REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DETECTION OF MYCOTOXIN LEVELS IN ANIMAL FEEDS AND RAW MILK SAMPLES BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Mohamed FATHI ABDALLAH

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This is to clarify that this study is full adequate in scope and quality as a thesis for the degree of Master of Sciences in the Programme of Pharmaceutical Toxicology.

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APPROVAL:

In the opinion of the above Examining Committee Members appointed by the Executive Council of the Institute of Health Sciences, this thesis was found to satisfy all the requirements as a thesis for the degree of Master of Sciences and therefore is accepted at the meeting of the Institute Executive Council of Health Sciences.

Prof. Dr. Diclehan ORHAN

Director of the Institute

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Thank you!
Mohamed
5th April, 2016

ABSTRACT

ABDALLAH, M.F. Detection of Mycotoxins Levels in Animal Feeds and Raw Milk Samples by Using High Performance Liquid Chromatography and Liquid Chromatography Tandem Mass Spectrometry. Hacettepe University Institute of Health Sciences, M.Sc. Thesis in Pharmaceutical Toxicology. Ankara, 2016. Mycotoxins, secondary toxic metabolites produced by several fungi, have a serious threat on human and animal health. Beside their health impact, mycotoxins represent a considerable economic obstacle. In the last few decades, a trend for multi-target methods for simultaneous assessment of mycotoxins has been greatly developed. These methods decrease the cost of analysis and save the time in comparison with single run analysis. The aim of the present thesis was to detect the natural co-occurrence of several fungal and bacterial metabolites in maize and feed in addition to aflatoxin M_1 (AFM_1) in raw milk provided from Upper Egypt using the state-of-the-art methodology such as liquid chromatography tandem mass spectrophotometry (LC-MS/MS) and high performance liquid chromatography equipped with florescence detector (HPLC-FLD) with post-column photochemical derivatization and immunoaffinity columns. The results showed the contamination with 115 fungal and bacterial metabolites in maize and feed together and AFM_1 in raw milk samples. This work is considered the first survey in Egypt covering a broad spectrum of fungal toxic metabolites including the traditional mycotoxins, emerging and modified mycotoxins. The obtained data can be used with the future surveys to establish a database for mycotoxin contamination levels in Egypt in order to minimize the possible health risks of mycotoxins.

Key words: Mycotoxins, immunoaffinity column, aflatoxin, fumonisins, zearalenone, deoxynivalenol, maize, animal feed, milk, emerging mycotoxins, HPLC-FLD, LC-MS/MS.

The thesis was partially supported by Hacettepe University Scientific Research Projects Coordination Unite (014 D06 301 002-620).

ÖZET

ABDALLAH, M.F. Hayvan Yem ve Çig Süt Örneklerinde Mikotoksin Düzeylerinin Yüksek Performansli Sıvı Kromatografisi ve Sıvı Kromatografisi Tandem Kütle Spektrometrisi Kullanılarak Belirlenmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Programı Yüksek Lisans Tezi. Ankara, 2016. Mikotoksinler, insan ve hayvan sağlığı üzerine ciddi bir tehdit oluşturan, çeşitli küf mantarları/küfler tarafından üretilen ikincil toksik metabolitlerdir. Sağlık üzerine etkilerinin yanı sıra önemli bir ekonomik engel oluşturmaktadır. Geçtiğimiz yıllarda mikotoksinlerin eşzamanlı değerlendirmesinde çoklu hedeflendirme yöntemlerinde önemli gelişmeler kaydedilmiştir. Bu yöntemler analiz sayısını azaltmakta ve tekli ölçüm yapılan analizlere göre daha fazla zaman kazandırmaktadırlar. Sunulan tez çalışmasının esas amacı, Yukarı Mısır bölgesinden toplanan çiğ süt örneklerinde aflatoksin M_1 (AFM_1) saptanmasının yanı sıra mısır ve yem örneklerinde doğal olarak oluşan küf ve bakteri kaynaklı metabolitlerin sıvı kromatografisi-kütle spektrofotometri (LC-MS/MS), immünoafinite kolon ve kolon sonu fotokimyasal dedektör bulunan floresans dedektörlü yüksek performanslı sıvı kromatografisi (HPLC-FLD) gibi gelişmiş teknolojik yöntemler kullanarak belirlemektir. Elde edilen sonuçlara göre mısır ve yem örneklerinde toplam 115 metabolit ile çiğ süt örneklerinde AFM_1 kontaminasyonu olduğu tespit edilmiştir. Bu araştırma mısır ve yemlerde oluşan bilinen, yeni görülen ya da değişikliğe uğramış mikotoksinleri içerecek şekilde küf kaynaklı toksik metabolitleri kapsayan, Mısır'dan sağlanmış örneklerde yapılmış geniş ölçekli ilk çalışmadır. Elde edilen veriler, gelecek araştırmalar ile birlikte Mısır'da mikotoksin kaynaklı sağlık risklerini en aza indirmek amacıyla hazırlanacak, mikotoksin kontaminasyon düzeylerini gösteren bir veri tabanı oluşturulmasında kullanılabilir.

Anahtar kelimeler: Mikotoksinler, immünoafinite kolon, aflatoksin, fumonisinler, zearalenon, deoksinivalenol, mısır, hayvan yemi, süt, yeni görülen mikotoksinler, HPLC-FLD, LC-MS/MS.

Bu tez kısmen Hacettepe Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından desteklenmiştir (014 D06 301 002-620).

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ABBREVIATIONS

15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivalenol
ACN	Acetonitrile
AFBO	AFB_1-8, 9-epoxide
AFs	Aflatoxins
AOZ	Aflatoxins, ochratoxin A, zearalenone
DON	Deoxynivalenol
DON-3-G	Deoxynivalenol-3-glucoside
EU	European Union
ELISA	Enzyme-linked immunosorbent assay
ENs	Enniatins
ESI	Electrospray ionisation
FAO	Food and Agriculture Organization
FBs	Fumonisins
FB_1	Fumonisin B_1
FLD	Fluorescence detector
HAc	Glacial acetic acid
HFB_1	Hydrolysed fumonisin B_1
HPLC	High performance liquid chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
LC-MS	Liquid chromatography / mass spectrometry
LC-MS/MS	Liquid chromatography / tandem mass spectrometry
m/z	Mass-to-charge ratio
NIV	Nivalenol
LOD	Limit of detection
LOQ	Limit of quantitation
OTA	Ochratoxin A

PBS	Phosphate buffered saline
PCD	Post-column derivatisation
Phe	Phenylalanine
QqQ	Triple quadrupole
RP	Reversed-phase
RT	Room temperature
sMRM	Scheduled multiple reaction monitoring
S/N ratio	Signal-to-noise ratio
SPE	Solid-phase extraction
T-2	T-2 toxins
TLC	Thin layer chromatography
UV	Ultraviolet
ZEN-14-G	Zearalenone 14-glucoside
ZEN	Zearalenone
α-ZEL	Alpha-zearalenol
β-ZEL	Beta-zearalenol

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1. INTRODUCTION AND AIM

The direct mycotoxin contamination in food commodities or the indirect one through the carry-over from contaminated feed into animal products to be consumed by human still remains an area of concern. Several outbreaks have been reported in human and animals after the consumption of mycotoxin-contaminated stuffs. Mycotoxin production and/or contamination in agricultural products can take place at different stages in food and feed chain: pre-harvest, during harvest and post-harvest under favorable conditions (temperature, moisture, water activity, relative humidity) (1 - 3). Moulds rarely grow uniformly throughout a commodity and a mycotoxin will not have an even distribution. Mycotoxins are commonly present in nuts, dried fruits, coffee, cocoa, spices, oil seed, dried beans, maize, wheat, and several other cereals. Not only food, but also animal feeds and animal products such as milk, cheese, yogurt, egg and meat are important sources for exposure (4, 5). The knowledge that mycotoxins have serious impacts on humans, animals and worldwide economy has also led to the establishment of regulations on mycotoxin levels in food and feed commodities. More than 100 countries had developed specific maximum limits by the end of 2003, representing approximately 87% of world population. However, still the majority of African countries have no specific mycotoxins regulations.

It is essential for food and feed safety to screen the commodities for a multitude of mycotoxins. On the one hand several fungi can co-colonise a given agricultural commodity, on the other hand fungi are able to produce several mycotoxins. Co-occurrence influences the overall toxicity because the toxicological effects of different mycotoxins might be additive, synergistic or antagonistic.

The scope of this thesis is the determination of a broad spectrum of mycotoxins and other fungal and bacterial metabolites occur naturally in animal feed and maize samples as well as aflatoxin M_1 (AFM_1) in raw milk samples in Upper Egypt using two analytical techniques applied for modern mycotoxins detection and quantification. The

structure of the presented work is divided into three main parts inside each chapter, from which one discussion and conclusion are drawn.

First objective was the determination of six of the most significant mycotoxins in the term of toxicity and incidence, AFB_1, AFB_2, AFG_1, AFG_2, ochratoxin A and zearalenone (AOZ abbreviation will be used to describe the six mycotoxins) in two different matrixes, commercial animal feed and maize in Assiut city in Upper Egypt. In addition, AFM_1 in raw dairy milk samples sold in shops for human consumption was also determined. The used chromatographic instrument was high performance liquid chromatography equipped with florescence detector (HPLC-FLD) for separation and quantitative determination with clean up step by using immunoaffinity cartridges (IACs) for better extraction of the target analytes from the matrix. An on-line post-column photochemical derivatization (PCD) has been equipped for sensitive multi-target analytes detection in feed and maize. The determination has been done at Pharmaceutical Toxicology Department, Hacettepe University, Republic of Turkey.

In continuation of our interest on the determination of more different mycotoxins in maize and feed commodities, different samples were collected from three different neighboring governorates in Upper Egypt, Minya, Assiut and Sohage. The samples were screened for broad spectrum of fungal and bacterial metabolites including the major traditional mycotoxins such as aflatoxins, ochratoxin A, zearalenone, and fumonisins, emerging mycotoxins such as fusaproliferin, beauvericin, enniatins and moniliformin, and the modified or masked fungal metabolites such as deoxynivalenol-3-glucoside and zearalenone 14-glucoside using the state-of-the-art methodology, high performance liquid chromatography tandem mass spectrophotometry (LC-MS/MS) which is the most advanced tools as the detection relays on the mass-to-charge ratio (m/z) of the target of interest using electromagnetic fields. The LC-MS/MS survey has been conducted at Center for Analytical Chemistry, Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences (BOKU), Austria.

2. LITERATURE REVIEW

2.1 Definitions and historical aspects

Mycotoxins are secondary toxic metabolites produced by more than 200 recognized filamentous fungi growing under a wide range of climatic conditions on different agricultural stuffs. They are named secondary as these metabolites are considered non-essential for fungal growth and/or reproduction. However, not all fungal metabolites are classified as toxic substances to human and animals (6, 7). The term mycotoxin is coming from the Greek word where fungus means “mykes” and the Latin word “toxicum” for poison.

All mycotoxins are low-molecular-weight natural products (MW ~700 Dalton) and the exact reason for the importance of mycotoxin production for fungi themselves is still unclear. A number of fungal genera, mainly *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps* and others have been found to produce around 300-400 toxic substances (8, 9). Some fungi have the ability of producing more than one mycotoxin and some mycotoxins can be produced by more than one mould species. Moreover, the multi-mycotoxin contamination commonly occurs in the nature (10, 11).

To date, however, above 300-400 of different mycotoxins have been discovered, a few of them represent significant food safety challenges. Extensive research on mycotoxins dates back to their discovery in 1960s when more than 100,000 turkey poults died due to an evidently new disease that swept through England. This mysterious phenomenon was called “turkey X disease”. Shortly, the fatalities were not only restricted to turkeys, but also extended to include young pheasants and ducklings. After performing a survey to detect the possible reasons, the main cause was linked to bird feeds contained peanut meal imported from Brazil. More investigations showed that those meals were heavily contaminated with unknown toxic agent and might be of fungal origin. Later on, the toxin producing fungus was identified as *Aspergillus flavus* (12, 13).

The diversity of mycotoxin structures induces several various toxic effects on mammals, poultry, and fish. Some are carcinogenic, mutagenic, teratogenic, nephrotoxic, hepatotoxic, estrogenic, hemorrhagic, neurotoxic, immunotoxic and dermotoxic. Table 2.1 shows some major mycotoxins and their mainly producing moulds. (14-17).

2.2 Major mycotoxins

2.2.1 Aflatoxins (AFs)

Aflatoxins (AFs) are difuranocoumarins produced mainly by two *Aspergillus species*, *A. flavus* and *A. parasiticus*. AFs nomenclature is composed of two syllables, the first one "a" was derived from the genes *Aspergillus*, the second one, "fla", from the species flavus and the term, "toxin", came from the adjective "toxic". Owing to their chemical structures (Figure 2.1 and 2.2), there are two main categories of AFs; the first category is difurocoumarocyclopentenone group which includes aflatoxins B_1, B_2 (AFB_1, AFB_2) and aflatoxins M_1, M_2 (AFM_1, AFM_2). The second category is difurocoumarolactone group which involves aflatoxins G_1 (AFG_1) and G_2 (AFG_2). The letters "B" and "G" refer to the blue or green fluorescence emit upon exposure of the mycotoxin to ultraviolet light. The four major naturally occurring aflatoxins (B_1, B_2, G_1 and G_2) are ubiquitous in animal feed stuffs.

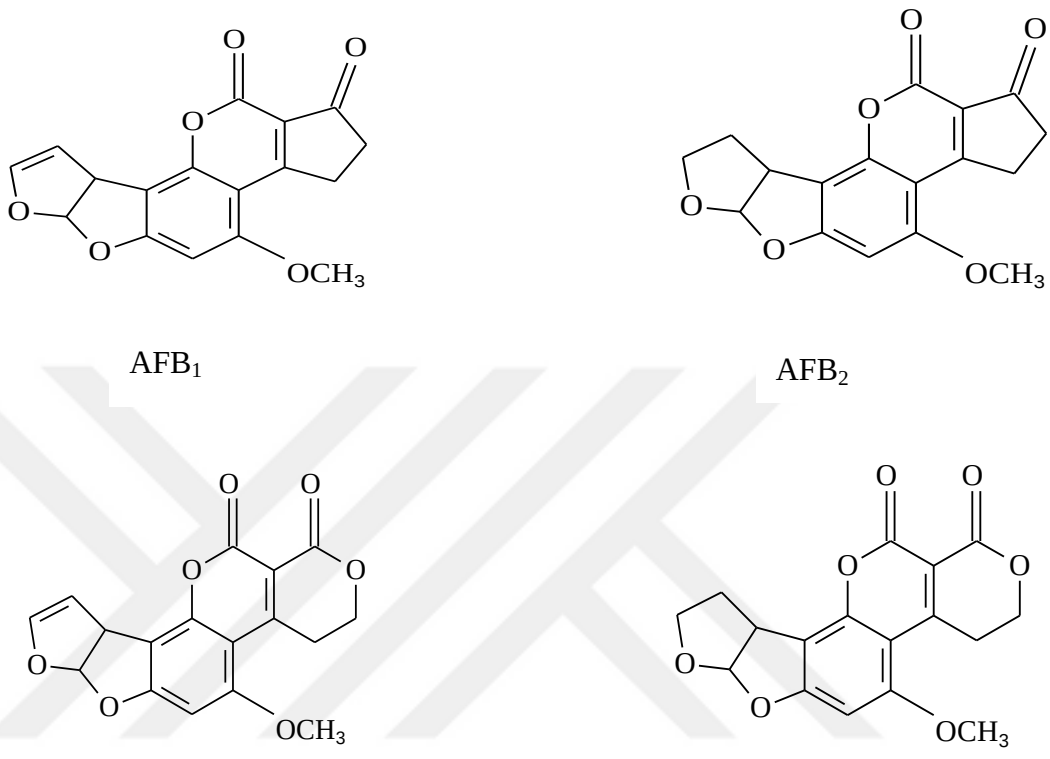


Figure 2.1. Chemical structures of major aflatoxins

Aflatoxins contaminate many commodities including peanuts, rice, maize, cottonseed, almonds, spices, and figs (11, 18 - 20). Among all discovered mycotoxins, AFs are by far the most intensively researched group due to their potent acute toxicological impacts and chronic hepatocarcinogenic effects in various susceptible animals.

Consumption of AFs contaminated agricultural stuffs is the main route of exposure. The liver is considered the main target organ for AFs toxicity including carcinogenicity. After ingestion, cytochrome P450 enzyme system in liver convert AFB_1 to the major carcinogenic metabolite AFB_1-8, 9-epoxide (AFBO) or to the major

monohydroxylated derivative AFM_1, and other metabolic products such as aflatoxicol, aflatoxin Q_1, and aflatoxin P_1 depending on the genetic predisposition of the species. The main CYP enzymes involved in AFs metabolism are CYP3A4, CYP3A5, CYP3A7, and CYP1A2. Evidences showed that the CYP1A2 enzyme is responsible for the conversion to aflatoxin M_1 (AFM_1) while CYP3A4 causes the production of an epoxide of AFB_1.

The produced unstable epoxide, AFBO, reacts with DNA guanine moiety of hepatocytes to form covalent bonds at the N-7 guanine residue leading to depurination (17, 21, 22). The reaction ends with gene mutation and cancer. Although, the liver is the primary target organ, under certain conditions, lung, kidney, and colon may be also affected. Adverse effects of AFs are anorexia, decreased feed intake and immune system suppression in both animals and humans. Immunosuppressive, hepatotoxic, carcinogenic, mutagenic, and teratogenic effects can be observed according to animal species, sex, age and AFs exposure level (type, dose and period) (6, 11, 23 - 25).

Poultry are more sensitive to AFs than mammals. In poultry, ducks are the most susceptible species then turkey poults and then chicken while in domestic animals; the order is canine, swine, calves, cattle and sheep. AFs play a role in hepatocellular carcinoma synergistically with hepatitis B or C virus. It has been claimed that the metabolism of AFB_1 changes in persons infected with hepatitis A and the incidence of hepatic carcinoma is elevated. Upon hepatitis A immunization, the rate of liver cancer cases may decrease.

The highest percentage of hepatocellular carcinoma incidences occurs in parts with frequent exposure to contaminated food and high rate of infection with hepatitis as Eastern and South Eastern Asia and Middle and Western Africa. AFs act as immune modulators, causing suppression of resistance to secondary infections. They can also affect testes and sperm quality which leads to infertility (20, 25, 26).

