MENDEL UNIVERSITY IN BRNO
Faculty of AgriSciences

PhD Thesis

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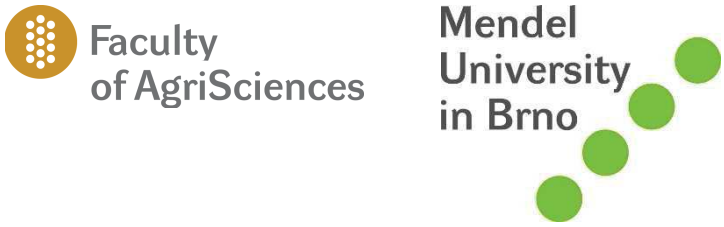

Mycotoxin contamination of spring barley in the chain barley grains–final products

PhD Thesis

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Brno 2017

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Acknowledgements

I would like to thank my supervisor, Assoc. Prof. Radim Cerkal PhD, for his valuable advice, comments, and suggestions that helped me to accomplish this work.
I am also grateful to my family for their patience, and being around me.

SUMMARY

Mycotoxins are toxic secondary metabolites of microscopic filamentous fungi. They represent a group of harmful worldwide occurring contaminants of food and feed. *Fusarium* mycotoxins are the most common contaminants of small grain cereals such as barley. The presented PhD study summarizes current knowledge in the field of *Fusarium* mycotoxins with a special emphasis on barley, malt and beer production.

A brief overview of the most common *Fusarium* mycotoxins in relation to barley is given in the first part of this PhD thesis. Mainly trichothecenes of A and B groups were discussed. Most research activities related to the first thematic part of the thesis were focused on the monitoring of mycotoxins in barley, malt and beer. In addition, the impact of the pre-harvest conditions (weather conditions, locality, barley variety, fungicide treatment) was evaluated. As a tool for mycotoxin determination, several analytical methods based on liquid chromatography coupled to mass spectrometry had to be developed and implemented within the presented thesis.

The second part of this work is focused on "masked" mycotoxins. "Masked" mycotoxins are formed as a results of detoxification processes in plants contaminated with mycotoxins. They became an issue of a high importance over the past ten year. Therefore, a lot of studies performed within author´s PhD studies were focused on: a) metabolism of mycotoxins in plants, b) development of analytical methods for indirect determination of "masked" mycotoxins as an effective tool to predict level of contamination in malt and beer.

The results obtained within this PhD thesis were published in 8 original papers and presented in a number of international conferences.

SOUHRN

Mykotoxiny jsou toxické sekundární metabolity mikroskopických vláknitých hub. Představují skupinu celosvětově se vyskytujících kontaminantů potravin a krmiv. Nejběžnějšími kontaminanty ječmene jarního a dalších obilnin jsou fusariové mykotoxiny. Předkládaná disertační práce shrnuje současné poznatky o fusariových mykotoxinech, jejich výskytu v zrnu ječmene a přestupu do sladu a piva během technologického zpracování.

První část disertační práce je zaměřena na fusariové mykotoxiny, které se nejčastěji vyskytují v zrnu ječmene jarního. Jedná se o trichoteceny typu A a B. V této části práce je nejvíce diskutovaná problematika výskytu mykotoxinů v zrnu ječmene, sladu a pivě. Také byl sledován a hodnocen vliv pěstebních postupů a dalších faktorů na výsledný výskyt mykotoxinů v zrnu. Mykotoxiny byly stanovovány pomocí kapalinové chromatografie s hmotnostní spektrometrií.

Druhá část předkládané práce se zabývá tzv. maskovanými mykotoxiny. Maskované mykotoxiny vznikají při detoxifikačních procesech v rostlinách jako odezva na mykotoxinovou kontaminaci. Jelikož znalosti o maskovaných mykotoxinech jsou minimální, staly se hlavním předmětem zájmu výzkumu v této oblasti. Pro bližší pochopení mechanismů vzniku těchto látek byl v rámci disertační práce studován metabolismus deoxynivalenolu a T-2 toxinu v rostlinách. V části věnované maskovaným mykotoxinům nebyla opomenuta ani problematika analytického stanovení těchto látek. Testovány byly různé přístupy nepřímých analytických metod pro stanovení celkového obsahu těchto látek bez znalosti jejich struktury.

Výsledky získané v rámci předkládané disertační práce byly publikovány v 8 vědeckých publikacích, v odborných časopisech a prezentovány na mnoha mezinárodních konferencích.

TABLE OF CONTENTS

1 INTRODUCTION

Beer is one of the most popular beverage worldwide. In Europe, 37% of the total alcohol is consumed in the form of beer. Czech Republic used to be a leading country in Europe, with an annual consumption of 146.6 litres per capita in 2014, followed by Austria (104.8 litres) and Germany (104.7 litres) (http://www.kirinholdings.co.jp/english/news/2015/1224_01.html#table3).

Malt and beer represent one of the most important trade commodities for the Czech food industry. Czech Republic is also a leading country in the field of barley breeding and production of barley of a high quality required for beer production.

Unfortunately, quality of barley grains might be decreased due to fungal infection. The most common barley disease is *Fusarium* head blight (FHB) caused mainly by *Fusarium* fungi developed under the field conditions. Beside a lost in grain yield, toxic secondary metabolites, mycotoxins, are usually formed.

In order to protect human and animal health, prevention and control of mycotoxin contamination of food and feed chain has become a major objective for farmers, producers, regulatory authorities, and researchers worldwide. Concerning barley (and other small-grain cereals), deoxynivalenol is considered as the most common mycotoxin. Fortunately, with the ongoing development of highly advanced analytical techniques, also other mycotoxins can be easily monitored. Therefore, other *Fusarium* mycotoxins have been already reported to be found in barley.

As mycotoxins are rather stable compounds, they can survive under the food processing conditions and pass into the final products. This issue has become of high concern since the existence of "masked" mycotoxins had been confirmed. These "masked" forms are formed in plants as a result of plant detoxification processes. Due to high incidence of deoxynivalenol, the most research activities in this field has been focused on deoxynivalenol-3-β-D-glucoside.

The mechanism of plant/fungi interaction is a subject of ongoing research. From the health risk perspective, it is fundamental to obtain knowledge about the entire spectrum of masked mycotoxins formed in plants, their stability during food processing, as well as their toxicological significance. In addition, effective analytical tools for their control in the food chain is required to collect occurrence data for future establishment of regulatory limits in food, in particularly for beer, for which no legislation limits have been laid down so far because of a lack of data.

2 MAIN OBJECTIVE OF THE PRESENTED PHD THESIS

The aim of this PhD thesis was to study mycotoxin contamination of spring barley and the follow-up products – malt and beer.

In line with that, sophisticated advanced analytical methods based on liquid chromatography coupled to mass spectrometry (LC-MS) were developed and validated for routine control of mycotoxins in barley, malt and beer.

Furthermore, a state-of-the-art approach consisting of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (U-HPLC-HRMS) was used for plant metabolism study of deoxynivalenol and T-2 toxin.

The following tasks were completed within this PhD thesis:

- **Metabolism of deoxynivalenol and T-2 toxin in small-grain cereals.**
  - The first comprehensive study about the fate of deoxynivalenol in *Fusarium* resistant and susceptible near isogenic wheat was conducted in **APPENDIX II**.
  - The metabolism of T-2 toxin in wheat and barley (**APPENDIX III**) was studied and the entire spectrum of novel "masked" mycotoxins elucidated.
- **Development of a fast screening method for determination of mycotoxins in barley.**
  - Various conditions of acidic and alkaline hydrolyses were tested in order to achieve a total hydrolysis of all masked forms of deoxynivalenol formed in barley to deoxynivalenol (a total "mycotoxin" pool). This approach could help to predict a final mycotoxin contamination in beer (**APPENDIX V**).
  - Various enzymes from glycoside hydrolase family 3 β-glucosidases were tested as well for the same purpose (**APPENDIX VI**).
- **An overview of analytical methods used for determination of mycotoxins in beer is summarized in APPENDIX VII.**
- **Assessment of mycotoxin contamination in barley, malt and beer.**
  - Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was used for determination of trichothecene of A and B groups in various varieties of spring barley and malt produced thereof (**APPENDIX IV**).
  - The impact of various pre-harvest conditions on the levels of *Fusarium* mycotoxins in spring barley and malt was assessed (**APPENDIX IV)**.

- A novel *Fusarium* mycotoxin – Pentahydroxyscirpene was discovered and identified. The natural occurrence was confirmed for the first time in barley harvested in the Czech Republic (**APPENDIX I**).
- A sensitive and robust LC-MS/MS based method was developed and validated for six different types of beer – pale, wheat, dark, bock and shandies (**APPENDIX VIII**).
- The validated LC-MS/MS method was used for the conduction of a survey of 374 beer samples collected originating from 38 countries (**APPENDIX IX**).

Note: The framed highlighted text refers to the author´s contribution to the discussed topics. Detailed description of conducted experiments and generated data are enclosed in the respective appendices.

3 MYCOTOXINS

Mycotoxins are natural toxic secondary metabolites produced by microscopic filamentous fungi which can invade a large scale of agricultural crops already during their growth on the field and/or in the follow-up stages of food and feed production chains worldwide.

It has been estimated that almost 25% of world's crops are affected by molds or fungal growth. The undesirable fungal infestation of crops results in high economic losses caused by lower yields. Further financial losses caused by mycotoxins due to decreases in the productivity of farm animal are, however, difficult to assess. There are approximately 100,000 described species and, like many other organisms, fungi still harbor many undescribed species. However, the total number of most commonly occurring species in foods/feeds and indoor environments is estimated around 175 (Samson et al., 2010). As for the genus *Fusarium*, Lesslie and Summerell (2006) included 70 species in their laboratory manual, but only a relatively small number of *Fusarium* species (15 spp.) are important with regard to food spoilage in its broad concept (Pitt and Hocking, 2009).

The most of toxinogenic species belong mainly to three fungi genera: *Fusarium*, *Aspergillus* and *Penicillium* (Moss 1992; Sweeney and Dobson 1999; Placinta et al., 1999; Medina et al., 2006). These fungi can produce a wide range of mycotoxins varying not only in chemical structures but also in the mode of toxicological actions. However, the highest health risk is attributed only to a limited number of them. Concerning high incidences (thus possible dietary exposures) together with their toxicity, aflatoxins (*Aspergillus*), ochratoxins (*Aspergillus* and *Penicillium*), trichothecenes, fumonisins and zearalenone (*Fusarium*) are of a high concern.

With regard to health hazard attributed to mycotoxins and their impact to the end consumers (and farm animals), many countries have set up regulations for their control in food chain. In the European Union the maximum limits for aflatoxins, deoxynivalenol, zearalenone, ochratoxin A, fumonisins and patulin in various foodstuffs have been established (European Commision (EC) No. 1881/2006, European Commision (EC) No. 1126/2007).

While quite extensive knowledge is available on occurrence of regulated mycotoxins, information about unregulated mycotoxin is lacking due to their insufficient monitoring. On this account the European Food Safety Authority (EFSA) has

recommended to regularly monitor not only regulated mycotoxins but also so-called "emerging mycotoxins" such as ergot alkaloids, beauvericin, and eniatins (www.efsa.europa.eu).

Besides free mycotoxins, mycotoxin conjugates in cereals represent an emerging issue in food safety. Nowadays, the most attention is being paid to deoxynivalenol-3-*β*-D-glucoside originating in plants as a result of detoxification processes (Berthiller et al. 2005; Berthiller et al., 2007; Vendl et al., 2009). Taking into account these compounds are, at least partly bioavailable, then the dietary exposure might be underestimated.

The following subchapters are focused on mycotoxins which were studied in this PhD thesis. A brief overview below summarizes their main producers, chemical structures and physicochemical properties.

3.1 *Fusarium* mycotoxins

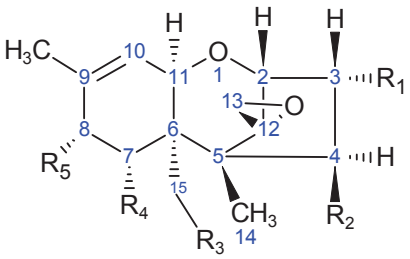
The most prevalent producers of mycotoxins growing under the temperate climatic conditions are *Fusarium* spp. The genus *Fusarium* includes several species which are important pathogens of maize and small-grain cereals. In addition to their pathogenity, some *Fusarium* spp. are also capable to produce mycotoxins which can be accumulated either in pre-harvested or in stored grains (Jestoi 2008). The mycotoxin pattern produced by *Fusarium* includes various groups of mycotoxins. Besides well-known trichothecenes, zearalenone and fumonisins, "emerging" enniatins and beauvericin must be also mentioned.

3.1.1 Trichothecenes

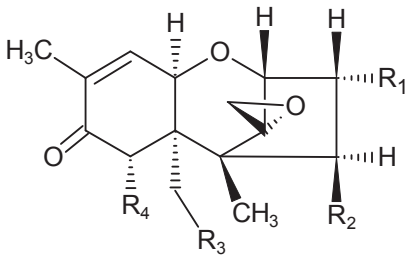
Trichothecenes are the largest group of mycotoxins known to date, consisting of more than 150 chemically related compounds. According to their chemical structures they belong to sesquiterpene compounds. The most important structural features causing the biological activities of trichothecenes are: a) the 12,13-epoxy ring, b) the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus, and c) the structure and position of the side-chain. These structural differences in the main skeleton of trichothecenes allow to divide these mycotoxins into four groups (**Figure 1**):

- Type A – characterised by a functional group other than a ketone at C-8
- Type B – characterised by a carbonyl function at C-8
- Type C – characterised by a second epoxide group at C-7,8 or C-9,10

- Type D – characterised by a macrocyclic ring between C-4 and C-15 with two ester linkages.



Type A



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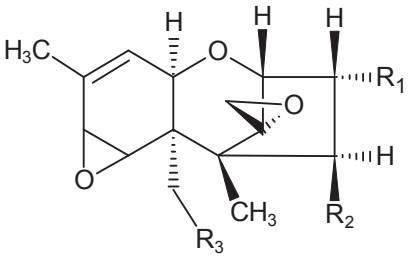

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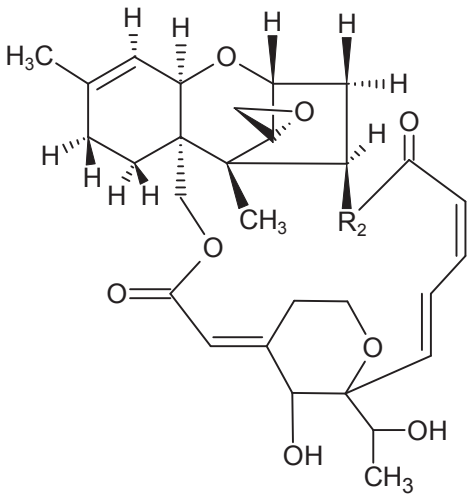

Type D

Figure 1 Basic skeletons of trichothecenes

Table I Chemical structures of some trichothecenes of A and B type occurring in foods and feeds

Type	Mycotoxin	Abbreviation	R_1	R_2	R_3	R_4	R_5
A	HT-2 toxin	HT-2	OH	OH	OAc	H	OIval
	T-2 toxin	T-2	OH	OAc	OAc	H	OIval
	Diacetoxyscirpenol	DAS	OH	OAc	OAc	H	H
	Neosolaniol	NEO	OH	OAc	OAc	H	OH
B	Deoxynivalenol	DON	OH	H	OH	OH	-
	3-acetyldeoxynivalenol	3ADON	OAc	H	OH	OH	-
	15-acetyldeoxynivalenol	15ADON	OH	H	Oac	OH	-
	Nivalenol	NIV	OH	OH	OH	OH	-
	Fusarenon-X	FUX-X	OH	OAc	OH	OH	-

Note: OAc, $-O-COCH_3$; OIval,$-O-COCH_3CH(CH_3)_2$

The most common trichothecenes occurring in cereals belong to the type A and B, and major representatives of these two types are listed in **Table I**.

Trichothecenes are colourless, mostly crystalline solids. They are relatively stable under temperatures below 120°C but application of temperatures higher than 200°C led to their partial degradation. Considering the solubility of trichothecene toxins in organic solvent, most of them are rather polar, and thus soluble in polar solvents, such as methanol, acetonitrile and their water mixtures. The less polar trichothecenes of type A may be soluble also in chloroform, diethylether and ethylacetate (Shepherd and Gilbert 1988).

Trichothecene contamination is economically important in wheat, barley, oats, and maize. The fungi cause a disease of wheat and barley known as *Fusarium* head blight (FHB) and a disease of maize known as *Gibberella* ear rot (**Figure 2ab**). Infected wheat spikelets exhibit premature bleaching as the pathogen progresses within the head and the developing grain becomes contaminated with mycotoxins. Maize ears infested with *F. graminearum* are often covered with a pinkish fungal mycelium (Edwards 2004).

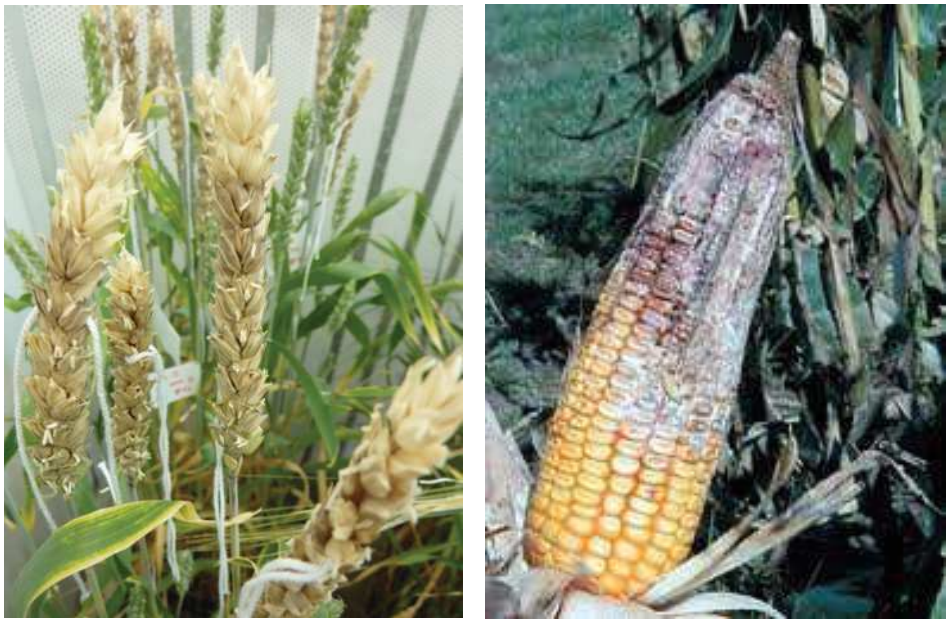

Figure 2 a) Wheat var. Remus inoculated with *F. graminearum* (Copyright, Elisabeth Varga); b) Gibberella ear rot

Deoxynivalenol

The most common trichothecene is DON. Due to its high incidence and levels it is often considered to be a marker of *Fusarium* mycotoxin contamination of cereals. DON is also the only one of trichothecenes for which maximum levels in various foodstuffs have been established by European Commission (European Commision (EC) 1881/2006). DON, chemically 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, is sometimes called vomitoxin because of its deleterious effects on the digestive system of humans and monogastric animals (Creppy 2002).

The main producers of DON are *Fusarium culmorum* and *F. graminearum* (Weidenbörner 2001).

Nivalenol

Nivalenol is the trivial name for (3α,4β,7α)-12,13-epoxy-3,4,7,15-tetrahydroxytrichothec-9-en-8-one. It was first successfully isolated from *F. nivale Fn-2B*, an atypical strain of *F. sporotrichioides*, in 1963 (Tatsuno et al., 1968), but it is also produced by other *Fusarium* spp. such as *F. poae* and to a lesser extent by *F. culmorum* and *F. graminearum* (Eriksen, 2003; Wiedenbörner, 2001). Although NIV was not common in the European countries and occurred rather in Asia, in the last few years its incidence in Europe was growing. On this account, NIV has been added on the list of contaminants for which the continuous occurrence data are requested by EFSA. At the

time of writing this thesis, the working group for *Fusarium* mycotoxins of EFSA panel for contaminants in the food chain were summarized available information and the occurrence data in order to assess the health risk for human related to the presence of nivalenol in food and feed.

New trichothecene mycotoxin, pentahydroxyscirpene (PHS) which is structurally related to nivalenol (owing hydroxyl group instead of ketone group (NIV) on the C8 compared) was identified (APPENDIX I). PHS was formed upon inoculation of rice with a NIV producing *Fusarium* strain in a level of roughly 10% of nivalenol. It was proposed that nivalenol and PHS share the common precursor 7,8-dihydroxy-3,4,5-triacetoxyscirpenol, which leads to the formation of PHS if the function of (a yet unknown) oxidoreductase is impaired. The molecular identification of the PHS producer IFA189 was reported. In addition other selected nivalenol-producing *Fusarium* strains and species were characterized regarding the ability to form PHS. Strain IFA189 was classified as *F. sporotrichioides* suitable for the production of fusarenon-X and nivalenol. However, molecular identification revealed that it is actually a *F. kyushuense*.

T-2 and HT-2 toxins

T-2 and HT-2 are produced by various *Fusarium* species, including *F. sporotrichoides*, *F. poae*, *F equiseti*, *F. acumninatum*, as well as species from the genera *Myrothecium*, *Acremonium*, *Verticimonosporum*, *Trichoderma*, *Trichothecium* and *Stachybotrys* (Weidenbörner, 2001). T-2 toxin is the trivial name for (3α,4β,8α)-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol 4,15-diacetate 8-(3-methylbutyrate).

T-2 is readily metabolised to HT-2 whose structure differs from T-2 only in the functional group at the C4-position. HT-2 is the trivial name for (3α,4β,8α)-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol 15-acetate 8-(3-methylbutyrate). Both toxins are found in different food and feed commodities, such as wheat, barley, oats, rice and corn and are toxic to all animal species as well as to humans (Weidenbörner 2001).

The increased incidence of HT-2 and T-2 resulted in proposal to set legislation limit for sum of HT-2 and T-2 in cereals but the establishment of the new regulation is still in

progress. However, based on all initiatives, investigation and assessment, EU recommendation on maximal levels of a sum of HT-2 and T-2 in various food and feed commodities has been released in 2013 (EC recommendation No. 2013/165/EU). For instance, the maximal recommended level of HT-2 and T-2 (in sum) should not exceed a level of 200 µg/kg in raw barley, including malting barley.

T-2 has been implicated as part of the alleged chemical warfare agent "yellow rain" in Southeast Asia and causes a fatal disease of humans known as Alimentary Toxic Aleukia (ATA); a disease that was particularly problematic in Russia in the 1940s. Symptoms of ATA in humans include skin pain, vomiting, diarrhoea, complete degeneration of bone marrow, and finally death (Li et al., 2011).

3.1.2 Zearalenone

Zearalenone (ZEA) is a mycotoxin that activates estrogen receptors resulting in functional and morphological alteration in reproductive organs. Swine are the most commonly affected domestic animals but cattle and poultry may also be affected. The clinical effects of zearalenone may include an enlarged uterus, swelling of the vulva and vagina (known as vulvovaginitis), enlarged mammary glands, anestrus (periods of infertility), and abortion. A commercially available derivative of ZEA (zeranol) was used as a growth hormone to increase weight gain of beef cattle. However, acute toxicity of ZEA is rather low, but it has been shown to be genotoxic, imunotoxic, hepatotoxic and hematotoxic (Zinedine et al., 2007).

ZEA, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone (**Figure** 3), is produced mainly by *F. culmorum*, *F. graminearum*, *F. semitectum* and *F. equiseti*. ZEA contamination is economically important in maize and hay, but the occurrence of ZEA has been reported also in wheat, barley and oat (Zinedine et al., 2007).

Concerning physico-chemical properties, ZEA is lipophilic, white crystallic solid. ZEA is not susceptible against high temperatures, the majority survives the temperature of 180°C for 30 min (Zinedine et al., 2007).

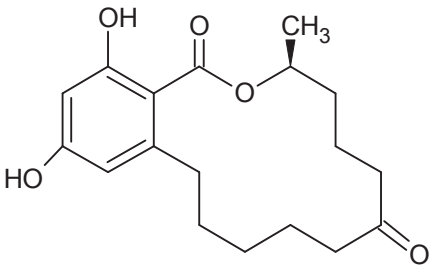

Figure 3 Chemical structure of zearalenone

4 MASKED MYCOTOXINS

4.1 Deoxynivalenol-3-glucoside

The issue of masked mycotoxins first caught an attention in the mid-1980s because in some cases of mycotoxicoses, clinical observation in animals did not correlate with low mycotoxin content determined in corresponding feed. This unexpected high toxicity was attributed to the occurrence of undetected conjugated forms of mycotoxins that were hydrolyzed to the precursor toxins in the digestive tracts of animals (Berthiller et al., 2005). Around the same period, the metabolic biotransformation of DON to less toxic derivatives in plants was for the first time hypothesized to occur in field corn inoculated with *Fusarium graminearum* and in naturally infected wheat (Berthiller et al., 2016).

Later, it was postulated that infected plants are probably able to reduce the toxicity of mycotoxins by chemical modification and/or by their inclusion into the plant matrix. This detoxification process includes the conjunction of mycotoxins to polar substances such as sugars, amino acids and sulfate and subsequent storage of conjugates into vacuoles (Berthiller et al., 2005). The first identified masked mycotoxin was conjugated form of DON and glucose-3-β-D-glucopyranosyl-4-deoxynivalenol (D3G), **Figure 4**.

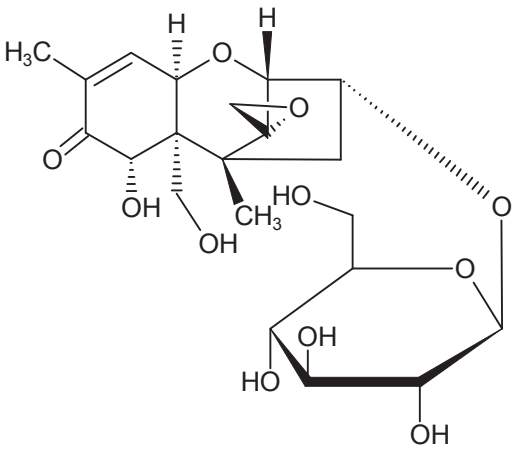

Figure 4 Chemical structure of deoxynivalenol-3-β-D-glucoside

Once D3G was unambiguously identified, studies intended for monitoring of its levels in crops were realized. D3G was detected in artificially infected wheat treated by DON during ear flowering. The presence of masked D3G beside DON in the samples of naturally contaminated wheat and maize was confirmed by Berthiller et al. (2005). The amount of DON bound in D3G was expressed as molar ratio calculated according to the following equation: [(Molecular weight(DON)/Molecularweight(D3G)×(concentration(D3G)/concentration(DON)]×100. In these samples, the levels of D3G ranged from 2.5 up to 7.4 mol % of parent DON. As published later, in some cases, D3G can reach even up to 46 mol % of total DON (Berthiller et al., 2009). Since 2007 its routine analysis has become possible for any research or control laboratories due to availability of analytical standard of D3G at the market (Romer Labs, Tulln, Austria). So far, D3G has been reported in wheat (grains, semolina and flour), maize (grains), oat (flour), barley, malt and beer (Berthiller et al., 2013).

4.1.1 Toxicology

Toxicological relevance of D3G is still a subject of ongoing research. As conjugation is known to be a detoxification process, it seem likely that D3G exhibits a lower acute toxicity compared to DON. This has been demonstrated *in vitro* when D3G showed a dramatically reduced ability to inhibit protein synthesis of wheat ribosomes compared to DON (Poppenberger et al., 2003).

Although D3G is less toxic for plants than DON, the toxicity of this substance to mammals is currently unknown. D3G might be cleaved to DON during digestion and this increasing the total DON burden of an individual. The latest studies on bioavailability of

D3G in rats showed that D3G is little bioavailable, hydrolysed to DON during digestion, and partly converted to deepoxy-deoxynivalenol and deoxynivalenol-glucuronide prior to excretion (Nagl et al., 2012). In urine of pigs orally administered with D3G, DON as well as deoxynivalenol-15-glucuronide A (D15GlcA) and deoxynivalenol-3-glucuronide B (D3GlcB) were found. However, when intravenously applied, majority of administered D3G was excreted in un-metabolised form via urine. Only traces of DON were detected. These results confirm the hyphothesis that D3G is almost completely hydrolysed in pigs, but only partially absorbed. The cleavage predominantly occurs in the digestive tract, while the toxin seems to be rather stable after systemic absorption. Compared to DON, the proportion of urinary excreted metabolites after oral D3G administration was reduced by factor of 2 (Nagl et al., 2014).

Biotransformation of DON in *Fusarium* resistant and susceptible near isogenic wheat lines was studied (Appendix II). The detected metabolites suggest that DON is conjugated to endogenous metabolites via two major metabolism routes: 1) glycosylation (D3G, deoxynivalenol-di-hexoside, 15-acetyl-deoxynivalenol-3-glucoside, deoxynivalenol-malonylglucoside) and 2) glutathione conjugation (deoxynivalenol-S-glutathione, deoxynivalenol-2H-S-glutathione, deoxynivalenol-S-cysteinyl-glycine, deoxynivalenol-S-cysteine). Furthermore, conjugation of DON to a putative sugar alcohol (hexitol) was found. This study confirms the hypothesis that D3G (although major metabolite) is not the only product of plant metabolism of DON, but the whole spectrum of other metabolites was detected. They might be cleaved in the gastrointestinal tracts in mammals, and thus contribute to the overall DON burden.

4.2 Other masked mycotoxins

A high interest of plant breeders, food and feed producers, toxicologists, and EU authorities had led to the extensive multidisciplinary research in this field.

Beside D3G, also other masked mycotoxins have been identified. For instance, zearalenone-14-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside, (4R)- and (4S)-4-hydroxy-ochratoxin A, β-glucosides of ochratoxin A. However, no extensive studies about their formation in barley have been performed so far.

Monoglucoside derivatives (HT2-3-glucoside and T2-3-glucoside) and diglucoside derivatives (HT2-di-glucoside) have been also reported to be formed *in planta* (Lattanzio et al., 2012; Nakagawa et al., 2013; Veprikova et al., 2012). Additionally, an extensive study of the metabolism of HT-2 and T-2 in wheat was done recently (Nathanail et al., 2015). Besides HT2-and T2-glucosides, HT-2-malonyl-glucoside and feruloyl-T2, as well as acetylation and deacetylation products were described (Nathanail et al., 2015).

A comprehensive study of the metabolism of HT-2 and T-2 in barley using isotope-assisted untargeted screening and quantitative LC-HRMS analysis was performed (Appendix III). This approach allowed to annotate 9 HT-2 and 13 T-2 metabolites. The metabolism routes included hydrolysis of acetyl and isovaleryl groups, hydroxylation as well as covalent binding of glucose, malonic acid, acetic acid and ferulic acid. Additionally, putative isomers of 15-acetyl-T2-tetraol-malonyl-glucoside, hydroxyl-HT2-glucoside, hydroxyl-HT2-malonyl-glucoside, HT-2-di-glucoside, HT2-malonyl-glucoside and feruloyl-T2 were revealed. HT2-3-O-β-glucoside was verified as the major metabolite of HT2 and T2 metabolism which reached its maximum already 1 day after toxin treatment and was subsequently further metabolized.

5 OCCURRENCE OF MYCOTOXINS IN BARLEY

The presence of mycotoxins in barley and other small-grain cereals is common. **Table II** displays an overview of studies reporting the mycotoxin contamination of barley. The list of mycotoxins is restricted to the most common toxins found in barley.

Table II Detected levels of some mycotoxins in barley

Country of origin	Harvest	Mycotoxins	Positive samples (%)	Detected levels (ug/kg)		Reference
				mean of positive samples	range/max. level	
Czech Republic	2008-2011	DON	66	87	213	Belakova et al., 2014
		ZEA	18	2	59	
		HT2+T2	36	36	145	
Czech Republic	2012	NIV	36	93	52–123	Bolechova et al., 2015
		DON	86	284	70–602	
		ZEA	9	193	181–204	
		HT2	45	14	6–57	
		T2	36	24	15–30	
Spain	2007, 2008	NIV	20	20	143	Ibanez-Vea et al., 2012
		DON	95	60	1111	
		3ADON	28	3	20	
		15ADON	57	6	65	
		FUS-X	2	10	17	
		HT2	24	15	201	
		T2	10	35	332	
		DAS	25	1	2	
Italy	2013	NIV	35	96	435	Beccari et al., 2016
		DON	7	39	109	
		D3G	n.d.			
		ZEA	12	2	2	
		FUS-X	2	154	154	
		HT2	65	36	263	
		T2	36	28	138	
		T2 triol	12	20	32	
		T2 tetraol	28	103	260	

The study enclosed as APPENDIX IV documents the incidence of A and B trichothecenes in various barley cultivars harvested in the Czech Republic in the years 2005–2008. As expected DON was detected each year with the overall incidence of 83% and concentration range of 5–181 µg/kg.

Since analytical standard of D3G became available, its co-occurrence with DON could have been confirmed. Contrary to an observation by Berthiller et al. (2009), D3G accompanied the parent DON not in all barley samples but only in 11% for the harvest in 2007. The levels of D3G ranged from 17 µg/kg to 107 µg/kg. When recalculation at D3G/DON molar ratio was applied, the percentage values were in range of 17.8–375.0. These values show that levels of masked analogue were higher than those of parent toxin. Despite the decline of trichothecene B type contamination in the following year, D3G was found in 17% of investigated samples. Interestingly, four out of six samples contained only D3G alone without co-occurrence of its parent toxin.

NIV was detected in all years as well. In 2007 its incidence culminated and NIV was found even in 96% of investigated samples with the mean level of 45 µg/kg and highest level of 140 µg/kg. The incidences in other experimental years were low, below 19% and the levels did not exceed 46 µg/kg.

APPENDIX IV also documents the rapid increase of incidence of HT-2 and T-2 in barley grains during investigated period (2005–2008). The incidence of HT-2 increased from 80% (concentration range was 13–83 µg/kg) to 100% with the levels of 60–718 µg/kg. Compared to HT-2, the incidences as well as levels of T-2 were lower. In the first year only 6% of samples were contaminated with T-2 ranging from 12–31 µg/kg. The highest contamination was also observed in the last year of the study, the incidence of T-2 was 86% with a concentration range from 6 µg/kg to 320 µg/kg.

Furthermore, a newly discovered trichothecene mycotoxin – pentahydroxyscirpene was found in naturally infected spring barley samples harvested in Kromeriz. The three positive samples contained 51 µg/kg PHS and 0.8 mg/kg NIV (PHS/NIV ratio 0.06) – variety Radegast, 65 µg/kg PHS and 1 mg/kg NIV (PHS/NIV ratio 0.06) – variety Bojos, as well as 310 µg/kg PHS and 4.6 mg/kg NIV (PHS/NIV ratio 0.07) in Malz, APPENDIX I.

6 FACTORS INFLUENCING THE LEVELS OF *FUSARIUM* TOXINS IN BARLEY

Although the complete elimination of mycotoxin contamination in agricultural commodities is currently not realistically achievable, a number of strategies have been developed to reduce the growth of mycotoxigenic fungi as well as to decontaminate and/or detoxify mycotoxin contaminated food and feed (Bakak et al., 2006).

Mycotoxins may occur in the field before harvest, during harvesting, or during storage and processing. Therefore, the prevention strategies are needed to be applied during all three periods of grain production, the pre-harvest, the harvest and the post-harvest ones (Bakak et al., 2006). In line with that, several codes of practice applicable for cereals, peanuts and apple products have been elaborated and accepted by Codex Alimentarius. The uniform guidance given in the General Code of Practice is recommended to follow from the beginning of the production chain to prevent mycotoxin contamination. However, local crops, climate and agronomic practices should be taken into account prior to implementation of provisions laid down in this Code (Codex Alimentarius Commission 2002).

In the European countries, the Commission Recommendation 2006/583/EC concerning prevention and reduction of *Fusarium* mycotoxins in cereals and cereal-based products has been set out. The control management of mycotoxin contamination should implement the principles of so-called Good Agricultural Practice (GAP) followed by Good Manufacturing Practice (GMP) involving the recommendations for handling, storage, processing, and the distribution of final products to customers (European Commission (EC) No. 586/2006).

6.1 Pre-harvest period

As mentioned above, the main mycotoxin hazard associated with small grain cereals is represented by *Fusarium* mycotoxins. Their producers, *Fusarium* fungi, occur widespread in nature. *Fusarium* spp. can be parasites, saprophytes or reveal even symbiotrophic properties, depending on the environmental conditions and the growth and health state of plants. *Fusarium* spp. may colonise the plant´s surface as epiphytes, without causing disease symptoms or loss of yield, feeding saprophytically on plant secretions and dead organic matter. On the other hand *Fusarium* fungi can also cause feared plant diseases, such as *Fusarium* head blight (FHB), *Fusarium* root rot and *Fusarium* crown rot. *Fusarium* diseases can be found on different plant organs of various

crop plants such as small grain cereals, maize, numerous vegetables, banana, lilies, trees, and more, all with specific disease patterns (Buerstmayr and Lemmens, 2015).

Concerning the small grain cereals, FHB can result in significant losses in crop yield and grain quality. Therefore, in the past many prevention strategies were rather focused on the reduction of FHB symptoms than on the elimination of mycotoxin contamination. However, the increasing interest in safe food has led to the studying of relationships between *Fusarium* fungi and mycotoxins accumulation. Many recently published studies dealing the effects of various pre-harvest factors are focused not only on the level of FHB disease, but also its correlation with mycotoxin contamination.

Measures that reduce the infection pressure and/or development of FHB on the crop contribute to disease control. Environmental factors and agronomical measures, such as the tilling practice and crop rotation, play an important role. FHB is best controlled by combining farming practices that reduce the disease pressure with planting resistant cultivars and applying fungicide or biocontrol measures if required. Agronomic measures that reduce the abundance of inoculum and therefore the disease pressure are for instance ploughing which removes crop debris from the soil surface and diverse crop rotations (Buerstmayr and Lemmens, 2015). Mainly because of economic reasons, popular farming practices rather promote the abundance of *Fusarium* inoculum and thus disease pressure. For example, the prevalence of maize-rich crop rotations in many parts of Europe and reduced or no-till farming practiced in many cereal-growing regions worldwide increase the abundance of *Fusarium* inoculum. Therefore, cultivation of resistant cultivars and the application of fungicides or biocontrol agents demand continuous attention.

6.1.1 Crop rotation

The effects of crop rotation have been studied in detail. They depend on the preceding crop, whether that crop is a potential host for the pathogens responsible for FHB, and the frequency of the crop concerned in the rotation. The shorter the rotation, the higher is the frequency of the disease. Therefore, head blight is most frequent when the susceptible crop occurs frequently within the rotation. The density of crop residues left in the field, their nutritional value and pathogen competition may also modify the effect of crop rotation (Champeil et al., 2004). Crops other than grass which are not hosts to *Fusarium* species, such as potatoes, sugar beet, clover, alfalfa or vegetables, should be used in rotation to reduce the occurrence of the inoculum in the field. For example, Dill-Macky and Jones (2000) showed that FHB disease severity and DON contamination of

grain was significantly different when the previous crop was maize, wheat or soya. While the highest DON levels were detected in grains growing after maize, the lowest contamination was observed when soya as a pre-crop was used. Soybean crops leave fewer residues than wheat crops, which in turn leave fewer residues than maize crops. Moreover, the amount of left crop residues regulates also the level of nitrogen in the soil which may increase the strength of *Fusarium* population resulting in higher production of mycotoxins (Champeil et al., 2004).

6.1.2 Plant resistance

The fully resistant cultivars against FHB have not been developed so far, hence the disease control relies on the complex agricultural strategies involving the choice of the most suitable cultivar for the intended growing locality.

Development of FHB-resistant cultivars is a major goal of numerous breeding programs across the world. Generally, the resistance/susceptibility of cultivar against FHB is determined by genetic pool responsible for the morphological characteristic of plant (Edwards 2004).

According to the findings from several studies, the mechanism of resistance against FHB can be explained as a defence to the initial penetration (type I resistance) and a resistance to the spreading of a pathogen in the host tissue (type II resistance) (Schröder and Christensen, 1963). Resistant cultivars show besides slower and later development of FHB (incubation period) also a delay in time of fungi sporulation (latent period). A type III resistance was described as insensitivity of plant to the toxin and defined as the ability of the resistant cultivar to degrade DON (Wang and Miller, 1988). The first evidence that resistant cultivars are able to metabolite DON by conversion to a glucoside was provided by Miller and Arnison (1986). Since D3G has been identified as a product of resistance conversion in *Arabidopsis thaliana* (Poppenberger et al., 2003), resistance to DON of different wheat cultivars has been studied based on D3G occurrence. For the first time, Lemmens et al. (2005) revealed that wheat cultivars with the high degree of DON resistance contained high levels of D3G.

6.1.3 Fungicide treatment

A part of an integrated control of FHB in cereal production involves the use of fungicides. Unfortunately, the efficacy of fungicide treatment is highly variable and difficult to predict. The reliability of fungicide protection depends not only on the active

ingredient but also on the way of application as well as on the right timing. In general, fungicide treatment is effective in case that the application is made several days prior to fungi infestation and entirely the active substance covers all ears. Other factors affecting the efficacy of fungicide are weather conditions in time of growth of fungi (Champeil et al., 2004).

Up to now, a wide range of chemical compounds have been tested against FHB pathogens *in vitro*, in glasshouse trials and under the field conditions. Of concern is the fact that a number of fungicides at sub-lethal concentrations stimulate mycotoxin production *in vitro* (D'Mello et al., 1998; Matthies et al., 1999). The findings from various field experiments are rather contradictory with regard to the ability of fungicides to reduce FHB together with reduction in mycotoxin formation. The use of PCR assays for quantification of individual species in the FHB complex provided data about the different activity of some fungicides against these pathogens. For instance, the triazole fungicides metconazole and tebuconazole are active against *Fusarium* species and their use led to the reduction of FHB as well as DON contamination (Edwards et al., 2001; Jennings et al., 2000). The strobilurin fungicide azoxystrobin is active against *Microdochium nivale* (a nontoxigenic species) which competes to other species in FHB complex. Therefore, the application of azoxystrobin led to the reduction of FHB symptoms on one hand, but the stimulation of DON production on the other (Jennings et al., 2000; Simpson et al., 2001).

In order to prevent the development of FHB and consequent formation of mycotoxins, the impact of weather, locality and different agricultural practices on the level of mycotoxin contamination in common malting barley cultivars as well as in newly bred lines was studied within four-year period. The obtained results were statistically evaluated and critically assessed in APPENDIX IV.

Weather was shown as a highly significant factor in barley contamination. The chances in mycotoxin pattern as well as in the concentrations of target toxins could be attributed to weather conditions. For instance, in 2005 NIV occurred rarely, however, high incidence and levels was observed in 2007. Similarly, HT-2 and T-2 were found in barley grains each year at low levels with extreme culmination even exceeded the most common DON in 2008.

Although statistical evaluation did not confirm significant differences between two growing localities, the spectrum of mycotoxins was rather broader in Zabcice than in Kromeriz. Concerning the cultivar resistance/susceptibility to mycotoxin contamination the presence/absence of hull was taken into account. While no significant impact was observed for NIV and DON, the differences in HT-2 and T-2 levels among hulled and covered cultivars were statistically significant. The highest levels of sum of these two toxins, 212 µg/kg and 208 µg/kg, were detected in two hulled cultivars, KM 2084 and Merlin, respectively. Fungicide treatment had no significant effect on the levels of mycotoxins except NIV. Different fungicide preparations based on tebuconazole not only failed in the reduction of mycotoxin contamination but even increased the contents of mycotoxins in samples. To produce safe and health grains, many factors are needed to be taken into consideration already during pre-harvest periods. Due to complexity of this issue, it is always hard to predict if the implemented strategy for the minimization of mycotoxin contamination will be sufficient enough.

7 FATE OF MYCOTOXINS DURING MALT AND BEER PRODUCTION, TOOLS FOR PREVENTION AND CONTROL

7.1 Technology

Beer, one of the most popular beverages around the world, may be also contaminated by mycotoxins which are transferred into this final product from contaminated malt.

Barley malt is produced by germinating grains under regulated conditions of moisture and temperature. Final malt is a source of amylase enzymes, which will break down starch in barley and adjunct grains to produce simpler sugars metabolized by yeast during beer fermentation. To germinate the grain, the barley moisture content is raised to 45% by steeping for 36–52 h at 12–20°C and involves several stages of immersion in aerated water followed by air-rests. The process rinses the barley during drain and fills cycles. After steeping, the seed is germinated at 15–20°C in humidified air. The germinated barley (green malt), is then dried to 4–5% of moisture in a kilning step. Kilning temperatures rarely exceed 90°C and humidity is controlled to prevent inactivation of desired enzymes. A wide range of chemical reactions occurs in barley during kilning. The products originated within these processes affect the final beer qualities, such as flavour, colour, odor and texture (Wolf-Hall, 2007).

Malt is ground to a small particle size and extracted with hot water within the process called mashing. The ground malt is mixed with water and heated at 70°C. During this process, the enzymatic complex coming from malt has the highest activity and cleave polysaccharides to simpler mono- and disaccharides which are fermented by yeasts. The final malt extract is filtrated to separate liquid portion called wort from solids. The spent grains, a by-product obtained within this step, are used as animal feed. The wort is boiled to kill spoilage organisms, then cooled, aerated and the yeast inoculation or pitch is added. The boiling process kills the contaminating fungi, but most mycotoxins will survive this process. The fermentation usually takes 5 to 7 days at 8 to 15°C. The beer is then aged, further carbonated and packaged (Wolf-Hall, 2007). Water soluble mycotoxins tend to remain in the wort.

7.2 Mycotoxin contamination of malt and beer

It seems that mycotoxin contamination of malt arises out of two different sources. Firstly, the initial barley can contain mycotoxins produced by fungi already before harvest. Further contamination can be caused by development of *Fusarium* fungi during malting process (Lancova et al., 2008).

Masked mycotoxins became an issue of high concern when the first beer study dealing with a fate of D3G revealed a significant increases of levels of DON and D3G during germination stage of malting. Authors` hypothesis was that it could be attributed to either their "de novo" formation by growing fungi or their release from the cell compartments by enzymatic activity, or combination of both (Lancova et al., 2008). Moreover, the malt was produced from barley contaminated with DON at very low levels (12 µg/kg and 238 µg/kg in naturally and artificially inoculated barley, respectively). The levels of D3G were below 5 µg/kg and 14 µg/kg. The levels in final malt was increased almost 4 times (natural contamination) and 2 times (artificial contamination) for DON. D3G was increased by factor of 9 in artificially contaminated malt (Lancova et al., 2008). The follow-up study with fungicide treated and untreated grains confirmed that the increase of DON and D3G was observed even when no alive *Fusarium* spores were detected by PCR (Zachariasova et al., 2012). Moreover, deoxynivalenol-di-glucosides and deoxynivalenol-tri-glucosides were detected in naturally infected green malt and their increase was observed during germination as well. The increase of D3G and corresponding oligoglucosides seems to be attributed to enzymatic activity in germinating grains. Masked forms of DON are either created by glucosidation (Maul et al., 2012) or released from the "masked mycotoxin pool" originating from the field (Zachariasova et al., 2012).

Further significant increase of DON and its masked forms in beer production was observed during mashing (Lancova et al., 2008; Zachariasova at al., 2012). The origination of this increase was likely related to cleavage of polysaccharides (Lancova et al., 2008).

7.3 Tools for prevention and control of mycotoxin levels in malt and beer

Mycotoxins are naturally occurring toxins. Therefore, to overcome their presence in small-grain cereals is almost impossible. However, following the rules of GAP together

with regular control of mycotoxin levels in raw materials should help to minimize health risk related to chronic exposure to these toxins.

Recent common analytical methods to mycotoxin control in cereals can be classified as screening and confirmatory methods. Fast screening methods represented mainly by Enzyme-Linked ImmunoSorbent Assays (ELISAs) or Lateral Flow Devices (LFDs) allow to obtain only semi-quantitative results. However, for the first informative control of the harvest it is sufficient enough.

The use of confirmatory methods is requested by the EU authorities (European Commission (EC) No. 2002/657/EC). Compared to the rapid screening methods, they are more expensive and require an experienced operator. Nowadays, mainly LC-MS is used for mycotoxin determination.

Analytical approaches used in beer analysis of mycotoxins were reviewed in APPENDIX VII. The analytical techniques discussed involve gas chromatography coupled with flame ionization detection, electron capture detection and mass spectrometry as well as liquid chromatography hyphenated to ultra-violet detection and mass spectrometry. Special attention was paid to samples preparation which represents a crucial step in beer analysis. In addition, also screening methods such as ELISAs are involved.

The final levels of DON and its masked form in malt and beer are affected by many factors. It depends on the barley variety, *Fusarium* infection extent and the way of plant growing (e.g. fungicide treatment). Therefore, apparently "mycotoxin free" barley grains might be subsequently processed in highly contaminated beer.

Our aim was to propose/develop a simple screening test based on chemical/enzymatic hydrolysis followed by commercially available ELISA kits. Such a test should have been a cheap and easy-to-use tool in prediction of "total mycotoxin pool" of raw barley grain and thus prevent production of beer with high level of mycotoxins.

Chemical hydrolysis of D3G, 3ADON and 15ADON using various anorganic acids and potassium hydroxide was investigated on barley, wheat and maize in order to cleave masked DONs into DON and determine indirectly as a "total DON" with a commercially available ELISA kit. Unfortunately, the main metabolite D3G is totally resistant to any acidic and alkaline conditions or it is rapidly destroyed without DON release (APPENDIX V). Therefore, it was concluded that chemical hydrolysis cannot be used for this purpose.

Indeed, more specific conditions needed to be used. Hence, the second study focused on the enzymatic cleavage was performed (APPENDIX VI). Ability of several fungal and recombinant bacterial β-glucosidases to hydrolyze D3G was tested. Furthermore, substrate specificities of two fungal and two bacterial glycoside hydrolase family 3 β-glucosidases were evaluated on a broader range of substrates. The purified β recombinant enzyme from *Bifidobacterium adolescentis* (BaBgl) displayed high flexibility in substrate specificity and exerted the highest hydrolytic activity towards 3-O-β-D-glucosides of DON, NIV, and HT-2. A Km of 5.4 mM and a Vmax of 16 µmol/min/mg were determined with D3G. Due to low product inhibition (DON and glucose) and sufficient activity in several extracts of cereal matrices, this enzyme has the potential to be used for indirect analysis of trichothecene-β-glucosides in cereal samples.

An LC-MS/MS method was developed for determination of DON, D3G and 3ADON in different types of beer (APPENDIX VIII). The simple sample preparation including degassing, precipitation of matrix compounds and reconstitution of the dried-down sample in solvent. Beer was categorized into pale, wheat, dark, bock and non-alcoholic beers and shandies. Final method was then optimized and validated for six different types of beer. Accuracy of the method was expressed as repeatability (4–16% considering all analytes and beer types), apparent recovery of 60–90% for DON, 39–69% for D3G and 96–124% for 3ADON. Limits of detection were in range of 1.0–2.9 µg/l for DON, 0.4–1.4 µg/l for D3G and 2.2–4.3 for 3ADON. Limits of quantification were estimated as 4.1–11.0 µg/l for DON, 1.3–4.1 µg/l for D3G and 6.0–11.0 for 3ADON. Dark and bock beer represented to most difficult matrix for analysis.

The validated method was applied to an extensive survey of 374 beer samples from 38 countries with a focus on Austria (156) and German (64) beers (APPENDIX IX). Beers were assigned to six categories – pale (217), wheat (46), dark (47), bock (20), nonalcoholic (19) and shandies (25). DON and its masked form D3G were found in 93% and 77% of samples, respectively. Whereas, 3ADON was not detected in any of tested beers. Non-alcoholic beer and shandies showed the lowest contamination – average levels were 2.7 µg/l and 4.4 µg/l for DON, and 1.5 µg/l and 3.2 µg/l for D3G. In bock beers characterized by a higher gravity, a significant trichothecene content of 12.4 µg/l (DON) and 14.8 µg/l (D3G) was found. The highest contamination was detected in a pale beer from Austria (89 µg/l DON and 81 µg/l D3G).

8 CURRENT REGULATION

Mycotoxins are regulated in more than 77 countries worldwide (FAO 2004), while regulations vary from country to country depending on the type of mycotoxin, matrix (type of food or feed) as well as the maximum levels. In the countries of European community, maximum levels for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone and fumonisins in various foodstuffs have been established by Commission Regulation (EC) No 1881/2006 (European Commission, 2006). The laid down levels of Fusarium toxins in maize and maize product has been amended a year later by Commission Regulation (EC) No 1126/2007 (European Commission, 2007).

The current maximum levels of *Fusarium* toxins for various foodstuffs are listed in **Table III**.

Maximum limits for HT-2 and T-2 in unprocessed cereals and cereal products for human consumption should have been laid down but they are not established yet due to lack of HT-2 and T-2 occurrence data. On this account the EFSA the scientific and control laboratories in one of its last "Calls for data" for providing the data concerning the levels of HT-2 and T-2 toxins in food and feed (http://www.efsa.europa.eu).

Maximum limits for masked mycotoxins are not established yet as well due to the lack of data. However, experts of EFSA´s panel on contaminants in the food chain are currently working on the Scientific Opinion on the risks for animal and public health related to the presence of deoxynivalenol, metabolites of deoxynivalenol and masked deoxynivalenol in food and feed (https://www.efsa.europa.eu/en/topics/topic/mycotoxins).

Table III Maximum levels for *Fusarium* toxins established by Commission Regulation (EC) 1881/2006 and (EC) 1126/2007

Mycotoxin	Food stuffs	µg/kg
DON	Unprocessed cereals other than durum wheat, oat, and maize	1250
	Unprocessed durum wheat and oats	1750
	Unprocessed maize, with exception of unprocessed maize intended to be processed by wet milling	1750*
	Cereals intended for direct human consumption, cereal four, bran and germ as end product marketed for direct human consumption with the exception of processed cereal-based foods, baby foods and infant food, and maize milling fraction mentioned below	750
	Pasta (dry)	750
	Bread (including small bakery wares), pastries, biscuits, cereals snacks and breakfast	500
	Processed cereal-based foods and baby foods for infants and young children	200
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	750*
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	1250*
ZEA	Unprocessed cereals other than maize	100
	Unprocessed maize, with exception of unprocessed maize intended to be processed by wet milling	350*
	Cereals intended for direct human consumption, cereal four, bran and germ as end product marketed for direct human consumption with the exception of processed cereal-based foods, baby foods and infant food, and maize milling fraction mentioned below	75
	Refined maize oil	400*
	Bread (including small bakery wares), pastries, biscuits, cereals snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100*
	Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
	Processed maize-based foods for infants and young children	20*
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	200*

Table III Continued

ZEA	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	300*
Sum of FB1 and FB2	Unprocessed maize, with exception of unprocessed maize intended to be processed by wet milling	4000*
	Maize intended for direct human consumption, maize-based foods for direct human consumption, with exception maize-based breakfast cereals and snacks, maize based foods for infants and young children	1000*
	Maize-based breakfast cereals and maize-based snacks	800*
	Processed maize-based foods for infants and young children	200
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	1400*
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	2000*

*Levels amended by EC 1126/2007

9 CONCLUSIONS AND FUTURE OUTLOOKS

The presented PhD thesis brought new knowledge in the fundamental and applied research in the field of mycotoxins. The issue of food and feed chain contamination with mycotoxins is very complex. Therefore interdisciplinary research is required to help to eliminate the presence of mycotoxin in cereals.

The main attention was paid to mycotoxin contamination of barley and its consequences, i.e. transfer of these contaminants into malt and beer. Nowadays, issue of a high importance is masked mycotoxins, especially when considering beer production. Masked forms of parent mycotoxins can be released during malting and brewing, hence the contamination of beer is much higher than expected (does not correspond with the used raw material).

Fusarium mycotoxins are formed already on the field. Therefore, the effective control of mycotoxin contamination has to start at the beginning of the production chain, i.e. the use of suitable cultivar for intended growing locality, fungicide treatment etc. The knowledge about the fungi/plant interactions, plant defence as well as detoxification of mycotoxins in plants and its mechanism are crucial for plant breeders. Investigation of DON and T-2 metabolism in barley and wheat was carried out and revealed that a broad spectrum of masked mycotoxins is actually formed. For this purpose, HPLC-HRMS techniques was required.

Accurate and sensitive methods are necessary for routine control at all stages of the food chain (beer production). Recently, mainly LC-MS/MS methods are used for daily control of mycotoxin contamination in cereals. However, also rapid/screening methods are feasible, in some cases even irrecoverable. Development of both types of the methods was one aim of this PhD thesis. Easy and fast method based on chemical and enzymatic cleavage of masked mycotoxins was developed and critically assessed. The one based on the enzymatic cleavage has a huge potential to be applied as a screening tool for malt producers. Sensitive and robust LC-MS/MS method was developed and validated for six types of beer. The method was then used for conduction of up to date biggest beer survey of 374. Data obtained within this study were reported to EFSA and were used in calculation of risk assessment related to the exposure of DON, D3G and 3ADON in a Scientific Opinion on the risks for animal and public health related to the presence of deoxynivalenol, metabolites of deoxynivalenol and masked deoxynivalenol in food and feed (https://www.efsa.europa.eu/en/topics/topic/mycotoxins).

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11 SYMBOLS AND ABBREVIATIONS

ATA	Alimentary Toxic Aleukia
15ADON	15-acetyldeoxynivalenol
3ADON	3-acetyldeoxynivalenol
D15GlcA	Deoxynivalenol-15-Glucuronide A
D3G	Deoxynivalenol-3-Glucoside
D3GlcB	Deoxynivalenol-3-Glucuronide B
DAS	Deacetoxyscirpenol
DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked ImmunoSorbent Assay
FHB	Fusarium Head Blight
FUS-X	Fusarenon-X
GAP	Good Agricultural Practice
GMP	Good Manufactural Practice
HT-2	HT-2 Toxin
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LFD	Lateral Flow Device
NEO	Neosolaniol
NIV	Nivalenol
PCR	Polymerase Chain Reaction
PhD	Philosofiae Doctor
PHS	Pentahydroxyscirpene
T-2	T-2 toxin
U-HPLC-HRMS	Ultra-high Performance Chromatography coupled to High Resolution Mass Spectrometry
ZEA	Zearalenone

12 LIST OF PUBLICATIONS

12.1 Journal papers in English language

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13 APPENDIXES

Appendix I

Varga E, Wiesenberger G, Frumann P, Malachova A, Svoboda T, Lemmens M, Adam G, Berthiller F, 2016. Pentahydroxyscirpene – producing strains, formation in planta, and natural occurrence. Toxins, 8, 295–309.

