DEVELOPMENT OF LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF AFLATOXINS AND FUMONISINS IN FOOD AND FEEDS

By

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DEDICATION

To

My husband who taught me that even the largest task can be accomplished if it is done one step at a time and for his patience and sacrifice during my study.

My parents who taught me that the best kind of knowledge to have is that which is learned for its own sake.

My daughter for her patience.
My sisters, brothers, nephew, & niece.

For their love and encouragement during my study.

WIJDAN

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Last but not least, thanks to all of them and to everyone who helped me, albeit his/her help was simple.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFs	Aflatoxins
CE	Capillary electrophoresis
CNTs	Carbon nanotubes
DLLME	Dispersive liquid-liquid microextraction
DSDME	Directly-suspeneded droplet microextraction
DW	De-ionized water
ELISA	Enzyme linked immunosorbent assays
ESI	Electrospray ionization
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FLD	Fluorescence detector
FMs	Fumonisins
g	Gram
GC	Gas chromatography
HF	Hollow fiber
HF-LPME	Hollow-fiber liquid phase microextraction

HPLC	High performance liquid chromatography
IAC	Immunoaffinity column
L	Liter
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase microextraction
MeOH	Methanol
2-ME	2-mercaptoethanol
MFC	Multifunctional column
mg	Milligram
µg	Micro gram
min	Minute
MIP	Molecularly imprinted polymers
µL	Micro liter
mL	Milliliter
µ-SPE	Micro-solid phase extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
N.D	Not detected
ng	Nanogram
NPs	Nanoparticles

ODS	Octadecylsilane
OPA	*o*-phthaldialdehyde
r^2	Coefficient of determination
RSD	Relative standard deviation
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UPLC	Ultra-performance liquid chromatography
ZEA	Zearalenone

LIST OF SYMBOLS

C_s	Analyte concentration in the final extract
C_w	Analyte concentration in the original sample solution
EF	Enrichment factor
EE	Extraction efficiency
H	Height of theoretical plates
K	Retention factor
L	Column length
N	Number of theoretical plates
n_s	Analyte mass in the final extract
n_w	Analyte mass in the original sample solution
R_s	Column resolution
t_R	Retention time
V_s	Volume of the concentrated extract
V_w	Volume of the original sample solution
W	Peak width

PEMBANGUNAN KAEDAH KROMATOGRAFI CECAIR UNTUK PENENTUAN AFLATOKSIN DAN FUMONISIN DI DALAM MAKANAN DAN MAKANAN HAIWAN

ABSTRAK

Penyediaan sampel secara baru menggunakan turus fungsi pelbagai (MFC) dan pengekstrakan fasa pepejal mikro (µ-SPE) untuk kromatografi cecair prestasi tinggi (HPLC) bagi penentuan aflatoksin (AFs) B_1, B_2, G_1 dan G_2 diterangkan. Untuk kaedah MFC, sampel itu mula-mula diekstrak menggunakan asetonitril:air (90:10) dan dimurnikan lagi menggunakan langkah tunggal MFC. Syarat-syarat yang optimum untuk pemencilan dan pemisahan kromatografi (menggunakan pengesan pendarfluor FLD) disiasat. Selepas pengoptimuman, kaedah yang dibangunk telah digunakan untuk penentuan AFs dalam empat puluh dua sampel makanan haiwan yang merangkaumi jagung (16), kacang soya (8), makanan bercampur (13), bunga matahari, gandum, canola, isirung sawit, kopra (1 setiap satu). Satu kaedah µ-SPE di ikuti dengan pemisahan dan pengesanan LC-MS/MS telah berjaya membangunkan dan dioptimumkan untuk penentuan AFs dalam makanan. Keadaan optimum adalah: bahan penjerap,C8; jisim penjerap, 20 mg; masa pengektrakan, 90 minit; kelajuan pengacau, 1000 rpm; isipadu sampel, 10 mL; pelarut nyahjerap, asetonitril; isipadu pelarut, 350 µL dan tempoh ultrasonikasi, 25 minit tanpa tambahan garam. Di bawah keadaan optimum, faktor pengkayaan 11, 9, 9 dan 10 masing-masing untuk AFG_2, AFG_1, AFB_2 dan AFB_1, telah dicapai. Kelinearan baik dan pekali korelasi telah diperolehi pada julat kepekatan 0.5-50 ng g^{-1} (r^2= 0.9988-0.9999). Kaedah ini digunakan untuk analisis 20

sampel melibatkan minuman malt (9) dan kopi dalam tin (11). Tiada AFs dikesan dalam sampel yang dipilih. Kaedah HPLC-FLD fasa terbalik telah dibangunkan secara berasingan untuk penentuan AFs (B_1, B_2, G_1 dan G_2) dan fumonisin (FMs) (FB_1 dan FB_2) menggunakan silika monolitik. Keadaan optimum bagi pemisahan AFs adalah: komposisi fasa gerak, asetonitril:metanol:air (15:22:60, v/v); suhu turus, 30 ^oC; kadar aliran, 1 mL min^{-1} dan isipadu suntikan, 15 µL manakala bagi FMs ialah: komposisi fasa gerak metanol: penimbal fosfat (78:22, v/v); suhu turus, 30 ^oC; kadar aliran, 1 mL min^{-1} dan isipadu suntikan, 10 µL. Pemulihan AFs yang dipaku ke dalam sampel–sampel makanan adalah 86.38-104.5 % dan RSD adalah <4.4%. Kaedah ini digunakan untuk penentuan AFs dalam sampel kacang tanah (9), beras (5) dan cili (10). Paras AFB_1 dan AFs melebihi had undang-undang yang disyorkan oleh Kesatuan Eropah dalam sampel cili dan kacang. FMs mula-mula diekstrak menggunakan asetonitril:air (50:50, v/v), dibersihkan menggunakan pengekstrakan fasa pepejal C18 dan diterbitkan sebelum turus *o*-ftaldialdehid bersama 2-merkaptoetanol. Lima puluh tiga sampel dianalisis termasuk 39 makanan dan makanan haiwan, dan 14 jagung dan beras yang inokulasi. Hanya 12.8 % daripada sampel makanan dan makanan haiwan telah dicemari dengan FB_1 dan FB_2. Pengesahan positif sampel terpilih telah dijalankan menggunakan LC-MS/MS, membabitkan penganalisis kuadrupel tripel dan dikendalikan dalam mod pemantauan tindakbalas pelbagai. Kelebihan yang ketara pemisahan ini adalah pengurangan yang ketara dalam masa pemisahan berbanding dengan turus jenis zarah konvensional (3.7 berbanding 17 minit untuk pemisahan AFs dan 4 berbanding 20 minit untuk pemisahan FMs).

