Mycotoxins in Vietnamese Pig Feeds

Contamination, Excretion in Pig Urine and Reduction of Aflatoxins by Adsorbents

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Abstract

The objectives of the study were to evaluate the mycotoxin contamination in feedstuffs and complete feeds for pigs, the capacity of local adsorbents to reduce the adverse effects of aflatoxin B_1 in piglets and methods for screening mycotoxins and their metabolites in pig urine as biomarkers for mycotoxin exposure. High incidences of aflatoxins (AF) and zearalenone (ZEA) in feedstuffs and pig feeds (83.3% - 100%) were detected. Aflatoxin B_1 was found in 100, 92, 92, 83, 100 and 96%, respectively, of samples of cassava chip, maize, rice bran, broken rice, and complete feeds for growing pigs and sows, with mean concentrations of 0.86, 77.5, 1.3, 1.6, 4.7 and 7.5 μg/kg, respectively. Of these, ZEA was detected in 8%, 33%, 36%, 21%, 67% and 67%, with average concentrations of 10.0, 163.5, 83.1, 31.4, 86.0 and 101.4 μg/kg, respectively. *In vitro* trial showed that the locally produced bentonite showed an ability to adsorb AFB_1 at 1.54 mg/g with the adsorption capacity of 12.7 mg/g. Feeding piglets an aflatoxin contaminated diet gave lower overall average daily weight gain, feed conversion efficiency, albumin and total protein compared to those given an aflatoxin free diet, while mean serum leukocyte and enzyme activities showed significant increases. The inclusion of bentonite at 0.4% or 0.5% in the aflatoxin contaminated diet restored the performance, feed efficiency and abnormal blood profiles of the piglets given AF. The urine analytical methods developed showed high sensitivity for screening ZEA, deoxynivalenol (DON), AF and their metabolites in pig urine with detection limits that ranged from 0.28 to 9.9 ng/ml. The recoveries of ZEA and DON and their metabolites ranged from 86% to 106% and those of AF and its metabolites ranged from 83 to 130%. Urinary excretion of AFB_1 in the feeding trial was 30% of the fed dose and the fraction of AFB_1 converted to AFM_1 in pig urine was 22%. The results from the survey of ZEA, DON and AF and their metabolites in pig urine samples collected from pig farms revealed that the pigs were exposed to ZEA, DON and AF contaminated diets. The toxins and metabolites for ZEA, DON and AF were found in 47, 73, and 80%, respectively, of the 15 urine samples analyzed.

Keywords: Aflatoxins, adsorbent, bentonite, urinary biomarker, deoxynivalenol, metabolite, pig feeds, pig urine, zearalenone, Vietnam.

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Dedication

To my parents
My wife Kim Huong
My daughters Dan Khanh, Van Khanh and Bao Khanh

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Thieu, N. Q., Ogle, B., Pettersson, H. (2008). Screening of aflatoxins and zearalenone in feedstuffs and complete feeds for pigs in southern Vietnam. *Tropical Animal Health and Production* 40(1), 77-83.

II Thieu, N. Q., Pettersson, H. (2008). *In vitro* evaluation of the capacity of zeolite and bentonite to adsorb aflatoxin B_1 in simulated gastrointestinal fluids. Accepted by *Mycotoxin Research* 24(3), X-Y.

III Thieu, N. Q., Ogle, B., Pettersson, H. (2008). Efficacy of bentonite clay in ameliorating aflatoxicosis in piglets fed aflatoxins contaminated diets. *Tropical Animal Health and Production* 40(8), 649-656.

IV Thieu, N. Q., Pettersson, H. (2008). Zearalenone, Deoxynivalenol and Aflatoxin B_1 and their metabolites in pig urine as biomarkers for mycotoxin exposure (manuscript submitted to *Mycotoxin Research*).

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Abbreviations

αZOL	Alpha zearalenol
βZOL	Beta zearalenol
ADG	Average daily gain
AF	Aflatoxins
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
ALB	Albumin
ALP	Alkaline phosphate
BLOB	Globulin
DeepoxyDON	De-epoxy deoxynivalenol
DON	Deoxynivalenol
ELISA	Enzyme-Linked Immunosorbent Assay
GGT	Gamma glutamyltransferase
GOT	Glutamic-oxalacetic tranaminase
HPLC	High Performance Liquid Chromatography
HSCAS	Hydrated sodium calcium aluminosilicate
IAC	Immunoaffinity column
LDH	Lactic dehydrogenase
SGFs	Simulated gastrointestinal fluids
TP	Total protein
ZEA	Zearalenone

1 Introduction

Vietnam is a tropical country and the climate is tropical monsoonal, hot and humid. These climate conditions are favourable for year-round agriculture but also create ideal environments for molds to develop and produce mycotoxins in agricultural products (Aidoo, 1993; CAST, 2003). Mycotoxins, which are secondary metabolites synthesized by a diverse number of fungal species, are toxic compounds that are known to produce a wide range of injurious effects on the health of humans and animals after acute or chronic exposure (Pier, 1981; Marquardt, 1996; Peraica *et al.,* 1999; CAST, 2003). Mycotoxins can be formed in the field during the growing season and increase during harvest, drying, storage or in transport and processing (Mirocha and Christensen, 1974; Scudamore, 1993).

The most well-known among the mycotoxins are aflatoxins (AF), which are usually the most predominant in foods and feeds in Southeast Asia (with respect to potency of toxicity, carcinogenicity and mutagenicity in humans and animals) (Eaton and Gallagher, 1994; Lopez *et al.,* 2002; Chin and Tan, 2006; Tan, 2008). The chronic effects at low dose exposure of AF consist of a number of adverse effects such as reduced growth rate and feed efficiency, decreased liver and kidney function, and suppression of the immune system, and can result in severe illnesses (Miller *et al.,* 1981; Sharma, 1993; Quezada *et al.,* 2000; Shivachandra *et al.,* 2003). Deoxynivalenol (DON), one of the trichothecene mycotoxins and commonly known as 'vomitoxin', is often considered to be responsible for reduced feed consumption or even feed refusal in animals when present in the diet (Arnold *et al.,* 1986; Bergsjö *et al.,* 1993 and Pestka, 2007). In addition, DON is also responsible for decreased body weight gain and reduced feed utilization efficiency and decreased concentrations of serum protein (Bergsjö *et al.,* 1993). The *Fusarium* mycotoxin zearalenone (ZEA) is known as a non-steroidal estrogenic mycotoxin that causes adverse effects on the reproductive system

of several animals, especially pigs (Farnworth and Trenholm 1983; D'Mello *et al.* 1999 and Zinedine *et al.* 2007). Pigs are found to be sensitive to mycotoxins, especially at nursing or nursery age. In general, mycotoxins cause reductions in feed intake, growth performance, reproductive performance and immune function of pigs even when the levels are relatively low (Miller *et al.,* 1981; Young *et al.,* 1990; Green *et al.,* 1990; Lindemann *et al.,* 1993; Marin *et al.,* 2002; Eriksen and Pettersson, 2004). Unfortunately, the contamination of foodstuffs and feedstuffs by mycotoxins is a worldwide problem and seems to be unavoidable, even though a considerable number of field and preservation studies have been done to prevent mycotoxins.

In Vietnam, the problem seems to be more serious, because most agricultural products such as rice (35.8 million tons per year), cassava (7.7 million tons per year), maize (3.8 million tons per year), soybean (258 thousand tons per year) and peanut (463 thousand tons per year) are harvested during or at the end of the wet season (Statistical Handbook of Vietnam, 2007). This situation, together with a lack of advanced post-harvesting techniques and equipment to preserve the harvest, results in delays in the drying of large quantities of agricultural products and consequently more favourable conditions are created for mycotoxins contamination (Wang *et al.,* 1995; Trung *et al.,* 2001). This was also demonstrated in a survey by Mien *et al.* (2003), who found 139 fungal species present in ingredients and complete feeds, of which 35 species have potential capacity to produce mycotoxins in high concentrations. The fungus *Aspergillus flavus* appeared in almost all feeds and ingredients.

However, these are the only studies that have been published about mycotoxin contamination in feeds and feedstuffs in Vietnam, and may not fully reflect the context of mycotoxin contamination in agricultural products of Vietnam. In addition, their impact on animal health, economic loss and the most effective way for detoxification of these mycotoxins have not been studied.

In the Vietnam context, a better comprehensive overview of all aspects of mycotoxin contamination in agricultural products and optimum methods to detoxify these mycotoxins in animal diets and to diminish their adverse effects on pig production, will have positive effects, not only on the performance of animals and production economics but also on human health.

For those reasons, the objectives of the study were:

- To determine the situation concerning mycotoxin contamination in feedstuffs and feeds for pigs in southern Vietnam, where a large of amount of feedstuffs and feeds are produced and consumed each year.
- To evaluate the capacity of locally produced adsorbents to adsorb aflatoxin B_1 in *in vitro* and *in vivo* conditions and counteract the toxic effects.
- To find appropriate analytical methods for measurement of AF, ZEA and DON and their metabolites in urine of pigs as biomarker for their exposure, and to determine the relationship between urinary biomarker of AFB_1 and levels in the feed of pigs.

2 Background

2.1 Occurrence of mycotoxins in feedstuffs and feeds

Mycotoxins include several groups of toxic secondary metabolites produced by a diverse number of fungal species, when colonizing agricultural products intended for human and animal consumption (CAST, 2003). A wide range of agricultural products such as rice, maize, wheat, barley, rye, cassava, soybean meal, peanut and cottonseed meal are commonly found to be contaminated by mycotoxins. Besides the considerable impact on animals and humans health, the frequent incidence of these mycotoxins in agricultural commodities has also a potential negative impact on the economies of the affected regions. FAO has estimated that as much as 25% of the world's food is contaminated with known mycotoxins (CAST, 2003). In the USA, Vardon *et al.* (2003) calculated that the potential annual cost of mycotoxin contamination of crops is estimated to range from $418 million to $1.66 billion. In addition, mitigation cost and livestock losses could add another $466 million and $6 million, respectively, to the mean simulated costs.

Since aflatoxins were discovered in 1960, many other mycotoxins have also been identified. However only some mycotoxins were found to occur significantly in naturally contaminated feeds and foods: aflatoxins, ochratoxin A, fumonisins, patulin, zearalenone, trichothecenes, citrinin and penicillic acid (Jelinek *et al.,* 1989). These mycotoxins are mainly produced by the fungi genera of Aspergillus, Fusarium and Penicillia (Mirocha and Christensen, 1974). The formation of mycotoxins in nature, according to CAST (2003), is in the field during the growing season, harvesting, drying, storage, transport and even when processing. However, natural formation of mycotoxins in certain geographical regions of the world is different,

depending on the environmental conditions (Some mycotoxins are produced more readily than others). In colder and more temperate regions like North America and Europe, the contamination of fusarium toxins such as deoxynivalenol, nivalenol, T-2, HT-2, zearalenone and ochratoxin A are a major problem (Snijders, 1990; Tanaka *et al.,* 1990; Placinta *et al.,* 1999; Scudamore and Patel, 2000; Jaimez *et al.,* 2004; Binder *et al.,* 2007). In tropical regions with warm and humid climate conditions like those existing in Southeast Asian, Latin American and African countries, the contamination of aflatoxins and fumonisins in agricultural commodities is commonly observed (Devegowda *et al.,* 1998). However, during certain seasons in these regions, natural occurrence of mycotoxins in foods and feeds might be different. ZEA, DON, fumonisin B_1 and ochratoxin A have also been detected in Southeast Asian countries, where AF is considered to be the major problem (Wang *et al.,* 1995; Yamashita *et al.,* 1995; Tan, 2008). Today, due to the globalization of food and feed trade, the occurrence of any type of mycotoxin contamination is likely to be observed in every region of the world (Binder *et al.,* 2007).

