DEVELOPMENT OF METHODS FOR THE DETERMINATION OF AFLATOXINS IN BROILER MIXED FEEDS

By

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Thesis presented for the degree of Doctor of Philosophy

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2006

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Janet Hemingway, for giving me the opportunity to study at Cardiff University, and for all her kind advice over the past three years. I would like to express my deep appreciation to Dr. Sumon Sakolchai, who suggested that I study at Cardiff and for his encouragement and advice during my study period.

Special thanks are extended to Mrs. Aruneepong Srisathaporn and Mr. Thanee Tessiri for technical advice and support in undertaking my research.

I am also indebted to all members of Professor Hemingway's research group at Cardiff, especially Dr. Graham Small, Dr. Patricia Penilla and Dr. John Vontas.

Thanks also to Mrs. Julie Harris for her kind assistance in many ways during my study.

Thanks to my wife, Fiat, for all her love and support. To the Great Spirit of my father, who I dedicate this thesis to.

Finally, I am grateful for the financial support of The Japan International Co-operation Foundation, especially Mr. Ryoichi Jinnai, the President of the Foundation; Mr. Yoshinori Imagawa, the Permanent Director and Mr. Susumi Akiyama, General Manager. Without this foundation, I would not have had the opportunity to study for this PhD.

ABSTRACT

DEVELOPMENT OF METHODS FOR THE DETERMINATION OF AFLATOXINS IN BROILER MIXED FEEDS.

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This study was undertaken to respond to the requirement of the emerging Thai broiler producers to comply to international standards in product aflatoxin contamination. Field work was established with farmers to improve their animal husbandry. A standard methodology for aflatoxin analysis was also established.

The efficiency of four commercial clean-up columns for the determination of aflatoxins in broiler mixed feeds were compared and contamination levels of aflatoxins in broiler mixed feeds marketed in Thailand were determined. The four clean-up columns used were from Varian, Vicam, Romer and Rhône. Two types of broiler feed, manufactured by the Charoen Pokphand (C.P.) company and the Betagro company were tested. Samples tested were standard aflatoxin solution, spiked broiler mixed feed and naturally contaminated feed. All samples were cleaned-up using the four columns followed by quantification of aflatoxins by HPLC. Comparative column efficiency was determined. Feed collected from the field over a 42 day period was examined for aflatoxin contamination using the Varian column.

There was variation in the efficiency of columns when different types of samples were used. There was also a difference in the column efficiency when different sources of feed samples were applied. The relative column efficiency for the clean-up of the standard aflatoxin solution was, in descending order, Varian, Vicam, Rhône and Romer columns. Based on the SAS analysis by CRD, the Varian column gave the best aflatoxin recoveries.

Aflatoxin recoveries from the four different columns were determined with spiked Betagro feed samples, Efficiency was in descending order, Varian, Vicam, Romer and Rhône columns. For the spiked C.P. feed the order was Vicam, Varian, Rhône and

Romer columns, respectively. There was a significant difference in column efficiency. The Varian and Vicam columns were significantly more efficient than the Rhône and Romer columns. The Varian and Vicam columns had the highest relatively efficiencies for both brands of broiler mixed feeds.

The natural contamination of aflatoxin determined in two brands of feed using the Varian columns was 14.41 to 18.40 p.p.b. for the C.P. feed and 11.33 to 18.18 p.p.b. for the Betagro feed when samples were collected from the delivery sacks. When samples were collected from feeding bins the range was 18.49 to 20.39 p.p.b. for the C.P. feed and 17.30 to 20.67 p.p.b. for the Betagro feed. Feed samples were of an acceptable quality and all broilers were normal. As the aflatoxin contamination levels detected in broiler mixed feeds were low, their relationship to changes in broiler physiology could not be determined.

The field study on broiler quality in 1998 indicated some abnormalities on the broilers but there was no such problem on the broiler's quality in 2000, which may be accounted by the instigation of the improved animal husbandry.

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LIST OF ABBREVIATION

AF	Aflatoxin
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemist Society
BCD	Before Column Derivatization
BF method	Best food method
C	Celcius
CB method	Contaminant branch
C.P.	Charoen Pokaphand company
CRD	Completely Randomized Design
EC method	Europian Community method
ECD	Electron Capture detection
FCR	Feed Conversion Ratio
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
GC	Gas Chromatography
GLC	Gas-Liquid Chromatography
GST	Glutathione S-transferase
HFB	Heptafluorobutyryl
HPLC	High Performance Liquid Chromatography
IAC	Immuno Affinity Column
ICA	Immunoaffinity Column Assay
LC	Liquid chromatography
LC-MS	Liquid Chromatography-Mass Spectrophotometry
MECC	Micellar Electrokinetic Capillary Chromatography
MFC	Multifunctional Column
MS	Mass spectrophotometry
NICI-MS	Negative-ion Chemical Ionization-Mass Spectrophometry
NP-TLC	Normal-Phase Thin Layer Chromatography
PCD	Post Column Derivatization
Pi	Phosphate

PH	Phenyl
PH method	Phenyl-bonded elut method
ppb	parts per billion
RIA	Radio Immuno Assay
RIC	Reconstructed Ion Chromatogram
RP-TLC	Reverse Phase Thin Layer Chromatography
SCF	Supercritical Fluid Chromatography
SCX	Strong Cation Exchanger
SPE	Solid Phase Extraction
TFA	Trifluoracetic acid
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
µl	microliter
UV	Ultraviolet
USDA	United States Department of Agriculture

CHAPTER 1

CHAPTER 1: INTRODUCTION

Background to this study:

Aflatoxins are important mycotoxins, which have a significant impact on the economy and on the health of animals and humans in most countries. Although aflatoxins have received much attention and intensive research has been undertaken on various aspects of their chemistry over the last four decades, problems with aflatoxins still exist. Most recent studies emphasize the need to develop new techniques to detect and quantify aflatoxins in animals and feed in order to achieve high sensitivity and specificity, and apply these techniques to determine the aflatoxin content in complex samples of food. In a developing country context animal husbandry techniques also need to be improved to avoid aflatoxin contamination of feed and poultry and allow farmers access to international markets for their produce.

There are many studies that show the harmful effects of aflatoxin on animal and human health, both at the acute and the chronic levels. In humans, aflatoxin induced liver cancer is a problem in Asia and Africa (Austwick, 1984). In animals aflatoxins cause hepatoxicity, teratogenicity, carcinogenicity and immune response deficiency (Wogan *et. al.*, 1971; Blunden *et. al.*, 1991; Jakubowska *et. al.*, 1984). Aflatoxins influence broiler performance and adversely affect the broilers producing a variety of symptoms in animals from chicken farms (Huff, 1980; Osbourne *et. al.*, 1975).

Various methods have been attempted to prevent and reduce aflatoxin contamination that occurs naturally in animal feeds (Kamimura, 1993). In many countries the tolerated limits of aflatoxin contamination in different commodities have been specified and these limits have been enforced to ensure that all imported food and feeds conform to these standards (Kamimura, 1993). Therefore, the determination of aflatoxin concentrations in various food, feeds, and commodities are of great importance.

Chemically, aflatoxins are a group of closely related mycotoxins consisting of a substituted coumarin structure and a fused bisfurano moiety. Twenty aflatoxins have been isolated and identified, four of them are naturally prevalent. These are aflatoxin B_1,

B_2, G_1 and G_2 (Kamimura, 1993). On exposure to long wavelengths, aflatoxins fluoresce making their detection possible by spectrophotometric techniques.

At present there are good methods to determine aflatoxin concentrations in simple food samples (Trucksess and Wood, 1994). These techniques include thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas liquid chromatography (GLC), and immunochemical methods, such as radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA) and immunoaffinity column assays (ICA) (Kamimura, 1993). However, determination of aflatoxins in more complex feeds is difficult. The matrix of raw materials and chemical constituents of many feeds necessitates purification and clean-up of samples prior to analysis. Since aflatoxin contamination in feeds normally occurs as very low concentrations (ppb levels) in complex chemical constituents clean-up methods need to be carefully controlled to ensure that levels can be accurately measured.

This study had two objectives:

1. To work with the farmers in large co-operatives in central Thailand to improve animal husbandry and reduce resultant aflatoxin contamination in their broilers.
2. Development of methods for extraction and clean-up of complex broiler feeds prior to aflatoxin analysis.

Earlier conventional method for aflatoxin extraction used solvent systems, which were appropriate for some feeds. Later, development of column chromatography and chemical adsorption procedures replaced extraction with conventional solvent systems. Most column chromatography steps employed silica gel or others stationary phase packing materials such as florisil, alumina and cellulose powder. The disadvantages of these methods are low recovery and poor reproducability (Coker *et. al.*, 1984; Stoloff and scott, 1984; Kamimura *et. al.*, 1985).

Recently development extraction and clean-up methods use solid-phase extraction (SPE), (referred to as liquid-solid extraction). The SPE technique is quick, solvent efficient and economical. Various commercial clean-up columns using the SPE concept are now available and have been used to determine the level of mycotoxin in many

contaminated feeds (Coker and Jones, 1985; Chu, 1991b; Scott, 1993b). Among the commonly-used columns, those from Varian, Vicam, Romer and Rhône are reported to allow efficient extraction of mycotoxins from many types of sample (Trucksess and Wood, 1994).

The use of such commercial columns for the extraction and clean-up of broiler mixed feeds has never been reported. In Thailand, extraction and clean-up methods for the determination of mycotoxin in animal feeds still employs conventional column chromatography and in some limited cases ELISA is used. The new commercial clean-up columns have never been used for aflatoxin determination, but the rapid development of the Thai broiler chicken export market means that the industry needs a more efficient method of aflatoxin determination.

Broiler mixed feeds, used for the production of broilers by Thai farmers, consist of raw materials that are easily contaminated by aflatoxins (Khajaroen *et. al.*, 1997; Charoenwai, 1999). These broiler mixed feeds are prone to natural contamination by aflatoxins. The aflatoxin contamination may occur during the manufacturing, transportation, or storage of the feeds, especially when they are exposed to extreme tropical conditions. In Thailand there are two major animal feed producers, the Charoen Pokphand (C.P.) company and the Betagro company, these companies supply large co-operative of broiler farmers who then supply their produce to the companies for export.

OBJECTIVES

The aims of this study were to

1. Work with farmers in the large co-operative groups sponsored by C.P. and Betagro to improve their methods of animal husbandry during rearing of broilers for the international export market.
2. Determine the column efficiency of 4 different commercially available columns for the extraction and clean-up of standard aflatoxin solutions and for aflatoxin-spiked broiler mixed feeds.

3. Compare the column efficiency among these different commercial clean-up columns with standard aflatoxin solutions and aflatoxin-spiked broiler mixed feeds from the two major feed producers.
4. Compare the recovery of total aflatoxins and the different individual aflatoxin subtypes.
5. Determine the degree of aflatoxin contamination naturally occurring in two sources of broiler mixed feeds from, the C.P. company and the Betagro company.
6. Examine the commercial characteristics, levels of aflatoxin contamination and performance of broilers fed on these mixed feeds.

CHAPTER 2

CHAPTER 2: LITERATURE REVIEW

Part I of this review highlights the methods used for the determination of aflatoxins, especially commercial clean-up columns. Part II presents a review of aflatoxin contamination in animal feeds and the effects of aflatoxins on animals and humans.

PART I

AFLATOXINS AND THE METHODS FOR THEIR ANALYSIS

Mycotoxins have drawn worldwide attention since 1960, when >100,000 poultry died in the UK from the liver disorder "Turkey X disease", caused by a mycotoxin (Blount, 1961). This event stimulated the active study of its cause, and eventually *Aspergillus flavus* was isolated from peanut meal used in their feed. In 1963, a highly toxic mycotoxin, named aflatoxin was identified from *A. flavus* as the compound responsible for poisoning the turkeys. This is one of the most strongly carcinogenic naturally occurring substances known.

Several mycotoxins are now known to be involved in the etiology of some human and animal diseases, stimulating the development of methodologies to study their detection and quantification. An awareness of the levels of mycotoxin contamination of natural products can only be obtained by developing good analytical methodologies for their detection in food, mixed feed and feed ingredients, animal tissues, blood, urine and milk.

