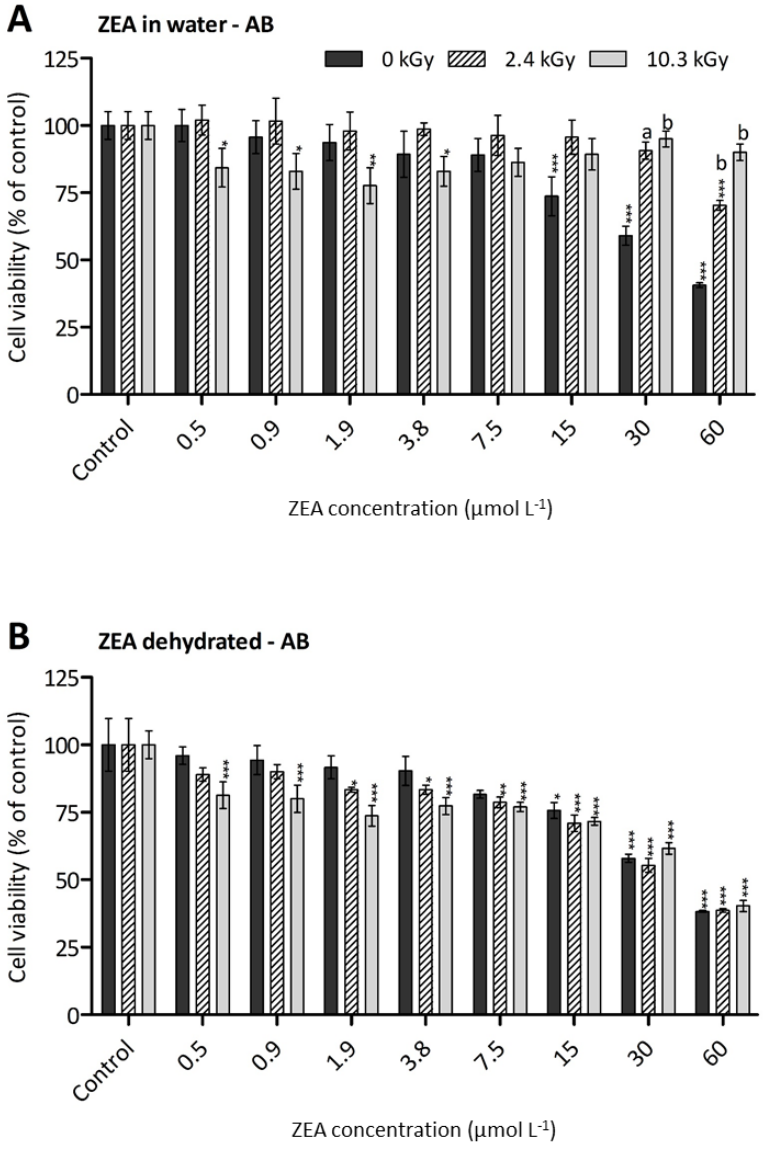

Figure 4.2 - Effect of ZEA samples on HepG2 cell viability assessed by means of the alamarBlue assay. Bars represent the mean and standard error of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as followed: ⋆ for $p < 0.05$, ⋆⋆ for $p < 0.01$, ⋆⋆⋆ for $p < 0.001$. Statistically significant differences with respect to non-irradiated sample (0 kGy) and the irradiated samples (2.4 kGy and 10.3 kGy) (one way ANOVA, Dunnett´s Post-hoc test) are indicated as followed: a for $p < 0.01$, b for $p < 0.001$, no letter for no statistical differences.

AlamarBlue is a commercial preparation of the dye resazurin, which is converted to a fluorescent form by viable cells. The diminishment of fluorescence indicates an impairment of cellular metabolism (Dayeh *et al.*, 2004). Comparing with controls, non-irradiated ZEA samples showed a significant reduction of fluorescence both in water and dried form. Regarding non-irradiated ZEA in water, although the trend was already discernible at concentrations as low as 0.9 µmol L^{-1}, the first statistical significant difference, respecting the vehicle control, was only detected at 15 µmol L^{-1}

(Figure 4.2A). Also, for dry non-irradiated ZEA, the trend was already perceptible at concentrations as low as 0.5 μmol L^{-1}, but the first statistical significant difference regarding the vehicle control was only detected at 15 μmol L^{-1} (Figure 4.2B).

ZEA samples irradiated at 2.4 kGy in the presence of water (Figure 4.2A), showed a significant statistical reduction of the toxic effect observed with non-irradiated ZEA. This result indicates that this radiation dose is effective to eliminate ZEA and its associated toxic effects for concentrations below 30 μmol L^{-1}. At the radiation dose of 10.3 kGy the toxic effect of ZEA was eliminated even at the higher ZEA concentration.

For dehydrated samples a different trend was observed (Figure 4.2B). In this case, the pattern observed with non-irradiated and irradiated samples was very similar. This result indicates that the reduction in toxicity after irradiation was low and similar for both doses of radiation. It is necessary to remember that, for samples irradiated under this condition, ZEA reductions of only 18 % and 21 % for 2.4 kGy and 10.3 kGy were observed, respectively. In others words the real ZEA concentration in samples was close and, thus a similar effect was obtained.

Figure 4.3A and Figure 4.3B show the cytotoxic effect of ZEA measured through the CFDA-AM assay. The CFDA-AM assay is based on the conversion of CFDA-AM to its fluorescent product 5-CF by cytosolic esterases, which are only retained in cells with intact plasma membrane (Dayeh *et al.*, 2005). For samples of ZEA in water, only the higher concentration of the non-irradiated sample presented significant statistical differences in respect to the vehicle control. Similar results were obtained for ZEA dry samples. With this reagent/dye, the decrease in fluorescence intensity was less prominent than with AB. This suggested that ZEA has a higher effect on the metabolic activity of the cell than in the plasma membrane activity. The same effect was observed by Ayed-Boussema *et al.* (2008), which verify that ZEA reduced HepG2 cells proliferation (IC_{50} about 100 μmol L^{-1}) and concluded that the reduction of cells is mainly due to apoptosis rather than necrosis only observed at high concentrations. These authors concluded that apoptosis is the major cause of ZEA-induced cells death and suggested that ZEA induces an activation of pro-apoptotic genes and does not interfere in anti-apoptotic proteins, causing an imbalance that leads to apoptosis. Furthermore, proteins and genes activated by ZEA lead to the disruption of mitochondrial membrane (Ayed-Boussema *et al.*, 2008) explaining the decrease of fluorescence when AB was used. With CFDA-AM, ZEA toxicity (dried and dissolved in water) was only reduced after irradiation with the highest dose (10.3 kGy) (Figure 4.3A and B). In relation to non-irradiated ZEA, CFDA-AM fluorescence was

increased respectively by 41% and 27% for the dried and dissolved conditions, showing significant reduction of toxicity.

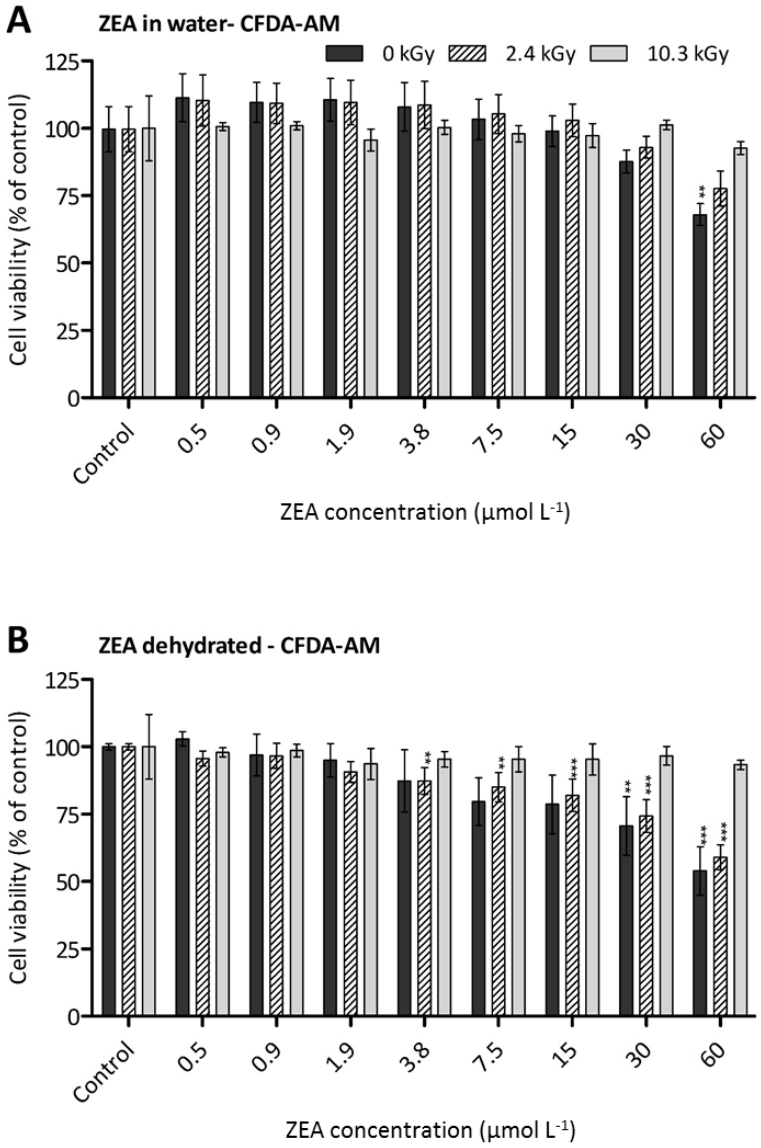

Figure 4.3 - Effect of ZEA samples on HepG2 cell viability assessed by means of the CFDA-AM assay. Bars represent the mean and standard error of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as followed: ⋆ $p < 0.05$, ⋆⋆ $p < 0.01$, ⋆⋆⋆ $p < 0.001$.

Figure 4.4A and Figure 4.4B show the cytotoxic effect of ZEA, measured through NR, on HepG2 cells after 48 h of incubation. The NRU assay is based on the accumulation of NR in functional lysosomes. In this case, the trend was similar to the AB assay for aqueous and dehydrated samples. With NR, ZEA toxicity of samples dissolved in water was reduced after irradiation with both doses and an increase of NR fluorescence of 23% and 53% at the 60 µmol L^{-1} ZEA

concentration was respectively observed with 2.4 and 10.3 kGy. In all these assays, the toxicity remaining in samples after irradiation is probably due to the presence of residual ZEA rather than to degradation products because they were not observed in HPLC chromatograms as explained before. Additionally, no toxigenic compounds were generated after irradiation because no increase of toxicity was observed.

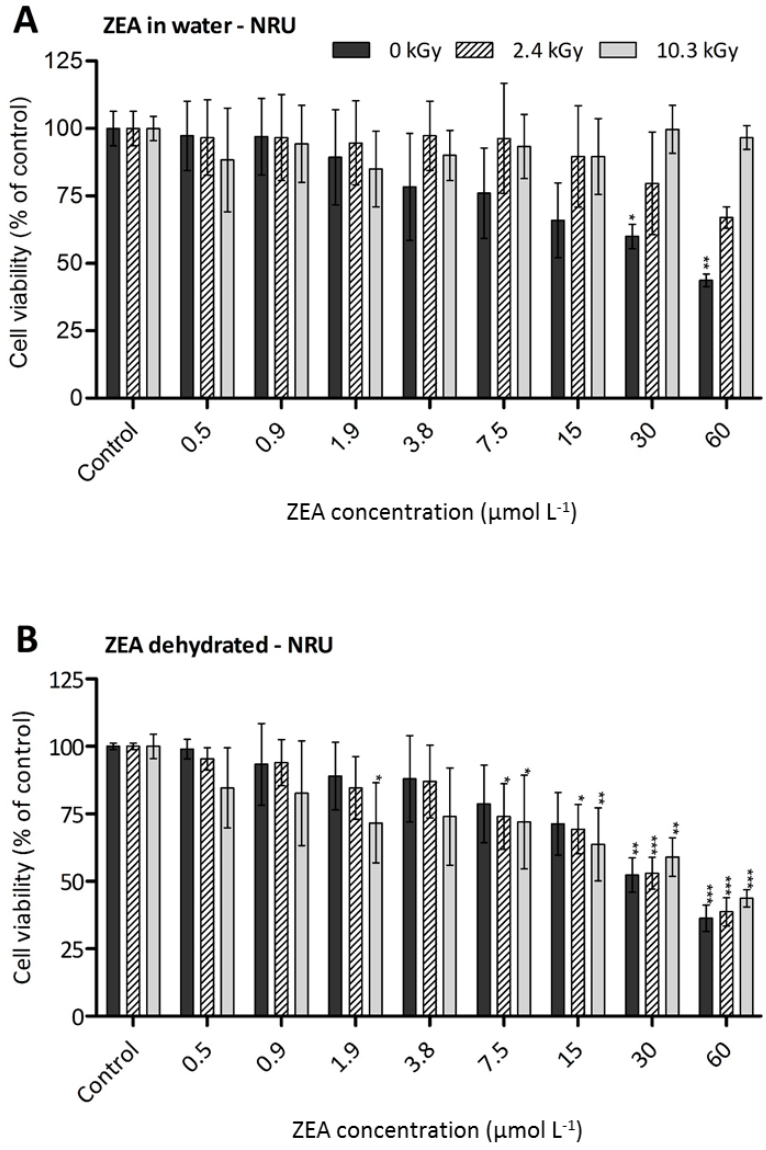

Figure 4.4 - Effect of ZEA samples on HepG2 cell viability assessed by means of the NRU assay. Bars represent the mean and standard error of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as followed: ⋆ $p < 0.05$, ⋆⋆ $p < 0.01$, ⋆⋆⋆ $p < 0.001$.

As it was mentioned above, ZEA detoxification by gamma irradiation is poorly studied and as a result, the are few studies about the toxicological safety of degradation products from irradiated

ZEA. Kalagatur and colleagues (2018) tested in macrophage cell line (RAW 264.7) the toxicity of irradiated and non-irradiated hydrated ZEA samples. The results of MTT assay and live/dead cells assay shown significant reductions of toxicity. These results corroborate findings from the current work.

4.3.3 Estrogenicity of irradiated ZEA

The estrogenic effect of ZEA and its metabolites are known and largely studied by several authors (Li *et al*,. 2012; Cortinovis *et al.*, 2013). The ideal detoxification method must decrease the estrogenicity of ZEA and of eventual degradation products. The dose-response curves shown in Figure 4.5A were obtained after exposure of hERα-HeLa-9903 cells for 24 h to irradiated ZEA aqueous samples with initial concentration of 3 μmol L^{-1}. Results reveal that the exposure to non-irradiated ZEA for 24 h produced an increase of the luminescence intensity, indicating an intensification of luciferase activity. This rise was already discernible at concentrations as low as 6.1E-05 μmol L^{-1}, but the first statistical significant difference, respecting the vehicle control, was only detected at 1.95E-03 μmol L^{-1}. All the irradiated sample doses showed no significant difference when compared to the vehicle control between them. This result shows that the two radiation doses tested in these conditions make a complete reduction of ZEA estrogenicity. These results corroborate with the HPLC results where no ZEA was detected (Figure 4.1). When the irradiation was made in dehydrated conditions (Figure 4.5B) the result was very different. In this case only a small reduction of the luminescence intensity was observed. Once again the results agree with HPLC results. In samples where the total destruction of ZEA by radiation was not observed, the estrogenicity was not reduced. In spite of this, no increase of estrogenicity with irradiation was verified.

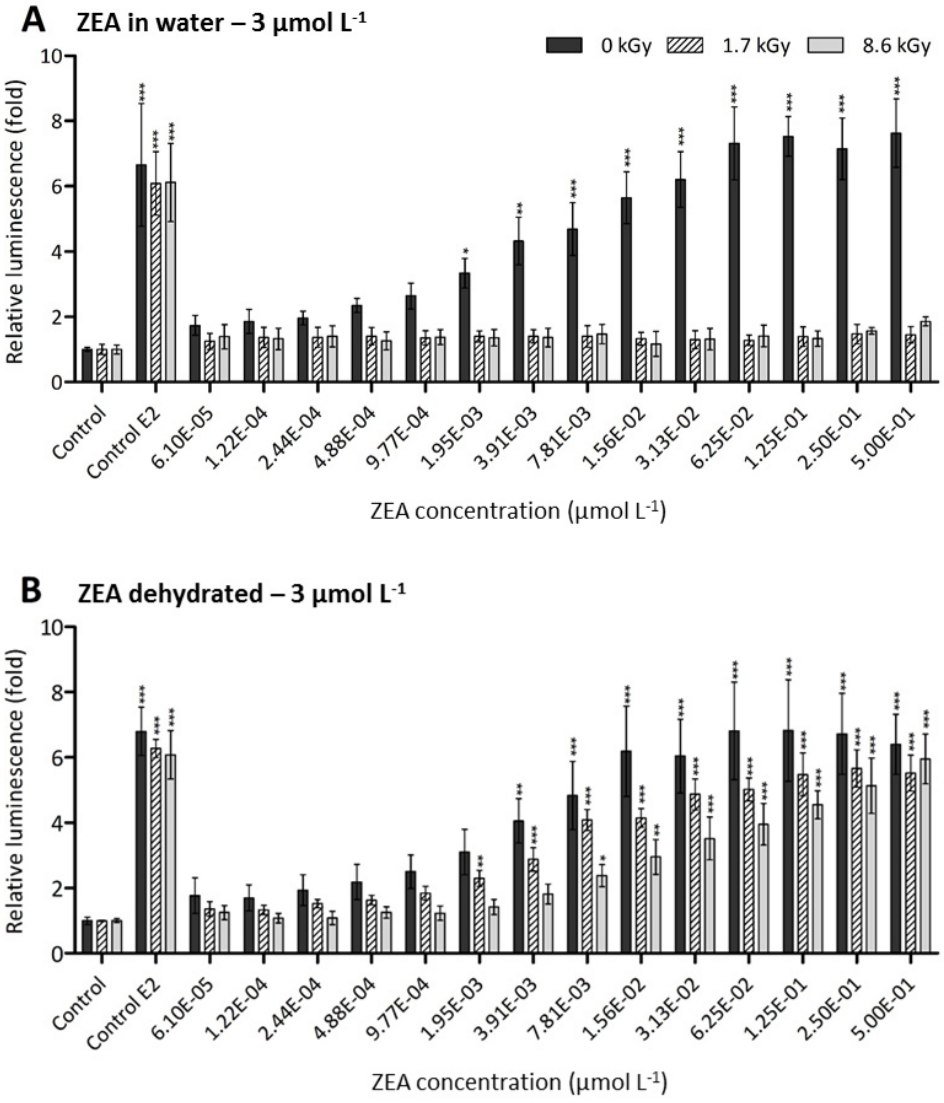

Figure 4.5 - Non-irradiated and irradiated ZEA (3 µmol L^{-1}) effect on hERα-HeLa-9903 cells, determined by luciferase activity. E2 (0.125 nmol L^{-1}) was used as positive control. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as followed: ⋆ $p < 0.05$, ⋆⋆ $p < 0.01$, ⋆⋆⋆ $p < 0.001$.

