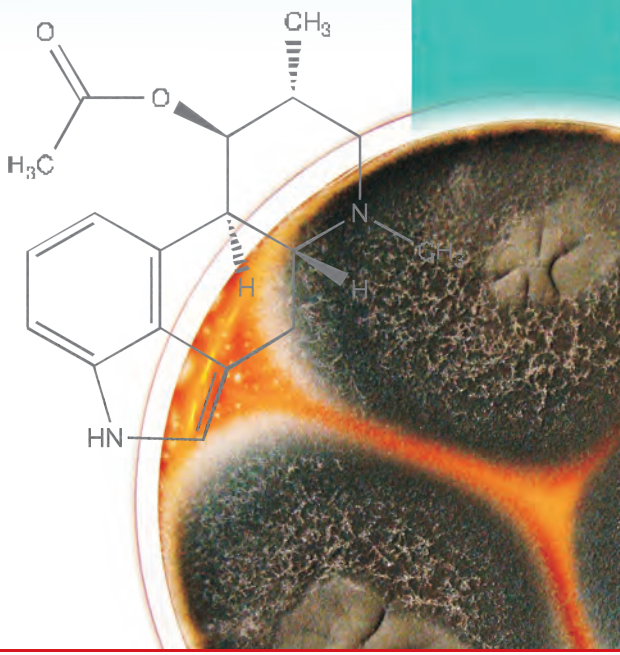
DEVELOPMENT AND APPLICATION OF AN ENZYME IMMUNOASSAY FOR THE DETECTION OF THE MYCOTOXIN FUMIGACLAVINE A

HADRI LATIF



INAUGURAL DISSERTATION
for the acquisition of the doctoral degree
at the Faculty of Veterinary Medicine
of Justus Liebig University Giessen
Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes

1. Auflage 2010

1st Edition 2010

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

From the Institute of Veterinary Food Science
Chair of Dairy Science
Justus Liebig University Giessen

Supervisor: Prof. Dr. Dr. habil. Ewald Usleber

Development and Application of an Enzyme Immunoassay for the Detection of the Mycotoxin Fumigaclavine A

INAUGURAL DISSERTATION
for the acquisition of the doctoral degree
at the Faculty of Veterinary Medicine
of Justus Liebig University Giessen
Germany

submitted by

HADRI LATIF
Veterinarian from Lasusua, Indonesia

Giessen 2010

With the permission of the Faculty of Veterinary Medicine of
Justus Liebig University Giessen

Dean : Prof. Dr. Martin Kramer

Referees : Prof. Dr. Dr. habil. Ewald Usleber
Prof. Dr. Dr. h. c. Bernd Hoffmann

Date of the Disputation: October 27th, 2010

Declaration

I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen,

Hadri Latif

Dedicated to My Dear Mother

CONTENTS

Contents i
List of Tables v
List of Figures vi

1 INTRODUCTION 1

2 LITERATURE REVIEW 3
2.1 Ergoline Alkaloid-Producing Fungi 3
2.1.1 *Aspergillus fumigatus* 3
2.1.2 *Penicillium roqueforti* 7
2.1.3 *Neotyphodium* spp. 7

2.2 Ergoline Mycotoxins 9
2.2.1 Lysergic Acid Derivatives 11
2.2.2 Clavine Alkaloids 11
2.2.2.1 Fumigaclavines 12
2.2.2.2 Other Clavines 14

2.3 Other Mycotoxins Produced by *A. fumigatus* 15
2.3.1 Gliotoxin 15
2.3.2 Fumitremorgins 16
2.3.3 Verruculogen 17
2.3.4 Fumigatin 18
2.3.5 Fumagillin 19
2.3.6 Helvolic Acid 20
2.3.7 Fumiquinazolines 21

2.4 Analytical Methods for Mycotoxins 25
2.4.1 Thin-Layer Chromatography 25
2.4.2 Gas Chromatography 27

2.4.3 Liquid Chromatography 29
2.4.4 Enzyme Immunoassay 32
2.4.4.1 Principle of EIA 32
2.4.4.2 Antibody production 32
2.4.4.3 Test formats 33
2.4.4.4 Enzyme conjugate 34
2.4.4.5 Detection systems 34
2.4.4.6 Application of EIA for mycotoxin analysis 35

3 MATERIALS AND METHODS 38
3.1 Materials 38
3.1.1 Laboratory Animals 38
3.1.2 Chemicals and Biochemicals 38
3.1.3 Toxins 39
3.1.4 Immunoreagents 39
3.1.5 Media 40
3.1.6 Buffers and Dilutions 40
3.1.7 Equipments and Instruments 40
3.1.8 Chromatographic and Fraction Equipments 41
3.1.9 Other Materials 41

3.2 Methods 42
3.2.1 Antibodies against FuA and EIA Development 42
3.2.1.1 Conjugate Synthesis 42
3.2.1.2 Rabbit Immunization 42
3.2.1.3 Antiserum Collection 43
3.2.1.4 Antibody Titer Determination 43
3.2.1.5 EIA Development 44
3.2.1.5.1 Method optimisation 44
3.2.1.5.2 Standard curve evaluation 45
3.2.1.6 Competitive Indirect EIA for FuA 45
3.2.1.7 Specificity of the Assay 46

3.2.2 Application Studies 47
3.2.2.1 Analysis of FuA in Silage 47
3.2.2.1.1 Sample collection 47
3.2.2.1.2 Sample preparation and analysis 47

3.2.2.2 Analysis of FuA in Tissue Samples from the Respiratory System of Birds with Aspergillosis 47
3.2.2.2.1 Sample collection 47
3.2.2.2.2 Sample preparation and analysis 48

3.2.2.3 Isolation and Characterization of Fumigaclavines 48
3.2.2.3.1 Fungal Cultivation and Toxin Isolation 48
3.2.2.3.2 EIA Analysis 49
3.2.2.3.3 HPLC Analysis 49
3.2.2.3.4 Immunochromatography Analysis 50

3.2.2.4 Combined Analysis of FuA and IsoFuA in Blue Cheeses 51
3.2.2.4.1 Competitive Direct EIA for Ergonovine 51
3.2.2.4.2 Specificity Pattern of FuA EIA and Ergonovine EIA 51
3.2.2.4.3 Analysis of Blue Cheese 52

4 RESULTS 54
4.1 Antibodies against FuA and EIA Development 54
4.1.1 Conjugate Synthesis 54
4.1.2 Antibody Production 56
4.1.3 EIA Development 57
4.1.4 Specificity of the Assay 60

4.2 Application Studies 61
4.2.1 Analysis of FuA in Silage 61
4.2.2 Analysis of FuA in Tissue Samples from the Respiratory System of Birds with Aspergillosis 61
4.2.3 Isolation and Characterization of Fumigaclavines 63
4.2.3.1 Fungal Cultivation 63

4.2.3.2 Toxin Isolation and EIA Analysis 65
4.2.3.3 HPLC Analysis 65
4.2.3.4 Immunochromatography Analysis 69
4.2.4 Combined Analysis of FuA and IsoFuA in Blue Cheese 71
4.2.4.1 Specificity Pattern of FuA EIA and Ergonovine EIA 71
4.2.4.2 Analysis of Blue Cheese 73

5 DISCUSSION 75
5.1 Antibodies against FuA 76
5.2 Development of EIA 77
5.3 Specificity of the Assay 78
5.4 Application Studies 79
5.4.1 Analysis of FuA in Silage 79
5.4.2 Analysis of FuA in Tissue Samples from the Respiratory System of Birds with Aspergillosis 79
5.4.3 Isolation and Characterization of Fumigaclavines 80
5.4.4 Combined Analysis of FuA and IsoFuA in Blue Cheese 81

6 SUMMARY 83
7 ZUSAMMENFASSUNG 85
8 REFERENCES 87
9 ABBREVIATIONS 116
ACKNOWLEDGEMENTS 118

LIST OF TABLES

Tab. 1 Some physiochemical properties of mycotoxins produced by *A. fumigatus* 23
Tab. 2 Examples of TLC methods used for the detection of mycotoxins 26
Tab. 3 Examples of GC methods used for the detection of mycotoxins 28
Tab. 4 Examples of LC methods used for the detection of mycotoxins 31
Tab. 5 Examples of protocols and detection limits using EIA for mycotoxin 37
Tab. 6 Relative cross-react of antibodies against FuA with FuB and some lysergic acid derivatives 60
Tab. 7 Recovery of FuA from artificially contaminated silage 61
Tab. 8 Recovery of FuA from artificially contaminated respiratory system tissue of birds (control samples) 62
Tab. 9 FuA in tissue samples from the respiratory system of dead birds with confirmed aspergillosis 62
Tab. 10 Different specificity pattern of FuA EIA and ergonovine EIA 72

LIST OF FIGURES

Fig. 1 Biosyntetic pathways of ergoline alkaloids in *C. purpurea* and *A. fumigatus* 4

Fig. 2 Comparative representation of the chemical structure of some ergoline alkaloids 10

Fig. 3 The basic structures of clavine alkaloids 12

Fig. 4 Structures of fumigaclavines 13

Fig. 5 Structure of gliotoxin 15

Fig. 6 Structures of fumitremorgins 17

Fig. 7 Structure of verruculogen 18

Fig. 8 Structure of fumigatin 19

Fig. 9 Structure of fumagillin 19

Fig. 10 Structure of helvolic acid 20

Fig. 11 Structures of fumiquinazolines 22

Fig. 12 Schematic of the microtiter plate and concentrations used in the checkerboard experiment 44

Fig. 13 UV spectrophotometry of FuA standard (5 µg/ml) dissolved in methanol 54

Fig. 14 Comparison of the UV spectrophotometry of FuA-KLH conjugate dissolved 1:20 in PBS, FuA and KLH 55

Fig. 15 Comparison of the UV spectrophotometry of FuA-BSA conjugate dissolved 1:20 in PBS, FuA and KLH 55

Fig. 16 Antibody titers of three rabbits after immunization with FuA-KLH conjugate 56

Fig. 17 Comparison of the standard curves of the competitive indirect EIA for FuA using the antiserum from rabbits 55 and 57 58

Fig. 18 Optimized standard curve of the competitive indirect EIA for FuA using plate coating with FuA-BSA and antiserum of rabbit 57 59

Fig. 19 Fungal growth on air sac of dead Humboldt Penguin with confirmed aspergillosis 63

Fig. 20 *A. fumigatus*: colonies 7 days at 25^{o}C on MEA (A), conidia and conidiophores (B) 64

Fig. 21 HPLC chromatogram of FuA standard (100 ng/ml) 66
Fig. 22 HPLC chromatogram of mycelium extract of *A. fumigatus* (diluted 1:1000 in methanol) 66
Fig. 23 Chemical transformation of FuA into FuB and FuC into FuD by alkaline hydrolysis 67
Fig. 24 HPLC chromatograms of mycelium extract of *A. fumigatus*, before hydrolysis (A) and after hydrolysis (B) 68
Fig. 25 Immunogram of HPLC eluate fractions of *A. fumigatus* extract after hydrolysis 70
Fig. 26 Standard curve of the competitive direct enzyme immunoassay for ergonovine 71
Fig. 27 Schematic diagram of different specificity pattern of antibodies against FuA and ergonovine 72
Fig. 28 HPLC chromatograms of blue-veined cheese extract, without and with FuA standard (500 ng/ml) 73
Fig. 29 Comparison of FuA and ergonovine EIA immunograms of HPLC eluate fractions of blue-veined cheese extract (sample 01) 74

1 INTRODUCTION

Fumigaclavine A (FuA) and the closely related compounds FuB and FuC are indole alkaloids belonging to the large group of ergolines (clavine alkaloids). Production of clavine alkaloids has been reported for species belonging to several fungal genera, including *Aspergillus* (*e.g.*, *A. fumigatus*), *Penicillium* (*e.g.*, *P. roqueforti*), *Claviceps* (*e.g., C. purpurea*), endophytic *Epichloë/Neotyphodium* species (*e.g.*, *Neotyphodium (*syn. *Acremonium) coenophialum*), and by an epibiotic clavicipitaceous fungus named IasaF13 (Lyons et al. 1986; Flieger et al. 1997; Panaccione 2005; Steiner et al. 2006; Frisvad et al. 2008, Ge et al. 2009). Due to the ubiquitous occurrence of clavine-producing fungi on and in plants, these alkaloids can potentially be found on (or in) all plant materials and in the environment.

Little is known about the effects of fumigaclavines, but in general clavine alkaloids are antagonists (or partial agonists) of serotonin, and therefore have neurological properties which are also used therapeutically (Pertz 1996). For other clavines, various pharmacological and toxicological properties have been reported. FuC has various pharmacological effects and caused concentration-dependent vasorelaxation in isolated rat aortic rings (Ma et al. 2006), improved concanavalin A-induced liver injury in mice via inhibiting TNF alpha production (Zhao et al. 2004), and improved experimental colitis in mice via downregulating Th1 cytokine production (Wu et al. 2005). Agroclavine interrupted early pregnancy in mice when given orally but not after subcutaneous application (Mantle 1969), and potentiated hippocampal EEG effects of weak combined magnetic field in rats (Vorobyov et al. 2009). Clavine alkaloids, but not lysergic acid derivatives also seem to have antibacterial effects (Schwarz and Eich 1983).

Analysis of FuA may therefore be interesting within a strategy of mycotoxin analyses and also could be indicative for presence and metabolic activity of one of the FuA producing fungal species listed above. Furthermore, FuA analysis may also be of interest considering infectious diseases caused by *A. fumigatus*. This fungus is an opportunistic pathogen in human and animals, and causes a wide

range of severe respiratory diseases in humans, including allergic reactions (allergic fungal sinusitis, allergic broncho-pulmonary aspergillosis; for review see, *e.g.*, Shah 2008) and necrotizing lung infections such as invasive aspergillosis, chronic necrotizing pulmonary aspergillosis, and aspergilloma (for review see, *e.g.*, Latgé 2001; Zmeili and Soubani 2007).

From an immunochemical point of view, clavine alkaloids are a very heterogenous group of compounds, because of the large variability of substituents at C-2, C-8, and C-9, and because of the existence of α- and β-epimeric forms at C-8 and C-9 for some compounds. Thus, the production of generic anti-clavine antibodies does not seem to be feasible. Since FuA is the most common clavine, and since it is commercially available as a standard substance, this study concentrated on FuA as the hapten. Until now antibodies against FuA have not been described.

The aims of this study were (1) to develop high-affinity antibodies against FuA, (2) to establish an enzyme immunoassay for trace level detection of FuA; and (3) to establish immunochemical methods of analysis for FuA in food (*e.g.*, cheese), feed (*e.g.*, silage), and biological material (*e.g.*, respiratory system tissue of bird).

2 LITERATURE REVIEW

2.1 Ergoline Alkaloid-Producing Fungi

Ergoline alkaloids represent one of the most interesting groups of secondary metabolites of microscopic fungi. They are produced by a wide range of fungi, predominantly by members of the grass-parasitizing family of the *Clavicipitaceae,* especially from the genus *Claviceps* (*e.g. C. purpurea*). However, the capacity to synthesize ergolines is not restricted to the genus *Claviceps*. Fungi of the genera *Acremonium/Neotyphodium* (*e.g.*, *Neotyphodium coenophialum*), *Aspergillus* (*e.g.*, *A. fumigatus*), *Penicillium* (*e.g.*, *P. roqueforti*), as well as certain plants of the *Convolvulaceae* family were also found to synthesize ergoline alkaloids (Flieger et al. 1997; Boichenko et al. 2001; Vinokurova et al. 2003a; Wang et al. 2004; Frisvad et al. 2008). The ergoline alkaloids of these divergent fungi share a four-membered ergoline ring system, but differ in number, type, and position of the side chain residues (Floss 1976; Coyle and Panaccione 2005).

Interestingly, ergoline alkaloid-producing fungi typically produce a characteristic profile of several ergoline alkaloids rather than a single end product (Panaccione 2005). Figure 1 shows the common steps in biosynthesis pathway of ergoline alkaloids in *A. fumigatus* and *C. purpurea*.

2.1.1 *Aspergillus fumigatus*

A. fumigatus Fresen. is a fungus of the genus *Aspergillus*, family Trichocomaceae, order Eurotiales, class Eurotiomycetes, phylum Ascomycota, and kingdom Fungi. It is a saprophyte fungus which survives and grows over a large variety of organic remains and whose most common ecological niche is soil (Marsh et al. 1979; Rementeria et al. 2005). *A. fumigatus* is found worldwide and has an essential role in recycling carbon and nitrogen. This fungal species has a very simple biological cycle, and grows faster than any other airborne fungi at 40^{o}C (Tekaia and Latgé 2005).

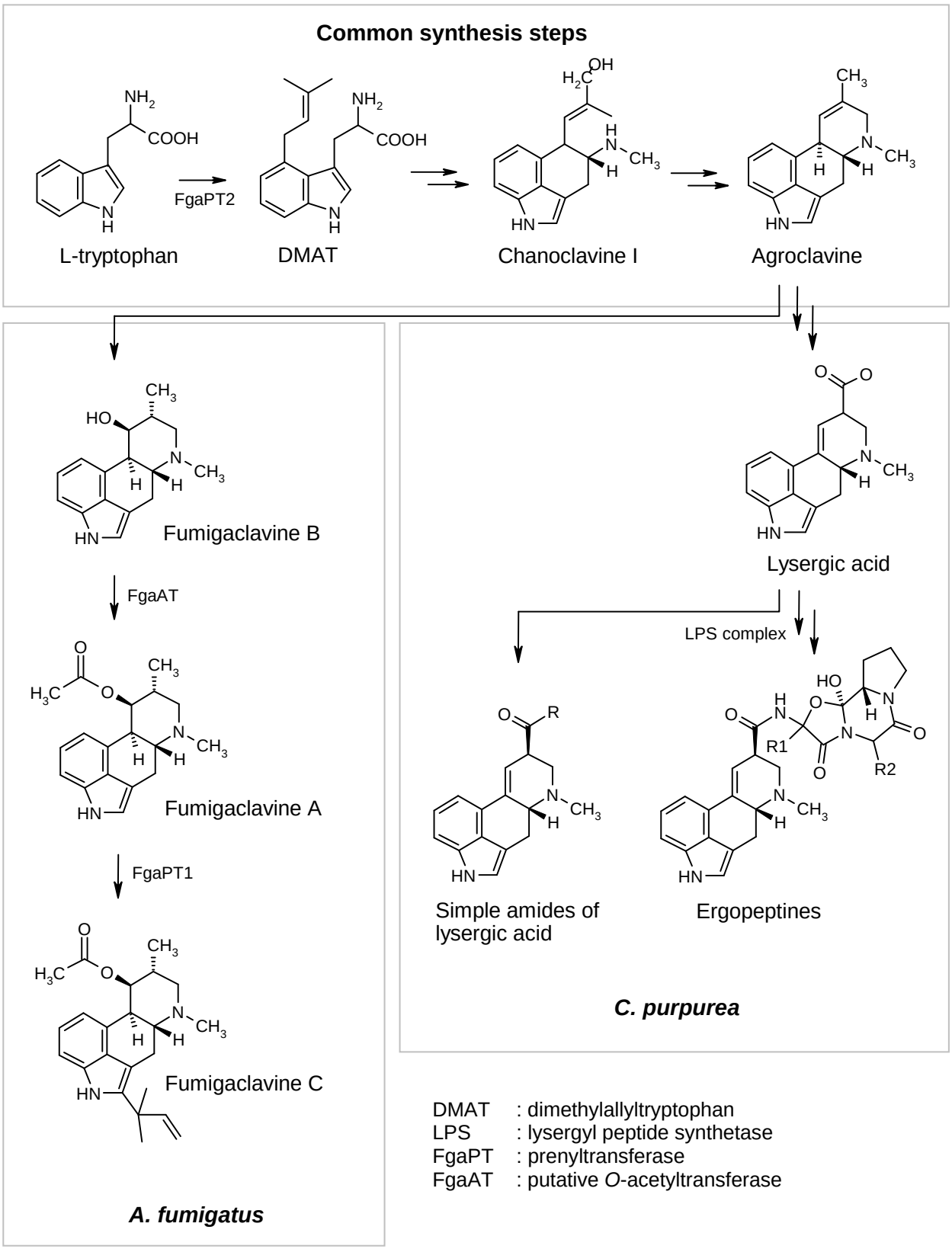


Figure 1. Biosyntetic pathways of ergoline alkaloids in *A. fumigatus* and *C. purpurea* (Tudzynski et al. 2001; Panacionne 2005; Li and Unsöld 2006; Rigbers and Li 2008).

At least 20% of *A. fumigatus*-specific genes appear to be functional and are involved in carbohydrate and chitin catabolism, transport, detoxification, secondary metabolism, and other functions that may facilitate the adaptation to heterogeneous environments such as soil or a living host (Fedorova et al. 2008). Much of the basic biology of *A. fumigatus*, including it is rapid growth rate, remarkable thermo-tolerance, numerous secondary metabolic pathways producing both toxic and useful metabolites, and pathogenicity in general are poorly understood (Ronning et al. 2005). Until recently *A. fumigatus* was only thought to reproduce asexually, as neither mating nor meiosis had ever been observed in the fungus. However, O'Gorman et al. (2009) reported for the first time that *A. fumigatus* possesses a fully functional sexual reproductive cycle, 145 years after it is original description by Georg W. Fresenius. These authors proposed the name *Neosartorya fumigata* for the teleomorph of *A. fumigatus*, although Hawksworth (2009) suggested that *A. fumigatus* should remain as the sole name for pragmatic reasons.

Genetically *A. fumigatus* seems to be very homogeneous and unique, and is clearly separated from related species based on genetic traits including beta-tubulin, actin, and calmodulin. Therefore the new taxonomies are based on a polyphasic approach using phenotypical characters together with multigene deoxyribonucleic acid (DNA) sequences. In a polyphasic approach, micro- and macromorphology, physiology, metabolites produced, and molecular data are all important, and in principle no particular method should be overemphasized (Samson et al. 2006).

The ubiquitous *A. fumigatus* is an opportunistic fungal pathogen, it is by far the most important pathogenic species within *Aspergillus* section Fumigati, both in humans and in animals (Tepšič et al. 1997; Stanzani et al. 2005; Li and Unsöld 2006; Orciuolo et al. 2007). *A. fumigatus* may also be regarded as the most important airborne pathogenic fungus (Brakhage and Langfelder 2002). It produces small-sized conidia (2-3 µm) which become airborne easily and allows them to be deposited deep in the lung (Cockrill and Hales 1999). *A. fumigatus* causes a wide range of diseases which include mycotoxicosis, allergic reactions,

and systemic diseases with high mortality rates (Rinyu et al. 1995; Rementeria et al. 2005). *A. fumigatus* produces a number of mycotoxins, including ergolines (clavine alkaloids) such as fumigaclavines and festuclavine (Spilsbury and Wilkinson 1961; Ohmomo et al. 1989; Panaccione and Coyle 2005; Coyle et al. 2007).

Since *A. fumigatus* is ubiquitous in silage (Cole et al. 1977; Santos et al. 2002), feeds may be contaminated with clavine alkaloids, although no data have been published. Likewise, natural occurrence of fumigaclavines in food seems to be probable, but only one study (Scott and Kennedy 1976) could be found in the literature.

Aspergillosis, which is mainly caused by *A. fumigatus*, presents a wide spectrum of clinical syndromes in the respiratory system. Invasive pulmonary aspergillosis (IPA) is a severe disease, and a major cause of mortality in severely immunocompromised patients. Critically ill patients without malignancy may also develop IPA without having the classic risk factors. Chronic necrotizing aspergillosis (CNA), which is locally invasive, is seen mainly in patients who are mildly immunocompromised or have chronic lung disease. Aspergilloma and allergic bronchopulmonary aspergillosis (ABPA) are non-invasive pulmonary diseases. Aspergilloma is a fungus ball that develops in a pre-existing cavity in the lung parenchyma, while ABPA is a hypersensitivity disease of the lungs that almost always affects patients with asthma or cystic fibrosis (for review see, *e.g.*, Zmeili and Soubani 2007; Shah 2008).

Among animals, birds kept in captivity are particularly affected by aspergillosis (Tell 2005). Various clavine alkaloids produced by *A. fumigatus* may be responsible for part of the immunosuppresive activity of the fungus and play an important role in the pathogenesis of aspergillosis.

2.1.2 *Penicillium roqueforti*

P. roqueforti is a common saprotrophic fungus from the family Trichocomaceae. It is the best studied representative of genus *Penicillium*. Formerly *P. roqueforti* was divided into two varieties, one used for cheese starter cultures (*P. roqueforti* var. *roqueforti*), and one ubiquitous patulin-producing variety (*P. roqueforti* var. *carneum*). Based on DNA ribosomal sequences, random amplified polymorphic DNA (RAPD) profiles, and secondary metabolite profiles, *P. roqueforti* was recently split into three species, nemely *P. roqueforti, P. carneum,* and *P. paneum* (Boysen et al. 1996). *P. roqueforti* is the most common species of the three, and due to it is resistance to organic acids and ability to grow at low pH, it is found as a contaminant of processed food such as bread, rye bread, beer, hard cheeses, and olives (Nielsen et al. 2006).

In cheese-making industry, *P. roqueforti* is commonly used to produce a variety of blue-veined cheeses. It was also the most frequently isolated fungus in silage (Ohmomo and Kitamoto 1994; Auerbach et al. 1998; O´Brien et al. 2006; O´Brien et al. 2008). Therefore, the ability of this fungus to produce mycotoxins, including ergoline alkaloids, has attracted much research.

Some clavine alkaloids have been identified in *P. roqueforti*, including IsoFuA, IsoFuB, festuclavine, and chanoclavine-I (Ohmomo et al. 1975; Scott and Kennedy 1976; Scott et al. 1976; Vinokurova et al. 2001).

2.1.3 *Neotyphodium* spp.

Ergoline alkaloids have gained considerable agricultural relevance not only due to contamination of cereals with sclerotia of the genus *Claviceps*, but also because of ergopeptine-producing endophytic fungi wich are involved in the intoxication with ergoline alkaloids and other toxins of animals held on pasture grassland in many parts of the world (for review see, *e.g.*, Tudzynski et al. 2001). These endophytes,

especially *Epichloë* spp. and their asexual forms *Neotyphodium* spp. (formerly *Acremonium* spp.*)*, which are living as symbionts intercellularly on grasses such as *Festuca,* can cause serious problems in livestock breeding, for example fescue toxicosis in cattle (Blankenship et al. 2001). In general, endophyte infection frequencies seem more variable in natural compared to pastoral situations, suggesting that expressions and benefits (protection against "herbivorous enemies") attributable to endophyte infection depend on the resource environment in which the host–endophyte complex was formed, and the conditions under which it is being grown (for review see, *e.g.*, Malinowski and Belesky 2006).

Neotyphodium spp. have been reported to produce ergoline alkaloids such as ergovaline (Lyons et al. 1986; Panaccione et al. 2003; Wang et al. 2004), lysergic acid amide, isolysergic amide**,** 8-hydroxylysergic acid amide, ergonovine*,* chanoclavine-I, and N-formylloline (Petroski et al.1992). In addition, other alkaloids such as lolitrem may be important in the context of anti-herbivorous alkaloids produced by endophytic fungi (Rasmussen et al. 2008).

2.2 Ergoline Mycotoxins

Mycotoxins are a group of secondary fungal metabolites which can cause a toxic response, termed a mycotoxicosis, if ingested by higher vertebrates and other animals (Sweeney and Dobson 1999). The most notorious mycotoxicosis in human history is ergotism, which is caused by consumption of grain, usually rye, contaminated with sclerotia of *Claviceps purpurea*. The toxic effects of ergoline alkaloids have been the cause of mass poisonings in both humans and animals. Ergoline poisoning in humans has been known for more than 2,000 years, and was responsible for numerous medieval epidemics of a disease then called St. Anthony's Fire was characterized by gangrenes of the extremities and neurological symptoms (convulsions, psychoses) (for review see, *e.g.* Desjardins and Hohn 1997; Tudzynski et al. 2001; Lee et al. 2009).

Ergoline alkaloids are indole compounds that are biosynthetically derived from L-tryptophan and represent the largest group of nitrogeneous fungal metabolites found in nature (Heinstein et al. 1971; Schiff 2006; Ahimsa-Müller et al. 2007). Over 80 ergoline alkaloids are known. The common structural feature is the ergoline ring system with a nitrogen atom at positions 1 and 6 (Figure 2). Because of their structural features, ergoline alkaloids can be divided into two groups, lysergic acid derivatives and clavine alkaloids (Floss 1976; Tudzynski et al. 2001; Metzger et al. 2009). Depending on the substituent at C-8 of ring D, ergoline alkaloids can also be divided in four groups, (1) the clavine alkaloids (*e.g.* agroclavine, lysergine, festuclavine, and fumigaclavines), (2) the simple lysergic acid derivatives (*e.g.* lysergic acid, ergometrine, also known as ergonovine), (3) the peptide alkaloids-cyclo ergoline alkaloids (*e.g.* ergolineamine, ergocristine, ergocornine), and (4) the lactam ergoline alkaloids (Flieger et al. 1997).

In general ergoline alkaloids interact with several monoamine receptors, resulting in cardiovascular, nervous, and reproductive disorders, and by affecting the immune system (Panaccione and Coyle 2005).

