The influence of strongly coloured foodstuffs on the performance of rapid *on-site* mycotoxin tests

Marieke Lobeau

Promoters: Prof. Dr. S. De Saeger and Prof. Dr. C. Van Peteghem

Ghent University
Faculty of Pharmaceutical Sciences
Department of Bioanalysis
Laboratory of Food Analysis

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A.	*Aspergillus*
Ab	antibody
ACN	acetonitrile
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
Ag	antigen
AOAC	Association of Official Analytical Chemists
a_w	water activity
BB	borate buffer
BEN	Balkan Endemic Nephropathy
BCR	Bureau Communautaire de Reference
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
CRM	certified reference material
CZE-LIF	capillary zone electrophoresis with laser induced fluorescence
DCC	N,N'-dicyclohexylcarbodiimide
DMF	dimethylformamide
DNA	desoxyribonucleic acid
DON	deoxynivalenol
EC	European Commission
ECD	electron capture detection
EDF	fluorescein-thiocarbamyl ethylenediamine
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme-multiplied immunoassay technique
ESI	electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations

FB_1	fumonisin B_1
FLD	fluorescence detection
FITC	fluorescein isothiocyanate
fn	false negative
fp	false positive
FPIA	fluorescence polarization immunoassay
F.	*Fusarium*
GAP	Good Agricultural Practices
GC-MS	gas chromatography-mass spectrometry
h	hour(s)
HACCP	Hazard Analysis and Critical Control Point
HAT	hypoxanthine aminopterine thymidine
HCG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
HPLC-FLD	high performance liquid chromatography-fluorescence detection
HRP	horse-radish peroxidase
HSA	human serum albumin
IA	immunoassay / immunoaffinity
IAC(s)	immunoaffinity column(s)
IARC	International Agency for the Research on Cancer
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
in.	inch
IOF	Industrieel Onderzoeksfonds Universiteit Gent
IR	infrared (detection)
IRMM	Institute for Reference Materials and Measurements
IV	intravenously
JECFA	Joint Expert Committee on Food Additives of the World Health Organization and the Food and Agriculture Organization
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
min	minute(s)
MIP(s)	molecular imprinted polymer(s)
ML(s)	maximum level(s)
mP	millipolarization

MRM	multiple reaction (reagent) monitoring
MS	mass spectrometry
MW	molecular weight
NHS	N-hydroxysuccinimide
NOAEL	no observed adverse effect level
OD	optical density
OTA	ochratoxin A
OTα	ochratoxin alpha
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMTWI	provisional maximum tolerable weekly intake
PHC	primary hepatocellular carcinoma
QCM	quartz cristal microbalance
P.	*Penicillium*
RAM	rabbit anti-mouse
RIA	radio immunoassay
RM	reaction mixture
RNA	ribonucleic acid
rpm	revolutions (rotations) per minute
RT	room temperature
SAX	strong anion exchange
SC	subcutaneously
SCF	Scientific Committee on Food
SCOOP	Scientific Cooperation on Questions relating to Food
SPE	solid phase extraction
spp.	species
SPR	surface plasmon resonance
TCTC(s)	trichothecene(s)
TDI	tolerable daily intake
TLC	thin layer chromatography
TMB	tetramethylbenzidine
tn	true negative
tp	true positive
UV	ultra violet
WHO	World Health Organization of the United Nations
ZEA	zearalenone

Introduction

Mycotoxins are secondary metabolites produced by moulds, present in a lot of feed and foodstuffs. Ingestion of these toxins can cause acute or chronic toxic effects (a.o. carcinogenicity, teratogenicity) in animals and humans. Because of these health risks, monitoring of mycotoxins in feed and food is necessary. As mycotoxins can never be completely removed from the food supply, the European Commission (EC) has recommended or set maximum levels (MLs) for mycotoxins in feed and food [1, 7, 8].

The most reliable methods for mycotoxin analysis are gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) and liquid chromatography (tandem) mass spectrometry (LC-MS(/MS)). These methods are expensive and time-consuming. For this reason, there is great demand for rapid detection methods, capable to be performed outside the laboratory. Rapid tests should be simple to use and results should be easy to interpret. It means that they consist of a simple sample extraction, minimal manipulations, little assay steps, short assay time and no or minimal use of toxic solvents. Ideal non-laboratory rapid tests are non-instrumental, making use of visual evaluations. The purpose is to make easily the distinction between positive and negative results.

Immunochemical membrane tests (dipstick, flow-through, lateral flow) are popular in the mycotoxin area. These are colorimetric tests and the developed colour on the detection zone of the membrane is indicative for the presence of the analysed mycotoxin. However, these membrane tests can only be used for a limited number of foodstuffs. For strongly coloured foodstuffs like roasted coffee, cocoa, spices, red wine and beer, the white membranes get fully coloured and interfere with the visual detection. Therefore, a clean-up step is necessary. Unfortunately, this counteracts the purpose of a rapid method, being performed outside the laboratory by non-analytically skilled personnel. This was the reason for designing a new format for coloured foodstuffs: the *clean-up tandem assay column* [9]. In this system, a clean-up layer and a detection layer are combined in one column. The clean-up layer adsorbs most of the sample interferences. For each foodstuff, the most appropriate clean-up layer has to be searched for. The other layer is the gel detection layer, containing antibodies, capable of capturing the analyte from the sample. After addition of a certain amount of mycotoxin-enzyme conjugate, there is competition for the antibodies in the gel layer with the analyte of the sample. Adding the substrate for the enzyme gives (blue) colour development, the colour intensity being

inversely proportional to the mycotoxin concentration in the sample.

Because more than one mycotoxin can be present in certain foodstuffs, the new format can also be useful for simultaneous detection of different mycotoxins in coloured food. The column then comprises one clean-up layer and more detection layers, separated by grids.

These rapid methods are qualitative tests and therefore cut-off levels are applied, based on the MLs set by the European Commission. The tests are developed in a way that the blue colour appearance is suppressed once the mycotoxin level in the sample reaches this cut-off level. Negative samples should give a blue coloured gel detection layer while the detection layers for positive samples should remain colourless.

The first two chapters in this thesis give basic information on mycotoxins and immunochemical methods. Chapter 3 exposes the objectives of this thesis. In chapters 4, 5, 6 and 7 the *clean-up tandem assay column* has been applied for roasted coffee, cocoa powder and spices. These chapters are manuscripts as published in international scientific journals using the peer review system. Therefore, some repetition of data can occur. Chapter 8 deals with a combined format of the membrane test and the *clean-up tandem assay column*. For this rapid membrane test, rapid clean-up methods were compared for red wine and beer. The paper is in preparation for submission. In chapter 9, the monoclonal antibody development is described of which the screening step was performed in our laboratory. Chapter 10 switches to a homogeneous immunoassay studying the fluorescence polarization immunoassay technique (FPIA), which is minimally affected by solution opacity or colour.

Chapter 1

Literature data on human exposure to ochratoxin A (OTA), deoxynivalenol (DON) and aflatoxin B_1 (AFB_1) by consuming coffee, cocoa, spices, wine and beer

1.1 Abstract

The three mycotoxins (OTA, DON and AFB_1) for which rapid methods were developed in this thesis are described in the first chapter: their chemical and physical properties, occurrence, fungal sources, toxicity and analytical methods.

Another part of this chapter deals with the human exposure to OTA, DON and AFB_1 determined on the one side by the contamination of the foodstuffs (coffee, cocoa, spices, wine and beer) and on the other side by the consumption of these products.

This chapter will make clear the reason why rapid tests were developed for these mycotoxins in the above mentioned foodstuffs.

1.2 The mycotoxins ochratoxin A, deoxynivalenol and aflatoxin B_1

Mycotoxins are a class of highly toxic compounds produced under particular environmental conditions, by several heterotrophic microorganisms, fungi or moulds, developing in many foodstuffs. Their presence depends on several factors, such as: fungal strain, climate and geographical conditions, cultivation technique and foodstuff conservation. Mycotoxins may occur in various vegetal products, such as cereals, dried fruits, coffee beans, cocoa and beverages, such as beer and wine [10].

Concerning the name 'mycotoxin', 'myco' means fungal (mould) and 'toxin' represents poison. In contrast to the bacterial toxins, which are mainly proteins with antigenic properties, the mycotoxins encompass a considerable variety of low molecular weight compounds with diverse chemical structures and biological activities [11]. These secondary metabolites are formed in the final stages of the exponential growth phase in contrast to the primary metabolites of fungi and other organisms which are those compounds that are essential for growth [12]. Like most microbial secondary metabolites, the functions of mycotoxins for the fungi themselves are still not clearly defined [11].

Fungi produce mycotoxins under stressful conditions such as changes in temperature, moisture or aeration and in the presence of aggressive agents [12]. Invasion of fungi and production of mycotoxins in commodities can occur under favourable conditions in the field (preharvest), at harvest and during processing, transportation and stor-

age. *Fusarium verticillioides* and *Fusarium graminearum* are fungi that are frequently found in the field. Most *Penicillium* spp. and *Aspergillus ochraceus* are storage fungi. Thus, most of the mycotoxin-producing fungi belong to three genera, namely *Aspergillus*, *Fusarium* and *Penicillium* [13]. However, not all species in these genera are toxinogenic. Genetics, environmental and nutritional factors, time of incubation, etc., greatly affect the formation of mycotoxins. In the field, weather conditions, plant stress, invertebrate vectors, species and spore load of infective fungi, variations within plant and fungal species and microbial competition all play important roles in mycotoxin production. During storage and transportation, water activity (a_w), temperature, crop damage, time, blending with mouldy components and a number of chemical factors - such as aeration (O_2, CO_2 levels), food substrate, pH, lack of specific nutrients, presence of inhibitors, minerals and chemical treatment - are important. In general, mould growth in food is necessary before subsequent onset of toxin production and optimal conditions for toxin formation generally have a narrower window than those for mould growth [11].

1.2.1 Ochratoxin A (OTA)

1.2.1.1 Chemical and physical properties

Ochratoxin A (OTA; $C_{20}H_{18}ClNO_6$, Mw=403.82 g/mol) (Figure 1.1) is a colourless, crystalline compound and its chemical name is L-β-phenylalanine-(R)-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl]carbonyl-(R)-isocoumarin. It is highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. The melting points are 90 °C and 171 °C, when recrystallized from benzene or xylene, respectively. OTA exhibits UV absorption: $\lambda \, {}^{MeOH}_{max}$ (nm) = 333. The fluorescence emission maximum is at 467 nm in 96 % ethanol and 428 nm in absolute ethanol. OTA has weak acidic properties. The pKa values are in the ranges 4.2-4.4 and 7.0-7.3, respectively, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part [14, 15].

OTA is a stable compound which is not destroyed by common food preparation procedures, as temperatures above 250 °C for several minutes are required to reduce the toxin concentration. Thus, raw and processed food commodities can be contaminated with OTA [16].

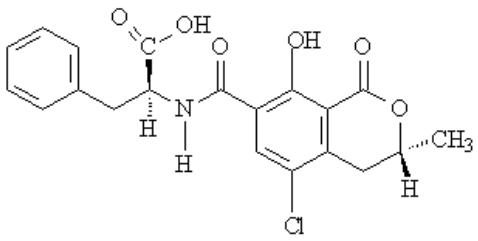

Figure 1.1: Chemical structure of OTA

1.2.1.2 Occurrence

For some time, cereals and animal products have been thought to be the major sources of human exposure to OTA [17]. However, OTA can occur in a large variety of commodities; cereals, beans, groundnuts, spices, dried fruits, coffee, cocoa, beer and wine [18].

The presence of OTA residues in animal products is of concern because OTA binds tightly to serum albumin and has a long half-life in animal tissues and body fluids. Thus, OTA can be carried through the food chain and the natural occurrence of OTA in milk, kidney, liver, blood serum, blood sausage, other sausage made from pork and poultry meat from animals fed with contaminated feed has been reported [11, 18].

1.2.1.3 Fungal sources

OTA has been reported in *A. ochraceus* and several related *Aspergillus* species [13, 19] but the first report of its natural occurrence and its potential importance was in a different source, *P. verrucosum* [20]. *A. carbonarius* was identified as a third major OTA source [21, 22]. In tropical and semitropical regions OTA is mainly produced by members of the *A. ochraceus* group. In temperate regions *P. verrucosum* predominates [23].

Most OTA producers are storage fungi, growing in a range of 4-37 °C and at a_w as low as 0.78. Optimal conditions for toxin production are narrower, with temperatures of 24-25 °C and a_w values > 0.97 (minimum a_w for OTA production is about 0.85) [11].

Whenever there is a problem in processing or storage of mixed feeds

and raw materials which allows fungal growth, the risk of OTA contamination should be taken into account [24].

1.2.1.4 Toxicity

OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several animal species and to cause kidney and liver tumors in mice and rats [25, 26, 27]. For these reasons, the International Agency for Research on Cancer (IARC) of the World Health Organisation (WHO) classified OTA in 1993 as possible carcinogen to humans (group 2B) based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans [28, 29, 30]. OTA is considered to be a weak nephro-carcinogen because a high level of toxin and an extended period of exposure are necessary to induce the tumors [31].

In all the mammalian species tested, the kidney is the major target for the toxicity of OTA. Besides the Balkan Endemic Nephropathy, elevated exposure to OTA should also be associated with human nephropathies in Algeria and Tunisia. Furthermore, high kidney failure rates have been observed in rural Scandinavian populations which may be due to the ingestion of pig meat contaminated with excessive amounts of OTA [23].

Liver necrosis and enteritis were also observed in animals [32, 33]. Studies show that OTA also causes liver cancer in rats [11] and acts as an immunosuppressor [33, 34] and a teratogen in test animals [35]. Mutagenicity of OTA was demonstrated using cell lines with stable human cytochrome P-450 enzymes [36] although OTA has not been shown to be mutagenic in early studies. The genotoxic status of OTA is still controversial because contradictory results were obtained in various microbial and mammalian tests, notably regarding the formation of DNA adducts [15].

The mechanisms involved in the toxicity of OTA indicate the following major effects: inhibition of protein synthesis, impairment of calcium homeostasis, induction of lipid peroxidation, oxidative stress and DNA damage, with species- and tissue-specific differences. The underlying mechanisms of OTA mediated pathologies have not been fully elucidated [15, 37].

OTA is relatively stable in human blood and its half-life may reach about 35 days in serum [38]. But wide species differences have been reported in the serum half-life of OTA [20]. It binds almost completely to plasma proteins and accumulates in kidney and liver tissue [26]. The

fact that the half-life of OTA in humans is 8-12 times longer than in rats is important for risk assessment. The primary source of excretion in rats is the urine but faecal excretion also occurs to some extent [23].

In all species examined, OTA is hydrolyzed (detoxified) to ochratoxin α, mainly by the bacterial microflora in the intestine. The capacity of the liver and kidney to hydrolyse OTA is low. However, OTA, which is excreted into the bile, can be hydrolyzed by the intestinal microflora and the resultant ochratoxin α may be absorbed. In this way OTA undergoes entero-hepatic circulation [39].

There is a great concern on OTA because it can be taken from different food sources. Although levels are usually low in each commodity, the concurrent intake of different contaminated food and drinks might provide a total amount of OTA near the provisional tolerable weekly intake (PTWI) set by the World Health Organization (WHO) at 100 ng/kg body weight [26, 38].

1.2.1.5 Analytical methods

Basic stages of mycotoxin analysis include sampling, extraction, clean-up and concentration, separation, detection and quantification. Ochratoxins are usually extracted with acidified organic solvents as OTA is present in dissociated form under neutral or alkaline conditions. Clean-up can be carried out by liquid-liquid partitioning using aqueous sodium hydrogen carbonate or by solid-phase extraction (SPE) [40]. Monoclonal antibody based immunoaffinity columns (IACs) have also been developed to substitute the traditional solid-phase clean-up. This method followed by conventional reversed-phase high performance liquid chromatography (HPLC) gives higher recoveries and lower detection limits from complex samples [41].

OTA is generally detected or quantified by using thin layer chromatography (TLC), HPLC with fluorescence detection (FLD) or gas chromatography (GC) [42, 43, 44].

Almost all validated and official (Association of Official Analytical Chemists, AOAC) methods for OTA determination are based on immunoaffinity clean-up and LC with FLD [45].

Liquid chromatography tandem mass spectrometry (LC-MS/MS) and capillary zone electrophoresis with laser induced fluorescence (CZE-LIF) for quantification of OTA after tandem clean-up on silica columns and IACs are alternatives [45].

Enzyme-linked immunosorbent assays (ELISA), based on antigen-

antibody reactions, have been developed for fast, specific and inexpensive screening of OTA in foodstuffs.

Lateral flow devices [46], flow-through enzyme immunoassays [47, 48, 49], array biosensors [50], fluorescence polarization immunoassays (FPIAs) [51], screen-printed electrode immunoassays and other immunochemical competitive biosensors based on surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) technologies [52] have been developed for rapid OTA determination [45]. Molecular imprinted polymer (MIP) is a novel technology under investigation as a potential alternative to IAC clean-up [18].

Certified reference materials (CRM) are available at the Institute for Reference Materials and Measurements (IRMM), which improve quality assurance in laboratories [18]. However, for OTA, only one is currently available (BCR471 (wheat, blank)).

1.2.2 Deoxynivalenol (DON)

1.2.2.1 Chemical and physical properties

Deoxynivalenol (DON; $C_{15}H_{20}O_6$, Mw=296.32 g/mol) (Figure 1.2) is a type B trichothecene (TCTC) mycotoxin. The term 'TCTC' is derived from trichothecin, the first compound isolated in this group. All the mycotoxins in this group contain a common 12,13-epoxytrichothecene (six-membered oxygen-containing ring) skeleton and an olefinic bond with different side chain substitutions. Based on the presence of a macrocyclic ester or ester-ether bridge between C-4 and C-15, TCTCs are generally classified as macrocyclic (type C) or non-macrocyclic. The non-macrocyclic TCTCs are further divided into two types: type A TCTCs, which contain a hydrogen- or ester-type side chain at the C-8 position and type B TCTCs, including DON, which contain a ketone [11].

Besides the 12,13-epoxy group, DON has also three OH functions, and an α,β-unsaturated keto group. Its chemical name is therefore 12,13-epoxy-3α,7α,15-trihydroxy trichothec-9-ene-8-one. DON crystallizes as colourless needles, with a melting range of 151-153 °C. The α,β-unsaturated keto function results in absorption of ultra-violet (UV) radiation of short wavelength but the UV spectrum of DON is not characteristic. As it is a type B TCTC, DON is soluble even in water and in polar solvents such as aqueous methanol, aqueous acetonitrile and ethyl acetate. DON is stable in organic solvents [53] but ethyl acetate and acetonitrile are the most suitable solvents, particularly for long-

term storage [54]. The 12,13-epoxy group is extremely stable to nucleophilic attack, DON is stable at 120 °C and it is not decomposed under mildly acidic conditions. The three hydroxyl groups can be derivatized (e.g. esterified), for instance before GC analysis [55].

Bretz *et al.* (2006) [56] studied the stability of DON under food-processing conditions such as cooking or baking by performing model heating experiments and screening the residue for degradation products. The findings indicate that the degradation of DON under thermal treatment might reduce the toxicity of DON contaminated food because of the formation of less cytotoxic products.

1.2.2.2 Occurrence

Contamination of DON occurs in wheat, barley and other commodities, including oats, sorghum, rye, safflower seeds and mixed feeds. DON has been found in cereals worldwide. With wet and cold weather during maturation, cereals are especially susceptible to *F. graminearum* infection, which causes so-called '*Fusarium* head blight' and simultaneously produces the toxin. The optimal temperature for DON production is about 24 °C. Outbreaks of DON in winter wheat in the US, Finland and Canada usually occur when continental chilly and damp weather favoring the fungal infection is followed by a humid summer favourable for toxin production. For other crops, such as corn and rice, a continental humid warm summer is more favourable. Depending on the weather conditions, the infestation of *F. graminearum* in wheat and corn and subsequent production of toxins in the field vary considerably from year to year as well as by regions. Thus, the levels of DON in these commodities are difficult to predict [11].

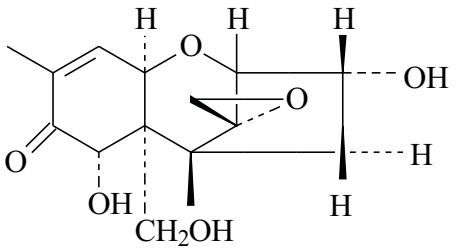

Figure 1.2: Chemical structure of DON

1.2.2.3 Fungal sources

TCTCs are a group of naturally occuring toxic tetracyclic sesquiterpenoids produced by many fungal species in the genera *Fusarium* (most frequently), *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium* and *Stachybotrys* [11].

DON is produced primarily by *F. graminearum* and other related fungi such as *F. culmorum* and *F. crookwellense* which are able to infect cereal crops in the field [56].

F. graminearum is the most widely distributed toxigenic *Fusarium* spp. It has an optimal growth range between 24 °C and 26 °C and grows at a minimum a_w of 0.90. Toxin production largely mirrors growth conditions [57].

1.2.2.4 Toxicity

DON may have adverse health effects after acute, short-term or long-term administration. After acute administration, DON produces two characteristic toxicological effects: decrease in feed consumption (anorexia) and emesis (vomiting) in pigs. Because of this, the name 'vomitoxin' for DON is also used. With respect to chronic effects, besides growth (anorexia and decreased nutritional efficiency), also the immune function and reproduction (reduced litter size) are adversely affected by DON in animals [58]. The toxin has a haemolytic effect on erythrocytes.

In 1993, IARC placed DON in the group 3 as not classifiable regarding its carcinogenicity to humans [28]. Accordingly, the results of a two-year study in mice did not suggest that DON presents a carcinogenic hazard [59]. Other studies revealed that DON inhibits the synthesis of DNA and RNA, as well as the protein synthesis at the ribosomal level, alters brain neurochemicals and has immunosuppressive properties depending on the dose and duration of exposure. DON has also been implicated in two large outbreaks of gastrointestinal illness in humans in India and China [60].

The pathological mode of action of DON is based on the 12,13-epoxy group attached to the TCTC ring and the position and structure of its side groups [61].

DON is rapidly metabolized in the animal organism and the carry-over into edible tissues, milk and eggs is very low. Thus, animal derived foods contribute only marginally to total human exposure to DON [62].

Although DON is one of the least acutely toxic TCTCs, it should be treated as an important food safety issue because it is a very common contaminant of cereals [63].

1.2.2.5 Analytical methods

Extraction of the relatively polar DON is usually performed by mechanical shaking or blending with aqueous acetonitrile or aqueous methanol although other solvents e.g. water/polyethylene glycol (PEG), chloroform-methanol and chloroform-ethanol have also been used. The main clean-up procedures are liquid-liquid partitioning, SPE, IACs and multifunctional clean-up columns [64].

Commonly used assays for the detection of DON include TLC, HPLC, GC and immunochemical methods such as ELISA [65]. DON forms a fluorescent derivative in the presence of aluminium chloride and this is the basis for several TLC methods [66, 67]. Detection of the UV absorbence of DON is the basis for several HPLC methods [68]. LC appears to be the most common technique for the determination of DON and many laboratories are applying LC and especially LC-MS for the analysis of TCTC mycotoxins [69]. The GC methods have employed either electron capture detection (ECD), mass spectrometry (MS) or infrared spectroscopy (IR) after derivatisation [70, 71, 72]. ELISA continues to provide a reliable alternative to more traditional chromatographic techniques for the analysis of TCTCs [69].

For rapid DON determination, membrane-based assays in a dipstick format [73, 74], flow-through enzyme immunoassays, lateral flow devices, FPIAs [65, 75, 76], fluorometric assays [77] and SPR methods [78, 79, 80] have been developed.

Wider availability of reference materials (BCR377 (maize flour, blank), BCR378 (maize flour, medium level), BCR396 (wheat flour, blank) and international comparative studies are needed to ensure improved quality assurance of the analytical methods [64].

1.2.3 Aflatoxin B_1 (AFB_1)

1.2.3.1 Chemical and physical properties

Aflatoxins have chemical structures containing dihydrofuranofuran and tetrahydrofuran fused with a substituted coumarin. At least 16 different structurally related toxins have been found [81]. AFB_1

($C_{17}H_{12}O_6$, Mw=312.3 g/mol) (Figure 1.3) is a 2,3,6α,9α-tetrahydro-4-methoxy-cyclopenta(c)furo(3', 2':4,5)furo(2,3-h)(l)-benzopyran-1,11-dione [23]. Of all the mycotoxins, AFB_1 is considered to be the most toxic/carcinogenic compound [28]. Other aflatoxins are less toxic ($B_1 > G_1 > B_2 > G_2$). Pure AFB_1 is a pale-white to yellow crystalline, odorless solid with a melting range of 268-269 °C. Aflatoxins are soluble in methanol, chloroform, acetone and acetonitrile. Consumption of AFB_1 contaminated feed by dairy cows results in secretion of AFM_1 in milk. AFM_1, a hydroxylated metabolite of AFB_1, is about 10 times less toxic than AFB_1 but its presence in milk is of concern for human health [69, 82, 83, 84, 85].

1.2.3.2 Occurrence

Aflatoxins occur all over the world in foods and in a wide variety of raw food materials like in cereals, maize, peanuts and peanut products, pistachio, tree nuts (Brazil nuts, almonds, pecans), cotton seeds, peppers, rice, pumpkin seeds, sunflower seeds and other oil seeds, copra, spices, dried fruits (figs, raisins) and yams [86, 87]. Among these products, frequent contamination with high levels of aflatoxins in peanuts, corn and cotton-seed, mostly due to infestation with mould in the field, are of the most concern [88]. It should be reiterated that resistance to aflatoxin contamination in the field does not guarantee that the commodities are free of aflatoxin contamination during storage. Inadequate storage conditions, such as high moisture and warm temperatures (25-30 °C), can create conditions favourable for the growth of fungi and production of aflatoxins. High levels of AFB_1 have been reported in some lots of rice, cassava, figs, spices, pecans and other nuts [11, 89, 90, 91].

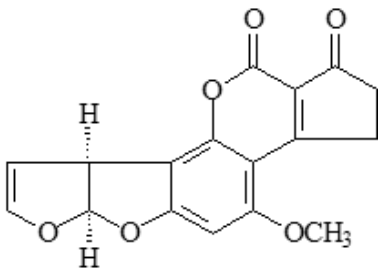

Figure 1.3: Chemical structure of AFB_1

1.2.3.3 Fungal sources

Aflatoxins (B_1, B_2, G_1, G_2) are produced by the fungi *A. flavus* and *A. parasiticus* in a number of important agricultural commodities in the field and during storage. *A. flavus* produces aflatoxins B_1 and B_2, while *A. parasiticus* gives rise to aflatoxins B_1, B_2, G_1 and G_2 [28]. Aflatoxins are also produced by some other *Aspergillus* spp. (*A. nomius*, *A. pseudotamarii*, *A. bombycis*), although they are not as important in economical terms as *A. flavus* and *A. parasiticus* [92]. Because the four major toxins were originally isolated from fungal cultures of *A. flavus*, the first few letters of the fungus name (A and fla) were used to create the toxin name. These toxins fluoresce either blue or green under UV light, and this distinguishes the B or G types of toxins [11].

1.2.3.4 Toxicity

In 1959 a severe outbreak of the Turkey "X" disease occurred at 500 locations in Great Britain, killing about 100 000 turkey poults. Brazilian groundnut meal was the toxic factor which served as a protein source in the feed. The toxic factor was produced by *A. flavus* and *A. parasiticus* which resulted in the name of aflatoxin [23]. There was a tragic outbreak of human aflatoxicosis in the eastern and central provinces of Kenya during the period April to September 2004. Three hundred forty one cases were diagnosed which resulted in 123 deaths and highlighted the continued threat posed by aflatoxin contamination of staple foods to human health in developing countries. Further aflatoxicosis outbreaks were reported in one of the same districts of Kenya in May 2005 [69].

The main target organ of aflatoxin is the liver. Typical symptoms for aflatoxicoses in animals include proliferation of the bile duct, centrilobular necrosis, fatty infiltration of the liver and hepatomas in addition to generalized hepatic lesions [93]. AFB_1 also affects other organs and tissues [94, 95, 96], including the respiratory system.

In addition to these acute (hepatotoxic) effects, carcinogenic effects of AFB_1 are of the most concern [93]. Epidemiological studies have shown a strong positive correlation between aflatoxin levels in the diet and primary hepatocellular carcinoma (PHC) incidence in some parts of the world, including certain regions of the People's Republic of China, Kenya, Mozambique, the Philippines, Swaziland, Thailand and the Transkei of South Africa [93, 97, 98, 99]. Whereas AFB_1 has been found to be a potent carcinogen in many animal species [98, 100, 101],

the role of aflatoxins in carcinogenesis in humans is complicated by hepatitis B virus (HBV) infections in humans [11, 102, 103, 104].

Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in laboratory animals and their acute toxicological effects in humans. Therefore, in 1987 the IARC accepted that aflatoxin B_1 should be classified as a group 1 carcinogen [105].

It was demonstrated that AFB_1 must be converted into its reactive epoxide to exert its effects and that protein binding plays an important role in its cytotoxicity [106].

Because of their presence in foods and evidence of their association with human carcinogenesis, aflatoxins are a serious threat to human health even after more than 40 years of research [93, 107].

1.2.3.5 Analytical methods

For aflatoxins, TLC [108], GC [109], HPLC [110] and LC-MS methods using atmospheric pressure photo-ionization [111] and electrospray ionization [112] have been reported. Several multi-toxin methods have been published which include determination of aflatoxins [113, 114, 115, 116]. Commercial IACs are used for clean-up after extraction with acetonitrile-water [69]. AOAC proposes a method based on derivatisation of AFB_1 and LC with fluorescence detection [117]. LC with UV detection [118] after derivatisation and micellar electrokinetic capillary chromatography [119] have also been used to investigate the production of aflatoxins.

ELISA methods are also available for the detection of aflatoxins [120].

Membrane-based assays in the format of a flow-through enzyme immunoassay [117], lateral flow devices [121, 122], fluorometric assays [123], electrochemical immunosensors [124] and SPR methods [52, 125] have been developed for rapid AFB_1 determination.

For AFB_1 the following certified reference materials are available: BCR264 (defatted peanut meal, high level), BCR401 (peanut butter, low level), BCR375 (compound feed, blank).

1.3 Exposure assessment

The mycotoxins OTA, DON and AFB_1 have been found in a lot of raw materials and in this way they can contaminate the industrial products.

By consuming contaminated food, humans are exposed to these toxic components. This is the most prominent route of exposure to mycotoxins and the effects of ingestion are relatively well-known through animal studies and epidemiologic evidence. However, exposure through skin contact and the inhalation of dust originating from contaminated cereals or food also may pose significant risks [126].

The term risk assessment, in the context of food safety, is used by many organizations to describe the process of assessing the health risks from a variety of agents that can be present in food. Risk assessment of mycotoxins is, in fact, the product of hazard assessment and exposure assessment. Data on the natural occurrence of mycotoxins in various commodities and food intake data are needed to enable exposure assessment [127]. Figure 1.4 shows the iterative process involved in issue identification, risk assessment and risk management. Out of this figure exposure assessment can be situated within the risk assessment of mycotoxins.

In the European Union (EU), efforts to assess exposure are undertaken within SCOOP (Scientific Cooperation on Questions relating to Food) projects, funded by the European Commission (EC). Because of variation between batches and crops, even within the same harvest year of a given commodity, data on mycotoxin contamination do not easily convert to accurate human exposure estimates [128]. However, the SCOOP projects are targeted to make the best estimates of intake of several mycotoxins by EU inhabitants [129].

Monitoring mycotoxin levels in blood, urine or breast milk provide an estimate of human exposure more specific to an individual than what can be ascertained using general occurrence levels in commodities. High incidence of OTA in blood is observed in different European and Nord-African countries like Denmark, Sweden, Italy, Norway, Morocco and Tunisia. Also in Canada, Japan, Chili and Switserland, OTA was found in blood [18, 130, 131, 132, 133]. Human exposure to DON can be evaluated in the urine [134]. OTA and AFM_1 were found in breast milk [135, 136, 137, 138]. For these analyses, the suitable, specific biomarker has to be searched for. Biomarkers are available for aflatoxins (AFB-N7-guanine in urine [139, 140], AFM_1 in milk [137]), OTA in serum [141], OTA in plasma [142], OTA in urine [143] and DON glucuronide in urine [134]. However, even when a specific biomarker of a given mycotoxin is identified in humans, it remains difficult to establish the relation with a given illness, because of genetic polymorphism, possible beneficial influence of diet and because other environmental

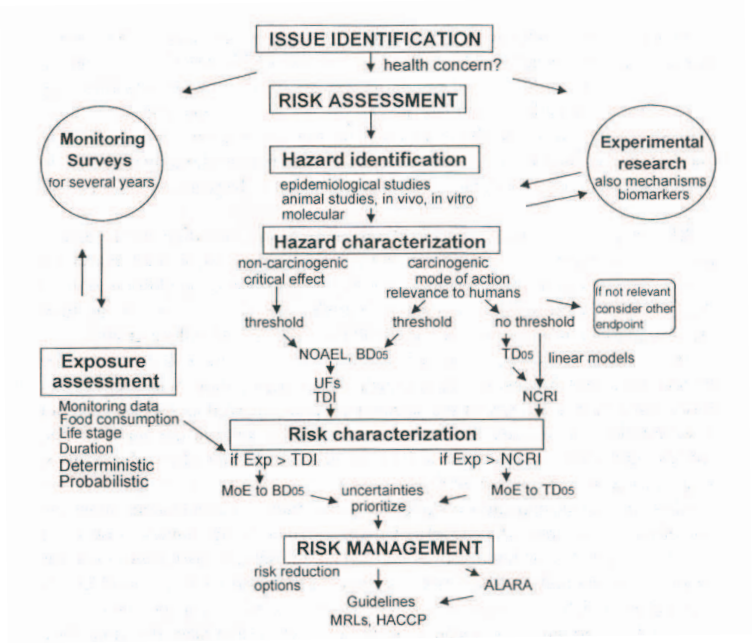

Figure 1.4: Iterative process involved in issue identification, risk assessment and risk management of mycotoxins in food and the relationship to monitoring surveys and toxicological and epidemiological research [3]
NOAEL: no observed adverse effect level, BD: benchmark dose, TD: tumorigenic dose, UFs: uncertainty factors, TDI: tolerable daily intake, NCRI: negligible cancer risk intake, Exp: exposure, ALARA: as low as reasonably achievable, MoE: margin of exposure , MRLs: maximum residue levels, HACCP: hazard analysis and critical control points

toxicants may interfere [59]. Moreover, sampling of neither blood nor urine nor breast milk allows for identification of the source of the mycotoxin intake into the human body [132, 144].

1.3.1 Natural occurrence of OTA, DON and AFB_1

1.3.1.1 Variety in mycotoxin level

A lot of factors are important when collecting information on the mycotoxin levels in food. These could be responsible for the differences between certain studies.

The growth of the fungi and the associated toxin production are closely correlated to the degree of moisture to which they are exposed, which itself is dependent on weather conditions at harvest and techniques for drying and storage [145]. All factors like temperature, a_w, pH, substrate, other microflora, the fungi and precautions have an influence on the growth of the fungus and its toxin production [132].

The knowledge on how the industrial process of the raw products affects the mycotoxin presence in foods is very important and can contribute to the reduction of mycotoxin levels in foodstuffs [146].

Another important factor is the presence of 'hot spots' of mycotoxins that may accidentally occur, even if the optimal required conditions for mould development have not been reached [147]. The random nature of the fungal contamination of raw materials and thus the uneven distribution of subsequent mycotoxin contamination means that sampling is a major issue. It is very difficult to obtain representative samples which are handled in bulk amounts. Statistically-based sampling plans for mycotoxins are available. For some beverages like wine, beer and coffee as consumed, sampling in terms of taking large sample sizes is unnecessary and here representative data are best obtained through analysis of large numbers of geographically varied samples [16].

Other factors affecting the variety in mycotoxin level can be found in the analysis of the mycotoxins. There may be matrix effects which can result in under-estimating the amount of mycotoxin, unless appropriate extraction methods for different foods are developed. As mycotoxins in foods occur frequently at low concentrations, with a number of samples below the limit of detection (LOD) and limit of quantification (LOQ) of the analytical method, it is important how these 'non-detects' are treated in the exposure estimation [3]. Another problem is the presence of conjugated mycotoxins, in which the toxin is usually bound to a more polar substance like glucose, referred to as masked

mycotoxins. These substances escape routine detection methods but can release their toxic precursors after hydrolysis [148].