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APPENDIX I

Varga E, Wiesenberger G, Frumann P, Malachova A, Svoboda T, Lemmens M, Adam G, Berthiller F

Pentahydroxyscirpene – producing strains, formation in planta, and natural occurrence

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Pentahydroxyscirpene—Producing Strains, Formation In Planta, and Natural Occurrence

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Abstract: Trichothecenes are a class of structurally diverse mycotoxins with more than 200 naturally occurring compounds. Previously, a new compound, pentahydroxyscirpene (PHS), was reported as a byproduct of a nivalenol producing *Fusarium* strain, IFA189. PHS contains a hydroxy group at C-8 instead of the keto group of type B trichothecenes. In this work, we demonstrate that IFA189 belongs to the species *Fusarium kyushuense* using molecular tools. Production of PHS in vitro was also observed for several isolates of other *Fusarium* species producing nivalenol. Furthermore, we report the formation of 4-acetyl-PHS by *F. kyushuense* on inoculated rice. Wheat ears of the variety Remus were infected with IFA189 and the in planta production of PHS was confirmed. Natural occurrence of PHS was verified in barley samples from the Czech Republic using a liquid chromatographic-tandem mass spectrometric method validated for this purpose. Toxicity of PHS to wheat ribosomes was evaluated with a coupled in vitro transcription and translation assay, which showed that PHS inhibits protein biosynthesis slightly less than nivalenol and deoxynivalenol.

Keywords: mycotoxins; LC-MS/MS; taxonomy; toxicity; cereals

1. Introduction

Mycotoxins are secondary metabolites of molds, which are toxic to humans and animals. While the Food and Agriculture Organization of the United Nations (FAO) estimated in the 1990s that about a quarter of all agricultural commodities worldwide are significantly contaminated with mycotoxins [1], more recent findings suggest an even higher rate [2]. In temperate regions of the world, *Fusarium* spp. are the most commonly occurring toxigenic fungi on crops. Thereof, the most important species is *Fusarium graminearum*, which can infect a wide range of cereals including wheat, barley, oats, rye, or maize [3]. Species within the *F. graminearum* complex can produce type B trichothecenes in planta [4,5]. Based on the different ability to hydroxylate C-4 either deoxynivalenol (DON, Figure 1a.) or nivalenol (NIV, Figure 1b) chemotypes are distinguished. DON producing

strains of *F. graminearum* contain a loss of function allele of the *TRI13* (cytochrome P450) gene [6]. Members of the DON chemotype are usually subdivided into 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) strains, based on which acetylated precursor accumulates in vitro, caused by allelic differences in the *TRI8* encoded esterase [7,8]. In most NIV strains 4-acetylnivalenol (fusarenon X, FUSX) co-occurs. During plant infection the acetyl groups are also removed by plant carboxylesterases [9]. Trichothecenes are chemically and thermally very stable and are readily carried over from raw cereals into processed food (e.g., [10]).

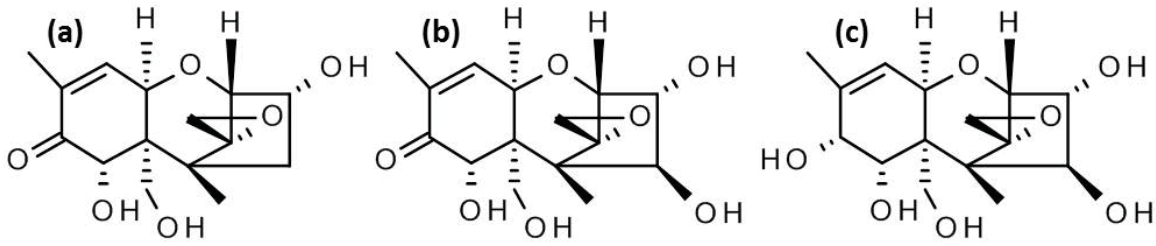

Figure 1. Chemical structures of (**a**) deoxynivalenol; (**b**) nivalenol; and (**c**) pentahydroxyscirpene.

In sensitive species, e.g., humans or pigs, type B trichothecenes elicit anorexia, abdominal distress, malaise, diarrhea, emesis, impaired weight gain, gastroenteritis, and immunotoxicity (reviewed by [11]). While the major mode of action is the binding of the toxins to eukaryotic ribosomes and interference with protein translation, also intracellular protein kinases are activated, which mediate both selective gene expression and apoptosis (reviewed by [12]). In 2010, the Joint FAO/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) decided to convert the provisional maximum tolerable daily intake (PMTDI) for DON to a group PMTDI of 1 μg/kg body weight (b.w.) for DON and its acetylated derivatives (3ADON and 15ADON) [13]. Likewise, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) established a tolerable daily intake (TDI) value of 1.2 μg/kg b.w. for NIV [14]. To cope with the threat for consumer safety, maximum levels in food are enforced for DON in many countries—including the European Union [15]—while currently no regulations for NIV are in force.

Only recently a novel mycotoxin, pentahydroxyscirpene (PHS, Figure 1c), which is structurally closely related to NIV was identified [16]. PHS was formed upon inoculation of rice with a NIV producing *Fusarium* strain in a level of roughly 10% of that of NIV. It was proposed that NIV and PHS share the common precursor 7,8-dihydroxy-3,4,15-triacetoxyscirpenol, which leads to the formation of PHS if the function of (a yet unknown) oxidoreductase is impaired. This intermediate is excreted and deacetylated before the keto group of NIV is formed. The occurrence and relevance in plant pathogen interaction and toxicological significance of this compound is yet unknown.

In this study, the molecular identification of the PHS producer IFA189 is reported and other selected NIV-producing *Fusarium* strains and species were characterized regarding the ability to form PHS. We furthermore investigated the in vitro toxicity of PHS to wheat ribosomes and whether PHS is formed in planta.

2. Results

2.1. Development and Validation of the Liquid Chromatographic-Tandem Mass Spectrometric (LC-MS/MS) Method

Mass spectrometric parameters were optimized for the target compounds (PHS, NIV, FUSX) using syringe injection of single analyte solutions and are summarized in Table 1. All analytes showed higher signal intensities in negative electrospray ionization mode. In case of PHS the deprotonated ion was chosen as precursor, whereas for the other two compounds the acetate adducts showed the highest signal intensity. While PHS is eluting quite early under reversed phase chromatographic conditions,

a retention factor k' of approximately 1.2 could be achieved with selecting 5% methanol (MeOH) as the starting mobile phase for the linear gradient.

Table 1. List of analytes with optimized mass spectrometric parameters.

Analyte	Retention Time (min)	*m/z* Precursor Ion (Declustering Potential in V)	Product Ions (Collision Energy in eV)
Pentahydroxyscirpene	4.0	313.1 (−110)	163.1 (−39) 175.0 (−30) 191.1 (−21)
Nivalenol	4.8	371.1 (−75)	59.1 (−42) 281.1 (−22)
Fusarenon X	6.1	413.2 (−70)	59.1 (−44) 263.0 (−22)

The method was validated for barley by spiking blank samples with PHS and NIV before extraction (with two different solvents) on five different levels in triplicate and after extraction on one level in five replicates. For the acetonitrile (ACN) based extraction, the determined apparent recoveries were 137% for PHS and 98% for NIV. A significant signal enhancement (174% for PHS and 114% for NIV) was observed. While rather uncommon for (more apolar) type B trichothecenes, the signal enhancement for the very polar PHS is seemingly caused by early eluting matrix components. The resulting extraction recoveries were 79% and 86% for PHS and NIV respectively, and relative standard deviations of less than 11% were achieved. Very similar results were obtained using the more polar MeOH based extraction method. There, the extraction recoveries were 103% and 91% for PHS and NIV, while the apparent recoveries were 173% for PHS (due to matrix effects) and 99% for NIV. Relative standard deviations were about 10% for both analytes. The suitability of the used acidified ACN:water mixture to also extract a variety of other mycotoxins [17] resulted in the preferred usage of this solvent.

2.2. Molecular Identification of IFA189 as Fusarium kyushuense

The PHS producing strain IFA189 had initially been received as *Fusarium sporotrichioides* suitable for NIV production (see discussion). To clarify its taxonomic status, we used the "Fusarium ID" approach described by Geiser et al. [18]. Part of Translation Elongation Factor 1α (TEF1α) was amplified using primers ef1 and ef2. The resulting polymerase chain reaction (PCR) product was sequenced with nested primers EF15fw and EF16rev [19] and the resulting sequence was blasted against sequences deposited at the Fusarium ID database [20] and at the National Center for Biotechnology Information [21]. A fragment of 646 base pairs (bp) showed 99.84% identity to TEF1α from *F. kyushuense* at Fusarium ID, while the blast search at NCBI revealed 100% identity to a 668 bp fragment form TEF1α of the same species (*Fusarium kyushuense* NRRL 6490, accession number AB674297.1). Thus we conclude that strain IFA189 is *Fusarium kyushuense*.

2.3. PHS Production by Other NIV Producers

NIV was first isolated from the "*Fusarium nivale*" strain Fn-2B (sometimes also termed Fn2B or FN-2B in various references), which eventually was recognized to be *F. kyushuense* (see conclusions below). The rice material used for feeding trials was obtained using this strain and also NIV for toxicological studies was purified from it. We therefore obtained all available *F. kyushuense* strains from the Agricultural Research Service (ARS) Culture Collection (NRRL) strain collection and tested them for PHS production. We furthermore set out to test whether NIV producing isolates from other species also produce significant amounts of PHS. Different known and suspected (based on genotyping) NIV-producing strains belonging to the genus *Fusarium* (*F. asiaticum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, and *F. kyushuense*) from our strain collections were selected for the screening

of PHS. The taxonomic classification of all used strains was confirmed by TEF1α sequencing. In extracts from five *F. graminearum* and one *F. equiseti* isolates the presence of PHS was confirmed, but at a far lower scale than in *F. kyushuense* extracts (Table 2). Also, three of the six *F. kyushuense* strains from the NRRL collection produced PHS on autoclaved rice.

Table 2. Screening for pentahydroxyscirpene (PHS) production of different *Fusarium* strains. Confirmed PHS producing strains are shown in bold letters.

Fusarium Strains	PHS (mg/kg)	NIV (mg/kg)	FUSX (mg/kg)
F. asiaticum SCK04 (#1) [1,2]	<0.15	4.84	67.0
F. asiaticum SCK04 (#1, PDA) [3]	<0.15	5.24	42.4
F. asiaticum SCK04 (#2) [2]	<0.15	2.65	12.1
F. asiaticum SCK04 (#2, PDA) [3]	<0.15	3.26	20.8
F. culmorum IFA450	<0.15	0.15	0.74
F. equiseti IFA33	<0.15	0.23	0.86
F. equiseti IFA34	<0.15	<0.012	<0.04
F. equiseti IFA63	<0.15	0.07	0.16
F. equiseti IFA64	<0.15	0.28	1.23
F. equiseti IFA157	<0.15	<0.012	0.05
F. equiseti IFA336	<0.15	<0.012	<0.04
F. equiseti IFA408	<0.15	0.036	0.15
F. equiseti IFA409	<0.15	0.10	0.33
***F. equiseti* IFA410**	**0.23**	**60.0**	**110**
F. graminearum DAGZ5	<0.15	0.67	20.0
***F. graminearum* DAGZ8, gray mycelium** [4]	**0.94**	**294**	**580**
F. graminearum DAGZ8, pink mycelium [4]	<0.15	5.00	212
***F. graminearum* DAGZ13**	**0.17**	**212**	**>6000**
***F. graminearum* DAGZ13 (PDA)** [3]	**0.29**	**238**	**>5000**
F. graminearum DAGZ22	<0.15	0.37	43.6
F. graminearum DAGZ23	<0.15	2.71	49.6
***F. graminearum* DAGZ24**	**0.49**	**88.8**	**788**
F. graminearum DAGZ25	<0.15	0.10	4.84
F. graminearum DAGZ29	<0.15	10.3	242
F. graminearum DAGZ31	<0.15	44.0	107
F. graminearum DAGZ36	<0.15	0.25	21.9
F. graminearum DAGZ37	<0.15	1.91	33.4
F. graminearum DAGZ39	<0.15	0.14	1.68
***F. graminearum* DAGZ46**	**1.44**	**399**	**1370**
F. graminearum DAGZ47	<0.15	7.72	820
***F. graminearum* DAGZ50 (#1)** [2]	**1.65**	**199**	**283**
***F. graminearum* DAGZ50 (#2)** [2]	**1.04**	**359**	**>1000**
***F. graminearum* DAGZ50 (PDA)** [3]	**2.93**	**>900**	**>3000**
F. graminearum DAGZ55	<0.15	32.2	378
F. graminearum DAGZ62	<0.15	13.4	133
F. graminearum DAGZ63 (#1) [2]	<0.15	<0.012	16.0
F. graminearum DAGZ63 (#1, PDA) [2,3]	<0.15	0.32	0.568
F. graminearum DAGZ63 (#2) [2]	<0.15	0.06	0.94
F. graminearum NRRL 26752	<0.15	1.46	2.42
F. graminearum NRRL 26752 (PDA) [3]	<0.15	1.26	1.48
***F. kyushuense* IFA189 (#1)** [2]	**110**	**1370**	**1980**
***F. kyushuense* IFA189 (#2)** [2]	**22.7**	**536**	**4560**
F. kyushuense NRRL 3509	<0.15	<0.012	<0.04
F. kyushuense NRRL 3510	<0.15	0.03	0.11
***F. kyushuense* NRRL 6490 (=Fn-2B)**	**10.0**	**404**	**2890**
***F. kyushuense* NRRL 6491**	**3.57**	**468**	**2930**
***F. kyushuense* NRRL 25348 (backup of 6490)**	**15.0**	**328**	**1440**
F. kyushuense NRRL 26204	<0.15	0.07	0.15

NIV—nivalenol; FUSX—fusarenon-X; [1] according to Kim et al. [22]; [2] two isolates (#1, #2) of the same strain were available; [3] PDA: potato dextrose agar, all other strains were originally cultivated on Fusarium minimal medium (FMM); [4] fungus on FMM medium was either grayish with air mycelium or reddish-pink without air mycelium, agar plugs were taken from either sector.

In one paper, it is claimed that *F. kyushuense* contains aflatoxin biosynthesesis genes and is able to produce aflatoxins [23]. Using the published primer sequences we were unable to obtain the described nor-PCR product in IFA189 or the strain used in the publication (NRRL 3509). At low stringency PCR fragments of the expected size (300 bp) were produced, isolated, and cloned into the vector pCR®4-TOPO®. DNA sequences from 12 transformants contained inserts with no homology to the nor1 gene from any *Aspergillus* species. Using LC-MS/MS analysis, neither aflatoxin B_1 nor aflatoxin G_1 were detected in extracts of the cultures (detection limit of the method 0.05 µg/kg), thus disproving the previous report.

2.4. Tentative Identification of 4-Acetyl-Pentahydroxyscirpene

As evident from Table 2, most strains produced more FUSX than NIV under the conditions used. FUSX, the presumed biosynthetic precursor of NIV, differs from NIV only by an acetyl-group at C-4. Likewise an acetylated form of PHS is likely to exist (see also Scheme 2 in [16]). Extracts of the *F. kyushuense* IFA189 rice cultures were measured with ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight mass spectrometer (QTOF). The mass spectrum of a peak at 6.35 min showed ions with m/z 355.1396 and m/z 401.1448. These ions closely match with the calculated $[M - H]^-$ ion (m/z 355.1398, $\Delta m = -0.6$ ppm) and the $[M + HCOO]^-$ (m/z 401.1453, $\Delta m = -1.2$ ppm) ion of a $C_{17}H_{24}O_8$ compound, thus verifying the expected sum formula. Tandem mass spectrometric measurements supported the hypothesis of a PHS metabolite since fragments of acetyl-PHS (Figure 2) matched those of PHS (see supporting information of [16]). The site of conjugation cannot be pinpointed by LC-MS/MS alone, but equally as with FUSX, the C-4 position seems very likely. Due to the lack of a standard, no absolute quantification of the identified acetyl-PHS was possible.

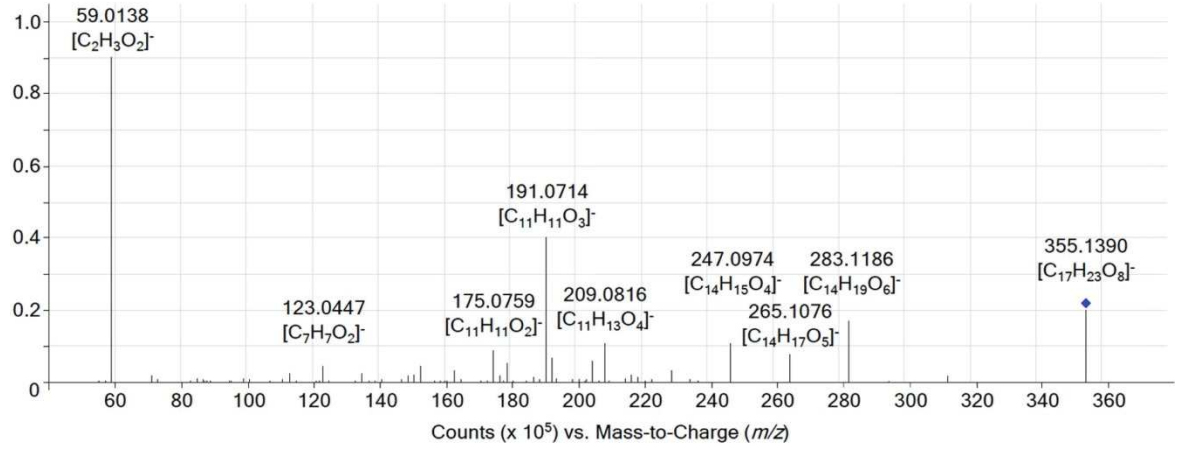

Figure 2. High resolution tandem mass spectrometric product ion scan of the tentatively identified 4-acetyl-pentahydroxyscirpene in negative electrospray ionization mode at a collision energy of 15 eV.

2.5. PHS-Impurity in Commercial NIV-Standards

Seemingly *Fusarium* strains able to produce (high concentrations of) NIV, also produce PHS. As the polarity of both substances is similar, it is conceivable that purified nivalenol standards might also contain PHS. Purity determination might be compromised by the low absorbance of PHS (no conjugated double bond) as well as the similar molecular mass (difference of 2 amu) and structure. Commonly used methods for purity determination of natural toxins, like type B trichothecenes, include LC-UV methods, nuclear magnetic resonance (NMR), or elemental analysis [24]. Given the nature of the compound, it is likely that those methods will fail to identify PHS as impurity in NIV. Therefore, we acquired commercially available NIV standards (solid or calibrants) to determine PHS. The certified reference material IRMM-316 (liquid calibrant) contained 0.2% PHS in relation to NIV. In the liquid

calibrants obtained from Romer Labs from 2006 to 2012, up to 20.8% PHS were detected, whereas all older and newer NIV-calibrants contained only up to 0.2% PHS. It has to be mentioned though that, despite the contamination with PHS, the measured concentration of NIV in all liquid calibrants from Romer Labs was within 98%–102% of the indicated NIV concentration. The IRMM standard, however, seemingly contained only 92% of the certified concentration. In the solid nivalenol hydrate standard (with an indicated purity of 98%) purchased from Santa Cruz (Santa Cruz, CA, USA) actually 3.7% PHS were quantified, while in solid NIV standards from *F. nivale* obtained from Sigma Aldrich (Vienna, Austria)(purity $\geq$ 98%) less than 0.1% PHS were determined by LC-MS/MS measurements.

2.6. Natural Occurrence of PHS

As a first step to test whether PHS is also produced in planta and might occur naturally, we inoculated wheat ears of the Fusarium susceptible variety "Remus" [25] at anthesis by injecting a spore suspension of strain IFA189 into two outer florets. After five weeks, the ears were visually inspected. The observed symptoms were much weaker than with *F. graminearum* and only local infection of single spikelets expressed as brown necrotic lesions were visible. Similar necrotic lesions were visible after the treatment of the wheat ears with solutions of NIV or PHS. A wheat ear treated with the spore suspension culture was milled, extracted, and measured with the LC-MS/MS method. The determined concentrations were 1.8 mg/kg for PHS and 12 mg/kg for NIV resulting in a PHS to NIV-ratio of 0.15.

Subsequently, we re-analyzed naturally infected grain samples with high NIV concentrations. The presence of PHS in naturally contaminated samples was for the first time confirmed in barley samples from Kromeriz (a town in the Zlin region of the Czech Republic). The three positive samples contained 51 µg/kg PHS and 0.8 mg/kg NIV (PHS/NIV ratio 0.06), 65 µg/kg PHS and 1 mg/kg NIV (PHS/NIV ratio 0.06), as well as 310 µg/kg PHS and 4.6 mg/kg NIV (PHS/NIV ratio 0.07).

2.7. Toxicity of PHS to Wheat Ribosomes

Trichothecenes bind to the large subunit of eukaryotic ribosomes and inhibit translation [26]. To test the toxicity of PHS towards plant ribosomes we performed in vitro translation experiments using a commercial wheat germ extract (Figure 3). The IC_{50} values for NIV, DON, and PHS are 0.75, 1.4, and 1.5, respectively. Therefore, we conclude that PHS efficiently inhibits translation and is only slightly less toxic than DON or NIV for plant ribosomes and thus is likely to contribute to virulence.

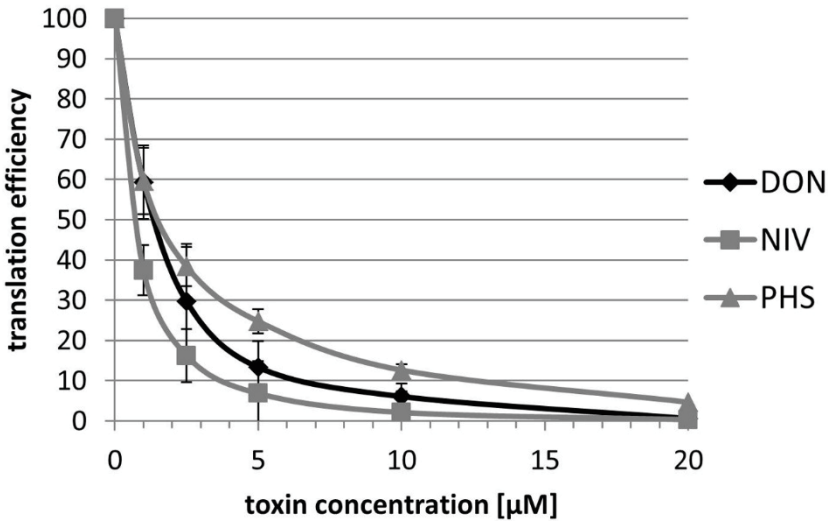

Figure 3. Inhibition of in vitro translation by DON, NIV, and PHS.

3. Discussion

Strain IFA189 was received about 20 years ago, at that time classified as *F. sporotrichioides* suitable for the production of FUSX and NIV (Prof. H. Pettersson, personal communication). Molecular identification revealed that it actually is a *F. kyushuense* strain, and most likely identical to Fn-2B [27]. The toxic principle nivalenol was isolated for the first time in 1968 [28] from isolate Fn-2B, at that time designated "*Fusarium nivale*" (*F. nivale* is now *Microdochium nivale* and considered to be non-toxinogenic [29]). Strain Fn-2B has a changeful taxonomic history. In 1984, it was described as *Fusarium tricinctum* producing NIV and FUSX [30]. It is deposited as NRRL 6490 (and backup NRRL 25348). IFA189 and these two strains produced the highest levels and proportion of PHS in our hands (see Table 2). In the monography of "Toxigenic *Fusarium* species" Fn-2B [31] was classified as *F. sporotrichioides*. Then, based on partial (low quality) ribosomal RNA sequences, it was proposed to be close to *Fusarium poae* in 1995 [32]. Eventually, in 1998 the new species *Fusarium kyushuense* was established, to which this strain belongs according to current molecular taxonomy [33].

The early toxicological experiments with NIV were performed with material derived from strain Fn-2B [34–36]. Potentially, PHS was present in a few percent, but this would have only slightly confounded the toxicological results, as PHS has, at the level of the ribosomal target, very similar effects as NIV both in animals and plants based on in vitro translation systems ([16] and this study). However, it is unknown if relevant differences between NIV and PHS in adsorption, metabolism, and excretion exist in animals. For some time, it was controversial whether nivalenol (or material co-eluting with NIV) is genotoxic, causing induction of chromosome aberrations and DNA damage (for review see: [14]). We now can exclude that contamination with aflatoxin—supposedly produced by *F. kyushuense* strains—is a cause for this, as previously claimed [23]. We were unable to detect aflatoxins, both in IFA189 and in NRRL 3509, the strain for which aflatoxin production had been reported. Furthermore, neither strain yielded a nor-1 specific PCR fragment. In agreement with these results, no aflatoxin biosynthesis genes were found in the unpublished whole genome sequence of the *F. kyushuense* strain NRRL 25348 (Robert Proctor, personal communication).

According to the information from the strain collection, NRRL 6491 (=old Fn-M) is producing FUSX, which we could confirm. Yet, *F. kyushuense* strains, e.g., NRRL 3510, are known, which produce primarily the type A trichothecenes T-2 toxin, HT-2 toxin and neosolaniol. In our hands, only a very low level of NIV was detected in extracts of this and other strains (Table 2). Potentially, in the *F. kyushuense* population, strains might co-exist that either hydroxylate only C-8 (either possessing a *TRI1* product like *F. sporotrichioides* [37], or a TRI1p that hydroxylates initially both C-7 and C-8 [38]). The C-8 hydroxy group is subsequently converted to the keto group in NIV or remains a hydroxy group in case of PHS. To produce PHS, this last step must be slow so that the acetylated intermediate can be excreted and deacetylated. Potentially, a variable mix of only C-8 or C-7 & C-8 hydroxylated trichothecenes might be produced. NRRL 3510 produces only minor amounts of NIV, while the NIV-producers make hardly any T-2 toxin, which does not support this speculation. Yet, strains are known which are able to simultaneously produce type A and B trichothecenes, as described for individual isolates of *F. poae* that can co-produce NIV and T-2 toxin plus neosolaniol [39,40] and potentially also PHS. Such "flexible" strains may have an advantage on highly variable host populations, as type B trichothecenes can form Michael adducts with glutathione, while type A trichothecenes lacking the conjugated keto-double bond cannot [41,42]. We could show that PHS is not only produced in culture, but also in planta. As NIV and PHS have similar toxicity for wheat ribosomes, there is seemingly only a small fitness price to pay for the potential advantage that the virulence factor produced by the pathogen can escape glutathione mediated detoxification, if PHS is formed rather than NIV. In this study, we have also presented evidence for the existence of the proposed 4-acetyl-PHS. This is expected if—like in case of NIV—the removal of the C-3 and C-15 acetyl group is faster than removal of the C-4 acetate, as in case of FUSX.

The finding that besides *F. kyushuense* also other high level NIV producing strains may generate a few percent of PHS as byproduct indicates that the toxicological burden of NIV contaminated

grain may be slightly higher than anticipated. Our study also shows that PHS can occur in naturally infected grain. Yet this effect is small and toxicologically most likely not relevant. NIV standards may be contaminated with a few percent PHS, which could potentially lead to a small systematic error. Yet, due to increasing awareness, hopefully PHS contamination of calibrants will be soon a matter of the past.

4. Materials and Methods

4.1. Chemicals and Reagents

MeOH and ACN (both LC gradient grade), as well as glacial acetic acid (p.a.) were purchased from VWR International GmbH (Vienna, Austria) and water was purified using a Purelab Ultra system (ELGA, LabWater, Celle, Germany). Ammonium formate was obtained as a 5 M aqueous solution from Agilent Technologies (Waldbronn, Germany). Ammonium acetate, formic acid (both LC-MS grade), crystalline NIV-standards from *Fusarium nivale* (#N-7769; discontinued) and certified reference material IRMM-316 (NIV in ACN; certified value 24.0 ± 1.1 µg/g; indicative value: 18.8 ± 0.9 µg/mL) were obtained from Sigma Aldrich (Vienna, Austria). Standards for NIV (10.1 µg/mL) and FUSX (10.2 µg/mL) were purchased as a mixed solution of type A and B trichothecenes as well as zearalenone (#002002) in ACN from Romer Labs GmbH (Tulln, Austria). The same supplier also delivered a certified NIV calibrant (100 µg/mL) in ACN (#002011). Solid nivalenol hydrate (sc-236183) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), weighted in using a Sartorius M500 P microbalance (Göttingen, Germany) and dissolved in MeOH to obtain a 1000 mg/L solution. PHS was previously purified and characterized by one- and two-dimensional nuclear magnetic resonance spectroscopy, as well as X-ray single-crystal diffraction [16].

4.2. LC-MS/MS Parameters

Analyses were performed on a 1290 UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Foster City, CA, USA). For chromatographic separation a Gemini C18 (150 × 4.6 mm, 5 µm) reversed phase column equipped with a guard column (4 × 3 mm) (both from Phenomenex, Aschaffenburg, Germany) were used at 25 °C. The eluents were composed of MeOH:water (eluent A: 5:95, *v:v*; eluent B: 98:2, *v:v*) and both contained 5 mM ammonium acetate. The flow rate was 1 mL/min and the default injection volume was 3 µL. After an initial holding time at 0% B for 1 min, a linear increase to 85% B within the next 6 min followed. Thereafter, the column was flushed with 100% B for 3 min, followed by re-equilibration for 2.4 min with the starting conditions.

The QTrap 5500 was equipped with a TurboV ion spray source and was operated in negative electrospray ionization mode. The following parameters were used: curtain gas 30 psi (207 kPa, nitrogen), collision gas (nitrogen) medium settings, ion spray voltage −4500 V, source temperature 550 °C, ion source gas 1 and 2 both 80 psi (552 kPa, zero grade air). Optimization of the MS/MS parameters was performed by syringe injection of single analyte solutions. Two (NIV, FUSX) or three (PHS) mass transitions were chosen in the selected reaction monitoring mode and the dwell time was set to 25 ms. The LC-MS instrument was controlled by Analyst software version 1.5.2 (Sciex, Foster City, CA, USA) and automatic and manual integration of the peaks were performed using the same (or higher) software version. Further data processing was carried out in Microsoft Excel 2010.

For high resolution mass spectrometric measurements, a 1290 UHPLC system coupled to a 6550 iFunnel QTOF (both Agilent Technologies (Waldbronn, Germany)) were used. Eluents were water and MeOH, both containing 0.1% formic acid and 1 mM ammonium formate. Chromatographic separation was performed on a Zorbax SB-C18 (150 × 2.1 mm, 1.8 µm, Agilent Technologies) equipped with a C18 UHPLC-guard column from Phenomenex at 30 °C. Starting conditions (10% B) were held for 0.5 min, afterwards a linear gradient to 35% B till 6 min was applied, followed by a wash step with 100% for 1 min and re-equilibration with the starting conditions for 2 min till the end of the run

at 9 min. The gas and the sheath gas temperature were 130 °C and 300 °C, respectively, the drying gas flow was 16 L/min and sheath gas flow 11 L/min. The nebulizer was set to 30 psig and the capillary voltage and nozzle voltages were 4 kV and 0.5 kV. Reference masses (m/z 112.9855 and 966.0007) were used to ensure the high mass accuracy. The mass range was m/z 100–1000 for MS and m/z 50–400 for MS/MS measurements and the acquisition rate was three spectra/s for both measurement modes. Instrument control and data evaluation was performed with MassHunter Workstation Software Version B.06.01 and MassHunter Qualitative Analysis Version B.07.00 (both Agilent Technologies, Waldbronn, Germany).

4.3. Sample Preparation and Method Validation

Two different extraction solvents were evaluated for the extraction of cereals. ACN:water:acetic acid (79:20:1, *v*:*v*:*v*), an extraction solvent which is often used in multi-mycotoxin analysis [43] was tested as well as a more polar mixture of MeOH:water (50:50, *v*:*v*). For the final method, the following procedure was applied: Cereal samples were ground using an Osterizer Blender (Sunbeam Oster Household Products, Boca Raton, FL, USA) and 5.00 ± 0.01 g of the homogenized sample were weighed into 50-mL polypropylene tubes (Sarstedt, Nümbrecht, Germany). After adding 20 mL of extraction solvent, the samples were briefly shaken by hand followed by an extraction for 60 min at room temperature on a rotary shaker (GFL3017, Burgwedel, Germany). Thereafter, the samples were allowed to settle for a few minutes and an aliquot of the clear extract (500 µL) was diluted with the same amount of water in an HPLC vial.

For method validation, PHS and NIV were spiked before extraction on five different levels (ranging from 10 to 1000 µg/kg) in triplicate and at 12.5 µg/L (equivalent to 100 µg/kg) after extraction in five replicates. A blank barley sample previously checked for the absence of the target analytes was used for the spiking experiments. Spiking before extraction was performed with a mixed solution in MeOH and the samples were stored overnight at room temperature to ensure the evaporation of the solvent. Thereafter, the samples were worked up together with a blank barley sample according to the sample preparation procedure described above. For the preparation of matrix spikes, the extract of the blank barley samples was spiked with the target analytes and diluted with water to achieve the same solvent composition as the samples. Furthermore, separate neat solvent standards (MeOH:water, 50:50, *v*:*v*) were prepared covering a concentration range from 0.38 to 250 µg/L (equivalent to 3 to 2000 µg/kg).

4.4. Molecular Classification of the Strain IFA189

For preparation of genomic DNA, we applied the CTAB extraction method [44] with minor modifications. Amplification of EF1α was performed as described in [18]. For sequencing reactions primers EF15fw and EF16rev [19] were used.

To investigate, whether strain IFA189 contains the aflatoxin-biosynthesis gene nor1, we utilized the primers nor_fw and nor_rev [23] for PCR-mediated amplification. The published conditions did not yield a PCR fragment of the expected size (300 bp). Therefore, we applied gradient PCR, which resulted in a 300 bp fragment. We isolated the fragment, cloned it into pTOPO, and prepared the plasmid DNA from 12 transformants, which were subsequently sequenced using the nor primers used for amplification.

4.5. Screening for PHS in Naturally Contaminated Cereals and in Known NIV Producing Strains

As PHS is likely to co-occur with the structurally related NIV, cereal samples measured positive for NIV with a previously published method [17] have been re-measured for PHS occurrence. Briefly, maize samples were originating from Malawi, Cameroon, Germany and Austria. Barley samples from an experimental field were provided by Prof. Radim Cerkal from the Czech Republic National Agency for Agricultural Research.

Furthermore, known or suspected NIV producing strains were cultivated as follows: The strains were grown on Fusarium minimal medium (FMM; 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.5 g/L KCl, 2 g/L $NaNO_3$, 30 g/L sucrose, 10 mg/L citric acid, 10 mg/L $ZnSO_4 \cdot 6\ H_2O$, 2 mg/L $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 0.5 mg/L $CuSO_4 \cdot 5\ H_2O$, 0.1 mg/L $MnSO_4$, 0.1 mg/L H_3BO_4, 0.1 mg/L $Na_2MoO_4 \cdot 2\ H_2O$) from [45] or potato dextrose agar (PDA, Sigma-Aldrich, Vienna, Austria) plates. For the preparation of solid rice medium, 200-mL jars were filled with ca. 10 g rice from a local store and 10 mL reverse osmosis water. The jars were incubated at room temperature for 1 h, before being autoclaved for 60 min at 121 °C. Small agar blocks (approx. 5 × 5 mm) of the FMM or PDA plates with mycelium were transferred to the solid rice medium. The cultures were incubated for two weeks at 20 °C in the dark and then stored at −20 °C until further processing. For extraction, 40 mL of MeOH-water (50:50, *v*:*v*) was added to each of the rice glasses, homogenized using an Ultra-Turrax T25 (IKA-Werke, Staufen, Germany), and extracted for 60 min on a GFL rotary shaker (Burgwedel, Germany). Homogenates were transferred to 50-mL polypropylene tubes and centrifuged (10 min at room temperature, 3200 g). An aliquot of each sample was transferred to 1.5-mL tubes and centrifuged again for 15 min at 20000× *g*, thereafter 800 µL of the supernatants were transferred to HPLC vials for analysis. If required, further dilutions were performed with MeOH-water (50:50, *v*:*v*).

To determine the presence or absence of aflatoxins, *F. kyushuense* strains IFA189 and NRRL 3509 were cultivated on rice. They were first sporulated in mung bean medium, which was prepared as follows: 10 g mung beans were added to 450 mL boiling water and cooked for 20 min. After removal of the mung beans, the extract was filtrated through a folded filter, filled up to 1 L and autoclaved for 20 min at 121 °C. Conidia were separated from mycelia by filtration through a glass-wool filter and sedimented overnight at 4 °C. After removal of the medium spores were re-suspended in water and counted in a Fuchs-Rosenthal chamber. Rice cultures in jars (see above) were inoculated with 10^5 spores and cultivated both under light (20 °C/55% humidity/24 h light) and dark (20 °C/24 h dark) conditions in triplicate for two weeks. Sample preparation was conducted as described above, except that ACN:water:acetic acid (79:20:1, *v*:*v*:*v*) was used for extraction. To achieve reasonably low limits of detection, extracts were purified with a MycoSep #226 column (Romer Labs). Afterwards, 4 mL of the clean extracts were evaporated to dryness under a gentle stream of nitrogen, re-dissolved in 1 mL MeOH-water (50:50, *v*:*v*), and transferred into HPLC vials. These samples were measured with a multi-mycotoxin-method [17].

4.6. Plant Experiments

Spring wheat ears of the German variety "Remus" ("Sappo"/"Mex"/"Famos") were grown in a glass house using standard conditions [46]. At anthesis, the ears were treated with a spore suspension of *F. kyushuense* containing 5 × 10^5 conidia/mL. The suspension was injected into the spikelets (10 µL/spikelet) using a syringe and 10 to 15 spikelets were treated per wheat ear. After treatment, the ears were covered with plastic bags for 24 h to provide a high relative air humidity and promote infection after inoculation. Five weeks after the treatment the wheat ears were harvested, frozen, and stored at −20 °C until further processing. Furthermore, wheat ears of the same variety were treated at anthesis with solutions of either NIV or PHS, again harvested after ripening and frozen at −20 °C. Each frozen wheat ear was ground into a fine powder in liquid nitrogen using a mortar. After homogenization of the samples, they were extracted with the fourfold amount of MeOH:water (50:50, *v*:*v*) for 90 min at room temperature, centrifuged, transferred to an HPLC-vial and measured with LC-MS/MS.

4.7. Toxicity Assays

In vitro translation assays with TnT® T7 Coupled Wheat Germ Extract System (from Promega (Madison, WI, USA)) were used as described in [47]. Seven independent assays were performed with 12 or 8 individual dilutions of PHS and NIV, respectively.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

(U)HPLC	(ultra-)high performance liquid chromatography
15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivalenol
ACN	acetonitrile
ARS	Agricultural Research Service
b.w.	body weight
bp	base pairs
CONTAM	European Food Safety Authority Panel on Contaminants in the Food Chain
DON	deoxynivalenol
EFSA	European Food Safety Authority
FAO	Food and Agricultural Organization of the United Nations
FMM	Fusarium minimal medium
FUSX	fusarenon-X
IC_{50}	half maximal inhibitory concentration
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
MeOH	methanol
NCBI	National Center for Biotechnology Information
NIV	nivalenol
NRRL	Agricultural Research Service Culture Collection strain
PCR	polymerase chain reaction
PDA	potato dextrose agar
PHS	pentahydroxyscirpene
PMTDI	provisional maximum tolerable daily intake
QTOF	quadrupole time-of-flight mass spectrometer
TDI	tolerable daily intake
WHO	World Health Organization

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APPENDIX II

Kluger B, Bueschl C, Lemmens M, Michlmayr H, Malachova A, Koutnik A, Maloku I, Berthiller F, Adam G, Krska R, Schuhmacher R

Biotransformation of the mycotoxin deoxynivalenol in Fusarium resistant and susceptible near isogenic wheat lines.

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RESEARCH ARTICLE

Biotransformation of the Mycotoxin Deoxynivalenol in Fusarium Resistant and Susceptible Near Isogenic Wheat Lines

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Abstract

In this study, a total of nine different biotransformation products of the *Fusarium mycotoxin* deoxynivalenol (DON) formed in wheat during detoxification of the toxin are characterized by liquid chromatography—high resolution mass spectrometry (LC-HRMS). The detected metabolites suggest that DON is conjugated to endogenous metabolites via two major metabolism routes, namely 1) glucosylation (DON-3-glucoside, DON-di-hexoside, 15-acetyl-DON-3-glucoside, DON-malonylglucoside) and 2) glutathione conjugation (DON-S-glutathione, "DON-2H"-S-glutathione, DON-S-cysteinyl-glycine and DON-S-cysteine). Furthermore, conjugation of DON to a putative sugar alcohol (hexitol) was found. A molar mass balance for the cultivar 'Remus' treated with 1 mg DON revealed that under the test conditions approximately 15% of the added DON were transformed into DON-3-glucoside and another 19% were transformed to the remaining eight biotransformation products or irreversibly bound to the plant matrix. Additionally, metabolite abundance was monitored as a function of time for each DON derivative and was established for six DON treated wheat lines (1 mg/ear) differing in resistance quantitative trait loci (QTL) *Fhb1* and/or *Qfhs.ifa-5A*. All cultivars carrying QTL *Fhb1* showed similar metabolism kinetics: Formation of DON-Glc was faster, while DON-GSH production was less efficient compared to cultivars which lacked the resistance QTL *Fhb1*. Moreover, all wheat lines harboring *Fhb1* showed significantly elevated D3G/DON abundance ratios.

Introduction

An effective response to stress conditions is crucial for sessile organisms such as plants which are exposed to external influences from the environment. In this respect, plants have developed

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a multitude of mechanisms to cope with abiotic (e.g. high intensity UV light, temperature changes, drought, salinity, xenobiotics) [1] as well as biotic stresses most commonly caused by microorganisms such as bacteria and filamentous fungi. Whereas biotrophic fungi depend on the living host, fungal pathogens with a predominantly necrotrophic lifestyle eventually kill their host cells by secreting toxic secondary metabolites and grow on the necrotic plant tissue [2].

Fusarium Head Blight (FHB, scab) is caused by various *Fusarium* species and occurs on wheat, and other small-grain cereals. It is responsible for severe yield and quality losses and considered to be a relevant global hazard for food safety and security [3]. Wheat, barley and other small grain cereals are affected by *Fusarium graminearum*, one of the most important species causing FHB [4]. The *F. graminearum* derived mycotoxin deoxynivalenol (DON) belongs to the group of trichothecene mycotoxins and plays a key role in FHB. DON acts as a virulence factor by inhibiting protein biosynthesis of the host [5]. As a consequence of fungal infection, the mycotoxin is a frequent contaminant of food and feed and has a great impact on both human and animal health [6]. Mechanisms of action and toxicological relevance of the toxin has been reviewed by Pestka in 2010 [7].

Mechanisms to counteract microbial toxins and other xenobiotics include their detoxification by conjugation to endogenous metabolites (e.g. by glucosylation, acylation, conjugation to amino acids and to the dedicated peptide glutathione). The resulting biotransformation products are subsequently transported to the vacuole or to the apoplast. Moreover, the toxin conjugates can be further incorporated into cell wall components [8]. So far, most knowledge about detoxification mechanisms of xenobiotics has been generated by metabolism studies of pesticides [9–12]. Despite the importance for fungal virulence and plant disease resistance, limited information exists regarding detoxification of mycotoxins *in planta*. Several reviews give an overview of the plant mediated metabolism involved in the detoxification of mycotoxins and the resulting conjugates [8,13,14]. These modified mycotoxins have an altered structure and different mass, making them difficult to detect by conventional analytical approaches and they have the potential to be reactivated to the parental toxin (e.g. by hydrolyzation in the intestinal tract [15,16]) and are therefore often designated to be masked mycotoxins. According to a recent proposal, the term masked mycotoxin should only be used for plant metabolites [17]. The difficulties in detection of conjugated mycotoxins lead to an underestimation of total mycotoxin content in contaminated food and feed [18]. With respect to DON, the probably most important detoxification reaction to reduce the toxicity of DON *in planta* is its conjugation to glucose first reported in *Arabidopsis thaliana* [19], and also reported for naturally *F. graminearum* inoculated and contaminated wheat [20]. Additionally, the occurrence of DON oligoglucosides such as DON-di-glucoside, DON-tri-glucoside and DON-tetra-glucoside in malt, beer and bread has been reported previously [21].

For detection of mycotoxins and their derived biotransformation products, liquid chromatography (LC) combined with mass spectrometry (MS) is currently one of the most powerful techniques due to its high selectivity and sensitivity. The concept of using isotopic patterns to study the metabolism of labeled tracers have long been known and has the advantage that the detected biotransformation products can be linked to the studied tracer substance [22]. Recently developed novel stable isotope labeling assisted approaches enable the automated, untargeted profiling of biotransformation products of xenobiotics, including mycotoxins *in planta* [23]. Kluger *et al*., reported the detection of novel GSH related conjugation products of DON in wheat such as DON-glutathione (GSH) and its corresponding degradation products DON-S-cysteinylglycine (DON-S-cys-gly) and DON-S-cysteine (DON-S-cys) [24]. Besides its putative relevance as masked mycotoxins, contributing to the overall toxicity, the formation of

conjugated mycotoxins may add to the current understanding of resistance mechanisms in host plants.

FHB resistance in wheat has been associated with more than 100 quantitative trait loci (QTL), with only few QTL genetically mediating FHB resistance [25], among them the most important *Fhb1* (formerly known as *Qfhs.ndsu-3BS*) [26] and *Qfhs.ifa-5A* [27]. *Fhb1* has been attributed to a more efficient glucosylation of DON to DON-3-*β*-*D*-glucoside (D3G). The gene mediating *Fhb1* resistance is still unknown. Lemmens *et al.* [28] reported that wheat lines harboring *Fhb1* showed an increased resistance to the phytotoxic effect of DON applied to flowering wheat ears. This increased resistance was associated with an increased D3G/DON concentration ratio in DON treated wheat kernels harvested after full ripening. Based on this observation, the mechanism underlying *Fhb1* based resistance was postulated to be attributed to either a gene encoding a UDP-glucosyltransferase (UGT) or exhibiting a regulatory function with respect to D3G formation. In a more recent metabolomics study Gunnaiah *et al.*, [29] also investigated the mode of action of the *Fhb1* QTL in wheat lines. Flowering wheat ears were infected with spores of *F. graminearum* and treated spikelets were harvested 72 hours after infection. In contrast to the findings of Lemmens *et al.*, measurements of DON and D3G content revealed that neither the DON content nor D3G/DON ratio in inoculated spikelets were affected by the presence of *Fhb1* under the tested conditions. The authors concluded that *Fhb1* is not related to DON glucosylation and from further metabolomics data they proposed that the formation of phenylpropanoid amides (PPA) was increased in *Fhb1* containing wheat lines. Thus, Gunnaiah *et al.* [29], concluded that PPAs serve as cell wall constituents of rachis tissue and *Fhb1* is confering FHB resistance by processes resulting in cell wall thickening, which leads to physical barriers and in turn prevents fungal growth from the infected spikelet into the rachis.

The study presented here is carried out in continuation of previously published work [24] that describes an untargeted stable isotope labeling assisted LC-HRMS based screening approach, which resulted in the detection of nine different DON biotransformation products in wheat. Here we present the tentative annotation of the remaining biotransformation products and show comparative metabolic kinetics of six different wheat lines: the resistant wheat variety 'CM-82036' harboring *Fhb1* and *Qfhs.ifa-5A*, the susceptible variety 'Remus' and 4 near isogenic lines (98% homology to 'Remus' with all possible combinations of both QTL) after treatment with DON.

Materials and Methods

Chemicals and reagents

Acetonitrile (ACN, HiPerSolvChromanorm, HPLC gradient grade) was obtained from VWR (Vienna, Austria); methanol (MeOH, LiChrosolv, LC gradient grade) was purchased from Merck (Darmstadt, Germany); formic acid (FA, MS grade) was obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and an ELGA Purelab Ultra-AN-MK2 system (Veolia Water, Vienna, Austria). Deoxynivalenol (DON) standard for LC-HRMS analysis was obtained from Romer Labs GmbH (Tulln, Austria) as a stock solution of 100 mg L^{-1} in ACN. Stock solution of deoxynivalenol-3-*β*-*D*-glucoside (D3G) at a concentration of 1.61 g L^{-1} was prepared as described by Berthiller *et al.*, [20].

Wheat lines and cultivation of wheat plants

For this study six different spring wheat (*Triticum aestivum* L.) lines were used. The resistant parent 'CM-82036–1TP-10Y-OST-10Y-OM-OFC' (abbreviated to 'CM-82036') has a very high level of resistance against FHB and against DON [28]. The second parent 'Remus' is a spring wheat cultivar highly susceptible to FHB and DON. Moreover, four different near

isogenic lines (NILs) were used, which had been developed from one BC5F1 plant with 'Remus' as recurrent parent (5 backcrosses), which differed in two validated QTLs related to the FHB resistance level (*Fhb1* and *Qfhs.ifa-5A*) [30]. The NILs C1–C4 used in this study contain different combinations of the resistance alleles. C1 (+*Fhb1*/+ *Qfhs.ifa-5A*), C2 (+*Fhb1*), C3 (+*Qfhs.ifa-5A*) and C4 (none) were cultivated in parallel with both parent lines as described below.

Seeds of the spring wheat lines were germinated and seedlings were planted in pots (diameter 23 cm) with homemade soil (mix of 500 L heat-sterilized compost, 250 L peat, 10 kg sand and 250 g rock flour) using a completely randomized block with five biological replications. Plants were grown in a greenhouse under environmentally controlled settings for light, temperature and relative air humidity. For a detailed description of the growth conditions during plant development, the reader is referred to the publication of Warth and co-workers [31]. From the start of DON application until the end of the experiment including sampling, the plants were illuminated for 16 hours/day and the temperature was set at 20°C during illumination and at 18°C at night. At the onset of anthesis each cultivar was treated using an aqueous DON solution as described in detail below.

DON- and mock treatment of wheat plants

At the beginning of anthesis plants of each cultivar ('CM-82036', C1, C2, C3, C4 and 'Remus') were treated either with an aqueous DON solution (5 g L^{-1}) or with water (mock) according to the following procedure: at time point zero 10 µL DON solution were injected in each of two adjacent spikelets in the lower part of a flowering ear. In total 20 spikelets were treated, by repeating the treatment with spikelets located above those treated before resulting in a total amount of 1 mg DON/wheat ear. 0, 12, 24, 48 and 96h post treatment, inoculated spikelets were sampled and immediately shock-frozen in liquid nitrogen (n = 5 replicates per treatment and time point) resulting in a total of 50 samples for each wheat line.

In order to establish a molar mass balance for DON and D3G, 10 wheat ears of the cultivar 'Remus' were inoculated with a total of 1 mg DON at anthesis using the same procedure as mentioned above. Treated spikelets were harvested after 0 hours and 96 hours after treatment (n = 5 wheat ears per time point), immediately immersed in liquid nitrogen and extracted by the same procedure as all other samples.

Sample preparation for LC-HRMS measurements

All wheat samples were milled in frozen conditions separately for 2 min at 30 Hz to give a fine powder by use of a ball mill (MM 301 Retsch, Haan, Germany) with pre-cooled (liquid nitrogen) 50 mL-stainless-steel grinding jars (Retsch) and a ø 25 mm-stainless steel ball (Retsch). 100 ± 5 mg of homogenized, frozen plant material were weighed into 1.5 mL-Eppendorf tubes. Extraction was performed by adding 1 mL of pre-cooled MeOH:water 75/25 (v/v) including 0.1% formic acid, vortexing for 10 s, and subsequent treatment in an ultrasonic bath (frequency 47 kHz, power: 105 W) at room temperature for 15 min [32]. Samples were centrifuged for 10 min at 19,000 g at 4°C.

An aliquot of the supernatant was transferred to another 1.5 ml-Eppendorf tube and pre-cooled water containing 0.1% formic acid was added to achieve a final MeOH:water ratio of 1:1 (v/v). Finally, the samples were vortexed for 10 s before transfer into HPLC vials for LC-HRMS measurements.

Preparation of standards

Monoglucosides of the DON-derivatives 15-Acetyl-DON (15-ADON) and 3,7,8,15-tetrahydroscirpene (THS) [33] were produced with a recently identified DON-specific glucosyltransferase [34]. The protein was expressed with the plasmid pKLD116 (N-terminal His6-tag and maltose binding tag in tandem; [35]) using *Escherichia coli* Rosetta (DE3) (Novagen, Madison, WI) as expression host. Protein purification was performed on a 5 mL His Trap FF Column (GE Healthcare, Vienna Austria) following the suppliers instructions. Glucosylation was performed using 1 mg mL^{-1} purified glucosyltransferase and 100 mM Tris/Cl (pH 7) at 37°C for an incubation time of 24 h. UDP-glucose was added to the substrate resulting in 1.5 molar excess.

LC-HRMS(/MS) analysis

Screening for DON and its biotransformation products.. LC-HRMS full scan measurements were carried out as described earlier [36] using the following instrumentation. A UHPLC system (Accela, Thermo Fisher Scientific, San Jose, CA, USA) coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source. Xcalibur 2.1.0 software was used to control the mass spectrometer and record the data. For HPLC separation, a reversed-phase XBridge C_{18}, 150 x 2.1 mm i.d., 3.5 μm particle size (Waters, Dublin, Ireland) analytical column was used at a flow rate of 250 μL min^{-1} at 25°C in a gradient program (injection volume 10 μL). Eluent A was water, eluent B was MeOH, both containing 0.1% formic acid (v/v). The chromatographic method held the initial mobile phase composition (10% B) constant for 2 min, followed by a linear gradient to 100% B within 30 min. This final condition was held for 5 min, followed by 8 min of column re-equilibration at 10% B. The ESI interface was operated in positive ion mode at 4 kV. The Orbitrap mass analyzer was operated in full scan mode with a scan range of *m/z* 100–1000 and a resolving power setting of 60,000 FWHM at *m/z* 400. Samples of individual wheat lines plus quality control samples were measured in separate sequences resulting in a total of six measurement sequences. Qualitative analysis of LC-HRMS data was performed using Xcalibur 2.1.0 QualBrowser software. For relative and absolute quantification of DON and its biotransformation products Thermo Xcalibur 2.1.0 QuanBrowser software was employed. All automatically integrated peak areas were inspected manually, and corrected if necessary.

For structure characterization of DON derivatives, LC-HRMS data files were inspected manually for the presence of DON and its nine biotransformation products, which had been formerly found in an untargeted profiling study [24]. Extracted ion chromatograms (EICs) of each biotransformation product were checked for chromatographic peak shape, retention time similarity of less than ± 0.2 min and mass deviation of less than ± 3 ppm from the exact mass of the proposed biotransformation product.

Structure annotation of DON conjugates.. LC-HRMS/MS product ion spectra of DON biotransformation products were recorded by using the same HPLC gradient program. Fragmentation was performed in the collision induced dissociation (CID) mode and subsequent fragment detection in the Orbitrap with a resolving power setting of 7,500 FWHM (at *m/z* 400) and an isolation width setting for the precursor of 3 u. All LC-HRMS/MS spectra were recorded in centroid mode, while *m/z* range and relative collision energy were adjusted to the respective precursors of DON biotransformation products.

Absolute quantitation of DON and D3G for establishment of mass balance.. For calibration, DON and D3G stock solutions were prepared separately at different levels and aliquots (15 μL) were spiked into 135 μL of an untreated 'Remus' sample extract resulting in concentration levels of 0, 1, 3, 5, 7 and 10 mg L^{-1}. Samples for the molar mass balance for DON and D3G

were prepared exactly as mentioned above but were further diluted 1:10 (v/v) with water containing 0.1% aqueous formic acid. In parallel the sample extract of the untreated 'Remus' wheat ear was diluted 1:10 (v/v) with water containing 0.1% aqueous formic acid.

Relative quantification of DON and its biotransformation products.. For relative quantification, data were normalized to facilitate a comparison of concentration levels across all six wheat lines. Therefore, within every measurement sequence the integrated peak area of the respective DON derivatives and for each experimental sample was divided by the average peak area of the respective metabolite in the aggregate control sample (preparation is described in the section "Quality control sample for LC-HRMS analysis") in the same measurement sequence. Finally, the peak area ratios of each experimental sample were normalized to the highest value found across all of the tested wheat lines (except for DON, where each time course was normalized separately for each cultivar). Average value and standard deviation of the normalized peak area ratio were calculated for each time point (n = 5) and for every wheat line, the resulting area ratios for each biotransformation product were plotted over time.

Univariate Statistical Analysis.. Time series plots were created with Python 2.6 (https://www.python.org/; last accessed 5. Aug. 2014) and matplotlib (v. 1.3; [37]). Significance testing (two-sided, non-paired t-test with 5% significance threshold) between the groups of wheat genotypes harboring QTL *Fhb1* (CM, C1, C2) or not (C3, C4, Remus) was performed with R (v. 2.15.2; [38]. A p-value of less than 5% is indicated with a '*' character.

Quality control sample for LC-HRMS analysis.. For quality control purposes, one aggregate control sample was produced and measured in regular intervals in parallel to each wheat line in order to compensate for variations during sample preparation and drifts of the mass spectrometer. To this end, aliquots of milled experimental wheat ears representing all six wheat lines, treatments (DON, mock) and time points (0, 12, 24, 48, 96h) were pooled together and milled again in the Retsch mill for homogenization. During preparation of experimental samples, control samples were prepared in parallel and measured at regular intervals throughout the respective LC-HRMS sequence. Furthermore, reference standards dissolved in pure solvent were included in each sequence to monitor mass precision, sensitivity and retention time drifts of the LC-HRMS measurement step.

Results and Discussion

Screening for DON and its biotransformation products

Previously, D3G [39] and DON-di-glucoside [21] have been described as wheat derived DON metabolites. In a more recent study, a novel screening strategy for the studying of metabolites derived from exogenous tracers using stable isotopic labeling has been presented. The screening approach was exemplified with the application of a 1+1 (v/v) mixture of native DON and U-^{13}C labeled DON to flowering wheat plants of the cultivar Remus as described previously [24]. Further detailed data evaluation of that study revealed a total of nine different DON derivatives, all containing the DON moiety with all 15 carbon atoms. These toxin derivatives, including the already published D3G, DON-GSH and two of its further degradation products (putatively identified on the basis of accurate mass and LC-HRMS/MS product ion spectra) served as candidates for a targeted screening in native DON treated wheat plants as described in this work. Based on LC-HRMS measurements using tolerance limits of ± 3 ppm for mass deviation compared to the exact mass postulated and ± 0.2 min for retention time similarity, the same nine DON biotransformation products were also observed in DON treated wheat samples in this investigation (see Table 1). DON and two of its biotransformation products have been identified based on authentic reference standards (marked with '**' in Table 1), while further five biotransformation products were additionally putatively identified based on accurate

Table 1. Overview of nine different DON biotransformation products in DON treated wheat samples. Initial annotation for all biotransformation products was performed based on the maximum mass deviation of ± 3 ppm and relative retention order (deviation retention time: ± 0.2 min) compared to the same biotransformation products annotated in the former study [24].

#	Name	RT (min)	Ion species	Theoretic mass (*m/z*)	Mass deviation (ppm)	Isotopic fine structure sulfur
1	DON-Hexitol (e.g.: Mannitol)	4.89	$[M+Na]^+$	483.1838	-2.9	
2	DON-S-cysteine*	5.59	$[M+H]^+$	418.1531	-2.9	x
3	DON-S-cysteinyl-glycine*	6.27	$[M+H]^+$	475.1746	-2.2	x
4	DON-glutathione (GSH) *	7.71	$[M+H]^+$	604.2173	-2.4	x
5	DON-di-hexoside	8.19	$[M+Na]^+$	643.2211	-2.7	
6	DON**	8.60	$[M+Na]^+$	319.1153	-2.2	
7	DON-3-β-D-glucoside**	9.16	$[M+Na]^+$	481.1682	-1.6	
8	"DON-2H"-glutathione*	11.48	$[M+H]^+$	602.2014	-2.2	x
9	DON-malonylglucoside*	11.73	$[M+Na]^+$	567.1686	-2.6	
10	15-acetyl-DON-3-β-D-glucoside**	13.71	$[M+Na]^+$	523.1786	-2.5	

* Annotation based LC-HRMS/MS spectra revealing characteristic fragment ions.