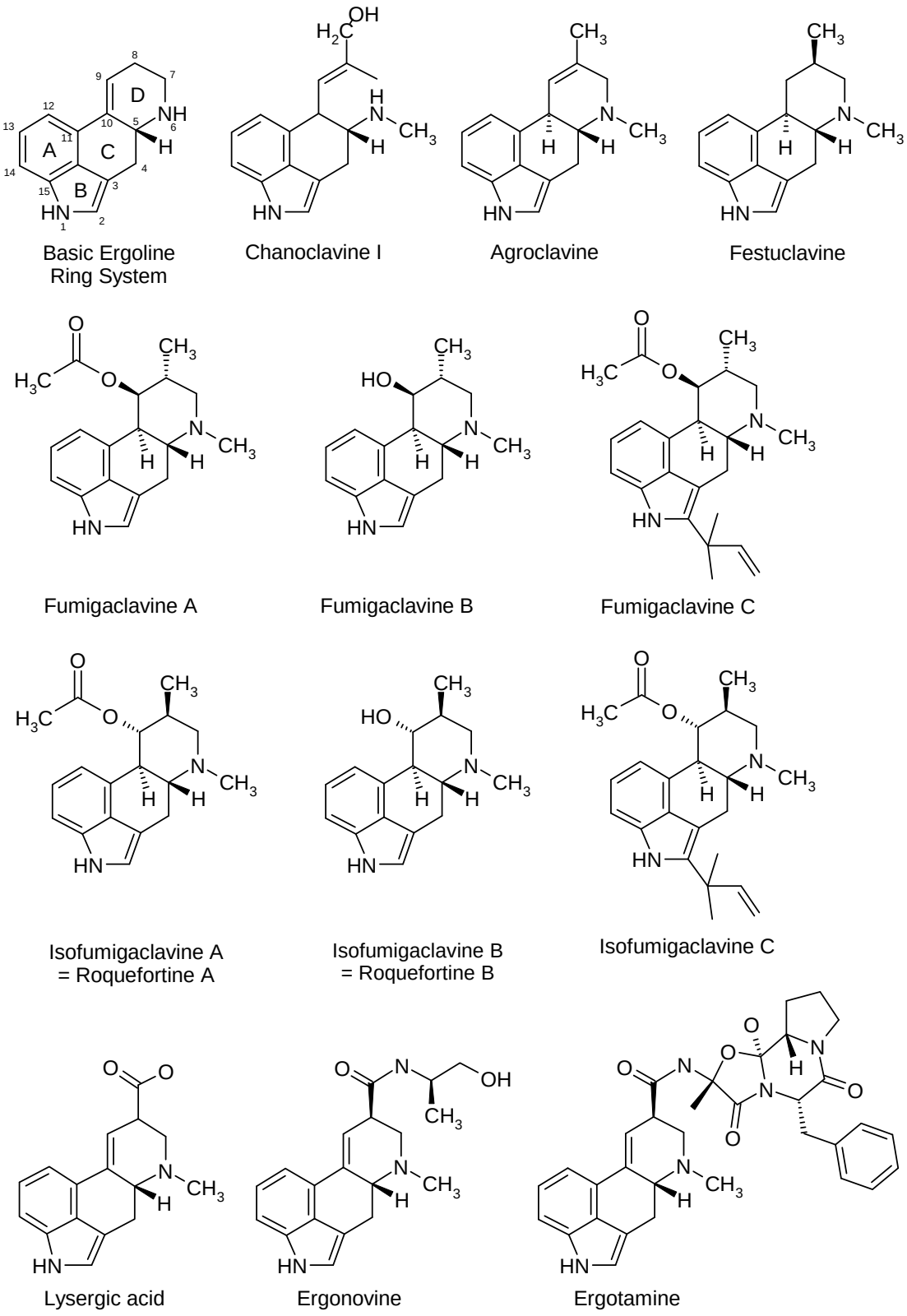


Figure 2. Comparative representation of the chemical structure of some ergoline alkaloids: clavines and lysergic acid derivatives.

2.2.1 Lysergic Acid Derivatives

Lysergic acid, also known as D-lysergic acid and (+)-lysergic acid, is a precursor of a wide range of ergoline alkaloids that are produced by the “ergot” fungus, namely *Claviceps* species. All lysergic acid derivatives reported to date are either lysergic acid amides (simple lysergic acid amides and paspalic acid, *e.g*., ergonovine, erginine) or lysergic acid peptides (sometimes also designated as ergopeptides or ergopeptines, *e.g*., ergotamine, α- and β-ergocryptine, ergocornine) (Floss 1976; Flieger et al. 1997; Schiff 2006).

Lysergic acid derivatives have a wide spectrum of pharmacological and toxicological activities. Depending on the substituent attached to the carboxy group at C-8 of ring D of the ergoline ring system, the lysergic acid pharmacophore has different affinities towards the various receptors of the noradrenaline, dopamine, and serotonin neurotransmitters. Since the C-8 bond may undergo isomerisation, all ergoline alkaloids exist as isomeric pairs, the –ine form (*e.g*., ergotamine) and, the –inine form (*e.g*., ergotaminine). The latter are pharmacologically inactive (Hofmann 1964). Chemical synthesis of the natural lysergic-acid-related compounds, such as ergometrine and ergopeptines, as well as of new semisynthetic derivatives (methylergometrine, methysergide, or cabergoline) used in therapy (for review see, *e.g.*, Tudzynski et al. 2001).

2.2.2 Clavine Alkaloids

Clavine alkaloids are the most simple ergoline alkaloids, they lack the amide-linked side chains on the ergoline ring system. Certain clavines provide a pathway to lysergic acid, whereas others may be products of shunts off the main ergoline alkaloid pathway. Still other clavines may be the ultimate pathway end product in their producing organism (for review see, *e.g*., Panaccione 2005).

Clavine alkaloids can be divided into three groups (Figure 3), one consisting of the alkaloids with an 8,9-ergolene skeleton, *e.g.* agroclavine, another consisting of those with a 9,10-ergolene skeleton, *e.g.* lysergol, and the third consisting of those with a hydroxyl group at the 9-position, *e.g.* fumigaclavines (Floss 1976; Rehacek 1980; Ninomiya et al. 1986).

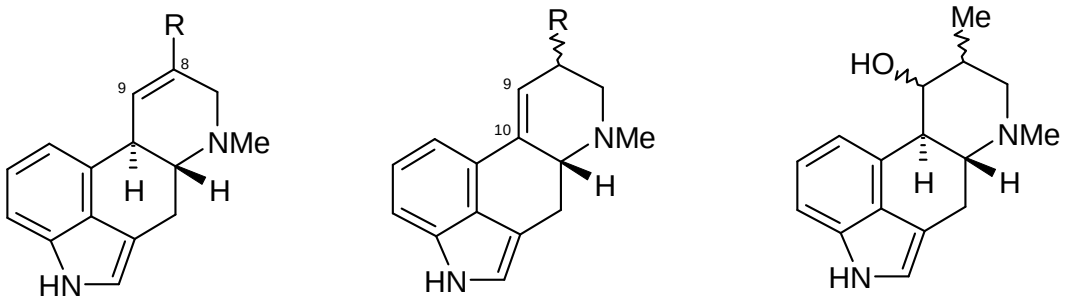


Figure 3. The basic structures of clavine alkaloids.

Although most clavine alkaloids have been isolated from members of the genus *Claviceps,* some clavines have also been found in other fungi, *e.g. Aspergillus* and *Penicillium* (Spilsbury and Wilkinson 1961; Vinokurova et al. 2001; Frisvad et al. 2007). For example, some clavine type alkaloids (fumigaclavines) are produced by *A. fumigatus*, but not by fungi belonging to genus *Claviceps* (*e.g. Claviceps purpurea*). Conversely, the amide and peptide derivatives of lysergic acid are produced by *C. purpurea*, but not by *A. fumigatus*. In contrast, agroclavine was identified in both fungal groups (Flieger et al. 1997; Li and Unsöld 2006).

2.2.2.1 Fumigaclavines

Fumigaclavines (FuA, FuB, and FuC, Figure 4) are clavine alkaloids which are mainly produced by *A. fumigatus* (Spilsbury and Wilkinson 1961; Ohmomo et al. 1989; Panaccione and Coyle 2005; Coyle et al. 2007; Frisvad et al. 2008), while the main producer of their 8,9-isomers (IsoFuA = roquefortine A; IsoFuB =

roquefortine B) are found in *Penicillium* species belonging to the *P. roqueforti* complex (Ohmomo et al. 1975; Scott et al. 1976; Nielsen et al. 2006). However, literature data concerning the question which isomers are produced by which fungus are to date somewhat confusing. For example, Lund (1995) reported that all isolates of *P. palitans* produced FuA but not IsoFuA. Vinokurova et al. (2001) described two strains of *P. roquefortii* Thom 1906, one producing FuA, the other IsoFuA. These authors also reported that some strains of *P. commune* Thom and of *P. clavigerum* Demelius were able to produce FuA and FuB (Vinokurova et al. 2003c). Finally, new clavines and other fungal ergoline alkaloids are isolated up today (Ge et al. 2009).

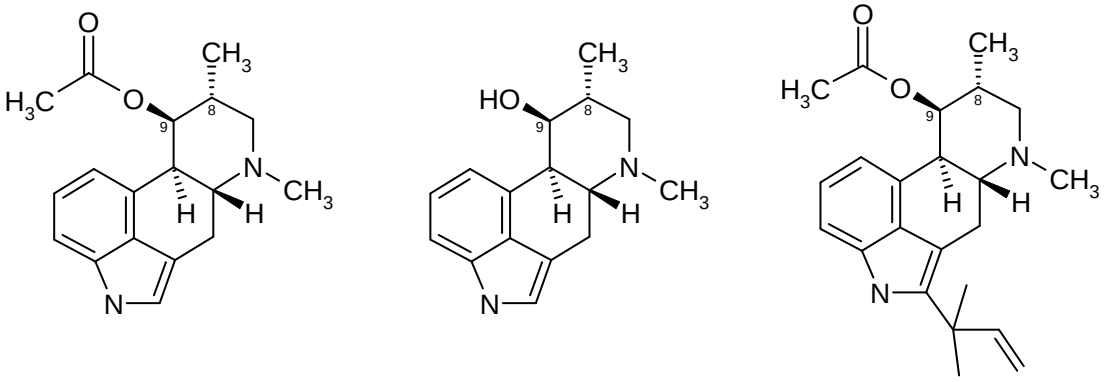


Figure 4. Structures of fumigaclavines.

Both FuA and FuC indirectly affect the peripheral and central nervous system (Lugauskas 2005). FuC exhibited potent concentration dependent vaso-relaxant actions in isolated rat aortic ring pre-contracted by high K^+ or phenylephrine with 50% effective dose (EC_{50}) values of 5.62 µmol/l and 1.58 µmol/l, respectively (Ma et al. 2006), and affects the lymphocytes by inhibiting their activation, proliferation and adhesion to extracellular matrices, and reducing their production of tumor necrosis factor-alpha (TNF-α) (Zhao et al. 2004). FuC also inhibited the expression and production of inflammatory cytokines, and the activity of matrix metallo-proteinase in sacral lymph node cells, colonic patch lymphocytes, and colitis

tissues from 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis mice (Wu et al. 2005).

Ingestion of crude extracts of *A. fumigatus* cultures (approximately 2.5 and 5.5 mg of FuC per kg body weight) by calves resulted in severe diarrhea, irritability, reduced feed intake, causes serious enteritis, and evidence of interstitial changes in the lungs. The oral 50% lethal dose (LD_{50}) of FuC was about 150 mg/kg in one day-old cockerels (Cole et al. 1977).

2.2.2.2 Other Clavines

The number of clavine alkaloids described in the scientific literature has proliferated greatly over the years, and new members continue to be added to the list. Little is known about the effects of clavine alkaloids in man and animal. However, some clavine alkaloids, including agroclavine, costaclavine, dihydrolysergol-l, elymoclavine, festuclavine, lysergene, lysergol, and pyroclavine are antagonism/partial agonism at 5-HT_{2A} receptors and antagonism at α_1-adrenoceptors in blood vessels (Pertz 1996). Agroclavine, festuclavine, and their derivatives were also reported as a potent cytostatic agents by Eich et al. (1984).

2.3 Other Mycotoxins Produced by *A. fumigatus*

A recent review concerning the metabolomics of *A. fumigatus* (Frisvad et al. 2008) includes a comprehensive list of mycotoxins produced by this fungus, but gives little information concerning their toxicity. Therefore some major toxins of *A. fumigatus* are described in the following chapters.

2.3.1 Gliotoxin

Gliotoxin (Figure 5) is a secondary metabolite which is produced by a number of *Aspergillus and Penicillium* species, and also by some species of *Gliocladium*, *Thermoascus,* and *Candida* (Nieminen et al. 2002; Lewis et al. 2005). However, the main producer seems to be *A. fumigatus* (Pattron 2006).

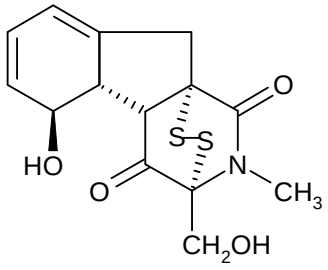


Figure 5. Structure of gliotoxin.

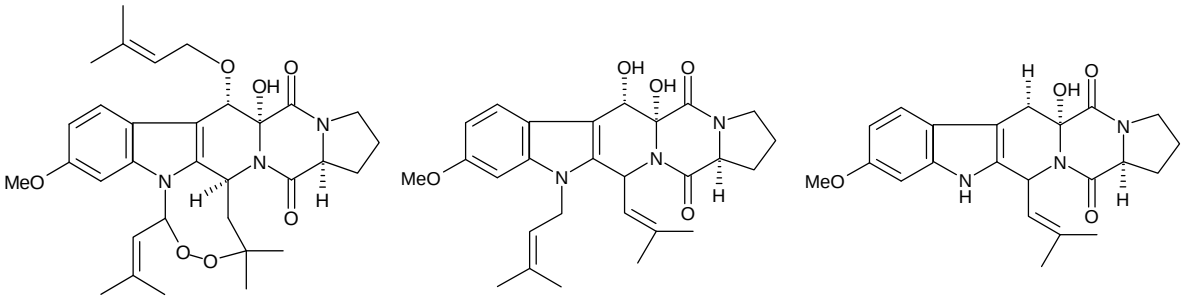
Gliotoxin is one of several members of the epipolythiodioxopiperazines (ETP), a class of cyclic dipeptides characterised by the presence of an internal disulfide bridge (Waring and Beaver 1996; Gardiner and Howlett 2005; Gardiner et al. 2005). It is extremely sensitive to alkalies and is altered rapidly even by mild alkaline reagents such as sodium bicarbonate, sodium sulfite, and sodium sulfide (Dutcher et al. 1945). The biological activity of gliotoxin is dependent on the presence of a strained disulfide bond that can react with accessible cysteine residues on proteins (Hurne et al. 2000).

Gliotoxin primarily has immunosuppressive activity (Sutton et al. 1994), and is now considered as a virulence factor in human and animal aspergillosis (Kamei and Watanabe 2005; Kwon-Chung and Sugui 2008; Hof and Kupfahl 2009). It is capable of inhibiting macrophage function (Eichner et al. 1986; Bertout et al. 2002) and thus may alter the immune response to *Aspergillus.* Gliotoxin induces apoptotic cell death in macrophages (Waring 1990), and affects spleen cells (Braithwaite et al. 1987). Gliotoxin has been detected in lung tissue samples of poultry (Richard and DeBey 1995; Richard et al. 1996), where it may facilitate fungal persistence and colonization of tissue. In addition, gliotoxin has been implicated for the destruction of lung parenchyma in invasive aspergillosis (Sutton at al. 1996) and the penetration of blood vessels in angio-invasive aspergillosis (Fraser 1993).

Gliotoxin inhibits phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembly and activation, a key component in host defence against filamentous fungi (Müllbacher and Eichner 1984). It also inhibits activation of nuclear factor kappa B (NF-kB; a transcriptional factor that is a key mediator of cytokine and inflammatory responses) in T and B cells (Pahl et al. 1996). Intraperitoneal injection of gliotoxin depressed platelet aggregation in mice at a dose of 0.25 mg/kg with LD_{50} value was about 5-10 mg/kg (Sakai and Watanuki 1987).

2.3.2 Fumitremorgins

A. fumigatus has been reported to produce fumitremorgins A, B, and C (Yamazaki et al. 1971; Cole et al. 1977). Fumitremorgins (Figure 6) are members of a group of prenylated indole alkaloids (Yamazaki et al. 1980a; Maiya et al. 2006). Fumitremorgin A is the most potent mycotoxin among the fumitremorgins (Nishiyama and Kuga 1986). It caused sustained tremor with intermittent convulsion (which was often fatal) in mice, rats, and rabbits after several tonic-clonic convulsions (Yamazaki et al. 1980a; Yamazaki et al. 1980b).



Fumitremorgin A Fumitremorgin B Fumitremorgin C

Figure 6. Structures of fumitremorgins.

From experiments with rabbits, Nishiyama and Kuga (1990) concluded that fumitremorgin A might activate some neurons in the midbrain reticular formation, and that convulsive burst discharges in peripheral motor nerves resulted from abnormal activation of these neurons. Fumitremorgen B also caused severe tremors and convulsion in experimental animals (Yamazaki et al. 1980b), and DNA damage in human lymphocytes (Sabater-Vilar et al. 2003). The LD_{50} value of fumitremorgin A in mice after intravenous administration was 185 µg/kg (Yamazaki et al. 1979).

2.3.3 Verruculogen

Verruculogen (Figure 7) is one of the tremorgenic mycotoxins produced by fungi belonging to the genera *Aspergillus* and *Penicillium.* Most producers of verruculogen were identified as *A. fumigatus,* and some *as A. fischeri* (Shreeve et al. 1983). Verruculogen is produced not only during myceliar growth of fungi, but was also found in the spores of *A. fumigatus* (Kosalec et al. 2005). It is also a member of the indole alkaloid group of mycotoxins (Abraham and Arfmann 1990). Like the fumitremorgins, verruculogen was found to induce tremor and muscular incoordination in laboratory and farm animals (Peterson et al. 1982). However, the effect of this mycotoxin upon health, especially of immuno-compromised patients,

is still unknown, as well as its influence on healthy persons (Kosalec et al. 2005). The intraperitoneal LD_{50} of verruculogen was 2.4 mg/kg in mice and 15.2 mg/kg in chicken. Orally administered LD_{50} values for this toxin were 126.7 mg/kg and 365.5 mg/kg in mice and chicken, respectively (Cole et al. 1972).

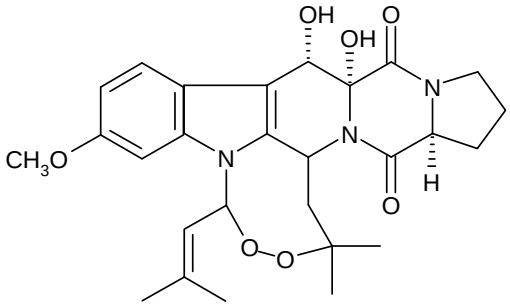


Figure 7. Structure of verruculogen.

Khoufache et al. (2007) reported that verruculogen modifies the electro-physiological properties of human nasal epithelial cells (HNEC). The role of these modifications in the colonization and invasion of the respiratory epithelium by *A. fumigatus* on first contact with the epithelium remains to be determined. The authors speculated that the presence of verruculogen at the cellular level could alter pulmonary clearance and thereby increase colonization and the risk of invasive aspergillosis.

2.3.4 Fumigatin

Fumigatin (Figure 8) has been originally isolated from the medium of a strain of *A. fumigatus* by Anslow and Raistrick (1938). It is a relatively small molecule, chemically it is a p-toluqunone derivative (Raistrick 1940; Pettersson 1963). Frisvad et al. (2008) list 21 fumigatin analogues within the metabolome of *A. fumigatus*, and report that 35% of the tested strains produced fumigatin.

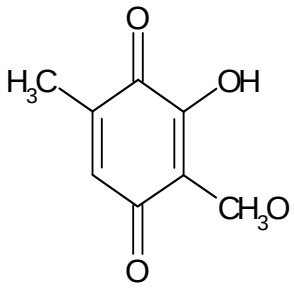


Figure 8. Structure of fumigatin.

Fumigatin is soluble in acetone, ether, chloroform, benzene, ethyl acetate, and alcohol, fairly soluble in water and slightly soluble in light petroleum (Anslow and Raistrick 1938). Fumigatin was found to have antibiotic activity (Waksman and Geiger 1944). It was cited to be toxic against experimental animals, but it is not clear to what extent these substance may cause disease conditions in animals under natural conditions (Austwick 1965).

2.3.5 Fumagillin

Fumagillin (Figure 9) is a cyclohexane derivative. It was first reported as an antiphage agent (H-3) by Hanson and Eble (1949) from *A. fumigatus*. Fumagillin is representative of a class of sesquiterpenes (Picoul et al. 2003). One of the unique structural feature of fumagillin is that possess two potentially reactive epoxide groups (Griffith et al. 1998).

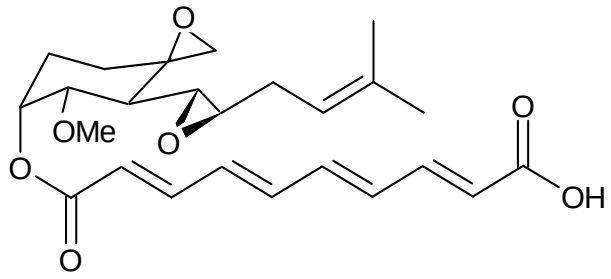


Figure 9. Structure of fumagillin.

Fumagillin was first described as an antimicrobial agent, but more recently, Ingber et al. (1990) discovered that this compound is a potent and selective inhibitor of endothelial cell proliferation of angiogenesis.

Fumagillin was reported to have cilioinhibitory effects on the human ciliated respiratory epithelium (Amitani et al. 1995). Stanimirovic et al. (2007) suggest that fumagillin has also genotoxic (clastogenic) potential in mammals *in vivo*.

2.3.6 Helvolic acid

The isolation of helvolic acid (Figure 10) from *A. fumigatus* has been described by Chain et al. (1943). Helvolic acid is soluble in chloroform, acetone, ethyl acetate, methanol, ethanol, alkali water, and pyridine (Chain et al. 1943; Tschen et al. 1997). The substance is stable at an active temperature of 22^{o}C-100^{o}C and is also stable at various pH levels (pH 3-11) (Tschen et al. 1997).

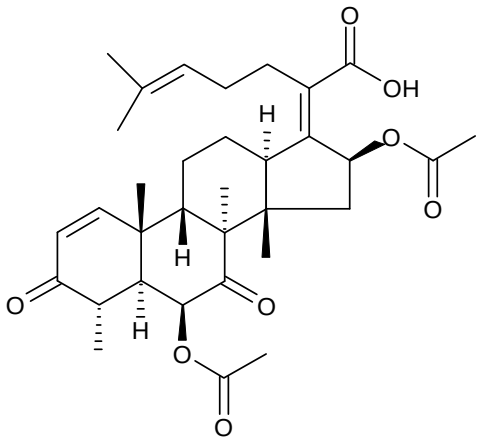


Figure 10. Structure of helvolic acid.

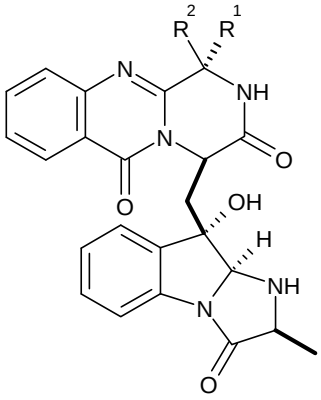
Information concerning the biological activity of helvolic acid is scarce. It has antibiotic (Chain et al. 1943) and phytotoxic (Tschen et al. 1997) properties. Mice weighing 20 g tolerate 5 mg helvolic acid given intravenously or 20 mg by mouth, but repeated injections cause severe liver damage (Chain et al. 1943). Mitchell

et al. (1997) have shown that helvolic acid inhibits the oxidative burst of macrophages. It is also cilioinhibitory but at high concentrations (Stanimirovic et al. 2007).

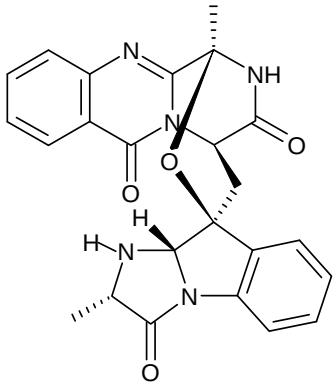
2.3.7 Fumiquinazolines

Fumiquinazolines (FQ) A-G (Figure 11) have been isolated from a strain of *A. fumigatus* which was originally separated from the gastrointestinal tract of the marine fish *Pseudolabrus japonicus* (Numata et al. 1992; Takahashi et al. 1995).

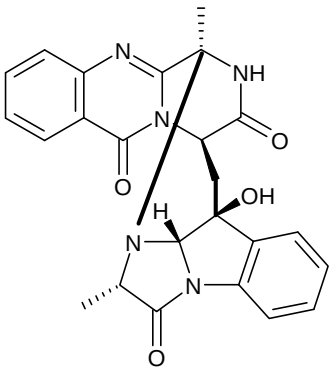
Biogenetically, these molecules are tri- or tetrapeptides derived from anthranilic acid, tryptophane, and ahnine. The fumiquinazolines must therefore be derived from D-tryptophane. Fumiquinazolines (A-G) exhibited moderate cytotoxicities *in vitro* against P388 lymphocytic leukemia cells with ED_{50} values of 6.1 µg/ml, 16.0 µg/ml, 52.0 µg/ml, 13.5 µg/ml, 13.8 µg/ml, 14.6 µg/ml, and 17.7 µg/ml, respectively (Biabani and Laatsch 1998).



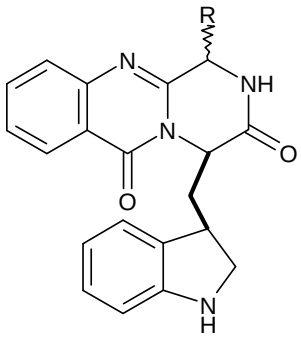
R^1 = Me, R^2 = H (FQA)
R^1 = H, R^2 = Me (FQB)
R^1 = Me, R^2 = OMe (FQE)



FQC



FQD



R = α-Me (FQF)
R = β-Me (FQG)

Figure 11. Structures of fumiquinazolines.

Table 1. Some physiochemical properties of mycotoxins produced by *A. fumigatus*

Mycotoxins	Molecular formula	Molecular weight	Melting point (°C)	UV absorbance properties, λ_{max} nm (log ε)	Reference(s)
Gliotoxin	$C_{13}H_{14}N_2O_4S_2$	326.4	191-218	268 in MeOH	McCorne 1954; Amitani et al. 1995
Verruculogen	$C_{27}H_{33}N_3O_7$	511.57	233-235	225, 275, 294 in MeOH	Cole et al. 1972; Vinokurova et al. 2003b
Fumitremorgin A	$C_{32}H_{41}N_3O_7$	579.67	206-209	226 (4.50), 278 (3.72), 296 (3.69) in EtOH	Yamazaki et al. 1980a
Fumitremorgin B	$C_{27}H_{33}N_3O_5$	479.56	211-212	226 (4.50), 278 (3.86), 295 (3.89) in EtOH	Yamazaki et al. 1980b
Fumitremorgin C	$C_{22}H_{25}N_3O_3$	379.45	128-132	224, 272, 292 in MeOH	Vinokurova et al. 2003b; Afiyatullov et al. 2004
Fumigaclavine A	$C_{18}H_{22}N_2O_2$	298.38	86	224, 275, 281, 292 in MeOH	Cole et al. 1977; Latif et al. 2009
Fumigaclavine B	$C_{16}H_{20}N_2O$	256.157	198-200	225 (4.49), 275 (3.79), 282 (3.82), 293 (3.72) in EtOH	Spilsbury and Wilkinson 1961; Ninomiya 1991
Fumigaclavine C	$C_{23}H_{30}N_2O_2$	366.49	194	225 (4.54), 277 (4.01), 283 (4.04), 292 (3.98) in MeOH	Cole et al. 1977
Fumigatin	$C_8H_8O_4$	168.147	97	262, 380 in cyclohexane	Packter 1965
Fumagillin	$C_{26}H_{34}O_7$	458.6	190-191	239, 336, 351 in chloroform	Hanson and Eble 1953
Helvolic acid	$C_{33}H_{44}O_8$	568.7	215	205, 230 in MeOH	Tschen 1997

Table 1. Continued

Mycotoxins	Molecular formula	Molecular weight	Melting point (°C)	UV absorbance properties, λ_{max} nm (log ε)	Reference(s)
Fumiquinazoline A	$C_{24}H_{23}N_5O_4$	445.177	178-183	208 (4.58), 226 (4.47), 234 (4.42), 256 (4.17), 264 (4.13), 278 (3.97), 306 (3.39), 318 (3.24) in EtOH	Snider and Zeng 2003
Fumiquinazoline B	$C_{24}H_{23}N_5O_4$	445.174	174-178	208 (4.74), 228 (4.63), 234 (4.59), 256 (4.30), 268 (4.19), 278 (3.97), 306 (3.39), 318 (3.24) in EtOH	Takahashi et al. 1995; Snider and Zeng 2003
Fumiquinazoline C	$C_{24}H_{21}N_5O_4$	443.159	179-182	207 (4.58), 225 (4.47), 260 (4.09), 271 (3.97), 282 (3.90), 304 (3.56), 317 (3.50) in EtOH	Takahashi et al. 1995; Snider and Zeng 2003
Fumiquinazoline D	$C_{24}H_{21}N_5O_4$	443.159	214-216	205 (4.30), 225 (4.26), 232 (4.24), 254 (3.91), 265 (3.84), 276 (3.77), 304 (3.43), 316 (3.54) in EtOH	Takahashi et al. 1995
Fumiquinazoline E	$C_{25}H_{25}N_5O_5$	475.51	168-170	210 (4.52), 226 (4.44), 234 (4.36), 256 (4.10), 278 (4.01), 304 (3.55), 317 (3.41) in EtOH	Lei and Zhou 2002; Snider and Zeng 2003
Fumiquinazoline F	$C_{21}H_{18}N_4O_2$	358.144	88-90	207 (4.71), 219 (4.73), 270 (4.13), 277 (4.13), 289 (3.99), 306 (3.78), 320 (3.66) in EtOH	Takahashi et al. 1995
Fumiquinazoline G	$C_{21}H_{18}N_4O_2$	358.143	119-121	208 (4.61), 220 (4.67), 273 (4.14), 278 (4.13), 288 (4.01), 307 (3.66), 323 (3.49) in EtOH	Takahashi et al. 1995

2.4 Analytical Methods for Mycotoxins

The fact that most mycotoxins are toxic in very low concentrations, and they are usually present in low concentrations in complex sample matrices, diversity of chemical structures and physicochemical properties, and they may occur in various combinations produced by a single or by several fungal species. Therefore, efficient analytical tools for the qualitative and quantitative analysis of toxic fungal metabolites in food, feed, and environmental samples are required (for review see, *e.g.*, Krska et al. 2008; Cigić and Prosen 2009).

