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EVALUACIÓN DE LA PRESENCIA DE OCRATOXINA A Y AFLATOXINAS EN ALIMENTOS

Tesis Doctoral

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Jorge Mañes Vinuesa, Catedrático del Departamento de Medicina Preventiva i Salud Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal y Juan Carlos Moltó Cortés, Profesor Titular del mismo Departamento,

Informan que:

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La toxicidad de ciertos hongos es conocida desde hace siglos, sin embargo el riesgo potencial de los metabolitos tóxicos de estos hongos no fue reconocido hasta 1850 cuando se descubrió la asociación entre ingestión de cereales infectados por *Claviceps purpurea* y las manifestaciones del ergotismo. Posteriormente, esta relación también fue encontrada entre la estachibotriotosicosis y el consumo de pan infectado por *Fusarium graminearum* y entre la aleukia alimentaría tóxica y la ingestión de granos infectados con *Fusarium poae* y *Fusiarium sporotrichioides* (1).

La contaminación de cosechas, alimentos y piensos por hongos constituye un problema higiénico sanitario a nivel mundial. Los hongos pueden invadir los alimentos desde el campo o en el almacén, provocando una disminución de la calidad nutritiva y organoléptica de los alimentos invadidos, además, ciertos hongos poseen la capacidad de sintetizar micotoxinas, substancias químicas altamente peligrosas para la salud humana y animal. En cierta manera todos los productos vegetales pueden servir como sustrato para el crecimiento fúngico con la posible contaminación por micotoxinas.

La exposición en humanos puede darse por la ingesta de alimentos contaminados con hongos micotoxigénicos, por la ingesta de carne y leche con

residuos de micotoxinas provenientes de animales alimentados con piensos contaminados y por exposición laboral dentro de la industria de cereales y frutos secos, y en los laboratorios de análisis e investigación (2).

De la gran variedad de hongos existentes en los diversos ecosistemas, los principales géneros que producen micotoxinas son *Aspergillus*, *Fusarium* y *Penicillium*, siendo las especies más reconocidas *A. flavus*, *A. parasiticus*, *A. ochraceus*, *F. moniliforme*, *F. roseum*, *P. viridicatum* y *P. expansum* por la generación de productos, altamente hepatotóxicas, nefrotóxicas, inmunodepresoras y cancerígenas para los animales domésticos, aves y seres humanos, con una gran implicación sanitaria y económica a nivel mundial (3).

Tabla I.1: Síndromes tóxicos asociados a micotoxinas

SÍNDROME	MICOTOXINAS RESPONSABLES
Hemorrágico	Aflatoxinas, patulina, rubratoxina, T-2 y ác glaucónico.
Hepatorenal	Aflatoxinas, ocratoxina A, fumonisinas, luteoskirina, ác. ciclopiazonico, rubratoxina y citrinina.
Nefropático	Ocratoxina A.
Reproductivo	Fusariogenina y zearalenona.
Subcutáneo	Zearalenona.
Nervioso	Fumonisinas, citreoviridina, patulina, fenicina y rubratoxina.
Gastrointestinal	T-2, staquibrotriotoxina, ácido oxálico, vomitoxina y derivados de tricotecenos.
Leucopénico	Fumonisinas, T-2 y diaceetoxyscirpenol.
Disminución del rendimiento zootécnico	Aflatoxinas, ocratoxina A y tricotecenos.
Inmunosupresión	Aflatoxinas, ocratoxina A y tricotecenos.

Actualmente se estima que el 25 % de los cereales del mundo están contaminados con micotoxinas conocidas, mientras que un porcentaje mayor

podría estar contaminado por toxinas aún no identificadas. No existe región alguna en todo el mundo que escape a la contaminación por micotoxinas y a su impacto negativo en la producción animal y salud humana. Entre las micotoxinas que generan una importante repercusión económica se encuentran las aflatoxinas, ocratoxinas, zearalenona, fumonisinas, tricotecenos, citrinina, patulina, sterigmatocistina y ergotoxinas (4).

La contaminación de alimentos por micotoxinas puede variar con las condiciones geográficas y climáticas, los métodos de producción y almacenamiento y el tipo de alimento. En todo el hemisferio Norte las micotoxinas más frecuentes son ocratoxina, vomitoxina y zearalenona, mientras que en Sudamérica, Centroamérica, Asia, África y Australia son aflatoxinas y fumonisinas (5).

En el campo, los cultivos, corren el riesgo de infectarse con diferentes hongos (*Fusarium*, *Alternaria*, *Cladosporium*, *Claviceps* y otros hongos endofíticos). Por lo tanto, en el momento de la cosecha estos alimentos pueden estar contaminados con micotoxinas tales como tricotecenos, zearolenona, fumonisinas, ácido tenuazónico, alternariol, alcaloides ergotamínicos, etc. Si en los silos se logra una correcta eliminación del aire en un período corto de tiempo, esta condición de anaerobiosis evita el crecimiento de hongos y la posterior síntesis de micotoxinas. Cuando la construcción y gestión del silo no es correcta se dan condiciones de aerobiosis que permiten la contaminación del material por crecimiento de hongos, especialmente con hongos de los géneros *Aspergillus* y *Penicillium*, que son potencialmente productores de toxinas tales

como aflatoxinas, esterigmatocistina, ocratoxinas, citrinina y patulina entre otras (6).

Tabla I.2: Principales mohos y micotoxinas encontradas en diversos alimentos (7)

MOHOS	MICOTOXINAS	ALIMENTOS
Aspergillus spp.	Aflatoxinas Esterigmatocistina Ocratoxina A	Cereales, maíz, arroz, frutos secos, semillas, legumbres, pasas, vino, Tejidos de animales y derivados lácteos.
Fusarium spp.	Tricotecenos Zearalenona Fumonisinas	Cereales.
Pencillium spp.	Patulina Citrinina Ocratoxina A Ácido ciclopiazónico	Frutas y zumos, arroz, queso y cereales.
Alternaria spp.	Alternariol Ácido tenuazónico	Frutas, legumbres y productos der. de manzanas y tomates.
Claviceps spp.	Alcaloides del Ergot	Cereales.

I.1.1.2. Estructura química

Desde el punto de vista estructural, las micotoxinas se pueden clasificar en 5 grupos:

1. Micotoxinas cumarínicas producidas por *Aspergillus spp.* como aflatoxinas y esterigmatocistina.

2. Micotoxinas lactónicas producidas por *Penicillum spp.* y *Aspergillus spp.* como ocratoxinas y patulina.

3. Micotoxinas lactónicas producidas por *Fusarium spp.* como la zearalenona.

4. Sesquiterpenos derivados de tricotecenos producidos por *Fusarium spp.* como nivalenol, deoxinivalenol, T-2 y diacetoxycirpanol.

5. Micotoxinas aminopolihidroxiladas producidas por *Fusarium spp.* como fumonisinas o por *Alternaria spp.* como fitotoxinas AAL o alpersinas.

Tabla I.3: Relación de micotoxinas con sus hogos productores y sus estructuras químicas (8)

MICOTOXINA	HONGO PRODUCTOR	ESTRUCTURA QUÍMICA
Aflatoxinas	*Aspergillus flavus* *A. parasiticus*	Anillo cumárico
Ocratoxinas	*Aspergillus ochraceus,* *Penicillum viridicatum*	Anillo lactónico
Fumonisinas	*Fusarium moniliforme*	Aminopolihidroxilada
Sterigmatocistina	*Aspergillus versicolor,* *A. nidulans*	Anillo cumárico
Zearalenona	*Fusarium tricinctum,* *F. moniliforme,* *F. roseum,* *F. gramineatum*	Anillo lactónico
Tricotecenos	*Fusarium spp.* *Trichothecium spp.*	Sesquiterpenos
Patulina	*Penicillum expansum* *P. patulum*	Anillo lactónico

I.1.2. Control y prevención de micotoxinas

Por el fácil crecimiento de hongos productores de micotoxinas en diversos sustratos de origen vegetal en condiciones ambientales adecuadas, la afectación de cosechas y su paso a través de la cadena alimentaría, así como sus características, tanto físico-químicas como toxicológicas hacen que el estudio de técnicas de prevención y análisis de peligros y control de puntos críticos adquieran una gran importancia en el intento de minimizar este problema.

I.1.2.1. Control antes de la cosecha

La prevención mediante la realización de unas buenas prácticas agrícolas durante la cosecha es el primer paso para asegurar un producto final inocuo y está orientada a afrontar factores críticos que potencian la producción de micotoxinas. Una vez infectado el cultivo en las condiciones reinantes en el campo, la proliferación de los hongos proseguirá durante las etapas posteriores a la cosecha, como son el almacenamiento y la transformación (9).

Algunas de las principales estrategias utilizadas son las siguientes:

1. Gestión del riego.

2. Empleo de variedades de plantas resistentes.

3. Manejo adecuado de los rastrojos o residuos agrícolas, destrucción de malezas y rotación de cultivos.

4. Control de la infestación por insectos de los granos y prevención de daños mecánicos de los productos.

I.1.2.2. Control durante la cosecha

Durante la cosecha es importante controlar, entre otras cosas, si el producto agrícola se ha desarrollado en el plazo previsto, evitando que el cultivo permanezca en el campo más tiempo del necesario, y si está limpio y seco. Este control es esencial para prevenir la formación de micotoxinas posteriormente durante el almacenamiento (10).

I.1.2.3. Control después de la cosecha y descontaminación

Los procedimientos de control después de la cosecha y de descontaminación representan un medio importante para evitar la exposición de los consumidores. La eficacia de cada método de elaboración deberá evaluarse para el producto en cuestión y para la toxina presente en el sistema (11).

Entre los criterios específicos para la evaluación y aceptación de determinados procedimientos de reducción de las micotoxinas o de descontaminación se incluyen los siguientes:

1. Inactivar, destruir o eliminar las toxinas.

2. No producir ni dejar residuos tóxicos en los alimentos o los piensos.

3. Conservar el valor nutritivo y la aceptabilidad del producto para alimento o pienso.

4. No alterar de modo apreciable las propiedades tecnológicas del producto.

5. Si es posible, destruir las esporas de los hongos.

A continuación se indican algunas estrategias habituales para después de la cosecha:

- Métodos físicos de eliminación y descontaminación de micotoxinas

Una vez que el producto contaminado llega a las instalaciones de elaboración, las primeras alternativas de control son su limpieza y segregación, donde se retira el material dañado o afectado sin alterar el producto. En algunos casos, son los mejores métodos para reducir la presencia de micotoxinas en los productos finales.

La degradación térmica permite reducir en cierto grado determinadas micotoxinas, sin embargo, algunas micotoxinas son termoestables y no se destruyen por completo a las temperaturas aplicadas durante la elaboración.

A pesar del rechazo por los consumidores de alimentos irradiados, no se ha encontrado un método plenamente satisfactorio para destruir las micotoxinas ya formadas, y la irradiación puede ser tomada en consideración como método eficaz para controlar los mohos productores de micotoxinas.

Un nuevo sistema para la prevención de la intoxicación con micotoxinas es la inclusión en los piensos de arcillas de reacción selectiva, que aglutinan firmemente estas sustancias en el aparato digestivo de los animales, reduciendo de modo apreciable su biodisponibilidad y la toxicidad asociada. Los resultados de estos estudios variaron considerablemente en función del tipo de adsorbente utilizado (arcillas, aluminosilicatos, zeolitas, glucomananos, carbón activo, polivinilpirrolidona,...), de la micotoxina estudiada y de la especie animal ensayada (12).

- *Descontaminación biológica*

Cabe señalar que los métodos biológicos que muestran propiedades efectivas de descontaminación son por lo general el resultado de compuestos específicos producidos por determinados microorganismos, ya sean enzimas o adsorbentes activos frente a micotoxinas (13).

- *Inactivación química*

Casi todos los estudios de este tipo se han centrado en las aflatoxinas relacionadas con los piensos. La amoniación es el método químico al que las

investigaciones han prestado más atención. Los resultados de una amplia evaluación de este procedimiento demuestran la eficacia e inocuidad de la amoniación como solución práctica para descontaminar piensos contaminados por aflatoxinas. Los dos procedimientos de amoniación utilizados principalmente para la contaminación por aflatoxinas en el maíz, el maní, las semillas de algodón y las harinas son el tratamiento a alta presión y alta temperatura, y el tratamiento a presión atmosférica y temperatura ambiente. Se ha informado sobre otros procedimientos químicos en los que se utilizan, por ejemplo, monometilamina y cal, urea y ureasa, cloruro sódico durante el tratamiento térmico, bisulfito sódico a diversas temperaturas, ozonización y ácido propiónico o sus sales (14).

I.1.3. Análisis de micotoxinas

La prevención y control mediante la aplicación del sistema del análisis de peligros y control de puntos críticos (APPCC) es el mejor método para evitar la entrada de las micotoxinas en la cadena alimentaría (10). Y para su diagnóstico y evaluación se precisan realizar análisis cualitativos rápidos y sensibles, además de exactos y reproducibles.

La toma de muestras es un punto importantísimo en el análisis de micotoxinas, debido a lo heterogéneo de la contaminación por estos tóxicos dentro de un mismo lote. Existe una normativa europea desarrollada expresamente para este fin, Directiva 98/53/CE y sus modificaciones (15), que

fija los métodos de toma de muestra y de análisis para el control oficial del contenido máximo de algunos contaminantes en los productos alimenticios.

Las técnicas para la detección y cuantificación de micotoxinas se encuentran en continuo desarrollo. La cromatografía de capa fina es un método de multidetección por el que se pueden investigar la mayoría de las micotoxinas de interés. La extracción de las micotoxinas se realiza en un único disolvente, utilizando posteriormente disolventes de desarrollo específicos y aplicando sistemas de identificación selectivas para cada una de las micotoxinas. La cromatografía de gases y la cromatografía líquida permiten la separación de estos tóxicos dependiendo de sus características químicas y, junto con sistemas de detección cada día más selectivos, evitan la realización de costosas purificaciones. Por otra parte se amplía el número de matrices sobre los que pueden aplicarse. Existen también métodos inmunoquímicos, como el análisis de inmunoenzimoensayo (ELISA) y las columnas de inmunoafinidad. Estos métodos simplifican el análisis y permiten el tratamiento de un mayor número de muestras (16).

Los análisis de micotoxinas constan de unas etapas predefinidas y comunes a todas ellas, que variarán dependiendo de su naturaleza química y de la matriz estudiada, estas etapas son:

Extracción: se basa en la solubilidad de las micotoxinas en disolventes orgánicos. Se pretende el paso de la micotoxina desde la matriz a una fase líquida con la cual trabajar.

Purificación: el método de limpieza se basa en tres diferentes procedimientos:

a) el uso de disolventes orgánicos mediante separación líquido-líquido

b) el uso de extracción en fase sólida

c) el uso de columnas de inmunoafinidad

Determinación: los métodos cromatográficos son los más utilizados y entre ellos la cromatografía líquida de alta resolución asociada a detectores de fluorescencia y de espectroscopía de masas.

I.1.4. Interés científico

El interés científico de las aflatoxinas y la ocratoxina A queda respaldado por la abundancia de estudios y congresos celebrados por todo el mundo, organizados por diversas instituciones tanto públicas como privadas, así como por la gran cantidad de artículos disponibles en la bibliografía y grupos de investigación dedicados a este tema. Dicho interés se debe a su toxicidad y a la dificultad para prevenir la contaminación en alimentos de gran consumo, por lo que suponen un riesgo para la salud pública y conllevan importantes repercusiones económicas y comerciales.

I.1.4.1.Toxicología

Desde los primeros brotes tóxicos en animales que dieron a conocer estas micotoxinas, la "enfermedad X" en pavos para aflatoxinas y la "nefropatía porcina" para ocratoxina A, así como la relación probada de aflatoxinas con casos de hepatocarcinomas y la de ocratoxina A con tumores renales en humanos, el estudio toxicológico de estos compuestos ha logrado grandes avances dando a conocer los diversos órganos y tejidos afectados por estas micotoxinas, así como su gravedad y evolución (17,18).

Entre los avances más destacables cabe citar el desarrollo de productos que interfieren con la absorción de las micotoxinas, muy útiles en la alimentación animal; se conocen los metabolitos y las vías de excreción más importantes, tanto en animales como en humanos; se han identificado los aductos de ADN y las dianas genéticas y metabólicas que dilucidan la tumorogénesis que provocan; se tiene en cuenta la sinergia tóxica de las combinaciones de micotoxinas presentes en los alimentos; y se estudian como diversos nutrientes o sustancias se oponen al efecto tóxico de las micotoxinas y la relación entre la situación nutricional del individuo y los efectos negativos producidos.

I.1.4.2. Seguridad alimentaría

Las micotoxinas, y concretamente aflatoxinas y ocratoxina A, representan un problema de primer orden en cuanto a seguridad alimentaria

debido a su toxicidad intrínseca, a la diversa cantidad de alimentos de gran consumo a los que afectan, productos vegetales directamente y productos animales indirectamente, por lo que se puede afirmar que la totalidad de humanidad y la cabaña animal se encuentra en posible riesgo.

Hoy en día, aunque estas micotoxinas tengan una delimitación geográfica concreta, el almacenamiento y transporte de las cosechas de alimentos susceptibles de presentar contaminación por todo el mundo, da lugar a un proceso de globalización del problema. El peligro se ve agravado en aquellos lugares donde la presencia de micotoxinas está favorecida por las condiciones medioambientales y la cultura agroalimentaria dando lugar a zonas de alto riesgo verificadas por estudios de afectación en humanos y animales.

Afrontar esta situación requiere actuaciones encaminadas a disminuir la tasa de estos contaminantes en la producción y la conservación de los alimentos para que redunde en el producto final, así como un sistema que vele por la protección de los consumidores.

I.1.4.3. Economía y sociedad

Debido a la gran cantidad e importancia de alimentos susceptibles de presentar contaminación por micotoxinas, las implicaciones económicas y sociales son enormes.

Por una parte, la cosecha afectada supone una merma de calidad del producto y una merma en su valor económico, en el mejor de los casos, se deberá recurrir a un tratamiento tecnológico para la disminución de la contaminación o bien recurrir directamente a la destrucción del producto. Por otra parte supone un problema sanitario, dado que de manera fraudulenta los productos contaminados en muchos casos se emplean en la fabricación de transformados destinados al consumo de los animales de granja, sin olvidar que en los países subdesarrollados, donde muchas comunidades no se pueden permitir esta pérdida, se comercializan para el consumo humano.

Las exigencias legislativas en cuanto a la presencia de estos contaminantes, que buscan proteger al consumidor, obligan a grandes inversiones en temas destinados a la prevención de la contaminación.

Como podemos ver en las tablas I.4 y I.5 existen valores máximos legislados en la Unión Europea para aflatoxinas y ocratoxina A en diversos grupos de alimentos. Además se encuentra en estudio fijar este límite, en el caso de la ocratoxina A, para café verde, frutos secos, cerveza, cacao y productos del cacao, vinos de licor, productos cárnicos, especias y regaliz.

A estas implicaciones socioeconómicas hay que añadir el veto, debido a la legislación proteccionista, de un país consumidor a un producto contaminado de un país productor, lo que puede llevar al desencadenamiento de crisis agrícolas y ganaderas con gravísimas repercusiones en estos sectores.

Tabla I.4: Valores máximos para aflatoxinas (19)

Producto	Contenido máximo (ng/g o ppb)		
	B_1	$B_1+B_2+G_1+G_2$	M_1
Frutos secos y derivados de consumo directo	2	4	
Frutos secos y derivados (excepto cacahuete) destinados a proceso de selección previo a su consumo	5	10	
Cacahuete destinado a proceso de selección previo a su consumo	8	15	
Cereales y derivados de consumo directo	2	4	
Cereales y derivados (excepto maíz) destinados a proceso de selección previo a su consumo	2	4	
Maíz y derivados destinados a proceso de selección previo a su consumo	5	10	
Leche			0'05
Especias (cayena, pimentón, chile, pimienta, nuez moscada, jengibre y cúrcuma)	5	10	
Alimentos infantiles a base de cereales	0'1		
Alimentos infantiles de base láctea			0'025
Alimentos dietéticos para lactantes	0'1		0'025

Tabla I.5: Valores máximos para ocratoxina A (20).

Productos	Contenido máximo (ng/g o ppb)
Cereales en grano	5
Productos derivados de cereales	3
Uvas pasas	10
Café tostado en grano o molido	5
Café soluble	10
Vino y mosto	2
Zumo y mosto de uva de consumo directo	2
Alimentos infantiles de base láctea o cereal	0'5
Alimentos dietéticos para lactantes	0'5

Otro punto a tener en cuenta, muy importante pero difícilmente cuantificable, es la derivada de la propia acción tóxica sobre el individuo, provocando una disminución en la calidad y esperanza de vida, y afectando de una manera importante a grupos de población muy susceptibles como son los niños, ancianos y enfermos en general.

I.1.4.4. Prevención

La prevención de la contaminación se encamina a lograr una disminución final en el producto de consumo, este proceso debe comenzar antes de la cosecha y extremar las precauciones en el almacenamiento y el transporte de alimentos.

También hay que tener en cuenta en este punto la necesaria prevención laboral dentro de la industria agroalimentaria para evitar la exposición de los trabajadores a estos tóxicos, siendo la más grave y estudiada la exposición por vía inhalatoria.

El control de puntos críticos y los procesos de descontaminación están sufriendo un gran desarrollo debido a la aplicación de la legislación y a lasnecesidades de importación-exportación a nivel mundial; pero tal vez se revela ineficaz en pequeñas comunidades agrícolas o zonas subdesarrolladas sin la capacidad económica necesaria para la adaptación a esta normativa, donde la prevención residiría en la educación de la comunidad dentro de un amplio programa de educación sanitaria y ambiental.

Otro tipo de prevención, a nivel individual, donde se hace necesaria la educación nutricional, reside en llevar una alimentación variada, con una ingesta adecuada de factores protectores frente a la acción tóxica de las micotoxinas, así como el consumo de productos frescos y de buena calidad.

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OBJETIVOS

Objetivos

OBJETIVOS

Las micotoxinas son compuestos orgánicos que pueden contaminar un gran número de alimentos, el crecimiento de los hongos que las producen depende de condiciones ambientales y del mismo alimento que les sirve de sustrato para su desarrollo. Entre los alimentos susceptibles de contaminación por hongos micotoxigénicos se encuentran muchos alimentos básicos y de gran consumo.

La ingesta de micotoxinas representa un grave problema de salud, destacando por su toxicidad las aflatoxinas y la ocratoxina A (OTA). Con el fin de salvaguardar la salud de los consumidores se han fijado basándose en los conocimientos toxicológicos actuales, unos niveles máximos permitidos para algunos alimentos, niveles que se encuentran en continua revisión. En el caso de las aflatoxinas, y debido a sus propiedades carcinógenas, su ingesta deberá ser tan baja como sea posible.

La contaminación de alimentos por dichas micotoxinas en concentraciones que superen los niveles máximos establecidos puede acarrear graves consecuencias económicas como la paralización de las exportaciones y la destrucción de la partida del alimento contaminado. Por ello se necesitan para su control métodos de análisis fiables, exactos y reproducibles, pero también económicos, rápidos y respetuosos con el medio ambiente, debido al elevado número de muestras a analizar.

Objetivos

El conocimiento del grado de contaminación actual de los alimentos consumidos en nuestro país permitiría estimar el nivel de ingestión de dichas micotoxinas por parte de la población.

El presente trabajo se propone como objetivos:

1. Estudiar el análisis de aflatoxinas y OTA siguiendo procedimientos de extracción con disolventes y con fases sólidas y de determinación con cromatografía líquida.

2. Investigar métodos sencillos que permitan la confirmación de resultados.

3. Explorar la utilidad de pruebas comerciales basadas en la técnica ELISA para detectar la presencia de micotoxinas.

4. Aplicar las columnas de inmunoafinidad para el aislamiento de las micotoxinas.

5. Validar y comparar los procedimientos estudiados.

6. Analizar distintos tipos de alimentos con los procedimientos de análisis seleccionados.

7. Realizar una aproximación a la estimación de las ingestas reales actuales de nuestra población.

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III.1.1. Aflatoxinas: generalidades

Las aflatoxinas son producidas por ciertas cepas de *Aspergillus flavus* y *Aspergillus parasiticus*. Estos hongos son ubicuos por lo que el riesgo de contaminación de alimentos y piensos es general. La contaminación por aflatoxinas depende de factores geográficos y zonales así como por las condiciones en las cuales se cultiva, cosecha y almacena el producto. Las condiciones óptimas para su formación son prevalentes en áreas con alta humedad y temperatura.

La actividad de agua óptima para la proliferación de *A. flavus* es alta (alrededor de 0'99), el valor máximo es al menos 0'998 y el mínimo no se ha determinado aún con precisión (aproximadamente 0'82). En general, parece que una actividad de agua alta favorece la producción de toxinas. Se ha notificado que *A. flavus* puede proliferar a temperaturas de 10 a 43ºC. La tasa de crecimiento óptima, hasta 25 mm al dia, se produce a una temperatura ligeramente superior a 30ºC. *A. flavus* produce aflatoxinas en el intervalo de temperaturas de al menos 15 a 37ºC, aunque esta producción es considerablemente mayor en el intervalo de 20 a 30ºC. Los efectos de la actividad de agua y la temperatura sobre el comportamiento de *A. parasiticus* son similares a los descritos anteriormente, con un valor mínimo de 0'83 para el crecimiento y 0'87 para la producción de alfatoxinas, así mismo se han notificado valores óptimos para el crecimiento y para la producción de aflatoxinas de 30 y 28ºC, respectivamente (1).

El uso de variedades de semillas resistentes, plaguicidas y procesos de secado y almacenamiento controlados pueden reducir la infestación por hongos, así como disminuir la contaminación por aflatoxinas. Las micotoxinas se eliminan parcialmente de los alimentos y piensos con las técnicas habituales de procesado y cocinado, y generalmente, se concentran en pequeñas porciones de las semillas que adquieren diferente color, así la segregación de estas semillas de color anómalo reduce los niveles de aflatoxinas. También se puede optar por la descontaminación química en piensos mediante agentes oxidantes y álcalis (2).

III.1.1.1. Estructura química

Las aflatoxinas químicamente son compuestos derivados de la isocumarina (bifuranocumarinas) que pueden estar acopladas a una ciclopentanona (aflatoxina B_1 y B_2) o a un anillo lactónico, (aflatoxina G_1 y G_2). En la figura III.1 se pueden observar las estructuras químicas de las aflatoxinas más comunes en los alimentos. Presentan fluorescencia al ser expuestas a luz ultravioleta de longitud de onda larga (365 nm). Se reconocen actualmente más de 20 aflatoxinas, siendo las más comunes en alimentos la B_1, B_2, G_1, G_2 y M_1. Son solubles en metanol, acetonitrilo, acetato de etilo y cloroformo, pero poco solubles en agua y en hidrocarburos. Son termoestables, alcanzando el punto de ebullición por encima de los 200°C (3).

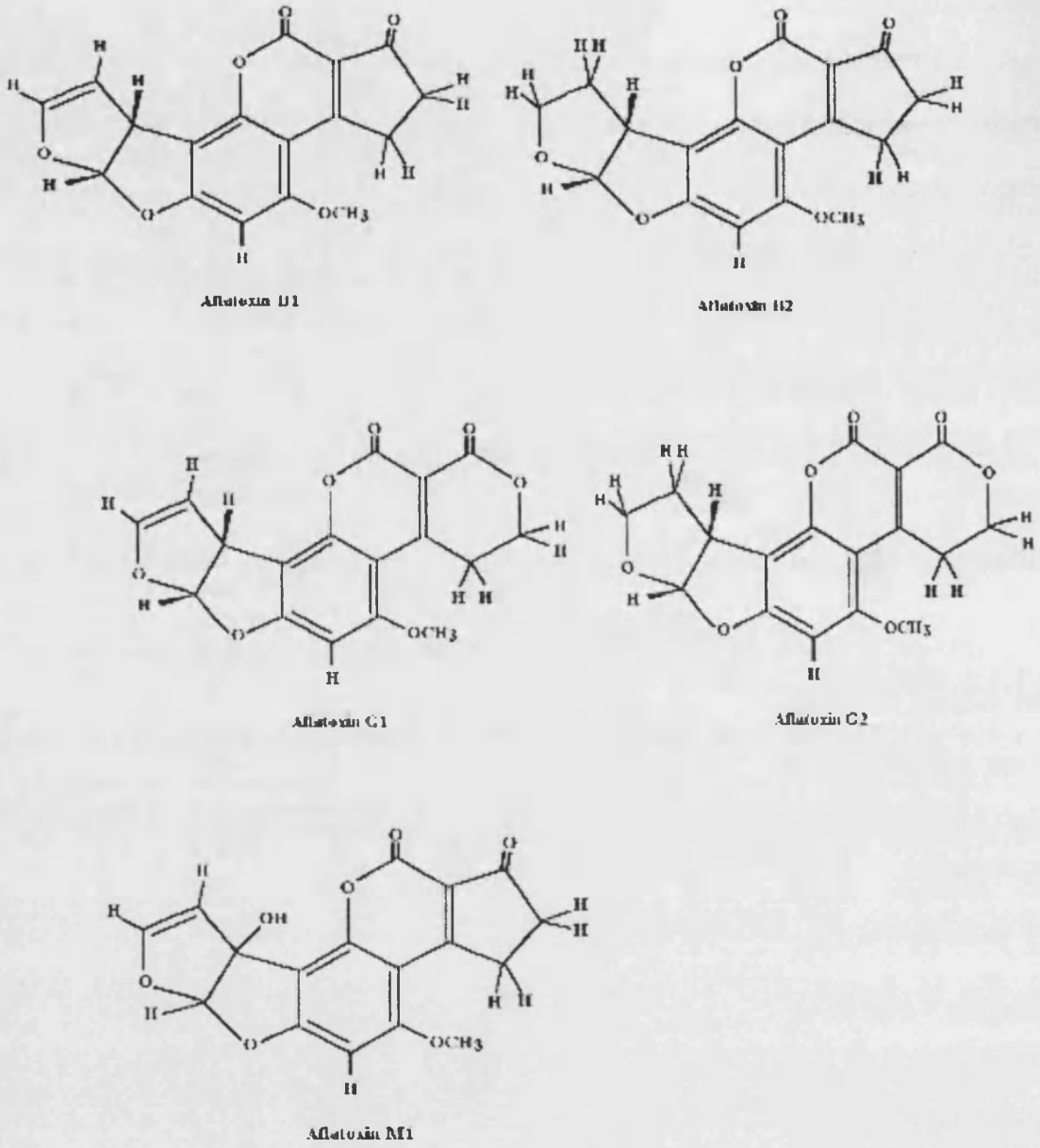


Figura III.1: Estructura química de las aflatoxinas B_1, B_2 , G_1, G_2 y M_1

III.1.1.2. Presencia en los alimentos

Cereales y frutos secos pueden ser colonizados por *Aspergillus spp.* antes de la cosecha pero generalmente la invasión se produce tras la

recolección y almacenamiento, antes del procesado, el periodo postcosecha implica un alto riesgo en lo referente a producción de aflatoxinas. Otro condicionante es el daño producido por insectos y que éstos sirvan como vectores para la infestación por *Aspergillus spp.*

En animales las aflatoxinas ingeridas se degradan metabólicamente; así, la aflatoxina B_1 se convierte en su derivado hidroxilado, aflatoxina M_1, la cual se excreta con la leche. La concentración de aflatoxina M_1 en leche de vaca es 300 veces menor que la concentración de aflatoxina B_1 presente en el pienso consumido. En estudios con cerdos alimentados con piensos contaminados durante varios meses se detectaron residuos de aflatoxinas en hígado, riñones y músculo (4).

Según los datos generados por el Sistema Mundial de Vigilancia FAO/OMS/PNUMA, a través del Programa de Vigilancia y Evaluación de la Contaminación de los Alimentos, dados a conocer en la Tercera Conferencia Internacional FAO/OMS/PNUMA sobre Micotoxinas celebrada en Marzo de 1999 en Túnez, se extraen dos conclusiones importantes en lo referente a la presencia de aflatoxinas de alimentos:

1. Un número considerable de estudios coinciden en que las aflatoxinas se encuentran en una gran variedad de alimentos, en particular frutos secos como los pistachos, castañas, almendras, nueces, anacardos, nueces de macadamia, avellanas y cacahuetes, en frutos desecados como los higos, en especias como el chile rojo, en el maíz y en semillas.

2. Cada vez se notifican más casos de presencia natural de aflatoxina M_1 en leche y productos lácteos y, aunque los niveles de contaminación no parecen constituir un grave peligro para la salud, los programas de vigilancia siguen siendo la estrategia fundamental para proteger a los consumidores.

En el congreso de la FAO de 1995, Aplicación del análisis de riesgos a cuestiones de normas alimentarías: Informe de la Consulta FAO/OMS de Expertos (OMS, Ginebra), se recogieron datos sobre micotoxinas referentes a 48 países, 33 de éstos suministraron datos sobre aflatoxina B_1 y aflatoxinas totales (B_1, B_2, G_1, G_2). De estos resultados se estimó un contenido medio de entre 4 y 8 μg/kg respectivamente en alimentos, con un rango de 0 a 30 μg/kg para B1 y de 0 a 50 μg/kg para el total de aflatoxinas. 17 países aportaron información sobre aflatoxina M_1 en leche con un contenido medio de 0'05 μg/kg y un rango de 0 a 1 μg/kg.

Los participantes de la "European Union Scientific Co-operation Assessment" (SCOOP) (5) sobre aflatoxinas en la revisión de datos suministrados por los países miembros y Noruega, llegaron a las siguientes conclusiones:

1. Las aflatoxinas se encuentrán en gran variedad de alimentos.

2. La mayoría de las muestras no contienen cantidades detectables de aflatoxinas.

3. Los métodos de muestreo son de extremada importancia en la estimación de los niveles de aflatoxinas.

4. Las diferentes formas en la elaboración de alimentos pueden significar una diferencia en la ingesta de aflatoxinas.

En alimentos vegetales la contaminación por aflatoxinas resulta directamente proporcional al crecimiento de los hongos; en especial, el maíz y los frutos secos son particularmente susceptibles. Por otra parte la leche, y posiblemente carne y huevos, pueden verse indirectamente contaminados a través de la absorción de las aflatoxinas contenidas en los piensos.

El nivel de exposición en el hombre depende tanto de los alimentos disponibles como de los hábitos de consumo de éstos, lo cual puede variar entre países de acuerdo a las condiciones locales, grupos étnicos y diferencias individuales.

Los niños son un grupo potencial de riesgo debido a que muchos alimentos infantiles suelen prepararse con deshidratados de leche y cereales, y además la cantidad de alimento consumida por kilo corporal de peso lleva a que los niveles de contaminación por aflatoxinas sean proporcionalmente más significativos que en adultos.

III.1.1.3. Efectos económicos

Los efectos económicos negativos de las micotoxinas en las industrias cerealista y ganadera han sido considerables, según estimaciones de la FAO, en 1991, en Asia sudoriental hubo un total de 1849 Tm de cacahuete, 16042 Tm de maíz, 12010 Tm de arroz y 2296 Tm de soja afectadas por micotoxinas. El coste directo de la contaminación por aflatoxinas en maíz y cacahuete en Tailandia, Indonesia y Filipinas se cifra en millones de dólares al año. Informes de la industria australiana del cacahuete han revelado que las pérdidas ocasionadas por aflatoxinas representan el 10% de la cosecha en una buena campaña y más del 50% en una campaña mala, con un coste en clasificación de las semillas por colores después del descascarillado y análisis químico de los niveles de aflatoxinas de al menos 1 millón de dólares al año. Datos publicados en los EEUU indicaron que este país había retenido, en un solo año, un total de 18.000 remesas de alimentos valoradas en 1.500 millones de dólares EEUU por afectación de aflatoxinas. Como resultado de los límites reglamentarios para la aflatoxina aprobados por la Unión Europea las importaciones de harina de cacahuete se han reducido de 0'91 millones de toneladas en 1979/80 a 0'4 millones en 1989/90. La exposición de animales de granja a piensos contaminados con micotoxinas ha dado lugar en ocasiones a brotes locales, afectando a aves de corral, ganado porcino, vacas lecheras y caballos, con un descenso en la producción, aumento de la mortalidad y aumento de los gastos en alimentación y cuidados veterinarios (6).

En un intento por armonizar las tolerancias, para la presencia de aflatoxinas en alimentos, actualmente vigentes en diferentes países, el Grupo de Trabajo Mixto OMS/FAO sobre Micotoxinas propuso un valor máximo de 15 μg/kg para las aflatoxinas totales en el cacahuete bruto, tomando como base una muestra de 20 kg. Muchos países consideran que el nivel de 15 μg/kg es un límite razonable, que está al alcance de los países productores por lo que facilita el comercio y que un nivel inferior como 10 μg/kg constituiría un obstáculo al comercio, dado que en la evaluación del JECFA (Joint FAO/WHO Expert Comité on Food Additives) se había llegado a la conclusión de que posiblemente no representaría una mejora importante para la salud pública. Sin embargo, las propiedades genotóxicas, la incertidumbre sobre la evaluación de riesgos y la insuficiencia de datos acerca de los efectos de un nivel de 10 μg/kg sobre el mercado justifican el principio de precaución y la tendencia al valor más bajo posible.