In the following sections the contamination of coffee, cocoa, spices, wine and beer is studied together with the possible influences on the mycotoxin contamination in each foodstuff.

1.3.1.2 Coffee

There is a large amount of literature documenting OTA contamination of green and processed coffee and reporting isolation of OTA producing fungi from coffee, starting in the 1970s. Studies have concluded that *A. ochraceus*, *A. carbonarius* and *A. niger* may all be responsible, though most studies have focused on *A. ochraceus* [149, 150]. The so-called dry processing of coffee, which is cherry drying, was identified as one of the steps during which OTA formation can take place, particularly under humid tropical conditions. Cherries contain sufficient amounts of water to support mould growth and OTA formation during the initial 3-5 days of drying on the outer part of the cherries [151]. In a study performed by Ilic *et al.* (2007) [152], the OTA problem was also suggested to occur most likely post-harvest because no OTA producing fungi were found in a fresh coffee bean sample analysed.

Despite the differences between studies, they all demonstrate considerable contamination of coffee products with OTA [153]. The OTA concentration in coffee beans can vary between 0.2 and 360 μg kg^{-1} [150]. Only 9 % of the samples in the study performed by Taniwaki *et al.* (2003) contained OTA above 5 μg kg^{-1}, which is the maximum level for OTA according to Commission Regulation (EC) No 1881/2006 of 19 December 2006 [1]. In the SCOOP study (2002) [4], only 1 % of roasted coffee samples contained OTA above 5 μg kg^{-1}.

Coffee bean source The OTA contamination and probably also the responsible species, are dependent on the region of coffee bean production. The OTA content was found to be higher in African samples (5-10 μg kg^{-1}) in comparison with Asian and American samples (mostly less than 5 μg kg^{-1}) [154]. In the SCOOP study, the mean OTA levels in green coffee were found to be higher in South Europe than in North Europe. This trend could be attributable to differences in hygienic quality of the imported green coffee [4].

Variability in OTA contamination could also be obtained during transportation of the green coffee because of changes in temperature,

relative humidity and moisture content [155].

Influence of roasting Regarding the stability of OTA, roasting of coffee gave the most variable results. Losses were reported in the range of 0-100 % [156, 157]. Results are influenced by several factors such as roasting conditions, non-homogenicity of natural coffee bean contamination, OTA levels, natural contamination versus spiking and analytical method performance [157]. The comparison of the European mean OTA levels in green and processed coffee samples in the SCOOP study (2002) [4] indicates a marked reduction of OTA to be probably attributed both to blending and to losses during the technological procedures usually adopted in South Europe, where more drastic roasting procedures are employed with respect to Northern countries. In a study performed by Suarez-Quiroz *et al.* (2005) [158], assays on the thermal stability of pure OTA showed that it should be found in larger quantities in roasted coffee. This suggested that OTA was masked by reactions with the coffee substrate during roasting. The absence of OTA in green coffee is therefore the best guarantee of safety [158].

Several recent studies reported that the way the coffee beverage was prepared also affects the OTA content, which could paradoxically be higher than that of the initial roasted coffee [158, 159, 160].

1.3.1.3 Cocoa

Experiments show that OTA is mainly formed in the initial stages of sun-drying of the cocoa beans. The drying period is critical to avoid the appearance of the mycotoxin in cocoa beans and not the period of storage [161]. OTA contamination is not correlated with the physical appearance of the cocoa beans i.e. mouldiness. So, it is not possible to sort out beans likely to be contaminated [162].

As reported by Serra Bonhevi (2004), OTA concentration levels found in cocoa product samples are ranging from 0.1 to 23.1 μg kg^{-1}. OTA levels in roasted cocoa powder samples were ranging from 0.1 to 2 μg kg^{-1} in 38.7 % of the samples. 54.8 % were found to contain $>$ 2 μg OTA kg^{-1}, among which 70 % contained $>$ 3 μg kg^{-1} [161]. Other studies report OTA concentrations in cocoa powder of $<$ LOD-2.4 μg kg^{-1} [4], 0.05-0.93 μg kg^{-1} [163], $<$ LOD-0.77 μg kg^{-1} [164] and $<$ LOD-4.4 μg kg^{-1} [161].

Processing of beans into finished products gives a reduction of OTA content. OTA is reduced by removal of the shell fraction; this pro-

cessing step is always applied. Further reduction (dilution) happens by mixing with sugar and other ingredients when producing finished products [162].

Influence of shelling The influence of the shelling process on the presence of OTA in cocoa beans has been investigated. Burdaspal and Legarda (2003) [163] have, taking into account a study carried out by CAOBISCO/ECA/ FCC (2003), pointed out the fact that shelling could reduce the OTA content with 25-50 %.

When a handmade cocoa shelling process was undertaken, almost 100 % of the shell was removed without any difficulties [146]. The apparent contradiction with the results obtained by CAOBISCO/ECA/FCC (2003) could be explained by the fact that during the industrial procedure, shells and nibs come into contact with each other and thus, nibs would be contaminated by OTA. In consequence, the facts that OTA appears in cocoa derivatives in spite of shelling can be explained due to industry failing to eliminate all of the shell or prevent the contact between cocoa nibs and shells. A well-controlled shelling process could achieve a very significant OTA reduction in the cocoa-derived products [146].

1.3.1.4 Spices

Spices are largely produced in countries where tropical climates (high ranges of temperature, humidity and rainfall) are favourable to mycotoxin contamination. Furthermore they are usually dried on the ground in the open air in poor hygienic conditions that even more promote growth of moulds and production of mycotoxins [165].

The contamination of spices by mycotoxins was especially reported in Ethiopia [166], Egypt [167], Portugal [165], India [168], Turkey [169], Hungary [87], Morocco [170], Russia, The Netherlands and Belgium [171, 172].

Aflatoxins are the leading toxins present in peppers (paprika, chilli, cayenne), nutmeg, mustard, ginger, black and white pepper and coriander. Ochratoxins have been reported also as natural contaminants in many spices [86]. The findings of Fazekas *et al.* (2005) [87] suggest that aflatoxin contamination very often occurs together with OTA contamination.

In India, the OTA contamination in chilli, a major component of cooked foods in this country, exceeded 20 μg kg^{-1} in over 26 % of the

market samples tested [173]. In the SCOOP project (2002) [4], a total of 361 data were presented on spices by four countries (Germany, Italy, Portugal and The Netherlands). Results indicate that also these food products are susceptible to OTA contamination (52 % positive). In Zinedine *et al.* (2006), the contamination level of OTA found in some analysed spices was quite high and the average of contaminated samples was around 50 % for several sorts of spices [170].

Concerning AFB_1, chilli and black pepper can be contaminated in the range 0.3-116.4 μg kg^{-1} according to a study performed by Bircan (2005) [169]. Colak *et al.* (2006) found 42.9 % of the spice samples contaminated with aflatoxins in the range of 0.3-46.8 μg kg^{-1} [174].

In the study performed by Tabata *et al.* (1992) [175], aflatoxins could not be effectively degraded or eliminated in either sauteing or boiling processes. Cooking experiments showed that aflatoxin levels in spiced sauces are not reduced by domestic cooking with either microwave or conventional gas oven heating [176].

1.3.1.5 Wine

A. carbonarius is supposed to play the main role for OTA synthesis in grapes because the percentage of positive strains and the amount of OTA produced *in vitro* are generally higher than in other black *Aspergilli*, in agreement with similar studies carried out in Europe [17, 22, 177]. Further research [178] revealed that *Aspergilli* section *Nigri* are present on bunches early in the season and their frequency increases going towards later grape growth stages. Blesa *et al.* (2006) [179] are giving a list of OTA concentrations in wines. In the SCOOP project (2002) [4], 2-4 % of the wine samples analysed were found to contain OTA in a concentration > 2 μg L^{-1} [180]. In Greek wine OTA was much more present: 11.5 % of the samples were found to have an OTA concentration ≥ 1 μg L^{-1} [181]. Chiodini *et al.* (2006) didn't find wine samples exceeding the level permitted by the European Union of 2 μg L^{-1} [182].

Influence of climate Within the red wines, the OTA concentrations are dependent on the geographical region. The levels are raising from the north to the south of Greece, from the north to the south of Italy, from the centre and the south-east of France to the Mediterranean Sea and from the north to the south of Europe. The climate seems to be the reason for these differences and not the variety in wine cultivation [183,

184, 185, 186, 187]. Also closeness to the sea seems to be an important factor (Visconti, personal communication).

Aspergillus spp. are responsible for the contamination in warmer regions. These can grow in vineyards with a mediterranean climate [179]. *Aspergillus section Nigri* spp. are more frequently isolated in hot and dry areas, while *A. carbonarius* showed higher incidences in hot and wetter areas [188, 189].

The OTA concentration can vary also from year to year because of the differences in weather conditions [179].

Influence of cultivation Two abiotic parameters (a_w and incubation time) had a significant influence on OTA accumulation for the *A. section Nigri* spp. in the study performed by Belli *et al.* (2004). High water activities seem to favour OTA production of these isolates and their growth [190].

Good Agricultural Practices (GAP) are very important to lower the contamination of the grapes or even to prevent. The fact that maximum amounts of OTA were found at the earlier growth stages of *A. section Nigri* isolates tested (optimum for *A. carbonarius*: 5 days) is an important hazard for OTA contamination in grapes. Consequently, carefully choosing the harvest dates and minimizing the harvesting and transport time to wine cellars become crucial. Moreover, damage on the grape skin produced at harvest or during transport may trigger the entrance of the fungi colonizing the berry surface, probably enabling production of maximum amounts of OTA. Therefore, minimizing mechanical damages of grape skins (caused by rain, birds, insects and fungi) during harvest and transport is a key factor in preventing OTA [190].

Concerning organically produced and conventional products, Chiodini *et al.* (2006) found that the concentration of OTA in organically produced wines was not significantly different from that in conventional products. In this case of analysed wine samples and used method, no difference could be found, wich is not a reason to say that organically and conventionally produced wines always have comparable OTA concentrations [182].

Influence of vinification The overwhelming majority of the analyses of OTA in different wines suggest that the incidence and concentrations decrease from the red to the rosé and white wine [44, 57, 184, 191]. Red wine is more susceptible to OTA contamination [57, 192, 193] due probably to the fact that red wine processing conditions produce intensive

contact with potential toxin-producing moulds [193]. In fact, in the red wines, the grapes are immediately processed and left mashed and the skin and the juice are put aside for several days, which obviously permits fungal growth and the production of toxins. However, de Cerain *et al.* (2002) [194] did not find a great difference between white and red wine probably due to the small number of samples and the great dispersion of the data, especially for red wines.

Blesa *et al.* (2006) reported that OTA producing moulds are inhibited by ethanol and the generally anaerobic conditions but the mycotoxin is alcohol resistant and therefore not degraded during wine making and storage. However, Fernandes *et al.* (2007) reported that vinification consistently reduced OTA concentrations in wine independent of the initial OTA concentration in grapes [195].

de Cerain *et al.* (2002) [194] suggested that the concentration of OTA present in wine could vary with time and demonstrated that it is stable in wine for at least one year.

1.3.1.6 Beer

If contaminated cereals are used for the brewing of beer, the present OTA and DON can survive the malting and the brewing process and in this way contaminate the beer.

OTA can be found in beer all over the world but in low concentrations. The use of contaminated ingredients on the one side and the low limit of detection of the used analytical methods on the other side, can explain the high incidence of the OTA contamination of beer. Studies [130, 147, 196, 197, 198] show OTA concentrations in beer varying between $<$ LOD and 182 ng L^{-1}. Several studies have shown that OTA can be found at high concentrations, up to 1530 ng L^{-1}, in a strong German beer [199]. Based on the findings of these studies, the detected exposure to OTA from beer consumption does not represent a serious health risk for the beer consumer [200, 201] but it is worthwhile to include beer in calculations of the total exposure of the consumer to OTA [197].

Several DON concentrations in beer were found in studies [147, 202, 203, 204], ranging between 4 and 221 μg L^{-1}. These values are not extremely high but beer consumption should be taken into account when evaluating the total exposure of DON to humans from cereal sources [204, 205].

The simultaneous occurrence of two or more toxins in cereals has

frequently been documented in literature, e.g. barley, malt, maize and wheat contaminated with DON, nivalenol and zearalenone [206, 207, 208], maize contaminated with fumonisin B_1, DON, T-2-toxin, nivalenol and zearalenone [209], wheat contaminated with DON and OTA [210] and barley contaminated with OTA and DON [211]. These findings highlight the problems associated with ingestion of multiple toxins that may have an interactive effect on the health of the consumer. The problem of multi-contamination should not be underestimated [147].

Influence of the malting and brewing process The steeping and germination stages of malting provide levels of moisture and solubilized and partially solubilized nutrients which can be utilized by micro-organisms in the original microflora of the grain [212].

Studies on the influence of malting and brewing on the OTA contamination have been performed. Scott and Kanhere (1995) [200] found a reduction of 9-13 % for OTA after alcoholic fermentation while for Baxter *et al.* (2001) [213] the reduction was 13-32 % for the same mycotoxin. This low level of contamination reduction is due to the polarity of the mycotoxin, which favours recovery in an aqueous medium, resulting in high contamination in beer as described by Scott (1996), Visconti *et al.* (2000) and Garda *et al.* (2005) [196, 214, 215].

Concerning DON, this toxin may be lost during steeping but the *Fusarium* mould is still capable of growth and mycotoxin production during steeping, germination and kilning. So, DON contamination of the beer can even increase during the malting process [202, 216].

Scott (1992) [217] mentioned experiments of fermentation using a malt contaminated with DON in which the results after 7-9 days of fermentation showed that DON was stable to the process. Schwarz *et al.* (1995) [218] detected 80-93 % of the DON present on the malt in the beer.

The same behavior was not observed in the study of Garda *et al.* (2005) [215] where the most polar mycotoxin (DON) did not generally present higher levels in the filtered sample. The mean value result in the treatments showed that 41 % of DON was transferred on to the filtered sample which would constitute the beverage. In the solid residue, 6 % of the initial contamination of DON was detected. The fermentation process caused a decontamination (formation of less cytotoxic products) of 53 % for DON , taking into account both the wort and the filtered sample.

Influence of alcohol content and fermentation Papadopoulou *et al.* (2004) reported a statistically significant correlation between alcohol levels and DON contamination, as well as a significant difference between bottom, top and spontaneous fermenting beers [204]. Beers with a higher alcohol level contained more DON. Spontaneous fermented beers were more contaminated than top fermented beers, which had a higher DON level than bottom fermented beers.

However, Tangni *et al.* (2002) [197] did not find a significant difference in DON contamination between strong beers (> 6 %) and beers with an alcohol percentage < 6 %.

Organic and conventional production Anselme *et al.* (2006) found that organic beers collected during 2003-2004 were more frequently OTA contaminated (95 %, n = 40) than their conventional counterparts (50 %, n = 40). Conventional beers were contaminated at a mean concentration of 25 ng L^{-1} (range: 19-198 ng L^{-1}), while organic beers contained a mean level of 182 ng L^{-1} (range: 18-1134 ng L^{-1}). High OTA contamination above the limit of 200 ng L^{-1} (up to 1134 ng L^{-1}) occasionally occurred in organically produced beers [147].

Of the former study and the one performed by Tangni *et al.*, 2002 [197], on beers found on the Belgian market, it appears that the level of OTA contamination is very variable either in organic or in the conventional brands but in both studies, the contamination tends to be higher for organic beer.

DON concentrations ranged from 2-22 μg L^{-1} (mean = 6 μg L^{-1}) in conventional beers, while organic beers ranged from 2-14 μg DON L^{-1} (mean = 4 μg DON L^{-1}) [147]. Thus, the DON contamination was not found to be different for conventional and organic beers.

1.3.2 Intake data

1.3.2.1 Limits

A Provisional Tolerable Weekly Intake (PTWI) of 100 ng OTA /kg body weight (approximately 14 ng/kg body weight/day) was established by the Food and Agriculture Organization (FAO)/WHO Joint Expert Committee on Food Additives (JECFA), based on the nephrotoxic effect in pigs in a sub-chronic study [219]. This PTWI is nearly equal to the upper limit of the proposed intake put forth by the European Commission Scientific Committee on Food (SCF) of 1.2-14 ng/kg body

weight/day (8.4-98 ng/kg body weight/week) [220]. The SCF concluded that OTA intake should be reduced as far as possible, such as below 5 ng/kg body weight/day. On the basis of the lowest observed adverse effect level (LOAEL) of 8 μg/kg body weight/day for early markers of renal toxicity in pigs (the most sensitive animal species), and applying a composite uncertainty factor of 450 for the uncertainties in the extrapolation of experimental data derived from animals to humans as well as for intra-species variability, a TWI of 120 ng/kg body weight was derived for OTA by the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) [16].

For DON, a tolerable daily intake (TDI) of 1 μg/kg body weight (or a Provisional Maximum Tolerable Daily Intake (PMTDI) in the terminology of JECFA) was established by the SCF and JECFA based on the no-observed adverse effect level (NOAEL) of 100 μg/kg body weight/day [221]. The TDI for DON is determined only temporarily (FAO/WHO 2001) because of the problem of multi-contamination and the sharing of common mechanisms of action, which may lead to significant interactions [147].

For DNA-reactive carcinogens like AFB_1, there is no treshold dose below which effects, such as initiation of the carcinogenic process, will not occur, and a TDI, which is based on a treshold such as the NOAEL, is generally not determined [3]. So, no TDI for aflatoxins was proposed by the JECFA in 1998 and other international expert groups have not specified a numerical TDI for aflatoxins [219].

1.3.2.2 Coffee

It is estimated that one cup of soluble coffee contains on average 2.5 ng OTA, which means that consumption of 28 cups per week by a 60 kg person would correspond to ca. 1 % of the PTWI established by the JECFA [222]. On the basis of results performed by Studer-Rohr *et al.* (1995) [223] a daily intake from coffee drinking of about 25 ng OTA per person, corresponding to approximately 0.4 ng/kg body weight /day (8 % of TDI) is easily possible (based on a daily use of 25 g ground roasted coffee, about 3 or 4 cups and a contamination level of 1 μg kg^{-1}).

In the SCOOP study (2002), the daily OTA intake through coffee comsumption was found to vary between 0.06 ng/kg body weight/day (Italy) and 0.42 ng/kg body weight/day (Finland). These are respectively 1.2 and 8.4 % of the TDI of OTA of 5 ng/kg body weight/day.

In Figure 1.5A, the contribution of each food commodity to the mean European total dietary intake of OTA is shown. Since almost all countries in the SCOOP study did not provide information for some food products, a tentative calculation of the overall intake from all OTA susceptible food commodities was performed by including surrogate values. These are shown in Figure 1.5B.

For coffee consumption these Figures show a contribution of 9-10 % to the total dietary OTA intake in Europe [4] (Figure 1.5).

1.3.2.3 Cocoa

Considering that roasted cocoa powder and chocolate were contaminated at average OTA levels of 2.41 and 0.63 μg kg^{-1}, respectively, and based on a mean consumption of 3.14 kg of chocolate and cocoa products per year in Spain, cocoa should contribute only a minor fraction to the OTA TDI [161]. The values are similar to the OTA concentrations found in different types of chocolates and cocoa powders commercially available on the European market [163]. An important remark out of the SCOOP study (2002) is that cocoa, including cocoa powder, provides a considerable contribution to the total intake in the UK; the contribution for the 1.5-4.5 years population was similar to that attributed to cereals [4]. The contribution of cocoa to the mean European total dietary intake of OTA is 4-5 % (Figure 1.5).

1.3.2.4 Spices

Spices were estimated to contribute to the total dietary OTA intake for 8 % in Europe [4]. Taking into account surrogate values the contribution of spices is only 3 % (Figure 1.5).

No data were found in literature concerning the contribution of spices to the total dietary intake of AFB_1.

1.3.2.5 Wine

In the SCOOP study (2002), the dietary intakes of OTA ranged from 0.02 ng/kg body weight/day (Portugal) (0.4 % of TDI) to 0.86 ng/kg body weight/day (Italy) (17.2 % of TDI). The contribution of wine to the total dietary OTA intake in Europe is 10-13 % [4] (Figure 1.5).

Chiodini *et al.* (2006) even mentioned a 15 % contribution of wine to the total daily OTA intake [182].

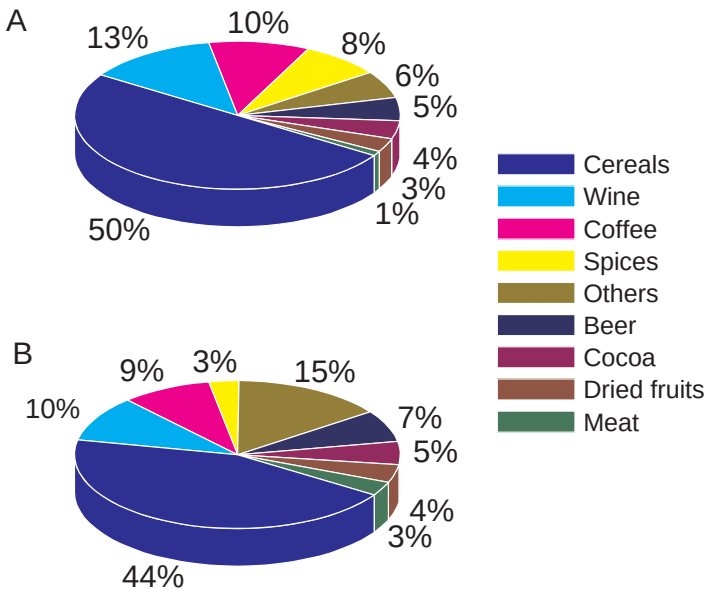

Figure 1.5: Contribution of each food commodity to the mean European total dietary intake of OTA (consumption data related to consumers only were employed for France, Norway and Sweden) A: Without surrogate values B: Surrogate values included [4]

1.3.2.6 Beer

The average OTA concentrations referred to all the surveyed beers or to Italian beers only in a study of Visconti *et al.* (2000) would be 0.020 or 0.010 ng mL^{-1}, respectively, corresponding to OTA daily intakes of 0.167 or 0.083 ng/kg body weight for a 60 kg person consuming daily 0.5 L of beer. These values are considerably lower than the PTWI established by JECFA and would lead to an estimate of about 1.2 % (for all surveyed beers) or 0.6 % (for Italian beers only) for the beer contribution to the level considered at risk for human health due to OTA exposure in Italy [196].

The mean beer consumption in Turkey is 10.5 L per person per year or 0.029 L per person per day. The contribution of beer to the mean daily intake of OTA is 0.19 ng/kg body weight/day for a 70 kg person [198].

Considering a daily intake of 0.3 L beer per capita in Belgium and the mean levels of OTA found in the study of Anselme *et al.* (2006), beer consumption contributes to 10 % of the TDI of 5 ng OTA/kg body weight for an adult of 60 kg [147].

According to a study performed by Harcz *et al.* (2007) [224], beer contributes for up to 1.76 ng OTA/kg body weight/day (35.2 % of the TDI) for organically produced beer and 0.23 ng OTA/kg body weight/day (4.6 % of the TDI) for conventionally produced beer, considering a mean daily intake of 0.64 L beer per capita in Belgium (consumer population) and the mean levels found. Considering a mean daily intake of 0.122 L beer per capita by the entire population, beer consumers' intake was 5.88 % of the TDI of OTA for organically produced beer and 0.8 % of the TDI of OTA for conventionally produced beer.

Miraglia *et al.* (2002) reported in the SCOOP study that beer contributes in Europe for 5-7 % to the total dietary OTA intake (Figure 1.5) [4].

Concerning DON, based on the results of Schothorst *et al.* (2003), the contribution from beers to the total DON intake is negligible, at least for the period covered by the survey. However, all the beers were probably prepared from cereals harvested in 2000, a year with a relatively low incidence of DON contamination. In years with high incidences of DON contaminated cereals, the situation could be totally different [203].

Papadopoulou *et al.* (2004) reported that in many European countries, the per-capita consumption was above 100 L in 2000 with a maximum of 159 L in the case of the Czech Republic. Considering the TDI

of 1 μg/kg body weight, a consumption of 159 L year^{-1}, beer with average contamination of 5.6 $\pm$ 2.6 ng mL^{-1} could contribute from 2.2 to 6 % to the TDI of DON of an average adult (60 kg) [204].

The study of Anselme *et al.* (2006) confirmed these findings; beer consumption contributes to 3 % of the TDI of 1 μg DON /kg body weight for an adult of 60 kg, considering a daily intake of 0.3 L beer per capita in Belgium and the mean DON levels found [147].

Harcz *et al.* (2007) [224] reported DON to contribute for 5 % of the TDI for organically produced beer and for 7 % of the TDI for conventionally produced beer for a person of 66 kg.

1.3.2.7 Legislation

From all the observations in the SCOOP study on OTA (2002) [4], the exposure seems to be in most cases quite below the value indicated by SCF. Nevertheless, some countries seem to be suffering from a more relevant contamination especially if specific groups of consumers are considered. Therefore, limits have to be set for mycotoxins in foodstuffs.

Several factors, both of a scientific and socio-economic nature, may influence the establishment of mycotoxin limits and regulations. These include: availability of toxicological data, availability of data on the occurrence of mycotoxins in various commodities and on the intake of these foodstuffs, knowledge of the distribution of mycotoxin concentrations within a lot, availability of analytical methods, legislation in other countries with which trade contacts exist and need for sufficient food supply [129].

The first two factors provide the necessary information for hazard assessment and exposure assessment respectively, the main ingredients for risk assessment. Risk assessment is the primary scientific basis for the establishment of regulations.

In Table 1.1, the maximum levels for OTA, DON and AFB_1 in the above mentioned foodstuffs (according to Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs) are summarized [1].

Table 1.1: Maximum levels for OTA, DON and AFB_1 [1]

Foodstuffs	**Maximum levels (μg kg^{-1})**		
Aflatoxins	**B_1**	**Sum of B_1, B_2, G_1 and G_2**	**M_1**
Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	5.0	10.0	-
Ochratoxin A			
Roasted coffee beans and ground roasted coffee, excluding soluble coffee		5.0	
Soluble coffee (instant coffee)		10.0	
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine		2.0[a]	
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices[b] and liquorice		-	
Deoxynivalenol			
Beer not listed			

[a] The maximum level applies to products produced from the 2005 harvest onwards, [b] For paprika powder a maximum level of 15 μg OTA kg^{-1} is proposed from the beginning of 2009

Chapter 2

Immunochemical methods for mycotoxin detection

2.1 Abstract

The general protocol for the development of immunochemical methods on mycotoxins includes four areas: (a) preparation of the immunogen: this includes the formation of a toxin derivative (if necessary) and conjugation to a protein; (b) production of antibodies: this includes immunisation, collection of the immune serum and purification of antibodies; (c) characterization of antibodies by determination of antibody titre and specificity; (d) development and application of immunochemical techniques [225]. Immunogens and toxin (derivative)-enzyme conjugates used during the PhD research were supplied by external firms. Monoclonal antibodies against OTA, DON and AFB_1 were also purchased. But in parallel, a first attempt to produce own anti-OTA monoclonal antibodies was made (chapter 9).

In this chapter, general information will be given on antibodies, immunochemical methods and rapid tests.

2.2 Antibodies

An antibody (Figure 2.1) is a bifunctional molecule that binds an antigen at antigen-combining sites and serves as a linker of the specific antigen to immune system cells. The basic structure of an antibody reflects this function in that there are (i) a highly variable domain that may interact with a vast diversity of antigens and (ii) a relatively constant domain that interacts with cells and effector systems of the body. This dual function is reflected in the organization of antibody genes: separate exons (gene segments) encode the variable and constant domains. Certain generalizations can be made about antibodies. (i) They are produced in response to antigenic stimulation. (ii) There are five classes (isotypes) of immunoglobulins (IgG, IgM, IgA, IgD, IgE). The immunoglobulin G (IgG) class is divided into four subgroups and IgA and IgM are divided into two subgroups. All known antibody molecules have either kappa (κ) or lambda (λ) light chains. (iii) Antibodies are heterogeneous in structure, in affinity with corresponding antigenic sites and in function *in vivo* and *in vitro*. (iv) All antibodies have the capacity to bind with their respective antigens. Antibodies may be classified according to their origin, their host specificity or the characteristics of the immunologic reactions in which they are involved. Most antibodies are found free and circulating in plasma, but some specific immunoglobulins, such as IgE, occur as cell-associated

or cytophilic antibodies [226].

2.2.1 Polyclonal antibodies

Animals can produce several different types of immunoglobulins which bind, at a specific site (epitope), the antigen or hapten. These immunoglobulins are synthesized by several cell types of lymphocytes. Such immune serum contains polyclonal antibodies which generally are not monospecific for the given antigen/hapten [225]. Polyclonal antisera have certain disadvantages including lack of uniform characteristics from batch to batch. Even when different batches are drawn from the same animal, there is a wide variation in class, affinity, specificity and reactivity among antibodies within an antiserum [226]. The source of these antibodies stops with the death of the immunised animals [225]. It should be noted, however, that this mixture of specificities that fingerprints and identifies the target antigen may be advantageous in certain applications [226].

2.2.2 Monoclonal antibodies

The aforementioned limitations of polyclonal antibodies justify attempts to produce monoclonal antibodies. A monoclonal antibody is defined as a uniform homogeneous antibody directed at a single epitope or antigenic determinant and produced continuously from one cell clone. Monoclonal antibodies have identical physical, biochemical and immunological properties [226].

Monoclonal antibodies offer at least four advantages [226, 227, 228]: (i) they are derived from one isolated clone and constitute a well-defined reagent, (ii) the production process is capable of yielding unlimited quantities of the same homogeneous reagent, (iii) they may be prepared with non-purified antigens and (iv) their affinity and specificity are defined.

2.3 Immunoassay

Immunochemical methods are based on the ability of antibodies to specifically bind different substances. The reversible association between antibodies and their corresponding antigens is called the immunological reaction. The binding forces involve hydrogen bonds and hydrophobic binding as well as weak molecular interactions like

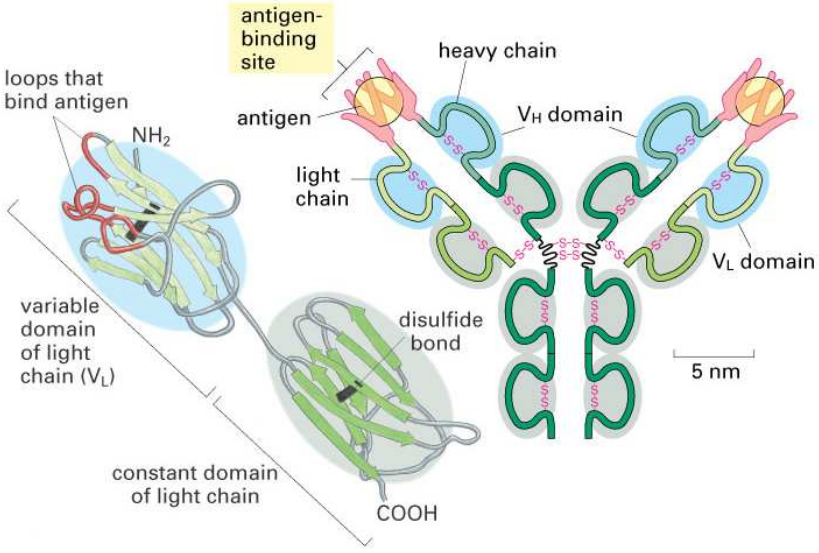

Figure 2.1: Structure of an antibody [5]

Coulomb and Van der Waals forces [225]. The main advantage of immunochemical analysis is the possibility to vary assay specificity using different antibodies [229].

Antigens are characterized by their immunogenicity as well as their antigenicity which resides in their ability to initiate the production of antibodies in animals and subsequently interact or bind with them. Small peptides and small non-peptidic molecules such as mycotoxins are not immunogenic and are called haptens. Once a hapten is conjugated to a protein, it becomes immunogenic.

Sensitive immunological methods were developed involving labeling techniques to measure the complex formation when the analyte is present at concentrations less than μg - mg mL^{-1}. Generally, the terms 'immunochemical methods' or 'immunoassays' are used to refer to such methods. Among different labeling techniques used in immunoassay, the best known are radioimmunoassay (RIA) and enzyme immunoassay (EIA) such as enzyme-linked immunosorbent assay (ELISA). Immunoadsorption methods are based on temporary immobilization of immunocomplexes on a solid support, immunoaffinity chromatography (IAC) is a means of retaining a given antigen from a complex matrix by using the anti-antigen antibodies [225].

2.3.1 Kinetics of antigen-antibody reactions

The kinetics of a reversible antigen-antibody reaction may be schematically represented as follows:

$$Ag + Ab \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AgAb$$

where Ag represents free antigen, Ab represents free antibody sites, AgAb is the antigen-antibody complex and k_1 and k_2 are the association and dissociation rate constants, respectively. The rate of formation of an antigen-antibody complex and the law of mass action is as follows:

$$\frac{d(AgAb)}{dt} = k_1(Ag)(Ab) - k_2(AgAb)$$

At equilibrum, the net rate is zero; therefore,

$$\frac{k_1}{k_2} = \frac{(AgAb)}{(Ag)(Ab)} = K$$

In these equations, Ag represents one of the often multiple antigenic sites on a given antigen molecule and Ab represents one or two or more antigen-binding sites on a given antibody molecule. As with a chemical reaction, there is an association and dissociation constant and the summed effect of the two yields an equilibrum constant, K. Antibody populations with high avidity are those with high K values and antibody populations with relatively low avidity have low K values [226].

2.3.2 Heterogeneous immunoassay

All of the heterogeneous immunoassays have at least one separation step to distinguish reacted from unreacted material. In the heterogeneous system, the antigen-antibody reaction does not affect the activity of the enzyme label.

2.3.2.1 Direct competitive immunoassay

A direct competitive immunoassay, with the antibody immobilized on a solid phase, consists of the following steps: the specific antibody is physically adsorbed or covalently attached to a solid-phase matrix. The unattached antibody is washed away and the labeled antigen is incubated in the presence of standard or sample antigen to be assayed. The sample is washed after the antigen and antibody have reacted and the solid-phase units containing the labeled antigen-antibody complex are detected. The signal is inversely proportional to the concentrations of standard or test antigen added [230]. If the antigen and the labeled antigen are added in a sequential way to the solid-phase linked antibody system, then this is called a sequential competitive immunoassay. In Figure 2.2, a signal is presented as a blue colour. The secondary antibody technique uses a secondary antibody which is attached to the solid phase and which is capable of capturing the primary anti-antigen antibody. After a wash step the method is further following the same principle.

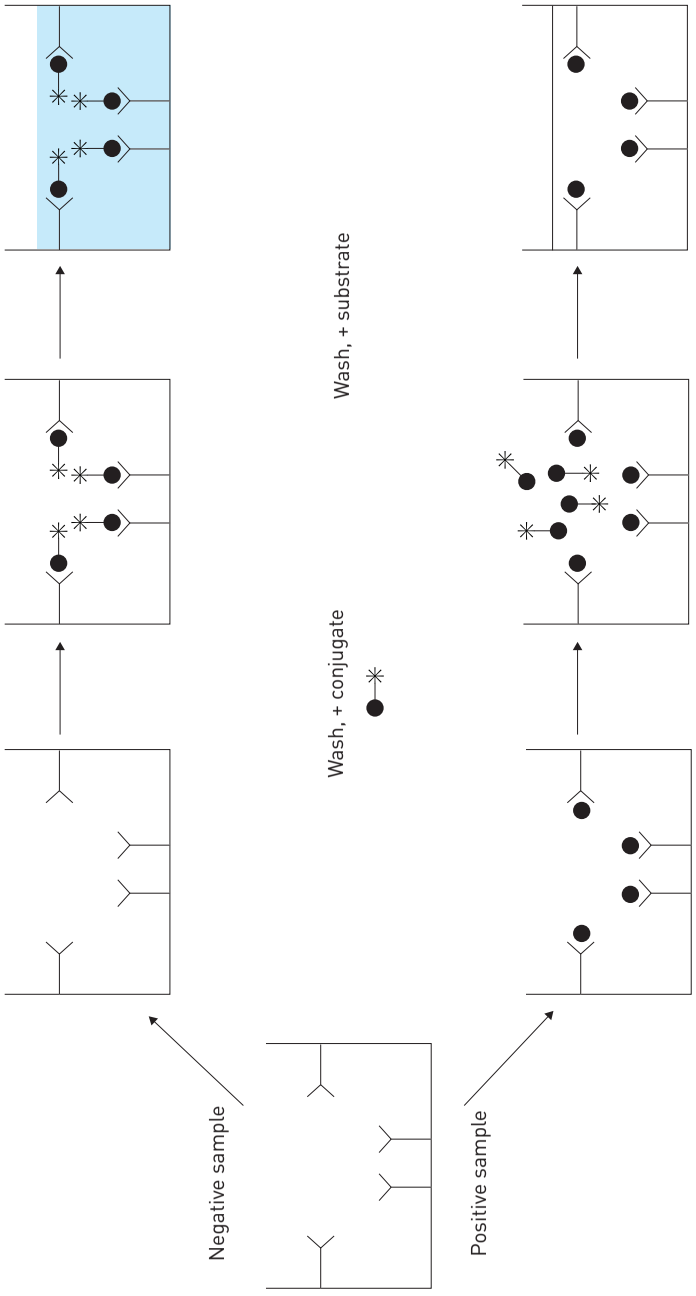

Figure 2.2: Direct immunoassay principle

2.3.2.2 Indirect competitive immunoassay

For indirect competitive immunoassays, the antigen is coated on the solid phase. The sample is added together with a constant and limited amount of labeled antibodies specific for the antigen (Figure 2.3). The signal (presented as a blue colour) is inversely proportional to the concentration of the standard or the antigen in the sample. The two-step method uses a labeled secondary antibody for detection. This was first described by Weller and Coons in 1954 and is still a popular method. The amount of bound analyte-specific antibodies is quantified by addition of a labeled antibody specific for the bound antibody. This labeled antibody is referred to as the detector antibody [231] (Figure 2.4). For ELISA it is important that the antibody enzyme conjugate is of high specific activity. This is achieved when the antibody is affinity purified and the enzyme conjugation chemistry preserves antibody specificity as well as enzyme activity [232].