Since mycotoxins display a wide diversity of chemical structure, there are no uniform methods of analysis either for mycotoxins collectively or for a specific toxin in different feeds. However, the main mycotoxins can now be readily identified qualitatively and quantitatively and most current investigations concentrate on increasing sensitivity,

accuracy and reproducibility of detection with a concomitant decrease in the time and cost of analysis.

STRUCTURE AND CHEMICAL PROPERTIES OF AFLATOXINS

Aflatoxins are a group of closely related mycotoxins with a substituted coumarin structure and a fused bisfurano moiety (Kamimura, 1993). Twenty aflatoxins have been isolated and identified (Cole and Cox, 1981). Four are naturally prevalent and have been designated aflatoxin B_1, B_2, G_1 and G_2 because of their characteristic blue (B) or blue-green (G) fluorescence after excitation with light at 365 nm, and their order of chromatographic elution. Aflatoxin B's are metabolized by animals and can be discharged in milk. The chemical properties of different aflatoxins (molecular formula, molecular weight, melting points, absorption wavelength and wavelength where fluorescence is emitted) are shown in Table 1.

Table 1. Chemical properties of the aflatoxins.

Aflatoxin	Molecular formula	Molecular weight	Melting point	362-363 nm absorption (ε)	Fluorescence emission (nm)
B_1	$C_{17}H_{12}O_6$	312	268-269	21,800	425
B_2	$C_{17}H_{14}O_6$	314	286-289	23,400	425
G_1	$C_{17}H_{12}O_7$	328	244-246	16,100	450
G_2	$C_{17}H_{14}O_7$	330	237-240	21,000	450
M_1	$C_{17}H_{12}O_7$	328	299	19,000 (357 nm)	450
M_2	$C_{17}H_{14}O_7$	330	293	21,000 (357 nm)	–
GM_1	$C_{17}H_{12}O_8$	344	276	12,000 (358 nm)	–
B_{2a}	$C_{17}H_{14}O_7$	330	240	20,400	–
G_{2a}	$C_{17}H_{14}O_8$	346	190	18,000	–
Aflatoxicol	$C_{17}H_{16}O_6$	314	230-234	14,100	425

The structures of various types of aflatoxins are illustrated in Figure 1.

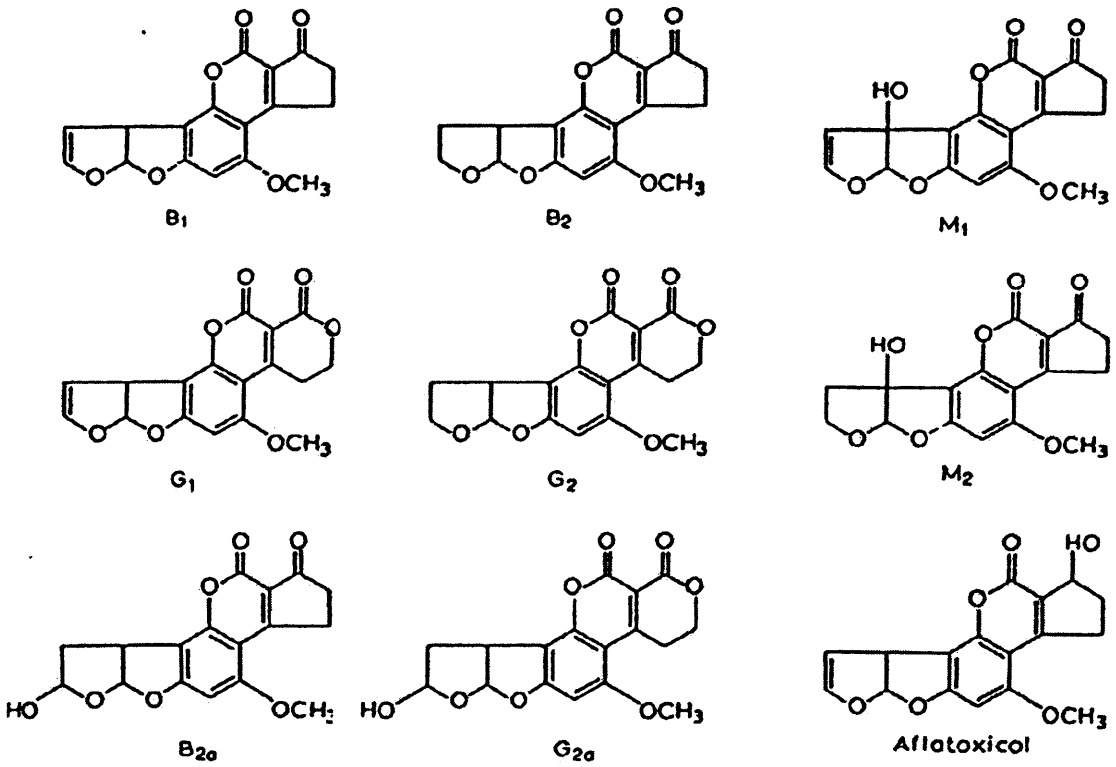


Figure 1. The Chemical structures of different aflatoxins.

METHODS FOR MONITORING AFLATOXIN CONTAMINATION OF FOOD.

Analytical procedures for determination of mycotoxin levels from any sample include three major steps: extraction, separation, and determination (Trucksess and Wood, 1994). Mycotoxins occur normally in food at very low concentration (ppb levels) in a complex chemical matrix. Prior to quantification, the mycotoxins must be extracted from such matrices. Mycotoxins and other co-extracted materials are then processed to remove non-mycotoxin contaminants in the clean-up process. Conventional extraction processes involve homogenization, either mechanically or manually, with a suitable solvent system.

Most mycotoxins, including aflatoxins, are soluble in slightly polar solvents and usually insoluble in completely non-polar solvents. Mycotoxins may exhibit differential binding to organic molecules and differing degrees of solubility in water. In practice, mycotoxins are extracted using mixtures of organic solvents such as chloroform, acetonitrile, methanol, acetone, ethylacetate or dichloromethane, often in combinations with small amounts of water or acids. The latter are used as aqueous solvents more easily penetrate hydrophilic tissues and enhance toxin extraction. With the correct proportions of water to solvent, the toxins are often more readily partitioned into the solvent (Trucksess and Wood, 1994). The first solvent system used for extraction was a mixture of chlorocarbon and water. However, this is now being replaced by methanol-water or acetonitrile-water systems.

The presence of pigments, fats and lipids in extracts from samples will reduce the efficiency of subsequent separation techniques. By adding non-polar solvents, such as hexane, to the extraction solvents, many of the fats and lipids are partitioned into the hexane and can be discarded, enhancing the efficiency of the mycotoxin extraction.

Although many contaminants may be partially removed during extraction, further clean-up of the extract is normally required. Column chromatography techniques for clean-up are

now widely practiced. The choice of column packing material depends on the contaminants and the particular mycotoxins. The types of column adsorbents used include silica gel, florisil, polyamide or Sephadex. The extracted samples are applied to the clean-up columns and, after washing the column with suitable solvents, that do not elute the mycotoxin, appropriate elution solvents can be applied to the column to elute and collect the mycotoxin.

The ultimate aim of the clean-up procedure is to remove most of the co-extracted material, reducing the chemical complexity of the final extract, which is used for detection and quantitation.

CONVENTIONAL METHODS FOR AFLATOXIN EXTRACTION

Various solvents have been used to extract aflatoxin from oilseeds, peanuts and cottonseed prior to their being quantitatively analyzed (Dollear, 1969; Hron *et al.*, 1992, 1994). Generally animal feeds are produced from different ingredients and extraction procedures need to be tailored to the specific feed matrix. The solvents used in the literature include 95 % ethanol, 90 % aqueous acetone, 80 % isopropanol, hexane-methanol, methanol-water, acetonitrile-water, hexane-ethanol-water and acetone-hexane-water. The solvent: sample ratio is also a crucial factor in the optimal recovery of the toxin (Cole and Dorner, 1994).

A variety of clean-up methods have been employed, including column chromatography, liquid-liquid extraction and chemical adsorption procedures. Clean-up methods originally employed column chromatography, with silica as the most popular packing material. Other stationary phases such as Florisil, alumina and cellulose powder have been used for column chromatography (Coker *et al.*, 1984; Stoloff *et al.*, 1984; Kamimura *et al.*, 1985). These methods are often laborious, time-consuming and costly. Their poor reproducibility and low recoveries, make them less than ideal as precise quantification methods.

The commonly used methods for aflatoxin extraction were developed by Best Food (BF) (Association of Official Analytical Chemists (AOAC), 1980), Contaminant Branch (CB) (AOAC, 1980a), Pons' (Pons *et al.*, 1966) and Romer (Romer, 1975). Selected methods from these sources are shown in Figures 2 and 3. These are standard methods accepted by AOAC for extraction and estimation of aflatoxin in groundnuts and groundnut butter. The Pons' method was developed for determination of aflatoxin in cottonseed products, but has been used for aflatoxin estimation from many other agricultural commodities. The method of Romer (1975) has been used for extraction and estimation of aflatoxins in mixed feed including groundnut meal.

Mehan *et al.* (1984) found marked differences in quantities of AFB_1 from groundnuts after extraction using the BF, CB, Pons' and Romer methods. The BF and Pons' methods were the most efficient. The CB method was slightly less efficient than the BF and Pons' methods while the Romer method extracted considerably lower amounts of AFB_1. The NaOH and KOH alkali treatment steps used in the clean-up may have been responsible for the low extraction efficiency of the Romer method.

Economy and speed of analysis are also important factors in choosing a method for mycotoxin analysis. Although the CB method is efficient (Chang *et al.*, 1979), it is a lengthy and expensive clean-up procedure. The BF method is the least expensive and time consuming compared to the other methods. The Pons' method was the next best in respect of cost and time requirements. The Pons' method is convenient for handling large numbers of samples, especially in the absence of centrifugation facilities.

Arim *et al.* (1995) compared the AOAC, The American Oil chemists' Society (AOCS) and the European Community (EC) methods for aflatoxin determination in copra meals for accuracy and practicality (cost, speed, equipment and skill requirement as well as exposure risk). They reported that the EC and the AOCS methods were the most appropriate for the analysis of copra meal aflatoxin.