2.1.1 Aflatoxins

Aflatoxins (AF) are toxic secondary metabolites produced by *Aspergillus* species, of which the most well-known are *Apergillus flavus* and *A. parasiticus*. It is known that *Aspergillus* species are widely distributed throughout the world. However the most favourable regions might be where high temperature and high humid conditions exist, as in the tropical regions (Aidoo, 1993; Devegowda *et al.,* 1998; CAST, 2003). There are four naturally occurring AFs: aflatoxin B_1 (AFB_1), B_2, G_1 and G_2, and all of them are toxic, mutagenic and carcinogenic compounds (CAST, 2003). Another aflatoxin is aflatoxin M_1, which is a metabolite of AFB_1. It occurs commonly in milk and is increasingly of concern as it can be a serious health hazard for children. AFB_1 is usually the most predominant in foods and feeds and the most toxic, as well as the most potent hepatocarcinogen known in experimental animals and humans (Newberne and Butler, 1969; Eaton and Gallagher, 1994; Lopez *et al.,* 2002). Many agricultural commodities are liable to infestation by aflatoxingenic fungi, and thereby contamination with aflatoxins. Among agricultural commodities, peanuts are found to have highest incidence of aflatoxins, as reviewed by Strange (1991). The reason why the incidence is more frequent in peanuts than in other agricultural commodities is not fully understood. However, it could be due to the fact that *A. flavus* dominates the mycoflora of peanut field soils, where the kernels develop and mature beneath the surface (Diener *et al.,* 1982). Other

agricultural commodities such as maize, rice and tree nuts are also found to be highly contaminated by aflatoxins. In Southeast Asian countries, AF occurrence in maize and rice/bran has been reported to have high incidence, especially in rice from the Philippines and Vietnam (Lipigorngoson *et al.,* 2003; Sales and Yoshizawa, 2005; Nguyen *et al.,* 2007). Recently, Binder *et al.* (2007) reported the results of a 2-year survey for mycotoxins in commodities, feeds and feed ingredients. The results showed that AFB_1 was present in most agricultural commodities and it was quite high in animal feed, not only in Asian and Pacific countries, but also in European and Mediterranean countries (possibly mainly from imported commodities). However, the AFB_1 concentration levels in agricultural commodities are lower in European (EC) countries than in Asian and Pacific countries. This might be a result of the stringent mycotoxin standard for exported/imported foods and feeds of EC countries. This means that developing countries are likely to export their best quality products, while keeping contaminated products domestically, which results in higher risk of aflatoxin exposure in those countries.

2.1.2 Zearalenone

Zearalenone, a β-resorcyclic acid lactone compound is also known to be produced by *Fusarium* fungi, primarily by *Fusarium graminearum*. However, this non-steroidal estrogenic toxin can also be produced by several other species, such as *F. culmorum, F. cerealis, F. equiseti and F. semitectum* (CAST, 2003). Zearalenone is characterized by its oestrogenic properties which result from the competitive binding of ZEA to estrogenic receptors (receptors for estradiol-17β) located in the uterus, liver, mammary gland and hypothalamus. It stimulates the target tissues and an estrogenic response is produced which can adversely affect normal reproductive function (Biehl *et al.,* 1993; Dänicke *et al.,* 2005b; Malekinejad *et al.,* 2006). The natural occurrences of ZEA in agricultural commodities have been reviewed by Yoshizawa (1991) and Zinedine *et al.* (2007). Among agricultural crops, maize has been the cereal grain most often contaminated with ZEA and, as a result, it is implicated in cases of hyperestrogenism in farm animals (Shotwell, 1991). ZEA has been reported in maize and poultry feed in American countries such as Argentina, Brazil and the USA (Yoshizawa, 1991; Resnik *et al.,* 1996; Silva and Vargas, 2001; Dalcero *et al.,* 1998). A similar situation was also observed in Europe by Veldman *et al.* (1992), Vrabcheva *et al.* (1996), Scudamore and Patel, (2000) and Jaimez *et al.* (2004). In Southeast Asia, ZEA has been detected in Indonesian maize-based food and feed (Nuryono *et al.,* 2005). Other cereal crops such as rice and

rice bran, wheat and wheat bran, soybean meal, maize gluten meal and finished feed have also found to be contaminated with ZEA (Chin and Tan, 2006; Tan, 2008). Many researchers have shown that ZEA often naturally co-occurs with other mycotoxins such as trichothecenes, AF, ochratoxin A and fumonisins (Tanaka *et al.,* 1990; Luo *et al.,* 1990; Yoshizawa, 1991; Park *et al.,* 1996; Scudamore and Patel, 2000; Vargas *et al.,* 2001; Binder *et al.,* 2007). The simultaneous occurrence of ZEA with DON has often been reported, as would be expected since both are produced by *Fusarium graminearum*. However, the co-occurrence of AF and ZEA is different, because aflatoxin is produced by an unrelated group of fungi, e.g. *Aspergillus flavus* and *A. parasiticus*. Therefore, the simultaneous occurrence of AF with ZEA or DON and NIV implies that the contaminated cereals were infected by at least two different fungi, possibly at different stages, in the field and/or during storage.

2.1.3 Trichothecenes

Trichothecene mycotoxins are a group of more than a hundred compounds produced by different fungi, but mainly from the *Fusarium* genus. The *Fusarium* fungi are known to occur throughout the world, especially in cooler regions, and colonize a wide range of agricultural crops, mainly cereals and commodities. The trichothecenes are chemically characterized by the presence of a ring structure with a 12, 13 epoxide and a variable number of hydroxyl or acetoxy groups in their structures. The 12, 13 epoxide ring is considered to be essential for the toxicity of the trichothecenes. Most of trichothecenes also have C-9, 10 double bonds, which is also important for the toxicity (Ehrlich and Daigle, 1987). The trichothecenes are often subdivided into four basic groups, with type A and B being the most important. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS). Type B trichothecenes consist of deoxynivalenol (DON) also known as vomitoxin, nivalenol (NIV) and fusarenon-X. Among the trichothecene mycotoxins, deoxynivalenol, and nivalenol are most commonly found in cereals such as wheat, rye, oat, maize and barley worldwide (Tanaka *et al.,* 1990; Placinta *et al.,* 1999; JECFA, 2001; Binder *et al.,* 2007; Driehuis *et al.,* 2008). The contamination of cereals, particularly maize and wheat, by DON is a serious problem in the USA and Canada. Animal health and reduced growth problems, involving the reproductive system, feed refusal, reduced weight gain, emesis and diarrhea, have been observed mainly in pigs fed on DON and ZEA contaminated maize and wheat (Trenholm *et al.,* 1981; Yoshizawa, 1991). Oats and barley are often found to be contaminated with T-2 and HT-2

toxins, mainly from Europe (Muller *et al.,* 1997; Binder *et al.,* 2007). In Southeast Asia, where the major contaminant is aflatoxins, trichothecenes have also been detected in maize, soybean meal, wheat, rice, maize gluten meal and finished feed (Chin and Tan, 2006). Recently, this situation was confirmed by Tan (2008), who also found DON in cereal crops and finished feed in Indonesia, Malaysia, Philippines, Thailand and Vietnam, but T2-toxin was not detected.

2.2 Determination of mycotoxins in feeds and feedstuffs

The identification and quantification of mycotoxins in feedstuffs and feeds are often based on the polarity, ultraviolet absorption, fluorescence, and ionic natural properties of mycotoxins. Today, many methods are available for determination of mycotoxins in agricultural commodities. These methods, in general, often consist of five major steps: sampling, sample preparation, extraction, clean-up and measurement of the toxins using chromatography. Each step requires a clear procedure and appropriate equipment to make the results meaningful (Langseth and Rundberget, 1998; CAST, 2003; Whitaker, 2004; Krska and Welzig, 2006; Pascale and Visconti, 2006).

Using solid–phase extraction (SPE) columns to cleanup mycotoxins has been applied for a long time. SPE is usually porous silica with the surface modified to provide selective adsorption of either the analyte or impurities. In principle, the analyte is retained on the column while the impurities pass through and are washed off. The analyte is then eluted by a stronger solvent. Today, many kinds of SPE are commercially available and cleanup should follow the recommendation of producers or method.

In recent years, the trend in the cleanup of mycotoxins is to use immunoaffinity columns (IAC) for cleanup of specific mycotoxins. In general, the IAC contain specific antibodies against the mycotoxins, which are attached to an inert support material and selectively bind the mycotoxins from the crude extract when passed through. The sample impurities are then washed off by water or phosphate buffer, and finally the analyte will be eluted by an organic solvent. Several studies have demonstrated that the IAC are very specific and efficiently remove sample impurities (Visconti and Pascale, 1998; Garcia-Villanova *et al.,* 2004; Castegnaro *et al.,* 2006; Pascale and Visconti, 2001, 2006). Therefore, several methods for various mycotoxins in a number of agricultural commodities using HPLC with IAC cleanup have been validated by collaborative studies. They have afterward been adopted as official or standard methods by AOAC International or the

European Committee for standardization. Today, IAC are commercially available for many individual mycotoxins such as AF, DON, HT-2 toxin, T-2 toxin, ZEA, ochratoxin A and B, moniliformin and fumonisins. Recently, IAC have also become commercially available in one cleanup run for multi-mycotoxins like AF and ochratoxin A; AF, ochratoxin A and ZEA; and AF and ZEA. However, mostly studies by the producer have up to now validated these columns.

The final step in analytical procedure is identification and quantification of analyte if present in the samples. This step is often performed with a chromatographic separation followed by suitable detection and quantification. In recent years, HPLC and GC methods are often chosen to detect and quantify mycotoxins in agricultural commodities. HPLC, coupled with fluorescence and/or UV detection is the most widely used method. It has been used in methods for many mycotoxins, such as aflatoxins, trichothecenes, zearalenone, ochratoxin A and fumonisins (Visconti and Pascal, 1998; Valenta, 1998; Valenta *et al.,* 2003; Stroka *et al.,* 2003; Garcia-Villanova *et al.,* 2004; Ip and Che, 2006).

Recently, GC and/or HPLC with mass spectrometry (MS) have successfully been used for quantification of multimycotoxins in a range of matrices such as foods, grain crops and body fluids in one LC-MS run (Razzazi-Fazeli *et al.,* 2003; Sforza *et al.,* 2006; Biselli and Hummert, 2005; Zöllner *et al.,* 1999, 2002, Ventura *et al.,* 2006). Actually, this multimycotoxin method has been developed to included aflatoxins, ochratoxin A, DON, NIV, fusarenon-X, fumonisins, ZEA and derivatives, T-2 and HT-2 and some other mycotoxins (Spanjer *et al.,* 2006).

Enzyme-linked immunosorbent assay (ELISA) is a rapid methodology also considered in recent years as suitable for screening mycotoxins in large number of samples due to its simplicity and specificity (Chu, 1984; Trucksess *et al.,* 1995; Park and Chu, 1996). This methodology is of particular interest for the screening of raw materials and for small laboratories which do not own chromatographic equipment, and it is also particularly suitable for feed mills. Additionally, the cleanup step is usually not necessary although the sensitivity is increased with suitable purification treatment. However, there are some disadvantages of immunoassay methods, as due to the simplification, they are usually of lower sensitivity and are also prone to cross-reactivity with related compounds (Zheng *et al.,* 2006; Krska and Welzig, 2006).