DEVELOPMENT OF LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF AFLATOXINS AND FUMONISINS IN FOOD AND FEEDS

ABSTRACT

New sample preparation methods (using multifunctional clean-up column (MFC) and micro-solid phase extraction (μ-SPE) for the high performance liquid chromatography (HPLC) determination of aflatoxins (AFs) B_1, B_2, G_1 and G_2 are described. For the MFC method, the samples were first extracted using acetonitrile:water (90:10) and further purified using a single step. Optimum conditions for the extraction and chromatographic separation (using fluorescence detector (FLD)) were investigated. After the optimization, the developed method was applied for the determination of AFs in forty two animal feeds samples comprising corn (16), soya bean meal (8), mixed meal (13), sunflower, and 1 sample each of wheat, canola, palm kernel, copra meals. A μ-SPE method followed by LC-MS/MS separation and detection was successfully developed and optimized for the determination of AFs in food. The optimum conditions were: sorbent material, C8; sorbent mass, 20 mg; extraction time, 90 min; stirring speed, 1000 rpm; sample volume, 10 mL; desorption solvent, acetonitrile; solvent volume, 350 μL and ultrsonication period, 25 min without salt addition. Under the optimum conditions, enrichment factor of 11, 9, 9 and 10 for AFG_2, AFG_1, AFB_2 and AFB_1, respectively were achieved. Good linearity and correlation coefficient was obtained over the concentration range of 0.5-50 ng g^{-1} (r^2 0.9988 - 0.9999). The method was applied to analysis 20 samples of malt beverage (9) and

canned coffee(11). No AFs were detected in the selected samples. Reversed-phase HPLC-FLD methods were independently developed for the determination of AFs (B_1, B_2, G_1 and G_2) and fumonisins (FMs) (FB_1 and FB_2) using monolithic silica based column. The optimized conditions for the separation of the AFs were: mobile phase composition of acetonitrile:methanol:water (15:25:60, v/v); column temperature, 30 ºC; flow rate, 1 mL min^{-1} and injection volume, 15 µL; while for FMs were: mobile phase composition of methanol:phosphate buffer (78:22, v/v); column temperature, 30 ºC; flow rate, 1 mL min^{-1} and injection volume, 10 µL. The recoveries of AFs that were spiked into food samples were 86.38-104.5% and RSDs were < 4.4%. The method was applied to the determination of AFs in peanut (9), rice (5) and chilli (10) samples. The levels of AFB_1 and total AFs levels exceed the legal limit established by the European Union in chilli and peanut samples. FMs were first extracted using acetonitrile:water (50:50, v/v), cleaned-up on a C18 solid phase extraction and were pre-column derivatized using *o*-phthaldialdehyde in the presence of 2-mercaptoethanol. Fifty three samples were analyzed including 39 food and feeds and 14 inoculated corn and rice. Only 12.8% of the food and feed samples were contaminated with FB_1 and FB_2. Positive confirmation of selected samples was carried out using LC-MS/MS, using triple quadrupole analyzer and operated in the multiple reaction monitoring mode. A significant advantage of these separations was the marked reduction in separation times compared to the conventional particle type column (3.7 vs 17 min for the AFs separation, and 4 vs 20 min for the FMs separation).

CHAPTER 1: INTRODUCTION

1.1. General overview

Mycotoxins are toxic low molecular weight metabolites (MW ~700) produced by fungi species, such as *Aspergillus, Fusarium,* and *Penicillum* that grow on a variety of agricultural commodities in humid and temperate conditions. The term mycotoxin is coined from the Greek word 'mykes' (meaning fungus) and the Latin word 'toxicum' (meaning toxic). Mycotoxins came to the forefront in 1960 when an outbreak of X-disease caused death to 100,000 turkey poultries. The reason behind the death was later attributed to the consumption of groundnuts that was infected by *Aspergillus (A.) flavus* and contaminated by aflatoxins (AFs). The ingestion of contaminated food, such as meat, eggs, milk and cheese by humans have been associated with several endemic diseases in Asia, Africa and Europe, such as the Kwarshiorkor and Reye's syndrome (damage to liver and kidney caused by AFs) and Balkan Endemic Nephropathy (tumors in the upper urinary tract caused by ochratoxin A) (Beltran *et al.*, 2009; Turner *et al.*, 2009).

Although more than 300 mycotoxins are known, only approximately 20 mycotoxins are expected to occur frequently in food and animal feedstuffs (Steyn, 1995). The common mycotoxins that contaminate food and feeds that are produced by different genera of fungi are: AFs, ochratoxin A (OTA), fumonisins (FMs), zearalenone (ZEA) and trichothecenes (Table 1.1). The chemical structure of mycotoxins varies

from the simple compound C4 (e.g. moniliformin) to the complex molecule (e.g. phomopsins and tremorgenicmycotoxins) (Zain, 2011).

Table 1.1 Commodities contaminated with mycotoxins and the relevant fungi (Paterson & Lima, 2010; Sherif *et al.*, 2009)

Mycotoxin	Fungus	Commodity
Aflatoxins	*Aspergillus flavus*, *Aspergillus parasiticus*	Peanuts, corn, wheat, cottonseed, copra, nuts, various foods, cheese, figs
Deoxynivalenol	*Fusarium culmorum*	Wheat, barley, corn
Ergo alkaloids	*Clavice pspurpurea*	Cereal grains
Fumonisins	*Fusarium verticillioides*	Feeds, corn, corn products
Ochratoxins	*Aspergillus flavus*, *Penicillium*	Cereal grain, dry beans, moldy peanuts, cheese, tissues of swine, coffee, raisins, grapes, dried fruits, wine, cocoa
Patulin	*Penicillium expansum*	Moldy feed, rotten apples, apple juice, wheat straw residue
Trichothecenes	*Fusarium sporotrichioides*, *Fusarium graminearum*	Corn, wheat, commercial cattle feed, mixed foods, barley, oats
Zearalenone	*Fusarium graminearum*	Corn, moldy hay, pelleted commercial food, water systems, oats, sorghum, sesame

The presence or production of mycotoxins in foods is largely dependent on physical, chemical, and biological factors (Figure 1.1). Chemical factors are contributed from the use of fungicides and/or fertilizers whereas physical factors include changes in climate conditions (e.g. temperature, humidity, drought, insect infection and rough handling) where all have been associated with fungal colonization and mycotoxins production.

The growth of each mycotoxin is dependent on climatic conditions. For example, hot and dry conditions are suitable for the production of AFs while hot and humid conditions are favorable to FMs formation. Cold and damp conditions are preferable to deoxynivalenol and trichothecenes production while temperate conditions are appropriate for patulin production. The occurrence of mycotoxins in the world is not limited to just hot or wet countries (Figure 1.2) (Paterson & Lima, 2010; Reddy *et al.*, 2010; Zain, 2011). Generally, it was found that the removal of mycotoxins from food during cooking or food preparation is not easy as it is thermally stable; however, mycotoxins content can be reduced (Cigic & Prosen, 2009).

