DEVELOPMENT OF ION TRAP HPLC-MS DETECTION METHODS FOR THE DETERMINATION OF PREVALENT MYCOTOXINS IN GRAIN AND APPLICATION TO REAL SAMPLES

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"Es ist nicht das Wissen, sondern das Lernen, nicht das Besitzen, sondern das Erwerben, nicht das Dasein, sondern das Hinkommen, was den größten Genuß gewährt." - Carl Friedrich Gauß (1777-1855)

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ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
a_w	water activity
bw	body weight
CID	collision induced dissociation
ELEM	equine leukoencephalomalacia
ESI	electrospray ionization
FAO	Food and Agricultural Organization of the United Nations
FB_x	fumonisin B_x
GC	gas chromatography
HACCP	hazard analysis and critical control point
HPLC	high performance liquid chromatography
ISTD	internal standard
JECFA	Expert Committee on Food Additives
LOAEL	lowest observed adverse effect level
LOEL	lowest observed effect level
LC-MS	liquid chromatography-mass spectrometry
LD_{50}	letal dose 50%
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
NOEL	no observed effect level
PEEK	polyetheretherketone
PMTDI	provisional maximal tolerable daily intake
PTWI	provisional tolerable weekly intake
RP	reversed phase
rrf	relative response factor
RSD	relative standard deviation
RSTD	recovery standard
RT	room temperature
SAX	strong anion exchange
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	selected reaction monitoring
TCA	tricarballylic acid
TDI	tolerable daily intake
WHO	World Health Organization

SUMMARY

The prevalent trichothecenes deoxynivalenol, nivalenol and HT-2 as well as zearalenone, zearalenol, ochratoxin A and citrinin are typical widespread mycotoxins in temperate climate zones mainly produced by the genus *Fusarium, Aspergillus and Penicillium*. Consequently, they co-occur frequently in the same agricultural commodities, especially in grain. Their simultaneous determination is highly desirable for screening method devices, due to the continuous need to protect the health of humans and animals by limiting their exposure to mycotoxins.

A rapid quantitative method for the simultaneous determination of the above mentioned mycotoxins was developed using HPLC coupled to an ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. It has been specifically developed for the rapid primary screening of cereal samples. The fast HPLC separation and APCI(+) detection which allows to detect mycotoxins of highly different polarity (lipophilic to ionic), make this method suitable for high sample throughput and routine analysis. Additionally, a fast and easy sample cleanup method was developed applying C18 and SAX SPE cartridges for the elution of mycotoxins with a wide range of polarity. Recoveries were in the range of 46-106 % and the limits of detection were between 25 and 125 ppb for different mycotoxins. Wheat and corn was chosen for the analysis of real samples.

Fumonisins belong to a substance class of mycotoxins, which was discovered recently (1988) and consists of long-chain methyl branched amino alcohols. Even nowadays the analysis of fumonisins is a big challenge not only because of the risk of the already known fumonisins in food, but also due to the fact that they form degradation and reaction products during food processing, which are not sufficiently characterized yet.

A method was developed for the quantification of fumonisins in corn based on reversed phase HPLC/MS and multiple MS. This lead for FB_1 to a improved LOD of 0.6 ppb and a LOQ of 8 ppb, which is a factor of five better compared to the former fluorescence method. Furthermore, a reduced chromatographic run time of up to 50 % could be applied successfully even for the analysis of real samples. Various corn products were examined for fumonisin contaminations. Products from the Swiss market were all below the tolerance level of 1 ppm.

To overcome matrix effects and guarantee to a reliable quantification of analytes an adequate ISTD was searched. Several approaches using uncomplicated and easy to perform synthesis was carried out adding protection groups to fumonisins. Unfortunately, the obtained products had either a too low stability under acidic conditions or the synthesis was not complete.

A total of 48 winter wheat samples from 1998 and 2000 were investigated by HPLC-MS for the presence of the trichothecenes nivalenol (NIV), deoxynivalenol (DON), neosolaniol (NEO), fusarenon-X (F-X), diacetoxyscirpenol (DAS), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), HT-2-toxin (HT-2) and T-2-toxin (T-2). Robustness of the applied technique under routine conditions was good and corresponding quality control information is given. The wheat samples were cultivated on a long-term field trial in

Switzerland with three different farming systems (biodynamic, bioorganic and conventional). DON was detected in 100%, NIV in 67% and HT-2 in 25% of all wheat samples. Only 48% of the detectable DON concentrations were above the limit of quantification (LOQ, 50 μg/kg). The range varied between 10-206 μg/kg. NIV and HT-2 were detected at concentrations around or below their LOQ (NIV: 100 μg/kg; HT-2: 10 μg/kg).

Statistically significant differences between the three farming systems could not be found although there were some indications that wheat from organic farming had lower DON contaminations than that from conventional farming. However, the results indicated that the presence and levels of (selected) trichothecenes may not be suitable as marker to differentiate between the three given farming systems. Furthermore, the results showed that even in years with low or nearly no fungal infection (warm and dry summers) low contaminations of DON in wheat are inevitable.

1. INTRODUCTION

Over the past decade, food protection from potentially hazardous residues has become a major public interest. Food regulation authorities, e.g. the Food and Agricultural Organization of the United Nations (FAO) created proposals for restrictions of unwanted residues in food. On a worldwide basis, at least 99 countries had mycotoxin regulations for food and/or feed in 2003 (see Figure 1.1), an increase of 30 % compared to 1995 (FAO 2003). Furthermore, regulations have become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology (Anklam et al. 2002; Gilbert et al. 2002).

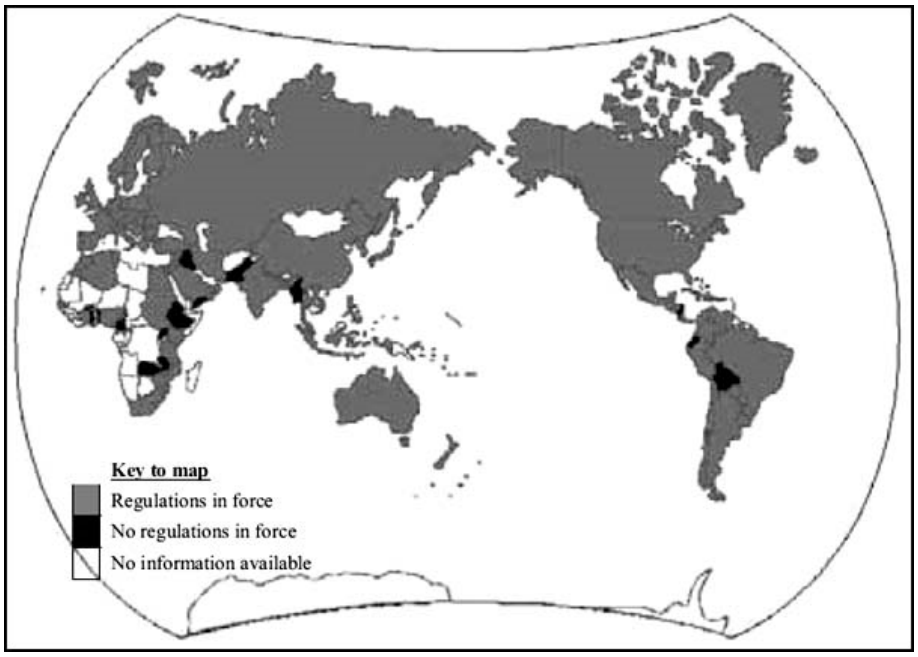

Figure 1.1: Countries with and without regulations for mycotoxins in 2003 (FAO 2003).

However, it is still a demanding task to provide food of the right quality that is nutritious and free from environmental contaminants, particularly in highly populated parts of the world. Within the large spectrum of natural toxins, mycotoxins cause considerable concern because of their ubiquity and potentially harmful effects on human and animal health. Moreover, the continuously increasing number of samples to be tested, related to the growing concern about food safety, requires methods that need to be simple, approved, inexpensive, fast and must meet safety standards of the laboratory waste problem.

Hazard Analysis and Critical Control Point (HACCP) is a system of food safety control based on the systematic identification and assessment of microbial, chemical and physical hazards in foods and the definition of means to control them. In order to develop effective HACCP-based integrated mycotoxin management programs, factors such as climate, farming systems, pre- and post-harvest technologies, public health significance of the contaminant, producer and processor compliance, availability of analytical resources and finally economy have to be considered (FAO 1979).

Mycotoxins are among the most widely studied natural toxins. Once, the infection occurred, they cannot be removed. However, risk analysis enables the definition of acceptable levels in food and feed supply: tolerances, guidelines and maximum residue levels. The Joint FAO/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) is the main organization for evaluating possible risks due to mycotoxins and various other contaminants in food and feed and provides FAO and WHO member countries with comprehensive scientific advice.

1.1 Metabolism of fungi

Most plant diseases are generated by fungal infection. Today about 300.000 different species of fungi are known. Among these, at least 10.000 are capable of causing plant diseases (Sachweh 1987). Fungi are chlorophyll-free, heterotrophic organisms that can be divided in macro- and micromycetes. During the vegetative development phase (trophophase) the primary metabolism synthesizes macromolecules and lipids as well as supplies energy and basic components for metabolism. Primary metabolites are mainly amino acids, vitamins and nucleotides, as well as products of the intermediary metabolism. Under a lack of nutrients or induced by a change of environmental conditions, a dysfunction of the metabolic equilibrium seems to be possible (Betina 1989). This dysfunction causes an enrichment of primary metabolites. Subsequently, they induce and activate the formation of enzymes, which convert them into secondary metabolites, e.g. mycotoxins. These metabolites are enriched in the cells, released into the surrounding medium or get further metabolized (“secondary metabolism”) (Reiss 1997).

In contrast to the primary metabolism, the secondary metabolism does not seem to be essential for organisms. Thus, nowadays a generally accepted theory for the task of the secondary metabolism does not exist, but there are presumable explanations like insufficient regularization of primary metabolites, the use of excess metabolites, decontamination reactions, defense effects by antibiotic inhibition of competitors or the prevention from being eaten by other microorganisms, including other fungi via formation of mycotoxins (Drogies 1997).

Furthermore, tremendous progress has been made in the past decade in understanding the genes that are associated with the production of various fungal secondary metabolites. The establishment of the "secondary metabolite cluster motif" and the identification of both pathway-specific and global regulators of these clusters resulted in the identification and manipulation of additional clusters. Coupling of secondary metabolism with morphological development of the fungus appears to be a universal constant in filamentous fungi and may indicate an underlying evolutionary mechanism important in fungal survival and possible aspects of pathogenesis (Yu et al. 2005).

1.2 Mycotoxins and mould species

Mycotoxins are secondary metabolites naturally produced by various endophytic moulds during plant growth or saprophytic moulds during storage of food or feed. Approximately 300 different mycotoxins formed by 350 species have been detected until now, which can be divided into 25 structural classes. When variations within species are considered, it is estimated that there are 10 000 mycotoxin producers which threaten foodstuff (Betina 1989).

A variety of these fungal metabolites, now known as mycotoxins, had originally been discovered as antibiotics, e.g. mycophenolic acid, penicillic acid, citrinin or patulin. Later, independent studies of their toxicity and isolation led to their characterization as mycotoxins. On the other hand, some compounds such as aflatoxins or ochratoxins had originally been recognized as mycotoxins and their antimicrobial properties were found only later (Betina 1989).

Mycotoxins are formed mainly by the species *Aspergillus*, *Fusarium* and *Penicillium* but only few of them are considered to be significant for human health due to their high concentrations or high frequency of occurrence in food (mainly cereals and groundnuts). Surveillance studies (Placinta et al. 1999) showed that the worldwide contamination of cereal grains and other feeds with *Fusarium* mycotoxins leads to wide spread problems of food supplies in many countries. The above mentioned moulds are probably the most prevalent toxin-producing moulds of the northern temperate regions and are commonly found in the temperate zones of America, Europe and Asia on corn, particularly maize, wheat and barley (Creppy 2002). These cereals are also the most important commodities concerning agricultural production (Josephs 1999).

The *Fusarium* species mainly produce the mycotoxins trichothecenes, fumonisins, zearalenone, moniliformin and fusarin C. They require temperatures between –3 and 37 °C with an temperature optimum between 25 and 30 °C and pH values which range from 2 to 10.5 (optimum at pH 6-7) and represent rather non-restraining conditions. Additionally, they need high moisture contents for growing (water activity coefficient a_w = 0.88-0.91) (Reiss 1997). Therefore, they usually affect crops before the harvest. Bad weather conditions in summer (e.g. heavy rainfalls, a low average temperature) and a late harvest are both factors leading to an increase of a possible damage of cereals. *Fusarium* species are also able to survive for years under disfavored climatic conditions and without the presence of suitable substrates because of their ability to form resistant chlamydospores (Prelusky et al. 1994).

In contrast to that, *Aspergillus* and *Penicillium* species mainly grow during storage. Mycotoxins produced by these species include aflatoxins, ochratoxins, patulin, aflatrem,

cyclopiazonic acid and fumitremorgins. The most well known mycotoxin, the potent human hepatocarcinogenic aflatoxin, is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. These moulds occur in warm climates and produce aflatoxin e.g. in drought-stressed maize and groundnuts in the field. They require moisture contents around a_w = 0.85 and temperatures of 12 to 42 °C with an temperature optimum of 35 to 37 °C (Reiss 1997) and pH values which range from 2 to >10.5 (optimum at pH 6.5-7.5).

1.3 Mycotoxin contaminations in food and feed

Mycotoxins pose a potential threat through the ingestion of food and feed products prepared from these commodities. A number of intoxications by mycotoxins (mycotoxicoses) have been described for intoxication of humans as well as farm animals. Human suffering includes ergot poisoning associated with ingestion of rye flour contaminated with ergot ("St. Anthony's fire"); cardiac beriberi is associated with *Penicillium* moulds in rice (yellow rice toxins); and alimentary toxic aleukia is associated with Fusarium moulds on overwintered wheat, millet, and barley. Although the adverse effects of moldy feeds was long known by livestock and poultry producers, an outbreak of "Turkey X disease" in Great Britain in 1960 was traced back to contaminated peanuts from Brazil. Aflatoxin was indicated as the cause of the death for more than 100.000 turkeys and 20.000 ducklings and pheasants. An overview of all mentioned mycotoxicoses is given by Betina (1989).

It is estimated by the FAO that at least 25% of the world's crops are contaminated with mycotoxins in both developing and developed countries (Prelusky et al. 1994). The global volume of such agricultural products as maize, peanuts, palm nuts and oilseeds, which are

high-risk commodities, is about 100 million tons - 20 million tons of which come from the developing countries (FAO 1996). Apart from crop losses, the costs on animal production are notable, although their evaluation is difficult. Factors influencing multiple aspects of chronic mycotoxicoses in food producing animals include reduced fertility, impaired immune status, lower feed utilization, reduced growth, etc. (Boutrif et al. 2001)

The health threat of mycotoxins in Central Europe is weather-related and estimated to be rather small due to the high standards of quality control in food production and processing. However, the problems increased in recent years due to a steadily growing import of food from tropical and subtropical areas that still have significant fungal contamination problems. Therefore, mycotoxin research was intensified the past years and consistently led to the detection of new substance classes, which may represent a substantial risk for human health.

Factors assisting the presence or generating the production of mycotoxins include storage, environmental and ecological conditions. The reasons for the production of mycotoxins are not clear up to now as described above. A single mould species may produce one or several mycotoxins and individual mycotoxins may be produced by different fungal species (Hussein et al. 2001). Moulds may grow well under a given set of conditions but not necessarily produce mycotoxins. However, it is supposed, that the formation of mycotoxins allows moulds to continue growing up. This results in a weakened immune system of the host plants and decreasing capability of resistance (Josephs 1999). Therefore, various factors for food and feed safety play an important role: injury-free harvest of the crop, cool and dry storage as well as the possible use of fungicides or fungi-resistant plant breeding. Additionally, insects are able to support an attack of cereals by moulds before harvest. Drying, heating, the use of

appropriate packing materials and the application of antidegradants are further suitable precautions against destruction and intoxication by moulds.

Although mycotoxin levels are usually low, possible interaction effects can occur. For example various additive (T-2 toxin/OTA and T-2 toxin/fumonisins) and synergistic (DON/Fumonisin B_1 and DAS/aflatoxins) effects have been reported (D' Mello et al. 1999). A carry-over of mycotoxins to animals and humans by metabolism has been observed in milk for ochratoxin A and for the transformation of aflatoxin B_1 to M_1, which is only one order of magnitude less toxic than B_1 (Creppy 2002).

1.4 Analytical methodology for mycotoxins

Important progress in the area of mycotoxin contamination of food and feed has coincided with the development of modern instrumental analysis. The need to analyze complex matrices for an increasing number of analytes and samples has caused an increasing sophistication of the analytical capabilities, especially of high performance liquid chromatography (HPLC). Therefore, this technique has emerged as a standard tool for the determination of mycotoxins in regulatory and research areas.

A number of gas chromatography (GC) and thin layer chromatography methods for mycotoxins still remain important, mainly since the coupling of GC with mass spectrometry (MS) provided a powerful, confirmatory tool. However, the technical difficulties of coupling HPLC to MS have been solved recently by advances in atmospheric pressure ionization techniques (see Chapter 1.6.2). Despite continuing advances in instrumental methods,

representative sampling and adequate extraction recovery still remain the main sources of analytical errors in mycotoxin analysis (Shepard 2001).

1.5 Sample pretreatment in food analysis

Sample preparation was often a neglected area, which over the years has received less attention compared to the chromatographic separation or detection stages. The more sensitive and accurate analytical techniques became, the more attention has been drawn lately to sample cleanup procedures (Buldini et al. 2002). According to Smith (2003) the trends in sample preparation over the recent years developed towards

- the ability to use smaller initial sample sizes even for trace analysis
- greater selectivity in extraction
- increased potential for automation or for online methods reducing manual operations and
- a more environmentally friendly approach (green chemistry) with less waste and the use of small volumes or no organic solvents.

In general, sample preparation consists of an extraction procedure that results in the isolation and enrichment of the compounds of interest from the matrix. Classical liquid extraction procedures are time consuming and are dependent on large amounts of solvents, which is expensive, generates considerable amounts of waste and potentially environmental and occupational hazards. Furthermore, they have limitations with respect to the range of polarity of the analytes, often provide very little selectivity and hardly offer possibilities for

automation. Therefore, over the past decade the search of alternatives to charcoal tubes, liquid-liquid and soxhlet extraction was the focus of research in developing more efficient sample pretreatment procedures. New technologies such as solid-phase extraction (SPE), supercritical fluid extraction (SFE) and sorbent traps are in general use nowadays. Additionally, new pressurized fluid extraction (PFE) approaches, including hot-solvent (accelerated solvent extraction) and hot-water extraction, microwave-assisted extraction, and microextraction approaches like solid-phase microextraction (SPME) followed by modern versions of solvent microextraction (such as single solvent drop approaches and other related techniques) also reduced solvent use (Pawliszyn 2003).

Different solid adsorbent materials to extract analytes from matrix solutions were developed in the 1970s and are now widely applied to many matrices including foodstuff. A widespread application of this sorbent technique is SPE using disposable cartridges. SPE sample pre-treatment can be done by means of trace enrichment, purification, solvent exchange, desalting, derivatization and class fractionation. Thus, the technique shows a high versatility, mainly due to the possible application of polar, hydrophobic and ionic interactions between analyte and sorbent. Furthermore, it has a high potential for automation (Bovanova et al. 2000; Rossi et al. 2000). The availability of cleaner and more reproducible manufactured sorbents compared to recent years facilitated its increasing range of applicability (Hennion 1999).

Generally, the extraction is performed online or offline in four steps: conditioning, retention, selective washing and elution. Method development in SPE is based on prediction from liquid chromatographic retention data or solvation parameters. It is accomplished by investigating

pH, ionic strength, sample volume, polarity, composition and flow rate of the solvents for conditioning, washing and elution as well as the physico-chemical properties of the sorbent bed (Buldini et al. 2002). A huge variety of sorbent materials are commercially available, e.g. aluminum, magnesium silicate, graphitised carbon and modified silica (RP-C18, NH_2, OH, etc.). Latest achievements in SPE include polymer based, mixed-mode and immunoactive (Hennion 1999) or molecular imprinted sorbent beds (Huck et al. 2000; Jodlbauer et al. 2002) for higher selectivity, the introduction of disk formats for better performance and faster extraction speed (Fritz et al. 2001) as well as innovations for automation and high throughput purposes.

1.6 LC-MS instrumentation

1.6.1 Impact of LC-MS for trace analysis in food science

Liquid chromatography combined with mass spectrometry (LC-MS) has during the past decade attracted increasing attention, especially in food, drug and environmental analysis, because the demand for sensitive and selective analyte detection methods in complex biological, environmental and food matrices can be met by this technique. Additionally, the ease-of-operation and the achieved level of automation makes this MS method an attractive tool in the areas mentioned above. In contrast to gas chromatography (GC)-MS, LC-MS is not limited to a small number of analytes with sufficient high volatility. A further advantage is that time-consuming and error affecting derivatization steps are only necessary in very few cases (Zöllner et al. 2003). The need for LC-MS methods has been further increased because

unambiguous analyte identification and accurate quantification are prerequisites in food and drug analysis, according to recent national and international laws and regulations.

Furthermore, tandem mass spectrometry (MS/MS) provides an advanced degree of certainty in analyte identification due to its high level of selectivity. It offers the possibility to eliminate the majority of potential interferences from the complex sample matrix, as well as from the mobile and stationary phase. From this point of view, it seems that sample preparation and chromatographic separation may be simplified or even eliminated to achieve the highest possible sample throughput (Zöllner et al. 2003). However, this is not the case since extensive cleanups and accurate separations still remain very important. Consequently, numerous LC-MS (and LC-MS/MS) applications have been developed or increasingly used to replace more laborious and time-consuming GC-MS methods.

1.6.2 LC-MS ionization techniques and interfaces

The coupling of HPLC with mass spectrometry has developed to a valuable analytic tool for the detection of polar, less volatile or thermally unstable compounds as well as to effectively reduce the complexity of mixtures within the last decade. For routine applications, the development of ion sources operating at atmospheric pressure ("Atmospheric Pressure Ionization", API) was an important improvement in MS technology. The interfaces for API, such as electrospray ionization (ESI) (see Chapter 1.4.2.1) and atmospheric pressure chemical ionization (APCI) (see Chapter 1.4.2.2), offer clear advantages in robustness and ease of use, and allow the development of routine and reliable LC-MS instrumentation for high sample

throughput. A further advantage is that the main part of HPLC eluent is separated before it enters the spectrometer, so that interferences of the high vacuum due to contamination can be avoided in most cases.

There are four main advantages of API techniques: i) they can handle flow-rates, that are typically used in LC; ii) they are suitable for the analysis of non-volatile, medium to highly polar and thermally unstable compounds as used in the liquid phase; iii) they are highly sensitive and approach sensitivities known from GC-MS and iv) they are comparatively robust and easy to use. Reviews are given by Niessen (1999; 2003). Table 1.1 shows the differences between ESI ionization (see Chapter 1.5.2.1) and APCI ionization (see Chapter 1.5.2.2).

Table 1.1 Differences between ESI and APCI ionization modes.

ESI	**APCI**
Flow rates between 0.005 to 1 ml/min	Flow rates between 0.2 to 2 ml/min
High spray voltage	High discharge current
Principle: ion evaporation	Principle: chemical ionization
Analysis of macromolecules until 200000 D, due to the presence of multiple charged species	Additional vaporizer to facilitate the evaporation of higher solvent amounts

1.6.2.1 Electrospray-ionization (ESI)

ESI is widely used for the analysis of polar and ionic compounds, which tend to form ion adducts. The formation of charges can be assisted by adding a salt or an acidic or basic buffer to the solvent. A schematic description of an ESI-interface and the mechanism of droplet evaporation is given in Figure 1.2.

The nebulization of liquid is achieved by the application of a high electric field resulting from a 3-5 kV potential difference between the ESI needle and the surrounding counter electrode, and the pneumatic nebulization. The resulting droplets shrink continuously by solvent evaporation during their flight. The remaining distance between the charges is steadily decreasing, resulting in a higher surface charge density and finally leading to a field-induced droplet disintegration ("Coulomb explosion"). This results in the formation of highly charged microdroplets. From the microdroplets, ions are ejected to the gas phase, either due to emission or desorption of preformed ions from the droplet surface (ion-evaporation-model) or due to the soft desolvation of preformed ions (charge-residue-model) (Niessen 2003). An overview is given by Bruins (1998) and Kebarle (2000).

