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FUNGAL POPULATIONS AND METABOLISM OF *Fusarium* MYCOTOXINS IN CEREALS: FROM OCCURRENCE TO ANALYSIS BY HYPHENATED ANALYTICAL TECHNIQUES

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Aims of the Thesis

The development of efficient and rapid methods for the detection of toxigenic fungi would be very helpful for early diagnosis and control of toxins. Therefore, the main objective of the presented thesis was the development and optimisation of analytical methods based on hyphenated techniques for the determination of mycotoxins, "masked mycotoxins" and volatile secondary metabolites in cereals, contaminated with fungi under controlled conditions. Current techniques available to detect the presence of mycotoxins in food products usually require an extended time lag between sampling and the corresponding results, and include different clean-up steps and eventually derivatization procedure.

This research project comprised several activities. First of all, a careful bibliographic search was aimed at the selection/evaluation of selected fungal species that can potentially produce new mycotoxins, as well as the study of potential modified mycotoxins and the identification of suitable analytical techniques for their extraction and detection.

Analytical methods based on hyphenated techniques were also developed for the separation (hight resolution liquid chromatography) and detection (MS/MS) of the mycotoxins. The use of appropriate chromatographic conditions was the strategy adopted for the accurate determination of the analytes.

The second part of the research project involved the study of the volatile components (VOCs) and metabolites released by the plant products (in particular wheat) as the consequence of a fungal attack from selected toxins-producing fungi. These studies were carried out under controlled conditions and the analytes of interest were monitored by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography (GC)-MS instrumentation in order to investigate the volatile compounds during wheat plants growth.

The main goal was to allow the identification of volatile biomarkers of early infection by toxigenic fungi, before the actual occurrence of toxin detectable levels; namely that volatiles that can be ascribed to the infection process. As well as the determination and study by conventional and advanced statistical approaches of the volatile component "fingerprint" in cereals for the identification of biomarkers of an early pathogen contamination.

The research results will be a valuable tool in the preservation of food production and protection of animal’s and human’s health, decreasing life risks. Moreover, the project results will have a positive impact on primary productions, improving the preservation of environment both in terms of cost and in terms of quality of life for food sustainability.

Abstract

Success in cereal production, with regard to fungal contamination at the pre-harvest stage, is strongly related to quality control assessment. The aim of this research has been to study how zearalenone (ZEN), a secondary metabolite produced by several *Fusarium* species, is metabolised and modified in cereals like wheat and barley. Furthermore, we have developped and optimised analytical methods for the identification of volatile organic compounds (VOCs) produced by the interaction between two selected toxigenic fungal species (*F. graminearum* and *F. culmorum*) and wheat plants. Among these VOCs the identification of biomarkers of early infection can help to prevent/reduce contamination in the food chain.

For ZEN were performed time course experiments. Wheat and barley were treated with ZEN. Samples were harvested at different time points and analysed with an UHPLC system coupled to a QTOF mass spectrometer. The LC-MS method was optimised to allow a fast analysis which is still able to separate the target metabolites. Regarding VOCs, wheat plants were inoculated using a spore suspension of the two fungi. Analyses were made at different growth stages. VOCs were sampled directly from the headspace of leaves and ears, by solid-phase microextraction, and analysed by GC-MS instrumentation.

Absolute amounts of ZEN and its metabolites could be plotted versus harvest time point after treatment. Based on the recovery in percent compared to the time point zero the majority of ZEN was not metabolised by the plants. The major metabolisation products were glucosides. Instead data obtained by VOCs analyses have been processed by using statistical approaches, such as PCA (principal component analysis), that underline very important differences among the samples. In particular the most of VOCs were detected during the early milk stage. Among substances identified, aliphatic alcohols, aldehydes and ketones were the most abundant. These compounds are biosynthesised on different pathways.

Regarding time course experiments it possible to conclude that most of ZEN remains unmetabolised, but we have observed some glucosides especially in barley, which has proven more active in producing glucosides than wheat. The approach developed in the study of VOCs has provided promising results to predict the potential of toxin formation by detecting the presence of both *Fusarium* species at early stages of fungal growth in wheat cultivars.

Introduction

1 Mycotoxins

1.1 Definitions and General Aspects

Mycotoxins are poisonous substances produced by various filamentous fungi (also commonly referred to as moulds). The word is derived from the Greek word “myces” (fungus) and the Latin word “toxicum” (poison) (Taniwaki and Pitt 2013).

Mycotoxins can be acutely toxic (e.g. causing vomiting, nausea or even death) or chronic toxic (e.g. cytotoxic, nephrotoxic, carcinogenic or mutagenic). The symptoms depend on factors like species, age, gender, nutritional and health status, as well as dose and length of exposure. However, information about the long-term effects of low doses is scarce (Bennett and Klich 2003).

Mycotoxins are secondary metabolites because their production is not essential for the growth of the fungus, but might provide some ecological advantages over other microorganisms competing for the same nutrients (Magan and Aldred 2007).

Fungi can either invade the living plants on the field (also called pre-harvest or field fungi) or can colonise the agricultural crop during storage (post-harvest). *Fusarium* or *Alternaria* species are examples for genera typically accounted to the first group, whereas *Aspergillus* or *Penicillium* species are classified as post-harvest fungi (Frisvad *et al.* 2007).

Under favourable conditions almost every agricultural commodity might be colonised by fungi, but food classes mainly affected by mycotoxin contamination include cereals, nuts and dried fruits. However, the presence of the fungus alone is not sufficient for toxin production, as also certain conditions have to meet. Factors influencing the mycotoxin production are related to the fungi itself (e.g. species, biodiversity, degree of invasion, competing pressure), to the crop varieties (e.g. resistance to fungal growth and/or mycotoxin production), agricultural practices (e.g. crop rotation, irrigation, fumigation systems) and climatic or storage conditions (especially concerning water activity and temperature, but also pH conditions) (Frisvad *et al.* 2007; Magan and Aldred 2007; CAST 2003).

In general mycotoxins are very stable molecules and it has to be kept in mind, that they can still be present as contaminants in food even when fungal growth is not visible. This is of particular relevance in the case of processed food and feed. However, several food processes might also reduce the mycotoxin contamination (e.g. milling, baking or extrusion) (Bullerman and Bianchini 2007).

The Food and Agriculture Organization of the United Nations (FAO) estimates that 25% of the global food and feed is contaminated by mycotoxins and that the annual losses due to fungal disease and mycotoxin contamination is as high as one billion US dollar in the United States alone (CAST 2003). Newer estimations are not available.

With regard to fungal contamination at the pre-harvest stage, it is of utmost relevance to develop diagnostic tools for early detection of toxigenic fungi, aiming to avoid contamination in the food chain (Girotti *et al.* 2012). Predicting the incidence of diseases and mycotoxin contamination and increasing the ability of producers to achieve good disease management may be useful for decision-making purposes: prevention/reduction of yield losses and hazards for human and animal health based on the correct time for spraying chemicals; the prediction of the final level of contamination and better organization of the post-harvest management.

In addition, it is important to emphasize that while it is easy, through direct measurements, assessing the impact of man-made contaminants, it is more difficult to evaluate the impact of non-anthropogenic contaminants on public health. Obligatory approval for synthetic compounds entering the food chain, such as pesticides and preservatives include their toxicological assessment, and guidelines for the application. These safeguards cannot be applied to naturally occurring toxins. Most importantly, only indirect control of the level of natural toxins in food commodities by good manufacturing practices, soil treatment, the use of resistant varieties and fungicide application is possible (Murphy *et al.* 2006).

For these reasons, the threat to the health of the consumer posed by natural toxicants appears more serious than the health risk posed by man-made pesticides, preservatives and other food additives (Mattsson 2007). In addition, the real danger of these toxins is certainly higher than the danger perceived by consumers.

1.2 Major Classes of Mycotoxins

Mycotoxins are not a new phenomenon and most probably they are older than humanity. Two examples of diseases caused by mycotoxins in prehistoric times are discussed in the recent article of Ramos *et al.* (2011): the first one is about ergotism, which is caused by alkaloids produced by *Claviceps purpurea.* The second one deals with alimentary toxic aleukia caused by trichothecenes. However, it was not until the early 1960s that the causing agents were identified one by one. During the investigations for the cause of the death of more than 100000 young turkeys in Great Britain the aflatoxins (AFs) were identified (Asao *et al.* 1963). Since this incidence known as “Turkey X disease”

the awareness about mycotoxins increased significantly worldwide. Nowadays, novel mycotoxin-commodity combinations are reported every year and several hundred mycotoxins are identified, but it is believed that a huge proportion is yet undiscovered.

It is beyond the scope of this thesis to discuss the biosynthesis, occurrence and toxicity of the individual toxins in detail. Instead, an overview of the major classes of mycotoxins is provided and sources of further information are given.

The major classes of mycotoxins with regard to occurrence and toxicity are as follows: aflatoxins (AFs), fumonisins (FBs), ergot alkaloids, ochratoxins, trichothecenes and zearalenone (ZEN) (CAST 2003).

Table1 provides a summary, including major producing fungi and associated effects, and figure 1 depicts one prominent mycotoxin of each class. Interested readers are referred to excellent reviews e.g. on the toxicity of mycotoxins in general (Bennett and Klich 2003), on the mycotoxicoses of major mycotoxins (Richard 2007), on the occurrence of mycotoxins in feed (Streit *et al.* 2013), on the occurrence, toxicology and exposure assessment of mycotoxins (Marin *et al.* 2013) or a critical review on occurrence and significance of mycotoxins (Didwania and Joshi 2013).

More insight into the biosynthesis of three groups - AFs, FBs and trichothecenes - is given by Woloshuk and Shim (2013). Further information about the risks for animals and public health related to certain mycotoxins can also be found in the EFSA Journal published by the European Food Safety Authority (EFSA 2014), e.g. most recently about nivalenol (EFSA 2013). Furthermore, the joint FAO/WHO Expert Committee on Food Additives (JECFA) publishes evaluations of certain contaminants e.g. on deoxynivalenol (JECFA 2011).

Concerning, the effects caused by the co-occurrence of mycotoxins, whether they are additive or synergistic very little is known so far, Grenier and Oswald (2011) provided the latest review regarding this topic.

Table 1: Major classes of mycotoxins.

Class	Common structures and examples	Main producer	Associated effects	Latest reviews (additional references)
aflatoxins	difurnocoumarins: aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1	*Aspergillus* spp. (*A. flavus, A. parasiticus*)	hepatotoxic, carcinogenic, growth suppression	Kensler *et al.* 2011; Yu 2012
A-trichothecenes	12,13-epoxytrichothec-9-ene: T-2 toxin, HT-2 toxin, 4,15-diacetoxyscirpenol	*Fusarium* spp. (*F. sporotrichioides, F. poae, F. oxysporum, F. langsethiae*)	alimentary toxic aleukia (vomiting, diarrhoea, haemorrhage)	Van der Fels-Klerx and Stratakou 2010; Li *et al.* 2011
B-trichothecenes	12,13-epoxytrichothec-9-ene (C-8 keto-group): deoxynivalenol, 3-acetyl-deoxynivalenol, nivalenol	*Fusarium* spp. (*F. graminearum, F. culmorum, F. cerealis*)	acute: vomiting, feed refusal chronic: anorexia, growth retardation, immunotoxicity	Pestka 2010; Sobrova *et al.* 2010; McCormick *et al.* 2011; Maresca 2013
fumonisins	diesters of propanotricarboxylic acid and long-chain polyhydroxyamines fumonisin B1, fumonisin B2, fumonisin B3	*Fusarium* spp. (*F. verticillioides, F. oxysporum, F. proliferatum*)	equine encephalomalacia, porcine pulmonary oedema, nephrotoxicity, oesophageal carcinoma	Voss *et al.* 2007; Scott 2012; Bryła *et al.* 2013
ochratoxins	dihydroisocoumarin moiety linked via amide to L-phenylalanine: ochratoxin A, ochratoxin B, ochratoxin C	*Aspergillus* spp. (*A. ochraceus, A. niger, A. carbonarius*) Penicillium spp. (*P. verrucosum*)	nephrotoxic, neurotoxic, teratogenic, immunotoxic, hepatotoxic, possibly carcinogenic, urinary tract tumours Balkan endemic nephropathy?	El Khoury and Atoui 2010; Amézqueta *et al.* 2012
patulin	polyketide	*Penicillium* spp. (*P. expansum*) *Aspergillus* spp. (*A. clavatus*)	immunological, neurological and gastrointestinal effects	Puel *et al.* 2010
zearalenone	macrocyclic β-resorcylic acid lactone	*Fusarium* spp. (*F. graminearum, F. culmorum, F. cerealis, F. equiseti*)	oestrogenic, hepatotoxic, haematotoxic, immunotoxic, genotoxic, possibly carcinogenic endocrine-disruptive effects	Zinedine *et al.* 2007; Maragos 2010; Metzler *et al.* 2010
Alternaria metabolites	dibenzopyrone derivatives: alternariol, alternariolmonomethylether, altenuene tetramic acid derivative: tenuazonic acid perylene derivatives: altertoxins I, II, III	*Alternaria* spp. (*A. alternata, A. tenuissima*)	convulsions, haemorrhage cyototoxic, fetotoxic, teratogenic, mutagenic, clastogenic, oestrogenic	EFSA 2011 (Müller and Korn 2013)
enniatins	cyclic hexadepsipeptides: enniatin A, enniatin A1, enniatin B, enniatin B1, enniatin B2, beauvericin	*Fusarium* spp. (*F. avenaceum, F. tricinctum, F. sporotrichioides, F. poae*)	cytotoxic	Tedjiotsop Feudjio *et al.* 2010; Santini *et al.* 2012
ergot alkaloids	ergoline ring ergosine, ergotamine, ergocristine, ergocryptine, ergocornine, ergocristam, ergometrine, agroclavines (in total ca. 50)	*Claviceps* spp. (*C. purpurea, C. fusiformis, C. africana*)	vascoconstriction and gangrene (St Anthony's Fire / Holy Fire) abdominal pains, vomiting, insomnia, hallucinations	Krska and Crews 2008; Alm and Elvevåg 2013

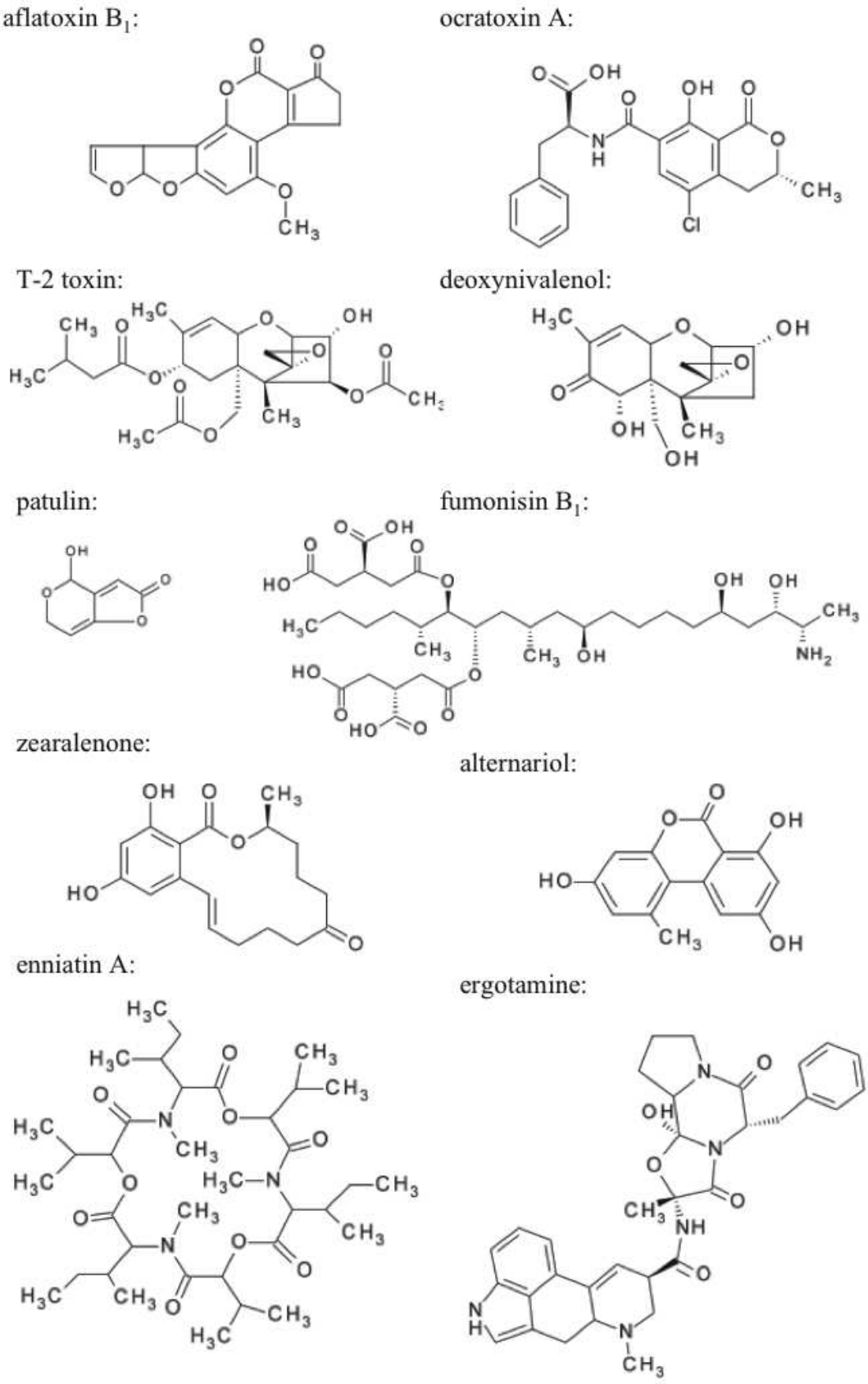


Figure 1: Structures of common mycotoxins.

The first mycotoxins identified were the **aflatoxins (AFs)** in the early 1960s (Asao *et al.* 1963). Kensler *et al.* (2011) provide an overview about these last 50 years with regard to AFs research. They are highly potent carcinogens and possess genotoxic properties. The International Agency for Research on Cancer (IARC) classified them as Group 1 carcinogens. Beside the chronic long-term effects, AFs have also acute effects including vomiting, abdominal pain, necrosis or fatty liver, but can even lead to death at higher doses (Marin *et al.* 2013). The incidence of these aflatoxicoses is low, but the effects are severe. For instance, 125 people died and 192 suffered because of aflatoxin contaminated maize (up to 8000 µg/kg) in Kenya in 2004 (CDC 2004). AFs are primarily a problem in hot and humid climates and are of primarily concern in cereals, nuts and spices. They are very stable, even at high temperatures used in food processing and hence might be a risk in processed food. The most important of the AFs is aflatoxins B_1 (AFB_1), followed by aflatoxins G_1 (AFG_1), aflatoxins B_2 (AFB_2) and aflatoxins G_2 (AFG_2). The monohydroxylated derivative of AFB_1, aflatoxins M_1 (AFM_1) is found in the milk of mammals after the consumption of AFB_1-contaminated food or feed. AFM_1 is classified as possible carcinogen for humans by the IARC (Marin *et al.* 2013).

More than 200 **trichothecenes** were identified from natural sources so far (Grove 2007). Chemically they are polycyclic sesquiterpenoids and share a tricyclic 12,13-epoxytrichothec-9-ene core structure. This characteristic backbone structure provides an ideal scaffold for different functional groups like hydroxyl- or acetyl-groups. Trichothecenes can be grouped into four classes, type A, B, C and D. In food and feed, type A (e.g. T-2 toxin (T-2), HT-2 toxin (HT-2) or 4,15-diacetoxyscirpenol), or type B trichothecenes (e.g. deoxynivalenol (DON), nivalenol) are by far the most prevalent. Type A trichothecenes possesses an oxygen function (other than keto-group) at C-8 and type B trichothecenes have a keto-function at C-8 (McCormick *et al.* 2011). Trichothecenes are potent inhibitors of the protein synthesis which is mainly related to the epoxide function. The most common trichothecene in Europe is DON along with its acetylated forms 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON). Acute symptoms of DON include vomiting and nausea; an associated long-term effect is for example growth retardation (Pestka 2010). Trichothecenes can be determined in a huge variety of food and feed matrices including all kinds of cereals. T-2 and HT-2 are primarily determined in oat and barley. They are attached to the outer hull hence cleaning, sorting and de-hulling might reduce the contamination considerable (Marin *et al.* 2013).

With regard to the **fumonisins (FBs)** more than 18 homologues classified into A-, B-, C- and P-type have been discovered so far. Fumonisins B_1 (FB_1) is the most prevalent one in the contamination

of food, followed by fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Bryła *et al.* 2013). Due to their structural similarity to the sphingoid bases sphinganine (Sa) and sphingosine (So) they are capable of disrupting the sphingolipid metabolism by the inhibition of the ceramide synthase. The toxicokinetic, mechanism of action and toxicity was reviewed by Voss *et al.* (2007). The food commodities most often contaminated with FBs are maize and maize-based products and colonisation is taking place already on the field. For more details the reader is referred to the reviews of Scott (2012) or Bryła *et al.* (2013).

Concerning **ochratoxins**, at least 15 natural occurring mycotoxins or metabolites thereof are known so far. Ochratoxin A (OTA) is by far the most important one with regard to food safety. Frequently contaminated food includes cereals, coffee, cocoa, grapes and spices and since OTA is very stable in the products thereof e.g. bread, coffee, chocolate or wine (Amézqueta *et al.* 2012). From a chemical point of view, OTA is similar to the amino acid phenylalanin. OTA is classified as potential human carcinogen (Group B2) by the IARC (IARC 1993) and the tolerable weekly intake (TWI) was set to 120 ng/kg body weight by EFSA (2006) and 100 ng/kg body weight by JECFA (2008). OTA producing fungi belonging to the genera *Aspergillus* and *Penicillium* are reviewed in Amézqueta *et al.* (2012), whereas the molecular biology of OTA biosynthesis can be found in El Khoury and Atoui (2010).

Patulin (PAT) is a polyketide and belongs to the smallest mycotoxins with a molecular weight of just 154 Da. PAT is mainly occurring in apples and apple-derived products (e.g. juice, cider, compotes), but also in pears and other fruits. The biosynthetic pathway of PAT is not fully understood so far (Puel *et al.* 2010). Its strong affinity to sulfhydryl groups might explain the inhibitory effect on several enzymes (Marin *et al.* 2013).

Zearalenone (ZEN), another polyketide, is a phenolic resorcyclic acid lactone. The primary toxicological effects associated with this mycotoxin are reproductive disorders and its oestrogenic activity (Zinedine *et al.* 2007). In animals ZEN is metabolised to α-zearalenol and β-zearalenol and then further to their glucuronic acid conjugates. ZEN is mainly produced on the field and can be detected in maize and to a lesser extent in wheat, barley, oat and rice. For further information on ZEN see the following reviews Zinedine *et al.* (2007), Maragos (2010) and Metzler *et al.* (2010).

Alternaria toxins are divided into three structural groups: the dibenzopyrone derivatives (e.g. alternariol, alternariolmonomethylether and altenuene), the tetramic acid derivatives (e.g. tenuazonic acid) and the perylene derivatives (e.g. altertoxin I and altertoxin II). Apart from these groups also tentoxin and AAL-toxins (*Alternaria alternata* toxins) exist. Occurrence data are scarce, but reports

exist for tomatoes, nuts, as well as fruits and their products, in particular beverages like fruit juices and wine (Marin *et al.* 2013). Toxic effects associated with Alternaria toxins include cytotoxicity, fetotoxicity and/or teratogenicity (EFSA 2011; Müller and Korn 2013).

Enniatins and beauvericin are accounted to the emerging Fusarium mycotoxins for which only limited data (mainly from Northern Europe and the Mediterranean region) is available (Santini *et al.* 2012). The prevalence determined in the surveys is usually high. They share cyclic hexadepsipeptides. Enniatin B is the member most commonly detected. From a toxicological point of view they are cytotoxic and since they are apolar they might be incorporated into the cell membranes forming cation selective channels (Marin *et al.* 2013).

The effects of **ergot alkaloids** are known since biblical times. In the middle ages the symptoms (gangrenous and convulsive effects) were called “St. Anthony’s Fire” or “Holy Fire”. Ergot alkaloids are produced by *Claviceps* species, in particular *Claviceps purpurea* and are characterised by a tetracyclic ergoline ring system. In contaminated grains, especially rye, sclerotia - dark-coloured, hard mycelial mass - are replacing the kernel. Nowadays, they are mainly a threat to animal health because the sclerotia can be separated from the grain with proper sorting devices which are state-of-the-art in the milling industry (Marin *et al.* 2013).

1.3 Metabolites of Mycotoxins

Beside the hundreds of known mycotoxins, more and more about the metabolism of mycotoxins by humans, animals or microorganisms or in plants has been explored in the last decade. For example, when the fungus is infecting the plant, the plant can defend itself by applying mechanisms to detoxify the produced mycotoxins. One approach is to attach polar substances (e.g. glucose) to the fungal metabolites. These modified metabolites are more water soluble and can then be transported to and stored in the vacuoles of the plant or incorporated into the cell wall. The term “conjugated” or “masked” mycotoxin is used for soluble metabolisation products, whereas the term “bound” or “hidden” mycotoxin is used for the insoluble forms. “Masked” mycotoxins were recently reviewed by Berthiller *et al.* (2013).