2.2.2 Aflatoxin M_1 (AFM_1)

Aflatoxin M_1 is the hydrolyzed metabolic form of AFB_1 in which ‘‘M’’ refers to milk of dairy animal that consume feed contaminated by AFB_1. Like all other aflatoxins members, it is a highly oxygenated heterocyclic compound (Figure 2.2).

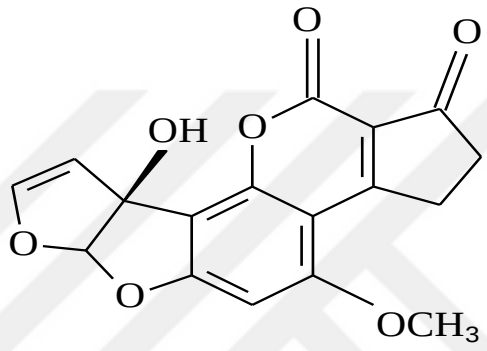


Figure 2.2. Chemical structure of aflatoxin M_1

Within 12 to 72 hours after the consumption of aflatoxin B_1 contaminated feed, AFM_1 can be detected in milk. It has been claimed that around 5% of digested AFB_1 is converted and excreted into milk of dairy animals. However, the excreted AFM_1 in milk is varying from animal to animal and from day to day in addition to a seasonal variation due to the nature of feed in summer and winter. AFM_1 was reported in pasteurized milk, ultra-high-treated milk, milk powder, some other milk-based products (cheese and yogurt), eggs, and meat.

Metabolization of AFB_1 to AFM_1 is considered a kind of detoxification process hence AFM_1 is acutely hepatotoxic as AFB_1, but its carcinogenicity is one tenth of the parent compound (18, 22, 27, 28). The International Agency of Research on Cancer (IARC) has classified AFB_1 as a human carcinogen, Group 1 while AFM_1 is categorized as possible human carcinogen; Group 2B as its carcinogenicity is 10 times less than the parent compound (29). Some previous reports detected AFM_1 in cereal grains (30, 31).

The presence of this metabolite might be derived from insects metabolizing AFB_1 contaminated grains (32, 33).

2.2.3 Ochratoxin A (OTA)

Ochratoxin A (OTA) is the most commonly encountered and toxic metabolite of ochratoxin group which includes three members, A, B and C (OTA, OTB, OTC). OTA is a fluorescent secondary metabolites produced by two genera of fungi, *Aspergillus* and *Penicillium*. The main OTA producing species are *A. ochraceus, A. carbonarius, A. melleus, A. sclerotiorum, P. verrucosum,* and *P. nordicum*. OTA nomenclature is derived from *A. ochraceus*, the first discovered fungus produces the toxin (13, 20, 34).

OTA has been detected in a wide range of different animal feed ingredients such as barley, maize, wheat and oats. The toxin is a frequent natural contaminant in food including various cereals and cereal products, coffee, spices, bean as well as dried fruits, grapes and grape based products such as wine. Moreover, edible animal tissues and milk have been reported to be contaminated with OTA (35, 36).

Because of the structural similarity to phenylalanine (Phe) (Figure 2.3), OTA inhibits all biological processes involving Phe, particularly Phe-tRNA synthetase and this leads to inhibition of protein synthesis as well as DNA and RNA (6, 25, 37, 38).

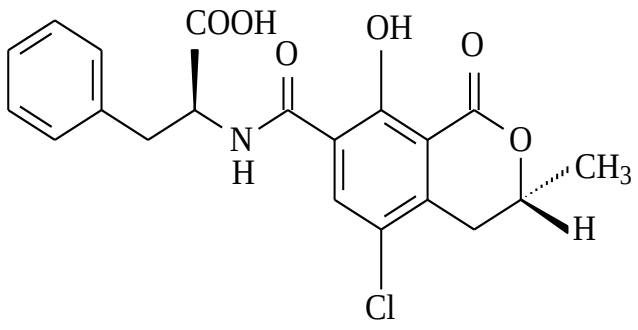


Figure 2.3. Chemical structure of ochratoxin A

OTA is a carcinogenic, genotoxic, immunotoxic and potent nephrotoxic agent. The main target organ in OTA toxicity is kidney. In Balkan countries, OTA causes high incidence of urinary tract carcinoma known as Balkan Endemic Nephropathy and urothelial tumors. Even though OTA is primarily acting on renal system, at high concentrations, it affects many organs including liver and brain manifested by multifocal hemorrhages (15, 39, 40).

In domestic animals, chronic renal failure is the main effect accompanied with OTA toxicity. OTA causes obvious immunosuppression with atrophy of immune organs, changes in number of immune cells and function, and malformation of cytokine production. Monogastric animals such as dogs and pigs are more susceptible to ochratoxicoses than chickens while ruminants are more resistant (16, 34).

2.2.4 Zearalenone (ZEN)

Zearalenone (ZEN) is a mycotoxin with hyperestrogenic effects produced by *Fusaria* mainly *F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F. crookwellense* and *F. semitectum*. They are frequent contaminants of several cereal crops worldwide and common soil fungi. Maize is mostly contaminated with ZEN producing moulds, but also other crops such as wheat, oats, barley, and rye throughout various countries are found to be invaded by these fungi.

Furthermore, milk contamination by ZEN and its metabolite has been reported (36, 41). Structurally (Figure 2.4), ZEN resembles 17β–estradiol and classified as non-steroidal oestrogenic mycotoxin. ZEN is metabolized into two diastereoisomeric zearalanols, α-zearalanol (α-ZEL) and β-zearalanol (β-ZEL). It has been found that α-zearalanol is three times more estrogenic than ZEN itself probably due to a greater binding affinity to estrogen receptors.

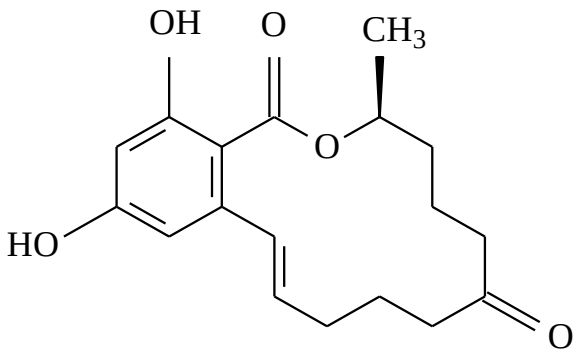


Figure 2.4. Chemical structure of zearalenone

Reproductive problems in domestic farm animals are the frequent disorders occur up on ZEN exposure. Several outbreaks of oestrogenic syndromes in farm animals have been reported. Although it is not common, in humans hyperoestrogenic syndromes can be observed (5, 11, 25, 42).

Beside the estrogenic effect, also hepatotoxic, haematotoxic, immunotoxic and genotoxic implications have been documented. Animal susceptibility shows a variation according to different factors such as species, sex, age, and reproductive state. Swine are the most susceptible farm animals to reproductive effects of ZEN. Prepubertal gilts show a hyperestrogenism, enlargement of the mammary glands while mature sows exhibit nymphomania and pseudopregnancy. Castrated males may develop enlargement of the prepuce and nipples. Immature boars demonstrate reduced or loss of libido and testicular atrophy. Ruminants may exhibit some adverse effects, reduced fertility and repeated breeding but generally are of low clinical incidence. ZEN is excreted into milk of pigs and cows as a result of exposure to high doses in feed (13, 39).

In humans, toxicity is mainly chronic in which ZEN and its metabolites can effectively stimulate mammary gland cells growth. Thus, it was suggested that ZEN may be implicated in breast cancer. There are some reported cases of precocious puberty in adolescent girls with ZEN exposure (42, 43).

2.2.5 Fumonisins (FBs)

Fumonisins (FBs) are a group of non-fluorescent hydrophilic mycotoxins, mainly produced by *Fusarium moniliforme, F. proliferatum, F. napiforme, F. dlamini* and *F. nygamai*. Maize and maize based animal feeds are mostly infected with fumonisins producing moulds, particularly when maize is imported from humid climates (39, 44, 45).

FBs contain a long hydroxylated hydrocarbon chain with a methyl and either acetyl amino or amino groups as shown in Figure 2.5. Fumonisins B_1, B_2, and B_3 are the most significant toxins among more than 28 fumonisin analogues. FBs are cancer-inducing toxins due to its similarity with sphinganine and sphingosine, the main constituent of sphingolipids.

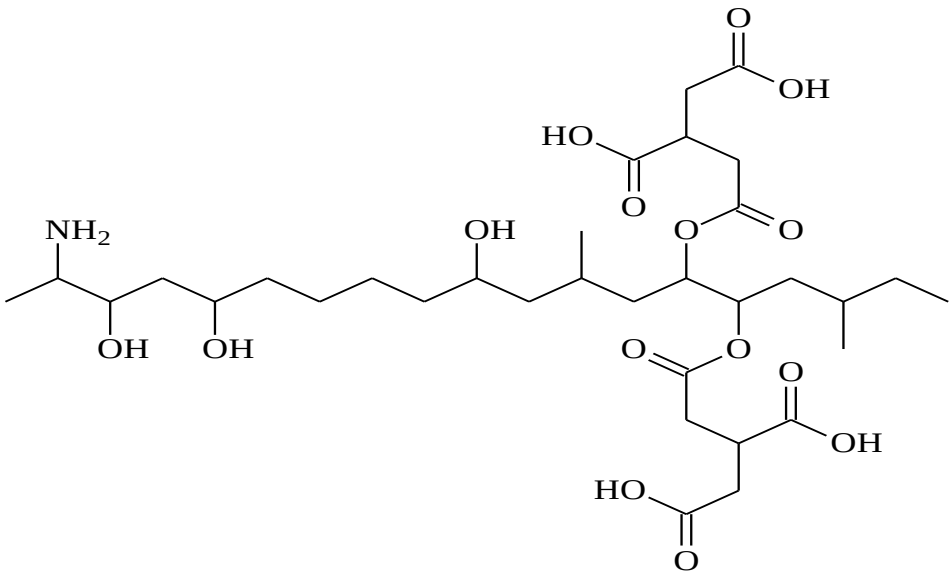


Figure 2.5. Chemical structure of fumonisin B_1

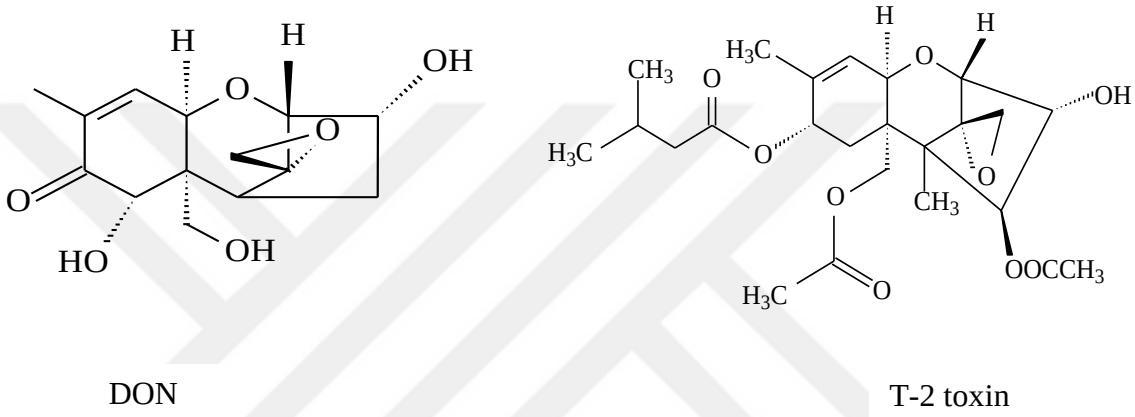
Disruption of sphingomyelin through inhibition of sphingolipid formation is considered as the main pathway of fumonisin toxicity (8, 11, 16). In mammals, equines and swines are the most susceptible species among domestic animals while poultry are more resistant than mammals. Equine leukoencephalomalacia is the fatal neurological disease in horses accounting for long term fumonisins exposure. Massive softening and liquefaction of the white matter of brain is the prominent post mortem lesion. In swine, Fumonisins B_1 (FB_1) causes cardiotoxicity and acute fatal porcine pulmonary edema. In addition to induced neurotoxicity, pulmoner toxicity and cardiotoxicity, FB_1 may also exert hepatotoxicity and nephrotoxicity (12, 15, 46).

Till now, there are uncertain impacts of FBs on human health. It is suggested that some types of esophageal and hepatic tumors and cardiac toxicity in humans are associated with FBs exposure through consumption of contaminated maize (7, 43, 45, 47, 48). FB_1 is a tumor promoter, but has no genotoxic effects and the experimental evidence proving or disproving the carcinogenicity of FBs on human cannot be found in literature. Therefore, is categorized as possible carcinogen to humans, Group 2B (29, 46).

2.2.6 Trichothecenes (T-2/HT-2, DON)

Trichothecenes (T-2/HT-2, DON) are mycotoxins produced mostly by different *Fusarium* species such as *F. culmorum, F. sporotrichioides, F. tricinctum, F. roseum, F. graminearum, F. nivale* and *F. sambucinum*. Although, some members of *Myrothecium, Spicellum, Stachybotrys, Cephalosporium, Trichoderma*, and *Trichothecium* are also able to produce trichothecenes. Maize, barley, wheat, oats, rye, soybeans, and fruits as well as animal feeds are mostly attacked by *Fusarium* (44, 49, 50). During the last 40 years, more than 180 trichothecene mycotoxins have been discovered. Structurally, trichothecenes (Figure 2.6) have been classified according to the difference of the functional group (keto-group, number of epoxy groups, and presence of a macrocyclic ring) into four groups. Type A involves T-2 toxin (T-2), HT-2 toxin (HT-2),

diacetoxyscirpenol (DAS) and neosolaniol; type B is represented by deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), and nivalenol (NIV); type C including crotocin; and type D or macrocyclics. Despite so many forms, a few numbers of trichothecenes have toxico potency. The most important are DON, T-2 and HT-2.



DON T-2 toxin

Figure 2.6. Chemical structure of some trichothecenes (DON and T-2)

Mechanism of toxicity is conducted through inhibition of protein synthesis by interaction with the 60S ribosomal subunit and the peptidyltransferase (8, 51, 52). Disruption of DNA and RNA is occurred through peptidyltransferase enzyme inhibition. It affects the actively mitotic cells such as intestinal epithelial cells, dermal, lymphoid and erythroid cells. Acute toxicity in humans shows abdominal cramps, nausea, vomiting and bloody diarrhea.

Alimentary toxic leukopenia is a disease caused due to long term exposure to trichothecenes, manifested by gastrointestinal problems followed by gastroenteritis, fever, immunosuppression, and lastly bronchial pneumonia and death. According to IARC, both DON and T-2 are classified as non-carcinogenic toxins to human, Group 3 (5, 10, 23, 24, 43, 53).

The most sensitive animal species is swine while cattle and birds are more resistant. Oral exposure to trichothecenes leads to feed refusal as a primary sign even after adding flavoring agents. Moreover, weight loss, anemia, and weakness may occur as consequences. In poultry, decreased egg production, abnormal feathering especially in broilers and some neurological signs are observed (9, 14, 26, 39).

2.2.7 Emerging mycotoxins

Emerging mycotoxins are group of mycotoxins that represented by fusaproliferin (FUS), beauvericin (BEA), enniatins (ENs), and moniliformin (MON) which are neither routinely determined, nor legislatively regulated in most of the countries that have regulations for traditional mycotoxins (54). Some species of *Fusarium* and other fungal genera including *Alternaria*, *Halosarpheia* and *Verticillium* are producing them. Enniatins A, A1, B and B1, have been most frequently detected in foods and feeds. These enniatins are structurally similar and have similar modes of action. Data about the toxic effects of emerging mycotoxins are still limited on their toxicity both *in vitro* and *in vivo* however, BEA and ENs are reported as cytotoxic and ENs are cholesterol acyl transferase enzyme inhibitors while FUS has phytotoxic properties in maize (55).

2.2.8 Modified mycotoxins

Modified mycotoxins form a great concern as they still remain undetected by conventional analytical methods and consequently this leads to underestimation of the total mycotoxin exposure as they can be converted back into their corresponding unmodified compounds by hydrolysis inside human or animals (56, 57). Gareis et al. (58) was the first one who detected these metabolites in plant and named them as masked mycotoxins as they cannot be detected by routine analysis. In addition to the plant, fungi and mammals are able to conjugate the parent metabolites with sugars or amino acids (59).

It is very important to differentiate between three different forms of mycotoxins, the free and unmodified forms such as DON, $AFB_{1,}$ and OTA. Matrix associated mycotoxins are fungal metabolites that physically dissolved or covalently bound matrix components or both effect such as fumonisines bound to starch and DON oligosaccharides. The third form, modified mycotoxins which have been modified either biologically or chemically. Biological modification occurs during phase 2 metabolism (conjugation phase) in plant or fungi or animals. Example for plant conjugates are DON-3-glucoside (DON-3-G) and zearalenone 14-glucoside (ZEN-14-G) which are named masked mycotoxins.

Chemical modification is done through thermal treatment during food and feed processing or non-thermal modification under alkaline conditions (60). Example for thermally modified mycotoxins is N-(carboxymethyl) fumonisin $B_{1.}$ Special situation for 3-ADON and 15ADON which are also formed during the normal biosynthetic pathway and may be considered as modified mycotoxins as well. The most frequently reported masked mycotoxins in food are DON-3-G, ZEN-14-G and zearalenone-14-sulphate (59).

Table 2.1. Some major mycotoxins, their main producing fungal species and the most frequently susceptible food and feed commodities

Mycotoxin	Species	Susceptible Commodities	Situation
Aflatoxins (B_1, B_2, G_1, G_2)	*Aspergillus flavus, A. parasiticus, A. nomius*	Peanuts, maize, wheat, rice, sorghum, spices, figs	Pre-harvest (field fungal attack) Post-harvest (damp storage conditions)
Aflatoxin M_1	Metabolite of aflatoxin B_1 in mammals	Milk and milk products	Animal consumption of the parent compound in feed
Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum, Aspergillus clavatus*	Cereals, beans, dried fruits, coffee and spices as well as wine and beer	Post-harvest (damp storage conditions)
Zearalenone	*Fusarium graminearum, F. culmorum, F. crookwellense*	Cereals and cereal products	Pre-harvest (field fungal attack)
Fumonisins (B_1, B_2, B_3)	*Fusarium moniliforme, F. proliferatum. F. napiforme, F. dlamini, F. nygamai*	Maize, maize products, sorghum	Pre-harvest (field fungal attack)
Deoxynivalenol	*Fusarium graminearum, F. cerealis, F. culmorum, F. roseum, F. poae*	Cereals and cereal products	Pre-harvest (field fungal attack)
Other trichothecenes (T-2 toxin)	*Fusarium langsethiae, F. poae, F. sporotrichioides, F. acuminatum, F. tricinctum*	Cereals and cereal products	Pre-harvest (field fungal attack)

2.3 Health and economic impacts

Mycotoxin contamination in animal feed and the potential transfer into animal products to be consumed by humans still remains a major problem alerting the entire world (3). Several outbreaks have been reported in humans and animals as a result of mycotoxicoses, different syndromes resulting from consumption of mycotoxin-contaminated food or feed or through respiratory, dermal, and other exposures to toxic fungal metabolites.