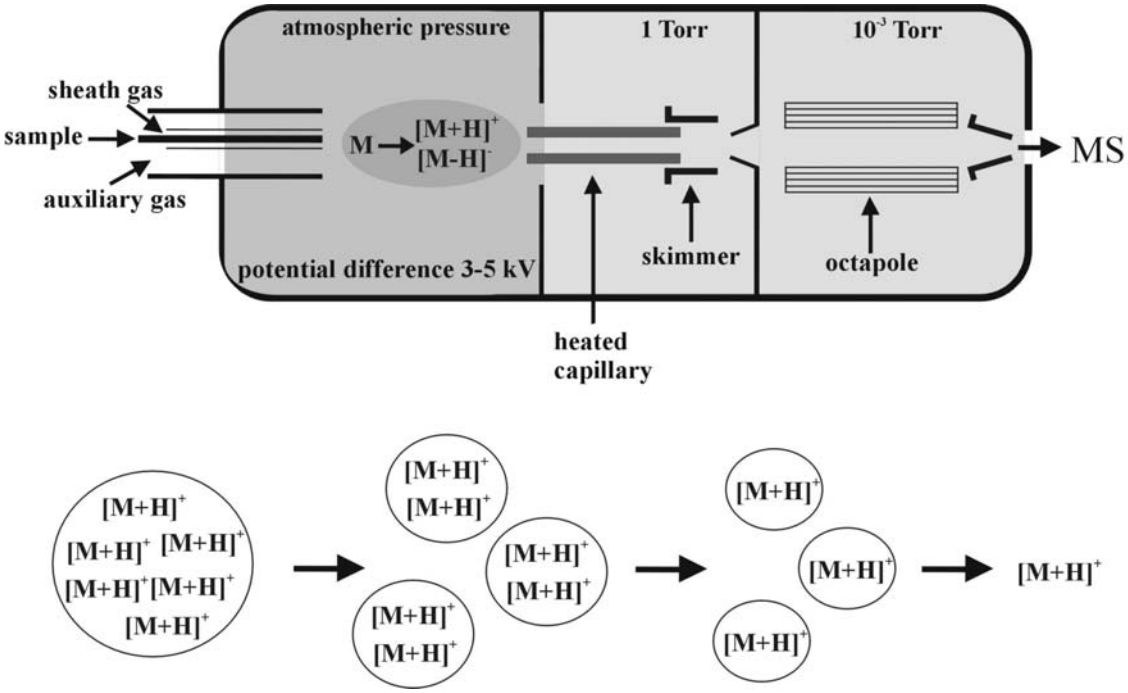

Figure 1.2: Schematic diagram of the components of an electrospray source and the mechanism of ion formation in ESI via droplet evaporation ("Coulomb explosion").

The signal intensity generated for a certain analyte depends on its ability to leave the droplet. This ability is caused by analyte specific properties like surface activity, solubility of the

analyte in a certain solvent, polarity as well as due to solvent properties such as surface tension and ionic strength (Cole et al. 1993). If the ionic strength in the solution is too high (Tang et al. 1991) or if there are other ions with a higher affinity to the droplet surface, ion suppression is the result. Tang et al. first described the mechanistic aspects of ion suppression in detail (Tang et al. 1993).

1.6.2.2 Atmospheric pressure chemical ionization (APCI)

APCI is the ionization technique of choice for the analysis of medium to less polar, small and thermally relative stable analytes. In contrast to ESI, solvent evaporation and analyte ionization are separated. The mobile phase is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube (up to 500 °C), i.e. solvent and analyte molecules are transferred into the gas phase. By applying a corona discharge from a needle (about 5 kV), reactive ions of the solvent are formed by ionization of the surrounding gases (N_2, H_2O, O_2) leading to hydronium ion-clusters $H^+(H_2O)_n$ (see Figure 1.3). Proton affinities of reagent gas ions relative to analyte ions strongly influence the sensitivity.

Assuming nitrogen is the sheath and nebulizer gas with atmospheric water vapor present in the source, then the type of primary and secondary reactions that occur in the corona discharge (plasma) region during APCI are as follows:

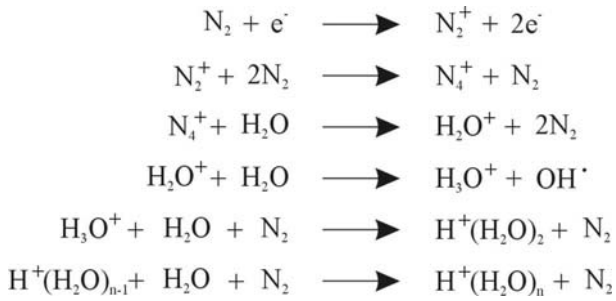

Figure 1.3: Primary and secondary reactions that occur in the corona discharge (plasma) region during APCI between reagent gas ions and analyte ions.

The high frequency of collisions results in a high ionization efficiency and thermalisation of the analyte ions. This results in spectra of predominantly molecular species by addition or abstraction of a proton ($[M+H]^+$ or $[M-H]^-$) and adduct ions with very little fragmentation (Rosenberg 2003). In contrast to ESI, APCI is able to handle higher flow rates in the range of 1 ml/min commonly applied in HPLC. Additionally, less or nearly no ion-molecule adducts (e.g. $[M+Na]^+$) are formed.

More details about APCI is given by Willoughby et al. (1998). A schematic description of an APCI-interface and the mechanism of APCI is given in Figure 1.4.

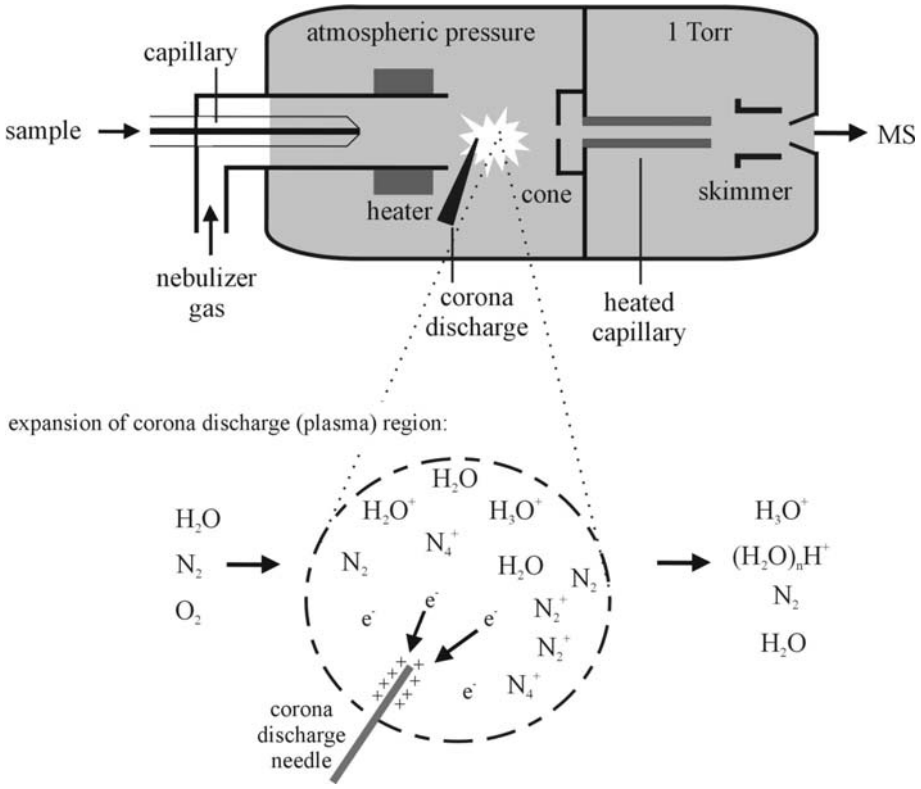

Figure 1.4: Schematic description of the atmospheric pressure chemical ionization (APCI) interface and the mechanism of ion formation in the corona discharge region.

1.6.3 Ion trap mass spectrometry

Ion trap mass analyzers become more and more important, mainly in combination with HPLC separations. The advantage of ion traps over quadrupole and magnet sector instruments is their ability to store ions. The ion trap consists of a ring electrode and two end-cap electrodes. The RF field applied to the electrodes is able to keep selected ions in stable trajectories. They are described by the Mathieu equation. If the field within the device is changed, the trajectories of simultaneously trapped ions of consecutive specific mass/charge ratio become sequentially unstable and ions leave the trapping field in order of their mass/charge ratio. The kinetic energy of the ions is removed by collision with helium atoms in the gas phase (at

approximately 0.1 Pa) focusing the ions towards the center of the trap. After ejection from the ion trap, ions strike a detector and provide an output signal. Figure 1.5 gives a schematic description of an ion trap mass analyzer.

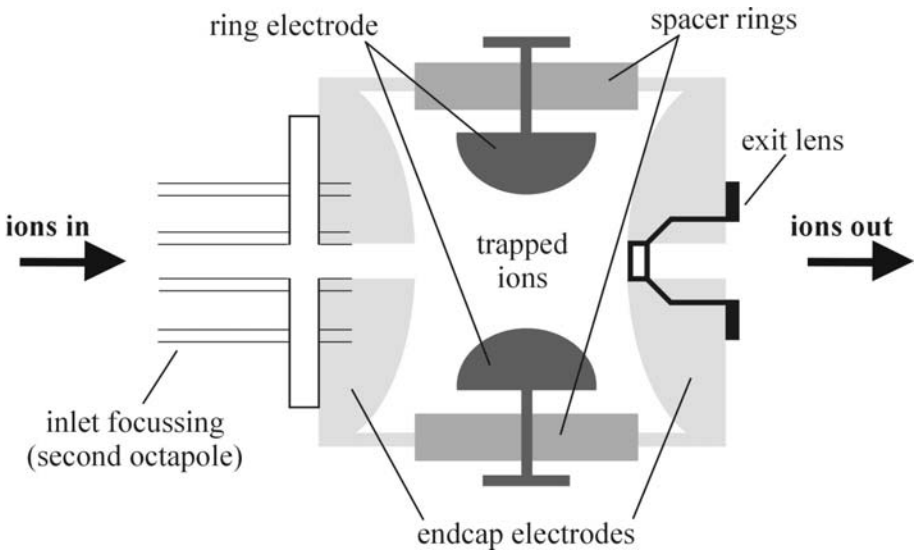

Figure 1.5: Schematic description of an ion trap mass analyzer.

The trap works discontinuous, i.e. there are certain cycles of filling the trap and of mass analysis. Due to the high scan speed, it can be employed for on-line detection nevertheless. It is important to guarantee an optimal filling of the ion trap; too many ions cause low resolution and if only less ions are trapped, sensitivity decreases. In order to guarantee the optimal filling of the trap the injection time for the analytic scan is determined by accomplishing a prescan ("automatic gain control"). A review is given by Jonscher et al. (1997) and March (2000).

The main advantages of the ion trap are its high sensitivity in full scan analysis and the possibility to record multiple mass spectrometry (MS^n) experiments. In the latter technique, trapped ions of a certain m/z ratio are isolated and additional collision-induced dissociations

(CID) lead to further fragment ions. If the fragment ion is stable enough and exhibits sufficient intensity, this process can be repeated several times (MS^3, MS^4, etc.). This represents an excellent tool for structure elucidation.

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2. EVALUATION OF BIODYNAMIC, BIOORGANIC AND CONVENTIONAL CULTIVATED WHEAT FOR TRICHOTHECENE CONTAMINATIONS

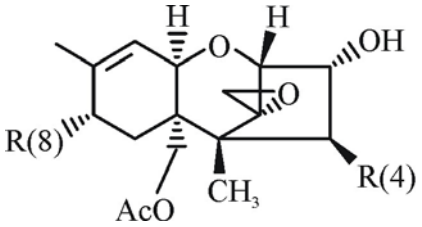
2.1 Introduction

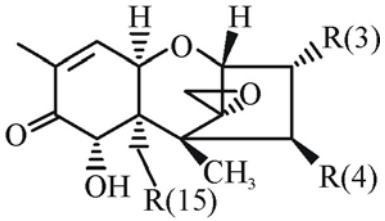
Over the past years, contamination of cereal grains and animal feed with *Fusarium* mycotoxins has been reported more frequently (Placinta et al. 1999). The responsible fungi infect cereals worldwide both during growth on the field and crop storage in humid environment. Besides the risk for human and animal health, this results in reduced crop yields, possibly additional costs for special treatments of infected grain and inferior food quality.

In recent years, not only the growing numbers of food scandals raised public awareness for our food quality. Thus, many people are changing their consumer behavior to an increased consumption of organic food, expecting them as healthier and possessing a higher nutrient content than conventional food. Therefore, the consumption of organic food has increased in Europe more than five-fold between 1993 and 2000 (Hamm et al. 2002). However, there is evidence that organic food often contains relatively high amounts of natural toxic compounds produced by fungi or plants, whereas the corresponding conventional food tends to contain more synthetic toxins as e.g. pesticides (Finamore et al. 2004), but there are only a few studies which evaluated detailed information about natural contaminants in dependence of the farming system.

2.1.1 Structure of trichothecenes

Trichothecenes are a group of more than 140 different mycotoxins, mainly produced by various species *of Fusarium* fungi (Grove 1993). They are the largest group of closely structural related toxins and are divided into four different groups according to their molecular structure. More than 148 natural members of this class have been reported (Lagana et al. 2001), although only a few of them play a significant role in cereal infestation. Their structure is based on a tetracyclic, sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system and they are subdivided into four different types (A-D) according to characteristic functional groups (see Figure 2.1).





Type A-trichothecenes			Type B-trichothecenes			
	R(4)	R(8)		R(3)	R(4)	R(15)
NEO	OAc	OH	**NIV**	OH	OH	OH
DAS	OAc	H	**DON**	OH	H	OH
HT-2	OH	i-Val	**F-X**	OH	OAc	OH
T-2	OAc	i-Val	**3-ADON**	OAc	H	OH
			15-ADON	OH	H	OAc

Figure 2.1: Structure of common trichothecenes. Abbreviations: NEO, neosolaniol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; T-2, T-2 toxin; NIV, nivalenol; DON, deoxynivalenol; F-X, fusarenon-X; 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol;

Type B-trichothecenes differ from type A by the presence of a carbonyl function at C(8). Type C is characterized by an additional epoxide function at C(7) and C(8) or C(9) and C(10), whereas type D includes trichothecenes containing a macrocyclic ring between C(4) and C(15). Epidemiological surveys have demonstrated that the predominant type A and B are

less toxic and widely distributed in cereals as natural contaminants, whereas the most toxic macrocyclic trichothecenes occur only rarely in food and feed (Krska et al. 2001; Tuomi et al. 1998).

2.1.2 Toxicity, occurrence and legislation

The toxicity of the trichothecenes is related to the epoxide function and shows a wide range of effects, such as feed refusal, skin irritation, emesis, diarrhea and multiple hemorrhages. Trichothecenes are also potent inhibitors of protein and DNA synthesis and are known to cause immunosuppression (Prelusky et al. 1994). The outbreaks of alimentary toxic aleukia reported in eastern Siberia (1913) and in southern Ural (1944) were due to a contamination with the type A trichothecene T-2 of grain harvested after a cold and wet winter. The most frequently detected trichothecene DON has a NOEL ("no observed effect level") of 0.1 mg/kg body weight (bw) (Creppy 2002). Some countries defined tolerance levels (from 5 ppb up to 1 ppm) for single trichothecenes (mainly DON), e.g. Switzerland provides a tolerance level for DON of 1 ppm on dry weight basis.

This diversity of trichothecenes causes a wide range of toxic effects in animals and humans such as food refusal, vomiting, anemia, hemorrhage and immunosuppression (Prelusky et al. 1994). However, only a limited number of the known trichothecenes have been identified in *Fusarium*-infected crops. Among these, deoxynivalenol (DON) is considered to be the most important *Fusarium* toxin in temperate zones (Bucheli et al. 1996) and is therefore together with NIV and ZON the most frequently investigated *Fusarium* toxin. In European agricultural commodities type A-trichothecenes usually occur less frequently and at lower concentrations

than DON (Krska et al. 2001), but only limited information is published on the natural occurrence of A-type trichothecenes such as T-2, HT-2, DAS and NEO until now.

2.1.3 State of the art in trichothecene analysis

The state of the art for the determination of A- and B-trichothecenes is represented by the use of multilayer cleanup columns followed by GC with electron capture detection or GC/MS after derivatization of the hydroxy groups to trifluoroacetyl, heptafluorobutyryl or trimethylsilyl derivatives. Various reviews have been published about applied analytical techniques (Krska et al. 2001; Langseth et al. 1998; Lin et al. 1998). HPLC in combination with post-column derivatization has been shown to be an interesting alternative. Recent studies about trichothecene determination by means of LC-APCI-MS (Berger et al. 1999; Razzazi-Fazeli et al. 2002) show the great potential of this technique for screening and quantification purposes of trichothecenes.

2.1.4 DOC trial

The DOC trial is a joint project of the Swiss Federal Research Station for Agroecology and Agriculture (FAL, Zürich-Reckenholz, Switzerland) and the Research Institute of Organic Agriculture (FiBL, Frick, Switzerland). It has been carried out since 1978 in Therwil in the northwestern part of Switzerland to compare biodynamic (D), bioorganic (O) and conventional (C) farming systems (see Table 1).

Table 2.1: Average input (1978-1998) of nutrients, pesticides and fossil energy to the different DOC trials. Soluble nitrogen is the sum of NH_4-N and NO_3-N. Input of active pesticides is based on years 1985-1991. Energy for production of machinery and infrastructure, in fuel and for production of mineral fertilizer and pesticides was calculated for 1985-1991 (Mäder et al. 2002).

Farm system*	Total nitrogen [kg N ha^{-1} $year^{-1}$]	Soluble nitrogen [kg N ha^{-1} $year^{-1}$]	Phosphorus [kg P ha^{-1} $year^{-1}$]	Potassium [kg K ha^{-1} $year^{-1}$]	Pesticides [kg active ingredients ha^{-1} $year^{-1}$]	Energy (GJ ha^{-1} $year^{-1}$)
D	99	34	24	158	0	12.8
O	93	31	28	131	0.21	13.3
C	149	96	43	268	6	20.9

* D (biodynamic), O (bioorganic), C (conventional)

The mentioned systems differ mainly in fertilization and plant protection. Furthermore, each farming system is subdivided into two treatments using different fertilizer intensities (trials 1 and 2). Crop and harvest are carried out in the same way for each treatment based on a crop rotation system (7 years). The trial is designed as a randomized block of fields including four replicates of each farming system. Together, there are 96 fields with a plot size of 5x20 m. The climate in Therwil is rather dry and mild with a mean precipitation of 785 mm per year and a mean temperature of 9.5 °C. The soil is a silty clay on loess (Besson et al. 1991).

2.2 Aim of work

The aim of this study was to evaluate the robustness and applicability of a recently developed HPLC-MS method for the quantification of trichothecenes (Berger et al. 1999) by studying both, the A- and B-type trichothecene contamination of winter wheat from two crop rotation periods, the third (1998) and fourth (2000) ones. The samples were cultivated on a long-term field trial in Switzerland (DOC trial) with three different farming systems (biodynamic, bioorganic and conventional). Furthermore, it should be investigated if trichothecene levels

were significantly different between the three different farming systems and if some compounds could therefore serve as biomarkers. A total of 48 winter wheat samples from the years 1998 and 2000 should be investigated by LC-MS for the presence of the trichothecenes. Based on their occurrence and levels in our region, the following mycotoxins have been selected for this study: Nivalenol (NIV), deoxynivalenol (DON), neosolaniol (NEO), fusarenon-X (F-X), diacetoxyscirpenol (DAS), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), HT-2-toxin (HT-2) and T-2-toxin (T-2).

2.3 Experimental

2.3.1 Chemicals and reagents

Mycotoxin standards of certified purity were purchased from Sigma Chemie (Buchs, Switzerland): DON, 3-ADON, DAS (> 99%); NIV, NEO (≥ 99%); F-X, 15-ADON (99%); T-2, verrucarol (≥ 98%) and HT-2 (> 97.5%). Hydrocortisone (purum, > 97%) was provided by Fluka Chemie (Buchs, Switzerland). Acetonitrile (190 far UV, > 99.9%) was obtained from Romil Ltd. (Cambridge, UK) and methanol (pestipur, > 99.8%) from SDS (Peypin, France). Water was obtained from an Elgastat Maxima HPLC water purification unit (Elga Ltd., Bucks, UK). Helium of 99.996% and nitrogen of 99.995% purities were used (Carbagas, Switzerland).

2.3.2 Wheat samples

A total of 48 winter wheat samples grown in 1998 and 2000 were selected from a long-term field trial in Therwil, Switzerland. 24 samples were collected per year (four per farming system and fertilizer intensity). To minimize interferences, samples were taken from a 20 m^2 area inside of the 100 m^2 replicate. 300 g of each were randomly collected and 100 g were ground with an ultracentrifugal mill ZM 100 (Retsch GmbH & Co. KG, Haan, Germany) at 18 000 rotations per minute and a 1 mm ring sieve. Samples were stored at 25 °C prior to analysis.

2.3.3 Extraction and sample cleanup

Various wheat samples were tested for the presence of verrucarol (VOL) and all of them were negative. To our knowledge there is no evidence in literature, that VOL is a naturally occurring trichothecene in wheat. Thus, 15 µg of the internal standard VOL in 150 µl of methanol were added to 10 g ground corn. The mixture was shaken for 2 h with 40 ml of acetonitrile/H_2O (84+16 v/v) on a wrist-action shaker LSL-V (Adolf Kühner AG, Birsfelden, Switzerland) with 200 movements per min and filtrated through folded cellulose filters of medium porosity (no. 311845, Schleicher & Schuell, Feldbach, Switzerland). A 4 ml aliquot was cleaned up on a MycoSep 227 trichothecene cartridge and a final cleanup was performed on a cleanup column No. 216 (both Romer Labs Inc., USA).

2.3.4 Separation and detection

Prior to analysis 1.5 µg of the recovery standard hydrocortisone in 150 µl of methanol/water (1+3 v/v) was added to the sample solution. HPLC separation was carried out on a C18 modified stationary phase (Nucleosil, 120 Å pore size, 3 µm particles, normal density, 125 mm column length, 2 mm inner diameter (i.d.), Macherey-Nagel, Oensingen, Switzerland). A linear binary gradient was applied (low-pressure binary gradient HPLC pump Rheos 4000; Flux Instruments, Basel, Switzerland) increasing from 25% to 98% methanol in water for 12 min, followed by 5 min rinsing with 98% methanol. Then, the methanol content was lowered to 25% within 1 min, and the column was re-equilibrated for 6 min. The flow rate of the mobile phase was 250 µl/min. An ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, USA) was used in the positive ion mode employing atmospheric pressure chemical ionization (APCI(+)). Mass spectra were acquired in the full-scan mode with a mass range of 150-500 u.

2.3.5 Quantification

Quantification was carried out using the mass chromatograms of the $[M+H]^+$ ions (internal and recovery standard, DON, HT-2) or the fragment ions *m/z* 294.9 + 312.7 for NIV. Limits of detection (LOD) were determined at a signal-to-noise ratio of 3:1 and limits of quantification (LOQ) at a ratio of 10:1. Sample extracts containing trichothecene amounts lower than 200 µg/kg were injected twice and the results averaged. Since recovery rates of trichothecenes hardly varied over a large concentration range, quantitative results were

corrected for recovery. Correction factors between the given toxin and the ISTD were not influenced by concentration over the whole calibration range (for NIV 1.2; DON 1.12 and HT-2 1.07 (Berger 2000)).

2.4 Results and discussion

2.4.1 Method performance

The applied methodology showed to be rather robust. Recoveries ranged from 74 to 107% with a mean recovery of 89% in 1998 and 91% in 2000 for DON. A typical coefficient of variation of the sample cleanup procedure was 4% (n=4) and of the whole method 8% (n=4). Differences between parallel determinations including extraction and cleanup did not exceed 20%, except for a few outliers. This somewhat higher value compared to method precision may be due to a slight to moderate heterogeneity of the samples, which is difficult to overcome. Retention times of DON varied less than 8% over a period of 12 h (n=10). Limits of detection were 10 μg/kg for NIV, 6 μg/kg for DON and 1 μg/kg for HT-2.

