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DISSERTATION

Development of DNA-based techniques for the rapid analysis of trichothecene and fumonisin producing *Fusarium* species and aflatoxins in maize

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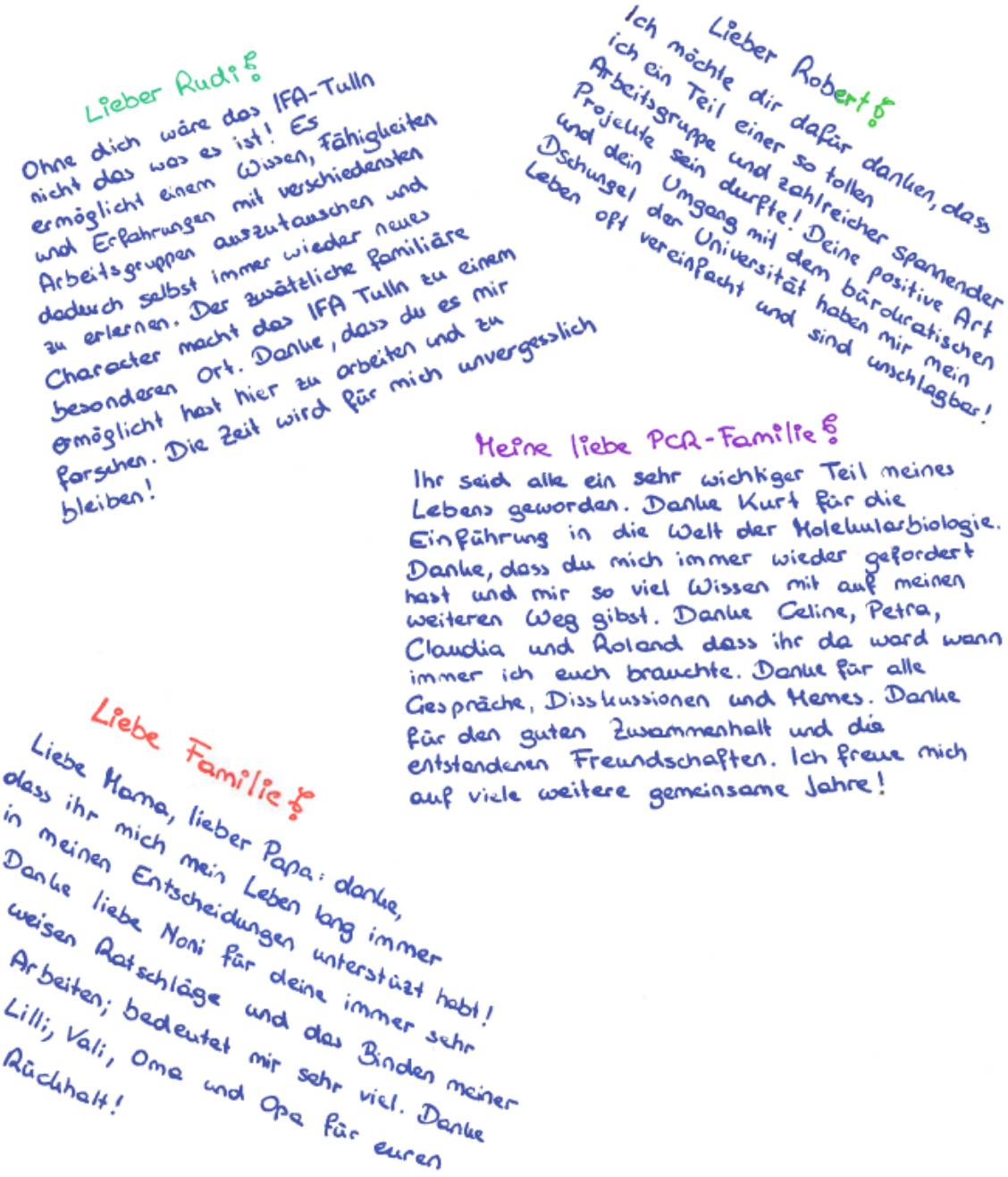
Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die Dissertation selbstständig verfasst, andere als angegebene Quellen und Hilfsmittel nicht benutzt und mich auch sonst keiner unerlaubten Hilfe bedient habe.

Tulln, August 2015 Viktoria Preiser

Danksagung



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Glossary

3-ADON	3-acetyl-deoxynivalenol
6FAM	6-carboxfluorescein
AB	antibody
ACN	acetonitrile
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
ATBR	aptamer-target binding readout
AuNP	gold nanoparticle
BHQ1	black hole quencher® 1
BSA	bovine serum albumine
CD	circular dichroism
CMO	carboxymethylhydroxylamine
C_Q	quantification cycle
CTAB	cetyltrimethylammonium bromide
Cy5	indodicarbocyanine
D3G	deoxynivalenol-3-glucoside
DAS	deacetoxyscirpenol
DIG	digoxigenine
DMF	dimethylformamide
DNA	desoxyribonucleic acid
DON	deoxynivalenol
dsDNA	double-stranded DNA
EDC	N-(3-dimethylaminopropyl)-N-carbodiimide
ELAA	enzyme linked aptamer assay
ELEM	equine leukoencephalomalacia
ELISA	enzyme linked immunosorbent assay
EURL	European Union Reference Laboratories
FAO	Food and Agriculture Organization

FAS	fatty acid synthases
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FDA	Food and Drug Administration
FLD	fluorescence detection
FRET	fluorescence resonance energy transfer
GMO	genetically modified organism
HEGL	hexaethylene glycol
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IARC	International Agency for Research on Cancer
IFA	Interuniversitäres Department für Agrarbiotechnologie
JOE	6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein
K_d	dissociation constant
LC-MS/MS	liquid chromatography tandem mass spectrometry
LFD	lateral flow device
LOQ	limit of quantification
MS	mass spectrometry
NA-LFD	nucleic acid lateral flow device
NEO	neosolaniol
NGS	next generation sequencing
NIV	nivalenol
NOR	norsolorinic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphor buffered saline
PKS	polyketide synthase
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RSD	relative standard deviation
RT	room temperature
SELEX	systematic evolution of ligands by exponential enrichment
ssDNA	single-stranded DNA

TAR	trans-activation response
TLC	thin layer chromatography
T_m	melting temperature
TNBSA	2,4,6-Trinitrobenzene Sulfonic Acid
UV	ultra violet
VER A	versicolorin A
VER B	versicolorin B
WHO	world health organisation
Z14G	zearalenone-14-β-D-glucopyranoside
ZEN	zearalenone

Abstract

The overall objective of this thesis was the development of DNA-based techniques for the rapid analysis of *Fusarium* species and aflatoxins in maize. The focus was laid on the mycotoxin classes trichothecenes and fumonisins as the most important mycotoxins produced by *Fusarium* sp. as well as aflatoxin B_1, produced mainly by *Aspergillus* sp., which is considered to be the most potent natural carcinogen known. Although tests for such analytes are available, many of these methods are time-consuming, cost-intensive and difficult to transfer into a more field-applicable approach.

The first part of the thesis addresses the development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize. The plant pathogenic fungus *Fusarium* causes considerable economic impact worldwide. The kernel size and weight are usually reduced but even more important are the numerous toxic metabolites produced by these fungi during the colonization of the plant. These mycotoxins have been related to toxic effects upon ingestion by humans and animals. Mycotoxin concentrations in infected plants can vary significantly between maize cultivars and are usually higher in susceptible plants than in more resistant cultivars. Hence, the determination of the resistance of new maize varieties is of high importance and usually combines anaylsis of mycotoxins by enzyme linked immunosorbant assay (ELISA), high performance liquid chromatography (HPLC) coupled either to ultra-violet (UV) and/or mass spectrometry (MS) detection methods and the visual scoring of disease symptoms. However, these methods are time-consuming, cost-intensive (especially HPLC-MS) and only indirect, because they provide no information about the biomass of a fungus in the sample. A new method to gain direct information about the DNA biomass of a fungus is the quantitative polymerase chain reaction (qPCR) technique, which is based on the quantification of the amount of organism specific DNA. Hence, an infection can be detected before any symptoms are visible. Besides the evaluation of the resistance of varieties, qPCR can be applied for *Fusarium* monitoring projects and as a screening method for food and feed contamination. In this study a sensitive quantification method for trichothecene and fumonisin producing *Fusarium* species in maize has been developed. This method enables the high-throughput screening of a large number of samples for *Fusarium* infection in relatively short time due to simultaneous quantification of the mycotoxin-related genes *tri5* (encoding for the fungal trichodiene synthase) and *fum1* (encoding for a polyketide synthase), responsible for the production of trichothecenes and

fumonisins, respectively. The newly developed multiplex method was applied to 24 maize field samples collected in Austria. Results obtained with the triplex assay were compared to the three singleplex qPCR runs to ensure that no loss of sensitivity occurs by using the simplified multiplex method. The new assay was found to be specific for either fumonisin or trichothecene producing *Fusarium* species. A limit of quantification (LOQ) of 0.32 pg DNA/µl for both *Fusarium* strains was found. Considering a genome size of 41.7 Mb for the fumonisin producing *Fusarium* strain *F. verticillioides* and a genome size of 36.2 Mb for the trichothecene producing *Fusarium* strain *F. graminearium* this represents approximately seven or eight genome equivalents, respectively. All samples were further analyzed for the trichothecenes deoxynivalenol (DON), DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) by liquid chromatography tandem mass spectrometry (LC-MS/MS) and compared with the qPCR results. This assay is the first report of the use of a multiplex qPCR for the quantification of trichothecene and fumonisin producing *Fusarium* species and was published in *Analytical Methods* (Appendix publication #1).

Aflatoxins represent another important class of mycotoxins. They are mainly produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1 (AFB_1) is the most prevalent and potent one in the class of aflatoxins. It possesses high toxicity and was classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC). Regulatory limits have been introduced for food and feed safety reasons in many countries and range from 1 µg/kg to 20 µg/kg. Therefore, rapid, sensitive and inexpensive analytical techniques are essential to detect and quantify AFB_1. Procedures based on chromatographic methods combined with MS and rapid screening approaches with immunoassays have been developed. However, these methods show some major drawbacks such as the requirement of skilled personnel, expensive equipment and sample pre-treatment, and the use of antibodies. Due to the limitations of antibodies used in immunoassays such as thermal instability, laborious and expensive production, aptamer-based analytical methods have been developed offering a promising alternative to antibodies. Aptamers with high affinity to AFB_1 have been developed in part two of this study. They have been selected using an iterative selection procedure named Systematic Evolution of Ligands by EXponential enrichment (SELEX) for their ability to bind to AFB_1 with high affinity and specificity. Sequences were obtained after four rounds of in vitro selection and were furthermore screened for their ability to bind AFB_1. Five unique sequences were obtained and additionally

characterized. The dissociation constant for each aptamer was determined by a fluorescence binding assay and was found to be 1.30 µM ± 0.14 µM for aptamer 1 (named Lib5_1e), 5.70 µM ± 0.54 µM for aptamer 2 (named Lib5_6e), 2.34 µM ± 0.13 µM for aptamer 3 (named Lib5_7e), 2.56 µM ± 0.13 µM for aptamer 4 (named Lib5_9e), and 4.95 µM ± 0.95 µM for aptamer 5 (named Lib5_10e). For AFB_1 biosensor development approaches a DNA fragment designed by Neoventures Biotechnology Inc. (Canada) in 2009 was used. Until now, a few aptasensors, based on this aptamer, for the rapid detection of AFB_1 have been developed. These assays are currently based on an indirect target detection format where a signal is produced if no target is present and *vice versa*. This study reports a new direct detection format for the target molecule AFB_1 with a nucleic acid lateral flow test strip. The idea claims AFB_1 induced unwinding of the aptamer's distinctive structure exposing a binding site for a complementary signaling DNA probe enabling the hybridization of the probe with the aptamer. Different incubation temperatures responsible for hybridization/dehybridization have been evaluated (room temperature, 37 °C, and 40 °C). The test results revealed that a temperature of 37 °C worked best. The developed aptasensor model demonstrates the potential of the structure switching ability of aptamers in terms of direct target detection. To the best of our knowledge, a dipstick assay based on an aptamer enabling an AFB_1 direct detection format has not yet been reported. A manuscript has been submitted to the journal *Analytical Methods* (Appendix publication #2).

In conclusion, within this thesis a multiplex qPCR method for the rapid screening of maize samples for all *Fusarium* species producing the most prevalent mycotoxins in maize, trichothecenes and fumonisins, have been developed. Furthermore, a rapid and simple aptamer dipstick assay using a direct detection format for AFB_1 has been designed. However, further optimization to increase the sensitivity and to speed-up the testing procedure is required.

Deutsche Kurzfassung

Ziel dieser Arbeit war die Entwicklung DNA-basierender Techniken für die rasche Analyse von Pilzen der Gattung *Fusarium* sowie Aflatoxin in Mais. Der Fokus wurde einerseits auf die wichtigsten Toxingruppen die von *Fusarium* Arten produziert werden gelegt. Dies umfasst Trichothecene und Fumonisine. Ein weiteres Kerngebiet dieser Arbeit umfasst die rasche Detektion des Mykotoxins Aflatoxin B_1, welches hauptsächlich durch *Aspergillus* Arten produziert wird und das stärkste, natürlich vorkommende Kanzerogen darstellt. Obwohl durchaus Testsysteme für diese Analyten existieren, sind viele der dabei angewandten Methoden zeitintensiv, kostspielig und lassen sich kaum als vor-Ort-Analysen einsetzten.

Das erste Kapitel behandelt die Entwicklung einer multiplex real-time PCR Methode zur Quantifizierung von *Fusarium* DNA Trichothecen und Fumonisin produzierender Arten in Mais. Der pflanzenpathogene Pilz *Fusarium* führt weltweit zu erheblichen wirtschaftlichen Einbußen. Die Korngröße sowie das Gewicht werden bei Befall reduziert, jedoch weitaus problematischer ist die mögliche Produktion einer Vielzahl toxischer, sekundärer Metaboliten. Diese Mykotoxine stehen in Verbindung mit Vergiftungserscheinungen bei Aufnahme durch Mensch und Tier. Der Mykotoxingehalt befallener Maissorten kann erheblich schwanken und ist in empfindlichen Sorten generell höher als in Pflanzen mit guter Resistenz. Daher ist eine genaue Beurteilung resistenter Maissorten hilfreich. Die Resistenzbewertung umfasst üblicherweise die Bestimmung des Mykotoxingehaltes durch Enzyme linked immunosorbent assay (ELISA), Hochleistungsflüssigkeitschromatographie (HPLC) gekoppelt mit einem ultra-violett (UV) Detektor und/oder einem Massenspektrometer und die visuelle Abschätzung des Toxingehalts durch die Bewertung der ausgebildeten Symptome. Diese Methoden sind sowohl zeit- als auch kostenintensiv und liefern keinerlei Informationen über die gebildete Pilzbiomasse. Eine neue Methode zur Bestimmung des Befallsgrades einer Pflanze bietet die quantitative Polymerase Kettenreaktion (qPCR). Sie ermöglicht das Erkennen einer Infektion noch bevor es zur Ausbildung von Symptomen kommt. Neben dem Einsatz zur Resistenzbewertung kann die qPCR auch für Monitoring Projekte sowie als Screeningmethode für Lebens- und Futtermittelkontaminationen eingesetzt werden. In dieser Arbeit wurde eine Methode zur Quantifizierung für Trichothecen und Fumonisin produzierende *Fusarium* Stämme in Maisproben entwickelt. Die Methode ermöglicht die Untersuchung einer Vielzahl an Proben in relativ kurzer Zeit durch die parallele Detektion der Gene *tri5* und *fum1*, welche jeweils für die Produktion von Trichothecenen beziehungsweise

Fumonisinen notwendig sind. Die entwickelte Methode wurde anhand von 24 natürlich infizierten Maisproben evaluiert. Alle Ergebnisse der multiplex Methode wurden mit den Einzelnachweisen verglichen und in Bezug auf ihre Empfindlichkeit untersucht. Es zeigte sich, dass der entwickelte Nachweis sowohl spezifisch für Fumonisin als auch für Trichothecen produzierende *Fusarium* Stämme ist. Die Bestimmungsgrenze liegt für beide *Fusarium* Stämme bei 0.32 pg DNA/µl. Des Weiteren wurden die Trichothecene Deoxynivalenol (DON), DON-3-Glucosid (D3G), Nivalenol (NIV), 3-Acetyl-DON (3-ADON), T-2 Toxin, HT-2 Toxin, Diacetoxyscirpenol (DAS) und Neosolaniol (NEO) sowie die Fumonisine Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2) und Fumonisin B_3 (FB_3) durch Flüssigkeitschromatographie gekoppelt an Tandem-Massenspektrometrie bestimmt und in Korrelation mit den qPCR bestimmten Ergebnissen gesetzt. Die entwickelte Methode ist der erste multiplex qPCR Test zum quantitativen Nachweis Trichothecen und Fumonisin produzierender *Fusarium* Stämme. Die Studie wurde im Journal *Analytical Methods* publiziert (Appendix Publikation #1).

Eine weitere wichtige Klasse der Mykotoxine sind unter anderem Aflatoxine. Diese werden vorwiegend von der Gattung *Aspergillus* und den darin enthaltenen Arten *Aspergillus flavus* und *Aspergillus parasiticus* produziert. Vorherrschend ist dabei Aflatoxin B_1. Es ist hoch toxisch und wurde von der International Agency for Research on Cancer (IARC) als kanzerogen für Menschen klassifiziert. Zum Schutz der öffentlichen Gesundheit wurden bereits in zahlreichen Ländern Grenzwerte festgelegt, welche sich im Bereich zwischen 1 µg/kg und 20 µg/kg befinden. Dieser Bereich muss zuverlässig nachgewiesen werden können und führt zu der immer stärker steigenden Nachfrage an schnelle, kostengünstige Methoden zur Detektion von AFB_1. Die zurzeit bestehenden Methoden basieren auf chromatographischen Verfahren gekoppelt an Massenspektrometrie sowie immunoanalytische Verfahren als rasche Screeningmethoden. Obwohl diese Methoden seit Jahrzehnten etabliert sind, weisen sie bedeutende Nachteile auf. Die benötigten Geräte verursachen hohe Kosten, müssen von geschultem Personal bedient werden und vor-Ort-Tests sind damit kaum realisierbar. Auch die Verwendung von Antikörpern geht mit einigen Einschränkungen einher. Antikörper sind thermisch labil und die Produktion ist aufwendig und kostenintensiv. Aptamer-basierte analytische Methoden bieten eine vielversprechende Alternative zu Antikörpern. In dem zweiten Teil dieser Arbeit wurden Aptamere mit hoher Affinität zu dem Zielmolekül AFB_1 entwickelt. Die Aptamere wurden mit einer Prozedur namens SELEX (Systematic Evolution of Ligands by EXponential enrichment) gewonnen. Nach vier SELEX Runden wurden die erhaltenen Sequenzen auf ihre Affinität zu AFB_1 evaluiert. Fünf

Aptamere wurden anschließend in Bezug auf ihre Dissoziationskonstante und ihr Faltungsverhalten charakterisiert. Es wurden Dissoziationskonstanten von 1.30 µM ± 0.14 µM für Aptamer 1 (Lib5_1), 5.70 µM ± 0.54 µM für Aptamer 2 (Lib5_6e), 2.43 µM ± 0.13 µM für Aptamer 3 (Lib5_7e), 2.56 µM ± 0.13 µM für Aptamer 4 (Lib5_9e) und 4.95 µM ± 0.95 µM für Aptamer 5 (Lib5_10e) ermittelt. Für die ersten Versuche zur Entwicklung eines Biosensors (Aptasensors) zur raschen Detektion von AFB_1 wurde ein DNA Fragment entwickelt von Neoventures Biotechnology Inc. (Canada) verwendet. Bereits bestehende Aptasensoren basieren auf einem indirekten Nachweisformat. Dabei entsteht ein Signal nur in der Abwesenheit des Zielmoleküls und *vice versa*. Im Rahmen dieser Dissertation ist ein erster Ansatz eines Teststreifens, basierend auf einem direkten Nachweisformat von AFB_1, beschrieben. Dahinter befindet sich die Idee, dass AFB_1 zu einer Strukturänderung des Aptamers führt. Dabei kommt es zur Freilegung einer Bindungsstelle und im darauffolgenden Schritt zum Binden einer signalgebenden Sonde. Um die geeignete Temperatur zum Binden (hybridisieren) und Lösen (dehybridisieren) dieser Sonde zu ermitteln, wurden die Temperaturen Raumtemperatur, 37 °C und 40 °C getestet. Die Ergebnisse zeigten, dass sich 37 °C am besten eignen. Der vorgestellte Ansatz bietet ein neues, noch einzigartiges direktes Nachweisformat für AFB_1 mit sofortiger Visualisierung *via* Teststreifen. Die ersten Ergebnisse wurden bei dem Journal *Analytical Methods* eingereicht (Appendix Puplikation #2).

Zusammenfassend wurde in dieser Dissertation eine multiplex qPCR Methode zur raschen Detektion der wichtigsten *Fusarium* Arten auf Mais, welche für die Produktion von Trichothecenen und Fumonisinen verantwortlich sind, entwickelt. Außerdem wurde ein erster Ansatz für ein direktes Nachweisformat für AFB_1 mittels Teststreifen gezeigt. Die Möglichkeit von Aptameren ihre Struktur verändern zu können trägt großes Zukunftspotential, allerdings sind für einen Prototypen und eine zukünftige kommerzielle Version noch zahlreiche Optimierungsschritte notwendig.

General Introduction

The introduction aims to provide readers who are not familiar with the field of study with basic information about the four main parts of the thesis. First, readers are introduced in the field of mycotoxins. Therefore, general aspects such as definitions and outbreaks attributed to mycotoxins in history as well as major mycotoxin classes and the legislative situation are addressed. Furthermore, essential genetic background information about mycotoxin production of the mycotxins trichothecenes, fumonisins and aflatoxins are given in part one. The second part deals with commonly used analytical methods for the identification and quantification of mycotoxins in food, feed and other biological matrices. Finally, recent advancements in molecular diagnostic methods are described. This includes the quantitative PCR (qPCR) techniques (part 3) based on the quantification of the amount of organism specific DNA as well as new analytical methods based on so called “aptamers” (part 4); a very promising tool in terms of simplicity with comparable performance to antibody tests.

1 Mycotoxins

1.1 General aspects

Mycotoxins are natural secondary metabolites produced by various filamentous fungi on agricultural commodities[1]. The most potent mycotoxins are produced by certain strains of the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Claviceps*. So far, several hundred different mycotoxins have been discovered with aflatoxins (mainly produced by *Aspergillus* sp.), ochratoxins (produced by *Aspergillus* sp. and *Penicillium* sp.), trichothecenes, fumonisins and zearalenone (produced by *Fusarium* sp.), patulin (produced by *Penicillium* sp.), and ergot alkaloids (produced by *Claviceps* sp.) as the most important ones. These compounds have been related to toxic effects upon ingestion by animals and humans[2]. Mycotoxins have a wide range of actions on animals and humans and can cause acute toxic effects like vomiting or diarrhea or chronic effects which are generally irreversible[3]. Symptoms depend on factors like the type of the mycotoxin, the host plant (e.g. health constitution, sex, age, etc.), as well as the length and dose of exposure[4].

Mycotoxin producing fungi can colonize the living plant at the field (preharvest) or during storage, transport, and processing (postharvest)[1]. Fungal growth and mycotoxin production are influenced by a series of complex factors and vary in the field more than compared to postharvest colonization of agricultural crops. The main conditions affecting the formation of mycotoxins are broadly related to the fungus itself including its constitution, the species, quantity of inoculum, etc., the constitution of the host plant as well as its resistance to fungal growth and/or mycotoxin production, etc., climatic and storage condition such as humidity and temperature, and agricultural measures[5-7]. Crop rotation, good agricultural practice[8], resistance breeding[9], and biocontrol[10] are agricultural measures done preharvest. Improvement of drying, milling, transport and storage procedures for food and feed to reduce the moisture content are interventions done postharvest[11].

Approximately, 25 % of the global food and feed is contaminated by mycotoxins and annual economic costs due to crop losses from aflatoxin, fumonisin, and deoxynivalenol contaminations are high as $1 billion, in the United States only[12].

1.2 Major classes of mycotoxins

Mycotoxins have been already mentioned in the Old Testament concerning deaths due to ergotism[13]. It is further assumed that some Egyptian tombs contained presumably ochratoxin A (OTA) were responsible for the deaths of several archeologists[14]. The term “mycotoxin”, is

derived from the Greek word "mukes" (meaning fungus) and the Latin word "toxicum" (meaning poisin)[1]. It originates from an unusual veterinary crisis near London where approximately 100,000 turkey poults died from an acute necrosis of the liver and hyperplasia of the bile[15]. This mystery, known as "Turkey X" disease was linked to the consumption of groundnuts infected with *Aspergillus flavus*. The research followed this event led to the isolation and identification of aflatoxins[16, 17] and since 1960 scientists started to search and characterize other toxic mold metabolites[18]. Till today over 400 different mycotoxins have been documented and the identification of new ones is still in progress[19]. The genera *Aspergillus* and *Fusarium* produce the most important in regard to animal and human health.

1.2.1 Aflatoxins

Aflatoxins structurally consist of five rings. Two furofuran moieties, an aromatic six-membered ring, a six-membered lactone ring, and either a five-membered pentatone (aflatoxins B_1 and B_2) or a six-membered lactone ring (aflatoxins G_1 and G_2)[20]. Aflatoxins are produced by serveral *Aspergillus* and *Penicillium* species, mainly *Aspergillus flavus* and *Aspergillus prasiticus*. These species are widespread in nature and can colonize many agricultural crop plants, including corn and groundnuts, as the most important ones[21-23]. *A. flavus* infects both crops while *A. parasiticus* is usually associated with peanuts[24]. Among several natural occurring aflatoxins, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) are the most prevalent ones. The letters "B" and "G" refer to the blue and green fluorescent colors produced under UV light[25]. Also milk can be an indirect source of aflatoxin. After ingestion of contaminated feed, AFB_1 is metabolically transformed into the hydroxylated aflatoxin M_1 (AFM_1)[4]. However, AFB_1 (Fig. 1) is mainly produced by toxigenic strains and is the most potent natural carcinogen known[26]. It possesses the highest toxicity and was classified as a group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC)[27]. Acute intoxication can lead to vomiting, necrosis and even death in higher dosage. Long-term effects on the other hand can result in liver cancer and immune suppression[28].

1.2.2 Trichothecenes

The first mycotoxin with the name trichothecin was isolated 1949 from the fungal species *Fusarium roseum*[29]. Till today, more than 200 different trichothecenes with toxic effects on humans and animals have been identified[30]. These metabolites are produced by a variety of fungal genera including *Fusarium*, *Phomopsis*, *Trichoderma*, and *Stachybotrys*. However,

Fusarium is considered as the most important genus involved in trichothecene production[31, 32]. Chemically, trichothecenes are polycyclic sesquiterpenoids and possess a common 12,13-epoxytrichothene backbone with various side chain substitutions. Due to their chemical structure they can be grouped into four classes, type A, B, C and D where typ B trichothecenes include the most prominent representative of this group, deoxynivalenol (DON)[33, 34]. DON is the most common mycotoxin found in small grains, especially wheat, barley and rye. It is mainly produced by *F. graminearum* and some related species[4, 24, 35]. When ingested in high dosage it causes vomiting, nausea and diarrhea. Therefore, DON (Fig. 1) was given the name "vomitoxin"[36]. Mycotoxins produced by *F. graminearum* are classified as group 3 agents (not classifiable as to its carcinogenicity to humans) according to the IARC[27].

1.2.3 Fumonisins

Fumonisins were first isolated and characterized in 1988 from *Fusarium moniliforme* (today known as *F. verticillioides*)[37]. They can be divided into four groups, A, B, C and P with the group B toxins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) playing an important role in carcinogenesis in humans. Especially FB_1 (Fig. 1) is associated with acute and chronic health issues, such as esophageal cancer. The major fumonisin producing species are *F. verticillioides* and *F. proliferatum*. Both are common fungal contaminants throughout the world[38, 39]. Fumonisins are linear and structurally similar to the sphingolipid intermediates sphinganine and sphingosine. They interfere with the sphingolipid metabolism by inhibiting the enzyme ceramidesynthase[33]. Hence, they can cause leukoencephalomalacia (ELEM), an uncommon disease that affects the liver and the brain, in equines and pulmonary edema in swine[40]. Consumption of fumonisin contaminated food by humans has been correlated with an increase in esophageal cancer. The World Health Organization (WHO) has reported immunosuppressive properties of fumonisins[41] and furthermore, the IARC classified fumonisins as group 2B agents (possibly carcinogenic to humans)[27].

1.2.4 Zearalenone

Zearalenone (ZEN) is a secondary metabolite mainly produced by *F. graminearum* and *F. culmorum*. These *Fusarium* species are regular contaminants of agricultural crops such as maize, wheat and barley worldwide[6, 33]. Compared to the other mycotoxins, it is hardly toxic but rather classified as nonsteroidal estrogen. ZEN (Fig. 1) binds to estrogen receptors in mammalian target cells and thus mimics an estrogenic effect. Furthermore, it can be

transformed into the reduced form zearalenol which has even increased estrogenic activity[4]. ZEN poisoning has been associated with hyperestrogenic syndromes (e.g. changes in the reproductive system), with pigs as the generally most affected livestock[1, 42]. It has been classified as a group 3 agent according to the IARC[27].

1.2.5 Ochratoxins

Ochratoxins including ochratoxin A (OTA) and ochratoxin B (OTB) are produced by fungal species belonging to the genera *Aspergillus* and *Penicillium*[1, 4, 24]. In contrast to OTA, OTB is less toxic and has been found only occasionally in foods[35]. During a comprehensive screening of fungal metabolites in 1965 OTA was discovered as a product of *A. ochraceus* which is the most important OTA producer and gives the toxin its name[43]. While OTA production associated with *A. ochraceus* usually occurs in warm climates, *P. verrucosum* mostly produces OTA in temperate climates[44]. OTA (Fig. 1) is a natural contaminant of cereal grains such as barley and wheat but it has also been found in maize, wine, coffee, tea and spices[45]. Primary effects are associated with the inhibition of an enzyme involved in phenylalanine metabolism[46]. Furthermore, it inhibits the mitochondrial adenosine triphosphate (ATP) production[47] and stimulates lipid peroxidation[48]. According to the WHO it possesses nephrotoxic, teratogenic, carcinogenic and immunotoxic properties[41]. The IARC classified OTA as possible carcinogenic to humans (group 2B carcinogen)[27].

1.2.6 Patulin

Patulin (Fig. 1) is produced by many different fungi belonging to the genera *Aspergillus*, *Penicillium*, *Paecilomyces* and *Byssochlamys*[1]. It was first isolated in the 1940s from *Penicillium patulum* (now named *Penicillium griseofulvum*), which has given the toxin its name. The first reported characteristics stated antibacterial, antiviral and antipotozoal activity. For this reason it was used as antibiotic. However, during the 1950s and 1960s studies showed that patulin is toxic to animals and plants. Therefore, it was reclassified as a mycotoxin[4]. It is the most dangerous mycotoxin in fruits (particularly apples and pears) and has been classified by the IARC as a group 3 agent[27]. Consumption of contaminated food affects the function of the liver, kidney and the overall immune system[49].

1.2.7 Ergot alkaloids

Ergot alkaloids are found in sclerotia (hardened fungal mycelium) of fungi belonging to the genus *Claviceps* and are common pathogens of various grass species. Apart from *Claviceps*, these toxins are also produced by *Aspergillus*, *Penicillium* and *Rhizopus* spp.[50]. Lysergic acid

is the common structure of all ergot alkaloids and lysergic acid alkaloids include the important ergotamine (chemical structure shown in Fig. 1). Symptoms caused by ergot alkaloids have been already mentioned 600 before Christ as "nocious pustule in the ear of grain"[51]. Human diseases caused by the consumption of contaminated cereals with ergot alkaloids are called ergotism or St. Anthony's Fire[4] named after monks of the Order of St. Anthony who were partially successfully treating this illness[52]. While ergotism is still important in veterinary medicine as it is causing abortion, gangrene and convulsions it has been almost eliminated as a human disease due to modern methods of grain cleaning[53].

