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BIO-ANALYTICAL DETECTION STRATEGIES FOR DEOXYNIVALENOL IN WHEAT DUST

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LIST OF ABBREVIATIONS

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15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
ACGIH	American Conference of Governmental Industrial Hygienists
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric pressure chemical ionization
ARAC	Aviation Rulemaking Advisory Committee
ARfD	Acute reference dose
ARL	Accept/reject limit
BSA	Bovine serum albumin
CBS	Carbonate-buffered saline
CC	Cyanuric chloride
CDI	N, N'-carbonyldiimidazole
cDNA	Complementary DNA
CDR	Complementarity determining region
CEN	European Committee for standardization
C_H1	Constant heavy 1
C_L	Constant light
CMO	Carboxymethyl oxime
CPI	Carboxypropyl imine
CR	Cross-reactivity
CTL	Cytotoxic lymphocyte
CTP	Cytidine triphosphate
CV	Coefficient of variation
dADP	Deoxyadenosine diphosphate
dATP	Deoxyadenosine triphosphate
DCC	N,N'-dicyclohexylcarbodiimide
dCDP	Deoxycytidine diphosphate
dCMP	Deoxycytidine monophosphate
dCTP	Deoxycytidine triphosphate
dGDP	Deoxyguanosine diphosphate
dGTP	Deoxyguanosine triphosphate

DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOM	Deepoxy deoxynivalenol
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3-β-glucopyranoside or deoxynivalenol-3-glucoside
dsb	Disulfide bond
dTDP	Deoxythymidine diphosphate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
E. coli	*Escherichia coli*
EC	European Commission
ECD	Electron capture detection
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ESI^+	Positive electrospray ionization
Fab	Fragment antigen binding
FCA	Freund's complete adjuvant
FHB	*Fusarium* head blight
FPLC	Fast protein liquid chromatography
FUS-X	Fusarenon-X
GAP	Good agricultural practices
GC	Gas chromatography
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
HA	Hemagglutinin
HAT	Hypoxanthine aminopterine thymidine
$HBS\text{-}N^+$	Buffer containing 0.01 M HEPES pH 7.4 and 0.15 M NaCl

HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HG	Hemiglutaryl
HILIC	Hydrophilic interaction liquid chromatography
HIS	Hexahistidine
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
IAC	Immunoaffinity column
IC_{50}	Half-maximal inhibitory concentration
ICP-AES	Inductively coupled plasma with atomic emission spectroscopy
IEC	Ion exchange chromatography
IMAC	immobilized metal-ion affinity chromatography
IMP	Intra-membrane particles
IP	Intraperitoneal
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
JECFA	Joint FAO/WHO Expert Committee on Food Additives
k_a	Association rate constant
k_d	Dissociation rate constant
K_D	Dissociation constant
K_{eq}	Equilibrium constant
KLH	Keyhole limpet haemocyanin
LB	Luria-Bertani
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass-to-charge ratio
MAPKs	Mitogen-activated protein kinases
MIP	Molecular imprinted polymer
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTBE	Methyl tertiary-butyl ether

MW	Molecular weight
MWCO	Molecular weight cut-off
n.a.	Not analysed
NHS	N-hydroxysuccinimide
Ni-NTA	Nickel-nitrilotriacetic acid
NIV	Nivalenol
OC	Operating characteristic
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing Tween 20
PCR	Polymerase chain reaction
PDA	Photo diode array
PDB	Protein data bank
PEG	Polyethylene glycol
QCM	Quartz-crystal microbalance
QuEChERS	Quick, easy, cheap, effective, rugged and safe
r	Correlation coefficient
RF	Replicative plasmid-like
ROS	Reactive oxygen species
RSD	Relative standard deviation
RSD_r	Intra-day precision or repeatability
RSD_R	Inter-day precision or within-laboratory reproducibility
RT-PCR	Reverse transcription polymerase chain reaction
RU	Response unit
s/n	Signal-to-noise ratio
SB	Super broth
SC	Subcutaneous
SCF	Scientific Committee for Food
scFv	Single-chain variable fragment
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel

SEC	Size-exclusion chromatography
Sec Ab-HRP	Secondary antibody labeled with horseradish peroxidase
SELEX	Systematic evaluation of ligands by exponential enrichment
SOC	Super optimal broth with catabolic repressor
SOE	Splicing by overlap extension
SPE	Solid-phase extraction
SPR	Surface plasmon resonance
SRM	Selected reaction monitoring
STC	Screening target concentration
StEP	Staggered extension process
TB	Terrific broth
TCA	Trichloroacetic acid
TDI	Tolerable daily intake
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran
TIS	Triisopropylsilane
TK	Thymidine kinase
TLC	Thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TMS	Trimethylsilyl
TOF	Time-of-flight
TRIS	Tris(hydroxymethyl) aminomethane
TRS	2-amino-2-hydroxymethyl-propane-1,3-diol
U	Uncertainty
UMP	Uridine monophosphate
UPLC	Ultra performance liquid chromatography
UTP	Uridine triphosphate
UV	Ultraviolet
V	Variance
V_H	Variable heavy
V_L	Variable light

INTRODUCTION AND OBJECTIVES

Effective food and feed control systems are essential to protect the health of consumers. Serious food and feed safety incidents during the last decades urged the European Commission and other countries across the world to review their food safety systems and to look for improved strategies to protect consumers against unsafe food.

One of the food safety issues includes the presence of trace levels of natural chemical contaminants including marine biotoxins and mycotoxins. Mycotoxins are secondary metabolites produced by a wide range of fungi on agricultural commodities in the field or during storage under specific environmental conditions. Despite extensive efforts during crop growth, harvesting or storage, it is not possible to completely avoid mycotoxin contamination. To understand the awareness of the Flemish population (including food consumers and producers) concerning mycotoxin contamination of food and feed, a questionnaire was developed and distributed online at the Agriflanders Fair for and by Flemish agriculture (2013).

Fusarium toxins called trichothecenes form the most commonly found mycotoxin group in Europe, among them deoxynivalenol occurs at highest rate. The mycotoxin deoxynivalenol, also known as DON or vomitoxin, is one of over 150 related compounds of trichothecenes and is of particular relevance to the wheat crop.

Analytical methods for mycotoxin detection and quantitation in grain cereals consists of a sampling, sample preparation and chromatographic or immunochemical detection step. The sampling step is considered as the most significant source of uncertainty of the test result. Mycotoxins are heterogeneously distributed in a cereal bulk lot, leading to the difficulty of selecting a representative test sample. To overcome the problem of heterogeneous distribution of mycotoxins in food and feed, the European MycoHunt project (FP7/2007-2013, grant agreement n°243633) suggested the testing of grain dust which is created when tons of bulk grains are moved and transported through closed systems *e.g.* (un)loading of a truck. Grain dust can be considered as a representative sample as it is continuously generated and sampled through the entire grain movement process.

The sampling of dust is not a novel technique. Tangni and Pussemier (2006) analysed settled grain dust for ochratoxin A and citrinin content and assessed the impact of the grain dust for its direct contribution as contaminant and inoculum in stored wheat. Krysinska-Traczyk *et al.* (2001) described and compared the sampling of grain and settled grain dust for the determination of *Fusaria* and fusariotoxin levels. As notable quantities of *Fusaria* and fusariotoxins were found in both grain and grain dust, the authors expressed their concern

regarding the risk of mycotoxicoses to agricultural workers exposed to grain dust. The inhalation of dust contaminated with mould spores and/or mycotoxins have been reported as the cause of acute and chronic respiratory diseases and certain infection diseases and cancer due to long-term inhalation [Halstensen *et al.*, 2004].

The purpose of the MycoHunt project was the combination of the grain (*i.e.* wheat) dust sampling technique with a fast and DON specific detection technique. For the detection, an amperometric immunoassay technology was selected. Immunoassays are based on antigen-antibody reaction and therefore anti-DON monoclonal antibodies were needed. All of the commercially available anti-DON antibodies show high cross-reactivity (> 100%) against one of the acetylated DON derivatives, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). Current legislation does not include maximum or guidance levels for both 3-ADON and 15-ADON, even though they are of toxicological importance. To comply with the legislation, specific anti-DON monoclonal antibodies were aimed for. During this research, an immunogen synthesis strategy was developed using different procedures. Also, different linkers with varying length and chemical structure between DON and the carrier protein were synthesized to be able to lower cross-reactivity of the monoclonal antibodies. Balb/C female mice (ethical approval according to ECD 10/08) were subjected to an injection with the different DON immunogens and monoclonal antibodies were developed using the hybridoma technology.

To even lower the cross-reactivity of the derived monoclonal antibodies, recombinant antibody technology was used. Using the gene sequence of the derived single-chain variable fragment (scFv) antibodies and the target DON chemical structure, modeling and docking studies were performed. These studies were able to visualize the amino acid importance within the complementary determining region (CDR) responsible for the specific target interaction.

As at the moment, no regulatory limits exist for mycotoxins in grain dust, a correlation was needed between the DON level in wheat dust versus the DON level in the corresponding wheat. First of all, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of DON in wheat dust. Using this method, the DON content of different wheat dust samples was measured and together with the DON levels of the corresponding wheat samples determined by the LC-MS/MS method described by De Boevre *et al.* (2012b), a correlation curve was prepared. As in the MycoHunt system, an immunoassay technology was developed, the suitability of the generated anti-DON

monoclonal antibodies was tested. Therefore an aqueous sample extraction and enzyme-linked immunosorbent assay (ELISA) method was developed and validated for the wheat and wheat dust matrix. The obtained ELISA results were compared to the LC-MS/MS results for DON determination in wheat and wheat dust and a general conclusion was made.

To summarize, the PhD research focused on the following objectives:

- To develop an alternative sampling method for wheat grain (Chapter 2);
- To synthesize different immunogens with varying length and chemical structure between DON and the carrier protein (Chapter 3);
- To develop monoclonal anti-DON antibodies using hybridoma technology (Chapter 3);
- To improve the specificity of the monoclonal anti-DON antibodies by using recombinant technology (Chapter 5);
- To make a correlation between the DON content in wheat dust versus wheat grain by the use of a developed and validated LC-MS/MS (Chapter 2) and ELISA method (Chapter 4).

CHAPTER 1

GENERAL INTRODUCTION AND PRINCIPLES

Adapted from

The Awareness about Mycotoxin Contamination of Food and Feed: a Survey in the Flemish Population

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CHAPTER 1: GENERAL INTRODUCTION AND PRINCIPLES

1.1 MYCOTOXINS: AN OVERVIEW

Mycotoxins are naturally occurring secondary metabolites produced by a wide range of fungi including *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp.. All of them are known to be invisible or cannot be detected by smell or taste [Binder, 2007]. They are produced by the specific fungus under optimal environmental conditions as high temperature and humidity. Furthermore, mycotoxin production occurs at different stages. *Fusarium* mycotoxins for example are produced on the field whereas *Aspergillus* and *Penicillium* mycotoxins are formed during storage [Savi *et al.*, 2013].

An obvious structural diversity can be seen over the five hundred different mycotoxins which have been discovered so far. This results in different chemical and physicochemical properties and as a consequence different biological effects. The toxic effects induced by mycotoxins depend on several factors, namely toxin-related (type of mycotoxin consumed, level of intake and duration of exposure, mechanism of action and metabolism) and animal-related factors (animal species, sex, age, breed, general health and immune status) [Bennett and Klich, 2003; Koppen *et al.*, 2010; Krska *et al.*, 2001; Binder, 2007]. In Table 1.1 an overview is given of different food and feed commodities together with the possible mycotoxin contaminants, the affected animal species and the most commonly found clinical effects on humans and animals. Since many plant and animal products are commonly used for the production of fish feed, water living animals also discover harm related to mycotoxin exposure. A decrease in growth, organ lesions and immunosuppression are symptoms commonly found in fishes exposed to subchronic levels of mycotoxins, whereas fish liver tumours are induced by chronic aflatoxin exposure. The co-occurrence of diverse mycotoxins can lead to antagonistic, additive or synergistic effects [Binder, 2007; Speijers and Speijers, 2004; Caruso *et al.*, 2013].

Mycotoxicoses, caused by the direct contamination of agricultural commodities and by a "carry-over" of mycotoxins and their metabolites into animal tissue after feeding with contaminated cereals, can be induced by either mycotoxin ingestion, inhalation or skin-contact [Stepien *et al.*, 2007; Koppen *et al.*, 2010; Krska *et al.*, 2001]. Through occurrence of outbreaks that affected animals and humans in the past centuries, it was possible to discover the presence of mycotoxins. From the 8^{th} to 16^{th} century there were epidemics of "Saint Anthony's fire", which were the result of eating rye contaminated with ergot alkaloids

produced by the mould *Claviceps purpurea*. The symptoms of Saint Anthony's fire include gangrene, burning sensations and hallucinations. Around 150 years ago, fumonisins were considered as a cause of disease and death of horses by consumption of maize which was contaminated with *Fusarium* species. Aflatoxins were discovered in the United Kingdom in 1960 just prior to Christmas. Hundred thousands of turkeys and other birds were found death after the consumption of contaminated groundnuts. This outbreak was called "Turkey X disease" [Bennett and Klich, 2003; Koppen *et al.*, 2010; Krska *et al.*, 2001]. In the mid 1980s conjugated or masked mycotoxins were discovered. Due to metabolism, plants are capable of transforming mycotoxins into conjugated forms such as zearalenone and deoxynivalenol glucosides. These masked forms are not detected during routine analysis, but are possibly hydrolysed during digestion which may contribute to cases of mycotoxicoses [Berthiller *et al.*, 2005; Binder, 2007].

Table 1.1 Summary of different food and feed commodities together with the possible mycotoxin contaminants, the most affected animals and the corresponding clinical effects

Mycotoxin	Food and feed commodities	Animals affected	Clinical effects
Aflatoxins	Maize, peanuts, tree nuts, dairy products	Swine, dogs, cats, cattle, sheep, young birds, humans	Liver damage, intestinal bleeding, carcinogenic and teratogenic effects
Ergot alkaloids	Rye, sorghum, pasture grasses	Cattle, sheep, humans	Nervous or gangrenous syndromes
Fumonisins	Maize, silage	Horses, swine, humans	Pulmonary oedema, leukoencephalomalacia, oesophageal cancer, neural tube defects, liver damage
Ochratoxins	Cereal grains, coffee, grapes	Swine, humans	Kidney and liver damage, cancer
Trichothecenes	Wheat, barley, oats, maize	Swine, dairy cattle, poultry, horses, humans	Immunological effects, inhibition protein synthesis, DNA damage, haematological changes, feed refusal, diarrhoea, vomiting, skin disorders
Zearalenone	Maize, hay	Swine, dairy cattle	Estrogenic effects, abortion, malformation of testicles and ovaries

Certain food processes enhance the cultivation of mycotoxin-producing fungi [Hazel and Patel, 2004]. *Fusarium* moulds are capable of growing and producing mycotoxins during the beer production process. Nowadays three reduction methods for mould growth during malting can be considered, namely irradiation, chemical and biological methods. However, their effect on malt quality and mycotoxin production of the surviving mould still needs to be evaluated in detail [Wolf-Hall and Schwarz, 2002]. During the lengthy process of winemaking, the skin of the grape fruit and juice are in close contact for more than 10 days. This contact gives the mycotoxin-producing fungi the opportunity to grow and secrete mycotoxins into the fermentation source. The coincidence of mycotoxins and mouldy fruits has been proved, however introducing mycotoxins into the winemaking process via intact fruits has not been previously reported [Jiang *et al.*, 2013; Serra *et al.*, 2005].

Besides fruit, also vegetables can be attacked by mycotoxigenic fungi. If consumers process a rotten fruit or vegetable, this form can still be a significant source of mycotoxins as most fungi survive food processing [Barkai-Golan and Paster, 2008; Monbaliu *et al.*, 2009a]. Due to the heat resistance of these fungi, they can even appear in coffee or tea. Their appearance in coffee can be the consequence of their presence during harvesting, preparation, transport or storage of coffee cherries and beans. Also herb tea or medicinal plants contain a number of micro-organisms, mainly originating from the flora of the plant [Halt, 1998; Batista *et al.*, 2003]. Dairy products can be contaminated with mycotoxins as well, due to the presence of fungal growth for fermentation or unintentional fungal growth. Although moulds are intentionally grown on cheese for the production of French Roquefort and Camembert, these fungi are not able to produce mycotoxins, but the presence of toxigenic strains due to environmental contamination could result in mycotoxin appearance [Prandini *et al.*, 2009]. Meat and fish can contain traces of fungi in a post-slaughter condition due to the contact with contaminated air, water, processing facilities or by addition of food ingredients [Hashem and Mohamed, 2011; Cook, 1995].

At the moment, the most common known source of mycotoxigenic fungi are cereals. Poor post-harvest management of grain cereals entering storage facilities can lead to rapid deterioration in grain quality, severely decreasing germinability and nutritional value of stored grains. The presence of fungal activity can contribute to previously mentioned effects and can result in contamination with mycotoxins [Magan *et al.*, 2003]. As these mycotoxins are very stable during food processing, they can be found in cereal end products such as bread or other bakery products [Molinie *et al.*, 2005; De Boevre *et al.*, 2012a].

The control of mycotoxin contamination starts with the implementation of good agricultural practices (GAPs) to prevent infection with the corresponding moulds. Preventive strategies should be implemented from pre- through post-harvest. Measures at the pre-harvest level include good planting and growing conditions *e.g.* soil treatment, crop rotation, irrigation. During the process of harvesting, functional harvest equipment and dry collection and transportation equipment would lower infection probability. Postharvest measures include the drying of the cereals and appropriate storage conditions [Magan *et al.*, 2003; Murphy *et al.*, 2006].

1.2 THE AWARENESS ABOUT MYCOTOXIN CONTAMINATION OF FOOD AND FEED: A SURVEY IN THE FLEMISH POPULATION

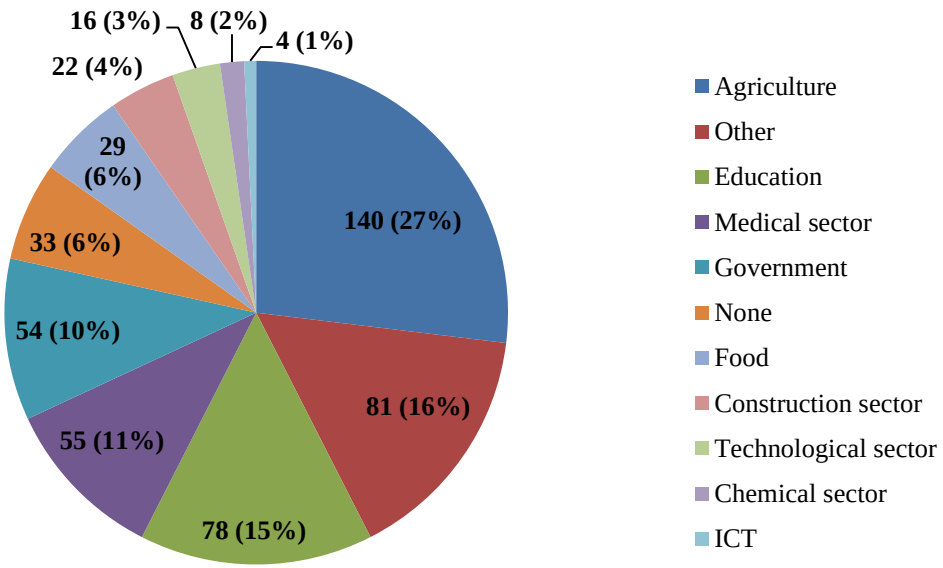
Knowledge of food and feed safety among consumers has various dimensions. A quantitative survey (*n* = 520) was set up to determine the awareness of the risks of mycotoxin contaminated food and feed throughout the Flemish population. In subchapter 1.2.2, all answers and considerations are presented and discussed.

1.2.1 Materials and methods

1.2.1.1 Objective and participants

To understand the awareness of the Flemish population concerning mycotoxin contamination of food and feed, a questionnaire was developed by the Centre of Excellence Food2know within Ghent University in cooperation with the Laboratory of Food Analysis. The questionnaire was distributed online at the Agriflanders Fair for and by Flemish agriculture from the 10th – 13th of January 2013. Of the in total 520 participants, 55% were women whereas 45% were men with the year of birth between 1924 and 1998. Young persons as well as elderly people participated in this survey. Most of the participants were married or lived together (67%) and 59% had children. Furthermore, the people included in the survey had different education levels, different jobs and were working in various sectors. In figure 1.1 an overview is given of the different working sectors of the participants and their profession.

A



B

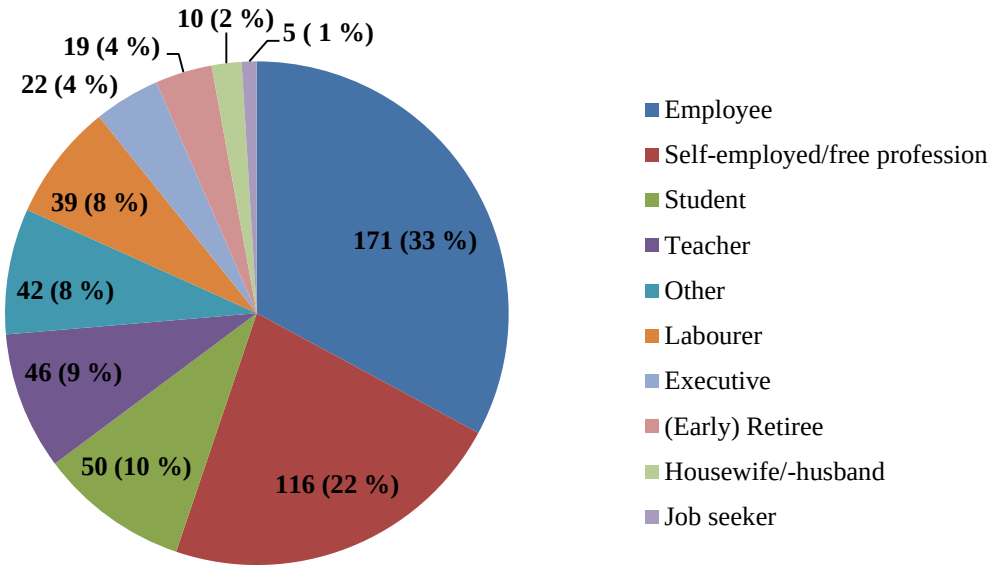


Figure 1.1 An overview of the different working sectors of the participants (A) and their profession (B)

1.2.1.2 Questionnaire

The complete questionnaire which was generated by using the online Qualtrics Survey Software, is displayed in Appendix A.

Overall, seven questions concerning food and feed safety in general and more specific concerning moulds and mycotoxins were posed. The first two questions dealt with the possibilities in which case a certain food or feed product can become unsuitable or dangerous to eat. A third question investigated the knowledge of the participants regarding mycotoxins. Furthermore, several food products were listed and the occurrence of mould growth was questioned. Consequently, participants were asked which actions they would take if a certain product is mouldy. Finally, two questions were stated to assess who is responsible according to the Flemish population to guard the safety of food and feed with respect to moulds and mycotoxins.

In addition to the above mentioned questions, some more general ones were addressed as well, including gender, year of birth, highest obtained degree, profession, working sector, marital status and number of children.

1.2.1.3 Statistics

All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 22.

1.2.2 Results and discussion

To start the questionnaire, people were asked in the first two questions in which cases food (question 1) or feed (question 2) cannot be eaten anymore or is even dangerous to eat. It was noticed that 444 of the 520 participants (85.4%) answered that food cannot be eaten when it is infected by moulds such as *Aspergillus* species. Therefore, it can be concluded that most people were aware of the presence and negative effects of the mould species. In addition, 110 of the 140 people (78.6%) who were working in the agricultural field stated that it is not trustworthy to eat mouldy food. Consequently, 21.4% of these people were not aware of the consequences of mould growth and correlating mycotoxin production. Regarding feed as a source of mould growth, a total of 411 of the 520 people (79.0%) answered that mouldy feed can be dangerous to animals and therefore should not be consumed anymore. In accordance with the answers concerning food, 111 of the 140 people (79.3%) working in the agricultural field did not trust mouldy feed. Compared to the results of the food matrix by the use of a Chi-squared test, in total a significantly higher number of people (χ^2 = 7.487, degree of freedom = 2, p-value = 0.025) would still feed the animals with a meal containing moulds.

Comparing the above described results with those concerning bacteria by the use of a Chi-squared test, shows that significantly (χ^2 = 62.600, degree of freedom = 2, p-value < 0.001) more people were aware of the potential negative effects of bacteria compared to moulds. In total 511 of 520 participants (98.3%) said it is possible for bacteria to cause these negative effects on food. Moreover, 134 of 140 people (95.7%) working in the agricultural field recognized these potential effects of bacteria. Furthermore, the presence and effects of bacteria in feed were found possible by 458 of 520 persons (88.1%) and 129 of 140 persons working in the agricultural field (92.1%). In conclusion one can state that the Flemish population is more aware of the negative effects of bacteria in food and feed then those concerning moulds.

In question 3, people were asked about the source of mycotoxin production. An overview of the answers is illustrated in Figure 1.2. A significantly (χ^2 = 581.191, degree of freedom = 6, p-value < 0.001) larger number of people (59.6%) picked out moulds as mycotoxin source compared to toxic plants (12.9%), bacteria (15.0%) and viruses (8.5%). But still in each category, the number of people who did not know the source of mycotoxin production was high, namely 38.3%. Between 29.3 and 39.3% of the people working in the agricultural sector did not know if toxic plants, bacteria, moulds or viruses are producers of mycotoxins, which is really a matter of concern.

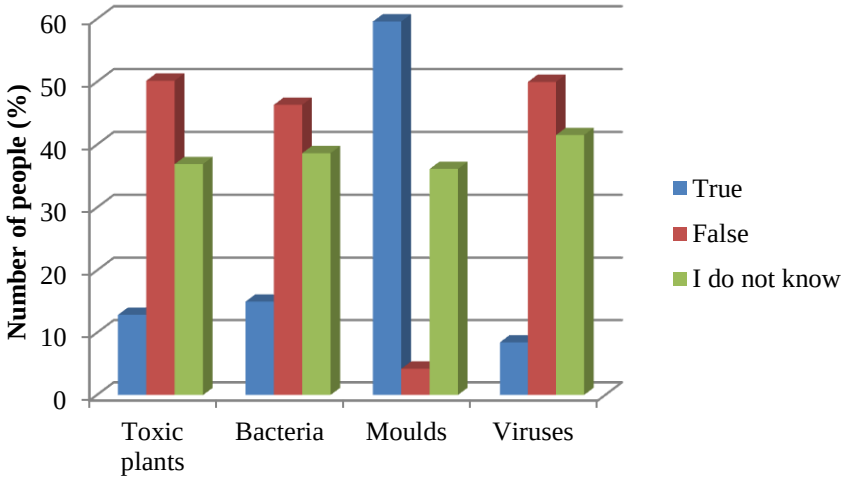


Figure 1.2 Percentage of people who found toxic plants, bacteria, moulds and viruses a source of mycotoxin production

In the second part of question 3, people were asked if mycotoxins could be harmful for humans and/or animals. The largest group of people, namely 376 of the 520 people (72.3%) and 369 of 520 people (71.0%), answered that mycotoxins could lead to respectively human and animal toxicity. But still 26.5% of the participants did not have an idea about toxicity of mycotoxins and 22% of these "not knowing" people were working in the agricultural field. People were also asked about the toxicity towards plants. It has been noticed by different researchers that the infection of plants can lead to plant cell death due to oxidative stress by the induction of increased reactive oxygen species (ROS) levels. Transcriptomic studies have identified genes encoding ROS scavenging enzymes for example peroxidases, that were up-regulated in plants in response to certain mycotoxins *e.g.* trichothecenes [Foroud *et al.*, 2012; Arunachalam and Doohan, 2013]. Based on the answers of the population, 73.3% of the people and 28.3% of persons working in the agricultural field did not have an idea about possible toxic plant effects or thought there are no toxic effects on plants. Also, the answers on possible toxicity towards water living animals are a matter of concern. The majority of the participants (57.5%) were not aware that mycotoxins are toxic towards fish. Hence, it is advisable to raise the knowledge of the Flemish population with regards to the harmful effects of mycotoxins.

As previously mentioned, mycotoxicoses can be induced by ingestion, inhalation or skin contact of mycotoxin contaminated food or feed. People were asked during this questionnaire about the possibility of mycotoxin exposure by inhalation. Of a total of 520 people, 354

persons (68.1%) did not know the answer or answered that this kind of exposure does not lead to mycotoxin toxicity. In all working fields, even in the agricultural sector, food sector and in education, the majority of the people were not aware of this exposure pathway.

The objective of question 4 was to check for consumers awareness of mould growth in food and feed. Figure 1.3 depicts the results of this question. The possible presence of moulds in fresh vegetables or fruits (85.2%), in dairy products (94%) and bread or other bakery products (93.8%) and animal feed (79.6%) was well known. The questionnaire showed that 48.8% of the people were not aware of the possible presence of moulds in beer or wine. First of all, during the malting process in beer production and during the fermentation process in winemaking, mould growth and mycotoxin production occur and even despite the existence of eradication processes, it is difficult to eliminate the mould growth [Jiang *et al.*, 2013; Serra *et al.*, 2005; Wolf-Hall and Schwarz, 2002]. Other sources of mould growth the population showed not being informed of, are coffee and tea (56.5%). The other food products mentioned in question 4 and Figure 1.3 are of less importance as they contain no or only traces of mould growth under normal storage conditions and will therefore not be discussed [Sulyok *et al.*, 2010].

In the following question, people were asked how to deal with mouldy products. The possible answers were: removal of the mouldy part so the remainder can still be consumed; moulds are not harmful so you can just eat it; after washing the product it can be consumed; after warming the product it can be consumed; you need to throw away the product. The results are illustrated in Figure 1.4. It has been shown that the removal of the fungal mycelium in fruits or vegetables, does not consequently lead to the elimination or reduction of the mycotoxin contamination. Careful selection, washing and sorting of fruits is the most important action in reducing the mycotoxin content of food products, but this still does not lead to a complete removal [Barkai-Golan and Paster, 2011; Drusch and Ragab, 2003]. As seen in Figure 1.4, 57.8% of the people thought it is not good to remove only the mouldy part of the food product, while throwing away the whole product was considered by 58.9% of the Flemish population. The majority of the people (86.6%) were aware of the harmful and toxic effects of moulds, which is an important consideration. The fact that mycotoxins can not be totally eliminated by washing or warming up the mouldy food product was known to respectively, 83.7% and 88.5% of the population. In general, it can be concluded that still more information should be distributed on how to handle mouldy food and feed products and which measures could be taken to eliminate or reduce mycotoxin exposure.

The last two questions focused on who could be considered responsible for food and feed safety related to mycotoxins. It is quite obvious that producers of food and feed products are primarily responsible. If they observe mould growth during harvest, measures should be taken, for example analysis of mycotoxins, and if this leads to a positive result, the total lot should be eliminated. As some unprocessed food and feed products escape this first control point or mycotoxin production occurs during the processing of food, grocers and even consumers should take extra precautions. Out of the last question, it became clear that the majority of the people wanted to have food or feed products tested for mould presence and mycotoxin contamination (84.8% and 79.6%, respectively). A total of 63.5% of the people knew that it is not possible to see mycotoxin contamination by eye and only 42.7% of the population thought it is possible to notice mould growth. For the final product, which is tested on mycotoxin presence, 51.7% of the participants did not mind to pay more for it.

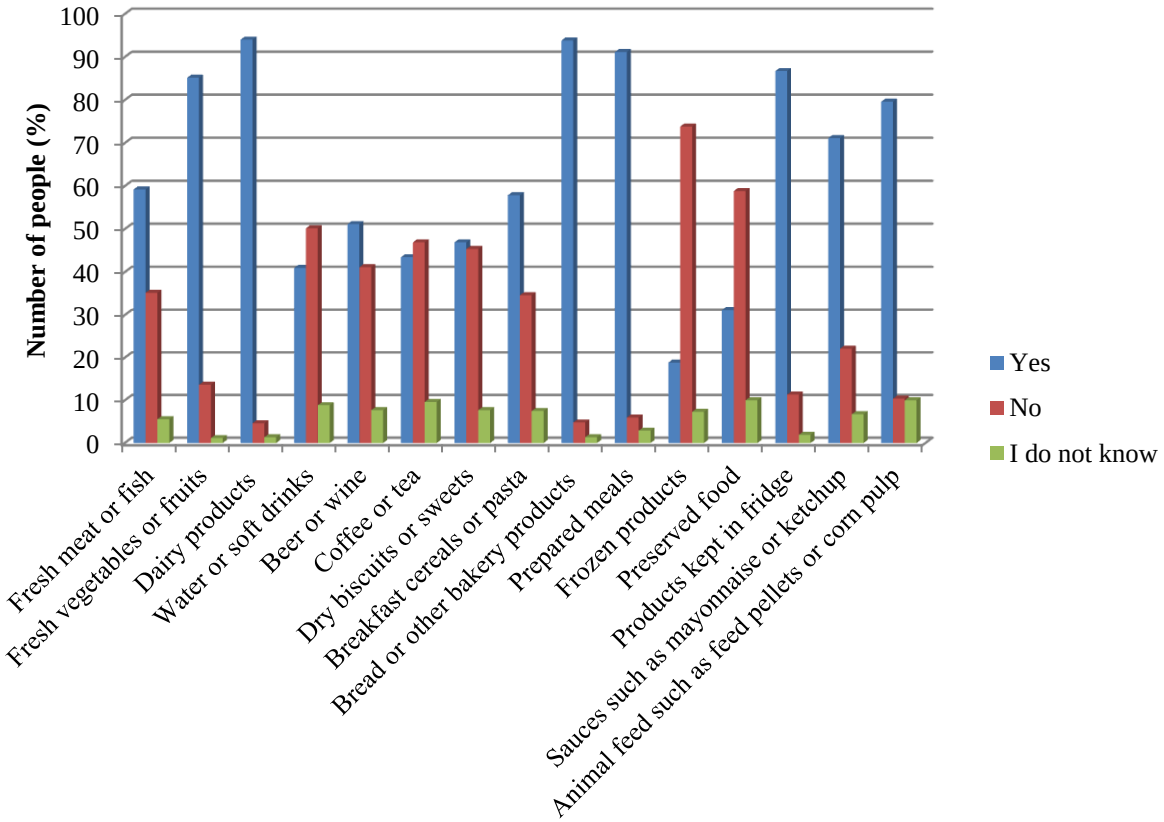


Figure 1.3 Consumers awareness of mould growth in food and feed

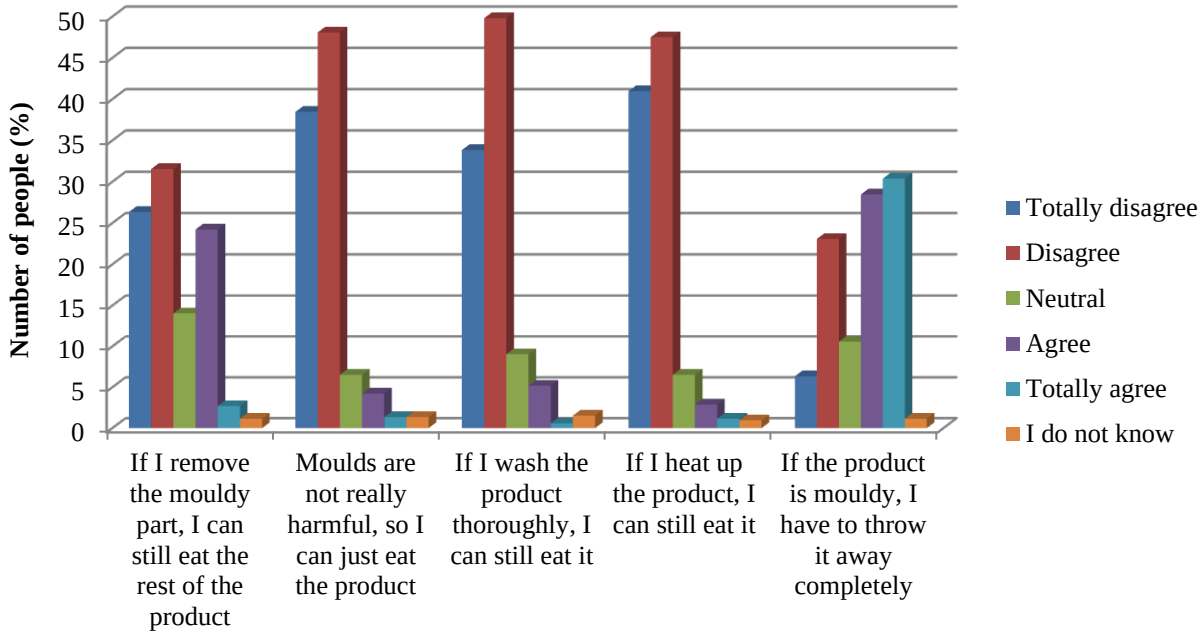


Figure 1.4 Frequency distribution of how people deal with mouldy products

1.2.3 Conclusion

The questionnaire which was distributed at the Agriflanders Fair for and by Flemish agriculture in January 2013, indicated some points of interest that should be considered in the future. In general, people were aware of the negative effects of moulds to cause unsuitable or harmful food or feed. However, these potential effects were better known for bacteria than for moulds. Although 59.4% of all participants stated that moulds are mycotoxin producers, still a large part, including people from the agricultural field, did not know by which organisms mycotoxins are produced. When it comes to addressing the toxic effects of mycotoxins, fewer persons were aware of these effects on plants and water living animals than for humans and other animals. In all cases, the toxic effects were not sufficiently recognized. Furthermore, the exposure pathways of mycotoxins, more specifically through inhalation, were not generally known. The participants were also not appropriately aware of the possible mould growth and presence of mycotoxins in beer, wine, coffee, tea and cereal derived products.

In addition, it can be concluded that consumers do not always take the correct measures when they come into contact with a mouldy food. Surprisingly, although 82.2% (average) of the participants preferred to have their food or feed tested for the presence of moulds and mycotoxins, only 51.7% was willing to pay more for it.

In summary, one can state that more awareness concerning mould growth, production of mycotoxins by moulds, their toxic effects on organisms, exposure pathways and how to deal with mouldy food is of high value and importance for the Flemish population. More information should be provided to increase the awareness of mycotoxin contamination of food and feed.

1.3 DEOXYNIVALENOL AND MASKED FORMS

1.3.1 *Fusarium* head blight

Trichothecenes are the most commonly found mycotoxin group in Europe produced by *Fusarium* species. Among the 150 related trichothecenes, deoxynivalenol (DON) also called vomitoxin, occurs at highest rate. Especially wheat, triticale and maize are vulnerable for *Fusarium* infection and are, therefore, frequently higher contaminated with DON compared to other cereals [Maragos *et al.*, 2006; Döll and Dänicke, 2011; Lancova *et al.*, 2008a; Krska *et al.*, 2001; Beyer *et al.*, 2006; Murphy *et al.*, 2006].

The infection of small grain cereals (*e.g.* wheat, barley, oats) by *Fusarium* species during anthesis causes *Fusarium* head blight (FHB). Until now, 17 species of *Fusarium* have been described to be potentially associated with FHB symptoms [Leonard and Bushnell, 2003]. In Europe, *F. graminearum* and *F. culmorum* are known as the most important FHB pathogens of wheat because of their high pathogenic character in comparison with other species and their high mycotoxin production level, in particular DON. As mentioned by Hooker *et al.* (2002), weather conditions during flowering are the main factors contributing to the variation in DON content. Rainfall and warm temperatures during wheat anthesis promote the FHB incidence [Edwards, 2004; Beyer *et al.*, 2006; Blandino *et al.*, 2012]. In addition, weather conditions during the vegetative growth of wheat during autumn and winter influence the FHB infection pressure [Landschoot *et al.*, 2012]. Besides weather conditions, good agricultural practices contribute to minimize the FHB problem.

FHB infection can be controlled by a combination of the following methods: crop rotation, tillage, choice of the wheat variety, and use of fungicides. Besides wheat, *Fusarium* species infect maize and after harvest crop debris provides an excellent source for the survival of pathogens. Therefore, including maize in crop rotation systems will increase the risk of FHB epidemics and DON contamination. Another method to control FHB infection is tillage (*i.e.* ploughing). No tillage and minimal tillage leave all or part of the crop residue on the soil surface after harvest and therefore favour FHB infection due to the capacity of *Fusarium spp.* to survive saprophytically on these crop residues. Ploughing decreases the inoculum level since *Fusarium spp.* are aerobic, and as such leads to a reduction in DON content. A third method which can be considered in order to reduce the FHB incidence is the wheat variety. At the moment, no complete FHB-resistant crop is known, even though the susceptibility of commercial wheat varieties to FHB pathogens and their DON content differs. The lack of an absolute type of resistance originates from the multigenic nature of FHB resistance encoded

by quantitative trait loci which are scattered throughout the genome. Consequently, cultivated wheat varieties display a continuous variation in resistance depending on the country of origin due to differences in the genetic pool within each country's breeding program and the different environmental and agronomic conditions in which crops are cultivated [Edwards, 2004; Miller *et al.*, 1998; Beyer *et al.*, 2006; Lori *et al.*, 2009; De Boevre *et al.*, 2012a]. The improvement of host genetic resistance is therefore seen as essential to achieve meaningful control although advances in this research are hampered by the multigenic nature of the resistance [Gilbert and Haber, 2013]. Besides crop rotation and variety choice, fungicides remain an indispensable measure to tackle the FHB problem. Research trials conducted in the nineties showed the effectiveness of the triazoles prothioconazole, metconazole and tebuconazole plus prothioconazole in suppressing FHB disease and controlling mycotoxins. The best control of the disease can be achieved with fungicide applications at wheat anthesis and the efficacy declines rapidly with time after and before the plant growth stage [Beyer *et al.*, 2006; Gilbert and Haber, 2013]. Although the impact of fungicides on fungal biomass is a straightforward reduction, the implication of fungicide application with regard to toxin production is a story that has not been fully elucidated and several research groups report on increased toxin production upon fungicide application or a selection towards specific chemotypes of the fungus [Audenaert *et al.*, 2010; Zhang *et al.*, 2013].

1.3.2 Chemistry

As illustrated in Figure 1.5A, trichothecene mycotoxins consist of a tetracyclic sesquiterpenoid structure, an epoxide ring structure at C_{12}-C_{13} and a double bond at C_9-C_{10}. Therefore they are called 12,13-epoxytrichothec-9-enes. Trichothecene mycotoxins are divided into 4 groups (A, B, C and D) based on the extra functional groups. Due to the presence of a carbonyl function at C_8, which is of importance for toxicological and immunological activity, DON (Figure 1.5B/C) belongs to group B trichothecenes. Next to the epoxy and the carbonyl function, the DON molecule consists of 3 extra reactive sites, namely the hydroxyl groups on C_3, C_7 and C_{15} of which the C_3 and C_{15} hydroxyl groups are considered as most chemical reactive [Li *et al.*, 2012; Maragos and McCormick, 2000].

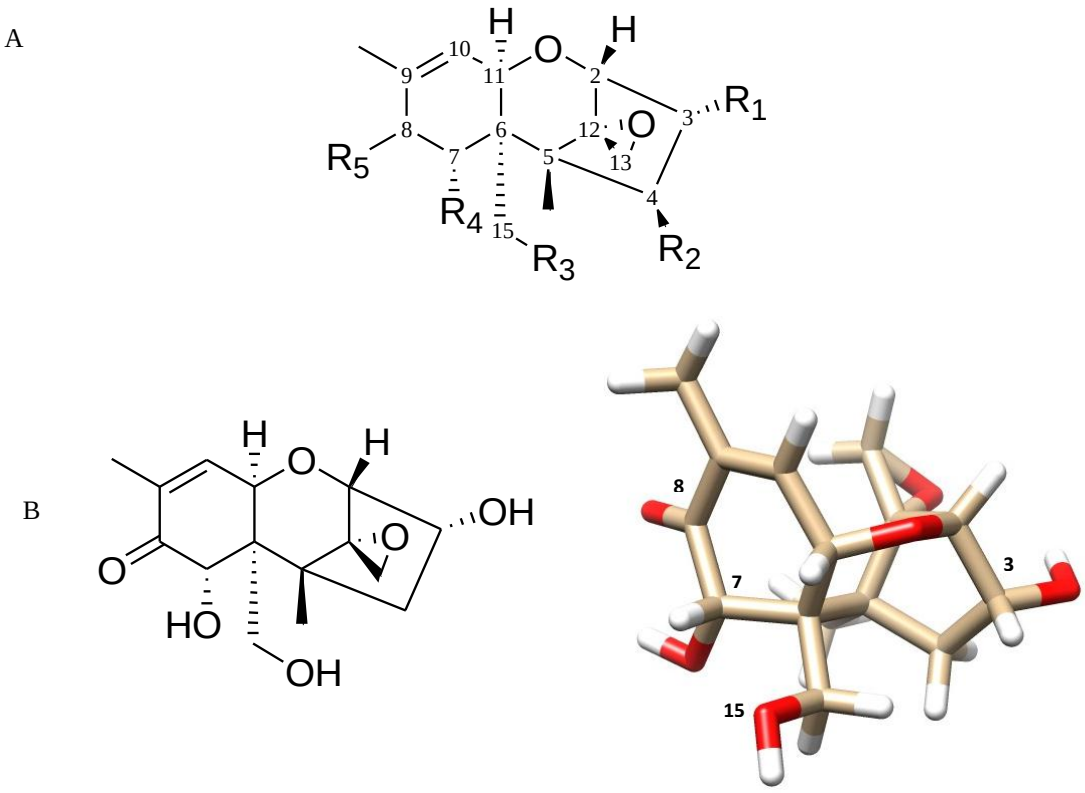


Figure 1.5 General structure of trichothecene mycotoxins (A) and stereochemical configuration (B) versus three-dimensional structure (C) of DON

1.3.3 Toxicity

Acute exposure to DON can lead to reduced food or feed consumption, abdominal distress, increased salivation, malaise, diarrhoea, leucopoenia, haemorrhage, shock and death in extremely high DON doses. The most common effects of prolonged dietary exposure to DON are decreased weight gain, anorexia, altered nutritional efficiency, immune function (enhancement and suppression) and reproductive effects (reduced litter size) [Pestka, 2007; Maragos and McCormick, 2000; Pestka and Smolinski, 2005].

The affection of the immune system with either immune suppressive or immune stimulative effects is depending on dose, exposure frequency and timing. A low DON dose leads to the transcriptional and post-transcriptional upregulation of the expression of cytokines, chemokines and inflammatory genes with concurrent immune stimulation. High DON dose exposure promotes leukocyte apoptosis and therefore can lead to immune suppression [Dänicke *et al.*, 2006; Krska *et al.*, 2001; Rocha *et al.*, 2005; Murphy *et al.*, 2006; Dietrich *et al.*, 2012; De Boevre *et al.*, 2012a; De Boevre *et al.*, 2012b; Zhou *et al.*, 2003]. The mechanism behind the immune system stimulation and suppression is the “ribotoxic stress

response". Due to the binding of DON to the eukaryotic 60s ribosomal subunit, mitogen-activated protein kinases (MAPKs) are phosphorylated and subsequently induced. This leads to an increase in transcription, messenger ribonucleic acid (mRNA) stability and cell apoptosis. Leukocytes, macrophages, B- and T-cells are especially sensitive to trichothecenes, like DON. In Figure 1.6, an overview is given of the trichothecene-induced toxicity [Zhou *et al.*, 2003; Casteel *et al.*, 2010; Pinton *et al.*, 2012; Pestka *et al.*, 2004].

The binding of DON to the eukaryotic 60s ribosomal subunit leads as well to interference of the activity of peptidyl transferase and subsequent inhibition of the initiation, elongation and termination of protein synthesis. DON is also known to induce DNA damage and cell death due to oxidative stress and an increase in ROS levels [Arunachalam and Doohan, 2013].

Equal DON toxicity was found within all evaluated animal species, although sensitivity was different due to differences in metabolism, absorption, distribution and elimination. The susceptibility of animals to DON follows this order: pigs > mice > rats > poultry ≈ ruminants [Pestka, 2007].

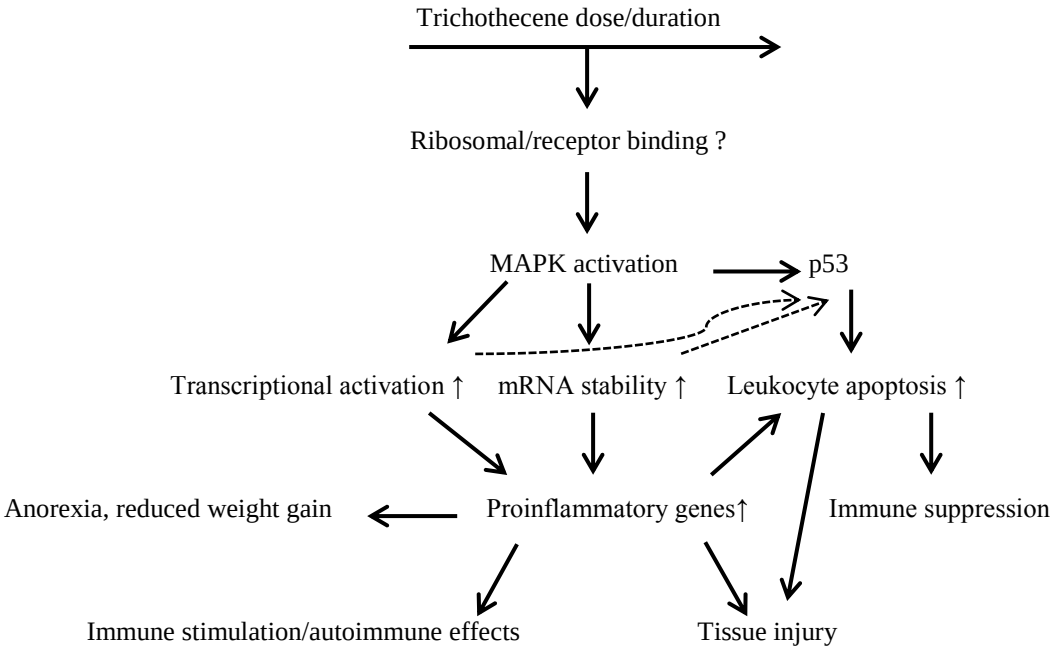


Figure 1.6 Mechanisms involved in trichothecene-induced toxicity [Pestka *et al.*, 2004]

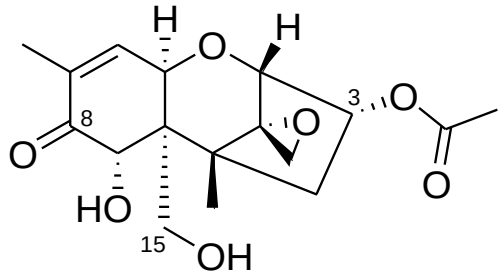
1.3.4 Masked forms

Masked mycotoxins are mycotoxin forms which are undetectable by conventional analytical techniques due to their structure change and therefore lead to an underestimation of the mycotoxin content. Two types of masked mycotoxins can be distinguished, namely the

extractable, conjugated and non-extractable, bound varieties. The extractable conjugated mycotoxins can be detected by appropriate analytical techniques when their structure is known and analytical standards are available. Bound mycotoxins first need to be liberated from the matrix by chemical or enzymatic treatment prior to chemical analysis. The conjugation of mycotoxins occurs during metabolic processes in fungi, living plants and mammals or during food processing [Berthiller *et al.*, 2009a; Berthiller *et al.*, 2013]. Further explanation will be given in relation to the masked DON forms.