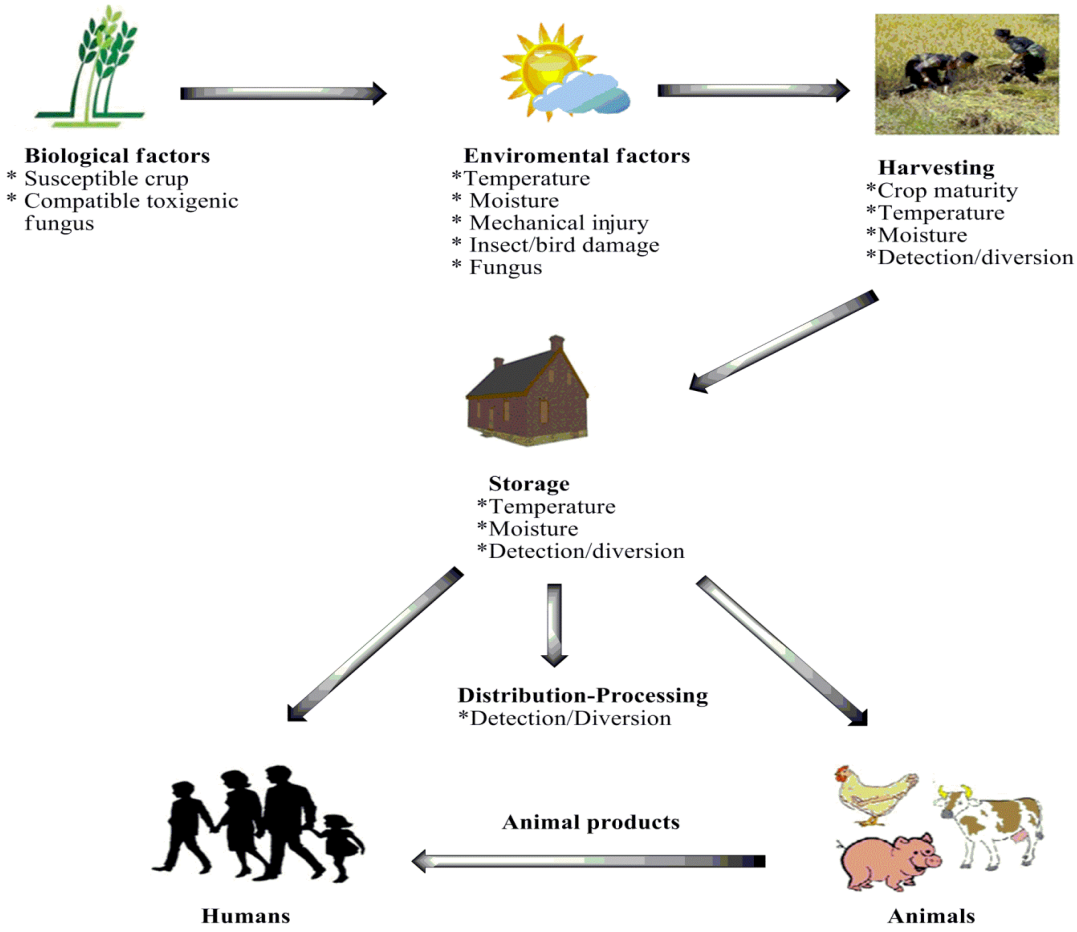
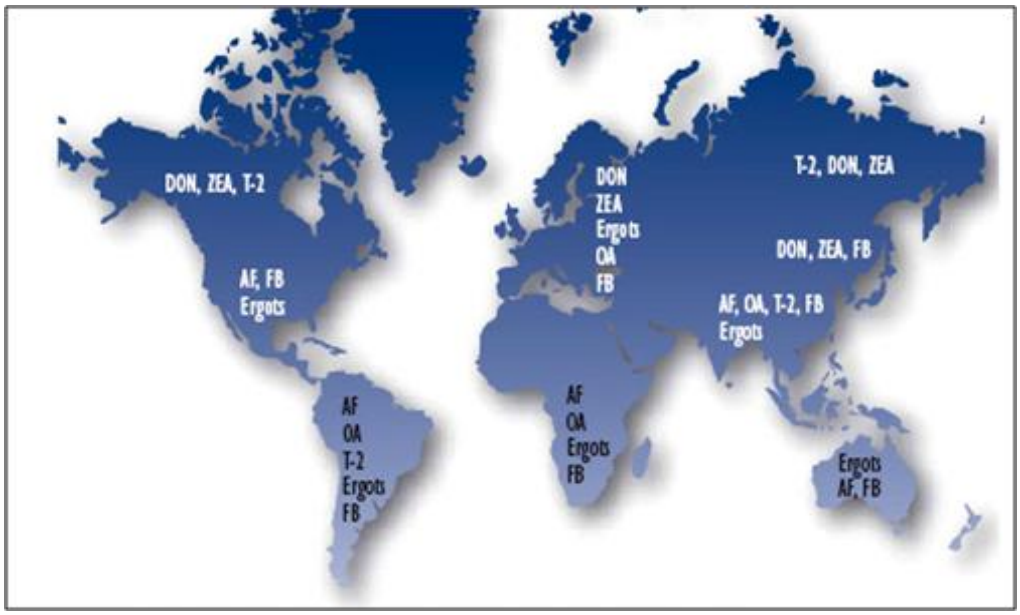


Figure 1.1 Factors affecting the presence of mycotoxins in foods and feeds (Paterson & Lima, 2010).

It was found that AFs, OTA, T-2 toxins, FMs and ergots mycotoxins are the most prevalent in feeds in South America while OTA was absent from the northern areas. In Africa and parts of Australia, feedstuffs are typically contaminated with AFs, FMs and ergots but AFs were not found in European countries. However, contaminated of feeds with DON, ZEA, T-2 toxin and FMs was found in some parts of Asia (Figure 1.2).



AF = aflatoxins, DON = deoxynivalenol, FB = fumonisins, OA = ochratoxinA, T-2 = T-2 toxin, ZEA = zearalenone

Figure 1.2 Global distributions of mycotoxins (Taylor-Pickard, 2009).

1.2. Toxic effects of mycotoxins on humans and animals

Table 1.2 explains the impact of the major mycotoxins on the health of humans and animals**.**

Table 1.2 Impact of the major mycotoxins on human's health and animals (Binder, 2007; Cigic & Prosen, 2009).