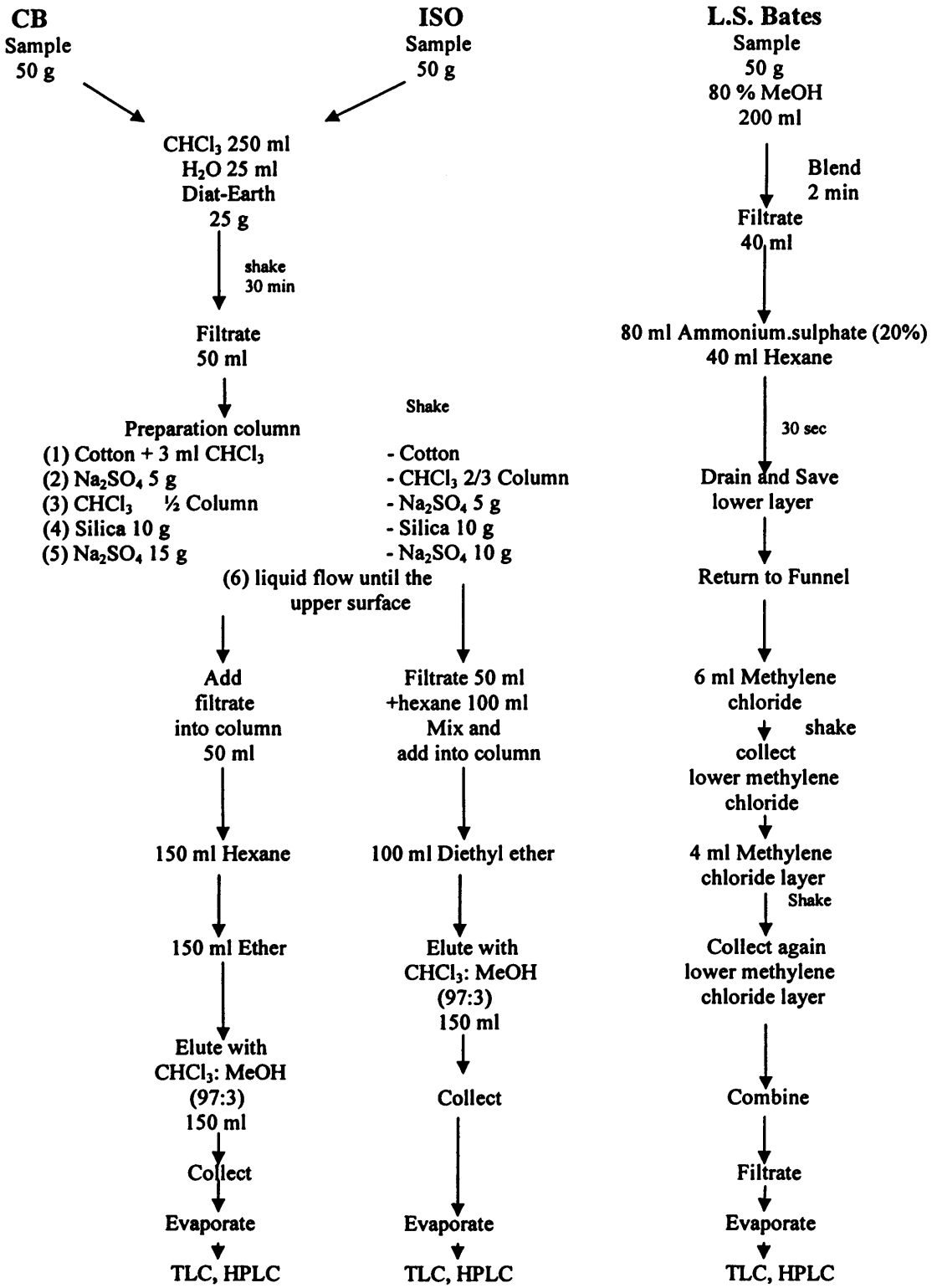


Figure 2. Diagrammatic representation of the steps involved in three traditional aflatoxin extraction methods, CB, EC (ISO) and L.S. Bates methods.

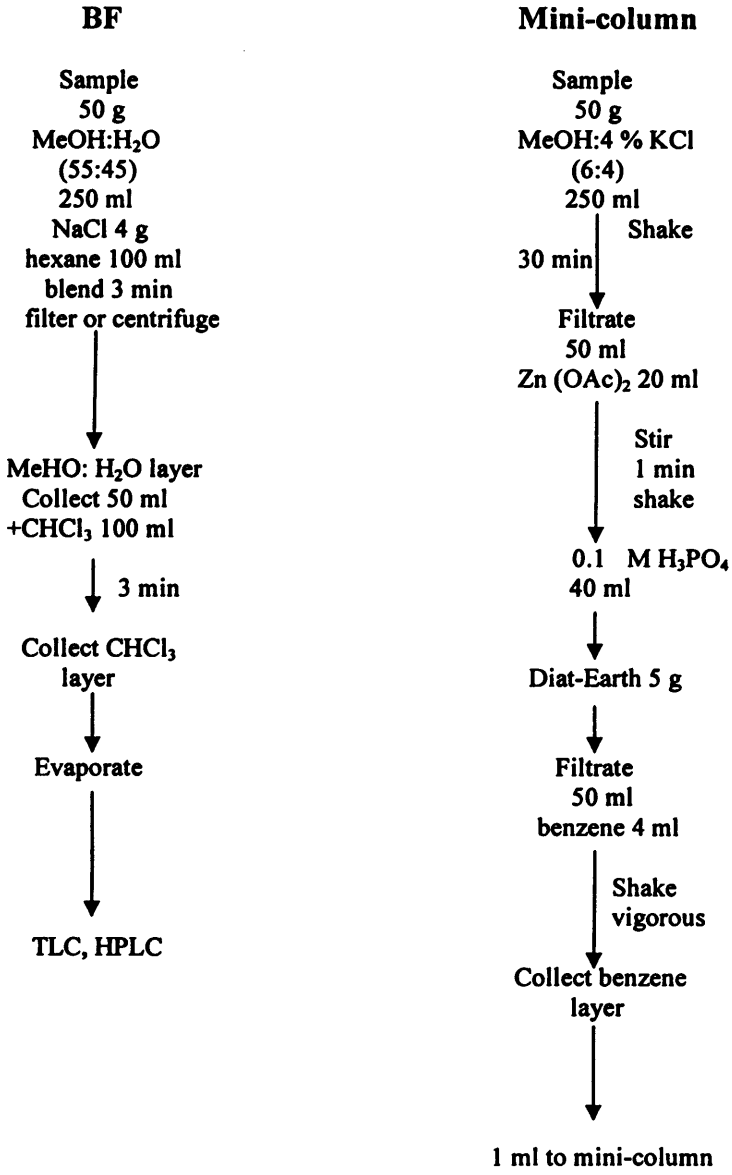


Figure 3. Diagrammatic representation of the steps involved in two traditional aflatoxin extraction methods, the BF and mini-column methods.

DEVELOPMENT OF NEW COMMERCIAL CLEAN-UP COLUMNS FOR AFLATOXIN EXTRACTION

The traditional procedures used in purification are column chromatography (silica gel) and liquid-liquid partitioning, involving large solvent volumes (>200 ml). Considerable time is required for preparing the adsorbent, packing the chromatographic columns, eluting the toxins from the columns, and evaporating the solvent. A significant recent improvement in the purification process is the use of solid-phase extraction (SPE). This method is quick, solvent efficient, and economical (Coker and Jones, 1988; Chu *et al.*, 1991; Holcomb *et al.*, 1992; Scott, 1993b). Examples of available commercial columns are Sep-Pak®, Bond-elut®, Aflatest®, Aflaprep®, Easi-Extract® and Multifunctional.

An SPE cartridge is a micro-column made of plastic tubing containing 100-500 mg 40-μm stationary-phase particles in the middle and plastic frits at both ends. Most aflatoxin SPE columns and cartridges contain silica gel (Kozloski, 1986), C_{18} bonded to silica gel (Van Egmond *et. al.*, 1988), florisil (Jewer *et al.*, 1989), phenyl, aminopropyl, aflatoxin antibody-agarose (Trucksess *et al.*, 1991) or strong anion exchange (quaternary ammonium) bonded phases. The bonded phase provides good clean-up of extracts containing fumonisins (Sydenham, 1992). One SPE column contains an inert hydrophilic diatomaceous earth and replaces liquid-liquid partitioning, for example, for the determination of trichothecences in grain (Scott *et al.*, 1986). In general, the adsorbent (bonded phase) material in the cartridges is prepared by reacting the hydroxyl group of silica with an organosilane to replace the hydrogen of the hydroxyl group with an appropriate moiety. The bonded phase, so created, takes on the physical properties of the bonded grouping (Engelhardt and Ahr, 1981) and can be employed with a wide range of solvent systems.

Usually a multi-cartridge vacuum manifold is used to pull extract and eluting solvent through the column. However, the antibody - agarose columns are quite fragile and require

the application of positive pressure with a piston syringe. The elution conditions for the cartridges are chosen to retain the mycotoxin on the adsorbent while the co-extracted contaminants are washed from the cartridge with the eluant; alternatively, the co-extracted materials are retained, while the aflatoxins are washed through the cartridge. SPE cartridges require less solvent than conventional column chromatography systems or liquid-liquid partitioning (Trucksess *et al.*, 1984; Hutchins *et al.*, 1989). The volume of eluate containing the mycotoxin is suitable for subsequent liquid chromatography injection, making automation of the analysis possible.

Many workers have reported development of automated and semi - automated methods for mycotoxin analysis that utilize various bonded phase adsorbents (Tomlins *et al.*, 1989; Hurst, 1984; Qian and Yang, 1984; Van Egmond *et al.*, 1991). Examples of bonded phases include ethyl (C_2), octadecyl (C_{18}), octyl (C_8), cyclohexyl (C_6), phenyl (non-polar), cyanopropyl, diol, and aminopropyl (polar). During SPE clean-up, partitioning of the mycotoxins and interfering compounds occurs between mobile and stationary phases.

Supercritical fluid chromatography (SFC) on fused silica GC capillary columns and LC packed columns has also been applied for separation of various Fusarium toxins (Young and Games, 1992). This type of technique has not received much attention in mycotoxin analysis from food, primarily due to supercritical fluid extraction problems (Engelhardt and Haas, 1993).

Multifunctional SPE columns are packed with a mixture of reverse phase, ion exclusion or ion exchange adsorbents. The extract containing aflatoxin is forced up through the column, co-extracted materials are retained and the aflatoxin is eluted up through the top of the column (Wilson and Romer, 1991). Aflatoxins B_1 and G_1 are derivatized to form water adducts with trifluoroacetic acid as a catalyst. The derivatives and the unreacted aflatoxins B_2 and G_2, are injected into a reverse phase liquid chromatography column. After separation the individual aflatoxins are determined by florescence detection.

Immunoaffinity clean-up methods are specific and sensitive (Candlish *et al.*, 1988; Trucksess *et al.*, 1991; Patey *et al.*, 1991; Sharman and Gilbert, 1991). They used monoclonal antibodies against aflatoxins bound to a gel material, such as Sepharose 4 B, in a small cartridge. When the extract is passed through the column, aflatoxin is bound to the recognition site of the immunoglobulin and extraneous material is washed from the column by water. The aflatoxin, in purified form, is then eluted and recovered using methanol or acetonitrile. Immunoaffinity columns are commercially available and have been routinely employed for determining aflatoxins in nuts, nut products, and dried fruit (Patey *et al.*, 1991; Sharman *et al.*, 1991), and for determining AFM_1 concentrations in milk (Mortimer, 1987) and cheese (Sharman *et al.*, 1989). These columns have the advantages of speed and simplicity compared to conventional clean-up, and have high specificity, producing extracts free of contaminants. A disadvantage is the slow constant column flow rate, which is tedious when carried out manually and can be a source of variable recoveries when not properly controlled (Patey *et al.*, 1991).

SOLID PHASE EXTRACTION

Solid phase extraction utilizes the same analyte/sorbent interactions that are exploited by high performance liquid chromatography (HPLC). Bond Elut extraction cartridges from the Varian company are packed with a variety of surface-modified bonded silica sorbents that selectively retain specific classes of chemicals from within a given matrix. As an example, the Bond Elut Strong cation exchanger (SCX) retains the cationic drug, amphetamine, from urine. The more specific the interaction between the sorbent and analyte, the cleaner the final extract.

Bonded silica sorbents are in many ways the ideal materials for chromatographic isolation, primarily due to the number of different functional groups that can be readily bonded to the silica surface. In addition, bonded silicas are rigid supports that do not shrink or swell; possess very large surface areas due to porosity; are stable under a wide range of aqueous and organic solvent conditions; and form a clean, substrate upon which to bond the functional groups.

Steps of solid phase extraction

The common goals of all extraction protocols are efficient clean-up, concentration, and solvent exchange (e.g. aqueous to organic) prior to analysis. Solid phase extraction achieves these goals in four simple steps (see Figure 4). They are:

1. *Conditioning*: Preparing the cartridge for reproducible interaction with the sample matrix by solvating the sorbent bed. This is done by passing a volume of a liquid, similar in nature to the sample matrix, through the column. A common example of cartridge conditioning would be to pass methanol, followed by water, through a C_{18} cartridge prior to extraction of an aqueous sample matrix.