III.1.2. Aflatoxinas: toxicidad

III.1.2.1. Toxicidad general

Las aflatoxinas son uno de los más potentes mutágenos y carcinógenos conocidos, siendo más tóxicas las aflatoxinas B_1 y G_1. Numerosos experimentos en animales han demostrado que las aflatoxinas son capaces de inducir cáncer de hígado en muchas de las especies estudiadas, además estudios epidemiológicos muestran la correlación entre la exposición a aflatoxina B_1 y un incremento en la incidencia de cáncer de hígado, aunque

entre estudios existe discrepancia a la hora de denominar a la aflatoxina B_1 como un factor de riesgo independiente o un factor de riesgo en asociación con otros factores como la hepatitis B.

Se han descrito varios casos de aflatoxicosis en animales en diversas áreas del mundo; aflatoxicosis en aves de corral, en cerdos, en vacuno, en perros y en humanos (7).

Diferentes investigadores han estudiado el potencial carcinógeno de las aflatoxinas in vivo usando animales de laboratorio, en la mayoría de estos estudios se comprobó una mayor incidencia de hepatocarcinogénesis, así como de otro tipo de tumores tales como: colon, riñón, laringe, sistema linfático, estómago, tráquea y esófago.

Las aflatoxinas aumentan la permeabilidad mitocondrial, interrumpen el transporte de electrones, declinando la respiración celular, y dañan las membranas de los lisosomas, provocando la liberación de hidrolasas con el consiguiente daño celular. Estudios en ratón demuestran que las aflatoxinas B_1 y G_1 causan una acumulación significativa de grasa en el hígado, un pequeño aumento de triglicéridos en sangre, inflamación hepatorrenal, necrosis por citotoxicidad en hígado y riñón y afectación de la vesícula biliar (8).

El metabolismo de las aflatoxinas ha sido estudiado por diversos autores para conocer las implicaciones celulares y metabólicas (9), para comparar el metabolismo de aflatoxinas en diferentes especies y para discernir

la implicación del citocromo P450, glutation-S-transferasa y epóxido hidrolasa en el metabolismo de aflatoxina B_1 (10).

Una gran variedad de vertebrados, invertebrados, plantas, bacterias y hongos son sensibles a la acción tóxica de las aflatoxinas pero su modo de acción no es completamente conocido (11), esta sensibilidad depende de:

1. La proporción de aflatoxina B_1 que es metabolizada a 8,9-epóxido en relación a otros metabolitos considerados menos tóxicos.

2. La relativa actividad de la fase II del metabolismo, la cual forma conjugados no tóxicos e inhibe la citotoxicidad.

3. La formación reversible de aflatoxicol, la cual podría generar una reserva metabólica de aflatoxina.

4. La inhibición de la formación de aflatoxicol por hormonas sexuales (17-cetoesteroides).

5. La relación de diversos factores nutricionales en la toxicidad de aflatoxinas, como es el caso del selenio y de la vitamina A.

6. Concomitancia de la infección por el virus de la hepatitis B y la exposición a aflatoxinas, ya que el virus de la hepatitis B y las aflatoxinas podrían ser cofactores en la etiología del cáncer de hígado.

III.1.2.2. Genotoxicidad

La aflatoxina B1 inhibe la ARN polimerasa-ADN dependiente, se une covalentemente al ADN tanto in vitro como in vivo e inhibe la síntesis de ARN, forma enlaces covalentes con el ADN e induce la transversión de guanina por timina en el codón 249 situado en el gen p53, tal mutación es seguida de la pérdida de los alelos funcionales de los genes supresores de tumores, fenómeno denominado pérdida de heterocigoticidad (12). La formación de aductos entre el ADN y el derivado epóxido de la aflatoxina B_1 ha sido bien caracterizada. Las aflatoxinas producen descenso en el recuento de espermatozoides así como un aumento de aberraciones en su morfología (13).

III.1.2.3. Citotoxicidad

Los metabolitos 2-hidroxi-2,3-dihidro derivados denominados hemiacetales se unen fuertemente a proteínas y son suficientemente reactivos in vivo para causar muchos de los efectos de la intoxicación por aflatoxinas. Los daños causados comprenden hemorragias en pulmones, riñones, glándulas suprarrenales, intestino delgado y colon, pequeñas áreas de degeneración grasa en el corazón, ascitis y edema abdominal y tejidos subcutáneos, vómitos, diarrea, coma, disminución de los niveles de fosfolípidos y glucosa y aumento de los ácidos grasos no esterificados en plasma, degeneración grasa y necrosis en hígado y edema cerebral con degeneración neuronal asociado al Síndrome de Reye en niños (14).

III.1.2.4. Otros efectos tóxicos

En ratas la continua exposición a bajos niveles de aflatoxina aumenta la susceptibilidad a las infecciones y tumorogénesis por inmunosupresión. Mediante experimentos se ha demostrado la capacidad inmunosupresiva de aflatoxina B_1 mediante exposición por inhalación (15).

En cuanto a la teratogeneicidad, se observó una mayor proporción de muerte o reabsorción de fetos en ratones tratados con aflatoxinas.

III.1.2.5. Exposición y riesgo

Existen diversos aspectos que relacionan la exposición a aflatoxinas y el riesgo de cáncer, pero se requieren más estudios experimentales y epidemiológicos para elucidar esta cuestión, de momento existen suficientes evidencias para la implementación de programas de control de aflatoxinas. Estos programas estarían destinados a reducir el nivel de contaminación por aflatoxinas con medidas como la educación de agricultores en lo referente a recolección y almacenamiento de las cosechas, precauciones y manejo de alimentos y piensos contaminados por aflatoxinas, mejora de los procesos tecnológicos de descontaminación de aflatoxinas, así como el llevar a cabo programas de análisis de alimentos y estudios epidemiológicos apropiados para salvaguardar la salud de la población (16). En diferentes especies y dependiendo del sexo varía la susceptibilidad a la intoxicación aguda por aflatoxinas y se observa que para valores de DL_{50} la potencia tóxica es $B_1 > G_1 > B_2 > G_2$.

III.1.3. Ocratoxina A: generalidades

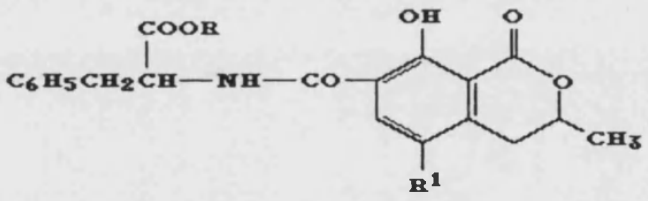
El grupo de sustancias conocidas como ocratoxinas (A, B y sus ésteres y α y β) son producidas por distintas especies de hongos de los géneros *Aspergillus* y *Penicillium*. Estos hongos son ubicuos por lo que la contaminación por estas sustancias de alimentos y piensos puede darse en cualquier parte del mundo. La ocratoxina A, que es el producto más tóxico del grupo y el más abundante en los alimentos, se conoce su presencia de forma natural en los alimentos desde 1969 (17), tanto en cantidad como en afectación, representa un problema de salud y seguridad alimentaria por las implicaciones toxicológicas, y económico por su impacto sobre las importaciones y exportaciones de alimentos.

La OTA se obtuvo por primera vez de *A. ochraceus*, pero investigaciones posteriores revelaron que varias especies de los géneros *Aspergillus* y *Penicillium* eran productoras de OTA y, de estos géneros, los productores más importantes son *A. ochraceus* y *P. viridicatum*. Para el desarrollo de *A. ochraceus* las condiciones ambientales deben situarse entre 20 y 30ºC con una actividad de agua a partir de 0'953, por esa razón, este hongo es el principal responsable de la contaminación por OTA en regiones de clima templado afectando a productos como el café, el cacao, las especias y la uva. Sin embargo el *P. viridicatum* se desarrolla entre 5 y 10ºC, por lo cual afecta a regiones de clima frío y a alimentos como los cereales (18).

III.1.3.1. Estructura química

La estructura química de la OTA tiene como base un anillo isocumarínico, con un cloro como sustituyente, unida por un enlace amida a un residuo de fenilalanina. Partiendo de esta molécula, la ocratoxina B corresponde al producto sin cloro, podemos encontrar los ésteres de OTA y OTB por adición del grupo metilo o etilo correspondiente al carboxilo de la fenilalanina, y también existen los productos de la hidrólisis del enlace amida de OTA y OTB, dando lugar a ocratoxina α y ocratoxina β respectivamente. Otros productos derivados son los producidos por la abertura del anillo lactónico. Cabe destacar, por sus implicaciones sobre la toxicidad de la OTA, el residuo de fenilalanina.

Figura III.2: Estructura química de las ocratoxinas



ocratoxina A: R= H, R^1= Cl

ocratoxina B: R= H, R^1= H

ocratoxina C: R= C_2H_5, R^1= Cl

metil éster de ocratoxina A: R= CH_3, R^1= Cl

metil o etil éster de ocratoxina B: R= CH_3 o C_2H_5, R^1= H

III.1.3.2. Exposición alimentaria

En 2002, la Comisión Europea estableció un grupo de cooperación científica, SCOOP Task 3.2.7, Assessment of dietary intake of Ochratoxin A by the population of EU Member Status (19), para proporcionar datos sobre exposición alimentaria a la OTA en la Unión Europea. Este grupo estimó las ingestas sobre la base de los datos nacionales de consumo; que oscilaban de 0'7 a 4'6 ng/kg/día, con una media de 1'8 ng/kg/día, también debido a su largo período de vida en la sangre humana (unos 35 días), los niveles plasmáticos constituyen un biomarcador útil de ingesta para varios días y partiendo de esos datos, se estimaron unas ingestas ligeramente inferiores, de entre 0'2 y 2'4 ng/kg/día, con una media de 0'9 ng/kg/día. La analogía en las estimaciones partiendo de los dos métodos sugiere que son ya conocidas las fuentes principales de OTA.

En primer lugar, los cereales son a todas luces los principales elementos que contribuyen a la exposición alimentaría, mientras que los grandes bebedores de vino tinto pueden recibir de esta fuente una ingesta importante. En segundo lugar, la fuerte contaminación de algunos productos, como uvas pasas y especias, da a entender que durante la producción y el almacenamiento pueden darse condiciones poco idóneas con posibilidad de mejora y, suponiendo un nivel análogo de contaminación, los cereales aportarían una ingesta de OTA superiores a las de Europa en varias regiones de Asia y de África.

III.1.3.3. Ingesta diaria

Ha habido diferentes criterios sobre la evaluación de riesgos de la OTA en las diferentes instancias, lo que depende en gran parte de que se considere que la carcinogeneidad de la OTA se produce, o no, a través de un mecanismo con umbral o sin umbral. Por lo tanto, la ingesta tolerable se ha estimado en 100 ng/kg/semana por la OMS, de 1'5 a 5'7 ng/kg/día por el Ministerio de Sanidad de Canadá y de 1'2 a 14 ng/kg/día por la Unión Europea. Estas diferencias se reflejan también en las medidas de gestión del riesgo que se han aplicado o propuesto, asignándose diferentes niveles máximos de contaminación a distintos productos y al mismo producto en diferentes países. La prevención de la contaminación en el origen se considera la medida más eficaz de sanidad pública.

El Comité Científico para la Alimentación de la Comisión Europea llegó a la conclusión de que la OTA es un potente agente nefrotóxico y carcinógeno y que tiene propiedades genotóxicas, por lo que sería prudente reducir la exposición lo más posible, procurando que las exposiciones sean "hacia el extremo inferior de la escala de ingestas diarias tolerables, por ejemplo, por debajo de 5 ng/kg/día".

III.1.3.4. Salud y comercio

Son evidentes las posibilidades de conflictos por lo que respecta a las normas sobre productos en el comercio internacional. Aunque puede ser justificable la

Tabla III.1: Ingesta de ocratoxina partiendo de datos europeos (19)

Alimento	Consumo g/día	Concentración media de OTA µg/kg	Ingesta diaria de OTA ng/kg p.corp.*	% de ingesta total
Cereales	226	0'5	1'9	54
Vino tinto	171	0'19	0'54	15
Café	29	0'9	0'43	12
Cerveza	234	0'07	0'27	7'6
Carne de cerdo	76	0'1	0'13	3'7
Uvas pasas	2.3	2'8	0'11	3'1
Especias	0.5	11	0'09	2'6
Aves de corral	53	0'03	0'03	0'9
Legumbres	12	0'1	0'02	0'6
Zumo (jugo) de uva	¿(50)?**	1'0	¿(0'8)?	¿(19)?
			Total 3'5	

*Se supone un peso corporal de 60 kg.

**No se dispone de datos fiables sobre consumo; no se ha tenido en cuenta en la cifra total de ingesta.

introducción de límites máximos para los cereales, dado que estos productos son la fuente principal de exposición, la mejor forma de asegurar la protección de la salud humana es a base de medidas preventivas para reducir al mínimo la contaminación por hongos productores de OTA y las condiciones que dan lugar a la producción de la toxina.

III.1.4. Ocratoxina A: toxicidad

III.1.4.1. Toxicidad general

Las primeras manifestaciones tóxicas achacadas a la OTA se deben a la nefropatía porcina, desarrollada en cerdos alimentados con piensos contaminados, tras las epidemias de 1963 y 1971 producidas por el consumo de piensos con un alto grado de humedad debido a las condiciones climáticas concurrentes (20). Las lesiones producidas en los cerdos incluyen atrofia tubular, fibrosis intersticial e hialinización glomerular; posteriormente se comprobó que la OTA daba lugar a nefrotoxicidad en todas las especies donde se ensayaba su toxicidad, que las lesiones producidas eran similares a las de la nefropatía endémica humana o nefropatía de los balcanes y se correlacionó altos niveles de contaminación por OTA en alimentos con áreas de prevalencia de este síndrome (21).

Sobre el metabolismo, la OTA disminuye el nivel de glucógeno hepático e incrementa la glucemia, debido a la inhibición del transporte activo de glucosa en el interior del hígado, a la supresión de la síntesis de glucógeno y a la aceleración de su paso a glucosa. A nivel mitocondrial, provoca su deterioro por deplección del ATP mitocondrial y actúa como inhibidor competitivo del transporte a través de proteínas de membrana. También inhibe la incorporación de ácido orótico en la formación de RNA a nivel renal y hepático, en este punto actúa sinérgicamente con otra micotoxina, la citrinina (22).

La absorción de la OTA se produce mayoritariamente en el estómago, una pequeña cantidad puede llegar al intestino donde en parte se hidroliza por la acción de carboxipeptidasas y alfa-quimotripsina en ocratoxina α, hidrólisis que es mayoritaria en rumiantes, lo cual les proporciona una cierta protección frente a este tóxico.

Los residuos de OTA se pueden encontrar en riñón, hígado, músculo y tejido adiposo en este orden de importancia.

La excreción se produce principalmente por orina y en mucha menor medida por las heces.

Se han realizado diversos estudios toxicológicos relacionados con la OTA y el Organismo Internacional de Investigaciones sobre el Cáncer ha publicado varias monografías detalladas, así como el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios. De las pruebas de que se dispone no hay lugar a duda que la OTA es nefrotóxica y cancerígena. También es teratógena e inmunotóxica, pues afecta a la inmunidad humoral y celular. A este respecto, la potencia varía notablemente según las especies y el sexo; la susceptibilidad frente a la OTA depende de la especie, el cerdo es la más sensible, y del sexo, las hembras sufren en mayor grado estos efectos, donde el órgano diana es el riñón aunque también existe una afectación del hígado. Los cambios en la función renal se caracterizan por una degeneración de la función tubular, debido a una atrofia de los túbulos proximales y distales y a la generación de fibrosis intersticial (23).

III.1.4.2. Genotoxicidad

La situación sigue siendo bastante confusa. La mayor parte de los ensayos a corto plazo sobre mutaciones génicas fueron negativos y, de forma análoga, fueron negativos o equívocos los ensayos para síntesis no programadas de ADN, intercambio cromático afín y aberraciones cromosómicas en células CHO.

Se ha afirmado que el tratamiento de ratas y ratones con OTA da lugar a la formación de aductos, pero hasta la fecha no se han caracterizado los aductos de ADN, ni hay certeza de que deriven directamente de metabolitos de OTA. Los análisis de las mutaciones inducidas refleja el efecto de un mecanismo oxidativo más bien que a la formación directa de aductos de ADN (24).

Se dan otras anomalías al adscribir la carcinogeneidad de la OTA a un mecanismo genotóxico directo, ya que no se ha aislado o identificado el metabolito mutágeno. Se conoce poco del metabolismo en mamíferos de la OTA in vivo y los datos de que se dispone no explican la selectividad del efecto tóxico por algunos órganos y la formación de tumores.

III.1.4.3. Citotoxicidad

La OTA es un potente inhibidor competitivo de la ligasa fenilalanina-tRNA, dando lugar a la inhibición de la síntesis proteica y, secundariamente,

de la síntesis de ARN y ADN. De hecho, los efectos tóxicos agudos de la OTA quedan inhibidos por la coadministración de fenilalanina. Los efectos carcinógenos y presuntamente genotóxicos de la OTA pueden también producirse por mecanismos secundarios como impedimentos a la reparación del ADN, fitotoxicidad crónica o inducción de tensión oxidativa, y pueden tener un umbral (25).

III.1.4.4. Otros efectos tóxicos

La OTA también provoca efectos teratogénicos que incrementan la mortalidad prenatal; en concreto, disminuye el peso fetal y da lugar a diversos tipos de malformaciones como son hidrocefalia y defectos cardiacos, entre otros.

III.1.4.5. Riesgo

El Centro Internacional de Investigaciones sobre el Cáncer ha llegado a la conclusión de que la OTA es un posible cancerígeno humano, clasificándola dentro del grupo 2A, pero que no existen suficientes pruebas de carcinogeneidad en los seres humanos y, como ya se indicó anteriormente, la demostración de una asociación causal con una nefropatía endémica sigue siendo poco convincente para algunos autores.

III.1.5. Análisis de aflatoxinas y ocratoxina A

III.1.5.1. Técnicas analíticas para aflatoxinas

Los métodos de extracción de aflatoxinas aprovechan en la solubilidad de estas sustancias en disolventes orgánicos de polaridad media, se suele utilizar cloroformo (26), metanol (27), acetona (28) y acetonitrilo (29), y mezclas de estos disolventes con agua.

La purificación se basa en la utilización de columnas de extracción en fase sólida, usando sílice, Florisil® u octadecilsílice (C_{18}) como fases extractivas. También se utilizan columnas de inmunoafinidad conteniendo anticuerpos específicos para las diferentes micotoxinas, con las que debido a la gran selectividad de esta técnica se consigue un alto grado de purificación del extracto (30).

La determinación y cuantificación de aflatoxinas se realiza mediante cromatografía líquida de alta resolución, aunque todavía se emplean métodos de cromatografía de capa fina con fines cualitativos para determinados niveles de contaminación, debido a su sencillez y bajo coste, y métodos de inmunoenzimoensayo para el análisis semicuantitativo cuando el número de muestras es muy alto. La detección se realiza aprovechando la fluorescencia nativa de las aflatoxinas, utilizando longitudes de onda de excitación entre 360 y 365 nm y de emisión entre 425 y 435 nm. También se utiliza la espectrometría de masas para la resolución del análisis en matrices complejas.

Para aumentar la fluorescencia de las aflatoxinas B_1, M_1 y G_1, y mejorar con ello los valores de sensibilidad de estos compuestos se utilizan diversos métodos de derivatización. Estos métodos pueden ser:

-derivatización con ácido trifluoroacético (31).

-derivatización postcolumna con iodo (32).

-derivatización postcolumna con tribromuro de piridinio (33).

-derivatización postcolumna con bromuro generado electro-químicamente (34).

III.1.5.2. Técnicas analíticas para ocratoxina A

Los métodos de extracción, el proceso de purificación y la detección con fluorescencia son similares a los métodos utilizados para las aflatoxinas.

Aprovechando las características de la molécula de la OTA, pK_a de 4´4 para el grupo carboxilo y pK_a de 7´1 para el grupo fenilo, es posible realizar una extracción selectiva con disolventes orgánicos en medio ácido; los disolventes más usados son cloroformo y tolueno, acidificados con ácido fosfórico o ácido clorhídrico. También puede realizarse la extracción mediante disoluciones acuosas en medio básico con bicarbonato sódico.

La determinación de OTA por fluorescencia utiliza unos valores de longitud de onda de excitación entre 330 y 333 nm y longitud de onda de emisión entre 445 y 470 nm. La confirmación de la presencia de OTA se realiza generando derivados fluorescentes, por reacción con metanol-clorhídrico (35), trifluoruro de boro (36) o amoníaco (37), o mediante espectrometría de masas (38).

III.1.6. Legislación

En la legislación europea queda patente la preocupación por ciertos productos tóxicos presentes en los alimentos desde un punto de vista sanitario "en interés de la salud pública, resulta esencial mantener el contenido de contaminantes en niveles aceptables desde el punto de vista toxicológico. Siempre que sea posible, la presencia de contaminantes debe reducirse cuidadosamente mediante buenas prácticas agrícolas o de producción, con el fin de alcanzar un nivel más alto de protección de la salud, especialmente para los grupos más sensibles de la población" y en su vertiente económica "vistas las disparidades existentes entre las legislaciones de los Estados miembros en lo que se refiere a los contenidos máximos de contaminantes en determinados alimentos, y las distorsiones de la competencia que éstas pueden acarrear, es necesario tomar medidas a escala comunitaria para garantizar la unicidad del mercado, respetando al mismo tiempo el principio de proporcionalidad" (39).

Según la legislación en cuanto a aflatoxinas y ocratoxina A podemos resumir que "son sustancias tóxicas para el ser humano y los animales y

pueden estar presentes en un gran número de productos alimenticios. El estado actual de los conocimientos científicos y técnicos y de las mejoras en las prácticas de producción y almacenamiento no permite eliminar completamente el desarrollo de estos mohos y, por consiguiente, la presencia de micotoxinas en los productos alimenticios. Por lo tanto, conviene fijar los límites en el nivel más bajo posible. Deben fomentarse los esfuerzos para mejorar las condiciones de producción, cosecha y almacenamiento con el fin de reducir el desarrollo de mohos" (40).

También tiene en cuenta un apartado importantísimo en este tipo de contaminación como es la toma de muestras y los métodos de análisis (41).

Tabla III.2: Contenido máximo de micotoxinas permitido en la Unión Europea para alimentos destinados a consumo humano

	Alimento	**Contenido máximo (μg/kg o μg/l)**		
Aflatoxinas		**B_1**	**$B_1+B_2+G_1+G_2$**	**M_1**
	Cacahuetes, frutos de cáscara y frutos secos	2-8[a]	4-15[a]	
	Cereales	2-5[a]	4-10[a]	
	Leche			0'05
	Alimentación infantil con base cereal	0'1		
	Alimentación infantil con base láctea			0'025
	Especias	5	10	
Ocratoxina			**A**	
	Cereales y derivados		3-5[a]	
	Uvas pasas		10	
	Alimentación infantil con base cereal		0'5	

[a] El contenido máximo permitido para algunos alimentos dependerá de si su consumo es directo o de si van a ser sometidos a un proceso de selección, u otro tratamiento físico, antes del consumo humano directo o de su uso como ingredientes en los productos alimenticios.

La aplicación de esta legislación ha llevado, en algunos casos, a obligar a un aumento de las garantías por parte de países exportadores de ciertos productos para su comercio en la Unión Europea como es el caso de países como Irán, Turquía o China afectando a productos como los higos, pistachos, avellanas, cacahuetes y productos relacionados (42).

Toda esta legislación se revisa y actualiza en cortos periodos de tiempo, y siempre que se produce un nuevo avance o se obtienen datos que así lo recomiendan, buscando un aumento en la seguridad de los consumidores (43).

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III.2. FACTORS AFFECTING TO THE PRESENCE OF OCHRATOXIN A IN WINES

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Abstract

Ochratoxin A (OTA) are synthesized mainly by different species of *Aspergillus* and *Penicillium* being its human toxicological effects reflected in different countries due to the consumption of different foods and beverages such as red, white, rosé and special wines. This review presents an overview of the direct (meteorological conditions, grape cultivation and wine-making techniques) and indirect (latitude, year of production, use of pesticides, presence of spoilage microorganisms, conditions of storage of the harvested grapes, type of maceration and conditions of fermentation) factors affecting the presence of OTA in wines.

III.2.1. Introduction

Ochratoxin A (OTA) is a mycotoxin discovered in 1965 by Van der Merwe et al. (1965)[1] as a secondary metabolite of *Aspergillus ochraceus* and more recently as a secondary metabolite of several other *Aspergillus* and *Penicillium spp.*[2]. This mycotoxin has a well documented nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties[3]; moreover, OTA is suspected as a possible cause of the two fatal human diseases known as urinary tract tumours (UTT) and Balkan-endemic nephropathy (BEN) (a tubulo-intersticial nephropathy leading to chronic renal failure at the age of about 50)[4-10]. Božić et al. (1995)[11] proposed the term endemic uropathy for the association between BEN and UTT. The International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2B)[12]. To date, OTA has been isolated in several foods and several beverages such as grapes and wines which have been reported to contain high levels of this mycotoxin[13,14]. The EU regulation for this mycotoxin in wine, which is being prepared, will probably set the acceptable limit of OTA at 1 μg/l[15].

Nowadays, the only reported species capable of producing OTA belong to the genera *Aspergillus* and *Penicillium.* These fungi were printed out in 1998 as responsible of the presence of this mycotoxin in grapes and as the source of the presence of OTA in wines[16]. Ochratoxigenic *Penicillium spp.* are scarcely detected in grapes because it does not attack grapes before harvest[17], but is prevalent in stored grapes where *P. expansum* is the most common

contaminant[18]. Several *Penicillium* species such as *P. aurantiogriseum*, *P. brevicompactum*, *P. citrinum* and *P. glabrum* among others[19,20] are isolated in stored grapes whereas *P. verrucosum* is the major responsible of the occurrence of OTA in foods[17]. The percentage of ochratoxigenic *Aspergillus* strains detected in grapes is shown in Table III.3. The section Nigri (black aspergilli) is considered the main producer of OTA. *A. carbonarius* is a black aspergilli frequently isolated in grapes that contained OTA[2].

Table III.3: Ochratoxigenic *Aspergillus* strains isolated from grapes in different countries

Country	Species (Section)	Incidence of ochratoxigenic strains	Reference
Argentina, Brazil	*A. carbonarius* (Nigri)	11/43 (25%)	21
	A. niger (Nigri)	24/101 (24%)	
	A. ochraceus (Circumdati)	2/5 (40%)	
Argentina	*A. niger var. niger* (Nigri)	19/44 (45%)	22
	A. niger var. awamori (Nigri)	5/15 (33%)	
	A. faetidus (Nigri)	1/4 (25%)	
France	*A. carbonarius* (Nigri)	14/15 (93%)	23
Italy	*A. biseriate*[a] (Nigri)	3/108 (3%)	24
	A. carbonarius (Nigri)	50/86 (60%)	
	A. fumigatus (Fumigati)	6/11 (50%)	
	A. ochraceus (Circumdati)	2/2 (100%)	
	A. uniseriate[b] (Nigri)	10/207 (5%)	
Portugal	*A. alliaceus* (Flavi)	1/1 (100%)	25
	A. carbonarius (Nigri)	38/39 (97%)	
	A. niger aggregate (Nigri)	12/294 (4%)	
	A. ochraceus (Circumdati)	1/2 (50%)	
Spain	*A. carbonarius* (Nigri)	38/39 (97%)	2

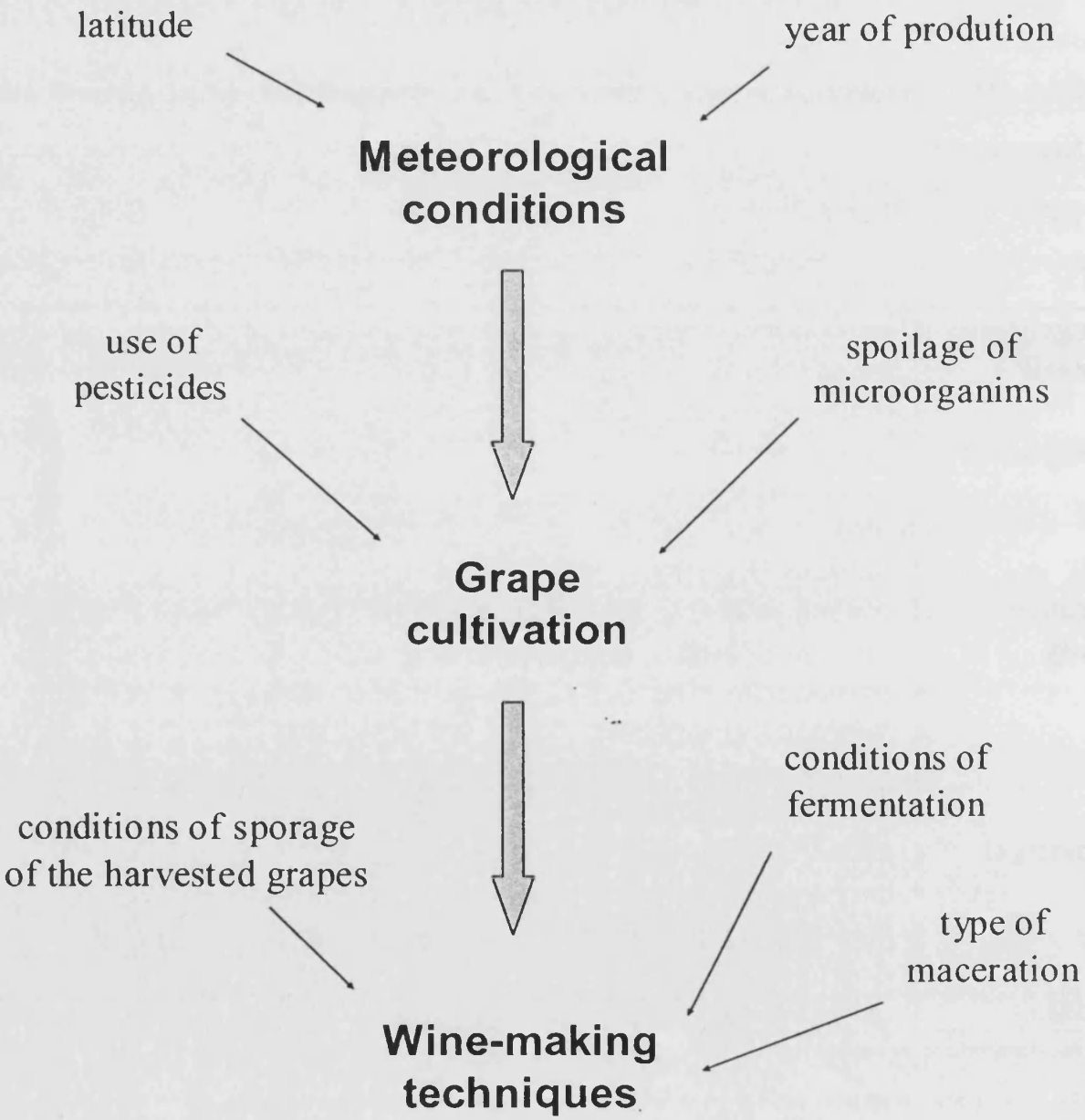
[a] The biseriate species included A. niger and A. tubingensis

[b] The uniseriate species included A. carbonarius, A. aculeatus and A. japonicus

III.2.2. Factors affecting to the presence of ochratoxin A in wines

Several factors, such as climate, grape cultivation and wine-making techniques, affect to the presence of OTA in wines. The Figure III.3 summarizes these factors.

Figure III.3: Direct and indirect factors affecting to the presence of OTA in wines



III.2.2.1. Climate

The rates of OTA contamination in raisins and consequently in wine vary depending indirectly on the latitude and year of production and directly on the meteorological conditions.

Latitude

Several authors suggested that a gradual increase of the contamination of OTA is reflected from north to south. Zimmerli and Dick (1996)[26] observed that the red wine samples of more southerly in Europe are higher in the frequency of occurrence and concentration of OTA. Otteneder and Majerus (2000)[27] confirmed the influence of the latitude on the level of contamination with OTA with red wine samples from Germany, France and Italy. Pietri et al. (2001)[28] and Visconti et al. (1999)[29] showed that the geographic region of origin had a strong influence on OTA contamination of red wines in Italy, with the more contaminated wines originating in the southern regions of Italy as compared to those produced in the north. Furthermore, a Swiss survey[30] revealed that Swiss males living south of the Alps had higher OTA concentrations in their sera than women and people living in the rest of the country. A possible explanation was that wine (whose consumption by males living south of the Alps is higher than north of the Alps) could be a source of OTA. A subsequent survey on wine obtained from retail outlets in Switzerland[26] showed a higher OTA concentration in red wines than in white and rosé wines. As far as red wines were concerned, it was deduced that the

amount of OTA was dependent on the latitude of the production region: the further south the origin, the more frequent the occurrence and the greater concentration. Considering the median values of OTA contamination of red wines for each region, a concentration gradient from north to south can be observed.

Nevertheless, this tendency is not so evident amongst the white and rosé dry wines, a fact that may be attributed to different wine-making procedures followed for red and white wines[31]. It is clear that the difference in latitude (from 46° to 36° parallel) and the consequent considerable climatic differences influence mould contamination and OTA production in a conclusive manner, especially in red wines. However, Soleas et al. (2001)[32] did not find evidence of a North-South gradient in concentration of OTA in red wines.

Year of production

Lopez de Cerain et al. (2002)[33] reflected very different contamination of OTA between the 2 years of harvest; 85% of OTA incidence versus 15% of wine samples. One year of harvest was worse than the other year, probably because of differences in the weather conditions (low temperatures and high incidence of storms) during the summer, which is led to lower production and several contamination problems with fungi such as *Oidium* and *Botrytis* that do not produce OTA.

Climatic conditions

The southern Europe favours the growth of ochratoxigenic *Aspergillus* species over *Penicillium*[26]. Black aspergilli are very resistant to sun exposure and to hot and dry environments. They are adapted to the conditions in the vineyards with Mediterranean climates, constituting a marked presence in the grapes of these locations[25].

In fact, OTA contamination in cool and temperature climates is due to *P. verrucosum*, where as *A. ochraceus* is more commonly associated with warmer and tropical climates. Sweeney and Dobson (1998)[34] commented that the involvement of *A. ochraceus* in the production of the OTA under tropical conditions should not be overlooked. Pitt and Hocking (1997)[17] believed that presence of OTA in foods from templated climate regions favours the growth of *P. verrucosum.*

In France[35], ochratoxicosis seems to be connected with the presence of *Aspergillus* species while, in Germany and in Scandinavia, it would be linked with the presence of *Penicillium spp.*, owing to their different temperature needs[36]. Filali et al. (2001)[37], Zimmerli and Dick (1996)[26] and Ospital et al. (1998)[16] found that the wines from the Mediterranean region contain high concentrations of OTA because the climate is characterized by high humidity and high temperature. In fact, ochratoxigenic *Penicillium spp.* grow well over a wide range of temperature (4-31°C), whereas *Aspergillus spp.* that produces OTA requires higher temperatures (12-39°C).

Before the wine is produced, the occurrence of OTA in the grapes is related with climate, which obviously depends on the latitude but also on the particular circumstances of a year in a well defined region[33].

III.2.2.2. Grape cultivation

Spoilage microorganisms and use of pesticides, which modifies the presence of OTA in wines, affect the grape cultivation.

Spoilage microorganisms

Several factors, such as grape variety, degree of berry maturity and physical damage, favour the spoilage and they affect to the growth of ochratoxigenic fungi in the grapes[18, 38,39]. Recently, the presence of OTA has been detected in soil[40] and the relation of this mycotoxin in the soil-water-grape system should be studied in detail as origin of ochratoxigenic fungi. Da Rocha Rosa et al. (2002)[21] observed that yeasts were a major component of the fungal population. The most frequent genera of filamentous fungi isolated were *Aspergillus, Penicillium, Botrytis, Phythophthora, Moniliella, Alternaria and Cladosporium* being some of them responsible of spoilage in the grapes. Zahavi et al. (2000)[41] found *A. niger* on the surfaces of healthy grapes and Barkai-Golan (1980)[19], Pitt and Hocking (1997)[17] and Snowdon (1990)[18] show that *Aspergillus spp.* is the responsible of post-harvest decay in the fresh fruit including grapes. In fact, Da Rocha Rosa et al. (2002)[21] obtained 8 of 48 and 16 of 53 *A. niger* strains from Argentinian and Brazilian grapes, respectively.

Since black aspergilli (mainly *A. carbonarius*) are the dominant fungi detected in dried wine fruits and have shown a consistent ability to produce OTA, they are the most probable source of this mycotoxin in these substrates. The occurrence of OTA in wine is linked to the presence of molds on grapes[42]. Pietri et al. (2001)[28] detected OTA in grapes in the beginning of ripening and in the harvesting. Furthermore, *Aspergillus spp.* were constantly associated with grapes in both sampling periods, while *Penicillium spp.* were present only at beginning of ripening in several vineyards in the south of Europe.

