Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

Programa de Doctorat amb Menció cap a l'Excel·lència en Ciències de l'Alimentació

REDUCCIÓN DE LA CONTAMINACIÓN POR HONGOS TOXIGÉNICOS Y MICOTOXINAS EN ALIMENTOS MEDIANTE EL USO DE ISOTIOCIANATOS

REDUCTION OF CONTAMINATION BY TOXYGENIC FUNGI AND MICOTOXINS IN FOOD THROUGH THE USE OF ISOTHIOCYANATES

Tesi Doctoral Internacional

València, 2019

Presentada per:
Juan Manuel Quiles Beses

Dirigida per:
Dr. Jordi Mañes Vinuesa
Dr. Giuseppe Meca

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

El **Dr. Jordi Mañes Vinuesa**, Catedràtic d'Universitat de l'àrea de Nutrició i Bromatologia i el **Dr. Giuseppe Meca**, Professor Titular de l'àrea de Nutrició i Bromatologia,

CERTIFIQUEN QUE:

El graduat en Ciència i Tecnologia dels Aliments **Juan Manuel Quiles Beses** ha realitzat baix la seua direcció el treball **"Reducció de la contaminació per fongs toxigènics i micotoxines en aliments mitjançant l'ús d'isotiocianats"**, i autoritzen la seua presentació per a optar al títol de Doctor per l'Universitat de València.

I, perquè així conste, expedeixen i signen el present certificat.

Burjassot (València), maig 2019.

Dr. Jordi Mañes i Vinuesa Dr. Giuseppe Meca

Aquesta tesi doctoral ha donat lloc a 5 articles, publicats a la següents revistes:

1. Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas. *Journal of Food Science and Technology, 52:12 (2015) 8315–8321. Impact factor: 1.797*

2. Influence of the antimicrobial compound allyl isothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust. *Food and Chemical Toxicology, 83 (2015) 222-228. Impact factor: 3.977*

3. Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population. *Food and Chemical Toxicology, 94 (2016) 19-24. Impact factor: 3.977*

4. Aflatoxins and *A. flavus* reduction in loaf bread through the use of natural ingredients. *Molecules, 23:7 (2018), 1638. Impact factor: 3.098*

5. Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation. *Toxins, 11:3 (2019), 137. Impact factor: 3.273*

El doctorand ha gaudit de la beca predoctoral del Programa “Atracció de talent” UV-INV-PREDOC16F1-384792 de la Universitat de València aixi com l’ajuda de mobilitat (2018) per la menció internacional del títol de Doctor, la qual es va realitzar a Bari (Itàlia) al Istituto di Scienze delle Prozuzioni Alimentari (ISPA-CNR) baix la direcció del Dr. Antonio Moretti.

Aixi mateix, aquesta Tesi Doctoral Internacional s’engloba dins dels següents projectes:

- Estudio de las micotoxinas y metabolitos en alimentos y muestras biológicas, de la toxicidad y de los procesos de descontaminación (AGL2013-43194-P).
- Mitigación, biomarcadores y toxicidad de micotoxinas legisladas y emergentes (AGL2016-77610-R).
- Reducción de la contaminación por hongos toxigénicos y micotoxinas en alimentos y piensos, mediante compuestos bioactivos de origen natural (GV2016-106).
- Red Nacional sobre las micotoxinas y hongos micotoxigénicos y de sus procesos de descontaminación - MICOFOOD (AGL2014-52648-REDT y AGL2017-90787-REDT).
- The European Project (H2020-Research and Innovation Action) MycoKey “Integrated and innovative key actions for mycotoxin management in the food and feed chain” (GA 678781).

  Programa Prometeo para grupos de investigación de excelencia (Prometeo 2018/126).

“*Filosófico es el preguntar,*

y poético el hallazgo”

María Zambrano (1904-1991)

Agraïments

Llegado a este punto, quiero acordarme de todas las personas que de una forma u otra han contribuido para que este día llegase. En primer lugar, dar las gracias a mis directores de tesis. Al Dr. Giuseppe Meca, por su confianza y apoyo a lo largo de todos estos años que me han permitido crecer tanto profesional como personalmente. Al Dr. Jordi Mañes, per permetre'm unir-me al grup de recerca i els sempre encertats consells que m'han permès portar a bon port aquesta tesi.

También quiero dar las gracias a la Dra. Guillermina Font, por su ejemplo de profesionalidad y rigurosidad científica.

Al personal del departamento de Medicina Preventiva, por generar un ambiente de trabajo tan positivo y por la ayuda que todos, en un momento dado, me habéis dado para llegar hasta aquí. Especialment a la Dra. Carmen Escriva, per rebre'mi sempre amb un somriure davant els meus mil i una dubtes.

Grazie al Dr. Antonio Moretti, per avermi accolto così gentilmente durante il mio periodo di ricerca all'Istituto di Scienze delle Produzioni Alimentari di Bari. Il lavoro con lui e il suo gruppo sarà sempre un ricordo che custodirò con affetto.

Gracias a todos aquellos estudiantes de máster, doctorado o erasmus con los que he compartido laboratorio a lo largo de estos años, por los buenos momentos vividos. A los que han formado parte de los *micointoxicados*: Bruno, Lucas, Keli, Marga, Carlos, Tiago, Raquel, y resto de compañeros. De todos y cada uno he aprendido mucho y me habéis ayudado a llegar hasta aquí.

A Noelia, per estar sempre ahi al llarg de tot aquest camí, i el que ens queda!

A Federica, la collega piu laboriosa, comprensiva e sensible incontrata finora.

Gracias a todos los amigos que durante este tiempo me han dado sus ánimos y me han ayudado a desconectar cuando lo necesitaba.

A Mariam, porque todo este trabajo no habría sido posible sin tener una persona como tu a mi lado. Gracias por tu amor y comprensión.

Por último, pero no menos importante, quiero agradecer a mis padres y a mi hermano Adrián, por apoyarme en todas las decisiones, por estar siempre cuando los necesito y porque no se las doy tanto como se merecen. Sin ellos este trabajo nunca habría existido.

Muchas gracias por todo.

Juanma.

TABLE OF CONTENTS

List of tables **XV**
List of figures **XVII**
List of abbreviations **XXI**
SUMMARY **1**
1. INTRODUCTION **7**
1.1. Micotoxinas **9**
1.1.1. Toxicidad 12
1.1.2. Legislación 14
1.1.3. Incidencia 17
1.1.4. Evaluación del riesgo 19
1.2. Isotiocianatos **21**
1.2.1. Glucosinolatos 22
1.2.2. Mirosinasa 23
1.2.3. Isotiocianatos 24
1.2.4. La mostaza como fuente natural de isotiocianatos 26
1.3. Los cereales **29**
1.3.1. Producción y consumo 30
1.3.2. Almacenamiento y conservación 32
1.3.3. Alteraciones microbiológicas del grano 33
1.3.4. Prevención y reducción de la contaminación por micotoxinas 34
1.4. El pan y productos derivados **38**
1.4.1. Definiciones y aspectos legales 39
1.4.2. Importancia nutricional 41
1.4.3. Vida útil y peligros biológicos 43
1.4.4. Métodos de conservación 45
1.5. Referencias **47**
2. OBJECTIVES **55**
3. RESULTS **59**
3.1. Ocurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population **61**

3.2. Influence of the antimicrobial compound allyl isothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust **81**

3.3. Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas **101**

3.4. Aflatoxins and *A. flavus* reduction in loaf bread through the use of natural ingredients **117**

3.5. Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation **145**

4. GENERAL DISCUSSION **171**

4.1. Micotoxinas en masas de pizza **174**

4.1.1. Análisis de micotoxinas en muestras de pizza refrigeradas comercializadas en Valencia 176

4.1.2. Estimación de la exposición a micotoxinas a través de pizzas refrigeradas y evaluación del riesgo 176

4.2. Reducción de hongos y micotoxinas **179**

4.2.1. Empleo de harina de mostaza 197

4.2.1.1. Validación del método de análisis de glusocinolatos 179

4.2.1.2. Determinación de glucosinolatos en harinas de mostaza 180

4.2.1.3. Actividad antifúngica de harinas de mostaza 181

4.2.1.4. Reducción de hongos y micotoxinas en pan de molde 183

4.2.1.5. Estudio de vida útil del pan de molde 185

4.2.2. Empleo de isotiocianatos 185

4.2.2.1. Reducción de hongos y micotoxinas en masas de pizza refrigeradas 186

4.2.2.2. Reducción de micotoxinas en tortitas de trigo 188

4.2.2.3. Dispositivo antifúngico para silos de cereales 189

4.2.2.3.1. Volatilización del gel de alil isotiocianato 190

4.2.2.3.2. Reducción de hongos y micotoxinas en cereales 190

4.3. Referencias **192**

5. CONCLUSION **197**

ANNEX **203**

LIST OF TABLES

1. INTRODUCTION

Tabla 1. Principales micotoxinas y especies fúngicas productoras 10

Tabla 2. Principales micotoxinas y calificación en función de su carcinogenicidad 14

Tabla 3. Contenidos máximos legislados de micotoxinas en alimentos 16-17

Tabla 4. Ingestas tolerables propuestas para las principales micotoxinas 21

Tabla 5. Principales GSs e ITCs asociados, y alimentos donde estos se encuentran de forma mayoritaria 23

Tabla 6. Composición nutricional de una muestra de harina de mostaza oriental 28

Tabla 7. Las tribus de los cereales 30

Tabla 8. Especies fúngicas toxigénicas presentes en almacenamiento de cereales 34

Tabla 9. Composición nutricional y valor energético de distintos tipos de pan 42

Tabla 10. Características de hongos alterantes del pan 44

Tabla 11. Coservantes más utilizados en pan 46

3. RESULTS

3.1. *Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population*

Table 1. Analytical parameters of the validation method. 67

Table 2. Mycotoxins detected in the analyzed refrigerated pizza dough samples, specifying the number of positive samples, the frequency, mean and range (positive > LOQ). 69

Table 3. Risk characterization of mycotoxins detected in the studied samples. 74

3.2. Influence of the antimicrobial compound allyl isothiocyanate against the Aspergillus parasiticus growth and its aflatoxins production in pizza crust

Table 1. Validation method parameters for the detection and quantification of sinigrin in oriental mustard flour. 90

3.3. *Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas*

Table 1. AFB_1, AFB_2, AFG_1 and AFG_2 reduction rates produced by the gaseous dispersion of ITCs produced by the enzymatic hydrolysis of the glucosinolates present in a) oriental and b) yellow mustard flour. The plastic bags were thermally sealed and incubated at 23 °C for 15 days. 110

3.4. Aflatoxins and *A. flavus* reduction in loaf bread through the use of natural ingredients

Table 1. Antifungal activity evidenced by no autoclaved and autoclaved YMF and OMF against mycotoxigenic fungi employed in this study (E = Direct water extract; L = Lyophilized water extract). Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++). 121

Table 2. MIC and MFC evidenced by the YMF. 124

Table 3. Shelf life, monitored in days, of the bread loaves treated with 4 different concentrations of the YMF and contaminated with (a) *Aspergillus flavus* (ISPA 8111) and (b) *Penicillium nordicum* (CECT 2320), in comparison with the commercial control produced with the additive E-281, and with a loaf bread prepared without any antimicrobial treatment. 132

3.5. *Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation*

Table 1. LODs, LOQs, recovery, and matrix effect (ME) (%) for AFB1, AFB_2, AFG_1, AFG_2, and OTA in corn, wheat, and barley. 150

4. GENERAL DISCUSSION

Tabla 1. Tratamientos de AITC probados en cereales 189

LIST OF FIGURES

1. INTRODUCTION

Figura 1. Síntesis de micotoxinas y curva de crecimiento 12

Figura 2. Número de notificaciones por micotoxinas en la UE de 2004 a 2017 18

Figura 3. Reacción de biotransformación de los GSs en ITCs y estructuras químicas de: a) AITC, b) PITC y c) BITC 25

Figura 4. Ejemplos de semillas de mostaza 27

Figura 5. Estructura y composición de un grano de trigo 29

Figura 6. Silo de grano moderno con dispositivo de aireación 33

3. RESULTS

3.2. *Influence of the antimicrobial compound allyl isothiocyanate against the Aspergillus parasiticus growth and its aflatoxins production in pizza crust*

Figure 1. Chemical structure of the main AFs. 82

Figure 2. LC-DAD chromatogram of sinigrin present in oriental mustard flour. 90

Figure 3. Population of *Aspergillus parasiticus* in fresh pizza crust treated treated with natural and synthetic preservatives (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, P ≤ 0.05 (*), P ≤ 0.001 (**), P ≤ 0.0001 (***). 92

Figure 4. Concentration and reduction rate of a) AFB_1 and b) AFB_2 present in fresh pizza crust contaminated with *Aspergillus parasiticus* and treated with several preserving treatments (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, P ≤ 0.05 (*), P ≤ 0.001 (**), P ≤ 0.0001 (***). 95

Figure 5. Concentration and reduction rate of a) AFG_1 and b) AFG_2 present in fresh pizza crust contaminated with Aspergillus parasiticus and treated with several preserving treatments (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***). 97

3.3. ***Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas***

Figure 1. General structure of aflatoxins B_1, B_2, G_1 and G_2. 102

Figure 2. Schematization of AFs reduction experiments carried out on wheat tortilla using gaseous dispersion of ITCs generated by enzymatic hydrolysis of GLCs contained in oriental and yellow mustard flours. a Control wheat tortilla contaminated by *Aspergillus parasiticus* CECT 2681 and wheat tortillas treated with a 0.1 b 0.5 and c 1 g of oriental mustard flours at 8 days incubation. Visible the green spots (fungal growth) are noticeable in the control group and absent in the treated groups. 106

Figure 3. AFs B_1, B_2, G_1 and G_2 produced by *Aspergillus parasiticus* CECT 2681 on wheat tortillas in the absence of oriental and yellow mustard (control group). 109

Figure 4. LC-MS/MS chromatograms of the AFB_1 and AFB_2 detected in wheat tortillas; a control and b treated with 1 g of yellow mustard flour. 111

3.4. Aflatoxins and *A. flavus* reduction in loaf bread through the use of natural ingredients

Figure 1. Chemical structures of the aflatoxins. 118

Figure 2. Antifungal activity of YMF lyophilized water extract against *P. nordicum* (CECT 2320) on PDA medium. 122

Figure 3. Population of (**a**) *A. flavus* and (**b**) *P. nordicum* in bread loaves treated with natural (YMF) and synthetic commercial (E-281) preservatives. Significantly different from the commercial control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***). 127

Figure 4. Concentration and reduction rate of AFB_1 present in bread loaves contaminated with *A. flavus* and treated with several preserving treatments (Black = synthetic commercial E-281 preservative, Grey = Different doses of natural YMF) at 10 days of incubation. Significantly different from the bread without treatment, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***). 130

Figure 5. Bread loaves contaminated with *P. nordicum* and treated with (a) 2.0 g/kg of sodium propionate (E-281); (b) not treated; (c) 2 g/kg of YMF; (d) 4 g/kg of YMF; (e) 6 g/kg of YMF and (f) 8 g/kg of YMF, after 10 days of incubation. 133

3.5. *Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation*

Figure 1. AITC detected in the headspace of the glass jar containing corn, wheat, and barley, used to simulate the storage of the cereals in a lab scale silo system. 148

Figure 2. Concentrations of AITC detected in corn, wheat, and barley after 1 (gray) and 30 (white) days of incubation. 149

Figure 3. Growth, in lab scale silo system, of the *A. flavus* in corn and of *P. verrucosum* in wheat and barley exposed to the vapor of the AITC after 1 and 30 days of incubation. Samples control (dark gray) and treated samples (clear gray). Significantly different from untreated cereal, $p \leq 0.05$ (*). 152

Figure 4. Aflatoxin B_1 detected in corn a); and ochratoxin A detected in barley b) treated with the AITC device in the lab scale silo system at 30 days of incubation. Samples control (dark gray) and treated samples (clear gray). Significantly different from untreated cereal, $p \leq 0.05$ (*). 153

Figure 5. Growth, in small-scale silo system, of the *A. flavus* in corn and of *P. verrucosum* in wheat and barley exposed to the vapor of the AITC after 1, 30, and 60 days of incubation. Control (dark gray) and treated samples (light gray). Significantly different from untreated cereal, $p \leq 0.05$ (*). 154

Figure 6. Ochratoxin A detected in wheat and barley treated with the AITC device in the small-scale silo system at 30 and 60 days of incubation. Samples control (dark gray) and treated samples (light gray). Significantly different from untreated cereal, $p \leq 0.05$ (*). 155

Figure 7. Lab scale silo system used for the treatment of corn, wheat and barley contaminated with *A. flavus* and *P. verrucosum* and treated with the AITC device. 162

Figure 8. Small-scale silo system used for the treatment of corn, wheat, and barley contaminated with *A. flavus* and *P. verrucosum*, and treated with the AITC device. (a) Theoretical silo design; and (b) the plastic drum used in this study. 162

LIST OF ABBREVIATIONS

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFC	Scientific Panel on food additives, flavourings, processing aids and materials in contact with food
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
AITC	Allyl isothiocyanate
ALARA	As Low As Reasonably Achievable
ANS	Panel on Food Additives and Nutrient Sources added to Food
a_w	Activity water
BEA	Beauvericin
BITC	Benzyl isothiocyanate
BPA	Buenas prácticas agrícolas (Good farming practices)
CAE	Código Alimentario Español
CECT	Colección Española de Cultivos Tipo (Spanish Type Culture Collection)
DON	Deoxynivalenol
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EN A	Enniatin A
EN A_1	Enniatin A_1
EN B	Enniatin B
EN B_1	Enniatin B_1
ENs	Enniatins
EO	Essential oil
FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisin B_1

FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FBs	Fumonisins
FID	Flame ionization detector
FUS	Fusaproliferin
GC	Gas chromatography
GSs-GSLs	Glucosinolates
GRAS	Generally recognized as safe
HCl	Hydrochloric acid
HCOOH	Formic acid
HEC	Hydroxyethyl cellulose
IARC	International Agency for Research on Cancer
ISPA	Istituto Scienze delle Produzioni Alimentari
ITCs	Isothiocyanates
JECFA	Joint Expert Committee on Food Additives
LC	Liquid-chromatography
LC-DAD	Liquid chromatography with a diode array detector
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOAEL	Lowest observed adverse effect level
LOD	Limit of Detection
LOQ	Limit of Quantification
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente (Ministry of Agriculture, Food and Environment)
MAP	Modified atmosphere packaging
ME	Matrix effects
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
NIV	Nivalenol
NOAEL	No observed adverse effect level

OMF	Oriental mustard flour
OTA	Ochratoxin A
PAT	Patulin
PBS	Phosphate buffer saline
pc	Peso corporal (body weight)
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ρ-HBITC	Para-hydroxybenzyl isothiocyanate
PITC	Phenyl isothiocyanate
PMTDI	Provisional maximum tolerable daily intake
PMTWI	Provisional maximum tolerable Weekly intake
R^2	Coefficient of determination
RASFF	Rapid Alert System for Food
RTS	Reglamentación Técnico-Sanitária (Technical-Sanitary Regulation)
SSE	Signal Suppression – Enhancer
TBA	Tetrabutylammonium hydrogensulfate
TCs	Thricothecenes
TDI	Tolerable daily intake
UE	Unión Europea (European Union)
UV	Ultraviolet
YMF	Yellow mustard flour
ZEA	Zearalenone

RESUMEN

El crecimiento de hongos es una de las causas más habituales del deterioro de los alimentos. Productos como los cereales, el pan y sus derivados son especialmente susceptibles ser contaminados con hongos toxigénicos y/o micotoxinas, lo que supone un problema de seguridad alimentaria. Por ello en la presente Tesis Doctoral se han estudiado la presencia de micotoxinas en 60 muestras de masas de pizza refrigeradas y el riesgo de exposición de la población a estos compuestos. Se detectaron aflatoxinas (AFs), zearalenona (ZEA), eniatinas (ENs) y beauvericina (BEA) en el 50, 100, 100 y 3 % respectivamente. Aunque el riesgo de exposición fue moderado, el 12 % de las muestras superaba los límites máximos legislados para AFs y la ZEA.

En base a estos datos, se planteó la necesidad de desarrollar estrategias basadas en el uso de sustancias naturales para reducir la presencia de hongos toxigénicos y micotoxinas en este tipo de productos y similares. Los isotiocianatos (ITCs) son compuestos bioactivos obtenidos por la hidrólisis de los glucosinolatos (GSs), metabolitos secundarios de vegetales del género *Brassica* como brócoli, coliflor, coles de Bruselas y por encima de todos, la mostaza. Se han evaluado distintas metodologías de aplicación de los ITCs a productos de panadería, como el uso de harina de mostaza en la formulación de panes, o la liberación de ITCs a partir de harina de mostaza en el interior de envases de tortitas de trigo y masas de pizza. Todos estos alimentos fueron contaminados con hongos toxigénicos de los géneros *Aspergillus* y *Penicillium* para estudiar la efectividad de los distintos tratamientos antifúngicos. Asimismo, se diseñó un dispositivo con base de gel de hidroxietil celulosa para la volatilización del ITC alil isotiocianato (AITC) en el interior de silos de cereales para reducir la contaminación fúngica en maíz, cebada y trigo.

Los resultados obtenidos reflejan la eficacia de los ITCs como conservantes de origen natural, observándose una eficacia frente a los hongos ensayados (*A. flavus*, *A. parasiticus* y *P. nordicum*) equivalente al de los conservantes clásicos como el ácido propiónico y sus sales, y en muchos casos superior (100 % de reducción) a dosis superiores a los 10 µL/L. Cuando se utilizó la harina de mostaza como ingrediente, la más efectiva fue la amarilla, rica en el ITC parahidroxibencil isotiocianato (ρ-HBIT), mientras que, si el efecto se buscaba por volatilización en el interior del envase, las mayores propiedades antifúngicas fueron mostradas por la harina oriental, cuyo ITC mayoritario es el AITC. La reducción de la síntesis de micotoxinas producidas por los hongos ensayados (AFs) fue proporcional a la inhibición del crecimiento fúngico. Finalmente, la capacidad antifúngica del AITC también se demostró en sistemas de silos de cereales simulados, con reducciones de entre 0,9 y 2 log en bidones de 100 L, tratados durante 60 días con 50 µL/L.

RESUM

El creixement de fongs és una de les causes més habituals del deteriorament dels aliments. Productes com els cereals, el pa i els seus derivats són especialment susceptibles de ser contaminats amb fongs toxigénics i/o micotoxines, el que suposa un problema de seguretat alimentària. Per això en la present tesi doctoral s'han estudiat la presència de micotoxines en 60 mostres de masses de pizza refrigerades i el risc d'exposició de la població a aquests compostos. Es van detectar aflatoxines (AFs), zearalenona (ZEA), eniatinas (ENs) i beauvericina (BEA) al 50, 100, 100 i 3 % respectivament. Tot i que el risc d'exposició va ser moderat, el 12 % de les mostres superava els límits màxims legislats per AFs i la ZEA.

D'acord amb aquestes dades, es va plantejar la necessitat de desenvolupar estratègies basades en l'ús de substàncies naturals per reduir la presència de fongs toxigénics i micotoxines en aquest tipus de productes i similars. Els isotiocianats (ITCs) són compostos bioactius obtinguts per la hidròlisi dels glucosinolats (GSs), metabòlits secundaris de vegetals del gènere *Brassica* com bròquil, coliflor, cols de Brussel·les i per sobre de tots, la mostassa. S'han avaluat diferents metodologies d'aplicació dels ITCs a productes de fleca, com l'ús de farina de mostassa en la formulació de pans, o l'alliberament d'ITC a partir de farina de mostassa a l'interior d'envasos de tortitas de blat i masses de pizza. Tots aquests aliments van ser contaminats amb fongs toxigénicos dels gèneres *Aspergillus* i *Penicillium* per estudiar l'efectivitat dels diferents tractaments antifúngics. Així mateix, es va dissenyar un dispositiu amb base de gel de hidroxietil cel·lulosa per a la volatilització de l'ITC alil isotiocianat (AITC) a l'interior de sitges de cereals per reduir la contaminació fúngica en blat de moro, ordi i blat.

Els resultats obtinguts reflecteixen l'eficàcia dels ITCs com a conservants d'origen natural, observant-se una eficàcia enfront dels fongs assajats (*A. flavu*s, *A. parasiticus* i *P. nordicum*) equivalent al dels conservants clàssics com l'àcid

propiònic i les seves sals, i en molts casos superior (100 % de reducció) a dosis superiors als 10 µL/L. Quan es va utilitzar la farina de mostassa com a ingredient, la més efectiva va ser la groga, rica en l'ITC parahidroxibencil isotiocianat (ρ-HBIT), mentre que, si l'efecte es buscava per volatilització a l'interior de l'envàs, les majors propietats antifúngiques van ser mostrades per la farina oriental, el ITC majoritari és el AITC. La reducció de la síntesi de micotoxines produïdes pels fongs assajats (AFs) va ser proporcional a la inhibició del creixement fúngic. Finalment, la capacitat antifúngica del AITC també es va demostrar en sistemes de sitges de cereals simulats, amb reduccions d'entre 0,9 i 2 log en bidons de 100 L, tractats durant 60 dies amb 50 µL/L.

SUMMARY

Fungi growth is one of the most common causes of food spoilage. Products such as cereals, bread and their derivates are especially susceptible to being contaminated with toxigenic fungi and / or mycotoxins, which is a problem of food safety. For this reason, in the present Doctoral Thesis, the presence of mycotoxins in 60 samples of refrigerated pizza doughs and the risk of exposure of the population to these compounds have been studied. Aflatoxins (AFs), zearalenone (ZEA), eniatins (ENs) and beauvericin (BEA) were detected in 50, 100, 100 and 3% respectively. Although the risk of exposure was moderate, 12% of the samples exceeded the maximum limits legislated for AFs and the ZEA.

Based on these data, there's a need to develop strategies based on the use of natural substances to reduce the presence of toxigenic fungi and mycotoxins in this type of products and the like. Isothiocyanates (ITCs) are bioactive compounds obtained by hydrolysis of glucosinolates (GSs), secondary metabolites of Brassica vegetables such as broccoli, cauliflower, Brussels sprouts and above all, mustard. Different methodologies for application of ITCs to bakery products have been evaluated, such as the use of mustard flour in the formulation of breads, or the release of ITCs from mustard flour inside the packages of wheat tortillas and pizza doughs. All these foods were contaminated with toxigenic fungi of the genera *Aspergillus* and *Penicillium* to study the effectiveness of the different antifungal treatments. In addition, a device with a hydroxyethyl cellulose gel base was designed for the volatilization of ITC allyl isothiocyanate (AITC) inside cereal silos to reduce fungal contamination in corn, barley and wheat.

The results obtained reflect the effectiveness of ITCs as preservatives of natural origin, showing an efficacy against the fungi tested (A. flavus, A. parasiticus and P. nordicum) equivalent to that of classical preservatives such as propionic acid and its salts, and in many cases superior (100 % reduction) at doses

higher than 10 µL/L. When mustard flour was used as an ingredient, the most effective was the yellow mustard, rich in ITC parahydroxybenzyl isothiocyanate (ρ-HBIT), while, if the effect was sought by volatilization inside the container, the highest antifungal properties were shown by the oriental mustard flour, whose main ITC is the AITC. The reduction of the synthesis of mycotoxins produced by the fungi tested (AFs) was proportional to the inhibition of fungal growth. Finally, the antifungal capacity of the AITC was also demonstrated in simulated grain silo systems, with reductions of between 0.9 and 2 log in 100 L plastic drums, treated for 60 days with 50 µL/L.

1. INTRODUCTION

Introducción

1. INTRODUCCIÓN

1.1. Micotoxinas

Por micotoxinas se conocen a una serie de compuestos tóxicos, de peso molecular medio (inferior a 700 Da) y cuyo origen se encuentra en el metabolismo secundario de distintos tipos de microorganismos del reino Fungi. La etimología de la palabra proviene de la unión de dos términos: "*myke*" del griego, que significa hongo, y "*toxicum*", del latín, que significa veneno (Soriano, 2007).

Ya desde la antiguedad se pueden encontrar referencias a micotoxicosis aunque se desconocía la etiología de esas patologías. Un ejemplo es el ergotismo o "fuego de San Antón", llamado así en la Edad Media porque uno de sus síntomas eran dolores comparables al de quemaduras. Esta enfermedad estaba causada por el consumo de pan de centeno contaminado por hongos y del que hasta 1850 no se identificarían como causantes a los alcaloides producidos por *Claviceps purpurea*, vulgarmente conocido como cornezuelo del centeno (Illana-Esteban, 2008).

Un siglo después, en 1960, tuvo lugar en el Reino Unido la muerte de miles de patos y pavos en la que fue conocida como "enfermedad X del pavo". Al descubrirse que la causa de dicha enfermedad era consumo de harinas de cacahuete contaminadas por micotoxinas, se produjo un amplio interés por el estudio de dichos compuestos y su impacto en la salud pública (Martínez-Larrañaga y Anadón, 2012).

Existen alrededor de una decena de géneros de hongos productores de micotoxinas, aunque los más importantes son *Penicillium*, *Aspergillus*, *Fusarium* y *Alternaría*. En la actualidad se han descrito más de 400 micotoxinas diferentes, y

muchas de ellas son de importancia por su habitual presencia en alimentos a nivel mundial (Tabla 1) (Streit *et al*., 2012).

Tabla 1. Principales micotoxinas y especies fúngicas productoras (Marín *et al.* 2013)

Micotoxina	Principales especies fúngicas productoras
Aflatoxinas B_1, B_2, G_1 y G_2 (AFB_1, AFB_2, AFG_1, AFG_2)	*Aspergillus parasiticus* *Aspergillus flavus*
Beauvericina (BEA)	*Fusarium sporotrichioides* *Fusarium poa* *Fusarium langsethiae* *Fusarium sección liseola,* *Fusarium avenaceum*
Deoxinivalenol (DON)	*Fusarium graminearum* *Fusarium culmorum* *Fusarium cerealis*
Eniatinas A_1, A_2, B_1, B_2 (ENA_1, ENA_2, ENB_1, ENB_2)	*Fusarium avenaceum* *Fusarium tricinctum*
Fumonisinas B_1 y B_2 (FB_1, FB_2)	*Fusarium verticillioides*
Ocratoxina A (OTA)	*Penicillium verrucosum* *Aspergillus ochraceus*
Patulina (PAT)	*Penicillium expansum,* *Aspergillus clavatus*
Toxinas T-2 y HT-2	*Fusarium acuminatum* *Fusarium poae* *Fusarium sporotrichioides* *Fusarium langsethiae*
Zearalenona (ZEA)	*Fusarium graminearum* *Fusarium culmorum* *Fusarium equiseti* *Fusarium cerealis* *Fusarium verticillioides* *Fusarium incarnatum*

Los alimentos que pueden encontrarse contaminados por micotoxinas son principalmente los de origen vegetal, como cereales, frutos secos, verduras y frutas, aunque también pueden encontrarse en alimentos derivados de ellos como son la cerveza, el vino y los zumos de fruta. En los alimentos de origen animal también se han encontrado micotoxinas debido al consumo de piensos contaminados (Richard, 2007).

Las micotoxinas son metabolitos secundarios, lo que significa que no son moléculas esenciales para la supervivencia del hongo como pueden serlo vitaminas o aminoácidos, sino que cumplen diversos tipos de funciones más complejas, como otorgar ventajas competitivas sobre otros hongos o bacterias cuando éstos crecen sobre un mismo sustrato (Fox y Howlet, 2008).

La mayoría de los hongos productores de micotoxinas son aerobios y mesófilos, por lo que necesitan oxígeno para desarrollarse y temperaturas en un intervalo de entre 25 y 30 °C. Su crecimiento puede dividirse en cuatro etapas: fase de latencia, de crecimiento exponencial, estacionario y de muerte. Es propio de los hongos el que la fase inicial de latencia sea singularmente larga, mientras que, transcurrido ese periodo inicial, el crecimiento es especialmente acelerado (Willey y Prescott, 2009). La formación de las micotoxinas tiene lugar en un periodo comprendido entre la etapa final de la fase de crecimiento exponencial y la etapa inicial de la fase de crecimiento estacionaria (Figura 1).

El otro parámetro junto con la temperatura que determina el crecimiento de los hongos y la formación de micotoxinas es la humedad. Los principales hongos toxigénicos requieren de una actividad de agua (a_w) mínima para su crecimiento de entre 0,78 (*A. flavus*) y 0,90 (*Fusarium spp*.) La a_w mínima para la formación de micotoxinas es ligeramente superior a la de crecimiento fúngico (0,80-0,95) aunque la óptima se sitúa entre 0,95-0,99 (Bennett y Klich, 2003).

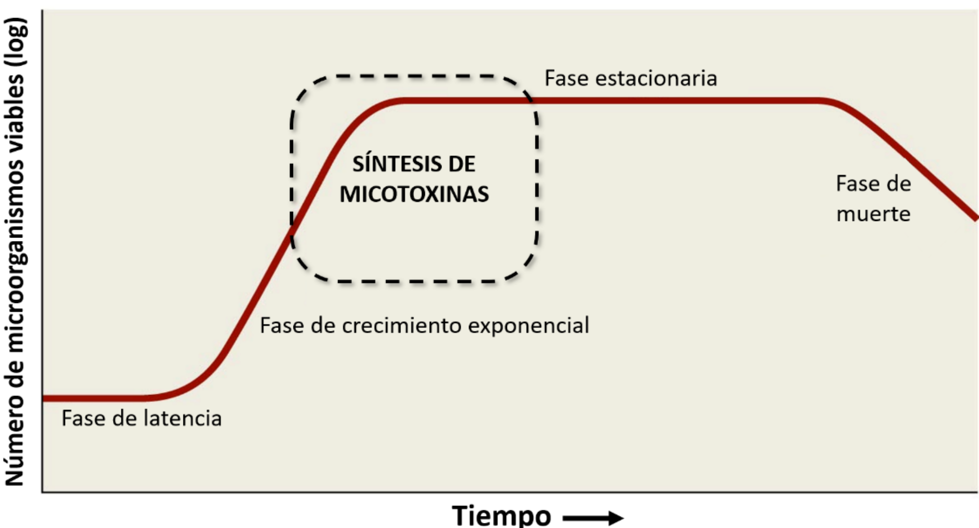


Figura 1. Síntesis de micotoxinas y curva de crecimiento

1.1.1. Toxicidad

La percepción generalizada de la opinión pública respecto a los riesgos tóxicos de los alimentos se centra mayoritariamente en aditivos y plaguicidas, pero la realidad es que las micotoxinas ocupan un lugar significativo entre los causantes de intoxicaciones agudas en animales de abasto y se trata de un posible responsable en la producción de efectos adversos a largo plazo en humanos.

Los efectos fisiopatológicos causados por las micotoxinas abarcan un amplio espectro: neurotóxicos, nefrotóxicos, hepatotóxicos y lesiones cardíacas en el caso de fumonisinas (FBs), trastornos gastrointestinales y neurológicos en el de la PAT, y nefropatía y tubulonefritis en el caso de OTA. La presencia habitual de las micotoxinas en alimentos a bajas concentraciones explica la mayor casuística de este tipo de afecciones asociadas a casos de exposición crónica (Marín *et al.*, 2013).

Asimismo, algunas micotoxinas tienen efectos carcinógenos y es por ello por lo que la Agencia Internacional de Investigación sobre el Cáncer (IARC) las ha clasificado de acuerdo con los siguientes grupos (Tabla 2) (Ruiz y Font, 2008):

- Grupo 1: agentes carcinógenos. Evidencia científica en humanos.
- Grupo 2: agentes potencialmente carcinógenos. Evidencia limitada.
    - Subgrupo 2A: agentes probablemente carcinógenos. Limitada evidencia en humanos, pero suficiente en estudios en animales.
    - Subgrupo 2B: agentes posiblemente carcinógenos. Limitada evidencia en humanos y en animales.
- Grupo 3: agentes posiblemente no carcinógenos. La evidencia indica que no es posible clasificarlo como tal, según la información científica disponible.
- Grupo 4: agentes probablemente no carcinógenos. Existen pruebas para demostrar que el agente no está asociado con el cáncer en seres humanos.

El Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) y la Autoridad Europea de Seguridad Alimentaria (EFSA), a partir de los datos toxicológicos como el nivel de mínimo efecto tóxico observable (LOAEL) o el nivel sin efecto adverso observable (NOAEL) han estimado la ingesta diaria tolerable (TDI) para las micotoxinas más importantes. A falta de datos fiables sobre los efectos de la exposición en humanos, se estiman otros indicadores como la estimación de la ingesta diaria tolerable máxima provisional (PMTDI) o la ingesta semanal tolerable máxima provisional (PMTWI). Estas dosis son las que posteriormente utilizan las autoridades para establecer los límites máximos de micotoxinas presentes en alimentos para consumo humano o animal.

Tabla 2. Principales micotoxinas y calificación en función de su carcinogenicidad (Ostry *et al*., 2017)

Micotoxina	IARC	Referencia
AFs	1	IARC, 2012
OTA		IARC, 1993
FB_1	2B	IARC, 1993
FB_2		IARC, 2002
PAT		IARC, 1987
DON	3	IARC, 1993
ZEA		IARC, 1993
T-2		IARC, 1993

1.1.2. Legislación

La última revisión de la FAO (2003), informó de la existencia de regulación sobre la presencia de micotoxinas en alimentos y/o piensos en 99 países de un total de 119 estudiados. Este dato supuso un aumento del 30 % respecto a la anterior evaluación de 1995. A fecha del último informe se considera que el 87 % de la población mundial habitaba en un país donde existía alguna normativa sobre micotoxinas, lo que significaba como mínimo, que tenían límites regulatorios para la AFB_1 o para la suma de las AFB_1, AFB_2, AFG_1 y AFG_2 en alimentos y/o piensos. En muchos casos además existen legislaciones específicas para otras micotoxinas como la aflatoxina M_1 (AFM_1), DON, T-2 y HT-2, FB_1, FB_2, FB_3, ZEA y PAT. A este respecto, la tendencia actual global en este campo pasa por aumentar el número

de micotoxinas reguladas en un mayor número de productos, mientras que los valores máximos tolerados se mantienen iguales o disminuyen (FAO, 2003).

Dentro de la Unión Europea (UE), el Reglamento EC 315/93 del Parlamento Europeo y del Consejo (CEE, 1993), por el que se establecen procedimientos comunitarios en relación con los contaminantes presentes en los productos alimenticios constituye la norma de referencia. Este reglamento armonizó las legislaciones previas de los Estados miembros, con el objetivo de garantizar la salud pública de los consumidores a través de unos niveles aceptables de contaminantes desde el punto de vista toxicológico.

Esta norma fue desarrollada a través del Reglamento CE n° 1881/2006 (CE, 2006a) por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. Esta norma ha sido actualizada en los años posteriores mediante los siguientes reglamentos:

- Reglamento (CE) n° 1126/2007, por el que se modifica el Reglamento (CE) n° 1881/2006 y se fija el contenido máximo de las toxinas de *Fusarium* en el maíz y sus productos derivados (CE, 2007a).
- Reglamento (UE) n° 105/2010 y (UE) n° 594/2012, por el que se modifica el Reglamento (CE) n° 1881/2006 y se fija el contenido máximo de OTA en productos alimenticios (UE, 2010a; UE, 2012a).
- Reglamento (UE) n° 165/2010 y (UE) n° 1058/2012, por el que se modifica el Reglamento (CE) n° 1881/2006 y se fija el contenido máximo de AFs en productos alimenticios (UE, 2010b; UE, 2012b).
- Reglamento (UE) n° 212/2014, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que concierne al contenido máximo de citrinina en complementos alimenticios basados en arroz fermentado (UE, 2014).

- Reglamento (UE) n° 2015/1940, por el que se modifica el Reglamento (CE) n° 1881/2006 en relación al contenido máximo de esclerocios de cornezuelo de centeno en determinados cereales (UE, 2015).

Los valores máximos que se legislan dependen de varios factores, como son sus efectos toxicológicos, el consumo poblacional, así como la edad de la población a la que van destinados estos alimentos y su posible uso por población con necesidades médicas especiales. Los intervalos de contenidos máximos en alimentos de micotoxinas legisladas se recogen en la Tabla 3.

Tabla 3. Contenidos máximos legislados de micotoxinas en alimentos

Micotoxina	Alimento	Límites máximos (µg/kg)
AFB_1	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, alimentos dietéticos destinados a usos médicos especiales, cereales y productos a base de cereales, frutos secos, frutos de cáscara, especias y cacahuetes.	0,1 - 8
Suma de AFs	Cereales y productos a base de cereales, frutos secos, frutos con cáscara, café y cacahuetes.	4 - 15
AFM_1	Preparados para lactantes y preparados de continuación, alimentos dietéticos destinados a usos médicos especiales, leche cruda, leche tratada térmicamente y leche para la fabricación de productos lácteos.	0,025 – 0,050
OTA	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, alimentos dietéticos destinados a usos médicos especiales, cereales y productos a base de cereales, zumo de uva, vino, café y uvas pasas.	0,5 - 10

Tabla 3. Continuación

Micotoxina	Alimento	Límites máximos (µg/kg)
Suma de FBs	Alimentos elaborados a base de maíz y alimentos infantiles para lactantes y niños de corta edad, alimentos a base de maíz y maíz no elaborado.	200 - 2000
PAT	Alimentos infantiles distintos a los elaborados a base de cereales para lactantes y niños de corta edad, zumo de manzana y productos sólidos elaborados a partir de manzana, bebidas fermentadas elaboradas con manzanas o que contengan zumo de manzana, zumos de frutas y néctares.	10 - 50
DON	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, cereales y algunos productos a base de cereales, cereales no elaborados.	200 - 1750
ZEA	Alimentos elaborados a base de maíz para lactantes y niños, alimentos elaborados a base de cereales, distintos al maíz, para lactantes y niños, maíz y productos a base de maíz y otros cereales no elaborados.	20 - 200

1.1.3. Incidencia

Para poder detectar de forma adecuada la presencia de micotoxinas en alimentos, son necesarias unas adecuadas metodologías analíticas que permitan comparar resultados. A este respecto, la UE publicó el Reglamento EC n° 401/2006 de la Comisión, de 23 de febrero de 2006 por el que se establecen los métodos de muestreo y de análisis para el control oficial del contenido de micotoxinas en los productos alimenticios (EC, 2006b).

La UE dispone de diversos mecanismos para controlar la inocuidad y seguridad de los alimentos que se consumen en los países miembros. El más importante de todos ellos es el Sistema de Alerta Rápida para Alimentos y Piensos (RASFF) cuyo fundamento jurídico se encuentra en el Reglamento CE nº 178/2002, en concreto los artículos 50, 51 y 52, los cuales establecen su ámbito de actuación y procedimental (CE, 2002).

Si se consulta el último de los informes que elabora y publica el RASFF anualmente, se observa como el número de notificaciones por micotoxinas ha sufrido un ligero repunte en el período comprendido entre los años 2014-2017, después de una importante bajada entre los años 2008-2014, debida principalmente a la implantación de sistemas de control de la calidad en origen (Figura 2).

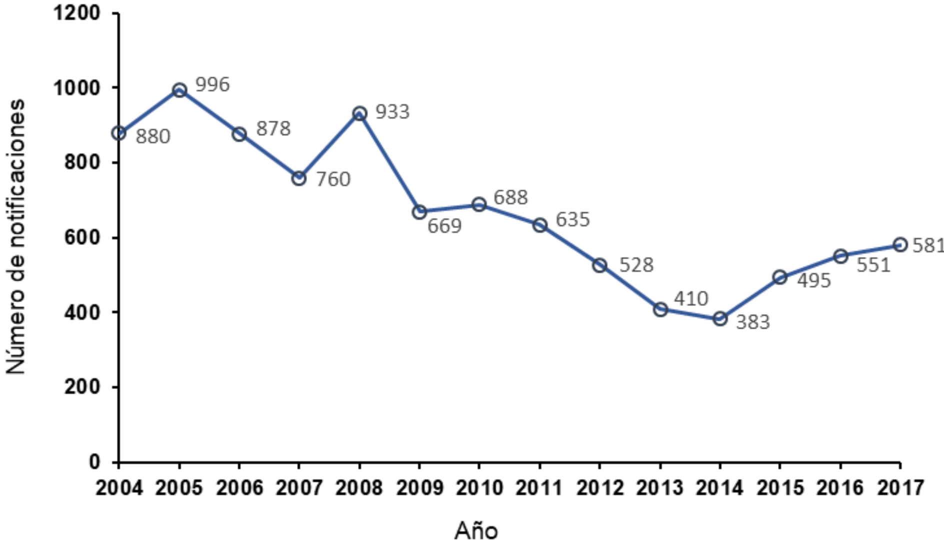


Figura 2. Número de notificaciones por micotoxinas en la UE de 2004 a 2017 (RASFF, 2017)

La mayor parte de las notificaciones por micotoxinas se deben a una serie de productos y de países recurrentes, como higos secos procedentes de Turquía, pistachos de Irán, y cacahuetes de India y China. De entre todas las micotoxinas, la AFB_1 es la responsable de aproximadamente el 90 % de las notificaciones, seguida por la OTA. Asimismo, y debido a la mayor importancia y menos niveles de micotoxinas legislados, los alimentos engloblan el 90 % de las notificaciones frente a un 10 % en el caso de piensos (RASFF, 2017).

1.1.4. Evaluación del riesgo

La evaluación del riesgo se define como la estimación de la probabilidad de que tengan lugar efectos adversos para la salud, conocidos o potenciales, resultantes de la exposición de los seres humanos a peligros trasmitidos por los alimentos, siendo la base científica de referencia para los reglamentos y normas legislativas (FAO, 2002).

Para llevar a cabo la evaluación del riesgo se debe seguir un procedimiento establecido y sistemático de cuatro etapas:

1. Identificación del peligro.
2. Caracterización del peligro.
3. Determinación de la exposición.
4. Caracterización del riesgo.