Because of the large chemical variability of mycotoxins, it is impossible to use one standard technique to detect all mycotoxins, as each will require a different method. Although recent reports claim the determination of nearly 100 different mycotoxins in a single run by LC-MS (Sulyok et al. 2007), the validity of such claims has yet to be proven. There are biological (*e.g., in vivo* or *in vitro* tests), physicochemical (*e.g.*, thin-layer chromatography, gas chromatography, and liquid chromatography), and immunoassay (*e.g.*, enzyme immunoassay) methods for mycotoxin analysis. Because biological methods are mostly qualitative or semiquantitative and they are often non-specific, their use in the surveillance of food and feed for mycotoxins is of minor importance. Biological methods may be useful in screening for mycotoxins, especially when the identity of the mycotoxins is not known (van Egmond and Paulsch 1986).

2.4.1 Thin-Layer Chromatography

Traditionally the most popular method used for mycotoxins analysis is thin-layer chromatography (TLC), which offers the ability to screen large numbers of samples economically (for review see, *e.g.*, Turner et al. 2009). TLC is based on the separation of compounds by how far they migrate on a specific matrix with a specific solvent. The distance that a compound will travel is a unique identifier for specific compounds, and a retention factor (Rf) has been determined for most mycotoxins. As with any detection system, a positive control containing purified

mycotoxins must be ran in parallel to ensure accuracy, since different chemicals can have a similar Rf (Muro-Cacho et al. 2004). Unfortunately, this method is often incapable of differentiating between ergot alkaloids and other indole-containing metabolites (Boichenko et al. 2001). Table 2 lists some examples TLC methods for the detection of several common mycotoxins.

Table 2. Examples of TLC methods used for the detection of mycotoxins

Mycotoxins	Protocol/detection	Matrix	Reference(s)
Aflatoxins and ochratoxin A	TLC with densitometry	Cereals	Braicu et al. 2008
Citrinin and patulin	Normal TLC	Grapes	Abrunhosa et al. 2001
Fumigaclavine A, fumitremorgins B and C, verruculogen	TLC agar plug method compered with HPLC	Isolated fungi	Tepšič et al. 1997
Fumigaclavines A and C, fumitremorgins	Normal TLC	Moldy silage	Cole et al. 1977
Fumigaclavines (B, C), fumitremorgins (B, C), gliotoxin	Normal TLC	Moldy silage	Santos et al. 2002
Gliotoxin	TLC, quantitated by HPLC	Rice cultures	Richard et al. 1989
Patulin	TLC using a charge coupled device	Apple juice	Welke et al. 2009
Fumonisin B1	Normal TLC compered with EIA	Maize	Shelby et al. 1994
Fusarium toxins	Normal TLC compered with EIA and HPLC	Maize and wheat	Schaafsma et al. 1998
Ochratoxin A	TLC with densitometry	Green coffee	Santos and Vargas 2002
Roquefortines	Normal TLC	Silage	Ohmomo and Kitamoto 1994
Verruculogen	Normal TLC	Airborne and clinical isolates	Kosalec et al. 2005
Zearalenone	TLC with densitometry	Corn	Shotwell et al. 1976

2.4.2 Gas Chromatography

The development of gas chromatography (GC) in the mid-1950s provided a convenient method of analysis of almost anything that could be volatilized (Vesonder and Rohwedder 1986). Most mycotoxins are not volatile and therefore have to be derivatised for analysis using GC. Several techniques have been developed for the derivatisation of mycotoxins. Chemical reactions such as silylation or polyfluoroacylation are employed in order to obtain a volatile material (for review see, *e.g.*, Turner et al. 2009)

Modern GC combines superior separation on the capillary columns with a variety of general and specific detectors. Various detection systems may be utilized as coupled to GC, but in most cases electron-capture detection (ECD) and mass spectrometry (MS) have been employed (Pittet 2005). GC-ECD and GC-MS were used to identify and quantify the presence of mycotoxins in food and feed samples, and many excellent sample-handling and analytical techniques have been developed, especially for trichothecenes and other *Fusarium* toxins (Croteau et al. 1994; Onji et al. 1998; Eskola et al. 2000; Tanaka et al. 2000; Nielsen and Thrane 2000).

Since the GC with detection systems are very specialized and costly, its availability may be limited as a routine method for mycotoxin analysis. Table 3 shows some examples of use of GC for the detection of several common mycotoxins. Nowadays GC is largely replaced with liquid chromatography methods, in particular with mass spectrometric detection.

Table 3. Examples of GC methods used for the detection of mycotoxins

Mycotoxins	Protocol/detection	Matrix	Reference(s)
Alternaria toxins	GC-mass spectrometry (GC-MS)	Apple juice	Scott et al. 1997
Deoxynivalenol	GC-Electron capture detection (GC-ECD)	Cereals	Eskola et al. 2000
Fusarium mycotoxins	GC-MS	Beers	Scott et al. 1993
Fusarium mycotoxins	GC-MS	Cereals	Onji et al. 1998
Ochratoxin A and deoxynivalenol	GC-MS and electronic nose	Barley grains	Olsson et al. 2002
Patulin	GC-MS in the selected ion monitoring mode	Apple juice	Llovera et al. 2005
Trichothecene toxins	GC-Ion trap detection	Cereals	Schwadorf and Müller 1991
Trichothecene toxins	GC-ECD	Corn	Croteau et al. 1994
Trichothecene toxins	GC-Tandem mass spectrometry (GC-MS/MS)	Fungal cultures	Nielsen and Thrane 2000
Trichothecene toxins and zearalenone	GC-MS	Cereals	Tanaka et al. 2000
T-2 toxin	GC-MS/MS	Water	Russo et al. 2007

2.4.3 Liquid Chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Chromatographic is performed using columns filled with stationary phase materials usually based on modified silica. Liquid chromatography generally utilizes very small packing particles and a relatively high pressure is referred to as high-performance liquid chromatography (HPLC) (IUPAC 1993). In a typical HPLC set-up screening for mycotoxins, C_{18} columns are commonly used, thus the equipment is run in the reverse phase mode (for review see, *e.g.*, Krska et al. 2008).

HPLC is used in combination with a variety of detectors, for example fluorescence detection (FLD, if necessary with pre-column or post-column derivatisation), ultraviolet detection (UV) or mass spectrometry (MS) as quantitative methods for the determination of mycotoxins. Such methods have been described for some mycotoxins, including aflatoxins, fumonisins, zearalenone, type-A (*e.g.*, T2-toxin) and -B trichothecenes (*e.g.*, deoxynivalenol), ochratoxin A, and gliotoxin in food and feed (Frisvad et al. 1989; Richard et al. 1989; Degelmann et al. 1999; Thimm et al. 2001; Meister 2003; Klötzel et al. 2005; Pena et al. 2005; Yentür et al. 2006, Toscani et al. 2007; Krska and Molinelli 2007). Determination of ergoline alkaloids in various matrices was also achieved by HPLC with UV, FLD, or MS (Jaussaud et al. 1998; Müller et al. 2006; Scott 2007; Reinhard et al. 2008).

In the last few years, the first quantitative liquid chromatography with tandem mass spectrometry (LC-MS/MS) method for *Penicillium* toxin, including roquefortine C, griseofulvin, mycophenolic acid, ochratoxin A, verruculogen, chaetoglobosin B, penitrem A, citrinin, rubratoxin B, cyclopiazonic acid, PR-toxin, patulin, and penicillic acid in food and feed samples was developed by Rundberget and Wilkins (2002). Using LC-MS/MS increased analytical methods for the simultaneous determination of different classes of mycotoxins (Sulyok et al. 2007). The main advantages this method are the general applicability and high sensitivity as well as an outstanding selectivity (Kokkonen and Jestoi 2009). Commonly, isotope labeled reference compounds are applied as internal standards for LC-MS/MS to

overcome matrix interference effects. Unfortunately today such standards are not commercially available for all mycotoxins (Biselli et al. 2005).

A rapid quantitative LC-MS/MS method for the simultaneous determination of the major *Fusarium* mycotoxins nivalenol, deoxynivalenol, fusarenon-X, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, and zearalenone in maize and wheat was developed by Schuhmacher et al. (2005). Zitomer et al. (2008) developed this method for the simultaneous analysis of fumonisins B1, B2, and B3 in maize with single-step extraction method.

Although LC-MS/MS offers sufficient selectivity and multianalyte capabilities, its realization in the field of multi-mycotoxin analysis has been hampered mainly by the chemical diversity of the different toxin classes, which include acidic (fumonisins), basic (ergoline alkaloids) as well as polar (moniliformin, nivalenol), and apolar (zearalenone, beauvericin) compounds. Therefore, compromises have to be made in the choice of extraction solvent and mobile phase, and the conditions may be far from optimal for certain analytes (for review see, *e.g.*, Krska et al. 2008). Table 4 lists some examples of LC methods for the detection of several common mycotoxins.

Table 4. Examples of liquid chromatography methods used for the detection of mycotoxins

Mycotoxins	Protocol/ detection	Matrix	Reference(s)
Aflatoxins	LC-MS	Nuts, cereals, dried fruits, spices	Nonaka et al. 2009
Aflatoxin M1	LC-MS/MS	Milk, milk powder	Chen et al. 2005
Alternaria toxin (tenuazonic acid)	HPLC-MS	Cereals	Siegel et al. 2009
Citrinin	HPLC-FLD	Fungal cultures, cheese extracts	Franco et al. 1996
Deoxynivalenol	HPLC-UV	Milk	Vudathala et al. 1994
Ergoline alkaloids	HPLC-MS/MS	Rye flour	Mohamed et al. 2006
Ergovaline	HPLC-FLD	Ovine plasma	Jaussaud et al. 1998
Fumonisins	HPLC-FLD	Corn silage	Kim et al. 2004
Gliotoxin	HPLC-MS	Building materials	Nieminen et al. 2002
Gliotoxin	LC-MS/MS	Lungs, sera	Lewis et al. 2005
Ochratoxin A	HPLC-MS/MS	Cheese	Zhang et al. 2009
Ochratoxin A	HPLC-FLD	Milk	González-Osnaya et al. 2008
Patulin	LC-MS	Fruit juice, dried fruit	Kataoka et al. 2009
Penicillium mycotoxins	LC–MS/MS	Foods, feeds	Rundberget and Wilkins 2002
Type A and B trichothecenes	HPLC-MS/MS	Maize	Berthiller et al. 2005
Type A and B trichothecenes	LC-MS/MS	Cereals, cereal-based food	Klötzel et al. 2005
Zearalenone	HPLC-FLD	Corn flour, cheese snacks	Oveisi et al. 2005
Zearalenone and its metabolites	HPLC-MS	Urine, plasma, faeces of horses	Songsermsakul et al. 2006

2.4.4 Enzyme Immunoassay/Enzyme-linked Immunosorbent Assay

2.4.4.1 Principle of the EIA

The basic principle of an immunoassay is the reaction between an antigen and its antibody to form a complex antigen-antibody. In general an enzyme immunoassay (EIA) consists of a series of antigen-antibody reactions which result in the binding of an enzyme-labeled antibody to a solid-phase (Yolken and Leister 1981).

The working principle of an EIA is analogous to radioimmunoassay (RIA), before measuring the enzyme activity, it is necessary to separate the bound and unbound labelled fractions of the reaction partners. The antigen-antibody reaction takes place on a special solid-phase, which can take many forms. These materials are able to adsorb the antigens or antibodies, which are chemically proteins, polysaccharides, or lipids. EIA is ideal systems for dealing with a wide range of studies in many biological areas. The main reason for this success is test flexibility, whereby reactants can be used in different combinations, either attached passively to a solid-phase support or in the liquid phase (Crowther 2009).

2.4.4.2 Antibody production

Antibodies are host proteins that comprise one of the principal effectors of the adaptive immune system. Their utility has been harnessed as they have been and continue to be used extensively as a diagnostic and research reagent (Lipman et al. 2005). The basic structure of all antibody or immunoglobulin molecules consists of four protein chains. There are five distinct classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. IgG has been more thoroughly investigated than the other immunoglaobulins. The basic molecular units consists of two identical heavy chains of approximately 50 kD and two identical light chains of about 23 kD are cross-linked each other by disulfide bonds (Terry et al. 1968; Yamaguchi and Harada 2003).

Since all immunoassays are based on the reaction of analyte with its specific antibody, the quality of the antibody is one of the most important factors for the development of sensitive assays. For mycotoxin EIAs this could mean high affinity for a specific toxin or for a group of chemically related toxins, depending on the individual need. Because mycotoxins are not immunogenic and are known as haptens or molecules that will not stimulate antibody production by themselves, earlier studies is focused on the development of methods of conjugation of mycotoxins to a protein or polypeptide carrier and optimization of conditions for antibody production (polyclonal antibodies) in rabbits or in other animals (Chu 1992; Fremy and Usleber 2003). The most frequently used carrier proteins for conjugation are bovine serum albumin (BSA), ovalbumin (OVA), conalbumin (CONA), thyroglobulin (TG), immunoglobulin (Ig), fibrinogen, or keyhole limpet hemocyanin (KLH) (Singh et al. 2004). High-quality antibody can be obtained by using the appropriate immunogen, immunization protocol, and screening procedures (Barna-Vetró et al. 1996).

2.4.4.3 Test formats

EIA make use of a wide variety of different solid phases on which one of the immunoreactants is immobilized. Most immunoassays for agrochemicals, however, utilize the 96-well microtiter plate from polyvinyl chloride (flexible plates) or polystyrene (inflexible rigid plates). The microtiter plate offers ease of handling, large sample capacity and simple test standardisation (Krotzky and Zeeh 1995; Crowther 2009). A key feature of the solid-phase EIA is that antigens or antibodies can be attached to surfaces easily by passive adsorption. This process is commonly called coating. Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between nonpolar protein substructures and the plastic matrix (Crowther 2009). Other formats include fluorescence polarization assays or membrane based rapid tests (Schneider et al. 2004).

2.4.4.4 Enzyme conjugate

Intrinsic to the EIA is the addition of reagents conjugated to enzymes. Assays are then quantified by the build up of colored product after the addition of substrate or a combination of substrate and dye. The analytical sensitivity of the EIA depends on the ability of the antibody to bind and the specific enzyme activity of the labeled immunoreactant, the conjugate. The linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if any chemical modification of the moieties involved alters the antigenic determinants or the reactive sites on antibody molecules (Crowther 2009).

Horseradish peroxidase (HRP), a holoenzyme of molecular weight 40,200, containing one ferritprotoprotein group per molecule, has been widely used for labeling antibodies and is now one of the most common marker enzymes in EIA (Porstmann et al. 1981; Nilsson et al. 1981; Porstmann et al. 1985; Abuknesha et al. 2005; Crowther 2009). Conjugates may be obtained commercially or made in individual laboratories. Great care must be exercised in using the appropriate reagent in any assay. Thus, the immunological implications of various reagents must be considered and information sought.

2.4.4.5 Detection system

Using spectrophotometric detection, the product of the substrate catalysis by enzyme is measured by transmitting light of a specific wavelength through the product and measuring the amount of adsorption of that light. Because different products are produced in EIA, care must be taken to select appropriate filters for the detection of the correct wavelengths (Crowther 2009).

The most commonly used substrates for horseradish peroxidase are a solution containing H_2O_2 and tetramethyl-benzidine (TMB), and for alkaline phosphatase, p-nitrophenyl-phosphate (PNP). Substrate reaction is assayed either at an

endpoint (the reaction is stopped by adding acid or base) or in a kinetic mode. UV/Vis-absorption in all wells on the microtiter plate can be assayed rapidly with compatible photometer (Krotzky and Zeeh 1995; Crowther 2009).

2.4.4.6 Application of EIA for mycotoxin analysis

Official regulations (maximum levels) for mycotoxins in foods and feeds is a major driver in terms of future requirements for analytical methods. This is an influencing factor not only in terms of demanding validated methods which are required for official purposes but also in terms of the needs for monitoring raw materials and quality assurance where rapid screening methods may be more appropriate (for review see, *e.g.*, Gilbert 2000).

EIA has become one of the most useful methods for the rapid monitoring of mycotoxins, especially for the screening of samples. The great advantages of this assay are speed, sensitivity, specificity, ease of operation, and high sample throughput (Barna-Vetró et al. 1996; Krska and Molinelli 2007).

Two types of EIA have been used frequently for the analysis of mycotoxins, and both types are competitive EIA. One type, direct EIA, binding of specific antibodies to a solid support, and the other system, indirect EIA, coated antigens to a solid support (Chu 1992; Goryacheva et al. 2009). Both competitive direct and indirect EIA employed in analysis of mycotoxins have their advantages and limitations. Direct EIA is quick since only one antibody is used and cross reactivity of secondary antibody in some cases is completely eliminated. However, they usually require high amounts of toxin for the preparation of enzyme conjugate, which in addition may be less stable. Indirect EIA has several advantages, in the sense that a wide range of labeled secondary antibodies are commercially available (for review see, *e.g.*, Turner et al. 2009).

Development of EIA for detection of trace level of mycotoxins in food, feed, and environmental matrices is now well documented in the literature. Table 5 shows some examples of protocols and detection limits using EIA for analysis of several common mycotoxins.

Table 5. Examples of protocols and detection limits using EIA for mycotoxins

Mycotoxins	Protocol	Detection limit	Sample/matrix	Reference(s)
Aflatoxin B1	Competitive direct EIA	15-17 pg/ml	Chicken liver	Gathumbi et al. 2003
Aflatoxin M1	Competitive indirect EIA	0.5 ng/ml	Milk and milk-based confectionery	Thirumala-Devi et al. 2002
Citrinin	Competitive indirect EIA	1-13 ng/ml	Wheat	Abramson et al. 1995
Deoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone	Competitive direct EIA	200 ng/g, 50 ng/g, and 20 ng/g, respectively	Wheat	Usleber et al. 1992
Ergoline alkaloids	Competitive EIA	10 ng/g	Seed and flour	Shelby and Kelley 1992
Fumonisin B1	Competitive indirect EIA	5 ng/g	Corn	Yeung, et al. 1996
Fumonisin B1	Competitive direct EIA	0.17 ng/ml	Corn	Usleber et al. 1994
Fumonisin B1	Competitive direct EIA	7.6 ng/g	Cereals	Barna-Vetró et al. 2000
Gliotoxin and helvolic acid	Competitive indirect EIA	5-10 and 10 µg/ml, respectively	Buffer solutions in EIA development	Fox et al. 2004
Ochratoxin A	Competitive direct EIA	0.5 ng/g	Cereals	Barna-Vetró et al. 1996
Ochratoxin A	Competitive indirect EIA	0.1 ng/ml	Chilie	Thirumala-Devi et al. 2000
Zearalenone	Competitive indirect EIA	1-50 ng/ml	Corn, wheat, pig feed	Liu et al. 1985
Zearalenone	Competitive indirect EIA	0.2 ng/ml	Barley and Job's-tears	Tanaka et al. 1995

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory Animals

Female Rabbit Chinchilla Bastard	Charles River, Kisslegg
Female Rabbit Holländer-Deutscher Riese	Manfred Bauer Kaninchen, Neuenstein

Immunization of rabbits were performed under permit number 265, Regierungspräsidium Gießen (Az: V54-19c20/15c GI 18/5).

3.1.2 Chemicals and Biochemicals

Acetic anhydride	Merck KGaA, 1.00042
Acetone	Merck KGaA, 1.00013
Acetonitrile	Merck KGaA, 1.14291
Acetonitrile	Merck KGaA, 1.15500
Ammonium sulfate	Merck KGaA, 1.01217
Bovine serum albumine	Sigma-Aldrich Chemie, A0281
Casein sodium salt	Sigma-Aldrich Chemie, C8654
Citric acid monohydrate	Merck KGaA, 1.00244
Dimethyl sulfoxide	Sigma-Aldrich Chemie, 472301
Di-Sodium hydrogen phosphate anhydrous	Merck KGaA, 1.06586
Ethyl acetate	Merck KGaA, 1.09623
Formaldehyde solution (37% [w/v])	Merck KGaA, 1.04003
Freund’s complete adjuvant	Sigma-Aldrich Chemie, F 5881
HCl	Riedel-de-Haën AG, 30721
Keyhole limpet hemocyanin	Calbiochem-Novabiochem, 374805
Methanol	Merck KGaA, 1.06007
Methanol	Merck KGaA, 1.06009
n-Heptane	Merck KGaA, 1.04365
Potassium dihydrogen phosphate	Merck KGaA, 1.04877
Potassium hydroxide	Merck KGaA, 1.05021
Pyridine	Sigma-Aldrich Chemie, P3776

Sodium carbonate	Merck KGaA, 1.06392
Sodium chloride	Merck KGaA, 1.06404
Sodium hydrogen carbonate	Merck KGaA, 1.06329
Sodium hydroxide	Merck KGaA, 1.06467
Sulfuric acid	Merck KGaA, 1.00729
3,3´, 5,5´-tetramethyl-benzidine	Sigma-Aldrich Chemie, T2885
Tween 20	Sigma-Aldrich Chemie, P1379

All chemicals used were of at least analytical grade

3.1.3 Toxins

Alpha-ergocryptine	Sigma-Aldrich Chemie, E5625
Ergonovine	Sigma-Aldrich Chemie, E7008
Ergotamine	Fluka Cheme, 45510
Fumigaclavine A	Alexis Biochemicals, ALX-630-110
Fumigaclavine C	Isolated from *A. fumigatus*
Fumigaclavines B and D	prepared from fumigaclavines A and C, respectively, by alkaline hydrolysis (Spilsbury and Wilkinson 1961)
Isofumigaclavine A	kindly supplied by Dr. Peter Scott, Ottawa, Canada
Gliotoxin	Fermentek Ltd.

3.1.4 Immunoreagents

Anti-ergonovine antibody (Institute of Veterinary Food Science, Chair of Dairy Science, Veterinary Faculty, Justus Liebig University)
Ergonovine-HS-HRP (Institute of Veterinary Food Science, Chair of Dairy Science, Veterinary Faculty, Justus Liebig University)
Polyclonal swine anti-rabbit immunoglobulins/HRP (DAKO A/S, P0217)

3.1.5 Media

Malt extract agar (MEA)	Oxoid, CM0059

3.1.6 Buffers and Dilutions

0.05 mmol/l sodium carbonate buffer (pH 9.6)
Phosphate-buffered saline (PBS, 0.01 mmol/l phosphate buffer containing 0.1 mmol/l NaCl, pH 7.4)
10% methanol/PBS (pH 7.4)
5% acetonitrile/PBS (pH 6.0)
1% and 2% casein sodium salt/PBS
Enzyme substrate/chromogen solution according to Gallati and Pracht (1985), containing 3 mmol/l H_2O_2 and 1 mmol/l 3,3´, 5,5´-tetramethyl-benzidine in potassium citrate buffer (0.2 mmol/l, pH 3.95)
1 mol/l sulfuric acid (H_2SO_4)

3.1.7 Equipment and Instruments

ELISA-auto-reader	Tecan GmbH
Spectrophotometer UV 1601	Shimadzu Germany GmbH
Ridawin software version 1.38	R-Biopharm AG
Magellan software version 1.3	Tecan Trading AG
Single channel pipette (0,5-10 µl, 10-100 µl, 100-1000µl)	Eppendorf AG
12-channel pipette 30-300 µl	Eppendorf AG
12-channel pipette 50-300 µl	Thermolabsystems
Pipette tip 10 µl, 200 µl, 1000 µl	Sarstedt
Vortex-Genie 2	Scientific Industries Inc.
Centrifuge (Multifuge 3 S-R)	Heraeus Christ GmbH
Centrifuge (5415 C)	Eppendorf AG

Balance (Basic Plus)	Sartorius AG
Balance (Master Pro LA)	Sartorius AG
Stomacher	Seward Stomacher, England

3.1.8 Chromatographic and Fraction Equipment

HPLC system (pump and auto injector)	Dionex, P580 Pump and ASI-100
UV detector (photodiode array detector)	Dionex, PDA-100
Fluorescence detector	Dionex, RF 2000
Column C_{18} (25 cm x 4 mm, 5 µm)	Supleco, 565513-U
Universal chromatography interface	Dionex, UCI-100
Fraction collector code 21 rack	Gilson, model 202
Fraction controller 201-202	Gilson, version C
HPLC software	Dionex, Chromeleon

3.1.9 Other Materials

Microtiter plate	MaxiSorp™ Surface, Nunc
Dialysis tubing 8/32, Ø 6 mm	Serva Electrophoresis GmbH, 44104
pH Indicator paper	Marck KGaA, 1.09535
Filter paper Ø 90 mm	Whatman GmbH
Syring filter cellulose 0,2 µm	VWR International

3.2 Methods

3.2.1 Antibodies against FuA and EIA Development

3.2.1.1 Conjugate Synthesis

The Mannich condensation reaction with formaldehyde was used to conjugate FuA to keyhole limpet hemocyanin (KLH) for immunization, and to bovine serum albumine (BSA) for use as coating antigen using a reaction protocol described earlier for the mycotoxin citrinin (Abramson et al. 1995). KLH (15 mg) from *Megathura crenulata* was dissolved in 2.0 ml of aqueous sodium acetate (0.1 mmol/l, pH 4.2). To this was added 2.0 mg of FuA in 0.1 ml of DMSO and 500 µl of 37% formaldehyde solution. After incubation for 3 h at 37^{o}C, the mixture was dialyzed at 4^{o}C for 3 days against three changes of phosphate-buffered saline (PBS; 0.01 mmol/l phosphate buffer containing 0.1 mmol/l NaCl, pH 7.4).

In a second conjugation experiment, BSA (4.4 mg) was dissolved in 1.4 ml of sodium acetate (0.1 mmol/l, pH 4.2). FuA solution (2.0 mg in 0.1 ml DMSO) was added, followed by 400 µl of 37% formaldehyde solution. After 3 h at 37^{o}C, each mixture was dialyzed at 4^{o}C for 3 days against three changes of PBS. Both FuA-KLH and FuA-BSA conjugates solution were stored in small portions at -18^{o}C until used.

3.2.1.2 Rabbit Immunization

A portion of the precipitate-containing FuA-KLH conjugate (600 µl, containing approximately 3 mg KLH), was made up to 1.5 ml with distilled water and emulsified with 4.5 ml of Freund's complete adjuvant. Three rabbits each received 2 ml of the emulsion described above intradermally at 20 sites on their backs. Booster injections using the same composition and amount of immunogen were given individually as soon as the relative antibody titer decreased.

3.2.1.3 Antiserum Collection

Antiserum was collected from rabbits from the *A. auricularis magna* every 2 weeks, starting 4 weeks after the initial immunization. After collection, blood was allowed to clot for 1 h at 37°C. The clot was then separated from the sides of the collection vessel and allowed to contract for 30 min at 4°C. The serum was removed from the clot, further purified by repeated centrifugation at 1,500*g* for 15 min at 4°C, and then stored frozen at -18°C until used. The relative antibody titer was determined for each individual serum and rabbit by competitive indirect EIA.

3.2.1.4 Antibody Titer Determination

The relative antibody titer was determinated for each individual serum and rabbit by competitive indirect EIA. The FuA-BSA conjugate was diluted 1:1,000 (v/v) in sodium carbonate buffer (0.05 mmol/l, pH 9.6), and 100 µl was added per well to microtiter plates (MaxiSorp™ Surface, Nunc, Denmark), which were kept overnight at ambient temperature. Free protein-binding sites were blocked with casein sodium salt (2% in PBS) for 30 min at ambient temperature before being washed three times with 0.85% NaCl solution containing Tween 20 (250 µl/liter) and decantation. Serial dilutions of antisera (in PBS) were prepared and 100 µl was added per well. After incubation for 1 h, the plate was washed three times with NaCl-Tween solution. Anti-rabbit IgG-HRP conjugate solution was diluted 1:1,000 in PBS, and 100 µl was added per well. After 1 h, the plate was washed three times with NaCl-Tween solution, and 100 µl of an enzyme substrate/chromogen solution was added per well. After 15 min, the reaction was stopped with 1 mol/l H_2SO_4 (100 µl per well), and the absorbance measured at 450 nm. Titer was defined as the antiserum dilution giving an absorbance value of 0.3 units under these conditions.

3.2.1.5 EIA Development

3.2.1.5.1 Method Optimisation

A chessboard or checkerboard experiment design under the conditions of competitive indirect EIA was used for optimisation of concentrations of antiserum and coating conjugate. Following the procedure as described in 3.2.1.4 with some modifications, different concentrations of the FuA-BSA conjugate were added to the microtiter plate with each row containing a different concentration (100 µl per well). After incubation overnight at ambient temperature, free protein-binding sites were blocked with casein sodium salt (2% in PBS) for 30 min before being washed three times with 0.85% NaCl solution containing Tween 20 and decantation. Each well in the half of plate were added with 10% methanol/PBS, and 1 µg/ml FuA standard solution, respectively (50 µl per well), and then different concentrations of the anti-FuA antiserum (50 µl per well) were added in both of the half of plate wich each column containing a different concentration. Thus each well in the half of plate had a different permutation of the amount of FuA-BSA conjugate and anti-FuA antiserum. Figure 12 shows a schematic of the microtiter plate and concentrations used in this experiment.