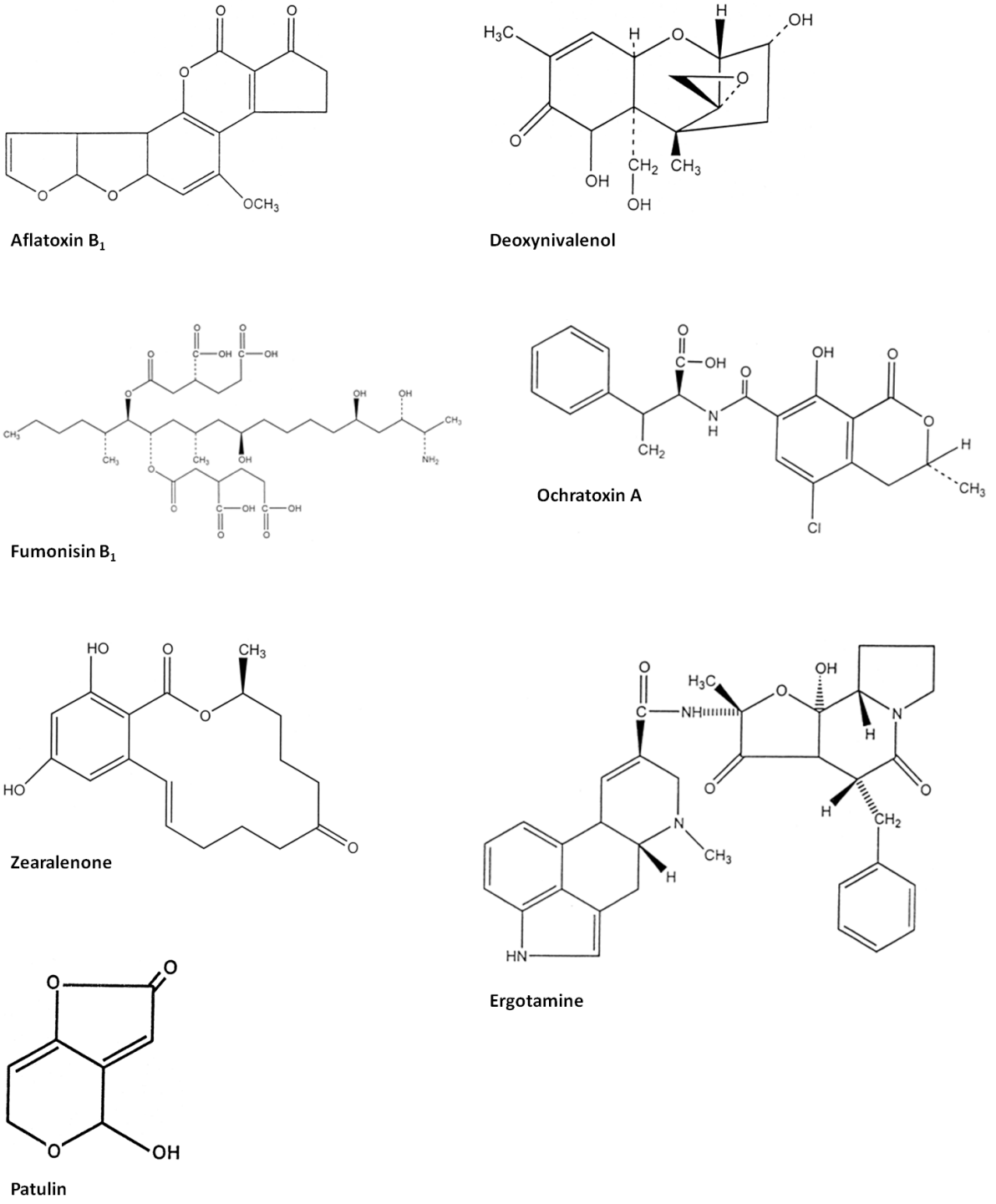

Fig. 1 Chemical structure of the major classes of mycotoxins

1.3 Masked mycotoxins

In a feeding trial in the mid-1980s animals fed with low-level mycotoxin contaminated feed showed unexpected symptoms of intoxication. This phenomenon was attributed to the presence of mycotoxin derivatives, a conjugated form of mycotoxins[54]. The term "masked mycotoxin" arose because the structure of the mycotoxin changes, whereby the metabolite becomes undetectable for conventional analytical methods. In general, masked mycotoxins

are less toxic compared to their parent compound. However, there is a potential risk of hydrolysis of the conjugate in the body back to their toxic precursor and thus increasing the toxic potency of the food. Conjugated mycotoxins can be found in plants, fungi and mammals. Furthermore, processing of food commodities can be another source of mycotoxin conjugate formation[55].

1.3.1 Plant conjugates

After infestation of a plant by a fungus and subsequent mycotoxin production, the plant induces a detoxification process chemically transforming the secondary metabolite to a masked mycotoxin. This transformation is catalyzed by plant enzymes[55]. During this detoxification process mycotoxins are bound to polar substances such as sugar molecules, amino acids or sulfates and are stored in the plants vacuoles[56]. The percentage of masked mycotoxins in a plant depends on its health constitution and genetic conditions. The healthier the plant the more toxins can be bound and stored by it. To detect modified mycotoxins they have to be liberated from the matrix[57]. So far, plant metabolites have been identified for DON, destrucins, fusarenon-X, fusaric acid, NIV, T-2 toxin, HT-2 toxin, OTA, and ZEN, but only the conjugate zearalenone-14-β-D-glucopyranoside (Z14G) and deoxynivalenol-3-glucoside (D3G) have been proven to occur in naturally infected cereals such as maize and wheat[55]. To date, D3G is the best studied masked mycotoxin regarding occurrence and toxicology.

1.3.2 Fungal conjugates

The fungus possesses the ability to excrete mycotoxins directly. The most prominent substances are 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON). Both substances can be found in *Fusarium* contaminated plants and are a biosynthetic precursor of DON[58, 59].

1.3.3 Food-processing conjugates

Generally, mycotoxins are very stable towards physical treatment, though especially heating or fermentation steps can potentially alter mycotoxins[58]. Lancova et al. (2008)[60] investigated the main trichothecenes 3-ADON, 15-ADON, DON, HT-2 toxin (HT-2), and ZEN in barley throughout the process from malt to beer. They reported the formation of high levels of D3G during the malting and brewing process.

1.3.4 Mammalian conjugates

Mycotoxin conjugates that arise from mammalian metabolism are commonly conjugated in the gastrointestinal tract and the liver during metabolization. They are further excreted in urine[61]. Common mycotoxin conjugates are DON glucuronide[62] and ZEN glucuronide[63], both have been detected in the urine of animals. These metabolisation products are often used as biomarkers to evaluate the total exposure of an individual towards specific mycotoxins. Warth et al. (2013)[64] investigated the metabolism of the major *Fusarium* toxins DON and ZEN in humans and summarized their recent approach. After a four days diet with food products containing 138 µg DON and 10 µg ZEN they found DON excretion and glucuronidations rates of 68 % and 76 %, respectively with DON-15-glucoronide as the main produced conjugate. In the case of ZEN an excretion rate of 9.4 % was determined and data showed that ZEN was mainly present as ZEN-14-glucoronide.

1.4 Legislation and regulatory limits

Contaminations of food and feed by mycotoxins have been characterized by the WHO as major sources of food-borne illnesses[41]. In several parts of the world, especially in developing countries, mycotoxins represent a significant food safety issue. Therefore, ensuring the food and feed safety has become of great national and international importance. Based on an international inquiry carried out in 2002 and 2003 by the Food and Agriculture Organization (FAO), approximately 100 countries had introduced maximum levels for mycotoxins in food and feedstuff[65, 66], covering about 87 percent of the world's inhabitants. The number of countries regulating myctoxins has significantly increased within the last years and is still growing. The maximum levels are affected by the toxicity of the respective mycotoxin, the frequency of occurrence in foodstuffs and the regularity intake of the product by the population[67]. Thus, the same mycotoxin can have different maximum levels in different foodstuffs. The situation is somewhat different for feed. Only AFB_1 is regulated with maximum levels[68]. For the remaining toxins, DON, FB_1, FB_2, OTA, and ZEN only non-binding guidance values are set for feed[69]. In the European Union (EU), Commission Regulation (EC) No 1881/2006[70] and its amendments EC Recommendation 2006/576[69], EU Recommendation 2013/165[71] and EC Directive 2002/32[68] setting maximum or guidance levels for the mycotoxins AFB_1, AFM_1, the sum of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), DON, the sum of FB_1 and FB2, OTA, Patulin, T-2 and HT-2 toxin, and ZEN in certain food and feedstuffs. An overview of these levels is provided in Table 1.

Table 1 Maximum levels for mycotoxins according to the European legislation

Mycotoxin	**Foodstuffs**[a]	**Maximum levels for food (unprocessed and processed) (µg/kg)**	**Maximum levels for feed (µg/kg)**
Aflatoxins (sum of B_1, B_2, G_1 and G_2)	groundnuts, nuts, dried fruit, all cereals and cereal products, maize, spices	4 – 15	-
Aflatoxin B_1		(0.1)[b] 2 – 8	5 – 20
Aflatoxin M_1	milk and milk-based products	(0.025)[b] 0.05	-
Deoxynivalenol	cereals, maize	(200)[b] 500 – 1,750	900 – 12,000
Fumonisins (sum of B_1 and B2)	maize	(200)[b] 400 – 2,000	5,000 – 60,000
Ochratoxin A	cereals, dried vine fruits, coffee and coffee beans, wine and wine-based products	(0.5)[b] 2 – 10	50 – 250
Patulin	fruit juices, apple products	(10)[b] 25 – 50	-
Rye ergot	-	-	1,000
Sum of T-2 toxin and HT-2 toxin	cereals	100 – 1,000	250 – 2,000
Zearalenone	cereals, maize	(20)[b] 50 – 200	100 – 3,000

[a] as defined in Regulation (EC) No 396/2005[72]
[b] intended specially for infants[70]

The maximum levels have a direct impact on all food and feed business operators and traders[73]. Therefore, regulative actions for mycotoxins are brought into harmony between countries with trade contacts. Harmonized regulations for some mycotoxins now exist for regions of Australia and New Zealand, the EU and Mercado Comun del Sur (MERCOSUR)[74]. This shall ensure that the foods and feeds meet the requirements of food law in the respective countries.

1.5 Genetics and molecular biology of mycotoxin biosynthesis

Mechanisms of metabolic pathways of mycotoxin production have been extensively studied over the last 50 years. Extensive knowledge about mycotoxin biosynthesis requires knowledge about its regulatory mechanisms. Besides biotic and abiotic factors, also genetic factors may affect mycotoxin formation. A better understanding of the mechanism of gene regulation in mycotoxin biosynthesis will help to identify inhibitors of fungal growth and toxin production and will eventually lead to new strategies to control mycotoxin contamination for a safer food and feed supply[75].

1.5.1 Biosynthesis of trichothecenes

Biosynthesis of trichothecenes starts with the cyclization of the initial substrate farnesyl-pyrophosphate to form trichodiene. Trichodiene is a low-toxic, essential precursor for the production of trichothecenes[76]. This reaction is catalyzed by the trichodiene synthase (Tri5). Tri5 is a homodimeric enzyme with a subunit of 45 kDa[77] and is encoded by the *tri5* gene. A series of oxygenation steps follows the trichodiene formation catalyzed by a cytochrome P450 monooxygenase encoded by *tri4*[78] leading to the intermediate isotrichotriol[79]. Then

isotrichodermol (C-3 –OH) is formed by a non-enzymatic isomerization and cyclization[80]. This metabolic intermediate is subsequently converted to isotrichodermin (C-3 –OR) by an acetyltransferase encoded by *tri101*[81]. A second and a third hydroxyl group is added (C-15 and C-4) and subsequently acetylated under the control of *tri3* and *tri7*, respectively[82-84]. In type A trichothecene producing *Fusarium* strains a fourth hydroxyl group at C-8 and an isovaleryl moiety are added (controlled by *tri1* and *tri16*). Finally, the acetyl group at C-3 is removed resulting in the production of T-2 toxin[85]. In type B trichothecene producing strains a hydroxyl group is added at C-7 and C-8 controlled by *tri1*[86, 87]. These groups are further converted to a keto group. The last step involves the removal of the acetyl groups at C-3 and C-15, by an esterase encoded by *TRI8* leading to the production of either 3-ADON or 15-ADON chemotypes[88]. Fig. 2 shows the trichothecene biosynthetic pathway in *Fusarium*.

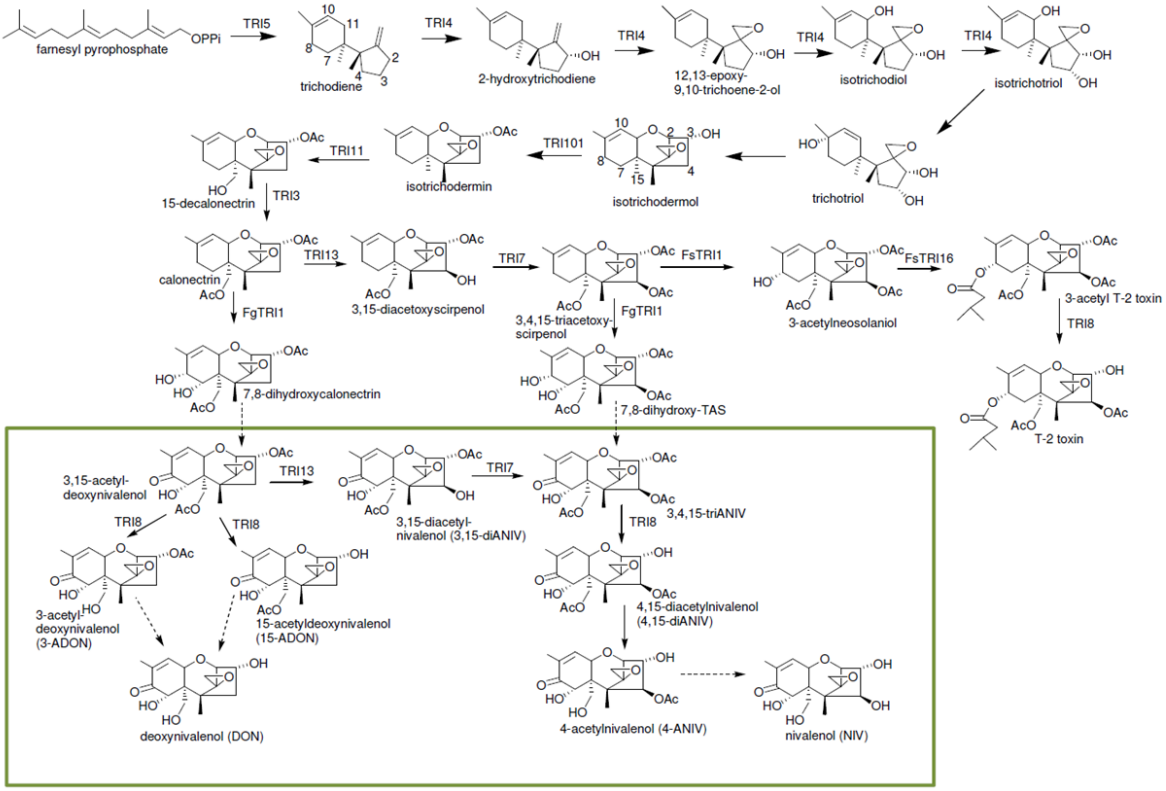

Fig. 2 Trichothecene biosynthetic pathway in *Fusarium* including all involved genes. Steps in the green box are performed for type B trichothecenes only[89]

1.5.2 Biosynthesis of fumonisins

Fumonisins are polyketide-derived secondary metabolites that are structurally similar to the sphingoid base backbone of sphingolipids[90]. These mycotoxins are synthesized by a cluster of genes designated as the *FUM* gene cluster[91]. The *FUM* gene cluster has a length of approximately 42 kb and contains 15 genes that are associated with fumonisin production[92].

FUM1, a polyketide synthase, FUM6, a chytochrome P450 monooxygenase and FUM8, an aminotransferase play essential roles in fumonisin biosynthesis[91, 93]. The first step of the biosynthesis is catalyzed by a polyketide synthase, controlled by *fum1*, which synthesizes the reaction from acetyl-CoA and eight malonyl-CoA to a polyketide. This step is followed by a condensation of alanine, controlled by *fum8* and an oxidation at C-14 and C-15 by the P450 monooxygenase encoded by *fum6*. Further steps are catalyzed by aminotransferases, dehydrogenases, monooxygenases and dioxygenases leading to the final product FB_1 (Fig. 3)[94].

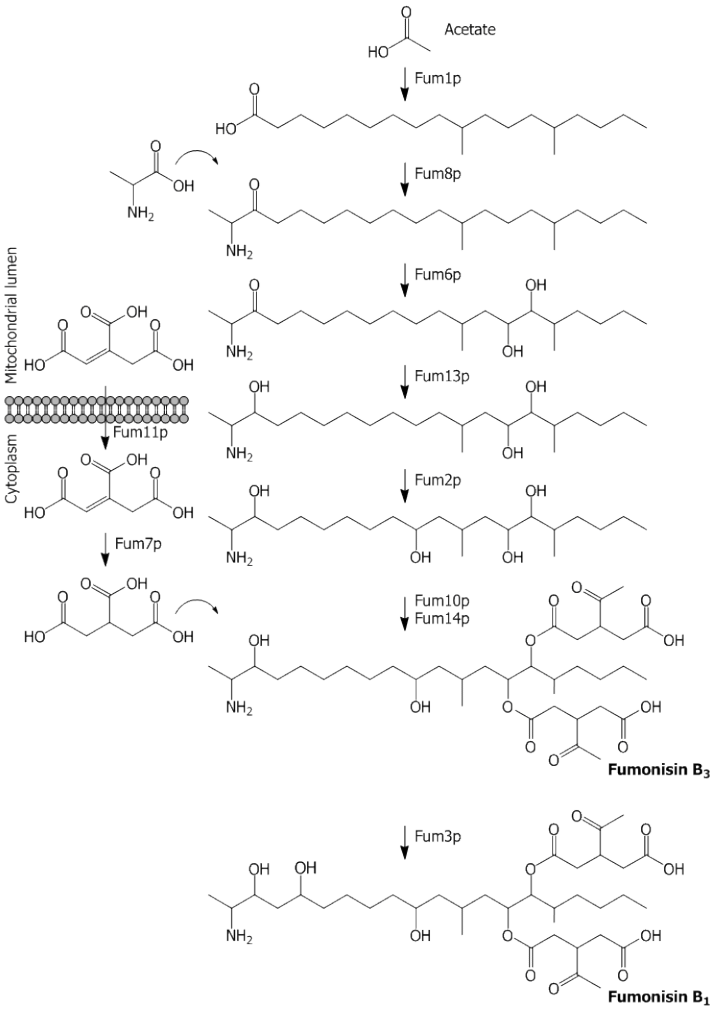

Fig. 3 Biosynthetic pathway for fumonisin B_1 production[95]

1.5.3 *Biosynthesis of aflatoxins*

The discovery of norsolorinic acid (NOR), the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway represents a milestone in the understanding of the chemistry of aflatoxin biosynthesis[96, 97] and led to the identification of other key aflatoxin intermediates[98]. The aflatoxin pathway gene cluster was discovered in *A. flavus* and *A. parasiticus*[99] and up to now

34 genes within an 82 kb DNA region have been identified as members of the cluster[100]. At the beginning a polyketide is synthesized from a hexanoyl starter unit catalyzed by two fatty acid synthases (FAS) and a polyketide synthase (PKS), controlled by the genes *aflA* (former fas-*1*), *aflB* (former *fas-2*) and *aflC* (former *pksA*) respectively[101]. Studies showed, that *pksA* is implicitly required for aflatoxin biosynthesis[102]. The produced product is noranthrone. The conversion from noranthrone to NOR is poorly understood but it has been estimated to be catalyzed by a monooxygenase or to occur spontaneously[103]. Based on NOR, versicolorin B (VER B) and versicolorin A (VER A) are formed involving several enzymatic steps and intermediates. Genes involved in this procedure encode for a reductase, dehydrogenase, oxidase, esterase, versicolorin B synthase (VERB) and desaturase[104, 105]. VER B is the critical branch point leading to the final products AFB_1, AFB_2, AFG_1 and AFG_2 (Fig. 4)[106].

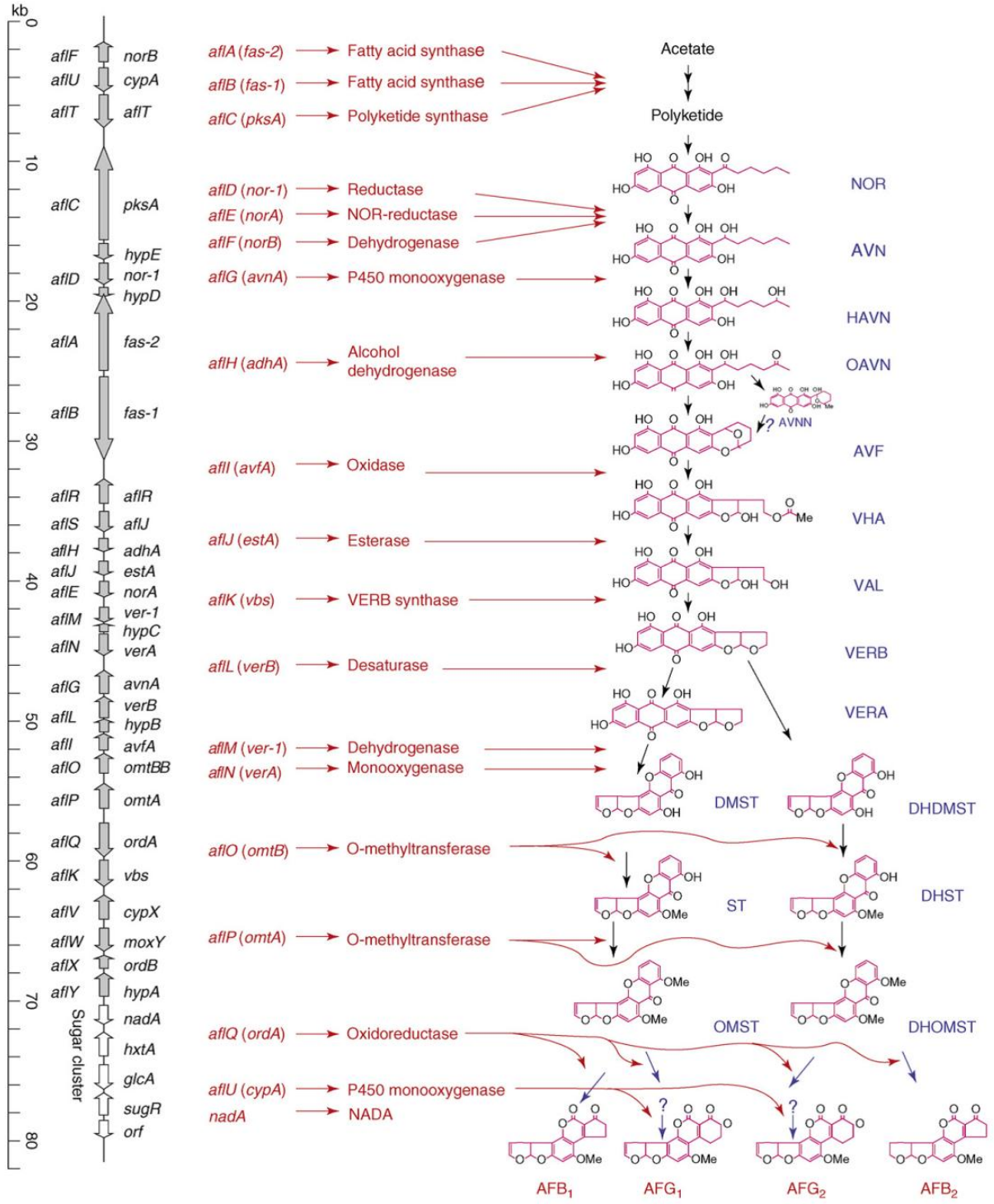

Fig. 4 An illustration of the aflatoxin biosynthetic pathway is presented on the right. The vertical line on the left side shows the aflatoxin gene cluster as well as the sugar cluster in *A. flavus* and *A. parasiticus*; new gene names are given on the left side of the vertical line and old gene names are presented on the right[107]

2 Mycotoxin analysis

Analytical techniques based on chromatographic separation (mostly liquid chromatography, LC) have been developed offering accurate and precise methods for the detection of fungal secondary metabolites[108]. Furthermore, highly sophisticated multi-mycotoxin methods based on LC coupled to tandem mass spectrometry (MS) being developed to allow the identification of currently more than 320 mycotoxins and other fungal and bacterial metabolites[109]. Since the number of countries setting maximum levels for certain mycotoxins in various matrices rises, the number of sample matrices and mycotoxins of interest increased. Hence, applications of simpler and cheaper solutions for the detection of mycotoxins are increasingly being required[108]. This led to the development of fast and accurate screening methods, based on immunological techniques (e.g. enzyme linked immunosorbent assay, ELISA) and DNA-based techniques, such as quantitative polymerase chain reaction (qPCR) and portable biosensors (e.g. based on aptamers) for on-site application[110].

2.1 Liquid chromatography – mass spectrometry (LC-MS)

Since Tswett introduced the liquid chromatography (LC) in 1906 it became one of the most widely applied analytical techniques[111]. This method includes any chromatographic procedure in which the moving phase is a liquid. LC is ideally suited for the separation of macromolecules, high-molecular-weight compounds and less stable products, such as fungal and plant metabolites, proteins, amino acids and so forth[112]. Chromatographic separation of the analytes occurs due to different interactions between the components in the matrix and the liquid mobile phase and/or the (usually) solid stationary phase. It takes the analytes different times to get from the position of sample introduction to the position at which they are being detected. To detect the separated analytes several possibilities exist including photometric detection (UV-Vis), fluorescence detection (FLD) and mass spectrometry (MS).

Generally, components of a mixture are identified and/or quantified on the basis of the chromatographic retention characteristics. However, more than one analyte can have identical retention times. This requires further information from an auxiliary technique – usually some form of spectroscopy[113]. The most widely used LC detectors are photometers based on ultra-violet (UV) and visible absorption, covering 70 % of all detections[111, 112]. An UV spectrum allows the class of compound to be identified but it is not possible to achieve complete certainty. MS allows more accurate identification and provides molecular weight as well as structural information of metabolites and sugar conjugates (such as "masked mycotoxins")[114]

The mass of a molecule is determined by measuring the mass-to-charge ratio (*m/z*) of its ions.

Once ions are formed by the loss or gain of charge from a neutral species, they are electrostatically directed into a mass analyzer. They are subsequently separated by their *m/z* and finally detected[115]. To increase the sensitivity of the analysis the separation capacity of LC was coupled with the selectivity and specificity of MS. Thereby, the mobile phase is removed and the analytes are transferred from the liquid to the gaseous phase[116].

The combination of chromatography and MS has attracted much interest over the last decades and become a widely used approach for mycotoxin analysis[110, 113]. Individual components are separated by LC (typically high-performance liquid chromatography, HPLC) followed by the detection with MS. The coupling of both methods enabled the development of highly selective, sensitive and accurate methods for mycotoxin determination[117].

Due to the fact that several fungi can colonize the same agricultural commodity or that fungi are able to produce several mycotoxins led to the development of analytical methods for the simultaneous determination of different classes of mycotoxins[110, 116]. LC-MS based mulit-mycotoxin methods allow the determination of a huge variety of diverse chemically analytes within a relatively short time[118]. In addition, sample throughput increases while costs per analysis decreases. Malachova et al.[109] developed a method that covers more than 320 fungal and bacterial metabolites.

Chromatographic procedures for the determination of mycotoxins are highly sensitive nevertheless, they are time-consuming, expensive and trained personnel are required. To overcome these drawbacks fast screening methods have been developed.

2.2 Immunochemical techniques

Immunological methods became very popular for mycotoxin screening since the late 1970s and ELISA is now a routinely used tool[119]. These binding assays are based on the use of monoclonal or polyclonal antibodies that bind to an antigen (e.g. a mycotoxin)[120]. Although they show high matrix dependence and lack accuracy at very low concentrations, the advantages are speed, ease of use, sensitivity and high sample throughput. Several ELISA kits are commercially available for qualitative, semi-quantitative or quantitative analysis for the major mycotoxins in cereal-based matrices (AFB_1, AFM_1, sum of aflatoxins, DON, FUM, HT-2 toxin, OTA, T-2 toxin and ZEN)[121-123]. In recent years the need for rapid screening test formats increased due to the rise of countries setting maximum levels for certain mycotoxins and the consequent increase of the amount of tested samples[108]. Hence, flow-through immunoassays and lateral flow devices (LFDs) have been developed[110]. Burmistrova et al.[124] developed a membrane-based flow-through immunoassay for the rapid mulit-detection of

FB_1, OTA, and ZEN. The sensitivity of the assay is sufficient for mycotoxin detection at the maximum limits set by the EU. LFDs can be described as immunochromatographic techniques based on an antigen-antibody reaction on a nitrocellulose membrane. Positive results are usually visualized by the formation of a red coloured band due to attached gold nanoparticles[125]. They allow qualitative or semi-quantitative determination of mycotoxins on a strip test within a few minutes. Such LFDs have been developed for selected mycotoxins in cereals, such as aflatoxins[126-128], fumonisin B_1[129, 130], DON and ZEN[131, 132], OTA[133], and recently also for the detection of AFM_1 in milk[134].

However, all immunoassays rely on the availability of appropriate antibodies. In many cases the production costs are high and adequate antibodies are not available for all analytes[135].

2.3 DNA-based techniques for the analysis of fungal species and mycotoxins

2.3.1 Polymerase chain reaction (PCR)

The principle of the polymerase chain reaction (PCR) was developed by Kary Mullis in 1983. This method enables the amplification of DNA fragments for the subsequent analysis via electrophoretic or fluorimetric techniques[136]. Nowadays, PCR is a standard analytical tool of significant relevance for various kinds of analyses with organisms as targets. PCR basically consists of recurring changes of temperatures, which are also described as cycles, in which a single DNA molecule can be amplified exponentially. One cycle typically involves three steps: a denaturation step, an annealing step and an extension step. Prior to the first cycle an initialisation step is induced. This step is only required for DNA polymerases that require heat activation. The following cycles start with the denaturation step in which the double stranded DNA (dsDNA) is separated into two single stranded DNA (ssDNA) strands by heating up the reaction to 95 °C. In the subsequent annealing step temperatures are lowered around 50 – 70 °C. This enables short oligonucleotides, so called primers, to bind to one of the separated strands indicating the starting point for the strand extension. The optimal annealing temperature is determined by the base composition and length of the primers and can be calculated according to formula (1):

$$T_m\,[°C] = 2\,°C \times (A+T) + 4\,°C \times (G+C) \qquad (1)$$

where T_m indicates the melting temperature and A, T, G and C the single nucleotides (adenosine, thymine, guanine and cyctosine, respectively).

Extension takes place at around 70 – 80 °C. During this step the thermostable DNA polymerase binds to the primer-template-complex and starts synthesising a new

complementary strand by adding desoxyribonucleotides in a 5' – 3' direction. Typically, 25 to 45 cycles are performed. The principle is displayed in Fig. 5[137].

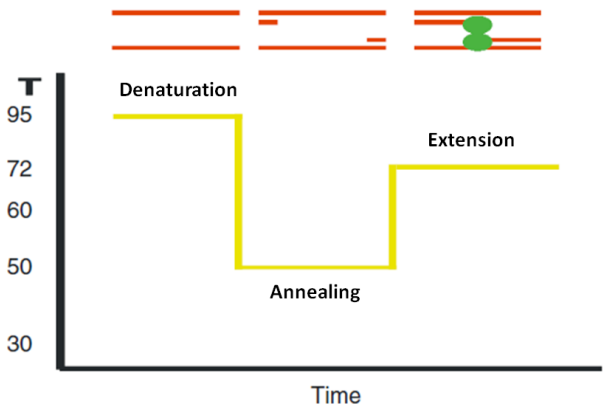

Fig. 5 Illustration of one PCR cycle.