2.4.2 Impact of climate conditions

Oldenburg et al. ranked factors influencing infection of grain (especially wheat). Climate had the greatest impact followed by infection pressure/tillage, corn as preceding crop, plant protection, cultivars and plant nutrition (Oldenburg et al. 2000). Additionally, Park et al. showed that growth of *Fusarium* fungi is mainly triggered by humidity and relatively low

temperatures (Park et al. 1996). The rather low concentrations found in this study may therefore be explained by the climatic conditions of the summers 1998 and 2000 not favoring fungal growth. The weather was warmer and in most cases also dryer than the 10 year's mean (see Figure 2-2). Especially at anthesis in the months May and June, where the ears are most susceptible to *Fusarium* infection (Döll et al. 2002), it was significantly warmer with less precipitation.

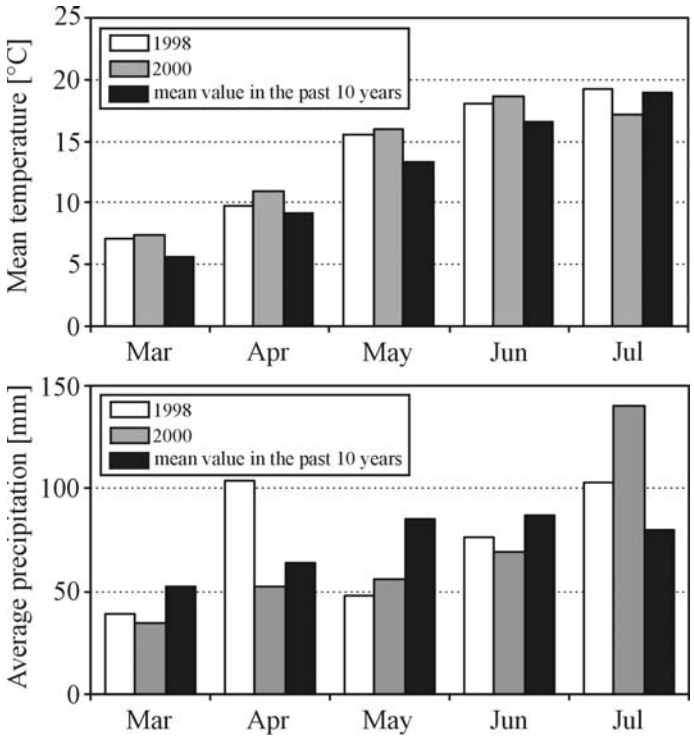


Figure 2.2: Monthly average temperatures and precipitation for March to July 1998 and 2000 in Liestal, Basel-Landschaft, Switzerland. Long term mean values (10 years) are given for comparison (Lufthygieneamt beider Basel, Liestal, Schweiz. Jahresdaten, Luftqualität und Meteorologie, Jahresrückblick (1998+2000)).

2.4.3 Trichothecene contents in wheat samples

In the 48 investigated samples only one type A-trichothecene (HT-2) and two type B-trichothecenes (NIV and DON) were present at detectable levels (see Table 2-2). DON was detected in all wheat samples from 1998 and 2000. However, concentrations were often below the LOQ of 50 µg/kg (52%) and can therefore only be considered as semi quantitative with a typical coefficient of variation of approx. 30%. They are presented in parenthesis.

Table 2.2: DON, NIV and HT-2 contents in wheat samples from the DOC trials in 1998 and 2000. The three different farming systems were biodynamic (D), bioorganic (O) and conventional (C) at two (1,2) different fertilizer intensities. Values are given in µg/kg. Concentrations of DON between the limit of detection (LOD) and limit of quantification (LOQ) are marked in parentheses.

	DON 1998	DON_{mean} 1998	DON s_d	NIV 1998	HT-2 1998	DON 2000	DON_{mean} 2000	DON s_d	NIV 2000	HT-2 2000
D1	(31)	40	18	<10	<1	(25)	27	24	1)	<1
	(22)			<10	<1	(10)			1)	<1
	(45)			<10	<1	61			1)	<1
	63			<10	<1	(10)			111	<1
D2	78	48	26	<10	<1	(41)	30	14	1)	<1
	(37)			<10	<1	(30)			1)	<1
	58			<10	2)	(40)			369	<1
	(18)			<10	<1	(10)			1)	<1
O1	142	75	51	1)	<1	(10)	48	44	1)	<1
	(45)			1)	<1	84			1)	<1
	84			1)	<1	(10)			1)	<1
	(27)			<10	<1	89			1)	<1
O2	110	74	32	<10	<1	(26)	46	34	1)	13
	(35)			<10	<1	85			1)	<1
	85			<10	2)	64			1)	<1
	67			<10	2)	(10)			136	<1
C1	129	105	60	1)	<1	(35)	52	23	1)	<1
	113			1)	2)	(40)			1)	<1
	(20)			1)	2)	(47)			1)	<1
	159			1)	13	85			1)	<1
C2	(21)	81	55	1)	<1	(29)	91	79	1)	14
	153			<10	<1	206			170	<1
	62			<10	2)	62			1)	<1
	87			<10	2)	66			177	2)

1) Value between LOD (10 µg/kg) and LOQ (100 µg/kg)
2) Value between LOD (1 µg/kg) and LOQ (10 µg/kg)

Figure 2-3 shows the frequency distribution of DON levels. The content in 13 % of the samples was below the detection limit of 10 µg/kg and in 86 % of the samples below 100 µg/kg. 46 % of the samples had contaminations above the LOQ, which is similar to the results obtained from Schollenberger et al. (1999) with 44 % of overall positive DON samples in German wheat.

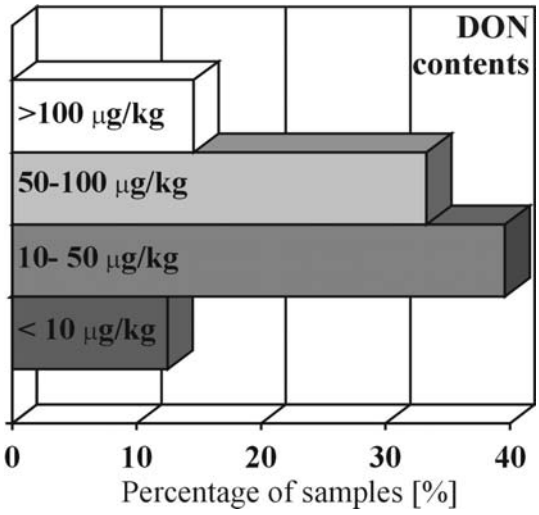


Figure 2.3: Frequency distribution of DON contents in µg/kg in wheat samples of the years 1998 and 2000. The total of samples was 48.

The range of DON concentrations was 10 to 206 µg/kg and the overall mean 60 µg/kg for both years and all farming systems. These values are comparable with the results by Noser et al. (1996), where wheat from the same region was investigated. DON contents below 300 µg/kg were reported for 1993 and 1994 and for 88% of the samples in 1995.

None of the observed DON levels in this study exceeded the Swiss tolerance value of 1 mg/kg on dry weight basis, the water content of 10-15% been taken into account. Moreover, the ubiquitous presence of DON in this region as documented by Bucheli et al. (1996) was confirmed due to its presence in every sample under investigation. This result shows that a low contamination of DON is inevitable in wheat. Berleth et al. (1999) found a comparable range of 14-184 µg/kg DON for wheat and rye from Germany in 1996. On the other hand, Schollenberger et al. (2002) reported DON contaminations of 15-1379 µg/kg in wheat samples from southern Germany harvested in 1999.

NIV and HT-2 were detected at concentrations around or below their LOQ (100 µg/kg for NIV and 10 µg/kg for HT-2, see Table 2-2). NIV was detected in 32 (67%) and HT-2 in 12 samples (25%) at levels similar to those observed by Müller et al. (1997b) and Schollenberger et al. (2002). However, NIV and HT-2 were found in a higher percentage of samples than for other studies from the same region (Müller et al. 1997a, Schollenberger et al. 1999; Müller et al. 1997b) where NIV was present in 11-64% and HT-2 in 0-13% of all samples. One sample from the year 2000 contained a comparable high NIV concentration of 369 µg/kg probably caused by an artifact, e.g. inhomogeneity of the wheat sample. Whitaker et al. (1998) showed that mycotoxins are likely to be inconsistent distributed in naturally contaminated grains, which comes from a random distribution of fungi on the fields. Therefore, an accurate sample selection and preparation are critical to generate useful data. In contrast to the studies mentioned above, this investigation did not detect further trichothecenes. Possible reasons could be climatic differences or the higher selectivity of the applied MS technique.

The average of DON concentrations was 30% higher in 1998 (interquartile range 52 µg/kg in 1998 and 39 µg/kg in 2000). Though temperature was lower in 1998 (see Figure 2-2), the data do not allow to consider the observed difference as significant. NIV was detected in 33% of the samples in 1998 compared to all the samples in 2000. For HT-2 there was a decrease in the number of positive samples from 38% in 1998 to 13% in 2000.

A survey of the DON contamination of the three farming systems (biodynamic, bioorganic and conventional) is presented in Figure 2-4. Mean values and standard deviations are given for 1998 and 2000. Slightly higher levels of DON have been observed for conventionally

grown wheat including concentrations below the LOQ (see Table 2-2). However, the difference was not statistically significant (P< 0.99 in 1998 and P< 0.95 in 2000). A similar trend was also found in preceding studies (Berleth et al. 1999; Bucheli et al. 1996). Schollenberger et al. (2002) reported higher concentrations in conventionally grown wheat (median 295 µg/kg) compared to biodynamic farming (median 120 µg/kg). Moreover, Döll et al. (2002) found more than 1 mg/kg DON in 23% of the samples of conventionally grown wheat compared to 9% in organically grown wheat. Similar to the study presented here, the data from the conventional farming system were more scattered.

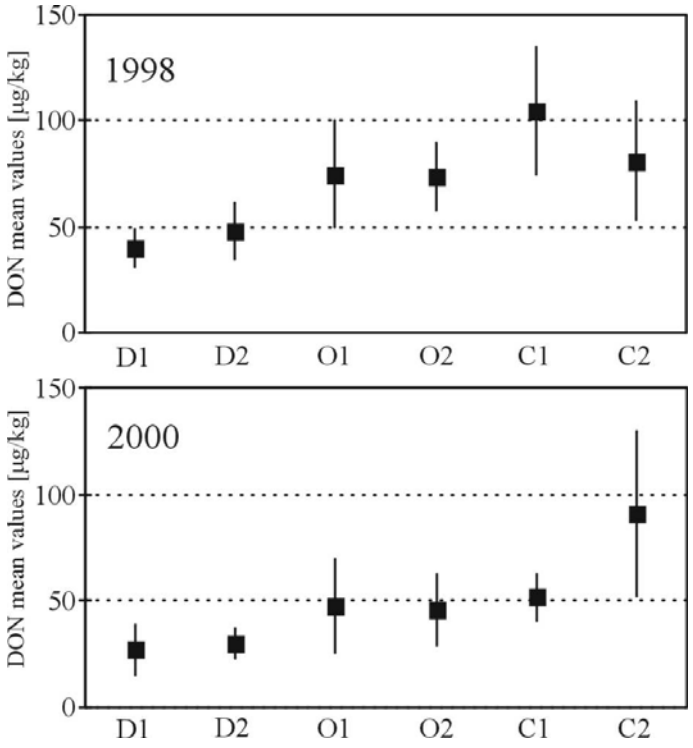


Figure 2.4: Mean DON concentrations (n=4) and standard deviations (s_d) in wheat samples from the farming systems biodynamic (D), bioorganic (O) and conventional (C) at two different fertilizer intensities (1 and 2) for 1998 and 2000.

The literature comparing mycotoxin content in wheat from organic and conventional farming contains quite contradictory information in some cases. An investigation from southern Germany (Marx et al. 1995) did not show any difference (occurrence in 88% and 76% of all samples and mean contents of 420 μg/kg and 486 μg/kg, respectively). Furthermore, Cirillo et al. (2003) found slightly higher DON contaminations in conventional corn and wheat products, while the amounts of rice based and mixed foodstuff showed slightly higher DON contaminations in organic products. Malmauret et al. (2002) reported significantly higher concentrations of DON in organically (median 106 μg/kg) than in conventionally grown wheat (median 55 μg/kg) in France. Additionally, they detected NIV, HT-2 and 3-ADON in organically grown wheat only (medians 10 μg/kg, 50 μg/kg and 10 μg/kg, respectively).

This study revealed more samples with NIV and HT-2 in conventionally farmed wheat in 1998. However, this was not the case for NIV in 2000. These results suggest that the farming system is not one of the major factors of trichothecene occurrence and concentration in grain. Other parameters like climate while anthesis or harvest (high moisture content of grain kernels at harvest), infection pressure/tillage, corn as preceding crop, plant protection, cultivars, plant nutrition or the random distribution of fungi on the fields might be more significant and should be considered for production of grain with low levels of mycotoxins.

2.4.4 Geographic distribution of DON contents

The geographic distribution of DON contents in the trial field is given in Figure 2-5. It shows that there is no region containing a increased regional accumulation of trichothecenes. This fact excludes the possibility of a punctual or local infestation of fungi. The mentioned two fertilizer intensities for each farming system are parallel to each other. It is obvious, that there is no correlation between the two intensities for both years.

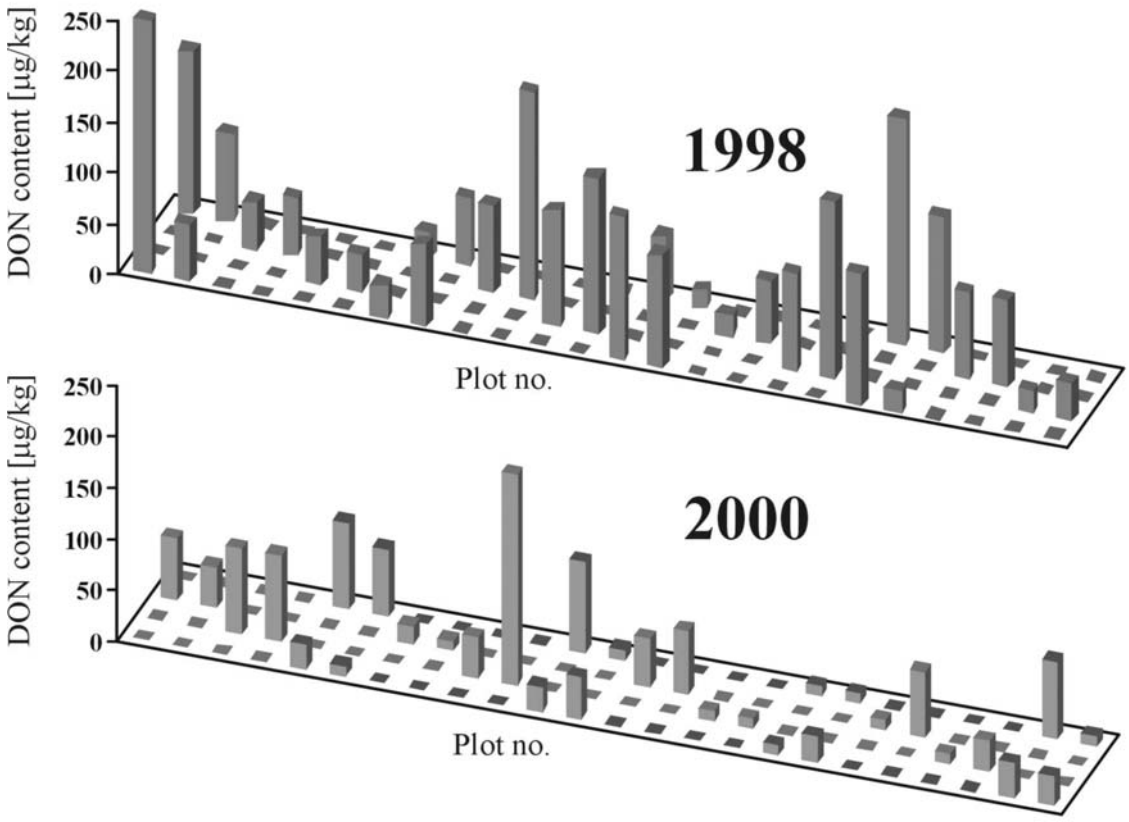


Figure 2.5: Geographic distribution of DON contents in µg/kg wheat in samples of the DOC trials of the years 1998 and 2000. All 96 plots of the trials are shown, starting with plot no. 1 in the left front. The numbers of the following plots are increasing to the right and backwards.

2.5 Conclusions

The presented study compared the levels of trichothecenes in biodynamic, bioorganic and conventional grown wheat of the years 1998 and 2000. In conclusion, a tendency of lower levels of DON in biodynamic wheat compared to bioorganic and conventional farming methods was observed for both years. However, the statistical significance could not be proven at a reasonable confidence level. Due to the low, rather scattered levels found in most samples at least one order of magnitude more samples would be required to draw any conclusion. Unfortunately, this would possibly exceed any cost frame when applying LC-MS. In general, the results of this study demonstrate that a conclusion about differences in quality of organic and conventional food requires further study and effort as a demand from the steadily growing market of organic products.

Nevertheless, the results indicated that the presence and levels of (selected) trichothecenes may not be suitable as marker to differentiate between the three given farming systems. Furthermore, the results showed that even in years with low or nearly no fungal infection (warm and dry summers) low contaminations of DON in wheat are inevitable. A difference between the two fertilizer intensities could not be observed in both investigations.

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3. DEVELOPMENT OF A LIQUID CHROMATOGRAPHY/ MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF FUMONISINS IN CORN

3.1 Introduction

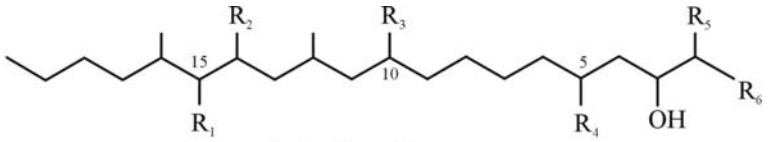
In 1988 Gelderblom et al. (Gelderblom et al. 1988) succeeded after eighteen years of research to isolate of a new class of mycotoxins, the so-called fumonisins. In the same year the structures of fumonisin B_1 (FB_1) and B_2 (FB_2) have been successfully elucidated by Benzuidenhout et al. (1988). Thereby, the predominant fungus of fumonisin production *Fusarium verticillioides* (*F. moniliforme*) was isolated from moldy corn during an outbreak of the epidemic cattle disease "equine leukoencephalomalacia" (ELEM) in South Africa in 1970.

Even nowadays the detection of fumonisins is a big challenge not only because of the risk of the already known fumonisins (see Chapter 1.3.2.) in food, but also due to the fact that they form degradation and reaction products during food processing, which are not sufficiently characterized yet. The example of hydrolyzed FB_1 (HFB_1) showed that the danger of contaminated food can be caused as well from a degrading product of the fumonisins (Hendrich et al. 1993). Thus, a better understanding of the reaction products of the fumonisins in food becomes an important task. This requires the development of an analytical method capable of detecting known fumonisins selectively and with high sensitivity so that reliable qualitative and quantitative analytical results are obtained. At the same time, this method must allow the analysis of novel structure-related toxins.

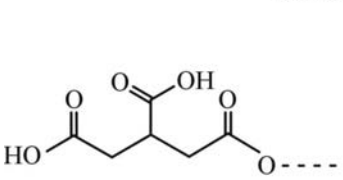
3.2 Fumonisins

Fumonisins are a class of water-soluble mycotoxins, which are mainly produced by *Fusarium moniliforme*, *Fusarium proliferatum* and *Aspergillus ochraceus*. The ubiquitous spread of these moulds is on corn plants, but fumonisins have been also detected in tea and medicinal plants (Martins et al. 2001; Omurtag et al. 2004), asparagus and garlic (Seefelder et al. 2002), as well as in rice, sorghum, barley and wheat (Kim et al. 2002). Their production results from stress situations of the fungus, e.g. drought. Therefore, higher concentrations of fumonisins can be observed in dry compared to more humid regions (Miller 2002). Today seventeen fumonisins are known as shown in Figure 3.1, three of them occur worldwide in higher concentrations: FB_1, FB_2 and FB_3.

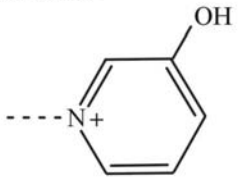
They are characterized by a 19 to 20 carbon aminopolyhydroxyalkyl-chain which is diesterified at the positions 14 and 15 with propane-1,2,3-tricarboxylic acid (tricarballylic acid, TCA). Another series of naturally occurring fumonisins (FP_1, FP_2 and FP_3) possess a 3-hydroxypyridin instead of the amino function (Musser et al. 1996). Hydrolyzed fumonisins generated by the loss of one or two TCA chains ($PHFB_{1a}$, $PHFB_{1b}$, and HFB_1) were also detected (Hopmans et al. 1993).



basic fumonisin structure



Tricarballylic acid (TCA)



3-Hydroxypyridinium (3HP)

	R_1	R_2	R_3	R_4	R_5	R_6	MW
FB_1	TCA	TCA	OH	OH	NH_2	CH_3	721
FB_2	TCA	TCA	H	OH	NH_2	CH_3	705
FB_3	TCA	TCA	OH	H	NH_2	CH_3	705
FB_4	TCA	TCA	H	H	NH_2	CH_3	689
FA_1	TCA	TCA	OH	OH	$NHCOCH_3$	CH_3	763
FA_2	TCA	TCA	H	OH	$NHCOCH_3$	CH_3	747
FA_3	TCA	TCA	OH	H	$NHCOCH_3$	CH_3	747
FAK_1	=O	TCA	OH	OH	$NHCOCH_3$	CH_3	603
FC_1	TCA	TCA	OH	OH	NH_2	H	707
FC_2	TCA	TCA	H	OH	NH_2	H	691
FC_3	TCA	TCA	OH	H	NH_2	H	691
FP_1	TCA	TCA	OH	OH	3HP	CH_3	800
FP_2	TCA	TCA	H	OH	3HP	CH_3	784
FP_3	TCA	TCA	OH	H	3HP	CH_3	784
$PHFB_{1a}$	TCA	OH	OH	OH	NH_2	CH_3	563
$PHFB_{1b}$	OH	TCA	OH	OH	NH_2	CH_3	563
HFB_1	OH	OH	OH	OH	NH_2	CH_3	405

TCA: tricarballylic acid 3HP: 3-Hydroxypyridinium MW: molecular weight

Figure 3.1: Structures of all known 17 fumonisins. FA, FB, FC and FP occur naturally, while the hydrolyzed fumonisins $PHFB_{1a}$, $PHFB_{1b}$ and HFB_1 are generated by food treatment.

More recently, also a number of fumonisins with amino-linked structures have been shown to be present at lower levels in food samples (Park et al. 2004). Most of them are not naturally occurring and are mainly formed in process-caused reaction products of the fumonisins by alkaline treatment or a Maillard-similar reaction with reducing sugars (Seefelder et al. 2001). Fumonisins are responsible for ELEM of horses and pulmonary edemas of pigs. Gelderblom et al. had been the first to prove their carcinogenic potential in rats by showing their potential

as tumor initiators and promotors (Gelderblom et al. 1991). Various studies of the intake of contaminated food by other animals, such as nonhuman primates, rabbits, hamsters, sheep and cattle exhibited in each case liver and kidney damage (Seefelder et al. 2002). So far, no data exist that verifies a threat to human health by exposure to fumonisins. However, a correlation between high fumonisin content and esophageal cancer in regions of South Africa (Marasas 2001) and China (Zhang et al. 1997) was confirmed in earlier studies. In contrast to other mycotoxins, only trace amounts of fumonisins have been found in animal tissue. It seems that residues present in meat, milk and eggs do not present a potential risk (Arranz et al. 2004).

The toxic effect of fumonisins is presumably based on their structural similarity to sphingolipids and they are therefore referred to have a "sphinganine analog mycotoxin" structure. However, fumonisins affect the lipid metabolism of the cell by inhibition of sphingosine N-acyl transferase (ceramide synthase). This leads to an accumulation of sphinganins in the cell and finally to sphinganine-induced cell death (Riley et al. 2001; Wang et al. 1992).