Mycotoxins	Major effects on humans' and animals' health
Aflatoxins (B_1, B_2, G_1, G_2)	Liver disease (hepatotoxic, hepatocarcinogenic, carcinogenic and teratogenic, mutagenic effects)
Citrinin	Hepatonephrotoxic, antifungal, antibiotic
Cyclopiazonic acid	Weight loss, nausea, diarrhea, giddiness, muscle necrosis, convulsions
Ergot alkaloids	Nervous or gangrenous syndromes
Fumonisins (B_1, B_2, B_3)	Liver and kidney tumors, oesophagel cancer, lung oedema (swine), leukoencephalomalacia (horses)
Ochratoxins (A, B, α)	Nephrotoxic, porcine nephropathic, mild liver damage, immune suppression
Patulin	Acutely toxic, genotoxic, carcinogenic, teratogenic, antibiotic
Trichothecenes (type A: T-2, H-2 toxin and Deoxynivalenol)	Immunologic effects, hematological changes, digestive disorders (emesis, diarrhea, reduced feed intake), dermal necrosis (poultry), hemorrhages of intestinal tissues, edema, weight loss
Zearalenone	Estrogenic effects, reproductive toxicity

1.3. Permissible limits in foods

Due to the adverse effects of mycotoxins, many countries have set maximum level for certain contaminants in foods. The latest amendments of the maximum levels of the important mycotoxins are listed in Table 1.3.

Table 1.3 Tolerance levels of mycotoxins in some foodstuffs.

Commodities	Maximum levels (µg kg^{-1})			References
Aflatoxins	AFB_1	AFs*	AFM_1	
Groundnuts (peanuts) and oil seeds and processed products intended for direct human consumption **	2.0	4.0	—	(European Commission, 2006b)
Dried fruits and processed products intended for direct human consumption **	2.0	4.0	—	(European Commission, 2010a)
All cereals and all products derived from cereals, including processed cereal products	2.0	4.0	—	
Dried fruits that are subjected to sorting or other physical treatment before human consumption**	5.0	10.0	—	
Maize and rice to be subjected to sorting or other physical treatment before human consumption**	5.0	10.0	—	
Certain species of spices (chilies, chili powder, cayenne and paprika, white and black pepper , nutmeg, ginger, turmeric)	5.0	10.0	—	
Almonds, pistachios and apricot kernels that are subjected to sorting, or other physical treatment before human consumption**	12.0	15.0		
Almonds, pistachios and apricot kernels intended for direct human consumption **	8.0	10.0		
Hazelnuts and Brazil nuts intended for direct human consumption **	5.0	10.0		
Raw milk, heat-treated milk and milk for the manufacture of milk-based products	___	___	0.05	
Infant milk	0.1		0.025	

*AFs = $B_1 + B_2 + G_1 + G_2$, ** or used as an ingredient in foodstuffs, AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1

Table 1.3 *Continued*

Commodities	Maximum levels (μg kg^{-1})	References
Ochratoxin A		
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3.0	(European Commission, 2006b, 2010b)
Dried vine fruit (currants, raisins and sultanas)	10.0	
Wine and grape juice	2.0	
Processed cereal-based foods and baby foods for infants and young children	0.5	
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0	
Soluble coffee (instant coffee)	10.0	
Spices (dried fruits thereof, whole or ground, including chillies, chilli powder, nutmeg, ginger, turmeric)cayenne and paprika, white and black pepper,	30 μg kg^{-1} as from 1.7.2010 until 30.6.2012 15 μg kg^{-1} as from 1.7.2012	
Deoxynivalenol		(European Commission, 2006b)
Cereals intended for direct human consumption, cereal flour, maize flour, maize meal and maize grits, pasta (dry),bran and germ	750	
Bread (small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500	
Processed cereal-based foods and baby foods for infants and young children	200	

Table 1.3 *Continued*

Commodities	Maximum levels (µg kg^{-1})	References
Patulin		
Fruit juices, concentrated fruit juices as reconstituted and fruit nectars	50.0	(European Commission, 2006b)
Sprit drinks, cider and other fermented drinks derived from apples or containing apple juice	50.0	
Apple juice and solid apple products, including apple compote and apple puree, for infants and young children	10.0	
Baby foods other than processed cereal-based foods for infants and young children	10.0	
Fumonisins	Sum of FB_1 and FB_2	
Maize snacks and maize based breakfast cereals	50	
Maize flour, maize meal, maize grits, maize germ and refined maize oil	1000	
Maize based foods for direct human consumption	400	
Processed maize-based foods and baby foods for infants and young children	200	
Zearalenone		
Cereals intended for direct human consumption and cereal flour bran	75	
Maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germ and refined maize oil.	200	
Bread (small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize based breakfast cereals	50	

1.4. Major groups of mycotoxins

1.4.1. Aflatoxins

The major AFs are aflatoxin B_1 (AFB_1), B_2 (AFB_2) (produced by *A. flavus* and *A. parasiticus*) G_1 (AFG_1) and G_2 (AFG_2) (produced by *A. parasiticus*) while M_1 (AFM_1) and M_2 (AFM_2) are the metabolic products of AFB_1 and AFB_2, respectively. The metabolites AFM_1 and AFM_2 are formed by cytochrome P4501A2 in humans and may be found in milk, urine, and feces obtained from livestock that have ingested contaminated food (Gurbay *et al.*, 2006). Hemiacetals, AFG_2a and AFB_2a are fluorescent products formed by derivatization of the weakly fluorescent aflatoxins AFB_1 and AFG_1 by using trifluoroacetic acid (TFA) (Akiyama *et al.*, 2001) (Figure 1.3). Other *Aspergillus* species, such as *A. bombycis, A. ochraceoroseus, A. nomius,* and *A. pseudotamari* are responsible for the production of other AFs. *A. flavus* is considered the most commonly contaminated fungi in agricultural commodities (Zain, 2011).

AFB_1 is classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002). On the other hand, AFs compound has been classified as possible carcinogens to humans. It has also been observed that the order of toxicity decreases in the following order: AFB_1> AFM_1> AFG_1> AFB_2> AFG_2 (Steyn, 1995). Wheat, sorghum, Brazil nuts, almonds, walnuts, pecans, dried fruits, legumes, peppers, potatoes, rice, copra, filberts, beer, medicinal herbs, meat products of animal feed, milk and milk products are reported to be infected by *Aspergillus* and contaminated with AFs (Lee *et al.*, 2004; Ventura *et al.*, 2004).