2.3 Detoxification of aflatoxins by using adsorbents

For several decades, research on the decontamination and detoxification of mycotoxins in agricultural products has been carried out to find appropriate methods which can be applied under practical conditions. However, methods for complete detoxification of mycotoxin contaminated commodities on a large-scale and under practical and cost-effective conditions are currently not available. An exception may be the case of cottonseed and peanut meal treated with ammonia (Gardner *et al.,* 1971; Park, 1993).

In most cases of mycotoxin contamination, mixing contaminated crops with batches of good quality materials leads to lower concentration of both moulds and toxins in the feeds and it is often applied at farm level (Charmley and Prelusky, 1994). However, since 1 January 1999, this practice has been banned in the EU countries (Karlovsky, 1999). In general, decontamination and detoxification of mycotoxins can be achieved by removal or elimination of the more contaminated fractions or by the inactivation of toxins present in the agricultural commodities through various physical, chemical and biological methods.

In recent years, using feed additives to adsorb mycotoxins and reduce toxic effects on farm animals has attracted interest, due to the fact that it is easy to perform in practice. Of the feed additive products, the largest group is adsorbents, with only one property: to adsorb mycotoxins in the intestinal tract and excrete them with faeces. Today, many feed additives are commercially available on the market, such as MycoSorb, NovaSil, MycofixPlus, Ergomix and Klinofeed, and are used in many countries.

Early in 1978, Masimango *et al.* (1978) screened a range of clays for adsorbing AFB_1 in a buffer solution, and the results showed high degree of adsorption by some clays, such as bentonite and montmorillonite. The clays originate from weathering of volcanic ash and zeolite, bentonite, montmorrillonite and sepiolite have been chosen due to their adsorptive properties and more importantly because they are available in most countries and are inexpensive. Today, various clays have been shown to have the ability to adsorb AFB_1 in *in vitro* studies (Phillips *et al.,* 1988; Ramos and Hernandez, 1996; Grant and Phillips 1998; Diaz *et al.,* 2002; Tomasevic-Canovic *et al.* 2003) and reduce the adverse effects of AFB_1 in *in vivo* studies (Lindemann *et al.,* 1993, 1997; Schell *et al.,* 1993a, 1993b and Shi *et al.,* 2005). Absorbent clay like hydrated sodium calcium aluminosilicate (HSCAS) has been extensively studied for adsorption of AFB_1 and the ability to retain AF on its structure (Phillips *et al.,* 1988). The mechanism for binding AF to HSCAS was proposed by Sarr *et al.* (1990, 1991), Phillips *et*

al. (1995) and Phillips (1999). The chemisorption of AF involves formation of a strong bond between a metal ion in the clay and the β-dicarbonyl of AF. Diaz *et al.* (2002) speculated that similar products would also bind AF by the same mechanism. HSCAS is a phyllosilicate, and montmorillonite is the main component. It has shown an ability to protect animals against the adverse effects of AF in chickens and pigs, and to reduce the carry over of AF into milk in the dairy cow, and importantly it does not interfere with the utilization of important vitamins and micronutrients in the diets (Harvey *et al.,* 1991; Lindmann *et al.,* 1993; Ramos and Hernandez, 1997; Huwig *et al.,* 2001; Diaz *et al.,* 2004; Pimpukdee *et al.,* 2004). Studies have, however, also confirmed that HSCAS does not protect animals against other mycotoxins (Phillips *et al.,* 2006). Other clays such as bentonite and zeolite also have the ability to protect animals against the adverse effects of aflatoxin. However, they have normally less capacity than HSCAS to absorb aflatoxin *in vitro*, but the efficacy *in vivo* in practical conditions is similar (Lindemann *et al.,* 1993; Galvano *et al.,* 2001; Huwig *et al.,* 2001). The differences of adsorptive capacity between HSCAS and other clays can probably be explained by the differences in processing and purification of the clays prior to marketing. On the other hand, the clay from each mine has a specific chemical composition and chemical attributes, and even within the same mine clays may vary by location (Diaz *et al.* 2002). Some other absorbents like activated charcoal and polymers e.g. cholestyramine and polyvinylpyrrolidone, have also been tested and found to bind AF and even trichothecenes, ochratoxin A and ZEA *in vitro*. However, *in vivo*, some absorbents have less or even no effect against mycotoxins, and others have not been tested (Ramos *et al.,* 1996; Alegakis *et al.,* 1999; Huwig *et al.,* 2001; Avantaggiato *et al.,* 2003). In the case of activated charcoal, the reason might be due to the fact that activated charcoal is a relatively unspecific adsorbent, and, hence, essential nutrients are also adsorbed, particularly if their concentrations in the feed are much higher compared to those of the mycotoxin.

Yeast cell walls, the products from an extracted cell wall fraction of *Saccharomyces cerevisiae*, were able to bind a large range of mycotoxins *in vitro* (Devegowda *et al.,* 1998). Feeding studies with chickens have shown that esterified glucomannan effectively alleviated the growth depression caused by AF, ochratoxin A, ZEA and T-2 toxin in a naturally contaminated diet (Aravind *et al.,* 2003). Recently, the binding mechanism of yeast cell walls for mycotoxins has been described by Yiannikouris *et al.* (2004a, b). Today, many yeast cell wall absorbents are commercially available. Products can be

found with only yeast cell wall or the combination of the yeast cell wall with clays.

2.4 Exposure of pigs to mycotoxins

Exposure to mycotoxins depends on the level of these toxins in different feeds, on the intake and on the feeding duration of these feeds. Traditional evaluation of human and animal exposure to mycotoxins is often based on direct analysis of feed and food, or more generally on occurrence data in food and feedstuffs combined with consumption data (Park *et al.,* 2004). The occurrence data, however, may not give accurate information on the intake of mycotoxins. Actual mycotoxin levels in food and feed may be quite different to occurrence data, or not all sources may have been taken into account. In the case of pigs for example, besides feed, they can consume bedding straw, soil and leftover feed on the floor of their pen, which may also contain mycotoxins. As such, the assessment of mycotoxin exposure at the individual level based on these parameters can be imprecise. Therefore, the analysis of blood, bile and urine for the residues, adducts and metabolites of mycotoxins is often used for proving an exposure of animals to these mycotoxins.

Exposure of animals towards mycotoxins, especially for AF, has been investigated through the metabolism and reaction of mycotoxins with protein or DNA of the host body, which could occur in intestinal cells and liver cells and through the blood excretion to urine. Aflatoxin, especially AFB_1, is often bound covalently with cellular DNA after microsomal oxidation to the reactive AFB_1-8, 9-epoxide to exert its hepatocarcinogenic effect (Eaton and Gallargher, 1994). These AF-adducts can be measured *in vivo* and are highly correlated to the carcinogenic potency of AFB_1. Biotransformation of AFB_1 also results in the production of more polar metabolites, such as aflatoxin M_1, Q_1 and P_1. Theses metabolites, AF-adducts and even AFB_1 may be conjugated with glucuronic acid and excreted via urine (Eaton and Gallargher, 1994; Essigmann *et al.,* 1982). The metabolism of other mycotoxins, such as trichothecenes, ZEA, ochratoxin A and fumonisins, has also been investigated, and the metabolite products or other products (sphinganine and sphingosine in the case of fumonisins) can be found in blood, bile and urine (Mirocha and Christensen, 1974; Olsen *et al.,* 1985a; Rotter *et al.,* 1994; Solfrizzo *et al.,* 1997; Tran *et al.,* 2003; Goyarts *et al.,* 2006). Fortunately, there is a correlation between mycotoxin intake and concentrations of some metabolites and adducts in blood and urine of animals and humans exposed to contaminated feeds and foods (Qian *et al.,*

1994; Solfrizzo *et al.,* 1997; Tran *et al.,* 2003; Döll *et al.,* 2003; Dänicke *et al.,* 2004a,b,c 2005a; Turner *et al.,* 2008). These metabolites and adducts could be considered as biomarkers for mycotoxin exposure and the measurement of these parameters will given an assessment of exposure to mycotoxins. However, the real life exposure of animals is often with various toxins due to the fact that feedstuffs and feeds are often contaminated by more than one fungus, and most fungal species produce two or more toxins. Additionally, due to the chemically diverse classes of mycotoxins, the evaluation of animal exposure to mycotoxins is more difficult and complex (Mirocha and Christensen, 1974).

It is important to know that the relationships between the feed levels and the excretion in body fluids are not clear for some mycotoxins and seem to be based on the metabolism capacity of a species or even individual animals of the same species, and body fluids (blood, bile and urine) taken for analyzing. In the case of ZEA, for example, due to the effect of enterohepatic cycling, the biological half-life of ZEA is prolonged and ZEA and its metabolites are concentrated in bile (Biehl *et al.,* 1993; Dänicke *et al.,* 2005b). Therefore, in this case, analysis of ZEA and its metabolites in bile fluid is a suitable way for evaluating the exposure of a pig at slaughter to this toxin (Döll *et al.,* 2003). For other mycotoxins, like AF and DON, analysis in urine might give a better evaluation of pig exposure.

At present, methods for the analysis of the mycotoxins and metabolites in a range of body fluids have been published with low detection and high precision for individual toxins (Olsen *et al.,* 1985b; Kussak *et al.,* 1995, 1998; Solfrizzo *et al.,* 1997; Jodlbauer *et al.,* 2000; Valenta *et al.,* 2003; Songsermsakul *et al.,* 2006). However, there are no methods for analysis of mycotoxin and metabolite clusters in pig urine as biomarkers for mycotoxin exposure like the methods developed and used in Paper IV. This approach is of interest for further studies in the future.

2.5 Effects of mycotoxin on pig health

Since AF caused the death of 100,000 turkey poults in 1960, extensive studies have been conducted to determine the effects of AF on various domestic animals. Aflatoxins, especially AFB_1 have been shown to cause cancer in animals and also potential cancer in humans (Newberne and Butler, 1969; Eaton and Gallagher, 1994). A hundred percent incidence of hepatic tumors in rats fed a diet containing 15 μg/kg AFB_1 continuously for 68 – 80 weeks was reported by Wogan and Newberne in 1967. Some years later, the same authors found that a dietary level of 100 ppb AFB_1 induced

hepatocellular carcinomas in all experimental rats raised for 54 – 88 weeks (Wogan *et al.,* 1974). In pigs fed a dose of 400 μg/kg of AFB_1, the histological lesions were seen in liver as well as variation in lymphocyte indices and liver specific serum enzymes, and with a high dose (800 ppb AFB_1) the histological lesions were more clearly seen, with hepatocellular degeneration plus elevated gama-glutamyl transpeptidase, aspartate aminotransferase and alkaline phosphatase (Miller *et al.,* 1981). A similar result was also obtained by Thieu *et al.* (2008a), who found that liver enzyme activities were significantly increased in piglets fed an AFB_1 contaminated diet (200 μg/kg). A "dose-related" decrease in weight gain was observed in weaning pigs fed a contaminated diet containing 280 μg/kg of AFB_1 (Marin *et al.,* 2002). The results of this trial also showed a reduced immune response induced by *Mycoplasma agalactiae*. However, at this dose no effects were seen on total red and white blood cell numbers, and on alteration of globulin, albumins or total protein concentration in serum. With regards to the performance of pigs, Lindemann *et al.* (1993) reported a quadratic decrease in average daily weight gain with increased (0, 140 and 280 ppb) dietary aflatoxin level and this growth decrease was also associated with a linear reduction in average daily feed intake and decrease in gain/feed.