In general, “masked” mycotoxins show a reduced toxicity compared to their parent compound, but there is the potential risk of metabolisation back to their toxic precursors. Hence “masked” mycotoxins might increase the toxicological potency of the food. Deoxynivalenol-3-glucoside (D3G) is the best studied “masked” mycotoxin with regard to occurrence and toxicology and was considered in the evaluation of deoxynivalenol by JECFA in 2011. JECFA concluded that occurrence data for D3G

(along with 3ADON and 15ADON) and absorption, distribution, metabolism and excretion studies (short ADME) on D3G should be conducted (JECFA 2011). For example it was shown by Berthiller *et al.* (2011) that the glucosylic bond is not cleaved by human cytosolic β-glucosidases, but fungal cellulases and cellobiases as well as several lactic acid bacteria isolated from the human gut were capable of cleaving D3G and releasing DON. An *in vivo* study investigating the metabolism of D3G in rats showed that while D3G was hydrolysed during digestion, it was less bioavailable and of lower toxicological concern than DON in this mammal (Nagl *et al.* 2012; Veršilovskis *et al.* 2012). Additionally, recent studies demonstrated that D3G is cleaved by microorganisms present in faeces samples from humans (Dall'Erta *et al.* 2013; Gratz *et al.* 2013).

Also in humans and animals biotransformation of mycotoxins is well known and most of the reactions occur in the gastrointestinal tract and the liver. However, not always a detoxification is achieved in this way: e.g. highly reactive and carcinogenic epoxides of AFs are formed as intermediates in the biotransformation of AFs. Similar to glucosidation in plants, glucuronidation - the conjugation of e.g. trichothecenes to glucuronic acid - can take place (reviewed in Galtier 1999). Due to glucuronidation, the polarity and excretion rate of the xenobiotic (e.g. DON) is enhanced. Furthermore, the binding to ribosomes is reduced in the case of trichothecenes resulting in a decreased toxicity. The degree of glucuronidation greatly varies between and within species (reviewed for DON in Maresca 2013). Deactivation pathways in humans and animals are often also assisted by microbial organisms present in the gastrointestinal tract: e.g. de-epoxidation of trichothecenes or hydrolysis of OTA (reviewed in Galtier 1999). For example, de-epoxy deoxynivalenol is reported to be at least 50-times less cytotoxic than DON (Sundstøl Eriksen *et al.* 2004). The metabolisation products are often used as biomarkers of exposure and recent approaches to investigate the metabolisation of mycotoxins by humans were summarised by Warth *et al.* (2013).

Beside microorganisms in the digestive tract of humans and animals, also bacteria and fungi present in soil and infested plant material have been isolated and tested for their ability to detoxify mycotoxins. Isolated microorganisms or the enzymes responsible for the detoxification reaction may serve as feed additives and help to lessen the impact of mycotoxin contamination in feed. Identified biotransformation reactions include acetylation, glucosylation, ring cleavage, hydrolysis, deamination and decarboxylation. McCormick (2013) provides an overview about the reported microbial detoxification of AFs, citrinin, FBs, OTA, PAT, ZEN and trichothecenes.

1.4 *Fusarium* Toxins and Volatiles

Fusarium species employ an arsenal of compounds as virulence factors, including certain trichothecene mycotoxins, to infect cereals such as wheat and barley (Desjardins *et al.* 1997). These two crop plants account for approximately 80% of the European small-grain production and may be severely contaminated with trichothecenes (Bottalico *et al.* 2002). Plants, however, are equipped with mechanisms to counteract the phytotoxicity of xenobiotics, including fungal toxins, leading to the formation of conjugated metabolites that are deposited in the apoplast or vacuole (Coleman *et al.* 1997).

Volatiles can be used as indicators of pathogenic activity and as taxonomic markers to differentiate between fungal species and between toxigenic and non-toxigenic fungal strains (Magan and Evans 2000).

Volatile organic compounds (VOCs) are typically classified on the basis of their origin as biogenic (BVOCs) or anthropogenic. BVOCs are biological terminal metabolites with strong volatility, containing important bio-information related with biological metabolism (Zhang *et al.* 2013). They mainly include alkanes, alkenes, alcohols, esters, aromatic compounds and terpenes (Biasioli *et al.* 2011). BVOCs play a relevant role in agro-industrial processes, and food sciences and technology as well as they are implicated, from crops to food, in most food chains.

Plants emit more than 100000 chemical products and at least 1700 of these are known to be volatile. A very large number of BVOC from plants are synthesized through a few common biosynthetic pathways. A vast array of volatile compounds are involved in stress-dependent signaling within a single plant as well as communication between plants and between plants and insects (Dicke 2009; Niinemets 2010). Anyway, the plant volatile emission is changed due to abiotic or biotic stresses (Niinemets 2010), and plants infected with different parasites start to emit different type of compounds (especially green leaf volatiles and secondary metabolites) as a result of indirect defense mechanisms (reviews in: Dicke 2009; Dicke and Baldwin 2010; Holopainen 2004).

There are only few papers regarding the volatile finger print of mycotoxins (some reviewed in Schnurer *et al.* 1999). In their work, Zeringue *et al.* (1993) found that headspace volatiles differed between aflatoxigenic strains and non-aflatoxigenic strains of *Aspergillus flavus* grown in liquid culture. This is basically the first attempt of using BVOC for detection of aflatoxins in food products. Even more, the recent work of De Lucca *et al.* (De Lucca *et al.* 2012; De Lucca *et al.* 2010) has shown distinct differences in the metabolites profiles in case of toxic and non-toxic strains of *A. flavus*. In

another recent work Girotti *et al.* (2012) has been shown that the trichodiene is a useful marker to detect an early infection caused by trichothecene producing *Fusarium graminearum*.

1.5 Legislation and Notification System

During the last years, regulations have been established in many countries to protect consumers from the harmful effects (EC Recommendation 576/2006) of such a fungal against human and animal health (Bennett and Klich 2003; Creppy 2002). Therefore, the development of efficient and rapid methods for the detection of mycotoxigenic species would be very helpful for early diagnosis and control of mycotoxins, such as aflatoxins, ZEN, DON, fumonisins and OTA, whose frequent co-occurrence with possible synergistic or additive interactions has been highlighted by Schatzmayr *et al.* (2006a and 2006b) and also by Streit *et al.* (2013) in a recent article.

Due to the awareness of the potential toxic effects and the widespread occurrence, more and more countries are establishing maximum or guidance levels for certain mycotoxins. Beside scientific factors (e.g. toxicological and occurrence data, sampling plans and analysis methods), socio-economic factors are influencing the levels. In 2003, approximately 100 countries (representing ca. 85% of the population worldwide) had such levels (FAO 2004; Van Egmond *et al.* 2007). Furthermore, harmonisation tendencies exist to facilitate trade e.g. by the European Union (EU) or MERCOSUR (Mercado-Cómun del Sur, Southern Common Market). In the EU, Commission Regulation (EC) 1881/2006 and its amendments set maximum levels for eleven mycotoxins or groups thereof (AFB_1, AFM_1, DON, OTA, PAT, ZEN, the sum of AFB_1, AFB_2, AFG_1 and AFG_2 and the sum of FB_1 and FB_2) in certain food categories, primarily cereals and cereal based foods.

The number of regulated mycotoxins has not changed since 2006, but the maximum levels of the individual mycotoxins were specified in greater detailed and extended to different matrices (EC Regulation 1881/2006). For the type A trichothecenes T-2 and HT-2 just recently a Commission recommendation was released providing indicative levels for the sum of these two toxins in cereals and cereal products (EU Recommendation 165/2013). For the feeding of animals, maximum levels are set for AFB_1 and rye ergots (EC Directive 32/2002) and guidance levels are set for DON, OTA, ZEN and the sum of FB_1 and FB_2 in feed material of certain farm animals (EC Recommendation 576/2006). An overview of these levels is provided in Table 2.

Table 2: European legislation - maximum, indicative or guidance limits for mycotoxins (all in µg/kg) according to EC Regulation 1881/2006 and amendments, EC Directive 32/2002, EC Recommendation 576/2006, and EU Recommendation 165/2013.

Mycotoxin	**Food matrices**	**Unprocessed food and food not for direct human consumption** [1)]	**Food for human consumption** [1) 2)]	**Feed and compound feed** [3)]
aflatoxins [4)]	groundnuts, oilseeds, nuts, dried fruits, cereals [5)], spices	10 – 15	4 – 10	-
aflatoxin B_1		8 – 12	(0.1) 2 – 8	5 – 20
aflatoxin M_1	milk and milk products	-	(0.025) 0.05	-
deoxynivalenol	cereals [5)]	1250 – 1750	(200) 500 – 750	900 – 12000
rye ergot	-	-	-	1000
sum of fumonisin B_1 and fumonisin B_2	maize	14000 – 4000	(200) 800 – 1000	5000 – 60000
ochratoxin A	cereals [5)], dried fruits, coffee, wine, juices, spices, liquorice	5	(0.5) 2 – 10 liquorice extracts up to 80	50 – 250
patulin	fruit juices, apple products	-	(10) 25 – 50	-
sum of T-2 toxin and HT-2 toxin	cereals [5)]	100 – 1000	15 – 200	250 – 2000
zearalenone	cereals [5)], maize oil	100 – 300	(20) 50 – 100 oil: 400	100 – 3000

1) Maximum levels, except for T-2 toxin and HT-2 toxin which are indicative values.
2) In parenthesis values for baby food or foods for infants and young children are provided.
3) Guidance levels, except for T-2 toxin and HT-2 toxin which are indicative values.
4) Sum of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2.
5) Cereals include wheat, rye, barley, oat, maize, as well as products thereof.

To facilitate the exchange of information and to ensure that non-confirmatory products are not distributed in or entering the European market the Rapid Alert System for Food and Feed (RASFF) was established in 1979. Since then it has been developed a quick, easy and effective tool to exchange information between the Member States of the EU (+ Norway, Liechtenstein, Iceland and Switzerland) as well as the European Commission which is of exceptional importance in the case of risk to human or animal health (DG SANCO 2009). It is accessible in an anonymous form (hence no trade names or the identity of the involved companies are revealed) also to the broad community via the RASFF-Portal (DG SANCO 2014). The majority of the initial notifications are border rejections hence the products

have not entered the European market. This is especially true for the hazard category “mycotoxins” in which more than 80% of all notifications are accounted to the category “border rejections” in 2012 (DG SANCO 2013a).

Compared to the previous years, the number of notifications concerning mycotoxins decreased in 2012 which is attributed to the decrease of more than 17% of notifications involving AFs (484 in 2012 instead of 585 in 2011). According to the RASFF annual report this decrease might be explained by the reinforced check regime for specific combinations of product and country of origin. The importance of mycotoxins as food and feed contaminants is pointed out in the top 10 list of the annual report. The top three in the list of notifications by country of origin are due to AFs in the product category “fruits and vegetables” of Turkey (134 notifications), “nuts, nut products and seeds” of China (59) and “feed materials” from India (58). In the list of the top 10 notifications by notifying country half of the list is due to AFs contamination (DG SANCO 2013a).

2 Chromatography and Mass Spectrometry for the Determination of Mycotoxins and Volatiles

2.1 General Analytical Aspects

The general analytical scheme for the determination of mycotoxins in food and feed are: sampling, homogenisation and subsampling, sample preparation (including extraction, clean-up and/or concentration) and detection. A brief overview of these issues is given in this section. The following dedicated subsection 2.2 explains how to determine and deal with matrix effects, the main obstacle of quantitative LC-MS methods. Furthermore, subsection 2.3 deals with aspects to be considered in the development of LC-MS based multi-mycotoxin methods.

All analytical methods, regardless of the used techniques, start with sampling and sample comminution until a sampling size suitable for further extraction is gained. The need of good sampling is often underestimated, but it is essential for accurate analysis. It is of exceptional importance especially for those mycotoxins which are produced during storage (like OTA and AFs) for which so called concentration "hot-spots" occur frequently. The heterogeneous distribution is expressed both as huge difference from one individual food item to the next (intrinsically) and within one sample of an individual food item (distributional) (Tittlemier *et al.* 2011). Thomas B. Whitaker is a pioneer in this field; he and his co-workers published numerous papers regarding the issue of sampling and sampling strategies for the determination of mycotoxins (e.g. Whitaker 2006). Furthermore, Meastroni and Cannava (2011) provide an overview about the sampling strategies to control mycotoxins. The methods of sampling and analysis for the official control are laid down in EC Regulation 401/2006. Furthermore, the Codex Alimentarius Commission (CAC) - a Joint FAO/WHO expert group - is working on sampling plans e.g. for AFs in dried figs or FBs in maize (CAC 2012).

Chemically, mycotoxins are low molecular weight substances and they show a huge diversity concerning physical and chemical properties which complicates to determine them by unified methods.

Sample preparation remains the key step in mycotoxin determination and limits the whole analytical process (Berthiller *et al.* 2014). What this means for the analysis of multiple mycotoxins is discussed in more detail in section 2.3. Sample preparation and clean-up approaches for mycotoxin analysis are summarised by Razzazi-Fazeli and Reiter (2011). For the dedicated analysis of certain analytes or groups thereof solid phase extraction (SPE), specifically tailored MycoSep columns or immunoaffinity columns (IAC) are applied in mycotoxin determination. SPEs are used since the late 1970s for sample clean-up and simultaneous enrichment. The sample is applied to a column packed e.g.

with octadecylsilyl derivatised silica (C_{18}). After a washing step to remove matrix components, the analytes are eluted from the column under different conditions (Arsenault 2012). Examples for SPE based sample preparation for mycotoxins can be found in the review of Turner *et al.* (2009). MycoSep columns are reversed SPE columns in which the matrix is retained on the column and the target analytes passes through.

This allows a one-step rapid purification within seconds, but allows no enrichment (Wilson and Romer 1991). A specific application of SPE is matrix solid phase dispersion (MSPD) combining extraction and clean-up in a single step, becoming a cheap, easy and versatile option. The sample and a solid support (e.g. C_{18}-material) are vigorously mixed, improving the disruption of the sample and hydrophobic and/or hydrophilic interactions that might lead to a selective enrichment (recently reviewed in Capriotti *et al.* 2013). Immunoaffinity columns show the highest specificity of all commonly used clean-up methods. The antibodies immobilised in the column can specifically recognise the antigen (i.e. the mycotoxin) and bind it until it is eluted (usually by applying organic solvents). IACs are typically for single analyte-use only, but nowadays, up to six different mycotoxins can be cleaned-up by one column containing different antibodies (e.g. Vaclavikova *et al.* 2013). Advantages and drawbacks of IACs specifically in the determination of mycotoxins in food are provided in the review by Castegnaro *et al.* (2006).

For recent developments regarding sample clean-up including immune-ultrafiltration, sol-gel immunoaffinity chromatography and the use of aptamers instead of antibodies see the review by Cichna-Markl (2011).

Aptamers are single stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences consisting of 20 to 90 oligonucleotides. Like antibodies they are able to bind molecules with high affinity and specificity, but are more stable than them (Cichna-Markl 2011; Vidal *et al.* 2013).

For the determination of mycotoxins several analytical approaches are possible that can be divided into immunological methods, methods based on chromatography and emerging methods. Immunological methods, similar to the IACs, are based on the recognition of the mycotoxin by antibodies and the quality of analysis is determined by the selectivity and specificity of this interaction. An overview about immunochemical methods for the rapid determination of mycotoxins in food and feed is provided by Goryacheva and De Saeger (2011). It is very important to check the cross-reactivity of the target analyte with similar ones (e.g. “masked” mycotoxins) when assessing the performance of these assays. This principle is used in enzyme linked immunosorbent assays (ELISA), as well as in lateral-flow immunoassays (LFIA, also called dip-stick assays). These LFIA are similar to pregnancy

tests in their setup and have the advantage that they are non-instrumental, rapid assays due to a one-step design (reviewed in Anfossi *et al.* 2013). Turner *et al.* (2009) provides examples of ELISA protocols for mycotoxin determination.

Within the chromatographic methods further classification into thin layer chromatographic methods (TLC) and methods based on gas (GC) or liquid (LC) chromatography or capillary electrophoresis is possible. The application of these different techniques for the determination of mycotoxins in food and feed is summarised by Shephard (2011). Various detectors are used after separation of the mycotoxins of interest from the matrix. Traditional single analyte methods were specifically dedicated to determine individual mycotoxins in certain commodities. They were based on the spectroscopic (e.g. OTA or AFs by UV-Vis) or fluorescent properties (e.g. ZEN by FLD) of the analytes after LC separation. Examples of protocols using TLC, HPLC and GC are provided by Turner *et al.* (2009). As reviewed by Li *et al.* 2013, determination of mycotoxins by means of MS is applied since the early 1970s, first in combination with TLC and GC and since the mid-1980s also in conjunction with LC separation. Gas chromatography was used especially in the last century. The drawback of GC is that derivatisation is required to render mycotoxins volatile. Advances in analytical chemistry, in particular with regard to the sensitivity and selectivity of the available mass spectrometers are the main reasons for the increased importance of LC-MS(/MS) methods (Li *et al.* 2013). The importance and powerful application of the combination of liquid chromatography with mass spectrometry is appreciated in the special issue entitled "State-of-the art of (UHP)LC-MS(-MS) techniques and their practical application" of the Journal of Chromatography A from May 2013 (edited by Guillarme and Veuthey 2013). It is shown that LC-MS is a widely used technology in different scientific fields including pharmacy and biological, environmental and food analysis. The diversity of LC-MS applications for food analysis was reviewed by Di Stefano *et al.* (2012).

Concerning the application of LC-MS for the detection of mycotoxins, we must highlight some important analytical aspects. In the interface the transition from the liquid phase coming from the LC-system to the gaseous phase, as well as the ionisation of the analytes takes place before they enter the MS. Atmospheric pressure ionisation (API) techniques can generate ions - as the name already indicates - at atmospheric pressure. This category includes atmospheric pressure electrospray ionisation (ESI) (Gross 2011). ESI is particular useful for the investigation of polar or even ionised molecules in positive as well as negative ionisation mode. It was developed by Malcolm Dole and his team in the 1960s and was improved by John Fenn, who also received the Nobel Prize in Chemistry in 2002 (shared with Koichi Tanaka and Kurt Wüthrich). ESI belongs to the so called "soft" ionisation

techniques in which the ions are created without decomposing the analyte. In negative mode ESI leads to the formation of deprotonated ions $[M-H]^-$ or adducts like $[M+CH_3COO]^-$, $[M+HCOO]^-$ or $[M+Cl]^-$. $[M-H]^-$ is also called pseudo-molecular ion, as the molecular mass of the neutral molecule is changed by about 1 amu (the mass of a proton). Also multiple charged ions are possible, particular for larger (bio-)molecules. It has to be kept in mind that for example double charged ions will appear in the mass spectrum at half of the total mass, since in the MS ions are separated according to their *m/z* (Gross 2011). Since in the case of sodium or potassium adducts the charge is not transferred to the fragments those are not widely used as precursors in tandem mass spectrometry. To avoid the formation of those adducts small amounts of volatile buffer substances (e.g. 5 mM ammonium acetate or ammonium formate) are frequently added to the mobile phases to shift the balance to ammonia adducts in positive mode (e.g. Berthiller *et al.* 2005). It is important that volatile buffers (like ammonium formate or ammonium acetate) and buffers with low ion strength are used during LC separation. Only a limited amount of charges is present in the ion source at a time and the target analytes are competing for them with co-eluting compounds. The difference between the ionisation efficiencies of the target analytes in the absence and presence of co-eluting matrix components is called matrix effect (reviewed in Gosetti *et al.* 2010). This issue is discussed in conjunction with possible compensation strategies in subsection 2.2.

As regards mass analysers, they can be grouped based on the separation principle into separation in time (e.g., quadrupole (Q)) and separation in place (e.g., Time of Flight (TOF)). The quadrupole consists of four rod electrodes which are cylindrically or hyperbolically shaped. To opposite rods direct current (DC) and an alternating current in a radiofrequency (RF) range is applied. RF and DC are adjusted in a way so only one *m/z* can pass on a stable trajectory through the rods. If several *m/z* values are measured the instrument operates sequentially, ideally from low to high *m/z* (Ardrey 2003; Gross 2011; Rodriguez-Aller *et al.* 2013). In TOFs the ions are separated by their *m/z* ratio while traveling through a field-free drift path (also referred to as flight tube). If all ions start with the same kinetic energy lighter ones reach the detector earlier than heavier ones. In linear TOFs the ions travel only once through the flight tube and the detector is on the opposite end of the drift path. In reflector TOFs, an electric field is applied at the end of the first drift path and the ions are reflected in the opposite direction. Hence the ions change their direction and travel a second time through the field free drift path before detected (Ardrey 2003; Gross 2011). Minor variations in starting kinetic energies can be compensated in this way and lead together with the longer flight path to higher achievable resolutions compared to linear TOFs. MS instruments operate under high vacuum so the generated ions

can reach the detector undisturbed. The vacuum requirements are different depending on the type of analyser. This high vacuum is usually reached in two pumping stages: first roughing pumps (either rotary van pumps or oil-free scroll pumps) generate a pre-vacuum. One or more high vacuum pumps (e.g. turbo molecular pumps) are then delivering the required vacuum. Tandem mass spectrometry combines two or more stages of mass analyses; each stage contributes to the improvement of one of the following parameters isolation, selectivity or structural information. Maybe the most popular examples are quadrupole time-of-flight (QTOF) and the triple quadrupole (QqQ) mass spectrometer. QTOFs are hybrid instruments combining a quadrupole and a TOF.

Especially, triple quadrupole tandem mass spectrometers are widely accepted, but in the last years a trend to HRMS is visible. Unambiguous identification and quantitation at trace levels in various food and feed matrices are possible nowadays.

Mycotoxins are typically separated using reversed-phase columns (in particular C_{18} or C_8 columns), hence the gradient elution starts with high water contents and the organic content is increased over the run. Since 2004, columns of fully porous sub 2 µm particles are available resulting in backpressures up to 1200 bar (120000 kPa). The advantage is a faster analysis or higher resolution with higher throughput. This requires also different equipment capable of withstanding the high pressure and the method is called ultra-high performance-liquid chromatography (UHPLC) (Snyder *et al.* 2010). On average, the peak widths at baseline are between 2 to 4 s in UHPLC applications, whereas they are around 10 to 20 s for HPLC separation. Since for quantitative purposes, 10 to 15 acquisition points are required to obtain a reliable peak shape and suitable performance with regard to reproducibility and precision, this might be a challenge for some MS systems depending on the duty cycle. Another aspect is, that the inlet and outlet column frits of UHPLC columns are reduced from ca. 2 µm in HPLC to around 0.2 µm in UHPLC hence there is a higher risk for column clogging and interruptions of the run than in HPLC application (Rodriguez-Aller *et al.* 2013).

"Masked" mycotoxins are usually not determined in routine analysis and at least three approaches for their determination exist. First, indirect methods based on the hydrolysis of the "masked" mycotoxins and detection of parent compound (sum of the parent compound present in the sample and the parent compound released from the "masked" forms during hydrolysis) can be applied. Hydrolysis is either performed by enzymatic approaches or under acidic or basic conditions (reviewed by Berthiller *et al.* 2013). Second, immunochemical methods with antibodies specific for the "masked" mycotoxins or with cross-reactivity to them can be used (reviewed by Goryacheva and De Saeger 2012). Third, direct methods for the determination e.g. with hyphenated chromatographic techniques

(reviewed by Cirlini *et al.* 2012) can be developed. The third approach is the most accurate one, but also that requiring the most information: the nature and structure of the "masked" compound has to be identified and for quantitation analytical standards have to be available.

Excellent reviews about the determination of mycotoxins in food matrices (especially by LC-MS) are those by Zöllner and Mayer-Helm (2006), about the trace analysis in biological and food matrices, Capriotti *et al.* (2012), about multi-mycotoxin methods, and most recently Li *et al.* (2013) on hyphenated chromatographic-mass spectrometric methods. Furthermore, De Saeger (2011) edited a book about the determination of mycotoxins and mycotoxigenic fungi in food and feed and the World Mycotoxin Journal publishes annually updates about mycotoxin analysis compiled by experts in the field (e.g. Shephard *et al.* 2013; Berthiller *et al.* 2014).

2.2 Matrix Effects

One of the major pitfalls of quantitative LC-MS methods are matrix effects (reviewed in Gosetti *et al.* 2010). This issue is known since the late 1990s and matrix effects were first extensively studied in pharmaceutical applications (e.g. Matuszewski *et al.* 1998; King *et al.* 2000). Matrix effects mean that in the presence of co-eluting matrix components the analyte signal might be lower (signal suppression) or higher (signal enhancement) compared to the analytes in neat standard solution and therefore result in under- or overestimation of the contamination levels (see Figure 2). The exact mechanisms behind are not fully elucidated yet. One theory is that interfering matrix components change the viscosity and surface tension of the droplets formed during ionisation. This is influencing solvent evaporation and the transfer of the analytes from the liquid to the gaseous phase (King *et al.* 2000; reviewed by Van Eeckhaut *et al.* 2009). It is important to keep in mind that also compounds which are not monitored might interfere during ionisation. Another theory hypothesises that in the ion source only a limited amount of charges is present at a given time, hence a competition process takes place - the molecules which are easier ionised are favoured over those which ionise worse. Finally, ion suppression might also be caused by neutralisation reactions occurring in the gas phase (Van Eeckhaut *et al.* 2009).