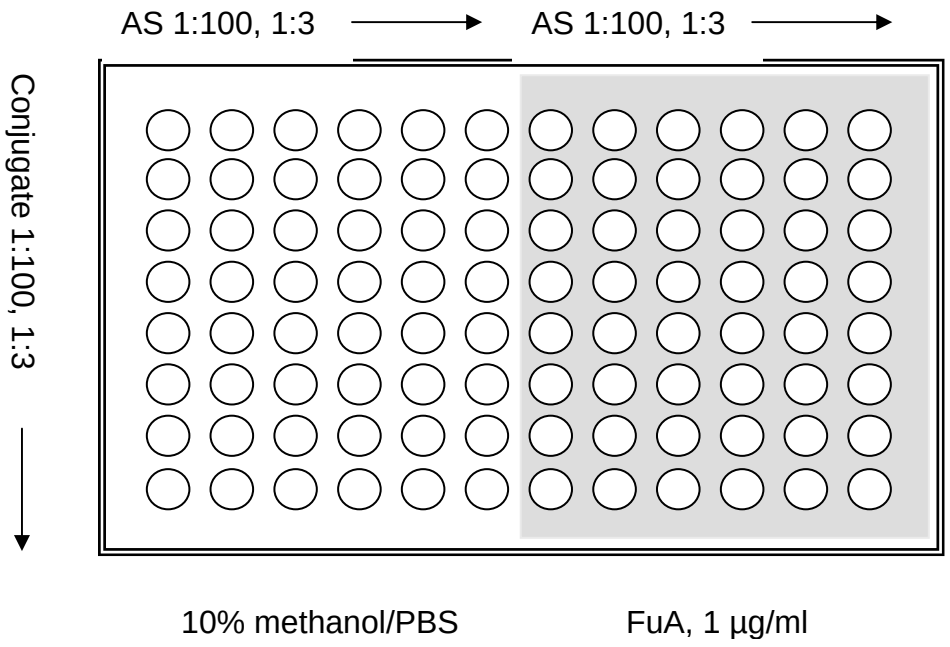


Figure 12. Schematic of the microtiter plate and concentrations used in the checkerboard experiment.

3.2.1.5.2 Standard Curve Evaluation

After optimizing the EIA reagents and assay procedures, the method was tested to detect the FuA standard in buffer solutions (10% methanol/PBS). The experiment with FuA was to perform a calibration curve. Four replicate wells were analyzed for each concentration of FuA. Measured absorbance (binding, B) values for FuA standard solutions were transformed and expressed as relative absorbance (B/B_0 x 100) values, using the absorbance value of the toxin-negative control as B_0. The 50% inhibition concentrations (IC_{50}) levels and detection limit of standard curves from different rabbit antisera were evaluated. Because of the relatively high sensitivity of the EIA standard curves obtained with serum of rabbit 57, all further experiments were performed with this antiserum (collected from week 12 after the initial immunization).

3.2.1.6 Competitive Indirect EIA for FuA

Competitive indirect FuA EIA was established for the detection of FuA. Briefly, the FuA-BSA conjugate was diluted 1:2,000 with sodium carbonate buffer (0.05 mmol/l, pH 9.6), and 100 µl was added per well to microtiter plates, which were kept overnight at ambient temperature. Free protein-binding sites were blocked with casein sodium salt (2% in PBS pH 7.4) for 30 min before being washed with 0.85% NaCl solution containing Tween 20 (250 µl/liter) and decantation. Afterward, to each well, serial dilutions of FuA standard solution or extracted samples (in 10% methanol/PBS; 50 µl per well) and anti-FuA antiserum obtained from rabbit 57 (diluted in PBS; 1:1,000, 50 µl per well) were incubated for 1 h. After a washing step with NaCl-Tween solution, anti-rabbit IgG-HRP conjugate solution (diluted 1:2,000 in PBS pH 7.4) was added (100 µl per well). After 1 h, the plate was washed, and 100 µl of enzyme substrate/chromogen solution was added per well. After 15 min, the reaction was stopped with 1 mol/l H_2SO_4 (100 µl per well), and the absorbance measured at 450 nm. A standard curve was established for FuA (4 replicates per standard concentration) in concentration range between 200

ng/ml and 26 pg/ml. Calculations of standard curve parameters were performed using EIA software (Ridawin or Magellan). The IC_{50} value was used to compare standard curves over a time period of several months.

3.2.1.7 Specificity of the Assay

Assay specificity was checked by competitive binding experiments with FuB and some lysergic acid derivatives, including ergonovine (also known as ergometrine), ergotamine, and alpha-ergocryptine under the conditions of competitive indirect FuA EIA as described in 3.2.1.6. The IC_{50} value of FuA and IC_{50} value of cross-reacting compound (CRC) were used as a reference for calculation of relative cross-reactivity (CR) according to the formula:

$$\text{Relative CR (\%)} = \frac{\text{FuA } IC_{50} : \text{FuA MW}}{\text{CRC } IC_{50} : \text{CRC MW}} \times 100$$

MW = molecular weight

3.2.2 Application Studies

3.2.2.1 Analysis of FuA in Silage

3.2.2.1.1 Sample collection

Corn silage and grass silage samples (*n*=24) were collected from various farms in Hesse, Germany (*n*=15) and from various farms from West Java Province, Indonesia (*n*=9). A representative silage sample (ca. 200 g) was collected in each farm. The samples were dried (for ca. 24 h at 40^{o}C) and blender-milled.

3.2.2.1.2 Sample preparation and analysis

Silage sample (5 g) was extracted by adding 50 ml of methanol/water (70:30, v/v) and shaking for 30 min. The extract was filtered through filter paper (Ø 90 mm). The extract solution was diluted in PBS (1:7, v/v) before applied on the FuA EIA. Further dilutions were made in 10% methanol/PBS.

In the recovery experiment, 5 g portions of silage were spiked with various amounts of FuA in methanol (25-1,000 ng/g sample), and extracted as described above.

Extracts were analyzed by the FuA EIA as described in 3.2.1.6. At least three different dilutions of silage extracts were analyzed, and four replicate wells were analyzed for all standard and extract solutions.

3.2.2.2 Analysis of FuA in Tissue Samples from the Respiratory System of Birds with Aspergillosis

3.2.2.2.1 Sample collection

Tissue samples from the respiratory system (lung, air sac) of dead birds with confirmed aspergillosis (“cases”, *n*=9), and of dead birds without pathological signs

of aspergillosis ("controls", *n*=7) were obtained from the Clinic for Birds, Reptiles, Amphibians, and Fish, Justus Liebig University, Giessen. A representative of respiratory tract sample (ca. 1-5 g, depend on the size of bird) was collected from each bird. Avian species included falcons (*Falco* sp.), parrots (*Psittacus erithacus* and *Eclectus roratus*), humboldt penguin (*Spheniscus humboldti*), common redstart (*Phoenicurus phoenicurus*), diamond dove (*Geopelia cuneata*), chicken (*Gallus domesticus*), and turkey (*Meleagris* sp.).

3.2.2.2.2 Sample preparation and analysis

One gram of tissue sample from respiratory system was homogenized with 10 ml of 10% methanol/PBS (v/v, pH 7.4) for 10 min in a Stomacher. If the total amount of tissue sample was less than 1 g, the solvent volume was reduced accordingly. Then the extract was centrifuged at 3,000*g* for 15 min at 4^{o}C. The supernatant was further purified by repeated centrifugation (11,000*g* for 15 min at ambient temperature). Dilutions of this extract were made in 10% methanol/PBS. In the recovery experiment, various amounts of FuA in methanol (5-100 ng/g sample) were spiked in to 1 g of samples in which the toxin was not present, and extracted as described above.

Extracts were analyzed by the FuA EIA as described in 3.2.1.6. At least three different dilutions of sample extract in 10% methanol/PBS were analyzed, and four replicate wells were analyzed for all standard and extract solutions.

3.2.2.3 Isolation and Characterization of Fumigaclavines from *A. fumigatus* Isolated from Air Sac Tissue

3.2.2.3.1 Fungal cultivation and toxin isolation

In some tissue samples of avian aspergillosis, fungal growth was visibly detectable. Fungal material was obtained by swabbing and plated on malt extract

agar (MEA). Plates were incubated for 7 days at 25°C. Fungi were preliminary identified as *A. fumigatus* by visual and microscopic evaluation (Schmidt and Wolff 1997).

A portion (~10 µg) of mycelium of *A. fumigatus* was mixed with 1 ml of methanol. The mixture was homogenized on a shaker (vortex) for 10 min, and then allowed to stand for at least 3 h at ambient temperature. The homogenate was mixed again (vortex) and then was centrifuged (11,000*g*, 10 min). The supernatant was collected and centrifuged again. Finally, the solution was filtered through a syringe filter containing cellulose (0.2 µm).

3.2.2.3.2 EIA Analysis

The purified fungal extract was diluted 1:10 with PBS. Further dilutions were made with 10% methanol/PBS. Diluted extracts were analyzed in the FuA EIA as described in 3.2.1.6. At least three different dilutions of toxin extract in 10% methanol/PBS were analyzed, and four replicate wells were analyzed for all standard and extract solutions.

3.2.2.3.3 HPLC Analysis

Confirmation of FuA in mycelium extract was performed by HPLC-UV using the method of Smedsgaard (1997) with slight modifications. Briefly, the toxins were detected using a photodiode array detector (200-600 nm), the detection wavelength for quantification was 280 nm. Additionally, a fluorescence detector set at 280 nm (Excitation) and 400 nm (Emission) was used in line after the diode array detector. The column was maintained at 40°C. A linear gradient starting from 85% water contained 0.1 g/l ammonium sulfate (solvent A) and 15% acetonitrile (solvent B) going to 100% acetonitrile in 40 min, then maintaining 100%

acetonitrile for 10 min, was used at a flow-rate of 1 ml/min. A portion (50 µl) of the mycelium extract (after 1:100 dilution in methanol) was injected into the HPLC system. FuA in mycelium extract was identified according to physicochemical characteristics (retention time, spectrum, fluorescence ability) and through comparison with FuA standard solution. FuB standard was used as prepared by deacetylation of FuA; FuC was estimated from the relative retention time given by Nilsen and Smesdsgaard (2003).

For deacetylation, the method of Spilsbury and Wilkinson (1961) with slight modifications was adopted for hydrolysis of FuA and FuC. Briefly, 0.1 ml of mycelium extract containing FuA (~3 µg/ml) and FuC (~10 µg/ml) was diluted in 0.8 ml methanol. To this was added 0.1 ml of 2 mol/l NaOH (equivalent to 0.2 mol/l NaOH in the final solution). After incubated for 16 h at ambient temperature, the pH was adjusted to approximately 7 with HCl (2 mol/l, 0.1 ml), and the deacetylated toxins analyzed by HPLC.

3.2.2.3.4 Immunochromatography Analysis (Combining HPLC-EIA)

Analysis of fumigaclavines in mycelium extract after hydrolysis was performed using a immunochromatography method. During analysis of mycelium extract by HPLC as described in 3.2.2.3.3, the HPLC eluate was fractionated based on a time setting of 1 min (corresponding to 1 ml per fraction). The entire process was automated using a Gilson Fraction Controller 201-202. The collected eluate from each fraction was diluted 1:10 in PBS. Detection of the fumigaclavines in HPLC eluate fractions was performed by FuA EIA.

3.2.2.4 Combined Analysis of FuA and IsoFuA in Blue Cheeses

3.2.2.4.1 Competitive Direct EIA for Ergonovine

A competitive direct ergonovine EIA as established previous by at the Institute was performed as described by Curtui et al. 2007. Briefly, the rabbit antiserum-ergonovine was diluted 1:2,000 (v/v) in sodium carbonate buffer (0.05 mmol/l, pH 9.6), and 100 µl was added per well to microtiter plate, which were kept overnight at ambient temperature. Free protein-binding sites were blocked with casein sodium salt (2% in PBS) for 30 min before being washed three times with 0.85% NaCl solution containing Tween 20 (250 µl/liter) and decantation. Then, 50 µl of serial dilution of ergonovine standard solution (in 5% AcN/PBS, pH 6.0) or extracted samples with 50 µl of ergonovine-HS-HRP conjugate (diluted 1:2,000 in 1% casein sodium salt/PBS) were incubated simultaneously for 2 h at ambient temperature. After washing with NaCl-Tween, 100 µl of an enzyme substrate/ chromogen solution was added per well. After 15 min, the reaction was stopped with 1 mol/l H_2SO_4 (100 µl per well), and the absorbance measured at 450 nm. The IC_{50} value of ergonovine and IC_{50} values of FuA, IsoFuA, and FuB were used as a reference for calculation of relative cross-reactivity according to the formula as described in 3.2.1.7.

3.2.2.4.2 Specificity Pattern of FuA EIA and Ergonovine EIA

To determine specificity pattern of antibodies against FuA and antibodies against ergonovine, competitive binding experiments under the conditions of the competitive indirect FuA EIA and the competitive direct ergonovine EIA as described in 3.2.1.6 and 3.2.2.4.1, respectively were used. The individual specificity pattern of the FuA EIA and the ergonovine EIA was checked by competitive binding experiments with some ergoline alkaloids including FuA, FuB, IsoFuA, and ergonovine.

3.2.2.4.3 Analysis of Blue Cheese

3.2.2.4.3.1 Sample collection

Blue-veined cheese samples (*n*=16) were purchased from retail shops in the area of Giessen, Germany. These samples included products originating from Germany (*n*=4), France (*n*=7), Italy (*n*=3), and Denmark (*n*=2).

3.2.2.4.3.2 Sample preparation and analysis

Blue-veined cheese sample containing plenty of mycelium (0.5 g) was homogenized with 2.5 ml of methanol and allowed to settle for at least 3 h at ambient temperature. The homogenate was mixed on shaker for 5 min and centrifuged (4,000*g*, 10 min, 4^{o}C). The supernatant solution was taken, further purified by repeated centrifugation. The solution was filtered through a syringe filter cellulose (0.2 µm). The aqueous solution washed 2 times with 2 ml of n-heptane to remove residual fat. This solution was directly used for HPLC. To check matrix interference, the aqueous solution was spiked with FuA standard solution. Sample solution for EIA was prepared by diluting the supernatant 1:10 with PBS. Further dilutions were made with 10% methanol/PBS as necessary.

Analysis of blue-veined cheese samples was performed by competitive indirect FuA EIA and competitive direct ergonovine EIA as described in 3.2.1.6 and 3.2.2.4.1, respectively. At least three different dilutions of cheese extracts were analyzed by both EIAs, and four replicate wells were analyzed for all standard and extract solutions.

3.2.2.4.3.3 Immunochromatography Analysis (HPLC-EIA)

Combining analysis of mycotoxins in blue-veined cheese samples were performed by using HPLC-EIA method. HPLC method was performed using the method as described in 3.2.2.3.3. A portion (50 µl) of the cheese extract was injected into the

HPLC system. During analysis of samples by HPLC, the HPLC eluate was fractionated. The entire process was automated using Gilson Fraction Controller 201-202 (version C). The fractionated HPLC eluate was collected based on a time setting of 2 min (with volume of 2 ml per fraction). The fractionated HPLC eluates from each fraction were diluted 1:10 in PBS and analyzed using the FuA EIA and the ergonovine EIA.

4 RESULTS

4.1 Antibodies against FuA and EIA Development

4.1.1 Conjugate Synthesis

UV spectrophotometric characterization of the FuA standard (98% purity, molecular weight: 298.4) dissolved in methanol revealed absorbance maxima at 224 nm, 275 nm, 281 nm, and 292 nm (Figure 13), which is largely in agreement with literature data for FuA/IsoFuA/roquefortine A (Scott et al. 1976; Frisvad and Thrane 1987; Kozlovsky et al. 2009).

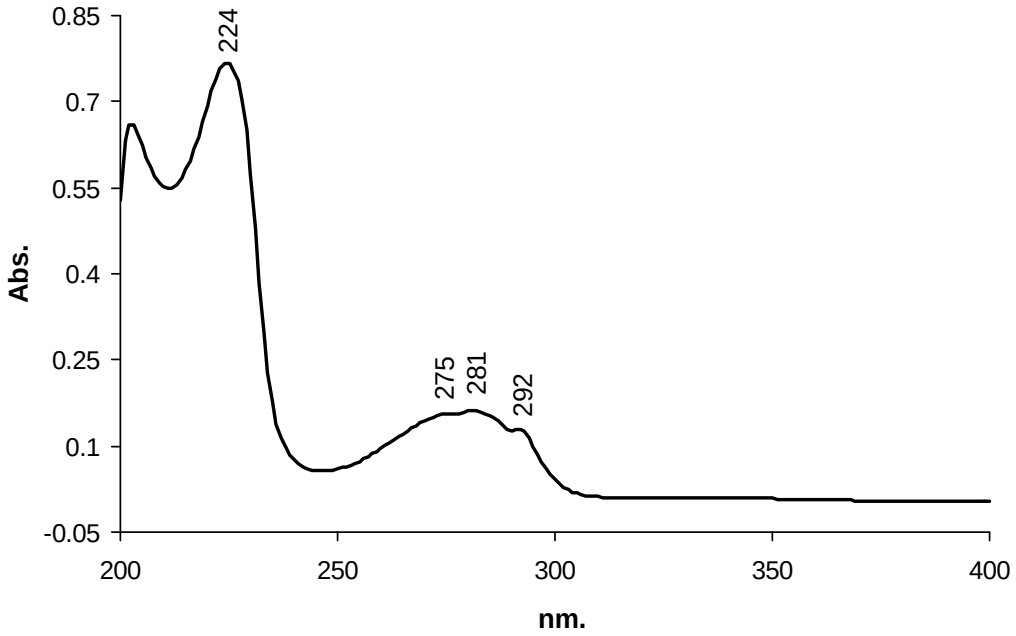


Figure 13. UV spectrophotometry of FuA standard (5 µg/ml) dissolved in methanol.

The FuA-protein conjugates were prepared using the Mannich condensation reaction. FuA-KLH was used as the immunogen, while FuA-BSA was used as the solid-phase antigen in the competitive indirect EIA. Spectrophotometric analysis of FuA-KLH and FuA-BSA conjugates are shown in Figures 14 and 15, respectively. Both conjugates showed increased UV absorption in the range of the UV maximum of FuA (~224 nm and ~280 nm). However, because of the overlay with

the UV absorbance of native proteins, quantification of the conjugation ratio was not possible.

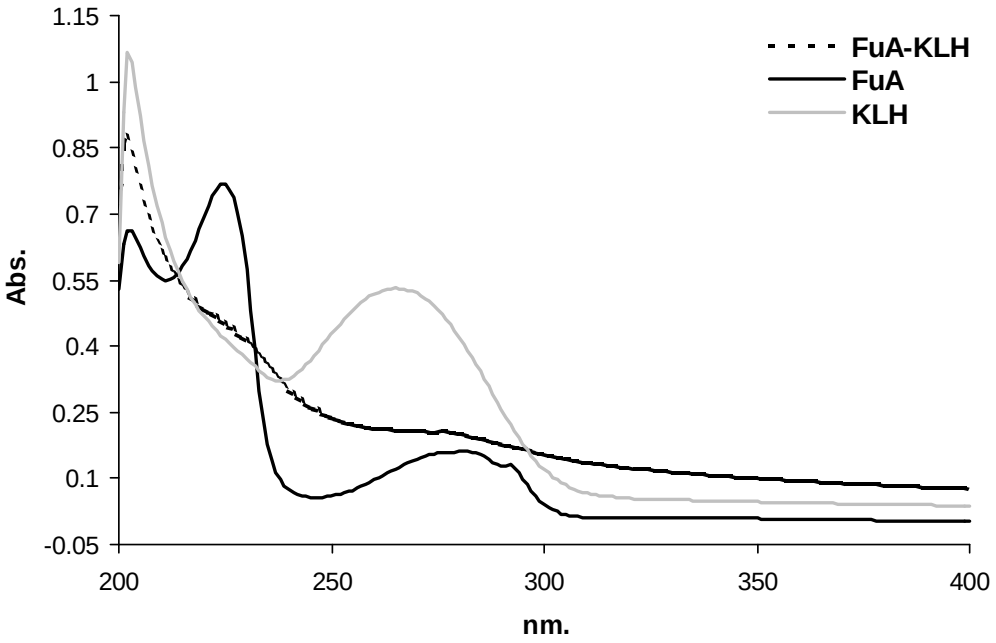


Figure 14. Comparison of the UV spectrophotometry of FuA-KLH conjugate dissolved 1:20 in PBS, FuA (5 µg/ml in methanol), and KLH (25 µg/ml in PBS).

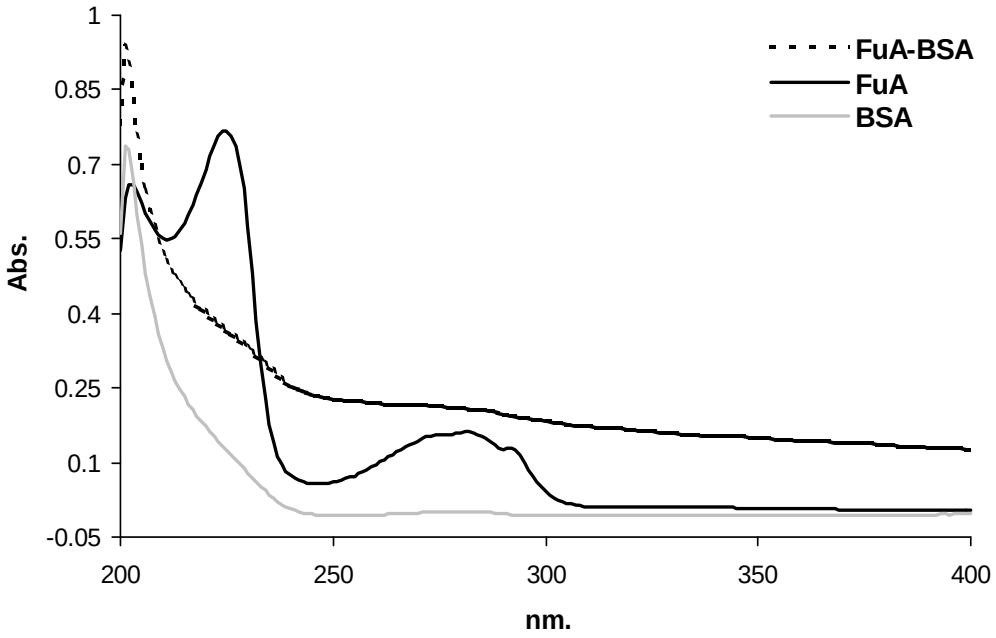


Figure 15. Comparison of the UV spectrophotometry of FuA-BSA conjugate dissolved 1:20 in PBS, FuA (5 µg/ml in methanol), and KLH (25 µg/ml in PBS).

4.1.2 Antibody Production

The FuA-KLH conjugate was used to immunize 3 rabbits (rabbits 55, 56, and 57). The titer development against FuA was monitored by competitive indirect EIA. Specific antibody titers could be obtained from rabbits 55 and 57, in both cases serum titers increased significantly after the first booster injection.

Antibody titers obtained from rabbits 55 and 57 increased until the second booster injection. However, the last booster injections did not increase further antibody titers from all rabbits. Resultant relative antibody titers from all rabbits during the first 36 weeks after initial immunization are shown in Figure 16.

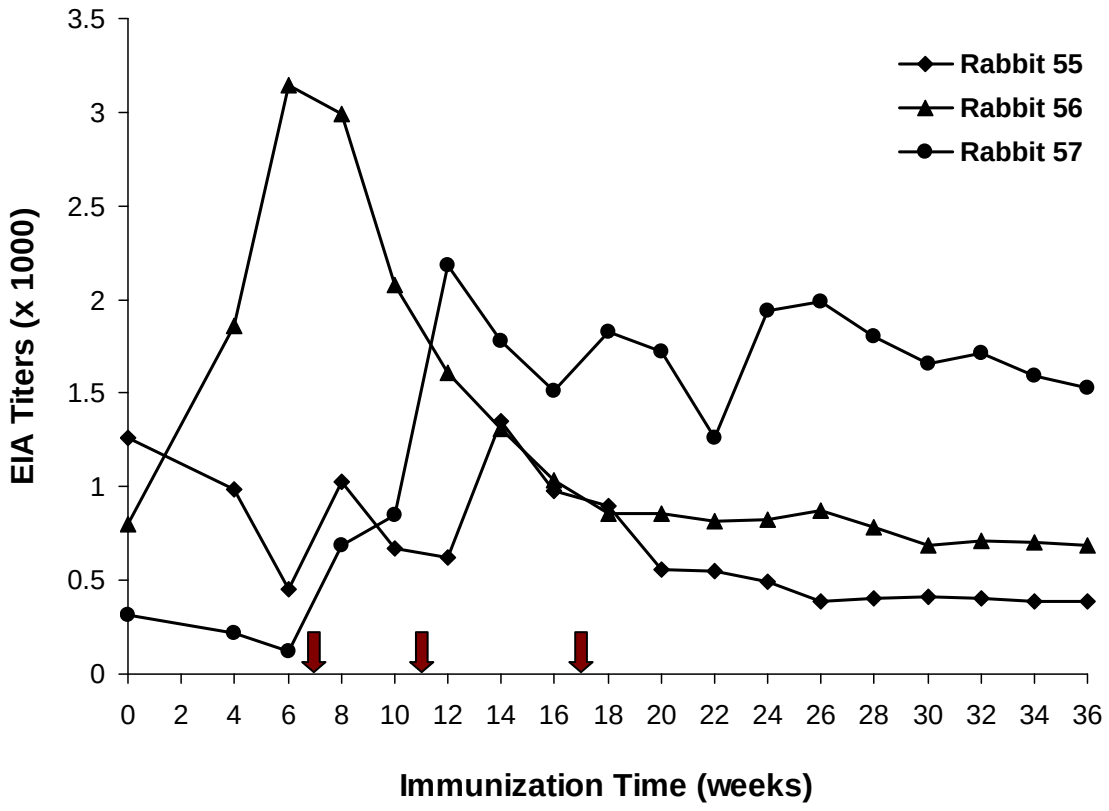


Figure 16. Antibody titers of three rabbits after immunization with fumigaclavine A-KLH conjugate prepared by formaldehyde condensation. Arrows indicate the time of booster injections.

Although solid-phase binding properties of sera obtained from rabbit 56 increased significantly 6 weeks after the initial immunization, with relative EIA titers of 1:3,200, no specific competitive binding inhibition by FuA could be achieved for these sera. Booster injections did not increase specific antibody production against FuA. Therefore EIA development was continued with sera from rabbits 55 and 57.

4.1.3 EIA Development

The optimization process and reagent concentrations by using competitive indirect EIA format found that FuA-BSA conjugate at a dilution of 1:2,000 (v/v) in sodium carbonate buffer (0.05 mmol/l, pH 9.6) to be optimum for coating the microtiter plates. The optimum solution of antiserum was 1:1,000 in PBS, while anti-rabbit IgG-HRP conjugate (commercially available) at a dilution of 1:2,000 in PBS gave optimum results.

All combinations of antiserum and conjugates that showed specific and sensitive results were used to carry out competitive indirect EIA for FuA. Evaluation of FuA standard curves, established for antisera from rabbits 55 (week 14) and 57 (week 12) over a period of several months, resulted in 50% inhibition concentrations (IC_{50}) of 20.6 ± 2.7 ng/ml (*n*=12) and 3.3 ± 0.3 ng/ml (*n*=23), respectively (Figure 17). Considering the overall test variability of competitive microtiter plate EIA, the realistic detection limit typically corresponds to a relative absorbance between the IC_{30} and IC_{20} value. For the FuA EIA using antiserum of rabbit 57, this would yield a detection limit of 0.2-0.5 ng/ml. The measuring range of the standard curve usually is from 30% to 80% relative absorbance (B/B_0 x 100) values. The standard curve obtained for FuA in buffer solutions (10% methanol/PBS) under these conditions is depicted in Figure 18. Since the polyclonal antibodies obtained from rabbit 57 enabled a nearly 6-fold better sensitivity in EIA compared with serum of rabbit 55, all further experiments were performed with serum of rabbit 57.

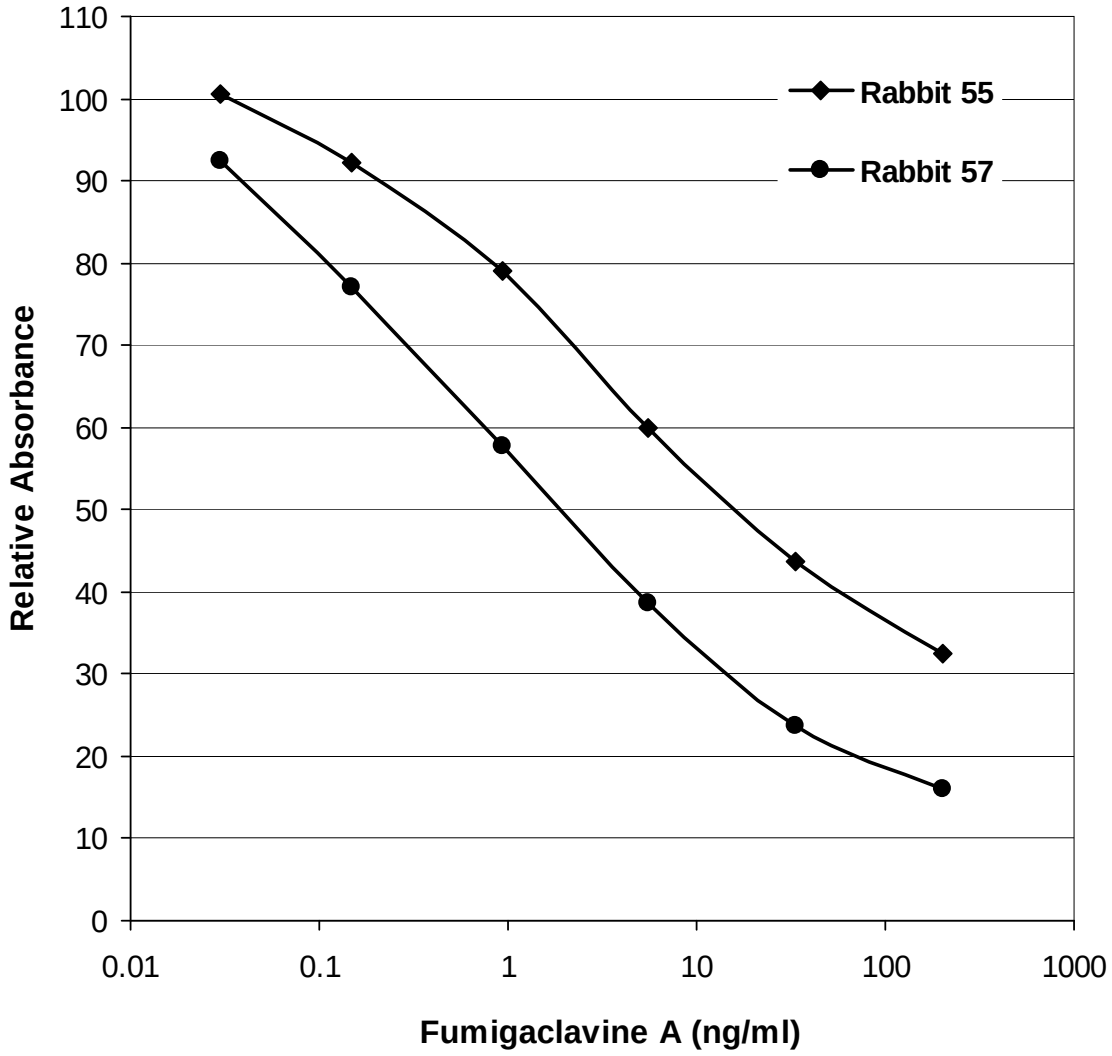


Figure 17. Comparison of the standard curves of the competitive indirect EIA for FuA using the antiserum from rabbits 55 and 57. Plates were coated with FuA-BSA (dilution 1:2,000). Sera (55; 57) were each used in a dilution of 1:1,000. Solid-phase bound antibodies were detected using anti-rabbit IgG-HRP in a dilution of 1:2,000.