The effect of irradiation on estrogenicity of ZEA samples with initial concentration of 60 µmol L^{-1} are presented in Figure 4.6A and Figure 4.6B, respectively. The irradiation of this high dose was not sufficient to eliminate ZEA estrogenicity in aqueous samples (Figure 4.6A) although it was adequate to reduce the cytotoxicity (Figure 4.2A). After irradiation with the two doses tested, dehydrated ZEA samples with initial concentration of 60 µmol L^{-1} (Figure 4.6B) presented the same trend as non-irradiated samples. As mentioned above, the reduction of ZEA concentration on these samples was only of 20%. Thus the concentration of ZEA in irradiated samples was very close to its initial concentration and, therefore, the effect was expected to be very similar. Also, possibly,

the estrogenicity observed in irradiated samples was due to the remaining ZEA. Once again, the results point out to significant impact of water during the radiation process to an efficient detoxification of ZEA.

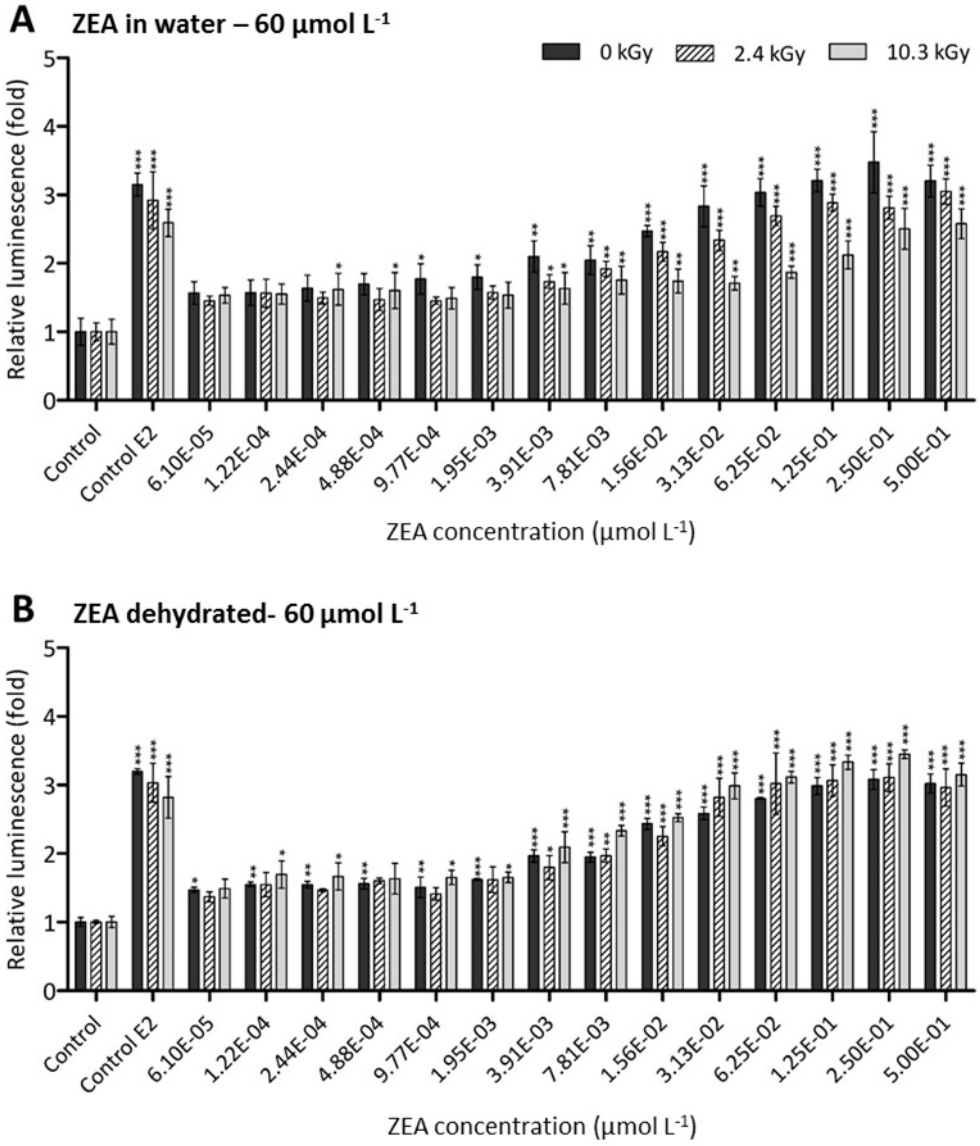

Figure 4.6 - Non-irradiated and irradiated ZEA (60 µmol L^{-1}) effect on hERα-HeLa-9903 cells, determined by luciferase activity. E2 (0.125 nmol L^{-1}) was used as positive control. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as followed: ⋆ $p < 0.05$, ⋆⋆ $p < 0.01$, ⋆⋆⋆ $p < 0.001$.

The modified Commission Regulation (EC) No 1881/2006 set the maximum ZEA levels allowed in different foods. The higher level is 400 µg/kg for refined maize oil. This concentration is equivalent to approximately 1 µmol L^{-1} and it is below the lower concentration tested (3 µmol L^{-1}) which the irradiation process was able to destroy. Therefore, within these

concentrations range, gamma radiation can potentially destroy substantial levels of ZEA and eliminate its toxicity in food and feed commodities, increasing their safety. However, the moisture content in food matrices is determinant in order to ensure the efficiency of irradiation process. Kalagatur *et al.*, (2018) studied detoxification efficiency of irradiation on ZEA fruit juices (orange, pineapple,and tomato) and verified that the reduction levels in these matrices were similar to the reduction of ZEA in water. These authors concluded that irradiation could be an efficient post-harvest food processing technique for detoxification of ZEA in fruit juices, despite the high dose of 10 kGy has minimally altered the quality of fruit juices.

ZEA is most commonly found in cereals and corn, and their typical moisture content is below 15%. So, the direct use of irradiation on grains may not destroy substantial levels of ZEA, especially if concentrations are very high. Nonetheless, the efficiency of irradiation may be increased if this technology is incorporated in some steps of well-established grain processing methods such as wet milling. This process involves a step where grain is soaked in water wherein the radiation can be applied. Of course, more research are needed on food samples naturally contaminated with ZEA in order to conclude about the applicability of this method of detoxification. The organoleptic and nutritional proprieties are also important points to investigate in order to evaluate if gamma radiation may affect important food characteristics. As it happens with others decontamination methods, it is necessary to see which foods are likely to be exposed to gamma radiation without compromising its properties.

4.4 Conclusions

In this chapter, gamma irradiation process was demonstrated to be a useful treatment for reducing ZEA levels *in vitro*. However, the presence of water during the irradiation process, such as observed for other researchers for other mycotoxins, play an important role on the efficiency of ZEA degradation. With respect to the cytotoxicity of ZEA radiolytic products, the results were promising since a decrease in ZEA concentration after irradiation was always followed by a reduction in toxicity especially when ZEA is in contact with water. The same results were observed for the estrogenic activity of irradiated ZEA. These results can indicate that irradiation process applied for ZEA detoxification can be a safe method to reduce ZEA health associated risk. More studies are needed about cytotoxicity and estrogenicity ZEA reduction with gamma irradiation.

References

Abdel-Rahman, G. N., Sultan, Y. Y., Salem, S. H., & Amer, M. M. (2019). Identify the Natural Levels of Mycotoxins in Egyptian Roasted Peanuts and the Destructive Effect of Gamma Radiation. Journal of Microbiology, Biotechnology and Food Sciences, 9(2):1174-1177

Abid-Essefi, S., Ouanes, Z., Hassen, W., Baudrimont, I., Creppy, E., & Bacha, H. (2004). Cytotoxicity, inhibition of DNA and protein syntheses and oxidative damage in cultured cells exposed to zearalenone. Toxicol In Vitro, 18(4):467-474.

ASTM. (1992). Practice for using the Fricke Reference Standard Dosimetry System, ASTM-E 1026 Annual Book of ASTM Standards (Vol. 12.02). Philadelphia, PA: ASTM.

Ayed-Boussema, I., Bouaziz, C., Rjiba, K., Valenti, K., Laporte, F., Bacha, H., & Hassen, W. (2008). The mycotoxin Zearalenone induces apoptosis in human hepatocytes (HepG2) via p53-dependent mitochondrial signaling pathway. Toxicol In Vitro, 22(7):1671-1680.

Ayed-Boussema, I., Ouanes, Z., Bacha, H., & Abid, S. (2007). Toxicities induced in cultured cells exposed to zearalenone: Apoptosis or mutagenesis? Journal of Biochemical and Molecular Toxicology, 21(3):136-144.

Aziz, N. H., Attia, E. S. A., & Farag, S. A. (1997). Effect of gamma-irradiation on the natural occurrence of *Fusarium* mycotoxins in wheat, flour and bread. Food / Nahrung, 41(1):34-37.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. Int J Food Microbiol, 119(1-2):140-146.

Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for Mold and Mycotoxin Control: A Review. Comprehensive Reviews in Food Science and Food Safety, 13(5):1049-1061.

Cetin, Y., & Bullerman, L. B. (2005). Evaluation of reduced toxicity of Zearalenone by extrusion processing as measured by the MTT cell proliferation assay. J Agric Food Chem, 53(16):6558-6563.

Čonková, E., Laciaková, A., Pástorová, B., Seidel, H., & Kováč, G. (2001). The effect of zearalenone on some enzymatic parameters in rabbits. Toxicol Lett, 121(3):145-149.

Cortinovis, C., Pizzo, F., Spicer, L., & Caloni, F. (2013). *Fusarium* mycotoxins: Effects on reproductive function in domesticanimals - A review. Theriogenology, 80:557-564.

Dayeh, V. R., Chow, S. L., Schirmer, K., Lynn, D. H., & Bols, N. C. (2004). Evaluating the toxicity of Triton X-100 to protozoan, fish, and mammalian cells using fluorescent dyes as indicators of cell viability. Ecotoxicology and Environmental Safety, 57(3):375-382.

Dayeh, V. R., Lynn, D. H., & Bols, N. C. (2005). Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, Tetrahymena thermophila. Toxicology in vitro, 19(3):399-410.

EC. (2006). Commission Regulation no 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (consolidated version from 12/12/2014). Official Journal of the European Union. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02006R1881-20140602.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhughenot, J., Pieters & R., Kleiner, J. (2002). Methods of in vitro toxicology. Food and Chemical Toxicology, 40(2–3):193-236.

Fernandes, A., Antonio, A. L., Oliveira, M. B., Martins, A., & Ferreira, I. C. (2012). Effect of gamma and electron beam irradiation on the physico-chemical and nutritional properties of mushrooms: a review. Food Chem, 135(2):641-650.

Ferrer, E., Juan-Garcia, A., Font, G., & Ruiz, M. J. (2009). Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. Toxicol In Vitro, 23(8):1504-1509.

Fink-Gremmels, J., & Malekinejad, H. (2007). Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone. Animal Feed Science and Technology, 137(3):326-341.

Gutleb, A. C., Morrison, E., & Murk, A. J. (2002). Cytotoxicity assays for mycotoxins produced by *Fusarium* strains: a review. Environ Toxicol Pharmacol, 11(3–4):309-320.

Hooshmand, H., & Klopfenstein, C. F. (1995). Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat, and soybeans with different moisture contents. Plant Foods for Human Nutrition, 47(3):227-238.

IARC. (2002). Evaluation of carcinogenic risks to humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Lyon, France: IARCPress Retrieved from http://monographs.iarc.fr/ENG/Monographs/vol82/mono82.pdf.

Iqbal, S. Z., Rabbani, T., Asi, M. R., & Jinap, S. (2014). Assessment of aflatoxins, ochratoxin A and zearalenone in breakfast cereals. Food Chem, 157:257-262.

Jalili, M., Jinap, S., & Noranizan, A. (2010). Effect of gamma radiation on reduction of mycotoxins in black pepper. Food Control, 21(10):1388-1393.

Jalili, M., Jinap, S., & Noranizan, A. (2012). Aflatoxins and ochratoxin a reduction in black and white pepper by gamma radiation. Radiation Physics and Chemistry, 81(11):1786-1788.

Ji, F., Xu, J., Liu, X., Yin, X., & Shi, J. (2014). Natural occurrence of deoxynivalenol and zearalenone in wheat from Jiangsu province, China. Food Chem, 157:393-397.

Jia, Z., Liu, M., Qu, Z., Zhang, Y., Yin, S., & Shan, A. (2014). Toxic effects of zearalenone on oxidative stress, inflammatory cytokines, biochemical and pathological changes induced by this toxin in the kidney of pregnant rats. Environ Toxicol Pharmacol, 37(2):580-591.

Kalagatur, N. K., & Kamasani, J. R. (2018). Assessment of detoxification efficacy of irradiation on zearalenone mycotoxin in various fruit juices by response surface methodology and elucidation of its in-vitro toxicity. Frontiers in Microbiology, 9:2937.

Keller, L., Abrunhosa, L., Keller, K., Rosa, C. A., Cavaglieri, L., & Venancio, A. (2015). Zearalenone and its derivatives alpha-Zearalenol and beta-Zearalenol decontamination by *Saccharomyces cerevisiae* strains isolated from bovine forage. Toxins (Basel), 7(8):3297-3308.

Lammel, T., Boisseaux, P., Fernández-Cruz, M. L., & Navas, J. M. (2013). Internalization and cytotoxicity of graphene oxide and carboxyl graphene nanoplatelets in the human hepatocellular carcinoma cell line Hep G2. Particle and Fibre Toxicology, 10:1-27.

Li, Y., Burns, K. A., Arao, Y., Luh, C. J., & Korach, K. S. (2012). Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor alpha and beta in vitro. Environ Health Perspect, 120(7):1029-1035.

Li, Y., Zhang, B., He, X., Cheng, W. H., Xu, W., Luo, Y., Liang, R., Luo, H., Huang, K. (2014). Analysis of individual and combined effects of ochratoxin A and zearalenone on HepG2 and KK-1 cells with mathematical models. Toxins (Basel), 6(4):1177-1192.

Maaroufi, K., Chekir, L., Ekue Creppy, E., Ellouz, F., & Bacha, H. (1996). Zearalenone induces modifications of haematological and biochemical parameters in rats. Toxicon, 34(5):535-540.

Marin, D. E., Taranu, I., Burlacu, R., Manda, G., Motiu, M., Neagoe, I., Dragomir, C., Stancu, M., Calin, L. (2011). Effects of zearalenone and its derivatives on porcine immune response. Toxicol In Vitro, 25(8):1981-1988.

Mutluer, B., & Erkoç, F. (1987). Effects of gamma irradiation on aflatoxins. Zeitschrift für Lebensmittel-Untersuchung und Forschung, 185(5):398-401.

OECD. (2009). Guidelines for the Testing of Chemicals. Retrieved from http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm.

Prouillac, C., Videmann, B., Mazallon, M., & Lecoeur, S. (2009). Induction of cells differentiation and ABC transporters expression by a myco-estrogen, zearalenone, in human choriocarcinoma cell line (BeWo). Toxicology, 263(2-3):100-107.

Riley, R. T., Voss, K. A., Pestka, J. J., & Williams, D. E. (2011). Developing mechanism-based and exposure biomarkers for mycotoxins in animals. In S. De Saeger (Ed.), Determining Mycotoxins and Mycotoxigenic Fungi in Food and Feed (pp. 262-265).

Rychlik, M., Humpf, H. U., Marko, D., Danicke, S., Mally, A., Berthiller, F., Klaffke, H., Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. Mycotoxin Res.