Several feeding studies of the effects of DON have been published, as reviewed by EFSA (2004a) and Eriksen and Pettersson (2004). In most cases naturally or artificially infected cereals have been mixed into the feed. Naturally infected feed had a stronger effect on the feed intake and weight gain than pure toxin in all these studies comparing the different sources of DON (Forsyth *et al.,* 1977; Rotter *et al.,* 1994; Trenholm *et al.,* 1994; Swamy *et al.,* 2002). This might result from the presence of other toxins and the presence of other compounds (as for example bacterial polysaccharides) in the materials, affecting the toxicity of trichothecenes and including taste aversion (Rotter *et al.,* 1996). Refusal and vomiting phenomena have been observed when high doses of DON were given to pigs (Vesonder *et al.,* 1976). Young *et al.* (1983) reported that complete feed refusal was observed at 12 mg DON/kg feed and vomiting at 20 mg/kg feed, and at low dose (1.3 ppm) caused a significant depression in feed intake and rate of gain. A similar result was also observed by Forsyth *et al.* (1977) who found that feed intake decreased by approximately 20% in pigs fed 3.6 ppm of DON and reached 90% at a dose of 40 ppm, thereby resulting in loss of weight. In addition, increased liver weights and decreased concentration of serum protein and albumin were also observed, together with a temporary fall in packed blood cell volume, serum calcium and serum phosphorus (Bergsjö *et*

al., 1993). On the other hand, delayed immune responses towards vaccinations have been observed one and two weeks after immunisation in pigs given 3 mg DON/kg feed (Rotter *et al.,* 1994).

Feeding the animals ZEA contaminated feed will cause negative effects on the reproductive functions. Initial studies suggested that an effective dietary ZEA concentration producing a clear oestrogenic effect in the pig would be higher than 1 mg/kg feed (Mirocha and Christensen, 1974). In more recent studies using crystalline ZEA, oestrogenic effects have been reported at concentrations ranging from 0.05 to 0.4 mg ZEA/kg feedstuff (EFSA, 2004b). Among livestock, pigs are sensitive to this oestrogenic compound especially, prepubertal gilts (Diekman and Green, 1992). Longer estrous cycle was observed when gilts were fed 20 mg ZEA from 6^{th} to 10^{th} and 11^{th} to 15^{th} day of the estrous cycle, with a range in inter-estrous interval from 28 to 74 days (Flowers *et al.,* 1987). A reduction of luteinizing hormone was also observed in gilts fed ZEA contaminated feed together with an increase in progesterone concentration. In another trial Green *et al.* (1990) also observed the decrease of luteinizing hormone, although the ZEA does not adversely affect subsequent reproduction if it is withdrawn from the diet two weeks before exposure to a boar. ZEA is also known to induce anoestrous in cycling females or delay return into oestrus after weaning of the sows. Reduction of embryonic survival occurred when sows were fed a diet containing a high level of ZEA and sometimes decreased foetal weight (Etienne and Dourmad, 1994). However, at concentration levels of either 5 or 10 ppm diet ZEA did not alter the proportion of sows returning to estrus, but the weaning to estrus interval increased (Young *et al.,* 1990).

3 Summary of materials and methods

3.1 Sample collection (Paper I and IV)

In Paper I, a total of 120 representative samples (1-2 kg per sample) was collected from local retail traders and feed companies in 8 provinces of southern Vietnam from January to February 2005. The collected samples included rice bran, broken rice, maize, cassava chip, and complete feeds for growing pigs and sows, and were stored in a freezer until analysed.

Fifteen urine samples (50 ml to 100 ml of each) were taken from different pig farms in Dongnai Province, Vietnam (Paper IV).

3.2 Analyses of aflatoxins and zearalenone in feedstuffs and complete feeds in Paper I

Aflatoxins were quantified by high performance liquid chromatography (HPLC). In summary, the samples were extracted by methanol/water by shaking and filtered. A portion of filtrate was then diluted by deionised water and cleaned up by immunoaffinity column. The immunoaffinity column was washed two times with 10 ml of deionised water and the aflatoxins were eluted by 1 ml methanol. The eluate was evaporated to dryness under a stream of nitrogen and was then derivatized with trifluoroacetic acid, mixed for 30 seconds and then diluted by mobile phase solvent (water:methanol:acetonitrile) for injection into the HPLC. Twenty microliter sample volumes were injected into the HPLC system (Shimadzu) and the aflatoxins were then separated by reversed-phase liquid chromatography. The aflatoxins were detected by fluorescence detector (Jasco 875) with the C_{18} 5µ (250x4.6) mm analytical column used. The excitation and emission wavelengths were 365 nm and 435 nm, respectively.

The detection limits were 0.13, 0.12, 0.09 and 0.15 ppb for AFB_1, AFB_2, AFG_1 and AFG_2, respectively.

Enzyme-Linked Immunosorbent Assay (ELISA) was used to analysis zearalenone according to the procedure suggested in the AgraQuant® Zearalenone Assay kit manual (RomerLabs, Singapore).

3.3 Analysis of biomarkers in urine of pigs in Paper IV

3.3.1 Equipment, chemicals and reagents

Incubator set at 37°C. EXtrelute® NT20 column, Merck. Rotary evaporator, Rotarvapor Büchi. Pipettete Biohite Electonic Finnpipet Electronic. Immunoaffinity columns: ZearalaTest™ columns, VICAM. DONPREP column, R-Biopharm, Rhône. AflaTest® WB column, VICAM. Vaccum manifold: IST Vacmaster. Evaporator Dri-Block DB-3, Bergman and Bewing.

All chemicals and solvents used in the study were pro-analysis grade or HPLC grade.

3.3.2 Sample extraction

The stored frozen urine samples were thawed and divided into aliquots for analyses. Five ml of urine sample was transferred to a testtube and 5.0 ml of sodium acetate buffer 0.2M pH 5.5 and 5000 units of β-glucuronidase (type H-1 or type H-2, Sigma) were added, and mixed. The samples were then incubated for 16 hours at 37°C. After incubation, 10 ml of sodium phosphate buffer (10 mM, pH 7) was added and applied onto an EXtrelut® NT20 column. The samples on the column were then extracted by passing 60 ml of ethylacetate:acetonitrile (9:1) into a 250 ml round-bottom flask. The extracts were then evaporated to dryness in rotary evaporator. For ZEA, the sample residue was dissolved with 2 ml of ethylacetate:acetonitrile (9:1) and transferred to a 10 ml vial. The flask was then washed with 1 ml of ethylacetate:acetonitrile (9:1) followed by 1 ml methanol and transferred to the sample vials. In the case of DON, the extract samples were dissolved by 3 ml of ethylacetate:acetonitrile (9:1) and transferred to a 10 ml vial. The round-bottom flasks were washed 2 times with 1 ml of methanol. The residues and rinses were combined and evaporated to dryness under nitrogen in heated dri-block at 65°C. In the case of aflatoxins, 10 ml urine was mixed with 10 ml of sodium acetate buffer (0.2M, pH 5.5) and the pH of the solution was adjusted to 4.5 by

adding an appropriate volume of acetic acid. β-glucuronidase (5000 units) was finally added to the solution and incubated at 37°C for 16 hours.

3.3.3 Clean up with immunoaffinity column

The IAC column was first allowed to reach room temperature prior to conditioning or loading the sample extracts. The ZEA and DON extracts were dissolved in 2 ml of acetonitrile:water (75:25) and 2.5 ml of water, respectively. For ZEA, the ZearalaTest™ column was conditioned by passing 15 ml of PBS at a speed of 1 drop/second by gravity prior to loading the ZEA sample. The ZEA dissolved sample (2 ml) was mixed with 18 ml of water and then passed through the ZearalaTest™ column at a flow rate of 1 drop/second by gravity. The column was then washed with 10 ml of water and dried by pushing air through with a hand pump syringe. ZEA and its metabolites were eluted with 2 ml of acetonitrile at a speed lower than 1 drop/second. Most of the applied elution solvent was collected by passing air through with a syringe.

In the case of DON, the dissolved sample was directly applied onto DONPREP column at a flow rate of 1 drop/second by gravity. The column was then washed with 3 ml of water and dried by hand pump syringe. The column was then eluted 3 times with 0.5 ml methanol at a speed of 1 drop/second by gravity with stop 1 minute after the first addition, and most of the applied elution solvent was collected by passing air through with a syringe.

After incubation, aflatoxin solution was directly applied on an AflaTest®WB column, which had previously been conditioned by 10 ml of water. The column was then washed by 10 ml of water, air dried using syringe pump, and toxins were eluted by 4 times 0.5 ml acetonitrile with stop 1 minute after the first addition. Most of the added elution solvent was collected by finally passing air through the column with a syringe. Flow rate of 1 drop/second by gravity was used for all steps.

The sample eluates were then evaporated to dryness under nitrogen on heated Dri-block at 65°C. The ZEA and DON samples were dissolved in 500 µl of their mobile phases and the solutions were then transferred to a small HPLC vial. In the case of aflatoxins, the dry residue was derivatized for 5 minutes by 100 µl of trifluoroacetic acid and then diluted by 900 µl acetonitrile:water (20:80, v:v) and transferred to a small HPLC vial.

All mycotoxins and their metabolites were finally analyzed by HPLC in separate systems.

3.3.4 High performance liquid chromatography

Liquid chromatograph: Hewlett Packard Serie 1050, with auto sampler and Chemstation for control and evaluation. Spectrophotometric LC-detector: Lambda-Max Model 481 LC, Waters. Schimadzu RF-530 Fluorescence HPLC monitor.

ZEA and its metabolites αZOL and βZOL were separated in a system using Novapak C18 4µ, 150x4mm, Waters, with a guard column Guard-pak Novapak C18 4µ, Waters. Mobile phase was acetonitrile:water (40:60, v:v) with a flow rate of 0.8 ml/minute. The injection volume was 100 µl ZEA and its metabolites αZOL and βZOL eluted after 3.6, 4.9 and 7.7 minutes, respectively, and were detected by both the fluorescence detector with excitation and emission wavelengths of 275 and 450 nm, respectively, and the UV detector at wavelength of 236 nm.

For DON and deepoxyDON a column Zorbax SB-AQ C18 3.5µ, 100x3 mm, Agilient with guard column of Zorbax C18 3.5µ, Agilent was used for separation. Injection volume was 100 µl and a solution of acetonitrile:water (5:95, v:v) was used as mobile phase A with a flow rate set at 0.4 ml/minute and 100% acetonitrile was used as mobile phase B for a washing gradient. The gradient started after 12 minutes and increased to 80 % of phase B at 17 minutes to 18 minutes and returned to 0% at 29 minutes. DON and deepoxyDON eluted after 6.8 and 11.4 minutes, respectively, and were quantified by the UV detector at wavelength of 220 nm.

The aflatoxins were separated on a Zorbax SB-C18 column, 3.5 µm, 3x100mm, Agilent. A gradient system was used with mobile phase A as acetonitrile:water (18:92, v:v) with flow rate of 0.4 ml/minute and 100% of acetonitrile as mobile phase B. The gradient started after 2 minutes and gradually increasing up to 10% phase B at 10 minutes and was maintained until 18 minutes, and then returned to 0% at 20 minutes. Injection volume was 100 µl. The aflatoxins AFM_1, AFG_1, AFB_1, AFG_2 and AFB_2 eluted after 3.4, 7.2, 9.6, 14.0 and 16.5 minutes, respectively and were quantified by the fluorescence detector at excitation and emission wavelengths of 365 and 435 nm, respectively.