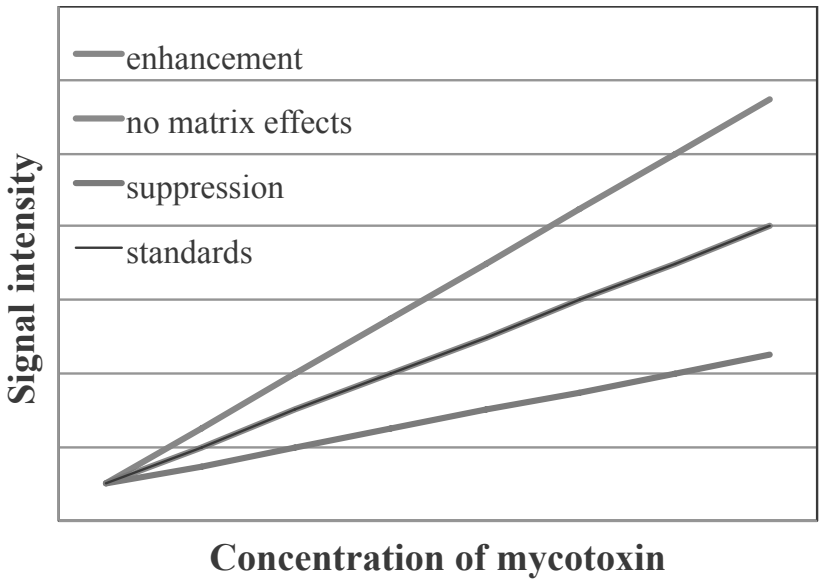


Figure 2: Matrix effects
Matrix effects are caused by co-eluting matrix components which influence the ionisation efficiency of the analytes. No matrix effects occur if the difference of the slope of the analytes in matrix (green line) is not significantly different than those in neat solvent (black line). If the slope is steeper in matrix-matched calibrations (blue line) compared to standards matrix enhancement is observed. A shallower slope (red line) compared to the standard characterises matrix suppression.

The extent of matrix effects (also termed absolute matrix effects) depends on the diversity and complexity of the sample, the extraction and clean-up procedures, the chromatographic conditions, the ion source dimensions and last but not least on the analyte itself. However, matrix effects may vary not only between different commodities (e.g. maize, wheat, barley), but also within one commodity e.g. because different varieties, different cultivation conditions, etc. affect the composition of a given crop. The variability of matrix effects between different samples of the same matrix is called relative matrix effects. Malachova *et al.* (2013) performed an inter-laboratory study to investigate these matrix effects in different food and feed samples. The determined absolute matrix effects ranged between 60 and 100% for cereal based feed and 7 and 187% for hay silage feed. They reported a high variability of matrix effects (41-72%) for DON, HT-2, T-2 and nivalenol in a sample set of wheat flour, crackers and rusks. However, for biscuits containing different ingredients relative matrix effects below 14% were observed for these analytes. Similar results were gained for AFs in maize with variabilities below 15% and for ergot alkaloids in wheat, rye and triticale with relative matrix effects of 19-28%. Furthermore, it was shown that to measure the analytes in negative ionisation mode (instead of positive) might help to reduce the matrix effects. Also to change LC conditions might have a positive influence and it was shown that to use any purification approach helps to lessen matrix effects of some compounds (Malachova *et al.* 2013). Concluding, it is not possible to predict whether matrix enhancement or suppression occurs and to which extent. Therefore, matrix effects might hamper the accurate and reliable quantitation which is especially of importance for the determination of regulated mycotoxins to ensure the enforcement of the set maximum levels.

The approaches to deal with matrix effects can be categorised into two groups. One option is to apply strategies reducing the amount of matrix components introduced into the system. This can be performed by various means, e.g. performing or modifying sample clean-up or by simply diluting the sample. The second option is to find an appropriate compensation strategy to deal with matrix effects (Gosetti *et al.* 2010), such as matrix-matched calibration, standard addition and internal calibration. In the following advantages and drawbacks of the individual approaches are discussed and the strategies how to assess matrix effects with the different approaches are shown.

Sample preparation and **sample clean-up** can only be applied when a limited number of analytes are of interest. The main issue is to minimise the losses of the target analytes during sample preparation, but to reduce the matrix load as much as possible. However, to apply extensive sample clean-up or specific clean-up procedures like IACs, does not mean that matrix effects are completely circumvented, as shown e.g. by Lattanzio *et al.* (2007). **Dilution** of the sample with solvents might significantly reduce matrix effects (e.g. Sulyok *et al.* 2006). However, it is not always feasible or only to a certain extent because mycotoxins usually occur in traces. Hence to achieve LODs and LOQs which are as low as possible is often a main goal in method development.

In the case of **matrix-matched calibration**, blank matrices are prepared in the same way as the samples. Just before analysis the analytes of interest are added to the raw extracts at different concentration levels so a calibration function can be calculated. The slope of this so called "matrix-matched calibration" is compared to the slope of the standards in neat solutions (e.g. methanol-water or acetonitrile-water mixtures) and multiplied with 100 to gain the signal suppression or enhancement (SSE) in per cent (see Formula 1b and Figure 2). Values above 100 indicate signal enhancement, and values below 100 signal suppression (Matuszewski *et al.* 2003). In a similar way, the apparent recovery and extraction recovery of the method might be assessed for validation purposes (see Formula 1a and Formula 1c). The only difference is that blank samples are already spiked before extraction on several levels.

$$(a)\ R_A\ (\%) = \frac{slope_{spiked\ sample}}{slope_{neat\ standard}} \times 100 \qquad (b)\ SSE\ (\%) = \frac{slope_{spiked\ extract}}{slope_{neat\ standard}} \times 100$$

$$(c)\ R_E\ (\%) = \frac{R_A}{SSE} \times 100 = \frac{slope_{spiked\ sample}}{slope_{neat\ extract}} \times 100$$

Formula 1: Calculation of apparent recovery R_A (a), matrix effects (b) expressed as signal suppression or enhancement (SSE) and extraction recovery R_E (c) (modified after Matuszewski *et al.* 2003).

Matrix-matched calibration was for example chosen by Beltrán *et al.* (2013) for the investigation of 18 mycotoxins in a total diet study involving 240 samples. For this purpose 24 matrices were spiked at two different concentrations to correct for matrix effects. For the application of matrix-matched calibration, blank samples for all analytes of interest are required. It is sometimes a challenge to find suitable, uncontaminated blank samples especially in the case of multi-mycotoxin applications. Therefore, it might be necessary to select several samples of one commodity as blank for certain analyte groups and hence increasing the number of samples to be analysed. For some analyte-matrix combinations it is hardly possible at all to find those blanks due to background contamination at low levels (e.g. DON in European maize). Another challenge is that the blank sample used for the preparation of the matrix-matched calibration should resemble the investigated samples as closely as possible. Additionally, matrix-matched calibrations are tedious and time consuming - especially if several different commodities are measured in one batch. Attention has also to be drawn to samples which are above the calibration curve and require further dilution. These additional dilution steps have to be performed with blank matrix extracts and not with neat solvents because otherwise the matrix effects will differ from non-diluted samples. Another drawback is that the differences within one commodity cannot be compensated by matrix-matched calibration.

Standard addition is another approach to compensate these intra-commodity differences. In general, this involves a two steps procedure: first the samples are measured after a normal sample preparation and are quantitated against neat standard solutions. In a second step, suspected positive samples are fortified with the target analytes either before extraction or just prior to analysis (depending on the approach) on one or more levels, hence at least two injections per positive sample are necessary. For the evaluation of actual concentration, the signal intensities of fortified and non-fortified samples are related to the added amount of analyte (see Figure 3 for details). A drawback of this approach is that false negative samples in which significant matrix suppression is observed might be missed. Fortifying all samples is time consuming (additional sample handling time and extended analysis-time), tedious and requires costly standard solutions. Additionally, especially for higher contaminated samples different dilutions might be necessary because the fortified samples are no longer in the linear range. Special attention is required to avoid a mix-up of samples and applied procedures for each individual sample. The approach of standard addition was for example chosen by Di Mavungu *et al.* (2009) for the investigation of mycotoxins in food supplements.

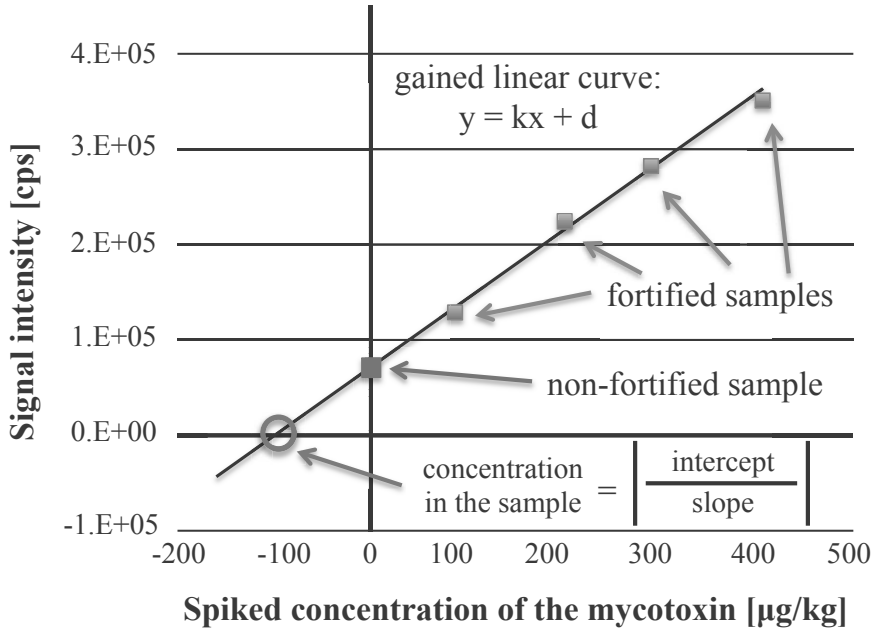


Figure 3: Standard addition
The obtained signal intensities of the non-fortified and fortified samples are plotted against the spiked concentration. A line is drawn through the gained data points. The intercept of this line with the x-axis provides the concentration of the mycotoxin as an absolute value of the negative result.
[cps – counts per second]

The last and maybe most promising approach to compensate matrix effects is **internal calibration**. Minimum demands for internal standards (IS) are that they are not present in samples and they have to be stable during sample preparation (if applied before extraction) and measurement. Additionally, the chemical-physical properties should be as similar as possible to the properties of the target analyte, but differentiation in the chosen detection system still must be possible (Gosetti *et al.* 2010). Depending on the point of addition, IS can also be used to compensate for losses during sample preparation if they are applied prior to extraction (see Figure 4). For instance the following losses can be compensated: incomplete extraction, pipetting errors, losses during clean-up (e.g. adsorption at surface walls), drying down or reconstitution (e.g. incomplete dissolution) and matrix effects. It is also possible to add the IS somewhere in-between sample preparation and then IS will compensate only losses in the subsequent sample handling steps. If they are applied just before analysis and are used for the compensation of matrix effects only, sample preparation should be very reproducible.

In practice, structural related or similar compounds, as well as, especially in the last years, stable isotopically labelled compounds are used as IS. The analyte concentration is assessed by the division of the signal intensity of the analyte and the IS multiplied with the concentration of the IS (see Figure 4b). The extent of matrix effects is assessed by the evaluation of the samples once with internal calibration and once without internal calibration which is possible with the same data set just by changing the evaluation settings. It is not necessary to measure the samples twice and hence preparation and analysis time compared to standard addition is saved. If the concentration values

obtained by internal calibration are higher, matrix suppression is observed for this analyte-matrix combination.

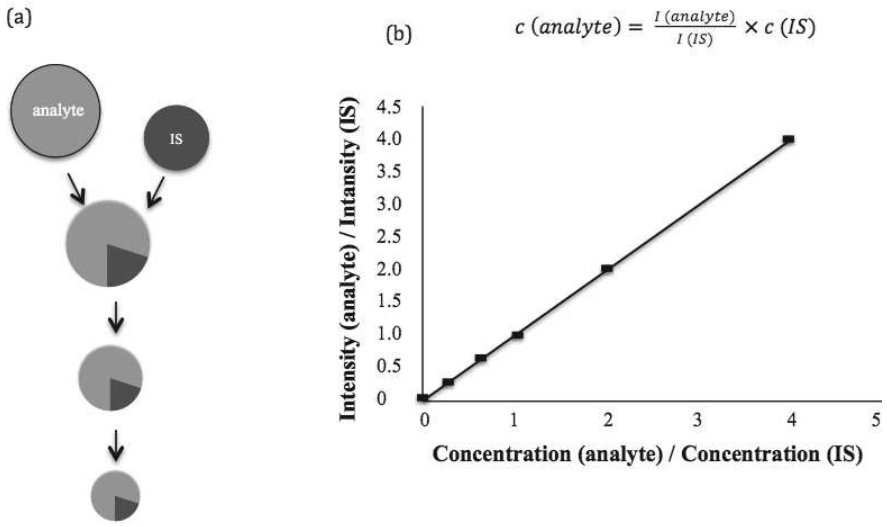


Figure 4: Internal calibration
Internal standards can compensate for any losses during sample preparation since the ratio between analyte (marked in green) and internal standard (IS, marked in blue) stays the same (a). For the calculation of the concentration the ratio of the signal intensity (I) of the analyte and the IS is plotted against the ratio of the concentration (abbreviation c). To obtain the concentration of the analyte divide the signal intensity of the analyte with those of the IS and multiply it with the concentration of the IS (b).

For example, zearalanone (ZAN) was used as IS for the determination of ZEN in wheat and corn (Pallaroni and von Holst 2003) or for multiple-mycotoxins in food and feed (Ren *et al.* 2007). ZAN differs from ZEN only by the absence of one double bond and hence is two mass units heavier than ZEN. However, Van Eeckhaut *et al.* (2009) recommend that the IS and the analyte itself should have a mass difference of at least three mass units to avoid interference of the signal contribution of the IS to the natural isotope. Other examples for structurally similar compounds used as IS for single analytes or groups thereof, are the trichothecene verrucarol (e.g. Berger *et al.* 1999), α-zearalanol (e.g. Royer *et al.* 2004) or mammalian metabolites like deepoxy-deoxynivalenol (e.g. Klötzel *et al.* 2005) or AFM_1 (e.g. Capriotti *et al.* 2010). A problem of these structurally similar compounds is that the retention time often slightly differs compared to the target analytes and hence matrix effects might also be different leading to incorrect compensation and ultimately to false results.

Stable isotopically (= non-radioactive) labelled IS provide a solution for this problem. In general, they have the same chemical and physical properties as the target analytes, but can be differentiated in the mass spectrometer by their different molecular mass. This procedure is often referred to as stable isotope dilution assay (SIDA) because the naturally abundant isotope is diluted due to the addition of the stable isotope labelled standards. The review of Rychlik and Asam (2008) provides an historical background of the use of SIDA in mycotoxin determination, defines the prerequisites and restrictions and summarises application and synthesis strategies. Generally, the use of

[^{13}C]- or [^{15}N]-labelled compounds is favoured compared to deuterium [^{2}H]- or [^{18}O]-labels. First, carbon and nitrogen are often part of the molecule backbone and the cleavage of C-C or C-N bonds are less likely. Second, isotope exchanges with the environment during sample preparation occur seldom which are especially a problem for deuterium isotopes. Third, the so called isotope effects (small physical or chemical differences of the isotopologues) are higher for deuterium than for [^{13}C] or [^{15}N] (Rychlik and Asam 2008).

The IS might be only partly labelled as shown by Bretz *et al.* (2005) with the synthesis of 3-d_3-acetyl-deoxynivalenol from deoxynivalenol or fully labelled ones. The characterisation, application and suitability of the first fully ^{13}C-labelled DON for the determination of this mycotoxin in maize and wheat without any sample clean-up were published by Häubl *et al.* (2006a and 2006b). Since stable isotope labelled IS are expensive they are most often applied just before analysis hence only for the correction of matrix effects in order to minimise costs. Another drawback is that although in the last ten years more and more IS became commercially available the number of isotopically labelled standards is still limited. For all mycotoxins regulated in the EU fully ^{13}C-labelled internal standards are available on the market. In 2011, the first SIDA-method covering the determination of nine mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, DON, ZEN, T-2, and HT-2) in cereal-based foods was published (Lattanzio *et al.* 2011). This method applied an acetonitrile-water (84:16, v/v) extraction followed by an SPE clean-up and the fully ^{13}C-labelled mycotoxins were applied prior to analysis as internal standard to compensate matrix effects.

2.3 Multi-Mycotoxin Methods by LC-MS

It is essential for food safety to screen food for a multitude of mycotoxins. On the one hand several fungi can co-colonise a given agricultural commodity, on the other hand fungi are able to produce several mycotoxins. Co-occurrence influences the overall toxicity because the toxicological effects of different mycotoxins might be additive, synergistic or antagonistic (e.g. McKean *et al.* 2006). Novel mycotoxin-commodity combinations are reported every year - this is both due to the increased use of multi-mycotoxin methods and due to the possibility of fungi to adapt to changing environmental conditions. LC-MS based multi-methods are a great option, allowing unambiguous determination of a huge variety of chemically diverse analytes within a relatively short time (Shephard *et al.* 2013). They are also a great tool to monitor changes in the regional fungal spread, for example caused by climate change (Magan *et al.* 2011, Paterson and Lima 2011). The major advantage over single-target methods is the increased sample throughput - the individual runs of multi-mycotoxin methods might be longer,

but provide more information compared to single or group target methods. The bottleneck of the analysis is shifting from sample preparation which required most of the time in traditional applications to data evaluation. The producers of mass spectrometers reacted and are developing specific software tools dedicated for multi-target analysis.

Spanjer (2011) provides an historic overview on the determination of mycotoxins by means of LC-MS with a special focus on multi-mycotoxin methods. In particular, 45 multi-methods published between 2002 and 2010 are summarised. All of these methods were based on RP-LC using C18-bonded silica columns as stationary phase. Discussion of all published multi-mycotoxin methods is beyond the scope of this work, but several important examples are described below. Berthiller *et al.* (2005) published the first triple quadrupole method for the determination of A- and B-trichothecenes, as well as ZEN in maize and one year later the first validated LC-MS/MS method for 39 mycotoxins in wheat and maize was published (Sulyok *et al.* 2006). Since then an exponential increase in published multi-mycotoxin methods using LC-MS is observed. More and more analytes are included in the analysis and the developed methods are validated for an increasing number of matrices. Static SRM in which all mass transitions are measured throughout the whole run, were replaced first by periods and then by so called dynamic or scheduled SRM methods. For example, the method developed by Sulyok and co-workers was extended over the years (Vishwanath *et al.* 2009) and currently is covering more than 300 mycotoxins and other fungal and bacterial metabolites (Malachova *et al.* 2014). This method has been extensively used for the (semi-)quantitative screening of mycotoxins in a huge variety of matrices (e.g. Sulyok *et al.* 2010; Abia *et al.* 2013).

There are several analytical challenges to be coped with in multi-mycotoxin methods: First, the number of relevant analytes is high and especially mycotoxins are characterised by a huge physical and chemical diversity. Concerning the molecular weight the target analytes range from very small molecules like moniliformin (98 g/mol) or 3-nitropropionic acid (119 g/mol) to beauvericin (783 g/mol) or the immunosuppressant drug cyclosporin A (1201 g/mol). With regard to the chemical properties, some mycotoxins are polar acidic (e.g. FBs) others are polar basic (e.g. ergot alkaloids) and even others are apolar (e.g. enniatins). This leads to one of the biggest challenges in multi-mycotoxin determination - extraction and sample preparation. Demands on the extraction procedures are fastness, simplicity, efficiency and that as few analytes as possible should be discriminated. Hence careful optimisation of extraction conditions is necessary. Individual results of the extraction of 39 analytes using eight different solvents are shown by Sulyok *et al.* (2006). Usually different mixtures of water and organic solvents (e.g. acetonitrile or methanol) with or without the addition of acid (either acetic or

formic acid) are applied (Capriotti *et al.* 2012). In a proficiency test involving the determination of up to eleven mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, OTA, DON, T-2, HT-2 and ZEN) in maize more than half of the 41 participating laboratories used mixtures of acetonitrile-water for extraction (De Girolamo *et al.* 2013). From the regulated mycotoxins, FBs are those with the lowest extraction recoveries in the published multi-mycotoxin methods because they require higher water contents than normally used. Marschik *et al.* (2013) investigated specifically the efficiency of different extraction methods used in multi-mycotoxin applications with regard to FBs.

They concluded that acetonitrile-methanol-water (1:1:2, v/v/v) is the extraction solvent of choice for the extraction of FBs from maize samples. FBs are also with another regard challenging analytes. To obtain stable retention times and ionisation efficiencies, they need slightly acidic conditions (Plattner 1999) which might hamper the analysis of other analytes. In RP chromatography applications, some analytes are difficult to retain due to their high polarity (e.g. moniliformin, 3-nitropropionic acid or kojic acid), others elute very late at a high percentage of organic solvent (e.g. equisetin or enniatins). Since high water content negatively influences the ionisation efficiency, conditions are not ideal for early eluting analytes. With regard to ionisation modes, some mycotoxins ionise in both polarities (e.g. OTA) to a similar extent, others are restricted to measurements in one specific polarity (e.g. AFs in positive ionisation mode) to achieve acceptable sensitivities (Spanjer 2011).

Concerning sample preparation, often the so called "dilute-and-shoot" approach is applied in multi-mycotoxin applications since it is not discriminating any analytes (e.g. Sulyok *et al.* 2006; Spanjer *et al.* 2008). In this approach sample clean-up is omitted and after extraction the extract is simply diluted with an appropriate solvent. Another possibility is to split up the extract, follow different sample clean-up procedures and then combine the extracts again prior to analysis (e.g. applied by Monbaliu *et al.* 2009). However, this is a more time-consuming and error prone approach. In the last years the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach which was invented for multi-pesticide analysis became also popular for multi-mycotoxin applications (Shephard *et al.* 2013). As an example, this approach was applied by Zachariasova *et al.* (2010) for the determination of several *Fusarium* mycotoxins in cereals or for the presence of 27 mycotoxins and other secondary metabolites in maize silage by Rasmussen *et al.* (2010). More specific sample clean-up procedures, such as solid phase extraction or IAC are not applicable for the determination of a huge variety of chemically diverse analytes. However, they are a valuable tool for the specific analysis of food for e.g. regulated mycotoxins. SPE was for example applied by Ren *et al.* (2007) or Lattanzio *et al.* (2008) and

IACs by Lattanzio *et al.* (2007) or Vaclavikova *et al.* (2013).

Another challenge of multi-mycotoxin methods is that they have to cover a large relevant concentration range. Some analytes may occur in traces in the low ng/kg range (e.g. AFs, enniatins), others in the mg/kg range (e.g. DON). Especially, the inclusion of AFB_1 and OTA for the determination of those mycotoxins in baby-food is a big challenge for multi-mycotoxin methods. The ionisation efficiency, extraction recovery and matrix effects of mycotoxins is quite different, whereas enniatins ionise quite well and LOQs in maize in the low ng/kg range were achieved by Malachova *et al.* (2014), far higher LOQs (254 µg/kg) were gained in the same application for e.g. patulin. The need for unambiguous identification in complex samples (e.g. spices) is another challenge (Rodriguez-Aller *et al.* 2013).

In general, to acquire two mass transitions for low resolution LC-MS/MS systems as recommended by EC Decision 657/2002 is enough to yield the required identification points. However, to check the metabolite pattern for plausibility and consistency is highly recommended to avoid false positive results. For example, AFs are usually accompanied by kojic acid and 3-nitropropionic acid, while *Myrothecium* metabolites in lemons or ergot alkaloids in tomatoes are very unlikely. In case of doubt, it is recommended to perform (enhanced) product ion scans to confirm the presence or absence of the mycotoxin. With this approach it was possible to avoid the false positive report of roridin A (a type D trichothecene) in oranges. Both SRM transitions and the retention time matched with the standard, but full MS/MS scans were not consistent with the standard. Developments in mass spectrometry made it possible to overcome most of these challenges in a satisfying way. However, it has to be kept in mind that multi-mycotoxin methods are always a compromise due to the sometimes conflicting chemical properties and the best LODs/LOQs are achieved with methods using the optimised conditions for the target analytes. (Spanjer 2011)

Furthermore, also the validation of multi-analyte methods is time- and cost-consuming. Validation is defined as: "*confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled*" (ISO 8402:1044 cited in EURACHEM 1998). It normally includes the determination of the following parameters: selectivity, specificity, accuracy, trueness and precision, as well as LOD and LOQ. The standards and recommendations available for single target methods are not always applicable for multi-analyte methods, in particular when the number of analytes is very high. For the multiple determination of mycotoxins no recommendations exists so far, but method validation and quality control procedures for the determination of pesticide residues in food and feed are available (latest update DG SANCO

2013b). In there, different commodity groups are defined, e.g. (a) high water content, (b) high oil content and very low water content, (c) high starch and/or protein content and low water and fat content and (d) "difficult or unique commodities". Malachova *et al.* (2014) chose one representative matrix of these four groups to validate their multi-mycotoxin method for almost 300 analytes. Since validation procedures for multi-mycotoxin methods are even more time and cost consuming than for single analyte methods, especially if several matrices are involved, lots of developed multi-methods are used for screening or semiquantitative screening purposes only.

Nowadays, most methods for mycotoxin determination are based on LC-MS, more specifically triple quadrupole mass spectrometers. The disadvantage of QqQs is that the target analytes have to be optimised before analysis and only what is known beforehand can be assessed. Hence they allow mainly targeted screening, covering a high linear range and good LODs, two essential parameters for mycotoxin determination. However, in the last years, the application of high resolution mass spectrometry showed an increased popularity. The biggest advantage of HRMS-instruments is that they operate in full scan mode and thus allow the screening for both known and unknown compounds. Even months or years after data acquisition it is possible to go back and check for the presence of some newly discovered mycotoxins or metabolites thereof without the need of additional LC-MS runs. Zachariasova *et al.* (2010) compared TOF-MS (R = 12000) with Orbitrap technology (R = 100000). They concluded that while for TOF-MS previous sample clean-up steps are necessary, crude extracts could be injected into the Orbitrap. Lattanzio *et al.* (2011) showed a comparison of a validated LC-MS/MS based triple quadrupole method with HRMS. They concluded that the performances are comparable, but HRMS has the advantage of post-data-acquisition. Ates *et al.* (2013) showed the development of an LC-HRMS based method for the determination of the *Fusarium* toxins DON, T-2, HT-2, ZEN, FB_1 and FB_2 in animal feed, maize and wheat. In addition to the full scan more, targeted MS/MS spectra for the six analytes were obtained for confirmation. Average recoveries ranged between 72 and 120% and relative standard deviations of 1 to 19% were obtained after spiking at three concentration levels in six replicates.