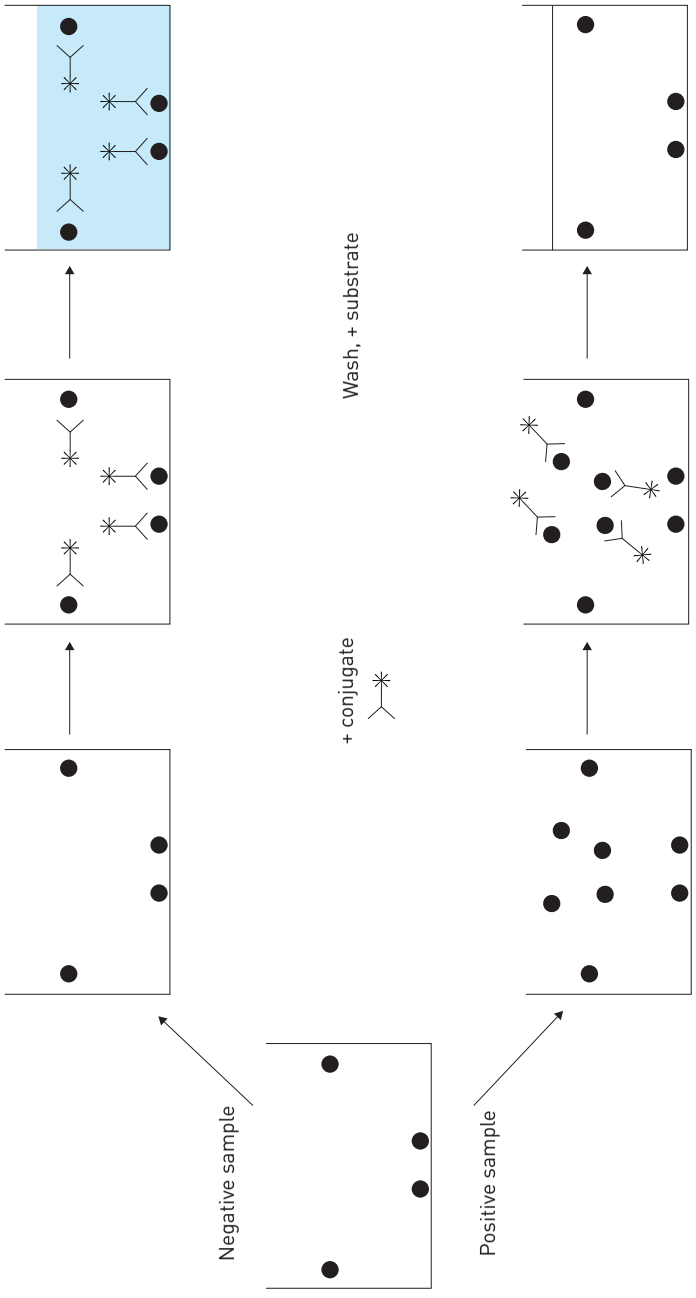

Figure 2.3: Indirect immunoassay principle, one-step method

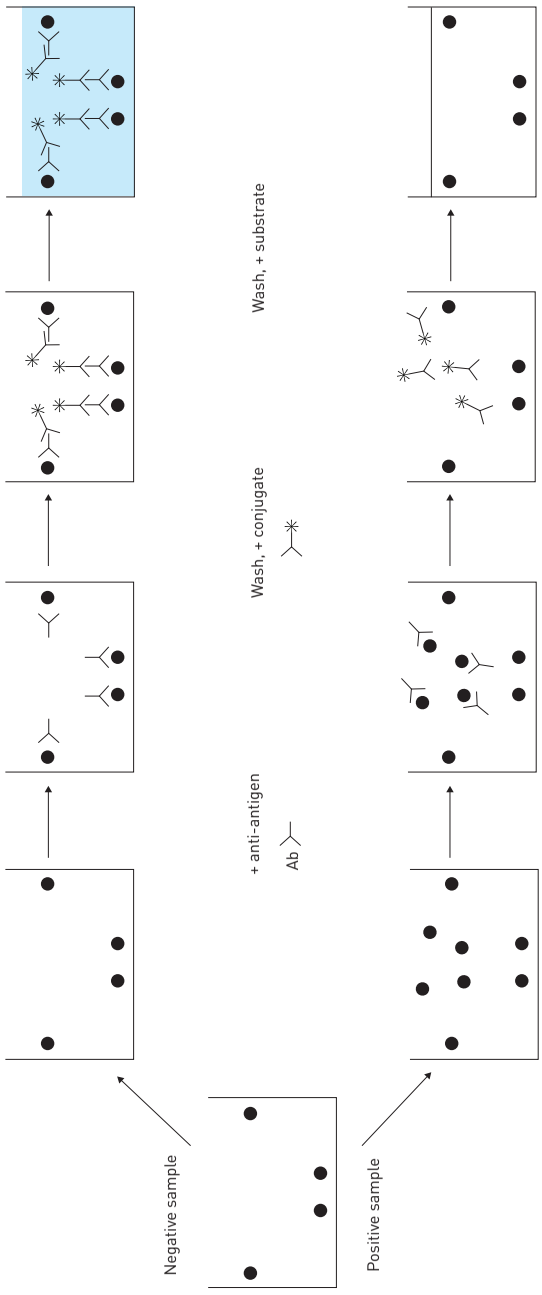

Figure 2.4: Indirect immunoassay principle, two-step method

2.3.2.3 Sandwich immunoassay

Many proteins have multiple epitopes, sufficiently well spatially separated, which allow two antibodies to bind at the same time. This forms the principle of the two-site immunometric assay or sandwich immunoassay. Binding of the two antibodies may occur sequentially or simultaneously. Sequentially, the sample is first incubated with the bound antibody, which reacts with the first epitope on the protein. The solid-phase is then washed to remove unreacted components and further incubated with the labeled detector antibody, which binds to the antibody-antigen complex. Unreacted excess of detector antibody is then removed by washing and the signal level emanating from the solid-phase is determined [233] (Figure 2.5). The signal (presented as a blue colour) is proportional to the concentration of the standard or the antigen in the sample. Similar as in Figure 2.4, the use of a labeled secondary antibody is also possible. Then, the amount of bound analyte-specific antibodies is quantified by addition of a labeled antibody specific for the bound antibody. As mycotoxins are haptens, low molecular toxins, this sandwich immunoassay is not applicable for these small molecules unless recombinant antibodies are used [234].

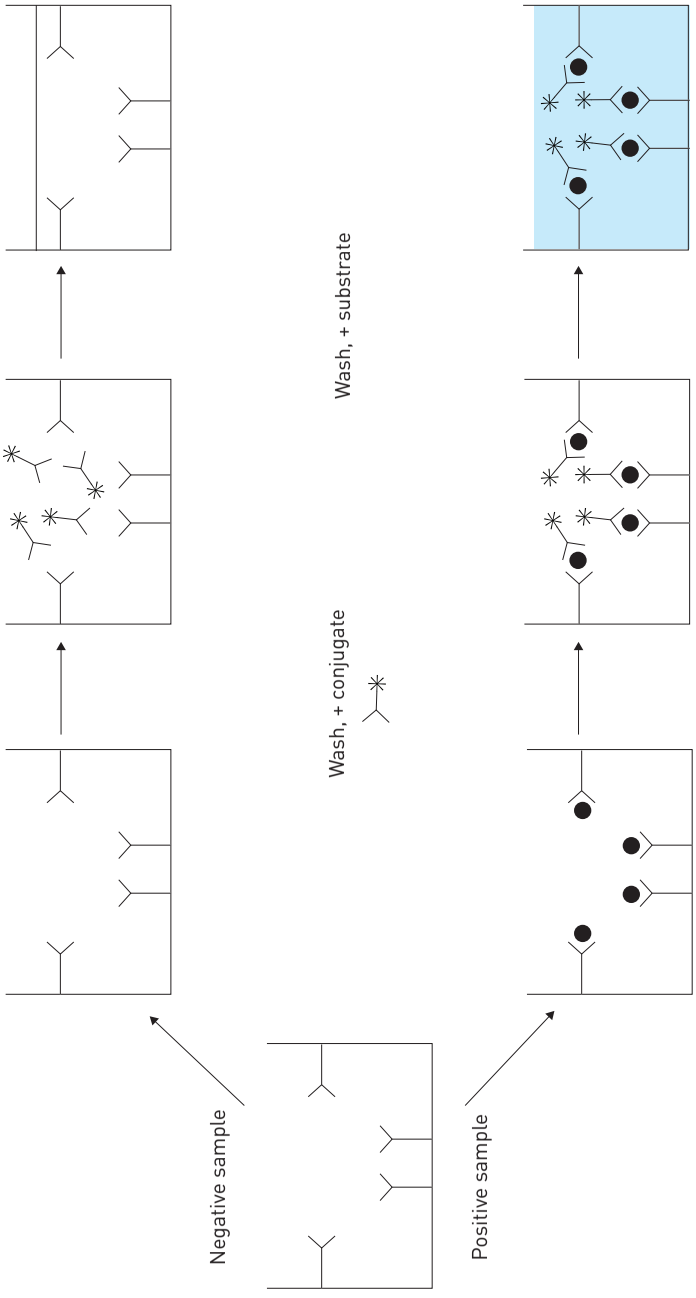

Figure 2.5: Sandwich immunoassay principle

2.3.2.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassays (EIA) can be defined as immunological procedures in which the antigen-antibody reaction is monitored by enzyme reactions. ELISA is a heterogeneous EIA.

ELISA can be formated in various configurations in which one of the reactants is immobilized onto a solid-phase matrix. In this method, the enzymatic activity in the bound or free fraction is quantitated by the enzyme-catalyzed conversion of a relatively non-chromatic or non-fluorescent substrate to a highly chromatic or fluorescent product [230].

2.3.3 Homogeneous immunoassay

A very promising way for the simplification of immunoassays for routine applications is a shift from heterogeneous methods (with separation) to homogeneous assays (without separation) [51]. The term 'homogeneous' may be applied to any system in which both the immunological reaction itself and the detection are carried out in a homogeneous solution. No washing step for separation of bound and free labels is required. On the other hand, the advantages of the separation steps include both the reduction of background signal noise and the elimination of interfering substances [235]. Enzyme-Multiplied immunoassay technique (EMIT) is a homogeneous immunoassay where the antigen-antibody interaction modulates the activity of the enzyme, thus eliminating the need for a separation step. Fluorescence polarization immunoassay (FPIA) (Chapter 8) is a widely used homogeneous technique, which meets the requirements of a simple, reliable, fast and cost-effective analysis. However, homogeneous immunoassay techniques like FPIA are generally known to be less sensitive than heterogeneous ones [51].

2.4 Rapid immunochemical tests

The old proverb 'time is money' is very appropriate to the use of rapid methods [236]. Rapid tests or screening methods are defined as 'methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples

for potential non-compliant results. They are specifically designed to avoid false compliant results' [237].

The conventional analytical methods (thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC)) applied to food or feed samples yield results within hours or days. Competition within the food and feed industry forces them to reduce cost, employ cheaper labour and deliver goods more rapidly. Additionally, increased analytical complexity in the food industry requires a rapid report for each individual contaminant. Thus, rapid methods for mycotoxins have become increasingly important.

Rapid methods are less expensive, easier to use and can be moved to an *on-site* environment. They can help to determine the effectiveness of food safety measures, to determine legal compliance, to achieve logistical and operational goals, to keep commodities and products moving rapidly through marketing channels, to save time and thus costs, to save investments in complex instruments and to employ staff with lesser technical training [238]. They generally require small sample sizes [236]. Most rapid methods provide qualitative or semi-quantitative results and are recommended for use in screening samples [238]. If necessary, fully quantitative analysis can be carried out on any samples that appear to be contaminated at levels close to the agreed limit [239].

As TDIs are established and legislative maximum levels (MLs) set for various mycotoxins, it is evident that rapid methods must be able to operate at high sensitivities. Antibody based techniques lend themselves to operating under the criteria of rapid methods as well as the low limits of detection. Processed food presents a significant challenge for antibody-based methods. The antibody based methods are more suitable for pre- and post-harvest analysis, at which time rapid/robust testing is required as a means to decide on the future use of a certain lot.

In the laboratory, microtiter plate ELISA still offers significant benefits in terms of speed, sensitivity and quantification [221].

In non-laboratory environments, e.g. in the field or at truck unloading stations, dipstick and lateral flow technologies which give a rapid visual result are more appropriate. However, the price of simplification and acceleration demanded by these techniques is loss of sensitivity [221].

It is important that methods to check compliance against the maximum tolerable levels for mycotoxins are appropriately validated, par-

ticularly to ensure that false negatives or positives do not occur [239]. Certified reference materials (CRM) play a key role in the validation [221].

2.4.1 Dipstick enzyme immunoassay

Dipsticks were probably the earliest potential ex-laboratory tests involving enzyme immunoassay. The dipsticks were made of plastic or a higher capacity matrix such as nitrocellulose or CNBr-activated paper bonded to a plastic stick. They were limited by the capacity of the plastic strip or by the diffusion of the samples and reagents through the matrix [240]. The dipstick, precoated with secondary antibodies, is immersed consecutively in solutions of primary antibodies, the sample, an analyte-enzyme conjugate and an enzyme substrate solution, in this way performing a direct competitive enzyme immunoassay using a secondary antibody [241].

2.4.2 Flow-through enzyme immunoassay

Valkirs and Barton (1985) have established this rapid technique in clinical chemistry [242] in which a monoclonal antibody coated membrane, incorporated into a cylindrical, disposable device, regulates sample and reagent delivery. In 1999, De Saeger and Van Peteghem [243, 244] described a flow-through enzyme immunoassay carried out in a device format consisting of a plastic bottom and top member. In the device, cotton wool was acting as an adsorbent which actively drew liquid reagents through the membrane. The membrane with coated rabbit anti-mouse antibodies and anti-horse radish peroxidase (HRP) antibodies (control) was held above the cotton wool, directed at the center of the top member for reagent access. The principle of the assay (direct competitive enzyme immunoassay using a secondary antibody) is schematically presented in Figure 2.6: a negative sample gives two coloured spots, a positive sample only one (the control spot). The flow-through assay is rapid, easy-to-use and is suitable for testing mycotoxins in the field. The method does not require any equipment.

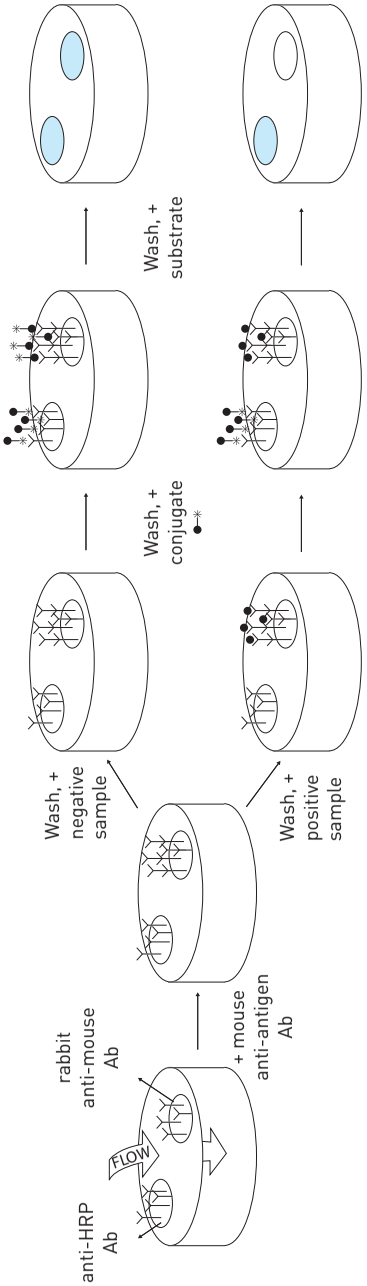

Figure 2.6: Flow-through immunoassay principle

2.4.3 Lateral flow immunoassay

There are many commercially available *in vitro* diagnostic test kits utilizing the principles of immunochromatography. The first major target analyte for this test format was (human) chorionic gonadotropin (HCG) for the detection of pregnancy.

The technology has been used for many years. However, its application in food analysis, especially mycotoxin testing, is quite recent [238]. A typical immunochromatography test strip is composed of a sample pad, a conjugate pad, a membrane, an absorbent pad and an adhesive backing.

The detector reagent, typically an antibody coupled to latex or a colloidal particle (carbon, gold), is deposited (but remains unbound) into the conjugate pad. When the sample is added to the conjugate pad, the detector reagent is solubilized and begins to move with the sample flow front up the membrane strip. Analyte present in the sample is bound by the antibody of the detector reagent. As the mixture passes over the zone to which the capture reagent (= antigen-protein conjugate) has been immobilized, the free antibody of the detector reagent is trapped (indirect competitive immunoassay). The colour of this test line is inversely proportional to the amount of analyte present in the sample (Figure 2.7). The strip may also contain a control line (secondary antibodies which bind the antibodies of the detector reagent) to indicate completion of the reaction. In this example, a negative sample gives two coloured lines while a positive sample gives only one (Figure 2.7). Movement of the sample by capillary action is maintained by the adsorbent pad at the far end of the strip [245].

2.4.4 Clean-up tandem immunoassay column

To overcome the problem of coloured membranes for strongly coloured food matrices, in this way interfering with the visual detection, a new format for a rapid test has been introduced. The clean-up tandem assay column comprises two superposed layers: a clean-up layer, capable of adsorbing at least part of the interfering fraction, and a detection layer where the direct immunoassay is performed. The clean-up tandem immunoassay column [9] has been applied in this work for the development of rapid tests for the detection of OTA in roasted coffee, OTA in cocoa powder, OTA in spices and the simultaneous detection of OTA and aflatoxin B_1 (AFB_1) in spices. The principle (direct competitive enzyme immunoassay using a secondary antibody) is explained in

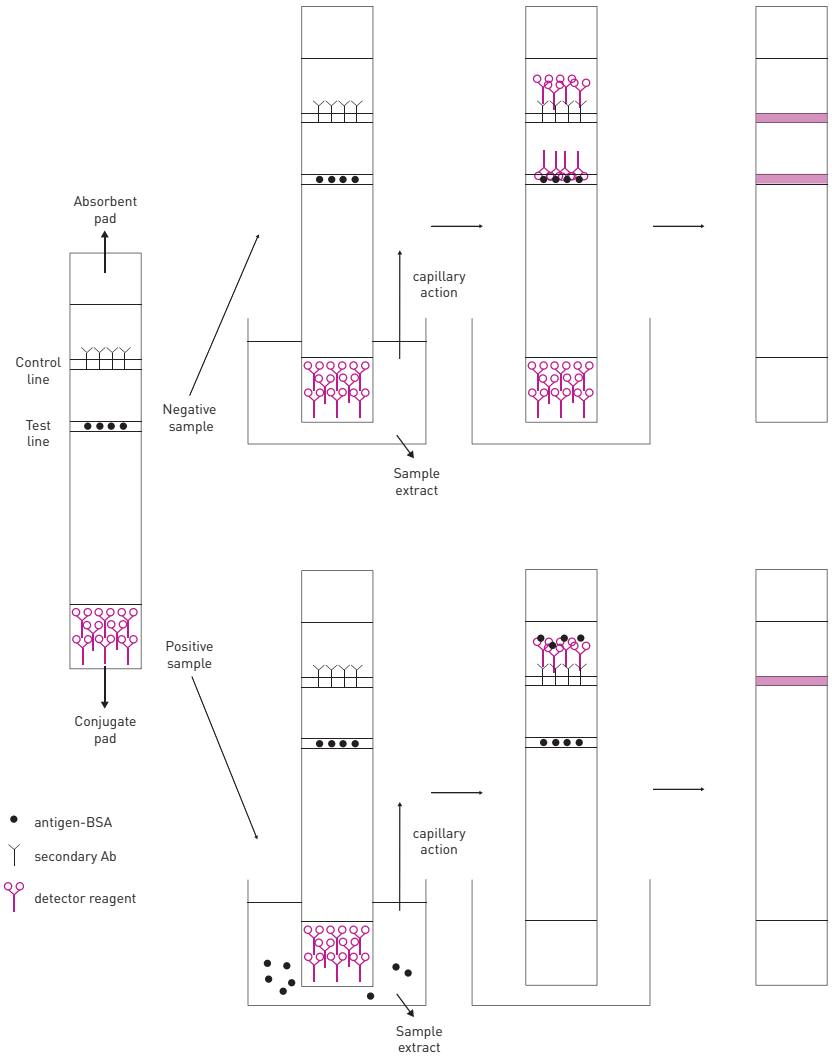

Figure 2.7: Lateral flow immunoassay principle

Figure 2.8: a negative sample gives a blue coloured detection layer, for a positive sample, the detection layer remains colourless. The set-up is explained in the respective chapters.

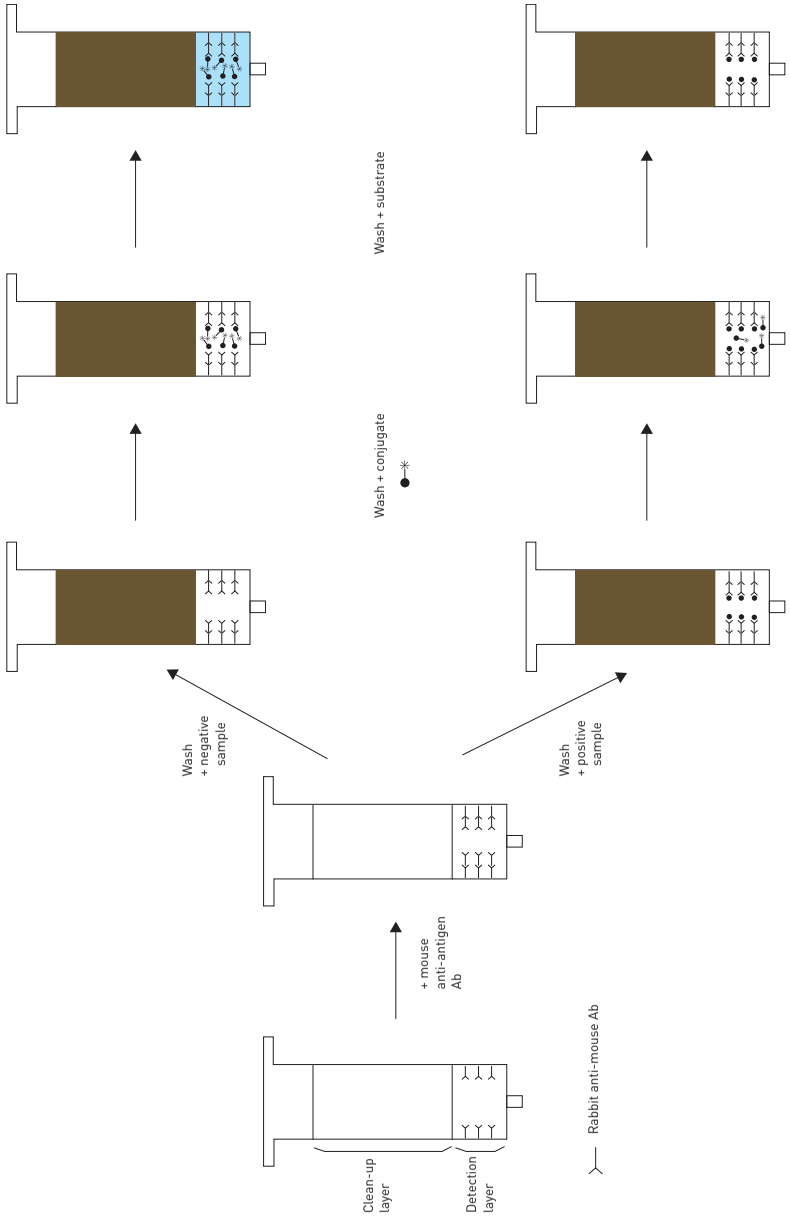

Figure 2.8: Clean-up tandem immunoassay principle

Chapter 3

Objectives of the thesis

Mycotoxin contamination of food and feed continues to be a problem, despite forces to implement good agricultural and manufacturing practices. Legislation for mycotoxins is growing but food safety can not wait until the harmonisation of European regulations on mycotoxins. Food safety requires the availability of analytical methods and evaluation of food products' contamination by mycotoxins.

In particular, rapid tests are becoming increasingly important because of the growing amount of analyses. For several foodstuffs, non-instrumental rapid membrane-based tests have been developed in our laboratory, such as flow-through enzyme immunoassays and lateral flow immunoassys. However, for strongly coloured foodstuffs, the membrane of these tests adsorb the colour and there is interference with the visual detection. A clean-up step has to precede the assay. Therefore, a new format, the *clean-up tandem assay column* was developed, in which clean-up and detection can be performed in one step.

The main objective of the present thesis was to develop rapid tests for the detection of mycotoxins in strongly coloured foodstuffs, applying the *clean-up tandem assay column*. Strongly coloured matrices, with problems of mycotoxin contamination (roasted coffee, cocoa, spices, red wine, beer), were selected and the immunoassay was developed for the mycotoxin(s) of concern in each of these.

Chapter 4

Development of a new clean-up tandem assay column for the detection of ochratoxin A in roasted coffee

4.1 Background and objectives

In 2002, Sibanda et al. [246] developed a flow-through method for the detection of ochratoxin A (OTA) in green coffee. When applying this membrane test for roasted coffee, problems of colour adsorption on the membrane and false results were observed. Therefore, a clean-up step had to precede the flow-through immunoassay. Several solid phase materials were tried out to serve as a suitable clean-up layer for roasted coffee. Aminopropyl derived silica seemed to be the most appropriate to adsorb matrix interferences while allowing OTA to directly elute. To avoid this extra clean-up step, a new format, the *clean-up tandem assay column* was developed. In this format, clean-up and detection are performed in one step.

In this work, primary monoclonal anti-ochratoxin A antibodies were bound to the detection layer gel. No binding was observed. Higher quantities of antibodies were needed. For primary monoclonal antibodies this was impossible in terms of cost-effectiveness and therefore, secondary polyclonal rabbit anti-mouse antibodies were covalently bound to the gel. The preliminary tests were performed without clean-up layer and with buffer standard solutions. Once blue colour development of the detection layer was observed for blank buffer solution, and also colour suppression for the fortified ones, roasted coffee extracts were applied. By adapting antibody and conjugate dilutions, sample volume and dilution of the extract, the test was developed and validated for a cut-off level of 6 μg kg^{-1}. The validation was based on Trullols et al. (2004) [2] and included determining the sensitivity in terms of false positive and false negative rates, the specificity, positive predictive value and negative predictive value.

The content of this chapter is reproduced from the paper published in *Analytica Chimica Acta*, 538:57-61, 2005, written by M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetró, and C. Van Peteghem.

4.2 Abstract

As the mycotoxin ochratoxin A (OTA) can occur in roasted coffee, regulations and maximum limits are necessary. The need for simple, rapid and inexpensive detection methods which require a minimum of equipment led to the development of a clean-up tandem assay column for the detection of OTA in roasted coffee. In this study the column comprised two superposed layers: a layer capable of adsorbing at least part of the interfering fraction of the sample and a detection layer containing antibodies that were able to capture the analyte. No expensive instruments were needed as results were visually evaluated. The cut-off level, i.e. the smallest mycotoxin concentration resulting in no colour development, was 6 μg kg^{-1}. Assay validation was performed using samples fortified with OTA. The method gave a low percentage of false negative results and no false positive results. Assay performance was evaluated by screening naturally contaminated samples using the clean-up tandem assay column. The described method offers a rapid, simple and cost-effective screening tool, contributing to more effective quality control procedures.

Keywords: ochratoxin A, roasted coffee, immunoassay, field test, column test

4.3 Introduction

Mycotoxins are secondary metabolites of low molecular weight, produced by certain types of moulds during their growth on food and feed. These are actually derived from other chemicals such as polypeptides, amino acids, phenols or terpenoids, which the fungi use in their normal metabolism. There are hundreds of known mycotoxins produced by moulds and additional toxins are discovered each year.

Ochratoxin A (OTA) (Figure 4.1) or N- [[(3R)-5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl] carbonyl] -L-phenylalanine belongs to the isocumarins having an amide linkage to L-phenylalanine [23]. OTA is produced by *Penicillium* and *Aspergillus* species.

It was first isolated from *Aspergillus ochraceus K. Wilh.* in 1965 by African scientists during laboratory screening for toxigenic fungi. OTA was found for the first time to occur naturally in a US maize sample in 1969. It seems that cereals and cereal products are the main contributors to OTA intake in northern climates e.g. in Europe. OTA also

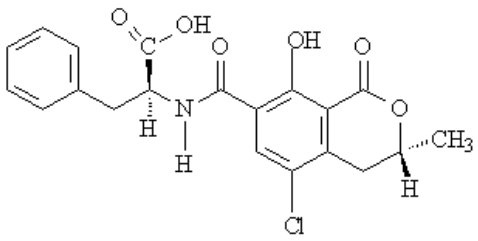

Figure 4.1: Chemical structure of ochratoxin A

occurs in a variety of other plant products, such as coffee beans, cocoa beans and dried fruit, all over the world. In addition, roasted coffee, beer, pig meat, blood products, wine, spices, grape juice and pulses are contributors to the intake of OTA [23].

OTA has nephrotoxic, teratogenic, immunotoxic and possibly also neurotoxic properties. It has been associated with nephropathy in humans and has a long half-life. Based on the available toxicological data, the International Agency for Research on Cancer (IARC) has classified OTA as a potential carcinogen for humans (class 2B carcinogens).

Once OTA has been formed it survives most food processing stages such as cooking, roasting and fermenting. Even temperatures as high as 250 °C are not sufficient for complete degradation of OTA. Regarding the stability of OTA, roasting of coffee gave the most variable results. Losses were reported in the range of 0-100 % [23, 156]. This may be due to several factors such as roasting conditions, non-homogenicity of natural coffee bean contamination, OTA levels, natural contamination versus spiking and analytical method performance. Since 'prevention is better than cure' to protect the consumer from the toxic effect of mycotoxins, the need for encouraging preventive actions such as good agricultural practice, good storage conditions, use of improved sorting procedures has to be stressed. But with current scientific and technical knowledge and despite improvements in production and storage techniques, it is not possible to prevent the development of these moulds altogether. Consequently OTA cannot be eliminated from food entirely. Limits should, therefore, be set. Commission Regulation (EC) No. 472/2002 of 12 March 2002 does not mention a maximum level for OTA in roasted coffee, although there are limits for raw cereal grains (5 μg kg^{-1}), cereal products (3 μg kg^{-1}) and dried vine fruit

(10 μg kg^{-1}). The Commission is discussing maximum limits for OTA in green and roasted coffee and coffee products, wine, beer, grape juice, cocoa and cocoa products and spices taking into account the investigations undertaken and the preventative measures applied to reduce the presence of OTA in these products [247]. It is obvious that the enforcement of these regulations requires accurate monitoring of commodities. Validated methods based on high performance liquid chromatography with fluorescence detection (HPLC-FLD) have been developed for the analysis of OTA in different food matrices. However, these cannot be applied effectively along the food processing chain, therefore, more rapid, cheap and reliable methods must become available. In particular, there is a need for field portable assay systems that can be conducted and interpreted by users which are as close to the source of contamination as possible. Most of them are basically designed as visual tests that require only low-cost instrumentation and offer an advantage of speed. Enzyme-linked immunosorbent assay (ELISA) has become a popular and useful screening tool thanks to the availability of polyclonal and monoclonal antibodies against OTA. In our research group the focus has been on rapid immunochemical field tests, for example, the flow-through and dipstick tests have been developed [49, 241].

The aim of this study was to develop a new clean-up tandem assay column (patent application pending [9]) for use as a field test for OTA detection in roasted coffee. The column (tube) comprised two superposed layers: a layer capable of adsorbing at least part of the interfering fraction of the sample and a detection layer containing antibodies capable of capturing the analyte. There is no requirement for expensive instruments and results are visually evaluated.

4.4 Materials and methods

4.4.1 Reagents and materials

OTA standard, casein sodium salt (casein) and Tween 20 were purchased from Sigma Chemical Co. (Bornem, Belgium). Rabbit anti-mouse immunoglobulin (Ig) G (No. Z259, protein concentration: 2,7 g L^{-1}) was supplied by DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Bio-Sil NH_2 (diameter 0.040-0.063 mm, pore size 90 Å) was from Bio-Rad Laboratories (Nazareth-Eke, Belgium).

OTA-horse radish peroxidase (OTA-HRP) conjugate was prepared by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary. Monoclonal antibodies against OTA were produced and characterized by the same institute. The antibody was an IgG1 with kappa light chains with a 9.3 % cross-reaction with ochratoxin B but none at all with ochratoxin α, coumarin, 4-hydroxy-coumarin and D,L-phenylalanine [248]. Phosphate buffered saline (PBS) 0.01 M, pH 7.4, was used to prepare the wash solution (PBS-Tween 0.05 %) and the assay buffer (PBS-casein 0.1 %). Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antibacterial preservative. Methanol was high pressure liquid chromatography grade and water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogen solution used was TMB One Component HRP Microwell Substrate obtained from Tebu-bio Laboratories (Le Perray en Yvelines Cedex, France). Stock solutions of OTA (1 mg mL^{-1}) and working solutions (100, 10, 1 ng μL^{-1}) were prepared in methanol and stored at -20 °C. Ederol filters No. 15, 110 mm were purchased from Binzer & Munktell Filter GmbH (Battenberg, Germany). Tubes (Bond Elut® reservoir, 1 mL) and polyethylene frits (1/4 in. diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium).

4.4.2 Safety

OTA has nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic and carcinogenic properties. Gloves should be worn when working with standards and samples. Solutions containing OTA are collected in accordance with the waste policy of Ghent University. Glassware and OTA contaminated waste are decontaminated by keeping them for 24 h in sodium hypochlorite solution (household bleach). Afterwards, decontaminated glassware should be washed with detergent and rinsed with water.