** Identification of compounds based on authentic standard with similar retention time and LC-HRMS/MS spectra (data not shown).

Biotransformation products have also been putatively identified in a previous study [24].

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m/z values and LC-HRMS/MS spectra (marked with '*' in Table 1). Evaluation of the isotopic fine structure revealed the presence of sulfur in four of the nine DON biotransformation products.

Three sulfur containing metabolites, namely DON-glutathione (GSH), DON-S-cysteinyl-glycine and DON-S-cysteine had already been putatively identified [24]. Herein, we putatively describe the characterization of the remaining DON biotransformation products. Based on

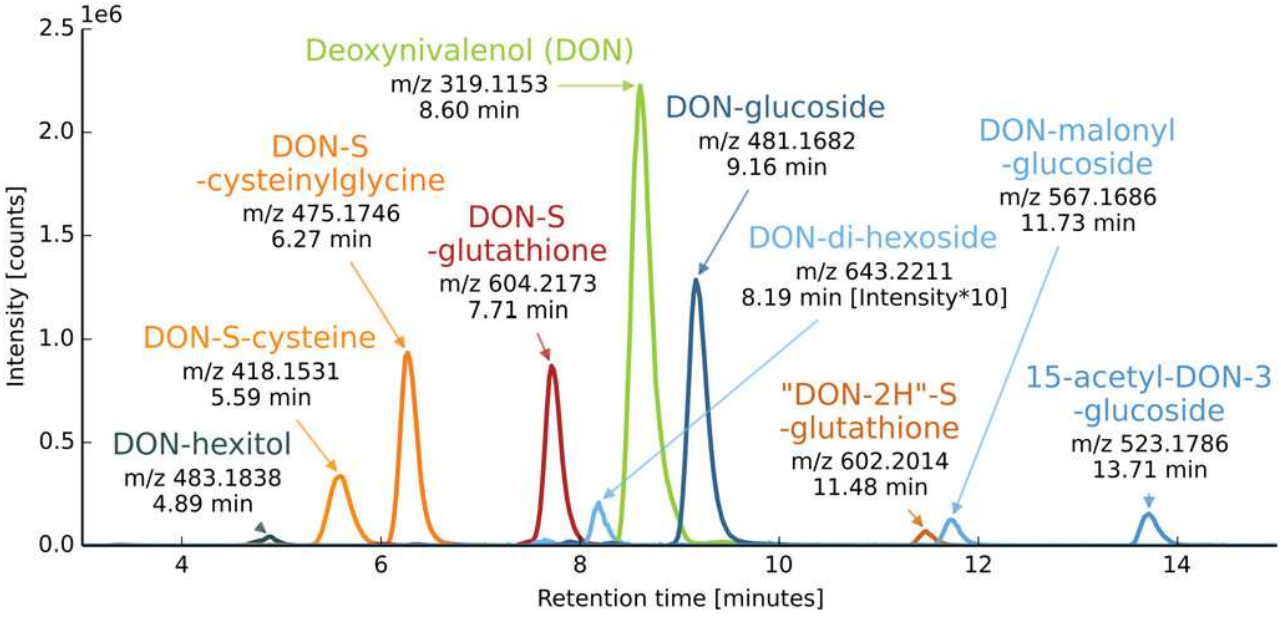

Fig 1. EICs of DON and its corresponding biotransformation products. EICs of accurate mass traces (± 3 ppm) of DON and its corresponding biotransformation products in a wheat sample harvested 96 hours post treatment with 1 mg DON. Due to low abundance, EIC intensities of DON-di-hexoside were multiplied by a factor of 10.

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accurate mass measurement and isotopic fine structure, one more GSH conjugate (#8) was detected, in which the glutathione moiety is intact, but the DON moiety is putatively oxidized by the loss of two hydrogen atoms. Thus, this compound was provisionally designated "DON-2H"-glutathione. Another DON derivative most probably constitutes to the toxin being conjugated to a hexitol (#1). Alternatively, this compound may be a derivative formed from D3G by introduction of 2H atoms. The remaining four biotransformation products have been annotated as D3G (#7), DON-malonylglucoside (#9), 15-acetyl-DON-3-glucoside (#10) and a DON-di-hexoside (#5) based on the arguments discussed below. Fig. 1 shows an overlay of EIC of DON and all corresponding biotransformation products in an extract of a DON treated wheat ear (1 mg, 96 h) of the cultivar 'Remus'.

Putative structure annotation of newly found DON conjugates

To confirm the proposed structures of DON-hexitol (#1), "DON-2H"-GSH (#8), DON-malonylglucoside (#9) and 15-acetyl-DON-3-*β*-*D*-glucoside (#10), LC-HRMS/MS product ion measurements of DON-treated wheat samples were carried out in Collision Induced Dissociation (CID) mode (Fig. 2). Due to low intensities of the precursor ion of #1 *m/z* 483.1824 (DON-hexitol, $[M+Na]^+$) no meaningful product ion spectra could be recorded. To verify whether this conjugate possibly consists of reduced DON, conjugated to a glucose moiety, standard 3,7,8,15-tetrahydroscirpene-3-glucoside was measured. This standard showed the same *m/z*, but had a different retention time in the chromatogram and therefore our data support the assumption that DON is conjugated to a hexitol. For the biotransformation product #5 *m/z* 643.2193 (DON-di-hexoside, $[M+Na]^+$) also no meaningful product ion spectra could be recorded due to low intensities. Thus, annotation for these two biotransformation products is based on accurate *m/z*, assumed ion species, number of DON derived carbon atoms per conjugate and evaluation of isotopic fine structure.

DON-malonylglucoside. Within the full scan mass spectrum, adducts at *m/z* 567.1671 $[M+Na]^+$ and *m/z* 589.1287 $[M+2Na-H]^+$ have been detected, therefore it was concluded that $[M+Na]^+$ is consistent with the sum formula $C_{24}H_{32}O_{14}Na$ (-2.6 ppm mass deviation). Evaluation of the LC-HRMS full scan spectra did not give evidence for mass signals corresponding to ^{15}N or ^{34}S isotopologs. LC-HRMS/MS measurements of precursor mass *m/z* 567.17 (corresponding to $[M+Na]^+$) using 18% relative collision energy revealed mass increments between two signals in the LC-HRMS/MS spectrum corresponding to neutral losses, Δ43.990 u, indicating the loss of carbon dioxide; Δ86.000 u and Δ248.054 u, corresponding to the loss of the malonylglucoside moiety minus water from the intact DON conjugate (Fig. 2a). Moreover, the intact DON molecule was observed (*m/z* 319.114, $[M+Na]^+$) in the product ion spectra. These findings are in good agreement with LC-HRMS/MS spectra of plant derived malonylglucoside conjugates such as flavanoids [40]. The conjugate is putatively identified as DON-3-MalGlc since it is assumed that this conjugate is formed from further metabolism of initially formed D3G.

15-acetyl-DON-3-*β*-*D*-glucoside. In the full scan mass spectrum, the mass 523.1773 which corresponds to the $[M+Na]^+$ ion of acetyl-DON-glucoside was detected. Subsequent LC-HRMS/MS measurements in the CID mode with 26% relative collision energy (Fig. 2b) revealed the neutral losses of Δ60.021 indicating a loss of an acetyl group and Δ162.053 ($C_6H_{10}O_5$) corresponding to the loss of glucose as already described by Dall'Asta *et al.* [39] for D3G. Together with the presence of the fragment at *m/z* 301.126, which corresponds to the sodium adduct of the acetylated DON, these findings suggest that both, the acetyl moiety as well as the glucose molecule are both covalently linked to the DON precursor at different positions. An authentic standard prepared in house by enzymatic in vitro synthesis using recombinant *Escherichia coli* expressed glucosyltransferase and 15-ADON as substrate (manuscript in

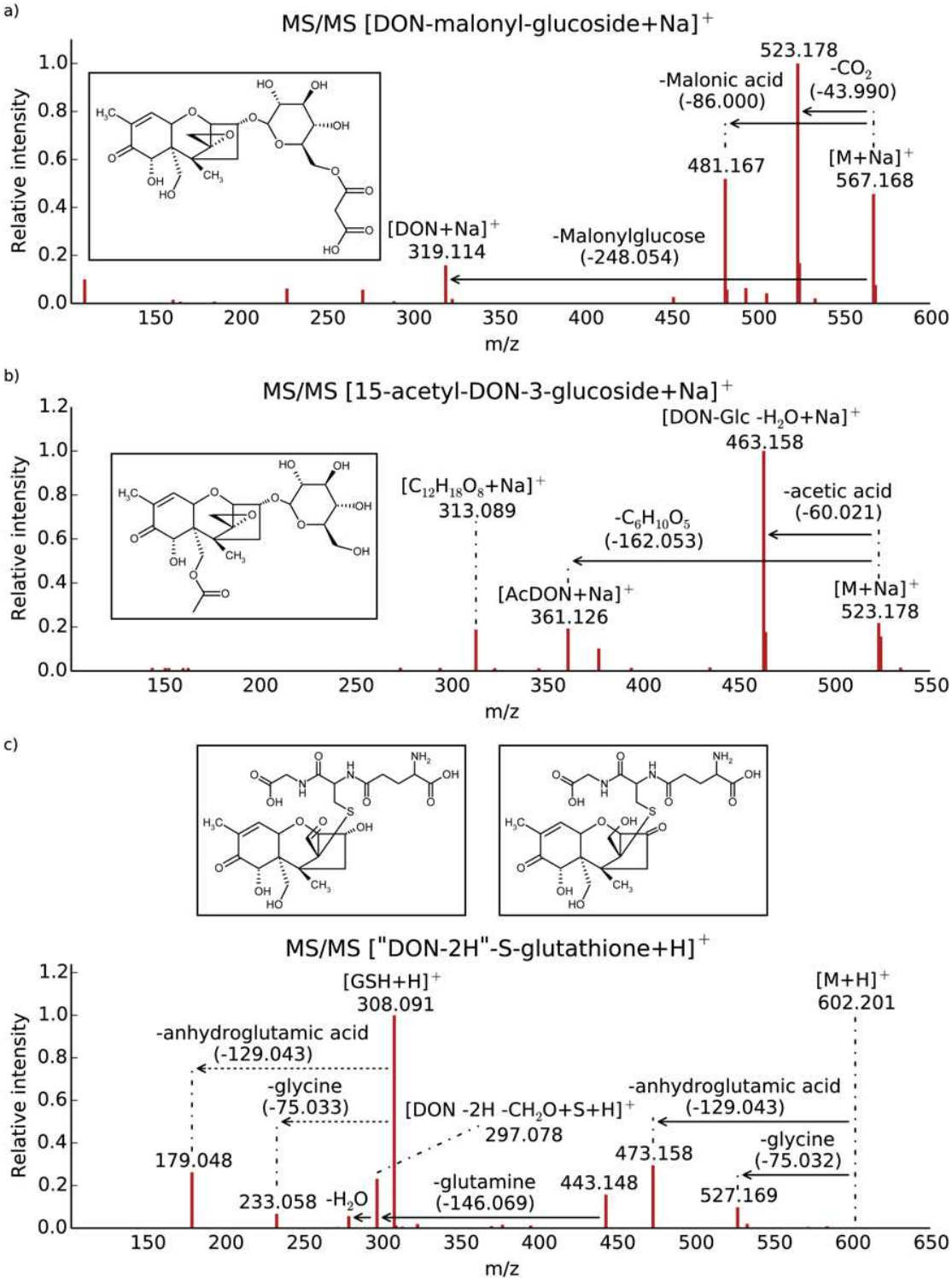

Fig 2. LC-HRMS/MS spectra of the DON biotransformation products. a) DON-malonylglucoside, b) 15-acetyl-DON-3-β-D-glucoside and c) "DON-2H"-S glutathione (GSH), all LC-HRMS/MS spectra are shown with a proposed structure formula.

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preparation), for which the same retention time and LC-HRMS/MS spectrum was observed, thus the compound was identified as 15-acetyl-DON-3-*β*-*D*-glucoside (15ADON3G). Since 15-ADON was not detected in the DON used for plant treatment and none of the other DON-Glc derivatives was observed in acetylated form, we assume that 15ADON3G presumably has been formed from acetylation of D3G.

"DON-2H"-S-glutathione conjugate. The mass of the observed intact molecule differed by Δ -2.016 u compared to DON-GSH. Analysis of its isotopic fine structure in LC-HRMS full scan spectra revealed the presence of sulfur within that molecule. LC-HRMS/MS measurements were performed at 18% relative collision energy. Observed fragments indicate the conjugation of an intact glutathione molecule to "DON-2H" (Fig. 2c). Observed neutral losses of Δ75.032 u (glycine), Δ129.043 u (anhydroglutamic acid) are in good agreement with our previous study [24], as well as the fragments of a glutathione moiety described by Levsen *et al*., [41]. The presence of GSH in #8 is further supported by the observation of a fragment to the protonated intact glutathione moiety (*m/z* 308.091) and its corresponding LC-HRMS/MS fragments. Interestingly the LC-HRMS/MS spectrum also showed the S-containing fragment at *m/z* 297.078, but lacked *m/z* 297.133 $[DON+H]^+$ indicating that in addition to GSH conjugation DON had been oxidized by the loss of two hydrogen atoms. Glutathione can form a Michael adduct at the C10 atom [42]. An alternative reaction described for glutathione S-transferases is the opening of the epoxide ring [43]. We speculate that after nucleophilic attack of the SH group of glutathione to the C12 atom of DON, the epoxide ring is opened. Subsequently an—OH group is oxidized to an aldehyde or ketone (-2H). This could occur at the primary alcohol newly formed by the epoxide opening (Fig. 2c right) or at the C3-OH of DON (Fig. 2c left). Such a conversion to a keto group by a bacterium has been described [44]. Alternatively, if only the glutathione adduct formation occurs at the double bond at C10, the C8-OH group may be converted into a keto group.

According to the current knowledge on metabolism of xenobiotics, we assume that during phase II metabolism DON is conjugated to endogenous molecules by two major metabolism routes, namely 1) glucosylation and 2) conjugation to the tripeptide glutathione (GSH) as well as the conjugation to a hexitol. These findings are in good agreement with former reports, which delineate conjugation of xenobiotics to glucose and GSH as major detoxification reactions *in planta*. While the detoxification of DON via the conjugation to D3G has already been reported earlier for *Arabidopsis* [19], maize cell suspension cultures [45] and naturally as well as artificially contaminated wheat [20], the conjugation of DON to GSH has only been reported recently [24]. It has been shown, that even the S-methyl adduct already leads to lower inhibition of protein synthesis, it may be assumed that the much bulkier cysteine or glutathione adduct also prevents interaction with the ribosomal target [42]. Our data suggest that the DON-GSH conjugate is stepwise degraded to DON-S-Cys-Gly and DON-S-Cys in wheat. Similar detoxification mechanisms have already been reported for glutathione S-conjugates of the herbicide alachlor in the vacuole of barley [11].

In addition, initially formed D3G is further metabolized to DON-MalGlc and DON-di-hexoside respectively. In agreement with our study, a MalGlc conjugate has already been reported as a detoxification product of 2,4-dichlorophenol in cell-suspension cultures of tobacco [12], and the malonylation of phenolic glucosides has also been reported to be a key reaction in xenobiotic metabolism of *Arabidopsis* [46].

In an earlier study, DON, D3G, DON-di-glucoside and further DON-oligoglycosides were detected in barley based products during beer production after pre-concentration of DON derivatives with immunoaffinity columns and LC-HRMS analysis [21]. In the present study no tri- or higher hexoside conjugates were detected which might be explained by the fact that no sample pre-concentration was employed. Thus, the presence of DON-di-hexoside suggests that

further oligoglucosides might be formed in wheat, but due to limited sensitivity were not detected by our approach.

Mass balance of DON and D3G in wheat

To further assess the metabolic rate of DON in wheat we have estimated a molar mass balance of DON and its main biotransformation product D3G for the investigated wheat samples. For both, DON (*m/z* 297.1334, $[M+H]^+$) and D3G (*m/z* 459.1862, $[M+H]^+$) matrix calibration was carried out at a concentration range from 1–10 mg L^{-1}. First the recovery rate of DON in wheat extracts was determined using five biological replicates harvested directly after treatment with 1 mg DON (≙ 3.38 μmol). On average 3.32 ± 0.07 μmol DON were detected in the samples, corresponding to a recovery rate of 98%. Thus, it can be concluded that DON is not irreversibly bound to wheat matrix within the first few minutes after application (sampling time point 0 h). 'Remus' wheat ears treated for 96 hours (n = 5) showed that 65% of the added DON (≙ 2.09 ± 0.21 μmol) were still present with 15% of the applied DON transformed to D3G (≙ 0.50 ± 0.08 μmol). Consequently, all other biotransformation products at most add up to a maximum of approximately 20% (≙ 0.62 ± 0.23 μmol) relative to the DON initially applied to the wheat spikelets. The molar mass balance demonstrates that the detected biotransformation products (including D3G) make up a significant percentage of the metabolized DON and thus both metabolism routes play an important role in the detoxification process of DON. Further detailed studies with respect to the occurrence of the different DON-conjugates in naturally contaminated cereals/wheat will have to be performed to investigate their toxicological relevance.

Time course kinetics of the formation of DON conjugates in DON treated wheat lines with different genetic background

The kinetics of the formation of the annotated biotransformation products in the investigated set of DON treated wheat lines have been elucidated with the aim to find QTL specific or QTL associated DON conjugation behavior. To this end, the parent wheat lines 'CM-82036' (resistant to FHB, harboring the resistance QTL *Fhb1* and *Qfhs.ifa*-5A) and 'Remus' (susceptible), and four near isogenic lines (genome 98% identical to 'Remus') C1 (+*Fhb1*/+ *Qfhs.ifa-5A*), C2 (+*Fhb1*), C3 (+*Qfhs.ifa-5A*) and C4 (no QTL) were studied. Integrated peak areas of each biotransformation product were normalized to the average EIC peak area observed for the respective biotransformation product in the control aggregate sample to account for changes of the MS sensitivity between different measurement sequences. For the ease of comparability of the respective biotransformation product across the different samples, the chromatographic peak areas were related to the maximum peak area in any of the tested wheat samples. Additionally, box plots were established for each wheat line 96 h after treatment. A non-paired t-test (5% global significance threshold) was performed for the univariate comparison of biotransformation products between wheat lines with and without harboring the resistance QTL *Fhb1*. With the exception of DON-di-hexoside, for which the abundance was too low, time course profiles could be established for every detected biotransformation product. For the mock treated samples none of the EICs showed a peak, confirming the high selectivity of the applied LC-HRMS approach (for raw data of integrated peak areas see S1 Table).

Degradation rate of DON. In this study DON was applied once (time point 0) and consequently DON levels continuously decreased over time in all wheat lines demonstrating that each of the tested wheat lines is capable of metabolizing DON (Fig. 3a). Wheat lines containing *Fhb1* ('CM-82036', C1, C2) showed a faster decrease of DON compared to the wheat lines without *Fhb1* (C3, C4, 'Remus'). The fastest DON metabolic transformation rate of all wheat lines was observed in 'CM-82036' indicating the efficient detoxification potential of this

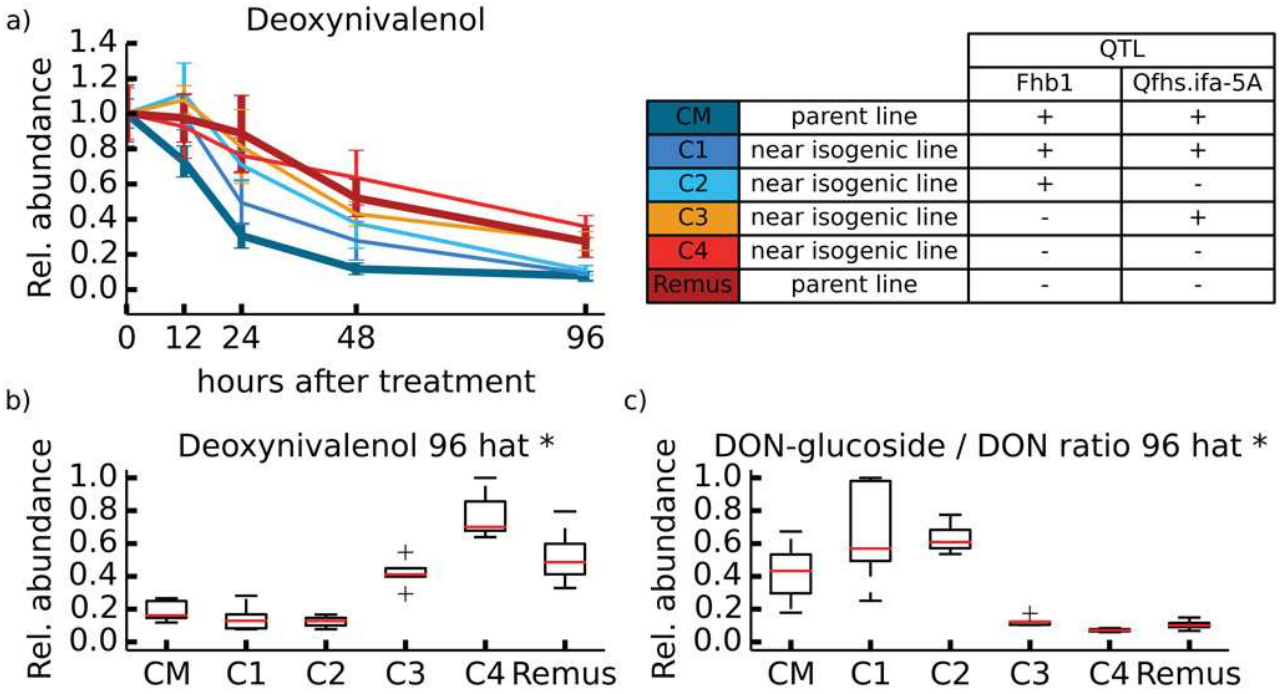

Fig 3. Overview on DON degradation. Time course for the degradation of DON (1 mg) of wheat lines 'CM-82036', C1, C2, C3, C4 and 'Remus'. Wheat ears were sampled 0, 12, 24, 48, and 96 hours after treatment (n = 5 biological replicates per time point and wheat line). a) Degradation rate of DON. b) boxplot of relative concentrations 96 h after DON treatment. c) DON-glucoside/DON ratio 96 h after DON treatment. * Significantly differing DON levels between wheat lines with and without resistance QTL *Fhb1* based on a non-paired t-test (5% global significance threshold).

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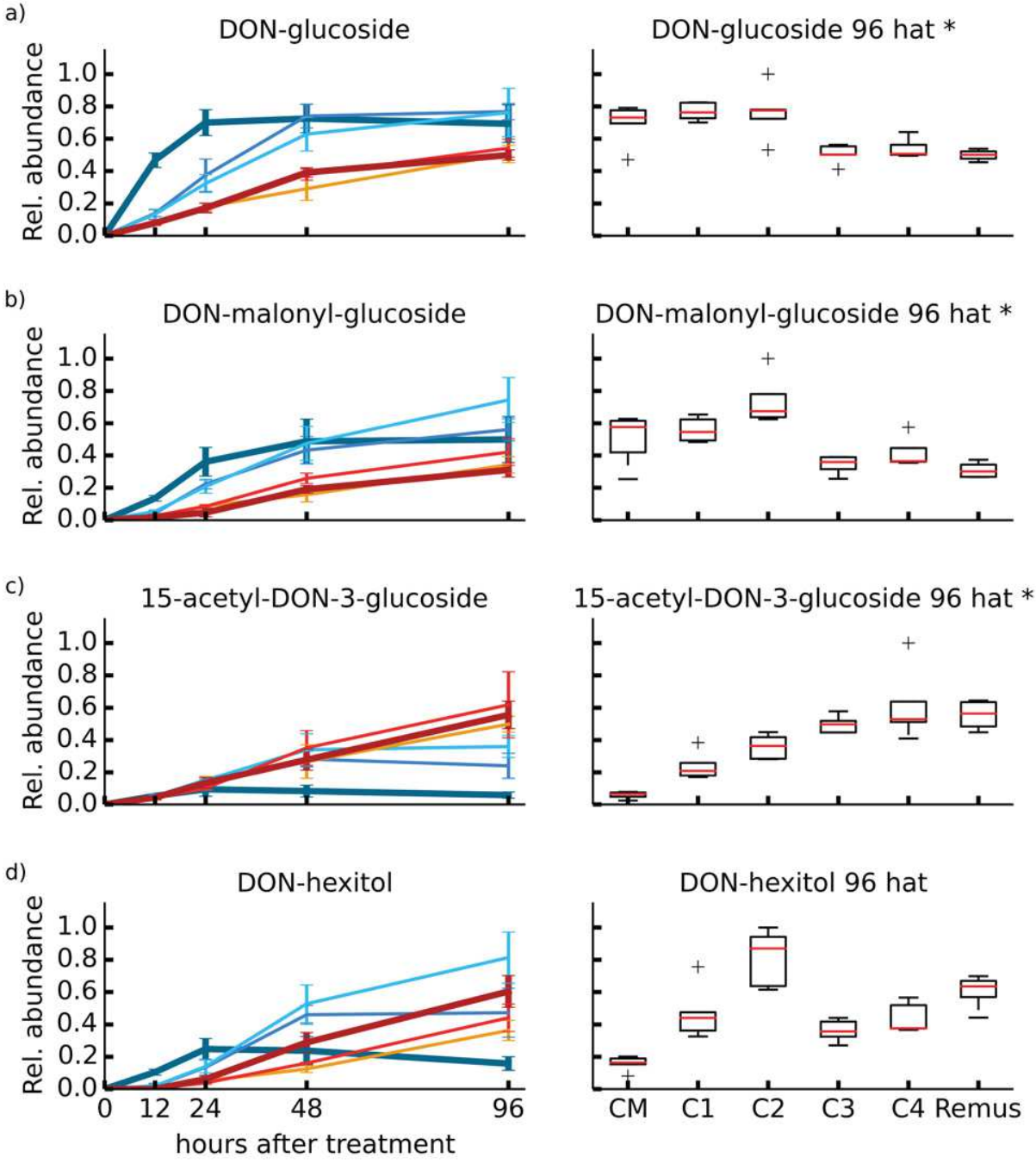
cultivar. After 96 h relative concentrations of DON (Fig. 3b) were observed to be significantly higher in wheat lines, which do not harbor the resistance QTL *Fhb1*. In contrast *Fhb1* harboring wheat lines showed significantly lower relative DON concentration levels after 96 h indicating the presence of an efficient mechanism to lower the toxin concentration. As shown in Fig. 3c, D3G/DON ratio under the mentioned test conditions is significantly higher in wheat lines 'CM-82036', C1 and C2, all harboring the resistance QTL *Fhb1*. Time course profiles of all biotransformation products (except DON-di-hexoside) of DON were examined with respect to the different combinations of FHB resistant QTL *Fhb1* and *Qfhs.ifa-5A* across the six wheat lines and are illustrated in Figs. 4 and 5 respectively.

Detoxification of DON via glucosylation/sugar alcohol conjugation. The resistant cultivar 'CM-82036' showed the fastest D3G formation rate of all tested wheat lines (Fig. 4a). The maximum D3G concentration was already reached 24 h after treatment with DON. Also the NILs C1 and C2, both harboring the resistance QTL *Fhb1*, showed higher formation rates of D3G compared to lines without that specific QTL. These findings support the earlier hypothesis that *Fhb1* influences the ability to detoxify DON via glucosylation [28]. In this respect DON-MalGlc (Fig. 4b), which is assumed to be derived from D3G showed a time course similar to D3G. Wheat lines carrying *Fhb1* showed faster formation of DON-MalGlc within 48 h after inoculation, and higher concentration levels 96 h after inoculation compared to those without *Fhb1*. D3G as well as DON-MalGlc showed significantly higher concentrations 96 h after treatment of wheat lines harboring *Fhb1*.

Interestingly, the time course for the formation of 15ADON3G (Fig. 4c) was different compared to that of the other DON-glucose derivatives D3G, DON-MalGlc. Concentration levels of 15ADON3G in C3, C4 and 'Remus' were increasing continuously during the observation



QTL	CM	C1	C2	C3	C4	Remus
Fhb1	+	+	+	-	-	-
Qfhs.ifa-5A	+	+	-	+	-	-

Fig 4. Detoxification of DON via glucosylation/sugar alcohol. Glucose/sugar alcohol related detoxification of DON. Relative formation rates for the biotransformation products DON-glucoside (a), DON-malonyl-glucoside (b), 15-acetyl-DON-3-glucoside (c) and DON-hexitol (d). Additionally, for each biotransformation product boxplots for relative metabolite abundance observed 96 h after DON treatment were generated. * Significantly differing biotransformation product levels between wheat lines with and without resistance QTL *Fhb1* based on a non-paired t-test (5% global significance threshold).

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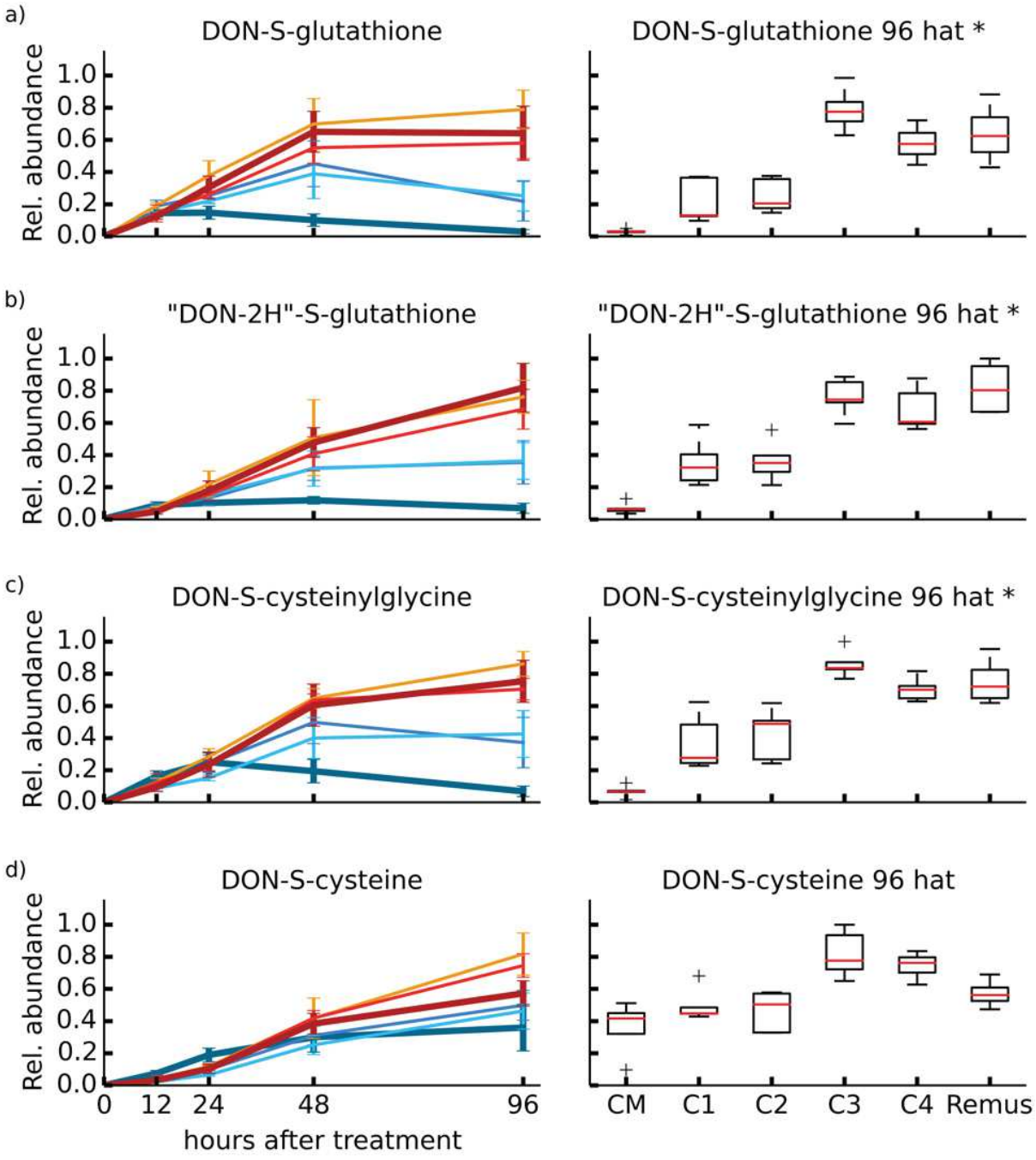

QTL	CM	C1	C2	C3	C4	Remus
Fhb1	+	+	+	-	-	-
Qfhs.ifa-5A	+	+	-	+	-	-

Fig 5. Detoxification of DON via glutathione pathway. Glutathione pathway related detoxification of DON. Relative formation rates for biotransformation products DON-S-glutathione (a) and its related degradation products "DON-2H"-S-glutathione (b), DON-S-cysteinylglycine (c) and DON-S-cysteine (d). Additionally, for each biotransformation product boxplots for relative metabolite abundance observed 96 h after DON treatment were generated. * Significantly differing biotransformation product levels between wheat lines with and without resistance QTL *Fhb1* based on a non-paired t-test (5% global significance threshold).

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period and showed significantly higher levels after 96 h than the wheat lines carrying *Fhb1*. This suggests that first 15-ADON is formed, in a competitive reaction, which is less pronounced in lines harboring *Fhb1*, thus, the subsequent formation of 15ADON3G is less favored in these wheat lines. The acetylation of DON is clearly performed by the plant, no ADON was present in the applied DON, and no 15ADON3G was observed in the samples harvested immediately after application with DON.

The kinetics of the putatively annotated DON-hexitol did not show a clear trend between wheat lines harboring *Fhb1* and without. Again the resistant cultivar 'CM-82036' showed the highest relative concentration 24 hours after treatment and then continuously decreased whereas all other wheat lines showed continuously increasing formation rates.

Detoxification of DON via the glutathione pathway. In contrast to glucosylated DON derivatives, detoxification to DON-GSH and its further degradation products, DON-S-Cys-Gly, DON-S-Cys and "DON-2H"-GSH was more efficient in 'Remus', C3 and C4, which all lack the resistance QTL *Fhb1*. Twelve hours after treatment all wheat lines roughly showed the same concentration levels of DON-GSH (Fig. 5a), while at later time points the cultivars harboring the QTL *Fhb1* showed significantly lower concentration levels for DON-GSH, DON-S-Cys-Gly and "DON-2H"-GSH. Only DON-S-Cys showed rising levels throughout the whole observation period indicating that this metabolite is being formed from DON-GSH via DON-S-Cys-Gly. One reason for the different kinetics between glucose- related and GSH- related metabolism might be that in the *Fhb1* harboring wheat lines, the formation of D3G is more efficient and thus the DON for the competing formation of DON-GSH and related derivatives is less available in these wheat lines. An apparent problem with this interpretation is that there is still a huge excess of unmetabolized DON detected in the presented experiment. Yet, this does not take into account that the intracellular concentration of DON may be much lower due to the action of drug efflux pumps, which are highly induced according to gene expression studies of DON treated barley [47], DON treated wheat [48] or *F. graminearum* inoculated wheat [49,50]. The pool of intracellular DON may therefore be limiting for the competing enzymes, so that DON can be metabolized preferentially into one or the other major pathway.

In this study DON was only applied as a single dose to all wheat lines and thus the resulting conclusions were made based on the described conditions. We conclude that all wheat lines under investigation have both, glucosylation-related as well as the glutathione pathway for the detoxification of DON. The detoxification of DON is faster in *Fhb1* harboring wheat lines, which can be mainly attributed to the formation of D3G and DON-MalGlc. The time course profiles as well as the relative concentrations 96 h after treatment show higher glucoside related biotransformation (D3G, DON-MalGlc) in *Fhb1* harboring wheat lines. The relative concentrations of GSH related metabolites (except DON-S-Cys) are significantly higher 96 h after treatment in wheat lines lacking *Fhb1*. In many plant-pathogen systems the dynamics of the plant resistance reaction has been described to be decisive for the outcome "diseased or resistant" [51]. As our findings clearly demonstrate that the presence of the QTL *Fhb1* was correlated with an increased metabolism of DON, it can be concluded that the speed of DON detoxification seems to be a decisive factor for resistance towards FHB enhancing the efficient glucosylation of DON.

Moreover, as our experiment employed treatment with pure toxin, the observed metabolic response *in planta* can be clearly attributed to DON only. It shall be noted however that the time course profiles and D3G/DON ratios obtained for the single dose DON treatment applied in this study, cannot directly be compared to the situation found during natural *F. graminearum* infection. Compared to single dose toxin treatment, continuous DON production as well as the release of additional fungal low molecular weight effectors and proteins, modulates the defense response under natural infection conditions. Transcription of UDP-

glucosyltransferases and glutathione-S-transferases have been shown to be highly DON inducible [47,50]. Whether these transcripts can be translated into active detoxification enzymes depends on the intracellular concentration of DON. While protein biosynthesis may be completely blocked at the site of DON application, lower levels due to diffusion should allow induction of the detoxification enzymes in neighboring tissue. In the study of Lemmens *et al.* two adjacent spikelets were treated with DON, and the whole ear was extracted after ripening (21 days). As both, infected as well as non-infected spikelets of DON treated wheat ears were included by Lemmens *et al.*, an efficient detoxification of DON could be expected at sites of relatively low toxin concentration (distant from the infected spikelets). The different rates of DON formation of the tested wheat lines can therefore explain the large differences in D3G/DON ratio found by Lemmens *et al.* In contrast, no significant difference in the D3G/DON ratio was found in inoculated spikelets and rachis between NILs differing in *Fhb1* 72 hours post inoculation (Gunnaiah *et al.*, 2012). This might be due to high local DON concentrations preventing an efficient DON metabolism directly in the infected tissue. In conclusion, both studies have an entirely different study design, with totally different toxico-kinetics and—dynamics (e.g. single application versus continuous DON production by the fungus, different tissue as well as time point of sampling), which could result in the described different findings. Another complication explaining seemingly contradictory results is that the advancing fungus, which overwhelms the initially inoculated spikelet regardless of the genotype, has the ability to efficiently reactivate D3G initially detoxified and stored in the vacuole with powerful β-glucosidase (data not shown). The capability of enzymatic hydrolysis of D3G has also been demonstrated for cellulase from *Trichoderma* and cellobiase from *Aspergillus* species [52].

Conclusion

In the present study, which aimed to study the metabolism of DON in wheat, four DON biotransformation products have been assigned to glucose- and another four to GSH-related DON detoxification pathways. Comparison of a set of six wheat lines, which carry different combinations of the two major resistance QTLs (*Fhb1* and Qfhs.ifa-5A) against FHB, revealed a faster D3G and DON-MalGlc formation in *Fhb1* lines. DON belongs to the group of trichothecene mycotoxins and is well known as a major virulence factor of *F. graminearum* most probably by inhibiting the biosynthesis of defense related proteins. Under our conditions, DON detoxification in wheat is clearly correlated with a major QTL for FHB resistance, *Fhb1*, and results in different formation rates of the respective DON biotransformation products.

Due to the treatment with pure toxin at the beginning of flowering in this study, all observed QTL specific effects can be clearly attributed to the presence of DON. However, these effects are not always evident in *Fusarium* infection experiments, which might also be attributed to the different experimental designs in the studies presented by Gunnaiah et al. [29] and Lemmens et al. [28] respectively. The occurrence of the detected conjugates in naturally *F. graminearum* infected cereals and their role as potential masked mycotoxins warrant further investigation in the future.

Supporting Information

S1 Table. Peak areas of DON biotransformation products used to establish the time courses, which are presented in this study.
(TXT)

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Author Contributions

Conceived and designed the experiments: BK CB ML HM AM AK IM FB GA RS. Performed the experiments: BK ML HM AM AK IM. Analyzed the data: BK CB AM FB RS. Contributed reagents/materials/analysis tools: BK CB ML HM AM AK IM FB GA RK RS. Wrote the paper: BK CB ML HM AM FB GA RK RS.

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APPENDIX III

Meng-Reiterer J, Varga E, Nathanail AV, Bueschl C, Rechthaler J, McCormick SP, Michlmayr H, Malachova A, Fruhmann P, Adam G, Berthiller F, Lemmens M, Schuhmacher R

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RESEARCH PAPER

Tracing the metabolism of HT-2 toxin and T-2 toxin in barley by isotope-assisted untargeted screening and quantitative LC-HRMS analysis

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Abstract An extensive study of the metabolism of the type A trichothecene mycotoxins HT-2 toxin and T-2 toxin in barley using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is reported. A recently developed untargeted approach based on stable isotopic labelling, LC-Orbitrap-MS analysis with fast polarity switching and data processing by MetExtract software was combined with targeted LC-Q-TOF-MS(/MS) analysis for metabolite structure elucidation and quantification. In total, 9 HT-2 toxin and 13 T-2 toxin metabolites plus tentative isomers were detected, which were successfully annotated by calculation of elemental formulas and further LC-HRMS/MS measurements as well as partly identified with authentic standards. As a result, glucosylated forms of the toxins, malonylglucosides, and acetyl and feruloyl conjugates were elucidated. Additionally, time courses of metabolite formation and mass balances were established. For absolute quantification of those compounds for which standards were available, the method was validated by determining apparent recovery, signal suppression, or enhancement and extraction recovery. Most importantly, T-2 toxin was rapidly metabolised to HT-2 toxin and for both parent toxins HT-2 toxin-3-*O*-*β*-glucoside was identified (confirmed by authentic standard) as the main metabolite, which reached its maximum already 1 day after toxin treatment.

Jacqueline Meng-Reiterer and Elisabeth Varga contributed equally to this work.

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Keywords Untargeted metabolite profiling · Stable isotopic labelling · Fast polarity switching · Type A trichothecenes · Masked mycotoxins

Introduction

HT-2 toxin (HT2) and T-2 toxin (T2) are secondary metabolites of fungi belonging to the genus *Fusarium* and are classified as type A trichothecene mycotoxins. Small grain cereals, oats, wheat and barley, are especially affected by type A trichothecene-producing fungi and consequently are prone to contamination with HT2 and T2 [1, 2]. The toxins differ structurally in an acetyl group at the C-4 position (see Electronic Supplementary Material (ESM) Fig. S1) but show similar toxicological effects such as immunotoxicity and haematotoxicity [3].

Plants employ various detoxification mechanisms to cope with the adverse effects of mycotoxins. For instance, phase I metabolism processes (enzymatic hydrolysis, oxidation and reduction) as well as phase II metabolism processes (covalent binding of, e.g. glucose, malonic acid, sulphuric acid, amino acids or glutathione) are used by the affected plants to inactivate xenobiotics [4]. Understanding the plant metabolism of mycotoxins and thus the resulting metabolic derivatives is becoming increasingly important for risk assessment. Up to now, there is no legislation for these so-called masked mycotoxins in food or feed, although studies [5, 6] have indicated that they might exhibit similar toxicity when cleaved during digestion [4]. Only limited knowledge exists about the biotransformation process of HT2 and T2 in plants. In an early article, Mirocha et al. [7] reported the occurrence of HT2, T-2-tetraol, 3′-hydroxy-HT-2 and 3′-hydroxy-T-2 formed in T2-treated *Baccharis* species. A few authors have described monoglucoside derivatives [2, 8–12] (HT2-Glc and T2-Glc) and diglucoside derivatives [10, 11] (HT2-di-Glc) to be formed in planta. Additionally, an extensive study of the metabolism of HT2 and T2 in wheat was recently performed in our lab [13]. Whilst the work presented here details the technical aspects of metabolite detection and characterisation, the study mentioned above focuses on the biological interpretation of HT2 and T2 metabolism in wheat and therefore complements the presented study.

Generally, the global untargeted analysis of endogenous metabolites and metabolic products of xenobiotics in biological systems constitutes a major challenge because of their chemical and physical diversity [14, 15]. Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is often used for metabolism studies. To cover as many metabolites as possible, it has been shown that measurements in positive and negative polarity should be performed which is ideally combined in time-saving fast polarity switching mode [16]. However, untargeted full scan LC-HRMS measurements produce large datasets which have to be interpreted properly. To this end, bioinformatic tools are frequently applied to extract relevant signals from raw data. Data interpretation with the single use of statistical methods such as principle components analysis (PCA) is limited to differential comparison between the tested experimental conditions and is partly error-prone [14]. One way to circumvent this problem is the employment of isotopic labelling approaches. Several authors have successfully performed metabolism studies employing stable isotopically labelled tracers [17–19]. In a further step, software tools to recognise specific characteristics of labelling in measurement data enable rapid and automated data evaluation [20, 21]. Bueschl et al. [22, 23] developed a programme to extract signals of labelled metabolites from LC-MS data. This procedure allows a truly untargeted analysis with the complete removal of unwanted signals coming from biological matrix, solvents, reagent impurities, background and instrument noise.

The objective of this study was to investigate the metabolism of the two major type A trichothecenes HT2 and T2 in barley. For this purpose, an untargeted method was applied by combining stable isotopic labelling, LC-Orbitrap-MS analysis in fast polarity switching mode and MetExtract data processing. After structure annotation, quantification experiments were performed with a Q-TOF instrument in order to study the kinetics of the metabolism of parent toxins and their major biotransformation products. To the best of our knowledge, this is the first demonstration of an automated fast polarity switching approach used to study the metabolism of a xenobiotic in planta. To cover the set of potential toxin derivatives as complete as possible, measurements in both polarities were essential resulting in complementary information, revealing novel metabolites.

Materials and methods

In general, three main experiments were carried out named hereafter qualitative screening, structure annotation and time course experiment (workflow presented in ESM Fig. S2).

Chemicals and standards

Methanol (MeOH) and acetonitrile (ACN) were purchased from VWR (Vienna, Austria). 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 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).

The following standard compounds (all purities specified by the supplier) were purchased from Romer Labs (Tulln, Austria): crystalline non-labelled HT2 (purity 92 %) and T2 toxin (purity 85 %) as well as uniformly labelled U-$^{13}C_{22}$ HT2 (purity 86 %; 99.3 atom% ^{13}C) and U-$^{13}C_{24}$ T2 (purity 98 %; 99.6 atom% ^{13}C). Barley test solutions of HT2 and T2 (qualitative screening experiment) were prepared by mixing non-labelled and labelled toxins (both 2,000 mg L^{-1}) 1+1 (*v*/*v*) to obtain a concentration of 1,000 mg L^{-1} per toxin in ACN/MeOH/H_2O 10+45+45 (*v*/*v*/*v*). All biological material treated with this mixture is later referred to as labelled barley samples or $^{12}C/^{13}C$ samples. Time course experiments were performed with a test solution of non-labelled HT2 and T2 toxin with 1,000 mg L^{-1} in ACN/H_2O 1+1 (*v*/*v*)+1 % Tween 20. Respective samples are later referred to as non-labelled or ^{12}C samples. For each experiment, test solutions solely containing the corresponding solvent mixtures (mock) were prepared to obtain blank samples. Analytical standards of HT2 and T2 toxin for quantification experiments were purchased from Romer Labs at concentrations of 100 mg L^{-1} (purity>99.9 %) and 101 mg L^{-1} (purity>99.9 %), respectively in ACN. Standard T2-α-Glc was prepared as described by McCormick et al. [12]. HT2-3-*O*-β-Glc and 3-acetyl-T2 were enzymatically produced or chemically synthesised within the scope of other studies (unpublished data). Highly pure standards of HT2 and T2 were used as raw materials, the final products were characterised by nuclear magnetic resonance measurements and the purities were estimated to be ≥95 %. Stock solutions of HT2-3-*O*-β-Glc, 3-acetyl-T2 and T2-α-Glc were prepared by dissolving in ACN to obtain concentrations of 1,000, 5,000 and 2,000 mg L^{-1}, respectively.

Cultivation of barley plants

For the qualitative screening and 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. 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 ears per treatment group) for both plant experiments.

During the whole experiment, the pots were watered if required (typically three times per week). 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 harvest of barley plants

Experiments included three treatment groups, HT2, T2 and mock, which were applied separately on different barley ears in triplicate. Treatment started at flowering stage of the respective ears. Test solutions were injected into the spikelets with an electronic pipette. To enhance toxin diffusion into the plants, small transparent plastic bags were internally wetted by spraying with purified water, placed over the barley ears after each treatment step and removed 24 h (±2 h) later. On the day of harvest, treated barley ears were cut, weighed, immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

Treatment for the qualitative screening experiment was based on pipetting 5 μL of the test solution (mixture of non-labelled and labelled toxins and mock) into spikelets of barley ears. The lowest four spikelets per ear (two per row) were treated, and 2, 4 and 5 days later, we continued with the next four spikelets above. Finally, on the sixth day, two more adjacent spikelets were treated resulting in a total of 18 treated spikelets or 90 μg applied toxin (native and labelled toxin). Treated plants were harvested on the seventh day resulting in a total of five time points per ear (treatment 7, 5, 3, 2 and 1 days before harvest) per treatment group. One extra time point was monitored in the form of a single treatment per treatment group. This was performed by treating 20 spikelets (two per row) of one ear with a total amount of 100 μg toxin and harvesting at full-ripening stage (approx. 8 weeks after treatment). Concerning all previously mentioned treatments, only treated parts of the ears were analysed.

The following treatment procedure was used for the time course experiments of HT2 and T2: For each toxin plus mock, in total, 20 spikelets (two per row) were treated with 10 μL of

the test solution (non-labelled toxins 1,000 mg L^{-1} and mock) on a single day. This resulted in 200 μg applied toxin per ear. Whole ears were sampled immediately (time point 0), 1, 3 and 7 days after treatment and at full-ripening stage (approx. 6 weeks after treatment).

Sample preparation

Frozen barley ears were milled with a ball mill (MM 301 Retsch, Haan, Germany) for 30 s at 30 Hz under cooled conditions (liquid nitrogen). Milled samples were weighed (100± 2 mg) into Eppendorf tubes. 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 with 200 rpm. After centrifugation for 10 min at 22,570×*g*, supernatants were transferred to HPLC vials. Raw extracts of labelled samples plus mock samples were diluted 1+3 (*v*/*v*) with 0.1 % aqueous FA prior to the LC-Orbitrap-MS measurement. For each treatment group, one extract was additionally measured undiluted as well as concentrated by a factor of 4 (evaporated to dryness and dissolved in 1/4 volume). For structure elucidation, LC-Q-TOF-MS/MS spectra were recorded with undiluted raw extracts of $^{12}C/^{13}C$, as well as ^{12}C (time course experiment) samples. Quantification of HT2, T2 and its metabolites was performed with ^{12}C sample extracts which were partially measured undiluted but also diluted 1+9 (*v*/*v*) and 1+49 (*v*/*v*) with ACN/H_2O 1+1 (*v*/*v*).

LC-HRMS(/MS) analysis

Qualitative screening

Labelled samples and mock samples were measured with an UltiMate 3000 HPLC system coupled to an Exactive Plus Orbitrap mass spectrometer (both from Thermo Fisher Scientific). Ten microlitres of sample solution was injected into the system. For chromatographic separation, a Zorbax SB-C18 column (150×2.1 mm, 3.5 μm; Agilent Technologies) was used at 25 °C and at a flow rate of 250 μL min^{-1}. Mobile phases consisted of H_2O (eluent A) and MeOH (eluent B), both containing 0.1 % FA (*v*/*v*) and 5 mM NH_4HCO_2. Gradient method 1 was as follows: 0–0.5 min, 10 % B; 0.5–20.0 min, 10–100 % B; 20.0–25.0 min, 100 % B; 25.0–25.1 min, 100–10 % B; and 25.1–30.0 min, 10 % B. Mass spectrometric analysis was performed in fast polarity switching mode using electrospray ionisation. Applied settings were similar to Kluger et al. [16] with some modifications: Automatic gain control was set to 5×10^5, and a maximum injection time of 500 ms was used. Full scan measurement was carried out in the scan range of *m*/*z* 130–1,300 with a resolution of 70,000 full width at half maximum (FWHM) at *m*/*z* 200. The instrument was calibrated with Pierce Ion Calibration Solution in both polarity modes prior to analysis. For data evaluation, software Thermo Xcalibur 2.2 was applied.

Structure annotation and time course experiment

Further qualitative and quantitative measurements were performed with a 1290 Infinity UHPLC system coupled to a 6550 iFunnel Q-TOF mass spectrometer (Agilent Technologies). The chromatographic method employed a Zorbax SB-C18 Rapid Resolution HD column (150×2.1 mm, 1.8 μm; Agilent Technologies), at 30 °C and at a flow rate of 250 μL min^{-1}. The same mobile phase composition was used as above described for Orbitrap measurements. For the acquisition of LC-HRMS/MS spectra, gradient method 2 was applied: 0–0.5 min, 10 % B; 0.5–20.0 min, 10–100 % B; 20.0–22.0 min, 100 % B; 22.0–22.1 min, 100–10 % B; and 22.1–25.0 min, 10 % B. To differentiate between isomers of formed toxin metabolites in the chromatogram and to compare with available standards, the longer gradient method 3 was used: 0–0.5 min, 20 % B; 0.5–3.0 min, 20–45 % B; 3.0–43.0 min, 45–65 % B; 43.0–43.1 min, 65–100 % B; 43.1–45 min, 100 % B; 45.0–45.1 min, 100–20 % B; and 45.1–50.0 min, 20 % B. Relative and absolute quantification experiments (time course experiments) were performed with a shortened gradient method 4: 0–0.5 min, 20 % B; 0.5–6.0 min, 20–100 % B; 6.0–8.0 min, 100 % B; 8.0–8.1 min, 100–20 % B; and 8.1–10.0 min, 20 % B.

LC-Q-TOF full scan mass spectra and LC-MS/MS spectra were acquired with 2 GHz in positive and negative mode within *m*/*z* 50–1,500 at a scan rate of 3 spectra s^{-1}. For all measurements, the following MS settings were used: capillary voltage, 4,000 V; nozzle voltage, 500 V; fragmentor voltage, 380 V; drying gas temperature and flow, 130 °C and 14 L min^{-1}, respectively; nebulizer, 30 psig; and sheath gas temperature and flow, 300 °C and 10 L min^{-1}, respectively. Precursor ion selection for fragmentation occurred in the quadrupole with an isolation width of *m*/*z* 1.3. Mass accuracy of Q-TOF instrument was checked and potentially optimised before analysis. Continually infused reference masses (positive *m*/*z* 121.0509, *m*/*z* 922.0098; 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.

Data processing by MetExtract

Acquired profile mode data were centroided and converted to the mzXML data format [24] with ProteoWizard [25] and successively processed with the in-house developed software MetExtract [16]. The tool

first searched for characteristic isotope patterns of native (^{12}C or M and $M+1$) and partially ^{13}C-labelled (^{13}C or M' and $M'-1$) metabolite ions (ion pairs) in each MS scan. The observed m/z difference between ^{12}C/^{13}C metabolite ion pairs corresponded to n-labelled carbon atoms originating from the uniformly ^{13}C-labelled tracer. This value had to show less than ±4 ppm deviation (based on preliminary evaluation of the mass accuracy from raw data) from the theoretical m/z difference ($\Delta m/z = n \times 1.00335$/charge). Moreover, the observed ratio of the ^{12}C/^{13}C ion pairs had to be approximately 1 (±0.5). Extracted ion chromatograms (EICs, m/z extraction window of ±5 ppm) generated for M and M' ions were recognised as chromatographic peaks with the algorithm of Du et al. [26] and had to show a minimum Pearson correlation of 0.75. Such extracted feature pairs were then convoluted into feature groups (i.e. metabolites) using a minimum Pearson correlation of 0.9.

Method validation for quantification

For in-house method validation, apparent recovery (R_A), signal suppression or enhancement (SSE, also known as matrix effects) and extraction recovery (R_E) were determined according to Sulyok et al. [27]. Two different barley blank matrices were used for this purpose: mock time point 1 day and mock time point ripen. A 1-mg L^{-1} (per toxin) stock solution including HT2, T2, HT2-3-*O*-*β*-Glc, 3-acetyl-T2 and T2-*α*-Glc in ACN was prepared. Blank samples were spiked at one level to obtain 300 μg L^{-1} per toxin in final matrix solution and were analysed in biological triplicate. Apparent recovery was evaluated by spiking stock solution before extraction to milled mock samples. Solvent was evaporated overnight, and extraction was conducted on the next day according to the procedure mentioned above. Matrix effects were determined by adding stock solution after extraction of blank mock samples to obtain 300 μg L^{-1} per toxin in undiluted matrix solution as well as in 1+9 (*v*/*v*) and 1+49 (*v*/*v*) dilutions (diluted with ACN/H_2O 1+1 (*v*/*v*)). After LC-Q-TOF-MS measurement, EICs of the target analytes ($[M+NH_4]^+$ adducts) were automatically extracted by MassHunter Quantitative Analysis software with a m/z extraction window of ±30 ppm (based on preliminary evaluation of the mass accuracy from raw data). R_A and SSE were provided by dividing the peak area of the respective metabolite obtained for spiked matrix sample by the area of a corresponding standard (mean value, derived from triplicate) and multiplying by the factor of 100. Extraction recovery was calculated by using the ratio of R_A to SSE. Mean values and relative standard deviations (RSDs) were calculated from R_A, SSE and R_E in triple determination.

Time course of metabolite formation—absolute and relative quantification

Absolute and relative quantification was performed with the UHPLC-Q-TOF instrument in positive full scan mode with chromatographic gradient method 4 and was based on EICs of ammonium adducts of the respective analytes (m/z extraction window of ±30 ppm). Where standards were available, absolute amounts of HT2, T2 and its metabolites could be plotted versus harvest time point after treatment. The following compounds were quantified: HT2, T2, HT2-3-*O*-*β*-Glc, 3-acetyl-T2 and T2-Glc. Although the T2 metabolite T2-Glc was annotated as T2-*β*-Glc, available standard T2-*α*-Glc was used for quantification assuming similar ionisation efficiency. External calibration was applied with concentrations at six levels in the range of 3–1,000 μg L^{-1}, and linear calibration curves were $1/x$ weighted. Biological replicates and different dilutions (mentioned above) of non-labelled 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) 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 ($n=3$).

Results and discussion

The qualitative screening of HT2 and T2 metabolites in barley was generally based on treatment of barley ears with a mixture of non-labelled and uniformly ^{13}C-labelled toxin, extraction, measurement of sample extracts with LC-Orbitrap-MS in fast polarity switching mode and data processing by MetExtract.

HT2 metabolism in barley

The total ion chromatogram (TIC) and EICs based on MetExtract data processing output of one representative labelled HT2 barley sample measured with LC-Orbitrap-MS is shown in Fig. 1. After automated data processing by MetExtract, HT2-derived metabolite peaks emerged clearly which are presented in form of EICs.