During each cycle the concentration of the DNA target is doubled. To check whether the PCR generated the desired amplicon, the new products can be size-separated by agarose gel electrophoresis and compared with a molecular weight marker[138, 139]. A more advanced approach is the quantitative real-time PCR (qPCR). Compared to conventional PCR-based formats real-time PCR combines amplification of target DNA with simultaneous detection of amplicons. The process can be monitored in "real-time" due to a fluorescence signal that is detected with a camera during each PCR cycle[140].

Since 1996 PCR-based assays have been set up for the major species and groups of mycotoxigenic fungi. Geisen[141] and Shapira et al.[142] published the first PCR assays for the detection of aflatoxin producers. Assays for the identification of trichothecene and fumonisin producing *Fusarium* species followed[143-146]. Since the early 2000s qPCR assays have been developed to gain direct information about the DNA biomass of a fungus based on the quantification of the amount of organism specific DNA[147-150]. In addition to existing species specific assays, tests were developed to quantify several fungal species in one reaction by the simultaneous detection of genes involved in the respective myctoxin production[151, 152].

2.3.2 Aptamers

Immunoassays, such as ELISAs and LFDs, are rapid methods of choice for mycotoxin analysis. They are based on the recognition of a fungal secondary metabolite by specific antibodies. A main drawback of antibody production is the complex procedure of preparation by immunization or cell cultures and the subsequent purification step. Hence, antibody preparation is cost-intensive and the quality of the product varies among the suppliers[153]. In

recent years promising aptamer-based analytical methods have been developed with similar functions as antibodies[154, 155]. The term aptamer is derived from the Latin word "aptus" meaning "fit" and the Greek word meros meaning "part"[156]. Aptamers are short, single-stranded oligonucleotides, DNAs or ribonucleic acids (RNAs), typically around 20 to 80 bases long. The particular sequence forms a defined three dimensional structure. Due to this the aptamers are able to recognize a particular molecule with high affinity and specificity (Fig. 6). The tertiary structure allows the aptamer to bind to its target via van der Waals forces, hydrogen bonding and electrostatic interaction. A so called aptamer-target-complex is formed[157].

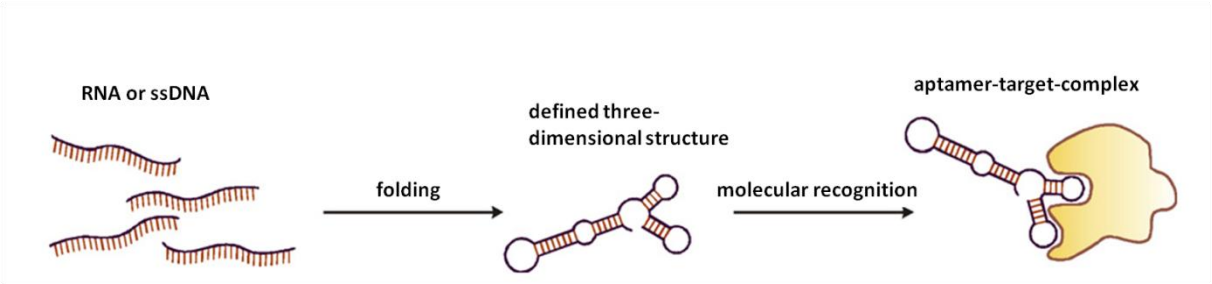

Fig. 6 Schematic representation of aptamer-target-complex formation[157]

Generally, the selection of aptamers is possible for virtually any molecular target[153]. They have been selected for small molecules such as mycotoxins[158, 159], peptides[160], proteins[161], viruses[162-164], bacteria[165-167], and even whole cells[168]. The great potential of aptamers has first arisen in the 1980s where research on the human immunodeficiency virus (HIV) showed that this virus evolved a short, structured RNA ligand the HIV trans-activation response (TAR) element that binds to the viral protein Tat as well as the cellular protein cyclin T1 to control viral gene expression and replication[169]. Similarly adenovirus has evolved a RNA aptamer to block one of the mammalian cell's antiviral strategies. These findings suggested that nucleic acid ligands might also be useful for therapeutic ends[170, 171]. There are various application fields of aptamers. They have been studied concerning their use as a diagnostic and therapeutic tool, in the development of new drugs, for food inspection and so forth[154]. In 2004 Macugen, an aptamer used for the treatment of age-related macular degeneration, was approved by the FDA[172].

Aptamers are referred to as "Next-Generation-Antibodies" due to that fact that these molecules overcome some of the drawbacks of antibodies. Proteins can be irreversibly denatured, whereas aptamers are thermally stable and can withstand repeated cycles of denaturation. Aptamers are produced in vitro and do not require the use of animals or cell cultures. Therefore, the production is easier, more cost effective and there are hardly any

batch to batch variations. Furhtermore, they can be targeted against small molecules such as mycotoxins and other molecules that do not cause a strong immune response. Aptamers can also bind molecules that cannot be recognized by antibodies (e.g. ions)[173]. Nevertheless, aptamers have also some disadvantages over antibodies. They are rather new and the development is in the early stages, they are faster excreted from living organisms than antibodies due to their smaller size and they strongly depend on pH and salt concentrations.

Aims of the Thesis

The presented thesis was performed within the Molecular Diagnostics Group under the supervision of Dr. Kurt Brunner at the Center for Analytical Chemistry (IFA-Tulln). The overall aim of this working group is the development of DNA-based techniques for the detection of undesired compounds in food and feed products including mycotoxin producing fungi, allergens and genetically modified organisms (GMOs).

Since the two topics discussed in this thesis are only distantly related, the thesis is divided into two main parts.

The **first chapter** describes the development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize. The work's task was to test the developed multiplex method with regard to following qualities:

- **Specificity:** Measurements of the genes responsible for the production of either trichothecenes (*tri5* gene) or fumonisins (*fum1* gene) should be neither influenced by different isolates nor by species. Furthermore, primers and probes need to be tested to be group specific for either fumonisin or trichothecene producing *Fusarium* species.
- **Sensitivity:** The determination of the minimum of fungal template DNA necessary for quantification should allow the comparison of the sensitivity of the multiplex assay to that of the singleplex method.
- **Repeatability:** The performance of the multiplex assay on different PCR thermal cyclers from various manufactures as well as the performance of the method by two different operators should demonstrate that the results and efficiencies obtained by multiplex qPCR are neither influenced by the instrument nor the operator.

  Furthermore, two sample extractions, two DNA precipitations as well as the performance of two quantifications either by the three singleplex assays or the developed multiplex method should ensure the repeatability of all parts of the method.

The assay has to be carefully evaluated to guarantee equal performance of the triplex assay compared to the singleplex assays. The multiplex assay will be finally used to study 24 samples with different levels of trichothecenes and fumonisins.

This work is based on previous research by the group of Dr. Kurt Brunner[150, 174]. Results have been published in Analytical Methods:

Viktoria Preiser, Daniela Goetsch, Michael Sulyok, Rudolf Krska, Robert L. Mach, Andreas Farnleitner and Kurt Brunner (2015) The development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize. Analytical Methods 7 (4): 1358-1365.

Chapter two addresses the main objective of this thesis: The development of aptamers for the rapid detection of aflatoxin B_1 (AFB_1) in maize.

First, chapter two aims at the selection of AFB_1 specific aptamers using the iterative SELEX procedure. Selected aptamers have to be identified and characterized by sequencing and the calculation of the folding structure and the determination of the dissociation constant (K_d). The evaluated parameters will be compared to previously published aptamers specific for AFB_1.

The study also aims at a rapid, sensitive, and simple aptamer dipstick assay using a direct detection format for AFB_1. Previously published assays are currently based on an indirect target detection format where a test line is formed if no target is present and *vice versa*. In this study the idea claims an AFB_1 induced unwinding of the aptamers's distinctive structure exposing a binding site for a complementary signaling DNA probe (Fig. 7). It is hypothesized that a hybrid is formed as a result of the AFB_1 induced structure switch, which might be then visualized via a simple lateral flow device. The developed test strip has to be carefully evaluated with regard to different temperatures responsible for hybrid formation.

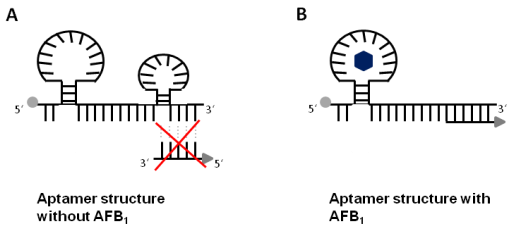

Fig. 7 The AFB_1 aptamer forms a distinct loop structure at the 3'- end according to Neoventures Biotechnology Inc. This structure impedes the binding of the complementary signaling DNA probe (A). It is assumed that due to target addition a structure switch is induced exposing a binding site for the DNA probe (B).

A manuscript has been submitted to Analytical Methods:

Viktoria Preiser, Antonia Tacconi, Robert L. Mach, Rudolf Krska, Sabine Baumgartner and Kurt Brunner (2015) A switch based aptamer biosensor: a new approach towards a direct detection format for aflatoxin B_1.

Chapter One

The development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize

Fungi of the genus *Fusarium* are common plant pathogens mainly associated with cereal foods and feeds. Despite great progresses in plant breeding, total resistance to this genus has not yet been achieved. Visual scoring of disease symptoms combined with the determination of mycotoxins are common approaches to identify new *Fusarium* tolerant lines. These methods are only indirect and do not obligatory determine the accumulated fungal biomass.

Aiming at a rapid and sensitive quantification method for trichothecene and fumonisin producing *Fusarium* species in maize, a multiplex qPCR assay was developed. Simultaneous quantification of the mycotoxin-related genes *tri5* and *fum1* enables high-throughput screening of a large number of samples in relatively short time. This method was applied to 24 maize field samples. All samples were analyzed for the trichothecenes deoxynivalenol (DON), DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) by LC-MS/MS and for the mycotoxin producers by the newly developed qPCR multiplex assay. The developed multiplex method was found to be specific for fumonisin as well as for trichothecene producing *Fusarium* species and showed a limit of quantification of 0.32 pg per µl for both *Fusarium* strains.

3 Introduction

3.1 Quantitative real-time polymerase chain reaction (qPCR)

3.1.1 General aspects

The qPCR was developed in 1992 by Higuchi et al.[175] and was introduced since then into various scientific fields to quantify nucleic acids. Real-time PCR assays are fast, easy to perform and combine amplification of target DNA with detection of the amplicon. The process of amplification is monitored in real-time by using fluorescent techniques. The two prevalent methods of detection are either the use of unspecific, DNA intercalating fluorescent dyes, or the usage of sequence specific oligonucleotides, so called probes, which are labelled with a fluorescent reporter[176]. The generated fluorescence signal reflects the amount of product formed[137].

The first **intercalating dye** used was ethidium bromide. Today SYBR Green I is the most frequently used dsDNA-specific dye. It is an asymmetric cyanine dye that binds to the minor groove of DNA (Fig. 8A). The advantage over ethidium bromide is a 100 times higher binding affinity. SYBR Green I is excited with blue light at a wavelength of 480 nm and emits light at 520 nm. A major drawback of intercalating dyes is that they bind to all present double-stranded DNA (dsDNA) strands including primer dimers or unspecific products giving false-positive results. To overcome this drawback a melting curve analysis of the product is frequently performed. Performing melting curve analysis the fluorescence signal decreases as a result of melting of double stranded PCR products. Multiple peaks indicate the formation of more than one product. The given melting temperature indicates the size of the product and the G/C content, which furthermore allows the discrimination between the desired product and unspecific amplicons or primer dimers[176].

Sequence specific probes increase the specificity of an assay due to the addition of a fluorophor-labelled oligonucleotide probe that is complementary to the product sequence. Hydrolysis probes like TaqMan® probes are complementary to the target DNA sequence. They are labelled on the 5'-end with a reporter fluorophor, such as fluorescin, and on the 3'-end with a quencher. Intact probes do not fluoresce because they are internally quenched due to spatial proximity of fluorophor and quencher. During the primer extension step the 5' – 3' exonuclease activity of the Taq DNA polymerase is exploited to cleave the TaqMan® probe (Fig. 8B). Thus, the reporter dye is separated from the quencher and the fluorescence increases[140, 176]. Other sequence specific probes commonly used for real-time PCR are hybridisation probes based on the increase of the fluorescence resonance energy transfer

(FRET) from a donor to an acceptor fluorophor and molecular beacons. One end of the probe is attached to a fluorophor and the other end is attached to a quencher. Only the middle part of the probe is complementary to the target DNA sequence, whereas the terminal nucleotides are self-complementary forming a stem-loop structure. Thereby, the activity of the fluorophor is quenched. During the annealing phase of the PCR the loop part of the probe binds to its complementary part, the stem is opened, quencher and fluorophor are separated and the signal increases[176].

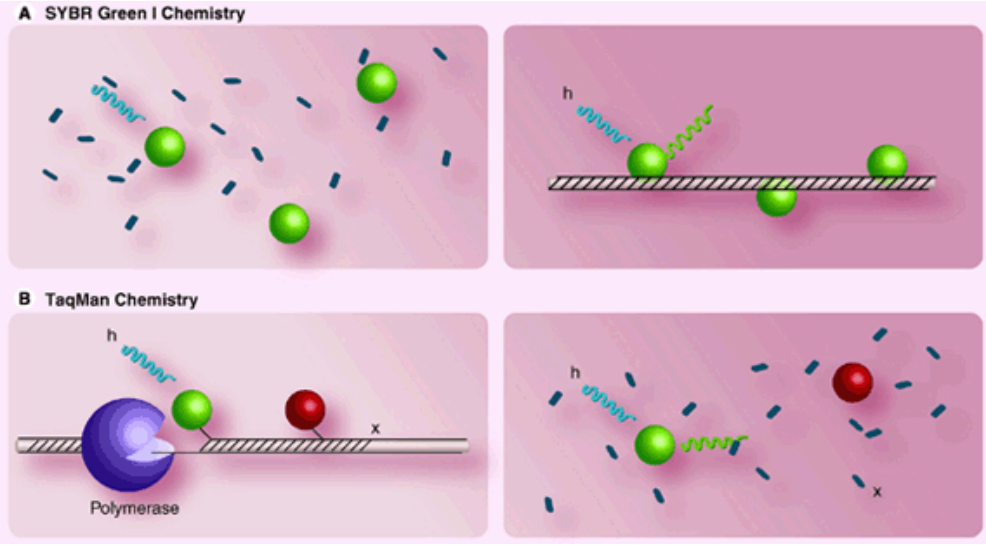

Fig. 8 Comparison of the intercalating dye SYBR Green I and the sequence specific TaqMan® probe[177]

To obtain quantitative results the monitored amplification process is analyzed by evaluating the amplification curves. Each curve consists of three distinct phases: an initial lag phase, an exponential phase or log-linear phase and a plateau-phase (Fig. 9).

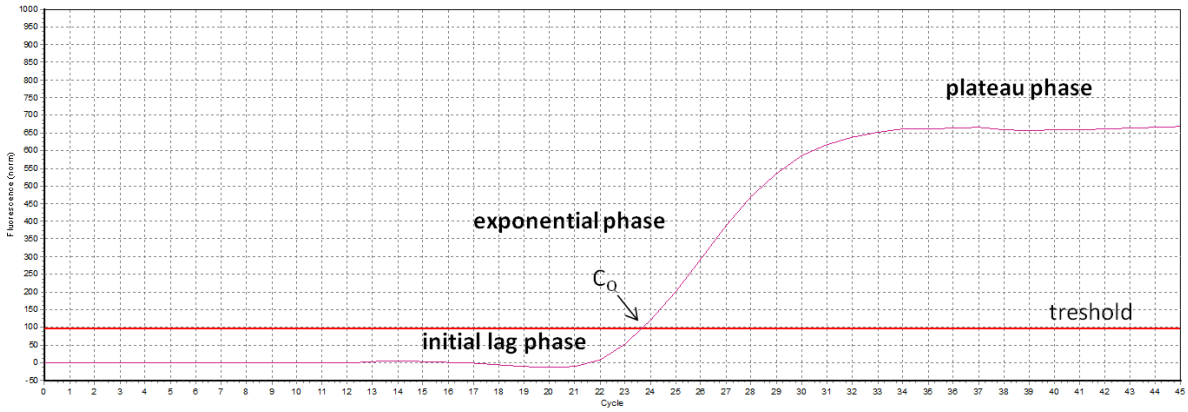

Fig. 9 The three distinct phases of a qPCR amplification curve

The calculation of the starting concentrations in qPCR analysis requires the setting of a fluorescence threshold and the determination of the quantification cycle (C_Q) value, which is the fractional cycle number that is required to reach this threshold. The C_Q values are

inversely proportional to the amount of initial target DNA in the sample. To quantify the unknown amount of target DNA in the samples, appropriate DNA standards with known concentrations are needed[152, 176]. Furthermore, for accurate quantifications it is crucial to determine the efficiency of the amplification reaction. The efficiency (ε) is calculated by following equation (formula (2)):

$$\varepsilon = \text{dilution factor}^{-\frac{1}{\text{slope}}} - 1 \qquad (2)$$

3.1.2 Real-time multiplex qPCR

Multiplex PCR assays are convenient methods to detect more than one target sequences in a single reaction. Although the establishment of a real-time multiplex qPCR is tedious due to a lot of optimization steps such as primer concentration minimization, the advantages are the decrease of reagent consumption and analysis time. To perform a multiplex qPCR it is important to have a thermocycler which is able to excite and detect different fluorescent dyes. Moreover, the used dyes must differ in their emission spectrum to distinguish between the formed products[178].

3.1.3 Detection of fungal biomass by application of a reference gene system

In order to normalize the results obtained from variable DNA extraction efficiencies a reference gene system was introduced by Brunner et al.[150]. Therefore, the plant gene *adh1*, encoding for the alcohol dehydrogenase of maize, was included in the measurements as a reference gene improving the repeatability of PCR tests. The protocol refers the fungal DNA to the amount of total extracted DNA from a sample. Formula (3) and (4) show the calculation of the infection grade of infested samples with either fumonisin or trichothecene producing *Fusarium* species.

$$\text{Infection}_{\text{fumonisin}}\ [\%] = \frac{\text{DNA from fumonisin producing species [ng]}}{\text{total DNA}^{a}\ [\text{ng}]} \times 100 \qquad (3)$$

$$\text{Infection}_{\text{trichothecene}}\ [\%] = \frac{\text{DNA from trichothecene producing species [ng]}}{\text{total DNA}^{a}\ [\text{ng}]} \times 100 \qquad (4)$$

[a] total DNA includes DNA from fumonisin and trichothecene producing species and maize DNA.

A similar strategy is applied by the European Union Reference Laboratories (EURL) for genetically modified (GM) food and feed for the determination of the relative content of GM events in the total maize DNA[179].

4 Materials and Methods

4.1 Materials and reagents

All chemicals and reagents used for extraction were ordered from Carl Roth GmbH (Karlsruhe, DE), whereas the primers and probes were obtained from Sigma Aldrich (St. Lous, MO). The Kapa qPCR master mix was ordered from PeqLab (Erlangen, DE).

Table 2 Equipment used for the development of a multiplex PCR for the simultaneous detection of fumonisin and trichothecene producing *Fusarium* species

Equipment	**Producer**
Analytical balance, MC1	Sartorius, DE
Absorption photometry, NanoVue	GE life sciences, UK
Centrifuge, Centrifuge 5430	Eppendorf, DE
Centrifuge, GS-6 Centrifuge	Beckman, US
Centrifuge, GS-15 Centrifuge	Beckman, US
Centrifuge tubes, 15 ml and 50 ml	Greiner Bio-one, DE
Eppendorf tubes, 1.5 ml and 2.0 ml	Eppendorf, DE
pH meter	WTW, DE
Pipettes, 10 µl, 20 µl, 100 µl, 1000 µl	Eppendorf, DE
Real-time PCR 96 well plate foils	Applied Biosystems, US
Real-time PCR 96 well plates	PeqLab, DE
Shaking device, MixMate	Eppendorf, DE
Shaking device, Thermomixer comfort	Eppendorf, DE
Thermocycler, 7500 Fast Real-Time PCR System	Applied Biosystems, US
Thermocycler, Mastercycler realplex² S	Eppendorf, DE
Thermocycler, Rotor-Gene® Q	Qiagen, DE
Vacuum pump, HetoVac VR-1	Heto Lab Equipment, DK
Water bath, GFL®	Müller-Scherr, Laborausrüstung, A

4.2 Sample and standard preparation

The fungal strains *F. verticillioides* and *F. graminearum* for standard preparation were provided by the Institute of Biotechnology in Plant Production at IFA-Tulln and the Institute for Chemical Engineering at the Vienna University of Technology.

Twenty-four ground maize samples grown in Styria (Austria) at four different locations (A, B, C and D) were obtained from the Center for Analytical Chemistry at the IFA-Tulln.

4.2.1 DNA extraction from fungal strains for standard preparation

The fungal DNA was obtained by phenol-chloroform extraction according to Peterbauer et al.[180]. In brief, fungal mycelia were homogenized with liquid nitrogen and subsequently

mixed with 600 µl of CTAB (cetyltrimethylammonium bromide) solution (1.4 M NaCl, 0.1 M Tris, 0.02 M EDTA, 2 % CTAB) and 2 % β-mercaptoethanol. The samples were incubated at 65 °C for 15 minutes. Then 450 µl phenol (pH 7.9) and 450 µl of chloroform:isoamylalcohol (49:1) was added. Tubes were mixed and centrifuged at 13,000 x g for 5 minutes at room temperature (RT). The aqueous upper phase, containing the DNA, was transferred into a clean tube and mixed with 400 µl chloroform:isoamylalcohol. The samples were centrifuged at 13,000 x g for 2 minutes at RT and the upper phase was again transferred into a clean tube. For DNA precipitation 0.7 volumes of isopropanol were added. Tubes were centrifuged for 20 minutes at 13,000 x g at RT. The supernatant was removed and the remaining pellet was washed with 500 µl 96 % ethanol (EtOH). Finally, the pellet was vacuum dried and resuspended in 50 µl Tris-EDTA (TE) buffer.

4.2.2 DNA extraction from maize leaves for standard preparation

Maize genomic DNA for DNA standard preparation was isolated from freshly grown maize leaves as described by Saghai-Maroof et al.[181]. Therefore, 3 g of fresh maize leaves were homogenized by liquid nitrogen. Afterwards, 9 ml of CTAB extraction buffer (50 mM EDTA, 700 mM NaCl, 1 % CTAB, 100 mM Tris, 140 mM β-mercaptoethanol) were added and the samples were incubated for 1 hour at 65 °C. Following incubation 4.5 ml chloroform-isoamylalcohol (24:1) were added and the tubes were centrifuged for 10 minutes at 1,500 x g at RT. The upper aqueous layer was transferred into a new flask and the last step was repeated. Subsequently, DNA was precipitated with 6 ml isopropanol. The precipitated DNA was collected with a glass hook and transferred into a tube containing 1.5 ml TE buffer. Fifty µl 5 M NaCl and 2.5 ml 99 % EtOH were added. Then the DNA was transferred into a tube containing 3 ml of 0.2 M Na-OAc and 76 % EtOH and immediately afterwards into a tube containing 1 M NH_4-OAc and 76 % EtOH and kept in the solution for 20 minutes. Finally, the DNA was transferred into a clean 1.5 ml tube containing 1 ml TE (pH 8) buffer.

4.2.3 DNA extraction from maize kernels

The protocol used for the DNA extraction from maize kernels is a modification of a method recommended by the EURL[179].

One gram of ground maize sample was weighed into a 15 ml flask and 6 ml of CTAB extraction buffer (1.4 M NaCl, 2 % w/v CTAB, 0.1 M Tris-base pH 8, 0.02 M EDTA pH 8, 1 % w/v polyvinyl pyrrolidone 40,000) were added and the samples were incubated for 1 hour at 70 °C. Tubes were inverted every 10 minutes during incubation to enhance the efficiency of

the extraction and the DNA yield. Next, the tubes were centrifuged at 2,978 x g for 15 minutes at RT. Subsequently, 500 µl of the supernatant were transferred into a clean tube followed by the addition of 55 µl of 10 % CTAB solution (10 % w/v CTAB, 0.7 M NaCl) and 550 µl of a mixture of chloroform-isoamylalcohol (24:1). The tubes were inverted 20 times and centrifuged at 7,200 x g for 5 minutes at RT. Thereafter, 350 µl of the upper aqueous layer were transferred into a clean tube and 1050 µl of precipitation buffer (1 % w/v CTAB, 0.05 M Tris-base pH 8, 0.01 M EDTA pH 8) were added. The solution was mixed and kept at RT for 30 minutes for precipitation. Then the samples were centrifuged at 7,200 x g for 15 minutes at RT. The supernatant was discarded and the pellet was washed twice with 200 µl 70 % EtOH. The pellet was vacuum dried for 30 minutes at 37 °C and then re-suspended in 100 µl 10 mM Tris buffer (pH 8). Finally, the samples were incubated in a thermomixer for 30 minutes at 65 °C at 1400 rpm to ensure solubilisation of the DNA and stored at -20 °C to avoid enzymatic degradation.
All samples were stored at -20 °C to avoid enzymatic degradation.

4.2.4 Determination of the DNA concentration

The concentration of the isolated DNA was measured photometrically by the NanoVue absorption photometer. Furthermore, the ratios between DNA and carbohydrates (260 nm : 230 nm) or DNA and proteins (260 nm : 280 nm) have been evaluated. A measured value between 1.8 and 2.2 ensures that the DNA is pure and no carbohydrates or proteins might later inhibit the PCR reaction. The DNA of all extracted samples was further diluted to a concentration of 50 ng/µl with poly(dIdC).

4.3 Development of the quantitative triplex qPCR assay

4.3.1 real-time PCR primers and dual-labeled probes

Three sets of specific primer pairs and the appropriate probes were used to amplify either maize DNA, DNA from fumonisin or DNA from trichothecene producing *Fusarium* species. The *adh1*, *fum1* and *tri5* probes were labeled at the 5'-ends with JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), Cy5 (indodicarbocyanine) and 6FAM (6-carboxyfluorescein), respectively and at the 3'-ends with a quencher (Black Hole Quencher® 1, BHQ1). JOE, Cy5 and 6FAM were chosen due to their distant absorbance spectra (643 nm, 520 nm, and 495 nm, respectively) that allows to distinguish between the particular signals. The primers and probe targeting maize DNA specifically ampliefied a 136 bp fragment of the *adh1* gene of maize. The *Fusarium* DNA of trichothecene producing species was detected by

the amplification of a 178 bp fragment of the *tri5* gene. Primers and the probe to quantify fumonisin producing species DNA were slightly modified from the study of Waalwijk et al. in 2008[182]. The amplified fragment has a length of 149 bp. Table 3 lists the used primers and probes with their sequence, the corresponding labeling as well as the fragment length that is amplified.

Table 3 Sequences of used primers and probes as well as the length of the fragment that is amplified

Target: maize		
Name	**Sequence (5' – 3')**	**Amplicon length**
adh1_fw	CGTCGTTTCCCATCTCTTCCTCC	136 bp
adh1_rev	CCACTCCGAGACCCTCAGTC	
adh1_probe	JOE-AATCAGGGCTCATTTTCTCGCTCCTCA-BHQ1	
Target: fumonisin producing *Fusarium* species		
Name	**Sequence (5' – 3')**	**Amplicon length**
fum1_fw	ATGCAAGAGGCGAGGAA	149 bp
fum1_rev	GGCTCTCAGAGCTTGGCAT	
fum1_probe	Cy5-CAATGCCATCTTCTTGAAACCT-BHQ1	
Target: trichothecene producing *Fusarium* species		
Name	**Sequence (5' – 3')**	**Amplicon length**
tri5_fw	GATTGAGCAGTACAACTTTGG	178 bp
tri5_rev	ACCATCCAGTTCTCCATCTG	
tri5_probe	6FAM-C[+C][+T][+T][+G]G[+G]CCA-BHQ1	

4.3.2 real-time PCR optimization and assay evaluation

Initially, the efficiency of the three primer sets was evaluated by testing them in separate reactions based on the protocols of EURL[179], Brunner et al.[150], and Waalwijk et al.[182]. The multiplex qPCR assay was optimized by empirically varying critical factors that affect multiplexing such as primer concentrations and annealing temperatures. All primers and probes have been tested with three concentrations: 0.1 pM/µl, 0.05 pM/µl and 0.025 pM/µl. A gradient PCR from 52 °C to 60 °C was performed with these primers to determine the optimal thermal cycling conditions. To test the reproducibility of the multiplex assay all analyses were performed on a 7500 Fast Real-Time PCR System as well as on a Rotor-Gene Q. A total reaction mix volume of 15 µl was used for all analysis. This mix contained 2 µl of template DNA, 7.5 µl Kapa probe fast master mix, 4.78 µl/3.34 µl water (singleplex and multiplex, respectively), 0.1 pM/µl or 0.05 pM/µl of forward and reverse primer, and dual labelled probe. The used temperature program consisted of an initial denaturation step at 95 °C for 1 min and 50 s, followed by 45 cycles of denatruation at 95 °C for 15 s and primer annealing and amplicon extension at 58 °C for 45 s. All samples were analysed in triplicates. To

quantifiy the unknown amount of target DNA in the samples, appropriate DNA standards with known concentrations have been produced. To obtain these standards *Fusarium* free maize DNA was used as background and then spiked with varying amounts of *F. graminearum* or *F. verticillioides* DNA to either quantify the amount *tri5* DNA or *fum1* DNA. Both DNA standards have been serially diluted by a factor of ten with maize DNA to reduce the amount of *Fusarium* DNA in a constant maize background DNA concentration.

The primer sets are the most sensitive part in the development of a multiplex qPCR assay. Therefore, the cross-reactivity of the primers and probes was analyzed by testing the different primer sets on different fungal species. *Fum1* primers were tested for the amplification of *tri5* producing *Fusarium* strains and *vice versa*. Furthermore, the specificity of all oligonucleotides was analyzed. The specificity of the *tri5* primers was already evaluated by Brunner et al.[150]. To prove equal efficiencies of the *fum1* primers for different fumonisin producing *Fusarium* strains, five different isolates belonging to three different *Fusarium* spp. were obtained by the Vienna University of Technology (internally numbered) and analyzed. Following species have been included: *F. proliferatum 23*, *F. proliferatum 353*, *F. proliferatum 2763*, *F. verticillioides* and *F. nygamai*. qPCR conditions were the same as mentioned above.

The sensitivity of the assay was evaluated by a dilution series of purified genomic DNA from *F. graminearum* and *F. verticillioides*. For that, 5 ng of DNA were serially diluted by a factor of five with maize DNA (50 ng/µl) to a concentration of $4.10 \bullet 10^{-9}$ ng/µl. This was done for the singleplex as well as for the multiplex method.

Each infected maize sample was divided into two sub-samples. Every sub-sample was extracted and the DNA was precipitated twice. To ensure the repeatability of all parts of the method each precipitate was quantified twice by the three singleplex assays as well as by the newly developed multiplex qPCR method (Fig. 10.)

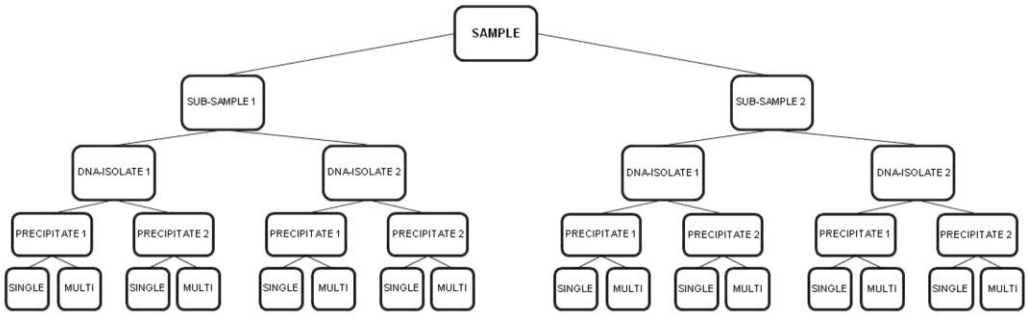

Fig. 10 Scheme of sample analyses. Every sample was divided into two sub-samples which were then used for DNA isolation. Furthermore, each DNA isolate was precipitated twice and finally, all precipitates were analyzed two times, either by the three singleplex assays or the multiplex method.