Use of pesticides

It is important for the growth of ochratoxigenic fungi in the grapes. Lo Curto et al. (2003)[43] suggested that the different OTA contents in wine samples can be considered to be an efficiency test of the pesticides used. In consequence, the phytosanitary control of crops during the cultivation of grapes is probably very important in order to prevent OTA contamination in wines. Using the principle of Good Agriculture Practices for applied pesticides in grapes helps to guarantee the presence of ochratoxigenic fungi[32, 44].

III.2.2.3. Winemaking

Several factors in the winemaking procedures such as conditions of storage of the harvested grapes, type of maceration and conditions of fermentation can be responsible of the presence of OTA in wines.

Conditions of storage of the harvested grapes

There are few studies about the storage conditions as a factor that affects to the presence of OTA in grapes and wines. Pitt and Hocking (1997)[17] suggested that *Penicillium* apparently do not attack grapes before harvest but are prevalent in stored grapes where *P. expansum* is a very contaminant[18].

Type of maceration

Occurrence of OTA in red, rosé, white and special wines are shown in Tables III.4, III.5, III.6 and III.7, respectively. Focusing a determined type of wine, the overwhelming majority of the analysis of OTA in different wines suggested that the incidence and concentrations decrease in the order red, rosé and white wine[26,27,29,37,46]. Red wine is more susceptible to OTA contamination[27,47,51] due to probably to that red wine processing conditions in which the elevated temperature and aerobic conditions associated with the dissolution of natural colorants produce intensive contact with potential toxin-producing molds[51]. In fact, in the red wines, the grapes are immediately pressed and left mashed and skin and juice are put aside for several days, which obviously permits fungal growth and production of the toxin. However, Lopez de Ceraín et al. (2002)[33]did not find a great difference between white and red wine probably due to the small number of samples and the great dispersion of the data, especially for red wines.

Tabla III.4: Occurrence of ochratoxin A in red wines

Origin	Incidence	Range (µg/L)	Reference
Morocco	20/20	0.04-3.24	37
Italy	14/96	0.010-3.177	28
Spain	13/28	0.050-0-316	33
Greece	8/8	0.002-2.35	45
Morocco	3/3	0.551-0.554	45
Spain-Portugal	6/6	0.002-0.5	45
France	12/12	0.002-3.4	45
European Community	1/1	1.54	45
Italy	1/1	0.892	45
Spain	66/72	<0.003-0.603	46
Spain	9/14	<0.003-0.022	26
Spain	3/6	<0.010-0-190	47
France	21/21	<0.01-0.27	16
Worldwide origin	40/89	<0.01-7.00	47
Worldwide origin	165/305	<0.01-3.31	27
Italy	37/38	0.01-7.63	29
Spain	3/6	<0.010-0.5	45
South Africa	9/9	0.07-0.39	48
Italy	5/5	0.23-0.91	48
France	8/8	0.004-0.452	46
Italy	6/8	0.003-0.191	46
Portugal	2/2	0.011-0.02	46
Hungry	1/1	0.005	46

Tabla III.4: (Continued)

Origin	Incidence	Range (µg/L)	Reference
Several countries[a]	96/580	0.05-0.20	32
Several countries[b]	79/79	<0.003-0.388	26
Greece	33/104	0.05-2.69	31
Italy	14/23	0.01-2.00	43
Spain	12/61	0.05-0.53	49

[a] Argentina, Australia, Canada, Chile, Central Europe, France, Germany, Italy, New Zealand, Portugal, South Africa, Spain, USA

[b] Algeria, France, Italy, Portugal, Spain, Switzerland

In this step of elaboration of wine, the use of fining is a common winery practice. It involves the addition of an adsorptive compound to reduce levels of certain compounds from wine. Enological fining agents have been shown to reduce the OTA level in wine during the ordinary clarification practice[52]. In particular, activated carbon[53,54] and potassium caseinate[53] have a good capacity to absorb OTA in model solutions, whereas bentonite has demonstrated low affinity for OTA[55].

Tabla III.5: Occurrence of ochratoxin A in rosé wines

Origin	Incidence	Range (µg/L)	Reference
Italy	7/8	0.01-1.15	29
Morocco	3/3	0.04-0.54	37
Spain	24/26	0.003-0.155	46
France	2/2	0.161	46
Italy	2/2	0.037-0.049	46
Portugal	1/2	0.003-0.010	46
Greece	7/20	0.05-1.16	31
France	2/2	<0.01-0.11	16
Several countries[a]	15/15	<0.003-0.123	26
Worldwide origin	22/55	<0.01-2.38	27
Worldwide origin	7/14	<0.01-2.40	47
Spain	8/21	0.05-0.46	49

[a] France, Italy, Spain

Tabla III.6: Occurrence of ochratoxin A in white wines

Origin	Incidence	Range (µg/L)	Reference
Italy	4/9	0.01-0.97	29
Spain	7/12	0.154-0.208	33
Morocco	7/7	0.028-0.18	37
Italy	3/3	0.01-0.08	48
South Africa	15/15	0.04-0.33	48
Spain	35/43	0.003-0.267	46
France	4/6	0.003-0.085	46
Italy	2/6	0.003-0.006	46
Portugal	2/4	0.003-0.020	46
Germany	0/8	nd	46
USA	2/2	0.010-0.019	46
Greece	55/118	0.05-1.72	31
Several countries[a]	14/362	0.05-0.10	32
France	4/4	<0.01-0.02	16
Portugal	64/64	<0.02	50
Several countries[b]	24/24	<0.003-0.178	26
Worldwide origin	15/60	<0.01-1.36	27
Worldwide origin	14/41	<0.01-1.20	47
Spain	4/24	0.05-0.76	49

nd = not detected

[a] Argentina, Australia, Canada, Chile, Central Europe, France, Germany, Italy, New Zealand, Portugal, South Africa, Spain, USA

[b] France, Italy, Switzerland

Tabla III.7: Occurrence of ochratoxin A in special wines

Type of wine	Origin	Incidence	Range (µg/L)	Reference
Marsala	Italy	1/1	0.29	29
Dessert wines	Italy	6/15	0.001-3.856	28
Moscatel and Malaga wines	Spain	13/14	0.003-2.54	46
Marsala wines	Italy	2/2	0.315-1.594	46
Jerez and Montilla-Moriles wines	Spain	23/27	0.003-0.254	46
Special wines	France	2/4	0.003-0.024	46
Special wines	Italy	3/4	0.003-0.040	46
Special wines	Germany	4/7	0.003-0.016	46
Special wines	Portugal	3/4	0.003-0.029	46
Special wines	Hungry	0/1	nd	46
Sparkling wines	Spain	10/12	0.003-0.037	46
Dessert wines	Greece	3/18	0.05-2.82	31
Retsina[a]	Greece	2/8	0.05-1.75	31
Port	Portugal	6/6	<0.003-0.017	26
Sherry	Spain	2/2	0.029-0.054	26
Marsala	Italy	2/2	0.044-0.337	26
Malaga	Spain	3/3	0.049-0.451	26
Vermouth	Italy	2/2	<0.003	26
Moscatel	Spain	3/7	0.05-0.40	49
Fondillón	Spain	3/6	0.05-0.38	49

nd = not detected

[a] Special kind of Greek wine with added resin from pine trees

In the final stage of the maceration process, good conditions exist for mould growth, as long as there is no fermentation and there are aerobic conditions[56]. This observation is confirmed on red grape juice, which also frequently contains elevated amounts of OTA[26,57]. This might be due to the fact that the grapes are treated with pectolytic enzymes in order to dissolve natural colorants. During this process there is a high temperature as well as no alcoholic fermentation. These combined factors facilitate mould growth and OTA production[27].

Conditions of fermentation

Yeast are predominant in natural flora on the surface of fresh grapes and are significant in winemaking because they carry out the alcoholic fermentation and may cause wine spoilage[58,59]. Their autolytic products may affect to sensory quality products and the growth of spoilage bacteria.

For ochratoxigenic fungi, OTA-producing molds are inhibited by ethanol and the generally anaerobic conditions but the mycotoxin is alcohol resistant and is not degraded during wine making and storage[56]. OTA is a weak acid with a pKa value for the carboxyl group of the phenylalanine moiety of 4.4[60]. This implies that OTA is partially dissociated at the pH of wine (ca. 3.5) and carries a negative charge that may interact with a positively charged surface. Zimmerli and Dick (1996)[26] suggested that OTA is probably formed prior to the alcoholic fermentation, assuming that OTA is not appreciably degraded during winemaking and storage. Lopez de Ceraín et al.

(2002)[33] suggested that the concentration of OTA present in wine could vary with time and demonstrate that is stable in wine for at least one year.

OTA producing moulds could also have been growing in the barrels and/or tanks or any other equipment before they come into contact with grapes, grape-juice or wine. Zimmerli and Dick (1996)[26] suggested that failure to remove mouldy fruits before further processing or mouldy equipment before it comes into contact with grapes or wine do not agree with good agriculture practices, and this could be a good reason for the occurrence of OTA in wine.

III.2.3. Conclusions

Among the factors affecting the presence of OTA in grapes and wines it is remarkable that a higher incidence and levels of OTA is found in red wines over these found in rosé, white and special wines, such higher incidence is more notorious for red wines produced in southern regions than for these produced in northern regions.

The Mediterranean climate favours the growth of ochratoxigenic *Aspergillus* in grapes whereas *Penicillium* predominates in countries with cooler climate.

Temperature, aerobic conditions and time of contact with the skin of grapes during the maceration procedures increase the OTA presence in wines,

whereas the use of adsorbents during the clarification process reduces the final level of OTA in wines which is not practically modified during further winemaking and storage.

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Estudio bibliográfico

III.3. CONTROL AND DECONTAMINATION OF OCHRATOXIN A IN FOOD PROCESSING: THE KEY OF TRACEABILITY

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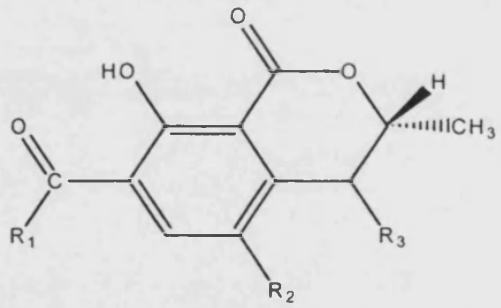
Abstract

Ochratoxin A (OTA) is a metabolite produced by some species of the genera *Aspergillus* and *Penicillium*. It is associated with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppresive properties. Some authors have suggested it is an aetiologic factor of the human disease known as Balkan Endemic Nephropathy and is hypothesised as a cause of testicular cancer. The International Agency for Research on Cancer (IARC) classified into group 2B as a possible human carcinogen. This review presents a briefly overview of the occurrence of OTA in different foods such as cereals, vegetables, cereal and vegetable products, meats, coffee, alcoholic beverages and spices from different countries. Furthermore, this paper reviews the application of traceability concept, including Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs) and Hazard Analysis and Critical Control (HACCP) system, for the control mycotoxin contamination. Finally, biological, chemical and/or physical decontamination procedures are reviewed for different foods and they can help to minimize or reduce the concentration of mycotoxin.

III.3.1. Introduction

Ochratoxins are isocoumarines grouped in ochratoxin A, B and C and their derivatives (Figure III.4) being rarely found in foods and feeds the chlorine free derivative of OTA, ochratoxin B and C. Ochratoxin α is generally considered to be non-toxic (Xiao et al., 1996), it has been reported to induce sister chromatid exchange at high concentrations (Follman et al., 1995).

Figure III.4: Structures of ochratoxins



Ochratoxin	R_1	R_2	R_3	
A		a	Cl	H
B		a	H	H
C		b	Cl	H
A methyl ester	c	Cl	H	
B methyl ester	c	H	H	
B ethyl ester	b	H	H	
4-hydroxy ochratoxin A	a	Cl	OH	
α		OH	Cl	H
β		OH	H	H

a= $C_6H_5CH_2CH(COOH)NH$-
b= $C_6H_5CH_2CH(COOEt)NH$-
c= $C_6H_5CH_2CH(COOMe)NH$-

Ochratoxin A (OTA) (R-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1-H-2-benzopyran-7yl)carbonyl]-phenylalanine) (Figure 1) was first described by Van der Merwe et al. (1965a,b) and is widely isolated in foods and feeds. OTA is a mycotoxin mainly produced by a single *Penicillium* species (*P. verrucosum*) and several related *Aspergillus* species as are *A. ochraceus*, *A. carbonarius* with a small percentage of isolates of the closely related *A. niger*. OTA is a moderately stable molecule and will survive most food processing (Harwig et al., 1983, Scott, 1991).

P. verrucosum grows only at temperatures below 30ºC and at a water activity as low as 0.8. These parameters are important to found this fungi in cool temperature regions being the source of OTA in cereals and cereal products in Canada and Europe. Furthermore, some animal products as are pig kidney and liver in that region have OTA due to the ochratoxigenic cereals are used in animal feeds and OTA is relatively stable in vivo (Pitt et al., 2001).

In other hand, *A. ochraceus* grows at moderate temperatures and at a water activity above 0.8. These parameters help to the presence of this fungi in tropics and subtropics regions and the occurrence of OTA in cereals and cereal products. A. carbonarius is associated as source of this mycotoxin in wines, dried vine fruits, coffee and maturing fruits as grapes (Pitt et al., 2001). Madhaystha et al. (1990) suggested that there is a clear preference for substrates and *P. verrucosum* produces more ochratoxin on carbohydrate rich cereals while A. ochraceus produces higher levels on oilseeds and protein rich seeds.

OTA is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppresive properties (Creppy, 1999; Marquardt and Frohlich, 1992) that may be implicated in the human disease Balkan Endemic Nephropathy (BEN) and in the development of urinary tract tumours in humans (Krogh and Elling, 1976; Pfohl-Leszkowicz et al., 2002). Recently, Schwartz (2002) offered a hypothesis of the OTA as a cause of testicular cancer by induction of adducts in testicular DNA. Furthermore, it is associated in the porcine nephropathy (Krogh, 1976). In 1993, the International Agency for Research on Cancer (IARC) classified into group 2B as a possible human carcinogen based on sufficient evidence in humans (IARC, 1993). III.3.2.

Sources of ochratoxin A in foods

Tables III.8,III.9 and III.10 reflect the occurrence of OTA in cereals and cereal products, alcoholic beverages and others foods (Miraglia and Brera, 2002). The sources of OTA in different foods are shown in Figure III.5.

In cereals including wheat, barley, oats, rye, corn and rice are susceptible to the contamination by OTA (Table III.8) since many of the toxigenic species of fungi are plant pathogens. The growth of fungi may occur on crops in the field during harvesting and drying and continue in storage, or it may origin in the storage (Abramson, 1991). Cereal products and animal feeds can be contaminated due to ochratoxigenic cereals.

Figure III.5: Sources of contamination of Ochratoxin A in foods and feeds

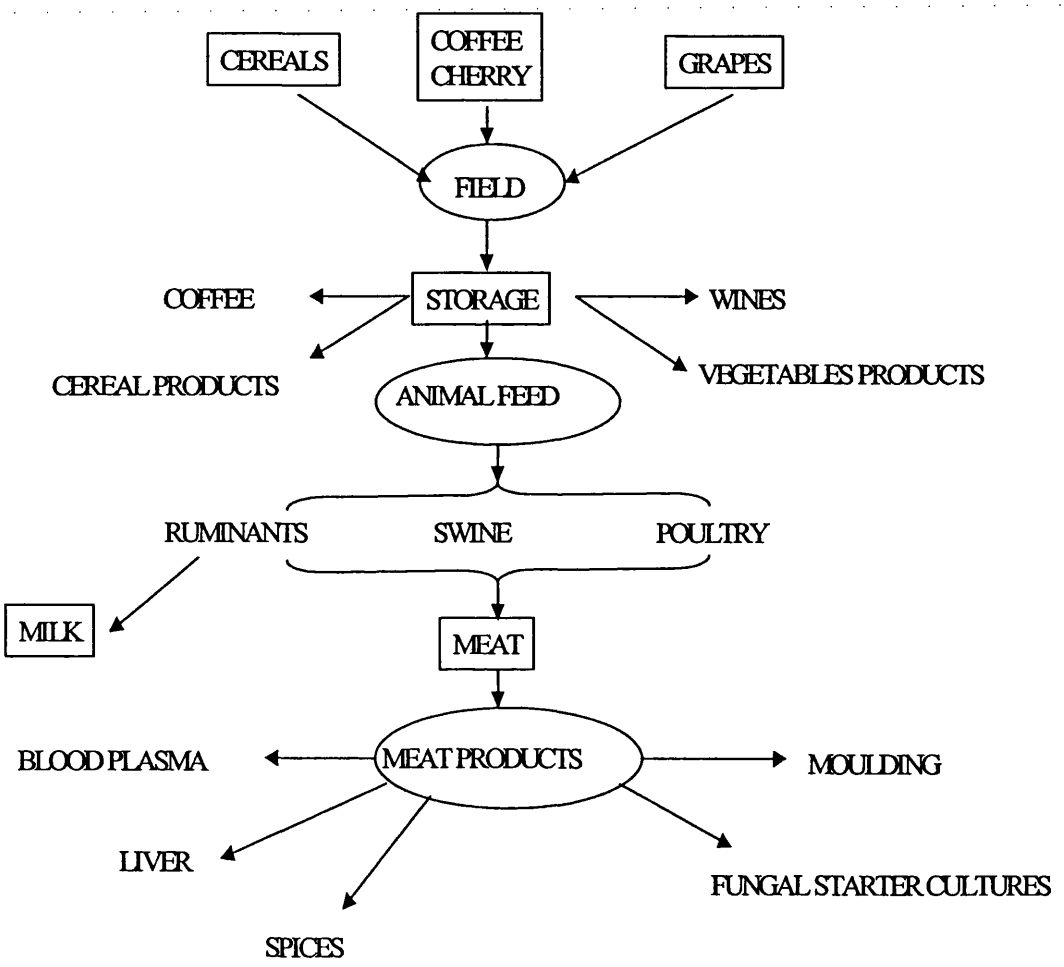


Table III.8: Occurence of ochratoxin A in cereals and cereals products

Food	Country	Incidence	Range (ng/g)	Reference
Barley	Finland	4/21	0.2-0.7	Miraglia and Brera (2002)
Barley	France	1/7	2	Miraglia and Brera (2002)
Barley	Germany	16/22	0.01-0.5	Miraglia and Brera (2002)
Barley	Italy	6/25	0.05-3.9	Miraglia and Brera (2002)
Barley	UK	7/67	0.1-6.4	Miraglia and Brera (2002)
Barley kernel	Finland	3/45	0.8-12.3	Solfrizzo et al. (1998)
Bread	UK	1/50	210	Osborne (1980)
Cereal products	UK	16/16	0.1-0.9	Patel et al. (1996)
Corn	Austria	3/27	5-100	Leibetseder (1989)
Corn	Egypt	1/3	12	Abdelhamid (1990)
Corn	France	1/18	0.2-1.1	Miraglia and Brera (2002)
Corn	Germany	14/31	0.01-3.35	Miraglia and Brera (2002)
Corn	Italy	7/49	0.05-4.9	Miraglia and Brera (2002)
Corn	Poland	2/123	25-400	Golinski et al. (1991)
Corn	Spain	1/30	0.5-2.5	Miraglia and Brera (2002)
Corn	USA	3/293	83-166	Shotwell et al. (1971)
Corn flour	UK	1/4	0.6	Patel et al. (1996)
Millet	Germany	24/26	0.01-0.831	Miraglia and Brera (2002)

Table III.8: (Continued)

Food	Country	Incidence	Range (ng/g)	Reference
Oat	Germany	24/29	0.01-0.55	Miraglia and Brera (2002)
Oat	Norway	15/72	0.1-4.2	Miraglia and Brera (2002)
Oat	Sweden	9/33	0.1-3.6	Miraglia and Brera (2002)
Oat	UK	1/22	5.9	Miraglia and Brera (2002)
Oat kernels	Finland	2/34	0.8-56.6	Solfrizzo et al. (1998)
Rice	France	2/16	0.2-1.4	Miraglia and Brera (2002)
Rice	Germany	2/22	0.1-0.28	Miraglia and Brera (2002)
Rye	Denmark	180/247	0.01-33	Miraglia and Brera (2002)
Rye	Finland	9/52	0.2-17	Miraglia and Brera (2002)
Rye	Germany	12/26	0.01-0.8	Miraglia and Brera (2002)
Rye	Germany	1/22	1.1	Wolff et al. (2000)
Rye	Norway	1/8	0.25-2.5	Miraglia and Brera (2002)
Rye	Sweden	33/47	0.1-27	Miraglia and Brera (2002)
Rye	UK	1/22	1.1	Miraglia and Brera (2002)
Wheat	Denmark	146/247	0.01-31.6	Miraglia and Brera (2002)
Wheat	Egypt	1/3	12	Abdelhamid (1990)
Wheat	Finland	7/125	0.2-3	Miraglia and Brera (2002)
Wheat	France	1/22	0.2-0.9	Miraglia and Brera (2002)
Wheat	Germany	10/27	0.01-0.26	Miraglia and Brera (2002)
Wheat	Norway	47/193	0.01-19.9	Miraglia and Brera (2002)
Wheat	Sweden	55/132	0.1-5.2	Miraglia and Brera (2002)
Wheat	The Netherland	1/31	8.7	Miraglia and Brera (2002)
Wheat	UK	6/138	0.1-6.3	Miraglia and Brera (2002)
Wheat	USA	31/297	5-115	Shotwell et al. (1976)
Wheat	USA	56/383	0.03-31.4	Trucksess et al. (1999)

The presence of OTA (Table III.9) in wines is due to direct factors such as meteorological conditions, grape cultivation and wine-making techniques. Furthermore, it is important know the indirect factors as are latitude, year of production, use of pesticides, presence of spoilage microorganisms, conditions of storage, types of maceration and conditions of fermentation (Blesa et al., 2004). The Table III.9 shows the occurrence of OTA in beer that is an important source of this mycotoxin.

Table III.9: Occurence of ochratoxin A in alcoholic beverages

Food	Country	Incidence	Range (ng/g)	Reference
Beer	Denmark	21/21	0.007-0.16	Miraglia and Brera (2002)
Beer	Finland	8/13	0.05-0.06	Miraglia and Brera (2002)
Beer	Germany	39/251	0.005-0.29	Miraglia and Brera (2002)
Beer	Portugal	3/7	0.005-0.006	Miraglia and Brera (2002)
Beer	Sweden	5/5	0.01-0.03	Miraglia and Brera (2002)
Dessert wine	Greece	3/18	0.05-2.82	Stefanaki et al. (2003)
Dessert wine	Italy	6/15	0.001-3.856	Pietri et al. (2001)
Red wine	Greece	8/8	0.002-2.35	Markaki et al. (2001)
Red wine	Hungry	1/1	0.005	Burdaspal and Legarda (1999)
Red wine	Italy	14/96	0.010-3.177	Pietri et al. (2001)
Red wine	Morocco	20/20	0.04-3.24	Filali et al. (2001)
Red wine	Morocco	3/3	0.551-0.554	Markaki et al. (2001)
Red wine	Portugal	2/2	0.011-0.02	Burdaspal and Legarda (1999)
Red wine	South Africa	9/9	0.07-0.39	Shepard et al. (2003)
Red wine	Spain-Portugal	6/6	0.002-0.5	Markaki et al. (2001)
Red wine	Spain	66/72	<0.003-0.603	Burdaspal and Legarda (1999)
Red wine	Spain	9/14	<0.003-0.022	Zimmerli and Dick (1996)
Red wine	Spain	3/6	<0.010-0-190	Majerus and Otteneder (1996)
Red wine	Spain	13/28	0.050-0-316	Lopez de Ceraín et al. (2002)
Red wine	Spain	12/61	0.05-0.53	Blesa et al. (2004)
Rosé wine	France	4/6	0.003-0.085	Burdaspal and Legarda (1999)

Table III.9: (Continued)

Food	Country	Incidence	Range (ng/g)	Referente
Rosé wine	Italy	4/9	0.01-0.97	Visconti et al. (1999)
Rosé wine	Italy	3/3	0.01-0.08	Shepard et al. (2003)
Rosé wine	Italy	2/6	0.003-0.006	Burdaspal and Legarda (1999)
Rosé wine	Morocco	7/7	0.028-0.18	Filali et al. (2001)
Rosé wine	Portugal	2/4	0.003-0.020	Burdaspal and Legarda (1999)
Rosé wine	Portugal	64/64	<0.02	Festas et al. (2000)
Rosé wine	South Africa	15/15	0.04-0.33	Shepard et al. (2003)
Rosé wine	Spain	7/12	0.154-0.208	Lopez de Ceraín et al. (2002)
Rosé wine	Spain	35/43	0.003-0.267	Burdaspal and Legarda (1999)
Rosé wine	USA	2/2	0.010-0.019	Burdaspal and Legarda (1999)
Rosé wine	Greece	55/118	0.05-1.72	Stefanaki et al. (2003)
Rosé wine	Spain	8/21	0.05-0.46	Blesa et al. (2004)
Special wines	France	2/4	0.003-0.024	Burdaspal and Legarda (1999)
Special wines	Italy	3/4	0.003-0.040	Burdaspal and Legarda (1999)
Special wines	Germany	4/7	0.003-0.016	Burdaspal and Legarda (1999)
Special wines	Portugal	3/4	0.003-0.029	Burdaspal and Legarda (1999)
Special wines	Hungry	0/1	nd	Burdaspal and Legarda (1999)
Special wines	Spain	6/13	0.05-0.40	Blesa et al. (2004)
Sparkling wines	Spain	10/12	0.003-0.037	Burdaspal and Legarda (1999)
White wine	France	2/2	0.161	Burdaspal and Legarda (1999)
White wine	Spain	4/24	0.05-0.76	Blesa et al. (2004)
White wine	France	2/2	<0.01-0.11	Ospital et al. (1998)
White wine	Greece	7/20	0.05-1.16	Stefanaki et al. (2003)
White wine	Italy	7/8	0.01-1.15	Visconti et al. (1999)
White wine	Italy	2/2	0.037-0.049	Burdaspal and Legarda (1999)
White wine	Morocco	3/3	0.04-0.54	Filali et al. (2001)
White wine	Portugal	1/2	0.003-0.010	Burdaspal and Legarda (1999)
White wine	Spain	24/26	0.003-0.155	Burdaspal and Legarda (1999)

In meat and meat products, the occurrence of OTA (Table III.10) is a result of either direct contamination with moulds or indirect transmission from animals exposed to natural contaminated feed. While the first route is not a problem of major concern, the carry-over of OTA in food of animal origin via feed must be regarded as more serious. In fact, organs or muscle meat from ruminants are of no importance due to the decomposition activity of the flora of the rumen. However, other farmed animals such as pig are known to be particularly sensitive to OTA and to show relatively high serum half-lives of 72-120 h (Galtier et al., 1991). The distribution within the tissues follows the pattern kidney>liver>muscle>fat. Apparently, differences were noted in the accumulation of OTA in blood serum and tissues following the intake of naturally-contaminated feed or crystalline toxin: concentrations in pigs are about three to five times higher with naturally contaminated feed (Madsen et al., 1982; Lusky et al., 1995). OTA concentrations greatly increased when blood or liver tissue was added to products such as blood sausages or Bologna-type sausages (Scheuer, 1989; Lusky et al., 1994) which could be due to the addition of naturally-contaminated spice mixtures and blood plasma (up to 10% of fresh blood plasma is allowed to be added to Bologna-type sausages). A survey conducted by Scheuer (1989) showed prevalences of 16-19% of the toxin in sausages with levels ranging between 0.1 and 3.4 μg/kg.

In the Table III.10 reflects the presence of OTA in coffee. A complete review carried out by Bucheli and Taniwaki (2002) reviewed the origin of this mycotoxin in coffee and suggested that A. ochraceus and A. carbonarius are the most potent OTA producers on this food.

Table III.10: Occurence of ochratoxin A in others foods

Food	Country	Incidence	Range (ng/g)	Reference
Baby food	Germany	63/97	0.01-2.13	Miraglia and Brera (2002)
Black pudding	UK	4/32	1-1.8	Miraglia and Brera (2002)
Chili sauce	UK	1/4	3.3	Patel et al. (1996)
Chocolate	UK	18/40	0.1-0.6	Miraglia and Brera (2002)
Cocoa	Germany	40/40	0.01-1.8	Wolff et al. (2000)
Cocoa	Germany	91/96	0.01-1.8	Miraglia and Brera (2002)
Cocoa powder	UK	39/40	0.2-2.4	Miraglia and Brera (2002)
Coffee	Europe	299/633	0.2-27.2	Stegen et al. (1997)
Coffee	UK	81/100	0.1-8	Patel et al. (1997)
Currants	UK	115/120	0.2-53.6	Miraglia and Brera (2002)
Dates	UK	1/20	0.2	Miraglia and Brera (2002)
Figs	UK	2/20	0.1-0.8	Miraglia and Brera (2002)
Fruit juice	France	1/19	3.45	Miraglia and Brera (2002)
Grape juice	Germany	75/90	0.01-5.26	Miraglia and Brera (2002)
Grape juice	Japan	2/12	0.003-0.006	Ueno (1998)
Grape juice	Spain	8/8	0.03-0.18	Miraglia and Brera (2002)
Grape juice	UK	19/20	0.01-2.10	Miraglia and Brera (2002)
Green coffee	Japan	5/68	3.2-17	Belitz and Maier (1991)
Ham	Germany	16/57	0.01-0.17	Miraglia and Brera (2002)
Instant coffee	Brazil	16/16	0.2-5.1	Leoni et al. (2000)
Milk	Norway	13/165	0.01-0.06	Miraglia and Brera (2002)
Milk	Sweden	5/36	0.01-0.03	Miraglia and Brera (2002)
Nutmeg	Portugal	3/3	0.2-8.5	Miraglia and Brera (2002)
Mixed feed	Australia	1/25	70000	Connole et al. (1981)
Mixed feed	Canada	4/51	48-5900	Abramson et al. (1983)
Olive oil	Italy	1/12	0.6	Miraglia and Brera (2002)
Pepper	Nether-lands	1/6	0.8	Miraglia and Brera (2002)

Table III.10: (Continued)

Food	Country	Incidence	Range (ng/g)	Reference
Pig kidney	France	3/300	1-5	Dragacci et al. (1999)
Pig kidney	UK	15/104	1-9.3	Miraglia and Brera (2002)
Pork edible offal	France	103/1011	0.05-6.1	Miraglia and Brera (2002)
Pork meat	Germany	8/58	0.01-0.14	Miraglia and Brera (2002)
Raisins	Finland	22/31	0.2-7	Miraglia and Brera (2002)
Raisins	UK	110/121	0.2-29.8	Miraglia and Brera (2002)
Roasted coffee	Brazil	23/34	0.2-6.5	Leoni et al. (2000)
Salami	Germany	29/68	0.01-0.19	Miraglia and Brera (2002)
Sausage	Germany	96/201	0.01-4.56	Miraglia and Brera (2002)
Sesame oil	UK	1/3	0.4	Patel et al. (1996)
Soya	Germany	5/13	0.01-0.09	Miraglia and Brera (2002)
Soya sauce	Japan	5/5	0.003-0.026	Ueno et al. (1998)
Spices	Italy	5/5	0.4-23.8	Miraglia and Brera (2002)
Spices	UK	1/4	2.6	Patel et al. (1996)
Sultanas	UK	104/120	0.2-25.1	Miraglia and Brera (2002)
Sweet pepper	Portugal	3/6	0.2-4.3	Miraglia and Brera (2002)
Vinegar	Germany	44/87	0.01-4.35	Miraglia and Brera (2002)

III.3.3. Control of ochratoxin A in food processing

The control of OTA in foods can be obtained by the application of traceability concept. Traceability is a total safety concept used to guarantee the control of foods (Soriano et al., 2003). This concept is grouped by several systems as are Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs) and Hazard Analysis and Critical Control Point (HACCP).

III.3.3.1. Good Agricultural Practices (GAPs)

Good Agricultural Practices (GAPs) comprise policies, procedures and methods used to minimize risk to public health from agronomic and harvesting practices of food products. GAPs join to GMPs must be working effectively within commodity system before HACCP is applied because if the GAPs and GMPs are not functioning effectively then the introduction of HACCP will be complicated, resulting in a cumbersome, over-documented system. Brera et al. (1998) suggested that appropriate irrigation procedures, crop turnover, adequate use of pesticides and biotechnology as is transgenic plants can help to minimize mycotoxin contamination. According to Bucheli and Taniwaki (2002), OTA contamination in coffee can clearly be minimized by following GAPs and a subsequent post-harvest handling consisting of appropriate techniques for drying, grading, transportation and storage of green coffee. Land used for crop or horticulture production should be fit for purpose being the application of pesticides, including fungal pesticides, suitable for legislated maximum residue levels (MRLs). In a recently study, Lo Curto et al. (2004) determined that synthetic pesticides can reduce OTA concentration from 96.5% in the grape sample treated with azoxystrobin to 88% in the sample treated with organic pesticides as are dinocap and penconazole. FAO (2001) reviewed this concept including Good Hygienic Practices (GHPs) and Good Storage Practices (GSPs) to make sure the food safety in the harvested commodity and stored commodity on the farm, respectively.

III.3.3.2. Good Manufacturing Practices (GMPs)

Good Manufacturing Practices (GMPs) are ways and means employed to minimize the risk to public health of OTA from improper handling, storage, distribution or processing of foods (Koek et al., 1983) and determine the hygienic aspect of food production. In the GMPs, the decontamination of OTA is the most important critical control point to guarantee the food safety. There are a number of conflicting reports which suggest that the production of mycotoxins is increased (Paster et al., 1985) or unaffected (Paster and Bullerman, 1988) after irradiation of fungi under various laboratory conditions. It appears that the fungal strain, condition of storage, humidity and irradiation dose affect mould growth and toxic production (Mitchell, 1988).

III.3.3.3. Hazard Analysis and Critical Control Point (HACCP)

Hazard Analysis and Critical Control Point (HACCP) is a system identifying and monitoring specific that can be applied to OTA as biological hazard. The hazard analysis serves as the basis for establishing critical control points (CCPs) that identify points in the food process which must be controlled in order to ensure the safety of the food and establish critical limits in each CCPs (FAO, 2001). According to the Wareing (1998), several measures as are irrigation, insect control, resistant varieties, timely harvesting and use of crib-storage prior to shelling and long-term storage, can be effective in reducing the risk of mycotoxin production. The application of this HACCP is a sequence of preliminary tasks and HACCP plan that can be developed according to the FAO (2001).

The preliminary tasks in the HACCP plan comprise:

(a) Scope of the HACCP study: The aim is to assure food safety during harvest and postharvest of food products.

(b) Assemble the HACCP team: The team must be multidisciplinary and include personnel with the necessary expertise, knowledge and experience, relating to the product and process being studied.

(c) Describe the product: This process includes all applicable information on the product, which will help in the hazard analysis and establishing CCPs.

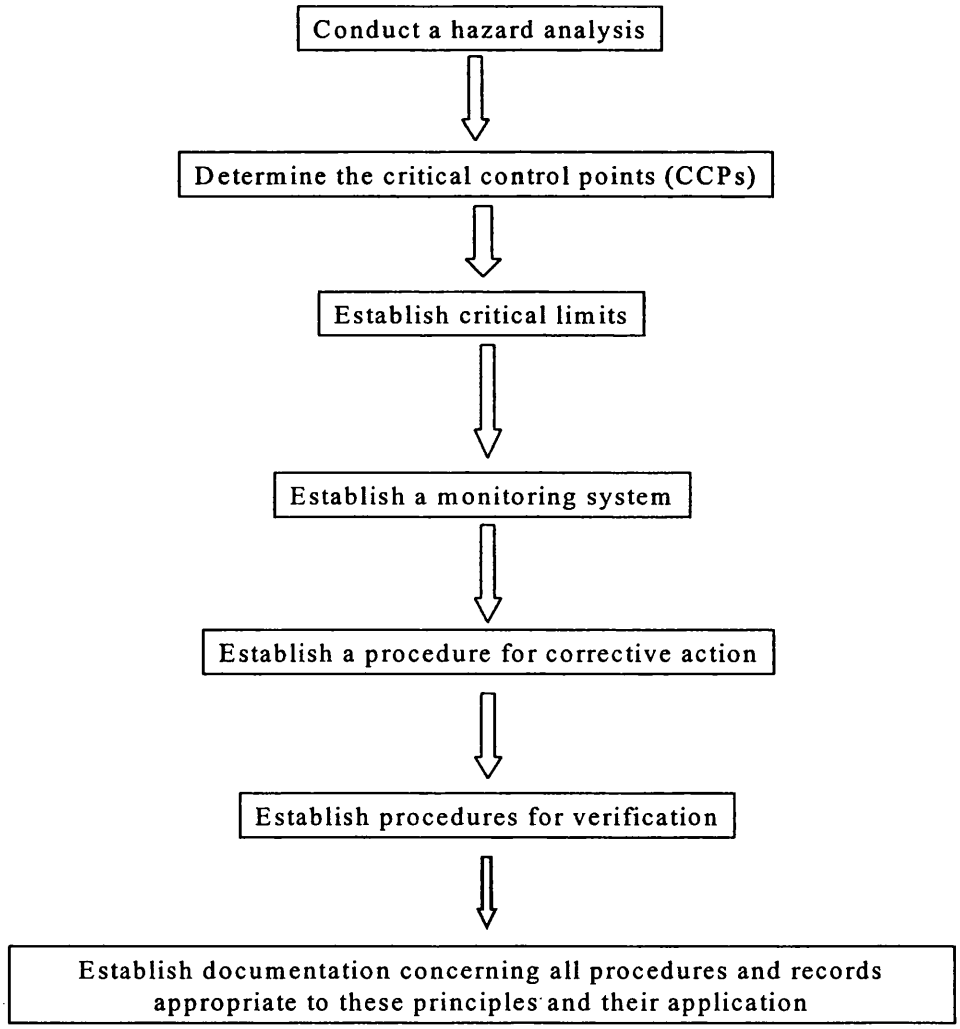
(d) Identify and describe the intended use and consumers of the food.