DON derivatives produced during fungal metabolization processes are known as 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). These DON derivatives are originating from 3,15-diacetyl deoxynivalenol and have been reported to occur together with DON in many *Fusarium*-contaminated cereals, but at lower levels [Pestka, 2010]. The structures of these DON derivatives are presented in Figure 1.7. In 2010 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered the toxicity of the acetylated derivatives equal to that of DON, but a recent study suggested the higher toxicity of 15-ADON should be taken into account [Pinton *et al.*, 2012; Berthiller *et al.*, 2009b; Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2011].

A



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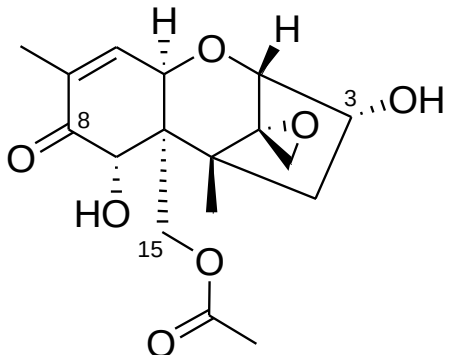

Figure 1.7 Chemical structure of 3-ADON (A) and 15-ADON (B)

Plants can protect themselves against mycotoxins, such as DON, by converting them to more polar metabolites and therefore inactivate and detoxify the mycotoxins. Miller *et al.* (1983) was among the first speculating increased DON levels due to an enzymatic conversion of an unknown precursor (conjugate). Later it was shown that deoxynivalenol-3-β-glucopyranoside (DON-3G) (Figure 1.8) could be formed from DON in maize cell suspension cultures and by *Arabidopsis thaliana* due to the presence of a gene encoding an UDP glucosyltransferase. The DON-3G formation is part of phase II reactions occurring in plants. During these reactions, hydrophilic components such as DON are conjugated to glucose or glutathione through the

involvement of the specific enzymes glucosyl- and glutathion-S-transferase, respectively. Phase II conjugates can be incorporated into the insoluble fraction of the plant cell wall or converted into a soluble form and transferred into the plant cell vacuoles (phase III metabolism) [Berthiller *et al.*, 2011; Berthiller *et al.*, 2009a; Poppenberger *et al.*, 2003].

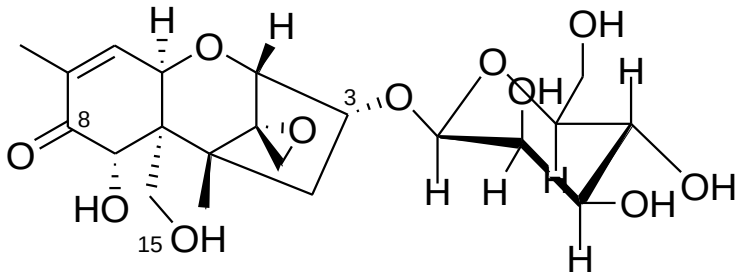

Figure 1.8 Chemical structure of DON-3G

Food processing can lead to an enormous increase in the production of masked DON conjugates. Lancova *et al.* (2008b) described a dramatic increase of DON-3G during malting and brewing experiments. Compared to the DON-3G content in input barley and malt, an increase of maximum 880% was observed. The presence of DON oligoglucosides, like di- and triglucosides in malts, beers and bakery products was described by Zachariasova *et al.* (2012). The nature of this increase in conjugated mycotoxins is still not completely known. A first explanation could be the production of extra mycotoxins which are efficiently conjugated by the metabolically highly active germling. Another possibility is the enzymatic release of bound mycotoxins present in the cell wall polymer fraction during the malting process.

There is concern that the masked DON-3G may be metabolised in the gastro-intestinal tract by mammals to DON and thus may contribute to the overall exposure to DON. This could increase the risk of exposure to DON levels above the tolerable daily intake (TDI) set by the Scientific Committee for Food (SCF) and mentioned in Commission Regulation 1881/2006/EC [Sasanya, 2008].

The mycotoxin conjugates that arise from mammalian metabolism, are not regarded as masked mycotoxins as they do not occur in food or feed. Mycotoxins are conjugated by mammals during their metabolization in the liver and are further excreted in urine. The described DON conjugates found in urine include DON-3-glucuronide and DON-4-sulfate. These conjugates can be used as biomarkers of exposure and via this way the total exposure of individuals towards certain mycotoxins regardless of their source and form of intake, can be monitored. Recently, a study was conducted to evaluate mycotoxin exposure through

different age and gender groups and different seasons and years within the Flemish population via the use of biomarkers [Heyndrickx *et al.*, 2014].

1.3.5 Legislation

In 2000 the European Union launched its "White Paper on Food Safety" as a legal basis for appropriate food and feed production and food safety control. This was followed by the introduction of the general Food Law in 2002 laying down general principles, procedures and requirements to assure and protect the human health in matters of food safety (178/2002/EC). One year later, in 2003, the European Food Safety Authority (EFSA) was set up to focus on risk assessment and scientific advice in the field of food safety questions.

In order to decrease the presence of DON in food and feed, the European Commission has set maximum levels and guidance levels for mycotoxins in products, respectively intended for human consumption (Commission Regulation 1881/2006/EC) and for animal feed (Commission Recommendation 2006/576/EC). An overview of the current DON maximum levels and guidance values for respectively food and feed is presented in Table 1.2.

Results from national monitoring programs on the presence of DON in food and feed are to be reported on a regular basis at the European level. In 2010, EFSA received a mandate from the European Commission to collect and analyse, on a continuous basis, all available data on DON in food and feed. The report analysing these data mentions the lack of data on DON-3G, 3-ADON and 15-ADON in order to characterize their potential contribution to the total DON exposure in a more efficient way. However, the EFSA CONTAM expert group on *Fusarium* mycotoxins is currently evaluating the DON masked forms.

In 2002, the SCF has set a provisional TDI for DON at 1 µg/kg body weight per day. The TDI was extended in 2010 by JECFA to the group of DON and its acetyl derivatives 3-ADON and 15-ADON and laid down an acute reference dose (ARfD) at 8 µg/kg body weight. The exposure assessments conducted to date at national or European level concluded that a high number of consumers and young children were exposed to DON at levels close to or even higher than the TDI [Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2011].

Table 1.2 Overview of the current DON maximum levels for food and guidance values for feed based on 1881/2006 (EC) and 2006/576 (EC), respectively

Foodstuffs: maximum levels	µg/kg
- Unprocessed cereals other than durum wheat, oats and maize	1 250
- Unprocessed durum wheat and oats	1 750
- Unprocessed maize	1 750
- Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits), bran as end products marketed for direct human consumption and germ, with the exception of foodstuffs listed in †	750
- Pasta (dry)	750
- Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
- † Processed cereal-based foods and baby foods for infants and young children	200

Feed: guidance values relative to a feeding stuff with a moister content of 12%	µg/kg
Feed materials	
- Cereals and cereal products with the exception of maize by-products	8 000
- Maize by-products	12 000
Complementary and complete feeding stuffs with the exception of:	5 000
- Complementary and complete feeding stuffs for pigs	900
- Complementary and complete feeding stuffs for calves (< 4 months), lambs and kids	2 000

1.4 ANALYTICAL METHODS FOR THE DETECTION OF DEOXYNIVALENOL AND ITS MASKED FORMS

Regarding food and feed safety, it is necessary to determine the mycotoxin content in different matrices. However, analysing grain samples for the presence of mycotoxins is not easy. A good sampling plan, which consists of sampling, sample preparation and analysis, is a prerequisite for correct classification of cereal lots. Because of the inevitable errors associated with each analysis step, the results should always be reported with an estimate of uncertainty. Precision and accuracy are the most important parameters related to the uncertainty. The variance (V), standard deviation (SD) and coefficient of variation (CV) are used as a measure to determine precision. Accuracy is associated with a bias, which is an influence that causes the deviation of the measured value from the true value. The final sample and analysis procedure needs to be selected to obtain high precision and high accuracy [Koch, 2004; Whitaker and Slate, 2012].

1.4.1 Sampling

A first step in the sampling plan is the sample selection. Heterogeneity remains the largest problem in mycotoxin analysis and the sampling step is generally considered as the most significant source of uncertainty. To overcome the problem of heterogeneous distribution of mycotoxins in food and feed, the sample taken for analysis should be an accumulation of many small portions (*i.e.* incremental samples).

The collection of laboratory samples out of a bulk lot can be performed in two different ways: dynamic or static collection. Dynamic sampling is the movement of the lot on a conveyor belt; sampling is performed by collecting the increments on different places at a fixed time-schedule. Static storage in containers, silos, load docks and cargo ships is more frequently used. Sampling is more difficult as several increments of all the layers of the bulk need to be charged. The mycotoxin concentration of the sample is determined as an estimation of the true mycotoxin concentration in the bulk lot or as a comparison to a defined accept/reject limit (ARL) that is usually equal to a maximum level or regulatory legal limit.

To obtain a homogeneous test sample, the different incremental samples need to be blended thoroughly. According to IUPAC, the degree of heterogeneity (*i.e.* opposite of homogeneity) is the determining factor of sampling error. Other sources of error and consequently uncertainty during the sampling step can be *e.g.* cross-contamination and the instability of

samples. By increasing the size of the sample and reducing the particle size followed by efficient mixing, the bias can also be reduced. Other measures to reduce the problem of heterogeneity together with sampling protocols are included in European Commission Regulation (EC) No. 401/2006 [Maestroni and Cannavan, 2011; Campbell *et al.*, 1986; Whitaker and Slate, 2005; Whitaker and Slate, 2012].

The relationship between the probability of accepting a lot with a specific mycotoxin concentration and the mycotoxin concentration of the bulk lot, is illustrated by the operating characteristic (OC) curve (Figure 1.9) [Whitaker and Slate, 2005]. The areas limited by the ARL and the OC curve describe the economic risk (Seller's risk) and the consumer risk (Buyer's risk). The Seller's risk is the risk of rejecting good lots (false positives) and the Buyer's risk is the risk of accepting bad lots (false negatives). The slope of the OC curve has a high economic and health relevance. When designing a sampling plan it is crucial to maximize the slope of the OC in order to reduce consumer and producer risks and minimize the risk of lot misclassification. The shape of the OC curve is defined by the sample size, degree of grinding, subsample size, the number of analysis and the ARL. Therefore these parameters can be used to reduce the Buyer's and Seller's risks associated with a sampling plan [Whitaker and Slate, 2005; Maestroni and Cannavan, 2011].

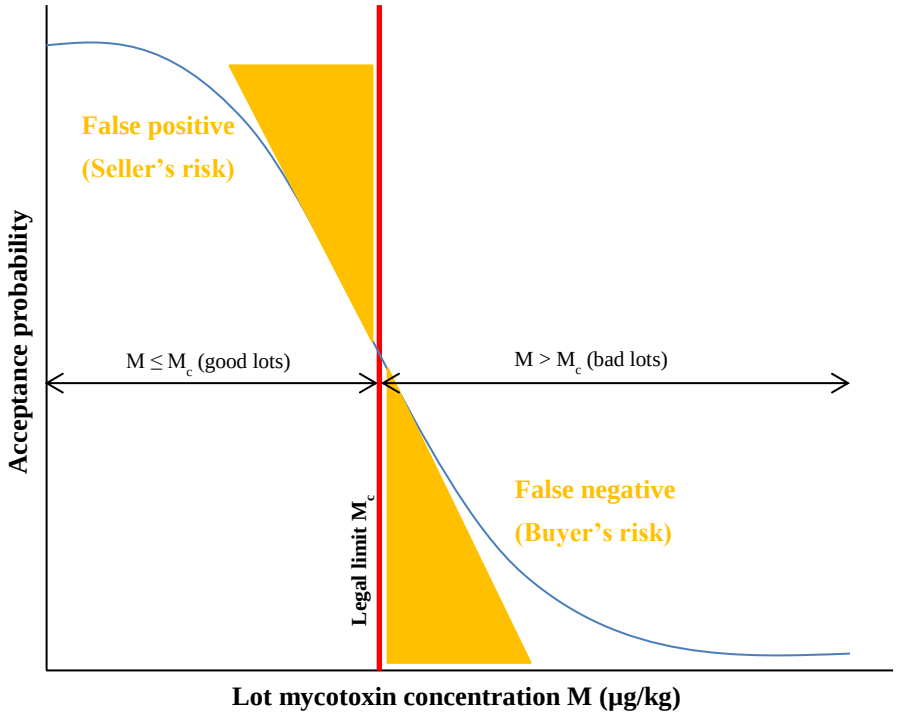

Figure 1.9 Operating characteristic curve for the acceptance probability of mycotoxin sampling in a bulk lot, defined with possible outcomes when lots are classified as accepted or rejected (Whitaker and Slate, 2005).

1.4.2 Sample preparation

The major goal of sample preparation is the elimination of matrix compounds and is a challenging process as a diversity of sample matrices can be contaminated with mycotoxins. Each sample matrix requires its own optimized sample preparation to generate accurate and consistent analytical results. Different conventional and modern sample pretreatment methods are currently used in different laboratories. Sample preparation normally includes two steps, namely extraction and clean-up. As DON is a polar mycotoxin, it can easily be extracted from food and feed matrices by shaking or blending with pure water or mixtures of water with other polar solvents such as acetonitrile or methanol. Trenholm *et al.* (1985) evaluated several extraction solvents for DON extraction such as methanol-water (50/50, v/v) or acetonitrile-water (84/16, v/v) for wheat and maize matrices and acetonitrile was found to lower matrix interferences.

Analyte enrichment is the next critical step after sample extraction. The most important procedures employed for clean-up of DON contaminated samples are liquid-liquid extraction (LLE), solid-phase extraction (SPE) and use of immunoaffinity columns (IAC). LLE is performed by shaking the sample extract with an immiscible solvent and therefore merging the analyte to its preferable solvent. SPE can either be used for analyte enrichment or matrix elimination. It can be applied as a reversed phase or a normal phase separation. Reversed phase SPE is characterized by a polar or moderately polar mobile phase and a non-polar stationary phase. In normal phase SPE the stationary phase is polar, while the mobile phase is non-polar. Retention of the polar DON analyte in the column takes place due to the interaction between its functional groups and the surface of the sorbent.

When the aqueous sample extract is applied to an IAC, the analyte molecules are bound to the antibodies which are linked to an organic carrier material. Subsequent to a washing step the toxins can be eluted due to the denaturation of the antibodies by the use of pure organic solvents [Krska *et al.*, 2001; Razzazi-Fazeli and Reiter, 2011].

1.4.3 Chromatographic methods

Because of the economic importance and from a food safety point of view a variety of analytical techniques have been developed for the separation and detection of DON in food and feed. Most commonly used chromatographic techniques include thin-layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC).

TLC was the first chromatographic method to be applied for DON determination and is still in use in many laboratories. Reagents such as sulphuric acid or *para*-anisaldehyde are necessary to visualize the non-fluorescent and short wavelength (λ_{max} 220 nm)-absorbing DON. In addition to the spray reagents which are specific to the 12,13-epoxy group, $AlCl_3$ is the most useful visualisation reagent for DON and other type B trichothecenes. TLC is a fast and low cost method, but due to low selectivity it is not sufficient for quantitative determination [Shephard, 2008; Trucksess *et al.*, 1984; Krska *et al.*, 2001]. Currently used quantitative methods for DON analysis include GC or LC based systems coupled to diverse detectors. The limitation of a GC method is the necessity of derivatization. Common derivatization reactions at the hydroxyl moieties of DON involve the formation of trimethylsilyl (TMS) ethers, or trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl ester derivatives. Methods were described using TMS ethers or fluoro acyl derivatives coupled to electron capture detection (ECD) [Eskola *et al.*, 2000; Radova *et al.*, 1998; Tacke and Casper, 1996] or mass spectrometric detection [Rodrigues-Fo *et al.*, 2002; Black *et al.*, 1987; Mirocha *et al.*, 1998]. When using ECD for DON detection a higher background signal and less sensitive results were obtained especially in complex samples, compared to MS detection. LC-analysis can be performed with fluorescence, ultraviolet (UV) (214-229 nm) or MS detection. For fluorescence detection a pre- or post-column derivatization is required, which makes this type of detection time-consuming. The use of UV detection is often limited by lack of specificity because of the need to use low wavelengths [Sano *et al.*, 1987].

Over the last few years, LC and GC techniques coupled to mass spectrometric detectors have been developed and established. Several of these developed chromatographic methods have been recognized as official as they have been interlaboratory validated in terms of repeatability, reproducibility, limit of detection (LOD) and limit of quantitation (LOQ). A number of international bodies such as Association of Official Analytical Chemists (AOAC), the European Committee for standardization (CEN) or the European Commission are involved in the validation procedures.

LC is a dynamic separation technique based on the difference in affinity between the mobile phase and the stationary phase. As DON is a small and polar compound, it will be ideally separated by the use of reversed-phase LC which is based on the separation of molecules up on their interaction with a hydrophobic stationary phase. Mostly, the hydrophobic stationary phase consists of silica particles with a diameter of 5 µm or less which surface is modified with a hydrophobic layer such as octadecyl (C_{18}) moieties. The DON containing extract is

applied on the LC-column in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between DON and the solid support resulting in desorption. The more hydrophobic the molecule, the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote desorption. Therefore DON will be separated from other interfering components based on the difference in hydrophobicity [Shephard, 2011]. In recent years, hydrophilic interaction liquid chromatography (HILIC) has been successfully applied for the separation of various polar components including mycotoxins [Sorensen *et al.*, 2007; Pereira *et al.*, 2009]. In HILIC mode, a high concentration (> 70%) organic phase is used as water will not retain the polar components on the stationairy phase. Increasing the amount of water will elute molecules in order of polarity.

Mass spectrometry (MS) provides a high detection selectivity owing to the ability to separate or filter ions according to their mass-to-charge ratios (*m/z*). MS includes ionization which implies a conversion of molecules into ions in the gas phase. Ionization methods used, are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). During ESI, the sample is dissolved in a polar solvent and pumped through a stainless steel capillary which caries between 2 000 and 4 000 V. The liquid aerosolizes at the exit of the capillary where ions leave the desolvating droplets and flow into the MS due to a combination of electrostatic attraction and vacuum. APCI differs from ESI as neutral analytes are transferred into the gas phase by vaporizing the introduced liquid in a heated gas stream. Both ESI and APCI methods were developed for DON identification and quantitation [Monbaliu *et al.*, 2009b; De Boevre *et al.*, 2012b; Toth *et al.*, 2011]. Literature data also switch between the positive and negative ionization mode. Lagana *et al.* (2003) reported data were the negative mode was preferred for type B trichothecenes such as DON, in contrast Bily *et al.* (2004) and Pallaroni *et al.* (2002) both preferred the use of positive ion mode for DON.

Prior to detection, the ions are separated based on their *m/z* value. During this research, a Quattro Premier XE MS/MS (Waters, Milford, MA, USA) was applied, consisting of two mass analysers or quadrupoles and a T-wave collision cell. Using selected reaction monitoring (SRM) a specific precursor ion is selected in the first quadrupole followed by destruction in the collision cell. Out of the fragment ions, a specific ion is detected in the second quadrupole. Based on the high accuracy and sensitivity of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in addition to the ability to use internal standards (*e.g.* deepoxy deoxynivalenol (DOM), ^{13}C-isotope-labelled internal standard) to correct losses of

DON during sample preparation and ion suppression effects in the mass spectrometer, this analytical method can be referred as a state-of-art technique for the measurement of DON [Langseth and Rundberget, 1998; Neuhof *et al.*, 2009]. Further advantages of LC-MS/MS include no need of derivatization prior to analysis, low detection limits, the ability to generate structural information of the analytes and the requirement of minimal sample pretreatment [Berthiller *et al.*, 2007; Spanjer, 2011].

1.4.4 Immunochemical methods

Immunochemical methods occupy a leading place in the screening group of methods for the determination of mycotoxins. They offer the advantage of selectivity, sensitivity, simplicity and rapidity, which are of importance for routine and on-site testing of mycotoxins. Moreover, these methods require simple instruments as most of them are based on photometric, fluorimetric, luminescence or electrochemical detection [Maragos and McCormick, 2000; Maragos *et al.*, 2006; Maragos *et al.*, 2002; Koppen *et al.*, 2010]. Common disadvantages are possible non-specific binding of components and cross-reaction with related compounds which can lead to an overestimation of DON-levels (false positive results) in comparison with chromatographic methods [Goryacheva and De Saeger, 2011]. Described immunochemical methods include enzyme-linked immunosorbent assay (ELISA), membrane-based immunoassay such as flow-through assay and lateral flow test, gel-based tests and fluorescence polarization.

1.4.4.1 Interaction principle

The interaction of an antibody with an antigen forms the basis of all immunochemical reactions. An IgG antibody, which is the type of antibody mostly used in immunochemical reactions, has a typical Y-shape (Figure 1.10) and contains two symmetrical parts each consisting of two polypeptide chains, namely a light and a heavy chain connected to each other via disulphide bridges between two cysteines. The light and heavy chain have a molecular weight of 25 and 55 kDa, respectively, and each chain contains a carboxy-terminal and amino-terminal end group. The amino groups of the variable heavy (V_H) and light (V_L) chain form the antigen binding site. The amino acids from the antigen binding site correspond to the amino acids of the hypervariable regions, also known as complementarity determining regions (CDRs).

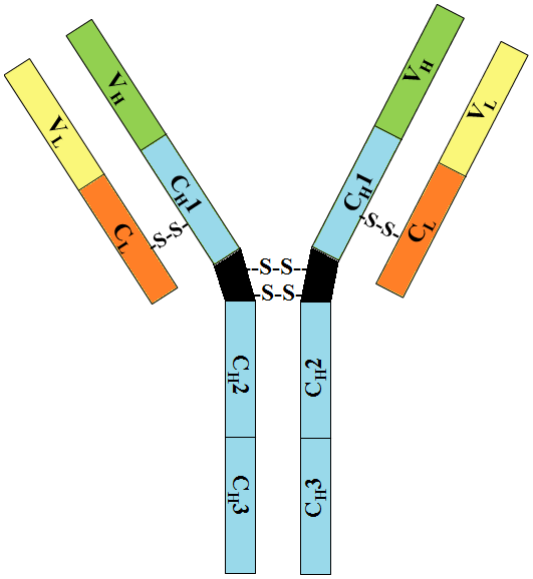

Figure 1.10 Structure of an IgG antibody

Interactions between the epitopes of the antigen and the CDRs of the antibody take place through physical non-covalent bonds, namely Coulombic bonds, Van Der Waals forces, Ca^{+2}-bridges and/or hydrogen bonds. An important property of antigen-antibody interaction is the affinity, which is the strength of the binding between the antigen and the antibody. As seen in the equilibrium reaction below, the affinity can be measured by the equilibrium constant (K_{eq}) or dissociation constant (K_D), which represents a measure of stability. At the beginning of an antigen-antibody binding, the reaction proceeds predominantly in one direction, but the reverse rate progressively increases until the forward and reverse speeds are equal. At this point, the reaction is said to have reached its equilibrium. At equilibrium, the ratio between the concentrations of the antigen-antibody complex and free antigen and antibody is constant and is characterized by K_{eq} (Equation 1.1), which typically assumes a value around 10^8 and 10^{10} and is the ratio of the dissociation (k_d) and association (k_a) rate constants. The constant K_{eq} is related to the change in free energy via Equation 1.2. In order for a reaction to proceed spontaneously, the free energy change must be negative. Therefore the higher the K_{eq}, the more negative the free energy change and the more preferable the antibody-antigen complex formation will be. Parameters that may be modulated to influence the affinity are: the surface tension, pH, ionic strength, dielectric constant and temperature.

Equilibrium reaction 1.1 Antibody + Antigen $\rightleftarrows$ Antibody-Antigen complex

Equation 1.1 $$\frac{[\text{Complex}]}{[\text{Antigen}][\text{Antibody}]} = \frac{k_a}{k_d} = K_{eq} = \frac{1}{K_D}$$

k_a is the association constant, k_d is the dissociation constant and K_{eq} is the equilibrium constant

Equation 1.2 $$\ln(K_{eq}) = -\frac{\Delta G}{R \times T}$$

K_{eq} is the equilibrium constant, ΔG is the free energy change, R is the universal gas constant (= 8.314472 J K^{-1} mol^{-1}) and T is the temperature (310 K at 37 °C)

Another important property in the antigen-antibody interaction is the specificity, which is a measure of the selectivity of the interaction between the antigen determinant and antibody-active sites compared to other chemically similar molecules. The ability of an antibody to react with similar antigenic sites on different substances is called cross-reactivity [Harlow and Lane, 1988; Reverberi and Reverberi, 2007; Vanoss *et al.*, 1986].

1.4.4.2 Enzyme-linked immunosorbent assay

In ELISA, the antigen-antibody interaction procedure is performed in 96-well polystyrene plates, where one of the immunoreagents is usually immobilized on the bottom and walls of the wells for separation of bound and free reagents. There are two main forms of ELISA, namely the direct and indirect form and each of them can be performed in a competitive or non-competitive way. The term competition describes an assay in which measurement involves the quantification of a substance by its ability to interfere with an established concentration of standard antigen [Goryacheva and De Saeger, 2011]. The principle of the different ELISA formats specific for DON screening is further explained.

For DON measurement using direct ELISA (Figure 1.11A), secondary antibody is coated to microtitre plates followed by the addition of primary anti-DON antibody. After DON is extracted from a ground sample with solvent, a portion of sample extract and an enzyme-coupled DON conjugate are added to the plate. Any DON in the sample extract or control standard is allowed to compete with the enzyme-conjugated DON for the antibody binding sites. A blue colour is developed in the plate when adding an enzyme substrate and the intensity of the colour is inversely proportional to the concentration of DON in the sample or standard. After stopping the enzyme reaction, the colour is optically measured using an

ELISA reader at a wavelength of 450 nm. The optical density (OD) is determined and compared to the ODs of the standards. For the indirect ELISA (Figure 1.11B), DON extracted samples or DON standard is added together with the primary anti-DON antibody to a microtitre plate coated with DON-protein conjugate. This reaction is followed by the addition of enzyme-coupled secondary antibody. The colour development and measurement occurs in the same way as for direct ELISA [Zheng *et al.*, 2006; Goryacheva *et al.*, 2009].

Currently, commercial ELISA test kits are available for the determination of DON contamination in several cereal target commodities (cereals, food, feed and beer). An overview of different commercially available ELISA test kits, namely RIDASCREEN® DON (r-Biopharm, Darmstadt, Germany), Deoxynivalenol EIA (Eurodiagnostica, Arnhem, The Netherlands), Veratox® 5/5 quantitative DON test (Neogen, Anderlecht, Belgium) and AgraQuant® DON (Romer Labs, Tulln, Austria) is shown with its major characteristics in Table 1.3. As seen, all of them are characterized by cross-reactivities towards the masked mycotoxins 3-ADON, 15-ADON and/or DON-3G. Even though these masked forms are not mentioned in current legislation (Commission Regulation 1881/2006/EC for food and Commission Recommendation 2006/576/EC for feed), based on the available toxicity data, it is important to measure their occurrence in food and feed [Goryacheva *et al.*, 2009; Zachariasova *et al.*, 2008]. ELISA test kits were validated for DON detection in several target commodities by several authors as mentioned in Table 1.3 [Lupo *et al.*, 2010; Yoshizawa *et al.*, 2004; Papadopoulou-Bouraoui *et al.*, 2004; Zachariasova *et al.*, 2008]. Validation generally includes the evaluation of the analytical range, detection limit, sensitivity, accuracy and precision as enforced by the AOAC.

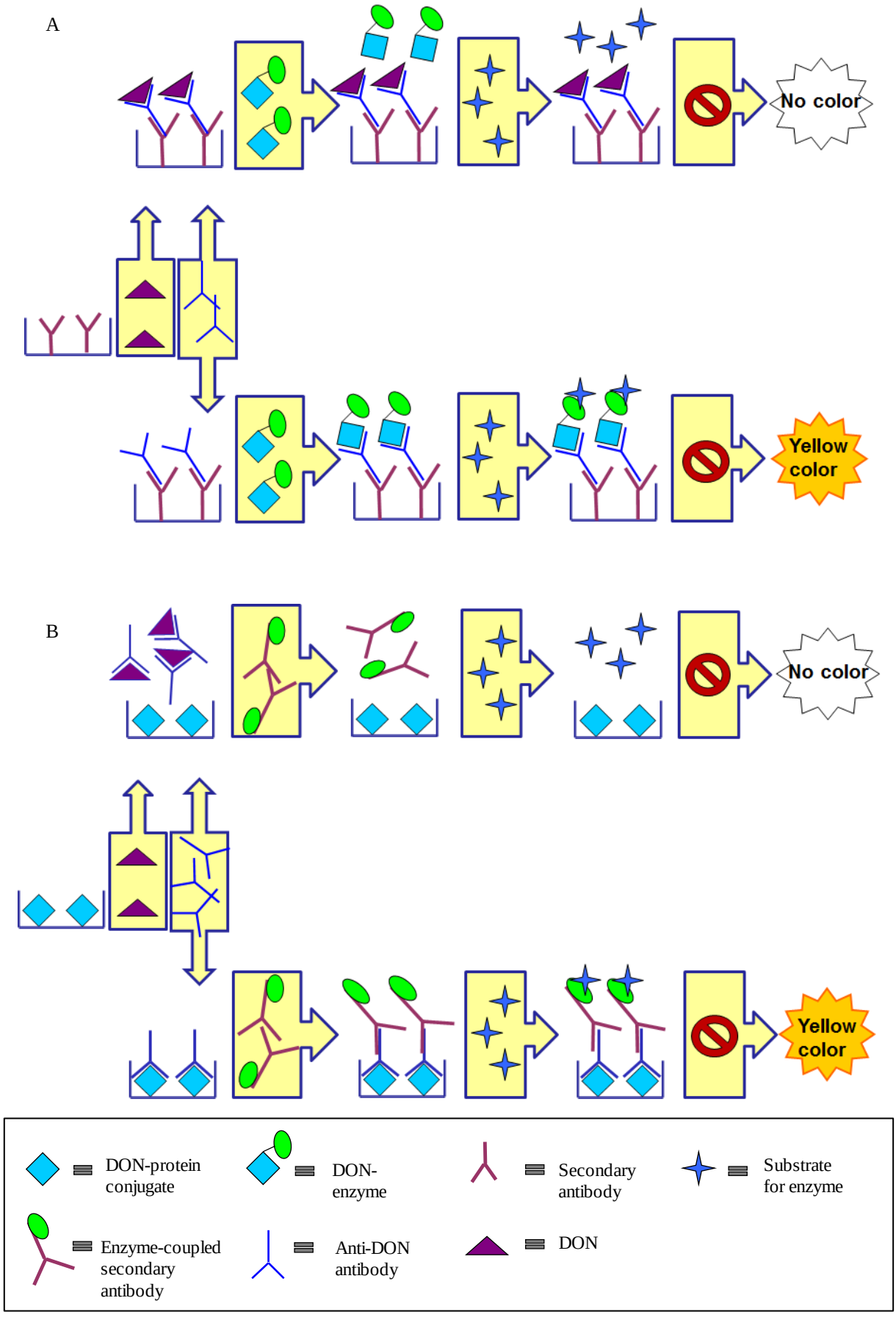

Figure 1.11 Competitive direct (A) and indirect ELISA (B) using anti-DON antibody

Table 1.3 Overview of commercial ELISA test kits for DON determination [Zachariasova *et al.*, 2008]

ELISA kit	Linear range (ng/mL)	Cross-reactivity (%)				Target commodities
		DON	3-ADON	15-ADON	DON-3G	
RIDASCREEN® DON	3.7-100	100	>100	19	*n.a.*	cereals, malt, feed and beer
Deoxynivalenol EIA	0.313-10	100	230	<0.1	115	cereals, food, feed, beer and silage
Veratox® 5/5-quantitative DON test	25-200	100	40	0	157	wheat, barley, maize, malted barley, oats and rice
AgraQuant® DON assay 0.25/5.0	12.5-250	100	770	2	52	wheat, barley, maize, oats, malted barley, rice and wheat flour

1.4.4.3 Membrane-based immunoassay

The flow-through assay and lateral flow test are the two most popular types of membrane-based immunoassay. The flow-through assay (immunofiltration test or immunofiltration assay or enzyme-linked immunofiltration assay) is based on the direct competitive ELISA principle with an anti-DON antibody-coated membrane surface. In contrast, the lateral flow test (strip test or dipstick test) is a membrane-based immunoassay format which consists of one step and does not require special instrumentation or reagents.

The procedure specific for DON screening by lateral flow test is the following. Firstly, a sample extract is added by dipping the strip test into the test sample or by dropping the sample onto the sample pad. Any DON present will bind to the anti-DON antibody colloidal gold particles present in the conjugate pad and as a complex they migrate along the membrane. When they pass the test zone containing DON-protein conjugate, free anti-DON antibody gold particles can be captured allowing coloured particles to concentrate and form a visible line. For negative samples (DON level lower than cut-off level) a colour will be developed. No coloured line will appear in the test zone for positive samples (DON level higher than cut-off level). Further on, a control zone is present which contains secondary antibody, which will be visible due to the anti-DON antibody gold particle-secondary antibody complex.

Confirmation of DON contamination can be performed by chromatographic analytical methods.

Several strip tests for DON determination have been developed and commercialized. Kolosova *et al.* (2008) describes the development of a lateral-flow colloidal gold-based immunoassay for DON determination using two different indicator ranges, namely 250-500 and 1 000-2 000 µg/kg. A multiplex dipstick was developed by Lattanzio *et al.* (2012) for the simultaneous determination of zearalenone, T-2/HT-2 toxin, DON and fumonisins in wheat, oats and maize. Cut-off levels for DON were set at 1 400 µg/kg for all different food commodities [Zheng *et al.*, 2006; Goryacheva and De Saeger, 2011]. Commercially available lateral flow test strips include RIDA®QUICK DON (r-Biopharm, Darmstadt, Germany) with a cut-off value of 0.5 mg/kg and validated for wheat, triticale and maize samples. The same cut-off level is mentioned for the AgraStrip® Quantitative DON test (Romer Labs, Tulln, Austria) using wheat samples. For maize samples a lower cut-off value of 0.2 mg/kg is described. Other commercial strip tests (DONCheck™, Cereal tester, Fleurus, Belgium and Deoxynivalenol test stip, Abraxis, Warminster, UK) have a higher cut-off value of 1 mg/kg.

Until now, only the use of colloidal gold particles was described for DON detection. Usleber *et al.* (1993) described the use of enzymes instead of colloidal gold as a label for the detection of 15-ADON. This could increase the sensitivity of the strip test to a detection limit of 5 ng/mL, even though extra steps were necessary during the measurement such as a washing and substrate addition step [Goryacheva and De Saeger, 2011].

1.4.4.4 Gel-based tests

The carrier membrane of a membrane-based immunoassay can be replaced by a sepharose gel to reduce matrix effects and increase assay sensitivity. The sepharose gel contains the immobilized anti-DON antibodies and is present in a small plastic cartridge as for SPE. When applying a sample extract on the column, the DON present in the extract binds to the antibody. Similar to the membrane-based assay, competition for binding with the antibody will be induced by adding DON-enzyme conjugate. A chromogenic substrate will induce colour development. Next to DON determination, the gel-based column can be used as an immunoaffinity column taking care of separation and preconcentration of DON analyte [Goryacheva and De Saeger, 2011; Schneider *et al.*, 2004; Zheng *et al.*, 2006].

Immunoaffinity columns are just like ELISA test kits and membrane-based immunoassays commercially available and validated for several food and feed matrices.

1.4.4.5 Fluorescent polarization

In the fluorescence polarization immunoassay format, DON present in the sample extract and DON labelled with fluorophore compete for anti-DON antibody binding sites. When DON-fluorophore binds to the antibody, the rotation of the tracer molecule is affected and therefore changes the orientation of fluorescence. Subsequently, polarization is determined and is inversely proportional to the amount of DON present in the sample. The differences with ELISA are that no enzyme reaction is involved and no separation of bound and free compounds is needed. Maragos and Plattner (2002) compared the use of fluorescent polarization with LC-UV for the determination of DON in wheat samples. Recoveries of 71.2% were determined when using fluorescent polarization immunoassay and a linear correlation between DON measured with fluorescent polarization and with LC-UV was found according to the correlation coefficient r (0.967), proving the working of fluorescent polarization for the screening of DON in wheat samples [Zheng *et al.*, 2006; Schneider *et al.*, 2004; Maragos *et al.*, 2002].

1.4.4.6 Other emerging technologies

In the last 20 years, the development of biosensors increased significantly due to the high demand for rapid and accurate methods to detect mycotoxins. Biosensors involve biological recognition elements such as enzymes, antibodies, nucleic acids or artificial receptors coupled to a transducer to translate a biochemical signal into a quantifiable electronic signal. Depending on the type of transducer, four groups of biosensors are mostly used for DON detection: colorimetric, surface plasmon resonance (SPR) sensors, mass-sensitive quartz-crystal microbalance (QCM) sensors and electrochemical sensors using amperometric, potentiometric or conductometric detection. During this research SPR was applied and will therefore be discussed in detail.

The use of an affinity-based SPR sensor system with less demanding sample clean-up steps as for other analytical methods, might be a useful alternative for screening and cost effective quantitative determination. SPR has been successfully used for many years in biochemical research, but is a quite new technique in food and feed analysis.

SPR is based on the measurement of interaction of light with the captured biomolecule on the sensor surface (Figure 1.12). The sensor glass surface consists of a thin gold film, where dextran molecules are attached to to be able to bind molecules and to make the surface hydrophilic. Biacore® technology is based on the SPR principle and the most versatile sensor chip used herein is the CM5 chip (GE Life Sciences, Diegem, Belgium), which consists of carboxymethylated dextran where molecules can be covalently coupled via amine, thiol, aldehyde or carboxyl groups.

At the interface between the two transparent media of different refractive indices, namely glass (high refractive index) and aqueous buffer (low refractive index), light coming from the side of higher refractive index is partly reflected and partly refracted. At a certain incident angle of polarized light, known as resonance or SPR angle, light is adsorbed by electrons (plasmons) in the metal film of the sensor chip, causing them to resonate. This results in a decrease in the reflected light intensity, which can be seen as a dark band that is displayed as a dip (angle shift) in the SPR reflection intensity curve. The shape and position of the registered dip provides information about the sensor surface. When an antigen-antibody interaction occurs at the sensor surface, the resonance angle position of the dark band changes due to a change in refractive index of the buffer solution, causing a shift in the SPR intensity curve. The resonance angle change is proportional to the amount of bound antigen-antibody. A sensorgram is built up out of the continuous monitoring of the resonance angle plotted as response units (RU) in function of time and delivers kinetic information of the antibody (*i.e.* affinity, selectivity) [Zheng *et al.*, 2006; Schneider *et al.*, 2004; Teeparuksapun, 2013; Schnerr *et al.*, 2002; Vidal *et al.*, 2013].

Research in using SPR technology for DON analysis by using anti-DON antibodies has been conducted and some results are quite promising. Schnerr *et al.* (2002) reports the use of a DON-biotin conjugate immobilized on a streptavidin coated SPR sensor surface. Wheat extracts were mixed with polyclonal anti-DON antibody and injected over the sensor surface of the Biacore® device. An LOD value of 2.5 pg/µL (corresponds to 7.5 µg/kg in naturally contaminated wheat samples) and an half-maximal inhibitory concentration (IC_{50}) value (*i.e.* measure for sensitivity) of 0.72 µg/mL was determined. The assay had a working range between 0.13 and 10 µg/mL, equivalent to 390 and 3 000 µg/kg of DON in wheat samples. Regeneration of the sensor surface to remove polyclonal antibody was performed using 6 M guanidinechloride in 10 mM glycine (pH 2.9). In Tudos *et al.* (2003), the use of a DON-casein sensor with monoclonal anti-DON antibody is described. An SPR system with an even

lower working range of 2.5 – 30 ng/mL was established, making it possible to quantify very low DON contamination levels. This was also done by Kadota *et al.* (2010) using a DON-BSA immobilised SPR sensor chip, obtaining an IC_{50} value of 14.9 ng/mL. A linear correlation (r = 0.98) was even found between the DON determined by LC-MS and SPR for several matrices, namely durum wheat, wheat-based breakfast cereals, maize, maize-based baby food and oats [Meneely *et al.*, 2010].

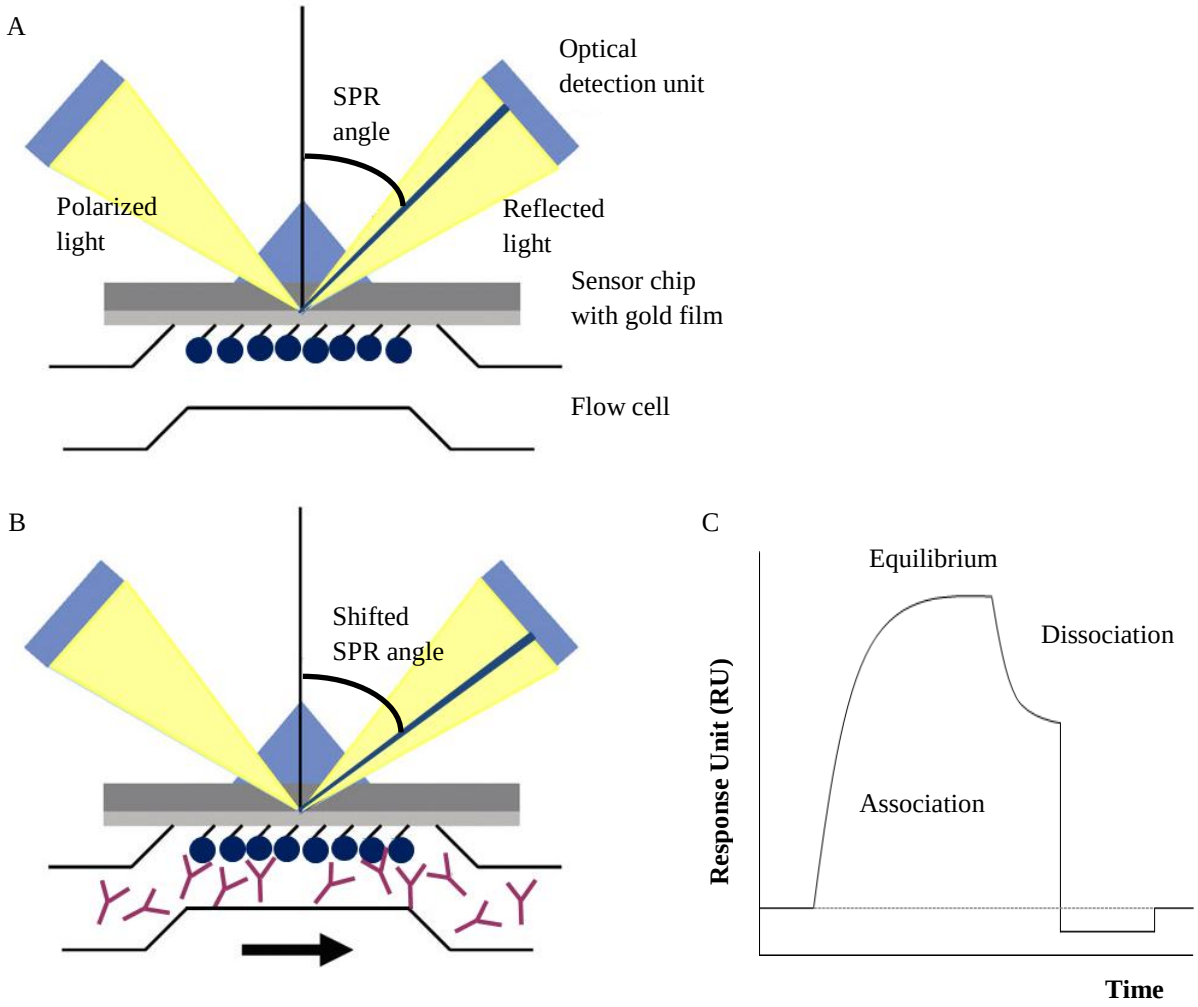

Figure 1.12 (A) Polarized light is applied on the surface of the sensor chip and is reflected. The intensity of the reflected light is reduced at a certain incident angle, the resonance or SPR angle. (B) Interacting substances near the surface of the sensor chip increase the refractive index, which alters the SPR angle. (C) A sensorgram is built up out of the continuous monitoring of the resonance angle plotted as response units (RU) in function of time.

Molecular imprinted polymers (MIPS) are alternative biomimetic receptors currently being researched. A MIP is a polymer in which monomers are polymerized in the presence of a template molecule. After removal of the template, binding sites or cavities containing functional groups remain which show an enhanced affinity for the chosen template molecule. MIPS are not only used for SPE of mycotoxins, but also in detection systems such as the described biosensors. Choi *et al.* (2011) describe the use of MIP technology for the detection of DON using an SPR transducer. As a result, DON was able to be detected between 0.1 and 100 ng/mL. The selectivity or specificity of the MIPS is improved compared to antibodies, but still not optimal as cross-reactivities were determined against 3-ADON and 15-ADON of respectively 19% and 44% [Goryacheva and De Saeger, 2011; Zheng *et al.*, 2006]. The commercially available AFFINIMIP® Deoxynivalenol (Polyintell, Val-de-Reuil, France) was recently developed for SPE applications in wheat, maize and oats matrices.

Another alternative for antibodies are aptamers. Aptamers are single-stranded oligonucleotides selected *in vitro* to bind to molecular targets ranging from large proteins to small molecules such as mycotoxins. The selection process used is the systematic evolution of ligands by exponential enrichment (SELEX). While antibodies have been the standard for several decades, aptamers have emerged owing to inherent advantages with respect to antibodies: immunization of animals is not required, they are more chemically and thermally stable, they are particularly useful in presence of organic solvents used for the extraction of mycotoxins [Vidal *et al.*, 2013; Goryacheva and De Saeger, 2011]. In literature no aptamers against DON are described, even though a patent filed at 25th of November 2011 (patent number CN102559686A) mentions the invention of a DON aptamer and describes its applications [Shuqing *et al.*, 2012].

1.4.5 Analytical methods for the detection of masked deoxynivalenol forms

The structural differences of the masked 3-ADON, 15-ADON and DON-3-G compared to their parent DON, induce changes in the physicochemical properties, such as solubility and polarity leading to differences in suitable experimental conditions for extraction and chromatographic analysis. Generally, two different approaches may be followed for the determination of masked mycotoxins: (a) the use of an analytical technique able to directly detect them (direct methods); or (b) the conversion of the masked mycotoxins into their parent forms by enzymatic/acidic/basic hydrolysis before the analysis (indirect methods).

For the sample preparation of masked DON forms, several researchers investigated the use of extraction solvents containing acetonitrile and water in different ratios, eventually acidified with acetic acid and/or formic acid and combined with a clean-up step using SPE or IAC [Berthiller *et al.*, 2005; Sasanya, 2008; Berthiller *et al.*, 2007; Zachariasova *et al.*, 2010; Vendl *et al.*, 2010]. Most emerging analytical methods are based on simple and rapid extraction procedures coupled to very selective and sensitive MS hyphenated chromatographic analysis without any clean-up steps [De Boevre *et al.*, 2012b]. In this context, a modified QuEChERS (quick easy cheap effective rugged and safe) sample preparation procedure, based on the partition between water and acetonitrile by the addition of salt mixtures (NaCl and $MgSO_4$), was recently proposed in combination with MS. Unfortunately, DON-3G showed poor recovery value, probably because of its high polarity which does not effectively allow the transfer into the acetonitrile layer [Zachariasova *et al.*, 2010; Cirlini *et al.*, 2012]. The majority of the described chromatographic methods use the direct approach for the determination of 3-ADON, 15-ADON and DON-3G as all three masked forms are commercially available as reference standards.

The use of immunochemical methods for the detection of the masked DON forms is based on the antibody characteristics. Monoclonal antibodies with specificity against 3-ADON were reported by Maragos and McCormick (2000) and Maragos *et al.* (2006) with cross-reactivities against DON and 15-ADON less than 15.9% and 2.5%, respectively. Other reported monoclonal antibodies showed higher cross-reactivity and therefore no specificity against one of the masked DON forms. Therefore, the sum of DON and its masked forms are determined instead of its individual occurrence. As an alternative, indirect detection approaches were described. Trifluoromethanesulfonic acid (TFMSA) was used by Zhou *et al.* (2008) and Tran and Smith (2011) to acidic hydrolyze the masked DON-3G conjugate and to measure total DON concentration in barley, wheat and maize. TFMSA was reported as alternative for trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) as the latter demanded very high reaction temperatures (> 130 °C). After TFMSA acidic hydrolysis, a DON increase of up to 75% was observed. Alternative DON-3G hydrolysis was performed under enzymatic conditions *e.g.* by the use of 1,3-β-glucanase [Nielen *et al.*, 2014]. Deacetylation of 3-ADON and 15-ADON to its parent mycotoxin was described as well by the use of enzymes, more specific by acetyl esterase [Mills *et al.*, 1990].

1.5 DEVELOPMENT OF MONOCLONAL ANTIBODIES

Monoclonal antibody production by somatic cell fusion or hybridoma technology was introduced by Köhler and Milstein in 1975 and is still the most common method for antibody development [Kohler *et al.*, 1976]. The technique involves the fusion of a normal antibody-producing B-cell with a myeloma cell to produce a hybrid cell or hybridoma. The hybridoma would possess the immortal growth properties of the myeloma cell while secreting the antibody produced by the B-cell. The resulting hybridoma could be cultured to provide polyclonal antibodies reacting against different antigens or different epitopes on a particular antigen. After screening and selection, a single hybridoma cell line is obtained producing large amounts of monoclonal antibodies. An overview of the general hybridoma production and monoclonal antibody development process is given in Figure 1.13.

