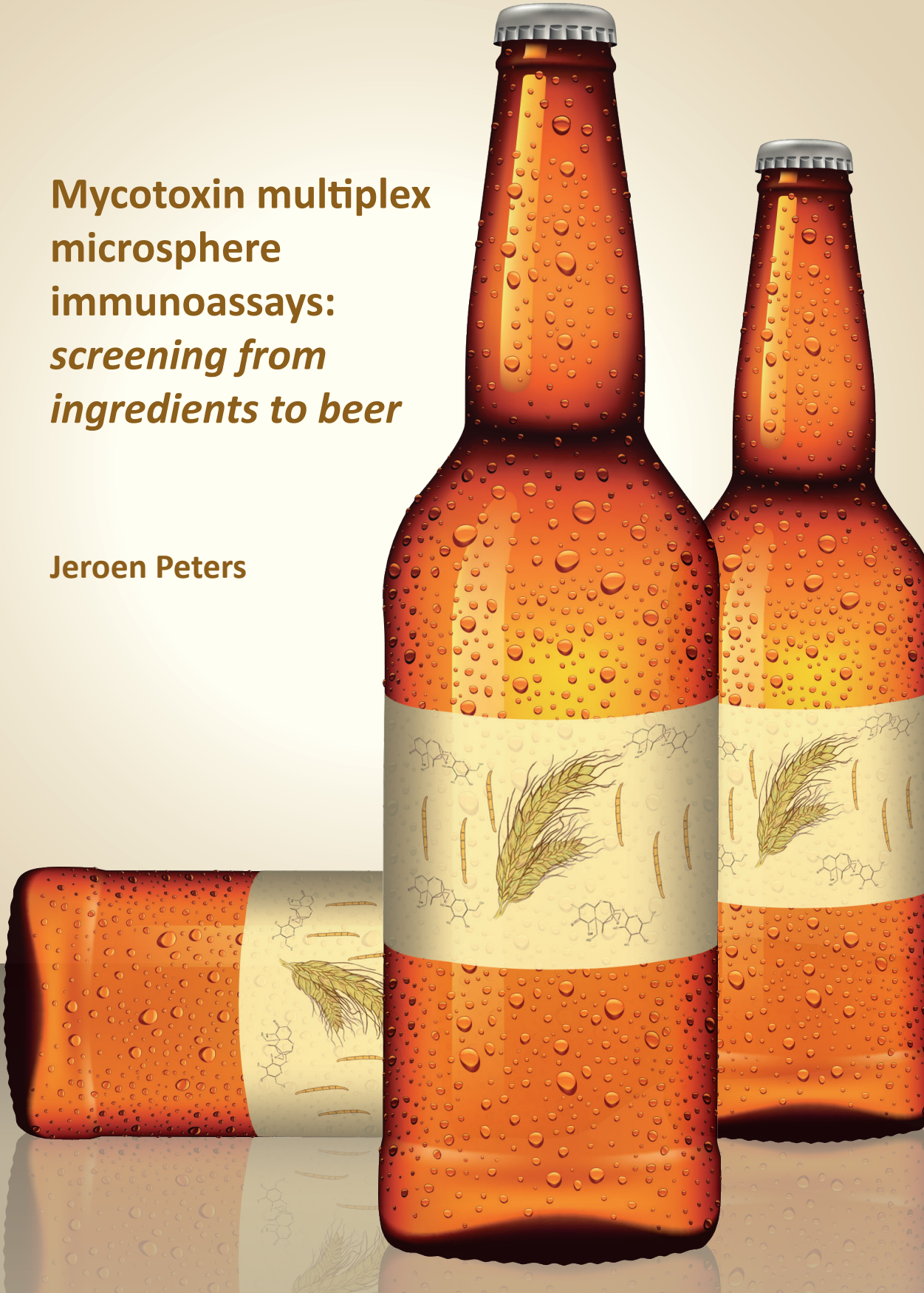
Mycotoxin multiplex microsphere immunoassays: *screening from ingredients to beer*

Jeroen Peters



Propositions

1. Once unmasked, masked mycotoxins should no longer be referred to as masked mycotoxins.
   (this thesis)

2. Regarding Maximum Levels for mycotoxins, beer cannot be considered as a processed cereal.
   (this thesis)

3. For the detection of food contaminants, multiplex immunoassays are not greater than the sum of their parts.

4. The sample extraction and preparation part of biosensing methods is often neglected when discussing rapid on-site analysis.

5. Aptamers for the detection of small molecules, are not the solution for the replacement of antibodies in standard immunoassay approaches for food safety.

6. Online collaborative research meetings are not the future.

7. Where flex-working at home decreases bonding from a distance, flex-desks decrease bonding at work.

8. Classic vinyl records sound superior to the ones currently produced.

Propositions belonging to the thesis entitled:

Mycotoxin multiplex microsphere immunoassays: screening from ingredients to beer.

Jeroen Peters

Wageningen, 30 May 2022

Mycotoxin multiplex microsphere immunoassays: screening from ingredients to beer

Jeroen Peters

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Mycotoxin multiplex microsphere immunoassays: screening from ingredients to beer

Jeroen Peters

Thesis

Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 30 May 2022
at 11 a.m. in the Omnia Auditorium.

Jeroen Peters

Mycotoxin multiplex microsphere immunoassays: screening from ingredients to beer

280 pages

PhD thesis, Wageningen University, Wageningen, NL (2022)

With references, with summary in English

DOI: https://doi.org/10.18174/568182

ISBN: 978-94-6447-195-3

We've come to this particular place tonight

'Cause we gotta look at things from every angle

We need some answers to some complicated questions

If we're going to get it right

Randy Newman – The Great Debate (from Dark Matter)

Table of contents

Abbreviations

General abbreviations

Ab(s)	Antibody (Antibodies)
ACN	Acetonitrile
BSA	Bovine Serum Albumin
CCD	charge-coupled device
DAS	Double Antibody Sandwich
EC	European Commission
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked immunosorbent assay
EU	European Union
FPIA	Fluorescence Polarization ImmunoAssay
HRMS	High Resolution Mass Spectrometry
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin
iSPR	imaging Surface Plasmon Resonance
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
LED	Light Emitting Diode
LFD	Lateral Flow Device
LFIA	Lateral Flow ImmunoAssay
LOC	Limit of Quantification
LOD	Limit of Detection
mAb(s)	monoclonal Antibody (Antibodies)
MeOH	Methanol
MES	2-(N-morpholino) ethanesulfonic acid
MIA	Micropshere ImmunoAssays
MIP	Molecular Printed Polymer
ML(s)	Maximum Level (s)
MQ	Milli Q water
NMR	Nuclear Magnetic Resonance
pAb(s)	polyclonal Antibody (Antibodies)
PBS	Phosphate Buffered Saline
PMT	PhotoMultiplier Tube
ppb	parts per billion
ppm	parts per million

Mycotoxin abbreviations

15ADON	15-acetyl-DON
3ADON	3-acetyl-DON
ADONs	Sum of acetyl-DONs
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
AFs	aflatoxins
AME	alternariol methyl ether
AOH	alternariol
D3G	deoxynivalenol-3-glucoside
DON	deoxynivalenol
EAs	ergot alkaloids
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FB_s	fumonisins
HT-2	HT2-toxin
HT2-3G	HT-2-toxin-3-glucoside
NIV	nivalenol
NIV3G	nivalenol-3-glucoside
OTA	ochratoxin A
PAT	patulin
STC	sterigmatocystin
T-2	T2-toxin
T2-3G	T-2-toxin-3-glucoside
TEA	tenuazonic acid
TEN	tentoxin
Z14G	zearalenone-14-glucoside
Z14S	zearalenone-14-sulphate
Z16G	zearalenone-16-glucoside
ZAN	zearalanone
ZEN	zearalenone

General abbreviations

ppt	parts per trillion
PTDI	Provisional Tolerable Daily Intake
rAb(s)	recombinant Antibody (Antibodies)
RIS	Russian Imperial Stout
RPE	R-Phycoerythrin
scFv	single-chain variable Fragment
S-NHS	N-hydroxysulfosuccinimide
SPR	Surface Plasmon Resonance
TDI	Tolerable Daily Intake
WFSR	Wageningen Food Safety Research
WHO	World Health Organization
xMAP	MultiAnalyte Profiling
%ABV	Percentage Alcohol By Volume

Mycotoxin abbreviations

α-ZAL	α-zearalanol
α-ZEL	α-zearalenol
β-ZAL	β-zearalanol
β-ZEL	β-zearalenol

CHAPTER

1

General Introduction

1. Introduction

1.1 Food safety and food safety control

According to an estimation of the World Health Organization, annually 600 million people get ill after consuming contaminated food, while 420,000 people even die from consuming contaminated food. The presence of microorganisms, parasites and chemical substances in food can cause over 200 different symptoms, that range from diarrhoea to cancers. Among these, diarrhoea is the biggest cause of all illnesses and deaths [1]. To prevent people getting ill, or dying, from eating contaminated foods, governmental bodies control food and feed commodities for a variety of contaminants. Food supply chains are a global network, which means that many commodities are imported from third countries into the European Union (EU). In 2020, the European Rapid Alert System for Food and Feed (RASFF) reported hazards in products originating from non-EU member states. The three highest-ranked contaminants were pesticide residues, pathogenic microorganisms and mycotoxins. These were detected in a wide range of matrices, e.g. nuts, fruits, herbs, fish and cereals. For mycotoxins, hazards from aflatoxins, ochratoxin A and deoxynivalenol were the most notified, with nuts being the most notified matrix. Germany and The Netherlands reported most notifications for aflatoxins in imported food. Figs from Turkey showed the highest incidence for aflatoxin contamination [2]. This underlines that monitoring of food for mycotoxins, and food contaminants in general, is crucial and that well-organized detection strategies should be in place to ensure food safety. Therefore, the EU has implemented General Food Law Regulation, to ensure a high level of consumer protection [3]. It defines requirements and procedures that are the basis for decision making in food, and feed, production. Food producers need to ensure their products are safe for human health. Next to that, through EU established food and feed safety regulations, control strategies in every country protect the health of humans and animals and guarantee that exported and imported commodities are safe to enter the food and feed processing and production chain and/or can be consumed directly [4]. To be able to guarantee safe food and feed, the development of methods for the detection of contaminants is important. Those methods preferably detect multiple relevant contaminants at the

same time. In this thesis, the development and application of screening-based methods for the detection of multiple mycotoxins are presented.

1.2 Mycotoxins

Mycotoxins are fungal metabolites produced by certain fungi species as a part of their plant invasive actions. The most prominent mycotoxin producing fungi that affect food products belonging to the families of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* [5]. Upon ingestion, mycotoxins can cause mycotoxicosis. The symptoms of mycotoxicosis depend on the class of mycotoxin and the intensity of the exposure. Synergistic effects may occur, when exposed to more than mycotoxin. Their effects on human and animal health can be acute or chronic [5]. These effects can be mild (immunosuppression, diarrhoea, reduced fertility) or severe (carcinogenic or even lethal) [6-8]. The suspected carcinogenic properties of mycotoxins are mainly supported by experimental studies, although some evidence comes from human epidemiological studies conducted in China and South-Africa [9]. New epidemiological studies could shed further light on the carcinogenic potency of a wider range of mycotoxins. Mycotoxins can be present in cereals, nuts, fruits, coffee, cacao, spices and several other commodities [10]. Contamination of mycotoxins can occur in the field and may continue, depending on environmental conditions, throughout storage, processing and transportation. Higher mycotoxin contaminations tend to occur in remote areas of the world where general knowledge is absent, poor handling and incorrect storage happen or because of the absence of regulations [5]. Cereal commodities are the basis for food and feed, and these can be contaminated by what are considered to be the most important mycotoxins in terms of agriculture and animal production; zearalenone (ZEN), deoxynivalenol (DON), T-2 toxin (T-2), aflatoxins (AFs), ochratoxin A (OTA) and fumonisins (FB_s). Since mycotoxin production in cereals is directly related to temperature and humidity, and wind is a factor for fungal distribution, weather conditions affect the occurrence of mycotoxins. Therefore, climate change raises concerns in relation to mycotoxin production. For example, increasing temperatures and decreasing humidity within Europe, may be the onset for higher incidences of AFs production, which is a direct health concern. On the other hand, these same conditions are less favourable circumstances for the production of OTA. However, research has shown that there

was a higher ochratoxigenic risk under higher carbon dioxide concentrations. These findings show that a change in mycotoxin occurrence is to be expected if climate change is not taken seriously [11-13]. In their world mycotoxin survey of 2020, BIOMIN monitored the mycotoxin prevalence and incidence in cereals for the six major mycotoxins in agricultural commodities used for livestock feed [14]. In total 21,709 samples, originating from 79 countries were analysed for their mycotoxin content. 67% of the samples analysed, contained more than one mycotoxin, while 22% of the samples contained only one mycotoxin. In 11% of the samples no mycotoxins were present based on the set limit of detection (LOD). For central Europe, 6,696 samples were analysed for the presence of ZEN, DON, T-2, AFs, OTA and FBs. Their presence was found in 47%, 60%, 32%, 7%, 15% and 46% of the samples respectively. The average contaminations found were 100 ppb for ZEN, 531 ppb for DON, 31 ppb for T-2, 6 ppb for AFs, 9 ppb for OTA and 31 ppb for FBs. The following paragraphs provide an overview over these various classes of mycotoxins, their occurrence and health effects, with the aim to display both the highly varied nature and the severity of their health effects. Additionally, the occurrence of these mycotoxins in beers will be discussed.

1.2.1 Aflatoxins

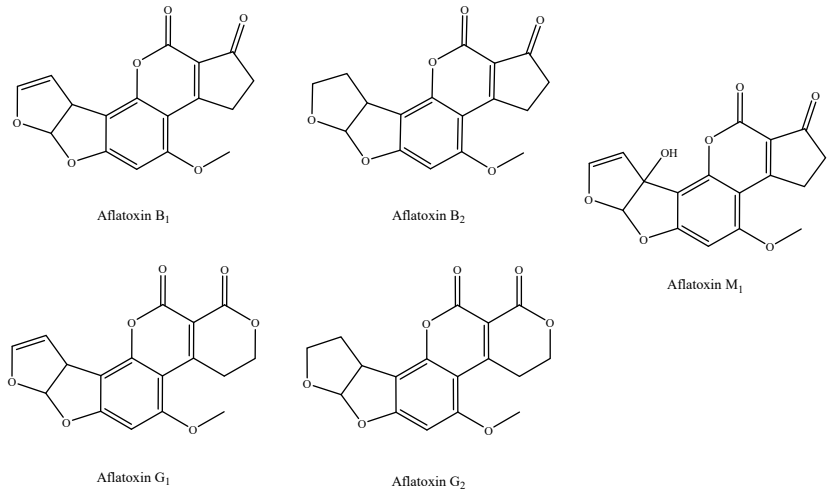


Figure 1. Chemical structures of the main aflatoxins

AFs derive their name from *Aspergillus flavus,* together with *Aspergillus parasiticus*, the main producer of AFs [15]. AFs were first discovered in 1961, when contaminated groundnut meal was fed to turkey poults, causing over a 100,000 deaths [16]. AFs occur in cereals like wheat, maize and barley [17-19], but also occur in commodities like nuts, spices, fruits, honey, milk, wine and beer [20-22]. The main aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (see Fig. 1). From this group, AFB_1 is the main occurring aflatoxin and at the same time the most toxic. AFB_1 is the most potent natural carcinogen that exists and can be metabolised to aflatoxin M_1 (AFM_1) and end up in the milk of lactating animals [23]. AFs, including AFM_1, are classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC), meaning that they are carcinogenic to humans [24]. Because of these risks, the EU has set maximum levels (MLs) for a wide range of food stuffs for AFs. The ML for all cereals, and all products derived from cereals (including processed cereal products), is set at 4 μg/kg. Exceptions are made for maize and rice (10 μg/kg) and food for infants (0.1 μg/kg) [25].

1.2.2 Fumonisins

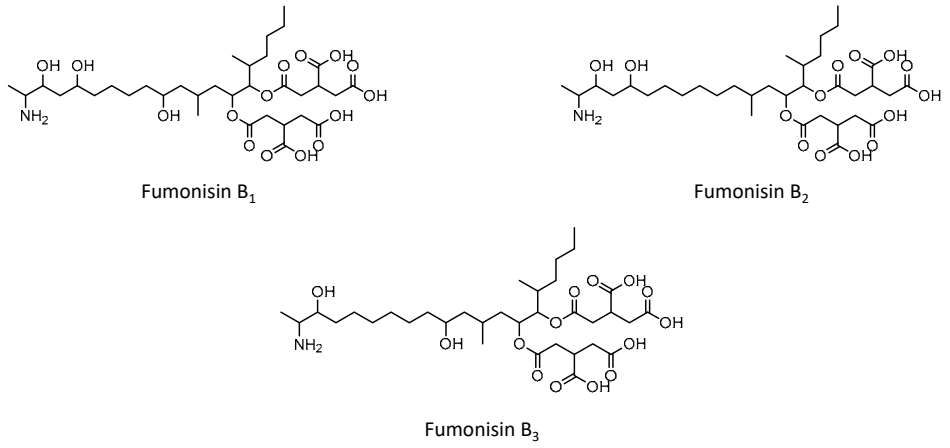
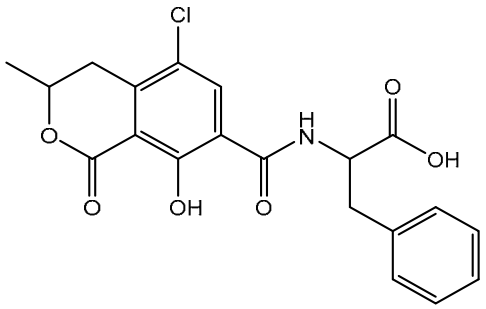


Figure 2. Chemical structures of the main fumonisins

FBs were first identified in cultures of *F. verticillioides* in 1988 [26]. There are 28 fumonisin analogues, which can be separated into four groups: fumonisins A, B, C and P [27]. The FBs most likely to contaminate grain commodities are, in decreasing

order off occurrence, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) [28] (see Fig. 2). FBs mainly occur in maize and sorghum [29] but its occurrence in other cereals, cannot be excluded [30]. Maize contaminated with FBs has been retrospectively attributed to the high incidence of oesophageal cancer in the Transkei region of southern Africa and in Northern China [31,32]. Evidence suggests that FB_1 is also responsible for leukoencephalomalacia (ELEM) in horses [33]. The IARC has classified FB_1 and FB_2 as group 2b carcinogens; meaning they are identified as being potentially carcinogenic to humans [24]. Because of these risks, the EU has set MLs for a wide range of food stuffs for the sum of FB_1 and FB_2. For maize and maize-based foods, intended for direct human consumption, an ML of 1,000 μg/kg was set. Exceptions were made for maize-based breakfast cereals and snacks (800 μg/kg) and for maize-based food for infants and young children (200 μg/kg) [25].

1.2.3 Ochratoxin A



Ochratoxin A

Figure 3. Chemical structure of ochratoxin A

Ochratoxins are produced by *Aspergillius ochraceous*, *Penicillium verrucosum*, and, in some geographical locations, *Aspergillus niger* [34]. Ochratoxin A (OTA) (see Fig. 3) was first identified from a culture of *Aspergillus ochraceus* grown on a sterile maize extract in 1965 [35]. There are three naturally occurring ochratoxin analogues: A, B, and C; and many more synthetic analogues have been defined. OTA however, is the most common and most toxic ochratoxin [36]. OTA occurs in grain products like wheat, barley and oats but also occurs in coffee, cacao and grapes. Indirect contaminations in pork and wine have been reported [5,37-41]. OTA poses a substantial risk to humans and animals. It is widely recognized as a causative agent

of renal failure, and has hepatotoxic properties at high concentrations. OTA causes nephrotoxic and teratogen effects and there is strong evidence that OTA was the causative agent of Balkan Endemic Nephropathy [42-44]. The IARC classified OTA as a possible carcinogen to humans (Group 2B) based on evidence for the carcinogenicity in experimental animals; however, they also noted there was "inadequate evidence'' in humans for carcinogenicity [24]. Recent information about the role of OTA in genotoxicity, oxidative stress and the identification of epigenetic factors involved in OTA carcinogenesis, could imply that OTA carcinogenicity is mediated by a mechanism that also operates in humans [45]. Because of its toxicity, the EU has set MLs for a wide range of food stuffs for OTA. For unprocessed cereals, an ML of 5 μg/kg has been set, while for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption, an ML of 3 μg/kg was set. An ML of 0.5 μg/kg was set for food intended for infants and young children [25].

1.2.4 Zearalenone

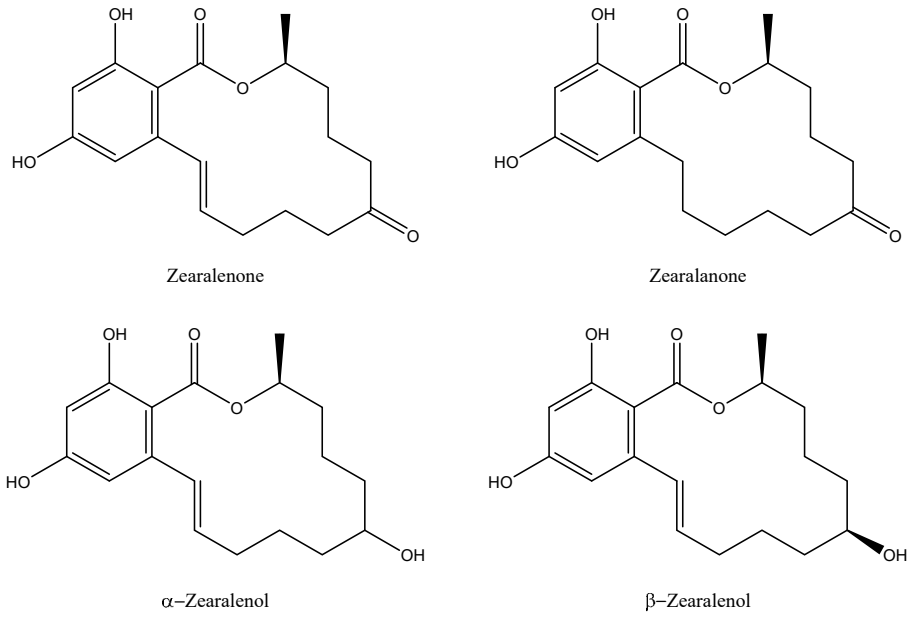


Figure 4. Chemical structures of the main zearalenone metabolites

ZEN is a non-steroidal oestrogenic compound [8] produced by *Fusarium graminearum* and *Fusarium culmorum,* and was first isolated in 1962 [46]. The main ZEN metabolites include α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) [47,48]. ZEN and its metabolites are frequent contaminants of maize, oats, wheat, barley, sorghum, millet and rice [8]. Although ZEN is labelled a mycotoxin, its toxicity is much less severe as for the other main mycotoxins. ZEN binds to the estrogen receptor in mammalian target cells. Especially in pigs, the intake of ZEN contaminated feed can lead to hyperestrogenic syndromes, possibly leading to disrupted conception and abortion at higher concentrations [5]. A synthetic ZEN derivative, Zeranol, was used as an anabolic growth promoter functional for all classes of cattle until it was forbidden by the EU [49]. The IARC has classified ZEN as not being carcinogenic to humans (group 3) [24]. However, a study demonstrated that ZEN stimulated the growth of human breast carcinoma cell lines, by the activation of estrogen receptors [50]. Because of the detrimental effects of ZEN on human health, the EU has set MLs for unprocessed and processed cereals. For cereals and cereal products intended for direct human consumption, a ML of 75 μg/kg was set except for maize which was 100 μg/kg. The ML for cereal-based food for infants and young children was set at 20 μg/kg [25].

1.2.5 Deoxynivalenol

DON is a class B trichothecene and is one of the most common mycotoxins that is globally found in cereal crops [51]. It was first isolated in the 1970's from *Fusarium* infested barley [52]. It is primarily produced by the *Fusarium* species, *F. graminearum and F. culmorum* [53] under favourable cool and moist climate. The main metabolites related to DON are 3-acetyl-DON (3ADON), 15-acetyl-DON (15ADON) and nivalenol (NIV). DON can occur in a wide range of cereal crops like wheat, barley, oats and maize [54]. DON has powerful emetic properties if consumed, hence the alternative name vomitoxin [55].

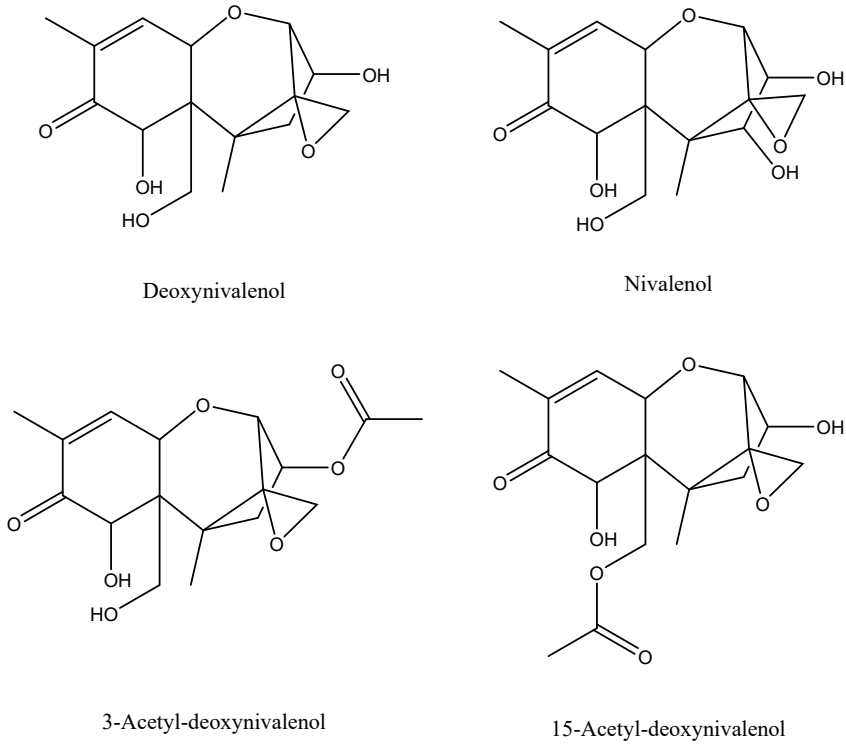


Figure 5. Chemical structures of the main type B trichothecenes

Its intake additionally causes symptoms like nausea, growth retardation, reproductive disorder and suppression of the immune system in humans and animals [56], with swine being the most susceptible to the toxic effects of DON [53]. More recently, DON is also believed to be active at the central nervous system level (brain) causing modified neurochemistry and neuronal activity [57]. DON is classified as non-carcinogenic (group 3) by the IARC [24]. Due to its toxic effects on human health, the EU has set MLs for both unprocessed and processed cereals. For unprocessed cereals, the MLs are 1250 or 1750 μg/kg, depending on the cereal type. For cereals and cereal products intended for direct human consumption, an ML of 750 μg/kg was set. The ML for cereal-based food for infants and young children was set at 200 μg/kg [25].

1.2.6 T-2 and HT-2 toxin

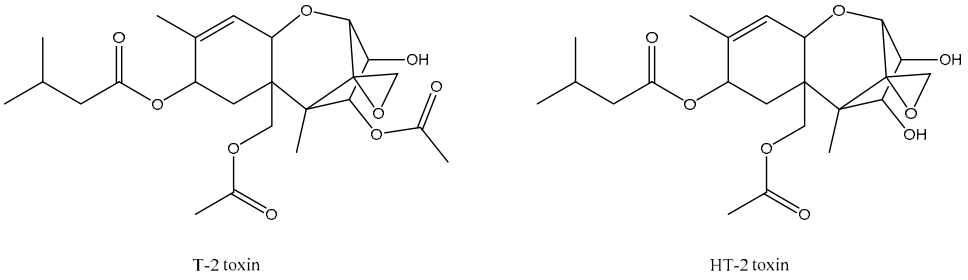


Figure 6. Chemical structures of the main type A trichothecenes

T-2 was first isolated in 1971 from corn infected with the *Fusarium tricinctum* strain [58]. T-2 is mainly produced by the *Fusarium* strains *F. sporotichiodes*, *F. poae* and *F. acuinatum* [59]. T-2 and HT-2, like DON and ZEN, belong to the family of trichothecenes, which are predominantly characterised by the presence of a tetracyclic structure. T-2 and HT-2 toxin (HT-2) belong to the type A trichothecenes. The T-2 and HT-2 producing fungi genera are known to be invasive pathogens of oats, rice, wheat, corn and barley [60]. T-2 and HT-2 toxin often co-occur in contaminated cereals, with the concentration of HT-2 even higher than T-2 [61]. Upon ingestion, T-2 is quickly metabolized to HT-2 and therefore they are considered to have equal harmful effects [62]. T-2 gained notoriety through its misuse in biological warfare. The suspected use of T-2 in Asian combat zones remains inconclusive, yet controversial [63]. The presence of T-2 and/or HT-2 in animal feed causes symptoms like emesis, vomiting, feed refusal, and weight loss. In humans, the effects of T-2 are severe skin irritation, weight loss, haemorrhage, necrosis erythema, edema, and dermal necrosis and is in severe cases even lethality [64,65]. The IARC has classified T-2 as not being carcinogenic to humans (group 3) based on the present toxicity studies [24]. For T-2 and HT-2, currently, no EU enforced MLs exist. However, indicative levels were set from which investigations of the factors leading to the presence of both mycotoxins should be performed. These levels were set for the sum of T-2 and HT-2. For cereals intended for direct human consumption, these levels ranged from 200 µg/kg (oats) to 15 µg/kg (cereal based foods for infants) [66].

1.3 Modified mycotoxins

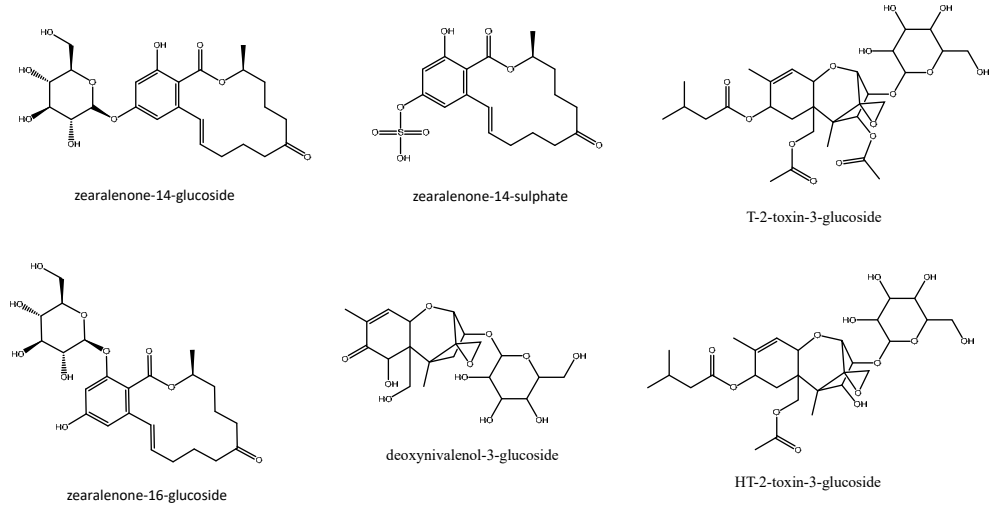


Figure 7. Chemical structures of common plant modified mycotoxins

As part of a detoxification strategy, the free forms of mycotoxins can be converted by living organisms like humans, animals, plants, yeast, bacteria and fungi. These conversions are initialized by biotransformation through the cytochrome P-450 system in all species. In phase I metabolism, the cytochrome P-450 system will mainly hydrolyse or oxidise the free mycotoxin. In subsequent phase II metabolism, transferase enzymes couples a polar group to the phase I metabolite. Some of these observed biotransformations include hydroxylation, glycosylation, oxidation, demethylation, sulfoxidation and epoxidation [67-69]. While phase I products may be more toxic than the original free form of the mycotoxin, phase II products are generally less toxic or non-toxic. In some cases the degradation of free mycotoxins may lead to less harmful products. A good example is the degradation of the toxic free form of OTA to its much lesser toxic breakdown product ochratoxin α (OTα) [70]. The metabolisation of the free mycotoxins will alter their structure and therefore they can escape routine instrumental analysis like liquid chromatography with tandem mass spectrometry (LC-MS/MS). If the modified mycotoxin has become non-toxic, then its detection in routine analysis is no longer relevant. However, if the modified form is equally toxic, or more toxic, then detection is crucial. More importantly is the fate of these modified mycotoxins once digested by

humans and animals. Well-known plant-modified mycotoxins are deoxynivalenol-3-glucose (D3G), nivalenol-3-glucose (NIV3G), T-2-toxin-3-glucoside (T2-3G), HT-2-toxin-3-glucoside (HT2-3G), zearalenone-14-β-D-glucopyranoside (Z14G), -16-β-D-glucopyranoside (Z16G), α-zearalenol-14-β-D-glucopyranoside (α-ZELG), β-zearalenol-14-β-D-glucopyranoside (β-ZELG) and zearalenone-14-sulphate (Z14S). All these plant-based conjugated mycotoxins are originally produced by *Fusarium* species [71,72]. There are many indications that these modified forms can be hydrolysed back to its original form after ingestion. Already in 1990, a study showed that, Z14G fed to pigs, only yielded ZEN and α-ZEL in both urine and faeces, indicating the (near-)complete hydrolysis of Z14G [73]. In other experiments the hydrolysis of Z14S and Z16G, back to its original form (ZEN) was shown when fed to pigs [74,75]. Kovalsky et al. [76] showed that Z16G added to a human faecal slurry was hydrolysed to ZEN. These experiments indicate that the presence of these conjugated forms of ZEN in the digestive tract, lead to additional toxicity and therefore should be detected along with the unconjugated toxins. D3G, probably the most studied modified mycotoxin, was submitted to cultures of the lactic acid bacteria *Enterococcus durans, Enterococcus mundtii* and *Lactobacillus plantarum* which all showed a high capability of hydrolysing D3G, releasing its original form, DON. This finding indicated that the hydrolysis in the intestines increases the bioavailability of DON and therefore should be weighed in risk assessment studies [77]. Based on hydrolysis studies with the gut bacteria *Bifidobacterium adolescentis*, *Butyrivibrio fibrisolvens*, *Roseburia intestinalis* and *Eubacterium rectale*, it can be assumed that also the T2-3G and HT-3G metabolites will be effectively hydrolysed back to their respective parent mycotoxins in human and animal intestinal tracts [78,79]. Formation of OTA-glycosides by a diverse range of plant cell suspension cultures has been previously reported [80,81]. Nevertheless, natural in-field contaminations with OTA-glycosides have not been reported so far. For FBs, hidden forms, that could be released upon hydrolysis of the cereals, were observed in several studies. These are not plant-modified forms, however in many cases, the amount of the hidden forms was higher than the free forms present. This shows that risk calculation could be underestimated for FBs [82].

Several surveys have elucidated the presence of modified mycotoxins in cereal crops and processed feed [69,83,84], with the highest incidence for D3G, which could be

partially related to the early availability of a commercial reference standard. A good example for better insights into the natural occurrence of modified mycotoxins in cereal grains, is brought forward by a Finnish nationwide survey of barley, oat and wheat samples [85]. The implemented instrumental analysis method (LC-MS/MS), covered 16 mycotoxins and modified mycotoxins. This survey showed the presence of the modified mycotoxins D3G, HT2-3G, NIV3G, Z14G, Z14S, Z16G, α-ZELG and β-ZELG in those cereal crops. In barley all these metabolites were present, with only HT2-3G and Z16G below the limit of quantification (LOQ). In barley D3G was present at an average concentration of 148 μg/kg in comparison to DON at 234 μg/kg. In oats only α-ZEL14G and β-ZEL14G were not present, while Z14G was present below the LOQ. In oats D3G was present at an average concentration of 806 μg/kg in comparison to DON at 2690 μg/kg. In wheat, only β-ZEL14G was not detected. In wheat, D3G was present at an average concentration of 174 μg/kg in comparison to DON at 866 μg/kg. In none of the researched samples, modified mycotoxins were present at a higher concentration then its free form. However, as shown for DON, the presence of modified mycotoxins can be rather substantial [85]. T2-3G was not part of the applied method,. However, in two other surveys, the natural occurrence of T2-3G in barley, wheat, and to a lesser extent in oats was shown. In several of those barley samples, T-2, HT-2, T2-3G and HT2-3G co-occurred, in one of those samples with respective concentrations of 13, 81, 14.5 and 77.8 μg/kg [86,87].

Due to the relevance designated to these modified mycotoxins, methods have been developed to release the native forms of the modified mycotoxins [88]. Additionally, the availability of commercial reference standards has increased in recent years. Acknowledging the relevance of these modified mycotoxins, the European Food Safety Authority (EFSA) has amended several mycotoxin specific Tolerable Daily Intake (TDI) levels. Currently there is an established group-TDI for the sum of DON metabolites (DON, 3ADON, 15ADON and D3G) of 1 μg/kg body weight per day [89]. The CONTAM panel of EFSA had already expanded the original TDI for ZEN, to a group health-based guidance TDI value of 0.25 μg per kg of body weight for ZEN and all of its phase I and phase II metabolites. Additionally they set potency factors, relating to the oestrogenic activity of the ZEN metabolites [90]. Considering the modified forms of T-2 and HT-2, the EFSA CONTAM panel found it appropriate

to establish a group TDI of 0.02 μg/kg body weight per day, for T2, HT2 and its modified forms [91].

Modified forms of mycotoxins have been assigned different names across scientific literature e.g. conjugated mycotoxins, bound mycotoxins, mycotoxin derivatives, hidden mycotoxins, but mostly masked mycotoxins, a term already put forward in 1990 [73]. In recent years, discussion started on how to name the modified free mycotoxins throughout all possible processes that can occur to them. Therefore, Rychlik et al. [92] presented a systematic definition consisting of four hierarchic levels: 1) the free unmodified mycotoxins produced by fungi, 2) biologically modified mycotoxins, 3) chemically modified mycotoxins and 4) matrix-associated mycotoxins. As a conclusion they suggested to use the term modified mycotoxins for future scientific wording and to use the term masked mycotoxins only for plant-modified mycotoxins.

2. Mycotoxins from field, to malt, to beer

2.1 Beer brewing

Malted barley is an essential ingredient for beer brewing. Harvested raw barley therefore goes through the processes of steeping (soaking in water), germination (induces α-amylase for starch conversion) and kilning (drying/roasting). After this step the shoots are removed from the kernels (Fig. 8). The malted barley is then milled to break the kernels into smaller fractions (grist), facilitating efficient extraction. Water is added to the grist, and mixed very well at high temperatures (~60 °C) with water (mashing). These conditions dissolve the starch and amylases, which will lead to the release of fermentable sugars. After mashing is complete, the liquid fraction (wort), is separated from the solid fraction (spent grains). At this point, in general, the wort is boiled to end the amylase activity and at the same time hops are introduced into the wort for bittering. After the wort is cooled down, yeast is added to form alcohol from the fermentable sugars. At this stage, in craft brewing, fresh hops may be added (dry-hopping). After fermentation is complete, the beer can be bottled or canned (Fig. 9). At this point industrial brewers will pasteurize their beer, which is not the case for real craft brewers [93].

Figure 8. Malting process at the Lo Vilot craft brewery and malthouse. Two row barley (A) is harvested from the field (B) and poured into the malting system (C), where the barley is soaked in water (D), germinates (E) and eventually is roasted in the same system. After the shoots are removed by a cyclonic separator, the malted barley is ready for brewing (F).

2.2 Beer versus Craft Beer

Beer is one of the oldest beverages known. With an annual total beer consumption of roughly 190 billion liters in 2019, beer was considered the most popular alcoholic beverage [94]. In recent years, the popularity of beer as a beverage, rapidly increased. This increased popularity, can be totally attributed to novel craft

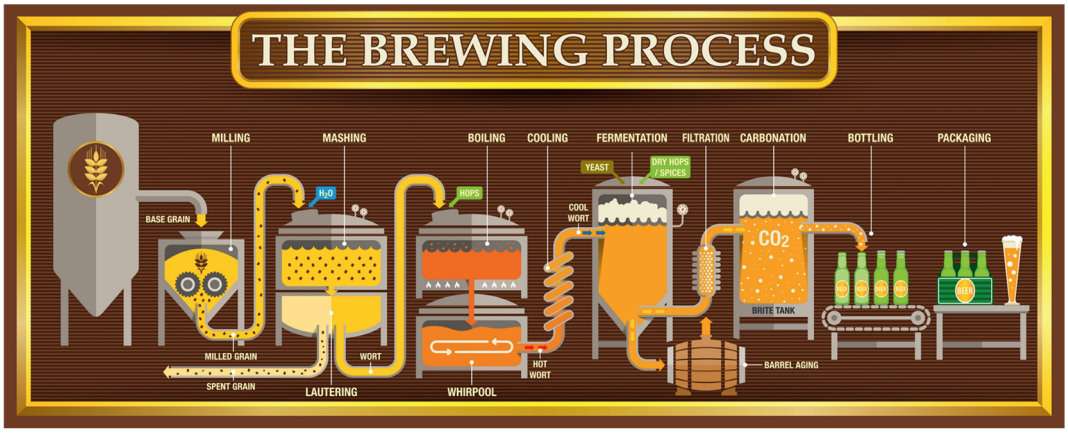


Figure 9. Scheme of a beer brewing process with craft beer elements

breweries, that set out to do something completely different then macro-brewers [95].In 2009, the US had a total of 1,653 breweries listed, of which 1,596 were considered craft breweries. This number had been pretty stable for the previous 10 years. But fast forward to 2020, the amount of breweries had increased to 8,884, of which 8,764 were considered craft breweries [96]. This was roughly a five-fold increase. In the Netherlands, a similar increase could be observed with the rise of breweries from a total of 181 in 2012, to 821 in 2020 [97]. In both cases, this explosion of new breweries could almost totally be contributed to the rise of new craft breweries. In fact, the pioneering US craft brewers are responsible for the most radical structural transformations ever taking place in American industry [98]. The increasing popularity of craft brewers and their beers did not go unnoticed by the industrial brewers, since craft beers was taking a larger market share every year. This eventually lead to industrial breweries adding certain craft beer style beers, mostly the ones cheapest to produce, to their core range. This step was then followed by large industrial brewers buying small popular craft brewers, e.g. AB InBev acquiring the Craft Brew Alliance. It also lead to investment companies buying shares in popular craft breweries (personal communication). The popularity of craft beer is based on high quality, original and very tasteful beers. These craft beers easily stand out from industrial produced beers, that are mostly produced low-cost and on a large-scale. Especially novel craft breweries, focus on innovation, producing newly invented styles, but also have alternative takes on classical and ancient beer styles. In general, beers are brewed with water, malted barley, hop and yeast, but in craft

brewing a wide selection of other ingredients are used: e.g. coffee, cacao, seaweed, tobacco, liquorice, candies, Christmas trees, nuts, whey, oysters, tomatoes, chili peppers, fruits, flowers and a range of spices [99,100]. The challenge to be unique and original in craft beer brewing still continues.

2.3 Mycotoxins: transfer from barley to barley malt to beer

The occurrence of mycotoxins in beer has been widely documented, and in recent years the focus also has been on craft beer [20,101-103]. Water, barley and malt are the main ingredients for beer brewing. Brewing water is not considered a source for mycotoxins. Although hop can be contaminated with *Fusarium* species [104], the presence of mycotoxin contaminations have never been reported. Surveys for mycotoxins in hops are rare and tend to focus on AFs and OTA. Neither AFs or OTA were traceable in hops, besides one incidence at 0.6 μg/kg for OTA [105-107]. Especially the malting process is prone to the formation of mycotoxins and modified mycotoxins. The malting of cereals and the subsequent brewing process, can both affect the breakdown, metabolization as well as the *de novo* synthesis of mycotoxins. Breakdown can occur because of temperature instability, metabolization by enzymes or microorganisms present in the beer at different stages. *De novo* synthesis of mycotoxins can occur through viable fungi still present in the barley [108]. Malted barley is by far the most used malted cereal in (craft) brewing, followed by malted wheat. Besides those two malts, other non-malted cereals like maize, rye and oats, are also added to the brewing process and therefore also can contribute to the total mycotoxin contamination (personal communication). Barley, especially grown for brewing purposes, can contain several *Fusarium* mycotoxins [109]. Additionally the mycotoxins AFs, OTA and FBs have been reported to be present in barley [110,111]. These contaminations are depending on the annual climate and the type of cultivar [112]. The question is, whether these mycotoxins from the original barley can be found back once the barley has gone through the malting and brewing process. In one study, barley contaminated with DON was submitted to all the stages of the malting and brewing processes. It was shown that from the original DON contamination of 5204 μg/kg in the green barley, 1414 μg/kg still remained [113]. In several other studies it was shown that, during the steeping phase of the malting process, initially the mycotoxin concentration for e.g. DON, D3G, ADONs and HT-

2 decreased, but then increased again during the germination phase. In one case, this resulted in a two-fold increase of the DON contamination, while for the modified mycotoxin D3G, this was roughly 10 fold [114]. An even much higher concentration of newly produced DON and D3G could be found in the shoots of the germinated barley. However, these shoots are removed at the end the malting process, so they do not contribute to the mycotoxin carry-over in the beer. These studies indicated that new (modified) mycotoxins were formed, which hint at the presence of viable *Fusarium* fungi, who are present in the barley grains. [113-115]. A study, where fungicide treated barley was compared to natural barley in the malting process, showed that for the former, no new mycotoxins were formed, while in the latter mycotoxins increased by almost 300% [112]. This evidence also suggested *de novo* synthesis of mycotoxins. The fact that *Fusarium* can still be present and viable, on field barley and produce new mycotoxins during malting was further proven by coupling specific DNA detection of fungal growth to the *de novo* production of new type B trichothecenes. It was shown that *Fusarium culmorum* and *Fusarium graminearum* showed significant growth and at the same time *de novo* synthesis of DON during the last two days of germination and the initial stages of kilning. It was also shown that growth of *Fusarium sporotrichoides* decreased during the malting process, which was linked to decreasing concentrations of *Fusarium* type A trichothecenes (T-2/HT-2) [116,117]. OTA and AFs are less well-studied considering their stability and *de novo* production during malting. In one study, high concentrations in the initial barley of OTA (400 – 800 μg/kg) were not detectable anymore after malting [118]. In a malting study, where wheat was artificially inoculated with *Aspergillus Flavus*, AFB_1 was produced in the initial wheat, but then decreased during germination. During kilning at 50 °C, the AFB_1 content increased again, but then later decreased again at the final stages of kilning at 80 °C with a final concentration of 240 μg/kg AFB_1 in the final malt [101]. This can be considered as a serious contamination, depending on the level of the *Aspergillus Flavus* infection of wheat in the field. More often the fate of OTA and AFs in beer brewing is investigated by adding the mycotoxins to the malted barley. However, if they already disappear in the malting process, they will not turn up in the final beer. Malted barley that is contaminated with mycotoxins, and modified mycotoxins, enters the brewing process as grist in the mashing phase, where it is mixed with

water and then heated (Fig. 9). In a study where barley malt was spiked with several mycotoxins, before undergoing a laboratory scale brewing process, most of the OTA, AFs and ZEN ended up in the spent grains (40 - 60%) while 16% (OTA), 5% (AFB_1) and 0% (ZEN) ended up in the final beer. From the other spiked mycotoxins, 61% DON, 71% NIV, 35% T-2, 52% HT-2 and 46% FB_1 alt was found back in the final beer [119]. Even though T-2, HT-2 and ADONs may be formed during the malting process, they do not show up, or are hardly detected in relevant concentrations, in large scale beer surveys [20,120,121]. However, in one study, the presence of ADONs in malts and their transfer to beer has been shown for different beer styles [122]. In a laboratory-controlled, in-line malting and brewing process, the ZEN contamination went from 3594 μg/kg in the initial barley, to 64 μg/kg in the wort, to below the LOD in the final beer. The majority of the initial ZEN was detected in the spent grains (3188 μg/kg). Generally, if any ZEN remains after mashing, it is further metabolized to β-ZEL during the fermentation process [123]. Therefore, ZEN is hardly reported in beer at significant concentrations, with exceptions for African traditional beers at higher concentrations, which is mainly caused by not filtering the spent grains, but instead include them in the beer. Occurrence of ZEN in European beers is hardly reported and if present in low concentrations. In one survey, all 44 samples were tested positive for ZEN by an EIA method with concentrations ranging from 0.35–2.0 μg/L. However, these were not confirmed by instrumental analysis [124]. Incidentally, ZEN is reported in European beers at high incidences (65% of beer samples), and at exceptional high concentrations, ranging between 8 and 63 μg/L, not yet found before in any other European survey [125].

In general, no TDI-exceeding concentrations are found in European beers for most mycotoxins. In a few cases, beers close to the TDI or exceeding the TDI were found in particular craft beer styles [20,120]. Although the spent grains are a waste product in brewing, they are used as animal feed and therefore can be detrimental to animal health. Moreover, these mycotoxins may still have an effect on humans after all, as farm animals are a part of the food supply chain.

3 Antibodies as biorecognition molecules for mycotoxins

3.1 Antibodies

Antibodies are a crucial part in immunoassays. For mycotoxin (immuno)assays, the most commonly used biorecognition molecules are antibodies. Infectious diseases in humans and mammals occur after the invasion of viruses, bacteria, fungi, or other microorganisms in the body. The immune system will start battling these infections to halt, and eventually recover from the infectious disease. Antibodies are a crucial factor in fighting those infectious diseases [126]. Upon infection, macrophages will phagocytise the invading microorganisms and present their antigens to the plasma B cells that are responsible for the first step in antibody generation. Antibodies are in the γ-globulin fraction of the bloodserum, which consists of the following immunoglobulins: IgG, IgM, IgA, IgD and IgE. For immunoassay purposes, the IgG class is the most used antibody of choice (Fig. 10).

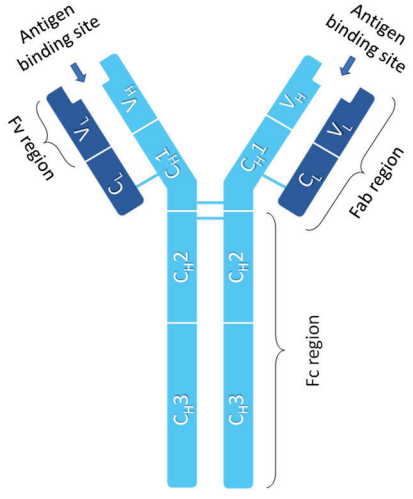


Figure 10. Schematic structures of an IgG antibody

It consist of 2 heavy (H) chains, each having 3 constant domains (C_H1, C_H2 and C_H3) and a variable domain (V_H). These H chains are linked together through 2 disulphide bonds. Attached to each H chain, by a disulphide bond, are 2 light (L) chains. These L chains consist of a constant domain (C_L) and a variable domain (V_L). The Fab

domains consist of the C_H3 and V_H domains of the H chains and the C_L and V_L of the L chain. The antigen binding sites are formed by the V_H and V_L at the end of each Fab fragment. The H chain parts that are below the hinge region, is called the Fc domain [127]. IgG based antibodies, from mouse and rabbits, are the most commonly used antibodies for mycotoxin detection assays. However, in some cases also IgA based mycotoxin detection assays were developed [128], as well as IgY (from chicken egg) based assays [129].

3.2 Monoclonal and polyclonal antibodies

In general, antibodies used in immunoassays are polyclonal antibodies (pAbs) or monoclonal antibodies (mAbs), mostly originating from mouse or rabbit. For the studies presented in this thesis, mAbs were used for all immunoassays. Both mAbs and pAbs need a immunization step to acquire a immune response. The first mAb was generated in 1975 using novel hybridoma technology. MAbs are produced by a single B-lymphocyte clone and are directed to a single epitope [130]. This makes them more specific than pAbs. However, pAbs can be more valuable in the detection of bigger molecules with the chance of generating multiple antibodies against different epitopes in the same serum fraction, making them suitable for setting up Double Antibody Sandwich (DAS) immunoassays. Another advantage of pAbs is, that they are relatively simple to produce. The disadvantage however, is that the serum fraction containing the pAbs will run out over time and new pAbs need to be generated. Due to batch to batch variation, the quality of the pAbs in new lots will differ [131]. In principle, the cell clones that produce mAbs, will last a lifetime and will always produce the same antibody. This way immunoassay sensitivity and selectivity are guaranteed. In the long run, the production of pAbs will require more laboratory animals compared to the production of mAbs. This is conflicting with new targets set by the EU considering to decrease the use of laboratory animals for antibody production [132]. Small molecules, mostly called haptens, are not capable of raising an immune response by themselves as xenobiotics. For this reason, they need to be conjugated to a carrier protein like keyhole limpet hemocyanin or the cholera toxin B subunit, through an activated group [131]. Immune responses against mycotoxins are generated by immunization with these mycotoxin-protein

conjugates, and can lead to the successful production of specific and high-affinity antibodies for suitable for mycotoxin detection by immunoassays.