(e) Develop a flow diagram that describes the process. It must be clear, simple description of all steps involved in the process from harvest until post-harvest.

(f) Verify the flow diagram: the HACCP team checks of the operation to verify the accuracy of the flow diagrams.

After these preliminary tasks, seven principles of HACCP are applied according to the FAO (2001) and they are shown in Figure III.6. The fact of incorporating the HACCP concept in the integrated mycotoxin management help to prevent the risk of exposure the toxins.

Figure III.6: Principles of the Hazard Analysis and Critical Control Point (HACCP) system



III.3.4. Decontamination of ochratoxin A in food processing

The value of the decontamination of OTA in food depends on the method used and the remaining toxicity of the treated sample (Beaver, 1991). According to the EMAN (2000), the ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic, or may reverse to reform the parent mycotoxin, or alter the nutritional and palatability properties of the foods. Methods for decontaminating the OTA content of food are physical, chemical and biological. In other hand, Alldrick (1996) suggested that methods of decontamination of OTA must satisfy two criteria: they must be compatible with existing national food safety legislation and the functionally of the cereal with regard to the final product quality must remain unaltered. Some physical methods of contamination are reflected in Table III.11.

Table III.11: Decontamination by physical methods

Methods	Sample	Reduction (%)	Reference
Cereals			
Autoclaving (30')	Oatmeal	61-67	Trenk et al. (1971)
Autoclaving (132°C 30')	Barley	85	Madsen et al. (1983)
Heating			
Milling	Barley	10-30	Chelkowski et al. (1981a)
Scouring	Wheat	>50	Brown et al. (1991)
Heating (210°C 5')	Biscuits	62	Osborne (1979)
Heating (210°C 5')	Biscuits	83	Levi et al. (1974)
Scouring	Cereals	44	Scudamore et al. (2003)

Table III.11: (Continued)

Methods	Sample	Reduction (%)	Reference
Vegetables			
Autoclaving (121°C 20')	Beans	20.1-76.7	Milanez and Sabino (1989)
Cooking	Beans	84	Milanez and Leitao (1996)
Coffee			
Roasting	Coffee	90	Micco et al. (1989)
Roasting	Coffee	69	Van der Stegen et al. (2001)
Roasting	Coffee	84	Blanc et al. (1998)
Roasting	Coffee	96	Micco et al. (1989)
Roasting	Coffee	79	Wilkens et al. (1999)
Roasting	Coffee	89	Wilkens et al. (1999)
Roasting	Coffee	90	Romani et al (2003)
Alcoholic beverages			
Heating (>90°C)	Beer	52-90	Krogh et al. (1974) Chu et al. (1975)
Activated carbon as fining agents	Wine	99.8	Galvano et al. (1998)
Potassium caseinate as fining agents	Wine	82	Castellari et al. (2001)
Meats and meat products			
Drying	Sausage	20	Bullerman (1981)
Frying	Kidneys	35	Josefson and Möller (1980)
Frying	Blood-pudding	32	Josefson and Möller (1980)
Frying	Muscular tissues	29	Josefson and Möller (1980)

III.3.4.1. Decontamination of cereals, vegetables and cereal and vegetable products

Decontamination by physical methods

From cereals are important to have information on how OTA is distributed within different fractions of the primary products being cleaning and scouring two effective methods for decontamination this mycotoxin in foods and feeds.

Chelkowsli et al. (1981a) have reported that dry cleaning and wet cleaning of contaminated lots of wheat and barley does not eliminate OTA from grains. In fractions >2.5 mm, concentrations of OTA reach 80-100% of the initial concentration in barley or wheat grains. In fact, OTA is mainly present not on the surface but in the inner, deeper parts of kernels and only 10-50% of the toxin can be extracted and removed from the surface of grain after chloroform extraction. Osborne et al. (1996) have shown that OTA is concentrated in the bran fraction of wheat. Cleaning and milling of wheat and barley did not remove OTA in naturally-contaminated samples and levels in flour and bran were similar. The results of milling the same grains show that the level of OTA is similar in flour and in bran. In fact, corn fractions containing 51% OTA higher than the corn bits (starch, fibre and gluten). OTA can slowly break down merely on storage of grains and grain products. Modern commercial milling of wheat to produce flour involves the removal of the starch-rich endosperm from the outer (bran) layers of the grain in a

sequential process coupled with grinding to produce the flour. White flour is essentially ground endosperm, while wholemeal flour represents the entire ground cereal produced by recombining the ground bran and endosperm at the end of the milling process. Chelkowski et al. (1981a) suggested that OTA is not removed from wheat during routine cleaning prior to milling and is equally distributed between flour and bran during milling. After milling the level of OTA in flour was similar to its concentration in bran. In pearl barley concentration of OTA stated only 10-30% of initial concentration in barley grain (Chelkowski et al. 1981b).

Furthermore, the effect of a scouring step (removal of the outer layers of the pericarp) while cleaning the grain prior to milling (Brown et al., 1991) had shown the potential of reducing in >50% of OTA. Furthermore, a reduction of pesticide and microbial contamination was obtained of flour by the use of such a process. Scouring removes a proportion of the pericarp (bran coat) prior to milling (Osborne et al. 1996). The results of the study confirmed that scouring reduced the OTA level in white and wholemeal flour three-fold for both the hard and soft wheat.

In other hand, the ratio temperature-time is other OTA decontamination procedure. Trenk et al (1971) shows that artificially-contaminated oatmeal and rice meal autoclaved for 30 min lost 67 and 61% of the initial OTA contents, respectively. El Banna and Scott (1984) observed an average destruction of 6% of OTA in polished wheat at 100ºC for 30 min. Madsen et al (1983) working on barley decontamination, verified an 85%

reduction in initial OTA contents when barley was heated in an autoclave, at 132°C for 30 min. The time necessary to reduce ochratoxin A content in dry or wet wheat by 50% ranges from 6 to 700 min at temperatures ranging from 250 to 100°C (EMAN, 2000).

For cereal products, scouring removed up to 44% of the OTA present, but only a small further loss occurred in the bread-making process. An overall reduction of about 75% could be achieved in white bread using a combination of cleaning scouring and removal of the bran and offal fractions. Maximum overall reduction in producing wholemeal bread was about 40% (Scudamore et al. 2003). Biscuits were achieved a 62% (Osborne, 1979) or 83% loss under 5 min heating at 210°C (Levi et al., 1974). It was suggested that the higher dough temperature and a much lower final moisture content assisted in the destruction of ochratoxin A. The fate of OTA has been reported during hydrotermic processes such as baking (Boudra et al., 1995) and breadmaking (Osborne, 1979). When OTA was added to white flour samples which were subsequently baked into bread (oven temperature of 220°C for 25 min), it was recovered without decomposition. When the experiment was repeated with biscuit baking, about two-thirds of the toxin was destroyed or immobilized. Osborne (1979) explained the difference in the OTA content in bread and biscuit by higher dough temperature achieved in the baking of biscuit and by the lower content in these biscuits. These explanations are supported by the findings of Boudra et al. (1995) who studied the descomposition of OTA in wheat under different thermal conditions and two moisture conditions (dry and wet). Their results show that OTA in a relatively thermostable mycotoxin and

that the presence of water (50%) increases the descomposition of OTA at 100°C and 150°C whereas its presence decreases this decomposition at 200°C. The presence of moisture can increase or decrease the stability of OTA during heating of cereals, depending on the temperature and possibly other variables (Boudra et al., 1995). No descomposition was observed in bread baking but it was considerable during biscuit baking. Also, cooking of wheat and cooking or canning of beans generally caused only small losses of OTA due to the heat itself.

For vegetables, Milanez and Sabino (1989) observed OTA losses of 20.1-76.7% after cooking artificially-contaminated beans in an autoclave for 20 min at 121°C. Milanez and Leitao (1996) observed that cooking caused a substantial reduction in the levels of OTA (up to 84%). This effect was even more pronounced when the bean grains were soaked in the water for 12 h before cooking under pressure, at 115°C for 45 min. Furthermore, processing of cocoa beans to dark chocolate largely destroyed OTA (Miraglia et al., 1993).

Decontamination by chemical methods

Studies show that formic, propionic and sorbic acids degrade OTA at concentrations ranging from 0.25% to 1% after exposure of 3-24 hours. Ammoniation almost completely decomposes OTA in corn, wheat and barley, while heating with 0.5% sodium hydroxide or autoclaving were nearly as effective treatments (Scott, 1996).

Chelkowski et al (1982) demonstrated that ammoniation grain can be used as feedstuff component without essential change of nutritive value during ammoniation and can be used to achieve decomposition of OTA to nondetectable level. Chelkowski et al. (1981b) studied that the addition of ammonia to final concentration 2% inactivates OTA and other mycotoxins such as aflatoxin, citrinin, penicillic acid and partially zearalelnon at temperature 20-50°C.

Solutions of monomethylamine or ammonia with calcium hydroxide used at 96°C were shown to descompose OTA in swine feed (Gerlach, 1992) or heating with alkaline hydrogen peroxide (Fouler et al., 1994).

A process commonly used on-farm in the United Kingdom, but specifically for cattle feed, is to treat wheat or barley with 2.5-3% caustic soda. This latter process is likely to be very effective in destroying ochratoxin A, which is known to be susceptible to alkaline hydrolysis (Scudamore, 1996).

Decontamination by biological methods

Ruhland et al. (1994, 1996a,b) reported that cell cultures of wheat, maize, tomato, soybean and other plants completely transformed OTA into a number of products. The transformation reactions included hydrolysis of ester and peptide bonds, methylation and hydroxylation. All plants investigated produced essentially the same transformation products. At least some of these transformations are known to lead to the loss of toxicity. Sweet potato tubers

converted OTA in vitro into at least five products distinguishable by thin layer chromatography (Fujita and Yoshizawa, 1989). However, these products were not characterized.

III.3.4.2. Decontamination of meats

Decontamination by physical methods

Only limited data on the natural occurrence of OTA in meat products are available. This mycotoxin seems to be very stable. OTA was moderately stable during drying of sausage, about 20% was lost from certain pig products during frying, and it was stable in frozen pig kidney but only partially recovered from cheese after storage at room temperature (Bullerman, 1981, Harwig et al., 1983). Production procedures such as heating and ripening as well as storage have no effects on the reduction of the levels of OTA in meat products (Scheuer, 1989; Lusky et al., 1994).

Frying or boiling could reduce the amount of OTA in Swedish blood-pudding, kidneys and muscular tissues by 32, 35 and 29%, respectively (Josefson and Möller, 1980). The heat stability of OTA in these foods mainly depends on the water content and the inner temperature of the heat-treated food. No loss could be found in adipose tissues.

Plank et al. (1990) studied several adsorbents and suggested that in vitro tests showed that the 1% addition of activated charcoal leads to complete

adsorption of OTA from aqueous solutions. This effect was not influenced by pH values ranging from 3 to 8. In contrast, adsorption by bentonite and hydrated sodium calcium aluminosilicate occurred primarily in the acid range (pH 3-4). Dietary addition of hydrated sodium calcium aluminosilicate (1%) and acid bentonite (1, 10%) to OTA contaminated feed (1 mg/kg) had no effect on the blood or tissue levels of the toxin in pigs. The addition of 1% activated charcoal caused a slight decrease of OTA in the blood, whereas a tenfold dosage resulted in a 50% to 80% reduction of OTA levels in both blood and tissue.

Reduction of OTA absorption via the dietary administration of activated charcoal (5%) was confirmed in 16 week feeding experiment. However, this experiment also showed the serum level of vitamin E to be lower than in the controls receiving adsorbent-free feed. Rotter et al (1989) demonstrated that the addition of charcoal to OTA contaminated diets appeared to be an ineffective method for reducing the toxic effects of OTA in growing chicks. Aravind et al. (2003) demonstrated that addition of dietary esterified glucomanan is effective in counteracting the toxic effects of naturally contaminated feed with OTA and other mycotoxins such as aflatoxin, zearalenone, T-2.

Several of these studies mention physical removal of OTA with the silverskins (chaff) as a possible mechanism for the observed reduction, but their data show that this can only partially be the explanation (Blanc et al. 1998). Another possible explanation was given by Studer-Rohr et al. (1995)

who showed partial isomerization of OTA at the C-3 position into a less toxic diastereomer. However, this also seems to be only a partial explanation. A third explanation might come from a study about thermostability of OTA in wheat under two moisture conditions (Boudra et al. 1995).

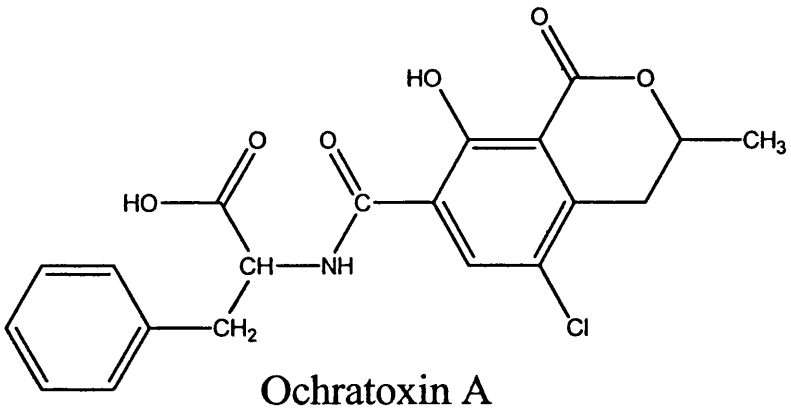
Decontamination by biological methods

Ruminant meat has a lower OTA levels due the activity of symbiotic microorganisms in the stomach of ruminants, particularly the protozoal fraction, hydrolysing by non-specific peptidases the peptide bond of OTA into phenylalanine and ochratoxin α (Figure III.7) (Hult et al., 1976; Kiessling et al., 1984; Pitout, 1969). In addition to the peptide bond, the lactone bond of OTA could be hydrolysed by esterases of microbial origin, resulting in the same product as alkaline treatment (Madsen et al., 1983). After opening the lactone bond, the toxicity of OTA towards Bacillus brevis disappears (Xiao et al., 1996) and its half-life in rat shortens significantly (Li et al., 1997). However, the lactone ring opening of OTA is reversible and the toxin can be regenerated under acidic conditions (Valenta and Richter, 1998).

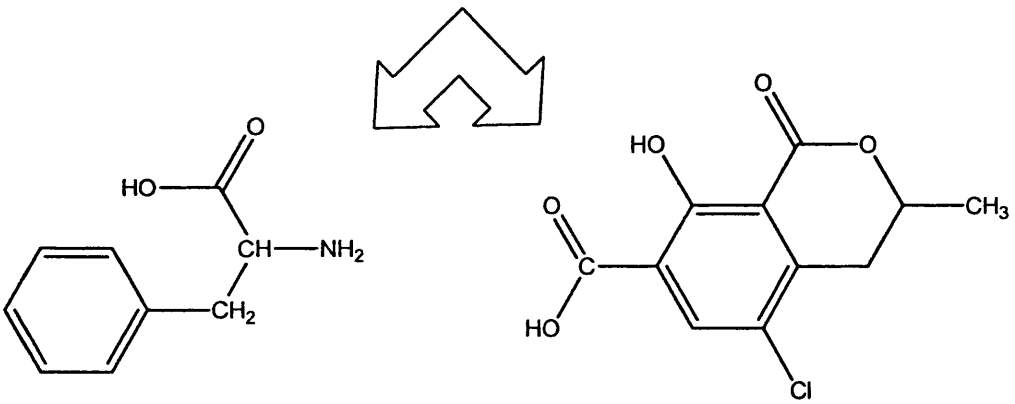
III.3.4.3. Decontamination of coffee

Prevention and reduction of OTA in green coffee can be achieved by avoiding the use of damaged and overripe cherries, refraining from inappropriate cherry storage in plastic bags, optimization of drying conditions and the reduction of green coffee defects (Bucheli et al., 2002). All these

Figure III.7: Degradation of Ochratoxin A in ruminants.



Ochratoxin A



Phenylalanine OTA α

measures are part of coffee post-harvest practices (Barel and Jacquet, 1994). In fact, there was little or no OTA production at 80% Equilibrium Relative Humidity, at 87% and 95% OTA production was high after different days of incubation. Under alternating temperatures OTA production was higher than at constant temperature, and alternating temperatures indirectly favoured OTA

production due to the condensation and a subsequent rapid increase in moisture content and water activity of the coffee beans (Palacios-Cabrera et al., 2004). Instant coffee is even more critical since it was shown to contain significantly higher levels of OTA than coffee prepared from roasted beans (Bresch et al., 2000).

The most important procedure for decontamination of coffee is physical method. Blanc et al (1998) demonstrated that the variability and the amount of OTA present in green coffee are drastically reduced during soluble coffee manufacture. A small proportion of OTA is eliminated during green cleaning, but the most significant reduction takes place during roasting. Table 4 shows that several authors obtained decontamination of OTA higher 68%. This OTA reduction that took place during the roasting process can be attributed to both thermal degradation and chaff removal (Romani et al. 2003). A 60% reduction of OTA was measured by Micco et al. (1989) after industrial decaffeination of a naturally-contaminated sample.

III.3.4.4. Decontamination of alcoholic beverages

Decontamination by physical methods

For beers, the amount of OTA in barley decreased significantly during ensiling (Rotter et al., 1990). There was a loss of toxin in both naturally contaminated barley and a mixture of clean barley with Aspergillus ochraceus mycelium. OTA in barley was also degraded during malting (Krogh et al.,

1974) and the final fermentation of beer brewing (Chu et al., 1975). The finding of ochratoxin α as a degradation product (Nip et al., 1975) indicates that peptidase activities, produced during malting, were responsible for ochratoxin degradation during the brewing process. In brewing industry in which cereal substrates are subjected to aqueous conditions at temperatures up to 90ºC have shown between 50 and 90% destruction of OTA (Krogh et al., 1974, Chu et al., 1975).

OTA appears to be destroyed during the malting of contaminated barley. Steps in the actual brewing process that gave the greatest losses of OTA in one study (Chu et al., 1975) were the malt mash (but no cooker mash), boiling of the wort with hops, and the final fermentation, so that overall losses were significant (72-86% in this study and 93-98% in another). However, a recent study showed little loss of OTA during fermentation of wort (Scott et al., 1995).

In other hand, fining is a common winery practice which involves the addition of an adsorptive compound to reduce levels of OTA. The use of enological fining agents has been shown to reduce the level of this mycotoxin. Activated carbon an insoluble powder formed by pyrolysis of different kinds of organic materials, shows different adsorbing properties depending on its origin. Surface area of activated carbons may vary from 500 to 2000 m^2/g and up to 3500 m^2/g for superactive carbons. Activated carbon has a good capacity to absorb OTA in model solution whereas bentonite has demonstrated a low affinity for OTA (Ramos et al., 1996). The use of activated carbons can

adsorbed until 99.86% of the OTA (Galvano et al., 1998). Castellari et al. (2001) studied several variety of fining agents such as activated carbon, silica gel, potassium caseinate, egg albumin and gelatine. The potassium caseinate was the highest specific adsorption capacity which removed up to 82% due to a high surface area per mass and low adsorption of total polyphenols. Phillips et al. (1988) shown that the use of aluminosilicate additives in feed is effective in absorbing some mycotoxins from feed in the digestive tract. In other hand, hydrates sodium calcium aluminosilicate and sepiolite were not effective in binding OTA (Galvano et al., 1998). Furthermore the use of 1 g of cholestyramine adsorbed 9.6 mg OTA from a solution (EMAN, 2000).

Decontamination by biological methods

Scott et al. (1995) fermented a worth containing added OTA and fumonisin B1 and B2. After 8 days fermentation using three different yeast strains (Saccharomyces), a maximal decrease was observed in the case of OTA 13 and 28%, respectively. The uptake of ochratoxin by yeast was 21%.

Cheng-An and Draughon (1994) screened bacteria and yeasts in order to detect microorganisms able to degrade ochratoxin A. Thirty seven bacteria , 10 strains of yeasts and 12 moulds were screened. Acitenobacter calcoaceticus was able to degrade ochratoxin A in ethanol minimal salts medium with an initial concentration of 10 μg per ml at both 25 and 30°C which end product of degradation of OTA by A. calcoaceticus is an α-OTA which is less toxic compound. Nevertheless, this study not implied the degradation of OTA in an in situ system using food or feed.

III.3.4.5. Decontamination of spices

Basilico and Basilico (1999) studied several spice essential oils of oregano, mint, basil, sage and coriander. Sage and coriander showed no important effect at any of the concentrations studied. However, at 1000 ppm of oregano and mint completely inhibited the fungal growth and OTA production up to 21 d, while basil was only effective up to 7d. At 750 ppm, oregano was completely effective up to 14 d, whereas mint allowed fungal growth but not OTA production up to 14 d. The use of different spices as method of decontamination is important due to that several meat products include this product in their processing.

Acknowledgments

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Estudio bibliográfico

ESTUDIO EXPERIMENTAL

Estudio experimental

IV.1. ANÁLISIS DE AFLATOXINAS

Estudio experimental

IV.1.1. ANALYSIS OF AFLATOXINS IN PEELED PEANUTS BY LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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IV.1.1.1. Introduction

Aflatoxins are the important mycotoxins, the very important aflatoxins are named B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), produced by moulds *Aspergillus flavus* and *Aspergillus parasiticus* and infect food and feed crops before and after harvest (Ellis et al. 1991). The International Agency for Research on Cancer classified the AFB1 as a carcinogen of group I (IARC, 2002). Aflatoxins have been found in several foods (Ellis et al. 1991) being peanut and their derivative products (Ali et al. 1999; Blesa et al. 2003; Candlish et al. 2001; Selim et al. 1996; Sobolev and Dorner, 2002; Whitaker et al. 1999) the main commodities to have high aflatoxin level. To protect the health of the consumers, the European Union established legal directives to control their levels in peanuts through the maximum tolerated levels that are 2 and 4 ng/g for AFB1 and total aflatoxins, respectively (Commission Regulation, 2001).

The extraction methods for aflatoxins are based on the solubility of these toxins in organic solvents The most frequently employed solvents are chloroform (Stroka et al. 2000), methanol (Akiyama et al. 2001), and acetonitrile (Drummer et al. 1999). Furthermore, several authors have used liquid-liquid partitioning (Vinitkektumnuen et al. 1997), immunoaffinity columns (Stroka et al. 2000) or solid-phase extraction (SPE) (Akiyama et al. 2001) cartridges or matrix solid phase dispersion (MSPD) (Blesa et al. 2003; 2004) as purification procedure. On the other hand, the chromatographic method most employed is the liquid chromatography coupled with

fluorescence or mass spectrometry detection (Jaimez et al. 2000; Papp et al. 2002).

The purpose of this work is to develop a method based on the extraction and clean-up steps for the determination of AFB1, AFB2, AFG1 and AFG2 in peeled peanuts and its application to real samples from Valencian port (Spain).

IV.1.1.2. Experimental

Chemical and reagents

Methanol, acetonitrile, hexane and chloroform were obtained from Merck (Darmstadt, Germany) and sodium chloride and anhydrous sodium sulphate from Panreac (Madrid, Spain) and trifluoroacetic acid (TFA) from Sigma (St. Louis, MO, USA). Deionised water (<8Ω cm resistivity) was obtained from a Milli-Q water purification system (Waters-Millipore, Milford, MA, USA). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

The aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) crystalline materials were purchased from Sigma. Stock standard solutions of aflatoxins with concentrations of 500 μg/ml were prepared in methanol, kept in security conditions at –20°C, wrapped in aluminium foil due to that the aflatoxins gradually breaks down under UV light and held for at most 3 months. Working solutions were diluted in acetonitrile and stored at –20°C.

As safety notes, soak all used laboratory ware and pipette tips in 10% solution of household bleach before discarding. Accidental spills of aflatoxins must be swabbed with 5% NaOCl bleach.

Samples and sampling

Random food samples were obtained from the port of Valencia (Spain) typically as a simple bag. All samples were stored in the dark and dry place at room temperature (18-23°C). The samples were divided with a subsample divider and a 200 g subsample was analysed.

Extraction procedure

A modified method of AOAC (2000) was used to extract aflatoxins in peanuts. Briefly, a portion of sample (50 g) was mixed with 100 ml of methanol-water (60:40, v/v) containing 4% of NaCl and blended thoroughly using a food processor. The homogenous mixture was filtered through a Whatman No. 5 filter paper, the filtrate transferred to a separation flask and washed with 20 ml hexane. Then, mycotoxins were extracted from the aqueous phase with 30 ml of chloroform and shaken for 1 min. The chloroform fractions were dried over anhydrous sodium sulphate and combined in a round-bottom flask and evaporated (50°C) till dry in a flash evaporator. The sample residue was dissolved in 5 ml of acetonitrile and evaporated to dryness on a Multiblock (60°C) under a stream of nitrogen before derivatization.

The derivatization procedure was prepared by adding 100 µl TFA to the evaporated solution of extract. The solution was homogenised, evaporated to dryness at 45°C and reconstituted in 1 ml of acetonitrile-water (1:1, v/v).

LC analysis

A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne Model 7125 injector (20µl loop) and a SRF-535 fluorescence detector. A LC column Kromasil SC-18 (5µm) (150 x 4.6mm. i.d.) (Scharlau, Barcelona, Spain) was used with mobile phase consisting of a mixture of water-acetonitrile (75:25, v/v) at a flow rate of 0.7 ml/min. Detection of aflatoxins was carried out using 365 and 435 nm as wavelengths for excitation and emission, respectively (Blesa et al. 2003, 2004).

For confirmation of aflatoxins, a Hewlett-Packard (palo Alto, CA,USA) HP-110 Series LC-MS system equipped with a binary solvent pump, an autosampler and a MS coupled with an analytical work station was used. The MS detector consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray. The LC-ESI-MS interface in positive ion mode operated with these conditions, 350°C gas temperature, 13.0 l/min drying gas flow, 40 p.s.i. nebulizer gas pressure and 4000 V capillary voltage. The fragmentor selected was 120 V. Using this interface, the ions obtained for AFB1, AFB2, AFG1 and AFG2 were the protonated molecule $[M+H]^+$ and the sodium adduct $[M+Na]^+$ at *m/z* 313, 315, 329 and 331, and 335, 337, 351 and 353, respectively. These pairs of *m/z* ions were, respectively, selected for

AFB1, AFB2, AFG1 and AFG2 identification. The mobile phase was a mixture water-methanol (55:45, v/v) at flow-rate of 0.7 ml. Finally, 20 µl were injected in each equipment (Blesa et al. 2003, 2004).

IV.1.1.3. Results and discussion

Method performance

The response linearity was obtained in triplicate with seven concentrations (0.4, 0.8, 1, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all >0.996. The values of intra-day repeatability (n=5) and inter-day reproducibility (5 different days) on 2.5 ng/g (5 ng/g for AFG2) and 20 ng/g for each mycotoxin calculated as relative standard deviation (RSD) ranged from 7 and 10% at the lower level and from 5 to 9% at the higher level. The accuracy was expressed as the percentage recovery obtained by the addition of 10 ng/g of the standard levels in triplicate to peanut samples, example of chromatogram with 10 ng/g, for each AF, standard solution is shown in Figure IV.1a. The average recovery was 81±7, 85±8, 92±9 and 93±7 % for AFB1, AFB2, AFG1 and AFG2, respectively. The limits of quantification (LOQ), (S/N 10:1), were 0.4, 0.7, 2 and 4 ng/g for AFB1, AFB2, AFG1 and AFG2, respectively.

Application to real samples

This study was carried out in cooperation with the Port Health

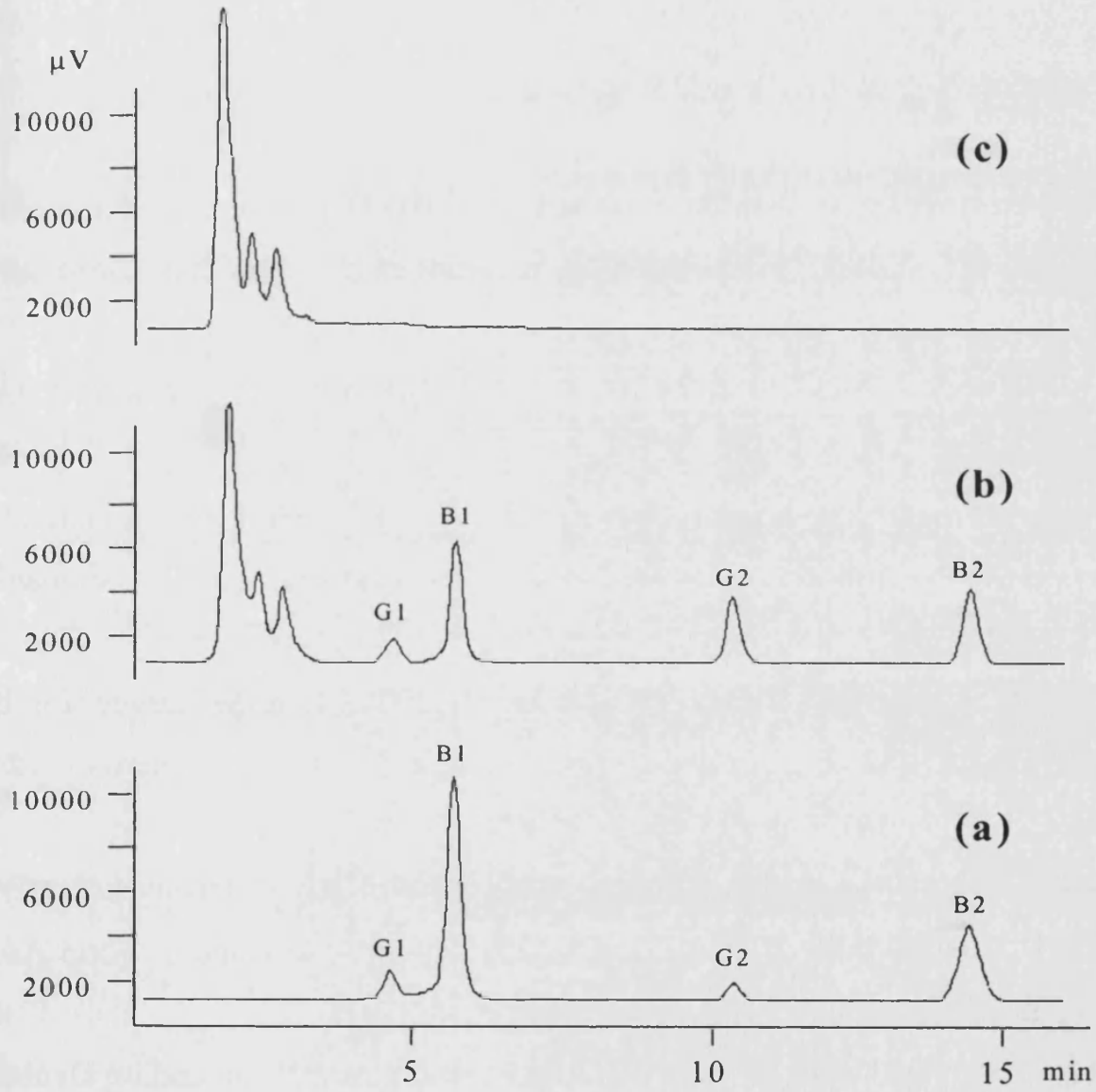
Authorities from Valencia (Spain) which is authorized by the European Union to receive peanuts from other countries (Commission Decision, 2003). In total 120 peanut samples were examined and concentrations of aflatoxins in positive samples are shown in Table IV.1. Three out of 120 samples were positive for these mycotoxins, for AFB1 in ranges of 1.2-5.4 ng/g, the sample with mayor contamination of AFB1 presented values for AFB2, AFG1 and AFG2 of 5.8, 9.9 and 38.3 ng/g, respectively, the chromatogram of this sample are depicted in Figure IV.1b, the Figure IV.1c shows a blank of peanut. All positive samples were confirmed by LC-ESI-MS. The obtained results show that 1 out of 3 positive samples contained aflatoxins at levels higher that the European legislated MRLs which are 2 and 4 ng/g from AFB1 and total aflatoxins levels in peanut products (Commission Regulation, 2001).

Table IV.1: Aflatoxin concentrations for imported peanuts from positive samples

Aflatoxin (ng/g)	**Sample**		
	1	2	3
AFB1	1.6	5.4	1.2
AFB2	-	5.8	-
AFG1	-	9.9	-
AFG2	-	38.3	-
Total aflatoxins	1.6	59.4	1.2

The sample which fail the EU legislation was destroyed under the supervision of the competent authority and the other contaminated samples entered in the food chain due to that physical treatments are possible to reduce the contamination (Rustom, 1997). This method is used due to the establishment of monitoring and surveillance programs for mycotoxins that requires suitably equipped laboratories, well-trained staff for both analytical and inspection activities, reliable analysis and sampling methods and application of analytical quality assurance programmes (Park et al. 1999, Sashidhar et al. 1992).

Figure IV.1: LC-fluorescence chromatograms obtained of (a) aflatoxins standard solution (10 ng/g for each aflatoxin), (b) peeled peanuts containing 5.4, 5.8, 9.9 and 38.3 ng/g of AFB1, AFB2, AFG1 and AFG2, respectively, and (c) blank of peeled peanuts.



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IV.1.2. DETERMINATION OF AFLATOXINS IN PEANUTS BY MATRIX SOLID PHASE DISPERSION AND LIQUID CHROMATOGRAPHY

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Abstract

A new method based on matrix solid phase dispersion (MSPD) extraction was studied to determine aflatoxin B1, B2, G1 and G2 from peanuts. Optimization of different parameters, such as type of solid supports for matrix dispersion and elution solvents were carried out. The method used 2 g of peanut sample, 2 g of C18 bonded silica as MSPD sorbent and acetonitrile as eluting solvent. Recoveries of each aflatoxin spiked to peanut samples at 2.5 ng/g (5 ng/g for aflatoxin G2) level were between 78 and 86% with relative standard deviations ranging from 4 to 7%. The limits of quantification ranged from 0.125 to 2.5 ng/g for the four studied aflatoxins using liquid chromatography (LC) with fluorescence detection. In addition, LC coupled to mass spectrometry with an electrospray interface (LC-ES-MS) was used for confirmation of aflatoxins present in real samples. Eleven peanut samples from different countries were analyzed by the proposed method and by using an enzyme-linked immunosorbent assay (ELISA). ELISA test is a good screening method for investigation of these mycotoxins in peanut samples.

IV.1.2.1. Introduction

Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are toxic metabolites produced by the food spoilage fungi *Aspergillus* particularly *A. flavus* and *A. parasiticus*. The AFB1 is listed as a carcinogen of group I by the International Agency for Research on Cancer [1]. Aflatoxins have been found as contaminants in agricultural and food products [2] being peanut [3-7] and their derivative products such as peanut butter [8] and oil [9], the main commodities to have high aflatoxin contamination. In the European Union, the aflatoxin B1 and the total aflatoxin level in peanut products are regulated with maximum residue levels (MRLs) that cannot be greater than 2 and 4 ng/g, respectively [10].

The most common solvents used for aflatoxins extraction are mixtures of chloroform-water [11], methanol-water [8] or acetonitrile-water [12]. For clean-up, the use of immunoaffinity columns [8] or solid phase extraction cartridges [13] which replaced the liquid-liquid partition procedures have been reported. Several chromatographic methods [14-18] have been used to analyze aflatoxins in foods being liquid chromatography (LC) with fluorescence detection (FD) [19] or mass spectrometry detection (MS) [20] the most employed. Niedwetzki and Geschwill (1994) developed an automatic work station for determination of aflatoxins [21].

Matrix solid phase dispersion (MSPD) is applied to the analysis of several residues [22-23]. However to date, MSPD has not been used for

analysis of aflatoxins from foods. The objective of this study was to apply the MSPD to the extraction of aflatoxins from peanuts and quantify the compounds by LC determination. The proposed method is applied to real samples and it is compared with the enzyme linked immunosorbent assay (ELISA) as a tool for routine analysis of aflatoxins in peanuts.

IV.1.2.2. Experimental

Chemical and reagents

Acetonitrile, acetone, ethanol, ethyl ether, hexane, methanol and methylene chloride were supplied by Merck (Darmstadt, Germany). HPLC-grade water was obtained by filtering deionised water through a 0.45 μm filter with a Waters-Millipore (Milford, MA, USA) system. Solvents and water were degassed for 20 minutes using a Branson 5200 (Branson Ultrasonic Corporation, Connecticut, USA) ultrasonic bath and solid phases used for MSPD were silica (40-60 μm), phenylsilica (50 μm), octylsilica (C8) (50 μm) and octadecylsilica (C18) (50 μm) bonded silica from Análisis Vínicos (Tomelloso, Spain).