Mean total fumonisin B levels (B_1, B_2 and B_3) of up to 3.4 ppm were found in corn products of Brazil (Van der Westhuizen et al. 2003). In Switzerland Pittet et al. (1992) determined concentrations up to 792 ppb. In 2001, a provisional maximum tolerable daily intake (PMTDI) for nephrotoxicity has been set by the 56th Joint FAO/WHO Expert Committee on Food Additives to 2 μg/kg body weight per day (FAO/WHO 2001). Switzerland proposed a maximum limit for fumonisins in corn of 1000 ng of the sum of FB_1 and FB_2 per g sample (Arranz et al. 2004). In Germany, maximum levels for the sum of FB_1 and FB_2

contaminations in various maize products and corn flakes range from 100 to 500 µg/kg (Engelhardt 2004).

3.3 State of the art in fumonisin analysis

Fumonisins are especially troublesome to analyze because they are relatively insoluble in organic solvents, are not separated easily by gas chromatography, and they do not absorb in UV range detectors used in liquid chromatography (Wilkes et al. 1998).

The application of SPE in fumonisin analysis is common and widely used for sample cleanup, employing strong anion exchange (SAX). Indeed, most detection methods for fumonisins show several difficulties. Using simple thin-layer chromatography (TLC), Gelderblom et al. (1983) isolated fumonisins from culture extracts for the first time. However, TLC is qualitatively useful, but not equally suitable for quantifying fumonisin levels. Furthermore, only few gas chromatography/mass spectrometry (GC/MS) methods exist, which are based on hydrolysis of the esterified side chain and derivatization of the fumonisin backbone with trimethylsilyl or trifluoroacetate (Shepard 1998). Although sensitive and selective, these methods involve expensive equipment and a tedious and time-consuming derivatization step, which may also cause additional problems by incomplete derivatization. Competitive enzyme-linked immunosorbent assays (ELISAs), which are easy to perform and do not need expensive equipment, can be used only for qualitative screening of FB_1. However, the results of this technique give false higher estimates than those of GC/MS and high-performance liquid chromatography (HPLC) methods (Sforza et al. 2005; Thielert et al. 1998). This seems to be associated with the cross-reactivities of antibodies used for these techniques (Park).

Most of the applied methods for fumonisin analysis are based on HPLC with fluorescence detection. Due to their UV-inactivity, a derivatization step is required, usually by *o*-phthaldialdehyde (OPA) (Shepard et al. 1990), but several other derivatization agents have been employed previously (Arranz et al. 2004). Although these methods are in general characterized by relatively high sensitivity and low detection limits (usually 5 to 30 ppb, (Dilkin et al. 2001; Solfrizzo et al. 2001)) disadvantages have been reported. For example, the derivatization of primary amines present as matrix constituents and fast degradation of the derivates complicate an accurate quantification. Additionally, only fumonisins containing a free amino function can be determined.

The use of HPLC combined with MS is now more frequently reported for qualitative and quantitative determination of fumonisins. They have become indispensable, as the variety of fumonisin representatives is rather high. Since a derivatization of the amino group is not necessary, this hyphenated methodology is also suitable for the sensitive detection of fumonisins, in which the amino group is blocked. Additionally, tandem mass spectrometry represents a versatile instrument for the characterization of new compounds of this class. Ionization is mainly performed by ESI in the positive ion mode (Doerge et al. 1994; Hartl et al. 1999; Josephs 1996; Lukacs et al. 1996). An overview of the determination of fumonisins is given by Shepard (1998) and Arranz et al. (2004).

One of the greatest limitations in recent fumonisin analysis is the lack of an ISTD for quantitative analysis. Since the matrix of corn samples is very complex, the application of an ISTD is actually an urgent need in the method development of fumonisin analysis. Recently, Paepens et al. (2005) published a method applying a “so-called” ISTD, however it is better

characterized as a recovery standard, because the substance is added after the sample cleanup. Only one method is described using an ISTD (Lukacs et al. 1996) for quantification. This standard is FB_1-d_6, a deuterated fumonisin, which is expensive, difficult and time-consuming to prepare due to its production via bacteria growth.

3.4 Aim of the work

The aim of this part of the thesis was to facilitate detection of FB_1 and FB_2 in corn using ion trap MS. A previously described method for the quantitative analysis using HPLC and fluorescence detection (Lötscher 2001) was used as starting point. Nowadays, pre-column derivatization with OPA/2-mercaptoethanol followed by fluorescence detection is the most commonly applied method. It has been adopted by AOAC International as an official first action method for corn analysis (Sydenham et al. 1996). However, this method could not be successfully applied for the analysis of corn-based food products such as corn bran flour, corn bran breakfast cereals, mixed baby cereals and corn flakes due to low recovery rates caused by an inadequate cleanup (Brera et al. 2004). Besides quenching a further disadvantage of fluorescence detection is the impossibility to determine N-acetylated fumonisins (A-series), which are sometimes present in samples at lower levels. Therefore, one aim of this work was its substitution by ion trap MS- and MS^n-detection.

Furthermore, MS detection enables the investigation of ionization and fragmentation behavior providing valuable additional information to the characteristics of a particular compound. Selective MS techniques such as e.g. MS/MS, selected ion monitoring (SIM) and selected

reaction monitoring experiments (SRM) should also be evaluated. In addition a full method validation should be carried out. Finally its applicability to real samples should be proven.

A further goal of this work was to find a suitable ISTD for the determination of fumonisins. Different substances should be examined. In case no applicable substance could be found, the ISTD should be synthesized by modifying FB_1.

3.5 Experimental

3.5.1 Materials

Water with a total organic content of <2 ppb was obtained from an Elgastat maxima HPLC water purification unit (Elga LTD., Bucks, Great Britain). Acetonitril, grade "190 far UV" (Romil, Cambridge, Great Britain) and Methanol of pestipur grade (SDS, Peypin, France) were used. Helium of 99.999 % purity and nitrogen of 99.995 % purity were purchased from Carbagas (Switzerland).

The following chemicals of certified purity (if specified) were purchased from Fluka Chemie (Buchs, Switzerland): sodium bicarbonate (≥99.7 %), ammonium formate (≥99 %), sodium hydroxide (99 %) trifluoroacetic acid (≥99.5%), (±)-2-aminopimelic acid (≥97%), hydrocortisone (≥97%), triethylamine (≥99.5 %), di-tert-butyl dicarbonate (BOC, ≥98 %), benzyl chloroformate (≥95 %) and silver nitrate (≥99 %). FB_1 (98 %), reserpine (98 %), cis-3-acetyl-2,2-dimethylcyclobutaneacetic acid (pinonic acid, PA, 98 %), ochratoxin B (OTB) and 2-dimethylaminohexadecanoic acid (DAHA) were provided by Sigma Chemie (Buchs,

Switzerland). *o*-Phthaldialdehyde (for fluorometry, OPA) was purchased from Merck (Darmstadt, Germany) and formic acid (≥98 %) from Riedel-de-Haen (Buchs, Switzerland). FB_2 (98 %) was obtained from ICN Biomedicals (Aurora, USA). FB_3 was purchased from PROMEC (Medical Research Council, Tygerberg, South Africa). 9-Fluorenylmethylchloroformate was purchased from Benn Chemicals (Dielsdorf, Switzerland). Solid phase extraction was carried out on a SAX Varian LRC Bond Elut column with 100 mg trimethylaminopropylchloride substituted silica gel as stationary phase. The columns possess a particle size of 40 µm, a pore size of 6 nm and a reservoir of 1 ml (Varian, Palo alto, USA, cat. no. 1210-2017). For filtration of the extracts, folded cellulose filters of medium porosity were used (Schleicher & Schuell, Feldbach, Switzerland, 150 mm i.d.).

3.5.2 Samples

All corn samples were obtained from Coop Quality Center (Pratteln, Switzerland) in 2002. Detailed information about the investigated corn samples is given in Table 3.1. After opening of the food package, all samples were stored at –18 °C.

Table 3.1. Food samples under investigation obtained from Coop Quality Center, Switzerland with corn content in % (dry weight).

Sample No.	Description	Corn content [%]
1	Coop Maisano bread mix for baking	38
2	Corn, raw material for Coop Maisano bread for baking	100
3	Coop Bio Popcorn	100
4	Coop Polenta Rustica	100
5	Coop Polenta Dorata Maisgriess	100
6	Maggi Polenta Ticinese	n.g.
7	Coop Maisano, corn bread, ground	n.g.
8	Old el paso Nachips, ground	73
9	Coop Cornflakes, ground	92
10	Bio Coop Naturaplan Cornflakes, ground	92
11	Corn, test material	n.g.

n. g.: not given

3.5.3 Instrumentation

For the preparation of reference solutions and real samples different balances from Mettler-Toledo Schweiz AG (Greifensee, Switzerland) were used: Mettler M3, Mettler AE 100 and Mettler AC 100. Real samples were extracted on a wrist-action shaker LSL-V from Adolf Kühner AG (Birsfelden, Switzerland) with 200 moves/min. For solvent reduction a self-constructed evaporation device consisting of a heated aluminum block and a nitrogen supply was used. pH values were determined with a pH-meter 744 of Metrohm (Herisau, Switzerland).

Samples were centrifuged in a Vaudaux Centrifuge 5412 (Binningen, Switzerland). An ultrasonic bath (Sonorex RK 100, Bandelin, Berlin, Germany) was used for the dissolution of sample residues in solvent. HPLC solvents were either degassed directly with helium for approx. 15 min or with a DG 1300 series vacuum degasser (Knauer, Berlin, Germany).

Samples were injected with a Valco Cheminert valve or a HTS-Pal auto-sampler (CTC Analytics, Zwingen, Switzerland). HPLC was carried out with a Rheos 2000 pump (Flux Instruments, Basel, Switzerland) equipped with a pulsation dampener.

A quadrupole ion trap spectrometer (LCQ, Finnigan, San Jose, CA, USA) was used for detection of fumonisins equipped with electrospray ionization in the positive ion mode (ESI(+)).

3.5.4 Methods

3.5.4.1 Sample extraction and cleanup

2 µg of ochratoxin B (OTB) were added to 10 g ground corn or corn products. The mixture was shaken for 2 h with 20 ml of methanol (MeOH)/0.1 M HCl (3+1) and filtrated once through folded cellulose filters. The pH value of the solution was adjusted to 6.5 with 0.1 M sodium hydroxide and 3 ml of the raw extract were centrifuged. The SAX column was conditioned with 2 ml of MeOH and 2 ml of MeOH/H_2O (3+1) allocated with 1 ml of raw extract and washed with 1 ml of MeOH/H_2O (3+1) and 1 ml of MeOH. The elution was performed by adding 2 ml of MeOH/1 % acetic acid. The flow rate was less than 1 ml/min. After neutralization of the eluate with sodium hydroxide (2 M and 0.2 M) the solution was dried under a constant flow of nitrogen at 50 ºC and re-dissolved in 40 µl of MeOH/H_2O (1+1). After a 5 min ultrasonic treatment, the sample was injected into the HPLC/MS.

3.5.4.2 Separation

A PEEK injection loop of 5 µl volume with an inner diameter of 175 µm was used for injection of samples into the HPLC/MS system. The flow rate of the mobile phase was 350 µl/min. Two commercially available HPLC columns were in use: First, the C_{18} reversed phase HPLC column Supersphere ODS1 with the following dimensions: particles 4 µm diameter, 10 nm pore size, 150 mm column length, 3 mm i.d from Interchrom/Interchim (Montluçon, France). Second, the Xterra MS HPLC column was obtained from Waters (Milford, USA) and had the following dimensions: particles 3.5 µm diameter, 12.5 nm pore size, 100 mm column length, 3 mm i.d. Pre-columns of 4 mm length and 4 mm i.d. were used for some of the real samples to retain matrix residues (Merck, LiChrosphere RP-18, particle size 5 µm, pore size 10 nm). Solvents used for chromatography were pure methanol (solvent A), 25 mmol ammonium formate buffer (pH 3.3)/methanol (1+1) (solvent B) and 10 mmol ammonium formate buffer (pH 3.3) (solvent C).

Two linear binary gradients were employed. First, the Supersphere ODS1 was conditioned 2 min with 70 % solvent A and 30 % solvent C, followed by an increase to 80 % of solvent A in 2 min and a rinsing for 4 min. Then, the methanol content was reduced to the starting conditions within 1 min and the column re-equilibrated for 2 min. Second, the Waters Xterra MS column was conditioned 3 min with 40 % solvent A and 60 % of solvent B, followed by an increase of solvent A to 60 % in 2 min and a rinsing period of 4 min. Then, the methanol content was reduced to the starting conditions within 1 min and the column was re-equilibrated for 2 min.

The peak symmetry factor $S_{10\%}$ was determined as follows:

$$S_{10\%} = \frac{b_{10\%}}{a_{10\%}}$$

$S_{10\%}$ peak symmetry factor at 10 % peak height
$a_{10\%}$ peak width at 10 % peak height at the front of the signal
$b_{10\%}$ peak width at 10 % peak height at the rear of the signal

3.5.4.3 MS-detection

Mass spectra in the full-scan mode were registered within a mass range of 235-800 u. For pneumatically assisted electrospray ionization, the spray capillary voltage was set to 4 kV and the nitrogen sheath gas flow to 30 arbitrary units (corresponding to ca. 300 ml/min). The following devices were optimized by the auto tune program to achieve maximum transmission of the $[M+H]^+$ ion of FB_1 (m/z 722): voltages of tube lens offset, octapole 1 offset, interoctapole lens, octapole 2 offset, octapole rf amplitude and heated capillary temperature.

The MS/MS experiments were accomplished with helium as collision gas. The fumonisin fragments and their intensities were observed at different collision energies. FB_1 was isolated at *m/z* 722 and FB_2 at *m/z* 706 with an isolation width of 1.5 u. Afterwards, the isolated quasi molecular ions were selected for further fragmentation using different collision energies. Relative collision energies were optimized for maximum intensity of the fragments *m/z* 352, 528, 546, 686 and 704 of FB_1 and *m/z* 336, 354, 512, 530, 688 of FB_2. SRM experiments were

performed with the following parameters. SRM-1 mode: $[M\text{-}TCA]^+$, *m/z* 546 for FB_1 and *m/z* 530 for FB_2, 29 % collision energy, 1.5 u isolation width. SRM-2 mode: $[M\text{-}H_2O]^+$, *m/z* 704 for FB_1 and *m/z* 688 for FB_2, 28 % collision energy, 1.5 u isolation width.

A "data dependent scan" of the complete mass spectrum of 235-800 u with 30 % collision energy and an isolation width of 2 u was recorded using an intensity threshold value by 60'000 counts.

3.5.4.4 Quantification

Quantification was based on both the internal and external standard method. For an external standard calibration a calibration curve was determined based on twelve calibration solutions. Hydrocortisone (HYC) was used as ISTD at concentrations between 1 and 10 µg/ml or OTB at concentrations between 0.1 µg/ml und 10 µg/ml.

Relative response factors (*rrf*) were calculated as follows:

$$rrf_i = \frac{(c_{ISTD}) \times (A_i)}{(c_i) \times (A_{ISTD})}$$

rrf_i	relative response factor of substance *i* relative to the ISTD
c_i	concentration of substance *i* in the reference solution
c_{ISTD}	concentration of the ISTD in the reference solution
A_i	signal area of substance *i* in the mass chromatogram of the reference solution
A_{ISTD}	signal area of ISTD in the mass chromatogram of the reference solution

The recovery R [%] of analyte in a sample was determined as follows:

$$R = \frac{(c_{ISTD}) \times (A_i)}{rrf_i \times (c_i) \times (A_{ISTD})} \times 100$$

c_{ISTD} concentration of the ISTD in the sample
c_i concentration of substance *i* in the sample
A_i signal area of substance *i* in the mass chromatogram of the sample
A_{ISTD} signal area of ISTD in the mass chromatogram of the sample
rrf_i relative response factor of substance *i*

The concentration of substance i (c_i) in the samples were determined as follows:

$$c_i = \frac{(c_{ISTD}) \times (A_i)}{(A_{ISTD}) \times rrf_i}$$

c_i concentration of substance *i* in the sample
c_{ISTD} concentration of the ISTD in the sample
A_i signal area of substance *i* in the mass chromatogram of the sample
A_{ISTD} signal area of ISTD in the mass chromatogram of the sample
rrf_i relative response factor of substance *i*

3.5.4.5 Adduct formation of FB_1 with Ag^+ ions

Solutions of FB_1 in methanol/H_2O (1+1) were infused in the presence of 5 and 10 mmol $AgNO_3$ with flow rates of 5 to 10 µl/min. Instrument parameters were optimized by the auto tune program to achieve maximum transmission of the $[M+H]^+$ ion of FB_1 (m/z 722) or the $[M+Ag]^+$ ion of FB_1 (m/z 828), respectively.

3.5.5 Synthesis of fumonisin derivatives

Synthesis was performed in a 1.5 ml Eppendorf vial. After the addition of the derivatization reagent, the reaction took place in a vortexer Top-Mix 94323 (from Heidolph Instruments,

Schwabach, Germany) selecting different time intervals. In case of higher reaction temperatures than RT, the mixture was heated in a water bath. Yields were determined by HPLC/MS proportionally by the reduction of the signal area of FB_1. If FB_1 was not detected any more in presence of an intensive product signal a yield of "100 %" was assumed. Synthesized solutions were used for maximum 8 h.

3.5.5.1 Derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl)

FMOC-FB_1 was synthesized according to Holcomb et al. (1993) and Herraez-Hernandez et al. (2001) by adding 100 µl buffer to a solution of FB_1 (6 µg in 500 µl CH_3CN/H_2O (1+1)). After addition of 100 µl 9-fluorenylmethylchloroformate (FMOC-Cl, 3 mg/ml in 100 % CH_3CN) the mixture was shaken for 5 min (see Table 3.2 for details). Buffers were obtained by preparing a 1 M solution of H_3BO_3 ($NaHCO_3$, respectively) in deionized water and adjusting the pH to 7.5 with 6 M NaOH. The reaction control was obtained by HPLC/MS. The complete removal of FB_1 and structure of the fumonisin derivative was confirmed by the molecular ions ($[M+H]^+$ of FB_1 at m/z 722 and FMOC-FB_1 at m/z 944; $[M+NH_4]^+$ of FMOC-FB_1 at m/z 961).

3.5.5.2 Derivatization with benzylchloroformate (Z-Cl)

Z-FB_1 was synthesized according to Bergmann et al. (1932) by adding 1 M Na_2CO_3, 0.5 M triethylammonium acetate (TEAA)/pH 7.5 or 1 M H_3BO_3/pH 7.5, respectively, to a solution of FB_1 (15 µg/ml) in CH_3CN or CH_3CN/H_2O (1+1). After addition of an eight to sixteen fold

excess of benzylchlorofomate (Z-Cl, 1 mg/ml) in 100 % CH_3CN, the mixture was shaken for various reaction times (see Table 3.2 for details). The complete removal of FB_1 and structure of the fumonisin derivative was confirmed by the molecular ions ($[M+H]^+$ of FB_1 at m/z 722 and Z-FB_1 at m/z 856; $[M+NH_4]^+$ of Z-FB_1 at m/z 873).

3.5.5.3 Derivatization with di-tert-butyldicarbonate (BOC_2O)

BOC-FB_1 was synthesized according to Tarbell et al. (1972) and Williams et al. (2003) by adding 1 M $NaHCO_3$ or NaOH/pH 9, respectively, to a solution of FB_1 in CH_3CN (15 µg/ml). After addition of a five to ten fold excess of BOC_2O (di-tert-butyldicarbonate, 200 µg/ml) in 100 % CH_3CN, the mixture was shaken for various incubation times (see Table 3.2 for details). The complete removal of FB_1 and structure of the fumonisin derivative was confirmed by the molecular ions ($[M+H]^+$ of FB_1 at m/z 722 and BOC-FB_1 at m/z 836; $[M+NH_4]^+$ of BOC-FB_1 at m/z 853).

3.5.5.4 Derivatization with o-phthaldialdehyde (OPA)

OPA-FB_1 was synthesized according to Sydenham et al. (1992) by addition of different amounts of OPA to a solution of 100 µl buffer (0.5 M H_3BO_3/pH 8.5) and 500 µl of FB_1 (10 µg/ml). The mixture was shaken for 5 min and in some cases heated. The complete removal of FB_1 and structure of the fumonisin derivative was confirmed by the molecular ions ($[M+H]^+$ of FB_1 at m/z 722 and OPA-FB_1 at m/z 852; $[M+NH_4]^+$ of OPA-FB_1 at m/z 869).

3.5.6 Stability test of fumonisin derivatives

The fumonisin derivatives with “100 %” yield were tested for their stability in an acidic medium. The reaction mixture was stored at room temperature and analyzed 13 times: after 0, 1, 5, 10, 30, 60, 90 min and after 5, 8, 24, 26, 28 and 30 h. Therefore, a 450 µl aliquot of MeOH/1 % acetic acid (acidic medium of the SPE elution solvent) was added to 50 µl of the reaction mixture. Immediately before injection, a 10 µl aliquot of the mixture was spiked with 100 pg OTB. The area ratio of the fumonisin derivative versus the OTB area was determined for each measurement. The stability of the fumonisin derivative was controlled by the added OTB.

Table 3.2: Overview of all applied reaction conditions of the derivatization of fumonisin B_1 (FB_1) as carbamate with FMOC-Cl (9-fluorenylmethylchloroformate), Z-Cl (benzylchloroformate) and BOC_2O (di-tert-butyldicarbonat) and as cyclic imide with OPA (*o*-phthaldialdehyde). (TEAA = triethylammonium acetate, ME = 2-mercaptoethanol, RT = room temperature)

	Reactants [µg]	Solvent [µl]	Buffer/pH	t_i [min]	Temp. [°C]
FMOC 1	FB_1: 6 FMOC-Cl: 300	500 CH_3CN	100 µl 0.1 M H_3BO_3/7.5	5	RT
FMOC 2	FB_1: 6 FMOC-Cl: 300	500 CH_3CN	100 µl 0.1 M H_3BO_3/7.5	5	RT
FMOC 3	FB_1: 6 FMOC-Cl: 300	500 CH_3CN	100 µl 1 M TEAA/7.5	5	RT
FMOC 4	FB_1: 6 FMOC-Cl: 450	500 CH_3CN	100 µl 1 M $NaHCO_3$	5	RT
FMOC 5	FB_1: 6 FMOC-Cl: 450	500 CH_3CN/ H_2O (1+1)	100 µl 1 M $NaHCO_3$	5	RT
FMOC 6	FB_1: 6 FMOC-Cl: 450	500 CH_3CN/ H_2O (1+1)	100 µl 1 M $NaHCO_3$	1	RT
Z1	FB_1: 3.75 Z-Cl: 150	250 CH_3CN	500 µl 1 M H_3BO_3/pH 7.5	5	RT
Z2	FB_1: 3.75 Z-Cl: 150	250 CH_3CN	500 µl 0.5 M TEAA/7.5	1	RT
Z3	FB_1: 3.75 Z-Cl: 150	250 CH_3CN	50 µl 1 M Na_2CO_3	10	RT
Z4	FB_1: 3.75 Z-Cl: 150	250 CH_3CN	50 µl 1 M Na_2CO_3	30	RT
Z5	FB_1: 3.75 Z-Cl: 300	250 CH_3CN	100 µl 1 M Na_2CO_3	10	RT
Z6	FB_1: 3.75 Z-Cl: 300	250 CH_3CN/ H_2O (1+1)	100 µl 1 M Na_2CO_3	30	RT
BOC1	FB_1: 6 BOC_2O: 100	500 CH_3CN	100 µl 1M $NaHCO_3$	5	RT
BOC2	FB_1: 6 BOC_2O: 100	500 CH_3CN	100 µl 1M $NaHCO_3$	10	RT
BOC3	FB_1: 6 BOC_2O: 100	500 CH_3CN	100 µl 1M $NaHCO_3$	30	RT
BOC4	FB_1: 6 BOC_2O: 100	500 MeOH	100 µl 1M $NaHCO_3$	10	RT
BOC5	FB_1: 6 BOC_2O: 200	500 CH_3CN	100 µl 1M $NaHCO_3$	30	RT
BOC6	FB_1: 6 BOC_2O: 200	500 CH_3CN	100 µl NaOH pH 9	30	RT
BOC7	FB_1: 6 BOC_2O: 200	500 CH_3CN	100 µl NaOH pH 9	30	50
OPA1	FB_1: 5, OPA: 60	500 CH_3CN/ H_2O (70+30)	100 µl 0.1 M H_3BO_3 /pH 8.5	5	RT
OPA2	FB_1: 5, OPA: 600	500 CH_3CN/ H_2O (70+30)	100 µl 0.1 M H_3BO_3 /pH 8.5	5	RT
OPA3	FB_1: 5 , OPA: 600	500 MeOH	100 µl 0.1 M H_3BO_3 /pH 8.5	5	RT
OPA4	FB_1: 5, OPA: 6000	500 CH_3CN/ H_2O (70+30)	100 µl 0.1 M H_3BO_3 /pH 9	5	RT
OPA5	FB_1: 5, OPA: 6000	500 H_3CN/H_2O (70+30)	100 µl 0.1 M H_3BO_3 /pH 9	5	60

3.6 Results and discussion

3.6.1 MS investigations

3.6.1.1 Ionization properties of fumonisins

The ionization of the fumonisins was investigated by ESI and APCI in the positive as well as in the negative ion mode. In APCI fumonisins could be detected only in the positive ion mode. The intensities were about 97 % lower with ESI(-) than with ESI(+), but less adducts were produced. The ESI(+) mode is more sensitive by a factor of 1000 compared to the APCI(+) mode. The LCQ was tuned for best signal-to-noise (S/N) ratio of the $[M+H]^+$ ion of FB_1 at m/z 722 and of FB_2 at m/z 706, respectively.