Salah-Abbes, J. B., Abbes, S., Haous, Z., & Oueslati, R. (2009). Raphanus sativus extract prevents and ameliorates zearalenone-induced peroxidative hepatic damage in Balb/c mice. J Pharm Pharmacol, 61(11):1545-1554.

Tatay, E., Meca, G., Font, G., & Ruiz, M. J. (2014). Interactive effects of zearalenone and its metabolites on cytotoxicity and metabolization in ovarian CHO-K1 cells. Toxicol In Vitro, 28(1):95-103.

Van Dyck, P. J., Tobback, P., Feyes, M., & van de Voorde, H. (1982). Sensitivity of aflatoxin B1 to ionizing radiation. Applied and Environmental Microbiology, 43(6):1317-1319.

Wang, F., Xie, F., Xue, X., Wang, Z., Fan, B., & Ha, Y. (2011). Structure elucidation and toxicity analyses of the radiolytic products of aflatoxin B1 in methanol-water solution. J Hazard Mater, 192(3):1192-1202.

Zhang Z, Xie Q, Che L. (2018). Effects of gamma irradiation on aflatoxin B1 levels in soybean and on the properties of soybean and soybean oil. Applied Radiation and Isotopes.139:224-230.

Zinedine, A., Soriano, J. M., Molto, J. C., & Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol, 45(1):1-18.

Chapter 5. Gamma Irradiation on Ochratoxin A control

5.1 Introduction

Ochratoxin A (OTA) is the most prevalent and toxic mycotoxin from the ochratoxin family. This mycotoxin is nephrotoxic and is classified in Group 2B (possible carcinogenic to humans) by the IARC (IARC, 2002).

OTA is produced by a large number of *Aspergillus* and *Penicillium* species. For example, it is produced by *A. carbonarius*, *A. cretensis*, *A. flocculosus*, *A. lacticoffeatus*, *A. niger*, *A. ochraceus*, *A. pseudoelegans*, *A. roseoglobulosum*, *A. sclerotioniger*, *A. sclerotiorum*, *A. steynii*, *A. sulphureus*, *A. westerdijkiae*, *P. nordicum* and *P. verrucosum* (Oliveira *et al.*, 2014). This high number of ochratoxigenic species is responsible for the widespread occurrence of OTA in many food and feedstuffs in several regions of the world. In cool and temperate regions, OTA is primarily produced by *Penicillium* spp. and, in tropical and semitropical regions, it is primarily produced by *Aspergillus* spp. (Ringot *et al.*, 2006).

Cereals such as wheat, maize, barley and rice are the most important sources of OTA (Oliveira *et al.*, 2014), but OTA can also be found in beans, nuts, coffee beans, fruits (Aziz and Moussa, 2002), spices (Ali *et al.*, 2015, bread, beer and wine (Abdu *et al.*, 2011; Di Stefano *et al.*, 2015). Additionally, because of its long elimination half-life, OTA can also be present in some of the organs and tissues of livestock (Duarte *et al.*, 2011). Li *et al.* (2000) verified that OTA was present in the bile and urine of rats that were fed with contaminated food. Pozzo *et al.* (2010) found OTA in swine blood. Chiavaro *et al.* (2002) found OTA in ham, and Markov *et al.* (2013) identified OTA in traditional Croatian sausages. OTA was also found in milk (Skaug, 1999) and in dairy products (Dall'Asta *et al.*, 2008). In humans, OTA was found in blood (Sangare-Tigori *et al.*, 2006; Karima *et al.*, 2010), urine (Gilbert *et al.*, 2001) and milk (Galvano *et al.*, 2008; Afshar *et al.*, 2013; Dehghan *et al.*, 2014).

After oral administration, OTA is quickly absorbed from the gastrointestinal tract and enters into the bloodstream, where it binds to serum proteins. OTA is subsequently metabolized in the liver and kidneys into many different products, and it is eliminated predominantly through the faecal and urinary routes (Ringot *et al.* 2006). Nonetheless, OTA can be reabsorbed in the kidneys and intestines, increasing its systemic redistribution towards the different tissues (Ringot *et al.* 2006). This characteristic of OTA greatly increases individual exposure to its toxic effects.

Although the mechanisms of OTA toxicity have not been fully elucidated, the available evidence suggests that OTA may disrupt phenylalanine metabolism, reduce gluconeogenesis, and induce apoptosis by inhibiting protein/DNA synthesis (Ringot *et al.* 2006; Li *et al.*, 2014). In addition, OTA can generate reactive oxygen species (ROS), which may cause protein and DNA damage and induce oxidative DNA lesions coupled with direct DNA adducts via quinone formation (Ringot *et al.*, 2006; Pfohl-Leszkowicz and Manderville, 2007; Li et a., 2014). In general, OTA is considered cytotoxic, nephrotoxic, immunotoxic, myelotoxic, teratogenic, carcinogenic, genotoxic and mutagenic (Pfohl-Leszkowicz and Manderville, 2007; Sorrenti *et al.*, 2013; Limonciel and Jennings, 2014; Bui-Klimke and Wu, 2015).

Because OTA constitutes an important risk for human and animal health, there is a pressing need for innovative strategies to reduce or eliminate OTA from food and feed, and, consequently, to improve food safety and prevent economic losses (Peng *et al.*, 2015; Ibarz *et al.*, 2015). Most important approaches currently in use are preventive and include common good agricultural and storage practices employed at the pre-harvest and post-harvest stages (Amezqueta at al., 2009 Abrunhosa *et al.*, 2010). Nevertheless, when OTA is still produced several decontamination methods can also be implemented to inactivate or remove this mycotoxin from contaminated materials (Temba *et al.*, 2016). In spite of current available strategies, there is still the need for the study, improvement, and implementation of innovative solutions.

Food irradiation is a physical method of preserving foodstuffs, and its contribution for the control of mycotoxins has been addressed by several researchers (Calado *et al.*, 2014). Gamma rays are the preferred source of radiation for food because of their penetrating capability and high effectiveness in inactivating a wide diversity of microorganisms. In the field of mycotoxicology, gamma radiation is known to exert direct action on mycotoxins by degrading them, and it has an indirect action through the inhibition or delay of fungal development (Calado *et al.*, 2014). Concerning OTA, some studies have shown good elimination of this mycotoxin (from 50%) from several food and feed commodities (Jalili *et al.*, 2010; Jalili *et al.*, 2012; Kumar *et al.*, 2012; Ben Mustapha *et al.*, 2014). However, there are also studies in which the elimination does not exceed 25% (Di Stefano *et al.*, 2014a; Di Stefano *et al.*, 2014b; Domijan *et al.*, 2015). Moreover, the degradation of mycotoxins by gamma radiation may form end-products that can still be toxic. Thus, studies involving the irradiation of mycotoxins should be complemented with toxicological assays to evaluate the safety of the radiolytic end products, individually or in total.

In vitro cytotoxicity assays using cells have the advantage of minimizing animal use, allowing for the testing of a wider range of chemicals and concentrations (Eisenbrand *et al.*, 2002; Li *et al.*, 2014). However, the use of cells in toxicity testing requires rapid and sensitive cell viability assays. Viability tests in microwell plates that employ fluorescent dyes have several desirable features. At present, many indicator dyes are commercially available, and therefore there is a large range of cellular parameters that can be monitored (Dayeh *et al.*, 2004). Examples of indicator dyes are Alamar Blue (AB) for changes in energy metabolism, carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for evaluating membrane integrity, and neutral red (NR) for evaluating lysosomal function (Dayeh *et al.*, 2005). OTA causes liver toxicity, and human hepatoma HepG2 cells were reported to retain many of the properties of primary liver cells; this human cell line has been used to test OTA toxicity (Li *et al.*, 2014).

The purpose of the present study is:

(i) to investigate the radiolytic effect of gamma radiation doses on OTA standards in its dried form or in a solution,

(ii) to evaluate the cytotoxicity of the irradiated OTA in water,

(iii) to test the radiolytic effects of gamma radiation on OTA-containing food matrices such as wheat flour, grape juice and wine.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

Methanol and acetonitrile were obtained from Merck (Lisbon, PT). An OTA standard (O1877-5MG) was purchased from Sigma-Aldrich (Sintra, PT). Ultraglutamine 1 (200 mmol L^{-1}) (L-Gln), foetal bovine serum (FBS), penicillin and streptomycin (P/S) (10,000 U mL^{-1}, 10 mg mL^{-1}), non-essential amino acids (NEAA) 100X, Trypsin-EDTA (200 mg L^{-1} EDTA, 17,000 U trypsin L^{-1}), and cell culture EMEM (Eagle's Minimum Essential Medium) were sourced by Lonza (Barcelona, ES). Phenol red-free Minimum Essential Medium (MEM) was purchased from PAN-Biotech (Aidenbach, DE). Alamar Blue, 5-carboxyfluorescein diacetate and acetoxy methyl ester (CFDA-AM) were purchased from Life Technologies (Madrid, ES). Neutral red (3-amino-7-dimethylamino-2-methyl phenanzine hydrochloride) solution (0.33%), sodium dodecyl sulfate (SDS), dimethyl sulfoxide

(DMSO) and glacial acetic acid were acquired from Sigma-Aldrich (Madrid, ES). Ethanol was purchased from Panreac (Barcelona, ES). High-purity water (<18 MΩ/cm) was obtained from a Milli-Q Element A10 Century (Millipore Iberia, ES). Wheat flour reference materials naturally contaminated with OTA at concentrations of 23.2 ± 3.3 μg kg^{-1} (O-W-823) and 93.7 ± 9.6 μg kg^{-1} (O-W-821) were from Trilogy, and were purchased from Ambifood (Porto, PT). Red wine (alcohol content of 13.5%) and red grape juice were purchased from a local Portuguese store. OTA immunoaffinity columns (OchraTestTM WB) were supplied by Vicam (Nixa, USA).

5.2.2 Preparation of samples containing OTA standards

A stock solution of OTA at a 1 mg mL^{-1} concentration was prepared by dissolving a commercial standard of OTA in 5 mL of methanol and stored at -20 °C until use. To prepare the OTA samples at 2.5 μmol L^{-1}, the appropriate amounts of stock were pipetted into clean amber 2-mL vials. The samples were then evaporated at 50 °C under a gentle nitrogen stream. To study the effects of irradiation, three different types of mycotoxin samples were prepared to obtain different moisture contents. A set of samples was left dry, another set was resuspended in 1 mL of deionized water (H_2O_{dd}) and the last set was resuspended in 1 mL of water/methanol (50:50, v/v). After being prepared, the samples were kept in amber vials and stored at -20 °C until they could be irradiated. For the cytotoxicity studies, OTA samples at a 200 μmol L^{-1} concentration in H_2O_{dd} were prepared as described above. An increase in the mycotoxin concentration was necessary to detect its cytotoxicity.

5.2.3 Preparation of food samples containing OTA

The moisture content of wheat flour reference materials was measured using a Radwag Mac 50/1/NH moisture analyser. The flour with 23.2 μg OTA kg^{-1} (Matrix A) contained 10.6% moisture, and the one with 93.7 μg OTA kg^{-1} (Matrix B) contained 11.2% moisture. Falcon tubes containing 2 g of each reference material were prepared in triplicate for non-irradiated control (0 kGy) and irradiated samples (gamma radiation doses of ≈ 2, 10 and 30 kGy) and each moisture content (≈ 11%, 15%, 20% and 35%) to be tested. The moisture of the reference materials was adjusted by adding H_2O_{dd} to the tubes, and the final moisture content of the samples was confirmed by using the moisture analyser mentioned before. Wine and grape juice samples were supplemented with

OTA standard at a concentration 20 and 100 μg L^{-1}. For each one, falcon tubes containing 10 mL of sample were prepared in triplicate to be irradiated at the same doses than that of wheat. After being prepared, the samples were stored at -20 °C until they could be irradiated.

5.2.4 Irradiation process

The irradiations were performed at room temperature in a Co-60 Precisa 22 experimental equipment (Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 165 TBq (4.45 kCi), and the instrument was located at Campus Tecnológico e Nuclear (Bobadela, Portugal). The average dose rate was 1.6 kGy/h, and it was previously determined by Frick reference dosimeter (ASTM, 1992). To monitor the process during the irradiation, two routine dosimeters (Amber Perspex dosimeters, Batch X, from Harwell Company, U.K.) were used in each irradiation experiment to estimate the highest and lowest doses absorbed by the samples. The absorbance and thickness of Amber Perspex dosimeters were measured in a UV-VIS Spectrophotometer (UV 1800, Shimadzu, USA) at 603 nm and in a micrometer (Mitutoyo America Corporation, USA), respectively, to estimate the dose according to a previous calibration curve. Samples of OTA (dried, aqueous and methanolic) were irradiated at the absorbed gamma radiation doses of 0.4, 0.9, 1.7, 2.5, 5.4 and 8.6 kGy. To evaluate the cytotoxicity after the irradiation, aqueous solutions of 200 μmol L^{-1} OTA were subjected to effective gamma radiation doses of 2.4 and 10.3 kGy. To the samples of wheat flour reference materials, grape juice and wine, gamma radiation doses of 2.4, 10.1 and 30.5 kGy were applied. Non-irradiated controls (0 kGy) were also prepared for each condition and sample type. All the samples were irradiated in triplicate. After irradiation, food samples were stored at -20 °C until analysis.

5.2.5 Determination of OTA in samples

After irradiation and before HPLC analysis, the dried OTA samples were resuspended in 1.5 mL of HPLC mobile phase, and aqueous and methanolic OTA samples were diluted with 0.5 mL of the mobile phase. The HPLC mobile phase was a mixture of water/acetonitrile/acetic acid (99:99:2, v/v/v) that was filtered and degassed with a 0.2 μm membrane filter (GHP, Gelman).

The determination of OTA in the wheat flour reference materials was performed according to a method recommended by the immunoaffinity columns supplier (Vicam) with few modifications. In

brief, 8 mL of acetonitrile:water (60:40, v/v) were added to each falcon tube, the samples were homogenized with an Ultraturrax (T-25, Ika) at 15,000 rpm for 30 s, and the extracts were centrifuged at 7,200 RCF for 10 min. Then, 5 mL of the supernatant was diluted in 20 mL of of phosphate-buffered saline buffer (PBS), and the solution was filtered using a PES syringe filter (0.45 μm, GVS). Ten mL of filtrate was passed through an OchraTest column at a flow rate of 1-2 drops/s, which was then washed with 10 mL of PBS buffer and 10 mL of H_2O_{dd}. The mycotoxin elution was performed by passing 2 mL of HPLC grade methanol that was collected in a vial. Finally, the residue was evaporated at 50 °C under a gentle stream of nitrogen and re-dissolved in 1 mL of mobile phase for HPLC analysis.

The determination of OTA in the grape juice and wine was also performed according to the method recommended by Vicam. Briefly, for matrices containing 20 μg L^{-1}, a sample of 5 mL was diluted with 15 mL of a solution containing PEG (1%) and $NaHCO_3$ (5%); for matrices containing 100 μg L^{-1}, a sample of 1 mL was diluted with 19 mL of a solution of PEG. The dilutions were mixed and filtered through a Whatman 1.5 μmol L^{-1} glass microfibre filter. Ten mL were passed through an OchraTest affinity column at a rate of about 1-2 drop/second. The column was washed with 5 mL of washing solution (2.5% NaCl and 0.5% $NaHCO_3$) and 5 mL of distilled water. Finally, the mycotoxin elution was done as previously described and a sample prepared for HPLC analysis.

The HPLC analysis was performed according to a modified version of the method described in (Serra *et al.*, 2004). The HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λ_{exc} = 333 nm and λ_{em} = 460 nm), a Varian 850-MIB data system interface and a Galaxie chromatography data system. The chromatographic separation was performed using a 21 min isocratic run on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 μm) that was fitted with a pre-column of the same stationary phase. The flow rate was set to 0.8 mL/min and the column temperature was set to 30 °C. The injection volume was 50 μL. The OTA was identified by its retention time (18.5 min) and quantified by comparing the peak areas with its respective calibration curve, which was prepared by dissolving the required amount of working standard solution in the mobile phase. The gain of the fluorescence detector was set to 100 or 1 depending on the OTA's working concentration range (0.012-2.5 μmol L^{-1} or 1.2-250 μmol L^{-1}), and for each case a calibration curve was prepared. The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times the signal-to-noise ratio, respectively. For the low working concentration range, the LOD and

LOQ were 0.004 µmol L^{-1} and 0.012 µmol L^{-1}, respectively. For the high working concentration range, the LOD and LOQ were 0.4 µmol L^{-1} and 1.2 µmol L^{-1}, respectively.

5.2.6 Fluorescence spectrum of irradiated OTA standards

To evaluate if the aromatic rings of the OTA molecule were degraded by irradiation and to check if any degradation product with an aromatic structure was formed, the fluorescence spectra of irradiated OTA standards were acquired using a Cytation 3 imaging reader (Biotek, USA) as follows. One hundred µL of each irradiated sample was transferred into a black 96-well microplate (Greiner); its emission spectra (λ_{em}) were acquired between 360 and 700 nm (excitation at 333 nm), and its excitation spectrum (λ_{exc}) were acquired between 280 and 400 nm (emission at 460 nm).