3.3 Antibodies in competitive immunoassays for mycotoxin detection

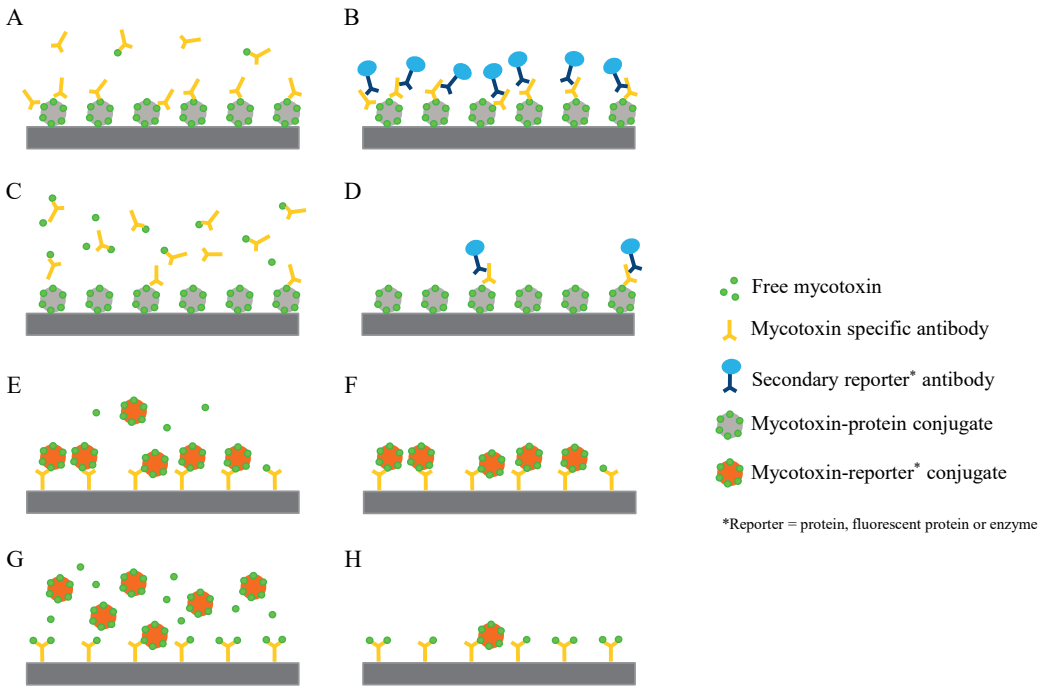


Figure 11. Competitive immunoassay formats for the indirect, and direct detection of mycotoxins. In an indirect format, mycotoxin-protein conjugates are coupled to a surface. Samples are mixed with mycotoxin specific antibodies and introduced to the surface. When a sample is not contaminated, or low contaminated with mycotoxins, the mycotoxin specific antibodies will interact predominantly with mycotoxin-protein conjugates on the surface (A). With the addition of a secondary reporter antibody, after washing, a high signal is generated (B). If there is a high mycotoxin contamination, the mycotoxin specific antibodies will predominantly bind to the free mycotoxins (C), generating a low signal after the addition of the secondary reporter antibody (D). In a direct format, the mycotoxin specific antibodies are coupled to a surface, and samples mixed with the mycotoxin-reporter conjugate are introduced. In case of no or low contamination (E), a high signal can be directly measured (F). In case of a high contamination (G), a low signal can be directly measured (H).

Since small molecules rarely have multiple antigenic properties, like proteins, the chance of generating multiple specific antibodies is very small. That is why for

mycotoxin immunoassays, competitive immunoassays are the standard. These competitive immunoassays, make use of specific mycotoxin-protein conjugates and are based on direct or indirect formats (Fig. 11).

4. Microspheres in mycotoxin immunoassays

Microspheres are micron-sized particles that can be implemented for immunoassay based detection of mycotoxins, where they can be the carrier for mycotoxin specific antibodies or mycotoxin-protein conjugates. This can be in in lateral flow devices [133], but also in more novel approaches like silicon photonic crystal microspheres-based detection [134]. A well-known microsphere-based detection technology is the Multi Analyte Profiling (xMAP) technology developed by Luminex. This xMAP suspension array consists of dual color-coded microspheres (also referred to as beads) that can be coupled with a wide range of biomolecules for assay development (Fig. 12). For the presented research, carboxylated microspheres were used. To couple antibodies or mycotoxin-protein conjugates to the microspheres, 1-ethyl-3-(3-dimethylamino) propyl carbodiimide (EDC)/ N-Hydroxysulfosuccinimide (S-NHS) chemistry is implemented [135]. EDC reacts with the carboxylic acids (-COOH) on the microspheres, to form an active-ester intermediate. By the addition of S-NHS, an NHS ester is formed which is more stable in aqueous solutions, enhancing the efficiency of the coupling reaction. After removing the EDC/S-NHS, the microspheres are incubated with the antibody or mycotoxin-protein conjugate of interest, which results in an amide bond formation between the microspheres and the biomolecules (Fig. 13).

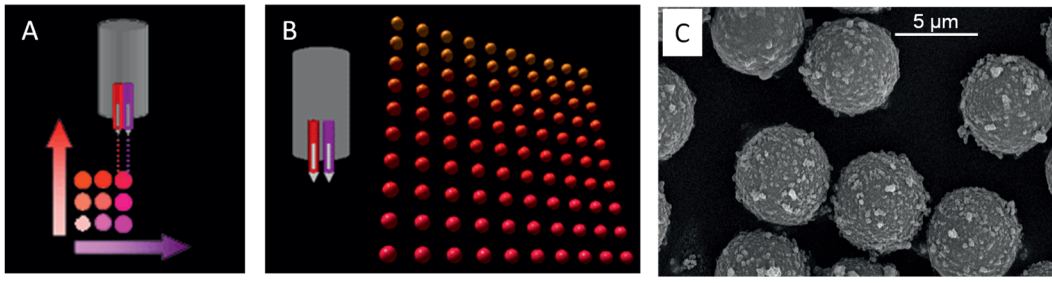


Figure 12. Polystyrene microspheres are loaded with unique amounts of a red and far-red fluorochrome (A), leading to a 100 unique microsphere sets (B), which are paramagnetic by scattered iron nano-particles on the surface (SEM image) (C).

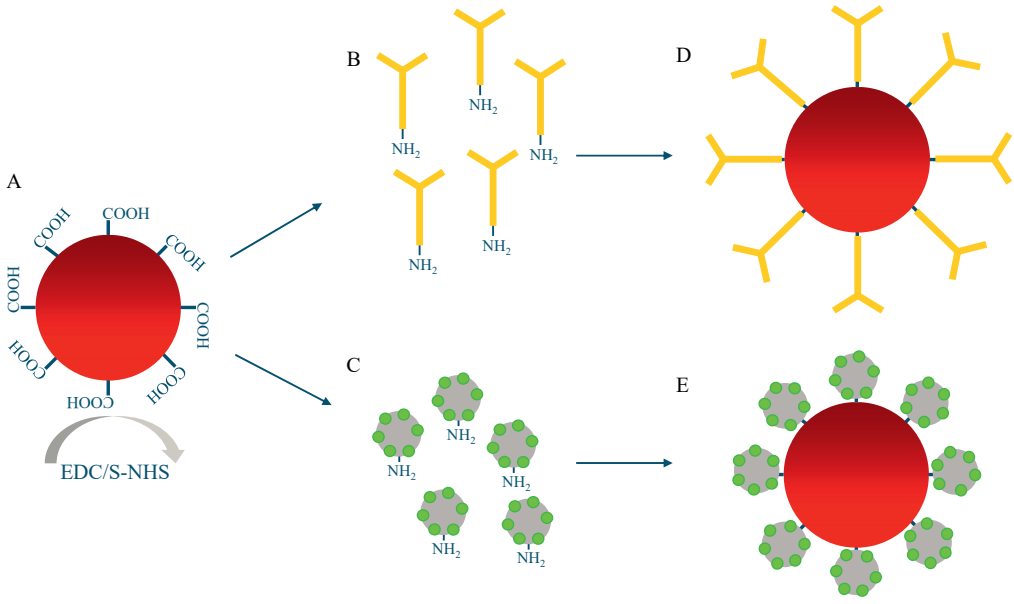


Figure 13. Microsphere activation and covalent coupling with biomolecules

The detection hardware of the xMAP suspension array-built assays, can be either through flow cytometer-based systems, or planar array analysers (Fig. 14). Since the microspheres can be differentiated, based on their specific red and far-red fluorescent emission, a single reporter label can be implemented independent of the multiplex number. Although the major research and application field for xMAP technology is clinical analysis, food-safety based microsphere immunoassays (MIAs) have also been developed for a wide range of contaminants e.g. marine toxins [136,137], coccidiostats [138], pesticides [139], persistent organic pollutants [140], antibiotics [141] and mycotoxins [142]. Initially, the developed mycotoxin assays were mainly based upon the indirect detection principle (Fig. 15A), where the mycotoxins are coupled as protein-conjugates to the microspheres and benchtop flow cytometer systems were applied for read-out of the results [143-145]. The subsequent introduction of paramagnetic xMAP microspheres allowed easier sample handling and faster washing steps in the general procedure [142]. The introduction of a dedicated planar array analyser for the detection of xMAP assays, has been a major step forward in robustness. This benchtop analyser was, unlike the previous flow cytometer-based systems, transportable and therefore implementable for point of need analysis. A direct 6- plex mycotoxin detection assay, using

fluorescent mycotoxin-protein conjugates (Fig. 15B), for the detection of all six main mycotoxins, metabolites and modified forms was developed and validated as described in chapter 4.

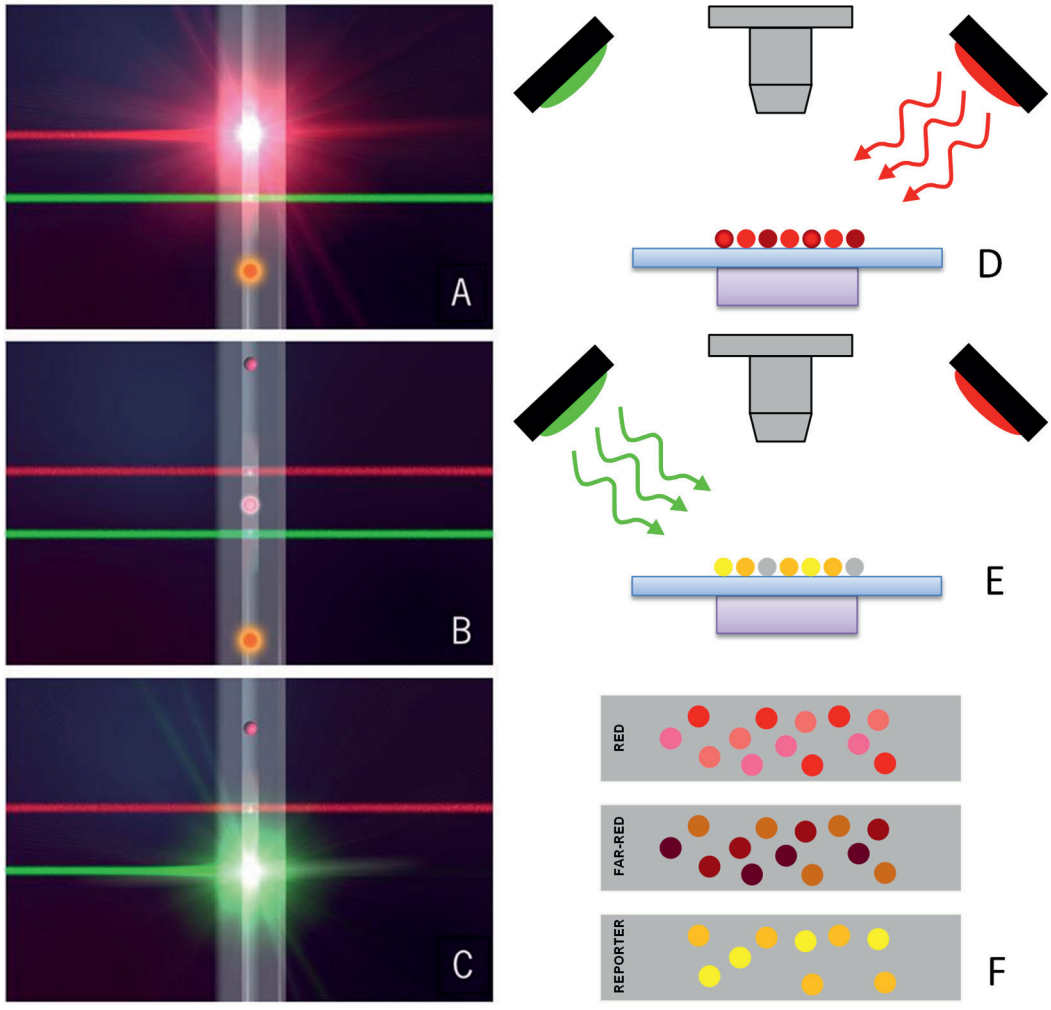


Figure 14. Flow cytometer and planar array based microsphere immunoassay detection. In the flow cytometer, a microsphere in the flow cell passes through the red laser it is classified by measuring a red and far-red emission (A). It continues to flow (B) and will pass through the green laser, where the reporter signal will be measured (C). In imaging planar array microspheres are trapped on a planar surface by magnetic force and illuminated by a red LED (D). Next, the microspheres are illuminated by a green LED (E). The CCD camera records red and far-red images for classification and reporter emission images during these steps (F).

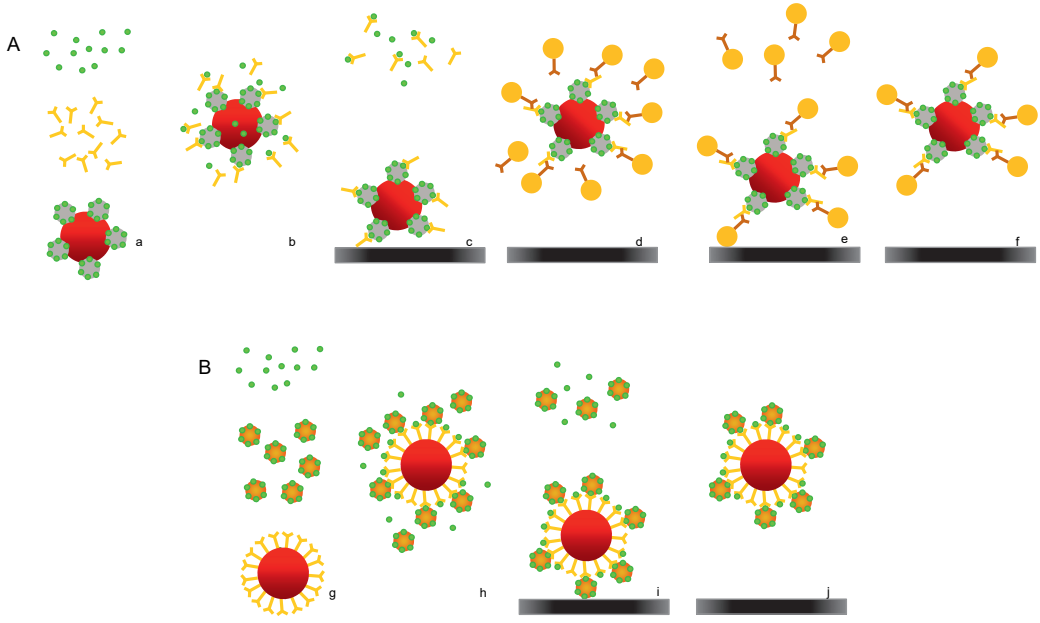


Figure 15. Microsphere immunoassay (MIA) formats. In an indirect assay (A), sample, antibodies and mycotoxin-BSA conjugated microspheres (a) are incubated in a single well, so competition between the conjugated mycotoxins on the microsphere and the free mycotoxins in the sample will occur (b). After incubation the microspheres are trapped by a magnet and the non-bound reagents washed away (c). The microspheres are then released and an goat-anti-mouse/R-Phycoerythrin (GAM-RPE) conjugate is added (d). After incubation this microspheres are trapped and non-bound GAM-RPE is washed away (e). After release, the microspheres are measured (f). In the direct assay format (B), sample, mycotoxin-RPE conjugate labels and antibody-coupled microspheres are incubated in a single well (g). Labelled and free mycotoxins compete for antibody binding on the microspheres (h). After incubation microspheres are trapped by a magnet and the non-bound reagents washed away (i). Microspheres are released and measured (j).

5. Confirmation by instrumental analysis

There are many toxic substances that can be found in food. To ensure safe food for everyone, they need to be reliably detected and quantified [146]. Instrumental analysis is a method that is fit-for-purpose for the detection of chemical food contaminates in food and feed matrices at EU legislative levels [147]. Confirmatory instrumental analysis-based mycotoxin detection can focus on only 1 type of mycotoxins (e.g. the detection of the four main AFs in peanuts and peanut products [148]), a matrix-dependent group of mycotoxins (e.g. the detection of 35 mycotoxins, as biomarkers for exposure, in pig urines [149], but can also be an extended range of analytes for a full mycotoxin screening (e.g. the detection of 87 mycotoxins and mycotoxin metabolites in a wide range of mouldy matrices [150]). With the determination of 295 fungal and bacterial metabolites in four different types of food matrices, show the power of this technique [151]. With roughly 300–400 mycotoxins known [152], LC-MS/MS mycotoxin multi-methods can be implemented for the detection of a wide array of mycotoxins in a dedicated matrix. The application of a multi-mycotoxin LC-MS/MS method to feed samples, revealed 139 different fungal metabolites in 83 feed samples [153]. In this thesis, LC-MS/MS-based methods have been applied for critical comparison of the developed multiplex screening assays, rather than comparing it to existing ELISA's. To confirm screening-based immunoassay results, a critical comparison to instrumental analysis data is crucial. For the critical comparison of the indirect 6-plex MIA (chapter 2) and the direct 3-plex MIA (chapter 3) results, LC-MS/MS confirmatory methods were implemented. A dedicated LC-MS/MS method was developed, focussing on the main six mycotoxins, as well as their modified forms, and implemented for the confirmation of specific samples from a global beer survey (chapter 6).

6. Scope and thesis outline

Mycotoxins occur in food and feed commodities and they transfer to processed products. Chances are high, that when a commodity is contaminated, there is likely more than one mycotoxin present [14]. For multi-contaminations in food and feed commodities, multiplex immunoassays are useful screening tools. The microsphere-based xMAP is a powerful technology that operates on commercially available flow cytometers and planar array analyzers, featuring demonstrated robustness and wide applicability in medical testing. For example, a current xMAP assay replaces 29 single human cytokine ELISA's by doing all 29 analyses, multiplexed in a single well of a microtiter plate. This thesis focusses on the development and application challenges of MIAs for the detection of mycotoxins and modified mycotoxins, using xMAP technology. The MIAs were developed in both indirect and direct competitive immunoassay formats, with the demands of being reproducible, sensitive and sufficiently accurate for semi-quantitative screening at MLs set by the EU. Additionally, the chosen format allows the easy extension of the multiplex assays with other relevant mycotoxins or contaminants for food and feed commodities at a later stage.

An indirect competitive multiplex immunoassay was developed for the detection of ZEN, DON, T-2, AFs, OTA, and FBs, and applied to a range of reference samples to check if it was fit for purpose as an inhibition-based screening assay for feed (**Chapter 2**). To further simplify and speed up the assay, a switch was made to a direct inhibition assay principle by the introduction of fluorescent mycotoxin-protein conjugates, thereby decreasing the incubation and washing steps in a 3-plex assay for the detection of ZEN, OTA and FBs. Additionally a faster type of microsphere dedicated flow cytometer was introduced for shorter measurement times. The shorter measurement times make the newly developed direct inhibition multiplex immunoassay approach faster and the new assay format requires less procedural steps than the previously developed indirect assay format. To prove functionality of the 3-plex assay, it was applied to naturally contaminated wheat, maize and feed samples which were all previously analysed by a confirmatory multi-mycotoxin LC-MS/MS method (**Chapter 3**). When the new direct competitive approach proved to be successful, the 3-plex was further extended to a 6-plex

method, to include all the main six mycotoxins in the direct format approach. For the 6-plex, the focus was set on barley as a sample matrix. A within-laboratory validation for screening assays was performed for the application of the 6-plex on barley samples. Additionally, the 6-plex was used to screen available barley reference materials. Moreover, because of their increasing relevance, the cross-reactions with available modified mycotoxins was determined. To ensure the possibility of future portable on-site screening at the point of need, all assays were performed on a transportable microsphere planar array analyzer (**Chapter 4**). Answers about mycotoxin contaminations in food and feed commodities, are preferably given before these commodities enter the production and processing chains. Therefore reliable pre-screening at the point of need can be crucial. Additionally, for governmental food safety inspectors, these on-site analysis need to be portable on-site. To make the developed microsphere immunoassays more suitable for portable on-site implementation, a 4-plex format that detects ZEN, OTA, T-2 and DON was chosen as a model assay. Changes to the previous method included: addition of reagents from dropper bottles, addition of samples by disposable micropipettes, simplified washing steps and rapid sample extraction, omitting centrifugation. The 4-plex assay was validated as a screening assay for barley (**Chapter 5**). Many beer surveys have been carried out for the occurrence of mycotoxins, but there was never a survey fully focused on the new wave of craft beers. Some did focus on smaller breweries, which may be considered as craft, but the focus on well-defined beer styles was mostly lacking. Besides that, the focus was often on the main mycotoxins, but did not zoom in on the presence of modified mycotoxins. To test if the 6-plex assay was fit for high-throughput screening of naturally contaminated samples, it was applied to a set of 1000 unique and very diverse global beer samples, of which 73% were craft beers. In the same research, confirmatory analysis was carried out focusing on the target mycotoxins, their metabolites and modified forms available at that stage (**Chapter 6**). Due to the increasing relevance of modified mycotoxins, and their (future) inclusion in EU regulations, screening methods need to be able to detect them and well-characterized reference standards should become available. With the aim of having our own source of modified mycotoxins, we set up a biotransformation method using *Cunninghamella* fungal strains and applied it to the main six mycotoxins, in an

attempt to produce relevant modified mycotoxin standards. The introduction of selective depleted media, allowed steering of the production to specific mycotoxin metabolites of interest (**Chapter 7**). Finally, all these developments, achievements and results were critically discussed. Additionally, ideas on how to make certain beer styles safer for consumption are proposed (**Chapter 8**).

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CHAPTER

2

Development of a multiplex flow cytometric microsphere immunoassay for mycotoxins and evaluation of its application in feed

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Adapted from:

Development of a multiplex flow cytometric microsphere immunoassay for mycotoxins and evaluation of its application in feed.
Mycotoxin Research 2011, 27, 63-72.

Abstract

A multi-mycotoxin immunoassay, using the MultiAnalyte Profiling (xMAP) technology, is developed and evaluated. This technology combines a unique color-coded microsphere suspension array, with a dedicated flow cytometer. We aimed for the combined detection of aflatoxins, ochratoxin A, deoxynivalenol, fumonisins, zearalenone and T-2-toxin in an inhibition immunoassay format. Sets of six mycotoxin-protein conjugates and six specific monoclonal antibodies were selected, and we observed good sensitivities and no cross-interactions between the assays in buffer. However, detrimental effects of the feed extract on the sensitivities and in some cases on the slopes of the curves were observed and different sample materials showed different effects. Therefore, for quantitative analysis, this assay depends on calibration curves in blank matrix extracts or on the use of a suitable multi-mycotoxin clean-up. To test if the method was suitable for the qualitative detection at EU guidance levels, we fortified rapeseed meal, a feed ingredient, with the six mycotoxins, and all extracts showed inhibited responses in comparison with the non-fortified sample extract. Contaminated FAPAS reference feed samples assigned for a single mycotoxin showed strong inhibitions in the corresponding assays, but also in other assays of the 6-plex microsphere immunoassay (MIA). In most cases, the presence of these other mycotoxins was confirmed by instrumental analysis. The 6-plex MIA can be easily extended with other mycotoxins of interest, but finding a suitable multi-mycotoxin clean-up will improve its applicability.

Introduction

Mycotoxins are secondary metabolites produced by fungi and often co-occur. Their presence in food and feed are serious threats to the health of humans and animals and monitoring is vital. The most common assayed mycotoxins are aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (FBs), zearalenone (ZEN) and T-2-toxin (T-2). Many methods are available for the detection of mycotoxins and, mainly, they can be divided into immunochemistry- and chromatography-based techniques, with enzyme-linked immunosorbent assay (ELISA) and liquid chromatography combined with mass spectrometry (LC-MS/MS) being the most popular. For the execution of legal tasks, often requiring high specificity, accuracy, sensitivity and good reproducibility, mycotoxins are frequently determined by LC-MS/MS. The simultaneous detection of several mycotoxins is a major advantage of this technique. Recently, a multi-mycotoxin LC-MS/MS-based method for the simultaneous detection of 23 mycotoxins was described [1]. However, this technique is less suitable for rapid and high throughput testing. It needs skilled personnel to handle the sophisticated machines and often requires sample clean-up, by the use of immunoaffinity (for specific mycotoxins) or solid phase extraction columns [2]. Therefore, LC-MS/MS methods are laborious and time-consuming and less practical for on-site testing.

Nowadays, ELISA is the most common immunoassay format used. ELISA test kits for the detection of the major mycotoxins are widely available on the market [3]. They allow easy and fast quantitative detection with good sensitivities and are suitable for the high throughput screening of samples and for on-site testing. For some sample materials, additional sample clean-up is necessary to avoid under or overestimates by disturbing matrix effects [4]. Other low-cost rapid immunoassay formats used are strip tests [5] and fluorescence polarization [6]. A major disadvantage of these rapid immunoassay formats is that they are not suitable for the simultaneous detection of several mycotoxins, although a duplex strip test [5] and a duplex microarray assay [7] have recently been described.

A new platform for robust multiplexed immunochemical detection is the MultiAnalyte Profiling (xMAP) technology from Luminex. It is an emerging

technology that uses small carboxylated polystyrene microspheres which are internally dyed with a red and an far-red fluorophore [8]. By varying the ratio of the two fluorophores, up to 100 different color-coded microsphere sets can be distinguished, and each microsphere set can be coupled with a different biological probe. The microspheres are detected and characterized by a dedicated flow cytometer [9], using a red laser (635 nm) for excitation and emission wavelengths are measured between 645 and 669 nm and >712 nm. After the microspheres are classified, the reporter signal is measured. The general reporter molecule used is R-Phycoerythrin (R-PE) which is excited by a green laser (532 nm) and the emission is measured at 580 nm [10]. This creates the possibility to simultaneously measure up to 100 different biomolecular interactions in a single well. There are different types of microspheres available such as the xTAG microspheres for DNA purposes, the generally used MicroPlex and the SeroMap microspheres with altered surface for problematic assays. All these varieties have a size of 5.6 μm. The superparamagnetic MagPlex microspheres (6.5 μm) used in this research are available in 500 unique colour codes. Scattered over the microsphere there are magnetite particles for response to a magnetic field which simplifies the work with food or feed samples. The microspheres can be coupled with a wide range of biomolecules like nucleotides, peptides, proteins, antibodies, receptors, polysaccharides and lipids [11]. The xMAP technology is already used in many fields and the number of applications is growing rapidly (http://www.luminexcorp.com/bibliography, 2010). The main xMAP applications are dedicated kits for medical testing like respiratory viruses, cytokine profiling, and neonatal screening (http://www.luminexcorp.com/products/assays/overview.html, 2010). However, the xMAP technology also allows you to develop your own customized assays. This has already been done in the field of nucleic acids [12], food proteins [13], antibiotics [14], polycyclic aromatic hydrocarbons [15], whole bacteria [10] and plant viruses [16] have also been assayed using this technology. More recently, an xMAP duplex immunoassay has been developed for the detection of the mycotoxins OTA and fumonisin B1 (FB_1) in grain products [17]. In other assays, the xMAP technology proved to be as sensitive as ELISA in comparative testing and is less labour-intensive and reduces costs [18].

In our multi-mycotoxin flow cytometric immunoassay xMAP approach, mycotoxin-BSA conjugates of aflatoxin B_1 (AFB_1), OTA, FB_1, DON, ZEN and T-2 Toxin (T-2) are coupled to the carboxylated paramagnetic microspheres. These six coupled microspheres and all six specific monoclonal antibodies (mAbs) against the same toxins are added to the sample. The free mycotoxins from the standards and/or sample extracts will inhibit the binding of the mAbs to the mycotoxin-BSA conjugates on the microspheres. After a magnetic capturing step, a secondary anti-mouse antibody coupled with R-PE is added as a detection molecule. This mixture containing six different microspheres will eventually pass through the flow cell and, upon laser illumination, the microspheres will be classified and its mean surface reporter signals (mean fluorescence intensities; MFIs) will be quantified.

After extensive testing for optimum dilutions/responses, sensitivity, specificity and cross-interactions, six mAbs were selected for this 6-plex MIA. We tested calibration curves for each mycotoxin in buffer and in an extract of a "blank" rapeseed meal (a feed ingredient). To see if the assay was able to perform at the EU guidance levels for feed, we fortified rapeseed meal with the pure mycotoxins prior to sample extraction. Other sample materials (feed and some feed ingredients) were also tested for their matrix influence on the 6-plex MIA. Finally, contaminated FAPAS reference feed samples were tested and the results were compared with instrumental analysis data.

2. Materials and methods

2.1 Instrumentation

The xMAP assay measurements were carried out on a Luminex 100 IS 2.2 system, consisting of a Luminex 100 analyzer, a Luminex sheath delivery system and a Luminex XY Platform, which is programmed to position a 96-well plate, using StarStation System software from Applied Cytometry Systems (ACS, Dinnington, Sheffield, UK). All washing steps were carried out on a Bio-Plex™ Pro II Wash Station (Bio-Rad Laboratories, Veenendaal, the Netherlands) using a magnetic plate carrier. Plates were incubated on a Dynatech microtiter vari-shaker (Alexandria, VI, USA). During the coupling procedures, the paramagnetic microspheres were

captured using the DynaMag-2™ magnet stand (Invitrogen Dynal, Oslo, Norway). Mixing for sample extraction was done in a REAX2 end-over-end shaker (Heidolph, Schwabach, Germany). All centrifuge steps were done in an Eppendorf 5810 R centrifuge using the A-4-62 rotor (VWR International, Amsterdam, the Netherlands).

2.2 Materials, reagents and standards

MagPlex microsphere sets with numbers 036, 038, 054, 086, 090, 100 and Sheath Fluid were purchased from Luminex (Austin, TX, USA). Monoclonal antibodies (mAbs) against AFB_1 (6G4), FB_1 (1D6), OTA (5E2), ZEN (88) and T-2 (8H2) were obtained from Soft Flow Biotechnology (Gödöllö, Hungary) and against DON (AB0222) from Aokin (Berlin, Germany). From Sigma-Aldrich (Zwijndrecht, the Netherlands), the AFB_1-BSA conjugate (A6655), 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), 2-(N-morpholino)ethanesulfonic acid (MES) and BSA were purchased. FB_1-BSA was a kind gift from Jules Beekwilder of Plant Research International (Wageningen, the Netherlands). BSA conjugates of OTA (CON003), DON (CON002), ZEN (CON005) and T-2 (CON004) were from Biopure (Tulln, Austria) as well as the solid standards of the mycotoxins AFB_1, OTA, DON, ZEN, FB_1 and T-2. Goat anti-mouse IgG-R-Phycoerythrin conjugate was obtained from Prozyme (Hayward, CA, USA). Skimmed milk powder (blotting grade blocker, non-fat dry milk) was from Bio-Rad Laboratories. Acetonitril was purchased from Biosolve (Valkenswaard, the Netherlands). Greiner Cellstar 96-well microtiter plates were used for all assays. All other chemicals were ordered from VWR. The "blank" rapeseed meal and other sample materials were supplied previously to and analyzed by RIKILT. Reference feed samples were ordered from FAPAS® (York, UK).

2.3 Coupling of mycotoxin-BSA conjugates to the microspheres

Each microsphere stock suspension (1.25×10^7 microspheres/ml) was vortexed vigorously for 5 min. From each stock, 500 µl (approximately 6 million microspheres) was taken and transferred to an Eppendorf tube. This tube was placed in the magnetic stand and microspheres were allowed to settle for 5 min. The

supernatant was removed and 500 µl of 100 mM monobasic sodium phosphate pH 6.2 was added with the tube still in the magnetic stand. After 2 min of settling, the supernatant was removed again and the microspheres were resuspended in 80 µl of the same sodium phosphate buffer. To this microsphere suspension, 10 µl of 50 mg/ml Sulfo-NHS and 10 µl of 50 mg/ml EDC were added. The suspension was incubated in the dark at room temperature for 20 min. During this incubation, the microsphere suspension was mildly vortexed every 5 min. After incubation, the tube was placed in the magnetic stand and microspheres were allowed to settle for 1 min. The supernatant was removed and the microspheres resuspended in 250 µl of 50 mM MES buffer pH 5.0 by pipetting up and down several times. The microspheres were captured again and the washing step with the same MES buffer was repeated. A previously prepared 500-µl solution of the mycotoxin-BSA conjugate at a concentration of 125 µg/ml in MES was then added to the microspheres. This suspension was incubated for 2 h in the dark at room temperature while gently rotating. The microspheres were captured and washed 2 times with 500 µl PBS-TBN (Phosphate Buffered Saline containing 0.1% BSA, 0.02% Tween-20 and 0.05% sodium azide, pH 7.4). For storage, the microspheres were resuspended in 500 µl of the same PBS-TBN. After overnight storage at 4°C, the microspheres were ready for use in the assays. The final concentrations of the microspheres were determined in the Luminex by counting diluted portions for 60 s with a flow rate of 60 µl/min.

2.4 The xMAP immunoassay

Mycotoxin standard stock solutions were prepared in an acetonitrile/water mixture (80:20; v/v) at concentrations of 100–200 µg/ml. For the calibration curves, necessary standard solutions were prepared from these stock solutions by serial dilutions in water. For the xMAP assay, 40 µl of standard solution or standard solution mixed (1:1; v/v) with sample extract (in the case of dose-response curves in sample extract) or 2 times in water-diluted sample extract was added to each well of a microtiter plate. Subsequently, 10 µl of concentrated PBM (5 times concentrated PBS containing 1% of skimmed milk powder) containing the mAbs of choice were added and incubated for 10 min on a shaker at room temperature. During the initial testing, each antibody was tested separately to check for cross-interactions. After this, the 6

microspheres sets coupled with different mycotoxins, around 1,000 microspheres of each set, were added in 10 µl of PBM and the assay was incubated for another 30 min at room temperature. The plate was then transferred to the washer for a total of 3 washing steps with PBS. The remaining volume after washing was approximately 30 µl. To each well, 70 µl of R-PE conjugated goat anti-mouse antibody (2.85 µg/ml in PBS) was added and the microspheres were brought back in suspension by pipetting up and down. The samples were incubated while shaking for 15 min. After incubation, the plate was again transferred to the washer for one final washing step with PBS. To each well, 70 µl of PBS was added and the microspheres were resuspended by pipetting up and down. The samples were measured in the Luminex analyzer for a total of 100 microspheres per set per sample with an average of 45 min for the measurement of a 96-well plate. During the measurement, the Luminex uses four detectors [three avalanche photodiodes (APDs) and one photomultiplier tube (PMT)]. Two APDs are used for the classification of the microspheres by measuring the emission signals (between 645 and 669 nm and >712 nm) from the two internal dyes excited by a red laser diode (633 nm). As shown in Fig. 1 (upper right part), the 6 microsphere sets are classified in the classifier plot. This laser diode is also used for the determination of side scatter signals of all the measured microspheres which correlates to the particle size. This side scatter signal is detected by the third APD, presented in the discriminator plot (Fig. 1, upper left part) and represents the total measured events. The PMT is used to measure the microsphere-bound reporter molecules after the excitation by a green laser (532 nm), and examples of the reporter signals are shown in Fig. 1 (the lower two parts) for the DON and ZEN assays in the 6-plex MIA. The software calculates the median reporter signal for each measurement.

2.5 LC-MS/MS multi-mycotoxin method

The amounts of the mycotoxins DON, FB_1, T-2, HT-2, OTA, ZEN and in some cases AFB_1 were determined using an in-house validated and accredited LC-MS/MS based method. In short, 2.5 g of sample material was extracted with 10.0 ml of extraction solvent (acetonitril/water/formic acid: 84/16/1; v/v/v). The mixture was shaken for 2 h and then centrifuged.

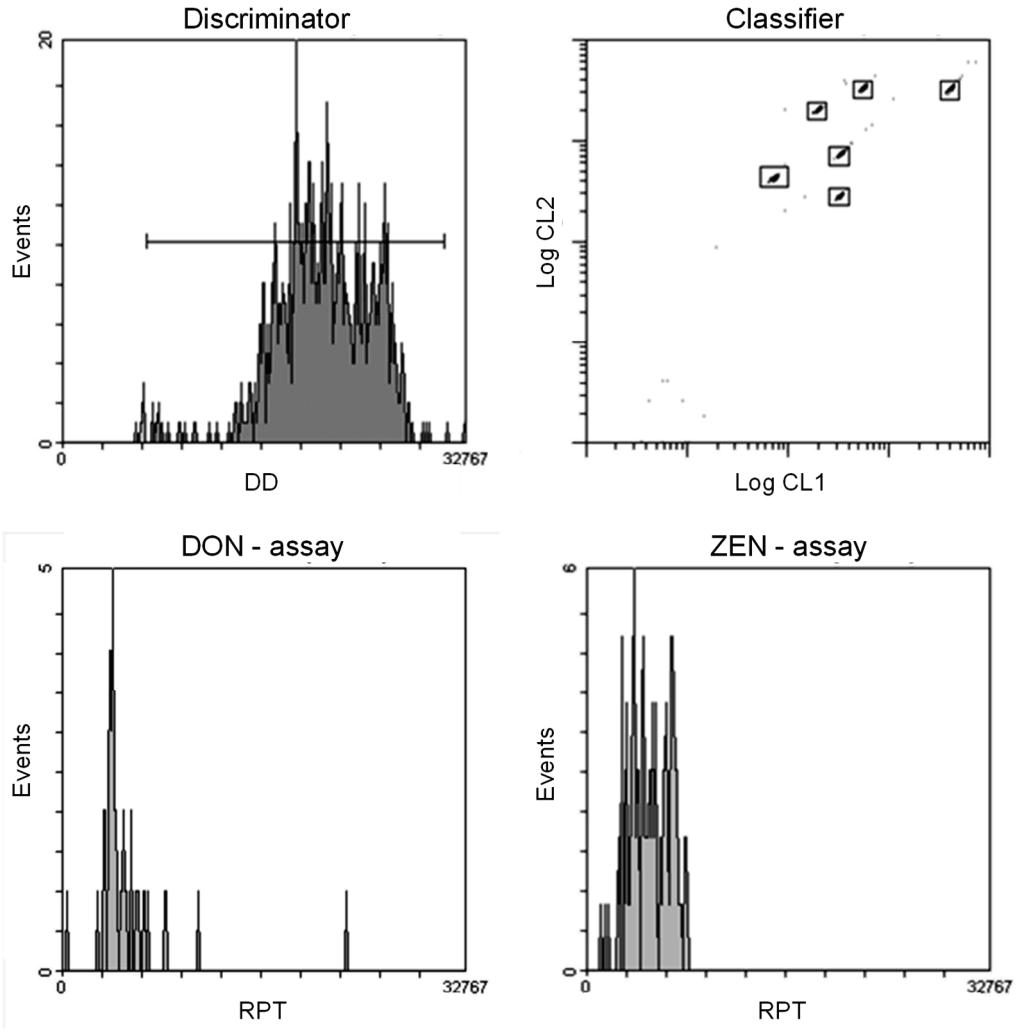

Figure 1. Typical flow cytometric output of the StarStation software showing all microspheres in the Doublet Discriminator (*DD*) plot (*upper left part*), classification and counting of the microspheres based on the log CL1 (red) and log CL2 (far-red) ratio in the Classifier plot (*upper right part*) and 2 of the 6 response plots (*lower two parts*) showing the reporter signals (*RPTs*) for the *DON* and *ZEN* assay in the multiplex flow cytometric immunoassay

The supernatant was diluted with water (1:1), and filtered prior to LC-MS/MS analysis with 5-µl injections and eluted using a water (eluent A)/95% methanol/water (v/v) (eluent B) gradient, both containing 1 mM ammonium formate and 0.53 mM formic acid, at a column temperature of 35°C. The LC-MS/MS system consisted of a Shimadzu Prominence system, a Phenomenex Synergi 4 µ Hydro RP

UPLC column (150 mm × 2 mm, 2.5 μm) and an AB SCIEX QTRAP® used in MS/MS-mode. The mycotoxin content was quantified with a standard addition procedure.

2.5 Fluorescent-HPLC (F-HPLC) AFB_1 detection

The AFB_1 content for some samples was previously determined in proficiency testing using an in-house validated and accredited HPLC-fluorescence-based method. In short, 20 g of sample material, 10 g of celite, 10 ml of water and 100 ml of chloroform were mixed for 30 min. After filtration, 2.0 ml of extract was evaporated until dryness. The residue was dissolved in 1.0 ml of methanol and the solution was diluted with 9.0 ml of water. The resulting solution was cleaned with Immuno Affinity Clean-up (IAC). The Fluorescent-HPLC system consisted of a Gilson pump and autoinjector, a Jasco fluorescence detector and a KOBRA-cell equipped with a Waters Symmetry C18 HPLC column (150 × 3.0 mm, 5 μm). For analysis, 100-μl extracts or reference solutions were injected and eluted using a water/methanol/acetonitril eluent (130/70/40; v/v/v) containing 1 mM KBr and 1 mM HNO_3. Quantitative analysis was performed by calculation versus a calibration curve.

2.6 Extraction of feed matrix

Sample extraction was performed according to an in-house LC-MS/MS protocol that was in use for the simultaneous detection of several mycotoxins. For each sample, 2 times 2.5 g was weighed and transferred to a 50-ml tube. To the first tube, 10 ml of double distilled water was added. To the second tube, 10 ml of acetonitril/water (84/16; v/v) mixture was added. Both tubes were then incubated for 2 h at room temperature while gentle mixing using an end-over-end shaker. The tubes were centrifuged at room temperature for 10 min at 2,000*g* using a swinging bucket rotor. The supernatants were combined in equal volumes and incubated for 1 h at 4°C. After incubation, the mixed sample extracts were again centrifuged at the same speed. The supernatant was diluted twice and used directly in the assays. The dose-response curves were made with standard solutions diluted in water, but also with mixtures (1:1; v/v) of the standard solutions and "blank" sample extract.

3. Results and discussion

Immunoassays for low molecular weight compounds use the direct (antibody-coated surfaces) or indirect (hapten-coated surfaces) competitive or inhibition assay

formats. We have chosen for the indirect inhibition assay format in which the binding of the mAbs to the mycotoxin-coated microspheres is inhibited by the mycotoxins in solution. For the coupling of proteins to the xMAP microspheres, standard protocols are available (Luminex) and, therefore, BSA was used as the carrier protein for the mycotoxins during the microsphere coupling. The final selection of mycotoxin conjugates and mAbs was compiled after a previously performed large-scale screening of reagents obtained from different suppliers (data not shown) and was based on maximum responses, sensitivities of the dose-response curves, specificities (cross-reactions with other mycotoxins), and cross-interactions between the assays. The optimal coupling concentration for the mycotoxin–BSA conjugates to the microspheres proved to be 125 μg/ml. The addition of mycotoxin-specific mAbs, at optimized dilutions, and a secondary anti-mouse R-PE reporter antibody showed significant fluorescence responses for each mycotoxin-coupled microsphere set ranging from 3,000 to roughly 6,000 MFI in buffer (Table 1).

Table 1. Average (*n*=2) maximum fluorescence intensity responses (MFI) obtained with the multiplex immunoassay format in buffer, using individual and the mixed antibodies

Antibody	Maximum Fluorescence Intensity (MFI) responses per assay[a]					
	AFB_1	OTA	ZEN	DON	FB_1	T-2
Mixed[b]	7200±100	4600±200	4600±200	8200±100	4500±200	4600±200
AFB_1	5000±200	9	21	25	35	5
OTA	1	3500±200	25	27	31	7
ZEN	13	15	5800±300	33	25	3
DON	1	13	19	5300±300	27	11
FB_1	5	7	29	27	3200±200	7
T-2	3	7	23	27	27	3100±100

[a] All data were obtained in multiplex microsphere setting, [b] A mixture of the six antibodies

The mAb stock solutions (1 mg/ml) were diluted from 600 to 30,000 times, resulting in final concentrations in the assay of 1.6 μg/ml for anti-AFB_1 and anti-FB_1, 0.83 μg/ml for anti-OTA, 0.67 μg/ml for anti-ZEN, 0.17 μg/ml for anti-DON, and 0.03 μg/ml for anti-T-2. The observed differences of signals depend on the coupling efficiencies of the mycotoxins to BSA and of the conjugates to the microspheres (influenced by the remaining free amino groups on the conjugates and the polar

changes of the protein surface by the mycotoxin molecules) and on the dilutions and affinities of the different mAbs. All the individual mycotoxin-specific mAbs were tested with the complete mixture of six mycotoxin-specific microsphere sets to see whether cross-interactions between the assays could be observed. Table 1 shows that the final selection of reagents did not show any remarkable cross-interactions between the assays. However, except for the ZEN assay, the responses for each specific microsphere set increased when all six antibodies were used simultaneously (mixed) in the 6-plex MIA. It seems that the presence of higher concentrations of antibodies increase the responses, probably due to the non-specific binding of antibodies to each other in the 6-plex MIA. Fortunately, this presumed non-specific binding had no negative effects on the dose-response curves because full inhibitions were still obtained (Fig. 2a). The dose-response curves in buffer, measured in triplicate over a 3-day period, showed good sensitivities for all mycotoxins when measured in multiplex setting (Fig. 2a). The concentrations at 50% relative response [or at 50% inhibition (IC_{50} values)] of the dose-response curves in the different assays, were 0.29, 0.33, 0.39, 1.6, 2.2 and 6.7 ng/ml for OTA, AFB_1, ZEN, FB_1, T-2, and DON, respectively. Compared to the ELISA data supplied by the manufacturers, the IC_{50} values of the multiplex for OTA, ZEN and T-2 were comparable and were two and three times lower for FB_1 and DON, respectively, and four times higher for AFB_1. This indicates that the AFB_1 assay can probably still be improved, for instance, by modifying or changing the buffer. The small error margins show the high precision of the multiplex assay in buffer. Unfortunately, the curves for AFB_1, OTA, ZEN and T-2 are very steep and therefore have limited dynamic ranges. The assays were tested for the described mycotoxins only but will also detect derivatives and other forms of these mycotoxins as shown from the manufacturer's data sheets. According to the suppliers specifications, the anti-AFB_1 mAb was reported to have cross-reactivity with the aflatoxins B_2 (76 %), G_1 (55 %) and G_2 (6 %) and the anti-FB_1 mAb with the fumonisins B_2 (FB_2, 91 %) and B3 (FB_3, 209 %). The anti-ZEN mAb cross-reacts with zearalanon (138 %), α-zearalenol (91 %), β-zearalenol (21 %), α-zearalanol (69 %) and β-zearalanol (6 %) and the anti-T-2 mAb with acetyl-T-2 (12.3 %), HT-2 (3.4 %) and iso-T-2 (2.5 %). The anti-OTA and anti-DON mAbs had no reported cross-reactivities. The reported cross-reactivities might differ per type of assay and sample material and still need to be tested with relevant samples in the 6-plex MIA final format.

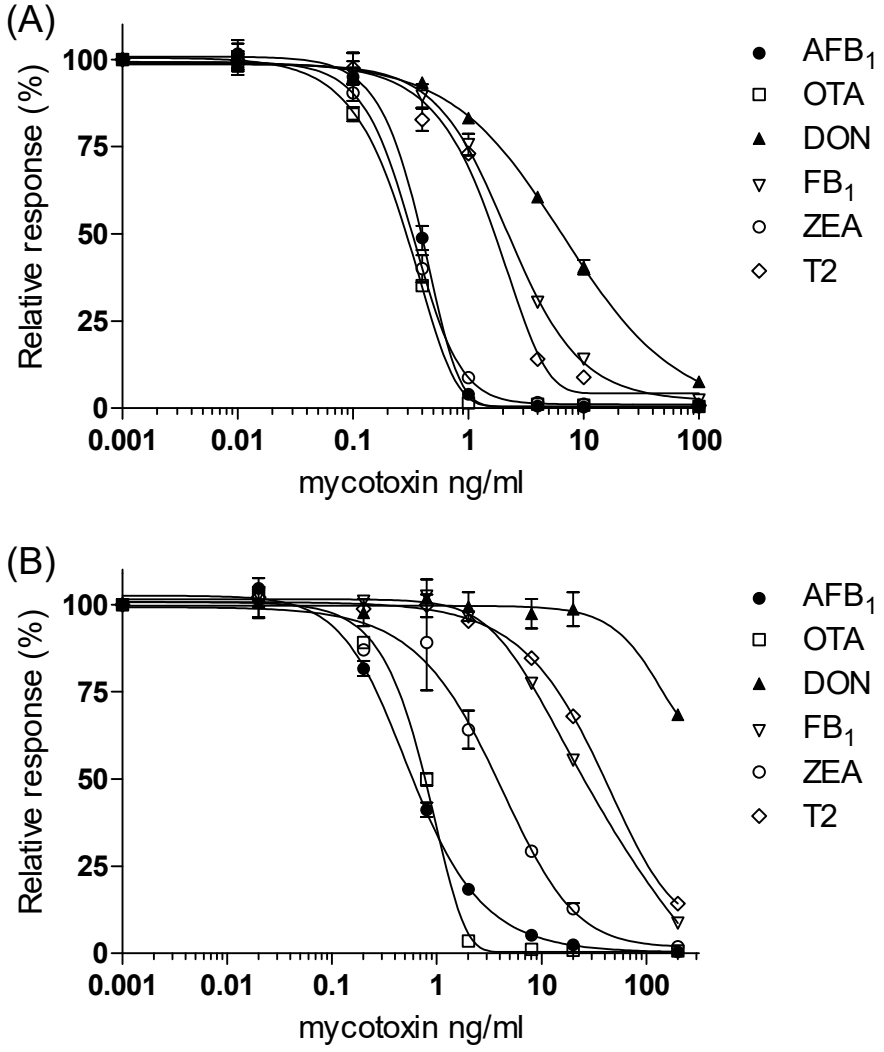

Figure 2. Average dose-response curves ($n = 9$) of the six mycotoxin assays in the multiplex microsphere inhibition assay format in buffer (**A**) and in two times diluted sample extract (**B**)

In general, cross-reactivities may lead to overestimated concentrations of the assayed mycotoxin. For instance, guidance values for aflatoxins in feed are just set for AFB_1 and there is no guidance level for the total aflatoxins, as is the case for food. This means that our assay could give overestimated results for AFB_1 due to relatively high cross-reactivities with AFB_2 and AFG_1. If critical, samples tested positive for aflatoxins in the 6-plex MIA should always be checked for the actual concentration

of AFB_1 using LC-MS/MS. Therefore, the AFB_1 assay is more qualitative than quantitative. The FB_1 antibody used in the assay has high cross-reactivities with FB_2 and FB_3. Like in food, there are combined guidance levels for FB_1 and FB_2 in feed. The concentration of FB_2 in feed is normally around 15–35% of the FB_1 concentration [19]. The high cross-reactivity for FB_3 seems not to be a major problem since it is rarely present in feed (ingredients) as was shown by previous LC-MS/MS measurements within our institute (data not shown). Its occurrence seems related to the presence of high FB_1 concentrations. For ZEN, the appearance of high concentrations of its metabolites in feed are very unlikely [20] and therefore will not contribute to substantial overestimations in this assay. In the case of T-2, the mentioned cross-reactions with HT-2 will slightly contribute to the total response when working with this mAb. If the simultaneous or single detection of HT-2 is desired another antibody is required or has to be added as a new parameter to the assay.

In this study, a rapeseed meal was chosen as the model "blank" feed sample material because, based on LC-MS/MS data available within RIKILT, mycotoxin concentrations were below the limits of detection (LODs) of this method for feed analysis (AFB_1 <0.005 mg/kg, DON <0.50 mg/kg, FB_1 <0.10 mg/kg, FB_2 <0.10 mg/kg, FB_3 <0.10 mg/kg, OTA <0.025 mg/kg, T2 <0.5 mg/kg, and ZEN <0.05 mg/kg). Therefore, it is still possible that this "blank" feed sample contains mycotoxins at levels below these LODs which might influence the screening assay. Measuring dose-response curves in the rapeseed meal extract showed significant decreases of the maximum responses, compared to the response in buffer, for most of the dose-response curves (up to 64%), and also had an effect on most of their sensitivities (Fig. 2b). The sensitivity for the DON curve was most influenced by the addition of the sample extract, and the addition of higher toxin concentrations will be necessary to produce a useful dose-response curve. The use of acetonitrile at a concentration of 14% showed no drastic influence on the total MFI for the DON assay when used as a blank sample. Also, for ZEN, a shift in sensitivity was observed and some toxin concentrations showed increased error margins. Besides a negative effect on the sensitivity and precision, the sample extract enlarged the dynamic range for ZEN. The same effect was seen for the AFB_1 curve. The OTA curve remained largely unaffected by the addition of sample extract. The effects on the maximum responses by the addition of different sample materials in the AFB_1 and OTA assays are shown in Fig. 3 which demonstrates that the AFB_1 assay is much more susceptible to matrix interference than the OTA assay. Each sample material shows a decrease of response

compared to the response in buffer. The same negative effects were observed for the ZEN and FB_1 assays but were most severe for the DON assay. The T-2 assay, like the OTA assay, remained largely unaffected. Therefore, for quantitative analysis, this assay depends on calibration curves in blank matrix extracts, which is difficult because of the varying content of feed, or on the use of a suitable multi-mycotoxin clean-up.

