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Analytical methodologies for evaluating mycotoxin contamination in food safety

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Introduction

Introduction

Mycotoxins are a structurally diverse group of mostly low molecular weight compounds, produced by the secondary metabolism of filamentous and microscopic fungi growing on agricultural commodities in the field or during storage.

Table I.1. Mycotoxins found in foods and foodstuffs.

Commodity	**Situation**	**Potential mycotoxins**
Cereals	pre-harvest fungal infection	deoxynivalenol, T2 toxin, nivalenol, zearalenone, alternariol, alternariol monomethyl ether, tenuazoic acid, fumonisins
Maize and peanuts	pre-harvest fungal infection	aflatoxins
Maize and sorghum	pre-harvest fungal infection	fumonisins
Stored cereals, nuts, spices	dump storage conditions (storage abuse)	aflatoxins and ochratoxin
Fruit juice	mould growth on fruit	patulin
Dairy products	animal consumption of mould contaminated feeds	aflatoxin M_1, cyclopiazonic acid, ochratoxin, compactin, cyclonaldic acid
Meat and eggs	animal consumption of mould contaminated feeds	patulin, citrinin, ochratoxin, cyclopiazonic acid, cyclopaldic acid, citromycetin, roquefortine, fumonisins
Oilseeds	pre-harvest fungal infection	tenuazonic acid, alternariol

Taken from Sweeney 1998

Mycotoxin occurrence in a wide range of food and animal feedstuffs has become of great concern worldwide because mycotoxins are often associated with acute or chronic diseases, called mycotoxicoses, in livestock and also in humans [Hussein 2001]. Acute effects are generally produced by high amounts of toxins present in food or feed, so that fatal incidents are usually restricted to the less developed areas of the world [Piva 2005], where resources for control are limited, or to livestock. Chronic effects are also of concern for the long-term health of the human population and must not be underestimated since many toxins are present in low amounts in daily intaken food [Sforza 2006].

The discovery of aflatoxins in the early 1960s led to the resurgence of interest in human mycotoxicoses. Mycotoxins induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins, ochratoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (ochratoxins), estrogenic (zearalenone), hemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (aflatoxins and phomopsins), dermotoxic (trichothecenes) and neurotoxic (ergotoxins, penitrems, lolitrems and paxilline), whereas others display antitumoral, cytotoxic, and antimicrobial properties [Steyn 1995].

In nature most cereal grains, oil seeds, tree nuts, and dehydrated fruits are susceptible to fungal contamination and mycotoxin formation. Then, the human ingestion of mycotoxins is due to the consumption of plant-based and animal-derived foods through the diet. In addition, toxicological risk also concerns possible mycotoxin metabolites formed in the food chain, e.g. aflatoxin M1 occurring in milk. The impact of mycotoxins on health depends on the amount of mycotoxin consumed, the toxicity of the compound, e.g. acute or chronic effects, the body weight of the individual,

the presence of other mycotoxins (synergistic effects) and other dietary effects [Kuiper-Goodman 1991]. Hsieh [Hsieh 1990] maintained that all of the following criteria have to be satisfied to link a mycotoxin to a specific human disease: occurrence of the mycotoxin in food supplies; human exposure to the mycotoxin; correlation between exposure and incidence; reproducibility of the characteristic symptoms in experimental animals; similar mode of action in human and animal models. The global nature of the mycotoxin problem is based on well-documented human mycotoxicoses [Piva 2005], such as ergotism in Europe, alimentary toxic aleukia (ATA) in Russia, acute aflatoxicoses in South and East Asia, and human primary liver cancer (PLC) in Africa and South East Asia. Ochratoxin A (OTA) is suspected to play a role in Balkan endemic nephropathy (BEN) in ex-Yugoslavia and chronic interstitial nephritis (CIN) in North Africa.

In terms of structural complexity, mycotoxins vary from simple C_4-compounds, e.g. moniliformin, to complex substances such as phomopsins [Culvenor 1989] and tremorgenic mycotoxins [Steyn 1985]. Under laboratory conditions, at least 300 mycotoxins have been produced by pure fungal cultures and chemically characterized. Fortunately, only about 20 mycotoxins are known to occur in foodstuffs at sufficient levels and frequency to be of food safety concern. These toxins are mainly produced by 5 genera of fungi: *Aspergillus, Penicillium, Fusarium, Alternaria,* and *Claviceps.* The mycotoxins produced by these fungi are: *Aspergillus* toxins, aflatoxin B**,** G, M, OTA, sterigmatocystin and cyclopiazonic acid; *Penicillium* toxins, patulin, OTA, citrinin, penitrem A (PA) and cyclopiazonic acid; *Fusarium* toxins (called fusariotoxins), deoxynivalenol, nivalenol, zearalenone, T-2 toxin, diacetoxyscirpenol, fumonisins and

moniliformin; *Alternaria* toxins, tenuazonic acid, alternariol and alternariol methyl ether; *Claviceps* toxins, ergot alkaloids.

A number of decontamination procedures have been investigated, broadly based on physical, chemical and biochemical principles. However, the general consensus now prevailing is that preventive measures offer greater potential than remedial procedures. In other words, the development and selection of cultivars of cereal and forage plants that are resistant to infection by toxigenic fungi is likely to be the long-term objective of any effort to control contamination with the associated mycotoxins [D'Mello 1999].

In this context the development and application of analytical methodologies play a fundamental role in assessing nature and level of mycotoxin contamination in food and feed.

Section I

Fusarium mycotoxins

Section I – *Fusarium* mycotoxins

In this section we describe the development of a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the simultaneous detection of the most important fusariotoxins, such as both type-A and -B trichothecenes, fumonisins, zearalenone and α-zearalenol, using an ESI interface by implementing a four-period switching for the ionization polarity. This method was designed and applied for the analysis of corn meal samples. The method here reported is truly multiresidue, since fusariotoxins were extracted, cleaned-up and analyzed simultaneously. In addition, it can be considered a primary screening method because it allows the identification of the target analytes, even if not under the best conditions for each class, and the complete analysis can be performed in about two hours. In addition, the fusariotoxins simultaneously extracted could be fractionated in classes, by reprocessing a new 5 mL aliquot of the extract. A specially designed fractionation during the clean-up step allowed us to collect three fractions that were then chromatographed separately. The primary screening method and the confirmatory method were evaluated in terms of accuracy, precision and method detection limits (MDLs). Furthermore, we applied the confirmatory method to evaluate performances of two ELISA-based methods for the analysis of deoxynivalenol and zearalenone, respectively.
Moreover, it is common knowledge that LC-MS/MS technique represents a powerful tool for compound identification, both in case of undiscovered toxic metabolites and of known mycotoxins, of which no analytical standard is available. Thus, we have tested the performance of the new Q

TRAP™ hybrid mass spectrometer operating in two different configuration modes combined in an IDA (Independent Data Acquisition) protocol for identification and quantitation of fumonisins FB_{1-4}.

Finally, this study also includes application of the LC-MS/MS methods developed to freshly harvested maize samples collected in 2002 and 2003 from representative Italian fields and from two experimental fields situated in Lazio region.

Chapter 1

Fusarium mycotoxins in cereals

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1.1. Classification of *Fusarium* mycotoxins

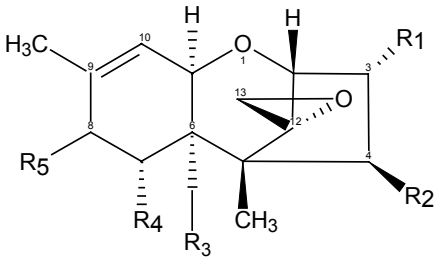
Agricultural products, mostly grains, are particularly susceptible to attack by fungi in the field and during storage, and to subsequent mycotoxin contamination [Doohan 2003]. The *Fusarium* genus has been reported among the most prominent pathogens of various cereals in both temperate and semitropical areas, including all European cereal-growing areas [Bottalico 1998, Creppy 2002]. Field contamination depends strongly on several factors including climatic conditions (rainfall, temperature, humidity), genetic susceptibility of maize cultivars to fungal infection, soil type, maize kernel damage by birds and, lastly, nutritional factors [Bakan 2002; Bosh 1992, Mubatanhema 1999]. Soft and durum wheat, and maize, seem to be especially susceptible [Langseth 1992]. The infestation of cereals with *Fusarium* genus has therefore been amply studied in many countries for its important implication in food safety [Kuiper-Goodman 1995, Placinta 1999, Quiroga 1995].

Fusarium genus is able to produce many structurally different mycotoxins, named fusariotoxins, including trichothecenes, macrocyclic lactones and fumonisins (figure 1.1) [Bottalico 1998, D'Mello 1999, Doohan 2003, Hussein 2001].

Trichothecenes are the largest and the most common group of mycotoxins produced by the *Fusarium* species [D'Mello 1999]. They are a group of over 148 closely related mycotoxins called sesquiterpenoids, which contain an olefinic group linking carbons 9–10, an epoxide between carbons 12 and 13, and a variable number of hydroxyl and acetoxy groups. According to

their functional moieties, trichothecenes are commonly classified in four groups (A, B, C, D).

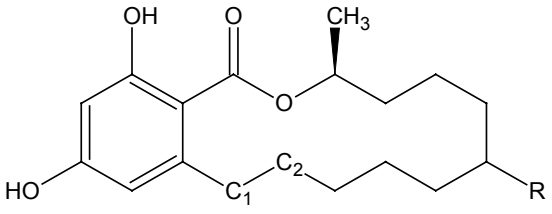
Figure 1.1. Structures and molecular weights (MWs, uma) of the investigated fusariotoxins.



Trichothecenes A and B

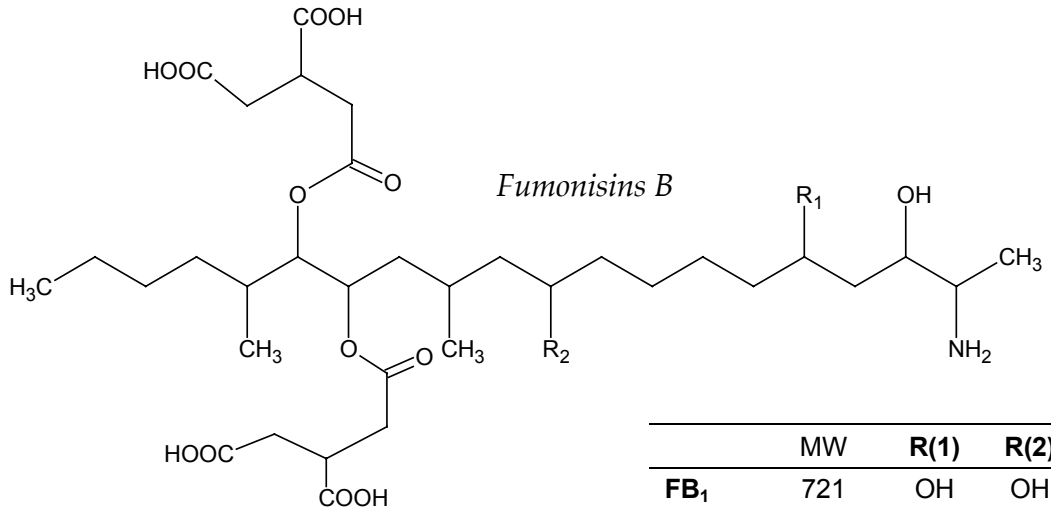
	MW	R_1	R_2	R_3	R_4	R_5
NIV	312	OH	OH	OH	OH	=O
DON	296	OH	H	OH	OH	=O
FUS X	354	OH	OAc	OH	OH	=O
3-ADON	338	OAc	H	OH	OH	=O
15-ADON	338	OH	H	OAc	OH	=O
NEO	382	OH	OAc	OAc	H	OH
DAS	366	OH	OAc	OAc	H	H
MAS	324	OH	OH	OAc	H	H
T-2	466	OH	OAc	OAc	H	i-Val
HT-2	424	OH	OH	OAc	H	i-Val
VER	266	H	OH	OH	H	H

OAc: acetyl i-Val: *iso*-valeryl



Zeranols

	MW	R	C_1–C_2
ZON	318	=O	C=C
α–ZOL	320	····OH	C=C
β–ZOL	320	◄OH	C=C
ZAN	320	=O	C—C
α–ZAL	322	····OH	C—C
β–ZAL	322	◄OH	C—C



Fumonisins B

	MW	R(1)	R(2)
FB_1	721	OH	OH
FB_2	705	OH	H
FB_3	705	H	OH
FB_4	689	H	H

Type-B trichothecenes differ from type A by the presence of an α,β-unsatured carbonyl group at position C-8. Both types include about 100 of the isolated toxins. Type C are characterized by an additional epoxide, and type D are macrocyclic trichothecenes. Although the number of characterized trichothecenes is large, only a few of them have been encountered as natural contaminants of cereal products. Trichothecene mycotoxins co-occur regularly throughout the world with particularly high concentration values in cereal grains from Poland, Germany, Japan, New Zealand and the Americas [Langseth 1998]. The type-A trichothecenes include: a) T-2 toxin (T-2) and HT-2 toxin (HT-2), mainly produced by strains of *F. sporotrichioides*, *F. acuminatum*, and *F. poae*; b) diacetoxyscirpenol (DAS), and monoacetoxyscirpenol (MAS), chiefly produced by strains of *F. poae*, *F. equiseti*, *F. sambucinum*, and *F. sporotrichioides*; and c) neosolaniol (NEO), mainly produced by strains of *F. sporotrichioides*, *F. poae*, and *F. acuminatum* [Bottalico 1998]. The type-B trichothecenes include: a) deoxynivalenol (DON, also known as 'vomitoxin') and its derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), produced by strains of *F. graminearum* and *F. culmorum*; and b) nivalenol (NIV) and fusarenon X (FUS X) produced by strains of *F. crookwellense*, *F. poae*, *F. graminearum,* and *F. culmorum* [Bottalico 1998].

Fusarium spp., mainly *F. graminearum, F. culmorum*, *F. crookwellense,* and *F. equiseti* [Bottalico, 1998] can also produce zearalenone (ZON), a macrocyclic lactone derivative of resorcilic acid. *Fusarium* spp. have indeed been found to generate at least five metabolites: α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zearalanone (ZAN)

[Lauren 1994; Richardson 1985]. In this thesis we call ZON and its derivatives 'zeranols', as van Bennekom and co-workers have proposed [van Bennekom 2002].

Finally, fumonisins are a group of structurally related mycotoxins mainly produced by *F. moniliforme* and *F. proliferatum*, the most common fungi colonizing corn crops throughout the world before harvest, during the time between harvesting and drying, and in storage [Sweeney 1998, Turner 1999]. These toxins are most frequently found in maize and maize-based foodstuffs [Soriano 2004] and feeds, and less in other grains (i.e. sorghum [Sweeney 1998] and rice [Cirillo 2003]). Of the structurally related fumonisins [Musser 1997, Sweeney 1998] described so far, the four type-B toxins FB_1, FB_2, FB_3 (FB_{1-3}) and, to a minor extent FB_4, are the most frequently occurring in nature [Lukacs 1996, Musser 1997].

1.2. Fusariotoxin adverse health effects

The presence of fusariotoxins in food and feed has long been recognized and extensively described as a potential human and animal health hazard [Creppy 2002, D'Mello 1999, Gutleb 2002, Hussein 2001, Soriano 2004].

Trichothecenes have been reported to cause a variety of toxic effects in both laboratory and farm animals, including skin inflammation, digestive disorders, hemorrhages in several internal organs, hemolytic disorders and depletion of the bone marrow, impairment of both humoral and cellular immune responses, and nervous disorders [IARC 1993, Rotter 1996]. Trichothecenes are commonly associated with several mycotoxicoses in livestock, including hemorrhagic and emetic syndromes, caused by type-A (T-2, DAS, MAS) and type-B (DON, NIV, FUS X) trichothecenes,

respectively. Furthermore, T-2 and DON were also implicated in human toxicoses. In particular, T-2 toxin and derivatives have been held responsible for Alimentary Toxic Aleukia (ATA) occurred in Russia in the last century due to human consumption of overwintered cereals and in China for toxicosises associated with consumption of contaminated rice. Nevertheless, by experimental evidences, IARC included these type of trichothecenes among the compounds not marked for their carcinogenicity to humans [IARC 1993].

Zearalenone (ZON) is an anabolic and uterotrophic macrocyclic lactone with estrogenic activity often associated with hypererstrogenism and infertility in swine, poultry and cattle [Minervini 2001]. The structure is flexible enough to allow ZON to adopt a conformation able to bind to the mammalian estrogen receptor, although with lower affinity than the natural estrogen 17-β-estradiol, thus resulting in severe effects on the reproductive system in several animal species, in particular pigs. The same activity is shown by the two isomeric metabolites such as α- and β-zearalenol (α- and β-ZOL), although α-ZOL is 2-4 times more active than ZON or β-ZOL [Hagler 1979]. As for trichothecenes, the estrogenic compounds produced by *Fusarium* were classified as not carcinogenic to humans by IARC [IARC 1993].

FB_1 and FB_2 cause animal diseases including equine leukoencephalomalacia (ELEM) [Kellerman 1990], porcine pulmonary edema (PEP) [Harrison 1990], nephrotoxicity and liver cancer in rats [Marasas 1995]. They are also suspected to be of epidemiological significance in the high incidence of human esophageal cancer in South Africa [Rheeder 1992], China [Chu 1994] and Italy [Franceschi 1990]. In addition, FB_1 is considered by IARC as a possible carcinogen to humans (class 2B) [IARC 2002]. The structural

similarity with sphingosins, which are components of the membrane sphingolipid complex, enables fumonisins to disrupt the biosynthesis of sphingolipids, and to provoke an increase in the concentrations of free sphingosine in blood and tissues, and of sphinganine/sphingosine ratio in serum and urine [Bottalico 1998]. The depletion of complex sphingolipids from the biological membranes may account for the toxicity, and perhaps the carcinogenicity, of the fumonisins [Riley 1996]. Human exposure occurs at levels of micrograms to milligrams per day, and is greater in regions where maize products are the dietary staple [IARC 2002]. The above mentioned links with animal diseases, and the possible exposure of humans through contaminated food supplies, have created an interest in the quantitative determination of fumonisins.

As further toxicological risk, additive effects may occur when more than one mycotoxin responsible for the same toxicity through the same mechanism of action are found together. In addition, synergistic effects may also occur; in this case the final toxic effect observed is greater than the sum of the toxic effects of each mycotoxin [D'Mello 1999, Speijers 2004]. This happens when one chemical increases the target site concentration of another, e.g., by increasing the absorption or decreasing the metabolic degradation, or if the compounds act at different stages of the same toxicity pathway. This fact means that in the field of screening for mycotoxins there is a need to develop accurate, rapid and efficient tests for monitoring more than one mycotoxin simultaneously.

1.3. Current legislative regulations

Mycotoxin levels occurring in food and feed are submitted to specific regulations in more than 77 countries in the world. Specifically, statutory limits vary country by country, associating closely a single mycotoxin with a well defined food commodity [Anklam 2002]. So far, maximum concentration levels mainly of aflatoxins and ochratoxin A have been extensively regulated [EC 2002b, EC 2003, EC 2004, EC 2005a].

In EU, risk assessment is assigned to the Scientific Committee on Food (SCF) that examines the indications from Member States, and then recommends tolerable limits for food safety consume. Table 1.1 reports the tolerable daily intake values (μg/kg body weight) proposed by SCF for some fusariotoxins [EFSA 2003, SFC 2003, EC 2005b].

Table 1.1. Tolerable daily intake values (TDI) proposed from SCF in 2003.

Fusariotoxin	TDI (μg/kg body weight)
$FB_1+FB_2+FB_3$	2
DON	1
NIV	0.7
ZON	0.2
T-2+HT-2	0.06

Fusariotoxin contamination levels vary considerably according to geographic areas, regions and years (ranging from a few ng/g to several μg/g) and, although their effects on health have been proved [FAO 2001], the first European legislative regulation regarding the maximum tolerable levels of these compounds in foodstuffs and feeds dates not earlier than June 6, 2005 [EC 2005b]. In addition, some points in the text of this recent regulation appear incomplete and provisional and will be updated not

beyond October 1st, 2007. Table 1.2 reports some of the maximum limits lastly introduced.

Table 1.2. Fusariotoxin limits set by the current European regulation (856/2005) for DON, ZON, fumonisins and HT-2+T-2 in food.

Fusariotoxin	**Food**	**Maximum level (μg/Kg)**
DON	Unprocessed durum wheat, oats and corn*	1750*
	Cereal flour, including maize flour, maize grits and maize meal	750
	Bread, biscuits, cereal snacks and breakfast cereals	500
	Young children, infant and baby foods	200
ZON	Unprocessed cereals	100
	Unprocessed maize, corn meal and flour	200*
	Maize snacks and maize breakfast cereal	50*
	Young children, infant and baby foods	20*
FB_1+FB_2	Unprocessed maize	2000*
	Maize grits, maize meal and maize flour	1000*
	Maize based finished products	400*
	Young children, infant and baby foods	200*
HT-2+T-2	Unprocessed cereals and cereal products	*to be fixed***

* Level which will apply if no specific level is fixed before 1 July 2007

** A maximum level will be fixed, if appropriate, before 1 July 2007.

The maximum levels set for unprocessed cereals apply to cereals placed on the market for first stage-processing. The maximum levels do apply for cereals harvested and taken over, as from the 2005/2006 marketing year, in accordance with Commission Regulation 824/2000/EC.

Before Regulation 856/2005, the European Commission recommended, as maximum levels of FB_{1-2}, 2000 μg/kg for unprocessed corn and 100 μg/kg for infant food [SCF 2003, Soriano 2004], whereas the US Food and Drug Administration has proposed the range 2000-4000 μg/kg as maximum levels of FB_{1-3} for human foods [FDA 2001]. In Italy, an official note from the Ministry of Health introduced the limit of 1000 μg/kg for FB_1 in corn

meal [IMoH 2004]. Moreover, Circular n.10 from the Ministry of Health [IMoH 1999], followed by Circular n.18 [IMoH 2000], set as guidelines the ZON limits at 20 and 100 μg/kg for baby foods and cereal grains, respectively.

Nevertheless, discussion and related suggestions about more and more appropriate fusariotoxin limits in food and feed to protect public health is nowadays still in progress [Miraglia 2005].

1.4. Analytical methodologies

Due to the widespread occurrence and toxicity of the *Fusarium* mycotoxins, rapid and reliable screening methods are required for their identification and quantitation in cereals to ensure safety and compliance with the current and forthcoming legislation. Thus, the availability of sound analytical methods for monitoring the presence of fusariotoxins along the food chain is of the utmost importance for keeping contamination under control.

Several analytical methods have been developed for determining fusariotoxins in cereal-based foods and feeds, milk, beer, biological fluids and *Fusarium* culture materials [Trucksess 2006]. These methods include thin layer chromatography (TLC), enzymatic assays, capillary gas chromatography-mass spectrometry (GC/MS), capillary electrophoresis-mass spectrometry (CE/MS), and high-performance liquid chromatography (LC) with UV or fluorescence detection, combined with derivatization where necessary, and with MS detection [Hines 1995, Krska 2001a, Krska 2001b, Sforza 2006, Trucksess 2006]. Among screening tests for fusariotoxins, competitive ELISA (enzyme-linked immunosorbent assay) methods are widely utilized [Christensen 2000, Krska 2001a, Krska 2001b,

Torres 1998, Trucksess 2006]. ELISA-based kits are simple to use and allow analysis of many samples per day [Gilbert 2000]. However, they are available for only few fusariotoxins, generally expensive, and may suffer from cross-reactivity phenomena giving rise to false positive results that must be confirmed by GC or LC coupled with mass spectrometry [Gilbert 2000].

ELISA kits for DON, ZON, and fumonisins are nowadays commercially available and they are commonly used by Italian public institutions as rapid screening tests to check DON, ZON and fumonisin contents in grains [Haouet 2003]. A drawback is, again, the event of both false positive and false negative results, therefore, confirmation by LC-based procedures of doubtful and/or positive ELISA results is required [Anklam 2002].

The need for rapid yes/no decisions has led to a number of new screening methods. In particular, rapid and easy-to-use test kits based on immunoanalytical principles or the generation of artificial macromolecular receptors employed in molecularly imprinted polymers (MIPs) have made good progress [Krska 2005]. Further research in mycotoxin analysis is pursued in the field of biosensors and electronic nose [Tognon 2005] and also the potential of near-infrared reflectance spectroscopic (NIRS) techniques as screening method was demonstrated [Berardo 2005].

The availability of sensitive and fast methods of analysis that can be used in situ or for decentralized tests is highly desirable. In this perspective, electrochemical methods, e. g. based on immunosensors and screen-printed electrodes, have shown important advantages compared to traditional methods currently in use, because of cost effectiveness, ease of handling and sensitivity [Alarcón 2006].

In the area of multiple mycotoxin analysis, the most promising development was observed in mass spectrometry. Recently, coupling of LC with MS has provided a powerful tool in both primary screening and confirmatory mycotoxin analysis [Núñez 2005, Sforza 2005]. In recent years, the initial, enthusiastic idea that atmospheric pressure ionization followed by tandem MS is a panacea for complex analytical problems has been revised. More and more experimental evidence [Bogialli 2003a, Kebarle 1993, Zöllner 2000] proves that, especially for multicomponent analysis in complex samples, the matrix effect can weaken the ionic signal to a large, unforeseeable extent. The yield of protonation (or deprotonation) of the analytes during electrospray ionization can be decreased by competition effects due to the co-presence of matrix components. As a consequence, sensitivity decreases and, unless ideal internal standards are available, time-consuming internal calibrations are required to achieve accuracy [Bogialli 2003b]. Selective extraction methods [Matuszewski 1998], suitable chromatography [Bogialli 2003a, 2003b], or both of them [Matuszewski 1998] could minimize analyte ion suppression. Triple-quadrupole (QqQ) MS/MS is recognized as a high sensitivity and selectivity technique for confirmation and quantitative purposes in analysis of complex matrices. Multiple reaction monitoring (MRM) with tandem mass spectrometry (MS/MS) enables selective and accurate analyses over a wide linear range [Zöllner 1999]. Confirmation is achieved by selecting at least two transitions in MRM mode [EC 2002a]. QqQ MS/MS can also be used for class identification in Precursor Ion Scan (PIS) or Neutral Loss scan (NL) modes. However, both scan modes involve a significant loss of sensitivity. As a consequence PIS and NL modes are not efficient acquisition modes for identifying compounds at trace levels.

Recently, a hybrid triple-quadrupole/ion trap mass spectrometer, the so-called Q TRAP™ [Hager 2003], was introduced. This instrument provides all the traditional MS/MS scan functions available using the QqQ configuration, and is also able to acquire full-scan MS/MS data using the third quadrupole as a linear ion trap (QqLIT) with enhanced sensitivity. By using a special protocol enabled by the Q TRAP™, PIS or NL mode has become a powerful tool for trace compound identification. By combining NL or PIS mode, as a survey scan, with LIT and QqLIT scan functions, as dependent scans, in an Information Dependent Acquisition experiment (IDA) [Yang 2000], compounds can be identified while collecting data "on the fly" during a single LC run.

For sample preparation, various techniques have been used for the extraction of mycotoxins from maize matrices and for the subsequent purification of the extracts [Krska 1998; Mateo 2002, Pallaroni 2003, Wilkes 1998]. Extraction of fusariotoxins from cereals is generally done using organic-aqueous solvent mixtures containing methanol or acetonitrile. Approaches reported in the literature [Kim 2002] for improving fumonisin extraction from corn and corn–based foods include addition of acid, enzymes, or EDTA to the extraction solvent, or increase of temperature. Among the clean-up procedures applicable for trichothecenes or zeranols, the most frequently used technique is solid-phase extraction (SPE), employing several adsorbent materials, such as charcoal-alumina [Romer 1986], ion-exchange resin [Mateo 2002], silica [Mateo 2002], Florisil [Mateo 2002, Tanaka 2000], Mycosep® columns [Mateo 2002, Schothorst 2001] and immunoaffinity columns [Zöllner 1999]. Fumonisin clean-up is mainly performed using a strong anion-exchanger (SAX) [Akiyma 1998, Lukacs

1996], C_{18} bonded silica [Dilkin 2001], or an immunoaffinity column [Newkirk 1998, Preis 2000].