4.3.3 Determination of Fusarium toxins by LC-MS/MS

All 24 maize samples were analyzed for the presence and concentrations of *Fusarium* metabolites by LC-MS/MS by Dr. Michael Sulyok at the Center for Analytical Chemistry, IFA-Tulln. The used method has been evaluated by Malachova et al.[109] and covers currently 320 metabolites. In brief, 5 g of sample were weighed into a 50 ml polypropylene tube (Sarstedt, DE) and extracted with 20 ml acetonitrile/water/acetic acid (79:20:1, v/v/v). Finally, the extracts were diluted in extraction solvent (1:1) and directly injected into the LC-MS/MS.

5 Results and Discussion

5.1 Development of a quantitative triplex PCR assay

Eighty-one different combinations of annealing temperatures and times have been evaluated together with three different oligonucleotide concentrations of 0.1 pM/µl, 0.05 pM/µl and 0.025 pM/µl. The oligonucleotides adh1_fw, adh1_rev and adh1_probe as well as the tri5_probe were set to 0.05 pM/µl; all other primers and probes were used at a concentration of 0.1 pM/µl. Finally, a gradient PCR from 52 °C to 60 °C was performed with these oligonucleotides to determine the ideal temperature program. The optimal thermal cycling conditions turned out to be 1 min 50 s at 95 °C as initial step, followed by 45 cycles of 15 s at 95 °C and 45 s at 58 °C. Experiments showed that the multiplex assay worked most efficiently with a two-step protocol.

5.2 Specificity and sensitivity of the multiplex qPCR assay

The efficiency of PCR amplification of the *tri5* gene has been demonstrated by Brunner et al.[150]. Therefore, different isolates from different trichothecene producing *Fuarium* species have been tested. All tested strains were amplified with an efficiency of 0.91 ± 0.41. Waalwijk et al.[182] evaluated the specificity of the *fum1* assay. They showed that no amplicons were generated from any of the non-fumonisin producers such as *F. equiseti*, *F. graminearium*, *F. oxysporum*, *F. semitectum*, and *F. subglutinans*. The specificity of the *fum1* assay was also tested in this study. Five different isolates from three different fumonisin producing *Fusarium* species were used provided by the Institute of Biotechnology in Plant Production at the IFA-Tulln and the Institute for Chemical Engineering at the Vienna University of Technology (*F. proliferatum 23*, *F. proliferatum 353*, *F. proliferatum 2763*, *F. verticillioides* and *F. nygamai*). All tested strains showed an amplification efficiency of 0.88 ± 0.43. Moreover, all isolates scored positive for the *fum1* product. Thus, it is suggested that the qPCR reaction is neither influenced by different isolates nor by species. Also no cross-reactivity was shown between fumonisin and trichothecene producing *Fusarium* strains, which confirms that the used primers and probes are group specific for either fumonisin or trichothecene producers (data shown in Table 4).

Table 4 Specificity of the multiplex real-time PCR assay against different fungal species

Fungal species	***fum1* detected**	***tri5* detected**
Fusarium proliferatum 23	+	-
Fusarium proliferatum 353	+	-
Fusarium proliferatum 2763	+	-
Fusarium verticillioides	+	-
Fusarium nygamai	+	-
Fusarium graminearum	-	+
Fusarium culmorum	-	+

To determine the sensitivity of the assay, the multiplex qPCR method was carried out using a dilution series of a mixture of pure fungal genomic DNA from *F. graminearum* and *F. verticillioides* in maize background with a starting concentration of 5 ng/µl. The DNA was diluted by a factor of five and used as template for the qPCR assay. The limit of quantification (LOQ) was found to be 0.32 pg of target DNA per µl for both *Fusarium* strains. The LOQ is defined as the minimum target concentration that remained within the linear regression line (Fig. 11). Due to high standard deviations lower concentrations could lead to false positive results. Considering a genome size of 36.2 Mb for *F. graminearum* and 41.7 Mb for *F. verticillioides*[183] this represents approximately seven or eight genome equivalents, respectively, calculated according to Staroscik[184]. Compared to the singleplex assays no loss of sensitivity was observed when applying the multiplex method. To compensate for varying extraction efficiencies all assays were run in combination with the maize *adh1* gene.

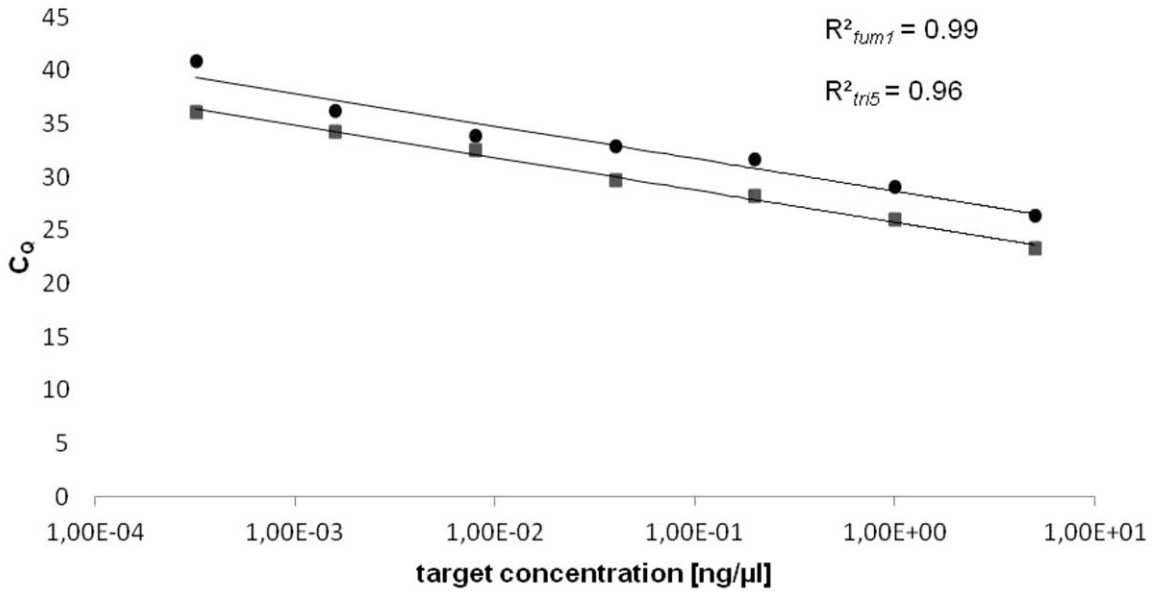

Fig. 11 Sensitivity of the triplex real-time PCR assay. • shows *F. graminearum* as the target DNA and ▪ shows the serial dilution of *F. verticillioides*. Both dilution series had a starting concentration of 5 ng per µl and have been 5-fold diluted. Following dilutions were used to generate a linear regression line: 5 ng/µl, 1 ng/µl, 200 pg/µl, 40 pg/µl, 8 pg/µl, 1.6 pg/µl and 0.32 pg/µl.

5.3 Reproducibility test of the multiplex qPCR assay

Reproducibility tests were performed to determine whether the instrument used for quantification or the operator affects the results. Therefore, all qPCR assays were run on two PCR thermal cyclers from different manufacturers; the 7500 Fast Real-Time PCR System from Applied Biosystems and the Rotor-Gene® Q from Qiagen. Fig. 12 shows the comparison of the *tri5* and *fum1* standards (concentrations: 5 ng/µl, 0.5 ng/µl and 0.05 ng/µl) either analyzed with the PCR system from Applied Biosystems or the Rotor-Gene® Q from Qiagen. The coefficient of determination was found to be $R^2 = 0.99$ for the *tri5* DNA standard correlation analyzed with the Applied Biosystems cycler and the Rotor-Gene® Q. The R^2 observed for the *fum1* DNA standard correlation between the two cyclers was found to be $R^2 = 0.97$.

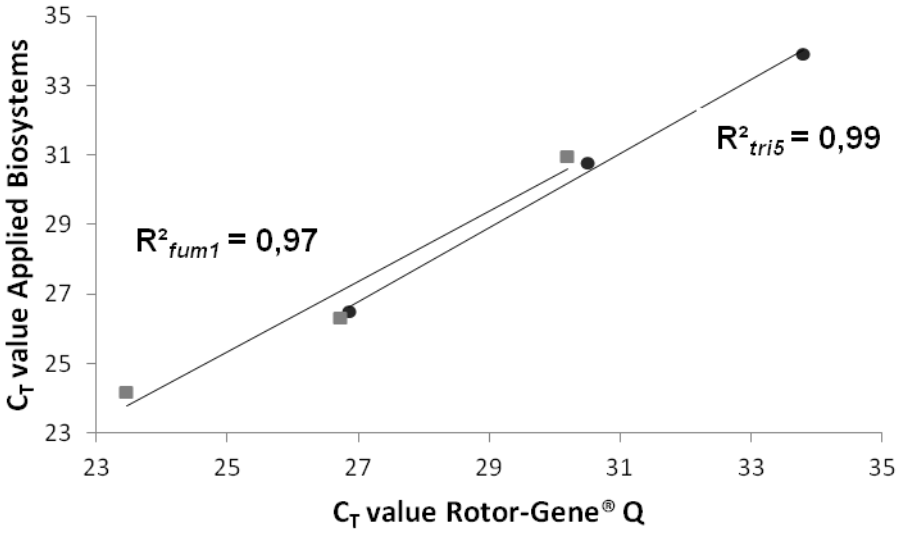

Fig. 12 Comparison of different PCR cyclers. ● shows the *tri5* standard quantified with either the cycler from Applied Biosystems or the Rotor-Gene® Q from Qiagen. ■ compares *fum1* standards either analyzed with the thermal cycler from Applied Biosystems or the Rotor-Gene® Q.

Furhtermore, two operators prepared the qPCR assays. Results obtained by operator one and operator two differ about 0.61 % and 1.62 % for trichothecene and fumonisin producing *Fusarium* species, respectively.

It could be shown that neither the instrument nor the operator had a significant influence on the obtained results.

5.4 Evaluation of the multiplex qPCR assay with Fusarium infested maize samples

The unknown amount of target DNA in *Fusarium* infested maize samples was quantified on appropriate DNA standards. Table 5 shows the C_Q values of the serially diluted DNA standards as well as the efficiencies of the multiplex assay. The calculated efficiencies were 0.94, 0.98 and 1.03 for the *tri5*, *fum1* and *adh1* target amplicons, respectively.

Table 5 C_Q values of serially diluted DNA standards and efficiencies for all three targets quantified simultaneously by the triplex PCR

Concentration of fungal DNA (*fum1* or *tri5*)	**C_Q for trichothecene producing isolates**	**C_Q for fumonisin producing isolates**	**Concentration of maize DNA standard**	**C_Q for maize DNA amount**
5 ng/µl	26.86 (± 0.23)	23.47 (± 0.21)	50 ng/µl	24.25 (± 0.23)
0.5 ng/µl	30.50 (± 0.43)	26.73 (± 0.17)	25 ng/µl	25.13 (± 0.12)
0.05 ng/µl	33.80 (± 0.30)	30.20 (± 0.53)	12.5 ng/µl	26.10 (± 0.28)
			6.25 ng/µl	27.18 (± 0.41)
Efficiency	0.94	0.98	Efficiency	1.03
R^2	0.9992	0.9997	R^2	0.9979

Twenty-four maize field samples were collected from four different locations in Austria (locations A, B, C and D) and analyzed for the presence of *Fusarium* DNA. The whole

analysis procedure, including the various steps of DNA extraction (Fig. 10) and the quantification by three singleplex PCR assays and by the triplex test developed in this study, was repeated twice. This enables a deeper insight into the repeatability of the whole method. Resutls showed low variations between the two sub-samples, the individual precipitates and no significant differences result from the PCR analysis. Generally, a mean total error of 23 % occurs over the whole analysis procedure. Going more into detail, 5 % of the observed error is caused by dividing the sample into two sub-samples, 10 % arises from the DNA precipitation step and 8 % of the error results from the multiplex qPCR method. To calculate the error rates, 48 values for sub-sample extraction, 96 values for precipitation and 192 values for qPCR analysis were included for each gene; *adh1*, *fum1* and *tri5*.

Finally, the infection of the samples was calculated based on formula (3) and (4) published by Brunner et al.[150] referring the fungal DNA to the total extracted DNA from a sample. All analyzed samples show a wide range of *Fusarium* infection and were positive for *fum1* DNA as well as *tri5* DNA. The grade of infection with fumonisin producing *Fusarium* species varies between $8.2 \cdot 10^{-4}$ ‰ and 0.22 ‰ whereas the range of infection with *tri5* producing *Fusarium* strains was much higher and ranged from 0.10 % to 3.33 %. The difference between the two groups might be due to that fact that fumonisin production appears commonly in southern areas and is reduced in cooler regions like Austria. Thus other toxins produced by other *Fusarium* species become more important and prevalent[185]. Due to the very low amount of *fum1* DNA detected in all samples the results inherently show a higher standard deviation. The mean relative standard deviation (RSD) for the samples for fumonisin producing species is 17.19 % for the singleplex and 23.30 % for the multiplex method. For the *tri5* assay, the mean RSD shows 7.17 % for the singleplex and 22.71 % for the multiplex method. All RSD values are referred to the complete analysis procedure, including DNA isolation and qPCR analysis. The loss of precision of the *tri5* multiplex method in contrast to the singleplex assay might be attributable to the interaction among the many oligonucleotides in the triplex assay. A good correlation of the results between the singleplex assay and the rapid multiplex test was shown (Fig. 13); therefore, a slight loss of precision is negligible. Furthermore, the costs and analysis time are reduced by a factor of three for the multiplex method.

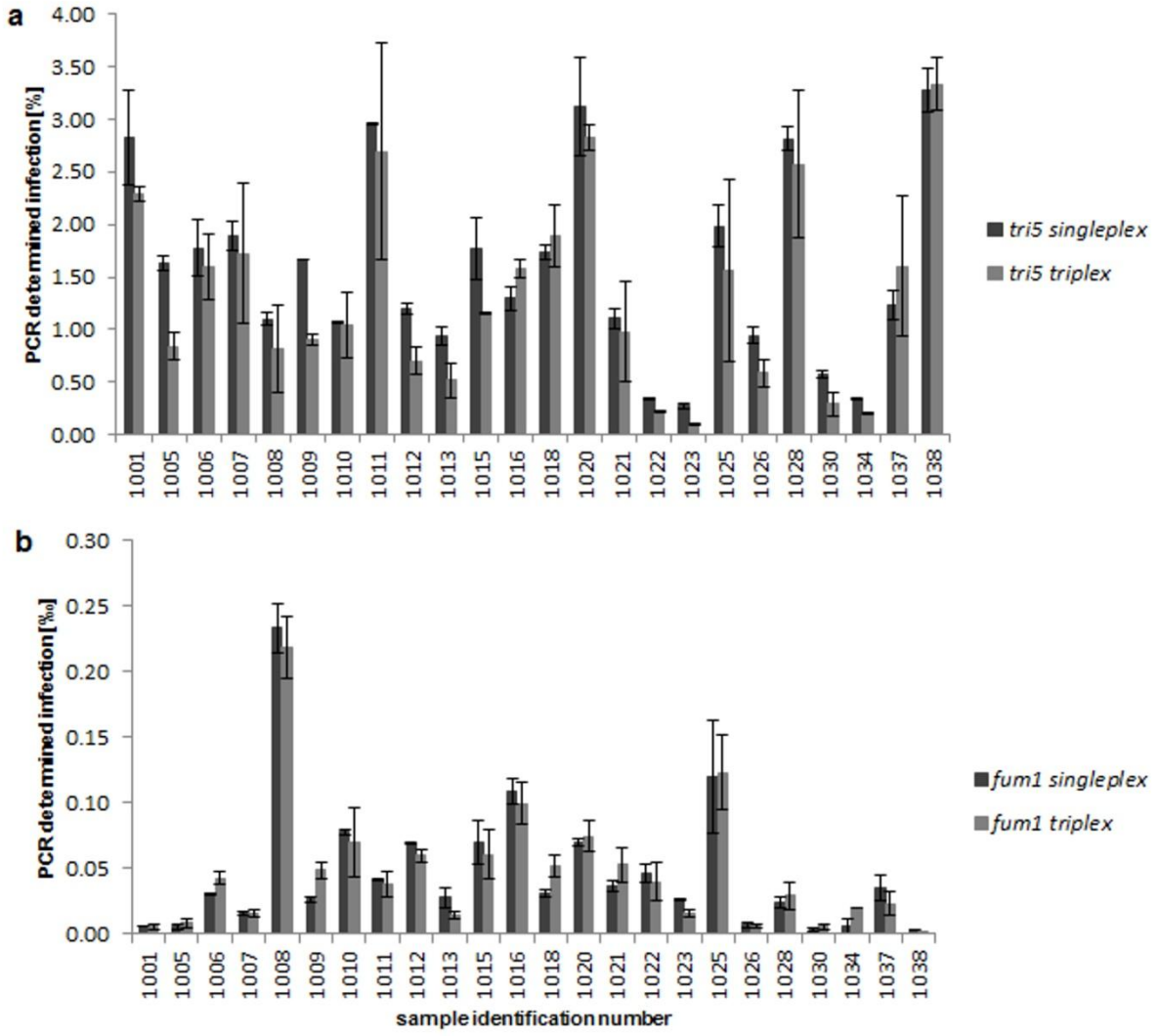

Fig. 13 Analysis of 24 Austrian field samples (internally numbered). The bars show the results obtained either by the singleplex assay (dark grey) or the multiplex assay (light grey). **(a)** PCR-determined infection grade of maize samples with trichothecene producing *Fusarium* species and **(b)** infection of samples with fumonisin producing species.

5.5 Comparison of LC-MS/MS determined mycotoxin concentrations with the real-time PCR determined Fusarium infection

The 24 maize field samples were used to analyze whether the qPCR results for *Fusarium* DNA correlate either with the trichothecene or the fumonisin content. Thus, the mycotoxin concentration of each sample was determined by LC-MS/MS according to Malachova et al.[109]. The qPCR determined infections obtained by the triplex method were plotted against the mycotoxin concentrations. The PCR primers for the *tri5* gene quantification are specific for all *Fusarium* species producing trichothecenes. For this reason, the *tri5* infection was not only compared to the prevalent DON concentration but rather to the total amount of trichothecenes including 3-ADON, DAS, DON, D3G, HT-2, NEO, NIV and T2. Results showed that the PCR determined infection grade correlates slightly better with the sum of the

eight toxins than with DON only. To compare the *fum1* infection with the fumonisin concentrations, the toxins FB_1, FB_2 and FB_3 were used as a sum parameter. The correspondence between the infection of a maize sample calculated according to formula (3) and (4) and toxin content determined by LC-MS/MS are shown in Fig. 14. The coefficient of determination was found to be $R^2 = 0.64$ for the correlation of the trichothecene content and the qPCR determined infection and $R^2 = 0.63$ for the fumonisin concentrations and the infection determined by the qPCR.

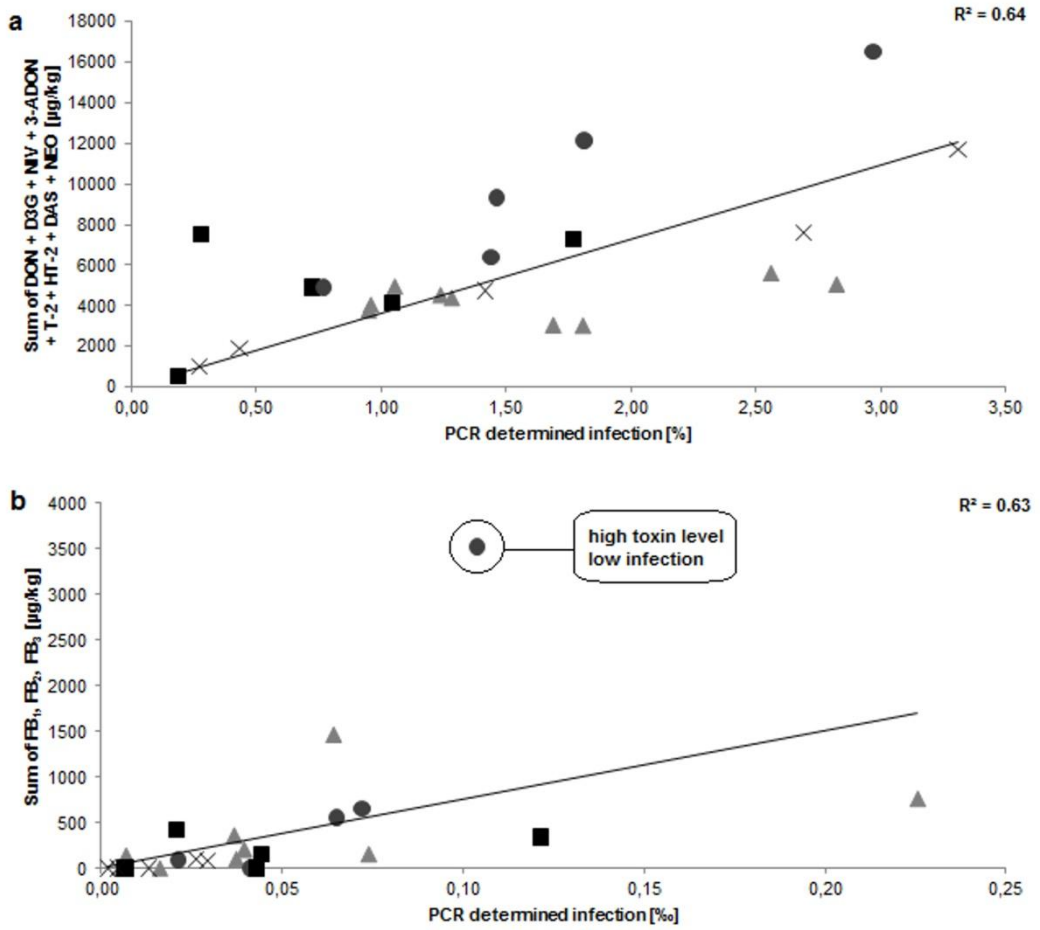

Fig. 14 Correlation between the mycotoxin concentration and infection grades determined by the multiplex qPCR method. The four different locations are labeled as follows: ■A, ●B, ▲C and ×D **(a)** Results for trichothecene producing strains; the type B trichothecenes DON, D3G, NIV and 3-ADON as well as the type A trichothecenes T-2, HT-2, DAS and NEO were used as a sum parameter **(b)** Correlation of the infection with fumonisin producing strains with the fumonisins FB_1, FB_2 and FB_3.

According to the correlation, it is obvious that *Fusarium* isolates present at location B produce presumably more toxin per fungal biomass, whereas at location C the situation is reverse. Therefore, the infection determined by qPCR analysis provides additional information which might be relevant for the determination of the resistance of new crop lines in breeding programs.

Currently, two methods are applied for the registration of new crop varieties: visual scoring and mycotoxin analysis. However, these methods only indirectly determine the resistance of plants which is defined as the pathogen growth on or in the plant. Moreover, experiments stated that the amount of fungal mycelia formed during infection not always correlates well with disease symptoms[150, 186] and also the amount of accumulated toxin does not necessarily reflect differences in resistance. Genotypes with good resistance based on visual scoring might show high or low toxin contamination and highly resistant wheat varieties can often not be classified by visual scoring anymore as the infection proceeds without any symptoms but still leads to high mycotoxin accumulation. The high sensitivity of the qPCR registers even minor amounts of fungal biomass[187].

6 Conclusion

In the last decade, several studies have described two distinct approaches for *Fusarium* quantification. On the one hand, species specific assays which quantify individual *Fusarium* species and on the other hand group specific assays which measure the abundance of a gene owned by a group of different species sharing a common biosynthetic pathway such as the genes for mycotoxin production[150, 182, 188]. Throughout the last decade two group specific quantitative PCR assays were used: one that enables the quantification of all trichothecene producing *Fusarium* species[154] and another one for all fumonisin producing *Fusarium* species[189]. However, until today these assays have been only available as two quantitative singleplex tests or as a duplex assay[151] which is only qualitative.

The here developed analysis techniques describe a multiplex qPCR assay which allows the quantification of all *Fusarium* species producing the most prevalent mycotoxins in maize, trichothecenes and fumonisins. Besides the two fungal toxin genes, *tri5* and *fum1*, the maize gene *adh1* was used as a reference gene to compensate for varying DNA extraction efficiencies, similar to the established system for GMO analysis. The newly developed multiplex qPCR assay allows rapid screening of high sample numbers in a relatively short time. The assay was designed to minimize costs by reducing the analysis time by a factor of more than 60 %. This method provides a perfect complementation to mycotoxin analysis to facilitate the classification of the resistance of new crop lines in breeding programs.

Chapter Two

The development of aptamers for the rapid detection of aflatoxin B_1 in maize

Aflatoxins are common contaminants in foods, particularly in the diets of people in developing countries. These toxins are secondary metabolites mainly produced by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. These species are widespread in nature and can colonize agricultural crop plants, such as corn and groundnuts. Among several natural occurring aflatoxins (B_1, B_2, G_1 and G_2), B_1 (AFB_1) is the most prevalent and potent of these toxins. Health concerns related to AFB_1 have increased over the years and maximum levels have been set between 1 µg/kg and 20 µg/kg in food. Therefore, rapid, sensitive and inexpensive analytical methods are essential to detect and quantify AFB_1. Currently, the most frequently used procedures are chromatographic based methods including high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) and rapid screening protocols with immunoassays (e.g enzyme linked immunosorbent assay, ELISA). However, these methods often require skilled personnel, expensive equipment (especially HPLC-MS based methods), extensive sample pre-treatment and the use of antibodies. To overcome these drawbacks aptamers for the detection of AFB_1 in maize have been evolved using an in vitro selection method named systematic evolution of ligands by exponential enrichment (SELEX). The most affine aptamers were further characterized concerning their sequence, the most likely folding structure and the dissociation constant. Furthermore, the present study demonstrates for the first time an approach for a rapid and simple aptamer dipstick assay using a direct detection format for AFB_1. The method is based on the capability of an aptamer to switch its structure after the addition of the target. Then a binding site for a complementary signaling DNA probe is exposed to enable the hybridization of the probe with the aptamer. This hybrid molecule is visualized *via* a simple lateral flow test. A test line is visible only when aflatoxin is present in a sample solution.

7 Introduction

7.1 Aptamer selection by Systematic Evolution of Ligands by EXponential enrichment (SELEX)

Target specific aptamers are artificially evolved by an iterative process named SELEX, *i.e.*, **S**ystematic **E**volution of **L**igands by **EX**ponential enrichment. This method was first described in 1990 by Tuerk and Gold[190] and simultaneously by Ellington and Szostak[156]. SELEX (or *in vitro* selection) is a technique used to isolate aptamers with high affinity for a specific target. Proper aptamers are selected from a library containing approximately 10^{12} to 10^{15} combinatorial oligonucleotide variations[191]. The SELEX procedure is characterized by the repetition of successive steps of target binding and removal of unbound oligonucleotides, followed by elution, amplification and purification of selected oligonucleotides[156, 190].

7.1.1 Starting random DNA oligonucleotide library

The starting point of each SELEX round is a synthetic random DNA oligonucleotide library consisting of 10^{12} to 10^{15} different ssDNA fragments. This library is comprised of a random region of about 20 to 80 mers flanked by primer binding sites[192] with specific sequences of 18 to 25 nucleotides (nt) (Fig. 15).

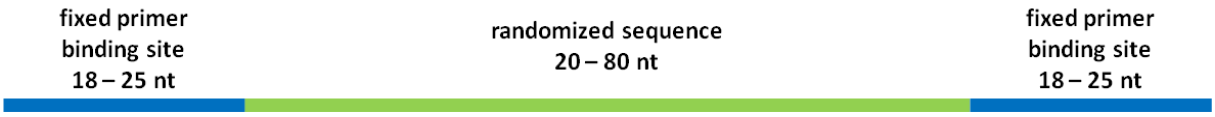

Fig. 15 Starting random ssDNA library

The size of the randomized region is an important aspect for designing the library. While short aptamer libraries are more cost-effective, longer randomized oligonucleotides on the other hand enable greater structural complexity. This might be important for targets that are not known to bind to nucleic acids[193]. However, a variety of studies showed that truncation of nonessential nucleotides, only retaining the functional part of the aptamer, often leads to a better formation of the aptamer-target-complex due to reduced steric hindrance[194-196]. Furthermore, nucleic acids can be chemically modified aiming at increase of complexity of a library, introducing new features like functional groups, to enhance stability or to increase the resistance to nucleases. Some other modifications can be used for the quantification of selected oligonucleotides during SELEX process or to monitor ssDNA generation for further selection steps[192].

7.1.2 Target immobilization

To ensure effective partitioning between target-bound and non-bound oligonucleotides the immobilization of the target molecule on a particular matrix or SELEX carrier is essential.

The use of **affinity chromatography** allows the immobilization of a target on column material like sepharose or agarose and is a conventional carrier used in the SELEX procedure. However, abundant amounts of target are necessary to obtain a highly efficient loading of the column[154, 197].

Another commonly used method of separation is the use of **magnetic beads**. This method requires only small amounts of target and enables a rapid and easy isolation of target-immobilized beads with a magnet[154, 198, 199].

Electrophoretic separation is an effective alternative to mechanical or magnetic separation. Thus, different compounds are separated based on their different speeds, which depend on individual sizes and charges under an electrical field[154, 198].

Ultrafiltration is a method of separation without target immobilization by the use of nictrocellulose filters with distinct molecular weight cut-offs[200].

The coupling chemistries depend on the functional groups that are available on both the target and the resin. Careful evaluation is crucial to ensure that the resin itself does not bind the DNA pool. The stringency of the selection conditions should therefore be increased during the SELEX procedure[197].

7.1.3 Aptamer selection

The *in vitro* selection of aptamers is mainly comprised of three steps that are repeated in order to find nucleotides that better bind to the target.

In the first step, **library generation/conditioning**, the strands from the DNA pool are converted into single stranded oligonucleotides that consist of a random sequence, usually 20 to 80 mers, flanked by primer binding sites with known sequence. The generated pool of oligonucleotides is then mixed with the target. In the second step, **binding and separation**, the target-bound library components are separated from unbound components, mostly coupled with different counter selection steps to ensure specificity of the selected aptamer and to increase the stringency of the method. The molecules that are not bound to the target are washed off and the bound ones are eluted. At the final step, **amplification**, the bound oligonucleotides are amplified by PCR to create a new library for the next selection round. These three steps are repeated up to 15 rounds to find aptamers of interest[154, 157, 201]. A scheme of the SELEX procedure is given in Fig. 16 in a simplified way.

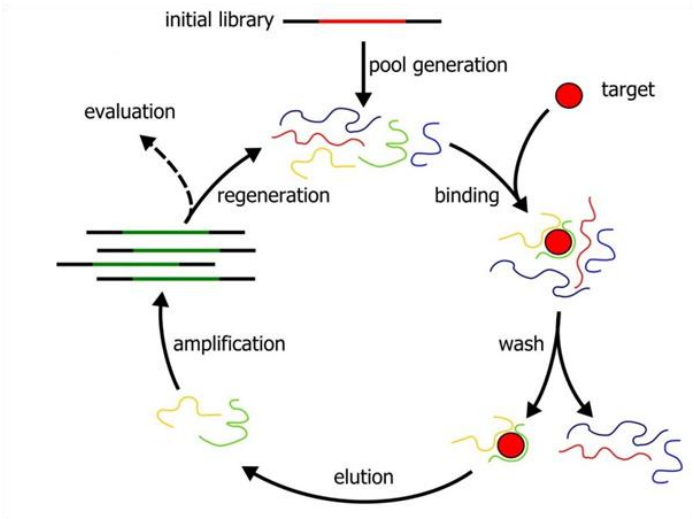

Fig. 16 In vitro selection of target-specific aptamers using SELEX technology

7.1.4 Single strand DNA generation methods

After the amplification step the enriched oligonucleotide pool is available as dsDNA. Before introducing the amplified dsDNA pool to the next cycle of the SELEX procedure single strand separation has to be carried out[192].

Asymmetric PCR is one of the most common forms of generating ssDNA in aptamer production. This method produces ssDNA due to an unequal concentration of sense and antisense primers used in the reaction. This process consists of two phases. The first phase is the exponentially phase where dsDNA is produced, followed by a linear phase that produces ssDNA due to that fact that the primer with the lower concentration is depleted after the exponential phase. The most critical aspect in this procedure is the ratio of primer amounts. Due to the production of both dsDNA and ssDNA a purification step is necessary since the dsDNA can bind non-specifically to the target molecules[202, 203].