The most intensive signal of FB_1 was the $[M+H]^+$ at m/z 722, whereas the adducts $[M+Na]^+$ at m/z 744 and $[M+K]^+$ at m/z 760 were observed with less signal intensity (12 % and 20 %, respectively). Ionization in the negative ion mode of ESI and APCI showed $[M-H]^-$ as the most intensive signal at m/z 720, $[M-2H+Na]^-$ at m/z 742, as well as the molecular ion $[M-2H]^{2-}$ at m/z 360. However, the presence of the double charged species led to a sensitivity loss. Due to the TCA chains of fumonisins, they can be detected in the negative ion mode, but the ESI(+) mode proved to be more sensitive. Additionally, the intensity between the different ions varied strongly in ESI(-). Consequently, ESI in the positive ion mode was chosen for further experiments. FB_2 and FB_3 led to the formation of similar ions as described for FB_1.

3.6.1.2 MS^n spectra

MS^2 to MS^5 spectra were recorded to study fragmentation behavior of fumonisins in the ion trap. Stable product-ions were obtained by collision induced dissociation (CID). Lukacs et al. observed similar fragmentations for triple quadrupole MS, even though different intensities for single product ions have been reported (Lukacs et al. 1996; Paepens et al. 2005). Figure 3.3 shows the ESI(+)-MS/MS spectra of the product ions of FB_1 at m/z 722 and FB_2 at m/z 706.

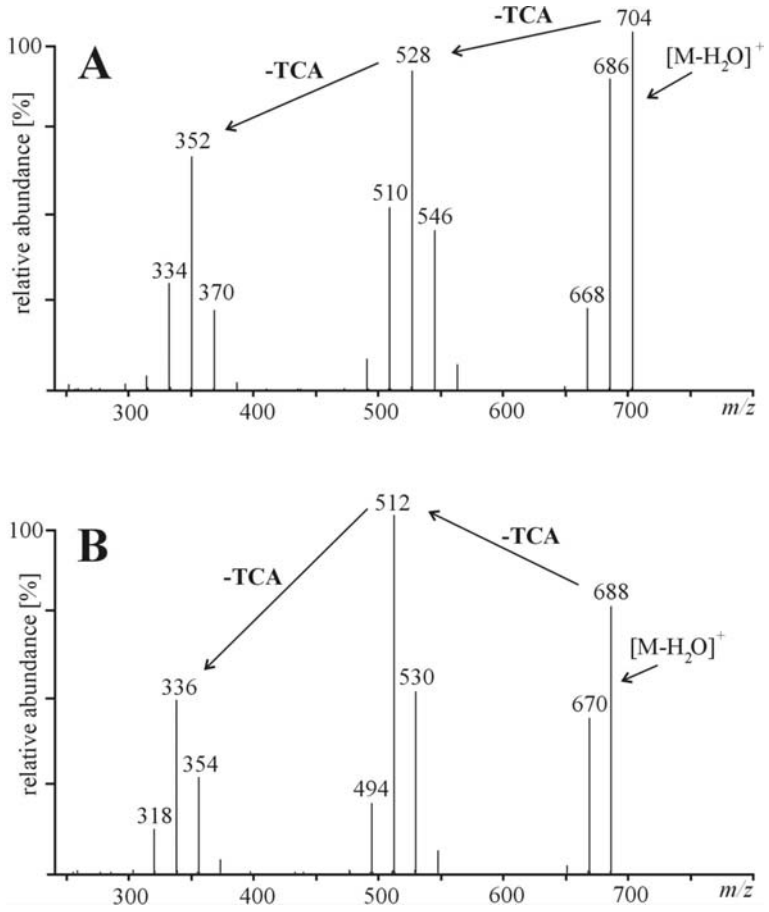


Figure 3.3: ESI(+)-MS/MS spectra of fumonisin B_1 (A) and B_2 (B) determined with an isolation width of 1.5 u and a collision energy of 50 %. A 10 µg/ml solution of both in CH_3CN/H_2O (1+1) was infused at a flow rate of 10 µl/min.

The following main fragments of FB_1 were intensive and reproducible: $[M-H_2O]^+$ at m/z 704, $[M-2H_2O]^+$ at m/z 686, $[M-TCA]^+$ at m/z 546, $[M-H_2O-TCA]^+$ at m/z 528 and $[M-H_2O-2TCA]^+$ at m/z 352. Fragmentation of FB_2 showed the following main fragments: $[M-H_2O]^+$ at m/z 688, $[M-TCA]^+$ at m/z 530, $[M-H_2O-TCA]^+$ at m/z 512, $[M-2TCA]^+$ at m/z 354 and $[M-H_2O-2TCA]^+$ at m/z 336.

For both fumonisins two losses of TCA and several of H_2O were observed. The loss of H_2O results presumably from both the hydroxy groups and the carboxyl groups. The loss of TCA is characteristic for fumonisins and, therefore, could be used as a "neutral loss" for a screening method (see Chapter 3.6.1.4). The intensity in dependence of the collision energy was examined for the main fragments. It is shown in Figure 3.4 for FB_1 (A) and FB_2 (B). Most intensive product-ions were found for both fumonisins as the $[M-H_2O]^+$ which was formed at collision energies of 28 % for FB_1 and 31 % for FB_2, respectively.

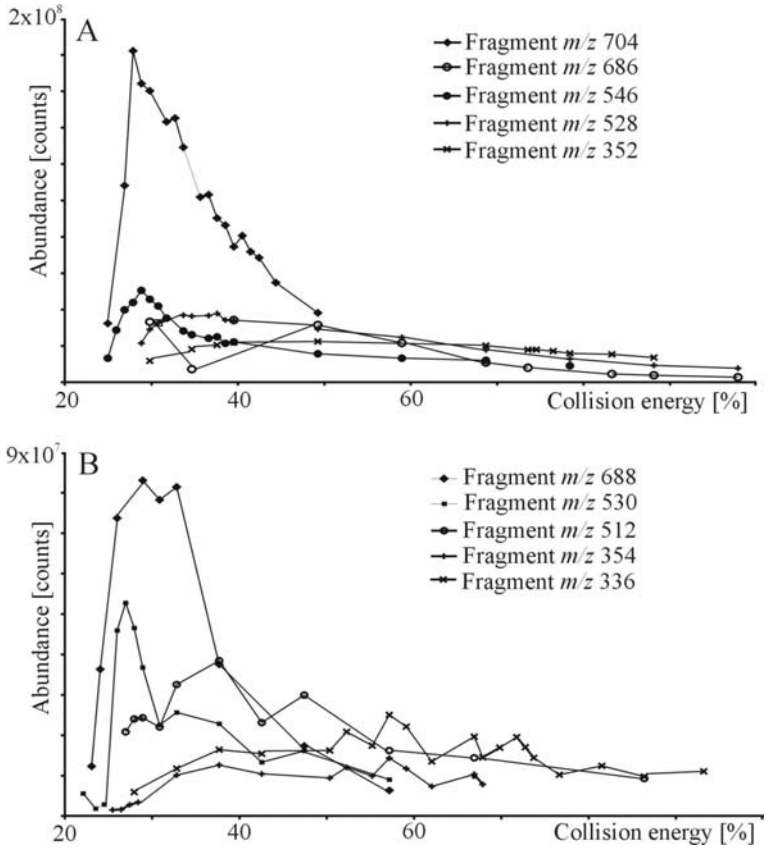


Figure 3.4: ESI(+)-MS/MS intensity of the main fragments of fumonisin B_1 (A) and B_2 (B) in dependence of the collision energy. Solutions of 5 µg/ml were infused for both.

The first loss of H_2O formed a stable fragment, stabilizing the charge by the TCA-groups. $[M\text{-}TCA]^+$-fragments showed a lower intensity. Their maximum was at a collision energy of 29 % for both fumonisins. Higher collision energies did not increase the intensity of other fragments. Table 3.3 gives an overview of the precursor and product ions in dependence of spray voltage, heated capillary temperature and collision energies that were used in the SRM mode for fumonisin detection.

Table 3.3: ESI(+)-MS/MS precursor and product ions used for the SRM mode of fumonisin detection with optimal source conditions of spray voltage, heated capillary temperature (HCT) and collision energy determined with an isolation width of 1.5 u.

Fumonisin	Precursor ion [m/z]	Spray voltage [kV]	HCT [°C]	Product ion [m/z]	Collision energy [%]
FB_1	722	4	250	**704 (100)**	28
				546 (23)	29
FB_2	706	4	210	**688 (100)**	31
				530 (28)	29

Bold: most abundant ion

The fragmentation pathway of FB_1 observed in the MS^n experiments of m/z 722 → 704 → 686 → 668 is similar to the neutral loss of three water molecules (18 u) and is most likely to happen. After cleavage of TCA (722 → 546) it could be seen that both a neutral loss of water (18 u) or of TCA (176 u) can occur.

3.6.1.3 Adduct formation of FB_1 with Ag^+ ions

Wax esters from jojoba oils (Medvedovici et al. 2002) and polycyclic aromatic hydrocarbons (Roussis et al. 2002) form silver adducts ions with higher response factors in ESI-MS. To increase the sensitivity of fumonisin detection, FB_1 was infused with 5 and 10 mmol $AgNO_3$-solutions. On the basis the isotopic distribution of silver (^{107}Ag 100 %, ^{109}Ag 93 %) stable $[M+Ag]^+$ adducts ions of the fumonisins were formed. In contrast to the mentioned studies, no increase of sensitivity was obtained compared to the $[M+H]^+$.

Figure 3.5 shows the ESI(+) mass spectra of FB_1 with silver adduct formation. The signal intensity of $[M+Ag]^+$ was approximately two times smaller compared to $[M+H]^+$. The

absolute intensity was approximately five times smaller compared to the molecular ion of Ag^+-free solutions. Higher concentrations of $AgNO_3$ did not improve signal intensities.

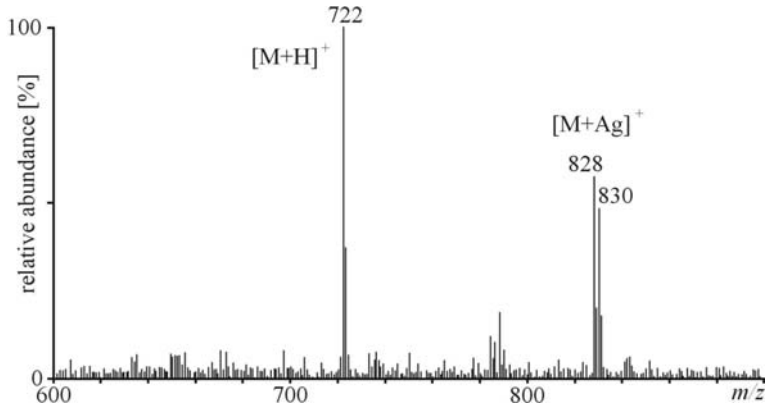


Figure 3.5: ESI(+) mass spectra of fumonisin B_1 (FB_1) and 5 mmol $AgNO_3$. 5 µg/ml of FB_1 and 0.75 mg/ml of $AgNO_3$ in CH_3CN/H_2O (1+1) were infused at a flow rate of 5 µl/min.

Since the adduct formation is based on addition of the Ag^+ ions with the π-electrons of the carboxylic groups of the analytes, it must be assumed that the isolated π -systems of the fumonisins (TCA rests) are not sufficient. The resulting adduct ion $[M+Ag]^+$ possesses no larger stability than the molecular ion $[M+H]^+$ and is therefore observed with a smaller intensity. A negative aspect of the use of Ag^+ ions is the possible reaction of the $AgNO_3$-solution with the buffer or with the matrix of the samples, whereby insoluble chloride salts may be formed that can be deposited in the ion trap. Additionally, the deposition of Ag_2O described in literature can have negative effects on the instrumentation (Medvedovici et al. 2002).

3.6.1.4 Neutral loss of fumonisins

A loss of 176 u could be detected for FB_1 and FB_2 under MS/MS conditions. This corresponds to a cleavage of one of the TCA groups. A "data dependent scan" was examined for a neutral loss *m/z* 176 as basis. The existence of TCA chains is characteristic for all naturally occurring fumonisins and those formed e.g. while food processing (see Figure 3.1). Figure 3.6 shows the mass chromatogram of FB_1 and FB_2 (solid line) and the chromatogram of the "neutral loss" function (dotted line) determined by a "dependent scan". The "neutral loss" function was more selective due to reduction of background interferences. This technique allows the detection of possible new fumonisin representatives with TCA chains, e.g. generated by food processing.

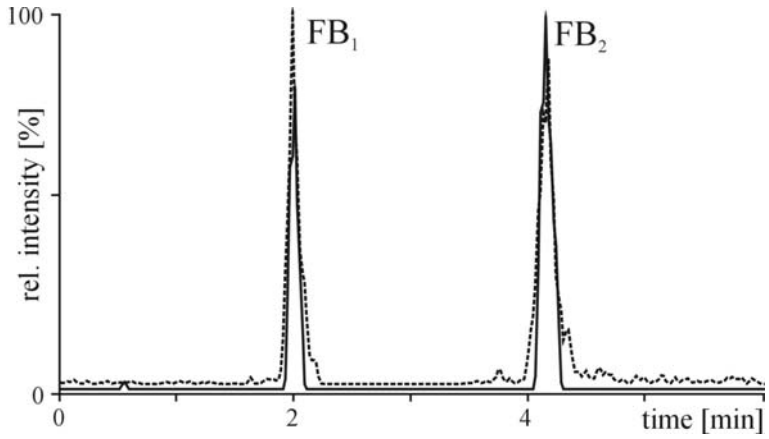


Figure 3.6:. Solid line: ESI(+)-mass chromatogram of FB_1 and FB_2 obtained by a “data dependent scan”. Dotted line: ESI(+) chromatogram of FB_1 and FB_2 of the “neutral loss” function of *m/z* 176. 25 ng of the analytes were injected.

3.6.2 Method development

3.6.2.1 Comparison of HPLC separations

Method development was accomplished using pure reference standards. Several commercially available HPLC columns were tested and the gradients adjusted correspondingly. Two columns were best suited for the determination of fumonisins (Supersphere ODS1 and Xterra MS). It was possible to reduce the separation time to 11 min for the Supersphere ODS1 and to 12 min for the Xterra MS (including flushing of the column and equilibration) while the elution order was the same for both. Analysis times for fumonisins in the literature are about 20 min or more (Faberi et al. 2005; Paepens et al. 2005), so that one HPLC/MS run could be shortened approximately 50 % without reducing the reliability of identification and quantification. The performance of both separation systems (column as well as corresponding gradient) was compared over five days on the basis of retention times and calculation of the peak symmetry factor *S*. The obtained values are given for the fumonisins FB_1 and FB_2 in Table 3.4.

Table 3.4: Comparison of the separation systems employing the columns Supersphere ODS1 (column 1) and Xterra MS (column 2). Retention time (R_t) and peak symmetry factor (*S*) with their relative standard deviations (RSD) are given for the fumonisins (FB_1 and FB_2).

Parameter	Column No.	FB_1	FB_2
R_t [min] (n = 10) ±	1	6.05 ± 0.07	9.60 ± 0.15
RSD [%]	2	1.95 ± 0.01	3.99 ± 0.01
S (n = 10)	1	0.88 ± 0.15	0.78 ± 0.08
± RSD [%]	2	0.78 ± 0.04	0.93 ± 0.03

It is obvious (see Table 3.4), that the chromatographic setup for column 2 gives a better separation of the fumonisins. Additionally, the Supersphere ODS1 column showed a constantly growing MS-background over a period of weeks and months. The sensitivity was significantly reduced by phthalates (*m/z* 279 and *m/z* 391) and column bleeding (*m/z* 295, 363, 431, 499 and 567). It can be concluded that it was caused by the plastic thread of the column as well as by a column material not completely suitable for subsequent MS detection. Since these effects were steadily increasing and could not be reduced, all following HPLC separations were performed with the Xterra MS column.

3.6.2.2 HPLC/MS performance validation

Instrument repeatability was good. Relative standard deviations of signal areas of 5 µg fumonisin were <10 % for 10 consecutive HPLC separations. Instrument detection (S/N of 3/1) and quantification limits (S/N of 10/1) were determined with pure reference substances.

HPLC/MS performance validation excluded FB_3 due to the similarity to the two other fumonisins FB_1 and FB_2, and mainly due to the high costs of FB_3 standards. Examinations were performed on both HPLC systems (column 1 and 2) as well as with the two different ISTDs OTB and HYC. Figure 3.7 shows the linear range of FB_1 and FB_2 after separation on column 1. The fumonisins were linear in a concentration range from 0.859 µg/ml to 22 µg/ml. Within a concentration range of 22 ng/ml to 90 ng/ml both fumonisins could be detected, but the response factors proved to be not linear. For this reason as well as because of the strong background contamination by phthalates and column bleeding column 2 was used for further separations.

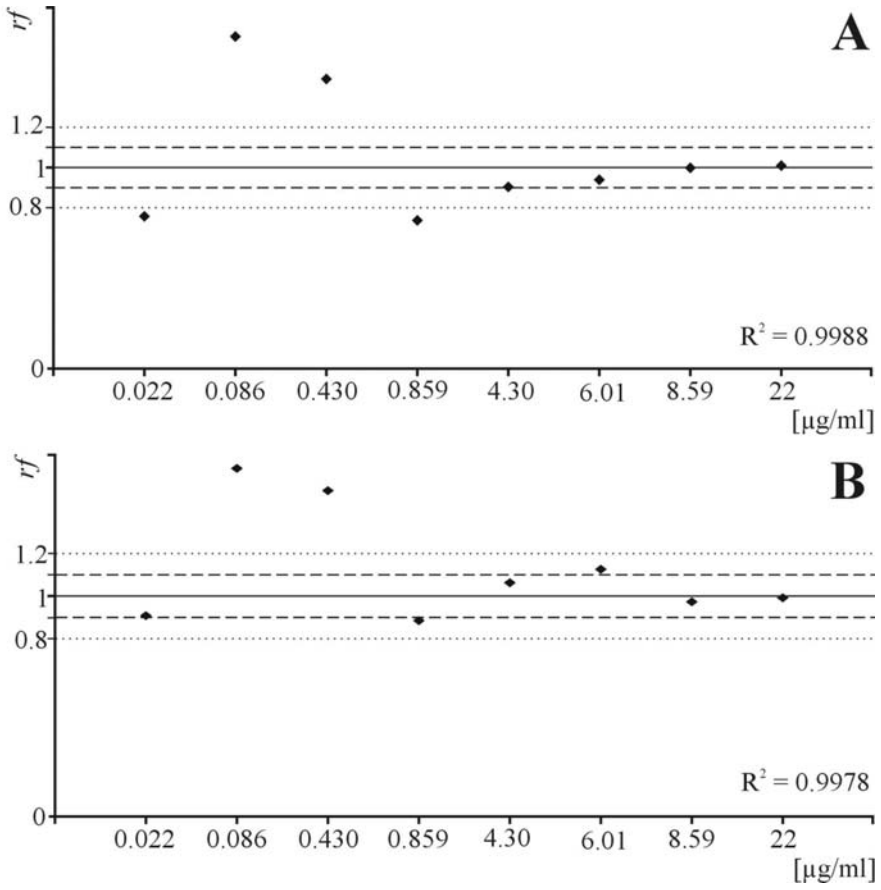


Figure 3.7: Linear range and correlation coefficients (R^2) of FB_1 (A) and FB_2 (B) with HYC as ISTD after separation on column 1 in the full scan mode. Data points represent averages of three measurements.

An overview of all concentration ranges and corresponding correlation coefficients for both columns is given in Table 3.5.

Table 3.5: Linear range and correlation coefficient (R^2) of FB_1 (A) and FB_2 (B) with OTB as ISTD (and HYC, see table) after separation on column 1 and 2 using the full scan and SIM (m/z 722 for FB_1 and m/z 706 for FB_2) mode. Concentrations of the analytes ranged from 22 ng/ml to 4.3 µg/ml. Data points represent averages of three measurements.

Column No.	Concentration range	MS mode	ISTD	R^2 FB_1	R^2 FB_2
1	1 ng/ml-90 ng/ml	Full scan	HYC	0.5986	0.5985
1	22 ng/ ml-22 µg/ml	Full scan	HYC	0.9988	0.9978
2	43 ng/ml-4.3 µg/ml	Full scan	HYC	0.8766	0.9784
2	0.859 µg/ml-22 µg/ml	Full scan	HYC	0.9495	0.9377
2	22 ng/ml-4.3 µg/ml	SIM	OTB	0.9933	0.9970

The linear range of column 2 showed even lower correlation coefficients with HYC as ISTD compared to column 1 (see Table 3.5). Better results were obtained by replacing the ISTD HYC with OTB. Figure 3.8 shows the linear range of FB_1 and FB_2 after separation on column 2. With OTB as ISTD the highest correlation coefficients could be obtained with a linear range between 86 ng/ml and 22 µg/ml.

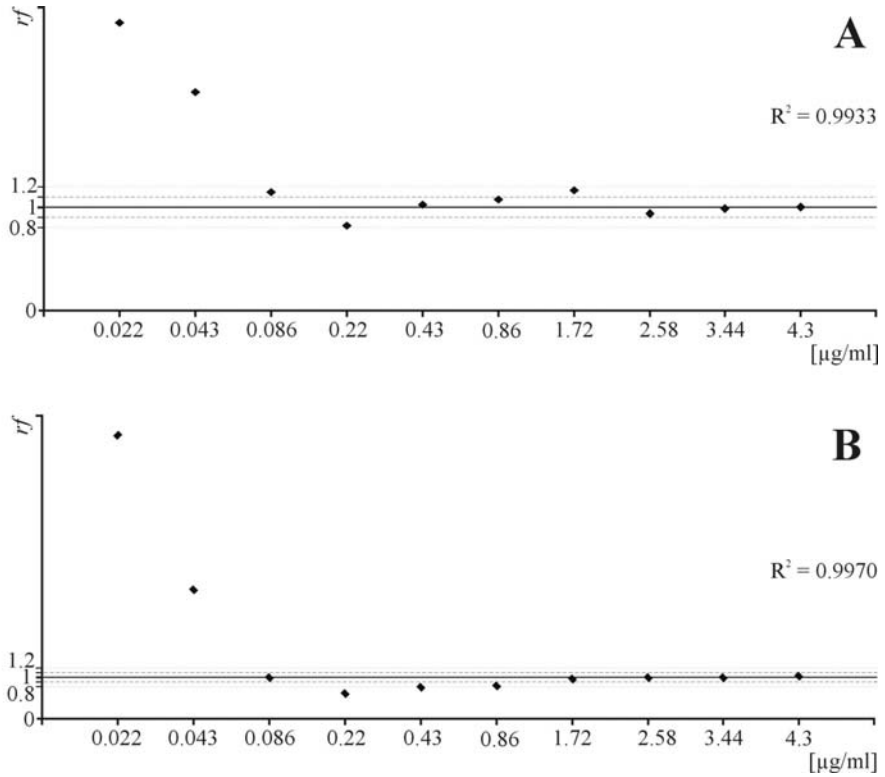


Figure 3.8: Linear range and correlation coefficients (R^2) of FB_1 (A) and FB_2 (B) with OTB as ISTD after separation on column 2 in the SIM (m/z 722 for FB_1 and m/z 706 for FB_2) mode. Data points represent averages of three measurements.