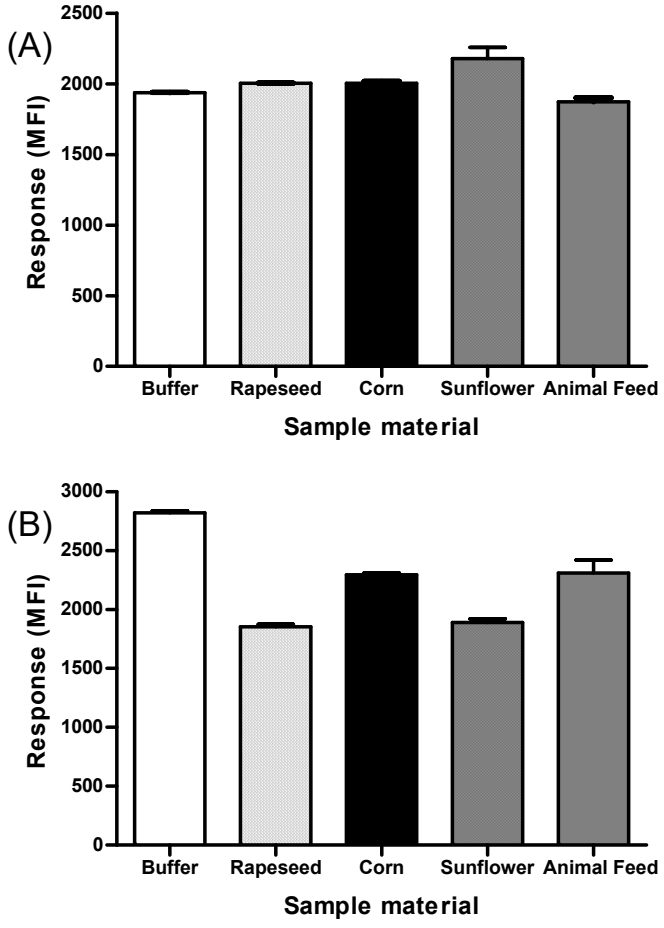

Figure 3. Average ($n = 3$) maximum responses (*MFI*) for the OTA (**A**) and AFB_1 (**B**) assays in different sample extracts and buffer

To test if the method was suitable for the qualitative detection, the "blank" rapeseed meal was fortified with the 6 mycotoxins at EU guidance levels [21,22] . All the fortified samples showed reduced responses (inhibition) in the specific assays when

compared to the non-fortified controls (Table 2). The samples fortified with other mycotoxins could also be considered as negative controls for the specific assays and all the responses in the fortified samples were found to be significantly lower (lower responses compared to the average responses minus three times the SD). In the case of the OTA, FB_1, ZEN and DON assays, there are strong inhibitions of the responses (99, 98, 89 and 56 %, respectively), but for the AFB_1 assay, and to a lesser extent the T-2 assay, there is less inhibition (15 and 34 %, respectively) at these relevant concentrations. The AFB_1 assay certainly needs some improvement which is probably best done by changing one of the two essential reagents. For the T-2 assay, it is less urgent to make changes.

Table 2. Average responses (MFI) obtained with the multiplex flow cytometric immunoassay (n = 3) for extracts of a "blank" rapeseed meal fortified with mycotoxins at EU guidance levels (Directive 2002/32/EC 7 May 2002, Commission recommendation 2006/576/EC 17 August 2006)

Mycotoxin added	Level of addition (μg/kg)	Responses (MFI) for each assay					
		AFB_1	OTA	ZEN	DON	FB_1	T-2
None	0	2100±70	2800±140	3600±150	980±30	1600±70	2000±50
AFB_1[a]	5	1900±110	2800±70	3800±140	1100±50	1600±50	2100±90
OTA[b]	50	2100±50	30±3	3700±240	980±30	1500±30	2000±80
ZEN[c]	100	2200±70	2800±120	420±20	1000±40	1700±60	2100±60
DON[d]	900	2300±130	2900±170	3900±220	460±50	1600±170	2200±160
FB_1[e]	5000	2300±50	2800±130	4000±130	1000±50	39±3	2300±60
T2[f]	10	2300±110	2900±180	3800±180	1100±60	1700±100	1400±80

Recommended guidance value of the EU: [a] Lowest level for feed for dairy cattle; [b] Lowest level for feed for pigs; [c] Lowest level for feed for young pigs; [d] Lowest level for feed for pigs; [e] Lowest level for FB_1 +FB_2 in feed for pig, horses, rabbits and pets; [f] The level for T2 was set at 10 ppb since there is no official guidance value

The guidance value we chose was very stringent. For example, Liesener et al. [23] used a level of 250 μg/kg based on the comparison of the toxicity of T-2 to DON. Furthermore, some east European countries have set the guidance level for T-2 at 100 μg/kg. It also becomes clear from the response ratio in Table 3 that the extraction

for T-2 is not optimal using the current protocol. This is not the case for AFB_1, the extraction of which seems to be optimal based on that ratio.

Table 3. Comparison of the responses (MFI) obtained with mycotoxin-spiked blank rapeseed sample (at guidance values) and with spiked blank sample extract indicating extraction efficiencies of the different mycotoxins using the multi-mycotoxin extraction procedure

Mycotoxin	MFI signal		Response ratio before and after extraction
	Fortified Supernatant [a]	Fortified raw material [b]	
AFB_1	1890	1976	1.04
OTA	30	26	0.87
ZEN	418	284	0.68
DON	459	529	1.15
FB_1	39	31	0.79
T2	1376	691	0.50

[a] Blank rapeseed meal extract (supernatant) was fortified with the different mycotoxins at the 100% extraction efficiency levels, [b] Rapeseed meal was fortified before extraction at EU guidance levels

The contaminated FAPAS reference feed samples (fortified or naturally contaminated, which was not clear from the sample information) were investigated and the average maximum responses of the rapeseed fortified dose-response curves were used to calculate the percentages of inhibition (Table 4). All four samples that were assigned for the presence of AFB_1 (7–23 μg/kg) showed strong inhibitions (90–98 %) in the AFB_1 assay, but three of them also in the ZEN assay (77–95 %) and one (T0470) in the DON assay (64 %). With LC-MS/MS, ZEN was found in two of these samples (52 and >200 μg/kg) and the DON sample contained a high concentration (1440 μg/kg). The two OTA assigned samples showed strong inhibited responses in the OTA assay (97 and 98 %), but also in the FB_1 assay (82 and 94 %) and one (T1758) in the T2 assay (62 %). With LC-MS/MS, FB_1 was found (77 and 164 μg/kg) but T-2 could not be detected with LC-MS/MS in that sample because of an interfering peak. LC-MS/MS data showed a high concentration of HT-2 in this particular sample (430 μg/kg, data not published). The ZEN assigned sample (T2225) showed a strong inhibition in the ZEN assay (94%) but also in the FB_1 and DON assay in which the LC-MS/MS found a low concentration of FB_1 (147 μg/kg) and a high concentration of DON (920 μg/kg).

Table 4. FAPAS reference samples and their relative xMAP responses, based on rapeseed meal fortified dose-response curves, obtained with extracts in the multiplex flow cytometric immunoassays compared to instrumental analysis results

Sample No.	Assigned Mycotoxin[a]	Assigned Value (µg/kg)	Percentage of inhibition (Luminex) and concentrations determined by instrumental analysis for the different mycotoxins											
			AFB_1		T-2		OTA		ZEN		FB_1		DON	
			% inh.	F-HPLC (µg/kg)	% inh.	LC-MS/MS data (µg/kg)	% inh.	LC-MS/MS data (µg/kg)	% inh.	LC-MS/MS data (µg/kg)	% inh.	LC-MS/MS data (µg/kg)	% inh.	LC-MS/MS data (µg/kg)
T0470	AFB_1	11.8	90	11.9[b]	16	<10	8	<5	95	>200	37	34	64	1,440
T2225	ZEN	274	0	<1[c]	17	<10	48	<5	94	>200	84	147	55	920
T2230	DON	894	0	<1[c]	8	<10	5	<5	40	160	97	2,880	52	710
T04102	AFB_1	18.1	96	23.7[b]	0	<10	0	<5	5	<5	0	<20	10	<100
T0486	AFB_1	23.0	98	34.6[b]	1	<10	12	<5	77	<5	14	<20	32	<100
T0478	AFB_1	7.28	93	8.7[b]	35	<10	97	<5	88	52	84	55	52	200
T1748	OTA	8.03	0	<1[c]	23	<10	97	8	44	n.d.	82	77	32	140
T2240	DON	775	29	2.6[c]	50	47	19	<5	60	10	94	115	59	810
T1758	OTA	41.4	34	<1[c]	62	n.d.[d]	98	48	63	11	94	164	45	250

[a]Samples came from single mycotoxin proficiency testing and were submitted for LC-MS/MS multi-mycotoxin analysis

[b]Previously determined values from FAPAS proficiency testing for AFB_1 using F-HPLC

[c]Values determined using the LC-MS/MS multi-method

[d]The quantification of T-2 in this sample was hampered by an interfering peak but a high concentration of HT-2 (430 µg/kg) was detected

The two DON-assigned samples showed strong inhibited responses in the DON assay (52 and 59 %) but with one sample (T2230) also in the FB_1 assay (97 %), in which the LC-MS/MS found 2,880 µg of FB_1/kg, and in the ZEN assay (40 %), in which the LC-MS/MS found 160 µg of ZEN/kg. The other sample (T2240) inhibited the T-2 assay (50 %), the ZEN assay (60 %) and the FB1 assay (94 %) and the LC-MS/MS found T-2 (47 µg/kg), ZEN (10 µg/kg) and FB_1 (115 µg/kg). These additional mycotoxins found in these reference feed samples, show the potential of the 6-plex MIA. However, one AFB_1 assigned sample (T0478) also caused strong inhibition in the OTA assay (97%) and another (T0486) in the ZEN assay, which could not be confirmed by LC-MS/MS.

The overall results of the qualitative 6-plex MIA look promising and will be further exploited in new research. Unfortunately, this research cannot be extended with the same reagents, because of the discontinuation of the supply of the mycotoxin-BSA conjugates from Biopure. Home-made mycotoxin–protein conjugates and conjugates from other suppliers are now under investigation in combination with the described mAbs. The performances of indirect and direct inhibition assays will be compared, as well as improvements in the assay's protocols (incubation time, temperature and buffer composition). The 6-plex MIA can be easily extended with other mycotoxins of interest, but finding a suitable multi-mycotoxin clean-up to remove matrix effects will improve its applicability.

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CHAPTER

3

Color-encoded paramagnetic microspherebased direct inhibition 3-plex flow cytometric immunoassay for ochratoxin A, fumonisins and zearalenone in cereals and cereal-based feed

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Adapted from:

Colour-encoded paramagnetic microbead-based direct inhibition 3-plex flow cytometric immunoassay for ochratoxin A, fumonisins and zearalenone in cereals and cereal-based feed. Anal Bioanal Chem 2013, 405, 7783–7794.

Abstract

A combined (3-plex) immunoassay for the simultaneous detection of three mycotoxins in grains was developed with superparamagnetic color-encoded microspheres, in combination with two microsphere-dedicated flow cytometers. Monoclonal antibodies were coupled to the microspheres and the amounts of bound mycotoxins were inversely related to the amounts of bound fluorescent labelled mycotoxins (inhibition immunoassay format). The selected monoclonal antibodies were tested for their target mycotoxins and for cross-reactivity with relevant metabolites and masked mycotoxins. In the 3-plex format, low levels of cross-interactions between the assays occurred at irrelevant high levels only. All three assays were influenced by the sample matrix of cereal extracts to some extent and matrix-matched calibrations are recommended for quantitative screening purposes. In a preliminary in- house validation, the 3-plex assay was found to be reproducible, sensitive and sufficiently accurate for the quantitative screening at ML level. The 3-plex assay was critically compared to liquid chromatography-tandem mass spectrometry using reference materials and fortified blank material. Results for the quantification of ochratoxin A and zearalenone were in good agreement. However, the fumonisin assay was, due to overestimation, only suitable for qualitative judgements. Both flow cytometer platforms (Luminex 100 and FLEXMAP 3D) performed similar with respect to sensitivity with the advantages of a higher sample throughput and response range of the FLEXMAP 3D and lower cost of the Luminex 100.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi as a part of their plant invasive actions. Their toxic effects can range from nausea to cancer. In 2010, BIOMIN monitored the mycotoxin prevalence in raw materials and animal feed worldwide as part of their annual 'mycotoxin survey program From more than 3,300 samples analysed, 55, 26 and 42% tested positive for the mycotoxins fumonisins, ochratoxin A and zearalenone, respectively [1]. Co-occurrence of mycotoxins in cereals can lead to increased toxicity by additive and synergistic effects [2]. Therefore, the risk for humans and animals increases and this calls for multiplex mycotoxin testing.

Fumonisins (FB's) most likely to contaminate commodities are, in decreasing order of occurrence, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) [3]. Maize and sorghum are the main commodities where FB naturally occur [4]. The maximum level (ML) set by the EU for FB_1+FB_2 in unprocessed maize is 2000 μg/kg [5]. Ochratoxin A (OTA) is the most common and most toxic ochratoxin [6], primarily occurs in grain products, although its presence in coffee, cacao, grapes and indirect contamination in pork have been noted [7,8]. The ML set by the EU for OTA is 5 μg/kg for all cereals [5]. Zearalenone (ZEN) is a non-steroidal oestrogenic compound [9] The main ZEN derivatives include α- zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) [10,11]. ZEN is a frequent contaminant of maize, oats, wheat, barley, sorghum, millet and rice [9]. The ML set by the EU for ZEN in unprocessed maize is 200 μg/kg and for other cereals 100 μg/kg [5]. Conjugated mycotoxins, in which the toxin is usually bound to a more polar substance like glucose, are referred to as masked mycotoxins. Because of their conjugation, they escape routine analysis, provided that there is no reference substance available. They can emerge by metabolization of the original molecule by living plants or food processing. Known plant conjugates for ZEN are zearalenone-14-β-D-glucopyranoside (Z14G), α-zearalenol-14-β-D-glucopyranoside (α-ZELG) and β-zearalenol-14-β-D-glucopyranoside (β-ZELG) and zearalenone 14-sulphate (Z14S) [12,13] and can appear in a variety of food and feed products [14].

Methods for the detection of mycotoxins are mainly based on chromatography and immunochemistry. A number of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods detect a large range of mycotoxins and their metabolites in a variety of food [15-17] and feed commodities [18]. In 2007, Sulyok et al [19] reported an LC-MS/MS method capable of detecting 87 analytes with just a single extraction step after which the diluted crude extract was measured directly. When applied, this method was able to detect 37 different metabolites in mouldy food samples. This method was then further extended with 99 fungal and bacterial metabolites [20]. Although the multiplex capacity and precision is high, and the need for clean-up is not always necessary [21], it cannot be considered as a rapid technology for a selection of mycotoxins.

The main immunochemical method used for the detection of mycotoxins is the enzyme-linked immunosorbent assay (ELISA) [22]. The ELISA format is robust, fast and has a high sample throughput. Sample purification is often minimal, even when screening raw materials. ELISA's for FB_1, OTA and ZEN were previously developed [23-25] and ELISA kits for common mycotoxins are available from a variety of suppliers [26]. Nowadays, lateral flow tests and dipstick tests are other popular immunochemical techniques for the detection of mycotoxins. They are rapid, can be carried out in just a few minutes, the sample preparation is often very short and they do not need equipment. However, they are less sensitive when compared to instrumental methods and the results are mostly qualitative, providing an answer on the presence or absence of a certain mycotoxin only [22]. Recently, a multiplex dipstick immunoassay for the indirect detection of ZEN, deoxynivalenol, T-2/HT-2 toxin and FB was developed, but omitting the most toxic mycotoxins. A photometric strip reader was used to get semi-quantitative results [27]. Using conventional SPR (surface plasmon resonance) several applications are known for single [28] and for multiple (4) mycotoxins [29]. A new approach is the multiplex detection of mycotoxins using the label-free imaging SPR (iSPR) technique. Using an IBIS iSPR, capable of reading out a spotted microarray sensor chip, Dorokhin et al [30] developed a method for the simultaneous detection of ZEN and DON. The used iSPR technology facilitates higher multiplexing capacity as was shown for allergens [31].

The demand for faster multiplex testing is high and new techniques are emerging. The proprietary xMAP (Multi-Analyte Profiling) technology is a suspension array platform based on color-encoded microspheres, often referred to as beads [32]. The microspheres have a carboxylated surface to facilitate the covalent coupling of biomolecules such as peptides, proteins, antibodies, polysaccharides, lipids and nucleotides. These microspheres can then be analysed in a microsphere dedicated flow cytometer [33]. Previously, two microsphere-based indirect immunoassays (MIAs) for mycotoxins were described [7,33] in which the mycotoxins were immobilized on the microspheres. In contrast, direct immunoassays having immobilized antibodies on the microspheres are rare. Very recently, Czeh et al [34] described a similar approach as in the present work, but using a different analyser with non-paramagnetic microspheres. Unfortunately, experimental data as well as detailed procedures are lacking in ref. [34] thus hampering a direct comparison with our results. Moreover, no LC-MS/MS or other confirmatory method was carried out using certified reference samples to verify or pre-validate that assay.

2. Materials and Methods

2.1 Instrumentation

For the measurement of the xMAP immunoassays, two different flow cytometers from Luminex (Austin, Texas, USA) were used. The Luminex-100 (consisting of a LX-100™ analyser, a sheath fluid delivery system and the XY platform) and the new FLEXMAP 3D which integrates all of these components in one machine. The LX-100 operates on XPONENT software version 4.0 and the FM3D on version 4.1. A Bio-Plex II Wash Station with magnetic plate support (Bio-Rad Laboratories, Veenendaal, the Netherlands) was used for all washing steps. For the retention of the MagPlex microspheres during the antibody-microsphere coupling process, a DynaMag-2 magnetic separator stand (Invitrogen Dynal, Oslo, Norway) was used. A Bühler TiMix 2 (Salm en Kipp, Breukelen, the Netherlands) was used for all microtiter plate incubation steps. A REAX 2 overhead shaker (Heidolph, Schwabach, Germany) was utilised for the agitation of samples during mycotoxin

extraction. Centrifugation of 50 ml Greiner tubes was done in an Eppendorf 5810R centrifuge using a A-4-62 rotor (VWR International, Amsterdam, the Netherlands) and high speed centrifugation of Eppendorf tubes with a Bio-Rad Model 16K Microcentrifuge (Bio-Rad Laboratories, Veenendaal, the Netherlands). A Vortex Genie 2 (Scientific Industries, New York, USA) was used to mix samples. Microsphere counting was done using a Bio-Rad TC10 automated cell counter (Bio-Rad Laboratories). For LC-MS/MS analysis, a Shimadzu Prominence high performance liquid chromatography system (Kyoto, Japan) was coupled with an AB SCIEX (Framingham, MA, USA) QTRAP 5500 triple quadrupole mass spectrometer, run in multiple reaction monitoring (MRM) mode. The probe temperature was set at 400 °C. Additional MS/MS acquisition details are provided in Table S1 (see Supplementary Material Chapter 2). A Restek (Bellefonte, Pennsylvania, USA) Ultra Aqueous C18 (100 x 2.1 mm) LC column was used. The chromatograms were integrated automatically with the Signal Finder integration algorithm of MultiQuant V2.0 software.

2.2 Chemicals and Reagents

The MagPlex microsphere sets MC10026, MC10036, MC10038 and sheath fluid were obtained from Luminex. Cellstar 96-wells culture microtiter plates (Greiner, Alphen a/d Rijn, the Netherlands) were used for all assays. Centrifugal filter units (50 kDa), used for buffer exchange and 30 kDa Amicon Ultra 4 centrifugal filter devices were purchased from Millipore (Bedford, MA, USA). Monoclonal antibodies (mAbs) against FB_1 and OTA were purchased from Soft Flow Biotechnology Ltd. (Gödöllő, Hungary). The anti-ZEN mAb as well as the FB_1 and OTA mycotoxins were purchased from Aokin AG (Berlin, Germany). Mycotoxin solutions of FB_1, FB_2, FB_3, OTA, OTB, ZEN, α-ZEL and β-ZEL were purchased from Coring System Diagnostix (Gernsheim a. Rhein, Germany). Z14G, α-ZELG, β-ZELG and Z14S were a kind gift of Dr. Franz Berthiller. The R-Phycoerythrin (RPE)-FB_1 and RPE-OTA conjugates were produced in-house using RPE from Moss (Pasadena, Maryland, USA). For RPE conjugate purification, Amicon Ultra 4 centrifugal filter devices were used. The RPE-ZEN conjugate was custom made by Aokin AG using the same Moss RPE. MES ((2-(N-morpholino) ethanesulfonic acid),

sulfo-NHS (N-hydroxysulfosuccinimide) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and caffeine-(trimethyl-$^{13}C_3$) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ethanol and all other solid chemicals were purchased from VWR International (Amsterdam, the Netherlands). Syringeless filter devices for sample clean-up were purchased from GE Healthcare (Rotterdam, the Netherlands). Acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, the Netherlands), formic acid (FA) from Merck (Whitehouse Station, NJ, USA) and Ammonium formate (AMF) from Fluka Analytical (Steinheim, Germany).

2.3 Matrix and reference samples

The blank maize and wheat materials were previously analysed at RIKILT using an accredited confirmatory LC-MS/MS method for feed samples (FB_1, FB_2 and FB_3 < 0.1 mg/kg, OTA < 0.025 mg/kg, ZEN, α-ZEL and β-ZEL < 0.05 mg/kg). Naturally contaminated maize and wheat materials were either laboratory stock or purchased from Coring System Diagnostix (Gernsheim a. Rhein, Germany) and R-Biopharm/Trilogy (Darmstadt, Germany) as reference materials. Additionally, naturally contaminated cereal-based feed samples were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium).

2.4 Methods

2.4.1 MAb coupling to the microspheres

The mAbs against the three mycotoxins were separately coupled to three different sets of the paramagnetic MagPlex™ microspheres according to a modified protocol originally provided by Luminex. Initially, the antibody stock solutions (1 mg/ml) were diluted to a concentration of 100 µg/ml using 50 mM MES buffer (pH 5.0), transferred to a 50 kD filtering unit, and centrifuged at 10,000 g for 10 minutes at RT. The concentrated antibody solution was then reconstituted in 500 µl using the same MES buffer. The original microsphere stock was vortexed for 1 minute to resuspend the microspheres. From the microsphere suspension, 1 ml (approximately 1.25 X

10^7 microspheres) was pipetted into an Eppendorf-tube. The Eppendorf-tube was then placed into the magnetic separator and, after a 1 minute magnetic separation period, the supernatant was carefully removed without disturbing the microspheres. The Eppendorf-tube was removed from the magnetic separator and the microspheres were resuspended in 100 µl double distilled water. The Eppendorf-tube was placed back into the magnetic separator and, after another 1 minute separation, the supernatant was removed. This wash step was repeated twice and the microspheres were resuspended in 100 µl of 100 mM monobasic sodium phosphate buffer (pH 6.2) and placed in the magnetic separator. The supernatant was discarded and the microspheres resuspended in 80 µl of the phosphate buffer. To this microsphere suspension, 10 µl of sulfo-NHS and 10 µl of EDC were added (both previously prepared at a concentration of 50 mg/ml with double-distilled water). This microsphere suspension was vigorously mixed by vortex and incubated in the dark at RT for a total of 20 minutes with a vortex pulse at 5 minute intervals. Following the incubation period, the tube was shortly centrifuged to make sure that all microspheres were at the bottom of the tube. After a 1 minute incubation in the magnetic stand, the supernatant was removed. Microspheres were resuspended in 250 µl 50mM MES (pH 5.0) and mixed by vortex. After magnetic separation, this step was repeated and the previously prepared mAb solution (500 µl) was added to the microsphere pellet and mixed by vortex. The activated microspheres and antibodies were then incubated in the dark at RT on a rotational shaker for 2 hours. Following the incubation, the tube was centrifuged for 30 seconds, placed into the magnetic separator stand and, after a 45 second separation period, the supernatant was removed. The tube was then removed from the magnetic stand and the microspheres were resuspended in 1 ml of Phosphate Buffered Saline-TBN (PBS, 0.1% Bovine Serum Albumin (BSA), 0.02% Tween-20 and 0.05% sodium azide, pH 7.4). The microsphere suspension was placed into the magnetic stand and, after a 45 second separation period, the supernatant removed. This wash step was repeated twice. Finally, the covalently modified microspheres were suspended in 1 ml PBS-TBN and stored in the dark at 4°C.

2.4.2 Microsphere counting

To make sure that the right number of microspheres of each set was added to the assay, coupled microspheres were counted with the Bio-Rad TC10™ automated cell-counter. The microsphere suspensions were diluted 10-fold with PBST (PBS and 0.02% Tween-20, pH 7.4) and 10 µl of each of the diluted suspension was applied to one of the counting chambers of a counter-dedicated slide. The slide was then inserted into the cell-counter for CCD imaging. The number of microspheres in the readout was then multiplied by a factor 10.

2.4.3 Coupling of OTA and FB_1 to R-PE

The OTA-RPE conjugation procedure was identical to the one described previously by Aqai et al [35]. The conjugation of FB_1 to RPE was based on the method of Szurdoki et al [31] with slight modifications: a glutaraldehyde buffer was prepared just before use by adding 400 µl of a 25% glutaraldehyde solution, 0.4 grams of NaCl and 5 ml of a 0.1 M Sodium Phospate solution (pH 7.5) to a 50 ml tube and the volume was adjusted to 50 ml using fresh double-distilled water. From this buffer, 3 ml was added to a dark glass vial. From a fresh 10 mg/ml RPE solution, 50 µl was added to the same glass vial. The reaction mixture was incubated for 24 hours with constant slow stirring and exchanged to PBS by using 30 kDa centrifugal filter devices with a total of 4 centrifuge steps at 3,000 g and a total volume of 12 ml PBS. The volume of the final concentrate was adjusted to 3 ml with PBS. To the modified RPE solution, 200 µl of FB_1 (10 mg/ml in methanol) was added drop wise. This reaction mixture was incubated overnight at room temperature. The next day, 16 µl of 0.2 M of L-lysine was added to the reaction, vortexed and placed in the fridge again for overnight incubation. The conjugate was purified by transferring the reaction mixture to a 30 kDa centrifugal filter device. The volume was adjusted to 4 ml with PBS and concentrated to 50 µl. This step was repeated three times and the remaining conjugate solution volume was adjusted to 500 µl with PBS.

2.4.4 Sample fortification

Blank maize and wheat materials were fortified at the maximum levels (MLs) for each mycotoxin for unprocessed cereals. For the fumonisins we chose to fortify with FB_1 at 2000 μg/kg which is the combined ML for FB_1+FB_2 in unprocessed maize. Fortification with OTA was done at 5 μg/kg which is the ML for all unprocessed cereals. For ZEN, we chose to fortify at 100 μg/kg which is valid for almost all unprocessed cereals. For the sample fortification, 2.5 g of sample was weighed in a 50 ml tube. The mycotoxin solutions (in 10% MeOH) were pipetted to the side of a near-horizontally positioned 50 ml tube (50 μl of 100 μg/ml for FB_1, 125 μl of 0.1 μg/ml for OTA and 25 μl of 10 μg/ml for ZEN) and the lid carefully placed on. The tubes were then shaken to allow the mycotoxins to mix with the sample material. The tubes were then un-capped and allowed to air dry for 60 minutes.

2.4.5 Sample extraction for xMAP assays

In a 50 ml tube, 10 ml of extraction solvent (80% MeOH) was added to 2.5 g of sample material. The tubes were then vortexed vigorously for 10 seconds and placed in the overhead shaker at a moderate speed setting for 30 minutes. The tubes were centrifuged at room temperature (RT) for 10 minutes at 4,000 g. The supernatant was transferred to another tube without disturbing the pellet. Before analysing, the supernatant was diluted 8-fold with double distilled water in order to reduce the influence of matrix effects and to ensure that quantitation (at MLs) occurred within the linear part of the curve. After dilution, the extracts were centrifuged once more at 12,000 g for 10 minutes at RT to remove non soluble components.

2.4.6 Calibration standards and matrix-matched standards

Initially, calibration standard solutions containing individual mycotoxins were prepared and tested in single assay format. For multiplex analysis, a multi standard was prepared including the three mycotoxins. The calibration curve ranges were determined according to the sensitivities of the assays. Serial dilutions were made from 1 mg/ml stock solutions. For FB_1, the highest calibration point was 10 μg/ml and for OTA and ZEN 1 μg/ml. From these

initial calibration points, the other calibration points were prepared by 10-fold serial dilutions with 10% MeOH in six steps. The eighth - and final - calibration point was a negative control consisting of 10% MeOH. For the matrix-matched standards, the blank sample extract was diluted 4-fold with double distilled water and combined (1:1) with the mycotoxin calibration standard solutions, thus resulting in an 8-fold dilution of the matrix material in total and a 2-fold dilution of the standards. Finally, the diluted samples with and without standard solutions were centrifuged at 12,000 g for 10 minutes at RT to remove non soluble components. Curve fitting and calculation of the fitting parameters was done using GraphPad Prism 5 (La Jolla, CA, USA).

2.4.7 Determination of specificity

To determine the specificity of the selected antibodies for a wide range of known metabolites and masked forms of the three target mycotoxins, calibration curves were made with the following compounds; Ochratoxin B (OTB), FB_2, FB_3, Z14G, α-ZELG, β-ZELG and Z14S. Stock solutions were diluted in 10% MeOH to get calibration curves under similar conditions as the original mycotoxins. These calibration curves of individual compounds were analysed in buffer and maize extracts using all microspheres and all mycotoxin-RPE conjugates. The cross-reactivity of these metabolites was calculated by dividing the concentration at 50% inhibition (IC50 value) of the target compound by the IC50 of the metabolite/masked form.

2.4.8 3-plex immunoassay

The final 3-plex immunoassay procedure started with the addition of 40 µl of a matrix-matched calibration standard solution (containing OTA, FB_1 and ZEN) or sample extract to a well of a low-protein binding 96-wells microtiter plate. Then, 10 µl of microsphere suspension containing 1,000 mAb-coupled microspheres for each of the three assays (3,000 microspheres in total per sample) were added to each well. After a 2 minute pre-incubation on a plate-shaker, 10 µl of the mixture of three mycotoxin-RPE conjugates, (diluted 100-200 times), were added to each well using PBST pH 7.4 as the assay buffer. The microtiter plate was then incubated at RT on a shaker for 15 minutes and

subjected to a single pre-programmed automated wash with PBST (previously optimised to retain the microspheres). To the residual volume left by the washer (30 µl), 70 µl PBST was added and the plate was mixed on a shaker for 1 min at RT. Finally, the plate was measured in the Luminex platform counting 100 microspheres per microsphere set. The overall measurement time for the assay (incubations, washing steps and the actual measurement) was 50 minutes for a 96-wells plate on the FM3D platform.

2.4.9 LC-MS/MS Analysis

The concentrations of FB_1, OTA, ZEN and their metabolites were determined by an in-house ISO 17025 accredited LC-MS/MS confirmatory method using multiple reaction monitoring. Briefly, the LC-MS/MS method was as follows. Electrospray ionisation was used as the soft ionisation mode in negative (ZEN and associated metabolites) and positive (the remaining) modes. Reference samples were analysed in parallel with fortified blank sample material in order to assess the validity of the results via monitoring of extraction efficiencies. Retention time and ion ratio were used as quality control parameters according to the SANCO/12495/2011 document for method validation and quality control procedures for pesticide residues analysis in food and feed [36]. LC-MS/MS sample preparation was as follows. An internal standard of $^{13}C_3$-caffeine (10 µg/ml) was added to the sample prior to extraction in order to monitor extraction and injection of each sample. From each sample 2.5 g of material was weighed and extracted with 10 ml ACN/H_2O/FA (84/15/1; (v/v/v)) for 2 hours on a horizontal shaker at room temperature. Following extraction, the tubes were centrifuged at 3,000 g for 10 minutes at RT. The supernatant was diluted (1:1) with Milli-Q water, vortexed, and refrigerated at 4°C for 30 minutes to allow for fat content to settle. Following refrigeration, the samples were filtered in polypropylene filter devices. The LC-MS/MS system was run with 5 µl sample injections. Mycotoxins were eluted from the LC column with an aqueous/organic gradient consisting of mobile phase A- water and B- MeOH/H_2O (95/5) at a column temperature of 35 °C. To each mobile phase, 1 mM ammonium formate (AmF) and 0.53 mM formic acid (FA) was added. The flow rate was set at 0.4 ml/min with a total runtime of 15 minutes.

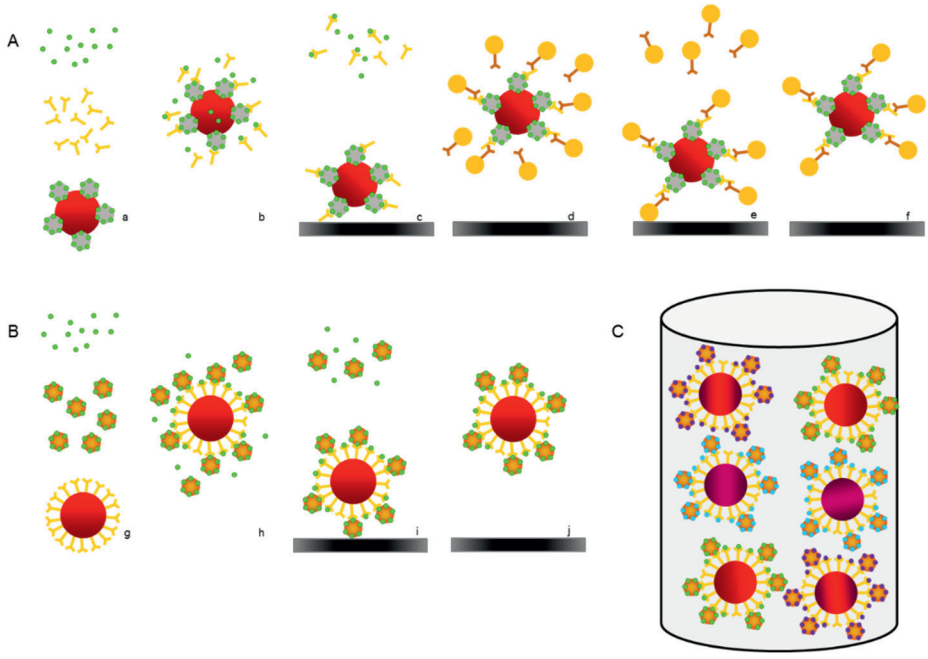

Figure 1. Comparison of the *direct* assay (this work) and *indirect* microsphere immunoassay [33]. In an indirect assay (A), sample, antibodies and mycotoxin-BSA conjugated microspheres (a) are incubated so that there is competition between the conjugated mycotoxins on the microsphere and the free mycotoxins in the sample (b). After incubation the microspheres are trapped by a magnet and the non-bound reagents washed away (c). The microspheres are released and an anti-mouse-RPE is added (d). After incubation the microspheres are trapped again and non-bound anti-mouse-RPE is washed away (e). After release, the microspheres are measured (f). In the much simpler direct assay presented in this work (B), sample, mycotoxin-RPE conjugate labels and antibody coupled microspheres are incubated (g). Labeled and free mycotoxins compete for antibodies on the microspheres (h). Then microspheres are trapped by a magnet and the non-bound reagents washed away (i). Microspheres are released and measured (j). This is done *all-in one* for 3 different mycotoxins in one well (C).

3 Results and Discussion

3.1 Building the 3-plex assay

The principle of the direct 3-plex assay is shown in Fig 1B. It shows that the direct format is more simple than the indirect formats (Fig 1A) previously developed [7,33]. After extensive testing of a variety of mAbs from different sources coupled at a fixed concentration to the microspheres, three of them

were selected based on the following criteria: good responses at low concentrations of the mycotoxin-RPE conjugates, low cross-interactions between the assays, desired cross-reactivity with other metabolites, and dynamic range of the assays which should match with the MLs established by the EU (Commission regulation No 1881/2006 foodstuffs) [37]. PBST was chosen as the optimal buffer for the 3-plex assay. The optimal dilutions of the mycotoxin-RPE conjugates were determined in single assay format and in all cases the highest dilution (approx. 1 ng reporter each, per well), while still retaining substantial absolute signal, gave the best dynamic range (data not shown). At this stage, the single assays were combined to create the 3-plex format. This means that 3 microspheres, 3 RPE reporter conjugates and 3 mycotoxin calibrants were present in a single well (Fig. 1C). This had an impact on the sensitivities of the assays. It resulted in a 3-fold decrease in sensitivity for the ZEN assay and a 2-fold decrease for the FB_1 and OTA assay based on the IC50's of the dose-response curves (see Supplementary Information, Fig. S1) most likely caused by combining the calibration curves and reporter conjugates all in one well. The incubation time was set at 15 minutes (the shortest incubation time tested) to comply with rapid testing. Longer incubation times (30 and 60 minutes) did not result in improved sensitivities. To check for cross-interactions between the assays, this 3-plex assay was also tested using the three antibody-coupled microspheres and the individual mycotoxin-RPE conjugates. The three microsphere sets were found to respond to their corresponding conjugates only (data not shown).

3.2 Cross reactivity with metabolites and masked forms

Although the antibodies used in the assay were raised against specific mycotoxins (FB_1, ZEN and OTA), depending on the chemistry of the conjugates used in immunization, cross-reactivity with metabolites and masked forms can occur because of strong homologies. To check for this, the 3-plex assay was used to record calibration curves of the designated mycotoxins and other relevant metabolites in buffer, as well as in maize extract. Percentages of cross-reactivityies were calculated at 50% inhibition. The FB_1 antibody shows a desirable high cross-reaction with FB_2 (61%) since legislation is for the sum of FB_1 and FB_2. The cross-reaction with FB_3 might

result in an overestimation but fortunately, that metabolite occurs at much lower concentrations than FB_1 (see section on naturally contaminated reference materials). The cross-reactivity of FB_3 is lower in maize matrix (44%) than in buffer (63%). Only OTA at a very high concentration (1 μg/ml, corresponding with 32 mg/kg in a cereal product) showed an impact on the FB_1 assay causing 40% inhibition. The OTA antibody showed high cross-reactivity for OTB (43% in maize), which is less desired since OTB is not as hazardous as OTA [35]. However, OTB occurs in much lower concentrations than OTA and therefore will not cause a significant problem [38]. FB_1 at a high concentration of 10 μg/ml and β-ZEL at 1 μg/ml showed inhibition in the OTA assay (20% and 30%). The ZEN antibody reacts in the ZEN assay with α-ZEL (66%) and to a much lesser extent with β-ZEL (13%). There is no legislation for α-ZEL and β-ZEL in cereals, so their cross-reaction might be considered undesirable. However, their occurrence is in much lower concentrations than ZEN itself [39]. All the other metabolites with a glucose or sulphate group located at the 14 position of the molecule have no cross-reactivity at all. Apparently the epitopes of the conjugated ZEN, α-ZEL and β-ZEL molecules used in our research are blocked for antibody recognition. These results are comparable to Dorokhin et al [30] who used the same antibody in a label-free iSPR approach, except the result for β-ZEL which differs substantially (10 fold). This might be due to the entirely different technique and the indirect assay approach used in that work. In the ZEN assay, inhibition of the signal is observed (30%) when FB_1 is present, but at a very high concentration (10 μg/ml) only.

3.3 Comparing the LX-100 and FM3D flow cytometers

In general, the new FM3D flow cytometer shows higher absolute responses (6- to 10-times, in high PMT voltage mode) when compared to the LX-100. As can be seen in Fig. S2(see Supplementary Information) the dose-response curves for all 3 mycotoxins are almost identical when the relative responses are plotted against the concentrations of the mycotoxins. Because of these results, we decided to conduct further research on the newer FM3D system because of its faster throughput, knowing that the developed assay would also perform well on the LX-100 when necessary. Previously, Bienenmann et

al [40] showed that both machines had good correlation when absolute responses were compared for a five-plex immunoassay for coccidiostats. Also other assays developed in our lab showed equal sensitivities on both machines. The FB_1 antibody initially used in Fig. S2 was later replaced by a more sensitive one.

3.4 Effects of wheat and maize matrix on calibration curves

Matrix-matched calibration curves are frequently used to compensate for any matrix effects and to avoid inaccurate quantitation. To check for the possible effects of maize and wheat extracts in our newly developed direct 3-plex assay method, we compared calibration curves in buffer with those in sample extracts. From Fig. 2 it becomes clear that only maize has a strong suppressive effect on the relative responses of the FB_1 and ZEN assays. The effect of wheat on the FB_1 curve is much less pronounced. OTA is not influenced by the maize extract but the wheat matrix yielded some sensitivity enhancement. These results show that matrix-matched calibration curves are to be preferred for quantitative data in this multiplex screening assay. According to the IC50 and LOD data, the 3-plex MIA developed shows LOD's for maize and wheat that are adequate for routine monitoring at ML. For OTA the sensitivities were; 0.7 and 3.4 μg/kg respectively (ML = 5 μg/kg), for ZEN; 5,8 and 32 μg/kg respectively (ML = 100 μg/kg) and for FB_1+FB_2; 170 and 1270 μg/kg respectively (ML = 2000 μg/kg). Maize, the material with the highest matrix effect on the dose-response curves, was not tested (or presented) in the work of Czeh et al [34], therefore we compared our wheat sensitivities with their results. Our OTA assay was 2 times more sensitive, while their ZEN and FB_1 assays were respectively 4- and 5-times more sensitive. Most importantly, our method met with the EU required ML's in grains [5].

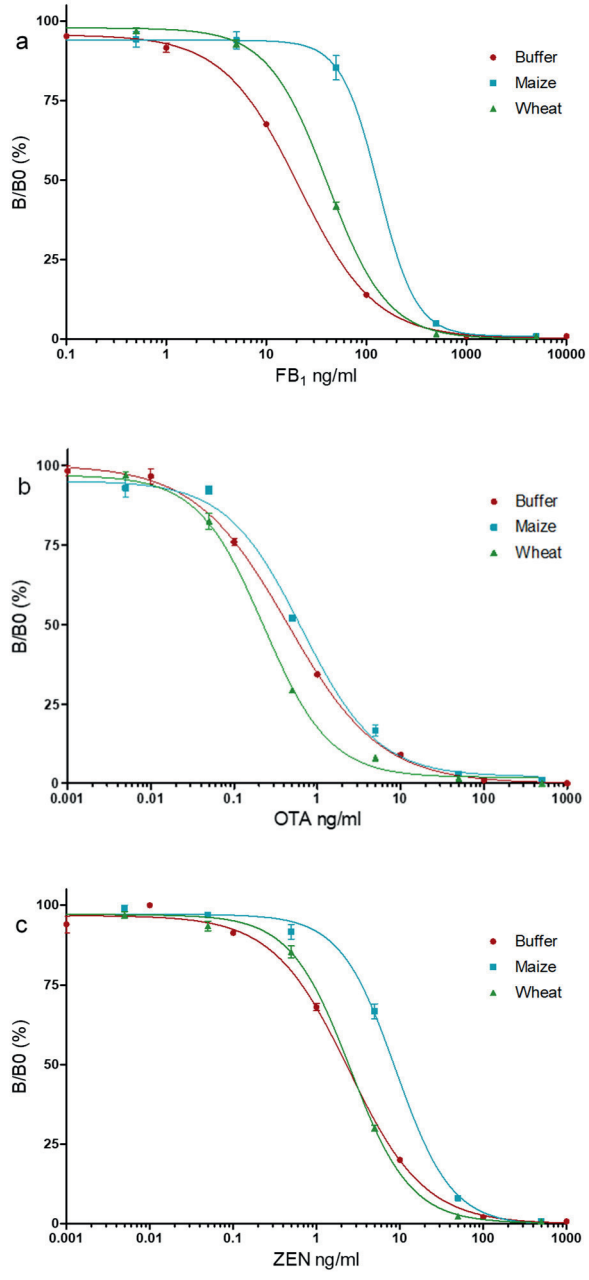

Figure 2. Dose-relative response curves for FB_1 (a), OTA (b) and ZEN (c) in the 3-plex MIA in buffer and maize and wheat -extracts (n=3)

3.5 Preliminary in-house validation using wheat and maize samples

Ground blank maize and wheat samples were fortified in triplicate by the addition of individual mycotoxins and their mixture. FB_1 was added at 2,000 ng/g, OTA at 5 ng/g, and ZEN at 100 ng/g, corresponding with the MLs targeted by EU legislation [37]. The same samples were also left unfortified and extracted, being the blank controls. After extraction, all samples were quantified in triplicate using the 3-plex MIA. The obtained results showed satisfying inhibition levels for all added mycotoxins and hardly any difference between the single and mixture fortification in maize and wheat (Table 1). The reproducibility of the triplicate analyses was ranging from 1 to 10 %RSD with an exception for the OTA mixture fortification in wheat (23 % RSD). In general, variations were slightly higher for the wheat extracts. The accuracy of the method was determined by quantitating samples using the aforementioned matrix-matched calibration curves. The experimental mycotoxin levels were compared with the initial fortification levels. The screening accuracies for OTA in wheat and maize were 50 and 70% respectively. For ZEN there was a 1.5-fold overestimation in maize while the accuracy in wheat was 84 and 74%, respectively. The accuracies for FB_1 were higher than expected, leading to a 4-fold overestimation in maize and 3-fold in wheat. The overestimation is reproducible and remarkable since the matrix-matched calibration curves have the same level of matrix components as the fortified samples analysed. To test for sample variance, six independent blank wheat samples (tested for feed MLs) were fortified at food ML levels (FB_1 2,000 ng/g, OTA at 5 ng/g, and ZEN at 100 ng/g). One sample a day was fortified in triplicate with each single mycotoxin and extracted. As a blank control, no mycotoxin was added. Also the controls were extracted in triplicate. The extracts were measured in triplicate in the 3-plex MIA. The results (Fig. 3) show that there is satisfactory inhibition in each assay and only slight variation in the relative responses between samples. Variation is probably due to the different blank wheat samples used for fortification. They were considered blanks according to an in-house *feed* LC-MS/MS method which does not exclude the presence of mycotoxins below the limits of detection.

Table 1. Reproducibility and accuracy of the 3-plex immunoassay (n=9) using single and mixed (all 3 mycotoxins) fortifications to wheat and maize with FB_1, ZEN and OTA at MLs

Fortified Mycotoxin(s)	Maize				Wheat			
	Fortification level (µg/kg)	Experimental level (µg/kg)	RSD (%)	Accuracy (%)	Fortification level (µg/kg)	Experimental level (µg/kg)	RSD (%)	Accuracy (%)
FB_1 single	2,000	8,100	2	405	2,000	5900	10	295
FB_1 mixed	2,000	8,020	2	401	2,000	5200	4	260
OTA single	5	3.46	4	69	5	2.5	4	50
OTA mixed	5	3.30	10	66	5	2.1	23	42
ZEN single	100	155	1	155	100	84	4	84
ZEN mixed	100	147	1	147	100	74	7	74

Another reason for the response variation could be that each independent sample was fortified, extracted and measured on a different day so inter-day extraction variation may occur.

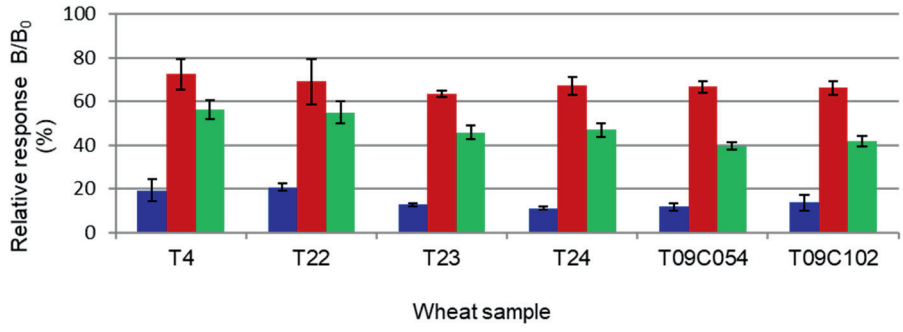

Figure 3. Relative responses (n=9) obtained in the 3-plex MIA by multi-fortification of 6 different blank wheat samples with FB1 (2000 µg/kg, blue bars), OTA (5 µg/kg, red bars) and ZEN (100 µg/kg, green bars)

3.6 Application for the analysis of incurred reference materials

An ISO 17025 accredited LC-MS/MS multi-mycotoxin method was used to analyse naturally contaminated maize (n=11) and wheat (n=6) reference samples and cereal-based feed samples (n=3) using matrix-matched calibration curves. This instrumental multi-mycotoxin method determines 33 mycotoxins and metabolites. A summary of the results, and critical comparison with the 3-plex MIA screening assay, is presented in Table 2. The immunoassay results for the OTA containing reference and mixed cereal samples showed similar accuracy's (~50%) as reported in the fortification experiments. In sample BRM 003022/M10482A, the immunoassay detects OTA at a concentration of 1 ng/g while it is not present in the LC-MS/MS measurement. This is probably not due to the sensitivity of the LC-MS/MS method since in 2 samples concentrations below 1 ng/g are detected. It may be caused by OTB which was not included in the LC-MS/MS method but will cross-react in the immunoassay. For ZEN, concentrations found in samples BRM 003022/M10482A and TR-O100/O-W-816 for the immunoassay correlate very well with the data found in the LC-MS/MS method.

Table 2. Comparison of the results obtained with the 3-plex MIA (n=3) and with LC-MS/MS (n=2) using extracts of naturally contaminated wheat, maize and feed samples

Sample ID	Material	Assigned mycotoxin (ng/g)	Mycotoxin concentrations (ng/g)									
			LC-MS/MS							3-plex immunoassay		
			FB_1	FB_2	FB_3	OTA	ZEN	α-ZEL	β-ZEL	FB*	OTA	ZEN**
BRM 003022/M10482A	wheat	DON / 877 ± 23	155	38	16	0	122.7	5.7	0	1763	1.0	142.6
TR-O100/O-W-813	wheat	OTA / 23.3 ± 3.2	141	41	22	27.1	0	0	8.8	2304	14.0	1.0
TR-O100/O-W-816	wheat	OTA / 101.8 ± 12.2	7	2	1	120.2	4.1	0	0	320	77.0	5.0
TR-O100/O-W-805	wheat	OTA / 3.2 ± 0.6	15	4	2	4.3	0	0	0	480	2.2	3.0
TR-D100/D-W-153	wheat	DON / 2,100 ± 200	1	1	0	0	3.4	0	18.8	203	0	21.4
TR-D100/D-W-163	wheat	DON / 500 ± 100	2	1	0	0	0.9	0.2	8.0	164	0	28.0
Mixed cereal 1	feed	OTA / NA***	100	23	16	76.1	6.6	0	0	1104	30.0	33.0
Mixed cereal 2	feed	OTA / NA***	79	21	13	266.0	7.9	0	19.0	1412	171.0	54.0
Mixed cereal 3	feed	OTA / NA***	142	36	23	706.6	9.9	0	0	1859	403.0	43.0
BRM 003012/M10195A	maize	DON / 2010 ± 290	703	197	82	0	133.5	3.5	0.	3104	0	358
BRM 003018/M10203C	maize	FB1 / 270 ±110 FB2 / <80 FB3 / <80	248	61	25	0	45.2	3.3	0	5181	0	79.0

*Sum of FB1, FB2 and FB3 according to antibody specificity, **Sum of ZEN, α-ZEL and β-ZEL according to antibody specificity, ***NA means not assigned for target mycotoxins

Table 2. Continued

Sample ID	Material	Assigned mycotoxin (ng/g)	Mycotoxin concentrations (ng/g)									
			LC-MS/MS							3-plex immunoassay		
			FB_1	FB_2	FB_3	OTA	ZEN	α-ZEL	β-ZEL	FB*	OTA	ZEN**
TR-D100/D-C-606	maize	DON / 1,100 ± 100	106	27	12	0.2	74.0	0	0	544	0	101
TR-Z100/Z-C-310	maize	ZEN / 59.4 ± 10.5	7,988	2,196	824	0	66.3	0	0	17728	0	68.0
BRM 003003/M09452Z	maize	FB1 / 2406 ±630 FB2 / 630 ± 116	2,123	644	271	0.2	21.6	0	0	15040	0	156
TR-MT100/T-C-973	maize	T-2 / 1153 ±160 H-T2 / 1381 ± 177	7,894	2,192	943	0	16.7	0	0	12384	0	49.3
TR-A100/A-C-274	maize	AFB1 / 7.3 ± 0.9	1,552	412	161	0	60.4	1.3	0	5376	0	77.4
BRM 003017/M10203B	maize	FB1 / 2630 ± 740 FB2 / 690 ± 340 FB3 / 310 ± 210	2,841	811	310	1.1	206.6	12.7	6.3	15424	1.3	184
BRM 003019/M10205A	maize	ZEN / 177.3 ± 64.8	1,842	560	215	0	161.5	28.4	27.6	15542	0	377
TR-A100/A-C-268	maize	AFB1 / 4.3 ± 0.5 AFB2 / 0.3 ± 0.1	1,548	309	170	0	29.4	0	0	5504	0	57.9
TR-A100/A-C-276	maize	AFB1 / 1.7 ± 0.3	1,165	300	125	0	18.9	0	0	4521	0	31.7

*Sum of FB1, FB2 and FB3 according to antibody specificity, **Sum of ZEN, α-ZEL and β-ZEL according to antibody specificity, ***NA means not assigned for target mycotoxins

Also for sample TR-O100/O-W-813 the correlation is good since the immunoassay value (1 ng/g) is based on the 13-17% cross-reaction of the mAb with the β-ZEL metabolite detected by the LC-MS/MS (8.8 ng/g). In contrast, much higher values for the immunoassay were found in samples TR-D100/D-W-153, TR-D100/D-W-163 and the mixed cereal samples (4 to 7-fold). For those samples, the overestimation cannot be explained by the presence of the α-ZEL and/or β-ZEL metabolites. Also it cannot be explained by the presence of the masked metabolites Z14G, α-ZELG, β-ZELG or Z14S since the ZEN mAb doesn't have any cross-reaction with those. For sample TR-O100/O-W-805 a concentration of 3 ng/g is determined with the immunoassay while the LC-MS/MS doesn't detect any ZEN metabolite. These overestimations might be caused by other possible masked forms with which the ZEN mAb might cross-react. Also Thongrussamee et al [25] found overestimations when 2 ELISA kits and HPLC were compared. In the 6 wheat reference samples, fumonisins were found by LC-MS/MS (ranging from 0 to 155 ng/g). As expected these concentrations were largely overestimated by the 3-plex MIA. The highest overestimation (100-fold) occurred at the lowest (according to LC-MS/MS) concentration (2 ng/g for FB_1 + FB_2). At the highest concentration found by LC-MS/MS, 193 ng/g (for FB_1 + FB_2), the immunoassay overestimation was approximately 10-fold. These overestimations are much higher when compared to the fortification experiments done for FB_1. Note that the FB_1 mAb is unable to distinguish between the metabolites and therefore the FB screening assay is indicative for the total level of fumonisins present. To study this overestimation issue further, a different antibody was tested under the same circumstances but again this resulted in high overestimations. After testing a range of buffers, PBST with a pH of 7.4 was chosen as the most optimal buffer. A more basic buffer ($NaHCO_3$, pH 9.6) had dramatic consequences for the ZEN assay although it was beneficial for the sensitivity for the OTA assay. Using MES buffer pH 5, as a more acidic approach, we found a 50 fold decrease for the OTA sensitivity and a 10 fold dcrease for the ZEN sensitivity. Furthermore, pH's outside the 5-10 regio will destabilize the

RPE making it lose its reporter fluorescence. Using this standard PBST buffer, a range of additives (fish gelatin (1%), ficoll (1%), polyethylene glycol (1%), polyvinyl alcohol (1%), polyvinylpyrrolidone (3%) and skimmed milk powder (1%)) were tested but without improved results. Omitting tween-20 from the buffer is not an option since this is necessary to keep the microspheres from clustering. Also the same extraction method but with acidified solvent had no effect. Extraction with ACN/water generally showed decreased overestimation for maize samples, but this improvement (reduced FB_1 overestimation), was not seen for the wheat samples (results not shown). Moreover, the ACN/water extraction had a negative impact on the sensitivities of the OTA and ZEN assay. Using MeOH/water/FA (80/20/0.1 v/v/v) showed the same overestimation as the MeOH/water extraction. Overestimation of FB_1 in immunoassays has been widely documented. Tejada-Simon et al [41] found consistently higher concentrations of FB_1 when compared to HPLC. Kulisek et al [42] described that extensively diluted samples yielded higher interpolated values for ELISA. Another issue causing inaccuracies might be the presence of bound fumonisins. Dall'Asta et al [43] detected bound fumonisins in gluten-free food products in even higher concentrations than the free forms. Furthermore, hidden fumonisins were also found in unprocessed food but in a non-covalent bound form [43]. These non-covalent bound hidden fumonisins are referred to as extractable hidden fumonisins. To date, physical characterization of the non-covalent interaction of fumonisins with matrix components was not carried out yet [44]. However, if we would apply a decision level (DL) for FB_1 of 4000 μg/kg for the 3-plex MIA, then it correlates quiet well with the LC-MS/MS data for maize. Looking at the maize samples that have concentrations of FB_1 and FB_2 around or above the ML of 2000 ng/g we would then see no false-negative results and just one false positive (BRM 003018/M10203C) (Table 2).