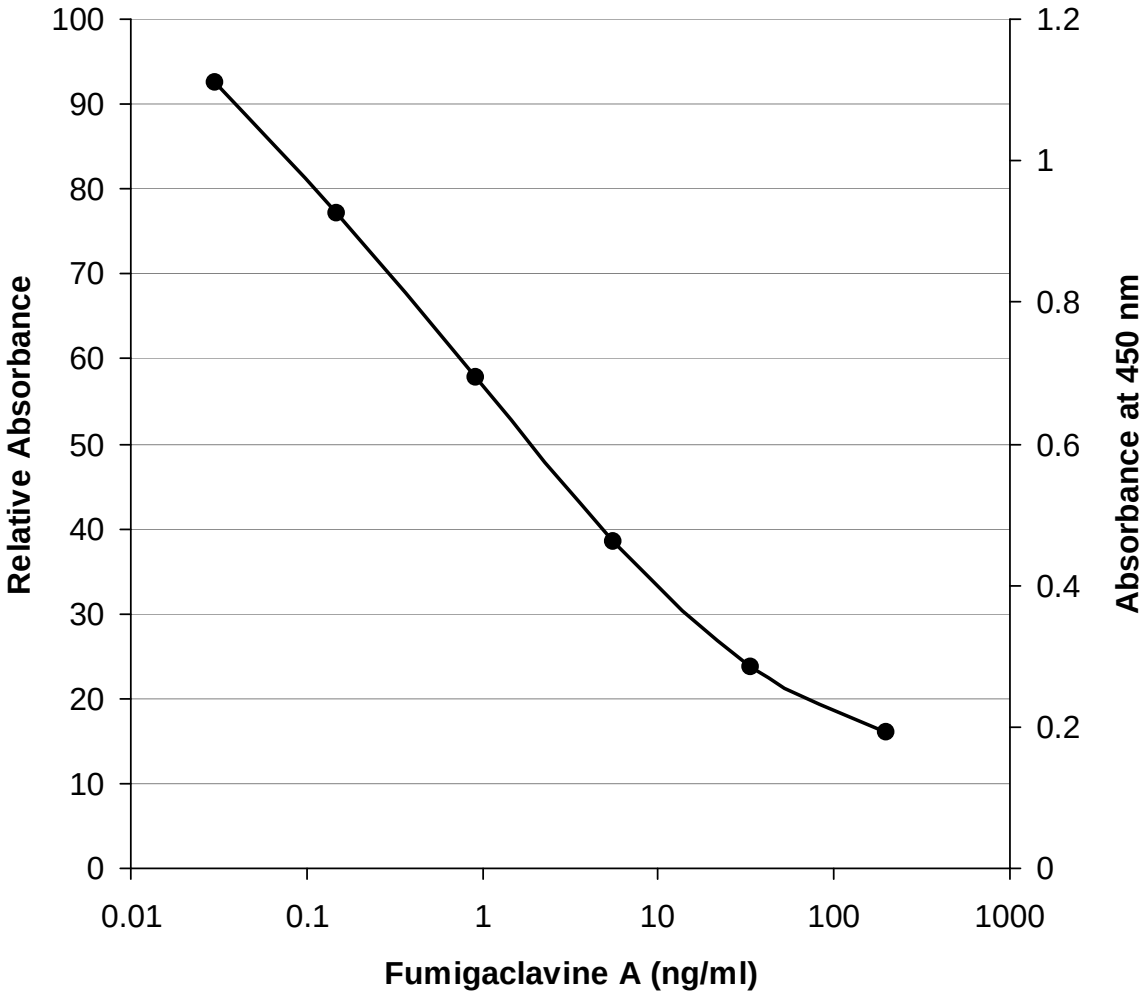


Figure 18. Optimized standard curve of the competitive indirect EIA for FuA using plate coating with FuA-BSA (1:2,000) and antiserum of rabbit 57 (dilution 1:1,000). B_0 was 1.2 absorbance units. Intraassay coefficient of variation (4 replicate wells per concentration level) ranged from 1.5% to 9.0%. The mean detection limit of the standard curve (80% B/B_0) was typically at 0.2-0.5 ng/ml.

4.1.4 Specificity of the Assay

The specificity of the assay was determinated against FuB and some lysergic acid derivatives (ergonovine, ergotamine, and alpha-ergocryptine). The assay was very specific for FuA, with low cross-reactivity with FuB. The assay did not cross-react with ergonovine, ergotamine, and alpha-ergocryptine at concentrations up to 10 µg/ml. Table 6 shows the IC_{50} values and relative cross-reactivity of antibodies against FuA with FuB and some lysergic acid derivatives under the condition of the competitive indirect FuA EIA.

Table 6. Relative cross-reactivity of antibodies against FuA with FuB and some lysergic acid derivatives

Compound	Molecular weight	FuA EIA	
		IC_{50} (ng/ml)	% CR
FuB	256.2	224.0	1.3
Ergonovine	325.4	>10,000	< 0.01
Ergotamine	581.5	>10,000	< 0.01
Alpha-ergocryptine	575.7	>10,000	< 0.01

4.2 Application Studies

4.2.1 Analysis of FuA in Silage

The competitive indirect FuA EIA was used for the detection of FuA in silage samples. The detection limit for FuA in silage was at 10 ng/g. Recoveries of FuA from artificially contaminated silage samples are summarized in Table 7. However, none of 24 silage samples contained detectable amounts of FuA (<10 ng/g).

Table 7. Recovery of FuA from artificially contaminated silage

FuA added	FuA found				
(ng/g)	mean (ng/g)	± SD (ng/g)	RSD (%)	recovery (%)	*n*
25	22.4	4.7	20.9	89.6	4
100	70.9	8.1	11.4	70.9	5
500	404.0	29.5	7.3	80.8	4
1,000	696.7	41.3	5.9	69.7	5

4.2.2 Analysis of FuA in Tissue Samples from the Respiratory System of Birds with Aspergillosis

The competitive indirect FuA EIA was used for the determination of FuA in tissue samples of the respiratory system of dead birds with confirmed aspergillosis. The detection limit of the assay for FuA in avian respiratory system tissue was at 1.5 ng/g. Recoveries of FuA from artificially contaminated control samples are summarized in Table 8.

Natural occurrence of FuA in respiratory system tissue of dead birds with pathological signs of aspergillosis was determinated. FuA was found in six (66%) tissue samples of aspergillosis cases (Table 9), while all control samples were negative.

Table 8. Recovery of FuA from artificially contaminated respiratory system tissue of birds (control samples)

FuA added	FuA found				
(ng/g)	mean (ng/g)	± SD (ng/g)	RSD (%)	recovery (%)	*n*
0	0				7
5	4.2	0.8	19.0	84.0	3
20	17.1	2.7	15.7	85.5	3
100	108.7	10.3	9.5	108.7	4

Table 9. FuA in tissue samples from the respiratory system of dead birds with confirmed aspergillosis

Sample Code	Species	FuA found (ng/g)
A1	Falcon (*Falco* sp.)	28
A2	Falcon (*Falco* sp.)	10
A3	Falcon (*Falco* sp.)	< 1.5
A4	Chicken (*Gallus domesticus*)	< 1.5
A5	African Grey Parrot (*Psittacus erithacus*)	14
A6	Common Redstart (*Phoenicurus phoenicurus*)	2.5
A7	African Grey Parrot (*Psittacus erithacus*)	4
A8	Humboldt Penguin (*Spheniscus humboldti*)	38
A9	Diamond Dove (*Geopelia cuneata*)	< 1.5

4.2.3 Isolation and Characterization of fumigaclavines from *A. fumigatus* isolated from air sac tissue

4.2.3.1 Fungal Cultivation

The dark green mycelium of fungus growth on tissue samples from the respiratory system of dead birds with aspergillosis was isolated from tissue samples A5 and A8 (Figure 19). In a previous experiment, FuA was found in both samples.

Cultivation of these fungi on MEA yielded fungal material which was typical for *A. fumigatus* (confirmed under the colonial morphology and the presence of characteristic conidia and conidiophores). Figure 20 shows the typical colony, conidia, and conidiophore of *A. fumigatus*.

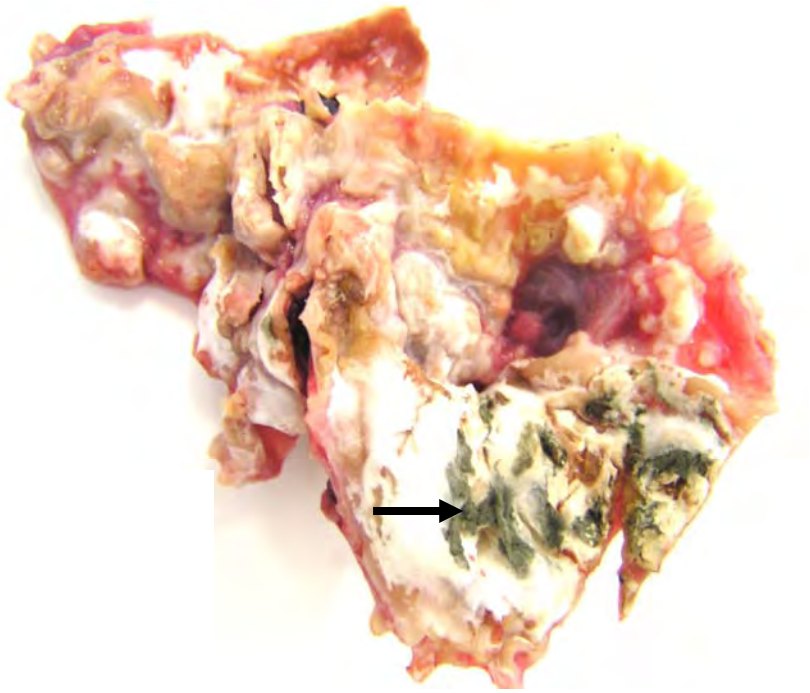
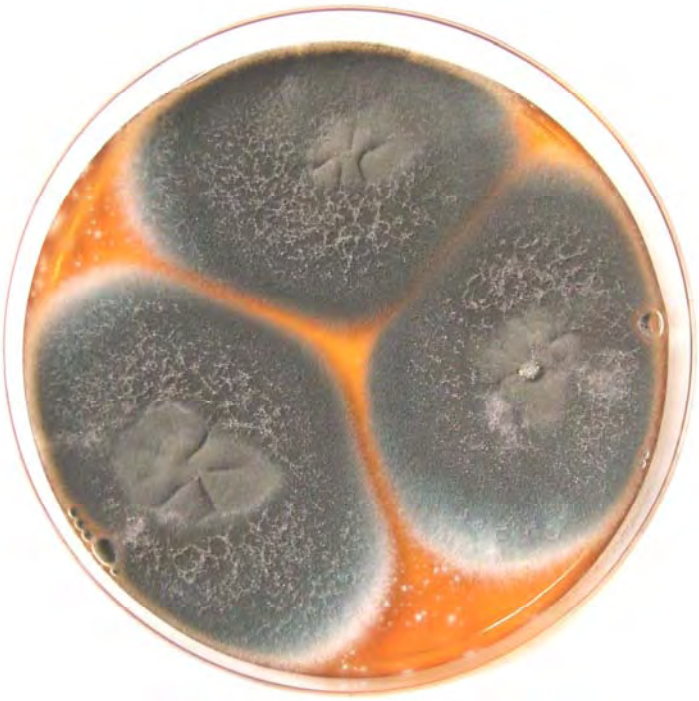


Figure 19. Fungal growth on air sac of humboldt penguin (sample A8) with confirmed aspergillosis.

A



B

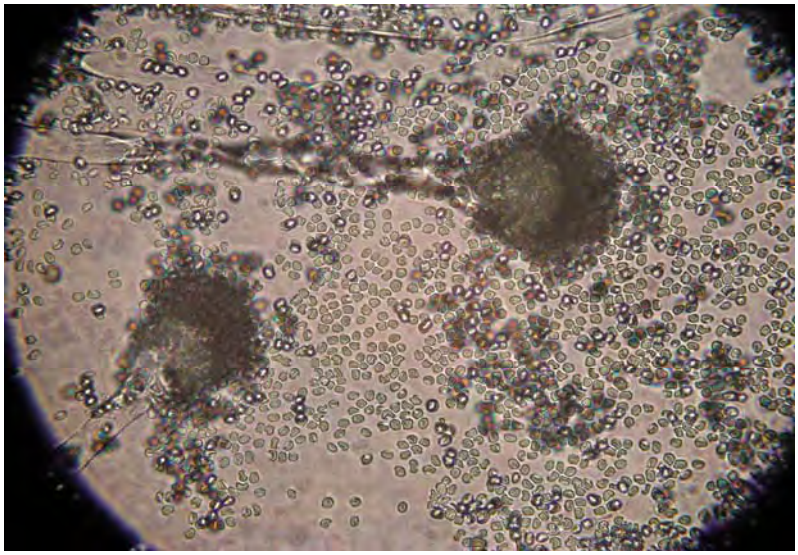


Figure 20. *A. fumigatus*: colonies 7 days at 25°C on MEA (A), conidia and conidiophores (B).

4.2.3.2 Toxin Isolation and EIA Analysis

Extraction of mycelium from two *A. fumigatus* isolates (A5, A8) were performed by methanol. When mycelium extracts were redissolved in 10% methanol/PBS, and assayed by FuA EIA, both mycelium extracts contained high amounts of FuA (up to 8 mg/g).

4.2.3.3 HPLC Analysis

By using HPLC, the identification of FuA in mycelium extracts of *A. fumigatus* was based on typical retention time (13.3 min), fluorescence ability, and spectra-maxima (224 nm, 281 nm) of FuA standard (Figure 21).

The presence of FuA was confirmed in mycelium extract of *A. fumigatus* (isolates A5 and A8) through comparison with FuA standard. Although FuC standard was not available, the presence of this toxin in mycelium extracts of *A. fumigatus* was confirmed by identification of retention time, spectra-maxima, and fluorescence ability of this toxin. FuA and FuC were found in both isolates. Some other unidentified compounds were also detected (Figure 22). However, the presence of gliotoxin was not detected from both *A. fumigatus* isolates (based on retention time, not fluorescent, and spectra-maxima of gliotoxin standard).

Alkaline treatment with NaOH proved to be an effective approach for hydrolysis of FuA and FuC. Hydrolysis of FuA and FuC gave deacetylfumigaclavine A and deacetylfumigaclavine C, respectively (Figure 23). Because deacetyl-fumigaclavine A was named FuB by Spilsbury and Wilkinson (1961), the name FuD is proposed for deacetylfumigaclavine C. HPLC chromatograms of mycelium extract from isolate A5, before and after hydrolysis are shown in Figure 24.

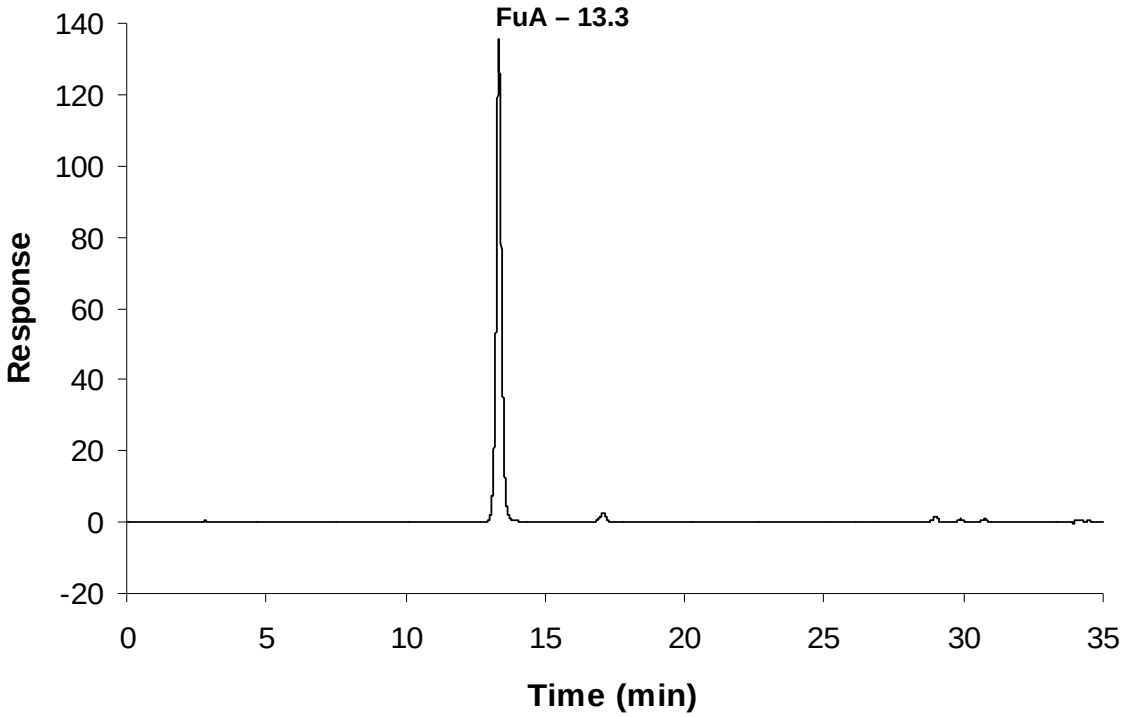


Figure 21. HPLC chromatogram (280 nm) of FuA standard (100 ng/ml).

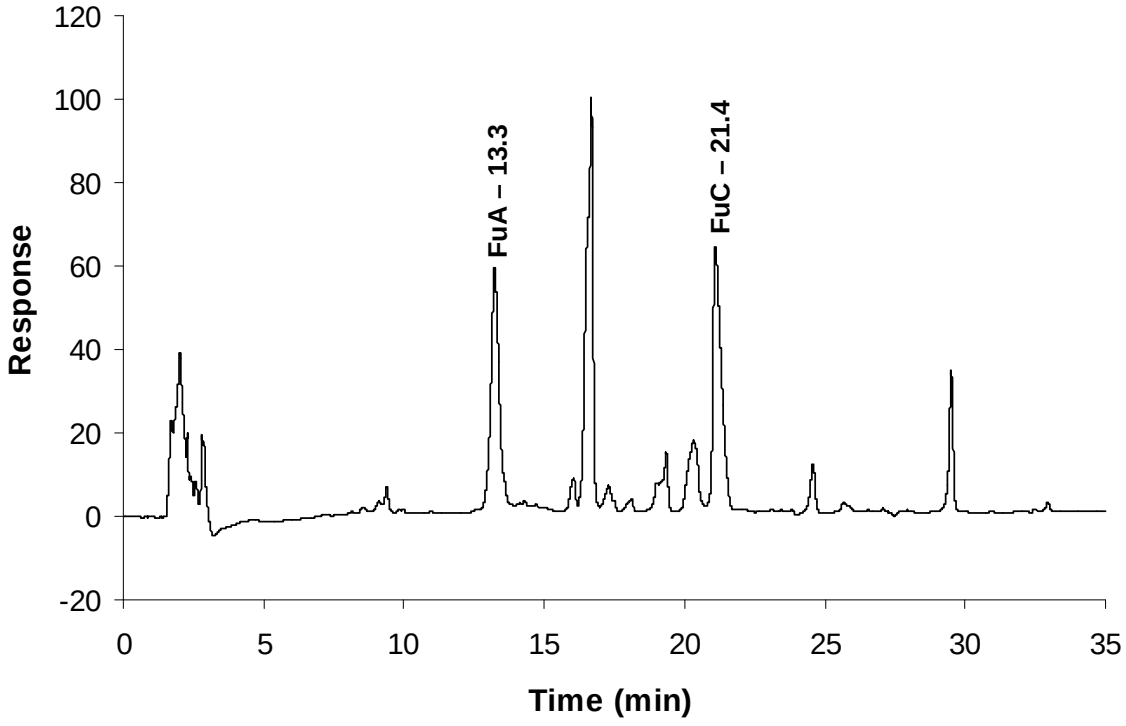
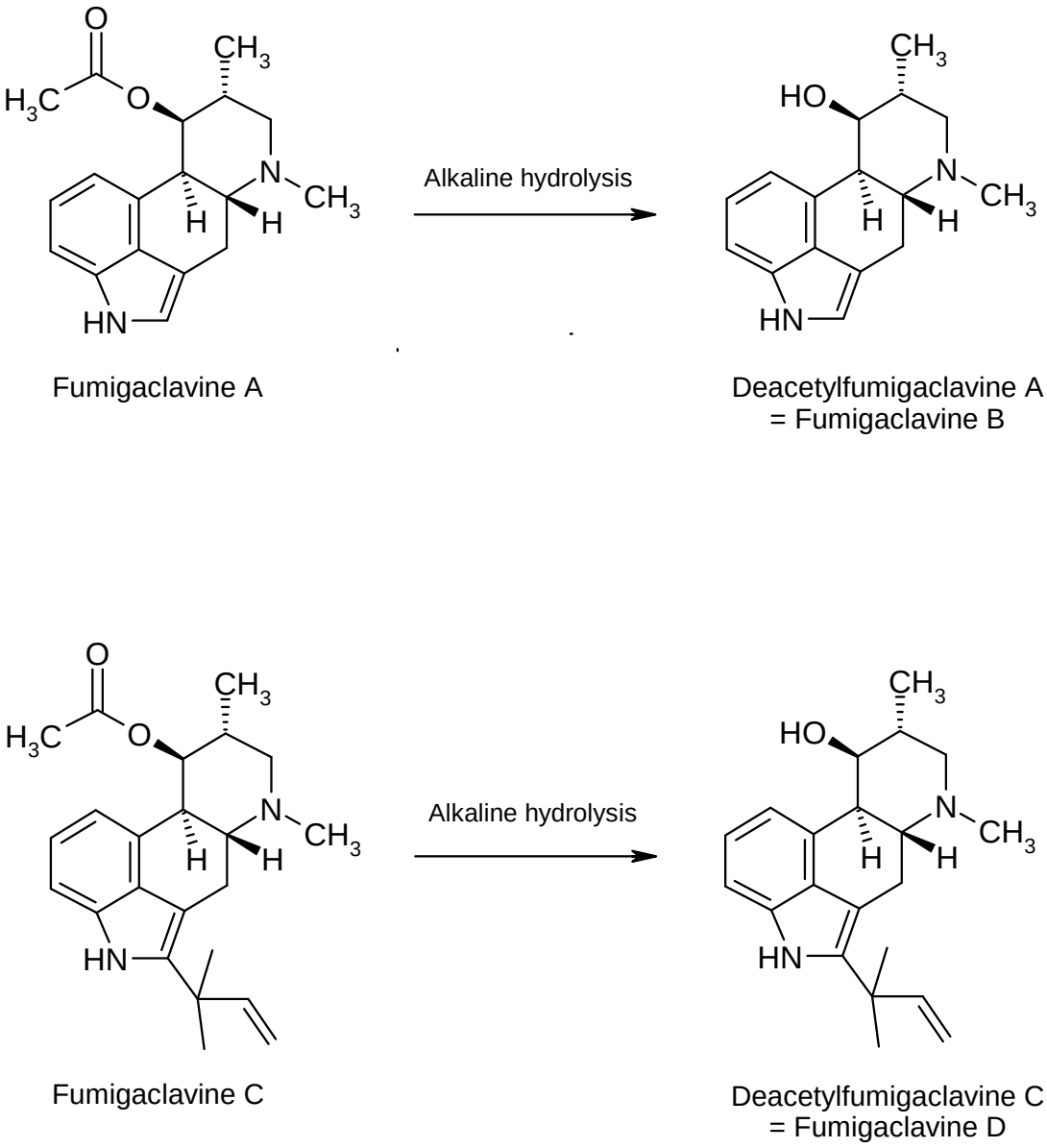


Figure 22. HPLC chromatogram (UV detector, 280 nm) of mycelium extract of *A. fumigatus* (diluted 1:1000 in methanol).



Fumigaclavine A

Deacetylfumigaclavine A
= Fumigaclavine B

Fumigaclavine C

Deacetylfumigaclavine C
= Fumigaclavine D

Figure 23. Chemical transformation of FuA into FuB, and FuC into FuD by alkaline hydrolysis.

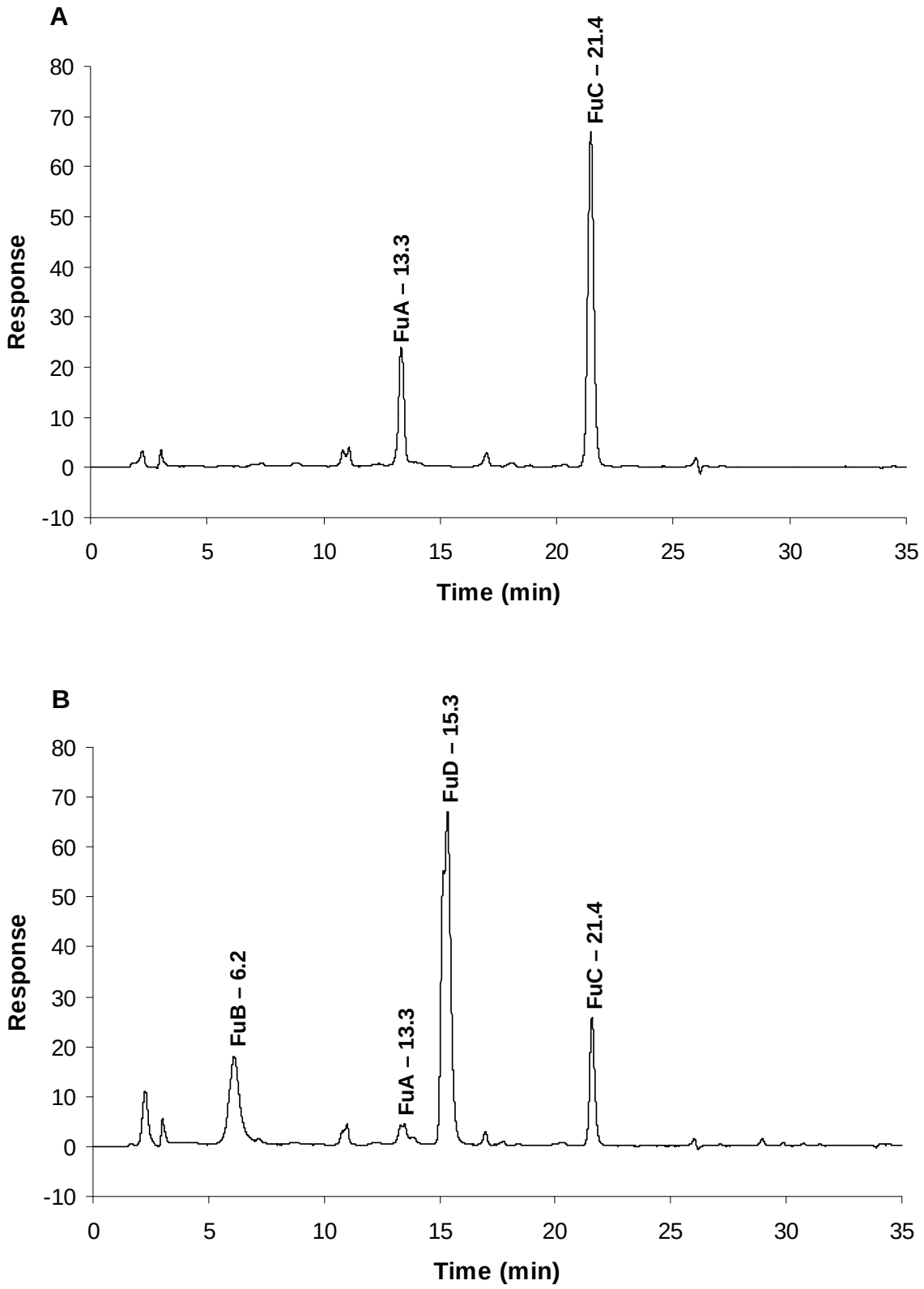


Figure 24. HPLC chromatograms (fluorescence detector) of mycelium extract of *A. fumigatus* (isolate A5), before hydrolysis (A) and after hydrolysis (B) of the extracts with NaOH.

4.2.3.4 Immunochromatography Analysis

Analysis of the HPLC eluate fractions of mycelium extract A5 (after hydrolysis) using FuA EIA detected not only FuA but also other fumigaclavines including FuB, FuC, and FuD. The retention times of the four compounds were 6.2 min (FuB), 13.3 min (FuA), 15.3 min (FuD), and 21.4 min (FuC), respectively. Because FuB, FuC, and FuD were not commercially available, an estimate to quantitate was made on these compounds, using the FuA standard. The amount of these compounds was each estimated by comparing the relative peak area with that of FuA, assuming that the absorptivity characteristics of all four compounds is nearly identical.

