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# Developing an HPLC-ESI-MS/MS method for simultaneous determination of mycotoxins in maize flour and other matrices

Master thesis

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## Contents

## 1. Abbreviations

3-AcDON - 3-acetyl-deoxynivalenol; 15-AcDON - 15-acetyl-deoxynivalenol; acetyl-CoA – acetyl-coenzyme A

AfB1 – aflatoxin B1; AfB2 – aflatoxin B2; AfG1 – aflatoxin G1; AfG2 – aflatoxin G2; AfM1 – aflatoxin M1

AOAC - Association of Official Analytical Chemists

CAST - Council for Agricultural Science and Technology

CEN – European Committee for Standardisation

DAD – diode array detector

DAS – diacetoxyscirpenol; DON – deoxynivalenol

ELIME array - Enzyme-Linked-Immuno-Magnetic-Electrochemical-array

ELISA - enzyme linked immunosorbent assay

ESI – electrospray ionisation

EU - European Union

F - Fusarium

FD - fluorescence detector

FHB - Fusarium head blight

FID – flame ionisation detector

GC - gas chromatography

HACCP - Hazard analysis of critical control points

HPLC - high performance liquid chromatography; LC – liquid chromatography

IAC – immunoaffinity column

LOD – limit of detection; LOQ – limit of quantitation

MS – mass spectrometry; MS/MS – tandem mass spectrometry

NIV - nivalenol

OTA - Ochratoxin A; OTB-Ochratoxin B; OTC-Ochratoxin C

PBS – phosphate buffer saline

QuEChERS – “quick easy cheap effective rugged and safe”

r – correlation coefficient

RSD – relative standard deviation (%)

spp.- species

TLC - thin layer chromatography

UPLC – ultra performance liquid chromatography

UV - ultraviolet

ZAN – zearalanone; ZON - zearalenone

## 2. Introduction

Mycotoxins are a group of chemical substances produced by different species of fungi. After infesting crops, fungi synthesize the toxins, which will be transmitted to the final food products. Some mycotoxins form derivatives inside the contaminated plants and organisms [1]. From the fungal species with the highest toxigenic potential, *Aspergillus, Fusarium, Penicillium, Alternaria* and *Claviceps* are the most important. [2]

The study of mycotoxins is very important due to their high level of toxicity and also because of the increasing legislative demands concerning mycotoxin content of different products.

Mycotoxins can be found in a wide variety of matrices, ranging from cereals, peanuts, spices, animal feeds, fruits and vegetables to meat, milk, eggs and many other derived products. Up to present days, around 400 mycotoxins have been registered [3], but not all of them manifest toxic behaviour towards plants or animals [4]. The classes of mycotoxins with relevance to health are: aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids [4]. Other mycotoxins with toxigenic potential against humans are: penitrem (a tremorgenic mycotoxin), patulin, cyclopiazonic acid and citrinin [5].

Common analytical methods for mycotoxins can be divided in two categories: screening methods and confirmatory (reference) methods [6]. The first category includes rapid methods such as ELISA, which has a wide range of applicability: aflatoxins, fumonisins, ochratoxin A, zearalenone, and trichothecenes. New screening techniques will be developed, for the purpose of being used in prevention strategies. Biosensor-based techniques with surface plasmon resonance detection [7] are beginning to be used. From the confirmatory methods, gas chromatography and high performance liquid chromatography, often with mass-spectrometric detection, are most commonly used in the present days. Thin layer chromatography was among the first methods used for mycotoxin analysis. Automation, high performance separation and generally lower detection limits are the advantages of GC and HPLC compared to TLC [6].

The purpose of the present work is developing an HPLC-MS/MS method together with extraction and sample cleanup steps for simultaneous analysis of aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A, zearalenone, T-2 toxin and HT-2 toxin.

# 3. Literature overview

## *3.1. Importance of mycotoxin level studies*

In this chapter, the main economical, legislative and health-related aspects concerning mycotoxins will be presented. Because of their toxicity, mycotoxins represent a threat to public health and can also cause major economical losses. Implementation of suitable regulations concerning mycotoxins has become an important issue, with influences on trade and food production.

Market losses caused by mycotoxins are a consequence of rejecting crops or disposing of the ones that don't fulfill certain requirements [8]. The mean annual economical losses caused by mycotoxins were estimated to $932million USD by the U.S. Food and Drug Administration (CAST, 2003). Mycotoxins surveyed were aflatoxins, fumonisins, and deoxynivalenol [9].

In developing countries, mycotoxins represent a higher risk to health than in industrial countries [8].

The implementation of legislation concerning mycotoxins requires validated analysis methods. Results obtained with these methods must have suitable accuracy, repeatability and reproducibility within and between laboratories. The reliability of results is necessary for proficiency testing trials, risk assessment, prevention strategies (HACCP) and legislative actions [6].

Although regulations might differ with respect to mycotoxin type, matrix or acceptable limits, harmonisation of the EU market is aimed. When there is need to set a legislative limit for a certain mycotoxin, official methods must fulfill certain requirements established by CEN and AOAC [6].

Method validation can be done nowadays for very low contamination levels [6], due to the sensitive methods used. The use of immunoaffinity cleanup associated with TLC and HPLC [10] and improved detection systems [6] solved the analytical problem of detecting very small concentrations.

European regulations concern different aspects: legislative limits of toxins in different products, choice of sampling and analysis methods, requirements for the laboratories designed with the official control of food products. Relevant examples of directives are shown below [6]:

• Directive 85/591/EEC that lays down the framework for sampling and analysis methods on community level (European Council, 1985).

• Directive 89/591/EEC and Directive 93/99/EEC which define general principles for the official control of foodstuffs (European Council, 1989).

• Directive 98/53/EEC that defines sampling methods and the statistical requirements (method performance) for analysis methods (European Commission, 1998a).

Mycotoxins are dangerous for human and animal health, considering their potential to cause various mycotoxicoses [11].The Fusarium toxins with the highest degree of toxicity for animals are trichothecenes, zearalenone and fumonisins [12].Aflatoxins, fumonisins, ochratoxin A, trichothecenes (e.g. nivalenol, deoxynivalenol, T-2 toxin), zearalenone and patulin are mycotoxins with relevant importance for health issues. Aflatoxin B1 is considered to be the strongest natural carcinogen in

animals [6].Since 1961, when they caused big losses in England, aflatoxins became a problem of great importance. Scientists took interest in their carcinogenicity and immunosuppressive nature [5].

On the background of chronic exposure at a large scale, different cases of mycotoxicoses occurred in Europe, Asia, New Zealand and South America [12].

Given their low concentrations in different food products, acute intoxications with mycotoxins are quite rare in humans. On the other hand, chronical exposure can lead to mycotoxicoses, in some cases because of a cumulative effect of the respective toxins [13].The contamination paths, either in humans or animals, are: inhalation, ingestion and skin contact [11].

There have been cases of acute intoxication with aflatoxins, despite their low level of food contamination. There is the possibility of potentially lethal aflatoxicoses [14, 15]. Recently, acute cases of aflatoxicosis in Kenya caused the death of 123 people [16-18].

Neurotoxic, carcinogenic, mutagenic and teratogenic effects of mycotoxins on animals have been proved by laboratory experiments [19,20].

## *3.2. Control of mycotoxin levels in crops and food products*

Mycotoxins are secondary metabolites of fungi. Their biosynthesis is influenced by storage conditions, climate and intrinsic factors such as fungal strain specificity [4]. This chapter will focus mainly on the aspects concerning the mycotoxin level control in crops, stored materials and also during processing the raw material. Prevention strategies, such as HACCP, involve monitoring the mycotoxin content from the crop field to the consumer's table. Testing protocols have to be set for all the important control points: fungal interactions with crop plants, harvesting and processing method, storage and product delivery.[5]

**Mycotoxin control before cereal harvesting**

A very important issue concerning mycotoxin levels in crops is the control of Fusarium head blight. Wheat and barley are among the plant species that are most commonly infected . FHB occurrence is caused mainly by *Fusarium culmorum, F. graminearum, F. avenaceum, F. poae* and *Microdochium nivale* [21]. DON, NIV, ZON and DON derivatives like (3-AcDON, 15-AcDON) are mainly associated to the disease [22,23].

In order to reduce the FHB incidence, different prevention methods, for example the use of fungicides, have to be applied. Calendaristic time and environmental conditions are factors that might facilitate the occurrence of the disease [21]. The time of crop flowering is considered to be favourable for crop infection. Fungal growth is intensified by a warm environment, with high degree of humidity [21]. Infection with *Fusarium* species can be prevented by using resistant cultivars, fungicides, biological control and cultural practices [21].

**Mycotoxins control after harvesting**

Mycotoxin levels in final products are also influenced by the processing methods.

A survey made by the UK Food Standards Agency [24,25] showed that trichothecenes are not completely destroyed in the processing stages. Different cereal products were analysed, and the highest DON and NIV content was found in breakfast cereals. (2261 µg/kg, respectively 260 µg/kg).

Cleaning by gravity separators [26] and milling of cereals are among the processing methods that can reduce the trichothecene content. In case of dry milling, the fractions with higher mycotoxin content (bran, wheatfeed) can be separated. Due to their high solubility in water, trichothecenes can be easily separated during wet milling. [27,28].Reducing the toxin content is strongly related to the infection pattern inside the grain. [29].

Other processing stages, such as baking, extrusion and brewing can also modify the mycotoxin concentration. Brewing steps can have different effects: steeping can lower DON level, but germination can increase it [30].

The study of water content [31] influence proved that for any temperature, an increased water activity value will increase the fungal growth rate [31]. Increasing temperature will also intensify fungal growth [31].

## *3.3. Methods for mycotoxin content determination in food products*

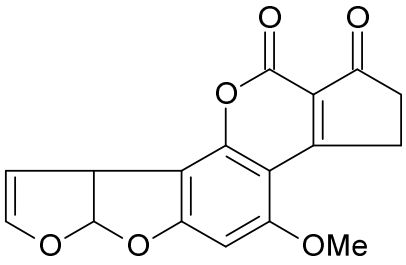
### 3.3.1. Chemical properties of mycotoxins

The chemical structures of mycotoxins are very diverse [32] and their molecular weight is generally low (under 700 Da) [32]. Because of their diversity in structure and properties, analysis methods have been developed for single toxins initially. Later, the advantages of simultaneous methods have been outlined.

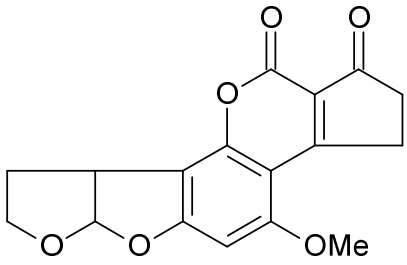
Aflatoxins

Aflatoxins (AfB1, AfB2, AfG1, AfG2) are difuranocoumarin compounds [33]. Aflatoxin M1 is a hydroxylated metabolite of aflatoxin B1, found mostly in animal tissues and fluids [5]. At present, there are 20 isolated aflatoxins [4]. The structure of aflatoxins is presented below [4]:

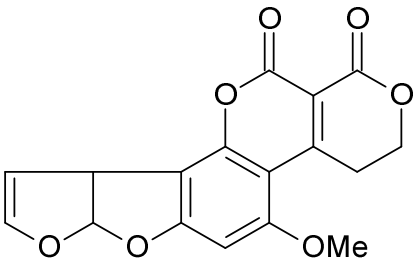
**Fig. 3.1. Structure of aflatoxins B1, B2, G1 and G2**.



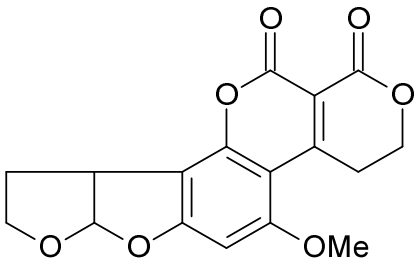

Aflatoxin B1




Aflatoxin B2




Aflatoxin G1




Aflatoxin G2

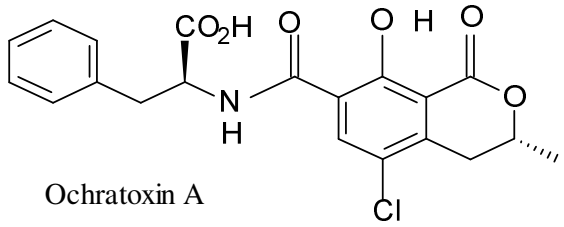
Aflatoxins are produced via a polyketide pathway by *Aspergillus* species [32]. Other aflatoxin metabolites are: aflatoxinM2, aflatoxinQ1, aflatoxinP1 and aflatoxinB1-8,9-epoxide [4,32].

Ochratoxins

Ochratoxins are 3,4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L-β-phenylalanine [34]. Ochratoxin A, the most important compound of this class, has fluorescent properties and is a secondary metabolite of *Aspergillus ochraceus* and *Penicillium verrucosum* [35]. The empirical formula for OTA is $C_{20}H_{18}O_6NCl$ and the molecular weight is 403.82 [36].

As for the biosynthetic pathway of OTA, it has not been determined completely, but in principle it refers to a shikimate pathway (from shikimic acid) and a pentaketide pathway, which describe the formation of the phenylalanine and the dihydroisocoumarin parts, respectively [36]. The first step of the pentaketide pathway represents the condensation between one acetate unit (acetyl-CoA) and four malonate units. Recently, it had been proved that a polyketide synthase is necessary for this condensation reaction [37].

**Fig. 3.2. Structure of Ochratoxin A [5].**




Ochratoxin A

Ochratoxin A is a white, crystalline substance, with high solubility in polar solvents [Chemical abstract specification (CAS) 303-47-9]. It is slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. The melting points are 90 and 171 °C, when recrystallized from benzene (containing 1 mol benzene/mol) or xylene, respectively [38]. OTA exhibits UV adsorption: $\lambda$MeOHmax (nm; $\varepsilon$) = 333 (6400) [39]. The fluorescence emission maximum is at 467 nm in 96% ethanol and 428 nm in absolute ethanol. The infrared spectrum in chloroform includes peaks at 3380, 1723, 1678 and 1655 $cm^{-1}$ [40]. OTA has weak acidic properties. The p*K*a values are in the ranges

4.2–4.4 and 7.0–7.3, respectively, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part [41–43].

The ochratoxin group also includes OTB, OTC, 4-hydroxyochratoxin A and Ochratoxin α [36].

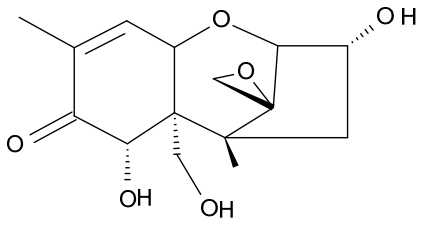
Trichothecenes

The structure of trichothecenes is based on a sesquiterpenoid ring, with functional groups that can differentiate the types of trichothecenes between them [44]. The toxic nature of these substances is due to an epoxidic ring at C12,13 position. The difference between type A and type B trichothecenes is the presence or absence of a carbonyl at C8, respectively [45]. Type A trichothecenes are: T-2 toxin, HT-2 toxin, diacetoxyscirpenol. Type B trichothecenes are: deoxynivalenol (vomitoxin) [46,47], nivalenol. Type C trichothecenes (crotocin, baccharin) have an epoxide group at the C7,8 or C9,10 position. Type D trichothecenes (satratoxin, roridin) contain a macrocyclic ring between the C4,15 positions [45].

Trichothecenes are non-volatile, resistant to light and temperature, and can be deactivated under strong acid or alkaline conditions [45]. There are bacteria and fungi that can degrade trichothecenes [48].

Type A trichothecenes have the tendency to cause acute mycotoxicoses, unlike type B trichothecenes, which rather induce chronical toxicoses [46,47].

**Fig. 3.3. Structure of deoxynivalenol [5].**

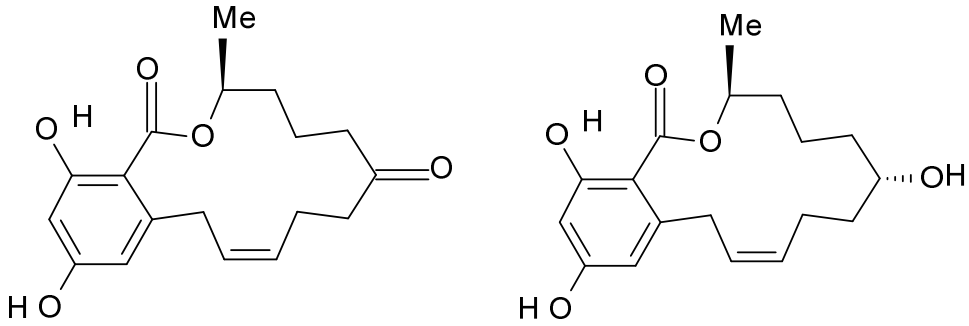



Zearalenone

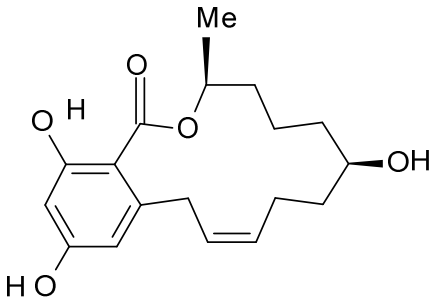
Zearalenone is biosynthesized through a polyketide pathway by different *Fusarium* fungi [1]. From a structural point of view, it is a resorcyclic acid lactone [1].

The structures of zearalenone and two derivates (α-zearalenol and β-zearalenol) are shown below [1].

**Fig. 3.4. Structure of zearalenone, α-zearalenol and β-zearalenol.**




zearalenone α-zearalenol




β-zearalenol

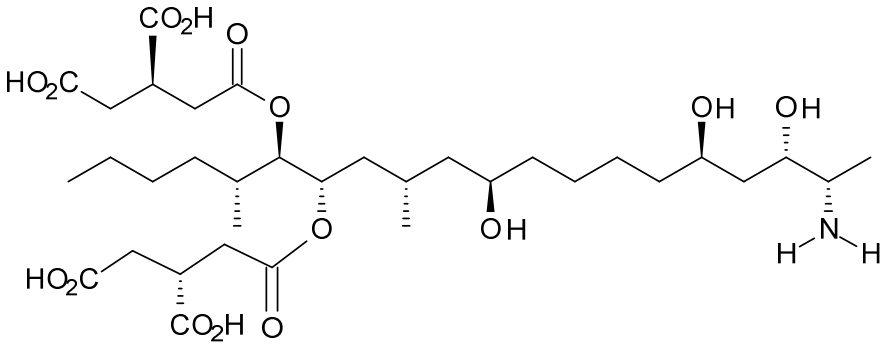
Other zearalenone derivates are: zearalanone, α-zearalanol, β-zearalanol.