Application of MetExtract to full scan Orbitrap-derived chromatograms of ^{12}C/^{13}C-HT2 barley samples revealed features which were grouped according to both retention time and peak shape similarity. Every such feature group represents

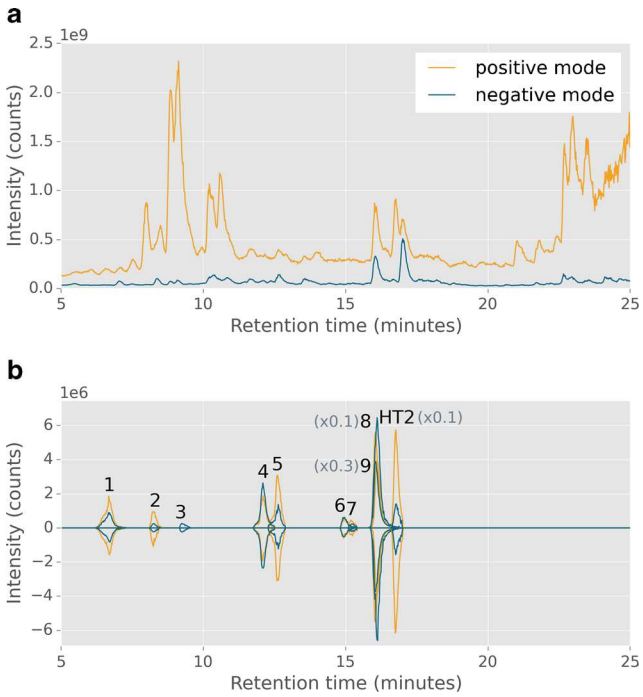

Fig. 1 Illustration of fast polarity switching measurement using an Exactive Plus Orbitrap instrument (**a**) and extracted ion chromatograms (*EICs*) based on MetExtract data processing output (**b**). One representative barley sample treated with a 1+1 (*v*/*v*) mixture of non-labelled and uniformly ^{13}C-labelled HT-2 toxin (5 time points per flowering ear) was used to depict positive (*orange*) and negative (*blue*) total ion chromatogram of Orbitrap measurement and EICs of non-labelled (*up*) and labelled (*down*) HT-2 toxin (*HT2*) as well as its feature groups (*metabolites*) obtained by MetExtract software. *Numbers* above EICs refer to HT-2 toxin metabolites listed in Table 1, and some EICs were scaled down for better visibility of the low abundant metabolites

a distinct metabolite plus unmodified parent toxin HT2. Table 1 summarises all annotated HT2 metabolites in barley. The accurate mass and ion species either of the chosen precursor for the follow-up LC-HRMS/MS experiments or the most abundant ion species for metabolites with low intensity are provided. Due to methodical limitations (e.g. LOD) and the strict criteria of MetExtract software, it is to be expected that especially low abundant metabolites might be missed in some samples. In most cases, this can be explained by the high degree of ^{13}C-isotopic enrichment of the parent toxins and thus of its metabolites resulting in missing isotopologues ($M'-1$) of the labelled metabolite form. Therefore, even MetExtract hits detected in a single measurement only were considered. On the other hand, MetExtract hits also included false positives whose number increased with higher sample concentration. These false-positive features were successfully excluded from further data evaluation either due to an impossible number of carbon atoms, implausible isotope pattern, imperfect coelution of labelled and corresponding non-labelled compounds or LC-HRMS/MS spectra which were not matching any toxin fragments.

Measurements in fast polarity switching mode showed that HT2 biotransformation products were found in both applied polarities, and different adducts were formed which facilitated the annotation of the molecular identity of the ion species and increased the probability of MetExtract detection of metabolites with low abundance. Mainly $[M+Na]^+$, $[M+NH_4]^+$ and $[M+HCOO]^-$ adducts were formed in full scan mass spectra, whilst for the tentative malonylglucoside (MalGlc) derivatives, $[M-CO_2+HCOO]^-$ adducts dominated in negative mode. Although positive and negative adducts were measured, the HT2 metabolite 15-acetyl-T2-tetraol-Glc[−2H] was recognised by MetExtract only in negative mode.

T2 metabolism in barley

MetExtract data evaluation of ^{12}C/^{13}C-T2 barley samples resulted in features and feature groups. In Table 2, all (putatively) identified T2 derivatives in barley are listed. A comparison with Table 1 depicts that all nine toxin metabolites detected in the HT2-treated samples were also detected in the T2-treated samples, due to fast conversion of T2 into HT2 in planta. For T2, four additional metabolites were detected, namely HT2, T2-Glc, 3-acetyl-T2 and feruloyl-T2. Since the untargeted approach enabled the detection of all HT2 metabolites in T2-treated samples, besides 15-acetyl-T2-tetraol-Glc, 15-acetyl-T2-tetraol-MalGlc and 15-acetyl-T2-tetraol-Glc[−2H], we manually searched for them by extracting EICs of corresponding ^{12}C- and ^{13}C-mass signals. This demonstrated that the ^{12}C/^{13}C signal ratios differed considerably from 1 (up to 6) leading to exclusion by MetExtract. Purity measurements of the T2 test solution showed a contamination with approximately 3 % non-labelled neosolaniol. Thus, the enhanced ^{12}C-mass signal might be due to neosolaniol which had been transformed into 15-acetyl-T2-tetraol and its derivatives. From the metabolism study in wheat [13], we got a hint about the presence of one additional T2 metabolite, namely feruloyl-T2. Since it was not recognised by MetExtract because of very low abundance ($M'-1$ isotopologue not detected), a manual screening was performed confirming its occurrence.

Fast polarity switching measurements showed that most T2 biotransformation products were detected in both polarities. On the contrary, under the tested conditions, T2, T2-Glc, 3-acetyl-T2 and feruloyl-T2 were only detected in positive mode as $[M+Na]^+$ and $[M+NH_4]^+$ adducts. Moreover, T2-triol-Glc was recognised by MetExtract only in negative ionisation mode.

Structure annotation of detected HT2 and T2 metabolites

Each metabolite is recorded by MetExtract with the number of C-atoms originating from the studied HT2 or T2. Structure

Table 1 HT-2 toxin and its (putative) biotransformation products in barley

	HT2 metabolites	RT (min)	*m/z*[d]	Ion[d]	Mass accuracy (ppm)	Positive/negative[e]	C-atoms[f]	Sum formula[g]
	HT2[a]	16.8	442.2428	$[M+NH_4]^+$	−1.7	+/−	22	$C_{22}H_{32}O_8$
1	15-Acetyl-T2-tetraol-Glc[b]	6.7	520.2380	$[M+NH_4]^+$	−1.6	+/−	17	$C_{23}H_{34}O_{12}$
2	15-Acetyl-T2-tetraol-MalGlc[b]	8.3	606.2381	$[M+NH_4]^+$	−1.9	+/−	17	$C_{26}H_{36}O_{15}$
3	15-Acetyl-T2-tetraol-Glc[–2H][c]	9.2	545.1867	$[M+HCOO]^-$	−1.6	+/−	17	$C_{23}H_{32}O_{12}$
4	Hydroxy-HT2-Glc[b]	12.1	620.2900	$[M+NH_4]^+$	−2.1	+/−	22	$C_{28}H_{42}O_{14}$
5	Hydroxy-HT2-MalGlc[b]	12.6	706.2906	$[M+NH_4]^+$	−1.5	+/−	22	$C_{31}H_{44}O_{17}$
6	T2-triol-Glc[b]	15.0	567.2402	$[M+Na]^+$	−1.8	+/−	20	$C_{26}H_{40}O_{12}$
7a	HT2-di-Glc[b]	15.2	793.3124	$[M+HCOO]^-$	−1.5	+/−	22	$C_{34}H_{52}O_{18}$
7b	HT2-di-Glc[b]	15.8	793.3124	$[M+HCOO]^-$	−1.5	+/−	22	$C_{34}H_{52}O_{18}$
8	HT2-3-*O*-*β*-Glc[a]	16.1	604.2950	$[M+NH_4]^+$	−2.3	+/−	22	$C_{28}H_{42}O_{13}$
9	HT2-MalGlc[b]	16.1	690.2950	$[M+NH_4]^+$	−2.6	+/−	22	$C_{31}H_{44}O_{16}$

RT retention time, *HT2* HT-2 toxin, *T2* T-2 toxin, *Glc* glucoside, *MalGlc* malonylglucoside

[a] Confirmation with standard by comparison of retention time, accurate mass and HRMS/MS spectra

[b] Annotation with accurate mass, sum formula calculations and HRMS/MS spectra

[c] Annotation with accurate mass and sum formula calculations

[d] Accurate mass and ion species either of the chosen precursor for the follow-up MS/MS experiments or the most abundant ion species for metabolites with low intensity are provided

[e] Detected polarity of compound either by untargeted approach or manually found

[f] Number of C-atoms derived from parent toxin

[g] Sum formula of neutral compound

annotation was performed by searching for typical conjugates, by calculating elemental formulas and by involving characteristic fragments detected in HRMS/MS spectra (Table 3). All toxin derivatives, for which LC-HRMS/MS spectra could be obtained in positive mode, showed fragments of HT2 or T2. The fragmentation patterns were very similar to previously reported MS/MS spectra of these toxins [2, 8, 10, 11, 28]. Since mainly Glc and MalGlc derivatives were found, typical fragments of these molecules were used for metabolite characterisation. Mass deviations of precursor ions did not exceed 5 ppm, whilst mass deviations of fragments did not exceed 16 ppm. Further LC-HRMS/MS measurements with pairs of corresponding non-labelled and labelled precursor ions provided additional information for structure annotation. By calculating mass differences between the corresponding fragments, the number of C-atoms remaining from the parent toxin was elucidated. In contrast, according to the native isotopic composition of the plant constituents, there was no $^{12}C/^{13}C$ mass shift for fragments derived from glucose, malonylglucose or ferulic acid moieties. As far as standards were available, retention time, accurate mass and MS/MS spectra were compared for identification of the biotransformation products. In the following part, annotation based on LC-HRMS/MS of the individual HT2 and T2 metabolites is described. Primarily, *m/z* values experimentally derived from the measurements of the HT2 samples are provided with the exception of those *m/z* values, which refer to T2-specific metabolites.

15-Acetyl-T2-tetraol metabolites (metabolites 1–3)

Our data suggest that barley plants metabolised HT2 or T2 to form putative 15-acetyl-T2-tetraol-Glc (**1**), 15-acetyl-T2-tetraol-MalGlc (**2**) and 15-acetyl-T2-tetraol-Glc[–2H] (**3**) by the cleavage of an acetyl group at C-4 position in the case of T2, the isovaleryl group at C-8 position and covalent binding of glucose and subsequently malonic acid. MetExtract recognised in full scan mass spectra adducts ($[\mathbf{1}+NH_4]^+$, *m/z* 520.2380; $[\mathbf{2}+NH_4]^+$, *m/z* 606.2381; $[\mathbf{3}+HCOO]^-$, *m/z* 545.1867) with a difference of Δ17.057 u (theoretical value) between the non-labelled and labelled metabolite form which corresponds to remaining 17 C-atoms of the HT2 backbone, indicating the loss of the isovaleric acid (isoval acid) moiety minus water (containing five carbon atoms, loss of 84.058 u; theoretical value). LC-HRMS/MS measurements of $[M+NH_4]^+$ and $[M+HCOO]^-$ adducts included characteristic fragments of HT2, glucose and malonylglucose moieties as well as fragment *m/z* 323.1489 (theoretical value) which corresponds to $[15\text{-acetyl-T2-tetraol}-H_2O+H]^+$ (positive LC-HRMS/MS spectra are shown in ESM Figs. S3 and S4). Since intensities for 15-acetyl-T2-tetraol-Glc[–2H] were too low to obtain meaningful LC-MS/MS spectra, in this case, structure annotation was based on sum formula calculations only. A comparison with 15-acetyl-T2-tetraol-Glc gives a difference of Δ2.014 (theoretical value) equivalent to two H-atoms. It is assumed that after the loss of isovaleric acid

Table 2 T-2 toxin and its (putative) biotransformation products in barley

T2 metabolites		RT (min)	*m/z*[d]	Ion[d]	Mass accuracy (ppm)	Positive/negative[e]	C-atoms[f]	Sum formula[g]
	T2[a]	17.9	484.2532	$[M+NH_4]^+$	−1.9	+	24	$C_{24}H_{34}O_9$
1	15-Acetyl-T2-tetraol-Glc[bh]	6.7	520.2384	$[M+NH_4]^+$	−0.9	+/−	17	$C_{23}H_{34}O_{12}$
2	15-Acetyl-T2-tetraol-MalGlc[bh]	8.3	606.2382	$[M+NH_4]^+$	−1.7	+/−	17	$C_{26}H_{36}O_{15}$
3	15-Acetyl-T2-tetraol-Glc[–2H][ch]	9.3	545.1872	$[M+HCOO]^-$	−0.7	+/−	17	$C_{23}H_{32}O_{12}$
4	Hydroxy-HT2-Glc[b]	12.1	620.2902	$[M+NH_4]^+$	−1.7	+/−	22	$C_{28}H_{42}O_{14}$
5	Hydroxy-HT2-MalGlc[b]	12.6	706.2912	$[M+NH_4]^+$	−0.7	+/−	22	$C_{31}H_{44}O_{17}$
6	T2-triol-Glc[b]	14.9	589.2500	$[M+HCOO]^-$	−0.3	+/−	20	$C_{26}H_{40}O_{12}$
7a	HT2-di-Glc[b]	15.2	793.3139	$[M+HCOO]^-$	+0.4	+/−	22	$C_{34}H_{52}O_{18}$
7b	HT2-di-Glc[b]	15.8	793.3139	$[M+HCOO]^-$	+0.4	+/−	22	$C_{34}H_{52}O_{18}$
8	HT2-3-*O*-*β*-Glc[a]	16.1	604.2953	$[M+NH_4]^+$	−1.8	+/−	22	$C_{28}H_{42}O_{13}$
9	HT2-MalGlc[b]	16.1	690.2952	$[M+NH_4]^+$	−2.3	+/−	22	$C_{31}H_{44}O_{16}$
10	HT2[a]	16.8	442.2428	$[M+NH_4]^+$	−1.7	+/−	22	$C_{22}H_{32}O_8$
11	T2-Glc[c]	17.0	651.2617	$[M+Na]^+$	−1.0	+	24	$C_{30}H_{44}O_{14}$
12	3-Acetyl-T2[a]	19.1	526.2640	$[M+NH_4]^+$	−1.3	+	24	$C_{26}H_{36}O_{10}$
13a	Feruloyl-T2[bh]	20.0	665.2557	$[M+Na]^+$	−1.7	+	24	$C_{34}H_{42}O_{12}$
13b	Feruloyl-T2[bh]	20.2	665.2551	$[M+Na]^+$	−2.6	+	24	$C_{34}H_{42}O_{12}$

RT retention time, *HT2* HT-2 toxin, *T2* T-2 toxin, *Glc* glucoside, *MalGlc* malonylglucoside

[a] Confirmation with standard by comparison of retention time, accurate mass and HRMS/MS spectra

[b] Annotation with accurate mass, sum formula calculations and HRMS/MS spectra

[c] Annotation with accurate mass and sum formula calculations

[d] Accurate mass and ion species either of the chosen precursor for the follow-up MS/MS experiments or the most abundant ion species for metabolites with low intensity are provided

[e] Detected polarity of compound either by untargeted approach or manually found

[f] Number of C-atoms derived from parent toxin

[g] Sum formula of neutral compound

[h] Not recognised by untargeted approach

minus water, most probably a double bond (i.e. a keto group at C-8 position) was formed.

Hydroxy-HT2 metabolites (metabolites 4 and 5)

Tentative hydroxy-HT2 metabolites were most probably formed by hydroxylation of the isovaleryl group and conjugation of a glucose (**4**) and subsequently a malonic acid (**5**) molecule, whilst parent toxin T2 additionally lost the acetyl group at C-4 position. $[M+NH_4]^+$ adducts ($[\mathbf{4}+NH_4]^+$, *m/z* 620.2900; $[\mathbf{5}+NH_4]^+$, *m/z* 706.2906) were measured in full scan mass spectra. The difference of ^{12}C and ^{13}C metabolite form was Δ22.074 u (theoretical value), indicating an intact HT2 molecule. Positive and negative LC-HRMS/MS measurements of $[M+NH_4]^+$, $[M+HCOO]^-$ and $[M-H]^-$ ions revealed characteristic fragments of HT2, glucose and malonylglucose moieties (positive LC-HRMS/MS spectra are depicted in ESM Figs. S5 and S6). Furthermore, overlaid MS/MS spectra of corresponding ^{12}C and ^{13}C-labelled precursor ions showed that *m/z* 485.2003 (**4**) and *m/z* 571.2020 (**5**) contain 17 C-atoms of HT2 in accordance with $[M-O-isoval\ acid+H]^+$. Additionally, in LC-HRMS/MS spectra of the negative mode (data not shown), a fragment of *m/z* 117.0558, corresponding to the $[M-H]^-$ ion of isovaleric acid plus one oxygen atom, was observed instead of *m/z* 101.0608 ($[M-H]^-$ of isovaleric acid; theoretical value) which is typical for HT2 and T2 metabolites containing an unmodified isovaleryl group. The direct measurement of the oxygenated isovaleric acid fragment therefore confirms that the hydroxyl group is located at the isovaleryl group of the detected HT2 derivatives. For the two metabolites of interest, the mass increments between fragment $[M-O-isoval\ acid+H]^+$ and HT2 fragment $[HT2-isoval\ acid+H]^+$ of Δ162.053 u (**4**) or Δ248.054 u (**5**) correspond to the loss of glucose minus water or the loss of malonylglucose minus water and verify the presence of glucose (**4**) or malonylglucose (**5**), respectively.

T2-triol-Glc (metabolite 6)

Barley transformed the parent toxins by cleavage of one acetyl group (C-15 position, in case of HT2) or two acetyl groups (C-4 and C-15 position, in case of T2), respectively.

Table 3 Characteristic LC-HRMS/MS fragment ions of HT-2 toxin and T-2 toxin, as well as the glucose and malonylglucose moieties used for structure annotation

m/z[a]	Ion	Sum formula[b]
HT2 fragments		
323.1489	[HT2 – isoval acid+H]$^+$	$C_{17}H_{22}O_6$
263.1278	[HT2 – isoval acid – acetic acid+H]$^+$	$C_{15}H_{18}O_4$
245.1172	[HT2 – isoval acid – acetic acid – H_2O+H]$^+$	$C_{15}H_{16}O_3$
233.1172	[$C_{14}H_{16}O_3$+H]$^+$	$C_{14}H_{16}O_3$
215.1067	[HT2 – isoval acid – acetic acid – H_2O – CH_2O+H]$^+$	$C_{14}H_{14}O_2$
185.0961	[$C_{13}H_{12}O$+H]$^+$	$C_{13}H_{12}O$
T2 fragments		
365.1595	[T2 – isoval acid+H]$^+$	$C_{19}H_{24}O_7$
305.1383	[T2 – isoval acid – acetic acid+H]$^+$	$C_{17}H_{20}O_5$
245.1172	[T2 – isoval acid – 2 acetic acid+H]$^+$	$C_{15}H_{16}O_3$
215.1067	[T2 – isoval acid – 2 acetic acid – CH_2O+H]$^+$	$C_{14}H_{14}O_2$
185.0961	[$C_{13}H_{12}O$+H]$^+$	$C_{13}H_{12}O$
Glucose moiety fragments		
145.0495	[Glucose – 2 H_2O+H]$^+$	$C_6H_8O_4$
127.0390	[Glucose – 3 H_2O+H]$^+$	$C_6H_6O_3$
161.0455	[Glucose – H_2O – H]$^-$	$C_6H_{10}O_5$
Malonylglucose moiety fragments		
249.0605	[Malonylglucose – H_2O+H]$^+$	$C_9H_{12}O_8$
231.0499	[Malonylglucose – 2 H_2O+H]$^+$	$C_9H_{10}O_7$
145.0495	[Glucose – 2 H_2O+H]$^+$	$C_6H_8O_4$
127.0390	[Glucose – 3 H_2O+H]$^+$	$C_6H_6O_3$
105.0182	[Malonic acid+H]$^+$	$C_3H_4O_4$
161.0455	[Glucose – H_2O – H]$^-$	$C_6H_{10}O_5$

HT2 HT-2 toxin, *T2* T-2 toxin, *isoval acid* isovaleric acid

[a] Exact mass

[b] Sum formula of neutral compound

MetExtract software recorded [**6**+Na]$^+$ adduct with *m/z* 567.2402 and [**6**+HCOO]$^-$ adduct with *m/z* 589.2500 as well as differences of Δ20.067 u (theoretical value) between the ^{12}C and ^{13}C peak pairs in LC-HRMS spectra due to a loss of C_2H_2O from the parent toxin HT2. The detected fragment [glucose−H_2O−H]$^-$ with *m/z* 161.0445 in negative MS/MS spectra of adduct [M+HCOO]$^-$ led to the conclusion that tentative T2-triol-Glc was formed.

HT2 and T2 glucosides (metabolites 7a, 7b, 8 and 11)

Glucosylation of HT2 and T2 in barley was observed, including identified HT2-3-*O*-*β*-Glc (**8**) as well as putative HT2-di-Glc (**7**) and T2-Glc (**11**) ([**8**+NH_4]$^+$, *m/z* 604.2950; [**7**+HCOO]$^-$, *m/z* 793.3124; [**11**+Na]$^+$, *m/z* 651.2617). HT2-3-*O*-*β*-Glc and HT2-di-Glc were formed in HT2- as well as in T2-treated (additional loss of acetyl group at C-4 position) ears with a difference of Δ22.074 u (theoretical value) between non-labelled and labelled metabolite forms. LC-HRMS/MS spectra of [M+NH_4]$^+$ (see ESM Figs. S7 and S8) and [M+HCOO]$^-$ precursor ions revealed characteristic fragments of HT2 and glucose moieties as well as one or two losses of glucose minus water with Δ162.053 u (theoretical value) calculated from [M+H]$^+$ adducts. The observed fragmentation patterns are consistent with the literature [2, 8, 9, 11]. Since the retention time, *m/z* of precursor ion and fragmentation pattern of the metabolite HT2-3-*O*-*β*-Glc was compared with those of the HT2-3-*O*-*β*-Glc standard, the structure could be confirmed. The application of another chromatographic method with the longer gradient (gradient method 3) showed that there was only one high metabolite peak which eluted at the same time (15.8 min) as the standard. However, the situation was different for HT2-di-Glc which was annotated by LC-HRMS/MS. Interestingly, two chromatographic peaks of HT2-di-Glc were detected by MetExtract at retention times 15.2 and 15.8 min (gradient method 1; [M+HCOO]$^-$, *m/z* 793.3124). It is assumed that HT2-di-Glc, as many other metabolites, is derived from HT2-3-*O*-*β*-Glc by the connection of the second glucose molecule via 1,4- or 1,6-glycosidic linkage resulting in two structural isomers. However, without

nuclear magnetic resonance measurements, we cannot exclude that the second glucose molecule is located at the available hydroxyl group at the C-4 position of HT2.

T2-Glc was exclusively formed in T2-treated barley. Within positive full scan mass spectra, MetExtract recognised differences of Δ24.081 u (theoretical value) between the ^{12}C and ^{13}C peak pairs originating from an intact T2 backbone. It was not possible to acquire a meaningful MS/MS spectrum due to the low abundance. A retention time comparison of T2-Glc formed in barley with standard T2-α-Glc in triplicate with the long gradient method (gradient method 3) revealed that the structure of metabolite T2-Glc differs from T2-α-Glc. Whilst the biotransformation product eluted at 22.2 min, the retention time of the standard was 21.4 min. Our findings contradict those of McCormick et al. [12], who reported the occurrence of T2-α-Glc in naturally contaminated oats and wheat. Since the glucoside is most likely formed by a family 1 UDP-glucosyltransferase, which are inverting enzymes, the formation of an alpha glucoside would be very surprising. The difference of retention times indicates that T2 is converted into T2-3-*O*-β-Glc in barley under the tested conditions because no other position of T2 is available to bind glucose. The elution order was in accordance with the mentioned study.

HT2-MalGlc (metabolite 9)

It is suggested that barley plants metabolise HT2 into HT2-3-*O*-β-Glc and subsequent into tentative HT2-MalGlc by covalent binding of malonic acid to the glucose moiety. Regarding T2 metabolism, the acetyl group at C-4 position is rapidly cleaved to form HT2 (see below). Within full scan mass spectra, $[\mathbf{9}+NH_4]^+$ adduct with *m/z* 690.2950 was measured. Since the differences of these precursor ions to its ^{13}C-mass signals were Δ22.074 u (theoretical value), it was obvious that the parent toxin HT2 was intact. Figure 2 depicts the LC-MS/MS spectrum of HT2-MalGlc in positive mode. Fragmentation of $[M+NH_4]^+$ and $[M-H]^-$ adducts show the typical fragments of malonylglucose moiety plus fragment *m/z* 425.2170 which corresponds to the putative loss of malonylglucose minus water (Δ248.053 u, theoretical value) from the $[M+H]^+$ adduct. These results compare favourably with those reported by Kluger et al. [29], who have described a similar fragmentation pattern of deoxynivalenol-MalGlc.

HT2 as T2 metabolite (metabolite 10)

T2 was rapidly transformed into HT2 in barley by the loss of an acetyl group at C-4 position. MetExtract detected HT2 with *m/z* 442.2428 $[\mathbf{10}+NH_4]^+$ in full scan mass spectra of T2-treated samples. Since 22 carbon atoms were annotated by MetExtract as well as retention time and LC-MS/MS spectra were in accordance with the authentic standard, HT2 was confirmed as T2 metabolite. LC-HRMS/MS measurement of the $[M+NH_4]^+$ adduct revealed typical fragments of HT2 as listed in Table 3 (LC-MS/MS spectra of HT2 and T2 are shown in ESM Figs. S9 and S10).

3-Acetyl-T2 (metabolite 12)

3-Acetyl-T2 was found only in T2-treated barley and was formed by the conjugation of an acetyl group at the C-3 position. Within positive full scan mass spectra, MetExtract recognised *m/z* 526.2640 $[\mathbf{12}+NH_4]^+$ adduct with a difference of Δ24.081 u (theoretical value) between the non-labelled and labelled metabolite form, indicating that 3-acetyl-T2 is formed from intact T2. Retention time, precursor ion mass and

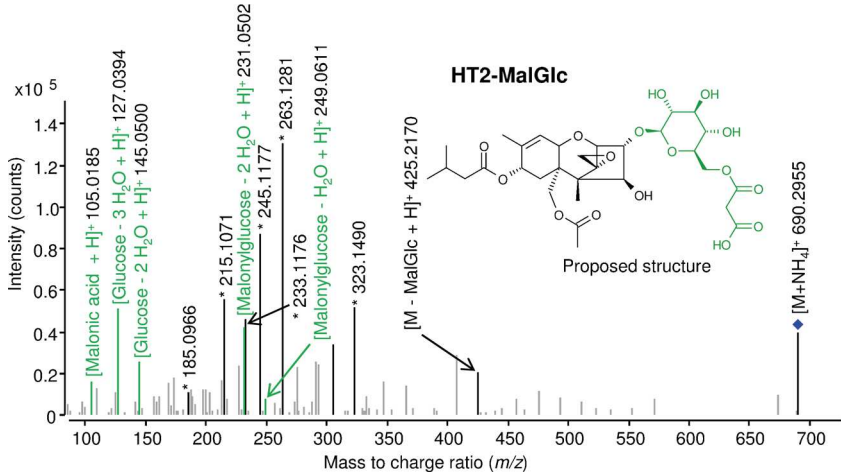

Fig. 2 LC-HRMS/MS spectrum of HT-2 toxin-malonylglucoside (HT2-MalGlc), an in planta metabolite of HT-2 toxin and T-2 toxin. Analysis was performed with a 6550 iFunnel Q-TOF LC/MS system in positive electrospray ionisation mode with a collision energy of 16 V. The ammonium adduct was chosen as precursor (marked with a *diamond*). Characteristic fragments used for annotation are *highlighted*, those fragments originating from the conjugate malonylglucose are displayed in *green* and characteristic HT-2 toxin fragments are marked with an *asterisk* (*)

LC-HRMS/MS spectra of the biotransformation product were in accordance with the standard 3-acetyl-T2. The fragmentation of the $[M+NH_4]^+$ adduct mainly generated the loss of isovaleric acid (theoretical value, Δ102.068 u; $C_5H_{10}O_2$), acetic acid (theoretical value, Δ60.021 u; $C_2H_4O_2$) and a ketene (theoretical value, Δ42.011 u; C_2H_2O) (positive ion mode LC-HRMS/MS spectrum is shown in ESM Fig. S11).

Feruloyl-T2 (metabolites 13a and 13b)

The presence of this metabolite suggests that ferulic acid was covalently bound to the parent toxin T2. Since we have found feruloyl-T2 initially in T2-treated wheat [13], we manually extracted EICs (*m/z* extraction window of ±5 ppm) from Orbitrap raw data of barley as well. Putatively identified feruloyl-T2 is formed from intact T2 in barley because corresponding EICs with a difference of Δ24.081 u ($[M+Na]^+$, *m/z* 665.2568 and *m/z* 689.3373; theoretical values) indicated the intact T2 toxin and showed perfect coelution as well as similar intensities. Two distinct EIC peaks of this metabolite were detected at retention times 20.0 and 20.2 min which might be due to the simultaneous presence of *cis*- and *trans*-ferulic acid conjugates of T2 toxin. Although the *trans*-form of ferulic acid is the main naturally occurring one, the *cis*-form might occur after light-induced non-enzymatic isomerisation [30]. Another isomer option would result from the addition of iso-ferulic acid instead of ferulic acid to T2. Figure 3 shows the fragmentation pattern of this compound. In addition to typical T2 fragments, mass signals of *m/z* 177.0549, *m/z* 145.0279 and *m/z* 117.0330 corresponding to [ferulic acid–H_2O+H]$^+$, [ferulic acid–H_2O–CH_3OH+H]$^+$ and [ferulic acid – H_2O–acetic acid+H]$^+$ were detected. Since HT2 is a major metabolite of T2, it can be hypothesised that ferulic acid is conjugated to the C-4 position of HT2, whilst the C-3 position is additionally acetylated by the plant or vice versa resulting in compounds with the same molecular weight and putatively similar LC-HRMS/MS spectra. However, with isotopic labelling approach, we can clearly differentiate between groups originating from plant or parent toxin. Since the T2 is fully preserved (Δ24.081 u between ^{12}C- and ^{13}C-mass signals), this hypothesis can be excluded. Thus, T2 appears to be directly and rapidly (see time course experiments) metabolised by the conjugation of ferulic acid at the C-3 position.

Screening of isomers of detected HT2 and T2 metabolites

The longer gradient method 3 was developed for LC-Q-TOF-MS analysis to confirm the structures of HT2-3-*O*-*β*-Glc and T2-Glc in comparison with standards. For being able to recognise additional isomers of HT2 and T2 metabolites, which potentially had not been chromatographically separated by LC-Orbitrap-MS analysis, method 3 was applied for the measurement of labelled samples. EICs of corresponding $^{12}C/^{13}C$ metabolite ion pairs of all detected biotransformation products were manually extracted, overlaid and checked to confirm coelution, similarity of elution profiles and intensities. As a result, EICs of each of the tentative 15-acetyl-T2-tetraol-MalGlc as well as HT2-MalGlc showed two peaks with one major and one smaller peak (eluting 0.2 or 0.85 min earlier, respectively, approx. 10 % intensity relative to main peak). The observation of these isomers may result from the conjugation of malonic acid to different hydroxyl groups of glucose. Interestingly, tentative hydroxy-HT2-Glc and hydroxy-HT2-MalGlc which are presumably derived from HT2-3-*O*-*β*-Glc were detected in barley in form of three isomers. One major peak and two smaller peaks (eluting 0.15 and 0.25 min earlier, respectively, approx. 10–20 % intensity relative to major peak) were revealed, suggesting that the

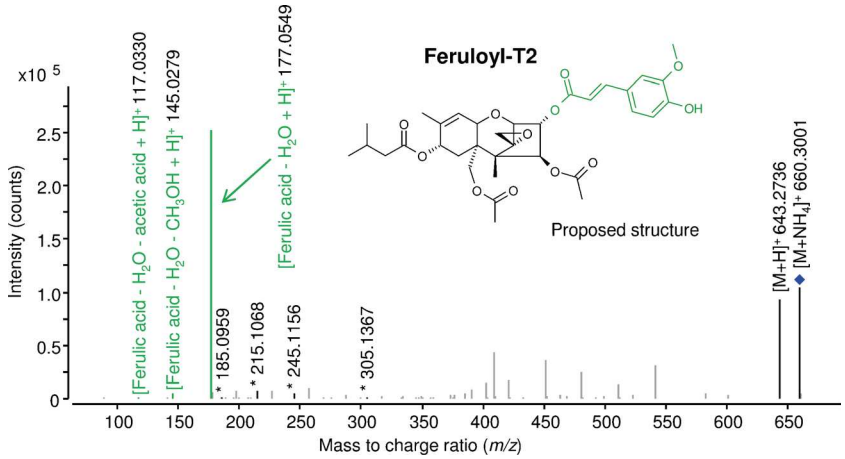

Fig. 3 LC-HRMS/MS spectrum of feruloyl-T-2 toxin (feruloyl-T2), an in planta metabolite of T-2 toxin. Analysis was performed with a 6550 iFunnel Q-TOF LC/MS system in positive electrospray ionisation mode with a collision energy of 5 V. The ammonium adduct was chosen as precursor (marked with a *diamond*). Characteristic fragments used for annotation are *highlighted*, those fragments originating from the conjugate ferulic acid are displayed in *green* and characteristic T-2 toxin fragments are marked with an *asterisk* (*)

additional hydroxyl group is located at different positions of the HT2 backbone or, in case of MalGlc derivative, malonic acid is conjugated to different hydroxyl groups of glucose.

Method validation for quantification

R_A, SSE and R_E were determined for HT2, T2, HT2-3-*O*-*β*-Glc, 3-acetyl-T2 and T2-Glc. R_A was very similar to SSE in undiluted matrix solution. Therefore, calculated R_E of the five quantified compounds was between 94 and 109 % with RSDs≤9 % (n=3). Matrix effects for 1+9 (*v*/*v*) and 1+49 (*v*/*v*) dilutions of the extraction solution were in the range of 98–114 % (RSDs≤4 %, n=3) and consequently negligible for the intended purpose to estimate the toxin metabolism rates. However, 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 (see ESM Table S12).

Time courses and mass balances of HT2, T2 and its metabolites

Time courses of quantified HT2, T2 and HT2-3-*O*-*β*-Glc are shown in Fig. 4. It was observed that the recovery of added HT2 and T2 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 HT2 (equal to 0.471 μmol) and T2 (0.429 μmol) at time point 0 was used as starting point to calculate percent yield of the respective derivatives formed at later time points. Figure 4 shows that HT2-3-*O*-*β*-Glc has been found to be the main metabolite of both HT2 and T2 toxin which reached its maximum already 1 day after toxin treatment.

Kinetics of HT2 metabolism

Regarding HT2, approximately 53 % (0.250±0.054 μmol) was transformed to HT2-3-*O*-*β*-Glc, whilst 25 % (0.116±0.033 μmol) remained unmodified as parent toxin within the first 24 h upon treatment. With increasing time, a decrease of HT2 and HT2-3-*O*-*β*-Glc was observed which ended in a content of 7 % (0.033±0.007 μmol) and 34 % (0.161±0.034 μmol) relative to the originally added HT2

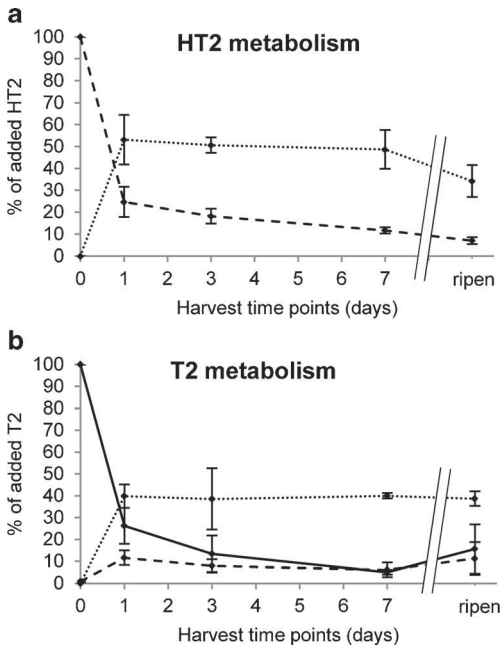

Fig. 4 Time courses of quantified HT-2 toxin (HT2, *broken line*), T-2 toxin (T2, *continuous line*) and HT-2 toxin-3-*O*-*β*-glucoside (HT2-3-*O*-*β*-Glc, *dotted line*) depicted separately for HT2- (**a**) and T2-treated (**b**) barley ears. Ears were treated with 200 μg toxin and harvested immediately, 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

after ripening, respectively. This finding confirms that HT2-3-*O*-*β*-Glc is further metabolised and correlates well with the relative quantification (Fig. 5) of HT2 metabolites. Taking a closer look at the formation of HT2 biotransformation products over time, hydroxy-HT2-Glc, hydroxy-HT2-MalGlc, HT2-di-Glc, HT2-MalGlc, as well as T2-triol-Glc (detectable only at one time point) show maximal abundance after ripening, leading to the assumption that they are derived from early formed HT2-3-*O*-*β*-Glc. Moreover, 15-acetyl-T2-tetraol-Glc and 15-acetyl-T2-tetraol-MalGlc were also found to be produced after 1 day. Although absolute quantification was not possible for HT2-MalGlc due to the lack of an authentic standard, comparison of EIC peak areas suggests that it belongs to the major biotransformation products.

Kinetics of T2 metabolism

One day after treatment of barley plants with 200 μg T2 (0.429 μmol), approximately 26 % of unmodified T2 (0.113±0.035 μmol) were still present, whilst 12 % (0.050±0.014 μmol) had been converted into HT2 and 40 % (0.171±0.023 μmol) into HT2-3-*O*-*β*-Glc. As the

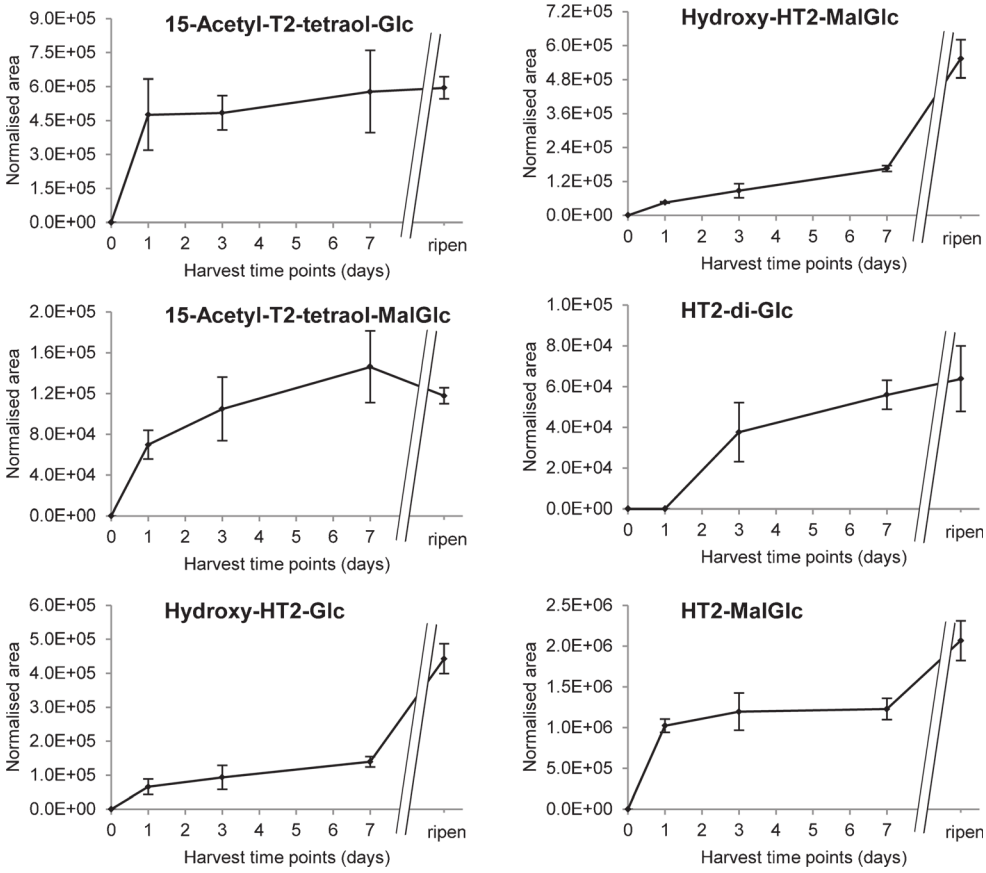

Fig. 5 Relative time courses of HT-2 toxin-derived metabolites. Barley ears were treated with 200 μg HT-2 toxin and harvested immediately, 1, 3 and 7 days after treatment and at full-ripening stage. Relative amounts (areas of extracted ion chromatogram peaks normalised by ear weight) are plotted versus 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. *HT2* HT-2 toxin, *T2* T-2 toxin, *Glc* glucoside, *MalGlc* malonylglucoside

sum of these three compounds amounts to 78 % of the initially added T2, all other biotransformation products of T2 made up a maximum of 22 % at this time point. 3-Acetyl-T2 was quantified with approximately 0.058 % (0.249±0.073 nmol) of the originally added T2, whilst T2-Glc reached a maximum of approximately 0.065 % (0.278 nmol; only one sample at time point 1 day showed levels>LOQ). Quantification of samples which had been harvested 3 and 7 days after treatment showed that the amount of T2 and HT2 had been further decreased, whilst the quickly formed HT2-3-*O*-*β*-Glc remained almost constant. It is assumed that T2 is very rapidly transformed into HT2 in planta and subsequently converted into HT2-3-*O*-*β*-Glc being the derivative which is then further metabolised. Time courses of relative abundance of T2 metabolites are shown in Fig. 6. Hydroxy-HT2-Glc, hydroxy-HT2-MalGlc, T2-triol-Glc (detectable only at one time point), HT2-di-Glc (detectable only at one time point) and HT2-MalGlc showed the highest amounts after ripening, suggesting that they are formed from HT2-3-*O*-*β*-Glc. Interestingly however, the conjugates carrying intact T2 toxin, namely T2-Glc (detectable only at one time point), 3-acetyl-T2 and feruloyl-T2, all attained their maximum levels after 1 day of incubation. This observation clearly supports the assumption that conjugation of T2 occurs immediately after application of T2 toxin, before the parent toxin is hydrolysed to HT2.

Detoxification of HT2 and T2

It was observed that barley modifies HT2 and T2 by using phase I as well as phase II metabolism processes. On the one hand, hydrolysis of the non-polar acetyl and isovaleryl groups and on the other hand hydroxylation and covalent binding of glucose and malonic acid occurred. Therefore, it is apparent that as part of the detoxification process, the HT2- and T2-treated plants try to inactivate these xenobiotics by transforming them into more polar compounds. Masuda et al. [31] reported that phytotoxicity of HT2 and T2 is comparable, whilst similar compounds without an isovaleryl group at the C-8 position induce only minor phytotoxic effects. Since glucosylation of the type B trichothecene deoxynivalenol was confirmed to be an important detoxification mechanism of plants [29], it is suggested that the glucosylated forms of HT2 and T2 are also less phytotoxic than the parent toxins. In contrast to

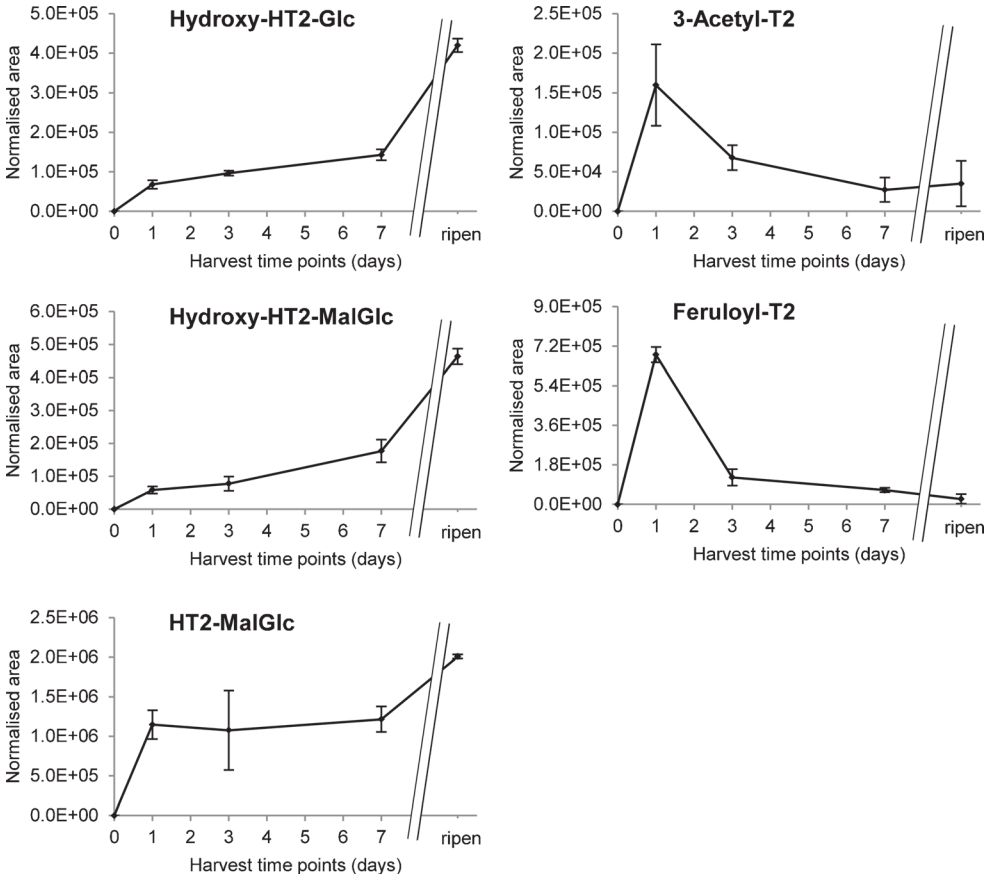

Fig. 6 Relative time courses of T-2 toxin-derived metabolites. Barley ears were treated with 200 μg T-2 toxin and harvested immediately, 1, 3 and 7 days after treatment and at full-ripening stage. Relative amounts (areas of extracted ion chromatogram peaks normalised by ear weight) are plotted versus 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. *HT2* HT-2 toxin, *T2* T-2 toxin, *Glc* glucoside, *MalGlc* malonylglucoside

deoxynivalenol plant metabolism, no glutathione conjugation was observed. A study [32] showing that trichothecenes with a hydroxyl group at C-3 position are more phytotoxic than those with an acetyl group indicates that acetylation of T2 and probably conjugation of ferulic acid at C-3 position provide an additional way for detoxification. This is also in good agreement with the observation that the metabolic modification at the C-3 of HT2 and T2 is potentially dominating (shown for conjugation of glucose to HT2). Further biological interpretation of HT2 and T2 plant metabolism is included in the study performed with wheat [13].

Conclusion

In this study, an analytical strategy based on isotopic labelling, LC-Orbitrap-MS analysis in fast polarity switching mode and data processing by MetExtract software was used for elucidation of HT2 and T2 metabolism in barley. Measurements in both electrospray polarities yielded complementary information which was shown to be highly valuable with respect to both coverage as well as annotation of metabolites. The qualitative data evaluation strategy with stable isotopic labelling and the application of MetExtract software enabled the untargeted analysis by exclusive extraction of HT2- and T2-derived biotransformation products and supported their structure annotation by supplying $^{12}C/^{13}C$ mass shifts in MS as well as MS/MS spectra. MetExtract provides an easy, rapid, sensitive and specific detection method. Since the Exactive Plus Orbitrap was not equipped with a collision cell, further MS/MS-based structure annotation experiments were performed with a LC-Q-TOF instrument. Therefore, it was possible to annotate and partly identify 9 HT2 and 13 T2 metabolites. The metabolism routes included hydrolysis of acetyl and isovaleryl groups, hydroxylation as well as covalent binding of glucose, malonic acid, acetic acid and ferulic acid. Additionally, putative isomers of 15-acetyl-T2-tetraol-MalGlc, hydroxy-HT2-Glc, hydroxy-HT2-MalGlc, HT2-di-Glc, HT2-MalGlc and feruloyl-T2 (two or three isomers for each) were revealed. As a result of the time course experiments, HT2-3-*O*-*β*-Glc was verified as the major metabolite of HT2 and T2 metabolism which reached its maximum already 1 day after toxin treatment and was subsequently further metabolised.

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Conflict of Interest The authors declare that they have no competing interests.

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APPENDIX IV

Malachova A, Cerkal R ,Ehrenbergerova J, Dzuman Z, Vaculova K, Hajslova J

Fusarium mycotoxins in various barley cultivars and their transfer into malt

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Fusarium mycotoxins in various barley cultivars and their transfer into malt

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Abstract

BACKGROUND: *Fusarium* toxins, secondary metabolites of toxinogenic *Fusarium* species, are found in a range of cereal grains. In this study the occurrence of the most commonest *Fusarium* toxins, namely nivalenol (NIV), deoxynivalenol (DON), deoxynivalenol-3-glucoside, fusarenon-X, 3- and 15-acetyldeoxynivalenol, HT-2 and T-2 toxins and zearalenone, in various barley cultivars harvested in 2005–2008 was monitored. The impact of weather, locality, fungicide treatment and barley cultivar (hulless or covered) on contamination was evaluated. The transfer of these mycotoxins into malt was assessed.

RESULTS: The most prevalent toxin was DON, which was found in 83% of samples (maximum level 180 μg kg^{-1}), while HT-2 was detected in 62% of samples (maximum level 716 μg kg^{-1}). Using analysis of covariance, weather was found to be the key factor in all years ($P < 0.001$). A relationship between cultivar and contamination was confirmed only for HT-2 ($P < 0.001$) and T-2 ($P = 0.037$), with higher levels of these toxins being observed in hulless cultivars. With the exception of NIV ($P = 0.008$), no significant relationship was found between fungicide treatment and contamination. No distinct trend regarding DON levels in malt was found, with both decreases and increases occurring.

CONCLUSION: The results show an inter-annual variation in mycotoxin occurrence in barley cultivars as well as differences in contamination of malt produced from fungicide-treated and untreated barley.

Keywords: *Fusarium* mycotoxins; barley cultivars; malt; fungicide treatment; deoxynivalenol; HT-2 and T-2 toxins

INTRODUCTION

Spring barley is an important crop in the Czech Republic, with a cultivation area of about 400 000 ha. According to data provided by the Czech Beer and Malt Association in 2007 (http://www.cspas.cz/index.asp?lang=2), only about 30% of harvested barley is used for malting, the majority serving as animal feed. In addition, barley rootlets, which are obtained at the final stage of malt production, are also used for animal feed.[1]

Unfortunately, barley, like other small-grain cereals, can be colonised already under field conditions by various toxinogenic fungi.[2,3] Among them, species of the *Fusarium* genus are the most common (during moist weather, their spores are windblown or splashed onto the heads of cereal crops). The plant disease known as *Fusarium* head blight (FHB), or scab, is widespread in moderate climatic zones around the world, including European cereal-growing areas. The species frequently associated with FHB of small-grain cereals in Europe are *F. graminearum* Schwabe, *F. culmorum* (W. G. Smith) Sacc. and *F. avenaceum* (Fr.) Sacc. At least 12 additional *Fusarium* spp. have been reported on wheat and barley grains, but they are generally regarded as less important.[4,5] In the Czech Republic the most widespread species responsible for FHB development in barley are *F. culmorum, F. poae* and *F. avenaceum*.[6] *Fusarium* infection may result in considerable yield loss, estimated to range between 10 and 40% of potential harvest; moreover, several quality problems can be encountered, such as reduced germinative capacity and lower weight of grains, which implies less endosperm and thus decreased malting yield.[5,7] Another adverse phenomenon experienced when infected grains are processed is beer gushing; in many cases the resultant impaired flavour is reported by consumers.[8,9]

One of the key problems associated with FHB is the formation of toxic secondary metabolites that pose a health risk to both humans and animals,[10] since they can be transferred across the beer production chain from barley to malt and thus to the final product.[11–13] Most *Fusarium* species that infect barley can produce one or more mycotoxins, but their contents do not necessarily correlate with infestation level.[14,15] For instance, *F. graminearum* and/or *F. culmorum* can produce deoxynivalenol, nivalenol and zearalenone, *F. poae* is considered to be the key producer of HT-2 and T-2 toxins, while the same type A

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trichothecenes have also been found in substrates infected by *F. sporotrichioides*.[16]

Although 'Good Agricultural Practice' conditions enabling full prevention/elimination of FHB in barley and other cereals have not been identified yet, much knowledge has been acquired and recommendations have been set up on the basis of various comprehensive field studies concerned with the susceptibility of cultivars to infection, the role of preceding crops, fertilisation and/or mineral composition of the soil, etc.[7,15,17,18] A lot of effort has also been expended on finding effective fungicides against FHB and their optimal application methods. However, a chemical treatment guaranteeing total prevention of mycotoxin hazard has not been found yet. Generally, fungicides with triazole chemistry (tebuconazole, metconazole and bromuconazole) seem to be the most effective. Depending on cultivar resistance, the aggressiveness of toxinogenic fungi and other factors mentioned above, efficacy exceeding 70% might be achieved.[19–21] In many cases the use of adjuvant together with fungicide was shown to significantly improve the penetration of active substances into plant tissue and thus increase the beneficial effect of treatment.[9,22]

The aim of the present four-year study was to evaluate the time trends in occurrence of the most abundant *Fusarium* toxins in various cultivars of spring barley grown in two localities in the Czech Republic and to critically assess the impact of factors such as weather conditions, growing locality, type of grain of barley cultivar and fungicide treatment on contamination levels in this important crop. Technological experiments focused on the transfer of mycotoxins from barley into malt were also conducted.

MATERIALS AND METHODS

Barley samples and agricultural design

A total of 148 barley samples harvested in the years 2005–2008 were examined for type A and B trichothecenes and zearalenone within the four-year study. A representative set of 12 different barley cultivars comprising both cultivars dedicated especially for beer production and cultivars suitable for food and feed processing was investigated. The characteristics of individual cultivars are summarised in Table 1. Each year except 2006 (see below) the same barley cultivars were obtained from two experimental localities, Zabcice and Kromeriz. In Zabcice, all 12 barley cultivars were obtained in two sets, one of which was grown under a low-input system and the other under a conventional system, whereas only a low-input approach was used in Kromeriz. The barley varieties were planted on approximately 1000 m^2 plots located in different areas of a 100 000 m^2 large field each year, and winter wheat was used as a pre-crop. The dates of sowing and harvest are listed in Table 2. Unfortunately, in 2006 the Zabcice harvest was completely destroyed by extremely high rainfalls during the harvest period, which caused the undesirable germination of grains in ears. Therefore, in that year, only pilot monitoring was carried out in the substitute localities Branisovice and Uhersky Ostroh, but the results were not included in the statistical assessment.

The conventional approach to growing is closely associated with the use of fungicide treatment. Thus, to evaluate the impact of fungicide treatment on mycotoxin levels, various fungicide preparations were applied each year to the conventional sample set in Zabcice. An overview of the fungicides and their active ingredients used in the study is given in Table 3. It should be noted that the fungicides were applied during the vegetation stage from the end of stem elongation to the beginning of heading in doses recommended by the manufacturers.

Characterisation of growing locality: experimental stations Zabcice and Kromeriz

Zabcice (16° 37′ N, 49° 01′ E) is situated near Brno in the south of Moravia (Czech Republic) on the Svratka River flood-plain in a maize production region (subregion K2), at a mean elevation above sea level of 184 m. Kromeriz (17° 22′ N, 49° 17′ E), at a mean elevation above sea level of 235 m, lies more to the north of Moravia in a sugar beet production region. The two localities differ from each other in soil type. Zabcice's dominating soil type is middle heavy to heavy gleyic fluvisols FLq and FLg, whereas Kromeriz's soil type is classified as black soil, type luvic soil. According to long-term meteorological records (reference period 1961–1990), Zabcice is considered as one of the warmest districts in the Czech Republic, with relatively mild winters, short periods of sunshine and limited precipitation in the vegetation period. The mean annual temperatures in Zabcice and Kromeriz are 9.2 and 9.1 °C respectively. The mean rainfall in Zabcice is lower (480 mm) than that in Kromeriz (568 mm). The differences in mean temperature (°C) and rainfall (mm) during the growing periods (March–July) in the years 2005–2008 are shown in Figs 1 and 2 for Zabcice and Kromeriz respectively. The drought periods in the climate diagrams are indicated by shading.

Malted barley

All barley cultivars from Zabcice harvested in 2005 that were suitable for beer production were micromalted at the Malting Institute of the Research Institute of Brewing and Malting (Brno, Czech Republic) in line with traditional procedures in three stages, i.e. steeping, germination and kilning (Table 4). The whole process was performed according to MEBAK.[24]

Mycotoxin analysis

Standards

Pure crystalline analytical standards of the *Fusarium* mycotoxins nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (FUS-X), 3- and 15-acetyldeoxynivalenol (sum of ADON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZON) and acetonitrile solution of deoxynivalenol-3-glucoside (DON-3-Glc) were purchased from Biopure (Tulln, Austria). Standard stock solutions (1 mg mL^{-1}) were prepared in acetonitrile and stored at −20 °C. To obtain matrix-matched standards, a composite working standard solution (1000 ng mL^{-1} of each analyte) was prepared by combining suitable aliquots of each standard stock solution and diluting them with methanol/water (50 : 50 v/v).

Chemicals and reagents

The organic solvents methanol and ammonium acetate (both of high-performance liquid chromatography grade) used for liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis and the acetonitrile used for extraction were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ultrapure water was produced by a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Mycotoxin analysis

The LC/MS/MS method used for analysis of the eight *Fusarium* toxins and DON-3-Glc in barley and malt was described in detail in a previous paper.[13] In the present study the purification step based on MycoSep TM 226 cartridges (Romer Labs, Tulln, Austria) was omitted in order to obtain higher DON-3-Glc recovery (this polar plant metabolite was more strongly retained by the sorbent compared with free toxins). The simplified procedure described briefly below was revalidated ($n = 5$).