5.2.7 Cytotoxicity studies

5.2.7.1 Cell culture and exposure

The HepG2 cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in 75 cm^2 Cell Star Cell Culture flasks (Greiner Bio-One GmbH, Frickenhausen, DE) in EMEM supplemented with 1% NEAA, 1% P/S, 1% L-Gln and 10% FBS (which is referred to as EMEM+ in the following text). The flasks were incubated at 37 °C in a humidified 5% CO_2 atmosphere and split twice a week using PBS/EDTA and trypsin.

A HepG2 cell suspension (5×10^5 cells mL^{-1}) with EMEM+ was seeded into transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, DE) by adding 100 µL of cell suspension to each well. The plates were incubated for 24 h and exposed to irradiated and non-irradiated mycotoxin samples for 48 h. The samples were prepared as previously described, dried in a speed vac concentrator (Savant SPD 111V) and dissolved in 1 mL of EMEM+ supplemented with 0.5% of DMSO (to improve the solubility of the residues). Then, a 1:4 dilution was performed with EMEM+, and the resulting solution was applied to a cell culture plate in which 6 successive serial dilutions (with a dilution factor of 2). As a positive control, a subset of wells was treated with increasing concentrations of SDS (15.6 - 500 µM, dilution factor 2/3). Cells that were treated with EMEM+ served as a negative control, whereas cells treated with 0.5% (v/v) DMSO/EMEM+ were the vehicle control.

5.2.7.2 AB, CFDA-AM and NR Uptake (NRU) assays

The AB, CFDA-AM and NRU assays were performed on the same set of cells. The assays were conducted according to the protocol described by Lammel *et al.* (2013). Prior to adding the reactants, the exposure medium was removed and the cells were rinsed twice with 200 µL of PBS. Then, 100 µL of phenol red-free MEM containing 1.25% (v/v) AB and 4 µM CFDA-AM was added to each well. The 96-well plates were incubated for 30 min in the dark as described above. The fluorescence intensity was subsequently measured at λ_{exc} = 532 nm and λ_{em} = 590 nm for the AB assay, and at λ_{exc} = 485 nm and λ_{em} = 535 nm for CFDA-AM assay using a microplate reader (Tecan Genios, Tecan Group Ltd., Männedorf, CH). After that, the medium was removed and the cells were washed once with PBS. One hundred µL of NR solution (0.03 mg/mL in phenol red-free MEM) was added per well, and the plates were incubated for 1 h in the dark as described above. After the incubation period, the NR solution was removed, the cells were rinsed twice with 200 µL of PBS, and the NR that was retained in the cells was extracted with 150 µL/well of an acidified solution composed of ethanol/Milli-Q water/acetic acid (50:49:1, v/v/v). The NR fluorescence was measured at λ_{exc} = 532 nm and λ_{em} = 680 nm. The fluorescence values were corrected for the cell-free control and normalized against the vehicle control. The fluorescence spectra of OTA samples (irradiated or not) did not show any significant fluorescence at the excitation/emission wavelengths that were used in the AB (532/590 nm), CFDA-AM (485/535 nm) and NRU (532/580 nm).

5.2.8 Statistical analysis

A statistical analysis was performed using SPSS Statistics for Windows (Version 22.0, Armonk, NY: IBM Corp.). To evaluate the significant differences between the OTA concentrations in non-irradiated and irradiated samples, the means were compared by using a two-way ANOVA analysis of variance followed by Duncan's posthoc test for the standard samples and Dunnett's test for the food matrices. The results of the cytotoxicity assays represent the means and standard errors (SEM) of at least three independent experiments, in which each treatment was applied in triplicate. Significant differences among treatments were determined by one-way repeated measures analysis of variance (rmANOVA, $p < 0.05$). A posthoc Dunnett's test was used to compare the different treatments with respect to the control group. All the data were tested beforehand for normality (Shapiro-Wilk's test, $p < 0.05$) and equal variance ($p < 0.05$).

5.3 Results and Discussion

5.3.1 OTA concentration after the irradiation of standards

The radiolytic effects of gamma radiation doses on samples containing 2.5 µmol L^{-1} of OTA are presented in Figure 5.1. There is a dose-dependent reduction of the mycotoxin, which was more pronounced in samples of OTA dissolved in water, can be observed. In the latter case, the lowest applied radiation dose (0.4 kGy) was sufficient for achieving a 25% reduction in the OTA concentration, while the highest dose tested (8.6 kGy) achieved a 98% reduction. However, the irradiation of dried OTA was less effective, and no significant reductions were observed for doses between 0.4 and 5.4 kGy. In its dried form, the maximum radiation dose tested (8.6 kGy) only eliminated 12% of the OTA. In the assays that were performed in water/methanol solutions, significant reductions in OTA were achieved only with radiation doses higher than 1.7 kGy, but the OTA reduction at 8.6 kGy was almost the same (97%) as it was in water (98%).

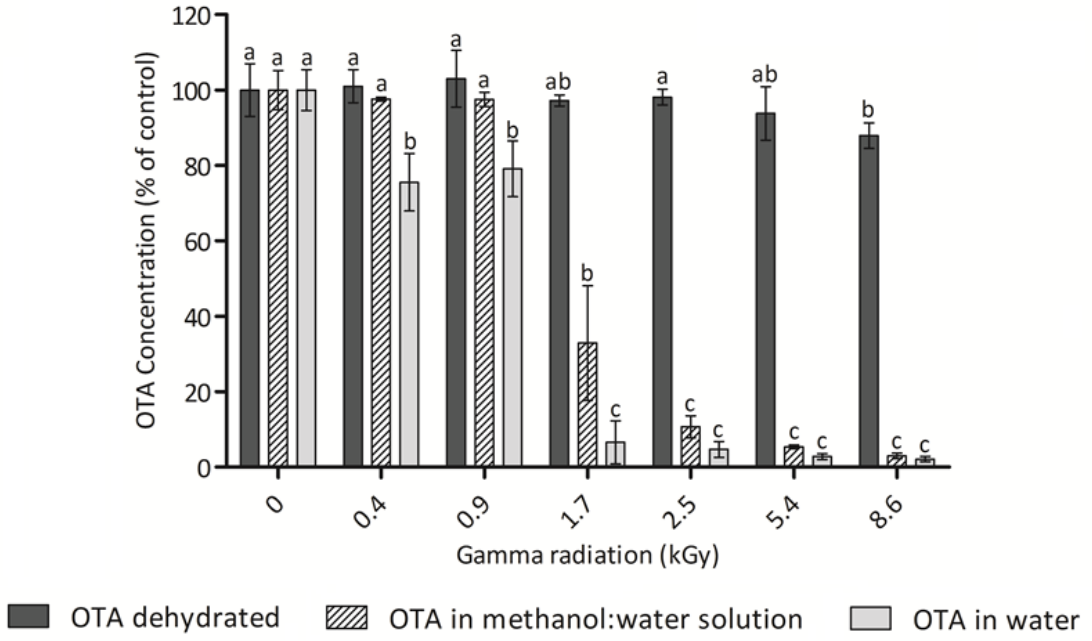

Figure 5.1 - Effects of incremental gamma radiation doses on the OTA concentration (2.5 µmol L^{-1}) under different conditions. Statistically significant differences for each sample of OTA (dried, aqueous and methanolic) are indicated with different letters, by analysis of variance (two-way ANOVA, Duncan´s post hoc test).

These results show that the presence of water during the irradiation process increases OTA elimination, and they are in accordance with the results of other studies. For example, Kumar *et al.* (2012) reported OTA concentration reductions in aqueous solutions of approximately 30%, 79%,

and 93% at 1, 2.5, and 5 kGy, respectively. In this case, the complete destruction of OTA was also observed at 10 kGy. These results are fully in accordance with the present data as it has obtained very similar reductions of approximately 20%, 95% and 97% with doses of 0.9, 2.5 and 5.4 kGy, respectively. Additionally, for methanolic solutions of OTA, the authors observed a lower elimination of mycotoxin (at most 24% when exposed to 10 kGy), and even lower reductions for dried OTA. In present results, it was observed a similar trend, with a decreasing degradation of OTA with the reduction in water content in the samples (dried<water:methanol<water).

The higher effect of gamma radiation in the presence of water can be explained by water radiolysis. During this process, the ionization of water causes the splitting of water molecules into positively charged water radicals (H_2O^+) and negative free solvated electrons (e–), which leads to the formation of the reactive species e–aq, H•, HO•, HO_2•, OH–, H_3O+, H_2, and H_2O_2 after several recombination and cross-combination reactions (Le Caër, 2011). These chemical species are reactive against double bonds, especially the ones found in aromatic or heterocyclic rings, and they initiate the hydrolysis of these compounds (Jalili *et al.*, 2012).

Although the water content has a very important role in the irradiation efficacy, the irradiation process can also be affected by other factors. The radiation dose, the type of mycotoxin, the mycotoxin concentration, and the existence of other compounds or matrix components are factors that also influence the success of irradiation (Calado *et al.*, 2014). For example, from the experiments that were conducted with 2.5 µmol L^{-1} and 200 µmol L^{-1} of OTA in water, it can be deduced that higher concentrations of mycotoxin require higher doses of irradiation to achieve satisfactory levels of elimination. In 2.5 µmol L^{-1} samples, OTA eliminations of 93% and 98% were achieved at doses of 1.7 kGy and 8.6 kGy, respectively; while for 200 µmol L^{-1} samples, radiation doses of 2.4 kGy and 10.3 kGy only led to reductions of 81% and 80%, respectively.

Another important difference that was observed between those two types of samples is the existence of detectable radiolytic products in irradiated 200 µmol L^{-1} samples that were not detected in the irradiated 2.5 µmol L^{-1} samples, nor in the non-irradiated ones. These radiolytic products were found to be more abundant in samples that were irradiated with low doses (Figure 5.2 b)) than in samples irradiated with higher ones (Figure 5.2 c)), indicating that the resulting products are themselves degraded by higher doses of radiation. These unidentified degradation products are present in small quantities but can, eventually, remain toxic and constitute an additional food safety risk if low doses of radiation are used for the treatment of food products.

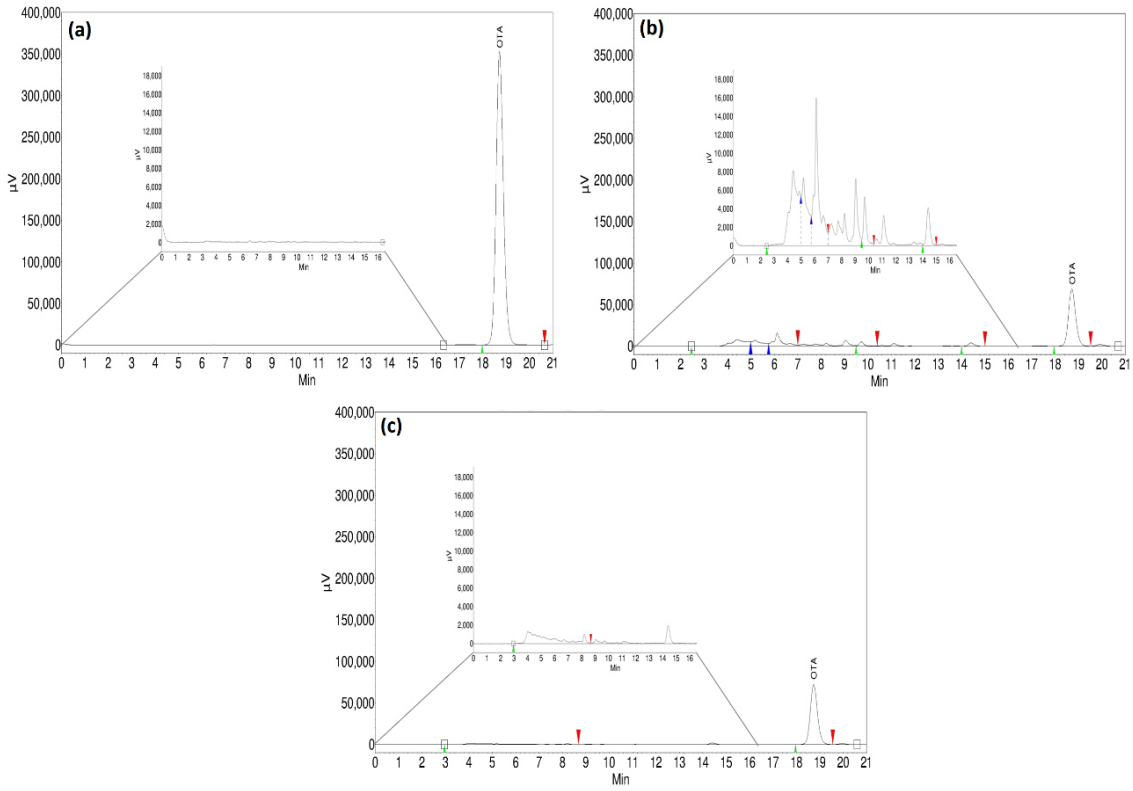

Figure 5.2 - HPLC-FL chromatograms corresponding to OTA (200 µmol L^{-1}) in water (a) before gamma radiation, (b) after 2.4 kGy gamma radiation and (c) after 10.3 kGy gamma radiation treatments.

In order to search for additional radiolytic compounds that could not be detected by HPLC-FL, fluorescence spectra were obtained. The spectra of the 2.5 µmol L^{-1} samples and of the 200 µmol L^{-1} samples are presented in Figure 5.3 and Figure 5.4, respectively.

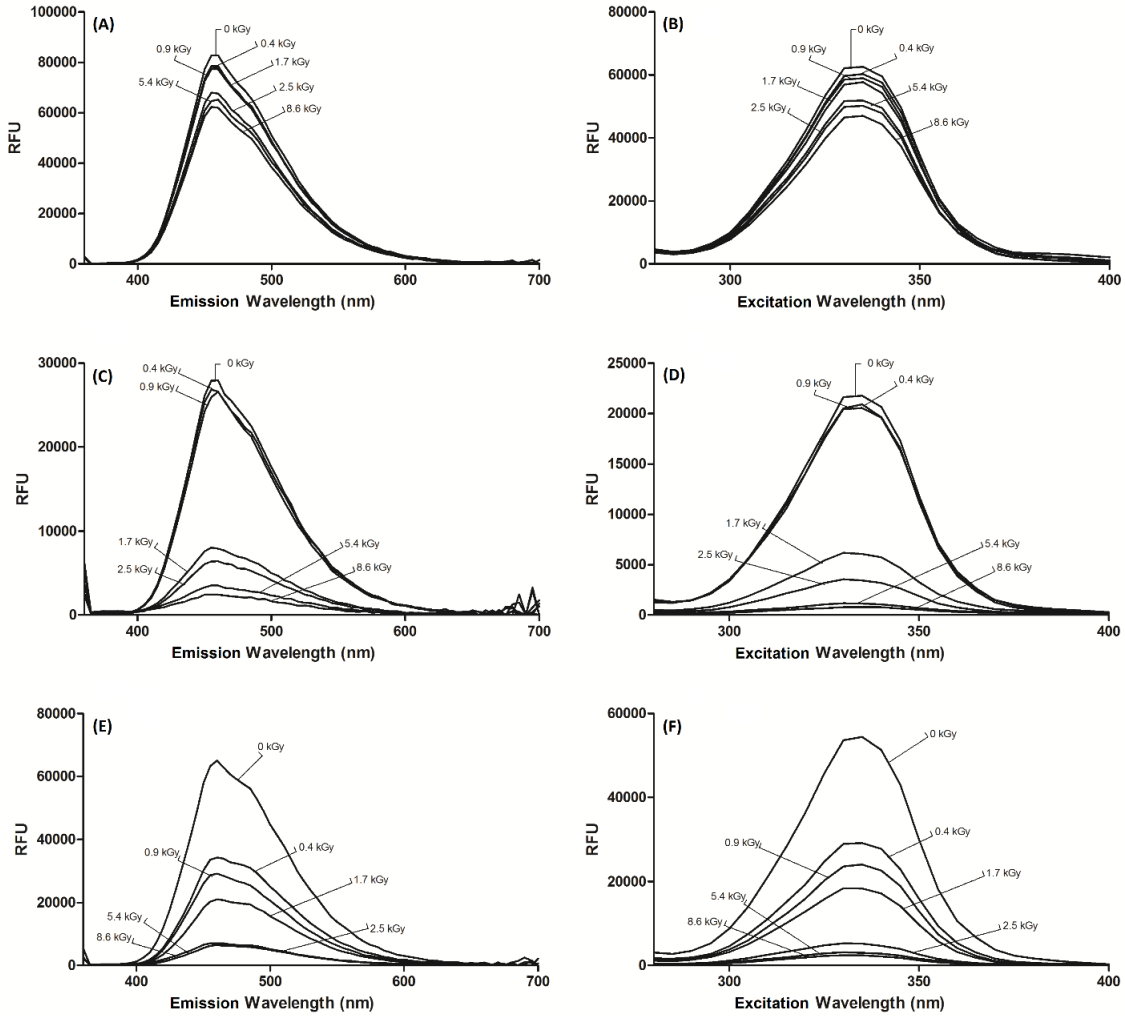

Figure 5.3 - Emission and excitation spectra of irradiated samples containing 2.5 µmol L^{-1} of: dehydrated OTA (A and B); OTA in methanol/water solution (C and D); and OTA in water (E and F).