Simultaneous determination of trichothecenes and zearalenone in GC-MS has already been performed, not only for confirmation but also for primary screening purposes [Krska 2001b]. The availability of sensitive detection, using ionization sources such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI), drastically improved the possibilities of employing LC-MS in the analysis of otherwise intractable polar and ionic analytes such as fumonisins. In addition, tandem mass spectrometry (MS/MS) results in enhanced performance, providing additional selectivity and increased sensitivity (based on S/N). For these reasons, more recently methods based on LC-MS and LC-MS/MS are rapidly spreading in routine analysis. Gilbert [Gilbert 2000] and Lukacs [Lukacs 1996] reported that in some cases LC-MS/MS methods enable rapid unequivocal screening for mycotoxins. In fact, the idea of applying LC-MS/MS as both screening and confirmation methods in food analysis is not new [Taylor 2002]. Rundberget *et al.* developed both LC-MS and LC-MS/MS methods for determining *Penicillium* mycotoxins in foods [Rundberget 2002]. Several analytical methods based on LC-MS or LC-MS/MS already exist for determining separately macrocyclic lactones, type-B trichothecenes, type-A trichothecenes and fumonisins, in cereals [Krska 2001a, 2001b, Shephard 1998].

In the literature, only a few studies describe the simultaneous analysis of some *Fusarium* mycotoxins belonging to different chemical families [Berger 1999, Biselli 2004, Cavaliere 2005, Dall'Asta 2004a, Mateo 2002, Royer 2004]. This is because the chemical structures of these contaminants pose a real challenge with respect to their simultaneous extraction from

the cereal matrix. Therefore, sometimes these methods do not involve common extraction and clean-up procedures and usually require consecutive injections under different analytical conditions [Cavaliere 2005, Mateo 2002]. In recent works, acetonitrile/water mixtures (acetonitrile 75-85%, v/v) with various extraction procedures are the most commonly used in extracting different classes of fusariotoxins [Berger 1999, Biselli 2004, Cavaliere 2005, Dall'Asta 2004a, Krska 2001b, Royer 2004]. With respect to purification of the extracts, Mycosep® cartridges, specifically developed for LC-UV or LC-fluorescence, although expensive, are often chosen also before LC-MS/MS to minimize matrix effects, and are used in tandem for multi-class determinations [Berger 1999, Biselli 2004, Dall'Asta 2004a, 2004b, Razzazi-Fazeli 2002, 2003, Royer 2004]. The main difficulties in devising a LC-MS(/MS) method for analyzing the fusariotoxins arise from the need for compatibility between chromatographic conditions suitable for fumonisins and ionization conditions suitable for the other classes. In fact, the few published works on such multi-residue methods do not include fumonisins.

Chapter 2

Materials and methods

Chapter 2 – Materials and methods

2.1. Reagents and chemicals

Standards of **trichothecenes A**, namely 4β,15-diacetoxy-3α-hydroxy-12,13-epoxy-trichothec-9-ene (DAS), 15-acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)-12,13-epoxy-trichothec-9-ene (HT-2), 15-acetoxy-3α,4β-dihydroxy-12,13-epoxy-trichothec-9-ene (MAS), 4β,8α,15-triacetoxy-3α-hydroxy-12,13-epoxy-trichothec-9-ene (NEO), 4β,15-diacetoxy-3α-hydroxy-8α-(3-methylbutyryloxy)-12,13-epoxy-trichothec-9-ene (T-2); of **trichothecenes B**, namely, 3α,4β,7α,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one (NIV), 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (DON), 3α,7α,15-trihydroxy-4β-acetoxy-12,13-epoxytrichothec-9-en-8-one (FUS X), 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-ADON), 15α-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one (15-ADON); of two **fumonisins (FBs)**, namely, 1,2,3-propanetricarboxylic acid, 1,1′-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester (FB_1), 1,2,3-propanetricarboxylic acid, 1,1′-[1-(12-amino-9,11-dihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester (FB_2); and of **macrocyclic lactones**: 2,4-dihydroxy-6-[10-hydroxy-6-oxo-trans-1-undecyl]benzoic acid μ-lactone (ZON), 2,4-dihydroxy-6-[6α,10-dihydroxy-trans-1-undecyl]benzoic acid μ-lactone] (α-ZOL), 2,4-dihydroxy-6-[6β,10-dihydroxy-trans-1-undecyl]benzoic acid μ-lactone (β-ZOL), 2,4-dihydroxy-6-[6α-10-dihydroxyundecyl]benzoic acid μ-lactone (α-Z), 2,4-dihydroxy-6-[6β-10-dihydroxyundecyl]benzoic acid μ-lactone (β-Z); and of the **internal standards (ISs)**, 2,4-dihydroxy-6-[10-hydroxy-6-oxo-undecyl]benzoic acid μ-lactone (ZAN), 4β,15-dihydroxy-12,13-epoxy-

trichothec-9-ene (VER), and 2-[(2,6dichlorophenyl)amino]benzene-acetic acid (diclofenac) were purchased from Sigma-Aldrich (Milan, Italy). Pure crystalline fusariotoxins were individually dissolved in acetonitrile at concentrations of 1 mg/mL, stored at –20 °C in amber glass vials, and brought to room temperature before use. Standard solutions are more stable in acetonitrile than in methanol for long time storage [Josephs 2003, Pettersson 2003; Shepherd 1988]. However, NIV was prepared in acetonitrile/water (70:30, v/v) at 0.20 mg/mL and FB_1 in acetonitrile/water (50:50, v/v) at 1 mg/mL because of their low solubility in organic solvent. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting them with a suitable solvent. These solutions were kept at 4 °C and renewed weekly.

All organic solvents were HPLC grade from Carlo Erba (Milan, Italy) and were used as received. Ultra-pure water was produced by a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Hydrochloric acid, formic acid and ammonia were purchased from Merck (Darmstadt, Germany).

2.2. Extraction and clean-up apparatus

Polytron homogenizer was from Kinematica (Luzern, CH). Bakerbond Octadecyl (40μm) was supplied by J.T.Baker (Deventer, Holland) and Carbograph-4 from LARA (Rome, Italy). Carbograph-4 is a graphitized carbon black (GCB) with a surface area of 210 m^2/g and particle size range of 120-400 mesh, similar to Carboprep 200 (Restek) and EnvicarbX (Supelco). C_{18} cartridges were prepared by filling 6 mL polypropylene

tubes with 100 mg of the adsorbent placed between two polyethylene frits. Immediately prior to use these cartridges were washed with 5 mL of acetonitrile/water (75:25, v/v). Carbograph-4 cartridges were prepared by placing 500 mg of the adsorbent inside 6 mL polypropylene tubes between two polyethylene frits. Before processing samples, Carbograph-4 cartridges were attached to a vacuum manifold apparatus and washed sequentially with 10 mL of dichloromethane/methanol (80:20, v/v) containing 50 mmol/L formic acid, 5 mL of methanol, 20 mL of acidified water (10 mmol/L hydrochloric acid) and 10 mL of water. Tubes, frits and the vacuum manifold were from Supelco (Bellefonte, PA, USA). PTFE syringe filters (0.45μm; 15 mm diameter) were purchased from Chemtek Analytica (Bologna, Italy). Oasis HLB™ cartridges (200 mg) were purchased from Waters (Milford, USA) and were used according to the manufacturer's instructions.

2.3. Sample preparation

2.3.1. Fusariotoxin extraction

One gram of corn meal was homogenized for 15 s with 10 mL of acetonitrile/water (75:25, v/v), using a Polytron homogenizer. Use of longer extraction time did not increase analyte recoveries, and indeed for some trichothecenes they slightly decreased, likely due to heating effects. The homogenized sample was then placed (using a pipette) on the top of the C_{18} cartridge that was positioned in the vacuum manifold. The vacuum was adjusted to the maximum and the extract was collected into a 25 mL volumetric flask. The extraction vessel was washed twice with 7 mL of the extracting solvent mixture, and these washings were also passed through

the C_{18} cartridge and collected in the volumetric flask; the volume was then adjusted to 25 mL.

For recovery studies, mycotoxin-free samples were artificially fortified as follows. One gram of sample was soaked in 1 mL of acetone solution containing a suitable amount of each analyte; the sample was then dried for 2 hours in a ventilated oven set at 40°C. The spiked sample was extracted as described above.

2.3.2. Fusariotoxin clean-up

A 5 mL aliquot of the extract was diluted to 500 mL with water and passed through the pre-conditioned Carbograph-4 cartridge at a flow rate of 20 mL/min. The cartridge was washed with 10 mL of water and the residual water content was further decreased by slowly passing 0.3 mL of methanol through the cartridge. The vacuum was adjusted to provide a flow rate of about 5 mL/min, and fusariotoxins were eluted with 1 mL of methanol followed by 8 mL of dichloromethane/methanol (80:20, v/v) containing 50 mmol/L formic acid. Both eluates were collected in the same vial, the solution containing VER and ZAN (ISs) in methanol was added, and the mixed solution was evaporated at 40 °C under a gentle flow of nitrogen to about 50 μL. The residue was diluted with 200 μL of water containing 10 mmol/L formic acid that had been adjusted to pH 3.8 with ammonia, and the solution was forced through a PTFE syringe filter. A 20 μL aliquot of the final solution was analyzed by LC-(ESI)MS/MS. For comparison purposes, a 5 mL aliquot of extract was diluted to 100 mL with water and passed through the pre-conditioned Oasis HLB™ cartridge, and the cartridge was then left to dry. Analytes were eluted

with 5 mL of methanol containing 1 mol/L ammonia, ISs were added, and the rest of the procedure described above was followed.

2.3.3. Selective clean-up for fusariotoxin classes

A new 5 mL aliquot of the extract was diluted to 500 mL with water and loaded on the pre-conditioned Carbograph-4 cartridge, as described in 2.2. The cartridge was washed with 10 mL of water and the residual water content was further decreased by slowly passing 0.3 mL of methanol through the cartridge. Fusariotoxins were fractionated sequentially and collected in three 1.4 cm i.d. round-bottom glass vials. Trichothecenes A and B were eluted with 8 mL of methanol (fraction A). Macrocyclic lactones were, then, eluted with 12 mL of dichloromethane/methanol (80:20, v/v) (fraction B). Finally, fumonisins were eluted from the cartridge with 8 mL of dichloromethane/methanol (80:20, v/v) containing 50 mmol/L formic acid (fraction C). Fraction A, spiked with IS solution (containing VER) was evaporated to dryness at 40 °C under a gentle flow of nitrogen and redissolved in 250 μL of acetonitrile/methanol/water (7:3:90, v/v). Fraction B, spiked with IS solution (containing ZAN), was evaporated at 40 °C under a gentle flow of nitrogen to about 50 μL and diluted with 200 μL of acetonitrile/methanol/water (35:15:50, v/v/v). Fraction C, spiked with IS solution (containing diclofenac) was evaporated to about 50 μL and diluted with 350 μL of methanol/water (50:50, v/v) containing 25 mmol/L formic acid. Both solutions were forced trough PTFE syringe filters. 20 μL of the three final solutions were injected into the HPLC column. In particular, fraction A was analyzed two times in different chromatographic conditions for trichothecenes A and B, respectively.

2.4. LC-MS/MS instrumentation

Liquid chromatography was performed using Perkin-Elmer series 200 micropumps (Norwalk, CT, USA) coupled with a Perkin-Elmer autosampler. The analytes were chromatographed on an Alltima C_{18} column (250 x 2.1 mm i.d., 5 μm particle size) from Alltech (Deerfield, IL, USA) with a Securityguard ODS, 4 x 2 mm i.d. precolumn supplied by Phenomenex (Torrance, CA, USA).

An Applied Biosystems/MDS SCIEX Q TRAP™ linear ion trap mass spectrometer (Concord, Ontario, Canada), coupled with ESI (TurboIonSpray) or APCI (heated nebulizer-Atmospheric Pressure Chemical Ionization) sources, was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAP™ can also operate in enhanced resolution scan (ER) and in enhanced product ion scan (EPI) modes. Applied Biosystems/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

2.4.1. LC-MS/MS of fusariotoxins

Fusariotoxins were separated using gradient elution with water as mobile phase A and methanol as mobile phase B, both containing 10 mmol/L formic acid and adjusted to pH 3.8 with ammonia. The pH value was measured using a pH meter for the water solution; after determining the suitable amount of ammonia to obtain pH 3.8 in water, the same amount was added to the methanol. B was linearly increased from 20% to 50% in 10 min, then to 80% in 15 min, and finally brought to 100% and held constant for 10 min. The flow rate was 200 μL/min and the column was

maintained in an oven (Timberline Instruments, Inc., Boulder, Colorado, USA) at 45°C.

The MS interface was operated in both negative (NI) and positive (PI) modes. The ESI needle voltage was set at 5500 V in PI and –4500 in NI, the curtain gas was set to 35 (arbitrary units), GS1 and GS2 were set respectively to 30 and 45, and the turbo-gas temperature was set to 450 °C. Nitrogen served both as turbo-gas and collision gas. Collisionally activated dissociation (CAD) MS/MS was performed in the collision cell (Q2), operating the collision gas set at medium value (arbitrary scale). Mass calibrations and resolution adjustments on the resolving quadrupoles were performed automatically using a 10^{-5} mol/L solution of PPG (polypropyleneglycol) introduced via a model 11 Harvard infusion pump. The peak-width was set on both resolving quadrupoles at 0.7 Th (measured at half height) for all MS and MS/MS experiments. Fusariotoxin spectra were preliminarily recorded by connecting the Harvard infusion pump to the interface. In order to optimize the tuning parameters for each compound, 5 ng/μL standard solutions in methanol/water (50:50, v/v) containing 10 mmol/L formic acid adjusted to pH 3.8 with ammonia, were infused at 10 μL/min. Depending on the behavior of the analytes in the ESI source, the protonated or deprotonated molecules or adducts were mass-selected by the first quadrupole and fragmented. From the MS/MS full scan spectra, of each analyte with the exception of 15-ADON, two suitable transition pairs were selected for acquisition in MRM mode. LC-(ESI)MS/MS conditions for detection of each mycotoxin are summarized in table 2.1.

Table 2.1. LC-MS/MS conditions and precursor ion/product ion pairs selected for acquiring fusariotoxins in ESI-MRM mode.

Analytes[b]	Period	IP	t_R (min)	DP (V)	EP (V)	RCE (%)[a]	Precursor Ion (m/z)	Product Ions (m/z)
NIV	1	–	5.54	-80	-9	13	357	311, 281
DON	2	–	7.85	-54	-7	13	341	295, 265
FUS X	3	+	9.94	48	10	10	372	355, 247
NEO	3	+	10.50	52	6	14	400	305, 215
VER (IS)	4	+	11.85	45	10	8	284	249, 231
3-ADON	5	+	12.98	100	11	13	339	231, 203
15-ADON	5	+	12.98	100	11	11	339	297
MAS	6	+	14.92	56	6	11	342	265, 107
DAS	7	+	17.67	60	8	13	384	307, 247
HT-2	8	+	21.31	80	8	14	442	263, 215
FB_1	8	+	21.71	98	10	38	722	334, 352
T-2	9	+	23.82	60	9	14	484	245, 215
FB_3	9	+	24.23	130	10	36	706	354, 336
α-ZOL	10	–	25.06	-115	-11	29	319	174, 160
ZAN (IS)	10	–	25.40	-110	-11	29	319	205, 161
ZON	10	–	25.92	-110	-11	29	317	175, 131
FB_2	11	+	26.58	130	10	36	706	354, 336

[a]Relative collision energy expressed as % with respect to the maximum voltage difference value between the high pressure entrance quadrupole (Q0) and collision cell quadrupole (RO2) (+ or – 130 V) permitted by the instrument.

[b]NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; NEO, neosolaniol; VER, verrucarol; 3- and 15-ADON, 3- and 15-acetyldeoxynivalenol; MAS, monoacetoxyscirpenol; DAS, diacetoxyscirpenol; FB_1, fumonisin B_1; HT-2, HT-2 toxin; FB_3, fumonisin B_3; T-2, T-2 toxin; α-ZOL, α-zearalenol; ZAN, zearalanone; ZON, zearalenone; FB_2, fumonisin B_2.

2.4.2. LC-MS/MS of trichothecenes A

Trichothecenes A were separated and detected in the same LC-(ESI)MS/MS conditions described above for the fusariotoxin analysis. In fact, both using mobile phase containing ammonium formiate at pH 3.8 and selecting the ions $[M+NH_4]^+$ as precursor ions are found to be the best conditions also for determining trichothecenes A class alone. In this case the use of the column oven could be avoided.

2.4.3. LC-MS/MS of trichothecenes B

Analysis of trichothecenes B was performed using a gradient separation. The initial composition of the mobile phase was 10% of acetonitrile/methanol (70:30, v/v) and the remaining 90% of water. The gradient was programmed to linearly increase the amount of organic phase up to 45% in 10 min, then immediately to 80% and held constant for 7 min. To purge the column the per cent of acetonitrile/methanol was increased to 100% and kept constant for 5 minutes. The flow rate was 200 μL/min.

In a preliminary phase of the work, the ESI and APCI sources were operated in both NI and PI modes. A turbo-gas of nitrogen heated at 350°C was used for ESI, and the nominal heating temperature for the heated nebulizer-APCI source was 450°C. DP, EP and RCE values were optimized for each compounds, whereas all other parameters were unchanged respect to fusariotoxin LC-MS/MS analysis (2.4.1). MS and MS/MS spectra were recorded by connecting the Harvard infusion pump directly to the ion sources. In order to optimize the tuning parameters, standard solutions of roughly 5 ng/μL for each compound, in an aqueous solvent of 50% methanol/ acetonitrile (30:70, v/v), were infused at 10 μL/min. The best ionization conditions turned out to be those using the ESI interface in the NI mode, so it was chosen for the final identification of the analytes. MRM mode was used for quantitation. LC-(ESI)MS/MS parameters for detection of each trichothecene B are summarized in table 2.2.

Table 2.2. LC-MS/MS conditions and precursor ion/product ion pairs for the acquisition in negative ESI(MRM) mode of trichothecenes B.

Analytes[a]	Period	t_R (min)	DP (V)	EP (V)	RCE[b] (%)	Precursor Ion (m/z)	Product Ions (m/z)
NIV	1	6.1	-92	-10	11	311	281
DON	2	7.9	-70	-9	15	295	265, 138
FUS X	3	9.7	-90	-9	27	353	187, 59
3-ADON	4	11.9	-62	-8	12	337	307, 173
15-ADON	4	12.1	-75	-10	11	337	219, 150

[a]NIV = nivalenol, DON = deoxynivalenol, FUS X = fusarenon X, 3-ADON = 3-acetyldeoxynivalenol, 15-ADON = 15-acetyldeoxynivalenol.
[b]see Table 2.1.

2.4.4. LC-MS/MS of zeranols

Macrocyclic lactones were separated in isocratic conditions. The mobile phase composition was set at 53% of acetonitrile/methanol (70:30, v/v) in water. To purge the column after each chromatographic run, the amount of acetonitrile/methanol was increased to 95% and held for 5 min. The flow rate was 200 μL/min.

The interface was operated in NI mode. A turbo-gas of nitrogen heated at 350 °C was used for ESI working. MS and MS/MS spectra were preliminarily recorded by connecting the Harvard infusion pump directly to the source. In order to optimize the tuning parameters for compounds not included in fusariotoxin LC-MS/MS (2.4.1) 5 ng/μL standard solutions, in methanol/acetonitrile (30:70, v/v) 50% in water, were infused at 10 μL/min. EP and RCE values were set for all zeranols to -11 V and 29% respectively. Other MS parameters were the same used in 2.4.1. From MS/MS full scan spectra, suitable transition pairs were selected for the

acquisition in MRM. LC-(ESI)MS/MS parameters for detection of zeranols are summarized in table 2.3.

Table 2.3. LC-MS/MS conditions and precursor ion/product ion pairs for the acquisition in negative ESI(MRM) mode of zeranols.

Analytes[a]	Period	t_R (min)	DP (V)	Precursor Ion (m/z)	Product Ions (m/z)
β-ZAL	1	10.3	-110	321	277, 161
β-ZOL	1	11.0	-115	319	174, 160
α-ZAL	1	14.8	-110	321	277, 161
α-ZOL	1	16.1	-115	319	174, 160
ZAN (IS)	2	24.1	-110	319	205, 161
ZON	2	25.5	-110	317	175, 131

[a]β-ZAL = β-zearalanol, β-ZOL = β-zearalenol, α-ZAL = α-zearalanol, α-ZOL = α-zearalenol, ZAN = zearalanone ZON = zearalenone.

2.4.5. LC-MS/MS of fumonisins B

Fumonisins were separated using gradient elution with water as mobile phase A and methanol as mobile phase B, both containing 25 mmol/L formic acid. After an isocratic step at 60% B for 3 min, B was linearly increased to 90% in 5 min, then brought to 100% and held constant for 10 min. The flow rate was 200 μL/min. Before use, the column was treated with a 0.1 mol/L EDTA solution, as reported by Liang et al. in an application to tetracycline [Liang 1998], to remove traces of metallic ions from the silica skeleton of the C_{18} stationary phase.

The ESI-MS/MS analyses were performed operating under the same mass spectrometric conditions adopted for the fusariotoxin acquisition (2.4.1).

Since standards of FB_3 and FB_4 were not easily available, we took maximum advantage of the power of the instrument for their identification in real samples. The Q TRAP™ was employed in QqQ, LIT, and QqLIT configurations; data were acquired using an IDA (Independent Data Acquisition) protocol. In this experiment a Neutral Loss scan (NL) was used as a survey scan to trigger the data collection, then an enhanced resolution (ER) scan mode of the most abundant ion, and an enhanced product ion (EPI) scan for the same ion were looped. The term "enhanced" indicates that the acquisitions are done using the LIT. Electrical and gas parameters were the same values as those set for FB_2. The characteristic neutral loss was set at 370 Da, and its precursor ions were sought in the m/z scan range 680-800. Scan rates in ER and EPI modes were respectively 250 and 4000 Th/s in the m/z scan ranges 680-800 and 250-800, with respectively step sizes of 0.03 and 0.12 Th . In addition, the LIT fill time was 50 ms, Q0 trapping was always set "ON", and two scans were summed. After identification of FB_3 and FB_4 in two contaminated corn meals via MS/MS spectra obtained by means of IDA experiments, further analysis of these fumonisins was performed in MRM mode on the basis of retention time (±2%) and two suitable MS/MS transition pairs, as for FB_1 and FB_2.

LC-(ESI)MS/MS parameters for the detection of each fumonisin are summarized in table 2.4. For the MRM analysis of FB_1 the most abundant fragment ions, $[M+H-2TCA-H_2O]^+$ and $[M+H-2TCA-2H_2O]^+$ (TCA = tricarballylic acid), were selected. For the other compounds the fragment ions resulting from consecutive loss of two molecules of TCA $[M+H-2TCA]^+$, and from the loss of an additional water molecule $[M+H-2TCA-H_2O]^+$, were used. Although the $[M+H-2TCA]^+$ ion signal was 30% less

intense than the $[M+H-2TCA-2H_2O]^+$ signal for FB_2, the first ion showed a higher S/N ratio and therefore was preferred. For FB_3 the $[M+H-2TCA]^+$ and $[M+H-2TCA-2H_2O]^+$ signal intensities were quite similar, while in the case of FB_4 the second ion was absent since there is only one hydroxyl group in the molecular structure (figure 2.1).

Table 2.4. LC-MS/MS conditions and precursor ion/product ion pairs selected for acquiring fumonisins in positive ESI(MRM) mode.

Compound	t_R (min)	DP (V)	EP (V)	RCE[a] (%)	Precursor ion (m/z)	Product ions (m/z)
FB_1	8.2	98	10	38	722	334, 352
FB_2	9.6	130	10	36	706	336, 354
FB_3	8.9	130	10	36	706	336, 354
FB_4	10.2	130	10	36	690	320, 338
Diclofenac (IS)	12.0	25	6	25	296	215

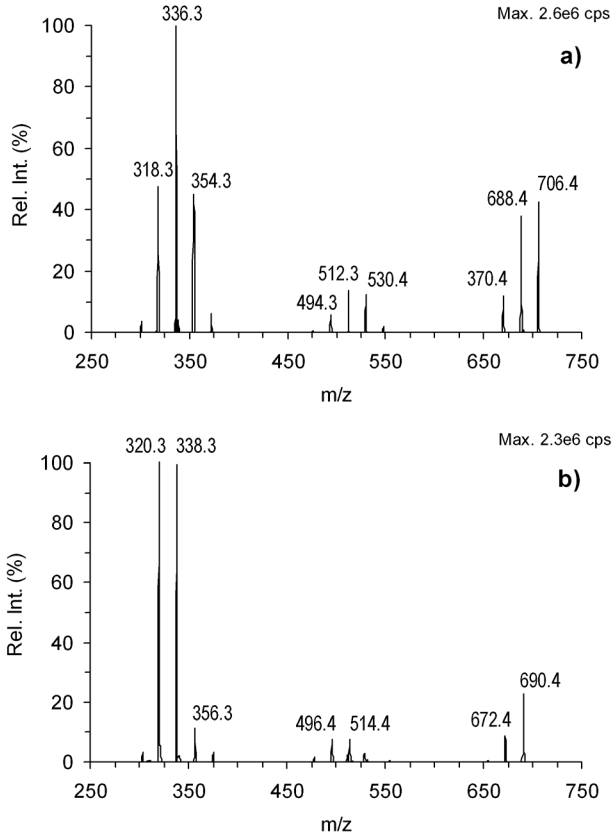
[a]see Table 2.1.

2.5. ELISA test

Two competitive enzyme immunoassays, RIDASCREEN® DON and RIDASCREEN®FAST Zearalenon, both provided by R-Biopharm AG (Darmstadt, Germany), were utilized for the analysis, respectively, of DON and ZON. The instructions given by the manufacturer were strictly followed for the correct use of the kits. The results of the analyses were obtained photometrically at λ = 450 nm using a Microplate Reader 550 (BIO-RAD) spectrophotometer. Recovery rate in cereal is between 85-110% for DON and 64-97% for ZON. Detection limits of the methods are,

respectively, 17 and 18.5 ng/g. Upper limit of linearity is 500 ng/g for both kits.

Figure 2.1. Enhanced Product Ion (EPI) spectra of fumonisin FB_3 (a) and FB_4 (b) generated during the IDA experiment for a naturally contaminated corn meal sample containing FB_3=61% of FB_2 and FB_4=16% of FB_2 (FB_2=0.24 μg/g).



2.6 Samples

Corn meal and maize- based product samples were purchased from Italian retail markets. ISC (Experimental Institute of Cereal Research, Rome and Bergamo, Italy) kindly provided the fusariotoxin-free corn and freshly harvested corn samples.

2.7. Quantitation and statistical evaluation

2.7.1 Fusariotoxins

Reference solutions were prepared by diluting appropriate volumes of the working standard solutions with the chromatographic mobile phase. Calibration was performed by spiking analyte-free samples after clean-up with known and appropriate volumes of the working standard and internal standard solutions. Samples were fortified at six levels to a concentration range of 0.004-4 ng/μL in 250 μL of final extract, and 20 μL were injected. This procedure was repeated three times. For each analyte the summed ion currents profile of both fragment ions was extracted from the LC-MRM dataset, and the plot of peak area versus injected amount was obtained by measuring the resulting peak areas. Method detection limit values were calculated on the basis of S/N = 3 for the summed signals of both fragment ions (with exception of 15-ADON).

Total recovery was assessed by spiking analyte-free corn meal samples, performing the extraction and clean-up procedures, measuring the peak areas, calculating the peak area ratios relative to the opportune IS added after clean-up, and comparing these data with those obtained by spiking the extracts of the same corn meal sample after clean-up. For fusariotoxin quantitation in real samples, corrections for recovery were made. The concentration of FB_3, for which a standard was not available to us, was evaluated as reported in 2.7.3. Briefly, after identification of FB_3 in naturally contaminated samples of corn meal via MS/MS spectra obtained using an information-dependent acquisition protocol provided by a QTRAP™ mass spectrometer (Applied Biosystems/SCIEX), FB_3 was quantitatively estimated using the ratio of its peak area to that for FB_2, the molecule most structurally similar to it. The values obtained as mole % of

FB_2 were transformed to μg/kg for internal consistency. Statistical evaluations were performed by ANOVA ($p = 0.05$).