Another frequently used method is the **streptavidin/biotin system**. The biotin-streptavidin bond is one of the strongest biological interactions. For this method one of the two primers is biotinylated. The resulting biotinylated PCR product is then immobilized onto streptavidin coated magnetic beads. Because of the high affinity of streptavidin towards biotin, the non-biotinylated strand is separated from the biotinylated one by alkaline treatment (NaOH). Thereafter the strands are treated with chloroform/phenol to remove streptavidin which could act as possible target in the next SELEX rounds, and the sample is subsequently concentrated by ethanol precipitation[202, 204].

A fast and efficient method of generating ssDNA relies on **enzymatic digestion** with the lambda exonuclease. This enzyme originates from bacteriophage I and is involved in the

repair mechanism of double-stranded breaks of the viral DNA[205]. It selectively digests the phosphorylated strand of dsDNA from the 5'-end to the 3'-end, whereas non-phosphorylated ssDNA remains[206]. For this method one of the used primers has to be phosphorylated at the 5'-end.

The principle of **size separation on denaturing urea polyacrylamide gel electrophoresis** (PAGE) is based on the labeling of one primer with a hexaethylene glycol (HEGL) spacer and a 20-nucleotides length of a string of adenosine (poly-dA). The modification impedes strand extension during amplification and a size difference of the amplicon strands is created. Thus, the strands migrate at different rates upon resolving on the urea-denaturing-PAGE[202].

Fig. 17 gives an overview about the above described methods.

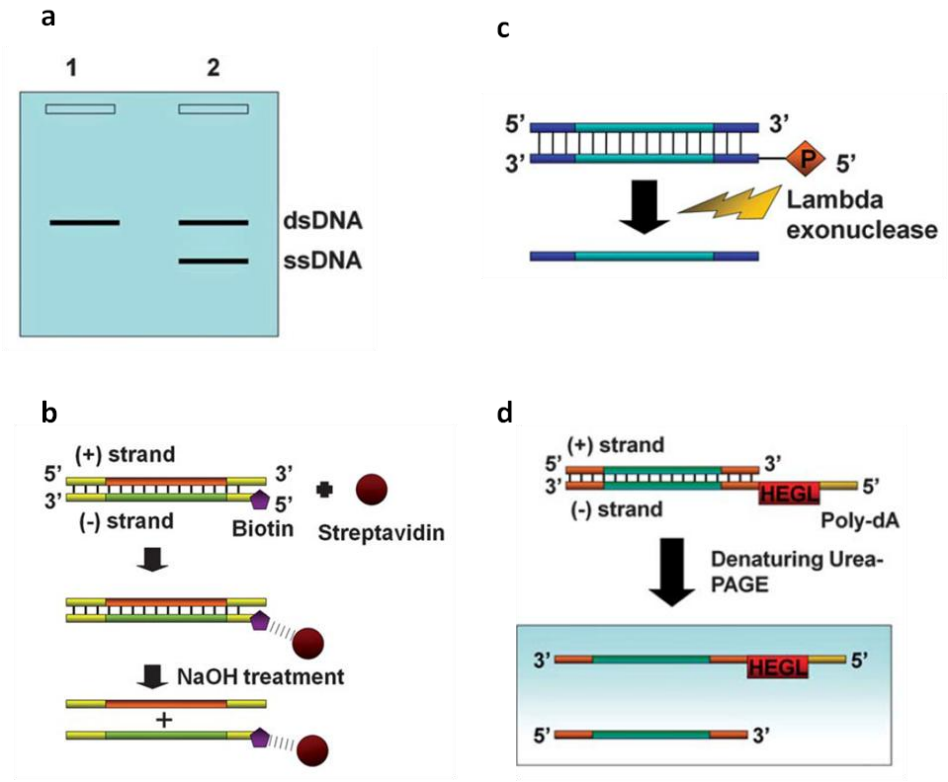

Fig. 17 Overview about possible methods for ssDNA generation. **(a)** Illustration of asymmetric PCR product separation on agarose gel electrophoresis. Lane 1 shows a symmetric PCR product (same amount of sense and antisense primer was used in the PCR); Lane 2 demonstrates an asymmetric PCR product with unequal amounts of primers. **(b)** Scheme of ssDNA generation from biotinylated PCR product using streptavidin. **(c)** Lambda exonuclease digestion due to a 5'-end phosphorylated PCR product. **(d)** Creation of a size difference of the PCR product. Therefore, the two strands migrate at different rates on the urea-denaturing-PAGE[202].

7.2 Characterization of aptamers

7.2.1 Sequencing methods

During the SELEX process the complexity of the initial library is reduced and the specific target-binding candidates are enriched. To obtain the sequence of the enriched aptamers, the

final pool is either cloned into a bacterial vector and subsequently sequenced conventionally by Sanger or directly by next-generation sequencing (NGS)[207].

Sanger sequencing is based on the use of chain-terminating dideoxynucleotides (ddNTPs) that lack the 3'-hydroxyl group. Besides, ddNTPs, which are mostly fluorescently labelled, the method furthermore requires a DNA template, a DNA polymerase and deoxynucleotides (dNTPs). The DNA polymerase ceases the extension of DNA due to the omitted 3'-hydroxyl group. After primer hybridization fragments of varying sizes are synthesized. Since four different chain-terminating nucleotides are used, all combinations of termination can be produced. Finally, the fragments are separated by HPLC and analyzed to get the DNA sequence[208, 209]. Sanger sequencing is a very accurate method and used routinely for sequencing of small amounts (500 bp to 1 kb)[210]. However, the method is time-consuming and cost-intensive.

A new generation of sequencing technologies is the so called **"next-generation" sequencing** (NGS). This technique enables rapid generation of data by sequencing massive amounts of DNA in parallel. The starting material is usually dsDNA that must be converted into a sequencing library. This library is created by fragmentation and size selection, to break the DNA templates into smaller fragments. Subsequently, synthetic DNA is added (adapter ligation) to the ends of the fragment, that serves as primers. The type of synthetic DNA used depends on the NGS platform[207]. One of the most widely used NGS platforms is the Illumina sequencing technology. According to this method, adapters are ligated to both ends of each DNA fragment. The ssDNA fragments are then bound to the inside surface of the flow cell channels. This flow cell channels contain two types of oligonucleotides complementary to the adapters added to the DNA fragment. Then unlabelled nucleotides and the polymerase are added. Solid phase bridge amplification is initiated and all fragments become double stranded. Subsequent the strands are separated by sodium hydroxid. The free ends bind again to the complementary primer to form a bridge. This process continous for 35 cycles and several million clusters of double stranded DNA are generated. After the last amplification cycle the sequencing starts at the appropriate primer by adding four labelled reversible terminators, primers and the DNA polymerase. The labelled bases bind one at a time, the emitted fluorescence is captured and the bases are identified[211]. A schematic illustration is given in Fig. 18.

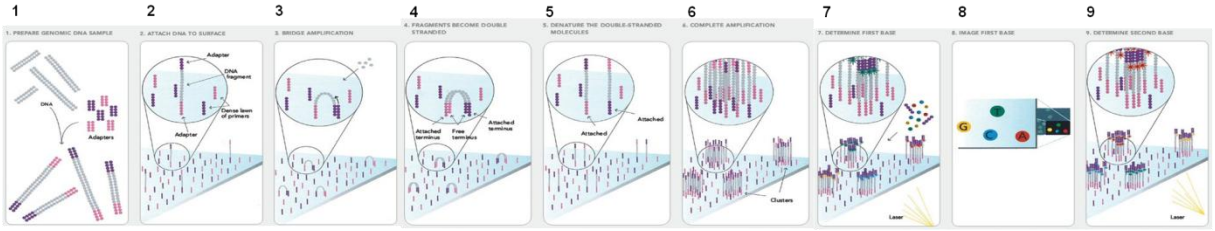

Fig. 18 (1) Adapters ligate to both ends of the DNA fragments. **(2)** Adapter binding to their complementary part on the flow cell surface. **(3)** Bridge amplification. **(4+5)** The dsDNA is separated by sodium hydroxide. **(6)** After complete amplification dense clusters are formed. **(7)** Sequencing starts at the sequencing primer and fluorescently labeled bases are added. **(8)** The fluorescence is captured and the bases are identified. **(9)** Process continuous till all bases are identified[212].

7.2.2 Sequence analysis

Sequence alignments are very useful to identify aptamers with homologous sequences and to evaluate the complexity of the selected oligonucleotide pool[213]. The program CLUSTAL X performs such alignments. Furthermore, secondary structure analyses provide information about relevant structures for target binding. For performing such analyses the online program *mfold* is typically used (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form). The *mfold* web server was developed by Michael Zuker (2003) and enables the prediction of nucleic acid folding structures by calculating the possible configurations of the oligonucleotide under consideration of stem-structures and loops[214].

7.2.3 Determination of the dissociation constant (K_d)

To assess the binding affinity of the aptamer to its target the affinity constant (dissociation constant K_d), as a characteristic parameter of an aptamer, is of major importance. The K_d-value of an aptamer-target-complex usually ranges from a low micromolar to a high picomolar level[215]. A low K_d-value indicates high affinity to the target. The equilibrium can be described by formula (5):

$$K_d = \frac{[A] \times [T]}{[C]} \qquad (5)$$

in which A is the concentration of the aptamer, T is the target concentration, and C is the concentration of the formed aptamer-target-complex[216].

The binding constant is typically determined by **direct titration**, where compound A is titrated with compound B and the formed product [AB] is in equilibrium with free [A] and [B] or **serial dilutions**, where saturated binding curves are generated and the K_d-value is further estimated by nonlinear regression analysis[217]. The signal generated due to binding affinity or structural change can be measured either by fluorescence or circular dichroism (CD).

7.3 Aptamer-based biosensors – Aptasensors

Aptamers are highly attractive for biosensor development due to their small size, high stability and binding affinity, and ease of modification[218, 219]. Significant advances have been reported in the development of aptamer-based sensing technologies, especially regarding the detection of proteins and small molecule targets[220]. Till now, several design formats coupled with various signalling methods have been published. Biosensor formats include:

- **Enzyme linked aptamer assays** (ELAAs) – they use an aptamer as recognition element and an enzyme for singal generation[221],
- **Nucleic acid lateral flow devices** (NA-LFDs) – the aptamer-target interaction is visualized on a test strip, and
- **Aptamer-target binding readout** (ATBR) – these sensors are usually composed of aptamers labelled with a fluorophore[222, 223] or an electroactive[224] moiety. Recently published work by Wang et al.[225] showed an alternative ATBR method using gold nanoparticles (AuNPs).

The signalling process includes calorimetric, fluorescent, electrochemical and chromatographic approaches. Each biosensor format can be combined with various signalling methods[221]. Concerning the aptamer-based detection of aflatoxin B_1 a few approaches have been described in literature.

Guo et al.[226] describe a simple and ultrasensitive aptasensor for the detection of AFB_1. An aptamer specific to AFB_1 was used as a molecular recognition probe, while its complementary DNA was used to generate a signal during qPCR amplification. The formation of the aptamer-target complex leads to a conformational change of the aptamer itself and in further consequence to the release of the complementary DNA. Thus, the amount of complementary DNA template for qPCR amplification is reduced, resulting in an increase of the C_Q value (Fig. 19a). This conformational change was furthermore recently exploited for biosensor development bei Guo et al.[227] for the analysis of potassium ions.

A chemiluminescence competitive aptamer assay was developed by Shim et al.[228] using a hemin/G-quadruplex horseradish peroxidise-mimicking DNAzyme (HRP-DNAzyme) linked with an aptamer specific for AFB_1. The developed assay format is similar to a competitive ELISA (Fig. 19b).

Furthermore, Shim et al.[229] developed an aptamer-based dipstick assay for the rapid detection of AFB_1. The assay format is based on a competitive reaction of a biotinylated AFB_1 specific aptamer between the target and cy5-labelled DNA probes. This assay is based on an indirect

target detection format where a test line is formed if no target is present and *vice versa* (Fig. 19c).

A label-free aptasensor for the rapid detection of AFB_1 was recently published by Luan et al.[230]. They used the fact that AuNPs preferably bind to ssDNA rather than to dsDNA. Consequently, ssDNA has the ability to enhance the stability of AuNPs against salt-induced aggregation. Following target addition the aptamer changes its random coiled structure to a folded rigid secondary structure, thus losing the ability to protect the AuNPs from salt-induced aggregation. As a result the colour changes from red to blue (Fig. 19d).

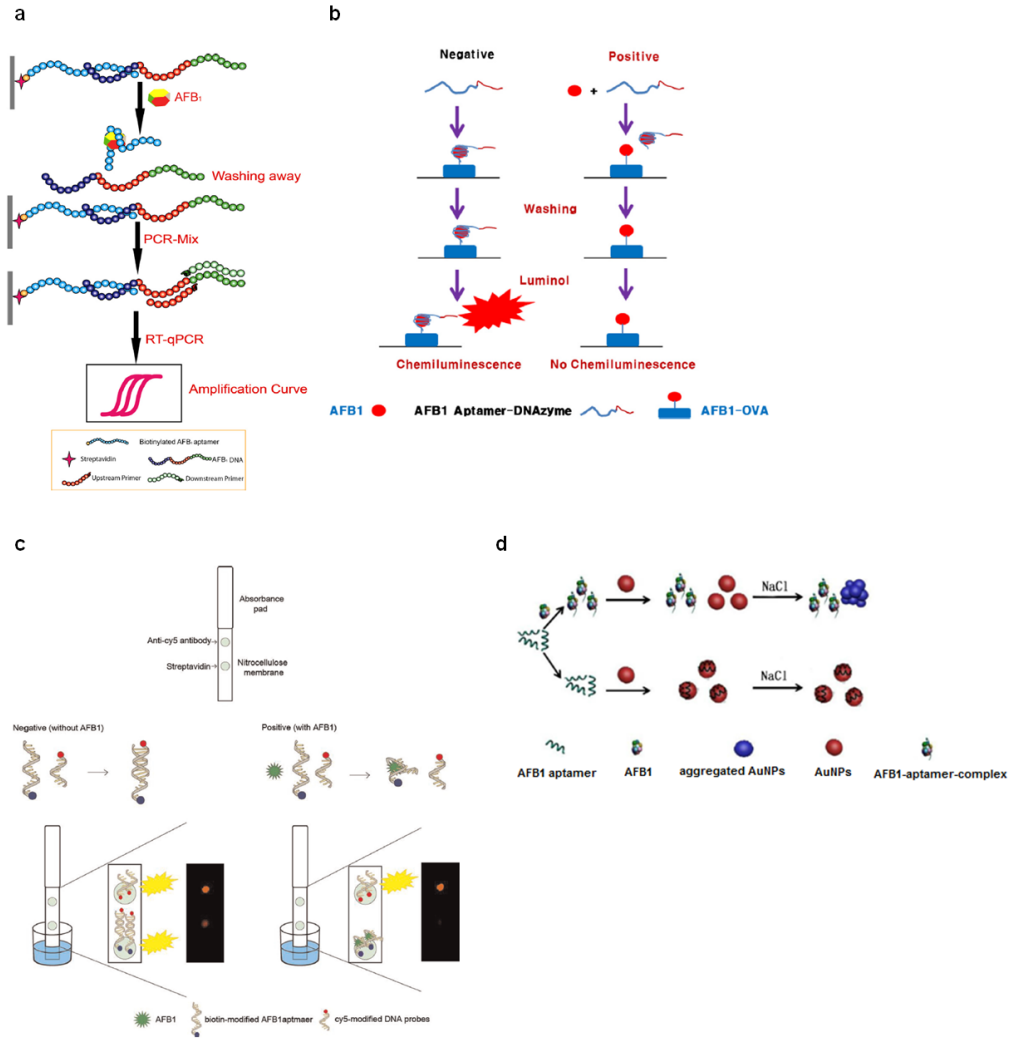

Fig. 19 Schematic illustration of the above described aptasensors. **(a)** Aptasensor developed by Guo et al.[226] **(b)** chemiluminescence competitive aptamer assay developed by Shim et al.[228] **(c)** aptamer-based dipstick assay developed by Shim et al.[229] **(d)** label-free aptasensor based on AuNPs developed by Luan et al.[230]

8 Materials and Methods

8.1 Materials and reagents

All chemicals and reagents used for buffer preparation were obtained from Carl Roth GmbH (Karlsruhe, DE), whereas the the ssDNA libraries and the primers were obtained by Eurofins MWG Operon (Ebersberg, DE). Further used chemicals and equipment are listed in Table 6.

Table 6 List of the equipment and reagents used for the development of aptamers specific for AFB_1 and following aptasensors as a rapid detection method

Equipment	**Producer**
Analytical balance, MC1	Sartorius, DE
Cellulose fiber pads	Millipore, US
Centrifuge, Centrifuge 5430	Eppendorf, DE
Centrifuge tubes, 15 ml and 50 ml	Greiner Bio-one, DE
Eppendorf tubes, 1.5 ml and 2.0 ml	Eppendorf, DE
Electrophoresis apparatus, BioRad PowerPAC Basic	BioRad, IT
Filter, Vivaspin 500, 5kDa MWCO PES	GE Healthcare, UK
Fluorescence reader, Infinite M200Pro	Tecan, CH
Glass fiber pads	Millipore, US
Laminated cards	Millipore, US
Magnetic separator, Ambion 6 Tube Magnetic Stand	ALT, GB
Molecular Imager, BioRad Universal Hood II	BioRad, IT
Nitrocellulose membrane	Millipore, US
pH meter	WTW, DE
Pipettes, 10 µl, 20 µl, 100 µl, 1000 µl	Eppendorf, DE
Plate reader, Sunrise® microplate reader	Tecan, AT
Real-time PCR 96 well plate foils	Applied Biosystems, US
Real-time PCR 96 well plates	PeqLab, DE
Shaking device, MixMate	Eppendorf, DE
Shaking device, Thermomixer comfort	Eppendorf, DE
Sprayer, Biojet Sprayer	Bio DOT, US
Thermocycler, 7500 Fast Real-Time PCR System	Applied Biosystems, US
Transilluminator, Mighty Bright	Hoefer Scientific Instruments, US
Water bath, GFL®	Müller-Scherr, Laborausrüstung, A
Reagents	**Producer**
Aflatoxin B_1 (2µg/ml)	Romer Labs®, A
Agarose	Sigma Aldrich, US
Anti-aflatoxin antibody (2.46 mg/ml)	Romer Labs®, A
Anti-mouse IgG	IFA Tulln, Center for Analytical Chemistry, A
Colloidal gold nanoparticles	BBI™ Solutions, UK
Dimethylformamide (DMF)	Sigma Aldrich, US
DNA Ladder, Ultra Low Range DNA Ladder	PeqLab, DE

Deoxynivalenol (100.1 µg/ml)	Romer Labs®, A
EDC (N-(3-dimethylaminopropyl)-N-carbodiimide)	Sigma Aldrich, US
FB_1 and FB_2 mix (50.3 µg/ml)	Romer Labs®
Lambda Exonuclease	New England Biolabs GmbH, DE
Magnetic beads, BioMag®Plus Amine	Polysciences, Inc., DE
Ochratoxin A (10.2 µg/ml)	Romer Labs®, A
PCR master mix, GoTaq® Colorless Master Mix	Promega, US
TNBSA (2,4,6-Trinitrobenzene Sulfonic Acid)	Thermo Scientific, DE
Zearalenone (10.9 µg/ml)	Romer Labs®, A

8.2 DNA library design

The starting DNA templates (listed in Table 7) contained a central randomized region of 27 nt flanked by defined primer binding sites for PCR amplification. In total, 5 different primer binding sites have been tested, resulting in 5 various libraries, namely Lib_1, Lib_2, Lib_3, Lib_4, and Lib_5. The length of the primer binding sites was set to 14 nt for Lib_1, Lib_2, and Lib_4 and 15 nt for Lib_3 and Lib_5. The lyophilized libraries and primers were resuspended in 10 mM Tris-HCl (pH 7.4) to a final concentration of 100 µM and stored at -20 °C until usage.

Table 7 ssDNA libraries used in the experiments composed of a randomized region flanked by two primer binding sites

Oligoname	**Sequence (5' – 3')**	**Length [bp]**
Lib_1	GACGGACTCGTTCA – N_{27} – CCGGATCTGTTACG	55
Lib_2	GCTGCACGCTATCT – N_{27} – GGGACGACAACAGA	55
Lib_3	TCACGGCTGTAGTGT – N_{27} – TATACATCGGCTGCT	57
Lib_4	TCCACCCACTCTCA – N_{27} – CGCCAAAAGAAATC	55
Lib_5	CGAGAGCAAGAGTTG – N_{27} – TAGCTAACGGACACG	57

8.3 Preparation of AFB_1-coated magnetic beads as a selection target

To allow binding and separating of target-bound oligonucleotides, the target was immobilized onto amine-functionalized (moiety -NH_2) magnetic beads with a bead particle diameter between 3-12 µm. AFB_1 was derivatized with O-carboxymethoxyamine to produce the protein-reactive oximinoacetate that enables due to the attached carboxygroup the coupling to the amine-group at the beads[231]. The derivatization was performed by DI Georg Häubl at Romer Labs®. In brief, AFB_1 and Carboxymethylhydroxylamine (CMO)-HCl were added to a mixture of pyridine-methanol-water. After incubation of the mixture over night at RT the formed product, AFB_1-CMO, was purified through preparative HPLC and the reaction was

controlled with thin layer chromatography (TLC). Fig. 20 shows the chemical scheme of AFB_1 derivatization.

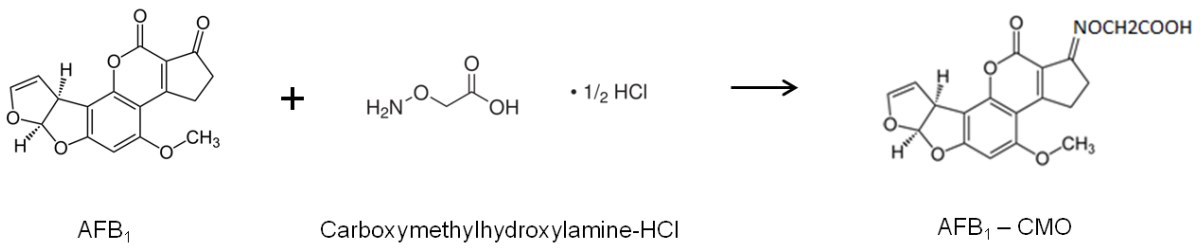

Fig. 20 Background information of the chemical reaction of AFB_1-CMO cross-link production

For AFB_1-CMO bead immobilization 13.17 mg (equals to 34.18 µmol) of purified AFB_1-CMO were dissolved in DMF – an organic solvent – and bound to 71.21 mg (equals to 17.10 µmol) of amine-activated magnetic beads. AFB_1-CMO was mixed in a ratio of 2:1 mole equivalent (eq) with the magnetic beads. The beads were cooled-down to approximately 4 °C and further reaction steps were carried out on ice. Subsequently, 115 mg (equals to 17.10 mmol or 500 eq referred to the amount of AFB_1-CMO) of EDC were added as a carboxyl activating agent for the coupling of primary amines in order to yield amide bonds. EDC was added incrementally in intervals of 7 min. The beads were then incubated at 4 °C over night. The chemical conjugation scheme is shown in Fig. 21. Following conjugation the beads were washed 60 times with 0.1 M hydrogen carbonate buffer ($NaHCO_3$) pH8 to remove unbound AFB_1-CMO excess. The supernatant of every tenth washing step was controlled under UV to verify the presence of AFB_1. Finally, the AFB_1-coated beads were dispersed in 1.548 ml SELEX binding buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$).

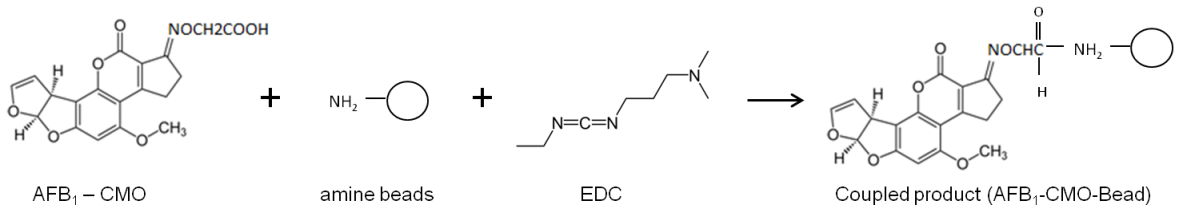

Fig. 21 Background information of the chemical reaction of AFB_1-CMO bead immobilization

The blank beads used for the counter selection were treated in the same way but without adding a ligand.

8.3.1 Evaluation of the coupling efficiency

To determine the amount of free or occupied primary amine groups respectively, three approaches have been tested:

i. Since aflatoxin B_1 fluoresces blue under **UV light** at approximately 365 nm the coupling efficiency was visualized with an UV transilluminator.

ii. A protocol to quantify free amine-groups using **TNBSA** was established by Habeeb (1966)[232]. Molecules with primary amine groups or hydrazine groups react with TNBSA in aqueaous solutions at pH 8.0 to form highly chromogenic adducts (trinitrophenyl derivatives, TNP). These colored derivatives can then be measured at 335 nm in order to quantify the amine content of a sample. To quantify the unknown amount of free amine groups on the magnetic beads a standard with known concentration was prepared. Therefore, 200 µg/ml (equals to 48 nmol $-NH_2$) of unmodified beads were serially diluted with binding buffer by a factor of two to a concentration of 3.125 µg/ml (equals to 750 pmol $-NH_2$). For quantification 500 µl of either only amine-functionalized beads or AFB_1-immobilized beads were treated with 0.01% (w/v) TNBSA and incubated for 2 h at 37 °C at 400 rpm. Afterwards, 250 µl of 10% sodium dodecyl sulphate (SDS) and 125 µl of 1 N HCl were added. The absorbance was then measured at 335 nm. Each sample was analyzed three times to ensure repeatability of the method.

iii. The third approach comprises the implementation of an **immunoassay**, based on a sandwich ELISA detection format, to determine the immobilization efficiency. Therefore, an antibody specific to aflatoxin (anti-aflatoxin antibody) and a mouse-specific horseradish peroxidase (HRP)-labeled antibody were used. Prior to antibody addition the surface of the magnetic beads was saturated with 1% bovine serum albumin (BSA) (w/v) for 1 h at 37 °C. This step impedes unspecific binding of antibodies to the beads. Next to saturation the beads were washed five times with 0.05 M phosphate buffered saline (PBS). Subsequently, 20 µl of either unmodified (blank beads) or AFB_1-modified beads were incubated with 5 µl of anti-aflatoxin antibody for 30 min at 37 °C. Thereafter, beads were washed with 0.05 M PBS and then 25 µl mouse-specific HRP-labeled antibody (1:10,000) were added. The mixture was furthermore incubated for 30 min at 37 °C. Finally, 25 µl colorless substrate solution (100 M citric acid pH 4, 190 mM potassium sorbate, 300 mM tetramethylbenzidine, 2.5 % (v/v) dimethylsulfoxide, 0.5 % (v/v) methanol, 30 % (v/v) H_2O_2) were added. In

the presence of AFB_1, HRP catalyzes a chemical reaction involving the substrate inducing a color change from colorless to blue.

8.4 In vitro selection of aptamers specific for the recognition of aflatoxin B_1

For the first SELEX round 2 nmol of the originally obtained ssDNA library were dissolved in 500 µl binding buffer to obtain a starting concentration of 4 pmol ssDNA per µl. The mixture was denatured by heating at 95 °C for 10 min and then cooled-down immediately on ice for 15 min and finally incubated for 7 min at RT. Meanwhile, modified and unmodified beads were washed eight times with 500 µl binding buffer to remove pieces of broken, not functioning bead components Subsequently, the denatured ssDNA library was added to a mix of 10^8 washed unmodified beads and a maize matrix extract spiked with 2,000 µg/kg FB_1 and FB_2, 1,250 µg/kg DON, 5 µg/kg OTA and 100 µg/kg ZEN and incubated for 1 h at RT. Counter selection steps were performed in every selection round. In round three and four the concentration of used unmodified beads for counter selection was decreased to $5•10^7$ to increase selection stringency. Details of each round are listed in Table 8. The supernatant collected after magnetic separation was introduced to 10^8 AFB_1-modified beads and incubated for 1 h at RT. After magnetic separation the beads were washed five times with 500 µl binding buffer to remove the unbound oligonucleotides. The volumes of binding buffer used for washing as well as the number of washing steps were increased during the selection procedure. Then, the bound ssDNA fragments were thermally dissociated and eluted with 50 µl elution buffer (40 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 10 mM Urea) at 80 °C for 7 min. The elution step was repeated three times and the eluates were pooled and used as templates for subsequent PCR amplification. Each reaction constituted the following: 0.3 pM/µl of forward primer, 0.3 pM/µl of 5'-phosphorylated reverse primer, 15 µl GoTaq master mix, 5.1 µl sterile-bidistilled water and 5 µl of template DNA. The PCR cycling conditions included an initial denaturation step at 95 °C for 2 min followed by 25 cycles of denaturation at 95 °C for 10 s, primer annealing at 43 °C for Lib_1 and Lib_4 and 50 °C for Lib_2, Lib_3 and Lib_5 for 30 s, extension at 55 °C for 30 s for Lib_1 and Lib_4 and 60 °C for Lib2_, Lib_3 and Lib_5 followed by an incubation step at 72 °C for 3 min after the last cycle. The amplified library was then converted to ssDNA by 10 units (U) of lambda exonuclease at 37 °C for 3 h. After ssDNA generation the enzyme was inactivated at 75 °C for 10 min and the originated products were visualized on a 2.5 % agarose gel. In total four rounds of in vitro selection were conducted.

Table 8 SELEX scheme

Round	Counter selection	$Bead_{modified}$ concentration	Incubation time	Washing step
1	Unmodified beads Toxin mix	10^8	1 h	5 times with 500 µl
2	Unmodified beads Toxin mix	10^8	30 min	8 times with 500 µl
3	Unmodified beads Toxin mix	$5•10^7$	30 min	8 times with 1,000 µl
4	Unmodified beads	$5•10^7$	15 min	8 times with 1,000 µl

8.5 Sequencing and structural analysis of the selected aptamers

Next generation sequencing (NGS) allows the possibility to track the copy number and enrichment-fold of more than 10^6 individual sequences through multiple selection rounds enabling the identification of thigh affine aptamers.

The aptamers obtained through SELEX were sequenced with the high-throughput sequencing platform Illumina (for characteristics see Table 9) by Microsynth (Balgach, CH).

Table 9 Characteristic parameters of the Illumina sequencing platform

Run time [h]	Read length [bp]	Throughput per run [Mb]	Error type	Error rate [%]	Advantages	Disadvantages
1 – 14	2 x 150	1,000 – 600,000	Substitution	> 0.1	High sequence yield	Expensive equipment, high DNA concentrations are required

Sequences obtained in selection round two and four were analyzed for Lib_5. For Lib_4 the selection rounds three and four were sequenced. First, to prepare the samples for sequencing, the aptamer pool from the respective round was PCR amplified. Approximately 0.5 µg PCR product with a concentration of 10 ng/µl was used for sequencing. The obtained sequences were counted and sorted by their counts, followed by enrichment analysis. Analyses were performed using the online tool Galaxy (https://usegalaxy.org/). To find conserved nucleotide regions the sequences were furthermore analyzed by ClustalX.

Secondary structures of the aptamers were obtained by the online program *mfold*[233] at 100 mM Na^+, 2 mM Mg^{2+} and 21 °C to determine the possible interacting regions of the aptamers.

8.6 Binding assay and measurement of the equilibrium dissociation constant (K_d)

The K_d values were measured for the sequences with the highest enrichment factor out of Lib5 from selection round two to selection round four. The characterized aptamer designed by

Neoventures Biotechnology Inc. (Canada) was used as control to assure the accuracy of the used method (aptamer listed in Table 12). Two different approaches have been tested to determine the dissociation constant K_d for the respective aptamer.