The aflatoxins B1, B2, G1 and G2 crystalline materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock standard solutions of aflatoxins with concentrations of 500 μg/ml were prepared in methanol, kept in security conditions at -20°C, wrapped in aluminium foil due to that the aflatoxins gradually breaks down under UV light and held for at least 3 months. Working solutions were diluted in acetonitrile and stored at -20°C.

As safety notes, soak all used laboratory ware, pipette tips and kit components in 10% solution of household bleach before discarding. Accidental spills of aflatoxins must be swabbed with 5% NaOCl bleach.

Extraction procedure

Samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2 g) of the sample was placed into a mortar (50 ml capacity) and 2 g of the C18 sorbent and 0.5 g of sand were added and gently blended for 5 minutes using a pestle, to obtain a homogeneous mixture. The mixture was introduced with 1 g of silica into a 100 x 9 mm i.d. glass chromatographic column with a coarse frit (No. 2) and covered with a plug of silanized glass wool in the top of the column. Then, 4 ml hexane followed by 1 ml diethyl ether and 4 ml methylene chloride was passed through and discarded. After that, aflatoxins were eluted with 20 ml acetonitrile. The eluate was evaporated to dryness with gentle stream of N_2 at 45°C. A volume of 2 ml of methanol was added, thoroughly mixed for 5 min and centrifuged at 5000 rpm for 10 minutes. The extract was filtered with a nylon acrodisk (0.45 μm), evaporated to dryness with N_2 at 45°C, redissolved with 100 μl of trifluoracetic acid (TFA) for 3 min, re-evaporated to dryness with N_2 at 45°C, and reconstituted in 1 ml of acetonitrile/methanol/water (1:1:1, v/v/v) for LC-FD and in 0.1 ml of methanol/water (1:1, v/v) for LC-ES-MS.

Recovery studies were carried out by spiking fresh samples (2 g) of raw peanut from local markets with 2.5 ng/g for aflatoxin B1, B2 and G1 and 5

ng/g for AFG2. For this study, samples were previously first analysed by ELISA, LC-FD and LC-ES-MS before being spiked and none of them were found of the studied aflatoxins.

The extraction procedure described above is based on the data obtained from different optimisation assays. They involved the study of different solid supports for matrix dispersion (silica, phenylsilica, C8 and C18) and also different solvents (ethanol, acetone, methanol, acetonitrile/water and acetonitrile) used for elution.

Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne Model 7125 injector (20 µl loop) and a SRF-535 fluorescence detector. A LC column Kromasil SC-18 (5 µm) (150 x 4.6 mm i.d.) (Scharlau, Barcelona, Spain) was used with a mobile phase consisting of a mixture of water-acetonitrile (25:75) at a flow rate of 0.7 ml/min. Detection of aflatoxins was carried out using 365 and 435 nm as wavelengths for excitation and emission, respectively.

For confirmation of aflatoxins, a Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series LC-MS system equipped with a binary solvent pump, an autosampler and a MS coupled with an analytical work station was used. The MS detector consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray (ES). Separations were carried out at room

temperature. The LC-ES-MS interface in positive ion mode operated under the following conditions, 350°C gas temperature, 13.0 l/min drying gas flow, 40 psi nebulizer gas pressure and 4000 V capillary voltage. The fragmentor selected was 120 V. Using this interface, the ions obtained for AFB1, AFB2, AFG1 and AFG2 were the protonated molecule $[M+H]^+$ and the sodium adduct $[M+Na]^+$ at m/z 313, 315, 329 and 331, and 335, 337, 351 and 353, respectively. These pairs of m/z ions were respectively selected for AFB1, AFB2, AFG1 and AFG2 identification. The mobile phase was a mixture water/methanol (55:45, v/v) at flow rate of 0.7 ml/min. Finally, 20 µl were injected in each equipment.

Enzyme linked immunosorbent assay (ELISA) analysis

Samples (10 g) were analysed by the Aflatoxin B-G ELISA kit (TECNA Diagnostics, Trieste, Italy) using the protocol of the manufacturer.

IV.1.2.3. Results and discusion

Method performance

For validation studies, peanuts were selected because they are the most susceptible commodity to high aflatoxin contamination [2] and they are greatly consumed in our country [24]. On the other hand, the elution solvents and the polarity of solid phases for the MSPD extraction must be adequately selected due to the possibility of interference from matrix components and the need for

determining aflatoxins at levels close to the limit of sensitivity of the instruments. For this reason, 20 ml of ethanol, methanol, acetone, acetonitrile and acetonitrile/water (9:1) were tested as elution solvents. Results are shown in Table IV.2. Although, the use of the different eluting solvents assayed produced similar recoveries, acetonitrile was considered the best for the extraction because it gave cleanest extracts and chromatograms.

Table IV.2: Average recoveries (%) and relative standard deviation (RSD%, in parenthesis) obtained with different elution solvents by using C18 MSPD extraction of raw peanuts spiked at 2.5 ng/g level (5 ng/g for AFG2)[a]

Solvent	**Aflatoxins**			
	B1	**B2**	**G1**	**G2**
Acetone	77 (5)	70 (7)	78 (5)	77 (4)
Acetonitrile	78 (4)	81 (6)	82 (6)	86 (7)
Acetonitrile/water (9:1)	78 (6)	70 (5)	81 (4)	81 (8)
Ethanol	69 (5)	65 (4)	71 (3)	63 (6)
Methanol	71 (3)	67 (7)	75 (4)	69 (5)

[a] n=5

Furthermore, silica, phenylsilica, C8 and C18 were checked as solid supports for MSPD. Recoveries obtained by using these solid phases are exposed in Table IV.3, it can be seen that the best recoveries for all aflatoxins obtained using C18 (with 20 ml of acetonitrile as elution solvent). The differences between the mean recoveries obtained with C18, phenylsilica and silica were of statistical significance, but not those between C18 and C8. The

C18 phase proved to be better peanut-dispersant than the other solid supports assessed due to their hydrophobic characteristics which provided high affinity for these compounds, moreover it produced chromatograms more clean than those generated by other phases. The use of silica failed to extract the studied aflatoxins and produced more heterogeneous results.

Table IV.3: Average recoveries (%) and relative standard deviation (RSD%, in parenthesis) obtained with different solid supports for matrix solid phase dispersion, using acetonitrile as elution solvent, of raw peanuts spiked at 2.5 ng/g level (5 ng/g for AFG2)[a]

Solid support	**Aflatoxins**			
	B1	**B2**	**G1**	**G2**
C18	78 (4)	81 (6)	82 (6)	86 (7)
C8	69 (6)	73 (5)	76 (5)	76 (4)
Phenyl	63 (5)	61 (4)	68 (7)	60 (8)
Silica	28 (9)	37 (7)	36 (10)	29 (9)

[a] n=5

Validation of the method was carried out according to these preliminary observations. Precision was calculated in terms of intra-day repeatability (n=5) and inter-day reproducibility (5 different days) on 2.5 ng/g (5 ng/g for AFG2) and 20 ng/g concentration levels for each analyte. The intra-day repeatability evaluated as RSD% ranged from 4 to 7% at the lower level and from 3 to 6% at the higher level. The inter-day reproducibility was lower than 8% for all instances. Linearity was verified in triplicate with seven concentrations (0.1,

0.25, 0.5, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all > 0.997. In order to investigate if natural constituents of raw peanuts interfere with quantification, parallel calibration graphs obtained from matrix-extracted and solvent-based standards were performed. The covariance analysis for each aflatoxin showed that the calculated F values were lower than the F Snedecor tabulated ones indicating that both straight lines were parallel ($P<0.05$) and hence matrix effect is negligible. Figure IV.2 shows the LC-FD chromatograms, obtained following the MSPD extraction procedure, for a raw peanut sample (A) and non-fortified (B). Figure IV.3 shows mass chromatograms obtained in ES positive ion mode from raw peanuts spiked (A) and non spiked (B). Table IV.4 reflected the limits of detection (LODs) (S/N 3:1) and limits of quantification (LOQs) (S/N 10:1) for MSPD-LC coupled with FD and MS in raw peanuts.

Table IV.4: Limits of detection (LODs) and quantification (LOQs) for aflatoxins

	Aflatoxin	Instrumental		MSPD method	
		LODs (ng/ml)	LOQs (ng/ml)	LODs (ng/g)	LOQs (ng/g)
LC-FD	**AFB1**	0.08	0.25	0.04	0.13
	AFB2	0.08	0.25	0.04	0.13
	AFG1	0.3	0.95	0.15	0.5
	AFG2	1.45	5	0.75	2.5
LC-ES-MS	**AFB1**	1.35	4	0.07	0.2
	AFB2	3.8	12	0.2	0.6
	AFG1	1.35	4	0.07	0.2
	AFG2	3.8	12	0.2	0.6

Figure IV.2: LC-fluorescence chromatograms obtained after MSPD extraction; (A) raw peanut extract fortified with 1.5, 0.75, 2.5 and 1 ng/g of AFG1, AFB1, AFG2 and AFB2, respectively and, (B) non-fortified extract

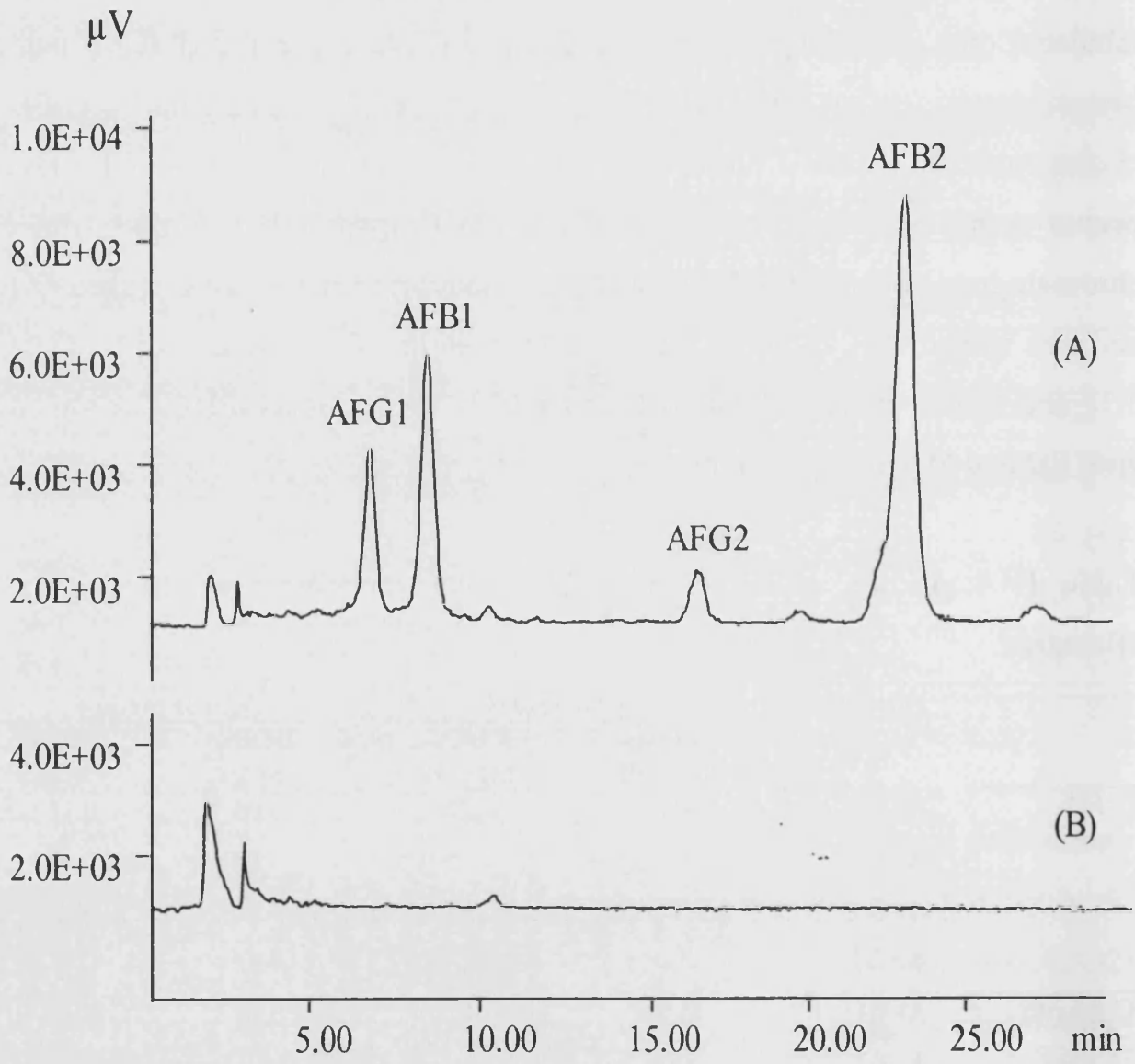
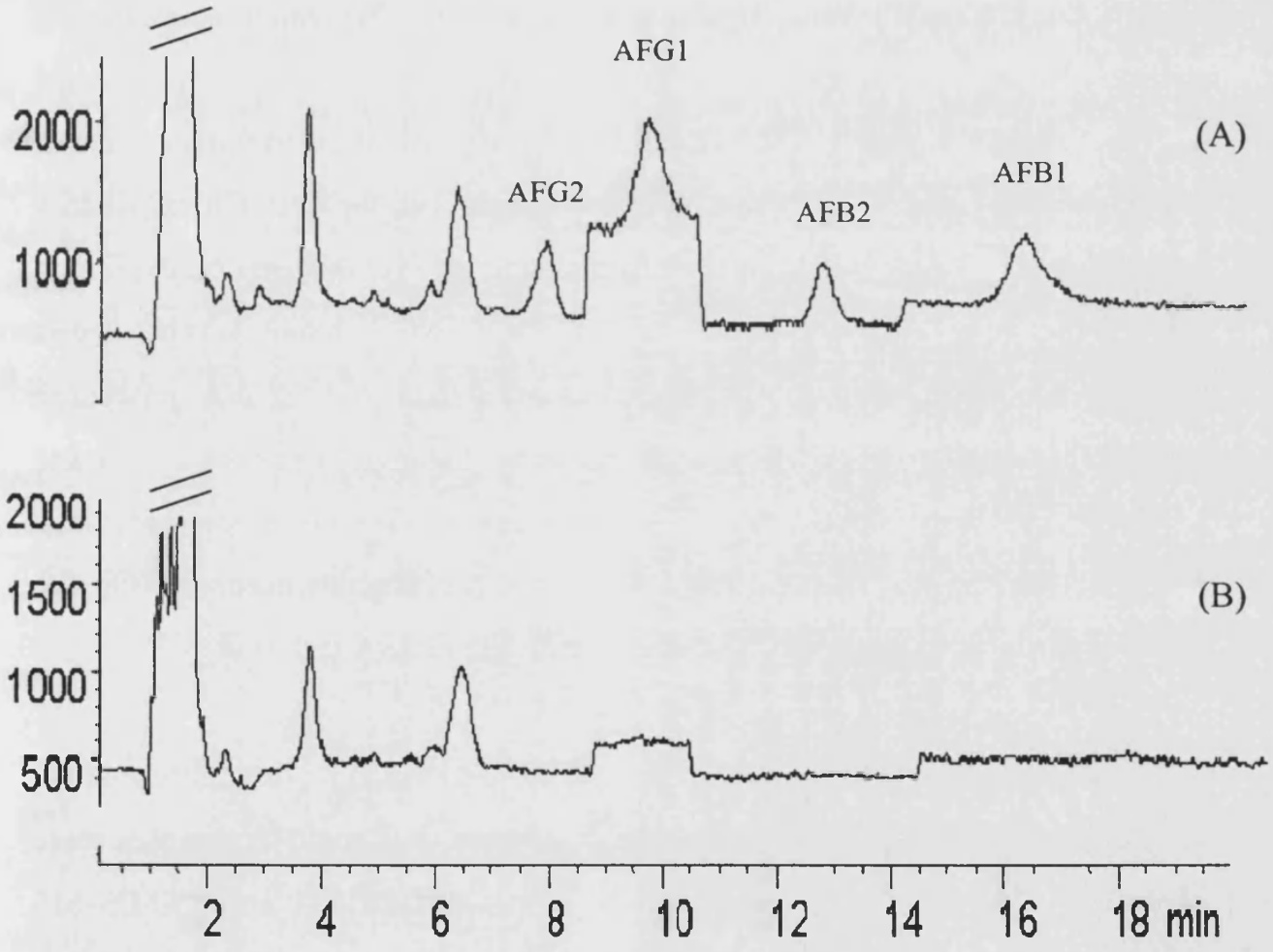


Figure IV.3: LC-ES-MS chromatograms obtained after MSPD extraction; (A) raw peanut extract fortified with 0.2 ng/g of AFG1 and AFB1 and 0.6 ng/g of AFG2 and AFB2 and (B) non-fortified extract



Application to real samples

The MSPD extraction followed by LC-FD determination, LC-ES-MS confirmation and ELISA test were applied to eleven types of peanuts (Table IV.5) from different countries (Brazil, China and USA) being three samples of unknown origin. All samples were obtained from Spanish supermarkets. In Table 4, the obtained results show that 4 samples (36.4%) contained aflatoxins at levels below the European legislated MRLs [10]. The analyses performed with these four samples by LC-FD and LC-ES-MS also showed the presence of aflatoxins. Table IV.5 shows that in a raw peanut sample from China, ELISA gave a positive result but LC-FD did not found AFB1, this can be explained because the cross-reactivity of the ELISA test with AFG1 which was present in such sample. According to the manufacturer's data, the ELISA procedure determines the aflatoxin B1 (100%) with a cross-reactivity of about 20, 33 and 2.3% for aflatoxin B2, G1 and G2, respectively. This cross-reactivity is a limitation for the use of the ELISA test for quantification purposes. On the other hand, no false negatives were found with the ELISA test.

Positive samples had occurrence of AFB1 and AFG1, but neither AFB2 nor AFG2 were detected in the analyzed samples. All positive samples were confirmed by LC-ES-MS. Figure IV.4 shows the LC-FD and LC-ES-MS chromatograms obtained by the MSPD procedure for a positive peanut sample.

Table IV.5: Incidence of aflatoxins in peanuts analyzed by ELISA and LC-FD

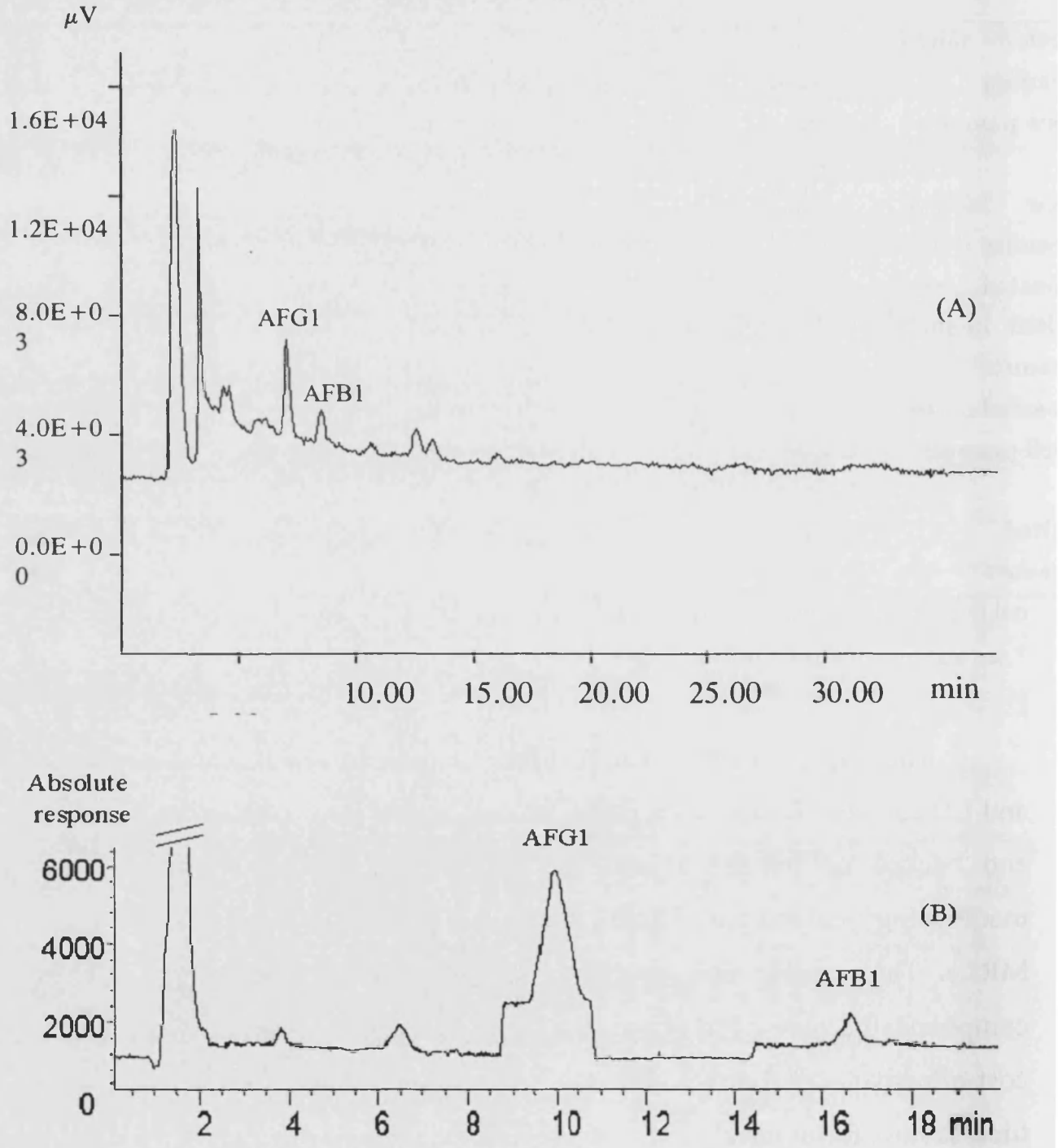
Sample	Country of origin	ELISA (ng/g)	LC-FD			
		AFB1 (ng/g)*	AFB1 (ng/g)	AFB2 (ng/g)	AFG1 (ng/g)	AFG2 (ng/g)
Peeled salted peanuts	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	0.15	0.15	n.d.	0.63	n.d.
Raw peanuts	China	0.13	n.d.	n.d.	0.61	n.d.
	USA	0.17	0.13	n.d.	n.d.	n.d.
Raw in-shell peanuts	Brazil	n.d.	n.d.	n.d.	n.d.	n.d.
Roasted and salted in-shell peanuts	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	n.d.	n.d.	n.d.	n.d.	n.d.
Roasted in-shell peanuts	Brazil	n.d.	n.d.	n.d.	n.d.	n.d.
	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	0.28	0.25	n.d.	1.68	n.d.
Salted peanuts	USA	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (below the quantitation limit).

* see text for cross-reactivity with AFB2, AFG1 and AFG2.

These results demonstrate that for aflatoxin analysis of peanuts, ELISA and LC can complement each other. ELISA can be employed as the initial test and backed up by MSPD and LC due to that they are an appropriate methodology for routine aflatoxin analysis in peanuts at concentrations below MRLs. This combination can be beneficial in the quantification of these compounds because of the large number of samples that can be analyzed in a cost-effective way. Furthermore, the application of MSPD is easy to handle, time-saving, fewer interferences and requiring less solvent.

Figure IV.4: Chromatograms of a positive peanut sample containing AFB1 (0.25 ng/g) and AFG1 (1.68 ng/g); (A) LC-FD and (B) LC-ES-MS



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IV.1.3. LIMITED SURVEY FOR THE PRESENCE OF AFLATOXINS IN FOODS FROM LOCAL MARKETS AND SUPERMARKETS IN VALENCIA (SPAIN)

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Abstract

Aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) were extracted by matrix solid phase dispersion with C18 silica and acetonitrile as eluting solvent, analysed by liquid chromatography with fluorescence detection (LC-FD) and confirmed by liquid chromatography with mass spectrometry using an electrospray interface (LC-ESI-MS) in 58 samples grouped as cereals, dried fruits, herbs and spices, pulses, snacks, nuts and nut products collected from local markets and supermarkets in Valencia (Spain). All samples analysed by the proposed method were previously studied with an enzyme-linked immunosorbent assay as a screening protocol for the fast detection of mycotoxins. The samples that containing residues (3/58) were hazelnut (0.42 and 0.52 μg/kg for AFB1 and AFG1, respectively), nut cocktail (0.29 and 0.47 μg/kg for AFB1 and AFG1, respectively) and pinhol (0.30 μg/kg for AFG1), such values were below the legislated maximum residue levels for the European Union.

IV.1.3.1. Introduction

Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are toxic metabolites produced by certain fungi, such as *Aspergillus* species and particularly *A. flavus* and *A. parasiticus*. These mycotoxins have a significant threat to both human and animal health due to that are potent carcinogens, teratogens and mutagens, besides the economic loss due to food contamination. AFB1 is listed as a group I carcinogen by the International Agency for Research on Cancer, especially implicated in the cause of human primary hepatocellular carcinoma (IARC, 2002). Aflatoxins have been found as contaminants of numerous samples including peanuts, cereals, herbs and spices (Malone et al. 2000, Otta et al. 2000). In the European Union, the aflatoxin levels are regulated with maximum residue levels (MRLs) that cannot be greater than 5 μg/kg for AFB1 and 10 μg/kg for total aflatoxin present in several spices as are paprika and nutmeg (Commission Regulation (EC) 472/2002) and 2 μg/kg for AFB1 and 4 μg/kg for total aflatoxin present in groundnuts, nuts, dried fruit, cereals and processed products thereof, intended for direct human consumption or as an ingredient in foodstuffs (Commission Regulation (EC) 257/2002). Furthermore, the presence of these mycotoxins have been reflected in the different countries such as Botswana (Siame et al., 1998), Brazil (Caldas et al., 2002), Cuba (Escobar and Regueiro, 2002), Egypt (Selim et al., 1996), Greece (Apergi et al., 1998), Japan (Taguchi et al., 1995), Portugal (Martins et al., 2001) and The Philippines (Ali et al., 1999). In Spain, the presence of aflatoxins has been found in corn-based (Sanchis et al., 1995), almonds and peanuts (Jiménez et al., 1991) and peanut samples (Blesa et al., 2003). This last study was carried out in samples from Valencia (Spain).

The procedures for extraction of aflatoxins from foods generally use chloroform or a mixture of methanol or acetonitrile with water. Clean up procedures include the use of immunoaffinity columns (IAC) or solid phase extraction (SPE) cartridges (Miraglia and Brera, 2000). The most extended chromatographic method is liquid chromatography (LC) coupled with fluorescence detection (LC-FD) or single (LC-MS) or tandem (LC-MS-MS) mass spectrometry detection (Jaimez et al. 2000, Papp et al. 2002). Furthermore, the thin layer chromatography (TLC) (Stroka et al. 2000) and enzyme linked immunosorbent assay (ELISA) (Nayak et al. 2001, Gunsen and Buyukyoruk 2002) procedures are commonly used to determination of aflatoxins in foods. In a previous study, the matrix solid phase dispersion (MSPD) that involves blending a small amount of sample with a solid support, followed by washing and eluting with a small amount of solvent to extract a wide range of compounds was applied for aflatoxin analysis in peanuts producing extracts that were clear enough to avoid further purification process (Blesa et al. 2003). In this way, the MSPD could be applied to other food samples.

The objective of this study was to provide information on the presence of aflatoxins in different food commodities collected in the local markets and supermarkets from Valencia (Spain).

IV.1.3.2. Experimental

Samples

Random food samples (1 kg) were obtained in the local markets and supermarkets from January to December of 2002, typically as a single bag and grouped into 6 categories: cereal, dried fruits, herbs and spices, nut and nut products, pulses and snacks. All samples were stored in dark and dry place at room temperature (18-23°C). The samples were divided with a subsample divider and a 200 g subsample was analysed (Commission Directive 2002/27/CE).

Chemical and reagents

All employed solvents were HPLC grade (Merck, Darmstadt, Germany) and HPLC-grade water was obtained by filtration of distilled water through a Milli-Q system (Millipore, Bedford, MA, USA). Solvents and water were degassed for 20 minutes using a Branson 5200 (Branson Ultrasonic Corporation, Connecticut, USA) ultrasonic bath and solid phases used for MSPD were C18 bonded silica (50 μm) from Análisis Vínicos (Tomelloso, Spain).

The aflatoxins B1, B2, G1 and G2 crystalline materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) being each stock standard solution prepared in methanol with concentrations of 500 μg/ml, kept in

security conditions at -20°C, wrapped in aluminium foil and less than 3 months. Working solutions were diluted in acetonitrile and stored at 4°C in darkness for a maximum of four days (Blesa et al. 2003).

Sample extraction

The method of Blesa et al. (2003) was used for extraction of aflatoxins. Briefly, samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2 g) of the sample was placed into a mortar (50 ml capacity) and 2 g of the C18 sorbent and 0.5 g of sand were added and gently blended for 5 minutes using a pestle, to obtain a homogeneous mixture. The mixture was introduced into a 100 x 9 mm i.d. glass chromatographic column with a coarse frit (No. 2) and covered with a plug of silanized glass wool in the top of the column. Then, 4 ml hexane followed by 1 ml diethyl ether and by 4 ml methylene chloride was passed through and discarded. After that, aflatoxins were eluted with 20 ml acetonitrile. The eluate was evaporated to dryness with N_2 at 45°C, and then 2 ml of methanol were added, thoroughly mixed for 5 min and centrifuged at 5000 rpm for 10 minutes. The extract was filtered with a nylon acrodisk (0.45 μm), evaporated to dryness with N_2 at 45°C, redissolved with 100 μl of trifluoracetic acid (TFA) for 3 min, re-evaporated to dryness with N_2 at 45°C, redissolved in 1 ml of acetonitrile/methanol/water (1:1:1, v/v/v) and 20 μl were injected for the analysis by LC-FD. Another extract was prepared as described about, but it was redissolved in 100 μl of methanol/water (1:1, v/v) instead of TFA, 20 μl of such solution was analyzed by LC-MS.

Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-6A system equipped with two LC-6A pumps, a Shimadzu RF-535 fluorescence detector, a Rheodyne model 7125 injector (20µl loop) and a Borwin data processor. A LC column Kromasil SC-18 (5 µm) (150 x 4.6 mm i.d.) (Scharlau, Barcelona, Spain) was used with a mobile phase consisting of a volumetric mixture of water-acetonitrile (25:75 v/v) at a flow rate of 0.7 ml/min. The aflatoxin concentrations in the final extract were calculated by comparing the peak areas with those obtained for standard solutions at λ_{ex} of 365 nm and λ_{em} of 435 nm. The limits of detection (LODs) (s/n 3:1) for AFB1, AFB2, and AFG1 and AFG2 were 0.04, 0.04, 0.15 and 0.75 ng/g, respectively. The limits of limit of quantification (LOQs) (s/n 10:1) for AFB1, AFB2, and AFG1 and AFG2 were 0.13, 0.13, 0.5 and 2.5 ng/g, respectively.

Confirmation of aflatoxins

For confirmation of aflatoxins, a Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series LC-MS system equipped with a binary solvent pump, an autosampler and MS coupled with an analytical workstation was used. The MS consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray (ES). Separations were carried out at room temperature with the Kromasil SC18 column as described before. The mobile phase was a mixture water/methanol (55:45, v/v) at flow rate of 0.7 ml/min. The LC-ES-MS interface in positive mode was operated under the following

conditions: 350ºC gas temperature, 13.0 l/min drying gas (N_2) flow, 40 psi nebulizer gas pressure and 4000 V capillary voltage. The fragmentor was set at 120 V. Using this interface, the ions obtained for AFB1, AFB2, AFG1 and AFG2 were the protonated molecule [M+H]+and the sodium adduct [M+Na]+ at m/z 313, 315, 329 and 331 and 335, 337, 351 and 353 respectively. These pairs of m/z ions were used for identification and determination of each aflatoxin. The LODs (s/n 3:1) for AFB1, AFB2, and AFG1 and AFG2 were 0.07, 0.2, 0.07 and 0.2 ng/g, respectively.

Enzyme linked immunosorbent assay (ELISA) analysis

An aliquot (10 g) of the sample was placed into a food processor to obtain a homogeneous mixture, then it was mixed with 50 ml of water/methanol (55:45, v/v) and 0.4 g of NaCl for 1 minute. Afterwards, 20 ml hexane was added and mixed for 3 minutes and discarded. Finally, the whole mixture was filtrated and the aqueous filtrate was collected. 1ml of the obtained filtrate was diluted 10 times with dilution buffer for ELISA analysis. Ninety-six-well polystyrene plates coated with antibodies anti-Aflatoxin B-G were from Tecna Diagnostics (Trieste, Italy). ELISA plates were washed with a microplate washer and absorbance was read at 450 nm with a M-2000 microplate reader from Comecta (Barcelona, Spain). ELISA test was performed by following the guidelines of the manufacturer.

IV.1.3.3. Results and discussion

ELISA test and MSPD extraction followed by LC-FD were applied to 58 samples grouped as cereals, dried fruits, herbs and spices, nut and nut products, pulses and snacks of different countries (Argentina, Australia, Brazil, China, France, India, Iran, Russia, Spain, Turkey and USA) being fifteen samples of unknown origin. To investigate if natural constituents of samples interfere with quantification, parallel calibration graphs obtained from matrix-extracted and solvent based standards were performed. The covariance analysis for each aflatoxin showed that the calculated F values were lower than the F Snedecor tabulated ones indicating that both straight lines were not parallel ($p<0.05$) and matrix effect is not negligible. For this reason, all analyses were conducted with spiked samples that were previously analyzed and did not contain aflatoxins. Recoveries obtained with MSPD method of raw peanuts spiked at 2.5-5 ng/g level for the AFB1, AFB2, AFG1 and AFG2 were 78, 81, 82 and 86%, respectively, such recoveries were considered as valid for analysing residues of aflatoxins in foods (Commission Directive 2002/27/CE). The Figure IV.5a shows the LC-FD chromatogram obtained following the MSPD extraction procedure for a raw peanut fortified with 0.5, 0.65, 0.75 and 2.5 μg/kg of AFB1, AFB2, AFG1 and AFG2, respectively.

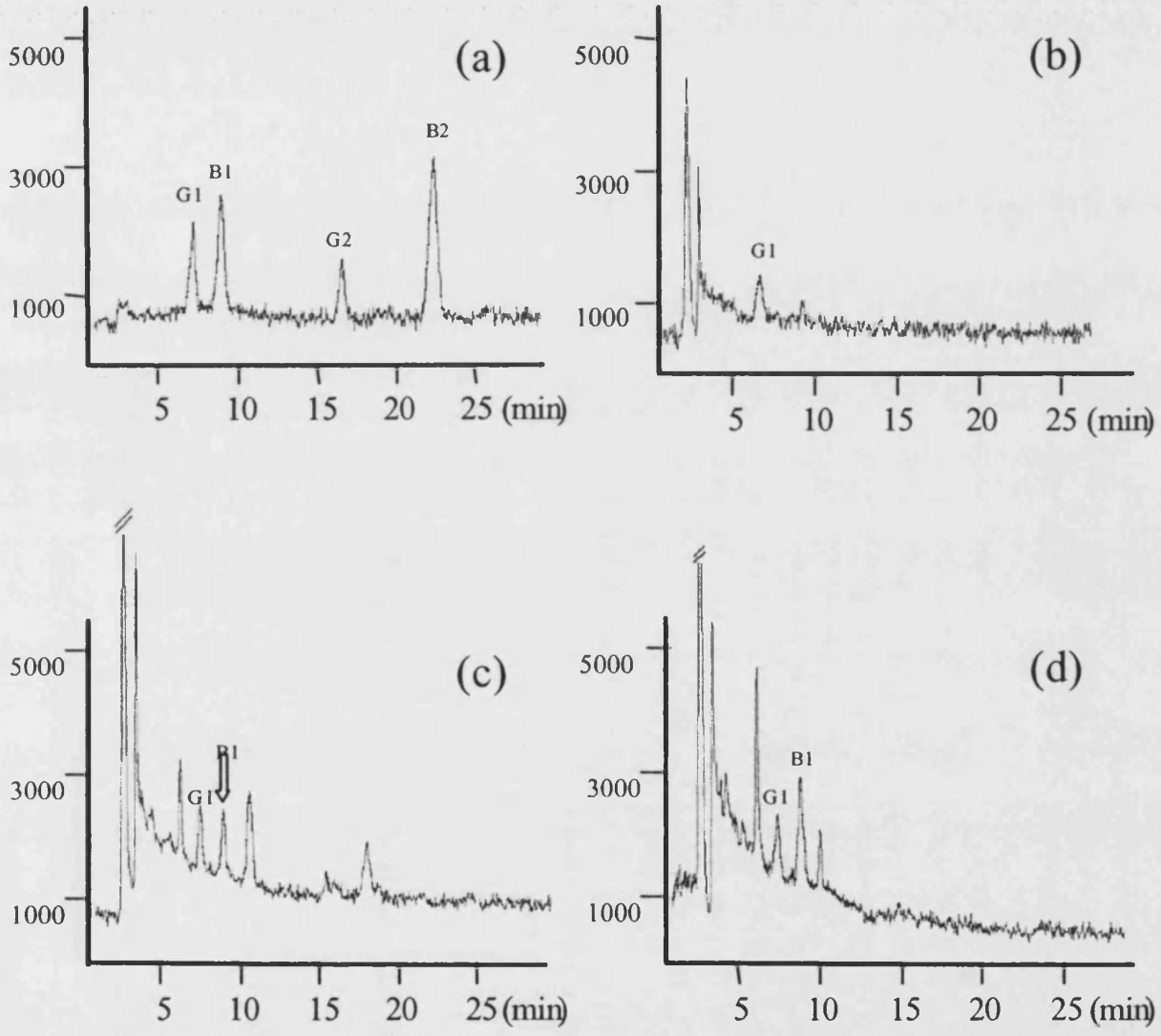


Figure IV.5: LC-FD chromatograms obtained after MSPD extraction; (a) raw peanuts extract fortified with 0.75, 0.5, 2.5 and 0.65 µg/kg of AFG1, AFB1, AFG2 and AFB2, respectively, and positive samples from (b) pinhol (containing 0.50 µg/kg of AFG1), (c) nut cocktail (containing 0.29 and 0.47 µg/kg of AFB1 and AFG1, respectively) and (d) hazelnut (containing 0.42 and 0.50 µg/kg of AFB1 and AFG1, respectively).