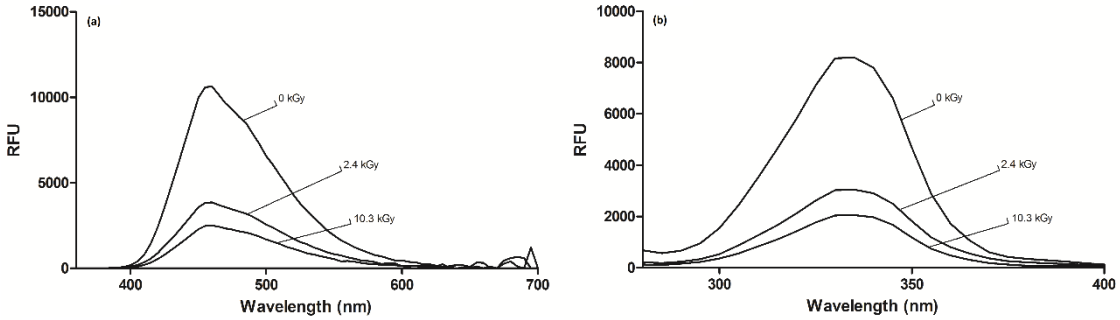

Figure 5.4 - Spectra of irradiated samples containing 200 µmol L^{-1} of OTA in water: (A) Emission spectra; and (B) excitation spectra.

These figures show that the excitation and emission spectra before and after the irradiation process are quite similar. Both spectra exhibit one single maximum (333 nm for excitation and 460 nm for emission) and no peak shift. A strong reduction in the fluorescence intensity that is dependent on the radiation dose is also evident in irradiated samples that contain water. These results strengthened the hypothesis that the aromatic rings of OTA are degraded by irradiation and show that no relevant compounds that still contain fluorescent aromatic rings were obtained after the process.

5.3.2 Cytotoxicity of irradiated OTA standards

There are several studies that claim gamma radiation as a promising method of reducing the mycotoxin contents of food and feed. Nonetheless, the decrease in mycotoxin toxicity after irradiation has been poorly studied. Therefore, cytotoxicity studies were conducted with irradiated and non-irradiated aqueous samples to evaluate if the degradation of OTA by irradiation corresponded to the elimination of its toxicity.

The cytotoxic effect of OTA standards on HepG2 cells after 48 h of incubation was measured through CFDA-AM, AB and NRU and is shown in Figure 5.5. The cytotoxicity assays were performed with serial half-dilutions of irradiated OTA samples dissolved in cell culture medium.

The CFDA-AM assay is an indirect measure of cell integrity. It is based on the conversion of CFDA-AM to its fluorescent product 5-carboxyfluorescein (5-CF) by cytosolic esterase, which is only retained in cells with an intact plasma membrane (Dayeh *et al.* 2005). With this assay, a non-significant decrease in cell viability was observed after OTA exposure (Figure 5.5a) – black bars). Similarly, no significant differences in cell viability were observed with solutions of irradiated OTA in which a degradation of approximately 80% was measured. These results suggest that the plasma membrane of HepG2 cells was unaffected by OTA or irradiated OTA, and that this assay is a poor discriminator of OTA toxicity.

Alamar Blue is a commercial preparation of the dye resazurin, which is reduced to a fluorescent form by viable cells. Its diminished reduction indicates an impairment in cellular metabolism (Dayeh *et al.*, 2005). Exposing HepG2 cells to non-irradiated OTA for 48 h resulted in a significant decrease in the fluorescence intensity, indicating a reduction in cell metabolic activity (Figure 5.5b) - black bars). However, the same trend in cytotoxicity was observed with irradiated samples. This

finding may be related to the residual content of OTA that was present in the samples. This test also showed poor efficiency for discriminating OTA toxicity between irradiation doses.

Neutral red (NR) is used to evaluate the lysosomal function because this assay is based on the accumulation of NR in functional lysosomes. The OTA cytotoxicity in HepG2 cells according to the NRU assay is presented in Figure 5.5 c) (black bars). Exposing HepG2 cells to non-irradiated OTA for 48 h resulted in a significant decrease in the fluorescence intensity and therefore reduced the cell viability, indicating clearly that OTA interferes with pinocytosis and cellular lysosomal activity. On the contrary, the irradiated OTA solutions showed a cytotoxicity that was significantly lower than that of the controls and from non-irradiated OTA. Differences were also evident between radiation doses. The cell viability was lower in samples that were irradiated with 2.4 kGy than in samples irradiated with 10.3 kGy, showing that the cytotoxicity of irradiated OTA is dependent on the radiation dose. However, an identical cytotoxicity would by expectable because the amount of OTA eliminated in both of these samples were quite similar (80 and 81%, respectively). The unexpected higher toxicity of 2.4 kGy samples can eventually result from the presence of OTA radiolytic products (unidentified peaks that were detected in Figure 5.5 b), which may themselves be toxic. Additionally, the full elimination of OTA toxicity was not observed after irradiation, even at a gamma radiation dose of 10.3 kGy, but a complete degradation of OTA was also not achieved after this irradiation treatment.

The use of different cytotoxicity assays should provide complementary information regarding the mechanism of toxicity (Simarro Doorten *et al.*, 2004). In comparing the three cytotoxicity assays used here, it was evident that the NRU is the most sensitive test for evaluating OTA cytotoxicity in HepG2 cells. The least sensitive test was the CFDA-AM. These results are in accordance with results obtained by Simarro Doorten *et al.* (2004), which verified that NRU is a more sensitive test than AB for measuring OTA cytotoxicity.

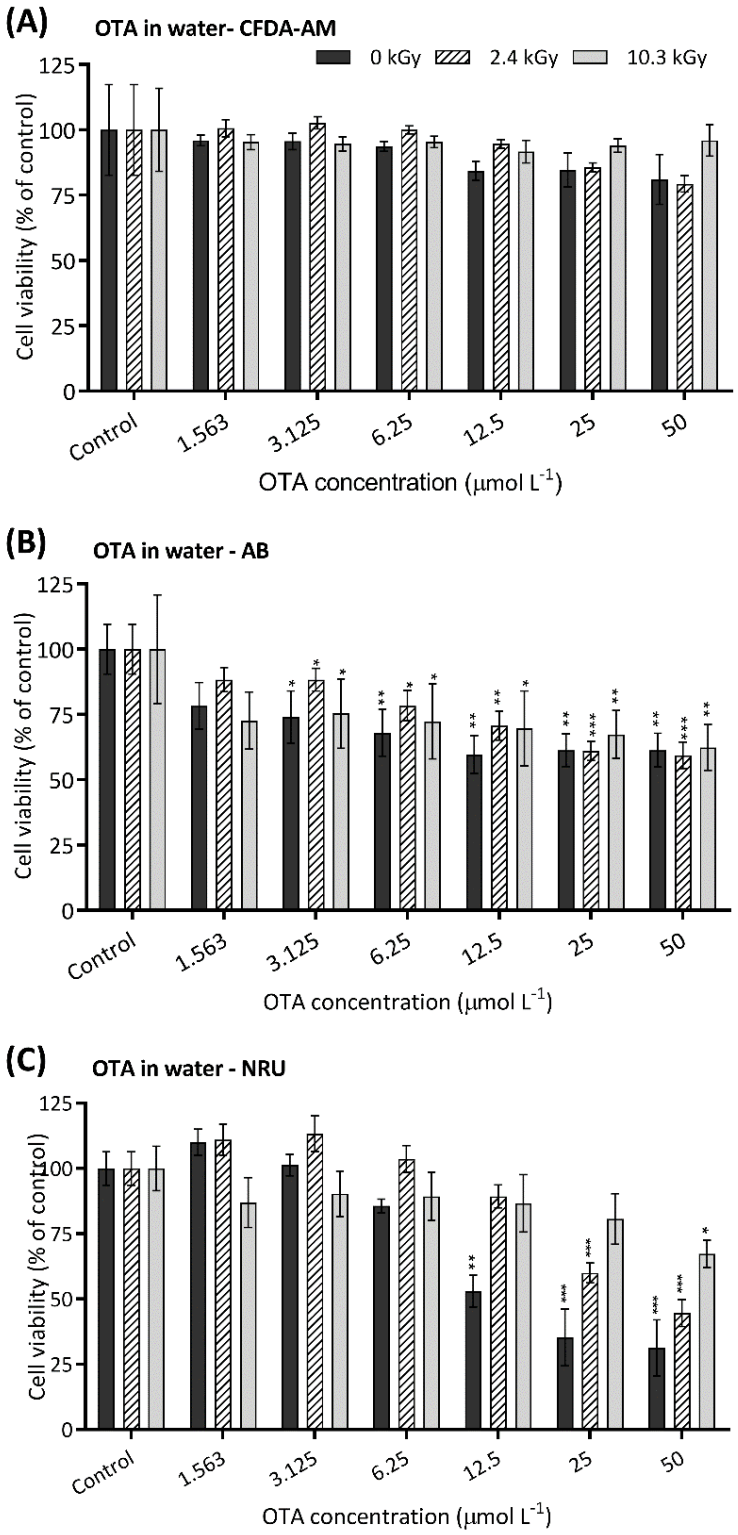

Figure 5.5 - Effects of OTA samples on HepG2 cell viability as assessed by means of (A), CFDA-AM (B) Alamar Blue (AB) and (C) Neutral Red uptake (NRU) assays. The bars represent the means and standard errors of the mean (SEM) of at least three independent repetitions. Statistically significant differences with respect to the vehicle control (one-way rmANOVA, Dunnett´s Post-hoc test) are indicated as follows: ⋆ for $p < 0.05$, ⋆⋆ for $p < 0.01$, ⋆⋆⋆ for $p < 0.001$.

There are only few studies that address the cytotoxicity of irradiated OTA. Kumar *et al.* (2012) verified a similar toxicity trend in intestinal epithelial cells with MTT assays. These authors verified that cells that were treated with irradiated OTA at 10 kGy have a 7-fold increase in cell viability when compared with cells treated with non-irradiated OTA. In our results, approx. a 2-fold increase in cell viability was verified with the NRU assay for the same dose. These differences could be related to the initial concentration of OTA that was used. Kumar *et al.* (2012) irradiated samples containing 6 μmol L^{-1} to 25 μmol L^{-1} of OTA, while in the current study samples contained 200 μmol L^{-1} of OTA. So, it may be expected that more residual OTA and OTA radiolytic products were present in the latter samples. The work of Domijan *et al.* (2019) may support this hypothesis. In this study, the authors only achieved an OTA cytotoxicity reduction of 20%, but used a much higher OTA concentration (50 mmol L^{-1}). Hence, the lower cytotoxicity reduction could be attributed to the higher OTA concentration tested. In a recent work, Maatouk and colleagues (2019) studied the toxicity of irradiated and non-irradiated OTA in HepG2 cells and verified the same toxicity for both of samples. These authors claim that observed toxicity trend could be explained by the nature and biological activity of OTA radiolytic products. Thus, more research of OTA irradiated with extraction, purification, and structural elucidation of radiolytic products are necessary to clarify the safety of this mycotoxin decontamination process.

5.3.3 Irradiation of food samples containing OTA

As confirmed in the first part of this study, water plays an important role in the efficiency of OTA radiolysis and on the subsequent reduction of its toxic effects. Therefore, it was hypothesized that water radiolysis may also promote the elimination of OTA and of its toxicity in foods, if enough moisture is provided. To test the effect of radiation on food matrices, assays with wheat flour, grape juice and wine containing OTA were performed. Two reference wheat flour materials with known certified initial concentrations of OTA (23.2 ± 3.3 μg kg^{-1} and 93.7 ± 9.6 μg kg^{-1}) were tested, at four different moisture levels (approx. 11%, 13%, 18% and 32%) for each reference material. Grape juice and wine with two different initial concentrations of OTA (approx. 20 μg kg^{-1} and 100 μg kg^{-1}) were also tested because they are liquid food matrices that may occasionally contain OTA.

The effects of gamma radiation on OTA-contaminated flour at different moisture levels are presented in Figure 5.6. For samples containing around 23 μg kg^{-1} of OTA (Figure 5.6 a), no

statistically significant reductions of the mycotoxin were observed for all the experimental conditions tested. However, significant reductions were observed for samples containing around 93 μg kg^{-1} of OTA (Figure 5.6 b). In this case, the efficiency of irradiation increased with the increase of wheat flour moisture and radiation dose, a maximum elimination of 24% with 30.5 kGy of radiation and 32% of moisture being achieved. Jalili *et al.* (2010) also observed more elimination of OTA and aflatoxins in food samples containing higher concentrations of mycotoxin than in samples containing lower amounts. This trend most likely results from the protective effect of food principal components. For the same amount of matrix, less mycotoxin will certainly be more protected from radiation effects than more mycotoxin. Even so, the elimination of OTA observed in wheat flour was drastically inferior to the one observed in pure water, suggesting that a large amount of water would be necessary to totally eliminate the mycotoxin from flour.

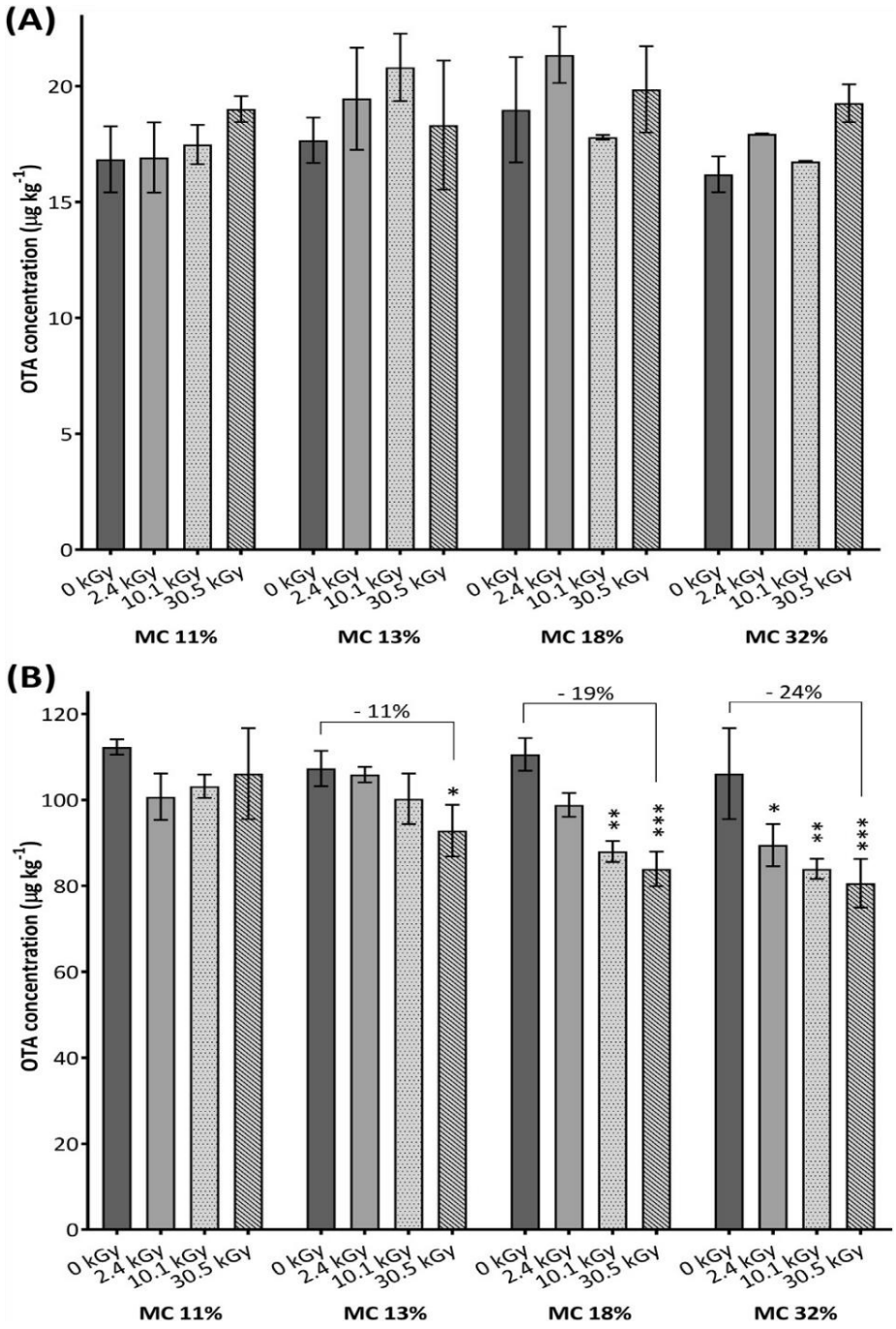

Figure 5.6 - Effects of gamma radiation on OTA reductions in naturally contaminated wheat flour with different moisture contents and initial OTA concentrations of 23.2 ± 3.3 µg/kg (a) and 93.7 ± 9.6 µg/kg (b). Statistically significant differences with respect to non-irradiated control (one-way rmANOVA, Tukey's multiple comparisons Post-hoc test) are indicated as follows: ⋆ for $p < 0.05$.

Considering the first findings of this study, gamma radiation can only be a practical solution for products containing a high of water content. The Commission Regulation n° 1881/2006 (2006) sets the maximum OTA level allowed in different foods for the European Union countries. In this document, there are many types of foodstuffs, most of which have a low level of moisture, namely cereals, and just a few of them have higher concentrations of water, such as fruit juices, wine, and

liquorice extracts. The highest limit for OTA is 80 µg/kg for liquorice extracts for use in particular beverages and confectionary. This concentration is approximately 10-fold below the lowest standard concentration that was irradiated in this study (2.5 µM), which was destroyed (>90%) by a radiation dose of 1.7 kGy in presence of water. Since there is not a clear evidence of the effect of the toxin concentration on its destruction by irradiation, and if water content will play a role on it, two liquid food samples that may occasionally contain OTA (grape juice and wine) were irradiated. Thus, it was possible to test whether the presence of higher water contents in the food matrix (between 80 and 90%) would be sufficient to improve substantially the elimination of OTA by radiation. The results obtained for grape juice and wine are presented in Figure 5.7.