2.7.2 Trichothecenes, zeranols and fumonisins

Analytes were quantified by external calibration procedure. Composite standard solutions were prepared at six concentration levels by diluting appropriate volumes of the working standard solutions. For each analyte, the combined ion current profiles for both fragment ions were extracted from the LC-MRM dataset. For each analyte the plot of peak area versus injected amount was obtained by measuring the resulting peak area and relating this area to that for the IS, with exception of trichothecenes B. The response of ESI-MS/MS was linearly related to injected amounts up to 100 ng ($R^2 > 0.985$). Correction for recovery was not done. Method detection limit values were calculated on the basis of S/N = 3 for the less intense fragment ion signal. Statistical evaluations were performed by ANOVA ($p = 0.05$).

2.7.3 $FB_{3\text{-}4}$ estimation

FB_3 and FB_4 were quantitatively estimated using ratios of peak areas to that for FB_2, the standard most structurally similar to them, and expressed as mol % of FB_2. Since the measurements were performed using MRM mode involving unknown fragmentation efficiencies, while the molar responses are more similar for the non-fragmented $[M+H]^+$ ions of these structurally related molecules, a correction factor (C_f) was used. This factor was obtained as follows. The same contaminated sample was submitted to LC-MS/MS analysis in MRM mode, and also to LC-MS analysis using the LIT in ER scan mode under trap conditions in which fragmentation was

reduced to its minimum. For FB_{2-4} the ion current profiles for the characteristic fragment ions were extracted from the LC/MRM dataset, and the $[M+H]^+$ ion current profiles were extracted from the LC/LIT dataset. Peak areas were measured and correction factors calculated as:

C_f (FB_n) = [Area MRM(FB_2)]/[Area MRM(FB_n)] x [Area LIT(FB_n)]/[Area LIT(FB_2)] (n =3 or 4)

Statistical evaluations were performed using ANOVA (p = 0.05).

2.8. Safety considerations

Fusariotoxins are dangerous compounds, consequently solution and extract should be handled with care. Gloves and protective clothing were worn as safety precautions during the handling of the compounds. Residues from analysis were disposed properly and glassware treated with 3% sodium hypochlorite before washing and reuse.

Chapter 3

Results and discussion

Chapter 3 – Results and discussion

3.1. Fusariotoxin extraction

The extraction procedure, as described in 2.3, was optimized after evaluating the performance of different techniques, such as pressurized liquid extraction (PLE), matrix solid phase dispersion extraction (MSPDE), ultrasonication and homogenization extraction, with different mixtures of solvents. The best compromise for simultaneous quantitative extraction in a short time was reached by homogenizing in acetonitrile/water (75:25, v/v) a previously ground and sieved maize sample. Acetonitrile/water has been shown to be the best extraction medium due to its suitability for isolation of analytes covering a wide range of polarity. After homogenization, the supernatant collection procedure includes extract filtration on C_{18} cartridge, allowing partial de-fatting of the matrix as a valid alternative to n-hexane partitioning [Krska 2001b, Mateo 2002]. With the solvent mixture used, the C_{18} material retains most of the phospholipids and triglycerides, while extracting the maize twice allows the quantitative recovery of target compounds. The proposed procedure is simple and fast (it requires 15 minutes at most to obtain 25 mL of clear extract) and effective. Recoveries were obtained by spiking, at a level of 250 ng/g, analyte-free samples, before and after the extraction step and following the rest of the procedure. In this way, the effect of extraction on total recovery can be isolated and evaluated by comparing the absolute peak areas for the same compound in samples spiked *ante* and *post* extraction. Using this set of conditions, fumonisin recovery rates were 84-88%, while all the other analytes were extracted with recoveries above

95%. Use of the extraction solvent mixture acidified with 50 mmol/L formic acid increased the fumonisin recoveries to over 95% (for details see 3.7.2 and table 3.10), but recovery rates for type-A trichothecenes decreased significantly. If necessary, fumonisin accuracies may be improved by using correction factors for extraction recovery rates.

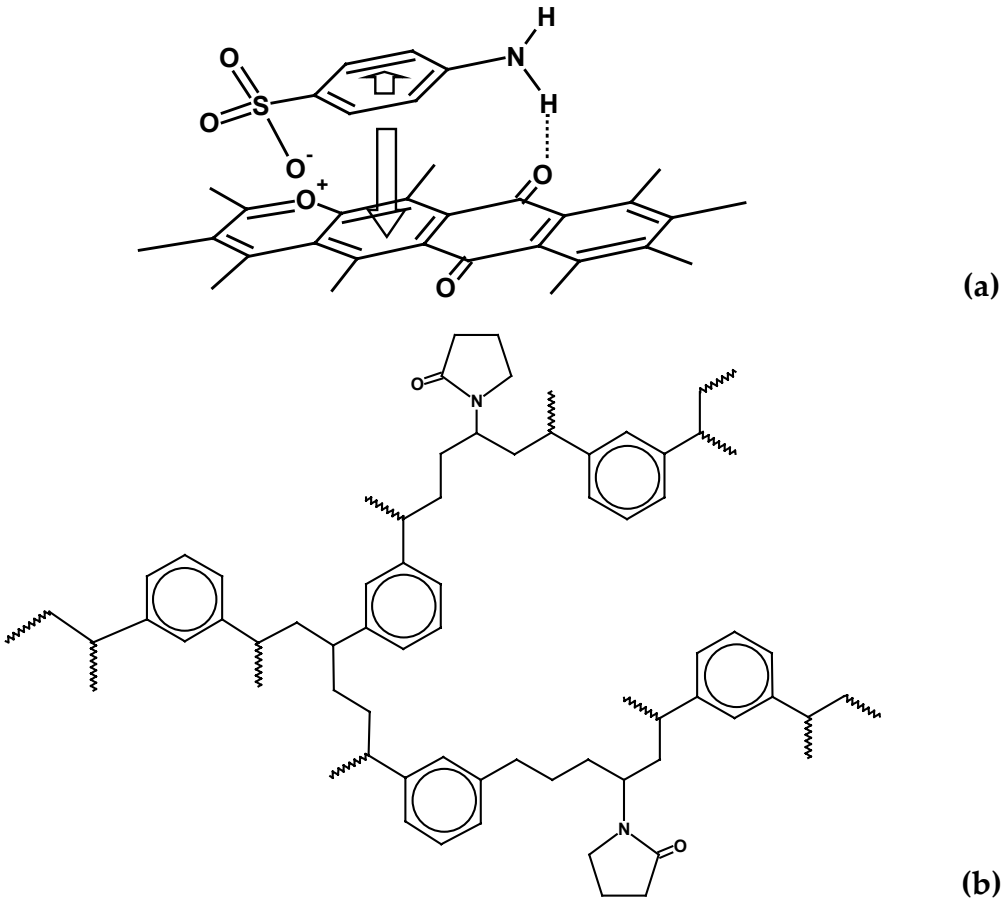
3.2. Clean-up

The amount of substances co-extracted from corn-based matrices was very high, and an efficient clean-up procedure was therefore essential.

3.2.1. Fusariotoxin clean-up

For clean-up purposes, two adsorbents suitable for the extraction of both polar and non-polar compounds, such as Oasis HLB™ and graphitized carbon black (GCB, Carbograph-4), were tested (Figure 3.1a and 3.1b).
The Oasis HLB™ cartridges are filled with hydrophilic-lipophilic balanced sorbents (divinylbenzene:N-vinylpyrrolidone 1:1). As formerly reported, GCBs can behave as sorbents with reversed-phase, polar interaction and ion-exchanger properties and, moreover, posses a particular affinity for aromatic compounds with respect to aliphatic ones [Altenbach 1995, Andreolini 1987, Crescenzi 1996]. Fusariotoxins were simultaneously eluted from Carbograph-4 using dichloromethane/methanol (80:20, v/v) containing 50 mmol/L formic acid.

Figure 3.1. Sketch showing (a) graphitized carbon black structure interacting with a model molecule and (b) Oasis HLB™ polymer structure.



The high content (80%) in dichloromethane of the eluting mixture was optimized as the minimum percent able to elute strongly retained compounds such as macrocyclic lactones. Moreover, the addition of formic acid allows recovery of compounds such as fumonisins that can establish electrostatic interactions with the GCB surface. An aliquot of 1 mL methanol was passed through the cartridge before elution to remove traces of water from the cartridge. This volume was collected together with the 8 mL aliquot of acidified dichloromethane/methanol (80:20, v/v) because the less-retained type-B trichothecenes, such as NIV and DON, began being eluted in the methanol wash. Water volumes for diluting the

extract aliquot before solid phase extraction clean-up were optimized to provide the highest recovery rates. These optimal volumes were determined to be 500 mL for the Carbograph-4 and 100 mL for Oasis HLB™.

Absolute recoveries from the clean-up procedures were obtained by comparing analyte-free sample extract aliquots spiked at a level of 250 ng/g both before and after clean-up. Recoveries obtained using both materials were comparable, and better than 82% for most of the intended analytes; however, NIV, FUS X, NEO and ADONs were insufficiently recovered from Oasis HLB™ (in the range 27-54%). In addition, by comparing samples spiked after clean-up with a reference solution, we observed generally stronger matrix effects when Oasis HLB™ sorbent was used than that for Carbograph-4. As shown from table 3.1, only the NIV signal was suppressed more when purification was performed on Carbograph-4. This material proved to be suitable for this application in terms of recovery and efficiency in removing interfering substances, even though the well-known loadability limitations of graphitized carbon blacks must be accounted [Andreolini 1987, Crescenzi 1996, Laganà 2003]. To avoid breakthrough for the less retained compounds, only 5 mL of the entire extract, added to 500 mL water, was submitted to the clean-up procedure. Dilution of the sample with an appropriate volume of water resulted in both lower matrix effect and higher recovery for the less retained compounds. In fact, the recovery of NIV decreased to 21% when the volume of dilution was 100 mL. This behavior has been already recognized [Andreolini 1987, Laganà 2003] and might be due to some kind of co-extracted molecules that can play a double role, namely, displacement from active adsorption sites during loading on Carbograph

cartridge and competition for ion desorption from charged droplets during the electrospray ionization process. The volume of 500 mL water was selected as the best compromise to maximize recovery and minimize signal suppression, and to obtain reasonable loading time.

Table 3.1. Matrix effects observed utilizing Oasis HLB™ and Carbograph-4 as sorbent in the purification step.

Analytes[b]	SPE sorbent	
	Oasis HLB™ (%)[a]	Carbograph-4 (%)[a]
NIV	91	53
DON	105	80
FUS X	59	77
3-ADON	56	86
15-ADON	49	82
NEO	57	83
MAS	75	86
DAS	84	93
HT-2	78	84
T-2	72	86
α-ZOL	18	64
ZON	23	68
FB_1	106	92
FB_2	74	86

[a]Absolute recoveries obtained by comparing analyte-free sample extract aliquots spiked at level of 250 ng/g after clean up with a reference solution (n = 3).

[b]NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; 3- and 15-ADON, 3- and 15-acetyldeoxynivalenol; NEO, neosolaniol; MAS, monoacetoxyscirpenol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; T-2, T-2 toxin; α-ZOL, α-zearalenol; ZON, Zearalenone; FB_1, fumonisin B_1; FB_2, fumonisin B_2.

The analytes did not show degradation or insolubility phenomena during the concentration step and the reconstitution of extracts before LC-MS/MS analysis.

3.2.2. Selective clean-up for fusariotoxin classes

For the above mentioned properties of GCBs (see 3.2.1), use of a particular type of GCB like Carbograph-4 allows separation of basic-neutral compounds from acidic ones (like fumonisins) by passing suitable eluent phases sequentially through the cartridge [Andreolini 1987]. In this work it was possible to separate sequentially, according to the procedure reported in 2.3.2, trichothecenes in the methanol fraction, zeranols in the dichloromethane/methanol (80:20, v/v) fraction, and fumonisins in the acidified dichloromethane/methanol (80:20, v/v 50 mmol/L formic acid). This clean-up procedure gave cleaner extracts than collecting a single fraction and drastically reduced ion suppression during ESI ionization process. When the intermediate acidic washing step was introduced, a small increase in ZON response was observed but fumonisin recovery rates decreased significantly. Naturally, also in this case to avoid the effect of breakthrough on the SPE cartridge, only 5 mL of the entire extract, added to 500 mL of water, were submitted to clean-up. Recoveries, obtained by comparing analyte-free samples spiked at level of 250 ng/g before and after clean up, were better than 82%.

3.3. Fusariotoxin screening method by LC-MS/MS

3.3.1. General remarks

In the case of multiresidue analyses, compromise conditions must often be accepted. As it is well documented in the literature, three experimental restrictions cannot be overcome in the case of these mycotoxins. First, due to the presence of four carboxylic groups in the molecular structures,

fumonisins are eluted as sharp and symmetrical peaks only under acidic chromatographic conditions [Plattner 1999], and can thus be detected only in positive ESI mode. We found that at a pH value not higher than 3.8, and in the presence of NH_4^+ ions, fumonisins are still eluted as sharp peaks. Second, type-A trichothecenes can give rise to intense $[M+Na]^+$ and $[M+NH_4]^+$ adducts. Sensitive MRM detection of this class of compounds requires the presence of NH_4^+ ions in the mobile phase [Razzazi-Fazeli 2002, Huopalahti 1997], although MRM of sodiated adducts of T2, HT2, DAS and NEO have been reported [Biselli 2004, Tuomi 1998]. Third, type-B trichothecenes are usually detected in negative ESI mode as $[M-H]^-$ ions without additives in the mobile phase (see 3.5.1), while formiate or acetate adducts definitely prevail when buffers containing these ions are used [Berger 1999, Biselli 2004, Voyksner 1987].

3.3.2. Optimization of MS/MS detection

Studies were conducted to select the most suitable precursor ions for the MRM acquisition mode. As far as fumonisins are concerned, MS and MS/MS spectra in presence of NH_4^+ ions were identical to those in solvents containing only formic acid as additive [Musser 1997]. For macrocyclic lactones, the precursor ion selected could be $[M+H]^+$ instead of $[M-H]^-$, but in this case the sensitivity was lower.

Type-A trichothecenes form abundant adducts with ammonium and alkali metal ions which are in the HPLC-grade solvents as impurities. Some authors have analyzed type-A and -B trichothecenes using LC-MS by selecting the $[M+Na]^+$ ion [Biselli 2004, Dall'Asta 2004a, Huopalahti 1997]. In our case, the expedient of adjusting the pH value by adding ammonia

increased the ammonium adduct abundance with respect to the sodium one. This is an advantage, because the $[M+NH_4]^+$ ion yields a fragmentation pattern more useful than that of $[M+Na]^+$ for MRM; sodiated fragments were clearly visible in the CID spectra of $[M+Na]^+$ ions of trichothecenes A, but Na^+ itself was the most abundant fragment ion in any case. In contrast, fragmentation of ammoniated adducts probably led to the loss of ammonia and subsequent losses of the following neutral masses [Razzazi-Fazeli 2002]: 18 u (water from OH groups), 30 u (formaldehyde from the epoxide ring), 60 u (acetic acid from acetyl groups), and 102 u (isovaleric acid from iso-valeryl groups). The most intense among these transitions were selected for acquisition in MRM mode, with the exception of MAS that gave an abundant ion at m/z 107, likely $[cresol-H]^+$.

In negative ESI mode in the presence of acetate or formiate, the $[M-H]^-$ ions of trichothecenes B were not detected; however, as for many other polar compounds containing neither acidic nor basic groups, trichothecenes B can form adducts with these anions. The $[M+HCOO]^-$ adduct was the most intense for NIV and DON and, although the formiate ion predominates in the CID spectrum, some less abundant fragments are available for detection in MRM mode. In contrast, 3-ADON gave a $[M+H]^+$ ion about three times more abundant than $[M+HCOO]^-$. The signals from 15-ADON were comparable in NI and PI acquisition modes; the $[M+H]^+$ ion was selected since better sensitivity was achieved in MRM mode. Although the two isomers are coeluted under our chromatographic conditions, as reported in table 2.1, they may be distinguished on the basis of different fragmentation pathways [Berger 1999]. Among type-B trichothecenes, only FUS X showed an abundant $[M+NH_4]^+$ ion; this is

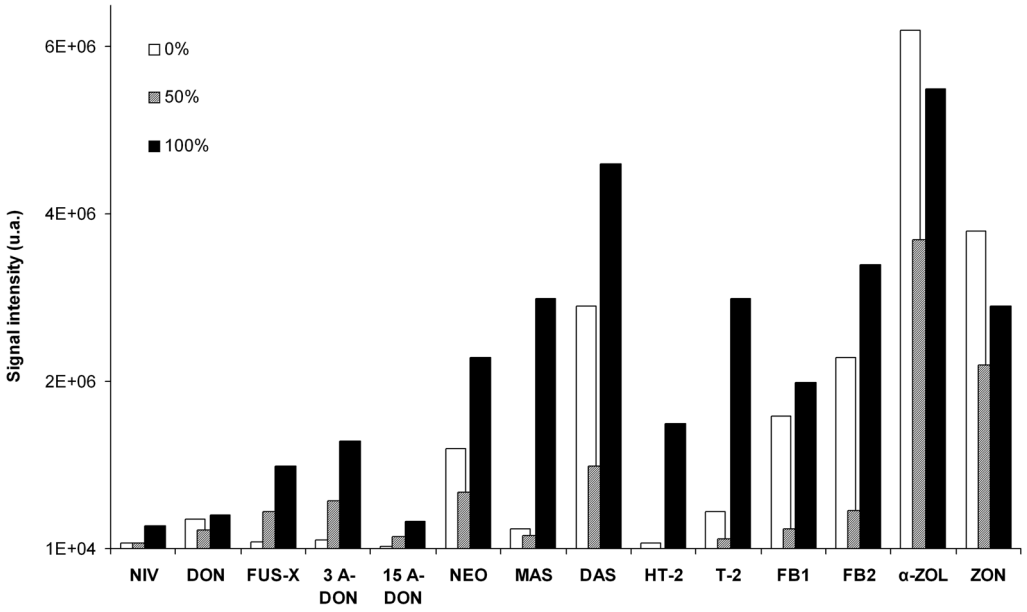
probably due to the acetyl side chain at C_4, syn with respect to the epoxide ring. Fragmentation of the $[FUS\ X+NH_4]^+$ ion was very similar to that of type-A trichothecenes.

In order to find the best conditions in terms of sensitivity, different concentrations of acetic and formic acids (5, 10, 20, 30 mmol/L) were prepared, and the pH was adjusted to 3.8 with ammonia. Then, the target analytes were chromatographed under the conditions reported in 2.4.1, and MS spectra were acquired in both PI and NI modes. Unresolved compounds, such as 3-ADON and 15-ADON, were injected separately. The most intense signal, with wide linear range, was obtained with 10 mmol/L formic acid for those compounds giving rise to adducts with the organic anion and NH_4^+, whereas the other compounds did not show any effect of the anions. The signal intensities decreased at higher electrolyte concentrations in an uneven manner and, as expected, decreases were larger for lower-response, more polar compounds (data not shown) [Cech 2001].

It should be emphasized that such conditions represent a compromise for the multiresidue analysis, and in particular the type-B trichothecene and macrocyclic lactone responses are much lower than in the optimum conditions. This can be deduced particularly from the limit of detection values obtained for NIV, DON, ADONs and ZON (see tables 3.3, 3.7, 3.9).

With the aim of optimizing the chromatographic separation, the effect of the organic modifier on the mass spectrometer response was investigated. Three different modifiers, such as methanol, acetonitrile and methanol/acetonitrile (50:50 v/v), were tested, with the other chromatographic conditions kept constant. The results are reported in figure 3.2.

Figure 3.2. Organic modifier effect on the ESI efficiency reported as signal intensity versus % of methanol in acetonitrile, each containing formic acid 10 mmol/L (adjusted to pH 3.8 with NH_3). For more details, see text.



The behavior of some compounds was somewhat unexpected and difficult to explain, especially the differences among trichothecenes B and the effect of mixing the two organic solvents. In practice, these results suggest that the use of solvents other than methanol in this separation before mass spectrometry is not advisable.

3.3.3. Optimization of LC separation

As reported in table 2.1, the selected precursor ions required the ESI interface to operate in four periods of polarity switching. The mass spectrometer used in this work is able to record analyte responses in both polarities during a single chromatographic run; however, the polarity switching time is 700 ms/cycle, resulting in a decrease in sensitivity. Therefore, parameters affecting the chromatographic separation were

carefully evaluated with the aim of separating the target analytes into retention time windows operating in one single polarity. Different kinds of reversed phase-columns, such as low and high coverage C_{18}, high-purity silica based C_{18}, and polar reversed phase, were tested. Among the columns tested, the best compromise in separation was obtained with the Alltima C_{18}. Moreover, the gradient was adjusted to improve the separation of the analytes eluted near the time of polarity switching. The chromatographic column was also kept in an oven at 45°C for two reasons, improving the separation and increasing the reproducibility of the retention times.

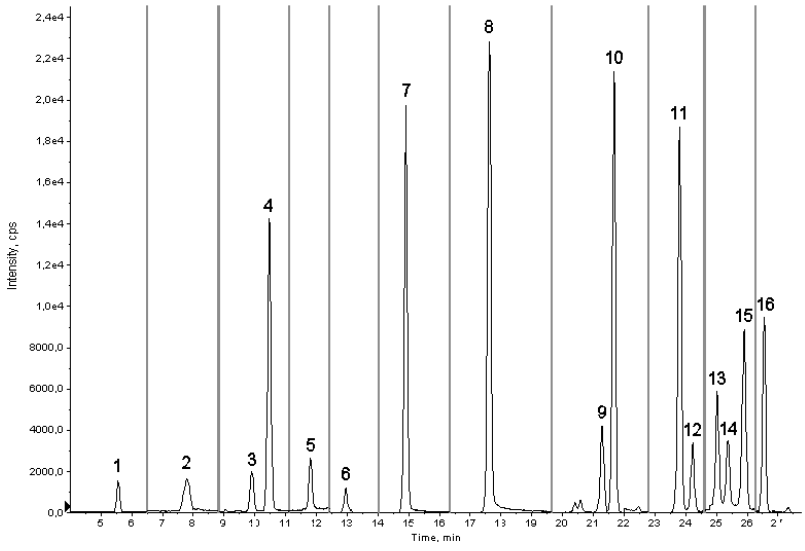
These conditions allowed the separation of all target compounds, with the exception of the 3-ADON/15-ADON isomers and the β-ZOL/FB_3 pair. Recently, Razzazi-Fazeli et al. obtained an adequate separation of the above-mentioned isomers by employing a Polar-RP column [Razzazi-Fazeli 2003]. We preferred to forego this separation, since it could be obtained only by mixing methanol and acetonitrile; moreover, *Fusarium spp*. strains rarely produce mixtures of 3- and 15-ADON [Berger 1999]. In addition, as already reported in 3.3.2, as these compounds fragment in different ways under ESI-MS/MS, a quantitative analysis of these compounds should be possible in real samples. Although α- and β-zearalenol are both derivatives of ZON which sometimes may be found in maize [Cavaliere 2005a], only α-ZOL was included in this method since, under the present conditions, β-ZOL is eluted with retention time of 24.1 min in complete overlay with the FB_3 positive ionization period; we decided that the detection of the second compound was preferred. Actually, α-ZOL has a 2-4 times higher estrogenic effect than ZON, while β-ZOL is less estrogenically active [Creppy 2002, D'Mello 1999]. Moreover,

according to previous work [Cavaliere 2005a], natural levels of β-ZOL are very low while, in contrast, FB_3 together with FB_1 and FB_2 has been often found in corn meal samples [Faberi 2005] and its determination is recommended by the Food and Drug Administration [FDA 2001].

Figure 3.3 depicts a chromatogram showing the separation and the response of the selected analytes, and table 3.2 reports the retention times with the %RSD obtained from 18 quality control samples analyzed during a working week.

The reproducibility of retention times was very good, and allowed us to handle a large number of samples without problems of separation instability. In the method optimization for fumonisin confirmation, we found slow increases in retention times and in peak tailing due to column deterioration under acidic mobile phase conditions. Considering the chelating ability of fumonisins, this effect may be due to traces of metal ions, likely Ca^{2+} and Mg^{2+}, bonded to silanol groups that remain free. Evidently, at pH 3.8 column ageing is slower than at more acidic pH values. Moreover, the NH_4^+ ions in the mobile phase at concentrations near 5×10^{-4}mol/L may compete effectively with divalent ions for silanol binding.

Figure 3.3. LC-(ESI)MS/MS chromatogram obtained on analyzing an analyte-free corn meal spiked with each fusariotoxin at 250 ng/g level. 1, nivalenol; 2, deoxynivalenol; 3, fusarenon X; 4, neosolaniol; 5, verrucarol (IS); 6, 3- and 15-acetyldeoxynivalenol; 7, monoacetoxyscirpenol; 8, diacetoxyscirpenol; 9, HT-2 toxin; 10, fumonisin B1; 11, T-2 toxin; 12, fumonisin B3; 13, α-zearalenol; 14, zearalanone (IS); 15, zearalenone; 16, fumonisin B2.



3.3.4. Accuracy, precision and method detection limits (MDLs)

Matrix-matched calibration was performed to take into account matrix effects on ionization efficiency. Calibration curves were obtained, as reported in 2.7.1, for a set of six different analyte-free samples spiked after clean-up. The ESI-MS/MS responses were linearly related to injected amounts up to 80 ng, and all calibration curves showed good linearity with coefficients of determination R^2 not lower than 0.988. Furthermore, the between-sample standard deviations of slopes and intercepts do not differ significantly ($p = 0.05$) from the within-sample values for all the compounds except NIV, that showed a tendency to have a specific matrix-dependence effect (between sample CV 12% and within sample CV 3.5% for the slope). Therefore, the matrix-matched calibration can be used

satisfactorily for quantitation purposes, considering this as a relatively rapid screening method.

Table 3.2. Retention time mean values (t_R), standard deviations s, and % relative standard deviations (%RSD) obtained in the liquid chromatographic conditions optimized for the fusariotoxin simultaneous analysis at T_{column} = 45°C (n = 18). For more details of the experimental conditions, see the text.

Analytes[a]	**t_R ± s (min)**	**RSD (%)**
NIV	5.54 ± 0.03	0.5
DON	7.85 ± 0.03	0.4
FUS X	9.94 ± 0.03	0.3
NEO	10.50 ± 0.03	0.3
VER	11.85 ± 0.03	0.2
3-ADON 15-ADON	12.98 ± 0.02	0.2
MAS	14.92 ± 0.02	0.1
DAS	17.67 ± 0.02	0.1
HT-2	21.31 ± 0.02	0.09
FB_1	21.71 ± 0.02	0.09
T-2	23.82 ± 0.02	0.08
FB_3	24.23 ± 0.02	0.08
α-ZOL	25.06 ± 0.01	0.04
ZAN	25.40 ± 0.02	0.08
ZON	25.92 ± 0.01	0.04
FB_2	26.58 ± 0.02	0.07

[a]NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; NEO, neosolaniol; VER, verrucarol; 3- and 15-ADON, 3- and 15-acetyldeoxynivalenol; MAS, monoacetoxyscirpenol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; FB_1, fumonisin B_1; T-2, T-2 toxin; FB_3, fumonisin B_3; α-ZOL, α-zearalenol; ZAN, zearalanone; ZON, zearalenone; FB_2, fumonisin B_2.

Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free corn meal samples at two concentration levels (100 and 1000 ng/g), the spiked samples were worked up as described in 2.3.1 and 2.7.1, and analyzed by LC-MS/MS. Method

detection limits (MDLs) were estimated with a sample spiked at the 25 ng/g level (100 ng/g for NIV, DON and 15-ADON). The ratio between the peak intensity under MRM LC-MS conditions and the background noise was calculated (S/N = 3). Results are reported in table 3.3.

Table 3.3. Recoveries, relative standard deviations (RSDs) and method detection limits (MDLs) for spiked blank corn meal samples.