4.4.3 Preparation of the clean-up tandem assay column

The gel of the detection layer was prepared following the principles and methods of Pharmacia LKB Biotechnology [249]. According to this procedure secondary rabbit anti-mouse antibodies were coupled to CNBr-activated Sepharose 4B. The hydroxyl groups on the sugar residues of this bead-formed agarose gel could be easily derivatised

for covalent attachment of a ligand. Immobilization of the ligands was done by the cyanogen bromide method as described below. The required amount of CNBr-activated Sepharose 4B (1 g freeze-dried material gives about 3.5 mL final gel volume) was weighed out, swollen and washed on a sintered glass filter using 1 mM HCl (200 mL g^{-1} gel). The secondary rabbit anti-mouse immunoglobulins (1.85 mL, 2.7 g L^{-1} ; 5 mg immunoglobulins) were added to 150 μL coupling buffer ($NaHCO_3$ buffer, 0.1 M, pH 8.3 containing NaCl 0.5 M) and this solution was mixed with 1 mL gel in an end-over-end mixer (Agitelec, J. Toulemonde and Cie, Paris, France) for 2 h at room temperature. The gel was washed with 5 mL coupling buffer to remove the excess of rabbit anti-mouse antibody. Then the remaining active groups were blocked by adding the coupled gel to a buffer with blocking agent (0.2 M glycine, pH 8.0) for 2 h at room temperature. The gel was washed with at least 3 cycles of alternating pH and at least five gel volumes of each buffer. Each cycle consisted of a wash with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl followed by a wash with coupling buffer pH 8.3 containing 0.5 M NaCl. The protein-agarose coupled gel was suspended in PBS (1/3, v/v) and stored at 4-8 °C. To prepare the gel for the detection layer the coupled gel/PBS-suspension was diluted five times with a suspension blocked gel/PBS (1/2, v/v) and PBS was further added to get a gel/PBS-suspension 1/3 (v/v). The blocked gel was prepared the same way as the coupled gel without the antibody coupling step, thus immediately blocking the gel with glycine. A polyethylene frit (grid) was first put in the 1 mL tube, followed by the addition of 200 μL of the detection layer gel/PBS-suspension. Another grid separated the detection layer and the clean-up layer (200 mg Bio-Sil NH_2) above. A third grid was placed above the clean-up layer (Figure 4.2).

4.4.4 Extraction of roasted coffee

The extraction methods described by [153, 250] were adapted. Fortified commercial roasted coffee samples (OTA concentrations of 0, 3, 4, 5, 6 and 7 μg kg^{-1}; 5 g) were extracted with a solution of 15 mL methanol/3% $NaHCO_3$ (80/20, v/v) by shaking at $\pm$ 200 rpm for 15 min with an Orbital Shaker SO3 (Stuart Scientific, UK). After filtration through an Ederol filter, 1.5 mL of the filtrate were diluted with 2.1 mL 3 % $NaHCO_3$ to reduce the methanol concentration, thus, avoiding the denaturation of the antibodies in the clean-up tandem assay column. This solution was completely used in the assay.

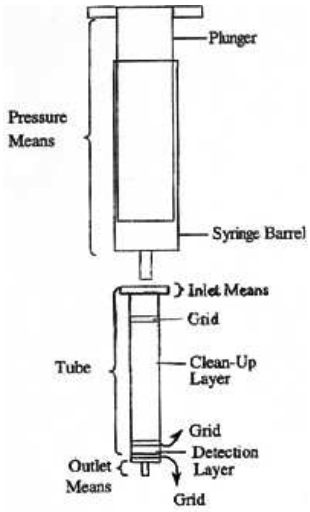

Figure 4.2: Clean-up tandem assay column set-up

4.4.5 Clean-up tandem assay procedure

The clean-up tandem assay set-up [9] is shown in Figure 4.2. In the clean-up tandem assay, each reagent was applied in sequence at the inlet or at the outlet means of the tube at a rate of $\pm$ 1 drop/2 seconds. First an aliquot of 100 μL primary mouse anti-OTA antibodies, diluted 1/75 in assay buffer, was applied at the outlet means, by drawing the solution into the syringe by pulling the plunger. In this way, the antibodies passed the detection layer first instead of the clean-up layer. This resulted in binding the monoclonal anti-OTA antibodies to the secondary antibodies immobilized on the gel. The monoclonal antibody solution was pressed out using the plunger and this was followed by a washing step with 3 mL PBS-Tween 0.05 % to remove unbound antibodies. Then the roasted coffee sample extract (3.6 mL) was applied onto the two layer adsorbent medium in which the clean-up layer was capable of actively adsorbing at least a part of the interfering fraction of said sample and the detection layer was capable of specifically retaining OTA. OTA, if present, was thus bound to the anti-OTA antibodies at the detection layer. After adding 6 mL wash solution at the inlet means of the column, 200 μL of an OTA-HRP conjugate dilution of 1/100 in assay buffer was drawn into the syringe through the outlet means by pulling the plunger (first contact with the detection layer instead of the

clean-up layer), followed by pressing it out of the tube. OTA-HRP conjugate was bound by free anti-OTA antibodies. A last washing step (3 mL) removed residual conjugate solution from the gel. Finally, 50 μL of colour substrate was aspirated through the outlet means. Owing to the competitive enzyme immunoassay test principle, the OTA from the sample and the OTA-HRP competed for the antibodies and colour intensity was inversely proportional to OTA concentrations in the sample. Colour was visually evaluated after five minutes. The smallest OTA concentration that resulted in no colour development was defined as the cut-off level. For samples contaminated with OTA equal or above this cut-off level, no blue colour appeared and they were considered to be positive. When a blue colour appeared, even substantially lighter coloured than the blank control sample, the sample was considered to be negative. Performing this clean-up tandem assay in series of maximum six samples took approximately 20 min, with a sample preparation of 15 min. This could be achieved with the use of a self-made device for six tubes.

4.5 Results and discussion

4.5.1 Clean-up tandem assay column set-up

The reason for binding secondary rabbit anti-mouse antibodies to the gel was that direct binding of the capture protein (the primary monoclonal anti-OTA antibody) could result in conformational changes that would reduce its affinity for the analyte [251, 252].

Sibanda *et al.* [246] compared various solid-phase materials for the clean-up layer. This phase had to remove the matrix interferences and the brown colour of the roasted coffee extract which could give false assay results. Only aminopropyl derived silica was capable of adsorbing the brown coffee colour and matrix interferences while at the same time allowing OTA to elute directly.

The reagents in the assay were applied in sequence at the inlet or at the outlet means of the tube at a rate of $\pm$ 1 drop/2 seconds. This rate was found to be optimal to allow enough OTA to pass the clean-up layer and reach the detection layer and at the same time allowing enough OTA to bind to the antibodies in the detection layer. An example of a serial of results is given in Figure 4.3. The intensity of the developed colour decreased with increasing concentrations of OTA. No blue colour developed at a concentration of 6 μg kg^{-1}, thus being the

cut-off level of the assay.

4.5.2 Optimization of the clean-up tandem assay method using fortified roasted coffee samples

An intralaboratory validation was performed determining the following performance characteristics: precision (in terms of false positive and false negative results), sensitivity, specificity and cut-off level.

For this qualitative screening test, precision was expressed as the number of false positive and false negative results as can be seen in Tables 4.1 and 4.2. The rates were determined at several concentrations including the cut-off level (0, 3-7 μg OTA kg^{-1}) [2].

Based on these results, epidemiological sensitivity (the ability to detect a true positive result) and epidemiological specificity (the ability to detect a true negative result) were respectively 92 and 100 %. The predictive value of positive results (fraction of positive results that are true positive) was 100 % and the predictive value of negative results (fraction of negative results that are true negative) was 97 %. These results are shown in Table 4.3.

According to AOAC [253], analyses become uninterpretable from lack of confidence in the presence or absence of the analyte when false positives and/or false negatives exceed about 10% of all values, unless all positive laboratory samples are reanalysed by a confirmatory method with a lower limit of determination than the method under study. As can be seen in Table 4.3, 10% was not exceeded.

Gel preparation was a source of error and this, therefore, compromised robustness. Preparing the gel in larger volumes would favour the repeatability. Reagent dilutions (anti-OTA and OTA-HRP) had to be reconfigured for every new gel in order for the assay to reach the 6 ppb cut-off level.

Given that European maximum limits for OTA in roasted coffee are under consideration, the cut-off level can be adjusted accordingly as these regulations become effective.

4.5.3 Screening of naturally contaminated roasted coffee samples

Coffee samples naturally contaminated with OTA at 0.8 μg kg^{-1}, 1.5 μg kg^{-1} and 5.4 μg kg^{-1} (HPLC-quantified and supplied by Nestlé) were analysed applying the above mentioned clean-up tandem assay column. No false results were obtained.

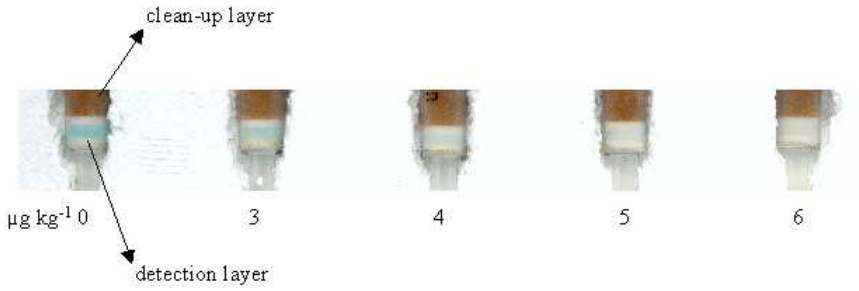

Figure 4.3: Clean-up tandem assay. A serial of results: the detection layer of each column is shown and the colour can be evaluated. Above the detection layer a little part of the clean-up layer (brown, the layer adsorbed the colour of the coffee) is visible. The OTA concentrations (μg kg^{-1}) of the samples roasted coffee are mentioned below the tubes.

Table 4.1: Contingency table for evaluating the performance parameters in qualitative analysis [2]

	Samples fortified at 6 μg kg^{-1} or more	**Samples fortified at less than 6 μg kg^{-1}**	**Total**
Positive (no colour)	tp[a]	fp[b]	tp + fp
Negative (blue colour)	fn[c]	tn[d]	fn + tn
Total	tp + fn	fp + tn	N[e]

[a] tp = true positive, [b] fp = false positive, [c] fn = false negative, [d] tn = true negative, [e] N = total amount of samples

Table 4.2: Contingency table with numerical values as performed with the clean-up tandem assay column

	Samples fortified at 6 μg kg^{-1} or more	**Samples fortified at less than 6 μg kg^{-1}**	**Total**
Positive (no colour)	11	0	11
Negative (blue colour)	1	30	31
Total	12	30	42

Table 4.3: Performance parameters.

False positive rate[a]	0 %
False negative rate[b]	8 %
Sensitivity rate[c]	92 %
Specificity rate[d]	100 %
Positive predictive value[e]	100 %
Negative predictive value[f]	97 %

[a]false positive rate = fp/tn+fp, [b]false negative rate = fn/tp+fn, [c]sensitivity rate = tp/tp+fn, [d]specificity rate = tn/tn+fp, [e]positive predictive value = tp/tp+fp, [f]negative predictive value = tn/tn+fn

4.6 Conclusion

The clean-up tandem assay column for the detection of OTA in roasted coffee uses simple equipment and limited operational steps. The result of the analysis can be binary only: presence/absence or yes/no response indicating whether OTA is present or not above the 6 μg kg^{-1} level. The assay allows a rapid method of screening samples whether negative or positive coupled with a simple and fast sample preparation step. It can be concluded that the described clean-up tandem assay can be used as a screening tool for OTA in roasted coffee to contribute to consumers' health protection.

4.7 Acknowledgements

This work was financially supported by Bijzonder Onderzoeksfonds Ghent University (011D02803). Special thanks to Dr. Alain Pittet from Nestlé Research Center, Lausanne, Switzerland, who supplied us with naturally contaminated samples.

Chapter 5

Application and validation of the clean-up tandem assay column for the screening of ochratoxin A in cocoa powder

5.1 Background and objectives

Cocoa powder gives problems when performing membrane tests for ochratoxin A detection due to colour interferences. Therefore, the *clean-up tandem assay column* was applied for this matrix. Several sorbents were tested to serve as a clean-up for cocoa powder. Aminopropyl derived silica was evaluated to be the most suitable. There is no legislation for the validation of screening methods for mycotoxins. Based on Trullols et al. (2004) [2], the validation of this cocoa powder test included determining the same characteristics as for roasted coffee. These were compared with the values obtained by constructing a performance characteristic curve [117] and good agreement was observed. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, applied for the determination of the blank cocoa powder sample and 10 commercially available cocoa powders, was not developed during the PhD thesis. A method, available for routine analysis of ochratoxin A (OTA) in the laboratory, was applied [254].

The content of this chapter is reproduced from the paper published in *Food Additives and Contaminants*, 24(4):398-405, 2007, written by M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetró, and C. Van Peteghem.

5.2 Abstract

A rapid antibody based assay for the detection of ochratoxin A in cocoa powder is described, involving sequential clean-up and visual detection of the toxin ('clean-up tandem assay column'). The screening test was developed to have a cut-off level of 2 μg kg^{-1} and was shown to have false positive and false negative rates of 10 and 2 %, respectively. Analysis of six samples can be carried out in the field in approximately 30 min by untrained workers. Using the proposed rapid screening test, ten retail cocoa powders were found to contain no detectable levels of ochratoxin A ($<$ 2 μg kg^{-1}). These samples were also found to be negative ($<$ 2 μg kg^{-1}) when analysed using an LC-MS/MS method.

Keywords: ochratoxin A, cocoa powder, immunoassay, qualitative test, validation

5.3 Introduction

Consumption of cocoa powder can contribute to the intake of the mycotoxin ochratoxin A (OTA). In cocoa beans OTA is most notably produced by *Aspergillus carbonarius* and *Penicillium verrucosum* at a minimum water activity of 0.85. OTA is mainly formed during the initial stages of sun drying, therefore, the drying period, rather than the storage period, is critical to avoid the appearance of OTA in cocoa beans [255].

There is no doubt that prevention of contamination at source is the best procedure for controlling contamination. However, despite improvements in production and storage techniques and current scientific and technical knowledge, it is not possible to entirely eliminate these moulds and, consequently, OTA contamination of food. Therefore, strict European Union limits are set for ochratoxin A [256] for a variety of food products, ranging from 2 to 10 μg kg^{-1}. For cocoa and chocolate, regulatory limits are still under discussion; the most likely limits will be in the range 1-2 μg kg^{-1} for raw cocoa materials and finished products [161].

Few conventional methods have been published for OTA determination in cocoa beans [161, 257, 258, 259] or cocoa-derived products [163, 260]. Clean-up of extract solutions, using immunoaffinity columns (IAC) combined with high performance liquid chromatography (HPLC), provides sensitive and selective results, but are laborious, time-consuming, require sophisticated equipment and can not be used

in the field.

Most commercial test kits for OTA detection use ELISA methods [261]. Other rapid assays described in the literature include array biosensors, fluorescence polarization immunoassays, screen printed electrode immunoassays, surface plasmon resonance biosensors and quartz crystal microbalance biosensors [45]. Very few rapid field-assays which can be used without special laboratory equipment are available (lateral flow devices, membrane-based flow-through portable immunoassays and test cards) [46, 238, 244, 262, 263]. However, the application of visual detection methods is difficult for intensively coloured products such as cocoa powder. As there are no commercial field tests for this food matrix, we developed a laboratory-made clean-up tandem assay column (patent application pending [9]), for the rapid detection of OTA in cocoa powder. In this column, clean-up and detection are performed in one step enabling visual evaluation.

5.4 Materials and methods

5.4.1 Reagents and materials

OTA standard, casein sodium salt (casein), Tween 20 and tetramethoxysilane were purchased from Sigma (Bornem, Belgium). Rabbit anti-mouse immunoglobulins (IgG) (protein concentration: 2,7 g L^{-1}) were supplied by DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased from Amersham Biosciences AB (Uppsala, Sweden). NH_2 derived silica (Bio-Sil NH_2, diameter 0.040 mm), Bond Elut® SAX-columns, Bond Elut® Si-columns, tubes (Bond Elut® reservoir, 1 mL) and polyethylene frits (1/4 inch diameter) were supplied by Varian Belgium (Sint-Katelijne-Waver, Belgium). Bakerbond™SPE C_{18} columns were purchased from JT Baker (Deventer, Holland). OchraTest™ immunoaffinity columns were supplied by Vicam (Watertown, MA, USA).

OTA-horse radish peroxidase (HRP) conjugate was prepared by the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary. Monoclonal antibodies against OTA were produced and characterized by the same Institute. The antibody was an IgG1 of kappa light chains with a 9.3 % cross-reaction with ochratoxin B, but none at all with ochratoxin α, coumarin, 4-hydroxycoumarin and D,L-phenylalanine [248]. Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was used to prepare the wash solution (PBS-Tween 0.05%) and

the assay buffer (PBS-casein 0.1%). Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Methanol was HPLC-grade and water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogen solution used was Colorburst^{TM}Blue TMB/Peroxide (ALerCHEK, Portland, ME, USA). Stock solutions of OTA (1 mg mL^{-1}) and working solutions (100, 10 and 1 ng μL^{-1}) were prepared in methanol and stored at -20 °C. Ederol filters (no. 14, 110 mm) were purchased from Binzer & Munktell Filter GmbH (Battenberg, Germany).

5.4.2 Blank cocoa powder sample

The OTA concentration in a commercially available cocoa powder sample, which was intended for use in the development of the clean-up tandem assay column, was determined using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with immunoaffinity clean-up.

Extraction of the cocoa powder sample (20 g) was carried out by adding 50.0 mL MeOH/3% $NaHCO_3$ (80:20, v/v) to the sample and shaking for 30 min at $\sim$ 250 rpm with an Orbital Shaker (SO3; Stuart Scientic, Stone, UK). The extract was centrifuged for 10 min at 3600 rpm and the supernatant (10.0 mL) diluted with 40.0 mL PBS-buffer. This solution (50.0 mL) was filtered through an Ederol filter and passed through the OchraTest^{TM} column at the rate of gravity. The column was washed with 5.0 mL water (under gravity) and OTA eluted with 3.0 mL MeOH. The eluate was evaporated to dryness under a N_2 stream and redissolved with 300 μL mobile phase for LC-MS/MS analysis.

A Waters Alliance 2695 XE high performance liquid chromatography (HPLC) system coupled to a Micromass Quatro micro triple quadrupole mass spectrometer was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 x 3.2 mm (Alltech, Deerfield, IL, USA), while the guard column was an Alltima C_{18}, 5 μm, 7.5 x 3.2 mm (Alltech). Injection volume was 20 μL. The mobile phase consisted of variable mixtures of formic acid in acetonitrile (0.3 %, solvent A) and formic acid in water (0.3 %, solvent B) at a flow of 0.3 mL min^{-1}. The gradient elution programme was: isocratic conditions with 10 % A/90 % B for 3 min, linear gradient from 10 to 100 % A for 6.5 min and isocratic conditions with 100 % A for 4.5 min. Then, within

1 min, the concentration of A was brought back to 10 % and maintained for 15 min. The mass spectrometer was operated in the positive electrospray ionisation (ESI+) mode using multiple reaction monitoring (MRM). Capillary voltage was 3.7 kV and nitrogen was used as spray gas. Source and desolvation temperatures were set at 120 and 350 °C, respectively. The precursor ion was *m/z* 404 and the cone voltage was 35 V. Product ions and collision energy were determined. Collision gas was argon. For OTA, product ions were at *m/z* 239 (collision energy 24 eV) and *m/z* 341 (collision energy 20 eV).

5.4.3 Extraction solution

The optimal composition of the extraction solution used for the clean-up tandem assay procedure was determined by high performance liquid chromatography (HPLC) coupled to fluorescence detection (FLD). Blank cocoa powder samples (5 g) were fortified (5, 15 and 25 μg kg^{-1}, n = 3 for each concentration level) with OTA the night before using the aforementioned working solutions in MeOH. They were extracted with 15 mL of a MeOH/3 % $NaHCO_3$ solution at different ratios (100:0, 95:5, 90:10, 80:20, 75:25, 50:50, v/v) [264] by shaking for 5 min at $\sim$ 200 rpm with an Orbital Shaker (SO3; Stuart Scientic). The suspension was filtered through an Ederol filter and the filtrate was analysed by HPLC-FLD.

Sample extracts (50 μL) were injected with an autosampler and separated on a Supelco Discovery C_{18} (5 μm, 25 cm x 4.6 mm) column (Supelco, Bellefonte, PA, USA) at room temperature. The mobile phase used was acetonitrile/water/acetic acid (54:44:2, v/v) at a flow rate of 1 mL min^{-1}. The HPLC system consisted of a Waters 600 Controller, a Waters 610 fluid unit and a Waters 474 scanning fluorescence detector (333 nm excitation wavelength, 470 nm emission wavelength). Sample OTA concentrations were quantified by comparing sample peak areas to those of standards and extraction recoveries determined.

5.4.4 Clean-up tandem assay column: Clean-up layer

Various solid-phase materials - aminopropyl derived silica (NH_2 derived silica), octadecyl derived silica, silica (silanol groups), strong anion exchange-silica (SAX) - were evaluated as to their ability to adsorb the brown colour and other matrix interferences [246], while at the same time allowing OTA to elute directly.

Blank cocoa powder samples (5 g) were fortified (20, 40, 80 and 160 μg kg^{-1}) with OTA the night before using the aforementioned working solutions in MeOH and extracted with 15 mL of MeOH/3% $NaHCO_3$ (95:5, v/v) by shaking for 5 minutes at $\sim$ 200 rpm with an Orbital Shaker (SO3; Stuart Scientic). The suspension was filtered through an Ederol filter.

The filtrate was diluted to reduce the methanol concentration, thus avoiding the denaturation of the antibodies in the clean-up tandem assay column. Milli-Q water and a 3% $NaHCO_3$ solution [250] were evaluated as dilution solutions by comparing the partition coefficients (K_d) for the dispersion of OTA between the selected solid phase (i.e. NH_2 derived silica; see Results: Clean-up layer) and the MeOH/3% $NaHCO_3$ (95:5, v/v) mobile phase. The filtrate (1.5 mL) was diluted with 2.1 mL Milli-Q water or with 2.1 mL of a 3% $NaHCO_3$ solution. This dilution was extracted over 200 mg of NH_2 derived silica without application of pressure [264]. The eluate from the column was collected and the OTA concentration measured by HPLC-FLD. The second fraction, obtained by washing the column with 4 mL of MeOH, was evaporated to dryness and redissolved in 300 μL of the mobile phase for HPLC analysis. The partition coefficient (K_d) was calculated using the formula K_d = [OTA wash fraction]/[OTA frontal elution fraction] or K_d = [OTA solid phase]/[OTA mobile phase].

5.4.5 Clean-up tandem assay column: Sepharose gel detection layer

The secondary rabbit anti-mouse antibodies were coupled to the Sepharose gel following the principles and methods of Pharmacia LKB Biotechnology [249, 264].

5.4.6 Clean-up tandem assay column: Sol-gel detection layer

Encapsulation of the monoclonal mouse anti-OTA antibody was carried out according to Zühlke *et al.* (1995) [265]. Hydrochloric acid (0.4 mL, 0.04 M), Milli-Q water (1.5 mL) and 6.8 mL tetramethoxysilane (TMOS) were mixed and sonicated under ice-cooling for 30 min. This silica sol (1 mL) was added to 1 mL of ice-cooled PBS pH 7.6 containing the amount of the antibodies to be immobilized (10 μL of 2 mg mL^{-1} primary anti-OTA antibody) in a beaker. The gel was weighed and the beaker was stored at 4 °C without capping. The drying process was stopped after 20 h incubation resulting in a clear glass. After

a weight loss of $\sim$ 30 %, the glass was ground in a mortar and packed into a column. The sol-gel glass immunoadsorber was then stored in PBS at 4 °C.

5.4.7 Preparation of the clean-up tandem assay column

A polyethylene frit (grid) was first put in the 1 mL tube, followed by the addition of 200 μL of the detection layer Sepharose gel suspended in PBS or 50 mg of sol-gel powder. Another grid separated the detection layer and 200 mg clean-up layer above. A third grid was placed above the clean-up layer [264].

5.4.8 Clean-up tandem assay procedure

The clean-up tandem assay procedure was the same as mentioned in Lobeau *et al.* (2005) [264]. In the clean-up tandem assay, each reagent was applied in sequence at the inlet or at the outlet of the tube at a rate of $\sim$ 1 drop s^{-1}. These reagents were, respectively, primary mouse anti-OTA antibodies (100 μL), wash solution (3 mL), diluted sample extract (3.6 mL), wash solution (3 mL), OTA-HRP conjugate (200 μL), wash solution (3 mL), colour substrate (50 μL) (see Reagents and Materials section). For columns filled with sol-gel powder, the application of the diluted cocoa powder extract (3.6 mL) onto the column was the first step.

The test was developed to have a visual cut-off level of 2 μg OTA kg^{-1} cocoa powder (EC proposed limit for cocoa powder) by optimizing the dilutions of the primary mouse anti-OTA antibodies and the OTA-HRP conjugate. In this way, the absence of a developed blue colour indicated a positive result with an OTA level above 2 μg kg^{-1}.

5.4.9 OTA analysis of 10 different commercial cocoa powder samples

Ten cocoa powder samples commercially available on the Belgian market were analysed in triplicate using the clean-up tandem assay column. The results were compared with a LC-MS/MS method with immunoaffinity clean-up, as described above.

5.5 Results and discussion

5.5.1 Blank cocoa powder sample

The OTA concentration in the tested cocoa powder sample was found to be less than 0.1 μg kg^{-1} (0.0955 μg kg^{-1}) with the LC-MS/MS method. The clean-up tandem assay column was further developed with this essentially blank sample.

5.5.2 Extraction solution

Lobeau *et al.* 2005 [264] used MeOH/3 % $NaHCO_3$ (80:20, v/v) as solvent for the extraction of OTA from roasted coffee. For OTA extraction from cocoa powder, different ratios of MeOH/3 % $NaHCO_3$ (v/v) were examined and recoveries were determined using the described HPLC-FLD method. Large amounts of 3 % $NaHCO_3$ in the extraction solution resulted in dark filtrates. Less than 20 % of a 3 % $NaHCO_3$ solution was necessary to avoid this interfering colouration. The best recovery (100.2 $\pm$ 7.1 %) was found with MeOH/3 % $NaHCO_3$ (95:5, v/v).

5.5.3 Clean-up layer

Only SAX-silica and NH_2 derived silica were found to be able to adsorb most of the colour of the cocoa powder. Recoveries were comparable for both. NH_2 derived silica was chosen because of the easier availability.

Dilution of the methanolic filtrate was necessary to avoid denaturation of the antibodies in the column. Milli-Q water and a 3 % $NaHCO_3$ solution were tested as dilution solvent and compared by calculating the partition coefficients (K_d) for the dispersion of OTA between the NH_2 derived silica solid-phase and the MeOH/3 % $NaHCO_3$ (95:5, v/v) mobile-phase. OTA should elute directly to the detection layer, so, the partition coefficient should be as low as possible.

Partition coefficients are shown in Table 5.1. On average lower coefficients (K_d = 0.4828 $\pm$ 0.086) were obtained using 3 % $NaHCO_3$ than with Milli-Q water (K_d = 0.9806 $\pm$ 0.488. Therefore, 1.5 mL of the sample extraction filtrate was diluted with 2.1 mL of a 3 % $NaHCO_3$ solution [264]. This was a reduction in MeOH content to $\sim$ 40 %.

Table 5.1: Calculated partition coefficients for the distribution of OTA between the aminopropyl solid-phase and the MeOH/ 3 % $NaHCO_3$ (95:5, v/v) mobile phase (n = 3)

OTA concentration (μg kg^{-1})	OTA concentration solid phase (μg kg^{-1})	OTA concentration mobile phase (μg kg^{-1})	Partition coefficient (K_d = solid/mobile)
Dilution with a 3 % $NaHCO_3$ solution			
20	3.99	6.68	0.5974
40	8.40	18.41	0.4563
80	18.23	46.55	0.3915
160	44.93	92.43	0.4861
Average			0.4828 ± 0.086
Dilution with Milli-Q water			
20	2.79	7.34	0.3806
40	10.15	11.92	0.8519
80	28.07	18.30	1.534
160	42.54	36.80	1.156
Average			0.9806 ± 0.488

5.5.4 Clean-up tandem assay column set-up

The reason for binding secondary rabbit anti-mouse antibodies to the Sepharose gel was that direct binding of the capture protein (the primary monoclonal anti-OTA antibody) gave no binding results, probably due to conformational changes that reduced the anti-OTA antibody's affinity for the analyte [233, 252].

In most cases the immobilization reaction can only be applied using highly purified ligands and often leads to conformational changes of bioligands and, therefore, decreased affinity for the analyte. This problem can be avoided when ligands are immobilized in the pores of a sol-gel glass matrix [252]. This method was compared with the Sepharose method. However, when applied as a gel in the clean-up tandem assay column, it was found to be unsuitable. The colour substrate seemed to react non-specifically with polar silane groups in the gel and colour development immediately appeared only on the side where the substrate made the initial contact with the gel. This result was observed for both negative and positive samples.

An example of a series of results for the clean-up tandem assay column using Sepharose gel is given in Figure 5.1. The dilution of the primary mouse anti-OTA antibodies and the OTA-HRP conjugate

were, respectively, 1/300 and 1/500 in assay buffer. The intensity of colour development decreased with increasing concentrations of OTA. No blue colour developed at a concentration of 2 μg kg^{-1}. Therefore, this was the cut-off level of the assay.

The analysis of six samples can be carried out in the field in approximately 30 min by untrained workers.

Dilution of the filtrate with 3 % $NaHCO_3$ resulted after a few minutes in precipitation and gelation of the proteins in the cocoa powder due to the alkaline pH. Because this could compromise the flow of the sample through the column, dilution with Milli-Q water was found to be more appropriate, contrary to the results reported in the Results: Clean-up layer section.

5.5.5 Validation of the clean-up tandem assay method using fortified cocoa powder samples

An intra-laboratory validation was performed with blank cocoa powder samples fortified at 0, 1, 2, 3, 4 and 5 μg OTA kg^{-1} the night before using the aforementioned working solutions in MeOH. The following performance characteristics were determined: precision (in terms of false positive and false negative results), sensitivity, specificity, positive and negative predictive values and unreliability region [2, 117].

For this qualitative screening test, precision was expressed as the number of false positive and false negative results, as can be seen in Table 5.2. Based on these results, sensitivity, i.e. the ability to detect a true positive result, and specificity, i.e. the ability to detect a true negative result, were, respectively, 98 and 90 %. The predictive value of positive results, i.e. fraction of positive results that are true positives, was 93 % and the predictive value of negative results, i.e. fraction of negative

Figure 5.1: Series of results for clean-up tandem assay. The detection layer of each column is shown and the colour can be visually evaluated. A small part of the clean-up layer is visible above the detection layer. The OTA concentrations (μg kg^{-1}) of the cocoa powder samples are indicated below the tubes.

results that are true negatives, was 97 %. The unreliability region, the region in which false results were obtained, was between 1 and 2 μg kg^{-1}. These results are shown in Table 5.3. For mycotoxins, criteria for screening methods are not available, but, according to Commission Decision 2002/657/EC, the requirement for a screening method is as follows: only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of < 5 % at the level of interest shall be used for screening purposes. In case of a suspected non-compliant outcome, the result shall be verified by a confirmatory method.

5.5.6 Performance characteristic curve

Performance characteristics were also estimated by a curve [117]. Blank cocoa powder samples were fortified at 0, 0.5, 1, 1.5, 2, 3, 4 and 5 μg kg^{-1} OTA the night before using the aforementioned working solutions in MeOH. At each concentration level, the percentage of positives, P(X), and the percentage of negatives, N(X) was calculated. With these data, a graph of percentage positives versus the concentration levels tested was plotted. The performance characteristic curve was obtained by fitting the experimental results from Table 5.4 to a sigmoidal function that minimized the root mean square of the residuals (Figure 5.2). To obtain the performance parameters, the probabilities of committing false-positive (α) and false-negative (β) errors, which could be accepted, had to be fixed. Usually, these values are fixed at 5 % and correspond to the horizontal lines plotted on Figure 5.2, α = 5 % and 100-β = 95 % [266]. Once the probabilities of error were fixed, all performance parameters were calculated. This was possible using the equation of the sigmoidal curve:

$$y = y_0 + \left(\frac{a}{1 + e^{\frac{-(x - x_0)}{b}}} \right)$$

with: $a = 100.4838, b = 0.2642, x_0 = 1.3449, y_0 = -1.0662$

The unreliability region was defined by its upper and lower limits. The upper limit corresponded to the concentration at which the 100-β line crossed the P(X) curve (2.16 μg kg^{-1}). Therefore, 2.2 μg kg^{-1} was the cut-off as well as the detection limit of our assay, corresponding to the visual observation. The lower limit corresponded to the concentration at which the α line crossed the P(X) curve (0.62 μg kg^{-1}). Therefore, the unreliability region was between 0.6 and 2.2 μg kg^{-1}. The

Table 5.2: Contingency table with numerical values as performed with the clean-up tandem assay column for the detection of ochratoxin A in cocoa powder

	Samples fortified at 2 μg kg^{-1} OTA or more	**Samples fortified at less than 2 μg kg^{-1} OTA**	**Total**
Positive (no colour)	55 (tp)	4 (fp)	59
Negative (blue colour)	1 (fn)	36 (tn)	37
Total number of samples	56	40	96

tp = true positive; fp = false positive; fn = false negative; tn = true negative

Table 5.3: Qualitative performance parameters for the detection of ochratoxin A in cocoa powder using the clean-up tandem assay column

False positive rate	10 %
False negative rate	2 %
Sensitivity	98 %
Specificity	90 %
Positive predictive value	93 %
Negative predictive value	97 %
Unreliability region	1-2 μg kg^{-1}

false positive rate = (fp/tn+fp) x 100, false negative rate = (fn/tp+fn) x 100, sensitivity = (tp/tp+fn) x 100, specificity = (tn/tn+fp) x 100, positive predictive value = (tp/tp+fp) x 100, negative predictive value = (tn/tn+fn) x 100

Table 5.4: Concentration levels of ochratoxin A tested, with probabilities of positive, P(X), and negative, N(X), results calculated for each concentration level

Concentration, μg kg^{-1}	Number of samples	P(X)	N(X)
0	20	0	100
0.5	12	0	100
1	20	25	75
1.5	12	58	42
2	14	100	0
3	14	93	7
4	14	100	0
5	14	100	0

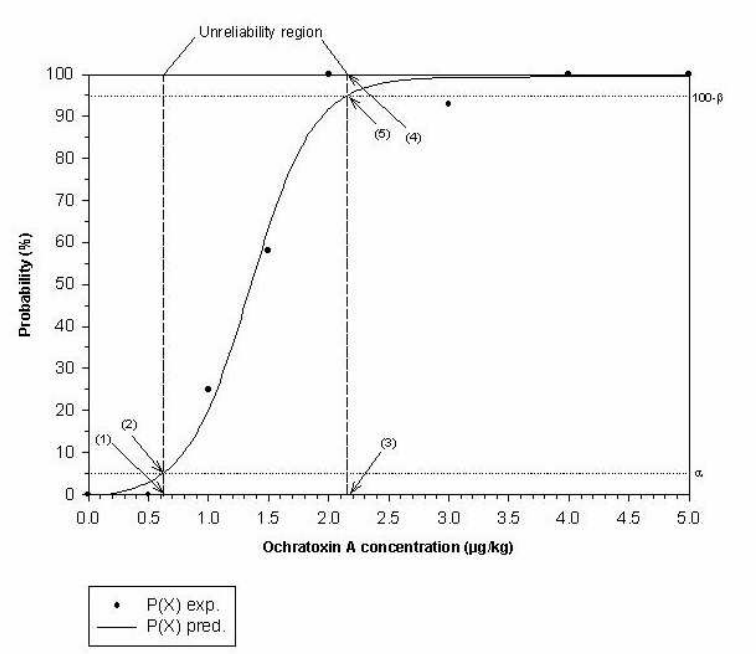

Figure 5.2: Experimental performance characteristic curve. Probability of positive responses, P(X), were plotted versus concentration levels tested. (1) fp = P(X); (2) $X_{0.05}$ where specificity = N(X) = 100-P(X); (3) $X_{0.95}$, cut-off limit, detection limit; (4) fn = 100-P(X); (5) sensitivity = P(X) = 100-β

sensitivity rate at 2.2 μg kg^{-1} was equal to 95%, as it corresponded to the point where P(X) = 100-β. The false negative rate (fn) at 2.2 μg kg^{-1} was equal to 5% as fn = 100-P(X). Similarly, the specificity rate of 0.6 μg kg^{-1} was equal to 95%, as it corresponded to the point where N(X) = 100-P(X). The false positive rate (fp) at this concentration level was also 5 % because fp = P(X). Gel preparation was probably a source of error and gave the false negative result at the 3 μg kg^{-1} concentration level. Preparing the gel in larger volumes would favour the repeatability and minimize false negative results.

5.5.7 OTA analysis of 10 different commercial cocoa powder samples

All ten commercially available cocoa powder samples were found to be negative (less than 2 μg kg^{-1}) using the clean-up tandem assay column. These qualitative results were compared with the quantitative results obtained using the above mentioned LC-MS/MS method. Results are shown in Table 5.5.