La primera etapa, corresponde con la identificación de los agentes tanto físicos, químicos o biológicos capaces de causar efectos nocivos para la salud por su presencia en alimentos, que en este caso es la posible presencia de micotoxinas.

Respecto a la segunda etapa, “caracterización del peligro”, se busca evaluar la naturaleza de los efectos nocivos para la salud de los peligros identificados previamente. Para ello, se utilizan los valores toxicológicos de referencia para las micotoxinas (TDI, PMTDI, PMTWI) establecidos por la JECFA y la EFSA (Tabla 4).

Para llevar a cabo la tercera etapa, “determinación de la exposición”, se busca conocer tanto la concentración del peligro de interés presente en los alimentos, como el consumo de dichos alimentos por la población. Esto puede realizarse adoptando distintos planteamientos, como los estudios de dieta total o los estudios concretos de determinados alimentos o grupos de ellos.

Finalmente, en la cuarta etapa o “caracterización del riesgo”, se determina un valor (cuantitativo y/o cualitativo) de la probabilidad de que la población pueda padecer los efectos adversos por la exposición a esta sustancia. Para ello, se calcula la ingesta diaria estimada (EDI) expresada en µg/kg peso corporal (pc) por día de las distintas micotoxinas encontradas en las muestras analizadas mediante la siguiente fórmula:

$$[\mathrm{EDI} = (C \cdot K) / bw]$$

Siendo "C" la media de la concentración de micotoxina encontrada en el alimento expresada en µg/Kg, "*K*" el consumo medio de ese alimento expresado en g/día y "*bw*" el peso medio del grupo de edad a estudiar. Finalmente, esta EDI se compara con las TDI mediante la siguiente ecuación:

$$[\%\ \mathrm{EDI\text{-}TDI} = (\mathrm{EDI}/\mathrm{TDI}) \cdot 100]$$

Algunas substancias, como las AFs, no poseen TDI por estar demostrada su carcinogenicidad (IARC Grupo I), por lo que se debe recurrir al principio de “tan bajo como sea razonablemente alcanzable” (ALARA) mientras que otras, como es el caso de las micotoxinas emergentes de *Fusarium* (ENs, BEA) es porque no se

poseen todavía estudios concluyentes de toxicidad in vitro. En estos casos, se recurre a la TDI de micotoxinas producidas por hongos del mismo género como el DON, el nivalenol (NIV) y los tricotecenos (TCs) T-2 y HT-2.

Tabla 4. Ingestas tolerables propuestas para las principales micotoxinas (Rodríguez, 2015)

Micotoxina	Ingesta tolerable	Referencia
OTA	0,017 µg/Kg p.c. diarios	EFSA, 2006
PAT	0,4 µg/Kg p.c. diarios	EFSA, 2002
$\Sigma(FB_1+FB_2+FB_3)$	2 µg/Kg p.c. diarios	FAO/WHO, 2012a
Σ(DON+3-ADON+15-ADON)	1 µg/Kg p.c. diario	EFSA, 2013a
NIV	1,2 µg/Kg p.c. diarios	EFSA, 2013b
Σ(T-2+HT-2)	0,1 µg/Kg p.c. diarios	EFSA, 2011a
ZEA	0,25 µg/Kg p.c. diarios	EFSA, 2011b

1.2. Isotiocianatos

Los isotiocianatos (ITCs) son unas sustancias naturales que destacan por su actividad biocida (fungicida, bactericida, e insecticida), así como antioxidante e inmunomoduladora. Los ITCs son generados mediante una reacción enzimática a partir de la hidrólisis de los glucosinolatos (GSs), compuestos presentes de forma natural en las plantas crucíferas.

Los cultivos de crucíferas han sufrido diversas modificaciones a lo largo del tiempo respecto a su ancestro común. Según el tipo de vegetal se ingieren una parte u otra de la planta: las hojas en la col, las yemas en las coles de bruselas, la raíz en el nabo, las flores en la coliflor, y las semillas en la mostaza. Algunos de estos alimentos pueden considerarse como funcionales por su potencialidad para mejorar la salud y reducir el riesgo de contraer enfermedades, más allá de sus propiedades nutricionales (Pal Vig *et al*., 2009).

1.2.1. Glucosinolatos

Los GSs son metabolitos secundarios presentes mayoritariamente en vegetales del orden *Brassicales*. Químicamente son moléculas derivadas de aminoácidos, con la adición de azufre y una glucosa (Hounsome *et al*., 2008). Su formación se divide en tres fases: primero, algunos aminoácidos alifáticos y aromáticos pueden ser expandidos insertando en sus cadenas laterales grupos metileno. A continuación, el aminoácido (modificado o no) se reconfigura para dar la estructura general de los GSs, y por último, éstos sufren las transformaciones que les darán las características propias de cada uno de los más de 120 GSs descritos (Halkier y Gershenzon, 2006).

La ingesta de productos ricos en GSs se ha relacionado con la posible prevención de determinados tipos de enfermedades, debido a que intervienen en la protección contra el estrés oxidativo y la activación de las enzimas relacionadas con la detoxificación de agentes carcinógenos (Johnson, 2002). Los GSs también tienen la particularidad de ser los precursores de los ITCs, una de las sustancias naturales con mayor número de propiedades bioactivas (Tabla 5).

Tabla 5. Principales GSs e ITCs asociados, y alimentos donde estos se encuentran de forma mayoritaria (Pal Vig *et al*., 2009)

R =	GS	ITC	Alimento
CH_3-	Glucocapparin	Methyl ITC	Alcaparras
CH_2=CH-CH_2-	Sinigrin	Allyl ITC	Mostaza oriental
CH_2=CH(CH_2)$_2$-	Gluconapin	3-butenyl ITC	Repollo
CH_2=CHC(OH)HCH_2-	Progoitrin	Goitrin	Coles de bruselas
C_3CS(=O)(CH_2)$_4$-	Glucoiberin	Iberin	Brócoli
C_6H_5-CH_2-	Glucotropaeolin	Benzyl ITC	Berros
p-HO-C_6H_4-CH_2-	Sinalbin	*p*-Hidroxy-benzyl ITC	Mostaza amarilla
H_3CS(=O)(CH_2)$_4$-	Glucoraphanin	4-Methyl-sulphinyl-butyl ITC	Coliflor

I.2.2. Mirosinasas

En los vegetales, los GSs se encuentran en vacuolas específicas distintas de donde se encuentra la enzima responsable de catalizar la reacción de transformación en ITCs. Cuando la planta sufre un daño físico, sustrato y enzima entran en contacto produciéndose los ITCs. La mirosinasa es una enzima β-tioglucósido glucohidrolasa (EC 3.2.1.147), responsable de la fase inicial de transformación de los GSs a ITCs. Una condición indispensable para que esta catálisis tenga lugar es la presencia de H_2O.

Debido a la gran variedad de GSs y a la elevada especificidad entre enzima y sustrato, existen variaciones entre los parámetros de actividad de las distintas mirosinasas en función del vegetal de origen. Por ejemplo, enzimas obtenidas a partir de semillas de mostaza blanca (*Sinapsis alba*) mostraron un pH óptimo de 4,2 - 6 y una temperatura óptima de 60 °C (Travers-Martin *et al*., 2008). Otros

estudios también han confirmado la inhibición enzimática a temperaturas superiores a 75 °C. Por otro lado, la actividad enzimática se mantiene cuando es sometida a presiones hidrostáticas de hasta 600 mPa (Van Eylen *et al*., 2005).

I.2.3. Isotiocianatos

Cuando las mirosinasas degradan los GSs, se forma una molécula intermedia (aglicona), a partir de la cual se constituyen los diferentes productos finales posibles, como son epitionitrilos, nitrilos, tiocianatos e ITCs (Figura 3).

Estos últimos poseen una gran variedad de propiedades (Pal Vig *et al*., 2009), entre las que destaca su actividad biocida (fungicida, bactericida, insecticida y frente a pequeños invertebrados), así como herbicida, antioxidante, y anticancerígena, (Higdon *et al*., 2007). Se han estudiado numerosos ITCs, como el benzil isotiocianato (BITC) (Sofrata *et al*., 2011) y el fenil isotiocianato (PITC) (Abreu *et al*., 2014), aunque de todos ellos, el más estudiado por la potencia de sus efectos es el alil isotiocianato (AITC).

El AITC es un compuesto volátil obtenido a partir de la hidrólisis del GS sinigrina, característico de las semillas de mostaza oriental (*Brassica juncea*) y del que se ha comprobado su eficacia inhibiendo el desarrollo de hongos de los géneros *Penicillium* (Tunc *et al*., 2006) y *Alternaria* (Sellam *et al*., 2007), así como las bacterias *Escherichia coli* O157:H7 (Nadarajah *et al*., 2004), *Listeria monocytogenes* (Lara-Lledó *et al*., 2012) o *Salmonella* sp. (Chen *et al*., 2012). El modo de acción por el que el AITC produce estos efectos aún no se conoce con exactitud, pero diversos estudios han demostrado daños a nivel de membrana plasmática, descenso de los niveles de adenosin trifosfato e inhibición de enzimas como la tiorredoxina reductasa y la acetato quinasa (Wilson *et al*., 2013).

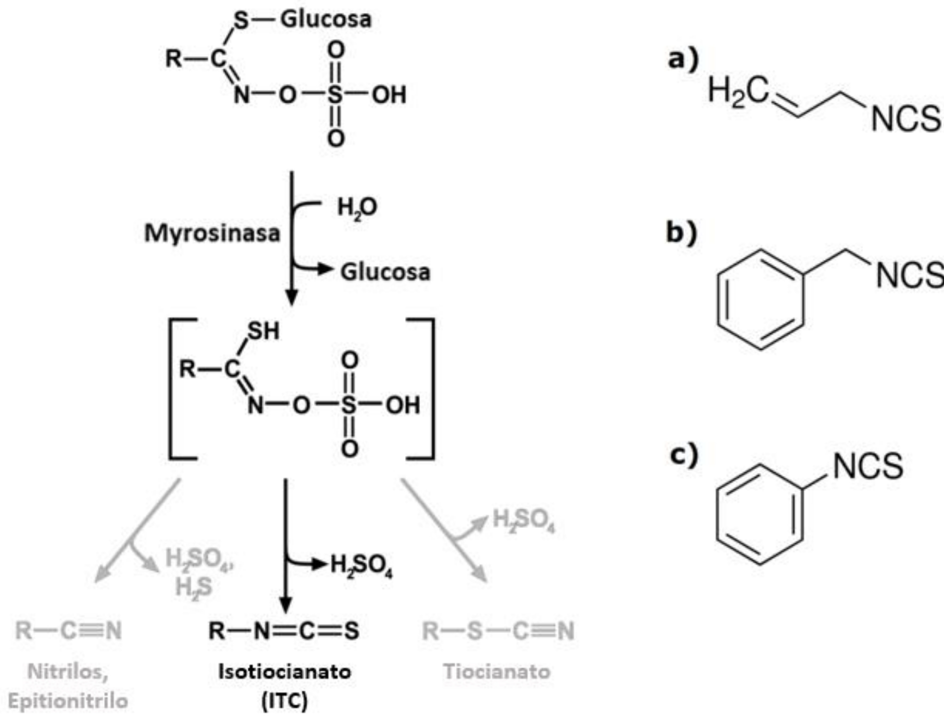


Figura 3. Reacción de biotransformación de los GSs en ITCs y estructuras químicas de: a) AITC, b) PITC y c) BITC (Ruhr-Universität Bpchum, 2009, con modificaciones)

Estas propiedades hacen del AITC un candidato ideal para ser utilizado como conservante, por lo que en 2005 la empresa Mitsubishi desarrolló un informe para conseguir que la Food and Drugs Administration de EE. UU. (FDA), lo reconociera como compuesto seguro para su uso en la industria alimentaria (GRAS). Las conclusiones fueron positivas y por tanto el AITC quedaba exento de los requisitos de aprobación previa a la comercialización (FDA, 2005). El uso de AITC ha sido evaluado por el JECFA y por la Comisión Técnica de Aditivos Alimentarios, Aromatizantes, Auxiliares Tecnológicos y Materiales en Contacto con los

Alimentos (AFC), este último perteneciente a la EFSA, que declararon que no existían problemas de seguridad en los niveles de ingesta diaria maximizados. Con esta información, y a petición de la Comisión Europea, el Panel de Aditivos Alimentarios y Fuentes de Nutrientes añadidos a los Alimentos (ANS), emitió un dictamen científico sobre la seguridad del AITC cuando se utiliza como conservante de alimentos a través de su adición en el embalaje de ciertos alimentos. Una de las conclusiones de este dictamen fue fijar la Ingesta Diaria Admisible (ADI) de AITC en 0,02 mg/Kg de pc/día (EFSA, 2010).

I.2.4. La mostaza como fuente natural de isotiocianatos

A nivel normativo, el Código Alimentario Español (CAE) define a la mostaza como "Semillas de la mostaza blanca «*Sinapis alba*», mostaza negra «*Brassica nigra*», o de especies afines" (CAE, 2003). Las semillas de mostaza se han usado como condimento desde hace miles de años, pudiéndose encontrar en textos Sumerios de hace 3000 años las primeras referencias escritas a su utilización (Mejía-Garibay, 2011). Debido a sus propiedades beneficiosas también eran utilizadas en el campo de la medicina, puesto que con su harina se preparaban los sinapismos: cataplasma de mostaza de uso externo y local, de consistencia blanda, y que se aplica sobre todo caliente para conseguir efectos calmantes y antiinflamatorios en las afecciones pulmonares y cutáneas (Ribera, 1893).

Existen tres tipos principales de plantas y por tanto de semillas de mostaza (Figura 6), cada uno con sus propias características (McGee, 2010):

- Mostaza blanca (*Sinapsis alba* = *Brassica hirta*): también llamada mostaza amarilla. De origen mediterráneo y nativa de Europa, produce semillas grandes y de color claro. Su GS e ITC principales son la sinalbina y el para-

hidroxibencil isotiocianato (ρ-HBITC), respectivamente. El ρ-HBITC es poco volátil en comparación con el resto de los ITCs y sus efectos tanto organolépticos como biocidas, también son menores. Se consume mayoritariamente en EE. UU. (Figura 4a).

- Mostaza negra (*Brassica nigra*): de origen mediterráneo y oriunda de Eurasia produce semillas pequeñas y con cáscara oscura. Posee elevadas concentraciones de sinigrina, pero su cultivo es muy complicado por lo que ha sido substituida por la mostaza oriental (Figura 4b).
- Mostaza oriental *(Brassica juncea)*: también llamada mostaza marrón. Se trata de un híbrido entre mostaza negra y nabo (*Brassica napa)*. Produce semillas grandes y pardas, con menor concentración de sinigrina que la mostaza negra, pero más fácil de cultivar y cosechar. Se consume mayoritariamente en Europa (Figura 4c).

Figura 4. Ejemplos de semillas de mostaza: a) blanca, b) negra y c) oriental

La otra forma de consumo de la mostaza es en forma de harina. Para obtener la harina de mostaza someten las semillas a una etapa de secado suave a 32 °C para evitar la desnaturalización de las mirosinasas. A continuación, se produce una molienda con rodillos de distintos tamaños con el objetivo de reducir el tamaño de las partículas, así como de separar el salvado de la harina. Por último, el producto se tamiza para retirar la fracción grosera de la harina fina (Cui y Eskin,

2000). Su composición nutricional puede variar debido a que generalmente está enriquecida con aceite, pero una aproximación sería la presentada en la Tabla 6.

Tabla 6. Composición nutricional de una muestra de harina de mostaza oriental (Abul-fald *et al*., 2011)

Componentes	**%**
Agua	5,0
Proteínas	32,5
Lípidos	36,3
Minerales	3,9
Fibra	6,3
Hidratos de carbono	16,6
AITC	0,5

Uno de los puntos importantes a tener en cuenta en los alimentos que contienen mostaza, es que se encuentra incluida en la lista de sustancias alergénicas establecida por la UE mediante la Directiva 2007/68/CE de la Comisión de 27 de noviembre de 2007, y por tanto es obligado informar de su presencia en el etiquetado (CE, 2007b). En el caso de la mostaza oriental, el alérgeno principal se denomina Bra-j-1, y es una proteína de 16 kDa, formada por 129 aminoácidos, no glicosilada, rica en cisteína, con una estructura tridimensional compuesta en su mayoría por α-hélices, resistente a la proteólisis y a la desnaturalización por calor (Vereda, 2008).

1.3. Los cereales

Por cereales se entiende a las semillas o granos comestibles producidos por las plantas de la familia Gramineae, las cuales se caracterizan por generar frutos de una única semilla (Figura 5). Esta semilla esta formada por una cubierta o cáscara llamada pericarpo que la rodea y se adhiere fuertemente a ella. En el interior de este pericarpo se encuentra el embrión o germen y el endospermo. A efectos de la alimentación humana, se define a los cereales como los frutos maduros, enteros, sanos y secos de vegetales pertenecientes a la familia de las Gramíneas (Dendy y Brokway, 2001).

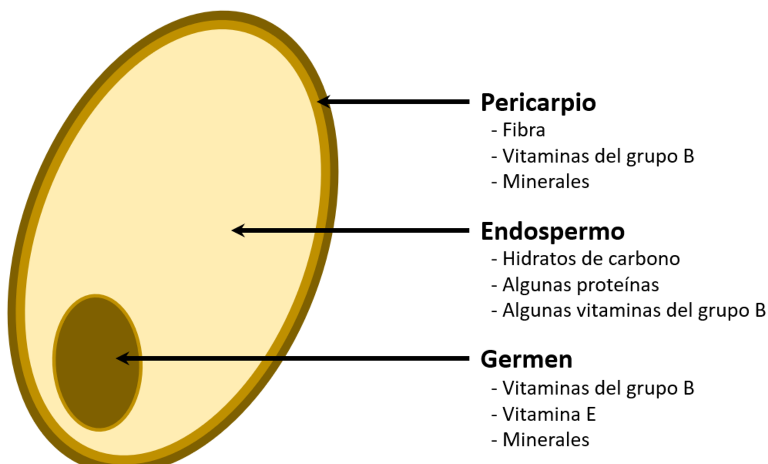


Figura 5. Estructura y composición de un grano de trigo

Los diferentes tipos de cereales suelen ser reconocibles cuando alcanzan un estado de madurez adecuada que permite su recolección, pero no es tan fácil en las primeras etapas de su desarrollo vegetativo. La familia Gramineae engloba siete tribus principales (Tabla 7), donde se sitúan los cereales mayoritariamente

utilizados para la alimentación humana: arroz (*Oryza sativa*), avena (*Avena sativa*), cebada (*Hordeum vulgare*), centeno (*Secale cereale*), maíz (*Zea mays*), mijo (*Panicum millaceum*), sorgo (Sorghum vulgare), trigo (*Triticum aestivum* y *Triticum durum*) y triticale (híbrido de centeno y trigo) (Dendy y Brokway, 2001).

Tabla 7. Las tribus de los cereales

Tribu	Especie típica
Hordeae	Trigo Cebada Centeno
Maydeae	Maíz
Oryzeae	Arroz
Aveneae	Avena
Andropogoneae	Sorgo
Zinanieae	Trigo silvestre

1.3.1. Producción y consumo

En términos de producción, el maíz, el trigo y el arroz son los de mayor importancia por su elevado volumen, seguidos a cierta distancia por la cebada. Si tenemos en cuenta los datos de la FAO, la estimación de la producción de cereales en 2018 fue de 2.608,6 millones de toneladas, lo que supone un descenso del 1,9 % respecto a la producción de 2017, pero solo del 0,2 % respecto a los de 2016. Por continentes, Asia se sitúa en la cabeza de la producción (1.154,6 millones de toneladas anuales, el 44,3 % de la producción mundial) seguida por América del

Norte (496,7 millones de toneladas, el 19,0 %), Europa (496,2 millones de toneladas, el 19,0 %), América del Sur (196,3 millones de toneladas, el 7,5 %), África (192,1 millones de toneladas, el 7,4 %), Centroamérica (42,6 millones de toneladas, el 1,6 %) y Oceanía (30,1 millones de toneladas, el 1,2 %). En términos de desarrollo humano, el 58,6 % de la producción de cerales tiene lugar en países desarrollados, mientras que el 41,4 % se produce en países en vías de desarrollo (1527,9 y 1080,7 millones de toneladas respectivamente). Finalmente, por productos, la producción quedó distribuida de la siguiente forma: maíz con 1074 millones de toneladas (41,2 %), trigo con 728,3 millones de toneladas (27,9 %), arroz con 514,9 millones de toneladas (19,7 %) y cebada con 140 millones de toneladas (5,4 %). Asimismo, el pronóstico de la FAO de las existencias de cereales para las cosechas de 2018/2019 es de una proporción entre los remanentes globales y su utilización (Stock-to-use) del 28,3 %, cuando en 2017/2018 fue del 30,5 % (FAO, 2018).

La distribución del consumo de cereales se debe a diversos factores entre los que destacan los relacionados con la producción y con los hábitos culturales. En Asia, el cereal consumido de forma mayoritaria es el arroz, mientras que en América lo es el maíz, y en Europa el trigo. Tradicionalmente, el trigo ha sido el cultivo mayoritario en los países desarrollados, mientras que gran parte de la producción de maíz era destinada a la alimentación animal. De la misma forma a mediados de siglo XIX el 90 % del arroz mundial se producía en países en vías de desarrollo, aunque actualmente las diferencias entre cultivos son menos acusadas. A modo de referencia, en 2014 el cereal más consumido en España fue el trigo (60,9 Kg/persona/año) principalmente en forma de pan (35,9 Kg/persona/año), productos de panadería (12 Kg/persona/año) y pasta (4 Kg/persona/año). El consumo de arroz fue de 3,9 Kg/persona/año mientras que el

de maíz es difícilmente cuantificable, aunque su consumo tiende a aumentar. El consumo de cebada se limitó esencialmente a su uso para la fabricación de cerveza (García-Villanova y Guerra, 2015).

1.3.2. Almacenamiento y conservación

Una de las consecuencias de la elevada producción cerealística mundial y del exceso entre producción y consumo, es que los granos pasan una mayor proporción de su tiempo en los procesos de transporte y almacenamiento frente a los de transformación o consumo. Los granos que se descascarillan manteniendo las glumas intactas, como el arroz, la cebada y el centeno, se almacenan bien ya que las glumas son una cubierta protectora que dificulta la penetración de los insectos. Las excepciones son los insectos perforadores de granos y los gorgojos. Los granos de maíz se almacenan mejor si están secos, limpios y enteros. Si los granos están limpios, muchos insectos no llegarán a desarrollarse porque prefieren para sus larvas, al menos en las etapas iniciales, polvo de grano o grano troceado que deje expuesto el embrión (Dendy, 2001).

En condiciones de almacenamiento óptimas, protegidos tanto de las inclemencias meteorológicas como de roedores e insectos, los cereales pueden conservarse en buen estado durante años. Este tiempo puede aumentar aun más si las condiciones son las óptimas (atmósfera y humedad controladas). El almacenamiento moderno tiene lugar en silos de acero de hasta 10.000 toneladas con puntos de acceso para introducir y extraer el cereal, así como de un sistema de desecación por aire para controlar la humedad y en algunos casos sistemas de atmósfera controlada (Figura 6) (Hoseney, 1991).

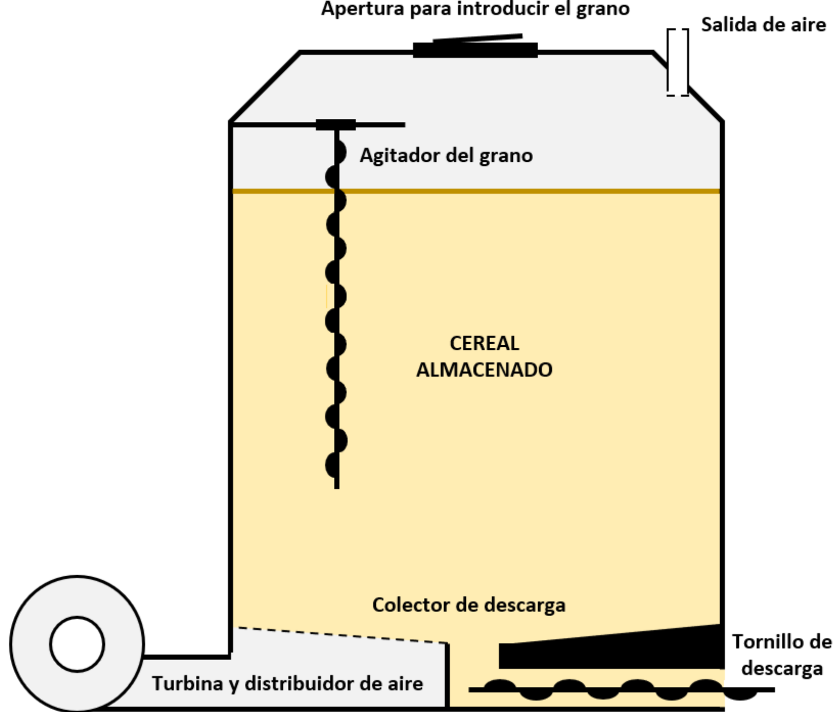


Figura 6. Silo de grano moderno con dispositivo de aireación

1.3.3. Alteraciones microbiológicas del grano

La alteración y el deterioro de los cereales almacenados puede tener lugar tanto por la infestación de insectos, artrópodos y vertebrados (principalmente roedores), como por el crecimiento de microorganismos. Aunque en todos los casos se producen pérdidas económicas, es la proliferación microbiana la causa más importante de alteraciones debido a que puede suponer un riesgo para la salud pública de los consumidores.

Los microorganismos más significativos respecto a la alteración y conservación de los granos son los hongos, puesto que pueden crecer con humedades muy inferiores a las que las bacterias necesitan. Las pérdidas

producidas por el crecimiento de las bacterias solo tienen lugar en granos en equilibrio con humedades relativas del 100 %. La mayoría de los hongos no pueden sobrevivir con un contenido de humedad en el grano inferior al 17 %, pero los más peligrosos son aquellos que pueden sobrevivir y crecer al 17-18 % de humedad relativa, entre los que se incluyen especies productoras de micotoxinas de los géneros *Aspergillus*, *Penicillium* y *Fusarium* (Tabla 8). De todas ellas, la más importante es el *Aspergillus flavus*, por la elevada toxicidad de las AFs que produce (apartado 1.1.3) (Dendy, 2001).

Tabla 8. Especies fúngicas toxigénicas presentes en almacenamiento de cereales. (Dendy, 2001)

Especie fúngica	Micotoxina
Aspergillus flavus	AFs
Aspergillus ochraceus	OTA
Penicillium verrucosum	OTA
Fusarium Graminearum	ZEA, TCs

1.3.4. Prevención y reducción de la contaminación por micotoxinas

El principal mecanismo para evitar la presencia de micotoxinas en alimentos es la prevención. En el caso de los cereales y sus productos derivados, el objetivo es reducir al mínimo el desarrollo de hongos toxigénicos, tanto durante el crecimiento de los vegetales como durante su almacenamiento. El sistema de prevención de micotoxinas en origen se basa en la aplicación de las Buenas Prácticas Agrícolas (BPA) (FAO/WHO, 2012b).

Las BPA constituyen una serie de directrices y pasos para controlar la contaminación por micotoxinas en el campo. Sin embargo, la mayoría de los factores que influyen en dicha contaminación son los relacionados con las condiciones climáticas, por lo que difícilmente pueden ser controlados. El Codex Alimentarius ha establecido un Código de Prácticas con las siguientes pautas y recomendaciones para garantizar unas BPA en la prevención y/o reducción de la contaminación por micotoxinas (Codex Alimentarius, 2003):

- Elaboración de un plan de rotación de cultivos.
- Selección del momento adecuado para la plantación de los cultivos.
- Selección de variedades vegetales y semillas resistentes al desarrollo de los hongos toxigénicos y a las plagas de insectos.
- Control del suministro de agua mediante sistemas de riego.
- Uso de fertilizantes y/o acondicionadores del suelo para garantizar un pH adecuado.
- Empleo de fungicidas adecuados frente a hongos productores de micotoxinas.
- Uso correcto de agentes químicos (insecticidas, herbicidas, etc.) para prevenir la infestación de insectos, malas hierbas y otros factores que pueden influir en el desarrollo de hongos y la formación de micotoxinas.
- Comprobación de que, en el momento de recolección de los cereales, estos no poseen una humedad superior al 15 %.
- Uso de maquinaria adecuada de recolección que evite daños físicos a los granos y su contacto con el suelo.
- Transporte en contenedores limpios, secos, y libres de insectos o contaminación fúngica visible.

A todas estas prácticas previas a la cosecha pueden añadirse otras anteriores al almacenado, como, por ejemplo:

- Añadir fases de secado a los cereales con contenidos de humedad superiores al 15 %.
- Aplicar etapas de limpieza a los granos, para eliminar granos dañados y materias extrañas.
- Control de temperatura (< 20 °C) y humedad (a_w < 0,7) del silo, así como posible uso de atmósferas modificadas (nitrógeno, monóxido o dióxido de carbono, etc.) para prevenir la proliferación de las esporas fúngicas que puedan quedar en los granos.

Cuando los mecanismos de prevención no son suficientes para evitar la presencia de micotoxinas, es posible aplicar otras estrategias para descontaminar las matrices alimentarias al máximo. Estas estrategias pueden clasificarse en tres procedimientos (Luo *et al*., 2018):

1. Métodos químicos: estos métodos pueden alterar las características organolépticas y nutricionales de los alimentos, así como a dar lugar a la formación de nuevos compuestos químicos cuya toxicidad puede ser desconocida. Por todo ello, el Reglamento (CE) 1881/2006 prohibe hoy en día, la detoxificación de micotoxinas mediante la aplicación de tratamientos químicos en la UE (CE, 2006a). Aun así, se han estudiado el empleo de diversas sustancias químicas de síntesis, como ácidos y bases, agentes clorados, agentes reductores y oxidantes (Jard *et al*., 2011), así como de sustancias naturales como los isotiocianatos o los compuestos fenólicos (Azaiez *et al*., 2013; Pani *et al*., 2014).
2. Métodos biológicos: estos métodos se basan en la adición de microorganismos para que absorban o transformen las micotoxinas. Se ha

estudiado la utilización de levaduras, bacterias ácido-lácticas y de otros hongos para reducir la presencia de AFs, ZEA, OTA, PAT, TCs (DON, T-2 y HT-2) y BEA (Ji *et al*., 2019). Cuando el objetivo es la transformación de micotoxinas, estos métodos sufren de la misma problemática que los químicos, con la aparición de compuestos derivados de la detoxificación microbiana. Asimismo, son métodos de difícil aplicación en la industria por las condiciones que requieren.

3. Métodos físicos: la descontaminación de micotoxinas en cereales por métodos físicos incluye varios procedimientos como la clasificación y separación, inmersión y lavado, irradiación, así como la inactivación por temperatura. La clasificación y separación de los granos de maíz puede reducir la contaminación por FBs y AFs (Broggli *et al*., 2002), mientras que tratamientos de inmersión y lavado, pueden eliminar hasta el 80 % de las AFs (Fandohan *et al*., 2005; Bethke *et al*., 2014) y reducir significativamente la OTA (Sudamore y Banks, 2004). Radiaciones ultravioletas (UV) son efectivas para la descontaminación de AFs, OTA, DON y T-2 cuando son aplicadas sobre capas delgadas de cereales (Peraica *et al*., 2002). Varios estudios han evidenciado la absorción de micotoxinas mediante carbón activado (PAT, ZEA, DON y NIV) y arcilla de bentónica (AFB_1 y AFM_1) los cuales son sustancias aptas para su adición en piensos (Liu *et al*., 2011; Magnoli *et al*., 2011). La inactivación térmica es un tratamiento que puede adaptarse de forma sencilla a las distintas etapas de los procesos tecnológicos de los cereales y sus productos finales. La eficacia en la reducción dependerá de factores como el tipo de micotoxina (AFs, OTA y FBs son micotoxinas termoestables), su concentración en el producto, así como de la temperatura y tiempo de aplicación (Milani y Maleki, 2014).

1.4. El pan y productos derivados

El pan constituye la base de la alimentación desde hace unos 7500 años aproximadamente. Inicialmente no se parecía al pan tal y como hoy lo conocemos, sino que era una pasta plana y ligeramente cocida, no fermentada, elaborada solo con agua y granos de trigo machacados, puesto que no se había desarrollado la producción de harina. Este alimento fue rápidamente aumentando su valor dentro de las sociedades antiguas, ya que podía elaborarse durante todo el año, poseía un alto valor nutritivo para la dieta de la época y además podía transportarse fácilmente. Todo indica que fue la civilización egipcia (hace unos 5000 años) la primera en producir panes fermentados al observar como en las masas elaboradas durante el día anterior se producían burbujas de aire que aumentaban el volumen consiguiendo una textura esponjosa. Otro avance significativo en el desarrollo de los procesos de panificación fue el uso por parte de los galos de la espuma de la cerveza como cultivo iniciador de la fermentación. En el siglo XIX las levaduras de las cervecerías fueron reemplazadas por las procedentes de las destilerías de alcohol de cereales. A finales del siglo XIX, a raíz de los trabajos de Pasteur, se desarrolla una industria específica para la producción de levaduras que culmina en 1920 con un moderno método de producción de levaduras de panadería (*Saccharomyces cerevisiae*), que evita la producción de etanol (Chiron y Godon, 1996).

En la panificación moderna influyen dos tendencias hasta cierto punto contrapuestas. Por un lado, los cambios en el estilo de vida y la difusión de los congeladores y de los hornos microondas han conllevado un aumento de la demanda de panes de más cómoda preparación y adecuados para su almacenamiento en congeladores. Por otro lado, existe también una cierta demanda de alimentos lo más parecidos posible al alimento tradicional.

En el año 2016, los hogares españoles consumieron 1.521,3 millones de kilos de pan y gastaron 3.683,8 millones de euros en estos productos, lo que significa 7 kilos de consumo y 83,9 euros de gasto por habitante. El consumo más notable se asocia al pan fresco normal y al congelado. Durante los últimos cinco años, el consumo de pan ha caído 1,2 kilos por persona y el gasto ha descendido 50 céntimos de euro per cápita. En el periodo 2012-2016, el consumo y el gasto más elevados tuvieron lugar en el año 2013 (37,3 kilos y 86,9 euros por consumidor). En la familia de pan, la evolución del consumo per cápita durante el periodo 2012-2016 ha sido diferente para cada tipo de producto. Durante el año 2016, los hogares españoles consumieron 269,8 millones de kilos de bollería y pastelería, y gastaron 1.375,1 millones de euros en dichos productos. En términos per cápita, se llegó a 6,1 kilos de consumo y 31,1 euros de gasto (Mercasa, 2017).

1.4.1. Definiciones y aspectos legales

La sección 4ª del CAE, "Productos de panadería", engloba al pan y a los productos de bollería. Al pan, lo define como "producto resultante de la cocción de una masa obtenida por la mezcla de harina de trigo, sal comestible, agua potable, fermentada por la adicción de levaduras activas" mientras que para los productos de bollería la definición es la siguiente: "aquellos elaborados de masa panaria fermentada y cocida a la que se han añadido complementos panarios en cantidades que modifiquen sus características básicas" (CAE, 2003).

Para cada uno de estos productos, se ha desarrollado una legislación específica en forma de Reglamentación Técnico-Sanitaria (RTS). La RTS para la fabricación, circulación y comercialización de pan y panes especiales considera tres categorías o tipos de productos de panadería (RD, 2002):

- Pan común: de consumo habitual en el día, elaborado con harina de trigo al que únicamente pueden añadirse coadyuvantes tecnológicos y aditivos autorizados para este tipo de pan. Para su comercialización puede denominarse pan bregado (o de miga dura, español o candeal) o como "pan de flama" (o de miga blanda).
- Pan especial: es aquel no incluido en el apartado de pan común y que reúne alguna de las condiciones que se detallan a continuación.
    - Incorporación de cualquier aditivo y/o coadyuvante tecnológico de panificación, autorizados para panes especiales.
    - La utilización como materia prima de harina enriquecida.
    - Que se haya añadido cualquier ingrediente de los siguientes, de forma que eleven suficientemente su valor nutritivo: Gluten de trigo, salvado o grañones; leche entera, concentrada, condensada, en polvo, total o parcialmente desnatada, o suero en polvo; huevos frescos, refrigerados, conservados y ovoproductos; harinas leguminosas en cantidad inferior al 3% en masa de la harina empleada, harinas de malta o extracto de malta, azúcares comestibles y miel; grasas comestibles; cacao, especias y condimentos; pasas, frutas u otros vegetales naturales.
    - Que no lleve microorganismos propios de la fermentación, voluntariamente añadidos.
    - Los panes especiales pueden recibir las siguientes denominaciones: pan integral, pan con grañones, pan de Viena y pan francés, pan tostado, biscote, colines, pan de otro cereal, pan enriquecido, pan de molde y pan rallado.

- Productos semielaborados:
  - Pan precocido: masa de pan común cuya cocción ha sido interrumpida antes de llegar a su finalización, siendo sometida posteriormente a un proceso de conservación autorizado.
  - Masa congelada: masa de pan común, que fermentada o no, y/o formada su pieza o no, ha sido posteriormente congelada.
  - Otras masas semielaboradas: masas de pan común, que fermentadas o no, y/o formadas sus piezas o no, han sido posteriormente sometidas a un proceso de conservación autorizado, distinto de la congelación, de tal manera que se inhiba, en su caso, el proceso de fermentación.

Los productos semielaborados, una vez finalizado su proceso de elaboración se clasifican en alguna de las variedades de pan común o especial definidas en la RTS. También existen ciertas denominaciones de venta que se pueden encontrar en los establecimientos panaderos y que son reguladas mediante Reglamentos específicos del Parlamento, del Consejo y de la Comisión de la UE. Algunos de estos productos son, el pan “sin sal”, “con bajo (o muy bajo) contenido de sal”, “alto contenido en fibra”, “fuente de fibra”, “sin gluten” (CE, 2006c).

1.4.2. Importancia nutricional

El pan común es un alimento energético que proporciona entre 244 y 285 kcal/100 g (Tabla 9). Los panes de miga dura son más energéticos que los panes de miga blanda, debido al menor contenido en agua, 29 y 39 %, respectivamente. El componente mayoritario son los hidratos de carbono, en concreto el almidón. La riqueza en proteínas se encuentra entre el 7 y el 10 %. En los panes de Viena, el

valor nutricional puede ser superior si se utilizan leche o derivados lácteos en su preparación. Los panes son alimentos bajos en grasa (≃ 1 %), a excepción del pan de molde ya que suele adicionarse. El contenido en minerales de valor nutricional es bajo, con una cantidad inferior a 25 mg/100 g para el calcio y de 1,5 mg/100 g para el hierro. La adición de sal suele proporcionar un contenido en sodio próximo a 500 mg/100 g, aunque si la masa es congelada o de fermentación controlada, el contenido será mayor ya que esta se adiciona en más cantidad para retrasar el inicio de la fermentación Las vitaminas hidrosolubles, tiamina (0,06-0,12 mg/100 g), riboflavina (0,03-0,06 mg/100 g) y niacina (0,5-1 mg/100 g), son las que se encuentran de forma habitual. La concentración media de fibra está próxima al 2,5 % para los panes normales y 4,3 % para los de molde (García-Villanova y Guerra, 2015).

Tabla 9. Composición nutricional y valor energético de distintos tipos de pan

	Blanco	Blanco de molde	Blanco tostado	Integral	Multicereal
Energía (Kcal)	244-276	266-285	380	230-267	255-265
Agua (%)	29-38	32-38	7	30-37	32-34
Proteínas (%)	7,3-9	7,8-10	10	8-9	11,5
Lípidos (%)	0,4-1,2	5	4,3	1,4-22	3,8
Hidratos de carbono (%)	50-58	48-52	75	44-53	44-46
Fibra (%)	1,6-3,5	3,2-5,2	2,7	4,4-9	6,5
Minerales (%)	1,6	-	-	1,7	-

El pan de trigo integral presenta un valor nutricional superior; así, el contenido en fibra es de dos a cinco veces superior al del pan normal, y la sustitución del pan común por el integral satisface gran parte de las necesidades de fibra dietética. El contenido en vitaminas (tiamina, riboflavina y niacina) también es superior, y aparecen cantidades pequeñas de ácido fólico, vitamina B_6 y vitamina E. El contenido en sodio se mantiene constante, aumenta ligeramente el calcio y se dobla la concentración de fósforo, potasio y hierro. En cuanto al contenido en macronutrientes, los hidratos de carbono están en una proporción ligeramente inferior, con una digestibilidad similar, las proteínas prácticamente igual, y es ligeramente superior el contenido de lípidos. El contenido en agua es similar, y el valor energético ligeramente inferior (García-Villanova y Guerra, 2015).

1.4.3. Vida útil y peligros biológicos

El pan es un producto perecedero que mantiene sus máximas propiedades organolépticas durante un periodo corto de tiempo desde que se hornea. Transcurridas unas horas comienzan a influir sobre el pan una serie de factores que hacen que pierda su "frescura" y condicionan su consumo. Estos agentes causantes se pueden clasificar en dos categorías: por un lado, están los procesos fisicoquímicos que provocan un endurecimiento de la miga, y por otro, el crecimiento de microorganismos en la superficie del pan. Los causantes del deterioro microbiológico del pan, odenados de menor a mayor importancia son, las levaduras, las bacterias del genero *Bacillus*, y los hongos (Cauvan, 2015).

Cuando una masa de pan se somete a la cocción, las altas temperaturas del horno (entre 180 y 250 °C), eliminan los microorganismos que pueda haber en ella, de forma que la posterior contaminación se debe a la presencia de esporas

en la atmósfera del lugar donde se llevan a cabo los procesos de enfriamiento, corte, envasado y almacenamiento (Ponte y Tsen, 1978).

En un pan entero, la baja humedad de su corteza generalmente impedirá el crecimiento de los hongos en su superficie, sobre todo en ambientes secos, mientras que, si el envasado se realiza con atmósferas con humedades relativas superiores al 90 %, o sin dejar que el pan se enfríe adecuadamente, los hongos pueden desarrollarse rápidamente. En panes cortados existe mayor riesgo de crecimiento fúngico, puesto que la miga es un sustrato adecuado por sus nutrientes y poseer una a_w superior a 0,90. Los hongos alterantes de pan más habituales son los de los géneros *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor*, *Neurospora* y *Rhizopus* (Tabla 10).

Tabla 10. Características de hongos alterantes del pan (Seiler, 1992)

Hongo	Color de la colonia	Aspecto de las colonias
Penicillium spp.	Verde azulado	Planas, crece muy lentamente.
Aspergillus niger	Negra	Algodonosas, con cabezas de esporas a menudo visibles.
Aspergillus flavus	Verde oscuro	-
Aspergillus candidus	Crema	-
Aspergillus claudus	Verde pálido	-
Cladosporium spp.	Verde muy oscuro	Planas, crecen lentamente.
Neurospora sitophila	Rosa	Muy algodonosas, con rápido crecimiento.
Rizophus nigricans	Gris oscuro	Muy algodonosas, con rápido crecimiento.
Mucor spp.	Gris	-

Por último, los distintos hongos que pueden contaminar el pan y su velocidad de crecimiento dependen de factores como la diversidad y concentración de las esporas fúngicas en el ambiente, la temperatura de conservación y los ingredientes de la preparación.

1.4.4. Métodos de conservación

Existen diversas técnicas tanto físicas como químicas para conseguir una adecuada conservación del pan y sus productos derivados. Las más utilizadas son las siguientes:

- Irradiación: su objetivo es la destrucción de toda la carga microbiológica contaminante presente en la superficie del pan, incluso en forma de esporas. Los tipos de irradiación que pueden utilizarse son la radiación de microondas, la radiación infrarroja y la radiación con luz UV (260 nm). El uso de radiación de microondas e infrarroja están limitados al efecto de calentamiento que producen, cosa que no sucede con la radiación UV (Grandison, 2012).
- Envasado con atmósferas modificadas (MAP): Esta técnica combina la sustitución del aire del interior de un envase por un gas o mezcla de gases, con el uso de materiales resistentes a la difusión de dichos gases. El uso de dióxido de carbono a concentraciones superiores al 20 % es efectivo para retardar el crecimiento de hongos, mientras que a niveles del 100 % el efecto antifúngico se maximiza mediante la anaerobiosis. En ocasiones se utiliza una mezcla de gases (60 % de dióxido de carbono y 40 % de nitrógeno) para evitar un posible colapso del envase por la absorción en el pan del dióxido de carbono. El MAP tiene la ventaja de que aumenta la

vida útil de los productos hasta 400 % sin afectar a sus cualidades organolépticas (Brennan y Day 2012).

- Conservantes:
  - Aditivos de síntesis: Los conservantes más habitualmente utilizados en el pan para evitar o reducir el crecimiento microbiano, es el ácido propiónico y sus sales (Tabla 11). Estos ácidos actúan distorsionando el equilibrio del pH de los microorganismos. La principal ventaja de estos compuestos es que sus organismos diana son los hongos y las bacterias, y en mucho menor medida las levaduras utilizadas en la fermentación.
  - Ingredientes naturales: Actualmente, existe una tendencia al estudio de nuevos compuestos de origen natural para su uso como conservantes. Dentro de estas sustancias bioactivas se encuentran antioxidantes, metabolitos de fermentos ácido-lácticos, especias (en forma de ingrediente o de aceites esenciales), etc. Es dentro de esta última categoría donde se englobarían el uso de la mostaza y/o de los ITCs.

Tabla 11. Conservantes más utilizados en pan

Conservante	Cantidad recomendada
Ácido propiónico	0,1 %
Propionato cálcico	0,2 %
Propionato de sodio	0,2 %

1.5. Referencias

Abreu, A.C., McBain, A.J., Simões, M., 2012. Plants as sources of new antimicrobials and resistance-modifying agents. Natural Product Reports, 29, 1007-1021.