3.4 Experimental design, animals, diets and feeding

In Paper II, the trial was designed to use single concentration adsorption and isotherm adsorption to evaluate the capacity of local zeolite and bentonite to adsorb AFB_1 in simulated gastrointestinal fluids according to the procedure described by Grant and Phillips (1998). Simulated gastrointestinal fluids (SGFs) were made according to Avantaggiato *et al.* (2003) and Stippler *et al.*

(2004). All chemicals and solvents used in the study were pro-analysis grade or HPLC grade. Pure crystal AFB_1 was dissolved in methanol (1mg/ml) and the appropriate volume of solution was transferred to the 16 x 125 mm test tubes, which contained appropriate concentrations of sorbent in 10 ml SGF. The test tubes were then capped and placed on an electric shaker at 280 rpm for 8 h. Then the samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was measured by a Vicam series-4 Fluorometer according to the recommendation of the Vicam manual and checked by HPLC. Adsorption data were assayed to fit the Freundlich isotherm according to Ramos and Hernandez (1996) and Jansen Van Rensburg (2005).

In vivo evaluation of efficacy of bentonite (Paper III) was conducted by using 48 crossbred piglets (sex balanced), which were randomly assigned to one of four treatments: 1) 0 g of bentonite and 0 mg of AF/kg feed (control, basal diet); 2) 4 g of bentonite per kg feed plus 200 μg AF/kg feed (AF+Bento 4); 3) 5 g of bentonite per kg feed plus 200 μg AF/kg feed (AF+Bento 5) and 4) 0 g of bentonite plus 200 μg AF/kg feed (AFA). Aflatoxin contaminated broken rice used in this trial was produced through the fermentation of cooked broken rice by *Apergillus flavus* using the method described by Shotwell *et al.* (1966).

In Paper IV, four crossbred (Landrace x Yorkshire x Duroc) castrated male pigs with an average body weight of 40 kg were used to study the excretion of AFB_1 and metabolites in urine, with three different levels fed to the pigs. The pigs were individually placed in stainless steel metabolism cages and given AFB_1 contaminated feed three times a day with the daily feed allowance restricted to 2 kg. Feed was formulated to meet the recommended critical nutrient concentrations (National Research Council, 1998). Water was supplied *ad libitum*. Feed refusals and spillage were recorded, and used to correct the AFB_1 intake data. Pig urine was collected daily and the volume was recorded. The urine was then mixed and sampled, and frozen at -18°C until analysis. Pure crystal AFB_1 was dissolved in methanol (20 ml) and sprayed on 2 kg of feed. The feed was then left overnight in an oven at 65°C to evaporate the methanol. The contaminated feed was well mixed to get homogeneity and an appropriately quantity was mixed with non-contaminated feed to give the desired concentration. The experiment was conducted with three periods (pigs were given 100, 200 and 300 μg AFB_1/kg of feed for period I, II and III, respectively), with 6 days per period, including 2 days of being given contaminated feed and 4 days of non-contaminated feed. Urine was sampled from the first day until two days after the last ration of contaminated feed was given to the pigs.

3.5 Statistical analysis

The data were analysed by using the General Linear Models (GLM) procedures of Minitab 14.12 statistical software (Minitab 2004) for analysis of variance (ANOVA). Tukey pair-wise comparisons were performed to determine differences between treatment means when the F- test was significant at $p < 0.05$.

4 Summary of results

4.1 Screening of aflatoxins and zearalenone in feedstuffs and complete feeds for pigs in southern Vietnam (Paper I)

The results show very high incidences (83.3% - 100%) of mycotoxins in all analyzed feedstuffs and complete feeds for pigs. AF and ZEA were found in all commodities analyzed. Of the 120 analyzed samples, AFB_1 was found with the highest incidence (112 samples) followed by ZEA (51), AFB_2 (38), AFG_1 (15). None of the samples was contaminated with AFG_2. AFB_1 was found in 100, 92, 92, 83, 100 and 96%, respectively, of samples of cassava chip, maize, rice bran, broken rice, and complete feeds for growing pigs and sows, with mean concentrations of 0.86, 77.5, 1.3, 1.6, 4.7 and 7.5 μg/kg, respectively. AFB_2 and AFG_1 were found in low concentrations and co-occurred with AFB_1 in all samples where they were found. ZEA was detected in 8%, 33%, 36%, 21%, 67% and 67%, respectively, of the samples of cassava chip, maize, rice bran, broken rice, and complete feeds for growing pigs and sows, with average concentrations of 10.0, 163.5, 83.1, 31.4, 86.0 and 101.4 μg/kg, respectively. There was co-occurrence of AF and ZEA in the present study, and approximately 46% of AF positive samples were simultaneously contaminated with ZEA. The highest co-occurrence ratios were found in complete feeds for sows (70%), followed by complete feeds for growing pigs (67%), rice bran (41%), maize (36%), broken rice (25%) and cassava chip (8%).

4.2 Ability of locally produced clays to eliminate adverse effects of aflatoxin B_1 with *in vitro* and *in vivo* experiments (Paper II and III)

The results in single concentration adsorption in the *in vitro* trial showed that more than 70% of AFB_1 was adsorbed by zeolite in SGFpH3, but only 20% in SIFpH7. In contrast, bentonite exhibited low adsorption (30%) in SGFpH3, but adsorbed more than 80% AFB_1 in SIFpH7. Linearity between the increased amount of AFB_1 adsorbed on sorbents and the decrease of sorbent concentration in solution was observed for bentonite and HSCAS, except for zeolite in SGFs at pH7. The isotherm adsorption trial revealed that the observed maximum amounts of AFB_1 adsorbed on bentonite and HSCAS were 1,540 and 1,557 μg/g, respectively. The adsorption capacities of bentonite and HSCAS for AFB_1 were 12,708 and 13,055 μg/g, respectively. Locally produced zeolite and bentonite showed an ability to adsorb AFB_1 in SGF fluids, although the adsorptive capacities were variable and lower than the commercial HSCAS. Bentonite has a better ability to adsorb AFB_1 than zeolite in SIFpH7, and this suggests that this sorbent can be a candidate for continuing evaluation by *in vivo* study.

The *In vivo* experiment showed that piglets fed the control diet and the AF contaminated diets plus clay appeared clinically normal throughout the study, whereas the piglets given the feed containing AF alone had roughened hair-coats, consumed less feed and did not gain weight as quickly as piglets in the three other treatments. The mean final live weights of piglets fed the AF contaminated diet were significantly lower ($p<0.01$) than of those fed the control diet, whereas these values in piglets fed the diets with AF plus bentonite were similar to those of the control diet. When compared to the control treatment, the overall ADG were lower in piglets fed the diet with AF alone ($p<0.01$), whereas the AF contaminated diet with bentonite gave similar ADG values to the control treatment. The present study also shows a poor feed efficiency for the AF alone diet ($p<0.05$) overall. The highest FCR (1.93 kg of feed/kg of gain) was found in the AF alone treatment, while the control treatment had lower FCR (1.66 kg of feed/kg of gain). The inclusions of 0.4% and 0.5% bentonite in the AF contaminated diet increased the feed conversion efficiency to that of the control treatment. Mean serum leukocyte was increased ($p<0.05$) in piglets fed the diet with AF alone and diets containing AF plus bentonite when compared to those in the control treatment in the first period. However, the difference was significant ($p<0.01$) only between the AF alone treatment and the control treatment. In the second period, mean leukocyte levels for the control, AF+Bento 4, AF+Bento 5 and AF alone treatments were 11.7,

11.2, 11.7 and 16.8 thousand per μl, respectively and were significantly higher ($p<0.01$) in the AF alone treatment compared to the other treatments. The mean concentrations of serum ALB and TP were lower ($p<0.01$) in the AF alone treatment compared to those in the control treatment in both periods. When bentonite was added to the diets with AF, these values were similar to those of the control treatment. There were significant ($p<0.01$) increases in the mean serum activities of GOT, GPT, GGT, ALP and LDH enzyme in piglets fed the AFA diet compared to those given the control diet in both periods. In the case of piglets fed the diet with AF plus bentonite, however, these values were found to be similar to those of piglets fed the control diet. There were no significant differences in growth performance, feed consumption, feed conversion efficiency and blood profiles between piglets fed diets with AF plus either 0.4% or 0.5% bentonite clay. The findings in the present study provide critically needed confirmation that bentonite has the ability to reduce the adverse effects of AF.

4.3 Zearalenone, deoxynivalenol and aflatoxin B_1 and their metabolites in pig urine as biomarker for mycotoxin exposure (Paper IV)

With regards to methods used, good results were obtained with the detection limits for ZEA, αZOL, ßZOL, DON and DeepoxyDON of 6.9, 1.2, 9.9, 2.4 and 1.6 ng/ml urine, respectively. Detection limits of AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 were 0.36, 0.28, 1.0, 0.5 and 0.5 ng/ml urine. The recoveries of ZEA and its metabolites were all high, and ranged on average from 86% to 106% with low relative standard deviation, that ranged from 2% to 6% for both UV and fluorescence detections. The recoveries of DON and DeepoxyDON were 92% and 94%, respectively. The mean recoveries of AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 were 80, 86, 83, 85 and 130%, respectively, and the relative standard deviation ranged from 5 to 10%.

No difference in urine concentration of AFM_1 and AFB_1 after feeding pigs AFB_1 at three levels (127, 227 and 327 μg/kg) could be measured. Approximately 30% of ingested AFB_1 was excreted in urine through three periods as the parent compound AFB_1 and the metabolite form AFM_1, which was dominant and accounted for 77% of total excretion. The proportion of AFB_1 excreted in urine as the AFM_1 form was 22% of the AFB_1 dose.

In the present study, using the current set of methods for measuring the biomarkers of mycotoxins in 15 collected pig urine samples, the toxins and metabolites were found for ZEA, DON and AF in 47, 73 and 80%, respectively (Table 3, Paper IV). ZEA, DON, AF and their metabolites were detected in 14 of the 15 pig urine samples. AFG_1 and AFG_2 were not detected. AFB_1 and AFM_1 were detected in 12 urine samples followed by AFB_2 (10 samples), DON (9 samples), αZOL (7 samples), and DeepoxyDON (6 samples), and ZEA and βZOL (1 sample). Average concentration levels of AFM_1, AFB_1 and AFB_2 were 4.12, 0.61 and 0.35 ng/ml, with ranges of 0.69 – 7.85, 0.37 – 1.55 and 0.28 – 0.48 ng/ml, respectively, in pig urine. DON and DeepoxyDON were detected in 10 pig urine samples, with means concentrations of 10.3 and 10.3 ng/ml and ranges of 2.4 – 26.5 and 1.7 – 37.9 ng/ml, respectively. ZEA and βZOL were found in one sample, 6.9 and 10.0 ng/ml, respectively, and average mean concentration of αZOL was 2.8 ng/ml with a range from 1.2 to 3.9 ng/ml. There was co-occurrence of AF and DON; AF, ZEA and DON; and ZEA and DON in 4, 5 and 2 urine samples, respectively. The results on the occurrence of ZEA, DON, AF and their metabolites in pig urine samples collected from pig farms revealed that the pigs were exposed to ZEA, DON and AF contaminated diets.