Analytes[f]	**Recovery**[a]**±RSD**[b] **(%)**	**Recovery**[c]**±RSD**[b] **(%)**	**MDL**[d] **(ng/g)**
NIV	83±10	84±7	27[e]
DON	85±9	87±6	40[e]
FUS X	88±6	90±5	13
3-ADON	99±8	104±6	7
15-ADON	95±11	98±8	30[e]
NEO	90±8	93±6	9
MAS	89±5	92±4	3
DAS	97±7	98±5	5
HT-2	91±7	94±4	12
T-2	92±8	97±4	14
α-ZOL	93±9	96±6	2
ZON	91±10	93±5	10
FB_1	82±7	85±5	5
FB_2	81±6	83±5	3

[a]Spiking level was 100 ng/g
[b]RSD was calculated for 6 samples
[c]Spiking level was 1000 ng/g
[d]Spiking level was 25 ng/g
[e]Spiking level was 100 ng/g
[f]NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; 3- and 15-ADON, 3- and 15-acetyldeoxynivalenol; NEO, neosolaniol; MAS, monoacetoxyscirpenol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; T-2, T-2 toxin; α-ZOL, α-zearalenol; ZON, Zearalenone; FB_1, fumonisin B_1; FB_2, fumonisin B_2.

The recovery of the method was above 80% for all compounds and the precision ranged from 4-11%. This method unfortunately lacks an internal standard for each and every type of precursor ion [Royer 2004]; in spite of that, precision is quite good, and therefore correction factors for recovery can be used for samples analyzed in duplicate.

When compared with results obtained from confirmatory methods, in which the compounds were determined using the optimized conditions for each class of compounds, the present MDLs for trichothecenes B (except FUS X) were dramatically increased; also, MDLs higher by factors of 2.5-3 were obtained in the present work for macrocyclic lactones (see table 3.9). Considering the levels of these compounds commonly found (table 4.1) and the corresponding regulatory requirements [EC 2005b] these higher MDLs do not represent a drawback. Moreover, a confirmatory analysis can be performed using an aliquot of the same extract with the same chromatographic column, simply by changing the clean-up procedure and the mobile phase and, for trichothecenes B, mass spectrometric conditions, as reported in 2.4.3. The same considerations apply to fumonisins, for which MDLs were 2-3 times higher than those achieved under the best conditions (see table 3.10). In contrast, trichothecenes A were detected here with MDLs that are better than those reported in the literature for LC-MS [Berger 1999, Dall'Asta 2004a, Razzazi-Fazeli 2003] and quite comparable with those obtained by Biselli *et al.* for LC-MS/MS [Biselli 2004].

3.3.5. Effect of subsampling

To ensure sample representativity, ten or more grams per sample are usually extracted [Razzazi-Fazeli 2002, Dall'Asta 2004], and part of the extract is submitted to the subsequent analytical steps. Such amount is hardly compatible with the extraction procedure proposed, which makes use of a high solvent/sample ratio and a statical-dynamical extraction mode. Our rationale was that if a sample is homogeneous enough, a subsample as small as one gram may be representative. Thus, six 1 g subsamples of a

naturally contaminated corn meal were independently processed and analyzed for 5 fusariotoxins (DAS, ZON, FB_1, FB_2 and FB_3) able to cover a wide concentration range. The between-subsample precision resulted in the range 6-12%, and was then not significantly different from the between-day precision (p=0.05) of the method for the same analytes (see table 3.3).

3.3.6. Corn meal samples

The applicability of the new method was tested by analyzing five corn meals (popular brands) collected from stores located in Rome. Analyses were performed in duplicate, and results are reported in table 3.4.

Table 3.4. Concentration levels of trichothecenes B, fumonisins and macrocyclic lactones found in 5 naturally contaminated corn meal samples.

	Fusariotoxin concentration (μg/kg)				
Analytes[b]	**1**	**2**	**3**	**4**	**5**
NIV	–[a]	–	–	–	–
DON	–	–	1210	754	343
FUS X	–	–	82	71	–
3-ADON	–	–	75	–	43
15-ADON	–	–	–	–	–
α-ZOL	–	–	–	24	–
ZON	–	–	52	83	94
FB_1	2060	1850	2100	1920	1250
FB_2	196	221	208	185	138
FB_3	114	131	129	111	190

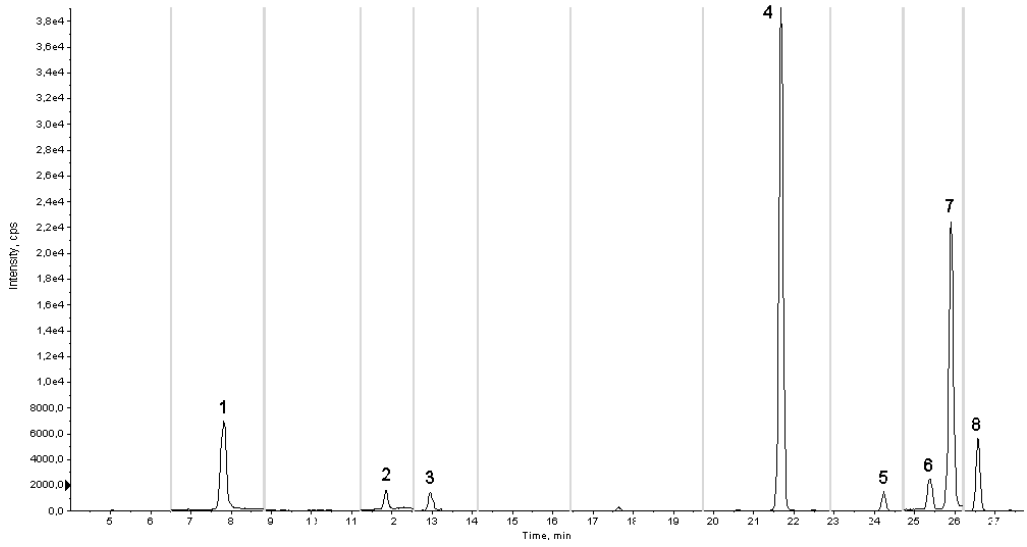
[a] –: < method detection limit.

[b]NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; 3- and 15-ADON, 3- and 15-acetyldeoxynivalenol; FB_1, fumonisin B_1; FB_3, fumonisin B_3; FB_2, fumonisin B_2; α-ZOL, α-zearalenol; ZON, zearalenone.

All samples turned out to be contaminated, and toxins belonging to three different classes were found in 3 samples out of 5. Type-A trichothecenes

levels were below the MDL values in all cases and are not included in table 3.4. As an example, figure 3.4. reports the chromatogram of a naturally contaminated corn meal sample.

Figure 3.4. LC-(ESI)MS/MS chromatogram obtained on analyzing a naturally contaminated corn meal sample. 1, deoxynivalenol; 2, verrucarol (IS); 3, 3-acetyldeoxynivalenol; 4, fumonisin B1; 5, fumonisin B3; 6, zearalanone (IS); 7, zearalenone; 8, fumonisin B2.



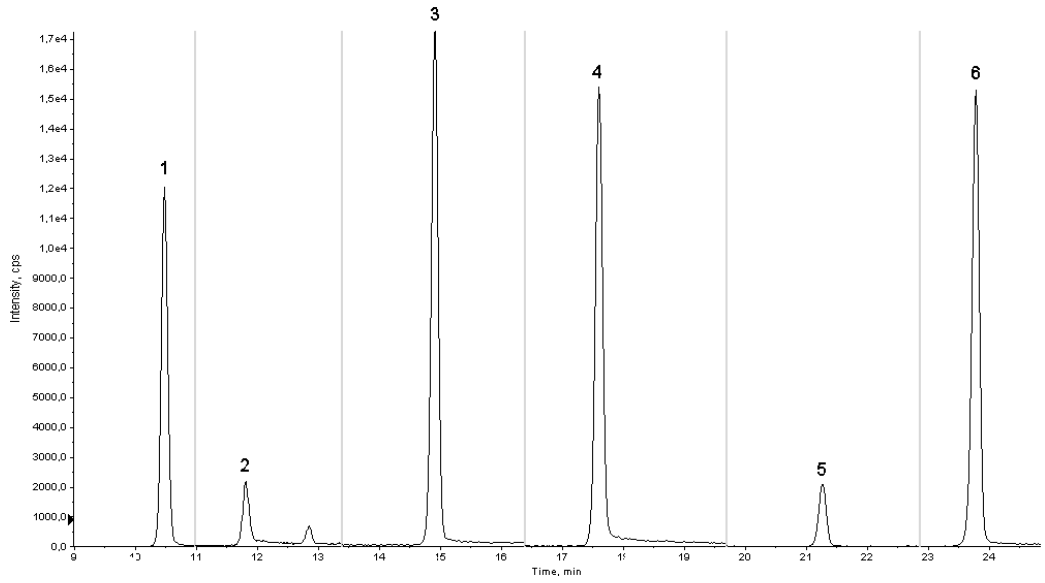
3.4. LC-MS/MS confirmatory method for trichothecenes A

3.4.1. Accuracy, precision and method detection limits (MDLs)

Calibration curves were obtained, as reported in 2.7.2, for a set of six analyte-free samples spiked after clean-up. Samples were fortified to have a concentration range of 0.004-0.4 ng/μL in 250 μL of final extract. Figure 3.5. shows a typical chromatogram obtained from a corn meal sample spiked at 250 ng/g. The procedure was repeated three times. All calibration curves show good linearity with determination coefficients R^2 not lower than 0.993 for all trichothecenes A. Furthermore, slope and

intercept do not differ significantly (p = 0.05) from calibration in standard solution (external calibration procedure) for all target compounds. Therefore, the method appears to suffer slightly from ion suppression phenomena due to coeluting matrix compounds, reflecting the efficiency of the selective clean-up procedure, whereas all other conditions remained unchanged. In the chromatographic traces, this can be also observed from S/N values, which are increased 1.5-3 times with respect to those obtained for the fusariotoxin method (data not shown).

Figure 3.5. LC-(ESI)MS/MS chromatogram obtained on analyzing an analyte-free corn meal spiked with each trichothecenes A at 250 ng/g level. 1, neosolaniol; 2, verrucarol (IS); 3, monoacetoxyscirpenol; 4, diacetoxyscirpenol; 5, HT-2 toxin; 6, T-2 toxin.



Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free maize samples at three levels (25, 250 and 500 ng/g) and the spiked samples were worked up and analyzed by LC-MS/MS as described in the experimental section. Recoveries obtained for the three spiking levels do not differ significantly and were above 84% (RSD% ≤ 8%) for all target compounds. MDLs, calculated using

a signal-to-noise ratio of 3 (the ratio between the peak intensity under MRM LC-MS conditions and the background noise) were estimated with a sample spiked at level of 25 ng/g. They were in the range 3-9 ng/g. Results are reported in table 3.5. When necessary, both accuracy and precision may be improved by internal calibration [Bogialli 2003a, 2003b].

Table 3.5. Recoveries, relative standard deviations (RDSs) and method detection limits (MDLs) for trichothecenes A obtained from artificially contaminated corn meal samples.

Analytes[a]	**Recovery**[b]**±RSD**[c] **(%)**	**MDL**[d] **(ng/g)**
NEO	93±8	3
MAS	89±5	4
DAS	91±6	3
HT-2	86±5	9
T-2	85±3	4

[a]NEO, neosolaniol; MAS, monoacetoxyscirpenol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; T-2, T-2 toxin.
[b]Spiking level was 250 ng/g.
[c]RSD was calculated for 6 samples.
[d]Spiking level was 25 ng/g

3.5. LC-MS/MS confirmatory method for trichothecenes B

3.5.1. Liquid chromatography/mass spectrometry

In literature there are different approaches to detecting trichothecenes B by LC-MS/MS [Plattner 2003, Razzazi-Fazeli 2003]. We evaluated two different interfacing systems for our analytes, ESI and APCI. These two interfaces complement one another well with respect to polarity and molecular mass of analytes, and polarity of LC eluent [Zhou 1996]. Initial

experiments were performed in both PI and NI modes using both interfaces; results of this preliminary study are presented in table 3.6.

Table 3.6. Effect of carrier modifiers and LC-MS interfaces on the intensities of $[M-H]^-$ ion.

	% Relative Intensity											
	ESI						**APCI**					
	w/a/m[a]		w/a/m 10 mM ammonium acetate		w/a/m 10 mM formic acid		w/a/m		w/a/m 10 mM ammonium acetate		w/a/m 10 mM formic acid	
	PI[b]	NI[c]	PI	NI	PI	NI	PI	NI	PI	NI	PI	NI
NIV	100	800	247	98	101	152	–	5	–	–	–	14
DON	100	638	57	123	116	2	–	5	–	5	–	15
FUS X	100	217	829	203	291	–[d]	–	55	–	18	–	30
ADON	100	207	11	145	214	11	32	10	2	1	–	26

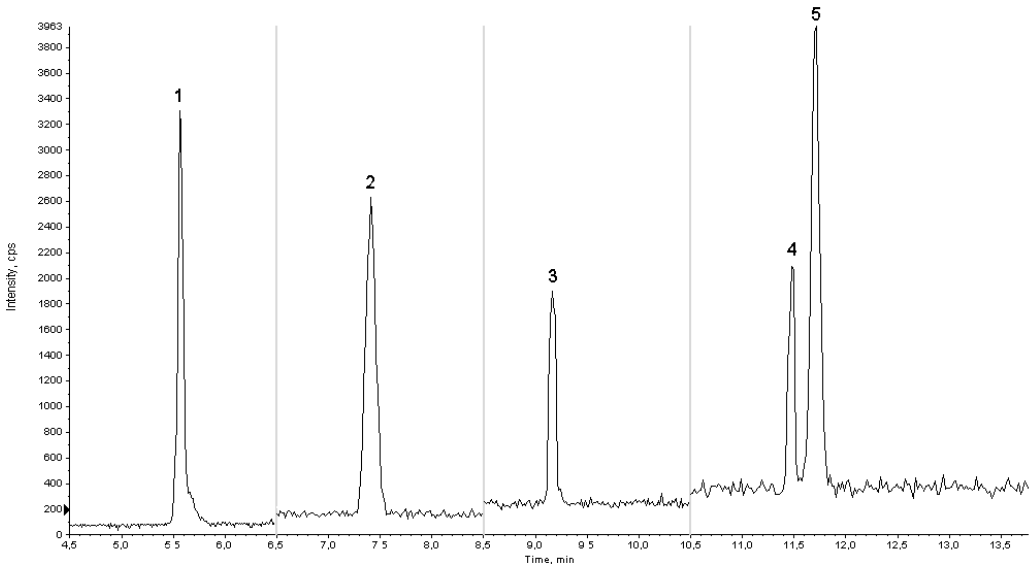
[a]w/a/m = water/acetonitrile/methanol
[b]PI = Positive ionization.
[cb]NI = Negative ionization.
[d]–: non detectable.
NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; ADON, 3- and 15-acetyldeoxynivalenol.

The most valuable spectral data for each trichothecene were obtained using the ESI system in NI mode, with a neutral mobile phase composed of a mixture of water/acetonitrile/methanol. The $[M–H]^-$ ions of all trichothecenes were found to be more abundant than the corresponding $[M+H]^+$ ions; the presence of hydroxylic groups favors deprotonation of the molecules in the gas phase. Furthermore, an excessive degree of fragmentation of the $[M+H]^+$ ions was observed, but this was not true of the $[M–H]^-$ ions. As shown in table 3.6, the APCI responses are lower than those obtained with ESI. In NI mode the type-B trichothecenes have very specific fragmentation patterns. The $[M–H]^-$ ion was the base peak in the ESI mass spectra for all the tested compounds. Other significant ions corresponded to additions of two water molecules, $[M–H+2H_2O]^-$, as also

reported elsewhere [Razzazi-Fazeli 1999]. In the MS/MS spectra of the $[M–H]^-$ ions of all the molecules, the most characteristic ion is due to the loss of 30 Da; this is assigned to the cleavage of the epoxy group and expulsion of ketene to form fragment ions $[M–H–CH_2O]^-$. This is the main fragment for NIV, but is less abundant for FUS X; other fragment ions were observed, but have not been interpreted as yet. The quantitation was performed using these $[M–H–CH_2O]^-$ fragment ions. For a selective determination of DON, FUS X and ADONs we used two characteristic transitions, and for NIV one transition did at least allow its positive identification in the matrix. In order to compare the efficiencies of the two interfaces in the analytical identification we evaluated the effect of a postcolumn mobile phase modifier, such as ammonium acetate or formic acid, to improve the ionization efficiency and therefore the ion abundance. The evaluation was performed by post-column addition of formic acid (10mM) or ammonium acetate (10mM) to the mobile phase (see table 3.6). For almost all the tested compounds acidic additions led to significant enhancement of the $[M+H]^+$ ions, while, in NI mode, it strongly reduced the signal intensity. The addition of ammonium acetate, on the other hand, decreases the $[M+H]^+$ ion signal and in the NI mode it had only a small effect on $[M–H]^-$ signals. Therefore, modifier addition was not used since neutral conditions gave the highest sensitivity and excellent reproducibility.

Thus, fraction A (containing trichothecenes A and B) obtained from selective clean-up and treated as described in 2.3.3 was analyzed again, under neutral mobile phase and negative ESI-MS conditions for trichothecenes B. Figure 3.6 illustrates the LC-MS/MS chromatogram of a spiked sample obtained operating the MRM mode.

Figure 3.6. LC-(ESI)MS/MS chromatogram of a spiked (100 ng/g) corn meal sample for the five trichothecenes B acquired in MRM mode. 1, nivalenol; 2, deoxynivalenol; 3, fusarenon X; 4, 15-acetyldeoxynivalenol; 5, 3-acetyl-deoxynivalenol.



Plattner used verrucarol (VER), a trichothecene A obtained by hydrolysis of macrocyclic precursors, as internal standard [Plattner 2003], but in PI mode. In this work quantitation was performed using external calibration, as done by other authors [Huopalahti 1997, Langseth 1998, Mateo 2002].

3.5.2. Accuracy, precision and method detection limits (MDLs)

Calibration curves were obtained, as reported in 2.7.2, for a set of six analyte-free samples spiked after clean-up. Samples were fortified to have a concentration range of 0.004-4 ng/μL in 250 μL of final extract. The procedure was repeated three times. All calibration curves show good linearity and coefficients of determination R^2 were above 0.994. Furthermore, slope and intercept do not differ significantly ($p = 0.05$) from calibration in standard solution (external calibration procedure) for all the compounds except NIV (-8%). Therefore, the method appears to suffer

slightly from ion suppression phenomena by virtue of sample clean-up efficiency and good chromatography.

Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free maize samples at three levels (25, 100 and 3000 ng/g) and the spiked samples were worked up and analyzed by LC-MS/MS as described in 2.3.2, 2.3.3 and 2.4.3. Recoveries obtained for the three spiking levels do not differ significantly and were above 88% (RSD% ≤ 10%) for all target compounds, except for NIV (recovery = 79%). MDLs (S/N = 3) were estimated with a sample spiked at level of 25 ng/g. They were in the range 2-8 ng/g, except FUS X value (12 ng/g). Results are reported in table 3.7. When necessary, both accuracy and precision may be improved by internal calibration [Bogialli 2003a, 2003b].

Table 3.7. Recoveries, relative standard deviations (RDSs) and method detection limits (MDLs) obtained from artificially contaminated maize samples.

Analytes[a]	**Recovery**[b]**±RSD**[c] **(%)**	**MDL**[d] **(ng/g)**
NIV	79±8	6
DON	89±10	2
FUS-X	95±6	12
3-ADON	97±3	3
15-ADON	96±3	8

[a] NIV = nivalenol, DON = deoxynivalenol, FUS X = fusarenon X, 3-ADON = 3-acetyldeoxynivalenol ; 15-ADON = 15-acetyldeoxynivalenol.
[b] Spiking level was 100 ng/g.
[c]RSD was calculated for 6 samples.
[d] Spiking level was 25 ng/g.

3.5.3. Analysis of naturally contaminated corn meal samples

The applicability of the developed technique was investigated by analyzing several naturally contaminated samples. A set of maize samples was collected from farms located in different Italian areas, immediately after the harvest. Results of this test are shown in table 3.8.

Table 3.8. Analytical data[a] corresponding to the analysis of trichothecenes B in maize samples collected from different Italian farms.

	NIV			FUS X		
Sample origin	**Positive/ total**	**Mean of positive**	**Range**	**Positive/ total**	**Mean of positive**	**Range**
Alessandria	2/10	27.5	10-45	0/10	—[b]	—
Bergamo	3/15	77.7	20-196	2/15	43.5	22-65
Brescia	0/5	—	—	0/5	—	—
Milano	5/12	66.6	7-171	4/12	91.5	32-210
Novara	0/15	—	—	0/15	—	—
Macerata	1/12	40.0	40	1/12	26.0	26
Roma	1/14	12.2	12	1/14	11.8	12
Terni	2/10	134.5	29-240	1/10	419.0	419
	DON			**3-ADON**		
Sample origin	**Positive/ total**	**Mean of positive**	**Range**	**Positive/ total**	**Mean of positive**	**Range**
Alessandria	3/10	206.7	50-310	2/10	32.0	15-49
Bergamo	3/15	262.0	36-530	3/15	123.0	23-224
Brescia	1/5	62.0	62	1/5	33.0	33
Milano	5/12	443.2	4-773	3/12	51.0	3-103
Novara	4/15	313.0	30-672	4/15	204.0	19-514
Macerata	2/12	170.5	132-209	2/12	28.5	8-49
Roma	7/14	130.4	24-871	4/14	7.6	2-19
Terni	0/10	—	—	1/10	22.0	22

[a]Concentrations in ng/g.
[b]—: < MDL
NIV = nivalenol, DON = deoxynivalenol, FUS X = fusarenon X,
3-ADON = 3-acetyldeoxynivalenol.

Less than 30% of the total samples were positive, with average contents ranging between a few ng/g and about 900 ng/g. It was observed that DON was the predominant toxin, its concentration in the range 4–871 ng/g. The frequencies of contamination by 3-ADON, FUS X and NIV are

less than that of DON. Moreover it should be noted that, in samples contaminated with DON, the other three toxins were commonly found. These analytical data are in good agreement with those found in other countries [Müller 1997, Quiroga 1995, Tanaka 1988]. Besides DON, appreciable amounts of the monoacetates were detected in highly contaminated samples. Finally, 15-ADON was never detected.

3.6. LC-MS/MS confirmatory method for zeranols

3.6.1. Liquid chromatography/mass spectrometry

ZON and the other macrocyclic lactones were eluted along an isocratic run, with the aim of separating the pairs of isomers α-ZOL from β-ZOL and α-ZAL from β-ZAL. Whereas ESI gave similar or slightly less sensitivity for zeranols than APCI, ESI interface was selected also for this analysis [Huopalahti 1997, Laganà 2001, Plattner 2003]. An additional reason for the choice of the ESI interface was that it was found to be much more rugged, maintaining sensitivity after many injections of dirty samples and requiring significantly less cleaning and maintenance than the APCI interface.

3.6.2. Accuracy, precision and method detection limits

Calibration curves were obtained, as reported in the 2.7.2, for a set of six analyte-free samples spiked after clean-up. Samples were fortified to have a concentration range of 0.004-4 ng/μL in 250 μL of final extract. The procedure was repeated three times. All calibration curves show good linearity with determination coefficients R^2 not lower than 0.986 for ZON

and its metabolites. Furthermore, slope and intercept do not differ significantly (p = 0.05) from calibration in standard solution (external calibration procedure) for all the compounds. Therefore, the method appears to suffer slightly from ion suppression phenomena due to coeluting matrix compounds, reflecting the efficiency of the sample clean-up procedure and the chromatographic separation.

Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free maize samples at three levels (25, 100 and 3000 ng/g) and the spiked samples were worked up and analyzed by LC-MS/MS as described in 2.31, 2.3.3 and 2.4.4. Recoveries obtained for the three spiking levels do not differ significantly and were above 90% (RSD% ≤ 12%) for all target compounds. MDLs, calculated using a signal-to-noise ratio of 3 were estimated with a sample spiked at level of 25 ng/g. They were in the range 3-5 ng/g. Results are reported in table 3.9. When necessary, both accuracy and precision may be improved by internal calibration [Bogialli 2003a, 2003b].

Table 3.9. Recoveries, relative standard deviations (RDSs) and method detection limits (MDLs) for zeranols obtained from artificially contaminated corn meal samples.

Analytes[a]	**Recovery**[b]**±RSD**[c] **(%)**	**MDL**[d] **(ng/g)**
β-ZAL	103±10	3
β-ZOL	89±5	3
α-ZAL	106±12	4
α-ZOL	100±10	5
ZON	95±7	3

[a] β-ZAL = β-zearalanol, β-ZOL = β-zearalenol, α-ZAL = α-zearalanol, α-ZOL = α-zearalenol, ZON = zearalenone.
[b] Spiking level was 100 ng/g.
[c] RSD was calculated for 6 samples.
[d] Spiking level was 25 ng/g.

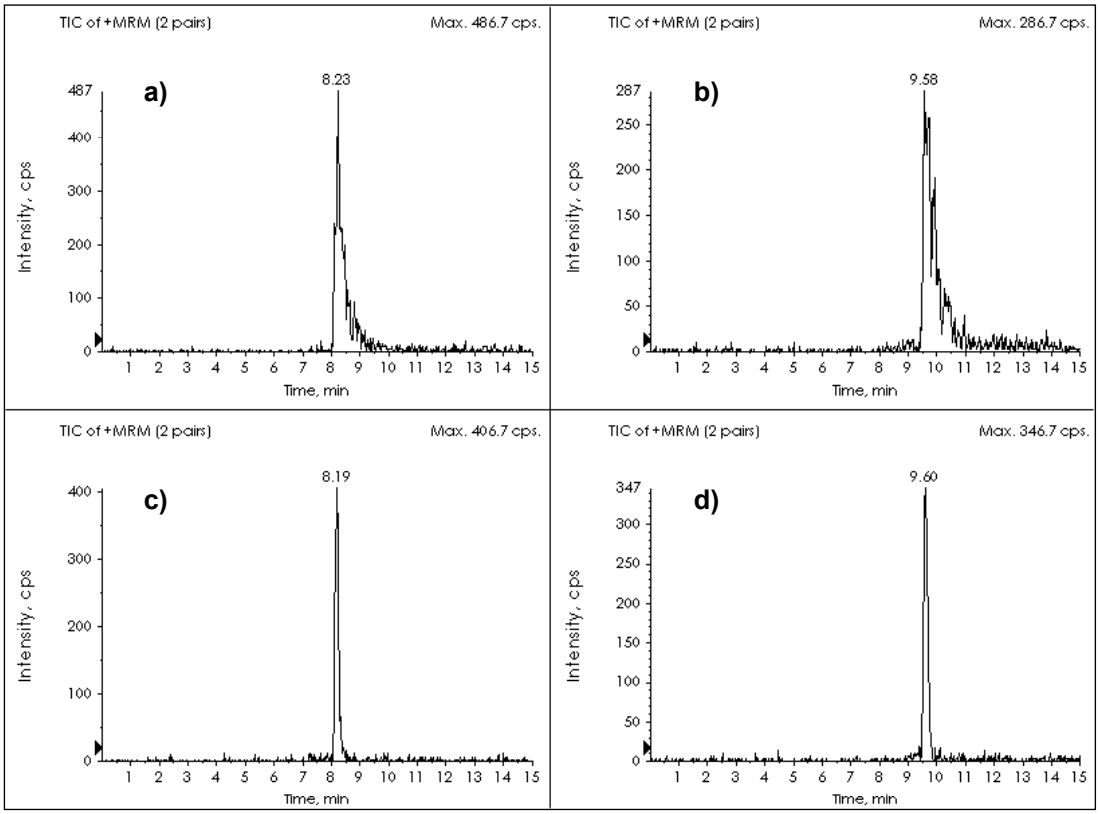
3.7. Fumonisins

3.7.1. Liquid chromatography/mass spectrometry

The elution of fumonisins from an LC column packed with reversed-phase silica-based materials as sharp and symmetrical peaks requires both an acidified mobile phase [Plattner 1999] and a pre-treatment of the LC column with EDTA. Probably, deprotonated fumonisins form complexes with traces of metal ions present on the packing material; these impurities could be responsible for the badly tailed peaks and poor accuracy for fumonisin quantitation at low concentrations. Increasing the acidity decreases the tailing, but at the expense of sensitivity. A very good quality column may display this behavior only after appreciable wear in acidic conditions, when the impurities became accessible. The EDTA treatment was found to be effective in re-establishing good elution profiles as shown in figure 3.7, even after 3 months of continuous use of a column. The composition of the organic modifier, and the nature and concentration of the acid added to the mobile phase, were optimized to provide the best compromise between chromatographic separation efficiency, peak shape, and mass spectrometric response (data not shown).