Abul-Fadl. M.M., El-Badry, N., Ammar, M.S., 2011. Nutricional and Chemical Evaluation for Two Different Varieties of Mustard Seeds. World Applied Sciences Journal, 15(9), 1225-1233.

Azaiez, I., Meca, G., Manyes, L., Luciano, F.B., Fernández-Franzón, M., 2013. Study of the chemical reduction of the fumonisins toxicity using allyl, benzyl and phenyl isothiocyanate in model solution and in food products. Toxicon. 63, 137-146.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clin. Microbiol. Rev. 16(3), 497-516.

Bethke, N.W., Conard, C.A., Fosdick, L.E., Fox, E.J., Grunig, D., Kirkvold, S.W. *et al*., 2014. Method and apparatus for reducing aflatoxin-contaminated corn. US Patent Publication, n° 8919569 B2.

Brennan, J.G., Day, B.D.F., 2012. Packaging. In: Food processing handbook. Wiley-VCH, 225-280.

Broggli, L.E., Resnik, S.L., Pacin, A.M., Gonzalez, H.H.L., Cano, G., Taglieri, D., 2002. Distribution of fumonisins in dry-milled corn fractions in Argentina. Food Additives & Contaminants, 19, 465-469.

CAE, 2003. Código Alimentario Español y disposiciones complementarias. Tecnos, pp: 810.

Cauvain, S.P., 2015. Bread: The Product. In: Technology of breadmaking. Springer US, 21-49.

CE, 2002, Reglamento (CE) n° 178/2002 del Parlamento Europeo y del Consejo, de 28 de enero de 2002, por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria. DOCE, L 31/1.

CE, 2006a. Reglamento (CE) n° 1881/2006 de la Comisión, de 19 de diciembre de 2006, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE, L 364/5.

CE, 2006b. Reglamento (CE) n° 401/2006 de la Comisión, de 23 de febrero de 2006, por el que se establecen los métodos de muestreo y de análisis para el

control oficial del contenido de micotoxinas en los productos alimenticios. DOUE, L 70/12.

CE, 2006c. Reglamento (CE) n° 1924/2006 del Parlamento Europeo y del Consejo, de 20 de diciembre de 2006, relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos, DOUE, L 404/9.

CE, 2007a. Reglamento (CE) n° 1126/2007 de la Comisión, de 28 de septiembre de 2007, que modifica el Reglamento (CE) n° 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a las toxinas de Fusarium en el maíz y los productos del maíz. DOUE, L 255/14.

CE, 2007b. Directiva 2007/68/CE de la Comisión, de 27 de noviembre de 2007, que modifica el anexo III bis de la Directiva 2000/13/CE del Parlamento Europeo y del Consejo por lo que se refiere a determinados ingredientes alimentarios. DOUE, L 310/11.

CEE, 1993. Reglamento (CEE) n° 315/93 del Consejo, de 8 de febrero de 1993, por el que se establecen procedimientos comunitarios en relación con los contaminantes presentes en los productos alimenticios. DOCE, L 37/1.

Chen, W., Jin, T.Z., Gurtler, J.B., Geveke, D.J., Fan, X., 2012. Inactivation of *Salmonella* on whole cantaloupe by application of an antimicrobial coating containing chitosan and allyl isothiocyanate. International Journal of Food Microbiology, 155, 165-170.

Chiron, H., Godon, B., 1996. Historia de la panificación. En: La panificación. Montagud. 20-61.

Codex Alimentarius, 2003. Código de prácticas para prevenir y reducir la contaminación de los cereales por micotoxinas, CAC/RCP 51-2003. URL disponible en: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCAC%2BRCP%2B51-2003%252FCXC_051s.pdf

Cui, W., Eskin, N.A.M., 2000. Procesamiento y propiedades de los productos y componentes de la mostaza. En: Alimentos funcionales: aspectos bioquímicos y de procesado. Abribia, 233-262.

Dendy, D.A.V., 2001. Almacenamiento y transporte de los granos y sus cereales. En: Cereales y productos derivados, química y tecnología. Acribia, 29-70.

Dendy, D.A.V., Brokway, B.E., 2001. Introducción a los cereales. En: Cereales y productos derivados, química y tecnología. Acribia, 1-28.

EFSA, 2002. Report of experts participating in Task 3.2.8, 2002. Assessment of dietary intake of Patulin by the population of EU Member States. https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_patulin_3.2.8_en.pdf

EFSA, 2006. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to ochratoxin A in food. EFSA Journal, 365, 1-56.

EFSA, 2010. Scientific Opinion on the safety of allyl isothiocyanate for the proposed uses as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). EFSA Journal, 8(12), 1943.

EFSA, 2011a. Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. EFSA Journal, 9(12), 2481.

EFSA, 2011b. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. EFSA Journal, 9(6), 2197.

EFSA, 2013a. Scientific Report. Deoxynivalenol in food and feed: occurrence and exposure. EFSA Journal 11(10), 3379.

EFSA, 2013b. Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. EFSA Journal, 11(6), 3262.

Fandohan, P., Zoumenou, D., Hounhouigan, D.J., Marasas, W.F., Wingfield, M.J., Hell, K., 2005. Fate of aflatoxins and fumonisin during the processing of maize into food products in Benin. International Journal of Food Microbiology, 98, 249-259.

FAO, 2003, Mycotoxin regulations in 2003 and current developments. URL disponible en: http://www.fao.org/3/y5499e/y5499e07.htm

FAO, 2004. Reglamentos a nivel mundial para las micotoxinas en los alimentos y en las raciones en el año 2003. Estudio FAO Alimentación y Nutrición. URL disponible en: http://www.fao.org/3/a-y5499s.pdf

FAO, 2018. Informe trimestral mundial: Perspectivas de cosechas y situación alimentaria. URL disponible en: http://www.fao.org/3/CA2726ES/ca2726es.pdf

FAO/WHO, 2002. Evaluation of certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Aditives. Geneva. URL disponible en: http://apps.who.int/iris/handle/10665/42448

FAO/WHO, 2012a. Safety Evaluation of Certain Food Additives and Contaminants. Fumonisins. WHO Food Additives Series 65, pp. 325-794.

FAO/WHO, 2012b. Prevención y Reducción de la Contaminación de los alimentos y piensos. URL disponible en: ftp://ftp.fao.org/codex/publications/Booklets/Contaminants/CCCF_2012_ES.pdf

FDA, 2005. GRAS Notice of Food and Drug Administration. Notification of Claim for General Recognition of Safety of Allyl Lsothiocyanate in a Food Shelf Life Extension and Anti-spoilage System. Mitsubishi-Kagaku Foods Corporation.

Fox, E.M., Howlett, B.J., 2008. Secondary metabolism: regulation and role in fungal biology. Current Opinion in Microbiology, 11, 481–487.

García-Villanova, B., Guerra, E.J., 2015. Cereales y productos derivados. En: Composición y calidad nutritiva de los alimentos (Tratado de nutrición). Editorial Medica Panamericana, 179-230.

Grandison, A.S., 2012. Irradiation. In: Food processing handbook. Wiley-VCH, 153–178.

Halkier, B.A., Gershenzon, J., 2006. Biology and Biochemistry of Glucosinolates. Annual Review of Plant Biology, 57, 303-333.

Higdon, J.V., Delage, B., Williams, D.E., Roderick, H., Dashwood, R.H., 2007. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. Pharmacological Research, 55, 224–236.

Hoseney, R.C., 1991. Principios de ciencia y tecnología de los cereales. Acribia, pp: 321.

Hounsome, N., Hounsome, B., Tomos, D., Edwards-Jones, G., 2008. Plant Metabolites and Nutritional Quality of Vegetables. Journal of food science, 73(4), 48-65.

IARC, 1987. Monographs on the evaluation of carcinogenic risks to humans: Overall evaluations of carcinogenicity: An updating of IARC Monographs. International Agency for Research on Cancer, 1–403.

IARC, 1993. Monographs on the evaluation of carcinogenic risks to humans: some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. International Agency for Research on Cancer, 56, 1–599.

IARC, 2002. Traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs on the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer, 82, 1–556.

IARC, 2012. Monographs on the evaluation of carcinogenic risks to humans: chemical agents and related occupations. A review of human carcinogens. International Agency for Research on Cancer, 100F, 224–248

Illana-Esteban, C., 2008. El cornezuelo del centeno (I): Biología, historia y ergotismo. Boletín de la Sociedad Micológica de Madrid, 32, 293-306.

Jard. G., Liboz, T., Mathieu, F., Guyonvarch, A., Lebrihi, A., 2009. Adsorption of zearalenone by *Aspergillus* japonicas conidia: New trends for biological ZON decontamination in animal feed. World Mycotoxin Journal, 2, 391-397.

Ji, C., Fan, Y., Zhao, L., 2016. Review on biological degradation of mycotoxins. Animal Nutrition, 2(3), 127-133.

Johnson, I.T., 2002. Glucosinolates: Bioavailability and Importance to Health. International Journal for Vitamin and Nutrition Research, 72(1), 26-31.

Lara-Lledó, M., Olaimat, A., Holley, R.A., 2012. Inhibition of *Listeria monocytogenes* on bologna sausages by an antimicrobial film containing mustard extract or sinigrin. International Journal of Food Microbiology, 156, 25-31.

Liu, Y.L., Meng, G.Q., Wang, H.R., Zhu, H.L., Hou, Y.Q., Wang, W.J. *et al*., 2011. Effect of three mycotoxin adsorbents on growth performance: Nutrient retention and meat quality in broilers fed on mould-contaminated feed. British Poultry Science, 52, 255-263.

Luo, Y., Liu, X., Li, J., 2018. Updating techniques on controlling mycotoxins - A review. Food control, 89, 123-132.

Magnoli, A.P., Texeira, M., Rosa, C.A., Miazzo, R.D., Cavaglieri, L.R., Magnoli, C.E. *et al*., 2011. Sodium bentonite and monensin under chronic aflatoxicosis in broiler chickens. Poultry Science, 90, 352-357.

Marín, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology, 60, 218-237.

Martínez-Larrañaga, M.R., Anadón, A., 2012. Micotoxinas. En: Toxicología alimentaria. Díaz de Santos, 289-307.

McGee. H., 2010. La cocina y los alimentos. Debate, pp: 941.

Mejía-Garibay, B., López-Malo, A., Guerrero-Beltrán, J.A., 2011. Mostaza: características químicas, botánicas y sus aplicaciones en el área de alimentos. Temas Selectos de Ingeniería de Alimentos, 5(1), 32-40.

MERCASA, 2017. Informe sobre Producción, Industria, Distribución y Consumo de Alimentación en España. URL disponible en: http://www.mercasa-ediciones.es/alimentacion_2017/pdfs/alimentacion_en_espana_web_2017_150px.pdf

Milani, J., Maleki, G., 2014. Effects of processing on mycotoxin stability in cereals. Journal of the Science of Food and Agriculture, 94(12), 2372-2375.

Nadarajah, D., Han, J.H., Holley, R.A., 2005. Inactivation of *Escherichia coli* O157:H7 in packaged ground beef by allyl isothiocyanate. International Journal of Food Microbiology, 99, 269-279.

Ostry, V., Malir, F., Toman, J., Yann Grosse, Y., 2017. Mycotoxins as human carcinogens—the IARC Monographs classification. Mycotoxin Res, 33, 65–73.

Pal Vig, A., Rampal, G., Thind, T.S., Arora, S., 2009. Bio-protective effects of glucosinolates. LWT - Food Science and Technology, 42, 1561-1572.

Pani, G., Schem, B., Azara, E., Balmas, V., Jahanshiri, Z., Carta, P., *et al*., 2014. Natural and natural-like phenolic inhibitors of type B trichothecene in vitro production by the wheat (*Triticum sp*.) pathogen *Fusarium* culmorum. Journal of Agricultural and Food Chemistry, 62(22), 4969-4978.

Peraica, M., Domijan, A.M., Jurjevic, Z., Cvjetkovic, B., 2002. Prevention of exposure to mycotoxins from food and feed. Archives of Industrial Hygiene and Toxicology, 53, 229-237.

Ponte, J.G., Tsen, C.C., 1978. Bakery products. In: Food and beverage mycology. AVI Publishing Co, 191-223.

RASFF, 2017. Annual report. URL disponible en: https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2017.pdf

RD, 2002, Real Decreto 1202/2002, de 20 de noviembre, por el que se modifica la Reglamentación técnico-sanitaria para la fabricación, circulación y comercio del pan y panes especiales, aprobada por el Real Decreto 1137/1984, de 28 de marzo. BOE 280.

Ribera, 1893. Elementos de historia natural. Cátedra de eméritos, pp: 472.

Richard, J.L., 2009. Some major mycotoxins and their mycotoxicosis - an overview. Int J Food Microbiol, 119(1-2), 3-10.

Rodríguez, Y., 2015. Estudio analítico y de exposición a micotoxinas de *fusarium.* Universitat de València, pp: 467.

Ruhr-Universität Bpchum, 2009. Department of Plant Physiology: Glucosinolate metabolism http://homepage.ruhr-uni-bochum.de/markus.piotrowski/

Ruiz, M.J., Font, G., 2008. Toxicidad y evaluación de riesgos. En: Micotoxinas en alimentos. Díaz de Santos. 15-28.

Scudamore, K.A., Banks, J.N., 2004. The fate of mycotoxins during cereal processing, In: Meeting the mycotoxin menace. Proceedings of the 2nd World Mycotoxin Forum, 17–18, 165-181.

Seiler, D., 1992. Mould growth on bread. In: Basic guide to product spoilage and hygiene. Chipping Campden, 117–119.

Sellam, A., Iacomi-Vasilescu, B., Hudhomme, P., Simoneau, P., 2007. *In vitro* antifungal activity of brassinin, camalexin and two isothiocyanates against the crucifer pathogens *Alternaria brassicicola* and *Alternaria brassicae*. Plant Pathology, 56, 296-301.

Sofrata, A., Santangelo, E.M., Azeem, M., Borg-Karlson, A.M., Gustafsson, A., Putsep, K., 2011. Benzyl Isothiocyanate, a Major Component from the Roots of *Salvadora Persica* Is Highly Active against Gram-Negative Bacteria. PLoS ONE, 6(8), 23045.

Soriano, J.M., 2007. Introducción. En: Micotoxinas en alimentos. Díaz de Santos, 3-14.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Martin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P., 2012. Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed-Focus on Europe. Toxins, 4(10), 788-809.

Travers-Martin, N., Kuhlmann, F., Müller, C., 2008. Revised determination of free and complexed myrosinase activities in plant extracts. Plant Physiology and Biochemistry, 46(4), 506-516.

Tunc, S., Chollet, E., Chalier, P., Preziosi-Belloy, L., Gontard, N., 2007. Combined effect of volatile antimicrobial agents on the growth of *Penicillium notatum*. International Journal of Food Microbiology, 113, 263-270.

UE, 2010a. Reglamento (UE) n° 105/2010 de la Comisión, de 5 de febrero de 2010, que modifica el Reglamento (CE) n° 1881/2006, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a la ocratoxina A. DOUE L 35/7.

UE, 2010b. Reglamento (EU) n° 165/2010 de la Comisión, de 26 de febrero de 2010, que modifica, en lo que respecta a las aflatoxinas, el Reglamento (CE) n° 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE L 50/8.

UE, 2012a. Reglamento (UE) n° 594/2012 de la Comisión, de 5 de julio de 2012, por el que se modifica el Reglamento (CE) n° 1881/2006 de la Comisión, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios, en lo concerniente a los contenidos máximos de los contaminantes ocratoxina A, PCBs no similares a las dioxinas y melamina en los productos alimenticios. DOUE L 176/43.

UE, 2012b. Reglamento (UE) n° 1058/2012 de la Comisión, de 12 de noviembre de 2012, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que respecta al contenido máximo de aflatoxinas en los higos secos. DOUE L 313/14.

UE, 2014. Reglamento (UE) n° 212/2014 de la Comisión, de 6 de marzo de 2014, por el que se modifica el Reglamento (CE) n° 1881/2006 en lo que concierne a los contenidos máximos del contaminante citrinina en complementos alimenticios basados en arroz fermentado con levadura roja *Monascus purpureus*. DOUE L 67/3.

UE, 2015. Reglamento (UE) n° 2015/1940 de la Comisión, de 28 de octubre de 2015, por el que se modifica el Reglamento (CE) n° 1881/2006 en relación con el contenido máximo de esclerocios de cornezuelo de centeno en determinados cereales no elaborados y con las disposiciones sobre seguimiento y presentación de informes. DOUE L 283/3.

Van Eylen, D., Indrawati, O., Hendrickx, M., Van Loey, A., 2006. Temperature and pressure stability of mustard seed (*Sinapis alba L.*) myrosinase. Food Chemistry, 97, 263–271.

Vereda, A., 2008. Estudio clínico e inmunológico de alergia a la mostaza. Universidad Complutense de Madrid, pp: 159.

Willey, J.M., Prescott, L.M., 2009. Microbiología. McGraw-Hill Interamericana de España. pp: 1088.

Wilson, A.E., Bergaentzlé, M., Bindler, F., Marchioni, E., Lintz, A., Ennahar, S., 2013. *In vitro* efficacies of various isothiocyanates from cruciferous vegetables as antimicrobial agents against foodborne pathogens and spoilage bacteria. Food Control, 30, 318-324.

2. OBJECTIVES

Objetivos

2. OBJETIVOS

El **objetivo general** del presente trabajo es la aplicación de isotiocianatos (ITCs) para reducir la presencia de hongos toxigénicos y micotoxinas en productos de panadería.

Para conseguir este objetivo se han planteado los siguientes **objetivos específicos**:

1. Evaluar el riesgo de exposición a micotoxinas presentes en muestras de masas de pizza refrigeradas procedentes de comercios de Valencia.
2. Caracterizar la actividad antifúngica de diversos extractos de harinas de mostaza amarilla y oriental.
3. Estudiar el uso de harina de mostaza amarilla para alargar la vida útil de panes de molde contaminados con *Aspergillus* y *Penicillium.*
4. Evaluar la utilización de alil isotiocianato (AITC) para inhibir el crecimiento fúngico y la síntesis de micotoxinas en masas de pizza refrigeradas contaminadas con *Aspergillus.*
5. Estudiar la utilización de AITC y para-hidroxibencil isotiocianato (ρ-HBITC) para reducir la contaminación de aflatoxinas (AFs) en tortas de trigo contaminadas con *Aspergillus.*
6. Diseño de un dispositivo liberador de AITC y su evaluación como herramienta para reducir la contaminación fúngica y de micotoxinas en cereales durante su etapa de almacenamiento en silos.

2. OBJECTIVES

The **general objective** of this work is the application of isothiocyanates (ITCs) to reduce the presence of toxigenic fungi and mycotoxins in bakery products.

To achieve this objective, the following **specific objectives** have been set:

1. To evaluate the risk of exposure to mycotoxins present in samples of refrigerated pizza doughs from shops in Valencia.
2. Characterize the antifungal activity of various extracts of yellow and oriental mustard flours.
3. Study the use of yellow mustard flour to extend the shelf-life of mold breads contaminated with *Aspergillus* and *Penicillium*.
4. To evaluate the use of allyl isothiocyanate (AITC) to inhibit fungal growth and mycotoxin synthesis in refrigerated pizza doughs contaminated with *Aspergillus*.
5. Study the use of AITC and para-hydroxybenzyl isothiocyanate (ρ-HBITC) to reduce the contamination of aflatoxins (AFs) in wheat tortitas contaminated with *Aspergillus*.
6. Design of an AITC release device and its evaluation as a tool to reduce fungal and mycotoxin contamination in cereals during its storage in silos.

3. RESULTS

Resultados

Food and Chemical Toxicology, 94 (2016) 19-24

3.1. Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population

Juan Manuel Quiles, Federica Saladino, Jordi Mañes, Mónica Fernández-Franzón, Giuseppe Meca*

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n,

46100 Burjassot, Spain

1. Introduction

Mycotoxins are a group of secondary metabolites produced by fungi, mainly by the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Fusariu*m, and *Claviceps*. Mycotoxins may contaminate cereals and other food commodities at pre-harvest, harvest and post-harvest and their presence is largely dependent on environmental factors that affect fungal growth (Zain, 2011).

The risk of human exposure to mycotoxins is related to consumption of contaminated food, which may cause diseases and can also lead to death (Erdogan, 2004). A limited number of more than 400 known mycotoxins are generally considered to play important roles in food safety (Reddy et al., 2010, Streit et al., 2012) because they can be responsible for pathophysiological changes like neurotoxicity, nephrotoxicity, hepatotoxicity, neurological cardiac lesions, gastrointestinal disorders, Balkan endemic nephropathy, tubulonefritis and so forth (Marin et al., 2013). There is much concern about chronic effects of mycotoxins by low levels of exposure, and some of them have been classified by the International Agency for Research on Cancer (IARC, 2013) as human carcinogens [Group 1: aflatoxins (AFs)] or probably human carcinogens or possible carcinogen to humans [Group 2B: fumonisin B1 (FB_1) and ochratoxin A (OTA)].

The most important mycotoxins detected in food are: AFs, OTA, trichothecenes (TCs) (type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)), zearalenone (ZEA), FB_1 and fumonisin B2 (FB_2), and the emerging mycotoxins fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs) (Marin et al., 2013).

Maximum levels of contamination have been established by The European Commission for some mycotoxins found in cereals and derived products, in

particular: 2 μg/kg for aflatoxin B_1 (AFB_1) and 4 μg/kg for total AFs (European Commission, 2010); 750 μg/kg for DON (European Commission, 2006); 1000 μg/kg for the sum of FB_1 and FB_2 in maize-based foods for direct human consumption (European Commission, 2007); 3 μg/kg for OTA (European Commission, 2012); 25 μg/kg for T-2 y HT-2 (Commission Recommendation, 2013); 75 μg/kg for ZEA (European Commission, 2006).

Monitoring studies of mycotoxins in several foodstuffs should be continuously conducted. They are necessary to collect and evaluate the presence of mycotoxins in food and feed and, thus, to obtain reliable information about the real exposure of human population to these toxic compounds (Rodríguez-Carrasco et al., 2013).

Pre-cooked pizza dough is considered a potential substrate for fungal development, because it is a product based on cereals with intermediary moisture content. These fungi can grow and affect nutritional and sensory properties of the food products and, above all, if the species are toxigenic, they may produce mycotoxins. Food preparation procedures could inactivate the fungi but do not guarantee the removing or reduction of the mycotoxins already produced by the fungi because they are relatively stable to cooking and processing. The contamination can also occur during packaging and inappropriate storage. Even in samples kept at refrigerated temperatures, up to the end of shelf life, mold and yeast growth was no inhibited and mycotoxins were produced (Pinho and Furlong, 2000).

The aim of this study was to: a) determine and quantify the occurrence of different legislated and no legislated mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEA, FB_1, FB2, FUS, BEA and ENs) in refrigerated pizza dough samples

commercialized in Spain and b) to estimate the daily intake of these mycotoxins among Spanish population to carry out the deterministic risk assessment.

2. Materials and methods

2.1. Chemical and reagents

Methanol was purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Molsheim, Francia). Chromatographic solvents and water were filtered through a 0.45 µm cellulose filter from Scharlau (Barcelona, Spain). Formic acid (HCOOH) and ammonium formate were obtained from Sigma–Aldrich (St Louis MO., USA).

2.2. Analytical standard

AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEA, FB_1, FB_2, FUS, BEA, ENB, ENB_1, ENA and ENA_1, were obtained from Sigma–Aldrich (St Louis MO., USA). All stock solutions were prepared by dissolving 1 mg of the mycotoxin in 1 mL of pure methanol, obtaining a 1 mg/mL solution. These stock solutions were then diluted with pure methanol in order to obtain the appropriated work solutions. All solutions were stored in darkness at −20 °C.

2.3. Sampling

A total of 60 refrigerated pizza dough samples of eight brands and all from different lots were randomly purchased from different supermarkets located in Valencia (Spain) from March to June 2015. All the samples were stored at −20 °C

before sample extraction. Refrigerated pizza dough ingredients were: wheat flour, water, vegetable fat (palm, rape), salt, wheat starch, alcohol and yeast.

2.4. Mycotoxin extraction

The method used for mycotoxins analysis is based on that described by Serrano et al. (2013) with some modifications. The pizza doughs were finely ground with an Oster Classic grinder (220–240 V, 50/60 Hz, 600 W; Madrid, Spain). Five grams of each homogenized sample were weighed in a 50 mL plastic tube and 25 mL of methanol were added. The extraction was carried out using an Ultra Ika T18 basic Ultra-turrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4000 rpm for 5 min at 5 °C and the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The residue was dissolved with 5 mL of methanol and was evaporated to dryness by nitrogen gas at 35 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was resuspended with 1 mL of methanol, vortexed, filtered through 13mm/0.22 µm nylon filters and injected into liquid chromatography associated with tandem mass spectrometry (LC-MS/MS). All the extractions were carried out in triplicate.

2.5. LC-MS/MS analysis

The liquid-chromatography analysis system was an Agilent 1200 Chromatograph (Agilent Technologies, Palo Alto, CA, USA) which consisted of a binary LC-20AD pump, a SIL-20AC homoeothermic auto sampler and a CMB-20A controller Analyst Software 1.5.2 was used for data acquisition and processing. The separation of mycotoxins was performed on a Gemini NX C18 column (150 ×

2.0 mm I.D, 3.0 µm, Phenomenex, Palo Alto, CA) at room temperature (20 °C). The mobile phase was composed of solvents A (5 mM ammonium formate and 0.1% formic acid in water) and B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min, then kept constant from 1.5 to 4 min, increased to 90% from 4 to 10 min, increased again to 100% from 10 to 14 min and finally return to the initial conditions and requilibrate during 10 min. The injection volume was 20 µL. A 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were *m/z* 313.3/241.3–284.9 for AFB_1, *m/z* 315.3/259.0–288.4 for AFB_2, *m/z* 329.7/243.3–311.1 for AFG_1, *m/z* 331.1/313.0–245.0 for AFG_2, *m/z* 801.2/784.1–244.1 for BEA, *m/z* 657.3/196.1–214.0 for ENB, *m/z* 671.2/214.2–228.1 for ENB_1, *m/z* 699.4/210.2–228.2 for ENA, *m/z* 685.4/214.2–210.2 for ENA_1, *m/z* 722.4/334.3–352.3 for FB_1, *m/z* 706.4/336.2–318.3 for FB2, *m/z* 404.0/102.0–239.0 for OTA, *m/z* 319.0/282.9–301.0 for ZEA, *m/z* 355.0/175.0–246.7 for FUS.

Analytical parameters of the validation method are showed in Table 1.

Table 1. Analytical parameters of the validation method.

Mycotoxin	LOD (µg/Kg)	LOQ (µg/Kg)	Recovery (%)	SSE (%)
AFB_1	0.08	0.2	70 + 13	37
AFB_2	0.08	0.2	64 + 12	29
AFG_1	0.16	0.5	62 + 16	27
AFG_2	0.3	0.9	66 + 15	34
ZEA	7.8	26.1	55 + 7	106
ENA	2.5	7.5	66 + 8	14
ENA_1	0.5	1.5	65 + 5	21
ENB	0.03	0.1	72 + 12	49
ENB_1	0.06	0.2	75 + 5	49
BEA	7	20	50 + 11	32

LOD = limit of detection, LOQ = limit of quantification, SSE = Signal Suppression – Enhancer

2.6. Dietary exposure

In order to estimate the risk associated to the intake of mycotoxins contained in refrigerated fresh pizza dough, a deterministic approach was carried out based on Rodríguez-Carrasco et al. (2013). The mean values of the mycotoxins concentration, obtained in the analyzed samples (considering positive and negative ones), were related with the Spanish consumption data of refrigerated pizzas. Spanish consumption of refrigerated pizza was 1.53 g/day and it was obtained from the database of the Spanish Agriculture, Food and Environment Minister (MAGRAMA, 2015). On the other hand, considering that adolescents are great consumers of pizza, if they take two portions of 65 g in a week the consumption will be 18.57 g/day. The EDI of the mycotoxins, detected in the analyzed samples, was calculated and expressed as µg/kg bw per day:

$$EDI = (C \times K) / bw$$

where “C” is the average concentration of mycotoxin detected in food expressed as µg/kg, “K” represents pizza consumption expressed in kg per day and “bw” is the average weight of the age groups studied. The EDI for the studied mycotoxins was assessed for the population divided into three age groups: 12–14, 15–18 and 19–64 years old. The weights used for the three groups were respectively 50, 65 and 70 kg. The average concentrations of each mycotoxin were calculated considering all contamination data including the positive and negative ones. The values below quantification limit were established as half of the quantification limit. Then, the EDI was compared with the tolerable daily intake (TDI), when available, to develop the health risk characterization:

$$\% \text{ EDI} - \text{TDI} = (\text{EDI}/\text{TDI}) \times 100$$

The TDI is a value set by the relevant agencies (EFSA, JECFA) and indicates the quantity of a substance that can be consumed over a lifetime without risk to health. This data is determined using various risk factors and toxicological parameters, like the “no observed adverse effect level” (NOAEL). Some substances do not have an established TDI, for example: AFs due to their demonstrated carcinogenicity (IARC Group I) and other mycotoxins because there are still no conclusive studies about their toxicity in vitro. For this reason, risk assessment of ENs and BEA were done with TDI values of other mycotoxins produced by fungi of the same genus as DON (1 µg/kg bw per day), NIV (1.2 µg/kg bw per day), sum of T-2 and HT-2 (0.1 µg/kg bw per day). TDI for ZEA was set at 0.25 µg/kg bw per day (EFSA, 2014).

3. Results and discussion

3.1. Occurrence of mycotoxins in fresh refrigerated pizza dough samples

Occurrence data of mycotoxins in fresh pizza dough samples are shown in Table 2 and evidenced the presence of nine compounds: AFB_1, AFB_2, AFG_1, ZEA, ENA, ENA_1, ENB, ENB_1 and BEA.

Table 2. Mycotoxins detected in the analyzed refrigerated pizza dough samples, specifying the number of positive samples, the frequency, mean and range (positive > LOQ).

Mycotoxin	Positive samples		Legislated maximum level		Mean	Range
(n = 60)	n	Frecuency (%)	n	Frecuency (%)	(µg/Kg)	(µg/Kg)
AFB_1	14	23 %	7	12 %	4.09	1.03-9.50
AFB_2	19	32 %	-	-	0.50	0.34-0.67
AFG_1	6	10 %	-	-	0.79	0.53-1.05
AFs	30	50 %	4	7 %	2.36	0.34-10.02
ZEA	60	100 %	7	12 %	77.78	28.64-176.28
ENA	5	8 %	-	-	14.96	14.96-14.96
ENA_1	60	100 %	-	-	4.54	1.64-14.19
ENB	60	100 %	-	-	3.37	0.23-10.54
ENB_1	60	100 %	-	-	1.69	0.23-13.40
ENs	60	100 %	-	-	8.87	1.93-27.75
BEA	2	3 %	-	-	22.39	22.39-22.39

Considering the co-occurrence of mycotoxins analyzed, 70% of the samples were contaminated with at least five mycotoxins, 58% with at least six and 12% with at least nine. It has to be highlighted that 100% of the samples were contaminated with at least four mycotoxins, all produced by *Fusarium* species (Marin et al., 2013): ZEA, ENA_1, ENB and ENB_1. In particular, ENs are emerging mycotoxins and their co-occurrence has been evidenced in different foods:

cereals commercialized in Spain (Meca et al., 2010); cereal products and multicereal food from Italy (Juan et al., 2013) and organic and conventional pasta collected in Spain (Serrano et al., 2013). In one study performed in infant formulas, samples resulted contaminated with three or more ENs (Serrano et al., 2012a). Most of the studies in the scientific literature evidences the simultaneous contamination of ENs, BEA and FUS, while the contamination of ZEA is studied together with other mycotoxins like AFs, OTA, FBs, DON (Klarić et al., 2009, Vidal et al., 2013, Iqbal et al., 2014) and only one study evaluated the co-occurrence of ZEA and ENs in maize (Adejumo et al., 2007).

The contamination frequencies of the analyzed samples with AFs were 23%, 32%, 10% and 50% for AFB_1, AFB_2, AFG_1 and sum of AFs, respectively. The average concentration of the positive samples was 4.09 µg/kg for AFB_1, 0.50 µg/kg for AFB_2, 0.79 µg/kg for AFG1 and 2.36 µg/kg for the sum of AFs. AFs, in particular AFB_1, are considered as the most strong natural genotoxic carcinogen, classified as "carcinogenic to humans" by the IARC (2013). Taking into consideration only the contaminated pizza doughs above the LOQ, AFB_1 content ranged between 1.03 and 9.50 µg/kg and the samples contaminated with AFB_1 were 14 of the 30 samples contaminated with at least one AF. Fifty percent of the pizza samples contaminated with AFB_1 (12% of total samples) exceeded the legislated limit of 2 µg/kg. AFs were detected in forty-one percent of the breakfast cereals analyzed by Iqbal et al. (2014) and the 16% of the samples were contaminated above the maximum level permitted by the legislation. Fifty percent of AFs positive samples of cereals and cereal-based products (ranged between 5.5 and 66.7 µg/kg) analyzed in the study of Serrano et al. (2012b) were contaminated by AFB_1 and fourteen out 265 total cereal samples were contaminated with AFB_1 exceeding the EU maximum limits. In wheat based complementary foods for infants and

young children AFB_1 was detected in concentrations between 0.24 and 29.0 µg/kg while total AFs between 0.33 and 33.92 µg/kg (Blankson and Mill-Robertson, 2016).

The AFB_2 content in positive samples ranged from 0.34 to 0.67 µg/kg, while AFG_1 was detected with concentrations from 0.53 to 1.05 µg/kg. The levels of AFB_2 detected by Serrano et al. (2012b) in cereals samples ranged between 5.6 and 26.0 µg/kg while the contents of AFG_1 ranged between 25.0 and 62.2 µg/kg. Furthermore, in the study of Serrano et al. (2012b), different from our study, AFG_2 was also detected with values ranged from 4.2 to 18.7 µg/kg.

AFs were not detected in wheat and oat based bran supplements analyzed by Vidal et al. (2013). Iqbal et al. (2014) focuses only on the occurrence of AFB_1 and total AFs in breakfast cereals, but no information was reported about the individual occurrence of AFB_2, AFG_1 and AFG_2. In refrigerated pizza dough samples sum of AFs minimum and maximum concentrations were 0.34 µg/kg and 10.02 µg/kg, respectively. Seven percent of total samples analyzed resulted above the legislated limit for the sum of AFs (4 µg/kg). The concentrations of the sum of AFs evidenced by Klarić et al. (2009) in cereal samples ranged between 2.0 and 4.5 µg/kg. Eight percent of the total breakfast cereals analyzed by Iqbal et al. (2014) exceeded the EU maximum limits established for the sum of AFs.

ZEA was detected in 100% of refrigerated pizza doughs with values that ranged from 28.64 to 176.28 µg/kg and the average content was 77.78 µg/kg. Twelve percent of total samples exceeded the EU legislated limit of 75 µg/kg. The occurrence of ZEA in wheat and wheat products is recognized all over the world such as in wheat supplements sold in Spain (Vidal et al., 2013) and Serbia (Skrbic et al., 2012), in wheat derived products of Pakistan (Iqbal et al., 2014), in wheat produced in Brasil (Almeida et al., 2013) and in wheat from Romania (Alexa et al.,

2013). According to the data obtained by Klarić et al. (2009) in cereal samples, ZEA is more frequent in cereals than other mycotoxins like OTA and AFB_1. Klarić et al. (2009) detected concentration levels of ZEA from 12.5 to 1182 µg/kg. In the study of the occurrence of ZEA in different commodities from Catalonian market (Spain), the maximum value detected for ZEA by Cano-Sancho et al. (2012) was 22.8 µg/kg, none of the food samples resulted above the limit. Juan et al. (2013b) detected ZEA in Italian wheat and wheat products with concentration values between 2.35 and 27.15 µg/kg.

Concerning the distribution of ENs, all pizza dough samples were contaminated by at least three ENs with concentration values ranged from 1.93 to 27.75 µg/kg. ENA_1, ENB and ENB_1 were detected in 100% of the samples. The average contents in positive samples were 4.54, 3.37, and 1.69 µg/kg respectively, whereas 14.19, 10.54 and 13.40 µg/kg were the maximum concentrations detected of these mycotoxins. The mean concentration of ENA detected was 14.96 µg/kg and it was detected in 8% of total samples. In the study of the occurrence of ENs in Italian cereal products by Juan et al. (2013) the incidences of ENB, ENB_1, ENA and ENA_1 were 70%, 26%, 13% and 9%, respectively. The mean levels detected by Juan et al. (2013) were 133.60, 8.10, 4.50 and 8.30 µg/kg for ENB, ENB_1, ENA and ENA_1, respectively. In Italian wheat and wheat based products the ENs were detected in concentrations between 5.3 and 97 µg/kg (Juan et al., 2013b).

BEA was detected only in two samples with concentrations of 22.39 µg/kg in one sample and below the quantification limit in the other one. Only 6% of the total (265) cereals products belonging to Spain, Italy, Morocco and Tunisia and analyzed by Serrano et al., 2012a, Serrano et al., 2012b) were contaminated with BEA. In a study on Portuguese food products only one of 61 samples resulted

contaminated with BEA (Blesa et al., 2012). BEA was detected in 5 of 57 samples of Italian wheat and wheat based products with a concentration range of 9.6–35 µg/kg (Juan et al., 2013b).

3.2. Estimation of the daily intake

In Table 3 are shown the exposure estimates for three different age groups and for high consumers based on the reported mycotoxins occurrence.

For AFs the EDI data ranged from 5.08E-05 µg/kg bw per day for the 19–64 years old age group to 6.89E-05 µg/kg bw per day for 12–14 years old. The mean value of exposure to the sum of AFs showed by Sirot et al. (2013) in the study of French total diet was 1.90E-06 µg/kg bw per day. EDI values of total AFs through the consumption of wheat based complementary foods for infants and young children ranged from 0.153 to 1.034 µg/kg bw per day for infants and from 0.094 to 0.819 µg/kg bw per day for young children (Blankson and Mill-Robertson, 2016). For the AFs the comparison between the EDI and the TDI cannot be assessed because these mycotoxins are classified as carcinogenic for human.

On the other side, for the other mycotoxins detected was possible to compare the EDIs obtained to the TDIs.

The values of EDI obtained for ZEA varied from a minimum of 9.63E-04 µg/kg bw per day for 19–64 years old to 1.31E-03 µg/kg bw per day for 12–14 years old. The exposure values of ZEA obtained by Cano-Sancho et al. (2012) of the commodities most susceptible to ZEA contamination and commonly consumed in Catalunia were below the TDI. They did not find difference between sexes but divided the population into five age groups.

Table 3. Risk characterization of mycotoxins detected in the studied samples.

	Mycotoxin	Mean (µg/Kg)	Age groups 12-14	15-19	20-64	High consumers
AFs	EDI (µg/Kg bw/day)	2.32	6.89E-05	5.40E-05	5.08E-05	8.34E-04
ZEA	EDI (µg/Kg bw/day)	43.99	1.31E-03	1.02E-03	9.63E-04	1.58E-02
	TDI (µg/Kg bw/day)		0.25			
	% EDI-TDI		0.52	0.41	0.39	6.32
ENs	EDI (µg/Kg bw/day)	13.06	3.88E-04	3.04E-04	2.86E-04	4.69E-03
	DON TDI (µg/Kg bw/day)		1.00			
	NIV TDI (µg/Kg bw/day)		1.20			
	T-2 + HT-2 TDI (µg/Kg bw/day)		0.10			
	% EDI-TDI (DON)		0.04	0.03	0.03	0.47
	% EDI-TDI (NIV)		0.03	0.03	0.02	0.39
	% EDI-TDI (T-2 + HT-2)		0.39	0.30	0.29	4.69
BEA	EDI (µg/Kg bw/day)	13.75	4.08E-04	3.20E-04	3.01E-04	4.94E-03
	DON TDI (µg/Kg bw/day)		1.00			
	NIV TDI (µg/Kg bw/day)		1.20			
	T-2 + HT-2 TDI (µg/Kg bw/day)		0.10			
	% EDI-TDI (DON)		0.04	0.03	0.03	0.49
	% EDI-TDI (NIV)		0.03	0.03	0.03	0.41
	% EDI-TDI (T-2 + HT-2)		0.41	0.32	0.30	4.94

- Mean concentration of mycotoxins were calculated assuming ½ LOQ in no detected samples.

- EDI: Estimated Daily Intake, TDI: Tolerable Daily Intake.

The EDI values obtained for adolescents (13–18 years old) and adults (20–65 years old) ranged from 9.00E + 02 to 2.20E + 03 µg/kg bw per day. EDI value obtained by Juan et al. (2013b) for ZEA considering the consumption of wheat and wheat based products was 0.075 µg/kg bw per day. Our results are based on the worst scenario as negative samples were considered half of the LOQ. However, it is widely considered that this scenario overestimates contamination and exposure levels, but other proposed scenarios could underestimate the real exposition. ZEA risk characterization, evidenced in our study, ranged from 0.39% for 19–64 years old to 0.52% for 12–14 years old. Percentage of EDI-TDI obtained in the study of exposure estimation of Spanish population to *Fusarium* mycotoxins through wheat based products intake ranged between 0.01 and 0.11% in adults of 70 kg (Rodríguez-Carrasco et al., 2013).

The EDI calculated for ENs ranged from 2.86E-04 µg/kg bw per day to 3.88E-04 µg/kg bw per day for 19–64 years old and 12–14 years old, respectively. The EDI of ENs obtained by Blesa et al. (2012), using total data of cereal consumption in Portugal and assuming an average adult body weight of 60 kg, was 4.842E-01 µg/kg bw per day. The EDI values for ENA, ENA1, ENB and ENB_1, calculated through the consumption of wheat and wheat products in Italy ranged between 0.003 (ENB_1) and 0.134 (ENB) µg/kg bw per day (Juan et al., 2013b). The lowest value of %EDI-TDI was 0.02% obtained for 19–64 years old, if we consider the TDI of NIV. The highest value was 0.39% calculated for 12–14 years old using TDI of T-2 + HT-2.

Considering the mycotoxin BEA, the minimum value of EDI was 3.01E-04 µg/kg bw per day for 19–64 years old and the maximum was 4.08E-04 µg/kg bw per day for 12–14 years old. BEA EDI obtained by Serrano et al. (2013) of Spanish population to emerging *Fusarium* mycotoxins present in pasta was 5.00E-04 µg/kg

bw per day. Through the consumption of Italian wheat and wheat based products the EDI of BEA resulted of 0.018 µg/kg bw per day (Juan et al., 2013b). In our study, the maximum percentage of BEA risk assessment was 0.41% obtained for 12–14 years old, using T-2 + HT-2 TDI.

Considering that the 12–14 age group is the class of population of great consumers and with the highest values of EDI, the dietary exposure of these adolescents was also calculated in a worse scenario assigning a consumption of two portions of pizza doughs in a week. The EDI values obtained, in this scenario, were 8.34E-04 µg/kg bw per day for AFs, 4.69E-03 µg/kg bw per day for ENs, 4.94E-03 µg/kg bw per day for BEA and 1.58E-02 µg/kg bw per day for ZEA. The values of % EDI-TDI resulted about tenfold higher than the ones calculated using the date of consumption of MAGRAMA for this class of population: 6.32% for ZEA; ranges from 0.39 to 4.69% for ENs and from 0.41 to 4.94 for BEA considering NIV TDI and T-2 + HT-2 TDI, respectively.

4. Conclusions

To our knowledge, this is the first report on the presence of different legislated and no legislated mycotoxins in refrigerated pizza dough samples commercialized in Spain.

None of the 60 samples analyzed was free from mycotoxins; four mycotoxins showed a frequency of 100%. There were samples in which the concentration of mycotoxin, as for ZEA and AFs, exceed the legislated limits. The presence of AFs in 30% of the samples was of toxicological concern, especially the samples contaminated with levels of AFB_1 above the maximum limit permitted. The intake

of AFs should be reduced as low as possible because of the demonstrated carcinogenicity of these compounds in human.

Even though the presence of mycotoxins in all samples, in some cases above the limit of legislations, the EDIs calculated were all lower than the value of TDI. However, to have a complete view of the risk assessment, it should be considered mycotoxins intake of the whole dietary. Furthermore, greater attention should be taken to children and adolescent who are exposed to a higher risk due to their high consumption and their lower weight.

References

Adejumo, T.O., Hettwer, U., Karlovsky, P., 2007. Survey of maize from south-western Nigeria for zearalenone, alpha- and beta-zearalenols, fumonisin B1 and enniatins produced by *Fusarium* species. Food Addit. Contam. 24, 993e1000.

Alexa, E., Dehelean, C.A., Poiana, M.A., Radulov, I., Cimpean, A.M., et al., 2013. The occurrence of mycotoxins in wheat from western Romania and histopathological impact as effect of feed intake. Chem. Cent. J. 7, 1e119.

Almeida, G.C.F., Barbosa, I.P.T., Sega, R., Machinski, M.J., 2013. Occurrence of zearalenone in wheat- and corn-based products commercialized in the State of Paraná, Brazil. Braz. J. Microbiol. 44, 371e375.

Blankson, G.K., Mill-Robertson, F.C., 2016. Aflatoxin contamination and exposure in processed cereal-based complementary foods for infants and young children in greater Accra, Ghana. Food control. 64, 212e217.

Blesa, J., Marin, R., Lino, C.M., Mañes, J., 2012. Evaluation of enniatins A, A_1, B, B_1 and beauvericin in Portuguese cereal-based foods. Food Addit. Contam. Part A 29, 1727e1735.

Cano-Sancho, G., Marin, S., Ramos, A.J., Sanchis, V., 2012. Occurrence of zearalenone, an oestrigenc mycotoxin, in Catalonia (Spain) and exposure assessment. Food Chem. Toxicol. 50, 835e839.

Commission Recommendation, 2013. Commission recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. Off. J. Eur. Union L 91/12.