Although the 3-plex MIA principle, described in this work, seems similar to the work of Czeh et al [34] there are some crucial differences. Besides sensitivity (discussed above) we used certified reference materials designated

to single mycotoxins (and in case of FB_1 also the FB_2 and FB_3 metabolites) in combination with a confirmatory LC-MS/MS method to get full information about all relevant metabolites present for a comprehensive comparison of the 3-plex MIA results. The cross-reactivity of the coupled antibodies with known metabolites of the targeted mycotoxins were not researched nor mentioned in ref [34] while they can lead to undesired under or overestimation of the original target mycotoxins.

To conclude, the developed direct inhibition multiplex immunoassay approach is faster and requires less procedural steps than the previously developed indirect assay format [33]. Moreover, because of the color-encoded microsphere concept, more mycotoxins can be easily added at a later stage in order to extend the application range of this rapid assay.

Acknowledgements

The authors would like to thank Frank Mallwitz from Aokin AG for fruitful discussions considering the RPE conjugate production. This research was carried out in the MultiTox project (PID091008/2.6A), financed by the Dutch Ministry of Economic Affairs, SenterNovem and the Province of Gelderland.

Chapter 3: Supplementary Material

Available for free at:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3765849/

Fig. S1: Comparison of the single and 3-plex MIA based on dose-response curves.

Fig. S2: Comparison of the LX-100 and FM3D flow cytometers using the 3-plex MIA* with mixed dose-response curves (*using a different FB_1 antibody than described in this paper).

Table S1: List of mycotoxin metabolites and the used parameters of the LC-MS/MS method

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CHAPTER

4

6-plex microsphere immunoassay with imaging planar array detection for mycotoxins in barley

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Adapted from:

6-Plex microsphere immunoassay with imaging planar array detection for mycotoxins in barley. The Analyst 2014, 139, 3968-3976.

Abstract

Mycotoxins are produced by fungi as secondary metabolites. They often multi-contaminate food and feed commodities posing a health risk to humans and animals. A fast and easy to apply multiplex screening of these commodities could be useful to detect multi-contamination. For this, we developed a semi-quantitative 6-plex microsphere immunoassay (MIA) using a suspension array of paramagnetic colour-coded microspheres combined with imaging planar array detection for the mycotoxins aflatoxin B_1, ochratoxin A, zearalenone, deoxynivalenol, T2-toxin, HT-2 toxin and fumonisin B_1. Mycotoxin specific monoclonal antibodies were coupled to different sets of microspheres and mycotoxins conjugated to the fluorescent protein R-Phycoerythrin served as reporter molecules. Competition between free mycotoxins in the sample and mixed reporter molecules for antibody binding sites on mixed microspheres created a multiplex direct inhibition immunoassay. The reagents were selected for no or low cross-interactions between the assays and cross-reactions with metabolites and possible modified forms were determined. A within-laboratory validation was carried out using blank and spiked barley samples. Furthermore, the 6-plex MIA was used to screen available barley, and malted barley, reference materials. The validation showed very high inter and intra-day precision for all samples with a maximum relative standard deviation value of 10%. The screening assay allows easy and rapid multiplex detection of the target mycotoxins in barley according to EU legislation. With a cut off factor of 50%, based on the EU maximum levels, we were able to screen at 2 μg/kg for aflatoxin B_1, 2.5 μg/kg for ochratoxin A, 625 μg/kg for deoxynivalenol, 50 μg/kg for zearalenone, 1000 μg/kg for fumonisin B_1 and 25 μg/kg for T-2 toxin. Thanks to the transportable planar array system, the developed 6-plex MIA has potential for future on-site testing. Future implementation of this method as a pre-screening tool, prior to instrumental analysis, is highly attractive since costly LC-MS/MS analysis of samples below the maximum levels can be avoided.

1. Introduction

Barley belongs to the grass family and comes in many varieties which can be clustered in two-row and six-row types, of which the latter is a naturally mutated form. It is known that the two-row varieties are often more resistant to Fusarium infection (known as Fusarium Head Blight or scab). The infection of barley, by Fusarium and other fungal species, can lead to huge economic losses as a consequence of reduced grain yield and quality. Consumption of food and feed products, produced from or with infected barley, can lead to mycotoxicosis in humans and animals. Barley, especially the two-row, is used as barley-malt in the beer brewing process and also as an ingredient for distilled spirits, syrups, coffee substitutes and other food and feed products. Therefore, accurate monitoring of barley crops for mycotoxins is essential [1-3]. Infected barley can contain the fungal metabolites; zearalenone (ZEN), deoxynivalenol (DON), T-2 toxin (T-2) and HT-2 toxin (HT-2) [4], aflatoxins (AFs) and ochratoxin A (OTA) [5] and even fumonisin B_1 (FB_1) [6]. These mycotoxins can have acute and chronic effects on human and animal health [7]. For instance, AFs are acutely toxic and also have mutagenic, teratogenic and carcinogenic properties, whereas ZEN is a non-steroidal oestrogenic compound causing diminished fertility amongst others [8,9]. The aforementioned mycotoxins are regulated by the European Union (EU) and maximum levels (MLs) are set for their occurrence in a variety of raw materials as well as in feed and food products [10]. The MLs in μg/kg in unprocessed cereals for food are 2 for aflatoxin B1 (AFB_1), 5 for OTA, 100 for ZEN, 1250 for DON and 2000 for FB_1 (unprocessed maize). Recently indicative levels were set at 200 μg/kg for the sum of T-2 and HT-2 by the the EU [11].

In a recent review, Streit et al. [12] showed that multi-mycotoxin contaminations in animal feed (monitored since 2004) occurred in 75–100% of the samples. These high numbers of mycotoxin co-occurrences underline that multiplex detection of mycotoxins is needed, especially for the ones legislated by the EU. As a consequence, instrumental mycotoxin multi-methods, often liquid chromatography with tandem mass spectrometry (LC-MS/MS)-based[13,14], gained popularity. In the field of rapid testing, new immunoassay-based multiplexed technologies are emerging which often

elaborate on existing ELISA principles. Lattanzio et al. [15], developed a multiplex dipstick immunoassay capable of detecting five major fusarium toxins (ZEN, DON, T-2/HT-2 combined and FB_1) in wheat, oats and maize using a single extraction step. The method was validated at full ML level with a false positive rate lower than 6% [16]. Ediage et al. [17] developed two different flow-through immunoassay formats with the antibodies coupled to the carrier material. The gel-based format was capable of detecting OTA, FB_1, DON and ZEN while the membrane-based format detected OTA, AFB_1, DON and ZEN. Using functionalized glass slides, Oswald et al. [18] immobilized OTA, AFB_1, FB_1 and DON onto the surface of a reusable assay-chip. Detection of the fluorescent signal, generated by a horseradish peroxidase enzyme labelled to a reporter antibody, was done in a stand-alone chip reader. Using a sandwich immunoassay approach, Mak et al. [19] developed a biosensing technology where magnetic nanotags (coupled to streptavidin for binding to biotinylated detection antibody) were used as a detection molecule. Using a capture-antibody, coupled to a spin-valve sensor for each mycotoxin, they were able to detect AFB_1, ZEN and HT-2 in real-time. Meneely et al. [20] developed a rapid surface plasmon resonance (SPR) immunoassay for the multiplex detection of T-2/HT-2 and DON. HT-2 and DON were covalently coupled thereby creating a mixed sensor chip surface. Detection of T-2 was possible due to the cross reactivity of the HT-2 mAb with T-2. The assay was applied to fortified cereals and fortified cereal-based products. Applying imaging surface plasmon resonance (iSPR), Dorokhin et al. [21] developed an indirect method for the simultaneous detection of DON and ZEN by immobilizing protein-mycotoxin conjugates as spots on an iSPR sensor chip. A novel microsphere indirect approach was presented by Deng et al. [22], using silica photonic crystal microspheres (SPCM) for the detection of AFB_1, FB_1 and citrinin. Mycotoxin-protein conjugates were coupled to the SPCM surface and the fluorescent signal, on the reporter antibody, was detected by an array fluorescent scanner. A similar approach was used by Xu et al. [23] replacing citrinin for OTA in their 3-plex SPCM assay format. Instead of using fluorescent labeled antibodies, they used an enzymatic conversion to generate a fluorescent molecule so the signal could be read on a microplate reader. A suspension array of color-coded microspheres (more often referred to as

beads) has the benefit of a single fluorescent reporter molecule. This principle has been applied for multiplex testing in food safety: multiplex flow cytometric immunoassays were developed for the detection of shellfish toxins[24], coccidiostats [25], pesticides [26], hormone biomarkers [27], persistent organic pollutants [28], antibiotics [29] and mycotoxins [30]. For mycotoxins, the developed assays were mainly based upon the indirect detection principle where the mycotoxins are coupled as protein conjugates to the microspheres. Using this approach with non-paramagnetic microspheres, Anderson et al. [31] developed a 2-plex for the detection of OTA and FB_1. Using the same bead format, Wang et al. [32] developed a 4-plex for the detection of AFB_1, DON, T-2 and ZEN and applied it to corn and peanut. Using the easier to handle paramagnetic microspheres, Peters et al. [30] developed a 6-plex for the indirect detection of AFB_1, DON, T-2, OTA, FB_1 and ZEN, and evaluated its application in feed samples. Recently, we suggested a much faster and more simplified direct approach using paramagnetic microspheres combined with unique fluorescent mycotoxin-reporter molecules [33]. Czeh et al. [34] developed a 6-plex assay using the same direct principle but on a different platform. However, all these previously reported mycotoxin assays used microspheres that were detected by flow cytometers.

In the present approach, we developed a semi-quantitative multiplex mycotoxin assay based on a transportable lower cost imaging planar bead array analyser. In the compact sized system, light-emitting diodes (LED's) and a CCD camera are utilized instead of lasers and photo multipliers in flow cytometer designs, yielding a transportable system. The paramagnetic microspheres in the sample pass through a flow chamber where the microspheres are trapped by a magnet and imaged (Fig 1.). After measurement, the microspheres are released, the flow chamber is washed and the next sample introduced. We validated the developed 6-plex MIA as a screening method for barley, according to the guidelines for screening methods for residues of veterinary medicines (initial validation and transfer) [35] and set the screening target concentration at 50% of the ML according to EU legislation.

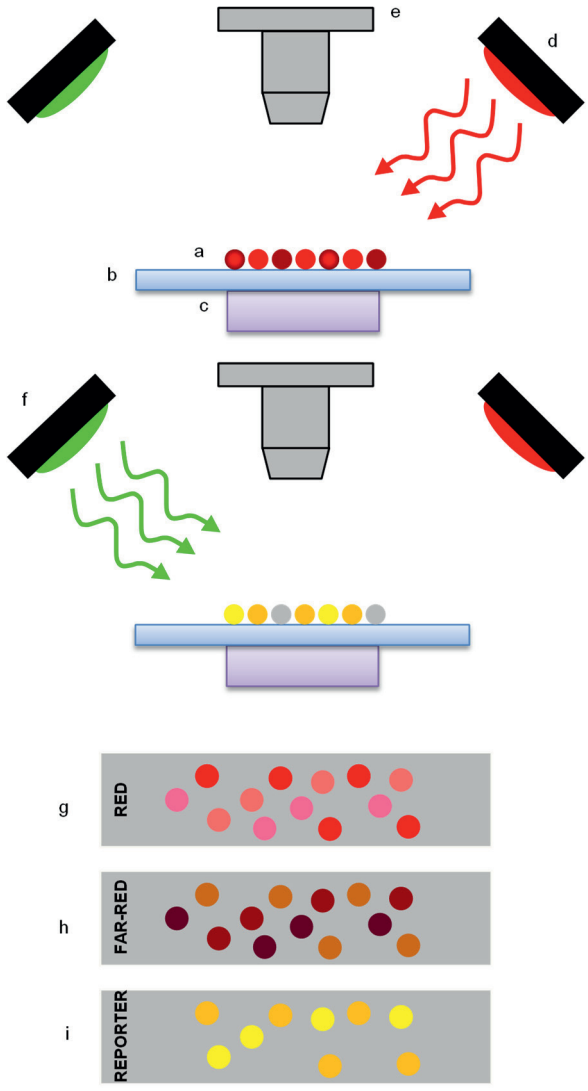

Figure 1. Imaging planar array detection principle. Paramagnetic color-coded microspheres (a) from the sample suspension are trapped on a planar surface (b) by magnetic force (c). For classification, the microspheres are illuminated by a red LED (d) and the CCD camera (e) records red and infra-red emission images (g and h). Next, the microspheres are illuminated by a green LED (f) and the CCD camera records a reporter image (i) from the reporter signal present on the microspheres. The software overlays the red, far-red and reporter images, and classifies each microsphere in the grid and quantifies the reporter signal that belongs to it.

2. Materials and methods

2.1 Instrumentation

A planar microsphere array analyser (MAGPIX) from Luminex (Austin, Texas, USA) was used and operated with XPONENT software version 4.2. A Bio-Plex II Wash Station (Bio-Rad Laboratories, Veenendaal, the Netherlands) with magnetic plate support was used for all washing steps. For the retention of the MagPlex microspheres during the antibody-microsphere coupling process, a DynaMag-2 (Invitrogen Dynal, Oslo, Norway) magnetic separator stand was used. A Bühler TiMix 2 (Salm en Kipp, Breukelen, the Netherlands) was used for all microtiter plate incubation steps and a REAX 2 overhead shaker (Heidolph, Schwabach, Germany) for mycotoxin extraction. Centrifugation of 50 ml Greiner tubes was done in an Eppendorf 5810R centrifuge (VWR International, Amsterdam, the Netherlands) using a A-4-62 rotor and high speed centrifugation of Eppendorf tubes with a Bio-Rad Model 16K Microcentrifuge (Bio-Rad Laboratories, Veenendaal, the Netherlands). A Vortex Genie 2 (Scientific Industries, New York, USA) was used to mix samples. Microsphere counting was done using a Bio-Rad TC10 automated cell counter (Bio-Rad Laboratories). Additional LC-MS/MS analysis was done as previously described [33].

2.2 Chemicals and Reagents

The paramagnetic color-coded microsphere sets MC10026, MC10036, MC10038, MC10042, MC10052 and MC10064 and drive fluid were obtained from Luminex. Cellstar 96-well culture microtiter plates and 50 ml tubes were from Greiner (Alphen a/d Rijn, the Netherlands). Monoclonal antibodies (mAbs) against FB_1 and OTA were purchased from Soft Flow Biotechnology Ltd. (Pecs, Hungary). The mAbs against ZEN, T-2, DON and AFB_1 were purchased from Aokin AG (Berlin, Germany). The R-Phycoerythrin (RPE)-FB_1 and RPE-OTA conjugates were synthesized in-house using RPE from Moss (Pasadena, Maryland, USA). The RPE-ZEN, RPE-T-2, RPE-AFB_1 and RPE-DON conjugates were custom made by Aokin AG using the same Moss RPE. Mycotoxin solutions of FB_1, OTA, AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1

(AFG_1), aflatoxin G_2 (AFG_2), T-2, HT-2, DON, deoxynivalenol-3-β-D-glucopyranoside (D3G), 3-acetyl-DON (3ADON), 15-acetyl-DON (15ADON), nivalenol (NIV) and ZEN, were purchased from Coring System Diagnostix (Gernsheim a. Rhein, Germany). MES ((2-(N-morpholino) ethanesulfonic acid), sulfo-NHS (N-hydroxysulfosuccinimide), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Methanol (MeOH) was purchased from Biosolve (Valkenswaard, the Netherlands). All other chemicals were purchased from VWR International (Amsterdam, the Netherlands).

2.3 Barley matrix and reference samples

Barley reference material surpassing the ML for ZEN (RMM-01-363a, 729 ± 244 μg/kg) was purchased from Aokin AG. Malted barley surpassing the ML for DON (D-MB-5851, 2400 ± 200 μg/kg) was purchased from R-Biopharm/Trilogy (Darmstadt, Germany). From a previous in-house screening, using an ISO 17025 accredited confirmatory LC-MS/MS method for feed samples, eight "blank" barley samples were selected (AFB_1 < 5 μg/kg, OTA < 25 μg/kg, DON < 500 μg/kg, T-2/HT-2 < 200 μg/kg, ZEN < 50 μg/kg, FB_1 < 100 and FB_2 < 100 μg/kg). A ninth barley sample (MB) was a blend of 3 blank samples (previously mixed) that each, independently, complied with the same reporting values. After a pre-screening and additional LC-MS/MS analysis 4 blank samples remained.

2.4 Antibody-microsphere coupling chemistry

The six mycotoxin specific mAbs were coupled to paramagnetic color-coded microspheres as described previously by Peters et al. [33]. Briefly, the antibody stock solutions were diluted and buffer exchanged, to a concentration of 100 μg/ml. The microspheres, approximately 1.25 X 10^7 in total, were washed and subsequently surface activated with EDC and sulfo-NHS to facilitate the coupling of the mAbs. The mAb solutions were then added to the activated microspheres and incubated together allowing covalent coupling. After coupling, the microspheres were washed to remove

the excess mAbs and the remaining activated carboxylic groups were blocked by BSA. The coupled microspheres were placed in storage buffer and the number of microspheres per ml was determined using a cell counter. The ready for use microspheres were stored at 4°C, in the dark.

2.5 Determination of specificity

Cross-reactivity of relevant metabolites and modified forms of mycotoxins were determined. Single mycotoxin calibration curves (ranging from 1 µg/ml to 1 pg/ml in 10-fold serial dilutions) for the available metabolites of AFB_1 (AFB_2, AFG_1 and AFG_2), DON (D3G, 3ADON, 15ADON and NIV) and T-2 (HT-2) were prepared in 10% MeOH. Using these calibration curves in the respective single assays, the concentration at 50% inhibition (IC50) was determined. The cross-reactivity was calculated by the following formula: (IC50 target/IC50 metabolite)*100.

2.6 Fortification of samples and extraction

Each blank barley sample was fortified with all six mycotoxins. The screening target concentration was set at half of the Maximum Level (½ ML), based on the EU MLs for mycotoxins in unprocessed cereals [10]. For T-2 and HT-2 we set a provisional ML of 50 µg/kg, which meant a screening level of 25 µg/kg This complied with the limit for analytical screening techniques recommended by the EU [11]. From each blank barley sample, 2.5 grams was weighed in duplicate in a 50 ml tube and one portion was multi-fortified using mycotoxin stock solutions in 80% MeOH (besides FB_1 in 50% MeOH) at the following concentrations; AFB_1 2 µg/kg, OTA 2.5 µg/kg, DON 625 µg/kg, ZEN 50 µg/kg, FB_1 1000 µg/kg and for T-2/HT-2 we chose to fortify at 25 µg/kg with just T-2. The mycotoxins were added by gently pipetting just above the sample touching the side of the tube. Subsequently, the tube was mixed and the sample allowed to air-dry for 30 minutes. To each blank and fortified sample, 10 ml of 80% MeOH was added and vortexed vigorously. Further extraction was done by placing the sample tubes in an overhead shaker. The total extraction time was 30 minutes at a moderate speed setting. After extraction, the sample tubes were placed for 30 minutes at 4°C to allow fat to

settle. Insoluble material was removed by centrifuging for 10 minutes at 4,000 g in a table centrifuge. Next, the extracts were diluted 4 times by adding 300 µl of double distilled water to 100 µl of extract. Insolubilities, formed as a consequence of this dilution step, were removed by centrifuging once more in a bench top centrifuge at 12,000 g. The naturally contaminated samples were extracted using the same method.

2.7 In-house validation of the 6-plex MIA

We prepared a microsphere suspension containing 1,000 mAb-coupled microspheres for each assay, making a total of 6,000 microspheres for each multiplex analysis. The reporter solution contained 6 fluorescent mycotoxin-RPE conjugates (each at an approximate level of 20 ng per sample). Both solutions were stored in the dark at 4°C until further use. In triplicate, 40 µl of the diluted sample extract was pipetted in a well of a 96 well plate. From here the protocol was followed as previously described [33]. Briefly, to 40 µl of sample extract, 10 µl of microsphere suspension was added and subsequently pre-incubated at room temperature for 2 minutes. Next, 10 µl of reporter solution was added to each sample well. The 96 well plate was then incubated, whilst shaking, at RT for 15 minutes. Washing was done using an automated washer with PBST (PBS pH 7.4 containing 0.02% (v/v) tween-20). The remaining buffer was adjusted to 100 µl with PBST as the washing buffer. The plate was briefly mixed on a shaker before measuring in the imaging planar array analyser. From each microsphere set, 50 microspheres were analysed at the same time.

3. Results and discussion

3.1 Development of the 6-plex assay

The 6-plex MIA discussed in this paper was designed on the basis of a previous developed 3-plex assay for the detection of OTA, ZEN and FB_1 [33] and further adjusted for the detection by a planar imaging array. To extend the previously developed, flow cytometer based, 3-plex immunoassay [33], three additional single immunoassays for the detection of the mycotoxins

AFB_1, DON and T-2 were developed and added. For this, suitable mAbs were selected and each coupled to a unique paramagnetic microsphere set in a previously determined optimal concentration (100 µg/ml). Since the analyser is capable of classifying the microspheres, only one type of fluorescent reporter was needed. Each target mycotoxin was coupled to RPE to create mycotoxin-reporter conjugates to be used in the competitive assay. Each assay was then optimized for maximum response and sensitivity by analysing serial dilutions of the RPE-mycotoxin reporter molecule while using a constant number of microspheres (1,000/set). Then, all the six RPE-mycotoxin reporter conjugates were tested for cross-talk by incubating them independently with a mixture of the six antibody-coupled microsphere sets, in one well. Different experiments were performed to overcome cross-interactions of reporters with a different antibody-microsphere besides its target microsphere. For example, the OTA-RPE that contained a spacer between OTA and RPE cross-interacted with the AFB_1 mAb-microsphere. This could not be solved by further dilution of the reporter. A newly synthesized OTA-RPE, without spacer, showed no cross-interaction. FPLC purification of the AFB_1-RPE reporter removed cross-interaction with both OTA-RPE and DON-RPE. The three single assays were then added to the previously developed 3-plex assay, consisting of microsphere-reporter couples for OTA, FB_1 and ZEN, to create the 6-plex MIA. The 6-plex MIA uses the same incubation times and buffer as for the aforementioned flow cytometry-based triplex assay. In the finalized direct 6-plex assay format, all six antibody-coupled microspheres and all six reporter molecules are incubated together in a single well with a sample extract or standard of choice for 15 minutes. During incubation, the free mycotoxins in the sample compete with the mycotoxin-reporter conjugates for antibody binding. After incubation, the microspheres are trapped by a magnet and the sample, as well as unbound assay components are washed away The microspheres are then released from the magnet and PBST buffer is added to facilitate measurement (Fig 2).

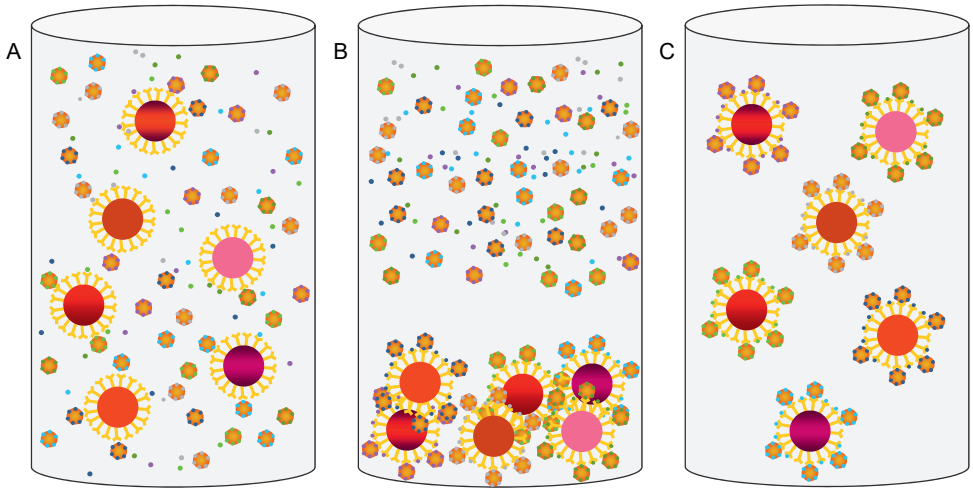

Figure 2. Principle of the 6-plex MIA. Sample (free mycotoxins), microsphere-coupled antibodies and mycotoxin-reporter conjugates are incubated in a single well (A). The microspheres are removed from the reaction by trapping them with a magnet at the bottom of the well, while the other assay components remain in solution and are washed away (B). The trapped microspheres are resuspended and measured in the imaging planar array system (C) as shown in Fig 1.

In the planar array analyser the microspheres are magnetically trapped in a flow chamber to create a monolayer array. After excitation by red light, red and infra-red images are recorded by the CCD camera. Next, a reporter image is recorded upon green light excitation (Fig 1). The measurement of a 96-well plate takes one hour, in which 48 samples (including calibrants) can be measured in duplicate. Although the strip test described by Lattanzio et al. [15] will be faster when applied as a pre-screening, the throughput of the assay is rather low when compared to our 6-plex MIA. In comparison, we can analyse 48 samples in a row fully automated, while strips are measured one by one. Besides this, the strip test does not analyse for the most potent mycotoxins AFB_1 and OTA. On the other hand, the strip-test uses minimal sample preparation and is not depending on sophisticated instrumentation as used in our research. The chip-based flow cell method developed by Oswald et al. [18] does include these most potent mycotoxins in a 4-plex approach, but the throughput of that is assay in its present setup is substantially lower with one sample measured in 11 minutes.

The presence of barley matrix had a strong effect on most of the 6-plex dose response curves (Fig 3). The OTA curve became more sensitive upon addition of the barley matrix, but especially for DON a large shift is observed. Previously, the authors showed that also wheat and maize can have an effect on the sensitivity of the independent assays in the multiplex [33]. This means that a specific blank matrix is necessary and that matrix based dose response curves are needed to avoid over- or underestimation in case of future quantitative analysis. A more suitable sample preparation may also help to solve the matrix dependence of the assay. Based on the multiplex matrix-based dose response curve data, the detection range was calculated by using the IC_{10} and IC_{90} values (Table 1). These values show good prospects for testing all mycotoxins at the MLs in food.

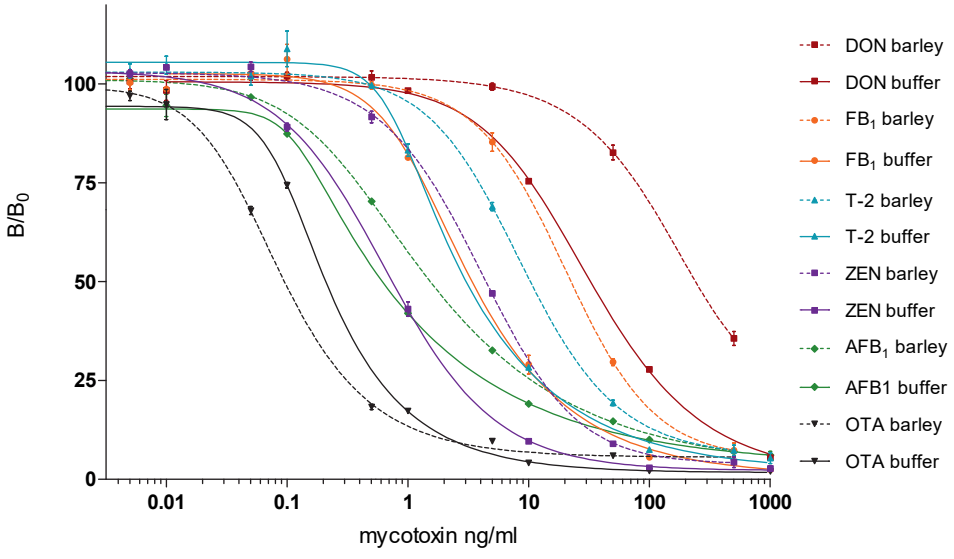

Figure 3. Average multiplex dose–response curves for the 6-plex direct inhibition assay in buffer and barley extract (n=3)

Table 1. Detection ranges based on the inhibitory concentrations of the multiplex matrix-matched dose-response curves (μg/kg)

	AFB_1	DON	FB_1	OTA	T-2	ZEN
IC_{10}	2460	-	3850	25.1	2528	736
IC_{50}	27.6	4240	360	1.6	168	71
IC_{90}	1.6	440	55	0.2	26	10

3.2 Cross-reactivity with other metabolites

The percentage of cross-reactivity was either determined experimentally or taken from literature (Table 2). The DON mAb reacts with the modified form D3G. Whether this wis desirable was still under discussion at this point [36,37]. On average the amount of D3G is about 20% of the amount of DON in cereal commodities, but much higher ratios have been observed in beer [38,39]. [38,39]The DON mAb also reacts strongly with 3ADON. This is not necessarily a problem, since 3ADON (as well as 15ADON) normally occurs in much lower concentrations than DON, depending on geographic location and where the infection is localized in the plant [40,41]. 3ADON is also less cytotoxic than DON. The antibody doesn't show cross-reactivity with 15ADON which is equally cytotoxic as DON. The AFB_1 mAb shows hardly any cross-reactivity with the AFB_2, AFG_1 and AFG_2 metabolites. While AFB_1 is the most predominant and the most toxic of the aflatoxins that can occur in barley, AFG_1 can also occur in relevant concentrations. This means that the developed assay was very specific for AFB_1 so significant amounts of other AFs may not be detected. Despite this, the current assay is still useful for screening, since there is a separate legislation for AFB_1 in food. Furthermore, the other metabolites, AFB_2 and AFG_2, are often only a small fraction of the total aflatoxin content [5]. The observed cross-reaction of the T-2 mAb with HT-2 is highly desired since they are considered equally toxic [42]. Although there is no EU regulation for T-2 and HT-2, the EU very recently set recommendations for analytical screening techniques [11].

Table 2. Cross reactions of mycotoxin metabolites in the 6-plex MIA

Metabolite	IC_{50} (ng/ml)	Cross-reactivity (%)
ZEN	0.13	100
α-ZEL	0.17	66
β-ZEL	0.90	13
Z14G	nd	< 1
α-ZELG	nd	< 1
β-ZELG	nd	< 1
Z14S	nd	< 1
DON	25	100
D3G	2	53
3ADON	35	776
15ADON	50	< 1
NIV	nd	< 1
AFB_1	0.7	100
AFB_2	26	2
AFG_1	4	7
AFG_2	224	< 1
FB_1	144	100
FB_2	236	61
FB_3	228	63
T-2	7.2	100
HT-2	7.9	88
OTA	0.13	100
OTB	0.20	74

3.3 In-house validation of the 6-plex MIA as a screening method

For successful validation, 20 blank samples, and 20 blank samples spiked at ½ ML, must show significantly different responses in order to have a CCβ value at ½ ML [35]. Available real blank samples (4 in total) were each fortified 5 times and analysed over 3 days to obtain the desired 20 samples. Background correction was done using blank sample MB which was co-

analysed as a daily reference over the validation period to obtain relative responses. With an overall assay time of 2 hours (including incubations, washing steps, pipetting and actual measurement) for 48 samples (in duplicate) and a direct output of results, the method can be considered very rapid. The intra- and inter day precision (%RSD) of the assay was determined by analysing both blank control and positively screened samples. This was done for the single MB sample as well as individual barley samples (Table 3). The precision was typically better than 5% RSD and only in 3 cases slightly above 10%.

Table 3. Intra- and interday precision of the assay (%RSD) for blank barley and fortified blank barley samples.

Sample	Precision	Nr. of analyses	%RSD for each mycotoxin assay					
			AFB_1	OTA	T-2	ZEN	DON	FB_1
MB blank	intraday	n=9	1.6	2.4	0.5	3.4	1.9	1.9
All blank	intraday	n=21	2.2	1.9	3.5	10.2	6.3	2.8
MB ½ ML	intraday	n=9	1.3	5.8	1.9	3.4	1.8	3.0
All ½ ML	intraday	n=21	2.1	5.0	2.4	4.3	5.3	2.8
MB blank	interday	n=27	3.2	1.8	3.7	3.4	2.2	1.6
All blank	interday	n=60	2.8	1.6	4.1	7.6	6.0	2.0
MB ½ ML	interday	n=27	3.0	11.2	4.3	5.2	7.2	10.9
All ½ ML	interday	n=60	2.6	7.5	3.8	4.5	7.0	9.2

n = the total number of samples analysed, All = all analysed barley samples, MB = mixed batch blank barley sample

When applying the most simple validation approach on our 6-plex MIA (annex 1, in the guidelines for the validation of screening methods [35]), the lowest response from the blank control samples should not overlap with the highest response from the fortified control samples. If this would occur then a sample should be classified as false compliant. When using 20 samples in total, only one sample (5%) is allowed to be false compliant according to the guidelines for screening methods[35]. The concentration level at 5% β error is

defined as the CCβ of the screening method. In five of the assays from the 6-plex, there was no overlap at all, except for one sample in the AFB_1 assay where the lowest blank signal was the same (93%) as the maximum value of the fortified control samples. This means that at ½ ML, the rate of false compliant results (β) is < 5% for the OTA, DON, ZEN, T-2 and FB1 assays and 5% for the AFB_1 assay. As a result, all assays in the 6-plex MIA comply with the EU validation requirements. Annex 2 in the guidelines for the validation of screening methods[35] offers a second approach to assess the validation parameters. For this we calculated the threshold value (*T*) by the following formula:

$$T = B - 1.64 \times SDb$$

where *B* is the mean response of the blank control samples and *SDb* the standard deviation of these responses.

The cut-off factor (*Fm*) was calculated by the following formula:

$$Fm = M + 1.64 \times SD$$

where *M* is the mean response of the fortified control samples and *SD* the standard deviation of these responses. The independent B/B_0 values obtained for the blank control- and fortified control samples were plotted in graphs, together with the threshold value and cut-off factor for each assay (Fig 4).

In all cases the *Fm* was smaller than *B* and also the *Fm* was smaller than *T*, meaning that each mycotoxin assay in the 6-plex was successfully validated. When validating the 6-plex assays at ML level, the differences between *Fm* and *T* were substantially larger (data not shown). A preliminary validation of the 6-plex MIA for wheat at MLs, suggests that the presented method will be amenable to other food and feed matrices as well.

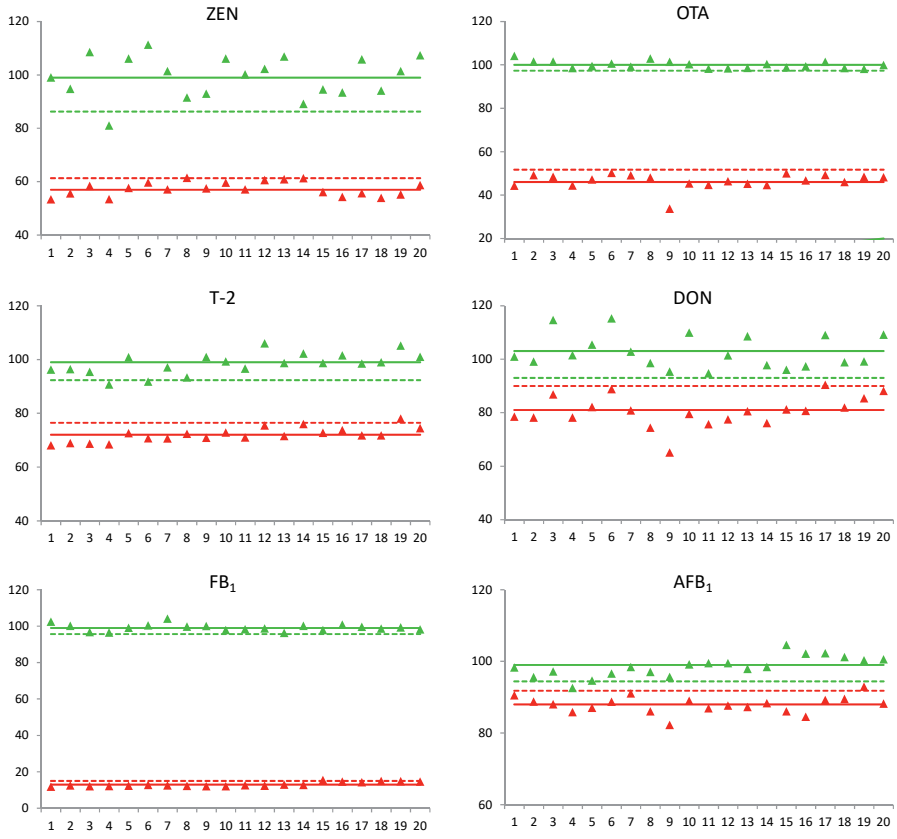

Figure 4. Validation graphs for each mycotoxin immunoassay in the 6-plex. Depicted are the relative responses (n=3) of the fortified (▲) and blank barley samples (▲),averages of the fortified (—) and blank samples (—), the cut-off factor (Fm) (⋯) and the threshold level (T) (⋯)

3.4 Application to naturally contaminated samples

To demonstrate the functionality of the validated assay, we applied the 6-plex MIA to naturally contaminated barley samples containing ZEN, T-2/HT-2, DON and OTA. The OTA sample (14 µg/kg) and the T-2/HT-2 sample (60 µg/kg) sample were amongst rejected "blank" samples found during an initial screening of in-house blank feed samples. Additionally, looking for commercially available samples with contaminations above the ML level, we found a malted barley sample (D-MB-5851) for DON (2400 ± 200 µg/kg) and

a barley sample (RMM-01-363a) for ZEN (729 ± 244 µg/kg, certified by Aokinmycontrol. These available samples (representing 4 mycotoxin contaminations) were extracted in triplicate as well as the aforementioned MB blank sample and the same MB sample fortified at ML for each specific mycotoxin (5 µg/kg for OTA, 100 µg/kg for ZEN, 1250 µg/kg for DON and 50 µg/kg for T-2/HT-2). The responses (Mean Fluorescence Intensities (MFIs)) of the naturally contaminated samples were then compared to the blank and fortified samples (Fig 6). All naturally contaminated and fortified barley samples were screened suspect in accordance with expectations. For DON, the contaminated sample did not give a lower response than the fortified blank sample (MB+). This is most likely due to the sample matrix being malted barley rather than regular barley, making the assay slightly less sensitive.

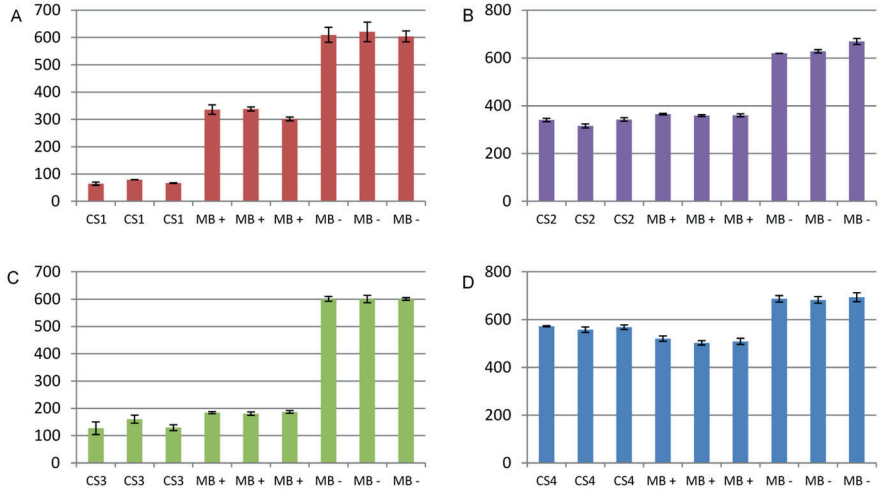

Figure 5. Screening of naturally contaminated barley samples CS1 (RMM-01-363a), CS2 (screening sample 1), CS3 (screening sample 6) and a malted barley sample CS4 (D-MB-5851) for the occurrence of ZEN (A), T-2 (B), OTA (C) and DON (D) together with a mixed blank barley sample (MB -) and the same blank sample fortified at ML level (MB +) (n=3) displayed as MFIs.

3.5 Suitability for on-site testing

The transportability of the system, and the subsequent performance of the 6-plex MIA, was demonstrated twice, but for the measurements of mycotoxins in beer samples. In both cases similar results were obtained as in the original laboratory. In order to show the potential of the developed assay for on-site testing, we extracted barley spiked at ½ ML for ZEN, as well as a ZEN reference sample and a blank barley sample. After a simplified 1 minute vortex step, sediment was allowed to settle for 1 minute. After dilution, the samples were submitted to the assay. Microspheres were captured by a handheld magnetic plate (instead of a sophisticated automated plate washer). Reagents were simply removed by reversing the plate directly followed by a firm, and short, vertical movement. Microspheres were resuspended by addition of PBST and the samples were directly measured. Results showed that this simplified extraction method worked, at least for ZEN (Supplementary material, Fig S1), thereby demonstrating that a dedicated simplified sample preparation for on-site testing is feasible.

4. Conclusion

The developed 6-plex MIA was capable of detecting all target mycotoxins at the MLs set for unprocessed cereals using barley as sample matrix. For T-2 we were able to screen below the EU recommended concentration of 200 μg/kg. At the same time the T-2 screening complied with the EU preferred limit of analytical screening for T-2/HT-2, which has been set at 25 μg/kg [11]. An improvement, for the future application of this assay in food screening, would be the addition of a new mAb capable of detecting all AFs. A future implementation of the developed 6-plex MIA as a semi-quantitative pre-screening method for regulated mycotoxins, prior to instrumental analysis (like LC-MS/MS), can enhance throughput and avoid the unnecessary submission of actual blank samples to the confirmatory method. Note that the same sample extract of positively screened samples may still be used for instrumental analysis. Furthermore the transportable instrument may

perform pre-screenings in a simple equipped on-site or mobile laboratory. This way, raw materials for food and feed can already be analysed prior to entering the food and feed supply chain. The multiplex capacity of the color-coded microspheres allows the addition of more mycotoxin assays up to a 50-plex. The feasibility of adding new assays will depend on the availability of sensitive and specific monoclonal antibodies, the preparation of the specific reporter conjugates and the prevention of cross-talk between the assays.

Acknowledgements

The authors would like to thank Theo de Rijk, Paul Zomer and Ed Boers from RIKILT Wageningen UR for supplying LC-MS/MS data of the used barley samples and additional LC-MS/MS analysis. Frank Mallwitz, Rüdiger Welz and Ursula Dahmen-Levison from Aokin AG for their help with supplying antibody resources and customized RPE conjugate production and our project partners Jan Bergervoet and Marjanne de Weerdt (Plant Research International, Wageningen, the Netherlands) and Nermin Sajic, Ron Verheijen and Piet van Wichen (EuroProxima, Arnhem, the Netherlands) for their helpful contributions. This research was carried out in the MultiTox project (PID091008/2.6A), financed by the Dutch Ministry of Economic Affairs, SenterNovem and the Province of Gelderland.

Chapter 4: Supplementary Material

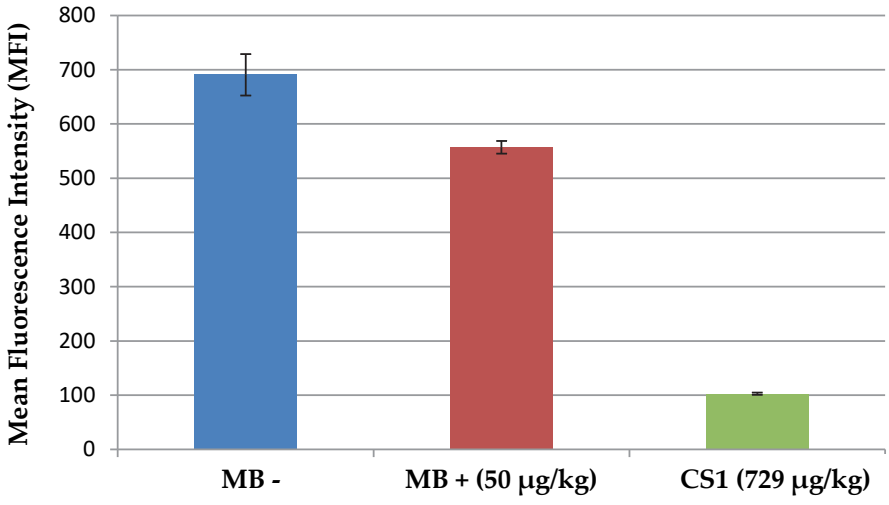

Figure S1. Application of a simplified extraction method to blank barley (MB), ZEN spiked barley (MB+, 50 µg/kg) and ZEN reference sample (CS1, 729 µg/kg).

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CHAPTER

5

Simplified multiplex paramagnetic microsphere immunoassay for portable onsite detection of mycotoxins in barley

Peters, J.; Bergervoet, J.H.W.; Haasnoot, W.; Nielen, M.W.F.

Abstract

Mycotoxins contaminate agricultural commodities. The early on-site screening of mycotoxin contaminations benefits food and feed production logistics and can help to avoid the unnecessary exposure of humans and animals. Since mycotoxins often co-occur, multiplex screening is desirable. We developed a simplified portable paramagnetic microsphere-based semi-quantitative 4-plex for the on-site detection of the mycotoxins ochratoxin A (OTA), zearalenone (ZEN), deoxynivalenol DON, T2-toxin (T-2) and HT-2 toxin (HT-2), using a imaging planar array analyser. In the competition format 4-plex screening assay, OTA, ZEN, DON and T-2 specific antibody-coupled microspheres and specific mycotoxin-R-phycoerythrin labeled reporter molecules were incubated together with sample extracts in a single well. Simplifications comprised; washing steps by a handheld magnetic plate, addition of pre-mixed reagents from dropper bottles, addition of samples by disposable fixed volume micropipettes, a sample extraction time of just 1 minute and omitting centrifugation. For the successful preliminary in-house laboratory validation of the 4-plex screening assay, we used blank and fortified blank barley samples. The initial preliminary in-house validation, using a laboratory sample extraction procedure, of the simplified 4-plex screening assay was successful at half the EU maximum levels and generally showed excellent inter and intra-day precisions. Preliminary in-house validation of the of the 4-plex screening assay, using an on-site extraction procedure, was successful at the EU maximum levels. The simplifications introduced in the developed 4-plex screening assay make it an useful tool for future portable on-site semi-high throughput screening of food and feed commodities.

1. Introduction

Mycotoxins contaminate commodities worldwide and pose a risk to human health when they end up in the food and feed production chain [1,2]. Early detection of mycotoxins in food and feed commodities can avoid the unnecessary exposure of humans and animals. Mycotoxin immunoassays are useful tools for early mycotoxin detection in commodities. A wide range of immunoassay formats have been developed and tested for the detection of mycotoxins in different commodities [3]. The implementation of these methods for on-site screening can contribute to a more efficient flow in the food and feed production chain. A simple immunoassay technique, suitable for on-site detection, is the lateral flow device (LFD). In general, incubation times for LFDs are short and read-out can be done either visually or with a dedicated reader. LFDs have been developed for the detection of single mycotoxin analytes [4], but also for multiplex mycotoxin detection [5]. Since mycotoxins often co-occur [6], multiplex screening is desirable. Lattanzio et al. [7] developed a strip test with 4 test lines capable of detecting 6 mycotoxins (zearalenone (ZEN), deoxynivalenol (DON), T-2 toxin (T-2) and HT-2 toxin (HT-2) and fumonisins (FBs)). Fluorescence polarization immunoassays can be rapid and field portable. Only recently a multiplex fluorescence polarization immunoassay, for the multi-wavelength detection of the mycotoxins DON, T-2 and fumonisin B_1 (FB_1) in maize, was reported [8]. Based on a portable nanostructured Imaging Surface Plasmon Resonance (iSPR) biosensor, Joshi et al. [9] developed a 6-plex mycotoxin immunoassay for the detection of DON, T-2, ZEN, FB_1, ochratoxin A (OTA) and aflatoxin B_1 (AFB_1) in barley. Based on infrared laser spectroscopy technology, Sieger et al. [10] developed a prototype instrument for the on-site screening of DON (in maize and wheat) and AFB_1 (in peanuts). Different multiplex immunoassays that use color-coded (or microspheres) for the detection of mycotoxins have been reported previously [3]. For example, Peters et al. [11] developed and validated a paramagnetic microsphere-based 6-plex for the detection of DON, T-2, HT-2, ZEN, FB_s, OTA and AFB_1 in barley and applied it for the screening of a wide range of beer samples for mycotoxin content. [12]. Although most of the above techniques have potential for (portable) on-site multiplex

detection, most of them are still in the prototype machine stage. Additionally, most of them are suitable for low sample throughput, but lack the potential for semi-high throughput analysis.

In this research, we developed a simplified semi-quantitative 4-plex paramagnetic microsphere-based immunoassay for the screening of DON, ZEN, OTA and T-2/HT-2 in barley using a robust portable imaging planar array format. Significant simplifications were realized: reduction of the extraction time from 30 to just 1 minute and omitting cold incubation/centrifugation steps, the use of low-cost fixed-volume disposable micropipettes for sample introduction, preparation of pre-mixed reagents in dropper bottles, and omitting automated washing steps by utilizing a handheld magnetic plate for microsphere capturing and washing. This simplified method was preliminary in-house validated for barley according to the guidelines for the validation of screening methods for residues of veterinary medicines [13] at 50% of the ML set by the EU for DON (625 μg/kg), ZEN (50 μg/kg) and OTA (2.5 μg/kg) [14]. For T-2 we chose to fortify at the EU preferred limit of analytical screening for T-2/HT-2 (25 μg/kg) [15]. The method, including the planar array analyser, is robust, suitable for semi-high throughput, transportable and only requires a bench and a (portable) power supply.

2. Materials and methods

2.1 Instrumentation

A planar microsphere array analyser (MAGPIX) from Luminex (Austin, TX, USA), operated with XPONENT software version 4.2 (Luminex) was used for all validation measurements. A magnetic plate separator with side clasps (Luminex) was used for microsphere capture and washing steps. For laboratory applications, A REAX 2 overhead shaker (Heidolph, Schwabach, Germany) was utilised for mycotoxin extraction. Centrifugation of 50 ml Greiner tubes was done in an Eppendorf 5810R centrifuge (VWR International, Amsterdam, the Netherlands) using a A-4-62 rotor. Assay incubation steps were performed on a Bühler TiMix 2 shaker (Salm en Kipp,

Breukelen, the Netherlands). A Vortex Genie 2 (Scientific Industries, New York, USA) was used to mix samples.