5 General discussion

5.1 Contamination of aflatoxins and zearalenone in feedstuffs and pig feeds in Vietnam

In Vietnam, the presence of mycotoxins in feedstuffs intended for animal feed was first shown by Wang *et al.* (1995), who found high incidences of AFB_1 in maize kernels and maize meal. In rice, the major staple food for humans in Vietnam, Nguyen *et al.* (2007) found AFB_1, citrinin and ochratoxin A in 51, 13 and 35%, respectively, of 100 rice samples. The findings of the present study also support previous studies that very high incidences (83.3% - 100%) of mycotoxins were observed in all analyzed feedstuffs and complete feeds for pigs.

High mean concentrations of AF have been detected in Indonesian maize, but the concentration of ZEA was rather low (Ali *et al.,* 1998). In contrast, Yamashita *et al.* (1995) found low concentrations of AF in maize imported from the Philippines and Thailand, whereas high mean levels were detected for ZEA. In Thailand, a similar average concentration of AFB_1 in maize to that found in the present study was reported by Lipigorngoson *et al.* (2003). Recently, Tan (2008) reported that in Southeast Asia AF was found in a low incidence of 20%, with mean of 72 μg/kg in 180 maize samples. The incidence and mean level of ZEA was higher than that of AF and a very high amount of 5,510 μg/kg was detected in one sample. Besides AF and ZEA, other mycotoxins such as DON, fumonisin B_1 and ochratoxin A were also observed in maize.

The high incidence of AF in maize found in the present study probably resulted from the harvesting season and lack of equipment for drying maize. In the south of Vietnam, maize is harvested twice a year, often in the middle or at the end of the rainy season. After harvest, maize is often sun-dried or

by charcoal heated forced air-dryers. Drying is often delayed during harvest time, due to the large quantities harvested and limited equipment and techniques. In Vietnam, maize is a main component of pig feeds, and a high proportion of maize containing aflatoxins and/or ZEA can affect the performance of pigs.

The present high incidence of AF found in cassava chip might have originated during the sun-drying of the cassava roots in the field or on the road for 2 to 4 days, which is the normal drying technique in Southern Vietnam. However, ZEA was found in only one sample at detection level, which is in contrast to the data reported by Veldman *et al.* (1992), who found all tapioca imported from Thailand and Indonesia to be contaminated with ZEA.

The high incidences of AF and ZEA in rice bran and broken rice probably resulted during the harvesting and drying of rice. In the Mekong Delta, rice is usually harvested twice a year. After harvest it is often put to dry on the roads for three to five days before being sent to the mills, where it is sorted and directed for human or animal consumption. This practice, together with the high relative humidity, provides good conditions for mould development and formation of AF in raw rice (Trung *et al.,* 2001). On the other hand, through rice processing, approximately 82% of the AF in raw rice will be transferred to rice bran and rice hulls, as reported by Sales and Yoshizawa (2005). In another survey, by Tan (2008), approximately 25% of rice samples were contaminated with AF. No survey of ZEA in rice bran and broken rice has been reported from Vietnam. Trung *et al.* (2001) could not find any strain of the main ZEA producers *Fusarium graminearum* and *F. moniliforme* in rice from the Mekong Delta. However, Chin and Tan (2006) found 17% of samples of rice and rice bran to be positive for ZEA in Asia. Recently, a high incidence of 25% in rice with a mean of 50 μg ZEA/kg was found in Southeast Asia (Tan, 2008). These results are comparable with that for rice bran and broken rice in the present study.

The present survey also revealed higher incidences of AF and ZEA in pig feeds (97.9%) compared with those in feedstuffs (90.3%). This can probably be explained by pig feeds being mainly based on maize, rice bran and cassava chip, which all have a high incidence of mycotoxins. This is also in accordance with data reported by Cespedes and Diaz (1997), who found the incidence of AF in complete feed in Colombia to be higher than in feedstuffs. A similar result was also reported by Tan (2008), who found finished feeds, comprising mainly poultry and swine feed samples, were contaminated by AF and ZEA with incidences of 22 and 37% and mean levels of 22 and 369 μg/kg, respectively.

Natural co-occurrence of AF and fumonisins in maize from tropical countries has been reported by several authors (Wang *et al.,* 1995; Yoshizawa *et al.,* 1996; Ali *et al.,* 1998), but has not been reported for other feedstuffs. Yamashita *et al.* (1995) found only 5 and 6% of maize from the Philippines and Thailand, respectively, to be co-contaminated with AF and ZEA. A slightly higher ratio (9%) of co-occurrence of AF and ZEA was found in maize from Indonesia (Ali *et al.,* 1998). The co-occurrence of AF and ZEA was also observed in the present study with approximately 46% of aflatoxin positive samples simultaneously contaminated with ZEA.

Although the AF and ZEA levels in the feedstuffs may be considered low in this study, they are still of concern because of the continuous high rates of inclusion of these feedstuffs in pig diets. Actually, high incidences of AF and ZEA were found in complete pig feeds, but the mean concentrations were low (less than 10 μg/kg), except for ZEA. The highest concentration of AFB_1 was detected in complete feed for sows (39 μg/kg). This level will not cause clear clinical effects, although over a long period of consumption it may result in chronic effects and carry-over to animal products (Lawlor and Lynch, 2001a). In contrast to AF, the highest level of ZEA 571 μg/kg was found in a complete feed for sows, and could have caused clinical effects. Pigs are the most sensitive species to ZEA, especially young gilts, and concentrations as low as 0.5 to 1 ppm may cause pseudo-oestrus and vaginal or rectal prolapse (Etienne and Dourmad, 1994, Lawlor and Lynch, 2001b). The present result indicates a potential high exposure of pigs to both AFB_1 and ZEA, and possible chronic adverse effects on gilt and sow health.

The high incidence of AF and ZEA in feedstuffs and pig feeds in southern Vietnam found in this study highlights the need for periodic monitoring of AF, ZEA and other mycotoxins, not only in feedstuffs and animal feeds but also in food for human consumption.

5.2 Ability of local adsorbents to reduce the adverse effects of aflatoxin B_1

5.2.1 Ability to adsorb AFB_1 by local zeolite and bentonite

In most *in vitro* studies, the adsorption reaction used to be done in an aqueous environment at neutral pH (Phillips *et al.,* 1988; Ramos and Hernandez, 1996; Diaz *et al.,* 2002). In the present study, simulated gastrointestinal fluids (SGFs) with different pH were used to carry out the experiment. The results from single adsorption of the reference adsorbent (HSCAS) showed no difference between the pH 3 and pH 7 of SGFs when

used in adsorption of AFB_1 to HSCAS. Additionally, the present result for HSCAS was higher than results obtained by Phillips *et al.,* (1988) and Scheideler (1993), and just slightly lower than those of Ledoux *et al.,* (1998). This suggests that the SGFs are suitable for performing an adsorption trial.

Adsorption capacity of clay to AFB_1 is often affected by many factors, such as the constituents of the clays, and components and pH of media in which the adsorptive reactions occur. Therefore, high variations in adsorptions can be observed. Vekiru *et al.* (2007) found the adsorption ability of clays to be variable when the adsorption test was performed in different environments such as acetic buffer, artificial gastric juice and real gastric juice media. The same authors also showed that the adsorption capacity of sorbents is often lower in the gastrointestinal tract compared to results obtained in acetate buffer solution. However, Diaz *et al.* (2002) found the AFB_1 binding ability of bentonite to be higher than 95%, with no significant pH effect on the AFB_1 binding. In agreement, Vekiru *et al.,* (2007) found that the adsorption ability of clays was slightly higher when the adsorption test was performed at a neutral pH compared to an acetic solution with pH 5. The finding in the present study showed an ability of zeolite and bentonite to adsorb AFB_1 in SGFs. However, in contrast to HSCAS, the local sorbents also showed a variation according to pH of SGFs. The differences in adsorptive capacity of sorbents among studies and sorbents can probably be explained mainly by the differences in processing and purification of clays prior to marketing. On the other hand, the clay from each mine has a different chemical composition and chemical attributes and even within the same mine clays may vary by location (Diaz *et al.* 2002).

It is known that the sorbent/toxin ratio may affect the maximal AFB_1 adsorption onto clay. The findings in the present study confirm this. A linear relation between the increased amount of AFB_1 adsorbed on clays and the decrease of clay concentration in the media was observed. However, this was only seen for HSCAS and bentonite. For zeolite it fluctuated and adsorbed below 450 μg/g AFB_1, which suggests that locally produced bentonite has a better adsorption capacity than zeolite.

Grant and Phillips (1998) reported an observed maximum amount of AFB_1 adsorbed on HSCAS of 114 mg/g and a similar result was also obtained by Vekiru *et al.* (2007) in isotherm adsorption studies. These results are far higher than those obtained in the present study for HSCAS and bentonite. This may be explained by the high concentration of sorbents used in the present study. In other studies, low sorbent concentration and high toxin levels have often been used to identify sorbents with a high AFB_1 adsorption capacity (Grant and Phillips, 1998; Shi *et al.,* 2006; Vekiru *et al.,*

2007). Possibly, this difference, together with the SIFpH7 medium resulted in low adsorptive capacity of clays in the present study. This was also found for HSCAS, which has been shown to have high adsorptive capacity in water and phosphate buffer, which has been used by others (Grant and Phillips 1998; Vekiru *et al.,* 2007).

The isotherm shapes of AFB_1 adsorption on HSCAS and bentonite can be categorized as an L type isotherm plot, but the curves did not reach a plateau. This can be seen by comparing the observed maximum amount with the maximum capacity of the fitted isotherm equation. The observed maximum of AFB_1 adsorbed onto HSCAS and bentonite was 11.9% and 12.1% of the maximum adsorptive capacity (k) of 13,055 and 12,708 μg/g, respectively, from the fitting of the data to the Freundlich adsorption isotherm equation. This suggests that HSCAS and bentonite do not clearly show a limited sorption capacity and even they adsorbed 97.6% and 96.5% of AFB_1 in stock solution, respectively. In agreement with the present results, Ramos and Hernandez (1996) and Desheng *et al.* (2005) found low adsorptive capacity of montmorillonite with a high sorbent concentration of 1 and 0.5 % (w/v), respectively, in the AF solution.

5.2.2 Ability of bentonite to ameliorate aflatoxicosis in piglets

It is well known that loss of appetite and subsequent reduced feed intake is often observed in piglets fed AF contaminated diets. The supplementation of clays such as bentonite and HSCAS has also been shown to improve feed intake (Schell *et al.,* 1993a; Lindemann *et al.,* 1993). In contrast, in another trial, Lindemann *et al.,* (1997) found no significant differences at any time in feed intake among control diet, an AF contaminated diet and AF contaminated diet supplemented with various clays. The finding in the present study also supported the general results, although feed intake of the piglets fed AF contaminated diets tended to be lower. The reasons for these differences are unclear, but may be related to the inclusion of AF substrate in the diet. The high inclusion of AF substrate in the diet may have affected palatability (Lindemann *et al.,* 1997).