LC-MS is a powerful tool for the determination of mycotoxins. Depending on the application, methods range from very accurate methods (e.g. using isotopically labelled standards) to screening methods with the purpose of monitoring a huge number of analytes in various matrices. Nowadays, triple quadrupole mass spectrometers are still the instrument of choice, but HRMS instrument are a valuable alternative especially for the screening of multiple analytes. Historically, quadrupole analysers and ion traps are most commonly used, but the importance of high-resolution mass

spectrometers (HRMS) is increasing. HRMS instruments include TOF, Orbitrap and FT-ICR mass spectrometers. Modern HRMS instruments are characterised by a resolution above 10000 and mass accuracies below 2 ppm (Holčapek *et al.* 2012). Among the benefits of HRMS are the acquisition of full-scan spectra which allow the screening of an indefinite number of analytes. Furthermore, it allows post-acquisition analysis even months or years after collection of the spectra. So it is possible to check old samples for the presence of a newly identified compound without repeated measurements. Additionally, HRMS can be used for confirmatory analysis or structural elucidations and identifications. The high mass accuracy together with the isotopic ratios allows the calculation of the elemental composition with high precision which reduces the number of possible sum formulas. A screening method without the need of reference compounds has the advantage that it is not necessary to prepare complex standard solutions and carry-over problems from the standard to the matrix are circumvented (Kaufmann 2012). While older generations of HRMS instruments were way behind QqQs with regard to sensitivity, the new developed TOFs can compete in that regard and are also characterised by a fast acquisition speed (Rodriguez-Aller *et al.* 2013). HRMS has developed to an important tool, especially in non-target analytics.

2.4 Methods for Volatiles Detection

The investigation of volatile organic compounds is not an easy task, because it needs to be undertaken in the context of fit for purpose food quality and safety systems. To date, great attention has been paid to the determination of the volatile fractions. This kind of determination can be carried out by analytical techniques (e.g., gas chromatography (GC) combined with mass spectrometry (MS)). Methods complementary to GC analysis are available, allowing assessment of the olfactory impact e.g. by an electronic nose (e-nose). Also, some innovative analytical techniques have to be considered.

Identification and quantification of odorant molecules exhaled by natural products of vegetable origin are attracting more and more attention to the scientific and the economic sectors.

The volatility depends on the chemico-physical properties of the substance itself, apart from the characteristics and the structure of raw materials. In particular, molecular weight plays a special role in that the higher it is, the lower is the release rate of the volatile substance. The correlation between molecular weight and persistence is of prime importance for low-molecular weight (LMW) compounds, as these can diffuse across the matrix more easily than other compounds. Furthermore, the structure of molecules - especially the position and the nature of their functional groups - seems to induce significant effects on the type and the strength of interactions with the matrix. Nonetheless,

several exceptions to this rule exist; double bonds, the nature and the number of functional groups, and the molecular weight must be taken into account, as must the polarity of the compound. In general, both polarity of the substance and matrix composition govern the ability of the substance to be released by the matrix and diffuse into the environment.

In 2003 De Roos ascribed the release kinetics of volatiles from the matrix to two major factors: volatility (thermodynamic factor) and resistance to mass transfer from the matrix to air (kinetic factor). The relative speed of release of a volatile compound is a measure of its ability to move into the gaseous phase when it is introduced as a solution or a mixture (Vernocchi *et al.* 2008). At equilibrium, this ability is expressed as the ratio of the air concentration (C_a) of the compound to its concentration in the matrix (C_p), according to the equation: $P_{ap} = C_a/C_p$, where P_{ap} is defined as the air-matrix partition coefficient, which strictly depends on product composition and temperature. The wide variety in chemical composition of volatiles and their different amounts in the same product make the analysis complex, especially considering that some substances are characterised by very low concentrations (Sanz *et al.* 2008; Tadeo and Bonomi 2002).

The selective extraction of a volatile from a given matrix is typically based on its volatility or solubility. Solvent extraction, e.g. makes exclusive use of solubility, while headspace analysis relies on volatility. Methods such as distillation or extraction aim at analysing the whole set of volatiles in the matrix, but they are unreliable in providing a self-consistent description of compounds responsible for olfactory perceptions (Wrolstad *et al.* 2004; Zarra *et al.* 2009). Accurate, precise, rugged, robust multi-component analytical methods characterised by high detection power and high throughput are thus keenly needed in research and control laboratories. These features are possessed to a large extent by some analytical techniques (e.g., gas chromatography (GC) combined with mass spectrometry).

Both static headspace (SH) and dynamic headspace (DH) techniques allow for the direct analysis of exhalations from raw materials with no disruption or alteration of their structure and with no use of chemical substances. By this technique, the overall volatile composition of a matrix can be exactly described.

As regards headspace methods for the analysis of volatile compounds released by foodstuffs, a key role is played by static (or equilibrium) headspace sampling. The sample is inserted into a sealed glass vessel, where it attains equilibrium with its vapor, which is especially rich in highly volatile components. The vapor is then injected into a chromatographic column, thus bypassing problems due to the introduction of poorly volatile substances or large masses of solvent. For a vapor at equilibrium with a liquid, the concentration of volatile components in the vapor phase is governed, under ideal

conditions, by Raoult's law according to the equation: $p_i = y\ p_o$, where p_i is the partial pressure of each single component in the vapor phase, p_o is the vapor pressure of each single component at the temperature of the vessel, and y is the mole fraction of each single component in the solution.

Headspace analysis allows chromatograms to be obtained, where each peak area (S) is proportional to the concentration of the corresponding component in the vapor phase and, therefore, in the liquid phase: $S = f\ y\ p_o$, where f is the detector response factor, experimentally determined. Since p_o is a constant (at a given temperature) for each component, the above equation can be simplified as follows: $S = f\ y$.

Headspace composition depends on the partition of volatiles between air and the various phases in the matrix (water, lipids). The detection power of this method, in terms of the amount of trapped volatile compounds, strictly depends on sample exposure time, as demonstrated by Piggott (Piggott 2000; Dijksterhuis and Piggott 2001).

As touched upon above, another approach for headspace sampling is the DH technique, which is based on dynamic conditions and is also called purge-and-trap (PAT) analysis. An inert gas passes through the thermostatted sample chamber as long as all or most volatile compounds are extracted from the sample. Many of the advantages of SH techniques are also shown by the DH approach, including easy sample preparation, absence of the solvent peak, ability to analyse only the volatiles, and automation. Moreover, the trapping stage of the analysis features enhanced detection power, thus allowing volatiles at the ng/g level to be routinely determined. From this viewpoint, if contaminants and instrument background are carefully minimised, the PAT techniques are capable of routine application in the pg/g range (Wampler 2001). In addition, a certain degree of selectivity as regards the volatiles collected is offered by sorbents, which, in turn, allows a combination of sorbent and temperature to be selected for collection and concentration of specific analytes of interest while skipping others. The overall analysis is thus substantially simplified. Some drawbacks of PAT are because the instrumentation is more complex, may be more expensive to purchase than other devices for sample introduction, and requires the monitoring of several steps (e.g., valving, heating zones) (Hui 2010; Washall and Wampler 1990; Chiralertpong *et al.* 2008).

In DH sampling, volatiles must be examined within a narrow temperature range, so, in order to gauge them, a trap must be resorted to (e.g., a suitable adsorbing or absorbing material (activated carbon, tenax)). The most common trapping methods are, e.g., cryogenic traps (also of the on-column type), adsorption beds (trapped compounds can be efficiently desorbed), and vapor columns. The assessment of the sorbent/analyte interaction and the selection of the best trapping material play key

roles at the developmental stage of any DH technique.

In this context, solid-phase micro-extraction (SPME) is of growing importance. With this approach only a very small amount of extraction solvent is used compared to the sample volume. This leads to partial removal of analytes into the extracting phase so that equilibrium is attained between sample and extracting medium. From a practical viewpoint, the extracting phase coats rods of various materials in a very stable fashion (Pawliszyn 1997). Most frequently, the extracting phase is a polymeric organic phase cross-linked and firmly attached to the support. One possible configuration features rods made of an optical fiber of fused silica (i.e. a chemically inert material). The fiber is protected against breakage by a coating of a polymer (e.g., absorption fibers with polydimethylsiloxane (PDMS) or polyacrylate (PA) and adsorption fibers with Carbowax (CAR) or divinylbenzene (DVB)). Some experimental findings showed that most of the other fibers achieve lower abundances of the extracted components than those obtainable by CAR-DVD-PDMS (Bicchi *et al.* 2004). The fiber is exposed to the sample matrix by sliding it outside of the protection tube into the matrix. This step can be performed either manually or automatically.

The amount of analyte extracted by the fiber coating is determined by the partition coefficient of the analyte itself between the sample matrix and the fiber-coating material. A variety of coatings with different polarities can be used for quantitative and qualitative analysis by SPME. Several important advantages are brought about by SPME when compared to traditional approaches to sample preparation. As said above, the absence of solvent in SPME is a distinctive property of this technique, as it makes the separation fast, with the ensuing high throughput and use of simple instrumentation, along with the fact that its environmental impact is minimised. Furthermore, the limited dimensions make SPME ideal for portable devices for field work. Its sensitivity and detection power are comparable to those of techniques based on liquid extraction. Although the analyte is extracted to a low extent from the matrix, the whole amount is transferred to the detector - in contrast to liquid extraction, which allows the majority of analyte to be transferred from the sample to the organic phase, even if only a portion of 1/100–1/1000 of the extracted analyte actually reaches the detector. SPME is generally used in a direct extraction mode or in a headspace configuration. Headspace-SPME thus affords selectivity significantly different from that of plain headspace analysis. This type of sampling requires neither solvent extraction (with the ensuing purification stages) nor complex PAT apparatus (Richter and Schellenberg 2007; Turner 2006). On the basis of the experimental data, the vapor-liquid partition coefficient of the volatiles under test can be calculated. Once trapped, solutes are extracted from the matrix, separately injected into a GC column and revealed by means of traditional flame

ionisation detection (FID) or specific MS detectors (Bucking and Steinhart 2002; Akiyama *et al.* 2009).

One of the basic requirements of GC is that substances under test must be as volatile as needed to be eluted and detected at the operating temperature, while the stationary phase must be sufficiently non-volatile and thermally stable in order to serve as the substrate on which separation occurs. The molecular mass operating range of GC spans the interval 2–1500 atomic mass units (amu), so that the compounds that can be separated by GC go from permanent gases (i.e. highly volatile substances) to volatile compounds (with a mass up to ~200 amu) and semi-volatile compounds (>200 amu). Column stability also implies reliability over a long time period, with the ensuing improved long-term reproducibility of determinations, this eventually leading to reliable analytical characterisation (Taylor and Lindforth 2010).

The stationary phase in the capillary column shows specific characteristics. It comprises an array of polymers (e.g., dimethylpolysiloxane and polyethylenglycol). Also, so-called chiral columns are available, by which cis- and trans-isomers of chemicals can be separated. In the latter case, the stationary phase generally comprises cyclodextrin derivatives.

In the carrier gas (e.g., He or N_2), very low amounts of the vaporised substance under test can be revealed and subsequently quantified by means of various detection techniques (CEN EN 13725:2003). The detection is performed by flame ionisation detection (FID) or MS. An FID device (detection power in the order of 10^{-11} g of material) measures the ionic currents generated by an H_2 microflame as a result of the combustion of the various components separated by the chromatographic column. Conversely, in the case of an MS system, not only does the spectrometer have a high absolute detection power (around 10^{-12} g of substance), but it also provides information on the fragmentation pattern for each eluted component, thus allowing the molecular weight of the compound to be ascertained along with its structure and functional groups. MS detection can be carried out in two different ways, namely, full scan mode (FSM) and the selected ion monitoring (SIM) (Bicchi *et al.* 2004; Turner 2006). A fairly reproducible MS fragmentation pattern (fingerprint) can be obtained by FSM. SIM is a highly powerful technique for trace analysis. With this approach, scanning of the whole spectrum is unnecessary and only a few ions are detected during the GC separation.

GC can be coupled not only to a fit-for-purpose detector (e.g. MS detector), but also to a suitable automated sample device (e.g., based on SPME, PAT, or headspace) (Marriott *et al.* 2009). Combination of GC with other techniques is indeed a versatile tool and the range of possible applications is wide. Precision is thus improved and throughput is high, this being of particular value in routine analysis in industry.

3 Materials and Methods

3.1 Time course experiments

Chemicals and standards

HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from VWR (Vienna, Austria). LC-MS grade formic acid (FA) and Tween 20 were obtained from Sigma-Aldrich (Vienna, Austria), whilst ammonium formate solution (5 M, NH_4HCO_2) was provided by Agilent Technologies (Waldbronn, Germany). All solvents were of LC gradient grade or higher. Purified water (H_2O) was produced by reverse osmosis and an ELGA Purelab Ultra Mk2 Analytic system from Veolia (Vienna, Austria).

Time course experiments were performed with a test solution of ZEN with 1000 mg L^{-1} in MeOH/H_2O (for wheat) or ACN/H_2O (for barley) 1+1 (v/v)+1% Tween 20. For each experiment, test solutions solely containing the corresponding solvent mixtures (mock) were prepared to obtain blank samples. Analytical standard of ZEN toxin for quantification experiments was purchased from Romer Labs (Tulln, Austria) at concentrations of 100 mg L^{-1} (purity>99.9%) in ACN. The analytical standard of ZEN-Glucosides were enzymatically produced from ZEN (ZEN-14-Glc and ZEN-16-Glc) or chemically synthesised (αZEL-14-Glc and βZEL-14-Glc from ZEN-14-Glc, αZEL-16-Glc and βZEL-16-Glc from ZEN-16-Glc) within the scope of the study. A highly pure standard of ZEN was used as raw materials, and the final product was characterised by nuclear magnetic resonance (NMR) measurements and the purities were estimated to be ≥95%.

Cultivation of plants

For the time course experiment, barley (*Hordeum vulgare* L. sensu lato) variety “Calcule” was selected. This is a two-row spring barley bred by Saatzucht Streng-Engelen GmbH & Co. KG (Germany). Seeds of the barley variety were germinated.

The time-course experiment was also conducted on the spring wheat (*Triticum aestivum* L.) variety “Remus” (“Sappo”/“Mex”/“Famos”), susceptible to Fusarium head blight (FHB) and DON, and the FHB-resistant variety “CM-82036-1TP-10Y-OST-10Y-OMOFC” (abbreviated to “CM-82036”) which originated from the cross of the varieties “Sumai-3” and “Thornbird” that possess awns (Lemmens *et al.* 2005). Seeds of Remus and CM-82036 were first germinated. No vernalization was required, but the wheat seedlings were routinely submitted to a cold treatment at 5 °C for 1 week to improve tillering.

Pots (diameter 23 cm) were filled with 7-L portions of a homemade substrate (mix of 500 L heat-sterilised compost, 250 L peat, 10 kg sand and 250 g rock flour). In each pot, five seedlings were planted. The experimental design was a completely randomised block with three biological replications (treatment of three individual barley/wheat ears per treatment group) for both plant experiments.

During the whole experiment, the pots were watered if required (typically three times per week). Water was applied until the substrate was completely wet and the water started to seep out through the holes in the bottom of the pot. The soil substrate contained sufficient minerals to support seedling growth. At the end of tillering, 2 g per pot of a mineral fertilizer (COMPO Blaukorn ENTEC, Münster, Germany; N/P/K/Mg: 14/7/17/2) was applied. To prevent mildew, the cabinet was treated twice during plant cultivation with sulfur overnight (sulfur evaporator, Nivola, Lisse, The Netherlands).

Plants for qualitative screening were grown in the greenhouse and after tillering transferred to a growth chamber with computer-controlled settings for light, temperature and relative air humidity. Light intensity was 560 µmol s^{-1} m^{-2} at 1 m above the soil. Relative air humidity was set between 60 and 70% during plant growth. Temperature (day/night) and duration of illumination (hours) varied according to the development stage of the plants: after planting until the end of tillering, 12 °C/10 °C/ 12 h; end tillering until mid-stem extension when the ear starts to swell, 14 °C/10 °C/14 h; mid-stem extension to start heading, 16 °C/14 °C/14 h; from the start of heading until start of flowering, 18 °C/14 °C/14 h; and from the start of flowering until the end of the experiments including application of the test solutions and sampling, 20 °C/18 °C/16 h.

The time course experiment was carried out exactly as described above with the following modifications: after tillering the plants remained in the greenhouse with computer-controlled settings for light, temperature and relative air humidity. Light intensity was 370 µmol s^{-1} m^{-2} at 1 m above the soil (measured after sunset).

Treatment and sampling of plants

Upon reaching the correct developmental stage, flowering ears were selected and individually labeled. Only one ear per plant was used in the experiment to prevent possible systemic effects of an earlier treated ear on other later flowering ears of the same plant. A woolen thread was used to label the lowest treated spikelet in the lower part of the ear (approx. 2/3 from the ear tip). In general, spikelets were treated by injecting 10 µL of the respective test solution into each of the outer floret (hence, 20 µL per spikelet) after placing the pipet tip in the spikelet using an electronic pipet Multipette Xstream (Eppendorf, Hamburg, Germany). After each treatment, the ear was covered for 24 h with a small

transparent plastic bag internally wetted by spraying ca. 2 mL of bidistilled water with a handhold sprayer. This assured a high relative humidity promoting diffusion of the mycotoxins into the plant cells.

Experiments included two treatment groups, ZEN and mock (methanol:water (50:50, v/v) and 1% TWEEN), which were applied separately on different ears in triplicate. 10 pairs of neighbouring spikelets (20 spikelets in total, two per row) were treated with 10 µL each in one treatment resulting in a single dose of 200 µg per ear. Wheat ears were collected at eight time points: 0 h, 6 h, 12 h, 1 day, 2 days, 3 days, 1 week, and at full-ripening stage (approximately 6 weeks after treatment). Instead barley ears were sampled immediately (time point 0), 1, 3 and 7 days after treatment and at full-ripening stage. On the day of harvest, treated ears were removed from the plants with a surgical scissor, weighed as a whole, and immediately frozen with liquid nitrogen to prevent any metabolic activity until analysis. All collected samples were stored at −80 °C until further processing.

Sample preparation

Frozen ears were ground into a fine powder using a ball mill (MM 301 Retsch, Haan, Germany). The samples were placed inside 10 mL stainless steel vessels, precooled with liquid nitrogen, and were milled for 30 s at 30 Hz under cooled conditions (liquid nitrogen). Milled samples were weighed (100±2 mg) into 1.5 mL Eppendorf tubes. After homogenization, samples were extracted by adding 500 µL ACN/H_2O/FA 79+20.9+0.1 (v/v/v), vortexing for 10 s and shaking on a rotary shaker in horizontal position (GFL 3017, Burgwedel, Germany) at room temperature for 90 min at 200 rpm (round per minute). After centrifugation for 10 min at 14000 rpm, using an Awel centrifuge MF 48-R (Blein, France), supernatants (ca. 200 µL) were transferred to HPLC vials containing microinserts.

For time course experiments, LC-Q-TOF-MS/MS spectra were recorded with undiluted raw extracts of samples. The samples were partly further diluted 1:5, 1:50 and 1:200 (v/v) in acetonitrile:water (79:21, v/v) to allow quantification of target analytes.

LC-HRMS(/MS) analysis: Structure annotation and time course experiments

Qualitative and quantitative measurements were performed with a 1290 Infinity ultra high performance liquid chromatography (UHPLC) system coupled to a 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer (both Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) interface. In the interface the transition from the liquid phase coming

from the LC-system to the gaseous phase, as well as the ionisation of the analytes takes place before they enter the MS.

Chromatographic separation was achieved on a reversed-phase Zorbax SB-C_{18} Rapid Resolution HD column (150×2.1 mm, 1.8 μm particle size; Agilent Technologies), kept at 30 °C. Mobile phases consisted of H_2O (eluent A) and MeOH (eluent B), both containing 0.1% FA (v/v) and 5 mM NH_4F, to promote the formation of ammonium adducts for improved fragmentation. For the acquisition of LC-HR-MS/MS spectra, gradient method was as follows: 0-0.5 min, 50% B; 0.5-7.5 min, 50-100% B; 7.5-10.0 min, 100% B; 10.0-10.1 min, 100-50% B; and 10.1-13.0 min, 50% B to re-equilibrate the column with the initial chromatographic conditions. During the first 1.5 min and after the 10 min point mark, the eluent flow was directed to the waste. The flow rate of the mobile phase was 0.25 mL/min, and the injection volume was set to 1 μL. To avoid carry over, the needle was flushed with acetonitrile:water (50:50, v/v) for 5 s immediately prior to injection. LC-Q-TOF full scan mass spectra and LC-MS/MS spectra were acquired with 2 GHz in negative ESI mode within m/z 60-1500 at a scan rate of 3 spectra s^{-1}. For all measurements, the source conditions were set as follows: capillary voltage, 4000 V; nozzle voltage, 500 V; fragmentor voltage, 380 V; drying gas temperature and flow, 130 °C and 16 L min^{-1}, respectively; nebulizer, 30 psig; and sheath gas temperature and flow, 300 °C and 11 L min^{-1}, respectively. Mass accuracy of Q-TOF instrument was checked and potentially optimized before analysis. Continually infused reference masses (negative m/z 112.9856, m/z 966.0007) were used for internal mass calibration during the measurement. Data were acquired with MassHunter Acquisition software B.05.01, and data evaluation was performed with MassHunter Qualitative and Quantitative Analysis B.06.00 (all Agilent Technologies), as well as Excel 2010 (Microsoft Co., Redmond, WA).

The mass spectrometric data are for examples displayed in the total ion chromatogram (TIC - see Figure 5a). The sum of the signal intensities of all scanned masses or mass transitions are plotted at each time-point. In the extracted ion chromatogram (XIC or EIC depending on the vendor, see Figure 5b) each selected mass or mass transition is displayed separately. Precursor and product ion specific parameters have to be optimised to achieve the best signal intensity prior to its use in SRM applications. The formed fragment ions depend on the structure of the molecule. Some chemical groups, like hydroxyl- or acetyl-groups are very easily cleaved off and quite low collision energies are necessary. For the generation of other fragment ions far higher collision energies might be necessary when the chemical bonds within the molecule are strong. The selection of the fragment ion should be made carefully, as non-specific mass transitions, like the loss of water, should be avoided if possible.

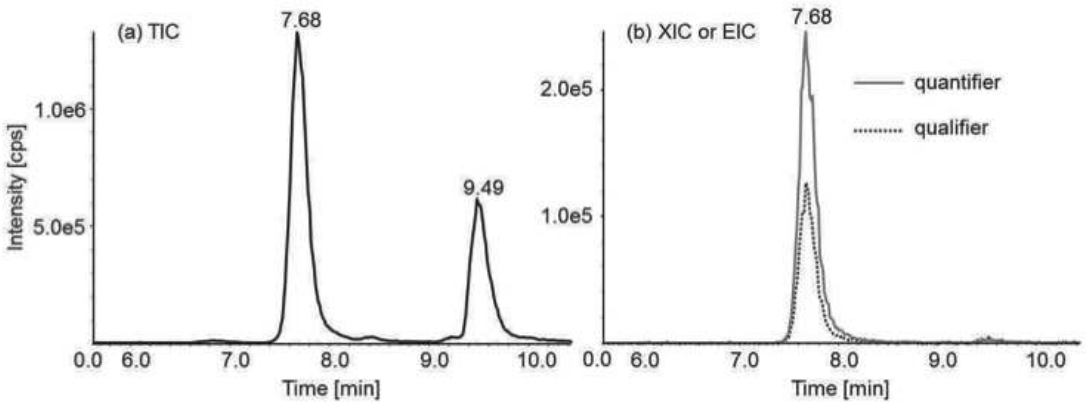


Figure 5: (a) Total ion chromatogram (TIC) and (b) extracted ion chromatogram (XIC or EIC)
Please notice the different scale of the y-axis in (a) and (b). In (a) all ions or mass transitions measured at the specific time-point are summed up and a total ion chromatogram is gained. In (b) only selected ions or mass transitions are displayed, each one separately. In general, per analyte two mass transitions are recorded in selected-reaction-monitoring mode – one is used as quantifier, the other one as qualifier.
[cps – counts per second]

3.2 Volatile Organic Compounds Experiments

Treatment and sampling of plants

Saragolla, a cultivar of durum wheat (*Triticum durum* Desf.) susceptible to attack by *Fusarium* fungi, was purposely contaminated under controlled conditions in order to obtained early infection indicators, before the actual occurrence of mycotoxin detectable levels. Grains were isolated, to verify the absence of any previous contamination, and planted in pots by using sterile soil. The cultivation of plants was exactly the same that was made for the time course experiments (see section 3.1).

The suspensions of the two selected fungi, *F. graminearum* and *F. culmorum*, were also prepared and used to inoculate, by spraying, the soil, seeds and plants, in different phenological phases: tillering, booting and bloom. Fungal cultures on liquid medium were incubated at 25 °C under constant agitation (200 rpm) for 10 days. After the incubation period, macroconidia were harvested by filtration through two layers of thin cheesecloth. The spore pellet was suspended in sterile distilled water to a final concentration of 3000 spores/mL, and the final liquid cultures were stored in a refrigerator. To preserve and keep alive the fungi, they were grown at 23 °C on plates containing PDA (Potato/Dextrose/Agar) as the culture medium, and the plates were periodically refreshed. Plants were grown in a climate chamber, while monitoring parameters such as temperature, humidity, ventilation and lighting.

Healthy and artificially infected plants by *F. graminearum* and *F. culmorum* were monitored until grain ripening. Samples were removed from the plants with a surgical scissor. All collected samples were immediately analysed and then stored at −20 °C to prevent any metabolic activity until further processing.