EFSA e European Food Safety Authority, 2014. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA J. 12 (12), 1e107, 3916. Available at: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3916.pdf.

Erdogan, A., 2004. The aflatoxin contamination of some pepper types sold in Turkey. Chemosphere 56, 321e325.

European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union L 364, 5e24.

European Commission, 2007. Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. Off. J. Eur. Union L 255, 14e17.

European Commission, 2010. Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. Eur. Union L 50, 8e12.

European Commission, 2012. Commission Regulation (EU) No 594/2012 of 5 July 2012 amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, no dioxin-like PCBs and melamine in foodstuffs. Off. J. Eur. Union, L 176, 43e45.

IARC, 2013. Agents Classified by the International Agency for Research on Cancer Monographs, Volumes 1e109. http://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.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, 257e262.

Juan, C., Ma~nes, J., Raiola, A., Ritieni, A., 2013. Evaluation of beauvericin and enniatins in Italian cereal products and multicereal food by liquid

chromatography coupled to triple quadrupole mass spectrometry. Food Chem. 140, 755e762.

Juan, C., Ritieni, A., Mañes, J., 2013b. Occurrence of *Fusarium* mycotoxins in Italian cereal and cereal products from organic farming. Food Chem. 141, 1747e1755.

KlariKlarić, M.Š., Cvetnic, Z., Pepeljnjak, S., Kosalec, I., 2009. Co-occurrence of aflatoxins, ochratoxin A, fumonisins, and zearalenone in cereals and feed, determined by competitive direct enzyme-linked immunosorbent assay and thin-layer chromatography. Arch. Ind. Hyg. Toxicol. 60, 427e434.

MAGRAMA, 2015. Alimentary Statistics - Database of Household Consumption. http://www.magrama.gob.es/es/alimentacion/temas/consumo-ycomercializacion-y-distribucion-alimentaria/panel-de-consumo-alimentario/base-de-datos-deconsumo-en-hogares/consulta.asp.

Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60, 218e237.

Meca, G., Zinedine, A., Blesa, J., Font, G., Mañes, J., 2010. Further data on the presence of *Fusarium* emerging mycotoxins enniatins, fusaproliferin and beauvericin in cereals available on the Spanish markets. Food Chem. Toxicol. 48, 1412e1416.

Pinho, B.H., Furlong, E.B., 2000. The occurrence of molds, yeasts and mycotoxins in precooked pizza dough sold in southern Rio Grande do Sul. Braz. J. Microbiol. 31, 99e102.

Reddy, K.R.N., Salleh, B., Saad, B., Abbas, H.K., Abel, C.A., Shier, W.T., 2010. An overview of mycotoxin contamination in foods and its implications for human health. Toxin Rev. e J. Toxicol. 29, 3e26.

Rodríguez-Carrasco, Y., Ruiz, M.J., Font, G., Berrada, H., 2013. Exposure estimates to *Fusarium* mycotoxins through cereals intake. Chemosphere 93, 2297e2303.

Serrano, A.B., Font, G., Mañes, J., Ferrer, E., 2013. Emerging *Fusarium* mycotoxins in organic and conventional pasta collected in Spain. Food Chem. Toxicol. 51, 259e266.

Serrano, A.B., Font, G., Ruiz, M.J., Ferrer, E., 2012b. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. Food Chem. 135, 423e429.

Serrano, A.B., Meca, G., Font, G., Ferrer, E., 2012a. Risk assessment associated to the intake of the emerging *Fusarium*mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin. Food control. 28, 178e183.

Sirot, V., Fremy, J.M., Leblanc, J.C., 2013. Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food Chem. Toxicol. 52, 1e11.

Skrbic, B., Zivancev, J., Durisic, N.M., Godula, M., 2012. Principal mycotoxins in wheat flour from the Serbian market: levels and assessment of the exposure by wheat based products. Food Control. 25, 389e396.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Martin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P., 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed-focus on Europe. Toxins 4 (10), 788e809.

Vidal, A., Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. Food Chem. Toxicol. 53, 133e138.

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc. 15 (2), 129e144.

Food and Chemical Toxicology, 83 (2015) 222-228

3.2. Influence of the antimicrobial compound allyl isothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust

Juan M. Quiles[a], Lara Manyes[a], Fernando Luciano[b], Jordi Mañes[a], Giuseppe Meca[a]*

[a]*Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain*

[b]*School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil*

1. Introduction

Aflatoxins (AFs) (Fig. 1) are highly toxic secondary metabolites produced by some species of *Aspergillus*, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Wejdan et al., 2010). AFs are of great concern because of their toxicity for the health of humans and animals, including carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Zinedine and Mañes, 2009). Aflatoxin B1 (AFB1) is the most potent hepatocarcinogen known for mammals and it is classified by the International Agency of Research on Cancer as a Group 1 carcinogen (IARC, 1993).

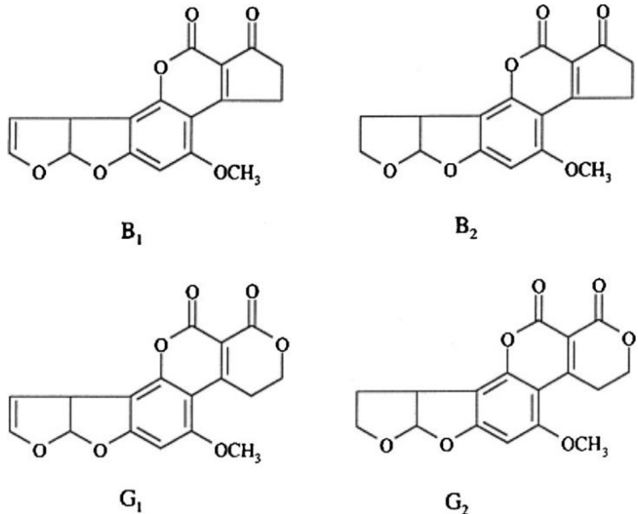


Fig 1. Chemical structure of the main AFs.

The process of mold contamination is complex and starts in the field where crops first become infected by Aspergilli residing on the soil and/or on decaying plant residues. Plant stress or damage allied to a warm and humid environment

increase susceptibility of crops to fungal growth (Cotty et al., 2008). These mycotoxin-producing species can grow on a wide range of agricultural commodities both in the field and during storage (Zinedine and Mañes, 2009). Major food products affected are nuts, dried fruits, spices, crude vegetable oils, cocoa beans, maize, wheat, barley and food products composed by cereal flours (Iamanaka et al., 2007, Imperato et al., 2011, Luttfullah and Hussain, 2011).

Fungal contaminations are usually determinant for the shelf-life of refrigerated pizza when it is stored aerobically. Pinho et al. (2000) have demonstrated that the most prevalent genus of contaminant fungi in pre-cooked pizza crust were *Penicillium* and *Aspergillus*. This was a study performed in Southern Brazil, where the authors also found the presence of ochratoxin A (confirmed, and aflatoxins B_1 and B_2(presumptive) in refrigerated pizza crusts after 45 days of production, which poses as a serious risk for human consumption.

Glucosinolates (GSLs) are β-thioglycosides found as secondary metabolites of Cruciferous plants, including Brussels sprouts, broccoli, cauliflower, cabbage, watercress, oilseed rape and mustard (Johnson, 2002, Thornalley, 2002). GSLs are converted to the corresponding aglycone by myrosinase hydrolysis (thioglucoside glucohydrolase, EC 3.2.3.147), which produces an unstable compound that is readily converted to isothiocyanates, thiocyanates or nitriles, depending on the substrate, pH and availability of ferrous ions. At physiological pH, isothiocyanates are the major product. Fungi growth inhibition by isothiocyanates has been reported since the late 1930's. These compounds are very unique in comparison to other essential oils, since they are only formed when the plant cell suffers some kind of injury such as insect bite, grinding, milling or fungi contamination in the presence of water (Luciano and Holley, 2009). Research studies have shown that ITCs exhibit biocidal activity against microorganisms including fungi, bacteria,

insects and nematodes (Bodnaryk, 1991, Mithen, 2001). In particular, allyl ITC (AITC), which is derived from sinigrin (2-propenyl glucosinolate), effectively inhibits a variety of pathogenic microorganisms, even at low concentrations (Lin et al., 2000, Luciano and Holley, 2009). The goals of this study were to evaluate: a) the antifungal activity of AITC, oriental mustard flour, potassium propionate and sorbic acid towards *A*. *parasiticus* inoculated in pizza crust and b) the influence of these preservatives the level of AFs produced.

2. Materials and methods

2.1. Chemicals

AFs B_1, B_2, G_1, G_2, sinigrin (98% purity), phosphate buffer saline (PBS) at pH 7, formic acid (HCOOH), allyl isothiocyanate (AITC), sorbic acid, potassium propionate, tetrabutylammonium hydrogen sulfate (TBA), ammonium formate, and sodium chloride (NaCl) were obtained from Sigma–Aldrich (St. Louis, USA). Oriental mustard flour was provided by G.S. Dunn Dry Mustard Millers (Hamilton, Ontario, Ca). Methanol was purchased from Fisher Scientific (New Hempshire, USA). Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. The strain of *A*. *parasiticus* CECT 2681, was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Buffered peptone water, potato dextrose agar (PDA) and potato dextrose broth (PDB) were provided by Oxoid (Madrid, Spain).

2.2. GSLs extraction and determination from oriental mustard flour

GSLs from oriental mustard flour were extracted using the method of Hontanaya et al. (2015) with modifications. Five grams of the flour were introduced in a 50 mL glass tube and autoclaved at 115 °C during 15 min to inactivate the enzyme myrosinase. Then, 25 mL of boiling distilled water were introduced in the same tube. The extraction of the GSLs was carried out using the Ultra turrax Ika T18 basic Ultraturrax (Staufen, Germany) during 3 min at speed 4. The mixture was centrifuged at 2500 rpm for 5 min at 4 °C and filtered through a filter paper (Whatman no. 4) into 50 mL screw-capped tubes. The extract was filtered again through a 0.22 µM filter. Separation and quantification of glucosinolates was performed using a Shimadzu LC system (Shimadzu, Japan), equipped with a Gemini C18 column (4.6 × 150 mm i.d. 5 µm; Phenomenex, Palo Alto, CA). Elution was carried out isocratically for 20 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water with 0.02 M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 20 µL. A UV detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of GSLs.

Commission Decision 2002/657/EC and 401/2006/EC were used as guidelines for the validation studies. All the parameters were evaluated by spiking blank samples (5, 10 and 15 mg of sinigrin), which were left to equilibrate overnight before the analysis. For identification purposes, retention time of compound in standards and samples were compared at tolerance of 70.5%. Method performance characteristics such as linearity, limits of detection (LOD), limits of quantitation (LOQ), matrix effect, recovery, repeatability and reproducibility were evaluated for sinigrin.

2.3. Samples preparation

The pizza crust recipe included 400 g of wheat flour, 3 g of sucrose, and 6 g of NaCl and 20 g of yeast for bakery products (Levital, Spain). The ingredients were kneaded manually for 5 min and the dough produced was left rising for 6 h at room temperature. Then, it was divided in small balls of approximately 40 g each, which were rolled out using a rolling pin. The pizzas were inoculated with 500 µL of a suspension containing 1×10^5 conidia/mL *A. parasiticus* CECT 2681. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^5 conidia/mL in PDB as reported Kelly et al. (2006). Inoculated pizzas received one of the following antimicrobial treatments:

1. Sorbic acid (E-200) and sodium propionate (E-281) were added to the dough at the concentration of 0.5, 1.0 or 2.0 g/Kg each. This is a common combination of preservatives used in fresh pizza crust in Spain. Crusts were packaged in 1 L plastic trays, covered with a plastic film (low oxygen transmission rate for modified atmosphere packaging) and hermetically sealed with a thermosealer (Domo, Valencia, Spain). All treatments below were packaged using this same process.

2. Addition of autoclaved oriental mustard flour (OMF) at the concentration of 0.7, 1.7 or 3.4% as a dry ingredient in the dough. Autoclaved or deheated OMF has been shown to have bactericidal activity in meat products (Lara-Lledó et al., 2012, Luciano et al., 2011, Cordeiro et al., 2013). Therefore, its potential fungicidal activity was also evaluated in this study.

3. Inoculated pizza crusts were introduced in the plastic tray together with small plastic packets containing 170, 425 or 850 mg of OMF [OMF (S)]. Packets were added with the same quantity of water (170, 425 or 850 µL)

to promote the conversion of sinigrin in AITC. Quantities of OMF were calculated to generate 2, 5 or 10 ppm of AITC inside the package.

4. Pizza crusts inoculated with *A. parasiticus* were packaged with paper filters (2.5 × 2.5 cm) soaked with AITC, giving a final concentration of 2, 5 or 10 ppm of the essential oil inside the package.
5. Packages containing inoculated pizza crust were added with plastic packets (similar to #3) containing paper filters (2.5 × 2.5 cm) soaked with AITC [AITC (S)], giving a final concentration of 2, 5 or 10 ppm of the essential oil inside the package.
6. The control group did not receive any antimicrobial treatment.

All plastic trays were closed hermetically and incubated at 4 °C during 30 d. Then, all packages were opened and samples were used to determine the fungal population and to quantify AFs using liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS).

2.4. Aflatoxins extraction

AFs extraction was performed using the method described by Hontanaya et al. (2015). Briefly, 5 g samples of the pizza crust were ground (Oster Classic grinder, Oster, Valencia, Spain) and placed in a 50 mL plastic tube. Then, 0.5 g of sodium chloride (NaCl) and 25 mL of a methanol/water (80:20, V/V) mixture were added. Samples were homogenized using Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500 × *g* for 5 min and the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach,

Switzerland). The residue was re-dissolved in 1 mL of extraction solvent, filtered through a 0.22 µM syringe filter and injected to the LC-MS/MS system.

2.5. AFs identification and quantification by LC-MS/MS

The liquid-chromatography analysis system consisted of a binary LC-20AD pump, an SIL-20AC homoeothermic auto sampler, a CTO-20A column oven and a CMB-20A controller and Analyst Software 1.5.2 was used for data acquisition and processing.

The mobile phase was composed by water +0.1% formic acid (Solvent A) and acetonitrile + 0.1% formic acid (Solvent B). The elution was carried out at a flow rate of 0.2 mL/min using the following gradient: from 0 to 1 min 10% of B, from 1 to 1.6 min the concentration of B was increased to 95% remained constant during 0.3 min. Afterwards, the column was re-equilibrated with 90% solvent B until the end of the run. The LC-MS/MS system was composed by an Alliance 2695 LC (Waters, Milford, MA) and a TQ mass spectrometer Quattro LC (Micromass, Manchester, UK). A Z-spray interface and Mass Lynx NT software 4.1 was used for data acquisition and processing. The ionization and fragmentation parameters used for the detection and quantification of the AFs were set according to Liu et al. (2013).

2.6. Determination of the fungal population

After incubation, 10 g of each sample were transferred to a sterile plastic bag containing 90 mL of sterile peptone water (Oxoid, Madrid, Spain) and homogenized with a stomacher (IUL, Barcelona, Spain) during 30 s. The mixture

was serially diluted in sterile plastic tubes containing 0.1% peptone water. Aliquots of 0.1 mL were plated in acidified (pH = 3.5) potato dextrose agar (Insulab, Valencia, Spain) and the plates were incubated at 25 °C for 7 d before microbial counting (Pitt and Hocking, 1997).

3. Results and discussion

3.1. Method performance and glucosinolates determination in oriental mustard flour

Mean recovery of fortified oriental mustard flour samples (n = 3) at three different levels of sinigrin (SN) (5, 10 and 15 mg) was of 84.6 ± 3.6% (Table 1). The value obtained for recovery and relative standard deviations of the method used is in agreement with the EU Commission Directive 2006/401/EC for methods to analyze bioactive compounds in foodstuffs (European Commission, 2006). Intra-day (n = 5) and inter-day (5 different days) variation values were 2.5 and 8.6%, respectively. These values are below 15%, which is the maximum variation for certification exercises of bioactive compounds. The detection limit (LOD) and the limit of quantification (LOQ) values were 0.05 and 0.15 mg/L, respectively.

Linearity, plotted as DAD response area against concentration estimated for the matrix matched standards, and matrix effects were studied using standard solutions and matrix matched calibrations. Calibration curves were built at eight different sinigrin levels, from LOQ to 100 times LOQ (from 0.1 to 300 ppm). Each level was prepared in triplicate. Slopes of standard solutions were compared with those obtained in matrix matched standards to assess the possible matrix effect on the chromatographic response. The results obtained showed that the matrix effect calculated for sinigrin was 87.4%

Table 1. Validation method parameters for the detection and quantification of sinigrin in oriental mustard flour.

Validation method parameters	Data
SN concentration (*n* = 9)	5.04 ± 0.22%
Limit of Detection (LOD)	15 ppb
Limit of Quantification (LOQ)	50 ppb
Matrix effect (n = 3)	88.19%
Recovery	84.67%
Intra-day variation (%)	2.5%
Inter-day cariation (%)	8.6%

Fig. 2 shows the LC-DAD chromatogram of sinigrin present in the oriental mustard flour used in this study, which presented a final concentration of 5.04 g/100 g of OMF. This glucosinolate is the precursor of AITC, which has been shown as potent natural antimicrobial in different food matrices, including chicken breast (Shin et al., 2010), ground beef (Nadarajah et al., 2005), dry-cured ham (Graumann and Holley, 2007), fermented dry sausage (Chacon et al., 2006) and bread (Nielsen and Rios, 2000).

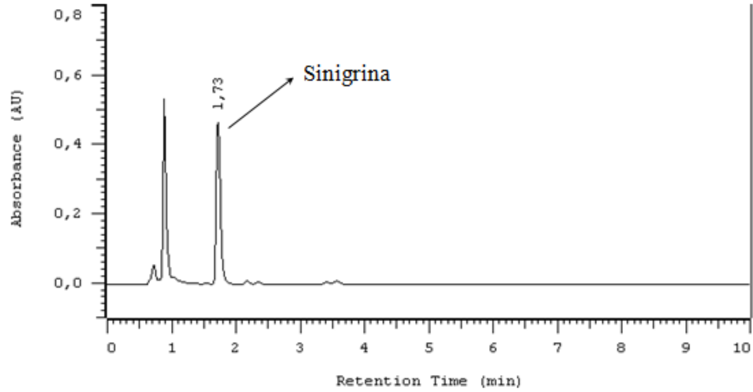


Fig 2. LC-DAD chromatogram of sinigrin present in oriental mustard flour.

3.2. Effect of OMF and AITC on A. parasiticus growth

The growing demand for safe foods without synthetic chemical preservatives has fostered research to investigate the effects of natural compounds against the growth of several pathogenic microorganisms. Both OMF and AITC have been described as potent antimicrobials (Nielsen and Rios, 2000, Nadarajah et al., 2005, Chacon et al., 2006, Luciano et al., 2011, Cordeiro et al., 2013). Nevertheless, this study evaluated the antifungal activity of the population of deheated OMF, AITC and sodium propionate + sorbic acid (commonly used in bakery products) towards *A. parasiticus* present in pizza crust. Crusts were tainted with ∼3,1 log conidia/g and kept at room temperature for 30 d at 4 °C prior to analysis.

None of the treatments was capable of completely inactivate *A. parasiticus* (Fig. 3). However, all preservatives were able to significantly retard the fungal growth in comparison to the control (8.99 CFU/g after 30 d) in a dose-dependent manner. In particular, the most effective treatment to inhibit *A. parasiticus* growth was AITC (S), followed by AITC in filter > OMF (S) > sorbic acid + sodium propionate > deheated OMF. Although deheated OMF has been found to reduce the initial levels of pathogenic bacteria in meat products by 5 log CFU/g (Luciano et al., 2011, Lara-Lledó et al., 2012), the same level of reduction was not found in the present study. The authors that used OMF in meat products suggested that the antimicrobial activity generated by this preservative was a result of bacterial myrosinase-like activity and a hurdling environment. Perhaps *A. parasiticus* lacks the capacity of converting sinigrin in AITC or the quantity of AITC formed was not lethal. Moreover, the pizza crust seems to be an optimal environment for the growth of this fungus, since its final population was extremely high (8.99 CFU/g).

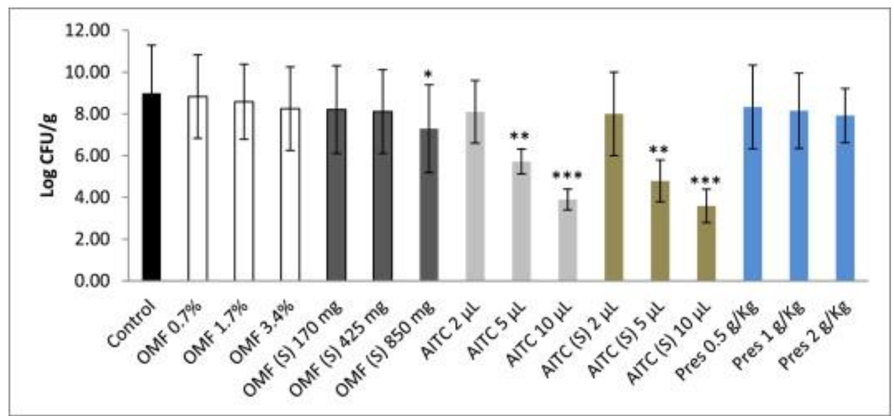


Fig 3. Population of *Aspergillus parasiticus* in fresh pizza crust treated treated with natural and synthetic preservatives (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, P ≤ 0.05 (*), P ≤ 0.001 (**), P ≤ 0.0001 (***).

The highest growth reduction was achieved by the use 10 ppm of AITC added to a filter paper or plastic sachet, resulting in >5 log CFU/g difference in comparison to the control. On the other hand, the *in situ*formation of AITC through the combination of OMF and water was not able to cause the same growth inhibition produced by pure AITC. This may have happened because sinigrin was not totally converted in AITC and/or other reaction products may have been formed, such as nitriles and thiocyanates (Cejpek et al., 2000). However, 850 mg of OMF was able to keep the fungal population 1.7 log CFU/g lower than the control and 0.63 log CFU/g lower than the highest dose of sorbic acid + sodium propionate after 30 d. Interestingly, the commercial preservative was not as efficient as the other compounds (except deheated OMF) to avoid *A*. *parasiticus* growth in fresh pizza crust.

Fungi growth inhibition by allyl isothiocyanate in bakery products has been reported before (Nielsen and Rios, 2000, Suhr and Nielsen, 2003, Azaiez et al.,

2013). However, to our knowledge, this is the first time that antimicrobial devices containing AITC were used to avoid mold growth in fresh pizza crust. Overall, the AITC filter was more effective in delivering the volatile oil than AITC or OMF sachets. Nielsen and Rios (2000) tested the ability of several essential oils (cinnamon, garlic, clove, vanilla, oregano and mustard) in inhibiting the growth of spoilage fungi commonly found in bread, namely *A. flavus*, *Endomyces fibuliger*, *Penicillium commune*, *Penicillium corylophilum*, *Penicillium discolor*, *Penicillium roqueforti*, *Penicillium polonicum*, *Penicillium palitans*, *Penicillium solitum* and *Pichia anomala*. Mustard oil containing 90–95% AITC was the most potent antimicrobial agent towards these microorganisms, with *in vitro* Minimum Inhibitory Concentrations ranging from 1.8 to 3.5 μg/mL. Sensory studies also showed that these concentrations were not noticeable or slightly noticeable in hot dog and rye bread treated for 24 h.

Mejía-Garibay et al. (2015) characterized the essential oil (EO) of black mustard (*Brassica nigra*) and quantified its antimicrobial activity, when applied by direct contact into the liquid medium or by exposure in the vapor phase (in laboratory media or in a bread-type product), against the growth of *Aspergillus niger*, *Aspergillus ochraceus*, or *Penicillium citrinum*. AITC was identified as the major component of *B. nigra* EO with a concentration of 378.35 mg/mL. When *B. nigra* EO was applied by direct contact into the liquid medium, it inhibited the growth of *A. ochraceus*and *P. citrinum* at 2 μL/mL, while μL/mL was necessary to inhibit *A. niger*. Exposure of molds to *B. nigra* EO in vapor phase showed that 41.1 μL/L of air delayed the growth of *P. citrinum* and *A. niger* by 10 days, while *A. ochraceus* growth was delayed for 20 days at the same concentration. Exposure to concentrations ≥47 μL/L of air was fungicidal to all species analyzed.

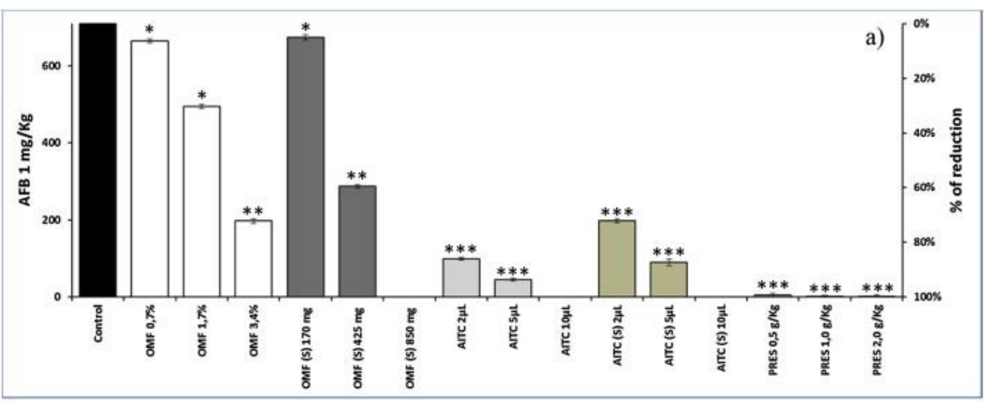
3.3. Effect of OMF and AITC on AFs production by A. parasiticus in pizza crust

As presented on Fig. 4, Fig. 5, *A. parasiticus* was able to produce 709.2 ± 3.5 mg/kg of AFB_1, 637.4 ± 5.9 mg/kg of AFB_2, 58.36 ± 3.5 mg/kg of AFG_1 and 0.54 ± 0.05 mg/kg of AFG_2 in fresh pizza crust after 30 d. All antimicrobial treatments derived from mustard were able to significantly ($P \leq 0.05$) reduce the levels of AFs in a dose-dependent manner. The highest reductions of AFs production were found for the crusts treated with 850 mg OMF(S), 10 µL of AITC in a paper filter and 10 and µL AITC(S), where no AFs were detected. AFs production was also greatly inhibited by the commercial preservative at all concentrations tested.

The only treatments that did not present significant difference in aflatoxin concentration in comparison to the control were the use of 170 mg OMF(S) and the incorporation of deheated OMF at 0.7% in the pizza crust. Interestingly, the commercial preservative composed by sorbic acid and sodium propionate did not inhibit the fungal growth after 30 d, but it reduced almost totally the production of AFs. Zhang et al. (2004) have demonstrated that increased levels of propionyl-CoA, which is a metabolite derived from propionate, impairs polyketide biosynthesis. In particular, the formation of sterigmatocystin is greatly inhibited by propionate in *Aspergillus nidulans*, and this polyketide is the precursor of AFs.

Other studies have reported the antimicrobial activity of deheated mustard flour towards foodborne pathogenic bacteria (Graumann and Holley, 2008, Luciano et al., 2011, Lara-Lledó et al., 2012). This activity was regarded to the higher content of antimicrobial substances in the deheated OMF, such as phenolic acids (released by the heat and pressure promoted by the autoclaving process) and Maillard reaction products (Luciano et al., 2011). Moreover, Rakariyathan et al. (2005) reported that several *Aspergillus* strains

were able to degrade sinigrin using an intracellular myrosinase with activity up to 3.19 U/mL in mustard extract broth. By a mechanism not fully understood, the deheated OMF may inhibit the growth and/or the production of mycotoxins by providing sinigrin, which may be intracellularly converted in AITC. This activity in combination with the other antimicrobials provided by the OMF may have caused the reduction in mycotoxin production seen on Fig. 4, Fig. 5.



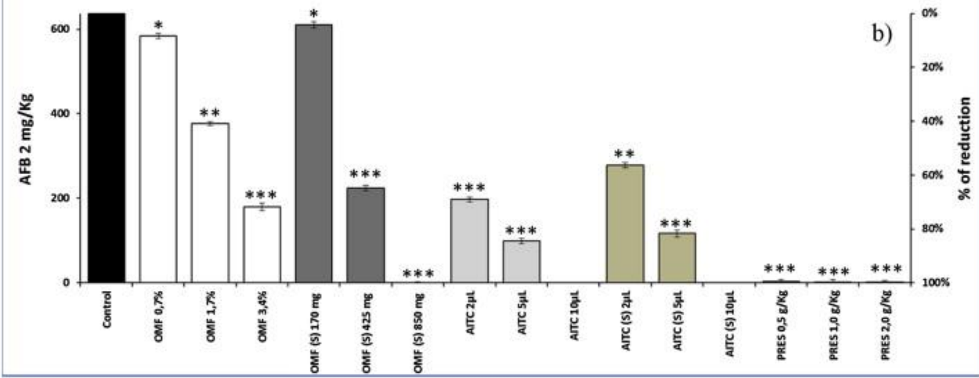
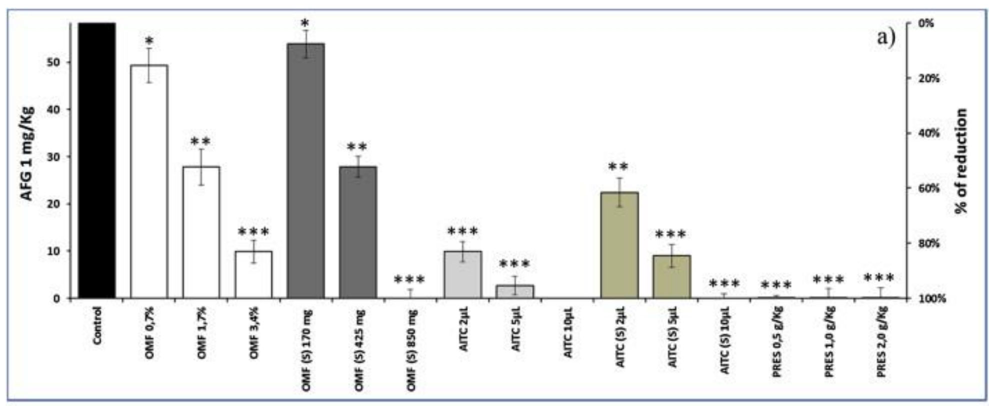


Fig. 4. Concentration and reduction rate of a) AFB_1 and b) AFB_2 present in fresh pizza crust contaminated with *Aspergillus parasiticus* and treated with several preserving treatments (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***).



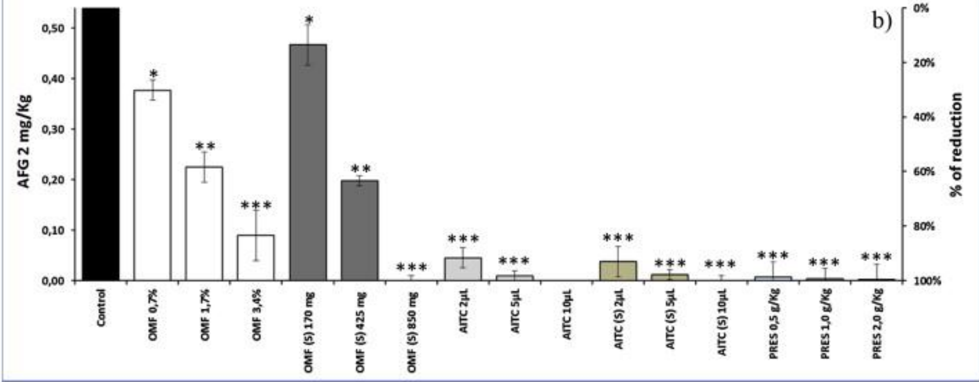


Fig. 5. Concentration and reduction rate of a) AFG_1 and b) AFG_2 present in fresh pizza crust contaminated with *Aspergillus parasiticus* and treated with several preserving treatments (Black = control, White = deheated oriental mustard flour added as ingredient, Dark gray = oriental mustard flour + water in a sachet, Light gray = AITC on filter paper, Cinnamon = AITC in a sachet, Light blue = sorbic acid + calcium propionate). Significantly different from the control, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***).

The reduction of AFs produced by *A*. *parasiticus* CECT 2681 in nuts (peanut, cashew, walnut, almond, hazelnut and pistachio) by isothiocyanates (ITCs) generated from the enzymatic hydrolysis of the glucosinolates (GLCs) present in oriental and yellow mustard flours was evaluated by Hontanaya et al. (2015). The ITCs reduced the *A*. *parasiticus* growth in all food products tested, where AFs B_1,

B_2, G_1 and G_2production was inhibited by 83.1–87.2% with the use of oriental mustard flour, whereas the yellow mustard flour was able to reduce 27.0–32.5% of the AFs concentration.

The concentrations of AITC used in the present study are quite low (10 ppm or less) and the use of these antimicrobial devices could help to avoid the growth of mycotoxinogenic fungi in pizza crust. Even if AITC change some of the sensory properties of the pizza crust, it will probably volatilize completely during baking.

4. Conclusions

Mycotoxin contamination of food and feed is a major concern in food and agricultural sector. Continuous research in the area of physical and chemical methods to eliminate mycotoxin is under continuous development, but there is a need to find safer natural agents to reduce the mycotoxin threat. This study demonstrates the efficacy of AITC-releasing devices to inhibit *A*. *parasiticus* growth and also production AFs during the storage of fresh pizza crust for 30 d. Further studies should be conducted to understand the impact of AITC on the sensory characteristics of the pizza crust and also to evaluate the ability of these devices in extending the product shelf-life.

References

Azaiez, I., Meca, G., Manyes, L., Fernandez-Franz_on, M., 2013. Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate in vitro and their use for fumonisisns reduction in bread. Food Control 32, 428e434.

Bodnaryk, R.P., 1991. Developmental profile of sinalbin (r-hydroxybenzyl glucosinolate) in mustard seedlings, Sinapis alba L., and its relationship to insect resistance. J. Chem. Ecol. 17, 1543e1556.

Cejpek, K., Valusek, J., Velisek, J., 2000. Reactions of allyl isothiocyanate with alanine, glycine, and several peptides in model systems. J. Agric. Food Chem. 48, 3560e3565.

Chacon, P.A., Muthukumarasamy, P., Holley, R.A., 2006. Elimination of Escherichia coli O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Appl. Environ. Microbiol. 72, 3096e3102.

Cordeiro, R.P., Luciano, F.B., Holley, R.A., 2013. Evaluation of deodorized yellow mustard concentrations for control of Escherichia coli O157:H7 viability in dry fermented sausage. Food control 33, 20e24.

Cotty, P.J., Probst, C., Jaime-Garcia, R., 2008. Etiology and management of aflatoxin contamination. In: Leslie, J.F., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxins: Detection Methods, Management Public Health and Agricultural Trade. CAB International, Oxfordshire, UK, pp. 287e299.

European Commission, 2006. Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Comm. L70, 12e34.

Graumann, G.H., Holley, R.A., 2007. Survival of E. coli O157:H7 during manufacture of dry-cured Westphalian ham surface-treated with allyl isothiocyanate or hot mustard powder. J. Sci. Food Agric. 89, 617e624.

Graumann, G.H., Holley, R.A., 2008. Inhibition of Escherichia coli O157:H7 in ripening dry fermented sausage by ground yellow mustard. J. Food Prot. 71, 486e493.

Hontanaya, C., Meca, G., Luciano, F.B., Ma~nes, J., Font, G., 2015. Inhibition of the Aflatoxins B1, B2, G1, and G2, production by Aspergillus parasiticus in nuts using yellow and oriental mustard flours. Food Control 47, 154e160.

Iamanaka, B., Castle de Menezes, H., Vicente, E., Leite, R., Taniwaki, M., 2007. Aflatoxigenic fungi and aflatoxins occurrence in sultanas and dried figs commercialized in Brazil. Food Control 18, 454e457.

IARC, 1993. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.

Imperato, R., Campone, L., Piccinelli, A.L., Veneziano, A., Rastrelli, L., 2011. Survey of aflatoxins and ochratoxin a contamination in food products imported in Italy. Food Control 22, 1905e1910.

Johnson, I.T., 2002. Glucosinolates: bioavailability and importance to health. Int. J. Vit. Nutr. Res. 72, 26e31.

Kelly, S., Grimm, L.H., Bendig, C., Hempel, D.C., Krull, R., 2006. Effects of fluid dynamic induced shear stress on fungal growth and morphology. Process Biochem. 41, 2113e2117.

Lara-Lledo, M., Olaimat, A., Holley, R.A., 2012. Inhibition of Listeria monocytogenes on bologna sausages by an antimicrobial film containing mustard extract or sinigrin. Int. J. Food Microbiol. 156, 25e31.

Lin, C.M., Preston, J.F., Wei, C.I., 2000. Antibacterial mechanism of allyl isothiocyanate. J. Food Prot. 63, 727e734.

Liu, S., Qiu, F., Kong, W., Wei, J., Xiao, X., Yang, M., 2013. Development and validation of an accurate and rapid LC-ESI-MS/MS method for the simultaneous quantification of aflatoxin B1, B2, G1 and G2 in lotus sedes. Food Control 29, 156e161.

Luciano, F.B., Holley, R.A., 2009. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against Escherichia coli O157:H7. Int. J. Food Microbiol. 131, 240e245.

Luciano, F.B., Belland, J., Holley, R.A., 2011. Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward Escherichia coli O157:H7 during dry sausage ripening. Int. J. Food Microbiol. 145, 69e74.

Luttfullah, G., Hussain, A., 2011. Studies on contamination level of aflatoxins in some dried fruits and nuts of Pakistan. Food Control 22, 426e429.

Mejía-Garibay, B., Palou, E., L_opez-Malo, A., 2015. Composition, diffusion, and antifungal activity of black mustard (Brassica nigra) essential oil when applied by direct addition or vapor phase contact. J. Food Prot. 78, 843e848. Mithen, R.F., 2001. Glucosinolates and their degradation products. Adv. Bot. Res. 35, 213e262.

Nadarajah, D., Han, J.H., Holley, R.A., 2005. Inactivation of Escherichia coli O157:H7 in packaged ground beef by allyl isothiocyanate. Int. J. Food Microbiol. 99, 269e279.

Nielsen, P.V., Rios, R., 2000. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. Int. J. Food Microbiol. 60, 219e229.

Pitt, J.I., Hocking, A.D., 1997. In: Pitt, J.I., Hocking, A.D. (Eds.), Fungi and Food Spoilage, Vol. II. Blackie Academic and Professional, London.

Rakariyathan, N., Butrindr, B., Niamsup, H., Shank, L., 2005. Screening of filamentous fungi for production of myrosinase. Braz. J. Microbiol. 36, 242e245.

Shin, J., Harte, B., Ryser, E., Selke, S., 2010. Active packaging of fresh chickenbreast, with allyl isothiocyanate (AITC) in combination with modifiedatmosphere packaging (MAP) to control the growth of pathogens. J. Food Sci. 75, 65e71.

Suhr, K.I., Nielsen, P.V., 2003. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. J. Appl. Microbiol. 94, 665e674.

Thornalley, P.J., 2002. Isothiocyanates: mechanism of cancer chemopreventive action. Anti-Cancer Drugs 13, 331e338.

Wejdan, S.K., Bahruddin, S., Chew, B.Y., Nor, H.H., Abdussalam Salhin, M.A., Muhammad Idiris, S., 2010. Determination of aflatoxins in animal feeds by HPLC with multifunctional column clean-up. Food Chem. 118, 882e886.

Zhang, Y.Q., Brock, M., Keller, M.P., 2004. Connection of propionyl-CoA metabolism to polyketide biosynthesis in Aspergillus nidulans. Genetics 168, 785e794.

Zinedine, A., Mañes, J., 2009. Occurrence and legislation of mycotoxins in food and feed from Morocco. Food Control 20, 334e344.

J Food Sci Technol. 2015 Dec; 52(12): 8315–8321.

3.3. Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas

Juan Manuel Quiles[1], Lara Manyes[1], Fernando Bittencourt Luciano[2], Jordi Mañes[1], Giuseppe Meca[1]*

[1]*Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain*

[2]*School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil*

1. Introduction

Aflatoxins (AFs, Fig.1) are naturally occurring mycotoxins produced by many species of *Aspergillus*, most notably *A. flavus* and *A. parasiticus*, during their growth on foods and animal feed (Williams et al. 2004). They are listed as group I carcinogens by the International Agency for Research on Cancer (IARC), which primarily affect the liver (Lee et al. 2004). Although more than 20 AFs have been identified, the major AFs of concern are known as B_1 (AFB_1), B_2, G_1 and G_2. Among them, AFB_1 is normally the most prevalent toxin and the most toxic (Delmulle et al. 2005; Zhang et al. 2009). As a result, many countries have regulatory limits for AFB_1 levels in agricultural products. The European Union (EU) sets the limits for AFB_1 and for total aflatoxins (B_1, B_2, G_1 and G_2) in nuts, dried fruits, cereals and spices. These limits vary according to the product, but range from 2 to 8 µg/kg for AFB_1 and from 4 to 15 µg/kg for total aflatoxins (Van Egmond 1995).

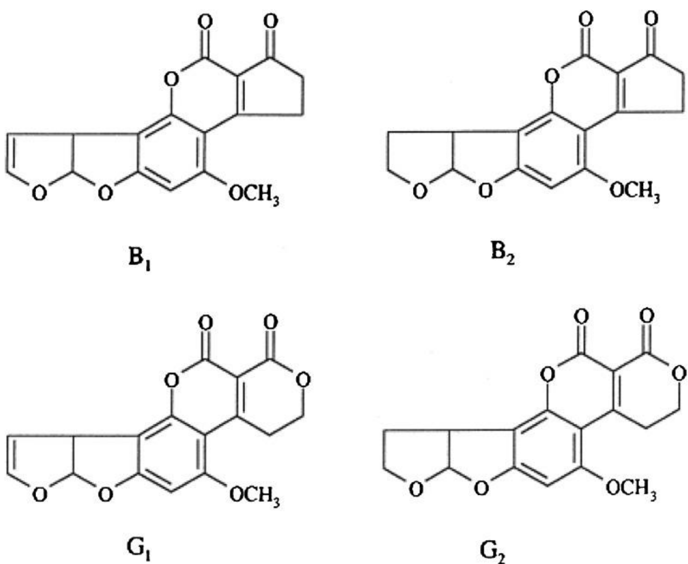


Fig 1. General structure of aflatoxins B_1, B_2, G_1 and G_2

Human exposure to AFs can occur directly from ingestion of contaminated foods or indirectly by the consumption of meat/dairy products from animals previously exposed to AFs. AFs are extremely toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated in human hepatic and extra hepatic carcinogenesis (Iqbal et al. 2012).

Isothiocyanates (ITCs) consist of aliphatic and aromatic compounds generated from the hydrolysis of glucosinolates (GSLs) by myrosinase in cruciferous vegetables such as cauliflower, broccoli, cabbage, horseradish and mustard (Ciska and Pathak 2004; Delaquis and Mazza 1995; Whitmore and Naidu 2000). Myrosinase and GSLs are physically separated within plant cells. Hydrolysis of GSLs occurs when plant tissues are disrupted in the presence of moisture, forming ITCs, nitriles, thiocyanates or epithionitriles depending on environmental conditions (Al-Gendy et al. 2010; Delaquis and Mazza 1995; Mari et al. 2008). Studies have shown that ITCs exhibit biocidal activity against microorganisms including fungi, bacteria, nematodes and insects. In particular, it has been demonstrated that allyl ITC (AITC), which is formed from sinigrin (2-propenyl glucosinolate), effectively inhibits a variety of pathogenic microorganisms, even at low concentrations (Lin et al. 2000; Luciano and Holley 2009). The potential of AITC as a natural antimicrobial has been shown in different foods, including chicken breast (Shin et al. 2010), ground beef (Chacon et al. 2006a; Nadarajah et al. 2005), dry-cured ham (Graumann and Holley 2007), fermented dry sausages (Chacon et al. 2006b) and tuna meat (Hasegawa et al. 1999). The goals of this study were to study a) the quali-quantitative GSLs composition of yellow and oriental mustard flours and b) the reduction of AFs production by *A. parasiticus* in wheat tortilla treated with ITCs generated from mustard flours.

2. Materials and methods

2.1. Chemicals

AFs B_1, B_2, G_1, G_2, sinalbin and sinigrin (98 % purity), phosphate buffer saline (PBS) at pH 7, formic acid (HCOOH), allyl isothiocyanate (AITC), para-hydroxybenzylisothiocyanate (ρ-HBITC), tetrabutylammonium hydrogen sulfate (TBA), ammonium formate, and sodium chloride (NaCl) were obtained from Sigma–Aldrich (St Louis MO., USA). Oriental and yellow mustard flours were provided by G.S. Dunn dry mustard millers (Hamilton, ON, Canada). Methanol was purchased from Fisher Scientific (Hudson, NH, USA). Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. *Aspergillus parasiticus* CECT 2681, was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain).

2.2. Glucosinolates extraction and determination from oriental and yellow mustard flours

GSLs from oriental and yellow mustard flours were extracted using the method described by Prestera et al. (1996) with few modifications. Twenty grams of either mustard flour was introduced in a 50 mL glass tube and autoclaved at 115 °C during 15 min to inactivate the endogenous myrosinase. Samples were added to different Erlenmeyers containing 200 ml of boiling distilled water (100 °C) and the mixtures were stirred for 10 min at 350 rpm. Then, the suspensions were cooled until they reached room temperature, centrifuged at 2500 rpm for 5 min at 4 °C and filtered through filter paper (Whatman no. 4) into

50 ml screw-capped tubes. The extracts were filtered through a 0.22 μM filter and injected into liquid chromatography diode array detector apparatus (LC-DAD). Separation and quantification of glucosinolates was performed using a Shimadzu LC system (Shimadzu, Japan), equipped with a C18 column Gemini (4.6 × 150 mm i.d. 5 μm; Phenomenex, Palo alto, CA). Elution was carried out isocratically for 20 min at a flow rate of 1 ml/min, using a solvent system containing 20 % (*v*/*v*) acetonitrile and 80 % water +0.02 M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume used was 20 μl. A diode array detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of GSLs.