Mycotoxin contamination can appear in the field before harvest, post-harvest or during processing and storage. They induce carcinogenic, mutagenic, teratogenic, immunotoxic, hepatotoxic, and estrogenic effects in human and animals. Mycotoxins have various acute and chronic effects and their severity depends on species and other factors within the same species such as the extent of exposure, age, sex, physiological and nutritional status of the individual and possible synergistic effects of other contaminants to which the living organism is exposed. The number of people or farm animals affected by mycoses and mycotoxicoses is still unspecified.

Some major mycotoxins effects with their pathological findings are summarized in Table 2.2. Mycotoxicoses often diagnosed when large numbers of population or when the whole animal or poultry farm is involved (5, 23, 53). IARC categorized the mycotoxins into different classes according to their potential carcinogenicity. Some are potent animal and presumed human carcinogens (29). Beside their health impact, mycotoxin contamination leads to economic losses including loss of human and animal life, health care and veterinary care costs, loss of livestock production, loss of forage crops and research costs focusing on relieving the impact and severity of these contaminants (19).

The economic impact of AFs contamination has become a worldwide concern on the agricultural markets affecting approximately 25% of the world's food supply as estimated by the Food and Agriculture Organization (FAO) of the United Nations (61).

The contamination of food and feed by the major mycotoxins, e.g. AFs, OTA, FBs, DON, T-2, HT-2, ZEN, and ergot alkaloids are of the greatest health and agro-economic importance throughout the world as a significant source of food and feed-borne illnesses.

Table 2.2. Toxic effects of some major mycotoxins in different animals

Mycotoxin	**IARC Classification**	**Major effects**	**Clinical and pathological signs in susceptible animals and poultry**
Aflatoxins	1	Carcinogenic, hepatotoxic and impaired immune system	Reduced productivity; inferior egg shell and carcass quality; increased susceptibility to infectious disease.
Aflatoxin M_1	2B		
Ochratoxin A	2B	Carcinogenic, nephrotoxic, hepatotoxic, neurotoxic and teratogenic	Kidneys are grossly enlarged and pale due to nephrotoxicity; fatty livers in poultry; shell decalcification/thinning.
Zearalenone	3	Fertility and reproduction (estrogenic activity) and disrupts endocrine system	Swollen, reddened vulva, vulvovaginitis, anestrus vaginal prolapse and sometimes rectal prolapse in pigs; feminization and suppression of libido; suckling piglets may show enlargement of vulvae; fertility problems.
Fumonisins	2B	Carcinogenic, hepatotoxic, central nervous system damage and immuno-depressants	Equine leukoencephalomalacia , porcine pulmonary edema, liver damage in poultry.
Deoxynivalenol	3	Immunotoxic and alimentary toxic leukopenia	Decreased feed intake and weight gain in pigs; feed refusal and vomiting at very high concentrations.
Other trichothecenes (T-2 toxin)	3	Immuno-depressants, gastrointestinal haemorrhaging and hematotoxicity	Reduced feed intake; vomiting, skin, and gastrointestinal irritation; neurotoxicity; abnormal offspring; increased sensitivity to infection; bleeding.

International Agency for Research on Cancer. 1: carcinogenic to humans AFs; 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans; 3: not classifiable as to its carcinogenicity to humans; 4: probably not carcinogenic to humans.

2.4 Prevention and control of factors influencing mycotoxins

Mycotoxins remain challenging to control due to their strong stability even during food and feed processing rendering their total elimination impossible or impractical. In order to reduce the risks associated with mycotoxin-contaminated feed and food, strict regulations must be applied through the use of: I) rigorous monitoring including strategies to decrease the mycotoxin production in agricultural stuffs pre and post-harvest and II) systematic protocols to diminish their exposure and modulate the metabolism of the toxins to reduce the toxic effects. This requires a better understanding of the ecology of mycotoxin producing organisms, animal production regimes, and feed chain systems.

Pre-harvest precautions include efficient agricultural practice which involves, the wise use of fungicides and insecticides to prevent fungal and insect invasion, irrigation to avoid moisture stress, harvesting of plants in maturity when moisture content is lowest. Also improvement of plant genes to resist fungal attack through genetic engineering and effective breeding programs are recently used. Another novel way, using of biocompetitive fungi in which non-toxigenic fungi are cultivated in the field to substitute naturally occurring toxic fungi has been considered. This approach gives considerable results for aflatoxins in some agricultural products, for instance, peanuts and maize (1, 40, 62, 63).

Post-harvest precautions for controlling the fungal activity in stored agricultural stuffs are based on two important factors, temperature and moisture. These parameters are affected by geographical location and other circumstances such as drying, aerating, turning over the grains and transport. Therefore, it is critical to keep storage equipment and other transporters free of insect and vector activities, water condensation, and water leakage to prevent fungal invasion. In some situations, food and feed can get contaminated with mycotoxins without being moulded to become associated with their produced secondary metabolites (1, 62, 63). A variety of chemical, biological, and

physical approaches have been developed to control mycotoxin contamination will be mentioned in the following paragraphs.

2.4.1 Physical treatment

Washing with water or sodium carbonate, dehulling, sorting of contaminated grains, heating at high temperature, wet and dry milling, and irradiation treatment such as ultraviolet (UV), X-rays or microwave have been employed to reduce mycotoxin contamination. Additionally, multiple processing and/or decontamination schemes carried out on adsorbent materials, organic and inorganic binders succeeded in reducing mycotoxin concentrations to acceptable levels. These compounds bind the toxin during digestion process resulting in reduction of toxin bioavailability.

Examples for inorganic adsorbents are bentonites, zeolites, diatomaceous earth, clays, modified clays, and activated charcoal. For organic adsorbents, fibers from plant sources like alfalfa and oat fibers, extracted cell wall fraction of *saccharomyces cerevisiae* and beta-D-glucan fraction of yeast cell wall. These dietary additives offer one of the greatest potentials for preventing toxicity in digestive tract where the bounded toxins can be excreted via urine or feces (48, 62, 64, 65).

2.4.2 Chemical treatment

Various chemicals including acids, bases, oxidizing agents, and different gases have been examined for detoxification of mycotoxins. Even so, chemical degradation of mycotoxins seems to be somehow practical, most of them can reduce the nutritive value and palatability of the feed and increase the possibility of toxic by-products formation. In addition, they are expensive and time consuming due to their need for additional cleaning treatments. Many organic compounds especially propionic acid, fungal growth inhibitor, form the core stone of many commercial antifungal agents used in animal feed industry. Extensive research has been done to consider ozonation and ammoniation as a practical method for decontamination of mycotoxins (63, 65 - 68).

2.4.3 Biological approaches and biocontrol

Biological control is the use of living agents to control pests or plant pathogens. For example, *Eubacterium* and other certain types of isolated yeasts have been successfully used management of mycotoxins in particular for AFs and OTA in food and animal feeds. Recent studies indicate that molecular approaches may offer an insight into the interactions of mycotoxin-producing fungi and other organisms including mycotoxin-degrading microbes. Interestingly, some aflatoxin producing fungi from *Aspergillus species* have the capability to degrade their own synthesized mycotoxins (40, 52, 69, 70).

However, none of these approaches individually fulfills the required efficacy, safety and cost needed for mycotoxin decontamination from agricultural products. Under all circumstances, detoxification processes should eliminate or inactivate mycotoxins, generate no toxic products, keep the nutritional value, induce no modification to the technological characters of the product and if possible destruct mould spores (26, 65, 71).

2.5 Global regulations and harmonisation studies

The possible economic and health risks caused by mycotoxins have presupposed the urgent need to control their levels. According to the geographical and climatic variations, different limits are being set to monitor and control mycotoxin levels. However, other various factors influence the decision-making process of setting limits such as data about human and animal susceptibility, the levels of exposure, as well as the availability of methods of sampling and analytical capability (17, 72). These regulations exist in at least 100 countries for major mycotoxins in different commodities. Current regulations are increasingly based on scientific opinions of authoritative bodies, for example the FAO/WHO Joint Expert Committee on Food

Additives of the United Nations and the European Food Safety Authority (3, 72 - 74, 75).

Up to date, the major source of food and feed all over the world is cereal grains. Maize is primarily used for feed manufacturing in some countries such as Egypt. In Africa, most of regulations exist for aflatoxins with exception a few countries have detailed regulations. The current Egyptian regulatory limits include total aflatoxins and aflatoxin B_1 in both maize and animal feed are 20μg/kg and 10 μg/kg respectively (76). Other commodities are shown in Table 2.3.

Table 2.3. Current Egyptian regulations in maize and animal feed

Commodity		Aflatoxin (s)	Level (μg/kg)
Food	peanuts & cereals, except maize	AFB_1	5
		Total AFs	10
	Maize	AFB_1	10
		Total AFs	20
Feed	Feed for animal and poultry	AFB_1	10
		Total AFs	20
Dairy	Milk, dairy products	M_1	Zero

2.6 Analytical methods for mycotoxin detection

The growing concern over food and feed safety has led to development of several methodologies for mycotoxin detection. Since the potential health effects may occur at very low concentrations, sensitive and reliable methods for their detection are highly required. Proper sampling, homogenization, extraction, and clean-up which may involve sample concentration are generally the most common steps in many analytical procedures. All the mentioned phases are variable according to the type of matrix, degree of mycotoxin contamination and the effect on the reliability of measuring

mycotoxin levels. Sampling is a very critical step for mycotoxin detection in both feed and food matrix. However, for homogeneous matrix like milk and other dairy products, sampling errors are reduced dramatically. Sample preparation is the principle time consuming factor and takes approximately two-thirds of the total time of an analysis. In general, sample preparation and the applied analytical methods must be precise and reproducible to provide reliable results (77 - 79).

For a rapid specific screening of mycotoxin type, immunology-based methods such as enzyme-linked immunoassay (ELISA) or radioimmunoassay (RIA) are the best approaches as they depend on specific antibodies beside their relatively low cost, easy application and their results could be comparable with those obtained by other conventional methods such as thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (4, 6, 80). The application of antibodies either in a bound form in immunoaffinity columns (IACs) or in a free form through immune-ultrafiltration for the clean-up have changed the way of many food and feed contaminant detections including mycotoxins (4, 81).

Due to synergistic effects of co-occurring mycotoxins, a number of studies have investigated multi-mycotoxin methods for detection of several classes of fungal metabolites. These methods can save time and costs compared to single target analysis if the sample has to be tested for different mycotoxins. Most of them are based on HPLC coupled with mass spectrometer (MS) detector beside a fewer number of studies used HPLC coupled with ultraviolet (UV) or fluorescence (FLD). A number of toxins already have natural fluorescence (e.g., OTA, AFs) and can be detected directly in HPLC–FLD, but many major mycotoxins are not UV and/or FLD active such as fumonisins and trichothecenes and hence they should undergo derivatization step firstly in order to be detected. Different chemical agents can be used for derivatization for example using of o-phthaldialdehyde naphthalene-2,3-dicarboxaldehyde and 9-(fluorenylmethyl) chloroformate fumonisins. Derivatization step can be performed by employing either

pre- or post-column according to the available equipments and number of the mycotoxins that will be detected.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) has become the most emerging analytical tool for the assessment of mycotoxins and their metabolites including masked mycotoxins as there is no need for derivatization, low detection limits, gathering information about the analytes structures, minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. The principle of liquid chromatography mass spectrometry (LC-MS) is the detection of the analytes according the chromatographic retention and to their mass-to-charge ratio (*m/z*) by utilizing electromagnetic field and in tandem mass (MS/MS) there are two steps of mass spectrometric selection with a fragmentation phase in between (77,78, 80).

A liquid chromatography mass spectrometry-Electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the determination of 39 mycotoxins including the major mycotoxins and their derivatives in cereals was developed (82). Without any clean up "dilute and shoot", the diluted raw extract was injected into the LC-MS/MS system through two consecutive chromatographic runs, once in the positive and once in the negative electrospray ionisation (ESI) mode for the best sensitivities for all analytes. Later on, the analytical method was continuously developed, allowing the determination of more mycotoxins to cover 295 bacterial and fungal metabolites including all regulated mycotoxins (83). However, sensitive clean up procedures such as IAC or solid phase extraction (SPE) cannot be applied to such a huge number of chemically diverse metabolites. Another drawback of multi-mycotoxins is that they have to cover a relevant concentration range.

However, such high-end instruments are not easily available in developing countries like Egypt where HPLC with FLD or UV could be an alternative tool because they are less expensive and no complex laboratory requirements are needed. In addition, using a fluorescence detector might still be superior in the area of quantitative

determination, where the influence of matrix effect (signal suppression or signal enhancement compared to the analytes in standard solution and therefore wrong estimation" under or over" of the contamination amount will occur) is negligible in comparison with possible problems that can arise with HPLC–MS quantification especially when no clean-up steps are performed (82, 84).

Rahmani et al. (85) reported a method suited for analysis of maize using commercial multiple-analyte IAC containing antibodies against AFs, OTA and ZEN and gradient elution with fluorescence detection of six mycotoxins. Another method for nine mycotoxins, including the above mentioned mycotoxins, DON and FB_1 and FB_2, has been developed for maize and validated in-house using three separated clean-up steps and a single HPLC run with fluorescence detection with post column photochemical derivatization for aflatoxins and UV detection for DON (86). However, in some instances, IACs antibodies can recognize a group of mycotoxins having a similar structure (e.g., AFB_1, AFB_2, AFG_1 and AFG_2) as reported in addition to its cost (87).

For mycotoxins screening in animal products e.g milk and milk products, lots of articles have presented different methods for determination either single or multi targets detection especially AFM_1 with the application of IACs which provide a combination of extraction and clean-up stages (36, 88, 89).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Name	Company name
Acetonitrile	Sigma-Aldrich, J. T. Baker
Glacial acetic acid	Riedel-de Haën, Sigma-Aldrich
Aflatoxin M_1 standard	Sigma-Aldrich
Aflatoxins (B_1, B_2, G_1, G_2) mix standard	Sigma-Aldrich
Ammonium acetate	Sigma-Aldrich
Di potassium hydrogen phosphate (K_2HPO_4)	Merck
Elga Purelab ultra analytic system	VeoliaWater
Hypochloride solution	Birka
Potassium di hydrogen phosphate (KH_2PO_4)	Merck
Methanol	Sigma-Aldrich, J. T. Baker
Nitric acid	Sigma-Aldrich
Ochratoxin A standard	Sigma-Aldrich
Phosphoric acid	Sigma-Aldrich
Sodium chloride (NaCl)	Merck
Standards of fungal and bacterial metabolites, n= 600	Romerlabs, Sigma-Aldrich, Iris Biotech GmbH , Enzo Life Sciences, Fermentek, BioAustralis, BioViotica and LGC Promochem GmbH
Sulfuric Acid	Sigma-Aldrich

Tween 20	Merck
Zearalenone standard	Sigma-Aldrich

3.1.2 Laboratory tools and instruments

Apparatus name	**Company name**
AFM_1 HPLC IACs	Vicam
Agilent ChemStation software	Agilent
Analyst® software version 1.5.1	AB Sciex
Analytical scale	Laboratory LC 4800 P, Denver Instrument, Schimadzu Libror EB-330D
AOZ HPLC IACs	Vicam
Automatic pipettes	BioHIT
Laboratory blender	Osterizer® Blender, Waring commercial
Centrifuge	Hettich Universal Rotina 420 R
Distilled water apparatus	mes mp minipure
Fluted filter paper	Vicam
Freezers and refrigerators	Liebherr, Arçelik
High Performance Liquid Chromatography system with Fluorescence Detector	Agilent 1100 Series
Autosampler	G 1313A
Pump	G 1311 A
Fluorescence detector	G 1312 A
Columns	
(25 cm × 4.6 mm, 5µm Particle size)	

Octadodecil silica gel C18	Hichrom
C18	ACE
C18 security guard cartridge (4 × 3 mm i.d.)	Hichrom
Personal Computer	HP
Printer	HP
Horizontal shaker	Edmond Bühler BH 2
LC-MS/MS system	
High Performance Liquid Chromatography System	Agilent 1290 Series
C18 Column (150 × 4.6 mm i.d., 5 μm particle size)	Phenomenex
C18 security guard cartridge (4 × 3 mm i.d.)	Phenomenex
QTrap 5500 LC-MS/MS System	Applied Biosystems
Personal Computer	HP
Printer	HP
Magnetic stir plate	Dottingen
Microfilter nylon 66	Vicam
Microfiber filter paper	Vicam
Mobile phase filtration apparatus	Neuberger
MultiQuant TM 2.0.2	AB Sciex
Oven	Dedeoğlu
pHmeter	Cyberscan pH 500
Photochemical reactor (PhCR)	Vicam

Rotary shaker	GFL 3017
Vortex	Mixer VTX-3000L, VF2
Water purification unit	Thermo scientific, Barnstead™

3.1.3 Solutions preparation

Preparation of phosphate buffered saline pH 7.4

Phosphate buffered saline (PBS) with 0.01% Tween-20 was prepared by mixing two solutions; the first one was prepared through dissolving 296.1 mg of K_2HPO_4 and 4.5 g of NaCl (to have isotonic NaCl 0.9%) in 500 mL purified water by magnetic stir bar for 3 to 5 min. Afterwards, 45 µL of Tween-20 was added to the solution. Second solution was prepared as the first one, but K_2HPO_4 was replaced by 231.3 mg of KH_2PO_4. Adjustment of pH to 7.4 by mixing the two solutions in one glass bottle and the pH meter was used to measure the solution pH to ensure that the desired pH was achieved. The prepared PBS solution was kept at room temperature (RT).