Table 1. Characterisation of barley cultivars examined in study

Cultivar	Type of grain	Height of vegetation	Resistance to lodging	Resistance to disease				Year of national listing	Country of cultivar origin	Use of cultivar
				Blumeria graminis	*Puccinia hordei*	*Pyrenophora teres*	*Rhynchosporium secalis*			
Amulet	Covered	+	+++	+	+	–	–	1995	Czech Republic	Exclusive cultivar suitable for Czech beer production[b]
Bojos	Covered	+++	++	+++	++	++	+	2005	Czech Republic	Exclusive cultivar suitable for Czech beer production[b]
Jersey	Covered	+++	+	–	++	–	–	2000	The Netherlands	Exclusive cultivar for beer production[b]
Malz	Covered	++	+	+	+	–	–	2002	Czech Republic	Exclusive cultivar suitable for Czech beer production
Merlin	Hulless	++	+++	–	–	–	–	1995	Canada	Cultivar suitable for animal feeding
Prestige	Covered	++	++	+	–	+	–	2002	France	Exclusive cultivar for beer production
Sebastian	Covered	+	++	+	++	–	–	2005	Denmark	Exclusive cultivar for beer production
Tolar	Covered	+++	++	+	–	–	++	1997	Czech Republic	Standard cultivar for beer production
AF Lucius[a]	Hulless	++	+++	++	++	++	++	2009	Czech Republic	Cultivar dedicated for special and whole-grain products for human consumption and also for animal feeding
KM 1057[a]	Hulless	–	++	++	++	++	++	Not registered	Czech Republic	Cultivar suitable for food purposes
KM 2283[a]	Hulless	+	+++	++	++	++	++	Not registered	Czech Republic	Cultivar tested for specific food industry application (extruded products, special flour extraction of soluble fibre)
KM 2084[a]	Hulless	+	+++	+++	+	–	–	Not registered	Czech Republic	Cultivar with similar attributes to AF Lucius

Key: +, low; ++, medium; +++, high; –, no available information.
[a] Cultivars developed by Agricultural Research Institute Kroměříž, Ltd and by Agrotest Fyto, Ltd.
[b] According to EC No 1014/2008.[23]

Table 2. Sowing and harvest dates of barley in different years

Locality	Sowing/harvest	2005	2007	2008
Zabcice	Sowing	30 March	17 March	10 March
	Harvest	27 July	16 July	12 July
Kromeriz	Sowing	1 April	28 March	31 March
	Harvest	30 July	20 July	21 July

Table 3. Overview of fungicide treatments

Fungicide(s)	Active ingredient(s)
Year 2005	
Horizon 250 EW	Tebuconazole
Mirage 45 EC	Prochloraz
Years 2007 and 2008	
Falcon 460 EC	Tebuconazole
	Triadimenol
	Spiroxamine

Extraction

Homogenised ground samples of barley and malt (12.5 g) were extracted by shaking them with 50 mL of acetonitrile/water (84 : 16 v/v) for 1 h, then the crude extracts were filtered (Filtrak No. 390, VEB Freiberger, Berlin, Germany). Aliquots (4 mL) of the filtered extracts were evaporated under vacuum to dryness, redissolved in 1 mL of water/methanol (1 : 1 v/v) and passed through a 0.2 µm microfilter (Alltech, Deerfield, IL, USA) before further analysis.

LC/MS/MS

An HP 1100 Binary Series high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with an LCQ Deca mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA) was used for mycotoxin analysis. A sample volume of 30 µL was separated on a Synergi RP reverse phase column with polar

end-capping (150 mm × 3 mm × 4 µm; Phenomenex, Torrance, CA, USA) held at 40 °C and operated under gradient conditions. Ammonium acetate (10 mmol L^{-1}) in water (mobile phase A) and methanol (mobile phase B) at a flow rate of 0.5 mL min^{-1} were used for gradient elution of sample components, which was started with 20% B, then changed linearly to 70% B over 8 min and held at 70% B until 16 min, and the post-run was 6 min with 20% B.

Identification and quantification of target analytes were performed using MS/MS with atmospheric pressure chemical ionisation (APCI) in negative ion mode (NIV, DON, DON-3-Glc, FUS-X, sum of ADON and ZON) or positive ion mode (HT-2 and T-2).

The capillary and vaporiser temperatures were 150 and 325 °C respectively, the nitrogen sheath and auxiliary gas flow rates were 1.2 and 3 L min^{-1} respectively and the discharge needle voltage was 6 kV.

Helium was used as the gas for collision-induced dissociation. Ions were scanned in the selected reaction-monitoring mode. APCI-positive ions in the form $[M + NH_4]^+$ were used for type A trichothecenes. The ion transitions, i.e. precursor ion > product ions (quantifier, Q; identifier, I), were HT-2: 442 > 424 (Q), 322 (I) and T-2: 484 > 305 (Q), 245 (I). For type B trichothecenes, fragmentation of $[M + CH_3COO]^-$ in the case of NIV: 371 > 311 (Q), 281 (I), DON: 355 > 295 (Q), 265 (I), DON-3-Glc: 517 > 457 (Q), 427 (I), sum of ADON: 397 > 337 (Q), 307 (I), FUS-X: 413 > 353 (Q), 263 (I), and fragmentation of $[M + H]^-$ in the case of ZON: 317 > 273 (Q), 299 (I) were performed. Figure 3 shows the chromatogram of the matrix-matched standard at a level of 50 µg kg^{-1} of each analyte. Limits of detection (LOD) and quantification (LOQ), recoveries and repeatabilities (relative standard deviations) for both matrices (barley and

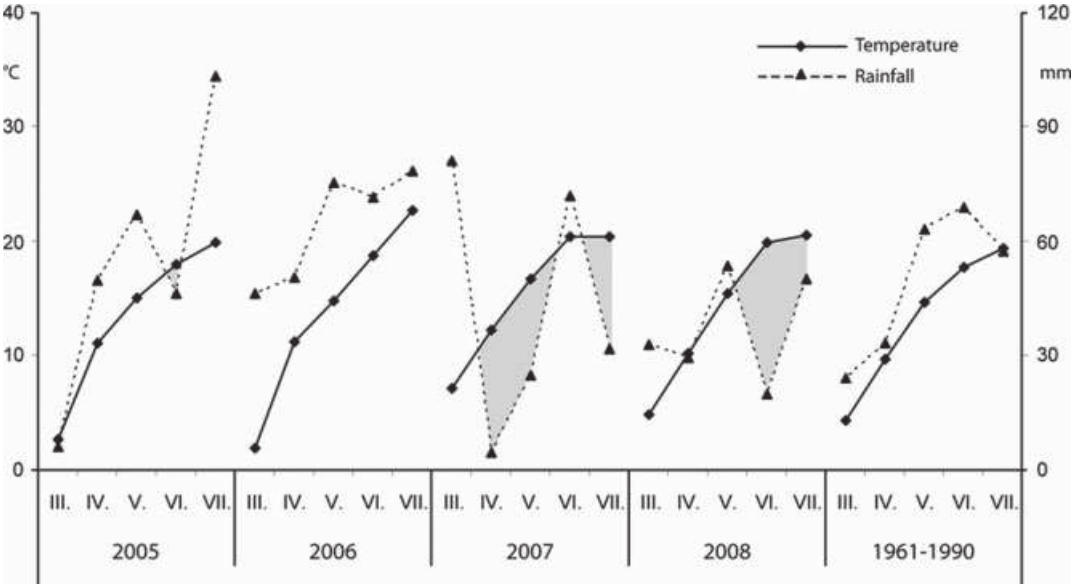


Figure 1. Four-year and long-term climate diagram for Zabcice.

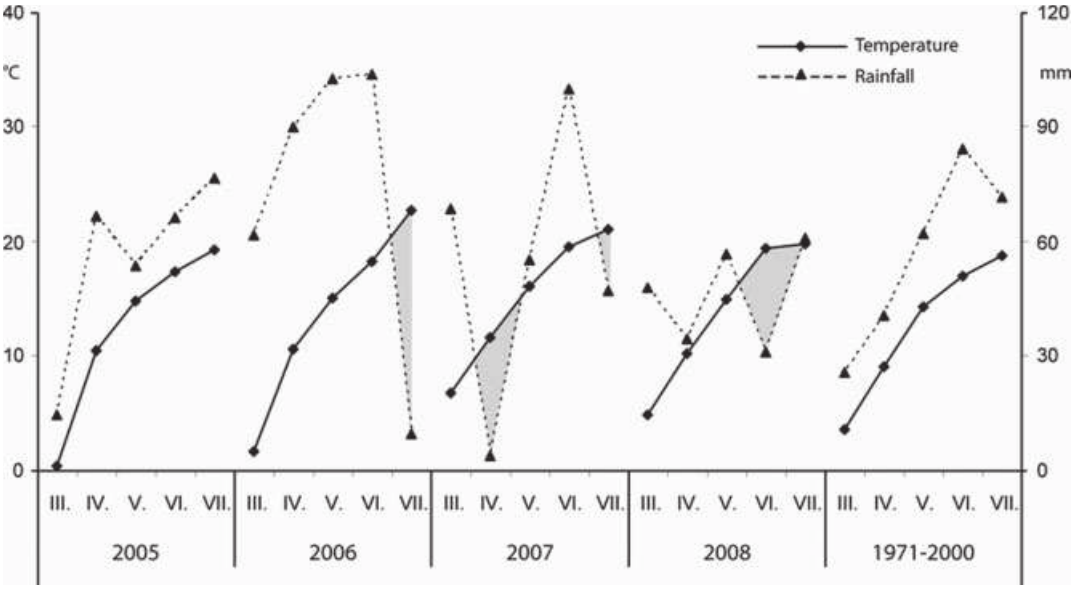


Figure 2. Four-year and long-term climate diagram for Kromeriz.

Table 4. Conditions and schedule of malting process

Malting stage		Duration of stage (h)	Temperature of ingoing air (°C)	Temperature of outgoing air (°C)
Steeping	Wetstep	5	14.5	Not measured
	Airrest	19		
	Wetstep	4		
	Airrest	20		
	Wetstep	24[a]		
	Airrest			
Germination		72	14.5	Not measured
Kilning		1	14.5–55.0	14.5–25.0
		11	55	25.0–35.0
		1	55.0–60.0	40.0–45.0
		1	60.0–65.0	45.0–50.0
		2	65.0–70.0	50.0–55.0
		1	70.0–75.0	55.0–65.0
		1	75.0–80.0	65.0–78.0
		4	80	78

[a] Water content was adjusted to 455 g kg^{-1} by steeping or spraying.

Table 5. Performance characteristics of LC/MS/MS method

Parameter	NIV	DON	DON-3-Glc	FUS-X	Sum of ADON	HT-2	T-2	ZON
Barley								
Recovery (%)[a]	75	97	98	91	83	98	97	92
Repeatability (%)[a]	4.0	4.2	7.0	7.1	6.3	7.4	5.3	6.6
LOD (μg kg^{-1})	5	1	1	1	1	5	0.5	0.5
LOQ (μg kg^{-1})	10	5	5	10	5	10	5	5
Malt								
Recovery (%)[a]	68	99	96	90	85	98	99	91
Repeatability (%)[a]	6.8	5.9	6.6	8.9	8.6	4.2	5.3	7.9
LOD (μg kg^{-1})	5	1	1	1	1	5	0.5	0.5
LOQ (μg kg^{-1})	10	5	5	10	5	10	5	5

[a] $n = 5$.

malt) obtained within the validation process are summarised in Table 5.

Statistical analysis

Analysis of covariance (ANCOVA) was performed to evaluate the impact of various factors on mycotoxin contamination using a linear model function in R software.[25] Since this approach combines analysis of variance and regression, it allows the inclusion of both quantitative and qualitative variables in the model. The linear dependence of the factors mean temperature and precipitation during vegetation, harvest year, fungicide treatment, barley cultivar and locality on mycotoxin levels in barley was assessed using forward stepwise selection, in which elimination and inclusion of variables are based on Akaike's information criterion (AIC).[26] For statistical calculations, samples below LOQ and LOD were assigned the values LOQ/2 and LOD/2 respectively. The *F* statistics in ANCOVA were calculated at the significance level $\alpha = 0.05$. Since the high number of factors included in the model increases the probability of 'statistical fishing' and the number of free model parameters, testing of significance was not performed for interactions between factors.

RESULTS AND DISCUSSION

Fusarium toxins in 12 barley cultivars

Changing climatic conditions together with continuous innovation of agricultural practices have resulted in some changes in the spectrum of pathogen species invading cereals in the field and consequently of secondary metabolites left in the crop at the time of harvest. Thus differences in mycotoxin pattern have to be taken into consideration when proposing strategies aimed at laboratory control of food/feed safety. In most of the previous studies conducted in the Czech Republic, mainly DON-dedicated enzyme-linked immunosorbent assay (ELISA) kits were employed for the detection of grain contamination caused by *Fusarium* species, so information on the occurrence of other toxic secondary metabolites of these fungi was not provided. Thanks to the growing use of advanced LC/MS techniques, a broad spectrum of the most important *Fusarium* toxins can now be monitored within a single analysis. Of eight target analytes involved in the present study, the only one that was not detected (LOD 0.5 μg kg^{-1}) in any of the 148 barley samples examined was ZON. The occurrence of this oestrogenic mycotoxin in barley is relatively rare; in one of few studies reporting its presence in this cereal, only 10% of 339 examined samples contained detectable ZON levels.[27] An overview of the aggregated data on *Fusarium* toxins determined in the present four-year study is shown in Table 6. DON, the most common trichothecene (type B), was detected in 83% of barley samples at a mean level of 28 μg kg^{-1}. In none of the samples examined did the DON level exceed the maximum limit of 1250 μg kg^{-1} specified for unprocessed barley intended for human consumption.[28] The frequency of DON did not differ significantly from that found in a previous monitoring study focused on cereals grown in the Czech Republic,[29] in which DON was detected in all 56 barley samples harvested in 2001 and 2005; on the other hand, the mean levels (37 and 157 μg kg^{-1} respectively) were somewhat higher than those detected (13–42 μg kg^{-1}) in the present study conducted in 2005–2008. It should be noted that DON levels determined in simultaneously conducted Czech projects employing ELISA kits for monitoring this mycotoxin tended to differ from our data. For instance, a significantly higher mean level of DON (307 μg kg^{-1}) was found in 50 samples of barley collected in 2007.[30] It can be assumed that, in addition to the large variability in toxinogenic fungi in the growing localities,[31] antibody cross-reactivity, documented for many commercial ELISA kits, could also be responsible for some overestimation.[32]

Regarding other *Fusarium* toxins, acetylated derivates of DON, i.e. 3- and 15-ADON, were found only in one sample at a low level of 46 μg kg^{-1} (sum of ADON). Starting in 2007, when a DON-3-Glc standard was introduced on the market, this DON conjugate (masked DON) was included in the list of analytes investigated in our study. In the first year, DON-3-Glc exceeded the LOQ of 5 μg kg^{-1} in only 11% of 36 samples examined, the maximum level found being 107 μg kg^{-1} (the DON-3-Glc/DON molar ratio

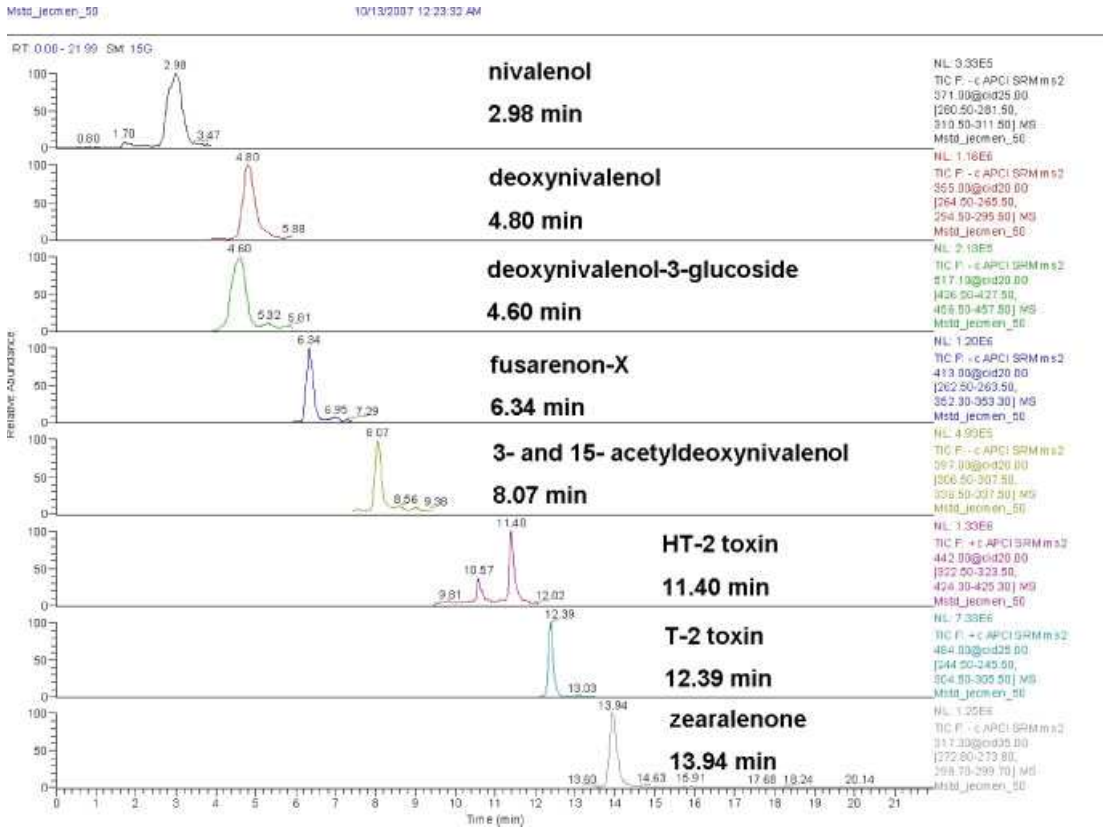


Figure 3. Chromatogram of target mycotoxins (matrix-matched standard at level of 50 μg kg^{-1} of each analyte).

in this sample was 3.9). In 2008, DON conjugate was detected in 17% of samples, but the maximum level was only 14 μg kg^{-1}. To our knowledge, masked DON has not been investigated in barley until now; only data on its occurrence in wheat and maize are available.[33]

Another major type B trichothecene, NIV, was detected in 34% of samples at a mean level of 21 μg kg^{-1}. The highest incidence was observed in 2007 when NIV was found in 96% of 36 samples at a mean level of 45 μg kg^{-1}. In 2008, NIV incidence decreased, with only 19% of 36 samples being positive. In any case, in line with Directorate-General for Health and Consumers (DG SANCO) recommendations,[34] more attention should be paid to this toxin in order to get sufficient data for assessment of the health risk associated with NIV dietary intake (although NIV is rarely the predominant *Fusarium* toxin, it is known to be more acutely toxic than DON).[35] Until now, no Czech study has reported on this mycotoxin in barley, though there are several other studies dealing with NIV levels in this cereal.[2,5,27,36,37] The main reason for the limited data on NIV might be not only the unavailability of a NIV-dedicated immunoassay for its easy monitoring but also the fact that its control is not required by current legislation. FUS-X, an acetylated form of NIV, was detected only in 2007 in two samples, the cultivars KM 1910 and Tolar, which contained NIV at levels of 140 and 104 μg kg^{-1} respectively. HT-2 and T-2, the key type A trichothecenes, were the most abundant contaminants after DON. In the entire sample set, their occurrence was 62% (mean level 110 μg kg^{-1}) and 50% (mean level 30 μg kg^{-1}) respectively. Currently, European legislation limits for combined levels of HT-2 and T-2 are being set up; the introduction of a limit of 200 μg kg^{-1} for unprocessed barley is expected. Considering this value, it was exceeded (sum of HT-2 and T-2) in 58% of samples collected in 2008.

Impact of critical factors on mycotoxin contamination of barley

The incidence and concentration of mycotoxins in barley varied widely within the four-year experimental period. Since, as mentioned above, many factors may play a role in *Fusarium* infestation and subsequent mycotoxin production in cereals, sophisticated statistical tools are needed for their overall, comprehensive assessment. In accordance with previously published studies,[7,15,17,18,38] weather in the respective locality in the particular harvest year (mean monthly air temperature in combination with total monthly precipitation), growing locality, susceptibility (mycotoxin content taken as criterion) of the particular barley cultivar to *Fusarium* infection, and fungicide treatment were employed in our study for the identification of essential preharvest factor(s) impacting levels of the most important target mycotoxins (NIV, DON, HT-2 and T-2). As mentioned above, the results from 2006 were not included in the statistical assessment.

Based on AIC, weather and fungicide treatment were selected as the factors with the highest importance and therefore entered into the model for variation calculation by ANCOVA. In the case of HT-2 and T-2, only weather and barley cultivar seemed to have a significant effect on their concentration according to AIC.

Weather conditions

Although weather conditions during vegetation varied greatly year-by-year in both growing localities in a similar way, the impact of this factor on the extent of barley contamination, regardless of the *Fusarium* toxin considered, was highly significant

Table 6. Overview of mycotoxins in spring barley harvested within 2005–2008

	2005		2006[a]		2007		2008	
Mycotoxin	Positive/total number of samples	Mean (μg kg^{-1})	Positive/total number of samples	Mean (μg kg^{-1})	Positive/total number of samples	Mean (μg kg^{-1})	Positive/total number of samples	Mean (μg kg^{-1})
DON	33/36	42	37/40	13	34/36	40	19/36	17
DON-3-Glc	–	–	–	–	4/36	6	6/36	2
Sum of ADON	0/36	<LOD	0/40	<LOD	0/36	<LOD	1/36	1
NIV	7/36	4	4/40	2	33/36	45	7/36	4
FUS-X	0/36	<LOD	0/40	<LOD	2/36	4	0/36	<LOD
HT-2	29/36	25	7/40	17	19/36	27	36/36	262
T-2	2/36	4	9/40	8	31/36	16	31/36	34
ZON	0/36	<LOD	0/40	<LOD	0/36	<LOD	0/36	<LOD

[a] Includes data from Kromeriz and substitute localities Branisovice and Uhersky Ostroh.

($P < 0.001$). However, the large inter-annual changes in mycotoxin incidences shown in Fig. 4 are not easy to interpret. While in the unusually dry year 2007 (Figs 1 and 2) the occurrence of NIV was unusually high, in another vegetation season with low precipitation (the winter of 2007–2008 was very mild) an increase in T-2 and HT-2 levels was observed. The changed mycotoxin pattern may be attributed for instance to competition between the various toxinogenic *Fusarium* species.[7] In this context the NIV-producing *F. graminearum* chemotype[39] might have prevailed in 2007 over the otherwise more common DON producer, which prefers warm and humid conditions (optimal growth at 25–28 °C and $a_w = 0.97$). The above-mentioned high incidence of T-2 and HT-2 was obviously associated with relatively low mean temperatures in May and July in 2008 during barley anthesis, which are conditions favourable mainly for type A trichothecene producers such as *F. sporotrichioides* and *F. poae* (optimal growth at 20–25 °C and $a_w = 0.99$).[40] The latter pathogen was found together with *F. graminearum* on barley grains obtained from this harvest.[31]

Considering the mean levels of mycotoxins found within the three-year experimental period, the differences between the two growing localities were not significant, although the spectrum of mycotoxins was rather broader in Zabcice than in Kromeriz. Besides local weather conditions, this could be attributed to differences in soil microclimatic conditions and composition between growing localities, the impact of which on mycotoxin contamination is still unclear.[7]

Barley cultivars

The resistance/susceptibility of cereals to *Fusarium* infection is an issue widely investigated in many projects. Although a lot of promising results have been achieved and the principles governing various scenarios have been at least partly explained, no cultivars fully resistant to FHB are available at present. In any case, physiological features of the barley plant such as height of vegetation, resistance to lodging or type of grain (hulless *versus* covered) and days to maturity of the barley cultivar (calculated from sowing date to harvest) are important factors in this context.

As a factor representing the impact of barley cultivar on mycotoxin levels, type of grain was entered into the statistical model. Interestingly, no impact was confirmed in the case of DON and NIV levels, but the presence/absence of hull was found to be statistically significant for HT-2 ($P < 0.001$) and T-2 ($P = 0.037$) levels in barley, with mean levels of both these toxins in hulless cultivars being twice as high as those in covered cultivars (Fig. 5). Considering the whole experimental period, the highest mean levels of 212 and 208 μg kg^{-1} (sum of HT-2 and T-2) were found in the cultivars KM 2084 and Merlin respectively. It should be noted that, up to now, no study concerning the impact of grain morphological features on type A trichothecene levels has been published, and only one study has been dedicated to the relationship between the presence of hull and levels of type B trichothecenes (DON, sum of ADON and NIV).[41] While the authors did not observe any differences in the case of NIV, levels of DON and its acetylated forms were significantly higher in covered barley cultivars, which are typically employed for malt production, than in hulless cultivars.

Fungicide treatment

Generally, the results of studies assessing the impact of fungicides on mycotoxin levels in treated crops are rather contradictory. In any case, besides the type of active ingredient and the method and time of its application, both weather conditions at the time of fungicide use and the extent and aggressiveness of *Fusarium* species invasion also play an important role.

In the present study, three different fungicide preparations with different active ingredients (Table 3) were applied. However, no statistically significant impact ($P = 0.103$) on DON (Fig. 6) or on HT-2 and T-2 was found. On the other hand, the use of fungicide treatment was statistically significant ($P = 0.008$) for NIV contamination level (Fig. 6).

Regarding the major *Fusarium* toxic secondary metabolite DON, in 2005 a combination of two fungicide preparations (containing tebuconazole and prochloraz respectively) was used for barley protection. In line with an earlier study by Milus and Parsons,[42] who reported an 'enhancing' effect on DON levels in wheat treated against FHB with tebuconazole, also in many barley samples treated with the same active ingredient-containing fungicide, DON levels were higher than those determined in untreated barley. The most pronounced increase was found in the cultivar Jersey, where levels of DON were nine times higher.

With regard to opposite observations,[43] good efficiency of tebuconazole-based preparations applied at mid-anthesis of barley was found in terms of both DON producer elimination (decrease in *F. culmorum* DNA) and DON levels; no general

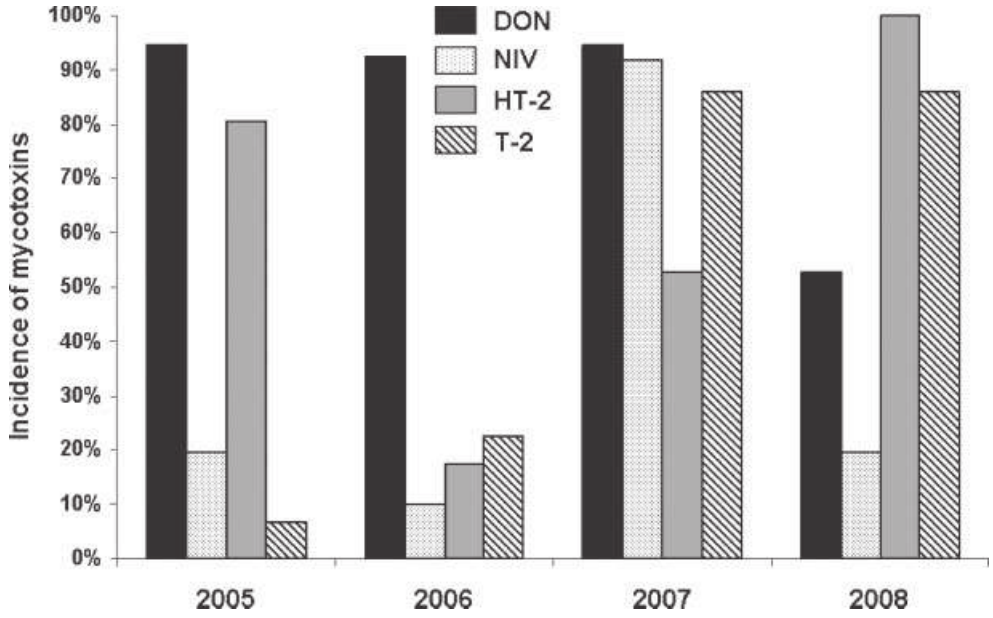


Figure 4. Inter-annual changes in DON, NIV, HT-2 and T-2 incidences.

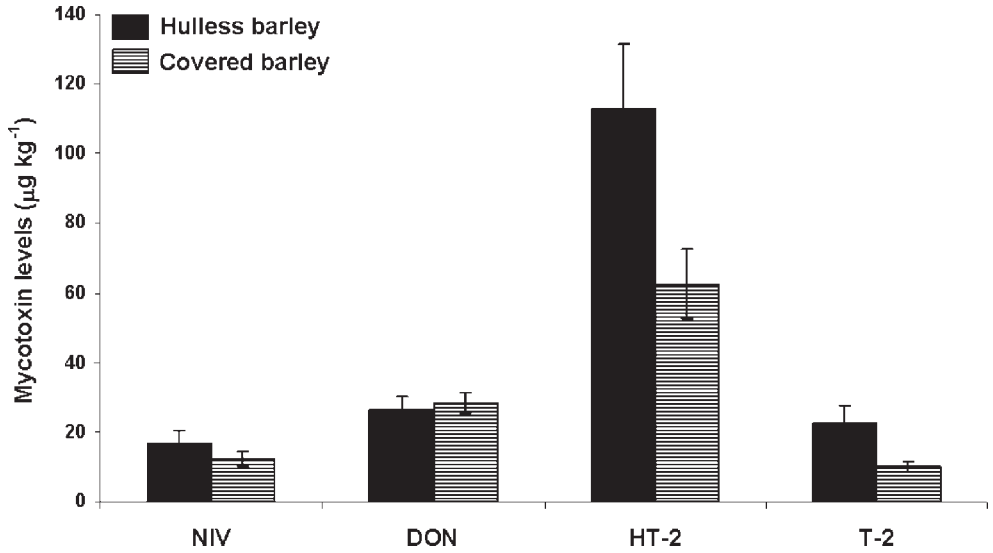


Figure 5. Differences in NIV, DON, HT-2 and T-2 mean levels between hulless and covered barley cultivars within three-year experimental period (error bars express standard error of means of respective toxin levels).

conclusion could be drawn in this particular year (2005). Considering the results of previous studies concerned with possible strategies of FHB elimination/reduction, most evidence on a DON-stimulating effect is available for azoxystrobin, which is otherwise known to be an effective fungicide against infection. Unfortunately, this fungicide was not used in any of our field experiments. In any case the complexity of this issue is shown by another study in which no DON-stimulating effect of azoxystrobin was observed when its mixture with tebuconazole was employed for cereal crop treatment.[44] Similar trends (often an increase in DON levels) were observed in the following years in our study. However, owing to the generally lower levels of DON, the differences were not statistically significant, so an unbiased assessment of the effectiveness of the fungicide preparations was not possible.

As regards NIV, the impact of fungicide treatment on its elevated levels in barley was statistically significant ($P = 0.008$) in all years. The most illustrative failure of fungicidal protection (in the case where Falcon 460 EC containing tebuconazole, triadimenol and spiroxamine was applied) was observed in 2007, when NIV levels were relatively high. The highest increase (by a factor of ten compared with untreated barley) in NIV concentration was documented for the cultivar KM 2283. Although no report on the effect of Falcon 460 EC in this context is available, our results are supported to some degree by Gareis and Ceynova,[45] who reported an increase in NIV content despite some decrease in FHB infection following treatment with tebuconazole-based fungicide.

Concerning the impact of fungicide treatment on HT-2 and T-2 concentrations, no statistical significance was found in our study. Interestingly, in contrast to the type B trichothecenes, the use of Falcon 460 EC resulted in a reduction in HT-2 as well as T-2 levels in barley harvested in 2008 when relatively extensive contamination by these toxins occurred.

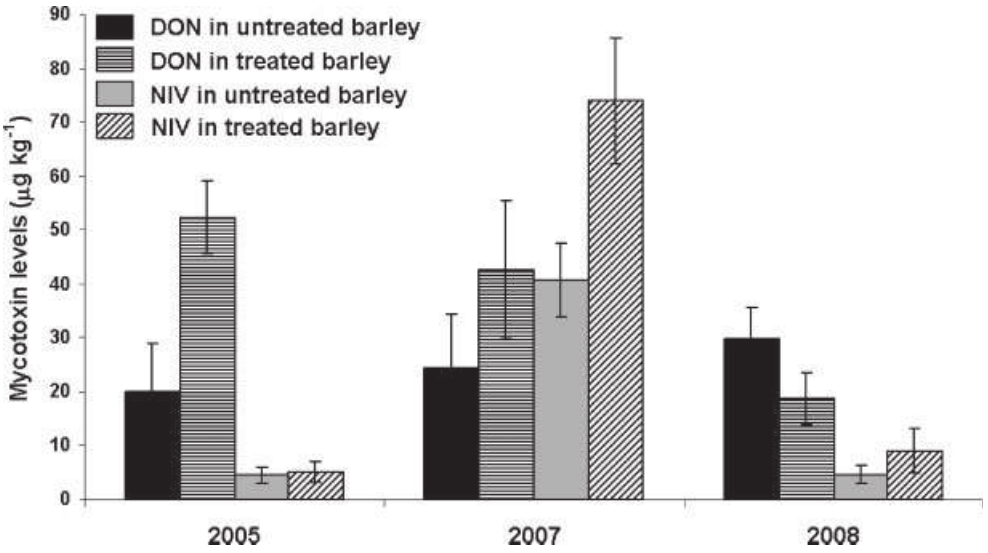


Figure 6. Comparison of DON and NIV mean levels determined in fungicide-treated and untreated (control) barley within three-year experimental period (error bars express standard error of means of respective toxin levels).

Mycotoxins in malt

Owing to their relatively high stability, trichothecenes survive most food-processing technologies, thus passing from raw materials such as barley into final products, beer in this particular case.[11,12] To characterise the transfer of *Fusarium* toxins from barley to malt, various cultivars harvested in 2005 in Zabcice were processed by micromalting technology simulating classic pale malt production (Table 4) and then analysed. As in the above studies, both decreases and increases in DON levels were observed in malted barleys (Fig. 7). Interestingly, with the exception of the hulless variety Merlin (not commonly used for malt production), in all fungicide-treated cultivars a decrease in DON content of up to 80% occurred during malting, while an increase in this major *Fusarium* toxin was observed in 70% of malts prepared from untreated (control) barleys. It should be noted, however, that the final levels of DON in the latter samples were higher in only 50% of malts when compared with products prepared from treated barleys. As discussed earlier, the use of fungicides (Table 3) resulted in some enhancement of crop contamination (Fig. 6). Although the limited number of samples does not allow any general conclusion on the impact of fungicides on DON behaviour during malting, it can be assumed that the distribution and forms of toxins in grains are somewhat different from those in 'naturally' contaminated barley. The available results suggest that, in treated barley, more DON is located on the surface parts of grains, so steeping allows its more efficient transfer into water.[46] In barley contaminated under 'natural' conditions, additional release of DON occurred during malting, due either to its *de novo* formation associated with fungal growth or to its release from masked forms (reported to be contained in cereals infected in the field).[33] Unfortunately, at the time of the malting experiments we were not aware of the potentially huge release of DON-3-Glc or of other interesting facts discovered in our follow-up experiments.[13]

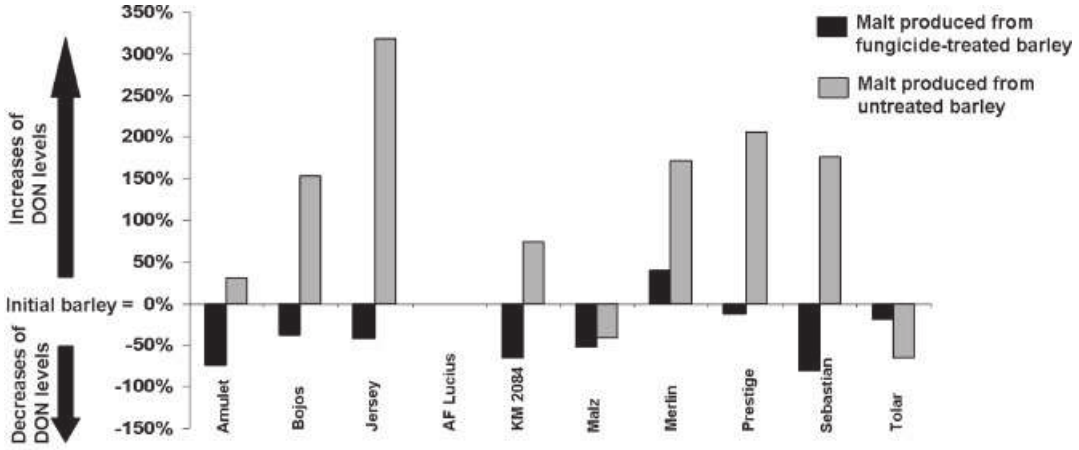
As regards type A trichothecenes, without exception, malting resulted in a decrease in their levels. Again, no general conclusions can be made on the basis of a single pilot study. Moreover, owing to the absence of literature data, our results cannot be critically assessed.

CONCLUSIONS

This four-year study has been aimed at extending knowledge on time trends and the impact of several factors on *Fusarium* toxins in various barley cultivars and malts prepared therefrom.

Although generalisation of the results obtained in our experiments is practically impossible owing to the large inter-annual variations observed, some suggestions for follow-up monitoring as well as research focus can be made.

1. DON, representing type B trichothecenes, remains the major *Fusarium* toxin in barley, but the incidence of type A trichothecenes HT-2 and T-2 seems to be growing. This trend might be due to the spread in particular growing localities of *Fusarium* species such as *F. poae* that were not common previously. The question arises as to whether the observed changes in weather and climate are one of the factors influencing the pattern of these pathogens in food crops.
2. Another interesting topic for further investigation is the role played by the hull (presence/absence) in the extent and distribution of mycotoxins in the grain. Our data indicated higher levels of contamination in hulless cultivars.
3. The outcome of fungicidal treatment was highly variable. None of the tested fungicides, although they represented various classes, guaranteed effective reduction of mycotoxin levels in treated barley. Contrary to expectation logically associated with the use of antifungal agents, the levels of DON were higher in most of the 'protected' barleys. On the other hand, some reduction in DON levels of fungicide-treated cultivars was found during malting in comparison with untreated cultivars. These results might indicate differences in the distribution of mycotoxins within the grain, which may result in various trends of DON changes during the malting process. It may be assumed that penetration into the internal parts is reduced by fungicides, so more efficient removal of mycotoxins during steeping may explain the observed differences.



Levels of DON in initial barley (μg kg^{-1})	Amulet	Bojos	Jersey	AF Lucius	KM 2084	Malz	Merlin	Prestige	Sebastian	Tolar
Fungicide treated barley	56	75	87	LOD	46	34	51	56	88	57
Untreated (control) barley	17	23	10	33	10	23	LOD	30	23	38

Figure 7. Decreases and increases (%) in DON levels after malting (total amounts of DON taken for respective barley cultivars, as in table below figure, are expressed as 0% in this figure).

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APPENDIX V

Malachova A, Stockova L, Wakker A, Varga E, Krska R, Michlmayr H, Adam G, Berthiller F

Critical evaluation of indirect methods for the determination of deoxynivalenol and its conjugated forms in cereals

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PAPER IN FOREFRONT

Critical evaluation of indirect methods for the determination of deoxynivalenol and its conjugated forms in cereals

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Abstract A critical assessment of three previously published indirect methods based on acidic hydrolysis using superacids for the determination of "free" and "total" deoxynivalenol (DON) was carried out. The modified mycotoxins DON-3-glucoside (D3G), 3-acetyl-DON (3ADON), and 15-acetyl-DON (15ADON) were chosen as model analytes. The initial experiments focused on the stability/degradation of DON under hydrolytic conditions and the ability to release DON from the modified forms. Acidic conditions that were capable of cleaving D3G, 3ADON, and 15ADON to DON were not found, raising doubts over the efficacy of previously published indirect methods for total DON determination. Validation of these indirect methods for wheat, maize, and barley using UHPLC-MS/MS was performed in order to test the accuracy of the generated results. Validation data for DON, D3G, 3ADON, and 15ADON in nonhydrolyzed and hydrolyzed matrices were obtained. Under the tested conditions, DON was not released from D3G, 3ADON, or 15ADON after hydrolysis and thus none of the published methods were able to cleave the modified forms of DON. In addition to acids, alkaline hydrolysis with KOH for an extended time and at elevated temperatures was also tested. 3ADON and 15ADON were cleaved under the alkaline pH caused by the addition of KOH or aqueous K_2CO_3 to "neutralize" the acidic sample extracts in the published studies. The published additional DON increase after hydrolysis may have been caused by huge differences in matrix effects and the recovery of DON in nonhydrolyzed and hydrolyzed matrices as well as by the alkaline cleavage of 3ADON or 15ADON after the neutralization of hydrolyzed extracts.

Keywords Masked mycotoxins · LC-MS/MS · Chemical hydrolysis · Wheat · Barley · Maize

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Introduction

Deoxynivalenol (DON) is the most frequently occurring mycotoxin worldwide, particularly in cereal crops such as wheat, maize, barley, oats, and rye, and less often in rice, sorghum, and triticale [1]. It belongs to the trichothecenes, a family of closely related compounds produced mainly by *Fusarium* spp. [1]. *F. graminearum* and *F. culmorum* are responsible for *Fusarium* head blight in wheat (scab) and *Fusarium* ear rot in maize [2], and are considered to be the most important producers of DON. A direct relationship between the incidence of *Fusarium* head blight and the contamination of wheat with DON has been established [3].

Plants have a versatile detoxification system that can deal with a wide range of xenobiotics. As DON interacts with vital cell functions of infected plants, it also represents a target for plant defence systems. Basically, detoxification mechanisms involve three major phases, consisting of chemical

modification (phase I and II metabolism) and compartmentation (phase III metabolism), resulting in the formation of so-called masked mycotoxins [4]. This term was introduced by Gareis et al. [5], and refers exclusively to plant metabolites [4]. Chemical modifications are achieved by linking polar moieties to the parent toxin, leading to a decrease in toxicity towards plants. The modified toxins are partly stored in vacuoles (extractable forms) or irreversibly bound to macromolecules (nonextractable forms) [4]. The major pathway in the biotransformation of DON is conjunction with a glucose moiety, forming deoxynivalenol-3-β-D-glucoside (D3G) [6]. Ever since an analytical standard of D3G first became commercially available, D3G has been reported as a co-contaminant with DON of cereals and cereal-based products, as reviewed by Berthiller et al. [4]. Moreover, increasing D3G levels have been observed during malting and brewing [7]. In addition, DON-diglucoside and oligoglycosylated DON conjugates with up to four bound hexose units were identified in malt and beer using several sample preconcentration steps prior to analysis performed by liquid chromatography–high-resolution mass spectrometry (LC-HRMS) [8]. Only recently, several other masked forms of DON (DON-*S*-cysteine, DON-*S*-cysteinyl-glycine, DON-glutathione) have been identified in wheat using an innovative strategy of untargeted screening based on stable isotope labeling followed by LC-HRMS measurements [9].

However, the formation of mycotoxin conjugates is not limited to plant defence mechanisms. For instance, the acetylated forms 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) are fungal precursors of DON, although they can also occur as intermediates during DON detoxification in plants [10]. Moreover, mycotoxins can also be modified by bacteria and mammals [4]. Thus, the term "modified mycotoxins" has been introduced to cover all structurally altered forms of mycotoxins, independent of their origin [11]. D3G and ADON can potentially be hydrolyzed to DON during food processing or in the digestive tracts of mammals, and thus contribute to the total dietary exposure to DON [8].

Little is known about the toxicological relevance of modified mycotoxins, but data from the literature show that conjugates of xenobiotics can be toxicologically significant. The Panel on Contaminants in the Food Chain of the European Food Safety Authority decided on a pragmatic approach to human risk assessment by assuming that all modified mycotoxins have the same toxicities as their parent compounds [12]. In line with that, the use of an indirect approach to determine mycotoxins in food and feed seems to be an attractive alternative to target analysis. Indirect methods aim to determine the entire "pool" of modified mycotoxins (extractable and nonextractable forms) in a sample by converting them into the parent toxin using chemical or enzymatic hydrolysis. The main advantages of this approach are that chemical standards of the modified forms are not required and as-yet undetected compounds can also be accounted for. A few indirect methods for the determination of modified forms have also been developed, as reviewed by Berthiller et al. [4]. Three of them deal with DON determination based on chemical hydrolysis (an overview is provided in Fig. 1) [13–15]. Liu et al. published an indirect approach based on trichloroacetic acid (TCA) hydrolysis followed by gas chromatography coupled with electron capture detection (GC-ECD) [13]. Similarly, trifluoroacetic acid (TFA) and trifluoromethanesulfonic acid (TFMSA) hydrolysis were used prior to total DON determination in barley [14] and in maize and wheat [15], respectively. GC-MS as well as GC-MS together with enzyme-linked immunosorbent assay (ELISA) were applied in the latter two studies.

The chemical hydrolysis of carboxylic acid esters is a pH-dependent process and is possible under either acidic or alkaline conditions. Acidic hydrolysis of esters involves initial protonation of the carbonyl oxygen. The polarization of the carbonyl group shifts some of the electron density away from the carbon atom, making it more electrophilic and therefore susceptible to the nucleophilic addition of water. Alkaline hydrolysis of esters proceeds via direct nucleophilic addition of the hydroxide ion to the carbonyl group, as it is a stronger nucleophile than water [16]. Glycosidic bonds can also be cleaved chemically. While glycosides are generally susceptible to acidic conditions, in some cases they are susceptible to basic conditions too. This sensitivity to acids is attributed to the sugar moiety, while the nature of the aglycon is more responsible for the instability of glycosides under basic conditions [17].

The aim of the study reported in the present paper was to provide a critical assessment of indirect methods for determining total DON based on acidic hydrolysis. The stability/degradation of DON, D3G, 3ADON, and 15ADON was tested using spiked samples of wheat, maize, and barley and by employing a liquid chromatographic tandem mass spectrometric (LC-MS/MS) method which was developed and validated for this purpose. The workflow of the experiments performed is also shown in Fig. 1.

Experimental

Chemicals and reagents

Methanol, acetonitrile (both LC gradient grade), glacial acetic acid (p.a.), potassium hydroxide (p.a.), and anhydrous sodium bicarbonate were purchased from VWR International GmbH (Vienna, Austria). Anhydrous potassium carbonate (p.a., ≥99 %) was obtained from Fluka Chemie AG (Buchs, Switzerland). Ammonium acetate (MS grade), trifluoroacetic acid (reagentplus®, 99 %), trichloroacetic acid (ACS reagent,

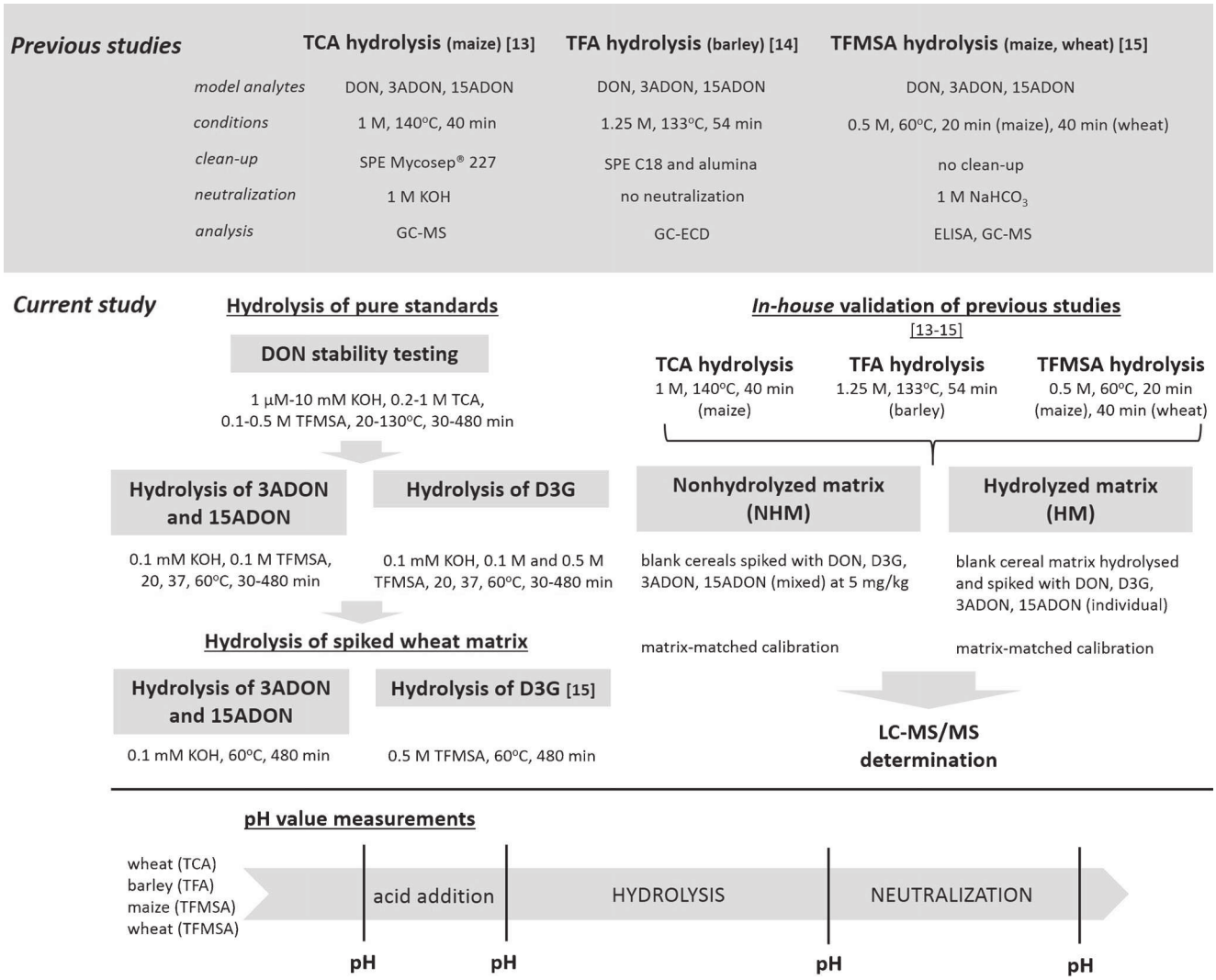

Fig. 1 Overview of three published chemical hydrolysis methods [13–15] for total deoxynivalenol (DON) determination in cereals, and the scheme of the experiments conducted in this study. *3ADON* 3-acetyl-deoxynivalenol, *15ADON* 15-acetyl-deoxynivalenol, *D3G* deoxynivalenol-3-glucoside, *TCA* trichloroacetic acid, *TFA* trifluoroacetic acid, *TFMSA* trifluoromethanesulfonic acid

≥99 %), and trifluoromethanesulfonic acid (reagent grade, 98 %) were obtained from Sigma–Aldrich (Vienna, Austria). Hydrochloric acid (fuming, 37 %, p.a.) was purchased from Merck KGaA (Darmstadt, Germany). Water was purified using a Purelab Ultra system (ELGA LabWater, Celle, Germany). Buffer solutions for calibrating the pH meter, disodium hydrogen phosphate/potassium hydrogen phosphate (pH 7.00 ±0.01, 20 °C), boric acid/potassium chloride/sodium hydroxide (pH 10.00±0.02, 20 °C), and citric acid/sodium hydroxide/hydrogen chloride (pH 4.00±0.01, 20 °C) were purchased from Merck KGaA. Standards for DON, 3ADON, and 15ADON were purchased from Romer Labs GmbH (Tulln, Austria) as stock solutions of 100.8 µg mL^{-1}, 100.1 µg mL^{-1}, and 103.3 µg mL^{-1}, respectively, in acetonitrile. D3G was initially purified from wheat plants treated with DON at anthesis [18] or prepared by enzymatic synthesis using a purified recombinant UDP-glucosyltransferase (Michlmayr et al., in preparation). A 200 µg mL^{-1} stock solution in methanol was used to prepare the standards. The concentration of D3G was verified with a liquid calibrant of D3G (purity>95 %) in acetonitrile obtained from Romer Labs.

Samples

Ground blank wheat, barley, and maize samples were used as model matrices for the spiking experiments. Wheat and maize were grown by Prof. Marc Lemmens at the Institute for Biotechnology in Plant Production (IFA-Tulln, BOKU, Austria) and a barley sample was kindly provided by Assoc. Prof. Radim Cerkal (Mendel University, Brno, Czech Republic). The artificially inoculated wheat sample of the variety "Sultan" used in this study was provided by the Crop Research Institute (Prague, Czech Republic).

Hydrolysis experiments with pure standards

The stability of DON was tested under various hydrolytic conditions. For each of the tested conditions, 35 μL of DON stock solution (100.8 μg mL^{-1}) were evaporated to dryness under a gentle nitrogen stream in triplicate and the residue was re-dissolved in 7 mL of the hydrolysis agent. Aqueous solutions of KOH (1 μM, 0.1 mM, 0.1 M), TCA (0.2 M, 0.5 M, 1 M), and TFMSA (0.1 M, 0.2 M, 0.5 M) were used for 30, 70, 120, 240, and 480 min at 20 °C, 37 °C, 60 °C, 100 °C, and 130 °C. TFA was not used in these initial experiments because it was not in stock. The following temperatures were maintained: (i) 20 °C (room temperature), (ii) 37 °C and 130 °C using a Kelvitron T type T6120 laboratory oven (Heraeus Instruments, Hanau, Germany), and (iii) 60 °C and 100 °C using an IKAMAG RET-G laboratory heater (IKA Labortechnik, Staufen, Germany). Afterwards, the hydrolyzed mixtures were neutralized with stoichiometrically equivalent amounts of HCl or KOH and measured with UHPLC-MS/MS. The neutrality of each solution was confirmed by pH measurements.

The conditions under which DON was found to be stable were used for further hydrolysis experiments with D3G, 3ADON, and 15ADON. Similarly, 1 μg of single dried-down standards of D3G, 3ADON, and 15ADON were prepared in triplicate and hydrolyzed with 4 mL of (i) D3G: 0.1 mM KOH, 0.1 M TFMSA, 0.5 M TFMSA at 20, 37, and 60 °C for 30, 70, 120, 180, 240, and 480 min; (ii) 3ADON and 15ADON: 0.1 mM KOH, 0.1 M TFMSA at 20, 37, and 60 °C for 30, 60, 120, 180, 240, 480 min as well as 16, 20, and 24 h. Neutralization after hydrolysis was performed with appropriate amounts of HCl or KOH. Again, neutrality was confirmed via pH measurements. Samples were subsequently measured with UHPLC-MS/MS.

Hydrolysis of conjugated DON in the wheat matrix

As naturally contaminated wheat samples containing only 15ADON or 3ADON (without large amounts of D3G) were not available, the experiments were performed with spiked samples. Both a naturally D3G-contaminated sample (containing only small amounts of 3ADON) and a D3G-spiked wheat sample were used.

3ADON, 15ADON: Blank wheat samples (0.500± 0.002 g) were weighed into an 8-mL vial and spiked with single standard solutions of 3ADON or 15ADON (500 μg kg^{-1}) in triplicate. Hydrolysis was performed with 4 mL of 0.1 mM KOH at 60 °C in the laboratory heater for 8 h. The samples were vortexed every 2 h during hydrolysis. Afterwards, 40 μL of 10 mM aqueous HCl were used for neutralization and the samples were cooled down. For extraction, 4 mL of acetonitrile were added to the samples in order to get a final extraction composition of acetonitrile:water 1:1, *v/v*. The samples were extracted on a GFL 3017 rotary shaker (Burgwedel, Germany) for 30 min. The extracts were transferred to HPLC vials and centrifuged on an Awel MF 48-R centrifuge (Blein, France) at 4500 rpm (3830×*g*) prior to injection. Moreover, further concentrations of KOH solution were tested. Therefore, 0.500±0.002 g of a blank wheat sample were weighed into an 8-mL vial and spiked with 3ADON and 15ADON, each present at 500 μg kg^{-1}, in triplicate. Hydrolysis was performed with 4 mL of KOH solution (2 mM, 10 mM, or 0.1 M) at 60 °C in the laboratory heater for either 240 or 480 min. The subsequent steps were the same as for the hydrolysis with 0.1 mM KOH.

D3G: The hydrolytic method of Tran and Smith [15] was followed with minor modifications. To decrease the cost of analysis, the whole sample preparation procedure was miniaturized (from 5 g to 0.5 g of sample), but all the conditions used (i.e., temperature, time of hydrolysis, ratios of sample weight/acid volume/extraction solvent volume/neutralization agent volume) remained the same. Blank wheat samples (0.500±0.002 g) were weighed into an 8-mL vial and spiked with 500 μg kg^{-1} D3G in triplicate. Afterwards, 3.6 mL of deionized water were added and the mixture was shaken on the rotary shaker for 30 min. Hydrolysis was performed with 100 μL of 0.5 M TFMSA at 40 °C for 40 min in the laboratory heater. The samples were neutralized with 1 M K_2CO_3. The final extracts were transferred to HPLC vials and centrifuged at 4500 rpm (3830×*g*) prior to injection. The same protocol was also applied to the naturally contaminated wheat sample containing 3520 μg kg^{-1} DON, 460 μg kg^{-1} D3G, and 67 μg kg^{-1} 3ADON. In order to verify the stability of DON in the matrix under these conditions, another blank sample was spiked with DON at a level of 3500 μg kg^{-1}.

In-house method validation

Sample preparation for the published indirect methods for the determination of DON and the total amount of DON after acidic hydrolysis with trichloroacetic acid [13], trifluoroacetic acid [14], and trifluoromethanesulfonic acid [15] was performed. Again, only 0.5 g of cereal were used in our case, keeping all other conditions and ratios as specified in the respective publications. Each method was validated for the matrices used in the respective paper, i.e., wheat [13], barley [14], and wheat and maize [15]. In addition, the matrix was validated before hydrolysis (nonhydrolyzed matrix, NHM) and after hydrolysis (hydrolyzed matrix, HM) in order to assure method accuracy.

Two sets of standard stock solutions in acetonitrile were prepared for spiking experiments: (i) individual solutions of DON, D3G, 3ADON, and 15ADON at a concentration of 10 μg mL^{-1} for the HM validation, and (ii) a mixed solution of all toxins used at level of 10 μg mL^{-1} for the NHM

validation. In addition, further dilutions were prepared from the 10 μg mL^{-1} mixed solution of all toxins for the preparation of solvent and matrix-matched standards.

Concerning the HM validation, each matrix (0.500± 0.002 g) was spiked with single standards of DON, D3G, 3ADON, and 15ADON at 5 μg kg^{-1} in triplicate and stored at room temperature for 2 h. Afterwards, the following method protocols were used. (i) TCA hydrolysis: 0.5 g of spiked sample were extracted with 5 mL of acetonitrile/water (84:16, *v/v*) for 30 min on a rotary shaker. Afterwards, 2 mL of deionized water and 1 mL of 1 M TCA were added. Solvolysis was performed at 140 °C in the laboratory oven for 40 min. After the mixture had cooled down, 0.5 mL of 1 M KOH were added for neutralization and the volume was adjusted to 10 mL with pure acetonitrile. (ii) TFA hydrolysis: 0.5 g of the spiked sample were extracted with 4 mL of acetonitrile/water (84:16, *v/v*) for 30 min on the rotary shaker. Hydrolysis was performed with 100 μL of 1.25 M TFA at 133 °C in the laboratory oven for 54 min. After cooling down, 125 μL of 1 M KOH were added for neutralization. The extract was mixed and transferred into an HPLC vial. (iii) TFMSA hydrolysis: 0.5 g of spiked sample were extracted with 3.6 mL of deionized water and shaken for 30 min on the rotary shaker. Afterwards, 100 μL of 0.5 M TFMSA were added and hydrolyzed for 40 min (wheat) or 20 min (maize) at 60 °C in the lab heater. The whole mixture was adjusted with 1 M sodium bicarbonate to 4 mL.

In addition, the pH was measured four times in each experiment: before acid addition, directly after acid addition, after hydrolysis, and after the addition of the neutralization agent. The pH was measured using a pH electrode on a Microprocessor pH 537 pH meter (WTW, Weilheim, Germany), which was calibrated with buffers of pH 4 and 7 (for acidic conditions) or buffers of pH 7 and 10 (for alkaline conditions).

Similarly, the NHM validation was performed using the same protocol as used for the HM validation but with two modifications. Firstly, samples were spiked with the mixed solution of all toxins. Secondly, water was used instead of acid in the protocol described above, and no KOH was added for neutralization.