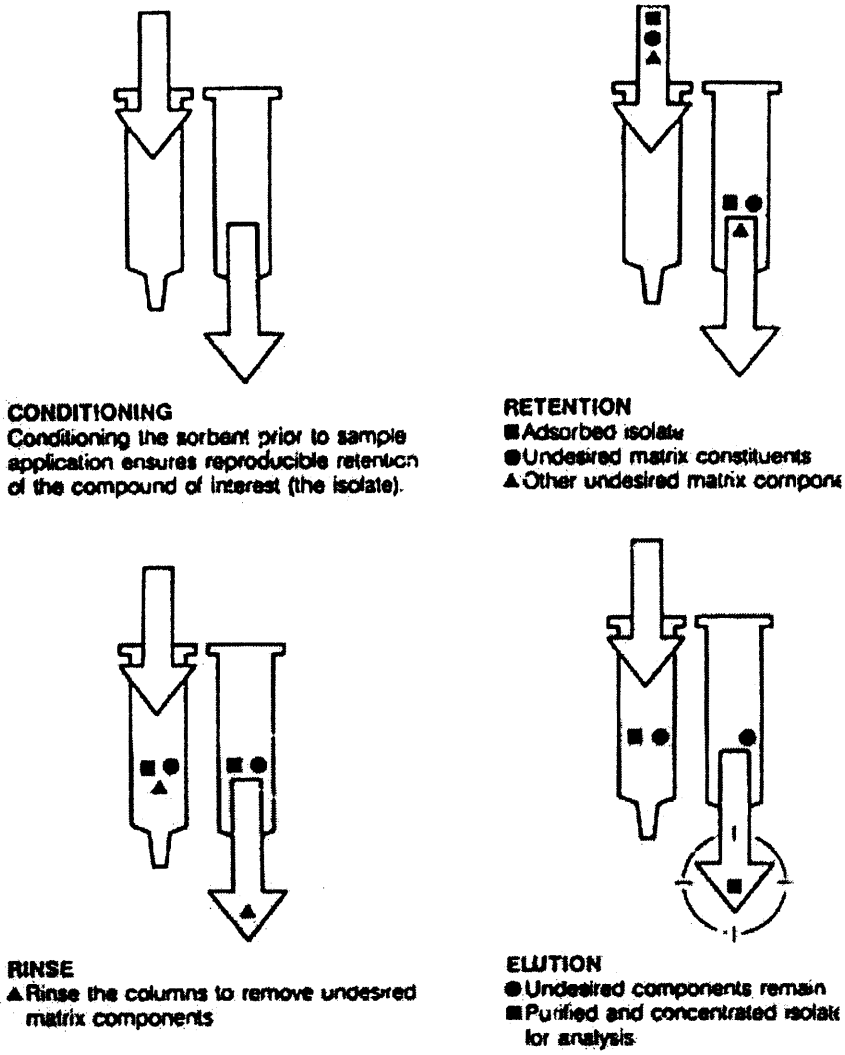


Figure 4. Principles of the different steps involved in solid phase extraction (from Varian's instruction, 1998).

2. *Retention*: Applying the sample to the conditioned cartridge results in the analyte, and perhaps other matrix components, being retained on the sorbent surface, due to one or more specific chemical interactions (e.g. Van der Waals or "non-polar" interactions between the hydrocarbon chain of an analyte and the hydrocarbon chain of a C_{18} bonded phase). Some matrix contaminants may pass through the cartridge unretained, hence cleaning up the sample even at the retention or loading step.

3. *Rinsing*: Passing solvents through the cartridge then removes additional contaminants while the analyte is retained within the sorbent bed. A common rinse solvent for a non-polar extraction on a C_{18} sorbent would be water.

4. *Elution*: Passing an appropriate solvent through the cartridge, which is specifically chosen to disrupt the analyte-sorbent interaction, results in selective elution of the analyte. To use a non-polar extraction example again, an organic solvent such as methanol is strong enough to disrupt the interaction between most non-polar analytes and a C_{18} bonded phase.

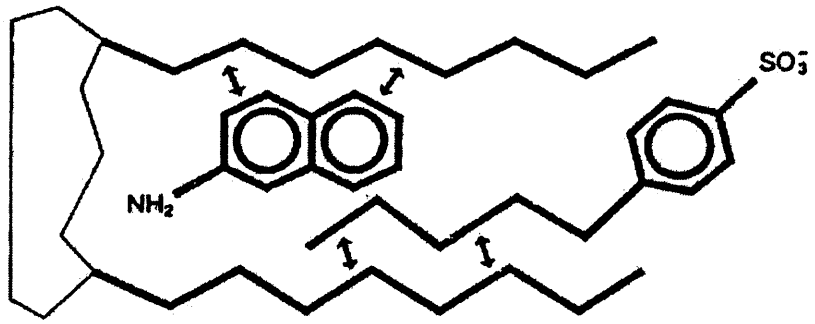
Selection of the extraction mechanism and sorbent

There are three general extraction mechanisms used in solid phase extraction: non-polar, polar, and ion-exchange. Sorbent mechanism selection is primarily based upon the functional groups present on the analyte and the composition of the sample matrix.

Each sorbent within a given extraction mechanism exhibits unique properties of retention and selectivity which may be quite specific for a given analyte. So even if an extraction calls for a non-polar extraction mechanism, it may still be necessary to test several sorbents to find the optimal balance between high recovery and efficient clean-up.

For example, both C_{18} and C_8 give acceptably high recoveries for non-polar analytes from an aqueous matrix, but the slightly increased polarity of C_8 may allow many matrix contaminants to pass through the cartridge, which would otherwise be retained on the more non-polar C_{18} sorbent. The end result is a cleaner final extract with the C_8 sorbent.

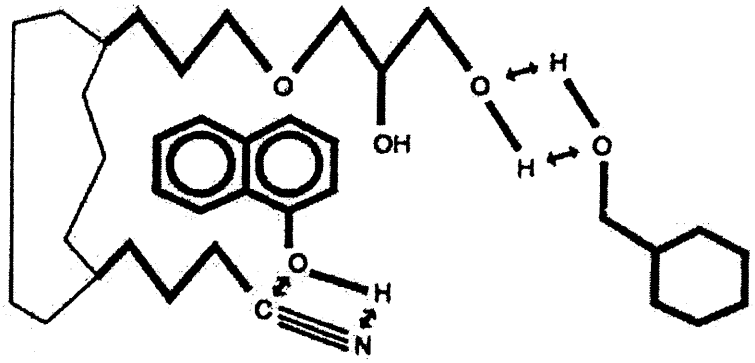
The Varian companies SPE offers several specialty phases; Certify I and Certify II for the extraction of drugs of abuse from urine and other aqueous biological matrices; PBA for cis-diols, sugars, amino acids, and nucleotides; EnvirElut for oil and grease and other non-polar environmental extractions.



Silica base Bonded functional group (C_8)

⟷ Van der Waals forces

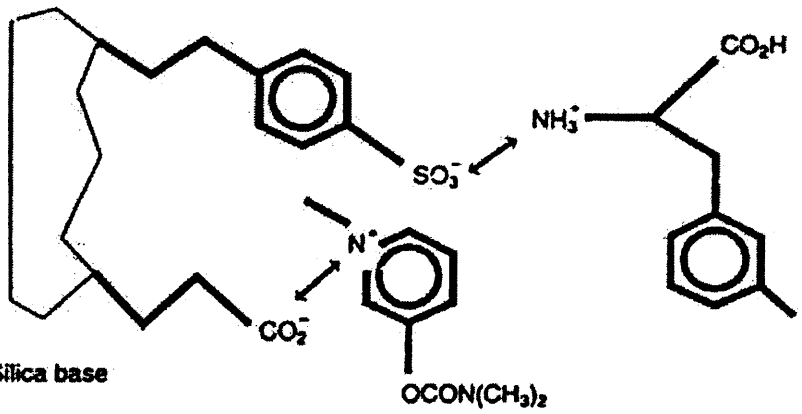
Interactions on non-polar phases



Silica base

⟷ Dipolar attraction or hydrogen bonding

Interactions on polar phases



⟷ Electrostatic attraction

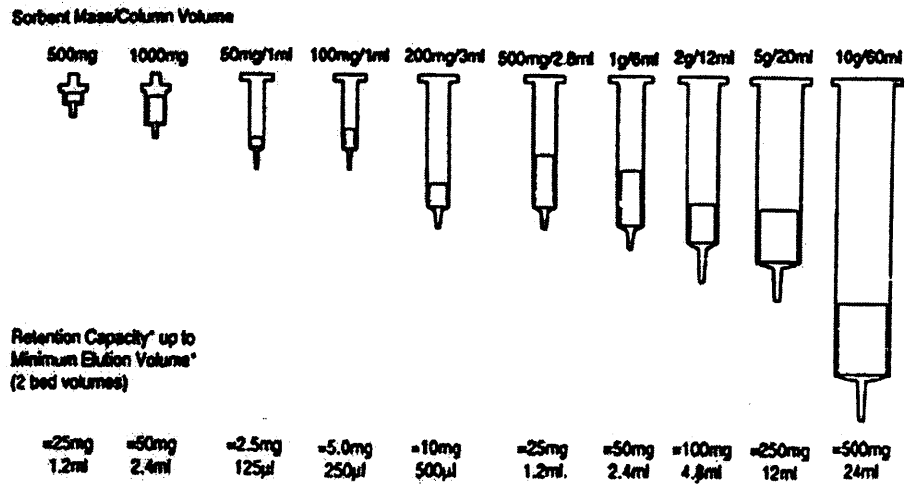
Interactions on ion-exchange sorbents

Figure 5. Interactions between analyte and functional groups of the bonded silica sorbents (from Varian's instruction, 1998).

Bond Elut cartridges are available in a variety of sizes, ranging from 50 mg to 10 g of sorbent. Smaller cartridges are useful for small samples or when the ability to elute the analyte in a very small volume is needed for maximum concentration.

Typical retention capacity for polar and non-polar sorbents is approximately 5 % of the sorbent mass (i.e., 5 mg for a 100 mg sorbent bed). However, this value must take into account additional compounds present in the sample matrix that are retained by the sorbent. Thus, the effective capacity for the analyte may be lower, and the cartridge capacity for each specific application should be tested.

While, larger sorbent amounts provide greater retention capacity, they also require more solvent to elute the compound from the column. Consequently, the analyte may be more dilute than if a smaller sorbent bed was used.



Approximate Values: Capacity (mg) = 5% of sorbent mass

Bed Volume = 120 µl/100 mg of sorbent

Figure 6. Capacity and elution characteristics of different sizes of Bond Elut columns (from Varian's instruction, 1998).

The minimum elution volume for a cartridge is defined as two bed volumes of elution solvent. A bed volume is 120 µl of solvent per 100 mg of sorbent. In some cases, less than two bed volumes can be used. However, such extractions are often very sensitive to flow rates and other variables and are not recommended because results are difficult to reproduce.

There are a variety of ways to process samples using Varian Bond Elut cartridges. Up to 24 samples may be processed manually using one of the Vac Elut vacuum manifolds. Single samples can be processed using a syringe to push solvents through the cartridge with an appropriate adapter. Cartridges can even be spun in a centrifuge using the centrifugal force to draw solvents through the sorbent bed.

Automated solid phase extraction is rapidly gaining popularity due to the tremendous productivity gains achieved with the successful combination of SPE and automated SPE hardware. The 96 well plate format is ideal for automated systems where a large number of samples need to be processed quickly. Varian's Bond Elut cartridges are the industry standard for automated SPE due to the flexibility of sorbent chemistries, syringe barrel tube configurations and extremely high manufacturing tolerances.

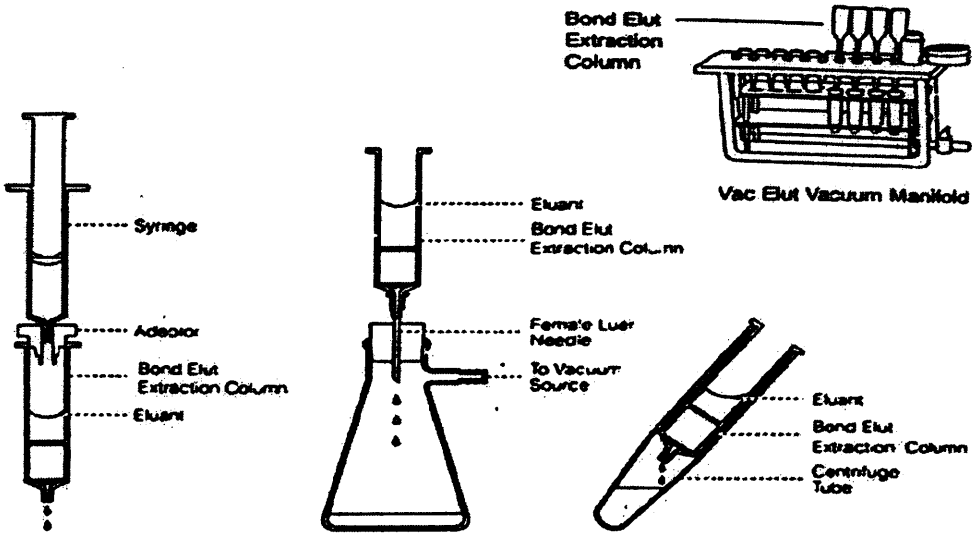


Figure 7. Methodologies involved in using Bond Elut (SPE) cartridge (from Varian's instruction, 1998).