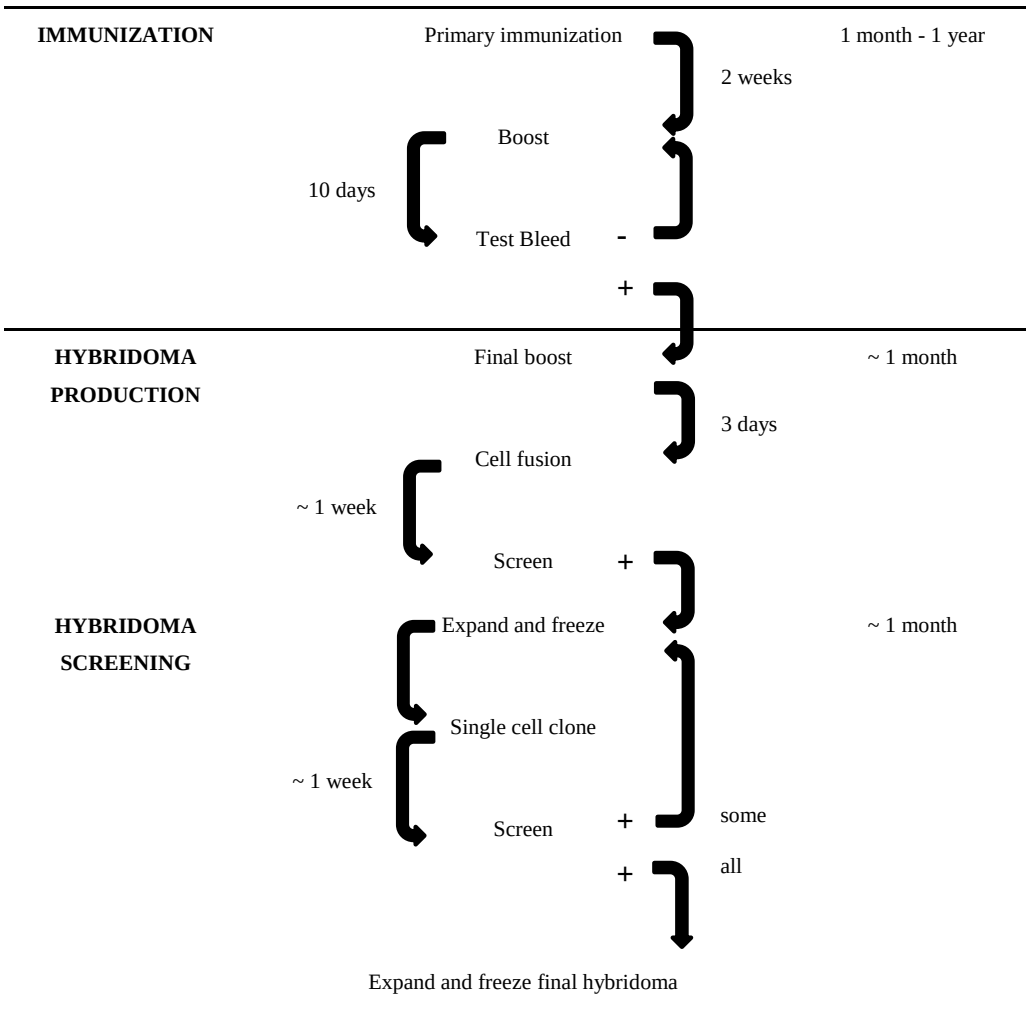

Figure 1.13 Stages of hybridoma production and monoclonal antibody development

1.5.1 Immunization

Antigens with a molecular weight below 1 000 Da (*e.g.* mycotoxins) are not immunogenic. They can be made immunogenic by coupling the analyte to a carrier high-molecular weight molecule (protein) such as bovine serum albumin (BSA), ovalbumin (OVA) or keyhole limpet haemocyanin (KLH). Several research groups already synthesized various DON immunogens by the synthesis of a linker between the C_3 and/or C_{15} chemical reactive hydroxyl group of DON and a protein. Linkers include hemisuccinates, hemiglutarates or direct linkages using the carbodiimide reaction [Casale *et al.*, 1988; Sinha *et al.*, 1995; Usleber *et al.*, 1991; Clare Mills *et al.*, 1990] resulting in one end product or a mixture of DON conjugates. As both hydroxyl groups are of similar chemical reactivity, the production of a single linker product is possible by the use of a protection scheme [Casale *et al.*, 1988] or the use of an acetyl DON derivative followed by deacetylation [Clare Mills *et al.*, 1990]. All of them describe the use of rabbits or mice as laboratory animals for the immune response generation. Other chemical reactive groups for the formation of a linker are the C_8 carbonyl and C_{12}-C_{13} epoxide groups. Previous researchers mentioned the use of carboxymethoxylamine hemihydrochloride for the coupling of the carboxymethyl oxime (CMO) linker at the C_8 carbonyl function of DON [Casale *et al.*, 1988; Usleber *et al.*, 1991]. Until now, the production of specific DON-antibodies by immunization of C_8 coupled DON immunogens in rabbits, mice and guinea pigs failed. This could indicate the immunogenic importance of the C_8 carbonyl for the generation of type B trichothecene antibodies. As the epoxide group of DON determines its toxicity and therefore its immunostimulative effect, no immunogens are described linking DON via its epoxide group to a protein [Karlovsky, 2011].

Injection of an immunogen requires the presence of an adjuvant, which enlarges the humoral antibody responses based on up to five of the following mechanisms of action: the “depot” effect, the antigen presentation effect, the antigen distribution or targeting effect, the immune activation/modulation effect and the cytotoxic lymphocyte (CTL) induction effect. Freund’s adjuvant is considered as the gold standard of adjuvants by many immunologists as the general immunostimulatory properties have not been surpassed by any adjuvant. Unfortunately, the use of Freund’s complete adjuvant (FCA) has been associated with a variety of lesions including localized injection site granulomas; subpleural, pleural, hepatic and renal granuloma formation; necrotizing dermatitis; and spinal cord compression from an injection site granuloma. This has led to numerous regulatory guidelines and recommendations concerning the use of FCA in laboratory animals. The Aviation

Rulemaking Advisory Committee (ARAC) guidelines for the use of adjuvants in research describe the use of alternatives for Freund's adjuvant such as other water-in-oil or oil-in-water emulsions, aluminium compounds or microparticles such as liposomes which serve as depot adjuvants. Guidelines are formulated as well for the correct preparation and injection of immunogens containing FCA. Examples of recommendations include the use of concentrations lower than 0.1 mg/mL of mycobacterial components and the preference of subcutaneous or intraperitoneal injection instead of other routes of injection [Stills, 2005; Harlow and Lane, 1988].

During the period of animal immunization, it is important to collect blood from the animal and to measure the serum titre with indirect ELISA after each boost injection to monitor the production of anti-DON antibodies. The obtained results will influence the decision to proceed with the fusion or to boost again. Figure 1.14 shows the kinetics of a typical immune response. Primary responses are often very weak. The humoral responses to the second and subsequent boosts are totally different. In this stage, the production of IgG antibodies increases exponentially, while the IgM fraction decreases [Harlow and Lane, 1988].

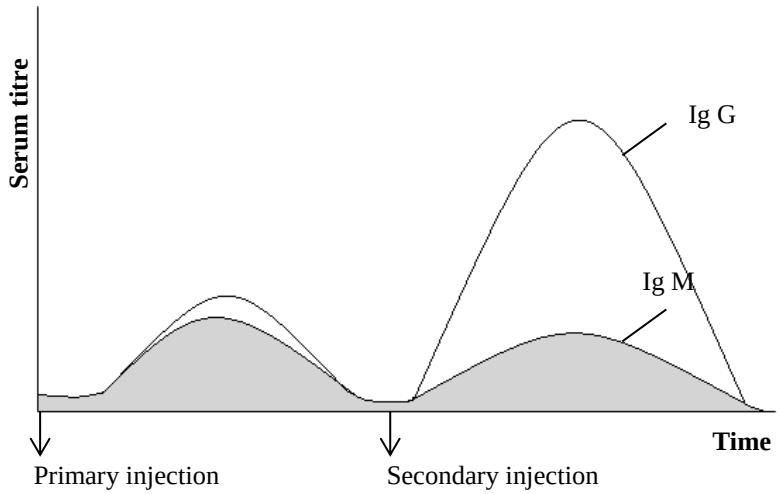

Figure 1.14 Kinetics of immune response during immunization

1.5.2 Hybridoma production and screening

Once a good immune response has been obtained, the construction of hybridomas is ready to start. Antibody-secreting cells are isolated from the appropriate lymphoid tissue, mixed with myeloma cells, centrifuged to generate good cell-to-cell contacts and fused by using polyethylene glycol (PEG). PEG dehydrates the cell membranes by competing for free water

and therefore makes the aqueous phase less polar and facilitates the solvation of lipid molecules creating a defect in the cell membrane bilayer. With the reduction of the electrostatic and hydration repulsion between bilayers due to the decrease in polarity of the medium, contact and fusion of the bilayers proceeds. Removal of PEG by dilution, leads to the fusion of intra-membrane particles (IMP) formed during the cell fusion process due to the replacement of PEG by isotonic buffer [Hui and Boni, 1990; Ahkong *et al.*, 1987].

The bone marrow and thymus constitute the primary lymphoid tissues and are responsible for the production and maturation of B-cells (B-lymphocytes or antibody-secreting cells). After maturation, B-cells join the blood system and travel to the secondary lymphoid organs, namely the lymph nodes and the spleen where the B-cells become activated by the presentation of antigens by macrophages and dendritic cells. As the spleen can be considered the largest mass of activated B-cells, this organ is used for cell fusion and hybridoma production.

Spleen cells alone can not survive *in vitro* and therefore need to be fused with myeloma cells. MOPC-21 was the first myeloma cell line used for spleen fusion experiments [Potter *et al.*, 1965]. As these cells are IgG producers just like spleen cells, mixed antibodies with dual specificity were derived after cell fusion. As a solution for this problem, Köhler *et al.* (1976) described a variant of MOPC-21 (P3-NS1-Ag4-1; abbreviated NS1) which lacked heavy chain synthesis due to a 50 pair deletion in the V_H framework region. Although NS1 myeloma cells could still make light chains, they were intracellular degraded and not secreted. The NS1 light chains could be rescued by binding to the introduced spleen heavy chains giving a not correctly folded and non-functional antibody. Subsequently several cell lines have been produced which synthesize neither heavy nor light chains, but which allow the production of antibody secreting hybridomas after spleen cell fusion [Goding, 1993]. These myeloma cell lines include Sp2/0-Ag-14 [Shulman *et al.*, 1978], X63-Ag8.653 [Kearney *et al.*, 1979] and NS0/1 [Galfrè and Milstein, 1981].

After the cell fusion procedure, fused cells (hybridomas) can be selected using hypoxanthine, thymidine and aminopterine (HAT). Used myeloma cell lines are deficient in hypoxanthine phosphoribosyltransferase (HPRT) or thymidine kinase (TK) required for the salvage pathway of nucleotide synthesis and will therefore die in HAT-containing medium because aminopterin blocks the normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage pathway. If myeloma and B-cells fuse, the resulting hybridoma will live indefinitely in culture because the B-cell supplies the

missing enzyme for selection in HAT-containing medium and the myeloma cell immortalizes the cell line. Unfused B-lymphocytes will only survive *in vitro* for approximately 1 week before they die [Harlow and Lane, 1988; Grimaldi and French, 1995]. An overview of the salvage pathway for nucleotide synthesis by using HAT is given in Figure 1.15.

After the production and culturing of the hybridoma in an appropriate medium, hybridoma need to be screened for the successful generation of high-affinity analyte-specific monoclonal antibodies. An ideal hybridoma screening method should be fast, reliable and easy to accomplish. For generating monoclonal antibodies against low-molecular-weight analytes (haptens, *e.g.*, pesticides, antibiotics and mycotoxins), one of the traditional methods for hybridoma screening is homologous indirect ELISA, *i.e.* the linker or spacer arm between the analyte and the protein of the coating antigen is the same as the immunogen. However, by doing so, a high proportion of false positives are frequently encountered [Cervino *et al.*, 2008]. The problem can be associated with the fact that many of the initially selected positive clones could have strong affinity to the spacer arms or the whole analyte-linker regions, which leads to poor or even no inhibition by the free target compounds [Kim *et al.*, 2003]. Since hapten heterology is often employed to modify the performance of competitive immunoassay for small molecules [Kim *et al.*, 2003; Wang *et al.*, 2011], it can also be introduced into the steps of hybridoma selection. To date, using heterologous ELISA for hybridoma screening is limited to very few kinds of monoclonal antibody generation [Liu *et al.*, 2010; Muldoon *et al.*, 2000; Shim *et al.*, 2010; Wang *et al.*, 2010]. During this research, homologous and heterologous ELISA were used and evaluated for the hybridoma screening.

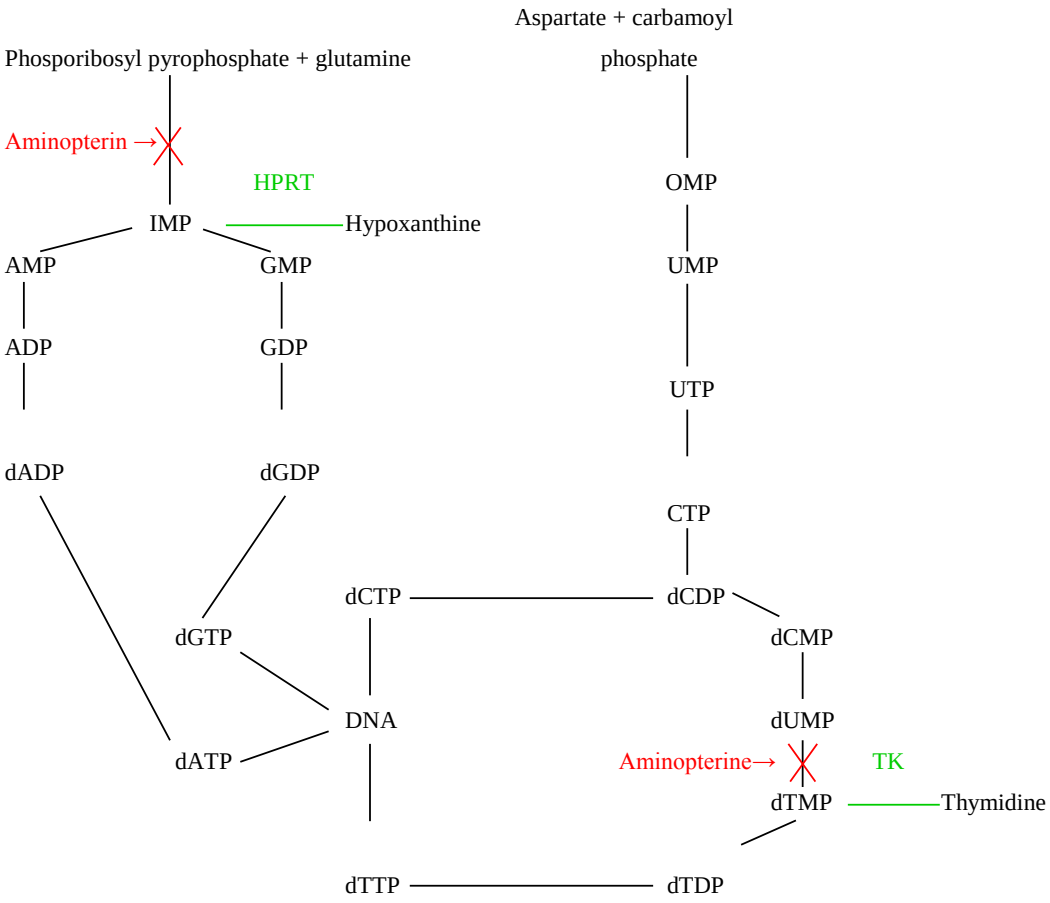

Figure 1.15 Salvage pathway for nucleotide synthesis using HAT. The ribonucleotide inosine monophosphate (IMP) forms the starting point for the synthesis of purine nucleotides, including adenine and guanine nucleobases. Orotidine monophosphate (OMP) forms the last intermediate in the biosynthesis of pyrimide nucleotides, including uracyl, cytosine and thymine nucleobases.

Adenosine monophoshate (AMP), adenosine diphosphate (ADP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), deoxyguanosine diphosphate (dGDP), deoxyguanosine triphosphate (dGTP), uridine monophosphate (UMP), uridine triphosphate (UTP), cytidine triphosphate (CTP), deoxycytidine diphosphate (dCDP), deoxycytidine monophosphate (dCMP), deoxyuridine monophosphate (dUMP), deoxythymidine monophosphate (dTMP), deoxythymidine diphosphate (dTDP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP), deoxyribonucleic acid (DNA).

1.5.3 Antibody purification

Antibody purification involves the enrichment or isolation of monoclonal antibodies from cell culture supernatant. Three different purification techniques exist based on physicochemical fractionation, class-specific affinity and antigen-specific affinity. Physicochemical fractionation includes size-exclusion chromatography (SEC), ammonium sulfate precipitation, ion exchange chromatography (IEC) and immobilized metal-ion affinity chromatography (IMAC). SEC is based on the separation of components by their molecular weight and

therefore antibodies can not be separated from proteins or other macromolecules present in the supernatant. Adding 40-50% of ammonium sulfate to the hybridoma supernant will salt out the present antibodies while other proteins or non-protein components remain in solution. Alternatives for ammonium sulfate include PEG and caprylic acid. Most often, this kind of purification is performed prior to column chromatography to improve the performance and extend the lifetime of the column. During IEC, buffers with different pH are used to positively or negatively charge proteins and withhold them on a conversely loaded column. The mechanism of IMAC is based on the interaction of proteins or peptides containing histidine residues to the nickel (Ni^{2+}) atoms on the column [Ayyar *et al.*, 2012]. The strategy is most often used to purify recombinant antibodies as they contain a terminal hexahistidine (HIS) tag. In the second purification technique, antibody separation is based on its affinity towards protein A, G or L. Protein A and G interact with the heavy constant region, whereas protein L has affinity towards the kappa light chain of an antibody. To accomplish antibody purification, protein A, G, A/G or L need to be covalently immobilized onto porous resins, such as agarose, or magnetic beads. The last purification format is based on antigen-specific affinity and for this kind of purification the antigen coupled to a protein is coated onto a surface.

For purification like IEC, IMAC or affinity purification using protein A, G, A/G or L prepared purification columns are commercially available for the manual or automatic purification using fast protein liquid chromatography (FPLC).

The choice of antibody purification method depends on several factors such as the species and the intended use. Bergmann-Leitner *et al.* (2008) evaluated the use of protein G sepharose, protein A/G sepharose, PEG and caprylic acid-ammonium sulfate precipitation. The highest yield and purity of antibody was obtained using either protein G, protein A/G or caprylic acid-ammonium sulfate precipitation for rabbit antibody purification, leaving functional antibodies. For human antibody purification, PEG precipitation leads to a concentrated antibody solution having high recovery rates and yields and an enhanced specific activity. For this origin of antibodies, protein G and protein A/G purification methods yielded statistically less functional antibody activities compared to the source material. The human or rabbit antibody avidity did not decrease during all purification methods.

For the purification of anti-DON monoclonal antibody, several purification methods are described. Maragos and McCormick (2000) describe the use of ammonium sulfate precipitation after lipoprotein removal by 5% sodium dextran sulfate and 11.1% calcium chloride for the purification of a developed anti-DON antibody. Other researchers describe the

single use of ammonium sulfate precipitation for anti-DON antibody purification [Casale *et al.*, 1988]. The use of affinity chromatography using protein A or G results also in pure and functional anti-DON antibodies [Kohno *et al.*, 2003; Xu *et al.*, 2010].

1.5.4 Antibody characterization

Most important parameters which need to be determined for the characterization of a purified monoclonal antibody include the antibody sensitivity and selectivity. Characterization is performed by the use of indirect or direct competitive ELISA or via the use of SPR technology. Calibration curves are set up where the absorbance values at 450 nm (for ELISA) or response units (for SPR) are set out in function of the competitor (antigen) concentration. Using this curve, the linear range for detection and the IC_{50} value as a measure for the antibody sensitivity can be determined. The selectivity of the antibody (or cross-reactivity) is measured by comparing IC_{50} values of chemical structurally related competitors.

Different researchers described the development and characterization of monoclonal anti-DON antibodies and an overview of the different antibodies with their IC_{50} values and cross-reactivities to structurally similar trichothecenes is presented in Table 1.4. As seen, for all of them cross-reactivities towards the masked mycotoxins 3-ADON, 15-ADON and/or DON-3G were observed, which is similar to the conclusion taken for the commercially available DON ELISA test kits (1.4.3.2). Even though legislation only considers sole DON levels, EFSA opinion highlights the importance of the determination of the total DON content of food and feed including its masked forms.

Another possible way to determine the sum of DON and its acetylated derivatives is mentioned by Xu *et al.* (1988) and Kohno *et al.* (2010). They reported the development of an antibody against tri-acetyl-DON, making it possible to determine the sum of DON and its acetylated derivatives after acetylation of all hydroxyl functions by the use of acetic anhydride.

Table 1.4 Overview of anti-DON antibodies: IC_{50} and cross-reactivity (CR) values

	[Maragos and McCormick, 2000] USDA Clone 4		[Maragos and McCormick, 2000] USDA Clone 22		[Lee *et al.*, 2013]		[Li *et al.*, 2012]		[Dos Santos *et al.*, 2011]	
Trichothecene	IC_{50} (µg/mL)	CR (%)	IC_{50} (µg/mL)	CR (%)	IC_{50} (µg/mL)	CR (%)	IC_{50} (µg/mL)	CR (%)	IC_{50} (µg/mL)	CR (%)
DON	0.013	100.0	0.018	100.0	0.023	100.0	---	100.0	---	100.0
3-ADON	0.173	7.5	0.003	632.0	*n.a.*	*n.a.*	---	34.0	---	< 0.5
15-ADON	0.004	325.0	558.0	0.3	5.5	0.4	---	< 1.0	---	333.0
NIV	1.751	0.7	> 20	< 0.1	6.0	0.4	---	< 0.1	---	5.0
T2	1.233	1.1	> 20	< 0.1	*n.a.*	*n.a.*	---	< 0.1	---	< 0.5
HT2	0.195	6.7	> 20	< 0.1	22.5	0.1	---	< 0.1	*n.a.*	*n.a.*
Fusarenon-X	*n.a.*	*n.a.*	> 20	< 0.1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
DON-3G	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
DOM	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*

1.6 DEVELOPMENT OF RECOMBINANT ANTIBODIES

In the mid-eighties, an innovative technology enabling the cloning of antibody genes and thereby bypassing the formation of hybridomas was introduced. In the new developed recombinant antibody technology, antibody genes are cloned directly from lymphocytes of immunized animals and expressed as an scFv or a fragment antigen binding (Fab) unit in bacteria. The expression of recombinant antibodies in bacteria is performed by the use of bacteriophages (phages) and therefore the term "phage display" was introduced by George Smith. By using this technology, large libraries of antibodies can be designed and built followed by the selection of appropriate antibodies (*e.g.* affinity, selectivity) through biopanning [Barbas *et al.*, 2001; Schmitz *et al.*, 2000; Tikunova and Morozova, 2009; Hoogenboom *et al.*, 1998]. Recombinant antibody technology has the advantage of low cost and high speed production of antibodies which are easy to manipulate and to adapt to different applications *e.g.* fusion with marker molecules for detection purposes.

1.6.1 Recombinant antibody generation through immunoglobulin cleavage

The cleavage of IgG molecules with papain yields Fab (Figure 1.16B) and F_c fragments as presented in Figure 1.16A. The smaller F_v antibody fragment is more difficult to produce by enzymatic digestion and is less stable than Fab fragments. Therefore a linker is introduced between V_H and V_L using recombinant technology to produce scFv (Figure 1.16C) improving its folding and stability. The size of the linker determines the association state of the scFv molecule. Linkers with more than 20 amino acids favour monomeric molecules, whereas dimers are favoured with linkers of around 5 amino acids. ScFv containing linkers with a size in between 5 and 20 amino acids, appear as a mixture of monomers and dimers [Barbas *et al.*, 2001]. The use of bispecific antibodies by linking two different scFv (diabody) or two different Fab ($F(ab')_2$) molecules or one scFv with one Fab molecule is described for the induction of double epitope binding (from different or the same antigen) [Lu *et al.*, 2002; Holliger and Winter, 1997]. Several clinical trials have been conducted using bispecific antibodies as mediators to redirect immune effector cells such as natural killer cells and T-cells towards tumour cells in order to enhance their destruction [Chames and Baty, 2009].

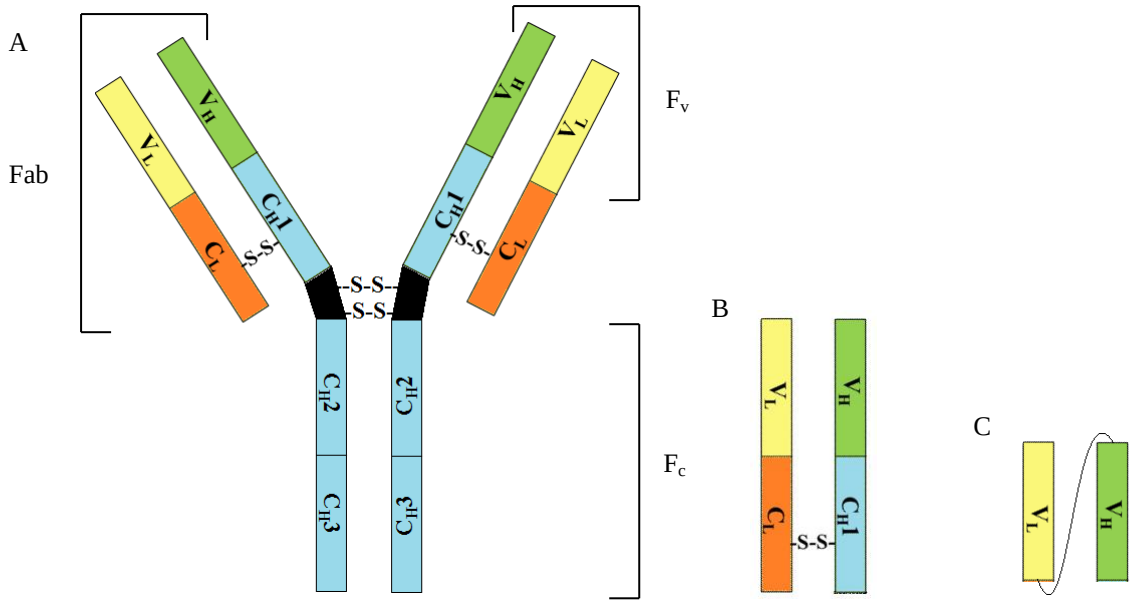

Figure 1.16 F_v, Fab and F_C fragments of an IgG molecule (A); Fab recombinant antibody (B); scFv recombinant antibody (C)

1.6.2 Recombinant antibody generation by polymerase chain reaction

The process of recombinant antibody production starts with the isolation of antigen-stimulated lymphocytes (B-cells) from the spleen of an immunized animal followed by mRNA extraction. Hybridomas are also a source of mRNA that predominantly or exclusively encodes a single antibody. After reverse transcription of mRNA into complementary deoxyribonucleic acid (cDNA), the cDNA is used as a template pool for the amplification of the antibody V_H, V_L, constant heavy 1 (C_H1) and constant light (C_L) chain genes. Specific complementary oligonucleotide primers are chosen to eventually include extra restriction sites and amplification by polymerase chain reaction (PCR) of antibody variable and constant chain genes is performed [Karu *et al.*, 1995]. For the amplification of light chain genes, two different batches of PCR primers need to be selected to distinguish between lambda (λ) and kappa (κ) light chain. The ratio between κ and λ chains varies significantly among different Ig classes or subclasses and animal species. In most murine species, which are the preferable laboratory animal species, a κ/λ ratio of 95/5 is observed. Reasons for this high ratio could be a reflection of the large κ variable region gene repertoire (> 200 genes) relative to the λ variable region repertoire (two genes) or the presence of specific genes regulating B-cell differentiation [Woloschak and Krco, 1987]. Isotyping of hybridoma light chains can be performed before hand and is recommended to avoid problems in monoclonal antibody

cloning during transcription of less or no functional variable region genes [Schaefer *et al.*, 2010].

After PCR amplification and subsequent gel-purification of V_H, V_L, C_H1 and C_L chain genes to remove any residual primer, the genes are further used for scFv and Fab synthesis. V_H and V_L chain genes are assembled using splicing by overlap extension (SOE)-PCR to form scFv recombinant antibody genes. The linker in between V_H and V_L domains is generated either by overlap of the two inner primers or by adding a linker primer whose sequence covers the entire linker or more. For Fab formation, C_H1 and C_L chain genes are first amplified in a phage vector to include extra gene sequences to give rise to separate polypeptide production. Then, SOE-PCR is performed for V_H – C_H1 and V_L – C_L coupling. A second SOE-PCR gives rise to the coupling of both SOE-PCR 1 reaction products and therefore the formation of the Fab recombinant antibody genes. The gel-purified SOE-PCR products are digested with restriction enzymes to create ends compatible with sites at the cloning phage vector [Horton *et al.*, 2013; Woloschak and Krco, 1987; Barbas *et al.*, 2001]. The total scFv synthesis by using PCR followed by SOE-PCR is illustrated in Figure 1.17.

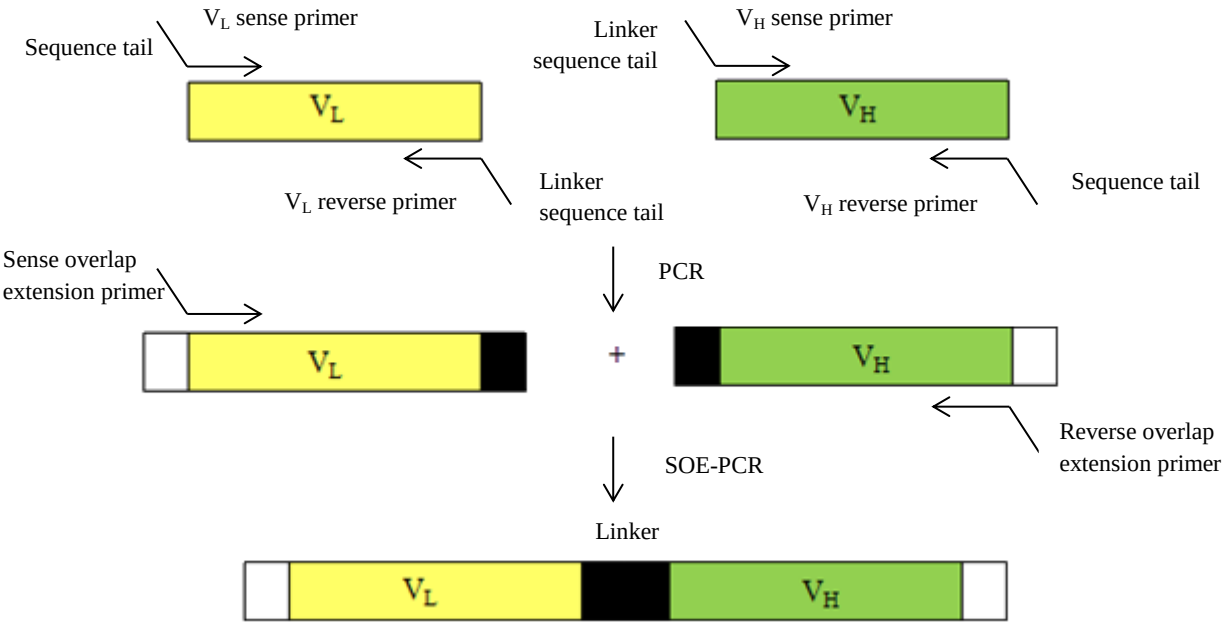

Figure 1.17 Procedure of scFv synthesis by the use of PCR and SOE-PCR adding a linker primer whose sequence covers the entire linker

The development of several scFv and Fab anti-DON recombinant antibodies from hybridoma cell lines producing monoclonal antibodies is described by previous researchers. Maragos *et al.* (2012) compared their developed scFv and the original anti-DON monoclonal antibody using direct ELISA and observed a lower sensitivity of the scFv (IC_{50} = 36.1 ng/mL) compared to the monoclonal antibody (IC_{50} = 13.8 ng/mL) even though no differences in cross-reactivities were determined. The results conclude no or little change in antibody selectivity and affinity when removing major framework regions and are conform the conclusion taken by Wang *et al.* (2007). These results are in contradiction with Choi *et al.* (2004) where lower affinity of the scFv antibody fragments ($K_D = 3.7 \times 10^{-6}$ M) was observed compared to the parental monoclonal antibody ($K_D = 8.8 \times 10^{-8}$ M) [Maragos *et al.*, 2012; Wang *et al.*, 2007; Choi *et al.*, 2004]. In Romanozzo *et al.* (2010), the application of a biotinylated anti-DON Fab fragment in an electrochemical immunosensor was evaluated in different sample matrices (wheat, breakfast cereal and baby-food). The authors considered the immunosensor as robust and insensitive to sample matrix with a quite low DON sensitivity (IC_{50} = 380 ng/mL).

1.6.3 Phage display technology

Phage display is currently the most widespread method next to the use of yeasts, ribosomes and bacterial cells for the display and selection of large collections of antibodies and for the engineering of selected antibodies [Sheedy *et al.*, 2007]. Phage display includes the use of filamentous phages which are visualised as flexible rods of about 1 µm long and 6 nm wide. Inside the rod of wild-type strain phages lies the single-stranded viral DNA with up to 6 407 nucleotides. At one side of the rod, five copies of the minor coat proteins pIII and pVI (genes III and VI, respectively) are present, while on the other side minor coat proteins pVII and pIX (genes VII and IX, respectively) are included. PVIII proteins are present over the total length of the phage particle [Smith and Petrenko, 1997; Barbas *et al.*, 2001]. The structure of a phage with its coat proteins is illustrated in Figure 1.18.

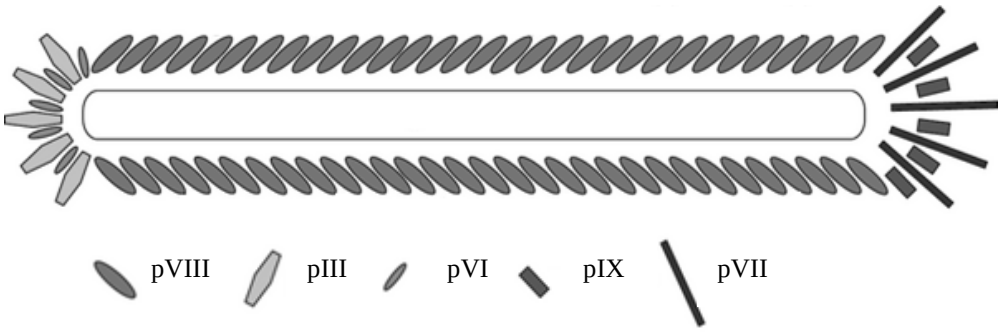

Figure 1.18 Structure of phage particle with pIII, pVI, pVII, pVIII and pIX coat proteins

Phages are viruses able to infect strains of *Escherichia coli* (*E. coli*) displaying an F pilus. Infection is initiated by attachment of the N-terminal domain of pIII to the tip of the pilus, which is the part of the phage that enters the cell first. As the process continues, the coat proteins of the phage dissolve into the surface of the bacterial cell, whereby the phage DNA enters the cytoplasm and turns into a replicative plasmid-like (RF) molecule by the *E. coli* replication enzymes. Double-stranded phage DNA is formed in the bacterial host and forms a template for transcription and translation of phage proteins. Continuous replication of the double-stranded DNA is followed by extrusion through the cell envelope by the use of coat proteins from the cell membrane and emerging as completed virions. These virions are secreted continuously without killing the bacterial host [Barbas *et al.*, 2001; Smith and Petrenko, 1997; Tikunova and Morozova, 2009]. An overview of the infection process is shown in Figure 1.19.

Smith and Petrenko (1997) showed that foreign DNA fragments could be inserted into filamentous phage gene III or gene VIII, which codes for the phage coat protein pIII and pVIII, to create a fusion protein with the foreign sequence in the amino-terminal domain. The foreign DNA fragments include the genetic material encoding scFv and Fab recombinant antibodies. Expression of the fusion product in the *E. coli* host and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle.

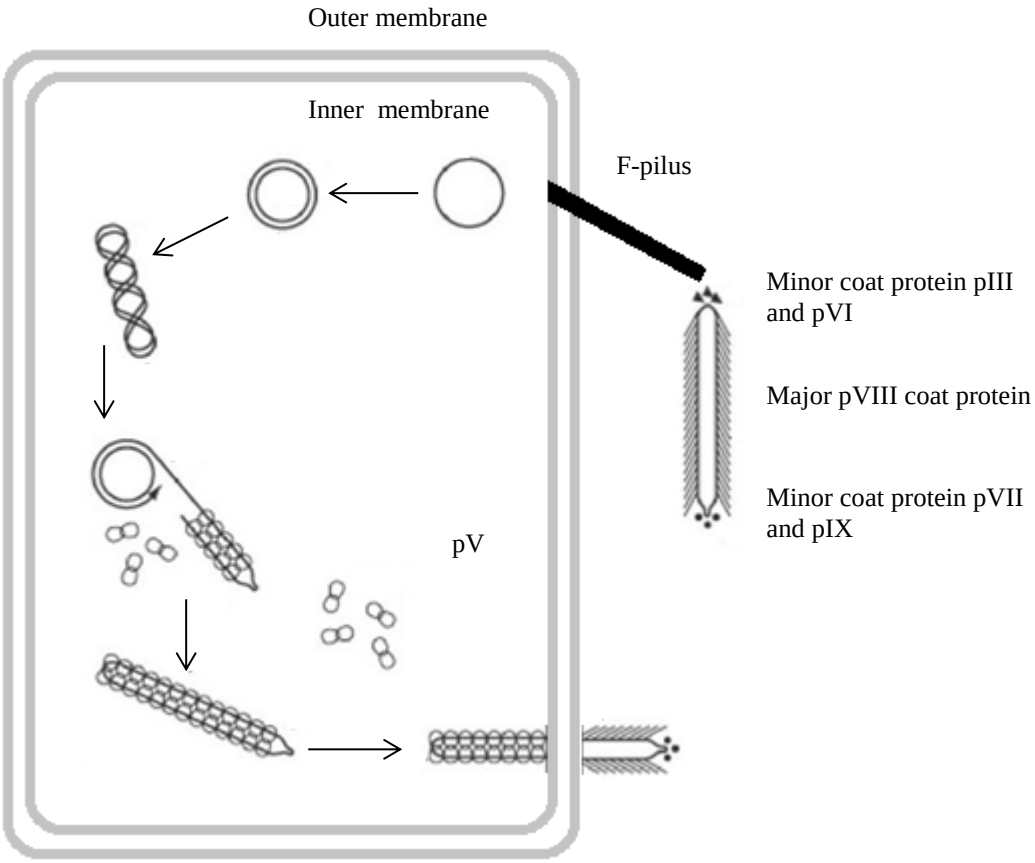

Figure 1.19 Infection process of *E. coli* with phage

Nowadays, small plasmid vectors or phagemids, which contain appropriate cloning sites and a phage packaging signal, have become a more popular type of display vectors. Phagemids have the advantage of having high transformation efficiencies and are therefore ideally suited for the generation of large antibody libraries. Other vector features include an antibiotic resistance marker, an origin of replication, a promotor region and an affinity tag to aid in purification. Helper phage (*e.g.* M13K07 or VCSM13) is also required to supply all necessary structural proteins for correct packaging of the phage particle. The *lacZ* promoter is the most common promoter used to control expression. Expression may be suppressed by the addition of the catabolic repressor glucose or induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) due to its binding to the *lac* repressor, thereby releasing the tetrameric repressor from the lac operator and allowing transcription of genes in the lac operon including the antibody genes.

The pComb3 phagemid vector containing the gene III is designed to express recombinant antibodies on the surface of filamentous phage or to express them as soluble proteins. The expression of soluble proteins requires the excision of gene III encoding the pIII coat protein.

The pComb3H phagemid vector (Figure 1.20A) is modified from pComb3 as the genes have been reversed and a single *lacZ* promoter is incorporated instead of the two separated *lacZ* promoters in the pComb3 phagemid. The transcription of the polypeptide chain is terminated by the *trp* transcription terminator followed by the direction of the chain to the periplasm due to the *ompA* and/or *pelB* signal peptide. Excision of gene III is necessary as well for the expression of soluble proteins. Another pComb3 variant is the pComb3X (Figure 1.20B), where the amber codon has been inserted between the 3' SfiI restriction site and the 5' end of gene III. This allows soluble protein expression without excising the gene III fragment. Two different tags, namely the HIS tag and the hemagglutinin (HA) decapeptide tag have been inserted for IMAC purification and detection using anti-HA secondary antibody, respectively. The pComb3 phagemid and analogues are used by several researchers for the expression of Fab, scFv, diabody or other proteins [Urushibata *et al.*, 2010; Andris-Widhopf *et al.*, 2000; Barbas *et al.*, 2001]. The pComb8 phagemid vector contains the gene VIII and has been used for the display of large fusion proteins such as Fab antibody [Wang *et al.*, 1997; Scholthof *et al.*, 1997; Barbas *et al.*, 2001]. The preferable use of the pComb3 or pComb8 vector for the expression of Fab is dependent from case to case. For both pComb3 and pComb8 vectors different restriction sites are included, such as the SfiI, SacI, SpeI and NheI as illustrated in Figure 1.20.

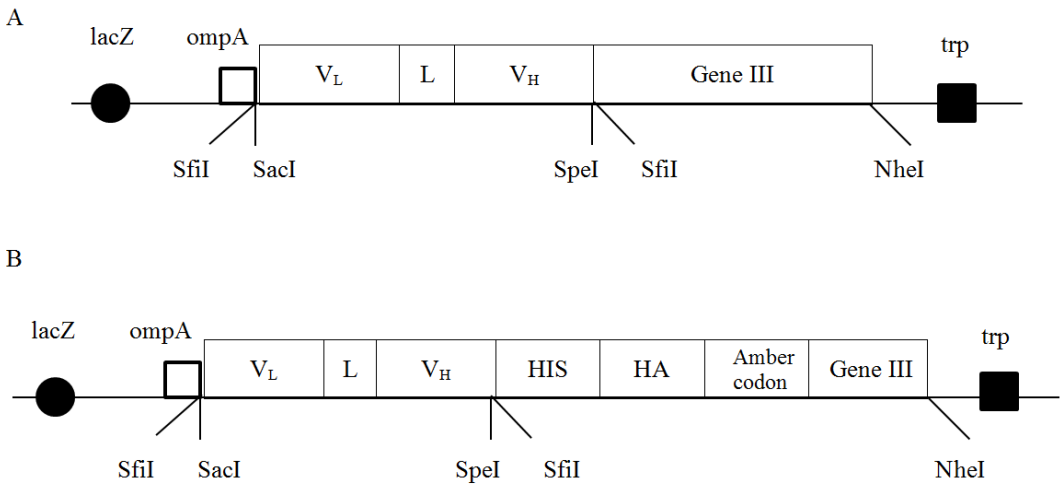

Figure 1.20 pComb3H (A) and pComb3X (B) phagemid vectors with *lacZ* promoter, SfiI/SacI/SpeI/NheI restriction sites, *trp* transcription terminator, *ompA* signal peptide, variable light gene chain (V_L), variable heavy gene chain (V_H), linker gene (L), gene III, HIS tag gene, HA tag gene and amber codon.

Another type of phagemid vector is the pET expression system, derived from the pBR322 plasmid and engineered to take advantage of the features of the T7 bacteriophage gene 10

promoting high-level transcription and translation. As the phagemid-encoded RNA polymerase is highly specific for the T7 promoter, only the target gene in the *E. coli* cell will be transcribed preventing transcriptional read-through of unwanted plasmid sequences and eliminating plasmid instability due to the transcription of toxic proteins. A wide variety of pET vectors (transcription and translation vectors) are available differing in leader sequences, expression signals, fusion tags and restriction sites.

After translation, the antibody polypeptide chain needs to be correctly folded by the addition of disulfide bonds. Inter-domain disulfide bonds link together heavy and light chain and are only present in Fab, whereas intra-domain disulfide bonds responsible for the immunoglobulin fold and stability are present in two copies in scFv and four copies in Fab. Due to the oxidizing environment and the disulfide bond (*dsb*) machinery, disulfide bond formation takes place in the periplasm of the *E. coli* cell (Figure 1.21). The first part of the *dsb* machinery includes the oxidative pathway in the periplasm of *E. coli* cells introducing disulfide bonds into newly translocated synthesized proteins catalyzed by DsbA and DsbB proteins. The periplasmic thiol-disulfide oxidoreductase DsbA and the cytoplasmic inner-membrane protein DsbB drive the formation of disulfide bonds by thiol-disulfide exchange. During thiol-disulfide exchange, the two cysteines of the DsbA are reduced followed by reoxidation of DsbA due to the presence of the DsbB membrane protein making DsbA ready to interact with another unfolded protein. To rescue misfolded polypeptides, an isomerization system is present in *E. coli* cells that proofreads and corrects non-native disulfides catalyzed by thiol-disulfide oxidoreductase DsbC. DsbC is maintained in a reduced active state by a continual flow of electrons from the cytoplasmic thioredoxin to DsbC through the DsbD cytoplasmic membrane protein [Heras *et al.*, 2009; Sevier and Kaiser, 2002; Guglielmi and Martineaum, 2009].

Cytoplasm production and folding of recombinant antibodies instead of periplasm folding is described by using mutant *E. coli* strains allowing the formation of stable disulfide bonds by the elimination of disulfide bond reduction. A mutant strain was selected containing knockouts in the thioredoxin and glutathione pathway (*trx*B and *gor*) responsible for the maintenance of the reducing environment with an extra point mutation in the *ahp*C gene taking care of normal growth of bacteria. This mutant *E. coli* strain allowed the expression of scFv and Fab fragments at levels comparable to that obtained in the periplasm [Guglielmi and Martineaum, 2009; Ritz and Beckwith, 2001].

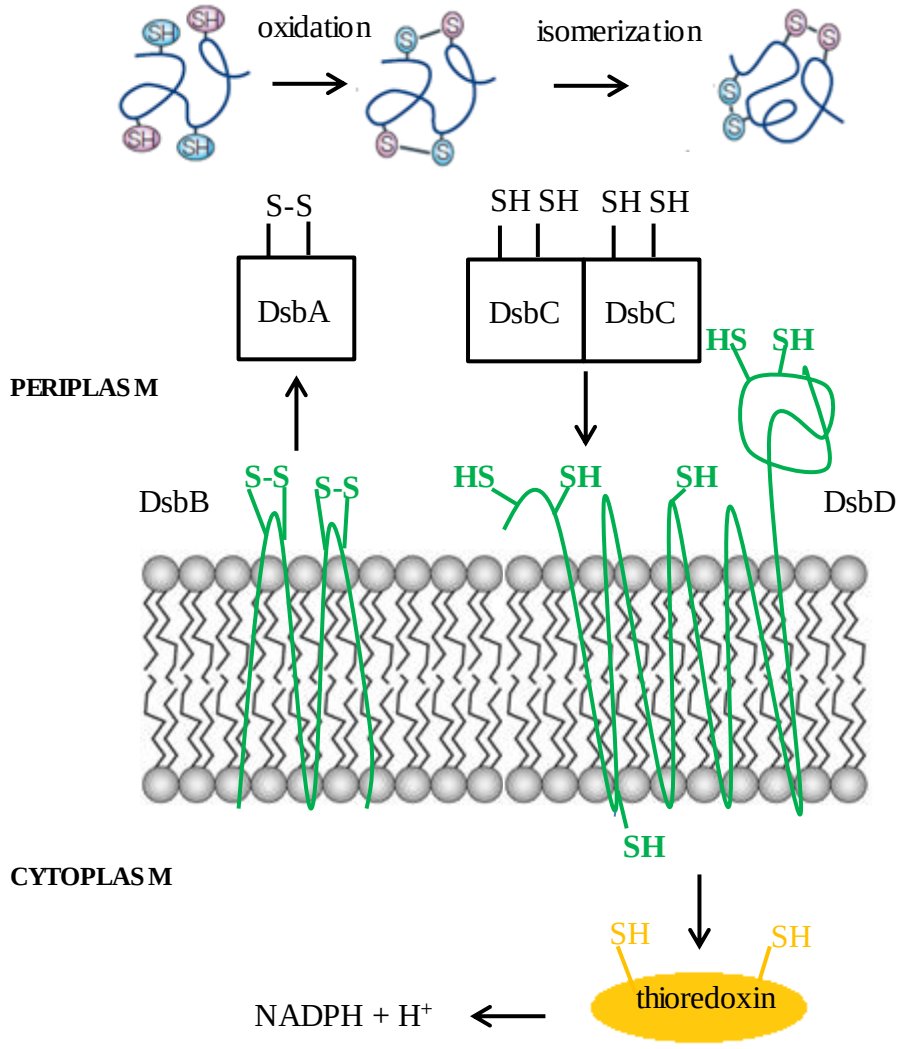

Figure 1.21 The *dsb* machinery for protein oxidation and isomerization in the periplasm of *E. coli* cells. The periplasmic thiol-disulfide oxidoreductase DsbA and the cytoplasmic inner-membrane protein DsbB drive the formation of disulfide bonds by thiol-disulfide exchange. To rescue misfolded polypeptides, an isomerization system is present that proofreads and corrects non-native disulfides catalyzed by thiol-disulfide oxidoreductase DsbC. DsbC is maintained in a reduced active state by a continual flow of electrons from the cytoplasmic thioredoxin to DsbC [Sevier and Kaiser, 2002].

In some situations (*e.g.* cellular stress), an accumulation of the antibody polypeptide chains into insoluble aggregates, known as inclusion bodies, can occur. A number of methods for the redirection of proteins from inclusion bodies into the soluble fraction or modification of the expression strategy to obtain soluble expression, are described. Techniques to limit the *in vivo* aggregation of recombinant antibody include the cultivation of transformed *E. coli* cells at low temperature, batch cultivation and co-overexpression of molecular chaperones. Specific to anti-DON scFv, Choi *et al.* (2003) described the use of DnaK-DnaJ-GrpE to improve the

cytoplasm solubility. Misfolded proteins can be engineered or pushed to gain soluble expression by the presence of affinity tags, expression of solubility enhancing maltose binding protein and N-utilizing substance A or by site-directed mutagenesis [Sorensen and Mortensen, 2005].

1.6.4 Biopanning of phage display library

Phage display libraries can be divided into three types, namely naive, immune and synthetic libraries. Naive libraries are generated from genetic material that has never been exposed to the antigen(s) prior to library construction, in contrast immune libraries are created using lymphocytes from an animal immunized with the antigen of interest. Synthetic libraries are made by the *in vitro* assembly of antibody gene segments and are used together with the naive libraries for any antigen without immunization [Sheedy *et al.*, 2007].

During biopanning using previously described immunochemical methods (1.4.3), the phage display library is subjected to affinity selection where recombinant antibodies are selected based on their affinity towards a specific antigen. In principle, very large naive or synthetic libraries offer the possibility to select high-affinity antibodies with any desired specificity without the need for immunization. If an antibody with desired affinity is not isolated from such a library, the affinity can be adapted through mutagenesis or immunization may be the best option.

One needs to decide whether to construct a library in the scFv or Fab antibody fragment format. The preparation of an scFv format has the following advantages: 1) less PCR steps are involved for scFv library construction, 2) the ability to form scFv diabodies enhances the avidity for the antigen and enhances the selectivity due to double epitope recognition, 3) higher scFv yield during *E. coli* culturing. The disadvantage of the scFv format is its property to multimerize, which is not the case for the Fab format as it is a stable, well-characterized protein fragment. The major disadvantage of Fab recombinant antibodies is the generally lower expression levels in *E. coli* [Barbas *et al.*, 2001].

Doyle *et al.* (2008) describe the panning of an immune phage library based on single-domain variable heavy chain antibody fragments derived from a llama immunized with DON-protein conjugates. A 15-ADON recombinant antibody was obtained by panning the library using DON-15-OVA conjugates. As most of the other described developed recombinant anti-DON antibodies are derived out of hybridomas producing a monoclonal anti-DON antibody, no

panning is necessary and therefore not described. Another novel technique is the screening of phage library based on mimotope DON peptides which bind to anti-DON monoclonal antibodies to form an alternative for mycotoxins in immunoassays and for the further development of antibodies [Yuan *et al.*, 1999].

1.6.5 Computational homology modeling and molecular docking

The ability to model recombinant antibodies based on the determined DNA and amino acid sequences and visualize antibody-antigen interactions in three-dimensional space is a powerful tool prior to antibody engineering. The structures of many antibodies have been determined by X-ray crystallography and are stored at the online Protein Data Bank (PDB). Because of the homology among antibody framework domains and the secondary structures of CDRs, it is possible to search for a computational model of a new antibody in order to predict which CDRs and other residues are important to epitope binding. During this research, the SWISS-MODEL web server was used to search for a recombinant antibody model based on the amino acid sequence input of the newly derived recombinant antibody [Schwede *et al.*, 2003]. The derived model can be further used for docking experiments predicting the conformation of an antigen-antibody complex from its separated components. Using docking servers or programs including SwissDock and UCSF Chimera, the PDB file of the model recombinant antibody needs to be loaded together with the PDBQ file of the target antigen. The docking server automatically generates possible antigen-antibody clusters based on physical non-covalent interaction, which are sorted by a scoring function and the lowest energy value (kcal/mol). Although computational docking is still in its infancy, there is no doubt that it will become more relevant and widespread in the coming years [Pedotti *et al.*, 2011; Karu *et al.*, 1995].

1.6.6 Antibody engineering

The antibodies selected after several rounds of biopanning are functional, even though affinity and specificity can be increased. Once the DNA and amino acid sequences of the selected recombinant antibodies are known and computational modeling and docking experiments are performed, *in vitro* mutagenesis can be applied to insert, delete, change one or several amino acids or exchange entire variable domains. These mutagenesis procedures can have an influence on affinity and/or specificity due to a change in orientation of the V_H and V_L

domains, a change in length of the CDRs that enlarge or shrink the binding pocket and an increase in flexibility of the CDRs [Karu *et al.*, 1995; Moore, 1989].