Fractions containing FuB, FuC, and FuD were obtained from HPLC separation of mycelium extract after alkaline hydrolysis. The concentration of toxins in these solutions were approximately 2 µg/ml (FuB), 3 µg/ml (FuC), and 7 µg/ml (FuD), as estimated from UV peak areas at the respective HPLC retention times. These fractions were analyzed by competitive indirect FuA EIA. From the concentration values obtained by EIA, the relative reactivities were calculated to be 100% (FuA), 1.3% (FuB), 12.6% FuC, and 0.2% (FuD). Therefore, the FuA EIA enables a relatively sensitive detection not only of FuA but also of FuC, which is in line with the similar structure of FuC at C-9 (Figure 2). An immunogram of mycelium extract of *A. fumigatus* after hydrolysis is given in Figure 25.

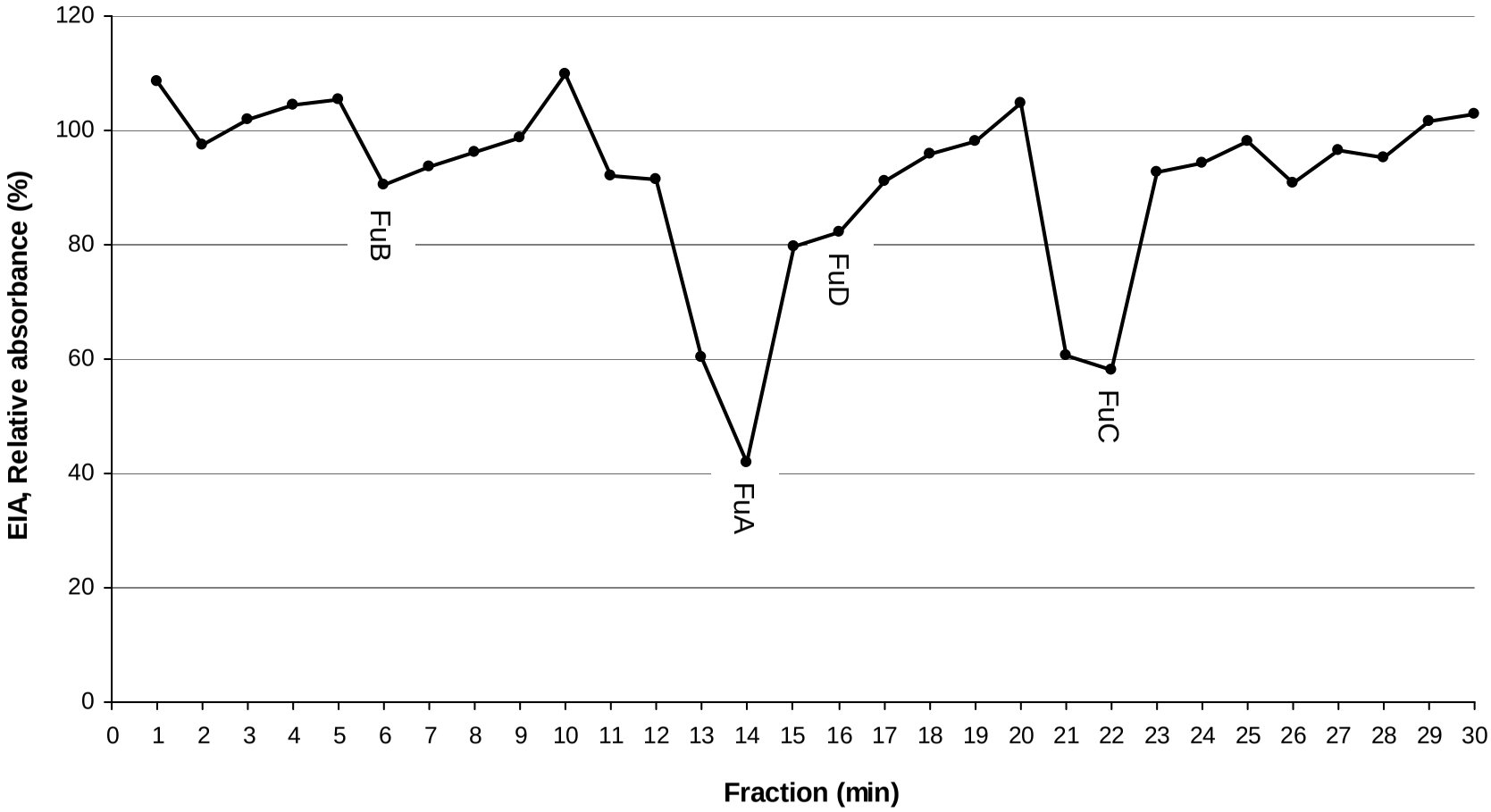


Figure 25. Immunogram of HPLC eluate fractions of *A. fumigatus* extract after hydrolysis. FuB (Fraction 6, 7), FuA (13, 14), FuD (15, 16), and FuC (21, 22) which were identified by HPLC retention times gave competitive binding inhibition in the FuA EIA.

4.2.4 Combined Analysis of FuA and IsoFuA in Blue Cheeses

4.2.4.1 Specificity Pattern of FuA EIA and Ergonovine EIA

To check whether FuA, IsoFuA, or both toxins may occur in blue cheeses, the differences in the specificity pattern of antibodies against FuA and against ergonovine were employed. Using competitive direct EIA format (typical standard curve is given in Figure 26), the anti-ergonovine antibody is able to detect several other clavine alkaloids, including FuA, FuB, and IsoFuA. Conversely, by competitive indirect EIA, anti-FuA antibody was only reactive with FuA and FuB, but did not cross-react with IsoFuA and ergonovine. A comparison of the specificity pattern of the FuA EIA and the ergonovine EIA are shown in Table 10 and Figure 27.

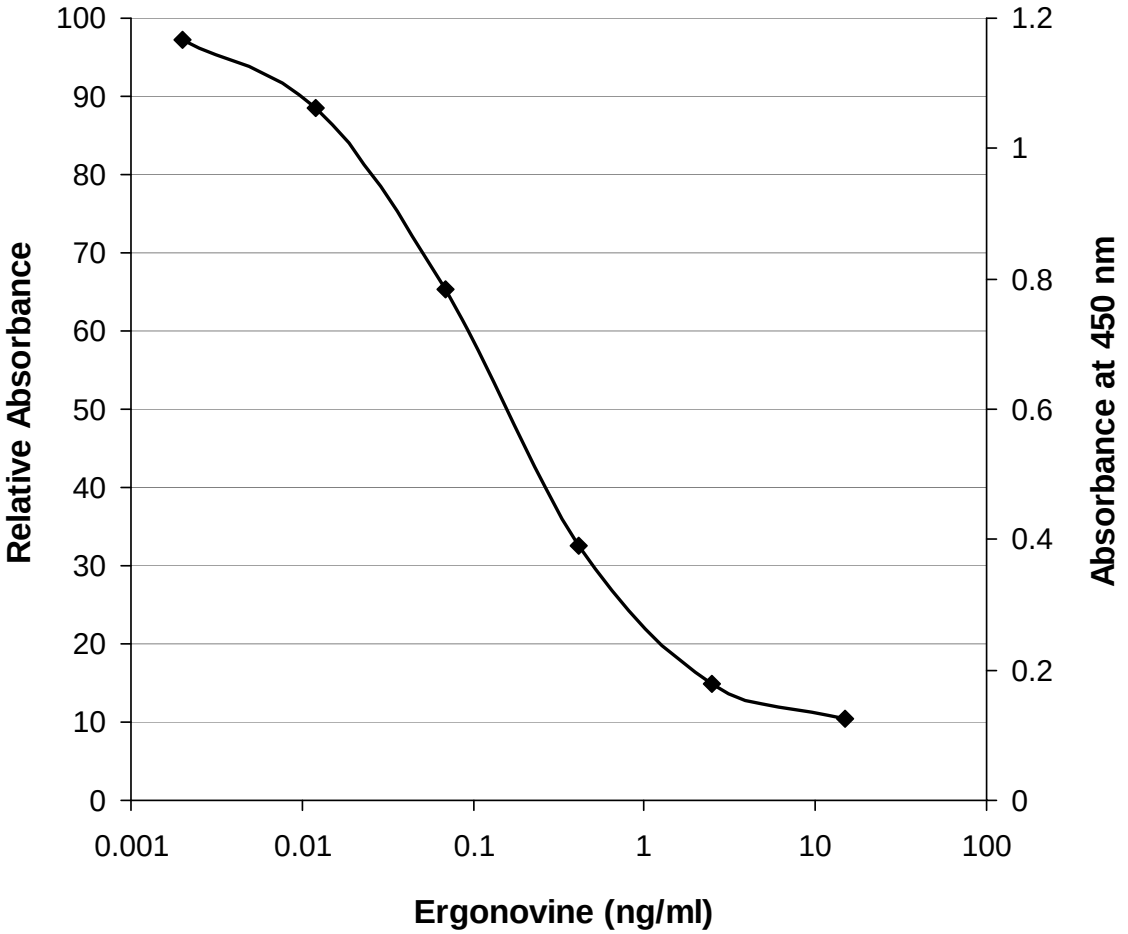


Figure 26. Standard curve of the competitive direct enzyme immunoassay for ergonovine. The IC_{50} of the EIA was 0.16 ng/ml with detection limit of standard curve varied between 0.02 to 0.05 ng/ml.

Table 10. Different specificity pattern of the FuA EIA and the ergonovine EIA

Compound	Molecular weight	FuA EIA		Ergonovine EIA	
		IC_{50} (ng/ml)	% CR	IC_{50} (ng/ml)	% CR
FuA	298.4	3.3	100	22.4	0.51
FuB	256.2	224	1.3	6.6	1.62
IsoFuA	298.4	>10,000	< 0.01	0.33	22.08
Ergonovine	325.4	>10,000	< 0.01	0.16	100

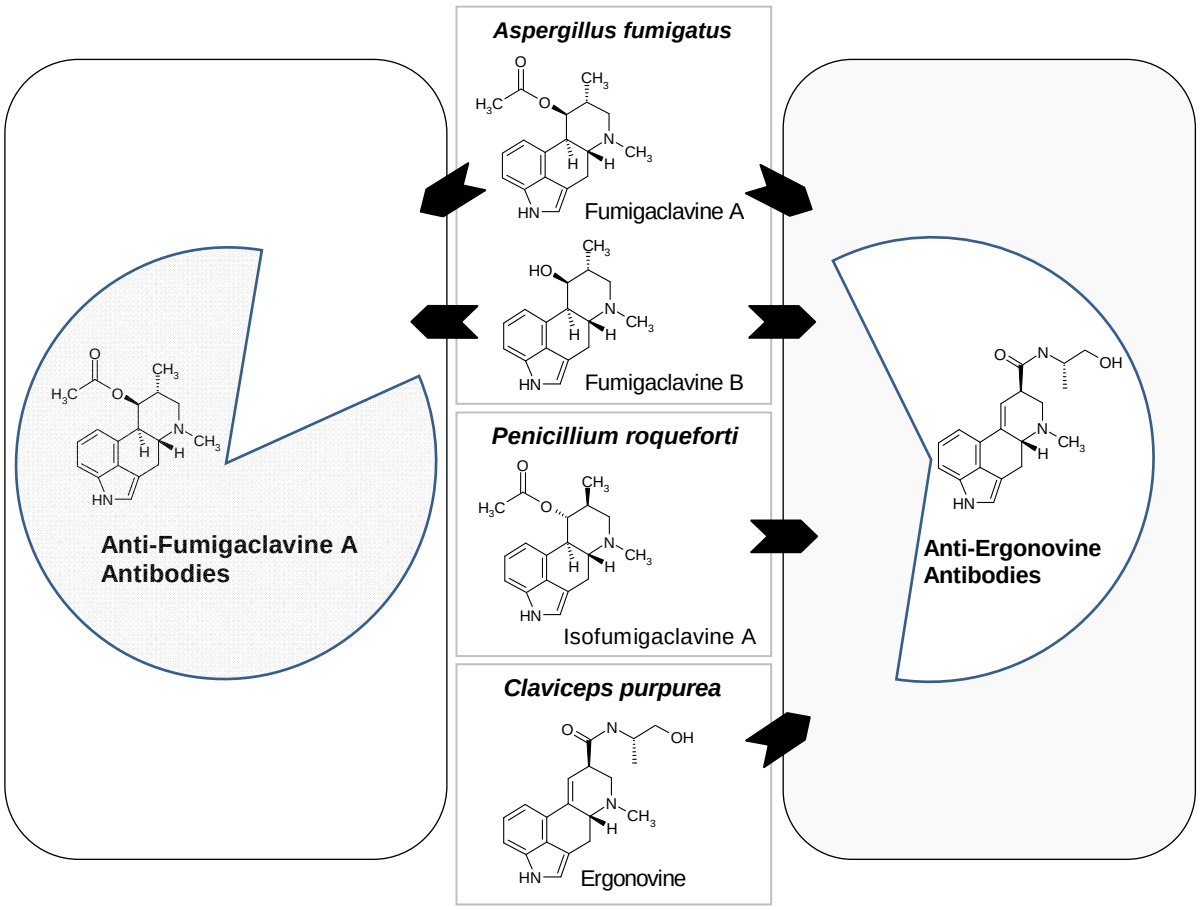


Figure 27. Schematic diagram of different specificity pattern of antibodies against FuA and antibodies against ergonovine: anti-FuA antibodies detect FuA and FuB, while anti-ergonovine antibodies detect FuA, IsoFuA, FuB, and other ergoline alkaloids.

4.2.4.2 Analysis of Blue Cheese

A variety of blue-veined cheeses from the German market such as Roquefort, Danish Blue, Bresse Blue, Bavaria Blue, Gorgonzola, and Cambozola were analyzed. Using the FuA EIA method, none of the 16 blue veined cheese samples contained FuA, while analysis of samples using ergonovine EIA found positive results in all cheese samples.

As shown in Figure 28, direct HPLC-UV analysis of raw blue cheese was not possible because of strong matrix absorbance. Only very high concentrations of FuA standard added to cheese extract yielded a clearly discernible peak at the retention time of FuA (15.6 min). However, analysis of fractionated HPLC eluates of cheese sample extracts in the FuA EIA and in the ergonovine EIA clearly confirmed that IsoFuA but not FuA was present in blue cheese (Figure 29). Fraction 8, corresponding to a HPLC run time of 14.1 – 15.9 min, which brackets the retention time of IsoFuA was clearly positive in the ergonovine EIA, but was negative in the FuA EIA. The immunograms of the ergonovine EIA and the FuA EIA obtained for one blue cheese are only qualitative but clearly demonstrate the analytical power of combining physicochemical separation and immunochemical detection.

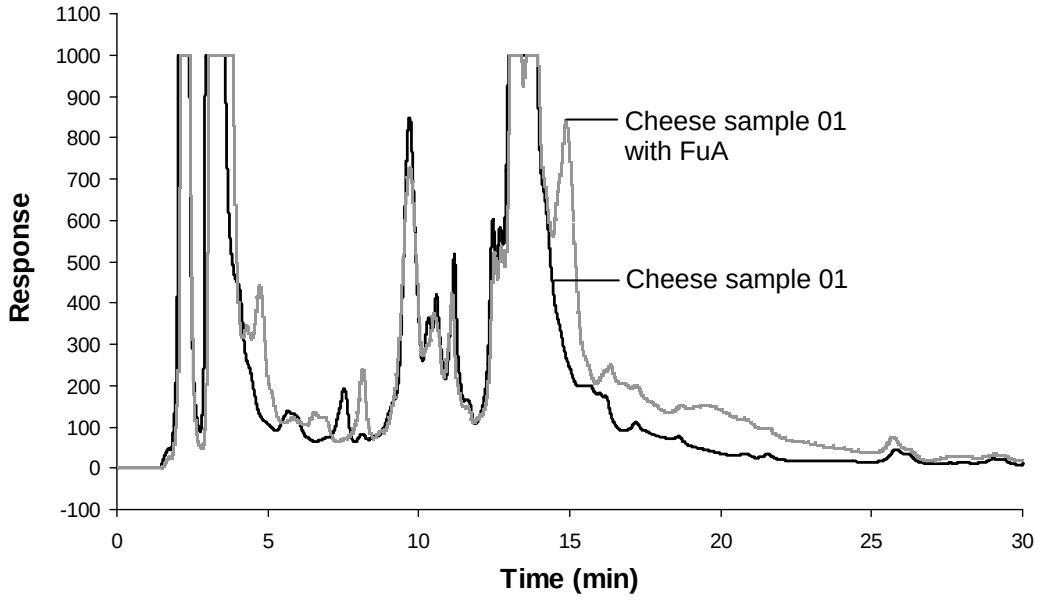


Figure 28. HPLC chromatograms of blue-veined cheese extract, without and with FuA standard (500 ng/ml).

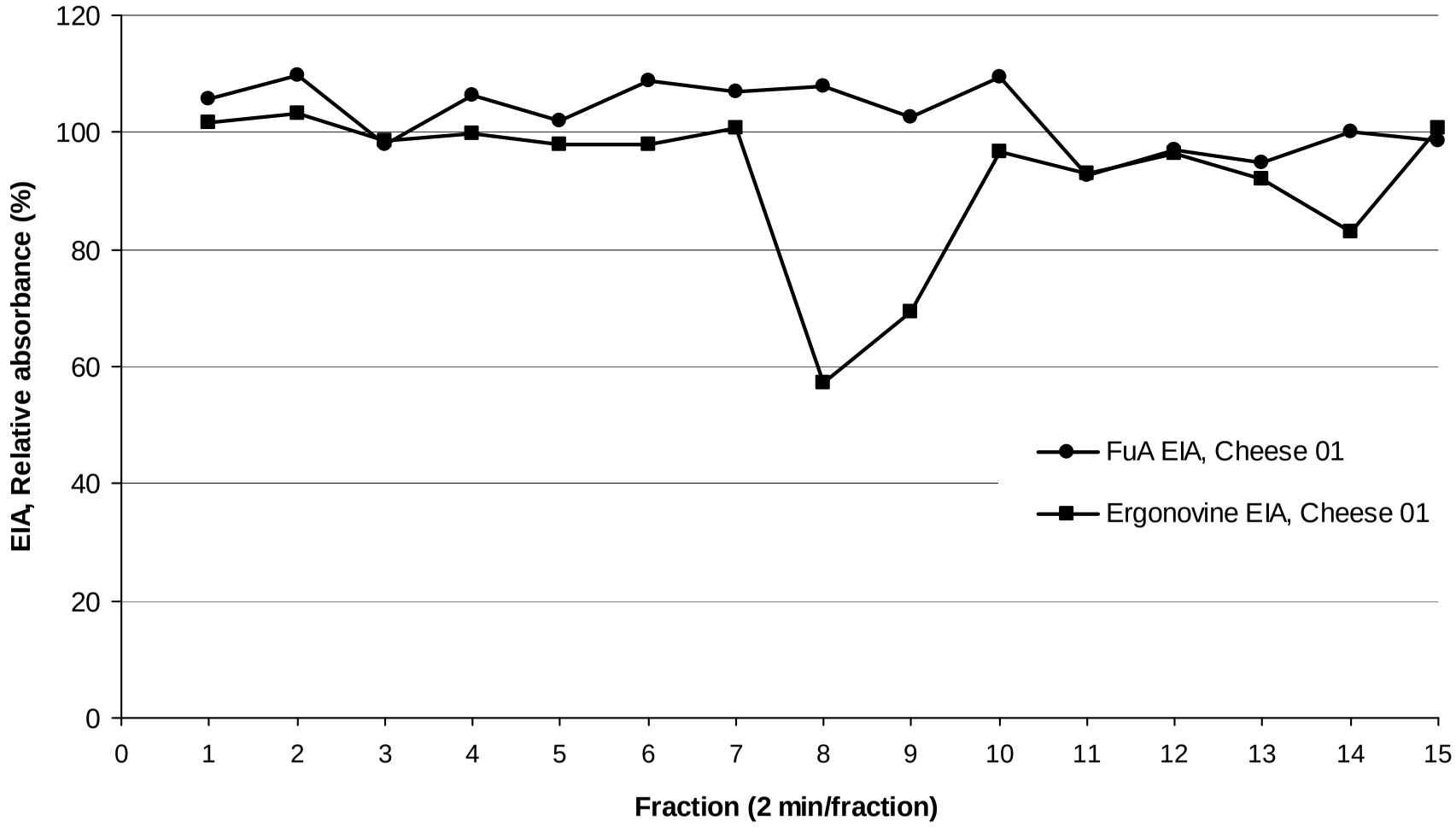


Figure 29. Comparison of the FuA EIA and the ergonovine EIA immunograms of HPLC eluate fractions of blue-veined cheese extract (cheese sample 01).

5 DISCUSSION

Ergoline mycotoxins probably comprise the largest group of mycotoxins. The number of potentially toxin producing fungal species (from at least four different genera) is also very high, and so is the variety of their habitats. It may therefore be concluded that ergolines are probably the most ubiquitous mycotoxins worldwide. However, relatively little research has been done in this field, compared with, for example, aflatoxins or trichothecenes. Likewise, no specific regulations for ergoline alkaloids in foods or feeds exist. There is an indirect regulation for *Claviceps purpurea* ergot set by the feed law (1000 mg/kg feed), but this does not directly refer to the alkaloid content. Furthermore, ergot (or any other fungus) cannot be detected anymore in milled and processed material. Hence, there is a need to provide specific analytical methods for ergolines. Depending on the individual demands, rapid and simple methods such as immunoassays may offer many advantages within an analytical strategy.

Methods described for ergoline alkaloids so far are mostly HPLC-based. Most method development has been reported for ergot alkaloids which have strong fluorescence properties, thus simplifying detection. Clavine alkaloids also do have UV absorbance and fluorescence, and should therefore be easy to analyse at trace level concentrations. However, very few methods have been described so far. Published research on immunochemical methods for ergolines is very scarce. The group of RA Shelby described immunochemical methods for some lyserg acid derivatives such as ergotamine (Shelby and Kelley 1990) and ergovaline (Shelby et al. 1998). Shelby and Kelley (1991) also developed monoclonal antibodies against ergonovine (ergometrine) which enabled sensitive detection of this alkaloid in enzyme immunoassay (50% inhibition value of the standard curve: 46 pg/ml). They also observed weak (0.05-0.1%) cross-reactions in this assay with festuclavine, agroclavine, and some other clavine alkaloids. However, immunochemical methods for clavine alkaloids, especially for fumigaclavines have so far not been reported.

5.1 Antibodies against FuA

A specific immune response against haptens can usually be achieved by coupling the haptens to high molecular weight protein. Like any other mycotoxin, FuA is a low molecular weight compound and is therefore nonimmunogenic. Therefore, in order to gain immunogenic properties it must be conjugated to a protein carrier (*e.g.*, KLH) before immunization (Chu et al. 1976; Krotzky and Zeeh 1995).

The FuA was conjugated to KLH and BSA using the Mannich condensation reaction. This reagent is very versatile and can be used to couple haptens through various functional groups, including primary and secondary amines (Wong 1991). Thus it may be assumed that FuA was conjugated to KLH and BSA via its indole nitrogen, although no definite proof for this could be obtained. Spectrophotometric analysis of the conjugates showed increased absorbance at UV wavelength maxima corresponding to FuA. However, quantitative determination of the conjugation ratios was not possible because of absorbance interference by turbidity and precipitates, which was mainly observed in the FuA-KLH, but to a lesser extent also in the FuA-BSA. However, the ultimate control of the success of immunogen synthesis lies with the ability to induce specific antibodies.

In this study, the induction of specific anti-FuA antibodies after immunization of rabbits with FuA-KLH was moderately efficient, as monitored by competitive indirect EIA. Although specific binding to FuA-BSA was measured for all sera, competitive binding inhibition through free FuA standard solution could only be achieved using sera from rabbits 55 and 57, respectively, because no specific antibodies against FuA were detected in the antiserum from rabbit 56. However, one rabbit (57) produced polyclonal antibodies which had very high apparent affinity for FuA in competitive binding inhibition assays.

5.2 EIA Development

Current methods of mycotoxin analysis usually include an extraction step and a clean-up step to reduce or remove sample matrix interferences, to improve the measurement of mycotoxins (Zheng et al. 2006; Krska et al. 2008). Because EIAs are easy to operate, require only simple equipment, and enable a high sample throughput, these assays have become one of the most useful tools for the rapid monitoring of mycotoxins (Schneider et al. 2004). However, immunochemical methods for fumigaclavines, including FuA have so far not been reported.

Using polyclonal anti-FuA antibodies and the FuA-BSA conjugate, competitive indirect EIAs were developed for analysis of FuA. Antiserum from rabbit 57 with an IC_{50} level and detection limit of the standard curve of 3.3 ng/ml and approximately 0.2 to 0.5 ng/ml, respectively had superior properties compared with serum of rabbit 55. It was used for the establishment of the immunoassay, because it yielded not only the most sensitive test system, but was also less affected by non-specific background color development.

The FuA EIA described here is the first one for a clavine alkaloid. Because this novel analytical method offered for the first time a possibility to check various matrices without time-consuming sample extract purification steps, a wide range of applications was tested. Analysis of FuA may be interesting within a strategy of mycotoxin analysis in food, feed, and environmental samples, but could also be useful to study toxin production by *A. fumigatus*.

5.3 Specificity of the Assay

The immunoassay specificity was initially studied with different ergoline alkaloid mycotoxins as competitive antigens. FuB and some other lysergic acid derivatives, including ergonovine, ergotamine, and alpha-ergocryptine were chosen because of basic structural similarities to FuA.

The IC_{50} for FuB, which differs from FuA only by the lack of an acetyl residue at C-9, was 224 ng/ml, corresponding to a relative cross-reactivity of 1.3%. Although other clavines were not available at this time, the relative poor reactivity of the closely related FuB indicated that cross-reactivity with other structurally related compounds, is probably very weak. No cross-reactivity was observed for lysergic acid derivatives (ergonovine, ergotamine, and alpha-ergocryptine). Obviously antibody binding is affected by structural differences of the substituent at C-9, and by α/β-epimeric forms at C-8 and C-9.

Reactivity data of FuC and FuD, obtained from extracts of *A. fumigatus*, grown on malt extract agar, with partial alkaline hydrolysis of FuC into FuD, showed that the acetate at C-9 is obviously important for antibody binding. FuC, which also has the acetate at C-9 and a bulky substituent at the indole ring, still has high reactivity of about 10% of that of FuA. After alkaline hydrolysis of the acetate, the resulting FuD retained only very weak reactivity (~0.2%). This shows that the FuA EIA could be used for the detection of FuA and FuC. It also indicates that the conjugation site of FuA in the immunogen is probably distal of the acetate, and most likely at the indole nitrogen, because antibody recognition was not strongly affected by modifications of the indole ring.

5.4 Application Studies

5.4.1 Analysis of FuA in Silage

Clavine alkaloid-producing fungi, in particular *A. fumigatus,* are ubiquitous in silage (Cole et al. 1977; O'Brien et al. 2006). However, natural occurrence of fumigaclavines in silage has so far not been reported. Previously, the determination of FuA from fungal material isolated from moldy silage was reported by Cole et al. (1977) and Santos et al. (2002). The present study reports the first direct analysis of FuA in silage samples.

Silage is a complex matrix, therefore FuA extraction was necessary before the samples could be tested by EIA. Sample extraction using methanol/water (70:30, v/v) was efficient enough for FuA, with recovery values between 70 and 90%. Because of the high sensitivity of the EIA, diluted extracts could be analysed to overcome matrix interference, and still a good detection limit of about 10 ng/g could be achieved. FuA was not found in any silage sample. Although the sample number was limited, the results indicate that FuA is probably not a widespread problem in silage.

5.4.2 Analysis of FuA in Tissue Samples from Respiratory System of Birds with Aspergillosis

A. fumigatus is an opportunistic pathogen in humans and animals, and causes a wide range of severe respiratory diseases including aspergillosis in birds (for review see, *e.g*., Rementeria et al. 2005). It was therefore interesting to see whether or not FuA is produced *in situ* during aspergillosis.

Using a simple sample preparation, FuA could be analysed in respiratory system tissue samples at concentration of 1.5 ng/g or higher. With this detection limit, the FuA EIA is sufficiently sensitive for FuA. Although FuA was found in 66% tissue samples of aspergillosis cases, the role of this mycotoxin in this disease remains

to be clarified. However, the determination of FuA in *Aspergillus*-infected birds, especially in aspergillosis cases, could be interesting, both for diagnostic purposes and to study its potential role within this disease complex. Further work will aim at analysis of FuA in blood serum from birds with aspergillosis. Although this is highly speculative at the moment, FuA could possibly be an indicator of this disease in live birds.

5.4.3 Isolation and Characterization of Fumigaclavines from *A. fumigatus* Isolated from Air Sac Tissue

A. fumigatus has been determined as the most pathogenic among the causative agents of aspergillosis in different avian species such as gulls (*Lorits* sp.), great rhea (*Rhea americana*), parrot (*Eclectus* sp.), penguin (*Spheniscus* sp.), turkey (*Meleagris* sp.), ostrich (*Struthio camelus*), and chicken (*Gallus* domesticus) (Pouldino 1952; Reissig et al. 2002; Lair-Fulleringeret et al. 2003; Xavier et al. 2007; Mayahi et al. 2008; Khosravi et al. 2008; Zafra et al. 2008).

Gliotoxin, a known as potential virulence factor of *A. fumigatus,* especially in human and animal aspergillosis, was not found by HPLC in mycelium extracts of *A. fumigatus* from aspergillosis cases. This was rather preliminary, because no specific attempts were made to optimize *A. fumigatus* cultivation for gliotoxin production. However, the production of high amounts of FuA by isolates of *A. fumigatus* from aspergillosis cases indicate that this compound could be of relevance. Speculatively, FuA (and maybe other fumigaclavines) could even play a role in disease causation by *A. fumigatus*, considering the vasoactive and immunomodulatory activity of the fumigaclavines (Ma et al. 2006; Wu et al. 2005).

5.4.4 Combined Analysis of FuA and IsoFuA in Blue Cheese

Relative cross-reactivities of FuA, IsoFuA, FuB, and ergonovine, when used as competitive inhibitors in the FuA EIA and the ergonovine EIA, indicated an interesting difference in the specificity pattern of both EIAs. It is interesting to note that antibodies obtained from rabbit immunized with FuA-KLH did not cross-react with ergonovine. Conversely, when the rabbit was immunized with ergonovine-KLH, the antibody cross-reacts with FuA.