Table 5.5: OTA analysis of 10 different commercial cocoa powder samples available on the Belgian market

Commercial sample	Qualitative result (clean-up tandem assay column, n = 3)	Quantitative result (μg kg^{-1}) via LC-MS/MS method
1	- - -	0.0678
2	- - -	0.0831
3	- - -	0.0579
4	- - -	0.0553
5	- - -	0.0781
6	- - -	0.0654
7	- - -	0.0622
8	- - -	0.0532
9	- - -	0.0774
10	- - -	0.0402

(+) = positive sample, ≥ 2 μg kg^{-1}, (-) = negative sample, < 2 μg kg^{-1}

5.6 Conclusions

A clean-up tandem assay column for the detection of OTA in cocoa powder was developed and the method validated by determination of its qualitative performance parameters. The column comprised two superposed layers; clean-up and detection were performed on one single column device. The result of the assay can be binary only: yes/no response, indicating whether OTA is present or not above the cut-off level. The test was calibrated to a cut-off level of 2 μg OTA kg^{-1} cocoa powder. Establishing this parameter by means of a performance characteristic curve resulted in a cut-off level of 2.2 μg OTA kg^{-1} cocoa powder. Therefore, the described clean-up tandem assay column can be used as a rapid screening tool for OTA in cocoa powder.

5.7 Acknowledgement

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Chapter 6

Approach for ochratoxin A fast screening in spices using clean-up tandem immunoassay columns with confirmation by high performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS)

6.1 Background and objectives

Spices were estimated to contribute to the total dietary OTA intake for 8 % in Europe [4]. Therefore, spices are a suitable matrix to screen for OTA contamination. As some spices are strongly coloured, the *clean-up tandem assay column* was most suitable as rapid non-instrumental test. For *Capsicum* spp. spices, the original set-up yielded no false results. Another set-up (top detection immunolayer set-up) was necessary for the spices ginger, nutmeg, black and white pepper. The method was validated by analysing non-fortified and fortified samples with the *clean-up tandem assay column*. These results were confirmed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, applied in the laboratory as a routine method for the detection of ochratoxin A (OTA) [254].

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6.2 Abstract

An approach for ochratoxin A (OTA) fast cost-effective screening based on clean-up tandem immunoassay columns was developed and optimized for OTA detection with a cut-off level of 10 μg kg^{-1} in spices. Two procedures were tested and applied for OTA detection. Column with bottom detection immunolayer was optimized for OTA determination in *Capsicum* spp. spices. A modified clean-up tandem immunoassay procedure with top detection immunolayer was successfully applied for all tested spices. Its main advantages were decreasing of the number of analysis steps and quantity of antibody and also minimizing of matrix effects. The total duration of the extraction and analysis was about 40 min for six samples. Chilli, red pepper, pili-pili, cayenne, paprika, nutmeg, ginger, white pepper and black pepper samples were analysed for OTA contamination by the proposed clean-up tandem immunoassay procedures. Clean-up tandem immunoassay results were confirmed by HPLC-MS/MS with immunoaffinity column clean-up. Among 17 tested *Capsicum* spp. spices, 6 samples (35 %) contained OTA in a concentration exceeding the 10 μg kg^{-1} limit discussed by the European Commission. All tested nutmeg (n = 8), ginger (n = 5), white pepper (n = 7) and black pepper (n = 6) samples did not contain OTA above this action level.

Keywords: ochratoxin A; spices; screening; immunoassay; high performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS)

6.3 Introduction

Mycotoxins are naturally occurring toxins produced by certain fungi that can grow on foods such as cereals, nuts, dried fruits, spices, legumes and others under certain environmental conditions. The European Commission has set the following limits for ochratoxin A (OTA): 10.0 μg kg^{-1} for dried vine fruits and soluble coffee, 5.0 μg kg^{-1} for raw cereal grains and roasted coffee, 3.0 μg kg^{-1} for cereals intended for human consumption, 2.0 μg L^{-1} for wine and grape juice and 0.5 μg kg^{-1} for baby foods and cereal-based foods intended for infants and young children [247, 256, 267]. For green coffee, cocoa and cocoa products, beer, some spices and meat products setting of limits still have to be considered [256].

Traditionally, extraction with aqueous acetonitrile or methanol,

followed by clean-up of the extract solutions using immunoaffinity columns (IAC) combined with high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD), provides sensitive and selective results for a wide range of foodstuffs: cereals [268], cereal-derived products [269], dried fruit [270, 271, 272], coffee [273], cocoa powder [274] and cocoa beans [259], chocolate [161], red pepper [87]. Acidified (orthophosphoric acid) chloroform is also a good extracting solvent for OTA extraction from cereals before C_{18} column clean-up and HPLC determination [275, 276], but it is now less popular with analysts as the use of halogenated solvents is discouraged on environmental and health grounds. For the OTA determination in liquid foodstuffs (wine [277, 278, 279], olive oil [280], beer [196] and grape juice [277, 281, 282]) HPLC-FLD preceded by extraction of OTA using IAC or solid sorbents [283, 284] is currently the most applied method. At present HPLC coupled with mass-spectrometry (HPLC-MS) has been applied for individual OTA detection [30, 285] or for simultaneous detection of various mycotoxins and their degradation products [114, 286]. However, HPLC methods are laborious, time-consuming, require sophisticated equipment and can not be used *in situ*.

For fast and inexpensive screening of OTA in foodstuffs some methods, based on the application of relatively simple equipment, have been developed: enzyme-linked immunosorbent assays (ELISA) [287, 288], electrochemical immunosensors [289], fluorescence polarization [51], surface plasmon resonance [52] or fluorescent [50] biosensors, TLC with densitometric quantification [290].

For *in situ* measurements some rapid tests that can be used without any special laboratory equipment were offered. A thin layer chromatography (TLC) with visual estimation of fluorescence under a UV lamp at 366 nm was applied for the OTA determination in green coffee [42]. A lateral flow device, based on a carrier membrane with immobilized antibodies, was developed for the OTA detection in fungal cultures [46]. A membrane-based flow-through enzyme portable immunoassay was involved for visual screening of roasted and green coffee samples for OTA levels [47, 49]. But application of methods with visual detection is difficult for intensively coloured products or extracts. For the rapid visual detection of OTA in intensively coloured media a device, containing both clean-up and detection immunolayers, was offered [9]. These clean-up tandem immunoassay columns were recently applied for the OTA detection in roasted coffee [264].

There are currently no legal limits for OTA in spices, however, a

European Commission has been discussing a limit of 10 μg kg^{-1} for OTA in spices such as *Capsicum* spp. (chillies, chilli powder, cayenne and paprika); *Piper* spp. (white and black pepper), *Myristica fragrance* (nutmeg); *Zingiber officinale* (ginger). The level and type of fungi contamination depend on the production region [291], moisture and temperature regimes of drying process, treatment, transport and storing [239, 292]. There are not many data concerning OTA levels in spices. As far as we know up to this moment there is no commercially available rapid test for OTA determination in spices. As most contaminated products nutmeg, paprika, chilli, coriander and pepper were indicated [180]. OTA contamination of spices has been reported for Hungarian commodities: almost 50 % of red pepper samples contained OTA, 10 % of which showing an OTA concentration of more than 10 μg kg^{-1} [87]. Aboul-Enein *et al.* reported an OTA concentration of 10.6 μg kg^{-1} for a red pepper sample [293]. Jørgensen described that the overall mean level of OTA in spices was 1.2 μg kg^{-1}. The maximum content reported was 24 μg kg^{-1} [180] and paprika and chilli were mentioned as the most contaminated spices. Thirumala-Devi *et al.* reported for Indian spices that 26 % of chilli samples [173], 54 % of black pepper samples [168], 8 % of ginger samples [168] were found to contain more than 10 μg OTA kg^{-1} sample and the maximum content reported was 120 μg kg^{-1} [173]. The purposes of this article were: (i) to develop a rapid cost-effective screening procedure for OTA contamination in spices; (ii) to optimize the clean-up tandem immunoassay columns for OTA determination in *Capsicum* spp. spices, ginger, nutmeg, black pepper and white pepper with a cut-off level of 10 μg kg^{-1}; (iii) to screen spice samples for OTA contamination; (iv) to confirm results by an HPLC-MS/MS method with IAC clean-up.

6.4 Experimental

6.4.1 Reagents and materials

OTA standard and Tween 20 were purchased from Sigma Chemical Co. (Bornem, Belgium). Rabbit anti-mouse immunoglobulin (IgG) (protein concentration: 2.7 g L^{-1}) was supplied by DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased from Amersham Biosciences AB (Uppsala, Sweden). Bio-Sil NH_2 (diameter 0.040 mm), tubes (Bond Elut® reservoir, 1 mL and 3 mL) and polyethylene frits (1/4 in. and 3/8 in. diameter) were supplied by Varian Belgium

NV/SA (Sint-Katelijne-Waver, Belgium).

OTA-horse radish peroxidase (OTA-HRP) conjugate was prepared by the Diagnostic Laboratory, Agricultural Biotechnology Center, Gödöllö, Hungary. Monoclonal antibodies against OTA were produced and characterized by the same institute. The antibody was an IgG1 with kappa light chains with a 9.3% cross-reaction with ochratoxin B but none at all with ochratoxin α, coumarin, 4-hydroxy-coumarin and D,L-phenylalanine [248]. Phosphate-buffered saline (PBS) 0.01 M, pH 7.4, was used as wash solution and assay buffer. Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Methanol was HPLC-grade and water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogen solution used was Colorburst^{TM}BlueTMB/Peroxide (ALerCHEK, Inc. USA). Stock solutions of OTA (1mg mL^{-1}) and working solutions (10, 1.0, 0.10 μg mL^{-1}) were prepared in methanol and stored at -20 °C. Ederol filters No. 15, 110 mm were purchased from Binzer and Munktell Filter GmbH (Battenberg, Germany). OchraTest^{TM} immunoaffinity columns were supplied by Vicam (Cereal Tester, Fleurus, Belgium).

6.4.2 Collection of samples

Capsicum spp. (17 samples), ginger (5 samples), nutmeg (8 samples), black pepper (6 samples) and white pepper (7 samples) were purchased from retail shops in Belgium, the Netherlands and Russia. Two samples of *Capsicum* spp. were purchased as pods; one of black and one of white pepper were purchased as seeds. For *Capsicum* spp. the name of spices depended on the manufacturing country, so we kept the original name.

6.4.3 HPLC-MS/MS

Before the HPLC-MS/MS determination a clean-up and preconcentration procedure was performed using OchraTest^{TM} columns. NaCl (2 g) was added to 10 g of ground red pepper and this mixture was extracted with 40 mL MeOH/water (80/20, v/v) for 1 h using an end-over-end shaker and centrifuged for 10 min at $\pm$ 4000 rpm. The supernatant (10 mL) was diluted with 40 mL of a PBS solution (pH 7.4), filtered and applied onto the OchraTest^{TM} column. The column was washed with 5 mL of PBS solution and 5 mL of water. OTA was eluted with 3 mL

of MeOH. This was completely evaporated with a gentle stream of nitrogen at 60 °C and redissolved in 300 μL of 50 % ACN (0.3 % formic acid), 50 % H_2O (0.3 % formic acid).

The Waters Acquity UPLC system coupled to a Micromass Quatro micro triple quadrupole mass spectrometer was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 x 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C_{18}, 5 μm, 7.5 x 3.2 mm (Alltech Associates). The column was kept at room temperature. The injection volume was 20 μL. The mobile phase consisted of variable mixtures of solvent A (acetonitrile with 0.3 %, v/v, formic acid) and solvent B (water with 0.3 %, v/v, formic acid) at a flow of 0.3 mL/min with a gradient elution programme: isocratic conditions with 10 % A:90 % B for 3 min, linear gradient from 10 to 100 % A for 6.5 min, and isocratic conditions with 100 % A for 4.5 min. Then, within 1 min, the concentration of A was brought back to 10 %. This was maintained for 15 min. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. Capillary voltage was 3.7 kV and nitrogen was used as spray gas. Source and desolvation temperatures were set at 120 and 350 °C, respectively. The precursor ion was *m/z* 404. The cone voltage was 35 V. Product ions and collision energy were determined [254]. Collision gas was argon. For OTA product ions were at *m/z* 239 (collision energy 24 eV) and *m/z* 341 (collision energy 20 eV). For OTA quantification negative *Capsicum* spp., *Piper* spp., ginger and nutmeg samples were fortified at 0.1, 1.0, 5.0, 15 and 30 μg kg^{-1} (= calibration curves).

6.4.4 Clean-up tandem immunoassay columns

For the OTA extraction from spices procedures described in [87] were adapted. NaCl (0.5 g) was added to 2.5 g of sample. This mixture was extracted with 7.5 mL MeOH/3 % $NaHCO_3$ water solution (80/20, v/v). After shaking for 15 min at $\pm$ 200 rpm with an orbital shaker the extract was filtered through an Ederol filter.

Two procedures for the clean-up tandem immunoassay were developed. For both procedures the 1 mL tubes were used. As the clean-up layer 200 mg of Bio-Sil NH_2 were applied.

In the first assay procedure columns with bottom detection immunolayer and top clean-up layer were used [264] (Fig 6.1a). For the detection immunolayer preparation, CNBr-activated Sepharose 4B with coupled secondary rabbit anti-mouse antibodies was mixed

with CNBr-activated Sepharose 4B with glycine blocked active groups in ratio 1:5. Their preparation was described in detail by Lobeau *et al.* [264]. Before the assay, coupled Sepharose and blocked Sepharose were diluted with PBS in ratio 1:3. Briefly, the procedure was started with binding of the primary mouse anti-OTA antibodies to the secondary rabbit anti-mouse antibodies, immobilized on the gel during the coupled gel preparation. For binding, 50 μL of primary mouse anti-OTA antibody solution (dilution 1/50) were applied into the column through the outlet means of the tube. In this way, antibodies were first in contact with the detection immunolayer instead of the clean-up layer. Unbound antibodies were removed by a washing step. All washing steps consisted of passing 2 mL of PBS solution through the inlet means of the tube using a plunger.

Sample extract (0.5 mL) was diluted with 1 mL of $NaHCO_3$ (3 %) solution to prevent destroying of the antibodies in the immunolayer. The extract was passed by plunger through the two layers: the clean-up layer in which Bio-Sil NH_2 adsorbent medium was capable of actively adsorbing at least a part of the interfering fraction and pigments of the extract, and the detection immunolayer, capable of specifically retaining OTA. OTA, if present, was thus bound to the anti-OTA antibodies at the detection immunolayer. To remove the interfering substances a second washing step was used. Then, through the outlet means 50 μL OTA-HRP conjugate solution (dilution 1/50) was applied. Before adding of 50 μL of chromogen substrate to the immunolayer a third washing step was carried out. As a result an intense blue colour was developed in the detection immunolayer for non-spiked samples. Detection time was 5 min after application of chromogen substrate.

In the modified (second) assay procedure the clean-up sorbent was placed on the bottom of the 1 mL tube above the polyethylene frit (Fig 6.1b). The next frit was put over this clean-up layer. The detection immunolayer was placed on top, and then the last frit. Before placing into the tube, the primary mouse anti-OTA antibodies were bound to the secondary rabbit anti-mouse antibodies in the assay gel, as follows: 0.5 mL of the coupled gel (CNBr-activated Sepharose 4B with coupled secondary rabbit antimouse antibodies, diluted with PBS 1:3) was added to 2.5 mL of the blocked gel (CNBr-activated Sepharose 4B with glycine blocked active groups, diluted with PBS 1:3) in a 3 mL tube with a bottom polyethylene frit. PBS excess was removed under gravitation. Then 400 μL of the primary mouse anti-OTA antibody solution (dilution 1/100) were added and carefully mixed. In 5 min, this solu-

tion was flowed through using a plunger and the gel was washed two times with 3 mL of PBS. Then 3 mL of PBS were added and the assay gel was mixed. PBS (200 μL) and then 200 μL of the prepared assay gel solution were placed on the second frit into the 1 mL tube, and finished with the last frit above.

For the assay procedure, 0.5 mL of the sample extract was diluted with 1 mL of $NaHCO_3$ (3 %) solution and drawn into the tube through the outlet means using the plunger as far as air passed through both layers. Then the extract was flowed through the column again, using the plunger. So, impurities from the extract remained on the lower part of the immunocolumn; and the extract was twice in contact with the detection immunolayer. To remove the interfering substances from the immunolayer the washing step with 3 mL PBS-Tween 0.05% through the inlet means of the tube was used. The next step was the application of 50 μL OTA-HRP conjugate solution (dilution 1/100) using a micropipette on the top detection immunoassay layer and removing the conjugate excess with 3 mL PBS-Tween 0.05%. The last step was the addition of chromogen substrate (50 μL) with a micropipette. Detection time was 3 min after substrate application.

6.5 Results and discussion

6.5.1 Clean-up tandem immunoassay optimization

The main requirement for screening methods is prevention of false negative results. For a testing procedure one sample of pili-pili, paprika, chilli, nutmeg, ginger, black pepper and white pepper were used. These samples were first analysed by HPLC-MS/MS and found to be OTA negative. To investigate possible matrix effects on the false negative results two tests were made for each sample: blank and fortified with OTA at 10 μg kg^{-1}. For black pepper, white pepper, nutmeg and ginger dilution of the extract with a 2 fold 3 % $NaHCO_3$ solution volume resulted in turbid solutions because of precipitated fats. The assay procedure with the bottom detection immunolayer was primarily applied. The outcomes for blank and fortified samples showed, that this procedure only allowed to obtain accurate results for *Capsicum* spp. spices. For ginger and nutmeg samples only some results were correct, while the developed blue colour of the detection immunolayer for blank samples was less intense than for *Capsicum* spp. spices. This could be the reason for the obtained false positive results. For black pepper and

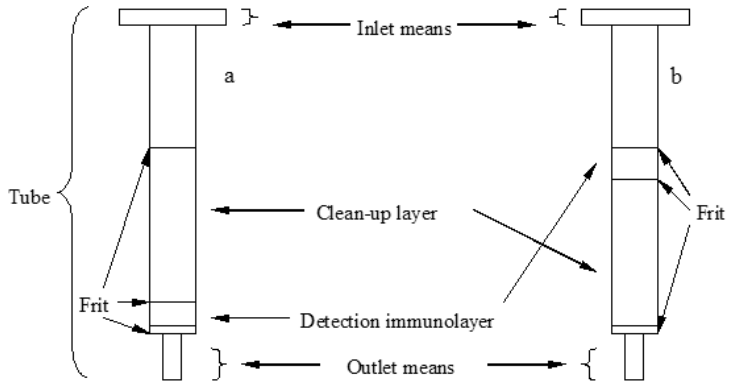

Figure 6.1: Clean-up tandem immunoassay column set-up. (a) Bottom detection immunolayer set-up. (b) Top detection immunolayer set-up.

white pepper all results for fortified samples were false negative: blue colour of the detection immunolayer developed after application of fortified sample extracts (results not presented). Thus for the OTA detection in ginger, nutmeg, black pepper and white pepper samples, the clean-up tandem immunoassay procedure had to be modified.

Among different tested procedures, the modified assay procedure described above with the top detection immunolayer was chosen as optimal. Development of the assay procedure with bottom detection immunolayer had shown that the application of the washing steps with PBS only (without Tween) allowed to avoid desorption of the pigments and other interfering compounds from the top clean-up layer and to prevent bottom detection immunolayer colouration. But if the detection immunolayer was situated on top of the column, the washing buffer firstly contacted this layer, and only afterwards with the clean-up layer. So, influence of desorption of interfering substances was impossible and PBS with Tween 0.05 % (v/v) was used as washing buffer. Applying this modified assay procedure for all blank spice samples resulted in the development of an intense blue colour of the detection immunolayer. No blue colour developed for all samples fortified at 10 μg kg^{-1}. Then for each sample (one for pili-pili, paprika, chilli, nutmeg, ginger, black pepper and white pepper), a set of fortified samples was prepared (0, 4, 6, 8, 10 μg kg^{-1}). The intensity of the developed colour decreased with increasing OTA concentrations. No blue colour developed at a concentration of 10 μg kg^{-1}, thus being the

cut-off level of the assay. An example of sets of results for pili-pili and black pepper are given in Fig 6.2.

The time for the clean-up tandem immunoassay procedure was less than 20 min for a set of six samples. This time is more than for membrane-based flow-through or lateral flow enzyme immunoassay kits, but clean-up tandem immunoassay columns could be applied for intense colour extracts. On the other hand, 20 min is less than the average ELISA duration. The total time for sample preparation was about 20 min, including extraction for 15 min. So the total time for preparation and analysis of six samples was only about 40 min.

For estimation of the stability of the columns with the top detection immunolayer several columns were prepared and stored at 4 °C. Sets of pili-pili samples fortified at 0, 6, 8, 10 μg kg^{-1} were analysed week after week with these columns. It was shown that the prepared columns gave reproducible results for at least 2 months: development of intense blue colour for all non-spiked samples, no colour development for all fortified with 10 μg kg^{-1} samples and weak blue colour development for samples fortified with 6 and 8 μg kg^{-1}.

The advantages of the modified assay procedure with the top detection immunolayer were:

- Binding of the primary mouse anti-OTA antibodies to the assay gel before placing in the column. It allowed to decrease the number of analysis steps, to reduce the quantity of antibodies and to have a more homogeneous distribution of antibodies inside the gel.

- Placing the clean-up layer on the bottom of the tube and the detection immunolayer on the top of the tube. It allowed to minimize the matrix effect and to enable OTA detection in ginger, nutmeg, black pepper and white pepper samples.

6.5.2 Application for OTA screening in spice samples

For OTA detection in the *Capsicum* spp. samples, the method using a column with bottom detection immunolayer was applied. For the black pepper and white pepper samples analysis, only the modified method using the column with the top detection immunolayer allowed to obtain correct results. For nutmeg and ginger, both methods were used and compared.

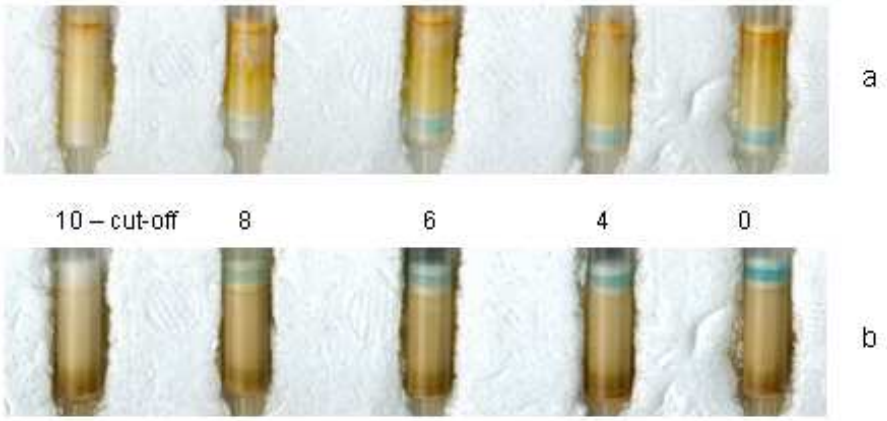

Figure 6.2: Clean-up tandem immunoassay. The detection and the clean-up layers of each column are shown and the colour can be evaluated. OTA concentrations (μg kg^{-1}) of the samples are indicated near the tubes. (a) Pili-pili extracts. Bottom detection immunolayer. (b) Black pepper extracts. Top detection immunolayer.

Generally, labels on spices do not contain detailed information about country of origin and production process. Therefore the influence for possible matrix effects was checked for each sample. To control it for all samples two portions of the extract were analysed: blank and fortified with 1.6 ng OTA per 0.5 mL of the extract (corresponding to 10 μg kg^{-1}). The assay procedure was repeated three times if all results for the blank portion of the extract were negative and 5 times in other cases. For one of the nutmeg samples, OTA detection was impossible because of an interfering intense brown colour on the detection immunolayer. Neither increasing of the extract dilution nor increasing of the washing buffer volume allowed to reduce intensity of that colour.

Table 6.1 presents results of the first assay procedure with the bottom detection immunolayer for the OTA determination in *Capsicum* spp., ginger and nutmeg extracts. It can be seen that all fortified *Capsicum* spp. extracts results were positive (no blue colour development of the detection immunolayer). For the fortified ginger and nutmeg extracts some false negative results (weak blue colour development of the detection immunolayer) were obtained. Therefore the column with bottom detection immunolayer could be applied for OTA determination only in *Capsicum* spp. spices.

Application of the modified assay procedure allowed to completely avoid false negative results for the ginger, nutmeg, black pepper and white pepper samples (Table 6.2). To show the universality of the modified clean-up tandem immunoassay column with the top detection immunolayer for the OTA detection in spices, *Capsicum* spp. samples were analysed and results were the same as for the column with the bottom detection immunolayer (results not presented).

Spices bought in Belgium (n = 25), the Netherlands (n = 10) and Russia (n = 8) during autumn-winter 2005-2006, were used. The results are summarized in Table 6.3. The number of samples was not enough for a precise evaluation of the total OTA contamination level for spices from different countries, but rough estimation showed that detected OTA levels were near to the reported data from Hungary - OTA contamination only for *Capsicum* spp. [87]. It is interesting to mention, that three of four *Capsicum* spp. bought in Russia, three of eight *Capsicum* spp. bought in Belgium and none of five *Capsicum* spp. bought in the Netherlands contained OTA above the 10 μg kg^{-1} limit.

6.5.3 HPLC-MS/MS confirmation

For the clean-up tandem immunoassay results confirmation and quantitative OTA determination in spice samples, the HPLC-MS/MS method was applied. A chromatogram from chilli sample (N 11) is presented in Fig 6.3. For *Capsicum* spp. spices all positive samples ($\geq$ 10 μg kg^{-1}), samples containing OTA but less than 10 μg kg^{-1} and also some negative samples were examined. With the clean-up tandem immunoassay columns no positive results were found for ginger, nutmeg, black pepper and white pepper. Some samples with negative OTA results were analysed by HPLC-MS/MS. Results are presented in Tables 6.1 and 6.2. The HPLC-MS/MS results confirmed both positive and negative clean-up tandem immunoassay results.

6.6 Conclusion

A rapid cost-effective *on-site* field assay was developed for OTA contamination screening in *Capsicum* spp. spices, ginger, nutmeg, black pepper and white pepper. Two procedures of assay with clean-up tandem immunoassay columns were optimized. The proposed modified assay procedure with top detection immunolayer has the advantages

Table 6.1: OTA determination in spices by the clean-up tandem immunoassay column with bottom detection immunolayer and HPLC-MS/MS

Spices	Clean-up tandem immunoassay		HPLC-MS/MS, (μg kg^{-1})
	Blank	Fortified (10 μg kg^{-1})	
***Capsicum* spp.**			
1 (Paprika)	- - -	+ + +	
2 (Paprika)	- - -	+ + +	
3 (Paprika)	- - -	+ + +	
4 (Paprika)	+ + + + +	+ + +	$>$ 30
5 (Paprika)	-± - - -	+ + +	2.6
6 (Pili-pili) (pods)	- - -	+ + +	nd
7 (Pili-pili)	+ + ± + +	+ + +	19
8 (Pili-pili)	- - -	+ + +	
9 (Red pepper)	+ + + + +	+ + +	18
10 (Red pepper)	+ + + + +	+ + +	30
11 (Chilli)	+ + + + +	+ + +	22
12 (Chilli)	- - -	+ + +	
13 (Chilli) (pods)	- - -	+ + +	nd
14 (Chilli)	- - -	+ + +	1.6
15 (Cayenne)	+ + ± + +	+ + +	13
16 (Cayenne)	- - -	+ + +	
17 (Cayenne)	- - -	+ + +	1.4
Ginger			
1	± - - - ±	+ ± ±	2.5
2	- - -	+± +	
3	- - -	+ + ±	nd
4	- - -	± ± +	nd
5	- - -	+ + +	
Nutmeg			
1	- - -	+ + ±	
2	Brown[a]	Brown[a]	
3	- - -	± ± +	1.1
4	± ± - ± -	+ ± +	2.1
5	- ± + ± ±	± ± ±	
6	+ + +	+ + +	1.2
7	- - -	+ + +	
8	- - -	± + +	1.5

(-) Blue colour development of the detection immunolayer, negative result; (±) weak blue colour development of the detection immunolayer, OTA concentration $<$ 10 μg kg^{-1} , negative result; (+) no blue colour development of the detection immunolayer; OTA concentration $\geq$ 10 μg kg^{-1}, positive result; (nd) no OTA detected. [a] Intense brown colour of the detection immunolayer. OTA detection is impossible.

Table 6.2: OTA determination in spices by the modified clean-up tandem immunoassay column with top detection immunolayer and HPLC-MS/MS

Spices	Clean-up tandem immunoassay		HPLC-MS/MS, (μg kg^{-1})
	Blank	Fortified (10 μg kg^{-1})	
Ginger			
1	- - ± ± -	+ + +	2.5
2	- - -	+ + +	
3	- - -	+ + +	nd
4	- - -	+ + +	nd
5	- - -	+ + +	
Nutmeg			
1	- - -	+ + +	
2	Brown[a]	Brown[a]	
3	- - -	+ + +	1.1
4	- - -	+ + +	2.1
5	- - -	+ + +	
6	- - ± - -	+ + +	1.2
7	- - -	+ + +	
8	- - -	+ + +	1.5
Black pepper			
1	- - -	+ + +	nd
2	- - -	+ + +	nd
3	- - -	+ + +	
4 (seeds)	- - -	+ + +	0.6
5	- - -	+ + +	
6	- - -	+ + +	nd
White pepper			
1	- - -	+ + +	nd
2 (seeds)	- - -	+ + +	nd
3	- - -	+ + +	
4	- - -	+ + +	nd
5	- - -	+ + +	
6	- - -	+ + +	nd
7	- - -	+ + +	

(-) Blue colour development of the detection immunolayer, negative result; (±) weak blue colour development of the detection immunolayer, OTA concentration < 10 μg kg^{-1} , negative result; (+) no blue colour development of the detection immunolayer; OTA concentration ≥ 10 μg kg^{-1}, positive result; (nd) no OTA detected. [a] Intense brown colour of the detection immunolayer. OTA detection is impossible.

Table 6.3: OTA contamination in spices

Species	Total/positive ($\geq$ 10 μg kg^{-1})	Positive (%) ($\geq$ 10 μg kg^{-1})
***Capsicum* spp.**	17/6	35
Ginger	5/0	0
Nutmeg	8/0	0
Black pepper	6/0	0
White pepper	7/0	0
Total	43/6	14

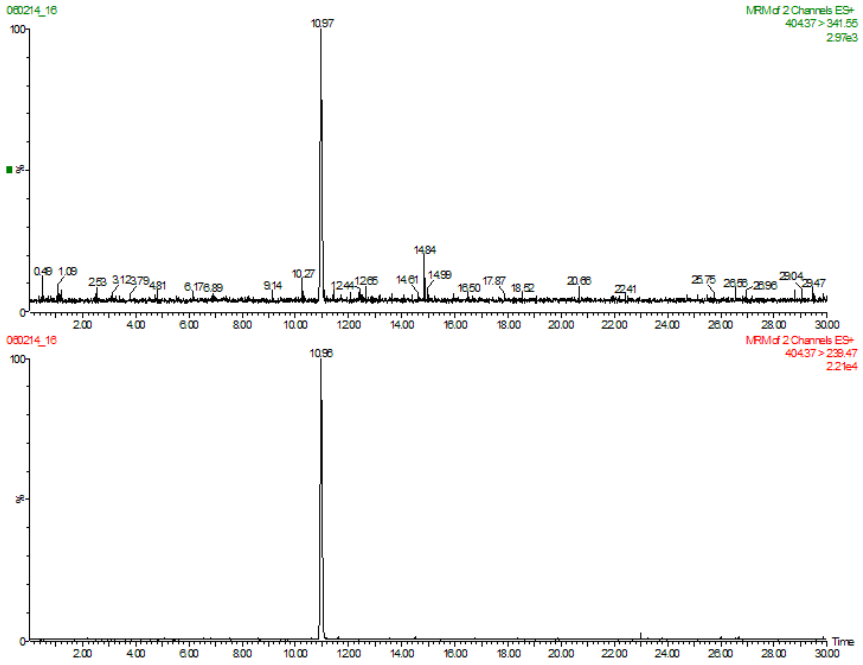

Figure 6.3: Mass chromatograms from chilli sample (N 11)

of universality for all target spices, minimization of spice's matrix interference and reduction of the assay steps. It does not need special equipment and expensive or commercially not available components neither for clean-up tandem immunoassay set-up preparation, nor for assay procedure.

It was found that 35 % of *Capsicum* spp. spices were contaminated with OTA at a level more than 10 μg kg^{-1}. The obtained clean-up tandem immunoassay results were confirmed by the HPLC-MS/MS method and complete agreement was shown. Therefore, the clean-up tandem immunoassay could be successfully applied for the simple rapid OTA contamination screening in spices. Most probably this technique could be universal for mycotoxins or other contaminants' fast *on-site* screening in intensively coloured foodstuffs, but it needs further research.

6.7 Acknowledgements

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Chapter 7

Simultaneous non-instrumental detection of aflatoxin B_1 and ochratoxin A using a clean-up tandem immunoassay column

7.1 Background and objectives

There is a great demand for rapid multi-analyte tests, reducing the number of analyses and therefore also reducing costs. As ochratoxin A (OTA) and aflatoxin B_1 (AFB_1) are often simultaneously found in spices, this matrix was suitable to develop a multi-mycotoxin screening test, based on the *clean-up tandem assay column*. The column comprised one clean-up layer (aminopropyl derived silica) and two detection immunolayers. The test was applied for simultaneous determination of AFB_1 and OTA in different spices and results were confirmed with a liquid chromatography tandem mass spectrometry (LC-MS/MS) method, used in the laboratory for routine analyses.

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7.2 Abstract

A set-up and simple method based on the clean-up tandem immunoassay approach was developed for the visual detection of two analytes. The method was based on a 1 mL column with one clean-up layer and two detection immunolayers. As detection immunolayers, CNBr-activated Sepharose 4B with coupled secondary rabbit anti-mouse antibodies was used. Different specific antibodies were coupled to each detection immunolayer. The analysis was realised in a competitive ELISA format with visual detection of the developed colour for each detection immunolayer and took 20 min for six sample extracts. The described method was applied to the simultaneous detection of aflatoxin B_1 and ochratoxin A in spices with cut-off levels at 5 and 10 μg kg^{-1}, respectively. Results were confirmed by LC-MS/MS with immunoaffinity column clean-up.

Keywords: multiple analysis, immunoassay, rapid methods, mycotoxins, ochratoxin A, aflatoxin B_1, spices

7.3 Introduction

One of the tendencies in present-day analytical chemistry is the simultaneous determination of multiple analytes in one analytical cycle for a given sample. Development of such methods is very important in the mycotoxin domain. Mycotoxins are present in different kinds of food and feed, and their presence should be controlled at various stages: before and post harvest, before and post processing. Some mycotoxins could simultaneously be present in the foodstuffs, so application of multi-mycotoxin assays allows to reduce the quantity of assays and, therefore, to lower costs and time for food and feed quality control. Recently some chromatographic methods for multiple mycotoxin determination were developed [113, 114, 115, 116, 294]. But these methods only yield results within hours and need expensive equipment and highly skilled personnel. Rapid methods for mycotoxin analysis have become increasingly important. At first for rapid simultaneous detection of several mycotoxins in food matrices, a thin layer chromatography (TLC) method was developed as an analytical screening procedure [295, 296].

Some rapid tests based on immunoassays with instrumental detection, such as a surface plasmon resonance biosensor [52] and a multiplexed assay [297] were also developed. Within the concept of flexible

on-site testing, the use of special equipment is prohibitive. But only few attempts have been made to prepare tests for simultaneous detection of several mycotoxins, which can be used without specific laboratory equipment: a membrane-based assay in the format of a dipstick [298], an 8-well immunofiltration test device for parallel simultaneous analysis [299], a line immunoblot assay [300], immunoblot approach - ELISAGRAM - combining separation by TLC and determination by ELISA [301], and an immunofiltration-based assay [302].

The goals of this study were: (i) to develop a clean-up tandem multiassay approach for the non-instrumental determination of at least two analytes in intense coloured foodstuffs; (ii) to optimize the clean-up tandem immunoassay columns for simultaneous OTA and AFB_1 determination in spices with cut-off levels of 10 and 5 μg kg^{-1}, respectively; (iii) to screen spice samples for AFB_1 and OTA contamination with clean-up tandem immunoassay and to confirm with multi-LC-MS/MS.