Limits of detection (LOD, signal-to-noise ratio (S/N) of 3:1) and limits of quantification (LOQ, S/N 10:1) were determined for the different MS detection methods. The results are shown in Table 3.6. The sensitivity in the SIM mode was in the same range as the SRM-2 mode. The loss of H_2O leads to more intensive product ions than the loss of TCA. Therefore,

SRM-2 is by a factor of 10 more sensitive than SRM-1 for FB_1 and a factor of 20 more for FB_2. The LOQs determined by SRM-2 of 2 ppb and 1 ppb for FB_1 and FB_2, respectively, were up to 10 times better than for most of the methods reported so far. Paepens et al. (2005) describe a multiple reaction method (MRM) with a LOD of 20 ppb and a LOQ of 40 ppb for FB_1. Results obtained on a triple-quadrupole instrument by Faberi et al. (2005) showed LODs (S/N: 3/1) of 2 ppb for FB_1 and 1 ppb of FB_2. Table 3.6 presents also the LODs and LOQs of a commonly used fluorescence method (Lötscher 2001) and the results obtained by MS and MS/MS.

Table 3.6: Comparison of the limits of detection (LOD, signal-to-noise ratio (S/N) of 3:1) and limits of quantification (LOQ, S/N 10:1) for HPLC/MS and a fluorescence method (Lötscher 2001) for fumonisins. SRM-1 and SRM-2 are based on the cleavage of TCA (tricarballylic acid) and H_2O, respectively.

		Fluorescence	**Full scan**	**SIM**	**SRM-1**	**SRM-2**
LOQ [ppb]	**FB_1**	40	86	8	42	2
	FB_2	40	86	8	11	1
LOD [ppb]	**FB_1**	2	5	0.6	12	0.6
	FB_2	2	5	0.8	5	0.5

Table 3.7 presents typical recovery rates and relative standard deviations obtained for corn spiked with different levels of fumonisins.

Table 3.7: Recovery rates and relative standard deviation (RSD) for corn spiked with fumonisins.

Recovery [%]	**FB_1**	**FB_2**	**FB_3**
Spiking level 250 ppb (n = 3) ± RSD [%]	82 ± 7.3	91 ± 10.5	91 ± 9.8
Spiking level 400 ppb (n = 1)	72	78	n.d.

n.d. = not determined

Recoveries were similar to Paepens et al. (2005), who reported mean recoveries of 84 % ± 10 %, 78 % ± 7 % and 85 % ± 9 % for FB_1, FB_2 and FB_3 respectively, for twelve replicates applying a similar extraction and sample cleanup system.

3.6.3 Analysis of real samples

Products were selected, which possessed a high proportion of corn. Table 3.8 gives an overview of the samples examined and their fumonisin content. The quantification was based on external calibration due to matrix interferences, which resulted in low recoveries of the ISTD OTB (mean recovery 5 %, RSD 3.7 %).

Table 3.8: Comparison of the concentrations in ppb of FB_1 and FB_2 in various corn samples obtained by fluorescence and MS detection The masses m/z 722 for FB_1 and m/z 706 for FB_2 were selected for the SIM mode. External calibration (n=12) was applied.

Sample	Fluorescence detection[a]		MS-detection[b]	
	FB_1 [ppb]	FB_2 [ppb]	FB_1 [ppb]	FB_2 [ppb]
Corn, test material	6887	2918	9069	3064
Corn, raw material for Coop Maisano bread mix for baking	<40	<40	<8	8
Coop Maisano, bread mix for baking	n.a.	n.a.	<8	<8
Coop Maisano, corn bread, ground	n.a.	n.a.	150	33
Coop Bio Popcorn	<40	<40	65	17
Coop Polenta Rustica	115	40	224	48
Coop Polenta Dorata	n.a.	n.a.	261	69
Maggi Polenta Ticinese	n.a.	n.a.	55	20
Old el paso Nachips, ground	n.a.	n.a.	49	19
Coop Cornflakes, ground	n.a.	n.a.	<8	12
Bio Coop Naturaplan Cornflakes, ground	n.a.	n.a.	16	<8

n. a.: not analyzed a: (Lötscher 2001), LOQ 40 ppb b: LOQ 8 ppb

Higher fumonisin contents have been found using MS detection in the SIM mode than with the fluorescence method, especially for FB_1. Deviations were up to 32 % for the corn test

material and up to 77 % for the "Polenta Rustica". The reason for this difference could be either inaccuracy of the calibration of FB_1, or a systematic error in the detection of FB_1 in the fluorescence or the mass spectrometric detection. However, the analogous analysis of FB_2 gave values that showed much better correspondence.

All food samples contained fumonisins below the tolerance value of 1 ppm. The values (see Table 3.8) were comparable to the average fumonisin content in corn products in Central Europe (Matthiaschk et al. 1999). The highest content represented the "Polenta Dorata" (see Figure 3.9) with a total fumonisin content of 330 ppb (FB_1+FB_2).

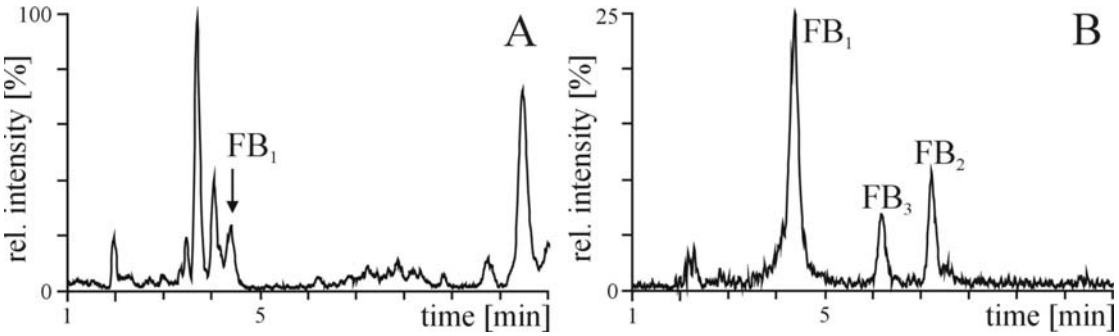


Figure 3.9: ESI(+) Base ion chromatogram (A) and extracted mass chromatogram (B) of the sample Polenta Dorata recorded in the full scan mode (see Table 3.6).

No fumonisins were detected in the bread baking mixture for Coop Maisano bread. Astonishingly, the corresponding bread showed comparatively high levels. This can be explained either due to a different, more contaminated batch of corn or due to a elimination or degradation of the interfering matrix parts by food processing.

This example underlines that the detection of the so-called “hidden fumonisins” in thermally processed food can be a problem. Kim et al. (2002) showed that fumonisins tend to bind to

various food constituents like cornstarch or glucose. This could explain the observed "loss" of fumonisins and the resulting recoveries of around 70 to 80 % (see also Chapter 3.6.2.2) as well as the lower levels of FB_1 and FB_2 in cornflakes and Nachips compared to unprocessed foodstuff. Here, it would be interesting to examine the products for hydrolyzed fumonisins. In two products, the raw material for corn bread and the cornflakes, only FB_2 could be identified. This result is unusual, because FB_1 represents the main component in most products.

An additional compound with a molecular ion $[M+H]^+$ of *m/z* 706 was identified as Fumonisin B_3 (see Figure 3.8), which could not be quantified due to the absence of a standard substance. It was present in all samples except "test material" and "raw material for Coop Maisano bread mix for baking". The observed amounts were lower than for FB_2.

3.6.4 Synthesis of ISTD

Several substances were tested for their applicability as ISTD, but none of them was capable. Therefore, it was tried to synthesize a suitable standard compound by modifying FB_1. The selected reactions should be performed under mild conditions and not require any complicated purification steps. Although the selected reactions were rather simple, it was difficult to find one yielding in a 100 % reaction. The amino function was selected for derivatization, because the carboxyl groups should remain unchanged to allow a further sample cleanup via anion exchange (SAX column). Additionally, the synthesis product had to be stable under the acidic conditions typical for sample cleanup. Figure 3.10 shows an overview of all applied derivatizations of FB_1 leading to a carbamate or a cyclic imide structure.

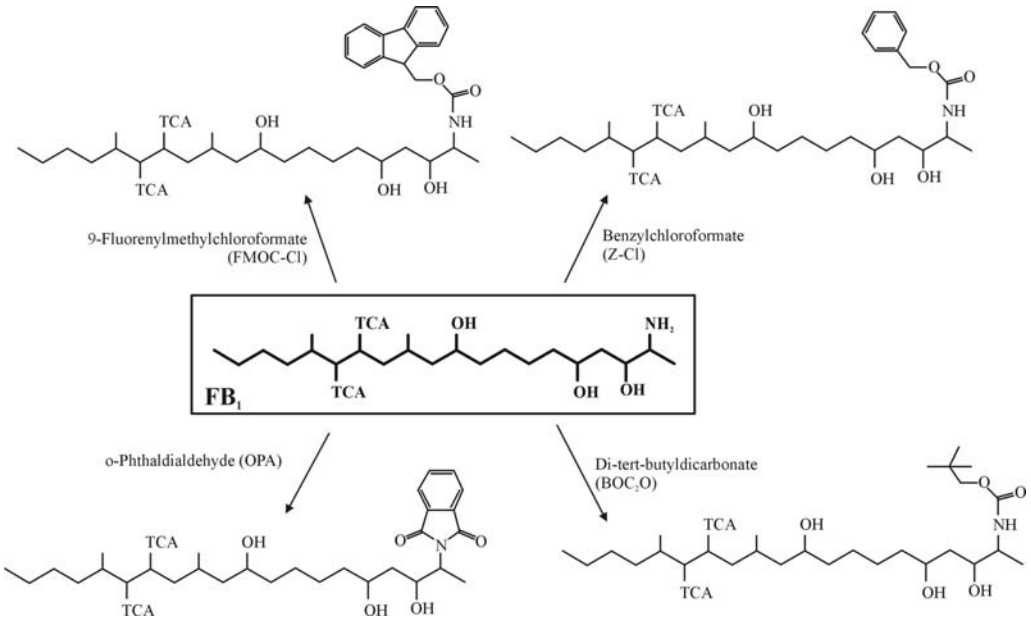


Figure 3.10: Overview of all applied derivatizations of fumonisin B_1 (FB_1). Reactions of FMOC-Cl, Z-Cl and BOC_2O give a carbamate and of OPA a cyclic imide. FMOC-Cl (9-fluorenylmethylchloroformate), Z-Cl (benzylchloroformate), BOC_2O (di-tert-butyldicarbonate), OPA (*o*-phthaldialdehyde), TCA (tricarballylic acid).

Table 3.9 gives an overview of all detected molecular ions and ion adducts and the most intensive ions. Table 3.10 gives an overview of the obtained yields of all tested derivatizations. Detailed information about the reaction conditions is given in Chapter 3.5.5.

Table 3.9: Observed molecular ions, ion adducts and most intensive ions of FB_1 derivatives obtained in the ESI(+) mode. Relative abundances are given in brackets. FMOC-Cl (9-fluorenylmethylchloroformate), Z-Cl (benzylchloroformate), BOC (tert-butoxycarbonyl), OPA (*o*-phthaldialdehyde).

Derivatives of FB_1	**$[M+H]^+$**	**$[M+NH_4]^+$**	**$[M+Na]^+$**
FMOC-FB_1	944 (14)	**961** (100)	966 (8)
Z-FB_1	856 (16)	**873** (100)	878 (20)
BOC-FB_1	836 (15)	**853** (100)	858 (11)
OPA-FB_1	852 (14)	**869** (100)	874 (10)

Bold: most intensive ion

Table 3.10: Yields of the derivatization reactions of FB_1. For detailed information about reaction conditions see Chapter 3.5.5. FMOC (fluorenyloxycarbonyl), Z (benzylchloroformate), BOC (tert-butoxycarbonyl), OPA (*o*-phthaldialdehyde).

Reaction No.	Yield [%]	Reaction No.	Yield [%]	Reaction No.	Yield [%]	Reaction No.	Yield [%]
FMOC1	10	Z1	40	BOC1	25	OPA1	70
FMOC2	5	Z2	10	BOC2	40	OPA2	100
FMOC3	50*	Z3	75	BOC3	40	OPA3	45
FMOC4	70*	Z4	80	BOC4	60	OPA4	100
FMOC5	100	Z5	85	BOC5	70	OPA5	100
FMOC6	100	Z6	100	BOC6	0		
				BOC7	0		

* Precipitation of $NaHCO_3$, see Chapter 3.6.5.1

3.6.5.1 Derivatization as carbamate

The FMOC-group is the most acid-stable of all derivatization reagents. However, FMOC-protection has hardly any stability in basic medium, so that traces of base lead to bond cleavage. FB_1 had to be dissolved in CH_3CN/H_2O (1+1) to avoid a precipitation of $NaHCO_3$. The reaction product FMOC-FB_1 gave a $[M+NH_4]^+$ at m/z 961 with a yield of 100 %. The reaction is complete within 5-10 min at room temperature. Figure 3.11 shows the ESI(+) mass spectra of the obtained FMOC-FB_1 derivatives. The intensity of the MS signals was stable for the first 9 h (see Chapter 3.5.5.3 for detailed reaction times), after that the derivative showed decomposition. These results are in contrast to the results obtained by Holcomb et al. (1993), which describe a stability of FMOC-derivatives for at least 72 h.

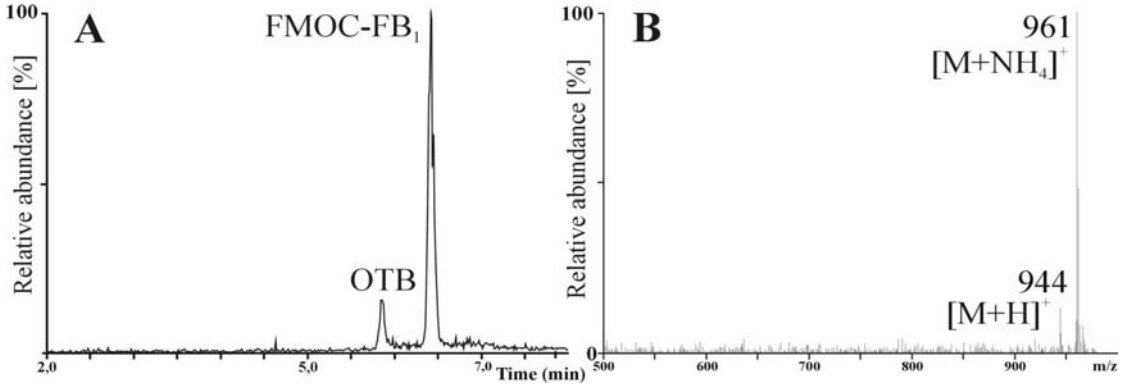


Figure 3.11: Derivatization of FB_1 with FMOC-Cl. ESI(+)-MS base peak chromatogram (A) and mass spectra of FMOC-FB_1 (B). Ochratoxin B (OTB) was used as ISTD.

The linear range of FMOC-FB_1 in a concentration range from 250 ng/ml to 10 µg/ml was determined with a correlation coefficient of 0.8746. Unfortunately, this seems to demonstrate that a quantification in this case based on a NH_4^+-adduct is not applicable. Therefore, this compound failed to be used as ISTD substance.

The reaction of FB_1 with benzylchloroformate (Z-Cl), as well as the derivatization by means of di-tert-butyldicarbonate (BOC_2O) also leads to a protection of the amino group as carbamate. Both derivatives were easily detectable in MS. The synthesis of Z-FB_1 was obtained with a 100 % yield. However, the received reaction products showed weak stability in acidic medium at pH values < 2. After 60 min of reaction time the derivatization product already started to decompose. The derivatization with BOC_2O did not yield in a 100 % reaction. An increase of reaction time and temperature showed no different results. Additionally, the derivatization was performed by means of ultrasonication in order to reduce the incubation time as proposed by Einhorn et al. (1991). These conditions did not result in higher overall yields. Both compounds therefore failed to be used as ISTD substances.

3.6.5.2 Derivatization as cyclic imide

The derivatization of FB_1 as cyclic imide using OPA succeeded in a quantitative yield of 100 %. The derivatives could be easily detected by MS. However, the derivatives showed low acidic stability at pH values < 2. OPA-FB_1 started to decompose after 30 min, which is in contrast with stabilities of OPA derivatives described by Rice et al. (1995).

3.7 Conclusions

The high sensitivity and selectivity of HPLC-ESI-MS/MS offers an advantageous tool for fumonisin determination at low levels. A LOD of 0.6 ppb and a LOQ of 8 ppb was obtained for FB_1 in the SIM mode, which is a factor of which is a factor of five better compared to the former fluorescence method. For SRM2 mode the improvement was even better by a factor of twenty. Furthermore, a 50 % reduced chromatographic run time could be applied successfully for the analysis of real samples. The detection of a neutral loss of fumonisins offers the possibility to determine possible new representatives of this substance class, e.g. generated by food processing.

Additionally, FB_3 could be identified as a further contaminant of corn in all samples (except test material and bread mix). However, its concentration was lower than for FB_2. All analyzed food samples contained fumonisins below the Swiss tolerance level of 1 ppm and represent typical values for fumonisin contamination in grain harvested in Middle Europe. This suggests that the endangerment by mycotoxins in Central Europe can be estimated keeping in

mind the small number of samples tested. However, the problems due to the rising import of food from tropical and subtropical areas in which fungal damage proves to be far more problematic are increasing.

OTB could not be recovered in a high number of real samples. This indicates that the corn matrix is very complex and can cause false results in the determination of recoveries. Regarding the high variability and complexity of food matrices in general, the application of an ISTD method is inevitable. Alternatively, ionization techniques have to be found which overcome matrix effects and ion suppression more effective.

The search for a suitable ISTD for fumonisin determination is still not solved, because the substances under investigation could not fulfill all given requirements. The substance of choice must offer a sufficient stability in acidic medium and show a proper linearity for a comprehensive quantitative analysis method making use of the advantages of ISTD methods.

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4. DEVELOPMENT OF A MULTIRESIDUE METHOD FOR PREVALENT FUSARIUM MYCOTOXINS WITH COMMON SPE SAMPLE CLEANUP AND DETECTION WITH APCI(+)LC/MS

4.1 Introduction

Placinta et al. (1999) found out that cereal grains and animal feed may be contaminated simultaneously by different substance classes of mycotoxins on a global scale. Marin et al. (2004) reported, that one fungal infection does not prevent the production of mycotoxins from other species. In fact, there is a massive exposure to multiple fungal infections in the field or during harvest and storage. Thus, several mycotoxins produced by different species can be found frequently in corn samples. This co-occurrence has also been described by a number of authors (Scudamore et al. 2000; Vargas et al. 2001; Vrabcheva et al. 2000). It increases the risk of a potential intoxication due to a number of additive or synergistic effects between several mycotoxins multiplying adverse influence on the associated health hazards for humans or animals (D' Mello et al. 1999). Therefore, rapid and reliable screening methods are required for the simultaneous identification and quantification of many mycotoxins to ensure food safety for human and animal consumption.

4.1.1 Mycotoxins

4.1.1.1 Zearalenone, Zearalenol

Zearalenone (ZON) and its derivatives (see Figure 4.1) are macrocyclic fungal metabolites, mainly found in maize, oat, barley, wheat and sorghum (Zöllner et al. 1999). ZON is one of the most frequently found *Fusarium* mycotoxins in European maize. This contaminant is a nonsteroidal, strongly estrogenic and anabolic acting mycotoxin with a relatively low acute toxicity (oral LD_{50} values of 4 to 20 g/kg bw; Kuiper-Goodman et al. 1987). However, the described properties of ZON may cause severe reproductive and infertility problems in farm animals, especially in pigs. Investigations about chronic ingestion demonstrated that its estrogenic properties are an important feature at levels down to 1.5 to 3 mg/kg (Placinta et al. 1999).

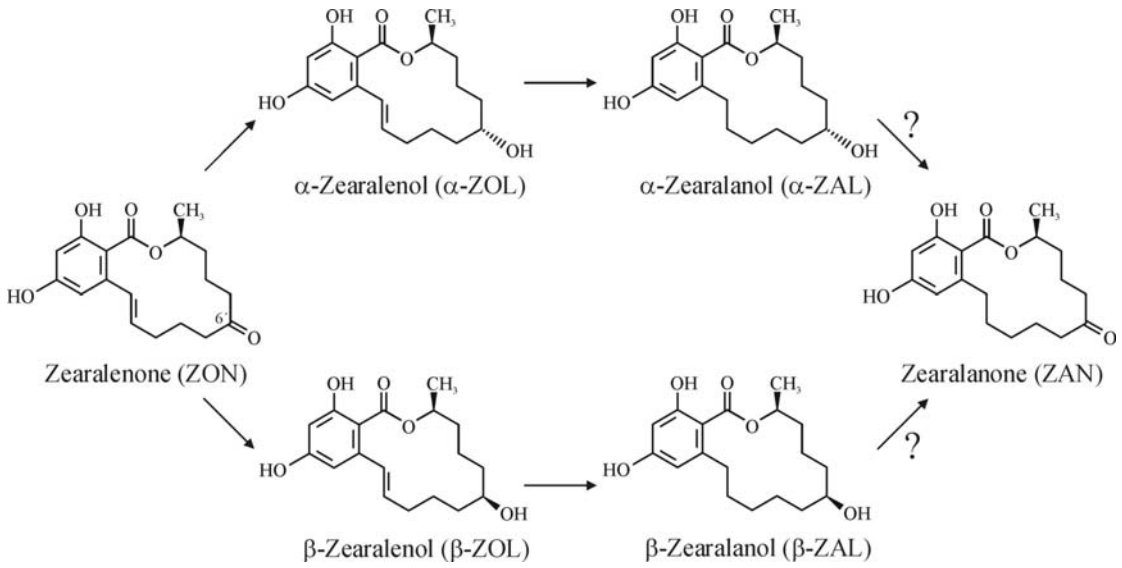

Figure 4.1: Structures of zearalenone (ZON) and its derivatives: α-zearalenol (ZOL), β-ZOL, α-zearalanol (ZAL, zeranol), β-ZAL (taleranol) and zearalanone (ZAN).

All other macrocyclic lactones are metabolites of ZON in mammals and show estrogenic, anabolic, immunomodulating, and antimicrobial properties at various degrees. α-ZOL has a three to four fold higher estrogenic activity than ZON and occurs naturally in cereals, however less frequent and in lower concentration levels than ZON. Both diastereoisomers of ZOL are produced by hydrogenation of the keto-function at the C(6') position. Labeling experiments suggest that ZOL is the precursor in the biosynthetic pathway leading to ZON formation (Schwadorf et al. 1992). Zearalanol (ZAL) consists of the two diastereoisomers α-ZAL (zeranol) and β-ZAL (taleranol). Zearalanone (ZAN) was first obtained by catalytic hydrogenation of ZON. Later it has been identified as a metabolite of the fungus *Fusarium reticulatum* (Bracher et al. 2001).

The PMTDI of ZON is 0.5 μg/kg bw based on the NOEL of 40 μg/kg bw per day (Creppy 2002). ZON shows only low acute toxicity after oral administration in mice, rats, and pigs (oral LD_{50} values from >4000 to 20 000 mg/kg bw (Kuiper-Goodman et al. 1987). Studies have shown that ZON and its derivatives possess highly estrogenic and anabolic properties, α-ZOL even three to four times higher than ZON (Josephs 1999). In Germany, maximum levels of ZON contaminations in cereals are regulated from 20 to 50 μg/kg (Engelhardt 2004).