2.3. Wheat tortillas inoculation and storage

Single wheat tortillas were introduced in multilayer polyethylene plastic bags (Saplex, Barcelona, Spain). Then, they were treated with three different quantities of oriental or yellow mustard flour (0.1, 0.5 and 1 g). The flours were introduced in a 50-mm petri dish bottom and added with 2 mL of water to promote the activation of the myrosinase and, consequently, the formation of ITCs (Fig. 2). The wheat tortillas were contaminated with 1 mL of *Aspergillus parasiticus* CECT 2681 grown in potato dextrose broth (PDB, Oxoid, UK) medium containing 10^6 conidia/ml. Conidial concentration was measured by optical density at 600 nm in sterile water and adjusted to 10^6 conidia/ml in PDB as reported Kelly et al. (2006). The control group did not receive any treatment with mustard flour+water. The plastic bag were thermally sealed and incubated at 23 °C during 15 days. Then, bags were opened and the tortillas were autoclaved (121 °C) and extracted for AFs quantification.

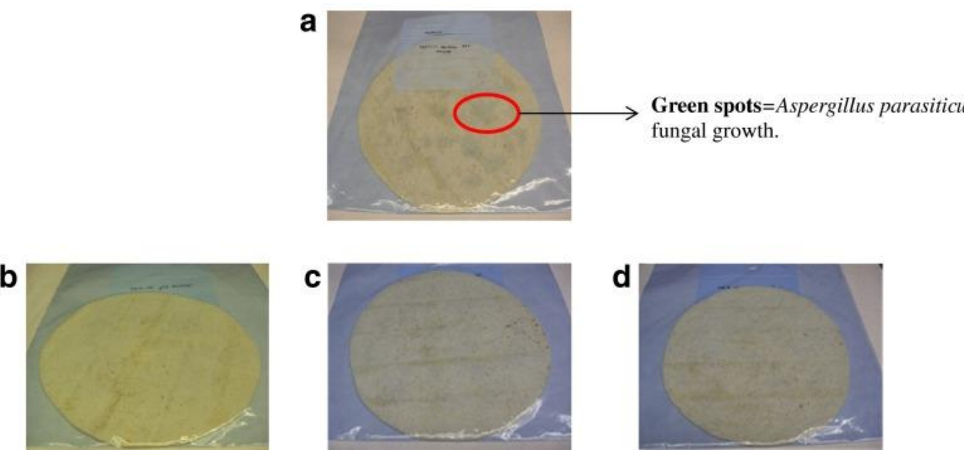


Fig 2. Schematization of AFs reduction experiments carried out on wheat tortilla using gaseous dispersion of ITCs generated by enzymatic hydrolysis of GSLs contained in oriental and yellow mustard flours. a Control wheat tortilla contaminated by *Aspergillus parasiticus* CECT 2681 and wheat tortillas treated with a 0.1 b 0.5 and c 1 g of oriental mustard flours at 8 days incubation. Visible the green spots (fungal growth) are noticeable in the control group and absent in the treated groups

2.4. Aflatoxins extraction

The aflatoxins extraction was carried out using the method described by Liu et al. (2013) with modifications. Briefly, an aliquot of 5 g of each finely ground wheat tortilla (Oster Classic grinder, Oster, Valencia, Spain) was weighed in a 50 mL plastic tube. Then, 0.5 g of sodium chloride (NaCl) and 25 mL of a mixture methanol/water (80:20, V/V) were added and the samples were extracted using Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500×*g* for 5 min and the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The residue was re-dissolved in 1 mL of the extraction solvent, filtered through a 0.22 µM filter and injected into LC-MS/MS.

2.5. AFs identification and quantification by LC-MS/MS

The liquid-chromatography analysis system consisted of a binary LC-20AD pump, a SIL-20AC homoeothermic auto sampler, a CTO-20A column oven, a CMB-20A controller and Analyst Software 1.5.2 was used for data acquisition and processing. The separation of AFs was performed on a Gemini NX C18 column (150 × 2.0 mm I.D, 3.0 μm, Phenomenex, Palo Alto, CA) at room temperature (20 °C). The mobile phase was composed of solvents A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) at a flow rate of 0.2 mL/min. After a hold time of 0.6 min, 10 % of B reached 95 % in 1.6 min and was kept constant for 0.3 min. Afterwards, the column was re-equilibrated with 10 % solvent A until the end of the run at 4.0 min. An API-4000 triple-quadruple MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were m/z 313.3/241.3–228.5, m/z 315.3/259.0–288.4, m/z 329.7/243.3–200.5, m/z 331.9/189.3–217.1 for AFB_1, AFB_2, AFG_1 and AFG_2 respectively.

3. Results and discussion

3.1 Glucosinolates in oriental and yellow mustard flour

The GSLs present in the yellow and oriental mustard flours were analytically characterized to predict the total amount of the GSLs that can be converted in ITCs through the action of myrosinase. Sinigrin was detected in the oriental

mustard flour at 5.2 %, whereas sinalbin was 4.6 % of the yellow mustard flour. Sinigrin and sinalbin are the precursors of the antimicrobial compounds allyl (AITC) and parahydroxybenzyl isothiocyanate (ρ-HBITC), respectively.

The addition antimicrobials that can act during the storage of bakery products to control the presence of the mycotoxigenic fungi is of great interest. Most bakery goods are added with propionate, sorbate or benzoate salts to avoid mold growth, but consumers have become more reluctant to the use of synthetic preservatives in their foods. All plants in the Brassicaceae family contain GSLs as secondary metabolites, and oriental and yellow mustard contains the GSLs sinigrin and sinalbin. Upon physical damage of the plant tissue, hydrolysis of GSLs is catalyzed by the endogenous enzyme myrosinase in the presence of moisture to produce the antimicrobials compounds AITC and ρ-HBITC (Delaquis and Mazza 1995; Ekanayake et al. 2006). The mechanism of action of these antimicrobial compounds is uncertain, but they may inhibit essential enzymes and cause membrane damage (Lin et al. 2000).

3.2. AFs reduction in wheat tortilla

The simulation of AFs contamination was carried out by inoculating the wheat tortillas with *Aspergillus parasiticus* CECT 2681 (AFs producer). As presented on Fig. 3, the control wheat tortilla contained 35.4 mg/kg of the AFB_1, 18.2 mg/kg of AFB_2, 6 mg/kg of AFG_1 and 3 mg/kg of AFG_2.

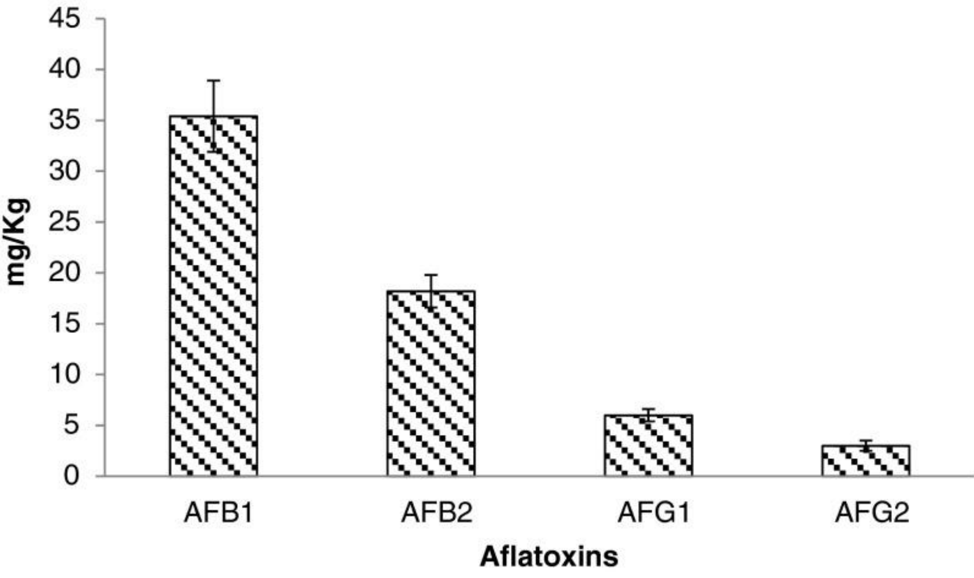


Fig 3. AFs B_1, B_2, G_1 and G_2 produced by *Aspergillus parasiticus* CECT 2681 on wheat tortillas in the absence of oriental and yellow mustard (control group).

As shown on Table 1, the AITC generated from oriental mustard flour was more efficient that ρ-HBITC to avoid the production of AFs. The highest reduction of the AFs (>90 %) was obtained employing 1 g of the oriental mustard flour. AFB_1 production were the most sensitive to AITC, where 0.1 g of oriental flour already produced a >74.8 % of these toxins. Aflatoxin production was affected quite evenly when 0.5 or 1 g of oriental mustard flour was used, with the exception of AFG_1 that was reduced by 54.17 ± 3.6 % with 0.5 g of the AITC-producing flour.

Table 1. AFB_1, AFB_2, AFG_1 and AFG_2 reduction rates produced by the gaseous dispersion of ITCs produced by the enzymatic hydrolysis of the glucosinolates present in a) oriental and b) yellow mustard flour. The plastic bags were thermally sealed and incubated at 23 °C for 15 days.

	% of reduction			
	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
a) Oriental mustard flour (g)				
Control	0.00	0.00	0.00	0.00
0.1	74.68 + 3.5	85.88 + 4.8	18.33 + 2.0	5.66 + 0.6
0.5	84.29 + 4.2	87.06 + 3.9	54.17 + 3.6	87.17 + 2.9
+1.0	94.00 + 5.0	93.82 + 3.8	90.00 + 4.0	96.23 + 3.6
b) Yellow mustard flour (g)				
Control	0.00	0.00	0.00	0.00
0.1	37.14 + 2.2	19.12 + 2.5	16.67 + 0.6	16.98 + 1.0
0.5	71.43 + 3.8	67.65 + 3.7	18.33 +0.7	60.38 + 3.4
1.0	72.29 + 4.1	82.35 + 1.9	35.83 + 2.7	77.36 + 2.9

AFs reduction was much lower with the use of yellow mustard. The mean reduction found ranged from 17.7 to 45.2 % for each aflatoxin. Again, % reduction was higher for AFBs than for AFGs. However, it is noteworthy that AFBs were produced in much higher doses that AFGs in the control group (Fig. 3). Figure 4 hows a LC-MS/MS chromatogram of the AFs present in wheat tortillas treated with yellow mustard flour. The mean AFs reduction acquired with yellow mustard was 1.5 fold lower than those found for oriental mustard. This phenomenon can be related to the stability of the isothiocyanates generated by the hydrolysis of the glucosinolates present in both flours used. In particular, p-hydroxybenzyl isothiocyanate is less stable in aqueous media and presents less volatility than allyl isothiocyanate (Luciano et al. 2011).

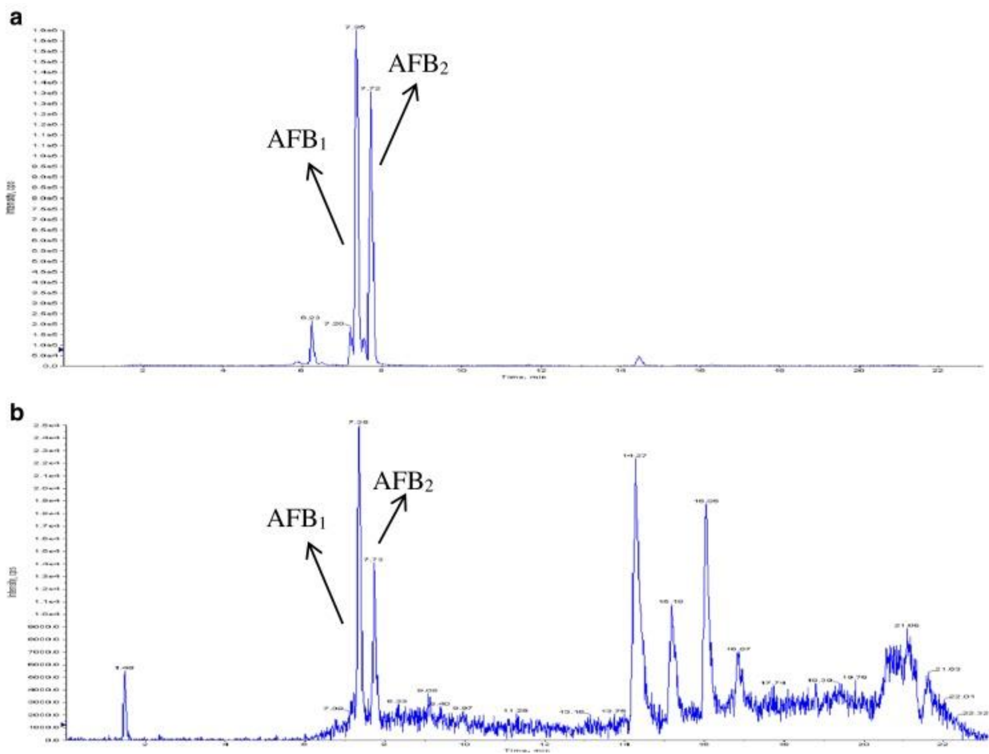


Fig 4. LC-MS/MS chromatograms of the AFB_1 and AFB_2 detected in wheat tortillas; a control and b treated with 1 g of yellow mustard flour.

This article can be considered the first where the application of isothiocyanates has been applied in wheat tortilla to reduce the *Aspergillus parasiticus* growth and consequently the content of AFs. Soher and Amal (2011) studied the effect of hydrochloric acid (HCl) on AFB_1 degradation in contaminated corn gluten under different HCl concentrations, hydrolysis temperatures and hydrolysis times. During the wet milling process the highest AFB_1 level (37.86 %) was found in corn gluten fraction. Treatment with 1 mol/L HCL at 110 °C produced a degradation of AFB_1 from 27.6 to 42.5 %.

Jubeen et al. (2012) investigated the effect of ultra violet irradiation on aflatoxins in ground and tree nuts. Samples of nuts were randomly selected from the retail market of Faisalabad with a moisture content of the nuts artificially increased to 10 ± 3 % and 16 ± 3 % to facilitate the mold growth. The samples were stored at a room temperature of 25–30 °C for 12 weeks. The stored nut samples were checked after 12 weeks and the fungi were found growing in all nuts along with considerable AFs production. AFs and mold contaminated samples were exposed to UV radiations of 265 nm for 15, 30 and 45 min. The fungicidal activity of UV radiation was more pronounced in nuts adjusted at high moisture level. The order of sensitivity for fungal disinfection by UV irradiation was walnut > almond = pistachio > peanuts. There was a proportional decrease in AFs levels with increased exposure time. Complete elimination of AFG_2 was achieved in all nut samples after 15 min exposure, while AFG_1 showed 100 % degradation only in almond and pistachio. After 45 min exposure to UV, AFB_1 showed maximum reduction of 96.5 % in almond and pistachio. The degradation of total AFs as well as that of AFB_1 by UV irradiation was found to follow first order kinetics.

Méndez-Albores et al. (2013) studied the effect of roasting and alkalization processes on the stability of AFB_1 and AFB_2, on cocoa samples contaminated with AFs at a concentration of 220.7 µg/kg and roasted at 250 °C for 15 min. Roasting conditions caused a notable reduction in the AFs content (up to 71 %). The resulting cocoa liquors contaminated with 63.9 ng/g were thermal-alkaline treated with sodium, potassium, and calcium hydroxide at three different concentrations (10, 20, and 30 g/kg). At a concentration of 10 g/kg, the AFs reduction was more effective when using NaOH and $Ca(OH)_2$ (up to 94 %) than when using KOH (up to 88 %). However, at concentrations of 20 and 30 g/kg, all of

the three chemicals were almost equally effective for AFs degradation (up to 98 %).

Azaiez et al. (2013) studied the reduction of the fumonisins (FBs) present in loaf bread contaminated with *Gibberella moniliformis* CECT 2987 by allyl (AITC), phenyl (PITC) and benzyl isothiocyanates (BITC). In addition, the antifungal activity of these ITCs toward *Fusarium* mycotoxigenic strains was also evaluated. The ITCs employed inhibited the growth of three mycotoxigenic *Fusarium* strains, reducing the mycelium size by 2.1 to 89.7 %, depending on the type of ITC and dose used. The ITCs used also reacted with the FB_2 produced by *G. moniliformis* in the bread, reducing its levels by 73–100 % depending on the dose and time of exposure.

Similarly to the present study, Hontanaya et al. (2015) studied the effect of ITCs generated by the enzymatic hydrolysis of the GSLs present in oriental and yellow mustard flours in liquid media and in nuts (peanut, cashew, walnut, almond, hazelnut and pistachio) contaminated by an aflatoxinogenic *A. parasiticus*. The ITCs reduced the *A. parasiticus* growth in both liquid media, where AFs reduction in ranged from 83.1 to 87.2 % using oriental mustard flour and 27.0 to 32.5 % with yellow flour. Nuts were only treated with oriental mustard flour and the mean AFs reduction ranged from 88 to 89 %.

4. Conclusions

The present study showed the capacity of ITCs generated by oriental and yellow mustard flours to reduce AFs naturally produced in wheat tortilla by *Aspergillus parasiticus*. This study shows that antimicrobial devices containing mustard flour and water can be used as natural preservatives for bakery products that are commonly contaminated by *Aspergillus* species. Further investigation will

be focused on use of active packaging, through an antimicrobial sachet or patch, as a form to introduce the production of ITCs in wheat tortilla bags.

References

Al-Gendy AA, El-gindi OD, Hafez AS, Ateya AM (2010) Glucosinolates, volatile constituents and biological activities of Erysimum corinthium Boiss. (Brassicaceae). Food Chem 118:519–524

Azaiez I, Meca G, Manyes L, Fernández-Franzón M (2013) Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate in vitro and their use for fumonisins reduction in bread. Food Control 32:428–434

Chacon PA, Buffo RA, Holley RA (2006a) Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against Escherichia coli O157:H7 in refrigerated, nitrogen packed, finely chopped beef. Int J Food Microbiol 107:231–237

Chacon PA, Muthukumarasamy P, Holley RA (2006b) Elimination of Escherichia coli O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Appl Environ Microbiol 72:3096–3102

Ciska E, Pathak DR (2004) Glucosinolate derivatives in stored fermented cabbage. J Agric Food Chem 52:7938–7943

Delaquis PJ, Mazza G (1995) Antimicrobial properties of isothiocyanates in food preservation. Food Technol 49:73–84

Delmulle BS, De Saeger SMDG, Sibanda L, Barna-Vetro I, Van Peteghem CH (2005) Development of an immuno-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. J Agric Food Chem 53:3364–3368

Ekanayake A, Kester JJ, Li JJ, Zehentbauer GN, Bunke PR, Zent JB (2006) Isoguard TM: a natural anti-microbial agent derived from white mustard seed. Acta Horticult 709:101–108

Graumann GH, Holley RA (2007) Survival of Escherichia coli O157:H7 in needle-tenderized dry cured Westphalian ham. Int J Food Microbiol 118:173–179

Hasegawa N, Matsumoto Y, Hoshino A, Iwashita K (1999) Comparison of effects of Wasabia japonica and allyl isothiocyanate on the growth of four strains of

Vibrio parahaemolyticus in lean and fatty tuna meat suspensions. Int J Food Microbiol 49:27–34

Hontanaya C, Meca G, Luciano FB, Mañes J, Font G (2015) Inhibition of the aflatoxins B1, B2, G1, and G2, production by *Aspergillus* parasiticus in nuts using yellow and oriental mustard flours. Food Control 47:154–160

Iqbal SZ, Asi MR, Ariño A, Akram N, Zuber M (2012) Aflatoxin contamination in different fractions of rice from Pakistan and estimation of dietary intakes. Mycotoxin Res 28:175–180

Jubeen F, Bhatti IA, Khan MZ, Zahoor-Ul H, Shahid M (2012) Effect of UVC irradiation on aflatoxins in ground nut (Arachis hypogea) and tree nuts (Juglans regia, prunus duclus and pistachio Vera). J Chem Soc Pak 34:1366–1374

Kelly S, Grimm LH, Bendig C, Hempel DC, Krull R (2006) Effects of fluid dynamic induced shear stress on fungal growth and morphology. Process Biochem 41:2113–2117

Lee NA, Wang S, Allan RD, Kennedy IR (2004) A rapid aflatoxin B1 ELISA: development and validation with reduced matrix effects for peanuts, corn, pistachio, and soybeans. J Agric Food Chem 52: 2746–2755

Lin CM, Preston JF, Wei CI (2000) Antibacterial mechanism of allyl isothiocyanate. J Food Prot 63:727–734

Liu S, Qiu F, KongW, Wei J, Xiao X, Yang M (2013) Development and validation of an accurate and rapid LC-ESI-MS/MS method for the simultaneous quantification of aflatoxin B1, B2, G1 and G2 in lotus seeds. Food Control 29:156–161

Luciano FB, Holley RA (2009) Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against Escherichia coli O157:H7. Int J Food Microbiol 131:240–245

Luciano FB, Belland J, Holley RA (2011) Microbial and chemical origins of the bactericidal activity of yellow mustard powder toward Escherichia coli O157:H7 during dry sausage ripening. Int J Food Microbiol 145:69–74

Mari M, Leoni O, Bernardi R, Neri F, Palmieri S (2008) Control of brown rot on stone fruit by synthetic and glucosinolate-derived isothiocyanates. Postharvest Biol Technol 47:61–67

Méndez-Albores A, Campos-Aguilar AZ, Moreno-Martínez E, Vázquez- Durán A (2013) Physical and chemical degradation of B-aflatoxins during the roasting and dutching of cocoa liquor. JAgric Sci Technol 15:557–567

Nadarajah D, Han JH, Holley RA (2005) Inactivation of Escherichia coli O157:H7 in package ground beef by allyl isothiocyanate. Int J Food Microbiol 99:269–279

Prestera T, Fahey JW, Holtzcaw WD, Abeygunawardana C, Kachinski JL, Talalay P (1996) Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolates. Anal Biochem 239:168–179

Shin JM, Harte B, Ryser E, Selke S (2010) Active packaging of fresh chicken breast, with allyl isothiocyanate (AITC) in combination with modified atmosphere packaging (MAP) to control the growth of pathogens. J Food Sci 75:65–71

Soher EA, Amal SH (2011) Fate of aflatoxin B1 in contaminated corn gluten during acid hydrolysis. J Sci Food Agric 91:421–427 J Food Sci Technol

Van Egmond HP (1995) Mycotoxins: regulations, quality assurance and reference materials. Food Addit Contam 12:321–330

Whitmore BB, Naidu AS (2000) Thiosulfinates. In: Naidu AS (ed) Natural food antimicrobial systems. CRC Press, Boca Raton, pp. 349–362

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004) Huma aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106–1122

Zhang D, Li P, Zhang Q, Zhang W, Huang Y, Ding X, Jiang J (2009) Production of ultrasensitive generic monoclonal antibodies against major aflatoxins using a modified two-step screening procedure. Anal Chim Acta 636:63–69

Molecules 2018, 23(7), 1638

3.4. Aflatoxins and A. flavus reduction in loaf bread through the use of natural ingredients

Juan M. Quiles[1, †], Raquel Torrijos [1, †], Fernando Luciano[2], Jordi Mañes[1], Giuseppe Meca[1]*

[1]*Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain*

[2]*School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil*

† **Authors have contributed equally to the study and should be considered co-first authors.**

1. Introduction

Aflatoxins (AFs) (Figure 1) are difuranocoumarin derivatives mainly produced through the polyketide pathway by two species of *Aspergillus* fungi which are especially found in areas with hot and humid climates. *Aspergillus flavus* is ubiquitous in Nature, preferring the colonization of the aerial parts of the plants (leaves, flowers) and usually producing group B AFs. *Aspergillus parasiticus* which produces both B and G AFs, is more adapted to soil environments and has more limited distribution [1]. *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* are also AF-producing species, but they are found less frequently [2].

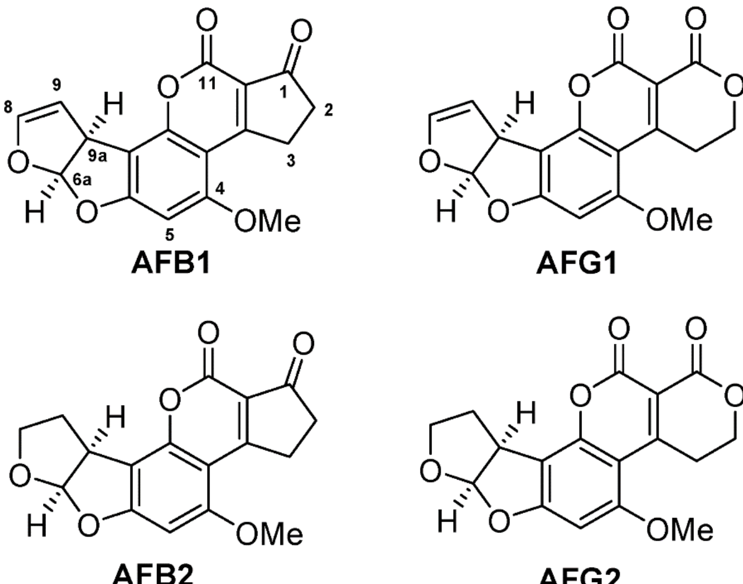


Figure 1. Chemical structures of the aflatoxins.

The four main AFs are AFB_1, AFB_2, AFG_1 and AFG_2. They can directly contaminate agricultural products and other foodstuffs under pre-and post-harvest conditions. AFB_1 is usually predominant in crops as well as in food products and it has been found to exhibit the greatest toxigenic potential [3]. The AFs can be classified from highest to lowest toxicity in the following order: AFB_1, AFG_1, AFB_2 and AFG_2, and this is probably explained by the presence of epoxidation at the 8,9 double bond, as well as by the greater power that accompanies the ring. AFB_1 is one of the most potent toxic carcinogens, it is a teratogen and a mutagen and it is listed as a Group I carcinogen by the International Agency for Research on Cancer (IARC) because it is a cause of human primary hepatocellular carcinoma [4].

Plants belonging to the *Brassicaceae* family are known worldwide for their rich bioactive composition, highlighted by glucosinolates, which are cleaved by an enzymatic reaction to give isothiocyanates (ITCs). These ITCs possess many properties, among which their biocidal activity (fungicidal, bactericidal and on insects and small invertebrates), as well as herbicidal, antioxidant and anticancer effects may be highlighted [5]. Both *Sinapis alba* (yellow or white mustard) and *Brassica juncea* (brown and oriental mustard) contain high levels of glucosinolates, which are cleaved by myrosinase (EC 3.2.1.147) in the presence of moisture, forming ITCs plus thiocyanates, nitriles and some other minor compounds. In oriental mustard, myrosinase forms allyl isothiocyanate (AITC) from the main glucosinolate, sinigrin. Its effectiveness inhibiting the development of fungi [6,7], as well as the bacteria *Escherichia coli* O157: H7 [8], *Listeria monocytogenes* [9] or *Salmonella* sp. [10] has been proven.

In yellow mustard, the ITC that is formed is *p*-hydroxybenzylisothiocyanate (p-HBIT) from the main glucosinolate sinalbine. Ekanayake et al. [11]

demonstrated that *p*-HBIT had significant antimicrobial activity against several foodborne pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Shigella boydii* and *Clostridium* spp.

The aim of the study was to evaluate the use of oriental and yellow mustard flours to reduce contamination by toxigenic fungi and their mycotoxins. First, the antifungal activity of yellow mustard flour (YMF) and oriental mustard meal (OMF) was studied against fourteen different fungal species. Next, the reduction of fungal growth, the formation of mycotoxins and the YMF improvement of the useful life of moldy bread contaminated with *A. flavus* and *P. nordicum* were analyzed.

2. Results and Discussion

2.1. YMF and OMF Antifungal Activity

Table 1 shows the antifungal activity in solid medium evidenced by the eight different extracts tested (YMF and OMF, without and with heat treatment, directly and after concentration) on the 14 mycotoxigenic fungi used in this study.

Table 1. Antifungal activity evidenced by no autoclaved and autoclaved YMF and OMF against mycotoxigenic fungi employed in this study (E = Direct water extract; L = Lyophilized water extract). Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++).

Strains	YMF		Autoclaved YMF		OMF		Autoclaved OMF	
	E	L	E	L	E	L	E	L
P. camemberti (CECT 2267)	-	+	-	+	-	-	-	-
P. roqueforti (CECT 2905)	-	+	-	+	-	+	-	-
P. nordicum (CECT 2320)	-	+	-	-	-	-	-	-
P. commune (CECT 20767)	-	+	-	-	-	-	-	-
P. brevicopactum (CECT 2316)	-	+	-	-	-	-	-	-
P. expansum (CECT 2278)	-	+	-	-	-	-	-	-
P. chrysogenum (CECT 2668)	-	+	-	-	-	-	-	-
P. solitum (CECT 20818)	-	+	-	-	-	-	-	-
P. digitatum (CECT 2954)	-	+	-	-	-	-	-	-
A. parasiticus (CECT 2681)	+	+	-	-	-	-	-	-
A. flavus (ISPA 8111)	-	+	-	-	-	-	-	-
A. carbonarius (ISPA 5010)	-	+	-	-	-	+	-	-
F. verticiloides (ISPA 1044)	-	+	-	-	-	-	-	-
F. graminearum (CECT 20486)	-	-	-	-	-	-	-	-

The non-autoclaved YMF was active only against *A. parasiticus*, whereas the concentrated extract of the non-autoclaved YMF showed the highest antifungal activity on the strains tested, and in particular on 13 of the 14 fungi studied (Figure 2). The autoclaved YMF extract was negative against all fungal strains tested, while the concentrated YMF extract was active against *P. camemberti* and *P. roqueforti*.

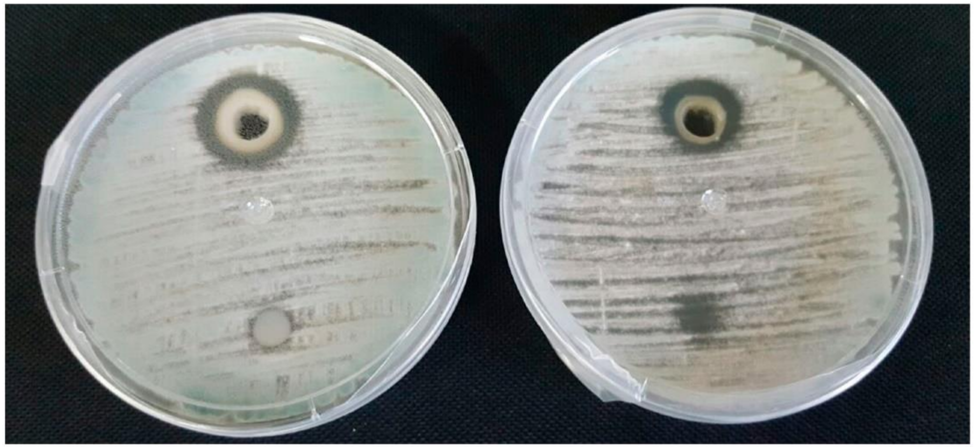


Figure 2. Antifungal activity of YMF lyophilized water extract against *P. nordicum* (CECT 2320) on PDA medium.

Considering the results of the antifungal activity of the OMF, only the concentrated extract of the non-autoclaved flour showed antifungal activity, against the strains of *P. roqueforti* and *A. carbonarius*. The other extracts of this bioactive ingredient tested did not shown any antifungal activity on the fungi tested at the incubation time employed. Considering the results of the antimicrobial activity on solid medium evidenced by the two matrices, the MIC

and MFC of the concentrated extract of the no-autoclaved YMF was determined using the 96-well microplate assay.

As evidenced in Table 2, the MIC of the concentrated extract of YMF (non-autoclaved) ranged from 238.2 (*P. camemberti*) to 15,000 µg/mL (*A. flavus, A. parasiticus* and *A. carbonarius*). Important results of the antifungal activity of the YMF were also evidenced against *P. roqueforti* and *P. digitatum* with MIC data of 476.5 and 937.5 µg/mL respectively. Considering the MFC data, the YMF presented inhibition data that ranged from 1875 (*P. nordicum*, *P. commune* and *P. brevicompactum*) to 15,000 µg/mL (*A. flavus*, *A. parasiticus* and *A. carbonarius).* In general, the *Aspergillus* strains tested were more resistant to the YMF extract in comparison with the employed strains of *Penicillium* and *Fusarium*. Considering the *Penicillium* strains tested, the microorganisms that showed a very low resistance to the mustard extracts tested in this study were, *P. camemberti* and *P. roqueforti*, that showed the lower MIC values.

Considering the other *Penicillium* strains, the MIC values detected for the YMF were always above 500 µg/mL. The lyophilized extract of the YMF was also active against the strain of *F. verticillioides*, a fumonisins (FBs) producer, showing MIC and MFC values of 1875.5 and 3750.5 µg/mL, respectively.

Table 2. MIC and MFC evidenced by the YMF.

Strain	MIC	MFC
	µg/mL	
P. camemberti (CECT 2267)	238.2	1906.2
P. roqueforti (CECT 2905)	476.5	1906.2
P. nordicum (CECT 2320)	937.5	1875.5
P. commune (CECT 20767)	937.5	1875.5
P. brevicopactum (CECT 2316)	937.5	1875.5
P. expansum (CECT 2278)	1875	7500.2
P. chrysogenum (CECT 2668)	937.5	3750.5
P. solitum (CECT 20818)	3750	3750.5
P. digitatum (CECT 2954)	937.5	7500.3
A. parasiticus (CECT 2681)	>15,000	>15,000
A. flavus (ISPA 8111)	>15,000	>15,000
A. carbonarius (ISPA 5010)	>15,000	>15,000
F. verticiloides (ISPA 1044)	1875.5	3750.5

Several authors have tested the application of the ITCs as antimicrobial substances, both directly and using mustard flours. In the first case, Mañes et al. [12] studied the antifungal activity of allyl isothiocyanate (AITC) against two mycotoxigenic strains of the genera *Aspergillus* and *Penicillium*. Tests of antifungal activity in a solid medium showed that 5 µL of AITC deposited inside a disc of sterile filter paper were sufficient to inhibit the growth of the *A.*

parasiticus fungus seeded on the surface of a PDA medium plate. The amount of AITC needed to inhibit the growth of the fungus *P. expansum*was greater, specifically 25 µL/L. Azaiez et al. [13] evaluated the antifungal activity of AITC, phenyl (PITC) and benzyl isothiocyanates (BITC) toward *Fusarium* mycotoxigenic strains. The ITCs employed in the study inhibited the growth of three mycotoxigenic *Fusarium* (*Gibberella moniliformis*), reducing 2.1–89.7% of the mycelium size depending on the time and the dose used (from 10 to 50 µL). The activity of ITCs against non-fungal pathogenic microorganisms transmitted by food has also been studied. Ekanayake et al. [11] demonstrated that ρ-HBIT had significant bactericidal activity against *E. coli*, *S. aureus*, *C. jejuni*, *P. aeruginosa*, *S. enteritidis*, *L. monocytogenes*, *S. boydii* and *C. perfringens* at a dose of 0.35–2.13 mM.

Other studies have demonstrated the antimicrobial capacity of mustard flours directly instead of ITCs. Kanemaru and Miyamoto [14] compared the antimicrobial effects of mustard and purified AITC at equal concentrations of AITC. They found that mustard was more effective against *E. coli* than purified AITC. They found that 0.1% mustard with 9.4 µg/mL of AITC was able to inhibit the growth of *E. coli* in culture medium within 24 h but 12.3 µg/mL of purified AITC was required to achieve the same level of inhibition. Mayerhauser [15] found that retail style mustards eliminated 6 log10 of *E. coli* O157:H7 from trypticase soy broth within a few hours at refrigerator or room temperature. More recently, Rhee et al. [16] showed that mustard flour alone or with acetic acid reduced 6 log10 of *E. coli* O157:H7 to 0.3 log10 in 24 h at room temperature.

2.2. Inhibition of Fungal Growth

The growing demand for safe foods without synthetic chemical preservatives has prompted scientists to investigate the effects of natural compounds against the growth of several pathogenic microorganisms [8,17,18]. The evaluation of the use of YMF in breads inoculated with *A flavus* and *P. nordicum* compared with breads produced with sodium propionate, the classical commercial additive for bakery products, showed that none of the treatments used completely reduced the *A. flavus* and *P. nordicum* growth (Figure 3a,b). However, some preservative treatments applied in this study were able to reduce the fungal growth of the mycotoxigenic fungi tested in comparison to the control experiment.

As shown in Figure 3a, the bread contaminated with *A. flavus*, stored during 10 days and produced with sodium propionate presented a fungal contamination of 6.30 Log/CFU/g and no statistically differences in the microorganism growth were observed in the bread treated with 2 and 4 g/kg of YMF (6.28 and 6.30 Log/CFU/g respectively). In the bread treated with 6 g/kg (6.06 Log/CFU/g) and 8 g/kg (5.78 Log/CFUg) a significant difference on the *A. flavus* was observed in comparison with the bread treated with the E-281, with a reduction of 0.24 and 0.52 Log/CFU/g respectively (47 and 67% CFU/g, respectively).

Related to the results of the *P. nordicum* growth in the loaf bread produced with sodium propionate and with the YMF, the results of the fungal growth along the incubation period are shown in Figure 3b. In particular the bread loaves produced with sodium propionate (at 10 days storage) presented a fungal contamination of 5.82 Log CFU/g, whereas in the bread loaves treated with 2 and 4 YMF, the level of contamination was 6.26 and 5.62 Log CFU/g respectively.

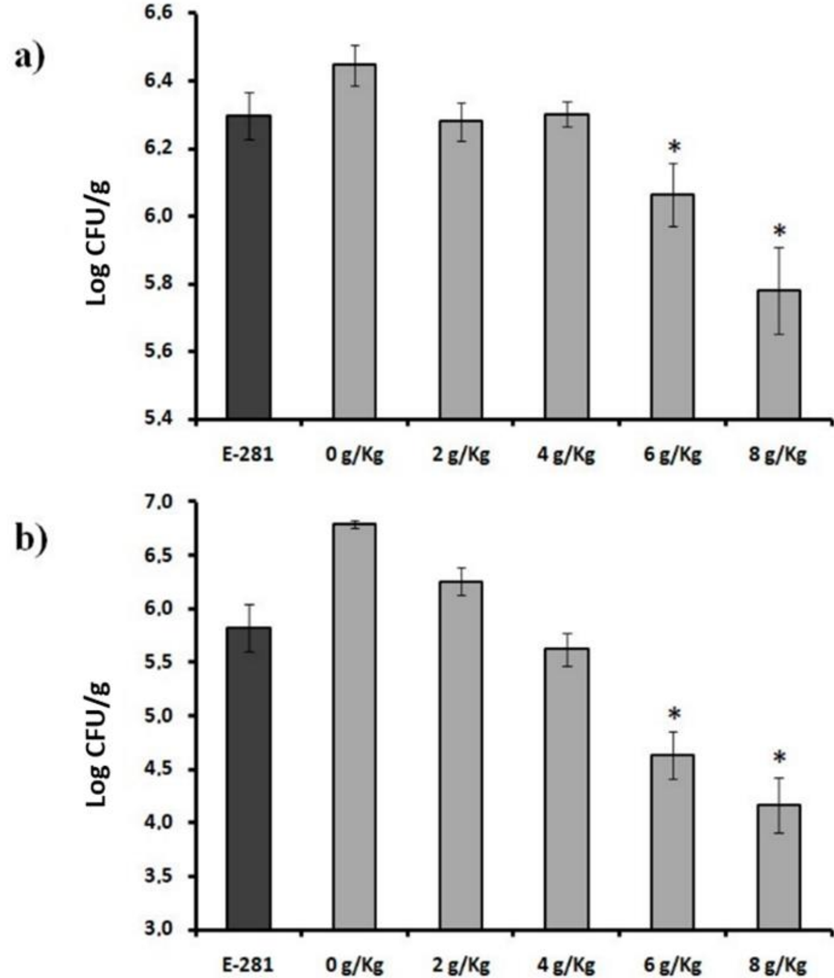


Figure 3. Population of (**a**) *A. flavus* and (**b**) *P. nordicum* in bread loaves treated with natural (YMF) and synthetic commercial (E-281) preservatives. Significantly different from the commercial control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

The bread treated with 6 and 8 g/kg of the YMF presented a statistically difference of *P. nordicum* growth of 4.63 and 4.16 Log CFU/g respectively in

comparison with the bread produced with the E-281, with a reduction of the fungal growth of the 1.19 and 1.66 Log/ CFU/g (94 and 97% CFU/g respectively).

The use of ITCs generated from mustard flours has been studied by several authors. Quiles et al. [19] tested active packaging devices containing AITC or OMF + water to inhibit the growth of *A. parasiticus* in fresh pizza doughs after 30 days of inoculation. The antimicrobial activities were compared with a control group (non-antimicrobial treatment) and a group added with a commercial preservative (sodium propionate). The growth of *A. parasiticus* was inhibited after 30 days with AITC on filter paper at 5 µL/L and 10 µL/L, and on of OMF at 850 mg + 1 mL of water. The use of yellow mustard as an ingredient has been reported for acid food matrices against pathogenic microorganisms. Graumann and Holley [20] demonstrated that the ρ-HBITC generated in situ by including ground yellow mustard powder as an ingredient in dry-fermented sausages inhibited the growth of *E. coli* O157:H7.

2.3. Mycotoxin Reduction

Mean recovery of fortified bread loaves samples (n = 3) at three different levels of AFB_1 and AFB_2 (5, 10 and 15 µg/kg) was of 84.6 ± 3.6% and 88.2 ± 3.3%, respectively. The values obtained for recovery and relative standard deviations of the method used agree with the EU Commission Directive 2006/401/EC for methods to analyze bioactive compounds in foodstuffs [21]. Intra-day (n = 5) and inter-day (5 different days) variation values were 2.5 and 8.6%, respectively. These values are below 15%, which is the maximum variation for certification exercises of bioactive compounds. The detection limit (LOD) and the limit of quantification (LOQ) values were 0.05 and 0.15 µg/kg,

respectively. Linearity, plotted as DAD response area against concentration estimated for the matrix matched standards, and matrix effects were studied using standard solutions and matrix matched calibrations.

Calibration curves were built at eight different mycotoxins levels, from LOQ to 100 times LOQ (from 0.1 to 300 ppm). Each level was prepared in triplicate. Slopes of standard solutions were compared with those obtained in matrix matched standards to assess the possible matrix effect on the chromatographic response. The results obtained showed that the matrix effect calculated for AFB_1 and AFB_2 were of 87.4 and 89.6% respectively.

The analysis of AFs in the bread inoculated with *A. flavus* (Figure 4), evidenced only the production by the mycotoxigenic fungi of the AFB_1. Analyzing the results shown in the figure, the breads produced with 2, 4 and 6 g/kg of YMF and contaminated with *A. flavus*, presented amounts of the AFB_1 not different from the statistical point of view in comparison with the control experiment, whereas in bread loaves treated with 8 g/kg of YMF no AFB_1 concentration was detected, confirming the antifungal potential of the ingredient employed.

The amount of the AFB_1 detected in the control experiment (a loaf of bread produced without any preservative ingredient) was of 5 mg/kg, whereas the loaves treated with sodium propionate showed a level of contamination of AFB_1 of 1.3 mg/kg. The bread loaves treated with 6 g/kg of YMF presented 1.1 mg/kg of the AFB_1 with a percentage of reductions of AFB_1 in comparison with the control of the 78%. Mycotoxin production by *P. nordicum* was not observed in bread loaf contaminated with this fungus.

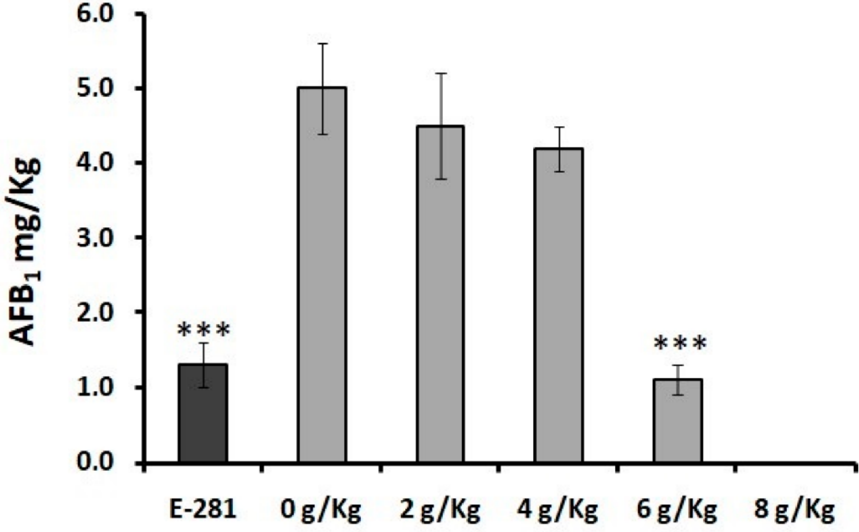


Figure 4. Concentration and reduction rate of AFB_1 present in bread loaves contaminated with *A. flavus* and treated with several preserving treatments (Black = synthetic commercial E-281 preservative, Grey = Different doses of natural YMF) at 10 days of incubation. Significantly different from the bread without treatment, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

Saladino et al. [22] used ITCs derived from OMF and YMF (0.1, 0.5 and 1 g of flour) to avoid the production of AFs in piadina (a typical Italian flatbread) contaminated with *A. parasiticus*. In addition, the antifungal activity of the isothiocyanates toward *A. parasiticus* was also evaluated. The mustard flours employed in this study inhibited the growth of *A. parasiticus*, reducing the mycelium size by 12.2–80.6%. The ITC produced in situ also reduced the AFs biosynthesis in Italian piadina. The use of YMF reduced the AFs content by 41–69.2%. The same authors [23] investigated the use the ITCs generated by the addition of water to OMF and YMF for the reduction of the formation of mycotoxins produced by strains of the genus *Penicillium* as *P. expansum*. The

patulin reduction (PAT) evidenced in the treated samples varied from 80 to 100%.