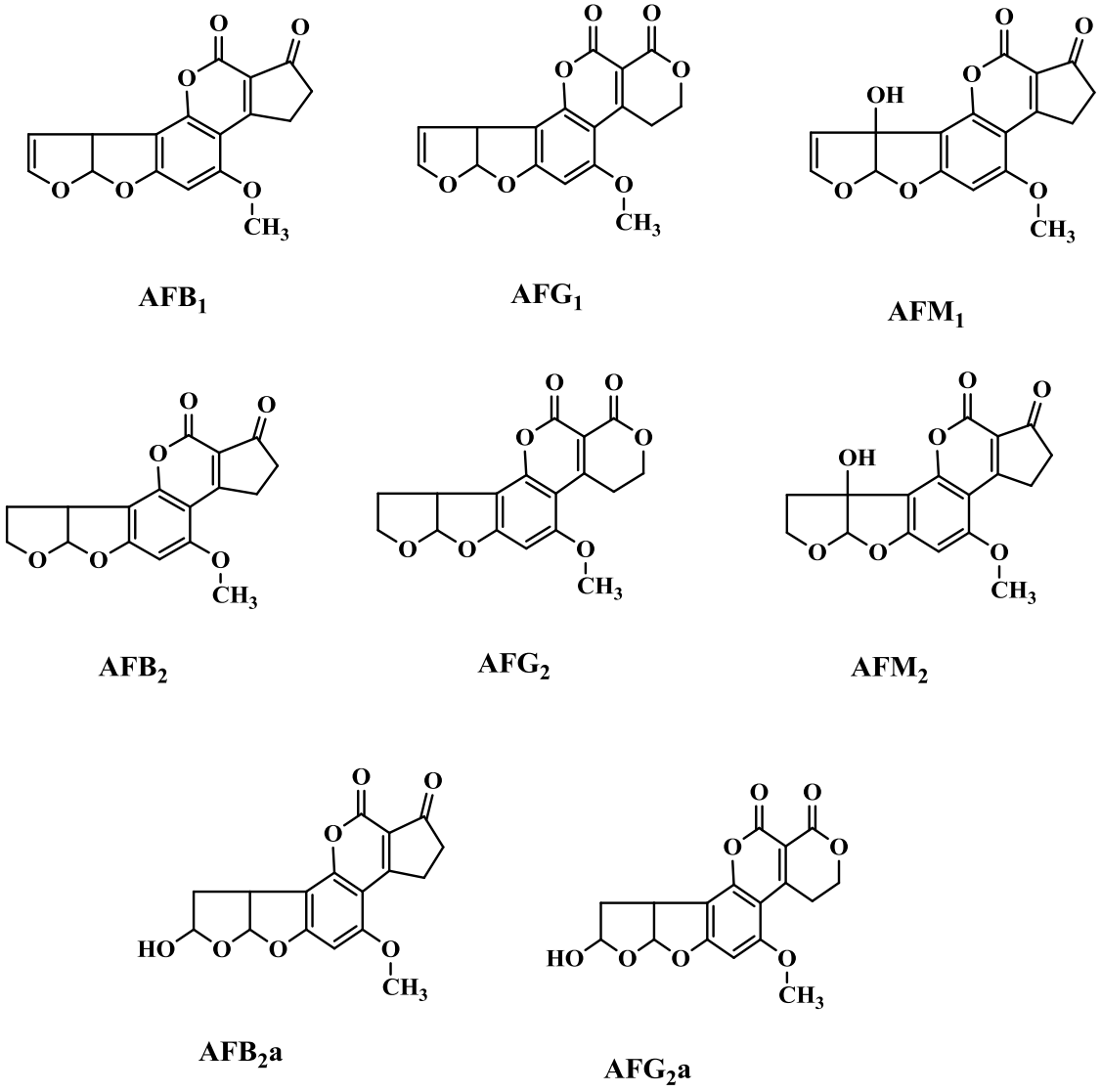


Figure 1.3 Chemical structures of AFs and their derivatives (Huang *et al.*, 2010).

1.4.2. Ochratoxins

Ochratoxins A, B and C (OTA, OTB and OTC) are secondary fungal metabolites produced by the genera of *Aspergillus* (e.g. *A. ochraceus*) and *Penicillium* (e.g. *P. verrucosum*) in semitropical and temperate climates. OTA is more toxic than OTB (without chlorine atom) and OTC (ethyl ester of OTA) (Figure 1.4) (Steyn, 1995; Zollner & Mayer-Helm, 2006). OTA was identified after the discovery of aflatoxins and was isolated from *Aspergillus ochraceus* (Sherif *et al.*, 2009).

Ingestion of food commodities that are contaminated with OTA (e.g., cereal and cereal products, fruits, coffee, cocoa beans, cassava flour, fish, milk, beer and wine) by animals and humans have been associated with many diseases. In animals, it led to the accumulation of OTA in pig blood and poultry tissue while in ruminants, OTA is rapidly metabolized to the non-toxic ochratoxin α and to 4- and 10-hydroxy OTA (Figure 1.4).

In humans, the consumption of food that is contaminated with OTA has been associated with tumors in the upper urinary tract, kidney disease and to the occurrence of Balkan endemic nephroropathy. Moreover, due to nephrotoxic, teratogenic and immunotoxic effects, the IARC has classified OTA as group 2B carcinogen (IARC, 2002). It was found that high levels of OTA are normally accompanied by a low or an absence of AFs and vice versa (Zain, 2011).