Circular dichroism (CD) evaluates the asymmetry of a system. Both enantiomers absorb circularly polarized light different. This technique allows the structural clarification of optically active chiral molecules by CD spectroscopy. The bases of DNA themselves are not chiral but become chiral within the sugar-phosphate backbone. Due to interaction of DNA with a ligand a CD signal is created[234]. For the K_d determination a stock solution of 2 ppm AFB_1 was individually diluted with acetonitrile (ACN) to concentrations of 20 ppb, 10 ppb, 5 ppb, 3 ppb, 2 ppb, 1 ppb, and 0.5 ppb while the aptamer concentration was kept constant at 0.5 µM. Each AFB_1 concentration was mixed with aptamer solution (resuspended in 100 mM NaCl) and incubated at RT for 1 h. For CD measurements following parameters have been used (Table 10):

Table 10 Parameters used for CD-based K_d determination

Wavelength	Data interval	Data points	Scans	Cuevette pathlength	Total reaction volume
350 nm – 200 nm	1 nm	151	3	2 mm and 10 mm	200 µl

The second approach uses a **fluorescence binding assay**. Therefore, 1.77 mg of AFB_1 were weighed in a 1.5 ml tube and dissolved in 100 µl ACN. The AFB_1 stock solution was then individually diluted with ACN to concentrations of 12 ppb, 6 ppb, 3 ppb, 1.5 ppb, 0.78 ppb, and 0.39 ppb while the aptamer concentration was kept constant at 2 µM. After the incubation of each AFB_1 concentration with aptamer for 1 h at RT the mixture was filtered through centrifugal filter devices with a cut-off of 5 kDa. Only AFB_1 which was not bound to the aptamer passes the membrane. The fluorescence of the eluate was measured with a fluorescence reader at the Institute of Environmental Biotechnology, IFA-Tulln. Excitation was measured at 365 nm and emission was measured 450 nm. Finally, the dissociation constant was calculated according to formula (5).

8.7 Development of a switch based aptamer biosensor for the detection of aflatoxin B_1

8.7.1 Evaluation of the minimal protecting amount of anti-DIG antibody to avoid salt-induced precipitation of the gold nanoparticles

First, the minimal amount of protein necessary to avoid a salt-induced precipitation of the colloidal gold nanoparticles (AuNPs) has been evaluated according to Horisberger and

Rosset[235]. In brief, the anti-DIG antibody (stock concentration 0.55 mg/ml) was diluted to a final concentration of 0.2 mg/ml with 0.05 M PBS (pH 7.5). Then following serial dilutions of the antibody (AB) have been prepared (Table 11):

Table 11 Scheme for AB dilution and calculated final AB concentration per ml AuNPs

µl AB	µl H2O	µg AB	µl AuNPs	µg AB/ml AuNPs
0	50	0	500	0
5	45	20	500	2
10	40	40	500	4
15	35	60	500	6
20	30	80	500	8
25	25	100	500	10
30	20	120	500	12
35	15	140	500	14
40	10	160	500	16
45	5	180	500	18
50	0	200	500	20

Afterwards, 500 µl of AuNPs were added to each solution and the mixture was incubated for 10 min at RT. Then 100 µl of 10 % NaCl were added and incubated for 5 min at RT. Subsequently, 200 µl of each dilution were added to a non-binding 96 well plate and the absorbance was measured at 600 nm (used as a reference) and at the 525 nm for the AuNPs with the microplate reader. Finally, the minimal protecting amount of anti-DIG antibody was evaluated.

8.7.2 Coupling of gold nanoparticles with anti-DIG antibodies

The anti-DIG AB amount necessary for coupling to 5 ml AuNPs has been calculated according to the minimal protecting amount evaluated before. After that, 4533 µl of AuNPs and 467 µl of anti-DIG antibody stock solution (0.55 mg/ml) were mixed and incubated for 20 min at RT at 6 rpm on the overhead shaker. Then 0.5 ml of 1 % (w/v) BSA solution was added and the mixture was incubated for 20 min at RT at 6 rpm on the overhead shaker. The solution was centrifuged at 15 °C and 9400 x g for 20 min and the supernatant was eliminated carefully. Five ml distilled water and 0.5 ml 1 % (w/v) BSA was added and the solution was again centrifuged at 15 °C and 9400 x g for 20 min. The supernatant was discarded to have about 500 µl left. Then 100 µl of 6 % (w/v) BSA has been added and the AuNPs were gently dissolved. The detection reagent was stored at + 4 °C until use.

8.7.3 Immobilization of the streptavidin and DIG as test line and control line, respectively

Prior to the assembly of the lateral flow strips, streptavidin (stock concentration: 1mg/ml) and digoxigenin (stock concentration: 1mg/ml) were sprayed as test line and control line, respectively (1 µg/cm, *i.e.* 450 ng per 4.5 mm strip) on the nitrocellulose membrane and dried over night at 24 °C. After drying, conjugate and absorbent pad were assembled with 2 mm overlapping between each component and the master card was cut into 4.5 mm wide strips.

8.7.4 Application of the developed test strip approach

The development of the aptasensor for the rapid detection of AFB_1 is based on a DNA fragment designed by Neoventures Biotechnology Inc. (Canada) in 2009[195]. The lyophilized aptamer and the complementary DIG labeled DNA probes were resuspended in 10 mM Tris-HCl (pH 7.0) to a final concentration of 100 µM and stored at -20 °C until usage (sequences are listed in Table 12).

Table 12 Listing of the developed digoxigenin (DIG) labelled complementary signalling DNA probes as well as the used biotinylated aptamer and its characteristics. The red bases marked in DIG_probe4 and DIG_probe5 indicate the base pair mismatches compared to the aptamer sequence

Name	**Sequence (5' – 3')**	**5' Label**	**Length [bp]**	**Match with aptamer sequence [%]**	**T_M [°C]**
AFB_1_aptamer	AAAAAAAAAAGTTGGGCACGTGTTGTCTC TCTGTGTCTCGTGCCCTTCGCTAGGCCCAC A[229]	Biotin	60		
DIG_probe1	AAATGTGGGCCTAGCGA[229]	DIG	17	100	39.4
DIG_probe2	AAATGTGGGCCTAGCG	DIG	16	100	38.2
DIG_probe3	AAATGTGGGCCTAGC	DIG	15	100	34.6
DIG_probe4	AAATGTGG**T**CCTAGCGA	DIG	17	94	35.8
DIG_probe5	AAATGT**T**GGC**T**TAGCGA	DIG	17	88	34.1

Before applying the sample on the lateral flow strip 10 µl AFB_1_aptamer (0.1 µM), 2 µl AFB_1 (2 µg/ml) and 10 µl signaling DNA probe (0.2 µM) were incubated for 30 min at 37 °C. Subsequently, 10 µl of the mixture were applied to the sample pad and the strip was placed into 150 µl running buffer (10 mM Tris, pH 7.0). Positive results were visualized as red lines within three to five min. Experiments were repeated five times to evaluate the repeatability of the postulated structure switch induced by AFB_1. A schematic principle of the test strip is shown in Fig. 22.

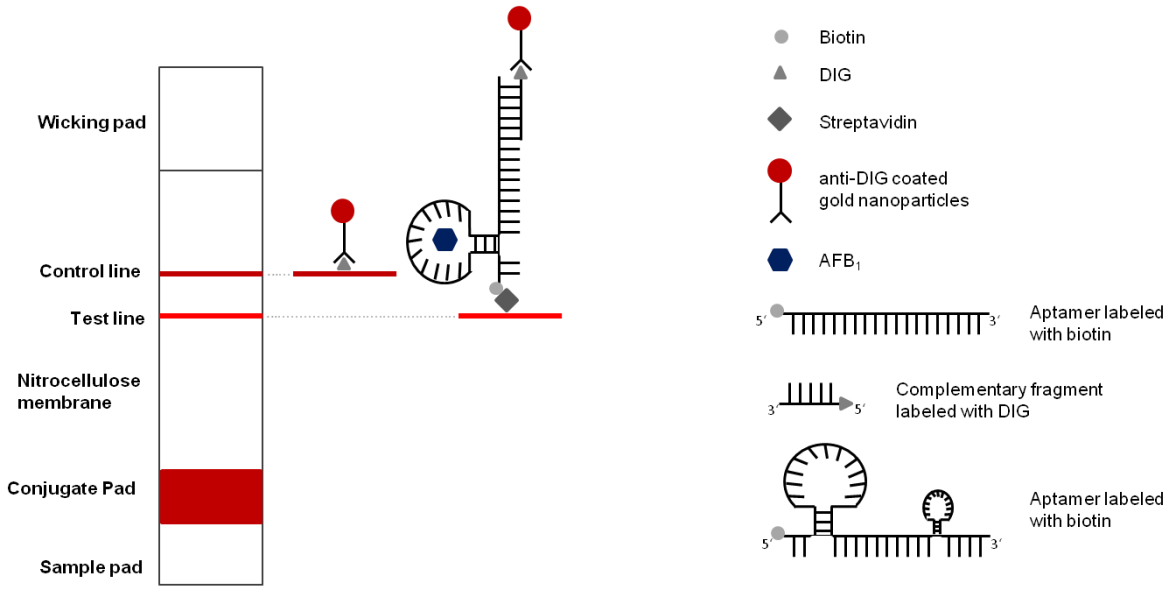

Fig.22 Schematic illustration of the principle of the test strip developed in this study for the visual detection of aflatoxin B_1 (AFB_1). The developed test strip is composed of a sample pad, a conjugate pad with anti-digoxigenin (DIG) coated gold nanoparticles, a nitrocellulose membrane containing immobilized streptavidin at the test line, DIG at the control line, and a wicking pad. The presence of AFB_1 evokes a structural switch of the biotinylated aptamer enabling the DIG labelled complementary fragment to bind. Accordingly, anti-DIG coated gold nanoparticles bind to DIG and biotin is captured by streptavidin at the test line. Hence, a red line is obtained. In the absence of the target, the loop structure at the 3'-end persists which in turn makes it impossible for the signaling DNA probe to bind. Therefore, no gold nanoparticles are attached and no signal is generated.

9 Results and Discussion

9.1 Immobilization and characterization of AFB_1-coated magnetic beads

Due to the derivatization of AFB_1 with O-carboxymethoxyamine a carboxy group was produced at the five-membered pentatone. The further coating process involved the coupling of the carboxy group of the AFB_1-CMO with the primary amine groups on the surface of the magnetic beads using EDC as a crosslinking agent. To remove unbound AFB_1, the beads were washed 60 times and the supernatants were controlled under UV light (Fig. 23). A significant decrease of fluorescence comparing tube one till four was observed. After the 30th washing step most of the unbound AFB_1 was removed and no significant difference in the fluorescence signal compared to the blank sample was discernible. Therefore, the optimum of washing steps was set to 30 for further immobilization approaches.

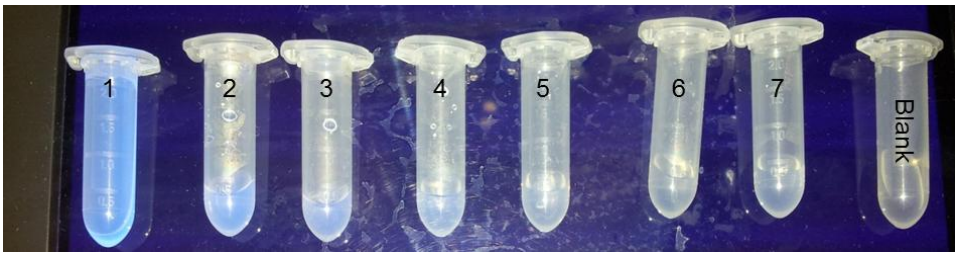

Fig. 23 Supernatants under UV light after the 1st (1), 10th (2), 20th (3), 30th (4), 40th (5), 50th (6), and 60th (7) washing step. The last tube contains $NaHCO_3$ as a reference.

Unfortunately, it was not feasible to determine the coupling efficiency using UV detection or the TNBSA protocol according to Habeeb[232]. No fluorescence was observed by UV detection due to the high absorbance of the magnetic beads. On the other hand, the quantification of free amine groups using the TNBSA protocol failed for every attempt. This is most likely attributed to the high limit of detection (LOD), which was previously reported to be in the milligram range[236]. Even the implementation of the immunoassay to determine coupling efficiency failed. Due to the binding of anti-aflatoxin antibody to its target a color change from colorless to blue is induced. This color change was only observed for unmodified beads (Fig. 24.) suggesting that the antibody is not specific to aflatoxin. Hence, it can be assumed that the anti-aflatoxin antibody preferably binds to the NH_2-moieties at the beads rather than to aflatoxin. Thus, it can be concluded that the majority of the amine groups is occupied by AFB_1.

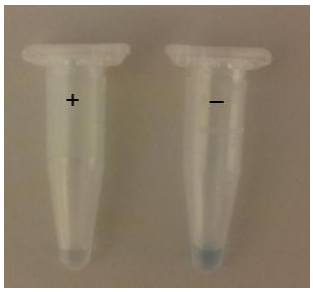

Fig. 24 Evaluation of the coupling efficiency with an immunoassay approach. (+) indicates the reaction of AFB_1 modified beads – a color change was expected due to the use of an anti-aflatoxin antibody. (-) shows the reaction of unmodified beads; no color change was expected.

9.2 In vitro selection of the ssDNA aptamers against AFB_1

ssDNA aptamers were chosen from a random library containing approximately $1.2 \cdot 10^{15}$ different ssDNA molecules through four rounds of in vitro selection. The conditions of the SELEX rounds are shown in Table 8. Prior to each SELEX round the ssDNA library pool was denatured at 95 °C and refolded at RT to enable the formation of a distinct secondary and tertiary structure. This structure provides the basis for high affine target binding. Additionally, non-specific sequences were eliminated by counter selection steps, including unmodified beads as well as a maize extract spiked with the mycotoxins DON, FB_1, FB_2, OTA, and ZEN resulting in a final amount that corresponds to the maximum values set by the European legislation. Weakly bound sequences were removed by an increased number of washing steps. To enhance the aptamers affinity for AFB_1, the selection conditions were made increasingly stringent by decreasing the incubation time and lowering the concentration of modified beads and consequently the amount of target. It turned out that excessive numbers, above 30, of PCR cycles lead to the amplification of non-specific products as well as oligomers of consecutive primer sequences. Therefore, the number of PCR cycles was finally optimized to 25 cycles for each round. A gradient PCR from 37 °C to 52 °C was performed for each library and the optimal thermal cycling conditions turned out to be 2 min at 95 °C, followed by 25 cycles of 10 s at 95 °C, 30 s at 43 °C for Lib_1 and Lib_4 and 50 °C for Lib_2, Lib_3 and Lib_5, 30 s at 55 °C for Lib_1 and Lib_4 and 60 °C for Lib_2, Lib_3 and Lib_5, and a final incubation step of 3 min at 72 °C. After ssDNA generation by enzyme digestion the ssDNA products were visualized via agarose gel electrophoresis and the concentration has been estimated. The agarose gel results showed a single band for Lib_4 and Lib_5 (Fig. 25) indicating that only products with the same size have been amplified. According to the used ultra-low range ladder the amplified products range between 50 and 75 bp long. Lib_1, Lib_2,

and Lib_3 showed more than one band indicating the amplification of more than one product and thus the production of unspecific products.

Therefore, only the selected oligonucleotides from Lib_4 and Lib_5 were used for sequencing and further characterization.

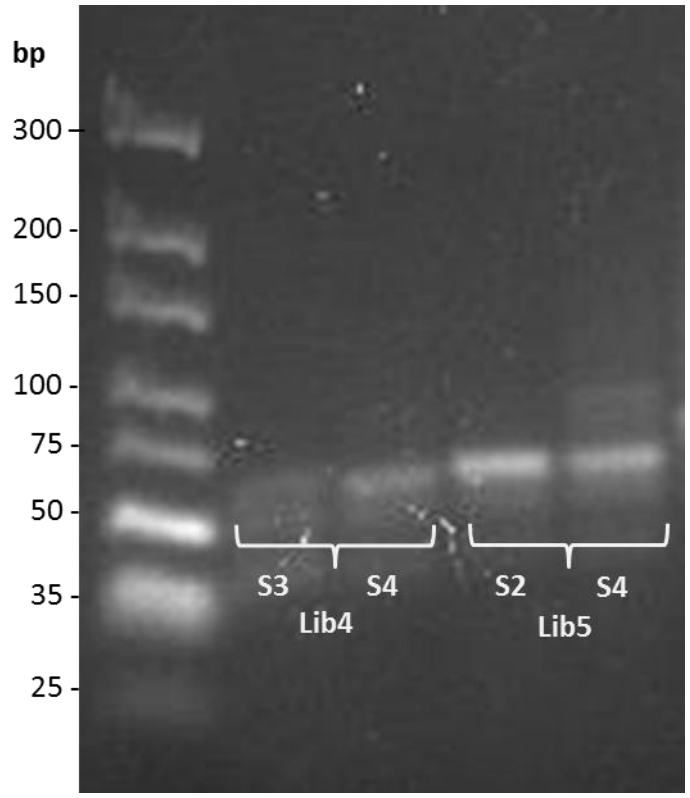

Fig. 25 Amplified products from Lib4 and Lib5 after selection round 2, 3, and 4

9.3 Sequence analysis

In total $7.9 \cdot 10^6$ reads have passed the filter of the entire sequencing run. The obtained sequences were analyzed with the online tool "Galaxy" (https://usegalaxy.org/). First, sequences that were not 55 bases or 57 bases (for Lib4 and Lib5, respectively) long were filtered out. Furthermore, reads that did not contain the correct primer on each end were eliminated resulting in approximately 3.7 to $15.4 \cdot 10^3$ sequences. Identic sequences were grouped and their abundance was counted. Finally, 1.716 and 562 sequence reads were observed for Lib4 and Lib5, respectively. A detailed list is given in Table 13.

Table 13 Passed filter reads of the entire sequencing run

Sample name	Raw tags	Filtered by length and primer sequence	Grouped sequences
Lib4 selection round 3	140.982	3.658	1.716
Lib4 selection round 4	218.834	5.161	
Lib5 selection round 2	209.492	15.389	562
Lib5 selection round 4	216.852	9.152	
Sum	**786.160**		

To identify high affinity sequences, the enrichment factor (ratio of copy numbers between two selection rounds) was calculated and the results were ranked in descending order. The top ten sequences of Lib4 and Lib5 are shown in Table 14. Results show, that aptamers selected from Lib4 consist predominantly of sequences made of forward primer concatamers. These artefacts were not used for further analysis. Hence only sequences obtained from Lib5, containing unique core structures were used for further analysis.

Table 14 Top ten enriched aptamer sequences for each library. The green and the blue marked bases indicate the forward and the reverse primer, respectively. The underlined part of almost all sequences from Lib4 indicates once more the sequence of the forward primer but on an unexpected position.

Lib4		
Aptamer name	Sequence of selected aptamer (5' – 3')	Enrichment factor (R4/R3)
Lib4_1e	**TCCACCCACTCTCA**AGGGACGGGCCT*TCCACCCACTCTCA*T**CGCCAAAAGAAATC**	41
Lib4_2e	**TCCACCCACTCTCA**AGGGACGGGCCT*TCCACCCACTCTCA*C**CGCCAAAAGAAATC**	23
Lib4_3e	**TCCACCCACTCTCA**CATACAATTCTGCAGGATACGTTGACA**CGCCAAAAGAAATC**	11
Lib4_4e	**TCCACCCACTCTCA**GCACCCAAAACA*TCCACCCACTCTCA*G**CGCCAAAAGAAATC**	10
Lib4_5e	**TCCACCCACTCTCA**CCGAACC*TCCACCCACTCTCA*ATGGA**CGCCAAAAGAAATC**	10
Lib4_6e	**TCCACCCACTCTCA**AGGGACGGGCCT*TCCACCCACTCTCA*G**CGCCAAAAGAAATC**	9
Lib4_7e	**TCCACCCACTCTCA**AGGGACGGGCCT*TCCACCCACTCTCA*A**CGCCAAAAGAAATC**	9
Lib4_8e	**TCCACCCACTCTCA**CCGAACC*TCCACCCAC*ATCTACCAACA**CGCCAAAAGAAATC**	9
Lib4_9e	**TCCACCCACTCTCA**GCACCCAAAACA*TCCACCCACTCTCA*AG**CGCCAAAAGAAATC**	9
Lib4_10e	**TCCACCCACTCTCA**ACCAGCTATTCGTCCAGTGTAAGGGTA**CGCCAAAAGAAATC**	8
	Lib5	**Lib4**
Aptamer name	Sequence of selected aptamer (5' – 3')	Enrichment factor (R4/R2)
Lib5_1e	**CGTGTCCGTTAGCTA**TTTGGGAGAACAAAAGGGATTACACAC**CAACTCTTGCTCTCG**	84
Lib5_2e	**CGTGTCCGTTAGCTA**GGCAAAGAAAATAAGACTGCATACCTT**CAACTCTTGCTCTCG**	79
Lib5_3e	**CGTGTCCGTTAGCTA**GCAGGGCACAACAGATTGGTATATACA**CAACTCTTGCTCTCG**	29
Lib5_4e	**CGTGTCCGTTAGCTA**TTGGTTCGTTCCATGCCCGGATATCTA**CAACTCTTGCTCTCG**	27
Lib5_5e	**CGTGTCCGTTAGCTA**GCACTTAAAGGGCACATGTGTTCACG**CAACTCTTGCTCTCG**	23
Lib5_6e	**CGTGTCCGTTAGCTA**TATTAGGTGAGGGAATGAGTCCTAGCC**CAACTCTTGCTCTCG**	16
Lib5_7e	**CGTGTCCGTTAGCTA**TGGAGAAGCTTCTCTGATCGCAATACG**CAACTCTTGCTCTCG**	15
Lib5_8e	**CGTGTCCGTTAGCTA**GCAGGGCGCAACAGATTGGTATATACA**CAACTCTTGCTCTCG**	14
Lib5_9e	**CGTGTCCGTTAGCTA**GCCCCAGATCGCTTATGAAACTGGAGA**CAACTCTTGCTCTCG**	12
Lib5_10e	**CGTGTCCGTTAGCTA**GGGCGTTATTAACAATCAAGGTTGGAT**CAACTCTTGCTCTCG**	11

It was observed that highly represented sequences in selection round two did not necessarily exhibit high enrichment between the rounds. As shown in Fig. 26 there are sequences with high copy numbers but minimal enrichment and on the other hand the sequences with the highest enrichment (Lib5_1e and Lib5_2e) appeared infrequently in selection round two. Thus, it is assumed that these sequences represent true high-affinity aptamers.

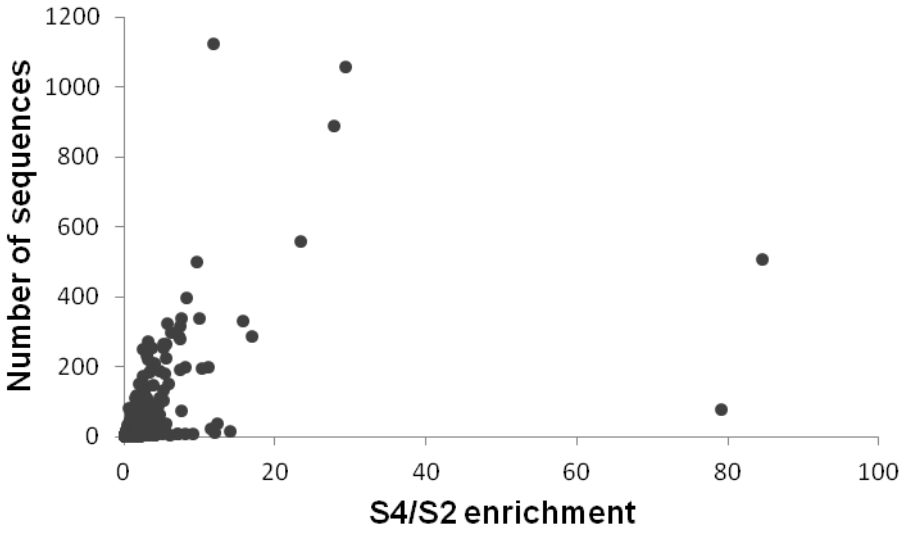

Fig. 26 The graph shows the number of sequences correlated with the enrichment fold of Lib5 from S2 to S4

9.4 Structural analysis of the selected aptamers

First, the obtained top ten sequences from Lib5 were aligned to identify core structures responsible for target recognition. As can be seen in Fig. 27 no sequence homology was found between the ten different sequences.

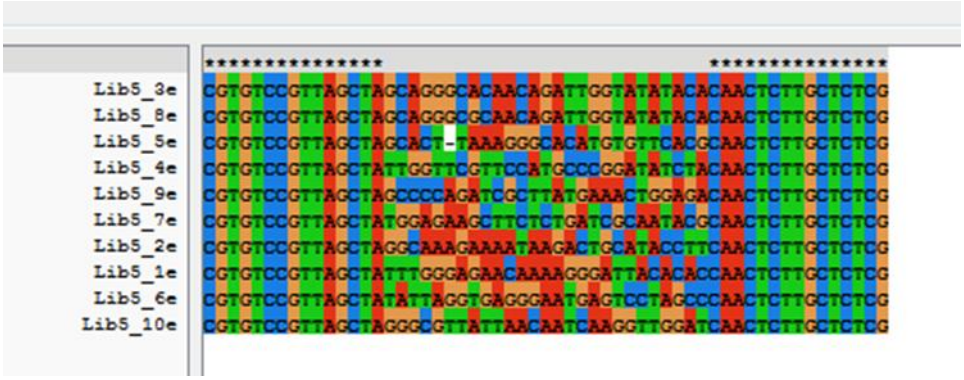

Fig. 27 Alignment of the obtained aptamers from Lib5. No similarities were found except of the primer regions.

Accordances were found by comparing the selected aptamers with the aptamer presented by Neoventures Biotechnology Inc. (GTTGGGCACGTGTTGTCTCTCTGTGT CTCGTGCCCTT CGCTAGGCCCACA) and the aptamer design by 吴淑庆 et al.[237] (AGCAGCACAGAGGTCAGATGGTGCTATCATGCGCTCAATGGGAGACTTTAGCTG CCCCCACCTATGCGTGCTACCGTGAA). The aptamers Lib5_1e, Lib5_6e, Lib5_7e, Lib5_9e and Lib5_10e showed the highest match of identity compared to the previously published aptamers and were therefore used for further characterization and experiments.

To find structural conformities or differences among the selected aptamers, the secondary structure of each sequence was predicted using the online *mfold* software[233], using the folding parameters of 100 mM Na^+, 2 mM Mg^{2+} and a temperature of 21 °C. The structures with the lowest minimum free energy can be found in Fig. 28. All structures show high complexity including between two or four prominent stem-loop motifs, very common to aptamers. It should be noted that some evidence has shown that higher complexity can lead to higher affinity[238]. Interestingly, the stem structures are formed within the 10^{th} and the 30^{th} base for all aptamers except Lib5_10e. Its stem structure formation starts at base number 32 and ends at base number 51. At this stage, however, it is unclear if one of the stem-loop motifs is involved in target binding, nor is it known whether these structures are built in the presence of the target molecule as a result of new molecular interactions.

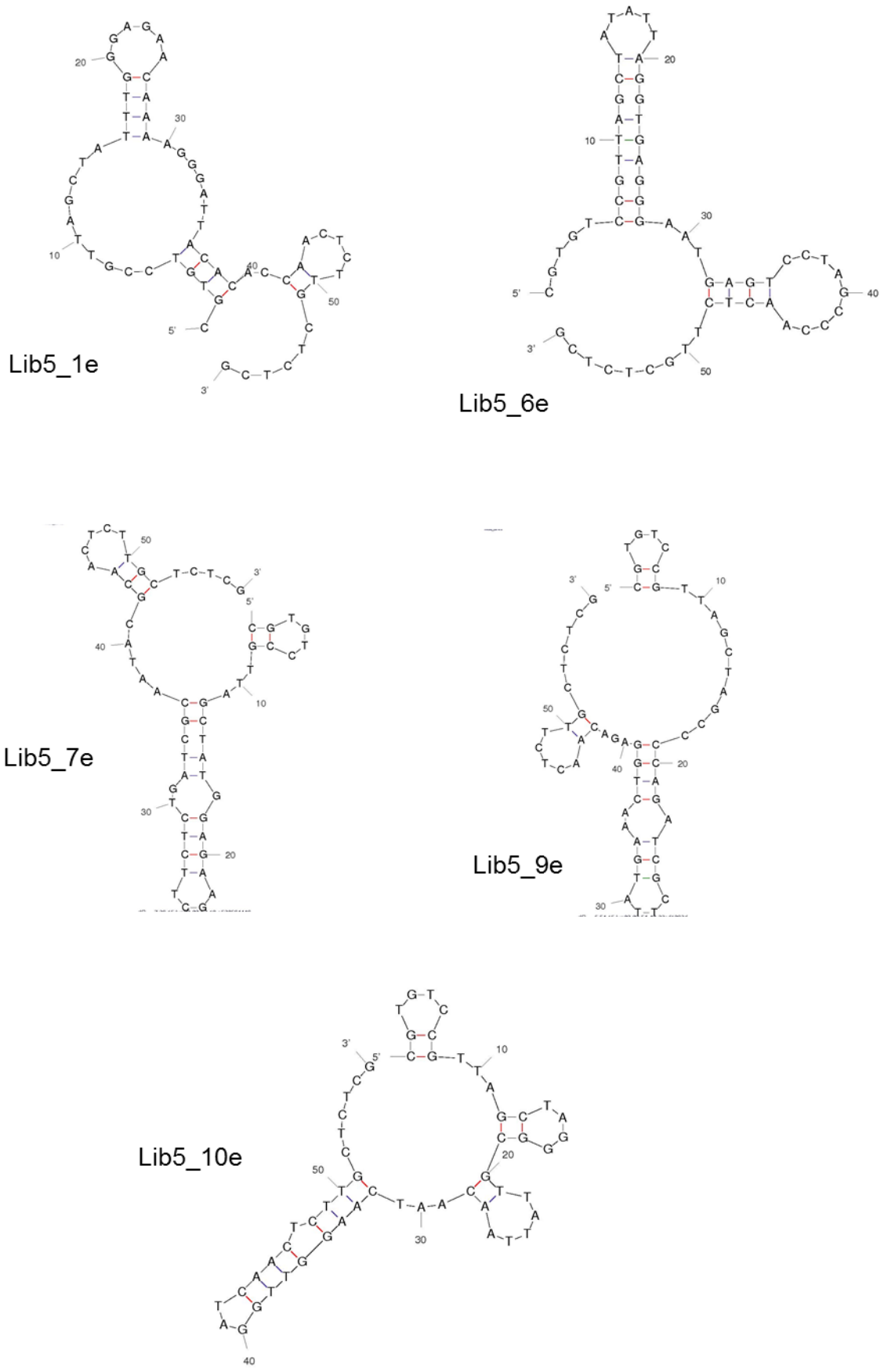

Fig. 28 Secondary structure prediction for the full length sequences generated by *mfold*[233]

9.5 Determination of the dissociation constant via fluorescence binding assay

No differences in the CD spectra have been observed using the AFB_1_aptamer from Neoventures incubated with target compared to the AFB_1_aptamer without target. Furthermore, no significant signal change occurred by changing the cuvette pathlength or the incubation temperature. As a result, no CD spectral records have been conducted with the aptamers selected from Lib5.

The measurement of the K_d via fluorescence binding assay was determined for the AFB_1_aptamer (published by Neoventures) as well as the selected aptamers Lib5_1e, Lib5_6e, Lib5_7e, Lib5_9e and Lib5_10e. The measured K_d values are listed in Table 15.