It is generally known that the greatest variation of antibody structure and thus antigen binding specificity lies in the three polypeptide loops forming the CDRs of each V_H and V_L. The third CDR of the V_H shows the largest diversity in composition and length and is, therefore, considered as most important amino acid sequence where mutagenesis can influence the specificity of the antibody [Wyat *et al.*, 1999].

Previous studies have revealed the importance of particular amino acids in the CDR regions of antibodies. The dominant role of tyrosine as a molecular contact mediator and serine or glycine for providing space and flexibility is mentioned in Koide and Sidhu (2009). The amphipathic character of tyrosine is able to tolerate the environmental hydrophilic or hydrophobic change that occurs during antigen-antibody complex formation. And this can be considered together with its large size enhancing non-covalent interactions and low side chain entropy as reasons to consider tyrosine as an important amino acid [Fellouse *et al.*, 2004]. Cysteine residues are also important for the formation of disulfide bridges and correctly folding of the antibody. Replacing disulfide bridges by valine-alanine pairs leaving a cysteine-free scFv recombinant antibody was found to increase the stability of the scFv, but seemed to be less active than its parent scFv [Worn and Pluckthun, 2001; Schier *et al.*, 1996].

The changes in amino acid chain can be introduced by several types of *in vitro* mutagenesis procedures grouped in three broad categories, namely random mutagenesis, site-directed mutagenesis and shuffling. Using random mutagenesis, mutations are randomly introduced throughout the gene by error-prone PCR or bacterial mutator strains. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations through out the entire gene sequence. The second possibility for random mutagenesis is the selection of antibodies from a library amplified in a bacterial mutator strain of *E. coli*.

Site-directed mutagenesis can be performed by the existing mutational hot spots. Chowdhury and Pastan (1999) have developed a theory introducing the presence of hot spot sequences in the DNA encoding the variable domains of antibodies. Mutations of the identified hot spot sequences led to a 15- to 50- fold increase in affinity, whereas mutations outside the hot spot only increased the affinity four times. Parsimonious mutagenesis is another type of site-directed mutagenesis developed by Balint and Larrick (1993). The principle of this method is to use mutagenesis codons to mutate about 50% of all targeted amino acids while keeping the

other 50% intact (wild type). Therefore, crucial amino acids are conserved in about 50% of the clones for one mutation and 25% for two mutations [Chames and Baty, 1998].

Shuffling of antibody genes can be accomplished by chain shuffling, DNA shuffling, staggered extension process (StEP) or variations of previously mentioned techniques. Using chain shuffling, a particular heavy and light chain from an antibody recognizing a specific antigen is shuffled against a complementary library of light and heavy chains. This type of mutagenesis is limited to antibodies selected from immune libraries [Kang *et al.,* 1991]. DNA shuffling was developed by Stemmer (1994) and involves the digestion of an antibody gene with DNase I to create a pool of random DNA fragments followed by annealing cycles in the presence of DNA polymerase. DNA shuffling offers several advantages over random and site-directed mutagenesis as it can be used for longer DNA sequences (> 1 kb) and gives the possibility to select clones with mutations outside the binding site of the antibody. StEP is a form of DNA shuffling developed in 1998 by Zhao *et al.* (1998) and consists of priming the template sequences followed by several cycles of denaturation and short polymerase catalyzed annealing. During each cycle, single DNA fragments can anneal to different templates based on sequence complementarity and extend further to create recombinant genes [Sheedy *et al.*, 2007].

The effect on antibody affinity and specificity after every possible mutagenesis procedure is determined by binding studies. A ranking is made of all antibody-bound phages of the secondary phage library and identification is performed of the amino acid change able to increase the strength of the antigen-antibody interaction [Xu *et al.*, 2012; Hoogenboom, 2005].

CHAPTER 2

THE DEVELOPMENT OF AN LC-MS/MS METHOD FOR THE DETECTION AND QUANTITATION OF DEOXYNIVALENOL IN WHEAT DUST

Adapted from

Sampling of Wheat Dust and Subsequent Analysis of Deoxynivalenol by LC-MS/MS

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Deoxynivalenol Content in Wheat Dust versus Wheat Grain: a Comparative Study

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CHAPTER 2: THE DEVELOPMENT OF AN LC-MS/MS METHOD FOR THE DETECTION AND QUANTITATION OF DEOXYNIVALENOL IN WHEAT DUST

SUMMARY

An LC-MS/MS method was developed and validated for the determination of DON in wheat dust. Extraction was carried out with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. Analysis was performed using a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Quattro Premier XE mass spectrometer. The method was validated according to the criteria mentioned in Commission Decision 2002/657/EC. Due to a high contamination level of wheat dust compared to wheat, LOD and LOQ levels were obtained of 358 µg/kg and 717 µg/kg, respectively.

A study, set up in the growing season 2011-2012, was designed to obtain quantitative data on the occurrence of DON in wheat grain and the corresponding wheat dust. The field experiment consisted of a complete randomized block design with five wheat varieties sown on a field on which maize was grown in the previous season. The impact of the tillage method and the influence of the wheat variety resistance on the DON content of wheat and wheat dust were investigated. The accumulation of DON in wheat dust was confirmed and a linear and sigmoidal relationship between the DON content in wheat dust versus wheat grain was determined. DON reduction was obtained by ploughing and by sowing moderately resistant wheat varieties. As wheat dust provides equal results and solves the problem of heterogeneity during sampling of conventional wheat matrix, the sampling of wheat dust can be considered as a promising alternative.

Keywords: LC-MS/MS, deoxynivalenol, wheat dust, validation, correlation

2.1 INTRODUCTION

The sample selection of grain cereals can be made more easy to perform, less labour-intensive, less costly with a lower error of uncertainty by sampling dust instead of parts of the cereal bulk lot [Maestroni and Cannavan, 2011; Whitaker and Slate, 2012; Whitaker and Slate, 2005]. Previously, the accumulation of mycotoxins on dust particles was confirmed [Krysinska-Traczyk *et al.*, 2001; Tangni and Pussemier, 2006]. Dust is created when grain is transported through closed systems such as the (un)loading of a truck. Also, accumulation in mills and storage facilities is described [Halstensen *et al.*, 2006; Nordby *et al.*, 2004]. Because of the small size of the dust particles and the accumulation of DON in the dust fraction, the proposed sampling technique is promising for the determination of DON in wheat.

The aim of the study described in *Chapter 2* was twofold. First of all to develop and validate an LC-MS/MS method for the determination of DON in wheat dust. The second aim was to obtain quantitative data on the occurrence of DON in wheat grain and dust taken into account the different methods controlling FHB infection like crop rotation, tillage (*i.e.* ploughing) and the choice of wheat cultivar.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and chemicals

LC-MS grade methanol was obtained from BioSolve BV (Valkenswaard, The Netherlands). Acetonitrile (Analar Normapur), *n*-hexane (Hipersolv Chromanorm) and ammonium acetate (analytical reagent) were purchased from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was obtained from Merck (Darmstadt, Germany). Ethyl acetate and dichloromethane were purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium).

DON (10 mg) and DOM (50 ng/µL) standards were purchased from Fermentek (Jerusalem, Israel) and Coring System Diagnostics (Gernsheim, Germany), respectively. The DON standard was dissolved in methanol in a concentration of 1 µg/µL and stored at 4 °C. Working solutions of DON (10 ng/µL) and DOM (2.5 ng/µL) were prepared in methanol and stored at -18 °C.

2.2.2 Determination of dust particle size and quantity

Dust fractions were laboratory fractionated by the use of a vibratory sieve shaker (Retsh, Aartselaar, Belgium). Freshly harvested wheat samples were cleaned by sieving, separating all grain particles smaller than 2.5 mm. A particle size distribution was built up with the particles smaller than 2.5 mm using different sieve pore diameters on the sieve shaker. The bottom fraction, which can be considered as dust, was further divided into > 100 µm, 50 - 100 µm and < 50 µm. The quantity of dust of each of these fractions was determined.

2.2.3 Dust collection

Dust was produced out of wheat samples by the use of a dust collection facility (Figure 2.1). Wheat samples were transferred through the dumping pit to the vertical elevator system via the Archimedes screw. At the highest point of the system the grains fall into the vertical bin on top of which the dust collection device (*i.e.* vacuum cleaner) was mounted. The dust was collected by removing the paper bag of the vacuum cleaner and after weighing the dust was transferred into closed containers.

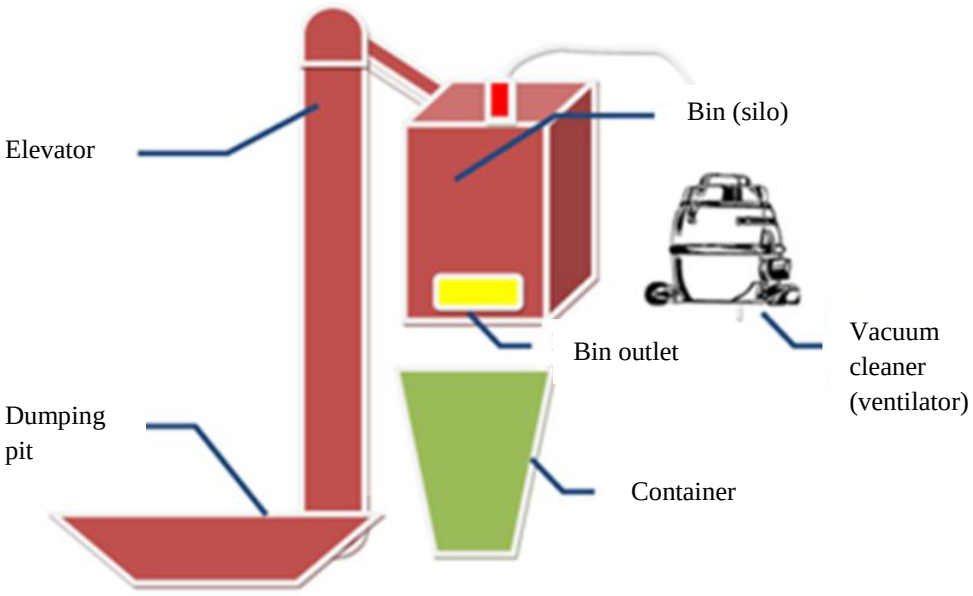

Figure 2.1 Schematic view on the laboratory dust collection facility

2.2.4 Sample preparation

One gram of dust sample was weighed in a Gosselin extraction tube (50 mL). Stock solution (20 µL) of the internal standard DOM (2.5 ng/µL) was added. The dust samples were vortexed (Labinco BV, Breda, The Netherlands) and soaked for 15 min. The extraction was performed with 10 mL acetonitrile/water/acetic acid (79/20/1, v/v/v) for 60 min using the Agitator decanter overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France) followed by centrifugation at 3 000 g for 15 min. The extraction residue was filtered using a Whatman No 4 filter (VWR International, Zaventem, Belgium). To the filtered residue 10 mL of hexane was added for defatting, followed by shaking for 15 min using the Agitator decanter overhead shaker. After centrifugation at 3 000 g for 15 min, the hexane layer was removed and the filtrate was evaporated until dryness under a stream of nitrogen at 40 °C. Then, the dry residue was redissolved in 100 µL injection solvent methanol/water/acetic acid (41.8/57.2/1, v/v/v) with 5 mM ammonium acetate. The redissolved sample was centrifuged for 10 min at 10 000 g prior to LC-MS/MS analysis.

2.2.5 LC-MS/MS analysis

LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI

interface by injecting a volume of 10 µL. Chromatographic separation was performed using an Acquity UPLC BEH C_{18} column (1.7 µm, 100 mm x 2.1 mm i.d.) equipped with a guard column of the same material (5 mm x 2.1 mm i.d.) supplied by Waters (Milford, MA, USA). The column was kept at 60 °C, and the temperature of the autosampler was set at 10 °C. A mobile phase consisting of water/methanol/acetic acid (94/5/1, v/v/v) containing 5 mM ammonium acetate (A) and methanol/water/acetic acid (97/2/1, v/v/v) containing 5 mM ammonium acetate (B) was used at a flow rate of 0.3 mL/min. The gradient elution programme started at 99% mobile phase A with a linear decrease to 50% mobile phase A in 3 min. A linear increase to 99% mobile phase B was established in 0.5 min. An isocratic gradient of 99% mobile phase B was initiated at 3.5 min for 2 min. Re-equilibration to 99% mobile phase A was established after 3 additional min. The duration of each UPLC run was 8.5 min. The mass spectrometer was operated in the positive electrospray ionization (ESI^+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures: 120 and 400 °C, respectively; capillary voltage: 3.2 kV; argon collision gas: 1.15 x 10^{-2} mbar; cone nitrogen and gas flow: 50 and 800 L/h, respectively. The data acquisition was performed using SRM. The optimized MS/MS parameters for the precursor ion and product ions of DON and DOM were determined. DON was set at *m/z* 297.10 ($[M + H]^+$) with product ions of *m/z* 231.20 (quantifier ion) and *m/z* 249.20, while DOM was determined at *m/z* 281.00 ($[M + H]^+$) with product ions of *m/z* 109.10 (quantifier ion) and *m/z* 137.00. The mycotoxins eluted at 2.54 min and 3.10 min, respectively. Masslynx version 4.1 and Quanlynx version 4.1. software (Micromass, Manchester, UK) were used for data acquisition and processing.

2.2.6 Method validation

The method was validated in terms of linearity, apparent recovery, LOD, LOQ, precision and accuracy based on the Commission Decision 2002/657/EC of 12 August 2002 concerning the performance of analytical methods and the interpretation of results. Although this EU legislation is not particularly established for dust analysis, it was chosen as a reference because of its clear criteria for LC-MS/MS contaminant analysis.

Blank wheat dust was spiked during 3 consecutive days at 6 different concentration levels of 250, 500, 1 000, 3 000, 9 000, 18 000 µg/kg with DON. DOM was added as internal standard and the quantitation was performed by the use of the relative standard peak area. A linear regression was applied.

The apparent recovery was calculated by the use of a matrix-matched calibration curve. In detail, blank dust samples were spiked with DON at different concentration levels and were analysed using the optimized LC-MS/MS method. The observed signal was plotted against the actual concentration. The measured concentration was determined using the calibration curve, and the apparent recovery was calculated by Equation 2.1.

Equation 2.1

% apparent recovery = measured concentration (µg/kg) / actual spiked concentration (µg/kg) x 100

LOD and LOQ were determined as 3, respectively 6 times the standard error of the intercept, divided by the slope of the standard curve. Also, the LOD and LOQ values were verified according to the IUPAC guidelines, stating that the signal-to-noise ratio (*s/n*) should be more than 3 and 10 times, respectively.

The intra-day precision or repeatability (RSD_r) of the method was determined by repeated analysis on the same day of dust samples spiked at different concentration levels (1 000, 3 000, 7 500, 9 000, 17 000 µg/kg). The same experiments were performed on 3 consecutive days for the determination of the inter-day precision or within-laboratory reproducibility (RSD_R) and accuracy. Out of the accuracy data, biases were evaluated and a conclusion was taken according to the acceptability of the method. All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 21.

2.2.7 Field trial and sample pretreatment

The experimental field trial was set up to investigate the influence of maize-wheat rotation, tillage method and wheat variety on the DON content of wheat grain and the corresponding wheat dust. Therefore, a randomized complete block design with two soil tillage (*i.e.* ploughing) systems, five wheat varieties and four replications was set up. During the growing season 2011, maize was grown on the experimental field. After harvesting the maize, one half of the field was ploughed before sowing, while on the other part wheat was sown after minimal tillage. In November 2011, five winter wheat varieties, namely Azzerti and Homeros (susceptible) and Sahara, Mulan and Tabasco (moderately resistant) were sown. One fungicide treatment was applied with a fungicide containing a strobilurin and a triazole. The field trial is explained in detail in Landschoot *et al.* (2013).

To estimate the DON content in the fresh harvested wheat derived from the different wheat varieties under the different tillage conditions, wheat grains were firstly analysed for DON presence using the Veratox® DON 5/5 ELISA test kit following the manufacturer's protocol (Biognost, Neogen, Leest, Belgium).

The mixing of the collected field samples (± 30-35 kg) was based on the fact that there was no significant influence of the pre-crop; only tillage or no tillage seemed to be important. So wheat derived from the same variety and with the same soil treatment were mixed to derive ten different wheat samples (± 150 kg). The ten samples were mixed in a vertical screw mixer and were divided into four subsamples (± 30-35 kg). From each subsample, 500 g was ground using the M20 grinder (Ika Werke, Staufen, Germany). The equipment was decontaminated after each milling step by the use of water and Disolol®. All subsamples were transferred to a laboratory bin equipped with a vacuum pump, sucking the dust from the wheat kernels.

2.2.8 Dust correlation study

The identity of DON within the wheat and wheat dust samples of all wheat varieties was controlled according to the identification criteria set in Commission Decision 2002/657/EC. Every analytical run consisted of a standard control mix, seven samples of the calibration curve and a maximum of 20 samples. All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 21.

The DON content of the wheat samples from the field trial was determined using the LC-MS/MS method described by De Boevre *et al.* (2012b). An extra batch of wheat samples (n = 12) was randomly collected in Belgium to enlarge the DON contamination range of the curve. From each wheat specimen, dust was produced and collected with the vacuum cleaner. The dust samples were analysed using the developed and validated LC-MS/MS method. Verifying a possible correlation, the Pearson correlation coefficient was determined for the batch of samples. Matrix effects of dust were evaluated in terms of peak response of wheat dust compared to wheat at DON levels between 300 µg/kg and 9 600 µg/kg.

2.3 RESULTS AND DISCUSSION

2.3.1 Determination of dust particle size and quantity

The particle size distribution of wheat particles smaller than 2.5 mm collected after cleaning of different wheat samples obtained by a vibratory sieve shaker, is presented in Table 2.1. Only 1.5% of the wheat sample (± 13 kg) showed to be smaller than 2.5 mm. When sieved on the vibratory system, 1.5% of this fraction was found to be smaller than 1 mm. The bottom fraction (dust), which was divided into > 100 µm, 50 - 100 µm and < 50 µm, was weighed and it was found that the < 50 µm fraction was most abundant (Figure 2.2). Therefore, it was decided to use this fraction for LC-MS/MS analysis.

Table 2.1 Size distribution for wheat particles smaller than 2.5 mm

Sieve pore diameter	Residual amount 1 (g)	Residual amount 2 (g)	Residual amount 3 (g)	Mean weight (g)	SD (g)	Percentage (%)	Cumulative sieve residue (%)
3.15 mm	17.02	11.25	9.82	12.70	3.81	6.35	6.35
2.0 mm	164.00	170.25	172.04	168.76	4.22	84.38	90.73
1.4 mm	14.09	12.89	13.28	13.42	0.61	6.71	97.44
1.0 mm	2.04	2.15	1.88	2.02	0.14	1.01	98.45
800 µm	0.78	1.03	0.75	0.85	0.15	0.43	98.88
710 µm	0.33	0.42	0.42	0.39	0.05	0.20	99.07
500 µm	0.51	0.64	0.76	0.64	0.13	0.32	99.39
400 µm	0.20	0.30	0.32	0.27	0.06	0.14	99.53
355 µm	0.08	0.12	0.13	0.11	0.03	0.06	99.58
bottom	0.43	0.58	0.53	0.51	0.08	0.26	99.84

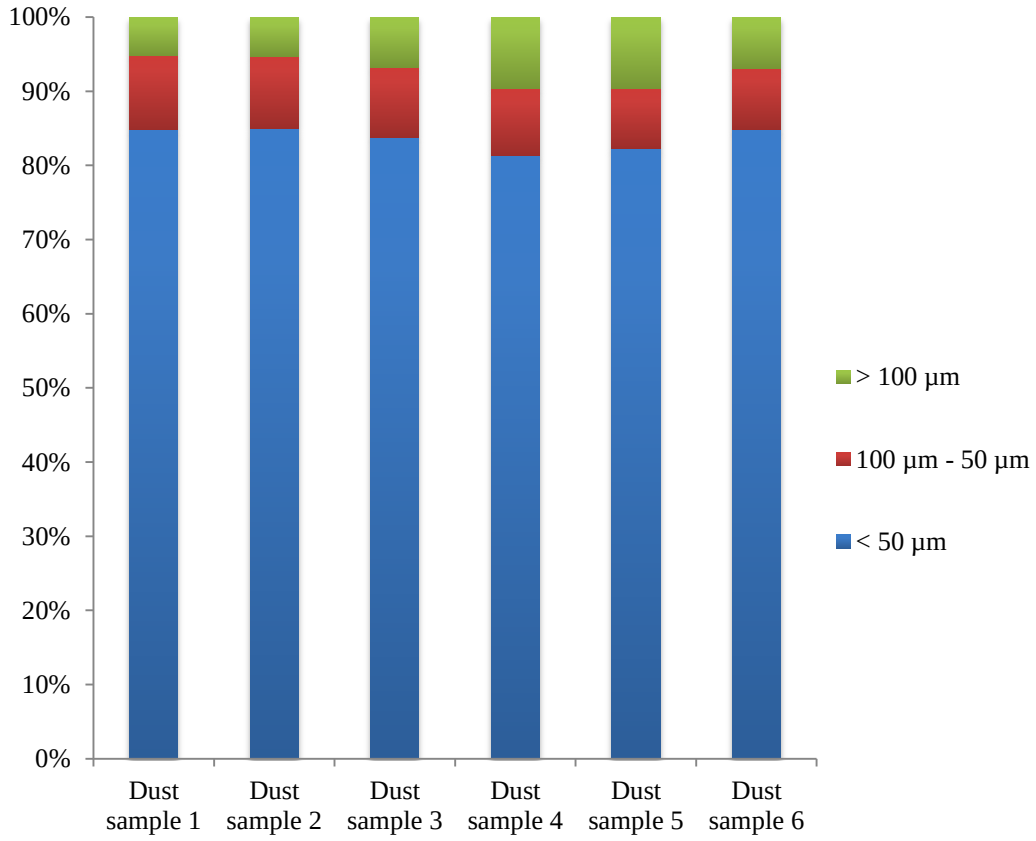

Figure 2.2 Cumulative distribution of the dust fractions in which three fractions can be distinguished: < 50 µm, 50 - 100 µm and > 100 µm

2.3.2 Optimization of sample preparation and clean-up

De Boevre *et al.* (2012b) described the use of LLE with acetonitrile/water/acetic acid (79/20/1, v/v/v) as extraction solvent and a hexane defatting step, as sample preparation for wheat matrix. A supplemental clean-up step with C_{18} columns 500 mg/6 mL (Achrom, Zulte, Belgium) was investigated. SPE was performed under acidic conditions by the use of acetonitrile/water/acetic acid (79/20/1, v/v/v) as extraction solvent. After the hexane defatting step, one part of the extract was filtered through a Whatman glass filter. Another part of the defatted extract was further purified by the use of a MultiSep 226 AflaZon+ column and acetonitrile/acetic acid (99/1, v/v) as washing solvent. Both purified extracts were combined and evaporated until dryness under a stream of nitrogen at 40 °C [Monbaliu *et al.*, 2010a; Monbaliu *et al.*, 2010b]. Additional sample preparation experiments were performed under neutral conditions. For these experiments, the extraction efficiency was tested by the use of methanol/water (90/10, v/v) and 100% ethyl acetate followed by 100% dichloromethane [Delmulle *et al.*, 2006]. All cleaned samples were firstly visually evaluated, as the colour was a measure of the dirtiness of the sample. Dust samples extracted under neutral conditions were

eliminated due to the obtained dirty extract and it was decided not to proceed with these extracts to protect the LC-MS/MS equipment.

2.3.3 Optimization of the LC-MS/MS method

For the chromatographic separation two different columns were compared: the ZORBAX Eclipse XDB C_{18} column and Symmetry C_{18} with a Symmetry C_{18} guard column. For the Zorbax Eclipse XDB C_{18} column, a mobile phase consisting of water/methanol (95/5, v/v) and methanol/water (95/5, v/v) buffered with 10 mM ammonium acetate and adjusted to pH 3 with glacial acetic acid was used [De Boevre *et al.*, 2012b]. The Symmetry C_{18} column was used in combination with a mobile phase consisting of water/methanol/acetic acid (94/5/1, v/v/v) containing 5 mM ammonium acetate and methanol/water/acetic acid (97/2/1, v/v/v) containing 5 mM ammonium acetate [Monbaliu *et al.*, 2010b]. Both columns were kept at room temperature and the flow rate was 0.2 mL/min and 0.3 mL/min, respectively. For the detection, MS/MS conditions were optimized for DON and DOM via direct injection into the mass spectrometer with a syringe pump at a flow rate of 10 µL/min. Cone voltages of 26 V and 37 V were applied and compared. In total two different sample preparation methods were combined with two different LC methods and two different MS/MS conditions. For the choice of the most optimal combination of sample preparation, LC and MS/MS conditions, the following factors were taken into account: the resulting relative peak area, the costs and time for sample preparation. According to the observed signal (expressed as relative peak area) for both extraction methods and LC-methods, a 4.4-fold higher signal was observed using no clean-up, cone voltage 26 V and the Symmetry C_{18} column. Adding a clean-up step increased the signal with a factor of 4.7, although it was decided not to add the SPE clean-up in the final sample preparation method for wheat dust. In conclusion, the most optimal results were obtained using the combination of the Symmetry C_{18} column for chromatographic separation and the MS/MS conditions with 26 V as cone voltage.

Another parameter for optimization was the total run-time for each LC. By using the Symmetry C_{18} column, it was only possible to have a chromatographic separation of DON and DOM in 18 min. Therefore, UPLC conditions were preferred. The Acquity UPLC BEH C_{18} column was suitable due to similar characteristics as the LC column. The same LC-MS/MS conditions were used, only the temperature of the column was set at 60 °C. Using these UPLC conditions, it was possible to reduce the time of each run to 8.5 min. The DON and internal standard DOM showed a retention time of 2.54 min and 3.10 min, respectively.

As a consequence, a sufficient separation of DON and DOM with 60 s difference (Figure 2.3), was obtained.

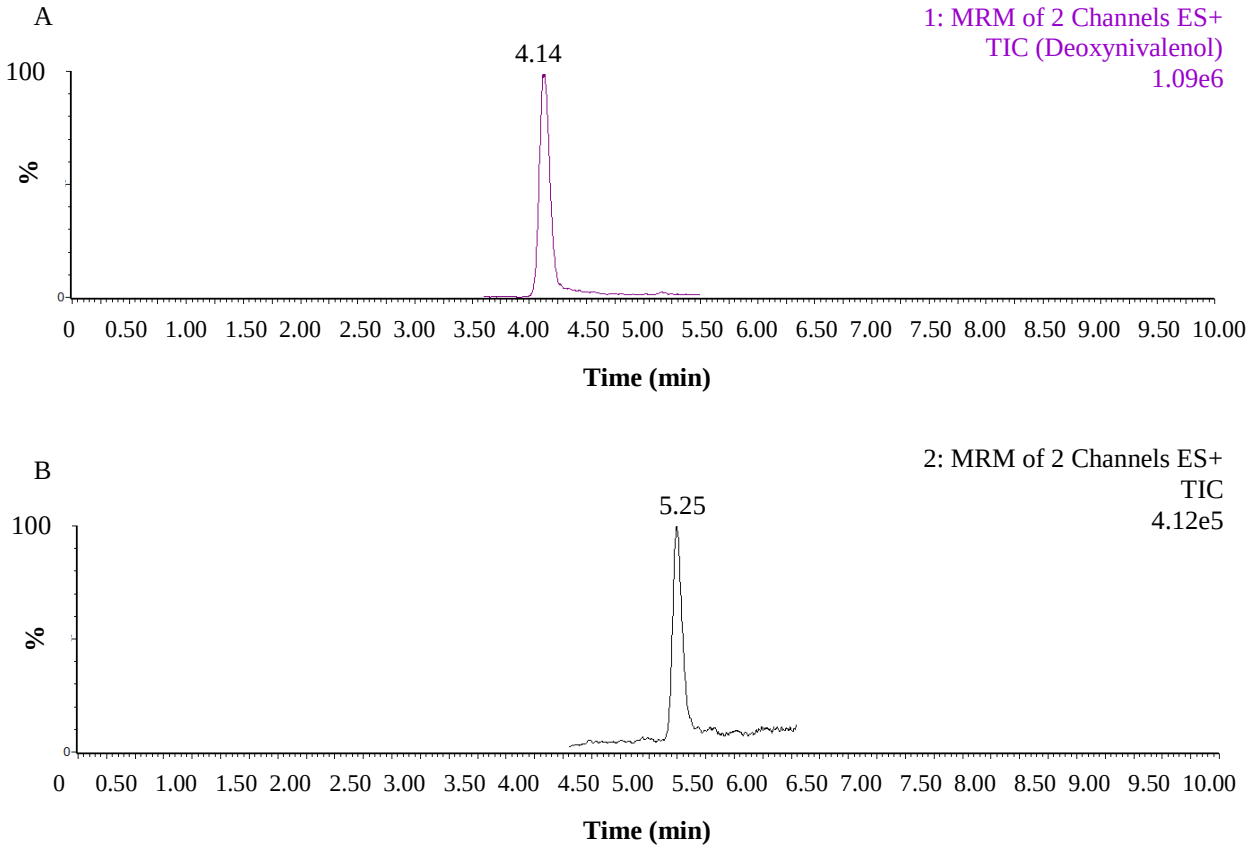

Figure 2.3 UPLC-MS/MS chromatogram of a spiked wheat dust sample: DON 1 000 µg/kg (A) and DOM 50 µg/kg (B)

2.3.4 Method validation

The LC-MS/MS method was successfully validated for DON in wheat dust based on the Commission Decision 2002/657/EC. A DON spiking range from 250 - 18 000 µg/kg was taken for the calibration curve, because of the high DON concentration in dust observed by ELISA and LC-MS/MS. The linearity was evaluated by the correlation coefficient and a lack of fit test. A correlation coefficient (r) of 0.998 and a p-value of 0.551 conclude that the calibration curve revealed good linearity. The apparent recovery ((RSD, %), n = 3) determined at medium level (1 000 and 3 000 µg/kg) was 97% (SD = 13) and 109% (SD = 4), respectively. The LOD and LOQ were 358 µg/kg and 717 µg/kg, respectively. During the precision study, RSDs were measured at five different concentration levels; however, only the data obtained at the medium and high level (7 500 and 17 000 µg/kg) were recorded. For the

intra-day precision an RSD_r of 6.87% and 2.09%, respectively, for the medium level and high level was observed. For the inter-day precision an RSD_R of 9.02% and 2.25%, respectively, was determined. The RSD_r and RSD_R data obtained are in agreement with the acceptable RSD values for repeatability according to Commission Decision 2002/657/EC. A maximum deviation of the DON concentration (accuracy) of 26% was observed for all spiking levels. Therefore the method can be accepted.

2.3.5 Analysis of field trial wheat and dust samples

To estimate the DON content in wheat dust, the dust fraction < 50 µm and non-sieved wheat grains were firstly analysed using the Veratox® DON 5/5 ELISA test kit [Zachariasova *et al.*, 2008]. According to the screening method, the DON content in the dust was a tenfold of the DON content in the wheat grain.

Four samples of each wheat variety and soil treatment were subjected to ELISA screening for DON determination and the results are presented in Table 2.2. The DON legal limit was exceeded in 87.5% of the samples and the highest DON concentration was found in the wheat variety Azzerti, sown after minimal tillage, with a mean DON concentration of 5 380 µg/kg. An independent-samples t-test revealed a lower DON content of wheat samples with soil treatment tillage for Homeros, Mulan, Sahara and Tabasco compared to the wheat samples without tillage (p-values respectively 0.007, 0.012, 0.008 and 0.019). For Azzerti, however, no significant difference (p-value = 0.228) was seen between tillage and no tillage.

The wheat samples showed DON concentrations between 1 450 µg/kg and 10 670 µg/kg, which means that each sample exceeded the limit for human consumption set at 1 250 µg/kg. As all LC-MS/MS results (Table 2.3) showed to be higher than the ELISA measurements (Table 2.2), an underestimation of the DON in wheat measured by ELISA can be concluded. However, the conclusions are analogous. The wheat variety Azzerti showed again the highest DON contamination level with a mean concentration of 7 596 µg/kg with tillage. A lower DON content of wheat samples with soil treatment tillage was discovered for Mulan, Sahara and Tabasco compared to the wheat samples without tillage by the use of an independent-samples t-test (p-values, respectively, of 0.002, 0.004 and 0.005). For Azzerti and Homeros no significant difference in DON level (p-values, respectively, of 0.972 and 0.122) was seen between tillage and no tillage. The resulting DON concentration of the corresponding wheat dust samples showed values above 8 000 µg/kg. This shows the accumulation of DON in

wheat dust. By the use of an independent-samples t-test, the wheat dust analysis results with regard to the tillage method (tillage and no tillage) were compared for each winter wheat variety. P-values of 0.388 and 0.471 for, respectively, Azzerti and Homeros, revealed no significant difference in DON content between tillage and no tillage for wheat dust. For Mulan, Sahara and Tabasco, p-values were determined of 0.043, 0.003 and 0.006 respectively. This means that for these wheat varieties a significantly lower DON content in wheat dust was observed in wheat sown after tillage.

Table 2.2 DON concentration (µg/kg) of the harvested wheat samples (*n* = 4) determined by ELISA

		Mean (µg/kg)	SD
Azzerti	No tillage	5 380	2 094
	Tillage	4 602	1 417
Homeros	No tillage	4 779	1 771
	Tillage	3 186	1 237
Mulan	No tillage	2 287	1 321
	Tillage	1 456	1 352
Sahara	No tillage	2 936	820
	Tillage	2 126	784
Tabasco	No tillage	3 689	1 268
	Tillage	2 479	1 477

2.3.6 Dust correlation study

The LC-MS/MS results for each wheat sample and the corresponding dust sample are presented in a scatterplot (Figure 2.4). For the extra batch of wheat samples (n = 12) a correlation between the DON concentration in dust versus the DON concentration in wheat was proved according to the correlation coefficient, r (0.970). For these samples, wheat dust showed a 13-fold accumulation of DON compared to wheat. In the field trial study the correlation between the DON content in wheat and dust was 0.829 (r) and a five-fold accumulation of DON in wheat dust was observed. This difference can be explained by the fact that the wheat samples from the different varieties and soil treatments showed higher

DON concentrations starting from 1 450 µg/kg. These extra results completed the calibration curve in the range above the limit for human consumption (1 250 µg/kg). When analysing the scatterplot, a logarithmic relationship between the DON content in wheat dust versus wheat grain was observed. Therefore, it can be concluded that the 13-fold accumulation of DON in wheat dust is present until 1 250 µg/kg for wheat (limit for human consumption) and 17 761 µg/kg for wheat dust. When a higher contamination level is observed, the sigmoidal curve with the following equation y = 5 935 ln(x) – 28 423, needs to be considered and a five-fold accumulation of DON in wheat dust is observed.

Table 2.3 DON concentration (µg/kg) of the harvested wheat and wheat dust samples (*n* = 4) determined by LC-MS/MS

		Wheat		Wheat dust	
		Mean (µg/kg)	SD	Mean (µg/kg)	SD
Azzerti	No tillage	7 573	623	22 723	3 524
	Tillage	7 596	1 069	21 973	5 253
Homeros	No tillage	5 502	615	25 799	3 737
	Tillage	4 157	1 293	25 833	4 967
Mulan	No tillage	4 274	767	14 789	902
	Tillage	1 483	997	8 182	2 205
Sahara	No tillage	4 701	618	26 622	2 795
	Tillage	2 534	728	17 123	933
Tabasco	No tillage	4 711	532	27 559	1 144
	Tillage	3 147	324	14 821	5 005

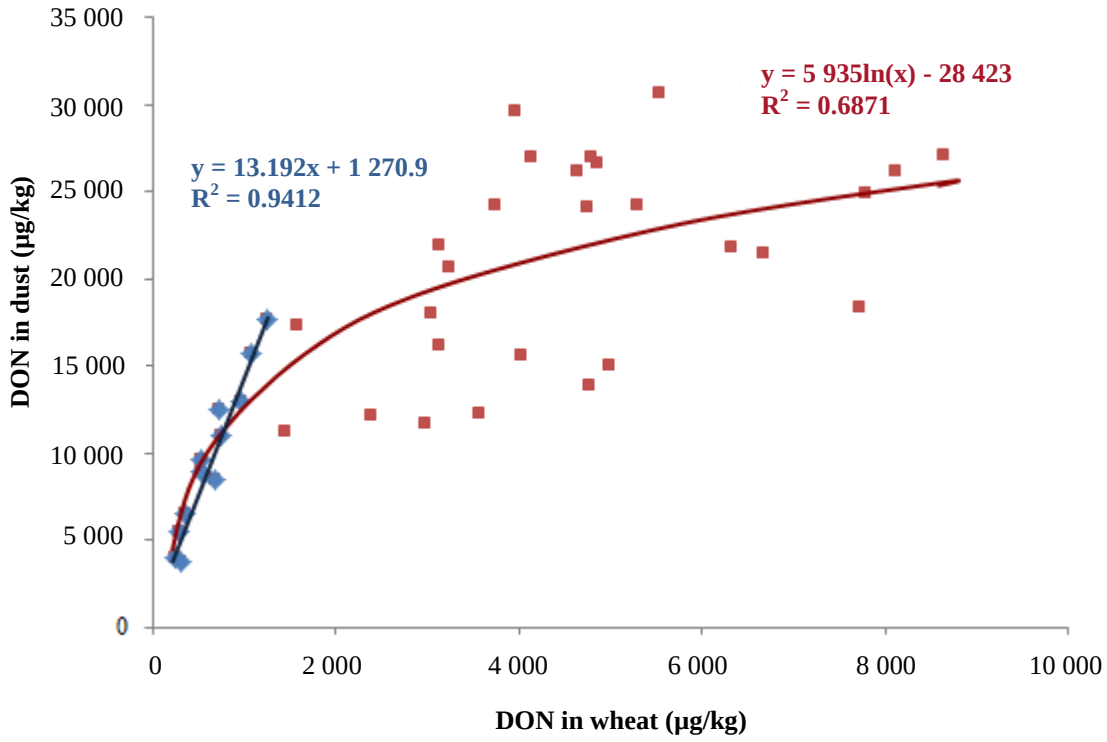

Figure 2.4 A scatterplot of the DON concentration in dust (y-axis) versus wheat (x-axis). For wheat samples with a DON contamination lower than 1 250 µg/kg (limit for human consumption), wheat dust showed a 13-fold accumulation of DON compared to wheat. With higher DON contaminated wheat, a sigmoidal relation of the DON content between wheat and wheat dust was observed.

As mentioned in Landschoot *et al.* (2013), maize-wheat rotation can be considered as an influencing factor on the DON content of the wheat crop during the next growing season. During the growing season 2012 wheat sown after maize reached a six times higher DON content compared to wheat sown after a crop different from maize. The susceptibility of the wheat variety had most influence on the DON content of wheat and wheat dust (Kruskal-Wallis test, p-value < 0.05). An independent samples t-test revealed that the moderately resistant varieties Mulan, Sahara and Tabasco had a significant lower DON content than the susceptible varieties Azzerti and Homeros. For wheat and wheat dust no significant difference in DON content was observed between tillage and no tillage for the susceptible wheat varieties Azzerti and Homeros. The determined matrix effects of wheat dust at different DON contamination levels had values between 0.814 and 1.038, which corresponds to a minor ion suppression in dust matrix compared to wheat matrix.

2.4 CONCLUSION

Based on the results of *Chapter 2*, it can be stated that an LC-MS/MS method was successfully developed and validated for the determination of DON in wheat dust. The developed LC-MS/MS method was used in an experimental field trial, where the influence of maize-wheat rotation, tillage method (*i.e.* ploughing) and wheat variety on the DON content of wheat grain and the corresponding wheat dust, was found.

Moreover, a significant correlation between DON in wheat dust and DON in wheat was found. However, this relationship is concentration-dependent, for concentrations up to 1 250 µg/kg a linear relationship can be used to predict the DON content in wheat dust. When a higher contamination level is observed, a sigmoidal curve is able to predict the DON content in a reliable way.

CHAPTER 3

THE DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST DEOXYNIVALENOL

Adapted from

Heterologous screening of hybridomas for the development of broad-specific monoclonal antibodies against deoxynivalenol and its analogues

Guo, Y.; Sanders, M.; Galvita, A.; Heyerick, A.; Deforce, D.; Bracke, M.; Eremin, S.; De Saeger, S.

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The immunogen synthesis strategy for the development of specific anti-deoxynivalenol monoclonal antibodies

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CHAPTER 3: THE DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST DEOXYNIVALENOL

SUMMARY

An immunogen synthesis strategy was designed to develop monoclonal anti-DON antibodies with limited cross-reactivity against structurally similar trichothecenes. A total of eight different DON immunogens were synthesized, differing in the kind of linker and the position of the linker on the DON molecule. After immunization, antisera from mice immunized with different DON immunogens were checked for the presence of interesting antibodies. Then, both homologous and heterologous ELISA were performed for hybridoma screening. Under this strategy, three monoclonal antibodies against DON and its analogues were generated. A new monoclonal antibody 13H1 could recognize DON and its analogues in the order of HT-2 toxin > 15-ADON > DON, with IC_{50} ranging from 1.14 to 2.13 µg/mL. Another monoclonal antibody 10H10 manifested relatively close sensitivities to DON, 3-ADON and 15-ADON, with IC_{50} values of 22, 15 and 34 ng/mL, respectively. Using an indirect ELISA format, decreased the 10H10 sensitivity to 15-ADON with 92%. A third monoclonal antibody 2A9 showed to be very specific and sensitive to 3-ADON, with IC_{50} of 0.38 ng/mL. Using both 2A9 and 10H10 monoclonal antibodies allows determining sole DON contamination.

Keywords: deoxynivalenol; monoclonal antibodies; immunogens; ELISA; cross-reactivity

3.1 INTRODUCTION

The objective of the study described in *Chapter 3*, was to improve the specificity of the ELISA assay by developing highly specific monoclonal anti-DON antibodies. Several antibodies to DON have been reported and all of them show high cross-reactivity against the acetylated derivatives 3-ADON and 15-ADON which reduces their specific character. Sensitivity of the produced antibodies, measured by the IC_{50} value ranged from approximately 20 ng/mL to 3 ng/mL [Casale *et al.*, 1988; Mills *et al.*, 1990; Sinha *et al.*, 1995; Maragos and McCormick, 2000; Usleber *et al.*, 1991; Xu *et al.*, 2010; Dos Santos *et al.*, 2011].

The high affinity of the monoclonal antibody to 3-ADON or 15-ADON is likely to derive from the chemistry used to prepare the immunogen. The ester linkage of 3-ADON or 15-ADON, as illustrated in Figure 1.7, may resemble the linkage of the DON-carrier protein immunogen. Therefore, it was decided to use different procedures, to make linkers with varying length and chemical structure between DON and the carrier protein.

Homologous and heterologous screening was performed to select the hybridoma producing anti-DON antibodies. Results indicated that monoclonal antibodies selected by heterologous ELISA were broad-selective to DON and its analogues, which were different from other previous anti-DON antibodies. To the best of our knowledge, it is the first report to employ hapten heterology for hybridoma screening to produce broad-specific monoclonal antibodies against DON and its analogues.

3.2 MATERIALS AND METHODS

3.2.1 Reagents and chemicals

DON, 3-ADON and 15-ADON standards were obtained from Fermentek (Jerusalem, Israel). Other mycotoxin reference standards namely nivalenol (NIV), HT-2 toxin, T-2 toxin, fusarenon-X (FUS-X) and DOM were purchased from Sigma-Aldrich (Bornem, Belgium), except that DON-3G was from Biopure Referenzsubstanzen GmbH (Tulln, Austria).

Colorburst™ blue 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution containing hydrogen peroxide was supplied by Alerchek (Springvale, Maine, USA). Rabbit anti-mouse immunoglobulins (anti-mouse IgG secondary antibody; protein concentration of 2.5 g/L) were purchased from DakoCytomation (Glostrup, Denmark). N,N'-carbonyldiimidazole, cyanuric chloride (CC), N,N'-diisopropylethylamine, glutaric anhydride, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), 1-butane boronic acid, sodium tetraborate, carboxymethoxylamine hemihydrochloride, γ-aminobutyric acid, N-hydroxysuccinimide (NHS), sulfo-NHS, tetrahydrofuran (THF), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetic acid, potassium carbonate, copper (II) sulfate, sodium ascorbate, 4.7-diphenyl-1.10-bathophenanthroline disulfonic acid disodium salt, arginine, glycine, aspartic acid, D-phenylalanine, lysine, TFA, triisopropylsilane (TIS), methyl tertiary-butyl ether (MTBE), PEG, bovine serum albumin (BSA), ovalbumin (OVA), horseradish peroxidase (HRP), rabbit anti-mouse IgG secondary antibody labelled with horseradish peroxidase (Sec Ab-HRP), phosphate buffered saline (PBS, 0.01 M, pH 7.4) powder, carbonate-buffered saline (CBS, 0.05 M, pH 9.6) capsule, complete and incomplete Freund's adjuvants, Tween 20 and skim milk powder were obtained from Sigma-Aldrich (Bornem, Belgium). Hydroxylamine, o-2 propynylhydrochloride was purchased from Focus Synthesis (San Diego, USA). O-propargyl-hydroxylamine hydrochloride was kindly provided by the Laboratory of Medicinal Chemistry (Ghent, Belgium). The Oasis HLB cartridges were obtained from Waters (Zellik, Belgium). Chlorotrityl chloride resin was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin and carbenicillin were purchased from Invitrogen (Merelbeke, Belgium). The CM5 sensor chips and ethanolamine-HCl were originating from GE Life Sciences (Diegem, Belgium). HBS-N^+ buffer containing 0.01 M 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.4 and 0.15 M NaCl was also bought at GE life Sciences. *n*-Hexane (Hipersolv Chromanorm) was purchased from VWR International (Zaventem, Belgium). Water was

obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium). Other chemicals and solvents were of analytical grade.

Nunc-Immuno™ F96-well microplates and Nunclon™ cell culture plates were from Nalge Nunc International (Roskilde, Denmark). Protein concentrators (9K molecular weight cut-off (MWCO), 20 mL) were purchased from Thermo Scientific (Rockford, USA).

3.2.2 Preparation of deoxynivalenol immunogens

3.2.2.1 Synthesis procedure

DON is a hapten due to its small size and therefore cannot elicit an immune response. Consequently, for the synthesis of immunogens, DON was coupled to a protein through a linker to increase the molecular weight. In search for specific anti-DON antibodies, different synthetic strategies were followed. Firstly, DON was coupled to a carrier protein via the C_3 and/or C_{15} position using a linker with a carboxyl function (Series I). To decrease the probability of cross-reacting monoclonal antibodies, a second DON series of immunogens was generated by coupling the carrier protein via a linker on C_3 and/or C_{15} without a carboxyl function (Series II). To reduce even further the possibility of cross-reacting monoclonal antibodies the linkage to the carrier protein was introduced through reaction with the C8 carbonyl function of DON, delivering a last series of immunogens (Series III). The used carrier proteins were BSA for the immunogen and OVA or HRP for the coating/competitive antigen in indirect or direct ELISA, respectively. The synthetic pathways underneath are described for BSA. The basic chemical structure of the three different series of immunogens is illustrated in Figure 3.1.

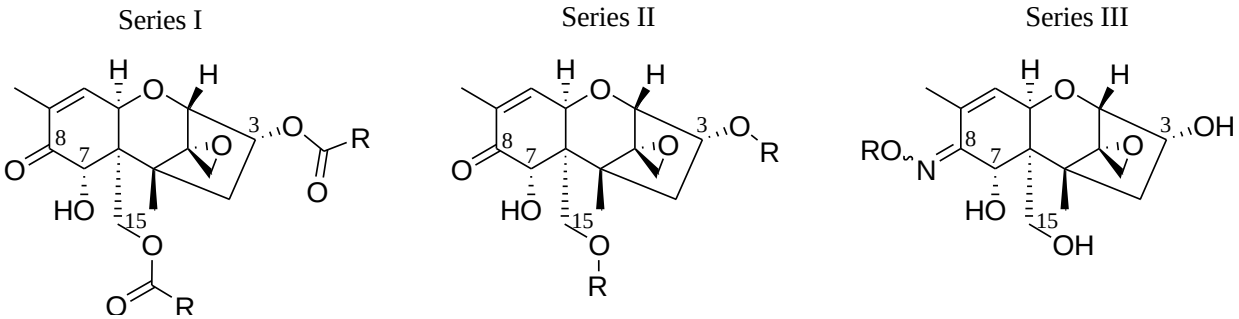

Figure 3.1 The three different series of immunogens

The first immunogen of the series I is DON-3,15-hemiglutaryl (HG)-BSA (Figure 3.6A) and was formed by the reaction of DON with glutaric anhydride in dry pyridine. Concisely, to a solution of 5 mg of DON (16.87 µmol) in 500 µL of dry pyridine, 100 mg of glutaric anhydride (0.88 mmol) was added and the solution was heated at 100 °C for 8 h. After evaporation, the residue was dissolved in 7.5 mL of chloroform and washed three times with 5 mL of 0.1M HCl. A purification step was performed using Oasis HLB® cartridges by a method derived from De Smet *et al.* (2010). Briefly, 5 µg of the crude DON-HG conjugate was dissolved in 1 mL of methanol/water (10/90, v/v) pH 2.3 and loaded on a preconditioned column. After loading, the sorbent was dried for 15 s by applying vacuum. DON-HG was eluted by washing the column with 1 mL of methanol/water (55/45, v/v) followed by 1 mL of methanol. The sorbent was dried for 15 s by applying vacuum. After purity determination by Time-of-Flight (TOF) MS, an aliquot of 1 mg of the synthesized DON-HG (1.92 µmol) was dissolved in 0.5 mL PBS using ultrasonication and transferred to 30 mg BSA (0.45 µmol) in 1 mL of PBS. Then 20 mg of EDC (0.10 mmol) was added to the above mixture and incubated for 5 h at room temperature with stirring [Mills *et al.*, 1990; Usleber *et al.*, 1991; Kohno *et al.*, 2003].

To obtain DON with one HG linker, namely DON-3-HG-BSA (Figure 3.6B), prior protection of the 7-OH and 15-OH groups as a cyclic boronate ester allowed to use similar reaction conditions as for DON-3,15-HG-BSA [Casale *et al.*, 1988]. The synthesis reaction is illustrated in Figure 3.2. Briefly, to a solution of 1 mg of DON (3.37 µmol) in 500 µL of dry pyridine, 3 mg of 1-butane boronic acid (0.029 mmol) was added and the solution was left stirring overnight at room temperature to yield 7,15-o-butylboronyl-DON. To the reaction product, 155 mg of glutaric anhydride (1.36 mmol) was added together with extra pyridine (500 µL) and the mixture was incubated for 3 h at 100 °C with stirring. After evaporation, the synthesized DON-HG (2.44 µmol) was dissolved in 500 µL of DMF together with 10 mg NHS (0.087 mmol) and 20 mg DCC (0.097 mmol) for activation of the carboxylic acid residues and the mixture was left agitating for 4 h at room temperature and during the night at 4 °C. The activated DON-HG hapten was added dropwise to 3 mg of BSA (0.044 µmol) in 2 mL of CBS buffer and left stirring for 2 h at room temperature and afterwards overnight at 4 °C.

Additionally in the first series of immunogens, common DON-BSA carbamate conjugates (Figure 3.6C and 3.6D) were synthesized by the N, N'-carbonyldiimidazole (CDI) coupling

reaction, adopted from published literature [Xiao *et al.*, 1995; Maragos and McCormick, 2000].