The present results also confirm and support the results of previous studies that AF are toxic to weaning pigs and that poor growth is the first and most consistent sign of AF intoxication (Miller *et al.,* 1981; Panangala *et al.,* 1986; Harvey *et al.,* 1988; Marin *et al.,* 2002). The addition of clays to AF contaminated diets restores the pig productive performance to that of AF non-contaminated diets (Ramos *et al.,* 1996; Ramos and Hernandez, 1997; Shi *et al.,* 2005). In agreement with these studies, the present study showed that the overall average daily gain (ADG) of the AF contaminated diets plus

0.4% and 0.5% bentonite was only 3.6% and 5.5% less than the control treatment, respectively. The ADG in the AF alone (AFA) diet was, however, 23.6% lower than the control treatment. The present results are also consistent with those of Lindemann *et al.* (1997), who found ADG to be 27.8% lower when piglets were fed a diet containing 500 μg AF/kg of feed. Addition of clays resulted in 47% to 60% recovery of the growth loss. Similar results were also observed when weaning pigs were fed an AF contaminated diet with 0.5% sodium bentonite or 0.5% calcium bentonite, which gave weight gain similar to those fed a control diet (Schell *et al.,* 1993a). Lindemann *et al.* (1993) also reported similar results for sodium bentonite, and this sorbent can be comparable with HSCAS, the sorbent which has been the most extensively studied.

The supplementation of clays to AF contaminated diets is known to improve poor feed efficiency in piglets fed AF contaminated diets (Lindemann *et al.,* 1993, 1997; Schell *et al.,* 1993b). The findings in the present study confirm these results, when inclusions of 0.4% and 0.5% of bentonite were used in the AF contaminated diet to pigs.

Besides growth performance and feed efficiency, changes in blood profiles are often observed in piglets fed AF contaminated diets. However, there are variations among experiments that seem to be due to dose level of AF in feed and time of exposure. Marin *et al.* (2002) found no effect on red blood cell number or on their relative number of lymphocytes, monocytes, neutrophils, basophils and eosinophils in blood as result of AF ingestion of low doses (140 and 280 ppb). Similarly, AF did not alter albumin (ALB), globulin (GLOB) and total protein (TP) in serum. However, AF had a biphasic effect on total white blood cell number. The low dose of AF (140 ppb) decreased the total white blood cells, while the high dose (280 ppb) had the opposite effect. In the present study, the mean levels of erythrocyte, haemoglobin and hematocrit were similar among treatments, but the leukocyte number was significantly increased in the AFA diet. This has also seen in a previous study by Miller *et al.* (1981). Additionally, lower serum TP, ALB and GLOB were observed in pigs fed an AF contaminated diet. Similar results were also reported by Harvey *et al.* (1988) and Lindemann *et al.* (1997). In agreement with these studies, the present findings showed that ingestion of feed containing 200 ppb AF reduced TP, ALB and GLOB. Fortunately, the inclusion of clay adsorbents such as bentonite in the AF contaminated diet had a positive effect, by restoring the abnormal blood profile (Schell *et al.,* 1993a, 1993b), and the present study also confirms this.

With regard to the serum enzyme activities, the present findings are consistent with previous studies showing increase of serum enzyme activities

in piglets fed AF contaminated diets compared to those fed a control diet (Miller *et al.,* 1981, 1982; Harvey *et al.,* 1988; Shi, 2005). In humans, an increase of serum enzyme activities, such as GOT, GPT, GGT, ALP and LDH, are indicators of inflammation or damage of the hepatic cells or biliary tract cells (Hargreaves *et al.,* 1961; Johnston, 1999). This may also be the case in pigs, and thus, Miller *et al.* (1981, 1982) found a linearity of the morphologic change in livers of pigs that had ingested AF and an increase in liver specific serum enzyme activities. This suggests that AF may cause morphologic changes in the liver of pigs which have ingested AF contaminated feed. The present study also shows that the addition of bentonite to the diet with 200 ppb AF gave similar serum enzyme activities to those for the control diet. Similar results were also obtained by Schell *et al.* (1993a, 1993b) and Lindemann *et al.* (1993, 1997), who reported that the inclusion of bentonite in AF diets reduced the adverse effects of AF.

The findings in the present study provide more evidence that bentonite has the ability to sequestrate AF adverse effects in piglets. Thus, the recovery of growth performance, feed efficiency, and serum biochemical and enzyme activities in the present study, may be explained by the reduced gastrointestinal absorption of AF in pigs. This is probably due to the strong bond formed between bentonite and AF, and the subsequent excretion of the complex in faeces, as seen in other studies (Sarr *et al.,* 1990, 1991; Phillips *et al.,* 1995, 1999; Diaz *et al.,* 2002).

5.3 Excretion of mycotoxins in pig urine and measurement of urinary biomarkers

5.3.1 Methods for urinary biomarkers in pigs

Exposure of pigs to mycotoxins results in excretion of their metabolites and parent toxins in urine (Eaton and Gallargher, 1994; Essigmann *et al.,* 1982; Olsen *et al.,* 1985a; Rotter *et al.,* 1994; Solfrizzo *et al.,* 1997; Tran *et al.,* 2003; Goyarts *et al.,* 2006). These toxins and metabolites in urine can be considered as biomarkers for mycotoxin exposure. The measurement of these parameters may give a good assessment of the exposure to mycotoxins.

Methods have been published for analysis of mycotoxins and metabolites in a range of body fluids, which may be used as biomarker methods (Olsen *et al.,* 1985b; Kussak *et al.,* 1995, 1998; Solfrizzo *et al.,* 1997; Jodlbauer *et al.,* 2000; Valenta *et al.,* 2003; Songsermsakul *et al.,* 2006). In Paper IV, new or modified methods partly based on these methods were developed for

screening mycotoxins and metabolites in pig urine as biomarkers for mycotoxin exposure.

It is known that the mycotoxins and their metabolites excreted in pig urine are normally conjugated with glucuronic acid to a high degree (Eaton and Gallargher, 1994; Essigmann *et al.,* 1982). Therefore, the pig urine has to be incubated with β-glucuronidase to cleave the glucuronide conjugated mycotoxins and increase the extractable mycotoxins. However, incubated pig urine may contain high amounts of salts, proteins and organic compounds, which can interfere with the adsorption on the immunoaffinity column and the separation on the HPLC column. An extraction column cleanup is thus preferred prior to application on the immunoaffinity columns. This has earlier been used in a method for DON (Valenta *et al.,* 2003) in pig urine and is now used for the cleanup of both ZEA and DON. However, for AF there were too high and variable losses during extraction and the rotary evaporation. The urine incubates for AF were instead further diluted and as in the method by Kussak *et al.* (1995) applied directly to a wide-bore immunoaffinity column.

This direct immunoaffinity cleanup of AF resulted in an impurity peak eluting just before AFM_1 in the HPLC separation. The amount of the impurity varied in different urine samples. It was separated from AFM_1, but had an influence on the quantification of AFM_1. It can be seen through the too high recovery obtained for AFM_1. A low initial acetonitrile concentration was used to obtain this separation and a gradient had to be used to get a faster elution and higher peaks of the other AF:s. This impurity was not seen by Kussak *et al.* (1995) in human urine. The difference could be due to the diets of the pigs or that Kussak *et al.* (1995) used bromide post-column derivatization.

After the immunoaffinity cleanup of DON a late eluting impurity also disturbed an isocratic HPLC separation of DON and DeepoxyDON. This has also been observed in earlier methods using immunoaffinity cleanup for DON in pig urine (Valenta *et al.,* 2003). A gradient wash, as in the other methods was used in the HPLC to eliminate the disturbance.

In agreement with the results of Olsen *et al.* (1985), the present results showed a weak response of βZOL compared to those of ZEA and αZOL when measured by fluorescence detector. In addition, the response of βZOL to fluorescence detector also had a high variation and was weaker than those to UV detector. The UV detection gave thus a more accurate and lower detection limit for βZOL.

Low and good detection limits were obtained for ZEA, DON and AF and their metabolites, and which were better or in the same range as those

for pig urine in previous studies reported by Olsen *et al.* (1985), Zöllner *et al.* (2002), Döll *et al.* (2003), Razzazi-Fazeli *et al.* (2003) and Valenta *et al.* (2003). The recoveries of ZEA and its metabolites were all high and ranged from 86% to 106% with a low relative standard deviation that ranged from 2% to 6% for both UV and fluorescence detections. The present results were slightly higher than those obtained in previous studies (Olsen *et al.* 1985b; Zöllner *et al.,* 2002; Döll *et al.,* 2003), where recoveries for ZEA, αZOL and βZOL ranged from 68% to 90%, with relative standard deviation from 2% to 10%. Good results on recoveries were also obtained for DON and DeepoxyDON, and these values were better than those obtained by Valenta *et al.* (2003), who had recoveries of DON and DeepoxyDON in urine of pigs of 82% and 81%, respectively.

A recovery that ranged from 83 to 130% with a relative standard deviation of 5 to 10% was obtained for AF and its metabolites in spiked urine of pigs. A high recovery for AFM_1 was observed, which ranged from 117 to 144% with mean of 130% of dose spiked in pig urine. This might have been affected by the impurity peak mentioned above. Better recoveries of AF and its metabolites were obtained by Kussak *et al.* (1995), who found the recoveries ranged on average from 96 to 103%. However, the relative standard deviation was high (from 1 to 21%).

5.3.2 Mycotoxin excretion in urine of pigs and the correlation with the concentration in diet

Several studies have shown that a main excretion pathway of ZEA, DON and their metabolites in pigs after exposure to a ZEA or DON contaminated diet is through the kidney into urine (Farnworth and Trenholm, 1983; Olsen *et al.,* 1985a; Dänicke *et al.,* 2004c, 2008; Goyarts *et al.,* 2006). Olsen *et al.* (1985b) found that 15.6% of ingested ZEA 5 mg/kg feed was excreted in urine as ZEA and αZOL within 8 hours. Most of the ZEA were found as glucuronide conjugates. The total concentrations of ZEA and αZOL found in the urine were between 50 and 159 ng/ml and 50 and 200 ng/ml respectively. A similar result was also obtained by Zöllner *et al.* (2002), who estimated that approximately 14% of ZEA intake was excreted in 8 hours via urine, either as parent toxin or as metabolites within 8 hours. However, Dänicke *et al.* (2005b) found the urine excretion of ZEA to be 30% within 6 hours after female pigs had been given a single bolus of 1 mg ZEA per kg body weight. Up to 70.4 % of the dose was excreted in urine within 72 hours. This delayed excretion is due to the entero-hepatic cycling of ZEA demonstrated by Biehl *et al.* (1993) and Dänicke *et al.* (2005b). The concentration of ZEA and αZOL in bile has been found to be higher and

prolonged compared to urine (Biehl *et al.,* 1993; Dänicke *et al.,* 2005a) and bile has therefore been suggested to be the best biological fluid for determination of exposure (Döll *et al.,* 2003).

In the dose-response study conducted by Döll *et al.* (2003), ZEA and αZOL in pooled urine samples were found to vary with the increased levels of ZEA in the diet. A better correlation was, however, found between diet levels and bile concentrations in individual samples. Also in the study of Dänicke *et al.* (2005a), with graded fusariumtoxins given to pigs, a significant linear increase in ZEA and αZOL concentrations in bile and urine were found due to increased dietary ZEA levels. If data on ZEA feed levels and urine concentrations from recent publications (Döll *et al.,* 2003; Dänicke *et al.,* 2005a, 2007; Goyarts *et al.,* 2007) are combined in a diagram, a positive trend line for increasing urine concentration can be found. The increase is 20 ng ZEA+ αZOL per ml urine for each 100 µg ZEA per kg feed, but the correlation is low.