Two types of calibration curves covering the concentration range 16.6–750 μg L^{-1} were prepared. Appropriate amounts of the mixed solution were evaporated under a gentle nitrogen stream and the standards were re-dissolved in 1 mL of acetonitrile/water 84:16, *v/v* for the solvent calibration curve. Similarly, matrix-matched calibrations for NHM and HM were prepared by re-dissolving dried-down standards in 1-mL blank extracts from the respective matrices.

Method performance characteristics were calculated according to the following equations:

Apparent recovery (R_A):

$$R_A(\%) = \frac{\text{slope (spiked samples)}}{\text{slope (neat solvent standard)}} \times 100. \quad (1)$$

Matrix effect as a signal suppression/enhancement (SSE):

$$\text{SSE } (\%) = \frac{\text{slope (matrix-matched standard)}}{\text{slope (neat solvent standard)}} \times 100. \quad (2)$$

Extraction recovery (R_E):

$$R_E(\%) = \frac{R_A}{\text{SSE}} \times 100. \quad (3)$$

DON was quantified after the hydrolysis of samples spiked with D3G, 3ADON, or 15ADON using the HM-matched calibration curve. The percentage of DON released was calculated as follows:

$$\text{DON release } (\%) = \frac{\text{quantified DON level}}{\text{spiked mod.toxin level}} \times \frac{M(\text{mod.toxin})}{M(\text{DON})} \times 100$$
$$M = \text{molar mass}\left(\text{g mol}^{-1}\right). \quad (4)$$

UHPLC-MS/MS analysis

A 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap 5500 MS/MS system from AB Sciex (Foster City, CA, USA) was used for analysis. Chromatographic separation was performed on an Acquity UPLC BEH column (50×2.1 mm, 1.7 μm, Waters, Milford, MA, USA) held at 40 °C. Water/acetic acid (99:1, *v/v*) was used as eluent A and eluent B consisted of methanol/water/acetic acid 95:4:1, *v/v/v*. Both eluents also contained 5 mM ammonium acetate. Gradient elution started at 10 % B, which slowly increased to 20 % B after 2 min, and then increased linearly to 40 % B in another 4 min. 90 % B was reached by performing a rapid increase for another minute. Re-equilibration for another 2 min at 10 % B was realized prior to further injection. The flow rate was 500 μL min^{-1} and the injection volume was 2 μL.

The QTrap 5500 was operated in electrospray ionization mode using a TurboV ion spray source with the following settings: curtain gas (CUR), 30 psi (207 kPa, nitrogen); collision gas (CAD, nitrogen), medium; ion spray voltage, −4500 V/+4500 V; temperature, 550 °C; sheath gas (GS1) and drying gas (GS2), both 80 psi (552 kPa, zero-grade air). Acquisition was performed in the selected reaction monitoring

mode and the chromatographic run was divided into two individual periods with a settling time of 50 ms. The mass spectrometric conditions for the individual analytes are provided in Table 1.

Results and discussion

Hydrolysis experiments with pure standards

The first criterion of an indirect method is that the final product (the compound which is released during the reaction and determined afterwards) has to be stable during the chosen hydrolytic conditions. Therefore, suitable conditions for hydrolysis must be found which leave DON intact. The reagents and conditions of the experiments on the pure standards were based on those used in previous reports [13–15, 19].

When KOH was used in the hydrolysis of acetylated conjugates of DON, it had no impact on DON stability at concentrations of 1 μM (pH 8) and 0.1 mM (pH 10) up to 100 °C for 8 h. Higher concentrations resulted in detectable DON degradation after 1 h. Higher temperatures resulted in even faster degradation. TCA caused fast DON degradation at each tested concentration. The conditions employed by Liu et al. [13], 1 M TCA at 130 °C, led to DON losses of almost 50 % after 30 min. 0.1 M and 0.2 M TFMSA had no impact on the stability of DON at temperatures of up to 60 °C. The conditions used by Tran and Smith [15], 0.5 M TFMSA at 37 °C for 4 h, seemed to be suitable, as losses of DON did not exceed 5 %. However, fast DON degradation was observed at temperatures exceeding 40 °C.

The conditions which passed the first criterion (no impact on DON stability) were used for the hydrolysis of individual standards of D3G, 3ADON, and 15ADON. As expected, D3G was stable at 0.1 mM KOH at any temperature and duration tested. Using 0.1 M TFMSA for the hydrolysis of D3G was also inefficient. Increasing the concentration of TFMSA to 0.5 M caused the rapid decomposition of D3G to unknown products at 37 °C and no release of DON was observed. Slow degradation of 3ADON was observed when 0.1 M TFMSA was used. The loss reached 11–23 % depending on the temperature applied (the higher the temperature, the greater the loss). In contrast, 0.1 M TFMSA had no effect on 15ADON regardless of the temperature used and time allowed. Hydrolysis of both 3ADON and 15ADON to DON was achieved in 1 mM KOH at 60 °C for 8 h. The hydrolysis rates for 3ADON and 15ADON were 90 and 95 %, respectively. There was gradual degradation of the DON formed at longer reaction times. Therefore, the following conditions were used as initial conditions for the subsequent experiments on the wheat matrix: 1 mM KOH, 60 °C, 8 h.

Alkaline hydrolysis of conjugated DON in the wheat matrix

Using 1 mM KOH at 60 °C for 8 h to hydrolyze 3ADON and 15ADON spiked into wheat did not prove to be successful. On the one hand, 3ADON and 15ADON were not completely hydrolyzed to DON, as only 32 % of the spiked 3ADON and 47 % of the spiked 15ADON were hydrolyzed. On the other, the levels of DON released were lower than expected (less than 25 %) and did not correspond to the amounts of hydrolyzed 3ADON and 15ADON. Moreover, it was revealed that the matrix buffers the pH of the hydrolytic agent from pH 10 to pH 6. Therefore, the concentration of KOH was increased and the experiment was repeated. The results are summarized in Table 2. Interestingly, the hydrolysis rates contradict those obtained in the first matrix experiment with

Table 1 Optimized tandem mass spectrometric conditions for deoxynivalenol (DON), DON-3-glucoside (D3G), 3-acetyl-DON (3ADON), and 15-acetyl-DON (15ADON)

Analyte	Retention time (min)	Period/ion	Precursor ion (*m/z*)	Product ions (*m/z*) [a]	DP (V)	CE (V)	CXP (V)	Dwell time (ms)
DON	1.20	1/ $[M+CH_3COO]^-$	355.1	58.9	−60	−52	−7	30
				295.1	−60	−14	−11	30
				265.1	−60	−22	−9	30
D3G	1.35	1/ $[M+CH_3COO]^-$	517.1	457.1	−80	−18	−17	30
				427.1	−80	−28	−15	30
3ADON	3.33	2/ $[M+H]^+$	339.1	203.0	116	19	8	30
				231.0	116	17	18	30
				213.0	116	19	10	30
15ADON	3.45	2/ $[M+H]^+$	339.1	321.0	126	13	14	30
				137.0	126	15	8	30
				261.0	126	17	12	30

DP declustering potential, *CE* collision energy, *CXP* collision cell exit potential

[a] The first product ion was used as quantifier, the other two transitions served as qualifiers

Table 2 Release of deoxynivalenol (DON) and residual recoveries of 3-acetyl-DON (3ADON) and 15-acetyl-DON (15ADON) after alkaline hydrolysis of a wheat blank sample spiked at 500 μg kg^{-1} with 3ADON and 15ADON

Analyte (%)	Hydrolytic conditions					
	2 mM KOH, 60 °C, 4 h	2 mM KOH, 60 °C, 8 h	10 mM KOH, 60 °C, 4 h	10 mM KOH, 60 °C, 8 h	0.1 M KOH, 60 °C, 4 h	0.1 M KOH, 60 °C, 8 h
DON	43	50	49	52	54	40
3ADON	91	94	92	88	4	4
15ADON	97	94	94	93	79	85

1 mM KOH. Increasing the concentration of KOH had only a small impact on the hydrolysis of acetylated DONs. For instance, using 2 mM KOH at 60 °C for 8 h hydrolyzed only 6 % of both 3ADON and 15ADON. Fast degradation of 3ADON without further DON release was observed with the highest KOH concentration tested (0.1 M). Furthermore, the molar sum of 3ADON, 15ADON, and DON was higher than the initial sum of these toxins before hydrolysis. This may have been caused either by the presence of additional (unknown) sources of DON in the sample or signal enhancement in LC-MS/MS.

Acidic hydrolysis of D3G in the wheat matrix

As no suitable conditions for the alkaline hydrolysis of D3G were found, the method of Tran and Smith using TFMSA [15] was applied to both spiked wheat samples and naturally contaminated wheat. Although D3G disappeared, no DON increase was observed. The reaction seemed to lead to the formation of other products. Interestingly, D3G degradation occurred to a lesser extent in the naturally contaminated sample (55 % of D3G initial levels detected after hydrolysis) compared to the spiked one where only 13 % of D3G were recovered. D3G seems to be better protected by the matrix in naturally contaminated samples. A negative control sample (sample spiked only with DON) was prepared in order to verify the stability of DON. The recovery of DON after hydrolysis was very low, yielding only 31 % of the expected amount. There are three theoretical reasons for this phenomenon: (i) DON is unstable during hydrolysis—which can be excluded based on the results reported above; (ii) low extraction efficiency of DON from the hydrolyzed matrix; or (iii) matrix effects in LC-MS/MS.

In-house method validation

As we did not find any reports of suitable conditions for acidic or alkaline hydrolyses of either acetylated DONs or D3G, our doubts over the effectiveness of previously published indirect methods for total DON determination [13–15] grew. In order to avoid the generation of biased data due to matrix effects, the developed LC-MS/MS method had to be properly validated. Therefore, sample preparation procedures used in published methods [13–15] were combined with the newly developed LC-MS/MS method and validated. The matrix was visibly changed after hydrolysis, so matrix effects must have influenced DON determination in the matrix before and after hydrolysis in different ways. Moreover, it was also suspected that the extraction recovery of DON from NHM was different from the extraction recovery of DON from HM. In order to compare the method accuracy for free (extractable) DON with the accuracy for total DON determination, we decided to validate both matrices (NHM and HM). The performance characteristics of the methods are summarized in Table 3.

Upon comparing the validation data for DON in NHM and HM, huge differences were observed in SSE and R_E values. Strong suppression of the DON signal in TCA-hydrolyzed wheat matrix and TFA-hydrolyzed barley matrix was observed compared to that seen in the respective NHM. In contrast, TFMSA hydrolysis of wheat and maize resulted in high DON signal enhancement when determined by LC-MS/MS. Although other analytical techniques (GC-ECD, GC-MS, and ELISA) were used as analytical tools in the previously published studies, matrix effects should have been taken into account during method development. GC-based methods, just like LC-MS/MS methods, can suffer from matrix effects or other difficulties which hinder accurate determination. It has been stated that GC-based techniques in particular (such as GC-ECD, GC–flame ionization detection, or GC-MS), when used for trichothecene determination, are prone to signal enhancement even when clean-up is performed [20, 21]. Moreover, aside from signal enhancement (the overestimation of a toxin due to matrix effects), other problems such as nonlinearity of calibration curves, poor repeatability, or memory effects from a previous sample injection [20, 21] could have led to the overestimation of DON in HM that was observed in the previously published studies [13, 14]. Further, the analytical methods used in these studies were not properly described. There is no information about method linearity and repeatability. More importantly, it is not clear whether the results were calculated using solvent- or matrix-matched calibration curves. In addition, the extraction recovery of DON is higher

Table 3 Validation data for the indirect approaches to DON determination using UHPLC-MS/MS

Agent (matrix)	Declared increase in DON after hydrolysis (%)	Analyte	Nonhydrolyzed matrix (NHM)				Hydrolyzed matrix (HM)*				DON released (%, n=3)
			R_E (%)	R_A (%)	SSE (%)	RSD (%)	R_E (%)	R_A (%)	SSE (%)	RSD (%)	
TCA (wheat)	13–63 published in [13]	DON	59	63	107	5	74	39	53	6	–
		D3G	82	46	56	1	31	20	65	4	n.d.
		3ADON	62	62	100	2	66	65	98	4	n.d.
		15ADON	60	61	102	3	67	61	91	1	n.d.
TFA (barley)	9–88 published in [14]	DON	101	97	96	2	120	67	56	21	–
		D3G	93	62	67	2	83	41	49	2	n.d.
		3ADON	100	100	100	4	119	111	93	1	n.d.
		15ADON	101	94	93	7	92	96	104	3	n.d.
TFMSA (wheat)	7–75 published in [15]	DON	94	126	134	19	55	82	149	6	–
		D3G	75	150	200	22	11	12	108	1	n.d.
		3ADON	80	103	128	6	86	45	52	9	33
		15ADON	82	76	93	6	47	8	17	10	40
TFMSA (maize)	8–70 published in [15]	DON	103	66	64	4	31	40	128	5	–
		D3G	97	70	72	4	75	54	72	4	n.d.
		3ADON	81	78	96	7	4	2	52	1	31
		15ADON	91	89	98	5	38	5	13	16	33

R_E extraction recovery, R_A apparent recovery, *SSE* signal suppression or enhancement, *RSD* relative standard deviation (repeatability, n=3, calculated from apparent recovery), *DON* deoxynivalenol, *D3G* DON-3-glucoside, *3ADON* 3-acetyl-DON, *15ADON* 15-acetyl-DON, *TCA* trichloroacetic acid, *TFA* trifluoroacetic acid, *TFMSA* trifluoromethanesulfonic acid, *n.d.* not detected, *except in the TFA hydrolysis procedure, the pH was not neutral after the addition of alkaline solution to the HM

after TCA and TFA hydrolysis than from NHM. Thus, the final free DON and total DON levels should have been corrected for recovery.

The indirect approach reported by Tran and Smith in 2011 [15] was verified using an AgraQuant® DON assay 0.25/5.0 test kit (Romer Labs Inc., Union, MO, USA). All samples were analyzed for free DON, 3ADON, and 15ADON by GC-MS, but D3G was not taken into account. After hydrolysis, no confirmation of the accuracy of the ELISA results was performed by GC-MS. The authors of this study nevertheless claimed that the increase in DON levels after hydrolysis was caused by the release of this toxin from masked forms other than 3ADON or 15ADON that were either present at low levels or below the limit of quantification. It was furthermore argued that D3G could not have contributed to the "total" DON level because it is unstable under the applied hydrolytic conditions. In general, antibody-based methods are considered to be useful tools for screening purposes, but the quantitative results obtained should be verified using a "confirmatory method" [22]. The main risk is of overestimating the results due to antibody cross-reactivity, which was most likely observed by the authors, rather than the release of DON. The results obtained in this study are doubtful for several reasons. First of all, AgraQuant® was found to be a kit that overestimates the DON content compared to LC-MS/MS results [23–25]. Two comprehensive studies performed on four commercially available DON ELISA kits revealed that DON overestimation is caused by both cross-reactivity with structurally related compounds and by matrix effects. Although the kit manufacturer stated that the cross-reactivity with 3ADON was >100 %, two studies reported it to be more than 300 % based on the solvent standard [24, 25]. Cross-reactivity with D3G was found to be 113 % in the latter study [25], while Tran and Smith [15] based their calculations on a mere 4.8 %, as specified by the manufacturer. Furthermore, a matrix contribution to DON overestimation was reported [24] several years before the published hydrolytic method of Tran and Smith [15]. A certified reference material of wheat was subsequently analyzed by LC-MS/MS, ELISA, and ELISA after MycoSep™ 226 clean-up [24]. The results obtained using the latter approach corresponded to those determined by LC-MS/MS, while analysis of the raw extract by ELISA resulted in a huge overestimate of the DON in addition to the effect caused by cross-reactivity with 3ADON, 15ADON, and D3G [24]. It is likely that the increase in DON levels after hydrolysis, considered to be due to the release of masked forms of this toxin, were actually caused by a combination of matrix effects (cross-reactivity of matrix co-extracts), the huge cross-reactivity of 3ADON, and the cross-reactivity of degraded D3G. In general, it is recommended that matrix-matched calibration curves should be used in ELISA analysis in order to decrease overestimation. It has been proven that every matrix behaves differently in terms of the cross-reactivity caused by matrix co-extracts [24, 25]. Based on our LC-MS/MS validation data, this is also true of nonhydrolyzed and hydrolyzed versions of the same matrix.

Although the authors' conclusion [15] that D3G is unstable under TFMSA hydrolysis has been confirmed, D3G may still have contributed to the total DON result. The unknown degradation products of D3G formed during hydrolysis may be cross-reactive to some extent.

Our doubts that none of the published indirect methods work properly were also confirmed by the fact that DON was not detected after TCA and TFA hydrolyses in the samples spiked with pure 3ADON, 15ADON, and D3G standards. This means that these compounds—if they were degraded at all—were not cleaved to DON. In addition, the stability of 3ADON and 15ADON towards acidic hydrolysis is supported by validation data. There are no differences in R_E and SSE for both acetylated forms of DON when comparing NHM with HM. However, in terms of the validation data for 3ADON and 15ADON in TFMSA hydrolysis, unexpectedly low values of R_E, R_A, and SSE were obtained in HM (Table 3). Moreover, released DON was detected in 3ADON- and 15ADON-spiked samples after the hydrolysis procedure. The initial experiments with pure standards revealed that 3ADON and 15ADON are fully stable under the acidic conditions used, but they can be cleaved under alkaline conditions. In order to explain those results, the next step was to perform a small-scale hydrolysis experiment and to measure the pH at each step of the procedure. The results are summarized in Table 4. Thus, in two of the three studies, 3ADON and 15ADON were most likely not cleaved during the acidic hydrolysis with TCA or TFMSA, but they were after the addition of an excess of the neutralization agent. The reason that this phenomenon was only observed in the validation data for TFMSA hydrolysis was the order of measurement of the samples. Validation samples of TFMSA hydrolysis were the last in the queue to be measured, >8 h after sample preparation ("neutralization"). Also, the matrix-matched standards of 3ADON and 15ADON prepared using blank extracts of hydrolyzed and "neutralized" matrices slowly decomposed during analysis due to the alkaline conditions present.

Conclusions

A critical assessment of three indirect methods for total DON determination based on acidic hydrolysis using TCA [13], TFA [14], or TFMSA [15] was carried out in this study. The first phase of the study focused on the stability/degradation of pure standards of DON, D3G, 3ADON, and 15ADON under various hydrolytic conditions, which was followed by experiments on a wheat matrix. As none of the hydrolytic conditions were found to be suitable for achieving the reliable decomposition of modified forms of DON to the parent toxin (DON), we decided to assess protocols for previously published indirect methods. The most important findings were:

- Validating both the method for "free DON" (NHM) and the method for "total DON" (HM) revealed huge differences in SSE as well as in R_E when DON was determined in NHM and HM. The authors of previous studies [13–15] did not take into account the changes caused to the matrix by hydrolysis. As no information on method performance characteristics and data evaluation or any corrections to the results (in terms of recovery and matrix effects) was provided, the observed DON increase was most likely caused by DON signal enhancement during HM analysis, signal suppression during NHM analysis, or both.
- The exclusion of D3G—the major masked form of DON—as a model toxin in indirect method development in previous studies [13–15] renders these methods unsuitable for routine use.
- An intended neutralization step using aqueous KOH or K_2CO_3 after hydrolysis led to alkalinization of the cereal extracts. Under those conditions, 3ADON and 15ADON (and potentially other modified forms) were cleaved to DON and thus further increased the level of DON (or further breakdown products). The increase in DON was not caused by acidic hydrolysis, as claimed by the authors.

In summary, the use of acidic or alkaline hydrolytic procedures for the indirect determination of total DON is strongly discouraged. Indirect methods using enzymatic hydrolysis show more promise. A 1,3-β-glucanase with high affinity for D3G was identified, but this enzyme was strongly inhibited under the matrix conditions employed here, and sample clean-up was required before enzymatic hydrolysis [26]. Recently, a highly efficient glucosidase from *Bifidobacterium* that is able to hydrolyze D3G in the matrix was identified [27]. Nevertheless, direct LC-MS/MS utilizing standards for known modified forms presently remains the method of choice to determine the levels of DON and its modified forms in cereals and cereal-based food.

Table 4 pH values obtained during indirect methods for total deoxynivalenol determination

Matrix	Before hydrolysis	After acid addition	After hydrolysis	After neutralization
Wheat (TCA)	6.18	1.16	1.61	13.22
Barley (TFA)	6.24	1.70	2.16	6.93
Wheat (TFMSA)	5.86	3.37	3.52	10.08
Maize (TFMSA)	6.11	3.15	3.97	10.07

TCA trichloroacetic acid, *TFA* trifluoroacetic acid, *TFMSA* trifluoromethanesulfonic acid

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APPENDIX VI

Michlmayr H, Varga E, Malachova A, Nguyen NT, Lorenz C, Haltrich D, Berthiller F, Adam G

A versatile family 3 glucoside hydrolase from Bifidobacterium adolescentis hydrolysis *β*-glucosides of the *Fusarium* mycotoxins deoxynivalenol, nivalenol, and HT-2 toxin in cereal matrices

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A Versatile Family 3 Glycoside Hydrolase from *Bifidobacterium adolescentis* Hydrolyzes β-Glucosides of the *Fusarium* Mycotoxins Deoxynivalenol, Nivalenol, and HT-2 Toxin in Cereal Matrices

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Glycosylation plays a central role in plant defense against xenobiotics, including mycotoxins. Glucoconjugates of *Fusarium* toxins, such as deoxynivalenol-3-*O*-β-D-glucoside (DON-3G), often cooccur with their parental toxins in cereal-based food and feed. To date, only limited information exists on the occurrence of glucosylated mycotoxins and their toxicological relevance. Due to a lack of analytical standards and the requirement of high-end analytical instrumentation for their direct determination, hydrolytic cleavage of β-glucosides followed by analysis of the released parental toxins has been proposed as an indirect determination approach. This study compares the abilities of several fungal and recombinant bacterial β-glucosidases to hydrolyze the model analyte DON-3G. Furthermore, substrate specificities of two fungal and two bacterial (*Lactobacillus brevis* and *Bifidobacterium adolescentis*) glycoside hydrolase family 3 β-glucosidases were evaluated on a broader range of substrates. The purified recombinant enzyme from *B. adolescentis* (*Ba*Bgl) displayed high flexibility in substrate specificity and exerted the highest hydrolytic activity toward 3-*O*-β-D-glucosides of the trichothecenes deoxynivalenol (DON), nivalenol, and HT-2 toxin. A K_m of 5.4 mM and a V_{max} of 16 μmol min^{-1} mg^{-1} were determined with DON-3G. Due to low product inhibition (DON and glucose) and sufficient activity in several extracts of cereal matrices, this enzyme has the potential to be used for indirect analyses of trichothecene-β-glucosides in cereal samples.

Infestation of cereals by phytopathogenic fungi is a global threat to the human food supply. In addition to economically devastating losses through yield and quality deterioration of agricultural products, contamination with mycotoxins poses a serious challenge for food safety (1). Among the most relevant mycotoxin producers worldwide are *Fusarium* species, causing *Fusarium* head blight disease of small-grain cereals (FHB; also known as *Fusarium* ear blight or scab). Trichothecene class mycotoxins inhibit eukaryotic protein synthesis and are important *Fusarium* virulence factors. Furthermore, they can cause apoptotic cell death and immunosuppression and trigger proinflammatory responses in humans and animals (1–3). The most important groups with regard to food safety are type A (T-2 toxin and HT-2 toxin [HT2]) and type B (nivalenol [NIV] and deoxynivalenol [DON]) trichothecenes (4). The type A trichothecene T-2 toxin possesses high acute toxicity and has caused fatal outbreaks of alimentary toxic aleukia in the last century (1). DON is the predominant trichothecene toxin produced by the *Fusarium graminearum* species complex and ranks among the most frequent contaminants of cereals. Although the acute toxicity of DON is lower than that of type A trichothecenes, its ubiquitous presence in *Fusarium*-infected cereals creates an important food safety issue (2). Acute symptoms of DON ingestion are gastroenteritis and emesis (hence, the colloquial term vomitoxin). Maximum levels for DON in cereal-based foodstuff are set in Commission Regulation 1881/2006 (5), and indicative levels are provided for the sum of T-2 toxin and HT-2 toxin in Commission Recommendation 2013/165/EU (6) in Europe to protect consumers.

The glycosylation of small molecules is a major route to inactivate endogenous and exogenous (xenobiotic) metabolites in plants (7–9). For example, formation of DON-3-*O*-β-D-glucopyranoside (DON-3G) is an important factor in plant defense against FHB and has been proposed to be the molecular basis of the still-unidentified *FHB1* gene (*Fusarium* head blight resistance quantitative trait locus) in wheat (10). DON-3G has been detected in a wide range of cereal commodities, with concentrations of about 20% relative to that of DON (11, 12). However, high regional and seasonal variations have been reported, and in some cases, the content of DON-3G even exceeded that of DON (13, 14). Evidence for glucosylated metabolites of NIV and type A trichothecenes has been presented as well (15–17).

Such glucoconjugates of plant origin have been termed masked mycotoxins (13, 18), implying that they escape detection through

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TABLE 1 Hydrolysis of DON-3G by fungal and bacterial β-glucosidases[d]

Preparation/source	Catalog no.	GH family	Protein in assay (mg ml^{-1})	DON-3G hydrolysis (%)	Hydrolysis measured as:	
					$\mu mol\ h^{-1}\ mg^{-1}$	$\mu mol\ h^{-1}\ ml^{-1}$
Aspergillus niger[a] (*An*Bgl)	E-BGLUC	3	0.19	24	6.9×10^{-3}	5.3×10^{-3}
Agrobacterium sp.[a]	E-BGOSAG	1	0.48	1.1	1.3×10^{-4}	2.4×10^{-4}
Thermotoga maritima[a]	E-BGOSTM	1	0.78	21	1.5×10^{-3}	4.6×10^{-3}
Phanerochaete chrysosporium[a] (*Pc*Bgl)	E-BGOSPC	3	1.1	88	4.3×10^{-3}	1.9×10^{-2}
Glucosidase from almonds[b,c]	G0395		5	Not detectable		
Glucosidase from *A. niger*[b,c]	49291		5	3.3	3.6×10^{-5}	7.3×10^{-4}
Novozyme 188[b]	C6105		Not specified	38		8.4×10^{-3}
Lactobacillus brevis (*Lb*Bgl)		3	1	100	$>5.12 \times 10^{-3}$	$>2.2 \times 10^{-2}$

[a] From Megazyme International (Wicklow, Ireland); concentrations in assays result from the supplied solution.
[b] From Sigma-Aldrich (Vienna, Austria).
[c] Supplied in solid form.
[d] Hydrolysis of DON-3G was performed with a 4-h reaction time, pH 7 (100 mM Tris-Cl), at 37°C.

routine analytical protocols, and reconstitution of the parental toxins through hydrolysis during food processing or in the digestive tract is possible. For example, most food-fermenting lactic acid bacteria possess β-glucosidase activities (19) and may release toxins from glucosylated precursors. DON-3G is known to be resistant to acidic hydrolysis, but it can be cleaved by several intestinal bacterial species, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (20). Evidence that DON-3G is almost completely hydrolyzed in the digestive tract of rats, pigs, and humans has been presented (21–23).

Sensitive analytical methods for direct detection and quantitation of β-glucosides of trichothecene toxins are of high interest but require expensive and sophisticated analytical equipment. Further challenges are discrimination in sample preparation and analysis due to different polarities compared to the parental toxins and the present unavailability of commercial analytical standards, except for DON-3G. Therefore, indirect detection of glucosylated mycotoxins through hydrolysis prior to measurement, preferably through the aid of hydrolytic enzymes, has been suggested as an alternative approach (13, 24). Chemical hydrolysis by super acids has been proposed (25–27) but found unsuitable for this purpose (62). A β-1,3-glucosidase active toward DON-3G was reported recently (24), but this enzyme was not suited for direct application in cereal samples due to strong end product inhibition.

The aim of this study was to identify a β-glucosidase with the capacity to efficiently hydrolyze trichothecene glucosides such as DON-3G in cereal samples. The substrate specificities of several fungal and bacterial glycoside hydrolase family 3 (GH3) members were investigated. Their possible usefulness for indirect analysis of masked mycotoxins was evaluated, and a particularly promising enzyme from *Bifidobacterium adolescentis* was identified.

MATERIALS AND METHODS

Cloning, expression, and purification of β-glucosidases. β-Glucosidase genes were amplified from genomic DNA of *Lactobacillus brevis* DSM 20054 (ATCC 14869) (*Lb*Bgl; GenBank accession number ERK40902.1, locus HMPREF0495_02581) and *Bifidobacterium adolescentis* DSM 20083 (ATCC 15703) (*Ba*Bgl; YP_910057.1; BAD_1194). The oligonucleotide primers used were *Lb*BglF (5′-GATATACATATGGACATCGAACGAACGC-3′), *Lb*BglR (5′-GTGGTGCTCGAGTTGACGTAATAAGGTGTTTGC-3′), *Ba*BglF (5′-GATATACATATGAGCGAAAACACCTATC-3′), and *Ba*BglR (5′-GTGGTGCTCGAGTTCGGCGGTTTCGG-3′). The restriction sites (NdeI and XhoI) used for cloning into the pET21a expression vector (C-terminal His_6 tag; Novagen, Madison, WI) are underlined. *Escherichia coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as the expression host. Protein production was carried out in terrific broth supplemented with ampicillin (100 mg $liter^{-1}$) by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM), which was added at an optical density at 600 nm (OD_{600}) of 0.5. Upon induction, incubation was continued for 16 h at 25°C with shaking (100 rpm).

Enzymes were purified from crude cell extracts on Ni^{2+}-charged chelating Sepharose fast flow (15-ml total volume; column dimensions, 2 cm^2 by 7.5 cm; GE Healthcare, Vienna, Austria) and eluted with imidazole according to the supplier's instructions. Anion exchange chromatography on Source 15Q (20-ml total volume; column dimensions, 2 cm^2 by 10 cm; GE Healthcare) was performed as a second purification step. Proteins were bound to the column in 25 mM Tris-Cl (pH 7.0) and eluted with 1 M NaCl in the same buffer by applying a linear gradient of 10 column volumes. Desalting/buffer change between the purification steps was performed by size-exclusion chromatography on a HiPrep desalting column (5 cm^2 by 10 cm; GE Healthcare). Enzyme activity of obtained fractions was assayed using the *p*-nitrophenol method as described below. The purified enzymes were stored in 25 mM Tris-Cl (pH 7) containing 150 mM NaCl at −80°C. All experiments reported in this study were conducted with the same batches of frozen enzyme(s).

SDS-PAGE with Coomassie blue staining was performed using the Mini-Protean system with precast gels (4 to 20%) from Bio-Rad (Vienna, Austria). The molecular mass marker used was a high-precision, dual-color marker (10- to 250-kDa range; Bio-Rad).

Enzyme assays. Commercial β-glucosidase preparations were obtained from Megazyme (Wicklow, Ireland) and Sigma-Aldrich (Vienna, Austria); a full description of the enzymes used is provided in Table 1. Serial enzyme dilutions were prepared with 25 mM Tris-Cl buffer, pH 7.0, supplemented with bovine serum albumin at 0.1 mg ml^{-1}.

Photometric enzyme assays were performed with chromogenic *p*-nitrophenyl (*p*NP) glycosides obtained from Sigma-Aldrich (Table 2). Standard assay conditions with these substrates were 10 mM substrate concentration, 100 mM Tris-Cl, pH 7.0, 37°C, 5-min reaction time. The assays were stopped by adding a 2-fold volumetric excess of 0.5 M Na_2CO_3. The absorption of released *p*-nitrophenol was measured at 400 nm on a Beckman Coulter DU800 spectrophotometer using a molar extinction coefficient of 18,300 $M^{-1}\ cm^{-1}$. pH dependence was determined with Britton-Robinson buffers ranging from pH 3.0 to 9.5 (28).

Assays with cellobiose, salicin, quercetin-3-β-D-glucoside, and *n*-octyl-β-D-glucoside were performed as described above but stopped by heat inactivation (90°C, 5 min). Enzyme activity toward these substrates was determined by quantifying released glucose through high-performance liquid chromatography (HPLC). Equipment and conditions were a HPLC Summit Dionex with a P680 pump and ASI-100 autosampler (all from Dionex, Sunnyvale, CA). Separation was performed on an Aminex HPX87-K column coupled to a Micro-Guard cation H cartridge (both

TABLE 2 Specific activities of GH3 glycosidases *Lb*Bgl, *Ba*Bgl, *An*Bgl, and *Pc*Bgl for synthetic and natural substrates[a]

Substrate	Concn (mM)	Sp act (μmol min^{-1} mg^{-1})			
		*Lb*Bgl	*Ba*Bgl	*An*Bgl	*Pc*Bgl
*p*NP-β-D-glucopyranoside	10	42 ± 2	49 ± 1	4.2 ± 0.1	22 ± 2
*p*NP-β-D-xylopyranoside	10	5.3 ± 0.1	29 ± 0	ND	ND
*p*NP-α-L-arabinofuranoside	10	0.34 ± 0.01	0.97 ± 0.01	ND	ND
*p*NP-β-D-galactopyranoside	10	ND[c]	0.73 ± 0.02	ND	ND
*p*NP-β-D-mannopyranoside	10	ND	ND	ND	ND
Cellobiose	10	0.71 ± 0.02	0.064 ± 0.001	17 ± 0	0.53 ± 0.01
Salicin	10	36 ± 1	43 ± 0	2.3 ± 0.2	1.3 ± 0.0
Quercetin-3-*O*-β-D-glucopyranoside[b]	0.7	ND	0.075 ± 0.005	ND	ND
n-Octyl-β-D-glucopyranoside	10	10 ± 0	28 ± 0	4.3 ± 0.0	8.4 ± 0.6
Deoxynivalenol-3-*O*-β-D-glucopyranoside	10	0.082 ± 0.003	11 ± 1	0.039 ± 0.002	0.026 ± 0.001
Nivalenol-3-*O*-β-D-glucopyranoside	1	0.0036 ± 0.0001	0.18 ± 0.02	0.0012 ± 0.0002	ND
HT-2-toxin-3-*O*-β-D-glucopyranoside	2	0.017 ± 0.000	3.5 ± 0.1	0.040 ± 0.000	<0.001

[a] Specific activities were determined at 37°C, pH 7.0 (100 mM Tris-Cl). All values represent the mean values from triplicate determinations ± standard deviations.
[b] Dissolved in 80% ethanol (20% in assay).
[c] ND, not detectable.

from Bio-Rad) at a temperature of 80°C. The mobile phase was H_2O with an isocratic flow of 0.5 ml min^{-1} with a 20-μl injection volume. Analytes were monitored with a Shodex RI-100 detector (Showa Denko, Tokyo, Japan). Calibration curves with cellobiose and/or glucose standards were prepared in the range from 0.05 to 5 g liter^{-1}.

DON-3G, nivalenol-3-*O*-β-D-glucopyranoside (NIV-3G), and HT-2-toxin-3-*O*-β-D-glucopyranoside (HT2-3G) were enzymatically prepared with a recombinant family 1 UDP-glucosyltransferase (29), purified by preparative HPLC, and structurally confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) methods (H. Michlmayr, A. Malachova, E. Varga, M. Lemmens, S. Newmister, I. Rayment, F. Berthiller, and G. Adam, unpublished data). Assays with these substrates were performed under the same conditions as those described above (100 mM Tris-Cl, pH 7.0, 37°C) but stopped by transferring 15 μl reaction mix to 135 μl methanol. The samples were centrifuged for 5 min at 20,000 × *g*, further diluted with deionized water, and transferred to HPLC vials. Cereal samples were finely ground in a coffee mill and extracted 1:4 (wt/vol) with 125 mM Tris, pH 7. Beer was degassed and the pH adjusted to 7.0 with 0.1 M KOH. Beer and cereal extracts were spiked with DON-3G at 12.5 mg liter^{-1}, and enzyme assays were done with 80 μl of spiked extracts and 20 μl enzyme solution (final concentration, 10 mg liter^{-1} DON-3G). The assays were stopped and prepared for analysis as described above.

Analysis of glucosides and released toxins by LC-MS. The screening of different enzymes for their hydrolytic activity against DON-3G was performed on an 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Applied Biosystems, Foster City, CA) with atmospheric pressure chemical ionization. The method was based on reference 30 with slight modifications concerning the LC conditions using an Zorbax eclipse XDB-C8 column (150 by 4.6 mm, 5 μm; Agilent Technologies).

For testing the specific activities and obtaining the kinetic constants, the method was transferred to a QTrap 4000 LC-MS/MS system (AB Sciex, Foster City, CA) to gain sensitivity. Furthermore, the following analytes were included in the method: NIV, NIV-3G, HT2, and HT2-3G. Chromatographic separation was achieved on a Gemini C_{18} column (150 by 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) at 25°C with a flow rate of 0.8 ml min^{-1}. The following water-methanol gradient (eluent A, 80:20 [vol/vol]; eluent B, 3:97 [vol/vol]; both containing 5 mM ammonium acetate) was used. Initial conditions at 0% B were a hold for 1 min, followed by a linear increase to 50% B within 5 min and an increase to 100% B within another 3 min. After holding with 100% B for 2.5 min, a fast switch to the initial conditions was performed, followed by column equilibration until 14 min. For the first 6.5 min the mass spectrometer was operated in negative electrospray ionization mode, whereas in the last 7.5 min the positive electrospray ionization mode was used. The following source settings were used: temperature, 550°C; ion spray voltage, 4 kV (positive mode) and −4 kV (negative mode); curtain gas, 30 lb/in^2 (207 kPa of >99% nitrogen); source gas one and two, both 50 lb/in^2 (345 kPa of zero-grade air); and collision gas (nitrogen) set to high. For quantitation, two selected reaction-monitoring transitions per compound were acquired with a dwell time of 25 ms. In the first period, the acetate adducts of the analytes (*m/z* 355.1 for DON, *m/z* 371.1 for NIV, *m/z* 517.3 for DON-3G, and *m/z* 533.1 for NIV-3G) were chosen as precursors, and the declustering potential (DP) was −40 V for DON and NIV, −50 V for DON-3G, and −60 V for NIV-3G. The following product ions were chosen as quantifier and qualifier, respectively: for DON, *m/z* 59.2 (collision energy [CE] of −40 V) and *m/z* 265.2 (CE of −22 V); for NIV, *m/z* 59.1 and 281.1 (CE of −38 V for both); for DON-3G, *m/z* 427.1 (CE of −30 V) and *m/z* 59.1 (CE of −85 V); and for NIV-3G, *m/z* 263.0 (CE of −30 V) and *m/z* 443.0 (CE of −26 V). In the second period, the ammonium adducts of HT2 (*m/z* 442.2; DP, 70 V) and HT2-3-G (*m/z* 604.4; DP, 51 V) were chosen as precursors, and the following product ions were selected: for HT2, *m/z* 215.1 (CE of 19 V) and *m/z* 197.1 (CE of 25 V); for HT2-3G, *m/z* 263.3 (CE of 27 V) and *m/z* 215.1 (CE of 25 V). A sample chromatogram of standards of DON/DON-3G, NIV/NIV-3G, and HT2/HT2-3G is shown in Fig. S1 in the supplemental material.

RESULTS

Substrate specificity and selectivity. Several commercially available β-glucosidase preparations and a previously described GH3 enzyme from *Lactobacillus brevis* (31, 32), here designated *Lb*Bgl, were assayed for their capacity to hydrolyze DON-3G at concentrations of 10 mg liter^{-1} (22 μM) within 4 h. Almond β-glucosidase (Sigma-Aldrich) apparently was inactive toward DON-3G. The highest conversion rates were observed with the β-glucosidases from *Aspergillus niger*, *Phanerochaete chrysosporium*, and *Lb*Bgl. The latter completely hydrolyzed DON-3G under these conditions.

It was previously reported that *B. adolescentis* is able to hydrolyze DON-3G *in vitro* (20). The genome of *B. adolescentis* ATCC 15703 (GenBank accession number NC_008618.1) contains six genes encoding putative GH3 hydrolases. BAD_1194 is the enzyme/gene with the highest amino acid sequence similarity to *Lb*Bgl (58% similarity and 41% identity by BLASTp [33]) and was

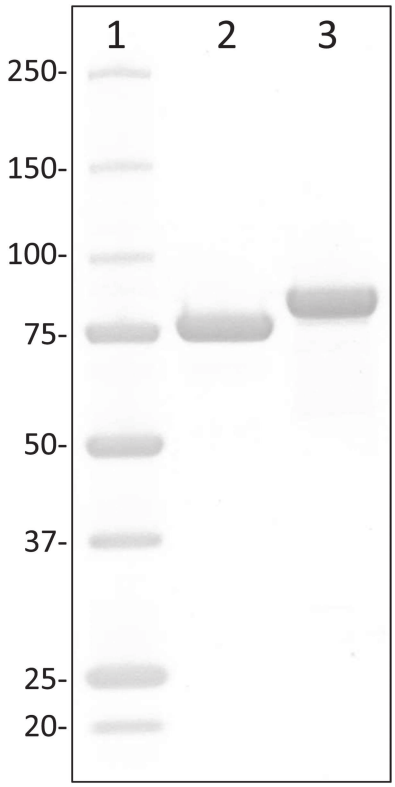

FIG 1 SDS-PAGE of two-step-purified His_6-tagged *Lb*Bgl and *Ba*Bgl with theoretical molecular masses of 83.6 and 82.2 kDa, respectively. Lane 1, Precision Plus protein standard (Bio-Rad), molecular mass in kilodaltons; lane 2, *Lb*Bgl (4 μg); lane 3, *Ba*Bgl (4 μg).

selected for biochemical characterization. This enzyme subsequently is referred to as *Ba*Bgl.

The substrate specificities of recombinant *Lb*Bgl and *Ba*Bgl (purified to apparent homogeneity) (Fig. 1) for several synthetic and natural substrates, including DON-3G and two other trichothecene-toxin β-glucosides available in our laboratory, were determined. The two fungal GH3 enzymes from *A. niger* (*An*Bgl) and *P. chrysosporium* (*Pc*Bgl) that were included in the DON-3G hydrolysis assays described above (Table 1) were characterized analogously to obtain a comparative view on the substrate range of these glycosidases. All assays were performed under conditions (37°C, 100 mM Tris-Cl, pH 7) that do not necessarily reflect the optimal reaction conditions of the individual catalysts. One reason for choosing these conditions was to compromise on a pH value that agrees with most of the commonly used inorganic and organic buffers, which is also relevant in practical terms. Second, all enzyme assays were performed under identical conditions to obtain a comparative view of the catalytic potential of different catalysts.

In assays with *p*NP-glycosides (Table 2), both fungal enzymes solely hydrolyzed *p*NP-β-D-glucopyranoside. The bacterial enzymes displayed additional β-D-xylopyranosidase and lower α-L-arabinofuranosidase side activities, which is consistent with the known possible functionalities of GH3 hydrolases (34). Concerning aglycon specificities, *An*Bgl clearly preferred cellobiose as the substrate and *Pc*Bgl displayed its maximum activities with the synthetic substrates *p*NP-β-D-glucopyranoside and *n*-octyl-β-D-glucopyranoside. Both bacterial enzymes demonstrated poor hydrolytic activity toward cellobiose and differed considerably in their substrate specificities, especially with regard to the trichothecene-β-glucosides. Among the tested enzymes, *Ba*Bgl showed the highest specific activities for DON-3G, NIV-3G, HT2-3G, salicin, quercetin-3-β-D-glucoside, and *n*-octyl-β-D-glucoside (Table 2). Kinetic constants obtained with selected substrates (Table 3) clearly confirm that both bacterial enzymes are highly inefficient with regard to cellobiose hydrolysis. Comparison of the k_{cat}/K_m values obtained with *p*NP-β-glucoside, *p*NP-β-xyloside, and DON-3G are a further indication that *Ba*Bgl is less selective than *Lb*Bgl. Although the affinities of *Lb*Bgl and *Ba*Bgl for DON-3G appear to be in a similar range as judged from apparent K_m values (2.8 and 5.4 mM, respectively) (Table 3), the catalytic efficiency (k_{cat}/K_m) of *Ba*Bgl exceeded that of *Lb*Bgl 80-fold.

Product inhibition and physicochemical characterization. Compared to the *Aspergillus* cellobiase *An*Bgl, the bacterial enzymes *Lb*Bgl and *Ba*Bgl were moderately inhibited by glucose (Fig. 2A): 50% activity reduction was estimated at 11 mM, 67 mM, and 180 mM glucose for *An*Bgl, *Lb*Bgl, and *Ba*Bgl, respectively. At 12 mM DON (3.5 g $liter^{-1}$), a concentration high above the DON levels that can be expected in contaminated cereal samples (milligram per liter range), DON did not appear to exert an inhibitory

TABLE 3 Kinetic constants of the GH3 glycosidases from *Lb*Bgl and *Ba*Bgl[a]

Enzyme and substrate	Kinetic constant			
	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}[b] (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
*Lb*Bgl				
*p*NP-β-D-glucopyranoside	0.63 ± 0.09	47 ± 1	66	104
*p*NP-β-D-xylopyranoside	2.6 ± 0.2	6.6 ± 0.2	9.3	3.5
Deoxynivalenol-3-*O*-β-D-glucopyranoside	2.8 ± 0.4	0.11 ± 0.00	0.15	0.053
Cellobiose	63 ± 4	5.3 ± 0.2	7.4	0.12
*Ba*Bgl				
*p*NP-β-D-glucopyranoside	1.1 ± 0.1	68 ± 2	94	87
*p*NP-β-D-xylopyranoside	4.2 ± 0.3	39 ± 1	53	13
Deoxynivalenol-3-*O*-β-D-glucopyranoside	5.4 ± 0.5	16 ± 1	22	4.0
Cellobiose	73 ± 12	0.50 ± 0.05	0.69	0.0094

[a] Kinetic constants from *Lb*Bgl and *Ba*Bgl were determined at 37°C, pH 7.0 (100 mM Tris-Cl).
[b] Calculations are based on the theoretical molecular mass of the His_6-tagged proteins: *Lb*Bgl, 83.6 kDa; *Ba*Bgl, 82.2 kDa.

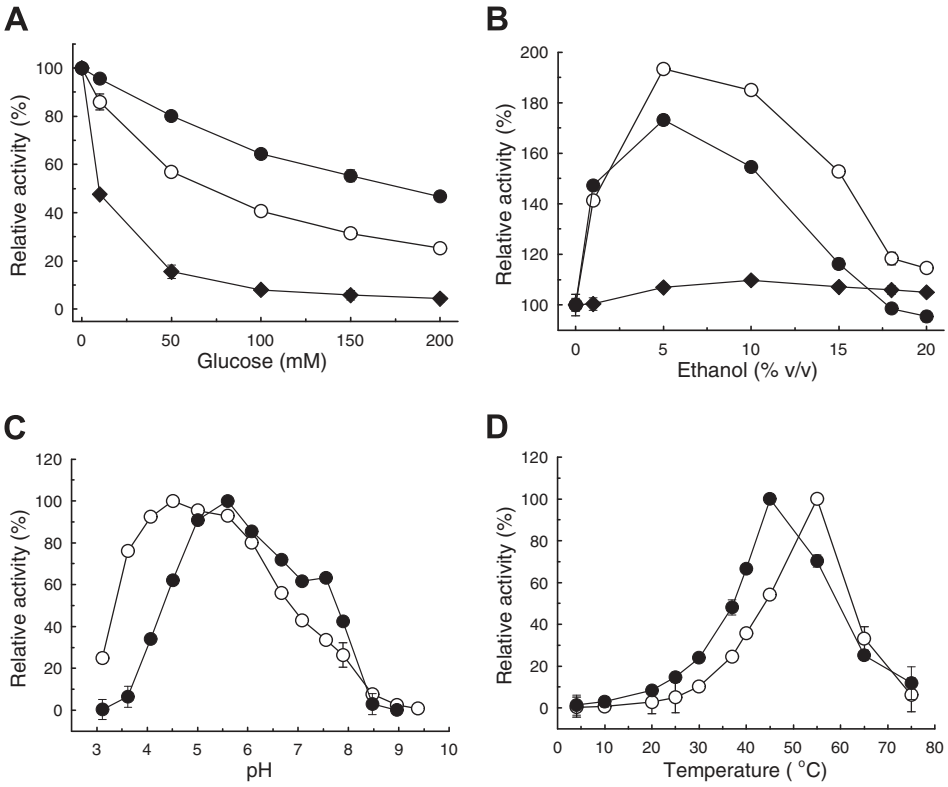

FIG 2 Influence of glucose (A) and ethanol (B) on activities of *Lb*Bgl (○), *Ba*Bgl (●), and *An*Bgl (◆) in the standard *p*NP-β-glucopyranoside assay (37°C, pH 7). pH (C) and temperature (D) dependence of *Lb*Bgl (○) and *Ba*Bgl (●). (C) Assays (37°C) performed with Britton-Robinson buffers (28) in the range of pH 3.0 to 9.5. (D) Standard assay (100 mM Tris, pH 7) performed at 4 to 75°C. All data represent the averages from triplicate determinations, and error bars indicate standard deviations. One hundred percent relative activity refers to standard assay conditions without additive (A and B) or maximum activity (C and D).

effect on *p*NP-β-D-glucoside hydrolysis by *Lb*Bgl and *Ba*Bgl (see Table S1 in the supplemental material). Increased activity in the presence of ethanol (<20% vol/vol) was observed with *Lb*Bgl and *Ba*Bgl but not with *An*Bgl (Fig. 2B).

The pH (Fig. 2C) and temperature (Fig. 2D) profiles of *Lb*Bgl and *Ba*Bgl indicate maximum hydrolytic activities in the range of pH 5 to 6 and 50 to 60°C. Inclusion of EDTA and salts of several mono- and divalent metal ions implied that *Lb*Bgl and *Ba*Bgl do not depend on metal ions for hydrolysis (see Table S1 in the supplemental material). Both enzymes were active in several commonly used organic and inorganic buffers (Table 4). At pH 7, highest activities were determined in citrate-phosphate buffer prepared according to McIlvaine (35), and the lowest activities were recorded in Tris-Cl buffer, which was used as the standard assay buffer in this study.

DON-3G hydrolysis in cereal samples. Judged from the catalytic efficiency with DON-3G and low product inhibition, the results described above indicated that *Ba*Bgl is an interesting candidate for the hydrolysis of DON-3G and possibly structurally similar trichothecene-glucosides in cereal samples. As a proof of concept, extracts of several cereal samples and degassed beer were spiked with DON-3G (10 mg liter^{-1}) to test the performance of *Ba*Bgl in realistic sample matrices. A naturally contaminated barley sample containing both DON and DON-3G was included as well (Table 5). *Ba*Bgl efficiently hydrolyzed DON-3G in all sample matrices (Table 5), as judged from the release of DON and the fact that DON-3G was not detectable after 15 min in all samples. While in beer DON-3G still was detectable, its levels were already below the limit of quantification (Table 5). Furthermore, NIV-3G and HT2-3G (both at 10 mg liter^{-1}) were completely hydrolyzed by *Ba*Bgl within 15 min in spiked wheat extract.

Sequence comparison of GH3 hydrolases. GH3 is a large and divergent enzyme family (at the time of writing, >6,000 sequences are in CAZy [34]) with considerable differences in known domain structures and arrangements (36). Figure 3 illustrates the relation-

TABLE 4 Relative activities of *Lb*Bgl and *Ba*Bgl in organic and inorganic buffers at pH 7[d]

Buffer	Relative enzyme activity (%)	
	*Lb*Bgl	*Ba*Bgl
Tris (100 mM)	100 ± 0	100 ± 2
McIlvaine[a]	158 ± 2	174 ± 1
Phosphate buffer (100 mM)	127 ± 2	143 ± 1
MOPS[b] (100 mM)	163 ± 2	158 ± 2
HEPES (100 mM)	145 ± 0	132 ± 1
Succinic acid (100 mM)	124 ± 2	135 ± 2
Britton-Robinson buffer[c]	136 ± 1	141 ± 1

[a] Citrate-phosphate buffer according to reference 35.
[b] Morpholinepropanesulfonic acid.
[c] Described in reference 28.
[d] Relative activities of *Lb*Bgl and *Ba*Bgl in organic and inorganic buffers at pH 7 were determined with *p*NP-β-D-glucopyranoside at 37°C. All values represent the means from triplicate determinations ± standard deviations.

TABLE 5 Hydrolysis of DON-3G in aqueous cereal sample extracts

Time (min)	Hydrolysis (mM) in extract of[a]:									
	Malt		Wheat		Rice		Beer[b]		Barley[c]	
	DON-3G	DON	DON-3G	DON	DON-3G	DON	DON-3G	DON	DON-3G	DON
0	23	ND	22	ND	22	ND	23	ND	15	39
5	ND	21	ND	21	<2.2	18	7.8	13	ND	50
10	ND	21	ND	20	ND	19	<2.2	19	ND	50
15	ND	23	ND	21	ND	20	<2.2	19	ND	49

[a] Hydrolysis of DON-3G by *Ba*Bgl (0.8 mg ml^{-1}) to DON in aqueous (100 mM Tris-Cl, pH 7) cereal sample extracts (1:5, wt/vol) spiked with 10 mg $liter^{-1}$ (22 μM) DON-3G. All values represent the means from triplicate determination, and concentrations are displayed in millimolars. ND, not detectable (signal-to-noise ratio below 3:1).
[b] Degassed and adjusted to pH 7 with 0.1 M KOH.
[c] Naturally contaminated sample. Concentrations refer to dry sample (concentration in assay, 1:5).

ships between the six putative GH3 enzymes encoded by the *B. adolescentis* genome relative to other GH3 hydrolases with known function and resolved crystal structure. BAD_0640 is a putative *N*-acetyl-β-D-glucosaminidase, and BAD_1197 could be related to GH3 xylosidases. BAD_1610 and BAD_1611 seem to belong to a different GH3 subclass with reversed domain arrangement. Together with BAD_1598 and *Lb*Bgl, *Ba*Bgl (BAD_1194) appears similar to β-glucosidase *Tn*Bgl3B from *Thermotoga neapolitana* (Fig. 3). *Tn*Bgl3B (37) consists of three domains, and overall sequence similarities suggest that *Lb*Bgl (36% identity, 97% sequence coverage) and *Ba*Bgl (37% identity, 99% coverage) are structured in a similar fashion. The architecture of *Tn*Bgl3 com-

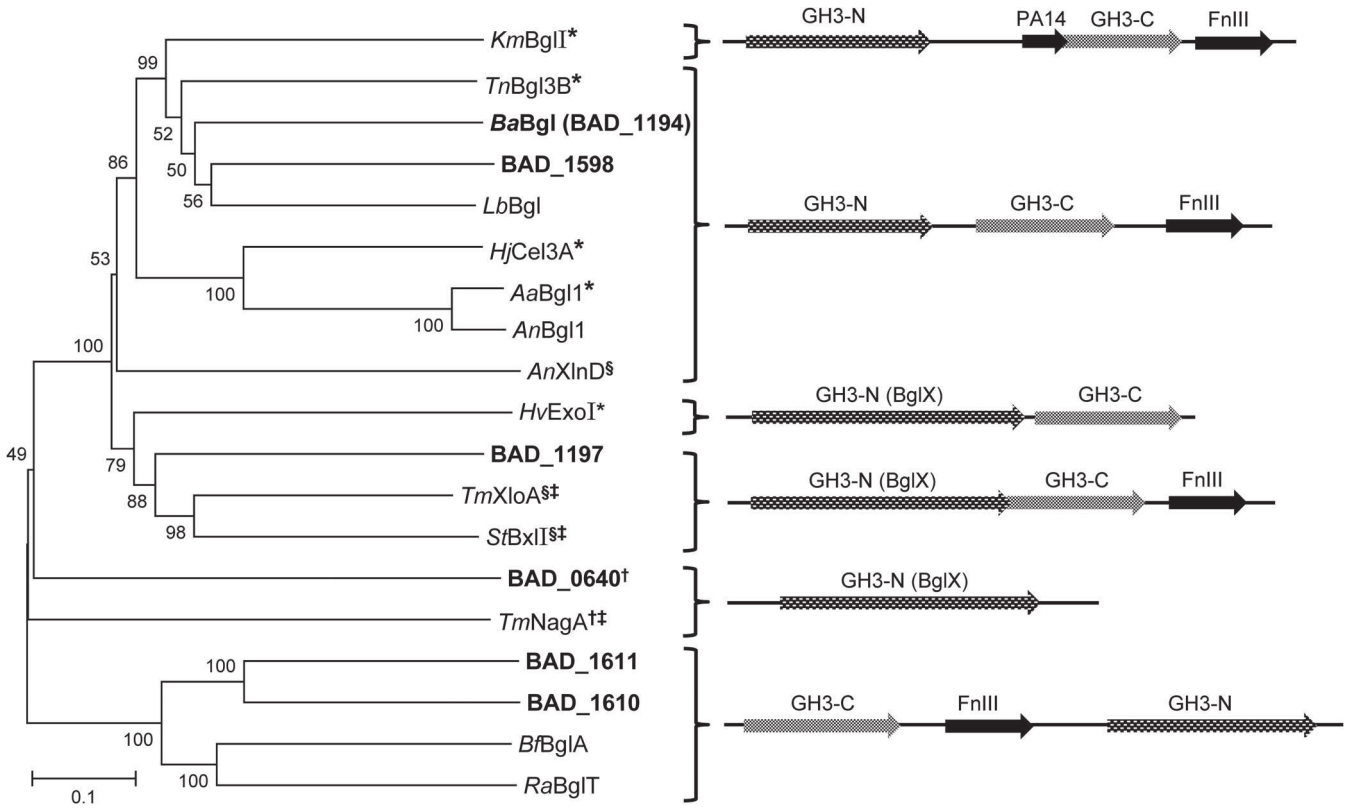

FIG 3 Neighbor-joining tree of putative and experimentally confirmed GH3 β-glucosidases and related enzymes. §, β-xylosidase; †, *N*-acetyl-β-D-glucosaminidase; *, three-dimensional structure is available from the references listed below; ‡, crystal structure not yet published (www.cazy.org). Information on domain organization as schematically depicted on the right is derived from the available crystal structures and by sequence alignments: GH3-N, conserved N-terminal GH3 domain (pfam00933); GH3-C, C-terminal GH3 domain (pfam01915); FnIII, fibronectin type III-like domain (pfam14310); PA14 domain (pfam07691); and BglX, glucosidase related hydrolases (COG1472). Sequences of *Bifidobacterium adolescentis* (NC_008618.1, loci BAD_0640, BAD_1194 [*Ba*Bgl], BAD_1197, BAD_1598, BAD_1610, and BAD_1611) are highlighted in boldface. *Km*BglI (*Kluyveromyces marxianus*, PDB entry 3AC0, reference 39), *Tn*Bgl3B (*Thermotoga neapolitana*, PDB entry 2X40, reference 37), *Lb*BglI (*Lactobacillus brevis*, ERK40902.1), *Hj*Cel3A (*Hypocrea jecorina*, anamorph of *Trichoderma reesei*, PDB entry 3ZYZ, reference 40), *Aa*BglI (*Aspergillus aculeatus*, PDB entry 4IIB, reference 38), *An*BglI (*Aspergillus niger*, CAB75696.1, reference 56), *An*XlnD (*A. niger*, CAB06417.1 reference 57), *Hv*ExoI (*Hordeum vulgare*, PDB entry 1EX1, reference 58), *Tm*XloA (*Thermotoga maritima*, AAD35170.1), *St*BxlI (*Streptomyces thermoviolaceus*, BAD02389.1, reference 59), *Tm*NagA (*T. maritima*, AAD35891.1), *Bf*BglA (*Butyrivibrio fibrisolvens*, P16084.1, references 36 and 60), and *Ra*BglT (*Ruminococcus albus*, CAA33461.1, references 36 and 61). Amino acid sequence alignment and phylogenetic analysis was performed with Mega 6. The Muscle algorithm was used for alignment, and the tree was constructed with the p-distance model and 1,000 bootstrap iterations.

prises an N-terminal TIM barrel-like $(\alpha/\beta)_8$ fold (domain 1, GH3 N-terminal domain; pfam00933) followed by a α/β sandwich fold (domain 2, GH3 C-terminal domain; pfam01915). Both domain structures are conserved within GH3, and the substrate binding pocket of *Tn*Bgl3B is located at their interface. Domain one contains the catalytic nucleophile (D242 in *Tn*Bgl3), and several conserved residues that have been identified as pivotal for glycon (β-D-glucopyranosyl-) binding and orientation. The acid/base catalytic residue (E458) is located on a less conserved region on domain two (37) (see Fig. S1 in the supplemental material). C-terminal domain three of *Tn*Bgl3B is a fibronectin type III-like fold (FnIII; pfam14310). This domain often is present in GH3 hydrolases (Fig. 3), and it also has been identified in eukaryotic GH3 β-glucosidases (38–40), but its function is unknown to date.