2.2 Chemicals and Reagents

Paramagnetic mycotoxin monoclonal antibody coupled microsphere sets and mycotoxin-R-Phycoerythrin (RPE) reporter conjugates were bought and/or prepared exactly as reported previously [11]. Droplet bottles (15 ml) were kindly provided by EuroProxima (Arnhem, the Netherlands). Disposable 100 µl micropipettes were a kind gift from Dr. Yirong Guo from the Institute of Pesticide and Environmental Toxicology, Zhejiang university, Hangzhou, China. Drive fluid for MAGPIX operation was obtained from Luminex. Assays were performed in Cellstar 96-well culture microtiter plates Greiner (Alphen a/d Rijn, the Netherlands). Mycotoxin extractions were performed in 50 ml Greiner centrifuge tubes. Mycotoxin solutions of OTA (10 µg/ml in acetonitrile) and T-2, DON, ZEN (each 100 µg/ml in acetonitrile) were purchased from Romer Labs (Oostvoorne, Netherlands). Methanol (MeOH) was purchased from Biosolve (Valkenswaard, the Netherlands). All other chemicals were purchased from VWR International (Amsterdam, the Netherlands). Based on the results of an in-house LC-MS/MS screening, using an ISO 17025 accredited confirmatory method, 4 barley samples (out of 9) were selected as blank control samples. One of those, MB, was a blend of 3 blank barley samples (previously mixed).

2.3 Fortification and extraction of barley samples

Blank barley samples were multi-fortified with OTA, DON and ZEN at 50% of the Maximum Level (½ ML) based on the EU MLs for mycotoxins in unprocessed cereals [14] and for T-2 at the EU preferred limit of analytical screening [15], as previously described [11]. In short, 2.5 grams of blank barley was fortified, using mycotoxin standard solutions, at the following concentrations; OTA 2.5 µg/kg, DON 625 µg/kg, ZEN 50 µg/kg and T-2 25 µg/kg. Divided over 3 days, 20 blank barley samples and the same 20 blank barley samples fortified at ½ ML levels, were extracted for 30 minutes with 10 ml of 80% MeOH using an overhead shaker. After 30 minutes incubation at

4°C, the tubes were centrifuged for 10 minutes at 4,000 g. Next, 300 µl of double distilled water was added to 100 µl of extract. This diluted extract was used for measurements.

2.4 Laboratory procedure of the mycotoxin 4-plex droplet immunoassay

A 4-plex microsphere suspension, sufficient for three 96-wells plates, was prepared and placed in a dropper bottle. This microsphere suspension contained approximately 400,000 mAb-coupled microspheres per ml PBS. Next, a reporter solution mixture was prepared, consisting of 4 fluorescent mycotoxin-RPE conjugates (each at an approximate concentration of 2 µg/ml in PBS), and placed in another dropper bottle. For washing purposes, dropper bottles were filled with PBST (PBS pH 7.4 containing 0.02% (v/v) Tween-20). The dropper bottles containing the microsphere suspension, reporter solution and PBST were stored in the dark at 4°C until further use. From each sample extract, 40 µl was pipetted in a well of a 96 well plate using a laboratory pipet. To each well, one drop of the 4-plex microsphere suspension was added, followed by one drop of the 4-plex reporter solution. The assay plate was placed on a shaker at room temperature (RT) for 15 minutes. The assay plate was then placed and clipped onto a magnetic plate separator, allowing the capture of microspheres for 1 minute. With the assay plate still attached to the magnetic plate separator, contents were removed by flicking the plate above the sink. Without removing the magnetic plate separator, 2 drops of PBST were added to each well. After 1 minute, the contents were removed as described above. The assay plate was released from the magnetic plate separator and 2 drops of PBST were added to each well. Next, the assay plate was briefly mixed on a shaker before analysis in the imaging planar array analyser. From each microsphere set, 50 microspheres were analysed in duplicate at the same time (Fig 1).

2.5 Portable mycotoxin 4-plex droplet immunoassay procedure

2.5 grams of a mixed blank barley sample (MB, consisting of 3 individual blank barley samples) was weighed in 50 ml tubes. Six tubes were left blank,

while the other 6 tubes were fortified with the target mycotoxins at ML level (1250 μg/kg DON, 100 μg/kg ZEN, 5 μg/kg OTA and 50 μg/kg for T-2). The tubes with fortified barley were left open at RT for 30 minutes for solvents to evaporate. The 6 blank and the 6 fortified barley samples, were divided for duplicate measurements over 2 days. To each tube, 10 ml of 80% MeOH was added and the tubes were manually shaken for 1 minute only to allow extraction of mycotoxins. After extraction, 40 ml of double distilled water was added. After brief mixing, the extracts were left to settle for 5 minutes at RT. Next, 2 drops (approx. 50 μl) of each barley extract were added to a well of a 96 well plate by a disposable micropipette. To each well, one drop of previously prepared microspheres and one drop of previously prepared reporters (each approx. 35 μl) were added from dropper bottles (Fig. 1). The assay was incubated at RT for 15 minutes on a platform shaker. Next, the 96 well plate was clipped on the magnetic support to allow capture of the microspheres for 1 minute. Frome here on we followed the laboratory procedure as described above. Furthermore the extraction time was reduced from 30 minutes to 1 minute and samples were directly diluted 5 times and then left to settle for 5 minutes at RT. Laboratory pipettes, for the addition of sample extracts and assay reagents, were replaced by low-cost fixed-volume disposable micropipettes, and dropper bottles that contained pre-mixed assay reagents. No modifications were made to the already rapid incubation time (15 minutes). In the well, the OTA, ZEN, DON and T-2 specific antibody-coupled microspheres and the specific mycotoxin-reporter molecules were incubated together in a single well with the sample extract (Fig. 1). Following a short 15 minutes incubation, the paramagnetic microspheres were trapped, washed and resuspended. After resuspension, the microspheres were measured in an imaging planar array analyser. Microspheres were classified upon red light excitation while the reporter signal intensity was measured upon green light excitation. The classification and reporter emission images were recorded by a CCD camera in the analyser (Fig. 1). Thanks to its robustness, compact size (16.5 cm W x 60 cm D x 43 cm) and moderate weight (17.5 kg), the imaging planar array analyser can be easily transported by car and used on-site using either a generator or a locally available mains supply.

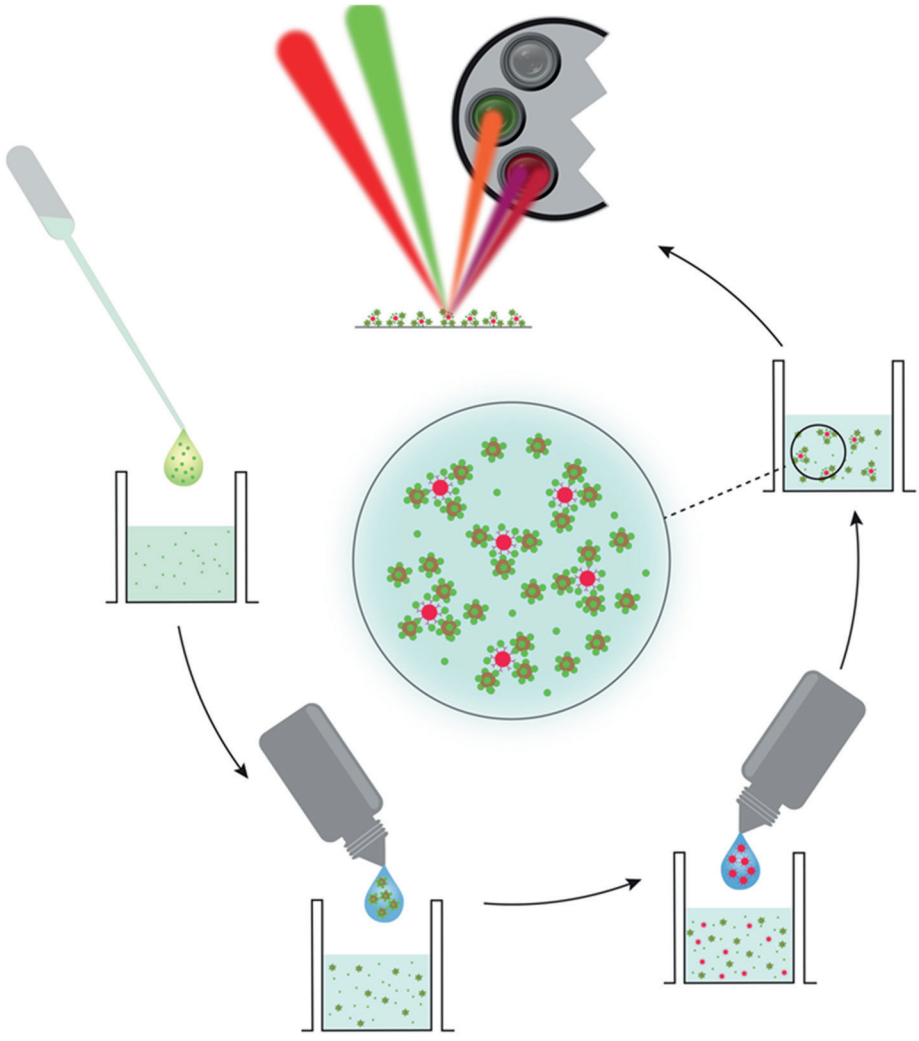

Figure 1. Principle of the paramagnetic microsphere-based screening, (for simplification only one assay in the 4-plex is shown). Samples are pipetted in a 96-wells plate, followed by the addition of mycotoxin-reporter molecules and mycotoxin antibody-coupled microspheres from dropper bottles. Sample and assay components are incubated, trapped and washed. After washing, the microspheres from the wells are trapped on a planar sample stage by a magnet and they are classified by red LED excitation while the reporter signal is measured by green LED excitation.

3.1 Simplification of the microsphere-based screening assay

The portable 4-plex mycotoxin screening assay, discussed in this paper, is a simplification of a previous developed paramagnetic microsphere-based mycotoxin multiplex screening assay [11]. For portable on-site suitability, we reduced the use of laboratory equipment. First, we simplified washing steps by replacing an automated magnetic washer with a handheld magnet and next omitted centrifuge steps after extraction. Instead of using an overhead shaker, extractions were carried out by manual shaking. Furthermore the extraction time was reduced from 30 minutes to 1 minute and samples were directly diluted 5 times and then left to settle for 5 minutes at RT. Laboratory pipettes, for the addition of sample extracts and assay reagents, were replaced by low-cost fixed-volume disposable micropipettes, and dropper bottles that contained pre-mixed assay reagents. No modifications were made to the already rapid incubation time (15 minutes). In the well, the OTA, ZEN, DON and T-2 specific antibody-coupled microspheres and the specific mycotoxin-reporter molecules were incubated together in a single well with the sample extract (Fig. 1). Following a short 15 minutes incubation, the paramagnetic microspheres were trapped, washed and resuspended. After resuspension, the microspheres were measured in an imaging planar array analyser. Microspheres were classified upon red light excitation while the reporter signal intensity was measured upon green light excitation. The classification and reporter emission images were recorded by a CCD camera in the analyser (Fig. 1). Thanks to its robustness, compact size (16.5 cm W x 60 cm D x 43 cm) and moderate weight (17.5 kg), the imaging planar array analyser can be easily transported by car and used on-site using either a generator or a locally available mains supply.

3.2 Preliminary in-house validation of the droplet 4-plex laboratory screening method

In-house validation was carried out using dropper bottles for the addition of assay reagents and washing steps, while the diluted barley extracts were added by laboratory pipettes. To prove that dropper bottles were fit for purpose, we tested their accuracy. From 3 randomly selected disposable

dropper bottles, 10 independent drops of PBST were weighed (Table 1). The average droplet weight values were 33.4 ± 1.3, 42.4 ±1.3 and 33.0 ± 1.4 mg. The dropper bottles, showed very little variation between drops, but dropper bottle 2 consistently produced bigger drops when compared to bottle 1 and 3. This means that for consistency in future applications, the dropper bottles should be pretested to minimize volume variation in the assay. However, one may argue that a 30 % systematic error is still acceptable for semi-quantitative immunoassay screening. Additionally, calibrators and blanks will be analysed with the same variation. For the current research dropper bottle 1 and 3 were selected.

Table 1. Accuracy of dropper bottles by weighing single drops (n=10) from 3 bottles

Drop no.	Weight per drop (mg)		
	Bottle 1	Bottle 2	Bottle 3
1	31	43	31
2	32	42	32
3	34	42	31
4	32	42	33
5	34	41	33
6	34	44	33
7	34	43	33
8	34	41	34
9	34	41	35
10	35	45	35
Average	33.4	42.4	33.0
St Dev	1.3	1.3	1.4

Validation was performed according to the guidelines for the validation of screening methods for residues of veterinary medicines [13]. To this end, 4 blank barley samples were analysed 5 times to yield a total of 20 blank samples (negative controls). Those same 20 blank barley samples were fortified at ½ ML (screen positive control) for OTA, ZEN and DON according to the MLs set by the EU, while for T-2, the EU preferred limit of analytical screening was followed [14,15]. The negative control and screen positive control samples, were analysed divided over 3 days. Relative responses were calculated using the MB sample as a daily reference. The intra- and interday

sample variability in the droplet assay (%RSD), was determined for the negative control and screen positive control samples. This was done for independent samples, as well as all samples together as a group (Table 2).

Table 2. Intra- and interday precision of the 4-plex screening assay (%RSD)

Sample	Precision	Nr of analysis	%RSD for each mycotoxin assay			
			OTA	T-2	ZEN	DON
MB blank	Intraday	n = 6	0.9	6.1	8.2	1.5
All blank	Intraday	n =14	1.3	6.9	18.8	3.9
MB ½ ML	Intraday	n = 6	5.0	6.0	5.2	2.1
All ½ ML	Intraday	n =14	4.6	5.7	5.9	5.0
MB blank	Interday	n = 18	1.6	5.2	4.4	4.8
All blank	Interday	n = 40	2.1	5.8	14.3	6.7
MB ½ ML	Interday	n = 18	4.2	7.3	10.7	5.6
All ½ ML	Interday	n = 40	3.5	8.6	11.1	6.1

n = total number of samples analysed (extracts x 2 replicates); All = all analysed barley samples; MB = mixed batch blank barley sample; OTA = ochratoxin A; T-2 = T-2 toxin; ZEN = zearalenone; DON = deoxynivalenol

Generally the %RSDs values were well below 10% for all assays in the 4-plex screening assay. In just a few cases the ZEN assay showed %RSD values above 10%. This was mainly the case for the all blank samples group, both for interday and intraday precision. However, did this did not hamper the validation of the ZEN assay. For all 4 assays in the multiplex screening assay, the validation was successful with regards to the criteria set by the official validation protocol [13]. In all cases the cut-off factor (*Fm*) was smaller than the average of the blanks (*B*). Moreover, in all cases, the *Fm* was smaller than the threshold (*T*), although for DON they were merely separated (Fig. 2). Upon repeating the validation at ML level, there was a much better separation of the *Fm* and *T* in the DON assay (Fig. 3). For all targets, the false-negative rates at ½ ML, defined as the *CCβ*, were below 5%. Guidelines used in this

research are highly similar to those presented in the EU commission regulation 519/2014 [16], which has a less strict calculations of the cut-off factor but with analysis over 5 different days instead of 3.

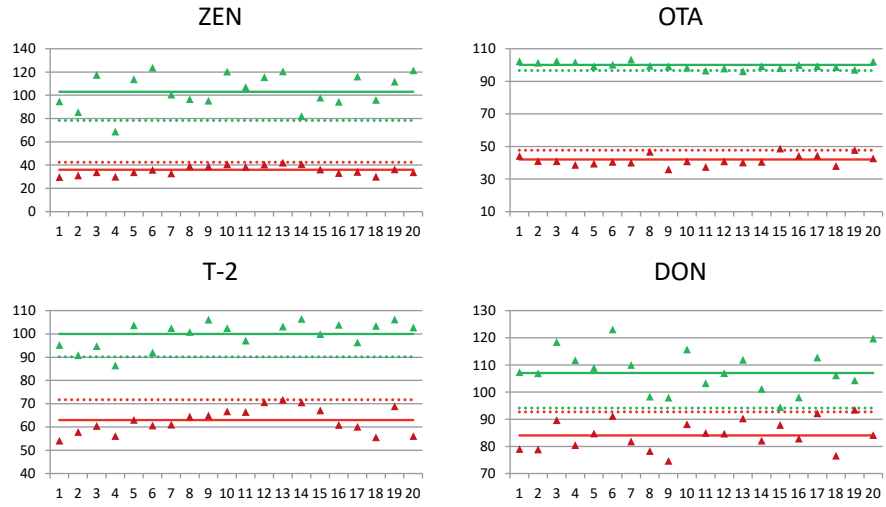

Figure 2. Validation results for the 4-plex mycotoxin screening assay at ½ ML with all values (n=2) as relative responses. Depicted are the fortified (▲) and blank barley samples (▲), averages of the fortified (—) and blank samples (—), the cut-off factor (Fm) (···) and the threshold level (T) (···)

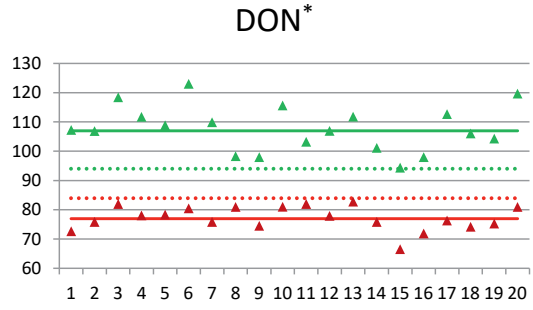

Figure 3. Validation result for the DON assay in the 4-plex mycotoxin screening assay at ML with all values (n=2) as relative responses. Depicted are the fortified (▲) and blank barley samples (▲), averages of the fortified (—) and blank samples (—), the cut-off factor (Fm) (···) and the threshold level (T) (···)

3.3 Further simplification of the droplet assay for field use

To further simplify the 4-plex screening assay for portable on-site screening suitability, we changed the extraction from 30 minutes in an overhead shaker to a 1 minute manual mixing. Furthermore we skipped the 30 minutes incubation at 4 ℃ and omitted centrifuging. Instead, the sample was allowed to settle for 5 minutes. Laboratory pipettes were replaced by disposable fixed-volume micropipettes. The accuracy of these micropipettes was tested in the same manner as the dropper bottles. From 3 disposable micropipettes, 10 independent drops of PBST were weighed (Table 3). The respective average values were 25.7 ± 1.8, 25.3 ± 1.9 and 24.8 ± 1.6 mg. These results show that the micropipettes have very little variation within drops and in between themselves. With the aforementioned modifications implemented, we investigated the functionality by analysing 6 blank barley samples and 6 fortified blank barley samples (at ML levels) for the target mycotoxins using the same blank barley sample (MB). These samples were analysed divided over 2 days and the difference in absolute responses between the blank and fortified blank barley samples were reported (Fig. 4).

Table 3. Accuracy of micropipettes by weighing single drops (n=10) from 3 pipettes

Drop no.	Weight per drop (mg)		
	Pipet 1	Pipet 2	Pipet 3
1	22	21	23
2	26	27	24
3	24	24	25
4	27	25	28
5	27	27	23
6	26	26	25
7	24	25	23
8	27	28	25
9	26	25	26
10	28	25	26
Average	25.7	25.3	24.8
St Dev	1.8	1.9	1.6

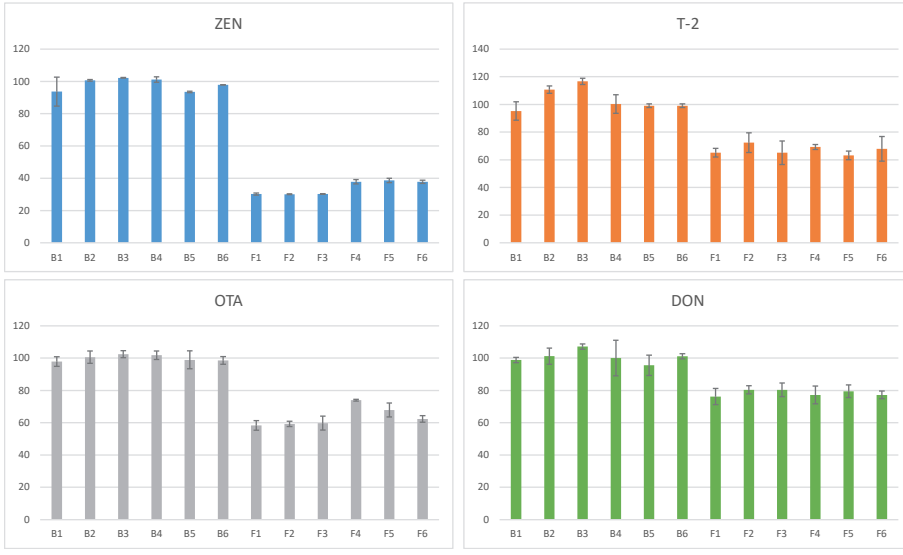

Figure 4. Mycotoxin 4-plex screening results for blank (B1 – B6) and fortified barley samples (F1 – F6) using 1 minute extractions and no centrifugation divided over 2 days. Relative responses (B/B0) are displayed on the y-axis.

In accordance with expectations, also the further simplified method showed significant differences between the blanks and the fortified barley samples. As previously seen with the in-house validation, DON is the least sensitive assay in the 4-plex, showing the least signal inhibition between the blanks and the fortified barley samples. However, the differences between the blanks and the fortified samples in the DON assay proved to be significant (student's T test, alpha factor 0.01). A more sensitive antibody, or an antibody which is less susceptible to matrix effects, could improve this assay and make it more suitable for the 4-plex.

4. Conclusion

The preliminary in-house validation of the simplified 4-plex screening assay in barley was successful at ½ ML for DON, OTA and ZEN. For T-2 the screening assay complied with the EU preferred limit of analytical screening for T-2/HT-2 (25 µg/kg) [15]. The most simplified 4-plex method (using one minute extractions and omitting cold incubation\centrifugation) was successfully applied to a mixed blank and fortified mixed blank barley

sample. With a rapid and simple 1 minute extraction, a single assay incubation step of just 15 minutes and the measurement of 96 samples within one hour, the simplified 4-plex method can be considered as rapid. Therefore, the developed 4-plex screening method is a good alternative for LFDs, whenever rapid on-site semi-high throughput multiplex analysis of mycotoxins is desired. Moreover, the chosen multiplex assay format facilitates the easy addition of other mycotoxin targets, as well as other contaminants, relevant for the commodities of interest. A point of attention is the low inhibition in the DON assay due to possible matrix interference. To make the DON assay more robust, a new antibody should be selected and implemented for future applications. Furthermore, the authors suggest that possible future developments of the portable planar array microsphere analyser should be towards a handheld battery operated device. This anyway seems to be a logical next step towards the demand for multiplex point-of-care analysis.

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CHAPTER

6

Mycotoxin profiling of 1000 beer samples with a special focus on craft beer

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Adapted from:

Mycotoxin profiling of 1000 beer samples with a special focus on craft beer.
PLoS ONE 2017 12(10)

Abstract

Currently beer is booming, mainly due to the steady rise of craft breweries worldwide. Previous surveys for occurrence of mycotoxins in beer, were mainly focussed on industrial produced beer. The present survey reports the presence of mycotoxins in craft beer and how this compares to industrial produced beer. More than 1000 beers were collected from 47 countries, of which 60% were craft beers. A selection of 1000 samples were screened for the presence of aflatoxin B_1, ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), T-2 and HT-2 toxins (T-2 and HT-2) and deoxynivalenol (DON) using a mycotoxin 6-plex microsphere immunoassay (MIA). For confirmatory analysis, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and applied. The 6-plex screening showed discrepancies with the LC-MS/MS analysis, possibly due to matrix interference and/or the presence of unknown mycotoxin metabolites. The major mycotoxins detected were DON and its plant metabolite deoxynivalenol-3-β-D-glucopyranoside (D3G). The 6-plex MIA reported the sum of DON and D3G (DON+D3G) contaminations ranging from 10 to 475 μg/L in 406 beers, of which 73% were craft beers. The popular craft beer style imperial stout, had the highest percentage of samples suspected positive (83%) with 29% of all imperial stout beers having DON+D3G contaminations above 100 μg/L. LC-MS/MS analysis showed that industrial pale lagers from Italy and Spain, predominantly contained FBs (3 – 69 μg/L). Besides FBs, African traditional beers also contained aflatoxins (0.1 - 1.2 μg/L). The presence of OTA, T-2, HT-2, ZEN, β-zearalenol, 3/15-acetyl-DON, nivalenol and the conjugated mycotoxin zearalenone 14-sulfate were confirmed in some beers. This study shows that in 27 craft beers, DON+D3G concentrations occurred above (or at) the Tolerable Daily Intake (TDI). Exceeding the TDI, may have a health impact. A better control of brewing malts for craft beer, should be put in place to circumvent this potential problem.

1. Introduction

Beer production and consumption is booming like never before, mainly due to the increasing popularity of craft beer. Craft beer is produced by small, independent and traditional breweries according to the definition of the Brewers Association. The number of craft breweries continues to grow, claiming a larger market share every year. In the US alone, a few hundred new craft breweries emerge annually. The total amount of craft breweries in 2014 saw an increase of 19.4% compared to 2013. Of the 3,464 breweries operating in the US, 3,418 were classified as craft breweries [1]. The same phenomenon is seen in other parts of the world. In the Netherlands, 108 new breweries emerged just in 2015, bringing the total brewery count to 382 [2]. The reason for the popularity of craft brewers is that they tend to focus on flavour and tradition, combined with innovation rather than on large-scale and low-cost production. This development already started with the Campaign for Real Ale (CAMRA) in Britain, 43 years ago [3]. Some craft breweries also produce similar beer styles as industrial brewers (e.g. pilsner beers). The vast majority of the craft breweries however, produce ancient beer styles, adjusted classic styles or even newly invented styles. Whereas "regular" beers are brewed almost exclusively with water, malted barley, hop and yeast, craft brewers add a wide range of different ingredients to the brewing process. Some examples are coffee, cacao, tobacco, liquorice, nuts, tomatoes, chili peppers, fruit and a range of spices [4,5]. A quick count on one of the most popular websites for craft beer [6], shows that there are currently 83 unique beer styles. Because new styles are regularly being invented, this number will likely increase in coming years.

Mycotoxins are fungal metabolites with acute and/or chronic health effects on animals and humans. These effects include diarrhoea, reduced fertility, immunosuppression, cancer and even death [7-9]. Mycotoxins contaminate a wide range of cereals, including wheat [10], maize [11] and oats [12]. Barley is one of the key ingredients in beer and is prone to mycotoxin contamination [13-15]. Occurrence of mycotoxins in beer has been extensively surveyed, utilizing both instrumental analysis as well as immunoassays. A selected overview [16-45] is presented in Table 1. The most reported mycotoxins in

beers, at relevant levels, are the type B trichothecene deoxynivalenol (DON), its plant metabolite deoxynivalenol-3-β-D-glucopyranoside (D3G) and fumonisins (FBs). DON and D3G were mainly reported in European beers, while FBs were mainly reported in beers from Africa and Southern Europe. In general, very high contaminations for all mycotoxins, besides T-2 toxin (T-2) and HT-2 toxin (HT-2), were reported previously in African beers. Ochratoxin A (OTA) was mainly reported in European beers, while aflatoxins (AFs) were mainly reported in African and Asian beers. T-2 toxin (T-2), HT-2 toxin (HT-2) and zearalenone (ZEN) were rarely reported. Most beer surveys in Table 1 lack detailed information about specific beer styles, country of origin or alcohol content. The presence of mycotoxins in hops, a key ingredient in beer, has rarely been investigated [46]. Furthermore, mycotoxins may also be introduced in beer upon the addition of commodities other than cereals. The risk of mycotoxin contamination may therefore be higher in craft brewing, where a wide range of commodities are added at various stages of the brewing process [47]. The question therefore arises, whether these new and revived craft beer styles contain more, or different, mycotoxins compared to regular commercial beers. Additionally, the changing climate may contribute to altered levels of mycotoxins in field crops [48,49] which eventually will lead to altered levels of mycotoxins in beer. In this work we present a large-scale survey for mycotoxin occurrence in 1000 beer samples with a unique outlook on the upcoming and strongly expanding craft beer market. Beer samples of many different beer styles (representing 60% craft beers) were collected throughout the world, but with a detailed focus on European countries. This selection of 1000 samples was investigated for mycotoxin contamination and to elucidate possible differences between industrial beers and craft beers. Furthermore this survey aimed for a detailed look into the possible occurrence of conjugated (masked) mycotoxins in beer. To facilitate the fast mycotoxin multiplex screening of 1000 beer samples, a previously developed 6-plex microsphere immunoassay method [50,51] for the detection of DON and D3G, aflatoxin B_1 (AFB_1), OTA, the sum of T-2 and HT-2 , fumonisins (sum of fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) and ZEN in barley, was modified and adapted for beer samples. To confirm the

Table 1. Overview of previous mycotoxin beer surveys

Mycotoxin	No. beers analysed	No. samples positive	Mycotoxin concentration range (µg/L)	Beer style	Highest contamination (Country)	Alcohol content (% ABV)	Year	Ref
AFB_1	116	13	0.0005 – 0.083	-	India	-	1999	[16]
	304	12	0.0012 – 0.23	-	India	-	2005	[17]
	422	271	0.00007 – 0.038	-	Ghana	-	2013	[18]
AFB_2	116	5	0.0012 – 0.0086	-	India	-	1999	[16]
	304	4	0.0156 – 0.032	-	India	-	2005	[17]
AFs	422	273	0.00007 – 0.04518	-	France	-	2013	[18]
	5	5	0.0088 – 0.0345	African traditional	Malawi	-	2011	[19]
	35	3	12 – 400	African traditional	South Africa	-	2002	[20]
T-2	30	17	0.2*	-	-	-	2014	[21]
HT-2	30	2	0.6*	-	-	-	2014	[21]
	154	14	25.1 – 38.2	Wheat	Germany	-	2015	[22]
ZEN	23	1	<LOQ	Adjunct lager	USA	4.7	2000	[23]
	91	10	0.46 – 0.55	-	Ireland	-	2013	[24]
	44	44	0.35 – 2.0	-	-	-	2016	[25]
	46	28	12.5 – 200	African traditional	Nigeria	-	1985	[26]
	44	21	20 – 201	African traditional	Botswana	-	2005	[27]
	35	7	2.6 – 426	African traditional	South Africa	-	2002	[20]
β-ZEL	23	1	0.264	Adjunct lager	USA	4.7	2000	[23]

Table 1. Continued

Mycotoxin	No. beers analysed	No. samples positive	Mycotoxin concentration range (μg/L)	Beer style	Highest contamination (Country)	Alcohol content (% ABV)	Year	Ref
OTA	116	107	0.0017 - 0.066	-	Belgium	-	1999	[16]
	61	30	0.010 – 0.135	-	Belgium	>6%	2000	[28]
	106	72	0.005 – 0.189	-	Denmark	-	2011	[29]
	150	42	0.1 – 8.1	-	-	-	2004	[30]
	19	10	1.5 – 2340	African traditional	South Africa	-	2002	[20]
	88	73	0.007 – 0.204	-	Germany	-	2005	[31]
	20	0	<LOQ	-	-	-	2011	[32]
	35	17	0.04 – 0.350	-	Tunisia	-	2013	[33]
FB_1	106	32	0.1 – 30.3	-	Italy	-	2011	[29]
	120	105	0.5 - 340	African traditional	Cameroon	-	2011	[34]
	18	18	38 - 1066	African traditional	South Africa	-	2005	[35]
	9	9	1522*	African traditional	Malawi	-	2014	[19]
	53	8	29 - 285	-	Brazil	-	2015	[36]
FB_2	106	19	0.2 – 3.9	-	Italy	-	2011	[29]
	18	17	8 – 135	African traditional	South Africa	-	2005	[35]
	9	8	251*	African traditional	Malawi	-	2014	[19]
FB_3	18	12	8 - 128	African traditional	South Africa	-	2005	[35]
	9	6	229*	African traditional	Malawi	-	2014	[19]
$FB_1 + FB_2$	72	64	157.2*	-	Spain	-	2012	[37]
	29	12	0.3 – 12.7	-	USA	-	1999	[38]
Total FBs	32	14	4.8 – 85.5	-	Spain	-	1998	[39]

Table 1. Continued

Mycotoxin	No. beers analysed	No. samples positive	Mycotoxin concentration range (μg/L)	Beer style	Highest contamination (Country)	Alcohol content (% ABV)	Year	Ref
DON	313	272	4.0 – 56.7	-	Belgium	-	2004	[40]
	20	18	5.1 – 35.9	Strong Pale Lager	-	9.0	2008	[41]
	15	15	5.6 – 62.2	-	-	-	2012	[42]
	176	113	1.0 – 35.9	Light beer	-	-	2009	[43]
	-	-	1.0 – 16.0	Dark beer	-	-	2009	[43]
	106	70	0.7 – 18.6	-	Croatia	-	2011	[29]
	120	107	140 - 730	African traditional	Cameroon	-	2011	[34]
	91	91	6.0 – 70.2	-	Poland	-	2013	[24]
	217	118	5.4 – 89.3	Pale beer	Austria	4.9	2013	[44]
	46	36	5.2 – 49.6	Wheat	Germany	4.9	2013	[44]
	47	14	11.1 – 45.0	Dark beer	Germany	5.3	2013	[44]
	20	18	6.5 – 27.1	Bock beer	Germany	11.0	2013	[44]
	19	5	3.2 – 26.1	Non-alcoholic	Serbia	0.5	2013	[44]
	25	13	4.2 – 12.7	Shandy	Serbia	2.0	2013	[44]
	61	14	200 - 360	Busaa	Kenya	-	2014	[45]
	154	92	24.5 – 47.7	-	Spain	-	2015	[22]
	53	17	127 - 501	-	Brazil	-	2015	[36]
	44	33	2.2 - 20	-	-	-	2016	[25]
ADONs	20	15	5.1 – 27.6	Strong Pale Lager	-	9.0	2008	[41]
	176	88	1.0 – 25.0	Light beer	-	-	2009	[43]
	176	88	1.0 – 24.0	Dark beer	-	-	2009	[43]
D3G	20	19	4.0 – 25.8	Pale Lager	-	5.0	2008	[41]
	15	15	6.0 – 82.1	-	-	-	2012	[42]
	176	130	1.4 – 37.0	Light beer	-	-	2009	[43]
	-	-	1.5 – 26.0	Dark beer	-	-	2009	[43]

Table 1. Continued

Mycotoxin	No. beers analysed	No. samples positive	Mycotoxin concentration range (μg/L)	Beer style	Highest contamination (Country)	Alcohol content (% ABV)	Year	Ref
D3G	217	142	3.6 – 81.3	Pale beer	Austria	4.9	2013	[44]
	46	32	3.5 – 28.4	Wheat	Germany	4.9	2013	[44]
	47	28	4.2 – 26.2	Dark beer	Germany	5.3	2013	[44]
	20	20	2.4 – 33.3	Bock beer	Germany	11.0	2013	[44]
	19	9	1.6 – 6.6	Non-alcoholic	Serbia	0.5	2013	[44]
	25	20	1.8 – 7.9	Shandy	Austria	2.2	2013	[44]

Mycotoxin abbreviations: AFB1 (aflatoxin B1), AFB2 (aflatoxin B2), AFM1 (aflatoxin M1), AFs (aflatoxins), T-2 (T-2 toxin), HT-2 (HT-2 toxin), ZEN (zearalenone), β-ZEL (β-zearalenol), OTA (ochratoxin A), FB1 (fumonisin B1), FB2 (fumonisin B2), FB3 (fumonisin B3), FBs (fumonisins), DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON).

presence of mycotoxin contaminations in a subset of the screened beer samples, a dedicated multi-mycotoxin liquid chromatography tandem mass spectrometry (LC-MS/MS) method for beer was developed. This method includes several conjugated mycotoxins, as well as mycotoxin metabolites, such as aflatoxin M_1 (AFM_1), ochratoxin B (OTB), nivalenol (NIV) and zearalenone 14-sulfate (Z14S).

This unprecedented survey reveals the discovery of the conjugated mycotoxin Z14S in beer and confirms that high DON and D3G contaminations can specifically occur in craft beer. The sum of these DON and D3G contaminations (DON+D3G) contribute to surpassing the tolerable daily intake (TDI) of DON upon moderate beer consumption.

2. Materials and Methods

2.1 Instrumentation

The 6-plex MIA was performed on a flow cytometer platform (FM3D) or on a planar microsphere array analyzer (MAGPIX), both running on XPONENT

software (all from Luminex, Austin, USA). Mycotoxin concentrations were calculated using the xMAP dedicated Bio-Plex manager software 6.0, combined with Bio-Plex results generator 3.0 (Bio-Rad Laboratories, Veenendaal, the Netherlands). A Bio-Plex II Wash Station (Bio-Rad) with magnetic plate support was used for all washing steps. Incubation of the 6-plex MIA was done on a Bühler TiMix 2 shaker (Salm en Kipp, Breukelen, the Netherlands) at room temperature (RT). Beer samples were degassed at RT using the Ultrasonic Cleaner (VWR International, Amsterdam, the Netherlands) at maximum power and centrifuged in an Eppendorf 5810R centrifuge (VWR) equipped with an A-4-62 swinging bucket rotor. All confirmatory analyses of mycotoxins in selected beer samples were done on an AB Sciex (Nieuwerkerk a/d IJssel, the Netherlands) QTRAP 5500 tandem mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source, operated in positive and negative multiple reaction monitoring (MRM) mode. The MS system was coupled to a Shimadzu ('s Hertogenbosch, the Netherlands) Prominence Liquid Chromatography (LC) system, equipped with a Restek (Interscience, Breda, the Netherlands) Ultra Aqueous C18 (100×2.1 mm) column (see Supporting Information (S.I.)). Integration of reconstructed MRM chromatograms was done with MultiQuant V2.0 software using the Signal Finder integration algorithm (AB Sciex). Monoclonal antibodies (mAbs) against AFB_1, ZEN, T-2 and DON were purchased from Aokin AG (Berlin, Germany), while the FB_1 and OTA mAbs were purchased from Soft Flow Biotechnology Ltd. (Gödöllő, Hungary). The R-Phycoerythrin (RPE)-FB_1 and RPE-OTA conjugates were produced in-house using RPE from Moss (Pasadena, MD, USA). The remaining RPE-mycotoxin conjugates were synthesized by Aokin.

2.2 Chemicals

Cellstar 96-well culture microtiter plates, 10 and 50 mL tubes were from Greiner (Alphen a/d Rijn, the Netherlands). Sheath fluid (FM3D) and drive fluid (MAGPIX) were both purchased from Luminex (Austin, USA). The following mycotoxins and metabolites were purchased from Biopure (Tulln, Austria): FB_1, FB_2,FB_3, OTA, OTB, AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M1 (AFM_1), T-2, HT-2, DON, D3G, 3-

acetyl-DON (3ADON), 15-acetyl-DON (15ADON), NIV, ZEN, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL). Zearalenone-14-β-D-glucopyranoside (Z14G), α-zearalenol-14-β-D-glucopyranoside (α-ZELG), β-zearalenol-14-β-D-glucopyranoside (β-ZELG) and zearalenone 14-sulfate (Z14S) were produced according to [52] or isolated from *Fusarium* inoculated rice. Syringeless filter devices (Mini-UniPrep, PTFE) for sample clean-up were purchased from GE Healthcare (Rotterdam, the Netherlands). Acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, the Netherlands), formic acid (FA) from Merck (Amsterdam, the Netherlands) and ammonium formate (AMF) from Fluka Analytical (Steinheim, Germany). All other chemicals were purchased from VWR International (Amsterdam, the Netherlands).

2.3 Beer samples

A total of 1000 beer samples, from 42 different countries (Table 2), were selected. Sample collection was mainly random and depending on access and availability, i.e. , sampling was not intended to be statistically representative. Primary goal was to collect as much as possible craft beer samples to be able to make a comparison to industrial produced beer and have a more detailed look on craft beer itself. Secondary goal was to cover many different beer styles. At the start of this research, it was still difficult to collect a representative number of international craft beers due to poor availability. Craft beers were mainly collected from bars, restaurants, supermarkets, specialized craft-beer shops and during craft-beer festivals between 2011 and 2014, while industrial beers were mainly collected in supermarkets. Craft beer samples also included vintage beers (beers produced before 2011, often cellared for conditioning). Based on local contacts, additional samples from the USA, China and several African countries were sent to the authors. African beer samples were both commercial (bought in South African supermarkets) and traditional opaque home-brews (collected on site in town villages in Northern South Africa).

Table 2. Origin of the 1000 global beer samples investigated

Country	Region	No.	Country	Region	No.	Country	Region	No.
Australia	Oceania	5	India	Asia	1	Poland	Europe	27
Austria	Europe	4	Ireland	Europe	3	Portugal	Europe	2
Belarus	Europe	1	Italy	Europe	28	Russia	Asia	1
Belgium	Europe	203	Jamaica	North-America	1	Scotland	Europe	12
Canada	North-America	6	Japan	Asia	9	Slovakia	Europe	1
China	Asia	5	Kenya	Africa	2	South Africa	Africa	46
Czech Republic	Europe	24	Latvia	Europe	1	Spain	Europe	48
Denmark	Europe	55	Malaysia	Asia	1	Sweden	Europe	5
England	Europe	38	Mexico	South America	4	Switzerland	Europe	3
Finland	Europe	1	Namibia	Africa	3	Trinidad & Tobago	North-America	1
France	Europe	16	Netherlands	Europe	209	Turkey	Europe	1
Germany	Europe	87	Nigeria	Africa	1	Ukraine	Europe	1
Greece	Europe	2	Norway	Europe	14	USA	North-America	124
Iceland	Europe	1	Peru	South America	1	Zimbabwe	Africa	2

From each beer, 10 mL was collected in a 50 mL tube and degassed by sonication at maximum power for 10 minutes at RT. After sonication, the beer samples were transferred to a 10 ml tube, centrifuged at 3200g and stored at -20°C. The designated beer styles of these samples were grouped into 20 main beer styles (Table 3).

2.4 Screening of beer samples

The mycotoxin paramagnetic 6-plex MIA method used, was a new adaptation of a previously described method [51]. The adapted mycotoxin 6-plex MIA involved a simplified extraction method and its performance in beer samples was validated on the planar array imaging platform. The stored beer samples were defrosted, mixed by inversion, and centrifuged for 10 minutes at 3200 g to pellet yeast or other insoluble matter. The supernatant was then diluted 8 fold using methanol-water (1:9 v/v) (10% MeOH). To 40 μL of the diluted beer samples a 10 μL mixture of microspheres, previously coupled with mycotoxin specific monoclonal antibodies, was added followed by the addition of 10 μL of a mixture of mycotoxin specific reporter molecules (mycotoxins coupled to RPE). The final buffer composition in the assay was phosphate buffered saline, 0.02 % Tween 20, pH 7.4. Sample and assay components were incubated for 15 minutes, to allow competition between the mycotoxin-RPE conjugates and the free mycotoxins in the samples for antibody interaction. The microspheres were then trapped by a magnet and washed followed by analysis on one of the microsphere dedicated platforms (Fig 1). Since the beer styles investigated are very diverse in composition, it is rather impossible to find a suitable common blank beer. As a practical solution, we selected a dark ale as blank beer for all screening assays, following confirmation of the absence of mycotoxins by LC-MS/MS. This blank beer was used to prepare beer-based multi-mycotoxin calibration curves for AFB_1, OTA, ZEN, DON, T-2 and FB_1. First, the blank beer was diluted 4-fold with 10% MeOH and subsequently mixed (1:1, v/v) with each standard of the mycotoxin used for the construction of calibration curves, resulting in a final 8-fold dilution of the matrix content and a 2-fold dilution of each standard.

Table 3. Grouping of individual beer samples into beer style groups and fraction of craft beers

Group	%ABV*	Styles	Number analysed	Number craft beers	Percentage craft beers
Non/low alcohol	< 3	Pale lager, Low alcohol, Non alcoholic	36	3	8
Pale Lager	3 – 5	Pilsener, helles lager, Adjunct lager, premium, Zwickel, California Common	166	11	7
Strong Pale Lager	6 - 14	Strong Pale lager, Imperial Pils	8	-	0
Pale Ale	4 – 9	Blond, Belgian Pale Ale, American Pale Ale, Amber Ale, Irish Ale, English Pale Ale, Mild Ale, Kölsch	94	63	67
Strong Pale Ale	9 - 15	Tripel (Abbey, Trappist), Barley Wine (American, English), Strong Ale (Belgian, American, English)	69	56	81
India Pale Ale	≤ 7.5	Bitter, Premium Bitter (ESB), India Pale Ale (Black, White)	42	37	88
Double India Pale Ale	≥ 7.5	India Pale Ale (Imperial, Triple, Double)	29	29	100
Dark lager	3 - 5	Schwarzbier, Dunkel	28	1	4
Dark Ale	6 – 9	Old Ale, Scotch, Dubbel (abbey, Trappist)	36	22	61
Strong Dark Ale	9 - 13	Quadrupel, Abt	44	28	64
Stout	< 8	Stout (Milk, Foreign, Oatmeal, Sweet), Porter	54	40	74
Imperial Stout	≥ 8	Stout (Imperial, Export), Porter (Imperial, Baltic)	126	123	98
Sour ales	4 - 13	Geuze, Lambic (Fruit, Faro, Unblended), Sour Ale, Gose, Wild Ale, Flanders Red, Flanders Oud Bruin, Berliner Weisse	82	72	88
Fruit/Vegetable/ Spice	5 - 16	Various styles	37	25	68
Saison	4 - 11	Saison, Bière de Garde	13	10	77
Smoked	5 - 11	Various styles	16	12	75
Wheat	5 - 8	Weizen, Weizen (Dunkel, Bock), Wit, Belgian White, Wheat Ale	42	14	33
Bock	5 - 12	Bock (Helles, Doppel, Dunkel, Lente)	38	19	50
Eisbock	9 - 40	Eisbock	6	5	83
African traditional	< 3	Mqombothi, Sorghum	34	33	97
		TOTAL	1000	589	59

*Percentage alcohol by volume

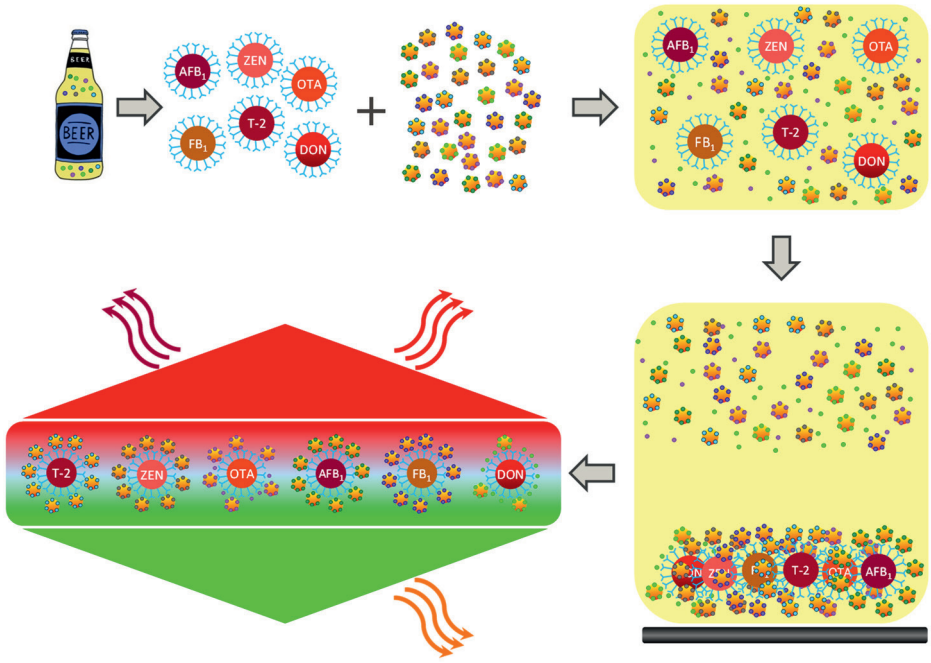

Figure 1. Overview of the 6-plex mycotoxin immunoassay: Mycotoxin mAb coupled paramagnetic microspheres and a mixture of mycotoxin specific reporter molecules (mycotoxins coupled to R-phycoerythrin) are added to a diluted beer sample. Competition occurs between the free mycotoxins present in the beer and the added mycotoxin-reporter molecules for antibody interaction on the microspheres. Next, the microspheres are captured at the bottom of the well by magnetic force. Remaining, non-interacting, assay components are removed by washing. Microspheres are then measured using a red light source for mycotoxin assay classification and a green light source for quantification of the reporter signal.

Using these multi-mycotoxin calibration curves (S1 Fig), the mycotoxin concentrations in 1000 beer samples were calculated. To this end, the dedicated Bio Plex manager software (Bio-Rad Laboratories, Veenendaal, the Netherlands) built for automated curve and data fitting, was used. The results of the triplicate sample measurements are displayed as a concentration range combined with a standard deviation to give a better idea about the performance of the multiplex method. The mycotoxin monoclonal antibodies (mAbs) in the 6-plex MIA were previously tested for cross-reactivity [50,51]. These results showed, amongst others, cross-reactivity of D3G in the DON immunoassay.

2.5 Confirmation of screening results by LC-MS/MS

From the 1000 beer samples that were screened with the mycotoxin 6-plex MIA, 100 beer samples were selected for confirmatory LC-MS/MS analysis. The first set of samples submitted for confirmatory analysis, was based on mycotoxin contamination results revealed by the 6-plex MIA. It included mostly high contaminations, as well as some blanks, revealed by the screening. Furthermore, the first selection was also based on covering a wide range of beer styles. Based on the confirmatory results of the first selection, a more detailed selection of new (previously screened) beers was made. The focus was on observed contamination trends in certain beer styles. Since craft beer was the main focus of this survey, we aimed for a total of 70% of craft beers in the final LC-MS/MS selection. For confirmatory analysis we adapted an existing ISO 17025 accredited LC-MS/MS method for feed. This adapted LC-MS/MS method (S1 Text) contained all the relevant mycotoxins, as well as a selection of available mycotoxin metabolites and conjugated forms, relevant to the 6-plex screening method targets (S1 Table). Since the variation in beer matrices is very diverse, especially in craft beer, we chose to use a single point standard addition method for quantification. To this end, 100 µl of degassed beer sample was diluted with 100 µl of the standard solution. The diluted sample was filtered through a syringeless filter device and 5 µl was injected. The limit of quantification (LOQ) was set at 10 times below the standard addition level. The upper quantification limit was set arbitrarily at 2 times above the standard addition level. The concentrations for standard additions can be found in the S1 Table. The limit of detection (LOD) was set at a signal-to-noise ratio of 3:1 based on the peak-to-peak noise around the retention times of the analytes in the reconstructed MRM chromatograms. To this end, we utilized MultiQuant V2.0 software (AB Sciex) using the Signal Finder integration algorithm.

3. Results and Discussion

3.1 Scope of the survey

Until now, most published beer surveys for mycotoxins are lacking relevant information on the beer styles (Table 1). Occasionally, information about the country of origin is supplied [29] and sometimes beers are grouped on the basis of their alcohol content [43]. More recently, Varga et al [44] provided detailed information about alcohol content, country of origin and beer style categories. Because of the serious style expansion by craft brewers, we chose to elaborate even further on the beer style categories while also focusing on alcohol content and origin. In our large-scale survey of 1000 beer samples from all over the world (S2 Fig), there was a strong focus on Europe with a total of 787 beers screened. Within Europe the emphasis was on beers from the Netherlands (209), Belgium (203) and Germany (87) (S2 Fig). Furthermore, nearly 60% of all the beers analysed were craft beers. A flow chart overview of the general survey approach is given in Fig 2.

3.2 Performance of the 6-plex MIA as a screening method

A mycotoxin 6-plex MIA, previously applied as a qualitative screening assay for barley [51], was adapted for beer (Fig 1). The extraction protocol was simplified and the method was made suitable for the fast and semi-quantitative detection of mycotoxins in beer. The performance of the adapted 6-plex MIA was bench-marked against the previously developed, and in-house validated screening assay for barley [51], by determining the intra- and interday precision for beer samples. The intra- and interday relative standard deviation (%RSD) for multi-mycotoxin fortified samples in dark ale, based on the B/B_0 values, were determined for each fortified sample and compared to the previous method (S2 Table).

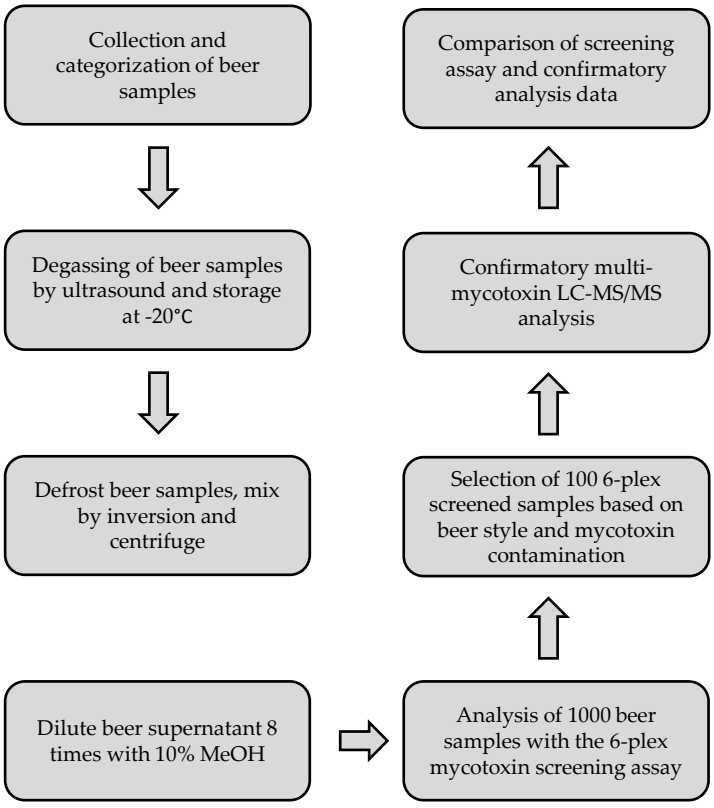

Figure 2. Schematic overview of the general approach

Also in beer, for most mycotoxins, the %RSD values were well below 10%. For T-2 the %RSD values were below 20%. This may indicate that the beer matrix interferes somewhat more with the T-2 assay than barley did. For quantification we prepared multi-mycotoxin dose-response curves in dark ale and checked the variation by comparing intra- and interday IC50s (S3 Table). Based on these dose-response curves, the mycotoxin concentrations in the multi-mycotoxin fortified samples were determined by the Bio Plex manager software. The intra- and interday quantitative precision is displayed in S4 Table.