Some classes of toxins (for example aflatoxins, Ochratoxin A, citrinin) have natural fluorescence and can be detected with HPLC–FD methods [49].

Fumonisins

The structure of fumonisins is based on a hydroxylated hydrocarbon chain. This chain contains methyl and amino groups in case of fumonisins B1 and B2. Fumonisins A1 and A2 contain methyl and acetyl groups [50].

**Fig. 3.5. Structure of fumonisin B1 [5].**



### 3.3.2. Quantitative and semiquantitative ELISA

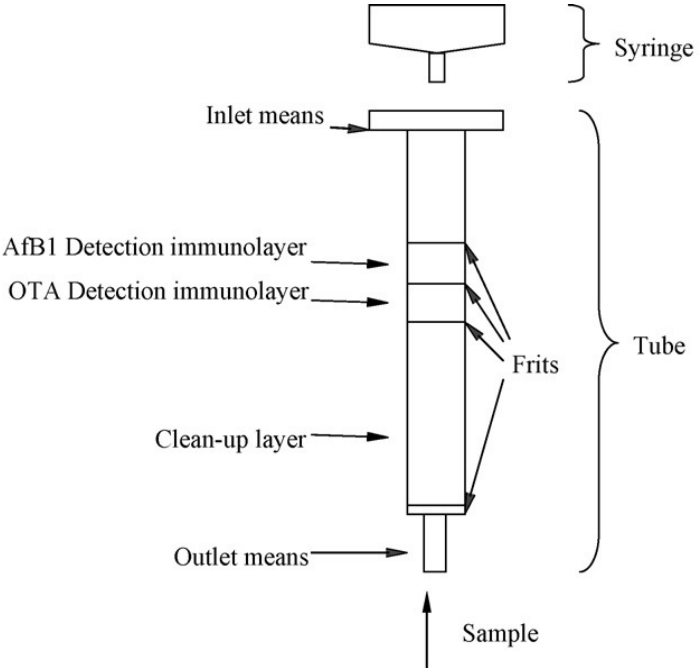
#### 3.3.2.1. Different immunoaffinity assays

Due to its simplicity, adaptability and sensitivity, enzyme linked immunosorbent assay has become a routine method for mycotoxin analysis. In many cases, spectrophotometric detection is used. Different ELISA trials and immunoassay methods are presented below, together with their performance characteristics [51,52].

A cleanup tandem immunoassay was used as screening method for Aflatoxin B1 and Ochratoxin A in spices [53]. Detection limits for the two toxins were 5 µg/kg and 10 µg/kg, respectively. The detection limit of 5 µg/kg for Aflatoxin B1 coincided with the legal limit set by the European Union for this toxin. The mycotoxin presence was detected visually and matrix effects were investigated. No false negative results were observed after cleanup. The results were confirmed by an LC-MS/MS method and showed that 32% of the Capsicum spp. spices contained OTA above detection limit, and 23% of the Capsicum spices were contaminated with AfB1.

The figure below shows the structure of the column used in the immunoassay, with one cleanup layer and two different detection immunolayers containing specific antibodies for each of the mycotoxins.

**Fig. 3.6. Column structure.**




Antibody-based analysis techniques can involve instrumental absorbance reading or rapid „on-site" testing [54]. The second method involves the use of antibodies fixed on a membrane [55,56] and it can be efficient even for multitoxin analysis. Low limits of detection were obtained with non-instrumental (visual) testing for different mycotoxins, in a test where spiked wheat samples were

analysed. LOD values were: 10 µg/kg for aflatoxin B1, 50 µg/kg (ochratoxin A), 3500 µg/kg (DON), 100 µg/kg (T-2 toxin), and 50 µg/kg (fumonisin B1). [57].

Sensitivity of the non-instrumental method is lower than sensitivity of the microtiter plate (instrumental) method. If analyte detection in the microgram per kilogram range is required, high-quality antibodies have to be used [54].

Extraction prior to ELISA is done with organic solvents (water in case of DON) and is followed by filtration in order to remove the solid particles. This extraction method is suitable for cereal samples or flour, but problems might appear with more complex matrices [54].

The results of a simultaneous enzyme immunoassay were compared with the ones obtained with individual ELISA performed for Aflatoxin B1 and Ochratoxin A in chili samples [58].
The simultaneous method proved to be cost-effective and rapid, also reducing the dilution error and avoiding laborious cleanup. The analyte concentrations were detected either visually, either by densitometry. The extraction solvent was methanol:water (80:20) v/v, only 2 fold diluted for simultaneous analysis and 10 fold diluted for individual ELISA [59,60,61,62].

The results are summarized in the following table, showing good accuracy and precision.

**Table 3.1. Comparison between simultaneous immunoassay and individual ELISA.**

| Analytical characteristic | Simultaneous method | | Individual ELISA | |
|---|---|---|---|---|
| | AfB1 | OTA | AfB1 | OTA |
| Detection limit (µg/kg) | 2 | 10 | 2 | 2 |
| CV, intra-assay (%) | 6-8 | 5-7 | 4-6 | 5-7 |
| CV, inter-assay (%) | 8-11 | 8-11.4 | 6-9 | 7-11 |
| Assay time | 6 min | | 35 min | 2 h 15 min |
| Average recovery (%) | 92-120 | 93-110 | 95-120 | 90-105 |

The analysis of Aflatoxin M1 in milk products by indirect competitive ELISA yielded accurate results [63], with intra-day and inter-day variation coefficients of 2.3-4.6% and 3.2-17.2%, respectively. For a spiking range of 0.1-3.2 ng/ml, the recovery values were between 80% and 128%. Linear range was 0.04-5 ng/ml, with an LOD of 0.04 ng/ml. The results outlined the high sensitivity of monoclonal antibodies.

Deep-red ground pepper samples were analysed by enzyme-linked immunosorbent assay, using previous immunoaffinity cleanup for Aflatoxin B1. Before the cleanup step, samples were extracted with 70% methanol, filtered and diluted. After aflatoxins were retained by cleanup column, they were eluted with methanol at slow flow rate and diluted with phosphate buffer solution. ELISA determination followed, and analyte concentration was detected by means of absorbance measurement, at 450 nm. Recovery was between 50-70%, detection limit was 0.025 µg/kg, and average coefficient of variation was 8% [64].

In order to obtain the required sensitivity and specificity, an electrochemical immunosensor was developed for the determination of Aflatoxin B1 in corn samples using indirect competitive ELISA approach [51]. Magnetic beads were used as immobilization support. Before the ELIME array, the samples were extracted with acetonitrile/water (84:16, v/v) and cleaned with a MycoSep column. Electrochemical detection was used in this experiment, and the detection limit was 0.6 ng/ml. The recovery and precision of the method were determined using four Certified Reference Materials, from which two replicates were analysed. Recovery was found to be between 95 and 114%, while relative standard deviation was between 11 and 26% [51].

In order to determine the total aflatoxin content in stored cassava chips, direct competitive ELISA was used. Extraction was done with methanol:water (70:30 v/v) and starndard aflatoxin solutions at 20 µg/kg were used as controls. Detection limit value was smaller than 5 µg/kg [65].

Competitive ELISA was also used to estimate the total aflatoxin content, as well as Aflatoxin B1 concentration, in mahua seeds. Extraction with 70% aqueous methanol and immunoaffinity cleanup were also involved in the experiment. As it was previously shown in other trials, detection was done by means of absorbance measurement [66].

Unlike in previous trials, no cleanup step was involved when total aflatoxin content was determined in peanut samples, as well as concentrations for individual aflatoxins B1, B2, G1 and G2. Samples were extracted with 75% methanol, diluted 10 times with PBS-methanol buffer and then analysed. Recovery values ranged from 87.5% to 102.0% [67].

#### 3.3.2.2. ELISA vs. Chromatography

The results of a cleanup tandem immunoassay [53] were confirmed by a LC-MS/MS method [53] for aflatoxin B1 and Ochratoxin A analysis in spices. Samples were extracted with MeOH/water (80/20, v/v); the extract was diluted, filtered and purified with an AflaOchra HPLC column. Toxins were eluted with methanol and redissolved in MeOH/water (30/70, v/v). Chromatographic separation was improved by the applied gradient [53]. Positive electrospray ionization and multiple reaction monitoring were used, and the precursors' mass to charge ratios were: AfB1-313; AfB2-315; AfG1-329; AfG2-331; OTA-404.

The results showed that, for all the samples, the AfB1 concentration was significantly higher than the concentrations of AfB2, AfG1 and AfG2.

In a different experiment [68], results from LC-MS/MS and ELISA were compared. B trichothecenes and macrocyclic lactone mycotoxins were extracted from maize using acetonitrile/water (75:25, v/v) [69]. Extraction contribution to the total recovery was evaluated by spiking the samples before and after the extraction step. The recovery of extraction was above 92%. Before chromatographic analysis, extracts were purified by Graphitized Carbon Black cartridge. The conditions and elution gradient [69] used in LC-ESI-MS/MS analysis did not allow chromatographic

separation of 3-Acetyl-DON and 15-Acetyl-DON, which were evaluated as a sum. A C18 and a guard column were used in the analysis. Using a different kind of column (Polar RP) [70], the separation of the two isomers was possible. Isocratic elution was used for macrocyclic lactones. Ionisation was done in negative mode [69]. The recovery (obtained using artificially contaminated maize samples) and detection limits were as follows:

**Table 3.2. Recovery and detection limit for deoxynivalenol and zearalenone [68].**

| Analyte | Recovery±RSD (%) | Detection limit (ng/g) |
|---|---|---|
| deoxynivalenol | 89±10 | 2 |
| zearalenone | 95±7 | 3 |

Competitive ELISA was done with separate kits for each mycotoxin (deoxynivalenol and zearalenone), using spectrophotometric detection at 450 nm. Detection limits for DON and ZON were 17 ng/g and 18.5 ng/g, respectively. Upper limit of linear range was 500 ng/g for both kits, and recoveries were between 85–110% for DON and 64–97% for ZON. The comparison showed that results from ELISA and LC-MS/MS were in concordance for zearalenone, and cross reactivity of antibodies with zearalenone metabolites did not affect the analysis. On the contrary, in case of DON, eight cases of false positives in case of ELISA method were outlined by LC-MS/MS.

The results of ELISA and HPLC were compared for Ochratoxin A analysis in wine [71]. Immunoaffinity cleanup was done prior to HPLC. Fluorescence detection and a non-polar column were used. Mobile phase for HPLC was: methanol:water: acetic acid (70:30:2).

For the ELISA analysis, cleanup with bicarbonate was used to eliminate the interference of chromogenes [72]. Detection was done at 450 nm. An additional extraction with chloroform, followed by a concentration step, improved the detection limit. Another way of eliminating the interferences would be using IAC before ELISA.

Linear regression analysis showed a good correlation between the results obtained with the two methods, with $r = 0.821$. Correlation was better at high OTA concentrations.

However, ELISA has certain disadvantages: the possibility of false positives caused by cross-reactivity and the possibility of false negatives. Confirmation with HPLC is necessary [6].

With respect to method sensitivity, ELISA is known to have detection limits that are comparable to those obtained with HPLC [6].

Although the commercial ELISA kits have low LOD values (nearly 11 μg kg−1), that comply with EU requirements, these LOD levels are not suitable for the trace analysis of Aflatoxin B1 in baby foods [73].

### 3.3.3. HPLC

Lately, the HPLC methods for mycotoxin analysis have gained more attention, due to their efficiency and high sensitivity [74]. Among different detector types, tandem mass spectrometry provides high selectivity, low quantitation limits and accurate analysis [73]. This chapter focuses mostly on HPLC-MS/MS methods encountered in a large amount of literature sources [75,76]. Another important aspect in HPLC analysis is the type of column used, given the wide range of physicochemical properties of the analytes. The extraction solvents, eluents and types of gradient used are also of extreme importance for the chromatographic separation [73]. Due to the complexity of food matrices, a large amount of HPLC methods use immunoaffinity cleanup, which will be discussed in detail in the following chapters. A tendency towards developing methods for simultaneous mycotoxin determination has been noticed [75].

For example, a LC-MS/MS method has been developed for the simultaneous analysis of 90 mycotoxins in cereals [75]. Two chromatographic runs were carried out, for each ionization polarity. Extraction was carried out with acetonitrile/water/acetic acid (79/20/1, v/v/v). Afterwards, the extract was diluted in a proportion of 1:1 (v/v) with acetonitrile/water/acetic acid (20/79/1, v/v/v) in order to adjust the solvent strength. No cleanup step was involved. The limits of detection ranged from 0.03 μg/kg (enniatin B) to 30 μg/kg (nivalenol).

High sensitivity, good correlation coefficients and linear range were achieved with an UPLC-MS/MS multimethod [73].

HPLC coupled to tandem mass spectrometry can provide higher selectivity than TLC [77] or HPLC-DAD [78]. These methods are selective enough for single target analysis in foodstuffs, but they are not appropriate for a large number of analytes [79].

Even in the case of HPLC, cleanup is necessary. Incomplete extraction and matrix effects can lead to an underestimation of the actual concentration in the samples. An HPLC-MS/MS method which avoids cleanup is more appropriate for semi-quantitative screening [79].

When the same type of sample was analysed in two different laboratories with different methods (HPLC-FD and HPLC-MS), the limits of quantitation for fumonisins were: between 70-90 μg/kg for fluorescence detection and 100 μg/kg for mass spectrometric detection. For T-2 toxin, the LOQ obtained with TLC was 125 μg/kg, and the LOQ obtained with HPLC-MS was 30 μg/kg [80].

HPLC with fluorescence or ultraviolet detection provides high sensitivity, low LOD and good automatic operation, and is considered to be the standard method of the European Committee for Standardization (CEN) [81].

Also, when an UPLC – MS/MS method was validated for multitoxin analysis, a comparison was done with a HPLC–FD standard reference method. The results confirmed the method's accuracy and applicability for quantitation purposes [73].

However, MS/MS detection method in combination with HPLC is efficient in providing information about the structures of the analytes and also low detection limits can be achieved. This method can cover a wide range of analyte polarity [75].

Analyte derivatization is necessary in case of the use of fluorescence detectors [82,83]. An advantage of MS/MS used with HPLC is that, unlike in gas chromatography, polar compounds can be analysed without the need of derivatization [75].

**3.3.4. Gas Chromatography**

GC can be used as a tool for simultaneous mycotoxin analysis, but the low volatility of the analytes and the necessity of derivatization reduce its attractiveness.

The analysis of seven trichothecenes and zearalenone was achieved with a GC-MS method. Cleanup was achieved with a Florisil column, from which all 8 toxins were eluted with a chloroform–methanol (9:1) mixture. Derivatization was necessary because of the method limitations, and the average recovery of the 8 toxins was more than 81%. Recovery was obtained by spiking corn and wheat at 50 ng/g. Limits of detection were 10 ng/g for DON and NIV and 5 ng/g for the other toxins [84].

Trichothecenes were analysed in wheat also by GC with flame ionization detection. This detection type provides large linearity range, it is easy to use and cost-effective [85].Extraction was done with acetonitrile-water (84:16, v/v) and cleanup with charcoal and ion exchange resins was used. The analytes were derivatised to trimethylsilyl ethers. A combination of two chromatographic columns was used to avoid peak overlapping. The method proved to have good linearity and a detection limit of 25 µg/kg. Quantitation limit for the trichothecenes was 75 µg/kg. The relative recovery, determined by using certified reference materials, was 105%, and the relative standard deviation was was 5.8% [85].

For the determination of trichothecenes, gas chromatographic methods with electron capture detection or mass spectrometry detection are most commonly used nowadays [86].

The disadvantages of GC/MS are related to volatility and thermal stability of the analytes [87].

**3.3.5. Other methods**

**3.3.5.1. Thin layer chromatography**

TLC method was used for the analysis of T-2 toxin, DAS (diacetoxyscirpenol) and DON in grains [88]. The samples were extracted with organic solvents and concentrated. Then, they were applied on TLC plates together with standard solutions. In case of extract for DON analysis, purification was done with a column packed with sodium sulphate–florisil–sodium sulphate (10:20:15, w/w/w). Detection was done using long wave UV light.

The mean recoveries obtained were 85%, 90% and 93%, for T-2, DAS and DON, respectively.

The coefficients of variation were 11.3% for T-2, 8.1% for DAS, and 9.1% for DON. Detection limits were: 0.1 mg/kg for T-2 toxin and DAS, and 0.01 mg/kg for DON.

A limit of quantitation of 125 µg/kg was obtained for T-2 toxin in feeds with a TLC method involving previous cleanup with Mycosep #227 and Multisep #219 cartridges [80]. Samples were evaporated, redissolved in toluene:acetonitrile 97:3 and applied to a reverse phase C-18 TLC plate. A methanol/water/acetic acid solution was used to develop the plate, which was then dried and dipped into methanol/sulphuric acid 90:10. Then, it was heated at 150 °C and toxins were detected by using UV light. A different value for the quantitation limit (30 µg/kg) was obtained in case of LC-MS analysis.

Aflatoxins were analysed in corn samples using silica gel G 60 plates and toluene: ethyl acetate: chloroform: formic acid (70:50:50:20, v/v/v/v) as mobile phase. Extraction was done with methanol containing 4% KCl. The extract was cleaned with 30% ammonium sulfate, diatomaceous earth and two partitions with chloroform. Cleanup was followed by evaporation and redissolution steps. For a 5–20 µg/kg spiking range, the recoveries were: 97% for AfB1, 93% for AfG1, 93% for AfB2 and 94% for AfG2. Detection limit was 4 µg/kg [89].

Because of its poor separation ability and unsatisfactory accuracy, TLC method has limited applicability [73].

#### 3.3.5.2. Near Infrared Spectroscopy

Near Infrared Spectroscopy can be used as a non-destructive screening method for aflatoxin B1 in maize and barley [90]. Two types of spectrophotometers were compared in this trial: a dispersive instrument, scanning over a range of 400–2500 nm, and a Fourier transform near infrared spectrophotometer with a range of 1112–2500 nm. Better spectral information was obtained with the Fourier transform spectrophotometer. Detection of AfB1 at 20 ppb level was achieved [90].