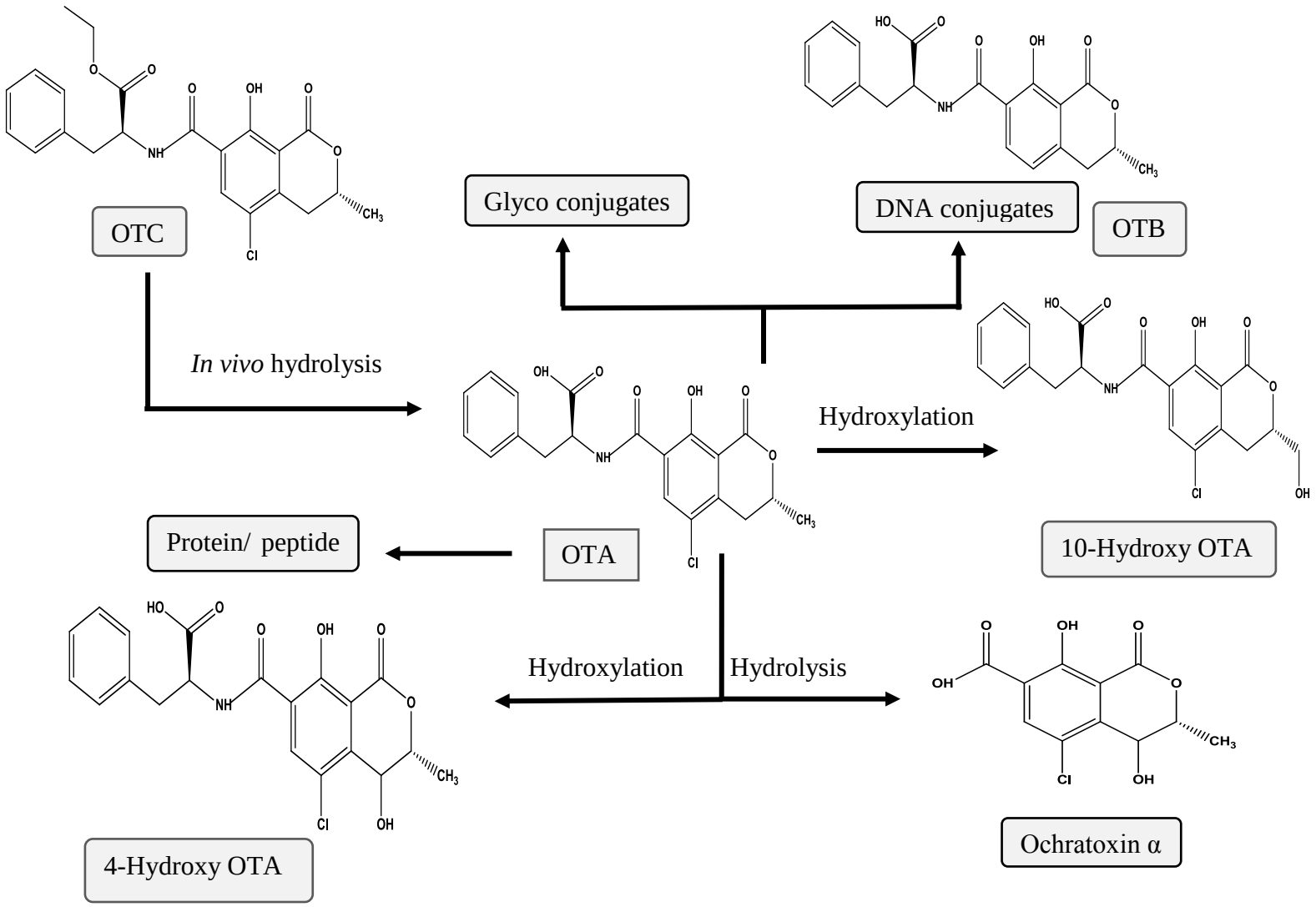


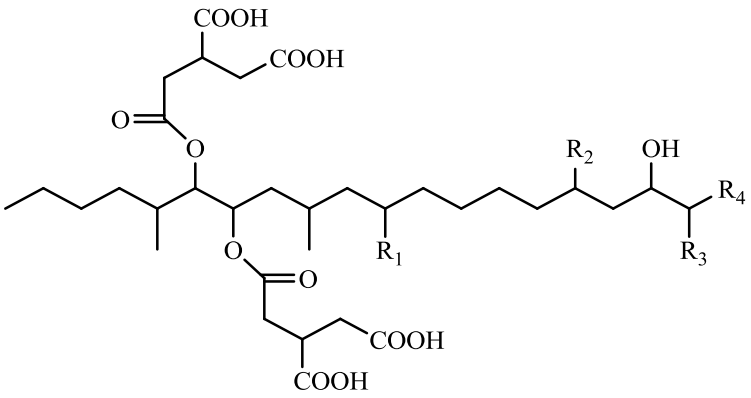
Figure 1.4 *In vivo* metabolism of ochratoxin A (Zollner & Mayer-Helm, 2006).

1.4.3. Fumonisins

Fumonisins are non-genotoxic carcinogens produced by *Fusarium (F.) moniliforme* (*F. verticillioides* and *F. proliferatum*) strains. They are classified into four major groups, A, B, C and P-series and are the most abundant analogs produced by *F. moniliforme.* These compounds are composed of related homologues that differ with regard to the presence or absence of hydroxyl group at C-5 and C-10 of the C-20 aminopentol backbone. On the other hand, group P-fumonisins can be characterized by the natural modification of N-linked 3-hydroxypyridine moiety with hydrolysis and oxidation of the ester group at position C-15. They are highly water-soluble as they lack aromatic-like structures that are common to all other mycotoxins (Figure 1.5) (Murphy *et al.*, 2006; Zollner & Mayer-Helm, 2006).

Among the 28 different fumonisin analogs that have been characterized, only the B-series fumonisins (B_1, B_2, B_3 and B_4) are the most abundant and fumonisin B_1 accounts for approximately 70% of the total fumonisins found in nature (Wang *et al.*, 2008a; Wang *et al.*, 2008b). Fumonisin B was first identified and characterized in 1988 (Bezuidenhout *et al.*, 1988). It occurs at high levels in corn and corn-based products, especially FB_1 followed by FB_2 (Silva *et al.*, 2009).

Based on the toxicological and carcinogenic effects on humans, the IARC has classified FB_1 to be a probable cause for human cancer (Group 2B) that is similar to OTA (Abramovic *et al.*, 2005; D'Arco *et al.*, 2008; Silva *et al.*, 2009).



Fumonisins	R_1	R_2	R_3	R_4
FA_1	OH	OH	CH_3CONH	CH_3
FA_2	H	OH	CH_3CONH	CH_3
FB_1	OH	OH	NH_2	CH_3
FB_2	H	OH	NH_2	CH_3
FB_3	OH	H	NH_2	CH_3
FB_4	H	H	NH_2	CH_3
FB_5	OH	H	NH_2	CH_3
FC_1	OH	OH	NH_2	H
FC_3	OH	H	NH_2	H
FC_4	H	H	NH_2	H
FD*	H	H	H	H
FP_1	OH	OH	3-hydropyridine	H
FP_2	H	OH	3-hydropyridine	H
FP_3	OH	H	3-hydropyridine	H

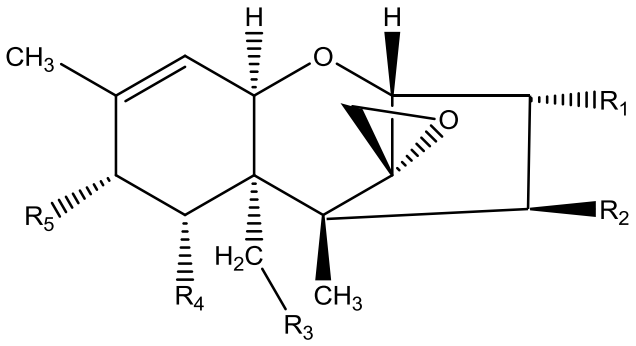
*Hydroxy group between R_1 and R_2 replaced by hydrogen atom

Figure 1.5 Chemical structure of fumonisins group (Zollner & Mayer-Helm, 2006).

1.4.4. Trichothecenes

Trichothecenes are a group of more than 200 structurally related sesquiterpenoid metabolites produced by a number of fungal genera such as; *Fusarium, Myrothecium, Phomopsis, Stachybotrys, Trichoderma,* and *Trichothecium*. Structurally, they contain tetracyclic, sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system. They are divided into four main groups: group A (without a carbonyl group at position C-8); group B (with a carbonyl group at C-8) (Figure 1.6); group C (with a second epoxy group) and group D (with a macrocyclic structure) (Steyn, 1995; Zollner & Mayer-Helm, 2006).

Types A and B trichothecenes occur in grains, such as maize, oats, barley and wheat while types C and D (more toxic) trichothecenes occur only rarely in foods. Toxins, such as T-2 and HT-2 are subscribed as type A whereas deoxynivalenol (DON) and nivalenol (NIV) are subscribed as type B trichothecenes. The toxic effects of trichothecenes in human and animals include anorexia, gastroenteritis, emesis and hematological disorder (Sherif *et al.*, 2009; Zollner & Mayer-Helm, 2006).