Besides the FB_1 assay, which showed overestimations up to a factor 3.9, the other assays performed satisfactory with only slight overestimations with a factor ranging from 1.1 to 1.7. The overestimations in the FB_1 assay were not unexpected and reported previously [50]. Moreover, the mycotoxin mAbs used in the 6-plex, in most cases, show cross-reactivity with other metabolites as was reported in detail previously [51] . Therefore, we chose to confirm selected results (see method section for selection criteria) of the screening assay by an LC-MS/MS based method. The complete mycotoxin 6-plex screening data for all 1000 beers, grouped by beer style groups, can be found in the S5 Tables in the supplementary information.

3.3 Performance of the LC-MS/MS confirmatory method

A multi-toxin ISO 17025 accredited LC-MS/MS method for feed was successfully adapted for beer. In order to make the LC-MS/MS method fit for purpose, 23 irrelevant mycotoxins and metabolites were removed from the original 40 targets and 8 new mycotoxin metabolites and conjugated forms of interest were added. The standard addition quantification method was found fit for purpose, following fortification of 5 diverse beer styles (pale lager, adjunct lager, dark ale, sour ale and imperial stout) with all mycotoxins and metabolites. This resulted in adequate detection of precursor and product ions in the respective reconstructed MRM chromatograms. Average variation of LC retention times were well below 0.2 minutes and the deviations in MS/MS product ion intensity ratios, when comparing beer samples to non-matrix-matched standards (in 10% MeOH), were typically below 30% (occasionally below 50%). For DON, higher deviations occurred mainly at low concentrations (< 10 μg/L) and were not linked to any specific beer style. For both methods, the 6-plex MIA and the LC-MS/MS, we chose to simply dilute the beer samples rather than to perform concentration or clean-up steps. Therefore, potential matrix effects were only reduced by dilution in both the 6-plex MIA and LC-MS/MS. Since the beer styles analysed vary strongly in composition and gravity, variable matrix interference and signal suppression (or enhancement) were to be expected. As a consequence, some mycotoxin standard additions (mainly in imperial stouts) were hardly visible in the reconstructed MRM chromatograms for some of the beer samples. These

samples were not considered nor reported. Please note that the sample dilution used for LC-MS/MS is smaller than for the 6-plex MIA, since the standard addition method allows correction for matrix effects. The actual volume of the beer sample analysed in the LC-MS/MS is 2.5 µl while the 6-plex screening uses 5 µl.

3.4 Occurrence and discussion of specific mycotoxins

3.4.1 Aflatoxins

The 6-plex data suggests the presence of AFB_1 in several beer samples with concentrations ranging from 0.1 to 3.7 µg/L (S5 Tables). In particular imperial stouts (S5 Table H), as well as some other dark beers, showed AFB_1 contaminations. However, when analysed by LC-MS/MS, none out of the 28 selected AFB_1 suspected beers could be confirmed. Since the AFB_1 immunoassay showed almost no cross-reaction with AFB_2, AFG_1 or AFG_2 [51], contamination with these metabolites was highly unlikely. A possible explanation for the screening results could be the presence of the structurally related sterigmatocystin (STC), whose presence in beer has been reported [53]. However, additional testing showed that STC had no cross-reactivity in the AFB_1 assay. Further research is needed to elucidate the origin of the observed suspect screening results, but it seems plausible that matrix effects yielded the false positive results in the immunoassay for these beers. On the other hand, LC-MS/MS analysis revealed 5 beers positive for AFs (Table 4) that were not screened suspect with the 6-plex MIA. The 8 times dilution of the sample in the immunoassay may be the reason for this, compared to a 2 times dilution in the LC-MS/MS analysis. From those 5 beers, 4 were African traditional beers and one a pale lager from Zimbabwe. All positive beers were contaminated with AFB_1 (0.1 – 1.2 µg/L) and three of them also contained AFB_2 (0.1 – 0.2 µg/L). In one traditional beer also AFM_1 was detected. This may indicate the use of milk or milk derived products in this particular beer. Milk products can be used in certain beer styles (e.g. whey in milk stouts) and therefore can be a source of AFM_1 contamination. However, nowadays milk stouts are often produced by the addition of lactose to beer [5]. In this particular sample, being home-brewed, the chance of cross-contamination

from other sources (which is common in African domestic brewing) may provide a plausible explanation. AFG_1 or AFG_2 were not detected in any beer sample. Contaminations of AFs in African traditional beer have been previously reported [19,20], while AF contaminations in European beers is rarely reported [18]. Occurrence of AFs in beer is of the highest toxicological concern and therefore consumption should be avoided at any time, considering that the IARC classified aflatoxins as carcinogenic to humans (Group 1) [54].

3.4.2 T-2 and HT-2 toxins

We obtained signals for T-2/HT-2 in many beer samples in the 6-plex MIA (S5 Tables). However, only in 3 out of the 31 T-2/HT-2 suspected beer samples (and 4 out of all 100 beers submitted for confirmatory analysis) the presence of T-2 or HT-2 toxins was confirmed by LC-MS/MS (Table 5). The highest values being 2.3 μg/L and 3.4 μg/L respectively. These values are lower than those recently found by Rodríguez-Carrasco et al [22]. In their survey 14 (out of 154) samples contained HT-2, all with levels between 24.2 – 38.2 μg/L. All those samples, from 2013, came from Germany and were from the wheat beer style. In our case, the presence of T-2 and HT-2 (as determined by LC-MS/MS) seemed not style nor country/region dependent. Our results suggest that several suspect immunoassay screening results may indicate either false positives or possible modified forms of T-2 and/or HT-2. Regular consumption of these beers will not lead to exceedance of the TDI of 0.1 μg/kg BW for the sum of T-2 and HT-2 [55] (14 μg/L for a person of 70 kg BW drinking one 0.5 L bottle of beer per day). Beer sample 356, an imperial stout, had the highest T-2/HT-2 contamination (57 μg/L) in the 6-plex MIA, but this was not confirmed by LC-MS/MS. In preliminary follow-up research, we analysed this sample using high resolution LC-MS/MS analysis and found indications that two forms of HT-2 glycosides may be present in this beer sample (results not shown). It has been reported previously [56] that HT-2 glycosides were present in wheat. Due to the lack of proper standards, quantification of these HT-2 glycosides was not possible. For the same reason the cross-reaction of HT-2 glycosides in the 6-plex MIA could not be determined, making it impossible to verify if the T-2/HT-2 suspect results are

Table 4. Beer samples with confirmed aflatoxin contaminations (µg/L)

Combined Style	Sample Number	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)		
					average	range	SD	AFB_1	AFB_2	AFM_1
African Traditional	421	yes	South Africa	?	nd	nd	nd	0.1	<LOD	<LOD
African Traditional	423	yes	South Africa	?	nd	nd	nd	1.2[1]	0.2	0.1
African Traditional	429	yes	South Africa	?	nd	nd	nd	1.0	0.1	<LOD
African Traditional	452	yes	South Africa	?	nd	nd	nd	0.2	0.1	<LOD
Pale Lager	280	no	Zimbabwe	5	nd	nd	nd	0.2	<LOD	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, SD = standard deviation, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, AFB_1 (aflatoxin B_1), AFB_2 (aflatoxin B_2), AFM_1 (aflatoxin M_1)

Table 5. Beer samples with confirmed T-2 and HT-2 contaminations (µg/L)

Combined Style	Sample Number	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)	
					average	range	SD	T-2	HT-2
Stout	138	no	Czech Republic	10.5	nd	nd	nd	<LOQ	<LOD
Sour Ale	291	yes	Belgium	5	12.9	12.7 - 13.1	0.2	2.3	<LOD
Strong Pale Ale	382	yes	Belgium	10	1.2	0.9 – 1.6	0.4	<LOD	3.4
Pale Lager	698	no	Poland	5.2	0.3	0.1 - 0.4	0.1	0.7	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, SD = standard deviation, nd = not detected, LOD = limit of detection, LOQ = limit of quantification, T-2 (T-2 toxin), HT-2 (HT-2 toxin)

based on the presence of glycosides or other possible conjugated forms. The metabolites T-2 triol and T-2 tetraol were not detected in any beer sample and therefore not the cause of the suspect screening results.

3.4.3 Zearalenone

For ZEN, concentrations up to 5.6 μg/L were found in the 6-plex MIA (S5 Tables). From the 3 selected ZEN suspect beers, 1 was confirmed as positive when analysed further by the LC-MS/MS method. LC-MS/MS analysis of selected negative immunoassay samples revealed 6 additional ZEN contaminations, although 5 out of 6 results were below the LOQ of 0.3 μg/L (Table 6). Also β-ZEL was found in 4 samples, in all cases below the LOQ of 2 μg/L and in two cases co-occurring with ZEN. Z14S was detected in 12 beer samples in Table 6. From those samples, 3 were above the LOQ (0.5 μg/L) while 9 samples had concentrations between the LOQ and LOD. Z14S was not found in African traditional beers. Z14S co-occurred with ZEN four times and in 2 beer samples ZEN, β-ZEL and Z14S co-occurred. In a previous survey, including conjugated mycotoxins, Z14S was not detected in beer [57]. *α*-ZEL, *α*-ZELG, β-ZELG and Z14G were not detected in any beer sample. ZEN was previously detected in high concentrations (up to 426 μg/L) in African traditional beers [20]. Using immunoassays, Bauer et al [25] and Kuzdraliński et al [24] both detected ZEN in beers (concentrations up to 2.0 μg/L), but these were not confirmed by instrumental analysis. Therefore it remains unclear whether ZEN metabolites were contributing to the ZEN values reported. The ZEN mAb in our 6-plex MIA showed no cross-reactions to Z14S. None of the contaminated samples in our survey would lead to exceedance of the TDI for ZEN, set by EFSA at 0.25 μg/kg BW [58], under normal circumstances (35 μg/L for a person of 70 kg BW drinking one 0.5 L bottle of beer per day). This also counts if we add up the sum of all ZEN metabolites in a single sample. Therefore ZEN is not a major risk factor in the confirmed beers.

Table 6. Beer samples with confirmed ZEN and ZEN metabolite contaminations (µg/L)

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)		
					average	range	SD	ZEN	β-ZEL	Z14S
African traditional	407	yes	South Africa	4	nd	nd	nd	<LOD	<LOQ	<LOD
African traditional	416	yes	South Africa	?	nd	nd	nd	<LOD	<LOQ	<LOD
African traditional	417	yes	South Africa	?	nd	nd	nd	<LOQ	<LOD	<LOD
African traditional	430	yes	South Africa	?	nd	nd	nd	<LOQ	<LOD	<LOD
Bock	238	no	Poland	10	nd	nd	nd	<LOQ	<LOD	0.7
Dark Lager	124	no	Czech Republic	3.8	nd	nd	nd	<LOQ	<LOQ	0.5
Dark Lager	132	no	Czech Republic	4.7	nd	nd	nd	<LOD	<LOD	<LOQ
Imperial Stout	183	yes	USA	15	nd	nd	nd	0.3	<LOQ	0.5
Imperial Stout	631	yes	Norway	14	nd	nd	nd	<LOQ	<LOD	<LOQ
Imperial Stout	644	yes	Netherlands	11	nd	nd	nd	<LOD	<LOD	<LOQ
Imperial Stout	771	no	Poland	8	nd	nd	nd	<LOD	<LOD	<LOQ
Non/Low Alcohol	121	no	Czech Republic	0	0.5	0.4 - 0.6	0.1	<LOD	<LOD	<LOQ
Pale Ale	97	yes	Netherlands	5.5	nd	nd	nd	<LOD	<LOD	<LOQ
Pale Lager	698	no	Poland	5.2	nd	nd	nd	<LOD	<LOD	<LOQ
Stout	707	yes	Denmark	7	nd	nd	nd	<LOD	<LOD	<LOQ
Strong Pale Ale	768	yes	Norway	10	nd	nd	nd	<LOD	<LOD	<LOQ

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, ZEN (zearalenone), β-ZEL (β-zearalenol), zearalenone 14-sulfate (Z14S)

3.4.4 Ochratoxin A

The 6-plex MIA assay showed several OTA suspect samples with indicative levels ranging from 0.1 – 1.6 μg/L (S5 Tables). From the 25 selected OTA suspect samples, 6 samples were confirmed positive by LC-MS/MS and 5 samples had OTA concentrations ranging from 0.3 – 0.6 μg/L (Table 7).

Table 7. Beer samples with confirmed OTA contaminations (μg/L)

Combined Style	Sample No	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)
					average	range	SD	OTA
Bock	325	yes	Norway	8.5	0.8	0.8	<0.1	0.6
Dark Ale	361	yes	Norway	4.5	0.4	0.3 - 0.4	0.1	0.3
Double India Pale Ale	330	yes	Norway	10	0.7	0.7 - 0.8	0.1	0.5
India Pale Ale	300	yes	England	6	0.3	0.3 - 0.4	<0.1	<LOQ
Pale Ale	380	yes	Norway	6	0.4	0.3 – 0.4	0.1	0.4
Strong Pale Ale	353	yes	England	11	0.6	0.4 – 0.7	0.1	0.4

n = number of replicates, %ABV = percentage alcohol by volume, LOQ = limit of quantification, OTA (ochratoxin A)

Remarkably, from these confirmed OTA positives, 4 beers were from the same Norwegian brewery. The other beers confirmed positive were from England, but originated from different breweries. Note that sampling of all these positive craft beers had occurred in the same year (2011) and at the same craft beer festival. The OTA contaminations found in our survey were in beers from European origin. They were slightly higher than those previously found (in European beers) by Visconti et al [28] and Bertuzzi et al [29] but considerably lower than the contaminations found by Odhav en Naicker [20] in African traditional beers. OTB was not detected in any beer sample. OTA is of high toxicological concern, since it is possibly carcinogenic to humans (Group 2B) [59]. In 2006, EFSA established a Tolerable Weekly Intake (TWI) for OTA of 120 ng/kg BW per week which can be translated to an average of 17 ng/kg BW per day [60]. The beers in our survey confirmed positive for OTA do not surpass this derived TDI under normal circumstances (2.4 μg/L for a person of 70 kg BW drinking one 0.5 L bottle of beer per day).

3.4.5 Fumonisins

From previous research it is known that the 6-plex MIA overestimates the fumonisin content [51]. As a result, several false suspects became apparent when we compared the FBs immunoassay data (S5 Tables) with the LC-MS/MS data. Higher false suspect concentrations were typically found in darker style beers (e.g. imperial stouts, dark lagers and ales). Confirmatory analysis showed that pale lagers contaminated with FB_1 were mainly from Spain and Italy (Table 8). In some countries, mostly for economic reasons, pale lagers often contain other cereals besides barley. The declared information on the label of some of these supermarket beers purchased in Spain and Italy, revealed that they contain maize as an adjunct. In fact, Italian and Spanish beers brewed with higher amounts of barley (or 100% barley) are often considered specialty beers in these countries. The use of maize in pale lagers (or any other beer) increases the risk of contamination with FBs. These data may suggest a Mediterranean trend. However, in a preliminary screening of available Greek commercial and craft beers in 2015, we did not find any pale lager that contained FBs (results not published). The highest LC-MS/MS FB_1 contamination detected in our survey was for an Italian pale lager (51 μg/L), followed by a Spanish non-alcoholic beer that contained (28 μg/L). In only 4 beer samples we were able to detect FB_3 and in 2 beer samples this was above the LOQ (1 μg/L). FB_2 was not detected in any beer sample. Fumonisin contaminations of Italian beers (30 μg/L) and Spanish beers (85 μg/L) were reported previously [29,39]. Besides pale lagers, mainly African traditional home-brews were prone to FB_1 contamination. The highest contamination in that category was 36 μg/L with another two beers close to this contamination level (30 and 28 μg/L respectively) (Table 8). There are agro-ecological and cultural reasons for this. First, most of Africa is hot and humid thus ideal for *Fusarium* infection and growth. Further, Shepard et al [35] have shown that in the Eastern Cape, the best maize is selected for cooking while the mouldy maize is then used for brewing beer. It is believed that infected maize adds a desirable taste to the final beer. Beers from two Spanish breweries were sampled again approximately 2 years later, and analysed only for FBs using LC-MS/MS (S6 Table).

Table 8. Beer samples with confirmed FB contaminations (μg/L)

Combined Style	Sample Number	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)	
					average	range	SD	FB_1	FB_3
African Traditional	272	yes	South Africa	?	27	24 - 31	4	3	<LOD
African Traditional	278	yes	Zimbabwe	6	376	357 - 393	18	28[1]	<LOQ
African Traditional	407	yes	South Africa	4	30	28 -31	1	11	<LOD
African Traditional	416	yes	South Africa	?	5	3-7	2	4	<LOD
African Traditional	417	yes	South Africa	?	11	10 - 12	1	4	<LOD
African Traditional	420	yes	South Africa	?	10	8 - 11	1	5	<LOD
African Traditional	421	yes	South Africa	?	24	20 - 27	4	7	<LOD
African Traditional	423	yes	South Africa	?	84	82 - 88	3	36[1]	<LOD
African Traditional	427	yes	South Africa	?	15	13 - 16	2	3	<LOD
African Traditional	428	yes	South Africa	?	15	14 - 16	1	2	<LOD
African Traditional	429	yes	South Africa	?	25	25 - 26	1	30[1]	<LOD
African Traditional	430	yes	South Africa	?	16	16 - 17	1	3	<LOD
African Traditional	451	yes	South Africa	?	28	27 -29	1	27[1]	<LOD
African Traditional	452	yes	South Africa	?	33	31 - 35	2	13	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, FB_1 (fumonisin B_1), FB_3 (fumonisin B_3)

Table 8. (continued)

Combined Style	Sample Number	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)	
					average	range	SD	FB_1	FB_3
Non/Low Alcohol	398	no	Spain	0	34	32 -35	1	28[1]	<LOD
Pale Ale	217	no	Poland	5.7	7	3 - 9	3	12	<LOD
Pale Lager	48	no	Germany	5	20	19 - 21	1	20	<LOD
Pale Lager	280	no	Zimbabwe	5	11	10 -13	2	11	<LOQ
Pale Lager	386	no	Spain	4.8	7	6 - 8	1	25[1]	<LOQ
Pale Lager	388	no	Spain	5.4	22	20 -24	2	16	<LOQ
Pale Lager	399	no	Spain	5.5	36	32 - 39	4	9	<LOD
Pale Lager	488	no	Italy	4.7	71	64 - 76	6	51[1]	<LOD
Pale Lager	493	no	Italy	4.7	59	58 - 63	3	15	<LOD
Pale Lager	498	no	Italy	4.5	56	54 - 59	2	11	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, FB_1 (fumonisin B_1), FB_3 (fumonisin B_3)

Like the previous results, the cheapest pale lagers (from brewery #2) had the highest FB_1 (56 μg/L) and total FB contamination (69 μg/L). Beers from brewery #1, showed lower FB contaminations this time (14 and 17 μg/L respectively) at the sampling two years later. Besides FB_1, all beers contained FB_2 and FB_3 in this reassessment. With the FB concentrations found in our survey, the TDI is not easily exceeded. EFSA has set a group TDI of 2 μg/kg BW per day (sum of FB_1, FB_2 and FB_3) [61]. If we take into account an average body weight of 70 kilogram for an adult [62], then a person would need to drink more than 2 litres per day of the highest contaminated beer (69 μg/L) before reaching the TDI [61]. At that consumption level, alcohol intake is definitely a more serious risk. Nevertheless, daily exposure to FB_1 through beer should be avoided as much as possible, since consumers may be exposed to other dietary sources of FBs as well. FBs are of high toxicological concern, since they are possibly carcinogenic to humans (Group 2B) [59]. The incidence of human oesophageal cancer and the occurrence of *Fusarium verticillioides* (and its mycotoxins FBs), has been associated with regions where corn is produced and consumed as staple food [63]. Franceschi et al [64] reported significant associations, in males, between maize consumption and oral cancer in northern Italy. In a case-control study, 80% of the patients diagnosed with oesophageal cancer indicated to be regular consumers of African traditional beers. Based on these findings, Segal et al [65] concluded that the consumption of these African traditional beers was a major risk factor. However, the African traditional beers analysed in our survey, did not have that extreme FBs contaminations compared to those previously reported in literature [19,35].

3.4.6 Type B trichothecenes: DON, D3G, ADONs and NIV

The 6-plex MIA reports results for the sum of DON and D3G (S5 Tables). This is beneficial since previous research seems to indicate [66-69] that D3G is of toxic relevance. Recently a request was made to EFSA for a scientific opinion on the risks for animal and human health related to the presence of deoxynivalenol, its metabolites and masked deoxynivalenol (D3G) in food and feed [70]. Therefore, it is plausible that D3G will be added to the total DON group (of DON and its acetylated derivatives) for risk assessment. The

majority of the beers screened (60%) had contamination levels below 10 µg/L of DON+D3G, while beers with contaminations above 100 µg/L occurred less frequent (6%) (Fig 3).

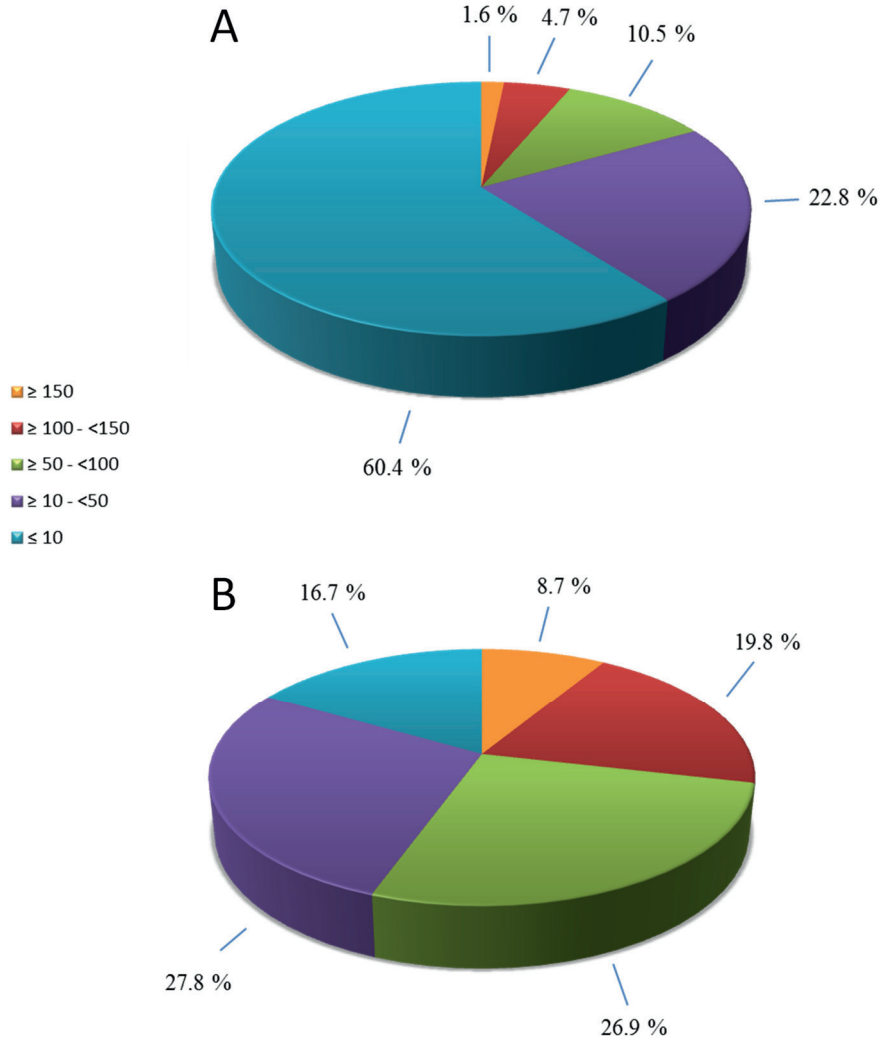

Figure 3. Occurrence of different DON+D3G contamination levels (µg/L) in all beer styles (A) and in the imperial stout beer style (B), based on the 6-plex screening results and the percentage of total beers

From the 406 beers that have DON+D3G contaminations above 10 µg/L, 73% were craft beers and these had a higher average contaminations (63 µg/L) compared to industrial produced beers (39 µg/L). The popular craft beer style imperial stout did not follow this trend. Only 17% of all imperial stouts screened had DON+D3G contamination levels below 10 µg/L, while 29% had DON+D3G contaminations above 100 µg/L (Fig 3). The highest overall DON+D3G contaminations were present in imperial stout, eisbock and stout (475, 308 and 169 µg/L, respectively) beers. The highest average group DON+D3G contaminations, based on beer style, were imperial stout, eisbock and African traditional with 86, 81 and 65 µg/L respectively, while the saison, pale lager and non/low alcohol beer styles had the lowest average contaminations (19, 23 and 23 µg/L, respectively) (S7 Table). The highest contamination incidences were found in eisbock, imperial stout and dark lager (83, 83 and 68%, respectively) (S7 Table), while the lowest contamination incidences were found in the sour ales, saison and pale lager beer styles (7, 8 and 13% respectively (S7 Table).

The screening results revealed a clear correlation between the alcohol content (%ABV) and the DON+D3G contamination (Fig 4).

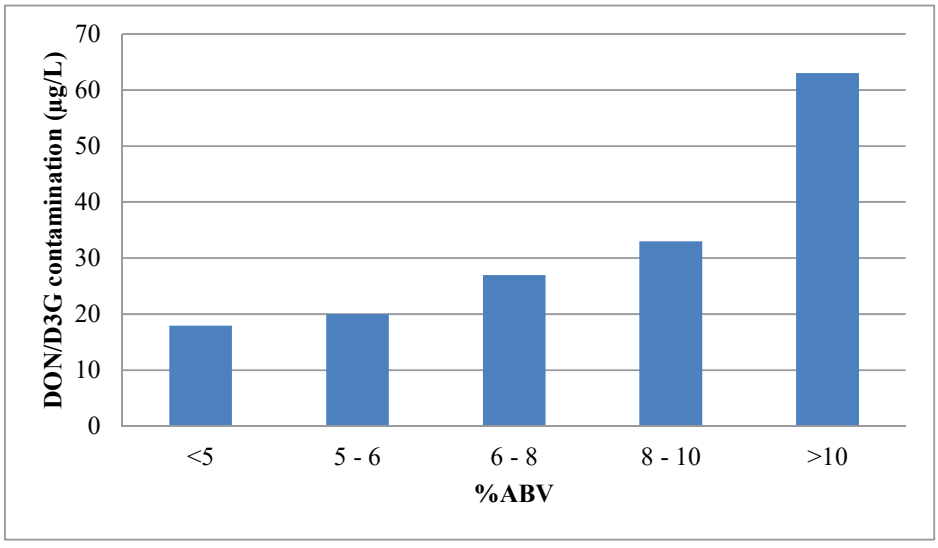

Figure 4. Correlation of DON+D3G contaminations (µg/L) relative to the %ABV based on the 6-plex screening results

The same positive correlation was reported in previous mycotoxin beer surveys [40,43,44]. For high %ABV beers, a higher input of grains is needed to deliver the fermentable sugars and with that comes a higher risk of mycotoxin contamination. LC-MS/MS data showed that, within the selected 100 beers for confirmation, 26 beers were contaminated with only DON above the corresponding LOD and 13 beers with only D3G. In contrast, in 38 beers both DON and D3G were detected. In 19 beers the concentration of D3G was higher than that of DON (Table 9). Varga et al [44] previously reported molar D3G/DON ratios (corrected for molecular mass) between 0.11 and 1.25 with an average of 0.56. In our survey, the molar D3G/DON ratios ranged from 0.10 to 2.60 with an average of 0.79. The highest ratio observed was for a pale lager from Poland. Generally, in this survey, beers having a D3G/DON ratio higher than 0.60, are almost all craft beers. DON was also present in African traditional beers, but in these beers no D3G contaminations were observed. The absence of D3G in these beers, may indicate that sorghum malt, often used in these traditional beer styles [71], does not have the potential to conjugate DON to D3G. Like the screening assay, LC-MS/MS analysis showed that beer sample 183 had the highest contamination for both DON and D3G with contaminations of 412 and 619 μg/L respectively. Since these concentrations were more than 2 times higher than the standard addition, we decided to reanalyze this particular beer sample following a 10 times dilution. Reanalysis showed that it contained 309 μg/L of DON and 535 μg/L of D3G. These concentrations reconfirmed that this imperial stout had the most extreme contamination in the entire survey. Recently, Piacentini et al [36] surveyed Brazilian craft beers using liquid chromatography with fluorescence detection and found beers with high DON contaminations (17 samples, range 127 – 501 μg/L). Unfortunately the surveyed beer styles were not further defined than ales and lagers. Previously, DON concentrations as high as 501 μg/L had only been reported in African traditional beers [34,45].

Table 9. Beer samples with confirmed trichothecene B contaminations (µg/L)

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)			
					Average	Range	SD	DON	D3G	ADONs	NIV
African Traditional	272	yes	South Africa	?	nd	nd	nd	10	<LOD	<LOD	<LOD
African Traditional	407	yes	South Africa	4	nd	nd	nd	16	<LOD	<LOD	<LOD
African Traditional	416	yes	South Africa	?	nd	nd	nd	68	<LOD	<LOD	<LOD
African Traditional	417	yes	South Africa	?	107	94 - 118	12	139	<LOD	<LOQ	<LOD
African Traditional	420	yes	South Africa	?	107	96 - 118	11	133	<LOD	<LOQ	9
African Traditional	421	yes	South Africa	?	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
African Traditional	423	yes	South Africa	?	10	4 - 22	10	10	<LOD	<LOD	<LOD
African Traditional	427	yes	South Africa	?	91	83 - 104	12	140	<LOD	<LOQ	9
African Traditional	428	yes	South Africa	?	43	36 - 51	8	99	<LOD	<LOQ	8
African Traditional	430	yes	South Africa	?	57	46 - 75	15	121	<LOD	<LOQ	<LOD
Bock	238	no	Poland	10	97	90 - 102	6	64	97	<LOD	<LOD
Bock	325	yes	Norway	8.5	53	47 - 59	6	40	23	<LOD	<LOD
Dark Lager	124	no	Czech Republic	3.8	106	102 - 112	5	41	68[1]	<LOD	<LOD
Dark Lager	132	no	Czech Republic	4.7	17	8 - 33	14	24	36	<LOD	<LOD
Double India Pale Ale	330	yes	Norway	10	78	73 - 80	4	67	48	<LOD	<LOD
Eisbock	351	yes	Belgium	39	41	36 - 48	6	32	32	<LOD	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON), NIV (nivalenol)

Table 9. Continued

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)			
					Average	Range	SD	DON	D3G	ADONs	NIV
Fruit/Vegetable/Spice	57	no	Germany	2.5	99	93 - 103	5	<LOQ	<LOD	<LOD	<LOD
Imperial Stout	11	yes	Norway	15.5	140	138 - 143	3	104	56[1]	<LOD	<LOD
Imperial Stout	183	yes	USA	15	475	429 - 516	45	412[2]	619[1]	<LOD	<LOD
Imperial Stout	259	yes	Norway	11	22	15 - 28	7	23	11	<LOD	<LOD
Imperial Stout	317	yes	Norway	9	62	60 - 66	3	116	69[1]	<LOD	<LOD
Imperial Stout	356	yes	Netherlands	11	45	34 - 55	11	28	15	<LOD	<LOD
Imperial Stout	581	yes	USA	9.6	167	166 - 169	2	146	88[2]	<LOD	<LOD
Imperial Stout	589	yes	USA	9.6	85	76 - 99	12	43	22	<LOD	<LOD
Imperial Stout	596	yes	USA	13	280	251 - 322	37	73	113[1]	<LOD	<LOD
Imperial Stout	607	yes	Denmark	10.9	152	131 - 170	20	14	27	<LOD	<LOD
Imperial Stout	631	yes	Norway	14	125	108 - 141	17	182	43	<LOD	<LOD
Imperial Stout	644	yes	Netherlands	11	21	16 -26	5	<LOD	13	<LOD	<LOD
Imperial Stout	674	yes	USA	10.5	56	53 - 61	4	26	61[1]	<LOD	<LOD
Imperial Stout	678	yes	Denmark	13	34	31 - 38	3	<LOD	20	<LOD	<LOD
Imperial Stout	748	yes	USA	11	101	97 - 105	4	32	45	<LOD	<LOD
Imperial Stout	764	yes	USA	11	21	17 - 25	3	<LOD	49	<LOD	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON), NIV (nivalenol)

Table 9. Continued

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)			
					Average	Range	SD	DON	D3G	ADONs	NIV
Imperial Stout	767	yes	USA	8	2	1 - 3	1	<LOD	9	<LOD	<LOD
Imperial Stout	771	no	Poland	8	21	19 - 23	2	<LOD	39	<LOD	<LOD
Imperial Stout	780	yes	Canada	8.5	54	45 - 81	8	38	81[1]	<LOD	<LOD
India Pale Ale	62	yes	USA	7.1	16	7 - 23	9	<LOQ	12	<LOD	<LOD
India Pale Ale	449	yes	USA	7.5	5	3 - 10	6	16	18	<LOD	<LOD
India Pale Ale	479	yes	Belgium	7	84	80 - 87	4	64	12	<LOD	<LOD
Non/Low Alcohol	121	no	Czech Republic	0	36	25 - 60	20	16	16	<LOD	<LOD
Pale Ale	54	yes	Belgium	8	4	1 - 9	4	<LOQ	<LOD	<LOD	<LOD
Pale Ale	97	yes	Netherlands	5.5	92	85 - 99	7	40	82[1]	<LOD	21
Pale Ale	176	yes	France	6.5	nd	nd	nd	<LOD	8	<LOD	<LOD
Pale Ale	217	no	Poland	5.7	26	18 - 32	7	20	6	<LOQ	<LOD
Pale Ale	380	yes	Norway	6	8	2 - 14	7	9	<LOD	<LOD	<LOD
Pale Lager	55	no	Germany	5.2	17	13 - 20	3	12	22	<LOD	<LOD
Pale Lager	208	no	Poland	5	31	28 - 36	4	15	20	<LOD	<LOD
Pale Lager	399	no	Spain	5.5	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
Pale Lager	488	no	Italy	4.7	nd	nd	nd	<LOD	9	<LOQ	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON), NIV (nivalenol)

Table 9. Continued

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)			
					Average	Range	SD	DON	D3G	ADONs	NIV
Pale Lager	493	no	Italy	4.7	nd	nd	nd	<LOD	6	<LOQ	<LOD
Pale Lager	698	no	Poland	5.2	70	65 - 74	5	13	53[1]	<LOD	<LOD
Smoked	369	yes	Netherlands	11	94	70 - 101	17	23	14	<LOD	<LOD
Sour Ale	119	yes	Belgium	5	nd	nd	nd	13	<LOD	<LOD	<LOD
Sour Ale	310	yes	Belgium	8	25	22 - 27	3	29	21	<LOD	<LOD
Sour Ale	343	yes	Belgium	6	nd	nd	nd	14	<LOD	<LOD	<LOD
Sour Ale	597	yes	Italy	9	14	4 - 40	22	<LOD	7	<LOD	<LOD
Stout	138	no	Czech Republic	10.5	49	31 - 70	19	<LOQ	42	<LOD	<LOD
Stout	160	yes	Sweden	7.5	135	116 - 157	21	26	30	<LOD	<LOD
Stout	647	yes	USA	6.4	41	28 - 50	11	30	<LOD	<LOD	<LOD
Stout	707	yes	Denmark	7	66	58 - 75	8	<LOD	52[1]	<LOD	<LOD
Strong Dark Ale	159	yes	Belgium	8	19	0 - 47	nd	10	18	<LOD	<LOD
Strong Dark Ale	189	no	Belgium	10	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
Strong Dark Ale	444	yes	Belgium	10.2	40	34 - 43	5	25	35	<LOD	<LOD
Strong Pale Ale	5	yes	Denmark	10	50	47 - 54	3	26	<LOD	<LOD	<LOD
Strong Pale Ale	17	yes	Denmark	12	79	53 -94	23	25	41	<LOD	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON), NIV (nivalenol)

Table 9. Continued

Combined Style	Sample No.	Craft	Country	%ABV	Screening 6-plex immunoassay (n=3)			Confirmatory analysis LC-MS/MS (n=1)			
					Average	Range	SD	DON	D3G	ADONs	NIV
Strong Pale Ale	18	yes	Netherlands	10.5	25	18 - 36	9	<LOD	21	<LOD	<LOD
Strong Pale Ale	84	no	Belgium	9	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
Strong Pale Ale	150	yes	USA	11.5	76	61 - 89	14	<LOD	25	<LOD	<LOD
Strong Pale Ale	353	yes	England	11	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
Strong Pale Ale	382	yes	Belgium	10	32	27 - 38	6	32	<LOD	<LOD	<LOD
Strong Pale Ale	508	yes	England	10.2	42	36 - 50	7	23	21	<LOD	<LOD
Strong Pale Ale	768	yes	Norway	10	7	4 - 11	3	<LOD	20	<LOD	<LOD
Strong Pale Lager	460	no	Austria	14	41	32 - 48	8	12	17	<LOD	<LOD
Wheat	113	no	Netherlands	5	36	29 - 44	7	<LOQ	<LOD	<LOD	<LOD
Wheat	228	no	Germany	5.6	3	2 - 4	2	<LOQ	<LOD	<LOD	<LOD
Wheat	454	no	Germany	5	17	7 - 26	9	10	<LOD	<LOD	<LOD
Wheat	540	no	Belgium	4.9	nd	nd	nd	<LOQ	<LOD	<LOD	<LOD
Wheat	550	no	Netherlands	5	26	12 - 36	13	<LOQ	4	<LOD	<LOD

n = number of replicates, %ABV = percentage alcohol by volume, nd = not detected, [1] Value is above the upper quantification limit, ? = %ABV unknown, LOD = limit of detection, LOQ = limit of quantification, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside) and ADONs (sum of 3-acetyl-DON and 15-acetyl-DON), NIV (nivalenol)

The LC-MS/MS method used was not able to distinguish between 3ADON and 15ADON (ADONs) and therefore the ADON contaminations found should be considered as the sum of both (Table 9). In 5 African traditional beers ADONs were detected and in 3 of those also NIV was present. All these mycotoxin levels were below the LOQ (10 μg/L for ADONs and 5 μg/L for NIV). ADONs were also detected in one pale ale (Poland) and 2 pale lagers (Italy), again all below the LOQ. In one Dutch pale ale, NIV was detected at a concentration of 21 μg/L. These results indicate that NIV and ADONs are not frequent contaminants of beers, with the exception of African traditional beers (5 out of 14). Previously, Kostelanska et al [43] found ADONs concentrations as high as 25 μg/L and described them as common contaminants present in 50% of 176 beers that were analysed. On the other hand, Bertuzzi et al [29] could not find any ADONs in 106 beer samples analysed while Varga et al [44] did not find any 3ADON in 374 beer samples surveyed.

Taking into account that the DON mAb used in the 6-plex MIA has 60% cross-reaction to D3G, we compared the 6-plex and the LC-MS/MS data. In 23 beers, both in 6-plex and LC-MS/MS, no DON+D3G was detected. From the 77 beers confirmed positive for DON and/or D3G (Table 9) by LC-MS/MS, 14 beers were negative in the 6-plex MIA. Concentrations for DON+D3G in these samples were generally low with the exception of African traditional beer sample 416 (68 μg/L) suggesting a beer specific interference in the immunoassay. For 27 beers, the 6-plex values for DON+D3G were below those found by LC-MS/MS analysis with an average factor of 0.7. For imperial stouts (9 beers) this factor was the same. For 32 beers, the 6-plex values for DON+D3G were above those found by LC-MS/MS. In average, the immunoassay values were a factor 2.1 higher. This value excludes beer 57, a fruit/beer mix, which showed a 24 times overestimation. After reanalysis, with both the 6-plex MIA and LC-MS/MS, this beer still showed the same overestimation. The addition of grapefruit juice to this beer seems to be responsible for high matrix interference in the 6-plex MIA. For the imperial stouts (9 beers) within the group of 32 beers, the average 6-plex values for DON+D3G were a factor 2.3 higher when compared to LC-MS/MS. This was mainly attributed to a few extremes (Table 9).

Imperial stouts show the highest DON+D3G contaminations in our survey, and since both USA and European imperial stouts were well presented, a geographical comparison was made. In total, 52 imperial stouts from the USA and 74 imperial stouts from Europe were screened. The mean DON+D3G contaminations (based on beers with contaminations higher than 10 μg/L) were 93 μg/L and 64 μg/L respectively. This suggests that USA imperial stouts have higher DON+D3G contaminations compared to European ones. This may be attributed to malt usage. Imperial stout is a high gravity style and is mostly pitch-black because of the specific taste-defining malts used. These malts are often, but not limited to, brown malt, caramel malt, chocolate malt and roast malt. This may suggest that these colored malts are responsible for the high DON+D3G contributions, since the strong pale lagers (e.g. barley wines) seem to suffer less of high DON+D3G contaminations compared to the imperial stouts. When comparing identical styles divided by lighter and darker colors (pale vs. dark) (S3 Fig), dark lagers clearly have higher DON+D3G contaminations compared to pale lagers. Dark ales tend to have higher contaminations (higher than 50 μg/L) compared to pale ales, but pale ales have more contaminations higher than 25 μg/L. Comparison of strong pale lagers and strong dark lagers shows minor differences. These comparisons show that for some beer styles higher contaminations can be associated to beer color. The DON+D3G contaminations in 27 beers from this survey, are equal to, or exceed the TDI of 1μg/kg BW for DON (140 μg/L for a person of 70 kg BW drinking one 0.5 L bottle of beer per day). Personal risk, related to exceeding the TDI for DON, based on the DON+D3G contaminations for selected beers in this survey, is presented in table 10. Additionally, the consumption of multiple bottles and the likely additional exposure via the daily diet (bread, pasta, breakfast cereals) further increases the risk. Unlike FBs, AFs and OTA, DON is not grouped as a (possible) carcinogenic mycotoxin. Its intake causes symptoms like vomiting, nausea, growth retardation, reproductive disorders and suppression of the immune system in humans and animals [72]. More recently DON is also believed to be active at the central nervous system level (brain) causing modified neurochemistry and neuronal activity [73].

Table 10. Personal risk based on the tolerable daily intake (TDI) and the DON+D3G contamination in selected beers

Body Weight (kg)	DON+D3G concentration (LC-MS/MS)											
	Dark lager (132)# Czech Republic 60 μg/L			Pale ale (97)# Netherlands 122 μg/L			Imperial stout (631)# Norway 225 μg/L			Imperial stout (183)# USA 1031 μg/L		
	0.33 L	0.5 L	1.0 L	0.33 L	0.5 L	1.0 L	0.33 L	0.5 L	1.0 L	0.33 L	0.5 L	1.0 L
50	-	-	+	-	+	+	+	+	+	+	+	+
70*	-	-	-	-	-	+	+	+	+	+	+	+
100	-	-	-	-	-	+	-	+	+	+	+	+

- below or equals TDI, + above TDI, # Beers from the survey with DON+D3G contaminations as determined by LC-MS/MS * default average BW (body weight) set by EFSA for the European adult population, DON (deoxynivalenol), D3G (deoxynivalenol-3-β-D-glucopyranoside)

Conclusion

To our best knowledge, this survey is the largest ever performed for the occurrence of mycotoxins in beer. It is for certain the most extensive screening for mycotoxins in craft beers to date. The applied mycotoxin 6-plex screening method facilitated fast and easy screening of 1000 global beer samples, whilst the developed beer-dedicated LC-MS/MS method proved to be very useful for quantitative confirmatory analysis. The effectiveness of the 6-plex mycotoxin immunoassay screening approach, without any sample clean-up, was hampered by matrix interferences. This occurred particularly at low concentrations and certain beer styles. It caused false suspect samples for AFB1, FBs, T-2/HT-2 mainly in dark beer styles. For type B trichothecenes the chosen approach lead to over- and underestimations, particularly in a few imperial stouts. Therefore, a blank reference beer for imperial stouts is desired in the 6-plex assay. It will help to improve mycotoxin determination in this complex beer style. But still, even then large matrix background variations can be expected. Older recipes just contain malts while newer recipes show the addition of coffee, cacao and other adjuncts. For a further reduction of over- and underestimations, a suitable clean-up procedure may be considered for future 6-plex screening of mycotoxins in beer. Additionally, averaging data from replicates in the LC-MS/MS standard addition method may lead to a better quantitative comparison. Furthermore, the use of logarithmic dose-response curves in the 6-plex immunoassay, compared to a narrow linear range for the standard additions used in LC-MS/MS, will always contribute to a less accurate quantification in the 6-plex immunoassay.

Until now, there are no Maximum Levels set for the occurrence of mycotoxins in beer. We agree with the conclusion drawn previously by Varga et al [44] about their extensive DON survey in beer, stating that setting maximum levels for DON and its metabolites in beer helps to protect beer drinkers from consuming highly contaminated beers. Based on beer samples in the presented survey, exceeding the TDI for DON, future research should additionally focus on malts and/or grains used for high gravity beer styles like imperial stout. It seems that the darker malts and/or roasted malts in imperial stouts, combined with the high gravity, contribute to the high DON+D3G

levels. Color comparison of similar styles, with pale and dark varieties, partially supports this hypothesis. Current malt certificates are often lacking information, therefore, it is suggested that malts used in high gravity beer styles should be analysed in more detail, especially for DON+D3G. With that detailed information brewers should be able to judge until what gravity the malts are safe to use. It is proposed that stricter maximum mycotoxin levels, or better specified levels, are applied for malts that are used for brewing high gravity beers. Furthermore, small craft breweries should consider the implementation of cheap, reliable, easy and fast on-site mycotoxin assays to control the purchased malts and adjuncts as well as their final products. In the end, on-site mycotoxin testing may not always be feasible for small starting breweries. Therefore they should be able to rely on the proper control of the purchased malts. With the craft beer market consistently expanding, and with many craft breweries producing imperial stouts, quality control management seems a necessary step.

Acknowledgments

The authors would like to thank Diana Chan, previously a student of Nottingham Trent University, for her contribution to the beer screening. Ron Hoogenboom of RIKILT Wageningen University & Research (Institute of Food Safety) and Martien Spanjer of the Netherlands Food and Consumer Product Safety Authority (NVWA) for critical reading of the manuscript. Menno Olivier of Brouwerij de Molen for constructive discussions regarding craft brewing and the supply of relevant information. Milena Zachariasova of the Institute of Chemical Technology, Prague for sending a selection of Czech beer samples.

Chapter 6: Supplementary material

All supplementary material can be found free of charge at:

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0185887#sec020

S1 Text. LC-MS/MS conditions.

S1 Fig. Multi-mycotoxin dose-response curves in dark ale.

S2 Fig. Global beer samples surveyed by region. European beer samples surveyed by country.

S3 Fig. Comparison of DON+D3G contaminations in similar beer styles.

S1 Table. Mycotoxin specific MS/MS settings and standard addition levels.

S2 Table. Intra- and interday precision of the 6-plex assay.

S3 Table. Intra- and interday IC50s based on multi-mycotoxin dose-response curves.

S4 Table. Intra- and interday determinations of mycotoxin concentrations using the 6-plex assay.

S5 Tables. Total 6-plex immunoassay screening data for A) African traditional beers B) bock beers C) dark ale beers D) dark lager beers E) double India pale ale beers F) eisbock beers G) fruit/vegetable/spice beers H) imperial stout beers I) India pale ale beers J) non/low alcohol beers K) pale ale beers L) pale lager beers M) saison beers N) smoked beers O) sour ale beers P) stout beers Q) strong dark ale beers R) strong pale ale beers S) strong pale lager T) wheat beers.

S6 Table. Reassessment of Spanish breweries for FB contaminations by LC-MS/MS analysis.

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CHAPTER

Controlled production of zearalenoneglucopyranoside standards with *Cunninghamella* strains using sulphate depleted media

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Adapted from:

Controlled Production of Zearalenone-Glucopyranoside Standards with Cunninghamella Strains Using Sulphate-Depleted Media. Toxins 2021, 13, 366.

Abstract

In recent years, conjugated mycotoxins have gained increasing interest in food safety, as their hydrolysis in human and animal intestines leads to an increase in toxicity. For the production of zearalenone (ZEN) glycosides reference standards, we applied *Cunninghamella elegans* and *Cunninghamella echinulata* fungal strains. A sulphate depleted medium was designed, for the preferred production of ZEN glycosides. Both *Cunninghamella* strains were able to produce zearalenone-14-β-D-glucopyranoside (Z14G), zearalenone-16-β-D-glucopyranoside (Z16G) and zearalenone-14-sulphate (Z14S). In a rich medium *Cunninghamella elegans* preferably produced Z14S, while *Cunninghamella echinulata* preferably produced Z14G. In the sulphate depleted medium a dramatic change was observed for *Cunninghamella elegans*, showing preferred production of Z14G and Z16G. From 2 mg of ZEN in sulphate-depleted medium, 1.94 mg of Z14G and 0.45 mg of Z16G were produced. Following preparative Liquid Chromatography-Mass Spectrometry (LC-MS) purification, both fractions were submitted to ^{1}H and ^{13}C NMR and High-Resolution Mass Spectrometry (HRMS). These analyses confirmed that the purified fractions were indeed Z14G and Z16G. In conclusion, the presented research shows that a single *Cunninghamella* strain can be an effective and efficient tool for the controlled biotransformation of ZEN glycosides and other ZEN metabolites. Additionally, the biotransformation method was extended to zearalanone, β-zearalenol and other mycotoxins.

1. Introduction

Zearalenone (ZEN) is a non-steroidal oestrogenic mycotoxin produced by *Fusarium* spp. It occurs in grain commodities, and can cause reproductive disorders in farm animals and lead to hyperoestrogenic syndromes in humans [1]. Because of its toxicity, the European Commission established a tolerable daily intake (TDI) for zearalenone of 0.25 μg/kg of body weight [2]. In 2016, the CONTAM panel of EFSA expanded this TDI to a group health-based guidance TDI value of 0.25 μg per kg of body weight for ZEN and all of its phase I and phase II metabolites. Additionally, potency factors relating to the oestrogenic activity of the metabolites were assigned to all the ZEN metabolites [3]. The main occurring ZEN metabolites include α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) [4,5]. Conjugated mycotoxins, often referred to as masked mycotoxins, are biologically modified phase II metabolites produced by plant biotransformations as a detoxification process [6]. Well-known ZEN plant conjugates are zearalenone-14-β-D-glucopyranoside (Z14G), zearalenone-16-β-D-glucopyranoside (Z16G), α-zearalenol-14-β-D-glucopyranoside (α-ZELG), β-zearalenol-14-β-D-glucopyranoside (β-ZELG) and zearalenone-14-sulphate (Z14S) [7,8]. The occurrence of Z14G in wheat was reported by Schneweis et al. [9] when they analysed 24 Bavarian wheat samples. In total 10 samples contained Z14G with concentrations ranging from 17 to 104 μg/kg. In their survey of cereal-based foods, De Boevre et al. [10] found the conjugated mycotoxins Z14G, Z14S, α-ZELG and β-ZELG with maximum concentrations of respectively 369, 45, 192 and 206 μg/kg. Streit et al. [11] analysed 139 feed samples of which 49% contained Z14S. Nathanail et al. [12] analysed different commodities of Finnish cereal grains and detected Z14G, Z16G, α-ZELG, β-ZELG and Z14S in oats, with the highest concentrations being respectively 9.6, 15.1, 5.1, 0.7 and 220 μg/kg. In processed food, Peters et al. [13] detected the presence of Z14S in beers, with concentrations ranging from 0.5 to 0.7 μg/L. Borzekowkski et al. [14] showed that some tempeh products, acquired from Indonesian markets, contained ZEN, α-ZEL and Z14S.

Conjugated mycotoxins can be hydrolysed into their free forms leading to increased toxicity [6]. Already in 1990, Gareis et al. [15] showed that, when Z14G was fed to pigs, only ZEN and α-ZEL were found back in the urine and faeces, indicating hydrolysis of Z14G. Additional research indicated the hydrolysis of Z14S, Z14G and Z16G when fed to pigs [16,17]. Dellaflora et al.[18] showed that Z14G was hydrolysed to ZEN in bovine blood, while Versilovskis et al. [19] discovered that Z14G fed to rats, was successfully hydrolysed to ZEN. Kovalsky et al. [20] showed that Z16G was hydrolysed to ZEN using human faecal slurry. These experiments indicate that the presence of these conjugated forms of ZEN lead to additional toxicity and therefore should be detected along with the unconjugated toxins.

To be able to monitor the presence of conjugated mycotoxin forms in food and feed products, enzymatic deconjugation methods or available reference standards are essential. Biotransformation of ZEN with plants and microorganisms has been successfully applied to produce secondary metabolites. Berthiller et al. [21] spiked *Arabidopsis thaliana* plant seedlings with ZEN and found 17 different ZEN conjugates, including glucosides. Next, the responsible *Arabidopsis thaliana* UDP-glycosyl transferase gene was expressed in *Saccharomyces cerevisiae* and this was used for the direct biotransformation of ZEN yielding Z14G. Part of the produced Z14G was then efficiently chemically modified to α-ZELG and β-ZELG [22,23] . Using a cloned UDP-glycosyl transferase gene from barley, expressed in yeast, Kovalsky et al. [20] were able to produce Z14G and Z16G. Alternatively, root and leaf cultures, made from two durum wheat varieties, selectively produced a wide variety of putative conjugated ZEN metabolites, including malonyl, sulphate, glucoside, maltoside and other di-glycoside forms [24,25].