The results of real samples are shown in Table IV.6. Aflatoxins were present in several samples analysed (3 of 58) such are hazelnut, nut cocktail and pinhol. Figures IV.5b, IV.5c and IV.5d show chromatograms of aflatoxins found in pinhol, nut cocktail and hazelnut samples, respectively. Figure IV.6 shows the LC-ESI-MS chromatogram by the MSPD procedure for a positive hazelnut sample.

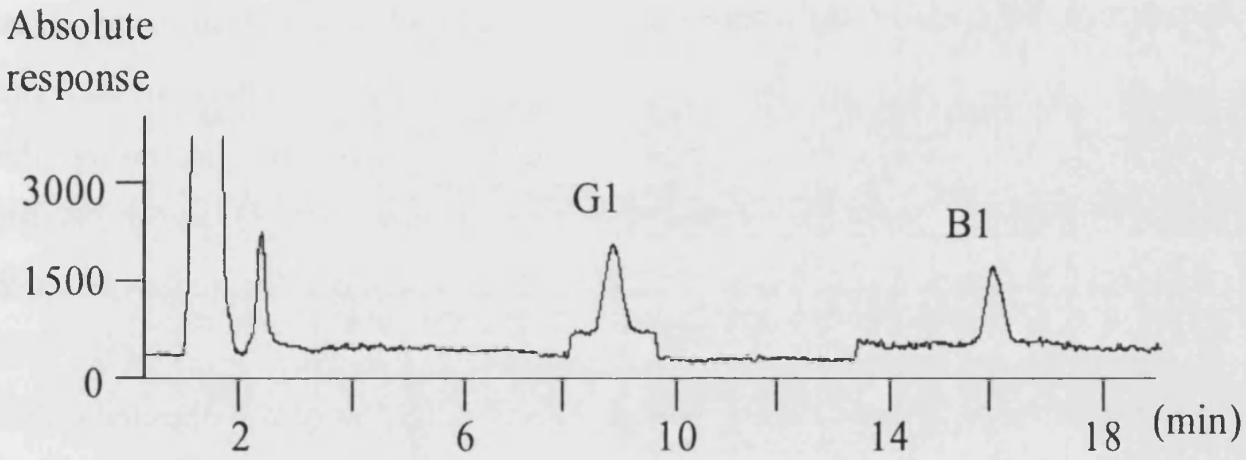
Table IV.6: Incidence of aflatoxin B1 and total aflatoxin in nut and nut products samples analysed by ELISA and LC-FD, respectively

Samples	Country of origin (Incidence)	ELISA* AFB1 (μg/kg)	LC-FD (μg/kg)			
			AFB1	AFB2	AFG1	AFG2
Cashew nut	India (0/1)	nd	nd	nd	nd	nd
Chestnut	Spain (0/1)	nd	nd	nd	nd	nd
Hazelnut	Unknown (1/2)	0.61	0.42	nd	0.52	nd
	Spain (0/3)	nd	nd	nd	nd	nd
Macadamia nut	China (0/1)	nd	nd	nd	nd	nd
Nut cocktail	Unknown (1/1)	0.37	0.29	nd	0.47	nd
Peanut	USA (0/4)	nd	nd	nd	nd	nd
Pistachio	Iran (0/6)	nd	nd	nd	nd	nd
Raw almond	Brazil (0/1)	nd	nd	nd	nd	nd
	Unknown (0/3)	nd	nd	nd	nd	nd
	Spain (0/3)	nd	nd	nd	nd	nd
Pinhol	China (1/1)	0.09	nd	nd	0.50	nd
	Spain (0/1)	nd	nd	nd	nd	nd
Raw walnut	Unknown (0/3)	nd	nd	nd	nd	nd
	China (0/1)	nd	nd	nd	nd	nd
Toasted almond	Unknown (0/2)	nd	nd	nd	nd	nd
	Spain (0/1)	nd	nd	nd	nd	nd
Walnut	Unknown (0/1)	nd	nd	nd	nd	nd

nd = not detected (below the limit of quantification)

- see text for cross-reactivity with AFB2, AFG1 and AFG2.

Figure IV.6: LC-ESI-MS chromatogram of a positive hazelnut sample containing AFB1 (0.42 μg/kg) and AFG1 (0.52 μg/kg)



Considering the origin of positive samples as are hazelnut, nut cocktail, and pinhol only one sample from China and other two samples from unknown origin were positive in the ELISA and LC-FD and LC-MS.

An apparent discrepancy with the results from Chinese pinhol can be seen in Table IV.6, following the ELISA test, 0.09 μg/kg of AFB1 was quantified, whereas LC-FD determined 0.50 μg/kg of AFG1. That discrepancy can be explained for the cross-reactivity of the ELISA procedure, in fact, the manufacturer's data indicated that the ELISA procedure determines the aflatoxin B1 (100%) with a cross-reactivity of about 20, 33 and 2.3% for AFB2, AFG1 and AFG2, respectively. Furthermore, no false negatives were found with the ELISA test in all studied samples. Positive samples had occurrence of AFB1 and AFG1, but AFB2 and AFG2 were not detected either by LC-FD or by LC-MS.

Some interferences were observed when using the LC-FD for parsley and toasted sunflower seed samples, in such occasions, the ELISA assays and the analysis performed via LC-MS did not detect the presence of aflatoxins.

The studied aflatoxins were not detected in any of the cereal product samples. Other authors such as Taguchi et al. (1995) and Siame et al. (1998) did not detect aflatoxins in corn products and rice from Japan and in corn from Botswana, respectively. In contrast, aflatoxins were found in Basmati rice in a range from 0.1 to 2.4 μg/kg (Patel et al. 1996) and in several corn products, such as corn grits that were contaminated with aflatoxin at a mean level of 44

μg/kg in The Philippines (Aly et al. 1999). In Cuba, the 17.04% of different samples presented AFB1 corresponding the biggest percentage to sorghum with 83.3% of incidence, in addition corn, oat, wheat and soy had percentages of contamination with AFB1 of 23.3, 10.7, 25 and 4.6% respectively (Escobar and Regueiro 2002).

The analysis of herbs and spices, pulses, snacks and dried fruit samples were negative in our study. Other authors found levels of AFB1 between 1 and 20 μg/kg in paprika samples in Portugal (Martins et al. 2001) whereas this mycotoxin was not detected in any garlic samples in UK (Patel et al. 1996) and in Japan (Taguchi et al. 1995). Martins et al (2001) suggested that spices contribute little to a direct health hazard posed by aflatoxin compounds due to the small amount of the consumed spices. According to other studies (Abou-Arab et al. 1999, Lwellyn et al. 1992), these samples are not ideal substrates for aflatoxin formation taking into account their essential oils, which may inhibit the aflatoxin production. The presence of aflatoxins in spices is indicative of a contamination after harvesting and drying process. Analysis of samples of Egyptian foods (Selim et al 1996) reflected that the highest mean concentration of aflatoxin B1 was found in herb and medicinal plants (49 μg/kg) followed by cereals (36 μg/kg), spices (25 μg/kg), nuts and seeds (24 μg/kg) and dried vegetables (20 μg/kg). The presence of aflatoxins in Egypt was associated with the prevailing climate conditions (humid and warm weather), the agricultural practices and storage conditions.

In the analysis of nut and nut products (Table IV.6), nut cocktail, pinhol and hazelnut contained aflatoxins. In other countries, 0.1 μg/kg of AFB1 was found

in shelled peanuts whereas this mycotoxin was not isolated in almond, cashew, macadamia, pistachio and walnut samples in Japan (Taguchi et al. 1995). Scholten and Spanjer (1996) studied the presence of aflatoxins in pistachio, their results suggested that the shells contained less than 1% of the AFB1 found in the edible kernel, concentrations of AFB1 in pistachio shells, even in seriously contaminated samples are negligible. In Brazil, aflatoxins were detected in 19.6% of the samples such are raw peanuts and its products, popcorn, maize and Brazilian nuts. The highest levels of aflatoxin contamination (34.7%) were found in peanuts and its products, with levels of 1706 mg/kg of total aflatoxins (Caldas et al. 2002). In Greece, nuts, seeds and dried fruits showed no evidence of considerable total aflatoxin contamination (Apergi et al. 1998).

Only one sample of raisins (55.3 μg/kg) and one walnut (12.9 μg/kg) were found to exceed the maximum permitted levels in this country (10 μg/kg).

In other studies, the highest incidence of aflatoxin B1 occurred in nuts and seeds (82%) followed by spices (40%), herbs and medicinal plants (29%), dried vegetables (25%) and cereal grains (21%) (Patel et al. 1996).

In the literature, AFB1 is generally found in greater amounts than the other aflatoxins, followed by AFB2, AFG1 and AFG2. However, Aly (2002) found a higher percentage of AFG1 (86.4%) and AFG2 (78.4%) and a lower percentage of AFB1 (16.3%) and AFB2 (14.7%) in starch fraction of corn and Apergi et al. (1998) found only AFG1 (12.9 μg/kg) in a walnut sample. In fact,

the presence of aflatoxins should be different among food samples due to different production, processing, storing and management practices common in agriculture.

Acknowledgements

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Commission Regulation (EC) No. 257/2002 of 12 February 2002 amending Regulation (EC) No 194/97 setting maximum levels for certain contaminants in foodstuffs and Regulation (EC) 466/2001 setting maximum levels for certain contaminants in foodstuffs. L 41: 12-15

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IV.2. ANÁLISIS DE OCRATOXINA A

IV.2.1. RAPID DETERMINATION OF OCHRATOXIN A IN CEREALS AND CEREAL PRODUCTS BY LIQUID CHROMATOGRAPHY

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Abstract

A new method based on extraction with octylsilica (C8) followed by liquid chromatography coupled with fluorescence detection (LC-FD) was studied to determine ochratoxin A (OTA) from cereals and cereal products. Optimization of different parameters, such as, type and amount of solid phase, type and volume of eluent and amount of sample were carried out. Recovery of OTA from rice samples spiked at 10 ng/g level was of 86% with relative standard deviation of 5%. The limits of detection and quantification of the proposed method were 0.25 and 0.75 ng/g, respectively. Furthermore, LC-FD after of OTA methylation and liquid chromatography coupled to mass spectrometry with an electrospray interface (LC-ESI-MS), were used for confirmation of OTA in all studied samples. The proposed method was applied to 62 samples of cereals and cereal products and the presence of OTA was found in 7 samples.

IV.2.1.1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced widely in cereals and cereal products. In temperate climates, OTA is mainly produced by *Penicillium verrucosum* although *Aspergillus ochraceus* and related species also produced OTA in cold and wet climates [1]. Experimentally OTA has been shown to be a teratogenic, potent renal carcinogen, immunosupressive, enzyme inhibitor and it has effects on lipid peroxidation and has been implicated in Balkan nephropathy in humans. OTA is listed as a possibly carcinogen of group 2B by the International Agency for Research on Cancer (IARC) [2]. In the European Union [3], the OTA level in cereals and cereal products are regulated with maximum residue levels (MRLs) that can not be greater than 5 and 3 μg/kg, respectively.

Several analytical methods for determining OTA in cereals and cereal products have been reported. These methods generally involve liquid extraction with several solvents, such as mixtures of dichloromethane-citric acid [4], acetonitrile-water [5], methanol-phosphoric [6] and methanol-sodium chloride [7]. A clean up procedure is frequently used and it usually employs solid phase extraction columns such as anion exchange (SAX) [8], silica [9], C18 [10] and immunoaffinity columns (IACs) [5, 11-13]. IACs play a predominant role in separation and purification of OTA from crops. Detection and determination is performed by immunochemical methods as enzyme linked immunosorbent assay (ELISA) [4], and chromatographic procedures such as thin layer chromatography with densitometry detection [14], gas chromatography with

mass spectrometry detection [15] and especially by liquid chromatography with fluorescence (LC-FD) [5, 9, 16], mass spectrometry (LC-MS) [17] or tandem mass spectrometry (LC-MS-MS) detection [18]. Residue analysis must be simple and rapid to prevent distribution of harmful products, and economic enough to allow developing programmes for monitoring OTA over a wide number of food samples. However, only a few of the methods found in the literature comply with these premises.

The objective of this study is to optimise a simple method based in the use of C8 solid phase to extract and purify OTA in cereals and cereal products in which determination is carried out by LC-FD. Furthermore, two methods for confirmation, LC-FD after methylation of OTA and LC-MS of this mycotoxin in these products were used. Finally, the proposed method was applied to 62 cereals and cereal products.

IV.2.1.2. Experimental

Chemical and reagents

OTA crystalline material was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock standard solution with concentration of 500 μg/ml was prepared in methanol, kept in security conditions at -20°C, wrapped in aluminium foil, due to that OTA gradually break down under UV light, and held for less than 3 months. Standard working solutions were prepared by appropriate diluting in the same solvent and stored in glass-stopped tubes at -20°C.

Dichloromethane, acetonitrile, methanol and chlorhydric acid (37%) were supplied by Merck (Darmstadt, Germany), diethylamine and formic acid (98-100%) by Scharlau (Barcelona, Spain). Deionised water (< 8 MΩcm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 minutes using a Branson 5200 (Branson Ultrasonic Corporation, Connecticut, USA) ultrasonic bath.

Solid phases used were C8 (50 μm), C18 (50 μm), silica (40 μm), phenyl (50 μm) and neutral aluminium oxide from Análisis Vínicos (Tomelloso, Spain), ENVI-Carb from Supelco (Bellefonte, PA, USA) and Lichrolut® EN from Merck.

Samples

Random food samples were obtained in the cereal packers and supermarkets for cereals and cereal products, respectively, from May 2003 to September 2003, typically as a single bag. All samples were stored in the dark and dry place at room temperature (18-23°C). The samples were divided with a subsample divider and a 200 g subsample was analysed [19].

Extraction procedure

Samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2.5 g) of the sample was placed into a mortar (50 ml

capacity) and was gently blended with 1.5 g of the solid phase (C8) for 5 minutes using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 x 9 mm I.D. glass chromatographic column with a coarse frit (No. 2) and covered with a plug of silanized glass wool at the top of the column. OTA was eluted with 20 ml methanol-formic acid (99:1, v/v) with a vacuum manifold. The eluate was evaporated to 3 ml with a gentle stream of N_2 at 45°C, and then, it was filtered through a nylon acrodisk (0.45 μm) and centrifuged at 5000 r.p.m. for 10 minutes. The extract was filtered again and evaporated to 0.5 ml with N_2 at 45°C.

Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-6A system LC equipped with two LC-6A pumps, a Rheodyne Model 7125 injector (20 μl loop) and an SRF-535 fluorescence detector was used. A LC column Nova-Pack C18 (5 μm) (150 x 3.9 mm I.D.) was used with a mobile phase consisting of a mixture of methanol-formic acid 0.1 M (70:30, v/v) at a flow rate of 0.4 ml/min. Detection of OTA was carried out using 334 and 464 nm as wavelengths for excitation and emission, respectively. The limit of detection (LOD) (s/n 3:1) and limit of quantification (LOQ) (s/n 10:1) were 0.25 and 0.75 ng/g respectively.

IV.2.1.3. Confirmation procedures

Liquid chromatography – fluorescence detector after methylation of OTA

The method of Zimmerli and Dick (1995) [12] was used. Briefly, 200 µl of the extract was diluted to 2.5 ml methanol and 0.1 ml concentrated HCl were added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200 µl methanol-formic acid 0.1 M (70:30, v/v). 90% of the OTA was methylated with this method. The LC analysis was identical to that described above.

Confirmation by liquid chromatography – mass spectrometry

OTA was conducted using a Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series LC-MS system equipped with a binary solvent pump, an autosampler and a mass detector (MS) coupled with an analytical workstation (HP Chem software). The MSD consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray interface (ESI). The LC-ESI-MS interface in negative ion mode operated under the following conditions, 350°C gas temperature, 13.0 l/min drying gas nitrogen flow, 50 p.s.i. nebulizer gas pressure and 3500 V capillary voltage. Using a fragmentor of 120V, the ions selected for OTA identification were ($[M-H]^-$) and ($[M-H-CO_2]^-$) at *m/z* 402 and 358, respectively. The sample extract was injected into the LC using the same conditions specified above. The LOD and LOQ were 0.30 and 0.95 ng/g, respectively.

IV.2.1.4. Results and discussion

Method performance

The following modifications were made to optimize the analysis from cereals and cereal products, (a) type and (b) volume of eluent, (c) type of solid phase and (d) amount of solid phase and (e) amount of sample used. The optimization was tested in rice.

(a) Type of eluent; 20 ml of dichloromethane, acetonitrile, methanol, methanol-diethylamine (99:1, v/v) and methanol-formic acid 1% (99:1, v/v) were tested (n=5) as elution solvents by using 2.5 g of C8 solid phase and 1.5 g of rice fortified with 10 ng/g level. The best recoveries and relative standard deviation (86 and 5 %, respectively) were found with the methanol-formic acid solution.

(b) Eluent volume; Table IV.7 reflects that the maximum recovery was reached with 20 ml and it was not improved by using higher volumes. For this reason, 20 ml was selected.

(c) Type of solid phase; C8, C18, silica, phenyl, Lichrolut-EN, ENVI-carb and aluminium oxide were studied (n=5). For ENVI-carb and aluminium oxide, recoveries were lower than 20%. The values obtained for the other solid supports are shown in Table IV.8. The best recoveries for OTA analysis were obtained using C8 and methanol-formic acid as elution solvent. The differences

Table IV.7: Mean recoveries (± relative standard deviation) obtained with different volumes of methanol-formic acid as eluting solvent for rice samples spiked at 10 ng/g level (n=5)

Eluent volumes (ml)	Recovery (RSD) %
5	68 (8)
10	72 (6)
15	75 (7)
20	86 (5)
25	84 (5)
30	78 (6)
35	85 (8)
40	73 (10)

between the mean recoveries obtained with C8, LiChrolut-EN, ENVI-carb and aluminium oxide were of statistical significance, whereas the recoveries obtained with C8, C18, silica and phenyl were close. The tested C8 was chosen because their chromatograms are more clean than those generated by other phases.

Table IV.8: Percent mean recoveries and relative standard deviation (%, in parenthesis) obtained with different solid supports in rice samples spiked at 10 ng/g level (n=5)

Solid support	OTA
C8	86 (5)
C18	81 (8)
Silica	79 (12)
Phenyl	81 (10)
Lichrolut-EN	22 (9)

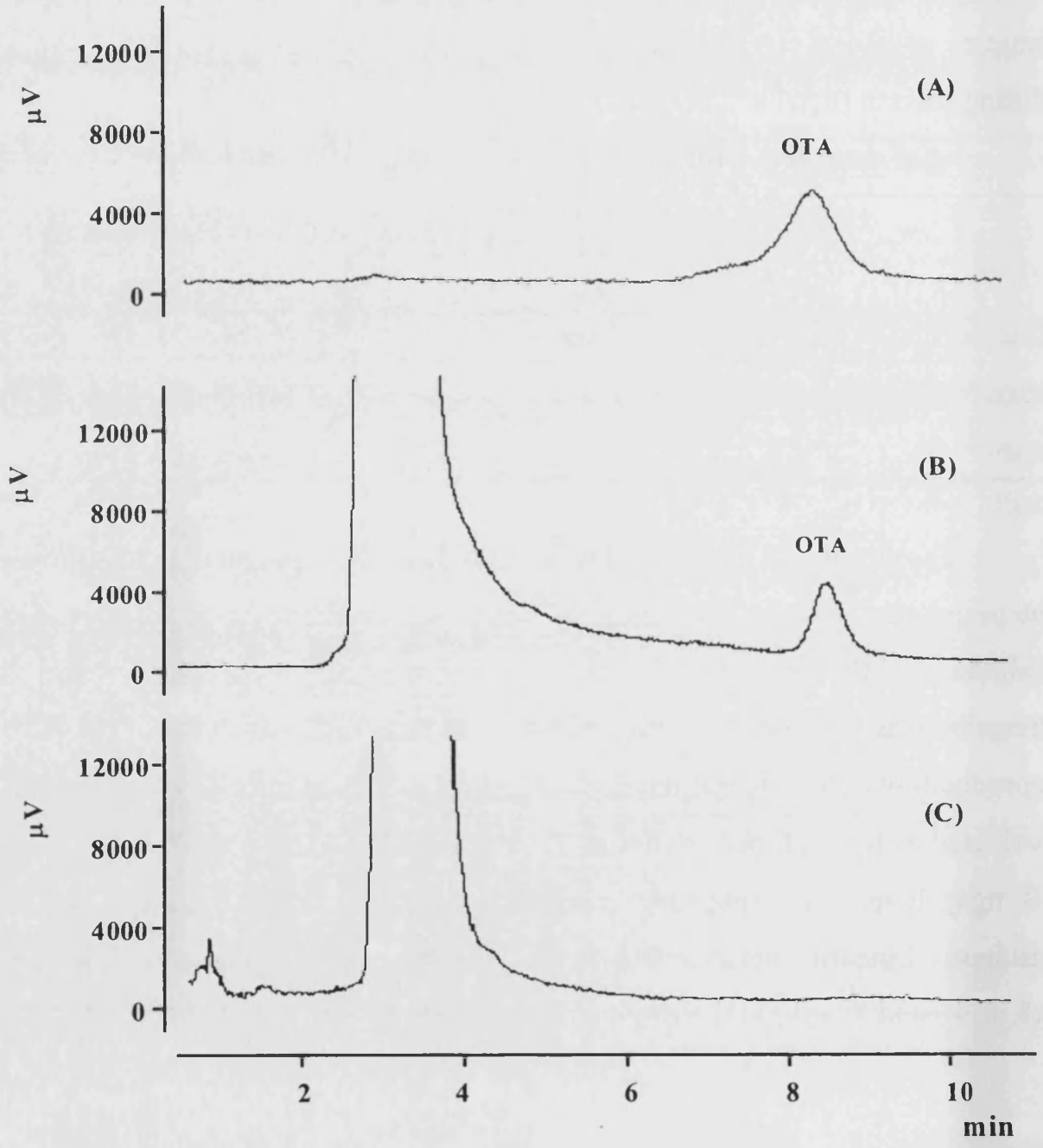
(d) y (e) Amount of solid phase and samples; Results reported in Table IV.9 shows that the best recoveries are obtained using 1.5 g of C8 solid phase and 2.5 g of sample.

Table IV.9: Percent mean recoveries and relative standard deviation (%, in parenthesis) obtained with different amounts of C8 solid phase and of rice samples spiked at 10 ng/g level, using methanol-formic acid (99:1, v/v) as eluting solvent (n=5)

g of sample + g of C8	**OTA**
0.5 + 0.5	69 (6)
2 + 1.5	74 (7)
2 + 2	78 (11)
2.5 + 1.5	86 (5)
3 + 1	77 (5)

The Figure IV.7 shows the LC-FD chromatogram obtained following the proposed extraction procedure for rice fortified with OTA at 10 ng/g level. Validation of the method was carried out according to the parameters selected. Precision was calculated in terms of intra-day repeatability (n=5) and inter-day reproducibility (5 different days) at 10 ng/g level. The intra-day repeatability evaluated as RSD ranged from 4 to 7% at 3 ng/g level and from 3 to 6% at the 10 ng/g level. The inter-day reproducibility was lower than 8% for all instances. Linearity was verified (n=3) with seven concentrations (0.25, 0.75, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all >0.998.

Figure IV.7: LC- fluorescence chromatograms obtained from rice with the proposed extraction procedure: (A) standard of OTA and (B) fortified with 10 ng/g of OTA and (C) a rice non-fortified sample extract



In order to investigate if natural constituents interfere with quantification, parallel calibration graphs obtained from matrix-extracted and solvent-based standards were performed. The covariance analysis for OTA according to the calculated F values were lower than the F Snedecor tabulated ones indicating that both straight lines were not parallel ($p<0.05$) and matrix effect is not negligible. For this reason, all analyses were conducted with spiked samples that were previously analyzed and did not contain OTA. Recoveries obtained for all cereals and cereal products spiked at 10 ng/g level (n=5) analyzed with the proposed method ranged from 77 to 89%.

The results of the study reflected that the extraction of OTA using 2.5 g of sample and 1.5 g of C8 solid phase gave clean chromatogram profiles and recoveries that were considered as valid for analyzing residues of OTA in foods according with European specification [19].

Application to real samples

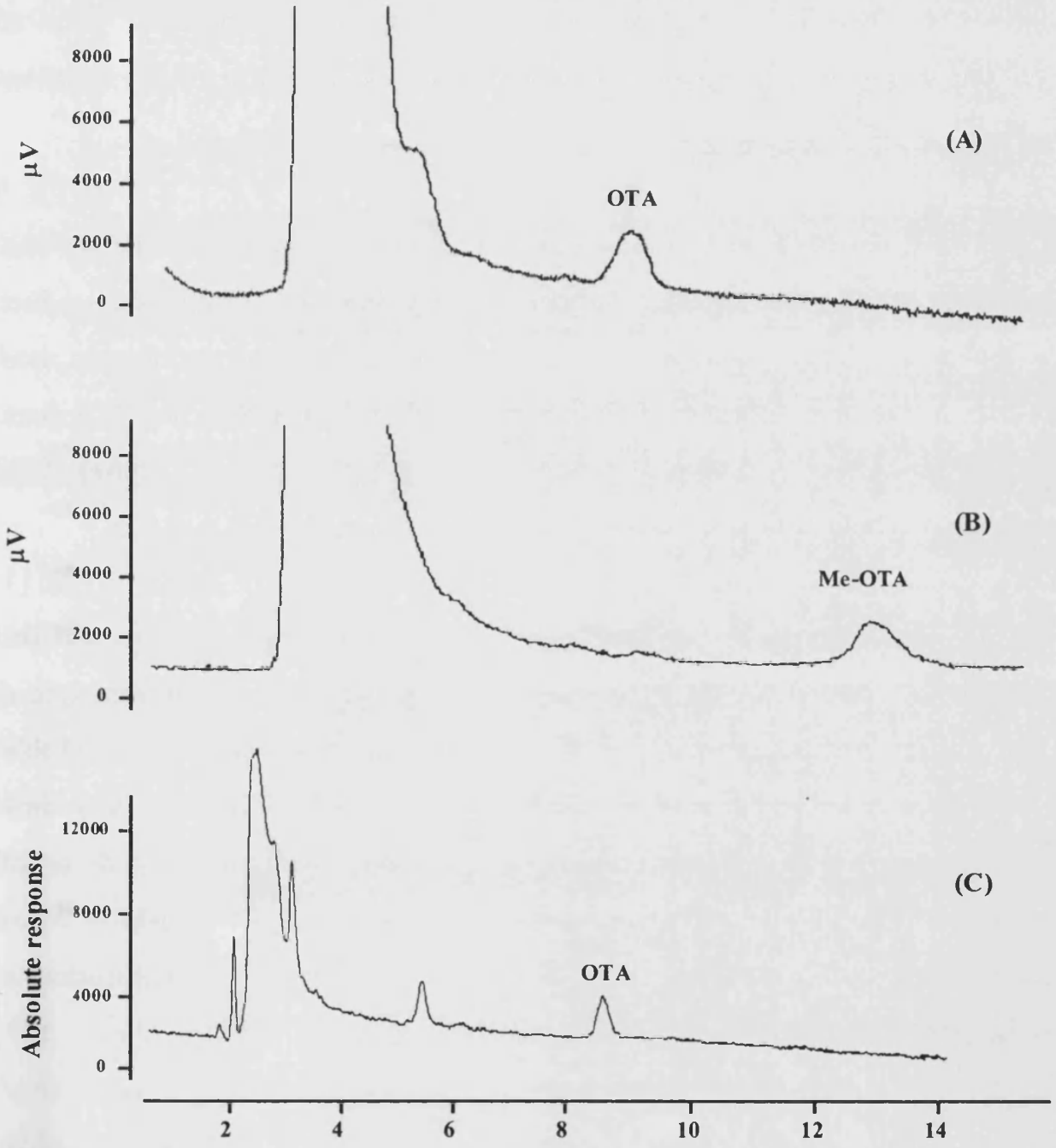
The proposed method of extraction followed by LC-FD was applied in 62 cereals and cereal products (Table IV.10). The selection of these products was done because they are well known for susceptible to fungal growth and toxin production [1] and are greatly consumed in Spain [20]. Figure IV.8 shows the LC-FD, LC-FD after methylation procedure and LC-ESI-MS chromatograms obtained by the proposed extraction procedure for a positive cereal product sample.

Table IV.10: Incidence of ochratoxin A in different samples analyzed by the proposed method

Sample (cereal)	N° of samples	N° of samples with OTA level			Value of OTA (ng/g)
		<LOD	LOD- LOQ	>LOQ	
Cereals					
Rice	9	9	0	0	-
Wheat	5	5	0	0	-
Barley	5	5	0	0	-
Cereal products					
Pasta (wheat)	6	6	0	0	-
Flour (wheat)	15	15	0	0	-
Flour (corn)	3	3	0	0	-
Cookie (wheat)	4	1	3	0	-
Bread (wheat)	4	4	0	0	-
Breakfast cereals (wheat)	2	1	0	1	1.88
Breakfast cereals (corn)	4	3	1	0	-
Breakfast cereals (wheat and corn)	2	1	0	1	1.51
Breakfast cereals (wheat and oat)	2	1	0	1	5.35
Breakfast cereals (whole wheat)	1	1	0	0	-*

* False positive

Figure IV.8: Chromatograms of a positive sample of breakfast cereal (wheat and oat) containing OTA (5.35 ng/g); (A) LC-FD, (B) LC-FD with methylation of OTA and (C) LC-ESI-MS



For cereal grains, the presence of OTA was not detected in any samples (Table IV.10). In the literature, several authors did not find this mycotoxin in cereals samples [9, 21]. However, concentration of OTA higher than MRLs (5 ng/g) [7, 22-24] was found in cereal samples. The occurrence of OTA in cereal grains is considered to depend principally on the condition of the grain at harvest, how carefully the grain is dried and the quality of the storage facilities [1].

For cereal products, the obtained results are shown in Table IV.10. The presence of OTA was detected and confirmed in 3 out 43 (7 %) cereal product samples. One sample of breakfast cereals, elaborated with wheat and oat, was found to contain 5.3 ng/g of OTA; this level is higher than the European legislated MRLs (3 ng/g) [3]. According to the Skaug et al. (2001) [25], breakfast cereals could be important contributors to dietary OTA intake.

The presence of problems due to the co-extractive substances in the matrix have been observed by Wood et al. (1995) [11] thus, a confirmation of the positive analysis is necessary. The breakfast cereal of whole wheat (Table IV.10) shows the presence of an interfering signal by LC-FD at the same time of retention of OTA. This problem was resolved with the procedures of confirmation, LC-FD after methylation of OTA and LC-ESI-MS. As a consequence, such breakfast cereal sample (whole wheat) was classified as a false positive.

In conclusion, a number of advantages reflects the use of presented extractive

method followed by determination with LC-FD and confirmation of positive samples by methylation or LC-ESI-MS herein with respect to the more utilized methods for determining OTA in cereals and cereals products based in solvent extraction and IACs. In particular, the use of hazardous solvents and expensive IACs are avoided and rapid sample preparation and clean up give to convenient analysis time-saving.

Acknowledgements

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Estudio experimental

IV.2.2. THE CONCENTRATION OF OCHRATOXIN A IN WINES FROM SUPERMARKETS AND STORES OF VALENCIAN COMMUNITY (SPAIN)

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by fungi species belonging to the genera Aspergillus and Penicillium being isolated in alcoholic beverages. The aim of this work is developed and applied a procedure for the analysis of OTA in wines. An analytical method based on immunoaffinity column (IAC) for clean-up, liquid chromatography with fluorescence detection (LC-FD) and LC-FD after of OTA methylation was used to determine the occurrence of OTA in wines. Recoveries of this mycotoxin spiked to red wines at 0.5 ng/ml level were >90% with an average of relative standards deviations of 4%. Furthermore, 116 wine samples from designation of origin and 3 samples from food stores of Valencian Community (Spain) were examined for the occurrence of OTA being the levels of this mycotoxin ranged from <0.01 to 0.76 ng/ml. Finally, the estimated daily intake of OTA in this study was 0.15 ng/kg b.w. day^{-1}.

IV.2.2.1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungi species belonging to the genera Aspergillus (e.g. A. ochraceus and A. carbonarius) and Penicillium (e.g. P. verrucosum). This mycotoxin shows nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties [1]. Furthermore, it may be implicated in the human disease Balkan Endemic Nephropathy (BEN) and in the development of urinary tract tumours in humans [2]. In 1993, the International Agency for Research on Cancer (IARC) classified into group 2B as a possible human carcinogen [3].

In temperate areas such the Mediterranean region, the natural occurrence of OTA is detected in different kinds of foods and beverages, including wines [1]. The use of immunoaffinity column (IAC) as clean-up methodology followed by liquid chromatography (LC) with fluorimetric detection has become the most popular procedures for OTA analysis in wines [4-8]. Several studies from African [9-11], American [12, 13] and European [7, 12, 14-17] wines have demonstrated level of contamination of OTA. Valencian Community is a Mediterranean place which the production of wines with designation of origin is around 1200000 hectolitre/year [18].

This designation of origin is designed by the Control Board, an independent agency of control and certification, according to the article 10 of the European Regulation 2081/92 [19], which will guarantee that the wine protected comply the requirements established of food safety and quality.

The objective of this work was to obtain data on the occurrence of OTA from 119 wines from Valencian Community (Spain) with designation of origin and other wines which is obtained from food stores, in order to evaluate its potential contribution to the dietary OTA exposure of consumers of these wines. The relevance of this paper is based on the determination of OTA in wines together with the analysis of wines from Valencian Community. To date, the occurrence of OTA in wines from this place has not been carried out.

IV.2.2.2. Experimental

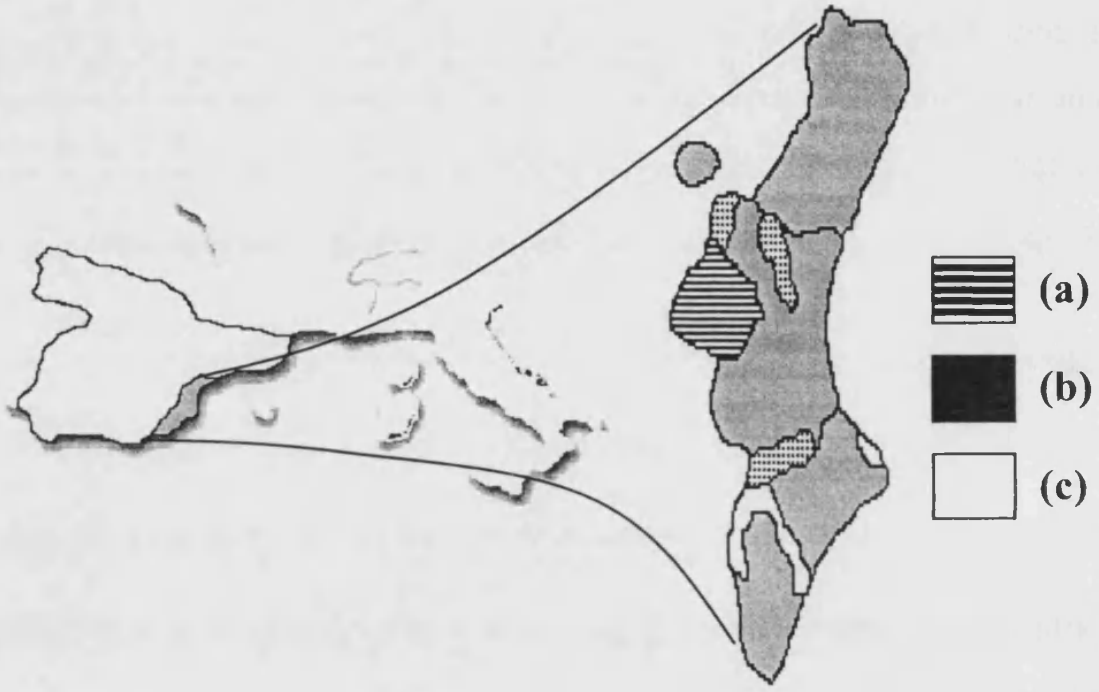
Samples

Samples of dessert, red, rosé and white wines from designation of origin from Valencian Community (Spain) (Figure IV.9) were purchased in supermarkets in Valencia and three white wine samples were obtained from food stores in a small village of Valencia. All samples were taken during July 2003 and stored at 4°C until their analysis. All information on the samples was taken from the bottle labels.