## *3.4. Typical approaches on sample preparation depending on analytical method*

### 3.4.1. Immunoaffinity cleanup – an important step in sample preparation

The most important advantages of IAC cleanup [91] are listed below:

(i) provision of clean extracts due to the specificity of the antibody;

(ii) applicability to complex matrices;

(iii) good precision, accuracy and sensitivity of analytical methods;

(iv) rapid clean up;

(v) limited use of organic solvents.

The efficiency of an immunoaffinity cleanup column was compared to the efficiency of a multi-functional column for the analysis of OTA in cereals, raisins and green coffee beans [92]. The samples were analysed by HPLC and the chromatograms corresponding to the multifunctional column contained overlapping peaks. This pointed to the lack of specificity of the multi-functional column.

Contrasting with these results, the IAC chromatograms contained only the OTA peak, with no interferences [92].

Cross-reactivity is another significant aspect related to the use of immunoaffinity cleanup. When the analytes have similar structures (for example Aflatoxins B1, B2, G1, G2), there is good cross-reactivity and a single antibody can be used [93]. However, recovery values can be different, for example 80% for Aflatoxin G2 and more than 90% for Aflatoxin B1 [93]. Some manufacturers specified cross-reactivity also for T-2 toxin, HT-2 toxin and DAS.

When the co-occuring analytes are structurally different, cleanup columns with mixed antibodies have to be prepared.

**Extraction step**

Water, acetonitrile, methanol, methanol-acetonitrile mixtures and aqueous solutions of acetonitrile or methanol can be used as extraction solvents for mycotoxins [73,94]. In case of OTA analysis, HPLC and TLC require extensive sample preparation [94]. Generally, for OTA analysis, the matrix is acidified before extraction with organic solvents [95-97]. Aflatoxins extraction from food matrices is usually done with chloroform or aqueous solutions of methanol or acetonitrile [98].

**Cleanup step**

Sample cleanup can be achieved using immunoaffinity columns [99] or solid phase extraction cartridges [98]. Due to its specificity, IAC has the tendency to replace solid phase extraction technique.

[100,101]. Another method of sample purification is the liquid–liquid extraction [102].

There are also trials where the cleanup step has been avoided [75,76].

The latest generation of LC-MS/MS instruments using electrospray ionization allows the analysis of crude plant extracts without any previous clean-up [103,104].

Single- and multi-toxin IAC can be used for mycotoxin analysis in food matrices in association with LC–MS or LC–MS/MS methods, at the expense of GC–MS and GC–MS/MS [100].

Some modifications were made to the original QECERS method for the purpose of trichothecenes analysis in wheat flour [94]. After they were homogenized, the samples were extracted with methanol:acetonitrile (85:15, v/v). Magnesium sulphate and sodium chloride were added and the extract was centrifuged and filtered. LC-ESI+/MS analysis was carried out, using a Luna C18 column. The five trichothecenes (T-2, HT-2, DAS, DON and NIV) were not detected in the studied samples. The analytes formed Na adducts with the same mass-to charge ratios: 355, 319, 389, 447 and 489, for NIV, DON, DAS, HT-2 and T-2, respectively.

**Table 3.3. Performance characteristics obtained for wheat flour samples spiked with five trichothecenes at 500µg/kg [94]:**

| Mycotoxin | LOD, µg/kg | LOQ µg/kg | Recovery (%) | Repeatability (RSD, %) (n=5) | Reproducibility (RSD, %) (5 different days) |
|---|---|---|---|---|---|
| DON | 3 | 10 | 86 | 6.7 | 8.2 |
| NIV | 30 | 100 | 100 | 6.5 | 8.1 |
| DAS | 1.5 | 5 | 108 | 3.4 | 8.1 |
| T-2 | 1 | 4 | 93 | 4.0 | 5.6 |
| HT-2 | 5 | 18 | 104 | 4.7 | 9.0 |

## *3.5.Comparison of analytical methods for mycotoxins*

Different analysis methods have been developed for mycotoxin analysis in food products and feeds. HPLC and ELISA have proven to be highly efficient for mycotoxin determination in a wide variety of matrices. GC has also been used, mostly with MS detector [84]. In association with HPLC, tandem mass spectrometry provides high selectivity and sensitivity [73]. Flame ionisation detection (in GC) and fluorescence detection (in HPLC) have also been used [81,85].

A very important aspect concerning mycotoxin analysis is sample preparation and cleanup. Cleanup steps are essential for any analysis method [66,72, 84, 100] in order to eliminate the interferences. The accuracy of a certain analysis method critically depends on the type of cleanup applied. Immunoaffinity cleanup is an efficient sample purification method, particularly in the case of HPLC-MS/MS.

In order to reduce analysis time, simultaneous analysis of different mycotoxin types is usually desired. The use of a cleanup column with different types of antibodies, corresponding to each toxin category, can be a solution to this problem.

The need for derivatization is a disadvantage of some methods such as HPLC-FD and gas chromatography. Cross-reactivity may occur in ELISA trials. In some cases, ELISA results are confirmed by HPLC-MS/MS [53], but in other cases [68] there is the possibility of false positives. For this reason, HPLC-MS/MS has to be used for confirmation purposes [6].

As a general conclusion of the literature survey HPLC-ESI-MS/MS together with an efficient sample cleanup step seems to the method of choice for mycotoxin analysis in food samples. The choice of HPLC-ESI-MS/MS with immunoaffinity cleanup can be explained in terms of method accuracy and sensitivity. Low LOD values can be obtained, due to mass spectrometry sensitive detection, enhanced by concentration steps during sample preparation. The linear range, recoveries and standard deviations obtained with HPLC-ESI-MS/MS [76], as well as immunoaffinity column specificity, are also reasons for choosing this method. The second MS step from mass spectrometric analysis offers additional selectivity to the method and leads to improved signal to noise ratios [105].

# 4. Experimental

## 4.1. Apparatus

Chromatographic analysis was performed with a reversed-phase HPLC-MS/MS system including a Waters 2690/2695 Separations module and a triple quadrupole Waters QuattroMicro API mass spectrometer. Electrospray ionisation in positive mode was used. Data analysis was carried out using QuanLynx 4.1 (Waters) software. A reversed-phase Sunfire C18 3,0x150 mm (3,5 µm) column was used for chromatographic separation. The guard column was Sunfire C18 3,0x20 mm (3,5 µm).

A Mettler Toledo AB204-S balance was used, and pH was determined with a Mettler Toledo SevenMulti pH meter.

A centrifuge, orbital shaker and a rotatory evaporator were also necessary for sample preparation.

## 4.2. Laboratory ware

For the sample preparation and cleanup, the following glassware and materials were used: volumetric flasks (100 ml, 1000 ml), beakers, graduated cylinders (20 ml, 50 ml), plastic flasks, glass vials, Erlenmeyer flasks, glass funnels, weighing funnels, Whatman 4 paper filter, Eppendorf pipets, volumetric pipets (1 ml, 1,5 ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml), automatic pipets (1 ml). VICAM Myco 6 in 1 cleanup columns, fitted plastic reservoirs and cuvette rack were used during sample cleanup.

### 4.2.1.VICAM Myco 6 in 1 column

VICAM Myco 6 in 1 is an immunoaffinity column designed to capture the following toxins: aflatoxins B1, B2, G1 and G2, ochratoxin A, fumonisins B1 and B2, deoxynivalenol, zearalenone , T-2 and HT-2. Other quantitation methods can also be applied to the methanol eluate. A LC-ESI-MS/MS method using this type of column was optimized [106] and the corresponding procedure was already validated for multitoxin analysis of corn samples.

## 4.3. Chemicals

For the sample extraction and cleanup, the following chemicals were used: phosphate buffer saline solution, HPLC grade methanol and deionized water. HPLC grade methanol, deionized water, ammonium acetate and formic acid were necessary for chromatographic analysis. Mycotoxin standards were used for spiking solution preparation and for calibration curves.

PBS solution was prepared with KCl, NaCl, $KH_2PO_4$, $Na_2HPO_4*12H_2O$ and deionized water [107]. The following amounts of salts were dissolved in 1 l of deionized water: 0.20 g KCl, 8.00 g NaCl, 0.20 g $KH_2PO_4$ and 2.92 g $Na_2HPO_4*$ $12H_2O$ [107]. Aflatoxins, T-2 toxin and HT-2 toxin standards were purchased from Supelco, while ochratoxin A and zearalenone standards and ammonium acetate were produced by Fluka. Methanol was purchased from J.T. Baker and the salts necessary for PBS, the formic acid and acetic acid were obtained from Merck.

## 4.4. Procedure description

Maize flour samples were purchased from the local grocery shop. 10 grams of sample were weighed and extracted with 50 ml phosphate buffer saline solution. Eight parallels were analysed. Before extraction, 6 samples were spiked with 1 ml spiking solution. The spiking solution contained the mycotoxins of interest in low concentrations, at maximum acceptable levels, as follows:

**Table 4.1. Spiking solution concentrations.**

| Mycotoxin | Spiking concentration |
|---|---|
| AfG1 | 0.020 µg/ml |
| AfB1 | 0.020 µg/ml |
| AfG2 | 0.020 µg/ml |
| AfB2 | 0.020 µg/ml |
| HT-2 toxin | 0.5 µg/ml |
| T-2 toxin | 0.5 µg/ml |
| Ochratoxin A | 0.02 µg/ml |
| Zearalenone | 0.5 µg/ml |

Extract A

After adding the extraction solvent, the maize flour samples were kept on the orbital shaker for 60 min at 300 rpm. Centrifugation was done at 4200 rpm for 20 min. After centrifugation, the solid was removed and the liquid extracts were transferred in glass vials wrapped in aluminium foil, in order to avoid chemical decomposition of aflatoxins B1 and G1. No filtration was done.

Extract B

35 ml HPLC grade methanol was added on the solid residues, in order to obtain an extraction solvent of 70% methanol. The samples were shaken for 60 min. at 300 rpm, and centrifuged for 20 min. at 4200 rpm. 10 ml of extract were diluted with 90 ml PBS and then filtered with Whatman 4 filter paper.

Immunoaffinity cleanup

50 ml of extract B were passed through VICAM Myco 6 in 1 column, at a speed of about 1 drop/second, until air passed through the column. Then the VICAM 6 in 1 was washed with 20 ml PBS, at a rate of about 1-2 drops/second, in order to remove the methanol traces.

After this washing step, 5 ml PBS extract (extract A) were passed through the column at a speed of 1 drop/second, until air passed through the column. Removing the methanol traces, before applying extract A on the column, was done for the purpose of improving mycotoxin recoveries.

After passing extract A, the column was washed with 20 ml deionized water, at a rate of 1-2 drops/second, for the purpose of removing PBS traces and matrix interfering compounds. Then the mycotoxins were eluted with 3 consecutive applications of 1,5 ml of HPLC grade methanol, at a rate of 1 drop/second. Towards the end of the first elution, after most of the methanol had passed through the cleanup column, applying air pressure to the column was stopped. The column was left undisturbed for 5 min., with a methanol layer above the resin bed.

The methanolic eluate was evaporated to dryness using a rotary evaporator. The residue was dissolved in 0.5 ml of solution containing 80% eluent A and 20% eluent B, and used for chromatographic analysis. Injection volume was 10 µl.

Eluent composition was as follows: eluent A consisted in a 5 mM $CH_3COONH_4$ aqueous solution containing 0.1%HCOOH, with a pH of 3.2. Eluent B was a 5 mM $CH_3COONH_4$ methanolic solution containing 0.1%HCOOH.

The chromatographic conditions are shown in the following table:

**Table 4.2. Chromatographic conditions for HPLC-ESI-MS/MS analysis of maize flour samples.**

| | |
|---|---|
| Guard column | Sunfire C18 3,0x20 mm (3,5 µm) |
| Analytical column | Sunfire C18 3,0x150 mm (3,5 µm) |
| Column temperature | 35 deg C |
| Sample temperature | 20 deg C |
| Eluent flow rate | 0.4 ml/min |

**Table 4.3. MS-detector settings for HPLC-ESI-MS/MS analysis of maize flour samples.**

| | |
|---|---|
| Capillary voltage | 2,35 kV |
| Extractor voltage | 3 V |
| RF lens voltage | 0.3 V |
| Source Temp. | 135 deg C |
| Desolvation Temp | 350 deg C |
| Desolvation gas | 600 l/h |
| Cone gas | 50 l/h |

**Table 4.4. Gradient program for HPLC-ESI-MS/MS analysis of maize flour samples.**

| Time(min) | % A | %B |
|---|---|---|
| 0 | 80 | 20 |
| 2 | 80 | 20 |
| 4 | 70 | 30 |
| 15 | 5 | 95 |
| 16 | 0 | 100 |
| 18 | 0 | 100 |
| 18.1 | 80 | 20 |
| 22 | 80 | 20 |

Eluent A: 5 mM $CH_3COONH_4$ aqueous solution containing 0.1%HCOOH, pH = 3.2

Eluent B: 5 mM $CH_3COONH_4$ methanolic solution containing 0.1%HCOOH

Retention times for the analysed toxins and mass to charge ratios of their corresponding parent ions were as follows:

**Table 4.5. Characteristics of analysed ions and corresponding parameters.**

| | Retention time (min) | Parent ion m/z | Q-ion m/z | Secondary Ion m/z | Cone voltage (V) | Fragmentation energy (eV) |
|---|---|---|---|---|---|---|
| Aflatoxin B1 | 13.4 | 313 | 285 | 241 | 40 | 26 |
| Aflatoxin B2 | 13.0 | 315 | 259 | 287 | 40 | 32 |
| Aflatoxin G1 | 12.5 | 329 | 243 | 213 | 39 | 35 |
| Aflatoxin G2 | 12.0 | 331 | 245 | 257 | 39 | 35 |
| Ochratoxin A | 16.9 | 403,8/405,8 | 238.8 | 240.8 | 20 | 10 |
| Zearalenone | 16.9 | 318.8 | 300.8 | 282.8 | 22 | 25 |
| T-2 toxin | 16.0 | 484 ($NH_4$-adduct) | 245 | 304 | 19 | 10 |
| HT-2 toxin | 15.1 | 442 ($NH_4$-adduct) | 263 | 215 | 20 | 15 |

The chromatograms obtained for each mycotoxin will be presented in Annex I, for two ion transitions.

# 5. Results and discussion

## *5.1. Results*

Eight maize flour samples were analysed, from which six samples were spiked and two samples were blank. The spiking mixture contained the mycotoxins of interest at the maximum accepted levels. Aflatoxins, ochratoxin A, zearalenone, T-2 toxin and HT-2 toxin were determined with reasonable accuracy in all the analysed samples.

Two sets of data were obtained, corresponding to two different chronological dates. The results are presented in Tables 5.1 and 5.2.

**Table 5.1. Mycotoxin concentrations found in spiked maize flour samples.**

| | Spike 1 (µg/kg) | Spike 2 (µg/kg) | Spike 3 (µg/kg) | Spike 4 (µg/kg) | Spike 5 (µg/kg) | Spike 6 (µg/kg) |
|---|---|---|---|---|---|---|
| Aflatoxin B1 | 1.79 | 1.71 | 1.74 | 1.90 | 1.75 | 1.72 |
| Aflatoxin B2 | 0.97 | 0.97 | 0.96 | 0.97 | 0.99 | 0.95 |
| Aflatoxin G1 | 1.67 | 1.69 | 1.67 | 1.73 | 1.66 | 1.61 |
| Aflatoxin G2 | 0.72 | 0.68 | 0.69 | 0.72 | 0.64 | 0.70 |
| Ochratoxin A | 1.46 | 1.37 | 1.56 | 1.46 | 1.39 | 1.56 |
| Zearalenone | 60.5 | 59.5 | 58.5 | 63.0 | 62.0 | 60.0 |
| T-2 toxin | 20.1 | 18.8 | 20.1 | 18.3 | 23.3 | 19.2 |
| HT-2 toxin | 49.0 | 51.5 | 46.0 | 51.5 | 42.0 | 54.0 |
| Sum of T2-HT2* | 69.1 | 70.3 | 66.1 | 69.8 | 65.3 | 73.2 |

**Table 5.2. Mycotoxin concentrations found in spiked maize flour samples.**

| | Spike 1 (µg/kg) | Spike 2 (µg/kg) | Spike 3 (µg/kg) | Spike 4 (µg/kg) | Spike 5 (µg/kg) |
|---|---|---|---|---|---|
| Aflatoxin B1 | 1.10 | 1.15 | 1.36 | 1.36 | 1.38 |
| Aflatoxin B2 | 1.84 | 1.65 | 2.04 | 1.90 | 1.82 |
| Aflatoxin G1 | 1.37 | 1.08 | 1.35 | 1.28 | 1.32 |
| Aflatoxin G2 | 1.66 | 1.55 | 1.80 | 1.56 | 1.56 |
| Ochratoxin A | 1.57 | 1.49 | 1.59 | 1.42 | 1.19 |
| Zearalenone | 35.35 | 31.60 | 37.35 | 35.50 | 33.00 |
| T-2 toxin | | 20.00 | 28.00 | 20.00 | 27.50 |
| HT-2 toxin | 66.00 | 37.00 | 53.00 | 52.50 | 42.50 |
| Sum of T2-HT2* | 66.00 | 57.00 | 81.00 | 72.50 | 70.00 |

*

According to the Vicam 6 in1 producer T-2 is transformed during the sample preparation into HT-2 [107].

Recoveries were determined by spiking of maize flour, from 6 replicates. The recovery and standard deviation values are shown in Tables 5.3 and 5.4.

**Table 5.3. Average concentrations for each toxin in maize flour and the corresponding spiking level, recovery, standard deviation and relative standard deviation.**

| | Average | Spiking level | Recovery | St. dev | Rel. st.dev | LOD |
|---|---|---|---|---|---|---|
| | µg/kg | µg/kg | % | µg/kg | % | µg/kg |
| Aflatoxin B1 | 1.76 | 2.0 | 88 | 0.07 | 4.1 | 0.21 |
| Aflatoxin B2 | 0.97 | 1.0 | 97 | 0.01 | 1.2 | 0.03 |
| Aflatoxin G1 | 1.67 | 2.0 | 83 | 0.04 | 2.4 | 0.12 |
| Aflatoxin G2 | 0.69 | 1.0 | 69 | 0.03 | 4.0 | 0.09 |
| Ochratoxin A | 1.46 | 2.0 | 73 | 0.08 | 5.4 | 0.24 |
| Zearalenone | 60.6 | 50 | 121 | 1.66 | 2.7 | 7.3 |
| T-2 toxin | 20.0 | 50 | 40 | 1.79 | 8.9 | 5.4 |
| HT-2 toxin | 49.0 | 50 | 98 | 4.37 | 8.9 | 13 |
| Sum of T2-HT2* | 69.0 | 100 | 69 | 2.90 | 4.2 | 8.7 |

*According to the Vicam 6 in1 producer T-2 is transformed during the sample preparation into HT-2 [107].