Figure S2 in the supplemental material (residue numbering refers to that of the *Tn*Bgl3B sequence) highlights the conserved amino acid residues identified in GH3 β-glucosidases (37, 40) compared to three GH3 β-xylosidase sequences. Notable differences are that D58 and W243 appear not to be conserved in GH3 xylosidases. Both residues have been shown to be crucial for glucose accommodation in the glycon binding subsite of *Tn*Bgl3B.

DISCUSSION

In addition to the primarily analytical objective of this study, the substrate specificities of two bacterial and two fungal GH3 hydrolases were of interest. While the two fungal GH3 enzymes (*A. niger* and *P. chrysosporium*) appear to be specific β-glucosidases, *Lb*Bgl and *Ba*Bgl possess considerable β-D-xylosidase and low α-L-arabinofuranosidase side activities. Glycon recognition motifs usually are highly conserved within GH families, yet the mechanistic details determining β-glucosidase or β-xylosidase activity in GH3 so far have not been clarified sufficiently. Sequence alignment of *Lb*Bgl and *Ba*Bgl (see Fig. S2 in the supplemental material) does not indicate differences in conserved glucose binding residues compared to other GH3 β-glucosidases. However, it was reported that aglycon binding also can have an effect on glycon orientation (41), and the side activities of *Lb*Bgl and *Ba*Bgl could be related to their broad aglycon specificities.

In contrast to the conserved glycon recognition motifs, the aglycon binding sites of glycosidases usually are much less defined. Aglycon accommodation at the active site is determined mainly by hydrophobic interactions, which allows for additional freedom in substrate positioning (42, 43). While this accounts for flexibility, it currently is not possible to predict aglycon specificities solely based on sequence analyses. Understanding the biochemical functionality of such β-glucosidases requires evaluation of a broad range of possible substrates which often is limited by unavailability or poor solubility of analytes of interest. It is further possible that some enzyme classes have been evolutionarily tailored to be versatile. Therefore, the quest to identify the true substrate (i.e., thermodynamically ideal substrate in terms of affinity or catalytic efficiency) may not always yield the best results, especially with regard to a possible physiological function. Thus, the true substrate of *Ba*Bgl most likely has not been identified in this study. DON-3G hydrolysis by *Ba*Bgl appears to be caused by its unselective nature rather than by a particular preference for this structure, which is also reflected in the relatively high K_m value (5.4 mM for DON-3G). This stands in contrast to a 1,3-β-glucanase (catalog no. L9259 [product discontinued]; Sigma-Aldrich) recently reported to possess surprisingly high affinity (K_m of 4.5 μM) for DON-3G (24). However, this enzyme was almost completely inhibited by glucose at low concentrations (10 μM). Consequently, inconvenient sample cleanup was necessary to eliminate glucose originally present in the sample prior to enzymatic treatment.

*Ba*Bgl exerted adequate hydrolytic activity with DON-3G, NIV-3G, and HT2-3G and was able to hydrolyze these compounds in cereal samples as well. Its unspecific nature suggests that *Ba*Bgl or functionally related glycosidases are able to hydrolyze an even wider spectrum of masked mycotoxins. Although DON is the most frequently occurring trichothecene toxin worldwide (44), regional and seasonal variations of *F. graminearum* chemotypes and of other *Fusarium* species (45, 46) imply that occurrences of different trichothecenes and their (putative) β-glucosides underlie high geographic and seasonal fluctuations. For example, nivalenol is more prevalent in Asian countries (47), and high incidences of T-2 toxin and HT-2 toxin have been reported recently in northern European countries (48); *F. graminearum* strains isolated in the United States can produce a previously unknown type A trichothecene (NX-3) (49).

Practical applications of *Ba*Bgl may involve hydrolysis of glucosylated trichothecene toxins in aqueous cereal extracts prior to analysis. In principle, this would be of interest for immunological detection methods, such as enzyme-linked immunosorbent assay (ELISA) kits that are widely used for rapid estimation of DON levels in cereals. However, the utility of this approach is limited by the high cross-reactivities of DON-specific antibodies with DON-3G, which have been reported to range from 52 to 157% (50, 51). For example, assuming a cross-reactivity of 50%, the increase of the signal intensity due to the enzymatic cleavage of DON-3G to DON would be visible only if more than 20% DON-3G compared to the level of DON on a molar basis is present in the sample. The reason for this is the repeatability of ELISA methods (typically 10 to 20% relative standard deviation [RSD]), which would make it impossible to distinguish between the DON content before and after enzymatic hydrolysis. A more suitable application could be the hydrolysis of glucoconjugated trichothecenes prior to analysis by conventional HPLC and gas chromatography methods, which show better repeatabilities (often 5 to 10% RSD). This should reveal an increase in the parental toxin content equivalent to the molar concentration of the masked compound.

As a final remark, it is worthwhile to note that previous studies investigating glycoside hydrolase activities of bifidobacteria were concerned mainly with their positive implications. Examples include the metabolism of prebiotics (52) and a considerable number of publications on the release of isoflavones (phytoestrogens) and other potentially health-beneficial plant metabolites from their β-glucoside precursors (for examples, see references 53–55). One aim of this study was to point out the possible functional and structural diversity of (bacterial) GH3 β-glucosidases. By considering this and the high number of putative GH3 genes reported in individual *Bifidobacterium* genomes (52), it appears that a wide range of β-glucosidase functionalities in these bacteria can be expected. Therefore, it is crucial to consider that such intestinal species possess the capability to increase the bioavailability not only of health-beneficial plant metabolites but also of masked dietary toxins.

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This work is dedicated to the memory of Klaus D. Kulbe.

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APPENDIX VII

Malachova A, Varga E, Schwartz-Zimmermann H, Berthiller F

Analytical strategies for the determination of deoxynivalenol in its modified forms in beer: A mini Review

Kvasny Prumysl, 61 (2), 46–50 (2015)

Analytical Strategies for the Determination of Deoxynivalenol and its Modified Forms in Beer: A Mini Review

Analytické postupy stanovení deoxynivalenolu a jeho derivátů v pivu. Minireview

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Reviewed Paper / *Recenzovaný článek*

Malachová, A. – Varga, E. – Schwartz-Zimmermann, H. E. – Berthiller, F.: Analytical strategies for the determination of deoxynivalenol and its modified forms in beer: A mini review. Kvasny Prum. 61, 2015, No. 2, pp. 46–50

The aim of this review is to provide a brief overview of analytical methods used for the determination of deoxynivalenol and its modified forms deoxynivalenol-3-β-D-glucoside, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in beer. The analytical methods discussed involve gas chromatography coupled with flame ionization detection, electron capture detection and mass spectrometry as well as liquid chromatography hyphenated to ultra-violet detection and mass spectrometry. Special attention was paid to sample preparation. Immunochemical methods such as enzyme-linked immunosorbent assays (ELISAs) which represent efficient tools for fast screening of beer with no sample purification are also discussed.

Malachová, A. – Varga, E. – Schwartz-Zimmermann, H. E. – Berthiller, F.: Analytické postupy stanovení deoxynivalenolu a jeho derivátů v pivu. Minireview. Kvasny Prum. 61, 2015, č. 2, s. 46–50

Přehledové sdělení shrnuje stručný přehled analytických metod používaných pro stanovení deoxynivalenolu a jeho derivátů deoxynivalenol-3-β-D-glukosidu, 3-acetyldeoxynivalenolu a 15-acetyldeoxynivalenolu v pivu. Teprve nedávno byly v pivu identifikovány také oligoglykosidy deoxynivalenolu. Mezi analytickými metodami jsou uvedeny jak plynová chromatografie ve spojení s plamenoionizační detekcí, detekcí elektronového záchytu či hmotnostní spektrometrií, tak kapalinová chromatografie se spektrofotometrickou detekcí, a v neposlední řadě také kapalinová chromatografie ve spojení s hmotnostní spektrometrií. Diskuse je převážně zaměřena na přípravu vzorků před vlastní analýzou. Závěrečná část článku je věnována imunochemickým metodám. Nejrozšířenější imunochemickou metodou je tzv. ELISA (Enzyme-Linked Immunosorbent Assay), která představuje velice efektivní nástroj pro rychlou analýzu díky své jednoduchosti a přímé aplikaci tekutých vzorků bez předchozího přečištění.

Malachová, A. – Varga, E. – Schwartz-Zimmermann, H. E. – Berthiller, F.: Analytische Methoden für die Bestimmung von Deoxynivalenol und dessen modifizierten Formen in Bier. Kvasny Prum. 61, 2015, Nr. 2, S. 46–50

Dieser Review bietet einen kurzen Überblick über analytische Methoden für die Bestimmung von Deoxynivalenol und dessen modifizierten Formen Deoxynivalenol-3-β-D-Glukosid, 3-Acetyl-Deoxynivalenol und 15-Acetyl-Deoxynivalenol in Bier. Die verwendeten Methoden umfassen Gaschromatografie in Verbindung mit Flammenionisationsdetektion, Elektroneneinfangdetektion und Massenspektrometrie, sowie Flüssigkeitschromatografie mit Spektrophotometrischer Detektion oder Massenspektrometrie. Besonderes Augenmerk wurde auf die Probenvorbereitung gelegt. Immunochemische Methoden, wie z.B. enzymgekoppelte Immunadsorptionstests (ELISA), die eine effektive Möglichkeit für das Screening von Bier ohne Probenaufreinigung darstellen, werden ebenfalls diskutiert.

Keywords: *DON, deoxynivalenol-3-glucoside, masked mycotoxins, HPLC-MS, GC-MS, ELISA*
Klíčová slova: *DON, deoxynivalenol-3-3-glukosid, maskované mykotoxiny, HPLC-MS, GC-MS, ELISA*

1 INTRODUCTION

Deoxynivalenol (DON) is a naturally occurring toxic secondary metabolite, predominantly produced by *Fusarium* species. The main producers of DON are *F. graminearum* and *F. culmorum*. They are important plant pathogens invading crops already on the field and the associated disease Fusarium head blight is characterized by pre-mature bleaching of wheat ears (EFSA, 2013). As a consequence, high economic losses due to lower yields of crops and a frequent DON contamination of wheat, barley, oat, rye and maize have been reported worldwide (Edwards, 2004). Fusarium infected grains pose a serious problem during food processing such as beer gushing or fermentation inhibition in bread making (Champeil et al., 2004). Moreover, DON was found to be stable under food processing conditions. Thus, it can be passed on from raw cereals into the final products (Bullerman and Bianchini, 2007; Hazel and Patel, 2004). The effects of food processing on mycotoxin levels in final products have become more important since the existence of so-called "masked mycotoxins" was confirmed. Masked mycotoxins are formed in plants as a defense against the parent toxin's deleterious effects. This detoxification process involves chemical modification of the parent toxin by a linkage to small polar molecules (sugars, amino acids, sulfate), leading to a decreased toxicity for plants. Masked mycotoxins are either stored in vacuoles or further incorporated (bound) into macromolecules (Berthiller et al., 2013). The major bio-transformation pathway of DON is the conjunction with glucose leading to formation of deoxynivalenol-3-β-D-glucoside (D3G) (Berthiller et al., 2009). So far, D3G has been found along with DON in wheat, maize, and barley, as reviewed by Berthiller et al. (2013). The first studies dealing with the effects of food processing on D3G revealed that alike DON, D3G can also be transferred into the final products, beer and bread. (Lancová et al., 2008, Kostelanská et al., 2011). Moreover, it was revealed that D3G is likely to be further released from cell structures during malting and brewing because its levels in beer were increased compared to the used raw material (Lancová et al., 2008). Some recent survey studies on commercially available beers confirmed that D3G is a common contaminant in this beverage. The incidence of D3G was even higher than DON (Kostelanská et al., 2009; Varga et al., 2013). The average levels of D3G and DON in contaminated beer reported by Varga et al. (2013) were 9.5 µg/l and 13.6 µg/l, respectively. Beside D3G also acetylated forms of DON, 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), might occur in beer. They are the fungal precursor of deoxynivalenol and are also formed as intermediates of the detoxification of DON in plants. However, their incidence in beer is very low (Kostelanská et al., 2009; Varga et al., 2013). Although DON belongs to the mycotoxins for which maximum levels are regulated by the European Commission (EC 1881/2006; EC 1126/2007), no limit for DON in beer has been established so far. Recently, it was revealed in various animal species that 3ADON and 15ADON are hydrolyzed to DON *in vivo*. Hence, their toxicity is similar to that of DON (reviewed by Wu et al., 2010). Therefore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a group provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg body weight (b.w.) for the sum of DON, 3ADON and 15ADON (JECFA, 2011). The bioavailability of D3G is a subject of ongoing research. The latest studies on rats and pigs indicated that D3G partly contributes to the total DON intake (Nagl et al., 2012; Nagl et al., 2014). Authors of another study dealing with the bioavailability of masked mycotoxins confirmed that DON conjugates were effectively deconjugated by the human colonic microbiota releasing parent DON (Dall'Erta et al., 2013). Due to a high incidence of D3G

Table 1: Overview of some instrumental analytical methods for determination of deoxynivalenol (DON) in beer

Reference	Method	Ionization	Clean-up	LOD (µg/l)	LOQ (µg/l)[a]	SSE (%)	Recovery (%)	RSD (%)	Number of analytes in the method	Other analytes in the method
Scott et al., 1993	GC-MS	EI	SPE	0.1–1.5	n.e.	n.e.	58–125	n.e.	5	NIV, ZEA, a-ZEL, b-ZEL
Schothorst and Jekel, 2003	GC-FID		SPE	8	25	n.e.	92	3.2	8	TRICH
Bertuzzi et al., 2011	GC-MS	EI	SPE	0.5	1.5	n.e.	92	2.4	13	TRICH, AFs, FBs, OTA
Balajová and Rauová, 2008	HPLC-UV		IAC	6	7	n.e.	90–105	11.4	1	
Anselme et al., 2006	HPLC-UV		IAC	2	6	n.e.	n.e.	n.e.	1	
Omurtag and Beyoğlu, 2007	HPLC-UV		SPE	125	625	n.e.	n.e.	n.e.	1	
Lancová et al., 2008	HPLC-MS/MS	APCI	LLP, SPE	1	5	n.e.	117	>10	8	TRICH, D3G
Malachová et al., 2012	HPLC-MS/MS	ESI	LLP	1.0–2.9	3.0–11.0	n.e.	60–90	5–12	3	D3G, 3ADON
Romero-Gonzáles et al., 2009	UHPLC-MS/MS	ESI	SPE	0.14	0.45	55–162[b]	74	8.7	12	TRICH, FBs, AFs, OTA, ZEA
Tamura et al., 2011	UHPLC-MS/MS	ESI	QuEChERS, SPE	n.e.	5	n.e.	94	5.5	15	TRICH, ZEA, PAT, AFs, FBs, OTA
Al-Taher et al., 2012	UHPLC-MS/MS	ESI	no	10	50	61–89	n.e.	4.27–14.16	11	TRICH, ZEA, FBs, AFs, OTA
Zachariášová et al., 2010	UHPLC-HRMS	APCI	LLP	n.e.	3	n.e.	93–104	4.9	32	D3G, TRICH, ZEA, FBs, ENN, BEA, AFs, AT, EA, OTA, STER
Rubert et al., 2011	HPLC-HRMS	ESI	SPE	16–20	48–60	83–105	78–97	11	18	TRICH, FBs, AFs, OTA, BEA

[a] LOQ=LCL (lowest calibration level) in HRMS methods; [b] SSE evaluated on three types of beer, validation done only for pale beer; n.e.-not evaluated
3ADON-3-acetyl-deoxynivalenol; AFs-aflatoxins; a-ZEL-a-zearalenol; APCI-atmospheric pressure chemical ionization; AT-Alternaria toxins; BEA-beauvericin; b-ZEL-b-zearalenol; D3G deoxynivalenol-3-β-D-glucoside; EA-ergot alkaloids; EI-electron impact ionization; ENN-enniatins; ESI-electrospray ionization; FBs-fumonisins; FID-flame ionization detection; GC-gas chromatography; HPLC--high performance liquid chromatography; HRMS-high resolution mass spectrometry; IAC-immunoaffinity chromatography; LLP-liquid-liquid partitioning; LOD-limit of detection; LOQ-limit of quantification; MS-mass spectrometry; MS/MS-tandem mass spectrometry; NIV-nivalenol; OTA-ochratoxin A; PAT-patulin; QuEChERS-Quick, Easy, Cheap, Effective, Rugged, Safe; RSD-repeatability; SPE-solid phase extraction; SSE-signal suppression/enhancement; STER-sterigmatocystin; TRICH-trichothecenes; UHPLC-ultra high performance liquid chromatography; UV-ultra violet detection; ZEA-zearalenone

Table 2: Performance characteristics of the analytical methods for deoxynivalenol-3-β-D-glucoside (D3G), 3-acety-ldeoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON)

Reference	D3G					3ADON (15ADON)				
	LOD (µg/l)	LOQ (µg/l)	Recovery (%)	SSE (%)	RSD (%)	LOD (µg/l)	LOQ (µg/l)	Recovery (%)[a]	SSE (%)	RSD (%)
Schothorst and Jekel, 2003	not reported	not reported	not reported	not reported	not reported	8	25	102[b]	n.e.	2.7
Bertuzzi et al., 2011	not reported	not reported	not reported	not reported	not reported	0,5 (1)	1.4 (2.8)	90 (90)[b]	n.e.	2.3 (2.6)
Lancová et al., 2008	1.0	5.0	102	n.e.	<10	5.0	10.0	116	n.e.	<10
Malachová et al., 2012	0.4–1.4	1.3–4.1	42–66[b]	n.e.	6–16	2.2–4.3	6.8–11	96–109[b]	n.e.	4–14
Zachariášová et al., 2010	n.e.	2–3[a]	96–111		6.3		8–18[a]	99–112		14
Rubert et al., 2011	not reported	not reported	not reported	not reported	not reported	25–30	75–90	80–101	101–107	12

[a] LOQ=LCL (lowest calibration level) in HRMS methods; [b] Apparent recovery evaluated (extraction recovery and SSE is included in the value); n.e. – not evaluated
LOD-limit of detection; LOQ-limit of quantification; SSE-signal suppression/enhancement; RSD-repeatability

in cereals and cereal-based products and preliminary evidence about its bioavailability, the European Food Safety Authority (EFSA) has initiated the work on evaluation of human and animal risks related to DON, metabolites of DON and masked DON (EFSA, 2014). On this account, more occurrence data about DON and its conjugated forms are requested.

In this context this mini-review focuses on the analytical methods used for the determination of DON in beer with an emphasis on sample preparation techniques. In addition, analytical approaches for the determination of D3G, acetylated DONs and other masked DON--metabolites in beer are discussed.

2 ANALYTICAL METHODS FOR THE DETERMINATION OF DON IN BEER

In general, analytical approaches to determine DON in beer do not differ from procedures applied to cereals. Beer might be considered as an easy matrix for analysis in comparison to cereals because DON is already homogenously dissolved in liquid. Therefore, in principle, direct analysis of beer could be an option. However, several difficulties have to be taken into account in beer analysis. Beer is a complex matrix containing a wide range of low molecular compounds of various structural classes with different polarities which might interfere with the analysis of target compounds (Zachariášová et al., 2010). In addition, the levels of DON (and also other mycotoxins) in beer are approximately ten times lower compared to raw cereals. Therefore, a suitable analytical method has to be capable of detecting low levels (< 5 µg/l for DON).

2.1 Instrumental analytical methods

Until recently, analytical methodology for the determination of DON in beer relied predominantly on gas chromatography (GC) coupled either with conventional detection such as electron capture detector (ECD) (Molto et al., 2000), flame ionization detector (FID) (Schothorst and Jekel, 2003) or mass spectrometry (MS) (Scott et al., 1993, Bertuzzi et al., 2011). Liquid chromatography hyphenated to spectrophotometric detection (LC-UV) has been used to a lesser extent (Anselme et al., 2006; Balajová and Rauová, 2008; Omurtag and Beyoğlu, 2007). Current trends in mycotoxin determination led to the development of fast, highly efficient and accurate analytical methods based on liquid chromatography coupled with mass spectrometry (HPLC-MS) (Romero-Gonzáles et al., 2009; Tamura et al., 2011; Malachová et al., 2012). An overview of some analytical methods used for the analysis of beer is provided in Table 1.

2.1.1 Gas chromatography

For several decades, GC used to be the first choice in trichothecene determination. However, polar, non-volatile compounds such as DON require a derivatization step prior to GC separation. In addition, extensive clean-up is important in order to remove matrix components reacting with the derivatization agent, interfering in final analysis or both. A two-step clean-up first on a cartridge with a hydrophilic stationary phase, then on a C18-cartridge was employed in a GC-MS method for the determination of DON along with nivalenol (NIV), zearalenone (ZEA), α-zearalenol and β-zearalenol in beer (Scott et al., 1993). In that study it was shown that heptafluorobutyric (HFB) derivatization resulted in a better sensitivity than derivatization with trimethylsilylation (TMS). The validated method was finally applied to the analysis of 50 beer samples. Due to the high sensitivity of the method, DON was detected in the range of 0.33 µg/l to 50.3 µg/l. In 2003, a combination of two multifunctional cartridges MycoSep®227 and MycoSep®216 instead of C18 was used for the purification of beer prior to TMS derivatization followed by GC-FID (Schothorst and Jekel, 2003). Likely due to poor sensitivity (limit of quantification – LOQ of 25 µg/l), in only three out of 21 beers DON could be determined. In another study, multifunctional columns Trichothecene EP were used for beer purification and TMS-derivates were detected by GC-MS with a quantitation limit of 1.5 µg/l for DON (Bertuzzi et al., 2011). In 2000, a GC-ECD method achieving an LOQ of 2 µg/l for DON was used for the trichothecene occurrence survey of Argentinian beers (Molto et al., 2000). In that study, the sample preparation protocol of Scott et al. (1993) described above was employed. Concerning the modified forms of DON, an advantage of GC technique is the baseline separation of 3ADON and 15ADON which is tricky in reversed-phase LC. Some of the above described methods included also acetylated DONs, detailed method specifications for these conjugates are summarized in Table 2. For instance, 3ADON was included in the method by Schothorst and Jekel (2003). Likewise, Bertuzzi et al. (2011) included the acetylated DONs in a validated GC-MS method. However, neither 3ADON nor 15ADON were detected in any beer samples above the limit of detection (LOD) of 0.5 µg/l and 1.0 µg/l, respectively.

2.1.2 Liquid chromatography

High performance liquid chromatography currently represents a dominating strategy in mycotoxin determination in general. Besides multi-analyte LC-MS methods covering groups of compounds with a wide range of physicochemical properties, single-analyte methods based on HPLC-UV were developed for DON determination in beer (Anselme et al., 2006; Balajová and Rauová, 2008; Omurtag and Beyoğlu, 2007). Unlike the GC-based methods, UV-detection does not require any derivatization, but needs to be performed at the absorption maximum of 220 nm. The major drawback of HPLC-UV methods is the high background noise caused by matrix compounds. Thus, involvement of a specific purification step is necessary to achieve low limits of detection. The best option is the use of immunoaffinity chromatography (IAC). Beer is loaded directly (Balajová and Rauová, 2008) or after pre-concentration (Anselme et al., 2006) onto an IAC cartridge containing anti-DON antibodies. Matrix passes through and the analyte of interest is retained on the column. The cross-reactivity of the used antibodies determines whether only DON or also DON conjugates are retained. Following a washing step, DON is eluted using acetonitrile or methanol. Typical LOQs of IAC-HPLC-UV methods are below 10 µg/l. A non-specific clean-up based on two-step purification using aluminacelite-charcoal and C18-cartridge cannot be recommended for beer analysis by HPLC-UV. The LOD of 625 µg/l achieved for DON is too high for detection of trace levels in beer (Omurtag and Beyoğlu, 2007).

High and ultra-high performance liquid chromatography (HPLC/UHPLC) hyphenated with various mass spectrometric (MS) detectors have become the most frequently used techniques in the field of mycotoxin analysis. So far, a few (U)HPLC methods coupled with tandem mass spectrometry (MS/MS) have been developed and validated for beer (Lancová et al., 2008; Romero-Gonzáles et al., 2009; Tamura et al., 2011; Malachová et al., 2012).

An HPLC-ion trap method with the ion trap operated in MS^2 mode was developed and validated to study the transfer of DON, D3G, 3ADON and other trichothecenes from barley to malt and beer (Lancová et al., 2008). Furthermore, the method was applied to a survey involving the determination of these three analytes in 176 beers (Kostelanská et al., 2009). A simple sample preparation involving shaking with acetonitrile and Celite® and pre-concentration of purified beer by evaporation resulted in LOQs of 5 µg/l for both DON and its glycosylated form. Tamura et al. (2011) applied the QuEChERS technique for the analysis of beer using commercially available QuEChERS cartridges. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method had originally been introduced for pesticide analysis, but has since then also been applied in many others fields. The method consists of extraction with acetonitrile in a disposable tube, followed by salting out and removal of water from the acetonitrile phase with NaCl and $MgSO_4$. Purification is then achieved by dispersive solid phase extraction in which the extract is shaken with either a primary-secondary amine (PSA), silica gel alone, or with PSA plus C18 or graphite carbon black (GCB). In the method of Tamura et al. (2011), the QuEChERS extract was further cleaned through C18-cartridges. A UHPLC-triplequadrupole-MS (UHPLC-QqQ-MS) in selected reaction monitoring mode was used as a final analytical tool. The method was applied to a set of 24 Japanese beers in which DON was not detected at levels higher than 5 µg/l. In another study, two types of SPE cartridges, Oasis HLB and C18, were tested for the purification of beer prior to the simultaneous determination of DON, type-A trichothecenes, aflatoxins, fumonisins, OTA and ZEA by UHPLC-QqQ-MS/MS (Romero-Gonzáles et al., 2009). In the development of multi-analyte methods, the sample preparation (extraction and clean-up) is a compromise to allow the determination of all involved analytes. Therefore, C18-cartridges were selected for further method development and validation due to better recoveries of aflatoxins. Although the validation was carried out for one pale beer only, the matrix effects were studied on three types of beer (non-alcoholic, normal and special beer). No differences were observed when comparing normal and non-alcoholic beer, but a huge signal enhancement (overestimation of results) was revealed for special beer. Therefore, the use of matrix-matched calibration was recommended for analysis of different types of beer to overcome the matrix-induced underestimation or overestimation of results despite the inclusion of a purification step. Variable influences of matrix components in different types of beer were also confirmed by another study (Malachová et al., 2012). The HPLC-MS/MS method for the determination of DON, D3G and 3ADON in six beer categories including pale, wheat, dark, bock, non-alcoholic beer and shandies was developed and in-house validated. The simplicity of the sample preparation method which consisted of degassing, precipitation of matrix components with cold acetonitrile and reconstitution of dried-down sample in solvent indicated that the analysis would be af-

fected by matrix effects. Therefore, matrix matched calibrations based on spiked samples prepared from three beers of each category were used to compensate matrix effects. The method was applied to so far the largest survey study on beers where not only DON, but also D3G and 3ADON were monitored (Varga et al, 2013). Another option to cope with matrix-induced signal suppression/enhancement in LC-MS is a stable isotope dilution assay (SIDA). The approach is based on the addition of ^{13}C-labelled internal standards to the samples prior to LC-MS analysis. Stable isotopically labelled standards exhibit identical chemical and physical properties as the target analytes, but they are not present in the naturally contaminated samples (reviewed by Rychlik and Assam, 2008). The first multi-mycotoxin method covering all mycotoxins regulated in maize based on the combination of 'dilute and shoot' approach and SIDA has been developed for maize (Varga et al., 2012). The application of the SIDA approach for beer and wine analysis was shown by Al-Taher et al. (2013). An aliquot of degassed beer (wine) was mixed with an appropriate amount of ^{13}C-labelled standards and solvent mixture (acetonitrile:water, 30:70, v/v) prior to UHPLC-QqQ-MS/MS analysis. The method was validated for beer and wine and applied to 76 beer samples. Indeed, the use of ^{13}C-DON as an internal standard compensated the signal suppression for DON (Al-Taher et al., 2012). It is worth noticing that SIDA is an approach for compensation of matrix effects, not for their removal. Therefore, as neither a clean-up nor preconcentration of the samples was carried out, the method did not reach the LOQ required for DON in beer analysis (Table 1). Concerning the application of SIDA in determination of conjugated forms of DON, only ^{13}C-labelled 3ADON is commercially available so far.

High resolution MS (HRMS) enables a comprehensive profiling of a sample. High mass resolving power together with high mass accuracy plays an important role in the unbiased identification and reliable quantification of target analytes. In addition, retrospective data mining is possible by means of non-targeted search. The applicability of two types of HR mass spectrometers, the *Time of Flight* (TOF MS) and the *Orbitrap* (Orbitrap MS) for the determination of mycotoxins in beer was assessed (Zachariášová et al., 2010). To avoid contamination of the LC-MS system and to reduce matrix effects, the beer was purified by precipitation of matrix components with acetonitrile. The supernatant was then preconcentrated by evaporation and reconstituted in solvent. Such prepared spiked beer samples were used for the assessment of detection capability of TOF MS and Orbitrap MS. The TOF MS instrument operated at its maximum attainable mass resolving power (12,500 FWHM) was able to detected DON only at levels above 25 µg/l. While using the 'ultra-high' mass resolving power of 100,000 FWHM, Orbitrap MS enabled detection of even 5 µg/l. The final method was validated on UHPLC-Orbitrap MS. The target analytes were identified with a mass deviation less than 5 ppm. Moreover, acquisition of both positive and negative ions within a single run was possible when using 'high resolution' mode of 50,000 (FWHM). Validation data obtained for three different types of beer (pale lager, non-alcoholic and dark lager) revealed that both DON and D3G could be detected at levels > 3 µg/l for all beer types. Lowest calibration levels (LCL, better suited for HRMS than LOQ) for 3ADON were almost three times higher in pale lager and non-alcoholic beer (8 µg/ml for both) and even eight times higher in dark lager beer compared to the LCL for DON and D3G (Zachariášová et al., 2010). Recently, the application of hybrid linear ion trap-HRMS to the determination of 18 mycotoxins in beer purified using Oasis HLB cartridges was studied by Rubert et al. (2011). Validation was performed on four types of beer (stout, red ale, ale and pale lager beer). Despite a mass resolving power of 100,000 FWHM, the LCLs for DON and 3ADON were too high for beer analysis (*Table 1, Table 2*).

A unique strategy of highly specific immunoaffinity based clean-up followed by UHPLC-HR (Orbitrap) MS was applied in the identification of novel DON oligoglycosides in malt and beer (Zachariášová et al., 2012). Two complementary approaches were used for structure elucidation: (i) MS monitoring of specific fragment ions formed upon in-source fragmentation of respective pseudomolecular ions and (ii) specific enzymatic hydrolysis reaction. It is supposed that DON oligoglycosides are released from starch after the enzymatic cleavage during malting and brewing. They occurred at trace levels compared to DON in beer (Zachariášová et al., 2012). Therefore, in order to achieve detectable levels for the subsequent structure elucidation, a specific pre-concentration of the samples was essential. First, a cascade of three DON immunoaffinity cartridges without cross-reactivity to D3G was used in order to remove DON. Then, DON oligoglycosides were trapped using the DON immunoaffinity cartridges with high cross-reactivity to D3G. Such a pre-concentrated sample was then analyzed. The separation of the analytes was performed by hydrophilic interaction liquid chromatography and reversed phase liquid chromatography. It was revealed that DON oligoglycosides containing up to four bound hexose units occurred in malt, beer and also other cereal-based products contaminated with DON. Due to lack of standards, quantification was not possible though.

2.2 Immunological methods

Enzyme-linked immunosorbent assays (ELISA) represent a group of methods for rapid screening of single analytes. Nowadays, several ELISA kits are commercially available for DON determination.

Two ELISA kits (both indirect competitive assays), Ridascreen® DON and EZ-Quant® High Sensitivity (HS) DON were used for extensive DON screening in 313 retail beers (Papadopoulou-Bouraoui et al., 2004). Beer samples were applied directly after degassing (cloudy beers were first filtered before analysis). All samples were analyzed in duplicates. Moreover, GC-MS was used as a confirmatory method for the analysis of 33 selected beer samples of different contamination levels to ensure the accuracy of results obtained by ELISA. In the first step, both ELISA kits were tested on DON spiked samples of pale and dark beer. The average recoveries of 111% and 134% for pale and dark beer, respectively, were obtained for Ridascreen® DON. Similar recovery values (115% for pale beer, 138% for dark beer) were calculated for EZ-Quant® High Sensitivity (HS) DON. DON was found in 87% of tested beer samples (4-56 µg/l). The average contamination of the sample set was 13.5 µg/l. A comparison of the results obtained by ELISA with those by GC-MS revealed that both ELISA kits systematically tended to overestimate the DON content likely due to the cross-reactivity of antibodies (Papadopoulou-Bouraoui et al., 2004). Ridascreen® DON was used also in one recently published DON survey of 99 beer samples in Poland. As in the latter study, beer was analyzed directly after degasing without any purification. DON was detected in all beers tested in a range of 6-70.2 µg/l (Kuzdraliński et al., 2013).

A comprehensive assessment of the applicability of four commercially available ELISA kits for the analysis of DON and its conjugated forms in beer was performed in the study of Zachariášová et al., 2008. Altogether 20 different types of beer were analyzed by all four ELISA kits (Ridascreen® DON, Veratox 5/5 DON®, Deoxynivalenol EIA and AgraQuant® DON Assay 0.25/5.0) in duplicate. The obtained results were than compared to those determined by LC-MS/MS. The cross-reactivity of antibodies to DON conjugates was tested in aqueous solution and in beer spiked with D3G, 3ADON and 15ADON. For all tested kits, a cross-reactivity to D3G was documented. The highest response for D3G dissolved in water was observed in Ridascreen® DON, the lowest in Veratox 5/5 DON®. The cross-reactivity for 3ADON was more than three times higher compared to the values declared by the producers (Ridascreen® DON). The cross-reactivity for spiked beer samples was even higher than in the case of aqueous solutions of D3G, 3ADON, and 15ADON standards. The follow-up experiments with certified reference material of wheat which was analyzed with and without purification using Mycosep® 226 by means of ELISA as well as by a validated LC-MS/MS method revealed that not only DON conjugates, but also matrix contributes to the overestimation of DON results obtained by ELISA (Zachariášová et al., 2008). The issue of cross-reactivity of immunochemical methods for mycotoxin detection towards masked mycotoxins have been summarized in recently published review paper by Zachariášová et al. (2014).

3 CONCLUSIONS

Contamination of beer with DON and its conjugated forms poses a potential risk for human health. Indeed, beer can significantly contribute to the total daily intake of DON. For this reason, it is important to regularly monitor the levels of DON, D3G and acetylated DONs in this commodity. However, development of a rapid and simple analytical method for detection of these toxins at low levels in such a complex matrix as beer is not an easy task. As concerns the cost of analysis and the complexity of the method, ELISA represents an efficient and cheap analytical tool for rapid screening of total DON contamination in beer. However, the accuracy of the results has to be confirmed by validated methods based on GC-MS or LC-MS. Besides the high cost of equipment and time-consuming analysis, instrumental analytical methods (GC-MS, LC-MS/MS and LC-HRMS) require an experienced operator. To date, they represent the only techniques for accurate beer analysis.

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APPENDIX VIII

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Development, validation and application of an LC-MS/MS based method for the determination of deoxynivalenol and its conjugates in different types of beer

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Development, validation and application of an LC-MS/MS based method for the determination of deoxynivalenol and its conjugates in different types of beer

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Abstract

After water and tea, beer is the third most popular beverage worldwide. Brewed from malted cereal grains, beer is known to be potentially contaminated with mycotoxins. Some studies have shown that not only the *Fusarium* mycotoxins deoxynivalenol (DON) and 3-acetyl-DON (3-ADON), but also the conjugated mycotoxin deoxynivalenol-3-glucoside (D3G) can be found in beer on a regular basis, albeit usually at low concentrations. The aim of this work was to develop the first triple quadrupole LC-MS/MS based method for the determination of DON, D3G and 3-ADON in beer and to perform an in-house validation. The simple sample preparation includes degassing, precipitation of matrix compounds and reconstitution of the dried-down sample in solvent. Since different kinds of beer exist and method performance parameters will likely differ, we categorised the samples into pale, wheat, dark, bock and non-alcoholic beers, as well as shandies, and validated all six matrices. Although three individual beers for each category were spiked at eight levels prior to sample preparation, the repeatability of the overall method was still excellent with relative standard deviations from 4-16% for all analytes and types of beer. Limits of detection were in the sub- or low-µg/kg range. Apparent recoveries of 60-90% for DON, 39-69% for D3G and 96-124% for 3-ADON were obtained for the different types of beer, with dark and bock beers being the most difficult matrices. To prove the applicability of the method, ten beers of each category were analysed. While average concentrations of 6.6 µg/l for DON and D3G were found, no 3-ADON was detected in any of the samples.

Keywords: masked mycotoxins, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol

1. Introduction

Deoxynivalenol (DON) and its acetylated forms, 3- and 15-acetyl-deoxynivalenol (3-ADON and 15-ADON), are type B-trichothecene mycotoxins formed by *Fusarium* spp. in grains and cereal products. Toxic effects in humans and animals include growth retardation, reproductive disorders, suppression of the immune system, nausea, and vomiting. In plants, DON causes for instance inhibition of germination and growth retardation (reviewed by Rocha *et al.*, 2005). Plants can attach polar groups, like sugars, to trichothecenes, thereby enhancing their water solubility and favouring their transport to the vacuolar space (Bowles *et al.*, 2006; Engelhardt *et al.*, 1999). This transfer withdraws mycotoxins from the cytosol, resulting in reduced toxicity for plants. In this way, 'masked mycotoxins' are formed (reviewed by Berthiller *et al.*, 2009b). The main plant metabolite of DON is deoxynivalenol-3-glucoside (D3G) (Berthiller *et al.*, 2005), but formation of higher glucosides (di- and triglucosides of DON) has also been shown (Zachariasova *et al.*, 2009).

As 3- and 15-ADON are hydrolysed to DON *in vivo* (reviewed by Wu *et al.*, 2010), their toxicity is similar to that of DON. In the case of D3G, research on adsorption, distribution, metabolism and excretion has just started. However, one *in vitro* study reported cleavage of D3G to DON by intestinal lactic acid bacteria, suggesting that

hydrolysis may equally take place *in vivo* (Berthiller *et al.*, 2011). Therefore, besides DON also its acetylated and glucosylated metabolites should be included in routine methods for food and feed.

Several methods for the determination of DON and its conjugated forms have been published and applied to a variety of cereal based foods and beverages (e.g. Berthiller *et al.*, 2009a; Desmarchelier and Seefelder, 2011; Kadota *et al.*, 2011; Kostelanska *et al.*, 2009, 2011; Sasanya *et al.*, 2008; Vendl *et al.*, 2009; Zachariasova *et al.*, 2010a,b). Beer proved to be an interesting matrix, showing greater D3G to DON and 3- plus 15-ADON to DON ratios than the raw ingredients. This was explained by the release of D3G from higher glycosides or insoluble complex carbohydrate or protein structures during germination and brewing (Kostelanska *et al.*, 2009; Lancova *et al.*, 2008).

However, most studies on beer reported only concentrations of the aglucone DON. Still, even for the determination of just DON, various different sample preparation and analysis methods have been reported. Sample preparation methods range from simple dilution of beer with acetonitrile (ACN) in different ratios (Kostelanska *et al.*, 2009; Lancova *et al.*, 2008; Zachariasova *et al.*, 2010a) over reversed and/or normal phase solid phase extraction (SPE) (Bertuzzi *et al.*, 2011; Omurtag and Beyoglu, 2007; Romero-Gonzalez *et al.*, 2009; Schothorst and Jekel, 2003; Scott *et al.*, 1993) and a modified QuEChERS approach (Tamura *et al.*, 2011) to immunoaffinity chromatography (IAC) clean-up (Anselme *et al.*, 2006; Belajova and Rauova, 2008; Kostelanska *et al.*, 2011; Rudrabhatla *et al.*, 2008). Analysis methods include ELISA (Niessen and Donhauser, 1993; Papadopoulou-Bouraoui *et al.*, 2004; Ruprich and Ostry, 1995), GC-FID (Schothorst and Jekel, 2003), GC-MS (Bertuzzi *et al.*, 2011; Scott *et al.*, 1993), HPLC-UV (Anselme *et al.*, 2006; Belajova and Rauova, 2008; Omurtag and Beyoglu, 2007), and HPLC-MS/MS (Kostelanska *et al.*, 2009, 2011; Lancova *et al.*, 2008; Romero-Gonzalez *et al.*, 2009; Rubert *et al.*, 2011; Rudrabhatla *et al.*, 2008; Tamura *et al.*, 2011; Zachariasova *et al.*, 2010a).

All methods based on LC-UV, GC-FID and GC-MS are preceded by expensive IAC or at least different SPE clean-up steps. ELISA methods, on the other hand, are applied directly after dilution of the beer samples. However, the drawback of ELISA methods is cross-reactivity of the used antibodies not only with DON conjugates, but also with matrix compounds, often resulting in overestimation of DON concentrations (Zachariasova *et al.*, 2008). Despite the selectivity and sensitivity of LC-MS/MS, most of the published methods still include a clean-up step by SPE or IAC. Simple dilution with ACN (with or without addition of celite®) to remove proteins and co-precipitating compounds has been reported only by one research group (Kostelanska *et al.*, 2009; Lancova *et al.*, 2008; Zachariasova *et al.*, 2010a).

A major challenge of such a 'dilute and shoot' approach arises from matrix effects, resulting in suppression or enhancement of the MS signal due to co-eluting matrix compounds (e.g. discussed in Sulyok *et al.,* 2006). Therefore, matrix-matched calibration is usually required for beers, for which selection of representative beer samples for method validation is mandatory. However, to the best of our knowledge, the variation of matrix effects within one category of beer (e.g. within pale, dark or non-alcoholic beer) has never been evaluated. Finally, just a few methods are capable of determining also D3G in (pale and dark) beer (Kostelanska *et al.*, 2009, 2011; Zachariasova *et al.*, 2010a), using ion trap, orbitrap or time of flight mass spectrometers.

The aims of our study were: (1) to develop a simple, accurate and robust triple quadrupole MS based method for the determination of DON, D3G and 3-ADON; (2) to validate this method for six different categories of beer or beer-based matrices (pale, wheat, dark, bock and non-alcoholic beer, as well as shandy); and (3) to apply the developed method to the analysis of ten beers of each category (60 beers in total).

2. Materials and methods

Reagents and samples

Methanol, ACN (both LC gradient grade) and glacial acetic acid (p.a.) were purchased from VWR International GmbH (Vienna, Austria) and ammonium acetate (MS grade) was obtained from Sigma Aldrich (Vienna, Austria). Water was purified using a Purelab Ultra system (ELGA LabWater, Celle, Germany).

Standards for DON and 3-ADON were purchased from Romer Labs GmbH (Tulln, Austria) as stock solutions of 100.1 mg/l and 103.3 mg/l, respectively, in ACN. D3G was previously purified from wheat plants treated with DON at anthesis (Berthiller *et al.*, 2005) and a 200 mg/l stock in methanol was used for the preparation of the standards. Stocks of DON, D3G and 3-ADON were combined to a working solution containing 1 mg/l of each analyte in ACN. Stock as well as working solutions were stored at -20 °C. For the preparation of neat standards, the required volumes of working solution or dilutions thereof in ACN were transferred into HPLC vials, evaporated to dryness under a gentle stream of air and reconstituted in methanol:water (1:1, v/v).

The matrix 'beer' was categorised into six groups, namely pale, wheat, dark, bock and non-alcoholic beer, as well as shandy. For method development and application, ten beer samples of each category were purchased in Vienna (Austria) in December 2011. The majority of the beers were produced in Austria as well. They were stored in a dark and cold environment until analysis.

Sample preparation

Approximately 15 to 20 ml of each beer was transferred to 50 ml polypropylene tubes (Sarstedt, Wr. Neudorf, Austria) and degassed in a Branson 3210 ultrasonic bath (Branson Ultrasonics BV, Soest, the Netherlands) for approximately 10 min. Until further sample preparation, the samples were stored at 4 °C in the dark. 1.00 ml of degassed beer was transferred into a 15 ml polypropylene tube (Sarstedt) and 5.25 ml of ice-cold ACN was added. After vortexing, an aliquot was transferred to a 2 ml reaction vial (Carl Roth, Karlsruhe, Germany) and centrifuged for 10 min at 10,000 rpm (8,940×*g*) using a Beckman GS-15 centrifuge equipped with a F2402 rotor (Beckman Coulter Inc., Brea, CA, USA). 500 µl of the supernatant was then transferred into an HPLC vial and evaporated under a gentle stream of air. The residue was reconstituted in 160 µl of methanol:water (1:1, v/v) and transferred into a micro-insert for HPLC-MS/MS analysis (VWR International GmbH, Vienna, Austria). Hence, a concentration of e.g. 10 µg/l in the measurement solution corresponds to 20 µg/l of a given analyte in beer.

HPLC-MS/MS analysis

For HPLC-MS/MS analysis a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap 5500 MS/MS system from AB Sciex (Foster City, CA, USA) was used. Chromatographic separation was achieved on a Gemini RP-C18 column (150×4.6 mm, 5 µm) equipped with a guard column (4×3 mm) (both from Phenomenex, Aschaffenburg, Germany) at 23 °C. The eluents were composed of different mixtures of methanol:water:acetic acid (solvent A: 10:89:1, v/v/v; solvent B: 97:2:1, v/v/v) and contained 5 mM ammonium acetate. After an initial isocratic period at pure solvent A for 2 min, the percentage of solvent B was linearly increased to 50% within 3 min, followed by a linear increase to 80% B within further 5 min. A steep gradient to 100% B within 1 min was then followed by a hold time of 1.9 min at 100% B, a change to pure solvent A within 0.1 min and a final 2 min hold time for column re-equilibration, resulting in a total run time of 15 min. The flow rate was 1 ml/min and the injection volume was 5 µl.

The QTrap 5500 was operated in negative electrospray ionisation mode using a TurboV ion spray source with the following parameters: curtain gas (CUR) 30 psi (207 kPa, nitrogen), collision gas (CAD, nitrogen) medium settings, ion spray voltage -4,500 V, temperature 550 °C, sheath gas (GS1) and drying gas (GS2) both 80 psi (552 kPa, zero grade air). In the multiple reaction monitoring mode (MRM), two (3-ADON) or three (DON and D3G) mass transitions were recorded and the dwell time was set to 50 ms. Optimisation of the MS/MS parameters was performed by syringe injection of single analyte solutions.

In the final method, the following MRM transitions were chosen for DON: 355.1 to 265.1 (quantifier, declustering potential (DP) -60 V, entrance potential (EP) -10 V, collision energy (CE) -22 V, cell exit potential (CXP) -9 V); 355.1 to 58.9 (CE -52 V, CXP -7 V); and 355.1 to 295.1 (CE -14 V, CXP -11 V). Ion ratios were calculated by dividing the peak areas of the different qualifiers by those of the quantifier. They were as follows: 3.8 (58.9/265.1) and 2.2 (295.1/265.1). Despite the higher intensities, the quantifier yielded the lowest limits of detection (LODs) due to lower noise. For D3G the monitored MRM transitions were: 517.1 to 427.1 (quantifier, DP -80 V, EP -10 V, CE -28 V, CXP -15 V); 517.1 to 58.9 (CE -85 V, CXP -7 V, ion ratio 1.2); and 517.1 to 457.1 (CE -18 V, CXP -17 V, ion ratio 1.4). Again the quantifier yielded the lowest LODs, despite lower absolute intensities. For 3-ADON the chosen transitions were: 397.3 to 307.1 (quantifier, DP -70 V, EP -10 V, CE -10 V, CXP -20 V); and 397.3 to 58.9 (CE -10 V, CXP 38 V, ion ratio 4.7). Here, the qualifier would be more sensitive, but the more selective mass transition was used as quantifier.

Analyst® software version 1.5.2 (AB Sciex) was used to control the LC-MS/MS instrument, as well as for automatic and manual integration of the peaks. Further data evaluation was carried out in Microsoft Excel 2007.

Method validation

Prior to method validation, several beer samples of each group were randomly selected and analysed for their mycotoxin content. Three beers of each category with low contaminations were selected for the spiking experiments. Spiking was carried out at eight levels ranging from 2.5 to 100 µg/l of DON, D3G and 3-ADON in beer. The required volumes of working solution or dilutions thereof in ACN were transferred into 15 ml polypropylene tubes and evaporated to dryness under a gentle stream of air. Subsequently, the analytes were reconstituted in 1 ml of degassed beer and the above described sample preparation procedure was applied.

Calibration functions of neat solvent standards and of spiked samples were established by plotting peak areas versus analyte concentrations in the measured solutions and performing linear regression. The intercept of each spiked sample calibration function was subtracted from the peak areas of each individual spiking level of the respective calibration function to correct for possible basic contaminations. Subsequently, a new linear regression line was constructed from the corrected peak areas for all three beers of one category and the analyte concentrations. The slope of this new regression line was divided by the slope of the neat solvent calibration function to obtain the apparent recovery (R_A). To calculate the method repeatability for each category of beer, each corrected peak area of the validation was divided by the corresponding spiking concentration.

From the obtained values, the relative standard deviation (RSD) was calculated and used to measure the repeatability over the whole concentration range. The LOD (signal-to-noise (S/N) = 3) and limit of quantification (LOQ, S/N = 10) were derived from the quantifier only. These values were estimated for each category of beer by subtracting the S/N of non-spiked beer (due to the absence of blank beers in some categories) from that of a low spiking level. For each category, the mean value was calculated.

Quantification of deoxynivalenol, deoxynivalenol-3-glucoside and 3-acetyldeoxynivalenol in beer samples

Samples of one category of beer were evaluated on the basis of one combined calibration function established from the intercept corrected data points of the three spiked blank beer samples of this category. Average LOD and LOQ values of three individual beers of one category were used for quantitative evaluation. For the calculation of the average DON and D3G content, 50% of the determined LOD and LOQ in the specific beer category were used for values below the LOD and LOQ, respectively.

3. Results and discussion

Optimisation of MS/MS conditions

Method development started with the optimisation of MS/MS parameters for DON, D3G, 3-ADON and 15-ADON under electrospray ionisation (ESI) conditions in both negative and positive mode. Concerning the MS/MS optimisation, direct infusion of a 100 µg/l standard solution of each individual analyte in ACN:water (1:1, v/v) into the source was used. The compound optimisation software tool was applied to obtain MRM transitions with optimised MS parameters. Signal intensities of the resulting MRM transitions were greater in negative than in positive mode for all compounds except 15-ADON. Since 3-ADON and 15-ADON only differ in the location of the acetyl-group, they have the same molecular mass and a similar fragmentation pattern. In addition, liquid chromatographic separation of 3- and 15-ADON is very difficult to achieve within a reasonable HPLC runtime. However, there is one specific MRM transition for 3-ADON (*m/z* 397.3 to 307.1) and ionisation of 3-ADON is about 10 times greater than that of 15-ADON in negative mode. Therefore, we decided to include only 3-ADON in addition to DON and D3G and to monitor all selected analytes in the negative ionisation mode.

Although most of the previously published studies determining trichothecenes employ ESI, in some applications atmospheric pressure chemical ionisation (APCI) interfaces showed better ionisation efficacies (Berthiller *et al.*, 2005; Zachariasova *et al.*, 2010a). Therefore, also MS/MS parameters for an APCI interface were optimised by flow injection analysis. A combined standard solution of 100 µg/l in ACN:water (1:1, v/v) was injected directly into the mobile phase containing 25% of aqueous methanol with a flow rate of 450 µl/min. Source parameters (CUR, evaporation temperature, GS1 and GS2), as well as compound specific parameters (DP and EP) were optimised with APCI, while MRM transitions, CE and CXP obtained from ESI optimisation were taken also for APCI. As the signal intensities for all selected MRM transitions were lower with APCI, we decided to use ESI for the final method.

Optimisation of LC conditions

Separation of the target analytes was performed using the same HPLC column and eluents as already used for multiple mycotoxin analysis (Sulyok *et al.*, 2006). Initially, acetic acid was omitted from the eluents and a shorter gradient efficiently separated all three analytes within 6 min including column equilibration (0 min: 30% B, 2 min: 40% B, 2.5 min: 100% B, 3.9 min: 100% B, 4 min: 30% B, 6 min: 30% B). However, pre-validation experiments with matrix-matched standards of pale beer revealed that the separation of target analytes and interfering matrix components was not sufficient and analyte signal intensities using this short method were strongly affected by matrix effects. In order to reduce the degree of matrix effects and thus to decrease the limits of detection in beer, the initial steep and short gradient was flattened and extended to 9 min (0 min: 10% B, 0.1 min: 10% B, 4 min: 100% B, 6.9 min: 100% B, 7 min: 10% B, 9 min: 10% B). In addition, eluents were acidified with 1% acetic acid (v/v) because of literature reports stating improvement of signal intensities under acidic conditions (Tamura *et al.*, 2011), even in negative ion mode. This method enabled more efficient separation of DON and 3-ADON from co-eluting matrix components in all types of beer, resulting in apparent recoveries of 62-97% for DON and 74-122% for 3-ADON. However, the early eluting D3G still suffered from severe matrix effects, in particular in pale, bock and wheat beer (R_A <30%). Therefore, further changes in gradient design were necessary for reliable quantification of this masked mycotoxin at low levels. Best results were obtained with the 15 min gradient method described in the materials and methods section. Finally, this method was validated and used for the analysis of beer samples to demonstrate the applicability of the method. The retention times of the analytes in the final method were: D3G 5.79 min, DON 5.91 min, 3-ADON 7.31 min (see Figure 1A, 1C and 1E for chromatograms obtained with a 1.25 µg/l solution of all analytes).

Sample preparation

Our aim was to develop a high-throughput sample preparation method with minimum sample work-up. First, all beers were degassed in an ultrasonic bath. To ensure that sonication does not degrade trichothecenes

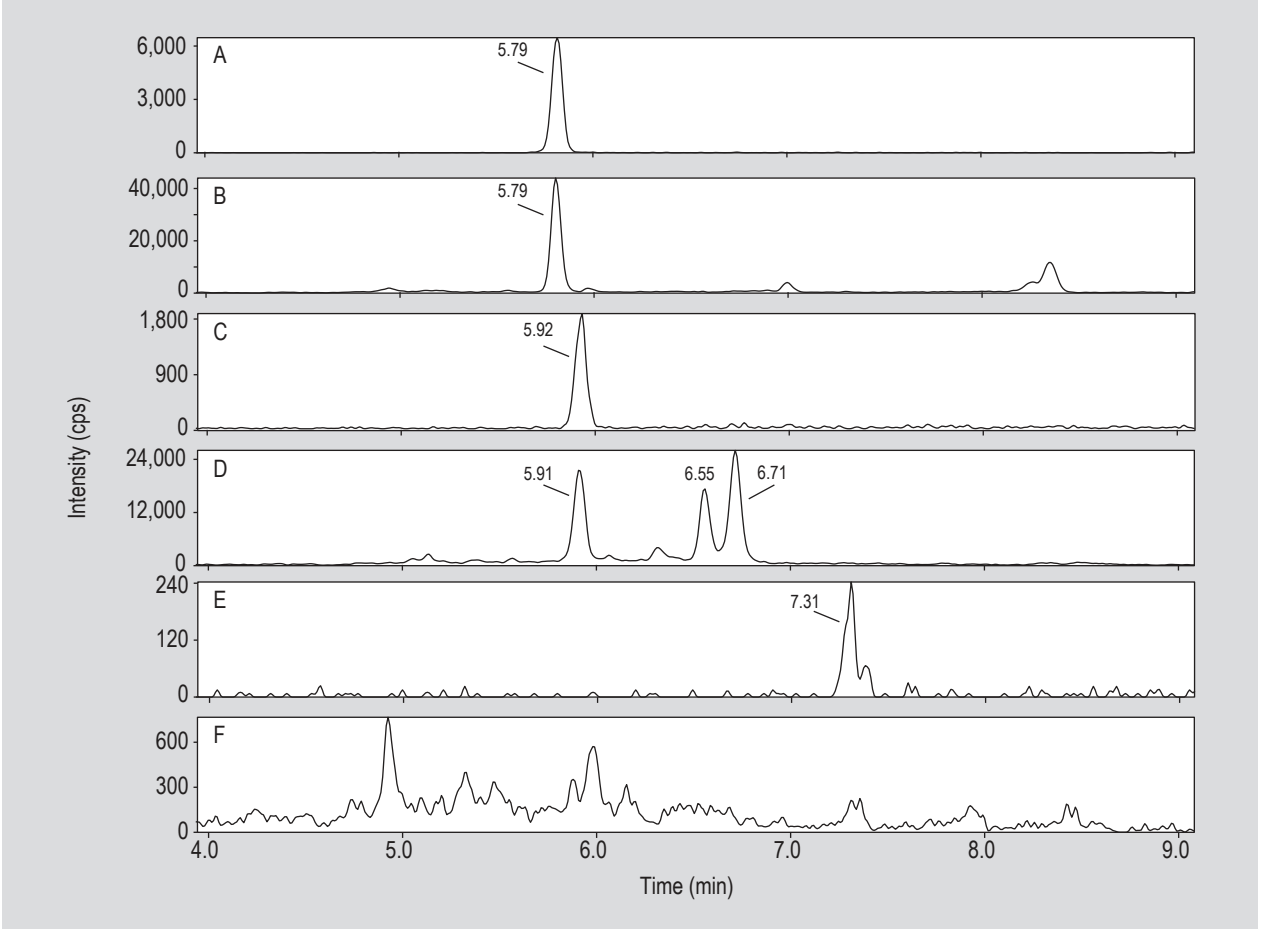

Figure 1. LC-MS/MS (MRM) chromatograms of a liquid standard containing 1.25 µg/l of D3G (A), DON (C) and 3-ADON (E) and chromatograms of a naturally contaminated beer sample from Austria containing 19 µg/l D3G (B), 30 µg/l DON (D) and 3-ADON below the detection limit (F). In all cases the quantifier transition is shown. Several matrix peaks can be observed in the MRM chromatograms, e.g. at 6.55 and 6.71 min for the DON transition, but are clearly separated from the analytes.

in beer, as reported by Lindner (1996) for cereals, pale beer was spiked at a level of 20 µg/l and was placed in the ultrasonic bath. Prior to sonication and after 10, 20, 30, 40, and 60 min an aliquot was drawn. The RSD calculated from the peak areas of the respective spiked samples was below 2% for all target analytes, proving their stability under the chosen conditions. As direct injection of degassed beer is not feasible due to matrix complexity (Zachariasova *et al.*, 2010a), a fast purification/precipitation step involving addition of cold ACN at a ratio of 1:1 (v/v) was tested. However, significant losses (more than 50%) of all investigated analytes were observed, probably due to co-precipitation and a low desorption rate from solid particles. Follow-up experiments therefore focused on the increase of the ACN volume compared to beer and the following ratios were tested: 1:3, 1:4, 1:5.25 (degassed beer:ACN, v/v). An evaporation step was required because the higher dilution factors would result in unacceptable high limits of detection. Additionally, it is not feasible to inject sample solutions containing such a high percentage of ACN due to peak distortion. Therefore, methanol:water (1:1, v/v) was chosen as reconstitution solvent. The increased ratio of ACN helped to avoid co-precipitation of mycotoxins under all three tested conditions. To keep the time consuming evaporation step as short as possible, it was decided to use an azeotropic ratio of beer:ACN (1:5.25, v/v) for the final method. Whereas reconstitution of residues after evaporation in the volume corresponding to undiluted beer samples was accompanied by severe matrix suppression, a dilution factor of two yielded acceptable recoveries and was used for the final method. To conclude, the results of our experiments to optimise sample preparation are in line with the method developed by Zachariasova *et al.* (2010a), except that a higher dilution factor (2 for the triple quadrupole method versus 0.8 for the orbitrap method) was required.