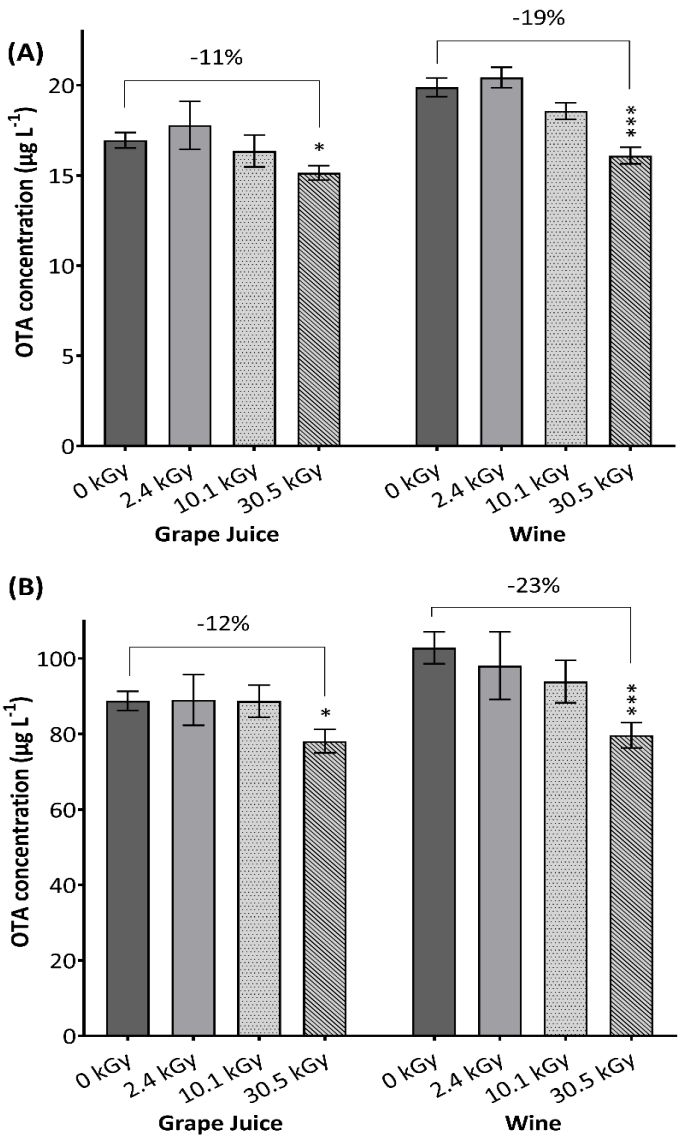

Figure 5.7 - Effects of gamma radiation on OTA in grape juice and wine containing initial OTA concentrations of (A) 20 µg L-1 and (B) 100 µg L-1. Statistically significant differences with respect to non-irradiated control are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-way ANOVA, Dunnett's posthoc test).

Surprisingly, it was found that the eliminations of OTA in these matrices are also low, between 11 and 23%, depending on the matrix and radiation dose tested. These findings clearly show that irradiation has a limited effect on OTA in real food samples, even if they are liquid matrices with water contents higher than 80%. The presence of scavengers on food matrices that may react with free radicals from water radiolysis are probably protecting the mycotoxin, reducing the radiation damage. These compounds, which are present in greater amounts than OTA, may also be absorbing the radiation energy, reducing the amount of primary reactive radicals generated from water radiolysis. For example, in grape juice and wine a reduction of their red colour with increasing doses of radiation (Figure 5.8) was clearly observed, suggesting that radiation had a destructive effect on anthocyanins and other flavonoids that are usually the main source of red colour in grape-derived products. The degradation of other flavonoids (kaempferol and quercetin) by gamma radiolysis has been demonstrated (Marfak *et al.*, 2003a; Marfak *et al.*, 2003b). The protective effect of food matrix components may also explain why the elimination of OTA was lower in grape juice (up to 12%) than in wine (up to 23%). The higher content of dissolved solids (°Brix) and total solids in grape juice compared to wine may have protected better OTA from the effects of radiation.

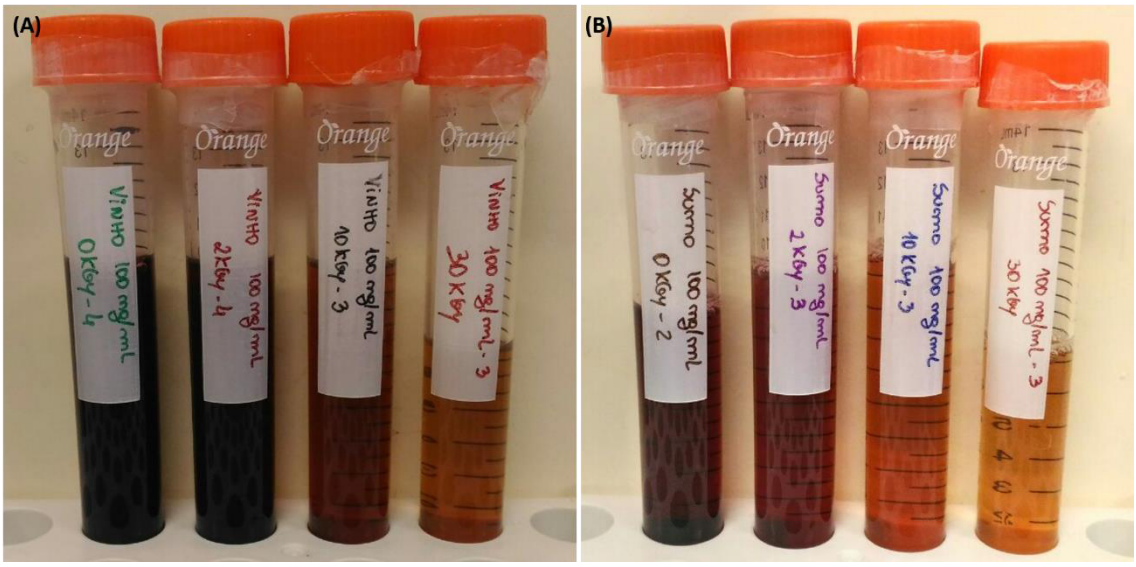

Figure 5.8 - Red colour reduction induced by gamma radiation in (a) wine and (b) grape juice containing 100 µmol L^{-1} of OTA.

In summary, it can be concluded from present data that gamma radiation can effectively eliminate OTA in water solutions using a dose as low as 1.7 kGy. However, its effect on real food samples is quite moderate even in food matrices that contain high moisture content, not exceeding 24%. Additionally, a dose of radiation 3 times higher than the maximum dose recommended for most of the food products (10 kGy) was necessary to achieve those levels of reduction. These results

challenge data from Mehrez and co-authors (2016), which reported OTA reductions of 35.5% and 47.2% with an irradiation dose of 8 kGy in wheat grains containing 14% and 16% of moisture, respectively. In this case, the authors irradiated full grains of wheat that were spiked with OTA, instead of naturally contaminated wheat flour as in the current study. It is more likely that the uneven distribution of OTA in wheat grains may have affected the subsequent quantifications of the mycotoxin by Mehrez *et al.* (2016). Even with an extremely homogeneous matrix such as wheat flour, high variability in the OTA determinations was obtained as explained above. In the current study, certified reference materials were used, in which the homogeneous distribution of OTA is guaranteed. The importance of using homogeneous matrices is also found in other publications and present data is in agreement with these studies. Di Stefano and collaborators conducted irradiation studies with OTA in poultry feed (Di Stefano *et al.*, 2014b) and almond samples (Di Stefano et a., 2014a), and they obtained reductions of only 23.9% with a dose of 15 kGy. In these studies, the poultry feed samples were blended and almonds were crumbled in order to obtain homogeneous matrices before the irradiation process. In addition, Domijan *et al.* (2015) verified an OTA reduction of only 22.5% with a 10 kGy dose on different dry-cured meat products that were previously homogenized with knife mills. It can be concluded that the homogenous distribution of the mycotoxin is a critical point when real food samples are irradiated and studied. Without this guarantee, it is not possible to state that the reductions observed for OTA are caused by the irradiation process.

It is clear that the increase in wheat flour moisture of up to 35% was not sufficient for improving the radiolysis of OTA. Even though the role of water activity in the elimination of OTA has been observed in raw agricultural commodities in other studies, the results are very contradictory. For example, Jalili *et al.* (2010) verified that the irradiation of black pepper that was artificially contaminated with OTA at 60 kGy only reached an elimination of 52%, indicating that the low water content of this product (approx. 9-12%) was the primary reason. However, the same authors later observed an increase in OTA degradation with increasing water contents in the peppers (the elimination increased from 46 to 55% at a dose of 30 kGy when the moisture content increased from 12 to 18%) (Jalili *et al.*, 2012). However, for radiation doses similar to the ones used in our study (≤10 kGy), no significant reductions were observed in peppers at all the tested moisture levels (Jalili *et al.*, 2010, Jalili *et al.*, 2012). Furthermore, our results are in accordance with the observation by Hooshmand and Klopfenstein (1995) for other mycotoxins (aflatoxin B1, T2-toxin and zearalenone) in feed grains (wheat, maize and soybeans). In the latter study, the authors

showed that moisture contents of 9, 13 and 17% had no significant effects on the irradiation efficiency.

As explained above, the presence of water is not the only factor that compromises the irradiation process, but the matrix composition is another important factor. For example, in their study about the effect of gamma radiation on different dry-cured meat products, Domijan *et al.* (2015) verified that the most substantial reductions in OTA were obtained in samples containing a higher fat content and not in samples containing more water. Nonetheless, it is also predictable that the macro-components in food will compete with OTA for primary reactive radicals that protect the mycotoxin from radiolysis. This hypothesis may explain the inefficacy of gamma radiation in the elimination of OTA from wheat flour, despite of increasing its water content, and the inefficacy in the elimination of OTA from juice and wine.

5.4 Conclusions

In conclusion, gamma irradiation was shown to eliminate significant amounts of OTA when this mycotoxin is dissolved in water but its elimination resulted only in a 2-fold decrease of sample toxicity. It was also shown that dried OTA is extremely resistant to radiation doses of up to 8.6 kGy. In food matrices, OTA was also found more radioresistant than in pure water. In spite of increasing the moisture content of wheat flour, the maximum elimination achieved was 24% with a radiation dose of 30 kGy. In liquid matrices such as grape juice and wine, no further improvements in OTA elimination were achieved. Therefore, it can be concluded that gamma radiation is not a feasible technology for the detoxification of OTA in foods.

References

Abdu, S., Ali, A., & Ansari, S. (2011). Cytotoxic effect of Ochratoxin A on the renal corpuscles of rat kidney: could Ochratoxin A cause kidney failure? Histology and histopathology, 26(4):543.

Abrunhosa, L., Paterson, R. R. M., & Venâncio, A. (2010). Biodegradation of ochratoxin A for food and feed decontamination. Toxins, 2(5):1078-1099.

Afshar, P., Shokrzadeh, M., Kalhori, S., Babaee, Z., & Saeedi Saravi, S. S. (2013). Occurrence of Ochratoxin A and Aflatoxin M1 in human breast milk in Sari, Iran. Food Control, 31(2):525-529.

Ali, N., Hashim, N. H., & Shuib, N. S. (2015). Natural occurrence of aflatoxins and ochratoxin A in processed spices marketed in Malaysia. Food Additives and Contaminants: Part A, 32(4):518-532.

Amezqueta, S., Gonzalez-Penas, E., Murillo-Arbizu, M., & De Cerain, A. L. (2009). Ochratoxin A decontamination: A review. Food Control, 20(4):326-333.

ASTM. (1992). Practice for using the Fricke Reference Standard Dosimetry System, ASTM-E 1026 Annual Book of ASTM Standards (Vol. 12.02). Philadelphia, PA: ASTM.

Aziz, N. H., & Moussa, L. A. A. (2002). Influence of gamma-radiation on mycotoxin producing moulds and mycotoxins in fruits. Food Control, 13(4–5):281-288.

Ben Mustapha, M., Bousselmi, M., Jerbi, T., Ben Bettaieb, N., & Fattouch, S. (2014). Gamma radiation effects on microbiological, physico-chemical and antioxidant properties of Tunisian millet (Pennisetum Glaucum L.R.Br.). Food Chemistry, 154:230-237.

Bui-Klimke, T. R., & Wu, F. (2015). Ochratoxin A and human health risk: a review of the evidence. Critical reviews in food science and nutrition, 55(13):1860-1869.

Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for Mold and Mycotoxin Control: A Review. Comprehensive Reviews in Food Science and Food Safety, 13(5):1049-1061.

Chiavaro, E., Lepiani, A., Colla, F., Bettoni, P., Pari, E., & Spotti, E. (2002). Ochratoxin A determination in ham by immunoaffinity clean-up and a quick fluorometric method. Food additives and contaminants, 19(6):575-581.

Dall'Asta, C., De Dea Lindner, J., Galaverna, G., Dossena, A., Neviani, E., & Marchelli, R. 2008. The occurrence of ochratoxin A in blue cheese. Food Chemistry, 106(2):729-734.

Dayeh, V. R., Chow, S. L., Schirmer, K., Lynn, D. H., & Bols, N. C. (2004). Evaluating the toxicity of Triton X-100 to protozoan, fish, and mammalian cells using fluorescent dyes as indicators of cell viability. Ecotoxicology and Environmental Safety, 57(3):375-382.

Dayeh, V. R., Lynn, D. H., & Bols, N. C. (2005). Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, Tetrahymena thermophila. Toxicology in vitro, 19(3):399-410.

Dehghan, P., Pakshir, K., Rafiei, H., Chadeganipour, M., & Akbari, M. (2014). Prevalence of ochratoxin A in human milk in the khorrambid town, fars province, South of iran. Jundishapur journal of microbiology, 7(7).

Di Stefano, V., Avellone, G., Pitonzo, R., Capocchiano, V. G., Mazza, A., Cicero, N., & Dugo, G. (2015). Natural co-occurrence of ochratoxin A, ochratoxin B and aflatoxins in Sicilian red wines. Food Additives and Contaminants: Part A, 32(8):1343-1351.

Di Stefano, V., R. Pitonzo and G. Avellone, (2014a): Effect of gamma irradiation on Aflatoxins and Ochratoxin A reduction in almond samples. Journal of Food Research, 3.

Di Stefano, V., R. Pitonzo, N. Cicero and M. C. D'Oca, (2014b): Mycotoxin contamination of animal feedingstuff: detoxification by gamma-irradiation and reduction of Aflatoxins and Ochratoxin A concentrations. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 31:2034-2039.

Domijan, A.-M., Marjanović Čermak, A. M., Vulić, A., Tartaro Bujak, I., Pavičić, I., Pleadin, J., Markov, K. & Mihaljević, B. (2019). Cytotoxicity of gamma irradiated aflatoxin B1 and ochratoxin A. Journal of Environmental Science and Health, Part B, 54(3):155-162

Domijan, A. M., Pleadin, J., Mihaljevic, B., Vahcic, N., Frece, J., & Markov, K. (2015). Reduction of ochratoxin A in dry-cured meat products using gamma-irradiation. Food Additives and Contaminants: Part A, 32(7):1185-1191.

Duarte, S. C., Lino, C. M., & Pena, A. (2011). Ochratoxin A in feed of food-producing animals: an undesirable mycotoxin with health and performance effects. Veterinary microbiology, 154(1-2): 1-13.

EC. (2006). Commission Regulation no 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (consolidated version from 01/04/2016). Official Journal of the European Union Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02006R1881-20160401.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhughenot, J., Pieters & R., Kleiner, J. (2002). Methods of in vitro toxicology. Food and Chemical Toxicology, 40(2–3):193-236.

Galvano, F., Pietri, A., Bertuzzi, T., Gagliardi, L., Ciotti, S., Luisi, S., Bognanno, M., La Fauci, L., Lacopino, A. M., Nigro, F., Li Volti, G., Vanella, L., Giammanco, G., Tina, G.L & Gazzolo, D. (2008). Maternal dietary habits and mycotoxin occurrence in human mature milk. Molecular nutrition and food research, 52(4):496-501.

Gilbert, J., Brereton, P., & MacDonald, S. (2001). Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples. Food additives and contaminants, 18(12):1088-1093.

Hooshmand, H., & Klopfenstein, C. F. (1995). Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat, and soybeans with different moisture contents. Plant Foods for Human Nutrition, 47(3):227-238.

IARC. (2002). Evaluation of carcinogenic risks to humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Lyon, France: IARCPress Retrieved from http://monographs.iarc.fr/ENG/Monographs/vol82/mono82.pdf.

Ibarz, R., Garvín, A., Azuara, E., & Ibarz, A. (2015). Modelling of ochratoxin A photo-degradation by a UV multi-wavelength emitting lamp. LWT - Food Science and Technology, 61(2):385-392.

Jalili, M., Jinap, S., & Noranizan, A. (2010). Effect of gamma radiation on reduction of mycotoxins in black pepper. Food Control, 21(10):1388-1393.