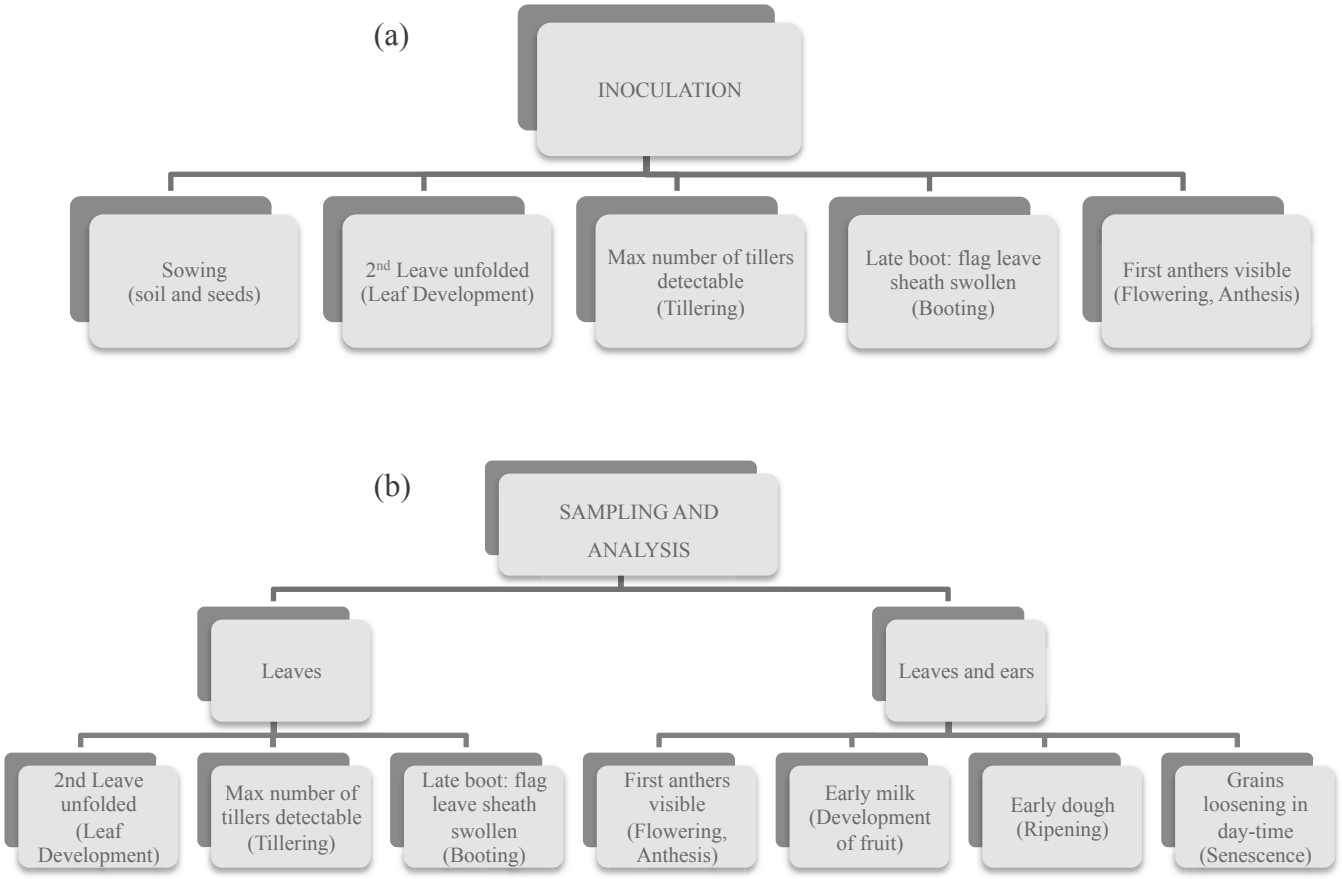


Figure 6: rappresentation of the different phenological phases of inoculation (a) and sampling/analysis (b).

HS-SPME/GC/MS analysis

Analytical procedure for analysis of wheat samples was based on Headspace-Solid-Phase Microextraction (HS-SPME) and Gas-Chromatography coupled to mass spectrometry (GC/MS). Three SPME fibres polydimethylsiloxane 7 μm (PDMS), polydimethylsiloxane 100 μm (PDMS) and polyacrylate 85 μm (PA) were tested. Fibers were purchased from Supelco and thermally conditioned in accordance with the manufacturer's recommendations before first use.

Definitely, the SPME fiber coating used in this study was made of polydimethylsiloxane

(PDMS), 100 μm thickness and 23 gauge. Volatile organic compounds (VOCs) were sampled directly from the head space of wheat leaves and ears. Samples and controls were transferred to a 20 mL glass headspace sample vial and were kept at 40 ºC for 10 min before exposing the fiber. Then, a headspace sampling/extraction time of 30 min was adopted.

A Gerstel MPS autosampler (Gerstel, Baltimore, MD, USA) mounted to an Agilent 6890N gas chromatograph (Little Falls, DE, USA) paired with an Agilent 5975 mass selective detector (MSD) was the analytical system. SPME injections were in splitless mode using a SPME injection sleeve (0.75 mm I.D) at 250 °C for 660 s. During this time, the thermal desorption of analytes from the fiber occurred in a HP-INNOWax column (60 m×0.25 mm I.D., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The oven temperature was initially held at 40 °C for 1.0 min, then it was programmed to 230 °C at 6 °C min^{-1} with a final holding time of 15 min before returning to the initial temperature (40 °C). Helium carrier gas was used with a total flow of 1.2 mL min. The total cycle time was 47.67 min. The MS detector was set in the electron impact mode at 70 eV and operated in scan mode (mass range 46-350); the transfer line to the MS system was maintained at 240 °C. The identify of peaks was assigned using the NIST 11 Library. The samples were analysed in triplicate and blank runs were made with empty glass vial before and after each analysis. Different parts of plant, culms, leaves, spikes and crops, were analysed, depending on the phenological phase in which the sampling was made in order to understand how the spread of the fungus occurred.

In addition to VOCs analysis, the degree of the fungal contamination was evaluated by fungal re-isolation on plate, of the inoculated part of plants, and real-time polymerase chain reaction (RT-PCR) analysis. These confirmed the presence of pathogens in all the inoculated samples. Tables 3 and 4 show the level of contamination (expressed in pg of DNA of fungi/mg of sample) of some samples from plants inoculated in different growth stages by *F. culmorum* and *F. graminearum*, respectively and sampled/analysed in successive growth stages. As it is possible to note the level of DNA is low. This evidence cannot rule out the occurrence of a contamination with a consequent production of toxins. In fact, as discussed in chapter 5 an important presence of the toxin levels was observed also in samples with a very low DNA content.

INOCULUM BY *F. culmorum*	SAMPLING	PART OF PLANT	pg DNA *Fc*/mg
SOIL	tillering	leaf	41.13
SOIL	booting	leaf	0.04
SOIL	booting	ear	4.81
SOIL	early milk stage	leaf	0.03
SOIL	early milk stage	grain kernel	15.82
SOIL	early dough stage	ear	341.91
SOIL	early dough stage	grain kernel	267.81
SOIL	senescence	grain kernel	327.12
SEEDS	senescence	ear	91.68
SEEDS	senescence	grain kernel	48.54
TILLERING	tillering	leaf	0.12
TILLERING	tillering	leaf	0.41
TILLERING	early milk stage	leaf	0.01
TILLERING	early milk stage	ear	167.76
TILLERING	early dough stage	ear	189.47
TILLERING	senescence	leaf	0.03
TILLERING	senescence	grain kernel	12.29
BOOTING	booting	ear	22.38
BOOTING	early milk stage	grain kernel	35.21
BOOTING	senescence	grain kernel	20.57
BLOOM	early milk stage	ear	2.81
BLOOM	senescence	grain kernel	1.83

Table 3: RT-PCR of different kind of samples (leaf, ear or grain kernel) from plants inoculated in different growth stages by *F. culmorum* and sampled/analysed in the successive growth stages. The level of contamination is expressed in pg of DNA of *F. culmorum* (*Fc*)/mg of sample.

INOCULUM BY *F. graminearum*	SAMPLING	PART OF PLANT	pg DNA *Fg*/mg
SOIL	early dough stage	ear	0.48
SOIL	senescence	ear	0.42
SOIL	senescence	grain kernel	3.86
SEEDS	tillering	leaf	0.01
SEEDS	early dough stage	grain kernel	0.14
SEEDS	senescence	ear	82.06
TILLERING	early milk stage	ear	0.01
TILLERING	senescence	grain kernel	0.02
BOOTING	senescence	grain kernel	3.09
BOOTING	senescence	grain kernel	0.03
BLOOM	early milk stage	grain kernel	0.03
BLOOM	early dough stage	ear	0.13
BLOOM	early dough stage	grain kernel	0.01
BLOOM	senescence	ear	5.18
BLOOM	senescence	grain kernel	0.03
BLOOM	senescence	grain kernel	3.91

Table 4: RT-PCR of different kind of samples (leaf, ear or grain kernel) from plants inoculated in different growth stages by *F. graminearum* and sampled/analysed in the successive growth stages. The level of contamination is expressed in pg of DNA of *F. graminearum* (*Fg*)/mg of sample.

4 Results and Discussion

4.1 Zearalenone Metabolites

Data evaluation is currently based on the accurate mass and the retention time, which both matched with the available standards (all the structures are shown in Figure 12). All the considered compounds were chromatographically separated (Figure 7) and quantified.

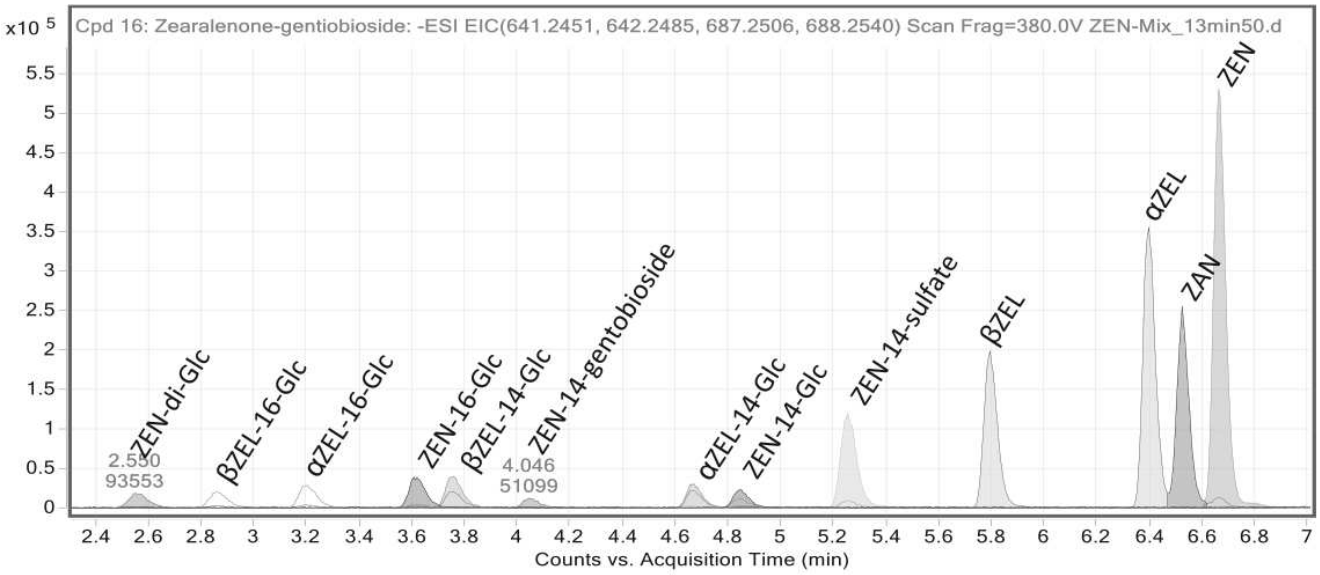


Figure 7: HR-HPLC-MS chromatogram of a ZEN-metabolites standard mix.

LC-HRMS(/MS) analysis: Validation of the Method for Quantification Purposes.

For those analytes for which analytical standards were available (ZEN, αZEL, βZEL, ZEN-14-Glc, ZEN-16-Glc, αZEL-14-Glc, αZEL-16-Glc, βZEL-14-Glc, βZEL-16-Glc, ZEN-14,16-di-Glc, ZEN-14-gentiobioside, ZEN-14-sulfate), an in-house validation was performed. Mock samples of the variety Remus were weighed (100 ± 2 mg) using the biological triplicates (1 day and full ripening) and were spiked with a solution containing all target analytes in acetonitrile before extraction on one level (1500 µg/kg, corresponding to 300 µg/L in the "undiluted" sample extract). The samples were stored overnight at room temperature to ensure solvent evaporation and to achieve equilibrium between the analytes and the matrix. The next day, the samples were extracted according to the procedure described above. Calculating ratios of the EIC (target m/z ± 30 ppm) peak area achieved in these samples with those in a neat solvent standard (300 µg/L, mean value from triplicate analysis) multiplied with 100 resulted in the apparent recovery (R_A). In the fully ripened samples, lower recoveries (56-124%) and

higher relative standard deviations (up to 31%) were observed than in the samples harvested 1 day after treatment (74-132% and up to 3.5%, respectively).

For the evaluation of matrix effects (expressed as signal suppression or enhancement, SSE), mock samples of the same type (again in biological replicates) were extracted, and the raw extracts of the undiluted samples and 1:10 and 1:50 (v/v) diluted samples were spiked at a concentration of 300 µg/L. A comparison of EIC abundance values with the neat solvent standard (300 µg/L) provided the SSE values of the respective dilution. For undiluted samples, SSE values between 88 and 144% (RSD≤5%, n=3) were observed due to matrix components which affect the ionisation process of the coeluting metabolites of interest. The extraction recovery was calculated by dividing R_A with the SSE of the undiluted sample and multiplying with 100. For the calculation of the corresponding relative standard deviation, the propagation of uncertainty was taken into account.

For the quantification of ZEN and its metabolites, neat solvent standards ranging from 3 to 300 µg/L for ZEN monoglucosides, from 3 to 1000 µg/L for the aglucons and 1 mg/kg for the other compounds were prepared in acetonitrile-water (50:50, v/v). They were measured along with the different dilutions of the sample extracts, and calibration was performed using 1/x weighted calibration curves. External calibration was applied with concentrations at six levels in the range of 3-1000 µg L^{-1}, and linear calibration curves were 1/x weighted. In the middle of the range the curves result linear and in all the case R^2>0.99.

Biological replicates and different dilutions (mentioned above) of samples were analysed. Metabolite levels in respective matrices corresponding to a signal-to-noise (S/N) ratio of 10 served as limit of quantification (LOQ). Concentration values were multiplied by ear weight to obtain results in microgram/ear or subsequently in micromole/ear, respectively. Based on the estimated method precision, matrix effects were only corrected if below 85% and above 115%. For other biotransformation products, relative quantification was carried out by integrating peaks of EICs above a S/N of 3 in matrix (limit of detection, LOD, that is below than required legal limit) and performing normalisation by ear weight. Thus, time courses of normalised metabolite peak areas were graphically displayed. Each time point value was presented as mean value ± standard deviation (n = 3).

LC-HRMS(/MS) analysis: Time courses and mass balances of ZEN and its metabolites

Time courses of quantified ZEN and its metabolites are shown in Figures 8 and 9. Where standards were available, the absolute amounts of ZEN and its metabolites were plotted versus harvest

time point after the treatment. The majority of ZEN, was not metabolised by the plants.

It was observed that the recovery of added ZEN at time point 0 (harvest and quenching immediately after treatment) deviated significantly from the expected 100%. We presume that the time period allowed for the toxin to diffuse into the plant cells was too short, and thus, sample handling for harvest and quenching resulted in a loss of toxin solution (possible wash off of toxin by liquid nitrogen and the contact with gloves and scissors also contributed to losses). Standard deviations for time point 0 (n = 3) were considerably higher than those of any other time point which supports this assumption. Therefore, the theoretically added toxin amount of 200 µg ZEN at time point 0 was used as starting point to calculate percent yield of the respective derivatives formed at later time points.

Figure 9 shows that ZEN-Glucosides (especially ZEN-16-Glc) have been found to be the main metabolites of ZEN toxin which reached their maximum already 1 day after toxin treatment.

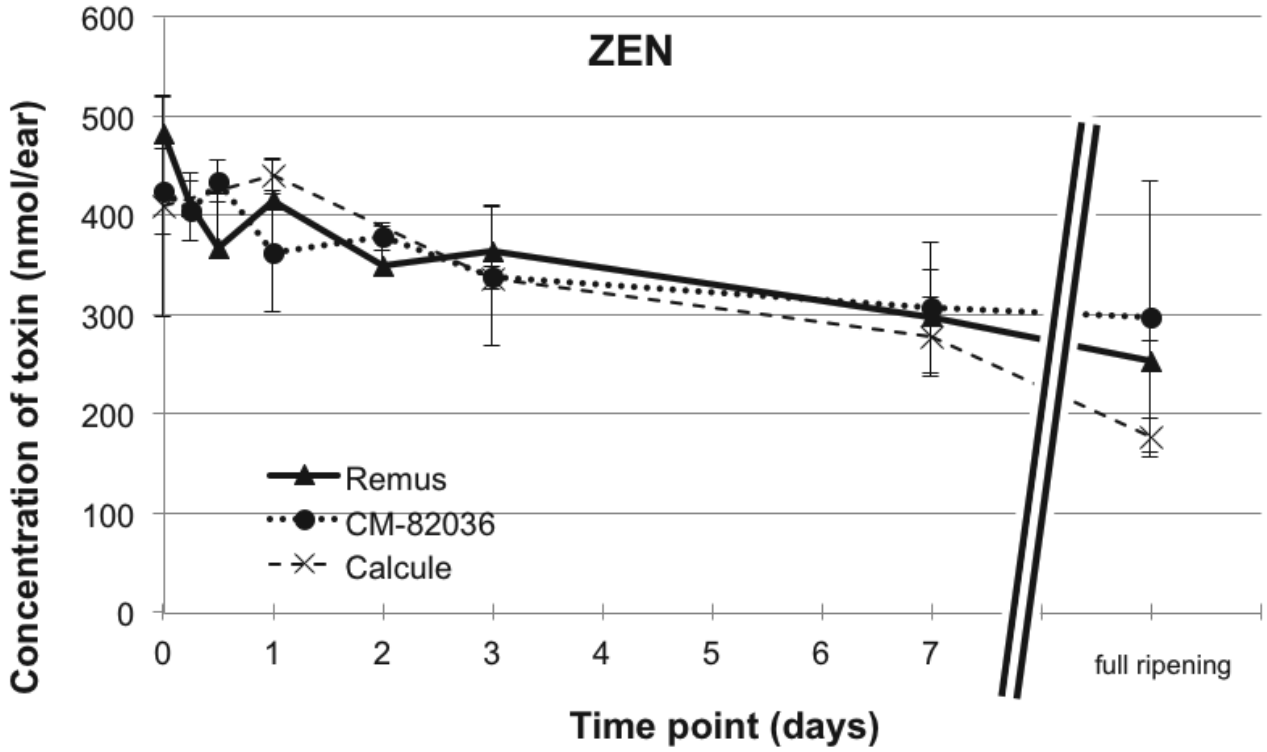
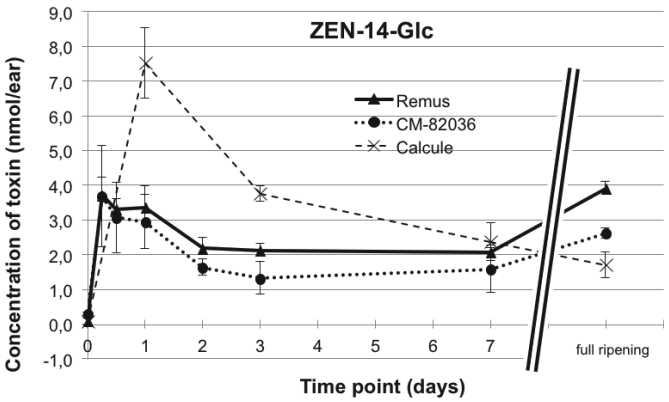
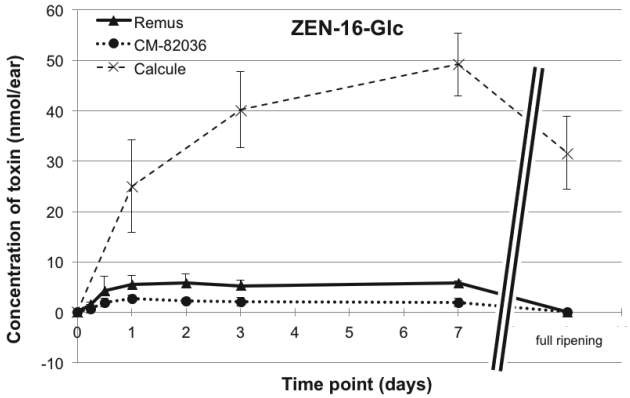
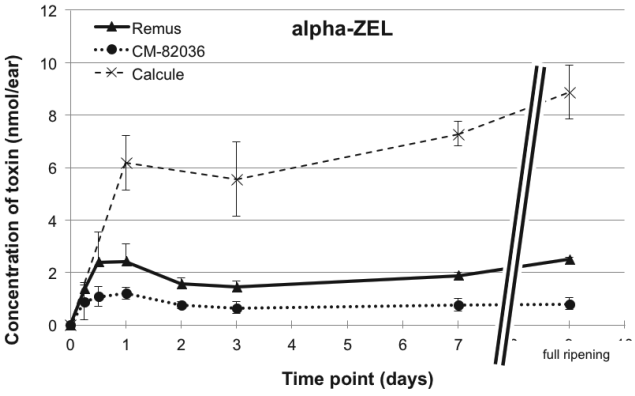


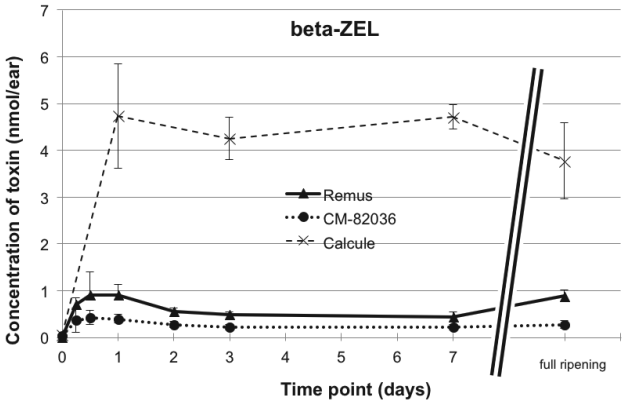
Figure 8: Time courses of quantified ZEN in Remus (continuous line), CM-82036 (dotted line) and Calcule (broken line). Ears were treated with 200 µg toxin and the whole ears (n = 3) were harvested as follows: wheat - immediately after treatment (0 h), after 6 h, 12 h, 1 day, 2 days, 3 days, and 1 week, as well as at full ripening (ca. 8 weeks after treatment); barley - 1, 3 and 7 days after treatment and at full-ripening stage. For each ear, absolute analyte concentrations were measured and related to the amount of theoretically added toxin and plotted versus harvest time point after treatment. Analysis was performed with a 6550 iFunnel Q-TOF LC/MS system. Error bars refer to the standard deviation of biological triplicates.

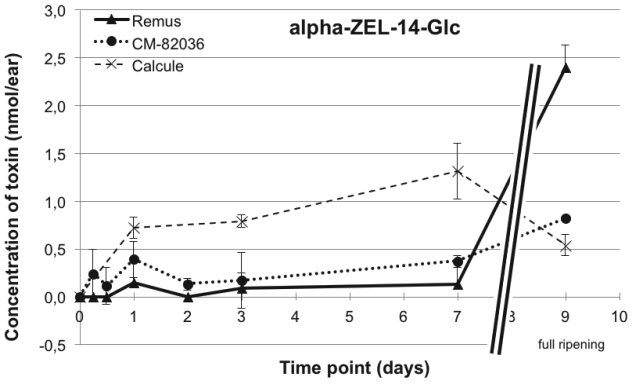
In figure 9 can be also observed that most of the ZEN-metabolites in both wheat and barley plants reached their maximum already 1 day after toxin treatment (however within the first 7 days after the treatment).