Trichothecenes	R_1	R_2	R_3	R_4	R_5
Type A					
T-2	O-isovaleryl	H	O-acetyl	OH	OH
HT-2	O-isovaleryl	H	O-acetyl	O-acetyl	OH
Type B					
Desoxynivalenol(DON)	=O	OH	OH	OH	OH
3-Acetyl DON	=O	OH	OH	H	OH
Fusarenon X	=O	OH	OH	H	O-acetyl
Nivalenol(NIV)	=O	OH	O-acetyl	H	OH

Figure 1.6 Chemical structures of type A and B trichothecenes (Zollner & Mayer-Helm, 2006).

1.5. Other Mycotoxins

1.5.1. Zearalenone

Zearalenone (ZEA) is produced by *Fusarium* species (*F. graminearum* or *F. culmorum*) that has estrogenic and anabolic properties in farm animals such as cattle, sheep and pigs. It is a non-steroidal compound that is known as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone (Figure 1.7) (Richard, 2007; Zain, 2011).

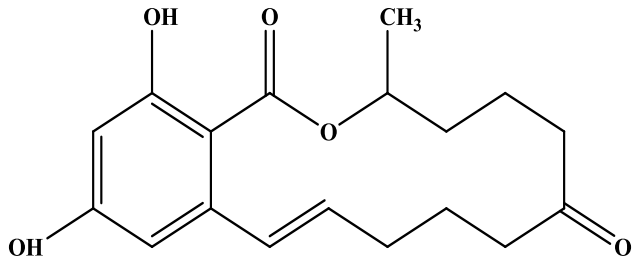


Figure 1.7 Chemical structure of zearalenone (Zain, 2011).

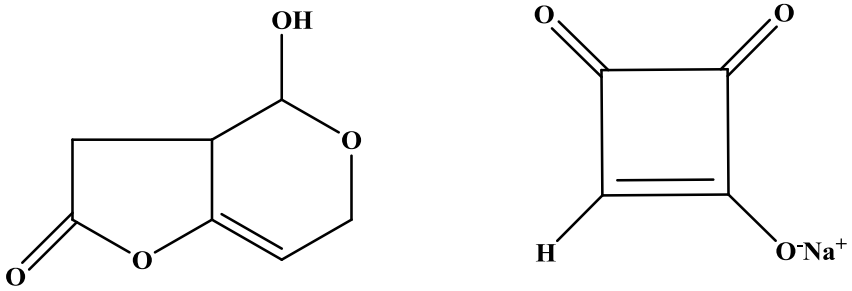
ZEA is commonly found in corn, wheat, barley, oats, sorghum and sesame in moist cool conditions. Due to estrogenic activity, ZEA can conjugate with estrogen receptors, leading to hormonal changes. The latter help to stimulate the growth of human breast cancer cells which contain estrogen response receptors and increase the plasma level of cholesterol and triglyceride in female and hyperestrogenism in children (due to the occurrence of ZEA in milk and milk products during feeding livestock with food containing ZEA). It is of low acute toxicity in mice, rats and guinea pigs. It is also known as immunotoxic and genotoxic agent that can induce DNA-adduct formation (Manes & Zinedine, 2009; Sherif *et al.*, 2009)

1.5.2. Moniliformin

Moniliformin (MON) is produced mainly by *Fusarium proliferatum* growing on corn kernel. It is a potassium or sodium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Figure 1.8). In animals, MON causes some pathological affects i.e. myocardial degeneration and necrosis, while in humans MON was associated with Keshan disease (myocardial impairment) that was reported in China and South Africa (Zain, 2011; Zollner & Mayer-Helm, 2006)

1.5.3. Patulin

Patulin (PAT) is 4-hydroxy-4H-furo[3,2c] pyran-2(6H)-one (Figure 1.8) that is produced by different fungi species including some of *Penicillium* (such as *P. clavifome*, *P. expansum* and *P. patulum*), some *Aspergillus* (including *A. clavatus, A. terreus* and others) and *Byssochlamys* (*B. nivea* and *B. fulva)* in apple juice, moldy bread, sausage, fruit (bananas, pears, pineapple, grapes and peaches) and cider. It was identified and isolated during 1940 from *Penicillium patulum* (Reddy *et al.*, 2010). The effects of PAT include the inhibition of DNA synthesis, direct effects on plasma membrane, cellular glutathione level and mitochondrial function. According to immunosuppressive and carcinogenic properties of PAT, the IARC concluded that no evaluation could be made on the carcinogenicity to animals (Sherif *et al.*, 2009).



Patulin Moniliformin

Figure 1.8 Chemical structures of patulin and moniliformin (Reddy *et al.*, 2010; Zain, 2011).

1.6. Detoxification of mycotoxins

Due to the toxic and carcinogenic effects of mycotoxins, different methods have been recommended to reduce or eliminate these toxic compounds from contaminated food. Such methods include the following: biological methods e.g., an nontoxigenic strains of *A. flavus* and *A. parasiticus* or other nontoxicgenic moulds, chemical methods such as treatment with ammonia, sodium bisulfate, calcium hydroxide, formaldehyde, and physical methods (e.g., extraction, adsorption, heating and radiation) (Jalili *et al.*, 2010). Another approach to reduce bioavailability of the mycotoxins involves the use of adsorbent materials with the capacity to tightly bind and immobilize toxins in the gastrointestinal tract of animals (Soriano & Dragacci, 2004).

Oxidation by ozone has been used for the detoxification of AFs in foods. The action of ozone involves the formation of primary ozonides and then their rearrangement into monozonide derivatives (e.g. aldehyde, ketone, and organic acid). The ozonation process occurs at the 8,9 double bond of the furan ring of AFs during the electrophilic attack (Inan *et al.*, 2007).

Gamma ray treatment was applied to reduce mycotoxins in spices (Jalili *et al.*, 2010). Some microorganisms, such as lactic acid bacteria (LAB) are widely used for degradation toxic compounds available in foods, such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, amino acid pyrolysates, hydrocarbons and mycotoxins. LAB was applied to detoxify OTA, AFs and PAT from food (Fuchs *et al.*, 2008; Topcu *et al.*, 2010).

Treatment with ammonia is an example of a chemical method used for the detoxification of AFs in food. The mechanism action of ammonia on the AFB_1 includes the destruction of lactone carbonylic ring and the formation of non-fluorescent phenol known as aflatoxin D_1 (AFD_1) (Figure 1.9) (Piva *et al.*, 1995).

Extrusion cooking of cereal products, adding nutritionally inert sorbents, or using *Rhizopus* strains (i.e. *R. stolonifer*, *R. oryzae*, and *R. microsporus*) are the most common approaches that have been reported to reduce ZEA toxicity (Zinedine *et al.*, 2007b).