An improvement in peak shape due to the addition of ammonium ion to the mobile phase was already observed in the optimization of the screening method (see 3.3.3.).

Figure 3.7. Peak shape for fumonisin FB_1 and FB_2 before ((a) and (b)) and after ((c) and (d)) treatment of LC column with EDTA solution.



3.7.2. Accuracy, precision and method detection limits

Calibration curves were obtained as described in the 2.7.2 for a set of six analyte-free samples spiked after they had been subjected to the extraction and cleanup procedures. These matrix-matched calibration samples were fortified so as to have a concentration range of 0.005–5 ng/μL in 400 μL of final extract. The procedure was repeated three times. All calibration curves showed good linearity with correlation coefficients in the range 0.992–0.995. Furthermore, slopes and intercepts did not differ significantly ($p = 0.05$) from calibrations obtained using standard solutions in clean solvent. Therefore, the method does not appear to suffer from ESI suppression phenomena by virtue of the sample cleanup efficiency and

good chromatography. Furthermore, diclofenac can thus be added after cleanup and, although not very similar to fumonisins, is suitable as the IS in the subsequent analytical steps.

Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free maize based samples, such as corn meal, cornflakes and popcorn. As suggested by contamination values found in the literature and in the present work, corn meal was spiked with FB_1 and FB_2 at two levels, 'high' (5000 and 100 ng/g) and 'low' (1000 and 20 ng/g); cornflakes and popcorn were spiked only at low levels (100 ng/g FB_1 and 20 ng/g FB_2). MDLs were estimated by measuring S/N for samples spiked at levels of 20 and 10 ng/g for FB_1 and FB_2, respectively, and extrapolating to S/N = 3. The noise (N) used in the calculation is based on 3σ of the baseline signal and was directly provided by the instrument software. The spiked samples were worked up as described in 2.3.1 and 2.3.3, analyzed by LC-MS/MS (2.4.5), and results are reported in Table 3.10.

Analyte recoveries ranged between 91 and 105%, and were independent of both matrix and spiking level. The precision calculated is the reproducibility during a complete working week.

Table 3.10. Recoveries, relative standard deviations (RDSs) and method detection limits (MDLs) for fumonisins B_{1-2} obtained from artificially spiked maize-based samples.

Analytes	CORN MEAL			CORNFLAKES		POPCORN	
	Recovery[a] ±RSD[b] (%)	Recovery[c] ±RSD[b] (%)	MDL[d] (ng/g)	Recovery[c] ±RSD[b] (%)	MDL[d] (ng/g)	Recovery[c] ±RSD[b] (%)	MDL[d] (ng/g)
FB_1	98±4	101±6	2	105±3	1	93±6	1
FB_2	95±5	97±8	1	95±4	0.5	91±5	0.5

[a]Spiking levels were 5000 ng/g for FB_1 and 1000 ng/g for FB_2
[b]RSD was calculated for 6 samples
[c]Spiking levels were 100 ng/g for FB_1 and 20 ng/g for FB_2
[d]Spiking levels were 20 and 10 ng/g respectively for FB_1 and FB_2.

3.7.3. Identification of FB_3 and FB_4 in corn meal samples

Two corn meal samples contaminated with high levels of FB_1 and FB_2 were re-analyzed making use of the IDA protocol that looped three experiments, including MS and MS/MS full scans, for class identification of fumonisin compounds on the basis of the 370 Da characteristic neutral loss. If the unknown compound is a fumonisin the $[M+H]^+$ ion undergoes collision-induced dissociation resulting in consecutive loss of two TCA residues. Moreover, due to the presence of at least one hydroxyl group, MS/MS spectra of fumonisins include lower-mass fragment ions arising from loss of a water molecule [Josephs 1996, Lukacs 1996].

In the IDA protocol, the three experiments that were looped included MS and MS/MS full scans, thus providing the maximum amount of information from a single LC injection. Fumonisins are identified with the NL scan as a survey scan, using the mass spectrometer in the QqQ mode, and confirmed with an EPI scan as the dependent scan using the instrument in the hybrid QqLIT configuration. The survey and dependent scans were performed sequentially, and repeated for the entire duration of

LC analysis (each cycle was 1.6 ms). Although the NL scan range was restricted in order to increase the sensitivity since the fumonisin molecular weight range was known, this mode is still not as sensitive as ion trap mode. Thus its role is solely to identify the precursor ions for the ensuing EPI experiment. In addition, the large working step size (0.5 Th) used for the NL scan is responsible for the poor mass accuracy. One way to improve the accuracy in selecting precursor mass for very weak signals and at fast scanning rates is to include an ER scan between the NL scan and the EPI scan. In ER scan mode the mass assignment accuracy showed bias less than 0.1 Th, and peak resolving power ranged between 3000–3500 (FWHM). Finally, the EPI experiment was performed on candidate precursor ions using selection criteria including mass range, ion abundances, and mass tolerance. Using this 'on the fly' experiment we were able to confirm the presence of FB_3 and FB_4 at retention times of 8.9 and 10.2 min in all three experiments on the corn meal samples. Figure 2.1 illustrates the EPI spectra of FB_3 (top) and FB_4 (bottom). Under the conditions used we were unable to obtain EPI spectra of any other fumonisins belonging to other series. This may be due to very low concentrations of the other fumonisins produced by *Fusarium* species [Musser 1997] or to experimental conditions unsuitable for detecting those fumonisin types that might undergo other more intense constant neutral losses.

3.7.4. Comparison between MRM and EPI modes

Triple-quadrupole instruments are generally used in MRM mode for accurate, precise and sensitive quantitation and confirmation of mycotoxins at trace levels in food matrices. Low detection limits can be

achieved, but fragmentation pattern information is partially lost. It has been reported that, using the QqLIT mass spectrometer in EPI scan mode, full-scan spectra can be obtained for an extracted ion chromatogram (XIC) peak with a S/N about half that for the corresponding transition in MRM [Kim 2002, Xia 2003]. We substantially confirm this finding, since our S/N values for XIC range between ½ and ¼ the S/N values in MRM. The increased corresponding MDLs and lower limits of quantitation may not be a drawback in practice because of the high contamination levels allowed (see 1.3). Obviously, full-scan product ion spectra (EPI data) contain more structural information about the target compound. However, MRM acquisition mode is preferred for routine analyses. Unlike in a quadrupole, the LIT is able to trap and then accumulate ions by appropriate setting of the LIT fill time parameter, resulting in a requirement for relatively frequent cleaning of the LIT.

3.7.5. Quantitative evaluation of FB_3 and FB_4

Quantitation by LC-(ESI)MS/MS without access to a standard substance is a challenge. Comparison with structurally similar molecules is, at best, only approximate. MS/MS fragmentation, in particular, can be strongly influenced by relatively small structural differences; note that choice of the analogous transitions in MRM results in a response of FB_2 that is about twice that of FB_1. Responses of related compounds in LC-MS mode may be more closely related when some conditions are fulfilled, namely, no matrix effect, negligible fragmentation, absence of direct interferences, very similar desorption constants from charged droplets [Cech 2001]. Correction factors for differences in MRM responses, relative to those for MS without fragmentation, were calculated as reported in 2.7.3; these

values turned out to be: $C_f(FB_3) = 1.1$ and $C_f(FB_4) = 2.4$, and will be used for quantitative evaluation of commercial samples. As for the other conditions mentioned above, we can only suppose these to be approximately satisfied, and for this reason we decided to report FB_3 and FB_4 as mol % response with respect to FB_2.

3.7.6. Analysis of commercial samples

The applicability of the developed method was investigated by analyzing several maize-based products collected from stores located in Rome. Figure 3.8 shows a typical chromatogram for a corn meal sample, and results are reported in table 3.11.

The levels of FB_{1-4} found in the analyzed commercial corn meal samples exceed US FDA guidance levels [FDA 2001] for human foods and the EU regulation [EC 2005b] for unprocessed corn in 8 out of 15 cases. In the two samples of steam-treated corn meal (precooked corn meal), the total abundance of FB_{1-3} is below the average values found for unprocessed corn meal. Finally, levels for cornflakes and popcorn, that can be considered "infant food" in a broad sense, do comply with the EU regulation [EC 2005b] for infant food.

Figure 3.8. LC/ESI-MS/MS chromatogram in MRM mode resulting from analysis of an Italian corn meal sample. Extracted ion current profile of (a) FB_1 (1.1 μg/g), (b) FB_3 at 8.9 min (56% of FB_2) and FB_2 at 9.6 min (0.14 μg/g), (c) FB_4 (16% of FB_2) and (d) diclofenac as internal standard.

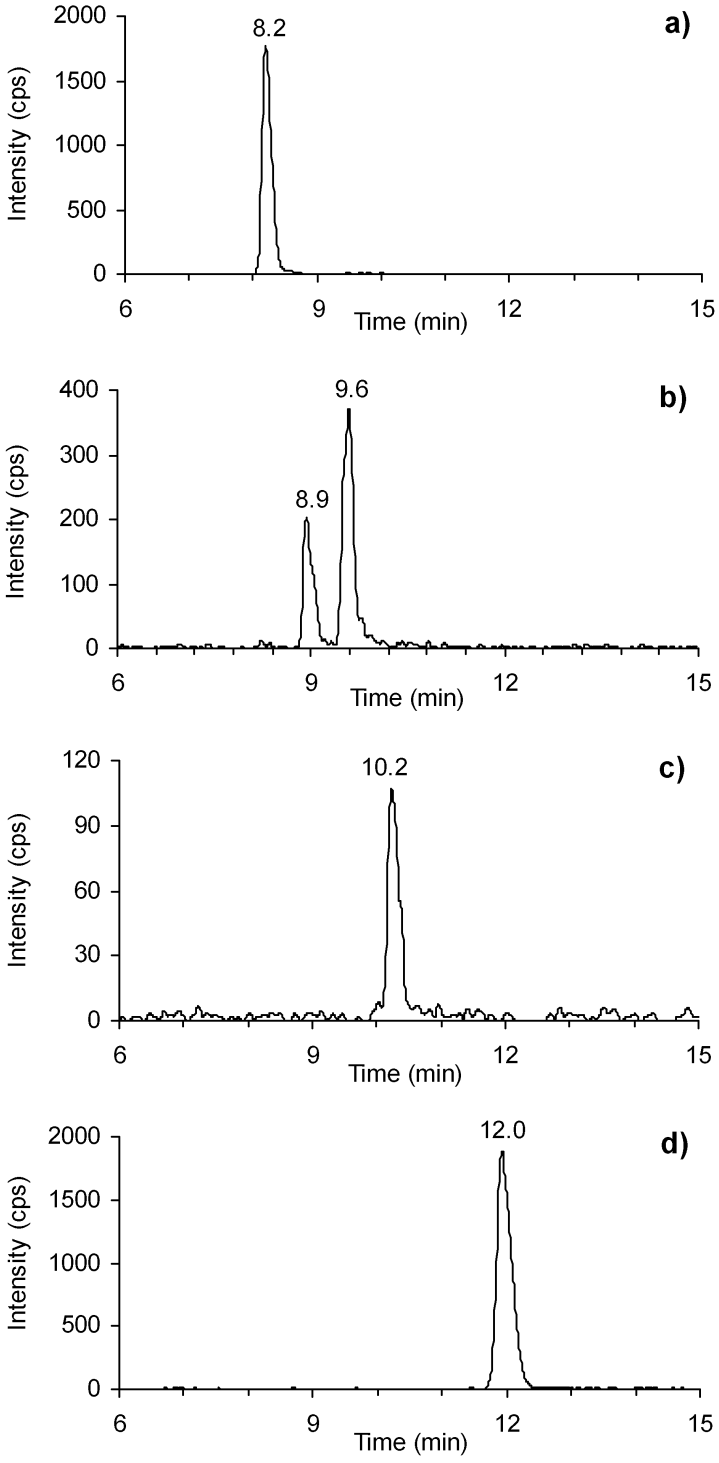


Table 3.11. Contamination levels (incidence, mean and range) of fumonisins FB_{1-4} in the corn-based products analyzed.

Sample	FB_1			FB_2		
	Incidence (pos/tot)	Mean (μg/g)	Range (μg/g)	Incidence (pos/tot)	Mean (μg/g)	Range (μg/g)
Corn meal	15/15	1.5	1.1-2.4	15/15	0.18	0.11-0.26
Corn meal steam-treated	2/2	0.89	0.85-0.92	2/2	0.073	0.067-0.078
Cornflakes	4/5	0.021	0.007-0.032	0/5	–[b]	n.d.[c]
Popcorn	3/4	0.064	0.042-0.079	1/4	0.013	0.013

Sample	FB_3			FB_4		
	Incidence (pos/tot)	Mean (mol % of FB_2)[a]	Range (mol % of FB_2)	Incidence (pos/tot)	Mean (mol % of FB_2)[a]	Range (mol % of FB_2)
Corn meal	15/15	59	52-63	15/15	17	13-23
Corn meal steam-treated	2/2	58	57-59	2/2	16	13-18
Cornflakes	0/5	–	n.d.	0/5	–	n.d.
Popcorn	1/4	35	43	0/4	–	n.d.

[a] Concentrations of FB_3 and FB_4 are reported as % mol calculated with respect to the FB_2 response.
[b] –: not calculated
[c] n.d.< MDL

3.8. Comparison between ELISA and LC-MS/MS methods

Table 3.12 shows data obtained from 25 randomly selected samples of freshly harvested maize and analyzed by both techniques, ELISA for DON and ZON, and LC-MS/MS, for trichothecenes B and zeranols.

Table 3.12. Comparison of data obtained analyzing maize samples with both ELISA and LC-MS/MS methods.

	Trichothecenes B (ng/g)		
	ELISA	*LC-MS/MS*	
Sample (#)	DON	DON	$\Sigma_i X_i$[a]
1	191	–	433
2	27	–	–
3	–[c]	–	29
4	61	–	660
5	–	–	23
6	29	–	–
7	–	–	7
8	154	20	48
9	>500	2060	5560
10	>500	1050	2020
11	42	–	2620
12	20	–	487
13	74	–	427
14	44	–	–
15-25	–	–	–
	Zeranols (ng/g)		
	ELISA	*LC-MS/MS*	
Sample (#)	ZON	ZON	$\Sigma_i Y_i$ [b]
1	–	8	8
2	54	46	46
3	97	86	101
4	385	384	395
5	–	10	18
6	251	223	238
7	379	414	429
8	59	68	68
9	184	161	174
10	–	6	6
11-25	–	–	–

[a] $\Sigma_i X_i$: sum of deoxynivalenol (DON), nivalenol, fusarenon X, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol.

[b] $\Sigma_i Y_i$: sum of zearalenone (ZON), α and β- zearalenol, α and β-zearalanol.

[c] – < MDL.

Concerning ZON, only 7 samples were found contaminated at level above MDL value of the ELISA-based method (18.5 ng/g). Regression analysis (x = LC-MS/MS) gave y = 0.93x+17 and R^2 = 0.982. Cross-reaction event with α-ZOL, β-ZOL and ZAN has been reported for this commercial ELISA test kit [Krska 2001a]. We did not find any overestimation that could be due to cross-reactivity of ELISA antibody with metabolites of ZON, probably because they were present at very low concentration in all samples (no more than 15 ng/g as total).

Conversely, in the case of DON, the LC-MS/MS method evidenced 8 cases of false positives and 1 case of overestimation (table 3.12). This fact can be ascribed partly to cross-reaction phenomena of the antibody towards other trichothecenes B. Cross-reactivity rates reported in the ELISA kit instructions are: NIV 4%, 3-ADON > 100%, 15-ADON 19% and FUS X < 1%. Indeed the same maize samples, analyzed by LC-MS/MS, were proved to be randomly contaminated by NIV and ADONs. An other drawback of ELISA method is the restricted linear dynamic range that is 17-500 ng/g. DON concentration values in the samples 9-10 exceeded the upper limit of these range, but it has not been possible to reanalyze both samples. By LC-MS/MS the linear dynamic ranges are from MDLs until at least 5,000 ng/g, keeping in mind that 1/5 of the 1 g extract is cleaned-up and then 1/5 of the purified extract is analyzed.

Therefore results obtained from ELISA and LC-MS/MS for ZON were in good agreement. Moreover, simultaneous presence of ZON and its metabolites in significant amounts was never found even by other authors [Müller 1997, Schollenberger 2002], so that it is unlikely that cross-reaction may occur. On the contrary, for DON the LC-MS/MS should be used to confirm positive samples detected by the ELISA-based method. In

addition, by LC-MS/MS NIV, DON, ADONs and FUS X can be unequivocally identified from the mass spectra and quantified simultaneously. This is important if the different toxicity of trichothecenes is taken into account.

3.9. Comparison between fusariotoxin screening method and confirmatory methods for fusariotoxin classes

The analytical methods that will be compared are based on the strategy of screening and confirmation. Screening methods are methods that are used to detect the presence of an analyte or class of analytes at the level of interest. Confirmatory methods are methods that provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest [EC 2002a]. When necessary, for a more stringent confirmatory purpose, in which the identity of the molecule might have to be confirmed with greater confidence, it would be preferable to have the entire fragmentation spectrum of a molecule, e.g. by using Q TRAP mass spectrometer in quadrupole-linear ion trap configuration (for details see ochratoxin A application in 7.2).

A set of 24 corn meal samples, kindly provided by ISC (Bergamo, Italy) were analyzed both with the screening LC-MS/MS method and with four confirmatory methods for fusariotoxins classes, after reprocessing 5 mL aliquot of the same extract, as described in chapter 2 and discussed in chapter 3. MDL values reported for screening method were calculated on the basis of S/N = 3 for the sum of transitions monitored; conversely, those for confirmatory methods were obtained on the basis of S/N = 3 for the less intense ion transition. The identity of NIV could not be confirmed by the

EU criteria [EC 2002a] because the MS/MS spectrum contained one diagnostic ion only (*m*/*z* 311 → 281). For these reasons the one positive result for NIV was excluded.

During screening 156 positive results were obtained (42 for trichothecenes A, 26 for trichothecenes B, 16 for zeranols and 72 for fumonisins), i.e. 156 times a fusariotoxin of interest was detected with S/N ≥ 3 for the summed fragment ion current values. Confirmatory analysis was found to be successful in 130 cases (72 of which regarded fumonisins) or about 83% of all cases.

As for the identification, not unexpectedly, concentration played an important role, since most of discrepancies between screening and confirmation results concerned fusariotoxins at concentration levels below 25 ng/g, i.e. the range of MDL values (most of them included trichothecenes A). For this reason the 6 false negatives resulted from the screening method can be explained. In addition, during screening 40 cases were doubt, for which the S/N values of the less intense transition signal were < 3. In 28 of these 40 cases, fusariotoxins were then confirmed by using the specific LC-MS/MS method. The failures were caused by improper ion ratios or by the absence of the second ion, because its intensity was too low (or completely absent, if it was not the target analyte). In this case the interference effect from eventually coeluting substances became predominant in the screening method. Finally, confirmatory analysis was necessary for 15-ADON (only 1 fragment ion was monitored during screening). All cases (11), in which 15-ADON occurrence had resulted ≤ 3*MDL value, were not confirmed, while for the others (7) the presence of 15-ADON could be demonstrated with a mean inaccuracy of –250%.

As for quantitation, the discussion about agreement between screening and confirmation data in term of inaccuracy % has been developed with reference to the lowest regulatory limits recently introduced for DON, ZON and FB_1+FB_2 (see table 1.2). For DON only four samples were contaminated with levels above 750 ng/g and two samples above 200 ng/g. FB_1+FB_2 were detected and confirmed in all samples above 200 ng/g. For both DON and fumonisins the mean inaccuracy were found to be -12%. ZON was present in ten samples at concentrations above 50 ng/g, but only six cases were confirmed. The mean inaccuracy was +24%. In addition the confirmatory method detected one false negative case.

Finally, confirmatory methods allowed us to detect also fumonisin B_4 (24 positive cases out of 24) and zearalenone metabolites such as β-ZOL (3 out of 24).

3.10. Conclusions

This multiresidue method has been specifically developed as a relatively rapid primary screening method. It allows simultaneous identification and quantification of the major mycotoxins that can arise from the various species of the *Fusarium* genus potentially colonizing *Zea mays* in only two hours, even if not in the optimum LC-MS/MS conditions. Some compromises have been made, such as the overlay between 3- and 15-ADON isomers, and FB_3 determination at the expense of β-ZOL. An important advantage of the method is the clean-up on Carbograph, which in suitable conditions allows both simultaneous multiresidue analyses or selective analyses for target fusariotoxins. The selective analysis of a new aliquot from the same extract, together with optimized chromatographic

and mass spectrometric conditions, can be performed for more sensitive and accurate confirmatory analysis. For both screening and confirmatory methods, limits of detection were at least twenty times below law values set for corn meal.

The technical capabilities of the Q TRAP™ mass spectrometer were exploited to estimate the amount of FB_3 and FB_4, for which standards were not available to us. The bias error for FB_3, that is an isomer of FB_2 with a closely similar retention time, should be low. Moreover, FB_4 has one hydroxyl group less and the error may thus be higher. However, results for the samples tested here suggest that FB_4 is always a very low percentage of the total FBs so that, unless particularly high toxicity of this compound is discovered, an uncertainty of 20-30% is still acceptable.

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Chapter 4

Application to real samples

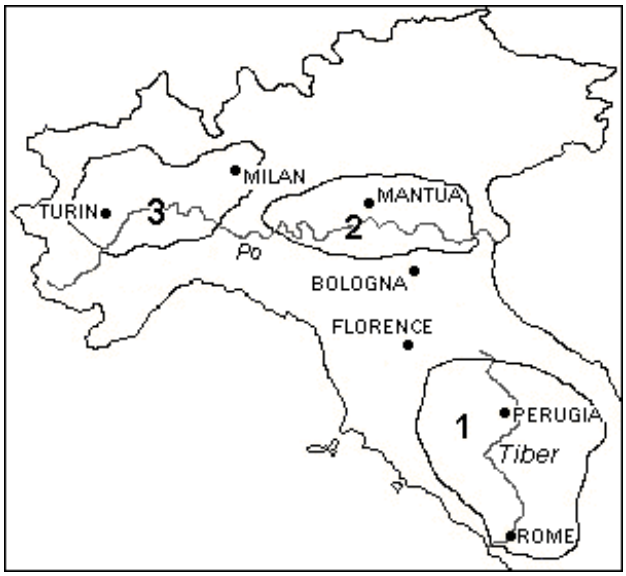
Chapter 4 – Application to real samples

4.1. Sampling

4.1.1. Field experiments in 2002

Researchers from ISC (Bergamo, Italy) conducted sampling during the 2002 harvest season and kindly provided us with 78 maize samples. 46 maize samples (from three hybrid types belonging to FAO 400, 500 and 600 classes) were from twelve villages scattered in the countryside across central and northern Italy. They were harvested from private farms "in open field". In this case management practices were determined by the farmer co-operator according to local practice and needs. We divided the samples into three groups depending on their geographical origin which is linked to area climatic conditions: 1- central Italy, 2- Po Valley, and 3- north-western Italy (figure 4.1).

Figure 4.1. *1*, *2* and *3* corn sown sampling areas across central and northern part of Italy.



Area 1 is hilly with little rain, but muggy, area 2 is a wide valley between Apennines and Alps and very humid, area 3, placed in the North of Po Valley, is drier than area 2, but very rainy. Meteorological data relating to the months between April-October 2002 were acquired from weather reports edited by UCEA (Ufficio Centrale di Ecologia Agraria) for the Department of Agriculture. These data came from ten stations located through the whole studied area. In the April-October period mean rainfall index in area 1 was around 77 mm/month with mean humidity 53-83%, in area 2 around 103 mm/month with humidity 75-81% and in area 3 around 120 mm/month with humidity in the range 52-75%. Besides this, the most important data are the abundant and unusual rainfalls occurred in Italy during the July-August period.

In the same period, ISC (Rome) provided us with a further 32 samples coming from corn cultivated in two experimental fields in Lazio, one in Northern Rome, and the other in Torreinpietra (a village to the West of Rome). The former is located in a valley in which humidity stagnation can occur, while the Torreinpietra one is near the sea. Field experimental design was carried out employing controlled farming conditions, (same irrigation, fertilizing, etc.). In both sites the area selected for sowing was divided into equal parcels each of 9.6 m^2; three replicate plots were planted for each maize hybrid. 5 random ears of corn were sampled from each parcel and then three sub-samples were put together in a single sample for analysis. 8 different maize hybrids belonging to three different FAO precocious flowering groups (FAO 300, 400 and 500) were sowed. In particular, the 32 maize samples were divided into two batches (16x2), belonging to the different sampling sites: Northern Rome and Torreinpietra. Each batch was, in its turn, divided into two sets (8x2),

depending on different sowing and harvest days: the first sowed on 8 April and harvested on 9 September 2002 ("normal harvest"), the second sowed on 30 April, and harvested on 1st October 2002 ("delayed harvest"). Samples were sent to our laboratory as soon as collected and were ground, sieved and extracted on arrival and analyzed in order to avoid external factors that could favor mold growth and mycotoxin development.

4.1.2. Field experiments in 2003

For the 2003 harvest season, 40 corn meal samples were kindly provided by ISC (Bergamo). Samples came from five sites in northern Italy (Vigone, Torino; Caleppio, Lodi; Luignano, Cremona; Palazzolo, Udine; and Rottaia, Udine). For each site, samples were collected in two replicates for four hybrid types belonging to FAO 300, 500, 600 and 700 classes. Growing, harvesting and sampling procedures were the same as in 2002 described in 4.1.1.

4.2. Results and discussion

4.2.1. Maize samples collected in 2002 from Italian fields

The second part of this study was achieved by collecting 46 maize samples "in open field" directly from some Italian private farms across the midlands and northern part of Italy. The authors' intent was to sample corn grown in a large variety of geographical locations to investigate the occurrence extent of some fusariotoxins in the field. These data are still partial because, as explained in the experimental section, the exceptionally

high rainfall index leveled differences among the selected sampling areas and probably amplified the occurrence levels.

The peculiarity of this work lies in evaluating the occurrence of some fusariotoxins produced by fungi on corn grown just before harvest. For this reason these data cannot be compared with other analogues in the literature as well explained by Müller et al. [Müller 1997]. Some authors conducted specific studies on mycotoxin contamination of freshly harvested cereals: on fumonisins in Brazilian corn [Almeida 2002], fumonisin B1, moniliformin and ZON in a Zimbabwean one [Mubatanhema 1999], trichothecenes B, moniliformin in Polish wheat [Grabarkiewicz-Szczęsna 2001], and *Fusarium* toxins in German barley [Müller 1997]. Other authors analyzed fusariotoxin levels in genetically modified maize grown in France, Spain [Bakan 2002] and the United States [Hammond 2004]. Data obtained in the present study are summarized in table 4.1.

As far as trichothecenes B are concerned, over 40% of the samples were found contaminated by DON, which was the most abundantly detected mycotoxin (up to 3430 ng/g), followed by 3-ADON (26%, maximum value 3500 ng/g). The frequencies of NIV (15%) and FUS X (13%) contamination were less than DON's and, in particular when FUS X was found, the levels were no more than 420 ng/g. In addition, co-occurrence of DON and 3-ADON was observed in 23%, NIV and FUS X in 10%, while NIV and DON, FUS-X and DON co-occurred only in 2%. The prevalence of DON resulting from our gross screening across Italian fields is not surprising and may be attributed, partially, to the large diffusion of *Fusarium* strains in temperate regions producing, as described by some authors [Bottalico 1998, Müller 1997, Mubatanhema 1999], high contents of DON. This fact is

further amplified by a wet 2002 summer. It has to be noted that the highest mean value of DON (1330 ng/g) and the highest incidence (100%) were found in the samples coming from the northern-western Italy (area 3), where , as a general rule, fluctuations over the day-night cycle (up to 15°C) are higher than in areas 1 and 2.