There is a wide variety of fungal species, and among them, species with unique biochemical pathways. With these biochemical pathways, they are able to produce a wide variety of phase I and phase II metabolites. This ranges from important pharmaceuticals (e.g. antibiotics) to natural toxins (e.g. mycotoxins) [26,27]. These biochemical pathways are also an effective tool to metabolize chemical compounds that are administered to the fungus. Coupling of sugars, or sulphates, to the chemical compound will increase its

polarity and makes it easier for the fungus to secrete the conjugated compound into the culture medium [28]. *Cunninghamella* and *Rhizopus* are families of filamentous fungi occurring in soil and plants, and are well studied in in-vitro biotransformation models. Some observed biotransformations include hydroxylation, glycosylation, oxidation, demethylation, sulfoxidation and epoxidation [29,30]. These fungi have been successfully applied to a wide range of compounds. Applying several strains of the *Rhizopus* family, Brodell et al. [31] and Borzekowski et al. [32] showed that these strains were able to successfully bio-transform ZEN into Z14S, Z14G, Z16G and α-zearalenol-sulfate, and that each had its own preferred pathway as shown by the produced metabolites. El-Sharkawy et al. [33,34] applied liquid cultures of the *Cunninghamella bainieri* and *Thamnidium elegans* strains to metabolize ZEN. Besides α-ZEL, β-ZEL and β-zearalanol (β-ZAL), the metabolites Z14S and Z14G were produced.

Aiming for the efficient and selective production of ZEN glycosides, two *Cunninghamella* strains, namely *Cunninghamella echinulata var. elegans (C. elegans)* and *Cunninghamella echinulata var. echinulata (C. echinulata)* were ordered based on their ability to glycosylate compounds as reported previously [33,35,36]. With the specific aim of steering the biotransformations to the preferred target glucose-conjugated ZEN metabolites, we applied sulphate depleted media to avoid excess sulphate metabolite production. Even though comprehensive optimization studies were not undertaken yet, besides prolonged exposure of the fungi to ZEN, we were able to effectively produce the biologically modified [37] Z14G and Z16G. The successful controlled production of ZEN-glycosides, was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), both 1D and 2D, and high resolution mass spectrometry (HRMS) analysis. In addition, the functionality of the controlled glycosylation directed biotransformation by the selected *Cunninghamella* strains was also tested for ZAN, β-ZEL and a range of other common mycotoxins.

2. Results and Discussion

2.1 Small scale biotransformation of ZEN

For initial experiments, we adopted a protocol applied in quercetin biotransformation [35]. Using non-optimized small scale cultures of *C. elegans* in potato dextrose broth (PDB) medium, 25 ug/mL ZEN was added to a 3-day culture and incubated for 144 hours. Supernatant samples were analysed using HRMS. This experiment revealed that *C. elegans* was also capable of metabolising ZEN. Metabolites formed included the desired Z14G and Z16G conjugates, as well as Z14S but the parent compound ZEN was not completely metabolized (Fig. 1A and 1B).

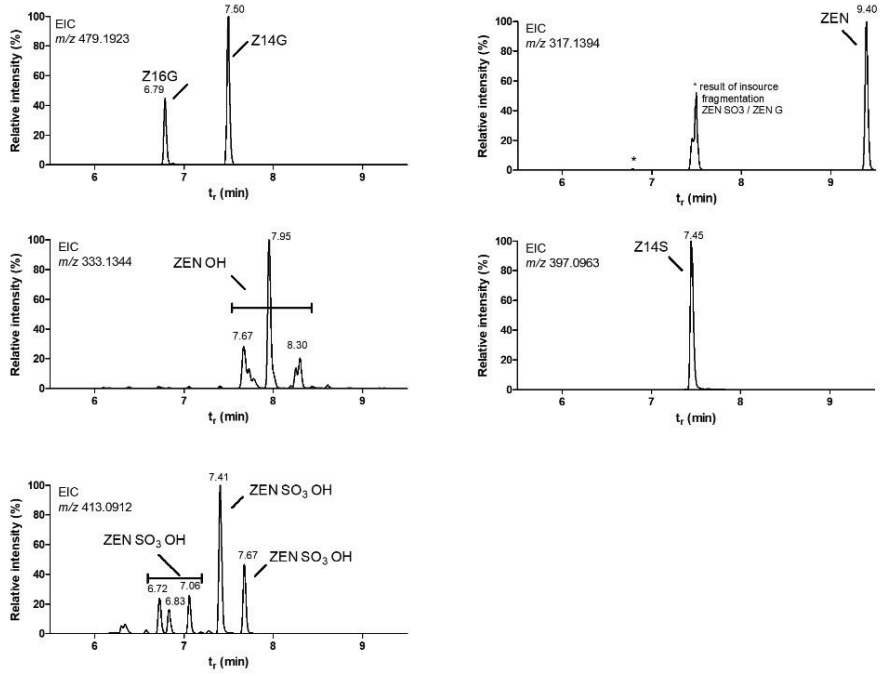


Figure 1A. Formation of various ZEN metabolites by Cunninghamella elegans. Tentative identified with LC-HRMS based on the exact mass of both the precursor ions and specific fragments (*m*/*z* 131.0505 and 175.0403). Extracted ion chromatograms of the precursor ions are shown.

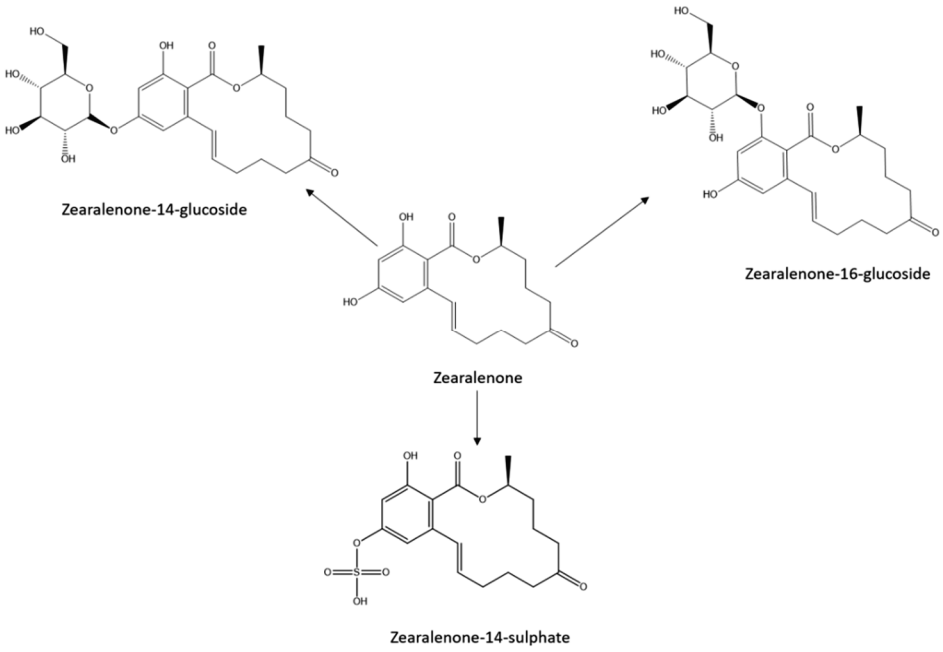


Figure 1B. Chemical structures of ZEN and the main phase II metabolites produced by *Cunninghamella elegans*

Next, a dedicated LC-MS/MS method was set up using reference and purified standards of ZEN, Z14G, Z16G and Z14S (Fig. S15). The retention times and MS/MS characteristics of each molecule were determined. This allowed the selective quantification of each target conjugate during analysis. Because of identical product ion masses, LC retention time was crucial for the identification of the Z14G and Z16G metabolites. Separation of Z14G and Z14S, not realized in the initial HRMS runs, was satisfactory. With the biotransformation incubation times adjusted to 336 hours, and the ZEN concentration adjusted to 5 μg/mL, biotransformation was more optimal and *C. elegans* seemed to remain viable, as visually observed by continued growth of the culture. Next, fungal cultures of *C. elegans* and *C. echinulata* were fortified with ZEN in PDB and in the modified Czapek-Dox (MCD) sulphate depleted media. With the depletion of sulphates in the MCD medium, we intended to direct the biotransformation towards the glycosylated metabolites

while minimizing the Z14S production. Fig. 2 shows the LC-MS/MS chromatograms of the respective supernatants of those biotransformations.

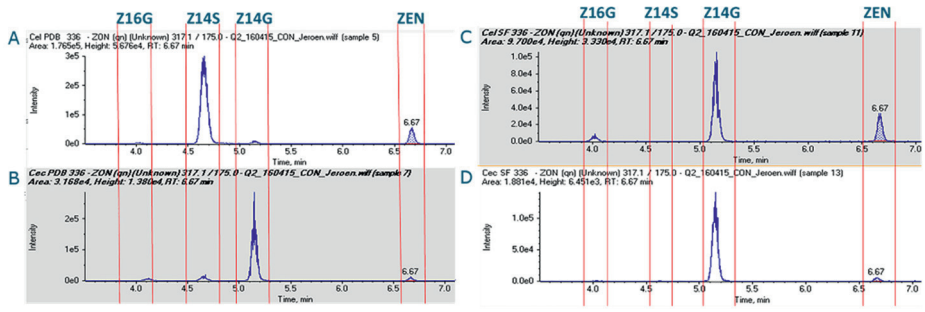


Figure 2. LC-MS/MS chromatograms (*m*/*z* 317.1 →175.0) for the production of Z14G, Z16G, and Z14S metabolites by *Cunninghamella elegans* in PDB medium (A) and synthetic depletion medium (C) and *Cunninghamella echinulata* in PDB medium (B) and synthetic depletion medium (D)

As each conjugate contains ZEN, the *m*/*z* 175.0 product ions will appear in the chromatograms at their respective retention times. Focussing on the rich PDB medium, *C. elegans* predominantly produces Z14S, with only minor traces of Z14G and Z16G. *C. echinulata* predominantly converts ZEN into Z14G, whilst also producing small amounts of Z16G and Z14S. The *C. elegans* and *C. echinulata* cultures that were washed and then transferred to the minimal sulphate MCD media showed interesting differences in the biotransformation of ZEN when compared to the PDB cultures. Most prominent is the shift from the production of Z14S by *C. elegans* in PDB to the production of Z14G and Z16G. Moreover, no significant amount of Z14S is produced. The *C. echinulata* strain mainly produces Z14G in the MCD medium. This shift is less dramatic when compared to *C. elegans*. Based on these results we decided to continue the production of ZEN glycosides using the *C. elegans* strain and the MCD medium.

2.2 Upscaled production of ZEN-glycosides

To increase the production, *C. elegans* cultures were upscaled 40 times, where 2 mg of ZEN was introduced from a stock solution into 400 mL of MCD in a 2 litre flask. For efficiency comparison, another 2 litre flask containing 400 mL of PDB was also fortified with 2 mg of ZEN. After 2 weeks incubation,

supernatants were analysed by LC-MS/MS using standard curves in MCD and PDB media for calibration. Based on these standard curves it was calculated that from 2 mg ZEN, 1.96 mg of Z14G and 0.45 mg Z16G was produced in MCD medium. On a molar ratio this meant that 65% of ZEN was metabolized to Z14G, while 14% ZEN was metabolized to Z16G. In PDB medium, those efficiencies were only 3% for the production of Z14G and 2% for Z16G (Table 1).

Table 1. Calculated efficiencies for ZEN-glycosides production from 2 mg of ZEN in potato dextrose (PDB) and sulphate depleted growth medium (MCD) (n.d. = not determined)

		Potato dextrose broth medium (PDB)			Sulphate depleted medium (MCD)		
Compound	MW	Amount (μg)	Amount (μM)	Conversion (%)	Amount (μg)	Amount (μM)	Conversion (%)
ZEN	318.4	2000	6.28	n.d.	2000	6.28	n.d.
Z14G	480.1	80	0.167	3	1960	4.08	65
Z16G	480.1	56	0.117	2	440	0.92	15

The calculated recoveries only refer to the supernatants of the cultures, as extraction from mycelia was not considered. The large-scale experiment was then repeated in two 2-liter Erlenmeyer flasks, with each 2 mg of ZEN spiked in 400 mL of MCD in order to generate sufficient metabolites for the subsequent steps. While the efficiency of Z14G production was comparable to the research of Borzekowski et al. [32], the Z16G production was a bit less efficient. However, in the current research, only a single fungal strain was sufficient to selectively produce two different ZEN-glycosides. This single *C. elegans* strain could also predominantly produce Z14S by using a rich growth medium.

2.3 NMR and HRMS analysis of purified Z14G and Z16G fractions

Prior to NMR analysis, the produced ZEN glycosides were purified by preparative LC-MS after liquid-liquid extraction by an external propriety method. Purified Z14G and Z16G fractions were collected over several runs and subsequently pooled and freeze-dried. Next, the freeze-dried Z14G and Z16G fractions were each dissolved in 2 mL of 50:50 v/v ACN/H_2O. All NMR spectra and corresponding data are deposited in the Supplementary Materials. The ZEN chemical structure numbering is shown in Fig. S1. The ^{1}H, ^{13}C and DEPT NMR spectra of ZEN are shown in Fig. S2-S4. 2D-NMR techniques provided the identity of each peak (Fig. S5). The ^{1}H and ^{13}C NMR spectra for Z14G are shown in Fig. S6 and S7. The ^{13}C spectrum is similar to ZEN, except for the addition of the glucose peaks between 100 and 60 ppm, surrounding the peak of C2 at 73 ppm. Due to the similarities in the spectra between ZEN and Z14G, assigning the peaks was easy, and the clear coupling between C14 and C19 seen on the HMBC confirmed the compound as Z14G, see Fig. S8 and S9. Just like in ZEN, C14 and C16 could be identified by whether they only coupled to the hydrogen at C15, or also to the one at C13. For Z16G, the ^{1}H NMR spectrum (Fig. S11) had more overlapping peaks than those for ZEN and Z14G, and the ^{13}C NMR spectrum had a wavy baseline due to the small amount of material (Fig. S11). The peaks for the alkene hydrogens were different from Z14G, both having a chemical shift near 6.2 ppm, instead of C11 having a much higher chemical shift as in ZEN and Z14G. The peak in the ^{1}H NMR for the hydrogen on the anomeric glucose carbon, C19, was hidden under the solvent peak at 3.3 ppm. A small shoulder can be seen to the left of the peak, as shown in Fig. S12, and the identity of this as a compound peak was shown by the HSQC coupling between the C19 peak (at 102.8 ppm on the ^{13}C NMR spectrum (Fig. S11) and the shoulder (Fig. S13). The key HMBC and COSY couplings are shown in Fig. S13 and S14. Further data specification can be found in tables S1 – S6. Additionally, the purified standards were also submitted to LC-HRMS to obtain high resolution MS and fragmentation spectra. These spectra are shown in Fig. S15 and S16 of the supplementary information. Besides the deprotonated molecular ion, both

Z14G and Z16G produced a formic acid adduct. For the fragmentation spectra, these formic acid adducts were isolated and fragmented. From the MS spectra it became clear that Z14G also showed intense radical anions. The obtained *m*/*z* values for all deprotonated ions, adducts and fragments were within a 5 ppm mass error.

2.4 Feasibility of biotransformation for other ZEN metabolites

In a short feasibility study, ZAN and β-ZEL were spiked independently to both *Cunninghamella* cultures, in PDB and MCD media, at a concentration of 5 μg/mL. The fortified cultures were incubated for 2 weeks as described previously and the supernatants were analysed using a standard HRMS approach with ZAN, β-ZEL and β-ZELG as available reference standards. Tables S7 and S8 show the ions of the putative ZAN and β-ZEL metabolites formed based on HRMS analysis. Results indicate that ZAN is metabolized to zearalanone-sulphate and two forms of zearalanone-glucoside (Fig. 3A and B), while β-ZEL shows three possible glucoside metabolites and a sulphate metabolite (Fig. 4A and B). This suggests that besides β-ZELG (standard was available, glucose moiety at position 14) also β-zearalenol-16-glucoside and β-zearalenol-7-glucoside are formed. In both biotransformations, only one putative sulphate conjugated metabolite is observed. Based on previous research [32], it is presumable that the ZAN and β-ZEL metabolites have the sulphate moiety attached on position 14. However, upscaled production of all metabolites produced, followed by NMR analysis is necessary to confirm this.

2.5 Feasibility of biotransformation for entirely different mycotoxins

In additional exploratory experiments, using the same approach as for ZEN and its metabolites, the *Cunninghamella* biotransformation strategy was applied to the mycotoxins deoxynivalenol (DON), aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), T2-toxin (T-2) and ochratoxin A (OTA). For DON, AFB_1, FB_1 no obvious biotransformations were observed at the conditions

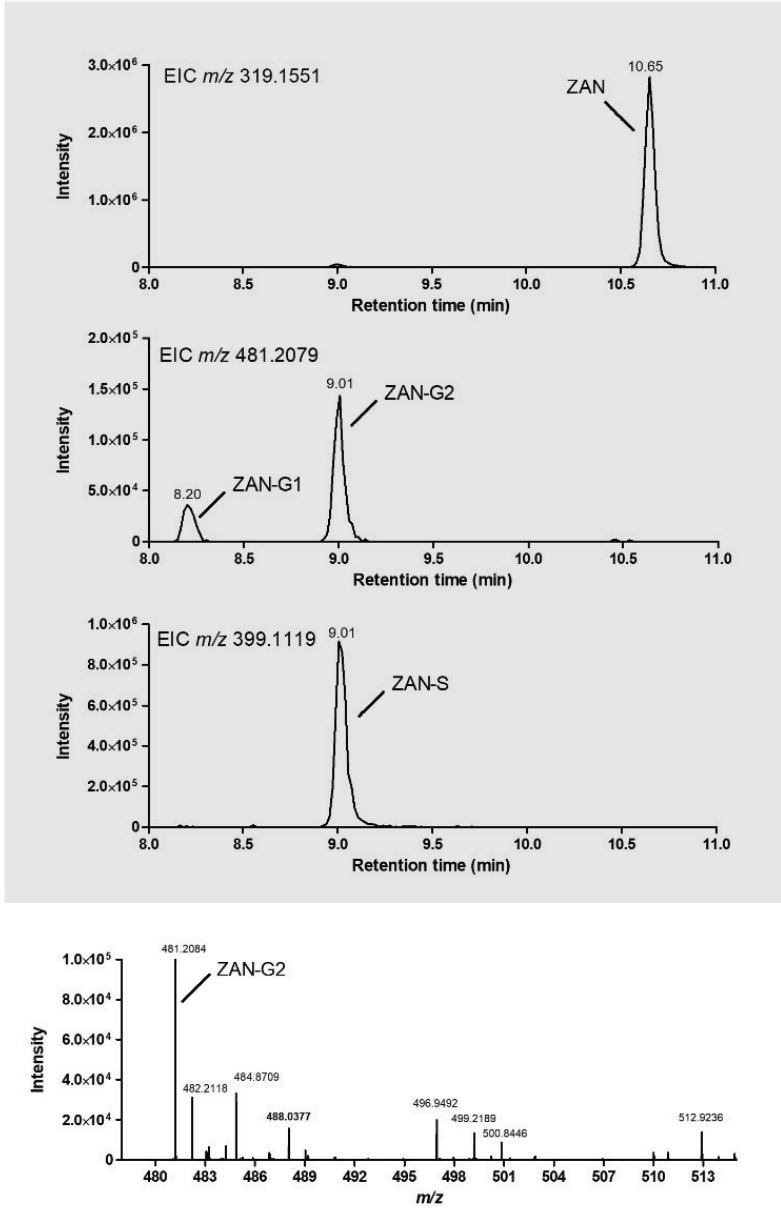


Figure 3A. LC-HRMS analysis of ZAN biotransformation by *Cunninghamella elegans* in PDB

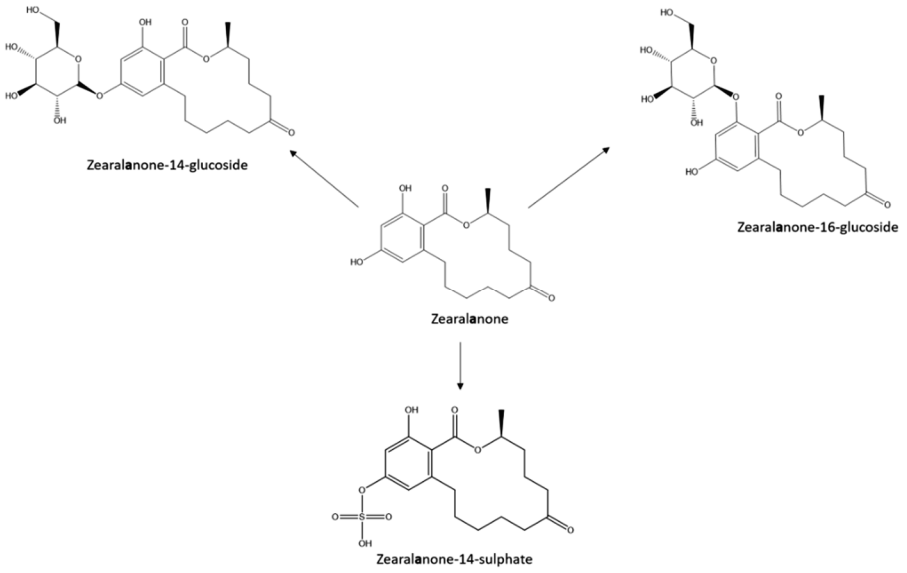
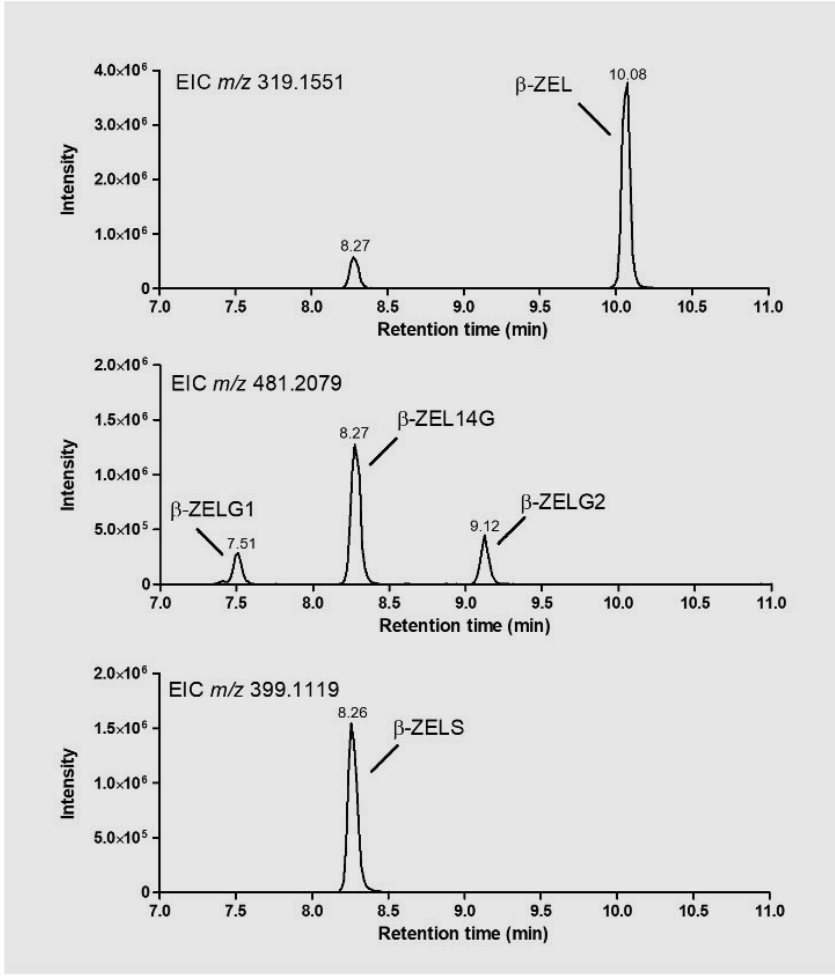


Figure 3B. Chemical structures of ZAN and the putative sulphate and glucose phase II metabolites produced by *Cunninghamella elegans*

previously used for ZEN. No predicted conjugates (e.g. hydroxy, sulphate, glucose) were found in HRMS analysis; moreover, no decrease in mass balance was observed versus the initial concentrations of the fortified mycotoxins. McCormick et al. [38] previously reported the formation of T2-glycosides by certain yeast strains, but both *Cunninghamella* strains applied in our research were not able to produce these target glycosides. However, upon addition of T-2, we did observe two other biotransformations: to HT2-toxin and to hydroxy-T2 Toxin. The degradation of OTA by *Rhizopus* strains was previously reported [39]. Formation of OTA-glycosides by plant cell suspension cultures was also previously reported [40,41]. *C. elegans* very effectively transformed OTA to hydroxy-OTA within 96 hrs after fortification, but no phase II metabolites were observed, probably because the new OH group at the lactone ring is sterically hindered while the phenolic OH moiety is involved in hydrogen bonds with the adjacent carbonyl groups.



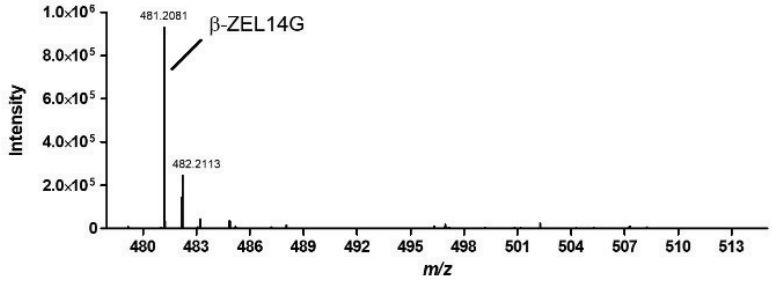


Figure 4A. LC-HRMS analysis of β-ZEL biotransformation by *Cunninghamella echinulata* in PDB

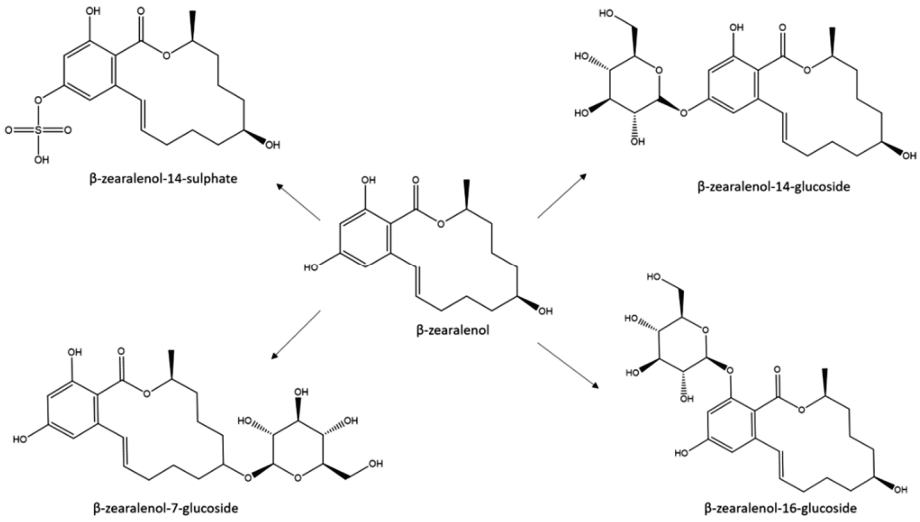


Figure 4B. Chemical structures of β-ZEL and the main phase II metabolites (and putative metabolites) produced by *Cunninghamella elegans*

3. Conclusion

The implementation of sulphate depleted media in *Cunninghamella* based biotransformation of ZEN, proved to be a successful method for steering the reaction towards the preferred abundant production of ZEN-glycosides. Although in general the transformation efficiencies were satisfying, ZEN was not fully metabolized by the fungal culture and still present in the supernatant. To further optimize future production, the implementation of larger culture volumes in dedicated bio processors, tweaking temperatures and especially aeration [42], while fortifying at lower ZEN concentrations may lead to more optimal production of ZEN glycosides. Additionally, the fungal matter could also be extracted to further increase biotransformation efficiency. Besides the effective glycoside production, it is at the same time an effective tool to produce the Z14S metabolite when using the standard PDB growth medium in combination with *C. elegans*. The HRMS run of a sub-optimal biotransformation (Fig. 1a), revealed several phase I and phase II ZEN metabolites, including ZEN-hydroxy's. These most likely are α-ZEL and β-

ZEL. Subsequent phase II metabolism may turn these into α-ZELG and β-ZELG, as shown by the formation of 3 glucose metabolites from β-ZEL in preliminary experiments. However, based on the mass balance, it seems that Z14S, Z14G and to a lesser extent Z16G are the conjugates that are preferably produced under the conditions investigated. Besides transformation efficiency optimization, future research could also focus on the implementation of a wider range of *Cunninghamella* strains and determine their preferred pathways in rich and selective growth media.

The developed method is easy implementable, does not need extensive microbiological experience and does not have complex work schemes. It may be a useful tool for production of metabolites, in case novel, or emerging toxin metabolites are discovered and reference standards are not commercially or scientifically available.

4. Materials and methods

4.1 Instrumentation

Fungal culture streaks were grown in a temperature controlled incubator (Van Tol laboratorium techniek, Kerkdriel, The Netherlands). All sizes of liquid fungal cultures, were grown in a Innova 44 rotary shaker (New Brunswick Scientific, Edison, USA) and centrifuged in an Eppendorf 5810R centrifuge (Eppendorf, Nijmegen, The Netherlands) equipped with an A-4-62 swinging bucket rotor. The formation of ZEN conjugates was monitored on an AB Sciex (Nieuwerkerk a/d IJssel, the Netherlands) QTRAP 6500 tandem mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source, operated in negative ion multiple reaction monitoring (MRM) mode. The MS system was coupled to a Shimadzu ('s Hertogenbosch, the Netherlands) Prominence Liquid Chromatography (LC) system, equipped with a Restek (Interscience, Breda, the Netherlands) Ultra Aqueous C18 (100×2.1 mm) column. Integration of reconstructed MRM chromatograms was done with MultiQuant V2.0 software using the Signal Finder integration

algorithm (AB Sciex). Produced ZEN-glycosides were purified according to a propriety method of the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). NMR measurements for ZEN and Z14G were performed using a Bruker Avance III 400MHz NMR spectrometer, recorded by Topspin software at 25 °C against internal standard TMS at 0.00 ppm. For Z16G, the ^{1}H and ^{13}C NMR spectra were recorded at a probe temperature of 300 K on a Bruker Avance-III-600 spectrometer, equipped with a cryo-probe located at MAGNEFY (MAGNEtic resonance research FacilitY, Wageningen, The Netherlands). 1D and 2D COSY, HMBC, and HMQC spectra were acquired using standard pulse sequences delivered by Bruker. For the HRMS experiments of the produced ZEN-glycosides a Q-Exactive Orbitrap mass spectrometer equipped with a HESI-II electrospray source was used (Thermo Scientific, San Jose, CA, USA). The HRMS system was coupled to an Ultimate 3000 UHPLC LC system (Thermo Scientific, San Jose, CA, USA) equipped with a 100 × 3 mm ID, 3 μm Atlantis T3 analytical column (Waters, Milford, MA, USA). Extracted ion chromatograms were constructed with the Thermo Scientific Xcalibur software. GraphPad Prism 4 was used for building graphics where possible.

4.2 Materials

Fungal strains *Cunninghamella echinulata var. elegans* (ATCC 9245, deposited name *Cunninghamella blakesleeana* Lendner) and *Cunninghamella echinulata var. echinulata* (ATCC 9244, deposited name *Cunninghamella bainieri* Naumov) were ordered from LGC (Wesel, Germany) and BCCM (Brussels, Belgium). Potato dextrose broth (PDB), potato dextrose agar (PDA) and 2 litre Erlenmeyer flasks for growing large scale cultures, were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Fungi were plated in petri dishes and small scale cultures were grown in disposable 50 ml tubes, both from Greiner (Alphen a/d Rijn, the Netherlands). ZEN, ZAN and β-ZEL in solid form were purchased from Fermentek (Jerusalem, Israel). Z14G and Z16G standards were kindly provided by Dr Franz Berthiller (IFA Tulln, Austria) while the Z14S standard was kindly provided by Dr Matthias Koch (BAM, Germany). Syringeless filter devices (Mini-UniPrep, PTFE) were purchased from GE Healthcare (Rotterdam, the Netherlands). Acetonitrile

(ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, the Netherlands), acetic acid, formic acid and ammonium formate from Merck (Amsterdam, the Netherlands) and ammonium acetate from Fluka Analytical (Steinheim, Germany). For the HRMS experiments, ACN, MeOH and water, all of UHPLC-MS purity grade were purchased from Merck. All other chemicals were purchased either from VWR International (Amsterdam, the Netherlands) or Sigma-Aldrich (Zwijndrecht, the Netherlands).

4.3 Fungal starter cultures

For small scale cultures, fungal mycelia were transferred from PDA plates to 50 ml tubes, containing 10 ml of PDB, by an inoculation loop. After inoculation the tubes were closed and vortexed vigorously. For large-scale cultures, fungal mycelia were first transferred from PDA plates into a 50 ml tube containing 10 ml of PDB. After inoculation, the tube was closed and vortexed vigorously. Next, 5 ml of fungal suspension was transferred to 400 ml of PDB in a 2 litre Erlenmeyer flask. The tubes and flasks were then placed in a rotary shaker and incubated at 27 °C while shaking at 200 RPM for a total of 3 days.

4.4 Biotransformation of ZEN in liquid fungal cultures

After 3 days of stress-free growth, ZEN at a final concentration of 5 µg/ml was added to the fungal cultures in PDB using a stock solution of 1 mg/ml of ZEN (prepared by dissolving 5 mg of solid ZEN in 5 ml of MeOH). After the addition of ZEN, the fungal cultures were further incubated with a final optimal biotransformation time of 2 weeks. For steering the biotransformation towards the preferable production of ZEN-glucosides, a new growth medium was developed. The new medium was based on Czapek-Dox medium, a growth medium based on inorganic salts, sugar and water. All sulphate based salts were omitted from the modified Czapek-Dox medium (MCD) and were not replaced by other salts. The adjusted medium then consisted of 30% dextrose, 2% $NaNO_3$, 0.5% KCl and 1% K_2HPO_4 in double-distilled water, set at pH 7.3. The adjusted biotransformation procedure was as follows. After 3 days of stress-free growth, fungal cultures were centrifuged at 3000 g for 10 minutes and the supernatant (PDB) was removed. The fungal mycelia were

then washed 1 time with double-distilled water and again centrifuged at 3000 g for 10 minutes. The fungal mycelia were reconstituted in the original culture volume using MCD medium and fortified at 5 μg/ml ZEN. These fortified cultures were then further incubated for 2 weeks at 27 °C in a rotary shaker set to 200 RPM.

4.5 LC-MS/MS analysis of produced conjugates

An LC-MS/MS method was developed based on available ZEN, Z14G, Z16G and Z14S reference standards (Fig. S17). Before LC-MS/MS measurements, the fungal cultures were centrifuged at 3000 g for 10 minutes and the supernatant was collected. The supernatant was filtered through a syringeless filter device and 5 μl was injected into the LC system, applying a flow of 0.40 mL/min and a column temperature of 35°C. A gradient was applied (Table S9) using running buffer A that consisted of 5 mM ammonium acetate + 0.1% acetic acid in water and running buffer B that consisted of 5 mM ammonium acetate + 0.1% acetic acid in MeOH/H_2O v/v 95/5. Detection of ZEN, and its main formed phase II metabolites, was performed in a negative MRM mode according to the settings shown in Table S10. Concentrations of the produced metabolites, were calculated by creating dose response curves (from 10 ng/mL to 1 μg/mL) of the available standards in the fungal growth medium.

4.6 NMR analysis of produced conjugates

Initially, 10 mg of ZEN was dissolved in deuterated methanol (MeOD), and ^{1}H, ^{13}C, ^{13}C-DEPT, HSQC, COSY and HMBC analyses were carried out to identify the various peaks. Then the produced conjugates were also dissolved in MeOD, and a ^{1}H NMR analysis was carried out to determine if enough conjugated ZEN metabolites were available. Next, ^{1}H, ^{13}C, ^{13}C-DEPT, HSQC, COSY and HMBC analysis was carried out on the 400 MHz NMR spectrometer. Since the amount of Z16G was very low (< 0.5 mg), a 600 MHz NMR spectrometer fitted with a cryoprobe was used for ^{1}H, ^{13}C, HSQC, COSY and HMBC analysis.

4.7 LC-HRMS analysis of the ZEN metabolites by Cunninghamella and collection of HRMS spectra of the purified ZEN glycosides

For the separation of ZEN metabolites in the fungal extract, after filtration, 10 µL was injected on the analytical column which was kept at 40°C. The ZEN metabolites were separated with a gradient elution (Table S11) using running buffer A that consisted of 2 mM ammonium formate and 0.002 v/v% formic acid in water and running buffer B that consisted of 2 mM ammonium formate and 0.002 v/v% formic acid in acetonitrile/water v/v 90/10. The flowrate was kept constant at 0.3 mL/min. The HRMS operated in negative ionization mode, the capillary temperature was set at 250°C with a spray voltage of 3.5 kV. A full scan event followed by an all ion-fragmentation scan event was applied. The full scan data was recorded with a *m/z* range of 120-1200 with a resolution setting of 35,000, the automatic gain control (AGC) set at 5e5 and the maximum injection time (IT) set at 200 ms. For the all ion fragmentation, a resolution setting of 17,500, an AGC of 3e6 and an IT of 200 ms and a stepped normalized collision energy (NCE) of 40 and 60 was used. The MS/MS spectra were obtained by infusing the purified standard in a diluted 2 mM ammonium formate and 0.002 v/v% formic acid in MeOH/water v/v 95/5 at a flowrate of 10 µL/min. Spectra were obtained at a resolution setting of 70,000. For the MS/MS fragmentation the formic acid adduct $[M+FA-H]^-$ *m/z* 525.19720 +/- 2 Da was isolated and fragmented with 50 NCE.

Acknowledgments

We would like to thank Mathias Koch and Martina Werneburg of the Bundesanstalt für Materialforschung und –prüfung (BAM) institute for their advice, assistance and hospitality considering the purification of the produced ZEN metabolites. Monique de Nijs and Willem Haasnoot for stimulating discussions and Marco Blokland for supporting additional HRMS analysis, all from Wageningen Food Safety Research. We are grateful to Marianne Drijfhout for her contribution to the NMR studies on ZEN and the ZEN-glycosides.

Chapter 7: Supplementary Material

Supplementary Materials are available online, free of charge at:

https://www.mdpi.com/article/10.3390/toxins13060366/s1

Figure S1: Numbering of atoms for ZEN and its glucosides

Figure S2: 1H-NMR spectrum of ZEN

Figure S3: 13C-NMR spectrum of ZEN

Figure S4: DEPT spectrum of ZEN

Figure S5: The most important 2D-coupling signals in ZEN

Figure S6: 1H-NMR spectrum of Z14G

Figure S7: 13C-NMR spectrum of Z14G

Figure S8: Key HMBC coupling between C14 and C19 in Z14G

Figure S9: Key HMBC and COSY interactions in Z14G

Figure S10: 1H-NMR spectrum of Z16G

Figure S11: 13C NMR spectrum for Z16G

Figure S12: Z16G 1H NMR showing the shoulder on the MeOD peak indicating the proton on the anomeric C19 which couples to C16

Figure S13: Key HMBC coupling between C16 and C19 in Z16G

Figure S14: Key HMBC and COSY interactions in Z16G

Figure S15: Applied ZEN reference standards in the developed LC-MS/MS method

Figure S16: HRMS fragmentation spectra of Z14G and Z16G

Table S1: 1H NMR data of ZEN

Table S2: 13C NMR data of ZEN

Table S3: 1H-NMR data of Z14G

Table S4: 13C-NMR data of Z14G

Table S5: 1H-NMR data of Z16G

Table S6: 13C-NMR data of Z16G

Table S7: Calculated and HRMS established ions (m/z) of ZAN biotransformation metabolites

Table S8: Calculated and HRMS established ions (m/z) of β-ZEL biotransformation metabolites

Table S9: LC gradient, Table S10: Mycotoxin specific MS/MS settings for the negative ionization mode

Table S9: LC gradient (HRMS).

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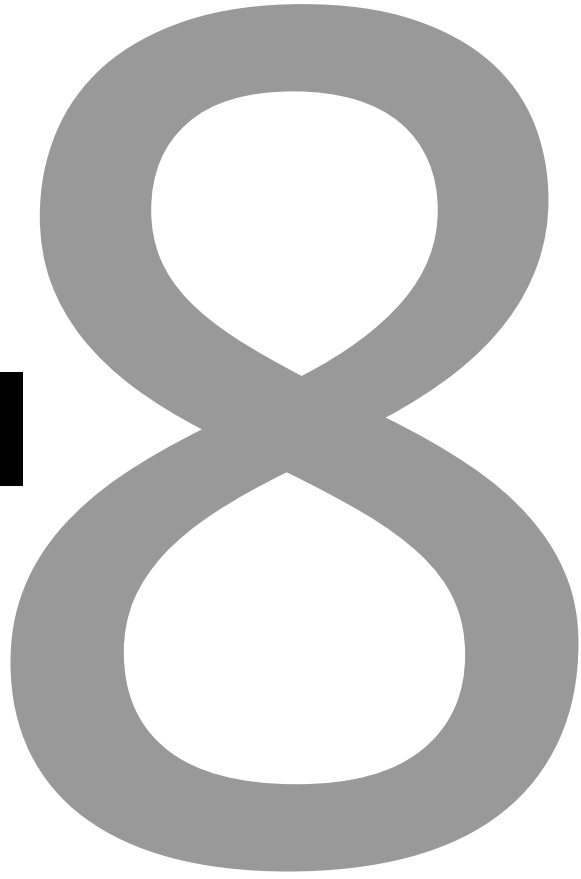
CHAPTER



General discussion and future perspectives

This discussion chapter is divided in four parts. In part I, the general performance of the developed xMAP mycotoxin multiplexes is discussed and the possibilities for future extension of the developed multiplex with emerging mycotoxins, as well as other targets, are considered. In part II, the xMAP technology is critically compared to other technologies and immunoassay formats for mycotoxin detection. Additionally, based on new EU recommendations, other biorecognition molecules are discussed for their suitability for implementation in the developed multiplex and other immunoassay formats in general. In part III, findings of chapter 6 are further discussed on the basis of a unique craft beer recipe. Based on calculations, we show how high DON+D3G contaminations can be expected in high gravity beers. New recommendations for MLs in malts and/or craft beer are discussed. In part IV, the suggestions for future research of the previous parts are summarized.

Part I

1. General Discussion

At the biosensors laboratory of WFSR, xMAP is a preferred technology for building multiplex immunoassays for application in the field of food, feed and environmental safety. The multiplex mycotoxin detection methods presented in the research chapters of this thesis, were all developed on this xMAP technology. The initial indirect competitive immunoassay format in chapter 2, where mycotoxin-protein conjugates were coupled to the microspheres, was successfully replaced by a direct approach where the antibodies (Abs) were coupled to the microspheres and fluorescent mycotoxin-protein conjugates were successfully introduced as reporter molecules. The introduction of this direct competitive approach, shortened the assay incubation from 1 hour time to only 15 minutes, by omitting the need for a secondary reporter antibody. The application of the developed assays on the flow cytometer-based systems is entirely lab-based, while application of the planar array analyser (MAGPIX) system has the potential for portable on-site use, due to the easy transportation and simplified operation. On-site

applications of the MAGPIX have been previously demonstrated for the detection of antibiotics in chicken feathers and for the detection of marine toxins at the point of need. A serious advantage is that the developed assays can easily be exchanged between the different xMAP analysers, resulting in the same sensitivities and only differing in sample measurement times. For all the developed assays in this thesis, broad-range calibration standard curves were implemented. They resulted in dose-response curves with a large dynamic range. From the research chapters in this thesis, it becomes clear that some of the developed xMAP assays are prone to matrix effects depending on assay/matrix combination. For example, quantification of beer samples was performed using matrix-matched addition in a standard beer, however, the beer styles assessed differed dramatically from each other in terms of malt gravity and composition. So beer style-matched dose-response curves for each beer type might lead to more accurate quantitation of samples. To this end, proper blank matrix materials need to be available. This does not seem a very realistic option, so sample pre-treatment should be considered. Besides this, the dose-response curves were based on a logarithmic scale, meaning that a small variation in response can easily result in less accurate quantitation when compared to LC-MS/MS.

The sensitivity of the developed assays is important for allowing detection of legislated mycotoxins at their maximum levels (MLs). Even though it has become a habit to acquire antibodies that result in the most sensitive assays for our targets of interest, this is not always beneficial. Of course, when we want to detect AFB_1, we preferably want to be able to detect it at the ML set for baby food (50 ppt), and preferably we want it even more sensitive (5 ppt), so that an extract with organic solvents can still be 10x diluted before immunoassay measurement. However, in some cases, we do not need such a high sensitivity. This becomes clear in chapter 4 where the mycotoxin 6-plex is successfully validated for all six mycotoxins in barley. From those results, it becomes clear that the FB assay is very sensitive, which will result in many positive screening results, even with concentrations that are substantially below the MLs set for FB. Besides their sensitivities, broad range Abs are

becoming more relevant when MLs and/or TDIs are set for the main mycotoxins, including their metabolites and modified forms. From 2016 onwards, the TDIs for DON, ZEN and T-2 were expanded to group-based TDIs, including metabolites and their modified forms. Not all the Abs in the current mycotoxin 6-plex were able to detect all these modified forms that are currently included in the extended TDI's. For future immunoassays, Abs should thus be implemented, or generated, for the detection of the missing modified forms. Another option can be a preincubation step with enzymes that are able to cleave the modified mycotoxins, so their original form is released [1,2]. Additionally, sensitivity improvements of the mycotoxin 6-plex may be necessary as MLs in food, as set in Regulation (EC) No 1881/2006 and its amendments, are expected to be lowered. These amendments are expected to include new proposed limits for the sum of T-2 and HT-2 and proposed lower limits for DON and OTA, in combination with proposed limits for new matrices. This will require a critical check of our developed mycotoxin 6-plex, to see if all assays are still fit for purpose for the detection at the newly proposed limits.

The diversity of the feed and beer samples assessed in this research were expected to have variable matrix effects, and therefore, confirmatory analysis of screening results was chosen over a comparison with available mycotoxin ELISAs. On the basis of LC-MS/MS results, we can indeed ascribe some false positive samples in the mycotoxin 6-plex to matrix effects. This was the case for some AFB_1-positive screened beers. Further investigation would be necessary, to elucidate what exactly interacted with the AF Ab. There is a possibility that the Ab cross-reacts with an unknown metabolite produced during the beer brewing process. The diversity and complexity of the beer matrices also had their effects in the LC-MS/MS confirmatory analysis, leading to matrix interferences and signal suppression. This especially was the case with the high gravity beer styles. Confirmatory analysis was based on a simple dilution of the beer sample, combined with matrix-matched standard addition. If more time and capacity would have been available, a

beer-dedicated beer sample pretreatment would have been developed to decrease matrix effects.

In hindsight, we do regret the removal of 23 mycotoxins, classified as not relevant for the confirmatory analysis of the mycotoxin 6-plex, from the initial LC-MS/MS method. These mycotoxins might have shed light on their presence , expected or unexpected, in craft beer.

2. Extension of the mycotoxin multiplex

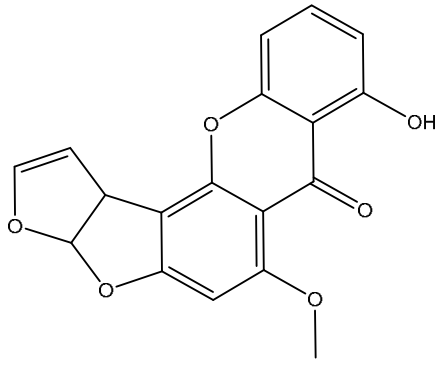
The applied xMAP technology, theoretically allows the development of multiplex assays up to a 500 targets when using the fastest flow cytometer (FM3D) and up to 50 targets when using the MAGPIX planar array analyser. For both platforms, the number of detectable targets can still be increased when broad-range Abs are applied (e.g. Abs that detect both T-2 and HT-2). Although the 500-plex capacity may be used to its fullest in molecular clinical detection (2 × a 500-plex) [3], while some impressive multiplexes for protein profiling (384-plex) also have been reported [4]. However, immunoassay multiplex methods for the detection of 500 targets are not very realistic in food and feed safety screening methods. That is why we believe that the multiplex capacity of the MAGPIX is sufficient for food and feed safety detection assays. If we take the transportable MAGPIX with its 50-plex capacity as the model instrument, many extensions of the mycotoxin 6-plex and combinations with other food contaminants can be considered. The most logical step is to develop or acquire antibodies that are capable of not only detecting the original mycotoxins, but also their relevant modified forms and metabolites (e.g. all ZEN metabolites and modified forms). For further extensions of the 6-plex we should ask ourselves, what is the purpose for the extension and who needs those extensions?

2.1 Emerging mycotoxins

Screening assays for mycotoxins normally focus on the six main mycotoxins for which harmonised legal limits in food are set (or recommended), i.e. AFs, OTA, FBs, DON, T-2/HT-2 and ZEN. Besides these mycotoxins with legal limits, there are also the so-called emerging mycotoxins. For these emerging

mycotoxins, MLs and TDIs are being considered, but the lack of occurrence- and toxicity data makes it hard to take decisions on these. Additionally, they are less well-studied from a toxicological point of view [5,6]. As a result, food and feed producers do not yet display significant interest in introducing these emerging mycotoxins in novel screening methods. However, for future extension of the presented mycotoxin 6-plex, some emerging mycotoxins should seriously be considered.

2.1.1 Sterigmatocystin



Sterigmatocystin

Figure 1. Chemical structure of sterigmatocystin

Sterigmatocystin (STC) is a precursor to AFB_1, produced by *Aspergillus* fungi and has carcinogenic properties in animals, and is therefore classified as a group 2B carcinogen by the IARC [7]. Therefore, STC seems to be a valid candidate for future extension of the mycotoxin 6-plex. STC has been previously detected in in barley, wheat, rye and rice at concentrations ranging from 0.05 to 5.0 μg/kg [48] with the highest incidences in rice and oats [8]. STC has also been detected in beer, at concentrations ranging from 0.018 to 8 μg/L [9,10]. Abs for the detection of STC have already been generated and applied in ELISA-based methods [11], which supports the possibility of adding STC to the mycotoxin 6-plex.

2.1.2 Ergot mycotoxins

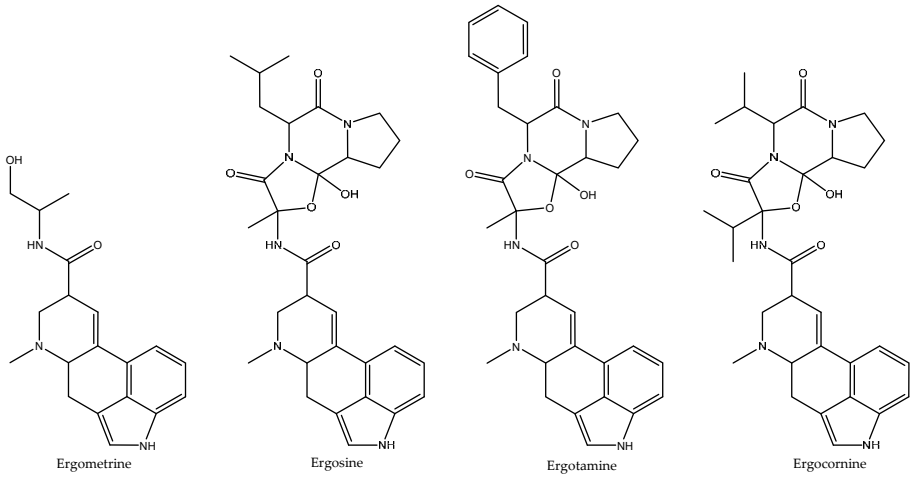


Figure 2. Chemical structures for a selection of ergot mycotoxins

Ergot mycotoxins, more often called ergot alkaloids (EAs), are produced by the fungus *Claviceps purpurea.* The fungus produces crescent-shaped bodies, called ergot sclerotia, that contain a mix of several ergot mycotoxins. EAs can be transferred from the ergot sclerotia to cereals during cleaning and subsequently to flour. EAs can have a wide range of toxicological effects, with the most extreme being gangrene [12]. Recently, a new EU Commission Regulation [13] was published, in which MLs were set for the sum of 12 defined EAs in milling products. EA's predominantly occur in rye and triticale, but also in wheat, oats and barley [14,15]. In barley contaminations as high as 54 μg/kg have been reported for the sum of EAs [14]. The presence of EAs in beer has been rarely assessed or reported. But evidence suggests that EAs do not survive the malting and subsequent brewing process, as concluded from experiments in which beer was brewed from malt that contained 0.1% (w/w) of ergot sclerotia [16]. Several Abs for EAs have been generated for scientific purposes, but also commercial Abs are available [17,18] for the detection of EAs. This shows that the mycotoxin 6-plex can be extended for EAs. However, the specific challenge for EAs is in the broad-range of the common EAs like ergometrine, ergosine, ergotamine,

ergocornine, ergocristine and ergogkryptine and there isomers [19,20]. With MLs for EAs currently in place, the extension of the 6-plex with EAs seems a logical step, especially when milling products will be assessed.