Preparation of potassium phosphate buffer pH 3.5

Potassium phosphate buffer pH 3.5 was prepared through dissolving 2.04 g of KH_2PO_4 in one liter of purified water by magnetic stir bar for 3 to 5 min. The pH 3.5 was adjusted by pipetting a few drops of phosphoric acid and the pH meter was used to measure the solution pH to ensure that the desired pH was achieved. The prepared buffer was kept at RT (if it was used in the same day) and at refrigerator (if the analysis was in the following day) and was filtered before HPLC analysis by mobile phase filtration apparatus.

Preparation of 0.1% glacial acetic acid

Glacial acetic acid (HAc) for sample extraction was prepared by dilution of 50 µL of HAc in 49.950 mL of purified water in appropriate laboratory bottle and it was ready for usage.

Preparation of eluents and solvents for LC-MS/MS

The preparation of eluents A and B was carried out as follow (see Table 3.1 for volumes). Firstly, in order to prepare 5 mM of ammonium acetate, 0.962 g was weighted and pre-dissolved in purified water (50 mL). Then, the volume of the component with the highest percentage (i.e. water for eluent A, methanol for eluent B) was put into an appropriate bottle (for example 2.5 liters). Afterwards, ammonium acetate pre-dissolved in water was added, followed by methanol and acetic acid. The bottle was shaken and finally, the remaining volume of water or methanol was added. The mixture was shaken well before usage. Ammonium acetate should be dissolved in water because it does not dissolve in methanol and dissolving is better to be done in a small bottle (e.g. 50 mL) because the turbulences are larger and accelerate the procedure.

Table 3.1. Preparation of mobile phases A and B for LC-MS/MS

Component	Acetic acid	Ammonium acetate	Methanol	Water
Eluent A	25 mL	5 mM (~ 0.962 g)	250 mL	2225 mL
Eluent B	25 mL	5 mM (~ 0.962 g)	2425 mL	50 mL

Extraction and dilution solvents are mixture of water, acetonitrile, and acetic acids (see Table 3.2 for volumes). It is crucial to add acetic acid and the other remaining component after acetonitrile or water during the preparation of extraction and dilution

solvents. Attention should be given to the fact that while mixing, cooling happens and condensing of water (from the air) makes the bottle a little bit slippery.

Table 3.2. Preparation of extraction and dilution solvents for LC-MS/MS

Component	Acetic acid	Acetonitrile	Water
Extraction solvent	10 mL	790 mL	200 mL
Dilution solvent	10 mL	200 mL	790 mL

Preparation of mycotoxin standard solutions for HPLC-FLD application

Firstly, three stock solutions of AFs, OTA and ZEN mycotoxins were prepared. Both aflatoxins (B_1, G_1 1000 ng/mL and B_2, G_2 290 ng/mL) and ZEN (50000 ng/mL) were diluted in methanol (1:10, v/v) to achieve (B_1, G_1 100 ng/mL and B_2, G_2 29 ng/mL) and (5000 ng/mL), respectively while OTA (50000 ng/mL) was diluted in methanol (1:100, v/v) to reach (500 ng/mL). Then 144 µL of AFs mix, 288 µL of OTA and 96 µL of ZEN were mixed with 972 µL of methanol and 1500 µL of 0.1% acetic acid to have at the end 3 mL (the second stock solution) with the following concentrations B_1, G_1 (4.8 ng/mL) and B_2, G_2 (1.39 ng/mL), ZEN (160 ng/mL) and OTA (48 ng/mL).

Afterwards, other working standard solutions were prepared by diluting the second stock solution with an equal amount of methanol and 0.1% acetic acid (1:1, v/v) to achieve different concentrations of mycotoxins mixtures (see Table 3.3). Another method was also used through adjustment of the injection volume directly from the second stock solution into HPLC instrument to have 8 different points as shown in Table 3.3. It is important to dilute the standard with 0.1% HAc directly before the injection time. All stock and working standard solutions were stored in amber vials at refrigerator. The standard solutions were used to calculate the LC detector response and recovery studies.

Table 3.3. Standard solution for AOZ method

Standard	AF B_1, AF G_2 (ng/mL)	AF B_2, AFG_2 (ng/mL)	OTA (ng/mL)	ZEN (ng/mL)
STD1 (12.5 µL)*	0.024	0.00696	0.24	0.8
STD2 (25 µL)*	0.036	0.01044	0.36	1.2
STD3 (100 µL)*	0.048	0.01392	0.48	1.6
STD4 (12.5 µL)	0.06	0.0174	0.6	2
STD5 (25 µL)	0.12	0.0348	1.2	4
STD6 (50 µL)	0.24	0.0696	2.4	8
STD7 (75 µL)	0.36	0.1044	3.6	12
STD8 (100 µL)	0.48	0.1392	4.8	16

*diluted (low) concentration

Preparation of AFM_1 standard solutions

Standard solutions of AFM_1 with the concentrations of 0.02, 0.05, 0.1, 0.15 and 0.2 ng/mL acetonitrile were prepared from a working solution of concentration 2 ng/mL and were used to obtain the calibration curve. Another method was also used through adjustment of the injection volume acetonitrile into HPLC instrument as the following:

- STD1 (10 µL): 0.02 ng/mL
- STD2 (25 µL): 0.05 ng/mL
- STD3 (50 µL): 0.1 ng/mL
- STD4 (75 µL): 0.15 ng/mL
- STD5 (100 µL): 0.2 ng/mL

Preparation of standard solutions for LC-MS/MS

Individual stock solutions of the standards were prepared by dissolving the solid substance in acetonitrile or methanol or methanol/water 1:1 (v/v) or acetonitrile/water 1:1 (v/v) or only water according to the solubility and preference. The stock solutions were combined or grouped into 61 working solutions and stored at -20°C. Then a mastermix stock solution was prepared approximately every 3 months by mixing the 61 combined working solutions in appropriate concentrations. Before preparing the mastermix, all working solution groups were brought to RT in a dark environment (cupboard) and thoroughly mixed by using a Vortex. The prepared mastermix contains more around 600 fungal and bacterial metabolites in different concentrations and it was serially diluted for every sequence after bringing the mastermix from -20°C to RT and dark environment as well (Sulyok, et al., unpublished). Only fuminisin mix (B_1 and B_2) and fumonisin B_3 were separately stored and were not added to mastermix. The preparation should be done as fast as possible and exposure to sunlight should be avoided to prevent analyte degradation.

To obtain appropriate concentrations of the standard solutions (STD 1-1000) the multi-analyte stock solution was diluted with dilution solvent (ACN/H_2O/HAc; 20:79:1 v/v/v) as follows:

- STD 1: 300 µL mastermix + 260 µL dilution solvent
  + 20 µL fumonisin Mix (B_1, B_2)
  + 20 µL fumonisin B_3
- STD 3: 300 µL STD 1 + 700 µL extraction + dilution solvent (1:1 v/v)
- STD 10: 100 µL STD 1 + 900 µL extraction + dilution solvent (1:1 v/v)
- STD 30: 30 µL STD 1 + 970 µL extraction + dilution solvent (1:1 v/v)
- STD 100: 20 µL STD 1 + 1980 µL extraction + dilution solvent (1:1 v/v)
- STD 300: 300 µL STD 100 + 700 µL extraction + dilution solvent (1:1 v/v)
- STD 1000: 100 µL STD 100 + 900 µL extraction + dilution solvent (1:1 v/v)
- Blank: Extraction + dilution solvent (1:1 v/v)

3.2 Methods

3.2.1 Sampling

Samples collection for HPLC-FLD (AOZ and AFM_1)

All samples, maize (n=61) and commercial animal feed (n=17) were randomly collected between summer 2013 and winter 2014 (each 250 g) from formal and informal markets as well as from farms located across Assiut governorate in Upper Egypt as shown in Figure 3.1 (area 2). In addition to 20 raw milk samples (each 250 mL) intended for human consumption were purchased from different local markets in the same area as in Figure 3.1 (area 2), Assiut governorate, Upper Egypt during summer season of 2013, between July and August 2013.

Afterwards, samples were transported in polystyrene boxes with cooling gel (pre-frozen to -20°C) to Ankara, Turkey for extraction and HPLC-FLD analysis. All samples were kept at -20°C at the laboratory of the department until use. After the analysis and data evaluation have been done, all the samples were discarded in an appropriate way.

Samples collection for LC-MS/MS

A total of 156 samples of maize (n=79) and commercial animal feed (n=77) were randomly collected and purchased (each 250 g) from formal and informal markets as well as from farms located across three different governorates (Minya, Assiut and Sohage) in Upper Egypt. As shown with colors and numbers in Figure 3.1, samples from Assiut city (area 2) were collected between 2014 and 2015 and the other samples from remaining cities (Minya city, area 1) and (Sohage city, area 3) were collected during summer 2015, between July and September 2015. Afterwards, samples were transported in polystyrene boxes with cooling gel (pre-frozen to -20°C) to Center for Analytical Chemistry, Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences (BOKU), Austria for extraction and LC-MS/MS-based

analysis. During the whole period of analysis, all samples were kept at -20°C at storage cooling room inside IFA-Tulln. After the analysis and data evaluation have been done, all the samples were discarded in an appropriate way.

Figure 3.1. Map of Egypt showing the sampling areas in Upper Egypt (Map modified after NordNordWest Version of 10 March 2014). https://commons.wikimedia.org/wiki/File:Location_map_Egypt.svg published under the licence of Creative CommonsAttribution-ShareAlike 3.0 Unported (CC BY-SA 3.0),http://creativecommons.org/licenses/by-sa/3.0/deed.en

3.2.2 Pre-treatment of samples

Samples extraction for AOZ method

In general, samples were prepared by mixing them with an extraction solution, blending, filtering and passing through immunoaffinity columns (IACs). Each sample (250 g) of maize and feed was ground by a laboratory blender for 1-2 minutes. Afterwards, solid–liquid extraction has been done according to the manufacturer's instructions published by Göbel and Lusky. (90) and Rahmani et al. (85) with minor modifications (will be mentioned later) using acetonitrile and purified water (80:20, v/v). A 25 g of each sample was weighted and placed with 5 g of NaCl (adding NaCl is modified step) in laboratory blender. NaCl was added in the step of extraction for the purpose of salting out. 100 mL of acetonitrile and purified water (80:20, v/v) were added to the mixture and blended for 2 minutes at high speed. The mixture was filtered twice through two different filter papers, fluted filter paper and microfiber filter paper obtained from Vicam (Sweden) and (Germany), respectively (filtrations are modified steps).

After the first filtration, 10 mL of the extract was diluted with 40 mL PBS with 0.01% Tween-20. It has been reported that using PBS with 0.01% tween-20 is the best solution to obtain clear methanol elutes from the column with high recovery rate in comparison with other washing solutions (91). The mixture was mixed well before the second filtration was performed. The filtered extract was gathered in flask then taken in a syringe barrel, which was attached with IACs. A 20 mL of the filtrate were passed by syringe barrel to the AOZ HPLC IACs obtained from VICAM (bounded with specific antibodies to aflatoxins, ochratoxin A and zearalenone) at a flow rate of about 1-2 drops per second. It is important to not let the column completely dry out. Afterwards, the columns were washed with 10 mL PBS with 0.01% Tween-20 solution at about 1-2 drops per second flow rate followed by 10 mL of purified water. After the water wash, the column was dried gently by passing air through the column for two to three seconds.

The column was washed with water to rid the immunoaffinity column of impurities. By passing 1.5 mL of HPLC grade methanol through the column, the toxins were removed from the antibodies and collected in a glass tube. Finally, 1.5 mL 0.1% HAc solution was passed through the column at a rate of 1 drop per second or less for the completely remove of all bounded toxins and collected in the same the glass tube with HPLC grade methanol. After vortex, the solution was divided into two HPLC injection vials of equal amount and submitted for HPLC-based analysis.

Samples extraction for AFM_1 detection

The extraction and determination of AFM_1 has been done according to the AOAC Official Method 2000.08 reported by Dragacci et al. (89). Samples are prepared by centrifuging and separating out the fat layer. Each raw milk sample (250 mL) was centrifuged at 4000 RPM for 15 minutes at RT. Afterwards; samples were filtered twice through two different filter papers, fluted filter paper and microfiber filter paper obtained from Vicam (Sweden) and (Germany), respectively. The filtrate was gathered in flask then taken in a syringe barrel, which was attached with the IACs. Skimmed (defatted) milk samples (each 50 mL) were passed by syringe barrel the through Afla M_1^{TM} immunoaffinity column obtained from VICAM (Watertown, Massachusetts, USA) for clean-up at a rate of about 1-2 drops/second until air comes through column. The column is then washed with 10 mL of purified water at a rate of 1-2 drops/second to rid of impurities.

The washing step was repeated once again until air comes through the column. Afterwards, AFM_1 was eluted from the column (i.e. the toxin is eluted from the antibody) by passing 1.25 mL of acetonitrile: methanol mixture solution (3:2, v/v) at a rate of 1 drop for every 2-3 seconds followed by an equal amount of purified water (1.25 mL purified water through column at a rate of 1 drop for every 2-3 seconds). The final solution (2.5 mL) was collected in a glass cuvette then transferred into HPLC injection vial for analysis.

Samples extraction for LC-MS/MS method

Each sample (250 g) of maize and feed was ground by Osterizer® blender for 1-2 minutes. The samples were weighed (5.00 ± 0.01 g for each) into a 50-mL polypropylene tubes with conical bottom and the extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added at a ratio of 4 mL per gram of sample (i.e., 20 mL for each 5 g samples). Samples were extracted for 90 min on a GFL 3017 rotary shaker in a horizontal position at RT at 180 RPM. Centrifugation was not necessary due to sufficient sedimentation by gravity. For this, they were left in an upright position for at least 5-10 min in order to allow any suspended particles to settle down and a supernatant of 500 µL of raw extract was transferred into autosampler vials and diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v), and 5 µL of the diluted raw extract were injected without any further manipulation into LC-MS/MS as described in Sulyok et al. (82).

Apparent recoveries of the analytes were taken from the analysis of 10 individual spiked samples (5 maize and 5 feed). Multi-component standard (100 µL) containing more than 600 analytes were added to 0.25 g of the respective sample (for the lower level of the initial validation, only 20 µL were used). The used method, screening of more than 600 fungal and bacterial metabolites (Sulyok, et al., unpublished), is an extension for the published paper by Malachová et al. (83) who developed a method for screening 295 fungal and bacterial metabolites. The spiked samples were left overnight in the dark at RT for evaporation of the solvent. Afterwards, they were extracted using 1 mL of extraction solvent as described above.

3.2.3 Instruments parameters

HPLC-FLD parameters for AOZ

Chromatographic separation was performed by using an Agilent 1100 HPLC equipped with ACE® C18 column (25 cm × 4.6 mm, particle size 5µm) and a C18 4×3 mm i.d. security guard cartridge. The HPLC was coupled with florescence detector for quantitative determination with on-line post-column photochemical derivatization. The photochemical reactor (PhCR) from Vicam (Taiwan) was used for aflatoxins (AFB_1 and AFG_1) to enhance the sensitivity and/or selectivity of fluorescence detection response. The photochemical reactor was placed between the HPLC analytical column and the detector.

The chromatographic parameters in this analysis are described in Ofitserova et al. (86). However, the author detected 9 mycotoxins per run including the mycotoxins of interest in this work and consequently the gradients in the present study were modified. The mobile phase consisted of methanol /acetonitrile/ sodium buffer pH 3.5 (28:15:57, v/v/v) as shown in Table 3.4.

Table 3.4. HPLC pump gradient program

Time (minutes)	Methanol %	Acetonitrile %	Potassium buffer (pH 3.5%)	Max pressure (bar)
0	15	28	57	250
15	15	28	57	250
20	0	60	40	250
40	0	60	40	250
50	15	28	57	250

The HPLC pump flow rate was set at 0.8 mL/min and column temperature at 40°C. The HPLC pump gradient and fluorescence detector programs are presented in Tables 3.5. The injection volume was 100 µL and the running time was 50 minutes. The equilibration time was 10 minutes.

Table 3.5. Fluorescence detector program

Time (minutes)	λ ex	λ em
0	360	460
25	335	460
36	275	460
50	360	460

Ex: excitation; EM: emission

The required excitation and emission wavelengths were as follow: aflatoxins λex = 360 nm; λem = 460 nm, ochratoxin A λex = 335 nm; λem = 460 nm and zearalenone λex = 275 nm; λem = 460 nm.

HPLC-FLD parameters for AFM_1

For AFM_1, the chromatographic separation was performed at 25 ± 1°C using HPLC equipped with a Hichram® ODS2 column (250 mm × 4.6 mm i.d., 5 µm particle size) and a C18 (4×3 mm i.d.) security guard cartridge. The mobile phase consisted of methanol/water/acetonitrile (22:62:16; v/v/v) in isocratic elution and was adjusted through HPLC software (92). The fluorescence detector was adjusted with excitation and emission wavelengths of 360 nm and 430 nm, respectively. The injection volume was 100 µL. The flow rate was 1 mL/ min, the retention time was 10.7 ± 0.2 min and the running time was 15 min.

LC-MS/MS parameters

The chromatographic method, chromatographic and mass spectrometric parameters are described elsewhere (83). Briefly, LC-MS/MS screening of target microbial metabolites was performed with a QTrap 5500 LC-MS/MS System equipped with Turbo Ion Spray ESI source and a 1290 Series HPLC System. Chromatographic separation was performed at 25°C on a Gemini® C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 × 3 mm i.d. security guard. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min.

Further linear increase of B to 100% within 9 min was followed by a hold time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1 mL/ min. ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The sMRM detection window of each analyte was set to its expected retention time ± 23 and ± 27 second in the positive and the negative modes, respectively. The target scan time was set to 1 second.

The settings of the ESI source were as follows: source temperature 550°C, curtain gas 30 psi (206.8 kPa of maximum 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage − 4500 and 5500 V, respectively, and collision gas (nitrogen) medium. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin and 3-nitroproponic acid which exhibited only one fragment ion). This yielded 4.0 identification points according to European Union Commission decision 2002/657 (93).

In addition, the LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively. Analyst® software version 1.6.2 was used to control the LC-MS/MS instrument, whereas MultiQuant ™ 3.0.2 was used for automatic and manual integration of the peaks. Quantification was performed using linear, 1/x weighed calibration functions deriving external calibration.