4.1.1.2 Ochratoxin A

Ochratoxin A (OTA, Figure 4.2) is the most toxic and frequent representative of a group of mycotoxins produced mainly by *Penicillium* and *Aspergillus* species. OTA occurs naturally in a variety of plant products such as cereals (Birzele et al. 2000), beans (Hurst et al. 1998) and

dried fruits (MacDonald et al. 1999) all over the world but mainly in cooler temperate regions of Canada and Europe (Creppy 2002). But this compound has also been detected in products such as coffee, beer, and wine (Soleas et al. 2001) as well as in the kidney (Jörgensen et al. 1999), liver (Jimenez et al. 2001) and blood (Scott et al. 1998) from mammals by carry-over from animal feed (Scudamore et al. 1997). Transfer to milk has been demonstrated in rats, rabbits and humans, whereas little is transferred to the milk of ruminants due to metabolism of OTA by the rumen microflora (Creppy 2002). Studies have shown that OTA contamination is mainly associated with storage (Beretta et al. 2002), so that suitable post-harvest conditions (temperature and moisture) are important.

OTA is a phenylalanine derivative of a substituted isocoumarin with carcinogenic, nephrotoxic and teratogenic properties. The high affinity of OTA to proteins allows its accumulation in organs as shown in animal studies (Hagelberg et al. 1989). Additionally, OTA is suspected to be the cause of chronic kidney disease in South-Eastern Europe, known as “Balkan endemic nephropathy“ and of urinary tract tumors (Pfohl-Leszkowicz et al. 2002).

The EU Scientific Committee on Food defined a tolerable daily intake (TDI) of 16 ng/kg bw in 1991 based on a Lowest Observed Adverse Effect Level (LOAEL) of 8 µg/kg bw/day and applying a safety factor of 500. More recently, efforts have been made to reduce this value to 5 ng/kg bw (Scientific Commission on Food 1998). Furthermore, a provisional tolerable weekly intake (PTWI) of 112 ng/kg bw was established on the basis of deterioration of renal function in pigs, for which the lowest observed effect level (LOEL) was 8 µg/kg bw per day. Maximum levels for OTA contaminations are in Germany regulated between 0.5 to 10 µg/kg (Engelhardt 2004).

4.1.1.3 Citrinin

The mycotoxin citrinin (CIT) is formed by moulds of the species *Aspergillus* and *Penicillium*. Both moulds are able to produce CIT and OTA simultaneously, which show similar nephrotoxic, teratogenic and carcinogenic effects (Reiss 1997). They are estimated to act synergistically (Creppy et al. 1980). Furthermore, CIT has antibiotic, bacteriostatic, antifungal and antiprotozoal properties. The oral LD_{50} in rats and mice is 67 mg/kg bw and 35 mg/kg bw, respectively (Betina 1989). CIT mainly occurs in cereals such as wheat, barley, rye, oats, corn and rice (Reiss 1997). The chemical structure of CIT is given in Figure 4.2.

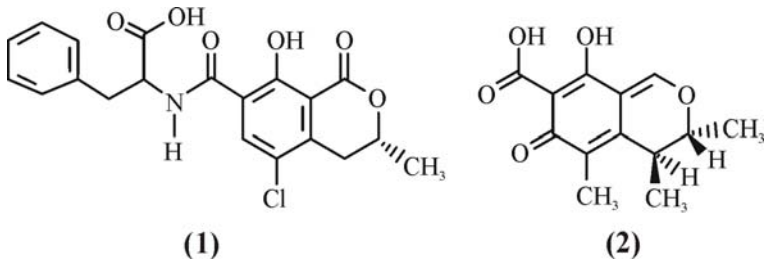

Figure 4.2: Structures of Ochratoxin A (1, OTA) and Citrinin (2, CIT)

4.1.2 State-of-the-art in multiresidue mycotoxin detection

Today, several methods for mycotoxin determination in food matrices exist that often combine chromatography with mass spectrometric (MS) detection. The main advantage of the latter is its specificity, which makes LC/MS or GC/MS to favored techniques. Furthermore, the extensive technical improvements in the field of LC/MS during the last years shifted mycotoxin analysis to LC/MS and LC/MS/MS applications. Trichothecenes (see Chapter 2) and fumonisins (see Chapter 3) represent analytes, where LC/MS methods are mandatory for

qualitative and quantitative detection due to the presence of different toxins with similar structure and subsequently similar chromatographic behavior.

However, the detection of structurally different mycotoxins or classes within a single analysis still represents a big challenge. The complex composition of food and feed matrices as well as the different physical and chemical properties of single mycotoxins are the main problems that need to be solved. So far, only a limited number of multiresidue methods have been described in literature using MS detection. The simultaneous determination of trichothecenes and ZON by GC/MS by Tanaka et al. (2000) and Onji et al. (1998) was used for confirmation purposes as well as for primary screening. However, it requires an additional derivatization step. Further multi mycotoxin techniques for grain are based on a splitted sample cleanup (Plattner 1999; Plattner et al. 1983; Rajakylä et al. 1987; Sörensen et al. 2005). A big challenge for a common sample cleanup is to cover the strongly varying polarities of different mycotoxins. To compensate the reduced specifity, detectors are needed with a high degree of selectivity such as MS. Until now, the idea of a single cleanup was only realized by Cavaliere et al. (2005) and Berthiller et al. (2005).

4.2 Aim of the work

Today, a huge variety of techniques and methods are available for the determination of single mycotoxins or their substance classes. Therefore, the aim of this work was to develop a LC/MS method that allows the simultaneous determination of further widespread mycotoxins (ZON, ZOL, OTA and CIT) in addition to the determination of the prevalent trichothecenes (DON, NIV and HT-2). A screening method for grain should be available, which is on the one

hand simple and reliable and on the other hand covers mycotoxins of widely varying polarity. The final aim was to establish a quantitative multiresidue method of universal applicability allowing a fast and sensitive routine analysis. The essential simplification in comparison to the methods described in literature, was the development of a sample cleanup based on common extraction and subsequent SPE procedures. Furthermore, the developed method should be applicable for different grains such as wheat and corn.

4.3 Experimental

4.3.1 Materials and standard solutions

Water with a total organic content of <2 ppb was obtained from an Elgastat maxima HPLC water purification unit (Elga LTD., Bucks, Great Britain). Acetonitril, grade "190 far UV" (Romil, Cambridge, Great Britain) and Methanol of pestipur grade (SDS, Peypin, France) were used. Helium of 99.999 % purity and nitrogen of 99.995 % purity were purchased from Carbagas (Switzerland). The following chemicals of certified purity (if specified) were purchased from Fluka Chemie (Buchs, Switzerland): ammonium formate (≥99 %), trifluoroacetic acid (≥99.5 %) and hydrocortisone (HYC, ≥97 %). CIT (≥99 %), DON (>99 %), HT-2 toxin (>97,5 %), NIV (98 %), OTA (≥99 %), OTB, verrucarol (VOL, ≥98 %), ZOL (≥99 %) and ZON (≥99 %) were provided by Sigma Chemie (Buchs, Switzerland). N-Hexane for pesticide residue analysis was purchased from Scharlau (Barcelona, Spain), formic acid (≥99 %) and ammonia (≥32 %) from Merck (Darmstadt, Germany).

Solid phase extraction (SPE) was carried out on a variety of different phases. Table 4.1 gives an overview of all investigated SPE columns. Folded cellulose filters of medium porosity were used for filtration (Schleicher & Schuell, Feldbach, Switzerland, 150 mm i.d.).

Table 4.1: Supplier, Charge and reservoir volume of the tested SPE columns.

SPE phase	Description	Supplier/ Catalogue number	Sorbent mass [mg]	Volume [ml]
Bond Elut C18	Non-polar C18 packed sorbent bed	Varian/1210-2001	100	1
Bond Elut C18	Non-polar C18 packed sorbent bed	Varian/1210-2096	200	1
Bond Elut SAX	Strong anion exchange packed sorbent bed	Varian/ 1210-2017	100	1
NH_2	Amino-substituted sorbent bed	Macherey-Nagel/ Chromabond 730 031	100	1
Bond Elut C2	Non-polar C_2 packed sorbent bed	Varian/2210-2060	50	1
Bond Elut C8	Non-polar C_8 packed sorbent bed	Varian/1210-2100	100	3
Bond Elut PH	Phenyl-substituted sorbent bed	Varian/1411-3005	100	10
Certify II (Certi)	Mixed mode sorbent bed	Varian/1211-3063	100	10
Nexus	Polymeric phase sorbent bed	Varian/ 1211-3101	60	10
CN	Cyano-substituted sorbent bed	Macherey-Nagel/ Chromabond 730 061	100	1
Easy	polystyrene divinyl-benzene copolymer	Macherey-Nagel/ Chromabond 730 752	100	1

The mycotoxin standard solution (M8) contained the following concentrations: CIT 3 µg/ml, DON 20 µg/ml, HT-2 4 µg/ml, NIV 10 µg/ml, OTA 20 µg/ml, OTB 15 µg/ml, VOL 20 µg/ml, ZOL 30 µg/ml, ZON 10 µg/ml, HYC 4 µg/ml.

4.3.2 Instrumentation

Different balances from Mettler-Toledo Schweiz AG (Greifensee, Switzerland) were used for the preparation of reference solutions and real samples: Mettler M3, Mettler AE 100 and Mettler AC 100. Real samples were extracted on a wrist-action shaker LSL-V from Adolf Kühner AG (Birsfelden, Switzerland) with 200 movements per min. A self-constructed evaporation device consisting of a heated aluminum block and a nitrogen supply was used for solvent reduction. pH values were determined with a pH-meter 744 of Metrohm (Herisau, Switzerland). HPLC solvents were degassed directly with helium for approx. 15 min. Samples were injected with a Valco Cheminert valve or a HTS-Pal auto-sampler (CTC Analytics, Zwingen, Switzerland). HPLC was carried out with a Rheos 4000 pump and a Rheos 2000 pump equipped with a pulsation dampener (both pumps originated from Flux Instruments, Basel, Switzerland). A quadrupole ion trap spectrometer (LCQ, Finnigan, San Jose, CA, USA) was used for detection of mycotoxins equipped with atmospheric pressure chemical ionization and detection in the positive ion mode (APCI(+)).

4.3.3 Methods

4.3.3.1 Extraction and cleanup

1 ml of standard solution M8 was added to 10 g of ground corn or wheat and extracted in 40 ml CH_3CN/H_2O (84+16) for 2 hours in a 500 ml bottle on a action wrist shaker with 200 movements per minute. Subsequently, the crude extract was filtered and 4 ml aliquots were

concentrated on a heated aluminum block (40 °C) under a nitrogen flow. The residue of each aliquot was dissolved in 500 ml MeOH and some samples were shaken with 1 ml hexane to remove the fatty components of the matrix. The organic layer was separated with a syringe.

Standard solutions of M8 were used for the determination of the mycotoxin recovery during the method development of the SPE sample cleanup. Various conditioning, washing and elution steps as well as a number of different SPE column materials were examined for maximum recovery rate. The residue from SPE sample cleanup was immediately analyzed with LC/MS. SPE phases were conditioned with 1 ml MeOH and 1 ml H_2O (2 ml H_2O pH 8.5 for Nexus, no conditioning for Easy). The washing was performed with 1 ml H_2O (1 ml MeOH for Easy), and the elution of analytes was done with 3 ml MeOH (C2, C8, PH, Certi, Nexus), 3 ml MeOH 1 % TFA (C18, CN), 3 ml 10 mmol ammonium formate buffer (pH 1 for SAX and pH 2 for NH_2) and 1.5 ml H_2O (Easy).

4.3.3.2 Separation and detection

PEEK injection loops of 5 µl and 20 µl volume with an i.d. of 175 µm were used for the injection of samples to LC/MS. The flow rate of the mobile phase was 250 µl/min. Two commercially available HPLC columns were selected: The Xterra MS HPLC column was obtained from Waters (Milford, USA) and had the following dimensions: particles 3.5 µm diameter, 12.5 nm pore size, 10 cm column length, 3 mm i.d. The Discovery HSF5 with a pentafluorophenylpropyl phase from Supelco (Buchs, Switzerland) had the following dimensions: particles 3 µm diameter, 12 nm pore size, 10 cm column length, 2.1 mm i.d. Other HPLC columns tested during method development were: Nucleosil CN (5 µm particle

diameter, 100 Å pore size, 250 mm length x 4 mm i.d.), Nucleosil Phenyl (5 µm, 100 Å, 125 x 4 mm), Nucleosil C8 HD (5 µm, 100 Å, 250 x 4 mm), Nucleosil C18 AB (3 µm, 100 Å, 125 x 3 mm), Nucleosil C18 Nautilus (5 µm, 100 Å, 125 x 3 mm) (all Nucleosil phases obtained from Macherey-Nagel, Oensingen, Switzerland), Discovery HS PEG (3 µm, 120 Å, 100 x 2.1 mm; obtained from Supelco, Buchs, Switzerland) and the Micra C18 NPS (1.5 µm, "non-porous" particles with only small porous surface, 30 x 3 mm; obtained from Micra Inc. Northbrook, USA). Table 4.2 shows the applied binary and ternary HPLC gradients. The first one was employed for the Discovery HSF5 column and the second for the Nucleosil and Xterra MS columns. The flow rate was 250 µl/min.

Table 4.2: Applied HPLC gradients. The binary gradient was used for the Discovery; the ternary gradient for the Nucleosil and the Xterra MS columns. The buffer consisted of 10 mmol ammonium formate (pH 2.5) Solvent composition is given in [% (v-v)].

Time	**Binary gradient**		**Ternary gradient**		
[min]	**Buffer**	**MeOH**	**H_2O**	**Buffer**	**MeOH**
0	75	25	75	-	25
1.5	75	25	75	-	25
2	-	-	65	10	25
5	-	-	-	50	50
10	-	-	-	15	85
12	2	98	-	2	98
17	2	98	-	2	98
18	75	25	75	-	25
24	75	25	75	-	25

The mass spectrometer was used in the positive ion mode employing APCI. Mass spectra were acquired in full scan mode (mass range m/z 235 to 800). Following instrument parameters were optimized: heater temperature 230 °C; nitrogen sheath gas flow 40 arbitrary units (approx. 400 ml/min); ionization current of corona discharge 1.5 µA. The autotune program optimized the voltages of the heated capillary, lenses and octapoles for maximum

transmission of the $[M+H]^+$ ion of ZON (m/z 319). The temperature of the capillary between the ionization chamber and the first vacuum stage was 150 °C.

4.3.3.3 Quantification

The signal areas of all mycotoxins were integrated in the extracted mass chromatograms of the $[M+H]^+$ ions. Only for HT-2, the $[M+NH_4]^+$ ion showed a higher abundance and subsequently a better recovery compared to the $[M+H]^+$ ion. Table 4.3 shows all analytes with their corresponding mass used for quantification.

Table 4.3: Masses used for the quantification of mycotoxins [m/z].

Mycotoxin	NIV	DON	VOL	HYC	HT-2	CIT	OTB	ZOL	OTA	ZON
$[M+H^+]$	313	297	267	363	442[1]	251	370	321	404	319

[1] corresponds to the $[M+NH_4^+]$

Quantification was based on the internal standard method. Hydrocortisone (HYC) was chosen as recovery standard (RSTD) and added before separation. OTB and VOL were selected as internal standard (ISTD) and added to the extraction solution prior to sample cleanup. VOL was applied for quantification of trichothecenes (NIV, DON and HT-2), ZOL and ZON. OTB was employed as ISTD for OTA and CIT. Signals were quantified above a signal-to-noise ratio of 3:1. Recovery was determined as follows (A_m, signal area of mycotoxin and A_{ISTD}, signal area of ISTD):

$$\text{Recovery}[\%] = \frac{A_m(\text{sample})}{A_m(\text{standard})} * \frac{A_{ISTD}(\text{standard})}{A_{ISTD}(\text{sample})} * 100$$

4.4 Results and discussion

4.4.1 Extraction and cleanup

4.4.1.1 C18 SPE phases

C18 SPE columns can be used for the sample cleanup of a wide range of compounds due to the hydrophobic behavior of the SPE sorbent. C18 sample cleanup showed the highest recoveries for all mycotoxins. The optimized SPE conditions for each step of the C18 sample cleanup for the method development for mycotoxins sample cleanup are summarized in Table 4.4. Recoveries for all mycotoxins on the C18 phase are summarized in Table 4.5.

Table 4.4: Optimized SPE conditions for the mycotoxin sample cleanup applying a C18 phase.

Analysis step	Optimized conditions
Equilibration	1 ml MeOH, 1 ml H_2O
Sample loading	100 µl in MeOH
Washing	1 ml H_2O
Elution	3.5 ml MeOH 1 % TFA
Evaporation	To dryness, at 40 °C
Sample solution	100 µl MeOH/H_2O (25+75)

Table 4.5: Mean recoveries and relative standard deviations (RSD) of mycotoxins on C18 SPE phases for sample cleanup (n=4).

Mycotoxin	Mean recovery [%]	RSD [%]
NIV	88	18
DON	86	10
VOL	79	9
HT-2	70	17
CIT	71	3
OTA	46	2
OTB	65	9
ZON	82	9
ZOL	106	11

It was observed that the flow rate of the elution solvent is of some importance. Highest recoveries were obtained with a flow rate of ca. 0.7 ml/min. It should not exceed 1 ml/min. Additionally, the recoveries were investigated in dependence of the volume of elution solvent. Figure 4.3 gives a summary for the analytes DON, ZON and CIT as typical representatives for each mycotoxin class. The final elution volume was 3 ml (together with a safety factor).

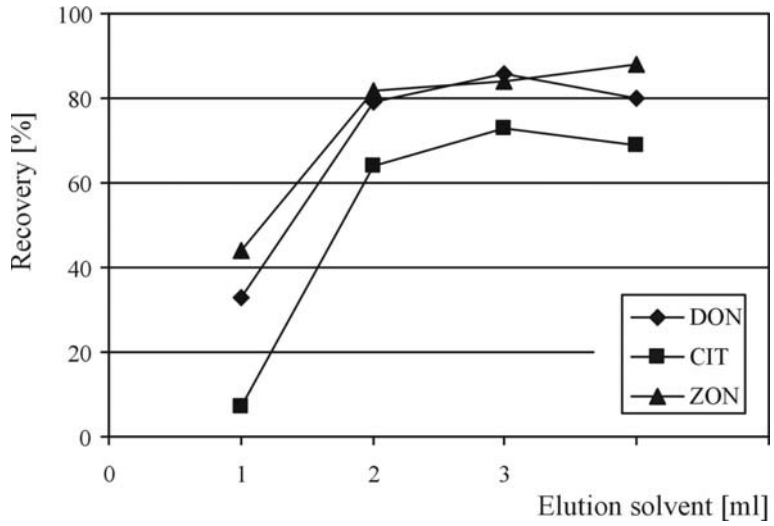

Figure 4.3: Dependence of the recovery from the volume of elution solvent for C18 SPE sample cleanup of mycotoxins. DON, CIT and ZON are shown as representatives for each substance class of the mycotoxins.

The recovery of the C18 phase could be increased by 10% by changing the elution solvent from CH_3CN to MeOH. Furthermore, the influence of the pH was investigated at pH 2.0, 5.5 and 8.5. Best recoveries were obtained at pH 2, where the highest interaction with the non-polar SPE surface was observed. Moreover, a change from 100 to 200 mg sorbent mass resulted in an increase of recoveries by 5 % to 9%. All mycotoxins had a recovery between 46 and 106 % on the C18 phase. However, the variation was relatively high for selected analytes (see RSD, Table 4.5). Nevertheless, the C18 sample cleanup was a suitable compromise even if the recovery of mycotoxins with an acid functional group was just satisfactory.

4.4.1.2 SAX SPE phases

SAX SPE columns showed the best and most stable recoveries among all tested SPE sorbents besides C18. SAX cleanup is specifically suited for the enrichment of acidic compounds. The low recoveries of the acidic mycotoxins using C18 cleanup resulted in an evaluation of the SAX SPE cartridge as a further option. The concept was to re-combine the final extracts of both cleanups prior to HPLC separation and MS detection. Table 4.6 summarizes the optimized conditions for the SAX sample cleanup of acidic mycotoxins. The obtained recoveries were 52 % for CIT, 86 % for OTA and 102 % for OTB (n=2).

Table 4.6: Optimized SPE conditions of the sample cleanup development applying a SAX phase.

Analysis step	Optimized conditions
Equilibration	1 ml MeOH, 1 ml ammonia formate buffer (10 mmol) pH 2[1]
Sample loading	100 µ in MeOH
Washing	1 ml H_2O
Elution	3 ml ammonia formate buffer (10 mmol) pH 1
Evaporation	To dryness, at 40 °C
Sample solution	100 µl MeOH/H_2O (25+75)

[1] pH values were adjusted with 1 M HCl.

4.4.1.3 Further SPE materials

The mixed phase Nexus consists of a styroldivinylbenzene methacrylate copolymer and exhibits mixed functionalities with a large specific surface (575 m^2/g). Thus, it is able to extract both polar and nonpolar analytes within a large pH range (Hennion 1999). Even charged organic compounds can be enriched due to ππ-interactions between analyte and polymer matrix as described by Guenu et al. (1996) for pesticides. The influence of the pH

value on the recoveries of mycotoxins was evaluated for pH 2, pH 5.5 and pH 8.5. Good results were obtained at pH 8.5 with an average recovery of 60-90% (see Table 4.9). Table 4.7 summarizes the obtained recoveries for various SPE materials. Further details of the SPE cleanup procedure are provided in chapter 4.3.3.1.

Table 4.7: Overview of mean recoveries on all tested SPE phases. Further details to the SPE cleanup procedure are provided in chapter 4.3.3.1.

SPE material	NIV	DON	VOL	HT-2	CIT	OTA	OTB	ZON	ZOL
C_2 (n=2)	5	15	n.a.	n.d.	3	15	22	61	60
C_8 (n=2)	21	49	n.a.	n.d.	12	30	18	65	69
NH_2 (n=1)	2	5	10	37	7	60	107	38	31
PH (n=1)	22	59	n.a.	65	24	19	10	92	90
Certi (n=2)	20	68	143	138	n.d.	2	3	79	72
CN (n=2)	4	5	12	169	n.d.	n.d.	n.d.	63	58
Nexus (n=2)	25	50	70	129	45	39	46	25	50
Easy (n=1)	98	66	n.a.	51	n.d.	n.d.	n.d.	24	36

n.a. not analyzed n.d. not detected

Among all tested SPE materials, the lowest recoveries were found for the cyano phase (CN). Except for HT-2 all mycotoxins were poorly enriched and consequently also detected in the washing solution. The C2 and C8 materials showed acceptable recoveries for ZON/ZOL, but not for trichothecenes or the acidic mycotoxins CIT and OTA/OTB. The phenyl-substituted SPE material (PH) also provided high recoveries for ZON/ZOL, but was inadequate for all other mycotoxins. The mixed phases Certify II (Certi) and Easy as well as the amino phase gave no satisfying results either.

4.4.2 HPLC Separation

Several commercially available HPLC columns with different C18 reversed phase materials were tested (see Chapter 4.3.3.2). Among these, the Waters Xterra MS (column 1) with its polymeric silica-based material (hybrid particle technology) represented a good compromise. The Supelco Discovery HSF5 (column 2) showed comparable results with changed elution order. Both columns were tested with a binary and a ternary gradient system (see Table 4.2). Figure 4.4 compares the HPLC separations on both columns.