Jalili, M., Jinap, S., & Noranizan, A. (2012). Aflatoxins and ochratoxin a reduction in black and white pepper by gamma radiation. Radiation Physics and Chemistry, 81(11):1786-1788.

Karima, H. K., Ridha, G., Zied, A., Chekib, M., Salem, M., & Abderrazek, H. (2010). Estimation of Ochratoxin A in human blood of healthy Tunisian population. Exp Toxicol Pathol, 62(5):539-542.

Kumar, S., Kunwar, A., Gautam, S., & Sharma, A. (2012). Inactivation of *A. ochraceus* spores and detoxification of ochratoxin A in coffee beans by gamma irradiation. J Food Sci, 77(2):44-51.

Lammel, T., Boisseaux, P., Fernández-Cruz, M. L., & Navas, J. M. (2013). Internalization and cytotoxicity of graphene oxide and carboxyl graphene nanoplatelets in the human hepatocellular carcinoma cell line Hep G2. Particle and Fibre Toxicology, 10:1-27.

Le Caër, S. (2011). Water Radiolysis: Influence of Oxide Surfaces on H_2 Production under Ionizing Radiation. Water, 3(1):235-253.

Li, S., Marquardt, R., & Frohlich, A. (2000). Identification of ochratoxins and some of their metabolites in bile and urine of rats. Food and Chemical Toxicology, 38(2):141-152.

Li, Y., Zhang, B., He, X., Cheng, W. H., Xu, W., Luo, Y., Liang, R., Luo, H., Huang, K. (2014). Analysis of individual and combined effects of ochratoxin A and zearalenone on HepG2 and KK-1 cells with mathematical models. Toxins (Basel), 6(4):1177-1192.

Limonciel, A., & Jennings, P. (2014). A review of the evidence that ochratoxin A is an Nrf2 inhibitor: implications for nephrotoxicity and renal carcinogenicity. Toxins, 6(1):371-379.

Marfak, A., Trouillas, P., Allais, D.-P., Champavier, Y., Calliste, C.-A., & Duroux, J.-L. (2003a). Radiolysis of kaempferol in water/methanol mixtures. Evaluation of antioxidant activity of kaempferol and products formed. Journal of Agricultural and Food Chemistry, 51(5):1270-1277.

Marfak, A., Trouillas, P., Allais, D. P., Calliste, C. A., Cook-Moreau, J., & Duroux, J. L. (2003b). Mechanisms of Transformation of the Antioxidant Kaempferol into Depsides. Gamma-Radiolysis Study in Methanol and Ethanol. Radiation Research, 160(3):355-365.

Markov, K., Pleadin, J., Bevardi, M., Vahčić, N., Sokolić-Mihalak, D., & Frece, J. (2013). Natural occurrence of aflatoxin B1, ochratoxin A and citrinin in Croatian fermented meat products. Food Control, 34(2):312-317.

Mehrez, A., Maatouk, I., Romero-González, R., Amara, A. B., Kraiem, M., Frenich, A. G., & Landoulsi, A. (2016). Assessment of ochratoxin A stability following gamma irradiation: experimental approaches for feed detoxification perspectives. World Mycotoxin Journal, 9(2):289-298.

Oliveira, P. M., Zannini, E., & Arendt, E. K. (2014). Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: from crop farming to cereal products. Food Microbiology, 37:78-95.

Peng, C., Ding, Y., An, F., Wang, L., Li, S., Nie, Y., Zhou, L., Wang, C. & Li, S. (2015). Degradation of ochratoxin A in aqueous solutions by electron beam irradiation. Journal of Radioanalytical and Nuclear Chemistry. 306(1):39-46.

Pfohl-Leszkowicz, A., & Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res, 51(1):61-99.

Pozzo, L., Cavallarin, L., Nucera, D., Antoniazzi, S., & Schiavone, A. (2010). A survey of ochratoxin A contamination in feeds and sera from organic and standard swine farms in northwest Italy. Journal of the science of food and agriculture, 90(9):1467-1472.

Ringot, D., Chango, A., Schneider, Y. J., & Larondelle, Y. (2006). Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chemico-biological interactions, 159(1):8-46.

Sangare-Tigori, B., Moukha, S., Kouadio, J. H., Dano, D. S., Betbeder, A. M., Achour, A., & Creppy, E. E. (2006). Ochratoxin A in human blood in Abidjan, Cote d'Ivoire. Toxicon, 47(8):894-900.

Serra, R., Mendonça, C., Abrunhosa, L. s., Pietri, A., & Venâncio, A. (2004). Determination of ochratoxin A in wine grapes: comparison of extraction procedures and method validation. Analytica Chimica Acta, 513(1):41-47.

Simarro Doorten, A. Y., Bull, S., van der Doelen, M. A., & Fink-Gremmels, J. (2004). Metabolism-mediated cytotoxicity of ochratoxin A. Toxicology in vitro, 18(3):271-277.

Skaug, M. A. (1999). Analysis of Norwegian milk and infant formulas for ochratoxin A. Food additives and contaminants, 16(2):75-78.

Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M., & Galvano, F. (2013). Toxicity of ochratoxin a and its modulation by antioxidants: a review. Toxins, 5(10):1742-1766.

Temba, B. A., Sultanbawa, Y., Kriticos, D. J., Fox, G. P., Harvey, J. J. W., & Fletcher, M. T. (2016). Tools for defusing a major global food and feed safety risk: Nonbiological postharvest procedures to decontaminate mycotoxins in foods and feeds. Journal of Agricultural and Food Chemistry, 64(47):8959-8972.

Chapter 6. Integration of Results

6.1 Introduction

As it was mentioned in the beginning of this thesis, mycotoxin contamination of food is an ongoing global concern. Mycotoxin contamination is considered an unavoidable and unpredictable problem, even when good agricultural, storage, and processing practices are implemented, posing a difficult challenge to food safety (Alshannaq and Yu, 2017). Additionally, many mycotoxins are not easily eliminated during food processing because of their stability against heat, physical, and chemical treatments (Pitt, 2000). Ponzilacqua *et al.* (2019) highlights that mycotoxin contamination of food and feed is difficult to control due to their various features including low molecular weight, non-immunogenic, thermostability, active in low concentrations, and a broad spectrum of toxicity. Acute or chronic mycotoxins exposure may trigger off a carcinogenic, nephrotoxic, tremorgenic, hemorrhagic, teratogenic and dermatological humans diseases (Al-Jaal *et al.,* 2019; Leitão, 2019). To reduce the potential risk of mycotoxin exposure, countries worldwide adopted strict legislations to control mycotoxin presence in food and feed. Although prevention of fungal contamination in agricultural products is the key to mitigation of the impact of mycotoxins on human and animal health, the current agricultural and manufacturing practices do not always allow this prevention. In addition, climate change is predicted to increase the presence of mycotoxins in agricultural commodities (Medina *et al.*, 2017) and the growing human population, estimated to exceed 9 billion by 2050 (Marroquín-Cardona *et al.*, 2014), make food more valuable. These issues are expected to exert pressure on the food system to increase the use of detoxification methods (Marroquín-Cardona *et al.*, 2014, Wielogorska *et al.*, 2019).

Decontamination of mycotoxins has been a continuing challenge for the food industry. Novel processing methods are continuously explored to achieve complete mycotoxins degradation in food products. Detoxification techniques appears to be the most attractive approach, and it can be classified into three types: physical, chemical, and biological processes, as summarized in Table 6-1.

Table 6-1 - Techniques applied for mycotoxin detoxification

Mycotoxin	Technique	Reference
OTA	Biopreservation agentes (lactic acid bacteria)	Taroub *et al.*, 2019
FB1	Biodegradation (novel bacterial consortium SAAS79)	Zhao *et al.*, 2019
AFB_1	Photosensitization	Temba *et al.*, 2019
AFB_1 **AFB_2** **AFG_1** **AFG_2**	Photocatalytic (titanium dioxide)	Magzoub *et al.*, 2019
B1(AFB1) **AFB1+B2 (TotAFIs)**	Cold plasma and gamma irradiation	Sen *et al.*, 2019
PAT	Biological degradation	Sajid *et al.*, 2019
PAT	Ozone	Diao *et al.*, 2019
OTA	Cold plasma	Casas-Junco *et al.*, 2019
OTA	Gamma Irradiation	Calado *et al.*, 2018
OTA	Gamma Irradiation	Pereira *et al.*, 2017

Novel processing technologies show significant potential for future applications in decontaminating mycotoxins in the food industry (Pankaj *et al.*, 2018). Gamma irradiation for mycotoxins reduction is one of these technics that have some advantages such as: no chemical additives, no chemical residues, deep penetration through food samples, the possibility of being applied to disinfestations, improvement of shelf life and safety of food products (Iqbal *et al.*, 2013; Panjak *et al.*, 2018). Although promising, there are a number of contrasting reports regarding the effect of gamma irradiation on mycotoxin in different foods and a lack of toxicity studies. Thus, further research is required for knowing the real effectiveness and potential of this detoxification technic.

6.2 Discussion

As it was mentioned in Chapter 1, it was decided to present chapters 3, 4 and 5 as self-sustaining works. However, the main conclusions obtained in each chapter can be integrated and discussed. In the next sections it will be analysed the results previously showed and compared to each other.

6.2.1 Mycotoxins concentration after irradiation

The study was performed with the main purpose of applying the same treatment to all mycotoxins to the same way, being possible the compare results from different mycotoxins. Tests carried out with the various mycotoxins tested under different conditions (dehydrated, water and methanol:water solution) showed that the presence of water is one of the most important factors for irradiation effectiveness. The six mycotoxins tested showed the same trend – increased mycotoxin degradation with increasing available water in solution. For all mycotoxins, irradiation in methanol:water solution showed an intermediary trend between dehydrated samples and water samples. This is expectable because the water availability increases from dehydrated samples to water:methanol solution, and finally to water. The radiolysis of water seems to play an important role in the effectiveness of detoxification with irradiation. All mycotoxins showed, with 2.5 kGy, degradation equal or above 95%, when irradiated in water.

Although all mycotoxins tested were partially detoxified by gamma radiation, mycotoxins presented different levels of sensitivity to irradiation. ZEA and AFG1 were found to be the most sensitive ones. In water, it was verified, with 0,4 kGy, a total reduction of these mycotoxins. In dehydrated condition, with the highest dose tested (8,6 kGy) a reduction of 87% to ZEA and 77% to AFG1 was observed. All mycotoxins revealed a high resistance to radiation when they were irradiated in dehydrated condition. With the higher irradiation dose, reductions of 16%, 14%, 42% and 17% was verified to AFB1, AFB2, AFG2 and OTA, respectively.

Besides the role of water in detoxification by gamma irradiation, the results point out that effectiveness of the method also depends on the mycotoxin present during irradiation.

When food commodities are contaminated with AFIs, commonly more than one aflatoxin is present. The presence of more than one type of mycotoxin can cause synergic effects between mycotoxins and can hinder the detoxification process. With the purpose of verify if synergistic effects between

the four AFIs affect degradation by irradiation, a solution containing the four AFIs was irradiated in the three conditions mentioned above. As it was observed for AFIs irradiated separately, higher radiation doses resulted in a higher degradation to all AFIs. Also, the trend that increase water content leads to increase degradation was observed. The obtained results could also indicate that the presence of the four mycotoxins enhances degradation since the obtained results for degradation were higher when compared to AFIs irradiated separately. These results can be explained by the higher concentration of AFIs in each solution. In fact, the way that samples were prepared led the concentration to increase 4-fold. The relation between the concentration and the performance of gamma irradiation, was not completely clear. In one hand, some published studies shown that increased concentration of mycotoxins resulted in increased degradation (Jalili *et al.*, 2010, Zhang *et al.*, 2018; Abdel-Rahman *et al.*, 2019). However, some other studies showed that the increase of concentration led to a decrease of degradation (Kalagatur *et al.*, 2018; Maatouk *et al.*, 2019). In the present work, different results were observed for different mycotoxins. For aflatoxins, as it was observed by Jalili *et al.* (2010), Zhang *et al.* (2018) and Abdel-Rahman *et al.* (2019), higher concentrations seems to improve degradation. For ZEA, as it was observed by Kalagatur *et al.* (2019), higher concentrations seem to decrease degradation. For OTA, results present in section 5.3.1, and as it was observed by Maatouk *et al.* (2010), showed that higher OTA concentration decreased degradation, in standards solutions. For contaminated flour, the trend was opposite but is in accordance with verified by Jalili *et al.* (2010), in powder black pepper.

6.2.2 Cytotoxicity of irradiated mycotoxins

The study of cytotoxicity of irradiated mycotoxins are crucial to claim the applicability of this method to detoxify contaminated food. Mycotoxin reduction could lead to the production of toxic by-products, and toxicological studies are needed to elucidate the safety of these decontaminated food matrices (Maatouk *et al.*, 2019).

Several cytotoxic effects of mycotoxins on humans and animals have already been evaluated (García-Herranz *et al.*, 2019; Juan-García *et al.*, 2019; Yirga *et al.* 2017). García-Herranz *et al.* (2019) detected high toxicity of Beauvericin (BEA), deoxynivalenol (DON) and OTA mycotoxins on fish (RTH-149 and PLHC-1) and on mammalian cell (H4IIE) lines. This study shows that the mechanism of toxicity at cellular level of DON and OTA seems to depend on species and tissue from which the cell lines employed in the cytotoxic assessment were derived from, and that the

plasma membrane was less affected. The cytotoxic effect of pure Citrinin (CTN) in embryonic kidney cells (HEK293) at a concentration of 60 μmol L^{-1} for 72 h was investigated by Yirga *et al.* (2017). According to the authors, a 50% of cell death was observed when compared to the control cells. However, there are relative few published studies that have evaluated the use of gamma irradiation technique for mycotoxins detoxification and their effect on mammal cells (Table 6-2).

Table 6-2 - Cytotoxicity of gamma irradiated mycotoxins

Mycotoxin	Cells	Reference
OTA	Human hepatoma - HepG2	Maatouk *et al.*, 2019
AFB1 **OTA**	Porcine kidney epitelial - Pk15 Human hepatoma - HepG2 Human neuroblastoma - SH-SY5Y	Domijan *et al.*, 2019.
ZEA	macrophage cell line of Mus musculus - RAW 264.7	Kalagatur *et al.*, 2018
OTA	Human hepatoma - HepG2	Calado *et al.* 2018.
OTA	Human intestinal epithelial - Int-407	Kumar *et al.* 2012.

Kumar *et al.* (2012) tested toxicity of irradiated and non-irradiated OTA samples in Int-407 cell line through MTT assays. The authors verified that due to the cytotoxic effect of OTA at 10 μg/mL, cell viability was significantly reduced, whereas, treatment with radiation exposed OTA significantly increased the cell viability by approximately 7-fold.

As it was described in chapter 5 of this thesis, more than 90% of OTA was degraded by gamma radiation doses above 2.5 kGy, occuring a 2-fold reduction in OTA Human hepatoma (HepG2) cytotoxicity.

Kalagatur *et al.* (2018) irradiated ZEA samples in water solution at 5 kGy and 10 kGy. These authors tested non-irradiated and irradiated ZEA samples at 3 μg/mL in RAW 264.7 cell line, and assessed the toxicity by determining the cell viability (MTT and live/dead cell assays), intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), nuclear damage, and

caspase-3 activity. Results confirmed that toxicity of ZEA was decreased with irradiation, leading authors to conclude that irradiation could be a potential post-harvest food processing technique for detoxification of ZEA mycotoxin in fruit juices.

Domijan *et al.* (2019) using stock solutions of AFB1 and OTA (50 mmol L^{-1}, in methanol) previously gamma irradiated (5 and 10 kGy), tested for cytotoxicity on Pk15, HepG2 and SH-SY5Y cell lines (MTT assay, 1-500 μ mol L^{-1} concentration range; 24 h exposure). Gamma irradiation even at 5 kGy had effect on AFB1 and OTA molecules; however, this effect was dependent on the mycotoxin. Since gamma irradiation at low dose reduced initial level of both mycotoxins, and gamma irradiated mycotoxins had lower toxicity in comparison to control (non-irradiated) mycotoxins, authors concluded that gamma irradiation could be used as decontamination method.

Maatouk *et al.* (2019) tested OTA samples irradiated in methanol solution with 2 and 4 kGy in HepG2 cells. The concentration tested in cells was 120 μmol L^{-1}. There was no significant difference in the mortality rates of HepG2 cells after exposure to irradiated and to nonirradiated OTA. These authors previously measured the decrease of OTA concentration in irradiated samples, and observed a poor detoxification (the maximal reduction observed was 15% at 4 kGy). Thus, low reductions of toxicity are explained by the poor OTA detoxification in samples.

In the present thesis, for all mycotoxins irradiated in water, a reduction of mycotoxin concentration and the reduction of cytotoxicity in HepG2 cells was observed. For irradiation of dehydrated samples, the cytotoxicity reduction was less pronounced; however, no increase of toxicity was observed in this condition. These results were in accordance with all published research. For this reason, it is possible to conclude that no toxic by-product was formed during the irradiation process and that this technique does not enhance mycotoxins toxicity.

Also the estrogenicity of ZEA was tested in hERα-HeLa-9903 cells. The results, as it was observed by cytotoxicity, shown no increase by irradiation process. For lower concentration in water solution, when degradation was higher, a pronounced reduction of estrogenicity was observed.