4. RESULTS

4.1 Determination of method performance parameters

4.1.1 LOD and LOQ for AOZ method by HPLC-FLD

Sensitivity of the method was tested by examining both limit of detection (LOD) and limit of quantitation or quantification (LOQ). LOD is the lowest absolute concentration of analyte that could be detected and it is calculated based on the concentration of the analyte in which ratio of signal to noise should be 3 (S/N=3) while LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy (S/N=10). The LOD and LOQ for the six target analytes were calculated (see Table 4.1).

Table 4.1. Limits of detection and quantification for AOZ method

	LOD (ng/mL)	LOQ (ng/mL)
AFB_1	0.04	0.13
AFG_1	0.13	0.38
AFB_2	0.03	0.09
AFG_2	0.03	0.09
OTA	0.02	0.07
ZEA	0.92	2.79

4.1.2 Recovery rate for AOZ method by HPLC-FLD

The sample extraction should allow good recoveries for all analytes of interest in the specific matrix. For calculation of the percentage of recovery, the concentrations of the spiked samples (maize and feed) were determined against calibration curves and the obtained concentrations were then divided by the spiked concentrations and finally multiplied by 100. Both maize (n=2) and feed (n=2) were spiked with 150 µL of AFs main stock (1000 ng/mL), 50 µL of 2nd dilution of OTA stock (5000 ng/mL), and 150 µL of ZEN main stock (50000 ng/mL). The spiked samples were left for 3 hours in the dark at RT for evaporation of the solvent. Afterwards, they were extracted as described before (see Section 3.2.2). The obtained recovery levels were as shown in Table 4.2.

Table 4.2. Recovery rates for AOZ method

Metabolites	AFB_1	AFG_1	AFB_2	AFG_2	OTA	ZEA
Recovery%	100	34	40.5	40.3	100	88.8

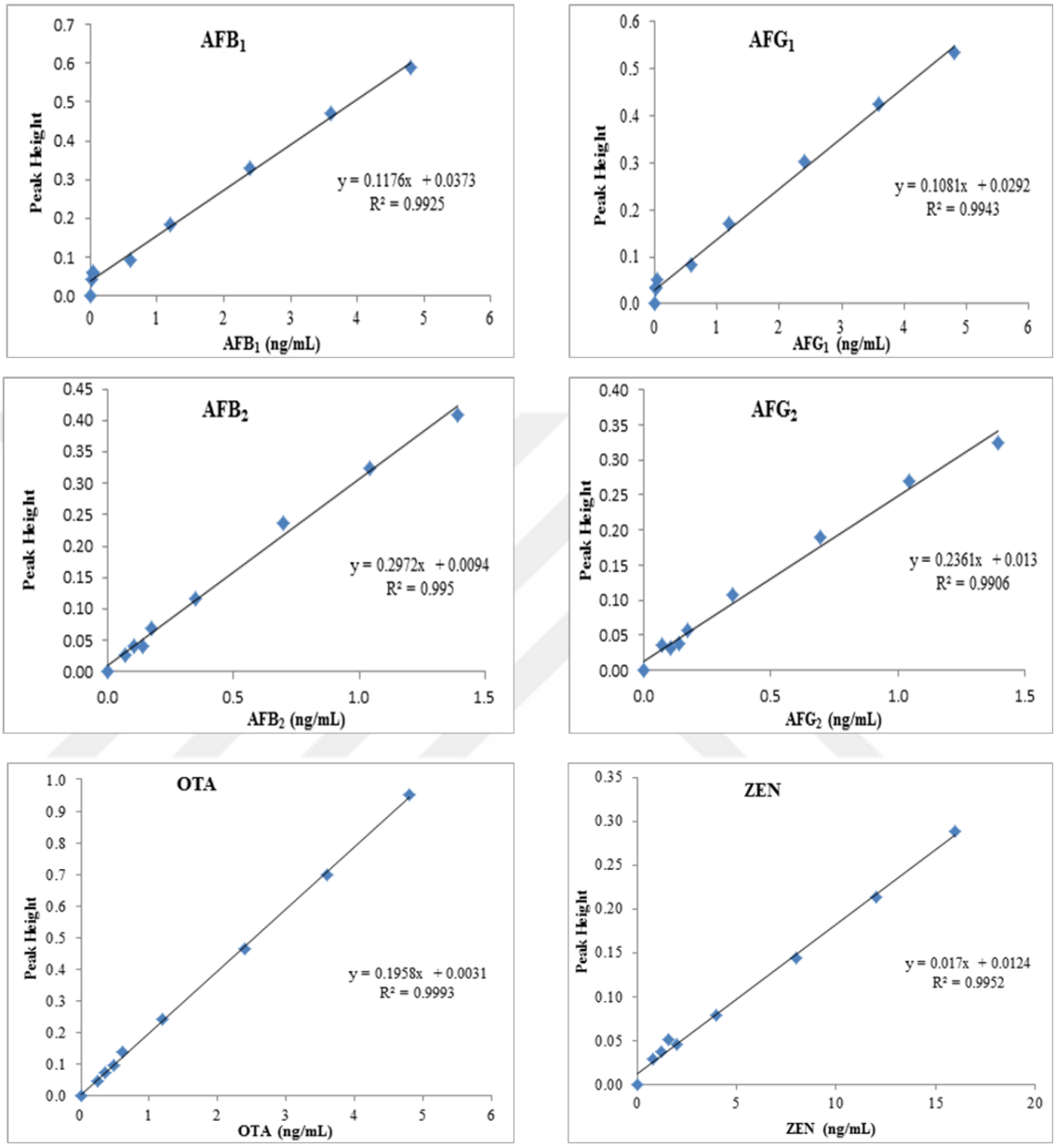


Figure 4.1. Calibration curves for aflatoxins (B_1, B_2, G_1, G_2), OTA, and ZEN obtained by AOZ HPLC-FLD method

4.1.3 LOD and LOQ for AFM_1 by HPLC-FLD

LOD and LOQ were calculated by the same way, from the signal-to-noise (S/N) ratios and defined as an S/N of 3 and 10, respectively. LOD was 0.008 ng/mL while LOQ was 0.026 ng/mL.

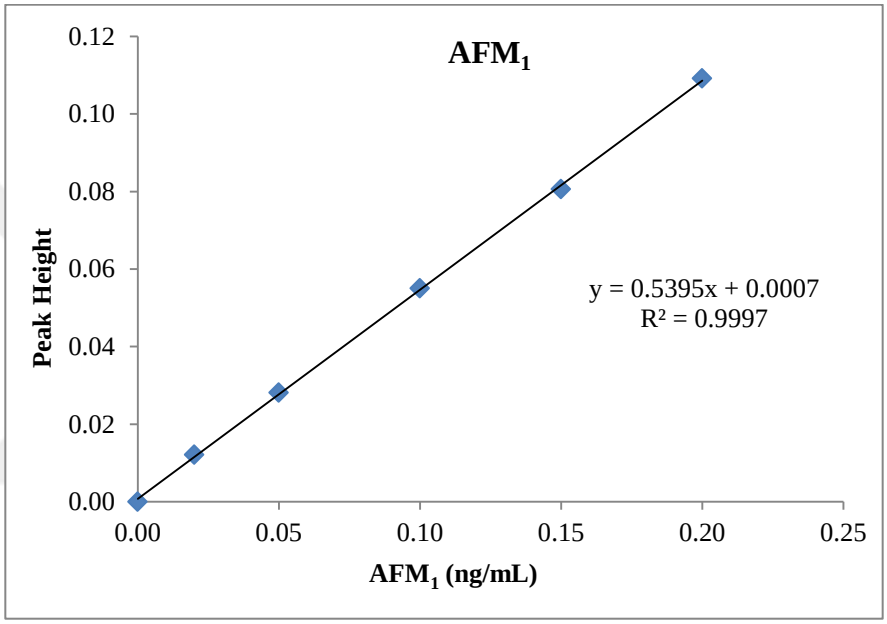


Figure 4. 2. Calibration curve for Aflatoxin M_1

The standard solutions of concentrations from 0.02 to 0.2 ng/mL AFM_1 were used to find calibration/standard curve as described by the following regression equation: y = 0.5395x + 0.0007, where y = peak height and x = amount of AFM_1. The results showed the linearity of the standard curve over the range studied. The coefficient of determination (R^2) was 0.9997 (Figure 4.2).

4.1.4 Recovery rate for AFM_1 by HPLC-FLD

Recovery was performed to determine the efficacy of the analytical method by spiking 50 mL of defatted milk sample with 200 µL of AFM_1 main stock (20 ng/mL). The spiked sample was left for 3 hours in the dark at RT. After incubation, they were extracted as described before in section 3.2.2. The recovery performed once and was found to be 143%.

4.1.5 LOD, LOQ and recovery rate for LC-MS/MS

LOD and LOQ were calculated also from the signal-to-noise (S/N) ratios and defined as an S/N of 3 and 10, respectively. For the analytes addressed by legislation, the respective limits of detection were far below the regulatory limits set in Egypt and the European Union (EU) for maize and animal feed.

For the determination of the variability of the apparent recovery, 10 individual samples (maize n=5) and (feed n=5) that were previously found to contain no or only traces of the detected metabolites were spiked at the one concentration level equivalent to the high level used for the initial evaluation, SDT 10 (i.e 1:20 dilution of mastermix). Therefore, 100 µL of the multi-component standard containing more than 600 analytes in addition to 66.6 µL of diluted fumonisins (B_1+B_2+B_3) were added to 0.25 g of the respective sample. The diluted fumonisins mix was prepared by mixing 80 µL of each stock solution of (fumonisin B_1, B_2) and (fumonisin B_3) with 640 µL of extraction dilution solvent (1:1, v/v). The spiked samples were left overnight in the dark at RT for evaporation of the solvent and distribution of analytes within the matrix. Afterwards, they were extracted using 1 mL of extraction solvent as described previously in the literature (82). Recovery rates for the detected metabolites by LC-MS/MS are shown in Table 4.9 and 4.10.

4.1.6 Multi-mycotoxins detection in feeds and maize by HPLC-FLD with IACs

In both feed and maize samples (n=78) the most occurred mycotoxins was AFB_1. In total 23 samples in feed and maize have been positively detected for AFB_1 contamination (Figure 4.3) and 7 samples for AFB_2, 4 samples for ZEN and only one sample was contaminated with OTA. The prevalence, mean and median values are shown in Table 4.3.

Table 4.3. Overview on the detected analytes in maize and animal feed by HPLC-FLD

Mycotoxin	AFB_1 (µg/kg)	AFB_2 (µg/kg)	AFG_1 (µg/kg)	AFG_2 (µg/kg)	OTA (µg/kg)	ZEN (µg/kg)
No. of positive samples	23	7	-	-	1	4
Max value	44.93	7.06	-	-	0.63	11.93
Min value	0.14	0.18	-	-	0.63	3.38
Median	0.93	1.59	-	-	0.63	8.61
Mean	6.28	1.94	-	-	0.63	8.14
Prevalence %	29	9	-	-	1	5

In feed (total n=17), 47 % (n=8) of the samples were contaminated with AFB_1 while in maize (total n= 61) 23% (n=15) have been positively detected for AFB_1 contamination. However, the maximum value of AFB_1 in feed was much lower than the detected one in maize. ZEN was detected only in feed samples while OTA has been found in one maize sample. The prevalence, mean and median values are shown in Table 4.4 and 4.5.

Table 4.4. Overview on the detected analytes in animal feed by HPLC-FLD

Mycotoxin	AFB1 (µg/kg)	AFB_2 (µg/kg)	AFG_1 (µg/kg)	AFG_2 (µg/kg)	OTA (µg/kg)	ZEN (µg/kg)
No. of positive samples	8	1	-	-	-	4
Max value	5.92	0.53	-	-	-	11.82
Min value	0.14	0.53	-	-	-	1
Median	0.78	0.53	-	-	-	8.45
Mean	1.56	0.53	-	-	-	8.14
Prevalence%	47	6	-	-	-	24

Table 4.5. Overview on the detected analytes in maize by HPLC-FLD

Mycotoxin	AFB1 (µg/kg)	AFB_2 (µg/kg)	AFG_1 (µg/kg)	AFG_2 (µg/kg)	OTA (µg/kg)	ZEN (µg/kg)
No. of positive samples	15	6	-	-	1	-
Max value	44.93	7.06	-	-	0.60	-
Min value	0.24	0.18	-	-	0.60	-
Median	1.35	1.76	-	-	0.63	-
Mean	8.79	2.21	-	-	0.63	-
Prevalence %	25	10	-	-	2	-

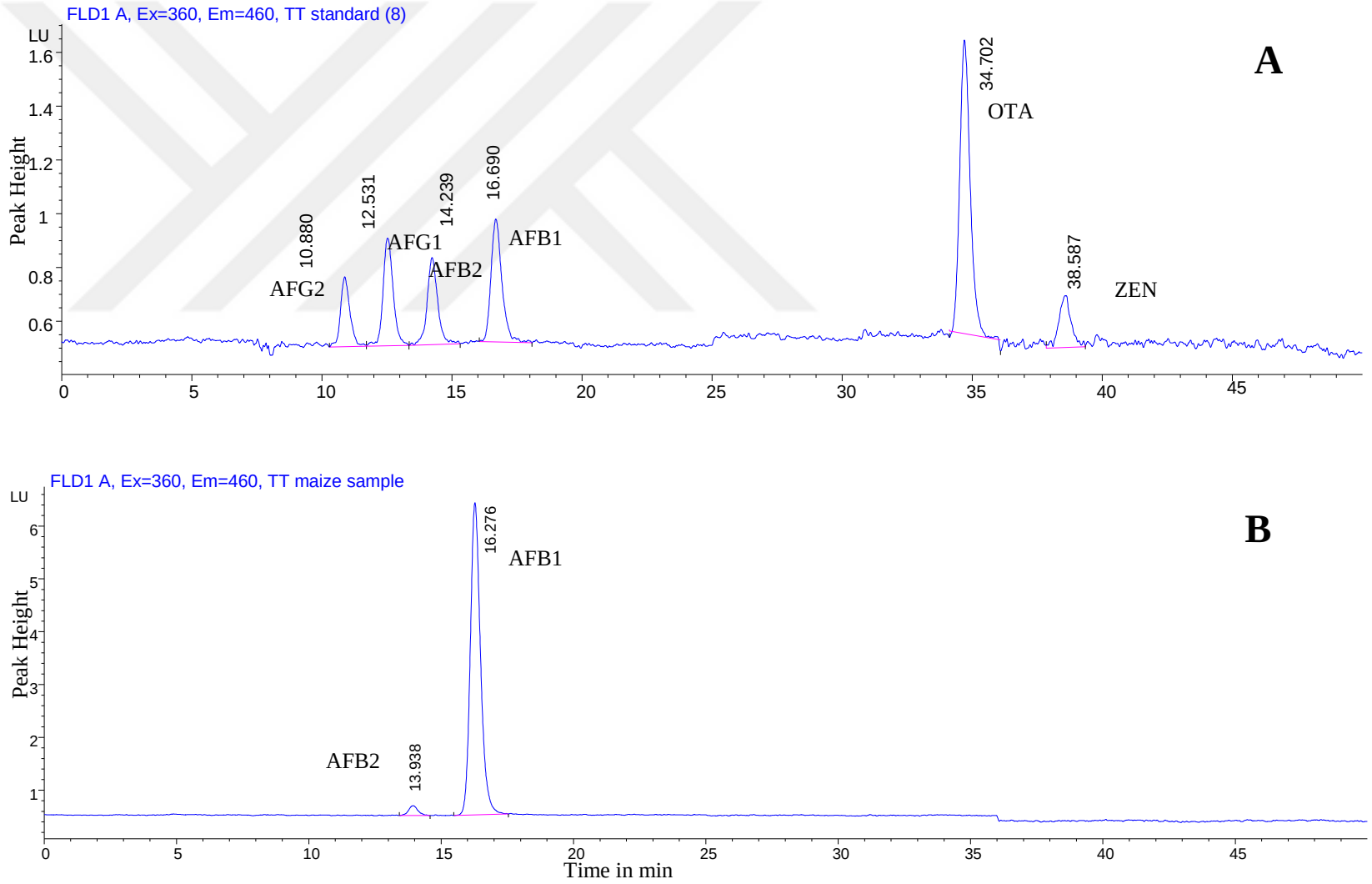


Figure 4.3. HPLC-FLD chromatographs of one standard solution (A) and contaminated maize sample (B) with AF (B_1, B_2)

4.1.7 AFM_1 detection in raw dairy milk samples by HPLC-FLD

The analytical results showed that AFM_1 is detectable in all examined milk samples (100%) and 14 samples (70%) were above the maximum permissible level in the EU (0.05 μg/kg). The concentrations ranged from 0.02 μg/kg to 0.19 μg/kg except only one sample was under limit of quantification. The median concentration and mean values were 0.08 μg/kg. Figure 4.4 shows a chromatograph of one contaminated samples with standard solution injection.