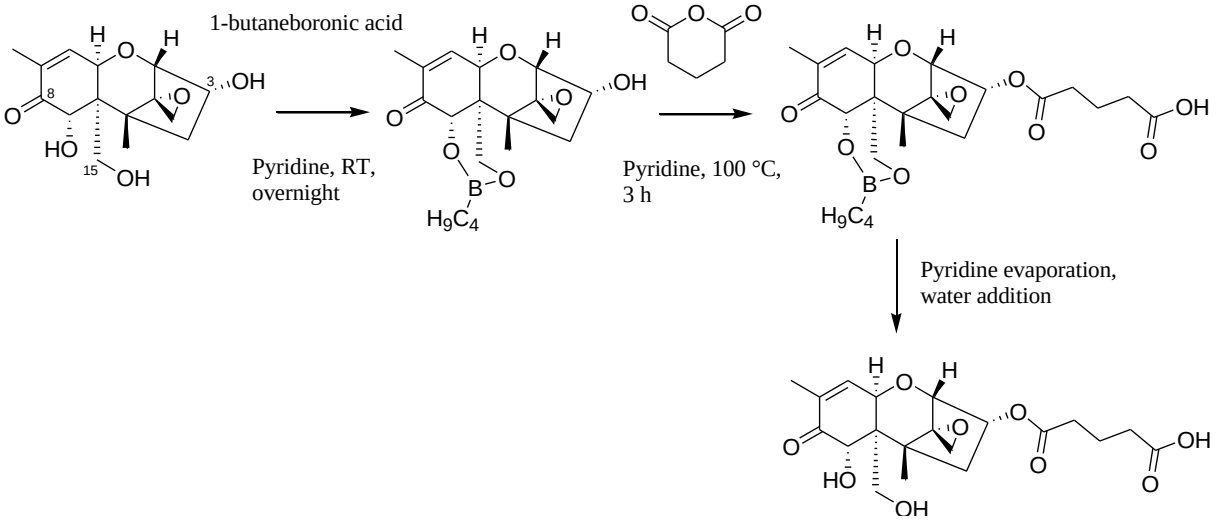

Figure 3.2 Pathway for DON-3-HG production using a chemical protection group

Within series II, DON-CC-BSA conjugates (Figure 3.6E and 3.6F) were synthesized by first dissolving 1 mg of DON (3.37 µmol) in 480 µL of cold acetonitrile (Figure 3.3). A solution of 620 µg of cyanuric chloride (3.36 µmol) in 1.24 mL of acetonitrile was prepared and cooled to -20 °C. The DON solution was added over 1 h to the vigorously stirred solution of cyanuric chloride. A solution of 870 µg N,N'-diisopropylethylamine (6.73 µmol) in 260 µL of cold acetone was added to the solution of DON and cyanuric chloride and stirred for 5 h at 55-60 °C followed by stirring for 16 h at room temperature. For coupling to BSA, a solution of 1.83 mg of BSA (0.027 µmol) in 1090 µL of 0.1 M sodium tetraborate (pH 9.2) was prepared. The solution was cooled to 4 °C and 3.5 mg of DON, a 10-fold molar excess with respect to the available amino groups was added and the resulting mixture was stirred for 1 h at 4 °C [Abuchowski *et al.*, 1977; Abuknesha and Griffith, 2005].

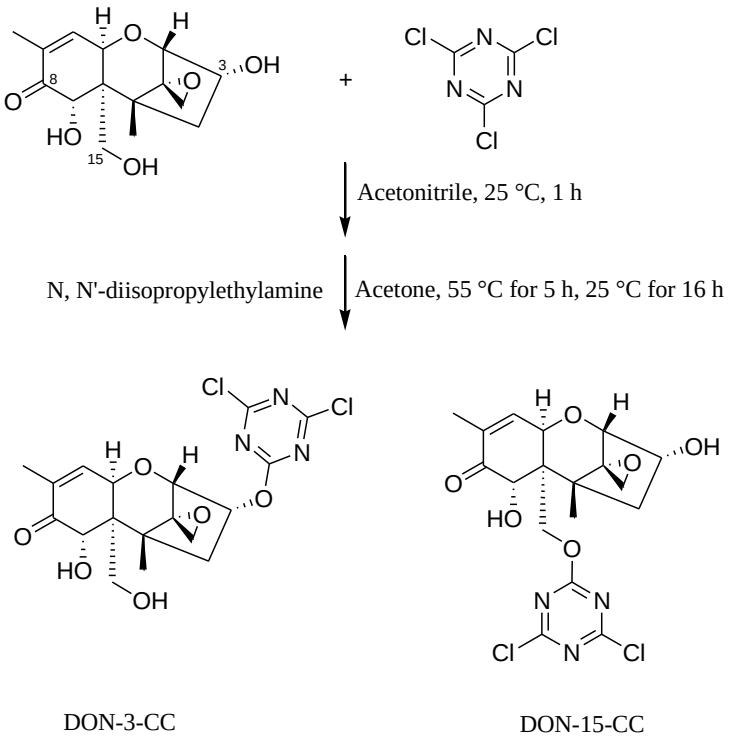

Figure 3.3 Synthesis of DON-CC

In an effort to further lower the chance for cross-reactivity against 3-ADON and 15-ADON, the C8 carbonyl function of DON was used as a reactive site to attach an appropriate linker via an oxime moiety (Series III). Hereto a CMO strategy was applied (Figure 3.6G) [Burkin *et al.*, 1999]. To investigate the effect of the length of the linker between DON and the protein and with the aim of lowering the immune response against the linker itself, we also introduced a carboxypropyl imine (CPI) linker at the C8 position of DON (Figure 3.6H). For this purpose, 0.5 mg of DON (1.69 µmol) was reacted with 750 µg of carboxymethoxylamine hemihydrochloride (5.88 µmol) in presence of 500 µL of dry pyridine for the DON-CMO synthesis and with 1.6 mg of γ-aminobutyric acid (15.50 µmol) in 1 mL DMF for the DON-CPI synthesis. After overnight reaction at room temperature, the reaction mixture was concentrated and the residue redissolved in 200 µl of water/DMSO (1/1.5, v/v). Next, 2 mg of sulfo-NHS (9.21 µmol) and 4 mg of EDC (0.021 mmol) were added together with 1.78 mg of BSA (0.026 µmol) and the volume was adjusted to 1 mL by further dilution with water. The reactions were performed for 2 h at room temperature followed by washing and purification as described for previous immunogen synthesis.

Finally click chemistry was adopted for linking C8 of DON without carboxyl function (Figure 3.6I). Following a modified procedure (Figure 3.4) of Ikuina *et al.* (2003), DON was

condensed to propargyl-hydroxylamine hydrochloride. Briefly, 2 mg of DON (6.76 µmol) was reacted with 1.81 mg of O-propargyl-hydroxylamine hydrochloride (25.52 µmol) in presence of THF/acetic acid (1/1, v/v) at 40 °C. After 2 h an additional 170 µg of O-propargyl-hydroxylamine hydrochloride was added and the mixture was stirred for an other 4.5 h to obtain DON-oxime. For the synthesis of azido-BSA, 10.686 mg of BSA (0.16 µmol) was dissolved in water together with 4 mg of potassium carbonate and 0.5 mg of copper (II) sulfate. Then, 1 mg of imidazole-1-sulfonylazide hydrochloride (4.79 µmol) was added and the reaction was left agitating overnight. For the click reaction, 0.5 mg of DON-oxime (1.43 µmol) was transferred to an aqueous solution of azido-BSA (1.42 mg) containing 25 µg copper (II) sulfate (10 mM), 20 µg sodium ascorbate (10 mM) each and 54 µg 4,7-diphenyl-1,10-bathophenanthroline disulfonic acid disodium salt (10 mM). The reaction went on for 14 h at room temperature [Ikuina *et al.*, 2003; Van Dongen *et al.*, 2009; Horak *et al.*, 2010; Goddard-Borger and Stick, 2007].

The click chemistry immunogen product was further expanded by the introduction of a N-azido cyclic peptide (630.3 g/mol) (Figure 3.6J). The cyclic peptide increases the distance between DON and BSA and renders the DON molecule more available for the immune system. Through solid phase peptide synthesis using 2-chlorotrityl chloride resin and a Fmoc/*t*Bu protection scheme, the amino acids glycine, aspartic acid, D-phenylalanine, lysine and arginine were succesively coupled to each other. After mild acid cleavage of the peptide from the resin, cyclization of the peptide was performed followed by treatment with TFA/TIS/H_2O (95/2.5/2.5, v/v/v) to cleave off the protecting groups in solution. The peptide was then precipitated in MTBE/hexane (1/1, v/v) and redissolved in methanol for a diazo transfer. For the click chemistry (Figure 3.5), 1 mg of DON-oxime (2.84 µmol) was reacted with 1.78 mg of cyclic peptide (2.84 µmol) under previously mentioned conditions. Under CDI reaction conditions the obtained DON-cyclic peptide (2.84 µmol) was coupled to 3.9 mg of BSA (0.058 µmol) in water/DMSO (1/5, v/v) for 3 h at room temperature [Dijkgraaf *et al.*, 2007; Dai *et al.*, 2000].

All DON immunogens were dialysed against 4 L PBS to remove low molecular weight substances and concentrated using Pierce Concentrator columns 20 mL/9K MWCO. An overview of the DON immunogens is given in Figure 3.6. The DON-BSA and DON-CC-BSA synthesis resulted in a reaction mixture containing BSA coupled via its linker to the 3-OH and 15-OH groups of DON. Due to their similarity in mass and chemical properties it was not possible to separate the reaction mixture prior to immunization. The DON-3,15-HG-BSA

synthesis did not resulted in a mixture of one and two hemiglutaryl linkers as an excess of glutaric anhydride was used.

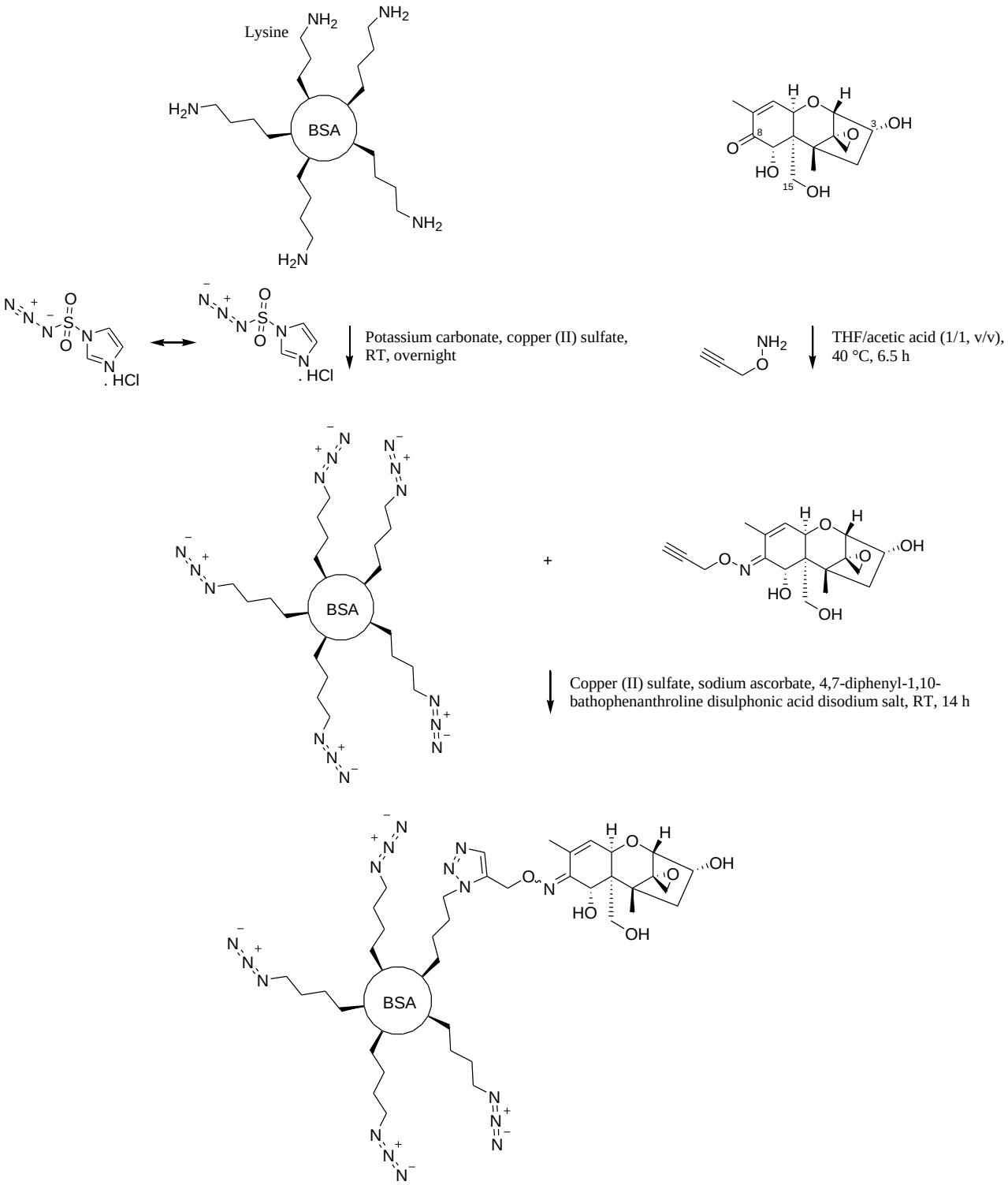

Figure 3.4 Synthesis of DON-azido-BSA by the use of click chemistry

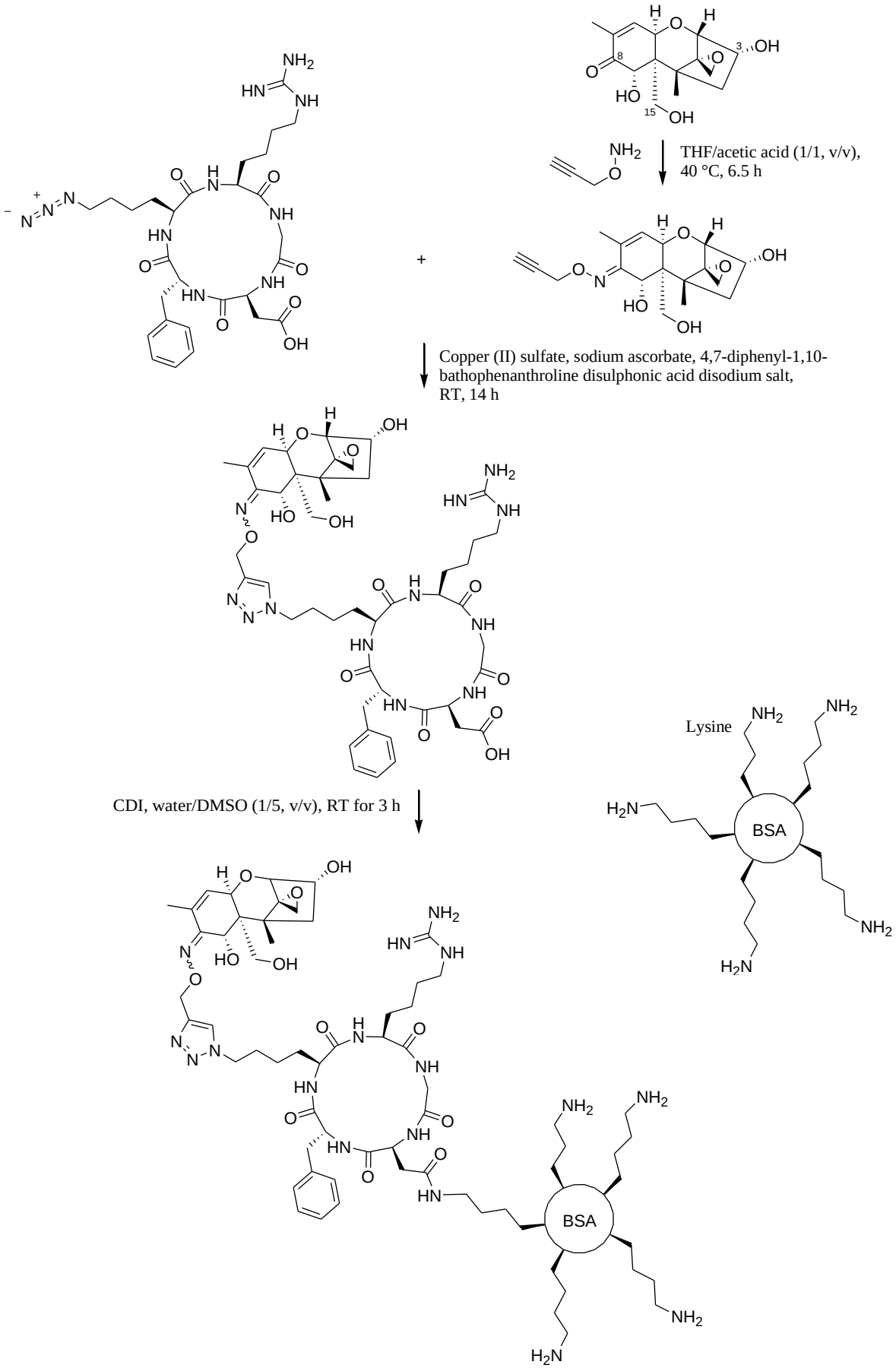

Figure 3.5 Synthesis of DON-cyclic peptide-BSA by the use of click chemistry

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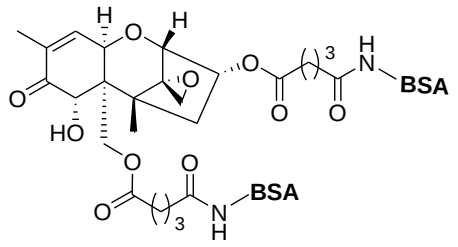

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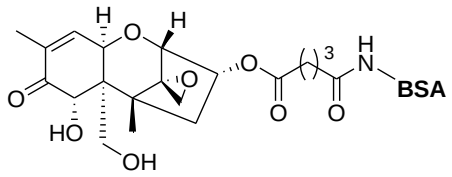

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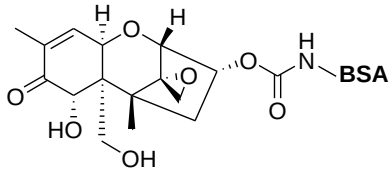

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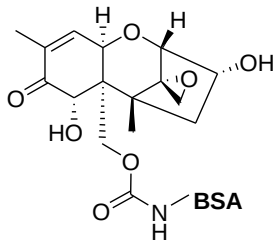

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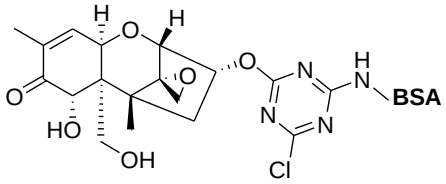

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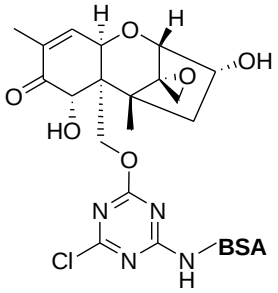

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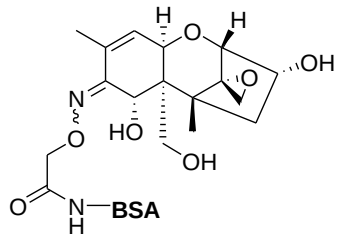

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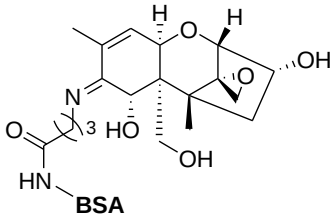


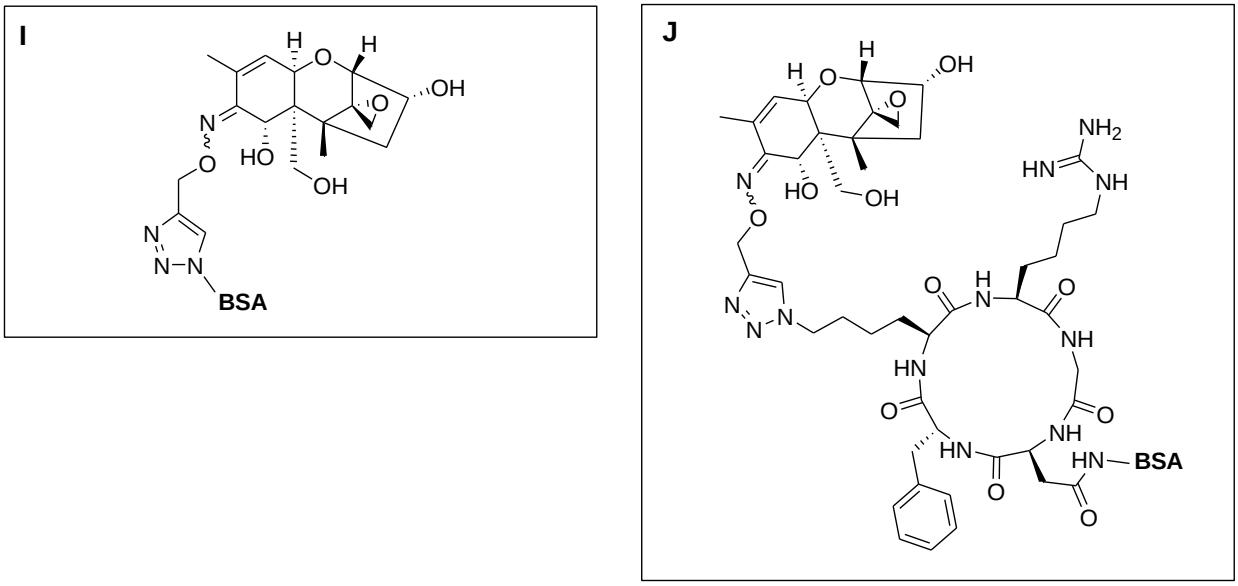


Figure 3.6 Overview of the DON immunogens only illustrating the amino group of the protein. A: DON-3,15-HG-BSA; B: DON-3-HG-BSA; C: DON-3-BSA; D: DON-15-BSA; E: DON-3-CC-BSA; F: DON-15-CC-BSA; G: DON-CMO-BSA; H: DON-CPI-BSA; I: DON-azido-BSA; J: DON-cyclic peptide-BSA.

3.2.2.2 Determination of synthesis success

The successful synthesis of the DON-3,15-HG-linker was confirmed by mass spectrometry LCT Premier XETM TOF (Waters, Milford, MA, USA), equipped with a standard ESI and modular LockSpray TM interface in the positive and negative electrospray ionization ($ESI^{+/-}$) mode. The purified DON-3,15-HG mixture was infused in acetonitrile/water (1/1, v/v) at 10 µL per min. The purity of the final product was assessed by HPLC and photo diode array (PDA) detection (190-400 nm) using a Phenomenex Luna 2.5 mm C_{18} (2)-HST column. A mobile phase consisting of eluents A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) was used at a flow rate of 0.4 mL/min. A linear gradient of 10-100% solvent B was applied over 9 min. Other mass measurements of DON-linker syntheses were performed by direct injection of the reaction product into the Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA). The mass spectrometer was operated in $ESI^{+/-}$ mode. Masslynx version 4.1 was used for data acquisition.

After coupling to the protein, the immunogen was characterized by the use of indirect competitive ELISA. All incubations, except for the first coating step, were carried out at 37 °C. After each incubation, the plates were washed three times (300 µL/well) with PBST (PBS containing 0.05% (v/v) Tween 20) using an automatic microplate washer "96PW" (TECAN, Salzburg, Austria).

3.2.3 Immunizations

To obtain antibody-producing B-lymphocytes against DON, 6 to 8-week-old Balb/C female mice (ethical approval according to ECD 10/08) were subjected to an injection with the different DON immunogens emulsified with complete or incomplete Freund's adjuvant. For each group of mice, 40-100 µg of the DON immunogen was administered and injections were repeated every 3-4 weeks. Antisera titres were checked one week after each boost injection. When mice reached sufficient antisera titres, three days before cell fusion a last injection was given with the immunogen diluted in PBS.

3.2.4 Cell fusion

Mice were sacrificed by cervical dislocation and cell fusion of mouse spleen cells and myeloma cells (NSO cells) was performed. PEG 1500 was added as fusing reagent and HAT for the selection of the fused cells. The cells were distributed into 96-well culture plates and cultured in a humidified, 37 °C, 5% CO_2 incubator. Culture supernatant was screened by indirect ELISA to determine the positive hybridomas producing antibody against DON. These hybridomas were further screened for the production of the target antibody and cloned by limiting dilution.

3.2.5 Hybridoma selection and cloning

Ten to twelve days after cell fusion, plates of hybridoma culture supernatants were initially screened by non-competitive indirect ELISA, based on homologous or heterologous DON antigens. The positive wells were picked out and screened again by competitive ELISA. Only those wells that gave substantial inhibition (more than 20%) by DON and had no cross-reactivity with OVA were selected. The interesting hybridomas were expanded, rechecked and subcloned by limiting dilution for 2-3 cycles to ensure the monoclonality. The resulting stable clones were expanded and stored in liquid nitrogen. For large-scale production of monoclonal antibodies, each cell line was cultured in a two-compartment bioreactor CELLine 350 (INTEGRA Biosciences AG, Zizers, Switzerland) for more than three months. The collected supernatant was further used for monoclonal antibody characterization.

3.2.6 Indirect ELISA

High-binding polystyrene 96-well microplates were coated with the prepared DON conjugates coupled to OVA diluted in CBS (100 µL/well). After incubation at 4 °C overnight, the plates were blocked with 2% (w/v) skim milk in PBS (300 µL/well) for 30 min. For competitive indirect ELISA analysis, standard solutions of DON and PBS control were added (50 µL/well), followed by adding 50 µL/well of diluted antibodies (antisera or culture supernatants) in PBS. For the characterization of the synthesized immunogens, in PBS diluted reference DON monoclonal antibodies clone 4 or 22 were used, kindly provided by C. Maragos (USDA). After shaking and incubation for 1 h, 100 µL/well of SecAb-HRP was added and incubated for another 1 h. Then, 100 µL/well of TMB substrate solution was added. The reaction was stopped after 15 min with 2 M sulphuric acid (50 µL/well), and the absorbance at 450 nm was measured by a Bio-Rad model 550 microplate reader (Richmond, CA, USA).

3.2.7 Direct ELISA

The microplates were coated with 100 µL/well of anti-mouse IgG secondary antibody diluted in CBS (5 µg/mL) by an overnight incubation at 4 °C. Then, the plates were blocked in the same way, mentioned above. For testing, antibodies diluted in PBS were added (100 µL/well). After 1 h of incubation and the washing step, standard solutions of DON and PBS control were added (50 µL/well), followed by adding another 50 µL/well of DON-CC-HRP or DON-HRP diluted in PBS. The plates were shaken and incubated for another 1 h, and colour development was carried out and measured as described in the procedure of indirect ELISA.

3.2.6 Characterization of monoclonal antibodies

3.2.6.1 By using ELISA

Checkerboard assays, in which antibodies were titrated against various amounts of coating antigens were conducted to select appropriate working concentrations for evaluation of assay sensitivities to DON. Standard competitive curves were obtained by plotting relative absorbance (ratio of absorbance measured at the standard concentration and zero concentration: $B/B_0 \times 100\%$) against the logarithm of analyte concentration. IC_{50} values (*i.e.*, analyte concentrations at which the maximum absorbances were inhibited by 50%) were

determined to assess the assay sensitivity. To evaluate the specificity or assay selectivity of the antibody, a set of DON analogues were utilized to perform cross-reactivity studies. IC_{50} of each tested compound was based on its corresponding competitive curve. Cross-reactivity (CR) values were calculated according to the following equation: CR(%) = [IC_{50} (DON) / IC_{50} (analogue)]×100%，where CR values were calculated using IC_{50} values with units of µg/mL. Similarly, CR_{molar} values were calculated using $IC_{50\ molar}$ values with units of nmol/mL.

3.2.6.2 By using SPR

The characterization of the obtained monoclonal antibody was performed on a Biacore 3000 (GE Healthcare, Uppsala, Sweden) SPR instrument. DON-OVA was immobilized on a CM5 sensor chip by the use of amine coupling. During the immobilization, HBS-N^+ buffer was used as a running buffer at a flow rate of 10 µL/min. The sensor chip was activated using a mixture of 400 mM EDC and 100 mM NHS in water. Then, DON-OVA diluted to 100 µg/mL with a 10 mM acetate buffer (pH 4.2), was loaded on the activated sensor chip. Finally, a 1 M ethanolamine-HCl solution (pH 8.5) was used as a blocking solution. The activation, immobilization and blocking was carried out for 2 547 s.

During all kinetic runs, the flow rate of HBS-N^+ buffer used for the association and dissociation was set at 30 µL/min. The regeneration of the sensor chip surface was performed using 10mM NaOH at 10 µL/min for 60 s. For the determination of the affinity of the monoclonal antibody towards the DON coated on the sensor chip, different concentrations of the antibody (31.25 ng/mL, 62 ng/mL, 125 ng/mL, 250 ng/mL and 500 ng/mL) were injected over the sensor surface. In order to check sensitivity and cross-reactivity, DON and 3-ADON were analysed by SPR. Each toxin was dissolved in HBS-N^+ buffer and mixed with 500 ng/mL of monoclonal antibody. Calibration curves for each toxin were obtained and IC_{50} values were calculated from the RU value at the midpoint of the calibration curve. Cross-reactivity values were calculated in the same way as for the ELISA results.

The BIAevaluation software was used for the calculations, using a 1:1 Langmuir model and local fitting of the data.

3.3 RESULTS AND DISCUSSION

3.3.1 Determination of synthesis success

When the desired synthesis products were obtained based on the exact mass measurements performed by direct injection into the Quattro Premier XE mass spectrometer or the LCT Premier XETM TOF mass spectrometer, further coupling to BSA and OVA or HRP was performed. The synthesized immunogens and coating antigens were characterized by competitive ELISA using reference monoclonal antibodies (clone 4 or 22, USDA). If the B_0 value was equal to or higher than 1 and decreasing B values were obtained when using increasing standard DON concentrations, the testing conjugates were confirmed and used for immunization or further ELISA experiments.

3.3.2 Determination of antisera titres by using indirect ELISA

Antisera titres were determined by an indirect homologous ELISA using the immunizing haptens coupled to OVA instead of BSA as coating antigen. By using the same format, the cross-reactivity against OVA was determined as well. An overview of all immunized mice and the corresponding antisera titre measurement is given in Table 3.1. The best antisera titres were obtained for the mice injected with DON-BSA and DON-CC-BSA. For DON-3,15-HG-BSA, high antisera titres were found, but the cross-reactivity against OVA was high. It was concluded that antibodies were probably formed against the protein instead of the target DON. This could be explained by the presence of two linkers on the DON molecule, which makes it less free for activation of the immune system of the mouse.

Table 3.2 lists the polyclonal antibody characteristics from the best DON-CC-BSA mouse. Judged from the working concentration of antigen and antiserum, it is clear that the polyclonal antibody had much higher affinity to the homologous hapten DON-CC, suggesting the strong recognition of both DON and the CC bridge region. But concerning the inhibition results, the DON-OVA coated ELISA was more sensitive to DON, which proved that the heterologous format did improve the assay sensitivity for DON.

Table 3.1 Overview of immunized mice with corresponding antisera titre

	Immunogen	Amount	Primary injection	Intraperitoneal boost injections	Final injection	Cell fusion	Antisera titre	Cross-reactivity to OVA
1	DON-3,15-HG-BSA	75 µg	SC + IP	3	/*	/*	+	+
2	DON-3,15-HG-BSA	75 µg	SC + IP	4	ok	ok	+	+
3	DON-3,15-HG-BSA	100 µg	SC + IP	4	ok	ok	+	+
4	DON-3,15-HG-BSA	100 µg	SC + IP	4	ok	ok	+	+
5	DON-CC-BSA	40 µg	SC + IP	4	ok	ok	++	-
6	DON-CC-BSA	40 µg	SC + IP	4	ok	ok	++	-
7	DON-CC-BSA	60 µg	SC + IP	4	ok	ok	++	-
8	DON-CC-BSA	60 µg	SC + IP	4	ok	ok	++	-
9	DON-3-HG-BSA	100 µg	SC + IP	7	/*	/*	-	-
10	DON-3-HG-BSA	100 µg	SC + IP	7	ok	ok	+/-	-
11	DON-azido-BSA	75 µg	SC + IP	4	/*	/*	+/-	-
12	DON-BSA	100 µg	SC + IP	4	ok	ok	++	-
13	DON-BSA	100 µg	SC + IP	4	ok	ok	++	-
14	DON-azido-BSA	75 µg	SC + IP	4	ok	ok	+/-	-
15	DON-azido-BSA	75 µg	SC + IP	4	/*	/*	+/-	-
16	DON-CMO-BSA	75 µg	SC + IP	6	ok	ok	+/-	-
17	DON-CMO-BSA	75 µg	SC + IP	7	ok	ok	+/-	-
18	DON-CMO-BSA	75 µg	SC + IP	7	ok	ok	+/-	-
19	DON-CPI-BSA	75 µg	SC + IP	5	ok	ok	+	-
20	DON-CPI-BSA	75 µg	SC + IP	5	/*	/*	+	-
21	DON-CPI-BSA	75 µg	SC + IP	7	ok	ok	+	-
22	DON-azido-BSA	75 µg	SC + IP	4	ok	ok	+/-	-
23	DON-azido-BSA	75 µg	SC + IP	4	ok	ok	+/-	-
24	DON-cyclic peptide-BSA	75 µg	SC + IP	4	ok	ok	+/-	-
25	DON-cyclic peptide-BSA	75 µg	SC + IP	4	ok	ok	+/-	-

** mouse died during procedure; subcutaneous (SC), intraperitoneal (IP)*

The antisera from the DON-BSA injected mice could recognize both DON-OVA and DON-CC-OVA very well, despite that the dose of DON-CC-OVA was higher than that of DON-OVA (Table 3.2). However, using the heterologous coating antigen did not promote the assay sensitivity for DON. By contrast, the polyclonal antibody derived from DON-BSA displayed stronger affinity to the free DON than that from DON-CC-BSA. The phenomena must be associated with the fact that DON-BSA contained a smaller linkage compared to DON-CC-BSA, leading to the reduced influence from the spacer arm. Given that the molecular weight of DON is less than 300 Da, the influence of the linkage arm is indeed crucial [Chappey *et al.*, 1994].

Table 3.2 Characteristics of antisera from the best DON-CC-BSA and DON-BSA mice

Immunogen	ELISA format	Serum dilution	Coating antigen (μg/mL)	Maxium absorbance	Inhibition by 2 μg/mL DON (%)
DON-CC-BSA	Homologous	1/16 000	DON-CC-OVA (1)	0.96	11.2
DON-CC-BSA	Heterologous	1/4 000	DON-OVA (4)	0.92	34.8
DON-BSA	Homologous	1/16 000	DON-OVA (0.5)	0.88	57.9
DON-BSA	Heterologous	1/16 000	DON-CC-OVA (4)	0.81	54.5

For DON-CMO-BSA, DON-CPI-BSA, DON-azido-BSA and DON-cyclic peptide-BSA a relative low titre, but no cross-reactivity against OVA, was determined. When the ELISA response of the serum of the immunized mice reached a plateau phase using a high serum dilution, the mice were sacrificed by cervical dislocation.

3.3.3 Hybridoma selection and subcloning

3.3.3.1 Hybridoma screening by indirect ELISA

The best DON-CC-BSA mouse was first sacrificed for hybridoma production. Due to a great amount of spleen cells, seventeen 96-well plates were used to distribute the fused cell suspension. Non-competitive indirect ELISA based on both DON-CC-OVA and DON-OVA were used for primary hybridoma screening. When using DON-CC-OVA (4 μg/mL) for homologous screening, almost all the wells were visually positive. This could be envisaged from the very high titre of antiserum and the satisfying fusion rate (every well contained at

least one cluster of hybridomas). In contrast, using DON-OVA (8 μg/mL) for heterologous screening, only a few wells in each plate manifested strong signals. These wells showed even deeper colour in the corresponding DON-CC-OVA-coated plate. In other words, these double-checked positive wells exhibited higher affinity to the DON part than other wells did. By contrast, it seemed that many hybridomas could secrete antibodies that recognize the whole DON-CC structure or the CC linker, which led to the high background of DON-CC-OVA coated ELISA, as the CC ring structure is also an important epitope for eliciting the immune response.

In total, 47 positive wells were quickly selected based on the results of DON-OVA coated heterologous ELISA. After adding the fresh medium overnight, they were rechecked by competitive indirect ELISA. Top 6 wells with both strong signal and substantial inhibition by DON were expanded and re-selected. Eventually, the best clone 13H1 was subjected to subcloning for three cycles to get a stable single-cell line.

3.3.3.2 Hybridoma screening by direct ELISA

Since the antiserum titre of the DON-BSA mice was very high, it could be foreseen that a great number of positive clones would be obtained when using indirect ELISA with DON-OVA for hybridoma screening. Moreover, the standard indirect ELISA screening can result in a large number of false positives, which gives rise to a lot of unnecessary workload and exorbitant waste of the precious experimental materials in the following procedures. With the aim of efficiently selecting the target clones that can secrete high-affinity monoclonal antibodies, direct ELISA was applied to screen hybridomas from the DON-BSA mice, although the sensitivity of direct ELISA was not as high as that of indirect ELISA [Cervino *et al.*, 2008]. However, from another point of view, only clones with high affinity to the target antigen should be detected as the positives by direct ELISA, reducing the number of clones with fairly low affinity.

For initial screening of DON-BSA hybridomas, non-competitive direct ELISA based on both DON-HRP and DON-CC-HRP were carried out. Likewise, much more positive wells were found when using the homologous tracer DON-HRP, compared to the heterologous tracer DON-CC-HRP. The wells, which were positive from the DON-CC-HRP-based ELISA, gave even stronger signals in the corresponding DON-HRP-based ELISA. Thus, the top 8 positive wells were easily chosen for further inhibition tests by competitive direct ELISA. Among

them, two clones 10H10 and 14D4 could recognize free DON very well and exhibited cross-reactivity to both 3-ADON and 15-ADON; two other clones 2A9 and 4B10 had much higher affinity to 3-ADON but very poor reactivity to DON. Subsequently, hybridomas 10H10, 14D4, 2A9 and 4B10 were subcloned to obtain the final stable single-cell lines. Given that 10H10/14D4 and 2A9/4B10 monoclonal antibodies possessed very similar characteristics, only clone 10H10 and 2A9 were used for large-scale culturing.

To be emphasized: the advantage of heterologous screening is that it can notably reduce the proportion of false positives, and hence one may efficiently get the interesting hybridomas with high affinity to the free target analyte. Consequently, it generates time and is labour-saving as well as being cost-effective.

As no hybridoma were found producing anti-DON antibodies out of the mice injected with DON immunogens belonging to the third series, the previous statement about the immunogenic importance of the C_8 carbonyl function in the DON molecule was confirmed [Usleber *et al.*, 1991].

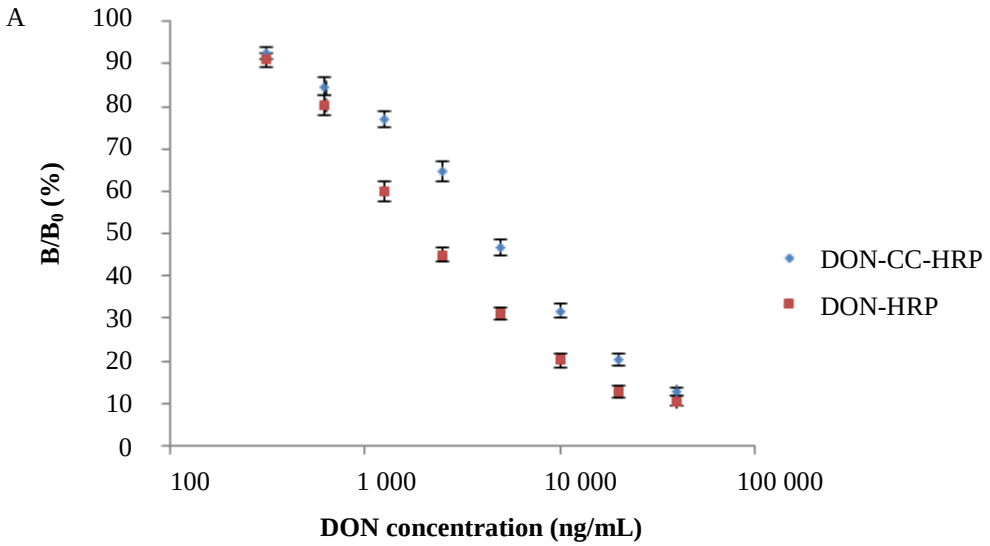
3.3.4 Characterization of monoclonal antibodies

3.3.4.1 By using ELISA

The clones 13H1, 10H10 and 2A9 were individually cultured in CELLine 350 bioreactors for large-scale production of monoclonal antibodies. In view of practical applications, the competitive direct ELISA format, a popular format in commercial ELISA kits, was used for monoclonal antibody characterization. After checkerboard titration, the standard curves were set up with the optimal working dilutions of monoclonal antibodies and tracers. As shown in Figure 3.7, for clone 13H1, the heterologous format based on DON-HRP did improve the assay sensitivity to DON, with IC_{50} value decreasing from 4.34 µg/mL (DON-CC-HRP-traced ELISA) to 2.13 µg/mL (DON-HRP-traced ELISA). In contrast, for clone 10H10, the heterologous format based on DON-CC-HRP was less sensitive (IC_{50} = 0.064 µg/mL) than the homologous format based on DON-HRP (IC_{50} = 0.022 µg/mL). For clone 2A9, only a homologous ELISA format was performed using DON-HRP and an IC_{50} value of 0.202 ng/mL was determined. These results confirmed that the degree of hapten heterology could influence the assay sensitivity of direct competitive ELISA, but whether improving or not, it depends on the specific case. The assay sensitivity may not be in parallel with the degree of hapten heterology, and this finding is similar to that of Wang *et al.* (2011).

For cross-reactivity measurement DON-HRP-traced competitive direct ELISA was used, including eight common DON analogues. Table 3.3 lists the cross-reaction results of monoclonal antibodies 13H1, 10H10 and 2A9.

It seemed that the 13H1 antibody could be considered as a unique broad-selective antibody, with even higher sensitivity to HT-2 toxin and 15-ADON than to DON, minor affinity toward NIV, DOM and DON-3G, and very low cross-reactivity against 3-ADON, while no obvious cross-reaction with T-2 toxin and FUS-X was found. Interestingly, this monoclonal antibody could recognize HT-2 toxin but not T-2 toxin, although both of them belong to type A trichothecenes (absence of a carbonyl at the C_8) and just differ at the C_4. The observations were mainly ascribed to the immunogen DON-CC-BSA, in which the CC linker must be located more at the C_{15} than at the C_3 and it significantly affected the electronic configuration of the DON molecule. Probably, the molecular property of the immunizing hapten DON-CC was more close to HT-2 toxin than to other tested compounds, which led to the fact that the generated antibody had the greatest affinity to HT-2 toxin. These results were in agreement with Muldoon et al.'s perspective that both steric and electronic features governed antibody recognition [Muldoon *et al.*, 2000].



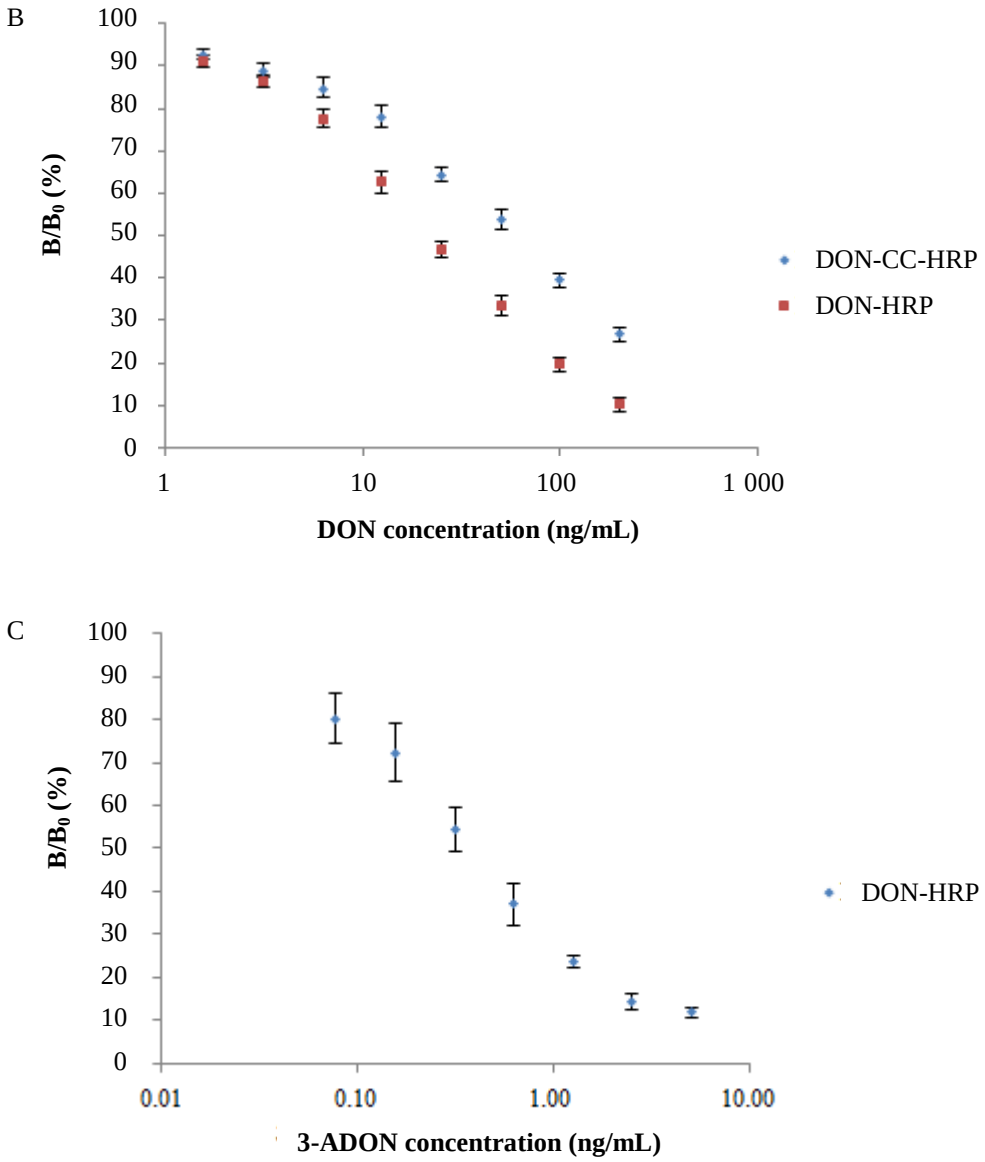


Figure 3.7 Standard curves of direct competitive ELISA with DON or 3-ADON based on different monoclonal antibodies (A: 13H1, B: 10H10 and C: 2A9) and tracers (*n* = 3). B/B_0: ratio of absorbance measured at the standard concentration and zero concentration. As the experiment was repeated on 3 consecutive days, the inter-day precision and accuracy was determined by the standard deviation and represented by the error bars on the calibration curve.

Table 3.3 Cross-reactivity of monoclonal antibodies 13H1, 10H10 and 2A9

		Clone 13H1				Clone 10H10				Clone 2A9			
Compound	MW	IC_{50} (µg/mL)	CR (%)	IC_{50} molar (nmol/mL)	CR molar (%)	IC_{50} (µg/mL)	CR (%)	IC_{50} molar (nmol/mL)	CR molar (%)	IC_{50} (µg/mL)	CR (%)	IC_{50} molar (nmol/mL)	CR molar (%)
DON	296	2.13	100	7.20	100	0.022	100	0.074	100	0.202	0.188	0.682	0.182
3-ADON	338	34.12	6	100.95	7	0.015	147	0.044	167	0.00038	100	0.00124	100
15-ADON	338	1.62	131	4.79	150	0.034	65	0.101	74	0.432	0.088	1.278	0.097
NIV	312	7.69	28	24.65	29	> 10	< 0.2	> 32	< 0.2	*n.a.*	*n.a.*	*n.a.*	*n.a.*
T-2 toxin	466	> 100	< 2	> 214.6	< 3	> 10	< 0.2	> 21	< 0.3	*n.a.*	*n.a.*	*n.a.*	*n.a.*
HT-2 toxin	424	1.14	187	2.69	268	> 10	< 0.2	> 24	< 0.3	*n.a.*	*n.a.*	*n.a.*	*n.a.*
FUS-X	354	> 100	< 2	> 282.5	< 3	> 10	< 0.2	> 28	< 0.3	*n.a.*	*n.a.*	*n.a.*	*n.a.*
DON-3G	458	15.67	14	34.21	21	0.072	31	0.157	47	0.025	1.498	0.055	2.271
DOM	280	5.47	39	19.54	37	0.079	28	0.282	26	*n.a.*	*n.a.*	*n.a.*	*n.a.*

Monoclonal antibody 10H10 showed better sensitivity to 3-ADON than to DON, moderate affinity to 15-ADON, and minor cross-reactions with DON-3G and DOM, but no cross-reactivity towards the other trichothecenes. It was interesting to observe that this anti-DON monoclonal antibody could recognize both 3-ADON (IC_{50} = 0.015 µg/mL) and 15-ADON (IC_{50} = 0.034 µg/mL) very well, and the differences among the three CR values were much smaller than those from other reported anti-DON monoclonal-based ELISAs [Li *et al.*, 2012; Maragos *et al.*, 2006; Maragos and McCormick, 2000; Tangni *et al.*, 2010; Xu *et al.*, 2010]. In other words, the discrepancies of the assay sensitivities to DON, 3-ADON and 15-ADON are so narrow that it is suitable to utilize this novel monoclonal antibody for simultaneous detection of the three mycotoxins. As a result, the "total" DON content can be rapidly estimated in one assay and an overall health risk of DON exposure for the consumers can be assessed. The broad-selectivity of this monoclonal was probably due to the involved strategy of heterologous screening for hybridomas instead of the homologous one. Previous researchers [Liu *et al.*, 2010; Muldoon *et al.*, 2000; Wang *et al.*, 2010] also indicated that application of this screening strategy increased the likelihood of isolating hybridomas secreting very broad-specific monoclonal antibodies.

A very sensitive anti-3-ADON antibody was developed out of clone 2A9 with an IC_{50} value of 0.38 ng/mL. Setting the monoclonal antibody activity for 3-ADON as 100%, the cross-reactivity values for DON, 15-ADON and DON-3G (Table 3.4) were determined as 0.188%, 0.088% and 1.498% and can be considered as negligible. In comparison with other previously reported monoclonal antibodies for 3-ADON such as the ones described at Maragos *et al.* (2006) and Maragos and McCormick (2000) (Table 3.4), the newly developed clone 2A9 is probably the most sensitive and specific antibody to 3-ADON [Baumgartner *et al.*, 2010; Casale *et al.*, 1988].

Note that when comparing target analytes to analogues with different molecular weight (MW), cross-reactivity values calculated from units of mole per volume should be more accurate than those from units of weight per volume (though the latter are often used) [Xu *et al.*, 2009], since cross-reactivity is a structure-related phenomenon in antibody-antigen binding. Generally, the gaps between CR and CR_{molar} values would not be too big, except that the antibody is broad-specific and shows high affinity to the analogue whose MW has a very significant deviation from that of the target analyte. Herein, as for the cross-reactivity of monoclonal antibody 13H1 to HT-2 toxin, the usual CR was 187%, while the CR_{molar} increased up to 268%. Also, as described in a few references [Ruprich and Ostrý, 2008;

Tangni *et al.*, 2010], some DON-ELISA tests displayed very high cross-reactivity to the masked form DON-3G and the corresponding CR_{molar} values must be much larger than the normal CR values. Hence, we suggest that evaluation of the cross-reactivity of a broad-specific antibody should be based on the CR_{molar} values as far as possible. Certainly, CR data obtained in the current work can be somewhat different from the values declared later in the future application studies (because of various assay formats, sample matrices and so on), but the general tendency should be similar.