RECENT STUDIES OF COMMERCIAL CLEAN-UP COLUMNS

Solid phase extraction and immunoaffinity procedures have greatly simplified the mycotoxin clean-up processes to produce high purity extracts that can be used with modern sensitive detection methods (Bradburn *et al.*, 1989; Bradburn *et al.*, 1990; Cavajal *et al.*, 1990; Patey *et al.*, 1991; Trucksess *et al.*, 1991). The procedures use relatively small volumes of solvent and can be used with automated sampling handling devices that reduce analysis time and increase throughput. The Aflatest immunoaffinity columns, coupled with solution fluorimetry or liquid chromatography with post-column derivatization has been adopted as the official first action method by the AOAC for the determination of aflatoxin in corn, raw peanuts and peanut butter (Trucksess *et al.*, 1991). The United States Department of Agriculture's Federal Grain Inspection Service has approved the Aflatest and Easi-extract test kits as alternative methods for the screening of maize samples (Emnett, 1989).

Phenyl bonded-phase cartridges were successfully used for the analysis of aflatoxin in cottonseed (Bradburn *et al.*, 1989) and maize (Bradburn *et al.*, 1990) when compared to the first action AOAC CB method. Both the bonded-phase and immunoaffinity columns gave better aflatoxin B_1 recoveries and had comparable precision to the standard AOAC (CB) method.

The development and application of solid phase extraction methods for the determination of aflatoxin from groundnut meals was reported by Roch *et al.* (1992). The phenyl-bonded phase clean-up with acetone-water from spiked groundnut meal extracts gave recoveries of 101.3 % and 101.8 % for aflatoxin B_1 and B_2, respectively. Higher recoveries of AFB_1 from naturally contaminated samples were recorded compared to the CB method, although the precision of the two methods did not differ at the 5 % significance level. Similar recoveries

of AFB_2 were recorded for both methods. The solid phase extraction method is less time consuming and more economical on solvents than the CB method.

A solid-phase clean-up method was used for the analysis of aflatoxin in groundnut cake extracted by a bonded-phase (PH) cartridge followed by HPLC quantification with fluorescence detection after post-column derivatization with iodine (Roch *et al.*, 1995). Average recoveries were 82-88 % with limits of detection of 2.7, 1.6, 2.5 and 3.2 ng/g for aflatoxins B_1, B_2, G_1 and G_2, respectively. This method (PH method) was compared with the CB method. The precision of the two methods was not significantly different at the 5 % level, but the PH method extracted significantly more aflatoxin B_1 from naturally contaminated samples than the CB method.

The efficiency of two different immunoaffinity columns and a phenyl-bonded phase column were evaluated during the extraction, clean-up and quantification of aflatoxin B_1 from sorghum and maize (Bradburn *et al.*, 1995). Maize is a simple matrix and comparable precision and accuracy were obtained for each of the methods. The sorghum matrix was complex and the bonded-phase procedure was the most accurate and precise method. The lower aflatoxin recovery from sorghum by immunoaffinity columns may be a solvent extraction problem.

After the introduction of the immunoaffinity method for aflatoxin analysis, several studies evaluated the efficiency of this and existing methods. Nine laboratories, from Denmark, Finland, Norway and Sweden evaluated an immunoaffinity column clean-up / liquid chromatographic determination of aflatoxin B and G in samples of peanuts, figs, maize gluten, soya expeller and copra spiked with aflatoxin concentrations ranging from 1.4 to 28.6 ng/g. From the 13 samples analyzed, 6 were pairs of blind duplicates. Although the results obtained were individually corrected for recovery, they were lower than expected in most cases. In some cases, the recovery was unacceptably low, particularly for aflatoxin G_2. Method repeatability and reproducibility were good, but generally better for the peanut and

fig samples. Results were better for aflatoxin B_1 and G_1 than for aflatoxin B_2 and G_2 (Barmark and Larsson, 1994).

The commercial EASI-EXTRACT immunoaffinity column method was compared with the CB method for aflatoxin B_1 from raw ground unskinned peanuts (Carvajal *et al.*, 1990). The EASI-EXTRACT immunoaffinity column recovered 93 % of B_1 from 10 ppb spiked samples and 95.5 % of B_1 from 50 ppb spiked samples, which was higher than the recoveries from the CB method. The immunoaffinity column also saved analytical time compared with the CB method. There were no interfering spots on TLC plates after EASI-EXTRACT as the antibody recovered B_1 specifically, enabling easier and more accurate quantification. The CB method and immunoaffinity methods were comparable with aflatoxin M_1 from milk (Mortimer *et al.*, 1987) and cheese (Sharman *et al.*, 1989).

Immunoaffinity columns were comparable to the approved EC method for M_1 in milk and milk powder in an inter-laboratory study organized by the International Dietary Federation (Tuinstra *et al.*, 1993). Comparison of aflatoxin B_1, B_2, G_1 and G_2 recoveries were made using animal feeds and maize (Roos *et al.*, 1997). The extraction and HPLC analysis of both procedures were comparable, hence a direct comparison of the performance of the alternative clean-up columns were made. The results were similar for both methods, but the immunoaffinity method had fewer manipulation steps. The immunoaffinity column was easier to use, less solvent was required and greater samples throughput was obtained.

Multifunctional columns. Wilson *et al.* (1991) used the Mycosep multifunctional column (MFC) for the determination of aflatoxin in agricultural products. MFC columns provide rapid one step extract purification. They retain particular groups of compounds that may interfere with quantification, while allowing compounds of interest to pass through. The method was successfully applied to corn, almonds, pistachios, walnuts, peanuts, Brazil nuts, milo, rice, cottonseed, corn meal, corn gluten meal, fig paste and mixed feeds (Trucksess *et al.*, 1994). The MFC column method is accepted by AOAC as described in the AOAC Official Method of Analysis (1995).

The proprietary packing material in the MFC column contains both lipophilic (non-polar) and charged (polar) active sites. Lipophilic sites remove fats and other non-polar compounds such as xanthophyl pigments. Charged sites consist of both dipolar and anionic exchange sites that remove proteinaceous compounds, carbohydrates and other polar compounds.

The MFC column differs from the affinity columns and the solid phase extraction (SPE) columns that have been used extensively for aflatoxin extract purification (Beebe, 1978; Hutchins *et al.*, 1989; Trucksess *et al.*, 1991). Both the affinity column and the SPE column clean-up methods require 3 steps of extract purification: retain aflatoxin on packing material of the column, washing to remove contaminants, and elution of the compound of interest, whereas the MFC column requires only 1 step with no wash or elution steps. The MFC column offers more versatility than the affinity column, which is selective for aflatoxin only. SPE columns can be used for the individual analysis of several mycotoxins, but each mycotoxin requires different clean-up steps. Moreover, with the MFC technology, irreversible adsorption or premature elution from the clean-up column is eliminated. Both of these phenomena may occur with SPE and affinity column clean-up. Recovery of aflatoxin from the MFC is significantly higher than the recovery of aflatoxin from affinity columns (Trucksess *et al.*, 1991). Recovery of total aflatoxin through the MFC column is typically above 95 %.

Choice of solvents for extraction of clean-up process. Conventional methods for aflatoxin analysis from food using TLC and HPLC involved chloroform extraction. There has been pressure to replace chloroform with solvents such as acetonitrile and methanol on environmental and toxicity grounds (Cole and Dorner, 1994). Akiyama *et al.* (1996) reported the use of multifunctional columns after non-chloroform extraction, derivatization with trifluoroacetic anhydride and determination of aflatoxin in nuts and corn using HPLC. Recoveries of aflatoxin B_1, B_2, G_1 and G_2 spiked in peanuts, various other nuts and corn at 1 or 10 ng/g were in the 82-102 % range.

QUANTITATIVE DETERMINATION OF AFLATOXINS

Although final extracts have been subjected to clean-up procedures they will still normally contain large amounts of co-extracted substances and require further separation with chromatographic techniques. The most widely and routinely used methods are one-dimensional and two-dimensional thin-layer chromatography (TLC) and HPLC. Since most mycotoxins are non-volatile, gas-liquid chromatography (GLC) has limited use, but is particularly important with the non-fluorescing trichothecene mycotoxins. Techniques for the determination of aflatoxins after the extraction and the purification steps are as follows:

(1) Thin-Layer Chromatography (TLC)

TLC was the most widely used analytical method for separating and identifying mycotoxins from concentrated extracts. TLC involves, applying a concentrated sample to a glass plate coated with silica gel on a baseline, separation by solvent migration, drying and characterization of the resultant spots. With every combination of solvents each mycotoxin will have a characteristic migration and separation pattern, giving a fixed Rf value.

The innovations in TLC analytical techniques for mycotoxins include two-dimensional chromatography, in which the sample is developed in one direction with a given solvent, dried and then developed in a second direction, perpendicular to the first, with a second solvent. Two-dimensional chromatography is particularly suitable for sample extracts containing large amounts of co-extracted substances. Thus, development in the first direction serves as a clean-up step, while the second direction is for the actual detection. TLC is still one of the most widely used separation techniques in aflatoxin analysis. The first adaptation of this technique was published by Eppley (1966). It is an AOAC official method (AOAC, 1990) and has been a method of choice for aflatoxins at levels as low as 1 ng/g.

TLC is often used to verify findings by newer, more rapid methods. Reliable quantitative tests are now available with TLC due to improvements in instrumentation and the availability of a wider variety of adsorbents for use as the stationary phases on TLC plates. For example, phases with small particle size and narrow particle size distribution have become available.

TLC is more popular in Europe than in the USA. The number of publications on TLC has declined, but this is not necessarily an indication of the extent to which TLC is being used. For example, TLC methods may be used routinely, but are not published unless they are being applied to new commodities or are improvements of previously published methods. Many TLC methods for aflatoxins in foods such as corn, peanuts, peanut butter, cottonseed, milk, meat, and eggs are included in the compendium of Official Methods of Association of Official Analytical Chemists.

There are four types of TLC development in which silica gel is used as the stationary phase: one solvent, two solvents, bi-directional, and two-dimensional. The one-solvent system is self-explanatory. In the two-solvent development, the plate is first developed with a solvent that removes the contaminants, then the plate is dried and developed with another solvent in the same direction to separate the toxins. In bi-directional TLC, extracts are spotted in the middle of the plate. After the first development with a non-polar solvent to remove the non-polar components, the top of the plate below the solvent front is removed and the plate is turned through 180 ° and developed with a more polar solvent system to separate the toxins. Two-dimensional TLC is a powerful technique that offers high resolution. This requires two solvents of different selectivity for the two developments. The test extract is spotted in one corner with reference standards on the two adjacent corners. The plate is developed in one direction, then rotated through 90 ° and developed in a second direction).

In the past decade, TLC plates pre-coated with bonded-phase silica gel, known as reverse-phase (RP)-TLC plates, have become available commercially. In the RP-TLC system, the

mobile phase is more polar than the stationary phase whereas, in the normal-phase (NP)-TLC (silica or alumina), the mobile phase is less polar than the coating medium. RP-TLC plates are made of a variety of bonded-phase adsorbents, including C_2, C_8, C_{12}, C_{18}, and diphenyl types. Quantitation of aflatoxins by RP-TLC is still in the developmental stage, although RP-TLC can be used to confirm the identity of aflatoxins separated on NP-TLC plates. This method can be used for screening, for example, 18 mycotoxins, including the aflatoxins, were identified using RP C_{18} or RP diphenyl TLC plates (Abramson *et al.*, 1989).