Chemical and reagents

LC-grade acetonitrile, acetic acid and methanol were supplied by Merck (Darmstadt, Germany). Clorhidric acid (HCl) was obtained by Scharlau (Barcelona, Spain) and phosphate-buffered saline (PBS) was bought to Sigma (St. Louis, MO; USA). Deionised water (<8 Ωcm resistivity) was obtained

Figure IV.9: Location of wines with designation of origin of (a) Utiel-Requena, (b) Valencia and (c) Alicante from Valencian Community.



from a Milli-Q water purification system (Waters-Millipore, Milford, MA, USA). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. The immunoaffinity column (IAC) used for OTA analysis was Ochraprep from R-Biopharm Rhône Ltd (Scotland, UK). OTA standard was purchased from Sigma. OTA crystalline material was purchased from Sigma. The standard solutions were made in methanol according to the concentration established using a UV spectrophotometer ($\varepsilon = 55000\ mol^{-1}\ cm^{-1}$), kept in security conditions at -20°C, wrapped in aluminium foil, due to that OTA gradually break down under UV light, and held for less than 3 months. Standard working solutions were

prepared by appropriate diluting in the same solvent and stored in glass-stopped tubes at -20°C.

Extraction procedure

Samples (10 ml) were diluted with 10 ml of PBS. The mixture was shaken for 5 min. The IAC was placed on a SPE vacuum manifold (Visiprep, Supelco) and preconditioned with 4 ml of PBS. Then, the mixture of the diluted sample (20 ml) was applied to the IAC column (1-2 drops per second), followed by a washing with 9 ml of PBS and 8 ml de-ionized water and then dried with air. The OTA was then slowly eluted from the IAC with 2 ml methanol into a glass vial; the eluate was evaporated to 1 ml with a gentle stream of N_2 and stored at 4°C until LC analysis. Finally, 150 µL was injected onto the LC column.

Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne Model 7125 injector (150 µl loop) and a SRF-535 fluorescence detector. A LC column Kromasil SC-18 (5 µm) (150 x 4.6 mm I.D.) (Scharlau, Barcelona, Spain) was used with a mobile phase consisting of a mobile phase of acetonitrile/water/acetic acid (50:49:1, v/v/v) at a flow-rate of 1 ml/min. Detection of OTA was carried out using 333 and 470 nm as wavelengths for excitation and emission, respectively.

Confirmation

Confirmation of positive samples was performed according to the method of Zimmerli and Dick [6]. Briefly, 200 µl of the extract was diluted to 2.5 ml with methanol and then 0.1 ml concentrated HCl was added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200 µl acetonitrile/water/acetic acid (50:49:1, v/v/v). 90% of the OTA was methylated with method. The LC analysis was identical to that described above.

IV.2.2.3. Results and Discussion

Method performance

The recovery for OTA on a red wine sample spiked at a level of a 0.5 ng/ml was 92±4% (mean ± SD, n=3) with a limit of detection (S/N 3:1) and quantification (S/N 10:1) of 0.01 and 0.05 ng/ml, respectively. The Figure IV.10c shows the LC-FD chromatogram obtained following the proposed method for a red wine fortified with 0.5 ng/ml. Precision was calculated in terms of intra-day repeatability (n=5) and inter-day reproducibility (5 different days) on 0.25 ng/ml. The intra-day repeatability evaluated as RSD ranged from 3 to 5%. The inter-day reproducibility was lower than 8% for all instances. Linearity was verified (n=5) with six concentration (0.05, 0.10, 0.25, 0.75, 1 and 5 ng/ml). The regression coefficients (r) were all >0.997. The results of the study reflected that the analysis gave cleanest chromatograms and recoveries

were considered as valid for analyzing residues of OTA in wines according with European signification [20]. This method presents several advantages as is good repeatability, avoid the common emulsions formed when red wines are treated with liquid-liquid extraction and reduces drastically the time of analysis compared with other procedures [6]. In fact, the average time for wine sample preparation was about 10 min.

Occurrence of OTA

In the present study, a total 116 wine samples from designation of origin from Valencian Community and 3 samples from food stores were analyzed and their results are shown in Table IV11. The levels of OTA ranged from <0.01 to 0.76 ng/ml. Figures IV.10d and IV.10e show chromatograms obtained with positive samples found from a dessert and a white wines, respectively. The European Union have proposed 2 ng/ml of OTA in wine as maximum residue levels (MRLs) [21]. Neither of the studied samples shown levels above the European regulatory limit. On the other hand, level of OTA decreased in the order red>rosé>dessert>white for wines with designation of origin.

The higher values for red, rosé and white wines were found of the most recently year of production and decreasing in accordance with the year. According to our results, López de Cerain et al. [17] suggested that OTA is stable in wines for at least one year and that the concentration of OTA in raisins and consequently in wine vary from one year to the other depending on

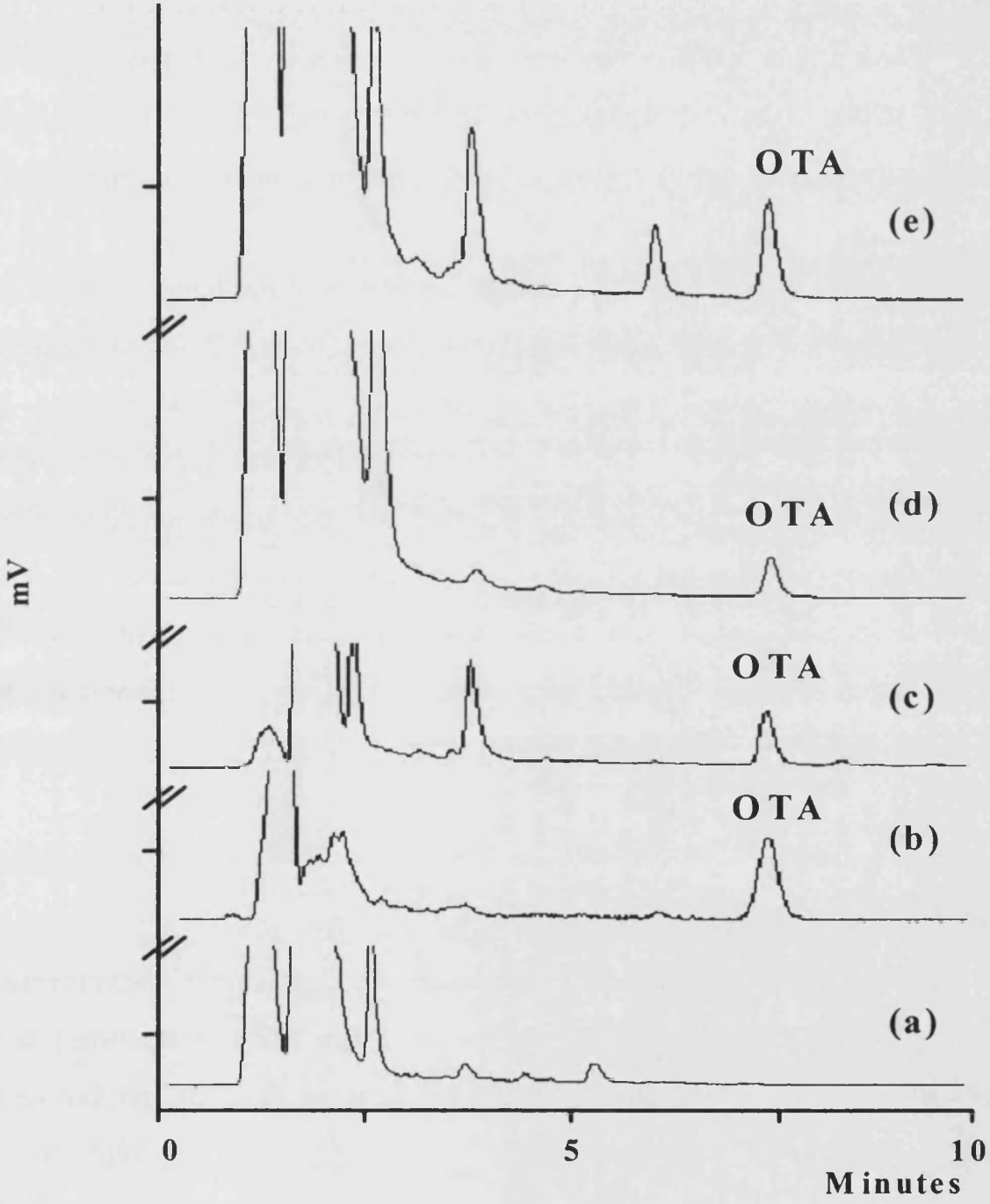
Table IV.11: Occurrence of OTA in Valencian Community wine samples

Origin of samples	Year of production	No. of samples	No. of samples without residue[a]	No. of samples with residue	
				LOD-LOQ[b]	>LOQ(ng/ml)
Dessert wine					
Valencia (Moscatel)	2001	7	3	1	3 (0.28, 0.31, 0.40)
Alicante (Fondillón)	2001	6	2	1	3 (0.10, 0.19, 0.38)
Red wine					
Alicante	1999	8	8	-	-
	2000	9	6	1	2 (0.20, 0.29)
	2001	11	4	3	4 (0.10, 0.11, 0.20, 0.51)
Utiel Requena	1999	10	8	1	1 (0.06)
	2000	10	7	1	2 (0.20, 0.40)
	2001	8	4	2	2 (0.39, 0.53)
Valencia	2001	5	3	1	1 (0.38)
Rosé wine					
Utiel Requena	2000	11	6	2	3 (0.25, 0.32, 0.40)
	2001	10	3	2	5 (0.11, 0.24, 0.29, 0.33, 0.46)
White wine					
Valencia	2000	11	11	-	-
	2001	10	9	-	1 (0.09)
Food stores	2003	3	-	-	3 (0.32, 0.48, 0.76)

[a] <LOD , <limit of detection (<0.01 ng/ml)

[b] LOQ, limit of quantification (0.05 ng/ml)

Figure IV.10: LC-fluorescence chromatograms obtained of (a) non-fortified wine, (b) OTA standard solution (1 ng/ml), (c) red wine fortified with 0.5 ng/ml and two positive wine samples containing (d) 0.38 and (e) 0.76 ng/ml from dessert and white wines, respectively, from Valencian Community



the meteorological conditions. In fact, several authors reflected that the wines from the Mediterranean region contain high concentrations of OTA due to that the climate is characterized by high humidity and high temperature [10, 14].

The higher value is obtained with white wines from food stores (0.76 ng/ml) versus wines with designation of origin. In some places, the vine grower sells his grapes to food stores which pay a part with money and the other with wine elaborated by themselves. This wine is called cold wine but is not wine with designation of origin. This food stores have not implemented food safety measures such as Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs) and the Hazard Analysis and Critical Control Point (HACCP) system [22]. It could explain the higher value for white wine in comparison with other wines. In other hand, wines of designation of origin (DO) are proposed by the Control Board which is an independent official authority in charge of prescribing and enforcing the regulations of a DO. These wines are given to a specific wine which meets certain requirements as its origin; the way it is processed and its quality.

Daily intake

For Valencian Community wines, the average sample contamination of OTA was 0.25 ng/ml, according to the data of our study. Assuming that wine consumption in this Community is about to 41.4 ml/day [23] and that an adult body weight 70 kg, the estimated daily intake of OTA in this study was 0.15 ng/kg b.w. day^{-1}. This value represent 3 and 0.9% of the Tolerable Daily Intake

(TDI) according to the Scientific Committee on Food of the European Commission (5 ng/kg b.w. day^{-1}) [24] and the WHO Committee of Experts on Food Additives (16 ng/kg b.w. day^{-1}) [25], respectively. In our study, the contribution to daily intake for these studied wines could be considered to be rather small in comparison with the daily intake values of OTA in France (2 ng/kg b.w. day^{-1}) [9], Greece (3.7 ng/kg b.w. day^{-1}) [15], Spain (0.3 ng/kg b.w. day^{-1}) [17], Sweden (0.2 ng/kg b.w. day^{-1}) [26], Switzerland (0.7 ng/kg b.w. day^{-1}) [27].

Conclusions

The use of IAC for clean-up followed by LC-FD has shown to be a technique with good analytical performance for the OTA determination in wines. The application of this procedure to analysis 119 wine samples from Valencian Community has demonstrated that none of them contained levels above the European MRLs. Furthermore, the estimated daily intake of OTA has reflected to be rather small in comparison with other countries.

Acknowledgements

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IV.2.3. ABSENCE OF OCHRATOXIN A IN SOY SAUCE

(Short Communication)

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Abstract

A method is described for the determination of ochratoxin A (OTA) in soy sauce using phosphate-buffered saline (PBS) extraction, an immunoaffinity clean-up, a liquid chromatographic determination with fluorescence detection (LC-FD) and confirmation with LC-FD after methylation of OTA. Recoveries of OTA spiked to soy sauce samples at 0.25 ng/ml level were 90% with relative standard deviations of 4%. The limit of detection was 0.01 ng/ml for OTA using the proposed method. Furthermore, the proposed method was applied to sixty soy sauce samples from China and Japan and none of them were found to contain OTA.

IV.2.3.1. Introduction

Soybean was introduced as a food source in the eastern half of north China in the 11th century B.C. (Hymowitz and Kaizuma, 1981). However, the use of soy sauce dates back about 2000 years (Hesseltine, 1965). Soy sauce is usually made from soybeans that are mixed with roasted grain (usually wheat, rice or barley) and fermented for several months. Once the aging process is completed, the mixture is strained and bottled (Aidoo et al., 1994; Valyasevi and Rolle, 2002).

Soy bean and roasted cereals as raw materials for elaboration of this sauce are a source of mycotoxigenic fungi and mycotoxins. Several mycotoxins have been detected in soy bean (el-Kady and Youssef, 1993; Ministry of Agriculture, Fisheries and Food, 1980; Pacin et al., 2002; Roy et al., 2000; Scudamore et al., 1997; Weidenenbörner, 2001). Cereals are contaminated by different mycotoxins, OTA among them (Egmond and van Speijers, 1994; Walker, 2002). On the other hand, aflatoxins have been determined in soy sauce (Wei et al., 1980; Sripathomswat and Thasnakorn, 1981). However, no studies have been found to determine OTA in soy sauce. This mycotoxin has been experimentally shown to be teratogenic, a potent renal carcinogen, immunosuppresive, an enzyme inhibitor, has effects on lipid peroxidation and has been implicated in Balkan nephropathy in humans. OTA is listed as a possible carcinogen of group 2B by the International Agency for Research on Cancer (IARC, 1993).

The objective of this study is to optimise a method to analyze OTA in soy sauce which was applied to 60 samples from China and Japan.

IV.2.3.2. Materials and methods

Samples

Soy sauce samples were obtained in supermarkets from September 2003 to July 2004 and stored at 4°C prior to analysis. All information on the samples was taken from the bottle labels.

Chemical and reagents

OTA crystalline material was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock standard solution with concentration of 500 µg/ml was prepared in methanol, kept in a closed container at –20°C, wrapped in aluminium foil, and held for less than 3 months. Standard working solutions were prepared by appropriate dilution in the same solvent and stored in glass-stopped tubes at –20°C.

Acetonitrile, acetic acid and methanol were supplied by Merck (Darmstadt, Germany) and formic acid (98-100%) by Scharlau (Barcelona, Spain). Deionized water (<8MΩcm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solvents and water were degassed for 20 minutes using a Branson 5200 (Branson Ultrasonic Corporation, Connecticut, USA) ultrasonic bath.

Extraction and clean-up procedures of the proposed method

The method is modified procedure of Brera et al. (2003). Briefly, sixty ml of phosphate-buffered saline (PBS) were added to a 10 ml portion of soy sauce. The mixture was shaken vigorously, filtered through Whatman GF/A glass microfibre filter and the filtrate collected. A 20 ml volume of the diluted and filtered sample was passed through an Ochraprep (Rhône Diagnostics Technologies, Glasgow, UK) immunoaffinity columns (IAC) at a flow-rate of about 1-2 drop/second. The IAC was washed with 10 ml of PBS and 9 ml of deionized water and then dried with air. OTA was eluted at 1 drop/second with methanol from the IAC until that 1.5 ml of methanol was recovered. The eluted extract was diluted with 0.5 ml deionized water, mixed vigorously and retained at ca. 4°C until HPLC analysis. This method is modified.

Liquid chromatography analysis

The chromatographic system consisted of a Shimadzu (Kyoto, Japan) SCL-6A system LC equipped with two LC-6A pumps, a Rheodyne Model 7125 injector and an SRF-535 fluorescence detector. Separation was carried out on a Nova-Pack (C18) column, 150 x 3.9 mm ID, with 5 μm particles. A 20 μl aliquot of the sample was injected onto the column. The mobile phase consisted of acetonitrile/water/acetic acid (50:49:1, v/v/v) pumped at a flow rate of 1 ml/min. The determination was performed at 334 nm (excitation wavelength) and 464 nm (emission wavelength).

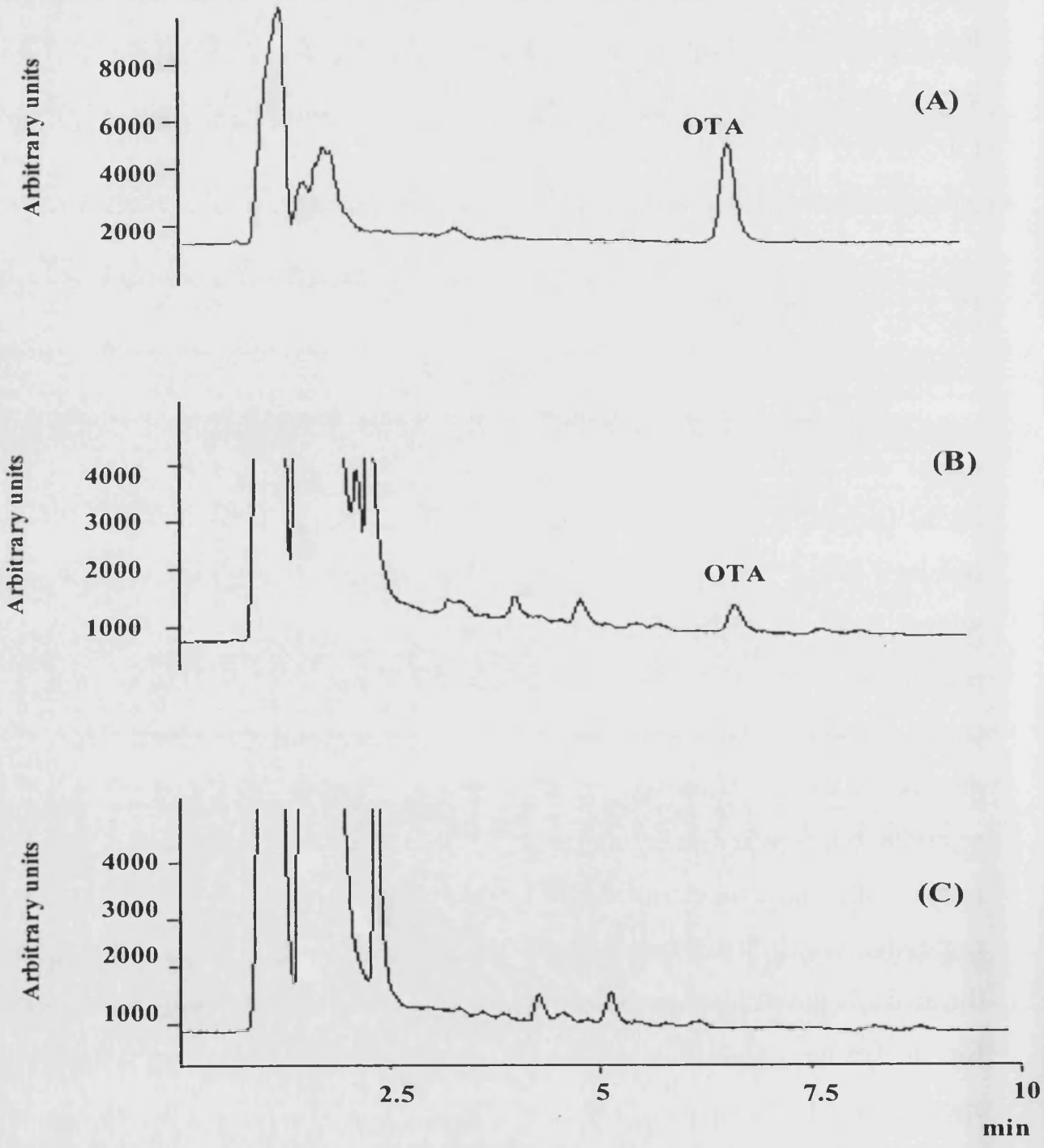
Confirmation

Confirmation of positive samples was performed according to the method of Zimmerli and Dick (1995). Briefly 200 µl of the extract was diluted to 2.5 ml methanol and 0.1 ml concentrated HCl as added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200 µl methanol-0.1 M formic acid (70:30, v/v). 90% of the OTA was methylated by this method. The LC analysis was identical to that described above.

IV.2.3.3. Results and discussion

The recovery for OTA on a soy sauce sample spiked at a level of a 0.25 ng/ml was 90 ± 4% (mean ± SD, n=3) with a limit of quantification of 0.1 ng/ml (signal/noise = 10:1). Figure IV.11B shows the LC-FD chromatogram obtained following the proposed extraction procedure for a soy sauce fortified with 0.25 ng/ml. Validation of the method was carried out according to these parameters selected. Precision was calculated in terms of intra-day repeatability (n=5) and inter-day reproducibility (5 different days) on 0.25 ng/ml. The intra-day repeatability evaluated as RSD ranged from 2 to 4%. The inter-day reproducibility was lower than 6% for all instances. Linearity was verified (n=5) with six concentrations (0.1, 0.25, 0.5, 0.75, 1 and 3 ng/ml). The regression coefficients were all >0.998. The results of the study reflected that the analysis gave clean chromatograms and recoveries were considered as valid for analyzing residues of OTA in soy sauce according with European specification (Commission Directive, 2002).

Figure IV.11: LC-fluorescence chromatograms of (A) OTA standard solution (1 ng/ml); (B) soy sauce fortified with 0.25 ng/ml of OTA; and (C) soy sauce non-fortified



The proposed method was applied to 60 soy sauce samples from China and Japan. OTA was not detected in any of the studied samples. A chromatogram of a real sample is shown in Figure IV.11C. All samples were fermented natural products. In Japan, synthetic soy sauce is not recognized by the government, which is produced in a matter of days through a hydrolytic reaction and seasoned with corn syrup, caramel coloring, salt and water. Nowadays, new and better microbial strains introduced into the process include the fungi *Aspergillus oryzae* (Beuchat, 1995) and *A. sojae* for koji production (Wood, 1994). To our knowledge, no other work on the determination of OTA in soy sauce has been published yet. However, several authors have detected aflatoxins and aflatoxigenic fungi in this sample.

As raw materials can contain OTA, it is important to have information on the frequency of occurrence and concentration of OTA in the soybean and roasted grain which is used for soy sauce production. The use of roasted grain (usually) in the elaboration of soy sauce can not pose a contamination risk for OTA due to two factors. First, wheat, rice or barley are grains with low concentration of OTA *versus* corn. Second, the roasting procedure can result in a reduction of more than 50% of this mycotoxin (Benford et al., 2001).

For soybean as raw material, poor storage may lead to increased contamination of OTA and the presence of OTA may increase or decrease in the soy sauce. El-Kady and Youssef (1993) detected the presence of several mycoflora in Egyptian soybean seeds such as *A. niger* and *A. alutaceus*. However, OTA was not detected and aflatoxins were detected in 35% of

soybean seed samples (5-35 μg/kg). Pacin et al. (2002) detected from soybean *Fusarium verticillioides*, *F. semitectum*, *A. flavus* and *A. ochraceus* among others and suggested that based on the toxigenic species recovered, OTA may pose a contamination risk for soybean. Ministry of Agriculture, Fisheries and Food. (1980) found OTA in the range of 50-500 μg/kg in soybeans and soy flour. On the contrary, Pineiro et al. (1996) and Scudamore (1996) did not detect OTA in soybean and soya as feed ingredients.

Two arguments may be sufficient to explain the absence of OTA in soy sauce. First, soy bean and roasted grain may be poor substrate for growth of ochratoxigenic fungi and elaboration of OTA. In fact, Bhumiratana et al. (1980) suggested that soybean has been shown to be a poor substrate for aflatoxin production, even for strains of *Aspergillus* which are high producers on other substrates. The second possibility is that *A. oryzae* and *A. sojae* can eliminate the presence of OTA due to their catabolic metabolism and competition with ochratoxigenic fungi. For these arguments, several studies will be developed to clarify the absence of OTA in soy sauce.

Acknowledgements

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Estudio experimental

IV.3.PRESENCIA SIMULTÁNEA DE AFLATOXINAS Y OCRATOXINA A

Estudio experimental

IV.3.1. A SURVEY OF AFLATOXINS AND OCHRATOXIN A IN SPANISH AND IMPORTED BEERS

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Abstract

In this work were applied The objective of this work was to apply the immunoaffinity columns (IACs) for enrichment and clean up of aflatoxins (AF) and ochratoxin A (OTA) from beers and quantify these mycotoxins by liquid chromatography coupled by fluorescence detection (LC-FD). For aflatoxins, recoveries of each aflatoxin spiked to beer samples at 0.25 ng/ml level were between 90 and 99.2% with relative standard deviations ranging from 2 to 6%. The limits of quantification (LOQs) ranged from 0.035 to 0.120 ng/ml for the four studied aflatoxins. For OTA, recoveries of this mycotoxin spiked to beers at 0.25 ng/ml level were >84% with an average of relative standard deviations around 2%, being the LOQ of 0.03 ng/ml.

The proposed method is applied to 37 beers from Spain and other countries (Argentina, Australia, Belgium, France, Germany, Ireland, Mexico, Netherlands, UK and USA), which were obtained from Spanish supermarkets. This application demonstrated that 1 out of 37 samples had 0.059 ng of AFB1/ml and 3 out of 37 samples contained OTA in a level of 0.036, 0.037 and 0.039 ng/ml. No co-occurrence of these mycotoxins was obtained in all studied samples. Furthermore, the contribution in our study to Spanish estimated daily intake of OTA for beer was 0.02 ng/kg b.w. day-1 and it could be considered to be rather small.

Estudio experimental

IV.3.1.1. Introduction

Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are toxic metabolites produced by the food spoilage fungi Aspergillus particularly A. flavus and A. parasiticus. AFB1, the most commonly occurring mycotoxins in this group, has been found to be one of the most potent naturally occurring carcinogens. In 1993, the International Agency for Research on Cancer (IARC) classified AFB1 into group 1 as a human carcinogen (10).

Ochratoxin A (OTA) is an other mycotoxin produced by certain toxigenic species of Aspergillus (e.g. A. ochraceus and A. carbonarius) and Penicillium (e.g. P. verrucosum). It has been shown to be hepatotoxic, nephrotoxic, teratogenic and carcinogenic to animals and has been classified as a possible human carcinogen (group 2B) by the IARC (11). Furthermore, it may be implicated in the human disease Balkan Endemic Nephropathy (BEN) and in the development of urinary tract tumours in humans. The Italian Ministry of Health has issued a directive setting guidelines for OTA in several foods, including beer for which a maximum level of 0.2 ng/ml has been fixed (14).

These mycotoxins occurs in various foodstuffs and beverages including a variety of cereals, beans, groundnuts, spices, dried fruits, pig kidney and blood, coffee, cocoa, wine and beer (1-5, 15-27). The Codex Alimentarius Commission based on limited European studies, suggested that beer is the fourth major source of human exposure to OTA following cereals, red wine and coffee (26).

The objective of this work was to analysis of 37 Spanish and imported beer samples applying the immunoaffinity columns (IACs) for the extraction and clean-up of mycotoxins followed by liquid chromatography with fluorescence detection. The daily intake of OTA was estimated.

IV.3.1.2. Experimental

Samples

In this study, 37 beer samples were obtained from supermarkets from Valencia (Spain) during October-December 2003. Samples were composed of 10 Spanish beer samples and 27 beer samples imported from several countries (Table 1). They were stored at 4°C until opened for analysis. All information on the samples was taken from the bottle labels.

Chemical and reagents

LC-grade acetonitrile, acetic acid and methanol were supplied by Merck (Darmstadt, Germany). Clorhidric acid (HCl) was obtained from Scharlau (Barcelona, Spain) and trifluoroacetic acid (TFA) and phosphate-buffered saline (PBS) was bought to Sigma (St. Louis, MO; USA). Deionised water (<4 MΩcm resistivity) was obtained from a Milli-Q water purification system (Waters-Millipore, Milford, MA, USA).

Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. The IAC used for OTA and aflatoxins analysis were OchraTest and AflaTest, respectively, from Vicam (Watertown, MA, USA). OTA and AFB1, AFB2, AFG1 and AFG2 crystalline materials were purchased from Sigma.

Stock standard solutions of OTA and aflatoxins were prepared in methanol, kept in security conditions at -20°C and wrapped in aluminium foil due to that the OTA and aflatoxins gradually breaks down under UV light and held for at least 3 months. Working solutions were diluted in methanol and stored at -20°C.

As safety notes, all used laboratory ware, pipette tips and kit components was soaked into 10% solution of household bleach before discarding it.

Extraction procedure

Beer was filtered through glass microfiber filter and degassed for 30 minutes by using ultrasonic bath. The extraction procedure for aflatoxins was carried out by minor modification of the method of Scott and Lawrence (22). 35 ml degassed beer was added to AflaTest column fitted with a reservoir that was previously preconditioned with 1 ml of distilled water. The column was then washed with 1 ml of distilled water. Aflatoxins were eluted with 2 ml methanol, this eluate was evapored to dryness in a thermoblock at 45° C under

a gentle stream of N2 and derivatized with TFA as follows; the extract was dried and then with 100 µl of TFA by mixing for 3 minutes, re-evaporated to dryness with N2 at 45° C and reconstituted with 1.5 ml of mobile phase.

In the other hand, OTA was carried out by a procedure described for the analysis of OTA in wine (4) with minor modification. 20 ml degassed beer was diluted with 20 ml PBS and added to an OchraTest column fitted with a reservoir and that was previously preconditioned with 5ml PBS. The column was washed with 8 ml PBS and 9 ml distilled water in this order. OTA was eluted with 2 ml methanol, evaporated to dryness with gentle stream of N2 at 45° C, and reconstituted with 1.5 ml of mobile phase.

Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-6A system equipped with two LC-6A pumps, a Shimadzu RF-535 fluorescence detector, a Rheodyne model 7125 injector (50 µl loop), LC column Kromasil SC-18 (5 µm, 150 x 4.6 mm i.d.) (Scharlau, Barcelona, Spain) and a Borwin data processor was used. For aflatoxins the mobile phase was a mixture of water-acetonitrile (75:25, v/v) at a flow rate of 0.7 ml/min, and λexc 365 and λem 435 nm were set in the detector. For OTA the mobile phase was acetonitrile-water-acetic acid (49.5:49.5:1, v/v/v) at a flow rate of 0.5 ml/min and λexc and λem of 333 and 460 nm, respectively, were set for detection.

Confirmation

Confirmation of positive samples was performed according to two procedures. For AFB1, the positive sample was confirmed by the method of Scott and Lawrence (21). Briefly, the extract from IAC was dissolved in mobile phase without derivatization of trifluoroacetic acid and observe absence of AFB1 peak. For OTA, the positive beer was confirmed by the method of Zimmerli and Dick (27). Briefly, 200 µl of the extract was diluted to 2.5 ml methanol and 0.1 ml concentrated HCl was added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200µl mobile phase, 90% of the OTA was methylated with this method. For confirmation of both AFB1 and OTA, the LC analysis was identical to that described above.

IV.3.1.3. Results and discussion

Method performance

Validation of the method was carried out with 0.250 ng/ml concentration levels for each mycotoxin. Precision was calculated in terms of intra-day repeatability (n=3) and inter-day reproducibility (5 different days). The intra-day repeatability evaluated as RSD ranged from 2.1 to 2.8%. The inter-day reproducibility ranged from 4 to 12.2%. Linearity was verified in triplicate with 5 concentrations (0.05, 0.075, 0.100, 0.125 and 0.250 ng/ml for AFB1, AFG1 and OTA, and 0.125, 0.150, 0.175, 0.250 and 0.500 ng/ml for AFB2 and AFG2). The regression coefficients were all >0.98.

Average recoveries ranged from 84.6 (OTA) to 99.2 % (AFB2) and relative standard deviations were from 2.3 to 6 %. The limits of quantification (LOQs) (S/N 10:1) were 0.030, 0.035, 0.045, 0.075 and 0.120 ng/ml for OTA, AFB1, AFG1, AFB2 and AFG2, respectively.

Occurrence of aflatoxins and OTA

The results of the analysis of 37 different brands of beer produced in Spain or imported from several countries worldwide are summarized in Table IV.12. Any mycotoxin was detected in the imported beer samples. In addition, no co-occurrence of aflatoxins and OTA was detected in studied beer samples.

AFB1 was detected in one Spanish beer (0.059 ng/ml) (Table IV.12) (Figure IV.12b). Scott and Lawrence (21) confirmed the presence of AFB1 in one sample of Mexican beer at 0.049 ng/ml. In the study of Okoye and Ekpenyong (18), the presence of AFB1 was detected in 32 of 40 Nigerian native beer with a range from 1.7 to 138 ng/ml.

For OTA in our study, three Spanish beers were found to contain a range of 0.036-0.039 ng/ml (Table IV.12) (Figure IV.12d). These levels of OTA are lower than the Italian MRLs (0.2 ng/ml) (14). Our results are slightly higher than the study of Legarda and Burdaspal (13) which detected a mean value of 0.026 and 0.018 ng/ml for Spanish alcoholic and unalcoholic beers. Nakajima et al. (15) detected OTA in 95.5% of Japanese beers and in 91.5% of

non-Japanese beers at mean concentrations of 0.012 and 0.010 ng/ml, respectively.

Of 61 beers analyzed by Visconti et al. (25), 50% were positive for OTA, with a mean value of 0.035 ng/ml. Similar results with a mean level of 0.037 ng/ml were obtained by Tangni and Larondelle (23) in 100% of the tested beers commercialized in Belgium. This same author detected OTA in Belgian and imported beers with average levels of contamination about 0.033 and 0.032 ng/l, respectively Tangni et al. (24). Assaf et al (1) detected OTA with a mean value of 0.19 ng/ml in beers collected from different regions of Lebanon. However, OTA was not found in Moroccan beers (9). In the other hand, El Dessouki (8) hypothesised that strong beers (>6% alcohol) are much more contaminated in OTA than pale beers (<6% alcohol) this is in contrast with the present study that show that the positive samples are pale beers. The study of Visconti et al. (25) and Soleas et al. (22) show no difference between these types of beers.