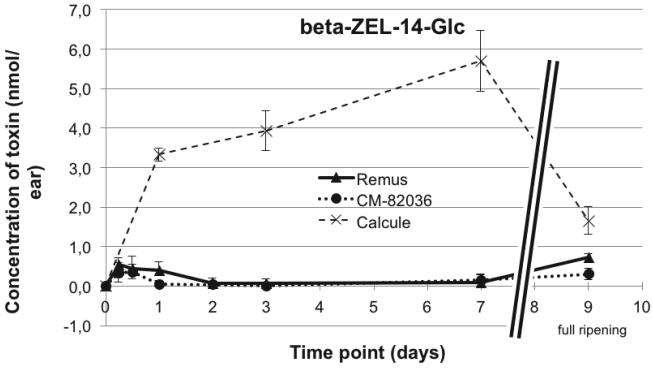












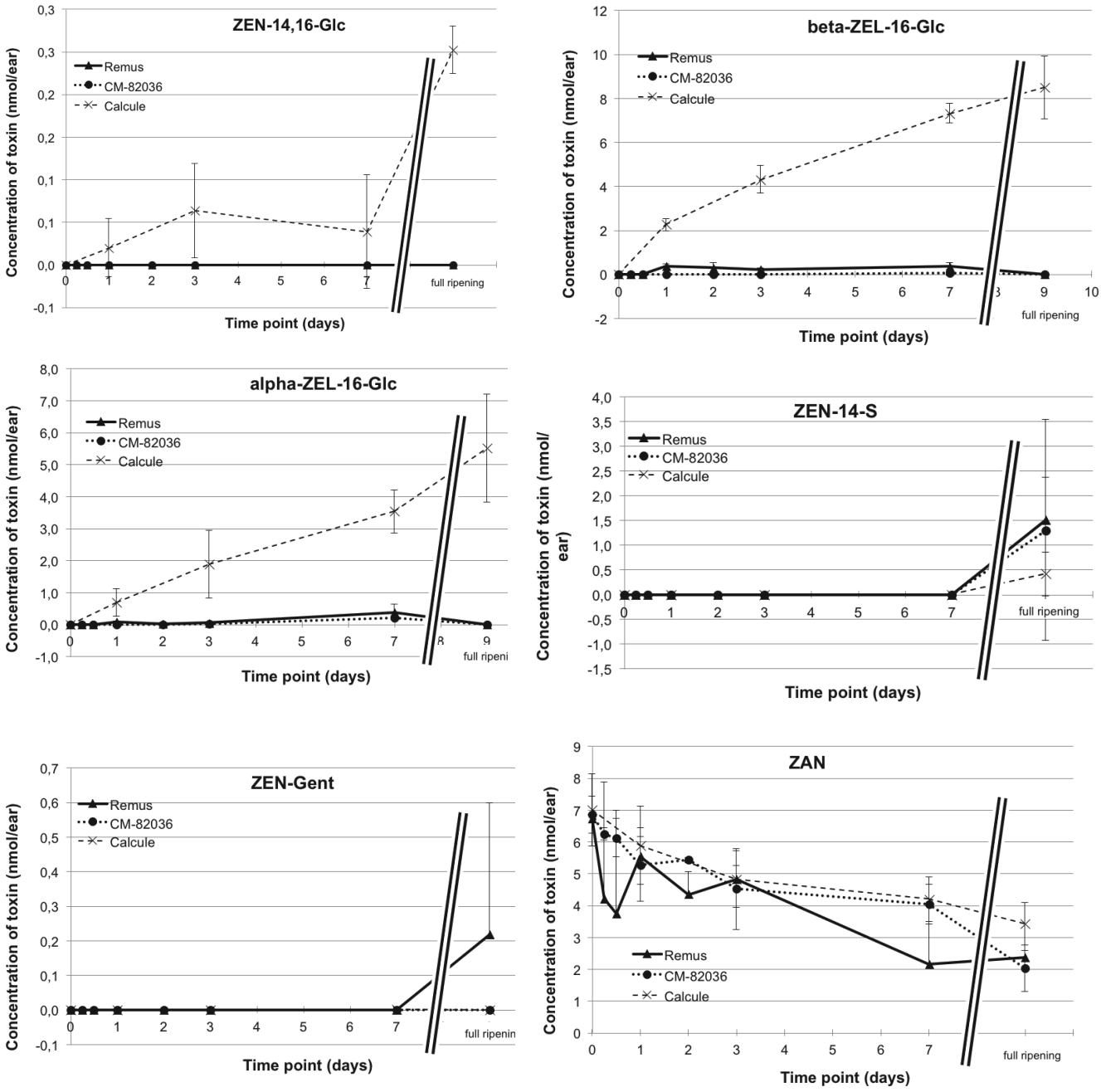


Figure 9: Time courses of quantified ZEN-metabolites in Remus (continuous line), CM-82036 (dotted line) and Calcule (broken line). Ears were treated with 200 µg toxin and the whole ears (n = 3) were harvested as follows: wheat - immediately after treatment (0 h), after 6 h, 12 h, 1 day, 2 days, 3 days, and 1 week, as well as at full ripening (ca. 8 weeks after treatment); barley - 1, 3 and 7 days after treatment and at full-ripening stage. For each ear, absolute analyte concentrations were measured and related to the amount of theoretically added toxin and plotted versus harvest time point after treatment. Analysis was performed with a 6550 iFunnel Q-TOF LC/MS system. Error bars refer to the standard deviation of biological triplicates. Please notice the different scale of the y-axis in the graphs.

The major metabolisation products, were ZEN-14-Glc, ZEN-16-Glc, αZEL and βZEL, most of the other compounds occurred only in traces. For all the metabolites the best results were obtained for barley that forming 3-20 times more ZEN-16-Glc than ZEN-14-Glc (Figure 10).

In total, approximately the 26% of ZEN was metabolised as αZEL, βZEL or glucosides, from barley; less significant amounts were recovered from the two varieties of wheat: ca. 3% from Remus and ca. 12% from CM-82036.

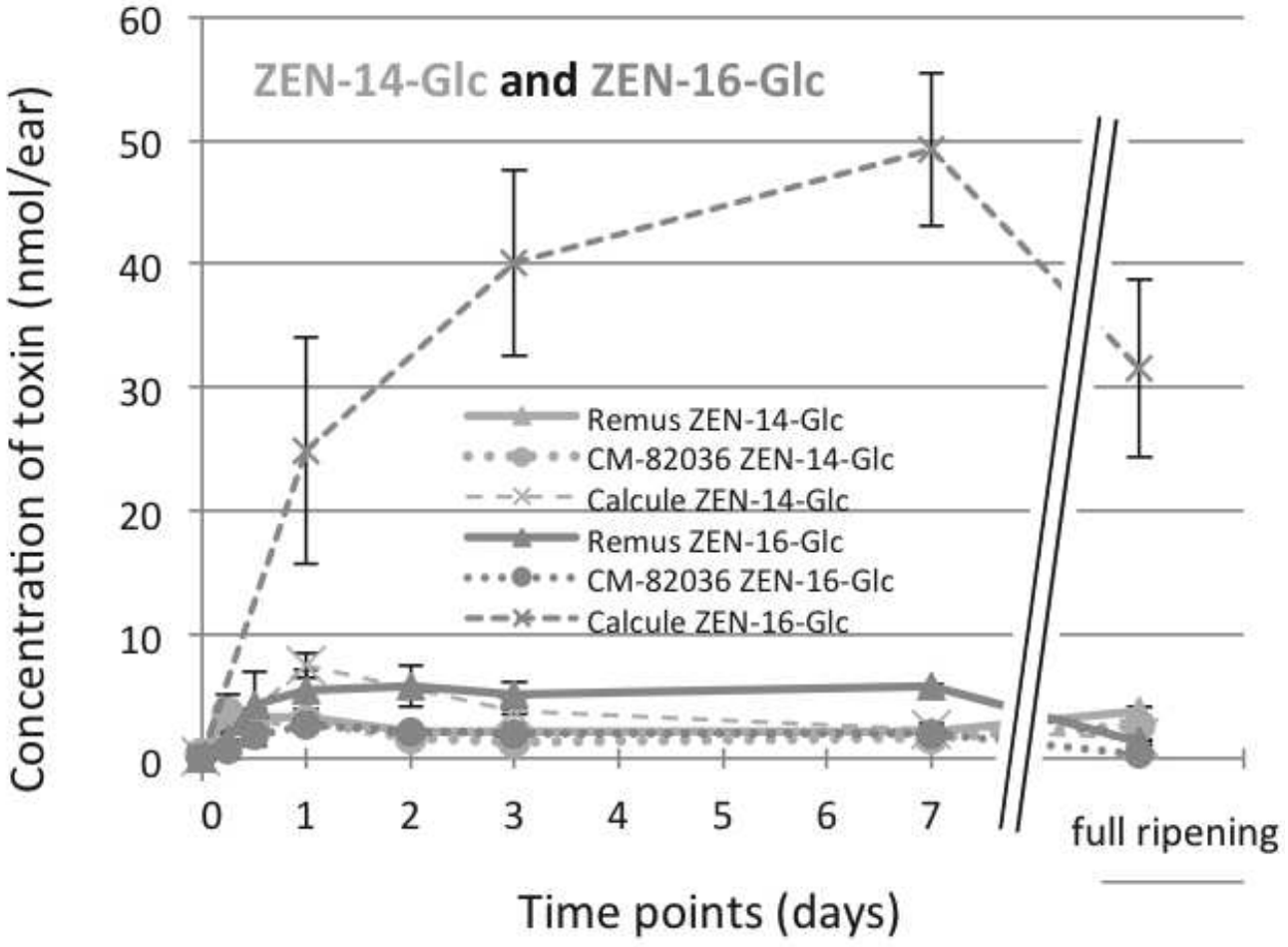


Figure 10: Results from the time course kinetics experiment. After exposure to a single dose of 200 µg/ear ZEN toxin, the whole ears (n = 3) were harvested immediately after treatment (0 h), after 6 h, 12 h, 1 day, 2 days, 3 days, and 1 week, as well as at full ripening (ca. 8 weeks after treatment). For the two major ZEN-metabolites ZEN-14-Glc and ZEN-16-Glc, the absolute amounts (in nanomoles per ear) are provided together with the standard deviation of the biological triplicate. These values are based on quantitative measurements using the respective analytical standards; the individual ear weights were taken into consideration.

Detoxification of ZEN

It was observed that plants modify ZEN by using phase I as well as phase II metabolism processes. The variety of the identified ZEN metabolites, in total 17 different substances, is summarised in Figure 11. The proposed pathway shows metabolic activation (phase I) and conjugation (phase II) processes. The later occurrence of malonylglucosides and disaccharides suggests that they derive from the respective monoglucosides, which is also in agreement with the metabolism of other xenobiotics in plants (Cole and Edwards 2000). Therefore, it is apparent that as part of the detoxification process, the ZEN-treated plants try to inactivate these xenobiotics by transforming them into more polar compounds. As part of their metabolism, plants are capable of transforming xenobiotics into a huge variety of conjugated forms.

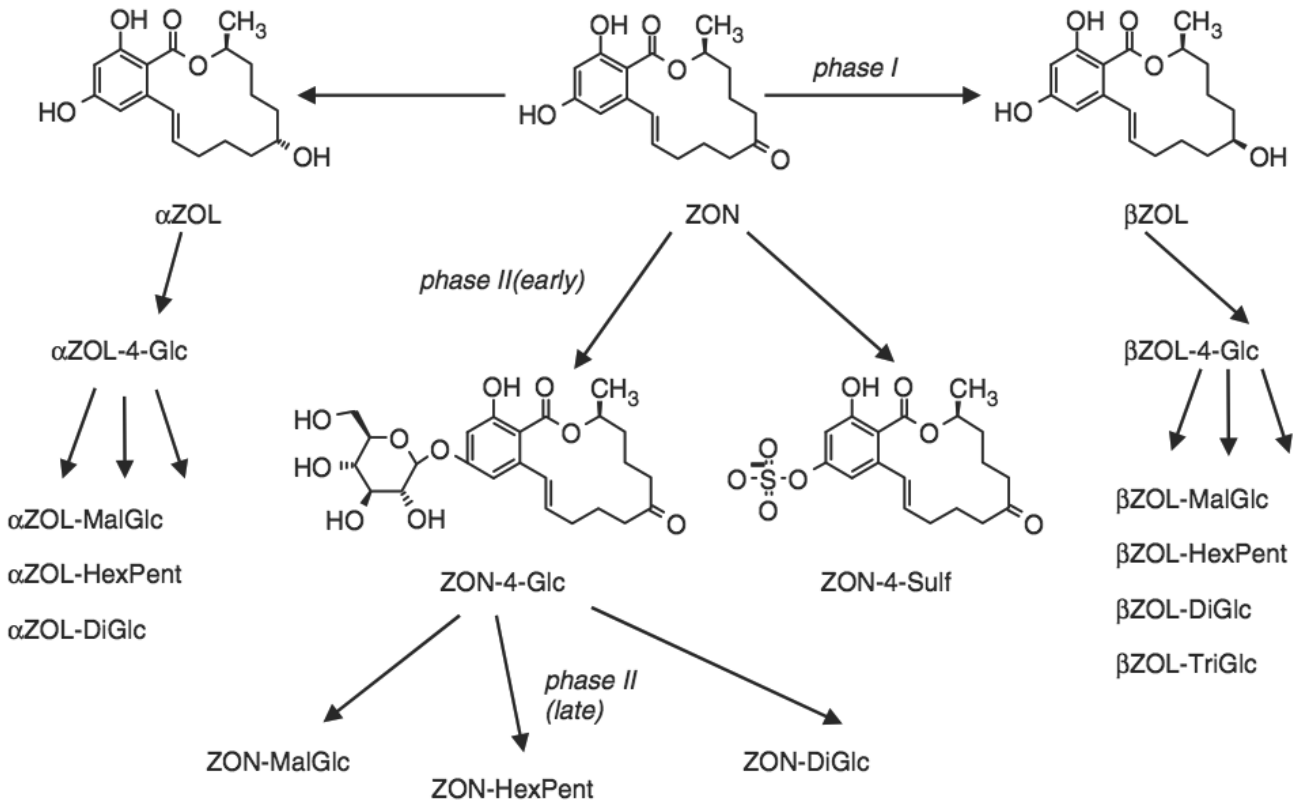
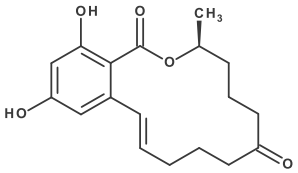


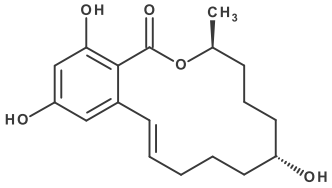
Figure 11: Proposed biotransformation pathway of zearalenone, covering both phase I and II metabolism (Berthiller *et al.* 2006).
The abbreviations for these compounds have changed during the last years. Now we use ZEN for zearalenone, instead of ZEA or ZON. Such as ZEL for zearalenol, instead of ZOL (the proposed ones by Metzler *et al.* 2011 on Mycotoxin Research).

Zearalenone
(ZEN)



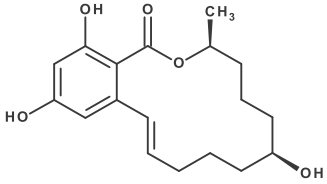
Formula Weight : 318.36(1)
Formula : $C_{18}H_{22}O_5$

alpha-zearalenol
(α-ZEL)



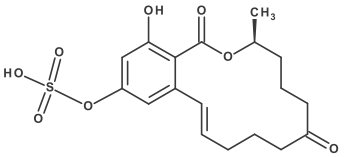
Formula Weight : 320.38(1)
Formula : $C_{18}H_{24}O_5$

beta-zearalenol
(β-ZEL)



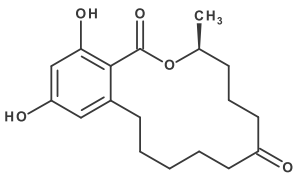
Formula Weight : 320.38(1)
Formula : $C_{18}H_{24}O_5$

zearalenone-14-sulfate
(ZEN-14-S)



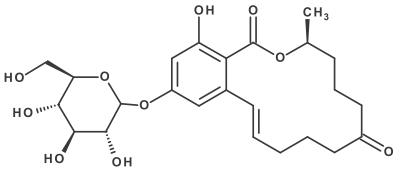
Formula Weight : 398.43(2)
Formula : $C_{18}H_{22}O_8S$

Zearal**a**none
(ZAN)



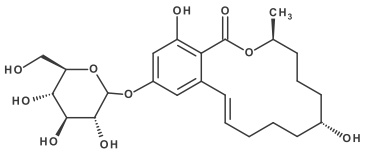
Formula Weight : 320.38(1)
Formula : $C_{18}H_{24}O_5$

zearalenone-14-glucoside
(ZEN-14-Glc)



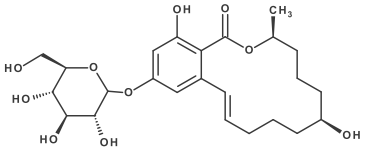
Formula Weight : 480.50(2)
Formula : $C_{24}H_{32}O_{10}$

alpha-zearalenol-14-glucoside
(αZEL-14-Glc)



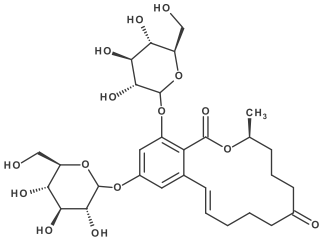
Formula Weight : 482.52(2)
Formula : $C_{24}H_{34}O_{10}$

beta-zearalenol-14-glucoside
(βZEL-14-Glc)



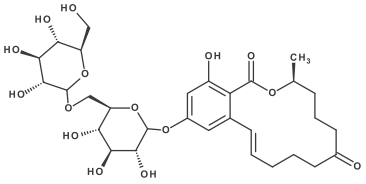
Formula Weight : 482.52(2)
Formula : $C_{24}H_{34}O_{10}$

zearalenone-14,16-di-glucoside
(ZEN-14,16-Glc)



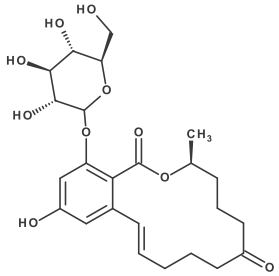
Formula Weight : 642.64(2)
Formula : $C_{30}H_{42}O_{15}$

zearalenone-14-gentiobioside
(ZEN-14-Gent)



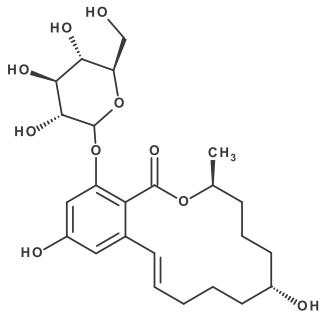
Formula Weight : 642.64(2)
Formula : $C_{30}H_{42}O_{15}$

zearalenone-16-glucoside
(ZEN-16-Glc)



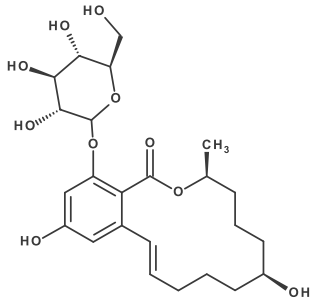
Formula Weight : 480.50(2)
Formula : $C_{24}H_{32}O_{10}$

alpha-zearalenol-16-glucoside
(αZEL-16-Glc)



Formula Weight : 482.52(2)
Formula : $C_{24}H_{34}O_{10}$

beta-zearalenol-16-glucoside
(βZEL-16-Glc)



Formula Weight : 482.52(2)
Formula : $C_{24}H_{34}O_{10}$

Figure 12: Structures of ZEN metabolites, of which standards were available.

4.2 Volatile Organic Compounds

A total of 48 volatile organic compounds (VOCs) were isolated and identified by SPME-GC-MS from wheat leaves and ears: 13 alcohols (3-methyl-1-Butanol; 1-penten-3-ol; 2-penten-1-ol; 1-hexanol; 3-hexenen-1-ol; 3-hexen-1-ol; 2-hexenen-1-ol; 2-hexen-1-ol; 1-octen-3-ol; 2,6-dimethyl-cyclohexanol; 2,4-dimethyl-cyclohexanol; benzyl alcohol; eugenol), 9 aldehydes (hexanal; 2-hexenal; 2-pentenal; 2-heptenal; 2-octenal; 2,4-heptadienal; 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde; heptadecanal; tetradecanal), 4 esters (acetic acid, 2-ethylhexyl ester; hexadecanoic acid, ethyl ester; 3-oxo-2-pentyl-cyclopentaneacetic acid, methyl ester; triethyl phosphate), 4 furans (2-ethyl-furan; 2-pentyl-furan; cis-2-(2-Pentenyl)furan; 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone), 5 hydrocarbons (tridecane; 3-methyl-tridecane; 3-methyl-tetradecane; 1-pentylheptyl-benzene; methylmethylene-cyclopropane), 10 ketones (3-octanone; 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-3-buten-2-one; 2-pentadecanone; 1-(3-cyclohexen-1-yl)-ethanone; 2,2,6-trimetyl-cyclohexanone; 2,3-octanedione; 6-methyl-5-hepten-2-one; 3,5-octadien-2-one; 2-undecanone; 2,4,4-trimethyl-cyclopentanone), and 3 terpenes (7-oxabicyclo[4.1.0]heptane; 4-methyl-1,4-hexadiene; 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde). Very important differences were found during the different growth stages of wheat. In particular during tillering a significant number of volatiles were formed; the most of VOCs were detected during the early milk stage, but also during the early dough stage and the senescence. Among substances produced by wheat plants/fungi interactions aliphatic alcohols, aldehydes and ketones dominate. These compounds represent both primary and secondary metabolites and are biosynthesised on different pathways. Terpenoids are derived from mevalonate that is a precursor found in the fungi metabolism. Fatty acids and keto acids are utilised in the production of alcohols and esters.

Alcohols and Ketones

Alcohols and ketones have been detected as metabolites of filamentous fungi (Kamiński *et al.* 1974). When strains of Fusaria spp. cultivated on wheat were investigated main volatile compounds comprised 1-octen-3-ol, 1-penten-3-ol, 2-penten-1-ol, 1-hexanol, 3-hexen-1-ol, 2-hexen-1-ol. Tressl *et al.* (1982) observed that linoleic acid is the natural precursor of l-octene-3-ol. The same authors have proposed linolenic acid as a precursor to 8-carbon components containing two double bonds (i.e., 2,3-octanedione or 6-methyl-5-hepten-2-one). Enzymes catalysing the formation of l-octen-3-ol include lipooxygenase, hydroxyperoxide cleavage enzyme, and alcohol oxidoreductase which reduces 1-octen-3-one to l-octen-3-ol (Tressl *et al.* 1980). Wurzenberger *et al.* (1982; 1984) suggested that linoleic acid

is oxidised by a lipooxygenase enzyme in the presence of atmospheric oxygen. As a result, 10-hydroperoxide is formed. A lyase catalysed cleavage of 10-hydroxyperoxide yields the eight carbon fragments where l-octen-3-ol is the predominant compound. The oxygen present in the hydroxyl group of l-octene-3-ol originates from atmospheric oxygen. Chen *et al.* (1984) have shown that a reductase system reduce l-octen-3-one to l-octen-3-ol. Apart from eight carbon alcohols, those possessing shorter chains are common volatile metabolites of fungi. The most often detected alcohol (Larsen *et al.* 1995) was l-butanol-3-methyl. The presence of ketones in fungal cultures has been noted for the most filamentous fungi. The synthesis of methyl ketones has been reviewed by Forney *et al.* (1971) and Kinsella *et al.* (1976). The ability of fungi to convert fatty acids into methyl ketones is common for many genera in the fungal kingdom. Medium-chain fatty acids are converted into methyl ketones, which are one carbon shorter, by fungal spores and mycelium. It follows the β-oxidation pathway for fatty acid oxidation up to the production of β-ketoacyl CoA, which then may be deacylated and decarboxylated to form the methyl ketone or to undergo further β-oxidation. When oxygen is limited the methyl ketones may act as terminal hydrogen acceptors to produce secondary alcohols.

Aldehydes

Polyunsaturated fatty acids, such as linoleic and linolenic, are substrates for aldehydes having 6 to 12 carbon atoms formation, and the enzymes involved in this biosynthesis are lipooxygenase, hydroperoxide lyase and hydroperoxide dehydrase (Jeleń & Wasowicz 1998). Strains of *Fusarium* spp. inoculated on wheat plants are able to produce several aldehydes compounds comprising hexanal, 2-hexenal, 2-pentenal, 2-heptenal, 2-octenal, 2,4-heptadienal, heptadecanal and tetradecanal.

Esters

Numerous esters (e.g. triethyl phosphate; acetic acid, 2-ethylhexyl ester; hexadecanoic acid, ethyl ester) have been detected in cultures of *Fusarium*. As early as 1923, it had been suggested (Omelianski 1923) that microorganisms can produce ethyl esters in the reaction of organic acids with ethanol. It has been proposed that production of esters is associated with microorganism lipolytic ability in yielding free fatty acids (Pereira *et al.* 1958). Released free fatty acids may then react with alcohols. Examining acetate formation, it was shown that the acid moiety of the ester may arise in three possible pathways: by activation of monocarboxylic acids, by oxidative decarboxylation of 2-oxo acids, or by intermediates of long-chain monocarboxylic acid synthesis (Nosträm *et al.* 1964).

Furans

Furanones are known to exhibit antifungal activity and there are a number of papers describing synthesis, structure-activity relationship study and modifications of various antifungal furanones. In our work the furans that we found were 2-ethyl-furan; 2-pentyl-furan; cis-2-(2-Pentenyl)furan; and 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone, supposedly produced by inoculated plant, like antifungal. Many naphthoquinone derivatives have been previously obtained from fungi in the genus *Fusarium* isolated from various sources (Boonyapranai *et al.* 2008).

Terpenoids

Volatile terpenes presented in fungal cultures belong to a group of mono- and sesquiterpenoids. In most species 4-methyl-1,4-Hexadiene, 7-Oxabicyclo[4.1.0]heptane, and 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde were detected. The presence of monoterpenes in *Fusarium* spp. (Jeleń 1995) has been documented. Biosynthesis of terpenoid compounds has been a subject of several monographies (Bernfeld 1963; Bucher 1959). Biogenesis of terpenoids has been historically associated with the "isoprenoid rule". Biochemical reactions that lead from acetate to isoprenoid substances can be divided into a few stages; the formation of mevalonate from acetate, formation of isopentenyl pyrophosphate, and finally, the polymerisation of isopentenyl pyrophosphate (Richards *et al.* 1964). The first step of terpenes biosynthesis from acetate is the formation of 4-carbon unit - acetoacetyl-CoA from two molecules of acetyl coenzyme A. Acetoacetyl coenzyme A can, as a result of carbonyl group reduction, yield β-hydroxybutryl coenzyme A. Alternatively, an acquisition of another acetyl coenzyme A, a fragment at the carbonyl group will lead to β-hydroxy-β-methylglutaryl coenzyme A and a free coenzyme A. β-hydroxy-β-methylglutaryl coenzyme A can also be produced from leucine. The important development in the biosynthesis of terpenoids was the discovery of 3,5-dihydroxy-3-methyl pentanoic acid (mevalonic acid) (Skeggs *et al.* 1956). Mevalonic acid (as mevalonate) is related to β-hydroxy-β-methyl glutaryl coenzyme A by reduction of one of the carboxyl groups to a primary alcohol.