Hontanaya et al. [24] studied the reduction of AFs produced by *A. parasiticus* in nuts using ITCs (AITC and p-hydroxy benzyl isothiocyanate (ρ-HBITC)) produced by the enzymatic hydrolysis of glucosinolates sinigrin (SG) and sinalbin (SA) present in OMF and YMF. The reduction of AFB_1, B_2, G_1 and G_2 observed ranged from 83.1 to 87.2% and from 27.0 to 32.5% after nuts exposure to AITC and ρ-HBITC respectively.

2.4. Shelf Life Analysis

Considering the results of the shelf life improvement of the bread loaves treated with the sodium propionate and with different concentrations of YMF, it's possible to underline several important data.

In particular, considering the results on the mycotoxigenic fungi *A. flavus*, the control bread (bread produced with any preservative compound or ingredient) showed a visible fungal growth at 2 days of incubation (Table 3a), whereas the bread treated with sodium propionate (E-281), presented an evidence of the fungal growth at 3 days of incubation. The bread loaves produced with 2 and 4 g/kg of the YMF presented the same pattern of the *A. flavus* growth recorded for the experiment carried out with the E-281. The bread loaves produced with 8 g/kg of YMF, did not shown any fungal growth during the incubation period used in this study.

Table 3. Shelf life, monitored in days, of the bread loaves treated with 4 different concentrations of the YMF and contaminated with (**a**) *Aspergillus flavus* (ISPA 8111) and (**b**) *Penicillium nordicum* (CECT 2320), in comparison with the commercial control produced with the additive E-281, and with a loaf bread prepared without any antimicrobial treatment.

a)

Treatment	Days									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
E-281	–	–	+	+	+	+	+	+	+	+
YMF 0 g/kg	–	+	+	+	+	+	+	+	+	+
YMF 2 g/kg	–	–	+	+	+	+	+	+	+	+
YMF 4 g/kg	–	–	+	+	+	+	+	+	+	+
YMF 6 g/kg	–	–	-	+	+	+	+	+	+	+
YMF 8 g/kg	–	–	–	–	–	–	–	–	–	–

b)

Treatment	Days									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
E-281	–	–	–	–	+	+	+	+	+	+
YMF 0 g/kg	–	+	+	+	+	+	+	+	+	+
YMF 2 g/kg	–	+	+	+	+	+	+	+	+	+
YMF 4 g/kg	–	–	–	+	+	+	+	+	+	+
YMF 6 g/kg	–	–	–	–	–	–	–	–	–	–
YMF 8 g/kg	–	–	–	–	–	–	–	–	–	–

Considering the shelf life improvement observed in the bread loaves contaminated with *P. nordicum* and treated with the antimicrobial ingredients tested (Table 3b), the bread produced with E-281, presented a visible fungal growth at 5 days of incubation, while the control bread and the loaves produced with 2 and 4 g/kg of YMF, presented a microbial growth at 2 and 4 days respectively. In the breads baked using 6 and 8 g/kg of YMF, it was not observed any *P. nordicum* growth during the incubation period used (Figure 5).

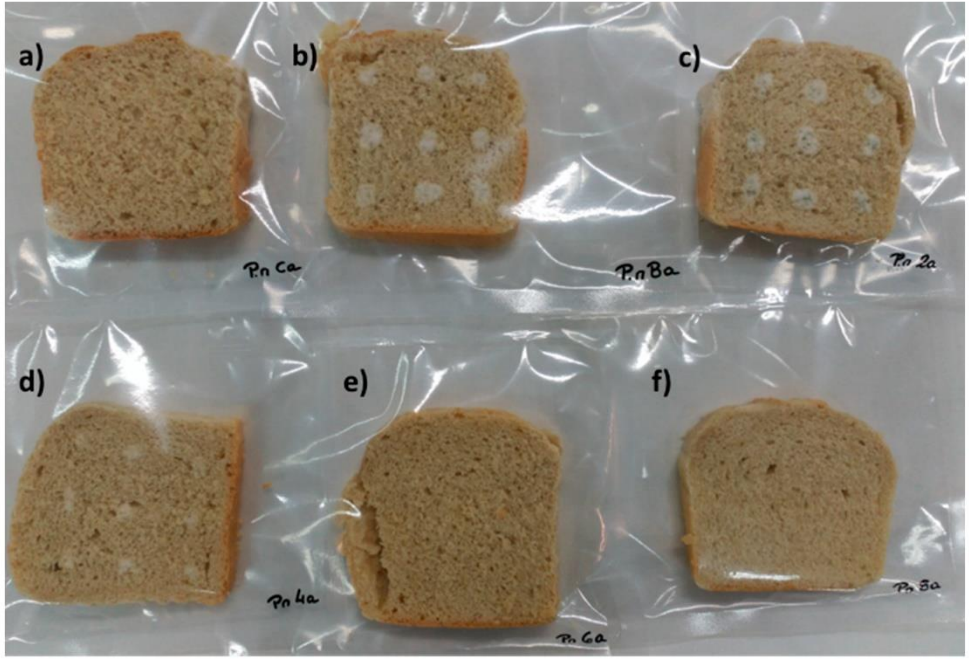


Figure 5. Bread loaves contaminated with *P. nordicum* and treated with (**a**) 2.0 g/kg of sodium propionate (E-281); (**b**) not treated; (**c**) 2 g/kg of YMF; (**d**) 4 g/kg of YMF; (**e**) 6 g/kg of YMF and (**f**) 8 g/kg of YMF, after 10 days of incubation.

3. Materials and Methods

3.1. Chemicals and Microorganisms

AFs B_1, B_2, G_1, G_2, sinigrin (98% purity), formic acid (HCOOH), sodium propionate, tetrabutylammonium hydrogen sulfate (TBA), ammonium formate, and sodium chloride (NaCl) were obtained from Sigma Aldrich (St. Louis, MO, USA). YMF and OMF were provided by G.S. Dunn Dry Mustard Millers (Hamilton, ON, Canada). Methanol was purchased from Fisher Scientific (Rockingham County, NH, USA). Deionized water (<M18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., Danbury, CT, USA) ultrasonic bath. The strains of *Aspergillus parasiticus*CECT 2681, *Penicillium camemberti* CECT 2267, *Penicillium roqueforti* CECT 2905, *Penicillium nordicum* CECT 2320, *Penicillium commune* CECT 20767, *Penicillium brevicopactum* CECT 2316, *Pencillium expansum* CECT 2278, *Penicillium chrysogenum* CECT 2668, *Penicillium solitum* CECT 20818, *Penicillium digitatum* CECT 2954 and *Fusarium graminearum*CECT 20486 were obtained from the the Spanish Type Culture Collection (CECT, Valencia, Spain). The strains of *Aspergillus flavus* ISPA 8111, *Aspergillus carbonarius* ISPA 5010 and *Fusarium verticiloides* ISPA 12044 were obtained from the Institute of Sciences of Food Production (ISPA—CNR, Bari, Italy). Buffered peptone water, potato dextrose agar (PDA) and potato dextrose broth (PDB) were acquired from Oxoid (Madrid, Spain).

3.2. Extraction of the Water-Soluble Components from Mustard Flours

The water-soluble components of YMF and OMF were extracted using the method of Hontanaya et al. [24] with some modifications. Flour (5 g) was placed in a 50 mL glass tube and autoclaved at 115 °C for 15 min to inactivate the enzyme myrosinase. This matrix was used to confirm that the possible antimicrobial activity of the mustard flours was due to the ITCs. Experiments with untreated mustard flours were carried out directly using the flour matrices. 25 mL of distilled water was introduced into the same tube and the extraction was carried out using an UltraTurrax T18 basic mixer (Ika, Staufen, Germany) for 3 min at 11,000 rpm. The mixture was centrifuged at 2500 rpm for 5 min at 4 °C and filtered through a filter paper (Whatman No. 4) in 50 mL tubes with screw cap. Finally, the extract was filtered again through a 0.22 µM filter and kept under refrigeration at 4 °C. To test also the antimicrobial activity of the concentrated mustard extracts, half of them were lyophilized by depositing 25 mL of extract in 100 mL plastic containers in a Virtis SP SCIENTIFIC Sentinel 2.0 lyophilizer (Warminster, PA, USA).

3.3. Antifungal Activity Tests on Solid Medium

The method used to evaluate the antimicrobial activity of the components of water-soluble mustard meal was as follows. The lyophilized extracts of YMF and OMF were previously suspended in 1 mL of sterile water obtaining a concentrated extract. The fungi to be analyzed were grown on the surface with a cotton swab in 9 mm Petri dishes prepared with 20 mL of PDA. In each plate, 10 µL in surface and 100 µL in pre-prepared agar wells were added. The triplicates of each plate were incubated at 28 °C observing the development of fungal growth at 48 h.

3.4. Antifungal Activity Tests on Liquid Medium

The assay was performed in 96-well sterile microplates, using the modified method of Siah et al. [25]. The tests of antifungal activity in liquid medium were not carried out with OMF due to the results obtained in the tests in solid medium. The first column served as control of the medium. For this, 200 µL of PDB were added in each of the wells to verify the absence of contamination of the medium. The next column consisted of a control of the microorganism, to verify its viability, so 100 µL of PDB was deposited. The remaining (3–12) contained 100 µL of lyophilized YMF extract resuspended at doses between 30 to 15,000 ppm. Each well was inoculated with 100 µL of a 5×10^4 spores/mL suspension in PDB of the mycotoxigenic fungi described in the paragraph 2.1. The negative control consisted of inoculated medium without any treatment. The plates were incubated at 25 °C for 72 h in the dark. Four wells were used for each assay of each fungus (2 fungi per 96-well plate) and the experiments were performed in triplicate.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the YMF extract, where the fungi did not show any visible growth. For the determination of the minimum fungicidal concentration (MFC), after determining the MIC, the concentrations corresponding to the inhibitory and to higher concentrations, as well as the controls, were subcultured on PDA plates. After 72 h of incubation at 25 °C, MFCs' readings were made being MFC the lowest extract concentration in which a visible growth of the subculture was prevented.

3.5. Baking and Bread Treatment

The bread recipe included 600 g of wheat flour, 20 g of sucrose, 10 g of NaCl, 40 g of yeast for bakery products (Levital, Spain) and 250 mL of tap water. The commercial control was prepared adding to the ingredients sodium propionate (E-281) at 2 g/kg that is a common preservative used for loaf bread production in Spain. The studied breads were produced adding to the basic ingredients YMF at the concentration of 0, 2, 4, 6 and 8 g/kg of dry ingredient in the dough. The ingredients were kneaded manually for 10 min and the dough produced was left rising for 1h at room temperature. Baking was performed at 230 °C for 30 min in a deck oven (MIWE, Arnstein, Germany). The oven was pre-steamed (300 mL of water) before cooking. The loaves were kept for 30 min on cooling racks at room temperature. Loaves were cut in slices of 30 g each. The slices were inoculated with 100 µL of a suspension containing 1×10^5 conidia/mL of *A. flavus* or *P. nordicum*. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^5 conidia/mL in PDB as reported by Kelly et al. [26] and introduced in 1 L plastic trays. All plastic trays were closed hermetically and incubated at room temperature for 10 days. Each day until the analysis the bread slices were examined to determine the visible fungal growth and the shelf life evaluation. Then, all packages were opened, and samples contaminated with *A. flavus* were used to determine the AFB_1 content by liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS). Each bread was made in triplicate and from each bread 3 slices were analyzed (n = 9).

3.6. Aflatoxins Extraction

AFs extraction was performed using the method described by Hontanaya et al. [24] Briefly, the bread slices were finely grounded with a blender (Oster

Classic grinder, Oster, Valencia, Spain) and 5 g samples were placed in a 50 mL plastic tube. Then, 0.5 g of sodium chloride (NaCl) and 25 mL of a methanol/water (80:20, *v*/*v*) mixture were added. Samples were homogenized using an Ultra Ika T18 basic UltraTurrax (Staufen, Germany) for 3 min. The mixture was centrifuged at 4500 rpm for 5 min and the supernatant was evaporated to dryness with a Rotavapor R-200 (Büchi, Postfach, Switzerland). The residue was re-dissolved in 1 mL of extraction solvent, filtered through a 0.22 µm syringe filter and injected to the LC-MS/MS system.

Commission Decision 2002/657/EC [27] and 401/2006/EC [21] were used as guidelines for the validation studies. All the parameters were evaluated by spiking blank samples (5, 10 and 15 µg/kg of AFB_1 and AFB_2), which were left to equilibrate overnight before the analysis. For identification purposes, retention time of compound in standards and samples were compared at tolerance of 70.5%. Method performance characteristics such as linearity, limits of detection (LOD), limits of quantitation (LOQ), matrix effect, recovery, repeatability and reproducibility were evaluated for sinigrin.

3.7. AFs Identification and Quantification by LC-MS/MS

LC-MS/MS analyses were performed with a system consisting of Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP mass spectrometer (Applied Bio-systems, AB Sciex, Foster City, CA, USA) equipped with a turbo ionspray electrospray ionisation (ESI) interface. The instrument data were collected and processed using the Analyst version 1.5.2 software (AB Sciex, Foster City, CA, USA). Separation of analytes was performed using a reversed-phase analytical column (Gemini C18 column, 150 × 2 mm, I.D.

3 µm particle size), equipped with a security guard cartridge C18 (4 × 2 mm, I.D.; 5 µm) all from Phenomenex (Madrid, Spain). The mobile phases used were: Water with 0.1% of formic acid and 5 mM ammonium formate (Phase A) and methanol with 0.1% of formic acid and 5 mM ammonium formate (Phase B). The elution gradient was established initially with 10% eluent B. It was increased to 80% in 1.5 min and was kept constant until 4th min. Then it increased to 90% for 6 min. Subsequently, it was increased to 100% until the 14th minute and then reduced to 50% at the minute 17. Afterwards the initial conditions were maintained for 5 min. The flow rate was 0.25 mL min and MS/MS were achieved in the selected reaction monitoring (SRM) mode using ESI in positive mode. For LC-MS/MS analysis, scheduled SRM was used with a 120 s SRM detection window and 1 s of target scan time. The applied parameters were: ion spray voltage, 5500 V; source temperature,450 °C; curtain gas, 20; ion source gas 1 (sheath gas), 50 psi; ion source gas 2 (drying gas), 55 psi. Nitrogen served as nebulizer and collision gas. The ionization and fragmentation parameters used for the detection and quantification of the AFs were set according to Liu et al [16].

3.8. Determination of the Fungal Population

After incubation, each slice was weighed and transferred to a sterile plastic bag with sterile peptone water (Oxoid) in a 1:10 dilution and homogenized with a stomacher (IUL, Barcelona, Spain) during 30 s. The mixture was serially diluted in sterile plastic tubes containing 0.1% peptone water. Aliquots of 100 µl were seeded in acidified PDA (pH 3.5) (Insulab, Valencia, Spain) and the plates were incubated at 25 °C for 7 days before the microbial count [17].

4. Conclusions

The present study demonstrated the capacity of YMF extracts to inhibit or reduce the growth of several fungal strains belonging to *Aspergillus*, *Penicillium* and *Fusarium* species. Moreover, YMF used as an ingredient in bread preparation can significantly retard and reduce *A. flavus* and *P. nordicum* growth, improve the shelf life of the bread and reduce AFB_1 naturally produced on substrate by *A. flavus*, so this study shows that YMF can be potentially used as natural preservative for bakery products which are commonly contaminated by *Aspergillus* and *Penicillium* species. YMF could be a potential substitute to conventional preservatives used in bread satisfying the growing demand of the consumer for natural products free from common chemical preservatives.

References

1. EFSA-European Food Safety Authority. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *EFSA J.* **2007**, *446*, 1–127.
2. Peterson, S.W.; Ito, Y.; Horn, B.W.; Goto, T. *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *Aspergillus nomius*. *Mycologia* **2001**, *93*, 689–703.
3. Coulombe, R.A. Aflatoxins. In *Mycotoxins and Phytoalexins*; Sharma, R.P., Salunkhe, D.K., Eds.; CRC Press: Boca Raton, FL, USA, 1991; pp. 103–143.
4. Amano, K. Monographs on the evaluation of carcinogenic risks to humans. In *A Review of Biological Agents for Human Carcinogens*; International Agency for Research on Cancer: Lyon, France, 2012.
5. Pal Vig, A.; Rampal, G.; Thind, T.S.; Arora, S. Bio-protective effects of glucosinolates. *LWT Food Sci. Technol.* **2009**, *42*, 1561–1572.

6. Tunc, S.; Chollet, E.; Chalier, P.; Preziosi-Belloy, L.; Gontard, N. Combined effect of volatile antimicrobial agents on the growth of *Penicillium notatum*. *Int. J. Food Microbiol.* **2007**, *113*, 263–270.
7. Sellam, A.; Iacomi-Vasilescu, B.; Hudhomme, P.; Simoneau, P. In vitro antifungal activity of brassinin, camalexin and two isothiocyanates against the crucifer pathogens *Alternaria brassicicola* and *Alternaria brassicae*. *Plant Pathol.* **2007**, *56*, 296–301.
8. Nadarajah, D.; Han, J.H.; Holley, R.A. Inactivation of *Escherichia coli* O157:H7 in packaged ground beef by allyl isothiocyanate. *Int. J. Food Microbiol.* **2005**, *99*, 269–279.
9. Lara-Lledó, M.; Olaimat, A.; Holley, R.A. Inhibition of *Listeria monocytogenes* on bologna sausages by an antimicrobial film containing mustard extract or sinigrin. *Int. J. Food Microbiol.* **2012**, *156*, 25–31.
10. Chen, W.; Jin, T.Z.; Gurtler, J.B.; Geveke, D.J.; Fan, X. Inactivation of *Salmonella* on whole cantaloupe by application of an antimicrobial coating containing chitosan and allyl isothiocyanate. *Int. J. Food Microbiol.***2012**, *155*, 165–170.
11. Ekanayake, A.; Kester, J.J.; Li, J.J.; Zehentbauer, G.N.; Bunke, P.R.; Zent, J.B. Isogard™: A natural anti-microbial agent derived from white mustard seed. *ActaHorticulturae* **2006**, *709*, 101–108.
12. Mañes, L.; Luciano, F.B.; Mañes, J.; Meca, G. In vitro antifungal activity of allyl isothiocyanate (AITC) against *Aspergillus parasiticus* and *Penicillium expansum* and evaluation of the AITC estimated daily intake. *Food Chem. Toxicol.* **2015**, *83*, 293–299.
13. Azaiez, I.; Meca, G.; Manyes, L.; Luciano, F.B.; Fernández-Franzón, M. Study of the cemical reduction of the fumonisins toxicity using allyl, benzyl and phenyl isothiocyanate in model solution and in food products. *Toxicon* **2013**, *63*, 137–146.
14. Kanemaru, K.; Miyamoto, T. Inhibitory effects on the growth of several bacteria by brown mustard and allylisothiocyanate. *Nippon Shokuhin Kogyo Gakkaishi* **1990**, *37*, 823–829. (In Japanese)
15. Mayerhauser, C.M. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in retail mustard. *J. Food Prot.***2001**, *64*, 783–787.

16. Rhee, M.S.; Dougherty, R.H.; Kang, D.H. Combined effects of mustard flour, acetic acid, and salt against *Escherichia coli* O157:H7 stored at 5 and 22 °C. *J. Food Prot.* **2002**, *65*, 1632–1635.
17. Luciano, F.B.; Belland, J.; Holley, R.A. Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder to ward *Escherichia coli* O157:H7 during dry sausage ripening. *Int. J. Food Microbiol.* **2011**, *145*, 69–74.
18. Cordeiro, R.P.; Luciano, F.B.; Holley, R.A. Evaluation of deodorized yellow mustard concentrations for control of *Escherichia coli* O157:H7 viability in dry fermented sausage. *Food Control* **2013**, *33*, 20–24.
19. Quiles, J.M.; Manyes, L.; Luciano, F.; Manes, J.; Meca, G. Antimicrobial compound allylisothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust. *Food Chem. Toxicol.***2015**, *83*, 222–228.
20. Graumann, G.; Holley, R.A. Inhibition of *E. coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. *J. Food Prot.* **2008**, *71*, 486–493.
21. European Union. European Commission, Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union* **2006**, *L70*, 12e34.
22. Saladino, F.; Bordin, K.; Manyes, L.; Luciano, F.B.; Manes, J.; Fernandez-Franzon, M.; Meca, G. Reduction of the aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina by isothiocyanates. *LWT Food Sci. Technol.* **2016**, *70*, 302–308.
23. Saladino, F.; Manyes, L.; Luciano, F.B.; Manes, J.; Fernandez-Franzon, M.; Meca, G. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. *LWT Food Sci. Technol.* **2016**, *66*, 101–107.
24. Hontanaya, C.; Meca, G.; Luciano, F.B.; Manes, J.; Font, G. Inhibition of the Aflatoxins B_1, B_2, G_1, and G_2, production by *Aspergillus parasiticus* in nuts using yellow and oriental mustard flours. *Food Control* **2015**, *47*, 154–160.
25. Siah, A.; Deweer, C.; Morand, E.; Reignault, P.; Halama, P. Azoxystrobin resistance of French *Mycosphaerella graminicola* strains assessed by four in vitro bioassays and byscreening of G143A substitution. *Crop Prot.* **2010**, *29*, 737–743.

26. Kelly, S.; Grimm, L.H.; Bendig, C.; Hempel, D.C.; Krull, R. Effects of fluid dynamic induced shear stress on fungal growth and morphology. *Process. Biochem.* **2006**, *41*, 2113–2117.
27. European Union. European Commission, Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Union* **2002**, *L221*, 8e34.

Toxins 2019, 11(3), 137

3.5. Development of an antifungal and antimycotoxigenic device containing allyl isothiocyanate for silo fumigation

Juan Manuel Quiles[1, †], Tiago de Melo Nazareth[1,2, †], Carlos Luz[1], Fernando Bittencourt Luciano[2], Jordi Mañes[1], Giuseppe Meca[1]*

[1]*Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain*

[2]*School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil*

† **Authors have contributed equally to the study and should be considered co-first authors.**

1. Introduction

AFs are the foremost harmful category of mycotoxins naturally produced by *Aspergillus* species such as *Aspergillus nomius, Aspergillus flavus*, and *Aspergillus parasiticus* during pre-or postharvest of crops [1,2]. The most important and toxic aflatoxins are the AFB_1, AFB_2, AFG_1, and AFG_2 [3]. Among these compounds, the AFB_1 has been classified in Group 1 of the risk of the carcinogen molecules by the International Agency for Research on Cancer [4], and it has been implicated with the development of human hepatic and extra hepatic carcinogenesis [5]. Human exposure to AFs could be due to the intake of contaminated food or by the consumption of milk, meat, and eggs from animals that consumed contaminated feed [6]. The occurrence of AFs in foods from in the Spanish market has been previously reported in several products, such as cereals, pulses, dried fruits and nuts, snacks, breakfast cereals, bread, herbs, or spices [7,8].

OTA is one of the most dangerous mycotoxins and is produced by *Aspergillus* and *Penicillium* species, among which are *Aspergillus ochraceus* [9], *Aspergillus carbonarius* [10], and to a lesser extent *Aspergillus niger* [11] and *Penicillium verrucosum* species [12]. The contamination of food by the presence of OTA is common in Europe. In more than 50% of the 6476 foods analyzed, OTA amounts were detected above the detection limit of 0.01 mg/kg [13]. IARC considers OTA as a possibly carcinogenic compound in humans (Group 2B) [4]. OTA dietary average for citizens of the European Union has been experimentally established in a range of 0.9 (Germany) to 4.6 (Italy) ng/kg. Foods with the greatest OTA contamination are coffee, cereals, spices, and beer [14,15]. When these contaminated foods are ingested, OTA can cause a nephrotoxic, hepatotoxic, and teratogenic effects [16,17].

Methods for controlling mycotoxins are usually preventive, including good agricultural practice and drying of crops after harvest. Some researchers have reported that mycotoxins can be degraded by heat treatment, but the extent of mycotoxin degradation is dependent on temperature, time of exposure, and mainly the contamination level [18]. OTA is a stable molecule, which can resist roasting, brewing, baking, ammoniation, and heat treatment to some extent [19]. Likewise, AFB_1seems to be stable up to 150 °C [20]. For this reason, other methods of detoxification have been developed to prevent these mycotoxins in food and feed.

Isothiocyanates (ITCs) are products originated from the enzymatic hydrolysis of glucosinolates, which are sulfur-containing glucosides present in plants of the *Brassicaceae* family. These compounds contribute to the characteristic pungent taste of these vegetables [20] and have been reported as potent antimicrobials [21]. Allyl isothiocyanate (AITC), which is the most studied ITCs, was found to inhibit the growth of yeast, mold, and bacteria at very low levels [22], including molds from the genera *Aspergillus*, *Penicillium*, and *Fusarium* [23,24]. ITCs are characterized by the presence of a –N.C.S group, whose central carbon atom is strongly electrophilic [25]. This electrophilic nature enables ITC to readily bind to thiol and amino groups of amino acids, peptides, and proteins, forming conjugates [21], dithiocarbamate, and thiourea structures [26]. OTA contains a free and readily available amino group and AFs contains a carboxylic group. Therefore, ITCs could be good candidates to react with these mycotoxins.

The objective of this research was to investigate the efficacy of an antifungal device based on the natural compound AITC to reduce the growth of *A.*

flavus and *P. verrucosum* in cereals during storage and the mycotoxin production.

2. Results

2.1. AITC Concentration in Headspace and Cereals in Laboratory Scale Silo

Figure 1 shows the AITC present in the headspace of laboratory scale silos. The AITC concentration decreases gradually from 0.92 µL/L at day 1 to 0.25 µL/L at day 30. On the other hand, there was no significant difference in AITC concentration after day 7 up to day 30. These results suggest that either a fraction of AITC left the silo or it was absorbed to grains releasing a constant average concentration ranging from 0.37 to 0.25 µL/L for 30 days.

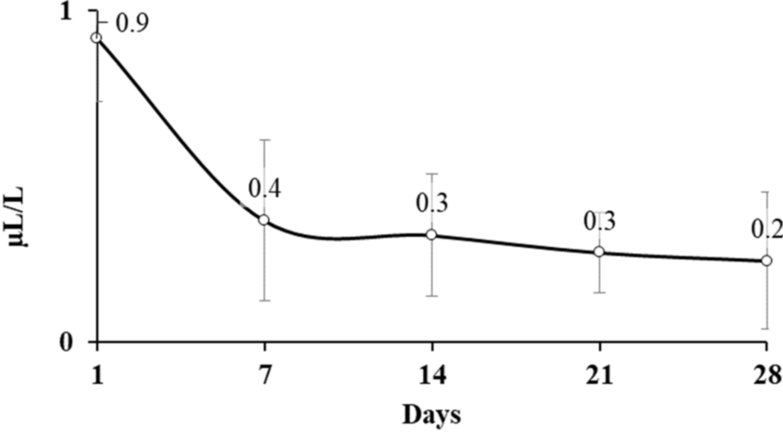


Figure 1. AITC detected in the headspace of the glass jar containing corn, wheat, and barley, used to simulate the storage of the cereals in a lab scale silo system.

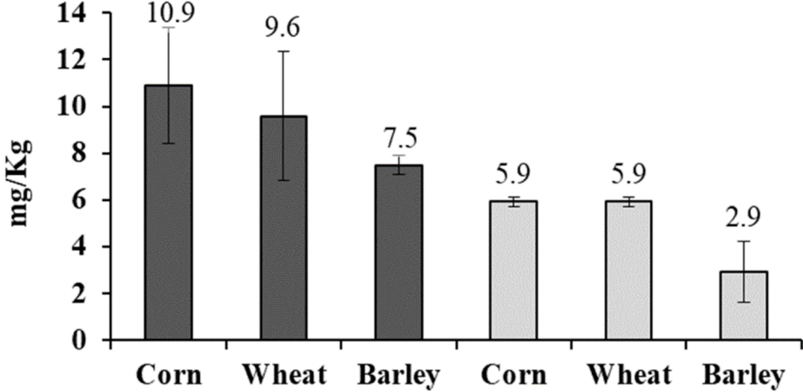


Figure 2. Concentrations of AITC detected in corn, wheat, and barley after 1 (gray) and 30 (white) days of incubation.

The residual concentration of AITC in barley, corn, and wheat was studied on day 1 and 30, and the results are shown in Figure 2. Corn was the most susceptible matrix to AITC penetration, showing 10.9 and 5.9 mg/kg of AITC at days 1 and 30, respectively. Barley grains showed a lower capacity to maintain AITC with 7.5 and 2.9 mg/kg absorbed at days 1 and 30, respectively. Wheat grains did not show a significant difference to barley and corn at day 1, with 9.6 mg/kg of AITC. However, at day 30, wheat grains showed 3 mg/kg more concentration of AITC than barley samples.

2.2. Validation Method for the Analysis of Mycotoxins in Cereals

To validate the analytical method, the following parameters such as linearity, recovery, repeatability, reproducibility, limits of detection (LOD) and quantification (LOQ), and the matrix effect for each mycotoxin analyzed were carried out. All the mycotoxins showed good linearity in the working range, with resolution determination coefficients (R2) greater than 0.9922.

Linearity was evaluated using paired matrix calibrations in triplicate at concentrations between 5 and 500 µg/kg. To calculate the matrix effect, the calibration slope from the matrix calibration curve was divided by the slope of the standard calibration curve and multiplied by 100. The value of the recovery was carried out in triplicate for three consecutive days using three addition levels: LOQ, 2 × LOQ, and 10 × LOQ.

The results were between 70.4% and 75.6% and the relative standard deviation (RSD) was less than 17%. The values for intraday repeatability (n = 3), expressed as the relative standard deviation of the repeatability (RSDr), varied from 7.5% to 11.6%; and the reproducibility between days (n = 5), expressed as the relative standard deviation of the reproducibility (RSDR), varied from 8.2% to 17.3% for the same linearity addition values. LODs and LOQs were calculated by analyzing blank samples enriched with the standard mycotoxins; these parameters have been assessed as the lowest concentration of the molecules studied that showed a chromatographic peak at a signal-to-noise ratio (S/N) of 3 and 10 for LOD and LOQ, respectively (Table 1).

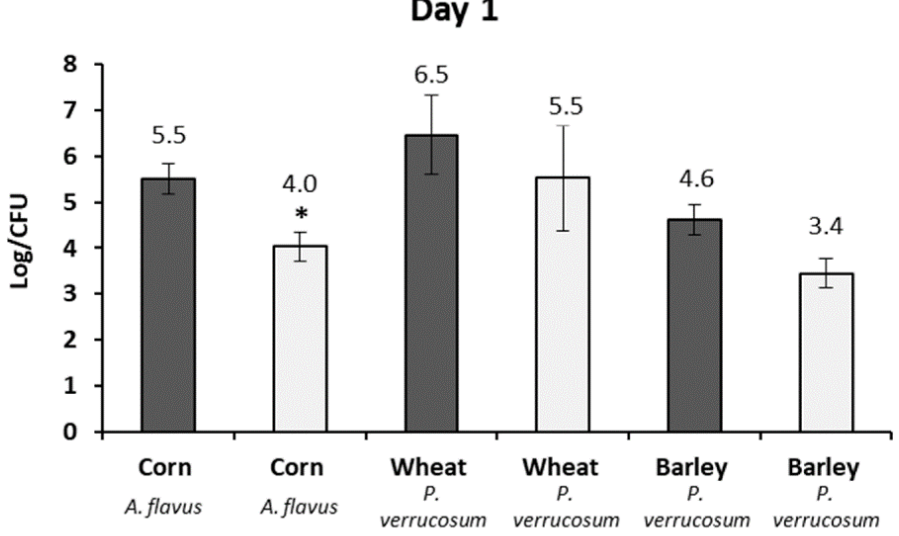
Table 1. LODs, LOQs, recovery, and matrix effect (ME) (%) for AFB_1, AFB_2, AFG_1, AFG_2, and OTA in corn, wheat, and barley.

Mycotoxin	**LOD (µg/Kg)**	**LOQ (µg/Kg)**	**Recovery (%)**	**ME (%)**
AFB_1	0.08	0.27	70.4	78.2
AFB_2	0.08	0.27	64.2	76.5
AFG_1	0.16	0.53	62.8	65.3
AFG_2	0.30	1.00	66.1	60.9
OTA	0.05	0.17	75.6	89.7

2.3. Fungal Growth and Mycotoxin Production in Lab Scale Silo System

The results of *A. flavus* and *P. verrucosum* growth on barley, corn, and wheat at days 1 and 30 are shown in Figure 3. At day 1, AITC treatment demonstrated a significant reduction in the fungal population of corn, wheat, and barley, reducing in 1.5, 1, and 1.2 log CFU/g, respectively. After 30 days of storage, the fungus growth in the control groups remained stable. However, the treatment reduced the population of fungi in wheat and barley to levels below our limit of detection, while in the corn the reduction was of 4.4 log CFU/g.

In correlation to fungal growth, the mycotoxin production was determined after 30 days of storage and the results are shown in Figure 4. *A. flavus* produced in the control corn 8.07 μg/Kg of AFB_1 at day 30. This value is above the limit of AFB_1 in foodstuffs set by the European Commission (EC 165/2010). Therefore, this cereal is classified as inappropriate for human consumption. The AFB_1 present in the treated corn was 0.12 μg/Kg, representing a reduction of 98.51%. Regarding the values of OTA, the reduction was not significant in barley, whereas in the wheat samples the OTA was not produced even in the control group, reaching values below our limit of detection.



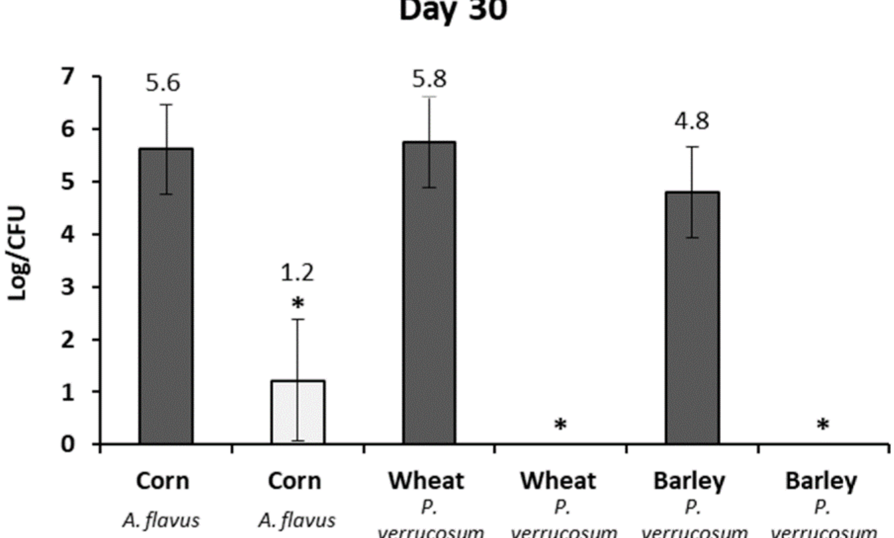


Figure 3. Growth, in lab scale silo system, of the *A. flavus* in corn and of *P. verrucosum* in wheat and barley exposed to the vapor of the AITC after 1 and 30 days of incubation. Samples control (dark gray) and treated samples (clear gray). Significantly different from untreated cereal, $p \leq 0.05$ (*).

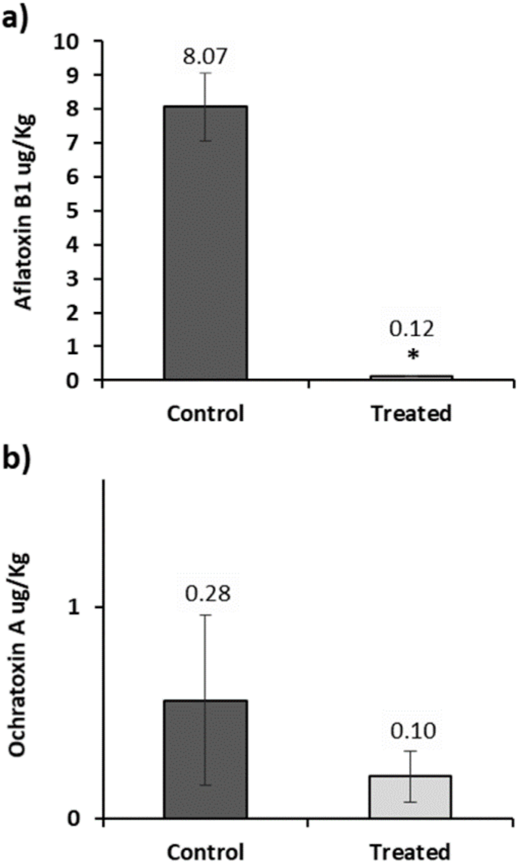


Figure 4. Aflatoxin B_1 detected in corn a); and ochratoxin A detected in barley b) treated with the AITC device in the lab scale silo system at 30 days of incubation. Samples control (dark gray) and treated samples (clear gray). Significantly different from untreated cereal, $p \leq 0.05$ (*).

2.4. Fungal Growth and Mycotoxin Production in a Small-Scale Silo System

After laboratory scale analysis in silos of 100 L containing 50 Kg of cereal. Barley, corn, and wheat were contaminated and then treated with a gel dispositive developed with 5 mL of AITC. The sampling was realized monthly and the results for microbiological analysis are shown in Figure 5.

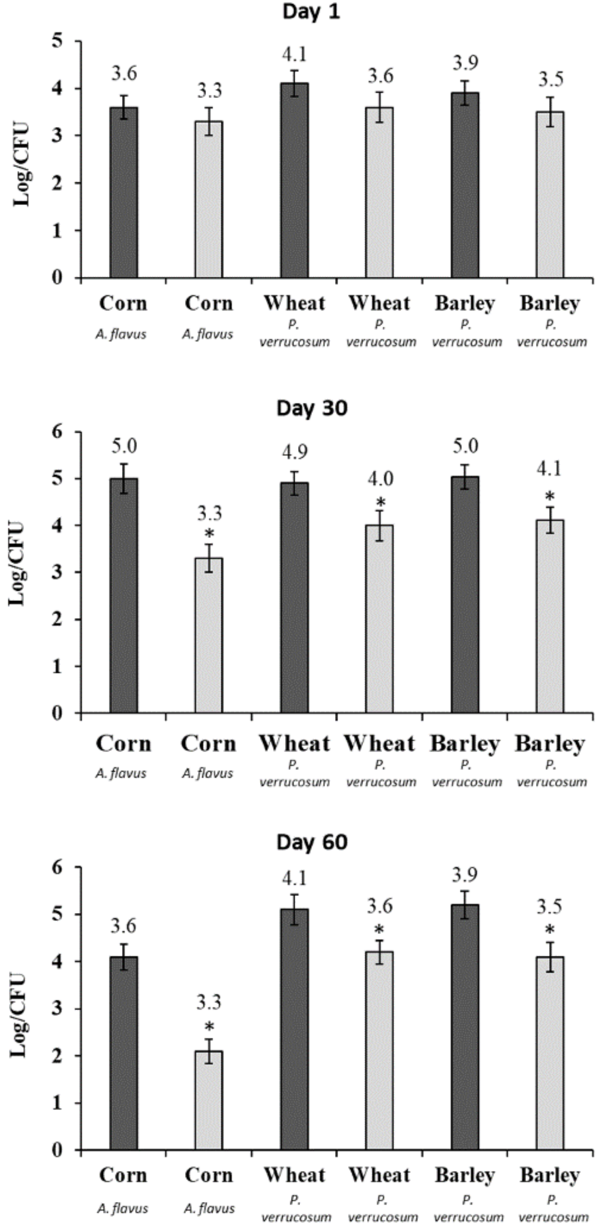


Figure 5. Growth, in small-scale silo system, of the *A. flavus* in corn and of *P. verrucosum* in wheat and barley exposed to the vapor of the AITC after 1, 30, and 60 days of incubation. Control (dark gray) and treated samples (light gray). Significantly different from untreated cereal, $p \leq 0.05$ (*).

At day 1, AITC treatment did not demonstrate a significant reduction in fungal population in corn, wheat, and barley. Similarly, to the laboratory scale silo, after 30 days of storage, the treatment with AITC was able to reduce significantly the fungal growth of *A. flavus* in corn and *P. verrucosum* in barley and wheat. In addition, at the end of the experiment (after 60 days of storage), AITC treatment demonstrated a significant reduction in the fungal population of corn, wheat, and barley, reducing in 2, 0.9, and 1.1 log CFU/g in comparison to the control group, respectively (Figure 5). Along with the fungal growth, the production of mycotoxins was determined at days 30 and 60 of storage (Figure 6).

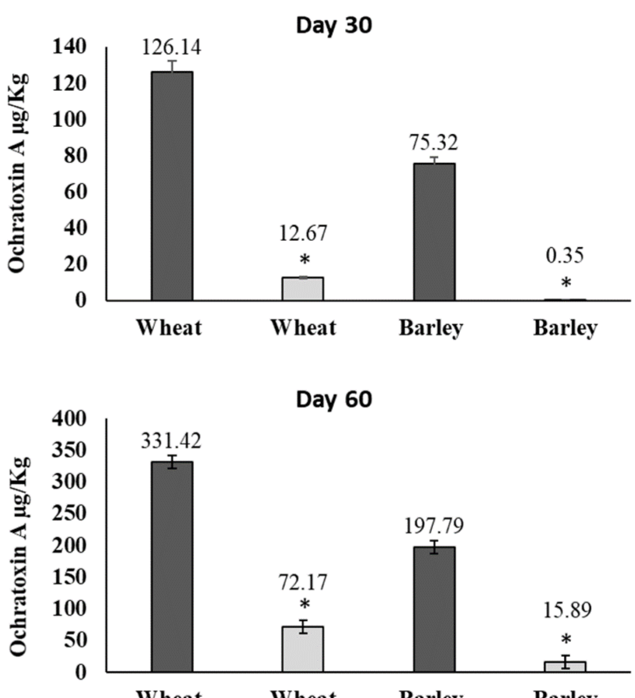


Figure 6. Ochratoxin A detected in wheat and barley treated with the AITC device in the small-scale silo system at 30 and 60 days of incubation. Samples control (dark gray) and treated samples (light gray). Significantly different from untreated cereal, $p \leq 0.05$ (*).

No AFs could be detected in corn contaminated with *A. flavus*. In the samples of wheat and barley contaminated with *P. verrucosum*, the OTA reduction was 90.0% and 99.5% for day 30 and 78.2% and 92.0% for day 60, respectively.

3. Discussion

Similarities and differences were identified comparing the results of the lab scale silo system with the results of small-scale silo system. Microbiologically, the exposure to AITC of cereals contaminated with *A. flavus* (corn) and *P. verrucosum* (wheat and barley) reduced, in all experiments, significantly the fungal population after 30 days. However, due to the lower concentration of AITC (50 µL/L) and the micro atmospheres generated by small-scale silo system, the AITC device could not completely inhibit the fungal growth when compared to the lab scale silo system results. In other words, a lower dose of AITC and higher headspace in the small silo system did not reduce the fungal population to the values below to our limit of detection (1.2 logs CFU/g). These results suggest that the effect of AITC is dose depending. In addition, the higher the grain volume, the higher should be the AITC concentration to achieve a total inhibitory effect.

Regarding the production of mycotoxins, differences between the two tests were observed, probably since a moderate inoculum was used to replicate actual contamination conditions of the field. Another difference among the experiments was the reduction of the potential maximum concentration of AITC in the headspace (500 µL/L in the lab silo and 50 µL/L in the small silo) due to issues of scaling and safety of the compound. Even so, in all analysis, a significant reduction in mycotoxin production could be observed among the control and the treated samples when both AFB_1 and OTA could be produced in matrices.

In the small-scale system, there was an increasing concentration of mycotoxins over time, even in the treated samples. These results could be explained by the presence of the fungal population in the cereals, which allows the mycotoxin production.

In particular, *A. flavus* and *P. verrucosum* depend on oxygen to grow. In our experiment, the headspace in the lab scale silo system and small-scale silo system was around 50% and 20%, respectively. The lower concentration of free oxygen could reduce the regular growth of *A. flavus* and consequently, avoid the AFB_1 production in the small-scale system. Moreover, the cereals in the small-scale system were not autoclaved, which increased the competitiveness among microorganisms by nutrients.

The application of the AITC to reduce the growth of the fungi mycotoxin producer has been studied previously by other authors. Manyes et al. studied the capacity of AITC produced by the volatilization of a standard solution of the oriental mustard essential oil to prevent the growth of the fungi *A. parasiticus* and *Penicillium expansum* [27]. In that study, Petri dishes were inoculated with the mycotoxigenic fungi *A. parasiticus* (producer of AFs) and P. expansum (producer of patulin), and the inhibition of micellar growth was observed when they were deposited in the center of the petri dish with 25 µL and 50 µL of AITC, respectively.

Okano et al. assessed the capacity of the AITC obtained by a commercial mustard seed extract to reduce the aflatoxins production by *A. flavus* during the corn storage [28], in a simulated silo condition. The AITC concentration in the headspace of the model system used by the authors reached the highest value of 54.6 µg/L on the day 14 of incubation and remaining stable until 21.8 µg/L until

the end of the incubation period. Also, the AITC reduced completely the visible growth of *A. flavus* and the AFs production in both sterilized and unsterilized corn

Delaquis et al. evaluated the capacity of the AITC to reduce the growth of *A. flavus* and *P. expansum* at concentrations of 0.1 µL/L [29]. The experimental model used was the one used in this study. In that case, 2 L flasks were used and inocula of 105 conidia/mL were placed in the presence of different amounts of AITC. These antifungal properties of the AITC were also confirmed by Suhr and Nielsen who inoculated pieces of bread with 106 spores/mL of *Penicillium roqueforti*, *Penicillium corylophilum*, and *A. flavus* and arranged them in closed systems in the presence of mustard essential oil (99% of AITC) [30]. Fungal growth inhibition was observed at concentrations of 1 µL/L. Other studies on the fungicidal activity of AITC against food-disrupting fungi observed the ability of the compound to penetrate the matrix and extend its effect over time. Winther and Nielsen showed that cheeses treated with AITC could absorb this compound, increasing its useful life from 4 to 28 weeks [31].