Table 15 Determined dissociation constants (K_d)

Name	**Dissociation constant (K_d)**
AFB_1_aptamer (Ref. Neo)	20.30 nM ± 0.08
Lib5_1e	1.30 µM ± 0.14 µM
Lib5_6e	5.70 µM ± 0.54 µM
Lib5_7e	2.43 µM ± 0.13 µM
Lib5_9e	2.56 µM ± 0.13 µM
Lib5_10e	4.95 µM ± 0.95 µM

9.6 Development of a switch based aptamer biosensor as a direct detection format for AFB_1

The first step towards on-strip application was the optimization of essential experimental parameters including the length as well as the base composition of the signaling DNA probe and the incubation temperature of the assay. These parameters are crucial for aptamer-target-complex formation and signal detection. It was assumed that if the fragment length of the complementary DNA is too long it favors the binding to the DNA probe rather than the target. On the other hand too short DNA probe fragments probably will not bind at all or are possibly easily dehybridized from its complementary strand by the slightest increase of temperature. Experiments focused on five different DIG labeled DNA probes listed in Table 12, starting with an oligonucleotide introduced by Shim et al.[229] named DIG_probe1 (17 mer) in this study. Results revealed that the DIG_probe1 has a strong binding affinity to the complementary part at the aptamer. Hybrids were produced in presence and absence of the analyte (Fig. 29-A). As a result two more signaling DNA probes were synthesized namely DIG_probe2 (16 mer) and DIG_probe3 (15 mer) with decreased fragment length. Attempts with decreased probe length did not improve the results (Fig. 29-A). Focusing on the base composition of the probes (Table 12) it was assumed that the five consecutive G/C bases

hinder the DIG labelled complements from dehybridization. Such regions are called GC-clamps. GC-clamps require high temperatures and often even a detergent is required to break the H-bonds between the base pairs. Nevertheless, some are unbreakable. Since false-positive results might be GC-clamp specific we slightly modified the sequence of the DNA signaling probe DIG_probe1 by introducing base pair mismatches. In contrast to the other porbes the new synthesized ones DIG_probe4 and DIG_probe5 include one (G→T) or two (G→T and C→T) base pair mismatches, respectively. This results in decreased binding affinity. As shown in Fig. 29-C, the DIG_probe5 was the only tested complementary sequence that reveals the ability to bind to the aptamer only if AFB_1 is present. Hence, the proposed structure switch seems to occur and allows the complementary probe to bind to the aptamer. Without target the loop structure at the 3'-end persists and thus impedes the binding of the signaling probe. These findings support the assumption and therefore, DIG_probe5 containing two base pair mismatches was chosen for further experiments. For the development of assays based on aptamers the incubation temperature responsible for hybridization/dehybridization is an important parameter. Shim et al.[229] recommend an incubation temperature of 37 °C for their aflatoxin aptamer tests. In this study RT and 40 °C were investigated as well. Especially the performance of the assay at RT would make this method immediately on-site applicable without the need for a thermal device. Experiments at RT showed a test line even with or without target wereas tests performed at 40 °C never gave a signal, neither with nor without target (Fig. 29-B). A closer look at the high melting temperature of DIG_probe5 of 34.1 °C reveals, that once the complementary sequence has bound to the aptamers, the dehybridization at RT will fail. On the other hand, the two strands can be easily denatured at 40 °C. Comparing the three tested temperatures, findings clearly showed that a temperature of 37 °C worked best (Fig. 29-C). To assure that the signal is solely derived from the aptamer-target-complex only aptamer, only DIG_probe5 and only AFB_1 were used as a control (Fig 29-D). The composition of the running buffer and the concentrations of the aptamer and the DIG labelled DNA probe were mainly derived from a previously reported assay[229] and slightly adjusted. It was observed that a 5-fold excess (0.5 µM) of the complementary DNA with regard to the aptamer concentration of 0.1 µM leads to a signal decrease at the test line which is attributable to the saturation of gold nanoparticles with DIG labelled DNA. On the contrary, a concentration of 0.2 µM showed a clear difference in signal intensities between AFB1 positive (4 µg/ml) and AFB1 negative (0 µg/ml) samples (Fig. 29-C). Therefore, 0.2 µM of DIG_probe5 was chosen as optimal concentration for further studies.

The AFB_1 concentration (4 µg/ml) used for this experiments was 60-times higher than the aptamer concentration to ensure a differentiation between positive and negative results.

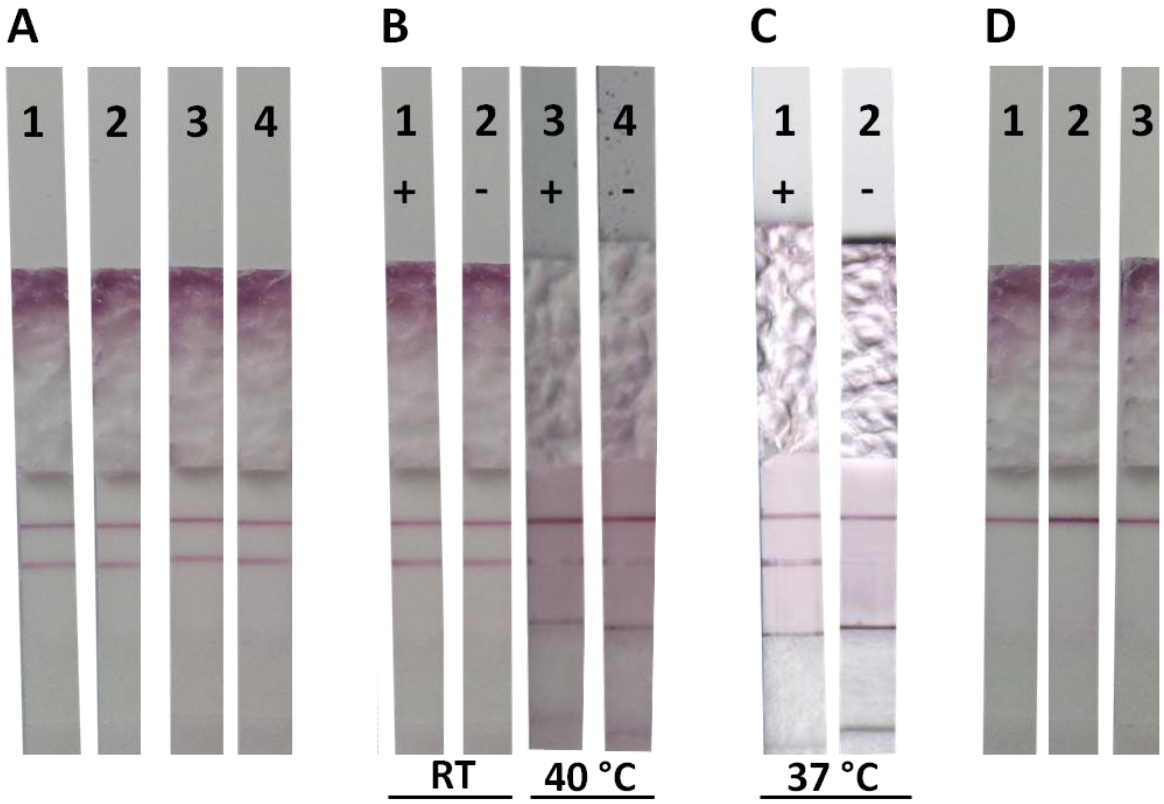

Fig. 29 Lateral flow strip results. + and – indicates whether AFB_1 was added or not. Findings with DIG_probe1 **(A-1)**, DIG_probe2 **(A-2)**, DIG_probe3 **(A-3)**, and DIG_probe4 **(A-4)** without the addition of AFB_1.Section **B** shows the experimental results performed with DIG_probe5 at different temperatures (RT, 40 °C). **C** shows dipstick test results conducted under optimized condtions (37 °C, DIG_probe5). **D** shows the tested control samples (1-only aptamer; 2-only DIG_probe5; 3-only AFB_1).

10 Conclusion

Four rounds of increasing stringent selection conditions were performed in order to obtain DNA sequences with high binding affinity to aflatoxin B_1 (AFB_1). Out of 786.160 sequence reads five individual sequences, that showed structural similarities with previously published aptamers[237], were obtained from the SELEX experiment. These five candidates (namely Lib5_1e, Lib5_6e, Lib5_7e, Lib5_9e and Lib5_10e) showed high enrichment over the performed selection rounds, thus indicating high-affinity aptamers. The selected sequences were further characterized concerning their sequence and the most likely folding structure. Furthermore, the equilibrium dissociation constant (K_d) was calculated to assess the binding affinity of the aptamer to its target. The dissociation constants were found to be 1.30 µM ± 0.14 µM for aptamer Lib5_1e, 5.70 µM ± 0.54 µM for aptamer Lib5_6e, 2.34 µM ± 0.13 µM for aptamer Lib5_7e, 2.56 µM ± 0.13 µM for aptamer Lib5_9e, and 4.95 µM ± 0.95 µM for aptamer Lib5_10e.

Additionally, an approach for a rapid aptamer dipstick assay using a direct detection format for AFB_1 was demonstrated. The proposed approach for a switch based aptamer biosensor in combination with a nucleic acid lateral flow immunostrip offers considerable advantages compared to previously published detection formats. It allows a direct detection format for AFB_1 and an immediate visualization *via* the developed test strip without the need of fluorescence read-out systems. Furthermore, it is a simple, user-friendly and cheap method that is as well attractive for on-site screening by untrained personnel. The described aptasensor is a model to demonstrate the potential of the structure switching ability of aptamers in terms of direct target detection. Moreover, a dipstick assay based on an aptamer enabling an AFB_1 direct detection format has not yet been reported.

Summary

This section provides the reader with a brief summary of the two topics discussed in this thesis.

The first part of this thesis aimed the development of a DNA-based technique for the rapid quantification of trichothecene and fumonisin producing *Fusarium* species in maize as a complementation to mycotoxin analysis to facilitate the classification of the resistance of new crop lines in breeding programs. The determination of the resistance of new maize varieties usually comprises a combination of mycotoxin analysis by ELISA or HPLC-MS and the visual scoring of disease symptoms. Although these techniques are accurate methods for the analysis of fungal secondary metabolites they have some major drawbacks. They are cost-intensive (especially HPLC-MS), time-consuming and only indirect methods, because no information of produced fungal biomass is provided. The quantitative PCR technique provides information about the DNA biomass of a fungus based on the detection of organism specific DNA. In the last decade several real-time qPCR assays for the quantification of *Fusarium* species have been described. These assays are based either on the quantification of individual *Fusarium* species or on the measurement of the abundance of a gene owned by a group of different species sharing a common biosynthetic pathway such as the genes for mycotoxin production[150, 182, 188]. In chapter one a sensitive quantification method for trichothecene and fumonisin producing *Fusarium* species in maize is described. The developed multiplex method allows the simultaneous quantification of all *Fusarium* species producing the most prevalent mycotoxins in maize, trichothecenes and fumonisins. Due to the simultaneous quantification of the genes responsible for trichothecene and fumonisin production the method allows high-throughput screening of a large number of samples in a relatively short time. Furthermore, costs are reduced by a factor of more than 60 % due to the reducing of the analysis time.

Chapter two addresses the development of aptamers for the rapid detection of aflatoxin B_1 (AFB_1) in maize. The high toxicitiy of AFB_1 led to the introduction of regulatory limits for foodstuff and feed and in further consequence to the need of rapid, sensitive and inexpensive analytical techniques to detect and quantify AFB_1. Existing techniques based on chromatographic methods combined with MS and screening approaches with immunoassays have some major drawbacks such as expensive equipment, the need of skilled personnel and

limitations of antibodies (e.g. thermal instability). In this study aptamers with high affinity to AFB_1 have been evolved offering a promising alternative to antibodies. Through an iterative selection procedure named Systematic Evolution of Ligands by EXponential enrichment (SELEX) five unique sequences were obtained after four rounds. They were furthermore characterized concerning their folding structure, their sequence and their ability to bind AFB_1. It could be shown that the binding affinity of all selected aptamers ranged between 1.30 µM and 5.70 µM.

For a first approach of an aptamer-based biosensor for the detection of AFB_1 a DNA fragment designed by Neoventures Biotechnology Inc. (Canada) was used. Already developed aptasensors, using this aptamer, are based on an indirect target detection format where a signal is only generated when no target is present[228, 229]. This study demonstrates for the first time an approach using a direct detection format for AFB_1. The method is based on the capability of the aptamer to switch its structure after the addition of AFB_1. This structure switch exposes a binding site for a complementary signaling DNA probe. The so produced hybrid molecule is then visualized *via* a lateral flow test and a test line is only generated when AFB_1 is present in a sample solution. Results revealed that a hybridization worked best at 37 °C for 30 min. However, for prototyping and future commercial application as a rapid screening method further optimization steps are required. Future work will involve the improvement of the sensitivity through reduction of target concentration. Further steps aim to lower the incubation time and temperature to speed-up the testing procedure as well as to provide a test feasible without a thermal device.

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Analytical Methods

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The development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize†

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Contamination of cereals with *Fusarium* species is one of the major sources of mycotoxin contamination in food and feed. Despite great progress in plant breeding, a complete resistance to *Fusarium* species has not yet been achieved. Visual scoring of disease symptoms combined with the determination of mycotoxins are common approaches to identify new *Fusarium* tolerant lines, but these methods are only indirect and therefore of limited use to determine the level of resistance against *Fusarium* spp. Aiming at a rapid and sensitive quantification method for trichothecene and fumonisin producing *Fusarium* species in maize, a multiplex qPCR assay was developed. This method enables high-throughput screening of a large number of samples for *Fusarium* infection in a relatively short time due to simultaneous quantification of the mycotoxin-related genes *tri5* and *fum1*. The multiplex method was applied to 24 maize field samples. All these were analyzed for the trichothecenes deoxynivalenol (DON), DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) by LC-MS/MS and for the mycotoxin producers by the new qPCR multiplex assay. The assay was found to be specific for fumonisin as well as for trichothecene producing *Fusarium* species. The limit of quantification was found to be 0.32 pg per µl for both *Fusarium* strains. To the best of our knowledge, this is the first report of the use of a multiplex qPCR for the quantification of trichothecene and fumonisin producing *Fusarium* species.

1 Introduction

One of the major problems in modern agriculture is the soil born plant pathogenic fungus *Fusarium* which causes considerable economic impacts worldwide.[1–4] The infection with this pathogen results in severe damage of numerous cultivable plants like maize, wheat and barley as the most important agronomic host plants.[5,6] Due to the global infestation of crops with *Fusarium* species, significant yield and quality losses arise. The kernel size and weight are usually reduced upon infection with *Fusarium*. But even more important are the numerous toxic metabolites which are produced during the colonization of the plant. These compounds have been related to toxic effects upon ingestion by humans and animals.[7,8] The most important trichothecene producing *Fusarium* species are *F. graminearum* and *F. culmorum*, whereas *F. verticillioides* and *F. proliferatum* are the major fumonisin producing species.[9–12] Mycotoxin levels in infected maize plants can vary significantly among maize cultivars and are usually higher in susceptible plants than in more resistant cultivars. Therefore, the determination of the resistance of new maize varieties is of high importance and usually combines the analysis of mycotoxins by enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) coupled either to UV and/or mass spectrometric (MS) detection methods[13,14] and the visual scoring of disease symptoms.[15] Methods to determine mycotoxin contents are highly sensitive but nevertheless time-consuming, cost-intensive (especially HPLC/MS) and only indirect, because they provide no information about the actual biomass of a fungus in a sample. Hill *et al.*[16] developed a *Fusarium*-specific ELISA to directly determine the *Fusarium* biomass. The authors analyzed artificially infected barley samples and found out that fungal biomass determination requires only one third to one fourth of the field replicates to acquire the same information on plant resistance as with visual scoring or DON analysis. Another method to determine the fungal biomass during infection of a plant deals with the measurement of the ergosterol content of cereal samples.[17–19] The analytical procedure to measure the ergosterol levels is as elaborate as toxin

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4ay02581d

measurements and therefore it is not a commercially applicable alternative to determine the *Fusarium* resistance of a plant. Recent studies focused on the development of more direct techniques to quantify *Fusarium* diseases. In general, two methods gained acceptance in the last decades: immunoassays and polymerase chain reaction (PCR). ELISA allows the direct measurement of *Fusarium* species biomass in infested plant tissue due to specific fungal antigens. It profits from its low costs and ease of sample preparation. Nevertheless, commercial antibodies for *Fusarium* are rare. Another interesting novel method to gain direct information about the biomass of a fungus is the quantitative PCR (qPCR), which is based on the quantification of the amount of organism specific DNA.[20–22] An infection of highly resistant plants can also be detected before any symptoms are visible.[20,23] Besides the application as a rapid and therefore inexpensive method for resistance evaluation, the qPCR can perfectly be applied for *Fusarium* monitoring projects and even as a screening method for food and feed contamination.[24] Furthermore, the PCR has the potential to analyze numerous samples in parallel, and common PCR instruments even allow the analysis of approximately 45 samples in duplicate within less than one hour. In addition to existing species specific assays,[20,21] tests were developed to quantify all trichothecene producing *Fusarium* species[22] or all fumonisin producing *Fusarium* species[25,26] in one assay by the detection of a gene essential for the respective mycotoxin production. However, to obtain reliable results, an elaborate optimization of the whole procedure is indispensable.

Bluhm *et al.*[25] developed a multiplex PCR assay that provides differential detection of fumonisin and trichothecene producing *Fusarium* species. Nevertheless, this published method is only qualitative and allows no quantification of fungal biomass. In this study, we describe for the first time the development and application of the quantification of three relevant target genes in parallel. The *tri5* gene, encoding for the fungal trichodiene synthase, was targeted to quantify DNA from trichothecene producing species. The trichodiene synthase is the enzyme catalyzing the first step of trichothecene synthesis.[27] The *fum1* gene encodes for a polyketide synthase and has been applied to measure DNA from fumonisin producing *Fusarium* species.[25] According to Brunner *et al.*,[28] a co-quantification of plant DNA compensates for variable DNA extraction efficiencies and is able to improve the repeatability of PCR tests. Therefore, the plant gene *adh1*, encoding for the alcohol dehydrogenase of maize, was included in the measurements as a reference gene. In order to develop a quantitative multiplex group specific assay for the detection of trichothecene and fumonisin producing *Fusarium* species, primers and probes from previously presented studies were used[24,28,29] and new qPCR conditions were developed to run all three assays in one reaction. The performance of the new method was evaluated with twenty-four maize samples collected in Austria and the novel triplex assay was compared to the three singleplex qPCR runs. Thereafter, all maize samples were analyzed for the trichothecene toxins DON, DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) by liquid chromatography tandem mass spectrometry (LC-MS/MS) and compared to the qPCR results.

2 Experimental

All chemicals and reagents used for extraction were obtained from Carl Roth GmbH (Karlsruhe, DE), whereas the primers and probes were ordered from Sigma Aldrich (St. Louis, MO). The Kapa qPCR master mix was obtained from PeqLab (Erlangen, DE).

2.1 Sample preparation

Twenty-four ground maize samples from four different locations (A, B, C and D) in Austria were obtained from the Center for Analytical Chemistry at the IFA Tulln. All samples were stored at −20 °C. The toxin contents of DON, D3G, NIV, 3-ADON, T-2, HT-2, DAS, NEO, FB_1, FB_2 and FB_3 were analyzed by LC-MS/MS and the fungal biomass was quantified by qPCR.

2.2 DNA extraction

The protocol used for the DNA extraction from maize kernels is a modification of a method recommended by the European Union Reference Laboratory (EURL).[30]

One gram of ground maize sample was weighed into a 15 ml flask and 6 ml of CTAB (cetyltrimethylammonium bromide) extraction buffer (1.4 M NaCl, 2% w/v CTAB, 0.1 M Tris–base pH 8, 0.02 M EDTA pH 8, 1% w/v polyvinyl pyrrolidone 40 000), preheated up to 70 °C, were added. The samples were incubated for 1 h at 70 °C. During the incubation, the tubes were inverted every 10 minutes to enhance the efficiency of the extraction and the DNA yield. Then the tubes were centrifuged at 2978 × *g* for 15 minutes at room temperature (RT). Subsequently, 500 µl of the supernatant were transferred into a clean tube. Then 55 µl of 10% preheated CTAB solution (10% w/v CTAB, 0.7 M NaCl) and 550 µl of a mixture of chloroform–isoamyl alcohol (24 : 1) were added and the tubes were inverted 20 times. The samples were centrifuged at 7200 × *g* for 5 minutes at RT. Thereafter 350 µl of the upper aqueous phase were collected and transferred into a clean tube and 1050 µl precipitation buffer (1% w/v CTAB, 0.05 M Tris–base pH 8, 0.01 M EDTA pH 8) were added. The solution was mixed gently and was kept at RT for 30 minutes for precipitation. The DNA was then collected by centrifugation at 7200 × *g* for 15 minutes at RT. The supernatant was discarded and the pellet was washed twice with 200 µl precooled 70% ethanol. The pellet was then vacuum dried for 30 minutes at 37 °C and the DNA was re-suspended in 100 µl 10 mM Tris buffer (pH 8). Finally, the solution was incubated in a thermomixer for 30 minutes at 65 °C to ensure solubilisation of the DNA and stored at −20 °C.

Maize genomic DNA was used as the DNA standard. It was isolated from freshly grown maize leaves as described by Saghai-Maroof *et al.*[31] DNA from *F. verticillioides* and *F. graminearum* used as fungal DNA standards was obtained by phenol–chloroform extraction according to Peterbauer *et al.*[32]

2.3 Real-time PCR primers and dual-labelled probes

Three sets of specific primer pairs and the appropriate probes were used to amplify either maize DNA, DNA from fumonisin or

from trichothecene producing *Fusarium* species. The *adh1*, *fum1* and *tri5* probes were labeled at the 5′-ends with JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein), Cy5 (indodicarbocyanine) and 6FAM (6-carboxyfluorescein), respectively and at the 3′-end with a quencher (Black Hole Quencher® 1 – BHQ1). The primers adh1_fw (5′-CGTCGTTTCCCATCTCTTCCTCC-3′) and adh1_rev (5′-CCACTCCGAGACCCTCAGTC-3′) specifically amplified a 136 bp fragment of the *adh1* gene of maize, which was quantified by the dual labelled probe adh1_probe (JOE-5′-AATCAGGGCTCATTTTCTCGCTCCTCA-3′-BHQ1). The *Fusarium* DNA of trichothecene producing species was detected by the amplification of a 178 bp fragment of the *tri5* gene. For the amplification and quantification, the primer pairs tri5_fw (5′-GATTGAGCAGTACAACTTTGG-3′) and tri5_rev (5′-ACCATCCAGTTCTCCATCTG-3′) as well as the tri5_probe, a locked nucleic acid (LNA) oligonucleotide (6FAM-5′-C[+C][+T][+T][+G]G[+G]CCA-3′-BHQ1) (bases in brackets are locked), were used. The primers and probes used for the *adh1* and *tri5* assay were published by the EURL[29] and Brunner *et al.*,[28] respectively. The primer pairs fum1_fw (5′-ATGCAAGAGGCGAGGCAA-3′) and fum1_rev (5′-GGCTCTCAGAGCTTGGCAT-3′) as well as the fum1_probe (CY5-5′-CAATGCCATCTTCTTGAAACCT-3′-BHQ1) to quantify fumonisin producing species DNA were slightly modified from the study of Waalwijk *et al.*[26] The amplified fragment has a length of 149 bp.

2.4 Real-time PCR optimization and assay evaluation

Initially, the three primer sets were tested in separate reactions based on the EURL,[29] Brunner *et al.*[28] and Waalwijk *et al.*[26] The optimization of the multiplex qPCR was performed by empirically varying critical factors that affect multiplexing such as primer concentrations and annealing temperatures. All analyses were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) as well as on a Rotor-Gene Q (Qiagen, Hilden, DE) to test the reproducibility of the assay. A total reaction mix volume of 15 µl containing 2 µl of template DNA, 7.5 µl Kapa probe fast master mix, 4.78 µl/3.34 µl water (singleplex and multiplex, respectively), 0.1 pM μl^{-1} or 0.05 pM μl^{-1} of dual labelled probe, and forward and reverse primers was used. The PCR cycling conditions included an initial denaturation step at 95 °C for 1 min and 50 s, followed by 45 cycles of denaturation at 95 °C for 15 s and primer annealing and amplicon extension at 58 °C for 45 s. PCRs were performed in triplicate for all samples.

The cross-reactivity of the primers used in the developed multiplex qPCR method was analyzed by testing the different primer pairs on different fungal species. Therefore, *fum1* primers were tested for amplification of *tri5* producing *Fusarium* strains and *vice versa*. The specificity of the *tri5* oligonucleotides was already evaluated by Brunner *et al.*[28] To prove equal efficiencies of the *fum1* primers for different fumonisin producing strains, five different isolates belonging to three different *Fusarium* spp. were analyzed (*F. proliferatum* 23, *F. proliferatum* 353, *F. proliferatum* 2763, *F. verticillioides*, and *F. nygamai*). These isolates were provided by the Vienna University of Technology and were internally numbered. The qPCR reagent concentrations and conditions were the same as mentioned above.

To evaluate the sensitivity of the assay, 5 ng of purified genomic DNA from *F. graminearum* and *F. verticillioides* were serially diluted by a factor of five with maize DNA (50 ng μl^{-1}) to a concentration of 4.10×10^{-9} ng μl^{-1}. This was done for the singleplex as well as for the multiplex method.

The infected maize samples were divided into two subsamples. Each sub-sample was extracted and the DNA was precipitated twice. Each precipitate was quantified twice by the three singleplex assays as well as by the newly developed multiplex qPCR method to ensure the repeatability of all parts of the method.

2.5 Determination of *Fusarium* toxins by LC-MS/MS

All maize samples were analyzed for the presence and concentrations of *Fusarium* metabolites by LC-MS/MS according to Malachová *et al.*[33] The analytical method has been extended to cover 320 metabolites, transferred to a more sensitive mass spectrometer. In brief, 5 g of sample was weighed into a 50 ml polypropylene tube (Sarstedt, Nümbrecht, DE) and extracted with 20 ml acetonitrile/water/acetic acid (79 : 20 : 1, v/v/v) for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, DE). The extracts were diluted in extraction solvent (ratio 1 : 1) and directly injected into the LC-MS/MS instrument.

3 Results and discussion

3.1 Development of a quantitative triplex PCR assay

During multiplex qPCR optimization, 81 combinations of different annealing temperatures and times have been evaluated together with three concentrations of oligonucleotides (0.1 pM μl^{-1}, 0.05 pM μl^{-1} and 0.025 pM μl^{-1}). For the oligonucleotides adh1_fw, adh1_rev, adh1_probe and tri5_probe, a concentration of 0.05 pM μl^{-1} was used. All other primers and probes were used at a concentration of 0.1 pM μl^{-1}. A gradient PCR from 52 °C to 60 °C was performed with these primers and the optimal thermal cycling conditions turned out to be 1 min 50 s at 95 °C, followed by 45 cycles of 15 s at 95 °C and 45 s at 58 °C. The multiplex assay worked most efficiently with a two-step protocol.

3.2 Specificity and sensitivity of the multiplex qPCR assay

The specificity of the *tri5* assay has been previously demonstrated by Brunner *et al.*[28] for wheat samples. All tested trichothecene producing strains were amplified with an efficiency of 0.91 ± 0.41. Waalwijk *et al.*[26] evaluated the specificity of the *fum1* assay and showed that no amplicons were generated from any of the non-fumonisin producers such as *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. semitectum* and *F. subglutinans*. The specificity of the *fum1* assay was also tested in this study using five different isolates from three different fumonisin producing *Fusarium* species, obtained and internally labelled by the Vienna University of Technology (*F. proliferatum* 23, *F. proliferatum* 353, *F. proliferatum* 2763, *F. verticillioides* and *F. nygamai*). The amplification efficiency for all tested strains

was found to be 0.88 ± 0.43. All isolates scored positive for the *fum1* product, suggesting that the qPCR reaction is neither influenced by different isolates nor by species. Furthermore, no cross-reactivity was shown between fumonisin and trichothecene producing strains, which confirms that the primers and probes are group specific for either fumonisin or trichothecene producers (data shown in Table 1).

To determine the minimum amount of fungal template DNA necessary for quantification and the sensitivity of the assay, the multiplex qPCR method was carried out using a dilution series of a mixture of pure fungal genomic DNA from *F. graminearum* and *F. verticillioides* in maize background DNA with a starting concentration of 5 ng μl^{-1}. The genomic DNA was serially diluted by a factor of 5 and used as a template. Due to high standard deviations for low target concentrations, no more than 0.32 pg of target DNA per µl are quantifiable for both *Fusarium* strains. The limit of quantification is defined as the minimum target concentration that remained within the linear regression line (Fig. 1). Lower concentrations could lead to false positive results. Considering a genome size of 41.7 Mb for *F. verticillioides* and 36.2 Mb for *F. graminearum*[34] this represents approximately seven or eight genome equivalents, respectively, calculated according to Staroscik.[35] Finally, no loss of sensitivity compared to the singleplex assays was observed when the multiplex method was applied. All assays were run in combination with the maize *adh1* gene for normalization.

3.3 Reproducibility test of the multiplex qPCR assay

To determine whether the instrument used for quantification or the operator affect the results, reproducibility tests were performed by conducting all qPCR assays on two PCR thermal cyclers from different manufacturers. Furthermore, two operators prepared the qPCR assays. Comparing the results obtained by multiplex qPCR as well as the qPCR efficiencies, it could be shown that neither the instrument nor the operator had a significant influence on the obtained results.

3.4 Evaluation of the multiplex qPCR assay with *Fusarium* infested maize samples

The calculation of the starting concentrations in qPCR analysis requires the setting of a fluorescence threshold and the determination of the quantification cycle (C_Q) value, which is the fractional cycle number that is required to reach this threshold.

Table 1 Specificity of the multiplex real-time PCR method against various fungal species

Fungal species	*fum1* detected	*tri5* detected
Fusarium proliferatum 23	+	–
Fusarium proliferatum 353	+	–
Fusarium proliferatum 2763	+	–
Fusarium verticillioides	+	–
Fusarium nygamai	+	–
Fusarium graminearum	–	+
Fusarium culmorum	–	+

The C_Q values are inversely proportional to the amount of initial target DNA in the sample. To quantify the unknown amount of target DNA in the samples, appropriate DNA standards with known concentrations are needed. To obtain these standards, that optimally reflect the natural conditions, *Fusarium* free maize DNA was used as background and then spiked with varying amounts of *F. graminearum* or *F. verticillioides* DNA. From both DNA standards, a dilution series was made by diluting them ten-fold with maize DNA to reduce the amount of *Fusarium* DNA in a constant maize background DNA concentration. Besides the quantification of the two different *Fusarium* DNAs, the maize specific gene *adh1*, encoding for an alcohol dehydrogenase, was included as a reference to normalize variations in DNA-extraction yields. A similar strategy is applied by the European Reference Laboratories for GM Food & Feed for the determination of the relative content of GM events in the total maize DNA.[29]

Table 2 shows the C_Q values of the serially diluted DNA standards as well as the efficiencies of the multiplex assay. The efficiency of the assay is derived from the standard curve and is calculated according to formula (1):

$$\text{Efficiency} = \text{dilution factor}^{\left(\frac{-1}{\text{slope}}\right)} - 1 \qquad (1)$$

The slope for the calculation of the efficiency is obtained by the linear regression line of the standard curve.

The calculated efficiencies were 0.94, 0.98 and 1.03 for the *tri5*, *fum1* and *adh1* target fragments, respectively.

To get a deeper insight into the repeatability of the whole analysis procedure, including the various steps of DNA extraction and quantification by three singleplex PCR assays and by the triplex test developed here, twenty-four maize field samples were collected from four different locations in Austria (locations A, B, C and D) and tested for the presence of *Fusarium* DNA. To identify the steps having the highest influence on the total error of the method, all samples were divided into two sub-samples. Each sub-sample was used for DNA isolation and the DNA was finally precipitated twice, as this can be considered as the most crucial step of the extraction procedure. Moreover, every precipitate of a sample was analyzed two times, either by the three separate singleplex assays or the multiplex qPCR method (Fig. 2).

The results show low variations between the two sub-samples, the individual precipitates and no significant differences result from the PCR analysis. In general, a mean total error of 23% occurs over the whole analysis procedure. Going more into detail, 5% of the observed error is caused by dividing the sample into two sub-samples, 10% arises from the DNA precipitation step and 8% of the error results from the multiplex qPCR method. For the calculation of the error rates, 48 values for sub-sample extraction, 96 values for precipitation and 192 values for qPCR analysis were included for each gene (*adh1*, *fum1* and *tri5*).