**Table 5.4. Average concentrations for each toxin in maize flour and the corresponding spiking level, recovery, standard deviation and relative standard deviation.**

| | Average | Spiking level | Recovery | St. dev | Rel. st.dev | LOD |
|---|---|---|---|---|---|---|
| | µg/kg | µg/kg | % | µg/kg | % | µg/kg |
| Aflatoxin B1 | 1.27 | 2.0 | 63 | 0.13 | 10.5 | 0.39 |
| Aflatoxin B2 | 1.85 | 2.0 | 92 | 0.14 | 7.6 | 0.42 |
| Aflatoxin G1 | 1.28 | 2.0 | 64 | 0.12 | 9.1 | 0.36 |
| Aflatoxin G2 | 1.62 | 2.0 | 81 | 0.11 | 6.6 | 0.33 |
| Ochratoxin A | 1.45 | 2.0 | 73 | 0.16 | 11.0 | 0.48 |
| Zearalenone | 34.6 | 50 | 69 | 2.26 | 6.5 | 9.1 |
| T-2 toxin | 23.9 | 50 | 48 | 4.48 | 18.8 | 13 |
| HT-2 toxin | 50.2 | 50 | 100 | 11.14 | 22.2 | 33 |
| Sum of T2-HT2* | 69.3 | 100 | 69 | 8.80 | 12.7 | 26 |

*According to the Vicam 6 in1 producer T-2 is transformed during the sample preparation into HT-2 [107].

Except zearalenone, no mycotoxins were found in the blank samples. In the case of zearalenone the found low values (2.3 µg/kg) were subtracted from the results when recovery was calculated.

Tentative LOD values were calculated based on the standard deviations *s* of the results of spiking experiments as follows: LOD = $x_m$ + 3 x *s*, where $x_m$ is the average content found in the blank samples.

**Table 5.5. Average recoveries and detection limits for the analysed mycotoxins.**

| | Recovery | LOD |
|---|---|---|
| | % | µg/kg |
| Aflatoxin B1 | 75.5 | 0.30 |
| Aflatoxin B2 | 94.5 | 0.23 |
| Aflatoxin G1 | 73.5 | 0.24 |
| Aflatoxin G2 | 75 | 0.21 |
| Ochratoxin A | 73 | 0.36 |
| Zearalenone | 95 | 8.2 |
| T-2 toxin | 44 | 9.4 |
| HT-2 toxin | 99 | 23 |
| Sum of T2-HT2* | 69 | 18 |

*According to the Vicam 6 in1 producer T-2 is transformed during the sample preparation into HT-2 [107].

During the validation experiments for T-2 and HT-2 toxin, the assumption that during sample preparation T-2 is transformed into HT-2 toxin [107] was taken into account. As a consequence in this case, the detected sum of those toxins could be used instead of single toxins, with average recovery. However this assumption was not confirmed in light of the results of the FAPAS proficiency test 2261 that was performed later on. The disagreement between the FAPAS test and the Vicam specifications consisted in the fact that in the case of FAPAS, T-2 had a certain value for recovery, but in Vicam specifications it was not detected at all [107]. In the FAPAS proficiency test 2261, oat-based test material was treated and analysed in five replicates as described in the experimental section. The assigned value for proficiency test was 164 µg/kg for T-2 toxin and 257 µg/kg for HT-2 toxin. Results of analysis in the laboratory are presented in Table 5.6. The results are not recovery-corrected.

**Table 5.6. Results of the FAPAS proficiency test 2261.**

| | Number of replicates | Average conc. µg/kg | σ µg/kg | Recovery | Consensus values µg/kg | Target st. dev. µg/kg | Datapoints | z-score |
|---|---|---|---|---|---|---|---|---|
| T-2 toxin | 5 | 50.2 | 8.1 | 40% | 164 | 34.5 | 59 | -3.3 |
| HT-2 toxin | 5 | 218 | 16.8 | 100% | 257 | 50.5 | 57 | -0.8 |

The result of the HT-2 toxin is good, but the result of the T-2 toxin is unacceptable. By looking at the recovery of the T-2 toxin it is evident that the disagreement between the measured value and the consensus value comes form the low recovery. Correcting with recovery if the recovery is low and its cause is unknown is not considered good practice [108]. Therefore the result of T-2 toxin was not recovery-corrected. If, however, such correction would be carried out then the corrected value would be 125.5 µg/kg and z-score would be -1.1. This indicates that the determined recovery most probably is adequate.

For the procedure developed in this work, the linear range was 1-500 ng/ml. Squared correlation coefficients ranged between 0.995 and 0.999 for 4 point calibration curves.

There is not enough data for carrying out comprehensive uncertainty analysis. However, using the Nordtest uncertainty estimation approach [109] a tentative preliminary estimate can be obtained for the HT-2 Toxin. The relative standard deviation of the spiking experiments 8.9% can be used as an estimate of $u(R_w)_{rel}$. The absolute difference between the found value and the consensus value 39 µg/kg can be used as $RMS_{bias}$ and the $u(Cref)$ can be found as the standard deviation of the mean value of the participating laboratories giving 6.7 µg/kg. The resulting $u(bias)$ value is 39.6 µg/kg and $u(bias)_{rel}$ = 18.2%. The relative combined standard uncertainty estimate is $u_c$ = 20%. This uncertainty estimate is quite realistic keeping in mind the complexity of the analysis and the low levels of the analytes.

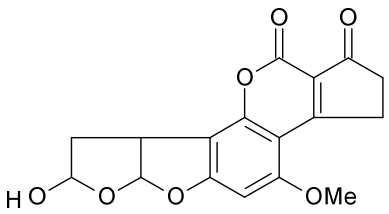
## 5.2. Discussion

The performance of the developed method was found adequate for mycotoxin monitoring in food for all mycotoxins except the T-2 toxin. The recovery of the T-2 toxin is not fully acceptable for routine work and further effort is necessary to improve the performance of the method for this toxin.

The calculated LOD values are tentative because they were found at higher concentration levels that the actual LOD. At the same time, in instrumental analysis, the higher is the analyte level, the higher also is the scatter of the parallel results and thus the standard deviation. Therefore it is not expected that the actual LOD values are much higher. Of course, in the future LOD determination have to be repeated at lower analyte levels.

It is known that aflatoxins are unstable when exposed to ultraviolet light. Also, it is stated that hydrogenation of aflatoxin B1 and G1 yields aflatoxin B2 and G2 respectively [110]. When a test solution containing aflatoxins B1 and G1 was exposed to the light for a week and analyzed on LS-MS/MS system couple of interesting chromatograms were produced (annex G, fig. 1,2). These multiple peaks might correspond to compounds formed by hydrogenation and/or hydroxylation of aflatoxin B1 and G1 [111]. During chromatographic elution, these compounds would have different retention times due to the differences in polarities. The presence of hydroxyl groups reduces retention time. The compound presented in Figure 5.1 can be obtained by addition of a water molecule to the double bond of aflatoxin B1 [111].

**Fig. 5.1. Aflatoxin B1 decomposition product.**




After giving away one water molecule, an unprotonated compound with the mass of 312 would be obtained [111], corresponding to a precursor of m/z = 313.This fact shows the importance of keeping the aflatoxin B1 and G1 standards in suitable conditions without unnecessary exposure to the light, in order to prevent decomposition. In the samples analysed such a phenomenon was not noticed, probably due to the fast procedure and use of aluminum foil for wrapping the extract vials.

**Developing an HPLC-ESI-MS/MS method for simultaneous determination of mycotoxins in maize flour and other matrices**

Dana-Maria Bunaciu

## 6. Summary

A method for simultaneous analysis of 8 mycotoxins – aflatoxins B1, B2, G1, G2, Ochratoxin A, Zearalenone, T-2 toxin and HT-2 toxin – was developed using an HPLC-ESI-MS/MS method with immunoaffinity sample cleanup. Approximate quantitation limits were the following: 2 µg/kg for aflatoxin B1, 1 µg/kg for aflatoxin B2, 2 µg/kg for aflatoxin G1, 1 µg/kg for aflatoxin G2, 2 µg/kg for ochratoxin A, 50 µg/kg for zearalenone, 50 µg/kg for T2 toxin, 50 µg/kg for HT2 toxin and 100 µg/kg for the sum of T2 and HT2. Sample preparation involved sequential extraction with PBS and methanolic solution. The mycotoxins were determined with reasonable accuracy in the spiked maize samples. With the exception of T-2 toxin, recoveries ranged between 69 and 121%, while relative standard deviation values were between 1.2 and 8.9%. Since matrix effects were not investigated, the results are presented as apparent recoveries.

A tentative uncertainty estimation was carried out for the HT-2 toxin, which yielded relative combined standard uncertainty as 20%.

Method's accuracy relies on antibodies specificity and high selectivity and sensitivity of detection. The possibility of simultaneous determination of the eight mycotoxins significantly reduces the analysis time.

Based on the results presented in this thesis further experiments will be performed in Health Board Tartu laboratory, in order to develop a method with required LoD-s and LoQ-s for other types of food samples. The further goal will be an accredited method by the fall of 2010.

**HPLC-ESI-MS/MS meetodi arendus mükotoksiinide koosmääramiseks maisijahus ja teistes maatriksites**

Dana-Maria Bunaciu

## 7. Summary (in Estonian)

Töötati välja metoodika kaheksa mükotoksiini – aflatoksiinid B1, B2, G1, G2, Ochratoksiin A, Zearalenoon, T-2 toksiin ja HT-2 toksiin – määramiseks toidus. Proovide ettevalmistamisel kasutati ekstraktsiooni PBS ja metanooli abil. Proovilahuseid puhastati enne analüüsi immunoafiinsuskolonnil. Määramine viidi läbi HPLC-ESI-MS/MS meetodiga. Metoodika määramispiirideks saadi: 2 µg/kg aflatoksiin B1 jaoks, 1 µg/kg aflatoksiin B2 jaoks, 2 µg/kg aflatoksiin G1 jaoks, 1 µg/kg aflatoksiin G2 jaoks, 2 µg/kg Ochratoksiin A jaoks, 50 µg/kg Zearalenooni jaoks, 50 µg/kg T2 toksiini jaoks, 50 µg/kg HT-2 toksiini jaoks ja 100 µg/kg T2 ja HT-2 summa jaoks. Välja arvatud T-2 toksiin, olid saagised 69 kuni 121% suhteliste standardhälvetega 1.2 kuni 8.9%. Kuna maatriksiefekte ei uuritud, siis on saagised antud näivate saagistena.

Tehti ka tentatiivne määramatuse hinnang HT-2 toksiini jaoks ja saadi suhteliseks liitstandardmääramatuseks 20%.

Metoodika täpsus põhineb proovide puhastamisel kasutatavate antikehade spetsiifilisusel ning määramismetoodika selektiivsusel ja tundlikkusel. Erinevate toksiinide koosmääramine vähendab oluliselt analüüsiks kuluvat aega.

Töös toodud tulemustele baseerudes keskendub Terviseameti Tartu labor metoodika edasisele arendamisele, mille lõppeesmärgiks on nõutavate avastamis- ja määramispiiride saavutamine ka teiste toiduproovide analüüsiks, et saada 2010. aasta sügiseks uus akrediteeritud analüüsimetoodika.

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# 9. Annexes

A) Important mycotoxin categories, together with the fungal species that produce them

B) Mycotoxin incidence and contamination levels in Europe

C) Maximum mycotoxin levels according to European legislation

D) Health effects of mycotoxins in humans

E) Ultra-performance liquid chromatography tandem mass spectrometry for simultaneous mycotoxin analysis

F) Methods used in order to prevent crop infection

G) Chromatogarphic peaks obtained for Aflatoxin B1 and Aflatoxin G1

H) Calibration graphs

I) Chromatograms obtained for the analysed toxins for two ion transitions

J) Spectra of analysed mycotoxins

K) Other mycotoxin structures

A) Important mycotoxin categories, together with the fungal species that produce them

**Table 1. Important mycotoxin categories, together with the fungal species that produce them [3].**

| Mycotoxin | Fungal species |
|---|---|
| Aflatoxins | Aspergillus flavus, A. parasiticus, Penicillum frequentans |
| Ochratoxins | A. ochraceus, A. carbonarius, P. verrucosum, P. cyclopium, P. veridicatum |
| Trichothecenes | Fusarium tricinctum, F. sporotrichoides |
| Fumonisins | F. moniliforme, F. proliferatum |
| Zearalenone | F. graminearum, F. tricinctum, F. oxysporum |
| Nivalenol | F. nivale |

B) Mycotoxin incidence and contamination levels in Europe

**Table 2. Incidence of mycotoxin contamination in feed grain and animal feed [80] sourced in Europe and the Mediterranean Region (%).**

| | DON | T-2 | ZEA | Fumonisins | Afla B1 | OTA |
|---|---|---|---|---|---|---|
| Maize | 81 | 6 | 63 | 56 | 21 | 0 |
| Wheat | 62 | 22 | 92 | 100 | 0 | 42 |
| Barley | 50 | 20 | 11 | 0 | 0 | 3 |
| Oat | 82 | 81 | 0 | 0 | 0 | 0 |
| Finished feed | 56 | 13 | 27 | 30 | 32 | 73 |
| Soybean meal | 41 | 0 | 6 | 50 | 0 | 0 |
| Other feed ingredients | 73 | 43 | 26 | 39 | 37 | 68 |

Arithmetic means of concentrations found in the analysed samples and values for maximum levels detected are presented in the table below.

**Table 3. Mycotoxin contamination levels (µg/kg) detected in samples from different regions in Europe and the Mediterranean [80].**

| | Northern Europe, (µg/kg) | | Central Europe, (µg/kg) | | Southern Europe and Mediterranean, (µg/kg) | |
|---|---|---|---|---|---|---|
| | Mean | Max | Mean | Max | Mean | Max |
| DON | 559 | 5510 | 571 | 8020 | 304 | 3036 |
| T-2 | 137 | 1776 | 190 | 829 | 30 | 60 |
| Zearalenone | 180 | 970 | 273 | 1392 | 174 | 2348 |
| Fumonisins | 432 | 530 | 580 | 580 | 757 | 3120 |
| Aflatoxin B1 | 10 | 60 | 47 | 311 | 67 | 656 |
| Ochratoxin A | 8 | 10 | 231 | 530 | 6 | 28 |

C) Maximum mycotoxin levels according to European legislation [112]

**Table 4. Maximum levels for aflatoxins in foodstuffs (µg/kg).**

| | Product | AfB1 | AfB1, B2, G1, G2 | M1 |
|---|---|---|---|---|
| 4.1 | All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 4.2, 4.3, 4.4 | 2.0 | 4.0 | - |
| 4.2 | Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs | 5.0 | 10.0 | - |
| 4.3 | Processed cereal-based foods and baby foods for infants and young children | 0.10 | - | - |
| 4.4 | Dietary foods for special medical purposes intended specifically for infants | 0.10 | - | 0.025 |

**Table 5. Maximum levels for zearalenone in foodstuffs (µg/kg).**

| | Product | Concentration |
|---|---|---|
| 5.1 | Unprocessed cereals other than maize | 100 |
| 5.2 | Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling | 350 |
| 5.3 | Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 8.4, 8.5, 8.6, 8.7 and 8.8 | 75 |
| 5.4 | Maize intended for direct human consumption, maize based snacks and maize-based breakfast cereals | 100 |
| 5.5 | Processed cereal-based foods (excluding processed maize based foods) and baby foods for infants and young children | 20 |
| 5.6 | Processed maize-based foods for infants and young children | 20 |
| 5.7 | Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 200 |
| 5.8 | Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 300 |

**Table 6. Maximum levels for ochratoxin A in foodstuffs (µg/kg).**

| | Product | Concentration |
|---|---|---|
| 6.1 | Unprocessed cereals | 5.0 |
| 6.2 | All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs listed in 9.3 and 9.4 | 3.0 |
| 6.3 | Processed cereal-based foods and baby foods for infants and young children | 0.50 |
| 6.4 | Dietary foods for special medical purposes intended specifically for infants | 0.50 |

D) Health effects of mycotoxins in humans

**Table 7. The main clinical manifestations of mycotoxicoses [11].**

| Mycotoxin | Localisation | Symptome |
|---|---|---|
| Aflatoxins | Vascular | hemorrhage |
| Aflatoxins | Digestive | liver necrosis, intestinal hemorrhage |
| Ochratoxin A | Urinary | renal malfunction |
| T-2 toxin | Cutanate | irritation, necrosis |
| Zearalenone | Genital | sterility |
| Fumonisins | Respiratory | pulmonary edema, fever |

**Table 8. Mycotoxin-producing fungi of relevance to children's health [113].**

| Fungus | Mycotoxins | Associated health effects |
|---|---|---|
| *Aspergillus flavus, Aspergillus parasiticus* | Aflatoxins | Vomiting, hepatitis,<br>Liver cancer |
| *Fusarium verticillioides, Fusarium proliferatum, Aspergillus ochraceus* | Fumonisins | Vomiting<br>Neural tube defects<br>Esophageal cancer |
| *Fusarium culmorum, Fusarium graminearum, Fusarium cerealis* | Deoxynivalenol | Vomiting |
| *Fusarium sporotrichiodes* | T-2 toxin | Alimentary toxic aleukia<br>Vomiting, hemorrhage |
| *Aspergillus ochraceus, Aspergillus niger* | Ochratoxins | Balkan nephropathy<br>Renal cancer |
| *Fusarium graminearum* | Zearalenone | Estrogenic effects, cervical cancer (suspect) |

E) Ultra-performance liquid chromatography tandem mass spectrometry for simultaneous mycotoxin analysis [73]

Samples (corn, wheat, peanut products) were extracted with an 84% (v/v) acetonitrile aqueous solution. Then they were filtered, purified with Mycosep 226 Aflazon+ Multifunctional cartridges and concentrated [73]. Different aspects were considered for method optimization. Four reversed-phase chromatographic columns and three different cleanup cartridges were tested. Reducing the column length and particle size shortened the elution time. The recovery for cleanup step was higher for the Mycosep 226 purification cartridge. Methanol was chosen as mobile phase component, because the use of acetonitrile reduced method sensitivity. To improve ionization efficiency and sensitivity, 10 mmol/l of ammonium acetate combined with methanol was used as mobile phase for the positive ionization mode; the mobile phase for the negative ionization mode was composed of methanol and 0.1% (v/v) of aqueous ammonia. 10 mycotoxins were analysed in positive mode and negative mode was used for 7 mycotoxins [73]. AfB1, AfB2, AfG1, AfG2, AfM1, OTA, DON, NIV, T-2, HT-2 and ZON were among the analysed toxins. Linear gradient elution was applied and quantitation was done using an internal standard for ZON (ZAN was used as internal standard) and an external standard for the other 16 toxins.