Table 4.1. Incidences, mean of positives and concentration ranges (ng/g) of target fusariotoxins in maize samples collected from different farms placed in the north-center of Italy

Analytes[a]	Central Italy			North-western Italy			Po Valley		
	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)
NIV	6/31	607	12-2440	1/11	–	200	0/4	–	–
DON	6/31	712	5-2060	11/11	1330	45-3430	3/4	387	68-967
FUS X	5/31	137	26-420	1/11	–	34	0/4	–	–
3-ADON	5/31	711	6-3500	7/11	31	7-95	0/4	–	–
ZON	6/31	93	8-384	7/11	228	41-969	0/4	–	–
α-ZOL	1/31	–[b]	9	4/11	14	7-33	0/4	–	–
β-ZOL	3/31	8	6-11	5/11	20	8-33	0/4	–	–
α-ZAL	0/31	–	n.d.[c]	0/11	–	n.d.	0/4	–	–
β-ZAL	2/31	7	6-8	0/11	–	n.d.	0/4	–	–

[a] NIV = nivalenol, DON = deoxynivalenol, FUS X = fusarenon X, 3-ADON = 3-acetyldeoxynivalenol, ZON = zearalenone, α-ZOL = α-zearalenol, β-ZOL = β-zearalenol, α-ZAL = α-zearalanol, β-ZAL = β-zearalanol.
[b] – Not calculated.
[c] n.d.< MDL.

ZON was detected in 30% of the samples, usually at low levels, while its derivatives were rarely encountered, as reported in previous works [Bakan 2002, Müller 1997, Schollenberger 2002]. We found α and β-ZOL in

28% of the samples with concentration values up to 33 ng/g. α-ZAL were never detected.

Although there was random contamination in the field samples collected, however the few samples did not allow us to find significant correlations between hybrid type and mycotoxin content.

4.2.2. Maize samples coming from experimental fields collected in 2002

Finally, we also tried to investigate the way in which field contamination, when it occurred, was dependent on weather and environmental factors. Although two different irrigations on maize crops in Lazio were scheduled, because of considerable summer rainfall, the second irrigation was eliminated. Thus, it was impossible to determine any difference in mycotoxin prevalence between well-watered and stressed maize. After a hot and dry June, in July and August, generally arid months in Mediterranean regions, abundant and unexpected rains had fallen, enhancing the harvests, but, probably encouraging mold growth.

Four sets (32 samples) of maize samples, kindly provided us by ISC of Rome, were collected from two experimental fields (northern Rome and in Torreinpietra) selected for their different microclimatic conditions (see experimental section). The results are summarized in table 4.2. Quite similar low levels of mycotoxins were detected in both sets coming from Torreinpietra and in the first set from northern Rome. Notably, in the second set of the northern Rome samples, the level of mycotoxins, especially trichothecenes B, was seriously increased if compared with the first one. NIV varied from the mean value of 135 ng/g to 1920 ng/g, 3-ADON from 14 ng/g to 2060 ng/g, DON and ZON from "not detected" to 2040 ng/g and 273 ng/g respectively.

Table 4.2. Incidences, mean of positives and maximum levels (ng/g) of fusariotoxins found in maize samples coming from two experimental fields, located in Torreinpietra and in northern Rome, after the normal harvest and delayed harvest.

Torreinpietra

Analytes[a]	normal harvest			delayed harvest		
	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)
NIV	1/8	–[b]	16	1/8	–	28
DON	1/8	–	19	1/8	–	17
FUS X	1/8	–	27	1/8	–	24
3-ADON	1/8	–	6	1/8	–	9
ZON	2/8	10	11	2/8	13	16
others	1/8	–	8	1/8	–	7

Northern Rome

Analytes[a]	normal harvest			delayed harvest		
	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)
NIV	2/8	135	240	2/8	1920	3270
DON	0/8	–	n.d.[c]	2/8	2040	2140
FUS X	1/8	–	382	2/8	104	181
3-ADON	2/8	14	23	1/8	–	2860
ZON	0/8	–	n.d.	3/8	273	412
others	0/8	–	n.d.	6/8	9	13

[a] NIV = nivalenol, DON = deoxynivalenol, FUS X = fusarenon X, 3-ADON = 3-acetyldeoxynivalenol, ZON = zearalenone, others = sum of α and β- zearalenol, α and β-zearalanol.
[b] –: not calculated.
[c] n.d.: < MDL.

Presumably this is due to the stagnant humidity typical of the northern Rome field, located in a valley, and to low minimum temperatures recorded in the 2nd and 3rd decade of September. The influence of temperature on simultaneous production of DON and ZON by *Fusarium*

strains was investigated by other authors [Martins 2002]. In particular, ZON production is favored by cold weather in high moisture conditions [Hollinger 1999].

Therefore the maize harvested in October remained longer in the field between the end of summer and the beginning of autumn; the humidity and the rainfall contributed in mold development while thermal fluctuations favored fusariotoxin production. This trend was partially reflected by the data from the northern-western area (table 4.1), where low minimum temperatures are usually reached before the normal harvest time. On the contrary, Torreinpietra, being near the sea, has a windier climate with higher minimum temperatures, therefore, has proved to be a site less susceptible to contamination when the corn harvest is delayed.

In conclusion, the simultaneous occurrence of mycotoxins assayed in a large part of just-harvested corn demonstrates the importance of improving farming and agronomic practice during cultivation. Indeed, the findings of the present study fortify the need for regular screening of cereals for *Fusarium* toxins, particularly in years with abundant precipitation late in the summer.

4.2.3. Maize samples collected in 2003 from Northern Italian fields

The 2003 sampling of freshly harvested maize coming from Northern Italian fields completed the survey about the occurrence extent of fusariotoxins in the field. Data obtained for trichothecenes B and zeranols substantially confirmed the results of the 2002 sampling. However, with respect to 2002, these toxins were found with less frequency and abundance, probably because of meteorological conditions characterized by drier (scarce rainfalls) and cooler climate.

Moreover, although trichothecenes A are fusariotoxins typical of cold climate (i.e. Northern Europe), we detected them at trace concentration levels (≤ 23 ng/g). DAS was the most detected trichothecene A (61%), followed by MAS (24%). T-2, HT-2 and NEO were found in three samples.

Fumonisins FB_{1-3} were found in all the analyzed samples (40), and it is therefore possible to discuss the sampling site and hybrid class effects. Concentration levels (mean and range) for fumonisins are reported in table 4.3.

Table 4.3: Mean values and concentration ranges (mg/kg) of FB_{1-3} in maize samples collected in 2003 from different farms placed in Northern Italy.

Site		Analyte		
		FB_1	**FB_2**	**FB_3**
Caleppio (LO)				
	Mean[a]	15.4	6.66	2.39
	Range[a]	5.58-37.6	1.06-21.7	0.71-5.59
Luignano (CR)				
	Mean[a]	20.3	7.58	3.69
	Range[a]	8.34-27.34	3.19-17.1	1.52-6.66
Vigone (TO)				
	Mean[a]	4.62	1.70	0.74
	Range[a]	2.1-12.7	0.32-5.5	0.20-1.87
Rottaia (UD)				
	Mean[a]	21.32	10.67	3.37
	Range[a]	1.97-69.4	0.50-42.2	0.21-11.3
Palazzolo (UD)				
	Mean[a]	4.88	1.42	0.73
	Range[a]	0.27-16.6	0.14-4.38	0.05-2.97

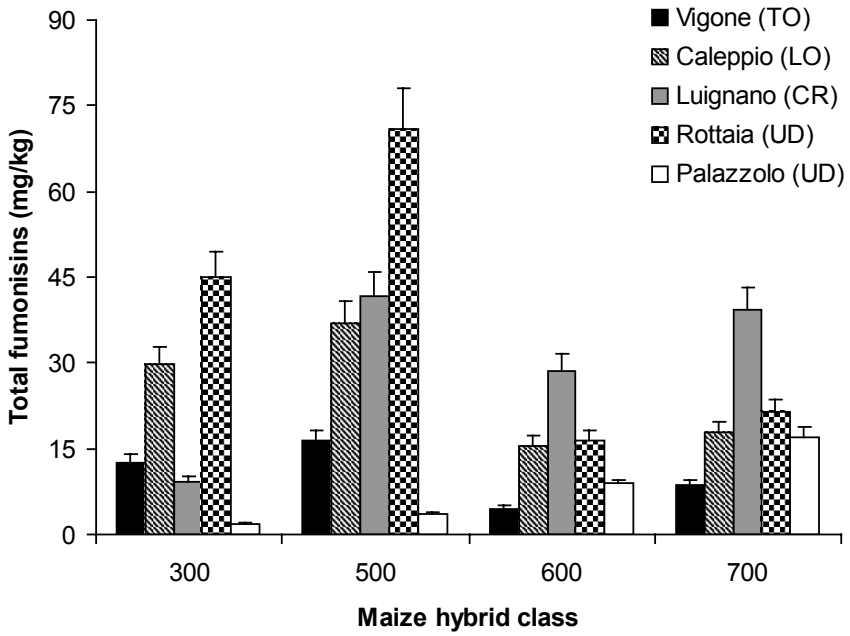
[a]values are expressed in mg/kg

As reported by other authors [Paepens 2005], there is a strict correlation between the occurrence of these fumonisins, probably related to their

synthetic pathways from *Fusarium* metabolism [Bottalico 1998, Sweeney 1998].

In Figure 4.2. the total fumonisin occurrence found in the 5 target sites for 4 maize hybrid classes (i.e. 300, 500, 600 and 700) is summarized in form of histogram. For each sampling site, data show that the different hybrid class maize samples were contaminated according this order: 500 > 700 ≅ 300 > 600. In particular, the samples that resulted most contaminated belonged to the 500 hybrid class and came from Rottaia, whereas the least contaminated samples were found in the 300 hybrid class coming from Palazzolo.

Figure 4.2: Total fumonisin occurrence found in the five target sampling sites for 4 hybrid classes of maize samples harvested in 2003.



4.3. Conclusions

The delicate negotiations on the legislative regulations regarding the maximum tolerable levels of fusariotoxins in food is currently in progress. This means that detailed information about contamination levels, and fast, simple analytical methodologies capable of high performance are now required. The work for the determination of fusariotoxins described in this section meets these requirements, and this chapter summarizes some results, obtained by analyzing freshly harvested maize, highlighting the fact that several environmental factors can influence mould growth and mycotoxin production, that type-B fumonisin contamination is a general and significant problem for corn meal and simultaneous contamination can occur. In view of this, the development and selection of cultivars of cereal and forage plants that are resistant to infection by toxigenic fungi should be the long-term objective of any effort to prevent contamination in future.

Section II

Ochratoxin A

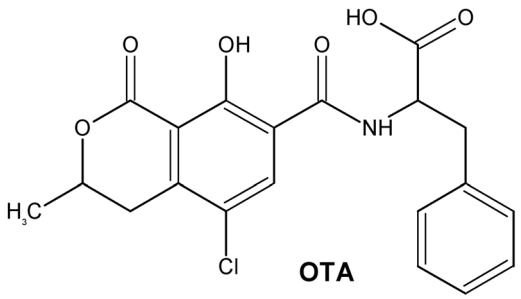
Chapter 5

Introduction

Section II – Ochratoxin A

Chapter 5 – Introduction

Ochratoxin A, 7-(L-β-phenylalanyl-carbonyl)–carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin (figure 5.1) is a widespread mycotoxin produced by the secondary metabolism of several *Aspergillus* and *Penicillium* species, mainly by *P. verrucosum, A. ochraceus* [Visconti 1999, Jornet 2000, Lau 2000] and *A. carbonarius* together with a low percentage of the closely related *A. niger* [WHO 2002, Serra 2003]. These fungi differ in their ecological niches, in the commodities affected and in the frequency of their occurrence in different geographical regions. They can infect various plants and plant products such as cereals (wheat, barley, maize and oats), legumes, groundnuts, spices, dried fruits, coffee, cocoa, grapes. Consequently, contamination of ochratoxin A (OTA) can occur also in their derived products such as beer, wine, bread and bakery products [WHO 2002].



OTA

M.W. = 403.8

OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several animal species and to cause kidney and liver

tumors in mice and rats [Visconti 1999, Becker 1998, Leitner 2002, Dall'Asta 2004b]. For these reasons, in 1993 the International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogen to humans (group 2B) [IARC 1993, Peraica 1999].

With current scientific and technical knowledge, and despite improvements in production and storage techniques, it is not possible to prevent the development of these moulds altogether and very difficult OTA removal [Bejaoui 2005]. Consequently, OTA cannot be eliminated from food entirely. Limits should therefore be set as low as reasonably achievable. The World Health Organization (WHO) has set a provisional tolerable weekly intake level for ochratoxin A at 100 ng/Kg body weight [JEFCA 2001], taking its potentially carcinogenic effect into account. Regulatory levels have been established within the European Union for products of wide consumption [EC 2002b]. Regulatory levels have been also discussed as regards wine [SCF 1998], for which a maximum residue limit of 2.0 μg/L has been recently fixed [EC 2005a]. In 1999 a maximum guidance level of 0.2 μg/L for beer has been set by the Italian Ministry of Health [IMoH 1999].

The occurrence of OTA in wine samples has been reported in several studies mainly dealing with European wines, showing a considerable level of contamination with high toxin concentration and incidence. Generally, red wines contain higher amounts of this mycotoxin than white and rosé wines [Battilani 2002] and Southern European countries together with North Africa seems to be more affected by the OTA contamination [Dall'Asta 2004b]. Differences are attributed to climatic factors, grape cultivation, wine-making techniques and storage conditions.

Reversed-phase liquid chromatography coupled with fluorescence detection (LC–FLD), preceded by immunoaffinity column (IAC) clean-up, is currently the most applied method for OTA determination in wine and beer [Jornet 2000, Leitner 2002, Dall'Asta 2004b, Shephard 2003, Monaci 2004, Visconti 2001]. Recently *Aresta et al.* have developed a sample preparation procedure based on solid-phase microextraction [Aresta 2006]. An automated method using a robotic sample processor has been recently developed [Brera 2003] to reduce analysis time. Immunochemical methods based on enzyme-linked immunosorbent assay (ELISA) are also used [Barna Vetro 1996]. However, problems arising from matrix complexity, such as cross-reaction, can lead to erroneous results. This problem may be overcome by employing mass spectrometry (MS) as detection technique after LC [Becker 1998, Leitner 2002, Reinsch 2005, Zöllner 2000].

State-of-the-art, on-line SPE (solid phase extraction)-LC-MS provides high precision, sensitivity and a higher sample throughput as compared to off-line SPE [Henion 1998]. Several technical devices for coupling on line SPE with LC are commercially available, but also simpler home-made apparatus may offer good performances [Jemal 1999]. At our best knowledge there is only one published paper, dealing with the determination of fumonisins [Newkirk 1998], that makes use of an on-line SPE-LC-MS method for determining these mycotoxins in corn-based feed in which the SPE is realized by coupling an immunoaffinity capture column to a polymeric resin-filled trap.

SPE-LC-MS/MS methodologies have been developed especially for drug monitoring [Koal 2004], pharmacokinetic application [Jemal 1999, Lee 1999, Schellen 2003] and analysis of pesticides in water [Asperger 2002]. In

order to achieve very high throughput methods, short (20-50 mm) columns working at low resolution condition are often adopted for biological samples. SPE systems coupled with these stressed mini-columns are equally very fast-operating and a total time of 2.5 min or less could be achieved [Jemal 1999, Koal 2004, Lee 1999]. Such a considerable shortening in time of analysis is, at least in part, obtained at the expense of selectivity and limit of detection (only very low enrichment factors are possible) relying on MS/MS for accurate quantitation at a very low level.

The fact that in the electrospray ionization (ESI) process co-extracted and co-eluted matrix components can decrease the yield of analyte ion production by competition is now a well recognized effect. The use of an internal standard (IS) that undergoes the same signal suppression as the analyte surely eliminates the inaccuracy problem, but the signal is still weakened. Narrowing the peak by very fast elution may compensate for ion production decrease only as long as the extra analytical column contribution to peak width becomes the limiting factor. Limits of detection reported in a recent work for compounds well amenable of positive ESI were about 0.1 ng/mL in blood [Koal 2004]. Moreover, handling a more complex matrix like urine, good performances were obtained with runtimes longer than 10 min, although deuterated I.S. were used [Kato 2003].

The aim of this work was to develop and evaluate an "on-line" SPE-LC method coupled to ESI-MS/MS for confirmation analysis of OTA in wine and beer. The pursued characteristics of this method were: 1) high performances, 2) total automation, 3) simple apparatus and 4) high throughput [Bacaloni 2005].

Chapter 6

Materials and methods

Chapter 6 – Materials and methods

6.1. Chemicals and materials

Standard of ochratoxin A was purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile and methanol, both LC grade, as well as ethanol, formic and acetic acids were supplied by Carlo Erba (Milan, Italy). Deionized water was further purified using a Milli-Q apparatus (Millipore, Billerica, MA, USA).

A standard stock solution (200 ng/μL) was prepared by dissolving OTA in acetonitrile. Working standard solutions were prepared at different concentrations immediately before use by diluting the stock solution with suitable volumes of water/ethanol (85:15, v/v) 10 mmol/L formic acid. All solutions were kept at –20° C and allowed to equilibrate at room temperature before use.

The cartridge employed for on-line SPE extraction was a 4.0 x 4.0 mm i.d. LiChroCART 4-4 ™ containing 100 mg of C_{18} (5 μm average particle size), purchased from Merck (Darmstadt, Germany). For sample filtration PTFE syringe filters (0.45 μm; 15 mm diameter) were employed (Chemtek Analytica, Bologna, Italy).

6.2. Wine and beer samples

Bottled and boxed Italian wine samples (glass or Tetrapak™ packaging) were purchased from local stores. A total of 66 wine samples were analyzed: 43 red, 16 white and 7 rosé. Bottled and canned beer samples

were also purchased from local stores. A total of 18 beer samples (of Italian and foreign origins) were analyzed.

6.3. Instrumentation

The liquid chromatography system used was a series 200 apparatus from Perkin-Elmer (Norwalk, CT, USA). The system consisted of a binary LC micropump, a binary LC pump, two vacuum degassers and an autosampler equipped with a 200 μL loop. The chromatographic column was a 150 mm x 1 mm i.d. Alltima (Alltech, Deerfield, IL, USA) filled with C_{18} reversed-phase packing, 5 μm average particle size, equipped with a Securityguard 4 x 2 mm i.d. precolumn (Phenomenex, Torrance, CA, USA).

Electrospray mass spectrometry (ESI-MS/MS) was carried out on a Q TRAP™ quadrupole-linear ion trap instrument equipped with a TurboIonSpray (TISP) interface and with a built in eight port valve (Applied Biosystems/MDS Sciex, Concord, ON, Canada).

The LC-MS system was controlled by Analyst software (1.3.2 version, Applied Biosystems/MDS Sciex).

6.4. Sample preparation and analytical conditions

Ten milliliter aliquots of beer samples were previously degassed by ultrasonication for 30 minutes. After this step, the analytical procedure was the same for both alcoholic drinks. Before analysis, wine and beer samples were filtered on 0.45 μm PTFE filters, and without any further

processing a 200 μL aliquot was injected directly into the apparatus by the autosampler. The schematic of the on-line system is shown in figure 6.1.

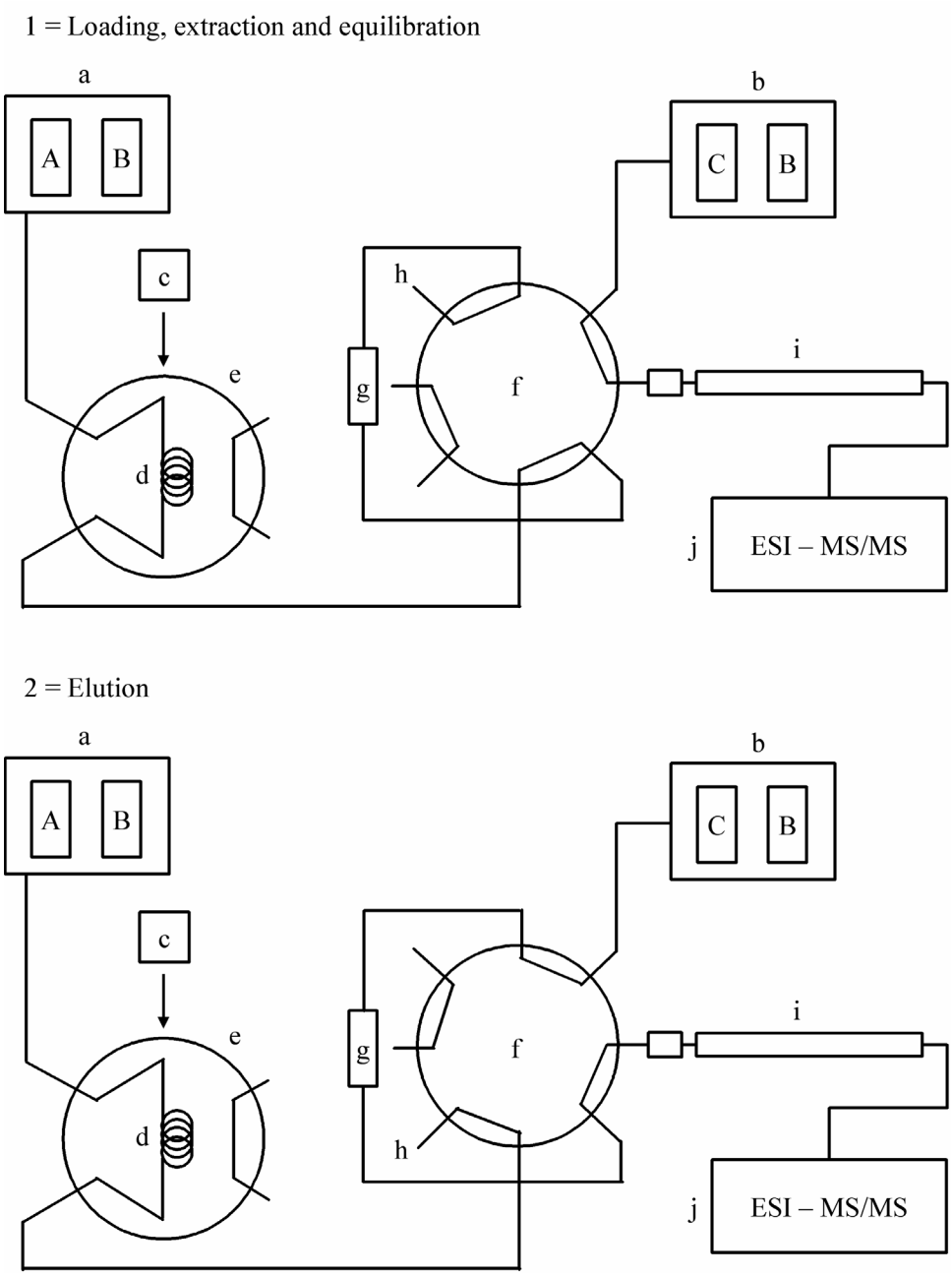
The two alternate positions of the software-controlled eight port valve allowed flow switching in the SPE extraction cell. The LC binary pump (pump 1) was used to deliver a high flow (1 mL/min flow-rate) through the extraction column to load the sample and wash out very polar matrix compounds and subsequently to flush and equilibrate the extraction column. Instead, the LC binary micropump (pump 2) was used to deliver a low flow (50 μL/min flow-rate) to elute the analyte from the extraction column in back flushing mode and from the chromatographic column and subsequently to flush and equilibrate the latter one. Mobile phases used for sample loading and washing were ethanol (B) and water (A). Mobile phases for analyte elution were methanol (C) and water (A). All the above cited mobile phases contained 10 mmol/L formic acid. Gradient profiles and valve position are reported in table 6.1.

Table 6.1. Gradient profiles of the two LC binary pumps and valve positions.

	PUMP 1 Φ^a **= 1 mL/min**	**Valve position**	**PUMP 2** Φ^a **= 50 μL/min**
Time[b] (min)	**% B[c,d]**		**% C[c,e]**
0-1	15	1[f]	60
1-3	15	2[g]	60
3-3.5	15	2[g]	60→63
3.5-8	15→85	1[f]	63→90
8-10	85	1[f]	90
10-24	15	1[f]	60

[a] Flow-rate.
[b] Time starts after autosampler injection.
[c] Solvent A was water containing 10 mmol/L formic acid.
[d] Ethanol containing 10 mmol/L formic acid.
[e] Methanol containing 10 mmol/L formic acid.
[f] Loading, extraction and equilibration position.
[g] Elution position.

Figure 6.1. Schematic of the on-line system.



1 = position used for sample loading, extraction and cartridge regeneration; 2 = position used for OTA elution from the extraction cartridge. Mobile phases: A = ethanol, B = water, C = methanol, (all mobile phases contained 10 mmol/L formic acid). A = pump 1 (1 mL/min flow-rate); b = pump 2 (50 μL/min flow-rate); c = autosampler; d = 200 μL loop; e = injection valve; f = eight port valve; g = SPE extraction/concentration column (4.0 x 4.0 mm i.d.); h = waste; i = analytical column (150 x 1 mm i.d.); j = mass spectrometer.

The column effluent was directly transferred into the mass spectrometer. Taking advantage of the two different analyzers available on Q TRAP™,

data acquisition and quantitation were performed working either in multiple reaction monitoring (MRM) mode or in enhanced product ion (EPI) mode. Instrument tuning parameters were suitably adjusted by infusing at 10 μL/min flow-rate an OTA standard solution, 10 ng/μL, prepared in methanol/water (50:50, v/v) containing 10 mmol/L formic acid. Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a 10^{-5} mol/L solution of PPG (polypropyleneglycol) introduced via a Model 11 Harvard infusion pump. The peak-width was set on both resolving quadrupoles at 0.7 Th (Thomson) measured at half height for all MS and MS/MS experiments. Mass spectra recorded in full-scan mode were obtained by scanning over the range *m*/*z* 100-450 in 2.6 s. TISP interface was operated in the negative ionization mode, by applying to the capillary a voltage (IS) of –4200 V. Nitrogen was used as curtain gas (CUR), while air was used as nebulizing (GS1) and turbo spray gas (GS2, heated at 350°C), with the optimum values set respectively at 20, 20 and 40 (arbitrary values). Collisionally activated dissociation (CAD) MS/MS was performed in the collision cell (Q2), operating at medium pressure (arbitrary value) of nitrogen as collision gas. Declustering potential (DP) was set at –30 V, while entrance potential (EP) was set at –10 V; relative collision energy (RCE) was optimized at 19% (for details see table. 2.1).

Operating in MRM mode, Q1 (first quadrupole) and Q3 (third quadrupole) resolutions were set at unit and the collision cell exit potential (CXP) was set at –2 V. Two transitions were monitored for the deprotonated molecular ion $[M-H]^-$ of OTA: *m*/*z* 402→358 and *m*/*z* 402→211. However, only the former was used for quantitation, while the latter was used for confirmation purpose. Operating in EPI mode, LIT

(linear ion trap) fill time was optimized at 50 ms and Q3 entry barrier at 8 V, while the scan rate was set at 4000 amu/s. For quantitation purpose the *m*/*z* 358 product ion (0.5 Th width) was selected.

6.5. Calibration curve, quantification and evaluation of method performance

Calibration solutions were prepared daily by dissolving a suitable volumes of OTA standard stock solution in water/ethanol (85:15, v/v), containing 10 mmol/L formic acid, at 15.0, 5.0, 1.0, 0.1 and 0.025 ng/mL concentration levels. For quantification, a calibration curve was constructed by plotting the observed peak area against the OTA concentration. The linear regression equation was obtained in duplicate by least-square analysis from the five point calibration curves. The calibration solutions were injected, and then the curves plotted, at the beginning and the end of each analytical batch.

The on-line SPE-LC-ESI-MS/MS method was evaluated for the following parameters: linearity, within-day and between-day accuracies and precisions, matrix effect, specificity, limit of identification (see later for definition), limit of quantification and performance stability.

Linearity in the working range was evaluated from a calibration curve on five points, each in triplicate (concentration range 0.05-25 ng/mL). Accuracy and precision were measured using, as quality check samples, simulated wine samples (SWSs) at three different known concentration levels (1.0, 3.0 and 15.0 ng/mL) prepared in water/ethanol (85:15, v/v) containing 10 mmol/L formic acid from a separate dilution of the standard stock solution. Each SWS was analyzed in triplicate per single batch in five

different days. Specificity was assessed by analyzing several different wine or beer samples in MS/MS EPI mode and comparing the obtained spectra with that of a standard solution.