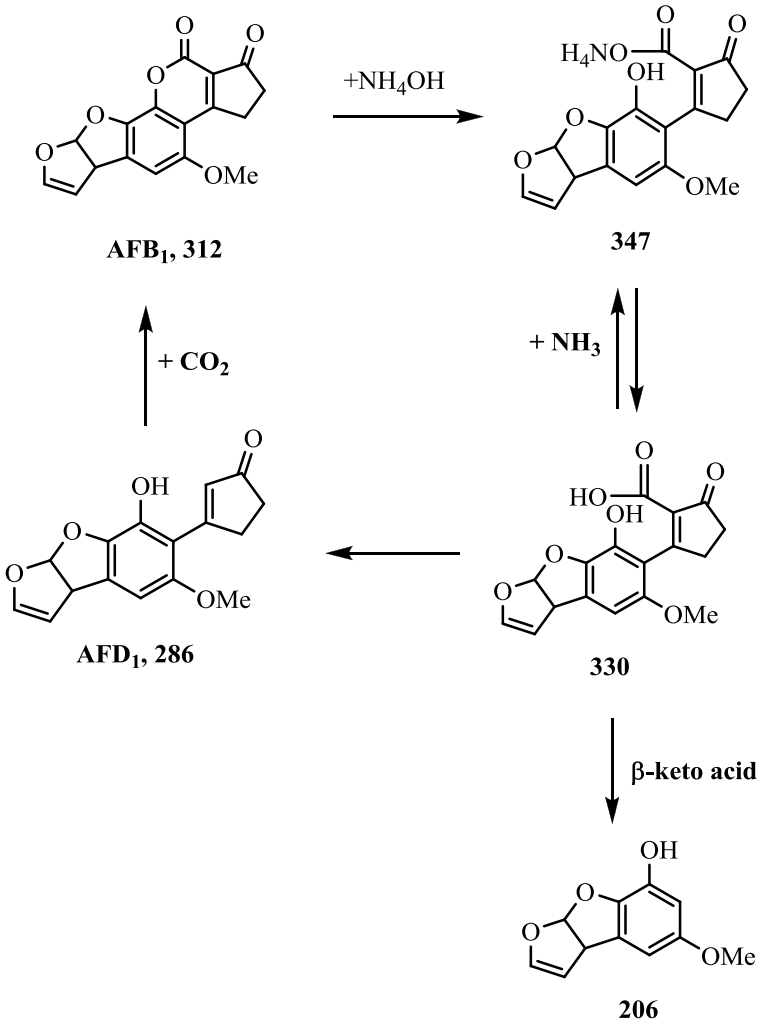


Figure 1.9 Ammoniation of AFs (Piva *et al.*, 1995).

1.7. Analytical determination of mycotoxins

Due to the contamination of agricultural commodities with mycotoxins at low levels, sensitive and reliable methods are required for their detection. The determination of mycotoxins is affected by various factors, such as the complexity of the sample matrix, the level of the analyte and the heterogeneity of mycotoxins in the sample (Krska *et al.*, 2005). The major steps in the analysis include sampling, sample preparation, extraction, clean-up/pre-concentration (e.g. liquid-liquid extraction, solid phase extraction, supercritical fluid extraction and microextraction techniques) and finally the analytical determination. The latter is done by using one of the conventional analytical techniques such as thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and liquid chromatography (LC). Immunological methods such as enzyme-linked immune assay (ELISA), direct fluorimetry, fluorescence polarization, biosensors and screening methods (e.g., using strips) are also used. The LC methods are used with different detectors, such as UV, fluorescence (FLD), mass spectrometric (MS) and evaporative light scattering detectors (ELSD) (Fernandez-Cruz *et al.*, 2010).

1.7.1. Sampling and sample preparation

Sampling and sample preparation are one of the most crucial steps for the qualitative and quantitative determination of mycotoxins. This is due to the heterogeneous distribution of mycotoxins in agricultural commodities. Organizations such as the Food Agricultural Organization (FAO, 2004), the Food and Drug

Administration (FDA, 2010), UK Food Standard Agency (Food Standards Agency, 2007), and the European Commission (EC) have reported different methods of sampling. In Commission Regulation No. 401/2006 (European Commission, 2006a), the EC has laid down the methods of sampling and the official analysis of various mycotoxins in foodstuffs. It involves sampling methods for the analysis of AFs, OTA, PAT and *Fusarium* toxins in different commodities i.e. cereal, dried fruit, fruit juices, wine, groundnuts and nuts, spices, milk and coffee, baby foods and food intended for children and young children.

AFs are usually distributed heterogeneously with large particle size in some food products (e.g., in dried figs or groundnuts). In general, some commodities have low number of contaminated particles, but the level of contamination of these particles is very high. Thus, to obtain similar representativeness for batches of food products with large particle sizes, the sample weight should be larger than when the batches are with smaller particular size (Krska *et al.*, 2008). Large quantity of samples reduces the sample error, but at the same time it is not easy to get a homogenized sample.

Dry grinding and slurry mixing are important steps for sample preparation. Dry milling is favored as it is fast, easy to apply and able to hold the sample till 4 Kg. It is especially suitable for samples of high oil content or butter of 10 Kg (Spanjer *et al.*, 2006). Slurry mixing is another way to obtain finer particles and a more uniform particle distribution. It can avoid clogging of samples with high oil content. It is considered to be time consuming when preparing and cleaning the equipment.

Moreover, it can handle samples till 10 Kg with a lower coefficient of variation (Velasco & Morris, 1976).

1.7.2. Sample extraction

Analytical determination of mycotoxins requires a suitable solvent to isolate the mycotoxins from the sample matrix. Extraction from food matrices is affected by different parameters, such as the polarity of metabolites, solvent type, composition of solvent mixture, solvent to sample ratio, type of matrix (e.g., processed or unprocessed), extraction method, physical aggregating of the sample, and the type of clean-up used afterwards (Shephard, 2008).

Polar solvents or mixture of solvents (e.g., acetonitrile (ACN), methanol (MeOH), chloroform, ethyl acetate, methanol:water (MeOH:DW), acetonitrile:water (ACN:DW), hexane-acetonitrile, dichloromethane-ethanol mixtures) are most frequently applied. For example, the extraction of AFs from food matrices was carried out by using chloroform which have been used in the previous methods and has since been replaced by aqueous mixtures of polar organic solvents (Papp *et al.*, 2002). The mixtures, ACN:DW or MeOH:DW at different ratios have been used for extracting type-A and type-B trichothecenes from grain (Royer *et al.*, 2004). In the case of ZEA, a mixture of ACN:DW, ethyl acetate, MeOH, chloroform has been reported to extract from cereals, grains and animal tissues (muscle) (Jodlbauer *et al.*, 2000; Kleinova *et al.*, 2002; Zollner *et al.*, 2002), Extracting FMs from food matrices has been achieved by using a mixture of ACN:DW or MeOH:DW (Shephard, 1998).