The ionization efficiency and the chromatographic separation are also affected by the sample solvent medium before injection. After the purified extract was dried by nitrogen gas at 50°C, it was redissolved in a mixture of methanol and 10 mmol/l ammonium acetate (1:1, v/v) [73].

The correlation coefficients were bigger than 0.99 for all 17 mycotoxins [73]. Linear range for positive ions was 0.05–20 ng/ml; linear range for negative ions was 0.5–50 ng/ml. Intra-day precision was 3.52–7.02% and inter-day precision was: 4.02–9.24%. Recoveries ranged from 70.6% to 119.0% for three different spiking levels. LOQ values were between 0.01 μg/kg and 0.70 μg/kg, lower than the values mentioned in EU legislation.

F) Methods used in order to prevent crop infection

**Dehulling**

It has been shown that hulless barley species contain a smaller DON amount than the covered ones [114].

**Fungicides**

At present days, fungicides have limited efficiency, even when the products are applied in optimal conditions. The efficiency of the best products currently available is still estimated to be only 60-70%. There are many aspects that influence fungicide activity: product choice, application timing and application rate [21].

The difference in activity of a fungicide towards different fungal species has been noticed. For example, a trial outlined the efficacy of azoxystrobin against *M. nivale*, as well as the differential activity of tebuconazole towards fungal species [22]. According to this study, fungicides also showed selective activities towards mycotoxins; for example, in 2002, the use of 90 g metconazole/ha significantly reduced the DON level, but it was not efficient against nivalenol.

Treatments with fungicides in combination with insecticides reduced mycotoxin levels in crops but were not efficient against mycoflora [115]. Trichothecene synthesis inhibitors, such as ferulic acid, can reduce the trichothecene yield to 85% [116]. Phenolic compounds, for example caffeic acid and vanillic acid, can be efficient against some Fusarium species. Low water activity values and high concentrations of phenolic compounds are required for the complete reduction of fungal growth [117].

**Biological control**

Biological control can also be used against crop infection by Fusarium species. Antagonistic micro-organisms compete with the fusaria for nutrients. For this purpose, they can produce antibiotics or cell-wall degrading enzymes which attack competitors. Antagonists may also have faster growth under optimal and sub-optimal conditions, better survival during unfavourable conditions, or faster re-growth after periods ufavorable for growth. It has been shown that different species of yeasts and bacteria also had antagonistic activity, and prevented FHB occurrence [21].

G) Chromatographic peaks obtained for Aflatoxin B1 and Aflatoxin G1

**Fig. 1. Chromatograms for aflatoxin B1 obtained for a test solution.**

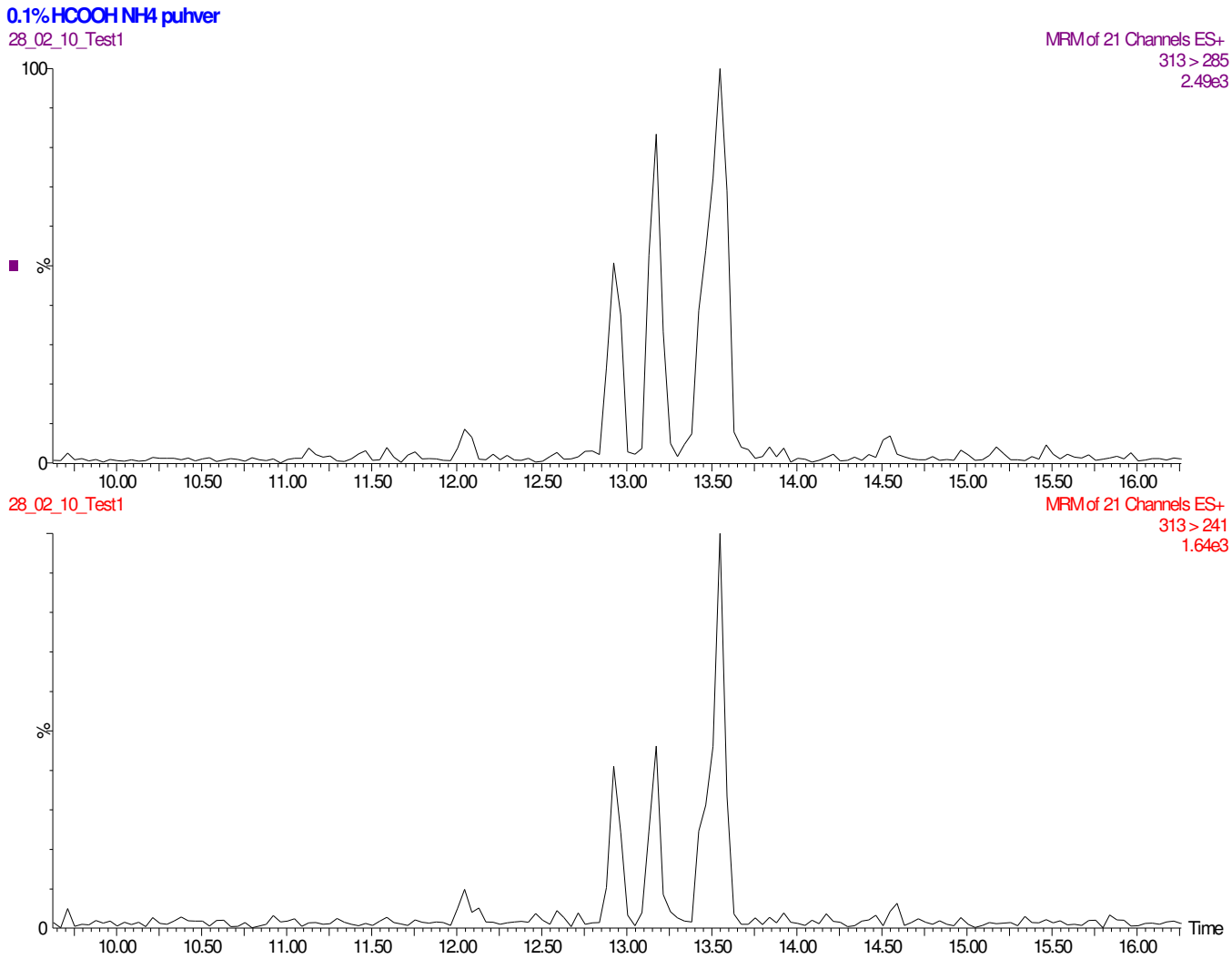
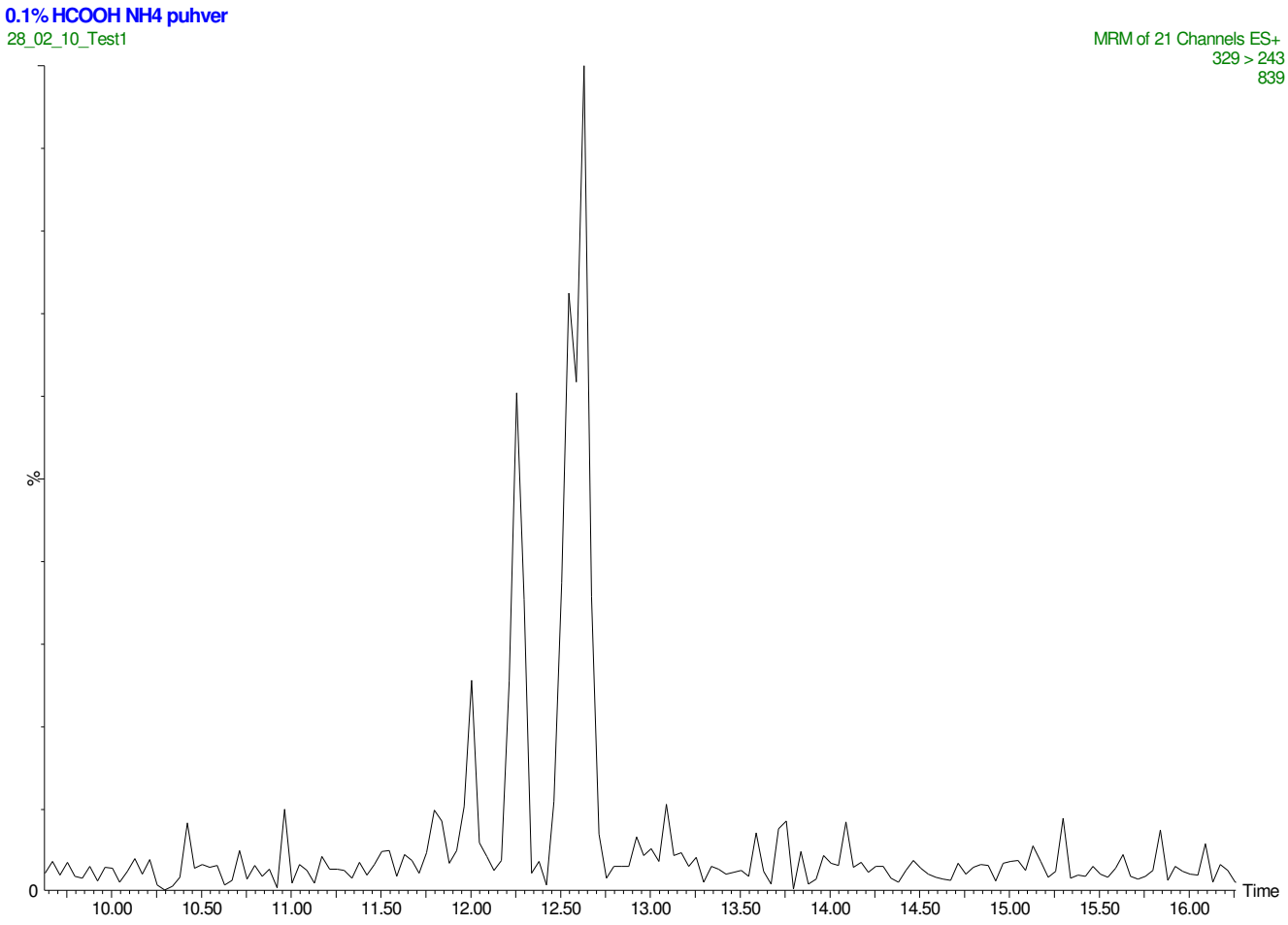
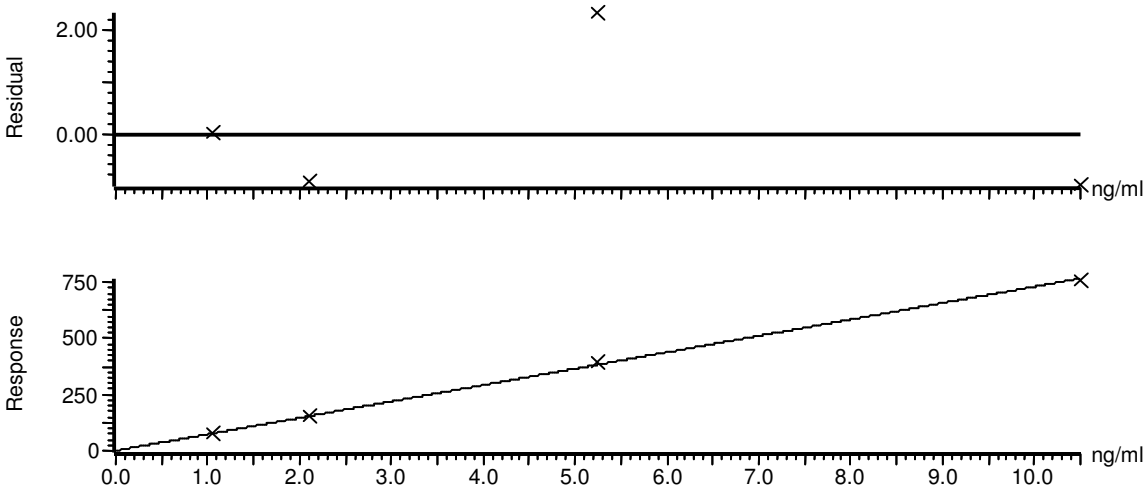


**Fig. 2. Chromatogram for aflatoxin G1 obtained for a test solution.**



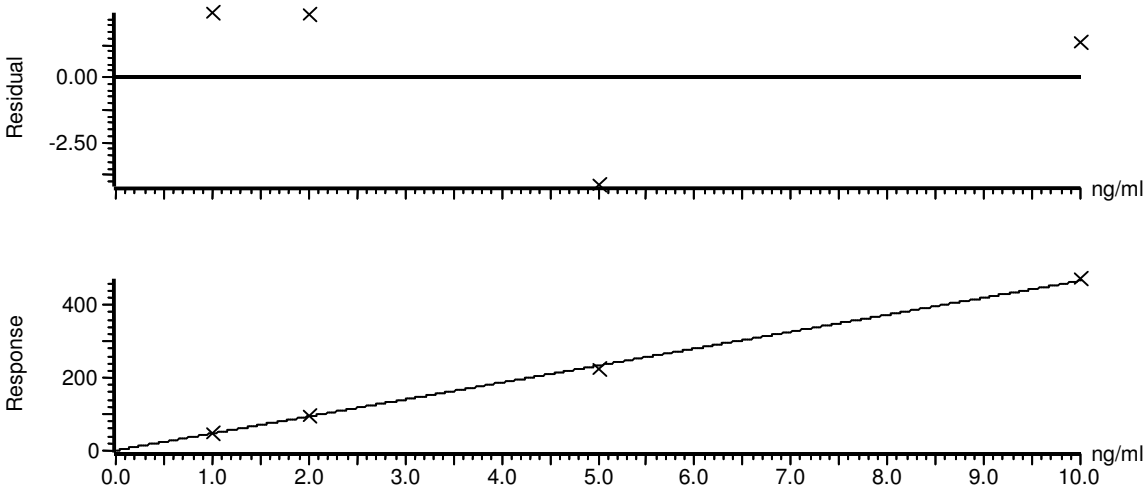
## Fig. 3. Calibration graph for aflatoxin B1

Compound name : Aflatoxin B1
Correlation coefficient : r = 0.999842 , r^2 = 0.999684
Calibration curve : 73.0702 * x + 0.34786
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



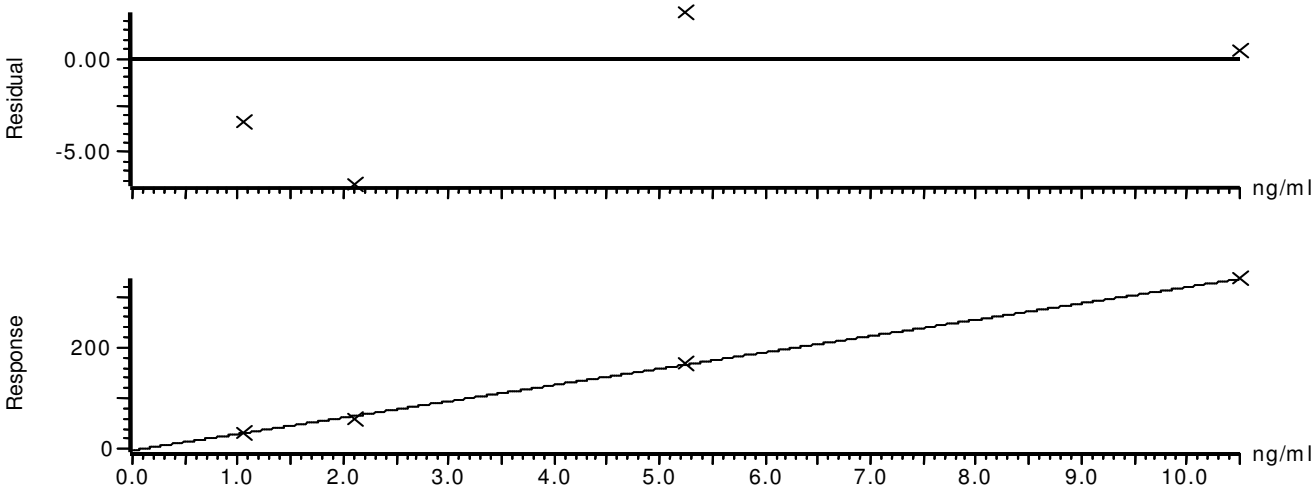

## Fig. 4. Calibration graph for aflatoxin B2

Compound name : Aflatoxin B2
Correlation coefficient : r = 0.999489 , r^2 = 0.998979
Calibration curve : 46.5311 * x + 0.977831
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



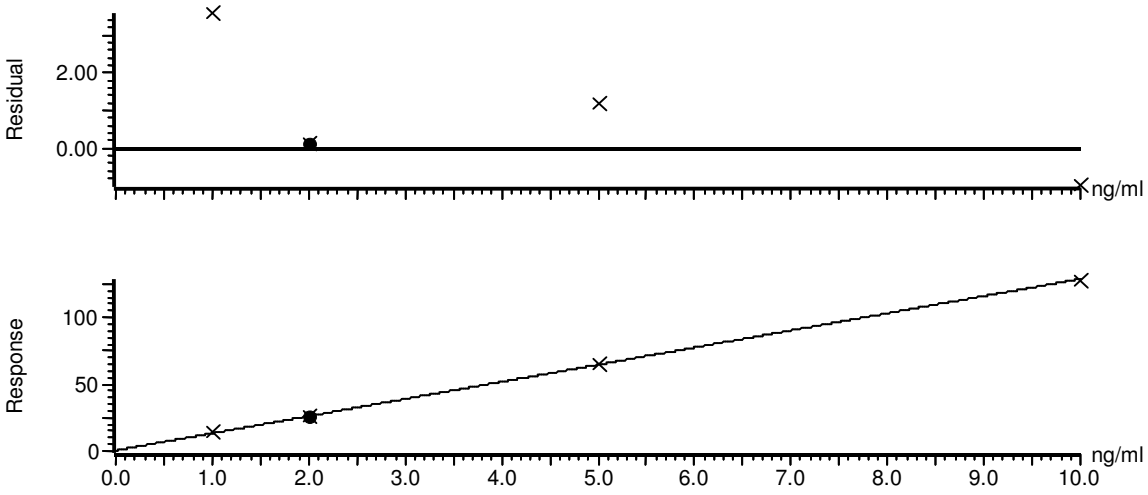
## Fig. 5. Calibration graph for aflatoxin G1