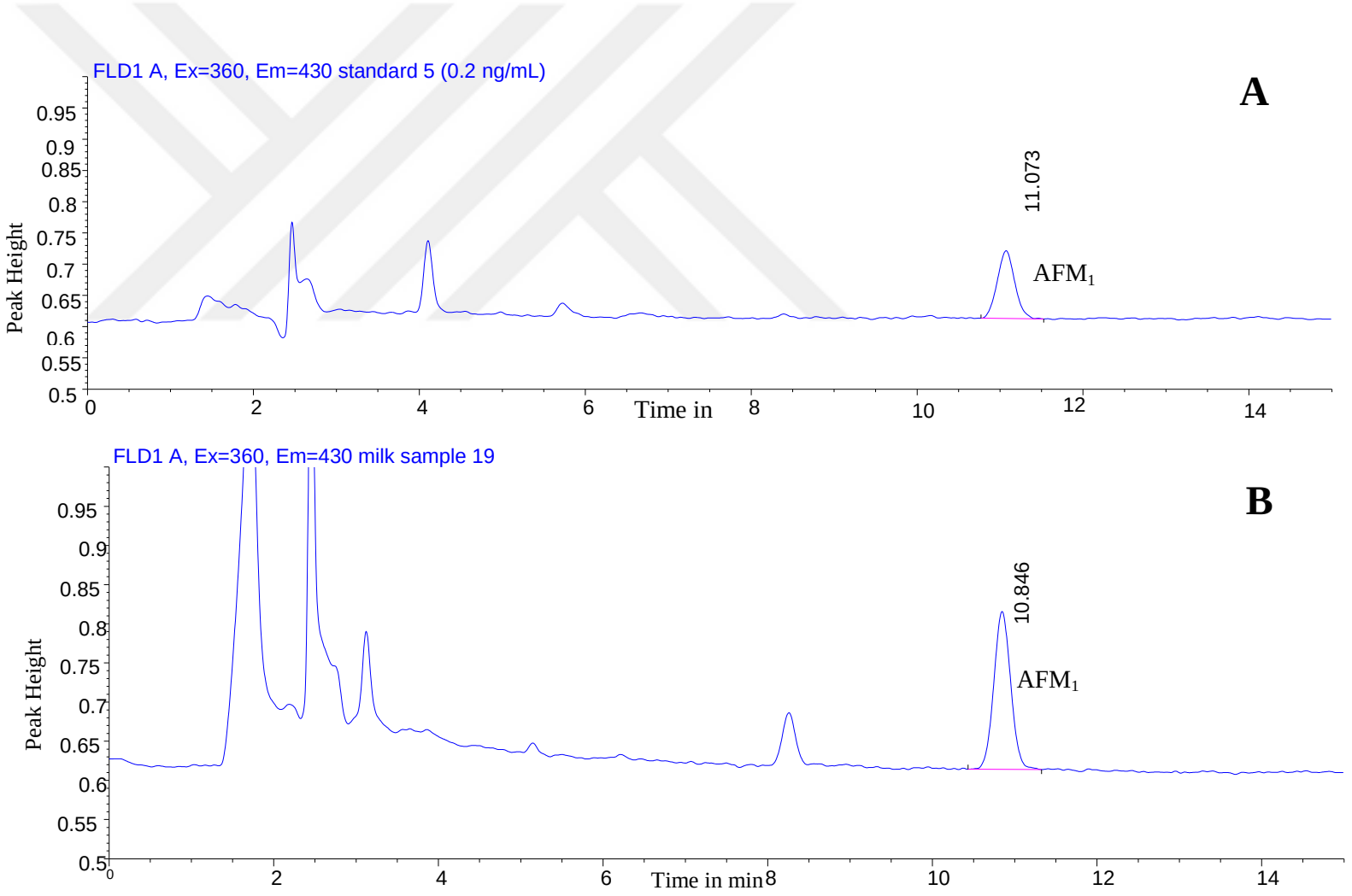


Figure 4.4. HPLC-FLD chromatograph of one standard solution (A) and contaminated milk sample (B) with aflatoxin M_1

4.1.8 Multi-mycotoxins detection in feeds and maize by LC-MS/MS

Overall, multimycotoxin analysis using LC-MS/MS revealed the contamination of maize and animal feed by 115 different fungal and bacterial metabolites. All major mycotoxins and their derivatives (aflatoxin B_1, aflatoxin B_2, aflatoxin M_1, fumonisin B_1, fumonisin B_2, fumonisin B_3, fumonisin A_1 precursor, deoxynivalenol, 3-acetyl-deoxynivalenol, deoxynivalenol-3-glucosid, nivalenol, T-2 toxin, HT-2 toxin, ochratoxin A, zearalenone, alpha-zearalenol, and beta-zearalenol) have been quantified in both commodities. In addition to some of *Fusarium* metabolites (apicidin, aurofusarin, beauvericin, butenolid, enniatin A, enniatin A1, enniatin B, enniatin B1, equisetin, epi-equisetin, fusaproliferin, moniliformin, culmorin, and 15-hydroxyculmorin), aflatoxin precursors (averantin, averufanin, averufin, norsolorinic acid, sterigmatocystin, seco-sterigmatocystin, and versicolorin A), *Alternaria* metabolites (alternariol, altertoxin-I, alternariolmethylether, macrosporin, tentoxin, and tenuazonic acid), other *Aspergillus* metabolites (3-nitropropionic acid, citrinin, dihydrocitrinone, and kojic acid), other *Penicillum* metabolites (griseofulvin), metabolites from other fungal species (cytochalasin H and cytochalasin J, emodin, skyrin, radicicol, tryptophol, and brevianamid F) and bacterial metabolites (nonactin) have been found. The detected metabolites in positive and negative mode (Figure 4.5 and 4.6) were 54 metabolites and 61 metabolites, respectively (see Table 4.6). On average 26 different metabolites were detected per sample. Maximum number of analytes per samples was 54 while the minimum one was 4 analytes.

In feed samples, 97 metabolites have been positively identified while in maize were 82 metabolites in total. There were some mycotoxins are detected only in maize e.g. AFB_2, Aflatoxin G_2, AFM_1 (see Figure 4.7 for AFB_1 and AFM_1) and OTA as presented in Table 5. Only in feed samples, alpha-zearalenol, beta-zearalenol, HT-2 toxin and T-2 toxin were positively identified with other mycotoxins (see Tables 4.8 and 4.9). OTA was only detected in 2 samples in maize and was below limit of detection in other maize samples and feeds. The prevalence of the major mycotoxins metabolites in

feed as shown in Table 6 were, ZEN detected in 71 (92%) samples, FB_1 59 (67%), DON 55 (71%), T-2 toxin 19 (24%), and AFB_1 3 (4%). In maize, the incidence of the major mycotoxins metabolites as shown in Table 7 were, FB_1 detected in 40 (50%) samples, AFB_1 13 (16%), ZEN 10 (12%), and DON 6 (7%).

Table 4.6. Overview on the detected analytes in maize and animal feed by LC-MS/MS in positive mode

Positive mode		
15-Hydroxyculmorin	Diplodiatoxin	O-Methylsterigmatocystin
3-Acetyldeoxynivalenol	Elymoclavine	Oxaline
7-Hydroxypestalotin	Enniatin A	Pestalotin
Aflatoxin B1	Enniatin A1	Questiomycin A
Aflatoxin B2	Enniatin B1	Quinocitrinine A
Aflatoxin M1	Enniatin B	Rugulovasine A
Asperglaucide	Ergometrinine	Siccanin
Aurofusarin	Fumonisin B1	Sterigmatocystin
Beauvericin	Fumonisin B2	T-2 toxin
Brevianamid F	Fumonisin B3	Tryptophol
Citrinin	Fusaproliferin	Viridicatin
Cordycepin	Fusapyron	Xanthotoxin
Culmorin	Griseofulvin	
cyclo(L-Leu-L-Pro)	HT-2 toxin	
cyclo(L-Pro-L-Tyr)	hydrolysed Fumonisin B1	
cyclo(L-Pro-L-Val)	Infectopyron	
Cytochalasin H	Kojic acid	
Cytochalasin J	Monocerin	
Decalonectrin	N-Benzoyl- Phenylalanine	
Destruxin A	Nonactin	
Destruxin B	Ochratoxin A	

Table 4.7. Overview on the detected analytes in maize and animal feed by LC-MS/MS in negative mode

Negative mode		
3-Nitropropionic acid	Dihydrocitrinone	Neoechinulin A
alpha-Zearalenol	Dihydroxymellein	Nivalenol
Alternariol	DON-3-glucoside	Norlichexanthone
Alternariolmethylether	Emodin	Norsolorinic acid
Altersolanol	EpiEquisetin	Quinadoline A
Altertoxin-I	Equisetin	Quinolactacin A
Apicidin	Fallacinol	Radicicol
Ascochlorin	Fellutanine A	Rugulosin
Asperphenamate	Fumonisin A1 Vorstufe	seco-Sterigmatocystin
Averantin	Fusarin C	Siccanol
Averufanin	Griseophenone B	Skyrin
Averufin	Griseophenone C	Tentoxin
Berkedrimane B	Ilicicolin A	Tenuazonic acid
beta-Zearalenol	Ilicicolin E	Usnic acid
Butenolid	Integracin A	Versicolorin A
Chlorocitreorosein	Iso-Rhodoptilometrin	Violaceol II
Citreorosein	LL-Z 1272e	Zearalenone
Cladosporin	Lotaustralin	
Curvularin	Macrosporin	
Dehydroaustinol	Meleagrin	
Demethylsulochrin	Methylsulochrin	
Deoxynivalenol	Moniliformin	

Table 4.8. Overview of the only detected analytes in animal feed and in maize by LC-MS/MS

Metabolites only in feed
alpha-Zearalenol
Altersolanol
Altertoxin-I
Apicidin
Berkedrimane B
beta-Zearalenol
Chlorocitreorosein
cyclo(L-Leu-L-Pro)
Cytochalasin H
Cytochalasin J
Decalonectrin
Dehydroaustinol
Destruxin A
Destruxin B
Elymoclavine
EpiEquisetin
Fusaproliferin
Griseofulvin
Griseophenone B
Griseophenone C
HT-2 toxin
Infectopyron
Lotaustralin
Methylsulochrin
Nonactin
Quinadoline A
Rugulosin
Rugulovasine A
seco-Sterigmatocystin
Siccanin
Sterigmatocystin
T-2 toxin
Tenuazonic acid
Violaceol II

Metabolites only in maize
3-Acetyldeoxynivalenol
7-Hydroxypestalotin
Aflatoxin B_2
Aflatoxin G_2
Aflatoxin M_1
Averantin
Averufin
Butenolid
Citrinin
Dihydrocitrinone
Integracin A
Kojic acid
Meleagrin
Moniliformin
Norsolorinic acid
Ochratoxin A
Pestalotin
Radicicol
Viridicatin

Table 4.9. Overview on the prevalence and concentrations of selected analytes in animal feed by LC-MS/MS

Feed (n =77)							
Row Labels	Positive	Prevalence %	Median (µg/kg)	Maximum (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery %
15-Hydroxyculmorin	68	88	83.2	820	1.3	4.4	108
3-Nitropropionic acid	2	2.6	5.6	< LOQ	3	10	56
Aflatoxin B_1	3	3.9	5.3	11	0.72	2.4	59
alpha-Zearalenol	5	6.5	7.3	8	1.3	4.5	84
Alternariol	63	82	1.9	95.6	0.4	1.4	74
Alternariolmethylether	73	95	8.9	245	1.2	3.8	63
Asperglaucide	76	99	47.7	591	1.1	3.6	98
Asperphenamate	75	97	5.1	45.5	0.1	0.33	102
Aurofusarin	56	73	355	3005	13	42	82
Beauvericin	68	88	6.45	91	0.85	2.8	83
beta-Zearalenol	28	36	3.9	60	1.2	3.5	87

Brevianamid F	66	86	44.2	353	7.8	25	93
Cladosporin	17	22	2.8	83	0.22	0.66	70
Culmorin	53	69	6.77	64	0.26	0.86	106
cyclo(L-Pro-L-Tyr)	77	100	307	4244	1.4	4.7	90
cyclo(L-Pro-L-Val)	74	96	294	12100	3	9.8	65
Deoxynivalenol	55	71	171	1516	9.5	31	86
DON-3-glucoside	8	10	49.4	239	5.9	19	61
Emodin	71	92	1.96	76	0.45	1.5	76
Enniatin A	67	87	0.38	1.86	0.03	0.11	99
Enniatin A1	70	91	0.19	6.4	0.01	0.03	142
Enniatin B	72	93	0.21	16	0.14	0.46	110
Enniatin B1	70	91	0.41	25	0.04	0.12	85
EpiEquisetin	60	78	2.32	34.5	0.38	1.25	106
Equisetin	68	88	1.12	16	0.05	0.18	174

Fellutanine A	58	75	24.7	812	4.4	14	39
Fumonisin B_1	59	76	459	2409	2.6	8.5	93
Fumonisin B_2	53	69	55.4	260	1	3.3	88
Fumonisin B_3	42	54	106	310	3.8	11	69
Fusaproliferin	44	57	198	1381	6.7	22	100
HT-2 toxin	10	13	5.47	32.3	1.7	5.7	89
hydrolysed Fumonisin B_1	31	40	10.2	34.3	2.9	9.6	68
Ilicicolin A	20	26	0.3	13.2	0.15	0.50	45
Ilicicolin E	26	34	0.06	3.31	0.02	0.08	77
Iso-Rhodoptilometrin	49	64	0.5	3.3	0.11	0.35	75
Macrosporin	65	84	2.55	1060	0.04	0.12	70
Monocerin	42	54	1.35	29.8	0.64	2.1	90
Neoechinulin A	62	80	48.5	1085	1.67	5.5	34

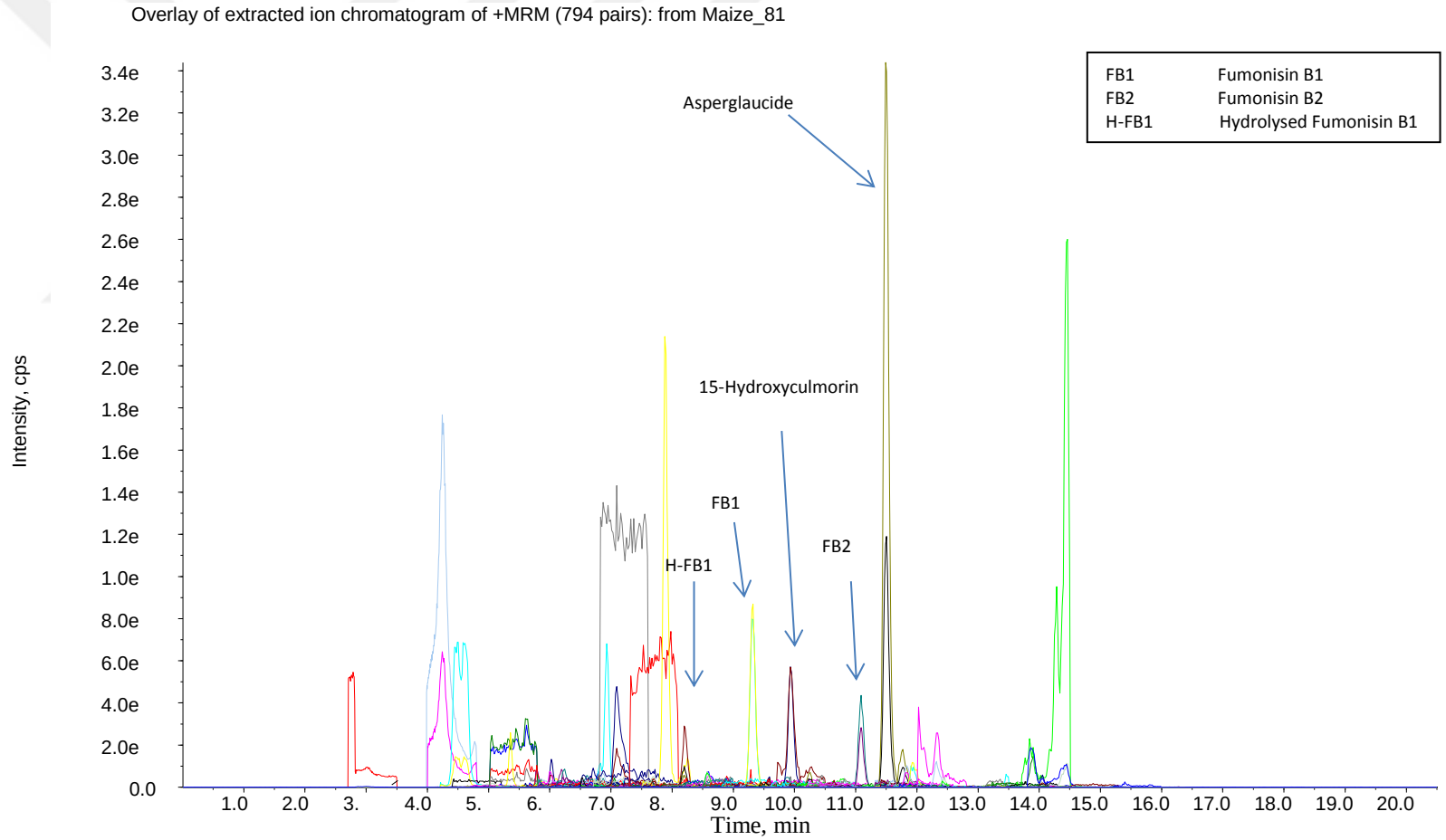
Nivalenol	44	57	18.7	146	0.66	2.2	77
Norlichexanthone	38	49	0.95	26.3	0.35	1.1	45
T-2 toxin	19	25	2.83	39.5	1.05	3.5	87
Tentoxin	59	77	2.2	133	0.21	0.69	98
Tenuazonic acid	20	26	17.5	292	0.93	3.1	200
Tryptophol	69	90	331	10878	29	96	95
Xanthotoxin	12	16	4.1	24.7	0.5	1.6	79
Zearalenone	71	92	33.2	791	0.64	2.1	86

Table 4.10. Overview on the prevalence and concentrations of selected analytes in maize by LC-MS/MS

Maize (n=79)							
Row Labels	Positive	Prevalence %	Median (µg/kg)	Maximum (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery %
15-Hydroxyculmorin	18	23	0.64	245	0.12	0.38	95
3-Nitropropionic acid	46	58	6.11	823	0.15	0.5	82
Aflatoxin B_1	13	16	4.81	197	0.3	0.98	69
Aflatoxin B_2	4	5	7.65	9.8	0.42	1.4	72
Aflatoxin M_1	4	5	1.21	2.69	0.1	0.32	82
Alternariol	4	5	0.38	< LOQ	0.15	0.50	83
Alternariolmethylether	5	6	0.3	< LOQ	0.16	0.54	97
Asperglaucide	55	70	0.6	825	0.03	0.11	95
Asperphenamate	66	83	0.13	49.5	0.01	0.03	98
Aurofusarin	7	9	123	1858	8.3	27.3	62
Beauvericin	50	63	0.64	71.6	0.06	0.19	111
Brevianamid F	5	6	1.3	4.9	0.79	2.6	84
Butenolid	38	48	2.3	529	0.34	1.1	53