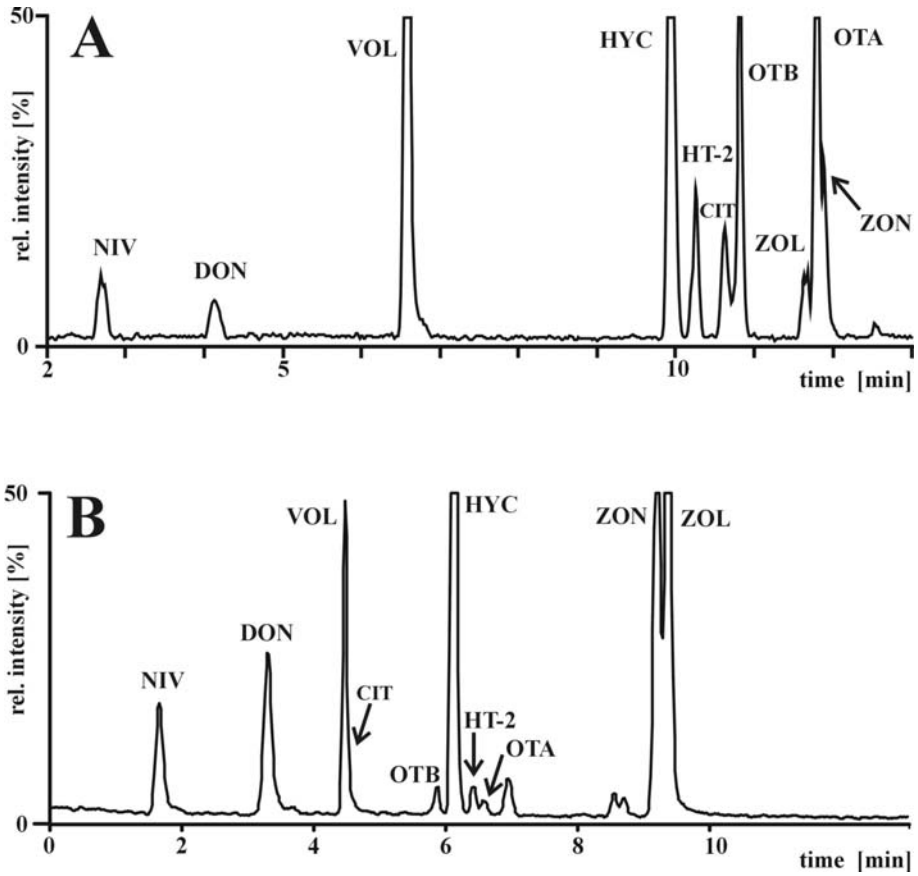

Figure 4.4: Comparison of the separation on two selected HPLC columns. APCI(+) extracted mass chromatograms of mycotoxin standard solution M8 are shown. A ternary gradient was used for the columns Xterra MS (A) and Supelco Discovery HSF5 (B).

The optimized conditions allowed the separation of all target compounds. A short chromatographic separation time of 12 min (column 1) and 10 min (column 2) was achieved. Finally, an overall run time of 24 min including flushing and reconditioning was used. The retention times of OTA and OTB decreased by almost 50 % by changing to column 2. Moreover, shorter retention times and better peak shapes could be achieved for most mycotoxins and ZON, ZOL, and OTA were separated successfully. So far, this is the fastest HPLC separation compared to other published multiresidue methods (Cavaliere et al. 2005; Mateo et al. 2002), which allows high sample throughput in routine analysis.

As described before, the analytes of interest cover a wide range of polarity. Therefore, a compromise had to be found in terms of pH of the mobile phase and the peak shape of the acidic mycotoxins (mainly OTA). The best buffer conditions were at a pH $\leq$3.2 with a salt concentration of 10 mmol. However, under these conditions the MS ion yield of the other mycotoxins decreased significantly as for example seen by the $[M+NH_4]^+$ adduct formation of trichothecenes. The effect was significantly higher for compounds with lower response and higher polarity. The separation of the mycotoxins was best by applying the ternary gradient. Mainly the polar mycotoxins NIV, DON and VOL had a better peak shape. Figure 4.5 shows the extracted mass chromatograms of a separation of the mycotoxin standard solution M8 on the Discovery HSF5 column.

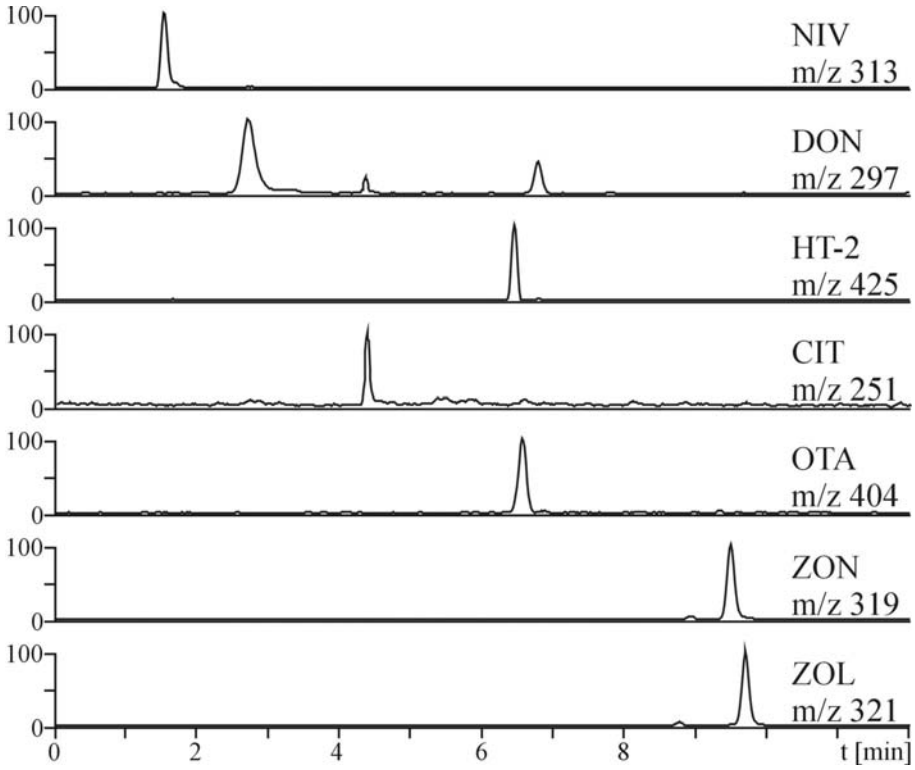

Figure 4.5: APCI(+) extracted mass chromatogram of a separation of the mycotoxin standard solution M8 on the Discovery HSF5 column. Concentrations were 3 µg/ml CIT, 20 µg/ml DON, 4 µg/ml HT-2, 10 µg/ml NIV, 20 µg/ml OTA, 15 µg/ml OTB, 20 µg/ml VOL, 30 µg/ml ZOL, 10 µg/ml ZON, 4 µg/ml HYC.

The reproducibility of the retention times was very good. Only OTA and VOL had strongly varying retention times caused by the significant sensitivity of these mycotoxins due to changes in pH and solvent composition. Table 4.8 summarizes the precision for 10 injections (column 1) and 4 injections (column 2) of standard solution M8. Although column 2 performed shorter retention times and accomplished the separation of ZON and ZOL, column 1 was selected for further HPLC analysis of real samples. This decision was based on the more stable retention times and the absence of coelution of VOL and CIT.

Table 4.8: Mean retention time (t_R), standard deviations s and relative standard deviations (RSD, [%]) of the chromatographic separation of standard solution M8 performed with the Xterra MS from Waters (column 1, n=10) and the Discovery HSF5 from Supelco (column 2, n=4) within 7 days.

Analytes	t_R[min]		±s [min]		RSD [%]	
	1	2	1	2	1	2
NIV	2.78	1.63	0.03	0.09	1.1	7
DON	4.32	3.48	0.08	0.14	2.0	10
VOL	6.6	4.56	0.31	0.07	4.6	5.3
HYC	10.14	6.26	0.05	0.16	0.5	3
HT-2	10.44	6.46	0.05	0.05	0.5	3.4
CIT	10.82	4.56	0.1	0.07	1.0	5.5
OTB	11	5.83	0.06	0.22	0.5	16
ZON	11.86	9.36	0.05	0.09	0.4	6.3
ZOL	12.07	9.17	0.05	0.12	0.4	8.5
OTA	12.12	6.74	0.26	0.16	2.1	11

4.4.2 Ionization and detection

Parameters influencing the ionization of the quasimolecular ions $[M+H]^+$ and $[M-H]^-$ were studied for all mycotoxins in both the positive and negative ionization mode. The greatest influence was observed by changing the vaporizer and capillary temperature. In general, the response of the A-type trichothecene (HT-2, see Figure 2.1) was better in the positive ionization mode. In contrast, B-type trichothecenes (DON and NIV, see Figure 2.1) as well as ZON and ZOL (see Figure 4.1) had higher ion signal intensities in the negative ion mode. However, OTA and CIT showed a reduced abundance in the negative ionization mode. Table 4.9 summarizes the optimized APCI ionization parameters for mycotoxin detection.

Table 4.9: Optimized APCI-ionization parameters for A- and B-type trichothecenes (TCT), zearalenone (ZON), zearalenol (ZOL), ochratoxin A (OTA)/citrinin (CIT) and the multiresidue method.

Parameter	TCT	ZON/ZOL	OTA/CIT	Multiresidue
Ionization mode	+	-	+	+
$[M+H]^+$/ $[M-H]^-$ for optimization	DON	ZON	OTA	DON
Discharge current/ Spray voltage [µA]	1.5	1.5	2.5	1.5
Vaporizer temperature [°C]	250	230	230	250
Sheath gas flow [Arb][1]	40	45	40	40
Auxiliary gas flow [Arb][2]	0	1	0	0
Heated capillary temperature [°C]	250	170	150	150

[1] One arbitrary unit corresponds to 250 ml/min [2] One arbitrary unit corresponds to 330 ml/min

During flow injections it was observed, that methanol instead of CH_3CN as solvent led to higher intensities for most of the mycotoxins except for ZON and ZOL. This has also been described by Cavaliere et al. (2005). Figure 4.6 shows the intensity differences of MS responses for the mycotoxins dissolved in CH_3CN or MeOH, respectively. MeOH was therefore used for further chromatographic separations.

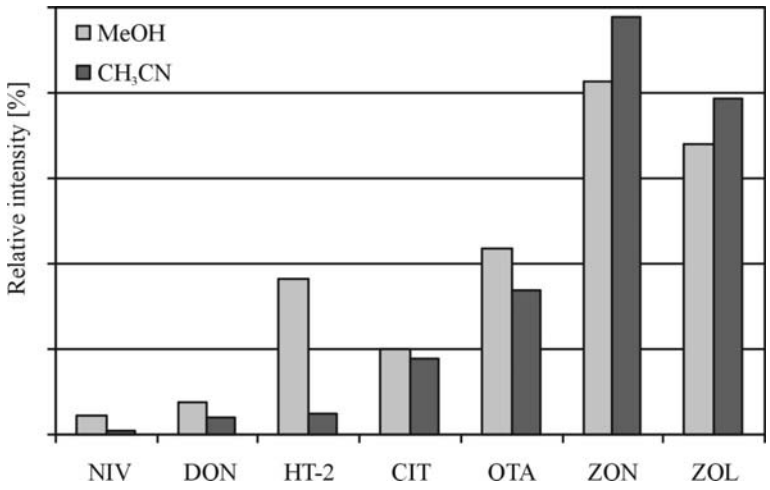

Figure 4.6: APCI-(+)-MS response of mycotoxins using MeOH or CH_3CN as organic solvent for flow injections. The concentrations of mycotoxins were 10 µg/ml for NIV, DON, CIT, OTA, ZON, and ZOL and 8 µg/ml for HT-2.

Two different buffers (ammonium formate and acetate) were tested for their suitability for mycotoxin separation. Various adduct formations were observed in ESI depending on the mycotoxin structure. Ions such as $[M-H]^-$, $[M+HCOO]^-$ and $[M+CH_3COO]^-$ were detected in the negative ionization mode and $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ in the positive ionization mode. The intensity of adduct ions was successfully minimized by changing the interface from ESI to APCI. Separations performed with ammonium formate as buffer system resulted in better peak shapes and more reproducible retention times. HPLC separations without any buffer systems were not feasible due to the presence of the acidic groups.

4.4.4 Method performance

The achieved mass spectrometric repeatability was good. Relative standard deviations of signal areas of the mycotoxin standard solution M8 were <10 % for 10 consecutive HPLC separations. Instrument detection limits at a signal-to-noise ratio of 3:1 were determined with pure reference substances and are provided in Table 4.10.

Table 4.10: Limits of detection (LOD) for the multiresidue analysis of the mycotoxins nivalenol (NIV), deoxynivalenol (DON), HT-2, zearalenone (ZON), zearalenol (ZOL), ochratoxin A (OTA) and citrinin (CIT). In addition, maximum and tolerance levels of Swiss food and typical values found in grain samples (Müller et al. 1997) are given.

Mycotoxin	LOD [ppb]	Maximum level*/ tolerance level** [ppb][1]	Typical values in cereals [ppb][2]
NIV	104		2-333
DON	125	1000**	2-4764
HT-2	125		8-288
ZON	50		1-311
ZOL	50		n.d.
OTA	25	5*	n.a.
CIT	94		n.a.

[1]Valid for Switzerland [2](Müller et al. 1997) n.d. not detected n.a. not analyzed

In particular response factors for type B trichothecenes (DON and NIV) were much lower than for the conditions optimized by Berger et al. (1999). It should be emphasized that a compromise had to be made to achieve a reasonable ionization yield for all selected mycotoxins. The obtained LODs were satisfactory except for OTA, which was above the Swiss tolerance level of 5 ppb.

In comparison to the single mycotoxin class determination of trichothecenes by ion trap MS (Berger et al. 1999), the obtained LODs of the multiresidue analysis are approximately a factor of 10-100 higher. The LODs are about 10 times higher compared to the specific methods by Pallaroni et al. (2003) for ZON and ZOL. According to the available knowledge, CIT has never been implemented into a general multiresidue screening in grain. Furthermore, only one multiresidue method for grain exists that included OTA as an acidic mycotoxin (Rajakylä et al. 1987). Here, the authors report LODs between 0.25 and 1 ppm for trichothecenes, ZON, OTA and Patulin using LC/MS thermospray. Therefore, the obtained LODs in this work are comparable good. Various other multiresidue methods included mainly trichothecenes and ZON/ZOL with LODs in the range of 2-100 ppb (Cavaliere et al. 2005; Mateo et al. 2002; Plattner et al. 1983; Berthiller et al. 2005).

Linearity tests were performed on the Discovery HSF5 column with OTB and VOL as ISTDs and HYC as RSTD. The correlation coefficients R^2 for a calibration curve based on seven measuring points were determined twice. R^2 ranged from 0.953 to 0.993 (NIV 0.985, DON 0.983, HT-2 0.974, CIT 0.993, ZON 0.982 and ZOL 0.953) except for OTA, which had a R^2 of only 0.355. The whole linear range of the method was determined from 180 ng/ml to 15 µg/ml. The absolute variation of the response factors was within ±10 %.

4.4.5 Spiked grain extracts

Corn and wheat samples determined to be mycotoxin-free were spiked with various amounts of mycotoxins and analyzed by the developed method described above in order to test the applicability of the developed multiresidue method for real samples. The corn matrix differed substantially from the wheat matrix employed for former experiments. It possesses an extremely higher background (approx. 90%, see Figure 4.7).

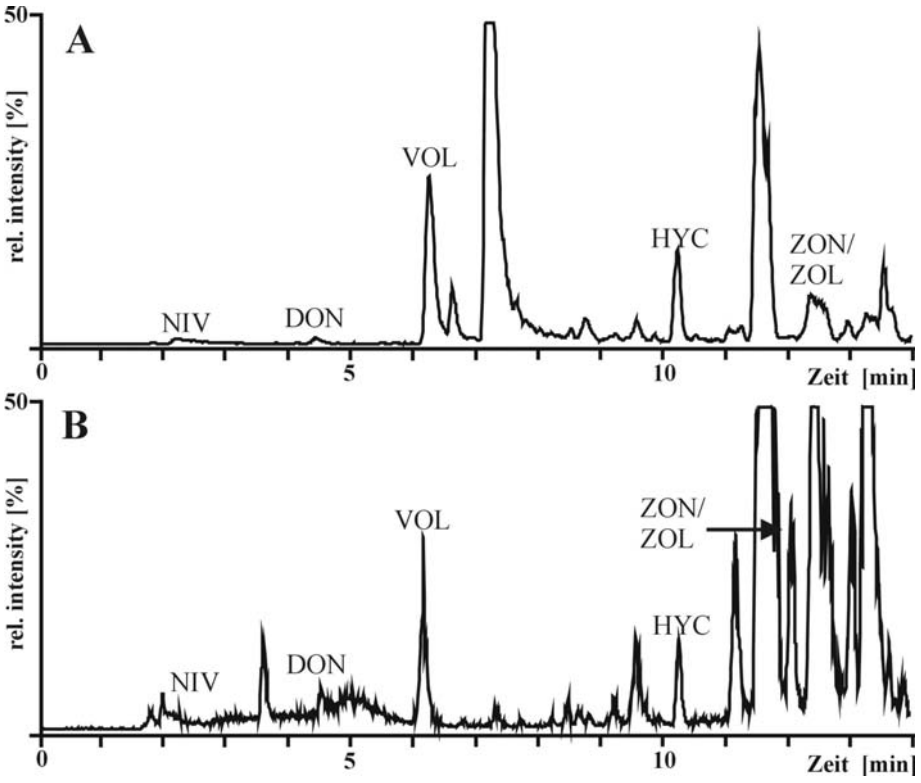

Figure 4.7: Real sample extract from a C18 cleanup of wheat (A) and corn (B) spiked with mycotoxins separated with column 1 (DON 4 µg/ml, NIV 2 µg/ml, VOL 4 µg/ml, HT-2 0.8 µg/ml, CIT 0.6 µg/ml, OTA 4 µg/ml, OTB 3 µg/ml, ZON 2 µg/ml, ZOL 6 µg/ml).

The cleanup of wheat and maize real samples showed that the matrix has an important influence on the recovery of single analytes. During cleanup of real samples also matrix coelution occurred, which decreased sensitivity and complicated quantification by signal

suppression. The attempt to remove the non-polar matrix of real samples by extraction with n-hexane did not lead to satisfying results.

The SPE phases (C18 phase and SAX) applied for cleanup only eliminated the polar matrix components with shorter retention times. Therefore, the matrix part from 0 to 10 minutes retention time is approximately 70 % (corn) and 40 % (wheat) lower after cleanup with SAX and 80 % (corn) and 40 % (wheat) lower for C18.

Both, the C18 and SAX SPE sorbents were applied. In general, the recoveries from corn were lower, compared to wheat. This might be due to the higher complexity of the corn matrix. Mycotoxins with higher retention times showed lower recoveries compared to mycotoxins eluting earlier (mainly ZON and ZOL). The reason for this is presumably the enhanced background for compounds that showed a later elution profile. HT-2, OTA and CIT could not be detected after cleanup with the C18 phase column. Moreover, it was not possible to determine CIT with SAX cleanup. OTA was detected with 87 % in wheat samples and 118 % in corn samples. Table 4.11 gives an overview of the obtained recoveries with C18 cleanup.

Table 4.11: Recoveries of the C18 cleanup of wheat and corn spiked with the following mycotoxins: DON 4 µg/ml, NIV 2 µg/ml, VOL 4 µg/ml, HT-2 0.8 µg/ml, ZON 2 µg/ml, ZOL 6 µg/ml. Recovery of each mycotoxin is given in percentage (n=2).

Mycotoxin	Wheat	Corn
NIV	95	64
DON	78	55
ZON	46	28
ZOL	35	16

4.5 Conclusions

A quantitative method for the simultaneous determination of prevalent mycotoxins that can arise from fungal infection in our region (DON, NIV, HT-2, ZON, ZOL, OTA and CIT) was developed using ion trap LC-APCI(+)-MS. It has been specifically developed for the rapid primary screening of cereal samples. The fast HPLC separation and APCI(+) detection which allows to detect mycotoxins of highly different polarities (lipophilic to ionic) make this method suitable for high sample throughput and routine analysis.

The greatest challenge of developing a multiresidue method was to find a common, simple and easy in use SPE sample cleanup procedure ensuring high recoveries for all mycotoxins. Among various SPE sorbents, SAX and C18 columns showed the best results for certain mycotoxins using standard solutions. A combined sample cleanup applying both, C18 and SAX material was therefore the most promising idea. The determined recoveries were overall satisfactory and the method showed a linear range from 180 ng/ml to 15 μg/ml except for OTA. Moreover, the LOD of OTA was above the tolerance level. Further investigations for the inclusion of this particular mycotoxin into the method should be considered or otherwise it should be omitted. The analysis of spiked extracts showed the complexity of grain matrix. Some mycotoxins showed low recoveries or could not even be detected. Here, further investigations about interfering compounds and their possible elimination would be necessary.

OTB and VOL proved to be suitable ISTDs for the quantification of trichothecenes. HYC served as a RSTD. Nonetheless, a suitable internal standard for the quantification of ZON and

ZOL, as well as the application of MS/MS could be an important improvement, in terms of lower LODs, less matrix interferences and a better accuracy of the method. Moreover, it would be valuable to test the applicability of the method to other cereals and different real samples, e.g. processed food.

4.6 Standard operation procedure (SOP) for the determination of prevalent Fusarium mycotoxins with common SPE sample cleanup and detection with APCI(+)LC/MS

1. Summary of the method:

A rapid quantitative method for the simultaneous determination of the widespread mycotoxins (ZON, ZOL, OTA and CIT) in addition to prevalent trichothecenes (DON, NIV and HT-2) was developed using HPLC coupled to an ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. OTB and VOL served as ISTDs and HYC as RSTD. Additionally, a fast and easy sample cleanup method was developed applying C18 and SAX SPE cartridges for the elution of mycotoxins with a wide range of polarity.

2. Samples:

The method was optimised for the determination of wheat samples.

3. Analytes:

The method covers the mycotoxins zearalenone (ZON), zearalenol (ZOL), ochratoxin A (OTA), citrinin (CIT), deoxynivalenol (DON), nivalenol (NIV) and HT-2.

4. Sample cleanup:

The following SPE columns were used: Bond Elut C18 (Varian catalogue no. 1210-2001; 100 mg sorbent mass; 1 ml volume) and Bond Elut SAX (Varian catalogue no. 1210-2017; 100 mg sorbent mass; 1 ml volume)

5. Equipment:

HPLC was carried out with a Rheos 2000 pump (Flux Instruments, Basel, Switzerland). A quadrupole ion trap spectrometer (LCQ, Finnigan, San Jose, CA, USA) was used for the detection of mycotoxins equipped with atmospheric pressure chemical ionization and detection in the positive ion mode (APCI(+)).

6. Chemicals:

CIT (≥99 %), DON (>99 %), HT-2 toxin (>97,5 %), NIV (98 %), OTA (≥99 %), OTB, verrucarol (VOL, ≥98 %), ZOL (≥99 %) and ZON (≥99 %) were provided by Sigma Chemie (Buchs, Switzerland). Ammonium formate (≥99 %), trifluoroacetic acid (≥99.5 %) and hydrocortisone (HYC, ≥97 %) were purchased from Fluka Chemie (Buchs, Switzerland). Water with a total organic content of <2 ppb was obtained from an Elgastat maxima HPLC water purification unit (Elga LTD., Bucks, Great Britain). Methanol of pestipur grade (SDS, Peypin, France) were used. Helium of 99.999 % purity and nitrogen of 99.995 % purity were purchased from Carbagas (Switzerland).

7. Selectivity:

High selectivity is given due to chromatographic separation on a reversed phase HPLC column and subsequent detection by mass spectrometry.

8. Linearity:

The correlation coefficients R^2 for a calibration curve based on seven measuring points were determined twice. R^2 ranged from 0.953 to 0.993 (NIV 0.985, DON 0.983, HT-2 0.974, CIT 0.993, ZON 0.982 and ZOL 0.953) except for OTA, which had a R^2 of only 0.355. The whole linear range of the method was determined from 180 ng/ml to 15 µg/ml.

9. Limits of detection (LOD):

Mycotoxin	**LOD [ppb]**
NIV	104
DON	125
HT-2	125
ZON	50
ZOL	50
OTA	25
CIT	94

10. Recovery:

The recoveries of the optimized conditions for the C18 sample cleanup are given in the following table:

Mycotoxin	**Mean recovery C18 [%] (n=4)**	**RSD [%]**
NIV	88	18
DON	86	10
VOL	79	9
HT-2	70	17
CIT	71	3
OTA	46	2
OTB	65	9
ZON	82	9
ZOL	106	11

The recoveries of the optimized conditions for the SAX sample cleanup of acidic mycotoxins were 52 % for CIT, 86 % for OTA and 102 % for OTB (n=2).

11. Precision and accuracy:

The absolute variation of the response factors was within ±10 %.

12. Interferences and limitations:

The determined recoveries were overall satisfactory except for OTA. Moreover, the LOD of OTA was above the tolerance level. Further investigations for the inclusion of this particular mycotoxin into the method should be considered or otherwise it should be omitted. The analysis of spiked extracts showed the complexity of grain matrix. Some mycotoxins showed low recoveries or could not even be detected. Further investigations about interfering compounds and their possible elimination are necessary.

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EIDESTATTLICHE ERKLÄRUNG

Ich erkläre, dass ich die Dissertation „ Development of ion trap HPLC-MS detection methods for the determination of prevalent mycotoxins in grain and application to real samples" nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Basel, den 16.12.2006

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