Compound name : Aflatoxin G1
Correlation coefficient : r = 0.999242 , r^2 = 0.998485
Calibration curve : 32.1548 * x + -2.43171
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



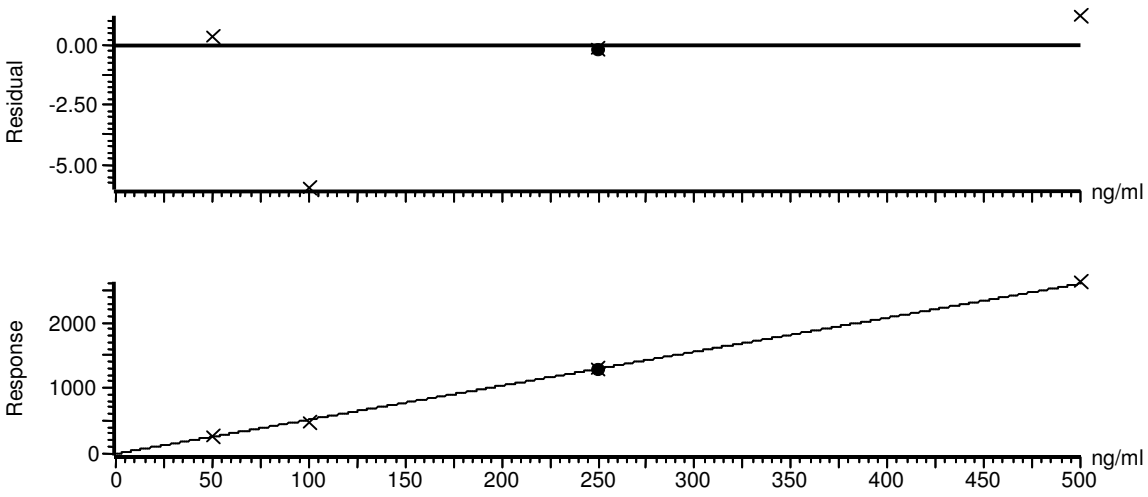

## Fig. 6. Calibration graph for aflatoxin G2

Compound name : Aflatoxin G2
Correlation coefficient : r = 0.999817 , r^2 = 0.999633
Calibration curve : 12.8367 * x + 0.503267
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



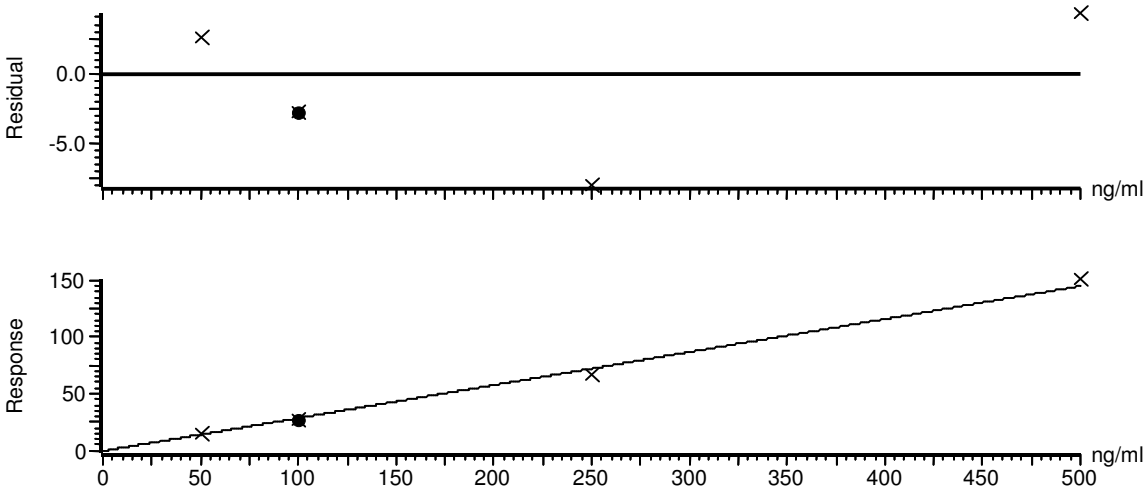
## Fig. 7. Calibration graph for T-2 toxin

Compound name : T2-toxin
Correlation coefficient : r = 0.999562 , r^2 = 0.999125
Calibration curve : 5.22811 * x + -11.8707
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



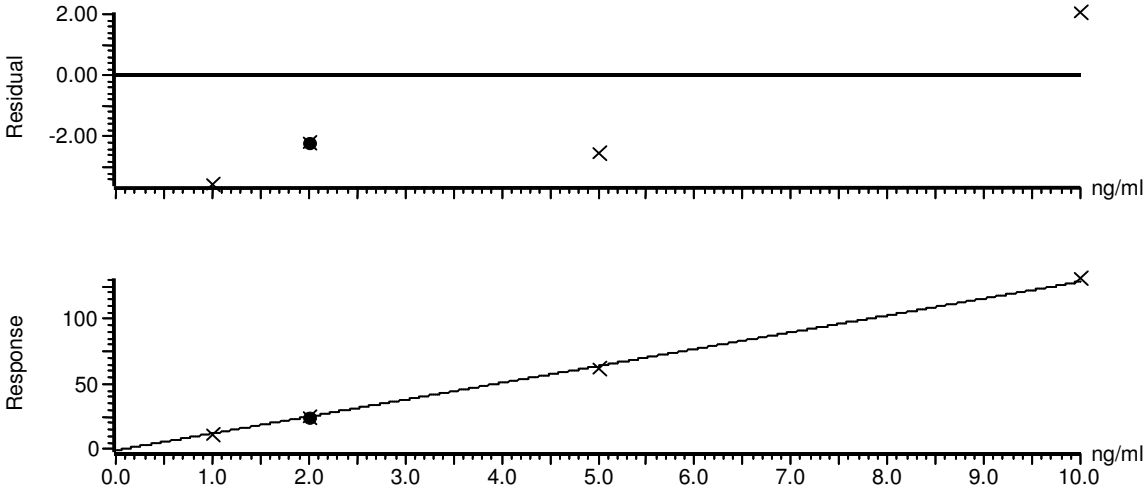

## Fig. 8. Calibration graph for HT-2 toxin

Compound name : HT2-toxin
Correlation coefficient : r = 0.997782 , r^2 = 0.995569
Calibration curve : 0.290844 * x + -0.561994
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



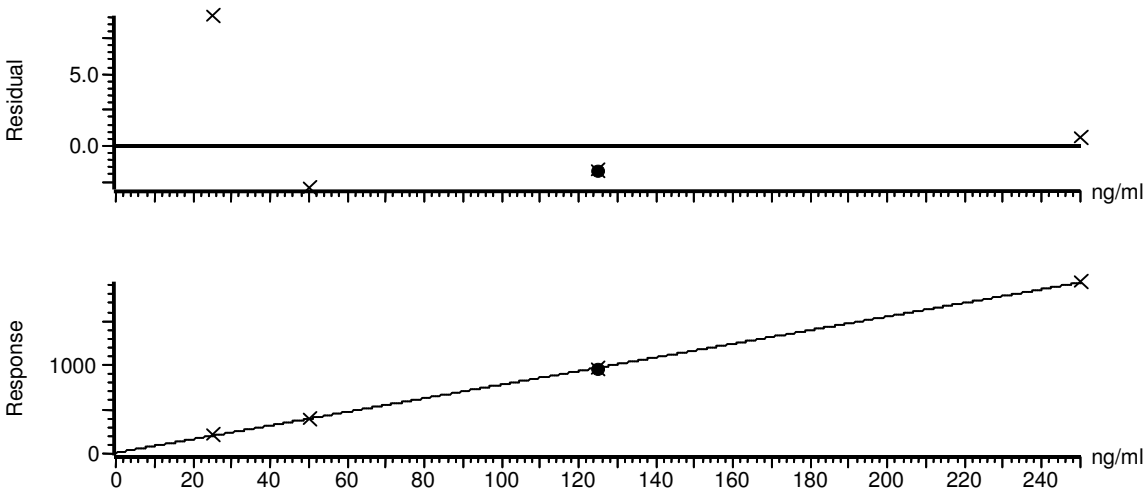
## Fig. 9. Calibration graph for ochratoxin A

Compound name : Ochratoxin A
Correlation coefficient : r = 0.999441 , r^2 = 0.998883
Calibration curve : 12.9145 * x + -0.807657
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None




## Fig. 10. Calibration graph for zearalenone

Compound name : Zearalenone
Correlation coefficient : r = 0.999421 , r^2 = 0.998843
Calibration curve : 7.69917 * x + 9.5829
Response type : External Std, Area
Curve type : Linear, Origin : Include, Weighting : 1/x, Axis trans : None



I) Chromatograms obtained for the analysed toxins for two ion transitions

**Fig. 11. Chromatograms obtained for two ion transitions of Aflatoxin B1 for a spiked sample (2 µg/kg).**

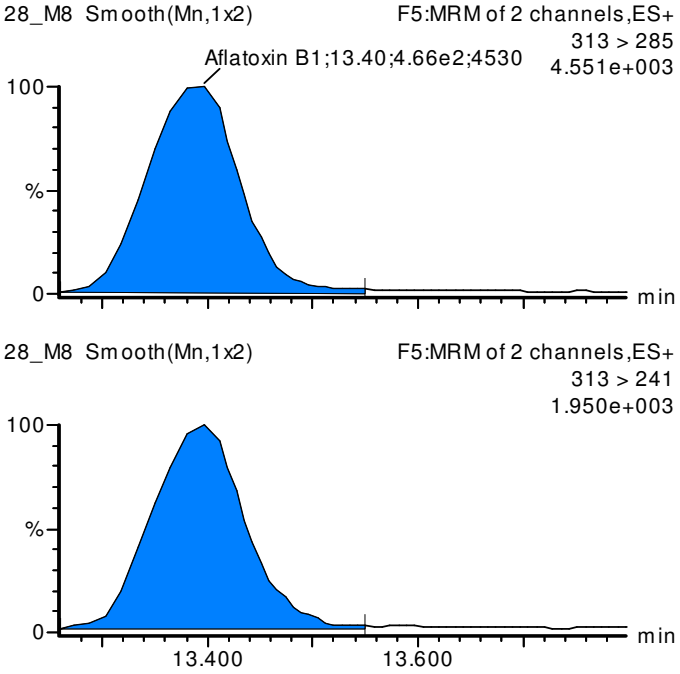



**Fig. 12. Chromatograms obtained for two ion transitions of Aflatoxin B2 for a spiked sample (1 µg/kg).**

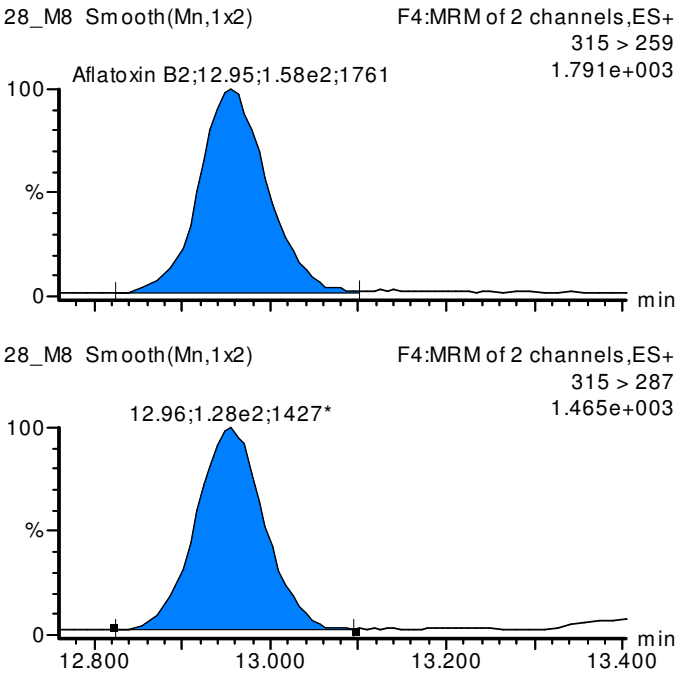


**Fig. 13. Chromatograms obtained for two ion transitions of Aflatoxin G1 for a spiked sample (2 µg/kg).**

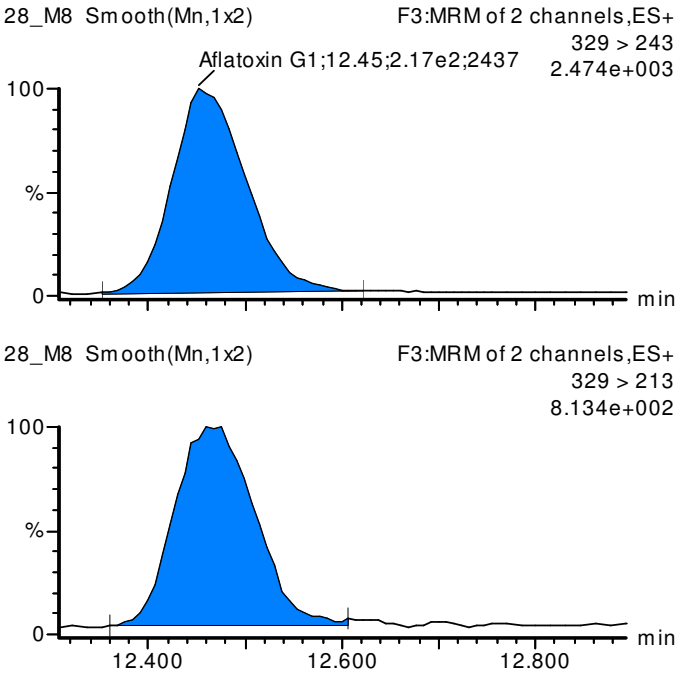



**Fig. 14. Chromatograms obtained for two ion transitions of Aflatoxin G2 for a spiked sample (1 µg/kg).**

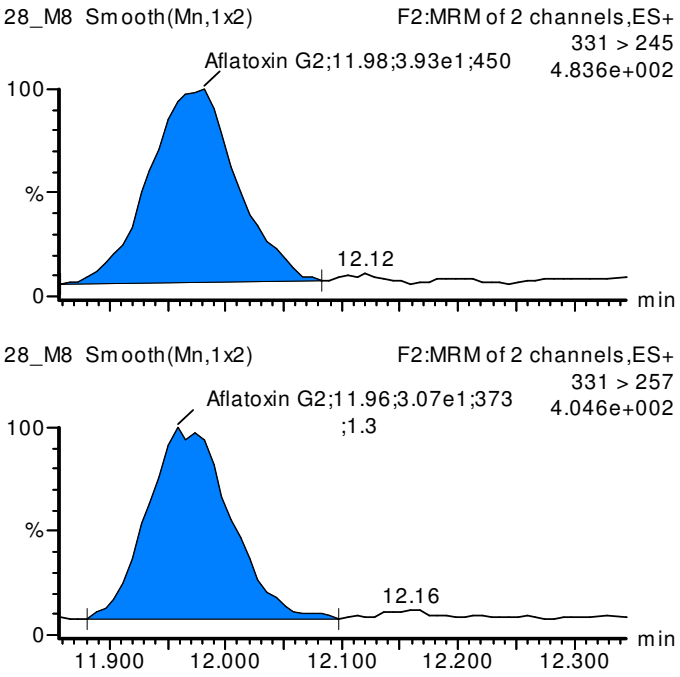


**Fig. 15. Chromatograms obtained for two ion transitions of T-2 toxin for a spiked sample (50 µg/kg).**

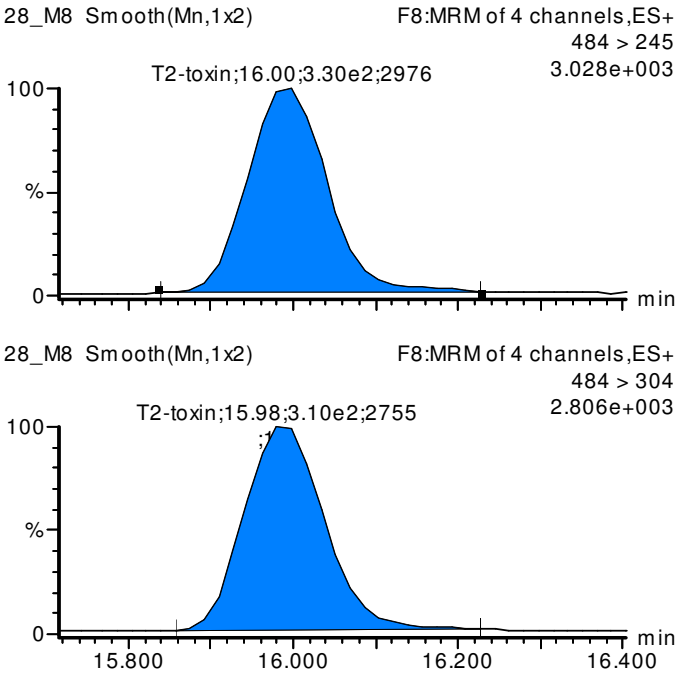



**Fig. 16. Chromatograms obtained for two ion transitions of HT-2 toxin for a spiked sample (50 µg/kg).**

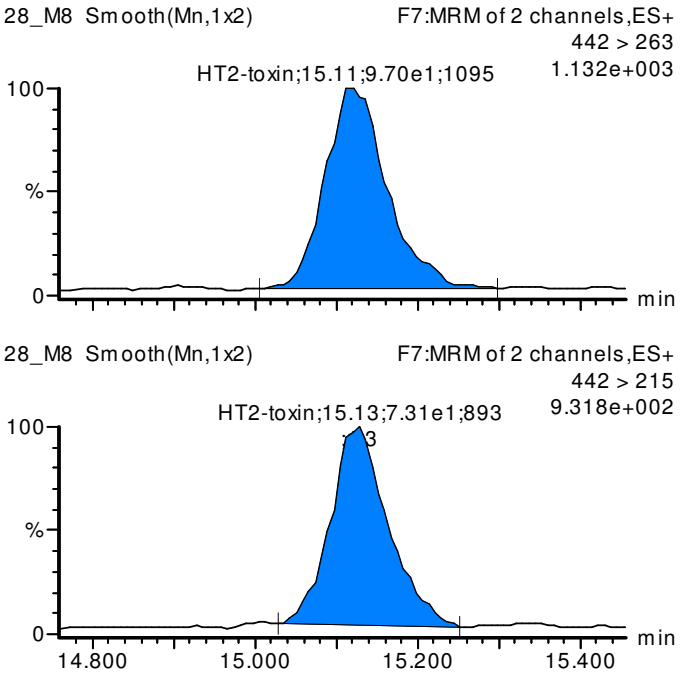


**Fig. 17. Chromatograms obtained for two ion transitions of Ochratoxin A for a spiked sample (2 µg/kg).**

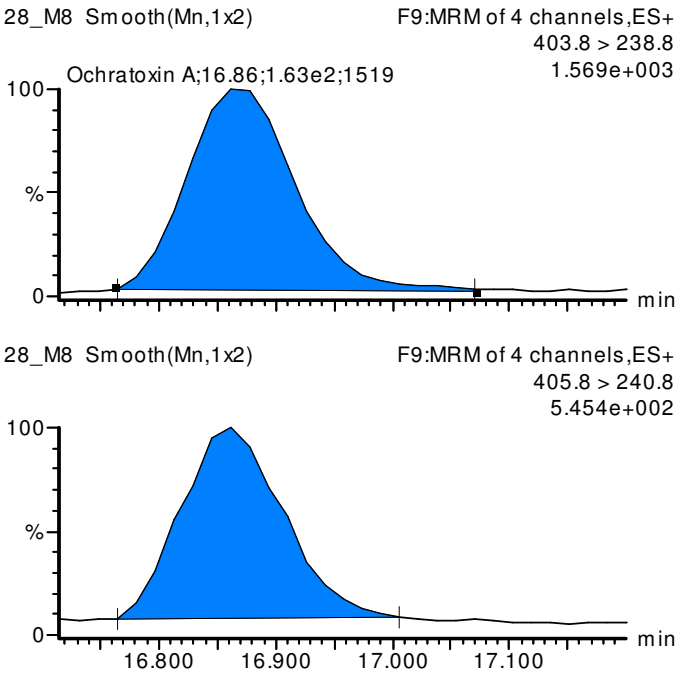



**Fig. 18. Chromatograms obtained for two ion transitions of Zearalenone – for a blank sample (corresponds to about 6 µg/kg).**