Quiles et al. developed an active packaging dispositive based on the AITC to reduce the sporulation of *A. parasiticus* and AFs production in fresh pizza doughs during 30 days of inoculation [32]. The antifungal activity of the AITC was compared with untreated samples (fresh pizza doughs without any preservative treatment) and with samples treated with sodium propionate, the classical preservative used in bakery products. After 30 days, the growth of *A. parasiticus* was inhibited with the treatment of AITC at 5 µL/L and 10 µL/L. The reduction of AFs was total at the dose of 10 µL/L.

Nazaret et al. evaluated the capacity of AITC to reduce the production of AFs, beauvericin and enniatin, by *A. parasiticus* and *Fusarium poae* in wheat flour [24]. The analysis of the results showed that the AITC concentration of 0.1 µL/L

reduced by 23% the production of the mycotoxins. Also, the application of the AITC at 10 µL/L completely reduced the biosynthesis of the mycotoxins studied during 30 days of incubation.

Tracz et al. evaluated the capacity of AITC at 50, 100, or 500 µL/L to avoid mycotoxin production in corn kernels [33]. Both treatments were able to avoid the production of 12 mycotoxins, including AFB_1 and Ochratoxin A. Saladino et al. analyzed the fungal growth and AFB_1 reduction by AITC (0.5, 1, or 5 µL/L) in loaf bread [34]. As result, the treatments of 1 and 5 µL/L reduced the AFB_1 concentration by above 60%. Our results corroborate with these studies, since the AITC at 50 µL/L demonstrated a fungicide and antimicotoxigenic effect, inhibiting the AFB_1 and OTA synthesis and the fungal growth of *A. parasiticus* and *P. verrucosum* in our small-scale assays.

4. Conclusions

The results obtained in this study showed the capacity of the AITC to reduce the growth of the fungi *A. flavus* and *P. verrucosum* in corn, wheat, and barley. The volatilization of the AITC in the headspace of the lab scale silo system was enough to avoid the *A. flavus* and *P. verrucosum* growth in all cereals tested. Moreover, the treatment with AITC device was able to reduce the AFB_1 and OTA production in corn and barley, respectively.

In the small-scale silo system, a significant reduction of the *A. flavus* and *P. verrucosum* growth was observed as well as an important reduction of the OTA produced by *P. verrucosum*. The application of the device based on the AITC could be an alternative method to reduce the growth of fungi mycotoxin producer in cereals during the storage phase.

For further studies, the tests carried out in this work will be staggered for 200-ton real silos with naturally contaminated barley and treated with AITC release devices.

5. Material and Methods

5.1. Chemicals and Microbial Strains

AFB_1, AFB_2, OTA (98% purity), AITC, and formic acid (HCOOH) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile have been obtained by Fisher Scientific (Hudson, NH, USA). Deionized water (<18 MΩ cm resistivity) was produced by a water purification system (Millipore, Bedford, MA, USA). All the chromatographic solvents were filtered through a 0.22 µm membrane filter Scharlau (Barcelona, Spain). Barley, wheat, and corn were provided by Tot Agro (Barcelona, Spain). The peptone water and dextrose potato agar culture medium were obtained from Liofilchem (Teramo, Italy). The strains of *A. flavus* ITEM 8111 were provided by the Microbial Culture Collection of Institute of Sciences and of Food Production (ISPA, Bari, Italy) whereas the *P. verrucosum* VTT D-01847, was obtained from the VTT Culture Collection (Espoo, Finland).

5.2. Laboratory Scale Silo System and Antifungal Treatment with the AITC Device

The silo simulation was carried out as shown in Figure 7. Glass jars of 1 L containing 300 g of cereals were contaminated with 104 conidia/g of *P. verrucosum* (barley and wheat) and *A. flavus* (corn). The cereals were stored for

three days to allow fungal adaptation, and treated with 500 µL/L (in the relation of volume of the jar) of AITC into a gel device.

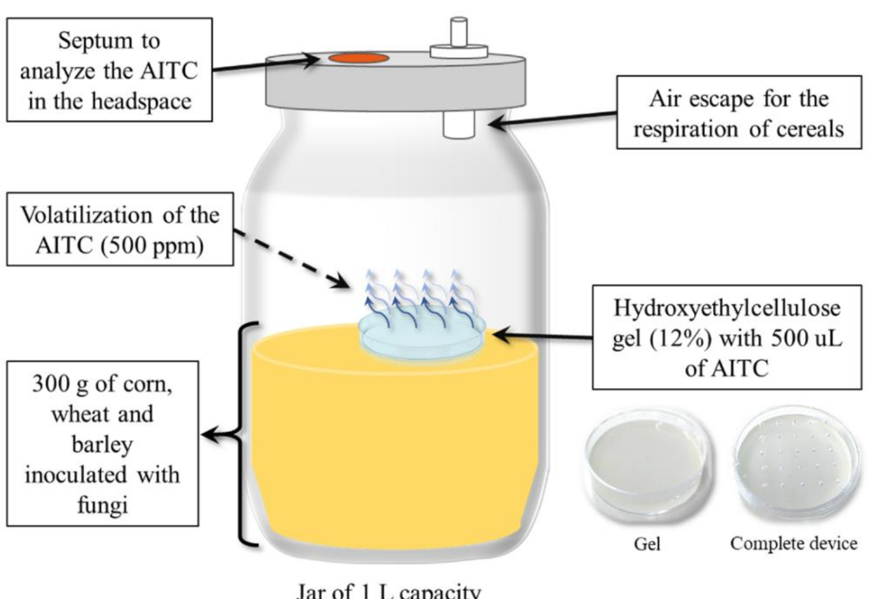


Figure 7. Lab scale silo system used for the treatment of corn, wheat and barley contaminated with *A. flavus* and *P. verrucosum* and treated with the AITC device.

The gel device was manufactured mixing 1.2 g of hydroxyethyl cellulose (gelling agent), 10 mL of water and 500 µL/L of AITC into a Petri dish. The lid of the Petri dish was previously perforated to facilitate the AITC volatilization, as shown in Figure 7. Posteriorly, the antifungal device was placed inside the jars. The jars were closed with adapted lids that contained a septum and an air escape, which allowed AITC analysis in the headspace and cereal respiration, respectively. The samples were stored for 30 days at room temperature. After that, the fungal growth, the mycotoxins contained in the grains, the AITC in the headspace, and the AITC adsorbed by the grains were determined.

5.3. AITC Device Application in a Small-Scale Silo System

Fifty kilograms of barley, corn, and wheat were placed inside plastic drums (100 L) separately. Each cereal was contaminated with 104 conidia/g of *P. verrucosum* or *A. flavus*. The barley and wheat were contaminated with *P. verrucosum* and the corn was contaminated with *A. flavus*. Then, the device described in Section 5.2 was adapted to the small-scale silo system. The petri dish was changed for a glass tapper wear, increasing the quantity of the pure AITC contained to 5 mL, in order to obtain 50 µL/L of this bioactive compound in the headspace of the silo. The device was located in the silo bottom and the grains were introduced in the upper part of the silo as shown in Figure 8.

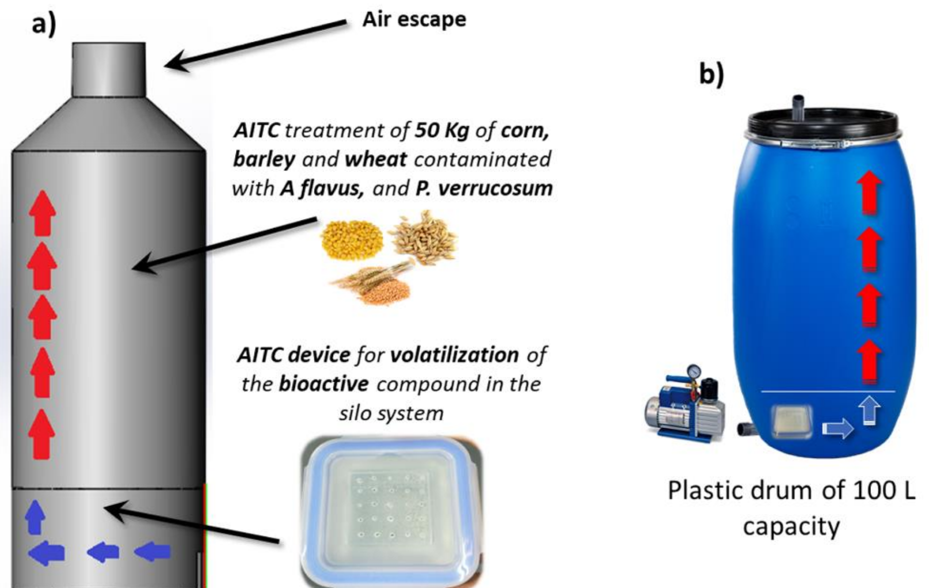


Figure 8. Small-scale silo system used for the treatment of corn, wheat, and barley contaminated with *A. flavus* and *P. verrucosum*, and treated with the AITC device. (a) Theoretical silo design; and (b) the plastic drum used in this study.

A metal grid separated the lower and the upper part of the silo in order to isolate the device and the AITC vapors from the stored cereals. The control group did not receive any antifungal treatment. The analysis carried out on the treated cereals was the same as described in Section 5.2.

5.4. Determination of AITC Concentration in the Headspace of the Laboratory Scale Silo

The AITC content in headspace was determined through a septum localized in the lip of a laboratory silo system (Figure 7). The air was recovered using a syringe of 1 mL, and aliquots of 200 µL were injected in a gas chromatograph (GC) with flame ionization detector (FID) (GC 6890, Agilent Technologies Inc., Santa Clara, CA, USA.). The chromatograph was equipped with a 30 × 0.25 mm CP-SIL 88 fused capillary column (Varian, Middelburg, Netherlands). The temperature of the detector arrived at 200 °C with a gradient of temperature that starts at 60 °C. This temperature was maintained for 1 min and increased 8 °C per min up to 100 °C, then maintained for 5 min and finally increased in 15 °C per min up to 200 °C. The gas utilized as the carrier was H2 at 5 mL/min. The ionization was realized with H2 at 40 mL/min and purified air at 450 mL/min.

5.5. Determination of AITC Concentration in the Cereals of the Laboratory Scale Silo

Extraction of AITC from cereals samples was conducted as described by Tracz et al. with some modifications [33]. Five g samples were weighed into 15 mL polyethylene tubes to which 10 mL of methanol was added. The mixture was shaken for 30 min in a water bath (40 °C) and for 10 min in an ultrasonic bath. The

samples were centrifuged at 4000 g for 5 min at 20 °C. The supernatant (8 mL) was collected and filtered through a 0.22 µm nylon membrane. 20 µL were injected in the LC system, 1220-Infinity (Agilent, Santa Clara, CA, USA) coupled with a diode array detector (LC-DAD) at 236 nm. A Gemini C18 column (Phenomenex, Torrance, CA, USA) 4.6 × 150 mm, 3 µm particle size at 30 °C was used as a stationary phase. The isocratic mobile phase consisted of water/acetonitrile (60:40, v/v) with a flow rate of 1 mL/min.

5.6. Mycotoxin Extraction and LC-MS/MS Analysis of Corn, Barley, and Wheat

The extraction of mycotoxins was carried out following the method described by Serrano et al. with some modifications [35]. Each cereal sample was crushed using a food grinder (Oster Classic Grinder 220e240 V, 50/60 Hz, 600 W, Oster, Valencia, Spain). The resulting particles were mixed, and three 5 g aliquots of each sample were taken in 50 mL plastic falcon tubes. 25 mL of methanol was added to each of these tubes and the samples were homogenized for 3 min by Ultra Ika T18 ultraturrax (Staufen, Germany) at 10,000 rpm. The extract was centrifuged at 4000 rpm during 5 min at 5 °C, and the supernatant was transferred to a plastic flask and evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The obtained residue was resuspended in 5 mL of methanol, transferred to a 15 mL plastic falcon tube and evaporated with nitrogen gas stream using a multi-sample Turbovap LV evaporator (Zymark, Hopkinton, MA, USA). Finally, the residue was reconstituted in 1 mL of methanol, filtered through a 13 mm/0.22 µm filter and transferred to a 1 mL glass chromatography vial. The liquid-chromatography system consisted of an LC-20AD pump coupled to a 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) using an ESI interface in positive ion mode. The mycotoxins were separated on a Gemini

NX C18 column (150 × 2.0 mm I.D, 3.0 mm, Phenomenex, Palo Alto, CA, USA). The mobile phases were the solvent A (5 mM ammonium formate and 0.1% formic acid in water) and solvent B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution was carried out using a linear gradient from 0 to 14 min. The injection volume set was of 20 mL, the nebulizer, the auxiliary and the auxiliary gas were set at 55, 50, and 15 psi respectively. The capillary temperature and the ion spray voltage were of 550 °C and 5500 V, respectively. The ions transitions used for the mycotoxin identification and quantification were: m/z 313.1/241.3 and 284.9 for AFB_1, m/z 315.1/259.0 and 286.9 for AFB_2, m/z 329.0/243.1 and 311.1 for AFG_1, m/z 331.1/313.1 and 245.1 for AFG_2, m/z 404.3/102.1 and 358.1 for OTA.

5.7. Determination of the Fungal Population

After the incubation time, 10 g of each sample was transferred to a sterile plastic bag containing 90 mL of sterile peptone water (Oxoid, Madrid, Spain) and homogenized with a stomacher (IUL, Barcelona, Spain) during 30 s. The suspensions formed were serially diluted in sterile plastic tubes containing 0.1% of peptone water. After that, aliquots of 0.1 mL were plated on Petri dishes containing acidified potato dextrose agar (pH 3.5) (Insulab, Valencia, Spain) and the plates were incubated at 25 °C for 7 d before microbial counting. The results were expressed in logs of colony-forming unit/g of cereal (log CFU/g). All analyses were conducted in triplicate.

5.8. Statistical Analysis

The Prism version 3.0 software (GraphPad corporation1, La Jolla, CA, USA, 1989) for Windows was used for the statistical analysis of data. The experiments were realized in triplicate and the differences among groups were analyzed by Student's t-test. The level of significance considered was $p \leq 0.05$.

References

1. Majeed, S.; Iqbal, M.; Asi, M.R.; Iqbal, S.Z. Aflatoxins and ochratoxin A contamination in rice, corn and corn products from Punjab, Pakistan. *J. Cereal Sci*. **2013**, *58*, 446–450.
2. Iqbal, S.Z.; Asi, M.R.; Ariño, A.; Akram, N.; Zuber, M. Aflatoxin contamination in different fractions of rice from Pakistan and estimation of dietary intakes. *Mycotoxin Res*. **2012**, *28*, 175–180.
3. Pittet, A. Natural occurrence of mycotoxins in foods and feeds—An updated review. *Rev. Med. Vet*. **1998**, *149*, 479–492.
4. IARC-International Agency for Research on Cancer. Monographs on the evaluation of carcinogenic risks to humans. In *A Review of Biological Agents for Human Carcinogens*; IARC-International Agency for Research on Cancer: Lyon, France, 2012.
5. Iqbal, S.Z.; Asi, M.R.; Ariño, A. Aflatoxins. *In Brenner's Encyclopedia of Genetics*, 2nd ed.; Science Direct: London, UK, 2013; pp. 43–47.
6. Hammami, W.; Fiori, S.; Al Thani, R.; Ali Kali, N.; Balmas, V.; Migheli, Q.; Jaoua, S. Fungal and aflatoxin contamination of marketed spices. *Food Control* **2014**, *37*, 177–181.
7. Blesa, J.; Soriano, J.M.; Moltó, J.C.; Mañes, J. Limited survey for the presence of aflatoxins in foods from local markets and supermarkets in Valencia, Spain. *Food Addit. Contam*. **2004**, *21*, 165–171.
8. Van de Perre, E.; Jacxsens, L.; Lachat, C.; El Tahan, F.; De Meulenaer, B. Impact of maximum levels in European legislation on exposure of mycotoxins in dried products: Case of aflatoxin B1 and ochratoxin A in nuts and dried fruits. *Food Chem. Toxicol*. **2015**, *75*, 112–117.

9. Van der Merwe, K.J.; Steyn, P.S.; Fourie, L. Mycotoxins. II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc. Perkin* **1965**, 7083–7088.
10. Téren, J.; Varga, J.; Hamari, Z.; Rinyu, E.; Kevei, F. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathologia* **1996**, *134*, 171–176.
11. Abarca, M.L.; Bragulat, M.R.; Castellá, G.; Cabañes, F.J. Impact of some environmental factors on growth and ochratoxin A production by *Aspergillus niger* and *Aspergillus welwitschiae*. *Int. J. Food Microbiol*. **2018**, *291*, 10–16.
12. Schmidt-Heydt, M.; Bode, H.; Raupp, F.; Geisen, R. Influence of light on ochratoxin biosynthesis by *Penicillium*. *Mycotoxin Res*. **2010**, *26*, 1–8.
13. Wolff, J.; Bresch, H.; Cholmakov-Bodechtel, C.; Engel, G.; Garais, M.; Majerus, P.; Rosner, H.; Scheuer, R. Ochratoxin A: Contamination of foods and consumer exposure. *Arch. Lebensmittelhyg*. **2000**, *51*, 81–128.
14. Petzinger, E.; Weidenbach, A. Mycotoxins in the food chain: The role of ochratoxins. *Livestock Prod. Sci*. **2002**, *76*, 245–250.
15. Rizzo, A.; Eskola, M.; Atroshi, F. Ochratoxin a in Cereals, Foodstuffs and Human Plasma. *Eur. J. Plant Pathol*. **2002**, *108*, 631–637.
16. Denli, M.; Perez, J.F. Ochratoxins in feed, a risk for animal and human health: Control strategies. *Toxins* **2010**, *2*, 1065–1077.
17. Reddy, L.; Bhoola, K. Ochratoxins-food contaminants: Impact on human health. *Toxins* **2010**, *2*, 771–779.
18. Jackson, L.S.; Katta, S.K.; Fingerhut, D.D.; DeVries, J.W.; Bullerman, L.B. Effects of baking and frying on the fumonisin B_1 content of corn-based foods. *J. Agric. Food Chem*. **1997**, *45*, 4800–4805.
19. Meca, G.; Blaiotta, G.; Ritieni, A. Reduction of ochratoxin A during the fermentation of Italian red wine Moscato. *Food Control* **2010**, *21*, 579–583.
20. Engel, E.; Baty, C.; Le Corre, D.; Souchon, I.; Martin, N. Flavor-active compounds potentially implicated in cooked cauliflower acceptance. *J. Agric. Food Chem*. **2002**, *50*, 6459–6467.
21. Luciano, F.B.; Holley, R.A. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against Escherichia coli O157:H7. Int. *J. Food Microbiol*. **2009**, *131*, 240–245.
22. Isshiki, K.; Tokuoka, K.; Mori, R.; Chiba, S. Preliminary examination of allyl-isothiocyanate vapor for food preservation. *Biosci. Biotechnol. Biochem*.

1992, *56*, 1476–1477.
23. Manyes, L.; Luciano, F.B.; Mañes, J.; Meca, G. In vitro antifungal activity of allyl isothiocyanate (AITC) against *Aspergillus parasiticus* and *Penicillium expansum* and evaluation of the AITC estimated daily intake. *Food Chem. Toxicol*. **2015**, *83*, 293–299.
24. Nazareth, T.M.; Bordin, K.; Manyes, L.; Meca, G.; Mañes, J.; Luciano, F.B. Gaseous allyl isothiocyanate to inhibit the production of aflatoxins, beauvericin and enniatins by *Aspergillus parasiticus* and *Fusarium poae* in wheat flour. *Food Control* **2016**, *62*, 317–321.
25. Zhang, Y. Cancer-preventive isothiocyanates: Measurement of human exposure and mechanism of action. *Mutat. Res*. **2004**, *555*, 173–190.
26. Cejpek, K.; Valusek, J.; Velísek, J. Reactions of allyl isothiocyanate with alanine, glycine, and several peptides in model systems. *J. Agric. Food Chem*. **2000**, *48*, 3560–3565.
27. Okano, K.; Ose, A.; Takai, M.; Kaneko, M.; Nishioka, C.; Ohzu, Y.; Odano, M.; Sekiyama, Y.; Mizukami, Y.; Nakamura, N.; et al. Inhibition of aflatoxin production and fungal growth on stored corn by allyl isothiocyanate vapor. *J. Food Hyg. Soc. Jpn*. **2015**, *56*, 1–7.
28. Delaquis, P.J.; Sholberg, P.L. Antimicrobial activity of gaseous allyl isothiocyanate. *J. Food Prot*. **1997**, *60*, 943.
29. Suhr, K.I.; Nielsen, P.V. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol*. **2003**, *94*, 665–674.
30. Winther, M.; Nielsen, P.V. Active packaging of cheese with allyl isothiocyanate, an alternative to modified atmosphere packaging. *J. Food Prot*. **200**6, *69*, 2430–2435.
31. Quiles, J.M.; Manyes, L.; Luciano, F.; Manes, J.; Meca, G. Antimicrobial compound allylisothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust. *Food Chem. Toxicol*. **2015**, *83*, 222–228.
32. Tracz, B.L.; Bordina, K.; Nazareth, T.M.; Costa, L.B.; Macedo, R.E.F.; Meca, G.; Luciano, F.B. Assessment of allyl isothiocyanate as a fumigant to avoid mycotoxin production during corn storage. *LWT* **2017**, *75*, 692–696.
33. Saladino, F.; Quiles, J.M.; Luciano, F.B.; Mañes, J.; Fernandez-Franzón, M.; Meca, G. Shelf life improvement of the loaf bread using allyl, phenyl and

benzyl isothiocyanates against *Aspergillus parasiticus*. *LWT* **2017**, *78*, 208–214.

34. Serrano, A.B.; Font, G.; Mañes, J.; Ferrer, E. Emerging *Fusarium* mycotoxins in organic and conventional pasta collected in Spain. *Food Chem. Toxicol*. **2013**, *51*, 259–266.
35. Pitt, J.I.; Hocking, A.D. *Fungi and FoodSpoilage*; Blackie Academic and Professional: London, UK, 1997; Volume II.

4. GENERAL DISCUSSION

Discusión general

4. DISCUSIÓN GENERAL

El trabajo de investigación llevado a cabo en esta tesis doctoral se ha centrado en el análisis de micotoxinas en masas de pizza refrigeradas y en el estudio de las propiedades antifúngicas y antimicotoxigénicas de los extractos de mostaza y de los isotiocianatos (ITCs); así como de los distintos métodos de aplicación para reducir la presencia de hongos toxigénicos y micotoxinas en pan y productos derivados a base de cereales.

Inicialmente se ha evaluado la exposición de la población valenciana a un conjunto de micotoxinas a través del consumo de masas de pizza, y el riesgo asociado a su exposición mediante el análisis de 60 muestras procedentes de pequeños comercios y grandes superficies de la ciudad de Valencia. La presencia de micotoxinas en las muestras analizadas confirmó la necesidad de estudiar nuevos mecanismos para reducir su presencia o evitar su formación mediante la inhibición del crecimiento de hongos toxigénicos en este tipo de productos. Los compuestos utilizados han sido los ITCs, sustancias volátiles de elevado poder antifúngico.

Asimismo, se han estudiado la reducción de hongos toxigénicos y micotoxinas en productos como masas de pizza refrigeradas, tortas de trigo y pan de molde utilizando diversas estrategias; como el uso de ITCs de síntesis química, ITCs generados por reacciones enzimáticas a partir de harinas de mostaza, y la inclusión directa de harinas de mostaza como ingredientes alimentarios. En este último caso también se evaluó la actividad antifúngica de diversos extractos de harina de mostaza.

Finalmente, se estudió el diseño de un dispositivo liberador de alil isotiocianato (AITC) basado en un gel de hidroxietilcelulosa (HEC) para su utilización como antifúngico en silos de cereales.

4.1. Micotoxinas en masas de pizza

4.1.1. Análisis de micotoxinas en muestras de pizza refrigeradas comercializadas en València

El análisis de muestras de masas de pizza refrigeradas evidenció la presencia de nueve micotoxinas: aflatoxinas B_1, B_2 y G_1 (AFB_1, AFB_2 y AFG_1), zearalenona (ZEA), eniatinas A, A_1, B y B_1 (ENA, ENA_1, ENB y ENB_1) y beaubericina (BEA). Respecto a las aflatoxinas (AFs), el porcentaje de muestras contaminadas fue del 23, 32, 10 y 50 % para AFB_1, AFB_2, AFG_1 y suma de AFs, respectivamente; mientras que, las concentraciones promedio de las muestras positivas fueron de 4,09 µg/kg para AFB_1, 0,50 µg/kg para AFB_2, 0,79 µg/kg para AFG_1 y 2,36 µg/kg para la suma de AFs. Teniendo en cuenta únicamente las 14 muestras contaminadas por encima del límite de cuantificación (LOQ), el contenido de AFB_1 osciló entre 1,0 y 9,5 µg/kg, y el 50 % de ellas excedió el límite legislado en la UE para las AFB_1 (2 µg/kg). Estas muestras supusieron un 14 % del total.

Otros autores también han referido la presencia de AFs en cereales y productos derivados. Serrano *et al*. (2012a) estudiaron la presencia de micotoxinas en 265 muestras de cereales y productos a base de cereales procedentes de países mediterráneos, de los cuales un 19 % de las muestras positivas estaban contaminadas por AFs. De estas muestras contaminadas con AFs, un 52 % contenía AFB_1 a concentraciones comprendidas entre 5,5 y 66,7 µg/kg. Iqbal *et al*. (2014) analizaron 237 muestras de cereales para el desayuno y detectaron AFs en el 41 % de ellas, con unos valores superiores a los límites legislados en el 16 y 8 % para AFB_1 y suma de AFs, respectivamente. En otros productos como complementos alimentarios a base de trigo se ha detectado AFB_1 en concentraciones comprendidas entre 0,24 y 29,0 µg/kg (Blankson y Mill-Robertson, 2016).

Se detectó ZEA en todas las masas de pizza, con valores que oscilaron entre 29 y 176 µg/kg, con un contenido promedio de 78 µg/kg. El 12 % del total de muestras excedió el límite máximo legislado por la Unión Europea (75 µg/kg). La presencia de ZEA en trigo y productos derivados se ha observado previamente. Según los datos obtenidos por Klarić *et al*. (2009) en muestras de cereales, la presencia de ZEA es más frecuente en cereales que otras micotoxinas como ocratoxina A (OTA) y AFB_1. En dicho estudio se detectaron concentraciones de ZEA comprendidas entre 12,5 a 1182 µg/kg. Cano-Sancho *et al*. (2012) evaluaron la aparición de ZEA en diferentes alimentos presentes en el mercado catalán (España), estableciendo un valor máximo de 22,8 µg/kg, pero ninguna muestra resultó por encima del límite máximo legislado.

Con respecto a la distribución de las eniatinas (ENs), todas las muestras de masa de pizza presentaban contaminación por al menos tres ENs (ENA_1, ENB y ENB_1) con valores que oscilaron entre 1,9 y 27,7 µg/kg. Los contenidos promedio en muestras positivas fueron 4,54, 3,37 y 1,69 µg/kg respectivamente, mientras que 14,2, 10,5 y 13,4 µg/kg fueron las concentraciones máximas detectadas de estas micotoxinas. La concentración media de ENA fue de 15 µg/kg y se encontró en el 8 % del total de las muestras analizadas.

Juan *et al*. (2013a) estudiaron la presencia de ENs en productos de cereales italianos, observando una incidencia de ENA, ENA_1, ENB y ENB_1 del 13, 9, 70 y 26 % respectivamente. Los valores medios detectados fueron de 4,5 µg/kg (ENA), 8,3 µg/kg (ENA_1), 133,6 µg/kg (ENB) y 8,1 µg/kg (ENB_1). Saladino et al. (2016a) analizó la presencia de micotoxinas en panes de molde, encontrando una incidencia del 96 % para la ENB, seguida de la ENB_1 (79 %) y la ENA_1 (18 %), mientras que la ENA no se detectó. Las concentraciones observadas oscilaron entre 0,2 y 54 µg/kg.

La BEA se detectó solo en dos muestras, una por encima del LOQ (22 µg/kg) y otra por debajo. De las 265 muestras de cereales y productos derivados analizados por Serrano *et al*., (2012a, 2012b) un 6 % estaban contaminados con BEA. En un estudio sobre productos alimenticios portugueses, solo 1 de las 61 muestras resultó contaminada con BEA (Blesa *et al*., 2012). Un análisis de trigo italiano y productos derivados detectó BEA en 5 de 57 muestras con un rango de concentración de 9,6– 35 µg kg (Juan *et al*., 2013b).

4.1.2. Estimación de la exposición a micotoxinas a través de masas de pizza refrigeradas y evaluación del riesgo

El nivel de exposición de la población a las micotoxinas a través de la dieta depende de la composición de dicha dieta y de la presencia de micotoxinas en los alimentos que la forman. La ingesta diaria estimada (EDI) se calcula multiplicando el dato de consumo de un determinado alimento por la media del valor de micotoxina encontrada en dicho alimento y se expresa en función del peso corporal medio del grupo poblacional a estudiar. Para este estudio se dividió la población en tres grupos de edad: 12–14, 15–18 y 19–64 años. Los pesos utilizados para los tres grupos fueron, respectivamente, 50, 65 y 70 kg. Las concentraciones promedio de cada micotoxina se calcularon considerando todos los datos de contaminación, incluidos los positivos y negativos. Los valores por debajo del LOQ se establecieron como la mitad del LOQ. Los datos del consumo español de pizzas refrigeradas se obtuvieron de la base de datos del Ministro de Agricultura, Alimentación y Medio Ambiente de España (MAGRAMA, 2015). Este valor fue de 1,53 g/día, aunque se consideró un grupo de grandes consumidores (12-14 años que consumen 2 porciones de pizza por semana) cuyo consumo se calculó en 18,57 g/día.

Finalmente, estas EDIs se han comparado con las ingestas diarias tolerables (TDIs) para desarrollar la caracterización del riesgo. Algunas sustancias no tienen una TDI establecida: en el caso de las AFs, debido a su probada carcinogenicidad, y en otras micotoxinas todavía no hay estudios concluyentes sobre su toxicidad. Por este motivo, la evaluación del riesgo de ENs y BEA se realizó utilizando los valores de TDIs de otras micotoxinas producidas por hongos del mismo género, como el deoxinivalenol (DON) con 1 µg/kg de peso corporal (pc) por día, el nivalenol (NIV) con 1,2 µg/kg pc/día, y la suma de T-2 y HT-2 (0,1 µg/kg pc/día). La TDI para ZEA es de 0,25 µg/kg pc/día (EFSA, 2014).

Para las AFs, los datos de EDIs oscilaron entre $5,08 \cdot 10^{-5}$ µg/kg pc/día para el grupo de edad de 19–64 años, a $6,89 \cdot 10^{-5}$ µg/kg pc/día para el grupo de edad de 12–14 años. El valor medio de la exposición a la suma de AFs mostrada por Sirot *et al*. (2013) en un estudio de la dieta total francesa fue de $1,90 \cdot 10^{-6}$ µg/kg pc/día. García-Moraleja *at al*. (2015) determinóde caff la EDI de AFs por consumo de café, la cual fue de $3,00 \cdot 10^{-6}$, $1,00 \cdot 10^{-6}$, $6,00 \cdot 10^{-6}$ y $1,40 \cdot 10^{-5}$ µg/kg pc/día para AFB_1, AFB_2, AFG_1 y AFG_2 respectivamente. Saladino *et al.* (2016a) obtuvieron una EDI para la suma de AFs por consumo de pan de molde de entre $8,00 \cdot 10^{-6}$ y $7,80 \cdot 10^{-5}$ µg/kg pc/día.

Los valores de EDI obtenidos para ZEA variaron entre $9,63 \cdot 10^{-4}$ y $1,31 \cdot 10^{-3}$ µg/kg pc/día para los grupos de 19–64 y 12-14 años, respectivamente. Comparando con otros estudios similares, las EDIs de ZEA obtenidas por Juan *et al.* (2013b) considerando el consumo de trigo y productos a base de trigo fue de $7,50 \cdot 10^{-2}$ µg/kg pc/día. El EDI de Saladino *et al.* (2016a), en panes de molde, osciló entre $2,38 \cdot 10^{-3}$ y $2,38 \cdot 10^{-3}$ µg/kg pc/día.

El EDI calculado para las ENs varió de $2,86 \cdot 10^{-4}$ y $3,88 \cdot 10^{-4}$ µg/kg pc/día para los grupos de 19–64 y 12–14 años, respectivamente. El EDI de las ENs obtenido

por Blesa *et al.* (2012), utilizando datos totales del consumo de cereales en Portugal y suponiendo que el peso corporal promedio de un adulto de 60 kg fue de $4{,}84 \cdot 10^{-1}$ µg/kg pc/día. Los valores de EDI para ENA, ENA_1, ENB y ENB_1, calculados a través del consumo de trigo y productos de trigo en Italia oscilaron entre $3{,}00 \cdot 10^{-3}$ (ENB_1) y $1{,}34 \cdot 10^{-1}$ (ENB) µg/kg pc/día (Juan *et al*., 2013b), mientras que el análisis de pan de molde proporcionó una EDI para la suma de ENs de $1{,}00 \cdot 10^{-3}$ µg/kg pc/día (Saladino *et al*., 2016a).

Respecto a la BEA, los valores de EDI fueron de $3{,}01 \cdot 10^{-4}$ y $4{,}08 \cdot 10^{-4}$ µg/kg pc/día para los conjuntos de 19–64 y 12–14 años, respectivamente. La EDI obtenida por Serrano *et al*. (2013) de BEA por consumo de pasta fue de $5{,}00 \cdot 10^{-4}$ µg/kg pc/día. Por consumo de trigo italiano y productos a base de trigo, el EDI de la BEA resultó de $1{,}80 \cdot 10^{-2}$ µg/kg pc/día (Juan *et al*., 2013b).

Todas las EDIs calculadas para las distintas micotoxinas presentes en masas de pizza se encontraron por debajo de sus respectivas TDIs. En concreto, el riesgo más alto de todas las micotoxinas es debido a la ZEA, con una EDI calculada del 0,52 % de la TDI (para el grupo de edad de 12-14 años). Aún así, si tenemos en cuenta el grupo de alto consumo (edad entre 12 a 14 años que consumen dos porciones de pizza semanales) el porcentaje de las EDIs respecto a las TDIs resultaron considerablemente elevados: 6,3 % para ZEA; 4,7 % (TDI de T-2 + HT-2) para la suma de ENs y de 4,9 % (TDI de T-2 + HT-2) para la BEA. Estos valores finales, junto con el porcentaje de muestras que superaban los límites legislados para AFs (14 %) indican que no podemos despreciar este alimento como factor de riesgo respecto a la ingesta de micotoxinas.

En especial si tenemos presente que no existen valores toxicológicos de referencia para la suma de diversas micotoxinas presentes en un mismo alimento, y por tanto ingeridas de manera conjunta. Por otra parte, también debe tenerse

en cuenta que estas mismas micotoxinas pueden encontrarse presentes en los alimentos no incluidos en el presente estudio.

4.2. Reducción de hongos y micotoxinas

4.2.1. Empleo de harina de mostaza

La mostaza son semillas de vegetales que pertenecen al orden *Brassicales* ricos en glucosinolatos (GSs), metabolitos bioactivos cuya hidrólisis enzimática da lugar mayoritáriamente a los ITCs, sustancias naturales que destacan por su actividad biocida (fungicida, bactericida e insecticida), lo que las hace ideales para su utilización como ingrediente funcional en sustitución de aditivos alimentarios sintéticos, de gran uso en el momento actual.

4.2.1.1. Validación del método de análisis de glucosinolatos

Para poder usar adecuadamente la harina de mostaza como fuente de ITCs se validó un método de determinación de los GSs en dicha matriz. Este método, basado en el descrito por Prestera *et al.* (1996) con algunas modificaciones, se basa en una extracción acuosa de harina de mostaza previamente desactivada térmicamente la enzima mirosinasa, homogenizada con Ultra-Turrax®, centrifugado, y filtrado del sobrenadante. El análisis se llevó a cabo en un un cromatógrafo líquido de alta resolución acoplado a un detector de fila de diodos (HPLC-DAD) a 227 nm. Para confirmar la validez del método se evaluaron la linealidad, la precisión mediante la repetibilidad (intradía) y reproducibilidad (interdia), la exactitud mediante la recuperación, la sensibilidad con los límites de detección (LOD) y del LOQ, y finalmente, el efecto matriz (ME).

La recuperación media de las muestras de harina de mostaza oriental fortificada (n = 3) a tres niveles diferentes de sinigrina (5, 10 y 15 mg) fue de 84,6

± 3,6%. El valor obtenido para la recuperación y las desviaciones estándar relativas del método utilizado están de acuerdo con la Directiva 2006/401/CE de la Comisión Europea para métodos de análisis de compuestos bioactivos en productos alimenticios (EC, 2006). Los valores de variación intradía (n = 5) e interdía (5 días diferentes) fueron de 2,5 y 8,6 %, respectivamente. Estos valores están por debajo del 15 %, que es la variación máxima para los ejercicios de certificación de compuestos bioactivos. Los valores de los LOD y LOQ fueron 0,05 y 0,15 mg/L, respectivamente, mientras que el ME fue del 87,4 %.

4.2.1.2. Determinación de glucosinolatos en harinas de mostaza

La harina de mostaza amarilla (YMF) y oriental (OMF) utilizada en los ensayos posteriores se caracterizó analíticamente utilizando el método descrito previamente para predecir la cantidad total de GSs que podía convertirse en ITCs a través de la acción de la mirosinasa. En la harina de mostaza oriental se detectó una concentración del 5,2 % del GS sinigrina, mientras que en la harina de mostaza amarilla se obtuvo un 4,6 % del GS sinalbina. Estos GSs, característicos mayoritariamente de estas matrices, son los precursores del AITC y del parahidroxibencil isotiocianato (ρ-HBITC), respectivamente. Estos datos son próximos a los de otros autores, como los de Tsao *et al*. (2002) que calculó un 5,12 % de sinigirna en mostaza oriental o Herzallah y Holley (2012), que obtuvieron concentraciones de entre 1,49 y 4,06 % de sinigrina en muestras de mostaza oriental.

4.2.1.3. Actividad antifúngica de harinas de mostaza

Previamente a la realización de los ensayos con las harinas de mostaza como ingredientes conservantes naturales, se procedió a estudiar la actividad antifúngica de diversos extractos de YMF y OMF en medio sólido y líquido frente a una batería de hongos.

En concreto se estudiaron ocho extractos distintos:

- Extracto acuoso de YMF
- Extracto acuoso de YMF tratada térmicamente
- Extracto concentrado de YMF
- Extracto concentrado de YMF tratada térmicamente
- Extracto acuoso de OMF
- Extracto acuoso de OMF tratada térmicamente
- Extracto concentrado de OMF
- Extracto concentrado de OMF tratada térmicamente

El tratamiento térmico tenía como objetivo la desactivación de la mirosinasa, y los extractos obtenidos a partir de dichas harinas se utilizaron como controles, con la finalidad de comprobar la existencia de actividad antifúngica de origen distinto al de los ITCs. Los extractos concentrados se obtuvieron mediante la liofilización de 25 mL del extracto acuoso y su posterior resuspensión en 1 mL de agua estéril. Se evaluó la actividad en medio sólido depositando 10 y 100 µL de cada extracto sobre placas sembradas con 14 hongos distintos: *A. parasiticus, A. flavus, A. carbonarius, P. camemberti, P. roqueforti, P. nordicum, P. commune, P. brevicopactum, P. expansum, P. chrysogenum, P. solitum, P. digitatum, F. graminearum* y *F. verticiloides.*

El YMF fue activo solo contra *A. parasiticus*, mientras que el extracto concentrado del YMF mostró la mayor actividad antifúngica en las cepas analizadas, en particular en 13 de los 14 hongos estudiados, dado que *F. graminearum* no se vio afectado. Respecto a los extractos de YMF tratados térmicamente, el no concentrado fue negativo contra todas las cepas fúngicas analizadas, mientras que el extracto concentrado fue activo solo contra *P. camemberti* y *P. roqueforti*. En el caso de la OMF, solo el extracto concentrado mostró actividad antifúngica contra las cepas de *P. roqueforti* y *A. carbonarius*. Los otros tres extractos de este ingrediente bioactivo no mostraron ninguna actividad antifúngica frente a los hongos ensayados.

Teniendo en cuenta estos resultados se procedió a determinar las concentraciones mínimas innhibitorias (MIC) y fungicidas (MFC) del extracto más efectivo (concentrado de YMF) en medio líquido, utilizando para ello el ensayo de microplaca de 96 pocillos.

Las MICs presentaron datos de inhibición que variaron entre 238 µg/mL (*P. camemberti*) y 15.000 µg/mL (*A. flavus, A. parasiticus y A. carbonarius*). Los resultados mejores se obtuvieron frente a hongos de los géneros *Penicillium*, como el ya citado *P. camemberti*, *P. roqueforti* (476 µg/mL) y *P. digitatum* (937 µg/mL). Las MFCs oscilaron desde 1.875 µg/mL para *P. nordicum*, *P. commune* y *P. brevicompactum* hasta los 15.000 µg/mL para *A. flavus*, *A. parasiticus* y *A. carbonarius*. En general, las cepas de *Aspergillus* fueron más resistentes que las cepas de *Penicillium* o *Fusarium*.

Varios autores han probado la aplicación de los ITC como sustancias antimicrobianas, tanto directamente como usando harinas de mostaza. En el primer caso, Mañes *et al*. (2015) estudiaron la actividad antifúngica AITC contra cepas toxigénicas de *Aspergillus* y *Penicillium*, consiguiendo inhibir el crecimiento

de los hongos *A. parasiticus* y *P. expansum* en placas de medio sólido expuestas a 5 y 25 µL de AITC, respectivamente. Azaiez *et al*. (2013) evaluaron la actividad antifúngica del AITC, fenil isotiocianato (PITC) y bencil isotiocianato (BITC) frente a cepas toxigénicas de *Fusarium*. Los ITCs empleados en el estudio inhibieron el crecimiento de *Gibberella moniliformis*, reduciendo 2,1–89,7 % el tamaño del micelio dependiendo del tiempo y la dosis utilizada (de 10 a 50 µL). Otros estudios han probado la capacidad antimicrobiana del uso directo de harinas de mostaza en lugar de los ITCs. Kanemaru y Miyamoto (1990) demostraron que el 0,1 % de mostaza con 9,4 µg/mL de AITC fue capaz de inhibir el crecimiento de *E. coli* a las 24 h, mientras que se requirieron 12,3 µg/mL de AITC para lograr el mismo nivel de inhibición.

4.2.1.4. Reducción de hongos y micotoxinas en pan de molde

Tras averiguar que la harina de mostaza con mayores propiedades antifúngicas en su uso directo era la amarilla, se evaluó su utilización como conservante natural preparando panes de molde con distintas concentraciones de YMF en su formulación (0, 2, 4, 6 y 8 g/Kg), así como panes control con propionato sódico (E-281) a 2 g/Kg. Todos los panes fueron inoculados con los hongos toxigénicos *A. flavus* (AFs) y *P. nordicum* (OTA), envasados y conservados a temperatura ambiente. Transcurridos 10 días de ensayo, se comprobó que ninguno de los tratamientos utilizados inhibió completamente crecimiento de los hongos inoculados, aunque alguno de ellos redujo su desarrollo en comparación con el experimento control.

La cuantificación de la reducción de la contaminación fúngica se realizó mediante homogeneización de las muestras en stomacher con agua peptonada estéril y la siembra de diluciones seriadas decimales en placas de medio PDA y

posterior recuento de las unidades formadoras de colonias (CFU). En el caso de la inoculación con *A. flavus*, el pan control presentó una carga fúngica de 6,30 log CFU/g. Se observaron diferencias significativas con los panes tratados con 6 g/Kg (6,06 log CFU/g) y 8 g/Kg (5,78 log CFU/g) de YMF, lo que supone una reducción de 0,24 log CFU/g (47 %) y 0,52 log CFU/g (67 %) respecto al pan tratado con propionato. En relación con los panes inoculados con *P. nordicum*, el crecimiento en los controles con propionato sódico fue de 5,82 log CFU/g. En este caso, también hubo diferencias significativas con los panes tratados con 6 g/Kg (4,63 log CFU/g) y 8 g/Kg (4,16 log CFU/g) de YMF, lo que equivale a una reducción de 1,19 log CFU/g (94 %) y 1,66 log CFU/g (97 %) para 6 y 8 g/Kg de YMF, respectivamente.

La determinación de las micotoxinas presentes en los panes contaminados se llevó a cabo mediante una extracción en metanol, con homogeneizado con Ultra-Turrax®, centrifugado y evaporación mediante rotavapor, resuspensión en metanol, filtrado (0,22 µm) y por último, inyección en un cromatógrafo líquido acoplado a un esprectrómetro de masas en tandem (LC-MS/MS). En los panes inoculados con *A. flavus* solo se detectó la presencia de AFB_1, en concentraciones de 1,3 mg/kg (E-281), 5 mg/kg (sin tratamiento), 4,5 mg/kg (2 g/Kg de YMF), 4,2 mg/kg (4 g/Kg de YMF), 1,1 mg/kg (6 g/Kg de YMF) mientras que en los panes tratados con 8 g/Kg de YMF no detectó la micotoxina, lo que confirma el potencial antitoxigénico del ingrediente empleado. Por último, no se detectó presencia de micotoxinas producidas por *P. nordicum* en los panes contaminados con dicho hongo.