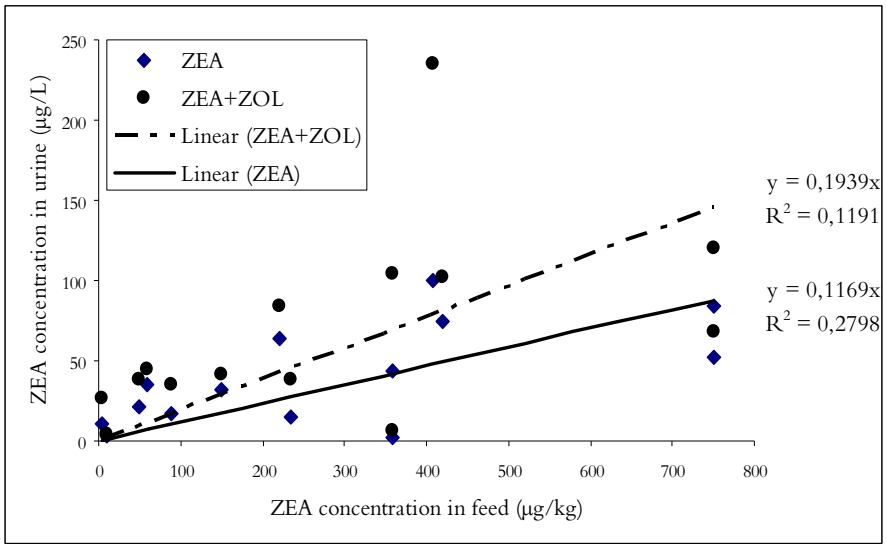

Figure 1. Correlation of urinary ZEA and its metabolite concentrations versus dietary ZEA concentration

The major excretion pathway for DON in pigs is through urine. Prelusky *et al.* (1988) found that 54-85% of radioactive labeled DON administered intra-gastric to pigs was excreted in urine within 24 hours. Similar results have been found in several more recent studies (Dänicke *et al.,* 2004a,b) when analyzing pig urine for DON and DeepoxyDON after DON consumption. About 40-60 % of DON in urine is glucuronide conjugated (Eriksen *et al.,* 2003; Dänicke *et al.,* 2005a), although this has not

been checked in most of the studies. The amount of DeepoxyDON in urine is normally only about 2-5 % of the DON in urine and 1-5% of the DON intake (Dänicke *et al.,* 2004a,b,c; Goyarts and Dänicke, 2006).

In a study with graded levels of DON in wheat to pigs Dänicke *et al.* (2004c) reported a linear increase in urine concentration of DON and DeepoxyDON with increasing dietary DON concentration. Döll *et al.* (2003) reported in a similar trial with DON in maize that DON and DeepoxyDON in pooled urine had a trend of increasing concentrations as diet contamination increased. The correlation was, however, not good. They pointed out that urine concentrations may be strongly influenced by the water consumption of the pigs. If data on DON feed levels and urine concentrations of DON and deepoxy-DON from recent publications (Döll *et al.,* 2003; Dänicke *et al.,* 2004a,b,c; Dänicke *et al.,* 2005a; Goyarts *et al.,* 2006, 2007) are combined in a diagram, a positive trend line for increasing urine concentration can be found. The increase is about 256 ng/ml when feed level is increased by 1 mg/kg.

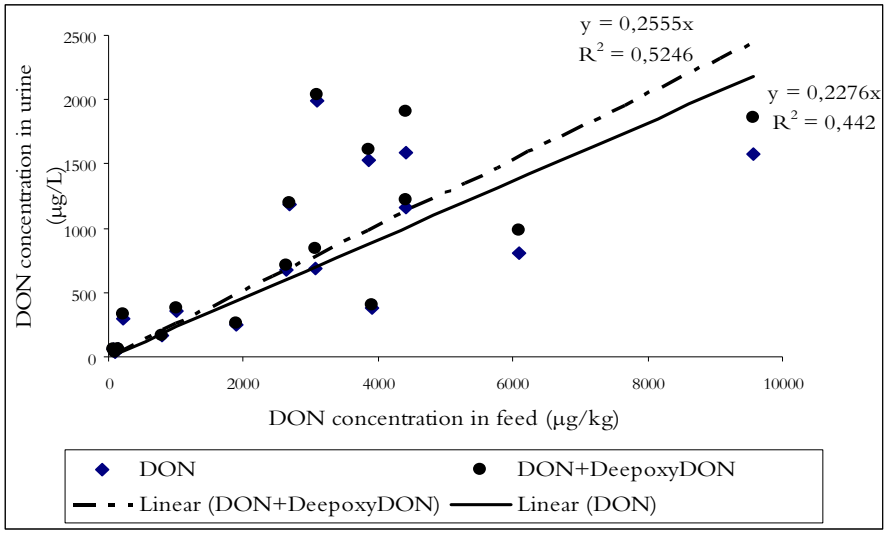

Figure 2. Correlation of urinary DON and DeepoxyDON concentrations versus dietary DON concentration

With regards to AF Lüthy *et al.* (1980) found that approximately 20% of the radioactive AFB_1 dose was excreted in the urine during 9 days after pigs were given AF. Tang *et al.* (1980) found AFB_1 and its metabolite AFM_1 in the range of 0.08 – 0.25 ng/ml and 0.21 – 0.42 ng/ml, respectively, in urine of pigs fed 26.48 µg AFB_1/kg body weight during 42 days. However, AFB_1 was not detected when pigs consumed 13.21 µg AFB_1/kg body weight,

whereas AFM_1 was found in the range of 0.3 – 0.54 ng/ml. In another trial, the average of AFB_1 and AFM_1 excreted in urine was found to be higher for AFM_1 (0.40 – 2.29 ng/ml) and lower for AFB_1 (0.05 – 0.12 ng/ml) in pigs fed diets with 12.7 μg AFB_1/kg body weight for 12 weeks (Ho, 1987). Neither AF nor its metabolite was detected in pig urine during the first week, and it was only found after one week of exposure to AFB_1. The studies on aflatoxin excretion in pig urine cited above are very few and relatively old. The relations between feed level and the excreted AF:s are not clear. A feeding study with different AFB_1 feed levels to pigs and determination of AF:s and metabolite concentration and excretion in urine was therefore conducted. The results are given below.

5.3.3 AFB feeding and urine excretion

By using current methods for measuring the biomarkers of AFB_1 in pig urine, AFM_1, AFB_1 and AFB_2 were detected at levels of 4-32, 1-35 and 0.4-0.9 ng/ml, respectively. Detailed results are given in Table 2 (Paper IV). The urine concentrations of AFB_1, AFM_1, and the sum did not differ between the diet levels. One pig excreted relatively high amounts of AFB_1 and low amounts of AFM_1 in urine on diet A, but had a similar ratio of AFM_1/AFB_1 to the other pigs on the other following diets.

Between 20 and 48% of the consumed AFB_1 dose was excreted into urine as AFB_1 and AFM_1. The proportion of AFB_1 converted to AFM_1 was on an average 22% of dose.

Low amounts of AFB_2 were also found in the pig urine. The concentration was low and similar on all diets. This may be a result of the excretion of the natural contamination of AFB_2 in the feed used for the trial. A conversion from AFB_1 can, however, not be excluded.

5.3.4 Pig urine screening in Vietnam

Pig urine from Vietnam was screened with the current biomarker methods for AF, ZEA and DON. The mycotoxins or their metabolites were detected in 14 of the 15 samples (Table 3, Paper IV). Both AFM_1 and AFB_1 were detected in 12 of the urine samples, while AFB_2 was only found in 10 of them. As expected, AFM_1 was found in the highest concentrations and in one sample 7.85 ng/ml was found. The results indicate that pigs in Vietnam are commonly exposed to AF. AF has also frequently been found in complete feeds and feedstuffs for pigs in Vietnam (Wang *et al.,* 1995; Thieu *et al.,* 2008b). The relation between AF levels in feed and pig urine concentrations is still unclear, but pigs with the highest urine concentrations

may have consumed feed containing AFB_1 in the range 50-100 μg per kg feed.

αZOL was found in seven urine samples, but it is remarkable that ZEA and βZOL were only co-occurring in one sample. Both αZOL and ZEA have been found together in urine from most feeding studies with ZEA to pigs and in similar concentrations (Farnworth and Trenholm 1981; Olsen *et al.,* 1985a; Zöllner *et al.,* 2002; Dänicke *et al.,* 2005a; 2007). Döll *et al.* (2003), have, however, found a higher ratio between ZEA and αZOL in weaning piglets. The ratio may be related to animal differences in the activity of the 3α-hydroxysteroid dehydrogenase, the enzyme which catalyses the reduction of ZEA as suggested by Olsen and Kiessling (1983). A possible explanation for the absence of ZEA in urine containing αZOL could thus be a different metabolism of ZEA in the pig breeds of Vietnam compared to European breeds. The αZOL concentrations in the urine samples were low and in the range 1-4 ng/ml, and it is impossible to conclude what feed level they may correspond to.

DON and DeepoxyDON occurred in 10 urine samples, but co-occurred only in four of them. DON was found alone in 5 and DeepoxyDON in 2 of the samples. The high DeepoxyDON concentration (37.9 ng/ml) in one of the samples is strange and co-occurred with relatively high ZEA, αZOL and βZOL concentrations. The two samples with DON + DeepoxyDON concentration around 33 ng/ml may correspond to DON feed levels just above 100 μg/kg if we assume a linear and similar excretion as found for European pigs.

There was a high co-occurrence of mycotoxins in the investigated urine samples. AF and DON+DeepoxyDON co-occurred in 9 samples and AF – αZOL occurred together in 5. All three toxin-metabolites were found in 3 samples.

6 General conclusions and implications

6.1 Conclusions

- High incidences of AF and zearalenone in feedstuffs and complete feeds for pigs in Vietnam were found, but the (mean and ranges) levels were low, except for maize and sow feed.
- Bentonite from Vietnam is able to adsorb more than 2 mg AFB_1 per g sorbent in the *in vitro* study and has a capacity to adsorb more than 12 mg AFB_1 per g sorbent, similar to HSCAS.
- Locally produced bentonite had the ability to restore the decreased performance and abnormal serum biochemical parameters caused by AF in piglets consuming AF contaminated feed.
- Analytical methods for ZEA, DON, AF and their metabolites in pig urine have been developed as biomarkers for exposure to the mycotoxins. They provide low detection and good recoveries.
- In the AFB feeding study, no difference in urine concentrations of AFM_1 and AFB_1 could be detected in pigs fed AFB_1 at three different levels (127, 227 and 327 μg/kg). Between 20 and 48% of consumed AFB_1 was excreted into pig urine as AFB_1 and AFM_1. The fraction of AFB_1 converted to AFM_1 and found in pig urine was on average 22% of dose.
- High incidences of AF, αZOL, DON and DeepoxyDON were observed in pig urine samples collected from pig farms in Vietnam. This showed that the pigs had been exposed to AF, ZEA and DON contaminated diets.

6.2 Implications

The findings of the present studies revealed that contamination of mycotoxins in feedstuffs and animal feeds is commonly observed in Vietnam and pigs are normally exposed to mycotoxins in their diets. These results highlight the need for periodic monitoring of AF and ZEA and also other mycotoxins, not only in feedstuffs for animals but also in food for human consumption. The use of locally produced bentonite at 0.4% in the diet, as used in the present study, may be a means for producers to limit or reduce the adverse effect of AF. However, an improvement in processing and purification of bentonite may be needed to enhance the surface area, and this will probably result in better adsorptive capacity for this sorbent.

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