Table 3.4 Comparison of cross-reactivity results between clone 2A9 and other anti-DON monoclonal antibodies

Compound	Clone 2A9 Direct ELISA		Mab 1-6.2.6 Indirect ELISA [Maragos *et al.*, 2006]		USDA Clone 22 Direct ELISA [Maragos and McCormick, 2000]	
	IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
3-ADON	0.38	100	1.70	100	2.88	100
DON	202.14	0.188	15.80	10.7	18.20	15.8
15-ADON	431.79	0.088	68.90	2.4	558.00	0.52
DON-3G	25.36	1.498	*n.a.*	*n.a.*	*n.a.*	*n.a.*

The previously mentioned characterization results were obtained by the use of direct competitive ELISA, which is a general method for the characterization of monoclonal antibody. Characterization of the 10H10 monoclonal antibody was also repeated by indirect competitive ELISA using DON-OVA (4 µg/mL) and DON-CC-OVA (4 µg/mL) coating and DON, 3-ADON and 15-ADON for competition. This comparison between direct and indirect ELISA is illustrated in Table 3.5. The sensitivity of the antibody towards DON and 3-ADON measured by indirect ELISA is approximately two times (DON-OVA coating) and five times (DON-CC-OVA coating) the value measured by direct ELISA. For 15-ADON, the sensitivity decreased at least ten times when using indirect ELISA. When looking to the CR_{molar} values for direct ELISA and indirect ELISA with DON-OVA coating, the same cross-reactivity is seen for 3-ADON, but the cross-reactivity towards 15-ADON lowered 6.7 times when using

indirect ELISA. It can even be concluded that the 10H10 antibody shows only cross-reactivity to 3-ADON when using indirect ELISA with DON-OVA coating. When using an indirect ELISA format with DON-CC-OVA coating, the cross-reactivity against 3-ADON increased three times and the cross-reactivity against 15-ADON decreased four times compared to the direct ELISA format. So, the characterization of the monoclonal antibody depends on the type of ELISA and the coating antigen used.

Table 3.5 Comparison between direct and indirect ELISA for antibody characterization (clone 10H10)

			IC_{50} (µg/mL)	CR (%)	IC_{50} molar (nmol/ mL)	CR molar (%)
Direct ELISA		DON	0.022	100	0.074	100
		3-ADON	0.015	147	0.044	167
		15-ADON	0.034	65	0.101	74
Indirect ELISA	DON-OVA	DON	0.040	100	0.135	100
		3-ADON	0.028	142	0.083	162
		15-ADON	0.421	9	1.250	11
	DON-CC-OVA	DON	0.113	100	0.383	100
		3-ADON	0.024	480	0.070	547
		15-ADON	0.637	18	1.885	20

3.3.4.2 By using SPR

The characterization of the 10H10 monoclonal antibody was performed by the use of SPR as well. After DON-OVA coating and blocking of the CM5 sensor surface of the Biacore 3000, a total amount of 12 000 RU of DON-OVA remained. The interaction between antibody and immobilized DON-OVA on the sensor chip was determined by monitoring real-time RU changes for a range of antibody concentrations (31.25 ng/mL, 62.5 ng/mL, 125 ng/mL, 250 ng/mL and 500 ng/mL) in HBS-N^+ buffer. The antibody was injected over the surface, followed by a dissociation phase which consisted of a flow of HBS-N^+ buffer. After the dissociation, the surface was regenerated by the injection of 10 µl 10 mM NaOH. In Figure 3.8, the SPR sensorgram is shown of the kinetic study using a flow of antibody only. The injection of the highest antibody concentration (500 ng/mL) gave an increase in signal of 100

RU. Decreasing the antibody concentration by half, decreased the RU in the same way. As seen in Figure 3.8, the association phase was followed by a constant dissociation phase which indicates a strong interaction between the antibody and the DON-coated surface. A dissociation constant K_D of 4.73×10^{-11} was obtained during these experiments. This K_D gives an idea about the minimum amount of antibody necessary for a particular experiment and therefore the affinity of the antibody to the antigen. This value is related to the sensitivity. As a K_D of 10^{-11} was determined, the antigen DON can be detected in a picomolar (pM) range. The sensorgram of the 10H10 monoclonal antibody was compared to the Langmuir model and it can be concluded that there is no significant difference between the resulted curve and the model curve ($\chi^2 = 0.489$).

Calibration curves were set up for DON and 3-ADON as this is the only cross-reacting mycotoxin using DON-OVA coating (Figure 3.9). The antibody (500 ng/mL) was first mixed with increasing toxin concentrations and the mixture was injected over the surface of the sensor chip. The portion of antibody, which did not bind to the mycotoxin but to the DON-OVA-immobilized sensor chip was detected by SPR. RU values obtained by SPR were correlated with the mycotoxin concentration. Out of the calibration curves, IC_{50} values of 3.17 ng/mL and 2.49 ng/mL and molar IC_{50} values of 10.71 ng/mL and 7.37 ng/mL were found for DON and 3-ADON, respectively. Relative to DON a cross-reactivity and molar cross-reactivity of respectively 127% and 145% was found for 3-ADON. So, there can be concluded that SPR gives more than 10 times better sensitivity values compared to indirect ELISA using the same coating antigen. Similar cross-reactivity values were found for SPR and direct or indirect ELISA.

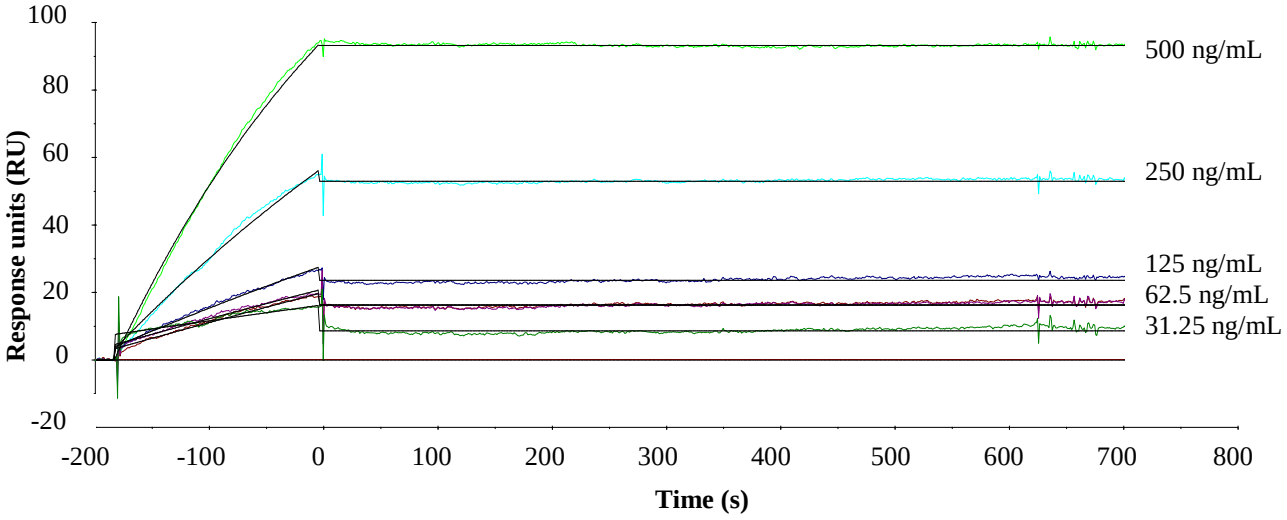
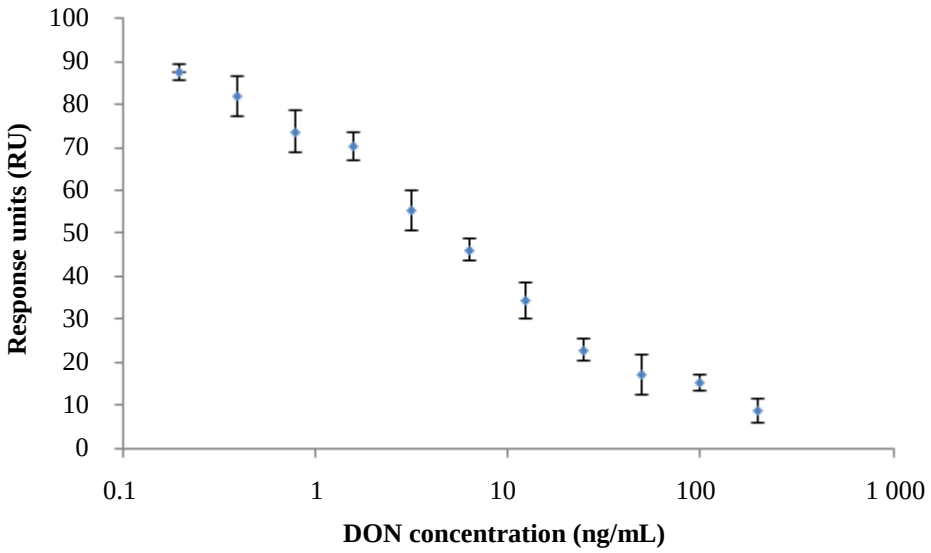


Figure 3.8 SPR sensorgram by monitoring the SPR angle (plotted as response units (RU)) in function of time injecting different concentrations of the 10H10 monoclonal antibody on a DON-OVA coated CM5 sensor surface. 1 RU corresponds to 0.0001° shift in SPR angle or to 1 pg/mm^2 of surface coverage. The association phase was followed by a constant dissociation phase which indicates a strong interaction between the antibody and the DON-OVA-coated surface.

A



B

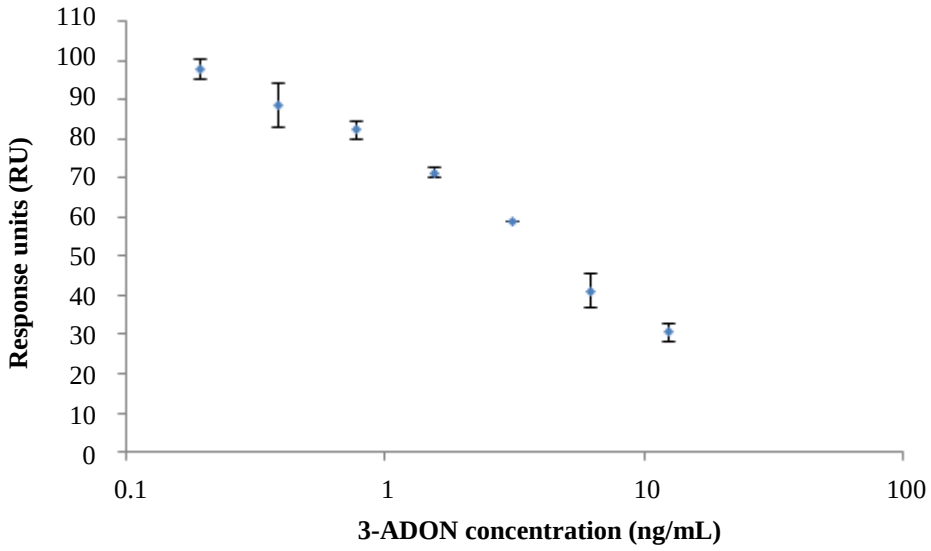


Figure 3.9 Calibration curve by monitoring the SPR angle (plotted as response units (RU)) in function of the concentration of competitor (antigen) DON (A) or 3-ADON (B) injected together with 500 ng/mL of 10H10 monoclonal antibody on a DON-OVA-coated CM5 sensor surface. 1 RU corresponds to 0.0001° shift in SPR angle or to 1 pg/mm^2 of surface coverage. As the experiment was repeated on 3 consecutive days, the inter-day precision and accuracy was determined by the standard deviation and represented by the error bars on the calibration curve.

3.4 CONCLUSION

In *Chapter 3*, the development and characterization of three anti-DON monoclonal antibodies is described. The superior monoclonal antibody 10H10 has relatively similar affinity to DON, 3-ADON and 15-ADON, with IC_{50} values of 22, 15 and 34 ng/mL, respectively. This is, so far, the first broad-specific monoclonal antibody against DON and its two acetylated forms, which can be used for simultaneous monitoring of the three mycotoxins. Thus, it could be further applied to different kinds of DON-related immunoassays, or immunoaffinity columns for sample clean-up before chromatographic analysis. The monoclonal antibody 2A9 can be called an anti-3-ADON monoclonal antibody, because of its very sensitive and specific characteristics towards 3-ADON. Furthermore, when applying monoclonal antibody 10H10 in an indirect ELISA using DON-OVA coating, almost no affinity for 15-ADON is observed. So, when monoclonal antibodies 10H10 and 2A9 are used together in an indirect ELISA format, the DON content without its acetylated derivatives can be determined.

By using a three-series synthesis strategy for the development of specific monoclonal antibodies against DON, previous statements were confirmed and new conclusions could be made. The place of the linker on the DON molecule is of importance for the immunogenic response. When a linker is positioned on the C_3 or C_{15} of DON for the synthesis of an immunogen, the produced antibody shows higher cross-reactivity against 3-ADON or 15-ADON, respectively. When coupling a linker to the carbonyl C_8 of DON, no high anti-DON immune response was observed. This emphasizes the immunogenic importance of the C_8 carbonyl function. The size of the linker does not seem to influence the immunogenicity of the injected conjugate. The kind of linker has an influence on the electrostatic configuration of the DON molecule and therefore on the antigenic determinant of DON which is available for the immune system of the mouse.

Finally, we conclude that it is possible to produce specific antibodies against trichothecene mycotoxins such as 3-ADON by synthesizing the proper immunogen with the suitable linker and screening the hybridomas carefully. By changing the ELISA format and/or coating antigen, it is possible to influence the selectivity and cross-reactivity of the monoclonal antibody.

CHAPTER 4

THE DEVELOPMENT AND VALIDATION OF AN ELISA METHOD FOR RAPID SCREENING OF DEOXYNIVALENOL IN WHEAT DUST

CHAPTER 4: THE DEVELOPMENT AND VALIDATION OF AN ELISA METHOD FOR RAPID SCREENING OF DEOXYNIVALENOL IN WHEAT DUST

SUMMARY

A sample preparation and ELISA method was developed and validated for the screening of DON in wheat and wheat dust. Extraction was carried out with water and was successful due to the polar character of DON. ELISA experiments were performed using the developed 10H10 anti-DON monoclonal antibody (*chapter 3*). The method was validated according to the criteria mentioned in Commission Regulation 519/2014/EC and Commission Decision 2002/657/EC. A small survey was executed on 16 wheat lots and their corresponding dust samples. The samples were analysed according to the developed sample preparation and ELISA procedure. A linear and logarithmic correlation (r = 0.887 and 0.928, respectively) were found for the DON concentration in dust versus the DON concentration in wheat. Therefore, it is possible to estimate the cereal contamination through dust analysis.

Keywords: deoxynivalenol, monoclonal antibodies, ELISA, wheat, wheat dust

4.1 INTRODUCTION

As mentioned in *Chapter 2*, DON accumulates in wheat dust. Former research highlighted the risk of developing respiratory diseases with people working in the agricultural sector, especially during the process of grain harvesting and threshing [Zukiewicz-Sobczak *et al.*, 2012]. Dust contaminated with mould spores and/or mycotoxins may cause asthma, allergic alveolitis, chronic bronchitis, allergic rhinitis, mucous membrane irritation, certain infectious diseases and cancer due to long-term inhalation [Pettigrew *et al.*, 2010; Krysinska-Traczyk *et al.*, 2001; Halstensen *et al.*, 2004].

The American Conference of Governmental Industrial Hygienists (ACGIH, 1997) has defined three grain dust mass fractions in relation to potential health effects: (1) the inhalable fraction (dust particle diameter of 100 µm that enters the airways region), (2) the thoracic fraction (dust particle diameter of 10 µm which deposits in the tracheobronchial regions) and (3) the respirable fraction (dust particle diameter of 4 µm which enters the gas-exchange regions). Thus, the smaller the dust particle size and the bigger the surface area, the more dangerous in terms of health risks. As indicated in Figure 2.2 the most abundant dust fraction in wheat has a particle size smaller than 50 µm. The health risk should surely be taken seriously.

At the moment, no fast screening technique exists for the determination of mycotoxins in grain dust as no regulatory limits for mycotoxins in grain dust and no mycotoxin absorption data after inhalation are yet available.

As mentioned in *Chapter 2*, there is a more homogenous distribution of mycotoxins in grain dust compared to grain cereals. Due to the possibility of continuously sampling through the entire grain movement process (recovery of only 20 g dust out of a wheat bulk lot of 100 kg), a more representative sample can be taken. For dust, a grinding step is not necessary prior to sample extraction making it less time-consuming than the conventional cereal sampling. *Chapter 4* describes the development and validation of a simple sample preparation and ELISA method for the determination of DON in wheat and wheat dust using the developed 10H10 monoclonal antibody.

4.2 MATERIALS AND METHODS

4.2.1 Reagents and chemicals

DON standard was obtained from Fermentek (Jerusalem, Israel). DON-HRP was prepared as previously described in *Chapter 3*.

Colorburst™ blue TMB substrate solution containing hydrogen peroxide was supplied by Alerchek (Springvale, Maine, USA). Rabbit anti-mouse immunoglobulins (anti-mouse IgG secondary antibody; protein concentration of 2.5 g/L) were purchased from DakoCytomation (Glostrup, Denmark). PBS (0.01 M, pH 7.4) powder, CBS (0.05 M, pH 9.6) capsule, Tween 20 and skim milk powder were obtained from Sigma-Aldrich (Bornem, Belgium). Water was obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium). Other chemicals and solvents were of analytical grade.

Nunc-Immuno™ F96-well microplates were from Nalge Nunc International (Roskilde, Denmark).

4.2.2 Dust collection

Dust was produced out of wheat samples by the use of a dust collection facility as mentioned in *Chapter 2* and illustrated in Figure 2.1.

4.2.3 Chemical composition of wheat dust

The chemical composition of two different batches of wheat dust was determined to better understand possible interfering components for the monoclonal antibody in ELISA. First, between 3 and 5 g of wheat dust was weighed in a cup and placed in an oven. Starting from room temperature, every 5 h the temperature of the oven increased with 5 °C until a temperature of 550 °C was reached. Afterwards, the cup was cooled down in an desiccator and the water together with the organic fraction was determined by calculating the difference in weight before and after incineration. Based on Dashek *et al.* (1986), the moisture content of spring wheat dust has a value between 4.97% and 8.08% depending on the duration of drying at 60 °C. Previous researchers have measured a percentage of ash between 7.9% and 28.5% [Parnell *et al.*, 1986; Martin, 1981]. Then, 100 mg of the ash was redissolved in 5 mL 6 M HCl followed by 5 mL 3 M HCl while heating. After cooling down, 50 mL of water was added and the solution was filtered by the use of a Whatman No 5 filter (VWR International, Zaventem, Belgium). The filtered

solution was analysed by inductively coupled plasma with atomic emission spectroscopy (ICP-AES) to determine and quantify the mineral composition of the inorganic fraction of the wheat dust.

4.2.4 Sample preparation

One gram of wheat and half a gram of the corresponding wheat dust was weighed in a Gosselin extraction tube (50 mL). The extraction was performed by adding 30 mL of water for 30 min and 50 mL of water for 20 min respectively, using the Agitator decanter overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France), followed by a short centrifugation at 3 000 g for 5 min prior to ELISA to remove small particles from the supernatant.

4.2.5 Direct ELISA

All incubations, except for the first coating step, were carried out at 37 °C, and after each incubation, the plates were washed three times (300 µL/well) with PBST using an automatic microplate washer "96PW" (TECAN, Salzburg, Austria). High-binding polystyrene 96-well microplates were coated with 100 µL/well of anti-mouse IgG secondary antibody diluted in CBS (5 µg/mL). After incubation at 4 °C overnight, the plates were blocked with 2% (w/v) skim milk in PBS (300 µL/well) for 30 min. For competitive direct ELISA analysis, an appropriate dilution of 10H10 monoclonal antibody (100 µL/well) was added to the 96-well plate, followed by an incubation step of 1 h. After this, standard solutions of DON and PBS control were added (50 µL/well) for the standard curve and wheat or wheat dust extract (non-diluted, 1/2, 1/5 and 1/10 dilution) (50 µL/well) for the unknown grain sample. DON-HRP (50 µL/well) was added for competition of binding to the antibody and this was incubated for another hour. Then, 100 µL/well of TMB substrate solution was added. The reaction was stopped after 15 min with 2 M sulphuric acid (50 µL/well), and the absorbance at 450 nm was measured by a Bio-Rad model 550 microplate reader (Richmond, CA, USA).

Standard competitive curves were obtained by plotting relative absorbance (ratio of absorbance measured at the standard concentration and zero concentration: $B/B_0 \times 100\%$) against the logarithm of analyte concentration. IC_{50} values were determined to assess the assay sensitivity. Matrix-matched standard competitive curves were made by spiking blank wheat and wheat dust samples at different DON concentration levels until 3 000 µg/kg and

15 000 µg/kg, respectively. Blank samples were considered as blank if no DON contamination was found after LC-MS/MS analysis.

4.2.6 Method validation

The ELISA methods for the determination of DON in wheat and wheat dust were validated according to the criteria for the validation of semi-quantitative screening methods mentioned in Commission Regulation 519/2014/EC and Commission Decision 2002/657/EC. Twenty blank wheat and wheat dust samples and twenty wheat and wheat dust samples contaminated around the screening target concentration (STC) were analysed over 5 different days. The DON concentration was determined using matrix-matched standard competitive curves. The determined validation parameters include the apparent recovery, analytical range, limit of detection, sensitivity and intra-assay accuracy and precision. The fitness for purpose of the screening methods was determined by the evaluation of the cut-off value and false suspect rate.

4.2.7 Collection of wheat and corresponding dust samples

Wheat samples (n = 16) were randomly collected in Belgium and Hungary in the framework of the European FP7 MycoHunt project ("Rapid biosensor for the detection of mycotoxin in wheat"). As only 20 g of dust can be recovered out of a wheat bulk lot of 100 kg, extensive efforts were made to receive a sufficient amount of DON contaminated wheat samples from grain millers, but only 16 samples could be obtained. Out of one part of every wheat sample, dust was aspirated by the use of the dust collection facility, while the other part of the wheat sample was ground.

4.2.8 Dust correlation study

Using the developed and validated sample preparation and ELISA method, the DON content of the 16 wheat and corresponding wheat dust samples was determined. All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 22. Verifying a possible correlation, the Pearson correlation coefficient was determined for the batch of samples.

4.3 RESULTS AND DISCUSSION

4.3.1 Chemical composition of wheat dust

The water plus organic fraction as determined by the difference in weight before and after incineration resulted in 27.94% and 11.65% for the two different wheat dust batches, which lies in the range mentioned by Martin (1981). The mineral composition of the inorganic fraction, is shown in Table 4.1. In batch 1, the most predominant minerals were Fe, K and P and for batch 2, most mineral components were present in a higher quantity with extremely high values for Fe, K, Mg, Na and P. Therefore it can be concluded that the water and organic fraction and the mineral composition of wheat dust can be different from batch to batch.

It is generally known that changing the composition of PBS buffer or PBST buffer by adding extra sodium or phosphate, can have an influence on the outcome of an ELISA. Therefore, it can be concluded that a different mineral composition of wheat dust can change the ELISA results in the same way.

Table 4.1 Mineral composition of wheat dust

Mineral	Concentration batch 1 (µg/g) (*n* = 2)	Concentration batch 2 (µg/g) (*n* = 2)
Cu	51	339
Fe	27 400	13 450
K	31 700	79 300
Mg	8 700	44 650
Mn	768	2 320
Na	1 900	11 100
P	10 400	46 150
S	3 630	9 870
Zn	700	3 380

4.3.2 Optimization of sample preparation

The wheat and wheat dust matrix effect on the sensitivity of the developed 10H10 monoclonal antibody was evaluated. The results are presented in Figure 4.1. As seen, the sensitivity of the antibody decreased from a value of 22 ng/mL to 56 ng/mL and 61 ng/mL in wheat and wheat dust extract, respectively. As the sensitivity was reduced by half when working in a cereal matrix, it can be concluded that a matrix effect needs to be considered for wheat and wheat dust. Therefore it was decided to use matrix-matched calibration curves during the optimization experiments and for the ELISA screening of contaminated wheat and wheat dust samples.

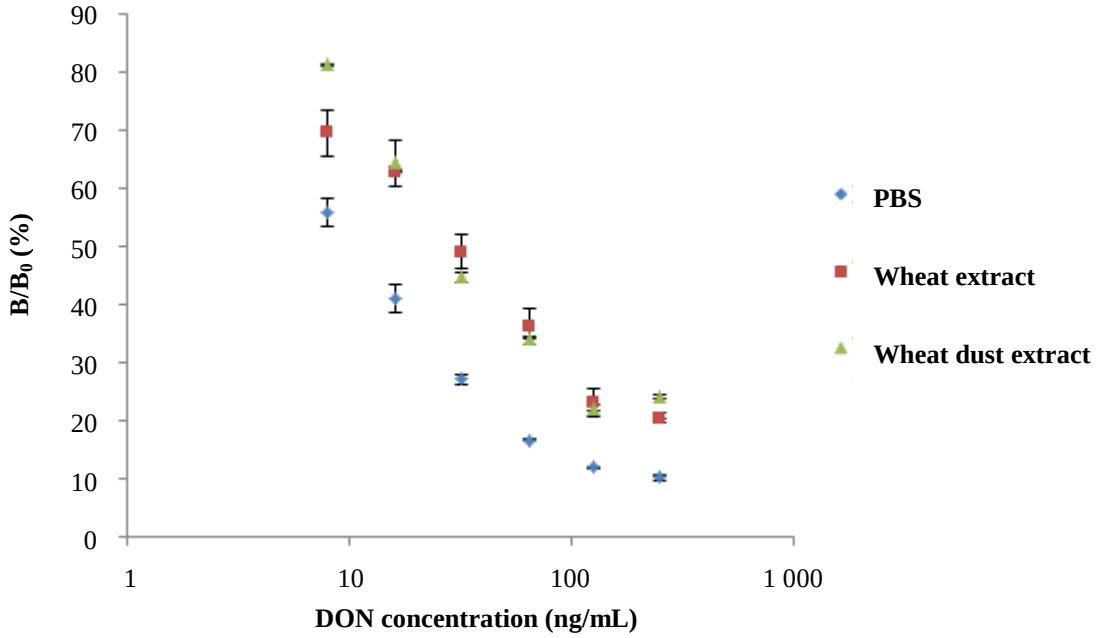


Figure 4.1 Calibration curve of DON in PBS, wheat and wheat dust extract with the 10H10 monoclonal antibody using a direct ELISA format. As the experiment was repeated on 3 consecutive days, the inter-day precision and accuracy was determined by the standard deviation and represented by the error bars on the calibration curve.

During the optimization experiments, blank wheat and wheat dust extracts were spiked with DON standard dissolved in acetonitrile (100 ng/µL) at a level of 3 000 µg/kg for wheat and between 4 000 µg/kg and 30 000 µg/kg for wheat dust. The best sample preparation conditions were determined based on the measured apparent recovery values. As DON is good water soluble (solubility of 11 g/L), there should be no problem of using water as extraction solvent.

First of all, half a gram of wheat dust was spiked with DON at a level of 4 000 µg/kg, 12 000 µg/kg and 20 000 µg/kg and extracted by overhead shaking for 1 h using 5 mL of water. After centrifugation, half of the supernatant was filtered using a Whatman No 4 filter (VWR International, Zaventem, Belgium) and diluted 1/10, 1/100 and 1/1 000 with water prior to direct ELISA. The ELISA results using the dust extracts without filtration are presented in Table 4.2. As seen for the 4 000 µg/kg spike, the best recoveries were found with the 1/100 diluted samples. This is normal as the linear range of the 10H10 antibody in wheat dust matrix lays in between 110 µg/kg and 4 060 µg/kg taken into account the 0.5 g of wheat dust and 5 mL of extraction solvent. Based on this linear range, only the 1/10 and 1/100 dilution would be quantifiable. As not much difference was seen between the use of filter or no filter, it was clear that small particles did not influence the antibody or the ELISA format. Therefore, no filter was used in further experiments. A very low recovery (below 32%) was observed for the spiked wheat dust samples at a level of 12 000 µg/kg and 20 000 µg/kg. Therefore it was concluded that a higher extraction volume or a certain percentage of methanol was necessary to extract large DON concentrations.

Table 4.2 Direct ELISA recovery results for wheat dust spiked at 4 000 µg/kg, 12 000 µg/kg and 20 000 µg/kg

Spiking level	Extraction ratio (g/mL)	Dilution	DON concentration (µg/kg)	Recovery (%)
4 000 µg/kg	1/10	1/10	2 060	51.60
		1/100	5 010	125.25
		1/1 000	52 110	1302.75
12 000 µg/kg	1/10	1/10	3 780	31.52
		1/100	1 980	16.53
		1/1 000	100 950	841.27
20 000 µg/kg	1/10	1/10	7 340	36.70
		1/100	4 510	22.54
		1/1 000	23 220	116.11

The same experiment was performed using only wheat dust samples spiked at 20 000 µg/kg extracting with water or water with 10% methanol (5 mL) to evaluate the influence of organic solvent on the extraction efficiency. No difference in recovery was observed between the use of water and water with 10% methanol as extraction solvent (p-value = 0.442) with a maximum recovery of 52%. When increasing the extraction volume of water up to 10 mL, 25

mL and 50 mL, the recovery increased with a maximum and optimal recovery value at 50 mL extraction volume and no subsequent dilution of supernatant prior to ELISA. All recovery values at spiking levels of 4 000 µg/kg, 8 000 µg/kg, 12 000 µg/kg, 16 000 µg/kg and 30 000 µg/kg lay in the range 75% - 153%. The extraction duration was the last parameter which was optimized. Spiked dust samples were extracted for 10, 20, 30 min and 1 h and the recovery was calculated and compared. A maximum recovery was reached after 20 min of water extraction.

For the optimization of wheat extraction, one gram of wheat was spiked with DON at a level of 3 000 µg/kg and extracted by overhead shaking for 1 h using 5 mL, 10 mL or 30 mL of water. The influence of centrifugation (3 000 g, 5 min) of wheat extracts prior to ELISA was determined and no difference was seen in the ELISA results when using a centrifuged extract or not (p-value = 0.098). As no difference in recovery was noticed between an extraction time of 30 min and 1 h (p-value = 0.488), it was decided to use the shorter extraction time of 30 min. We choose to use 30 mL of extraction volume in later experiments as an increase in recovery of 20% was observed compared to 5 and 10 mL of extraction solvent. In the optimal wheat sample preparation conditions the determined mean recovery was 100.17%.

4.3.3 Method validation

The ELISA methods were successfully validated for DON in wheat and wheat dust based on Commission Regulation 519/2014/EC and Commission Decision 2002/657/EC. A DON spiking range from 100 - 3 000 µg/kg and 500 - 15 000 µg/kg was taken for the wheat and wheat dust calibration curve, respectively. The linearity was evaluated by the correlation coefficient (r) and a lack of fit test.

The calibration curve showed good linearity for wheat, with a r of 0.998 and a p-value of 1.000. The apparent recovery ((RSD, %), n = 5) determined at 2 000 and 10 000 µg/kg was 96% (SD = 10) and 102% (SD = 7). A LOD determined as the IC_{10} (i.e. 10% inhibitory concentration) of 233 µg/kg or 4.67 ng/mL was calculated. The analytical range of the assay was set as 386 - 7 894 µg/kg based on the IC_{20} and IC_{80} value, though the maximum spiking level for the calibration curve was 3 000 µg/kg. During the precision study, relative standard deviations (RSDs) were measured at the different concentration levels; however, only the data obtained at the medium and high level (1 500 and 3 000 µg/kg) were recorded. For the intra-day precision an RSD_r of respectively, 3.87% and 7.93% for the medium level and high level was observed. For the inter-day precision an RSD_R of 7.50% and 8.73%, respectively was

determined. The RSD_r and RSD_R data obtained are in agreement with the acceptable RSD values for repeatability of quantitative methods according to Commission Decision 2002/657/EC. A maximum uncertainty (U) of the determined DON concentration was 22% for all spiking levels. For the determination of the fitness for purpose of the screening method, the STC was set at the maximum DON regulatory level in unprocessed cereals other than durum wheat, oats and maize (1 250 µg/kg) and the contamination level of the 20 positive control samples was taken around this STC, namely at 1 500 µg/kg. The calculations of the cut-off level and the rate of false suspected results were based on the relative responses of the blank samples and positive control samples. As a cut-off value a relative response of 0.58 or 1 167 µg/kg was measured, which approximately corresponds to the STC. For the determination of the false suspect results, a t-value of 5.68 was calculated which corresponds to the event that a result of a negative control sample is above the cut-off value. Based on this t-value and the degrees of freedom (19), a probability of false suspect samples for a one tailed distribution of less than 0.01% was determined.

The calibration curve for wheat dust revealed good linearity as well, giving a r of 0.998 and a p-value of 1.000. The apparent recovery ((RSD, %), n = 5) determined at 2 000 and 10 000 µg/kg was 96% (SD = 6) and 108% (SD = 5). The analytical range of the assay was set as 744 - 13 775 µg/kg and a LOD of 458 µg/kg or 9.15 ng/mL was calculated. RSDs were recorded at a medium concentration level of 2 000 µg/kg and a high concentration level of 8 000 µg/kg. For the intra-day precision an RSD_r of respectively, 7.33% and 9.28% for the medium level and high level was observed. For the inter-day precision an RSD_R of 8.16% and 9.47%, respectively was determined. A maximum uncertainty of the determined DON concentration was 29% for all spiking levels. As no maximum levels are yet established for DON in wheat dust, the STC was chosen at a higher concentration than for wheat based on the research results described in *Chapter 2* and was set at 8 000 µg/kg. The calculations of the cut-off level and the rate of false suspected results were based on the relative responses of the blank samples and positive control samples. As a cut-off value a relative response of 0.36 or 6 326 µg/kg was measured. For the determination of the false suspect results, a t-value of 7.32 was calculated which corresponds to the event that a result of a negative control sample is above the cut-off value. By the use of the t-value and the degrees of freedom (19), a probability of false suspect samples for a one tailed distribution of less than 0.01% was determined.

Based on the above described validation results, both ELISA methods show to be acceptable for their purpose. An overview of the validation results is given in Table 4.3.

Table 4.3 Overview of validation results of the wheat and wheat dust ELISA screening methods

	Wheat		Wheat dust	
Parameters	Spiking level (µg/kg)	Results	Spiking level (µg/kg)	Results
r		0.998		0.998
Apparent recovery	2 000	96 %	2 000	96 %
	10 000	102 %	10 000	108 %
LOD		233 µg/kg		458 µg/kg
Analytical range		386 – 7 894 µg/kg		744 – 13 775 µg/kg
RSD_r	1 500	3.87 %	2 000	7.33 %
	3 000	7.93 %	8 000	9.28 %
RSD_R	1 500	7.50 %	2 000	8.16 %
	3 000	8.73 %	8 000	9.47 %
U		< 22 %		< 29 %
False suspect result		< 0.01 %		< 0.01 %

4.3.4 Analysis of wheat and wheat dust samples

The DON content of wheat and dust samples (n = 16) was determined according to the described direct ELISA method. Each wheat and corresponding wheat dust sample was analysed once. All wheat samples were contaminated with DON in a range of 14 µg/kg to 1 113 µg/kg with a mean contamination level of 244 µg/kg (median = 75 µg/kg). Dust samples clearly showed higher levels in a range from 607 µg/kg to 14 043 µg/kg with a mean contamination of 5 012 µg/kg (median = 1 518 µg/kg). According to the Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, for DON in unprocessed cereals other than durum wheat, oats and maize the maximum limit was set at 1 250 µg/kg. No wheat samples exceeded this maximum limit.

4.3.5 Dust correlation study

The ELISA results for each wheat sample and the corresponding dust sample are presented in a scatterplot (Figure 4.2). A sigmoidal correlation between the DON concentration in wheat dust versus the DON concentration in wheat was proved according to the correlation coefficient, r (0.928) and a trend analysis of the residuals. When not considering the data

points with a DON concentration in wheat lower than 200 µg/kg (< LOD), a linear correlation (r = 0.889) was found. The slope of the trend line described a value of 5.023 which corresponds to a 5-fold accumulation of DON on small particles and is different to the slope (13.192) found after LC-MS/MS analysis of wheat and their corresponding wheat dust samples (*Chapter 2*). As limited wheat and corresponding wheat dust samples were obtained and only at a concentration level in wheat lower than 1 200 µg/kg, the uncertainty of the slope corresponding to the linear curve is high. Also, no conclusion can be taken about whether to use the linear or logarithmic curve.

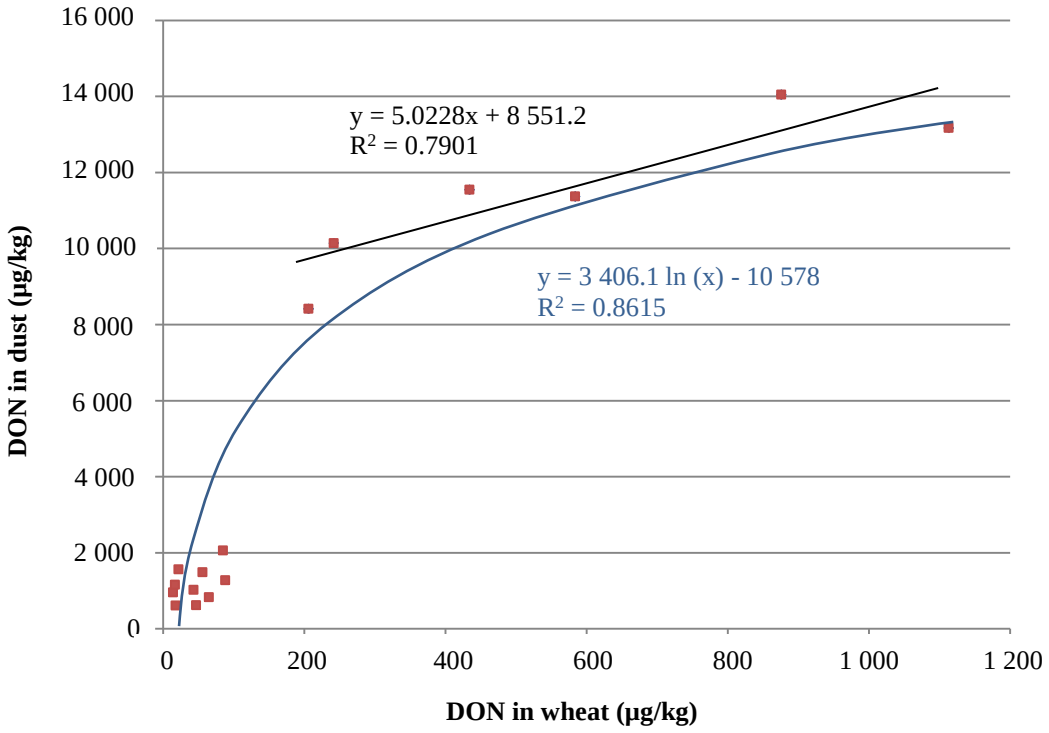


Figure 4.2 A scatterplot of the DON concentration in dust (y-axis) versus wheat (x-axis). A sigmoidal correlation between the DON concentration in wheat dust versus the DON concentration in wheat was observed (r = 0.928). When not considering the data points with a DON concentration in wheat lower than 200 µg/kg (< LOD), a linear correlation (r = 0.889) was found.

4.4 CONCLUSION

Based on the results of *Chapter 4*, it can be stated that the ELISA methods were successfully developed and validated for rapid screening of DON in wheat and wheat dust. The developed ELISA methods were used in an experimental field trial, where a linear and logarithmic (r = 0.889 and 0.928, respectively) correlation was found between the DON content in wheat dust versus the DON content in wheat. At the moment, no decision can be made about whether to use the linear or logarithmic calibration curve as more contaminated wheat and corresponding dust samples are necessary.

The chemical composition results of wheat dust reveal that certain wheat dust samples can have a very high mineral content. These minerals, like Fe, K and P can influence the ELISA results. As a high extraction volume (50 mL) was used, the concentration of these minerals and their influence on the developed ELISA format can be considered as negligible.

In general, the sampling of dust and subsequent ELISA analysis can be considered as a fast and easy-to-use technique which can be performed on-site. The determined ELISA results show together with the LC-MS/MS data described in *Chapter 2*, the possibility of estimating the DON content in cereals through the determination of DON in dust.

CHAPTER 5

THE DEVELOPMENT OF SPECIFIC RECOMBINANT ANTIBODIES AGAINST DEOXYNIVALENOL

CHAPTER 5: THE DEVELOPMENT OF SPECIFIC RECOMBINANT ANTIBODIES AGAINST DEOXYNIVALENOL

SUMMARY

There is a need for a sensitive screening technique for the determination of DON in cereal samples. Currently, only for DON maximum levels were set by the European Commission. Therefore, specific monoclonal antibodies against DON with minimal cross-reactivity against other mycotoxins like 3-ADON or 15-ADON need to be developed. By using the hybridoma technology, three different monoclonal anti-DON antibodies were developed but each of them showed cross-reactivity against structurally related trichothecene analogues. Therefore it was decided to use recombinant immunotechnology to synthesize more specific anti-DON antibodies. A recombinant scFv antibody was developed from the RNA of the hybridoma producing anti-DON monoclonal antibodies and checked for its binding properties towards DON. No specificity tests were carried out as too low *E. coli* expression levels were obtained to set up ELISA calibration curves. Using the Swiss Model program, molecular modeling of the constructed scFv was performed. The binding of the ligand DON to the model scFv was predicted by SwissDock.

Keywords: deoxynivalenol, recombinant antibodies, molecular modeling, docking

5.1 INTRODUCTION

The objective of *Chapter 5* was to improve the specificity of the 10H10 monoclonal anti-DON antibody and to relocate the specific character of the 2A9 monoclonal anti-DON antibody towards DON. Recombinant antibody technology involves the handling of key antibody fragments and expression in *E. coli*, allowing low-cost and high-speed production and purification, which are important advantages for medical, diagnostic and therapeutic applications [Choi *et al.*, 2004].

One of the most popular types of recombinant antibodies is the scFv, which is easily produced by several expression systems. In this chapter, the development of a DON scFv is described in detail. Repertoires of PCR amplified V_H and V_L chain antibody gene fragments were cloned into a phagemid vector to produce scFv-displaying phage particles. During this study, the phage display vector pComb3XSS was employed to express DON scFv on the surface of filamentous phage particles [Maragos *et al.*, 2012; Fitzgerald *et al.*, 2011; Fallecker *et al.*, 2013]. Based on the sequencing results of the developed DON scFv, modeling and docking experiments were carried out to evaluate the possible interaction sites and strength towards the DON antigen.

5.2 MATERIALS AND METHODS

5.2.1 Reagents and chemicals

Colorburst™ blue TMB substrate solution containing hydrogen peroxide was supplied by Alerchek (Springvale, Maine, USA). Expedeon InstantBlue™ stain and TMB membrane substrate were purchased from Thermo Scientific (Rockford, USA). PBS (0.01 M, pH 7.4) powder, CBS (0.05 M, pH 9.6) capsule, Tween 20, skim milk powder, RNase-free water, agarose, NaH_2PO_4, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, bromophenol blue, Tris(hydroxymethyl)aminomethane (TRIS), glycine, BSA, Triton X-100, lysozyme, imidazole, tryptone, yeast extract, NaCl, KCl, $MgCl_2.6H_2O$, $MgSO_4.7H_2O$, glucose, agarose, 3-(N-morpholino)propanesulfonic acid (MOPS), glycerol, KH_2PO_4, K_2HPO_4 and anti-HA-HRP were obtained from Sigma-Aldrich (Bornem, Belgium). DMEM, fetal bovine serum, penicillin, streptomycin, carbenicillin and Platinum Taq DNA polymerase High Fidelity were purchased from Invitrogen (Merelbeke, Belgium). TRIzol and SuperScript II reverse transcriptase kit, IPTG and One shot chemically competent TOP 10F' *E. coli* cells were bought at Life Technologies (Ghent, Belgium). The iScript Advanced cDNA synthesis kit for reverse transcription polymerase chain reaction (RT-PCR) was obtained from BioRad (Nazareth, Belgium). RNeasy mini kit, Qiashredder kit and nickel-nitrilotriacetic acid (Ni-NTA) resin were purchased from Qiagen (Manchester, UK). GoTaq G2 Flexi DNA polymerase and 1 kB DNA ladder were obtained from Mybio (Kilkenny, Ireland). T4 DNA ligase, deoxyribonucleotide triphosphate (dNTP) solution mix, Phusion High Fidelity DNA polymerase, SfiI restriction enzyme and Antarctic phosphatase with corresponding buffer were bought at New England Biolabs (Dublin, Ireland). XL1-Blue electroporation-competent *E. coli* cells were purchased from Agilent technologies (Cork, Ireland). Vivaspin 5-kDa cut-off columns were bought at GE Life Sciences (Diegem, Belgium). Nucleospin Extract II kit was obtained from Labquip Ireland (Dublin, Ireland). Other chemicals and solvents were of analytical grade. Water was obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium).

The *E. coli* growth media were prepared as follow. For the super optimal broth with catabolic repressor (SOC) medium, 20 g of tryptone, 5 g of yeast extract, 10 mL of 1 M NaCl and 2.5 mL of 1 M KCl were combined and water was added to 970 mL. The broth was autoclaved and cooled down to room temperature prior to the addition of 5 mL of 1 M $MgCl_2.6H_2O$, 5 mL of 1 M $MgSO_4.7H_2O$ and 10 mL of 2 M glucose (filtered solutions). To prepare Luria-Bertani (LB) broth, 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl were dissolved in 1

L of water. An amount of agar (15 g/L) was added to the LB broth for the preparation of agar plates. The super broth (SB) consisted of 10 g of MOPS, 30 g of tryptone and 20 g of yeast extract dissolved in 1 L of water. The terrific broth (TB) was prepared by mixing 12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol and adding water up to 900 mL. The LB broth or agar and SB and TB broth were autoclaved as well prior to use. For the TB broth extra nutrients were added including 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 dissolved in 100 mL of water, 5 mL of glycerol, 5 mL of 10% (w/v) glucose and 1 mL of 1 M $MgSO_4.7H_2O$.

Nunc-Immuno™ F96-well microplates were from Nalge Nunc International (Roskilde, Denmark).

5.2.2 First strand cDNA synthesis

The mouse hybridoma cell line 10H10 against DON and 2A9 against 3-ADON was established and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. For total mRNA isolation from the hybridoma cell line, TRIzol was used according to the manufacturer's instructions. Out of the derived mRNA, cDNA was synthesized using the iScript Advanced cDNA synthesis kit for RT-PCR containing reverse transcriptase and the corresponding reaction mix. Because of failure of correctly folded cDNA synthesis using this procedure, for the 2A9 hybridoma cell line, mRNA was extracted using the RNeasy mini kit combined with the Qiashredder kit. After confirmation of successful mRNA extraction using gel electrophoresis, cDNA was generated by reverse transcription using the SuperScript II reverse transcriptase kit.

5.2.3 Construction of scFv expression vector

The cDNA was used as a template for the amplification of V_H and V_L genes. Primers sourced from Eurofins MWG (Ebersberg, Germany) were compatible with the primers described by Barbas *et al.* (2001). For the amplification of 2A9 cDNA, a mix of V_κ5' MSCVK1-17 sense and V_κ3' MSCJK12-BL, MSCJK4-BL and MSCJK5-BL reverse primers and a mix of V_H 5' MSCVH1-19 sense and V_H3' MSCG1ab-B, MSCG3-B and MSCM-B reverse primers were used. For the 10H10 cDNA, the V_κ 5' MSCVK17 sense primer and the mix of V_κ3' MSCJK12-BL, MSCJK4-BL and MSCJK5-BL reverse primers and V_H 5' MSCVH5 sense primer and the mix of V_H3' MSCG1ab-B, MSCG3-B and MSCM-B reverse primers were

combined. The optimal $MgCl_2$ concentration for DNA amplification of the 2A9 and 10H10 cDNA using goTaq DNA polymerase was set at 4 mM and 3 mM. The PCR products were purified using the Nucleospin Extract II kit. The V_H and V_L purified fragments were joined via a glycine-serine linker by SOE-PCR using high fidelity Phusion Taq and 2 mM $MgCl_2$ for the 2A9 DNA and high fidelity Platinum Taq and 1.5 mM $MgSO_4$ for the 10H10 DNA to produce a 700-800 bp fragment that was cloned into the pComb3XSS vector via the SfiI restriction sites. The cyclic DNA of the pComb3XSS is presented in Figure 5.1.

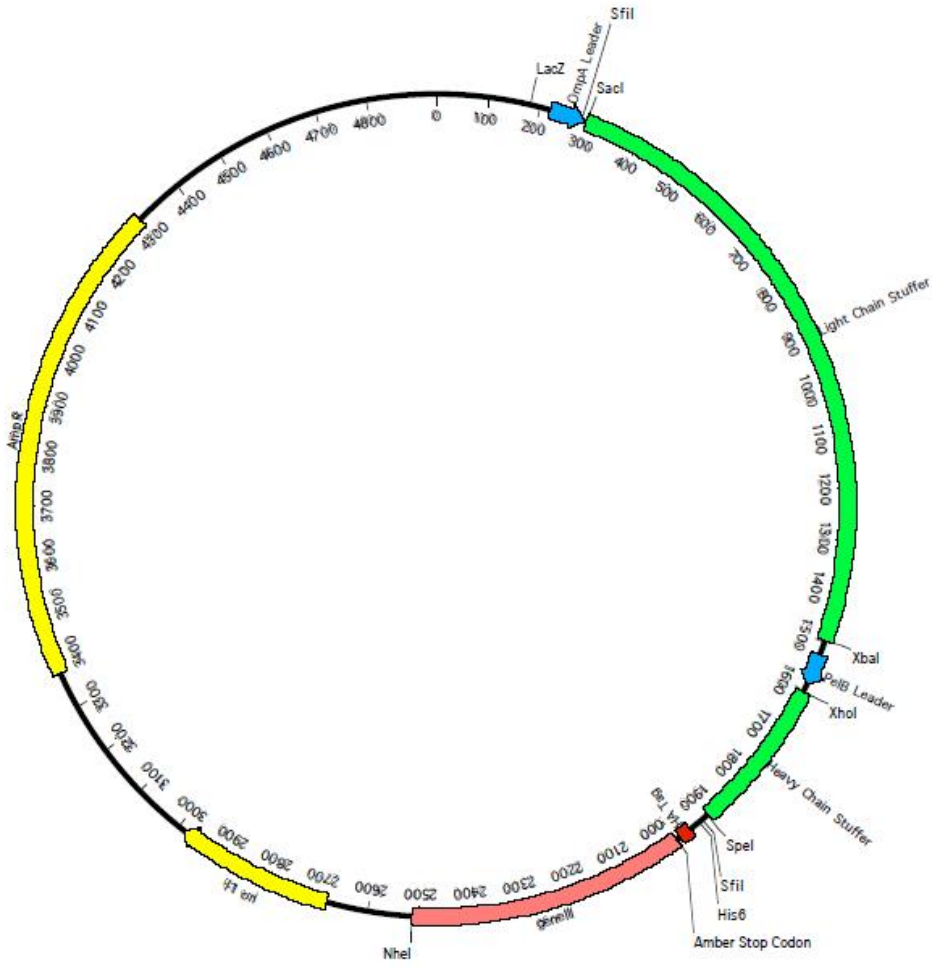


Figure 5.1 Cyclic DNA of pComb3XSS vector

First of all, the SOE-PCR products and the cyclic DNA of the pComb3XSS were digested using SfiI restriction enzymes. Subsequently, to 1 µg of the PCR products, 4 µL of NEB buffer and 16 units of SfiI enzyme was added and the volume was adjusted to 50 µL with water. For the pComb vector, 20 µg of the DNA was combined together with 20 µL of NEB buffer and 120 units of SfiI enzyme and the volume was adjusted to 100 µL with water. The digests were incubated for 5 h in a warm water bath at 50 °C. To the digested pComb vector,

5 µL of Antarctic phosphatase enzyme together with 10 µL of reaction buffer was added and this was incubated for 30 min at 37 °C and inactivated for 20 min at 65 °C. The digested SOE-PCR and pComb products were run on a 1% and 0.6% agarose gel, respectively. The digested SOE-PCR, pComb and stuffer were cut out and purified using the Nucleospin Extract II kit. The concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, USA).