The chemical structures of ergonovine and FuA differ at C-8 and C-9. However, using competitive direct ergonovine EIA, the anti-ergonovine antibody was able to detect FuA and the closely related compounds IsoFuA and FuB. These data strongly indicated that the basic ergoline ring system is detected by the anti-ergonovine antibody. Therefore, anti-ergonovine antibody has a cross-reactivity with several other ergoline alkaloids, including clavine alkaloids. In contrast, the FuA EIA did not detect ergonovine and IsoFuA. This would indicate that anti-FuA antibody is greatly influenced not only by an acetyl group at C-9 and an α/β-epimeric forms of FuA at C-8 but also an isomeric forms of FuA at C-8 and C-9. Thus, anti-FuA antibody did not react with IsoFuA and some lysergic acid derivatives, such as ergonovine.

P. roqueforti is commonly used to produce a variety of blue-veined cheeses, but on the other hand it has been reported that this fungus is able to produce some clavine alkaloids such as IsoFuA, IsoFuB, and festuclavine (Ohmomo et al. 1975; Scott and Kennedy 1976; Scott et al. 1976; Nielsen et al. 2006). Vinokurova et al. (2001) described two strains of *P. roqueforti* Thom 1906, one producing FuA, the other IsoFuA. Ohmomo et al. (1975) reported that they found “insignificant” levels of 0.2-3.6 mg/kg (IsoFuA and IsoFuB) in roquefort type cheese, but gave no further details. Until today, the only larger study on natural occurrence of clavine alkaloids in food was performed by Scott et al. (1977) who found IsoFuA and IsoFuB in blue cheese. IsoFuA was found in 11 out of 16 analyzed samples, at levels of 0.020-4.7 mg/kg, while only traces of IsoFuB were present in four samples.

In this study, the presence of IsoFuA in blue-veined cheese samples was detected using the ergonovine EIA. Since this assay also had cross-reactivity with some other clavine alkaloids, the positive results could not automatically be attributed to IsoFuA. However, with the development of a combined HPLC separation following by the FuA EIA and the ergonovine EIA (Immunochromatography analysis), the determination of toxins in blue-veined cheese was possible.

Analysis of HPLC eluate fractions was clearly demonstrated that IsoFuA was present in these samples. This is only the third report on IsoFuA in cheese, and the first one for more than 30 years. Since cheese production parameters influence toxin production by *P. roqueforti,* further studies should aim at a larger screening study, including quantitative analysis of IsoFuA.

In conclusion, in this study, the first antibodies against a clavine alkaloid have been developed. The EIA established for the determination of FuA is highly sensitive and easy to perform. Application of this EIA to determine FuA in food, feed, and biological material has been successful. Therefore the FuA EIA present a versatile analytical tool to enhance the study of ergoline alkaloids.

6 SUMMARY

The present study describes the development of specific polyclonal antibodies against fumigaclavine A (FuA) in rabbits, and the development of a highly sensitive enzyme immunoassay (EIA) for this mycotoxin. The Mannich condensation reaction with formaldehyde was used to conjugate FuA to keyhole limpet hemocyanin (KLH) as the immunogen, and to bovine serum albumine (BSA) for as the coating antigen. Conjugation of FuA to KLH with formaldehyde proved to be an effective approach for the preparation of immunogen for anti-FuA antibody production.

A competitive indirect EIA was optimized using antiserum obtained from one rabbit. The EIA was very sensitive for FuA, with a 50% inhibition concentration (IC_{50}) value of 3.3 ng/ml and a detection limit of the standard curve in buffer solutions of 0.5 ng/ml. The EIA was very specific for FuA with 1.3%, 12.6%, and 0.2% cross-reactivity with FuB, FuC, and FuD, respectively. IsoFuA and several other lysergic acid derivatives (ergonovine, ergotamine, and alpha-ergocryptine) were tested but did not cross-react in this assay.

The EIA was applied to the analysis of FuA in silage and in tissue from the respiratory system of birds with aspergillosis. The detection limit for FuA in silage was at 10 ng/g, average recoveries from artificially contaminated control samples were 77.8%. None of 24 analyzed silage samples contained detectable amounts of FuA. Although the number of samples was limited, the results indicate that there is not a widespread problem of FuA in silage.

The detection limit of the assay for FuA in tissue from the respiratory system of birds was at 1.5 ng/g, with average recoveries from artificially contaminated of 92.7%. FuA was found in 66% tissue samples of aspergillosis cases. Cultivation of fungal growth on respiratory tissue samples of aspergillosis cases on malt extract agar (MEA) yielded fungal material which was typical for *A. fumigatus*. When the mycelium was extracted and assayed by FuA EIA, high amounts of FuA was found (up to 8 mg/g mycelium). Further analysis of the mycelium extract by HPLC found

FuA, FuC, and other unidentified compounds. Hydrolysis of FuC which have been isolated from mycelium extract of *A. fumigatus* gave a new fumigaclavine derivative. FuD is proposed as the name of this compound.

Different specificity pattern of the FuA EIA and that of a previously developed ergonovine EIA were studied. In competitive indirect EIA, the anti-FuA antibody did detect neither ergonovine nor IsoFuA. Conversely, by competitive direct EIA format, the anti-ergonovine antibody was able to detect both FuA and IsoFuA. Using these assays in combination with HPLC separation of cheese extracts, a series of blue-veined cheeses from the German market were analyzed. None of 16 blue-veined cheese samples contained FuA. However, the presence of IsoFuA in blue-veined cheese samples was detected using the ergonovine EIA.

This is the first description of antibodies against FuA and the first development of an EIA for FuA. This is also the first report demonstrating that FuA is correlated with aspergillosis in birds. However, the role of this mycotoxin in this disease remains to be clarified.

7 ZUSAMMENFASSUNG

Die vorliegende Arbeit beschreibt die Herstellung spezifischer Antikörper gegen Fumigaclavin A (FuA) und die Entwicklung eines hochempfindlichen Enzym-immuntests (EIA) für dieses Mykotoxin. Die Mannich-Kondensation mit Formaldehyd wurde verwendet, um Fumigaclavin A an keyhole limpet hemocaynin (KLH) als Immunogen und an bovines Serumalbumin (BSA) als Beschichtungsantigen zukoppeln. Die Kopplung von FuA an KLH mittels Formaldehyd erwies sich als effektiver Ansatz zur Herstellung eines Immunogens zur Gewinnung von Anti-FuA Antikörpern in Kaninchen.

Ein kompetitiver indirekter EIA wurde erstellt und optimiert, unter Verwendung polykloner Antikörper eines Kaninchens. Der EIA war sehr empfindlich für FuA, mit einer 50%-Dosis von 3,3 ng/ml und einer Nachweisgrenze von 0,5 ng/ml (in Pufferlösung). Der EIA war relativ spezifisch für FuA, mit Kreuzreaktionen von 1,3%, 12,6% bzw. 0,2% Kreuzreaktion für FuB, FuC bzw. FuD. IsoFuA und einige andere getestete Lysergsäurederivate (Ergonovin, Ergotamin, und alpha-Ergokryptin) wurden ebefalls untersucht, zeigten aber keine Kreuzreaktion in diesem Testsystem.

Der EIA wurde zur Untersuchung von FuA in Silage und in Gewebsproben (Repirationstrakt) von Vögeln mit Aspergillose eingesetzt. Die Nachweisgrenze für FuA in Silage betrug 10 ng/g, die durchschnittlichen Wiederfindungsraten für FuA in künstlich kontaminierten Kontrollproben lagen bei 77,8%. Keine der 24 untersuchten Proben enthielt nachweisbare Gehalte an FuA. Obwohl nur eine begrenzte Probenanzahl untersucht wurde, zeigen die Ergebnisse, dass FuA kein weit verbreitetes Problem in Silage darstellt.

Die Nachweisgrenze für FuA in Gewebsproben des Repirationstrakts von Vögeln lag bei 1,5 ng/g, mit durchschnittlichen Wiederfindungsraten für FuA in künstlich kontaminierten Proben von 92,7%. FuA wurde in 66% der Proben von Vögeln mit *Aspergillose* nachgewiesen. Bei einer Kultivierung von Pilzisolaten des Atmungstraktes auf Malzextraktagar (MEA) entwickelte sich ein Mycel, das typisch

für *A. fumigatus* war. Bei Extraktion des Mycels und Untersuchung im FuA-EIA wurden große Mengen an FuA (bis zu 8 mg/g Mycel) nachgewiesen. Bei einer weiteren Untersuchung des Mycelextrakts mittels HPLC wurden FuA, FuC und andere, nicht identifizierte Komponenten gefunden. Eine Hydrolyse von FuC, das aus dem Mycelextrakt von *A. fumigatus* gewonnen wurde, ergab ein neues Fumigaclavin-Derivat. FuD wird als Name für diese Verbindung vorgeschlagen.

Zur Untersuchung von Blauschimmelkäse auf Clavin-Alkaloide wurden die unterschiedliche Spezifitäten des FuA-EIA und eines zuvor entwickelten Ergonovin-EIA ausgenutzt. Im kompetitiven indirekten EIA für FuA waren alle Proben negativ. Dagegen wurde bei Verwendung eines kompetitiven direkten EIA unter Verwendung von Anti-Ergonovin-Antikörpern hohe Meßwerte erzielt. Bei einer Untersuchung mit Hilfe dieser beiden Testsysteme und einer HPLC-Trennung von Käse-Extrakten wurden Blauschimmelkäse des deutschen Marktes untersucht. Keine der 16 untersuchten Proben enthielt FuA, jedoch wurde die Anwesenheit von IsoFuA in Blauschimmelkäse nachgewiesen.

Die vorliegende Arbeit beschreibt erstmals die die Entwicklung von Antikörpern gegen FuA und eines Enzymimmuntests für dieses Mykotoxin. Auch wird erstmalig eine Korrelation zwischen FuA-Gehalten in Gewebe des Repirationstrakts und der Aspergillose von Vögeln beschrieben. Die Rolle dieses Mykotoxins bei diesem Krankheitsbild bleibt allerdings noch zu klären.

8. REFERENCES

Abraham WR, Arfmann HA (1990) 12,13-Dihidroxy-fumitremorgin C from *Aspergillus fumigatus*. Phytochemistry 29: 1025-1026

Abramson D, Usleber E, Märtlbauer E (1995) An indirect enzyme immunoassay for the mycotoxin citrinin. Appl Environ Microbiol 61: 2007-2009

Abrunhosa L, Paterson RRM, Kozakiewicz Z, Lima N, Venâncio A (2001) Mycotoxin production from fungi isolated from grapes. Lett Appl Microbiol 32: 240-242

Abuknesha RA, Luk CY, Griffith HHM, Maragkou A, Iakovaki D (2005) Efficient labelling of antibodies with horseradish peroxidase using cyanuric chloride. J Immunol Methods 306: 211-217

Afiyatullov SS, Kalinovskii AI, Pivkin MV, Dmitrenok PS, Kuznetsova TA (2004) Fumitremorgins from the marine isolate of the fungus *Aspergillus fumigatus*. Chem Nat Compd 40: 615-617

Ahimsa-Müller MA, Markert A, Hellwig S, Knoop V, Steiner U, Drewke C, Leistner E (2007) Clavicipitaceous fungi associated with ergoline alkaloid-containing Convolvulaceae. J Nat Prod 70: 1955-1960

Amitani R, Taylor G, Elezis EN, Lewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R (1995) Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immun 63: 3266-3271

Anslow WK, Raistrick H (1938) XCI. Studies in the biochemistry of micro-organisms. LVII. Fumigatin (3-hydroxy-4-methoxy-2: 5-toluqui-none) and spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone) metabolic products respectively of *Aspergillus fumigatus* Fresenius and *Penicillium spinulosum* Thom. Biochem J 32: 687-696

Auerbach H, Oldenburg E, Weissbach F (1998) Incidence of *Penicillium roqueforti* and roquefortine C in silages. J Sci Food Agric 76: 565-572

Austwick PKC (1965) Pathogenicity. In: Raper KB, Fennell DI (eds). The genus *Aspergillus.* Williams and Wilkins, Baltimore: 82-126

Barna-Vetró I, Solti L, Téren J.; Gyöngyösi A, Szabó E, Wölfling A (1996) Sensitive ELISA test for determination of ochratoxin A. J Agric Food Chem 44: 4070-4074

Barna-Vetró I, Szabó E, Fazekas B, Solti L (2000) Development of a sensitive ELISA for the determination of fumonisin B1 in cereals. J Agric Food Chem 48: 2821-2825

Berthiller F, Schuhmacher R, Buttinger G, Krska R (2005) Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1062: 209-216

Bertout S, Badoc C, Mallie M, Giamimis J, Bastide JM (2002) Spore diffusate isolated from some strains of *Aspergillus fumigatus* inhibits phagocytosis by murine alveolar macrophages. FEMS Immunol Med Microbiol 33: 101-106

Biabani MAF, Laatsch H (1998) Advances in chemical studies on low-molecular weight metabolites of marine fungi. J Prakt Chem 340: 589-607

Biselli S, Wegner H, Hummert C (2005) A multicomponent method for *Fusarium* toxins in cereal based food and feed samples using HPLC-MS/MS. Mycotox Res 21: 18-22

Blankenship JD, Spiering MJ, Wilkinson HH, Fannin FF, Bush LP, Schardl CL (2001) Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media. Phytochemistry 58: 395-401

Boichenko LV, Boichenko DM, Vinokurova NG, Reshetilova TA, Arinbasarov MU (2001) Screening for ergot alkaloid producers among microscopic fungi by means of the polymerase chain reaction. Microbiology 70: 306-310

Boysen M, Skouboe P, Frisvad J, Rossen L (1996) Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology 142: 541-549

Braicu C, Puia C, Bodoki E, Socaciu C (2008) Screening and quantification of aflatoxins and ochratoxin a in different cereals cultivated in Romania using thin-layer chromatography-densitometry. J Food Qual 31: 108-120

Braithwaite AW, Eichner RD, Waring P, Mullbacher A (1987) The immuno-modulating agent gliotoxin causes genomic DNA fragmentation. Mol Immunol 24: 47-55

Brakhage AA, Langfelder K (2002) Menacing mold: the molecular biology of *Aspergillus fumigatus.* Annu Rev Microbiol 56: 433-455

Chain E, Florey HW, Jennings MA, Williams TI (1943) Helvolic acid, an antibiotic produced by *Aspergillus fumigatus*, mut. *helvola* Yuill. Br J Exp Pathol 24: 108-119

Chen CH, Li WJ, Peng KY (2005) Determination of aflatoxin m1 in milk and milk powder using high-flow solid-phase extraction and liquid chromatography-tandem mass spectrometry. J Agric Food Chem 53: 8474-8480

Chu FS, Chang FCC, Hinsdill RD (1976) Production of antibody against ochratoxin A. Appl Environ Microbiol 31: 831-835

Chu FS (1992) Recent progress on analytical techniques for mycotoxins in feedstuffs. J Anim Sci 70: 3950-3963

Cigić IK, Prosen H (2009) An overview of conventional and emerging analytical methods for the determination of mycotoxins-review. Int J Mol Sci 10: 62-115

Cockrill BA, Hales CA (1999) Allergic bronchopulmonary aspergillosis. Annu Rev Med 50: 303-316

Cole RJ, Kirksey JW, Dorner JW, Wilson DM, Johnson JC, Johnson AN, Bedell DM, Springer JP, Chexal KK, Clardy JC, Cox RH (1977) Mycotoxins produced by *Aspergillus fumigatus* isolated from silage. J Agric Food Chem 25: 826-830

Cole RJ, Kirksey JW, Moore JH, Blankenship BR, Diener UL, Davis ND (1972) Tremorgenic toxin from *Penicillium verruculosum*. Appl Microbiol 24: 248-256

Coyle CM, Kenaly SC, Rittenour WR, Panacionne DG (2007) Association of ergot alkaloids with conidiation in *Aspergillus fumigatus*. Mycologia 99: 804-811

Coyle CM, Panaccione DG (2005) An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. Appl Environ Microbiol 71: 3112-3118

Croteau SM, Prelusky DB, Trenholm HL (1994) Analysis of trichothecene mycotoxins by gas chromatography with electron capture detection?. J Agric Food Chem 42: 928-933

Crowther JR (2009) The ELISA guidebook. Humana Press, Vienna, Austria

Curtui V, Gassen C, Dewenter L, Schneider E, Usleber E (2007) Immunchemische Nachweisverfahren für Mutterkornalkaloide. Forschungsprojekt 03HS019, Abschlussbericht

Degelmann P, Becker M, Herderich M, Humpf H-U (1999) Determination of ochratoxin a in beer by high-performance liquid chromatography. Chromatographia 49: 543-546

Desjardins AE, Hohn TM (1997) Mycotoxins in plant pathogenesis - current review. MPMI 10: 147-152

Dutcher JD, Johnson JR, Bruce WF (1945) Gliotoxin. VI. The nature of the sulfur linkages. conversion to desthiogliotoxin. J Amer Chem Soc 67: 1736-1745

Eich E, Eichberg D, Muller EG (1984) Clavines - new antibiotics with cytostatic activity. Biochem Pharmacol 33: 523-526

Eichner RD, Al-Salami M, Wood PR, Mullbacher A (1986) The effect of gliotoxin upon macrophage function. Int J Immunopharmacol 8: 789-797

Eskola M, Boonzaaijer G, van Osenbruggen WA, Rizzo A, Tijmensen G (2000) A study of the suitability of gas chromatography-electron capture detection for the analysis of deoxynivalenol in cereals. Mycotox Res 16: 73-90

Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, White OR, Whitty BR, Youngman P, Wolfe KH, Goldman GH, Wortman JR, Jiang B, Denning DW, Nierman WC (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genetics 4: 1-13

Flieger M, Wurst M, Shelby M (1997) Ergot alkaloids – sources, structures and analytical methods. Folia Microbiol 42: 3-30

Floss HG (1976) Biosynthesis of ergot alkaloids and related compounds. Tetrahedron 32: 873-912

Fox M, Gray G, Kavanagh K, Lewis C, Doyle S (2004) Detection of *Aspergillus fumigatus* mycotoxins: immunogen synthesis and immunoassay development. J Microbiol Methods 56: 221-230

Franco CM, Fente CA, Vazquez B, Cepeda A, Lallaoui L, Prognon P, Mahuzier G (1996) Simple and sensitive high-performance liquid chromatography-fluorescence method for the determination of citrinin: application to the analysis of fungal cultures and cheese extracts. J Chromatogr A 723: 69-75

Fraser RS (1993) Pulmonary aspergillosis: pathologic and pathogenetic features. Pathol Annu 28: 231-277

Fremy JM, Usleber E (2003) Policy on characterization of antibodies used in immunochemical methods of analysis for mycotoxins and phycotoxins. J AOAC Int 86: 868-871

Frisvad JC, Filtenborg O, Thrane U (1989) Analysis and screening for mycotoxins and other secondary metabolites in fungal cultures by thin-layer chromatography and high-pertbrmance liquid chromatography. Arch Environ Contam Toxicol 18: 331-335

Frisvad JC, Rank C, Nielsen KF, Larsen TO (2008) Metabolomics of *Aspergillus fumigates.* Med Mycol: S1-S19

Frisvad JC, Thrane U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone indices and UV-VIS spectra (diode array detection). J Chromatogr 404: 195-214

Frisvad JC, Thrane U, Samson RA (2007) Mycotoxins producers, in Food Mycology. A multifaceted approach of fungi and food (Dijksterhuis J and Samson RA, eds.). CRC Press, Boca Raton

Gallati H, Pracht I (1985) Peroxidase aus Meerrettich: Kinetische studien und Optimierung der Peroxidase-Aktivitätsbestimmung mit den Substraten H_2O_2 und 3,3',5,5',-Tetramethylbenzidin. J Clin Chem Clin Biochem 23: 453-460

Gardiner DM, Howlett BJ (2005) Bioinformatic and expression analysis of the putative gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*. FEMS Microbiol Lett 248: 241-248

Gardiner DM, Waring P, Howlett BJ (2005) The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions, and biosynthesis. Microbiology 151: 1021-1032

Gathumbi JK, Usleber E, Ngatia TA, Kangethe EK, Märtlbauer E (2003) Application of immunoaffinity chromatography and enzyme immunoassay in rapid detection of aflatoxin B1 in chicken liver tissues. Poult Sci 82: 585-590

Ge HM, Yu ZG, Zhang J, Wu JH, Tan RX (2009) Bioactive alkaloids from endophytic *Aspergillus fumigatus*. J Nat Prod 72: 753-755

González-Osnaya L, Soriano JM, Moltó JC, Mañes J (2008) Simple liquid chromatography assay for analyzing ochratoxin A in bovine milk. Food Chem 108: 272-276

Goryacheva IY, Rusanova TY, Burmistrova NA, De Saeger S (2009) Imunochemical methods for the determination of mycotoxins - review. J Anal Chem 64: 768-785

Griffith EC, Su Z, Niwayama S, Ramsay CA, Chang YH, Liu JO (1998) Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. Proc Natl Acad Sci 95: 15183-15188

Hanson FR, Eble TE (1949) An antiphage agent isolated from *Aspergillus* sp. J Bact 58: 527-529

Hanson FR, Eble TE (1953) Fumagillin and preparation. United States Patent Office 2,652,356

Hawksworth DL (2009) Separate name for fungus's sexual stage may cause confusion. Nature 458: 29

Heinstein PF, Lee SL, Floss HG (1971) Isolation of dimethylallylpyrophosphate: tryptophan dimethylallyltransferase from the ergot fungus (*Claviceps* spec.). Biochem Biophys Res Commun 44: 1244-1251

Hof H, Kupfahl C (2009) Gliotoxin in *Aspergillus fumigatus*: an example that mycotoxins are potential virulence factors. Mycotox Res 25: 123-131

Hofmann A. (1964): Die Mutterkornalkaloide. Vom Mutterkorn zum LSD - Die Chemie der Mutterkornalkaloide. Reprint der Originalausgabe, 2nd Ed 2006. Nachtschatten Verlag, Solothurn

Hurne AM, Chai CLL, Waring P (2000) Inactivation of rabbit muscle creatine kinase by reversible formation of an internal disulfide bond induced by the fungal toxin gliotoxin. J Biol Chem 275: 25202-25206

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J (1990) Synthetic anlogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348: 555-557

International Union of Pure and Applied Chemistry (IUPAC) (1993) Nomenclature for chromatography (IUPAC Recommendations 1993). Pure Appl Chem 65: 819-872

Jaussaud P, Durix A, Videmann B, Vigie A, Bony S (1998) Rapid analysis of ergovaline in ovine plasma using highperformance liquid chromatography with fluorimetric detection. J Chromatogr A 815: 147-153

Kamei K, Watanabe A (2005) *Aspergillus* mycotoxins and their effect on the host. Med Mycol 43 Suppl 1: S95-S99

Kataoka H, Itano M, Ishizaki A, Saito K (2009) Determination of patulin in fruit juice and dried fruit samples by in-tube solid-phase microextraction coupled with liquid chromatography mass spectrometry. J Chromatogr A 1216: 3746-3750

Hawksworth DL (2009) Separate name for fungus's sexual stage may cause confusion. Nature 458: 29

Khosravi AR, Shokri H, Ziglari T, Naeini AR, Mousavi Z, Hashemi H (2008) Outbreak of severe disseminated aspergillosis in a flock of ostrich (*Struthio camelus*). Mycoses 51: 557-559

Khoufache K, Puel O, Loiseau N, Delaforge M, Rivollet D, Coste A, Cordonnier C, Escudier E, Botterel F, Bretagne S (2007) Verruculogen associated with *Aspergillus fumigatus* hyphae and conidia modifies the electrophysiological properties of human nasal epithelial cells. BMC Microbiol 7:5

Kim EK, Maragos CM, Kendra DF (2004) Liquid chromatographic determination of Fumonisins B1, B2, and B3 in corn silage. J Agric Food Chem 52: 196-200

Klötzel M, Schmidt S, Lauber U, Thielert G, Humpf HU (2005) Comparison of different clean-up procedures for the analysis of deoxynivalenol in cereal-based food and validation of a reliable HPLC method. Chromatographia 62: 41-48

Kokkonen MK, Jestoi MN (2009) A multi-compound LC-MS/MS method for the screening of mycotoxins in grains. Food Anal Methods 2:128-140

Kosalec I, Klaric MS, Pepeljnjak S (2005) Verruculogen production in airborne and clinical isolates of *Aspergillus fumigatus* Fres. Acta Pharm 55: 357-364

Kozlovsky AG, Zhelifonova VP, Antipova TV (2009) Clavine alkaloid biosynthesis by the fungus *Penicillium palitans* Westling 1911 isolated from ancient permafrost deposits. Appl Biochem Microbiol 45: 182-186

Krotzky AJ, Zeeh B (1995) Immunoassays for residue analysis of agrochemicals: proposed guidelines for precision, standardization and quality control. Pure Appl Chem 67: 2065-2088

Krska R, Molinelli A (2007) Mycotoxin analysis: state-of-the-art and future trends. Anal Bioanal Chem 387:145-148

Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, Macdonald S, Crews C (2008) Mycotoxin analysis: an update. Food Add Contam 5: 152-163

Kwon-Chung KJ, Sugui JA (2008) What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*?. Med Mycol: 1-7

Lair-Fulleringer S, Guillot J, Desterque C, Seguin D, Warin S, Chermette R, Bretagne S (2003). Differentiation of *Aspergillus fumigatus* isolates from breeding turkeys and their environment by genotyping with microsatellite markers. J Clin Microbiol 41: 1798-1800

Latgé JP (2001) The pathobiology of *Aspergillus fumigatus*. Trends Microbiol 9: 382-389

Latif H, Curtui V, Ackermann Y, Groß M, Usleber E (2009) Production and characterization of antibodies against fumigaclavine A. Mycotox Res 25: 159-164

Lee MR (2009) The history of ergot of rye (*Claviceps purpurea*) I: from antiquity to 1900. *J R Coll Physicians Edinb* 39:179-84

Lei J, Zhou J (2002) A marine natural product database. J Chem Inf Comput Sci 42: 742-748

Lewis RE, Wiederhold NP, Chi J, Xiang YH, Komanduri KV, Kontoyiannis DP (2005) Detection of gliotoxin in experimental and human aspergillosis. Infect Immun 73: 635-637

Li SM, Unsöld IA (2006) Post-genome research on the biosynthesis of ergot alkaloids. Planta Med 72: 1117-1120

Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F (2005) Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR J 46: 258-268

Liu MT, Ram BP, Hart LP, Pestka JJ (1985) Indirect enzyme-linked immunosorbent assay for the mycotoxin zearalenone. Appl Environ Microbiol 50: 332-336

Llovera M, Balcells M, Torres M, Canela R (2005) Parallel synthesis: a new approach for developing analytical internal standards. Application to the analysis of patulin by gas chromatography-mass spectrometry. J Agric Food Chem 53: 6643-6648

Lugauskas A (2005) Potential toxin producing micromycetes on food raw material and products of plant origin. Botanica Lithuanica, Suppl 7: 3-16

Lund F (1995) Diagnostic characterization of *Penicillium palitans, P. commune,* and *P. solitum.* Lett Appl Microbiol 21: 60-64

Lyons PC, Plattner RD, Bacon CW (1986) Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. Science 232: 487-489

Ma HY, Song YC, Mao YY, Jiang JH, Tan RX, Luo L (2006) Endophytic fungal metabolite fumigaclavine C causes relaxation of isolated rat aortic rings. Planta Med 72: 387-392

Maiya S, Grundmann A, Li SM, Turner G (2006) The fumitremorgin gene custer of *Aspergillus fumigatus*: identification of a gene encoding brevianamide F synthetase. Chem Bio Chem 7: 1062-1069

Malinowski DP, Belesky DP (2006) Ecological importance of *Neotyphodium* spp. grass endophytes in agroecosystems – review and interpretation. Grassland Sci 52: 1-14

Mantle PG (1969) Interruption of early pregnancy in mice by oral administration of agroclavine and sclerotia of *Claviceps fusiformis* (Loveless). J Reprod Fert 18: 81-88

Marsh PB, Millner PD, Kla JM (1979) A guide to the recent literature on aspergillosis as caused by *Aspergillus fumigatus*, a fungus frequently found in self-heating organic matter. Mycopathologia 69: 67-81

Mayahi M, Esmaeilzadeh S, Kiani R, Fatahinia M (2008) *Aspergillus fumigatus* infection in a green parrot. Comp Clin Pathol 17: 279-281

McCrone WC (1954) Gliotoxin. Anal Chem 26: 1662-1663

Meister U (2003) Detection of citrinin in ochratoxin A-containing products by a new HPLC method. Mycotox Res 19: 27-30

Metzger U, Schall C, Zocher G, Unsöld, Stec E, Li SM, Heide L, Stehle T (2009) The structure of dimethylallyl tryptophan synthasereveals a common architecture of aromaticprenyltransferases in fungi and bacteria. PNAS 106: 14309-14314

Mitchell CG, Slight J, Donaldson K (1997) Diffusible component from the spore surface of the fungus *Aspergillus fumigatus* which inhibits the macrophage oxidative burst is distinct from gliotoxin and other hyphal toxins. Thorax 52: 796-801

Mohamed R, Gremaud E, Richoz-Payot J, Tabet JC, Guy PA (2006) Quantitative determination of five ergot alkaloids in rye flour by liquid chromatography-electrospray ionisation tandem mass spectrometry. J Chromatogr A 1114: 62-72

Müllbacher A, Eichner RD (1984) Immunosuppression in vitro by a metabolite of a human pathogenic fungus. Immunology 81: 3835-3837

Müller C, Klaffke HS, Krauthause W, Wittkowski R (2006) Determination of ergot alkaloids in rye and rye flour. Mycotox Res 22: 197-200

Muro-Cacho CA, Stedeford T, Banasik M, Suchecki TT, Persad AS (2004) Mycotoxins: mechanisms of toxicity and methods of detection for identifying exposed individuals. J Land Use 19: 537-549