2.1.3 *Alternaria* mycotoxins

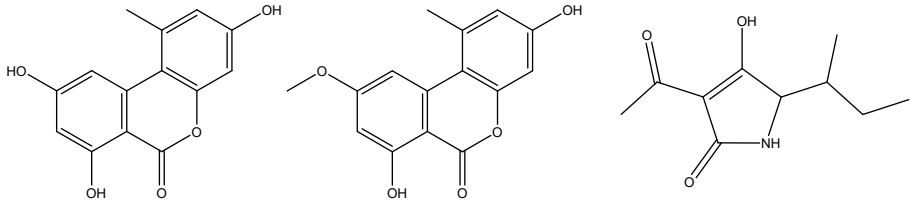


Figure 3. Chemical structures for a selection of *Alternaria* mycotoxins

A*lternaria* fungi contaminate a wide range of agricultural crops, from tomatoes to barley and can produce over 70 phytotoxins. From those phytotoxins, a selection has been designated and classified as mycotoxins with an effect on animal and human health. From those altenariol (AOH), alternariol methyl ether (AME), tentoxin (TEN), and tenuazonic acid (TEA) are the most studied [21-23]. A*lternaria* mycotoxins have been reported to be mutagenic and genotoxic and have been detected in cereals at concentrations ranging from 3 to 766 μg/kg [24]. In barley, intended for malting, TEA was present at a concentrations of 196 μg/kg, while AOH, TEN and AME did not pass 5 μg/kg [25]. Like with certain *Fusarium* toxins, the concentrations for AOH, AME, TEN and TEA can significantly increase during the malting process, and both TEA and TEN were transferred almost completely to the final beer [26]. Several surveys have focussed on the occurrence of *Alternaria* toxins in beer [15,27-29] with the highest concentrations reported for TEA of 175 μg/L, for AOH 23 μg/L and for TEN 11 μg/L. Through their mutagenic and genotoxic properties some *Alternaria* toxins are of concern for animal and human health, and therefore harmonised MLs might be set in the EU [29]. Their high toxicity and frequent occurrence, makes the addition of selected *Alternaria* toxins to the mycotoxin 6-plex relevant. Immunoassays for the

detection of several *Alternaria* toxins are available [30,31], which indicates that an extension of the current mycotoxin 6-plex is feasible.

2.1.4 Other emerging mycotoxins

Several other mycotoxins are considered for future implementation, based on their occurrence and suspected toxicity (e.g. citrinin, nivalenol, enniatins, beauvericin, moniliformin), provided that commercial or scientifically developed Abs are available. In case these specific antibodies are not yet available, they might be developed. For beer-based screening even patulin (PAT) may be considered, as it is mutagenic and teratogenic [32]. Even though PAT is very stable in beer brewing [33], it predominantly occurs in fruits and therefore is not a logical contaminant in standard beer brewing. However, it still may be present in spontaneous fermented lambic beers, that contain high doses of various fruits, added during barrel fermentation, and left to ferment together with the beer for a year or more [34]. A survey on lambic and other fruit-based beers may shed more light on this possibility, although lactic acid bacteria present during barrel fermenting may break down PAT [35]. But if not proven relevant for fruit containing beers, the development of a new fruit-based mycotoxin xMAP method can be developed, since fruits can be contaminated with PAT but also *Alternaria* toxins, OTA and AFs.

2.2 Multiplex extension with other targets

Besides extending the multiplexing capacity with emerging mycotoxins, it can also be extended with non-mycotoxin targets, relevant to the matrices that are analysed. Combining multi-methods can be a perfect tool for the relevant matrices. At WFSR, we are currently combining the xMAP multiplex assays for antibiotics and anthelmintics, to generate a new 24-plex for the application to diverse matrices. In principle, parts of this 24-plex could be added to the mycotoxin 6-pex for the screening of feed. Another combination may be a multiplex for mycotoxin and pesticide detection. For example, in barley, fungicides can be used to decrease the chance of fungal infection in the field, however, these fungicides are food contaminants themselves, and need to be

screened for as well from a food safety perspective. In a survey on barley, the co-occurrence of DON and fungicides azoxystrobin and carbendazim was confirmed [36]. Since we have already developed xMAP assays for the detection of these two fungicides, they could be implemented into the existing mycotoxin 6-plex panel for screening purposes. When focussing on craft beer, the inclusion of pesticides may also be an option. Glyphosate is the most frequently used herbicide worldwide and has been marked a probable carcinogen to humans by the World Health Organization (WHO) [37]. In beers from the Latvian market, glyphosate concentrations up to 150 μg/L were detected [38], while in Germany it was also discovered in a selection of weizen beers. Due to its frequent use and its toxicity, we think it makes sense to include it in future beer surveys, even though its use will be prohibited from the end of 2022 in the EU. Hops seem not to be an important source for mycotoxins in beer brewing, but they may be a source for pesticides, as hops are prone to a range of pests [39]. Especially the double and triple imperial pale ale (IPA), with their extreme hop content, may be worth looking into. Besides pesticides, also heavy metals may occur in beers and their concentrations are sometimes above the set limit [40]. Since craft breweries often operate in old industrial complexes, heavy metals may occur in higher concentrations in beer due to leaching from old piping. Additionally, they may leach from tanks or filtering units. Although some heavy metals may not be of a direct concern for food safety at low concentrations, they may still influence the flavour stability of beers [41]. This makes the introduction of heavy metal immunoassays relevant as a beer quality factor. Currently, we are developing a 5-plex for the detection of heavy metals in aquaponics water and craft beer, which can be considered for the extension of the mycotoxin 6-plex for craft beer screening.

For all the above discussed extensions of the mycotoxin 6-plex, the availability of relevant Abs is the main concern. Their affinities, cross-interactions and their cross-reactivities in the multiplex are important factors to take into consideration. Additionally, the costs for the multiplex will increase with the addition of each target Ab and respective conjugate. Of course, the purpose of the developed method is crucial and should not lose its focus of application. Additionally, where does multiplex screening end, and does the screening by

instrumental analysis start? If an xMAP method is solely developed for the purpose of a scientific publication, then every added target in the multiplex may be considered relevant. The same holds for specific development requests by potential end-users, who decide what is relevant for them. The xMAP multiplex can also be extended for pre-screening purposes, to select positive samples prior to a confirmatory method, which includes the same targets. It is easy to conclude that xMAP technology can never compete with analytical methods that are capable of detecting 87 mycotoxins and mycotoxin metabolites, or 295 fungal and bacterial toxins in one method, or the detection of 630 multiclass food contaminants, including 426 pesticides, 117 veterinary drugs and 21 mycotoxins [42-44]. Besides multiplex capacity and the complexity of developing such a large multiplex method on xMAP technology, the simple fact is that a large amount of Abs are not yet developed for many of these compounds.

Part II

3. Alternative mycotoxin detection platforms

In scientific literature, there are a wide range of techniques that were implemented for the (multiplex) detection of mycotoxins for a variety of food matrices, all presented with promising results: e.g. gel-based and membrane-based multiplex flow-through immunoassays for the detection of OTA, FB_1, DON, ZEN and AFB_1 [45], microarray immunoassay with specific Abs coupled to a nitrocellulose surface and biotinylated mycotoxin-protein conjugates for competitive detection of OTA, FB_1, ZEN and AFB_1, using streptavidin-HRP for chemiluminescent detection [46], silica photonic crystal microsphere (SPCM) suspension array for the detection of AFB1, FB1 and citrinin in cereals, where the antigen coupled to photonic crystals can be uniquely identified by their reflection under white light [47], a competitive luminescent assay where an anti-fumonisin Ab was conjugated to one subunit of the NanoLuc protein, while FB_1 was conjugated to the other subunit. By adding a sample that contains the FB_1 analyte, competition will occur with the subunit-FB_1 conjugate, which detaches from the NanoLuc protein complex, which loses its luminescence [48]. For a critical comparison of the current

mycotoxin 6-plex to the aforementioned assays, more details would need to know. For the moment, we would like to critically compare our developed xMAP-based multiplex with some commercially available formats and to some key biosensing formats (Table 1).

Enzyme-Linked Immuno Sorbent Assay (ELISA) is one of the oldest Ab-based immunoassays. Even though there have been many method developments in mycotoxin detection, ELISA still remains very relevant today, ELISAs for mycotoxin detection can be bought form several companies [49]. The immunoassay principle and the robustness of the assay is largely comparable to our developed xMAP method in this thesis. However, in our experience the dynamic detection range for xMAP is larger when compared to competitive ELISA's. More importantly, the advantage of the xMAP technology is that it allows multiplexing to a high order in the same well, while the traditional ELISA needs a different well for each single analyte. However, with some creativity, variations on the ELISA technology, like the multi-coating of a well, allow some form of multiplexing [50]**.** Membrane-spotted microarrays in a microtiter well, can also be an option and are often confused for ELISA's. This is because the use of similar microtiter plates, but in fact it is a protein array [51].

Lateral Flow Immunoassays (LFIAs), also known as striptests, is another popular immunoassay detection format. It became well-known to the general public through pregnancy and, more recently, covid tests. Literature shows that the development of (LFDs) for rapid, fast and on-site application for food safety is, together with clinical applications the most frequent developed [52]. Like for ELISA, lateral flow devices (LFDs) for the detection of mycotoxins can be bought form several companies [49]. Unlike ELISA, LFIAs are capable to detect multiplex mycotoxins in one sample [53] and allowed even higher multiplexing capacities through microarray spotting on the LFIA membranes [54]. Companies like Unisensor and R-Biopharm used to sell multiplex LFDs for the detection of mycotoxins, but currently they are not listed in their product portfolio anymore. This raises the question why these are not available anymore. It could be related to poor performance, stability issues, or simply that the market demand was lacking for such tests.

Table 1. Comparison of the xMAP mycotoxin multiplex on the MAGPIX platform with commercially available methods, popular biosensing formats and confirmatory instrumental analysis

Technology - abbreviation		Costs equipment	Single tests commercially available	Multiplex Test Commercially available	Multiplexing yes/no	Multiplex flexibility	Multiplex capacity	Sample Throughput*	Ease of use	On-site portability
Multi-Analyte Profiling	xMAP	25 k€	no	no	yes	high	50	H	M	yes
Enzyme-Linked Immuno Sandwhich Assay	ELISA	8 k€	yes	-	no	-	-	H	M	no
Fluorescence polarization immunoassay	FPIA	5 k€	yes	-	no	-	-	L	M	yes
Lateral Flow Immunoassay	LFIA	0[1] - 3.5 k€	yes	no[2]	yes	low	4[3]	L	H	yes
Biacore 3000	SPR	>200 k€	no	no	yes	low	4	L	L	no
IBIS	iSPR	180 k€	no	no	no	low	96	L	L	no
liquid chromatography-tandem mass spectrometry	LC-MS/MS	>250 k€	-	-	yes	high	>400	L	L	no

H = high, M = medium, L = low, [1]In case of visual readout, [2] Not commercially available anymore, [3]Based on commercial LFD

The latter does seem plausible, as a company like Charm, never really considered to add multiplex LFDs to their product catalogue. Nevertheless, LFDs are the ideal tool for point-of-need analysis, although they are still depending on reader instruments (sometimes combined with incubators) for semi-quantitative results. Nevertheless, these readers can be considered as low-priced compared to the MAGPIX instrument. The introduction of smartphone based read-outs of LFDs can reduce the need for expensive equipment [55]. Compared to our developed xMAP technology their throughput is low, which means a substantial manual labour when a high amount of samples has to be processed, both in the lab, as well as at the point-of-need.

Fluorescence polarization immunoassay (FPIA), is a technique that uses a mycotoxin-specific Ab, that can bind to the target mycotoxin, which is coupled to a fluorescent molecule (tracer). Upon binding of the Ab to the mycotoxin-tracer molecule, the rotation of the tracer in solution is slowed down. This decrease of rotation can be measured by applying polarized light. If there are mycotoxins present, less Ab will bind to the tracer molecules, so rotation rate is not slowed down. FPIA was implemented for the determination of DON in durum wheat-based products and ZEN in maize and OTA in rice, amongst others [56-59]. FPIA is considered a rapid method, since it does not require washing steps (when compared to ELISA). Aokin AG from Berlin initially automated the FPIA technique for the detection of mycotoxins, but currently is only selling manual devices for single measurements. Therefore the throughput for FPIA compared to our xMAP method can be considered as low. Additionally, FPIA only allows the detection of a single mycotoxin per analysis and therefore is not suitable for multiplex detection. However, the FPIA devices can be considered low-priced compared to the MAGPIX.

Optical biosensors come in many forms, ranging from Biacore biosensors (from the basic 4-channel approach, up to multiplexing capacity (up to 20 targets on 4 channels), to the more simple OpenSPR machine (2 targets on 2 channels), or an IBIS array-based SPR imaging (96 targets/chip array). Several SPR-based biosensor methods have been developed for the detection of

mycotoxins [60,61], however, these have not resulted in commercial applications. Unlike the MAGPIX instrument, most biosensors are costly and only suitable for lab-based detection and are limited in their transportability. Nevertheless, in recent years, biosensors are also developed with the aim of being transportable to the point of need. In several European H2020 projects, scientists work together to develop portable biosensors. For example, it has led to the development of a 6-plex immunoassay method on a portable iSPR biosensor for the detection of DON, ZEN, T-2, OTA, FB_1 and AFB_1 in barley [62]. Obviously the SPR-based sensors have the advantage over xMAP that they can be operated label free, and are ideal for Ab kinetics as well. Additionally, iSPR systems like IBIS [63] are also capable of multiplexing immunoassays. However, the xMAP technology presented in this thesis does have a few advantages compared to IBIS. It allows on the spot selection of a desired multiplex with already target-coupled microspheres. This can be the whole 6-plex, but may also be reduced to a 2-plex for the detection of only two mycotoxins of interest e.g. DON and ZEN. This can be decided shortly before the measurement, for a matrix of choice. This is possible since all unique target microspheres are coupled in a large single batch, then stored separately and they remain stable for up to 2 years. For iSPR new chips need to be coupled in advance, which leaves no room for flexibility. The same holds for later extension of the iSPR assays, when new analytes have to be added to the method. Moreover, additional costs for the IBIS iSPR should be taken into account, as it is depending on chip spotting equipment.

4. Other biorecognition molecules

Abs are an essential factor in biological research and medical diagnostics. In both, scientific and commercial mycotoxin detection applications, Abs play a crucial role. This is mainly because they are a reliable tool for the sensitive and robust detection of low and high molecular weight molecules . For this reason, they were also the obvious choice for the development of the mycotoxin multiplex presented in this thesis. However, recently, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) released a recommendation for animal-free alternatives to antibodies [64]. This new recommendation forces us to have an in-depth look at non-animal derived

biorecognition molecules for possible replacement of Abs in our current mycotoxin 6-plex, or for the possible extensions of the 6-plex. A range of alternatives can be considered, from Ab display libraries to in silico modeling [65]. In the following sections, these biorecognition molecules are evaluated and discussed for their suitability to replace Abs in the mycotoxin 6-plex, and popular immunoassay-based formats in general.

4.1 Recombinant Abs

Recombinant antibodies (rAbs) for mycotoxin detection are acquired from Ab repertoire libraries by phage display selection. These phage-display based libraries are combinatorial libraries that contain billions of Ab fragments [66]. RAbs generated by phage display, result in a single-chain variable fragment (scFv) that consists of the V_H and V_L fragments of the original Ab (Fig. 4A), which are connected by a flexible polypeptide linker to bring them close together to form an Ab binding site (Fig. 4B). These scFv fragments have already been applied for the detection of DON [67], HT-2 [68] and AFB_1 [69]. An even simpler structure based rAb, is derived from camelids IgGs (Fig. 4C), which only contain heavy chains [70]. The resulting variable fragment is called a nanobody (Fig. 4D). These generated nanobodies tend to be more resistant to higher temperatures and organic solvents and have been successfully applied for the detection of AFB_1 [71], OTA [72] and 15ADON [73]. In our own studies, we critically compared scFv Abs directed against OTA and HT-2, with xMAP technology, to Abs that were part of the 6-plex presented in this thesis. We found that the sensitivities of the OTA and HT-2 rAbs based assays, were respectively 100 and 50 times less sensitive compared to the classical Abs assays. Since these scFv's both were the most optimal derived from display libraries, optimizations in their binding affinity through gene editing will be necessary to be able to compete with the classical Abs. This does not necessarily mean that rAbs are not suitable (or favorable) for the detection of small molecules, but in our case we could not improve their sensitivities by general assay improvements. To fully answer to the reduction of test animals for Ab development, these scFv fragments should come from

naïve libraries that are amplified from non-immunized humans and animals. A successful example is the implementation of a scFv fragment from a human library directed against the noro virus [74]. However, since small components by themselves are less immunogenic, chances to pick up a perfect rAb is challenging.

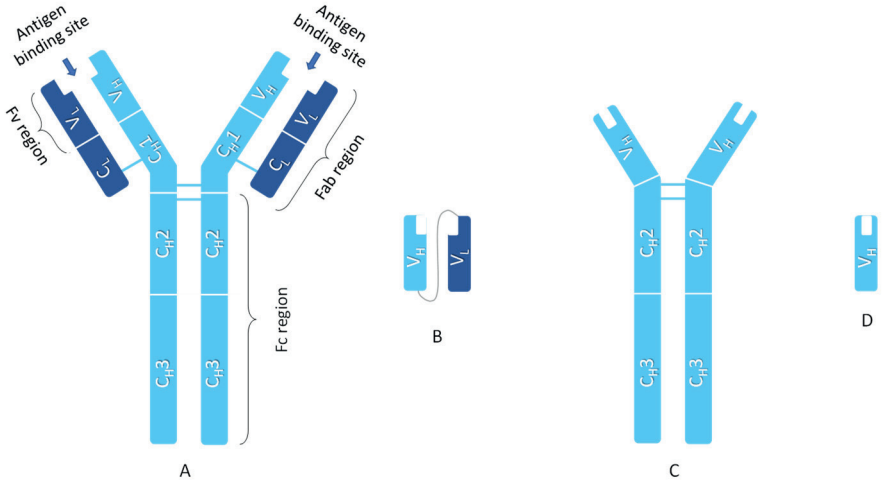


Figure 4. Schematic structures of an IgG antibody (A), a IgG derived recombinant antibody fragment, scFv (B), a camelid IgG (C) and its derived recombinant single chain nanobody (D).

4.2 Aptamers

Aptamers are single-stranded oligonucleotides (DNA, RNA) that are designed for detection purposes. By forming tertiary structures, these aptamers can bind to proteins, metals, and a wide range of small molecules. According to literature, aptamers are a promising tool in the detection of mycotoxins [75]. The best studied aptamers are those directed against OTA, AFB_1 and AFM_1 [76-78], while aptamers against ZEN [79], FB_1 [80] and T-2 [81] also have been reported. The main biosensing techniques used for aptasensor-based detection are fluorescence, colorimetry and electrochemistry [82]. Aptamers do have some advantages over traditional Abs in that they are cheaper to produce, without the use of test animals, can resist high temperatures and allow easy sequence adaption [83]. However, the

robust application of aptamers for mycotoxin detection in traditional ELISA and LFIA techniques is far from trivial and remains mainly research based [84,85]. For example, aptamers for OTA have been used, improved and reported successfully from 2008 onwards [86-88]. Yet none of these aptamers made it to a commercial application yet. All aptamer-based research at WFSR, where aptamers for the detection of mycotoxins were implemented in immunoassay-based formats, were not successful. Current WFSR research on aptamers, for the detection of heavy metals show the same problems as observed in biosensor-based immunoassays. However, an xMAP aptamer approach for heavy metals, that uses a DNA hybridization approach by complementary oligo's, shows some promising results for single assays, but complications are expected for constructing multiplex assays. This is mainly based on interaction speed, temperature dependence and buffer selectivity of the single aptamer assays. In general, the transfer of (multiplex) detection assays from a scientific publication to another preferred assay format is very challenging. If the goal is developing a rapid and robust (portable) on-site assay, which already is a challenge by itself, then it will become even more challenging with the introduction of aptamers.

4.3 Molecular imprinted polymers

Molecular imprinted polymers (MIPS) are a different class of biorecognition molecules, since they are chemically synthesized using the target molecule of interest as a template. Their application for mycotoxins mainly focusses on capturing and pre-concentration techniques [89-91]. Generally, MIPS have been integrated into many sensing formats, but so far these have not been commercially exploited [92]. In some cases, like for the determination of FB_1 in maize, the MIPS are directly used to replace Abs, to mimic an immunoassay based format [93]. For implementation in the xMAP based assays, the first challenge is to covalently couple them to the microspheres, while the second challenge is to design effective reporter-based mycotoxins for detection. MIPs have the advantage that they are stable over an wide pH-range, resist high temperatures and can resist organic solvents. However, the leaching of targets during measurements is a serious problem often encountered [94]. Generally, less flexibility can be expected in the MIP structure (lock and key), compared

to Ab structure (induced fit), which makes MIPS a very rigid detection molecule [95]. Because of these problems, it is not very likely that MIPS will seriously compete with Abs in our mycotoxin 6-plex in the foreseeable future.

4.4 Receptors

Cell-based receptor molecules are also applied for the detection of small analytes [96]. A receptor-based format has been applied for the detection of ZEN. ZEN differs from the other mycotoxins, because it is not directly toxic, but it has a potentially harmful hormonal activity. Through this activity it can bind to the estrogen receptor [97]. The effects of other mycotoxins on human receptors have been studied, but strong specific interactions were not reported [98], making them not suitable for broad-range mycotoxin detection. Therefore it is safe to say, that receptors will never play an important role in the multiplex detection of mycotoxins and their inclusion in xMAP mycotoxin assays do not need to be further considered.

Based on the EURL-ECVAM recommendation, scientist have seriously questioned the feasibility of abandoning the hybridoma based technology [99]. We have tried in several small innovative projects to replace mycotoxin Abs with well-described mycotoxin-binding aptamers in competitive immunoassay-like models. These were all unsuccessful, and the main problem was the poor interaction of the aptamers with the targets, but also the non-specific detection of mycotoxins by negative (random) control aptamers upon improvement efforts (e.g. increased salts and temperatures). Due to these unsuccessful results, a scientific publication was not considered. Based on literature, and our own experience, it is safe to say that the implementation of alternative options for animal derived mycotoxin Abs as biorecognition molecules is far from easy in popular rapid screening assays. There are many clever approaches for the application of aptamers for mycotoxin detection, but these really need to be extensively inter-laboratory validated against currently used technologies before we can consider them as a reliable replacement. In the short-term, the generation of rAbs from naive libraries seems to be the closest option to success for competitive

immunoassay approaches. Alternatively, we may also consider the genetic modification of antibody producing clones that were previously stored and considered as not good enough. Although these clones were generated by hybridoma technology, new immunisations can be avoided if genetic modification of these clones can be successful.

Part III

5. Does craft beer still need a closer look?

The demand for diversified craft beer is still increasing worldwide. Although the strong rise of craft breweries may have slowed down a bit in some countries in recent years, there is still strong expansion expected in other countries [100]. Pioneering craft brewers mostly get inspired from following the craft beer revolution in other countries, and then try to introduce it in their own country [101]. On the other hand, some countries have a strong historical brewing history, which makes the entries of new craft brewers more challenging [102]. Besides the passion for brewing very diverse tasteful beers, investing in craft beer and craft breweries is also seen as a good investment nowadays. In short, the craft beer revolution still continues and craft beer will become easier available worldwide. This can of course be considered as good news for lovers of high quality produced beers. On the other hand, in the light of the findings presented in chapter 6 of this thesis, the concerns for human health due to the absence of MLs set for (craft) beer should be taken into account and more style-defined surveys of craft beer should be seriously considered [103]. Even though beer seems to be a popular matrix for mycotoxin screening surveys, surveys with a strong focus on defined craft beer styles are really scarce [104]. Additionally, the transfer of mycotoxins from malt to beer have been well-studied, but there are very little widescale surveys that focus on brewery malts. Moreover, there is very little, to no, information on the type of malts assessed in brewery malt surveys [105-108].

5.1 Mycotoxins in brewery malts and their transfer to imperial stout

The most important conclusion of Chapter 6, was that imperial stout (also known as Russian Imperial Stout (RIS)), beers can be highly contaminated with DON and D3G. These high contaminations raised a concern, since TDIs for DON an its metabolites can easily be surpassed by drinking these beers. In total 5 Dutch RIS beers had DON+D3G contaminations between 100 and 140 μg/L (and even higher in RIS from other countries). Based on press-releases following the publication of chapter 6, discussions started with CRAFT, the Dutch organisation for small independent craft brewers in the Netherlands. As a conclusion of these discussions, 3 possible explanations were presented for the occurrence of such high amounts of DON+D3G in Dutch brewed RIS beers. One hypothesis was that the poor storage conditions in some of the smaller craft breweries, might be the reason for *de novo* mycotoxin production. This was especially assumed for some specialized malts that are not used on a daily basis. The second hypothesis was that craft brewers were provided with poorer quality malts, since craft brewers do not invest in mycotoxin control and no specified mycotoxin information was provided for malt batches that were bought by CRAFT brewers. The last hypothesis was that the imperial stout beer style, was prone to higher DON contaminations because of its high gravity, in combination with the darker malts.

These discussions led to a nationally funded Public Private Partnership project (AF18084), where LFDs for the detection of DON were introduced, for implementation in on-site measurements using improvised laboratory set ups (Fig. 5). The aim of this malt survey was to get an idea about the DON+D3G contaminations in malts from Dutch craft breweries and how these would relate to the final DON+D3G contaminations in RIS beers. In total 106 malt samples, from 11 maltsters located in the Netherlands, Belgium, Germany and the United Kingdom, were assessed for mycotoxin contamination. Many malt samples gave positive readings for DON, ranging from 50 to 350 μg/kg. However, these values were all below the quantitation range of 0.5 to 5.4 mg/kg and strong variations between sample repeats were observed. This

unfortunately meant that these values could not be implemented on a basic DON+D3G contamination calculation model based on a original RIS recipe.

Figure 5. Malt sampling at craft breweries for mycotoxin screening by on-site LFD measurements (A-E) and malt contents (F) for brewing an Imperial Stout beer (G).

5.2 Imperial stout: from recipe to final beer

To assess the magnitude of transfer of DON and D3G from malt to RIS beers, a simple calculation model was applied on the basis of an original RIS recipe, kindly provided by a Dutch craft brewery. With this recipe as a guideline, provisional calculations for DON+D3G contamination in RIS beers were made, based on its malt content. For this RIS beer, with an alcohol percentage of 9.6 %ABV, 8 different malts and adjunct grains are used: Munich, cara 120, melano, choco, aroma 100, black, roasted barley and wheat (Fig. 5). In total 837.5 kg of malt and adjunct grains are mashed with 1700 litres of brewing water. During the brewing process, the loss of water occurs at different stages, of which some do affect the mycotoxin concentration. During mashing the malts/grains will take up roughly 1 kg of water per kg of grains. This initial loss of volume is corrected for by flushing the spent grains with an equal volume of water (roughly 50% of the original volume) and then adding this

flush fraction to the remaining wort. This will establish the original brewing volume. More importantly, the flushing of the spent grains will release mycotoxins from the spent grains, which increases the total amount of DON and D3G in the wort, but will also dilute the overall concentration of DON and D3G in the wort. During boiling, roughly 100 litres of water will evaporate from the brewing system. This loss of volume will increase the mycotoxin concentration of the beer.

Since no hard data from our own assessment of malts were available, malt data from two malt surveys from literature were applied. For model 1, we used the malt data previously produced by Zachariasova et al. [105], who assessed 6 different malts (types not specified). The DON+D3G values for these malts ranged from 22 to 325 μg/kg. For model 2, we used the malt data previously produced by Ksieniewicz-Woźniak et al. [106], who assessed 87 barley malt samples from Poland, but unfortunately also did not specify the malt types. The highest contaminations reported for DON and D3G, were 348 and 410 μg/kg respectively. In both models the highest contamination was used as the main malt type. In model 1 we serially applied the other reported contaminations, while in model 2 we applied the average malt contamination from that survey. The results for the application of these survey data on the imperial stout brewing process are shown in Table 2 and 3. In the case of model 1, the theoretical initial concentration of DON+D3G based on 1700 litres of brewing water and a total DON+D3G amount of 246.85 mg is 145 μg/L. Taking all process steps into account and applying a 30 and 50% efficiency for the flushing step, the final DON+D3G contamination in the final beer ranges from 100 – 115 μg/L. For model 2, the theoretical value is 258 μg/L and with the final DON+D3G contamination in beer ranging from 178 – 206 μg/L. In both cases, these contaminations are substantial and can relate to the high DON and D3G contaminations previously reported in beer surveys (Chapter 6 of this thesis, [106,109,110].

Table 2. Malt DON+D3G contamination data [105] projected on an imperial stout recipe

Model 1: Total input of malt and grains			
Malt/grain type	Amount (kg)	DON+D3G (mg/kg)	Total DON+D3G (mg)
Munich	525	0.325	170.625
Cara 120	75	0.293	21.975
Melano	75	0.19	14.25
Choco	25	0.164	4.1
Aroma 100	37.5	0.22	8.25
Black	37.5	0.352	13.2
Roasted barley	25	0.293	7.325
Wheat	37.5	0.19	7.125
		Total DON+D3G	246.85

Model 1: Processing steps		
Process	Volume (L)	DON+D3G (µg/L)
Mashing (wort + grains)	1700	145
Wort (remainder)	850	145
Flush spent grains (30% efficiency)	850	44
Correction wort for added flush (30%)	1700	94
Loss of water through vapor	1600	100
Flush spent grains (50% efficiency)	850	73
Correction wort for added flush (50%)	1700	109
Loss of water through vapor	1600	115

Table 3. Malt DON+D3G contamination data [106] projected on an imperial stout recipe

Model 2: Total input of malt and grains			
Malt/grain type	Amount (kg)	DON+D3G (mg/kg)	Total DON+D3G (mg)
Munich	525	0.758	397.95
Cara 120	75	0.128	9.6
Melano	75	0.128	9.6
Choco	25	0.128	3.2
Aroma 100	37.5	0.128	4.8
Black	37.5	0.128	4.8
Roasted barley	25	0.128	3.2
Wheat	37.5	0.128	4.8
		Total DON+D3G	437.95

Model 2: Processing steps		
Process	Volume (L)	DON+D3G (µg/L)
Mashing (wort + grains)	1700	258
Wort (remainder)	850	258
Flush spent grains (30% efficiency)	850	77
Correction wort for added flush (30%)	1700	44
Loss of water through vapor	1600	178
Flush spent grains (50% efficiency)	850	129
Correction wort for added flush (50%)	1700	194
Loss of water through vapor	1600	206

In the calculations presented in Table 2 and Table 3, the stability of DON+D3G during mashing, boiling and fermentation is not included. However, if we look at previously published data on the stability of DON and D3G during these brewing steps, it can be concluded that no significant decreases are expected [111,112].

5.3 Can we stick with current MLs regarding malt as a processed cereal?

For future improvements regarding safer beers, we agree with the possible strategies that were previously put forward by Varga et al. [109], who found DON+D3G concentrations up to 170 μg/L in beer. These authors suggested that: a. brewing-malts should be responsibly monitored before the brewing process, b. official maximum levels for beer should be installed and c. the monitoring of DON and its metabolites in future beer surveys. The introduction of MLs for beer has been suggested more often (this thesis, [103]). However, they can have a big economic impact , especially for the smaller craft-brewers. Beer will be tested after the brewing process is finished, so if a batch of beer is tested above a set ML for DON+D3G, it would mean that the batch of beer is lost. This impact can be considered at its maximum for a high-gravity beer style like RIS. Although we think that the introduction of MLs for beer is a good idea for protection of consumers, introduction of stricter MLs for malts may be a better idea. In case of the presented RIS beer recipe in this chapter, the main contamination would most likely come from the 525 kg malt lot. However, if we follow the Commission Regulation No (EC) 1881/2006 [113] we observe that malts are not specified as such. If we assume that malt is a processed cereal for direct human consumption, then an ML of 750 μg/kg should be applied. This means that if the malts were properly screened at the malteries for mycotoxin content, with a 750 μg/kg decision level, that in the worst case a final DON+D3G concentration up to 300 μg/L can be expected in the final beer. This level could be higher, if for the non-malted cereals in the RIS recipe (wheat and roasted barley), the ML of 1250 μg/kg is implemented.

Additionally, as a general improvement, we suggest a change of industry standards. Maltsters should provide detailed mycotoxin analysis certificates

for the malts they provide to breweries. Based on those figures, breweries can then calculate the suitability of the malts for the beer styles they intend to brew, based on their own recipes. This way the mycotoxin concentrations in the final beers can always be kept below provisional beer MLs. This would improve consumer safety. Of course it goes without saying that first and foremost responsible beer consumption is the most important factor.

Part IV

6. Suggestions for future research

Based on the main topics discussed, we come with the following suggestions for possible future research:

1. Extension of the mycotoxin 6-plex with emerging mycotoxins should be considered, with the suggestion to start by adding sterigmatocystin, ergot alkaloids and *Alternaria* toxins.

2. Extension of the mycotoxin 6-plex with other targets than mycotoxins, relevant for the matrices intended for screening. For example, the addition of assays for pesticide detection, heavy metal detection, for the application in craft beer quality screening.

3. Most commercial and scientific mycotoxin detection assays are based on the use of antibodies. Now that the EU has serious plans of phasing out generating antibodies with laboratory animals, scientist should start to consider different options and a switch to novel (bio)recognition molecules becomes very relevant. The real challenge is to achieve a comparable robustness, sensitivity and ease of application as for LFDs, ELISA or xMAP in our case. Based on our own experience, this highly likely means not only the change of biorecognition element, but also a change of detection technology. Therefore, effort should be put in discovering and applying new easy to use technologies to get novel biorecognition molecules to function reliably for the detection of mycotoxins and other food contaminants.

4. Although there are many beer surveys reported, they are mostly not focussed on craft beer and if they do focus on craft beer, the beer styles surveyed are not well-defined. Therefore new beer surveys should always include craft beers and the styles assessed should be clearly listed to be able to better discuss mycotoxin occurrence. This should not be too hard, since nowadays craft beers are easily available, with the high gravity styles mostly in specialized beer shops, rather than in supermarkets.

5. Same as for style-defined beer surveys, there are not many surveys that focus on the malts intended for beer brewing. Therefore we also suggest that future malt surveys should also be more style-defined. This way it can become clearer, which type of malts are prone to higher mycotoxin contaminations.

6. Based on the presented calculations for RIS brewing, it becomes clear that the MLs set for cereals and/or processed cereals are not strict enough. Therefore we suggest that in future recommendations, additional MLs for malts and cereals intended for beer brewing are implemented. If this is not an option, then MLs for beer should be considered. Additionally, we urge maltsters to always supply a dedicated fact sheet for each malt, stating the mycotoxin levels. This way brewers can use recipe-based calculation models before starting brewing. In combination with previously mentioned MLs for beer, this may be an adequate solution to high mycotoxin incidences in certain beer styles.

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Summary

Food and feed can be contaminated with microorganisms, parasites and chemical substances, which can have light or severe toxicological effects. Therefore the European Union (EU) has established food and feed safety regulations. Combined with organized control strategies, they help to ensure the safety of food and feed that enter the production chain. Mycotoxins are produced by fungi and can be major food and feed contaminants. Upon ingestion, mycotoxins can cause mycotoxicosis. The symptoms of mycotoxicosis can be both acute and chronic, and range from nausea to cancer, and even death. Grain commodities are the basis for food and feed, and these can be contaminated by what are considered the most important mycotoxins in terms of agriculture and animal production; zearalenone (ZEN), deoxynivalenol (DON), T-2 toxin (T-2), aflatoxins (AFs), ochratoxin A (OTA) and fumonisins (FB_s). Contamination with mycotoxins can occur in the field and may continue during storage, transportation and processing. Methods for the detection of mycotoxins are available and can be mainly divided into immunochemistry- and chromatography-based techniques. The main aim of the research in this thesis, was to develop a multiplex immunoassay method capable of detecting the main 6 mycotoxins, and their modified forms, in grain-based feed and food commodities. For the development of the mycotoxin multiplex immunoassays, we implemented the multi-Analyte profiling (xMAP) technology from Luminex. This xMAP technology combines unique color-encoded microspheres in a suspension array format, which can be analysed by dedicated analyser platforms. Using the xMAP technology, a range of targets can be detected in one single sample, in one single well, with one single measurement. The developed multiplex should be able to screen for the main six mycotoxins at food and feed ML levels in a wide range of matrices.

In the introduction (**Chapter 1**), a more detailed look into the main mycotoxins is provided. Their history, toxicity, occurrence and EU recommendations and

legislation are summarized. A special focus is on the modified mycotoxins. These are the free forms of mycotoxins that are converted by living organisms like plants, bacteria and fungi. Their relevance and occurrence is shortly explained. In the presented research, barley is assessed as a food ingredient, while beer is assessed as a food product. Therefore, a closer look at the formation of mycotoxins in field barley and in malt production, and their transfer to beer is presented. The malting and brewing procedures are explained and the differences between industrial and craft beer is explained. Since antibodies are the key biorecognition molecules for the presented immunoassays in this thesis, they are shortly explained, followed by their implementation in the mycotoxin detection immunoassays. The previously mentioned xMAP technology, and how it was used for developing the multiplex assays in this thesis, are explained in detail. Last, but not least, the confirmatory instrumental analysis, which played an important role in this thesis, is explained.

In **chapter 2,** a semi-quantitative multiplex competitive immunoassay was developed for the detection of ZEN, DON, T-2, AFs, OTA and FB_s. In this format, protein-mycotoxins conjugates were coupled to unique microspheres, who were then incubated with free mycotoxins (in samples) and mycotoxin specific antibodies. For readout, the LX100 flow cytometer was used. The developed method showed no cross-interactions between the single assays. The developed 6-plex indirect immunoassay was applied to contaminated feed reference samples and inhibitions in the indirect 6-plex were compared to confirmatory instrumental analysis results of the same reference samples. Due to the discontinuation of the mycotoxin-protein conjugates, we had to look for new opportunities to continue our work. Instead of trying to find a new supplier for the mycotoxin-protein conjugates, the idea of producing fluorescent mycotoxin-protein conjugates was pursued. OTA, ZEN and FB_1 were coupled to R-Phycoerythrin (RPE), turning the indirect format into a direct format (**chapter 3**). With these 3 conjugates, a 3-plex xMAP immunoassay for the simultaneous detection of OTA, ZEN and FB_1 in grains

was developed. In the direct format, monoclonal antibodies (mAbs) were coupled to the microspheres and incubated with free mycotoxins and the fluorescent mycotoxin-conjugates. No significant cross-interactions were observed and the cross-reactivity with relevant metabolites and masked mycotoxins was determined. The triplex assay was applied to maize and wheat reference materials. The 3-plex results for OTA and ZEN were in good agreement, but serious overestimations were observed for FB_1. The 3-plex assay was also tested on a faster and higher throughput flow cytometer (FM3D). Besides faster measurements, the sensitivities for all assays were the same as on the LX100. All three assays were influenced by the sample matrix of cereal extracts which showed the need for matrix-matched calibrations.

As a next step, the developed direct 3-plex method was extended to a 6-plex, by the implementation of three new antibody-coupled microspheres and three new RPE conjugates: DON-RPE, T2-RPE and AFB_1-RPE. Additionally, we made a switch to a new detection platform, by introducing the MAGPIX planar array analyser (**Chapter 4**). All new reagents showed no relevant cross-interactions between the assays and cross-reactions with available mycotoxin metabolites and modified mycotoxins were determined. The new 6-plex direct multiplex was applied to barley samples, for a successful in-house validation as a screening method. The cut-off factors were set at 50% of the EU maximum levels (MLs) for food: 2 μg/kg for AFB_1, 2.5 μg/kg for OTA, 625 μg/kg for DON, 50 μg/kg for ZEN and 1000 μg/kg for FB_1. For T-2 a provisional ML of 25 μg/kg was applied, which complied with the EU preferred limit of analytical screening for T-2/HT-2. The validation showed very high inter and intra-day precision for all samples. The 6-plex was also used to screen available barley and malted barley reference materials. The developed 6-plex assay showed potential for future implementation as a semi-quantitative pre-screening method for regulated mycotoxins, prior to instrumental analysis (like LC-MS/MS). The introduced MAGPIX showed comparable results to the previously used flow cytometers.

In **Chapter 5** the development of a simplified portable paramagnetic microsphere-based semi-quantitative 4-plex, for the on-site detection of the mycotoxins OTA, ZEN, DON, T2 and HT-2, is presented. Since the MAGPIX planar array system is easily transported to the point-of-need, we tried to simplify the method from Chapter 4, by simplifying some laboratory methods that were part of the 6-plex. The simplifications introduced comprised; washing steps by a handheld magnetic plate instead of an automated washer, addition of pre-mixed reagents from dropper bottles, instead of laboratory pipettes, addition of samples by disposable fixed volume micropipettes, and a sample extraction time of just 1 minute, additionally omitting centrifugation. Initial preliminary in-house validation, using a laboratory sample extraction procedure, of the simplified 4-plex screening assay was successful at half the EU maximum levels and generally showed excellent inter and intra-day precisions. Preliminary in-house validation of the 4-plex screening assay, using the simplified on-site extraction procedure, was successful at EU maximum levels. The simplifications introduced, were a promising first step in taking the developed 4-plex screening assay from the laboratory to a point-of-need, for future on-site semi-high throughput screening of food and feed commodities.

In **Chapter 6**, the previously developed 6-plex (from Chapter 4) was implemented as a mycotoxin screening tool, in a unique global beer survey. Besides the high number of screened samples, 1000 in total, there was a really strong focus on beers from the emerging craft beer scene. Besides the application of the 6-plex, a confirmatory analysis method (LC-MS/MS) was developed and applied to a selection of samples based on the screening results. Confirmatory analysis of screening results proved to be relevant, since the 6-plex screening showed discrepancies with the LC-MS/MS analysis. This was possibly due to matrix interference and/or the presence of unknown mycotoxin metabolites. The major mycotoxins detected were DON and its plant metabolite deoxynivalenol-3-β-D-glucopyranoside (D3G). The 6-plex immunoassay reported the sum of DON and D3G (DON+D3G)

contaminations ranging from 10 to 475 μg/L in 406 beers, of which 73% were craft beers. The popular craft beer style imperial stout, had the highest percentage of samples suspected positive (83%) with 29% of all imperial stout beers having DON+D3G contaminations above 100 μg/L. LC-MS/MS analysis showed that industrial pale lagers from Italy and Spain, predominantly contained FBs (3 – 69 μg/L). Besides FBs, some African traditional beers also contained aflatoxins (0.1 - 1.2 μg/L). The presence of OTA, T-2, HT-2, ZEN, β-zearalenol, 3/15-acetyl-DON, nivalenol and the conjugated mycotoxin zearalenone 14-sulfate were confirmed in some beers. Chapter 6 shows that in 27 craft beers, DON+D3G concentrations occurred above (or at) the Tolerable Daily Intake (TDI) level, which may have a health impact.

The increased relevance of modified mycotoxins, and their inclusion in the process of the development of the presented mycotoxin multiplexes in this thesis, showed the importance of having access to novel reference substances. In **Chapter 7**, a first attempt was made to be self-sufficient in the supply of modified mycotoxins, by the introduction of *Cunninghamella* strains as bioconjugation models. Liquid media cultures of *Cunninghamella* were fortified with a range of mycotoxins. The chosen *Cunninghamella* strains were able to produce a range of ZEN metabolites, including zearalenone-14-β-D-glucopyranoside (Z14G), zearalenone-16-β-D-glucopyranoside (Z16G) and zearalenone-14-sulphate (Z14S). Additionally, we were able to steer the bioconjugation process in the direction of the ZEN-glycosides, by introducing a sulphate-depleted medium. From 2 mg of ZEN, in sulphate-depleted medium, 1.94 mg of Z14G and 0.45 mg of Z16G was produced by *Cunninghamella elegans*, while in the standard growth medium, almost all ZEN was modified to Z14S. Both Z14G and Z16G were identified and confirmed by ^{1}H and ^{13}C NMR and High-Resolution Mass Spectrometry (HRMS). Addition of other main mycotoxins to *Cunninghamella* cultures, resulted in the modification of T-2 to HT2-toxin and hydroxy-T2 Toxin, while OTA was very effectively modified to hydroxy-OTA.

Chapter 8 discusses the strengths, shortcomings of, and possible improvements to, the developed mycotoxin multiplexes. Possibilities for the extension of the current 6-plex are discussed and the focus is mainly on emerging mycotoxins. Additionally, other targets than mycotoxins, like pesticides are discussed for inclusion based on their relevance. The xMAP technology is also critically compared to other technologies that are capable of detecting mycotoxins, or are platforms suitable for developing mycotoxin detection assays. Since the EU wants to phase out the use of laboratory animals for the production of antibodies, the generation of new mycotoxin antibodies is under pressure. Therefore different (bio)recognition molecules are presented and their suitability for implementation in standard immunoassay-based approaches is discussed. Chapter 8 also focuses in more detail on the high DON+D3G contaminations in the craft beer style (Russian) imperial stout (RIS), that were found in the survey presented in Chapter 6. The transfer of mycotoxins from contaminated malts to a final RIS beer are defined on the basis of a simple gravity-based calculation model. The calculations are based on an actual RIS recipe from a Dutch craft brewer. The applied calculations are the basis for discussions regarding better-defined MLs for malts and/or MLs specifically defined for beer.

Curriculum Vitae

Jeroen Peters was born on August 13th 1967 in the city of Oss, The Netherlands. He followed a bachelor study in Biochemistry at the HAN University of Applied Sciences and graduated in 1990. After his study he fulfilled his military service, after which he started working at Wageningen University and Research (CPRO-DLO). In the cell biology department, he worked on the identification of cold-tolerance proteins of tomato, by comparative 2D gel electrophoresis. After a switch to the molecular biology department, he worked on the isolation of proteases and protease inhibitors from plants and insects, and the production and purification of protease inhibitors expressed in yeast and E.coli. At both, the molecular biology and developmental biology department he isolated genes and regulatory elements for building plant based expression-systems, for the localized secretion and production of proteins. With a strong desire to combine his passion for biochemistry and molecular biology and also look into new detection technologies within one research group, he made a switch to the Biointeractions department. Together with Mr. J. Bergervoet, he developed a range of molecular and immunochemical detection techniques for plant pathogenic bacteria and viruses, and additionally started implementing flow cytometry as a detection tool. It was in this department that he was introduced to the xMAP microsphere flow cytometric multiplex technology, that would become an essential part of this thesis. After a joint project with Dr. W. Haasnoot, he decided to move to Wageningen Food Safety Research (then RIKILT – Institute of Food Safety). Under the supervision of Dr. W. Haasnoot and Prof. Dr. M.W.F Nielen he started to focus on the multiplex detection of mycotoxins and modified mycotoxins. This eventually led to the publication of 5 manuscripts, all presented in this thesis. Currently he works as a researcher in the Bioassays and Biosensors group, focussing on the development of biorecognition-assays on a wide range of detection formats for food, feed and environmental contaminants. He specialised in the validation and application

of developed (multiplex) biosensors for the detection of pesticides, mycotoxins, drug residues (antibiotics) and marine biotoxins. He is currently the Work Package leader of the multiplex diagnostics group that focusses on contaminants and quality factors in the milk production chain within the H2020 MOLOKO project. He is also Work Package leader for the H2020 h-ALO project that focusses on portable rapid biosensing of heavy metals and pesticides in the Farm to Fork chain. A major part of his current research focusses on the immunochemical detection of pesticides harmful to bees, where the Institute of Pesticide and Environmental Toxicology of the Zhejiang University is an important partner. This research has led to the participation in the innovation work package of the H2020 B-GOOD project for healthy and sustainable beekeeping within the European Union and a Public Private Collaboration project with international plant trading companies, for developing point of need pesticide testing for transparent and sustainable production chains. He recently became an official international board member of the Food and Agricultural Immunology journal.

List of publications

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Acknowledgments

It has been a long ride, with many distractions, but I'm very happy that this thesis is finally a physical book. I would like to express my sincere gratitude to all the people who were involved in some way, actively or in the background.

Michel, thank you for being patient with me, especially for the moments where I was putting more effort into WFSR than into the PhD thesis. Highly appreciated. I remember that, no matter in what mood I entered your office, I always left it feeling positive and full of energy, ready to tackle any small or big problem that may have arisen. What I also remember, is the amazing speed with which you returned the revised manuscripts. Your comments were thought-provoking, with my absolute favourite being "grapjas". Under your supervision I have grown a lot as a researcher, and for that, I will always be grateful. Additionally, I would like to thank Han for welcoming me in his group and his critical contribution to the introduction and discussion of this thesis.

Willem, our pleasant interaction was the main reason I moved from Plant Research International (now WPR) to RIKILT (now WFSR). I remember the travels for the European projects, where you taught me the power of networking. Besides that, I really enjoyed our nice dinners and of course the evening walks in beautiful cities. I thank you for stimulating me, mostly with compliments and for convincing me to do this PhD.

Toine, I have to thank you for many things, maybe most of all for keep on pushing me to focus a bit less on WFSR and finally finish my thesis. I really appreciate how you sincerely monitored my well-being and for telling me to slow down at times. In addition, I would also like to thank Leen van Ginkel. I really felt your support and appreciate how you shaped/created a familiar environment where the Biosensors team could start a new life.

Jan B, besides a good friend and great colleague, I will always remember you as the person who bought the first Luminex analyzer in the Netherlands, because you strongly believed in its potential outside its medical applications. We both worked very hard to get it accepted as a powerful multiplex detection platform for microorganisms, and it makes me proud when I see how successful you managed to make it.

Nathalie. All of a sudden we saw ourselves with new responsibilities for the Biosensors team, which was a real challenge. But in the end, we managed very well. I really like how we openly discuss about a lot of matters, even if our opinions are sometimes miles apart. Besides that, I really appreciate you as a friend and how that never mixes with discussions we have about work.

I would like to thank my former colleagues Anniek, Dick, Susann, Adil, Anastacia, Sabina, Gerardo, Monique Ploum, Monique Bremer, Rubing (Ruby) but of course also the current colleagues in the Biosensor team and labs Richard, Alexander, Linda, Erik, Geert, Liza, Anouk, Gina, Gert, Ariadni, Xiaodie, Astrid, Gerlof, Piet, Andries and Qiaofeng (CiaoFun) and all other members of the B&B team. I also would like to thank my former students: Alex, Darren, Diana Chan, Alice, William James, William, Benjamin, Edward and Mang. Without a doubt, you were all dedicated and worked very hard. Some of your results found their way to the publications in this thesis, while other results were important to create new insights.

I would like to thank Ruud van Dam, Paul Zoomer and Ed Boers for their assistance in the performance of all instrumental analysis in this thesis. Additionally, I want to thank Theo de Rijk for always being easily approachable to discuss new ideas. For help beyond the call of duty, I would like to thank Arjen Gerssen. Even though you were very busy, you were always willing to help. Much appreciated. Monique de Nijs, thanks for your helpful comments throughout the time I was working on mycotoxins, and for always informing about my family. Very kind.

I would like to thank craft brewers Menno Olivier, Vincent Gerritsen and Niels Beekhuizen as project partners and advisors. My brewer friends from Catalunya, Quionia Pujol and Oscar Mogilnicki, for fruitful discussions and the supply of the pictures for this thesis. I deeply admire your full-circle brewing concept! Additionally, I would like to thank Ronald van Doorn for being a walking craft beer encyclopedia and a dear friend.

Of course I have to thank all of my close friends who enrich my life in many ways. No need for names, you know who you are.

Papa. You are the reason I got interested in science. I still remember how you built a "do it at home" chemistry set for me (thanks Organon). That really sparked my passion for chemistry and eventually was the reason I chose a biochemistry education. Your ongoing passion for science still amazes me every time we see each other. Stacks of new books about all kinds of science. I know you were really looking forward to the moment this thesis would (finally) be finished. Most of all I just would like to thank you for being a great dad and a great grandfather.

Ilja, lelijke zus ☺, without a doubt the best and sweetest sister in the world. Our relationship is effortless. We do not even need words to keep laughing for hours and just feel good.

Eva, Luna and Diana. Le donne più importanti della mia vita. Thanks for our warm and safe home, all the fun and for putting my feet back on the ground when necessary. Eva, thanks for your contribution to the cover of this thesis, love it! Diana, life was hard for you in the last 2 years during the covid crisis. Still you managed to stay strong and went from mother, to life-coach, to teacher and much more for our daughters. Words can't describe the admiration I have for you. Grazie per tutto.

Finally, I would like to dedicate this work to the sweet memory of my mother and stepmother. Miss you both, every day, still.

Overview of completed training activities

Discipline-specific activities	Organizer	Year
34th Mycotoxin Workshop	Society for Mycotoxin Research	2012
WMF meets IUPAC	World Mycotoxin Forum	2012
Planet xMAP	Luminex	2012
Recent Advances in Food Analysis (RAFA)	UCT/WFSR	2013
Advanced food analysis (4th edition)	VLAG	2013
35th Mycotoxin Workshop	Society for Mycotoxin Research	2013
LC-MS/MS course	WFSR	2013
Multiplex technology and ELISA training day	Evira Helsinki	2013
xSAMPLES Rotterdam	Luminex	2013
xSAMPLES Lisbon	Luminex	2013
Mycotoxin Summer Academy	IFA-Tulln	2014
Rapid Methods Europe	Bastiaanse communications	2018
xMAP connect Amsterdam	Luminex	2019

General courses	Organizer	Year
Writing for Academic Publication	L. McPhee	2010
Project management course	Wageningen Business School	2012
Techniques for writing and presenting a scientific paper	Wageningen Graduate schools	2012
Project and Time Management	Wageningen Graduate schools	2013
Personal Efficiency Program	PEP worldwide	2015
Advanced Course Guide to Scientific Artwork	WUR Library	2015
Intellectual Property Licensing	Forum Institute	2018
China Business Culture Training	Wageningen University & Research	2019
Advisory Acquisition	Hart voor Projecten	2021

Optionals	Organizer	Year
Preparing PhD research proposal	Wageningen Food Safety Research	2011
Colloquia	Organic Chemistry	2012-2016
Cluster/BU meetings	Wageningen Food Safety Research	2012-2021
Organizing WUR Luminex meetings	Wageningen Food Safety Research	2012-2014

The research described in this thesis was funded by the Dutch Ministry of Economic Affairs, SenterNovem and the Province of Gelderland (PID091008/2.6A).

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