For spices (*Capsicum* spp. spices, ginger, nutmeg, black pepper, white pepper and turmeric), the EU established the limit for total aflatoxins (sum of aflatoxins B_1, B_2, G_1, G_2) at 10 μg kg^{-1}, for AFB_1 as the most toxic and widespread aflatoxin at 5 μg kg^{-1} [303] and discusses a limit for OTA in *Capsicum* spp. spices, ginger, nutmeg, black pepper and white pepper at 10 μg kg^{-1}.

7.4 Experimental

7.4.1 Materials

AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and OTA standards and Tween 20 were purchased from Sigma Chemical Co. (Bornem, Belgium). Rabbit anti-mouse immunoglobulin (IgG) (protein concentration: 2.7 g L^{-1}) was supplied by DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased from Amersham Biosciences AB (Uppsala, Sweden). Aminopropyl derived silica, Bondesil NH_2 (NH_2) (mean particles diameter 0.040 mm), tubes (Bond Elut® reservoir, 1 and 3 mL) and polyethylene frits (1/4 and 3/8 in. diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium).

AFB_1-horse radish peroxidase (AFB_1-HRP) and OTA-horse radish peroxidase (OTA-HRP) conjugates were prepared by the Diagnostic Laboratory, Agricultural Biotechnology Center, Gödöllö, Hungary. Monoclonal antibodies against AFB_1 and OTA were produced and

characterized by the same institute. The anti-OTA antibody was an IgG1 with kappa light chains with a 9.3% cross-reaction with ochratoxin B but none at all with ochratoxin α, coumarin, 4-hydroxy-coumarin and D,L-phenylalanine. The anti-AFB_1 antibody was IgG2a with 79 % cross-reaction with aflatoxin M_1, 33 % with aflatoxin M_2, 76 % with AFB_2, 55 % with AFG_1, 6 % with AFG_2 and none at all with aflatoxin B_{2a} and aflatoxin G_{2a}. Phosphate-buffered saline (PBS) 0.01 M, pH 7.4, was used as assay buffer. PBS with 0.05 % Tween was used as wash solution. Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Methanol was HPLC-grade and water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogenic solution used was Colorburst^{TM}Blue TMB/Peroxide (ALerCHEK Inc. USA). Stock solutions of AFB_1 and OTA (1 mg mL^{-1}) and working solutions (10, 1.0, 0.10 μg mL^{-1}) were prepared in methanol and stored at -20 °C. Ederol filters No. 15, 110 mm were purchased from Binzer and Munktell Filter GmbH (Battenberg, Germany). AflaOchra HPLC^{TM} immunoaffinity columns were supplied by Vicam (Cereal Tester, Fleurus, Belgium).

7.4.2 Instrumentation

The Waters HPLC system coupled to a Micromass Quatro micro triple quadrupole mass spectrometer was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 mm x 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C_{18}, 5 μm, 7.5 mm x 3.2 mm (Alltech Associates, Deerfield, IL, USA).

7.4.3 Procedures

7.4.3.1 AFB_1 and OTA extraction

For the AFB_1 and OTA extraction from spices 2.5 g of sample were extracted with 7.5 mL MeOH/3 % $NaHCO_3$ water solution (80/20, v/v). After shaking for 15 min at $\pm$ 200 rpm with an orbital shaker (Orbital Shaker SO3, Stuart Scientific, UK), the extract was filtered through an Ederol filter. This extract (0.5 mL) was diluted with 1 mL of water or 3 % $NaHCO_3$ water solution.

7.4.3.2 Optimization of clean-up conditions

A polyethylene frit was put in a 1 mL tube, followed by the addition of 200 mg of NH_2. A second frit was placed above the clean-up layer. OTA and AFB_1 solutions (1.5 mL; 2.0, 4.0 and 6.0 ng mL^{-1}) in MeOH/water (25/75, v/v; pH 5.5) or MeOH/3 % $NaHCO_3$ water solution (25/75, v/v; pH 9.0) were applied on this clean-up layer. The recoveries of AFB_1 and OTA were calculated as a ratio of the peak areas after and before the clean-up step using an HPLC with fluorescence detection. The Waters Alliance 2695 XE HPLC system coupled to a Waters 474 Scanning Fluorescence detector was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 x 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C_{18}, 5 μm, 7.5 x 3.2 mm (Alltech Associates, Deerfield, IL, USA). The injection volume was 50 μL. Mobile phase composition was 50 % ACN, 48 % H_2O, 2 % acetic acid for OTA detection and 50 % ACN, 50 % H_2O for AFB_1 detection. Flow rate was 1 mL min^{-1}. The excitation/emission wavelengths were set at 333/470 nm for OTA and 365/455 nm for AFB_1.

7.4.3.3 Clean-up tandem immunoassay columns

The clean-up sorbent (200 mg of NH_2) was placed on the bottom of the 1 mL tube above the polyethylene frit (Figure 7.1). The next frit was put over this clean-up layer. The detection immunolayers were placed on top, separated by a frit, and then the last frit. Before placing into the tube, the primary mouse anti-AFB_1 or anti-OTA antibodies were bound to the secondary rabbit anti-mouse antibodies in the assay gel, as follows: 0.5 mL of coupled gel (CNBr-activated Sepharose 4B with coupled secondary rabbit anti-mouse antibodies, diluted with PBS 1:3) was added to 2.5 mL of blocked gel (CNBr-activated Sepharose 4B with glycine blocked active groups, diluted with PBS 1:3) in a 3 mL tube with a bottom polyethylene frit. PBS excess was removed under gravitation. Then 400 μL of the primary mouse anti-AFB_1 or anti-OTA antibody solution were added and carefully mixed. In 5 min this solution was flowed through using a plunger and the gel was washed two times with 3 mL of PBS. Then 3 mL of PBS were added and the assay gel was mixed. PBS (100 μL) and then 200 μL of the prepared assay gel solution with bound anti-OTA antibodies were placed on the second frit into the 1 mL clean-up tandem immunoassay column. Above this anti-OTA detection layer the third frit was placed. Assay gel solution

with bound anti-AFB_1 antibodies (200 μL) was put on top of the column and the column was finished with the last frit above.

For the assay procedure 0.5 mL of the sample extract was diluted with 1 mL of a $NaHCO_3$ (3 %) solution and drawn into the tube through the outlet means by pulling the plunger of a connected 20 mL syringe as far as air passed through all layers (Figure 7.1). So, impurities from the extract remained on the clean-up layer at the bottom part of the column. Mycotoxins, if present in the sample, bound with the antibody binding sites in the upper detection immunolayers. Then, to remove the extract from the column, it was flowed through the column again in the opposite direction, using the plunger. A washing step with 3 mL PBS-Tween 0.05% through the inlet means of the tube was used to remove the interfering substances from the immunolayers. The next step was the application of a mixture of OTA-HRP and AFB_1-HRP conjugates using a micropipette on the top frit and removing the excess of conjugates with 3 mL PBS-Tween 0.05%. Mycotoxin-HRP conjugates bound to the non-occupied antibody binding sites in the detection immunolayers. The last step was the addition of the chromogenic substrate with a micropipette. Colour intensities on the detection immunolayers were inversely proportional to mycotoxin concentrations in the sample. Colour was visually evaluated after 5 min of chromogenic substrate application. The smallest mycotoxin concentrations that resulted in no colour development on the detection immunolayers were defined as the cut-off levels. For samples contaminated with mycotoxins equal or above this cut-off level, no blue colour appeared and they were considered to be positive. When a blue colour appeared, even substantially lighter coloured than the blank control sample, the sample was considered to be negative.

7.4.3.4 LC-MS/MS

Before the LC-MS/MS determination, a clean-up and preconcentration procedure was performed using AflaOchra HPLC^{TM} immunoaffinity columns. Spice sample (10 g) was extracted with 40 mL MeOH/water (80/20, v/v) for 1 h using an end-over-end shaker and centrifuged for 10 min at 3452 g. The supernatant (10 mL) was diluted with 40 mL of water, filtered and applied onto the AflaOchra HPLC^{TM} immunoaffinity column. The column was washed with 10 mL of water. OTA and aflatoxins were eluted with 1.5 mL of MeOH. This was completely evaporated with a gentle stream of nitrogen at 60 °C and redis-

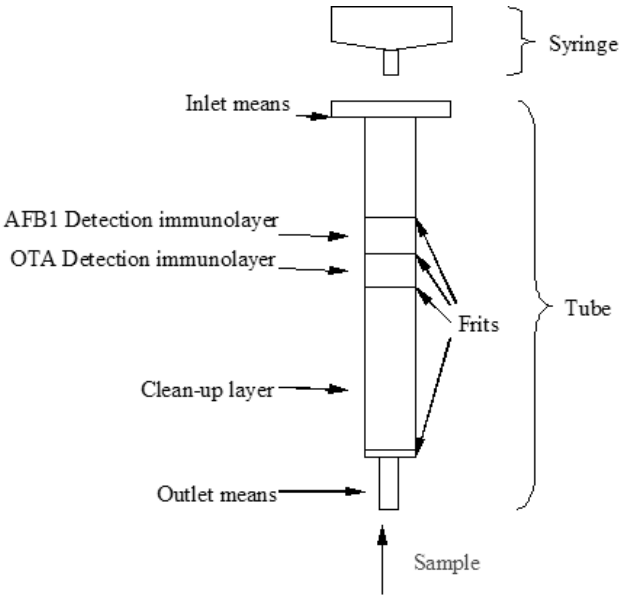

Figure 7.1: Clean-up tandem immunoassay column set-up with two detection immunolayers

solved in 200 μL of MeOH/water (30/70, v/v).

The mobile phase consisted of variable mixtures of water (solvent A) and methanol (solvent B), both with ammonium acetate (10 mM) and sodium acetate (20 μM), at a flow of 0.3 mL min^{-1} with a gradient elution programme: linear gradient from 70 to 50 % A for 1.5 min, linear gradient from 50 to 40 % A for 9.5 min, linear gradient from 40 to 15 % A for 1 min, isocratic conditions with 15 % A:85 % B for 7 min. Then, within 1 min, the concentration of A was brought back to 70 %. This was maintained for 15 min. The column was kept at room temperature. The injection volume was 20 μL.

The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode using multiple reagent monitoring (MRM). Capillary voltage was 3.7 kV and nitrogen was used as spray gas. Source and desolvation temperatures were set at 120 and 300 °C, respectively. The precursor ions (*m/z*) were 313 for AFB_1, 315 for AFB_2, 329 for AFG_1, 331 for AFG_2 and 404 for OTA. Cone voltages, product ions and collision energies were determined [4].

For aflatoxins and OTA quantification, blank chilli samples were fortified with AFB_1, AFB_2, AFG_1 and AFG_2 at 0.5, 1.0, 2.5, 5.0 and 12.5

μg kg^{-1} and with OTA at 1.0, 2.0, 5.0, 10 and 25 μg kg^{-1} (= calibration curves).

7.5 Results and discussion

7.5.1 Extraction of AFB_1 and OTA

For individual mycotoxin extraction from spices, two ways were previously described. An extraction with acidified chloroform was used for OTA extraction from red pepper [293] and also from black pepper, white pepper and spice mixture samples [87]. A more common way without chlorinated organic solvents is AFB_1 or OTA extraction with methanol (or acetonitrile) - water (or aqueous $NaHCO_3$ or KCl solutions) mixtures [111, 168, 304, 305]. For completeness of mycotoxin extraction, sodium chloride could be added to the spice sample before extraction [87, 171, 306].

In previous experiments with one of the red pepper samples it was shown that the recovery of OTA with MeOH/3 % $NaHCO_3$ (80:20 v/v) solution was 79 $\pm$ 5 % while in presence of NaCl (20 % w/w) it was 94 $\pm$ 6%. This difference is not important for rapid method development. Consequently, to simplify the assay procedure and to avoid salt weighing, extraction without additional salts was used.

7.5.2 Clean-up optimization

The main goal of adding the clean-up layer was to reduce matrix effects of sample extracts on the developed colour of the detection immunolayers. At the same time, sorption of the mycotoxins on the clean-up layer had to be as minimal as possible. As for mycotoxin extraction MeOH-water solutions are usually used, for selection of optimal clean-up condition methanol-water (pH 5.5) and methanol-$NaHCO_3$ (pH 9.0) solutions of mycotoxins were tested. For stability of detection immunolayers in the clean-up tandem immunoassay columns, PBS was used. To control possible PBS effects on the clean-up layer, dry NH_2 layer and NH_2 layer kept in contact with PBS for 1 h, 1 day and 10 days were tested.

Presence of $NaHCO_3$ dramatically influenced the OTA recovery from the NH_2 clean-up layer (Figure 7.2). Application of MeOH/3 % $NaHCO_3$ water solution (25/75, v/v) with pH 9.0 gave very high OTA recoveries ranging between 87 - 93 %, but application of MeOH/water

(25/75, v/v) with pH 5.5 resulted in very weak OTA recoveries (8 - 12 %). In the case of AFB_1 no pH effect was obtained, but recoveries from clean-up layer were slightly lower (77 - 90 %) than for OTA. So MeOH/3 % $NaHCO_3$ water solution was chosen for extraction and extract dilution before clean-up tandem multi immunoassay column application. It is interesting to mention that AFB_1 recovery slightly decreased when PBS was used for conditioning of the NH_2 layer, but this effect was not strong. The difference in AFB_1 recovery between an NH_2 layer conditioned for 1 h and for 10 days was 10 %. For OTA there was no difference.

7.5.3 Clean-up tandem immunoassay optimization

In our previous work, clean-up tandem immunoassay columns were developed for rapid OTA detection in highly coloured foodstuffs such as roasted coffee and spices [171, 264]. Now the goal was to develop a column with one clean-up layer and two (or theoretically more than two) detection immunolayers. For column preparation, two different immunogels were used - with coupled anti-AFB_1 and anti-OTA primary mouse antibodies.

As analytical signal for mycotoxin determination, the colour development of the detection immunolayers was used. The main requirements for obtaining reproducible multiassay results in this format are an equal colour intensity for both detection immunolayers and the same time for colour development. Primary parameters influencing colour intensity and time of its development are concentrations of monoclonal primary mouse antibodies and conjugates. Increasing of concentrations of antibodies and conjugates led to more intense developed colour in shorter time. But at the same time the sensitivity of assay decreased. Volume of the extract is also an important parameter. Increasing the extract volume could result in higher sensitivity of the assay but the possible matrix effects could become stronger. Besides for field test development minimization of the solution volume (especially for organic solution) is important. We used 0.5 mL of extract diluted with 1.0 mL of an aqueous $NaHCO_3$ (3 %) solution to reduce the methanol concentration, thus, avoiding the denaturation of the antibodies in the detection immunolayer. The approximate time for sample input was about 1-2 min.

Analysis procedures were developed to obtain cut-off levels for AFB_1 at 5 μg kg^{-1} and for OTA at 10 μg kg^{-1}. The optimal concen-

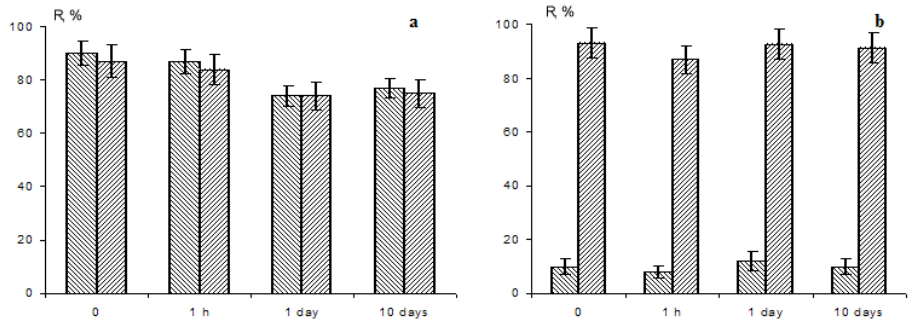

Figure 7.2: AFB_1 (a) and OTA (b) recovery from the aminopropyl derived silica clean-up layer with MeOH/water (25/75, v/v) (left columns) and MeOH/3%$NaHCO_3$ (25/75, v/v) solutions (right columns): dry layer and conditioned with PBS for 1 h, 1 day and 10 days

trations of monoclonal antibody solution were 10 μg mL^{-1} (dilution 1/100) for monoclonal anti-OTA antibodies and 2.5 μg mL^{-1} (dilution 1/400) for monoclonal anti-AFB_1 antibodies. The detection time was chosen as 5 min after chromogenic substrate application (100 μL) and was obtained with conjugate dilutions of 1/100 (100 μL) both for AFB_1-HRP and OTA-HRP.

For simultaneous AFB_1 and OTA determination, a set of mycotoxin concentrations was used for validation purposes. As mentioned before, the level of 5 μg kg^{-1} was legislated for AFB_1 in spices and therefore was chosen as cut-off level. A set of samples spiked with AFB_1 at 0, 2.0, 3.5, 5.0 μg kg^{-1} was used. According to a presumptive legal limit for OTA in spices, the cut-off level was set at 10 μg kg^{-1}. A set of samples spiked with OTA at 0, 4.0, 7.0, 10 μg kg^{-1} was used. Samples of chilli, nutmeg, black pepper and ginger fortified with AFB_1 and OTA were used. The intensity of the developed colours decreased with increasing concentrations of both mycotoxins (Figure 7.3). No blue colour developed at concentrations of 5 μg kg^{-1} for AFB_1 and 10 μg kg^{-1} for OTA.

7.5.4 AFB_1 and OTA simultaneous detection in spice samples

Forty four samples of spices were screened with the clean-up tandem multi immunoassay column. An absence of developed colour was interpreted as a positive result, development of blue colour showed a negative result. For a screening method it is very important to prevent

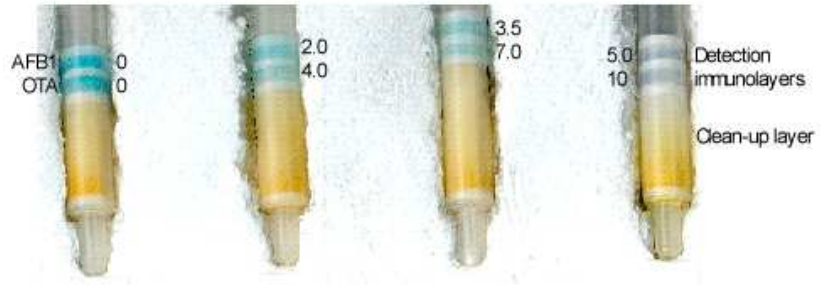

Figure 7.3: Clean-up tandem multi-immunoassay. Two detection immunolayers and one clean-up layer of each column are shown and the colour can be visually evaluated. AFB_1 and OTA concentrations (μg kg^{-1}) of the chilli samples are indicated near the tubes. Cut-off levels (= no colour development) were at 5 μg kg^{-1} for AFB_1 and 10 μg kg^{-1} for OTA.

false negative results. So for each analysed sample of spices the influence of possible matrix effects was checked. Therefore, one portion of extract was artificially contaminated with both mycotoxins (spike levels corresponded to 5 μg kg^{-1} AFB_1 and 10 μg kg^{-1} OTA) and analysed together with the non-spiked part of extract. It was shown that for all artificially contaminated samples there was no colour development of both detection immunolayers. So we could conclude that after clean-up there were no matrix effects (no false negative results) for *Capsicum* spp. spices, ginger, nutmeg, black pepper and white pepper samples.

Obtained results are presented in Table 7.1. All samples were also analysed with a clean-up tandem immunoassay column with only one detection layer (for OTA or AFB_1 only). The same results were obtained as for the column with two detection immunolayers. So we can conclude that neither immunolayers nor OTA-HRP and AFB_1-HRP conjugates interfered with each other during real sample analysis. This could give an assurance that the proposed approach could be useful for determination of not only two, but more different analytes in one clean-up tandem immunoassay column.

Table 7.1 shows that among the tested samples of spices only *Capsicum* spp. spices were contaminated with OTA and AFB_1 above the maximum level. In total 32 and 23 % of *Capsicum* spp. spices were

contaminated with OTA and AFB_1, respectively. These results are in agreement with literature data about spices contamination with mycotoxins [87, 165, 168, 169, 170, 173, 307]. It is interesting to mention that in five samples both AFB_1 and OTA, and in one paprika and one pili-pili sample only OTA, were detected above the maximum level. This finding suggests that aflatoxins and OTA contamination very often occurs together, as reported by Fazekas *et al.* [87].

Obtained results were confirmed by LC-MS/MS with AflaOchra HPLC^{TM} preconcentration for part of the samples. LC-MS/MS results confirmed both positive and negative clean-up tandem immunoassay results (Table 7.2). Figure 7.4 presents a LC-MS/MS chromatogram for one of the red pepper samples. It can be seen that AFG_1 and AFG_2 peaks are insignificant while the AFB_2 peak is small. For all tested spices the concentrations of aflatoxins B_2, G_1 and G_2 were much lower than for AFB_1. This is in agreement with other studies which showed that for spices contaminated with AFB_1, only few of them also contained AFB_2, AFG_1 and AFG_2 at levels much lower than for AFB_1 [165, 169, 170]. This indicates that aflatoxin B_1 contamination is much more significant in spices. So we suggest that for the development of the immunoassay screening test for aflatoxins the detection of only AFB_1 at 5 μg kg^{-1} is enough, besides very often anti-AFB_1 antibodies have high cross-reactivity for other aflatoxins and a positive result for AFB_1 is a sufficient motive for detail analysis of aflatoxins in the sample by LC-MS/MS or another technique.

Table 7.1: Aflatoxin B_1 and ochratoxin A content in spice samples after screening with the clean-up tandem multi-immunoassay column

Spices	**Amount, *n***	**Positive for AFB$_1$ (> 5 μg kg^{-1})**		**Positive for OTA (> 10 μg kg^{-1})**	
		n	**%**	***n***	**%**
Paprika	6	1	17	2	33
Red pepper and pili-pili	7	2	29	3	43
Chilli	4	1	25	1	25
Cayenne	5	1	20	1	20
Nutmeg	7	-	0	-	0
Black pepper	5	-	0	-	0
White pepper	5	-	0	-	0
Ginger	5	-	0	-	0
Total	44	5	11	7	16

n, number of samples

Table 7.2: Aflatoxin B_1 and ochratoxin A determination in spices by the clean-up tandem multi-immunoassay column and LC-MS/MS

Spices	Clean-up tandem multi immunoassay		LC-MS/MS		
	AFB_1 (cut-off 5 μg kg^{-1})	OTA (cut-off 10 μg kg^{-1})	AFB_1 (μg kg^{-1})	Total aflatoxins[a] (μg kg^{-1})	OTA (μg kg^{-1})
Paprika 1	-[b] - -	- - -	-[c]	-[d]	-[e]
Paprika 2	- - -	- - -	1.7	2.4	6.3
Paprika 3	+[f] + +	+[g] + +	6.8	8.0	17
Pili-pili 1	- - -	- - -	-	-	-
Pili-pili 2	- - -	- - -	-	-	1.2
Red pepper 1	+ + +	+ + +	8.2	9.1	15
Red pepper 2	+ + +	+ + +	5.4	6.4	30
Chilli 1	- - -	- - -	-	-	-
Chilli 2	- - -	- - -	-	-	-
Chilli 3	+ + +	+ + +	17	21	19
Cayenne 1	- - -	- - -	-	-	1.3
Cayenne 2	- - -	- - -	0.6	0.6	1.4
Ginger 1	- - -	- - -	-	-	-
Ginger 2	- - -	- - -	-	-	2.3
Ginger 3	- - -	- - -	-	-	1.0
Nutmeg 1	- - -	- - -	-	-	-
Nutmeg 2	- - -	- - -	-	0.8	-
Nutmeg 3	- - -	- - -	2.1	3.9	7.1
Black pepper 1	- - -	- - -	-	-	-
Black pepper 2	- - -	- - -	-	-	-
Black pepper 3	- - -	- - -	-	-	-
White pepper 1	- - -	- - -	-	-	-
White pepper 2	- - -	- - -	-	-	-
White pepper 3	- - -	- - -	-	-	-

[a] Total aflatoxin concentration was calculated as sum of AFB_1, AFB_2, AFG_1 and AFG_2 concentrations; [b] (-) - blue colour development of the detection immunolayer, negative result; [c] AFB_1 concentration < 0.5 μg kg^{-1} [d] concentration of each aflatoxin < 0.5 μg kg^{-1}; [e] OTA concentration < 1.0 μg kg^{-1}; [f] (+) - no blue colour development of the detection immunolayer; AFB_1 concentration ≥ 5 μg kg^{-1}, positive result; [g] (+) - no blue colour development of the detection immunolayer; OTA concentration ≥ 10 μg kg^{-1}, positive result.

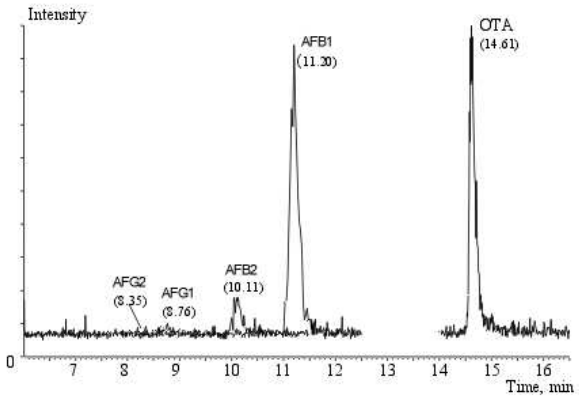

Figure 7.4: Chromatogram of the five mycotoxins from a naturally contaminated red pepper sample. Name of mycotoxin and retention time are indicated near the peaks.

7.6 Conclusion

An approach for two analytes rapid non-instrumental detection was developed. It was based on the column with one clean-up layer and two detection immunolayers. Application of the special clean-up layer allowed to use these clean-up tandem multi-immunoassay columns for intense coloured extracts, like spices extracts. Clean-up tandem multi-immunoassay columns were applied for rapid simultaneous aflatoxin B_1 and ochratoxin A determination with visual detection with cut-off level 5 and 10 μg kg^{-1}, respectively. The test was applied for simultaneous determination of aflatoxin B_1 and ochratoxin A in all sorts of spices, for which the EU limitation for aflatoxins exists and limitation for OTA is under discussion.

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Chapter 8

Influence of strongly coloured foodstuffs on the development of rapid membrane-based mycotoxin tests

8.1 Background and objectives

Considering advantages and disadvantages of the use of membranes (flow-through immunoassays) and columns (*clean-up tandem immunoassays*) for rapid mycotoxin detection, a new format for red wine and beer was developed. Membranes, spotted with antibodies (rapid and easy preparation), were placed at the bottom of columns (high sample volume application possible). Because membranes adsorb the colour of the matrix, giving interference with the visual detection, a rapid and easy clean-up has to precede the assay for strongly coloured foodstuffs. Aminopropyl derived silica seemed to give the best result as clean-up, both for red wine and beer. Cut-off levels according to EU legislation were not yet reached. Therefore, the methods will be further optimized.

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8.2 Abstract

Nowadays, several rapid membrane tests are commercially available for mycotoxin detection. These are qualitative tests with visual evaluation of coloured dots or lines. However, these membrane tests can only be used for a limited number of foodstuffs. Indeed, strongly coloured foodstuffs will interfere with the visual detection. E.g. red wine and beer will cover and colour the membrane such that visual detection becomes impossible.

Therefore, a suitable clean-up procedure must precede the assay. Of course, in view of the *on-site* applications, this procedure should be rapid and simple, with a minimum of handling steps.

In this paper, several easy-to-use clean-up methods were studied for red wine and beer. Aminopropyl derived silica was used for further development of a membrane test for ochratoxin A (OTA) detection in red wine and a multi-test for simultaneous detection of OTA and deoxynivalenol (DON) in beer.

Key words: Rapid test, red wine, beer, ochratoxin A, deoxynivalenol

8.3 Introduction

Because of their widespread natural occurrence in agricultural products and their diverse toxic effects, the presence of mycotoxins in foods and feed is considered to be potentially hazardous to humans and animals [61, 308, 309, 310, 311, 312, 313].

In wine the most important mycotoxin is ochratoxin A (OTA) which is not appreciably degraded during wine making, the fermentation process and storage. After cereals, wine is considered a major source of daily OTA intake [17] and could account for as much as 15 % of the total OTA intake [26]. Regulatory levels have been discussed with regard to wine, for which a maximum level (ML) of 2.0 μg L^{-1} has been recently fixed [1].

Beer shows a similar mycotoxin problem. If grains contaminated with mycotoxins are used for beer production, they may introduce them into the brewing process, e.g. if present in barley used for malting or in a cereal adjunct (maize, wheat, rice) or if *Fusarium* spp. grow during the germination of barley in the malting process [218]. The detected exposure to OTA from beer consumption does not represent a serious health risk for the beer consumer [201, 314]. However, it

is worthwhile to include beer in calculations of total human OTA exposure [197]. In Europe, an ML of 200 ng OTA L^{-1} [196] has been proposed.

Deoxynivalenol (DON) has been found at higher levels in beer [214]. As beer significantly contributes to the diet of a world-wide population [214], beer consumption should be taken into account when evaluating the total exposure dose of DON to humans from cereal sources [204, 205]. So far, no regulatory guideline for DON contamination in beer is available in terms of safe limit. Indeed, the recent European Commission Regulation No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, covers only cereals and cereal-based products, not including beer [1].

As consumers are exposed to serious health risks by consuming mycotoxin-contaminated food, this enforces the need to implement control and prevention measures [201].

Various methods including reversed-phase high performance liquid chromatography with fluorescence detection (HPLC-FLD) [27, 315, 316, 317, 318] or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [26, 30, 38, 319] have been published for the determination of OTA in wine and beer. For the detection of DON in beer, HPLC-UV [320], LC-MS/MS [321] or gas chromatography with flame ionisation detection (GC-FID) [203], electron-capture detection (EC) [202] or mass spectrometry [70, 322] is used. The complexity of the samples requires a pre-treatment step such as solvent extraction or immunoaffinity columns (IACs), which enables isolation of the toxin from the matrix. However, the application of specific stationary phases, especially IACs, is expensive and labour intensive.

Besides chromatographic methods, enzyme-linked immunosorbent assays (ELISA), based on antigen-antibody reactions, have been developed for the screening of OTA in wine [120], OTA in beer [198] and DON in beer [204, 205]. Other analytical developments are array biosensors for the detection of OTA in beverages [50].

The need for *on-site* rapid-response tests providing a binary yes/no response is growing. Such a field approach requires speed and minimal steps, not only for the assay but also for the preceding sample preparation. Sample dilution is commonly used in ELISA. Simple adjustment of pH (neutral) was successfully used before a biosensor assay for OTA in coffee but failed to improve assay results for red wine [50]. Alarcon *et al.* (2004) [289] used extraction with chloroform to reduce matrix interferences for OTA detection in wine with an electrochemical

immunosensor. High molecular weight polyethylene glycols (PEGs) (6000 and 8000 molecular weights) were applied to facilitate antibody binding activity in wine, beer and fruit juices [187, 323, 324]. However, almost no research was performed on decreasing sample colour and the resulting matrix interferences for *on-site* tests based on visual detection, such as membrane tests. Indeed, strongly coloured foodstuffs completely cover and colour the membrane, making visual detection impossible. Commercially available membrane-based tests for mycotoxin detection can therefore only be applied to a limited number of foodstuffs.

This paper deals with the selection of a suitable, simple and rapid clean-up method for red wine and beer. This was further applied to new membrane-based flow-through immunoassay columns for OTA detection in red wine and for the simultaneous detection of OTA and DON in beer.

8.4 Experimental

8.4.1 Reagents and materials

OTA and DON standards and gelatin from cold water fish skin were purchased from Sigma Chemical Co. (Bornem, Belgium). Polyethylene glycol (PEG) 6000 was from Federa (Brussels, Belgium). Rabbit anti-mouse immunoglobulins (IgG) (protein concentration: 2,5 g L^{-1}) were supplied by Dako (Heverlee, Belgium). Immunodyne ABC membranes, pore size 0.45 μm; nylon membrane filters, Nylaflo®, 0.45 μm, 47 mm; cellulose membrane filters GA-8 Supor 47 mm, 0.2 μm were from Pall France (Saint Germain-en-Laye, France). Tubes (Bond Elut® reservoir, 3 and 6 mL), polyethylene frits (1/4 in. and 3/8 in. diameter), Bond Elut® SAX columns (500 mg), Bond Elut® Si columns (500 mg), Bond Elut® C_8 columns (500 mg), Bond Elut® PH columns (500 mg), Bond Elut® CN columns (500 mg) and aminopropyl derived silica (NH_2 derived silica) (Bio-Sil NH_2; diameter 0.040 μm) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium). For NH_2 derived silica clean-up, tubes were filled with this bulk agent. Bakerbond^{TM}SPE C_{18} columns were purchased from JT Baker (Deventer, Holland). Kieselgel 60 (0.063-0.200 mm) (SiO_2) was from Merck (Darmstadt, Germany). Monoclonal antibodies against OTA were supplied by Soft Flow, Gödöllö, Hungary. Ochratoxin A-horse radish peroxidase (OTA-HRP) and deoxynivalenol-horse radish

peroxidase (DON-HRP) conjugates were supplied by Euro-Diagnostica (Arnhem, The Netherlands). DON antibodies (clone # 4) were supplied by Dr. Chris Maragos (USDA ARS, Mycotoxin Research Unit, Peoria, IL, USA). Ochratoxin A-alkaline phosphatase (OTA-AP) and deoxynivalenol-alkaline phosphatase (DON-AP) conjugates were prepared in the laboratory [325]; the standards were purchased from Sigma Chemical Co. (Bornem, Belgium), ImmunoPure AP (no. 31391) was supplied by Pierce (Rockford, IL, VS). Phosphate buffered saline (PBS) 0.01 M, pH 7.4, was used to prepare the assay buffer (PBS-casein 0.05 %), the blocking buffer (PBS-casein 2 %) and the washing buffer (PBS-Tween 0.2 %). Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). Stock solutions of OTA and DON (1 mg mL^{-1}) and working solutions (100, 10 and 1 ng μL^{-1}) were prepared in methanol and stored at -20 °C. The substrate chromogen solution used for the HRP-enzyme was Colorburst^{TM}Blue TMB/Peroxide (ALerCHEK, Inc., USA). BCIP/NBT-developer (5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue-terazolium) (BioFX Laboratories, Owing Mills, MD, VS) was used as substrate for the AP-enzyme. The wine and beer samples were obtained in local stores and were found to be negative for OTA (HPLC-FLD: OTA in wine, OTA in beer). DON contents were measured with LC-MS/MS and found to be 0.125 μg L^{-1} in a blond beer and 0.0625 μg L^{-1} in a dark beer.

8.4.2 Preparation of the flow-through column

The flow-through column for the analysis of red wine was prepared as follows. On an Immunodyne membrane, several circles of 1/4 inch diameter were drawn. These disks were completely covered with undiluted rabbit anti-mouse antibodies ($\pm$ 3 μL) and the membrane was dried for 30 minutes at 37 °C. Monoclonal mouse anti-OTA antibodies (diluted in assay buffer), 0.7 μL, were spotted in the middle of each 1/4 inch diameter disk of the membrane. The membrane was dried again for 30 minutes at room temperature. The remaining part of the membrane was blocked with blocking buffer for 30 minutes after which the membrane was dried at 37 °C for 30 minutes. Then the membrane disks were cut using scissors.

For the assays without the use of secondary antibodies, the anti-

OTA antibodies (diluted in assay buffer) (0.7 μL) were immediately spotted in the middle of the 1/4 inch membrane disks.

A frit (1/4 inch) was put at the bottom of a 3 mL Bond Elut® reservoir. A membrane disk covered this frit. A perforated frit (1/4 inch) was put on top of the membrane to fix it (Figure 8.1).

For beer, a multi-assay column was developed. On an Immunodyne membrane, several circles of 3/8 inch diameter were drawn. These disks were completely covered with undiluted rabbit anti-mouse antibodies ($\pm$ 5 μL) and the membrane was dried for 30 minutes at 37 °C. Monoclonal mouse anti-OTA antibodies and anti-DON antibodies (diluted in assay buffer), 0.7 μL, were spotted separately at two different places on each 3/8 inch diameter disk of the membrane and the membrane was dried for 30 minutes at room temperature. A pencil mark indicated the position of each spot. The remaining part of the membrane was blocked with blocking buffer for 30 minutes after which the membrane was dried at 37 °C for 30 minutes. Then, the membrane disks were cut using scissors.

For the assays without the use of secondary antibodies, the anti-OTA antibodies and anti-DON antibodies (diluted in assay buffer) (0.7 μL) were immediately spotted on the 3/8 inch membrane disks.

A frit (3/8 inch) was put at the bottom of a 6 mL Bond Elut® reservoir. A membrane disk covered this frit. A perforated frit (3/8 inch) was put on top of the membrane to fix it (Figure 8.1).