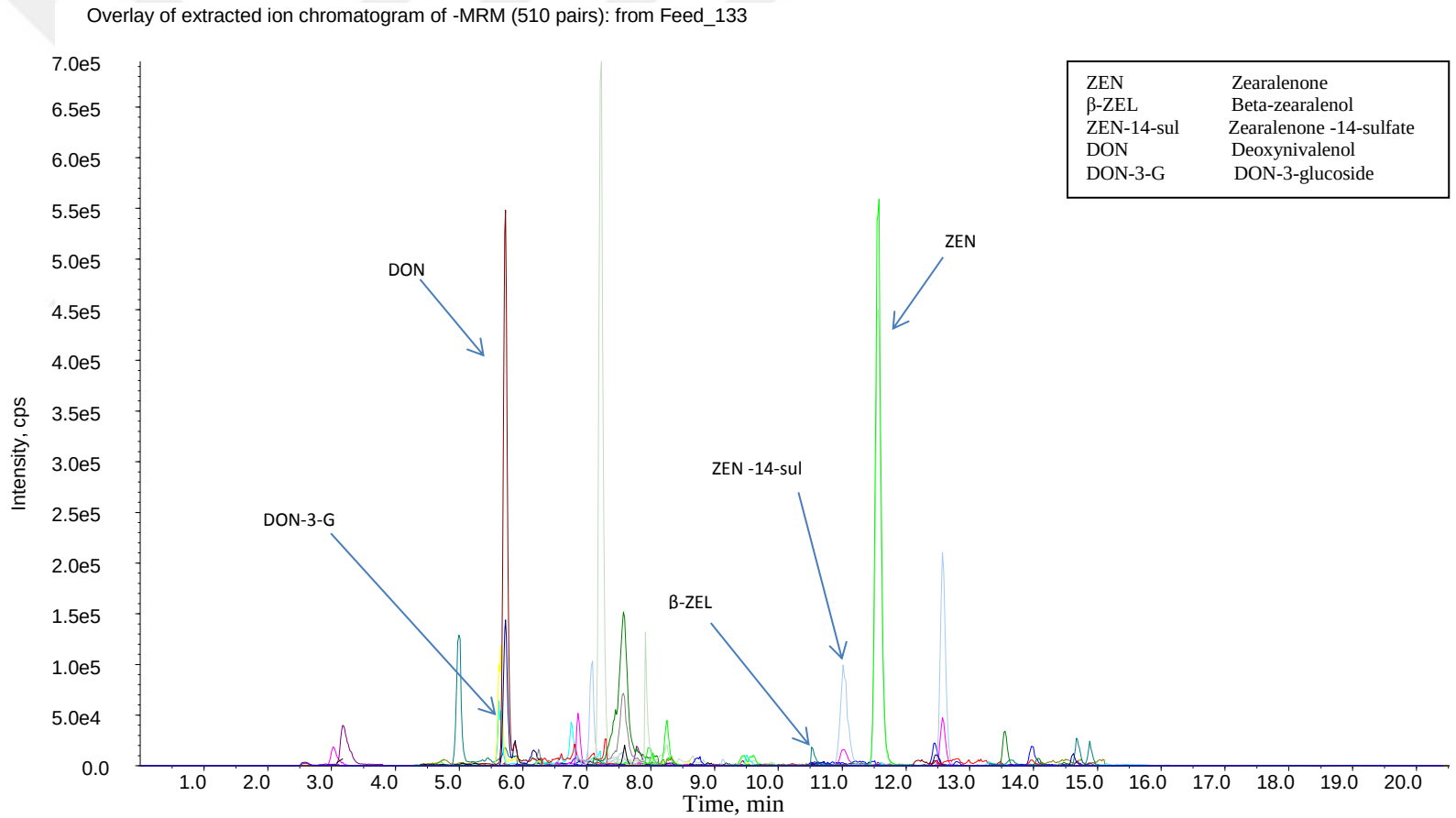
Cladosporin	6	8	1.9	12.2	0.23	0.74	80
Culmorin	6	8	5.3	15.5	0.3	0.99	105
cyclo(L-Pro-L-Tyr)	34	43	6.3	21.4	0.89	2.9	75
cyclo(L-Pro-L-Val)	79	100	13.2	73.5	0.97	3.2	72
Deoxynivalenol	6	8	311	807	26	86	103
Deoxynivalenol-3-glucoside	4	5	18.9	47.5	8.5	28	69
Emodin	45	57	0.15	66.6	0.071	0.25	84
Enniatin A	25	32	0.003	0.09	0.001	0.0033	93
Enniatin A1	28	35	0.04	0.09	0.011	0.033	101
Enniatin B	35	44	0.01	0.05	0.0011	0.0033	121
Enniatin B1	22	28	0.03	0.09	0.01	0.02	89
Equisetin	6	77	0.2	0.5	0.081	0.26	158
Fellutanine A	1	1	0.3	0.35	0.06	0.21	41
Fumonisin B_1	40	51	67.9	2453	1.04	3.4	85
Fumonisin B_2	14	178	4.68	386	1.3	4.3	81
Fumonisin B_3	6	8	16.8	286	1.5	4.9	81

Hydrolysed Fumonisin B_1	6	8	6.77	24	0.98	3.2	77
Ilicicolin A	15	19	0.37	0.96	0.2	0.65	38
Ilicicolin E	2	2.5	0.18	0.25	0.09	0.28	85
Iso-Rhodoptilometrin	16	20	0.22	6	0.06	0.21	82
Kojic acid	11	14	1565	25040	16	55	85
Macrosporin	17	21	0.89	382	0.09	0.28	89
Moniliformin	18	23	1.66	142	0.02	0.07	101
Monocerin	24	30	2.29	56.5	0.09	0.31	89
Neoechinulin A	8	10	9.66	34.7	1.1	3.8	45
Nivalenol	8	10	38.6	195	0.81	2.6	64
Norlichexanthone	1	1	0.22	0.22	0.1	0.34	68
Ochratoxin A	2	2.5	6.18	11.4	2.8	9.4	91
Tentoxin	2	2.5	0.02	0.04	0.01	0.03	97
Tryptophol	13	16	28.49	101	14	47.5	66
Xanthotoxin	3	4	0.34	0.74	0.25	0.83	84
Zearalenone	10	13	3.44	184	0.46	1.5	92



*cps: Count per second

Figure 4.5. Overlay of extracted ion chromatogram of + MRM



*cps: Count per second

Figure 4.6. Overlay of extracted ion chromatogram of - MRM

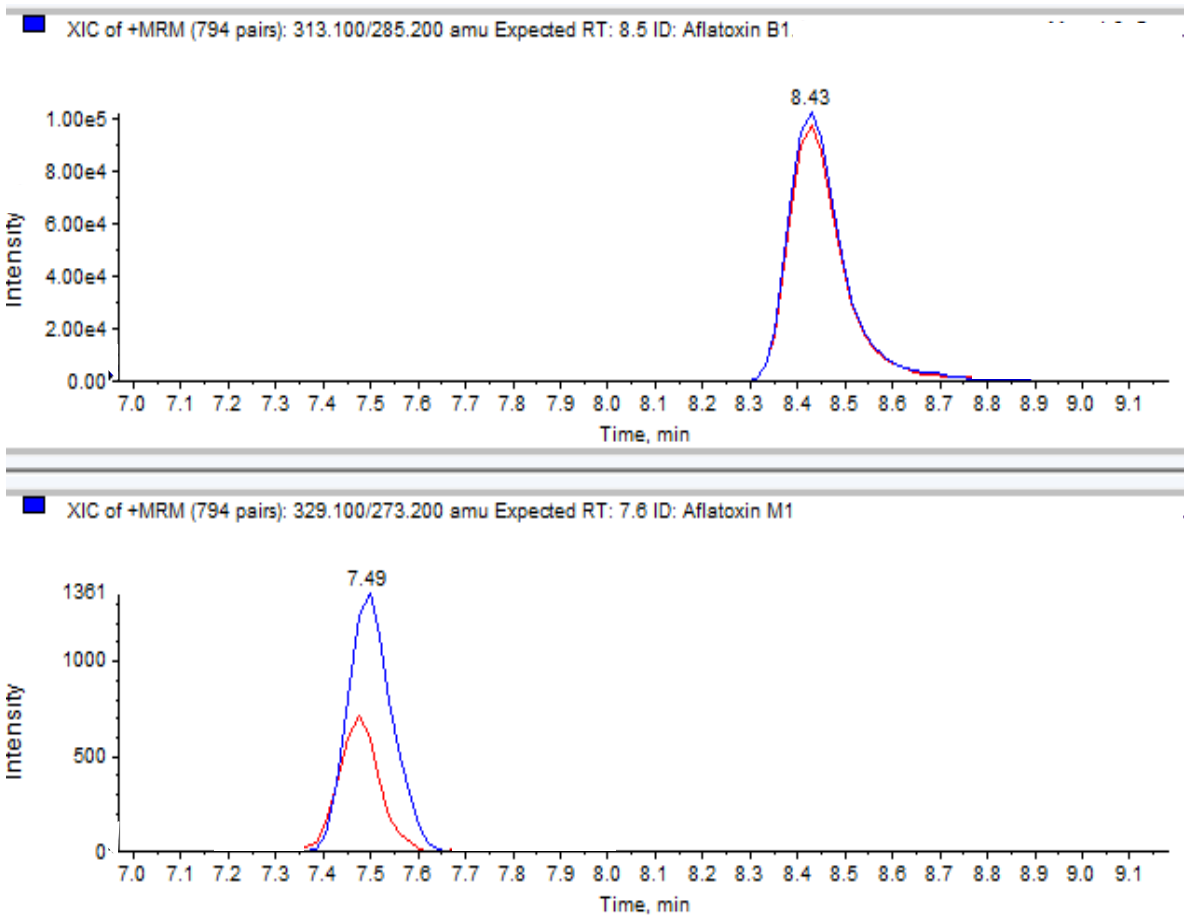


Figure 4.7. Smoothed extracted ion chromatogram for AFB_1 and AFM_1 in maize

5. DISCUSSION

Mycotoxins are surrounding us in our daily life. Food and feed contamination with mycotoxins is an alerting concern in both developed and developing countries. Based on their health and economic impacts, the number of nations that regulate mycotoxins has been expanded to be more than 100 nations over the last few years. These regulations differ among countries and may affect to a great extent on the patterns of feed and food trade (72 - 75, 94). It is well known that there is no enough data on multi-mycotoxin occurrence in maize and animal feed from the Egyptian markets, especially in the upper part of the country. Furthermore, the previous studies have emphasized on either single or a few of "classic" mycotoxins in maize and/or feed using less precise methods. For example, TLC detection of AFB_1 and ZEN in maize by El-Gohary (95) and Abd Alla (96), respectively. Total AFs using TLC in cereal grains including maize (97), total AFs in feedstuffs using ELISA (98), and fumonisins in maize from Assiut city using ELISA (99). Other previous reports between 1990 till 2013 are summarized in Table 5.1.

Table 5.1. Previous reports for mycotoxins detection in Egypt

Commodity	Area	Mycotoxin (s)	Detection method	Author
Feedstuffs	Dakahlia and Kafr El-Shikh	AFs, OTA, citrinin, ZEN and DON	TLC	Abdelhamid et al. (100)
Silage	Assuit and Sohag	Total AFs, T-2 toxins and sterigmatocystin	TLC	El-Shanawany et al. (101)
Maize	Cairo	Total AFs and FB_1 and FB_2	HPLC-FLD (two separated steps for extraction and sample clean-up)	Madbouly et al. (102)
Maize	Sohag	FB_1 and FB_2, ZEN, DON and T-2 toxin	TLC as qualitative and HPLC-FLD as confirmatory quantitative tool	Aboul-Nasr et al. (103)
Maize with other cereal grains	Cairo	ZEN	HPLC-FLD with IACs	El-Desouky and Naguib. (104)
Maize	10 governorates including Assiut	Total AFs, FB_1, OTA and ZEN	HPLC-UV (sample clean-up by IACs and SPE for AFs and FB_1, respectively)	Nooh et al. (105)

Also for AFM_1 in milk and milk products, ELISA technique was mainly used (98, 106 - 109). However, El-Sayed et al. (110) surveyed AFM_1 in milk from Cairo and Giza by using HPLC-FLD with IACs for clean-up purpose.

On the other hand, maize together with wheat and rice are the most important cereal grains in the world. The total amount of maize in Egypt imported from 53 different nations during the period from 2000 to 2009 is approximately 51,446,403 metric tons. Most of the imported maize is coming from USA, Argentina, Brazil, and Ukraine (i.e. Egypt imports maize from three different continents). With these amounts, Egypt is the third country among top maize importers after the Republic of Korea, Mexico over the last decade (111). However, only total AFs and AFB_1 up till now have been regulated for both maize and animal feed 20 μg/kg and 10 μg/kg, respectively (94, 112).

The regulatory limit for AFM_1 in milk according to some previous published papers based on Egyptian Regulations in 1990 that the milk sold in Egyptian markets should be free of AFM_1 (108, 109). Although, FAO mentioned in 2003 for Egypt total AFs and AFB_1 in feed and food, without AFM_1 (76).

The main aim of this work was the determination of multi fungal and bacterial metabolites in commercial animal feed used for farm animal feeding and maize as it is the main constituent in animal feed manufacturing in Egypt. Maize and feed as well as milk samples were collected from Assiut to be analyzed at Pharmaceutical Toxicology Research Laboratory, Hacettepe University by using HPLC-FLD for 6 major mycotoxins, AFB_1, AFB_2, AFG_1, AFG_2, ZEN and OTA after enrichment of the analytes through IACs application with PCD in addition to AFM_1 in raw dairy milk samples.

In continuation of our interest on the determination of different fungal metabolites in maize and feed commodities other samples from a wide zone, three different cities in Upper Egypt, were collected to be analyzed using LC-MS/MS for

more than 600 multi-fungal and bacterial metabolites. To date, no survey for the occurrence of such of this huge number of metabolites in feed and/or in maize have been done in Egypt using LC-MS/MS or even HPLC-FLD with photochemical derivatization and IACs for cleanup in the upper part of the country.

5.1 AFM_1 detection in raw dairy milk samples by HPLC-FLD

Aflatoxin M_1 in milk depends exclusively on the absorbed concentrations of the precursor AFB_1 in dairy milking animals. The determined levels of AFM_1 observed in the present study (20-190 ng/kg) was lower than the incidence of AFM_1 reported by other authors (106,109,110) and higher than (98, 107, 108) as shown in Table 5.2 which summarizes the previous reports in Egypt. In the present study, the detected concentration of AFM_1 in raw milk samples might be due to consuming of AFB_1 contaminated feeds by dairy animals in Upper Egypt or due to unsanitary conditions and contamination of the utensils used inside local markets for handling, boiling, and selling the raw milk to the population.

Table 5.2 Some previous reports for aflatoxin M_1 in Egypt

Sampling area	Detection method	Detected range (ng/kg)	Author (s)
Assiut	ELISA	0-15	Salem (98)
Ismailia	ELISA	10-200	Motawee et al. (106)
Alexandria	ELISA	23 – 73	Amer and Ibrahim (107)
Qena	ELISA	2-110	Ghareeb et al. (108)
Assiut	ELISA	99-500	Shaker and Elsharkawy (109)
Cairo and Giza	HPLC-FLD	5000-8000	El-Sayed et al. (110)

5.2 Multi-mycotoxins detection in feeds and maize by HPLC-FLD and LC-MS/MS

ZEN was the most common major mycotoxin in feed in which 92% of the samples were contaminated while in maize, ZEN infected only 12% of samples. Similar to this, 71% of feed samples were infected by DON while in maize only 7% of DON were contaminated. The high incidence of ZEN and DON in feed in comparison with maize suggesting that some other feed ingredients beside maize used in commercial feed manufacturing as a source of protein might be attacked with mycotoxin producing fungi either in the field or during storage. Adding to this, the detection of T-2 was only in feed (25% of samples) as shown in Tables 4.8 and 4.9. The contamination level of ZEN detected in the present study with maximum values 791 and 184 µg/kg in feed and maize, respectively was generally higher than the previous ones reported from maize in Cairo (2.15 µg/kg) (104), in some Egyptian districts (45.2 µg/kg) (96) and (3.5µg/kg) (105), and other surveys (100).

However, all the previous mentioned reports used either TLC or HPLC-UV for detection and no reports are available to date used LC-MS/MS regarding Egypt. For ZEN, α-ZEL, and β-ZEL detection by LC-MS/MS in poultry feeds, Abia et al. (113) reported lower ZEN (up to 600 µg/kg) and β-ZEL (Up to 28 µg/kg in comparison with feed from upper Egypt here, 59.57 µg/kg while α-ZEL was higher than the present survey, up to 20 µg/kg in feeds from Cameroon and 8.03 µg/kg for feeds from Egypt.

Our samples which have been analyzed by HPLC-FLD have ZEN only in 4 feed samples (5%) with a maximum value 11.93 µg/kg which is lower than other samples that have been analyzed by LC-MS/MS.

FB_1 was the most common major mycotoxin in both feed and maize. In feed was more likely to occur in feed (76.6% of feed samples) than in maize (50% of maize samples) (Table 4.9 and 4.10). However, fumonisins are associated almost exclusively

with maize and most probably the maize used in the feed was of inferior quality than the analyzed samples in our study. FB_2 and FB_3 have also been detected in feed and maize, but they are generally less prevalent in maize (17.7 and 7.6%, respectively) than in feeds (68.8 and 54.5%, respectively). In addition to, hydrolysed fumonisin B_1 (HFB_1) was determined in 7.6% of maize and 40% of feed samples. However, the maximum values of all FBs in feed were relatively less than in maize (FB_1, 2409 and 2453 µg/kg; FB_2, 259.8 and 386.4 µg/kg, respectively) except for FB_3 with HFB_1 (FB_3, 310.38, 286.5 µg/kg; HFB_1, 34.25 and 24 µg/kg, respectively). Salem and Ramadan (99) have reported higher levels of fumonisins (14.1 mg/kg) in raw corn samples from Assiut although they did not distinguish between the different types of fumonisins. Other studies have reported lower levels than reported here, FB_1, 947 µg/kg and FB_2, 310 µg/kg from Cairo districts in Madbouly et al. (102) and FB_1, 1915 µg/kg from Dakahlia city in Nooh et al. (105).

Aflatoxins (B_1, B_2, and M_1) were present in maize samples at different prevalence and contamination levels (Table 4.10). However, only AFB_1 was present in feed samples (Table 4.9). AFB_1 appeared to be present in a few number of samples in both feed and maize. The prevalence of AFB_1 in maize was lower relatively than in feed, AFB_1 was detected in 3 (3.9%) feed samples and 13 (16.5%) in maize. The maximum detected levels of AFB_1 were around twenty folds higher in maize (197.48 µg/kg) than in feed (11 µg/kg). Out of the positive samples in feed and maize, 1 and 5 samples, respectively were above the maximum Egyptian regulatory limits for AFB_1 (10 µg/kg) in maize and animal feed (76). AFB_2 in maize was detected in 4 samples with maximum level (9.7µg/kg). The current results confirm the presence of AFB_1 in maize from Egypt at levels higher than national and international limits as reported in maize from Cairo (19.2 µg/kg) (102) and maize from Assiut (21.8 µg/kg) (105).