Figure IV.12. LC-fluorescence chromatograms (a) obtained of beer fortified with 0.25, 0.25, 0.5 and 0.5 ng/ml of AFG1, AFB1, AFG2 and AFB2, respectively (b) of positive beer sample (0.059 ng AFB1/ml), (c) obtained of beer fortified with 0.25 ng OTA/ml, and (d) of positive beer sample (0.039 ng OTA/ml)

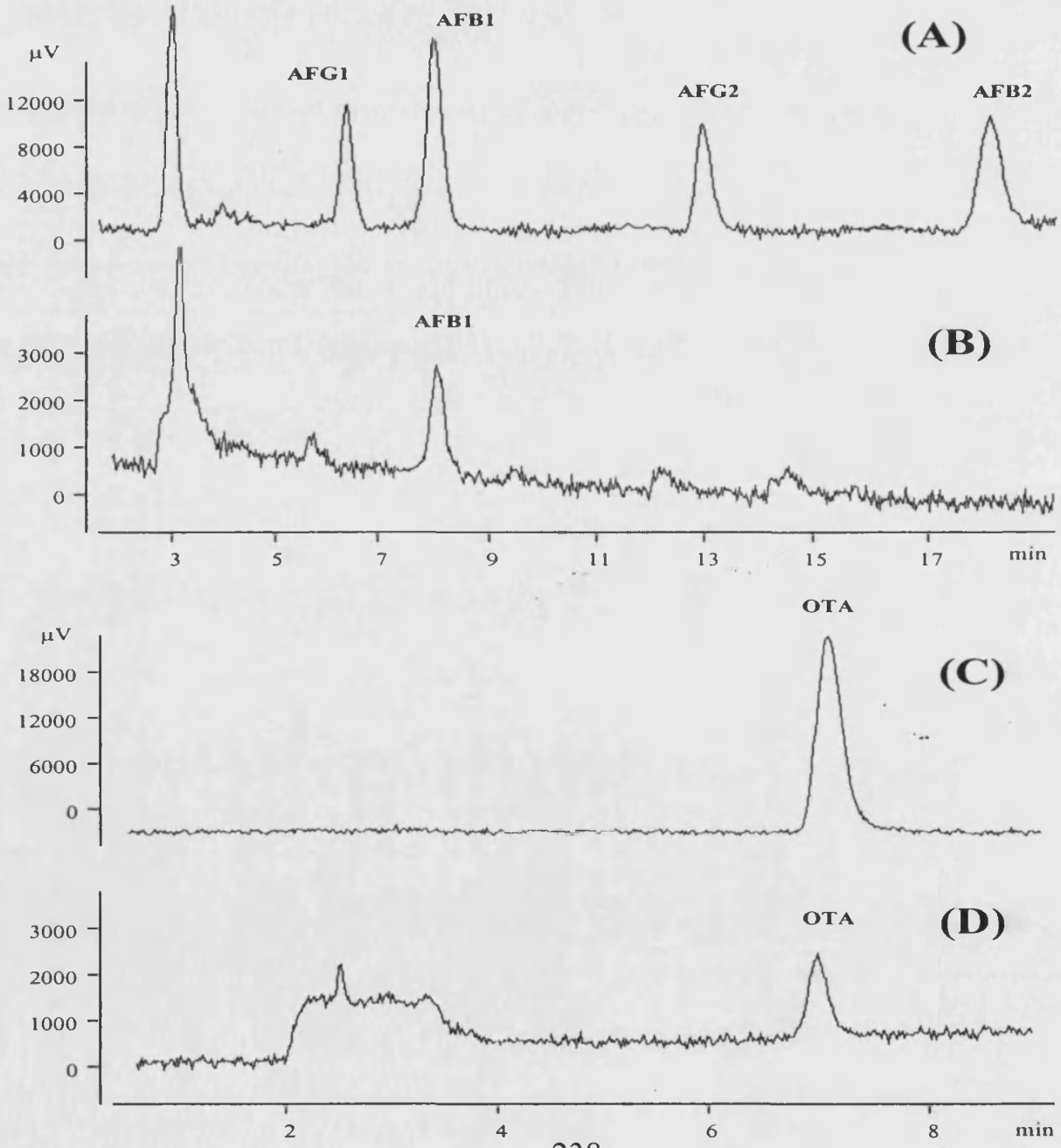

Table IV.12: Occurrence of aflatoxins and OTA in Spanish and imported beer samples

Origin of samples	Range of % alcohol	Aflatoxins		Ochratoxin A	
		Incidence	Concentration (ng/ml)	Incidence	Concentration (ng/ml)
Argentina	4.9	0/1	nd	0/1	nd
Australia	5.0	0/1	nd	0/1	nd
Belgium	4.5-9.0	0/9	nd	0/9	nd
France	5.9-6.6	0/2	nd	0/2	nd
Germany	4.8-12.5	0/7	nd	0/7	nd
Ireland	4.2	0/2	nd	0/2	nd
Mexico	4.5	0/1	nd	0/1	nd
Netherlands	5.0	0/1	nd	0/1	nd
Spain	4.2-5.5	1/7	0.059*	3/7	0.036 - 0.039
Spain	<1.0	0/3	nd	0/3	nd
UK	5.1	0/1	nd	0/1	nd
USA	4.7	0/2	nd	0/2	nd

nd: not detected

*AFB_1

In our study, it was observed that positive samples for OTA were detected in two different brewing companies located in the same city. Both companies had the same supplier of raw materials to elaborate the beers being

this fact probably the source of contamination due to that the supplier hadinadequate Good Agricultural Practices (GAPs) and/or Good Manufacturing Practices (GMPs). The degree of contamination depends upon the quality of the initial product (barley), storage conditions and the fate of OTA during brewing and fermentation. Although OTA does not survive malting, even if very highly contaminated barley malt is used. In consequence, adjuncts such as maize products (e.g. maize syrup and grits), rice, barley and wheat would be expected to be source of OTA contamination (2, 3, 5, 20). Okoye (17) detected AFB1 in a contaminated substrate into Nigerian native beer. Odav and Naicker (16) detected aflatoxins and OTA in South African traditionally brewed beers in a range from 200-400 and 3-2340 ng/ml, respectively.

Estimated daily intake of OTA

AFB1 is a genotoxic carcinogenic substance, for this mycotoxin there is no threshold below which no harmful effect is observed and therefore no admissible daily intake can be set (6). However, OTA daily intake was calculated. In our study, the OTA average concentrations referred to Spanish beers obtained is 0.003 ng/ml, corresponding to OTA estimated daily intake of 0.02 ng/kg b.w./day for a 70 kg person consuming daily 0.5 l of beer. These values are considerably lower than the provisional tolerable daily intake which are 14 and 5 ng OTA/kg b.w./day established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (12) and Scientific Committee on Food of the European Commission (7), respectively, and would lead to an

estimate of about 0.14 and 0.4%, respectively, for Spanish beer contribution to the level considered at risk for human health due to OTA exposure in Spain. In other countries, the consume of beer give values about to 0.167, 1.36, 0.23 and 0.26 ng/kg b.w. in the study of Visconti et al. (25), Assaf et al. (1), Tangni et al. (24) and Tangni and Larondelle (23), respectively.

In conclusions, the use of IAC for clean-up followed by LC-FD has shown to be a technique with good analytical performance for the aflatoxins and OTA determination in beers. The application of this procedure to analysis 37 beer samples from Spanish markets has demonstrated that none of them contained levels above the European MRLs. Furthermore, the estimated daily intake of OTA has reflected to be rather small in comparison with other countries.

Acknowledgements

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V. RESUMEN DE RESULTADOS Y DISCUSIÓN

La producción de cacahuete en España prácticamente ha desaparecido frente a la competencia de los países tropicales, de los cuales se importan 32000 Tm anuales. En cuanto al consumo de cacahuete dentro del mercado nacional la Comunidad Valenciana ocupa el segundo lugar con 0.84 g por persona y día. Respecto a otros frutos secos cabe destacar en la Comunidad Valenciana la producción de almendra con 8000 Tm anuales (1).

En el caso del estudio sobre la ocratoxina A, se optó por el análisis de cereales y derivados, debido a que según diversos estudios podrían representar hasta el 50% de la ingesta total de ocratoxina A. El análisis de vino, porque la uva supone un buen medio para el desarrollo de diversas cepas ocratoxigénicas, y porque representa un sector agroalimentario en expansión, con proyección internacional y con un consumo elevado entre ciertos entornos sociales. Y el análisis de salsa de soja, porque la soja representa un alimento cuyo consumo está generalizándose dada la proyección mediática desarrollada en los últimos años y teniendo buena aceptación ya sea como tal o sus diversos derivados.

Los cereales son, después de la leche, el segundo producto agrario más importante en la UE, con una producción en España, estimada para 2004, de 23'3 millones de Tm (trigo, cebada, maíz y arroz) a la cual la Comunidad Valenciana aporta 181000 Tm. En el caso del arroz, Asia produce el 90% del total mundial y dentro de la UE, España es tras Italia el segundo productor con 0'9 Tm de arroz blanco, donde la Comunidad Valenciana es el cuarto productor nacional, en la cual debido a su gran tradición culinaria con este producto su consumo anual por persona es de 7'4 Kg, frente al 4'7 Kg de

media nacional, y además dispone de una denominación de origen protegida (1).

España produce 49 millones de HL de vino (vino de mesa 66'9%, vino de D.O. 26'3%, espumosos y cavas 3'9% y otros 2'8%), ocupando el sexto lugar a nivel europeo y mundial en consumo de vino situándose por encima de la media europea. La Comunidad Valenciana es el cuarto productor de vino de España con 2'7 millones de HL y tiene tres D.O. (Utiel-Requena, Valencia y Alicante) de reconocido prestigio. El consumo de vino se reparte en 18'8 L por persona y año de vino de mesa, 7'5 L de vino de D.O. (4'4 L de tinto, 1'6 L de blanco y 1'4 L de rosado) y 1'1 L de espumosos, champanes y cavas, con un total de 27'3 L por persona y año, donde la Comunidad Valenciana se encuentra en la media de consumo nacional (1).

La soja es la leguminosa más utilizada del mundo, tanto para alimentación humana como animal, y los principales productores son Asia y los EEUU. La UE tiene un gran déficit de proteína vegetal por lo que debe importar grandes cantidades de soja y torta de soja.

Para el estudio de la presencia simultánea de aflatoxinas y ocratoxina A, se consideró la cerveza como una matriz adecuada, debido a la idoneidad de la cebada, y de otros cereales utilizados en su producción como el trigo y el arroz, como sustrato para hongos micotoxigénicos. Además de la existencia de estudios que encuentran estas micotoxinas en la cerveza.

En el caso de la cerveza, España produce 31 millones de HL y ocupa el décimo puesto de la UE en cuanto a consumo, con 3´4 L por persona y año por encima de la media per cápita europea. El consumo de cerveza en la Comunidad Valenciana es de 16´6 L por persona y año, 1´6 L más que la media española (1).

V.1.2.Métodos analíticos

(a). Toma de muestras y preparación de la muestra.
(b). Procedimientos de extracción y purificación.
(c). Determinación por cromatografía líquida.
(d). Detectores.
(e). Determinación de aflatoxinas y ocratoxina A.

(a). Toma de muestras y preparación de la muestra

La toma de muestras para aflatoxinas queda legislada en la Unión Europea por la Directiva 98/53/CE y para ocratoxina A por la Directiva 2002/26/CE cuya última modificación la constituye la Directiva 2004/43/CE (2).

En la toma de muestras es importantísimo conseguir que la porción utilizada para el análisis sea representativa del total de la muestra que se pretende evaluar. Este es un paso crítico en la determinación, ya que la contaminación por micotoxinas es en extremo heterogénea dentro de la

muestra, requiriendo una homogeneización laboriosa con procesos de triturado y mezclado, con el agravante que supone tratar de evaluar grandes cantidades de alimentos.

La elección en muchos casos de unidades de comercialización, bolsas o botellas, de los productos alimenticios estudiados, generalmente con un peso determinado, simplifica el muestreo y el procesado, pese al sesgo que supone el no poder acceder a la muestra original, ni saber si la distribución previa al envasado supone una homogeneización de la muestra. También nos proporciona una visión más real que la de los productos a granel, ya que muchas de estas muestras están directamente al alcance del consumidor en ese formato.

(b). Procedimientos de extracción y purificación

Los métodos de extracción y purificación buscan la consecución de un extracto útil para la determinación, este extracto debe estar libre de compuestos que interfieran con en analito de interés.

En la Tabla V.1, podemos observar desde una perspectiva cualitativa como cada uno de los métodos desarrollados se adecua a diversos parámetros, que se pueden considerar favorables o desfavorables para el desarrollo total del método.

Tabla V.1: tabla cualitativa (de + a + + + +) de diversos parámetros de los métodos de extracción y purificación

Consideración	Favorable		Desfavorable		
Variable	**Idoneidad**	**Ecología**	**Laboriosidad**	**Interferencias**	**Gasto**
ESL	+ + +	+	+ + + +	+ +	+ + +
DMFS	+ +	+ +	+ + +	+ +	+ +
CI	+ + + +	+ + + +	+		+ + + +
ELISA	+ + +	+ +	+ +	+	+ + +

ESL: extracción sólido-líquido

DMFS: dispersión de matriz en fase sólida

CI: columnas de inmunoafinidad

ELISA: enzime linked inmunosorbent assay

En la comparación de los métodos de extracción y purificación cabe tener en cuenta diversas realidades, unas a las que se debe amoldar obligatoriamente por ser el paso previo a la determinación, como son la idoneidad a la matriz y al analito en cuestión, así como conseguir evitar las interferencias, y otras de carácter de gestión ya sea desde un punto de vista ecológico o económico en términos de tiempo o en materiales fungibles.

Desde esta perspectiva, la ESL cuenta con el respaldo de su profuso uso en el análisis de diversas micotoxinas con protocolos perfectamente validados, en contra, la laboriosidad del proceso y la elevada cantidad de disolventes orgánicos requeridos, lo que aumenta el coste y la contaminación medioambiental, y la incapacidad en algunos casos de evitar las interferencias de la matriz.

La DMFS es un método de reciente aplicación a micotoxinas, con unos resultados aceptables; disminuye el coste, pero también disminuye la sensibilidad al utilizarse una pequeña porción de muestra, aunque simplifica los pasos propios de la extracción y consigue reducir el gasto en disolventes; también en algunos casos las interferencias provocan problemas.

Aunque se dispone de una amplia bibliografía referente a la DMFS para su uso en el análisis de diversos contaminantes, no es así en el caso de las micotoxinas, donde el método desarrollado para AFTx supone la primera referencia a este respecto y el proceso similar llevado a cabo con OTA tampoco encontraba precedente en la bibliografía. La puesta a punto de este método de extracción aplicado AFTx, consiste en identificar el tipo y cantidad de fase sólida, la cantidad mínima de matriz para alcanzar sensibilidad suficiente y el volumen y tipo de disolvente para la elución (ver tablas IV.2, IV.3, IV.7, IV.8 y IV.9). Los ensayos de recuperación se llevan a cabo con muestras adicionadas a diferente rango de concentración en función de que sean destinadas a la realización de ensayos de optimización o bien al cálculo de la exactitud y reproducibilidad del método propuesto.

Las CI, tienen la especificidad propia de las reacciones antígeno-anticuerpo, siendo en extremo selectiva frente al analito, como su principal característica. Este hecho provoca que el proceso de extracción sea sencillo y utilice poca cantidad de disolventes contaminantes; además puede ser aplicada a muy diversos tipos de muestras con pocas variaciones en el proceso y evita la práctica totalidad de las interferencias, con ello se consigue el procesado de un

gran número de muestras en un periodo aceptable de tiempo. El único inconveniente de las CI es el elevado coste de este tipo de columnas, problemas de caducidad y los requerimientos para su transporte y conservación.

El método basado en la técnica de ELISA, pese a ser una técnica semicuantitativa, requiere de un proceso de extracción similar al utilizado en la ESL, con las ventajas e inconvenientes de esta, pero permite el paso a la determinación simultánea de un gran número de muestras con un considerable ahorro de tiempo, con una especificidad elevada si bien es posible observar reactividad cruzada. Este método es muy valorado como técnica de "screening" de muestras contaminadas, para descartar aquellas que no superan un cierto nivel de micotoxinas en análisis de rutina. Su principal desventaja es que no es una técnica suficientemente selectiva ni cualitativa, por lo que se requiere confirmación.

(c). Determinación por cromatografía líquida

La cromatografía líquida es una técnica de separación ampliamente utilizada en el análisis de aflatoxinas y ocratoxina A debido a que las características físico-químicas de estos compuestos se adecuan a esta técnica y a que permite la utilización de detectores sensibles para su determinación, ya sea por fluorescencia o espectrometría de masas.

Para establecer las condiciones cromatográficas de trabajo, se estudiaron diversas mezclas y flujos de disolventes que proporcionaban

cromatogramas con picos bien resueltos. En todos los casos se eligió una columna con un relleno de octildecilsílice de 5 μm de tamaño de partícula, por permitir la correcta separación de analitos e interferencias y por emplear fases acuo-orgánicas compatibles con los sistemas de detección.

Los cromatogramas obtenidos tras la adecuación de los parámetros cromatográficos, para aflatoxinas y ocratoxina A, dan lugar a picos perfectamente resueltos y simétricos, además de un tiempo total de análisis aceptable, resultando provechosos para la cuantificación de estos compuestos.

En la tabla V.2 se puede observar los parámetros cromátográficos utilizados en el análisis de cada una de las micotoxinas tanto en la utilización del detector de fluorescencia como en el uso del detector de espectrometría de masas.

La variabilidad observada en el tiempo de retención puede ser debida al deterioro de la columna cromatográfica por el uso y también puede ser debida a cambios en la temperatura ambiental o de la fase móvil.

Tabla V.2: Condiciones cromatográficas

Fluorescencia	**AFB1**	**AFG1**	**AFB2**	**AFG2**	**OTA**	
Eluyentes (%, v/v)	H_2O / AcN (75:25)				MeOH / HCOOH 0'1 M (70:30)	AcN/H_2O /HCOOH (50:40:1)
Flujo (ml/min)	0'7				0'4	0.5* / 1**
Tiempo de retención aprox (min)	5'8–9'0	4'6–6'5	14'5–23'0	10'3–16'5	7'0 – 8'5 (13'0 Me-OTA)	
Espectrometría de masas	AFB1	AFG1	AFB2	AFG2	OTA	
Eluyentes (%, v/v)	H_2O / MeOH (55:45)				MeOH / HCOOH 0'1 M (70:30)	
Flujo (ml/min)	0'7				0'4	
Tiempo de retención aprox (min)	16'5	9'8	13'0	8'0	8'5	
Columna cromatográfica (ambos detectores)	C18 150 x 4'6 mm				C18 150 x 3'9 mm	

(* estudio OTA en cerveza)

(** estudio OTA en vino y soja)

(d). Detectores

La mayoría de los métodos propuestos para el análisis de aflatoxinas y ocratoxina A se basan en la cromatografía líquida con detector de fluorescencia, debido a que estas moléculas presentan la propiedad de la fluorescencia.

En el caso de AFB1 y AFG1 se hace necesaria la derivatización para obtener una mejor respuesta del detector, se utiliza el ácido trifluoroacético para conseguir los hemiacetales de dichas moléculas con una emisión fluorescente mayor, esta es una técnica sencilla y que ha sido muy utilizada por diversos autores, incluso como técnica de confirmación, ya que frente a un positivo por AFB1 o AFG1, al realizar el mismo análisis exceptuando la derivatización disminuye considerablemente o incluso desaparece el pico correspondiente a esa aflatoxina. En el caso de AFB2 y AFG2 no es necesaria la derivatización ya que la respuesta fluorescente de estos compuestos es suficiente para su detección. Para OTA la derivatización, mediante un proceso de metilación por reacción con ácido clorhídrico en metanol, se emplea como método de confirmación, ya que el producto resultante, metil-OTA, presenta un tiempo de retención distinto.

El uso de la detección por fluorescencia hace necesario conocer las longitudes de onda de emisión y de excitación (Tabla V.3) para cada molécula en el eluyente a utilizar, lo cual se consigue realizando los espectros en un espectrofluorímetro. El detector de fluorescencia es muy sensible y selectivo, útil siempre que la extracción evite la aparición de interferencias, que son relativamente frecuentes en matrices tan complejas como son los alimentos.

Tabla V.3: Longitudes de onda de excitación y de emisión

	AFB1	AFG1	AFB2	AFG2	OTA	
λexc (nm)	365				333*	334
λem (nm)	435				460*	464

(* estudio OTA en cerveza)

La cromatografía líquida asociada a detector de espectrometría de masas presenta unas características útiles en la determinación de aflatoxinas y ocratoxina A, ya sea como tal, como procedimiento de confirmación de positivos o para resolver interferencias en fluorescencia. Su uso se está generalizando en el análisis de estas micotoxinas a pesar del elevado coste del equipo y de su mantenimiento respecto al detector de fluorescencia.

La espectrometría de masas necesita la optimización de los parámetros característicos de esta técnica, como son la interfase a utilizar y su polaridad, el voltaje del fragmentador y del capilar, presión, temperatura y flujo del gas. Tras esto se debe seleccionar la relación masa-carga (m/z) a utilizar en la determinación (Tabla V.4).

Tabla V.4: Parámetros de la espectrometría de masas

	AFB1	**AFG1**	**AFB2**	**AFG2**	**OTA**
Interfase	API/ES*				API/ES*
Polaridad	positiva				negativa
Fragmentador (V)	120				120
Capilar (V)	4000				3500
Presión (psi)	40				50
Temperatura (°C)	365				350
Flujo (l/min)	13				13
m/z	$[M+H]^+$ / $[M+Na]^+$				$[M-H]^-$ / $[M-H-CO_2]^-$
	313/335	329/351	315/337	331/353	402/358

(* atmospheric pressure ionisation / electrospray)

(e). Determinación de aflatoxinas y ocratoxina A

Tras los estudios llevados a cabo para la optimización de los procesos de extracción y de los parámetros de los métodos de detección por CL-DF, el conjunto de datos obtenidos refleja que la determinación conlleva una buena respuesta lineal para concentraciones de AFTx entre 0'05 y 20 ng/g y concentraciones de OTA entre 0'05 y 20 ng/g, así como una reproducibilidad adecuada con coeficientes de variación inferiores a 7% intradía y 12% interdía para AFTx y 7% intradía y 8% interdía para OTA.

Para demostrar la capacidad de la técnica a la hora de determinar residuos de estas micotoxinas en las distintas muestras, se realizan ensayos de recuperación sobre muestras adicionadas, con concentraciones resultantes comprendidas entre 0'25 y 10 ng/g o ng/ml. Las recuperaciones oscilan entre 78 y 99% con coeficientes de variación inferiores al 7% para AFTx y 84 y 92% con coeficientes de variación inferiores al 5% para OTA. Los límites de cuantificación (S/N 1:10) obtenidos con las diversas metodologías desarrolladas proporcionan valores entre 0'035 y 5 ng/g o ng/ml para AFTx y valores entre 0'030 y 0'75 ng/g o ng/ml para OTA, son lo suficientemente bajos para la utilización de estos métodos en el análisis de estos residuos en las diferentes muestras estudiadas, teniendo en cuenta los límites máximos de residuos legislados y los valores encontrados en otros estudios. Todos estos valores se encuentran dentro de los rangos reflejados en la reglamentación europea sobre métodos de análisis para micotoxinas. En los cromatogramas obtenidos se aprecia la selectividad de la metodología utilizada en cada caso

con una buena resolución de los picos y la ausencia de interferencias, salvo en casos puntuales donde se aplicaron las técnicas de confirmación.

En la siguientes tablas (Tabla V.5 y V.6) podemos observar todos estos parámetros con más detalle, para cada uno de los métodos utilizados tanto en el análisis de aflatoxinas como en el de ocratoxina A.

Tabla V.5: Resumen características métodos analíticos para AFTx

Método de Extracción (muestra)	Recuperación (%) (concentración del ensayo)	Variabilidad (%) (concentración del ensayo)	Linealidad (ng/g)	LC (ng/g o ng/ml)	Reglamentación
ESL (Cacahuete)	81 y 93 (10 ng/g)	Intra: 5 - 7 Inter: 9 - 10 (2'5-20'0 ng/g)	0'4 - 20'0	0'4 B1 0'7 B2 2'0 G1 4'0 G2	Valor: 1-10 ng/g R(%): 70-110 RSD_R(máx): 2xHorwitz
ELISA (Frutos secos Cereales Especias Legumbres Frutas secas)	(*)100 % selectividad B1 (Reactividad cruzada 20% B2 33% G1 2'3% G2)		0'1 - 1'5	0'1 B1	Valor: <1 ng/g R(%): 50-120 RSD_R(máx): 2xHorwitz
MSPD (Frutos secos Cereales Especias Legumbres Frutas secas)	78 y 86 (2'5 ng/g (5'0 ng/g G2))	Intra: 3 - 7 Inter: < 8 (2'5 (5 G2)-20'0 ng/g)	0'1 - 20'0	0'25 B1 0'25 B2 0'95 G1 5'0 G2	Valor: 1-10 ng/g R(%): 70-110 RSD_R(máx): 2xHorwitz
CI (Cerveza)	90 y 99 (0'25 ng/ml)	Intra: 2 - 3 Inter: 4 - 12 (0'25 ng/ml)	0'05 - 0'250 B1 y G1 0'125 - 0'500 B2 y G2	0'035 B1 0'045 B2 0'075 G1 0'120 G2	Valor: <1 ng/g R(%): 50-120 RSD_R(máx): 2xHorwitz

Tabla V.6: Resumen características métodos analíticos para OTA

Método de Extracción (muestra)	Recuperación (%) (concentración del ensayo)	Variabilidad (%) (concentración del ensayo)	Linealidad (ng/g o ng/ml)	LC (ng/g o ng/ml)	Reglamentación
C8-MeOH-H^+ (Cereales)	86 (10 ng/g)	Intradía: 3 - 7 Interdía: < 8 (3'0-10'0 ng/g)	0'25 - 20'0	0'75	Valor: 1-10 ng/g R(%): 70-110 $RSD_r \leq 20\%$ $RSD_R \leq 30\%$
CI (Cerveza)	84 (0'25 ng/ml)	Intradía: 2 - 3 Interdía: 4 - 12 (0'25 ng/g)	0'05 - 0'25	0'03	Valor:< 1 ng/g R(%): 50-120 $RSD_r \leq 40\%$ $RSD_R \leq 60\%$
CI (Vino)	92 (0'5 ng/ml)	Intradía: 3 - 7 Interdía: < 8 (0'25 ng/g)	0'05 - 5'00	0'05	
CI (Salsa soja)	90 (0'25 ng/ml)	Intradía: 2 - 4 Interdía: < 6 (0'25 ng/ml)	0'1 - 5'0	0'1	

(*) En el caso del ensayo de ELISA, los parámetros que nos dan una idea de la robustez del método serían la selectividad y la reactividad cruzada.

R (%): recuperación en tanto por cien.

RSD_r: desviación típica relativa, calculada a partir de los resultados obtenidos en condiciones de repetibilidad, (s_r / X) x 100, donde X representa la media de los resultados de todos los laboratorios y muestras.

RSD_R: desviación típica relativa, calculada a partir de los resultados obtenidos en condiciones de reproducibilidad, (s_R / X) x 100, donde X representa la media de los resultados de todos los laboratorios y muestras. La precisión RSD_R puede calcularse aplicando el coeficiente de 0'66 a la precisión RSD_r correspondiente a la concentración que presente interés.

Ecuación de Horwitz: $RSD_R = 2^{(1 - 0'5 \times \log C)}$

$RSD_R = (s_R / X) \times 100$

C: tasa de concentración, 1= 100g / 100g, 0'001= 1000 ng/kg,...

V.2. Aplicación a muestras reales

La aplicación de los diversos métodos analíticos desarrollados a muestras reales, nos permite conocer la exposición real de la población por vía alimentaría a estos tóxicos y comparar con la situación existente en otros países, a la vez que ayuda a diseñar estrategias de prevención y a evaluar cómo las acciones llevadas a cabo en cuanto a seguridad alimentaría y las acciones legislativas están siendo efectivas o en su defecto revisarlas para su mejora.

El número total de muestras analizadas asciende a 473 con una incidencia de positivos de 66 muestras, lo que equivale al 13'95%.

Para aflatoxinas (Tabla V.7), del total de 226 muestras analizadas, 11 (4'9%) presentan residuos. En una muestra de cacahuete se encuentran presentes las cuatro aflatoxinas y tanto el nivel de AFB1 como el de aflatoxinas totáles supera el nivel máximo legislado, es la única muestra donde se da este caso; en tres muestras, una de cerveza y dos de cacahuete, se encuentra únicamente AFB1; en una muestra de piñón y otra de cacahuete se encuentra únicamente AFG1, y en cinco muestras, tres de cacahuete, una de avellana y una de mezcla de frutos secos encontramos AFB1 y AFG1 simultáneamente. Cabe destacar la mayoritaria presencia de AFB1, acompañada en muchas ocasiones de AFG1, y la relativa ausencia de AFB2 y AFG2. En proporción, los frutos secos representan el 74% de las muestras analizadas pero aglutinan el 90% de los positivos, esto reafirma la susceptibilidad de este tipo de alimento a la contaminación por aflatoxinas.

Tabla V.7: Resultados para aflatoxinas

Muestra	Número de muestras	Incidencia	Rango (ng/g)	Tipo de muestra contaminada
Frutos secos	167	10 (6'0%)	0'13-38'3	Cacahuete (6) Mezcla frutos secos (1) Piñón (1) Avellana (1)
Cereales **Frutas desecadas** **Especias** **Legumbres**	22	0 (0%)	- - -	- - -
Cerveza	37	1 (2'7%)	0'059	Cerveza con alcohol (1)
Total	226	11 (4'9%)	0'059-38'3	- - -

En España se ha detectado la presencia de aflatoxinas en maíz (3), almendras, cacahuetes y pistachos (4). Así también, productos de origen español exportados a otros países han presentado contaminación, como el caso de cinco muestras de pimentón, de un total de 61 muestras de varios países, analizadas en los puertos ingleses, que dieron positivo a análisis de aflatoxinas y ocratoxina A (5). En este aspecto, un buen resumen sobre la presencia y el control de micotoxinas lo representa el Sistema de Alerta Rápida para Alimentos y Piensos de la Comunidad Europea (6), RASFF sus siglas en inglés, el RASFF recibe en 2004 un total de 5562 comunicaciones de las cuales

888 corresponden a micotoxinas, de estas el 95% son debidas a aflatoxinas, el 3% a ocratoxina A y el 2% a fumonisinas; en el caso de las aflatoxinas esto supone un aumento respecto a 2003 (763 notificaciones) y casi el triple que en 2002 (288 notificaciones). Las micotoxinas son responsables del 4% de las notificaciones de alerta, son las que suponen un riesgo y conlleva acciones inmediatas de las autoridades sanitarias, y del 44% de las notificaciones de información, que tienen más bien un carácter evaluador de un posible riesgo. La mayoría fueron debidas a pistachos, cacahuetes y derivados, avellanas, higo desecado y pimentón. España, como país productor de origen, es el segundo país europeo, tras Italia, en número de notificaciones generadas por sus productos, con tres notificaciones en el grupo de productos vegetales y especias.

Según diversos estudios la presencia de aflatoxinas en alimentos es variable, dependiente de las condiciones climatológicas y de las condiciones de cultivo, recolección, almacenaje y transporte. Así, según Patel et al (1996) (7) la mayor incidencia de aflatoxinas ocurre en nueces y semillas, especias, vegetales desecados, hierbas y plantas medicinales y cereales, mientras que para el estudio de Selim et al (1996) (8) este orden sería hierbas y plantas medicinales, cereales, especias, nueces y semillas y vegetales desecados

En el presente estudio la ocratoxina A (Tabla V.8) se detecta en 51 (18%) de un total de 284 muestras, siendo el vino, el alimento con una mayor proporción de muestras contaminadas, representa el 41´9 % del número total de muestras y el 88´2 % de los positivos, aunque el mayor nivel de contaminación

se presenta en cereales con un valor medio de 2'91 ng/g para las muestras contaminadas, esto nos da una idea del elevado peso de estos dos alimentos, considerando su consumo, en la ingesta de OTA. También cabe destacar la ausencia de OTA en las muestras de salsa de soja analizadas, pese a ser una matriz teóricamente idónea para la presencia de este tóxico.

Tabla V.8: Resultados para ocratoxina A

Muestra	Número de muestras	Incidencia	Rango (ng/g)	Tipo de muestra contaminada
Cereales	68	3 (4'4%)	1'51-5'35	Cereal desayuno (3)
Vino	119	45 (37'8%)	0'06-0'76	Vino dulce (8) Vino tinto (21) Vino rosado (12) Vino blanco (4)
Soja	60	0 (0%)	- - -	- - -
Cerveza	37	3 (8'1%)	0'036-0'039	Cerveza con alcohol (3)
Total	284	51 (18 %)	0'06-5'35	- - -

Según el estudio llevado a cabo en la UE, sobre 5180 muestras de cereales se obtuvo un 55 % de positivos con una media de 0'5 ng/g con una gran variación entre los datos obtenidos por los diversos países y según el tipo de cereal; de vino se analizaron 1470 muestras resultando un 59 % de positivos y una media de 0'357 ng/g; y en el caso de la cerveza fueron 496 muestras donde el 39 % contenían ocratoxina A en un valor medio de 0'030 ng/g (9).

Por otra parte, la búsqueda de la presencia simultánea de AFTx y OTA en cerveza dio un resultado negativo, pese a que se han descrito en la bibliografía, y se ha corroborado en nuestro estudio, la presencia no conjunta de ambas micotoxinas en muestras de este tipo.

El estudio de Scott y Lawrence (10) detectó aflatoxina B_1 en una cerveza de origen mejicano y Okoye y Ekpenyong (11) hicieron lo mismo en 32 de 40 cervezas de origen nigeriano. En el caso de la ocratoxina A Legarda y Burdaspal (12) detectan ocratoxina A en cerca del 100 % de las muestras de cerveza analizadas tanto españolas como de importación, sin embargo para el estudio de Visconti (13) este valor disminuye al 50 %.

Ingestas diarias

La ingesta de micotoxinas representa un grave problema de salud, por lo que estudiar esta ingesta hace posible cuantificar de alguna manera el problema, así como servir de punto de referencia ante la aplicación de medidas encaminadas a la protección de la salud pública frente al consumo de alimentos que contienen estos tóxicos.

En el caso de las aflatoxinas, debido a sus propiedades carcinógenas, no existe un posible valor de ingesta máxima admisible por lo que su ingesta debe ser tan baja como sea posible ante la imposibilidad de que sea nula, así fue asumido por la ONU, ya en 1987, después de los estudios llevados a cabo por el Comité de Expertos en Aditivos Alimentarios (14).

Para ocratoxina A, en el 37ª Certamen del JECFA (Joint FAO/WHO Expert Committe on Food Additives) de 1990 se observó la necesidad de una ingesta semanal tolerable provisional, en el 44ª Certamen en 1995 se fijó esta ingesta en 100 ng/kg/semana, y se reiteró esta cantidad en el 56ª Certamen en 2001 (15).

En los estudios llevados a cabo, el referente a la presencia de ocratoxina A en vino nos brinda la oportunidad para calcular un valor de ingesta de este tóxico, debido al gran número de muestras analizadas de un alimento que es considerado como uno de los que participa con un porcentaje elevado a la ingesta total de ocratoxina A. En este estudio el valor medio de ocratoxina A fue de 0'25 ng/ml, asumiendo que el consumo de vino en la Comunidad Valenciana es de 41'4 ml/dia (16) y un peso para adulto de 70 kg, la ingesta diaria estimada resulta ser 0'15 ng/kg/dia, un valor por debajo del obtenido en estudios realizados en Francia (2 ng/kg/día) (17), Grecia (3.7 ng/kg/día) (18), España (0.3 ng/kg/día) (19), Suecia (0.2 ng/kg/dia) (20) o Suiza (0.7 ng/kg/día) (21).

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CONCLUSIONES

Conclusiones

VI. CONCLUSIONES

1.- La DMFS, utilizando C_{18} como absorbente y acetonitrilo como eluyente, puede emplearse como técnica de extracción de aflatoxinas en cacahuetes y otros frutos secos con recuperaciones medias del 82 % y coeficientes de variación del 6 %, y es aplicable al análisis por CL con detectores de fluorescencia y EM.

2.- La DMFS aporta ventajas sobre la clásica extracción con disolventes propuesta por la AOAC, como son el empleo de pequeñas cantidades de muestra y de disolventes, mayor rapidez y economía, mejoras en la seguridad toxicológica del analista y disminución de los vertidos contaminantes.

3.- Como procedimiento de detección de aflatoxinas por CL puede utilizarse la EM con interfase de ES en modo positivo, aventajando a la fluorescencia en que no requiere derivatización y en su mayor selectividad, pero con la desventaja de ofrecer una menor sensibilidad.

4.- El ELISA es útil para el análisis de aflatoxina B_1 como procedimiento de *screening*, pero obligatoriamente requiere el empleo de la CL con detectores de fluorescencia o EM para la confirmación y cuantificación, a causa de las reacciones cruzadas y el efecto de matriz.

5. Las aflatoxinas se encuentran presentes en un 2'5 % de los cacahuetes importados, de los que algo menos del 1 % superan las concentraciones

máximas permitidas por la UE. También se han detectado en alrededor del 8 % de los frutos secos y en el 3% de las cervezas presentes en el mercado, sin superar los límites establecidos.

6.- La DMFS, utilizando C_8 como absorbente y metanol-ácido fórmico como eluyente, puede emplearse como técnica de extracción de ocratoxina A en cereales y derivados con recuperaciones medias del 86 % y coeficientes de variación del 5 %, y es aplicable al análisis por CL con detectores de fluorescencia y EM.

7.- Como procedimiento de detección de ocratoxina A por CL puede utilizarse la EM con interfase de ES en modo negativo con la ventaja frente a la fluorescencia de su mayor selectividad, haciendo innecesario realizar una nueva cromatografía tras la derivatización para la confirmación, si bien presenta la desventaja de una sensibilidad ligeradamente inferior.

8.- La DMFS como procedimiento de extracción y purificación de ocratoxina A en cereales y derivados aporta, frente a las columnas de inmunoafinidad, la ventaja de su mayor economía; y la desventaja de su ligeramente inferior sensibilidad y selectividad.

9.- La ocratoxina A se encuentra presente en un 7 % de los derivados de cereales analizados, de los que algo más del 2 % superan las concentraciones máximas permitidas por la UE. También se ha hallado en el 39 % de los vinos valencianos y en el 8 % de las cervezas comercializadas es España, si bien en

ninguno de ellos se superan los contenidos máximos permitidos. No se ha detectado la presencia de ocratoxina A en salsa de soja comercializada en España.

10.- La ingesta diaria estimada de ocratoxina A aportada por el consumo de vino es de 0'15 ng/kg de peso corporal/día, y la aportada por el consumo de cerveza es 0'02 ng/kg de peso corporal/día; lo que representa el 3'4 % de la ingesta diaria tolerable fijada en la UE.

Conclusiones