(2) High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a modified form of TLC in which the stationary phase is improved by absorbents. HPTLC plates are smaller than conventional TLC plates, usually 10 x 10 cm or 10 x 20 cm The separation efficiency is typically 5,000 theoretical plates for 5 cm migration. Improvements have also been made in the instrumentation necessary to accommodate the smaller plate sizes, the small volumes of test solution applied, the extremely compact fluorescence signal of the aflatoxin spot, and the close migration of the toxin spots. HPTLC instruments for application of test solution, in which aflatoxin standard was used, plate development, and densitometry were evaluated. Optimum sensitivity, accuracy, and precision were obtained from HPTLC using a fully automated TLC sampler, an unsaturated conventional TLC glass chamber, and a monochromatic fluorodensitometer. Benzene-acetonitrile (98+2) was the most suitable spotting solvent. (Coker *et al.*, 1988).

Modern HPTLC differs from conventional TLC in several important aspects. Improvements include the evolution of high quality plates, automated sample preparation and automated plate quantification. An HPTLC plate is uniformly coated with a 0.1-0.3 mm layer of small particle size (2-10 μm) adsorbents. The small particle size results in rapid separation of the sample components. HPTLC is an open-bed system in which multiple samples and standards are applied, simultaneously, to the stationary phase.

Detection limits for aflatoxins using HPTLC quantification are typically in the low picogram range.

Modern HPTLC is a precise and accurate analytical tool with an efficiency comparable to HPLC (Shepherd and Gilbert, 1984; Roch *et al.*, 1992) and enzyme linked immunosorbent assay (ELISA) (Chu *et al.*, 1988; Mortimer *et al.*, 1988; Wilkinson *et al.*, 1988; Trucksess *et al.*, 1989; Patey *et al.*, 1989; Park *et al.*, 1989a; Chu, 1991). HPTLC is ideally suited to the analysis of a large number of samples. Up to thirty samples can be simultaneously chromatographed on a single 10 x 20 cm plate. HPTLC, used in conjunction with an SPE clean-up, offers a rapid and cheap method for aflatoxin analysis with reproducible quantification of ultra-trace levels of aflatoxin contamination.

HPLC in general affords better resolution than 1-dimensional HPTLC. HPTLC resolution is however, improved when multiple development procedures are used. However, the entire HPLC process can be automated, whereas the complete automation of HPTLC method is difficult, as plates must be handled manually. However, compared with HPLC, HPTLC offer a much faster sample throughput and does not require additional derivatization procedures. The capability of HPTLC methods to process samples and standards simultaneously yet independent under the same conditions, leads to statistical improvement in data handling, analytical precision and accuracy.

The solubility of the residue remaining after extraction and clean-up is an important consideration in selecting the spotting solvent. Most residues dissolve readily in chloroform. Compact spots can be obtained by reducing the rate of solvent delivery. A microcomputer has been interfaced to a fluorodensitometer to simplify the data handling procedure (Whitaker *et al.*, 1990).

(3) High Performance Liquid Chromatography(HPLC)

HPLC is a separation technique that has become increasingly used for the analysis of mycotoxins because it offers increased sensitivity and higher accuracy than many other methods.

The first chromatographic choice to be made in HPLC is the selection of column packing material. For solutes of intermediate or high polarity, such as most mycotoxins, a reverse phase (typically an ODS) column will usually give good results. Silica columns can often be employed for the same separation, but solvent selection and preparation is normally simpler with ODS packing.

In addition, excitation and emission wavelengths are solvent dependent. The former may readily by optimized by obtaining an UV spectrum of the toxin in the eluent of choice. Fortunately aflatoxins exhibit broad adsorption maxima and thus excitation wavelength selection is not critical, even when several toxins need to be determined. A wavelength of 365 nm is often a satisfactory compromise. However, where sensitivity is of concern, detection wavelengths should be chosen with care. The emission wavelength is dependent on mobile phase polarity and for B_1 may vary between 424 and 431 nm (and for G_1; 428 and 445 nm) in chloroform solutions containing increasing amounts of methanol. Emission of B_1 in aqueous eluents maximizes at wavelengths of up to 450 nm.

Trucksess *et al.* (1991) compared liquid chromatography (LC) with TLC. LC is similar to TLC in many respects, including analyte application, stationary phase, and mobile phase. LC and TLC complement each other. LC methods for the determination of aflatoxins in food include normal-phase LC (NPLC), reversed-phase LC (RPLC) with pre-column derivatization (BCD), RPLC followed by post-column derivatization (PCD), and RPLC with electrochemical detection. All these techniques, except electrochemical detection, use fluorescence detectors set at E_x 360 nm, E_m > 420 nm. Reviews of LC methodology only LC methods developed before 1986 for corn and peanuts (Beaver, 1989; Wilson, 1989).

In the early 1980s, most fluorescence detectors were not sufficiently sensitive to detect the native fluorescence of AFB_1 and AFB_2 at < 0.5 ng in a mobile phase eluted from a normal-phase silica gel column. The use of a detector flow cell, packed with silica gel, can enhance the fluorescence of AFB_1 and AFB_2 (Panalaks and Scott, 1977). In one study, the detection limit was 0.25 ng for AFB_1, 0.5 ng for AFG_1, and 0.2 ng for AFB_2 and AFG_2 in corn, when chloroform: cyclohexane: acetonitrile: isopropanol (75+22+3+0.2) was used as the mobile phase (Francis *et al.*, 1982). The detector cell required frequent repacking because the silica gel adsorbed contaminants irreversibly and caused elevated noise levels in the detector, decreasing the resolution, and lowering the fluorescence response of the toxins.

RPLC is an effective analytical technique that is frequently used to overcome the problems of NPLC. The stationary phase is usually a C_{18} chain chemically bonded to the silica gel support. The mobile phase is a mixture of water, methanol and acetonitrile. The column dimensions are 3.9 - 4.6 mm x 15 - 30 cm. The particle size is 5 - 10 μm with 9 - 12 nm pore size. One drawback of RPLC is that AFB_1 and AFG_1 do not fluoresce in an aqueous mobile phase. Consequently, pre-and post-column derivatization techniques are used to increase sensitivity (Beebe, 1978; Shepherd and Gilbert, 1984).

Pre-column derivatization procedures can be optimized by adding hexane and trifluoroacetic acid (TFA) to the extract (Tarter *et al.*, 1984), allowing the mixture to react for 5 min at room temperature, and adding aqueous acetonitrile to the test solution. After mixing, a portion of the aqueous layer is injected onto the column for separation and quantitation. The detection limit for aflatoxin in peanut butter is about 0.3 ng/g for AFB_1. The disadvantages of this technique are the occurrence of incomplete reactions and the formation of more than one derivative. The average recovery for added total aflatoxin at 10 - 30 ng/g levels in corn and peanut products was about 70 %. Although pre-column treatment of aflatoxins with trifluoroacetic acid is widely used for fluorescence enhancement of aflatoxins B_1 and G_1, aqueous iodine solution in a heated post-column reactor has also been employed for this purpose (Holcomb *et al.*, 1991). Post column derivatization with iodine allows detection of 0.7 ng AFB_1/g corn (Thiel *et al.*, 1986). In

this system, iodine is introduced as an aqueous solution into the eluant stream between the column outlet and the fluorescence detector. The disadvantage of this procedure is that it requires two pumps and a reaction coil kept at constant temperature (Trucksess *et al.*, 1991).

A modification of the second procedure provides the iodine from a column packed with solid iodine. Post-column addition of β-cyclodextrin also enhances fluorescence of aflatoxins B_1 and G_1 separated by reversed-phase LC. Another important mycotoxin, zearalenone, undergoes post-column fluorescence enhancement with aluminum chloride (Figure 8.) (Hetmanski and Scudamore, 1991).

Post-column derivatization by iodine has some disadvantages. The iodine reagent solution is not stable and has to be prepared daily. The post-column system requires an expensive pulse-less pump and a thermostated oven. A post-column derivatization method for the fluorescence detection of phenothiazines was developed using on-line electrochemically generated bromine (Kok *et al.*, 1986). This application used simpler hardware and avoided the use of unstable reagents (Kok *et al.*, 1986), it uses an on-line electrochemical cell to produce bromine, which enhances the fluorescence signal of AFB_1 and AFG_1 (Kok *et al.*, 1986). The method has been modified and used to analyze corn naturally contaminated with aflatoxins (D. M. Wilson, M. W. Trucksess, T. Urano, and Y. Kim unpublished observations). In this method, the bromine is produced from the bromide present in the mobile phase [water-methanol-acetonitrile (6+2+2) with 1 mM potassium bromide and 1 mM nitric acid] in a post-column electrode. This LC-PCD procedure is simple to use, but precautions must be taken to avoid damaging the electrochemical cell.

Two other PCD methods are less well established. One uses post-column enhancement with cyclodextrin (Francis *et al.*, 1988a), and is similar to PCD with iodine. The other uses an electrochemical detector, and is capable of reducing the background noise interference associated with other electrochemical methods (Duhart *et al.*, 1988) by pre-electrolyzing the mobile phase, switching to a glass-lined column, and using a better oxygen-removal

technique. The limit of detection is about 10 ng/g in peanut butter. The advantage of this procedure is that it does not require a separate derivatization step, as is common for fluorescence detection.

LC of mycotoxins has focussed on pre- and post-column derivatization reactions to improve sensitivity, on selective detection systems such as MS and diode array UV, and on incorporation of LC into automated methods (Lawrence and Scott, 1993). In addition some effort has been devoted to the chromatographic process itself. The number of acidic and basic mycotoxins for which ion pairing has been used in the LC separation has increased to include ochratoxin A, citrinin, moniliformin, tenuazonic acid, and ergot alkaloids. Microbore LC has not become a prominent technique for determination of mycotoxins.

Mycotoxins that are not naturally fluorescent or usefully UV absorbing require derivatization for LC detection. Fluorescence derivatization reagents, used pre-column, for fumonisins are o-phthaldialdehyde-mercaptoethanol, fluorescamine, 4-fluoro-7- nitrobenzo furazan, and naphthalene-2, 3-dicarboxaldehyde-potassium cyanide (Lawrence and Scott, 1993.) Various derivatization reagents have been evaluated for trichothecenes (Betina, 1989; Lawrence and Scott, 1993.) A post-column treatment, which uses no chemical reagents, involves UV photolysis of the trichothecenes deoxynivalenol, nivalenol, and fusarenon-X to oxidizable products which are detected amperometrically with good sensitivity.

LC-MS, especially LC-thermospray MS, is the subject of several reports covering aflatoxins B_1, B_2, G_1, and G_2, bisulfite adducts of aflatoxins B_1 and G_1, fumonisins, trichothecenes, zearalenone, patulin, and ochratoxin A (Lawrence and Scott, 1993.) The last four were determined by LC-thermospray MS in a single chromatographic run, with application to grain samples down to low ng/g levels. LC-MS has considerable potential for determination and confirmation of mycotoxins in food.

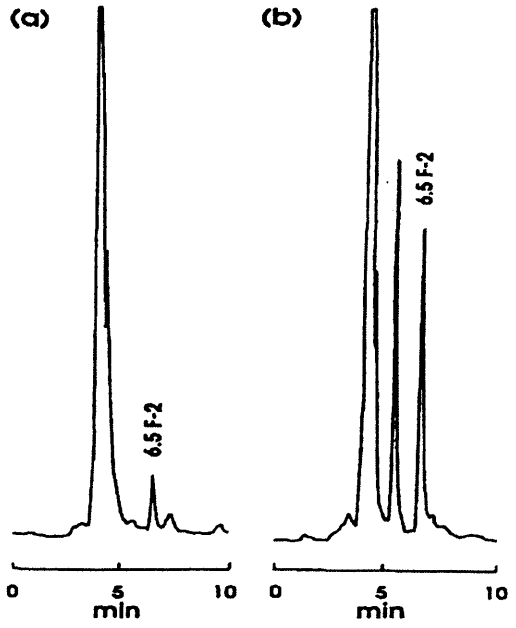


Figure 8. LC of blank corn extract with 100 ng zearalenone (F-2) /g added,
(a) without derivatization and (b) after post-column derivatization with 0.25 M aluminum chloride solution to enhance fluorescence response (Hetmanski and Scudamore, 1991).