Matrix effect on recovery was assessed by comparing the slope of the calibration curve obtained from the hydroalcoholic standard solution with the slope of calibration curves obtained by injecting wine (red or white) or degassed beer spiked at the same concentration level of the standard solution. The calculated mean recoveries were utilized for quantitative analysis of naturally contaminated samples.

To assess performance stability, a set of red wine samples spiked at five different concentration levels, covering the entire working range, plus a blank were processed by the on-line system. This set of samples was re-injected up to a total of 60 processed samples (10 cycles).

Chapter 7

Results and discussion

Chapter 7 – Results and discussion

7.1. General remarks

This method has been specifically developed for confirmation analysis purpose in compliance with the European Union laws in force [EC 2002a]. On this basis, a substance can be identified using LC-MS/MS, in MRM mode, by at least two transitions. For this reason, we prefer to indicate a "Method Identification Limit" (MIL), estimated as the minimum concentration giving a signal-to-noise (S/N) ratio = 3 for the second most intense MRM transition instead of a limit of detection. Moreover, this implied achieving a method quantification limit (MQL), S/N = 10, at least of 0.1 ng/mL for wine. To implement this objective we devised a non-laborious, reasonably low cost, high throughput, robust system suitable for routine use in specialized laboratory.

In order to assess the best conditions for the on-line extraction and analysis of OTA, each part of the analytical set up was optimized off-line in first instance; then, after the whole system was set up, mutual influence of each parameter was evaluated and re-optimized. In preliminary experiments conducted with SWSs we selected some fast conditions that failed when applied to actual wine sample. Irreproducibility, signal weakening and frequent clogging were the main problems. This was probably due to the fact that, like some food, wine is a complex matrix and requires selective extraction and efficient chromatographic separation to minimize ion suppression [Bogialli 2003c, Laganà 2003]. So, a new more selective set-up was planned and tested.

One advantage of automated on-line methodologies, if compared with off-line ones, is that sample manipulation is reduced to a minimum, and then more reproducible results are obtained. Another advantage is that sensitivity is usually enhanced since the whole sample, instead of an aliquot of the final extract such as in off-line systems, is transferred to the chromatographic system. In the methodology developed only filtration of the sample and loading of the autosampler were manually performed. A small volume of the sample (200 μL) was injected into the SPE trap and transferred to a 1 mm chromatographic column, with virtually no loss. Considering that in an analytical column like the one used in this study normally no more than 5 μL of sample can be injected without peak broadening, a 40 fold enrichment factor was achieved.

7.2. Method optimization: ESI-MS/MS conditions

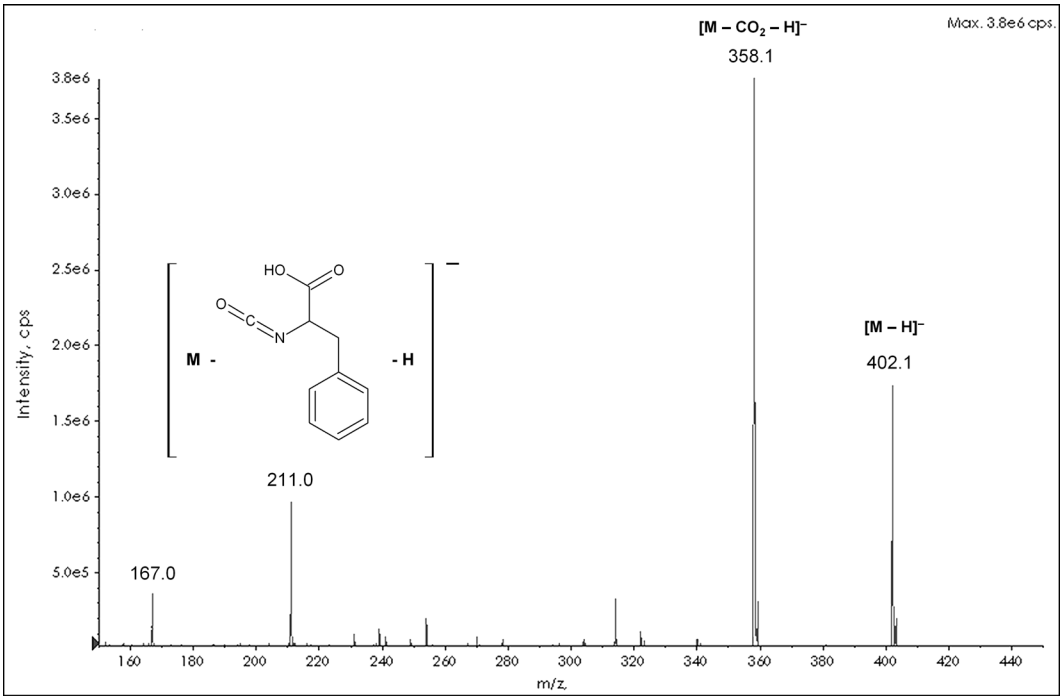
The presence of a carboxylic group in OTA structure, with regard to chromatographic separations, implies acid or buffer [Becker 1998] addition to mobile phases to avoid peak broadening and tailing. It is known that in these conditions OTA can be detected in positive ESI-MS [Lau 2000], because it forms either proton adduct at *m*/*z* 404 or sodium adduct at *m*/*z* 426, ions that can be monitored. However, using methanol and water as mobile phases and a relatively low concentration of formic acid as mobile phase modifier, we noticed that negative ionization gave a more intense $[M-H]^-$ ion at *m*/*z* 402.

Generally, in negative ESI the responses of compounds containing a carboxylic acid are decreased as formic acid concentration increases. On the other hand, the presence of acid helps to retain analytes on the column

and gives a better chromatographic resolution and lower matrix effect. We tried to find the best compromise between these two opposite tendencies. We noticed that the addition of 10 mmol/L formic acid to both mobile phases did not suppress the signal dramatically and gave symmetrical peaks. Moreover, by performing flow injection analysis in methanol/water (70:30, v/v) we surprisingly noticed that acid addition up to 1 mmol/L to mobile phases instead of suppressing the signal increased it slightly (data not shown). This is in agreement with recent studies performed by Wu et al. [Wu 2004] that described how ionization efficiency in negative ESI is influenced by mobile phase pH. In particular, it was observed that the intensity of the deprotonated ion signal of acidic substances sometimes is enhanced by acidification. The formation of charged droplets in electrospray, indeed, is achieved mainly through ion reduction on capillary surface. Positive ions, produced by protonation in an acidic environment, increase reduction process rate and enable the spray to carry more easily a negative charge excess, which then is transferred to the analyte. In figure 7.1 the fragmentation spectrum of the $[M-H]^-$ ion of OTA is shown. As can be seen at a RCE of 19% the two major fragments formed are: *m*/*z* 358, $[M - CO_2 - H]^-$ followed by the less intense *m*/*z* 211. For quantitative analysis only the transition *m*/*z* 402 → 358 was used, while both were selected for identification purpose.

At present, chromatographic separation followed by MRM mass spectrometric detection of two fragment ions is judged exhaustive to confirm the identity of a molecule. However, in view of a more stringent regulatory framework in which the identity of the molecule might have to be confirmed with greater confidence, it would be preferable to have the entire fragmentation spectrum of a molecule.

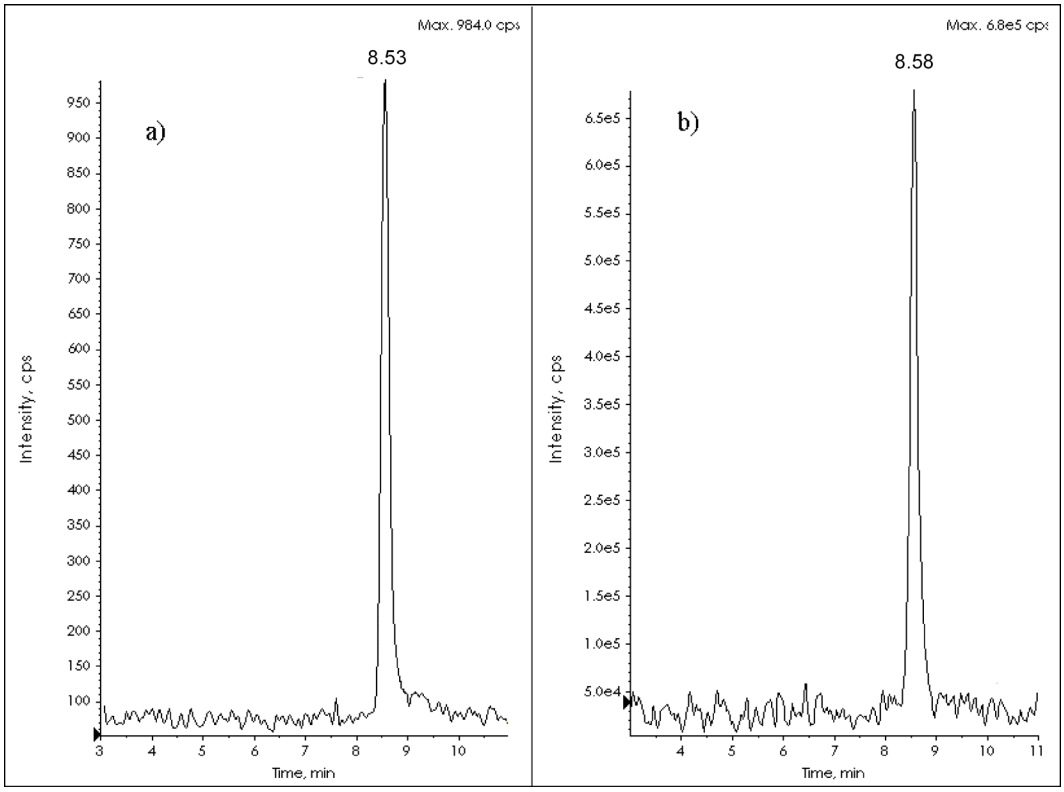
Figure 7.1. Enhanced Product Ion (EPI) spectrum of ochratoxin A $[M-H]^-$ ion (RCE = 19%).



Traditional triple-quadrupole instruments can perform product ion scan, but when this acquisition mode is utilized, the sensitivity is greatly diminished. To overcome this, we investigated the capability of the hybrid quadrupole-ion trap MS/MS system to address both qualitative and quantitative issues by taking advantage of the two available configurations of Q3. The system employed is a modified triple quadrupole where the Q3 region can be operated either as a conventional quadrupole mass filter or a linear ion trap with axial ion ejection. The instrument encompasses the functionality of an ion trap mass spectrometer, with its associated high sensitivity for product ion scanning, and that of a triple quadrupole mass spectrometer with the capabilities to perform MRM mode. An exhaustive description of the system and of its capabilities can be found in the work of Hager et al. [Hager 2002, Hager 2003]. Then, acquisition of EPI chromatograms of 402 *m/z* was made. The

extracted current of *m/z* 402 →358 transition (using a width of 0.5 *m/z)* was used for quantitative analysis. In figure 7.2 the MRM and EPI (402 → 358 *m/z*) chromatograms of a red wine spiked at 0.05 ng/mL level are shown.

Figure 7.2. 402→358 *m/z* MRM (a) and EPI (b) LC-MS/MS chromatograms obtained analyzing a red wine spiked at 0.05 ng/mL of OTA.



As can be seen, the sensitivity reached in EPI mode was lower than the sensitivity reached in MRM mode (S/N in MRM mode = 3.5 x S/N in EPI mode), but still sufficient to comply with current regulation requirements. Also in EPI mode the instrumental response was found to be linear in the range 0.05 to 25.0 ng/mL and no space-charge effects, typical of traditional quadrupole ion trap analyzer, were observed.

7.3. Method optimization: on line SPE-LC system conditions

The most critical parameters influencing method performance were: valve switching time and binary pump flow rate. These parameters were interdependent, because their combination influenced both recovery and chromatographic peak shape, and consequently the MQL. To optimize these parameters, the signal-to-noise ratio of OTA peak in the obtained chromatogram versus the two parameters, for a given amount injected, was monitored. A loading flow-rate of 1 mL/min and an 1.0 min loading time were found as optima (data not shown). Higher loading time gave analyte losses, while slower flow rates caused chromatographic peak broadening. Back-flushing elution of the extraction cartridge turned out to be better because it gave sharper chromatographic peaks. Regarding the sample volume injected, 200 μL was estimated sufficient to achieve a proper sensitivity; greater volumes were still amenable by the system with higher sensitivity (and lower MQL), but on the other hand the extraction cartridge duty cycle was diminished.

To avoid cartridge clogging after few analysis cycles when injecting wine samples, acidification of pump 1 mobile phases was necessary. Without acid addition the extraction cartridge underwent rapid clogging after few injections and, in addition, reproducibility was affected.

During method development, for quantification purposes, to achieve a better precision the use of ochratoxin B (OTB is like OTA with the chlorine atom missing) as internal standard was tested but no improvements were achieved, and on the contrary, a decrease of performance was noticed. This was, probably, due to the fact that the optimal SPE-LC conditions for the internal standard were different from those for OTA. External calibration procedure was therefore preferred.

7.4. Method performance

In the studied range (0.05-25 ng/mL), instrumental response was found to be linear showing a coefficient of determination (R^2) of 0.999 (table 7.1).

Table 7.1. Calibration curve parameters, recoveries relative to SWS and MILs[e] for OTA determination in beer and wine samples using the developed on-line automated system.

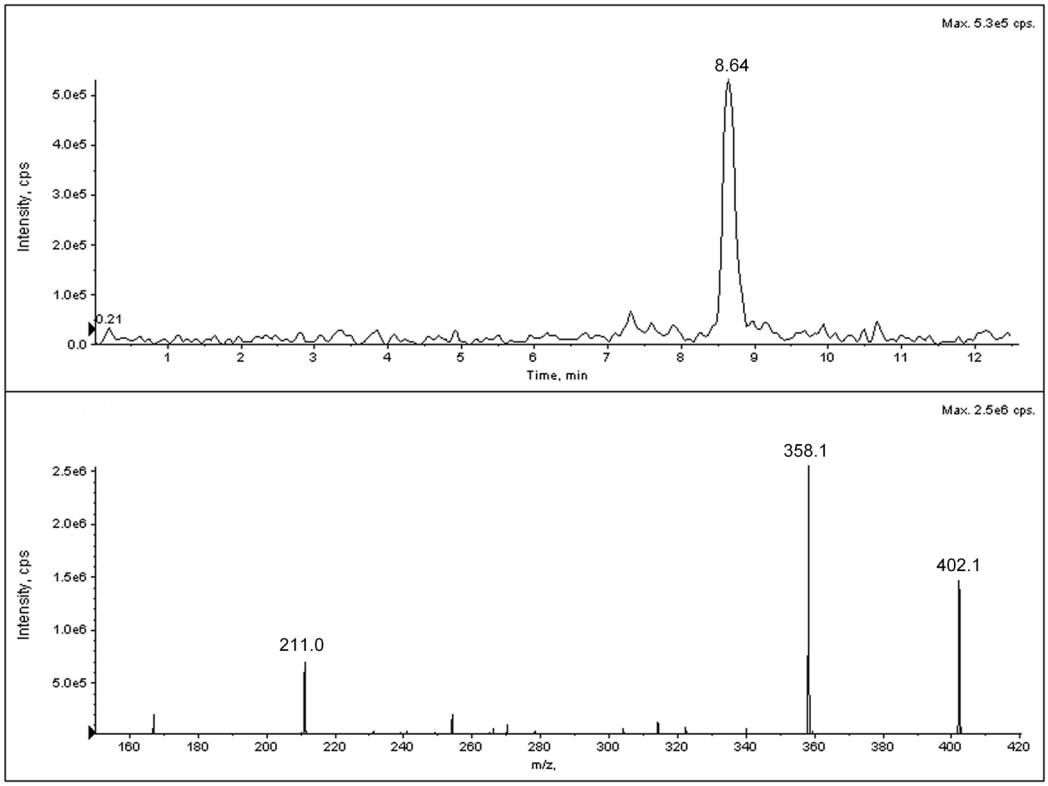
Calibration matrix[a]	Regression equation[b,c]	Coefficient of determination (R^2)	Recovery [d] ±RSD % (%)	MIL[c,e] (ng/mL)	OTA unspiked concn. (ng/mL)
SWS[f]	PA · 10^{-4} = 0.36 + 1.27C	0.999	–	0.007	–
	PA · 10^{-7} = 0.29 + 1.03C	0.998		*0.02*	
Beer	PA · 10^{-4} = 0.45 + 1.04C	0.999	82 ± 3	0.008	0.09
	PA ·10^{-7} = 0.37 + 0.85C	0.999		*0.02*	
White wine	PA ·10^{-4} = 0.44 + 0.96C	0.999	76 ± 4	0.01	0.09
	PA · 10^{-7} = 0.36 + 0.79C	0.999		*0.03*	
Red wine	PA · 10^{-4} = 0.63 + 0.94C	0.998	74 ± 4	0.01	0.31
	PA · 10^{-7} = 0.52 + 0.77C	0.998		*0.03*	

[a]Calibration from five points plus a blank, each in triplicate, in the range 0.05-25 ng/mL.
[b]PA = a + bC (PA: peak area; a: intercept; b: slope; C: concentration).
[c]Values in italic refer to EPI chromatograms, otherwise to MRM chromatograms.
[d]% signal respect to SWS. Means calculated from the ratio of the slopes of regression equation both in MRM and EPI mode.
[e]Method Identification Limit (see text for definition).
[f]Simulated Wine Sample.

The response was found linear in the same range also for wine and beer samples but, as can be noticed, total mean recoveries ranged from 82 to 74%. Signal suppression or analytical recovery related problems could be responsible for this matrix effect and individual contribution was not investigated. Analyzing several different types of wine and beer samples, matrix effect was noticed to be reproducible within each alcoholic beverage type, the relative standard deviation of the signal reduction coefficient found for each class of beverage was ≤ 4%. Thus, by using these coefficients, as correction factor, a good accuracy can be achieved. Specificity and high confidence in analyte identification are given by the

OTA fragmentation spectrum obtained in EPI mode. In figure 7.3 (top) the ion current profile for *m*/z 358 fragment ion from LC/EPI dataset of a beer sample naturally contaminated at 0.03 ng/mL is shown. In the bottom panel of figure 7.3 the background-subtracted EPI spectrum obtained at OTA retention time is also reported. As can be seen the characteristic fragment ions (namely 358, 314, 211 and 167 *m*/*z*) are all present. This demonstrates that with the new hybrid quadrupole-linear ion trap instrument, more stringent confirmatory data can be achieved without linearity range narrowing in instrumental response and with only a 3.5-fold decrease in S/N.

Figure 7.3. EPI scan of 402→358 *m*/*z* extracted current chromatogram (top) and EPI spectrum obtained analyzing a beer sample naturally contaminated at 0.03 ng/mL of OTA (bottom).



Within-day precision expressed as relative standard deviation (RSD %) evaluated from SWSs, as quality control samples, ranged from 4.8 to 6.1%. Between-day precision evaluated at the same concentration level ranged from 7.8 to 9.0%. The recovery % expressing the within-day and between-day accuracy ranged 96.3-105.5% and 95.3-109.4%, respectively. Values are reported in table 7.2.

Table 7.2. Precision and accuracy of the method for OTA determination on Simulated Wine Samples (SWS) using the automated on-line SPE-LC-ESI-MS/MS system[a].

OTA concn. in SWS (ng mL $^{-1}$)	Within-day precision (RSD %)	Between-day precision (RSD %)	Within-day accuracy (Recovery %)	Between-day accuracy (Recovery %)
1.0	6.1	8.1	104.2	107.6
3.0	4.8	7.8	96.3	95.3
15.0	5.3	9.0	105.5	109.4

[a] Three replicates of each Simulated Wine Sample (SWS) were done per single batch in five different days.

MIL was estimated as the minimum concentration giving a signal-to-noise (S/N) ratio = 3 for the second most intense MRM transition. The noise (N) used in the calculation is based on 3σ of the baseline signal and was directly provided by the instrument software. Since there is no meaning in quantifying an unidentified substance, although at MIL the S/N ratio of the most intense transition was 30, we set the MQL = MIL. The MILs were respectively 0.008 ng/mL for beer and 0.01 ng/mL for red or white wines. The second value is two hundred times lower respect to the maximum residue limit imposed by the current EU regulation for wine [EC 2005a].

The performance stability test made with 10 replicates of a red wine sample (alcohol 12% *v*/*v*, dried extract ~18 g/L) spiked at five concentration levels plus blank showed no carry over effect in blank samples during analysis, while values of relative standard deviation of signal intensities

(peak area counts) at the various concentration levels were in the range 4.7 to 7.7%. Thus, it can be assumed the apparatus is able to process up to 60 samples without performance deterioration. Consequently, this performance with minimum attendance (only daily routine instrument maintenance, sample filtration and autosampler loading are required) makes this method suitable to be employed in private and public laboratories involved in food safety controls.

7.5. Naturally contaminated wine and beer sample analysis

The method was tested, using MS/MS acquisition in MRM mode, in a survey of some wine and beer samples purchased in local stores. Sampling was made during April-June 2004 period. The purpose of the study was (other than to test the methodology) to have an indication of the possible average intake of OTA associated with wine consumption. Therefore the wines chosen were those commonly on sale in supermarkets in a medium price range. Also for beers the most common Italian and foreign brands that can be found on sale were chosen.

In table 7.3 the results of the survey are summarized. These are not surprising and in agreement with literature [Visconti 1999, Leitner 2002, Shephard 2003, Blesa 2004, Soleas 2001]. We planned this work, bearing in mind the WHO estimated tolerable weekly intake level for OTA of 100 ng/kg body weight [JEFCA 2001], when a lower MRL for wine in EU (0.5 μg/L, as originally proposed [SCF 1998]) was expected. A MRL of 2 μg/L means that a person of 70 kg body weight, drinking daily 250 mL of wine at still legal level of OTA, ingests with this beverage almost 50% of the tolerable intake. Among the wine samples analyzed, only 5/38 of red

wines and 1/7 of rosé wine have an OTA concentration between 0.5-2.0 μg/L, in agreement with other authors [Serra 2004]. This finding means that reasonable effort in improving wine-making practice may allow in a short time to revise high MRL without large economical consequence.

Table 7.3. Occurrence (incidence, mean of positives and range) of OTA in different wine and beer samples purchased in local stores in the period April – June 2004. The concentration levels were obtained using MS/MS acquisition in MRM mode.

Sample type	Incidence (pos/tot)	Incidence (%)	Mean (ng/mL)	Range (ng/mL)
Red wine				
Bottled	31/38	82	0.29	0.04-1.44
Boxed	5/5	100	0.33	0.17-0.87
White wine				
Bottled	8/13	62	0.16	0.03-0.42
Boxed	2/3	66	0.14	0.11-0.17
Rosé wine				
Bottled	5/7	71	0.32	0.10-0.82
Beer				
Bottled	5/12	42	0.07	0.02-0.14
Canned	3/6	50	0.09	0.04-0.12

7.6. Conclusions

In this section of the thesis, a completely automated and high-throughput method was presented, based on SPE-LC-(ESI)MS/MS for the determination of ochratoxin A in wine and beer samples. The method detection limit reached is at least two hundred times lower than current and proposed regulation. This result, in addition to the fact that up to 60 samples per day can be analyzed with minimum human intervention (only daily routine instrument maintenance and autosampler loading is required) makes it suitable for routine determination of ochratoxin A in wine and beer

samples. Consequently, this automated method could be employed in private and public laboratories involved in food safety controls. Finally, we demonstrated that with the new hybrid quadrupole-linear ion trap instrument, more stringent confirmatory data can be achieved without reduction of linearity range of instrumental response and with just a factor-three reduction in MDL.

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List of Papers

1) A. Laganà, R. Curini, G. D'Ascenzo, I. De Leva, A. Faberi, E. Pastorini.
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3) C. Cavaliere, G. D'Ascenzo, P. Foglia, E. Pastorini, R. Samperi, A. Laganà.
Determination of type-B trichothecenes and macrocyclic lactone mycotoxins in field contaminated maize.
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List of Abbreviations

15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
ADON	acetyldeoxynivalenol
AFM_1	aflatoxin M_1
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
ATA	alimentary toxic aleukia
BEN	balkan endemic nephropathy
C_{18}	*see* ODS
CAD	collisionally activated dissociation
C_f	correction factor
CID	collision-induced dissociation
CIN	chronic interstitial nephritis
CUR	curtain gas
CV	coefficient of variation
CXP	collision entrance potential
DAS	diacetoxyscirpenol
DON	deoxynivalenol
DP	declustering potential
EC	European Community
EDTA	ethylenediaminetetraacetic acid
ELEM	equine leukoencephalomalacia
ELISA	enzyme linked immunosorbent assay
EMAN	European Mycotoxin Awareness Network
EP	entrance potential
EPI	enhanced product ion

ER	enhanced resolution
ESI	electrospray interface/ionization
FAO	Food and Agriculture Organization
FB_1, FB_2, FB_3, FB_4	fumonisin B_1, B_2, B_3, B_4
FDA	Food and Drug Administration
FLD	fluorescence detection
FUS X	fusarenon X
FWHM	full width at half maximum
GC	gaschromatography
GCB	graphitized carbon black
GS1, GS2	gas 1, gas 2
HT-2	HT-2 toxin
IAC	immunoaffinity column
IARC	International Agency for Research on Cancer
IDA	independent data acquisition
IMoH	Italian Ministry of Health
IP	ionization polarity
IS	internal standard
IS	ion spray voltage
ISC	Istituto Sperimentale per la Cerealicoltura (Experimental Institute of Cereal Research)
IT	ion trap
LC	liquid chromatography
LIT	linear ion trap
MAS	monoacetoxyscirpenol
MDL	method detection limit
MIL	method identification limit
MIP	molecularly imprinted polymer
MQL	method quntification limit

MRL	maximum residue limit
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPDE	matrix solid phase dispersion extraction
MW	molecular weight
NEO	neosolaniol
NI	negative ionization
NIV	nivalenol
NL	neutral loss
ODS	octadecylsilica
OTA	ochratoxin A
OTB	ochratoxin B
P	probability
PA	penitrem A
PEP	porcine pulmonary edema
PI	positive ionization
PIS	product ion scan
PLC	primary liver cancer
PLE	pressure liquid extraction
PPG	polypropyleneglycol
PTFE	polytetrafluoroethylene
Q TRAP	quadrupole-linear ion trap instrument
Q1	first quadrupole
Q2 (q)	collision cell
Q3	third quadrupole
QqLIT	quadrupole-linear ion trap
QqQ	triple quadrupole
R^2	coefficient of determination

RCE	relative collision energy
RP	reverse phase
RSD	relative standard deviation
S	standard deviation
S/N	signal/noise
SAX	strong anion exchanger
SCF	Scientific Committee on Food
SPE	solid phase extraction
SRM	single reaction monitoring
SWS	simulated wine sample
T	temperature
T-2	T-2 toxin
TCA	tricarballylic acid
TISP	turbo ion spray
TLC	thin layer chromatography
t_R	retention time
UV	ultraviolet
WHO	World Health Organization
XIC	extracted ion current
ZAN	zearalanone
ZON	zearalenone
α-ZAL	α-zearalanol
α-ZOL	α-zearalenol
β-ZAL	β-zearalanol
β-ZOL	β-zearalenol

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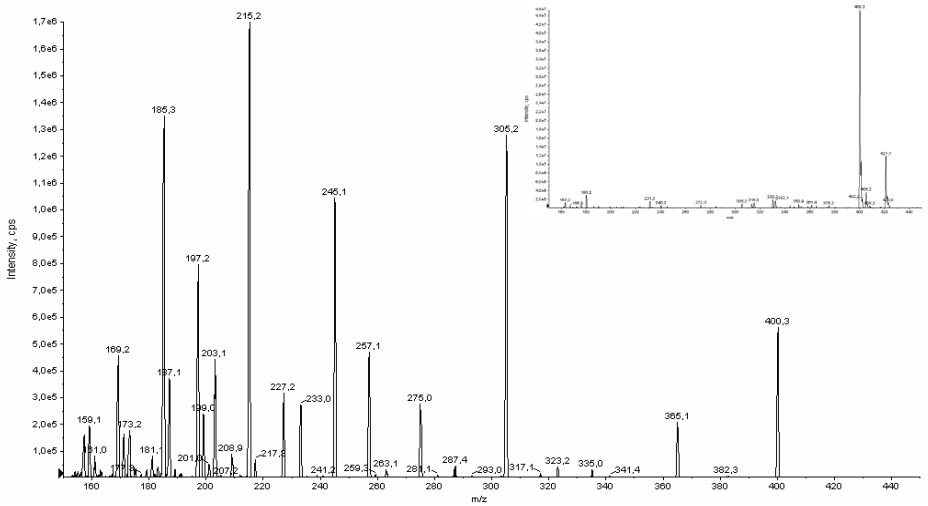
Supporting Information

MS/MS and MS (inset) spectra of trichothecenes A acquired in PI mode. The precursor ion selected is the $[M+NH_4]^+$ adduct.