Nielsen KF, Smedsgaard J (2003) Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography–UV–mass spectrometry methodology. J Chromatogr A 1002: 111-136

Nielsen KF, Sumarah MW, Frisvad JC, Miller JD (2006) Production of metaboltes from the *Penicillium roqueforti* complex. J Agric Food Chem 54: 3756-3763

Nielsen KF, Thrane U (2000) Fast methods for screening of trichothecenes in fungal cultures using GC-MS/MS. Mycotox Res 16, Suppl 2: 252-256

Nieminen SM, Kärki R, Auriola S, Toivola M, Laatsch H, Laatikainen R, Hyvärinrn A, Wright A (2002) Isolation and identification of *Aspergillus fumigatus* mycotoxins on growth media and some building materials. Appl Environ Microbiol 68:4871-4875

Nilsson P, Bergquist NR, Grundy MS (1981) A technique for preparing defined conjugates of horseradish peroxidase and immunoglobulin. J lmmunol Methods 41: 81-93

Ninomiya I, Hashimoto C, Kiguchi T, Naito T (1986) Photocyclization of enamides. XXV: Syntheses of despyrrole analogues of ergot alkaloids including elymoclavine, lysergol, isolysergol, lysergine, isolysergine, fumigaclavine, agroclavine, and lysergene. Chem Pharm Bull 34: 2799-2809

Ninomiya I, Kiguchi T, Hashimoto C, Naito T (1991) Photocyclization of enamides. XXXIII. Total syntheses of (±)-agroclavines, (±)-fumigaclavine B, and (±)-lysergene. Chem Pharm Bull 39: 23-30

Nishiyama M, Kuga T (1986) Pharmacological effects of the tremorgenic mycotoxin fumitremorgin A. Japan J Pharmacol 40: 481-489

Nishiyama M, Kuga T (1990) Central effects of the neurotropic mycotoxin fumitremorgin a in the rabbit. (II). Effects on the brain stem. Japan J Pharmacol 52: 201-208

Nonaka Y, Saito K, Hanioka N, Narimatsu S, Kataoka H (2009) Determination of aflatoxins in food samples by automated on-line in-tube solid-phase microextraction coupled with liquid chromatography mass spectrometry. J Chromatogr A 1216: 4416-4422

Numata A; Takahashi C; Matsushita T; Miyamoto T; Kawai K; Usami Y; Matsumura E; Inoue M; Ohishi H; Shingu T (1992) Fumiquinazolines, novel metabolites of a fungus isolated from a saltfish. Tetrahedron Lett 33: 1621-1624

O'Brien M, Egan D, O'Kiely P, Forristal PD, Doohan FM, Fuller HT (2008) Morphological and molecular characterisation of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. Mycol Res 112: 921-932

O'Brien M, Nielsen KF, O'Kiely P, Forristal PD, Fuller HT, Frisvad JC (2006) Mycotoxins and other secondary metabolites produced in vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. J Agric Food Chem 54: 9268-9276

O'Gorman CM, Fuller HT, Dyer PS (2009) Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature 457: 471-475

Ohmomo S, Kaneko M, Atthasampunna P (1989) Production of fumigaclavine B by a thermophilic strain of *Aspergillus fumigatus.* MIRCEN J 5: 5-13

Ohmomo S, Kitamoto HK (1994) Detection of roquefortines in *Penicillium roqueforti* isolated from moulded maize silage. J Sci Food Agric 64: 211-215

Ohmomo S, Sato K, Utagawa T, Abe M (1975) Isolation of festuclavine and three new indole alkaloids, roquefortine A, B, and C from the cultures of *Penicillium roqueforti*. Agr Biol Chem 39: 1333-1334

Olsson J, Börjesson T, Lundstedt T, Schnürer J (2002) Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. Int J Food Microbiol 72: 203-214

Onji Y, Aoki Y, Tani N, Umebayashi K, Kitada Y, Dohi Y (1998) Direct analysis of several *Fusarium* mycotoxins in cereals by capillary gas chromatography-mass spectrometry. J Chromatogr A 815: 59-65

Orciuolo E, Stanzani M, Canestraro M, Galimberti S, Carulli G, Lewis R, Petrini M, Komanduri KV (2007) Effects o*f Aspergillus fumigatus* gliotoxin and methylprednisolone on human neutrophils: implications for the pathogenesis of invasive aspergillosis. J Leukoc Biol 82: 839-848

Oveisi MR, Hajimahmoodi M, Memarian S, Sadeghi N, Shoeibi S (2005) Determination of zearalenone in corn flour and a cheese snack product using high-performance liquid chromatography with fluorescence detection. Food Add Contam 22: 443-448

Packter NM (1965) Studies on the biosynthesis of quinones in fungi - incorporation of 6-methylsalicylic acid into fumigatin and related compounds in *Aspergillus fumigatus* I.M.I. 9353. Biochem J 97: 321-332

Pahl HL, Kraub B, Schulze-Osthoff K, Decker T, Traenckner EBM, Vogt M, Myers C, Parks T, Warring P, Mühlbacher A, Czernilofsky AP, Baeuerle PA (1996) The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kB. J Exp Med 183: 1829-1840

Panaccione DG (2005) Origins and significance of ergot alkaloid diversity in fungi - mini review. FEMS Microbiol Lett 251: 9-17

Panaccione DG, Coyle CM (2005) Abundant respirable ergot alkaloids from the common airborne fungus *Aspergillus fumigatus*. Appl Environ Microbiol 71: 3106-3111

Panaccione DG, Tapper BA, Lane GA, Davies E, Fraser K (2003) Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. J Agric Food Chem 51: 6429-6437

Pattron DD (2006) Aspergillus, health implication & recommendations for public health food safety. Internet J Food Saf 8: 19-23

Pena A, Cerejo F, Lino C, Silveira I (2005) Determination of ochratoxin A in Portuguese rice samples by high performance liquid chromatography with fluorescence detection. Anal Bioanal Chem 382: 1288-1293

Perera KPWC, Day JB, Mantle PG, Rodrigues L (1982) Metabolism of verruculogen in rats. Appl Environ Microbiol 43: 503-508

Pertz H (1996) Naturally occurring clavines: antagonism/partial agonism at 5-HT_{2A} receptors and antagonism at α_1-adrenoceptors in blood vessels. Planta Med 62: 387-392

Peterson DW, Bradford HF, Mantle PG (1982) Actions of a tremorgenic mycotoxin on amino-acid transmitter release *in vivo*. Biochem Pharmacol 31: 2807-2810

Petroski RJ, Powell RG, Clay K (1992) Alkaloids of *Stipa robusta* (Sleepygrass) infected with an *Acremonium* endophyte. Nat Toxins 1: 84-88

Pettersson G (1963) The biosynthesis of fumigatin. Acta Chem Scand 17: 1323-1329

Picoul W, Bedel O, Haudrechy A, Langlois Y (2003) Progress in fumagillin synthesis. Pure Appl Chem 75: 235-249

Pittet A (2005) Modern methods and trends in mycotoxin analysis. Mitt Lebensm Hyg 96: 424-444

Porstmann B, Porstmann T, Gaede D, Nugel E, Egger E (1981) Temperature dependent rise in activity of horseradish peroxidase caused by non-ionic detergents and its use in enzyme-immunoassay. Clinica Chimica Acta 109: 175-181

Porstmann B, Porstmann T, Nugel E, Evers U (1985) Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase or beta-galactosidase?. J Immunol Methods 79: 27-37

Poulding RH (1952) Five cases of aspergillosis in immature gulls. Ibis 94: 364-366

Raistrick H (1940) Biochemistry of the lower fungi. Annu Rev Biochem 9: 571-592

Rasmussen S., Parsons AJ, Popay A, Xue H, Newman JA (2008) Plant-endophyte-herbivore interactions: more than just alkaloids?. Plant Signal Behav 3: 974-977

Rehacek Z (1980) Ergot alkaloids and their biosynthesis. Adv Biochem Eng 14: 33-60

Reinhard H, Rupp H, Zoller O (2008) Ergot alkaloids: quantitation and recognition challenges. Mycotox Res 24: 7-13

Reissig EC, Uzal FA, Schettino A, Robles CA (2002) Pulmonary aspergillosis in a great rhea (*Rhea americana*) - case report. Avian Dis 46: 754-756

Rementeria A, López-Molina N, Ludwig A, Vivanco AB, Bikandi J, Pontón J, Garaizar J (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. Rev Iberoam Micol 22: 1-23

Richard JL, DeBey MC (1995) Production of gliotoxin during the pathogenic state in turkey poults by *Aspergillus fumigatus* Fresenius. Mycopathologia 129: 111-115

Richard JL, Dvorak TJ, Ross PF (1996) Natural occurrence of gliotoxin in turkeys infected with *Aspergillus fumigatus* Fres. Mycopathologia 134: 167-170

Richard JL, LyonRL, Fichtner RE, Ross PF (1989) Use of thin layer chromatography for detection and high performance liquid chromatography for quantitating gliotoxin from rice cultures of *Aspergillus fumigatus* Fresenius. Mycopathologia 107: 145-151

Rigbers O, Li SM (2008) Ergot alkaloid biosynthesis in *Aspergillus fumigatus:* overproduction and biochemical characterization of a 4-dimethylallyltryptophan n-methyl-transferase. J Biol Chem 283: 26859-26868

Rinyu E, Varga J, Ferenczy L (1995) Phenotypic and genotypic analysis of variability in *Aspergillus fumigatus.* J Clin Microbiol 33: 2567-2575

Ronning CM, Fedorova ND, Bowyer P, Coulson R, Goldman G, Kim HS, Turner G, Wortman JR, Yu J, Anderson MJ, Denning DW, Nierman WC (2005) Genomics of *Aspergillus fumigatus* - review. Rev Iberoam Micol 22: 223-228

Rundberget T, Wilkins AL (2002) Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography–mass spectrometry. J Chromatogr A 964: 189-197

Russo MV, Veschetti E, Cinelli G, Avino P (2007) Short capillary traps in GC–GC tandem systems for direct analysis of T2 mycotoxin in aqueous samples. Chromatographia 66: 237-242

Sabater-Vilar M, Nijmeijer S, Fink-Gremmels J (2003) Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats. J Food Prot 66: 2123-2129

Sakai M, Watanuki M (1987) Inhibitory effect of gliotoxin on platelet aggregation-importance of disulfide structure. Agric Biol Chem 51: 2167-2170

Samson RA, Hong SB, Frisvad JC (2006) Old and new concepts of species differentiation in *Aspergillus*. Med Mycol 44: S133-S148

Santos VM, Dorner JW, Carreira F (2002) Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia 156: 133-138

Santos EA, Vargas EA (2002) Immunoaffinity column clean-up and thin layer chromato-graphy for determination of ochratoxin A in green coffee. Food Add Contam 19: 447-458

Schaafsma AW (1998) Analysis of *Fusarium* toxins in maize and wheat using thin layer chromatography. Mycopathologia 142: 107-113

Schiff PL (2006) Ergot and its alkaloids. Am J Pharm Educ 70, Article 98

Schmidt and Wolff (1997) Morphological characteristic of *Aspergillus fumigatus* strains isolated from patient samples. Mycoses 40: 347-351

Schneider E, Curtui V, Seidler C, Dietrich R, Usleber E, Märtlbauer E (2004) Rapid methods for deoxynivalenol and other trichothecenes. Toxicol Lett 153: 113-121

Schuhmacher R, Berthiller F, Buttinger G, Krska R (2005) Simultaneous determination of type A- & B-trichothecenes and zearalenone in cereals by high performance liquid chromatography-tandem mass spectrometry. Mycotox Res 21: 237-240

Schwadorf K, Müller HM (1991) Determination of trichothecenes in cereals by gas chromatography with ion trap detection. Chromatographia 32: 137-142

Schwarz G, Eich E (1983) Influence of ergot alkaloids on growth of *Streptomyces purpurascens* and production of its secondary metabolites. J Med Plant Res 47: 212-214

Scott PM (2007) Analysis of ergot alkaloids – a review. Mycotox Res 23: 113-121

Scott PM, Kanhere SR, Weber D (1993) Analysis of canadian and imported beers for *Fusarium* mycotoxins by gas chromatography-mass spectrometry. Food Add Contam 10: 381-389

Scott PM, Kennedy BPC (1976) Analysis of blue cheese for roquefortine and other alkaloids from *Penicillium roqueforti*. J Agric Food Chem 24: 865-868

Scott PM, Kennedy BPC, Harwig J, Blanchfield BJ (1977) Study of conditions for production of roquefortine and other metabolites of *Penicillium roqueforti*. Appl Environ Microbiol 33: 249-253

Scott PM, Merrien M-A, Polonsky J (1976) Roquefortine and isofumigaclavine A, metabolites from *Penicillium roquefortii.* Experientia 32: 140–142

Scott PM, Weber D, Kanhere SR (1997) Gas chromatography-mass spectrometry of *Alternaria* mycotoxins. J Chromatogr A 765: 255-263

Shah A (2008) *Aspergillus*-associated hypersensitivity respiratory disorders - review article. Indian J Chest Dis Allied Sci 50: 117-128

Shelby RA, Bridgman RC, Smith FT, Atigadda VR (1998) Determination of ergovaline in tall fescue by a specific monoclonal antibody. Food Agric Immunol 10: 339-347

Shelby RA, Kelley VC (1990) An immunoassay for ergotamine and related alkaloids. J Agric Food Chem 38: 1130-1134

Shelby RA, Kelley VC (1991) Detection of ergot alkaloids in tall fescue by competitive immunoassay with a monoclonal antibody. Food Agric Immunol 3: 169-177

Shelby RA, Kelley VC (1992) Detection of ergot alkaloids from *Claviceps* species in agricultural products by competitive ELISA using a monoclonal antibody. J Agric Food Chem 40: 1090-1092

Shelby RA, Rottinghaus GE, Minor HC (1994) Comparison of thin-layer chromatography and competitive immunoassay methods for detecting fumonisin on maize?. J Agric Food Chem 42: 2064-2067

Shephard GS, Sewram V (2004) Determination of the mycotoxin fumonisin B1 in maize by reversed-phase thin-layer chromatography: a collaborative study. Food Add Contam 21: 498-505

Shotwell OL, Goulden ML, Bennett GA (1976) Determination of zearalenone in corn: collaborative study. J AOAC 59: 666-670

Shreeve BJ, Patterson DS, Roberts BA, MacDonald SM (1983) Tremorgenic fungal toxins. Vet Res Commun 7: 155-160

Siegel D, Rasenko T, Koch M, Nehls I (2009) Determination of the *Alternaria* mycotoxin tenuazonic acid in cereals by high-performance liquid chromatography electrospray ionization ion-trap multistage mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine. J Chromatogr A 1216: 4582-4588

Singh KV, Kaur J, Varshney GC, Raje M, Suri CR (2004) Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. Bioconjugate Chem 15: 168-173

Smedsgaard J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760: 264-270

Snider BB, Zeng H (2003) Total synthesis of (−)-fumiquinazolines A, B, C, E, H, and I. Approaches to the synthesis of fiscalin A. J Org Chem 68: 545-563

Songsermsakul P, Sontag G, Cichna-Markl M, Zentek J, Razzazi-Fazeli E (2006) Determination of zearalenone and its metabolites in urine, plasma, and faeces of horses by HPLC–APCI–MS. J Chromatogr B 843: 252-261

Spilsbury JF, Wilkinson S (1961) The isolation of festuclavine and two new clavine alkaloids from *Aspergillus fumigatus* Fres. J Chem Soc 5: 2085-2091

Stanimirovic Z, Stevanovic J, Bajic V, Radovic I (2007) Evaluation of genotoxic effects of fumagillin by cytogenetic tests *in vivo*. Mutat Res 628: 1-10

Stanzani M, Orciuolo E, Lewis R, Kontoyiannis DP, Martins SLR, St John LS, Komanduri KV (2005) *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. Blood 105: 2258-2265

Steiner U, Ahimsa-Muüller MA, Markert A, Kucht S, Groß J, Kauf N, Kuzma M, Zych M, Lamshöft M, Furmanowa M, Knoop V, Drewke C, Leistner E (2006) Molecular characterization of a seed transmitted clavicipitaceous fungus occurring on dicotyledoneous plants (Convolvulaceae). Planta 224: 533-544

Sulyok M, Krska R, Schuhmacher R (2007) Application of a liquid chromatography–tandem mass spectrometric method to multi-mycotoxin determination in raw cereals and evaluation of matrix effects. Food Add Contam 24: 1184-1195

Sutton P, Newcombe NR, Waring P, Müllbacher A (1994) In vivo immunosuppresive activity of gliotoxin, a metabolite produced by human pathogenic fungi. Infect Immun 62: 1192-1198

Sutton P, Waring P, Mullbacher A (1996) Exacerbation of invasive aspergillosis by the immunosuppressive fungal metabolite, gliotoxin. Immunol Cell Biol 74: 318-322

Sweeney MJ, Dobson ADW (1999) Molecular biology of mycotoxin biosynthesis - mini review. FEMS Microbiol Lett 175: 149-163

Takahashi C, Matsushita T, Doi M, Minoura K, Shingu T, Kumeda T, Numata A (1995) Fumiquinazolines A-G, novel metabolites of a fungus separated from a *Pseudolabrus* marine fish. J Chem Soc Perkin Trans 1: 2345-2353

Tanaka T, Teshima R, Ikebuchi H, Sawada J, Ichinoe M (1995) Sensitive enzyme-linked immunosorbent assay for the mycotoxin zearalenone in barley and job's-tears. J Agric Food Chem 43: 946-950

Tanaka T, Yoneda A, Inoue S, Sugiura Y, Ueno Y (2000) Simultaneous determination of trichothecene mycotoxins and zearalenone in cereals by gas chromatography-mass spectrometry. J Chromatogr A 882: 23-28

Tell LA (2005) Aspergillosis in mammals and birds: impact on veterinary medicine. Med Mycol 43, Suppl 1: S71-S73

Tepšič K, Gunde-Cimerman N, Frisvad JC (1997) Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern. FEMS Microbiol Lett 157: 9-12

Tekaia F, Latgé JP (2005) *Aspergillus fumigatus*: saprophyte or pathogen?. Curr Opin Microbiol 8:385-392

Terry WD, Matthews BW, Davies DR (1968) Crystallographic studies of a human immunoglobulin. Nature 220: 239-241

Thimm N, Handl J, Binder M (2001) HPLC-MS method for A-trichothecenes. Mycotox Res 17, Suppl 2: 2006-209

Thirumala-Devi K, Mayo MA, Delfosse P, Reddy G, Reddy SV, Reddy DVR (2000) Production of polyclonal antibodies for ochratoxin A and its detection in chilies by ELISA. J Agric Food Chem 48: 5079-5082

Thirumala-Devi K, Mayo MA, Hall AJ, Craufurd PQ, Wheeler TR, Waliyar F, Subrahmanyam A, Reddy DVR (2002) Development and application of an competitive indirect enzyme-linked immunoassay for aflatoxin M1 in milk and milk-based confectionery. J Agric Food Chem 50: 933-937

Tomee JF, Kauffman HF (2000) Putative virulence factors of *Aspergillus fumigatus* - review. Clin Exp Allergy 30: 476-84

Toscani T, Moseriti A, Dossena A, Dall'Asta C, Simoncini N, Virgili R (2007) Determination of ochratoxin A in dry-cured meat products by a HPLC–FLD quantitative method. J Chromatogr B 855: 242-248

Tschen SMJ, Chen LL, Hsieh ST, Wu TS (1997) Isolation and phytotoxic effects of helvolic acid from plant pathogenic fungus *Sarocladium oryzae*. Bot Bull Acad Sin 38: 251-256

Tudzynski P, Correia T, Keller U (2001) Biotechnology and genetics of ergot alkaloids: mini review. Appl Microbiol Biotechnol 57: 593-605

Turner NW, Subrahmanyam S, Piletsky SA (2009) Analytical methods for determination of mycotoxins: A review. Analytica Chimica Acta 632: 168-180

Usleber E, Renz V, Märtlbauer E, Terplan G (1992) Studies on the application of enzyme immunoassays for the *Fusarium* mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone. J Vet Med B 39: 617-627

Usleber E, Straka M, Terplan G (1994) Enzyme immunoassay for fumonisin B1 applied to corn-based food. J Agric Food Chem 42: 1392-1396

Van Egmond HP, Paulsch WE (1986) Determination of mycotoxins. Pure Appl Chem 58: 315-326

Vesonder RF, Rohwedder WK (1986) Gas chromatographicmass spectrometric analysis of mycotoxins. In: Cole RJ, ed. Modern methods in the analysis and structural elucidation of mycotoxins, Orlando, Florida: 335-351

Vinokurova NG, Boichenko DM, Baskunov BP, Zelenkova NF, Vepritskaya IG, Arinbasarov MU, Reshetilova TA (2001) Minor alkaloids of the fungus *Penicillium roquefortii* Thom 1906. Appl Biochem Microbiol 37: 184-187

Vinokurova NG, Khmel'nitskaya II, Baskunov BP, Arinbasarov MU (2003a) Occurrence of indole alkaloids among secondary metabolites of soil *Aspergillus* Spp. Appl Biochem Microbiol 39: 192-196

Vinokurova NG, Boichenko LV, Arinbasarov MU (2003b) Production of alkaloids by fungi of the genus *Penicillium* grown on wheat grain. Appl Biochem Microbiol 39: 403-406

Vinokurova NG, Ozerskaya SM, Baskunov BP, Arinbasarov MU (2003c) The *Penicillium commune* Thom and *Penicillium clavigerum* Demelius fungi producing fumigaclavines A and B. Microbiology 72: 149-151

Vorobyova V, Yurkov I, Belova N, Lednev V (2009) Agroclavine potentiates hippocampal EEG effects of weak combined magnetic field in rats. Brain Res Bull 80: 1-8

Vudathala DK, Prelusky DB, Trenholm HL (1994) Analysis of trace levels of deoxynivalenol in cows milk by high pressure liquid chromatography. J Liq Chromatogr 17: 673-683

Waksman SA, Geiger WB (1944) The nature of the antibiotic substances produced by *Aspergillus fumigatus*. J Bacteriol 47: 391-397

Wang J, Machado C, Panaccione DG, Tsai HT, Schardla CL (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genet Biol 41: 189-198

Waring P (1990) DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol triphosphate levels. J Biol Chem 265: 14476-14460

Waring P, Beaver J (1996) Gliotoxin and related epipolythiodioxopiperazines. Gen Pharmac 28: 1311-1316

Watanabe A, Kamei K, Sekine T, Waku M, Nishimura K, Miyaji M, Kuriyama T (2003) Immunosuppressive substances in *Aspergillus fumigatus* culture filtrate. J Infect Chemother 9: 114-121

Welke JE, Hoeltz M, Dottori HA, Noll IB (2009) Quantitative analysis of patulin in apple juice by thin-layer chromatography using a charge coupled device detector. Food Add Contam 26: 754-758

Wong SS (1991) Chemistry of protein conjugation and cross linking. CRC Press, Boca Raton

Wu XF, Fei MJ, Shu RG, Tan RX, Xu Q (2005) Fumigaclavine C, an fungal metabolite, improves experimental colitis in mice via downregulating Th1 cytokine production and matrix metalloproteinase activity. Int Immunopharmacol 5: 1543-1553

Xavier MO, Soares MP, Meinerz ARM, Nobre MO, Osório LG, Filho RPS, Meireles MCA (2007) Aspergillosis: a limiting factor during recovery of captive magellanic Penguins. Brazil J Microbiol 38: 480-484

Yamaguchi H,·Harada A (2003) Antibody dendrimers. Top Curr Chem 228: 237-258

Yamazaki M, Fujimoto H, Kawasaki T (1980a) Chemistry of tremorgenic metabolites. I. Fumitremorgin A from *Aspergillus fumigatus*. Chem Pharm Bull 28: 245-254

Yamazaki M, Suzuki K, Fujimoto H, Sankawa U, Iitaka Y (1980b) Chemistry of tremorgenic metabolites. II. Structure determination of fumitremorgen B, a tremorgenic metabolite from *Aspergillus fumigatus*. Chem Pharm Bull 28: 861-865

Yamazaki M, Suzuki S, Kukita K (1979) Neurotoxical studies on fumitremorgin A, a tremorgenic mycotoxin, on mice. J Pharm Dyn 2: 119-125

Yamazaki M, Suzuki S, Miyaki K (1971) Tremorgenic toxins from *Aspergillus fumigatus* FRES. Chem Pharm Bull 19: 1739-1740

Yentür G, Er B, Özkan MG,·Öktem AB (2006) Determination of aflatoxins in peanut butter and sesame samples using high-performance liquid chromatography method. Eur Food Res Technol 224: 167-170

Yeung JM, Prelusky DB, Savard ME, Dang BDM, Robinson LA (1996) Sensitive immunoassay for fumonisin B1 in corn. J Agric Food Chem 44: 3582-3586

Yolken RH, Leister FJ (1981) Investigation of enzyme immunoassay time courses: development of rapid assay systems. J Clin Microbiol 13: 738-741

Zafra R, Pérez J, Préz-Écija RA, Borge C, Bustamante R, Carbonero A, Tarradas C (2008) Concurrent aspergillosis and ascites with high mortality in a farm of growing broiler chickens-case report. Avian Dis 52: 711-713

Zhang X, Cudjoe E, Vuckovic D, Pawliszyn J (2009) Direct monitoring of ochratoxin A in cheese with solid-phase microextraction coupled to liquid chromatography-tandem mass spectrometry. J Chromatogr A 1216: 7505-7509

Zhao Y, Liu J, Wang J, Wang L, Yin H, Tan R (2004) Fumigaclavine C improves concanavalin a-induced liver injury in mice mainly via inhibiting TNF-α production and lymphocyte adhesion to extracellular matrices. J Pharm Pharmacol 56: 775-782

Zheng MZ, Richard JL, Binder J (2006) A review of rapid methods for the analysis of mycotoxins. Mycopathologia 161: 261-273

Zitomer NC, Glenn AE, Bacon CW, Riley RT (2008) A single extraction method for the analysis by liquid chromatography/tandem mass spectrometry of fumonisins and biomarkers of disrupted sphingolipid metabolism in tissues of maize seedlings. Anal Bioanal Chem 391: 2257-2263

Zmeili OS, Soubani AO (2007) Pulmonary aspergillosis: a clinical update - review. Q J Med 100: 317-334

9 Abbreviations

ABPA	allergic bronchopulmonary aspergillosis
Abs.	absorption
BSA	bovine serum albumine
CNA	chronic necrotizing aspergillosis
CR	cross-reactivity
CRC	cross-reacting coumpound
^{o}C	degree Celsius
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC_{50}	50% effective concentration
ED_{50}	50% effective dose
ECD	electron capture detection
e.g.	*exempli gratia* (for example)
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme-multiplied immunoassay technique
et al.	*et alii* (and others)
EtOH	ethanol
ETP	epipolythiodioxopiperazine
FID	flame ionisation detection
FLD	flourecence detection
FuA	fumigaclavine A
FuB	fumigaclavine B
FuC	fumigaclavine C
FuD	fumigaclavine D
FQ	fumiquinazolines
GC	gas chromatography
HNEC	human nasal epithelial cells
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IC_{50}	50% inhibition concentration

IgG	immunoglobulin G
IPA	invasive pulmonary aspergillosis
IsoFuA	isofumigaclavine A
IsoFuB	isofumigaclavine B
IsoFuC	isofumigaclavine C
KLH	keyhole limpet hemocyanin
LC	liquid chromatography
LD_{50}	50% lethal dose
MEA	malt extract agar
MeOH	methanol
µg	microgram
mm	millimeter
ml	milliliter
MS	mass spectrometry
MS/MS	with tandem mass spectrometry
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa B
ng	nanogram
PBS	phosphate-buffered saline
pg	picogram
Rf	retention factor
RIA	radioimmunoassay
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor-alpha
TMB	3,3´, 5,5´-tetramethyl-benzidine
TNBS	2,4,6-trinitrobenzene sulfonic acid
UV	ultraviolet
v/v	volume per volume

Acknowledgements

I would like to thank the *Deutscher Akademischer Austausch Dienst* (DAAD) for supporting me with a doctoral scholarship. I am most grateful to my supervisor, Prof. Dr. Dr. habil. E. Usleber for giving me the opportunity to do the research for my 'Doktorarbeit' in his institute, and for his supervision, support, encouragement and invaluable suggestions in every steps of this research. I thank Dr. V. Curtui for giving me many inputs and directions in doing this research, especially during the initial stages of my work. I wish to give my gratitude to Prof. Dr. Mirnawati B. Sudarwanto, who introduced me to my supervisor.

I wish to thank Prof. Dr. E. Märtlbauer (Institute for Hygiene and Technology of Food of Animal Origin, Veterinary Faculty, Ludwig-Maximilians-University) for his recommendations to extend my scholarship. Many thanks to Dr. Peter Scott (Ottawa, Canada) for supplying the standard of isofumigaclavine A. I would like to acknowledge and thank to Prof. Dr. M. Lierz and his coworkers Dr. M. R. Hampel and TA N. Lorenz at the Clinic for Birds, Reptiles, Amphibians, and Fish, Justus Liebig University for providing tissue samples of birds with aspergillosis. My sincere thanks go to all staffs and members of the Chair of Diary Sciences, Institute of Veterinary Food Science, Veterinary Faculty, Justus-Liebig University, Giessen, especially Cornelia Eichmann, Christa Zeidler, Yvonne Ackermann, Madeleine Groß, Dr. Agnes Wescher, Renate Stumpf, Margit Keßler, and Karin Müller for their kindness and for all the kind help I have received.

All my deep gratitude to my dear mother, who always give me moral supports and encouragement from home. Special thanks to my beloved wife, Puji Rahayu, and my dear son, Engghar Fauzan Latif who have given me lots of supports, joys, and encouragement through out the time, who always shared the life around and outside the 'laboratorium world' with me and make survival and busy live in Giessen easier. I would also like to give my thanks to all the people, whom I might not have mentioned, for their friendships, supports, and helps in many different ways and for making the last three years in Germany an unforgettable experience.

édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-5547-9