Multi-mycotoxin LC with detection and determination by diode array UV was used successfully for screening fungal cultures (Lawrence and Scott, 1993). Up to 182 mycotoxins and other fungal metabolites were characterized in one study. There has only been limited application of diode-array detection for analysis of foods, for example, 14 mycotoxins were detectable in rice and corn when added at a level of 50 ng/g (Isohata and Hayakawa, 1992) and its potential for detection, identification, and quantification of Alternaria toxins in rice, corn, tomato and sunflower seeds has been demonstrated (Palmisano *et al.*, 1989). Alternariol and alternariol methyl ether occur naturally in sunflower seed at levels of 0.36 and 0.13 μg/g respectively (Palmisano *et al.*, 1989).

Another type of detection technique that has received increased attention is electrochemical detection. Applications include aflatoxins, deoxynivalenol, zearalenone and zearalenol, and the Alternaria toxins (Chu, 1991; Lawrence and Scott, 1993). An advantage of electrochemical detection is that no derivatization is necessary. However, minimum detectable amounts vary from 0.03 ng of altertoxin I, using positive and negative electrodes in series, and 0.02 ng of zearalenone, detected at a positive potential, to 5 ng for aflatoxin B_1, G_2, G_1 or G_2 by differential-pulse amperometric detection. of the use of this technique for food has been demonstrated for these and other mycotoxins. Quantitation of zearalenone from corn was possible at low ng/g levels, while for the altertoxins and other alternaria toxins, sub-μg/g concentrations were determined in various foods (Chu, 1991; Lawrence, 1991).

Automated LC methods have been developed for aflatoxin B_1 in cattle feed; aflatoxins B_1, B_2, G_1, and G_2 in peanut butter, dried figs, and animal feeding stuffs; aflatoxin M_1 in milk; and ochratoxin A in cereals and animal products (Lawrence and Scott, 1993). Both SPE and immunoaffinity columns have been used for the clean-up step. An attempt to reuse immunoaffinity columns by incorporating an online dialysis unit for milk gave very low (6 %) recoveries of aflatoxin M_1 from crude milk. Another automated dialysis system gave up to 69 % recoveries of aflatoxin M_1 from defatted milk using a C_{18} cartridge clean-up.

Micellar eletrokinetic capillary chromatography (MECC)

Micellar Electrokinetic Capillary Chromatography (MECC) is a technique capable of highly efficient liquid phase separations of neutral molecules, in addition to charged analytes, and has been applied to aflatoxins B_1, B_2, G_1 and G_2 (Cole *et al.*, 1992). Their separation is extremely rapid under conditions optimized for overall resolution and analysis time (Figure 9). The very small I.D. capillary columns used (25 and 50 μm) require on–column laser-based fluorescence for adequate detection sensitivity. Application to corn meal analysis has so far not been particularly useful, with a limit of detection of 1 μg/g, but modification of the work-up procedure may greatly improve this.

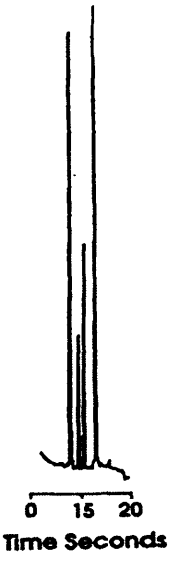


Figure 9. High-Speed separation of aflatoxins by micellar electrokinetic capillary chromatography (MECC). Elution order is G_2, G_1, B_2, B_1. Mobile phase composition: 0.05 M sodium deoxycholate, 0.01 M Na_2HPO_4, 0.006 M $Na_2G_4O_7$, 5 % acetonitrile. Applied voltage 36kV. Detection by laser–based fluorescence (Cole *et al.*, 1992).

(4) Gas Liquid Chromatography (GLC)

GLC is applicable to compounds that exert significant vapor pressure at temperatures below those of excessive pyrolysis. Such compounds can be converted to stable, volatile derivatives that can be separated by vapor phase chromatography. Since most mycotoxins are non-volatile, GLC has not been widely used. However, GLC combined with mass spectrometry is an effective method for identification and quantitation of mycotoxins (Kamimura *et al.*, 1985).

The best technique for the quantitation of trichothecene mycotoxins is GLC with electron capture detection (ECD) or mass spectrometric detection (MS). Sample preparation normally involves extensive clean-up by column chromatography on florisil. Trichothecenes are not sufficiently volatile for direct analysis by GLC. They must be derivatized through free hydroxyl groups on the molecules to form trimethylsilyl (TMS) ethers that are sufficiently volatile for GLC analysis. GLC with ECD and confirmation with MS has been successfully used for identifying trichothecenes, particularly deoxynivalenol and nivalenol in surveys of wheat, barley and their products.

The trichothecenes are the only mycotoxins for which GC is widely used, although a number of others can be determined in food by GC (Scott, 1993). Most trichothecenes possess hydroxyl (TMS) ether and heptafluorobutyryl (HFB) or trifluoroacetyl (TFA) esters can be readily formed. However, several workers have also determined trichothecenes without derivatization. As an example, capillary GC methods have been developed for trichothecene and its de-esterified analogue trichothecolone in grape juice and wine, with a detection limit of 50 ng/ml using flame ionization. A notable trend in GC of trichothecenes has been replacement of packed columns by capillary columns. Capillary column GC is essential for determination of multiple trichothecenes (de-oxynivalenol, nivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol, etc.) in grains, particularly with ECD. The preferred method for trichothecene determination in grains and grain foods is capillary GC-MS. An alternative approach to direct analysis of trichothecenes is to carry out alkaline hydrolysis to give parent trichothecene alcohols (e.g., T-2 tetraol, scirpentriol, and deoxynivalenol), which can be determined in hydrolysed grain or feed extracts by capillary GC after derivatization.

Another example of mycotoxins, in which GC-MS, and also GC-MS/MS, has been used, was ochratoxin A (Jiao *et al.*, 1992). Ochratoxin A was converted to O-methylochratoxin A methyl ester for quantitation (Figure 10). Deuterated internal standards are often employed in GC-MS methods, particularly for trichothecene determination.

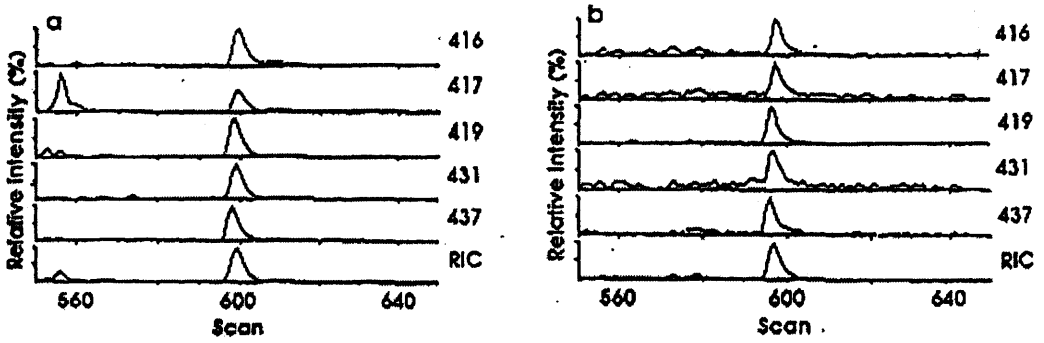


Figure 10. Multiple ion detection GC of O-methylochratoxin A methyl ester (m/z 431, 417 and 416) and its hexadeuterated analogue as an internal standard (m/z 437 and 419) in samples of (a) corn-peanut snacks and (b) bran (each estimated to contain 0.13 ng ochratoxin A/g). MS mode was negative ion chemical ionization. RIC = reconstructed ion chromatogram (Jiao *et al.*, 1992).

Heptafluorobutylation (HFB) of ochratoxin giving its derivatives is advantageous for both ECD and MS detection and its use for GC of mycotoxins other than trichothecenes has been reported. Patulin, zearalenone, and salframine have also been determined by GC of their HFB derivatives (Scott, 1993). Capillary GC of patulin HFB using ECD was applied to analysis of apple juice, with a sensitivity of 0.05 ng patulin and ≤ 10 ng/l apple juice (Tarter and Scott, 1991).

Since the discovery in 1981 that underivatized aflatoxin B_1 could be chromatographed by fused silica capillary GC-MS with on-column injection, there has been increased interest in this technique, particularly for confirmation purposes (Holcomb *et al.*, 1992; Scott, 1993). Aflatoxins B_1, B_2, G_1, and G_2 have now been separated. The limit of quantification is 1 ng for aflatoxins B_1 and B_2 and 2 ng for aflatoxins G_1 and G_2 by flame ionization detection; minimum detectable amounts by GC-MS were somewhat lower. Underivatized α-cyclopiazonic acid was also detected and separated from the aflatoxins by this technique (Scott, 1993).

The fumonisins are usually determined by LC (Sydenham *et al.*, 1992; Lawrence and Scott, 1993.) and no attempt has been made to determine them by GC, with or without derivatization. However, hydrolysis to C_{22} aminopolyols and formation of TMS or TFA derivatives of these allowed indirect capillary GC determination of fumonisins, preferably with MS detection (Scott, 1993). Tricarballylic acid, the other hydrolysis product of fumonisins, has also been derivatized and chromatographed by capillary GC-MS.

(5) Immunochemical methods

TLC and LC methods for determining aflatoxins in food are laborious and time consuming. These techniques require knowledge and experience to solve separation and interference problems. Through advances in biotechnology, highly specific antibody-based tests are now commercially available that can identify and measure aflatoxins in food in less than 10 min (Trucksess and Wood, 1994). These tests are based on the affinities of monoclonal or polyclonal antibodies for aflatoxins. The three types of immunochemical methods in use (Chu, 1990) are radioimmuno assay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column assay (ICA). The first two methods are based on competition between the unlabeled aflatoxin in the test solution and the labeled aflatoxin in the assay system for the specific binding sites of antibody molecules. Radioactive aflatoxin is use as a labeled ligand in the RIA and an aflatoxin-enzyme conjugate is used as ligand in the ELISA. In the ICA procedure, the antibody column traps or binds the aflatoxins, which are subsequently eluted from the column with methanol for quantitation.

RIA was developed in 1959 for the detection of insulin. In the competitive aflatoxin RIA, a specific antibody is incubated with a constant amount of radiolabeled toxin in the presence of varying amounts of toxin standard or unknown sample. Ammonium sulfate precipitation is used to remove the toxin-antibody complex from the solution. The toxin content of the sample is related inversely to the amount of unbound radioactive toxin remaining in the supernatant solution. Although RIA is very sensitive, it has several disadvantages. The radioisotopes used in the assays are hazardous, present disposal difficulties, and may have

short shelf lives; non-isotopic labels such as enzymes have been used in place of radioisotopes.

ELISA was developed for quantitative determination of mycotoxins in food (Chu, 1991). Although both direct and indirect competitive ELISAs have been used for aflatoxin determination, the direct assay is preferable for analytical purposes because it is simpler. This technique consists of a two-step process: (1) the reaction between the antibody and the toxin and (2) measurement of the reaction of the substrate with the enzyme attached to the toxin. Analyte isolation for the ELISA is simple. The test portion is extracted with methanol and water, and the filtrate is then diluted and analyzed. Diluted filtrate and aflatoxin peroxidase conjugate are added to the antibody-coated apparatus, the toxin antibody is formed, and the apparatus is washed with water. Substrate is added and the color is developed. The colour of the test solution is compared with that of the standards and controls.

Several immunoassay kits for aflatoxins are marketed under different trade names (CAST, 1989). No formal or standard criteria have been established for evaluation of the kits. Several organizations such as the AOAC, International Union of Pure and Applied Chemistry, Environmental Protection Agency, USDA, and FDA are engaged actively in developing evaluation guidelines.