NEO



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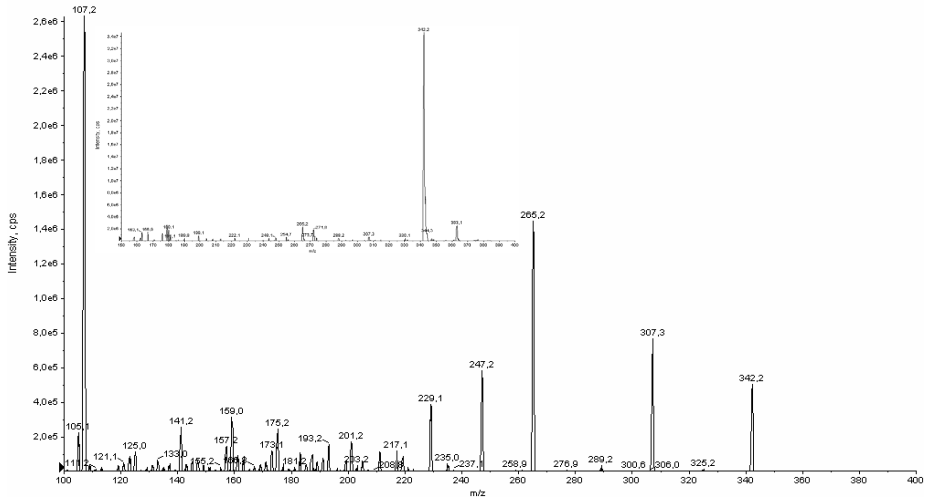

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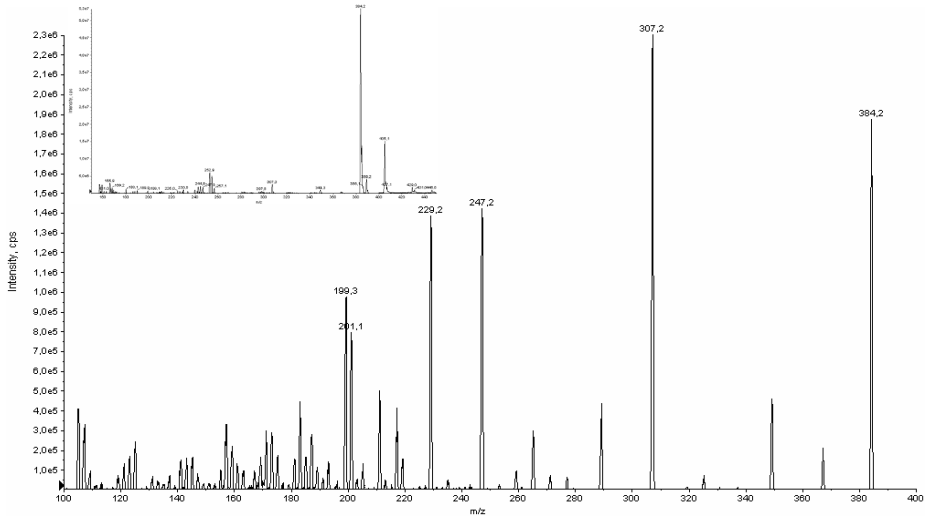
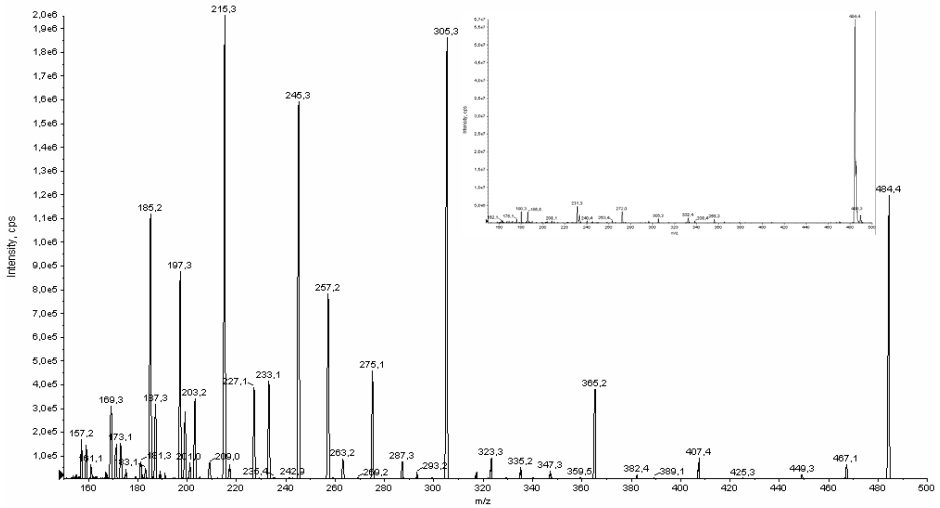
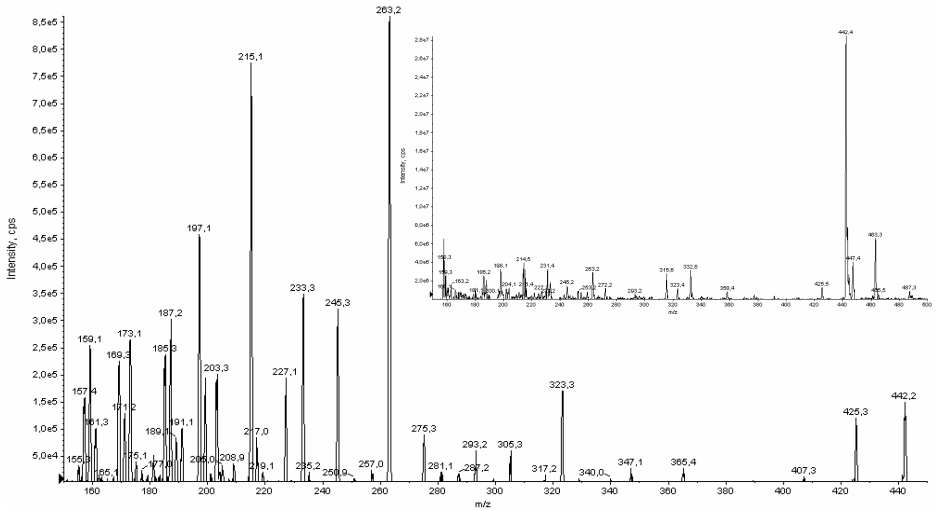



Intensity, cps
m/z
215,3
305,3
245,3
484,4
185,2
197,3
257,2
275,1
233,1
227,1
203,2
365,2
169,3
157,2
407,4
467,1
323,3
347,3
425,3
449,3

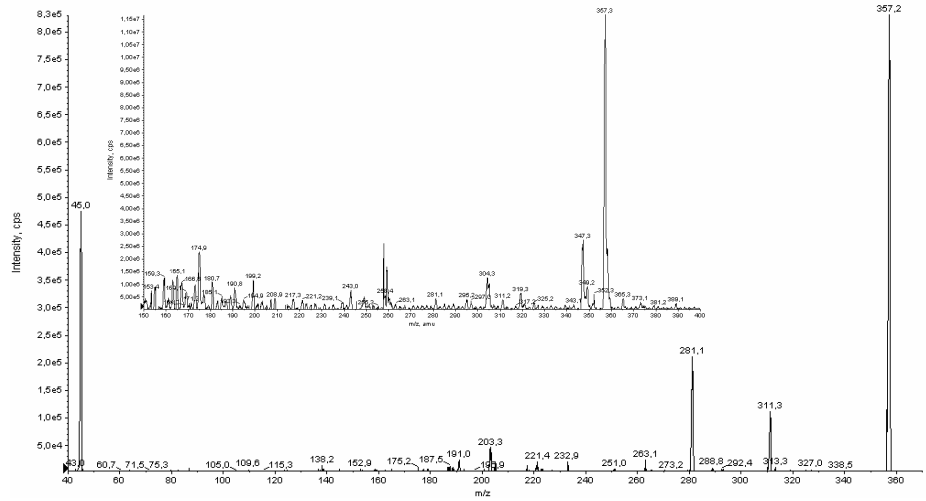
T-2


Intensity, cps
m/z
263,2
215,1
197,1
233,3
245,3
187,2
159,1
173,1
203,3
227,1
323,3
442,2
425,3
275,3
293,2
305,3
347,1
365,4
407,3

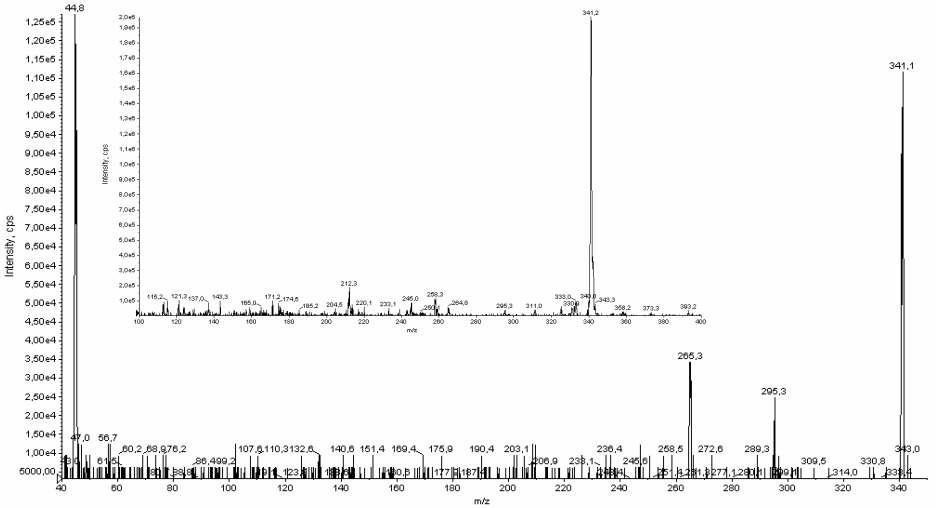
HT-2

MS/MS and MS (inset) spectra of trichothecenes B acquired selecting as precursor ion the $[M\text{-}HCOO]^-$ adduct for NIV and DON, the $[M+NH_4]^+$ adduct for FUS X and the $[M+H]^+$ ion for both ADONs.

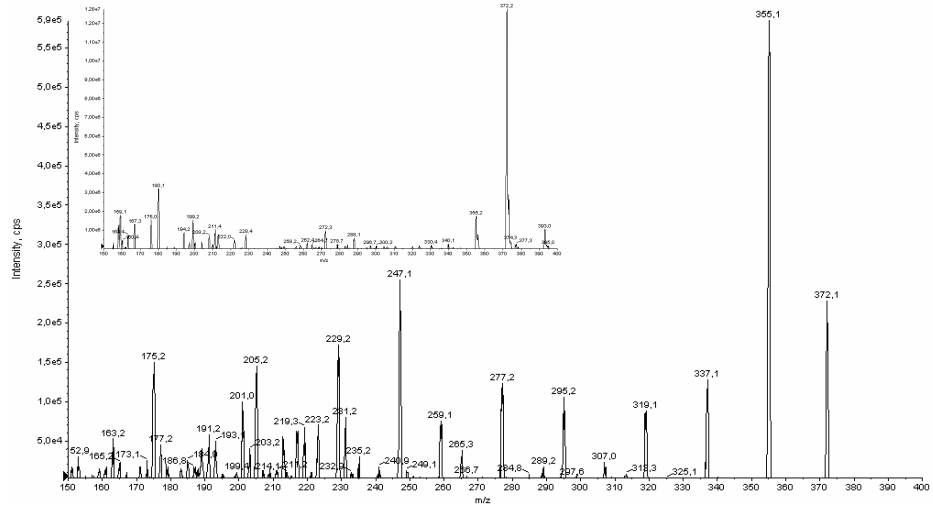
NIV

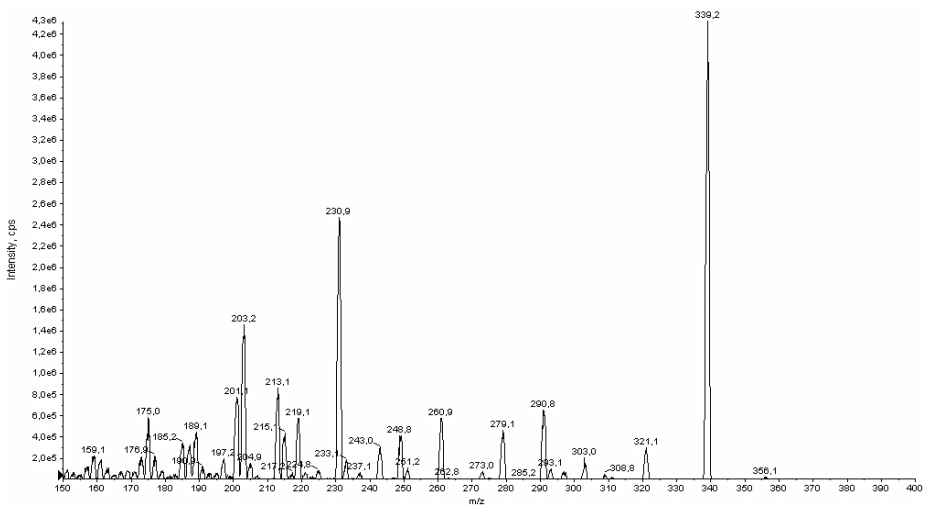


DON

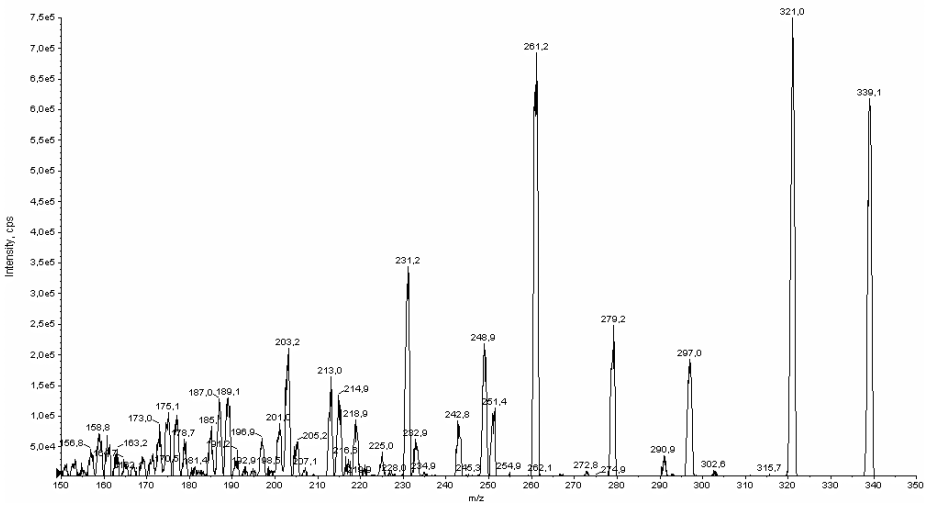


FUS X





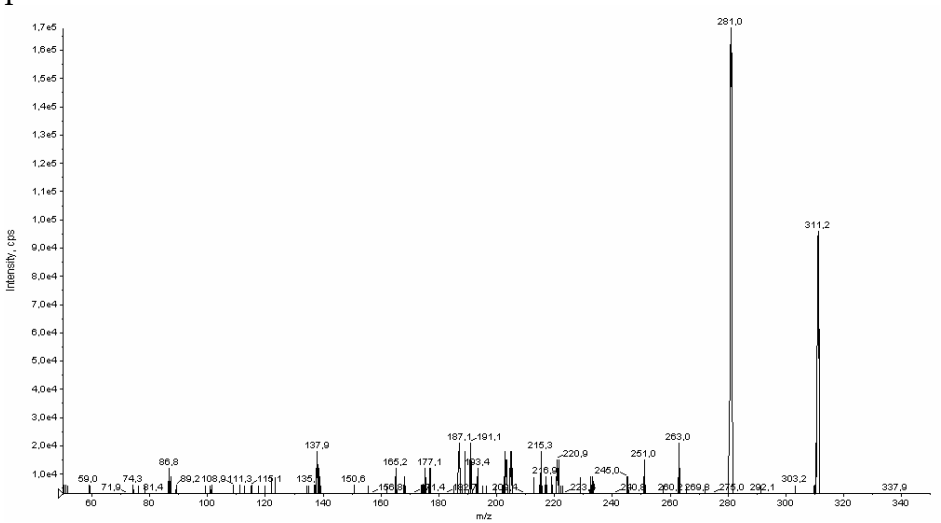
3-ADON



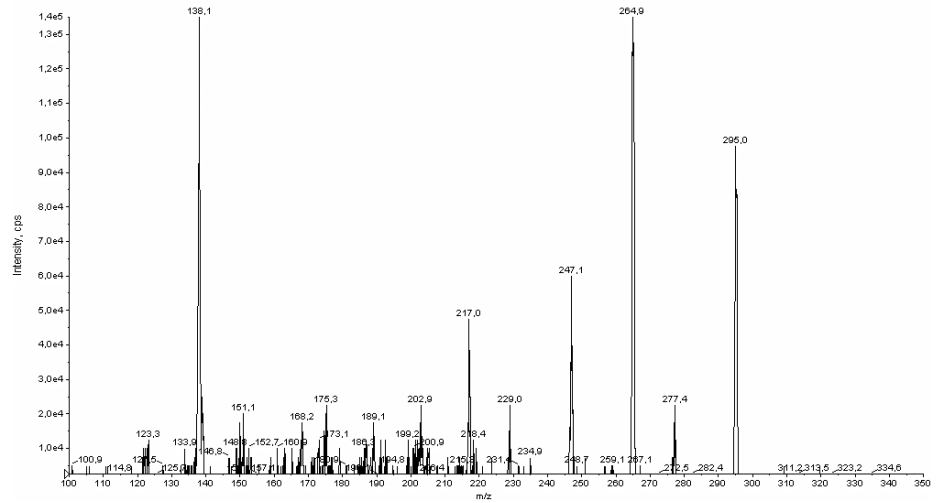
15-ADON

MS/MS spectra of trichothecenes B acquired in NI mode selecting $[M-H]^-$ as precursor ion.

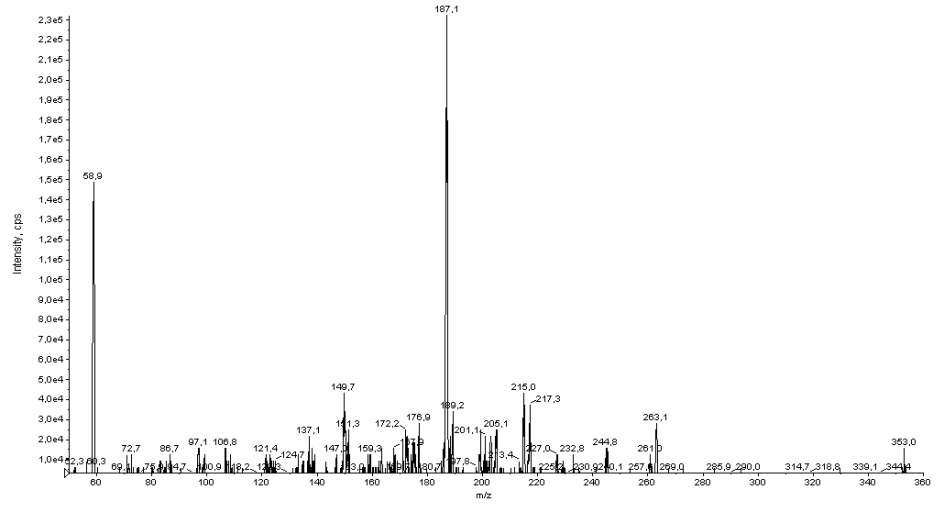
NIV

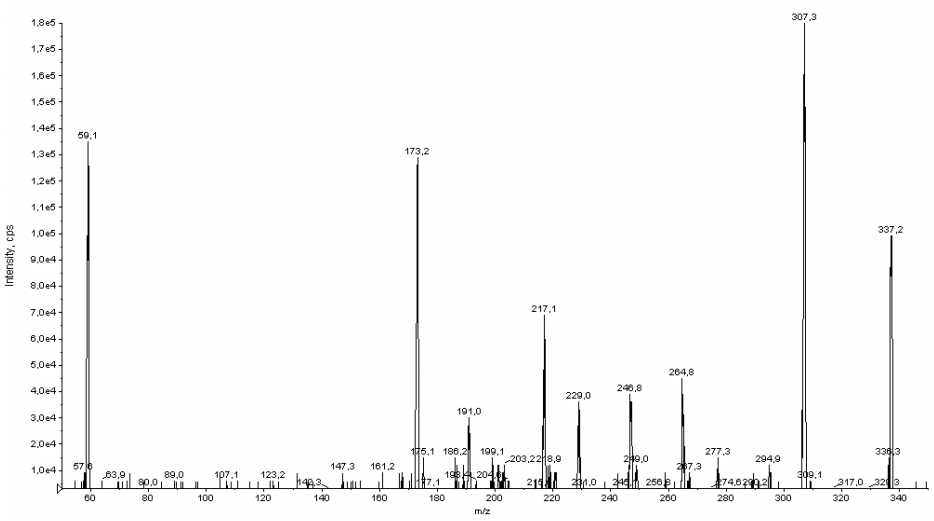


DON

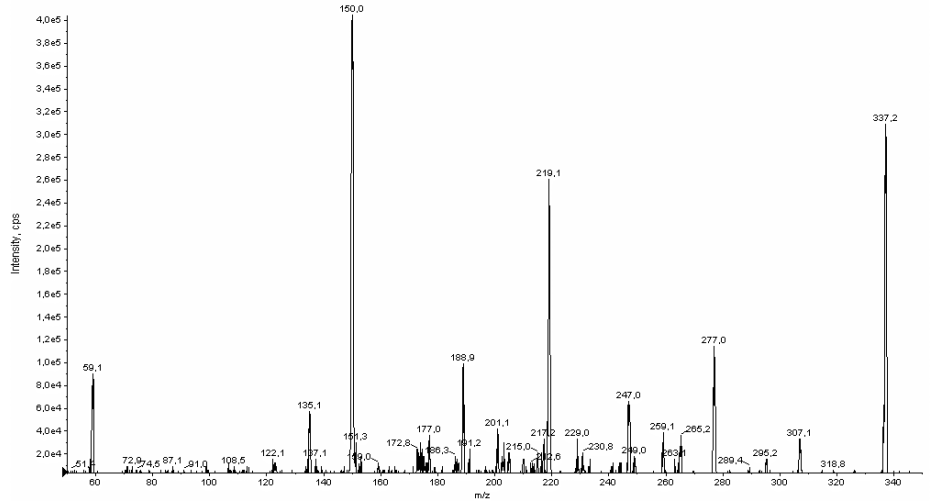


FUS X





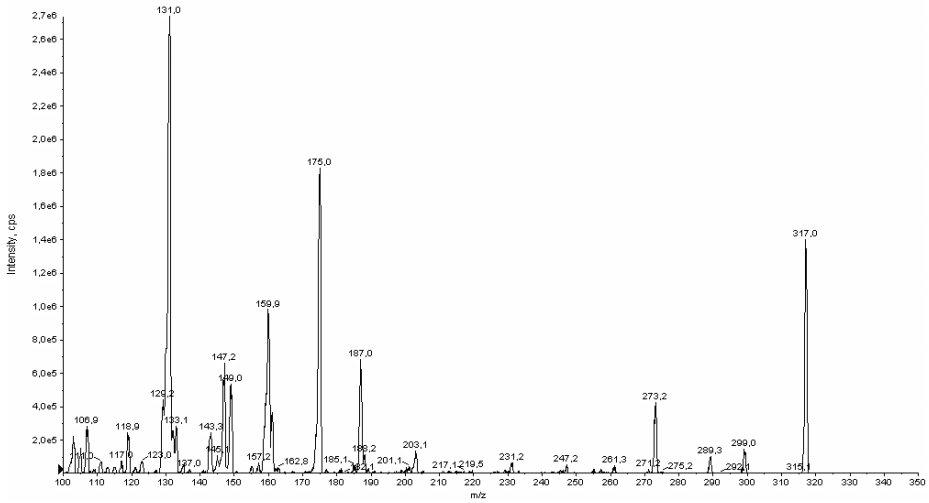
3-ADON



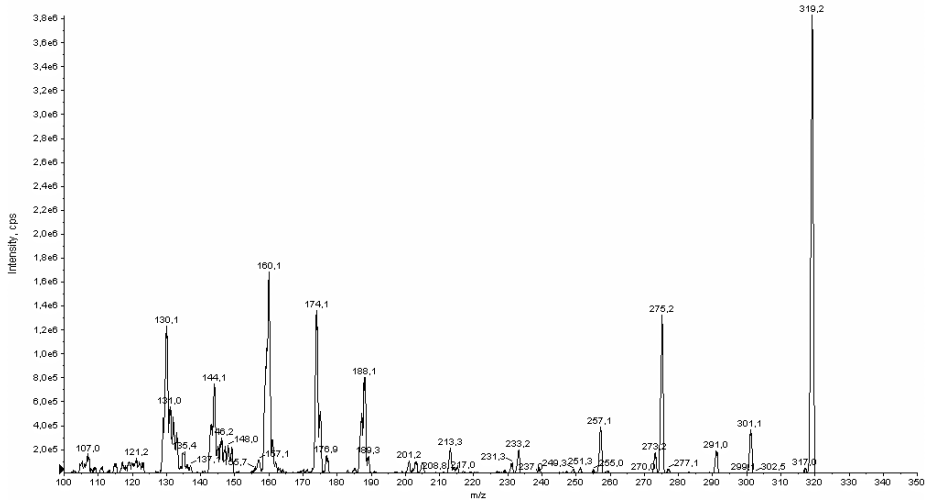
15-ADON

MS/MS spectra of zeranols acquired in NI mode selecting $[M-H]^-$ as precursor ion.

ZON

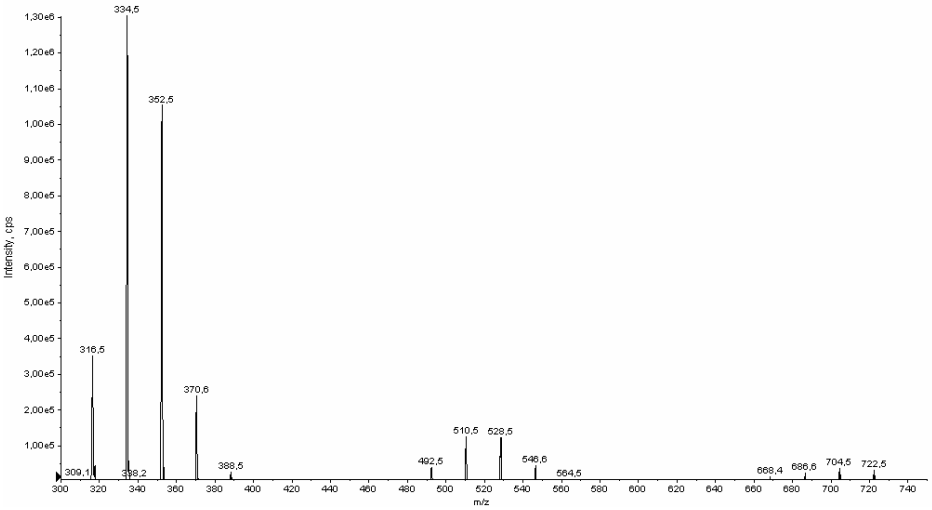


α- or β-ZOL



MS/MS spectra of B_1 and B_2 fumonisins acquired in PI mode selecting $[M-H]^+$ as precursor ion.

FB1



FB2