Method validation

It was decided to select three different beers (of each category) rather than to spike one beer in triplicate. This setup is likely to result in higher RSDs. However, it is better reflecting the real situation because it takes into account

the high variability in raw materials or ingredients used for the production of the individual beers.

In the current study, the apparent recoveries were evaluated (Table 1). The apparent recovery is composed of the recovery of the sample preparation and the mass spectrometric signal suppression or enhancement (see e.g. Sulyok *et al.*, 2006) and constitutes the overall recovery of the method. As revealed by pre-validation experiments, matrix effects were the main factor determining the R_A. Differences in the degree of matrix effects and consequently also in the R_A were expected especially in the case of dark beer, since a light dark, medium dark and an intense dark beer were chosen on purpose for method validation. Despite the high optical differences in the dark beers, the variabilities of R_A were only between 8% for DON and 13% for D3G. The RSDs for pale, wheat, bock and non-alcoholic beer were in a similar range or lower. The higher variability observed for shandy (16% for D3G, 12% for DON, 14% for 3-ADON) could be caused by different beer/lemonade ratios as well as by ingredients from the different used lemonades (grapefruit, lemon and black currant juice, respectively).

Despite the precipitation of matrix compounds, consistent signal suppression for D3G and DON dependent on the beer type was observed. As expected, the highest matrix effects occurred in the case of dark beer which is in agreement with Zachariasova *et al.* (2010a). LODs and LOQs were derived from the more specific transition (also used for quantification) which, in the case of 3-ADON, resulted in higher LOD and LOQ values than for the more sensitive (but less specific) transition.

Table 1. Performance characteristics of the developed LC-MS/MS based method.

Analyte	Beer category	R_A (%) ± RSD (%)[1]	LOD (µg/l)[1]	LOQ (µg/l)[1]
D3G	pale	64±12	0.4	3.5
	wheat	66±10	0.9	3.5
	dark	39±13	1.4	4.1
	bock	42±7	0.5	1.5
	non-alcoholic	69±6	0.4	1.4
	shandy	63±16	0.4	1.3
DON	pale	88±8	2.2	5.4
	wheat	83±10	1.0	4.5
	dark	60±8	2.9	11
	bock	75±11	1.2	4.1
	non-alcoholic	85±5	1.2	3.0
	shandy	90±12	1.5	3.9
3-ADON	pale	102±7	2.4	6.8
	wheat	104±10	2.2	8.2
	dark	96±11	4.3	11
	bock	101±4	3.6	9.2
	non-alcoholic	124±5	2.6	6.0
	shandy	109±14	2.7	10

[1] R_A: apparent recovery determined by spiking three different beers at eight levels per category; RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantification.

For D3G, excellent LOQs between 1.3 and 4.1 µg/l were achieved for all investigated matrices. While the LOQs for DON were slightly higher than those for D3G, still very reasonable values between 3.0 and 5.4 µg/l were obtained with the exception of dark beers (11 µg/l). LOQs for 3-ADON were close to 10 µg/l for all investigated matrices. Table 2 comprises published methods for the determination of DON (and if available also for D3G and 3-ADON) in beer, along with the reported LODs, LOQs and recoveries. Our developed method compares nicely to the performance characteristics of recently published LC-MS/MS based methods (Table 2).

Application to beer samples

The applicability of the method to commercially available beer samples was shown in the analysis of ten beer samples per category, hence 60 samples in total. With the exception of non-alcoholic beers, a high percentage of samples contained detectable levels of D3G and DON (see Table 3), but only about 7% of all measured beers contained D3G and DON concentrations above 10 µg/l. The average concentration of D3G and DON in all beers was 6.6 µg/l for both analytes. Maximum levels up to 32 µg/l D3G and up to 30 µg/l DON were observed.

These findings are in good agreement with the values reported by different research groups for samples from different harvest years. For instance, Kostelanska *et al.* (2009), determined average values of 8.5 µg/l D3G and 6.6 µg/l DON in beer samples from 2007. Likewise, Molto *et al.* (2002), and Scott *et al.* (1993), reported average DON concentrations of 5.0 and 5.4 µg/l, respectively, and maximum DON levels of 43 and 20 µg/l, respectively. A chromatogram from a pale beer showing high contamination of D3G (19 µg/l) and DON (30 µg/l) is shown in Figure 1 (B, D, F). Since 3-ADON was not detected in any of the samples, it is not included in Table 3. This finding is in accordance with Bertuzzi *et al.* (2011), who analysed 3- and 15-ADON by GC-MS and did not find any positive sample, despite excellent LODs of 0.5 and 1 µg/l for 3- and 15-ADON, respectively. All measured bock beer samples contained detectable concentrations of D3G and DON, and the average value of 16 µg/l D3G and 13 µg/l DON is twice as high as the average for pale beer. An explanation could be the higher gravity and thus a higher cereal content of this type of beer. The least contaminated group was that of non-alcoholic beers, which is in good agreement with the findings by Kostelanska *et al.* (2009). Also shandies, which

Table 2. Method comparison between published methods for deoxynivalenol and its conjugated forms.

Reference	Clean-up[1]	Measurement[1]	LOD (µg/l) [1,2]	LOQ (µg/l) [1,2]	Recovery (%) [1,2,3]
Ruprich and Ostry, 1995	none	ELISA	0.05	n.a.	80-90
Niessen and Donhauser, 1993	none	ELISA	50	n.a.	n.a.
Papadopoulou-Bouraoui *et al.*, 2004	none	ELISA	3.7	n.a.	111-134
Schothorst and Jekel, 2003	SPE; MycoSep®	GC-FID	8	25	DON: 92; 3-ADON: 102
Scott *et al.*, 1993	2-fold SPE	GC-MS	0.1-1.5	n.a.	103
Bertuzzi *et al.*, 2011	ACN, Trichothecene EP®	GC-MS	0.5	1.5	92
Anselme *et al.*, 2006	ACN; IAC	HPLC-UV	2	6	93
Belajova and Rauova, 2008	IAC	HPLC-DAD	6	7	101
Omurtag and Beyoglu, 2007	ACN/water + celite®; 2 fold SPE	HPLC-UV	125	625	83
Lancova *et al.*, 2008; Kostelanska *et al.*, 2009	ACN + celite®	HPLC-APCI-ion trap-MS/MS	DON: 1.0; D3G: 1.0; ADONs: 5.0	DON: 2.5-5.0; D3G: 2.5-5.0; ADONs: 5.0-10.0	DON: 84; D3G: 81; ADONs: 78
Zachariasova *et al.*, 2010a	ACN	UHPLC-APCI-Orbitrap-MS	n.a.	DON, D3G: 2-3, 3-ADON: 8-18	DON: 94-108; D3G: 88-111; ADONs: 89-112
Kostelanska *et al.*, 2011	IAC	UHPLC-ESI-TOF-MS	DON: 1.0; D3G: 1.0; ADONs: 1.0	n.a.	DON: 99; D3G: 97; ADONs: 95
Tamura *et al.*, 2011	modified QuEChERS	UHPLC-ESI-QqQ-MS/MS	n.a.	5	93
Romero-Gonzalez *et al.*, 2009	SPE	HPLC-ESI-QqQ-MS/MS	0.14	0.45	82-96; SSE: 46-135
Rubert *et al.*, 2011	SPE	HPLC-ESI-Orbitrap-MS	DON: 16-20; 3-ADON: 25-30	DON: 48-60; 3-ADON: 75-90	DON: 83-105; 3-ADON: 43-59
Rudrabhatla *et al.*, 2008	filtration, dilution, mixed IAC	HPLC-MS/MS-ESI-QqQ-MS	n.a.	n.a.	98

[1] Abbreviations used: n.a. = not available; SPE = solid phase extraction; ACN = acetonitrile; IAC = immunoaffinity chromatography; QuEChERS = Quick-Easy-Cheap-Effective-Rugged-Safe; ELISA = enzyme linked immunosorbent assay; SSE = signal suppression or enhancement; LOD = limit of detection; LOQ = limit of quantification; DON = deoxynivalenol; ADON = acetyl-deoxynivalenol; D3G = deoxynivalenol-3-glucoside.

[2] If only single values are given, the numbers refer to DON alone.

[3] The recovery value from the reference is provided, which is not necessarily R_A (as recoveries based on matrix matched standards are stated by various authors).

contain only approximately 50% beer, were contaminated at low levels. The molar ratios of D3G to DON (Table 4) were below or around 1 and support previous findings, that reported D3G contamination in the same range as the DON concentration (e.g. Kostelanska *et al.*, 2009). No significant differences between the different types of beer could be observed regarding the molar ratio.

The D3G/DON molar ratio (reflecting the different molecular masses of DON and D3G) ranged from 0.2-1.1, demonstrating that the molar concentration of D3G can be lower, equal or slightly higher than that of DON. Therefore, the concentration of free DON is not suitable for the prediction of the sum concentration of DON and D3G. Still, on average the concentration of D3G was about 62% of free DON on a molar basis.

4. Conclusion

We have developed an easy-to-use and well suited LC-triple quadrupole MS based method for the determination of DON, D3G and 3-ADON in various types of beer. Careful adaptation of precipitation conditions, the chromatographic gradient and the dilution factor was needed to achieve acceptable recoveries for all analytes despite the simple sample preparation. To our knowledge, this is the first work presenting performance characteristics for these analytes in six different beer categories (pale, wheat, dark,

Table 3. Application of the method to beer samples.

Analyte[1]	Beer category	All beers		No of samples		Contaminated beers	
		Average (µg/l)[2]	Median (µg/l)	<LOD[1]	Traces[3]	Average (µg/l)	Max. (µg/l)
D3G	pale	6.3	5.5	0	3	8.3	19
	wheat	6.4	5.9	1	2	8.6	15
	dark	7.4	6.9	0	3	9.6	16
	bock	16	12	0	0	16	32
	non-alcoholic	1.3	0.9	5	0	2.3	3.1
	shandy	2.3	2.3	0	4	3.5	5.5
	all	6.6	4.3	6	12	8.9	32
DON	pale	6.8	2.7	1	5	13	30
	wheat	12	9.2	1	1	14	27
	dark	5.0	5.4	4	4	11	11
	bock	13	13	0	0	13	22
	non-alcoholic	0.9	0.6	9	0	3.7	3.7
	shandy	2.1	1.9	2	7	6.4	6.4
	all	6.6	3.2	17	17	13	30

[1] Abbreviations used: LOD = limit of detection; DON = deoxynivalenol; D3G = deoxynivalenol-3-glucoside.
[2] For the calculation of the average D3G and DON content of all beers, 50% of the determined LOD and the limit of quantification (LOQ) in the specific beer category were used for values below the LOD and LOQ, respectively.
[3] Traces are defined as concentrations above the LOD but below the LOQ.

Table 4. Molar ratios of deoxynivalenol-3-glucoside/deoxynivalenol per beer category.

Beer category	n[1]	Average ± standard deviation	Range
Pale	4	0.6±0.2	0.4-0.8
Wheat	7	0.4±0.1	0.3-0.4
Dark	2	0.8±0.2	0.6-0.9
Bock	10	0.8±0.3	0.2-1.1
Non-alcoholic	1	0.5	-
Shandy	1	0.6	-
All	25	0.6±0.2	0.2-1.1

[1] n = number of beers used to calculate the molar deoxynivalenol-3-glucoside/deoxynivalenol ratio (both concentrations above the limit of quantification).

bock, non-alcoholic beer, and shandy). The determined method variability ranged between 4 and 16% and hence is in an excellent range for LC-MS/MS based methods. The differences between pale, wheat, non-alcoholic beer and shandy are minor in the case of D3G and DON, expressed by average apparent recoveries between 63 to 69% and 83 to 90% for D3G and DON, respectively. However, the apparent recoveries of dark and bock beer (39 to 42% for D3G and 60 to 75% for DON) are lower, emphasising the need for separate matrix matched standards for the different beer categories. In the case of 3-ADON, apparent recoveries between 96 and 124% and RSDs between 4 and 14% were observed. The average value of DON and D3G for all beers analysed in the small scale application study was 6.6 µg/l for both analytes. Furthermore, the average D3G/DON molar ratio was 0.7, showing that the masked mycotoxin D3G is present in beers in a similar molar concentration range as DON. The determined contamination is in line with other studies and not of big toxicological concern. None of the 60 samples contained 3-ADON above the LOD. Since the suitability of this in-house validated method for the different categories of beer was proven in this study, the method will be applied for a beer survey focusing on the DON and DON-conjugate (D3G and 3-ADON) content in more than 300 different beers from all over the world.

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APPENDIX IX

Varga E, Malachova A, Schwartz H, Krska R, Berthiller F

Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples

Food Additives and Contaminants: Part A, 1–10 (2012)

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Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples

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Beer is one of the most popular beverages worldwide. Malted cereal grains are among the basic ingredients and hence mycotoxin contamination might occur. Previous studies reported the presence of the *Fusarium* mycotoxins deoxynivalenol (DON) and 3-acetyl-deoxynivalenol (3ADON), as well as of the masked mycotoxin deoxynivalenol-3-glucoside (D3G) in beer. In the present survey, 374 beer samples from 38 countries with a focus on Austrian (156) and German (64) beers were analysed for the presence of D3G, DON and 3ADON. Beers were assigned to the following six categories: pale (217), wheat (46), dark (47), bock (20), nonalcoholic beers (19) and shandies (25). In total, 348 and 289 beers (93 and 77%, respectively) contained D3G and DON at the levels above the limit of detection, whereas 3ADON was not detected in any of the samples. Average concentrations of all beers were 6.9 $\mu g\,L^{-1}$ for D3G and 8.4 $\mu g\,L^{-1}$ in the case of DON. Nonalcoholic beers and shandies showed the lowest contaminations, 1.5 and 3.2 $\mu g\,L^{-1}$ for D3G and 2.7 and 4.4 $\mu g\,L^{-1}$ for DON, respectively. In bock beers characterised by a higher gravity, a significant trichothecene load of 14.8 $\mu g\,L^{-1}$ D3G and 12.4 $\mu g\,L^{-1}$ DON was found. The highest contamination (81 $\mu g\,L^{-1}$ D3G, 89 $\mu g\,L^{-1}$ DON) was detected in a pale beer from Austria, underlining the importance of this study for food safety. The molar D3G to DON ratio ranged between 0.11 and 1.25 and was 0.56 on average. Concluding, the average contamination of beer is not of toxicological concern for moderate beer drinkers. However, in the case of heavy beer drinkers, beer consumption may considerably contribute to the overall intake of DON, which might even lead to exceeding the maximum tolerable limits established for this *Fusarium* toxin.

Keywords: chromatography – LC/MS; survey; mycotoxins; mycotoxins – fusarium; mycotoxins – trichothecenes; beer; beverages

Introduction

Beer is the most popular alcoholic beverage worldwide. In Europe, 37% of the total recorded alcohol is consumed in the form of beer (WHO 2011). The leading country is the Czech Republic, with an annual consumption of 132 litre per capita in 2010 (Bierserver 2012), followed by Germany and Austria, with 107 (Die deutschen Brauer 2012) and 106 (Bierserver 2012) litre per capita, respectively. Barley is the most common cereal grain used in the production of beer. Similarly to other grains, barley can be infected by *Fusarium* species. These fungi are capable of forming the type B-trichothecene mycotoxins deoxynivalenol (DON) and its acetylated forms, 3- and 15-acetyl-deoxynivalenol (3ADON and 15ADON) on crop plants, like barley or wheat (reviewed by Foroud and Eudes 2009). The toxic effects of DON in humans and animals vary from nausea and vomiting, to growth retardation, reproductive disorders and the suppression of the immune system. On a cellular level, toxic effects include inhibition of protein, RNA and DNA synthesis, alteration of membrane structure and disturbance of mitochondrial functions and of cell division. In plants, symptoms are inhibition of germination and growth retardation (summarised by Rocha et al. 2005). Deoxynivalenol-3-glucoside (D3G) is the main plant metabolite of DON and shows reduced toxicity toward plants as a so-called "masked mycotoxin" (reviewed by Berthiller, Schuhmacher, et al. 2009).

Recent absorption, distribution, metabolism and excretion (ADME) studies reported rapid hydrolysis of acetylated DON *in vivo* (reviewed by Wu et al. 2010), resulting in a similar toxicity of acetylated and free DON in mammals. On the basis of these findings, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) converted the provisional maximum tolerable daily intake (PMTDI) for DON

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($1\,\mu g\,kg^{-1}$ body weight (bw)) to a group PMTDI of $1\,\mu g\,kg^{-1}$ bw for DON, 3ADON and 15ADON (JECFA 2011). Currently, D3G is not considered in the PMTDI because insufficient data on its toxicity are available. However, an *in vitro* study reporting partial cleavage of D3G by intestinal lactic acid bacteria to DON has recently been published (Berthiller et al. 2011), suggesting that D3G should also be monitored until more toxicological data are available.

DON is the only type B-trichothecene for which maximum levels have been set in certain foodstuffs within the European Union (European Commission 2012). However, the maximum levels for DON in cereals and cereal products are again the subject of discussion of the Codex Committee on Contaminants in Food (CCCF). As consensus on elaboration of maximum levels for DON and its conjugates in cereals and cereal-based products could not be reached (CCCF 2011), the CCCF called on Codex members to monitor DON and its derivatives in cereals in order to assess the relevance of DON conjugates. Several studies investigated the concentrations of DON, D3G, 3ADON and 15ADON in foods and beverages (e.g., Berthiller, Dall'Asta, et al. 2009; Kostelanska et al. 2009; Vendl et al. 2010; Bertuzzi et al. 2011; Desmarchelier and Seefelder 2011; Kostelanska et al. 2011; Malachova et al. 2011). Average molar ratios of D3G to DON were between 0.05 and 0.17 in cereals and cereal products (wheat grains, wheat flour, breakfast cereals and maize kernels) (Berthiller, Dall'Asta, et al. 2009; Desmarchelier and Seefelder 2011; Malachova et al. 2011). Average D3G/DON molar ratios ranged between 0.58 and 1.24 in light and dark beers containing between 4 and 5.9% alcohol (Kostelanska et al. 2009). Average molar ratios of ADONs to DON in the same beers were between 0.57 and 2.19. Another study by the same group of researchers confirmed these findings, reporting higher levels of D3G than of DON in all analysed samples of nonalcoholic and alcoholic beer from Czech and Austrian producers (Kostelanska et al. 2011).

Average concentrations of DON determined in the three largest recent studies investigating beers produced between 2003–2011 by gas and liquid chromatographic methods were $2.1\,\mu g\,L^{-1}$ (70 DON positive samples of 106 in total) (Bertuzzi et al. 2011), $5.0\,\mu g\,L^{-1}$ (59 of 80 samples) (Anselme et al. 2006) and $6.6\,\mu g\,L^{-1}$ (111 of 176 samples) (Kostelanska et al. 2009). The latest ones also reported average concentrations for D3G ($8.5\,\mu g\,L^{-1}$, 130 positive samples) and for 3- and 15ADON ($4.7\,\mu g\,L^{-1}$, 87 positive) in a total of 176 beer samples.

The aim of our work was to add to the current knowledge of DON and DON conjugate concentrations in beer by performing the largest survey of Austrian beers. To this end, DON, D3G and 3ADON should be quantitatively determined in a total of 374 beer or beer-based samples by a simple validated liquid chromatography tandem mass spectrometry (LC-MS/MS) based method. Beer samples from six different categories (pale, wheat, dark, bock, nonalcoholic beer and shandy), originating from 38 countries, should be evaluated, putting a strong emphasis on Austrian beers.

Materials and methods

Reagents and samples

Reagents were purchased from VWR International GmbH (Vienna, Austria) (glacial acetic acid [p.a.], LC gradient grade methanol and acetonitrile) and from Sigma Aldrich (Vienna, Austria) (MS grade ammonium acetate). A Purelab Ultra system (ELGA LabWater, Celle, Germany) was used for further purification of reverse osmosis water.

DON and 3ADON standards were obtained from Romer Labs GmbH (Tulln, Austria) as stock solutions of $100.1\,mg\,L^{-1}$ and $103.3\,mg\,L^{-1}$ in acetonitrile, respectively. The $200\,mg\,L^{-1}$ D3G stock in methanol had previously been prepared by treatment of wheat plants with DON at anthesis and subsequent purification of D3G by normal and reversed phase chromatography (Berthiller et al. 2005). A $1\,mg\,L^{-1}$ working solution of all three analytes in acetonitrile was prepared from the stock solutions and stored at $-20^{\circ}C$.

Three hundred and seventy-four beer samples produced in 38 countries were purchased in various stores in Austria (Vienna, Tulln, Königsbrunn am Wagram), Hungary (Sopron), Croatia (Čakovec, Osijek, Vukovar) and Serbia (Sombor) in December 2011 and January 2012. Until sample preparation, they were stored in a dark and cold environment. To each beer a two-letter country code was assigned, according to ISO 3166-1 alpha-2 (ISO 2011), followed by three digits of a continuous number within the country. The country indicated on the label as production place and not the head office of the brand was chosen for the code. In four cases, the country of origin was not given. Therefore, the country code "XX" was assigned. The vast majority of the beers originated from Europe (330), followed by several beers from America (19), Asia (13), Africa (7) and one beer from Australia. The collected beers were predominantly purchased in glass bottles but also in cans and some even in plastic bottles. The letter after the assigned number describes different lots of the same beer. Furthermore, 3 bottles each of 13 highly contaminated beers were resampled in June 2012.

The beer samples were classified into six groups, namely pale (217 samples), wheat (46), dark (47), bock (20), nonalcoholic (19) as well as shandy (25). Beer brewed with barley malt and exhibiting a light colour was defined as pale beer. Wheat beer was classified as

beer brewed with wheat malt alone or in combination with barley malt. Any beer exhibiting a darker, brownish colour and brewed from malt of any kind was allocated to the category of dark beer, regardless of the alcohol content. Beers were classified as bock beers when either the alcohol content was equal to or higher than 7 Vol. percentage of alcohol and/or the gravity was equal to or higher than 15°. As indicated by the name, nonalcoholic beer contained no alcohol or an alcohol content of less than 0.5 Vol. percentage. Shandies were mixtures of different ratios of beer and lemonade.

Sample preparation and LC-MS/MS analysis

Sample preparation, as well as LC-MS/MS parameters and instrumental setup, have been described in detail by Malachova et al. (2012). Briefly, beer samples were degassed for approximately 10 min by ultrasonication, diluted with cold acetonitrile (degassed beer + acetonitrile 1 + 5.25, v + v) and centrifuged. An aliquot (500 µL) was evaporated to dryness and reconstituted in 160 µL methanol + water (1 + 1, v + v), resulting in a total dilution factor of two. LC-MS/MS analysis (injection volume 5 µL) was performed on a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap 5500 MS/MS system from AB Sciex (Foster City, CA, USA). A Gemini RP-C18 column (150 × 4.6 mm, 5 µm) equipped with a guard column (4 × 3 mm) (both from Phenomenex, Aschaffenburg, Germany) were used for chromatographic separation in gradient mode at 23°C and at a flow rate of 1 mL min^{-1}. The eluents were composed of methanol + water + acetic acid (A: 10 + 89 + 1, v + v + v; B: 97 + 2 + 1, v + v + v) and contained 5 mM ammonium acetate. The TurboV ion spray source was operated in the negative electrospray ionisation mode and two (3ADON) or three (DON and D3G) mass transitions were monitored in the multiple reaction monitoring mode.

Data evaluation

The LC-MS/MS system was controlled by and peaks were integrated with Analyst® software version 1.5.2 (AB Sciex, Foster City, CA, USA). Further data evaluation was carried out using Microsoft Excel 2007. As explained in detail in our previous paper (Malachova et al. 2012), the beer samples were evaluated on the basis of one combined calibration function of three spiked beers per category. Average apparent recoveries for D3G were 64% (pale beer), 66% (wheat beer), 39% (dark beer), 42% (bock beer), 69% (nonalcoholic beer) and 63% (shandy). For DON, 88% (pale beer), 83% (wheat beer), 60% (dark beer), 75% (bock beer), 85% (nonalcoholic beer) and 90% (shandy) were recovered. In the case of 3ADON, the following recoveries were determined: 102% (pale beer), 104% (wheat beer), 96% (dark beer), 101% (bock beer), 124% (nonalcoholic beer) and 109% (shandy). Variabilities of recovery were between 4 and 16%, depending on analyte and category of beer. Limits of detection (LODs) and quantification (LOQs) were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively, for each category separately. These values can be found in Tables 1–6. Average D3G and DON values were calculated both for all beers and for beers contaminated above the LOQ. Half the values of LOD or LOQ of the respective category were assigned to values below the LOD and between the LOD and LOQ, respectively, for those calculations. The average molar ratio of D3G/DON was calculated only for those beers for which both analytes showed concentrations above LOQ.

Results and discussion

Beers of different categories yielded different validation parameters like apparent recoveries, LODs and LOQs (Malachova et al. 2012). Hence, samples were evaluated based on a combined calibration curve of three spiked beers per category. Concentrations of DON and D3G are presented separately for each category and sorted by the country of origin (Tables 1–6). The given alcohol content is that stated on the label. At the bottom of each table, the average of all beers and of the contaminated beers only, as well as the median of all beers of the respective category, are given. The ratio D3G/DON is shown only for beers containing DON and D3G above the LOQ. In Table 7, the data are summarised according to country of origin regardless of the beer category and an overview of the contamination range is given. In general, a high percentage of beers contained detectable levels of D3G (93%) and DON (77%), but more than three quarters of the samples contained less than 10 µg L^{-1} of each analyte. In contrast to previous findings by Kostelanska et al. (2009), 3ADON could not be detected in any of the samples. Hence, this column is not displayed in the tables. The absence of 3ADON might be due to the slightly elevated LODs (2.2 to 4.3 µg L^{-1}) compared to the two other mycotoxins (D3G 0.4 to 1.4 µg L^{-1}, DON 1.0 to 2.9 µg L^{-1}). However, Bertuzzi et al. (2011) could not confirm the presence of 3- and 15ADON in their GC-MS measurements of 106 beers (LODs of 0.5 and 1 µg L^{-1}, respectively), either. The overall average of all 374 beer samples regardless of the category was 6.9 µg L^{-1} for D3G and 8.4 µg L^{-1} for DON. Medians were 4.9 µg L^{-1} in the case of D3G and 5.6 µg L^{-1} for DON. Taking into consideration only the

Table 1. Analysed pale beer samples ($n = 217$; 147 samples > LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g L^{-1}$)	DON ($\mu g L^{-1}$)	Molar ratio D3G/DON
AT-001	5.2	6.5	5.4	0.78
AT-002	5.2	10.8	9.8	0.71
AT-005	4.9	6.6	6.4	0.66
AT-011	3.5	14.4	13.1	0.71
AT-012	5.2	9.7	7.6	0.83
AT-014	5.2	3.8	< LOQ	–
AT-017	5.2	6.2	10.3	0.39
AT-019	4.8	4.5	5.6	0.52
AT-020	4.8	7.7	8.7	0.57
AT-021	6.8	5.6	7.1	0.52
AT-022	5.2	4.5	< LOQ	–
AT-023	4.2	5.9	< LOQ	–
AT-027	5.5	8.5	7.9	0.69
AT-028a	5.2	7.3	7.8	0.60
AT-028b	5.2	5.5	5.5	0.64
AT-031	5.7	6.0	8.4	0.46
AT-035	4.8	10.0	9.3	0.70
AT-037	4.8	3.6	< LOQ	–
AT-038	5.0	4.0	< LOQ	–
AT-039	5.0	5.4	< LOQ	–
AT-040	5.0	5.2	5.8	0.57
AT-042	4.8	8.8	9.8	0.58
AT-043	4.8	9.5	10.4	0.60
AT-045	5.0	9.0	10.5	0.55
AT-047	5.2	3.8	< LOQ	–
AT-049	5.4	16.5	12.8	0.83
AT-050	5	4.9	6.5	0.48
AT-051	2.9	7.9	9.0	0.57
AT-053	5.0	7.4	10.7	0.45
AT-055	5.3	6.0	6.1	0.64
AT-059	5.1	7.0	6.5	0.70
AT-060	4.3	8.7	7.3	0.77
AT-062	5.0	< LOQ	6.7	–
AT-067	5.2	4.3	7.0	0.40
AT-071	5.2	6.3	6.6	0.62
AT-074b	5.2	3.7	< LOQ	–
AT-078	n.a.	10.2	9.1	0.73
AT-080	5.2	10.4	8.3	0.82
AT-084a	5.1	7.8	9.4	0.54
AT-084b	5.1	7.9	7.6	0.67
AT-085	5.2	14.6	23.2	0.41
AT-086	6.0	5.7	5.9	0.62
AT-087	5.1	40.2	29.6	0.88
AT-089	5.4	6.5	< LOQ	–
AT-092	4.9	8.3	6.3	0.85
AT-096	5.1	6.2	5.8	0.70
AT-097b	5.1	27.4	19.5	0.91
AT-101	5.2	14.7	23.5	0.40
AT-104	5.1	5.7	5.7	0.65
AT-104b	5.1	5.2	7.7	0.44
AT-104c	5.1	4.9	6.1	0.51
AT-107	4.2	6.4	7.4	0.56
AT-108	5.0	12.8	17.3	0.48
AT-110	4.0	8.8	13.9	0.41
AT-111	4.9	4.4	< LOQ	–
AT-113a	4.9	15.6	13.3	0.76
AT-113b	4.9	7.4	5.7	0.84
AT-120	5.0	9.0	6.6	0.89
AT-121	5.7	7.2	8.2	0.57
AT-122	4.2	8.2	5.6	0.94
AT-123	4.7	6.3	9.2	0.44
AT-125	4.9	17.4	15.7	0.72
AT-127	4.9	15.0	11.7	0.83
AT-128	5.0	11.2	10.5	0.69
AT-129	4.9	8.0	7.2	0.72
AT-130	5.0	5.1	< LOQ	–
AT-131a	5.0	9.3	19.0	0.32
AT-131b	5.0	10.1	7.8	0.83
AT-132	5.4	6.9	7.3	0.61
AT-133	4.9	**81.3**	**89.3**	0.59
AT-138	5.0	6.1	6.6	0.60
AT-139	3.0	3.8	< LOQ	–
AT-140a	5.4	6.2	7.3	0.55
AT-140b	5.4	4.9	< LOQ	–
AT-142	5.0	7.6	8.0	0.62
AT-146	5.3	3.8	< LOQ	–
BE-001	6.6	4.8	18.7	0.17
BE-003	5.0	< LOQ	9.0	–
CO-001	4.7	4.5	< LOQ	–
CU-001	5.4	11.1	6.4	1.11
CU-002	4.9	19.5	10.1	1.24
CZ-001	5.0	13.4	6.9	**1.25**
CZ-003	5.0	5.3	< LOQ	–
CZ-005	4.4	6.2	5.5	0.73
CZ-006	5.0	15.4	14.8	0.67
CZ-007	4.0	5.8	< LOQ	–
DE-004	4.8	9.7	12.5	0.50
DE-005	5.6	8.5	6.6	0.83
DE-014	4.9	< LOQ	5.5	–
DE-015	5.2	4.6	< LOQ	–
DE-029	5.5	5.1	6.8	0.48
DE-030	5.5	6.4	7.5	0.55
DE-031	6.0	5.6	7.4	0.50
DE-032	4.2	29.4	18.8	1.01
DE-033	4.9	4.3	< LOQ	–
DE-034	5.0	4.0	5.7	0.46
DE-044	4.9	6.2	7.1	0.57
DE-046	4.8	3.6	< LOQ	–
DE-052	4.9	15.4	11.6	0.86
DE-060	4.8	3.6	< LOQ	–
DE-062	5.2	4.4	< LOQ	–
DK-003	5.0	7.5	6.9	0.70
DK-006	5.5	4.8	< LOQ	–
DO-001	5.0	18.7	11.4	1.07
FI-001	5.2	5.2	5.6	0.59
FR-002	5.9	< LOQ	9.8	–
GB-006	5.0	4.8	< LOQ	–
GB-015	5.7	6.9	< LOQ	–
HR-001	4.5	6.0	5.6	0.69
HR-004	4.0	11.2	7.5	0.96
HR-005	4.5	6.4	6.9	0.61
HR-006	5.0	9.4	27.4	0.22
HR-009	4.5	4.9	7.1	0.45
HR-018	4.8	16.4	11.7	0.90
HR-020	4.8	6.5	< LOQ	–
HR-021	4.8	4.0	6.8	0.38
HR-022	4.8	6.2	8.6	0.47
HR-023	5.0	4.1	< LOQ	–
HR-026	n.a.	7.1	5.6	0.82
HU-001	4.5	10.7	8.3	0.83
HU-002	5	10.3	10.3	0.64

(*continued*)

(*continued*)

Table 1. Continued.

Beer	Alc (Vol.%)	D3G ($\mu g L^{-1}$)	DON ($\mu g L^{-1}$)	Molar ratio D3G/DON
HU-003	4.6	9.0	13.8	0.42
HU-008	5.2	9.6	9.3	0.67
HU-009	3	12.6	8.4	0.97
HU-011	4.5	16.1	12.9	0.81
HU-012	4.0	33.5	22.0	0.99
HU-013	5.0	5.0	<LOQ	–
HU-014	4.0	13.1	22.3	0.38
HU-015	4.0	21.6	13.9	1.00
HU-016	3.0	8.0	<LOQ	–
HU-017	4.5	11.2	28.0	0.26
HU-019	5.0	6.8	11.7	0.37
IE-003	4.3	5.5	5.7	0.62
IT-001	4.6	7.7	24.8	0.20
NL-002	5.0	5.3	6.7	0.52
PH-002	**6.9**	<LOQ	13.1	–
SI-001	4.9	10.1	19.3	0.34
SI-002	4.9	9.3	15.7	0.38
SI-003	4.9	6.3	24.1	0.17
SR-003a	5.0	18.2	33.6	0.35
SR-003b	5.0	11.5	30.6	0.24
SR-004	5.0	3.7	19.5	0.12
SR-005	5.0	16.8	31.8	0.34
SR-007	4.6	5.1	17.8	0.18
SR-008	4.8	9.4	22.1	0.27
SR-009	5.0	10.4	32.5	0.21
XX-003	4.8	6.0	5.4	0.72
Average all beers		6.7	7.5	–
Median all beers		5.2	5.6	–
Average cont. beers		9.3	12.0	0.61
No. of beers >LOQ		142	118	113

Notes: n.a. not available; LOQ = limit of quantification; LOD = limit of detection; D3G: LOD 0.4 $\mu g L^{-1}$, LOQ 3.5 $\mu g L^{-1}$; DON: LOD 2.2 $\mu g L^{-1}$, LOQ 5.4 $\mu g L^{-1}$; 3ADON: LOD 2.4 $\mu g L^{-1}$, LOQ 6.8 $\mu g L^{-1}$; highest values in this beer category marked in bold; 70 out of 217 samples below LOQ for all three analytes (number in parentheses is the number of noncontaminated beers): AT (15), DE (14), GB (7), HR (4), MX (4), BR (3), CH (2), NG (2), PE (2), TH (2), XX (2), AU (1), BE (1), BO (1), CN (1), CO (1), ES (1), KE (1), KR (1), NL (1), PH (1), SG (1), TR (1), TT (1).

contaminated beers above the LOQ, the average D3G content increased to 9.5 $\mu g L^{-1}$ and the average DON content reached 13.6 $\mu g L^{-1}$. These values are in good agreement with previous findings. For instance, Kostelanska et al. (2009) reported 8.5 $\mu g L^{-1}$ for D3G and 6.6 $\mu g L^{-1}$ for DON and Anselme et al. (2006) determined 5.0 $\mu g L^{-1}$ for DON. In our study, values above 40 $\mu g L^{-1}$ of DON were observed in 6 cases: a pale (AT-133; 89 $\mu g L^{-1}$) and a dark beer (AT-007; 41 $\mu g L^{-1}$) from Austria, two wheat beers (DE-061 50 $\mu g L^{-1}$ and DE-057 42 $\mu g L^{-1}$) and a dark beer (DE-058 45 $\mu g L^{-1}$) from Germany and a dark beer (SR-006 43 $\mu g L^{-1}$) from Serbia. In the case of D3G, only two pale beers from Austria contained values above 40 $\mu g L^{-1}$ (AT-133 81 $\mu g L^{-1}$ and AT-087 40 $\mu g L^{-1}$).

Table 2. Analysed wheat beer samples ($n = 46$; 36 samples >LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g L^{-1}$)	DON ($\mu g L^{-1}$)	Molar ratio D3G/DON
AT-006	n.a.	8.6	13.4	0.41
AT-008	7.1	11.0	24.0	0.30
AT-009	5.3	14.7	26.9	0.35
AT-010a	4.5	14.1	25.8	0.35
AT-010b	4.5	12.6	23.0	0.35
AT-018	5.3	<LOQ	17.8	–
AT-026	5.0	28.0	37.2	0.49
AT-033	5.2	5.5	8.9	0.40
AT-079	5.3	<LOQ	5.5	–
AT-091	5.0	12.0	20.4	0.38
AT-093	6.5	25.2	36.3	0.45
AT-095	5.2	4.0	6.4	0.40
AT-114	5.0	6.3	9.5	0.43
AT-117	5.1	7.6	19.6	0.25
AT-126	5.5	18.8	34.1	0.36
AT-136	5.0	9.5	14.5	0.42
AT-137	7.8	20.4	35.7	0.37
DE-003	5.5	9.2	16.6	0.36
DE-017	n.a.	<LOQ	5.5	–
DE-018	4.7	5.3	9.6	0.36
DE-020	5.3	6.6	12.2	0.35
DE-022	5.3	5.1	7.9	0.42
DE-026	5.0	3.7	5.8	0.41
DE-028	5.5	4.9	8.1	0.39
DE-036	5.5	<LOQ	5.2	–
DE-040	5.2	4.5	8.1	0.36
DE-043	5.5	8.9	14.0	0.41
DE-047	n.a.	6.5	8.0	**0.53**
DE-048	**8.2**	4.0	12.0	0.21
DE-049	5.4	4.8	6.8	0.46
DE-057	5.4	21.4	42.4	0.33
DE-061	4.9	**28.4**	**49.6**	0.37
DE-063	5.0	18.4	31.7	0.38
HR-015	5.0	17.1	22.6	0.49
HR-016	5.3	3.5	5.8	0.40
IT-002	4.8	16.9	29.7	0.37
Average all beers		8.4	14.6	–
Median all beers		5.4	9.5	–
Average cont. beers		11.5	18.4	0.38
No. of beers >LOQ		32	36	32

Notes: n.a. not available; LOQ = limit of quantification; LOD = limit of detection; D3G: LOD 0.9 $\mu g L^{-1}$, LOQ 3.5 $\mu g L^{-1}$; DON: LOD 1.0 $\mu g L^{-1}$, LOQ 4.5 $\mu g L^{-1}$; 3ADON: LOD 2.2 $\mu g L^{-1}$, LOQ 8.2 $\mu g L^{-1}$; highest values in this beer category marked in bold; 10 out of 46 samples below LOQ for all three analytes (number in parentheses is the number of noncontaminated beers): GB (6), DE (3), FR (1).

To ensure that our collected samples are representative for the whole batch, three bottles from the same lot were purchased in June 2012 ($n = 13$). The relative standard deviations of the concentrations ranged from 1.0 to 13% in the case of DON and from 0.8 to 5.2% in the case of D3G in these beers. As these values were lower than the method repeatabilities, we conclude that the distribution of the measured toxins is sufficiently

Table 3. Analysed dark beer samples ($n = 47$; 28 samples > LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g\,L^{-1}$)	DON ($\mu g\,L^{-1}$)	Molar ratio D3G/DON
AT-007	5.3	25.3	40.9	0.40
AT-032	4.5	9.9	11.1	0.57
AT-034	5.0	10.6	11.3	0.61
AT-052	4.7	11.4	< LOQ	–
AT-064	4.6	4.2	< LOQ	–
AT-068	5.2	11.0	< LOQ	–
AT-073	5.2	4.4	< LOD	–
AT-088	5.0	16.3	11.4	**0.93**
AT-090	5.0	16.6	22.5	0.48
AT-134	5.2	9.4	< LOQ	–
AT-135	5.0	24.4	29.3	0.54
AT-141	3.4	10.7	< LOQ	–
CZ-004	4.7	6.5	< LOD	–
DE-019	5.6	4.7	< LOQ	–
DE-021	7.3	9.8	15.6	0.41
DE-024	5.0	5.4	11.1	0.31
DE-035	5.1	9.1	< LOQ	–
DE-045	**7.9**	23.0	24.5	0.61
DE-058	5.3	**26.2**	**45.0**	0.38
DE-059	4.7	5.5	< LOQ	–
DE-064	n.a.	4.2	< LOD	–
DK-001	7.7	4.2	14.6	0.19
HR-003	7.2	5.8	21.1	0.18
HR-010	4.5	6.4	< LOQ	–
HR-019	5.2	12.9	< LOQ	–
IE-002	5.0	5.6	12.5	0.29
SR-006	6.2	10.2	42.6	0.15
XX-002	5.3	4.5	< LOQ	–
Average all beers		6.9	9.0	–
Median all beers		4.5	5.5	–
Average cont. beers		10.7	22.4	0.43
No. of beers > LOQ		28	14	14

Notes: n.a. not available; LOQ = limit of quantification; LOD = limit of detection; D3G: LOD $1.4\,\mu g\,L^{-1}$, LOQ $4.1\,\mu g\,L^{-1}$; DON: LOD $2.9\,\mu g\,L^{-1}$, LOQ $11\,\mu g\,L^{-1}$; 3ADON: LOD $4.3\,\mu g\,L^{-1}$, LOQ $11\,\mu g\,L^{-1}$; highest values in this beer category marked in bold; 19 out of 47 samples below LOQ for all three analytes (number in parentheses is the number of noncontaminated beers): GB (5), DE (2), DK (2), MX (2), NG (2), AT (1), HR (1), IE (1), JM (1), PE (1), TG (1).

homogenous to allow sampling of just one bottle of beer to still achieve representative results. Furthermore, the 13 chosen beers were of the same brand as the highly contaminated samples from January 2012. After five months, six of the thirteen beers were still contaminated with DON and D3G in the same high concentrations. However, seven beers showed lower contaminations, suggesting different raw materials for the production of these lots.

Kostelanska et al. (2009) and Papadopoulou-Bouraoui et al. (2004) reported a positive correlation between the mycotoxin levels and the alcohol content. These findings are also reflected in the presented study. The nonalcoholic beers showed the lowest percentage of contamination (47 and 26% positive samples

Table 4. Analysed bock beer samples ($n = 20$; all samples > LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g\,L^{-1}$)	DON ($\mu g\,L^{-1}$)	Molar ratio D3G/DON
AT-004	9.2	2.4	< LOD	–
AT-015	7.2	11.6	10.2	0.73
AT-016	7.2	6.9	8.4	0.53
AT-044	7.0	29.8	20.2	0.95
AT-048	7.5	32.2	21.8	0.95
AT-063	7.0	10.9	10.0	0.70
AT-069	7.3	18.0	14.7	0.79
AT-072	7.6	7.3	7.3	0.64
AT-094	**14.0**	5.8	18.9	0.20
AT-099	9.6	11.4	11.4	0.65
AT-106	7.2	12.5	7.2	**1.12**
AT-112	7.0	11.9	8.4	0.91
AT-119	8.3	6.9	6.5	0.69
AT-124	6.8	22.3	16.3	0.89
AT-143	6.8	24.5	17.9	0.88
CZ-002	7.6	26.7	19.0	0.91
DE-006	8.2	11.8	10.1	0.75
DE-016	11.0	**33.3**	**27.1**	0.79
DK-002	10.0	3.1	< LOD	–
DK-004	8.0	6.6	12.0	0.35
Average all beers		14.8	12.4	–
Median all beers		11.7	10.8	–
Average cont. beers		14.8	13.8	0.75
No. of beers > LOQ		20	18	18

Notes: LOQ = limit of quantification; LOD = limit of detection; D3G: LOD $0.5\,\mu g\,L^{-1}$, LOQ $1.5\,\mu g\,L^{-1}$; DON: LOD $1.2\,\mu g\,L^{-1}$, LOQ $4.1\,\mu g\,L^{-1}$; 3ADON: LOD $3.6\,\mu g\,L^{-1}$, LOQ $9.2\,\mu g\,L^{-1}$; highest values in this beer category marked in bold; all 20 samples contained at least D3G above the LOQ.

Table 5. Analysed nonalcoholic beer samples ($n = 19$; 9 samples > LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g\,L^{-1}$)	DON ($\mu g\,L^{-1}$)	Molar ratio D3G/DON
AT-003	n.a.	2.0	< LOD	–
AT-056	n.a.	3.1	3.7	0.54
AT-100	n.a.	2.8	< LOD	–
DE-008	n.a.	2.1	< LOD	–
DE-055	n.a.	1.6	< LOD	–
HR-007	0.5	2.6	3.8	0.44
HR-012	0.5	3.1	6.5	0.31
HU-007	0.5	3.0	3.2	**0.60**
SR-001	0.5	**6.6**	**26.1**	0.16
Average all beers		1.5	2.7	–
Median all beers		0.2	0.6	–
Average cont. beers		3.0	8.7	0.41
No. of beers > LOQ		9	5	5

Notes: n.a. not available; LOQ = limit of quantification; LOD = limit of detection; D3G: LOD $0.4\,\mu g\,L^{-1}$, LOQ $1.4\,\mu g\,L^{-1}$; DON: LOD $1.2\,\mu g\,L^{-1}$, LOQ $3.0\,\mu g\,L^{-1}$; 3ADON: LOD $2.6\,\mu g\,L^{-1}$, LOQ $6.0\,\mu g\,L^{-1}$; highest values in this beer category marked in bold; 10 out of 19 samples below LOQ for all three analytes (number in parentheses is the number of noncontaminated beers): AE (3), IR (3), AT (2), DE (2).

Table 6. Analysed shandies ($n = 25$; 20 samples > LOQ for at least one analyte).

Beer	Alc (Vol.%)	D3G ($\mu g\,L^{-1}$)	DON ($\mu g\,L^{-1}$)	Molar ratio D3G/DON
AT-013	2.2	3.3	< LOQ	–
AT-029	2.0	4.3	4.4	0.64
AT-030	2.0	4.8	5.0	0.62
AT-054	n.a.	1.9	< LOQ	–
AT-061	2.5	4.5	< LOQ	–
AT-070	2.9	1.8	< LOQ	–
AT-082	2.2	**7.9**	7.1	**0.72**
AT-083	2.0	5.5	6.4	0.55
AT-105	2.1	2.8	< LOQ	–
AT-145	2.5	2.8	< LOQ	–
BE-002	6.0	1.8	11.2	0.11
HR-013	2.0	4.0	6.3	0.41
HR-014	2.0	2.5	4.9	0.32
HR-017	2.0	2.9	< LOQ	–
HU-004	2.0	2.5	4.2	0.38
HU-005	2.0	5.0	7.5	0.43
HU-006	2.0	6.2	6.8	0.59
HU-010	2.0	3.5	4.2	0.53
HU-018	2.0	2.9	8.6	0.22
SR-02	2.0	5.4	**12.7**	0.27
Average all beers		3.2	4.4	–
Median all beers		2.9	4.2	–
Average cont. beers		3.8	6.9	0.45
No. of beers > LOQ		20	13	13

Notes: n.a. not available; LOQ = limit of quantification; LOD = limit of detection; D3G: LOD $0.4\,\mu g\,L^{-1}$, LOQ $1.3\,\mu g\,L^{-1}$; DON: LOD $1.5\,\mu g\,L^{-1}$, LOQ $3.9\,\mu g\,L^{-1}$; 3ADON: LOD $2.7\,\mu g\,L^{-1}$, LOQ $10\,\mu g\,L^{-1}$; highest values in this beer category marked in bold; 5 out of 25 samples below LOQ for all three analytes (number in parentheses is the number of noncontaminated beers): AT (5).

for D3G and DON, respectively). According to Kostelanska et al. (2009), this is probably a consequence of the different technological processes involved in the production of nonalcoholic beer compared to alcoholic beers. These involve earlier stopped fermentation, the use of specific yeasts and dilution of the beer.

Concerning the molar ratio of D3G to DON, no obvious differences among the different categories of beer were determined. The average for all beers ($n = 195$) was 0.56 ± 0.23 and single values ranged between 0.11 and 1.25 (Tables 1–6). These findings support the previously published data at a smaller scale from Kostelanska et al. (2009), who reported D3G/DON molar ratios between 0.58 and 1.24 in light and dark beers ($n = 176$). Compared to DON, the D3G content can thus be lower, equal or higher and it is not possible to predict the concentration of D3G by measuring DON alone. Cleavage of D3G in the gastrointestinal tract is likely as *in vitro* studies with intestinal lactic acid bacteria reported release of DON from D3G (Berthiller et al. 2011). Hence the concentration of D3G might have a significant impact and should be monitored to gain further occurrence data. Increased D3G/DON ratios in beer are due to a variety of processes in beer production. During the germination phase of malting, levels of DON and its conjugates increase compared to concentrations in the original barley grains. Reasons are the *de novo* production of DON and ADONs by growing fungi (Lancova et al. 2008), formation of D3G by glucosyltransferases from barley (Maul et al. 2012) and the release of D3G from higher glycosides and insoluble complex polysaccharide and protein structures (Lancova et al. 2008). The increase of D3G is due to high enzymatic activity and continues at the mashing stage of brewing (Lancova et al. 2008).

It is notable that only 2 out of 20 tested beer samples from the UK showed contamination above the LOQ (Table 7). These findings are in agreement with the reports of the Malters Association of Great Britain (MAGB) and Home Grown Cereals Authority (HGCA) of the UK. They state that the levels of mycotoxin contamination of malting barley is low in the UK, and malters in the UK allow a maximum limit of $500\,\mu g\,kg^{-1}$ for DON in barley due to the requirements of customers (Baxter and Muller 2006; Edwards 2007; MAGB 2011). Contamination with other mycotoxins still has to be investigated.

So far, no regulatory maximum level for any mycotoxin in beer has been set by the European legislation, but according to JECFA (2011), the PMTDI is $1\,\mu g\,kg^{-1}$ bw per day for the sum of DON, 3- and 15ADON. A poll conducted by an Austrian agency revealed that the average Austrian female and male (>16 years) weigh 67 kg and 81 kg, respectively (IMAS 2003). The Austrian brewery association reported a total beer consumption of $8.88\,10^8$ L in 2010 (Bierserver 2012). Having a total population of 8,404,252 on 1 January 2011 (Statistics Austria 2011), the per capita consumption of beer in Austria in 2010 was 105.6 L per year (Bierserver 2012). If only the Austrian population above the legal age of alcohol consumption (>16 years) is taken into consideration (7,075,911 at 1 January 2011, Statistics Austria 2011), the average per day consumption of beer is 0.34 L. In an average scenario, taking only the mean DON (and 3ADON) content of all beers ($8.4\,\mu g\,L^{-1}$) into account, the contribution of DON from beer to the PMTDI is 3.5% for males and 4.3% for females above 16 years. When considering also the D3G content and presuming a total cleavage to DON in the intestinal tract ($12.8\,\mu g\,L^{-1}$) and hence the same toxicity, these values rise to 5.4 and 6.6% for males and females, respectively. In 2000, Molto et al. calculated mean daily intakes of DON by beer consumption between $0.9\,ng\,kg^{-1}$ bw for males between 25 and 50 years and $16.4\,ng\,kg^{-1}$ bw for males between 18 and 24 years. Considering the PMTDI of $1\,\mu g\,kg^{-1}$ bw,

Table 7. Number of samples from each country of origin in the different contamination ranges (μg L^{-1}) of deoxynivalenol-3-glucoside (first number) and deoxynivalenol (second number).

Country[a]	No.[b]	< LOD[c]	LOD-LOQ[c]	LOQ-5	5–10	10–20	20–30	30–40	> 40
AT	156	2; 14	24; 38	29; 2	57; 56	32; 28	9; 12	1; 4	2; 2
DE	64	3; 17	21; 15	15; –	16; 16	3; 10	5; 2	1; 1	–; 3
HR	26	–; 4	5; 6	9; 2	8; 10	4; 1	–; 3	–	–
GB	20	6; 15	12; 5	1; –	1; –	–	–	–	–
HU	19	–	–; 2	4; 3	7; 6	6; 5	1; 3	1; –	–
SR	10	–	–	1; –	4; –	5; 3	–; 2	–; 4	–; 1
CZ	7	–; 1	–; 2	–	4; 2	2; 2	1; –	–	–
DK	7	–; 2	2; 2	3; –	2; 1	–; 2	–	–	–
MX	6	5; 5	1; 1	–	–	–	–	–	–
BE	4	–; 1	2; –	2; –	–; 1	–; 2	–	–	–
NG	4	2; 3	2; 1	–	–	–	–	–	–
AE	3	3; 3	–	–	–	–	–	–	–
BR	3	–; 3	3; –	–	–	–	–	–	–
IE	3	–	1; 1	–	2; 1	–; 1	–	–	–
IR	3	3; 3	–	–	–	–	–	–	–
PE	3	–; 3	3; –	–	–	–	–	–	–
SI	3	–	–	–	2	1; 2	–; 1	–	–
CH	2	–	2; 2	–	–	–	–	–	–
CO	2	–; 1	1; 1	1; –	–	–	–	–	–
CU	2	–	–	–	–; 1	2; 1	–	–	–
FR	2	–	2; 1	–	–; 1	–	–	–	–
IT	2	–	–	–	1; –	1; –	–; 2	–	–
NL	2	–; 1	1; –	–	1; 1	–	–	–	–
PH	2	–	2; 1	–	–	–; 1	–	–	–
TH	2	–; 2	2; –	–	–	–	–	–	–
AU	1	–; 1	1; –	–	–	–	–	–	–
BO	1	1; 1	–	–	–	–	–	–	–
CN	1	–; 1	1; –	–	–	–	–	–	–
DO	1	–	–	–	–	1; 1	–	–	–
ES	1	–	1; 1	–	–	–	–	–	–
FI	1	–	–	–	1; 1	–	–	–	–
JM	1	–	1; 1	–	–	–	–	–	–
KE	1	–; 1	1; –	–	–	–	–	–	–
KR	1	–	1; 1	–	–	–	–	–	–
SG	1	–	1/1	–	–	–	–	–	–
TG	1	1; 1	–	–	–	–	–	–	–
TR	1	–; 1	1; –	–	–	–	–	–	–
TT	1	–	1; 1	–	–	–	–	–	–
XX	4	–; 1	2; 2	1; –	1; 1	–	–	–	–
All	374	26; 85	97; 85	66; 7	107; 98	57; 59	16; 25	3; 9	2; 6

Notes: [a]Two letter country code according to ISO 3166-1 (ISO 2011). [b]Total number of samples from this country. [c]Limits of detection (LODs) and quantification (LOQs) depend on the category of beer.

the contribution of DON from beer to the PMTDI ranged between 0.09 and 1.6%. Strikingly, Warth et al. (2012) reported that 9 out of 27 Austrian volunteers already exceeded the PMTDI when consuming a regular diet.

Both the values obtained in the current study and the data published by Molto et al. (2000) demonstrate that DON ingested by moderate beer consumption is not of toxicological relevance. However, if a person consumes two large beers (1 L) per day, the contribution of DON from beer to the PMTDI of DON rises to 10.3% for males and to 12.5% for females if only DON is considered. If the sum of DON and D3G is taken into account, the values are 15.8 and 19.1% for males and females, respectively. In a high scenario, taking the 90th percentile of DON and D3G contamination of beers, the contribution to the PMTDI rises to 38.1 and 46.1% for males and females, respectively. In the worst case, consumption of one litre of the highest contaminated beer (89 μg L^{-1} DON) would already lead to exceeding the PMTDI of DON for an average individual.

Conclusion

To our best knowledge, the present survey is one of the largest ever performed to study the occurrence of DON in beer, and it is definitively the largest to study the occurrence of the masked mycotoxin deoxynivalenol-3-glucoside in this commodity. While the majority of the

beer samples was contaminated with DON and D3G below 10 $\mu g\,L^{-1}$, about a quarter of all beers showed contamination of both or either of the two analytes above that value. 3ADON was not detected in any of the samples due to higher LODs. Interestingly, the molar ratio of D3G/DON varied largely between 0.11 and 1.25 in different beers, suggesting that it is almost impossible to predict the concentration of D3G from the concentration of DON alone. While toxicological data for the significance of D3G are still lacking, the occurrence of DON in such a huge number of samples and especially in several highly contaminated beers is alerting. Fifteen out of 374 beers (4%) showed contamination levels above 30 $\mu g\,L^{-1}$, while in one beer almost 90 $\mu g\,L^{-1}$ of DON was quantified. The average values of DON and D3G (8.4 and 6.9 $\mu g\,L^{-1}$, respectively) were in line with other studies and well below any toxicological concern for moderate beer drinkers. However, for heavy beer drinkers, the contribution of this commodity to the daily intake of DON is not negligible. Under unfavourable conditions, the PMTDI can even be exceeded solely by beer consumption. We conclude also that future surveys should monitor the occurrence of DON, 3ADON and D3G in beer. While the overwhelming majority of breweries seem to very carefully and responsibly monitoring the grains used in beer production, official maximum levels in beer might help to protect consumers from highly contaminated beers.

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