8.4.3 Rapid simple clean-up methods for red wine

The following different clean-up methods were applied to red wine samples (2 mL) and compared in terms of colour reduction.
Gelatin: 5 drops of gelatin were added to the sample with a pipette, whether or not in combination with 2 g of silicon dioxide (SiO_2) or sample dilution with 2 mL of a 3 % aqueous $NaHCO_3$ solution;
Membrane filtration: the samples were filtrated through a cellulose/nylon membrane using a syringe and a membrane holder;
pH adjustment: the pH of the sample was adjusted with drops of a 3 % aqueous $NaHCO_3$ solution while evaluating the colour after each drop;
Various solid phase materials: NH_2 derived silica, silica (silanol groups), octadecyl derived silica (silica-C_{18}), strong anion exchange silica (SAX). Therefore, undiluted samples were applied onto clean-up columns, containing 500 mg of solid phase material. The colour of 2 mL red wine samples, diluted with 2 mL of an aqueous solution

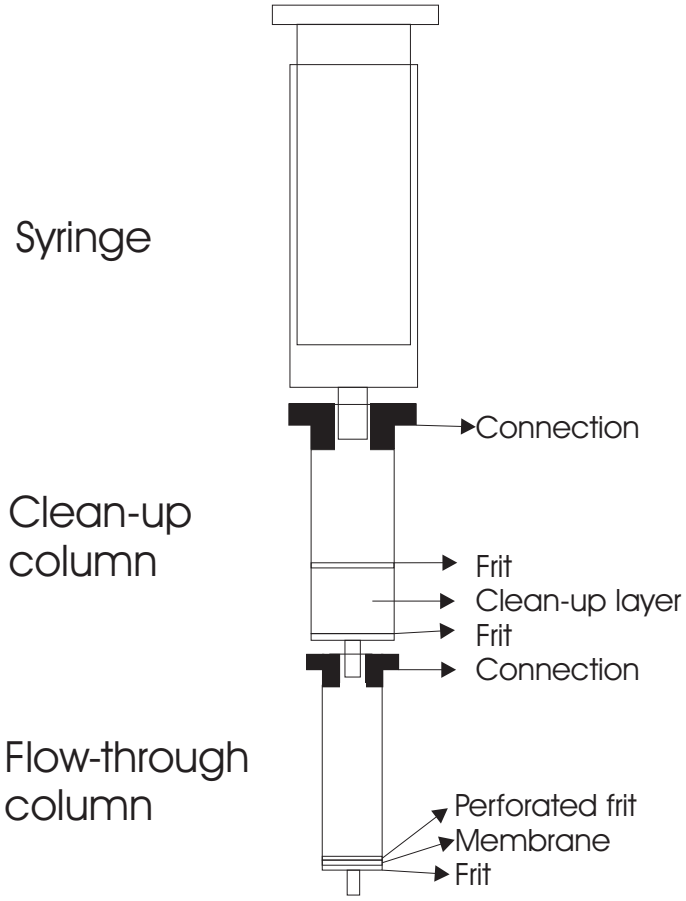

Figure 8.1: Set-up of the flow-through column connected with a clean-up column and syringe

containing 5 % $NaHCO_3$ and 1 % PEG 6000, was also evaluated after clean-up on 500 mg of sorbent.

To study the recovery of the most suitable materials, OTA fortified (5, 15 and 25 ng mL^{-1}) undiluted 2 mL samples and 2 times diluted samples were applied onto 500 mg of the sorbent. The eluate from the column was collected and the OTA concentration measured by high performance liquid chromatography-fluorescence detection (HPLC-FLD). Sample extracts (50 μL) were injected with an autosampler and separated on a Supelco Discovery C_{18} (5 μm, 25 cm x 4.6 mm) column (Supelco, Bellefonte, PA, USA) at room temperature. The mobile phase used was acetonitrile/water/acetic acid (54:44:2, v/v) at a flow rate of 1 mL min^{-1}. The HPLC system consisted of a Waters 600 Controller, a Waters 610 fluid unit and a Waters 474 scanning fluorescence detector (333 nm excitation wavelength, 470 nm emission wavelength). Sample OTA concentrations were quantified by comparing sample peak areas to those of standards. The recovery of OTA was calculated as a ratio of the OTA concentration after and before the clean-up step.

8.4.4 Rapid simple clean-up methods for beer

The following different clean-up methods were applied to degassed beer samples (3 mL) and compared in terms of colour reduction.

Zinc acetate: 1 g was added to the sample to precipitate the colour of the beer;

Gelatin: 5 drops of gelatin were added to the sample with a pipette, whether or not in combination with 2 g of SiO_2 or sample dilution with 2 mL of a 3 % aqueous $NaHCO_3$ solution;

Membrane filtration: the samples were filtrated through a cellulose/nylon membrane using a syringe and a membrane holder;

Clean-up in the assay: 2 mL of a cleaning solution containing trifluoroacetic acid or propionic acid or sodium bicarbonate was applied over the immunosorbent area of the assay membrane after applying the sample [326];

Various solid phase materials: NH_2 derived silica, octyl derived silica (silica-C_8), phenyl derived silica (silica-PH), silica-C_{18}, SAX, cyanopropyl derived silica (silica-CN). Therefore, undiluted beer samples were applied onto clean-up columns, containing 500 mg of sorbent.

8.4.5 Assay

The sample extract was put on top of the NH_2 derived silica clean-up layer (see Results: Optimal clean-up) in the clean-up column (Figure 8.1). By pressure of an adapted syringe, the sample was pushed through the clean-up column and interfering substances were adsorbed on the clean-up layer. Mycotoxin(s) present in the sample bound to the antibodies immobilized on the membrane of the flow-through column (8.4.2). The clean-up column was then disconnected. After adapting the syringe to the flow-through column, pressure was given to wash the membrane with washing buffer (2 mL). Then, the mycotoxin-enzyme conjugate(s) (diluted in assay buffer), 200 μL, was (were) added. After another washing step (1 mL), the substrate (100 μL) was added and colour development was evaluated. For the beer assay, two different enzyme conjugates were applied and the substrate for AP was first added. After colour development, the membrane was washed with water (100 μL) and the substrate for HRP was subsequently applied onto the membrane.

8.5 Results and discussion

8.5.1 New format: Flow-through column

Dipstick, flow-through and lateral flow immunoassays are membrane tests which are widespread in the rapid test area. But, strongly coloured samples cover and colour the membrane, in this way interfering with the visual detection. Therefore, a gel-based clean-up tandem immunoassay column [171, 264, 327] was developed. This consisted of a gel detection layer, replacing the membrane, and a clean-up layer, capable of adsorbing interferences. However, the preparation and binding of antibodies is more rapid and easier in a membrane format, compared to the previous described gel-based method. Therefore, the new format was membrane-based. Combining a membrane with a column permitted to apply larger sample volumes making enrichment of the analyte possible. To overcome the disadvantage of colouring the membrane, combining this new membrane format with a rapid and easy-to-use sample preparation was performed.

8.5.2 Optimal clean-up for red wine

An optimal clean-up removes compounds, responsible for the red wine colour such as the flavonoids. Also, it needs to be rapid and OTA extraction must be sufficient. It should be emphasized that the amount of colour reduction was not quantitatively measured, but visually evaluated.

Gelatin is a positively charged protein that can react with negatively charged polyphenolic compounds such as tannin in red wine, thereby reducing the astringency, bitterness and colour. Gelatin also reacts with negatively charged colloidal SiO_2 to form a dense coagulum with the destabilized silica, resulting in clarification [328]. Some drops of gelatin in a red wine sample gave precipitation and could already remove some of the colour after filtration. Still, the filtered sample was not suitable to undergo the membrane assay, neither when the wine sample was first diluted with a 3 % aqueous $NaHCO_3$ solution. The combination of gelatin and SiO_2 needed an overnight settling, making this a too slow method. The supernatants also remained coloured.

Cellulose membranes only adsorbed a limited amount of colour. Multiple nylon membranes gave better results but total decolourization was not possible.

Anthocyanins are very stable and highly coloured at low pH values and gradually lose colour with increasing pH. At pH 4-5, the anthocyanin is almost colourless [329]. Making the wine sample more alkaline using a 3 % aqueous $NaHCO_3$ solution, red colour changed to black, giving black coloured assay membranes. For red wine no pH value was found at which the sample was colourless.

Activated carbon is a good adsorbent for the anthocyanins, however it also removes OTA from wine [330].

Of the tested solid phase materials, only SAX and NH_2 derived silica resulted in almost colourless eluates. NH_2 derived silica clean-up produced a yellow-green, almost transparent eluate. SAX clean-up yielded a lighter coloured red eluate. Sáez *et al.* (2004) described that dilution with salts (5 % $NaHCO_3$, 1 % PEG 6000) gives a higher recovery [38]. Dilution of the red wine sample with salts yielded a black coloured solution after NH_2 derived silica clean-up and a light purple transparent sample after SAX clean-up.

The mean recovery of undiluted spiked red wine samples after NH_2 derived silica clean-up was 8 % while diluted spiked red wine samples yielded a mean recovery of 50 %. For clean-up with SAX, these values were 14 % and 20 %, respectively. NH_2 derived silica was chosen as

clean-up because of the best recovery. To obtain a colourless filtrate the sample volume was reduced (1 mL) so that dilution with the alkaline solution was possible (higher recovery).

8.5.3 Optimal clean-up for beer

An optimal clean-up removes pigments and other interferences from beer. It should also be rapid while not removing OTA and DON from the beer sample.

Zinc acetate can act as a precipitating agent for dyes and other components of beer [331]. Pal *et al.* (2005) used an *in-situ* clean-up in an assay for groundnut, wheat, corn and poultry feed samples [326]. These treatments however, and the use of gelatin and membrane filtration were not able to decolourize beer.

Of the tested solid phase materials, only NH_2 derived silica, silica-CN and SAX yielded colourless or pale yellow coloured filtrates. Because of the bulk availability in the lab, research was continued with the NH_2 derived silica.

8.5.4 Optimization of the assays

The secondary antibody technique was compared with direct spotting of primary antibodies on the membrane in regard to sensitivity. However, using secondary antibodies, a better sensitivity than 10 μg OTA L^{-1} red wine, 2 μg OTA L^{-1} blond beer and 50 μg DON L^{-1} blond beer (sensitivity reached using direct spotting, Figures 8.2 and 8.3) could not be achieved. Therefore, direct spotting was further applied.

For red wine 500 mg of NH_2 derived silica was used for clean-up of 1 mL sample diluted with 0.5 mL of salt solution (5 % $NaHCO_3$, 1 % PEG 6000). This yielded a colourless filtrate.

For beer, differences were noted between blond and dark beer. Blond beer (5 mL) was cleaned on 300 mg NH_2 derived silica, while for dark beer the amount was increased to 500 mg.

A high background colour was observed for the HRP assay. Therefore, PBS-Tween 0.2 % was used as washing buffer instead of PBS-Tween 0.05 % [264] to be sure that all unbound HRP conjugate was removed. To avoid this problem, OTA-AP was further used. For DON, the HRP-enzyme was further applied because of the better sensitivity. Applying DON-AP conjugate after a beer sample, containing 200 μg DON L^{-1}, even yielded a coloured spot.

For the beer multi-assay 2 different enzymes were used, giving 2 different coloured spots for OTA and DON. But, the substrates for HRP and AP could not be added together because of interference (no colour development). A washing step with water in between the addition of the two substrates could solve this problem.

Figures 8.2 and 8.3 show results of the assay for red wine and blond beer, respectively, both with direct spotting of the primary antibodies. For beer, a higher colour intensity was observed when buffer solution was used instead of beer. By increasing the sample volume, sensitivity increased but at the same time, more NH_2 derived silica was needed as clean-up. However, as studied in Lobeau et al. (2007) [327], during the clean-up there is partitioning of OTA between the eluate and NH_2 derived silica. Consequently, more clean-up resulted in a higher loss of toxins in the eluate. So, a compromise was made in order to adsorb the interferences, but not retaining the mycotoxin(s). In these assays the European ML of 2 μg OTA L^{-1} wine, the proposed level of 200 ng OTA L^{-1} beer and our proposed level of 50 μg DON L^{-1} beer were aimed for. Because of the above mentioned restriction, the OTA levels have not yet been reached. Anyway, the proposed OTA level for beer is extremely low.

An important remark is the problem of 'intra-matrix' variability for red wines and beers. Not every red wine interacted the same way with a particular clean-up layer. Figure 8.4 shows the results of 12 different red wine brands, cleaned on 500 mg of NH_2 derived silica. For most of the samples the clean-up yielded almost colourless filtrates while for brands 1 and 8, darker eluates were obtained. In Figure 8.5, the results are shown of a blond and a dark beer sample before and after NH_2 derived silica clean-up. A rapid method for a blond beer with the appropriate rapid clean-up, gave false results with interferences on the membrane for a dark beer with the same clean-up.

8.6 Conclusions

In this newly designed format, advantages on the one hand of membranes (rapid and easy preparation) and on the other hand of columns (possibility of using high sample volumes and higher sensitivity) are combined. For strongly coloured foodstuffs, membranes however show the disadvantage of adsorbing the sample colour, giving interferences with the visual detection. In this study the most suitable rapid clean-up was selected for red wines and beers. This was NH_2 derived

Figure 8.2: Result of a red wine assay: anti-OTA antibodies spotted in dilution 1/20, 0.7 μL, OTA-AP-dilution 1/500, 1 mL red wine sample diluted with 0.5 mL of salt solution, left side: no OTA, right side: spiked sample, OTA, 10 μg L^{-1}

Figure 8.3: Result of a blond beer assay: anti-OTA antibodies and anti-DON antibodies spotted in dilution 1/10, 0.7 μL, OTA-AP-dilution 1/500, DON-HRP-dilution 1/2200, 5 mL blond beer sample, left side: no mycotoxins, middle: assay with buffer solution, right side: spiked sample, OTA, 2 μg L^{-1}, DON, 50 μg L^{-1}

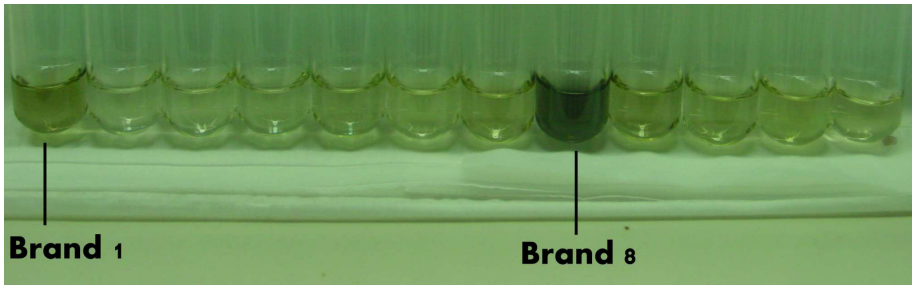

Figure 8.4: Intra-matrix variability: eluates of 12 different red wine brands, cleaned on 500 mg of NH_2 derived silica

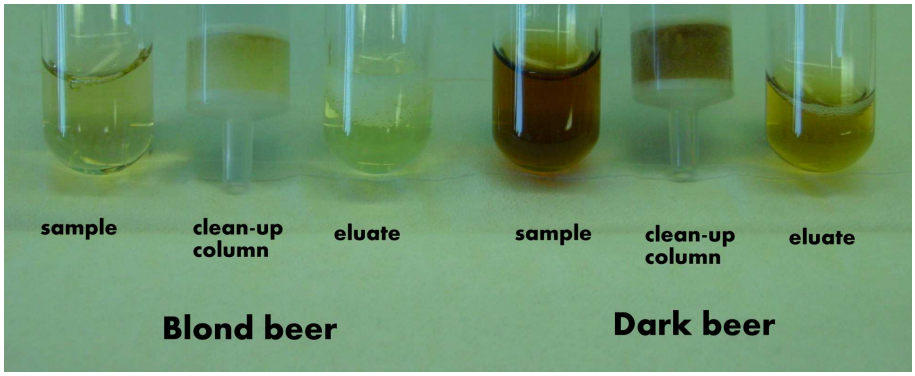

Figure 8.5: Intra-matrix variability: a blond and a dark beer sample before and after NH_2 derived silica clean-up

silica, which was further applied for the development of rapid membrane tests for OTA detection in red wine and the simultaneous OTA and DON detection in beer. The new format can also be applied for less coloured foodstuffs, as such increasing the sensitivity compared with other membrane formats.

8.7 Acknowledgements

This work was financially supported by Bijzonder Onderzoeksfonds (BOF) of the Ghent University (011D02803) and FWO Flanders Research Project No. G.0312.03. Special thanks go to Dr. Chris Maragos for providing the anti-DON antibodies.

Chapter 9

Monoclonal antibody development

9.1 Background and objectives

In 1975, Köhler and Milstein first reported a successful method for the production of monoclonal antibodies [332]. The technology introduced by them has led to an explosive application of monoclonal antibodies in research and clinical diagnostic medicine. Köhler and Milstein were awarded the Nobel Prize in medicine for their work, which was contributed to the biotechnology revolution. The use of monoclonal antibodies in clinical laboratories has been established in numerous assays in the fields of clinical chemistry, microbiology, immunology, haematology, surgical pathology and cytopathology [226]. They fused a specific antibody producing cell with a mutant myeloma cell line to produce hybrid cells which were selected in a particular culture medium and then screened for their specific antibody produced. The result is a cell line clone, which only produces a specific monoclonal antibody. These cells can be maintained *in vitro* over long periods of time and can generate large quantities of identical antibodies. They are - theoretically - immortal [225].

Figure 9.1 gives an overview of the monoclonal antibody development.

In this work, the purpose was to obtain monoclonal anti-OTA antibodies.

9.2 Materials and methods

9.2.1 Materials

OTA standard and OTA-BSA conjugate were purchased from Sigma Chemical Co (Bornem, Belgium). Stock solutions of OTA (1 mg mL^{-1}) and working solutions (100, 10 and 1 ng μL^{-1}) were prepared in methanol and stored at -20 °C. Rabbit anti-mouse immunoglobulins (IgG) (protein concentration: 2,7 g L^{-1}) were supplied by Dakocytomation (Heverlee, Belgium). OTA-HRP conjugate was prepared by the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary. Monoclonal antibodies against OTA were produced and characterized by the same Institute. The antibody was an IgG1 with kappa light chains with a 9.3 % cross-reaction with ochratoxin B but none at all with ochratoxin α, coumarin, 4-hydroxycoumarin and D,L-phenylalanine [248]. Phosphate buffered saline (PBS) 0.01 M, pH 7.4, was used for the dilutions (antibody and conjugate solutions) and for

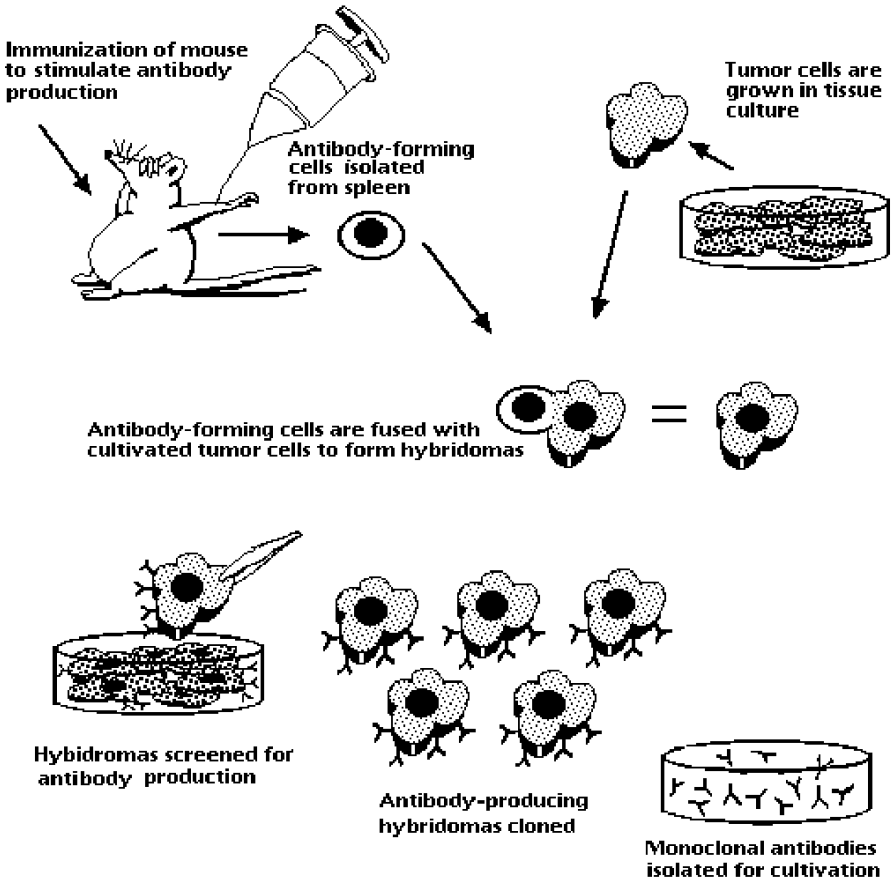

Figure 9.1: Monoclonal antibody development [6]

preparing the blocking buffer (PBS-2 % casein). Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogen solution used was Colorburst^{TM}Blue TMB/Peroxide (ALerCHEK, Inc. USA). Maxisorp 96-well ELISA-plates were supplied by Nunc (Denmark).

9.2.2 Immunisation scheme, fusion and cloning

The protocol for the immunisation, fusion and cloning, mentioned below, was performed by colleagues under the guidance of Prof. D. Deforce (Laboratory for Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University) and Prof. D. Elewaut (Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University). The screening of the sera and the supernatant fluids was done in our laboratory.

The immunisation scheme, the fusion and the cloning applied in Gyöngyösi *et al.* (1996) [248] to obtain anti-OTA antibodies were used as basis for the immunisation scheme.

Three six-week-old female Balb/c mice were immunized subcutaneously (SC) with 50 μg of OTA-BSA conjugate emulsified with an equal volume of complete Freund's adjuvant (CFA). After 2 weeks and after 4 weeks, the animals received intraperitoneal (IP) boosts of the same amounts of OTA-BSA conjugate as given earlier, emulsified with incomplete Freund's adjuvant (IFA). After 8 weeks, the mice received a final boost immunisation of 50 μg intravenously (IV). Three to four days after each immunisation, the sera were screened for anti-OTA antibodies using a direct competitive ELISA method. The serum of a non-immunized mouse served as control.

In the second experiment, the same scheme was followed for the immunisation of three six-week-old female Balb/c mice (marked at the ear, RR, R and LR) except for the boosts. The second and third boost were given SC. The mouse showing the best antibody titre in the serum (LR) was given a final boost immunisation (IV) of 10 μg after 12 weeks. The spleen of the mouse was removed aseptically 3 days after the boost and the splenocytes were fused with Sp2/O-Ag14 murine myeloma cell line using PEG 1600. Until 10 days after the fusion, the hybridomas were selectively grown in hypoxanthine, aminopterine, thymidine

(HAT) medium. The antibody secreting hybridomas were detected by direct competitive ELISA. Three weeks and eight weeks after the first fusion, the other mice received a final boost (10 μg IV (R) and 1 μg IV (RR), respectively) and fusion of the splenocytes with Sp2/O-Ag14 murine myeloma cell line was performed. The supernatant fluids of the hybridomas of the second fusion were also checked for antibody response using the same ELISA method as for the sera.

9.2.3 Screening of sera and supernatant fluids

The control ELISA method was first optimized using diluted purified antibodies (Ab) (produced at the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary) and diluted conjugate (from the same institute). The scheme of the ELISA, used for serum and supernatant fluid control, was:

1. coating plate: 10 μg mL^{-1} rabbit anti-mouse IgG; 150 μL/well
2. incubation overnight (37 °C)
3. 4 x wash with water
4. blocking 2 % casein 300 μL, 30 min
5. 4 x wash with water
6. add diluted sera (1/10 or 1/20) / supernatant fluid: 100 μL/well
   or
   add control (Ab): 100 μL/well
7. incubate 3-4 hours
8. 4 x wash with water
9. add 50 μL PBS (B_0)
   or
   add 50 μL OTA standard 1 μg mL^{-1} (B)
10. add 50 μL diluted OTA-HRP conjugate
11. incubate 1 hour (37 °C)
12. 4 x wash with water

13. add 150 μL TMB, incubation 15 min (in the dark)
14. add 50 μL 6N H_2SO_4 (stop reaction)
15. read optical density (OD) 450 nm

9.3 Results

Figure 9.2, where B/B_0 versus log OTA concentration is plotted, shows a dose-response curve using a purified anti-OTA antibody (produced at the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary) while optimizing the ELISA method, thus serving as an example for the antibodies, developed during the PhD research.

In the first experiment, the mice died after giving the final boost of 50 μg. Therefore, in the second experiment, the mice (LR and R) received a boost of only 10 μg.

Testing the supernatant fluids of the first 2 fusions (mice LR and R) yielded higher OD values for several wells. These hybridomas were cloned but when screening the supernatant fluids of these positives again with the direct ELISA method 10 days later, they did not indicate any antibody presence anymore.

As the expansion of the positive hybridoma cells after the first 2 fusions was too low, the final boost quantity was thought to be too high and to cause toxic effects. Therefore, the third mouse was given a final boost of 1 μg instead of 10 μg. The supernatant fluids of the hybridomas of the third fusion were not yet screened for antibody response.

9.4 On going experiments

An IOF-project (Industrieel Onderzoeksfonds Universiteit Gent, IOF06 /VAL/003, 'Productie van antilichamen voor de uitbouw en valorisatie van immunogebaseerde technologieën') was started to develop monoclonal antibodies against OTA, AFB_1, deoxynivalenol (DON) and zearalenone (ZEA). Immunisation quantities were based on literature and several concentration levels were tried out in several mice. A direct competitive ELISA is used for the testing of the sera and the supernatant fluids of the hybridoma cells.

A reason for the death of the hybridoma cells in the second experiment was searched for by fusing with spleen cells of non-immunized mice but the fusion was not successful. Mice were then immunized

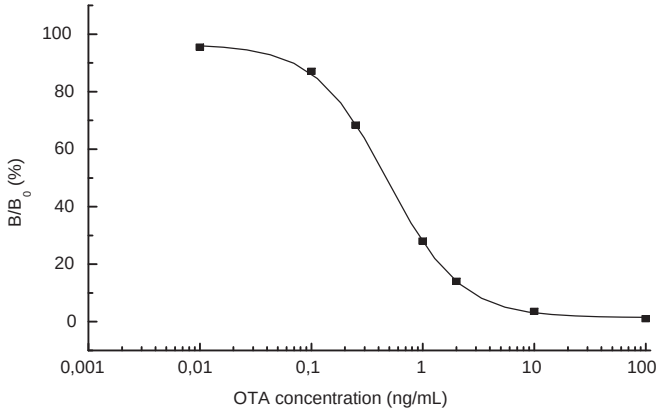

Figure 9.2: Dose-response curve with anti-OTA antibodies produced at the Agricultural Biotechnology Center, Diagnostic Laboratory, Gödöllö, Hungary

with human serum albumin (HSA) and after fusion of the spleen cells with myeloma cells the cells stayed alive and produced antibodies against HSA. Making use of these optimal conditions, the fusion and cloning experiments will be restarted for the anti-OTA antibody development.

Once positive hybridoma cells for the respective toxins are found, they will be cloned by the limiting dilution method. Ten days after the cloning, the supernatant fluids of the hybridoma cells will be tested for antibody response with the direct competitive ELISA method and these with the highest titer will be cloned again. The cloning and screening will be repeated 3 times to obtain a monoclonal cell line.

After further growth of the hybridoma cells, the monoclonal antibodies in the supernatant fluid will be purified using precipitation with ammonium sulphate and affinity chromatography.

9.5 Acknowledgements

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Chapter 10

Fluorescence polarization immunoassays for mycotoxin detection

10.1 Background and objectives

A bilateral scientific cooperation programme (project 01S04106, entitled: 'Strategy for express detection of mycotoxins in food products') between the Laboratory of Food Analysis (Faculty of Pharmaceutical Sciences, Ghent University) and the Department of Chemical Enzymology (Faculty of Chemistry, M. V. Lomonosov Moscow State University, Russia) gave me the opportunity to perform fluorescence polarization immunoassays (FPIAs) during a one month stay in Moscow. FPIA is minimally affected by solution opacity or colour and can serve for the detection of mycotoxins in strongly coloured foodstuffs. The goal was the development of a multi-method for the simultaneous detection of ochratoxin A (OTA) and aflatoxin B_1 (AFB_1) in paprika powder. We performed the first steps of this research, more specifically the synthesis of the mycotoxin tracers and their purification. After finding the optimal tracer dilution, dilution curves and competitive FPIAs in extraction solution (MeOH/3 % $NaHCO_3$ (80/20, v/v)) [172] were performed. The results are presented as the curves with fluorescence polarization units plotted against antibody dilution or standard concentration.The obtained results will be further used to study the matrix effect: fixed amounts of tracer solution and antibody solution at optimal concentration will be mixed with doubling dilutions of paprika powder extract solution. The optimal dilution will be chosen for further research with spiked samples.

10.2 Introduction

10.2.1 Use of FPIA

The use of fluorescence polarization (FP) was first described about 40 years ago [333, 334] and, due to improvements in instrumentation, is becoming more frequently used as a tool for modern analysis [65, 335].

FPIAs have been extensively used in the human clinical field to monitor therapeutic drugs and drugs of abuse for more than 20 years [75].

This technique has now found widespread use in other fields such as pesticides [336], animal disease [337] and environmental metal analysis [338].

In the mycotoxin field, until now, FPIAs have been developed for the determination of fumonisins and aflatoxins in grains [75, 339, 340],

deoxynivalenol (DON) in grains and wheat-based products [65, 75, 76], zearalenone in maize [341] and OTA in barley [51].

10.2.2 Principle

FPIA is a homogeneous assay technique based on differences in polarization of the fluorescence-labeled compounds (tracers) in the free and bound fractions: it involves the competition between free analyte and tracer for binding to a specific antibody [65].

When fluorescent molecules are excited by polarized light, the polarization of the emitted light depends on the random rotation that occurs during the excited state. With FPIA, the tracer is excited with polarized light. FP measures the sizes of the fluorescent molecules by measuring their rates of rotation in solution. The smaller the molecule is, the faster it rotates, because of lack of frictional drag, the lower the polarization value (Figure 10.1). When antibody binding occurs, rotation slows and the polarization signal increases [75, 337]. Polarization is dimensionless and does not depend directly upon the concentration of the fluorophore [65]. The fluorescence polarization value is indirectly proportional to the analyte concentration as the analyte is binding with a high molecular weight species-like antibody [229].

Anisotropy (r) and polarization (P) are both expressions for the same phenomenon and these values can be interconverted using the following equations [342]:

$$r = \frac{2P}{3 - P}$$

$$P = \frac{3r}{2 + r}$$

Anisotropy measurements provide information on the size and the shape of molecules or the rigidity of various molecular environments. Anisotropy measurements have been used to measure protein-protein associations and fluidity of membranes and for immunoassays of numerous substances. Anisotropy measurements are based on the principle of photoselective excitation of fluorophores by polarized light. Fluorophores preferentially absorb photons which electric vectors are aligned parallel to the transition moment of the fluorophore. The transition moment has a defined orientation with respect to the molecular

axes. In an isotropic solution, the fluorophores are oriented randomly. Upon excitation with polarized light, one selectively excites those fluorophore molecules which absorption transition dipole is parallel to the electric vector of the excitation. This selective excitation results in a partially oriented population of fluorophores (photoselection) and in partially polarized fluorescence emission. Emission also occurs with the light polarized along a fixed axis in the fluorophore. The relative angle between these moments determines the maximum measured anisotropy. The fluorescence anisotropy (r) and polarization (P) are defined by:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically and horizontally polarized emission when the sample is excited with vertically polarized light [342].

In general, a mycotoxin molecule or its analog is labeled with a suitable fluorophore to form a conjugate. Interaction of this conjugate with an antibody specific for that mycotoxin forms an immunocomplex that is larger than the conjugate and therefore gives a higher polarization. Addition of the free antigen to the antibody solution and subsequent addition of the conjugate results in a competition for antibody binding and a lower polarization value is observed, which is proportional to the amount of free toxin present (Figure 10.1). A standard curve with known concentrations of the mycotoxin is obtained and the unknown antigen concentration is calculated from the standard curve [75].

10.2.3 Advantages

FPIAs are easier, simpler and more economical to perform than traditional chromatographic methods, RIA or enzyme immunoassays [75]. FPIA shows two important differences with ELISA: the detection does not involve an enzyme reaction and separation of the bound and free compounds is not required. As a result, FPIAs do not require a washing step and do not require waiting for an enzyme reaction for colour development [238]. FPIA can yield results (qualitative and quantitative) in seconds to minutes without any extensive labour [75].

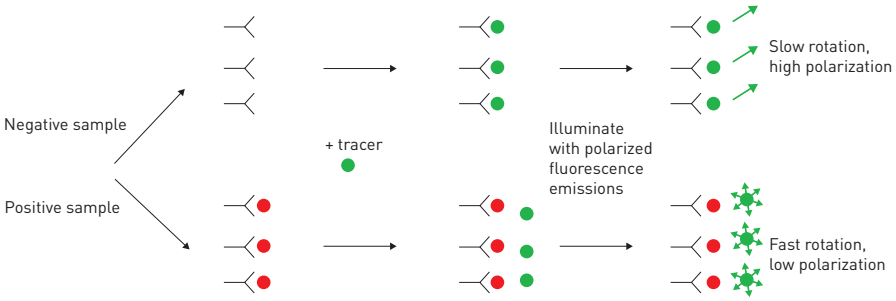

Figure 10.1: The fluorescence polarization immunoassay principle

The absence of washing and clean-up steps in FPIA makes this technique very attractive for mycotoxin analysis. FP is minimally affected by solution opacity or colour [65]. Since FPIA can work in oily, milky and coloured solutions, no special cleaning reagents or columns are needed [75].

10.2.4 Influences

The sensitivity of the assay depends upon the affinity of the antibody-antigen interaction, the instrument and the fluorophore properties [75, 343].

The tracer determines the intensity of emitted polarized light and also contributes to the competition for antibody binding. Thus, the lowest possible tracer concentration, which permits a reliable detection of the label and produces a minimum effect on the competition, should be used to develop a sensitive assay [51].

Besides antibody and tracer, response in the assay format is dependent on the time during which the sample is held between the addition of the tracer and the measurement of the signal (holding time). The disadvantage of using short holding times is the potential that small errors in holding time will result into large errors in measured toxin content. Such errors will be reduced by holding the samples for a longer period, allowing the samples to come to equilibrium before measuring the response. However, assays with longer incubation times are less sensitive [65].

10.3 Materials and Methods

10.3.1 Materials

N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), OTA standard and AFB_1 standard were purchased from Sigma Chemical Co. (Bornem, Belgium). Sodium borate buffer (BB) 50 mM, pH 8.0 with 0.1 % sodium azide, was used in all dilutions of the FPIA experiments. Methanol was HPLC-grade. Stock solutions of OTA and AFB_1 (1 mg mL^{-1}) and working solutions (100, 10 and 1 ng μL^{-1}) were prepared in methanol and stored at -20 °C. Working solutions (0, 4, 10, 25, 50, 100, 250, 500, 750 and 1000 ng mL^{-1}) in MeOH/3 % $NaHCO_3$ (80/20, v/v) were prepared for the calibration curves. Monoclonal antibodies against OTA were purchased from Soft Flow (Gödöllö, Hungary). Another monoclonal antibody, produced in Graduate School of Gyeongsang National University, South Korea, was also tried out in the FPIAs. Several AFB_1 antibodies, produced in the same institute in South Korea, were available. Thin layer chromatography (TLC) plates (Silica gel 60, fluorescent, 1 mm, 20 x 20 cm) were obtained from Merck Co. (Darmstadt, Germany). Fluorescein-thiocarbamyl ethylenediamine (EDF) was synthetised from fluorescein-isothiocyanate (FITC) and ethylenediamine dihydrochloride as previously described by Pourfarzaneh *et al.* (1980) [344] with modifications [229].

10.3.2 Apparatus

Measurements of fluorescence polarization and intensity were performed using TDx/FLx analyser (Abbott Laboratories, Irving, TX, USA) in semi-automatic PhotoCheck mode. TDx/FLx glass cuvettes (up to ten in one run) were loaded into the special carousel followed by the measurement of polarization (in mP units) and intensity (in conventional units) of fluorescence. The total time for measurement of ten samples was about 7 min.