Hydrocarbons

In fungal cultures, benzene derivatives are the frequently occurring hydrocarbons. A large group of methylated benzene derivatives have been detected in *Fusarium* species: 3-methyl-tridecane, 3-methyl-tetradecane, methylmethylene-cyclopropane. Diacetyl is mainly bacterial metabolite, but can also be produced by yeasts and filamentous fungi. For the benzaldehyde a biosynthetic pathway from

L-phenyloalanine (with the loss of amino group leading to trans-cinnamic acid), which subsequently yields benzoic acid which can be further reduced was proposed (Kawabe and Morita 1994). Also tridecane and 1-penthylheptil-benzene can be produced by *Fusarium* species.

4.2.1 Wheat Infections by *Fusarium graminearum*

In the table 5 a total of 20 compounds, the main VOCs found in leaves samples from plants inoculated by *F. graminearum*, were shown: 8 alcohols (3-methyl-1-butanol, 1-penten-3-ol, 2-penten-1-ol, 1-hexanol, 3-hexen-1-ol, 2-hexen-1-ol, 1-octen-3-ol, and 2,6-dimethyl-cyclohexanol), 4 aldehydes (hexanal, 2-hexenal, 2,4-heptadienal, and tetradecanal), 1 esters (triethyl phosphate), 2 furans (2-ethyl-furan, and 2-pentyl-furan), 3 ketones (2-pentadecanone, 2,2,6-trimetyl-cyclohexanone, and 2,3-octanedione), and 2 terpenes (7-oxabicyclo[4.1.0]heptane, and 4-methyl-1,4-hexadiene).

The reported compounds were found only in samples analysed during tillering and early milk stage. In particular, the leaves sampled in tillering were characterised mostly by alcohols and aldehydes. Instead, the VOCs detected in the leaves sampled during the early milk stage were primarily aldehydes.

In the table 6 a total of 22 compounds, the main VOCs found in ears sampled from wheat plants inoculated by *F. graminearum*, were shown: 7 alcohols (1-penten-3-ol, 2-penten-1-ol, 1-hexanol, 3-hexen-1-ol, 2-hexen-1-ol, 1-octen-3-ol, and 2,6-dimethyl-cyclohexanol), 4 aldehydes (hexanal, 2-hexenal, 2,4-heptadienal, and tetradecanal), 3 esters (2-ethylhexyl ester acetic acid, ethyl ester hexadecanoic acid, and methyl ester 3-oxo-2-pentyl-cyclopentaneacetic acid), 3 furans (2-ethyl-furan, 2-pentyl-furan, and 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone), 4 ketones (3-octanone, 2,3-octanedione, 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-3-buten-2-one, and 2-pentadecanone), and 1 terpenes (4-methyl-1,4-hexadiene).

The reported compounds were found in ears sampled and analysed during the tree different bloom stages (early milk, early dough and senescence). All these samples were characterised mostly by alcohols and aldehydes, but also by furans and ketones.

Compounds		Mean peaks area						
		TILLERING		EARLY MILK STAGE				
	Rt (min)	SOIL	SEEDS	SOIL	SEEDS	TILL.	BOOT.	BLOOM
Alcohols								
3-methyl-1-butanol	11.73	n.d.	n.d.	64183	n.d.	99908	73594	38011
1-penten-3-ol	10.6	441832	1107229	477753	118696	378482	533787	457173
2-penten-1-ol	14.44	50258	781389	168746	n.d.	224459	15371	167127
1-hexanol	15.05	n.d.	n.d.	n.d.	n.d.	588469	759992	n.d.
3-hexen-1-ol	15.81	987334	n.d.	105071	13203	81715	274227	86455
2-hexen-1-ol	16.27	n.d.	328011	431300	n.d.	590246	1992448	137050
1-octen-3-ol	17.20	1109227	576605	n.d.	n.d.	469407	180997	71100
2,6-dimethyl-cyclohexanol	20.73	n.d.	n.d.	66393	n.d.	2023025	3088978	n.d.
Total		2588651	2793234	1313445	131899	4455708	6919392	956916
Percentage (%)		42	76	6	12	24	17	19
Aldehydes								
hexanal	8.95	766348	n.d.	1512135	n.d.	1076171	3119016	259126
2-hexenal	12.17	1427731	635165	16474715	866168	5305518	18391083	3000904
2,4-heptadienal	18.43	197100	94857	175715	19068	1159725	1400961	384239
tetradecanal	28.39	1113428	n.d.	n.d.	n.d.	1259513	1038208	n.d.
Total		3504607	730022	18162565	885235	8800927	23949267	3644269
Percentage (%)		57	20	79	81	47	58	71
Esters								
triethyl phosphate	21.56	n.d.	n.d.	n.d.	n.d.	1344551	1754706	513684
Percentage (%)						7	4	10
Furans								
2-ethyl-furan	6.47	n.d.	n.d.	2734778	n.d.	2371067	6903608	n.d.
2-pentyl-furan	12.32	78542	109395	n.d.	60265	214283	213065	n.d.
Total		78542	109395	2734778	60265	2585350	7116673	
Percentage (%)		1	3	12	6	14	17	
Ketones								
2-pentadecanone	28.23	n.d.	n.d.	n.d.	n.d.	509119	n.d.	n.d.
2,2,6-trimetyl-cyclohexanone	14.52	n.d.	35829	n.d.	6923	13057	14276	n.d.
2,3-octanedione	14.48	n.d.	n.d.	n.d.	n.d.	177159	154356	n.d.
Total			35829		6923	699334	168632	
Percentage (%)			1		1	4	0	
Terpenes								
7-oxabicyclo[4.1.0]heptane	11.73	n.d.	n.d.	293499	n.d.	475619	474828	412678
4-methyl-1,4-hexadiene	16.45	n.d.	n.d.	404250	6010	280159	765102	99985
Total				697749	6010	755778	1239929	512663
Percentage (%)				3	1	4	3	10

Table 5: Main VOCs found in leaves samples from wheat plants inoculated by *F. graminearum*.

Compounds		Mean peaks area														
		EARLY MILK STAGE					EARLY DOUGH STAGE					SENESCENCE				
	Rt (min)	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM
Alcohols																
1-penten-3-ol	10.6	353914	517929	456510	472151	465435	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-penten-1-ol	14.44	200446	295270	182999	315608	208196	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-hexanol	15.05	654111	288763	331020	402604	415774	591205	549783	474088	628320	1360688	n.d.	n.d.	n.d.	n.d.	n.d.
3-hexen-1-ol	15.81	103087	87719	97640	108045	111430	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-hexen-1-ol	16.27	907757	644605	531616	526050	438578	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-octen-3-ol	17.20	394565	578178	570318	745612	540635	484794	580438	400883	442618	424699	346939	286164	139100	139332	1463713
2,6-dimethyl-cyclohexanol	20.73	398664	184353	1829558	2264647	2007037	3730312	2032283	2659135	3067242	2963784	3548258	3303985	2126777	1792634	1960527
Total		3012542	2596815	3999660	4834716	4187083	4806311	3162503	3534106	4138179	4749170	3895197	3590149	2265877	1931965	3424240
Percentage (%)		30	20	29	26	30	39	35	26	28	28	50	52	31	24	38
Aldehydes																
hexanal	8.95	446114	762150	675242	1018965	945641	535980	391184	972878	1407531	350143	672188	443348	1639423	1977457	1648786
2-hexenal	12.17	1464569	4892101	1313564	2788574	1465537	n.d.	n.d.	n.d.	n.d.	n.d.	157053	122365	n.d.	n.d.	205718
2,4-heptadienal	18.43	315051	315843	450988	575605	351445	885976	406181	1316289	1902156	1848625	1121040	849859	1396899	990688	1242682
tetradecanal	28.39	744292	783218	1102229	1484926	1159519	638930	570339	381601	384654	458178	n.d.	n.d.	n.d.	n.d.	n.d.
Total		2970025	6753311	3542022	5868069	3922142	2060886	1367703	2670767	3694341	2656946	1950280	1415571	3036322	2968145	3097185
Percentage (%)		29	52	26	32	28	17	15	20	25	16	25	21	42	37	35
Esters																
acetic acid, 2-ethylhexyl ester	15.76	93814	122262	92027	144910	136219	n.d.	n.d.	164452	95475	138995	n.d.	n.d.	n.d.	n.d.	n.d.
hexadecanoic acid, ethyl ester	31.89	240827	377223	461378	310551	210535	584487	504690	580127	558379	577317	n.d.	n.d.	n.d.	n.d.	n.d.
cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	32.55	212742	691319	355317	394791	330740	570240	665545	125564	304095	654931	380940	389765	456540	338748	525929
Total		547383	1190803	908721	850252	677493	1154727	1170235	870142	957949	1371242	380940	389765	456540	338748	525929
Percentage (%)		5	9	7	5	5	9	13	6	7	8	5	6	6	4	6
Furans																
2-ethyl-furan	6.47	1634654	739438	1351992	1893723	627587	n.d.	n.d.	514953	477043	661999	239724	388659	555028	714093	435167
2-pentyl-furan	12.32	280867	230534	415743	248493	235098	111649	94164	429102	617425	1025998	108093	66665	227128	149249	335629
5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	33.87	580276	416146	1137561	878516	545840	2610344	1932600	2762426	2352865	2062793	n.d.	n.d.	n.d.	n.d.	n.d.
Total		2495796	1386118	2905296	3020731	1408524	2721993	2026764	3706480	3447333	3750789	347817	455324	782156	863342	770796
Percentage (%)		25	11	21	17	10	22	22	28	23	22	5	7	11	11	9
Ketones																
3-octanone	12.9	70555	137140	75340	204105	170973	121426	120762	15912	29913	184523	n.d.	n.d.	n.d.	n.d.	n.d.
2,3-octanedione	14.48	193233	297933	197354	274402	152504	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)-3-buten-2-one	28	538754	133852	988810	1140375	446475	1278659	860910	1765435	1915204	2119989	780389	796493	595550	1293830	461262
2-pentadecanone	28.23	185080	272910	889359	1942184	3100934	301242	322142	911012	522704	2085539	370487	204557	141358	714290	697107
Total		987621	841834	2150862	3561065	3870885	1701326	1303814	2692359	2467820	4390051	1150876	1001049	736908	2008120	1158369
Percentage (%)		10	7	16	19	27	14	14	20	17	26	15	15	10	25	13
Terpenes																
4-methyl-1,4-hexadiene	16.45	90267	175196	102059	167804	94452	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Percentage (%)		1	1	1	1	1										

Table 6: Main VOCs found in ears samples from wheat plants inoculated by *F. graminearum*.

Multivariate analysis

In order to explore the variations of headspace components in different samples, from wheat plants inoculated during the different growth stages, the data were statistically analysed by principal component analysis (PCA) with a SCAN software from Minitab Inc. (State College, PA, USA).

PCA biplot of leaves samples, from wheat plants inoculated by *F. graminearum*, are showed in Figure 13 where the first and third principal components explain 33.6% and 10.4% of the variability, respectively. This analysis well evidences important differences among samples, which result grouped into four clusters: control samples (no inoculation) and those depending from the stage of sampling of inoculated plants. Samples collected during the early milk stage are grouped in the second and third

quadrants, where loadings of alcohols, aldehydes, esters, ketones and terpenes are present. The first quadrant contain the samples collected during the early dough and characterised especially by alcohols, hydrocarbons and ketones. Samples from the senescence stage are grouped in the fourth quadrant and they are characterised mostly by hydrocarbons. As expected, all controls (not inoculated samples) are located in the central zone of the plane, because the contribution of VOCs, is much lower than the other samples.

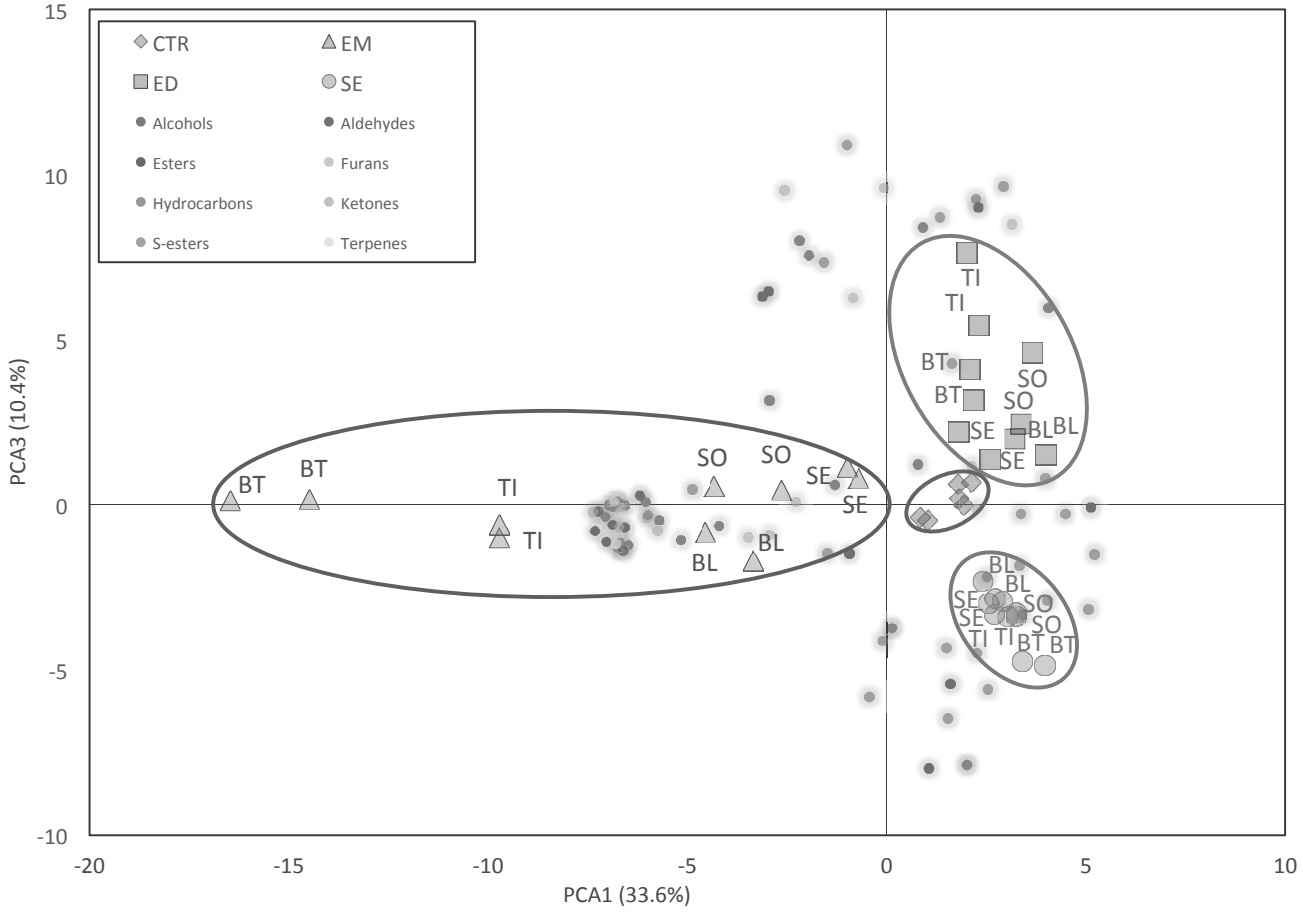


Figure 13: Principal component analysis (PCA) of the volatile profile of wheat leaves inoculated or not (CTR) by *Fusarium graminearum*. The inoculation with the fungus suspension were made by spraying the soil (SO), seeds (SE) or plants in different phenological phases: tillering (TI), booting (BT) and bloom (BL). Sampling and HS-SPME-GC-MS analyses were made at three different growth stages: early milk (EM), early dough (ED) and senescence (SE). First and third principal components have been selected.

PCA biplot of ears samples, from wheat plants inoculated by *F. graminearum*, are showed in Figure 14 where the first and second principal components explained 30.1% and 24.5% of the variability, respectively. Also in this case the analysis well evidences important differences among samples, which result grouped into four clusters depending from the inoculation or the stage of sample

collection. Samples collected during the early milk stage are grouped in the second quadrant where loadings of alcohols, aldehydes, ketones and terpenes are present. The first quadrant contain the samples from the early dough characterised especially by alcohols, aldehydes, ketones esters and thioesters. Samples collected in the senescence stage are grouped in the fourth quadrant and they are characterised especially by esters and hydrocarbons. All controls (not inoculated samples) are located in the third quadrant, and also in this case, as expected, the contribution of VOCs, is much lower than the other samples.

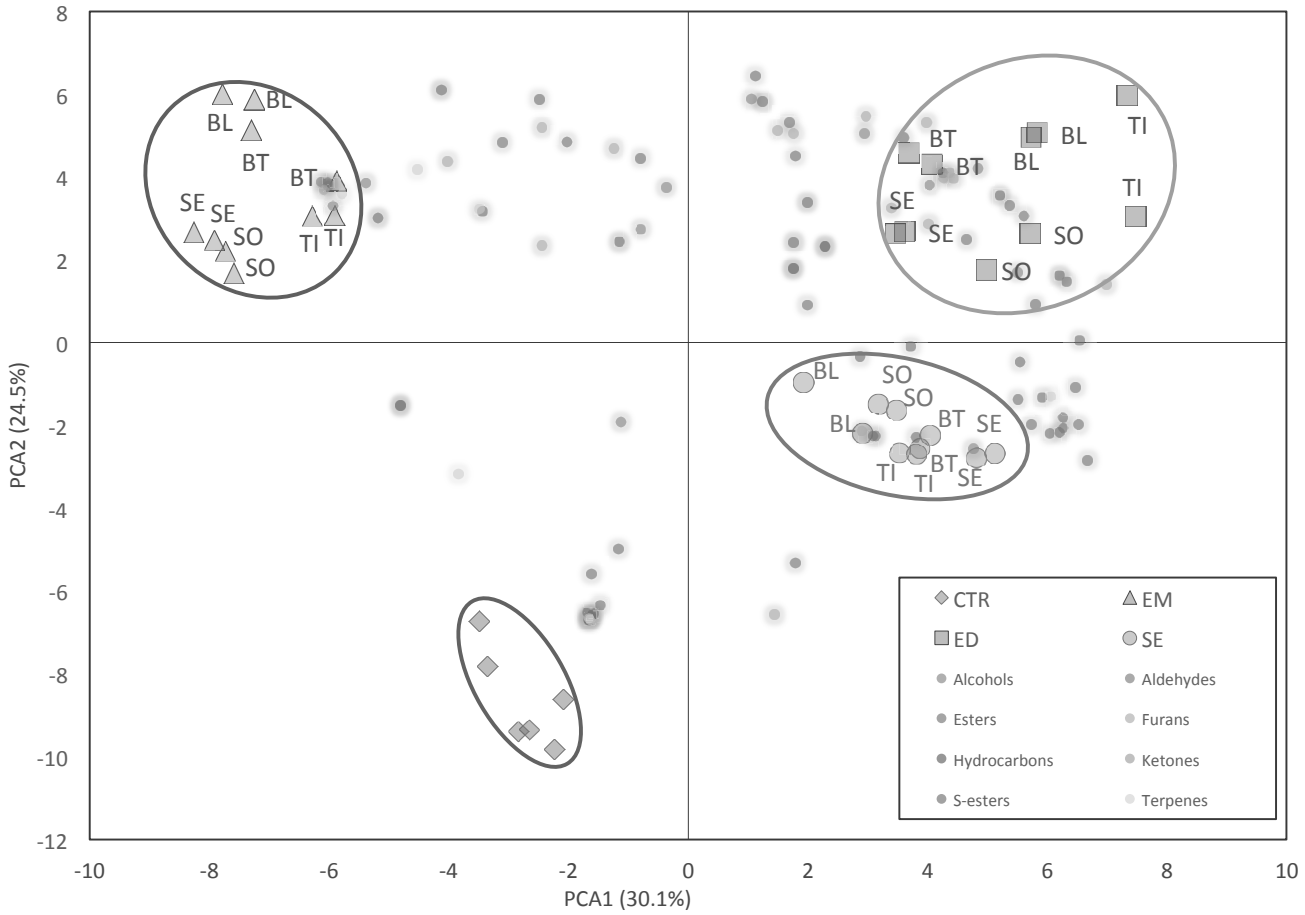


Figure 14: Principal component analysis (PCA) of the volatile profile of wheat ears inoculated or not (CTR) by *Fusarium graminearum*. The inoculation with the fungus suspension were made by spraying the soil (SO), seeds (SE) or plants in different phenological phases: tillering (TI), booting (BT) and bloom (BL). Sampling and HS-SPME-GC-MS analyses were made at three different growth stages: early milk (EM), early dough (ED) and senescence (SE). First and second principal components have been selected.

4.2.2 Wheat Infections by *Fusarium culmorum*

In the table 7 a total of 27 compounds, the main VOCs found in leaves samples from plants inoculated by *F. culmorum*, were shown: 10 alcohols (1-penten-3-ol, 2-penten-1-ol, 1-hexanol, 3-hexen-1-ol, 2-hexen-1-ol, 1-octen-3-ol, 2,6-dimethyl-cyclohexanol, 2,4-dimethyl-cyclohexanol, benzyl alcohol, and eugenol), 6 aldehydes (2-hexenal, 2-heptenal, 2,4-heptadienal, 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, heptadecanal, and tetradecanal), 2 hydrocarbons (tridecane, and methylmethylene-cyclopropane), 4 furans (2-ethyl-furan, 2-pentyl-furan, cis-2-(2-Pentenyl)furan, and 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone), 3 ketones (1-(3-cyclohexen-1-yl)-ethanone, 2,2,6-trimetyl-cyclohexanone, and 2,4,4-trimethyl-cyclopentanone), and 2 terpenes (7-oxabicyclo[4.1.0]heptane, and 4-methyl-1,4-hexadiene).

These compounds were found only in leaves sampled and analysed during the tree different bloom stages (early milk, early dough and senescence). All these samples were characterised mostly by alcohols (except for senescence), aldehydes, and furans.

In the table 8 a total of 19 compounds, the main VOCs found in ears samples from plants inoculated by *F. culmorum*, were shown: 5 alcohols (1-penten-3-ol, 2-penten-1-ol, 1-hexanol, 3-hexen-1-ol, and 2-hexen-1-ol), 6 aldehydes (2-pentenal, 2-heptenal, 2-octenal, 2,4-heptadienal, 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, and heptadecanal), 4 hydrocarbons (tridecane, 3-methyl-tridecane, 3-methyl-tetradecane, and 1-pentylheptyl-benzene), and 4 ketones (2,3-octanedione, 6-methyl-5-hepten-2-one, 3,5-octadien-2-one, and 2-undecanone).

Also in this case the compounds were found only in ears sampled and analysed during the tree different bloom stages (early milk, early dough and senescence). All these samples were characterised mostly by alcohols and aldehydes. Furthermore the VOCs detected during the early milk stage were also furans and ketones.

Compounds		Mean peaks area														
		EARLY MILK STAGE					EARLY DOUGH STAGE					SENESCENCE				
	Rt (min)	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM
Alcohols																
1-penten-3-ol	10.6	2765238	1288459	2571782	3737216	864545	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-penten-1-ol	14.44	638102	561250	622169	399832	473830	97771	35071	174513	66914	54048	n.d.	n.d.	n.d.	n.d.	n.d.
1-hexanol	15.05	248497	308707	251758	296076	212375	529675	843424	134858	104956	123277	n.d.	n.d.	n.d.	n.d.	n.d.
3-hexen-1-ol	15.81	8455967	12014760	10082914	8109946	4708950	16146002	16493033	214973	89353	119238	n.d.	n.d.	n.d.	n.d.	n.d.
2-hexen-1-ol	16.27	n.d.	n.d.	304600	188738	55290	125171	142505	182235	73491	46981	32982	70340	35303	27581	24587
1-octen-3-ol	17.20	n.d.	n.d.	84025	60013	15112	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2,6-dimethyl-cyclohexanol	20.73	13043	44418	131323	65460	35286	74531	24489	63202	59180	73775	43979	20477	47443	41519	47589
2,4-dimethyl-cyclohexanol	24.39	72861	78921	209418	129562	55967	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
benzyl alcohol	25.87	202736	352558	400032	250888	255189	78514	81984	32248	67466	155262	n.d.	n.d.	n.d.	n.d.	n.d.
eugenol	30.73	139209	111019	136260	95106	73534	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total		12535653	14760091	14794279	13332835	6750075	17051662	17620506	802028	461359	572579	76961	90817	82746	69100	72176
Percentage (%)		46	75	37	45	42	50	68	5	4	7	1	1	1	1	1
Aldehydes																
2-hexenal	12.17	8455967	41074	241382	68733	53284	241852	103979	463384	112495	134486	n.d.	n.d.	n.d.	n.d.	n.d.
2-heptenal	14.62	112965	56777	261120	540451	175282	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2,4-heptadienal	18.43	37240	55614	300510	97271	41054	98390	30367	350758	160171	216102	n.d.	n.d.	n.d.	n.d.	n.d.
2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	21.16	926075	909599	3791311	1457787	1254298	7433898	1162057	5723273	2803169	1735549	7524445	5759047	3597380	6218850	7894613
heptadecanal	26.6	138670	543778	243926	184057	75747	72122	92785	38277	34323	24234	n.d.	n.d.	n.d.	n.d.	n.d.
tetradecanal	28.39	348643	316165	1095859	1228218	217393	424532	317181	286252	80970	108686	62171	21000	33172	45345	54207
Total		10019560	1923007	5934106	3576515	1817058	8270793	1706368	6861943	3191127	2219056	7586616	5780047	3630552	6264194	7948820
Percentage (%)		36	10	15	12	11	24	7	39	31	28	64	63	48	61	76
Hydrocarbons																
tridecane	13.78	1042216	207440	5107732	2645306	1369053	2238564	1872568	2897863	2127816	1526387	n.d.	n.d.	n.d.	n.d.	n.d.
methylmethylene-cyclopropane	32.28	183385	498601	1894531	1527049	1561019	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total		1225601	706040	7002263	4172355	2930072	2238564	1872568	2897863	2127816	1526387					
Percentage (%)		4	4	18	14	18	7	7	17	21	19					
Furans																
2-ethyl-furan	6.47	201044	266713	616742	574306	237830	295752	191251	456144	106716	100097	204463	12625	60980	98844	108206
2-pentyl-furan	12.32	71828	97366	471165	344806	83760	260340	88488	617805	172601	216894	126634	121230	78727	80699	106086
cis-2-(2-Pentenyl)furan	14.02	1105687	393243	1320421	1053523	512506	629039	772727	1632977	1481510	480064	581854	485142	1172384	1061854	352395
5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	33.87	253415	98888	4201710	2752194	1379155	1985401	1441198	1363020	1385703	1389355	1523917	1341279	1583164	1769075	1239678
Total		1631974	856209	6610037	4724828	2213251	3170531	2493663	4069945	3146530	2186409	2436866	1960275	2895254	3010471	1806364
Percentage (%)		6	4	17	16	14	9	10	23	31	28	20	21	38	29	17
Ketones																
1-(3-cyclohexen-1-yl)-ethanone	12.86	202736	212438	813708	646527	353082	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2,2,6-trimetyl-cyclohexanone	14.52	446768	428648	1766595	996092	438893	803052	1134709	494869	373911	284094	n.d.	n.d.	n.d.	n.d.	n.d.
2,4,4-trimethyl-cyclopentanone	15.5	515114	234787	638090	839693	765307	860351	667501	421956	287266	423783	279446	635773	239345	248711	236349
Total		1164618	875872	3218393	2482311	1557281	1663402	1802210	916824	661176	707877	279446	635773	239345	248711	236349
Percentage (%)		4	4	8	8	10	5	7	5	6	9	2	7	3	2	2
Terpenes																
7-oxabicyclo[4.1.0]heptane	11.73	564900	261530	745431	668529	228065	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-methyl-1,4-hexadiene	16.45	407718	359813	1157488	558654	586634	1923844	423476	1962489	679584	729403	1544385	703967	736789	703345	452315
Total		972618	621342	1902919	1227183	814699	1923844	423476	1962489	679584	729403	1544385	703967	736789	703345	452315
Percentage (%)		4	3	5	4	5	6	2	11	7	9	13	8	10	7	4

Table 7: Main VOCs found in leaves samples from wheat plants inoculated by *F. culmorum*.