Ligation of the digested SOE-PCR into the digested pComb vector was performed overnight at room temperature using T4 DNA ligase enzyme and the corresponding buffer. In detail, 140 ng of the digested pComb vector was added to 70 ng of digested SOE-PCR insert together with 1 µL of T4 DNA ligase (400 units) in the appropriate buffer. The ligation efficiency of the pComb vector was tested by ligating it with the gel-purified stuffer fragment that was generated during SfiI digestion of the vector DNA (control 1). The amount of uncut or partly cut vector DNA was estimated by setting up a ligation reaction that contains only vector DNA (control 2). These two control ligations were performed using the same amount of pComb vector DNA.

Transformation of the ligated product into a commercially available strain of XL-1 Blue or One shot chemically competent TOP 10F' was performed. For the transformation into XL-1 Blue *E. coli* cells, a Gene Pulser Xcell electroporation system was used. The electroporation was performed at 2.5 kv, 25 µF, 200 Ω during 4.0 msec. The transformation into One shot chemically competent TOP 10F' *E. coli* cells was performed by incubation on ice and 42 °C for 30 min and 30 sec, respectively, followed by transfer of the cells on ice. The time lapse between applying the pulse and transferring the cells to the pre-warmed SOC growth medium was kept to an absolute minimum to ensure optimal recovery of the transformed *E. coli* cells. The transformed culture was serially diluted and plated on selective LB agar containing carbenicillin (100 µg/mL) plates.

5.2.4 Expression of scFv antibody

One colony of the *E. coli* cells was picked and the DNA was amplified using PCR to confirm the transfer of the DNA encoding the DON scFv. Another *E. coli* colony was transferred to 5 mL of TB growth medium containing carbenicillin (100 µg/mL) and cultured overnight at 37 °C while shaking at 240 rpm. Of the overnight culture, 1 mL was transferred into 400 mL of fresh SB medium containing carbenicillin (100 µg/mL) and grown at 37 °C with shaking

(240 rpm) until an OD_{600} of 0.6 was reached. Then, 1 mM IPTG was added to the culture and incubated overnight at 30 °C with shaking (240 rpm). The culture was spun at 1 466 g for 10 min to pellet the bacterial cells and the pellet was thoroughly resuspended in 15 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 7.5) and transferred to ice. This was sonicated on ice for 3 min (6 sec intervals) at an amplitude of 40 using a microtip Vibra Cell™ sonicator (Sonics, Newtown, USA). The cell debris was then removed by centrifuging at 13 201 g and 4 °C for 20 min and the lysate supernatant was filtered through a 0.2 µm filter prior to purification using IMAC.

5.2.5 Purification of scFv antibody

Purification of the filtered lysate was performed by IMAC using Ni-NTA resin. The Ni-NTA slurry was added to a 10 mL column and equilibrated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 7.5). Then, the filtered lysate was transferred to the equilibrated column and the flow through was collected. The column was washed with 8 mL of 50 mM NaH_2PO_4, 1 M NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and 20 mM imidazole (pH 7.5) followed by 16 mL of 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole (pH 7.5) and the flow-through was again collected. Elution of bound scFv was performed using a high concentration of imidazole by adding 5 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0). The eluate containing the scFv was buffer-exchanged against PBS using a 5 kDa cut-off Vivaspin column (GE Life Sciences, Diegem, Belgium). The scFv concentration was determined by the use of a Nanodrop ND-1000 spectrophotometer.

During the optimization of the expression and consequent purification procedures, the different flow-through and elution fractions were subjected to indirect competitive ELISA, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting assay.

5.2.6 Indirect ELISA

All incubations, except for the first coating step, were carried out at 37 °C, and after each incubation, the plates were washed three times (300 µL/well) with PBST followed by three times washing with PBS. High-binding polystyrene 96-well microplates were coated with the prepared DON conjugates coupled to OVA diluted in CBS (100 µL/well). After incubation at

4 °C overnight, the plates were blocked with 2% (w/v) skim milk in PBS (300 µL/well) for 30 min. In the first step, 100 µL/well of diluted scFv antibodies in PBS was added. After incubation for 1h, 100 µL/well of anti-HA-HRP was added and incubated for another 1 h. Then, 100 µL/well TMB substrate solution was added. The reaction was stopped after 15 min with 2 M sulphuric acid (50 µL/well), and the absorbance at 450 nm was measured by a Bio-Rad model 550 microplate reader (Richmond, CA, USA).

5.2.7 SDS-PAGE and Western blotting assay

Flow-through and elution fractions were mixed with loading buffer (5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 20 µg/mL bromphenol blue in 125 mM Tris pH 6.8) in a ratio 3/1 (v/v), heated at 98 °C for 5 min and separated by SDS-PAGE using electrophoresis buffer containing 5 mM Tris pH 8.3, 19.6 mM glycine and 0.01% (w/v) SDS. For SDS-PAGE analysis, the separated proteins were visualized by the use of Instant Blue stain. Prior to Western blotting the SDS-PAGE gels containing the separated proteins were electrophoretically transferred onto a nitrocellulose membrane at 20 V for 15 min. The nitrocellulose membrane was blocked with 5% (w/v) BSA in PBS for 45 min, followed by the addition of anti-HA-HRP and subsequent incubation for 1 h. Then TMB substrate solution was added and the colour development was observed.

5.2.8 ScFv antibody sequence analysis

The DNA sequencing of the scFv antibodies was performed at Source Bioscience (Tramore, Ireland). The CDR and FR regions of the V_H and V_L chain were determined at the Abnum server (http://www.bioinf.org.uk) by the use of the Kabat numbering system. A proteomic study was performed for the total scFv protein structure using the online ExPASy and PSIPRED program (http://bioinf.cs.ucl.ac.uk).

5.2.9 Modeling and docking experiments

Computational homology modeling was performed using the SWISS-MODEL web server. ScFv model templates were identified based on the sequence of the 10H10 scFv and the template with the greatest identity was selected. Using the CASTp server, surface accessible pockets for different molecules such as receptors and ligands were determined. The solvent

accessibility, which determines the arrangement of amino acid residues, was measured by the ASAView automated server [Ahmad *et al.*, 2004]. Molecular docking of the 10H10 model scFv and the DON ligand was performed using the online SwissDock and UCSF Chimera programs. Different clusters were generated sorted by FullFitness energy value (kcal/mol).

5.3 RESULTS

5.3.1 First strand cDNA synthesis

First of all, the RNA from the 2A9 and 10H10 hybridomas was extracted by using TRIzol and the cDNA was synthesized by the iScript Advanced cDNA kit. Further amplification of the 2A9 cDNA using the primers described by Barbas *et al.* (2001) did not succeed even though the cDNA pattern observed by gel electrophoresis indicated it to be correct. As the problem could be a non-correctly folded cDNA, RNA extraction and cDNA synthesis were repeated using the RNeasy mini and the Qiashredder kit, followed by SuperScript II reverse transcriptase. After this, a correctly folded cDNA was obtained which was further used for scFv construction. The electrophoretic separation of the RNA and cDNA on a 1% agarose gel together with a 1 kB DNA ladder, is illustrated in Figure 5.2. Compared to the description by Barbas *et al.* (2001), a normal pattern with two bands at a size of 850 bp and 1500 bp for RNA and a smear for cDNA, was observed.

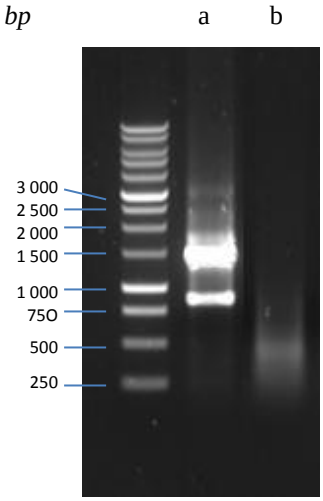


Figure 5.2 Agarose gel electrophoresis of 2A9 RNA (a) and cDNA (b)

5.3.2 Construction of scFv expression vector

For the construction of the 2A9 and 10H10 scFv, different parameters were optimized. Different $MgCl_2$ (1 mM, 1.5 mM, 2 mM, 3 mM and 4 mM) concentrations were used during the goTaq polymerase reaction. Using a combination of V_H 5' sense primers MSCVH1-19 or single MSCVH primers together with a combination of V_H3' reverse primers MSCG1ab-B, MSCG3-B and MSCM-B, it was possible to select the optimal primer composition for V_H amplification. The determination of a V_κ or V_λ light chain was performed using specific V_κ5'

sense/ $V_\kappa 3$' reverse primers and $V_\lambda 5$' sense/ $V_\lambda 3$' reverse primers respectively. As no amplification was observed using the combination of V_λ primers, the presence of V_κ light chains was concluded for both 2A9 and 10H10 antibody. The optimal primer composition for V_κ amplification was selected by using a combination of $V_\kappa 5$' sense primers MSCVK1-17 or single MSCVK primers together with a combination of $V_\kappa 3$' reverse primers MSCJK12-BL, MSCJK4-BL and MSCJK5-BL. Figure 5.3 shows the PCR amplification of 10H10 V_H and V_κ using the optimized conditions. The V_H and V_κ DNA products were derived using optimized PCR conditions and ran on an agarose gel, the bands were cut out and purified for further SOE-PCR reaction.

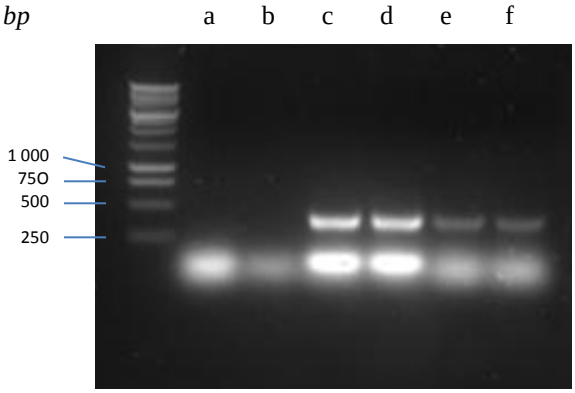


Figure 5. 3 Agarose gel electrophoresis after PCR amplification of 10H10 V_H and V_κ. Control (a, b), V_κ (c, d) and V_H (e, f).

During SOE-PCR, two different high fidelity polymerases were used. First of all, Platinum Taq was used for the linking of the V_H and V_κ of both 2A9 and 10H10 DNA products. A SOE-PCR product at a size of 750 bp was seen for 10H10 after agarose gel electrophoresis on a 1% gel (Figure 5.4). As for 2A9, no SOE-PCR product at this size was observed, it was decided to use another high fidelity polymerase enzyme, namely Phusion Taq. After SOE-PCR, 2 bands were observed for the 2A9 SOE-PCR at a size of 500 bp and 700 bp (Figure 5.4). The 2A9 and 10H10 SOE-PCR reactions were optimized using different concentrations of $MgCl_2$ (1.5 mM, 2 mM, 2.5 mM and 3 mM) and $MgSO_4$ (1.5 mM, 2 mM and 2.5 mM) for the Phusion Taq and Platinum Taq polymerase, respectively. The 2A9 (2 bands) and 10H10 (1 band) SOE-PCR products derived using optimized PCR conditions and ran on a 1% agarose gel were cut out and purified.

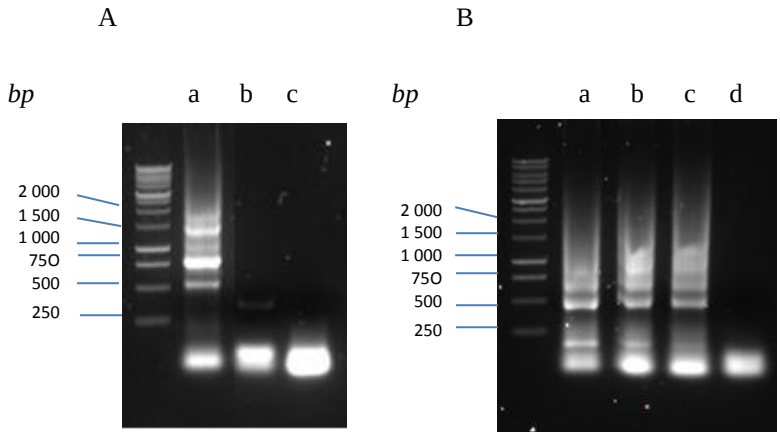


Figure 5.4 Agarose gel electrophoresis after SOE-PCR amplification. A: 10H10 SOE-PCR using (a) optimized PCR conditions, (b) no polymerase (control), (c) no V_H and V_κ. B: 2A9 SOE-PCR using (a) 1.5 mM $MgSO_4$, (b) 2 mM $MgSO_4$, (c) 2.5 mM $MgSO_4$, (d) no V_H and V_κ.

The purified pComb3XSS vector DNA and SOE-PCR were digested using the SfiI restriction enzyme and the digests were run on a 0.6% and 1% agarose gels, respectively. In Figure 5.5, the separation of two different amounts of the digested pComb vector on a 0.6% agarose gel is shown. Four different DNA bands are noticed. The first DNA band corresponds to the non-digested pComb and has a size of around 5 500 bp. Underneath, there is a second DNA band with a size of around 3 500 bp, which is the digested pComb. The two other bands with a size of around 1 700 bp and 2 300 bp are corresponding to the single and double cut stuffer.

Ligation of the digested SOE-PCR into the digested pComb vector was performed together with two control ligations to determine ligation efficiency (control 1) and the amount of uncut or partly cut pComb vector DNA (control 2). After transfer of the ligated products into the *E. coli* cells, the plated colonies were checked on the LB agar plus carbenicillin plates. More colonies were found on the first control plates concluding that a pComb vector of good quality and ligation efficiency was observed. The number of colonies found on the second control plates was less than 10% of the amount of colonies on the SOE-PCR-pComb ligated plate, which means that a low background was observed.

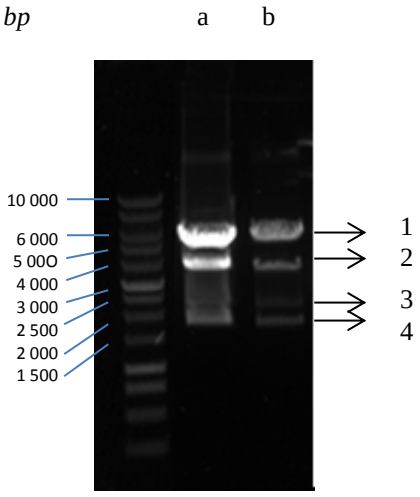


Figure 5.5 Agarose gel electrophoresis of a high (a) and low (b) amount of digested pComb3XSS vector with (1) the non-digested pComb vector (5 500 bp), (2) the digested pComb vector (3 500 bp), (3) single cut stuffer (2 300 bp), (4) double cut stuffer (1 700 bp).

5.3.3 Expression and purification of scFv

The transformed 10H10 XL-1 Blue *E. coli* cells were grown up in 2 mL of SB growth medium containing carbenicillin (100 µg/mL). Expression was performed overnight at 30 °C using 1 mM IPTG. The unpurified lysate from the expressed transformed XL-1 Blue *E. coli* cells was checked for the presence of 10H10 scFv using indirect ELISA. Therefore, the induced cultures were centrifuged at 825 g for 10 min. For cell lysis, the cell pellets were resuspended in 300 µL of PBS containing 1 mg/mL lysozyme followed by a freeze-thaw process. During this freeze-thaw process cells were frozen at -80 °C for 5 min followed by thawing in a 37 °C water bath for 3 min; this process was repeated twice. The cell debris was pelleted by centrifugation at 13 201 g for 5 min and the supernatant was used for indirect ELISA as this contains the scFv antibodies. For the indirect ELISA, 96-well microtitreplates were used coated with DON-OVA 4 µg/mL and 100 µL of the non-diluted supernatant was added to the plates. When measuring the optical density at 450 nm, no absorbance was observed. Another type of *E. coli* cells, namely One shot chemically competent TOP 10F' cells, were used for the expression of the 10H10 scFv antibody to see any difference in expression behaviour. The same process of cell lysis and ELISA screening was used and again no absorbance was observed at 450 nm.

The transformed 10H10 XL-1 Blue *E. coli* cell culture was scaled up using a volume of 400 mL of SB growth medium with carbenicillin (100 µg/mL). The SB medium was compared to other media, namely TB medium and overnight expressing medium in terms of scFv antibody

expression. Cell lysis was performed by dissolving the cell pellet in PBS containing 1 mg/mL lysozyme followed by the freeze-thaw process or by dissolving the cell pellet in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 7.5) followed by sonication. Purification of the filtered lysate was performed by IMAC using Ni-NTA resin under the conditions described in 5.2.5. The best scFv production conditions were observed using TB medium containing 100 µg/mL as *E. coli* growth medium. As not much difference was seen in the use of a freeze-thaw process and sonication for cell lysis, it was decided to further use the sonicator as this was more easy to handle with larger cell culture volumes. The concentration of the purified 10H10 scFv using optimal expression and purification conditions was set as 0.24 mg/mL as measured by the Nanodrop ND-1000 spectrophotometer. The different flow-through and elution fractions were subjected to SDS-PAGE and Western blotting (Figure 5.6). The different fractions that were analysed are the supernatant of the *E. coli* cells to see if the scFv antibody leaves the periplasmic region prior to cell lysis, the lysate, the flow-through, the flow-through after washing the Ni-NTA column and the eluate. Protein bands were observed at a size of around 27 kDa for the supernatant, the lysate, the flow-through and eluate (purified scFv). It can be concluded that the 10H10 scFv is present in the eluate even though some of the scFv antibody is lost due to the transfer of the scFv out of the periplasmic region of the *E. coli* cells before cell lysis and some is lost during purification. The purified 10H10 scFv diluted 1/10 was used in an indirect ELISA assay using different coating antigens, each of them belonging to the three different series of the immunogen synthesis strategy, namely DON-OVA, DON-CC-OVA and DON-CMO-OVA at a concentration of 4 µg/mL, 8 µg/mL, 16 µg/mL and 32 µg/mL. The results are presented in Table 5.1. As seen, the maximal absorbance occurred using DON-CC-OVA as coating antigen. As little difference in absorbance was seen between 8 µg/mL and 16 µg/mL in absorbance, it was decided to use the DON-CC-OVA 8 µg/mL as coating antigen in further experiments.

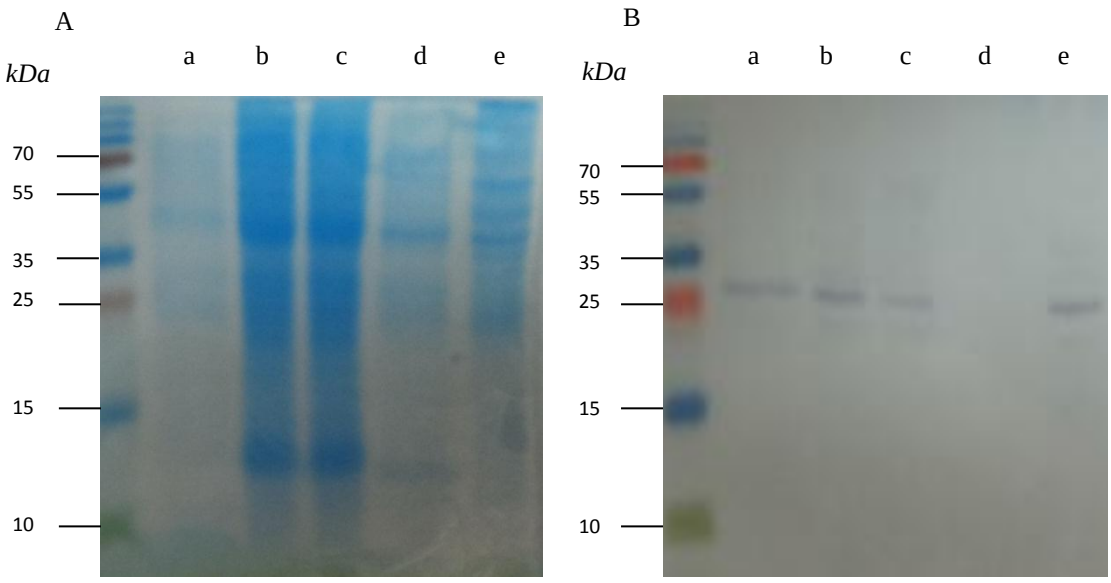


Figure 5.6 SDS-PAGE (A) and Western blot (B) of different flow-through and elution fractions during Ni-NTA purification of 10H10 scFv: (a) supernatant *E. coli* cells, (b) lysate, (c) flow-through, (d) flow-through after washing the column, (e) eluate (purified scFv).

Table 5.1 Indirect ELISA of purified 10H10 scFv using different coating antigens in different concentrations

Coating antigen	Coating concentration (µg/mL)	Absorbance (n = 2)
DON-OVA	4	0.174
	8	0.173
	16	0.194
	32	0.260
DON-CC-OVA	4	0.442
	8	0.568
	16	0.589
	32	0.565
DON-CMO-OVA	4	0.144
	8	0.112
	16	0.102
	32	0.099

The transformed 2A9 XL-1 Blue *E. coli* cells were grown in 2 mL of TB growth medium containing carbenicillin (100 µg/mL). Expression was performed overnight at 30 °C using 1 mM IPTG. The unpurified lysate from the 64 different expressed transformed XL-1 Blue *E.*

coli colonies was checked for the presence of 2A9 scFv using indirect ELISA with 96-well plates coated with DON-OVA 8 µg/mL or DON-CC-OVA 8 µg/mL. Therefore, the induced cultures were centrifuged at 825 g for 10 min. For cell lysis, the cell pellets were resuspended in 300 µL of PBS containing 1 mg/mL lysozyme followed by a freeze-thaw process. The cell debris was pelleted by centrifugation at 13 201 g for 5 min and the supernatant was subjected to indirect ELISA. The results are presented in Table 5.2. As seen, the absorbance values were generally higher for the DON-CC-OVA coating than for the DON-OVA coating. When a higher absorbance value was reached for one *E. coli* clone using DON-OVA coating, the signal was higher as well for the DON-CC-OVA coating. The highest absorbance value was seen for the cell clones C1, E1, G1 and H1. Therefore, these were further considered for large scale 2A9 scFv production.

To select optimal expression conditions for 2A9 scFv production, the first 8 clones (A1-H1) were selected and were grown up in 2 mL of TB growth medium containing carbenicillin (100 µg/mL). Expression was performed overnight at 25 °C or 30 °C using 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM or 4 mM IPTG. The unpurified lysate from the different expressed transformed XL-1 Blue *E. coli* colonies was checked for the optimal 2A9 scFv expression using indirect ELISA with DON-CC-OVA 8 µg/mL as coating antigen. The same experiment was performed for the 10H10 transformed XL-1 Blue *E. coli* cells to see if the use of a different induction temperature or IPTG concentration would increase small scale scFv production. The results are presented in Table 5.3. For the 2A9 transformed XL-1 Blue *E. coli* cells, maximum absorbance and therefore maximum scFv production was noticed for clone C1, E1 and F1 using an induction temperature of 30 °C and an IPTG concentration of 1 mM. For the 10H10 transformed XL-1 Blue *E. coli* cells, low absorbance values were seen for all scFv expression conditions. Some increasing trend in scFv production was observed using a lower induction temperature and an IPTG concentration of 0.1 mM, but the signal was too low to make any conclusion. So in general, it can be concluded that the 10H10 scFv production in small-scale is too low to be able to detect in an ELISA and the 2A9 scFv production can be increased by using an induction temperature of 30 °C and an IPTG concentration of 1 mM.

Table 5.2 Indirect ELISA of lysate 2A9 scFv of 64 different clones (A1-H8) using DON-OVA or DON-CC-OVA coating antigen 8 µg/mL

Coating antigen		1	2	3	4	5	6	7	8
DON-OVA	A	0.042	0.041	0.046	0.040	0.041	0.039	0.079	0.050
	B	0.043	0.042	0.066	0.052	0.041	0.040	0.050	0.041
	C	0.079	0.045	0.048	0.054	0.041	0.041	0.040	0.042
	D	0.044	0.043	0.043	0.042	0.049	0.062	0.053	0.067
	E	0.114	0.044	0.048	0.042	0.044	0.045	0.046	0.049
	F	0.088	0.041	0.056	0.052	0.047	0.042	0.040	0.061
	G	0.091	0.048	0.047	0.042	0.042	0.047	0.042	0.042
	H	0.139	0.048	0.050	0.112	0.045	0.042	0.041	0.044
DON-CC-OVA	A	0.042	0.041	0.044	0.040	0.040	0.039	0.098	0.052
	B	0.045	0.047	0.090	0.060	0.040	0.041	0.039	0.041
	C	0.165	0.045	0.046	0.063	0.044	0.042	0.040	0.042
	D	0.043	0.041	0.041	0.041	0.057	0.096	0.066	0.064
	E	0.295	0.050	0.061	0.081	0.045	0.055	0.070	0.057
	F	0.110	0.041	0.066	0.041	0.044	0.045	0.043	0.079
	G	0.159	0.055	0.048	0.042	0.048	0.041	0.042	0.043
	H	0.210	0.047	0.055	0.134	0.047	0.051	0.052	0.048

Table 5.3 Indirect ELISA of lysate 2A9 and 10H10 scFv obtained using different IPTG concentrations and induction temperatures during the expression of 8 different clones using DON-CC-OVA coating antigen 8 µg/mL

		Induction temperature 25 °C						Induction temperature 30 °C					
	IPTG conc	0.1 mM	0.2 mM	0.5 mM	1 mM	2 mM	4 mM	0.1 mM	0.2 mM	0.5 mM	1 mM	2 mM	4 mM
2A9	clone A1	0.044	0.043	0.042	0.041	0.039	0.040	0.040	0.041	0.039	0.039	0.039	0.040
	clone B1	0.050	0.045	0.043	0.042	0.042	0.040	0.040	0.046	0.042	0.046	0.042	0.041
	clone C1	0.105	0.072	0.091	0.084	0.076	0.056	0.085	0.093	0.077	0.157	0.148	0.103
	clone D1	0.085	0.045	0.044	0.041	0.041	0.040	0.041	0.041	0.039	0.040	0.041	0.039
	clone E1	0.076	0.079	0.072	0.111	0.067	0.067	0.070	0.074	0.115	0.214	0.195	0.161
	clone F1	0.091	0.097	0.092	0.075	0.071	0.086	0.069	0.093	0.069	0.171	0.100	0.191
	clone G1	0.086	0.081	0.086	0.076	0.089	0.071	0.084	0.085	0.093	0.116	0.173	0.102
	clone H1	0.093	0.103	0.080	0.070	0.066	0.076	0.083	0.083	0.063	0.103	0.069	0.072
10H10	clone A1	0.088	0.068	0.066	0.059	0.055	0.049	0.046	0.047	0.045	0.047	0.044	0.048
	clone B1	0.060	0.060	0.061	0.051	0.053	0.047	0.044	0.046	0.045	0.044	0.045	0.047
	clone C1	0.062	0.054	0.057	0.053	0.050	0.049	0.044	0.047	0.047	0.046	0.047	0.050
	clone D1	0.058	0.051	0.057	0.050	0.058	0.050	0.052	0.048	0.050	0.047	0.046	0.051
	clone E1	0.059	0.049	0.055	0.054	0.050	0.054	0.048	0.048	0.050	0.048	0.046	0.057
	clone F1	0.068	0.058	0.052	0.053	0.051	0.050	0.052	0.052	0.046	0.049	0.049	0.049
	clone G1	0.074	0.057	0.056	0.051	0.052	0.049	0.100	0.067	0.047	0.048	0.052	0.055
	clone H1	0.082	0.065	0.061	0.055	0.053	0.052	0.066	0.070	0.056	0.055	0.057	0.057

The transformed 2A9 XL-1 Blue *E. coli* cell culture was scaled up using a volume of 400 mL of TB growth medium with carbenicillin (100 µg/mL). Cell lysis was performed by dissolving the cell pellet in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 7.5) followed by sonication. Purification of the filtered lysate was performed as for the transformed 10H10 *E. coli* cells. The different flow-through and elution fractions were subjected to SDS-PAGE and Western blot analysis (Figure 5.7). The different fractions that were analysed are the lysate, the flow-through, the flow-through after washing the Ni-NTA column and the eluate. Protein bands were observed at a size of around 25 kDa for the lysate, the flow-through and eluate (purified scFv). For the Western blot, protein bands were observed at a much lower size (around 17 kDa) and the eluate did not seem to be pure as several protein bands of different sizes were observed. When analysing the lysate and purified 2A9 scFv in an indirect ELISA assay using DON-CC-OVA 8 µg/mL as coating antigen, only a signal was observed for the 2A9 lysate. It can be concluded that the majority of the 2A9 scFv is not binding to the Ni-NTA column and therefore gets lost during purification. As an alternative purification method $MgSO_4$ precipitation was tested, but this seemed to destroy the conformation and solubility of the antibody.

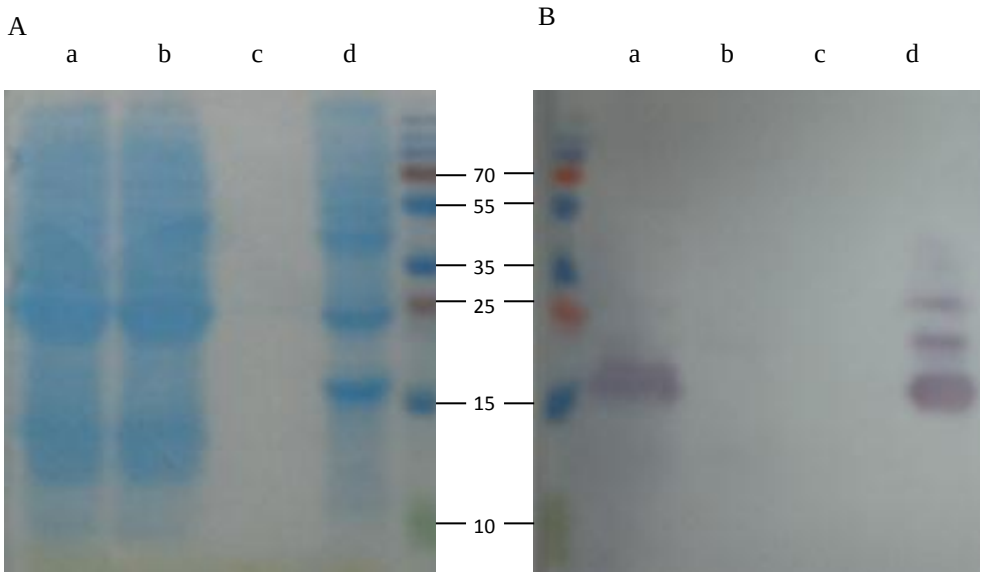


Figure 5.7 SDS-PAGE (A) and Western blot (B) of different flow-through and elution fractions during Ni-NTA purification of 2A9 scFv: (a) lysate, (b) flow-through, (c) flow-through after washing the column, (d) eluate (purified scFv).

5.3.4 ScFv antibody sequence analysis

Different purified and non-purified 2A9 XL-1 Blue *E. coli* samples were send off for sequencing, but from none of them a good sequence result could be obtained. The amino acid sequence of the 10H10 scFv antibody could be determined and is shown in Figure 5.8. The sequence has a total length of 243 amino acids including a flexible linker of glycine and serine residues, a HA decapeptide and HIS tag. The amino acid numbering and CDRs of the V_H (green) and V_L (blue) domains were determined according to Kabat *et al.* (2001). A total molecular weight of 26 251.9 Da and an isoelectric point of 8.28 was determined out of the ExPASy proteonomic study. The same molecular weight/size of protein was seen in the SDS-PAGE and Western blot analysis of the purified 10H10 scFv antibody, which confirms the previous results. Using the PSIPRED program, the secondary structure of the antibody was determined. The program detected 19 β-sheet folds, 20 random coils and no α-helices in the secondary structure of the scFv antibody. This is in agreement with the actual secondary structure of scFv antibodies.

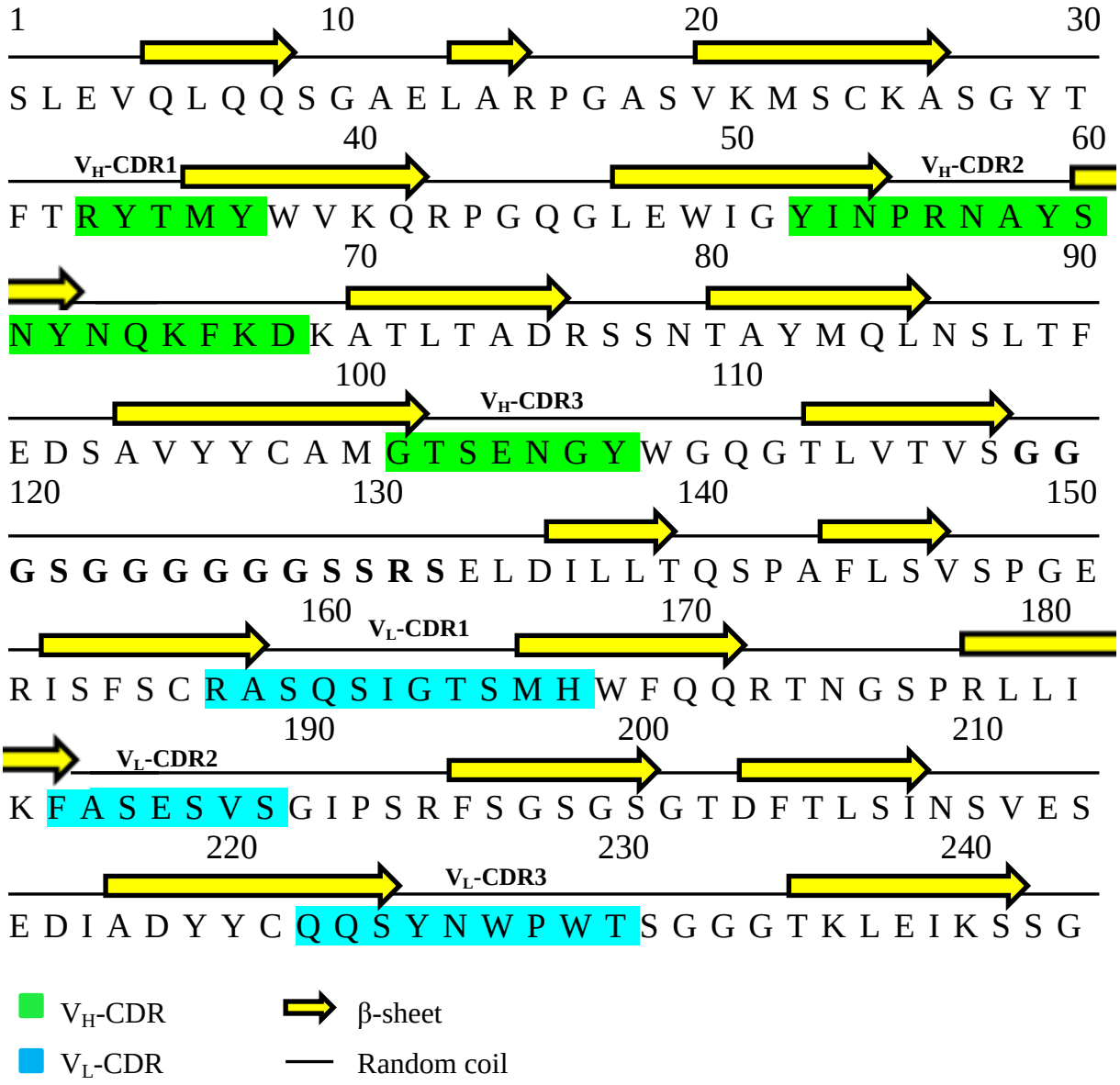


Figure 5.8 10H10 scFv amino acid sequence, mentioning the amino acid numbering, CDRs of the V_H and V_L domains and secondary protein structure according to the Kabat nomenclature

5.3.5 Modeling and docking experiments

Using the SWISS-MODEL web server, a computational homologue model of the 10H10 scFv was found with 61.92% identity. The homologue mouse scFv model is recognized in the protein databank (PDB) as 4H0G and is an scFv antibody which recognizes the antigen 2-amino-2-hydroxymethyl-propane-1,3-diol (TRS). The three-dimensional secondary structure of the model scFv and its antigen TRS is illustrated in Figure 5.9A. Also, a comparison is made between the three-dimensional structure of the TRS and DON antigen (Figure 5.9B/C). The secondary protein structure of the model scFv consists of 21 β-sheet folds, 22 random coils and 3 α-helices.

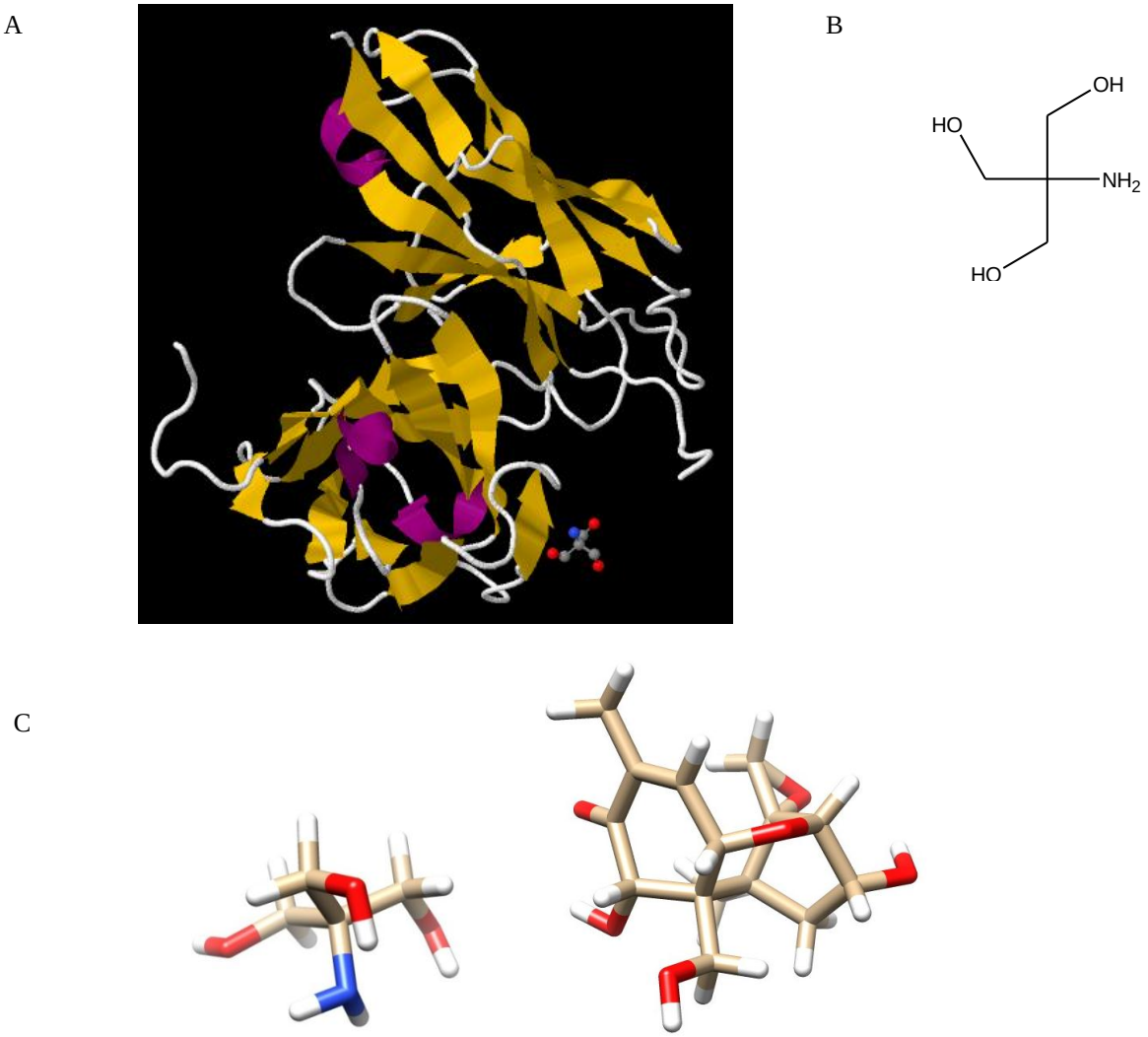


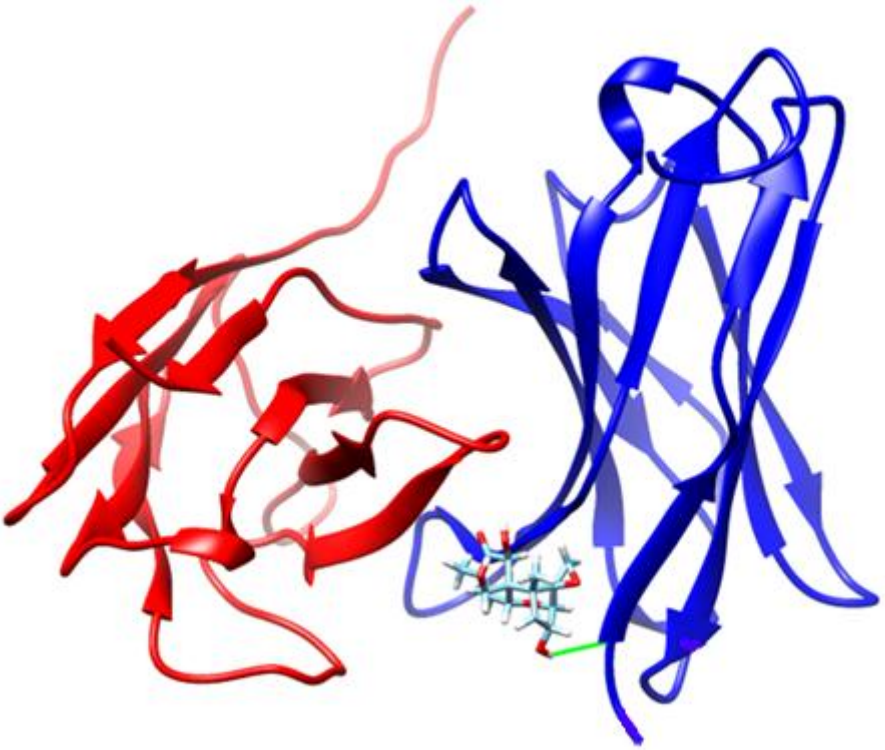
Figure 5.9 Three-dimensional structure of the model scFv-TRS interaction with the illustration of the β-sheet folds (yellow), random coils (white) and α-helices (purple) of the model scFv (A), two-dimensional structure of the TRS antigen (B) and three-dimensional structure of TRS and our target DON (C).

The CASTp automated server identifies surface accessible pockets for different molecules, including scFv receptors and ligands. When submitting the PDB code of the model scFv, a total of 42 different binding pockets at the model scFv were identified sorted by volume and area. The largest and most accessible binding pocket had a volume of 553.4 $Å^3$ and an area of 2070.2 $Å^2$. Based on this knowledge, the binding pockets of the model scFv suitable for binding with the target, namely the original TRS antigen or the DON antigen were determined using the online SwissDock and UCSF Chimera programs. A total of 33 and 62 different clusters binding in the different scFv pockets, were generated for the DON and TRS antigen respectively. The top 3 of most energetically enhanced interactions between the target and the model scFv is shown in Table 5.4. The FullFitness energy for all determined model scFv-DON clusters is negative and therefore favorable. The FullFitness values are higher than the ones for the model scFv-TRS interaction, but still very good. The Gibbs free energy is even lower than for the original scFv-target interact, which means that more energy will be released when DON binds to the model scFv. The three-dimensional structure of the energetic most favourable model scFv-DON cluster is presented in Figure 5.10. The first part of this figure illustrates the ribbons representation of the model scFv-DON cluster. As shown, a hydrogen bond is formed between the C_3 of DON and the glutamine amino acid of the model scFv. The second part of the figure represents the hydrophobic surface of the model scFv. The blue parts are the most hydrophilic parts and the colour goes from white to orange for more hydrophobic regions. Hydrogen bonds and hydrophobic interactions, together with electrostatic forces and Van der Waals bonds are responsible for the antibody-antigen interactions and give therefore an indication of the strength of the scFv-target cluster.

Table 5.4 Energetically favourable scFv clusters for the target DON and TRS

Antigen	Number of SwissDock clusters	Cluster rank	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
DON	33	1	-849.85	-7.14
		2	-849.83	-6.65
		3	-849.66	-6.77
TRS	62	1	-1100.13	-5.56
		2	-1099.95	-5.00
		3	-1099.02	-5.35

A



B

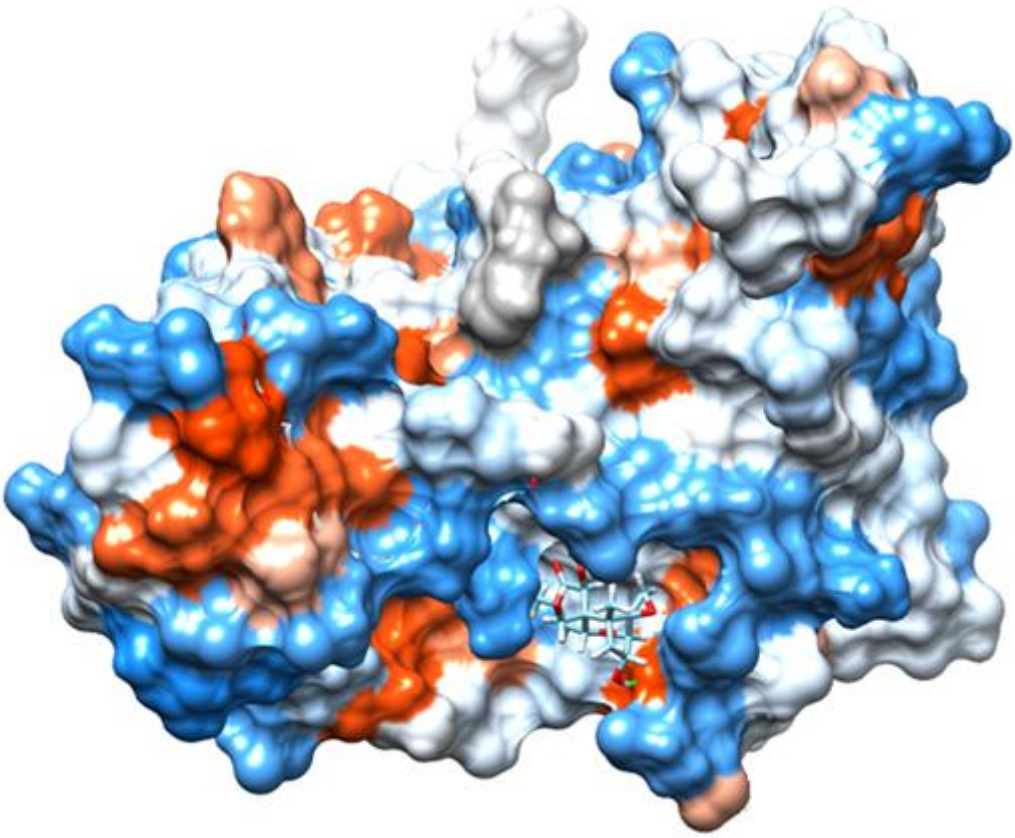


Figure 5.10 Three-dimensional structure of the model scFv-DON interaction: ribbons representation with V_L chain (red) and V_H chain (blue) (A) and hydrophobic surface from blue (hydrophilic) to orange colour (hydrophobic) (B).

The determination of solvent accessibility is an important step to three-dimensional structure prediction as it determines the packaging arrangement of amino acid residues. The solvent accessibility or accessible surface area was determined by the use of the ASAView automated server by uploading the PDB format of the model scFv. The results were given by the generation of a spiral plot sorting all amino acid residues (size of the circle) by their relative solvent accessibility (Figure 5.11). The blue and red colours represent the positive charged residues (R: arginine, K: lysine, H: histidine) and negative charged residues (D: aspartic acid, E: glutamic acid), respectively. The green circles stand for the polar uncharged residues (G: glycine, N: asparagine, Y: tyrosine, Q: glutamine, S: serine, T: threonine, W: tryptophan) and the yellow circles are the cysteine amino acids. All other hydrophobic residues (P: proline, A: alanine, V: valine, I: isoleucine, L: leucine, M: methionine, F: phenylalanine) are represented by a grey colour. When having a look at the model scFv in the Swiss-PDB viewer and UCSF Chimera programs, glutamine 5 (Q5) is responsible for the hydrogen bond (Figure 5.10) between the C_3 of DON and the model scFv. This Q5 residue is presented in the outer shelf of the spiral plot in a green circle at the left side (Figure 5.11), which confirms its accessibility.

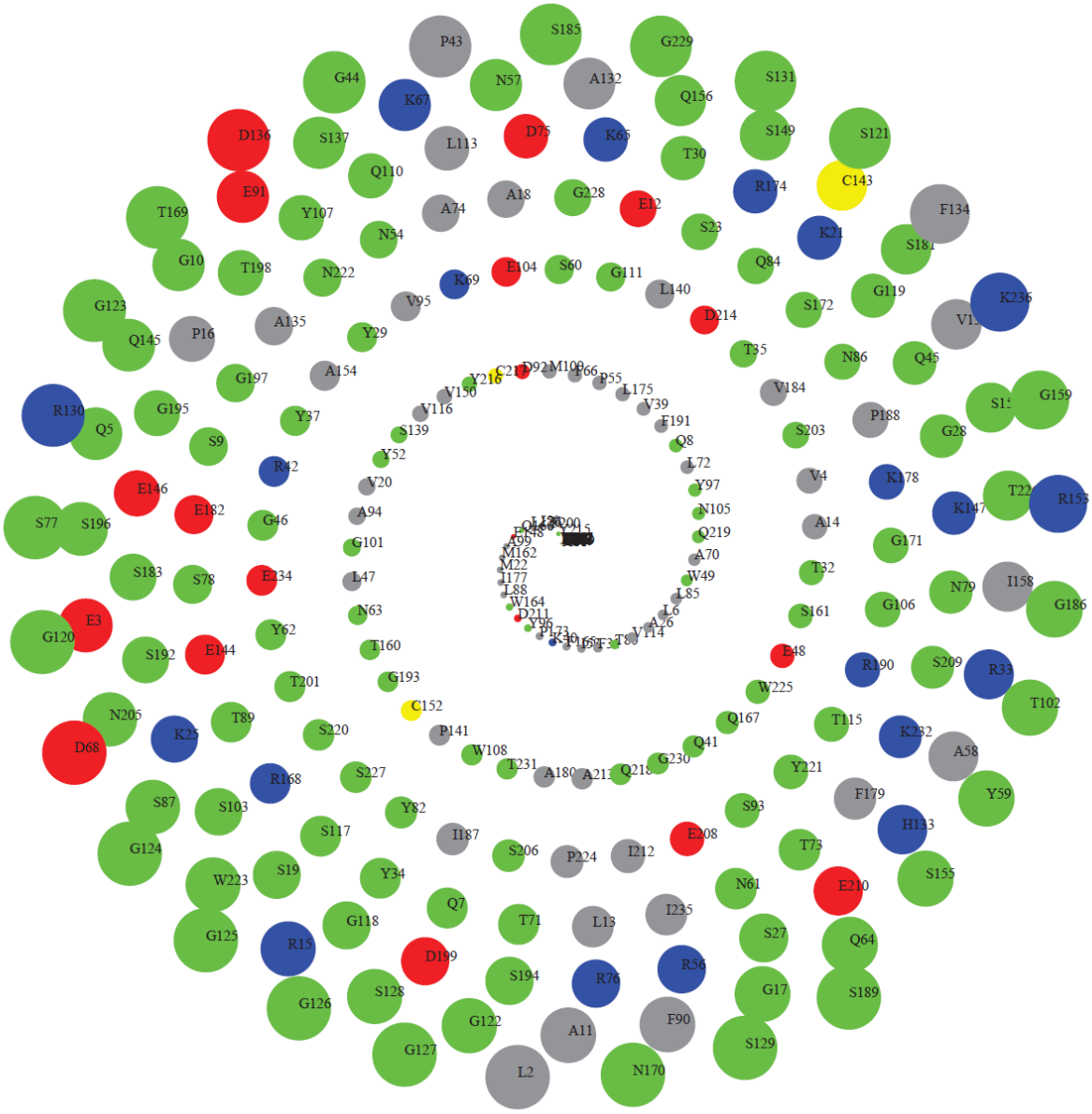


Figure 5.11 Spiral plot of amino acid residues of model scFv based on their solvent accessibility. Blue circles: positive charged residues, red circles: negative charged residues, green circles: polar uncharged residues, yellow circles: cysteine residues, grey circles: hydrophobic residues. The size of the circle is a measure of the solvent accessibility; maximum circle size represents maximum solvent accessibility.