Otros autores han estudiado el uso de mostaza amarilla y los ITCs derivados como conservantes en alimentos. Saladino *et al*. (2016b) utilizaron el ρ-HBITC derivado de la YMF (a partir de bolsitas con 0,1, 0,5 y 1 g de harina y adicionadas con agua) para reducir el contenido de AFs entre un 41 y 69 % en tortas italianas

contaminadas con *A. parasiticus*. Los mismos autores aplicaron dicha técnica (con 0,5, 1 y 2 g de harina) en tortas de trigo contaminadas con *P. expansum* y redujeron la formación de Patulina (PAT) entre un 85,5 y 99,1 % (Saladino *et al*., 2016c). Hontanaya *et al*. (2015) inhibieron el crecimiento de *A. parasiticus* inoculando en frutos secos mediante ρ-HBITC generado a partir de YMF, reduciendo la formación de AFB_1, AFB_2, AFG_1 y AFG_2 entre el 27,0 y el 32,5 %. Respecto a otras matrices y patógenos alimentarios, Graumann y Holley (2008) demostraron que el ρ-HBITC generado in situ al incluir polvo de mostaza amarilla como ingrediente en salchichas fermentadas inhibió el crecimiento de *E. coli O157: H7*.

4.2.1.5. Estudio de vida útil del pan de molde

De la observación diaria de los panes tratados con YMF e inoculados con *A. flavus* y *P. nordicum* se pudo evaluar la vida útil respecto a los panes tratados con E-281. En el caso del *A. flavus*, se observó la aparición de hongo visualmente a los días 2 (sin tratamiento), 3 (E-281, 2 y 4 g/Kg de YMF), 4 (6 g/Kg de YMF) mientras que en los panes con 8 g/Kg de YMF no se observó crecimiento fúngico en los 10 días. En el pan con *P. nordicum*, se detectó hongo visualmente a los días 2 (sin tratamiento, 2 y 4 g/Kg de YMF), 4 (4 g/Kg de YMF), y 5 (E-281), mientras que en los panes con 6 y 8 g/Kg de YMF el crecimiento fúngico no fue visible en 10 días.

4.2.2. Empleo de isotiocianatos

Además de utilizar la harina de mostaza, también se ha estudiado el uso de los ITCs, tanto generados por hidrólisis de GSs como de síntesis química en

diversas matrices alimentarias susceptibles de contaminación por hongos toxigénicos.

4.2.2.1. Reducción de hongos y micotoxinas en masas de pizza refrigeradas

En el siguiente ensayo se evaluaron distintas fuentes de AITC para inhibir el crecimiento del hongo toxigénico *A. parasiticus* y la formación de AFs en masas de pizza caseras y conservadas durante 30 días en refrigeración a las que se inoculó dicho hongo. Los tratamientos utilizados fueron los siguientes:

1. OMF tratada térmicamente a concentraciones de 0,7, 1,7 y 3,4 % para provocar una liberación del AITC generado por la actividad mirosinásica de los microorganismos de la masa.
2. OMF en pequeñas bolsas de plástico en cantidades de 170, 425 y 850 mg junto con agua para promover la conversión de la sinigrina a AITC y su volatilización en el interior del envase.
3. AITC en cantidades de 2,5, 5 y 10 µL depositado en la superficie de un papel de filtro situado en el interior del envase.
4. AITC en cantidades de 2,5, 5 y 10 µL depositado en la superficie de un papel de filtro que a su vez se coloca en el interior de una bolsa de plástico para ralentizar su difusión al resto del envase.

Las cantidades de OMF utilizadas en los tratamientos 1 y 2 se calcularon para generar una concentración de AITC potencial equivalente a los tratamientos 3 y 4 (2, 5 y 10 µL/L). También se añadieron masas tratadas con una combinación de ácido sórbico (E-200) y E-281 a concentraciones de 0,5, 1 y 2 g/Kg, así como un grupo de masas control sin tratamiento antimicrobiano alguno.

Ninguno de los tratamientos fue capaz de inhibir complematemte el crecimiento de *A. parasiticus*. Las masas control no tratadas presentaron una población de 8,89 log CFU/g, mientras que los tratamientos más efectivos fueron los 10 µL de AITC en bolsita (3,59 log CFU/g), seguido de los 10 µL de AITC en papel de filtro (3,9 log CFU/g), 5 µL de AITC en bolsita (4,80 log CFU/g), 5 µL de AITC en papel de filtro (5,72 log CFU/g), 850 mg de OMF con agua en bolsita (7,29 log CFU/g). El resto de los tratamientos, incluidos los conservantes E-200 y E-281, variaron entre 7,92 y 8,83 log CFU/g, y no mostraron reducciones significativas con respecto a las masas control no tratadas (8,89 log CFU/g). Respecto a la reducción de las AFs, todos los tratamientos con OMF o AITC fueron capaces de reducir significativamente los niveles de AFs de una manera dependiente de la dosis. Las masas de pizza sin tratamiento presentaron una contaminación AFs de 709 mg/kg (AFB_1), 637 mg/kg (AFB_2), 58 mg/kg (AFG_1) y 0,6 mg/kg (AFG_2). Los tratamientos que lograron una reducción promedio de las AFs superior o equivalente al uso de conservantes (entre el 99,4 y 100 %) fueron las tratadas con 850 mg de OMF con agua en bolsita, con 10 µL de AITC en papel de filtro y con 10 µL de AITC en papel de filtro en bolsita.

La inhibición del crecimiento de hongos habituales de productos de panadería mediante AITC ha sido estudiada previamente, aunque este estudio es el primero en utilizar dispositivos antimicrobianos con base de AITC en masas de pizza. Nielsen y Rios (2000) probaron la capacidad de diversos aceites esenciales (canela, ajo, mostaza, etc.) frente a hongos habituales en productos de panaderí́a como *A. flavus*, *P. commune*, *P. roqueforti*, *P. solitum*, entre otros. De todos ellos, el aceite esencial de mostaza rico en AITC (90-95 %) fue el compuesto antifúngico más potente, con unas MICs que oscilaron entre 1,8 y 3,5 µg/mL. Mejía-Garibay *et al*. (2015) utilizaron aceite esencial de mostaza negra (con 0,38 g/mL de AITC)

para inhibir el crecimiento de *A. niger*, *A. ochraceus* o *P. citrinum*, demostrando que la exposición a concentraciones de 41,1 µL/L del aceite esencial retrasó el crecimiento de *A. niger* y *P. citrinum* durante 10 días, y *A. ochraceus* durante 20 días, mientras que la MFC para todas las especies fue de 47 µL/L. Respecto a la reducción de micotoxinas, Saladino *et al*. (2016b) usaron el AITC derivado de la OMF (0,1, 0,5 y 1 g en bolsitas y adicionadas con agua) para reducir el contenido de AFs entre un 60 y 89 % en tortas italianas inoculadas con *A. parasiticus*. Los mismos autores aplicaron dicha técnica (con 0,5, 1 y 2 g de harina) en tortas de trigo contaminadas con *P. expansum* y redujeron la formación de Patulina (PAT) entre un 85 y 93 % (Saladino *et al*., 2016c). Hontanaya *et al*. (2015) inhibieron el crecimiento de *A. parasiticus* inoculado en frutos secos mediante AITC generado a partir de OMF, reduciendo la formación de AFB_1, AFB_2, AFG_1 y AFG_2 entre el 83 y el 87 %.

4.2.2.2. Reducción de micotoxinas en tortas de trigo

También se evaluó la utilización de ITCs para reducir la contaminación de micotoxinas en productos conservados a temperatura ambiente, como las tortas de trigo. Estas tortas se inocularon con el hongo toxigénico *A. parasiticus* y se mantuvieron a temperatura ambiente durante 15 días, tras lo cual se determinó la concentración de AFs. En este caso las muestras fueron tratadas con distintas concentraciones de OMF y YMF (0,1, 0,5 y 1 g) depositadas en placas Petri en el interior de los envases, a las que se añadió agua para promover la hidrólisis y volatilización de AITC y ρ-HBITC, respectivamente. En concordancia con el resto de los resultados, cuando la actividad proviene de la liberación de ITCs en el espacio de cabeza, el AITC fue más eficiente que el ρ-HBITC para evitar la producción de AFs. Teniendo en cuenta los tratamientos con 0,5 y 1 g de harina, las reducciones

promedio de AFs en las muestras analizadas oscilaron entre el 54 y el 87 % (0,5 g) y el 90 y el 96 % (1 g) para el AITC, y entre el 18 y el 71 % (0,5 g) y el 35 y el 82 % (1 g) para el ρ-HBITC. Este fenómeno puede relacionarse con mayor estabilidad química y volatilidad del AITC frente al ρ-HBITC (Luciano *et al*. 2011).

4.2.2.3. Dispositivo antifúngico para silos de cereales

Una vez comprobada la elevada capacidad antifúngica del AITC volatilizado en espacios de cabeza, se planteó el diseño de un dispositivo liberador de AITC para ser utilizado en los silos de cereales durante su fase de almacenamiento, etapa crítica por la posible alteración microbiana. Para ello se utilizó como matriz principal un gel de HEC al 12% adicionado con distintas cantidades de AITC en un envase de plástico perforado. Estos dispositivos se probaron en tarros de vidrio de 1 L (500 µL de AITC) y bidones de plástico de 100 L (5 mL de AITC), en los que se introdujo trigo y cebada contaminadas con *P. verrucosum* y maíz con *A. flavus* (Tabla 1).

Tabla 1. Tratamientos de AITC probados en cereales

<table>
<tr><th>Envase</th><th>Tratamiento</th><th>Cereal</th><th>Hongo</th></tr>
<tr><td rowspan="3">Bote de 1 L</td><td rowspan="3">500 µL de AITC
(500 µL/L)</td><td>Maíz</td><td>A. flavus</td></tr>
<tr><td>Trigo</td><td rowspan="2">P. verrucosum</td></tr>
<tr><td>Cebada</td></tr>
<tr><td rowspan="3">Bidón de 100 L</td><td rowspan="3">5 mL de AITC
(50 µL/L)</td><td>Maíz</td><td>A. flavus</td></tr>
<tr><td>Trigo</td><td rowspan="2">P. verrucosum</td></tr>
<tr><td>Cebada</td></tr>
</table>

4.2.2.3.1. Volatilización del gel de AITC

En el ensayo con tarros de 1 L, además de la reducción de carga fúngica y la inhibición de la síntesis de micotoxinas, se estudió la cinética de volatilización del AITC del dispositivo, así como su absorción por parte de los cereales. En el primer caso el ensayo se llevó a cabo mediante inyección de una fracción de aire del espacio de cabeza en un cromatógrafo de gases con detector de llama (GC-FID), mientras que el AITC captado por los cereales se determinó por extracción en metanol y posterior inyección en HPLC-DAD a 236 nm.

La concentración de AITC en el interior del tarro de vidrio disminuyó gradualmente desde 0,92 µL/L en el día 1 a los 0,25 µL/L en el día 30, sin que hubiese diferencias significativas desde los días 7 a 30. La concentración residual del AITC en los cereales se estudió los días 1 y 30 de tratamiento. El trigo fue la matriz más susceptible a la penetración de AITC, con 6,2 mg/Kg en el punto final del experimento, mientras que la cebada y el trigo mostraron valores de 5,9 y 2,9 mg/Kg, respectivamente.

4.2.2.3.2. Reducción de hongos y micotoxinas en cereales

La comparación de los resultados obtenidos en los modelos de 1 y 100 L mostró similitudes y algunas diferencias. A nivel microbiológico, la exposición al AITC de los cereales contaminados disminuyó la población de *A. flavus* y *P verrucosum* durante su tratamiento. En los tarros de 1 L, y transcurridos los 30 días de ensayo, el AITC redujo la población de *A. flavus* 4,4 log CFU/g y de *P. verrucosum* a niveles no detectables. En el caso de los bidones de 100 L, a tiempo final del experimento (60 días), el tratamiento con AITC demostró una reducción respecto a los controles no tratados en la población fúngica del maíz (*A. flavus*), trigo y cebada (*P. verrucosum*) de 2, 0,9 y 1,1 log CFU/g, respectivamente.

El análisis de micotoxinas en los tarros de 1 L evidenció una contaminación de 8,07 µg/Kg de AFB_1 en el maíz control, mientras que la concentración en el maíz tratado se redujo hasta 0,12 µg/Kg, es decir, un 98,5 %. El análisis de OTA en las muestras inoculadas con *P. verrucosum* no mostró una reducción significativa en el caso de la cebada, mientras que en el trigo no se detectó micotoxina en el grupo tratado ni en el control. En el caso de los bidones de 100 L, fue la AFB_1 la que no se detectó, aunque si se redujo la presencia de OTA en trigo y cebada un 78 y 92 %, respectivamente.

De forma general, el tratamiento con AITC redujo la contaminación fúngica y la síntesis de micotoxinas asociada a dicha contaminación. Las diferencias observadas y la menor efectividad en el modelo semiindustrial pueden explicarse por las diferencias metodológicas, dado que se utilizó una menor concentración de AITC (50 µL/L frente a 500 µL/L) por cuestiones de seguridad en la manipulación del compuesto y los cereales no fueron autoclavados, para recrear más fielmente las condiciones de un silo real, lo que pudo aumentar la competitividad entre otros microorganismos y modificar su crecimiento.

La aplicación del AITC para reducir el crecimiento de hongos toxigénicos y micotoxinas en cereales ha sido estudiada previamente por otros autores. Okano *et al*. (2015) evaluaron la capacidad antifúngica del AITC proveniente de extracto de semilla de mostaza comercial frente a *A. flavus* durante el almacenamiento de maíz en condiciones de silo simuladas, consiguiendo inhibir completamente el crecimiento visible del hongo y la producción de AFs con concentraciones de AITC de entre 54,6 y 21,8 µg/L. Tracz *et al*. (2017) también estudiaron la capacidad del AITC para evitar la producción de micotoxinas en maíz con tratamientos de a 50, 100 y 500 µL/L, inhibiendo la síntesis de 12 micotoxinas, incluyendo AFB_1 y OTA. Estos datos concuerdan con nuestros resultados, ya que la dosis de 50 µL/L de

AITC demostró su capacidad antifúngica inhibiendo el crecimiento de *A. flavus* y *P. verrusocum* y la síntesis de AFB_1 y OTA. Respecto a la absorción de AITC por las matrices alimentarias, Winther y Nielsen (2006) demostraron que la absorción en quesos influía positivamente en su vida útil, aumentándola de 4 a 28 semanas.

4.3. Referencias

Azaiez, I., Meca, G., Manyes, L., Fernández-Franzón, M., 2013. Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate in vitro and their use for fumonisins reduction in bread. Food Control 32:428–434

Blankson, G.K., Mill-Robertson, F.C., 2016. Aflatoxin contamination and exposure in processed cereal-based complementary foods for infants and young children in greater Accra, Ghana. Food control. 64:212-217.

Blesa, J., Marin, R., Lino, C.M., Mañes, J., 2012. Evaluation of enniatins A, A1, B, B1 and beauvericin in Portuguese cereal-based foods. Food Addit. Contam. Part A 29, 1727-1735.

Cano-Sancho, G., Marin, S., Ramos, A..J, Sanchis, V., 2012. Occurrence of zearalenone, an oestrigenc mycotoxin, in Catalonia (Spain) and exposure assessment. Food Chem. Toxicol. 50:835-839.

EC, 2006. Regulation (EC) Nº 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Comm. L70, 12e34.

EFSA, 2014. European Food Safety Authority. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA J. 12 (12), 1e107, 3916. Available at: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3916.pdf.

García-Moraleja, A., Font, G., Mañes, J., Ferrer, E., 2015. Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults. *Food and Chemical Toxicology*, 86, 225-233.

Herzallah, S., Holley, R., 2012. Determination of sinigrin, sinalbin, allyl- and benzyl isothiocyanates by RP-HPLC in mustard powder extracts. LWT—Food Sci Technol 47:293–299.

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.

Juan, C., Mañes, J., Raiola, A., Ritieni, A., 2013a. Evaluation of beauvericin and enniatins in Italian cereal products and multicereal food by liquid chromatography coupled to triple quadrupole mass spectrometry. Food Chem. 140, 755-762.

Juan, C., Ritieni, A., Mañes, J., 2013b. Occurrence of Fusarium mycotoxins in Italian cereal and cereal products from organic farming. Food Chem. 141, 1747-1755.

Kanemaru, K., Miyamoto, T., 1990. Inhibitory effects on the growth of several bacteria by brown mustard and allylisothiocyanate. Nippon Shokuhin Kogyo Gakkaishi 1990, 37, 823–829. (In Japanese)

Klarić, M.S., Cvetni, Z., Pepeljnjak, S., Kosalec, I., 2009. Co-occurrence of aflatoxins, ochratoxin A, fumonisins, and zearalenone in cereals and feed, determined by competitive direct enzyme-linked immunosorbent assay and thin-layer chromatography. Arch. Ind. Hyg. Toxicol. 60, 427-434.

MAGRAMA, 2015. Alimentary Statistics - Database of Household Consumption.http://www.magrama.gob.es/es/alimentacion/temas/consumo-ycomercializacion-y-distribucion-alimentaria/panel-de-consumo-alimentario/base-de-datos-deconsumo-en-hogares/consulta.asp.

Mañes, L., Luciano, F.B., Mañes, J., Meca, G., 2015. In vitro antifungal activity of allyl isothiocyanate (AITC) against Aspergillus parasiticus and Penicillium expansum and evaluation of the AITC estimated daily intake. Food Chem. Toxicol. 83, 293–299.

Prestera, T., Fahey, J.W., Holtzcaw, W.D., Abeygunawardana, C., Kachinski, J.L., Talalay, P., 1996. Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolates. Analytical Biochemistry, 239, 168-179.

Saladino, F., Quiles, J.M., Mañes, J., Fernández-Franzón, M., Luciano, F.B., Meca, G., 2016a. Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain. LWT - Food Science and Technology, 75, 697-701.

Saladino, F., Bordin, K., Manyes, L., Luciano, F.B., Mañes, J., Fernández-Franzón, M., Meca, G., 2016b. Reduction of the aflatoxins B1, B2, G1 and G2 in Italian piadina by isothiocyanates. LWT - Food Science and Technology 70, 302-308.

Saladino, F., Manyes, L., Luciano, F.B., Mañes, J., Fernández-Franzón, M., Meca, G., 2016c. Bioactive compounds from mustard flours for the control of patulin production in wheat tortillas. LWT - Food Science and Technology 66, 101-107.

Serrano, A.B., Font, G., Mañes, J., Ferrer, E., 2013. Emerging Fusarium mycotoxins in organic and conventional pasta collected in Spain. Food Chem. Toxicol. 51, 259-266.

Serrano, A.B., Font, G., Ruiz, M.J., Ferrer, E., 2012b. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. Food Chem. 135, 423-429.

Serrano, A.B., Meca, G., Fon,t G., Ferrer, E., 2012a. Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin. Food control. 28, 178-183.

Sirot, V., Fremy, J.M., Leblan, J.C., 2013. Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food Chem. Toxicol. 52, 1-11.

Tao. C., He, B., 2004. Isolation of intact glucosinolates from mustard seed meal to increase the sustainability of biodiesel utilisation. ASAE Annual International Meeting, pp 6703–6713.

Graumann G., Holley, R.A., 2008. Inhibition of *E. coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. J. Food Prot. 71, 486–493.

Hontanaya, C., Meca, G., Luciano, F.B., Manes, J., Font, G., 2015. Inhibition of aflatoxin B1, B2, G1 and G2 production by *Aspergillus parasiticus* in nuts using yellow and oriental mustard flours. Food control 47: 154-160.

Nielsen, P.V., Rios, R., 2000. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. Int. International Journal of Food Microbiology, 60, 219-229.

Mejía-Garibay, B., Palou, E., López-Malo, A., 2015. Composition, Diffusion, and Antifungal Activity of Black Mustard (*Brassica nigra*) Essential Oil When Applied by Direct Addition or Vapor Phase Contact. Journal of Food Protection, 4, 636-858.

Luciano, F.B., Belland J, Holley RA, 2011. Microbial and chemical origins of the bactericidal activity of yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening. Int J Food Microbiol 145:69–74

Okano, K., Ose, A., Takai, M., Kaneko, M., Nishioka, C., Ohzu, Y., Odano, M., Sekiyama, Y., Mizukami, Y., Nakamura, N. *et al*., 2015. Inhibition of aflatoxin production and fungal growth on stored corn by allyl isothiocyanate vapor. J. Food Hyg. Soc. Jpn. 56, 1–7.

Tracz, B.L., Bordina, K., Nazareth, T.M., Costa, L.B., Macedo, R.E.F., Meca, G., Luciano, F.B., 2017. Assessment of allyl isothiocyanate as a fumigant to avoid mycotoxin production during corn storage. LWT - Food Science and Technology, 75, 692–696.

Winther, M., Nielsen, P.V., 2006. Active packaging of cheese with allyl isothiocyanate, an alternative to modified atmosphere packaging. J. Food Prot. 69, 2430–2435.

5. CONCLUSIONS

Conclusiones

5. CONCLUSIONES

1. Se detectaron Aflatoxinas (AFs), zearalenona (ZEA), eniatinas (ENs) y beauvericina (BEA) en el 50, 100, 100 y 3 % de las masas de pizza refrigeradas analizadas.
2. Aunque los valores de ingesta diaria estimada (EDI) fueron más bajos que las ingestas diarias tolerables (TDI), el riesgo de exposición no debe despreciarse ya que el 12 % de las muestras superaba los límites máximos legislados para AFs y la ZEA.
3. Los ensayos en medio sólido demostraron la mayor actividad antifúngica de la harina amarilla frente a la oriental cuando la exposición se produce por contacto directo y no por la volatilización de los isotiocianatos. Extractos concentrados de harina de mostaza amarilla presentaron unas concentraciones mínimas inhibitorias (MIC) frente a diversos hongos de los géneros *Aspergillus*, *Penicillium* y *Fusarium* de entre 238 µg/mL para *P. camemberti* y 15.000 µg/mL para *A. flavus, A. parasiticus y A. carbonarius*.
4. Las concentraciones mínimas fungicidas (MFCs) oscilaron desde 1.875 µg/mL para *P. nordicum*, *P. commune* y *P. brevicompactum* hasta los 15.000 µg/mL para *A. flavus*, *A. parasiticus* y *A. carbonarius*.
5. La utilización de harina de mostaza amarilla como ingrediente en la formulación de panes de molde a dosis de 8 g/Kg fue capaz de reducir un 67 % el crecimiento de *A. flavus* y un 97 % el de *P. nordicum* respecto a los panes formulados con propionato sódico.
6. El uso de harina de mostaza amarilla como ingrediente en la formulación de panes de molde a dosis de 8 g/Kg también fue capaz de alargar su vida útil respecto a los panes formulados con propionato sódico 7 dias en el caso de *A. flavus* y 5 días en el caso de *P. nordicum*.

7. En los panes de molde contaminados con *A. flavus*, la utilización de harina de mostaza amarilla a 6 g/Kg redujo la formación de AFs en valores equivalentes a los panes con propionato sódico, mientras que en las muestras tratadas con harina de mostaza amarilla a 8 g/Kg no se detectaron AFs tras 10 días desde la inoculación.
8. Las masas de pizza refrigeradas contaminadas con *A. parasiticus* y expuestas a una atmosfera de alil isotiocianato (AITC) y harina de mostaza oriental produjo una reducción significativa del crecimiento fúngico. 10 µL de AITC y 850 mg de harina de mostaza oriental lograron una reducción de los valores de AFs igual o superior al empleo en la formulación de la masa de 2 g de ácido sórbico y propionato cálcico.
9. A iguales concentraciones, el AITC redujo un 96,2 % la presencia de AFs en tortas de trigo contaminadas por *A. parasiticus*, mientras que el p-hridroxibencil isotiocianato lo hizo en el 69,2 %, lo que justifica la mayor actividad antifúngica volátil de la harina de mostaza oriental frente a la amarilla.
10. El AITC en geles de hidroxietil celulosa presenta capacidad para reducir la población de *A. flavus* 4,4 log CFU/g e inhibir completamente el desarrollo de *P. verrucosum* en cereales contenidos en frascos a los 30 días.
11. El escalado del dispositivo de antifúngico de AITC en bidones de 100 L evidenció una reducción de la población de *A. flavus* en maíz y de *P. verrucosum* en trigo y cebada de 2, 0,9 y 1,1 log CFU/g respectivamente; así como una disminución de OTA del 78 % en trigo y del 92 % en cebada.

5. CONCLUSIONS

1. Aflatoxins (AFs), zearalenone (ZEA), eniatins (ENs) and beauvericin (BEA) were detected in 50, 100, 100 and 3% of the cooled pizza doughs analyzed.
2. Although the estimated daily intake (EDI) were lower than tolerable daily intakes (TDI), the risk of exposure should not be disregarded since 12% of the samples exceeded the maximum limits legislated for AFs and ZEA.
3. Tests in solid medium showed the highest antifungal activity of yellow mustard flour compared to the oriental mustard flour when the exposure is produced by direct contact and not by the volatilization of the isothiocyanates. Concentrated extracts of yellow mustard flour showed minimal inhibitory concentrations (MIC) against various fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium* of between 238 µg/mL for *P. camemberti* and 15,000 µg/mL for *A. flavus*, *A. parasiticus* and *A. carbonarius*.
4. The minimum fungicidal concentrations (MFCs) ranged from 1,875 µg / mL for *P. nordicum*, *P. commune* and *P. brevicompactum* to 15,000 µg/mL for *A. flavus*, *A. parasiticus* and *A. carbonarius*.
5. The use of yellow mustard flour as an ingredient in the formulation of loaf breads at doses of 8 g/kg was able to reduce the growth of *A. flavus* by 67% and that of *P. nordicum* by 97% compared to breads formulated with sodium propionate.
6. The use of yellow mustard flour as an ingredient in the formulation of loaf loaves at a dose of 8 g/kg was also able to extend their shelf-life compared to the breads formulated with sodium propionate by 7 days in the case of *A. flavus* and 5 days in the case of *P. nordicum*.
7. In loaf breads contaminated with *A. flavus*, the use of yellow mustard flour at 6 g/kg reduced the formation of AFs in values equivalent to the bread with

sodium propionate, while in the samples treated with yellow mustard flour at 8 g/Kg no AFs were detected after 10 days from the inoculation.

8. Refrigerated pizza doughs contaminated with *A. parasiticus* and exposed to an allyl isothiocyanate (AITC) and oriental mustard flour atmosphere produced a significant reduction in fungal growth. 10 µL of AITC and 850 mg of oriental mustard flour achieved a reduction of the AF values equal to or higher than the use in the dough formulation of 2 g of sorbic acid and calcium propionate.
9. At the same concentrations, the AITC reduced the presence of AFs in wheat tortitas contaminated by *A. parasiticus* by 96.2 %, while the p-hridroxybenzyl isothiocyanate did so in 69.2 %, which justifies the higher volatile antifungal activity of oriental mustard flour compared to the yellow mustard.
10. The AITC in hydroxyethyl cellulose gels has the capacity to reduce the population of A. flavus 4.4 log CFU/g and to completely inhibit the development of *P. verrucosum* in cereals contained in 1 L bottles at 30 days.
11. The scaling of the AITC antifungal device in 100 L plastic drums showed a reduction in the population of *A. flavus* in corn and *P. verrucosum* in wheat and barley of 2, 0.9 and 1.1 log CFU/g respectively; as well as a decrease of OTA of 78 % in wheat and 92 % in barley.

ANNEX

Anexo

Food and Chemical Toxicology 94 (2016) 19–24

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population

Juan Manuel Quiles, Federica Saladino, Jordi Mañes, Mónica Fernández-Franzón, Giuseppe Meca*

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form
18 May 2016
Accepted 19 May 2016
Available online 21 May 2016

Keywords:
Mycotoxins
Pizza dough
Deterministic risk assessment
Estimated daily intake
Tolerable daily intake

ABSTRACT

Mycotoxins are toxic metabolites produced by filamentous fungi, as *Aspergillus, Penicillium* and *Fusarium*. The first objective of this research was to study the presence of mycotoxins in 60 samples of refrigerated pizza dough, by extraction with methanol and determination by liquid chromatography associated with tandem mass spectrometry (LC-MS/MS). Then, the estimated dietary intakes (EDIs) of these mycotoxins, among the Spanish population, was calculated and the health risk assessment was performed, comparing the EDIs data with the tolerable daily intake values (TDIs). The mycotoxins detected in the analyzed samples were aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), zearalenone (ZEA), enniatin A (ENA), enniatin A_1 (ENA_1), enniatin (ENB), enniatin B_1 (ENB_1) and BEA (beauvericin) with average concentration of the positive samples of 4.09 μg/kg, 0.50 μg/kg, 0.79 μg/kg, 77.78 μg/kg, 14.96 μg/kg, 4.54 μg/kg, 3.37 μg/kg, 1.69 μg/kg and 22.39 μg/kg, respectively. The presence of ZEA, ENA_1, ENB and ENB_1 was detected in 100% of the samples, AFB_2 in 32%, AFB_1 in 23%, ENA in 8% and BEA in 3%. Twelve percent of the samples contaminated with AFB_1 and 12% of the doughs contaminated with ZEA exceeded the EU legislated maximum limits. The dietary intakes were estimated considering three different age groups of population, and the EDIs calculated for the mycotoxins detected in the samples were all below the established TDI.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Mycotoxins are a group of secondary metabolites produced by fungi, mainly by the genera *Aspergillus, Penicillium, Alternaria, Fusarium*, and *Claviceps*. Mycotoxins may contaminate cereals and other food commodities at pre-harvest, harvest and post-harvest and their presence is largely dependent on environmental factors that affect fungal growth (Zain, 2011).

The risk of human exposure to mycotoxins is related to consumption of contaminated food, which may cause diseases and can also lead to death (Erdogan, 2004). A limited number of more than 400 known mycotoxins are generally considered to play important roles in food safety (Reddy et al., 2010; Streit et al., 2012) because they can be responsible for pathophysiological changes like neurotoxicity, nephrotoxicity, hepatotoxicity, neurological cardiac lesions, gastrointestinal disorders, Balkan endemic nephropathy, tubulonefritis and so forth (Marin et al., 2013). There is much concern about chronic effects of mycotoxins by low levels of exposure, and some of them have been classified by the International Agency for Research on Cancer (IARC, 2013) as human carcinogens [Group 1: aflatoxins (AFs)] or probably human carcinogens or possible carcinogen to humans [Group 2B: fumonisin B_1 (FB_1) and ochratoxin A (OTA)].

The most important mycotoxins detected in food are: AFs, OTA, trichothecenes (TCs) (type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)), zearalenone (ZEA), FB_1 and fumonisin B_2 (FB_2), and the emerging mycotoxins fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs) (Marin et al., 2013).

Maximum levels of contamination have been established by The European Commission for some mycotoxins found in cereals and derived products, in particular: 2 μg/kg for aflatoxin B_1 (AFB_1) and 4 μg/kg for total AFs (European Commission, 2010); 750 μg/kg for DON (European Commission, 2006); 1000 μg/kg for the sum of FB_1 and FB_2 in maize-based foods for direct human consumption (European Commission, 2007); 3 μg/kg for OTA (European

* Corresponding author.
E-mail address: giuseppe.meca@uv.es (G. Meca).

http://dx.doi.org/10.1016/j.fct.2016.05.011
0278-6915/© 2016 Elsevier Ltd. All rights reserved.

Food and Chemical Toxicology 83 (2015) 222–228

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Influence of the antimicrobial compound allyl isothiocyanate against the *Aspergillus parasiticus* growth and its aflatoxins production in pizza crust

Juan M. Quiles [a], Lara Manyes [a], Fernando Luciano [b], Jordi Mañes [a], Giuseppe Meca [a, *]

[a] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100, Burjassot, Spain
[b] School of Agricultural Sciences and Veterinary Medicine, Pontifícia Universidade Católica, BR 376 Km 14, São José dos Pinhais, PR, 83010-500, Brazil

A R T I C L E I N F O

Article history:
Received 16 April 2015
Received in revised form
1 June 2015
Accepted 27 June 2015
Available online 3 July 2015

Keywords:
Aspergillus parasiticus
Aflatoxins
Sinigrin
Mustard essential oil
Fresh pizza crust
Antifungal activity

A B S T R A C T

Aflatoxins (AFs) are secondary metabolites produced by different species of *Aspergillus*, such as *Aspergillus flavus* and *Aspergillus parasiticus*, which possess mutagenic, teratogenic and carcinogenic activities in humans. In this study, active packaging devices containing allyl isothiocyanate (AITC) or oriental mustard flour (OMF) + water were tested to inhibit the growth of *A. parasiticus* and AFs production in fresh pizza crust after 30 d. The antimicrobial and anti-aflatoxin activities were compared to a control group (no antimicrobial treatment) and to a group added with commercial preservatives (sorbic acid + sodium propionate). *A. parasiticus* growth was only inhibited after 30 d by AITC in filter paper at 5 μL/L and 10 μL/L, AITC sachet at 5 μL/L and 10 μL/L and OMF sachet at 850 mg + 850 μL of water. However, AFs production was inhibited by all antimicrobial treatments in a dose-dependent manner. More importantly, AITC in a filter paper at 10 μL/L, AITC sachet at 10 μL/L, OMF sachet at 850 mg + 850 μL of water and sorbic acid + sodium propionate at 0.5–2.0 g/Kg completely inhibited AFs formation. The use of AITC in active packaging devices could be a natural alternative to avoid the growth of mycotoxinogenic fungi in refrigerated bakery products in substitution of common commercial preservatives.
© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Aflatoxins (AFs) (Fig. 1) are highly toxic secondary metabolites produced by some species of *Aspergillus*, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Wejdan et al., 2010). AFs are of great concern because of their toxicity for the health of humans and animals, including carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Zinedine and Mañes, 2009). Aflatoxin B1 (AFB1) is the most potent hepatocarcinogen known for mammals and it is classified by the International Agency of Research on Cancer as a Group 1 carcinogen (IARC, 1993).

The process of mold contamination is complex and starts in the field where crops first become infected by Aspergilli residing on the soil and/or on decaying plant residues. Plant stress or damage allied to a warm and humid environment increase susceptibility of crops to fungal growth (Cotty et al., 2008). These mycotoxin-producing species can grow on a wide range of agricultural commodities both in the field and during storage (Zinedine and Mañes, 2009). Major food products affected are nuts, dried fruits, spices, crude vegetable oils, cocoa beans, maize, wheat, barley and food products composed by cereal flours (Iamanaka et al., 2007; Imperato et al., 2011; Luttfullah and Hussain, 2011).

Fungal contaminations are usually determinant for the shelf-life of refrigerated pizza when it is stored aerobically. Pinho et al. (2000) have demonstrated that the most prevalent genus of contaminant fungi in pre-cooked pizza crust were *Penicillium* and *Aspergillus*. This was a study performed in Southern Brazil, where the authors also found the presence of ochratoxin A (confirmed, and aflatoxins B_1 and B_2 (presumptive) in refrigerated pizza crusts after 45 d of production, which poses as a serious risk for human consumption.

Glucosinolates are β-thioglycosides found as secondary metabolites of Cruciferous plants, including Brussels sprouts, broccoli, cauliflower, cabbage, watercress, oilseed rape and mustard (Johnson, 2002; Thornalley, 2002). Glucosinolates are converted to the corresponding aglycone by myrosinase hydrolysis

* Corresponding author. Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of València, Av. Vicent Andrés Estellés s/n, 46100, Burjassot, Spain.
E-mail address: giuseppe.meca@uv.es (G. Meca).

http://dx.doi.org/10.1016/j.fct.2015.06.017
0278-6915/© 2015 Elsevier Ltd. All rights reserved.

J Food Sci Technol
DOI 10.1007/s13197-015-1876-2

ORIGINAL ARTICLE

Effect of the oriental and yellow mustard flours as natural preservative against aflatoxins B_1, B_2, G_1 and G_2 production in wheat tortillas

Juan Manuel Quiles[1] · Lara Manyes[1] · Fernando Bittencourt Luciano[2] · Jordi Mañes[1] · Giuseppe Meca[1]

Revised: 7 May 2015 / Accepted: 13 May 2015
© Association of Food Scientists & Technologists (India) 2015

Abstract Reduction of the AFs produced by *Aspergillus parasiticus* CECT 2681 in wheat tortillas by isothiocyanates (ITCs) from oriental and yellow mustard flours was evaluated in this study. Polyethylene plastic bags were introduced with wheat tortillas contaminated with *A. parasiticus* and treated with 0, 0.1, 0.5 or 0.1 g of either oriental or yellow mustard flour added with 2 ml of water. The wheat tortillas were stored at room temperature during 1 month. The quantification of the AFs produced was analyzed by liquid chromatography (LC) coupled to the mass spectrometry detection in tandem (MS/MS). Gaseous allyl isothiocyanate (AITC) from oriental mustard was more effective than p-hydroxybenzyl isothiocyanate (p-HBITC) from yellow mustard to inhibit the production of AFs. More importantly, 1 g of AITC was able to reduce >90 % of AFs B_1, B_2, G_1 and G_2. p-HBITC is less stable and volatile than AITC, leading to a much lower AFs (average of 17.7 to 45.2 %). Further studies should investigate the use of active packaging using oriental mustard flour and water to reduce the production of AFs by *Aspergillus* species in bakery goods.

Keywords Aflatoxins · Glucosinolates · Isothiocyanates · Mustard flours · Mycotoxin reduction · LC-MS/MS

✉ Giuseppe Meca
giuseppe.meca@uv.es

[1] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

[2] School of Agricultural Sciences and Veterinary Medicine, Pontifícia Universidade Católica do Paraná, BR 376 Km 14, São José dos Pinhais 83010-500, Brazil

Introduction

Aflatoxins (AFs, Fig. 1) are naturally occurring mycotoxins produced by many species of *Aspergillus*, most notably *A. flavus* and *A. parasiticus*, during their growth on foods and animal feed (Williams et al. 2004). They are listed as group I carcinogens by the International Agency for Research on Cancer (IARC), which primarily affect the liver (Lee et al. 2004). Although more than 20 AFs have been identified, the major AFs of concern are known as B_1 (AFB_1), B_2, G_1 and G_2. Among them, AFB_1 is normally the most prevalent toxin and the most toxic (Delmulle et al. 2005; Zhang et al. 2009). As a result, many countries have regulatory limits for AFB_1 levels in agricultural products. The European Union (EU) sets the limits for AFB_1 and for total aflatoxins (B_1, B_2, G_1 and G_2) in nuts, dried fruits, cereals and spices. These limits vary according to the product, but range from 2 to 8 μg/kg for AFB_1 and from 4 to 15 μg/kg for total aflatoxins (Van Egmond 1995).

Human exposure to AFs can occur directly from ingestion of contaminated foods or indirectly by the consumption of meat/dairy products from animals previously exposed to AFs. AFs are extremely toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated in human hepatic and extra hepatic carcinogenesis (Iqbal et al. 2012).

Isothiocyanates (ITCs) consist of aliphatic and aromatic compounds generated from the hydrolysis of glucosinolates (GLCs) by myrosinase in cruciferous vegetables such as cauliflower, broccoli, cabbage, horseradish and mustard (Ciska and Pathak 2004; Delaquis and Mazza 1995; Whitmore and Naidu 2000). Myrosinase and GLCs are physically separated within plant cells. Hydrolysis of GLCs occurs when plant tissues are disrupted in the presence of moisture, forming ITCs, nitriles, thiocyanates or epithionitriles depending on environmental conditions (Al-Gendy et al. 2010; Delaquis and

Published online: 14 June 2015

Article

Aflatoxins and *A. flavus* Reduction in Loaf Bread through the Use of Natural Ingredients

Juan M. Quiles [1,†], Raquel Torrijos [1,†], Fernando B. Luciano [2], Jordi Mañes [1] and Giuseppe Meca [1,*]

1 Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; juan.quiles@uv.es (J.M.Q.); tocara@alumni.uv.es (R.T.); jorge.manes@uv.es (J.M.)

2 School of AgriculturalSciences and Veterinary Medicine, PontifíciaUniversidade Católica do Paraná, BR 376 Km 14, 83010-500 São José dos Pinhais, Brazil; fernando.luciano@pucpr.br

* Correspondence: giuseppe.meca@uv.es; Tel.: +34-963544959; Fax: +34-96354954

† These authors contributed equally to this work.

Received: 6 June 2018; Accepted: 3 July 2018; Published: 4 July 2018

Abstract: In this study, the antifungal activity of yellow mustard (YMF) and oriental mustard (OMF) meal extracts against 14 strains of fungi was tested on a solid medium. The results obtained with the YMF were next confirmed in liquid medium determining the minimum inhibitory concentration (MIC) and the minimum fungicide concentration (MFC). Finally, the use of YMF as a natural preservative to extend the useful life of bread was evaluated. Breads with different concentrations of YMF (2, 4, 6 and 8 g/kg) were prepared and contaminated with *Aspergillus flavus* ISPA 8111 and *Penicillium nordicum* CECT 2320. For 10 days the formation of mycelium was observed, and after that the fungal growth and the mycotoxins production was determined. The results obtained with the YMF were compared with breads treated with the commercial additive sodium propionate (E-281). The results showed a significant reduction of the fungal population using 6 g/kg and 8 g/kg of YMF in bread contaminated with *A. flavus* and with *P. nordicum* and an extensions of the breads shelf life of 7 and 5 days, respectively, in comparison with the control experiment. A reduction of 78% of AFB_1 was observed using 6 g/kg of YMF while no AFB_1 production was detected employing 8 g/kg of YMF in bread preparation.

Keywords: aflatoxins; shelf life; mustard flour; mycotoxin reduction; LC-MS/MS

1. Introduction

Aflatoxins (AFs) (Figure 1) are difuranocoumarin derivatives mainly produced through the polyketide pathway by two species of *Aspergillus* fungi which are especially found in areas with hot and humid climates. *Aspergillus flavus* is ubiquitous in Nature, preferring the colonization of the aerial parts of the plants (leaves, flowers) and usually producing group B AFs. *Aspergillus parasiticus* which produces both B and G AFs, is more adapted to soil environments and has more limited distribution [1]. *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* are also AF-producing species, but they are found less frequently [2].

The four main AFs are AFB_1, AFB_2, AFG_1 and AFG_2. They can directly contaminate agricultural products and other foodstuffs under pre-and post-harvest conditions. AFB_1 is usually predominant in crops as well as in food products and it has been found to exhibit the greatest toxigenic potential [3]. The AFs can be classified from highest to lowest toxicity in the following order: AFB_1, AFG_1, AFB_2 and AFG_2, and this is probably explained by the presence of epoxidation at the 8,9 double bond, as well as by the greater power that accompanies the ring. AFB_1 is one of the most potent toxic carcinogens,

Article

Development of an Antifungal and Antimycotoxigenic Device Containing Allyl Isothiocyanate for Silo Fumigation

Juan Manuel Quiles [1,†], Tiago de Melo Nazareth [1,2,†], Carlos Luz [1], Fernando Bittencourt Luciano [2], Jordi Mañes [1] and Giuseppe Meca [1,*]

1. Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain; juan.quiles@uv.es (J.M.Q.); demena@alumni.uv.es (T.d.M.N.); carlos.luz@uv.es (C.L.); jorge.manes@uv.es (J.M.)
2. School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil; fernando.luciano@pucpr.br

* Correspondence: giuseppe.meca@uv.es; Tel.: +34-963-544-959; Fax: +34-963-549-54

† Authors have contributed equally to the study and should be considered co-first authors.

Received: 29 January 2019; Accepted: 23 February 2019; Published: 1 March 2019

Abstract: The aims of this study were to evaluate the antifungal activity of the bioactive compound allyl isothiocyanate (AITC) against *Aspergillus flavus* (8111 ISPA) aflatoxins (AFs) producer and *Penicillium verrucosum* (D-01847 VTT) ochratoxin A (OTA) producer on corn, barley, and wheat. The experiments were carried out initially in a simulated silo system for laboratory scale composed of glass jars (1 L). Barley and wheat were contaminated with *P. verrucosum* and corn with *A. flavus*. The cereals were treated with a hydroxyethylcellulose gel disk to which 500 μL/L of AITC were added; the silo system was closed and incubated for 30 days at 21 °C. After that, simulated silos of 100 L capacity were used. Barley, wheat, and corn were contaminated under the same conditions as the previous trial and treated with disks with 5 mL of AITC, closed and incubated for 90 days at 21 °C. In both cases, the control test did not receive any antifungal treatment. The growth of the inoculated fungi and the reduction in the formation of AFs and OTA were determined. In the lab scale silo system, complete inhibition of fungal growth at 30 days has been observed. In corn, the reduction of aflatoxin B1 (AFB_1) was 98.5%. In the 100 L plastic drums, a significant reduction in the growth of *A. flavus* was observed, as well as the OTA formation in wheat (99.5%) and barley (92.0%).

Keywords: *Aspergillus flavus*; *Penicillium verrucosum*; AITC; fungal growth reduction; mycotoxin reduction

Key Contribution: The application of an allyl isothiocyanate device, as an antifungal system, to be applied in the storage of corn, wheat, and barley, to reduce the mycotoxigenic fungal growth and the mycotoxin biosynthesis.

1. Introduction

AFs are the foremost harmful category of mycotoxins naturally produced by *Aspergillus* species such as *Aspergillus nomius*, *Aspergillus flavus*, and *Aspergillus parasiticus* during pre- or postharvest of crops [1,2]. The most important and toxic aflatoxins are the AFB_1, AFB_2, AFG_1, and AFG_2 [3]. Among these compounds, the AFB_1 has been classified in Group 1 of the risk of the carcinogen molecules by the International Agency for Research on Cancer [4], and it has been implicated with the development of human hepatic and extra hepatic carcinogenesis [5]. Human exposure to AFs could be due to the intake of contaminated food or by the consumption of milk, meat, and eggs from animals that consumed contaminated feed [6]. The occurrence of AFs in foods from in the Spanish market has been