However, the few studies were insufficient to conclude about the decrease of toxicity by irradiation. As it was observed, the toxicity was different in distinct cell lines and differ with the viability of the test used. The next step can be the improve of knowledge about the process of mycotoxins detoxification. To reveal the precise process of mycotoxins detoxification, further research is needed on extraction, purification, structural elucidation and toxicity characterization of radiolytic products formed by each mycotoxin. Further knowledge about radiolytic product will provide insights on how

they can interact and affect the cell viability. The application of irradiation, as a detoxification method, requires further studies on detoxification of foods coupled with the study of irradiated foods cytotoxicity. This way, it will be possible to evaluate possible interations with the food components.

6.2.3 Irradiation of food samples

In this thesis, only OTA was tested in food matrices. As it was explained in Chapter 5, the irradiation of food matrices does not show a good degradation level. The results point out that the other components of food seem to hinder the OTA degradation by gamma radiation.

The results obtained with solid samples showed a low reduction (24%) of OTA, in a wheat flour sample with 32% of moisture content, at the higher irradiation dose tested (30.5 kGy). This result was observed for a set of samples with high OTA concentration (93 μg kg^{-1}). As it was explained above, some author reported the effect of higher mycotoxin concentrations on increased detoxification (Jalili *et al.*, 2010; Abdel-Rahman *et al.*, 2019). In this thesis, the reduction was higher in flour with 93 μg kg^{-1} than in flour with 23 μg kg^{-1} of OTA. Mehrez *et al.* (2016) obtained higher reductions in wheat: maximum reductions were 35.5 and 47.2% for moisture contents of 14 and 16%, respectively, at an irradiation dose of 8 kGy. Maatouk *et al.* (2019) observed for tunisian millet flour, OTA reduction of 62% at 4 kGy, leading the author to conclude that gamma irradiation can be a useful technology for the detoxification of OTA in millet flour.

Results summarized in section 6.2.1 about mycotoxins reduction in different condition by gamma irradiation, led to conclude that water improve the detoxification by gamma radiation. However, the result obtained in juice and wine irradiation point out that the abundant water was not sufficient to OTA detoxification. The colour changes observed in grape juice and wine due to irradiation, led to conclude that free radical formed by water radiolysis attack others compounds in juice and wine. These compounds, such as anthocyanins and other flavonoids, seem to sensitive to radiation. A similar study by Kalagatur and colleagues (2018), with irradiation of fruit juice contaminated with ZEA, showed a different result. Kalagatur *et al.* (2018) tested different ZEA concentrations and irradiation doses in distilled water, orange juice, pineapple juice and tomato juice. These authors verified reductions above 80% at 8.5 kGy when the initial ZEA concentration was 1.6 μg mL^{-1}. Minimal alterations in the quality of fruit juices was reported only for higher doses of 10 kGy.

These contradictory reports can be directly linked to the type of mycotoxin used to detoxify. The results obtained for several mycotoxins in this study show that the mycotoxins seem to present different sensitivity to gamma irradiation. Thus, the detoxification of mycotoxins needs more research about the type of food that it will be applied with focus in mycotoxin degradation, toxicity and sensorial characteristics.

The results of this thesis suggested the gamma irradiation cannot be useful to OTA detoxification in the food that was tested; however, it is important to remember that irradiation can be applied to food sanitizing. In this process, a reduction of mycotoxigenic fungus can improve the safety of food.

Iqbal *et al.* (2012) verified that, although gamma irradiation was not effective in reducing AFIs in hot pepper, it was sufficient to reduce total mould and *Aspergillus* counts in a dose-dependent relationship. A radiation dose of 6 kGy completely eliminated the population of total moulds and *Aspergillus* fungi. The author concluded that the distinct effectiveness of gamma radiation in moulds and aflatoxins can be explained by the target theory of food irradiation, which states that the likelihood of a microorganism or a molecule being inactivated by gamma rays increases as its size increases (Iqbal *et al.*, 2012).

Food irradiation can be used with some different goals, such as to prolong shelf life and reduce food losses, improve microbiologic safety, and/or reduce the use of chemical fumigants and additives, to reduce insect infestation of commodities, inhibit sprouting in tubers and bulbs, retard postharvest ripening of fruits, inactive parasites in meats and fish, eliminate spoilage microbes from fresh fruits and vegetables, extend shelf life in poultry and beef and sterilize foods and feeds. The preservation of nutritional and organoleptic properties in irradiated food is one area that need more research.

References

Abdel-Rahman, G. N., Sultan, Y. Y., Salem, S. H., & Amer, M. M. (2019). Identify the Natural Levels of Mycotoxins in Egyptian Roasted Peanuts and the Destructive Effect of Gamma Radiation. Journal of Microbiology, Biotechnology and Food Sciences, 9(2): 1174-1177.

Al-Jaal, B. A., Jaganjac, M., Barcaru, A., Horvatovich, P., & Latiff, A., (2019). Aflatoxin, fumonisin, ochratoxin, zearalenone and deoxynivalenol biomarkers in human biological fluids: A systematic literature review. Food and Chemical Toxicology: 2001–2018.

Alshannaq, A., & Yu, J. H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. International journal of environmental research and public health, 14(6):632.

Bennett, J. W. (1987). Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia. 100:3–5.

Calado, T., Fernández-Cruz, M.L., Verde, S.C., Venâncio, A., & Abrunhosa, L. (2018). Gamma irradiation effects on ochratoxin A: Degradation, cytotoxicity and application in food. Food Chemistry 240:463-471.

Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for mold and mycotoxin control: A review. Compr. Rev. Food Sci. F. 13:1049-1061.

Casas-Junco, P.P., Solís-Pacheco, J.R., Ragazzo-Sánchez, J.A., Aguilar-Uscanga, B.R., Bautista-Rosales, P.U., & Calderón-Santoyo, M. (2019). Cold plasma treatment as an alternative for Ochratoxin A detoxification and inhibition of mycotoxigenic fungi in roasted coffee. Toxins 11:337

Diao, E., Wang, J., Li, X., Wang, X., Song, H., & Gao, D. (2019). Effects of ozone processing on patulin, phenolic compounds and organic acids in apple juice. J Food Sci Technol. 56:957-965.

Domijan, A.M., Čermak, A.M.M., Vulić, A., Bujak, I.T., Pavičić, I., Pleadin, J., Markov, K., & Mihaljević, B. (2019). Cytotoxicity of gamma irradiated aflatoxin B1 and ochratoxin A. Journal of Environmental Science and Health, Part B. 54:155-162.

García-Herranz, V., Valdehita, A., Navvas, J.M., & Fernández-Cruz, M.L. (2019). Cytotoxicity against fish and mammalian cell lines and endocrine activity of the mycotoxins beauvericin, deoxynivalenol and ochratoxin-A. Food and Chemical Toxicology. 127: 288-297.

Maatouk, I., Mehrez, A., Amara, A. B., Chayma, R., Abid, S., Jerbi, T., & Landoulsi, A. (2019). Effects of Gamma Irradiation on Ochratoxin A Stability and Cytotoxicity in Methanolic Solutions and Potential Application in Tunisian Millet Samples. Journal of food protection, 82(8):1433-1439.

Iqbal, Q., Amjad, M., Asi, M.R. & Arino, A. (2012). Mold and aflatoxin reduction by gamma radiation of packed hot peppers and their evolution during storage. J Food Protect 75:1528-31.

Iqbal, S.Z., Bhatti, I.A., Asi, M.R., Zuber, M., Shahid, M. & Parveen, I. (2013). Effect of γ irradiation on fungal load and aflatoxins reduction in red chillies. Radiat Phys Chem 82:80-84.

Jalili, M., Jinap, S. & Noranizan, M.A. (2012). Aflatoxins and ochratoxin A reduction in black and white pepper by gamma radiation. Radiat Phys Chem 81:1786-88.

Juan-García, A., Tolosa, J., Juan, C., & Ruiz, M.J. 2019. Cytotoxicity, Genotoxicity and Disturbance of Cell Cycle in HepG2 Cells Exposed to OTA and BEA: Single and Combined Actions. Toxins.11:341. doi:10.3390/toxins11060341.

Kalagatur, N.K., Kamasani, J.R., Siddaiah, C., Gupta, V.K., Krishna, K., & Mudili, V. (2018). Combinational inhibitory action of Hedychium spicatum L. essential oil and γ-radiation on growth rate and mycotoxins content of Fusarium graminearum in maize: Response surface methodology. Front Microbiol. 9:1511.

Kumar, S., Kunwar, A., Gautam, S., & Sharma, A. (2012). Inactivation of A. ochraceus spores and detoxification of ochratoxin A in coffee beans by gamma irradiation. Journal of Food Science. 77:44–51.

Leitão, A.L. (2019). Occurrence of Ochratoxin A in Coffee: Threads and Solutions-A Mini-Review. Beverages. 5:36.

Magzoub, R.M.A., Yassin, A.A.A., Abdel-Rahim, A.M., Gubartallah, E.A., Miskam, M., Saad, B., & Sabar, S. (2019). Photocatalytic detoxification of aflatoxins in Sudanese peanut oil using immobilized titanium dioxide. Food Control. 95:206-214.

Marroquín-Cardona, A. G., Johnson, N. M., Phillips, T. D., & Hayes, A. W. (2014). Mycotoxins in a changing global environment – A review. Food and Chemical Toxicology, 69:220–230.

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A., & Magan, N. (2017). Climate change, food security and mycotoxins: Do we know enough? Fungal Biology Reviews, 31(3):143–154.

Mehrez, A.; Maatouk, I.; Romero-González, R.; Ben Amara, A.; Kraiem, M.; Garrido Frenich, A.; Landoulsi, A. (2016). Assessment of ochratoxin A stability following gamma irradiation: Experimental approaches for feed detoxification perspectives. World Mycotoxin J. 9:289-298.

Pankaj, S. K., Shi, H., & Keener, K. M. (2018). A review of novel physical and chemical decontamination technologies for aflatoxin in food. Trends in Food Science & Technology, 71: 73-83.

Pereira, E., Barros, L., Antonio, A.I., Verde, S.C., Santos-Buelga, C., Ferreira, I.C.F.R., & Rodrigues, P. (2017). Is Gamma Radiation Suitable to Preserve Phenolic Compounds and to Decontaminate Mycotoxins in Aromatic Plants? A Case-Study with Aloysia citrodora Paláu. Molecules. 22:347.

Pitt, J. I. (2000). Toxigenic fungi: which are important?. Sabouraudia, 38(1):17-22.

Ponzilacqua, B., Rottinghaus, G. E., Landers, B. R., & Oliveira, C. A. F. (2019). Effects of medicinal herb and Brazilian traditional plant extracts on in vitro mycotoxin decontamination. Food Control.100:24-27.

Sajid, M., Mehmood, S., Yuan, Y., & Yue, T. (2019). Mycotoxin patulin in food matrices: occurrence and its biological degradation strategies. Drug Metabolism Reviews. 1-48.

Sen, Y., Onal-Ulusoy, B., & Mutlu, M. (2019). Detoxification of hazelnuts by different cold plasmas and gamma irradiation treatments. Innovative Food Science & Emerging Technologies, 54:252-259.

Taroub, B., Salma, L., Manel, Z., Ouzari, H.I., Hamdi, Z., & Moktar, H. (2019). Isolation of lactic acid bacteria from grape fruit: antifungal activities, probiotic properties, and in vitro detoxification of ochratoxin A. Annals of Microbiology.69:17–27.

Tema, B.A., Fletcher, M.T., Fox, G.P., Harvey, J., Okothe, A.S., & Sultanbawa, Y.(2019). Curcumin-based photosensitization inactivates Aspergillus flavus and reduces aflatoxin B1 in maize kernels. Food Microbiology.82: 82-88.

Wielogorska, E., Ahmed, Y., Meneely, J., Graham, W. G., Elliott, C. T., & Gilmore, B. F. (2019). A holistic study to understand the detoxification of mycotoxins in maize and impact on its molecular integrity using cold atmospheric plasma treatment. Food chemistry, 301:125281.

Yirga, S.K., Ling, S., Yang, Y., Yuan, J., & Wang, S. (2017). The Preparation and Identification of a Monoclonal Antibody against Citrinin and the Development of Detection via Indirect Competitive ELISA. Toxins, 9: 110.

Zhang, Z., Xie, Q., & Che, L. (2018). Effects of gamma irradiation on aflatoxin B1 levels in soybean and on the properties of soybean and soybean oil. Applied Radiation and Isotopes.139:224-230.

Zhao, Z., Zhang, Y., Gong, A., Liu, N., Chen, S., Zhao, X., Li, X., Chen, L., Zhou, C., & Wang, J. (2019). Biodegradation of mycotoxin fumonisin B1 by a novel bacterial consortium SAAS79. Applied Microbiology and Biotechnology. 1-12.

Chapter 7. Conclusions and perspectives

7.1 General conclusions

The main purpose of this work was to verify the capacity of gamma radiation to degrade mycotoxins.

The primary assays, where gamma radiation was applied in aqueous solutions or pure mycotoxin, showed promising results. In these cases, gamma radiation was able to destroy mycotoxins when water was present. The formation of water radiolytic compounds during the irradiation process, seems to play a crucial role in mycotoxin degradation. These results point out that gamma irradiation is an effective method in mycotoxins degradation.

To assays with standards, mycotoxins irradiated in the presence of water are more sensitive to irradiation:

- Significant degradation was observed for doses ≥ 0.4 kGy for all mycotoxins tested;
- AFG1 and ZEA were not detected in samples after irradiation (≥ 0.4 kGy).

Irradiation in methanol:water solution, due to the less quantity of water, show a intermediated trend between the assays in water and in dehydrated conditions to all mycotoxins.

For assays with standards, for all mycotoxin tested, the irradiation on dehydrated condition led to lower degradation of mycotoxins. For the irradiation on dehydrated condition:

- The most sensitive mycotoxin was ZEA. For the higher dose, a reduction of 87 % was observed. Significant reductions of this mycotoxin were observed for doses ≥ 1.7 kGy.
- AFG1, the other sensitive mycotoxin, presented significant reductions for doses ≥ 0.9 kGy. For the higher dose, a reduction of 77 % was observed;
- For OTA and AFG2 significant reductions were observed for the higher dose tested. For this level of radiation, a reduction of 17% was observed to OTA and a reduction of 42% was observed to AFG2;
- For AFB1 and AFB2 no significant reduction was observed.

Although the mycotoxin sensitivity to radiation was different, in most of cases, ZEA and AFG1 were the more radio sensitive.

The cytotoxicity assays show that the degradation of mycotoxins was also accompanied by a reduction of the total toxicity. These results are very important for the application of this technique in food industry. With respect to the estrogenic activity of ZEA radiolytic products, the results were promising since a decrease in ZEA concentration after irradiation was always followed by a reduction in estrogenic activity especially when ZEA is in contact with water. These results can indicate that irradiation process applied for mycotoxins detoxification can be a safe method to reduce mycotoxins associated with health risk.

When gamma irradiation was applied in contaminated food, results were not promising. On solid food, although the humidity was around 30%, gamma radiation has not effective on mycotoxin concentration reduction. For OTA, the results of irradiation of wine and grape juice showed that this method is not indicated for these products. Although the content in water, the mycotoxin concentration does not decrease after the irradiation process. The free radicals that formed during irradiation attack other compounds. In case of wine and grape juice, the compounds in higher concentration, such as, antioxidants, are degraded instead of mycotoxin.

The application of gamma radiation to mycotoxins control needs more research in contaminated food products, since, although the degradation of mycotoxins separately was possible, the components of each food may interact by different way.

7.2 Perspectives to future work

Although this study shows that the degradation of mycotoxins separately was possible, the practical applicability of this method in food industry involves food irradiation, instead of mycotoxins standards irradiation. The food has several components that could interact with the efficiency of the gamma irradiation process. The application of this method in real contaminated food was important to assess the real effectiveness of gamma irradiation to detoxification food.

The test of ZEA reduction in food matrices could present good results of detoxification. Recent published studies show a good reduction of ZEA in liquid food and the present work shown that this mycotoxin is the most sensible to gamma irradiation. However, these mycotoxins are common in cereals, and it would be interesting to check the reduction in this type of food. On the same way, assays with AFIs in contaminated food products are required to evaluate if gamma irradiation can have applicability in real food.

The role of mycotoxin concentration on effectiveness of irradiation process does not shown a clear trend. Different behaviours were observed for different mycotoxins. This factor can be studied depth to design the degradation kinetics of each mycotoxin. The information can help to understand how each mycotoxin should be detoxified.

In the present work, tests of cytotoxicity and estrogenicity shown that a blend of radiolytic products and remaining mycotoxins presents a lower toxicity than non-irradiated mycotoxins. These experiments were carried out with mycotoxin standards dehydrated or in water. Again, the behaviour in real food can be distinct and the risk should be assessed. As it was mentioned above, the next step could be the characterization of all radiolytic products, test their toxicity and look for these components in real food irradiation.

Assays to prove the effectiveness of irradiation on deactivation of mycotoxigenic fungus is also required. Assays to verify the mycotoxin production capability after irradiation is very important to conclude about the feasibility of this method.

A critical analysis should be made to the available research to conclude about real applicability of gamma irradiation on detoxified food. If mycotoxin detoxify require high radiation doses and lead to degradation of nutritional and sensorial characteristics of food, this methodology could not be attractive. This technique can be more useful to inactivate the mycotoxigenic fungus and contribute to non-contamination of food.