AFB_1 level in feed was lower than that previously reported in feedstuff from different regions in Egypt where up to 400 µg/kg were detected (100) and from Assiut where 60 µg/kg have been detected (98).

AFM_1 may not be frequently detected in maize samples but it is common to find traces of this metabolite produced by *A. flavus* and *A. parasiticus* along with higher concentrations of AFB_1 (114). Previous surveys in Africa have reported the occurrence of AFM_1 in sorghum from Ethiopia and cassava samples from Tanzania with a maximum value 0.89 µg/kg and 0.3 µg/kg, respectively (30, 31). In the present study, AFM_1 have been quantified in 4 maize samples (5%) with a maximum value 2.69 µg/kg. AFM_1 in maize could be derived from insects metabolizing AFB_1 contaminated grains (32, 33).

Nevertheless, the occurrence of aflatoxins at levels as high as 197 µg/kg in some maize samples should be worth noting given the health risks associated with these toxins. In addition, there was similar prevalence of sterigmatocystin and other precursors (results are not shown).

Other samples in the present study which have been analyzed by HPLC-FLD, the prevalence was 47% of AFB_1 in feed and 23% in maize (see Table 4.4 and 4.5) with a maximum value 5.92 and 44.93 µg/kg, respectively which is lower than other samples analyzed by LC-MS/MS. OTA was detected in one maize sample, 0.6 µg/kg for median value (see Table 4.5)

Other major mycotoxins detected in the present study include DON and NIV. These two toxins were less prevalent in maize (7.6% and 10.1 %) than in feed (71.4 % and 57.1 %). Although DON occurred at higher levels in feed (maximum level was 1516.3 µg/kg DON) than in maize (maximum level was 807.2 µg/kg DON), NIV occurred at higher level in maize 195.2 µg/kg NIV then in feed 146.2 µg/kg NIV). T-2 and HT-2 have been detected only in feed samples with prevalence was 24.7% (19 samples) and 13 % (10 samples) with maximum levels 39.4 and 32.2 µg/kg, respectively.

The levels of DON and T-2 toxins detected in the present study (see Table 4.9 and 4.10) were generally higher than those reported by El-Sayed et al. (115), who detected up to 29.8 µg/kg DON and 12.7 µg/kg T-2 in maize samples and lower than T-2 levels reported by El-Maghraby et al. (116), who reported up to 30.4 µg/kg in maize from different Governorates of Upper Egypt.

The major conjugated form of DON, DON-3-G which forms up to 12% of the main toxin, was positively quantified in both feed and maize. The detection of the masked or modified mycotoxins such DON-3-G should be considered with the parent compound as there a possibility of the modified mycotoxin to be converted by hydrolysis up on ingestion inside the living organism. OTA was the only major mycotoxin that detected in 2 maize samples (see Table 4.10) at maximum value 11.4 µg/kg and was under level of detection in all other analyzed feed samples.

The commonly named emerging mycotoxins, other secondary toxic metabolites of *Fusarium* spp., such as fusaproliferin, beauvericin, enniatins, and moniliformin have been reported for the first time in Egypt. Up until now, only a limited number of surveillances have been published which have determined the emerging mycotoxins in different commodities especially from Africa. In the present study, the majority of feed samples and less than half of the maize were contaminated by enniatins (B, B1, A, and A1), while beauvericin occurred in a quite large number of samples in both commodities (88.3% of feeds and 63.3% of maize). The incidences enniatins B, B1, A and A1 were 72%, 70%, 67%, and 70% in feed and 35%, 22%, 25%, and 28% in maize, respectively. Nonetheless, their median levels were generally low (<1 µg/kg on average) in both commodities. However, the median and maximum values of enniatins in feeds were several folds higher than those reported in maize (see Table 6 and 7).

Our results in maize were higher in incidences and lower in contamination level than those reported by Oueslati et al. (117) in maize from Tunisia, up to 29.6 mg/kg enniatin A1 and up to 17 mg/kg enniatin B1, however, no other enniatins reported.

Beauvericin levels detected in the present study were up to 90.9 µg/kg in feed and 71.6 µg/kg maximum levels in maize. Beauvericin levels in the present study were at near range as detected by Abia et al. (118) in maize from Cameroon. However, more enniatins are detected in our samples (see Table 4.9 and 4.10)

On the other hand, moniliformin was detected only in maize with prevalence rate 22.8%, while fusaproliferin occurred in feed at higher prevalence rate, 57.1% of samples. Both of these metabolites occurred at higher levels (up to 142.1 µg/kg of moniliformin in maize and up to 1381.2 µg/kg of fusaproliferin in feeds). Moniliformin level was lower than detected by Adetunji et al. (119) in maize from Nigeria where up to 899 µg/kg were recorded.

Apart from these, there were broader pattern of fusarium metabolites including apicidin, aurofusarin, butenolid, equisetin, epi-equisetin, culmorin, and 15-hydroxyculmorin. Not only fusarium but also penicillium metabolites such as griseofulvin, and bacterial metabolites like nonactin have been detected (see Tables 4.6, 4.7, 4.8, 4.9, 4.10). Additionally, several fungal and bacterial metabolites have been detected. However, their quantifications were not possible due to lack of their standard solutions at the time of study.

The statistical calculation analysis using t-test showed that there is no significant difference between regions concerning the feed samples and between feed and maize in Assiut which was because of high standard deviation for the samples.

6. GENERAL CONCLUSION AND FUTURE WORKS

This study along with several other studies done before in Egypt and other different countries conclude that mycotoxins are still a serious problem altering both developed and developing countries. Different analytical (i.e., thin layer, gas and liquid chromatography) methods have been recommended to quantify various mycotoxins in food and feed. Although the present surveys show that the contamination rates in feed and maize were not alarming except for AFB_1 in maize and AFM_1 in milk, the necessity of further and permanent monitoring are highly recommended. Besides, the synergistic effects of the co-occurring mycotoxins should be determined as this may have a profound effect on human and animal health. These studies are the first reports on the multi-occurrence of a broad spectrum of mycotoxins and other fungal metabolites in feed in maize using reversed-phase liquid chromatography coupled with electrospray ionization triple quadrupole mass spectrometry and multi-mycotoxins contamination detection through HPLC-FLD with photochemical reactor and IACs for cleanup in Upper Egypt.

The survey presents very broad spectrum of mycotoxins including major mycotoxins such as AFs, FBs, ZEN, T-2, DON, OTA, and emerging mycotoxins such as enniatin A, enniatin B and beauvericin which are of great concern for human and animal even at low contamination level. These important surveys are required in order to establish an occurrence database for mycotoxins in Egypt to minimize the possible health risks accounting for mycotoxins exposure in addition to the economic impact on the world trade. More mycotoxins like FBs, ZEN, DON, and T-2 as well as emerging mycotoxins needed to be regulated by Egyptian authorities in order to decide whether maize and feed samples with these detected levels of contamination for example are allowed to be in Egyptian markets or not.

Moreover, to decide whether these commodities will be accepted for exportation to other countries in case it will fit with their maximum permissible level. National

cultivated maize is required to be analyzed and compared with imported one for better knowing the naturally growing fungi in the Egyptian environment and the chance for mycotoxin production with the others which can be imported from other countries such as Brazil, Ukraine and China as they are the largest exporting countries in maize to Egypt. Based on the above results, the present situation is hopeful and might represent the possibility of altering standard limit of different fungal and bacterial limits in maize and feed.

However, the results for AFM_1 in milk are expressing a great danger and strict regulation must be applied as soon as possible to animal feeding and to public shops selling milk and milk by product in Assiut as well as Upper Egypt. Consequently, more attention and strict legalisations are highly recommended in order to control dairy milk contamination with mycotoxins.

Furthermore, all the obtained results can be used for the future surveys to have a complete picture about mycotoxins likely to be occurred and collect more information about mycotoxins contamination in feed, maize as well as food commodities especially those are derived from animal origin. Reducing the maximum level of the regulated mycotoxins in food and feed commodities to a level accepted by international standards is important to both ensure the safety of consumers and to facilitate trade. The best strategy to achieve this can be done through preventing the contamination of crops by toxigenic fungi and associated mycotoxins. Work on appropriate control measure should also be given to lessen the impact of mycotoxins in case preventive strategies fail.

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APPENDIX

Evaluation letter for master thesis during "Erasmus stay" at Center for Analytical Chemistry, Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences (BOKU), Austria

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Tulln, March 2nd, 2016

Evaluation letter

To Erasmus office - Hacettepe Uniervsity,

Herewith we confirm that our Erasmus exchange master student Mohamed Fathi Abdallah from Hacettepe University, Turkey has achieved his studies including doing his master thesis in addition to attendance of some extra practical courses at IFA-Tulln Department, University of Natural Resource and Life Sciences Vienna (BOKU), Austria. During his six-month sojourn at our institute, Mohamed analyzed different maize and animal feed samples from Upper Egypt using LC-MS/MS to be inserted in his master thesis after coordination with the main supervisor at his home university, Hacettepe University, Turkey. The experimental part, results and discussion have been reviewed and accepted from our side.

Please, find below the detailed evaluation for the master thesis course according to the Austrian grading system at The University of Natural Resource and Life Sciences Vienna (BOKU).

- Student full name: - Mohamed Fathi Abdallah Abdelmohsen
- Date of birth: - 26.01.1990
- Student ID number: - 1541300
- Study program: - Master's program - Food Science and Technology- H066417
- Course title: - Master Thesis
- ECTS-credits: - 30 ECTS (earned)
- Assessment: 1 (excellent)
- Examination date: - 02/03/2016

Center for International Relations Universität für Bodenkultur Wien

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Examiners and supervisors for the thesis course at BOKU

Krska Rudolf, Univ.Prof. Dipl.-Ing. Dr.techn.

Head of Center for Analytical Chemistry, IFA-Tulln, BOKU

Sulyok Michael, Dipl.-Ing. Dr.techn.

Senior Scientist at Center for Analytical Chemistry, IFA-Tulln, BOKU

Center for International Relations Universität für Bodenkultur Wien

Austrian grading system for assessment
excellent (1), good (2), satisfactory (3), sufficient (4), unsatisfactory (5) successfully completed, not completed, pass, fail

This report is an official certificate for master thesis course only. Other courses attended by the student are available through BOKU online system.

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Curriculum Vitae

Curriculum Vitae

Mohamed Fathi Abdallah

Personal Data:

Date and Place of Birth: January 26th, 1990, Kuwait

Nationality: Egyptian

Education:

10.2013-04.2016	Research Assistant, Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Hacettepe University, Turkey
09.2015-02.2016	(6 months) Erasmus+ exchange master student, Center of Analytical Chemistry, Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences (BOKU), Austria
09.2014-12.2014	(4 months) Internship and research stay, Central Unit of Analytical Chemistry, Leibniz Research Centre for Working Environment and Human Factors (*IfADo*), Dortmund, Germany
10.2012-06.2013	Studying Turkish Language, Foreign Languages School. Ankara University, Turkey
03.2012-09.2012	(7 months) Research Assistant, Department of Toxicology, Faculty of Veterinary Medicine, Assiut University, Egypt
2006-2011	Bachelor of Veterinary Medicine with distinction, Faculty of Veterinary Medicine, Assiut University, Egypt

Grants and Fellowships:

- Erasmus + Exchange Scholarship, IFA-Tulln, BOKU, Austria (2015)
- Erasmus + Mobility Internship, *IfADo* Institute, Dortmund, Germany (2014)
- Turkish Government Scholarship for Master Degree, Ankara, Turkey (2012)
- Teaching and Research Assistantship, Faculty of Veterinary Medicine, Assiut University, Egypt (2012)

Publications:

- ***Abdallah M.F.***, Girgin G., Baydar T. Occurrence, Prevention and Limitation of Mycotoxins in Feeds. Animal Nutrition and Feed Technology Journal, (2015) 15: 471-490. ***(Review artilce)***
- ***Abdallah M.F.***, Karacaoglu E., Kılıçarslan B., Girgin G., Selmanoglu G., Baydar T. Influence of subacute melatonin treatment on antioxidant factors in the liver of female rats. Journal of Experimental and Applied Animal Science Volume 1, Number 3, pp. 359-368, 2015. ***(Reseacrh article)***
- ***Abdallah M.F***., Krska R., Sulyok M. Mycotoxin contamination in sugar cane grass and juice: Frist report on multi-toxins detection and exposure assessment in human. 5th International Symposium on Mycotoxins and Toxigenic Moulds: Challenges and Perspectives. May 11, 2016, Ghent, Belgium. ***(Poster presentation)***
- ***Abdallah M.F.***, Krska R., Sulyok M. Mini-survey of fungal and bacterial metabolites in dried dates palm from Egypt using LC-MS/MS. 38th Mycotoxin Workshop May 2-4, 2016, Berlin, Germany. ***(Poster presentation)***
- ***Abdallah M.F.***, Kilicarslan B., Girgin G., Baydar T. Multi-mycotoxins occurrence in maize and animal feed from Assiut Governorate, Egypt. 5th International Symposium on Mycotoxins and Toxigenic Moulds: Challenges and Perspectives. May 11, 2016, Ghent, Belgium. ***(Poster presentation)***
- ***Abdallah M.F.***, Kilicarslan B., Girgin G., Baydar T. Determination of aflatoxin M1 in raw milk samples from Assiut city, Egypt. 38th Mycotoxin Workshop May, 2-4, 2016 Berlin, Germany. ***(Poster presentation)***
- ***Abdallah M.F.***, Girgin G., Krska R., Baydar T., Sulyok M. Analysis of multiple mycotoxins in maize and animal feed in Upper Egypt using LC-MS/MS. 2. Linzer Kontaminantentagung. December 1-2, 2015, Linz - Austria. ***(Poster presentation)***
- ***Abdallah M.F.***, Karacaoglu E., Kılıçarslan B., Girgin G., Selmanoglu G., Baydar T. Preliminary Investigation of Subacute Melatonin Treatment on Rats.11th International Symposium on Pharmaceutical Sciences (ISOPS-11). June 9-12, 2015, Ankara - Turkey. ***(Poster presentation)***

FORM:

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GRADUATE SCHOOL OF HEALTH SCIENCES
THESIS ORIGINALITY REPORT

To the Hacettepe University
Graduate School of Health Sciences

Date: 28/4/2016

Name Surname:	Mohamed Fathi Abdallah
Student No:	N13121563
Department:	Pharmaceutical Toxicology
Program:	Pharmaceutical Toxicology
Status:	☒ Master ☐ Ph.D. ☐ Integrated Ph.D.

Thesis Title : Detection of Mycotoxin Levels In Animal Feeds and Raw Milk Samples by Using High Performance Liquid Chromatography and Liquid Chromatography Tandem Mass Spectrometry

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Name Surname and Signature
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ADVISOR APPROVAL

APPROVED.

28/04/2016

(Title, Name Surname, Date and Signature)
PROF.DR. TERKEN BAYDAR (Advisor)

ASSOC.PROF.GÖZDE GİRGİN (Co-Advisor)

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by Mohammed Fathi Abdallah

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1	Sulyok, M., F. Beed, S. Boni, A. Abass, A. Mukunzi, and R. Krska. "Quantitation of multiple mycotoxins and cyanogenic glucosides in cassava samples from Tanzania and Rwanda by an LC-MS/MS-based multi-toxin method", Food Additives & Contaminants Part A, 2015. Publication	3%
2	Chala, Alemayehu, Wondimeneh Taye, Amare Ayalew, Rudolf Krska, Michael Sulyok, and Antonio Logrieco. "Multimycotoxin analysis of sorghum (Sorghum bicolor L. Moench) and finger millet (Eleusine coracana L. Garten) from Ethiopia", Food Control, 2014. Publication	2%
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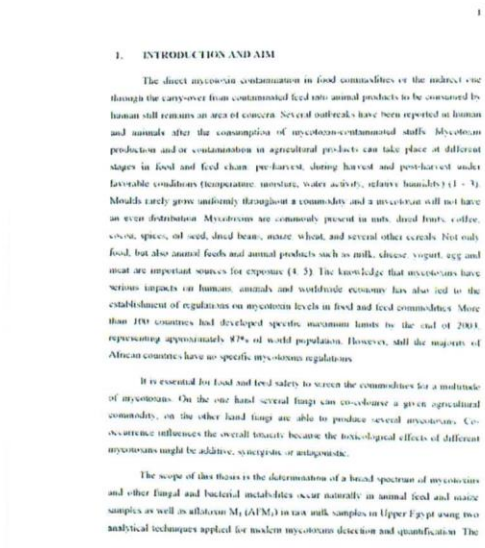

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1. INTRODUCTION AND AIM

The direct mycotoxin contamination in food commodities or the indirect one through the carry-over from contaminated feed into animal products to be consumed by human still remains an area of concern. Several outbreaks have been reported in human and animals after the consumption of mycotoxin-contaminated stuffs. Mycotoxin production and/or contamination in agricultural products can take place at different stages in feed and feed chain: pre-harvest, during harvest and post-harvest under favorable conditions (temperature, moisture, water activity, relative humidity) (1 - 3). Moulds rarely grow uniformly throughout a commodity and a mycotoxin will not have an even distribution. Mycotoxins are commonly present in nuts, dried fruits, coffee, cocoa, spices, oil seed, dried beans, maize, wheat, and several other cereals. Not only food, but also animal feeds and animal products such as milk, cheese, yogurt, egg and meat are important sources for exposure (4, 5). The knowledge that mycotoxins have serious impacts on humans, animals and worldwide economy has also led to the establishment of regulations on mycotoxin levels in food and feed commodities. More than 100 countries had developed specific maximum limits by the end of 2003, representing approximately 87% of world population. However, still the majority of African countries have no specific mycotoxins regulations.

It is essential for food and feed safety to screen the commodities for a multitude of mycotoxins. On the one hand several fungi can co-colonize a given agricultural commodity, on the other hand fungi are able to produce several mycotoxins. Co-occurrence influences the overall toxicity because the toxicological effects of different mycotoxins might be additive, synergistic or antagonistic.

The scope of this thesis is the determination of a broad spectrum of mycotoxins and other fungal and bacterial metabolites occur naturally in animal feed and maize samples as well as aflatoxin M_1 (AFM$_1$) in raw milk samples in Upper Egypt using two analytical techniques applied for modern mycotoxins detection and quantification. The