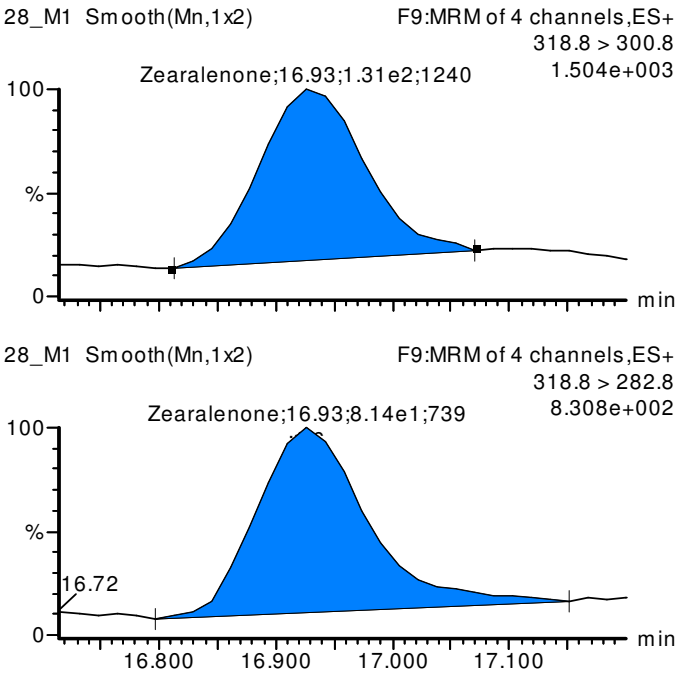
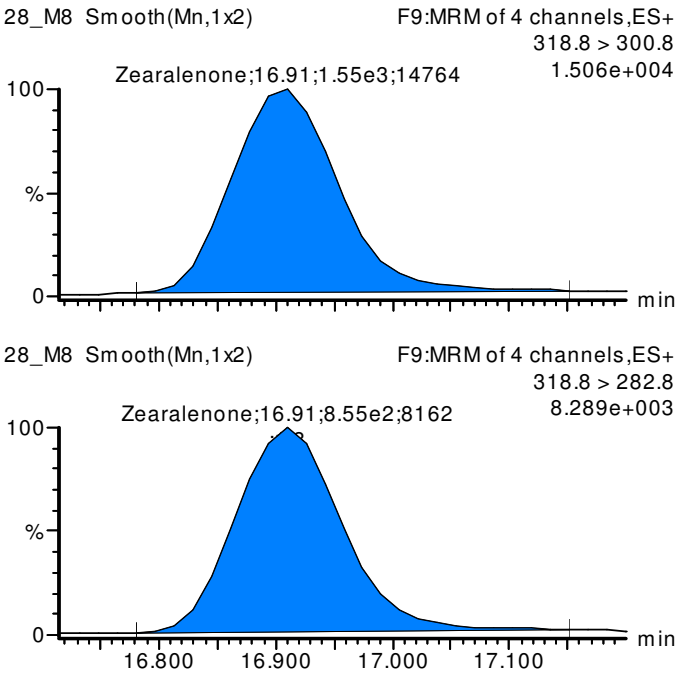