10.3.3 Synthesis of fluorescein-labeled tracer OTA-EDF

NHS (11.5 mg (100 μmol)) and DCC (20.6 mg (100 μmol)) were dissolved in 1 mL of pure dimethylformamide (DMF). NHS-DCC in DMF solution (100 μL) was added to 1 mg of OTA standard and this was mixed and incubated overnight at room temperature (RT). The white precipitate of dicyclohexylurea was formed. The reaction mixture

was then centrifuged at 8000 rpm for 10 min and the precipitate was discarded. The next day 0.5-1 mg (1-2 μmol) of EDF was added to the formed active ester solution. The reaction mixture (RM) was protected from light and stirred for 2 h at room temperature. The crude fluorescence-labeled tracer was kept at 4 °C.

The reaction mechanisms are as follows:

Ag-COOH + NHS + DCC → [Ag-CO-NHS]

[Ag-CO-NHS] + NH_2-EDF → Ag-CO-NH-CH_2-CH_2-NH-C(S)-NH-Fluorescein

Ochratoxin B (OTB) tracer, prepared by Eremin and Schneider (OTB-EDF RM 15/02/07) was also tried out in these experiments.

10.3.4 Synthesis of fluorescein-labeled tracer AFB_1-EDF

Since the aflatoxin molecule does not have an active functional group to directly react with a fluorophore, first an AFB_1-oxime was prepared using carboxymethoxylamine hemihydrochloride, that reacted with an amine derivative of fluorescein to give the aflatoxin tracer.

The reaction mechanisms are as follows:

Ag=N-OCH_2-COOH (oxime) + NHS + DCC → [Ag=N-OCH_2-CO-NHS]

[Ag=N-OCH_2-CO-NHS] + NH_2-EDF → Ag=N-OCH_2-CO-NH-CH_2-CH_2-NH-C(S)-NH-Fluorescein

In this study an AFB_1 tracer, prepared in 2001 (AFB_1-carboxymethyloxime (CMO)-EDF, Nesterenko I. RM 04/2001) was tried out in the FPIA.

10.3.5 TLC

A small portion of the tracer RM ($\pm$ 20 μL) was purified by TLC using chloroform ($CHCl_3$) as eluent (removing DMF). The plate was dried and again a purification by TLC using $CHCl_3$/MeOH (4:1, v:v) as eluent was performed. The major yellow bands were collected, eluted with methanol and stored at -20 °C in the dark.

10.3.6 Optimal tracer dilution

Several dilutions of the tracer (500 μL) were measured in the FPIA for their intensity and the dilution giving a value of 2000 was chosen for further experiments.

10.3.7 Dilution curves

To prepare antibody dilution curves, fixed amounts (0.5 mL) of tracer solution at appropriate concentration were mixed with doubling antibody dilutions (0.5 mL) in TDx/FLx glass cuvettes and immediately analysed using FPIA. The obtained results are presented as the curves with fluorescence polarization units plotted against antibody dilutions.

10.3.8 Competitive FPIA procedure

A quantity of 50 μL mycotoxin standard in extraction solution (MeOH/3 % $NaHCO_3$ (80/20, v/v) [172] (or sample extract), 0.5 mL tracer solution and 0.5 mL antibody solution in optimal dilution were added sequentially to the cuvette and mixed, immediately followed by measurement. The standard curves were plotted as fluorescence polarization versus the logarithm of the analyte concentration. The concentration of the toxin in (spiked) samples can be calculated after fitting the standard curve using the four-parameter logistic model.

10.4 Results

10.4.1 TLC

TLC was used for purification of the tracers. For ochratoxin Figures 10.2, 10.3 and 10.4 show the TLC plates for a bad synthesis, a successful synthesis in visible light and the latter one under UV light. The first Figure shows a thick band at the beginning while the other bands are much lighter coloured, indicating the bad synthesis. The band at R_f=0.9 of the OTB tracer synthesis (Figure 10.2), the band at R_f=0.7 of the first OTA tracer synthesis and the main band at R_f=0.7 of the second OTA tracer synthesis (Figure 10.3) were scraped and eluted with methanol. For AFB_1 (Figure 10.5), the bands at R_f=0.6 and R_f=0.7 were used.

10.4.2 Optimal tracer dilution

The tracer determines the intensity of emitted polarized light and also contributes to the competition for antibody binding. Thus, the lowest possible tracer concentration, which permits on reliable detection of a label and produces the minimum effect on the competition, should be used to develop a sensitive assay [51]. The dilution giving an intensity

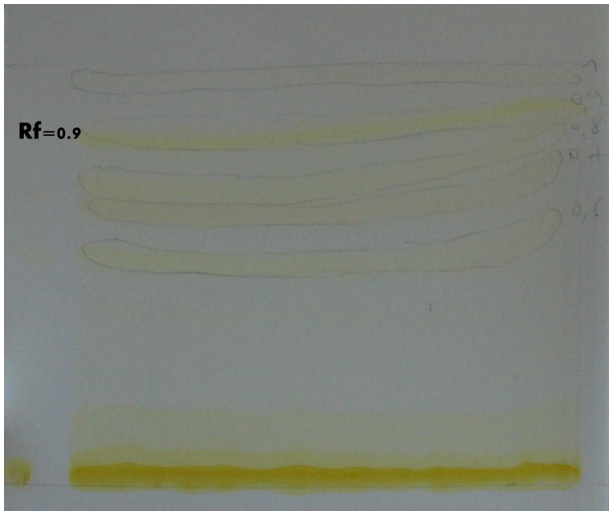

Figure 10.2: TLC of OTB tracer (bad synthesis)

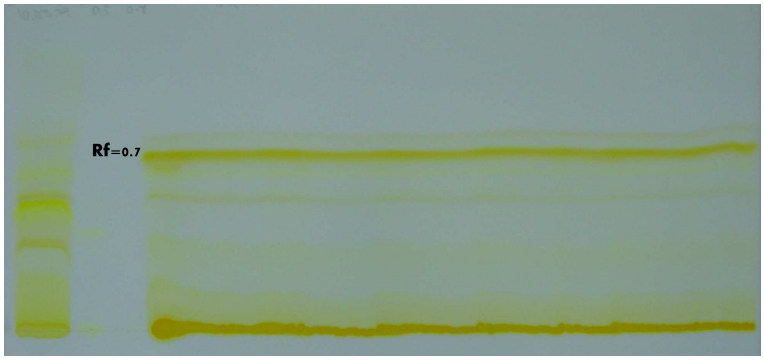

Figure 10.3: TLC of OTA tracer (successful synthesis), visible light

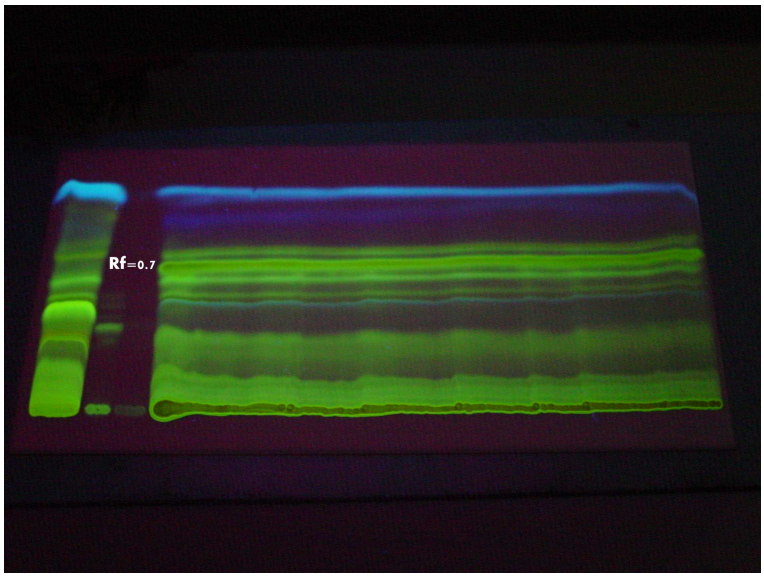

Figure 10.4: TLC of OTA tracer (successful synthesis), UV light

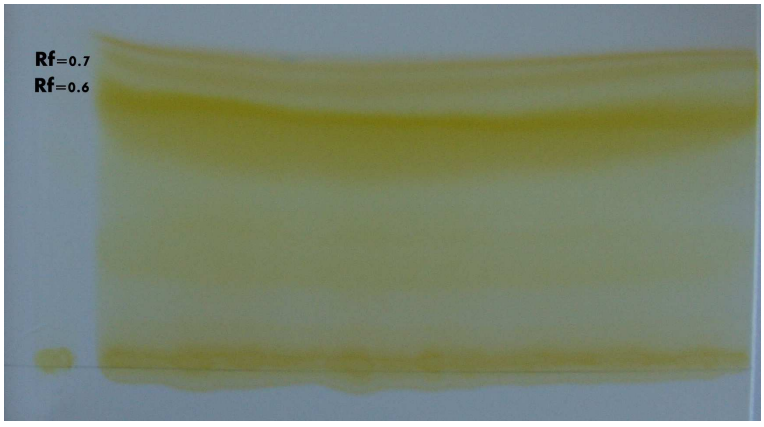

Figure 10.5: TLC of AFB_1 tracer

of 2000 in a volume of 500 μL should give an intensity of 1000 in the 1000 μL volume of the assays. This tracer concentration has a fluorescence intensity of $\pm$ 20 times higher than the background signal for BB (approximately 30-50 fluorescence intensity units).

For R_f=0.9 of the synthesis of the OTB tracer, R_f=0.7 of the first synthesis of the OTA tracer, R_f=0.6 and R_f=0.7 of the AFB_1 tracer synthesis, the dilutions of the tracers were 1/10 000, 1/4 000, 1/14 000 and 1/5 750, respectively. The optimal dilutions of R_f=0.67 and R_f=0.68 of the second purification of the AFB_1 tracer (R_f=0.6) were 1/1 000 and 1/15 000, respectively.

10.4.3 Dilution curves

For R_f=0.9 of the synthesis of the OTB tracer and R_f=0.7 of the OTA tracer, first synthesized, no dilution curves could be obtained because there was no increase in polarization signal when mixed with antibodies. Even after more purifications, no increase could be obtained. The OTA tracer of the new synthesis couldn't be checked because of problems of instrument availability. For AFB_1, Figure 10.6 shows the results of the two major bands of the TLC separation (R_f=0.6 and R_f=0.7). In Figure 10.7 the results of a second purification of the band R_f=0.6 are plotted. After control of the two major bands of the second purification (R_f=0.67 and R_f=0.68), the latter one seemed to give the highest value and the best sigmoidal curve (Figure 10.7). Optimal antibody dilution for construction of FPIA calibration curves was determined as that at which 70 %-75 % tracer binding was observed [345] (antibody dilution $\pm$ 1/2000).

10.4.4 Competitive FPIA

For OTA and OTB, it was impossible to make calibration curves because no increase in polarization signal was obtained when the tracer was mixed with antibodies. Competitive FPIA was only performed for AFB_1 standard in extraction solution (MeOH/3 % $NaHCO_3$ (80/20, v/v) [172] as for this toxin suitable dilution curves were obtained. For the standard solution, containing no AFB_1, the maximum binding signal was decreased using the optimal antibody dilution (1/2000). There was only a small difference in polarization signal for a blank sample and a sample, containing 1000 ng AFB_1 mL^{-1}. Therefore, the antibody was less diluted. Figure 10.8 shows a sigmoidal curve using an anti-

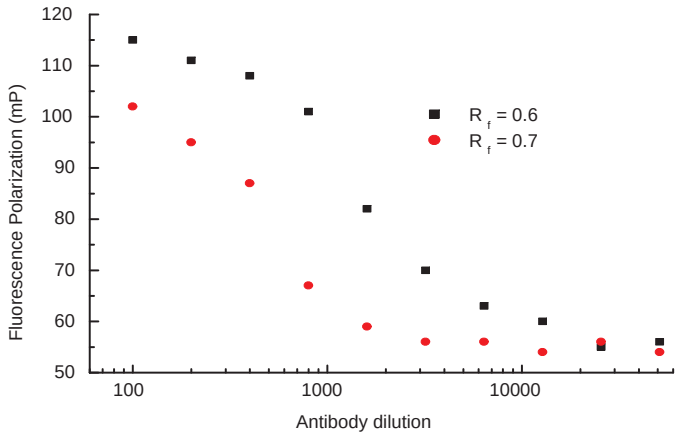

Figure 10.6: Results of first purification of the AFB_1 tracer

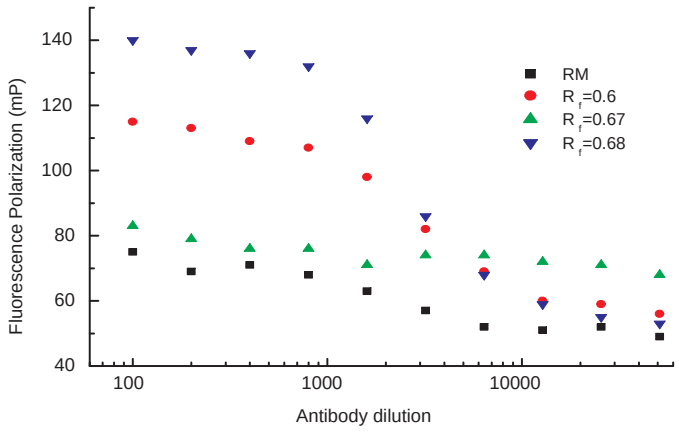

Figure 10.7: Results of second purification of the band at R_f=0.6 for the AFB_1 tracer

body dilution of 1/500. The assay is not sensitive as the IC_{50} value is $\pm$ 700 ng mL^{-1}.

10.5 Discussion

Shim *et al.* (2004) [51] studied the influence of several organic water-miscible solvents, commonly used for extraction, on the FPIA performance. Methanol didn't seem to give a decrease of maximum binding signal or assay sensitivity. It was shown that the near-optimal calibration curve could be obtained with the buffer containing up to 50 % (v/v) methanol. Only using 100 % methanol standard solutions caused a slight effect on the assay performance. Therefore, the presence of $NaHCO_3$ in the optimal extraction solution [172] in this case was suspected to give a decrease of maximum binding signal.

The signal in FPIA is related to the kinetics of the interaction between the antibody and the tracer or the antigen and the order of addition of the reagents [65]. Therefore, another option to adapt the sensitivity of this method is mixing the toxin with the antibody before addition of the tracer.

If the antibody used has a high affinity, it takes approximately 15 minutes to completely equilibrate the final FPIA values [75]. To control this phenomenon, Nasir *et al.* (2003) used a batch mode where all the samples and standards were first mixed with 1 mL of a pre-diluted antibody solution in buffer in separate test tubes. After adding the tracer to each tube, all the samples were incubated at room temperature for 15 minutes before reading [75]. Adapting the holding time in this assay could thus be favourable for the sensitivity.

For the extraction of OTA and AFB_1, MeOH was used since aflatoxins can only be extracted using a mixture of organic/aqueous solvent. The antibody used can have a high affinity for the tracer but it also has to be stable in the (diluted) organic solvents otherwise this could give unreliable results [75]. Other antibodies can be tried out to study this influence.

10.6 Conclusions

FPIA serves as a homogeneous, sensitive, specific and technically simple and inexpensive rapid method. Only the first steps for the development of a screening method were taken during the PhD study. The

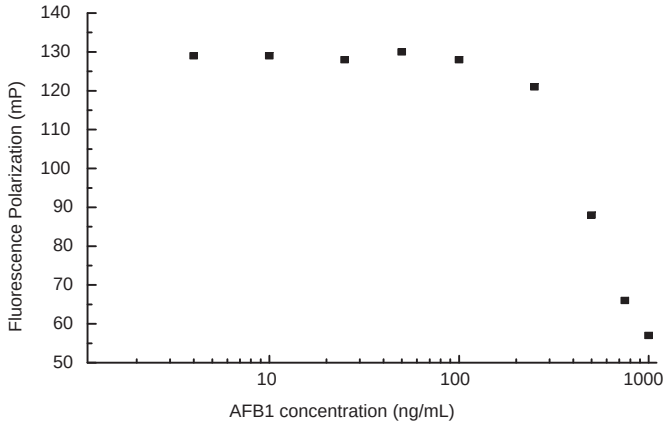

Figure 10.8: Competitive FPIA

first synthesis of the OTA tracer and the synthesis of the OTB tracer were not good. For the new synthesis of the OTA tracer, the TLC result seemed to yield a good tracer but the binding signal has to be tested with FPIA. The AFB_1 tracer yielded good dilution curves but when performing a competitive FPIA with standard in the extraction solution MeOH/3 % $NaHCO_3$ (80/20, v/v), the binding signal decreased. MeOH/3 % $NaHCO_3$ (80/20, v/v) seemed to be the optimal extraction solution for OTA and AFB_1 in spices [172] but adaptations will be necessary to increase the sensitivity of the method, i.e. high maximum binding signals. The effect of $NaHCO_3$ on the maximum binding signal will be studied and the optimal solution for simultaneous extraction of OTA and AFB_1 for FPIA has to be searched for once a suitable OTA tracer is found. Also influences of antibodies, sequence of addition and holding time will be adapted before further studying the matrix effect.

10.7 Acknowledgements

This research was supported by Bijzonder Onderzoeksfonds (BOF) of the Ghent University (011D02803) and Bijzonder Onderzoeksfonds, bilateral cooperation Flanders and Russia (project 01S04106 'Strategy for express detection of mycotoxins in food products').

Conclusions and perspectives

Given the growing importance of the mycotoxin problem, leading to more legislation, this work is an important contribution concerning the development of rapid, cheap and reliable mycotoxin tests. Because a more detailed legislation leads to more analyses, the interest in the field tests, described in this thesis, is extremely great. The food industry does not want to lose time, sending samples to a controlling laboratory. Membrane tests (lateral flow immunoassays, flow-through immunoassays) are known rapid tests. However, non-instrumental field tests for the detection of mycotoxins in strongly coloured foodstuffs were not available until recently.

The *clean-up tandem assay column*, applied in this work, has been designed for the detection of mycotoxins in roasted coffee, cocoa and spices. The column comprises at least two layers; a clean-up layer, capable of adsorbing at least part of the interfering sample fraction, and a detection layer for the visual colour evaluation whether the mycotoxin is present above or below a cut-off value. The format was also applied for the simultaneous detection of 2 mycotoxins. Aminopropyl derived silica was found to have the best possibilities as clean-up layer in all cases. The *clean-up tandem assay column* has proven to serve as a suitable rapid test for strongly coloured foodstuffs. For red wine and beer, advantages of membranes and columns were combined in a new format.

These rapid tests can be used *on-site*, by coffee/cocoa distributors, coffee/cocoa roasters, winegrowers, beer brewers.... No expensive instruments are needed.

Besides these heterogeneous methods, FPIA was also evaluated as a simple and rapid method for the analysis of mycotoxins, based on literature and preliminary results.

Future developments in the analysis of mycotoxins should be the further simplification of the assay procedure (decrease of number of assay steps, improving speed, user-friendliness, cost-effectiveness), miniaturization and portability for *on-site* mycotoxin tests. An example of the decrease of the number of assay steps is the development of a 3-step *clean-up tandem assay column* for the detection of ochratoxin A in spices [346]. High quality immunoreagents are necessary to compensate for the loss of sensitivity if further simplification of the method occurs. Another perspective in mycotoxin analysis is the development of multi-assays. Only few attempts were done for the simultaneous determination of multiple analytes in one analytical run for a given sample.

The use of recombinant antibodies can be another future option for diagnostic purposes of mycotoxins. Alternatives for antibodies in rapid tests are molecularly imprinted polymers (MIPs) and aptamers. MIPs are polymeric matrices capable of preferentially recognizing the template molecules used. These are more stable and simpler to produce (cheaper) than the conventional antibodies. Aptamers are synthetic oligonucleotides specifically selected to bind a certain target molecule. These have the same advantages of stability and ease of production as MIPs and both are options for future implementation in the development of rapid mycotoxin tests.

Summary

In **chapter 1**, the mycotoxins ochratoxin A (OTA), deoxynivalenol (DON) and aflatoxin B_1 (AFB_1) and their properties are described. Furthermore the human exposure to each mycotoxin by the consumption of coffee, cocoa, spices, wine and beer is evaluated.

Chapter 2 serves as a basis for the following chapters giving an overview of immunoassays and rapid immunochemical tests.

Chapter 3 exposes the objectives of this thesis.

In **chapter 4**, a clean-up tandem assay column was developed for the detection of OTA in roasted coffee. Aminopropyl derived silica was used as clean-up layer. The method, with a 6 μg kg^{-1} cut-off value, was validated determining several performance characteristics: false positive rate, false negative rate, sensitivity rate, specificity rate, positive predictive value and negative predictive value.

The detection of OTA in cocoa powder using the clean-up tandem assay column is explained in **chapter 5**. Aminopropyl derived silica seemed to be the most appropriate clean-up layer. A cut-off value of 2 μg kg^{-1} could be reached and qualitative performance parameters were determined. Ten commercially available cocoa powder samples were analysed with the described method and the results were verified with liquid chromatography tandem mass spectrometry (LC-MS/MS). No positive samples were found.

Some spices may be strongly coloured, making it impossible to analyse them with membrane tests based on visual detection. As for roasted coffee and cocoa powder, the clean-up tandem assay column could solve this problem (**Chapter 6**). Two assay procedures were optimized. In the first one, the clean-up layer (aminopropyl derived silica) was set above the detection layer, as applied in chapters 3 and 4. The modified assay procedure with top detection immunolayer could be used for all different target spices and resulted in less matrix interferences as well as in reduction of assay steps. Spices, available on Belgian, Dutch and Russian markets, were analysed with the clean-up tandem immunoassay column for the presence of OTA and confirmation was performed with LC-MS/MS. Of the *Capsicum* spp. spices, 35 % were found to be contaminated with OTA above the 10 μg kg^{-1} level.

In spices, the mycotoxins OTA and AFB_1 are most present. In view of the EU legislation for both OTA and AFB_1, it is desirable to have a single method of analysis for both toxins, using a single extraction and detection method. This would increase sample throughput and reduce costs. In **chapter 7**, the clean-up tandem assay column was applied

for the development of a multi-test, in this way having one clean-up layer (aminopropyl derived silica) and two detection immunolayers. The multi-method was developed with a cut-off level of 5 μg kg^{-1} for AFB_1 and 10 μg kg^{-1} for OTA. The test was applied for simultaneous determination of AFB_1 and OTA in different spices.

In **chapter 8**, a new format with visual detection was applied, i.e. a combination of a membrane test and the aforementioned column test. Advantages of both membrane and column were combined. The purpose was developing a 'flow-through column' for the detection of OTA in red wine and for the simultaneous detection of OTA and DON in beer. However, the disadvantage of a membrane is the adsorption of sample colour, in this way interfering with the visual detection. Therefore, a preceding rapid clean-up method with aminopropyl derived silica was used for red wine and beer. By increasing the sample volume, sensitivity could be improved but only to a limited extent because of saturation of the clean-up layer. Therefore, the sensitivity required for the set European maximum levels (MLs) for OTA could not be reached. An important conclusion for red wines and beers is the 'intra-matrix' variability.

In **chapter 9**, the screening step within the monoclonal antibody development process is exposed. Mice were immunized with an OTA-protein conjugate and after each immunisation the sera were checked for antibody response. After fusion of the splenocytes with myeloma cells, the supernatant fluids of the hybridomas were tested. Some of them gave positive antibody response. However, further optimization of the fusion protocol is necessary because after a second check no antibodies could be detected anymore.

A very promising way to the simplification of immunoassays for routine applications is a shift from heterogeneous methods (with separation) as used in previous chapters to homogeneous assays (without separation). **Chapter 10** deals with the fluorescence polarization immunoassay (FPIA) method, which is a homogeneous method where the detection does not involve an enzyme reaction and separation of the bound and free compounds is not required. The aim was to develop a multi-FPIA for the detection of OTA and AFB_1 in paprika powder. The development of an FPIA method for these mycotoxins includes the synthesis of the mycotoxin tracers. These were purified with thin layer chromatography (TLC) before making dilution curves to choose the optimal antibody dilution. For AFB_1 a suitable tracer was obtained. No sufficient sensitivity was observed with standard curves in MeOH/3 %

$NaHCO_3$ (80/20, v,v). Further optimization of the tracers and development of the method have to be performed.

Samenvatting

In **hoofdstuk 1** worden de mycotoxines ochratoxine A (OTA), deoxynivalenol (DON) en aflatoxine B_1 (AFB_1) en hun eigenschappen besproken. Ook wordt de blootstelling van de mens aan elk mycotoxine via consumptie van koffie, cacao, specerijen, wijn en bier geëvalueerd.

Hoofdstuk 2 dient als basis voor de volgende hoofdstukken. Hierin wordt een overzicht gegeven van de immunoassays en de vlugge immunochemische testen.

Hoofdstuk 3 geeft de objectieven van deze thesis weer.

In **hoofdstuk 4** werd een vlugge test ontwikkeld voor de detectie van OTA in gebrande koffie gebruik makend van de *clean-up tandem assay column*. Aminopropylgederivatiseerde silica werd gebruikt als opzuiveringslaag. De methode had een cut-off waarde van 6 μg kg^{-1} en werd gevalideerd door het bepalen van de volgende parameters: % vals positieven, % vals negatieven, gevoeligheid, specificiteit, positieve predictieve waarde en negatieve predictieve waarde.

De detectie van OTA in cacaopoeder met behulp van de *clean-up tandem assay column* wordt besproken in **hoofdstuk 5**. Hier bleek aminopropylgederivatiseerde silica de meest geschikte opzuiveringslaag te zijn. Een cut-off waarde van 2 μg kg^{-1} werd bereikt en kwalitatieve parameters werden bepaald. Tien commerciële cacaopoedermonsters werden geanalyseerd met de beschreven methode en de resultaten werden geverifieerd met vloeistofchromatografie-tandem massa spectrometrie (LC-MS/MS). Er werden geen positieve monsters gevonden.

Specerijen zijn vaak ook sterk gekleurde voedingsmiddelen die moeilijkheden opleveren bij analyse met membraantesten en visuele detectie. Zoals voor gebrande koffie en cacaopoeder kon de *clean-up tandem assay column* hier ook een oplossing bieden (**hoofdstuk 6**). Twee procedures met de *clean-up tandem assay column* werden geoptimaliseerd. Bij de eerste was de set-up gelijk aan die gebruikt in de hoofdstukken 3 en 4 (opzuiveringslaag (aminopropylgederivatiseerde silica) boven detectielaag). De gewijzigde procedure met de detectielaag bovenaan bleek volgende voordelen te hebben: universele bruikbaarheid voor alle specerijen, minder matrixinterferentie en reductie van het aantal stappen. Specerijen, aanwezig op de Belgische, Nederlandse en Russische markt, werden geanalyseerd met de *clean-up tandem assay column* naar de aanwezigheid van OTA en confirmatie gebeurde met LC-MS/MS. Van de *Capsicum* spp. specerijen was 35 % gecontamineerd met meer dan 10 μg OTA kg^{-1}.

De meest voorkomende mycotoxines in specerijen zijn OTA en AFB_1. Rekening houdende met de Europese regelgeving voor OTA en AFB_1 is het wenselijk om over één enkele methode te beschikken voor beide toxines, met dezelfde extractie en detectie. Dit zou de analysecapaciteit opdrijven en de kosten reduceren. In **hoofdstuk 7** werd de *clean-up tandem assay column* aangewend voor de ontwikkeling van een multi-test. Hierbij bestond de kolom uit een opzuiveringslaag (aminopropylgederivatiseerde silica) en twee detectielagen. De multimethode werd ontwikkeld met een cut-off waarde van 5 μg kg^{-1} voor AFB_1 en 10 μg kg^{-1} voor OTA. De methode werd toegepast voor de simultane detectie van AFB_1 en OTA in verschillende soorten specerijen.

In **hoofdstuk 8** werd een nieuwe opzet aangewend met visuele detectie; een combinatie van een membraantest en de boven vermelde kolomtest. Voordelen van een membraan werden gecombineerd met het voordeel van het gebruik van een kolom. Het doel was om een 'flow-through column' te ontwikkelen voor de detectie van OTA in rode wijn en voor de simultane detectie van OTA en DON in bier. Een nadeel aan het gebruik van een membraan is de adsorptie van de kleur van de stalen waardoor interferentie optreedt met de visuele detectie. Een voorafgaande snelle opzuiveringsmethode met aminopropylgederivatiseerde silica bleek noodzakelijk voor rode wijn en bier. Een verhoging van de gevoeligheid kon bereikt worden door het staalvolume op te drijven maar dit was beperkt omwille van de verzadiging van de opzuiveringslaag. Daarom werden de Europese maximale gehalten voor OTA niet behaald. Een belangrijke opmerking voor rode wijn en bier is de 'intra-matrix' variabiliteit.

In **hoofdstuk 9** wordt de screening besproken binnen het project van monoklonale anti-OTA antilichaamontwikkeling. Muizen werden geïmmuniseerd met een OTA-proteïne conjugaat en na elke immunisatie werden serumstalen getest voor antilichaamrespons. Na fusie van de splenocyten met myelomacellen, werden de supernatantia van de hybridoma's getest. Sommige gaven een positieve antilichaamrespons. Verdere optimalisatie van het fusieprotocol is echter noodzakelijk aangezien na een tweede screening van de hybridoma supernatantia geen antilichamen meer konden gedetecteerd worden.

Een veelbelovende manier voor de vereenvoudiging van immunoassays voor routineonderzoek is de overgang van heterogene methodes (met scheiding), gebruikt in vorige hoofdstukken, naar

homogene assays (zonder scheiding). **Hoofdstuk 10** behandelt de fluorescentie polarizatie immunoassay (FPIA) methode, die een homogene methode is waarbij de detectie geen enzymreactie inhoudt en scheiding van gebonden en vrije componenten niet nodig is. Het doel was om een multi-FPIA te ontwikkelen voor de detectie van OTA en AFB_1 in paprikapoeder. De ontwikkeling van een FPIA methode voor deze mycotoxines includeert de synthese van mycotoxinetracers. Deze werden opgezuiverd met dunnelaagchromatografie (DLC) vóór het maken van verdunningscurves om de optimale antilichaamverdunning te kiezen. Voor AFB_1 werd een gepaste tracer verkregen maar na het opstellen van standaardcurves in MeOH/3 % $NaHCO_3$ (80/20, v/v) werd geen goede gevoeligheid vastgesteld. Verdere optimalisatie van de tracers en ontwikkeling van de methode zijn dus noodzakelijk.

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CURRICULUM VITAE

Marieke Lobeau, geboren te Poperinge op 8 april 1979, behaalde in 2002 met onderscheiding het diploma van Apotheker aan de Universiteit Gent. In het kader van het Europese uitwisselingsprogramma *Erasmus* verbleef zij tijdens de 2de Proef een semester in Lyon (Frankrijk). In 2003 behaalde ze met onderscheiding het diploma van Industrieapotheker.
Sinds oktober 2003 is ze werkzaam als doctoraatsbursaal aan het Laboratorium voor Bromatologie (Faculteit Farmaceutische Wetenschappen) aan de Universiteit Gent, onder het promotorschap van Prof. Dr. Apr. Sarah De Saeger en Prof. Dr. Apr. Carlos Van Peteghem. De resultaten van het wetenschappelijk onderzoek, gefinancierd door het Bijzonder Onderzoeksfonds (BOF) van de Universiteit Gent, leidden tot meerdere publicaties. Ook droeg zij haar wetenchappelijk steentje bij met verschillende voordrachten en postervoorstellingen op nationale en internationale symposia. In het kader van een bilateraal project verbleef zij gedurende een maand in Moskou (Rusland).

Marieke Lobeau, born in Poperinge on April 8th 1979, graduated in 2002 with distinction as Pharmacist at Ghent University. In the framework of the European exchange program *Erasmus*, she staid for a semester in Lyon (France).
Since October 2003, she works as doctoral researcher at the Laboratory of Food Analysis (Faculty of Pharmaceutical Sciences) at Ghent University, with Prof. Dr. Apr. Sarah De Saeger and Prof. Dr. Apr. Carlos Van Peteghem as promoters. The results of this research, financially supported by the Special Research Fund of Ghent University, led to several publications. She presented also her results during several national and international symposia. In the framework of a bilateral project, she staid for one month in Moscow (Russia).

Courses, symposia and scientific stays during PhD

- 'Doctoraatsopleiding': Bioanalyse (1st year Master Pharmaceutical Sciences), academic year 2003-2004, 2nd semester, Prof. Dr. W. Lambert, Faculty of Pharmaceutical Sciences.
- 'Doctoraatsopleiding': Mycologie, 1st year Master Biology (optie plantkunde), academic year 2004-2005, 1st semester, Prof. A. Verbeken, Faculty of Sciences.
- 'Doctoraatsopleiding': Kwaliteitsbeheer en risicoanalyse in de agro- en voedingsindustrie, 3rd year Master Bio-engineer 'Landbouwkunde', academic year 2004-2005, 2nd semester, Prof. F. Devlieghere, Faculty of Bioscience Engineering.
- 'Doctoraatsopleiding': Laboratory Animal Science, academic year 2005 - 2006 (October 2005), Master, Prof. K. Hermans, Faculty of Veterinary Medecine.
- Danone Chair, The challenge of keeping mycotoxins out of the food chain, Prof. J. Gilbert, organised by Ghent University, 25th October 2004 - 2nd December 2004.
- Symposium: 'Mycotoxinen en voedselveiligheid : evolutie en perspectieven', organised by Ghent University (Laboratory of Food Analysis), Hogeschool Gent (Faculty of Biosciences and Landscape Architecture) and Toxi-Test, Ghent, 7th October 2004 (member of organising committee).
- S. De Saeger, L. Sibanda, C. Paepens, M. Lobeau, B. Delmulle and C. Van Peteghem. Novel developments in rapid mycotoxin detection. Abstracts of lectures and posters of the International Conference and Marketplace "Rapid Methods Europe 2005 for Food and Feed Quality Determination", Noordwijk (The Netherlands), 24-25 mei 2005, p. 84 (poster P12 - Approaches for Rapid Immunochemical Mycotoxin Detection).
- M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetró and C. Van Peteghem. Development of a new field test: a clean-up tandem assay column for the detection of ochratoxin A in roasted coffee. Abstracts of lectures and posters of the International Conference and Marketplace "Rapid Methods Europe 2005 for Food and Feed Quality Determination", Noordwijk (The Netherlands), 24-25 mei 2005, p. 86 (poster P14).

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- M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetró and C. Van Peteghem. Development of a NEW FIELD TEST: a clean-up tandem assay column for the detection of ochratoxin A in cocoa powder. Oral presentation, VIIIth International Conference of Agri-Food Antibodies 2005, Chester (United Kingdom), 6-9 september 2005, p. 20.

- Fifth International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerpen, 16 - 19 mei 2006 (member of organising committee).

- M. Lobeau, S. De Saeger, I.Y. Goryacheva and C. Van Peteghem. Screening of mycotoxins in strongly coloured food matrices. Oral presentation, Abstracts of lectures and posters of the International Conference and Marketplace "Rapid Methods Europe 2007 for biological and chemical contaminants in food and feed", Noordwijkerhout (The Netherlands), 29-30 January 2007, p. 42.

- Symposium Mycotoxins: Threats and risk management, organised by Ghent University (Laboratory of Food Analysis) and Hogeschool Gent (Faculty of Biosciences and Landscape Architecture) Ghent, 30th March 2007 (member of organising committee).

- Bilateral scientific cooperation: Strategy for express detection of mycotoxins in food products: scientific work during the month March 2007 in the Department of Chemical Enzymology, Faculty of Chemistry, Lomonosov Moscow State University, Moscow, Russia

Publications in A1 peer-reviewed journals

- M. Lobeau, S. De Saeger, L. Sibanda, I. Barna-Vetró, and C. Van Peteghem. Development of a new clean-up tandem assay column for the detection of ochratoxin A in roasted coffee. *Analytica Chimica Acta*, 538:57-61, 2005.
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- M. Lobeau, S. De Saeger, A. Kolosova, I.Y. Goryacheva, L. Sibanda, and C. Van Peteghem. Influence of strongly coloured foodstuffs on the development of rapid membrane-based mycotoxin tests. *Analytical and Bioanalytical Chemistry*, in preparation for submission.

Publications in journals without peer-review

- S. De Saeger, L. Sibanda, C. Paepens, M. Lobeau, B. Delmulle, I. Barna-Vetró, and C. Van Peteghem. Novel developments in rapid mycotoxin detection. *Mycotoxin Research*, 2:100-104, 2006.