Finally, the infection of the samples was calculated based on formula (2) previously published by Brunner *et al.*[28] referring the fungal DNA to the total extracted DNA from a sample,

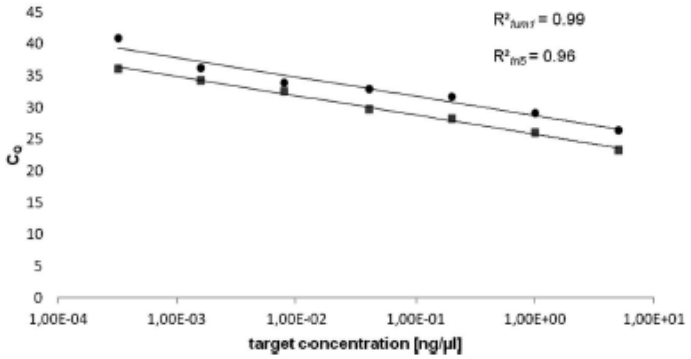

Fig. 1 Sensitivity of the multiplex real-time PCR assays. ● shows the serial dilution of *F. graminearum* and ■ shows *F. verticillioides* as the target DNA. Both dilution series had a starting concentration of 5 ng and have been diluted by a factor of 5. Following dilutions were used: 5 ng, 1 ng, 200 pg, 40 pg, 8 pg, 1.6 pg, and 0.32 pg.

Table 2 C_Q values of serially diluted DNA standards and efficiencies for the three targets quantified simultaneously by the multiplex PCR

Concentration of fungal DNA (*fum1* or *tri5*)	C_Q for trichothecene producing isolates	C_Q for fumonisin producing isolates	Concentration of maize DNA standard	C_Q for maize DNA amount
5 ng µl^{-1}	26.86 (±0.23)	23.47 (±0.21)	50 ng µl^{-1}	24.25 (±0.23)
0.5 ng µl^{-1}	30.50 (±0.43)	26.73 (±0.17)	25 ng µl^{-1}	25.13 (±0.12)
0.05 ng µl^{-1}	33.80 (±0.30)	30.20 (±0.53)	12.5 ng µl^{-1}	26.10 (±0.28)
			6.25 ng µl^{-1}	27.18 (±0.41)
Efficiency	0.94	0.98	Efficiency	1.03
R^2	0.9992	0.9997	R^2	0.9979

$$\text{Infection } [\%] = \frac{\textit{Fusarium}\text{DNA } [\text{ng}]}{\text{total DNA } [\text{ng}]} \times 100 \quad (2)$$

where "*Fusarium* DNA" is the DNA from trichothecene or fumonisin producing *Fusarium* species and "total DNA" is the sum of DNA from trichothecene producing *Fusarium* species, fumonisin producing *Fusarium* species and maize DNA.

The analyzed samples show a wide range of *Fusarium* infections, both for fumonisin and for trichothecene producing species. All analyzed samples were positive for *fum1* DNA and *tri5* DNA. Fig. 3 shows that comparable results were obtained by three singleplex assays and the rapid multiplex test. The grade of observed infection with fumonisin producing *Fusarium* species varies between 8.2×10^{-4} ‰ and 0.22‰, whereas the range of infection with *tri5* producing strains was much higher and ranged from 0.10% to 3.33%. This huge difference of infection between the two groups might be due to the fact that fumonisin producing strains commonly appear in southern regions. In cooler regions like Austria, fumonisin production is reduced and toxins produced by other *Fusarium* species become more important and more prevalent.[11] The results for the fumonisin producing group inherently show a higher standard deviation due to the very low amount of DNA detected in all samples. The mean relative standard deviation (RSD) for the samples for trichothecene producing species is 7.17% for the singleplex, and 22.71% for the multiplex method. For the *fum1* assay, the mean RSD shows 17.19% for the singleplex and 23.30% for the multiplex method. All RSD values are referred to the complete analysis method, including DNA isolation and the qPCR analysis. The loss of precision of the triplex *tri5* multiplex in contrast to the singleplex method might be attributable to the interaction among the many oligonucleotides in the multiplex assay. A slight loss of precision was therefore expectable and is negligible due to the considerable correlations of the results between the two methods. Furthermore, the costs and analysis time are reduced by a factor of three for the triplex method.

3.5 Comparison of LC-MS/MS determined mycotoxin concentrations with the real-time PCR determined *Fusarium* infection

The twenty-four previously extracted maize samples were now used to analyze whether the qPCR results for *Fusarium* DNA correlate either with the trichothecene or the fumonisin content. Therefore, the mycotoxin concentration of each sample was determined by LC-MS/MS. Five grams of sample were extracted with 20 ml acetonitrile/water/acetic acid (79 : 20 : 1, v/v/v) and analyzed according to Malachová *et al.*[33] The qPCR determined infections observed by the multiplex method were plotted against the mycotoxin concentrations (Fig. 4).

The PCR primers for the *fum1* and *tri5* gene quantification are specific for either all *Fusarium* species producing toxins belonging

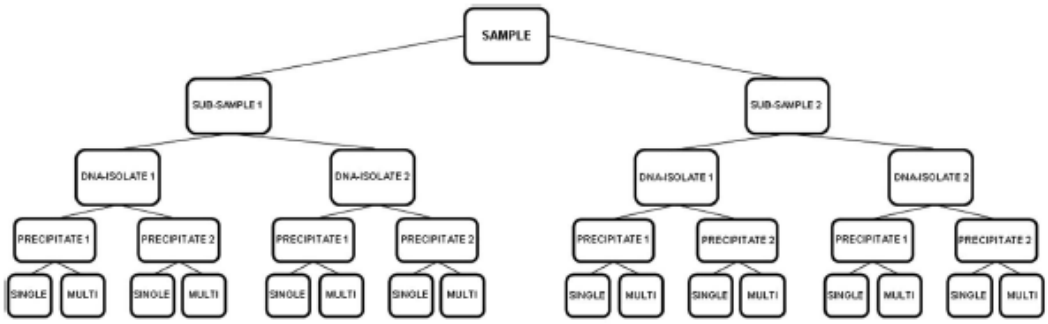

Fig. 2 Scheme of sample analyses to evaluate the repeatability of the crucial steps of the analysis procedure. Every sample was divided into two sub-samples which were then used for DNA isolation. Each DNA isolate was finally precipitated twice and analyzed two times, either by the three singleplex assays or the multiplex method.

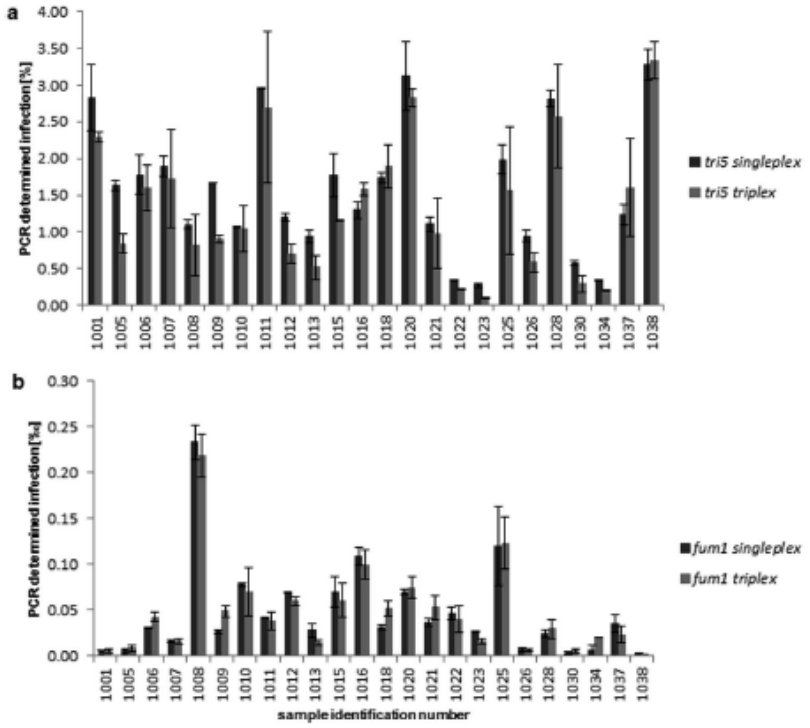

Fig. 3 Analysis of Austrian field samples. The bars show the results either obtained by the singleplex (dark grey) or the newly developed multiplex (light grey) method. (a) PCR-determined infection of maize samples with trichothecene producing species and (b) infection of samples with fumonisin producing *Fusarium* species.

to the class of fumonisins or the *Fusarium* species producing metabolites belonging to the group of the trichothecenes, respectively. For this reason, the *tri5* infection was not only compared to the prevalent DON concentration but also to the total amount of trichothecenes including DON-3-glucoside (resulting from *in planta* metabolisation of DON), NIV, 3-ADON and also the type A trichothecenes DAS, T-2, HT-2 and NEO.[36] It was found that the PCR results correlate slightly better with the sum of the eight toxins than with DON only. For the comparison of the *fum1* infection with the fumonisin concentrations, the toxins FB_1, FB_2

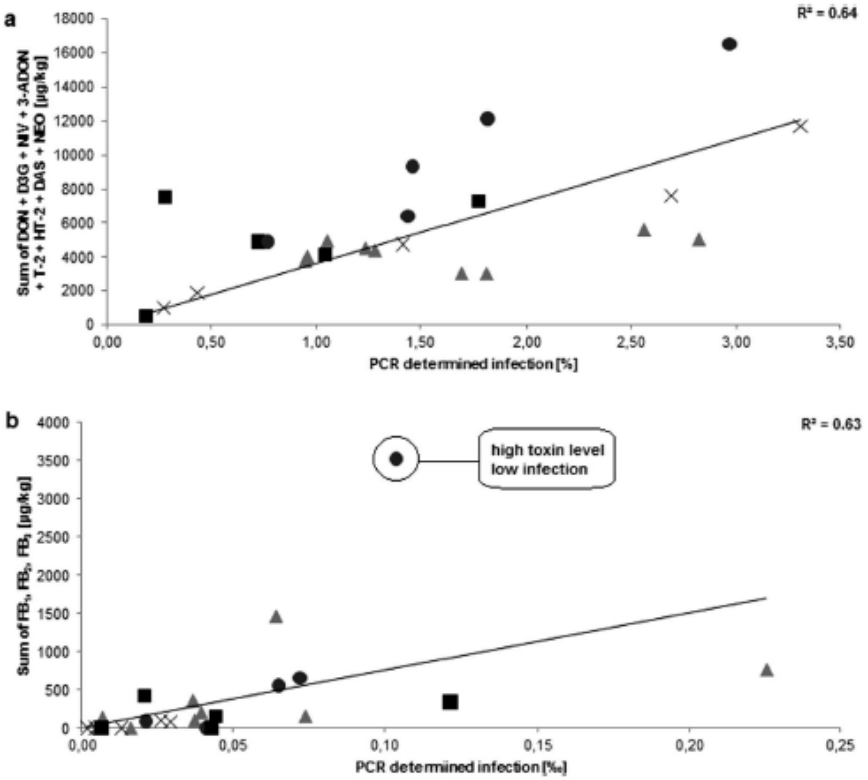

Fig. 4 Correlation between mycotoxin concentrations and PCR determined infection rates. The four different locations are labeled as follows: ■ (A), ● (B), ▲ (C) and ✕ (D). (a) Results for trichothecene producing strains; the type B trichothecenes DON, D3G, NIV and 3-ADON as well as the type A trichothecenes T-2, HT-2, DAS and NEO were used as a sum parameter. (b) Correlation of the infection with fumonisin producing strains with the fumonisins FB_1, FB_2 and FB_3.

and FB_3 were used as a sum parameter. Fig. 4 shows that a certain correspondence between the infection of a maize sample calculated according to formula (2) and the toxin content determined by LC-MS/MS can be observed. The coefficient of determination was found to be $R^2 = 0.64$ for the correlation of the trichothecene content and the PCR determined infection. Approximately the same R^2 was observed for the fumonisin concentrations and the infection determined by the qPCR.

Although a certain correlation between the mycotoxin content of a sample and the infection determined by qPCR can be observed, it is obvious that *Fusarium* isolates present at location B produce presumably more toxin per fungal biomass, whereas at location C the situation is converse. This fact demonstrates that the infection determined by qPCR analysis provides additional information which might be relevant for the determination of the resistance of new crop lines in breeding programs.

In general, two methods are applied for the registrations of new crop varieties: visual scoring and mycotoxin analysis. But these methods only indirectly determine the resistance of plants which is defined as the pathogen growth on or in the plant. Previous experiments stated that the amount of fungal mycelia formed during infection not always correlates well with disease symptoms[28,37] and also the amount of accumulated DON does not necessarily reflect differences in resistance. Genotypes with good resistance based on visual scoring might show high or low toxin contamination. Furthermore, highly resistant wheat varieties can often not be classified by visual scoring anymore as the infection proceeds without any symptoms but nevertheless leads to high toxin accumulation.[25] The supreme sensitivity of qPCR registers even minor amounts of fungal biomass.[37]

4 Conclusion

In the last decade, several studies have described two distinct approaches for *Fusarium* quantification, species specific assays which quantify individual *Fusarium* species or group specific assays which measure the abundance of a gene owned by a group of different species sharing a common biosynthetic pathway (*e.g.* for mycotoxin production).[26,28,38] Two group specific quantitative PCR assays were used frequently

throughout the last decade: one test which quantifies all trichothecene producing *Fusarium* species[23] and another one for all fumonisin producing species.[19] However, until now these tests have been only available as two quantitative singleplex tests or as a duplex assay[25] which is only qualitative.

In this study, we have developed for the first time a multiplex qPCR assay which allows the screening of maize samples for all *Fusarium* species producing the most relevant mycotoxins in maize, trichothecenes and fumonisins. Besides the two fungal toxin genes, *tri5* and *fum1*, the maize gene *adh1* is used additionally as a reference gene to compensate for varying DNA extraction yields, similar to the established system for GMO analysis.[26,27] The newly developed multiplex qPCR method is a high-throughput, reliable diagnostic tool that allows rapid screening of high sample numbers in a short time. This assay was designed to minimize costs by reducing the analysis time by a factor of more than 60%. This assay provides a perfect complementation to mycotoxin analysis to facilitate the classification of the resistance of new crop lines in breeding programs.

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Electronic Supplementary Material

Supporting Information

Table S1: Twenty-four maize samples were analyzed with qPCR as well as with LC-MS/MS to get information about the correlation of *Fusarium* DNA with mycotoxin content. This list provides all experimental data results.

Sample identification number	PCR determined infection (singleplex)		PCR determined infection (multiplex)		Trichothecenes [µg/kg]								Fumonisins [µg/kg]		
	tri5 [%]	*fum1* [‰]	*tri5* [%]	*fum1* [‰]	DON	D3G	NIV	3-ADON	T-2	HT-2	DAS	NEO	B_1	B_2	B_3
1001	2,83	0,00575	2,29	0,00559	3880	1733	7.92	70.3	2.69	16.3	< LOD	< LOD	< LOD	< LOD	< LOD
1005	1,63	0,00543	0,84	0,00834	3448	1083	16.5	80.8	6.64	11.3	< LOD	< LOD	115	< LOD	23.6
1006	1,78	0,03072	1,60	0,0428	2040	1015	132.8	74.9	2.95	13.3	0.51	< LOD	233	48.5	79.7
1007	1,89	0,01613	1,72	0,01634	2192	839	14.6	29	< LOD	3.55	0.76	< LOD	< LOD	< LOD	< LOD
1008	1,10	0,23280	0,82	0,21852	2680	1367	24.9	98.4	43.1	54.7	0.96	< LOD	558	174	29.6
1009	1,67	0,02620	0,90	0,04848	2960	1428	116	49.2	< LOD	3.77	1.36	< LOD	74.4	25.5	< LOD
1010	1,07	0,07766	1,04	0,0702	3240	1706	125	62.9	< LOD	< LOD	< LOD	< LOD	117	20.2	17.1
1011	2,95	0,04082	2,70	0,0382	3264	1794	204	97.6	< LOD	< LOD	< LOD	< LOD	161	23.5	21.1
1012	1,20	0,06864	0,71	0,05985	2776	1008	22.2	63.4	55.8	156	0.58	6.05	1115	243	106
1013	0,94	0,02776	0,51	0,01466	4064	853	218	139	< LOD	4.38	< LOD	< LOD	64.6	23.9	< LOD
1015	1,77	0,06958	1,16	0,06059	7864	1482	416	311	2.46	5.53	< LOD	< LOD	428	88.8	37.9
1016	1,30	0,10853	1,58	0,0994	5392	988	637	110	63.7	294	0.67	13.11	2488	691	341
1018	1,73	0,03080	1,89	0,0515	9280	2856	518	289	< LOD	7.45	< LOD	< LOD	< LOD	< LOD	< LOD
1020	3,12	0,06969	2,82	0,0744	13680	2856	821	337	3.16	17.6	< LOD	2.52	476	107	68.6
1021	1,11	0,03625	0,98	0,05262	3232	907	220	294	9.21	25	0.5	< LOD	97.7	38.2	22.1
1022	0,34	0,04620	0,21	0,03994	6496	1022	29.3	338	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1023	0,28	0,02658	0,10	0,01515	464.8	72	14.7	28	< LOD	< LOD	< LOD	< LOD	263	135	21.9
1025	1,98	0,11999	1,56	0,12291	5328	1963	102	327	2.01	< LOD	0.76	< LOD	241	74.1	24.6
1026	0,95	0,00705	0,59	0,00611	3912	1008	27.2	249	3.04	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1028	2,81	0,02357	2,57	0,02919	6432	1157	126	434	< LOD	< LOD	< LOD	< LOD	81.4	21.6	< LOD
1030	0,58	0,00391	0,29	0,00550	1616	277	234	93.6	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1034	0,34	0,00615	0,21	0,02	816	181	14.3	43.9	4.75	5.73	< LOD	< LOD	< LOD	< LOD	< LOD
1037	1,24	0,03570	1,60	0,023	3800	948	220.9	271	6.83	3.68	< LOD	< LOD	45.1	27.4	11.3
1038	3,28	0,00286	3,33	0,00082	8960	2734	208.6	960	9.12	30.5	< LOD	< LOD	< LOD	< LOD	< LOD

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ARTICLE TYPE

A switch based aptamer biosensor: a new approach towards a direct detection format for aflatoxin B_1

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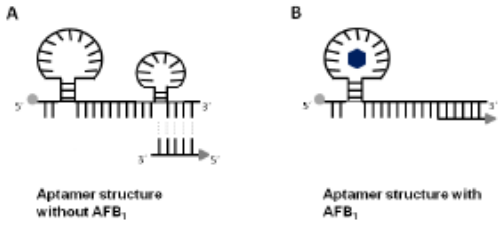
The present study demonstrates for the first time an approach for a rapid and simple aptamer dipstick assay using a direct detection format for aflatoxin B_1. Our proposed method is based on the capability of an aptamer to switch its structure after the addition of a target. Then a binding site for a complementary signalling DNA probe is exposed to enable the hybridization of the probe with the aptamer. This hybrid molecule is visualized *via* a simple lateral flow test. In contrast to all previously published assays, the test line is visible only when aflatoxin is present in a sample solution.

Aflatoxins are toxic secondary metabolites mainly produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus*. These species are widespread in nature and can colonize agricultural crop plants, such as corn and cereal grains.[1-3] Among several natural occurring aflatoxins (B_1, B_2, G_1 and G_2), B_1 (AFB_1) is the most prevalent and potent of these toxins.[4] It possesses the highest toxicity and was classified as a group 1 carcinogen (carcinogenic to humans) by the IARC.[5] Health concerns related to AFB_1 have increased over the years and regulatory limits have been introduced for food and feed safety reasons in many countries. These maximum levels are set between 1 µg/kg and 20 µg/kg for AFB_1 in food.[6] Therefore, rapid, sensitive and inexpensive analytical methods are essential to detect and quantify AFB_1. Various procedures based on chromatographic methods including high performance liquid chromatography (HPLC) combined with mass spectrometry (MS)[7-9] and rapid screening approaches with immunoassays, such as the enzyme linked immunosorbent assay (ELISA)[8, 10] have been developed. However, these methods often require skilled personnel, expensive equipment (especially HPLC-MS based methods) and extensive sample pre-treatment. Antibodies are the most popular class of molecules for molecular recognition and they have been in use for more than three decades. Nevertheless, the use of antibodies in immunoassays has some limitations, such as the limited stability at higher temperatures as well as laborious and expensive production.[11, 12] In recent years promising aptamer-based analytical methods have been developed with comparable performance as antibody tests. Aptamers are short, single stranded oligonucleotides that bind, due to their specific three-dimensional structure, to a target molecule with high affinity and specificity.[13-15] Compared to antibodies they have some significant advantages for analytical applications. They are thermally stable and can withstand repeated rounds of denaturation and renaturation, their production is cheap and there are barely any batch to batch variations. Furthermore, the chemical synthesis of aptamers does not depend on living animals or cell cultures.[16, 17]

Previous studies report that aptamers often undergo a conformational change upon binding to their analyte.[18-20] This structure switching mechanism can be exploited for biosensor development. Until now, a few aptasensors for the rapid detection of AFB_1 have been developed[21, 22] based on a DNA fragment designed by Neoventures Biotechnology Inc. (Canada) in 2009.[23] These assays are currently based on an indirect target detection format where a test line is formed if no target is present and vice versa. To the best of our knowledge, a structure switch of the aptamer developed by the Canadian company, as well as a direct format for AFB_1 detection has not yet been reported.

In the present study we describe a new direct detection format for the target molecule AFB_1 with a nucleic acid lateral flow teststrip. The idea claims an AFB_1 induced unwinding of the aptamer's distinctive structure exposing a binding site for a complementary signalling DNA probe (Scheme 1).



Scheme 1 The AFB_1 aptamer forms two distinct loop structures according to Neoventures Biotechnology Inc.[23] This characteristic structure impedes the binding of the complementary signalling DNA probe (A). We assume that due to target addition a structure switch is induced exposing a binding site for the DNA probe (B).

Our approach relies on hybridization of a biotin labelled aptamer with a complementary DIG labelled signalling DNA probe. A structure switch of the aptamer following AFB_1 addition results in the unwinding of the loop structure located at the 3'-end of the aptamer. This induced structure switch enables the complementary probe to bind resulting in the formation of

partially double-stranded dual-labeled DNA. Anti-DIG coated gold nanoparticles (40 nm) bind to DIG, while biotin on the other end is captured by streptavidin immobilized at the test line of the dipstick (principle described in Fig. 1). Hence, a red-coloured line is only obtained in the presence of AFB_1. If no target is present, the loop structure at the 3'-end persists and hinders the binding of the complementary probe to the aptamer. Therefore, no signal is generated.

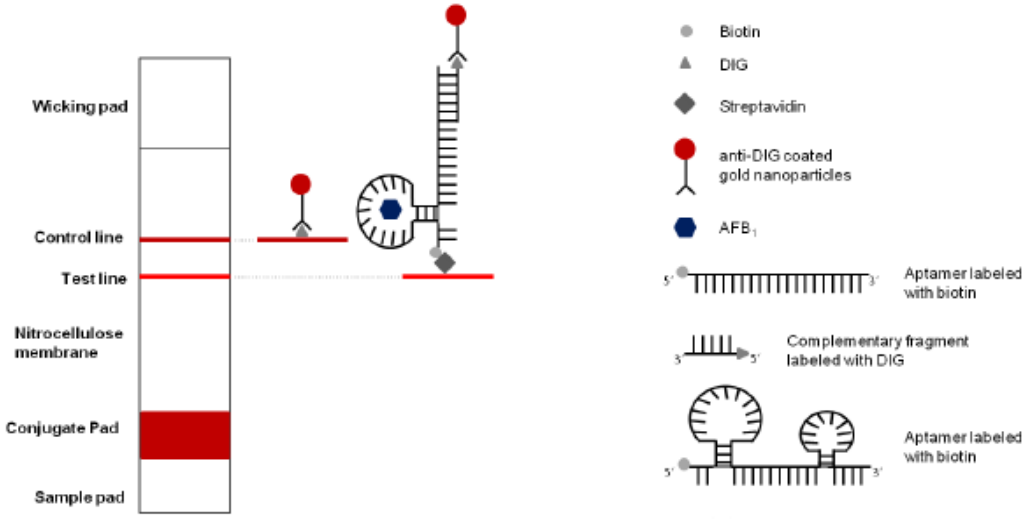


Fig. 1 Schematic illustration of the principle of the test strip used in our study for the visual detection of aflatoxin B_1 (AFB_1). The developed test strip is composed of a sample pad, a conjugate pad with anti-digoxigenin (DIG) coated gold nanoparticles, a nitrocellulose membrane containing immobilized streptavidin at the test line, DIG at the control line, and a wicking pad. The presence of AFB_1 evokes a structural switch of the biotinylated aptamer enabling the DIG labelled complementary fragment to bind. Accordingly, anti-DIG coated gold nanoparticles bind to DIG and biotin is captured by streptavidin at the test line. Hence, a red line is obtained. In the absence of the target, the loop structure at the 3'-end persists which in turn makes it impossible for the signalling DNA probe to bind. Therefore no gold nanoparticles are attached and no signal is generated.

To test the stated claim, the oligonucleotides listed in Table 1 were synthesized by Sigma Aldrich (St. Louis, MO) and used for the experiments. The target analyte AFB_1 was purchased from Romer Labs® (Tulln, Austria) and dissolved in acetonitril to a final concentration of 2 µg/ml. Furthermore, a test strip for the visualization of the signal was produced. The lateral flow strip is composed of a sample pad, a conjugate pad, a laminated nitrocellulose membrane and a wicking pad (Fig. 1). Before applying the sample on the lateral flow strip 10 µl AFB_1_aptamer (0.1 µM), 2 µl AFB_1 (2 µg/ml) and 10 µl signalling DNA probe (0.2 µM) were incubated for 30 min at 37 °C. Subsequently, 10 µl of the mixture were applied to the sample pad and the strip was placed into 150 µl running buffer (10 mM Tris, pH 7.0). Positive results were visualized as red lines within 3-5 min.

Table 1 Listing of the developed digoxigenin (DIG) labelled complementary signalling DNA probes as well as the used biotinylated aptamer and its characteristics. The red bases marked in DIG_probe4 and DIG_probe5 indicate the base pair mismatches compared to the aptamer sequence.

Name	Sequence (5' – 3')	5' Label	Length [bp]	Match with aptamer sequence [%]	T_M [°C]
AFB_1_aptamer	AAAAAAAAAAGTTGGGCACGTGTTGTCTCTCTG TGTCTCGTGCCCTTCGCTAGGCCCACA[21]	Biotin	60		
DIG_probe1	AAATGTGGGCCTAGCGA[21]	DIG	17	100	39.4
DIG_probe2	AAATGTGGGCCTAGCG	DIG	16	100	38.2
DIG_probe3	AAATGTGGGCCTAGC	DIG	15	100	34.6
DIG_probe4	AAATGTGGTCCTAGCGA	DIG	17	94	35.8
DIG_probe5	AAATGTTGGCTTAGCGA	DIG	17	88	34.1

The first step towards on-strip application was the optimization of essential experimental parameters including the length as well as the base composition of the signalling DNA probe and the incubation temperature of the assay. These parameters are crucial for aptamer-target-complex formation and signal generation. We assume that if the fragment length of the complementary DNA is too long it favours the binding to the DNA probe rather than the target. On the other hand too short DNA probe fragments probably will not bind at all or are possibly easily dehybridized from its complementary strand by the slightest increase of temperature. In our study we focused on five different DIG labelled DNA probes listed in Table 1, starting with an oligonucleotide introduced by Shim et al.[21] named DIG_probe1 (17 mer) in this study. Results revealed that the DIG_probe1 has a strong binding affinity to the complementary part at the aptamer. Hybrids were produced in presence and absence of the analyte (Fig. 2-A). As a result, two more signalling DNA probes were synthesized namely DIG_probe2 (16 mer) and DIG_probe3

(15 mer) with decreased fragment length. Attempts with decreased probe length did not improve the results (Fig. 2-A). Focusing on the base composition of the probes (Table 1) we assume that the five consecutive G/C bases hinder the DIG labelled complements from dehybridization. Such regions are called GC-clamps. GC-clamps require high temperatures and often even a detergent is required to break the H-bonds between the base pairs. Nevertheless, some are unbreakable. Since false-positive results might be GC-clamp specific we slightly modified the sequence of the DNA signalling probe DIG_probe1 by introducing base pair mismatches. In contrast to the other probes the new synthesized ones DIG_probe4 and DIG_probe5 include one (G→T) or two (G→T and C→T) base pair mismatches, respectively. This results in decreased binding affinity. As shown in Fig. 2-C, the DIG_probe5 was the only tested complementary sequence that reveals the ability to bind to the aptamer only if AFB_1 is present. Hence, the proposed structure switch seems to occur and allows the complementary probe to bind to the aptamer. Without target the loop structure at the 3'-end persists and thus impedes the binding of the signalling probe. These findings support our assumption and therefore, DIG_probe5 containing two base pair mismatches was chosen for further experiments.

For the development of assays based on aptamers, the incubation temperature responsible for hybridization/dehybridization is an important parameter. Shim et al.[21] recommend an incubation temperature of 37 °C for their aflatoxin aptamers tests. In our study room temperature (RT) and 40 °C for incubation were investigated as well. Especially the performance of the assay at RT would make this method immediately on-site applicable without the need for a thermal device. Experiments at RT showed a test line even with or without target whereas tests performed at 40 °C never gave a signal, neither with nor without AFB_1 (Fig. 2-B). A closer look at the high melting temperature (T_M) of DIG_probe5 of 34.1 °C reveals, that once the complementary sequence has bound to the aptamers, the dehybridization at RT will fail. On the other hand, the two strands can be easily denatured at 40 °C. Comparing the three tested temperatures, findings clearly showed that a temperature of 37 °C worked best (Fig. 2-C). To assure that the signal is solely derived from the aptamer-target-complex only aptamer, only DIG_probe5 and only AFB_1 were used as a control (Fig. 2-D).

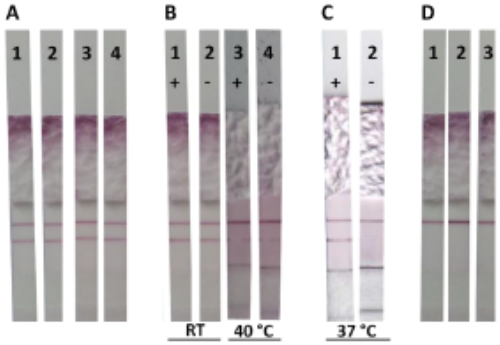


Fig. 2 Lateral flow strip results. + and – indicates whether AFB_1 was added or not. Findings with DIG_probe1 (A-1), DIG_probe2 (A-2), DIG_probe3 (A-3) and DIG_probe4 (A-4) without the addition of AFB_1. Section B shows the experimental results performed with DIG_probe5 at different temperatures (RT, 40 °C). C shows dipstick test results conducted under optimized conditions (37 °C, DIG_probe5). D shows the tested control samples (1-only aptamer; 2-only DIG_probe5; 3-only AFB_1).

The composition of the running buffer and the concentrations of the aptamer and the DIG labelled DNA probe were mainly derived from a previously reported assay[21] and slightly adjusted. We observed that a 5-fold excess (0.5 µM) of the complementary DNA with regard to the aptamer concentration of 0.1 µM leads to a signal decrease at the test line which is attributable to the saturation of gold nanoparticles with DIG labelled DNA (data not shown). On the contrary, a concentration of 0.2 µM showed a clear difference in signal intensities between AFB_1 positive (4 µg/ml) and AFB_1 negative (0 µg/ml) samples (Fig. 2-C). Therefore, 0.2 µM of DIG_probe5 was chosen as optimal concentration for further studies.

The AFB_1 concentration (4 µg/ml) used for this experiments was 60-times higher than the aptamer concentration to ensure a differentiation between positive and negative results. Experiments were repeated five times to evaluate the repeatability of the presumed structure switch induced by AFB_1.

Conclusions

The proposed approach for a switch based aptamer biosensor in combination with a nucleic acid lateral flow immunostrip offers considerable advantages compared to previously published detection formats. It allows a direct detection format for AFB_1 and an immediate visualisation *via* the developed test strip without the need of fluorescence read-out systems. Furthermore, it is a simple, user-friendly and cheap method that is as well attractive for on-site screening by untrained personnel. We like to emphasize that the described aptasensor is a model to demonstrate the potential of the structure switching ability of aptamers in terms of direct target detection. Moreover, a dipstick assay based on an aptamer enabling an AFB_1 direct detection format has not yet been reported.

However, for prototyping and future commercial application as a rapid screening method further optimization steps are required. Future work will involve the improvement of the sensitivity through reduction of target concentration. We also aim to lower the incubation time and temperature to speed-up the testing procedure as well as to provide a test feasible without a thermal device.

Acknowledgements

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Notes and references

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DOI: 10.1039/b000000x/

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