**Fig. 19. Chromatograms obtained for two ion transitions of Zearalenone – for a spiked sample (50 µg/kg).**



J) Spectra of analysed mycotoxins

**Fig. 20. Aflatoxin B1 MS/MS spectrum.**

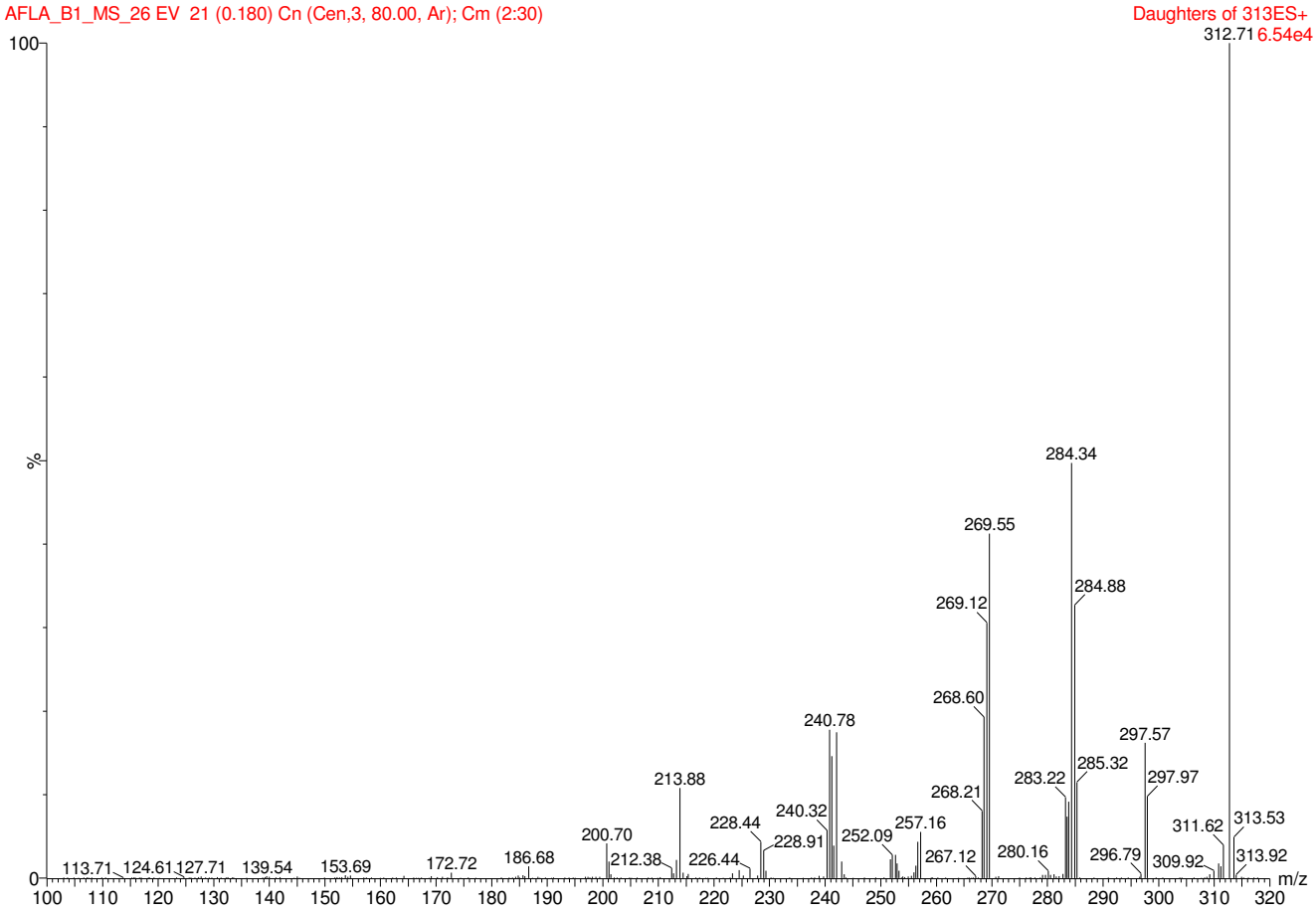


**Fig. 21. Aflatoxin B2 MS/MS spectrum.**

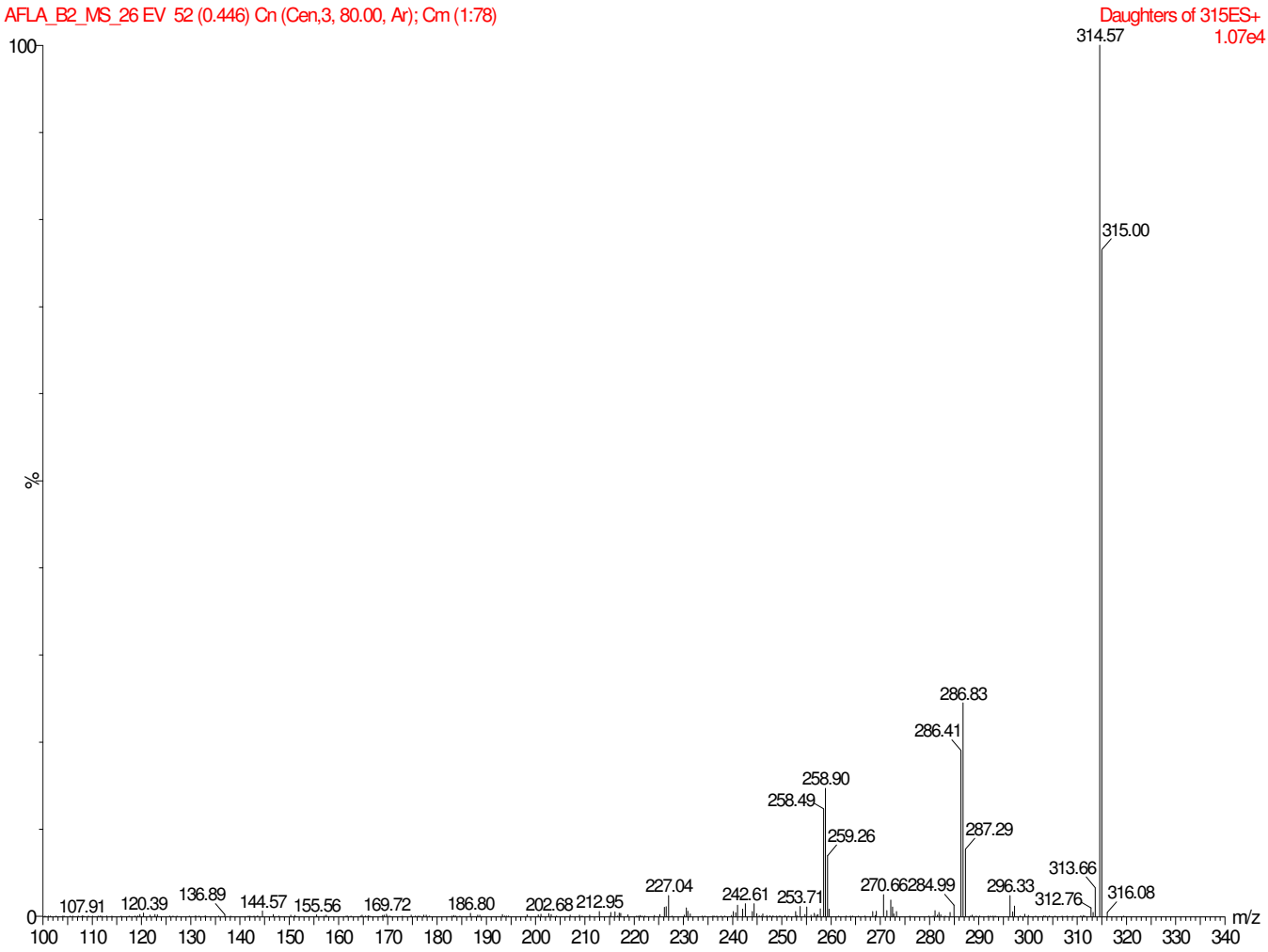


**Fig. 22. Aflatoxin G1 MS/MS spectrum.**

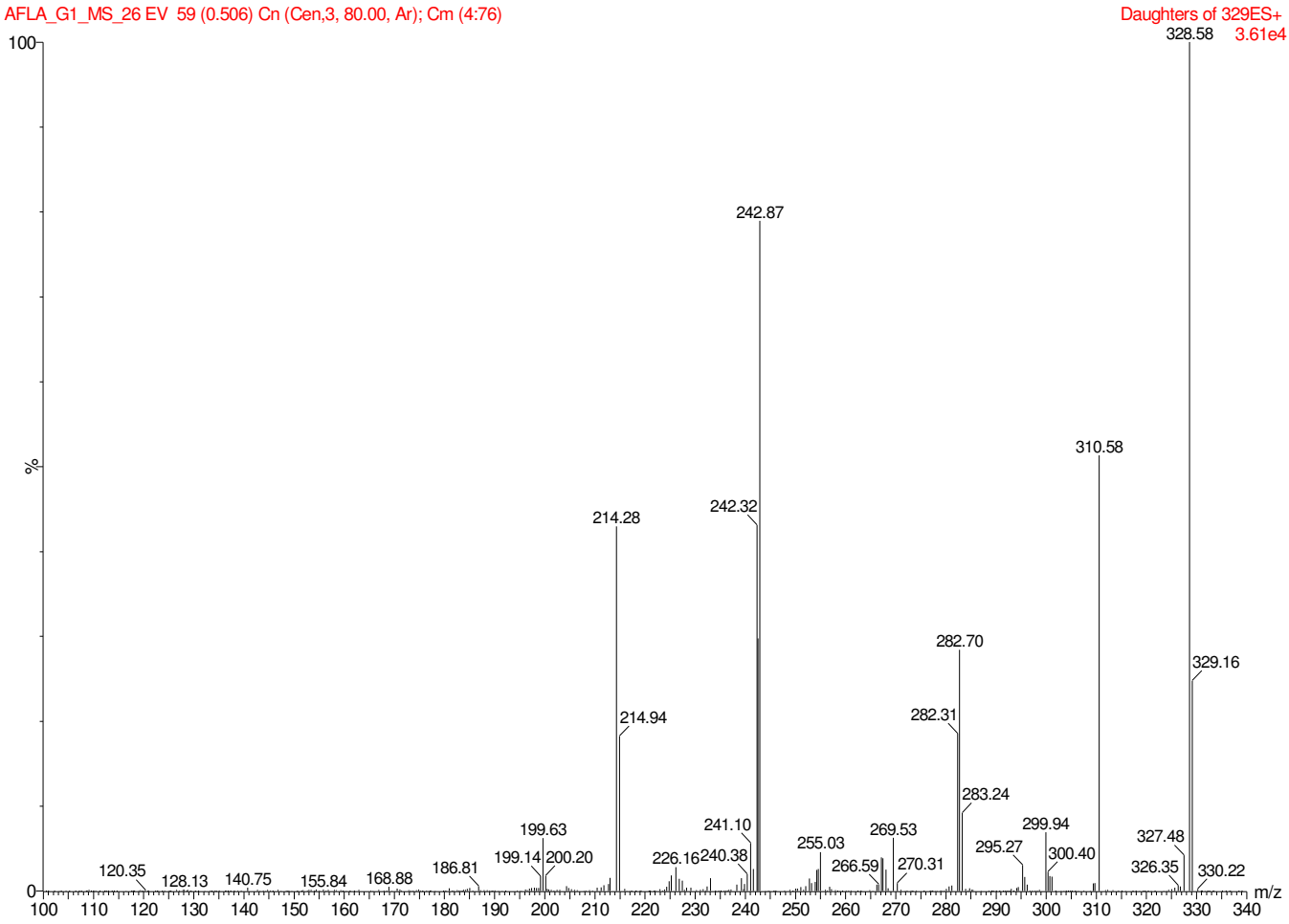


**Fig. 23. Aflatoxin G2 MS/MS spectrum.**

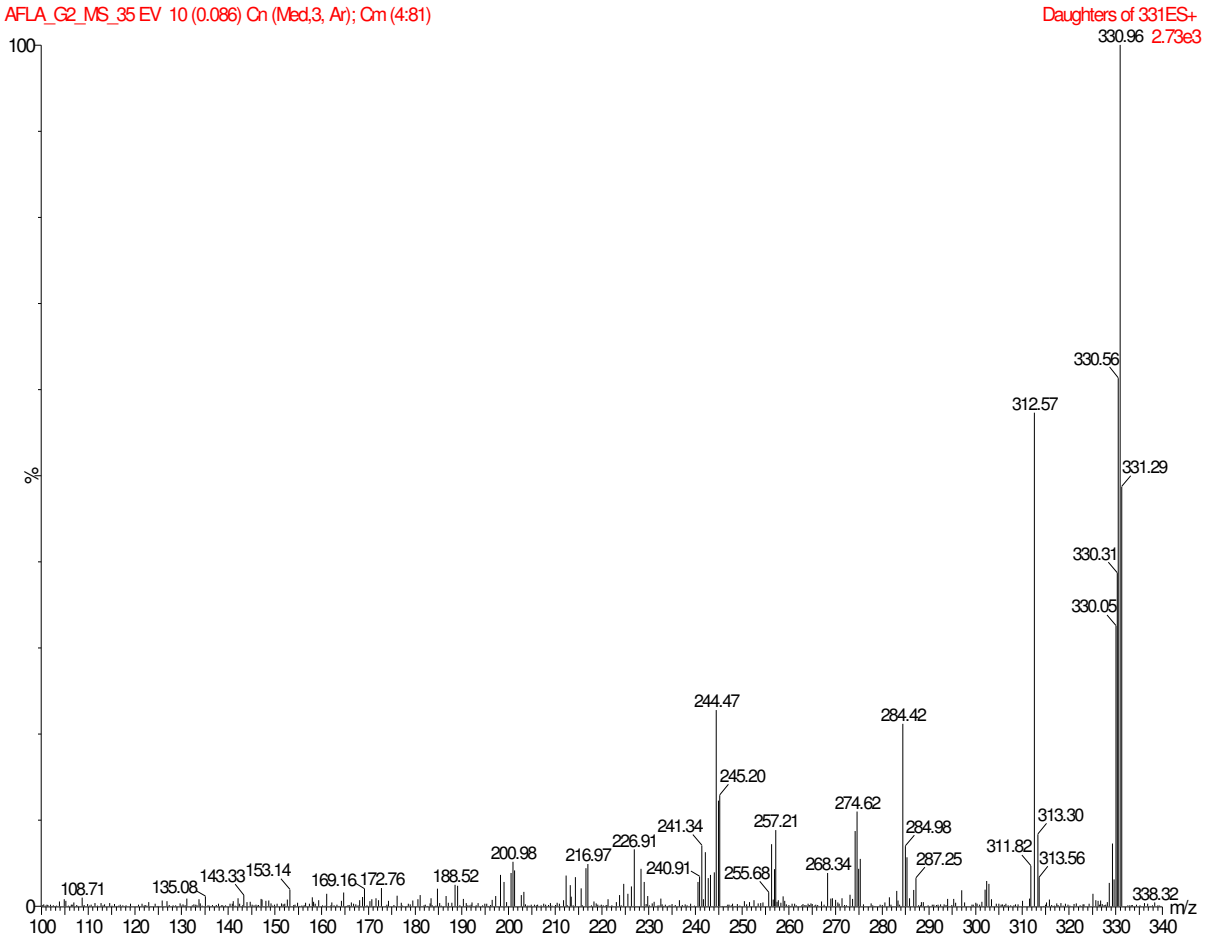


**Fig. 24. T-2-toxin MS/MS spectrum.**

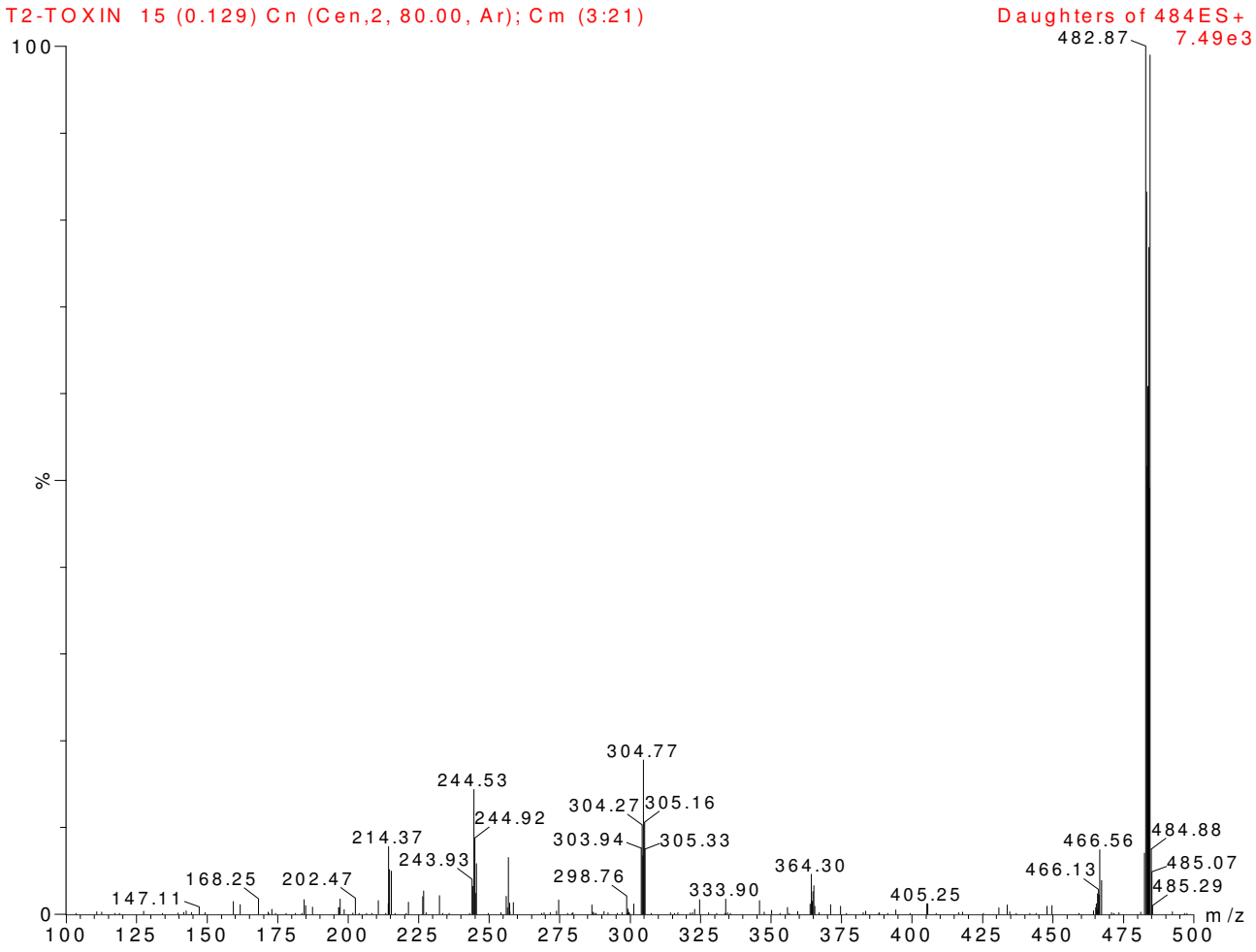


**Fig. 25. HT-2-toxin MS/MS spectrum.**

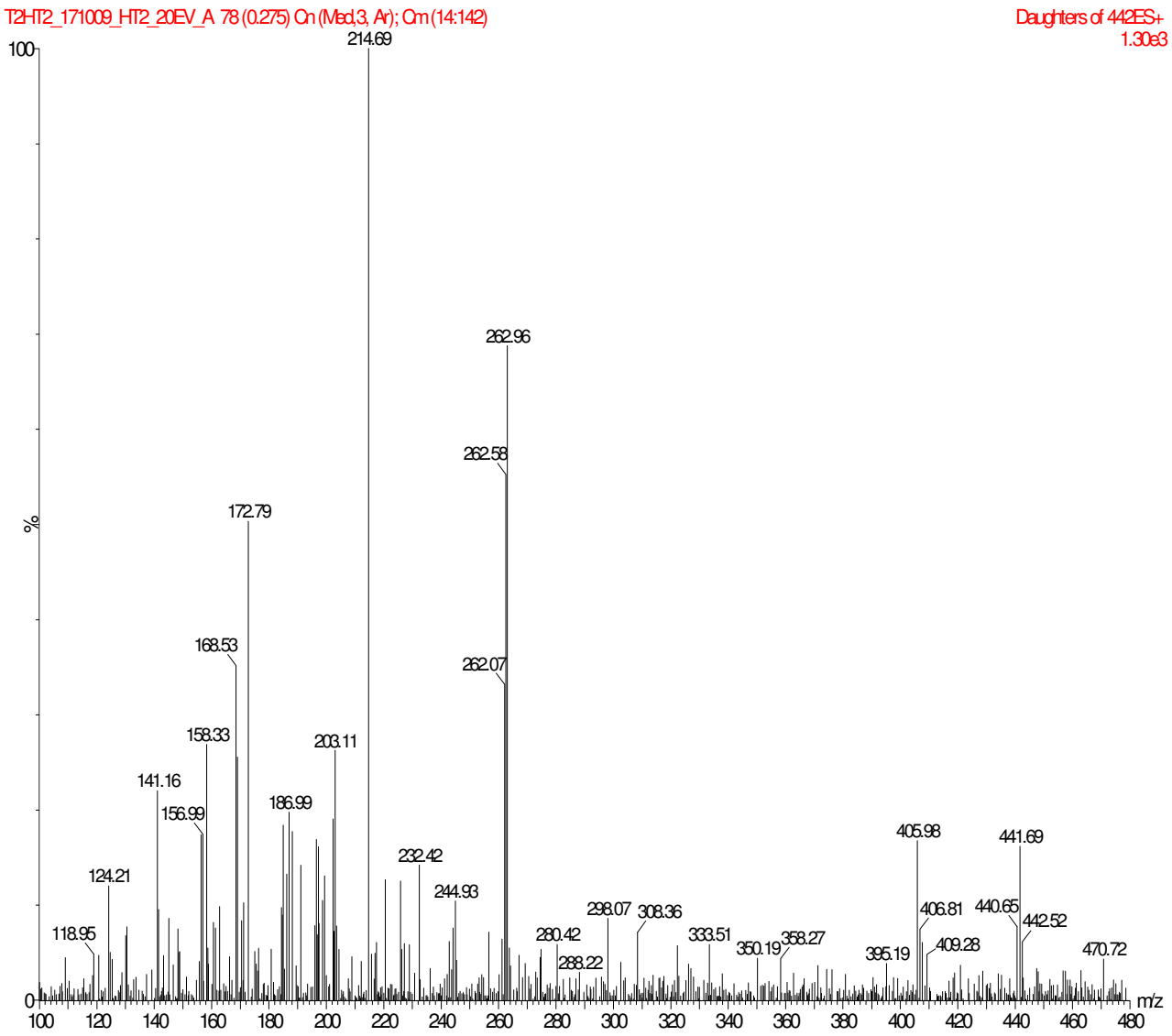


**Fig. 26. Ochratoxin A (m/z 404) MS/MS spectrum.**

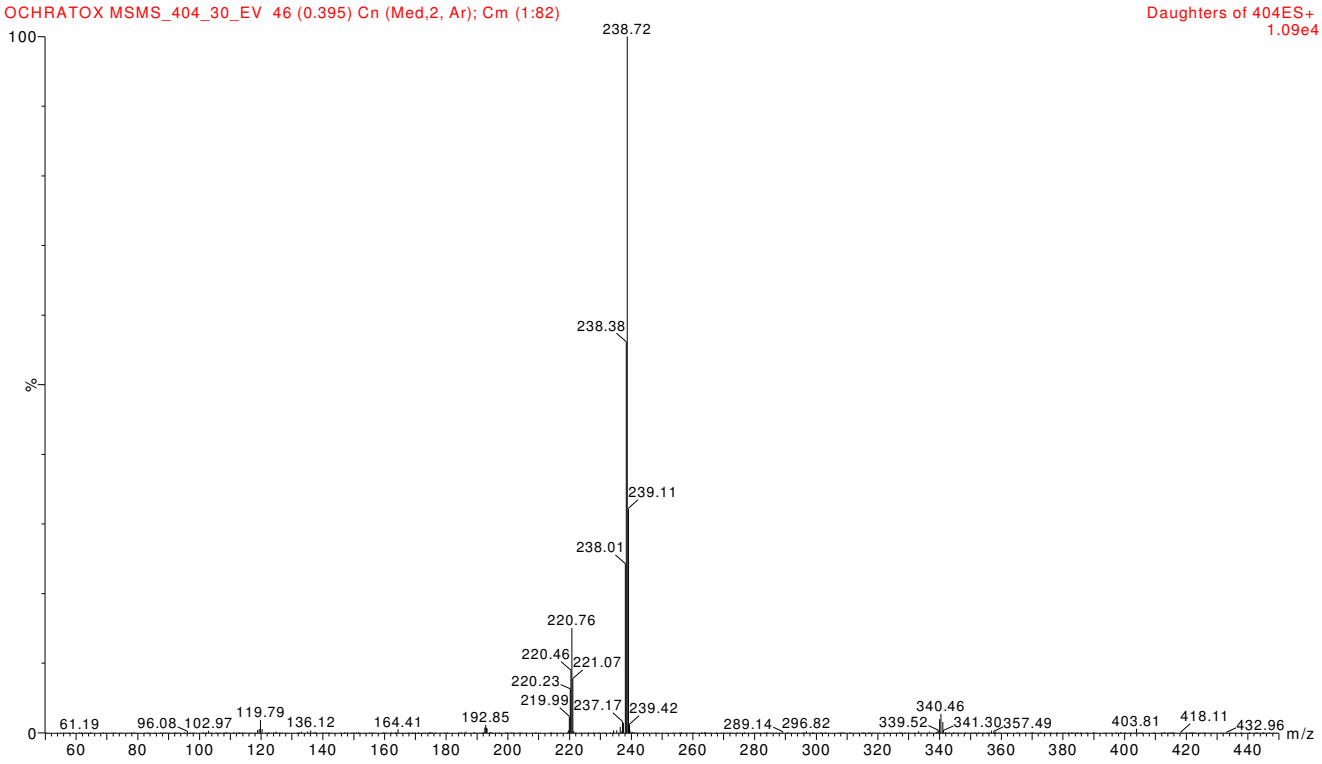
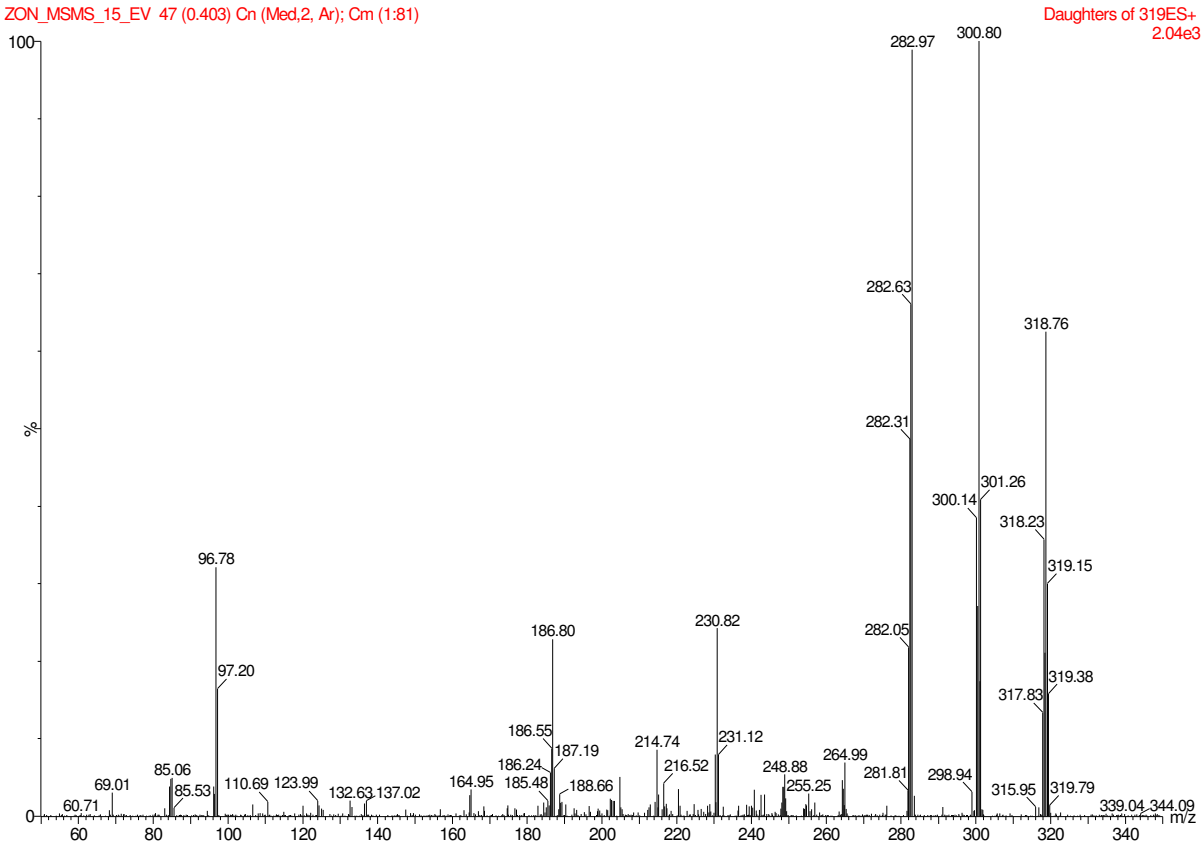



**Fig. 27. Zearalenone (m/z 319) MS/MS spectrum.**



K) Other mycotoxin structures

**Fig. 28. Structure of Aflatoxin M1 [4].**

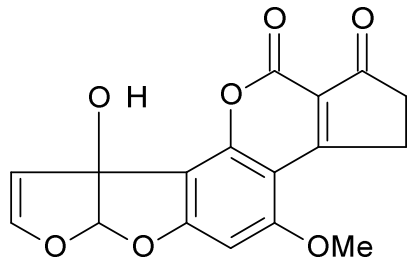



**Fig. 29. Structure of Aflatoxin M2 [4].**

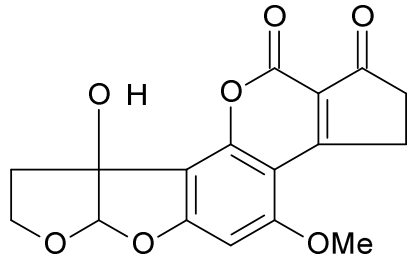



**Fig. 30. Structure of zearalanone [1].**

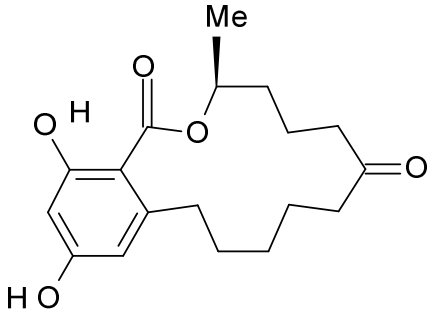



**Fig. 31. Structure of α-zearalanol [1].**

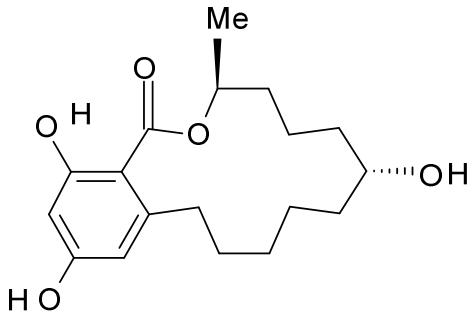


**Fig. 32. Structure of β-zearalanol [1].**