Compounds		Mean peaks area														
		EARLY MILK STAGE					EARLY DOUGH STAGE					SENESCENCE				
	Rt (min)	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM	SOIL	SEEDS	TILL.	BOOT.	BLOOM
Alcohols																
1-penten-3-ol	10.6	343119	248017	491241	387023	343168	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-penten-1-ol	14.44	86936	144729	282953	217985	202865	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-hexanol	15.05	193579	371257	496212	417815	748152	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-hexen-1-ol	15.81	76743	179993	113407	83835	125001	48371	49213	99610	116215	48889	n.d.	n.d.	n.d.	n.d.	n.d.
2-hexen-1-ol	16.27	180885	144540	398957	450104	953185	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total		881261	1088534	1782770	1556762	2372370	48371	49213	99610	116215	48889					
Percentage (%)		15	22	16	18	31	1	1	1	2	1					
Aldehydes																
2-pentenal	10.06	36037	52993	47783	48971	48565	n.d.	n.d.	187368	98997	88753	n.d.	n.d.	n.d.	n.d.	n.d.
2-heptenal	14.62	1072657	76251	47053	60924	91304	171154	30009	222402	199067	163882	n.d.	n.d.	n.d.	n.d.	n.d.
2-octenal	16.99	88813	76117	70356	87955	74999	737421	72088	322373	335123	255800	n.d.	n.d.	n.d.	n.d.	n.d.
2,4-heptadienal	18.43	167845	469082	480602	577370	478287	191460	583301	1404912	1651525	527187	279294	125780	1324391	1108376	752395
2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	21.16	58325	748263	3630851	2746735	1977992	1383594	1759271	1680592	1260000	2707545	n.d.	n.d.	n.d.	n.d.	n.d.
heptadecanal	26.6	260802	619434	783796	710106	661277	1087263	267570	1466532	796236	596785	214609	255651	707408	326841	377203
Total		1684477	2042139	5060440	4232060	3332423	3570892	2712238	5284177	4340947	4339950	493903	381430	2031798	1435216	1129598
Percentage (%)		29	42	45	50	43	62	79	71	79	80	65	65	87	87	80
Hydrocarbons																
tridecane	13.78	359552	168603	248612	199660	173574	25409	20748	92534	107703	94190	79113	17537	65265	39754	69146
3-methyl-tridecane	15.28	160720	131805	128284	162427	93348	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-methyl-tetradecane	16.8	299812	451798	211983	268267	213055	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-pentylheptyl-benzene	26.73	n.d.	n.d.	265697	143668	94791	545254	274390	249132	198649	283257	184890	184918	231453	166599	215755
Total		820084	752205	854575	774022	574767	570662	295137	341665	306351	377446	264003	202455	296718	206353	284901
Percentage (%)		14	16	8	9	7	10	9	5	6	7	35	35	13	13	20
Ketones																
2,3-octanedione	14.48	1592636	114192	28954	218186	227910	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-methyl-5-hepten-2-one	14.87	103021	68900	65558	98864	80176	95471	39055	130246	119107	93462	n.d.	n.d.	n.d.	n.d.	n.d.
3,5-octadien-2-one	18.90	83325	271324	1029507	560634	425856	1466649	333071	1607271	618937	580983	n.d.	n.d.	n.d.	n.d.	n.d.
2-undecanone	20.85	599337	505893	2374687	1082249	727921	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total		2378318	960308	3498706	1959933	1461862	1562119	372126	1737517	738043	674444					
Percentage (%)		41	20	31	23	19	27	11	23	13	12					

Table 8: Main VOCs found in ears samples from wheat plants inoculated by *F. culmorum*.

Multivariate analysis

PCA biplot of leaves samples, from wheat plants inoculated by *F. culmorum*, are showed in Figure 16 and underline that there are important differences. First and third principal components explained 28.3% and 25.2% of the variability, respectively. This analysis well evidences differences among samples, which result grouped into four clusters depending from the stage of harvesting. Samples harvested during the early milk stage are grouped in the second and third quadrants where loadings of alcohols, aldehydes, furans, ketones and terpenes are present. The first and the second quadrants contain the samples harvested during the early dough characterised especially by alcohols, aldehydes, esters, hydrocarbons and ketones. Samples harvested in the senescence stage are grouped in the third and fourth quadrants and they are characterised especially by alcohols and hydrocarbons. How could we expect, all controls (not inoculated samples) are located in the fourth quadrant, and also here the contribution of VOCs is much lower than the other samples.

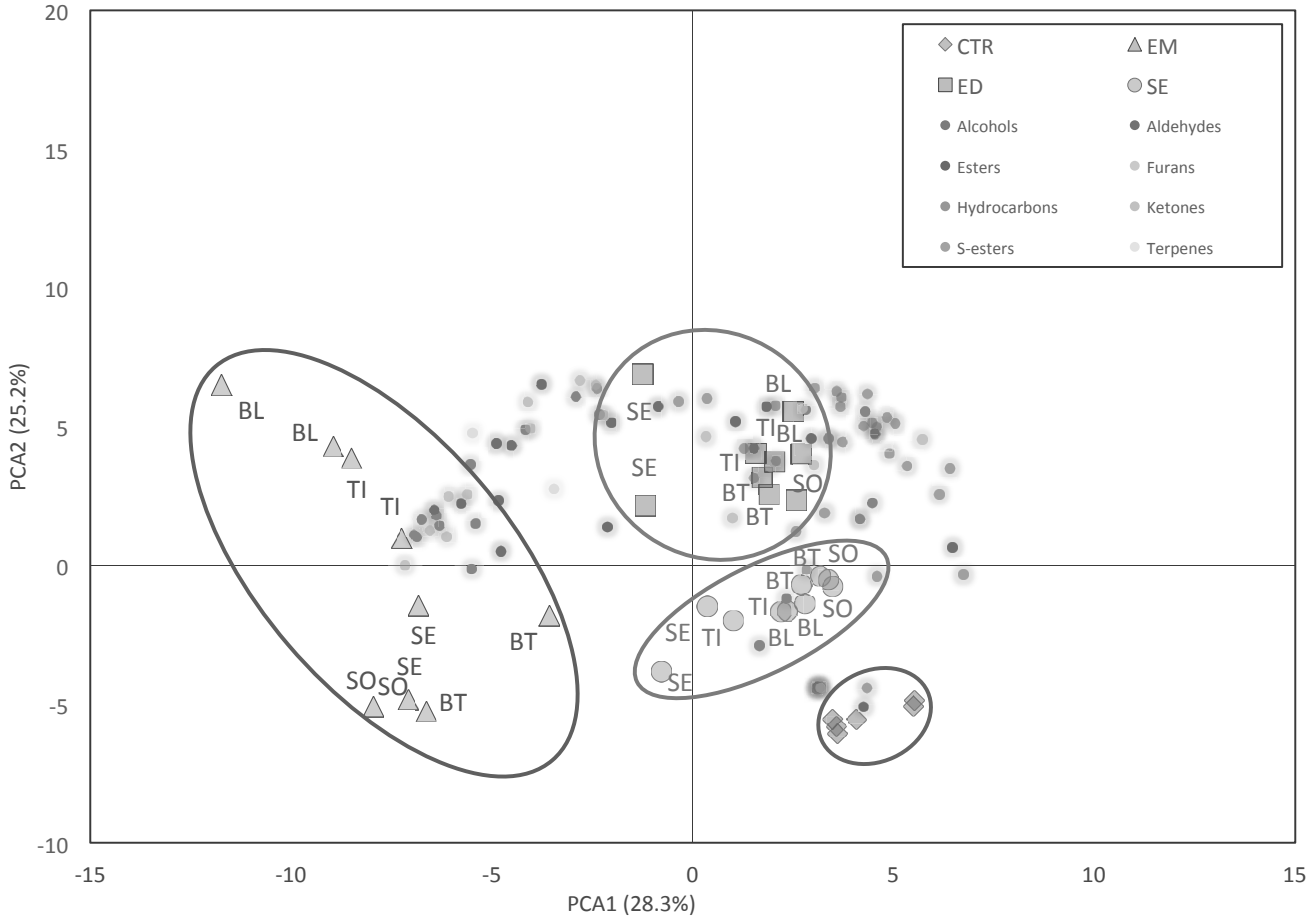


Figure 15: Principal component analysis (PCA) of the volatile profile of wheat leaves inoculated or not (CTR) by *Fusarium culmorum*. The inoculation with the fungus suspension were made by spraying the soil (SO), seeds (SE) or plants in different phenological phases: tillering (TI), booting (BT) and bloom (BL). Sampling and HS-SPME-GC-MS analyses were made at three different growth stages: early milk (EM), early dough (ED) and senescence (SE). First and second principal component have been considered here.

PCA biplot of ears samples, from wheat plants inoculated by *F. culmorum*, are showed in Figure 16 and underline that there are important differences. First and third principal components explained 33.7% and 20.6% of the variability, respectively. This analysis well evidences differences among samples, which result grouped into four clusters depending from the stage of harvesting. Samples harvested during the early milk stage are grouped in the second quadrant where loadings of alcohols, aldehydes, ketones and terpenes are present. Most of the samples harvested during the early dough stage are grouped in the first quadrant, and all the volatile organic compounds that we found can characterised them. Samples harvested in the senescence stage are grouped in the third and fourth quadrants and they are characterised especially by esters and hydrocarbons. All controls (not inoculated

samples) are located in the third quadrant, because there is no particular volatile organic compound that characterise them.

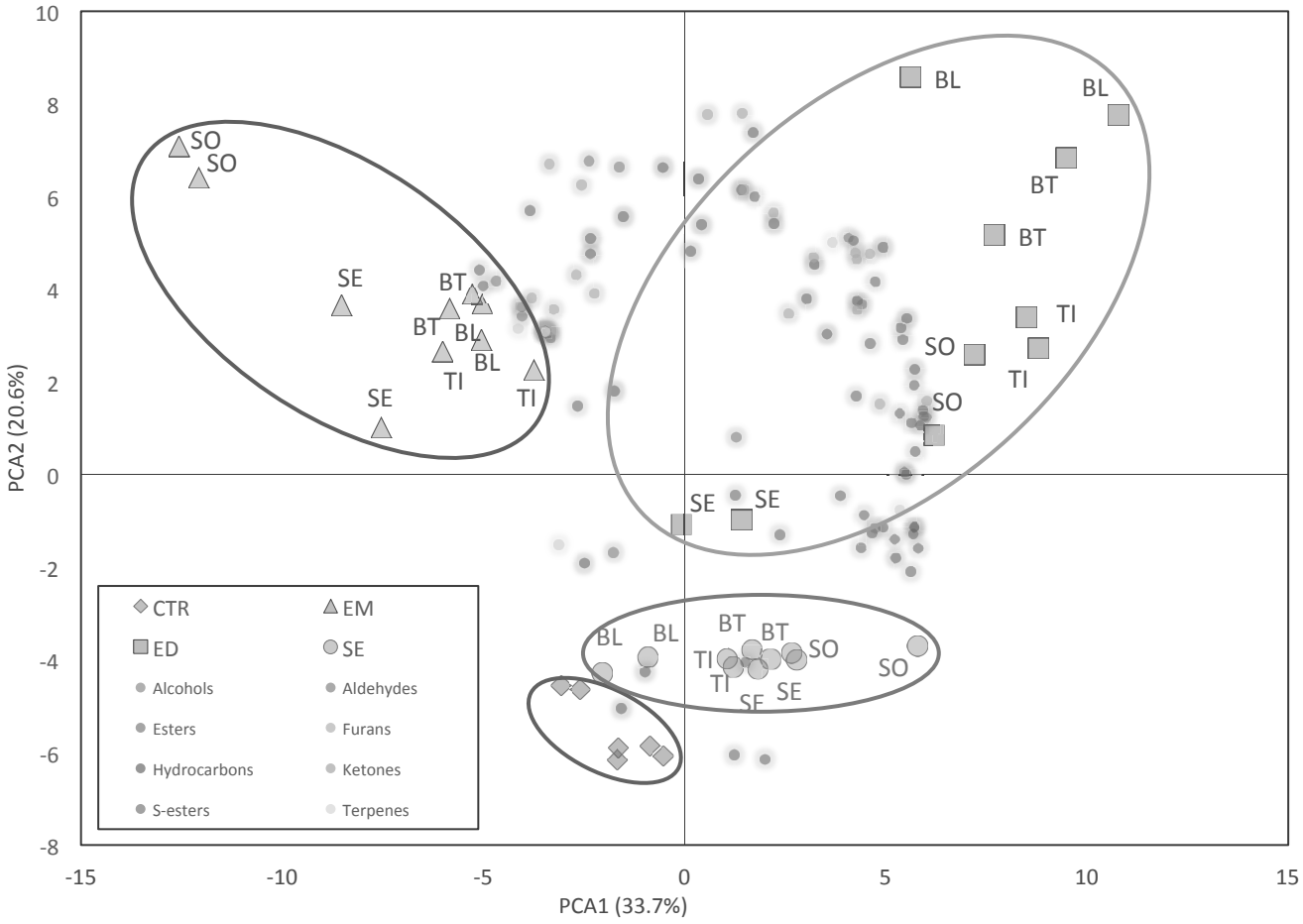


Figure 16: Principal component analysis of the volatile profile of wheat ears inoculated or not (CTR) by *Fusarium culmorum*. The inoculation with the fungus suspension were made by spraying the soil (SO), seeds (SE) or plants in different phenological phases: tillering (TI), booting (BT) and bloom (BL). Sampling and HS-SPME-GC-MS analyses were made at three different growth stages: early milk (EM), early dough (ED) and senescence (SE). First and second principal component have been considered here.

5 Conclusions

The project purpose was to give an innovative contribute in the field of fungal population and mycotoxins affecting plant products, through the development of integrated approaches based on hyphenated analytical techniques for the determination of new mycotoxins, "masked" mycotoxins and volatile organic compounds produced during fungal attacks.

ZEN Metabolites

The kinetic profiles of ZEN metabolites were studied with a time course experiment conducted in barley and in wheat lines susceptible (Remus) and resistant (CM-82036) to FHB. Treated ears did not show premature bleaching after a single exposure to 200 µg of ZEN, and in both wheat lines as well as in barley the same metabolites were determined. In ZEN-treated samples already after 12 h only ca. 26% of the initially added toxin could be determined. Most of ZEN remains unmetabolised, but some glucosides can be also observed especially in barley, which has proven more active in producing glucosides than wheat. As a result of the time course experiments, ZEN-Glc was verified as the major metabolite of ZEN metabolism which reached its maximum already 1 day after toxin treatment and was subsequently further metabolised. Although glucosylation of trichothecenes is considered a detoxification process, the resulting conjugates may be reactivated within the mammalian gastrointestinal tract, a fact that is currently regarded as the main health risk associated with "masked" mycotoxins (Dall'Erta *et al.* 2013; De Angelis *et al.* 2014; Nagl *et al.* 2014). Kinetic data revealed that detoxification progresses rapidly, resulting in the almost complete degradation of the toxins within 1 week after a single exposure in flowering ears.

VOCs

The approach developed in this study has provided important results to predict the potential of mycotoxin formation, by detecting the presence of *Fusarium* species, at early stages of fungal growth in wheat through the identification of volatile organic compounds as biomarkers of an early fungal contamination.

These behaviors can be explained considering the antifungal activity that can occur in inoculated wheat samples, leading to the production of alcohols (e.g., 2,6-dimethyl-cyclohexanol), aldehydes (e.g., hexanal and 2-hexenal), esters (e.g., triethyl phosphate), furans (e.g., 2-ethyl-furan), and many ketones and terpenes. These outcomes are in agreements with the results obtained by microbiological evaluations: fungal re-isolation on plate, from inoculated plants, and RT-PCR analysis.

Considering that the legal limit of DON for not processed durum wheat is 1.75 mg/kg (or μg/g) (EC Regulation 1881/2006), as we can see in the tabs below, some of the samples, reported as an example, contained significantly higher amounts of toxin then the maximum level established by law. However most of them contain a very low amount of toxin, very often lower then the legal limit.

The result is even more encouraging considering that even if the samples were found to be contaminated with high content of mycotoxins (see Tables 9), despite the very low level of chosen fungi inoculum, it has been still possible to identify volatiles biomarkers of early infection before the actual occurrence of mycotoxin detectable levels. Infact it is very important to note that in the samples in which the level of DON is lower than the LOD or, in any case, lower than the legal limit, the presence of fungi is already evident, for targeted interventions.

The project results can be considered a valuable tool in the preservation of food production and protection of animal and human's health. Moreover, results obtained can have a positive impact on primary productions, improving the preservation of environment both in terms of cost and in terms of quality of life for food sustainability.

(a)

Inoculum by *F. graminearum*	**Sampling**	**Part of plant**	**DON (μg/g)**
soil	early dough stage	ear	29.0
soil	senescence	ear	11.4
soil	senescence	grain kernel	< LOD
seeds	tillering	old leaf	9.1
seeds	early dough stage	grain kernel	< LOD
seeds	senescence	ear	9.3
tillering	early milk stage	ear	< LOD
tillering	senescence	grain kernel	< LOD
booting	senescence	grain kernel	0.2
booting	senescence	grain kernel	< LOD
bloom	early milk stage	grain kernel	< LOD
bloom	early dough stage	ear	8.1
bloom	early dough stage	grain kernel	< LOD
bloom	senescence	ear	3.6
bloom	senescence	grain kernel	< LOD
bloom	senescence	grain kernel	0.2
bloom	senescence	culms	3.7
control	senescence	grain kernel	< LOD

(b)

Inoculum by *F. culmorum*	Sampling	Part of plant	DON (µg/g)
soil	tillering	old leaf	18.9
soil	booting	leaf	< LOD
soil	booting	ear	< LOD
soil	early milk stage	leaf	< LOD
soil	early milk stage	grain kernel	< LOD
soil	early dough stage	ear	1.0
soil	early dough stage	grain kernel	< LOD
soil	senescence	grain kernel	< LOD
seeds	booting	ear	< LOD
seeds	early milk stage	ear	< LOD
seeds	early dough stage	ear	1.6
seeds	senescence	ear	1.4
seeds	senescence	grain kernel	< LOD
tillering	tillering	young leaf	< LOD
tillering	tillering	old leaf	< LOD
tillering	booting	ear	< LOD
tillering	early milk stage	leaf	0.7
tillering	early milk stage	ear	< LOD
tillering	early dough stage	ear	8.6
tillering	senescence	leaf	16
tillering	senescence	ear	8.8
tillering	senescence	grain kernel	< LOD
tillering	senescence	grain kernel	< LOD
booting	booting	ear	< LOD
booting	early milk stage	grain kernel	< LOD
booting	senescence	grain kernel	< LOD
bloom	early milk stage	ear	< LOD
bloom	senescence	ear	< LOD
bloom	senescence	grain kernel	< LOD
bloom	senescence	grain kernel	< LOD
bloom	senescence	culms	< LOD
control	early milk stage	ear	< LOD

Table 9: Chemical analysis for DON quantification in samples of wheat plants inoculated by *F. graminearum* (a) or *F. culmorum* (b). The analysis were carried out using the method by Pascale *et al.* (2014) based on the purification of the extracts with immunoaffinity columns and toxin determination using UPLC coupled with DAD detector. LOD = 0.03 µg/g.

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List of Abbreviations

15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivaleno
AAL	*Alternaria alternata* toxin
ACN	acetonitrile
ADME	absorption, distribution, metabolism and excretion
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
AFs	aflatoxins
amu	atomic mass unit
API	atmospheric pressure ionisation
BL	bloom
BT	booting
BVOC	biogenic volatile organic compound
c	concentration
C_{18}	octadecyl carbon chain-bonded silica
C_8	octacyl carbon chain-bonded silica
C_a	air concentration
CAC	Codex Alimentarius Commission of the Joint FAO/WHO Standards Programme
CAR	carbowax
CAST	Council for Agricultural Science and Technology
CDC	Centers for Disease Control and Prevention
CDL	Christian Doppler Laboratory for Mycotoxin Metabolism
CEN	Comité Européen de Normalisation (European Committee for Standardization)
CM-82036	CM-82036-1TP-10Y-OST-10Y-OMOFC (FHB-resistant variety of wheat)
C_p	concentration in the matrix
cps	counts per second
CTR	control
D3G	deoxynivalenol-3-glucoside
DC	direct current
DG SANCO	Directions générales de Santé et Consommateur (Health and Consumers Directorate-General of the European Commission)
DH	dynamic headspace
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DVB	divinylbenzene
EC	European Community
ED	early dough
EFSA	European Food Safety Authority
EIC	extracted ion chromatogram (other abbreviation XIC)
ELISA	enzyme-linked immunosorbent assay
EM	early milk

EN	European Norm
ESI	electrospray ionisation
EU	European Union
f	detector response factor
FA	formic acid
FAO	Food and Agriculture Organisation of the United Nations
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FBs	fumonisins
FHB	Fusarium head blight
FID	flame ionisation detector
FLD	fluorescence detection
FSM	full scan mode
FT-ICR	Fourier-Transform ion cyclotron resonance
GC	gas chromatography
Glc	glucoside
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HS-SPME	headspace solid phase microextracion
HT-2	HT-2 toxin
I	signal intensity
IAC	immunoaffinity column
IARC	International Agency for Research on Cancer
IS	internal standard
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LFIA	lateral-flow immunoassay
LMW	low-molecular weight
LOD	limit of detection
LOQ	limit of quantitation (also called limit of quantification)
m/z	mass-to-charge ratio
MeOH	methanol
MERCOSUR	Mercado-Cómun del Sur (Southern Common Market)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSD	mass selective detector
MSPD	matrix solid phase dispersion
NMR	nuclear magnetic resonance
OTA	ochratoxin A
PA	polyacrylate
P_{ap}	air-matrix partition coefficient
PAT	patulin
PAT	purge and trap
PCA	principal component analysis
PDA	Potato Dextrose Agar

PDMS	polydimethylsiloxane
P_i	partial pressure
P_o	vapor pressure
Q	quadrupole
QqQ	triple quadrupole
QTOF	quadrupole time-of-flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R	resolution
R_A	apparent recovery
RASFF	Rapid Alert System for Food and Feed
R_E	extraction recovery
RF	radiofrequency
RNA	ribonucleic acid
RP	reversed-phase
RT-PCR	real-time polymerase chain reaction
S	peak area
Sa	sphinganine
SE	seeds
SE	senescence
SH	static headspace
SIDA	stable isotope dilution assay
SIM	selected ion mode
SO	soil
So	sphingosine
SPE	solid phase extraction
SRM	selected reaction monitoring (replaced MRM)
SSE	signal suppression or enhancement
T-2	T-2 toxin
TI	tillering
TIC	total ion chromatogram
TLC	thin layer chromatography
TOF	time-of-flight
TWI	tolerable weekly intake
UHPLC	ultra high performance liquid chromatography
UV-Vis	ultraviolet-visible light spectrophotometry
VOC	volatile organic compound
WHO	World Health Organisation of the United Nations
XIC	extracted ion chromatogram (other abbreviation EIC)
y	mole fraction
ZAN	zearalanone
ZEL	zearalenol (before also ZOL)
ZEN	zearalenone (before also ZEA or ZON)