5.4 CONCLUSION

Based on the results of *Chapter 5*, it can be stated that two recombinant scFv anti-DON antibodies were developed out of the hybridoma producing the 2A9 and 10H10 monoclonal antibodies. Optimized conditions for culturing and expression of *E. coli* cells containing the pComb3XSS expression vector were set up, *e.g.* the growth medium, IPTG concentration and expression temperature. An indirect ELISA format was developed based on the different synthesized DON coating antigens described in *Chapter 3*. The best results were obtained using the DON-CC-OVA coating (heterologous) at a concentration of 8 µg/mL. As an absorbance of 0.568 was obtained using the 10H10 scFv, it can be concluded that the antibody did not loose its affinity for the DON antigen. Moreover, removing the constant domains of the antibody, leaving only one variable heavy and light chain did not decrease the ruggedness of the antibody structure. To characterize the scFv antibody in terms of specificity, a higher amount of soluble scFv antibody was needed.

Different purified and non-purified 2A9 XL-1 Blue *E. coli* samples were send off for sequencing, but from none of them a good sequence result could be obtained. The amino acid sequence of the 10H10 scFv could be determined and CDR regions of V_H and V_L were indicated based on Kabat *et al.* (2001). Based on this information, modeling and docking experiments were performed. A model scFv was found with 61.92% identity compared to the 10H10 scFv. Using this model, an assessment of DON-scFv possible interactions/clusters was performed.

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

GENERAL CONCLUSIONS

The doctoral research focused on the improvement of bio-analytical detection strategies for deoxynivalenol (DON) in cereals, especially in wheat.

First of all, there was a need for an alternative sampling method for cereals because of the heterogeneous distribution of mycotoxins within a bulk lot, which is the main cause of uncertainty of the test result. As an alternative for wheat grains, wheat dust was chosen characterized by its small particle size and production during the (un)loading of grain cereals and during harvesting. Wheat dust can be considered as a homogeneous and representative sample as it is continuously generated and sampled through the entire grain movement process. Another advantage of wheat dust is that no grinding step is needed prior to extraction and this makes it less time-consuming as normal wheat grain sample preparation.

During this research, wheat dust generated by a laboratory scale model, was characterized in terms of particle size and content (organic, inorganic and water fraction) and a mineral content of more than 10 000 µg/g was observed for Fe, K and P, independent of the wheat dust origin. The interference of these minerals on the detection results is dependent on the analytical method used (chromatographic versus immunochemical method) and the type and volume of the extraction solvent used prior to detection.

As wheat dust is easy to sample, this sampling method can be coupled to an immunochemical detection method for on-site DON measurement. Therefore anti-DON monoclonal antibodies are needed. The described and commercially available anti-DON monoclonal antibodies show high cross-reactivity against the acetylated derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). These modified forms (like all other modified mycotoxins) are not included in legislation even though their toxicity is equal or even higher (for 15-ADON) than DON.

A strategy was set up for the synthesis of DON-immunogens using different procedures and making different linkers with varying length and chemical structure between DON and the carrier protein to be able to lower cross-reactivity of the monoclonal antibodies. By using the hybridoma technology, Balb/C female mice (ethical approval according to ECD 10/08) were injected with the different DON immunogens to obtain antibody producing B-cells which were fused *in vitro* with myeloma cells to form hybridomas. Homologous and heterologous enzyme-linked immunosorbent assay (ELISA) hybridoma screening was performed whereby three anti-DON monoclonal antibody producing hybridomas were selected. The 13H1, 10H10

and 2A9 derived monoclonal antibodies were characterized in terms of sensitivity (IC_{50}) and cross-reactivity towards chemically related trichothecenes using direct/indirect ELISA and/or surface plasmon resonance (SPR) detection. Direct ELISA results showed that the 13H1 antibody could recognize DON and its analogues in the order of HT-2 toxin > 15-ADON > DON, with IC_{50} ranging from 1.14 to 2.13 µg/mL. The monoclonal antibody 2A9 proved to be very specific and sensitive to 3-ADON, with IC_{50} of 0.38 ng/mL. The 10H10 antibody on the other hand showed affinity for DON, 3-ADON and 15-ADON, with IC_{50} values of 22, 15 and 34 ng/mL, respectively. Therefore the 10H10 antibody can be considered as a broad-specific antibody, measuring total DON (DON, 3-ADON and 15-ADON) contamination. Compared to the commercially available anti-DON monoclonal antibodies, this is the first antibody which can determine DON, 3ADON and 15-ADON with a similar sensitivity. This is a very good monoclonal antibody from a researcher's point of view having knowledge about occurrence and toxicological data of 3-ADON and 15-ADON, not taking into account current legislation.

Using an indirect ELISA format for the characterization of the 10H10 antibody decreased the sensitivity to 15-ADON with 92%. SPR characterization of the 10H10 antibody resulted in a 10 times higher DON and 3-ADON sensitivity compared to indirect ELISA using the same coating antigen. So, depending on the used ELISA/SPR format, different characterization results were obtained. By using the combination of the indirect ELISA format with the 10H10 antibody and the direct ELISA with the 2A9 antibody, the single DON contamination can be determined.

As the developed DON immunogen synthesis strategy, combined with the hybridoma technology, did not deliver specific anti-DON monoclonal antibodies, recombinant antibody technology was used starting from the obtained 10H10 and 2A9 hybridoma. The single-chain variable fragment (scFv) synthesis and production in *E. coli* strains was optimized using different PCR (*e.g.* concentration of MgCl or $MgSO_4$, type of polymerase enzyme) and cell culturing (*e.g.* different growth media, temperature, isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentration) conditions. Indirect ELISA experiments gave absorbance values of around 0.6, revealing no decrease in ruggedness of the antibody structure and DON affinity. Based on the amino acid sequence of the scFv, modeling and docking experiments proved energetically enhanced DON-scFv interactions.

As no regulatory limits exist for mycotoxins in grain dust, a correlation was needed between the DON level in wheat dust versus the DON level in the corresponding wheat. Therefore, a liquid chromatography-tandem mass spectrometry method in electrospray positive mode (LC-

ESI^+-MS/MS) was developed and validated for the determination of DON in wheat dust. An extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v), followed by an additional hexane defatting step resulting in cleaner extracts and therefore increasing the sensitivity. The method was validated in terms of linearity, apparent recovery, limit of detection, limit of quantitation, precision and accuracy based on the Commission Decision 2002/657/EC of 12 August 2002 concerning the performance of analytical methods and the interpretation of results.

To determine the suitability of wheat dust in the measurement of the DON contamination level, an experimental field trial was set up during the growing season 2011-2012. The influence of maize-wheat rotation, tillage method and wheat variety (Azzerti, Homeros, Sahara, Mulan and Tabasco) on the DON content of wheat grain and the corresponding wheat dust, was investigated. For Azzerti and Homeros, known as susceptible wheat varieties, no significant difference in DON level for both wheat and wheat dust was noticed between tillage (*i.e.* ploughing) and no tillage. This is in contrast with the moderately resistant varieties Mulan, Sahara and Tabasco, where lower DON contents were observed with tillage as soil treatment. It can be concluded that the derived results are in accordance with the wheat variety resistance to *Fusarium* infection.

The data derived from the experimental field trial, together with LC-MS/MS results of an extra batch of wheat and wheat dust samples, gave rise to a scatterplot revealing a correlation between the DON content in wheat versus the DON content in wheat dust. For wheat samples with a DON contamination lower than 1 250 µg/kg (limit for human consumption), wheat dust showed a 13-fold accumulation of DON compared to wheat. With higher DON contaminated wheat, a sigmoidal relation of the DON content between wheat and wheat dust was observed.

As the MycoHunt system was based on immunoassay technology, the suitability of the developed 10H10 anti-DON monoclonal antibodies was tested. Therefore, a water-based extraction and a direct ELISA method was successfully developed and validated for the determination of DON in wheat and wheat dust. For the optimization of sample preparation conditions, blank wheat and wheat dust samples were spiked with DON at different concentration levels and evaluated in terms of recovery by a common ELISA procedure. A small survey was executed on raw wheat material and their corresponding dust samples (n = 16). A sigmoidal correlation between the DON concentration in wheat dust versus the DON concentration in wheat was observed (r = 0.928). When not considering the data points with a

DON concentration in wheat lower than 200 µg/kg (< LOD), a linear correlation (r = 0.889) was found. The slope of the trend line described a value of 5.023 which corresponds to a 5-fold accumulation of DON on small particles and is different to the slope (13.192) found after LC-MS/MS analysis of wheat and their corresponding wheat dust samples. As limited wheat and corresponding wheat dust samples were obtained and only at a concentration level in wheat lower than 1 200 µg/kg, the uncertainty of the slope corresponding to the linear curve is high. Also, no conclusion can be taken about whether to use the linear or logarithmic curve.

FUTURE PERSPECTIVES

Mycotoxins have already been known for many centuries, still multiple questions are left unanswered. Emerging technologies in sampling, sample preparation and especially mycotoxin analysis has led and is still leading to the disclosure of novel information *e.g.* the identification of masked mycotoxin forms and mycotoxin risk assessment after human and animal exposure.

Sampling and sample preparation

From the available literature and information obtained during the PhD-dissertation it became clear that there is really a need for alternative sampling procedures due to the very heterogeneous distribution of mycotoxins in agricultural commodities and products intended for human and animal consumption. As the mycotoxin concentration of a sample is determined as an estimation of the true mycotoxin concentration in the bulk lot, it is difficult to determine with 100% certainty the true contamination of the bulk lot. The problem of heterogeneity could be solved by the collection of incremental samples, the sampling of large amounts of cereals or using a smaller particle size of the starting material. As an ideal alternative for cereal grain sampling, grain dust was selected during this research (*Chapter 2*). This matrix is characterized by its readily availability without need for grinding (particle size < 50 µm) and could therefore be used for on-site mycotoxin measurement. For the determination of DON in wheat dust, a sample preparation method was developed prior to LC-MS/MS analysis and ELISA screening. In the future, this method could be extrapolated to other mycotoxins and other grain dust sources if the mycotoxin contamination occurs at the outer layers of the cereal. This could be the beginning of a more precise determination of the mycotoxin contamination level.

Analytical methods

Chromatographic methods

The need for multi-mycotoxin analyses is constantly rising and the technology of choice is LC-MS/MS. Further improvements of the LC-MS/MS instrumentation and its availability at a lower price will further contribute to LC-MS/MS, becoming the major tool in multi-contaminant analysis. Nevertheless this analytical technique is targeted and it pre-supposes a

knowledge of which mycotoxins might be present. The introduction and application of high resolution mass spectrometers such as time-of-flight and orbitrap offer new opportunities in mycotoxin research. This emerging method used for the untargeted screening of masked mycotoxins, has already led to the identification of new conjugates such as fusarenon X-glucoside, nivalenol-3-glucoside, T-2-3-glucoside, HT-2-3-glucoside and palmitoyl conjugates of zearalenone, DON and T-2 [Nakagawa *et al.*, 2012; Busman *et al.*, 2011; Chakrabarti and Ghosal, 1986]. Nowadays, this type of mass spectrometry is used in metabolomic studies. During metabolomics, conjugates or metabolites are identified revealing new detoxification routes of the fungus and unravelling new metabolization patterns of mycotoxins in biological fluids.

Immunochemical methods

Up to now, immunochemical methods are still important to monitor mycotoxin food and feed contamination even though the impact of cross-reactivity remains an unsolved problem. Hybridoma technology remains the method of choice for the production of antigen specific monoclonal antibodies. Over the past decade, the detailed knowledge of antibody structure and function has enabled antibody phage display of immune libraries to emerge as a powerful *in vitro* alternative to hybridoma methods for creating antibodies. The main advantage of phage display technology is the large diversity of variant antibodies ($> 10^{10}$ members) that can be screened for its selectivity and affinity towards the target antigen. Another advantage includes the ability to perform antibody engineering based on the antibody encoding DNA sequence thereby improving the antibody characteristics (*e.g.* affinity, specificity).

During the PhD-dissertation both antibody developing methods, hybridoma and recombinant technology, were used to prepare anti-DON antibodies. As described in *Chapter 3*, a broad-selective monoclonal antibody 10H10 to DON and its masked forms (3-ADON, 15-ADON and DON-3G) derived from traditional hybridoma technology, was picked out by heterologous ELISA. The achievement of a selective 3-ADON antibody (2A9) opened perspectives for the development of specific anti-DON antibodies. Out of the 10H10 and 2A9 hybridoma, recombinant scFv antibodies were synthesized. Indirect ELISA results revealed no loss in affinity towards their target antigen.

To be able to characterize the scFv in terms of specificity, the production of the soluble recombinant antibody should be upgraded. Different causes of low scFv levels together with

possible solutions are presented in literature. A high amount of inclusion bodies compared to soluble anti-DON scFv is described by Choi *et al.* (2003), who solved this problem by the coexpression of molecular chaperones DnaK-DnaJ-GrpE in the phage-transfected *E. coli* strains. In addition, expression of the GroEL-GroES molecular chaperone complex may corporate with DnaK-DnaJ-GrpE in a synergistic way to increase soluble production of some proteins. Another possibility to increase scFv production is the transfer of the gene sequence encoding the scFv from the pComb3XSS phagemid to a pET expression system known to speed up recombinant antibody production. As the ends of the gene sequence need to be compatible with restriction sites at the cloning vector and no similar restriction sites are present (except the sfiI restriction site) on the pComb3XSS and pET expression systems, the NdeI and XhoI restriction sites should be incorporated using the sense primers 5'-GCAACTCATATGCAGGTGAAGCTGCAGCAGTCT-3' (V_H), 5'-TCCGGCGGTGGTGG CAGCGGTGGCGGCGGTTCTCAGGCTGTTGTGACTCAGGAA-3' (V_L) and the reverse primers 5'-ACCGCTGCCACCACCGCCGGAGCCACCGCCACCTGAGGAGACGGTGAC CGTGGT-3' (V_H), 5'-CATTCTCTCGAGACCTAGGACA GTGACCTTGGT-3' (V_L).

Another point to consider is the loss of recombinant antibody during immobilized metal chelate chromatography (IMAC) purification. As the protein retention is based on the availability of the hexahistidine (HIS) tag, the recovery of the recombinant protein is dependent on the amino acid sequence and consequent protein folding. Several researchers describe the multi-position interactions between the immobilized metal ions and protein residues during proper IMAC purification conditions. None of them however could elucidate the exact interaction mechanism so far.

Total characterization of the 10H10 and 2A9 scFv recombinant antibodies can reveal changes in specificity compared to the monoclonal antibodies. The amino acid sequence differences between the complementary determining regions (CDRs) of both 10H10 and 2A9 scFv give an idea about the origin of their respective broad-selective and 3-ADON specific character. Altering these amino acids by performing *in vitro* mutagenesis studies followed by modeling and docking experiments, can therefore deliver specific antibodies against DON. Using antibody engineering, could even lead to the first specific antibody against the masked deoxynivalenol-3-glucoside (DON-3G).

Nowadays, hybridoma and recombinant technology is widely discouraged due to the animal pain and distress. Therefore promising alternatives to animal-based antibodies are developed including aptamers, molecular imprinted polymers (MIPS) and recombinant antibodies out of

naive or synthetic libraries, which can be created without using animals or animal tissue. However, to be able to synthesize and select the antibody substitutes, mycotoxin reference standards are needed. At the moment, masked mycotoxin reference standards are lacking. Promising techniques using detached leaves of maize and wheat obtaining large quantities in terms of mg of masked mycotoxins, have been described [De Boevre *et al.*, 2014].

Mycotoxin risk assessment

Risk assessment is an important tool to quantify the true exposure of mycotoxins and their masked forms through food and feed. Current mycotoxin exposure assessments are based on calculations combining mycotoxin occurrence data in food or feed with consumption data. As some important information is lacking, risk assessors often have to make estimates and consequently all risk assessments are uncertain to some degree. As an example, the mycotoxin exposure through inhalation is not taken into account. According to the data presented in *Chapter 2*, the mycotoxin content in dust is a multiple of the amount present in the corresponding wheat grain. As no mycotoxin absorption data are available after inhalation, the contribution to risk exposure can not be determined. Moreover, inter-individual and inter-species variation in kinetics (absorption, distribution, metabolization and excretion) exist, which further decrease the accuracy of the risk analysis.

Therefore, the determination of mycotoxin metabolization products or biomarkers can provide a more accurate measure at individual level. As at the moment only a few biomarkers have been validated for some mycotoxins, future research need to focus on the untargeted screening of new valuable biomarkers by the use of high resolution mass spectrometry. The biomarker approach can be used in several biological matrices like blood, urine, faeces, liver, kidney and breast milk by applying chromatographic or immunochemical analytical methods. This highlights the need for specific antibodies or antibody substitutes against these mycotoxin metabolization products and makes us return to the above mentioned suited recombinant antibody technology.

SUMMARY

The manuscript entitled “Bio-analytical detection strategies for deoxynivalenol in wheat dust” describes different biotechnological and analytical approaches for the detection of the mycotoxin deoxynivalenol (DON) in wheat dust transferable to other mycotoxins and other cereals.

In the general introduction presented in *Chapter 1* an overview of mycotoxins is shown together with the food and feed matrices where the mycotoxin contamination and related fungal infection can occur. To understand the awareness of the Flemish population (including food consumers and producers) concerning mycotoxin contamination of food and feed, a questionnaire was developed and distributed online at the Agriflanders Fair for and by Flemish agriculture (2013). Out of the results, it became clear that more information should be provided concerning mould growth, production of mycotoxins by moulds, their toxic effects on organisms, exposure pathways and how to deal with mouldy food or feed, to increase the awareness of mycotoxin contamination of food and feed.

Further on, the principle and characteristics of existing analytical and immunochemical methods are presented specific to DON detection. A detailed explanation is given about the use of traditional hybridoma and recombinant technology to prepare anti-DON antibodies.

In *Chapter 2*, an alternative sampling method for grain cereals, namely “dust sampling”, is described. Dust is created during the transport through closed systems such as the (un)loading of a truck and is produced during this research by a laboratory dust collection facility. Characterized by its small size, homogeneous mycotoxin distribution and readily availability for mycotoxin analysis, it forms the ideal alternative for grain sampling.

Combined with the sampling of wheat dust, a liquid chromatography-tandem mass spectrometry method in electrospray positive mode (LC-ESI$^+$-MS/MS) was successfully developed and validated for the determination of DON in wheat dust. An extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v), followed by an additional hexane defatting step. After filtration, the extract was evaporated and the residue was redissolved in mobile phase for injection. The method was validated in terms of linearity, apparent recovery, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy based on the Commission Decision 2002/657/EC of 12 August 2002 concerning the performance of analytical methods and the interpretation of results. Due to a high contamination level of wheat dust compared to wheat, LOD and LOQ levels were obtained of 358 µg/kg and 717 µg/kg, respectively.

An experimental field trial set up in the growing season 2011-2012, was designed to obtain quantitative data on the occurrence of DON in wheat grain and the corresponding wheat dust. The influence of maize-wheat rotation, tillage method and wheat variety (Azzerti, Homeros, Sahara, Mulan and Tabasco) on *Fusarium* head blight (FHB) infection determined by DON analysis was investigated. First of all, an important influence of maize-wheat rotation was observed as a six times higher DON content was found in wheat sown after maize. For the susceptible wheat varieties Azzerti and Homeros no significant difference in DON level for both wheat and wheat dust was noticed between tillage (*i.e.* ploughing) and no tillage. This was in contrast to the moderately resistant varieties Mulan, Sahara and Tabasco, where lower DON contents were observed using tillage.

The accumulation of DON in wheat dust was confirmed and a linear and sigmoidal relationship between the DON content in wheat dust versus wheat grain was determined. For wheat samples with a DON contamination lower than 1 250 µg/kg (limit for human consumption), wheat dust showed a 13-fold accumulation of DON compared to wheat. At higher DON contamination levels, a sigmoidal relation ($y = 5\,935 \ln(x) - 28\,423$) of the DON content between wheat and wheat dust was observed.

As wheat dust is readily available in contrast to grain cereals where adequate grinding needs to be performed prior to sample preparation, it forms an ideal basis for the development of a DON immunochemical screening method. As for this purpose antibodies are needed, monoclonal antibodies were developed using traditional hybridoma technology and this forms the basis of *Chapter 3*. A DON immunogen synthesis strategy was set up using different procedures and making different linkers with varying length and chemical structure between DON and the carrier protein. Balb/C female mice (ethical approval according to ECD 10/08) were subjected to an injection with the different DON immunogens to obtain antibody producing B-cells which were *in vitro* fused with myeloma cells using traditional hybridoma technology. For the hybridoma screening and selection, homologous and heterologous enzyme-linked immunosorbent assay (ELISA) was performed whereby three anti-DON monoclonal antibody producing hybridomas were selected. The 13H1, 10H10 and 2A9 derived monoclonal antibodies were characterized in terms of sensitivity and cross-reactivity towards chemically related trichothecenes using direct/indirect ELISA and/or surface plasmon resonance (SPR) detection. The 13H1 antibody derived using deoxynivalenol-cyanuric chloride-bovine serum albumin (DON-CC-BSA) immunization could recognize DON and its analogues in the order of HT-2 toxin > 15-acetyldeoxynivalenol (15-ADON) > DON, with a

sensitivity of 1.14 µg/mL, 1.62 µg/mL and 2.13 µg/mL, respectively. Whereas the 10H10 antibody derived from a deoxynivalenol-bovine serum albumin (DON-BSA) immunized mouse manifested relatively close sensitivities to DON, 3-acetyldeoxynivalenol (3-ADON), 15-ADON and deoxynivalenol-3-glucoside (DON-3G), with sensitivity values of 22, 15, 34 and 72 ng/mL, respectively. Using an indirect ELISA format for the characterization of the 10H10 antibody, decreased the cross-reactivity to 15-ADON from 65% till 9%. SPR characterization of the 10H10 antibody resulted in a 10 times better DON and 3-ADON sensitivity compared to indirect ELISA using the same coating antigen. Another DON-BSA immunized mouse gave rise to the third monoclonal antibody 2A9 which showed to be very specific and sensitive to 3-ADON, with a sensitivity value of 0.38 ng/mL.

In *Chapter 4*, the broad-specific anti-DON monoclonal antibody 10H10 was used to develop a direct ELISA method to determine DON contamination in wheat and wheat dust. As DON is a polar molecule and organic solvents can lead to protein denaturation, water was used as extraction solvent. A small survey was executed on raw wheat material and their corresponding dust samples (n = 16) using the optimized and validated extraction and ELISA procedure. A sigmoidal correlation between the DON concentration in wheat dust versus the DON concentration in wheat was observed (r = 0.928). When not considering the data points with a DON concentration in wheat lower than 200 µg/kg (< LOD), a linear correlation (r = 0.889) was found. As limited wheat and corresponding wheat dust samples were obtained and only at a concentration level in wheat lower than 1 200 µg/kg, the uncertainty of the slope corresponding to the linear curve is high. Also, no conclusion can be taken about whether to use the linear or logarithmic curve.

European legislation concerning DON contamination does not include the acetylated DON derivatives (3-ADON and 15-ADON) or other masked mycotoxins even though their toxicological importance is of concern. Until now, no specific anti-DON monoclonal antibodies were developed using the hybridoma technology. Therefore recombinant technology was used to synthesize single-chain variable fragment (scFv) antibodies out of hybridoma 10H10 and 2A9 and is described in detail in *Chapter 5*. Optimized conditions for culturing and expression of pComb3XSS transformed *E. coli* bacterial cells were selected based on a developed indirect ELISA procedure. High spectrophotometrically detected absorbance values revealed the ruggedness of the antibody structure after removing its constant domains. The amino acid sequence of the 10H10 scFv was determined and a distinction was made between the complementarity determining regions (CDRs) and

framework regions of variable heavy (V_H) and variable light (V_L) chain. Using the sequencing information, modeling and docking experiments were performed. A model scFv was found in the Swiss Model databank with 61.92% identity compared to the 10H10 scFv. Using this model, an assessment of DON-scFv possible and energetically enhanced interactions/clusters was performed.

The knowledge gathered through the complete PhD research gives rise to the formulation of general conclusions and future perspectives presented in *Chapter 6*, revealing further bio-analytical detection development and enhancement procedures.

SAMENVATTING

Het doctoraatsproefschrift getiteld "*Bio-analytical detection strategies for deoxynivalenol in wheat dust*" beschrijft verschillende biotechnologische en analytische procedures voor de detectie van het mycotoxine deoxynivalenol (DON) in tarwestof en is eveneens bruikbaar voor de detectie van andere mycotoxines in verscheidene graansoorten.

In de algemene inleiding voorgesteld in *Hoofdstuk 1*, wordt een overzicht weergegeven van de verschillende mycotoxines met de voedsel- en voedermatrices waarin ze voorkomen en de gerelateerde schimmelinfectie. Om een beeld te kunnen vormen betreffende het bewustzijn van de Vlaamse populatie (inclusief consument en producent) inzake mycotoxine contaminatie van voedsel en voeder, werd een enquête opgesteld en online ingevuld op de Vlaamse land- en tuinbouwbeurs Agriflanders (2013). Uit de resultaten bleek dat de mensen meer geïnformeerd moeten worden in verband met de schimmelgroei, productie van mycotoxines door schimmels, de toxische effecten op mens en dier, de blootstellingswegen en wat er te doen valt met beschimmeld voedsel of voeder. Op deze manier kan de kennis en het bewustzijn in verband met mycotoxine contaminatie van voedsel en voeder uitgebreid worden.

Verder in dit hoofdstuk worden de principes en kenmerken van bestaande analytische en immunochemische methodes voor de detectie van DON voorgesteld. Een gedetailleerde uitleg wordt gegeven in verband met het gebruik van de traditionele en recombinante technologie voor de aanmaak van anti-DON antilichamen.

In *Hoofstuk 2* wordt een alternatieve methode voor staalname van granen, namelijk de staalname van graanstof, voorgesteld en beschreven. Dit stof wordt geproduceerd tijdens het transport van granen doorheen smalle systemen zoals bij het laden of lossen van een vrachtwagen of schip. Tijdens dit onderzoek werd gebruik gemaakt van een speciaal ontwikkelde laboratoriumconstructie voor de productie en collectie van graanstof. Door de kleine diameter van de stofpartikels, de homogene mycotoxine distributie en de onmiddellijke beschikbaarheid voor mycotoxine analyse, vormt het een ideaal alternatief voor de staalname van graan.

Gecombineerd met de staalname van tarwestof, werd een vloeistof chromatography-tandem massaspectrometrische methode gebruik makend van de positieve *electrospray*-ionisatie (LC-ESI^+-MS/MS) succesvol ontwikkeld en gevalideerd voor de bepaling van DON in tarwestof. Een extractie werd uitgevoerd met acetonitrile/water/azijnzuur (79/20/1, v/v/v), gevolgd door een hexaanontvetting. Na filtratie werd het extract uitgedampt en het residu heropgelost in mobiele fase voor injectie. De methode werd gevalideerd inzake lineariteit, relatieve terugvinding, detectielimiet (LOD), kwantificatielimiet (LOQ), precisie en accuraatheid,

gebaseerd op Verordering Nr. 2002/657/EC inzake de performantie van analytische methodes en de interpretatie van resultaten. Omwille van de hoge contaminatie van tarwestof in vergelijking met tarwe, werden LOD- en LOQ-waarden bekomen van respectievelijk 358 µg/kg en 717 µg/kg.

Een experimentele veldtest werd opgesteld in het landbouwseizoen 2011-2012 voor het verkrijgen van quantitatieve data in verband met het voorkomen van DON in tarwe en het corresponderende tarwestof. Hierbij werd de invloed van maïs-tarwe wisselbouw, de akkerbewerkingsmethode en de tarwevariëteit (Azzerti, Homeros, Sahara, Mulan and Tabasco) op de *Fusarium* head blight (FHB) infectie nagegaan door bepaling van de DON-concentratie. Eerst en vooral werd een belangrijke invloed van de maïs-tarwe wisselbouw geobserveerd, aangezien een zes maal hogere DON-concentratie gevonden werd bij tarwe afkomstig van een akker waar voorheen maïs werd gezaaid. Voor de vatbare tarwevariëteiten Azzerti en Homeros werd geen significant verschil in DON-niveau geobserveerd bij het al dan niet bewerken van akkers voor zowel tarwe als tarwestof. Dit staat in tegenstelling tot de gemiddeld resistente variëteiten Mulan, Sahara en Tabasco waar een lagere DON-concentratie werd aangetroffen door het ploegen van de akker.

De accumulatie van DON in tarwestof werd bevestigd en een lineair en sigmoïdaal verband tussen de DON-concentratie in tarwestof versus tarwe werd bepaald. Voor tarwestalen met een DON-contaminatie lager dan 1 250 µg/kg (limiet voor humane consumptie), werd een 13 maal hogere DON-concentratie geobserveerd voor tarwestof ten opzichte van tarwe. Bij hogere DON-contaminaties bleek een sigmoïdale verhouding ($y = 5\ 935\ \ln(x) - 28\ 423$) tussen de DON concentratie in tarwe en tarwestof beter.

Aangezien tarwestof onmiddellijk beschikbaar is, in tegenstelling tot graankorrels die eerst vermalen moeten worden voor staalvoorbereiding, vormt dit een ideale basis voor de ontwikkeling van een DON-immunochemische screeningsmethode. Voor deze screeningsmethode zijn antilichamen nodig en deze monoclonale antilichamen werden ontwikkeld, gebruik makend van de klassieke hybridomatechnologie en dit vormt de basis van *Hoofdstuk 3*. Een DON-immunogen synthesestrategie werd ontwikkeld door gebruik te maken van verschillende procedures en verschillende verbindingen met variërende lengte en chemische structuur tussen DON en het carrier proteïne. Balb/C vrouwelijke muizen (Ethisch goedgekeurd volgens ECD 10/08) werden onderworpen aan een injectie met de verschillende DON-immunogenen om de B-cellen aan te sporen tot productie van antilichamen. Deze B-cellen werden *in vitro* versmolten met myelomacellen volgens de klassieke

hybridomatechnologie. Voor de hybridoma screening en selectie werden zowel homologe als heterologe *enzyme-linked immunosorbent assay* (ELISA) uitgevoerd, resulterend in de selectie van drie anti-DON monoclonale antilichaam-producerende hybridomas. De 13H1, 10H10 en 2A9 bekomen monoclonale antilichamen werden gekarakteriseerd naar gevoeligheid en kruisreactiviteit ten opzichte van chemisch gerelateerde trichothecenen door middel van directe/indirecte ELISA en/of *surface plasmon resonance* (SPR) detectie. Het 13H1 antilichaam afkomstig van *deoxynivalenol-cyanuric chloride-bovine serum albumin* (DON-CC-BSA) immunizatie kon DON en zijn analogen herkennen in de volgorde HT-2 toxine > 15-acetyldeoxynivalenol (15-ADON) > DON, met een gevoeligheid van respectievelijk 1.14 µg/mL, 1.62 µg/mL en 2.13 µg/mL. Daarentegen werd het 10H10 antilichaam bekomen via een *deoxynivalenol-bovine serum albumin* (DON-BSA) geïmmuniseerde muis, gekarakteriseerd door een gevoeligheid tegenover DON, 3-acetyldeoxynivalenol (3-ADON), 15-ADON en DON-3-glucoside (DON-3G) met waarden van 22, 15, 34 en 72 ng/mL respectievelijk. Het gebruik van indirect ELISA voor de karakterisatie van het 10H10 antilichaam, deed de kruisreactiviteit ten opzichte van 15-ADON dalen van 65% tot 9%. SPR-karakterisatie van het 10H10-antilichaam resulteerde in een 10 maal betere gevoeligheid voor DON en 3-ADON ten opzichte van indirect ELISA, gebruik makend van hetzelfde *coating* antigen. Een tweede DON-BSA geïmmuniseerde muis gaf aanleiding tot een derde monoclonaal antilichaam 2A9 dat een enorm grote specificiteit en gevoeligheid (0.38 ng/mL) vertoonde ten opzichte van 3-ADON.

In *Hoofdstuk 4* wordt het breed-specifiek anti-DON monoclonaal antilichaam 10H10 verder gebruikt voor het ontwikkelen van een directe ELISA-methode om de DON-contaminatie te bepalen in tarwe en tarwestof. Aangezien DON een polaire molecule is en organische solventen bovendien kunnen leiden tot denaturatie van proteïnen, werd water gebruikt als extractiesolvent. Een klein onderzoek werd uitgevoerd op tarwegranen en het corresponderend stof (n = 16), gebruik makend van de geoptimaliseerde extractie en ELISA procedure. Een sigmoïdale verhouding tussen de DON concentratie in tarwestof en tarwe werd gevonden (r = 0.928). Na het verwijderen van de datapunten overeenkomstig met een DON concentratie in tarwe lager dan 200 µg/kg (< LOD), werd een lineaire verhouding gevonden (r = 0.889). Door het beperkt aantal tarwe en overeenkomstige tarwestof stalen, is de onzekerheid van de lineaire regressie groot. Ook kan geen keuze gemaakt worden tussen de lineaire of logaritmische curve.

In de Europese wetgeving betreffende DON-contaminatie zijn de acetylderivaten 3-ADON en 15-ADON of andere gemaskeerde mycotoxines niet opgenomen, ook al zijn deze van toxicologisch belang. Tot nu toe werden geen specifieke anti-DON-antilichamen ontwikkeld door gebruik te maken van de klassieke hybridomatechnologie. Daarom werd de recombinante technologie gebruikt om *single-chain variable fragment* (scFv) antilichamen te synthetiseren, gebruik makend van de 10H10 en 2A9 hybridoma en dit is in detail beschreven in *Hoofdstuk 5*. Geoptimaliseerde condities voor het in cultuur en tot expressie brengen van pComb3XSS getransformeerde *E. coli* cellen werden geselecteerd gebaseerd op een ontwikkelde indirecte ELISA-procedure. Hoge spectrofotometrisch gedetecteerde absorptiewaarden onthulden de sterkte van de antilichaamstructuur na het verwijderen van zijn constante domeinen. De aminozuursequentie van het 10H10 scFv werd bepaald en een onderscheid werd gemaakt tussen de *complementarity determining regions* (CDR) and *framework regions* van de variabele zware (V_H) en variabele lichte (V_L) keten. Gebruik makend van deze sequentie-informatie werden *modeling* en *docking* experimenten uitgevoerd. Een model scFv werd gevonden in de Swiss Model databank met 61.92% gelijkenis ten opzichte van het 10H10 scFv. Door gebruik te maken van dit scFv-model werd een inschatting gemaakt van de DON-scFv mogelijke en energetisch gunstige interacties/clusters.

De verzamelde kennis doorheen het complete doctoraatsonderzoek heeft geleid tot het formuleren van een algemene conclusie en toekomstperspectieven waarin verder te ontwikkelen en te optimaliseren bio-analytische technieken worden voorgesteld. Dit staat uitgebreid beschreven in *Hoofdstuk 6*.

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Appendix A: Questions and potential answers on the survey about the awareness of mycotoxin contamination of food and feed

Question 1: In some cases food (for human consumption) can be unsuitable or even dangerous to eat. How could this occur according to you?

	Possible	Impossible	I do not know
By the presence of bacteria such as *Salmonella*	○	○	○
By the presence of viruses such as Norovirus	○	○	○
By the presence of moulds such as *Aspergillus*	○	○	○
Because the food is rancid	○	○	○
Because of chemical substances such as pesticides	○	○	○
Because of harmful substances produced by nature such as endotoxins	○	○	○
Because the expiration date has been exceeded	○	○	○
Because it does not smell good or look good anymore	○	○	○

Question 2: In some cases feed (for animal consumption) can be unsuitable or even dangerous to eat. How could this occur according to you?

	Possible	Impossible	I do not know
By the presence of bacteria such as *Salmonella*	○	○	○
By the presence of viruses such as Norovirus	○	○	○
By the presence of moulds such as *Aspergillus*	○	○	○
Because the feed is rancid	○	○	○

Because of chemical substances such as pesticides	○	○	○
Because of harmful substances produced by nature such as endotoxins	○	○	○
Because the expiration date has been exceeded	○	○	○
Because it does not smell good or look good anymore	○	○	○

Question 3: Mycotoxins are harmful substances produced by nature. Which of the following statements are correct or incorrect in your opinion?

	Correct	Incorrect	I don not know
Mycotoxins are produced by certain toxic plants	○	○	○
Mycotoxins are produced by certain bacteria such as *Salmonella*	○		○
Mycotoxins are produced by certain moulds such as *Aspergillus*	○		○
Mycotoxins are produced by certain viruses such as Norovirus	○	○	○
Mycotoxins are induced by burning food during preparation as well as during barbecuing	○	○	○
Mycotoxins are the consequence of pollution	○	○	○
Mycotoxins are toxic to plants	○	○	○
Mycotoxins are toxic to insects and animals living in water	○	○	○

Mycotoxins are toxic to humans	○	○	○
Mycotoxins are toxic to animals	○	○	○
Mycotoxins are visually detectable	○	○	○
All moulds produce harmful toxins	○	○	○
Mycotoxins can cause anomalies in newborns (animals and humans)	○	○	○
Mycotoxins are inhalable	○	○	○

Question 4: In some cases, food is not suitable to be eaten anymore because of the presence of moulds. Popularly one often says “there is growing hair on top of it”. In which products mould growth can occur in your opinion?

	Yes	No	I do not know
Fresh meat or fish	○	○	○
Fresh vegetables or fruits	○	○	○
Dairy products such as cheese	○	○	○
Water or soft drinks	○	○	○
Beer or wine	○	○	○
Coffee or tea	○	○	○
Dry biscuits or sweets	○	○	○
Breakfast cereals or pasta	○	○	○
Bread or other products from the bakery	○	○	○
Prepared meals	○	○	○

Frozen products	○	○	○
Preserved food	○	○	○
Products kept in fridge	○	○	○
Sauces such as mayonnaise or ketchup	○	○	○
Animal feed such as feed pellets or maize pulp	○	○	○

Question 5: In case you find out a product is mouldy, what can you do?

	Totally Disagree	Disagree	Neutral	Agree	Totally agree	I do not know
If I remove the mouldy part I can still eat the remaining of the product	○	○	○	○	○	○
Moulds are not really harmful so I can just eat the product	○	○	○	○	○	○
If I wash the product thoroughly I can still eat it	○	○	○	○	○	○
If I heat up the product I can still eat it	○	○	○	○	○	○
If the product is mouldy I have to throw it away completely	○	○	○	○	○	○
It is always clearly visible whether a product is mouldy or not	○	○	○	○	○	○

Question 6: Who has to supervise that no moulds or mycotoxins are present in food or feed? Please put in order; ranging from most important (1) to less important (4).

The government and food inspection

The producers of food or feed

I have to check this myself

The shops, store chains or the wholesalers who sell the food or feed products

Question 7: Who has to supervise that our food or feed does not contain moulds or harmful substances such as mycotoxins? How do you feel about following statements?

	Totally Disagree	Disagree	Neutral	Agree	Totally agree	I don't know
The government or producers have to test food (for human consumption) on the presence of moulds	○	○	○	○	○	○
The government or producers have to test feed (for animals) on the presence of moulds	○	○	○	○	○	○
The government or producers have to test food (for human consumption) on the presence of mycotoxins as they do for pesticides	○	○	○	○	○	○
The government or producers have to test feed (for animals) on the presence of mycotoxins as they do for pesticides	○	○	○	○	○	○
I can check myself if there are moulds present	○	○	○	○	○	○
I can check myself if there are mycotoxins present	○	○	○	○	○	○
I am willing to pay more for a qualitatively good product that is tested on the presence of moulds or mycotoxins	○	○	○	○	○	○

Finally, we would like to ask you a few more questions to be able to process the results as good as possible.

- What is your gender?

  Male

  Female

- In which year were you born?
- Which is your highest obtained degree?

  Primary school or no education

  Lower secondary school

  Higher secondary school

  University college

  University

  Post-university

- Which is your profession?

  Self-employed/free profession

  Employee

  Executive

  Labourer

  (Early) Retiree

  Student

  Job seeker

  Housewife/-husband

  Teacher

Other

- In which sector are you working?

  Agriculture

  Food

  Medical sector

  Chemical sector

  Technological sector

  Construction sector

  ICT

  Education

  Government

  Other

  None

- How is your marital status?

  Single

  In a relationship but not living together

  Married/living together

  Widow/widower

- Do you have children?

  Yes

  No

- If yes, how many?

CURRICULUM VITAE

Melanie Sanders was born on the 23th of April 1987 in Ostend, Belgium. In 2005 she obtained her diploma in Sciences and Mathematics at Immaculata Instituut De Panne, Belgium. In 2010 she graduated with distinction as a master in Pharmaceutical Sciences at Ghent University (Ghent, Belgium). The master thesis "The development of an LC-MS/MS method for the determination of ergot alkaloids in grain cereals" was accomplished at the Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University (Ghent, Belgium) under supervision of Dr. José Diana Di Mavungu and Prof. Dr. Sarah De Saeger.

In September 2010 she was admitted to the Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University (Ghent, Belgium) to start her PhD under promotorship of Prof. Dr. Sarah De Saeger.

The research received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement n°243633. Results of her research were published in a number of peer-reviewed scientific journals, and were presented during several national and international conferences. She worked during her PhD for 1 month at the department of Biotechnology, Lund University (Lund, Sweden) in the framework of the European FP7 MycoHunt project and 4 months at the school of Biotechnology, Dublin City University (Dublin, Ireland). She is also reviewer of several A1-peer reviewed journals. During her PhD-dissertation she supervised 2 master thesis students.

SCIENTIFIC OUTPUT

Master thesis

Development and validation of a new LC-MS/MS method for the simultaneous determination of six major ergot alkaloids and their corresponding epimers. Application to some food and feed commodities

Di Mavungu, J.D.; Malysheva, S.V.; Sanders, M.; Larionova, D.; Robbens, J.; Dubruel, P.; Van Peteghem, C.; De Saeger, S.

Food Chemistry 135 (1), 292-303 (2012)

PhD

Sampling of Wheat Dust and Subsequent Analysis of Deoxynivalenol by LC-MS/MS

Sanders, M.; De Boevre, M.; Dumoulin, F.; Detavernier, C.; Martens, F.; Van Poucke, C.; Eeckhout, M.; De Saeger, S.

Journal of Agricultural and Food Chemistry 61 (26), 6259-6264 (2013)

Heterologous screening of hybridomas for the development of broad-specific monoclonal antibodies against deoxynivalenol and its analogues

Guo, Y.; Sanders, M.; Galvita, A.; Heyerick, A.; Deforce, D.; Bracke, M.; Eremin, S.; De Saeger, S.

World Mycotoxin Journal 7 (3), 257-265 (2014)

Deoxynivalenol Content in Wheat Dust versus Wheat Grain: a Comparative Study

Sanders, M.; Landschoot, S.; Audenaert, K.; Haesaert, G.; Eeckhout, M.; De Saeger, S.

World Mycotoxin Journal 7 (3), 285-290 (2014)

The Awareness of Mycotoxin Contamination of Food and Feed in the Flemish Population

Sanders, M.; De Middeleer, G.; Vervaet, S.; Walravens, J.; Van de Velde, M.; Detavernier, C.; De Saeger, S.; Sas, B.

World Mycotoxin Journal (in press) (2014)

Novel multiplex fluorescent immunoassays based on quantum dot nanolabels for mycotoxins determination

Beloglazova, N.V.; Speranskaya, E.S.; Wu, A.; Wang, Z.; Sanders, M.; Goftman, V.; Zhang, D.; Goryacheva, I.Yu.; De Saeger S.

Biosensors and Bioelectronics 62, 59-65 (2014)

The immunogen synthesis strategy for the development of specific anti-deoxynivalenol monoclonal antibodies

Sanders, M.; Guo, Y.; Iyer, A.; Ruiz, Y.; Galvita, A.; Heyerick, A.; Deforce, D.; Risseeuw, M.; Van Calenbergh, S.; Bracke, M.; Eremin, S.; Madder, A.; De Saeger, S.

Food Additives and Contaminants: Part A (in press) (2014)

ACTIVE CONTRIBUTION

Oral presentations

ICAFA 2012 conference, Vienna, Austria (4-5 September 2012)

Sanders, M.; Guo, Y.; Galvita, A.; Heyerick, A.; Deforce, D.; Risseeuw, M.; Van Calenbergh, S.; Bracke, M.; Eremin, S.; Madder, A.; Hedström, M.; Mattiasson, B.; De Saeger, S.

A new Approach for developing monoclonal antibodies against deoxynivalenol

35th Mycotoxin Workshop, Ghent, Belgium (22-24 May 2013)

Sanders, M.; De Boevre, M.; Dumoulin, F.; Detavernier, C.; Guo, Y.; Martens, F.; Eeckhout, M.; De Saeger, S.

A promising grain sampling technique: the sampling of wheat dust and subsequent UPLC-MS/MS analysis

Trends in Food Analysis, Ghent, Belgium (19 September 2013)

Sanders, M.; De Boevre, M.; Dumoulin, F.; Detavernier, C.; Guo, Y.; Martens, F.; Eeckhout, M.; De Saeger, S.

A promising grain sampling technique: the sampling of wheat dust and subsequent UPLC-MS/MS analysis

Invited speaker at RME 2014, Noordwijkerhout, The Netherlands (31 March-2 April 2014)

Sanders, M.; Guo, Y.; Galvita, A.; Wootsch, A.; Uderszky, A.; Ágoston, A.; Martens, F.; Jantra, J.; Haesaert, G.; Hedström, M.; Mattiasson, B.; Eeckhout, M.; De Saeger, S.; Karacsonyi, E.

MycoHunt: a rapid biosensor for the detection of deoxynivalenol in wheat dust

Poster presentations

Mytox conference "Mycotoxins: Challenges and Perspectives", Ghent, Belgium (24 May 2011)

Sanders M.., Galvita A., Guo Y., Heyerick A., Deforce D., De Saeger S., Wootsch A., Uderszky A., Ágoston A., Orsolya K., Eeckhout M., Martens F., Haesaert G., Hedström M., Mattiasson B., Kumar A., Pikó T., Papageorgiou M., Vázquez P., Martin S., Martens J., Panagioths I., Fediuk W., Barbi F., Berta L., Bouchlas A., Lepez O., Baltzakis J., Marshall N.

MYCOHUNT: Development of a rapid, online biosensor for the detection of mycotoxin in wheat

KVCV conference "Mass spectrometry in Food and Feed", Ghent, Belgium (9 juni 2011)

Sanders M., Galvita A., Guo Y., Heyerick A., Deforce D., De Saeger S., Wootsch A., Uderszky A., Ágoston A., Orsolya K., Eeckhout M., Martens F., Haesaert G., Hedström M., Mattiasson B., Kumar A., Pikó T., Papageorgiou M., Vázquez P., Martin S., Martens J., Panagioths I., Fediuk W., Barbi F., Berta L., Bouchlas A., Lepez O., Baltzakis J., Marshall N.

MYCOHUNT: development of a rapid, online biosensor for the detection of Mycotoxin in Wheat

WMF meets IUPAC conference, Rotterdam, Belgium (5-8 November 2012)

Sanders M., Guo Y., Galvita A., Heyerick A., Deforce D., Risseeuw M., Van Calenbergh S., Eremin S., Madder A., De Saeger S.

Development of monoclonal antibodies against deoxynivalenol using different antigens

35th Mycotoxin Workshop, Ghent, Belgium (22-24 May 2013)

Sanders M., Guo Y., Galvita A., Heyerick A., Deforce D., Risseeuw M., Van Calenbergh S., Bracke M., Eremin S., Madder A., Hedstöm M., Mattiasson B. De Saeger S.

The development of monoclonal antibodies against deoxynivalenol

Trends in Food Analysis, Ghent, Belgium (19 September 2013)

Sanders M., Guo Y., Galvita A., Heyerick A., Deforce D., Risseeuw M., Van Calenbergh S., Bracke M., Eremin S., Madder A., Hedstöm M., Mattiasson B. De Saeger S.

The development of monoclonal antibodies against deoxynivalenol

Research Day, Dublin, Ireland (7 February 2014)

Sanders M., Guo Y., Galvita A., Heyerick A., Deforce D., Risseeuw M., Van Calenbergh S., Bracke M., Eremin S., Madder A., Hedstöm M., Mattiasson B. De Saeger S.

The development of monoclonal antibodies against deoxynivalenol

Tutorship bachelor theses

Pharmaceutical bachelor thesis: Vitamin C (2010-2011 / 2011-2012 / 2012-2013)

Tutorship master theses

Tiziana Micci (2011-2012)

Faculty of Pharmacy, University of Camerino, Camerino, Italy

Promotors: Prof. dr. S. Vittori and Prof. dr. S. De Saeger

The development of specific monoclonal antibodies against deoxynivalenol

Charlotte Campe (2012-2013)

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Promotor: Prof. dr. S. De Saeger

De ontwikkeling van specifieke monoclonale antilichamen tegen deoxynivalenol

Courses and workshops

Laboratory Animal Science I and II (4-15 October 2010)

Master course Biomedical Sciences, Ghent University, Ghent, Belgium

Advanced academic English: English proficiency for presentations (24 February, 3/10/17 March 2011)

University Language Centre, Ghent University, Ghent, Belgium

Quality research skills (22/23 November, 12 December 2011)

Departement onderzoeksaangelegenheden, Ghent University, Ghent, Belgium

Intensive training on mycotoxin analysis (27 August-7 September 2012)

Laboratory of Food Analysis, Ghent University, Ghent, Belgium

Intellectual Property and valorization (19 April-17 May 2013)

Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Mobility

The development of an amperometric immunosensor in the framework of the MycoHunt project (1-31 October 2012)

Department of Biotechnology, Lund University, Lund, Sweden

The development of a specific monoclonal antibody against DON using recombinant technology (1 November 2013-28 February 2014)

School of Biotechnology, Dublin City University, Dublin, Ireland

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