Three of the commercial test kits have been studied according to the AOAC guidelines: the Neogen Screen Kit, the Immuno Dot Screen Cup, and the Aflatest P immunoaffinity column (Park *et al.*, 1989a,b; Trucksess *et al.*, 1989, 1991). The first two tests are yes/no types whereas the Aflatest P is quantitative. Performance is assessed by examining the accuracy of classification of test samples as either positive or negative at a fixed aflatoxin level (20 ng/g).

Immunochemical methods are quite specific and can be used to screen for aflatoxins in grain and grain products. Some of the immunochemical methods are also capable of giving

quantitative results and are recognized as acceptable analytical methods by the AOCS (McKinney, 1989). Beginning with the AOCS, 1990-1991 Smalley Aflatoxin Series, immunoassay test kits have been included in the methodologies that can be used in the analysis of Smalley aflatoxin samples (peanut, corn, cottonseed, and nuts). Although the methods are specific, simple, fast, and cost effective, they cannot be coupled with mass spectrometry to confirm the identity of the aflatoxins being measured.

An ELISA method was applied to naturally contaminated mixed feed (Hongyo *et al.*, 1992). It used a highly sensitive and specific monoclonal antibody to aflatoxin B_1. The detection limit of the ELISA was approximately 100 pg/assay. A good correlation between this and TLC or LC methods was observed. However, for the mixed feeds, crude extracts cannot be applied directly to ELISA because of the occurrence of large non-specific reactions. However, if an LC sample preparation procedure is used before ELISA, high sensitivity can be achieved with the mixed feeds.

Although rapid immunologically-based screening tests and the ELISA methods are available for aflatoxin determination, these methods do not normally allow for simultaneous monitoring of both individual and total aflatoxin levels (Shepherd *et al.*, 1987; Koeltzow and Tanner, 1990). In such cases, HPLC analysis where aflatoxins are separated and individually quantified is more advantageous.

A commercial available ELISA system and HPLC method were evaluated simultaneously to analyze 178 samples of food for total aflatoxins (Azer and Cooper, 1991). High correlation coefficient values between the two methodologies were obtained (higher than 0.96) with nuts, nut products, peanuts and peanut butter. Poor correlations were obtained with grain and cereal samples.

CONFIRMATION OF AFLATOXIN IDENTITIES

Although analytical methods might consist of different extraction, clean-up, and quantification steps, the results of the analyses by such methods should be similar when the methods are applied properly. This agreement was illustrated by a study involving more than 20 European laboratories (Van Egmond and Wagstaffe, 1989, 1990) using reference materials (peanut butter naturally contaminated with aflatoxins) for validation and quality assurance of methods. One problem still to be solved is the confirmation of aflatoxin identity. Confirmation techniques involve either chemical derivatization or mass spectrometry (MS). TFA is the most common reagent used for chemical derivatization of aflatoxins. TFA is used as the catalyst to add water to the double bond of the vinyl ether function of AFB_1 and AFG_1. In TLC methods, TFA is added to the spots of the extracts and standard; the plate is dried at 40 °C for 10 min and developed with chloroform: acetone: 2-propanol (85+10+5). The fluorescent products of AFB_1 and AFG_1 are then observed at R_f values of 0.2 and 0.15. The identity of M_1 can be confirmed in a similar manner with minor modifications, i.e. a spotted plate is covered with a clean glass plate, heated at 70 °C for 8 min, and developed in a slightly more polar solvent (7 % 2-propanol). In the LC method, TFA, iodine, or bromine is used to derivatize aflatoxins before quantification; thus, no further chemical confirmation of identity is needed. The chemical methods for confirmation of identity are not as definitive as using MS techniques.

Confirming the identity of aflatoxins by MS requires additional clean-up steps such as TLC isolation or solid-phase extraction (Park *et al.*, 1985) because of the presence of impurities in the test extract. Another approach is to interface gas chromatography with mass spectrometry (GC/MS), i.e. to use GC to separate the impurities in the extract from the aflatoxins and use MS to confirm the identities of the aflatoxins. The first GC/MS method for AFB_1 used on-column injection at 40 °C (Trucksess *et al.*, 1984) and a 6-m x 0.2-mm methyl silicone-coated, fused-silica column. Immediately after the test extract was injected onto the column, the column temperature was raised to 250 °C in 4 min; the effluent was

analyzed by negative ion chemical ionization (NICI)-MS. The NICI mass spectrum of AFB_1 showed major ions at *m/z* 312 and 297.

Goto *et al.* (1988) used GC to analyze mixtures of four aflatoxins. The initial and final temperatures were set at 50 and 300 °C and the rate of heating was set at 15 or 20 °C/min. A 5 % phenylmethylsilicone column was used to separate AFB_1, AFB_2, AFG_1, and AFG_2 (2, 2, 4, and 4 ng), which were analyzed by GC with flame ionization detection. This technique coupled with MS may be used for quantitation and confirmation of aflatoxin identity.

A thermospray MS (TSMS) method was developed to characterize the reaction products of aflatoxins B_1 and G_1 with iodine in methanol-water (Holcomb *et al.*, 1991). About 4 μg of each derivative was injected into an LC/TSMS system. The mobile phase was 0.1M ammonium acetate in water and the flow rate was 1.2 ml/min. The vaporizer was set at 110 °C and the jet was set at 220 °C. The mass spectra showed *m/z* 471 and 488, which corresponded to the [M+H]+ derivatized AFB_1 and AFG_1. These results indicated that the reaction products were adducts of an iodine atom and a methoxy group to the furan ring.

An LC/TSMS method was developed for confirmation of identity of aflatoxins in peanuts (Hurst *et al.*, 1991). The column used was C_{18}, 5 μm, 4.6 mm x 25 μm, with a mobile phase of 0.1M ammonium acetate-methanol-acetonitrile (56+22+22) at a flow rate of 1.0 ml/min. The interface conditions used were T_{aux} 318°C, T_{block} 290 °C, and T_{tip} 185 °C. The detection limits (signal-to-noise ratio < 5) were 60, 40, 100 and 100 pg. AFB_1, AFB_2, AFG_1, and AFG_2, respectively. The mass spectra of AFB_1 and AFB_2 had strong MH+ peaks at *m/z* 313 and 315, respectively. Spectra of AFG_1 and AFG_2 had strong [MH+-44] peaks at *m/z* 285 and 287 respectively, in addition to the MH+ peaks at *m/z* 329 and 331.

Another approach to confirm the identity of aflatoxins is the use of tandem (MS/MS) mass spectrometry. The identity of aflatoxin M_1 isolated from milk after a disposable

immunoaffinity column clean-up was confirmed (J. E. Matusik, personal communication) after the eluate of 50 ml of milk was spiked at by 0.5 ng/ml M_1 and subjected to MS analysis. The test solution was introduced into the mass spectrometer via a direct exposure probe. The tandem instrument was operated in the daughter ion mode. The first quadrupole (Q_1), the mass filter, was set to pass the ion of interest at a particular *m/z*; the second quadrupole (Q2) acted as a collision cell, and the third quadrupole (Q_3) scanned the daughter ions formed in Q_2. The molecular ion at *m/z* 328 was selected in Q_1 and the collisionally activated decompositions occurred in Q_2. To increase sensitivity, Q_3 was set to monitor the following selected ions: m/z 328, 313,270, and 231. This procedure was able to identity M_1 at concenctrations of 0.05 ng/ml in 2 % low-fat milk.

METHODS OF AFLATOXIN ANALYSIS USED IN THAILAND

The Department of Medical Sciences, Ministry of Public Health Thailand (1999) reported the comparative analysis of aflatoxin contamination using mini-columns, TLC, ELISA and HPLC. The clean-up processes used were traditional solvent extraction, CB methods, BF methods and mini-column method. Suprasert (1997) compared the use of ELISA, CB, BF and mini-column methods for aflatoxin analysis at a level of 20 ppb. aflatoxin contamination. The use of commercial solid phase extraction columns has never been reported in Thailand. Commercial clean-up columns were introduced to analyse mycotoxins in Thailand in 1998 (by Trucksess and Stack at an "Aflatoxin Analysis Work shop" in February 1998). However, commercial columns have not been routinely applied in Thailand.

PART II

AFLATOXIN CONTAMINATION IN FOOD AND FEEDS

Mycotoxins can be produced during harvesting, production, distribution and storage of food (Kamimura, 1993). Mycotoxin-producing fungi can grow on agricultural products at any time if the temperature, humidity and other conditions are favorable for their growth. Consequently, the occurrence of mycotoxins in agricultural commodities depends on factors such as the region, season, and the conditions under which a crop is grown, harvested, and stored.

When ingested by a human or animal, agricultural products contaminated with mycotoxins can cause mycotoxicosis. There are two routes of poisoning. Either crops contaminated with mycotoxins can be ingested directly, or mycotoxins contaminated meat, internal organs, eggs, or cows milk may be ingested by humans.

Crops grown under warm and moist conditions in tropical or subtropical countries are much more prone to mycotoxin contamination than those grown in temperate zones, due to rapid growth of fungi in the former environments. However, certain toxigenic fungi, such as Fusarium species, can proliferate at low temperatures and produce mycotoxins. Over 100 fungal species produce mycotoxins associated with naturally occurring diseases in animals and humans. Although toxigenic fungi and their spores are ubiquitous, mycotoxicosis is primarily a problem in areas that have high rainfall, relative humidity and temperature. In addition to specific growth conditions, the fungal spoilage of crops and their grains are enhanced by drought, insect damage, cracked kernels during harvesting, and the presence of excessive chaff in the harvested grain. Mature fruits and vegetables are also highly susceptible to invasion by toxigenic fungi because they are high in moisture and nutrient content. Many fruits become easily injured as they approach full maturity and therefore are vulnerable to fungal attack (Kamimura, 1993).

Surveys have shown that *Aspergillus flavus* and *A. parasiticus* (Kamimura, 1993), which produce aflatoxin, are often found in the tropical and subtropical zones. Most of the products contaminated with aflatoxin come from tropical countries. Contamination by mycotoxins, especially aflatoxin, occurs with crops used in food and feeds often during the period in which these products are stored.

In many crops, the aflatoxigenic species of the *Aspergillus* group include *A. flavus*, *A. parasiticus* and *A. nomius*. The normal sources of *A. flavus* inoculum are airborne, soilborne and insect-vectored propagules. For some crops, for example, corn, peanuts and cottonseed, *A. flavus* colonization may occur anytime after flowering. Aflatoxin contamination of pre-harvest crops depends on the environmental conditions, especially temperature and moisture. Insect damage often results in increased aflatoxin contamination. The nutritional requirements for *Aspergillus* growth and aflatoxin production include a good supply of nitrogen, lipid, carbohydrates and some trace metals. Aflatoxin B_1, B_2, G_1, and G_2 all occur in pre-harvest crops, with B_1 and B_2 being the most common (Wilson and Payne, 1994).

The factors that increase susceptibility to aflatoxin contamination during storage are moisture content and temperature of the products (Wilson and Abramson, 1992). Water activity roughly corresponds to the relative humidity equilibrium in stored products. Fungi will not generally grow at a water activity below 0.70. At a water activity slightly above 0.70, fungi will grow slowly and subsequent increases in the water content, allow a more rapid growth of the fungi. Rapid fungal growth may raise the temperature of the stored products. Aflatoxin contamination is affected by the commodity, temperature, oxygen availability, and the initial fungal inoculum density. Aflatoxin contamination of susceptible crops in storage is primarily a result of storage of the commodity at water activity above 0.85. Insufficient drying, insect and rodent activity, moisture migration, roof leaks, wind-driven rain and other warehousing problems may contribute to *A. flavus* group growth and localized areas of heavy aflatoxin contamination.

SAMPLING AND SAMPLE PREPARATION

Specific sampling plans have been developed and tested rigorously for some commodities such as corn, peanuts, and tree nuts; sampling plans for other commodities have been modeled on these. The U.S. Department of Agriculture (USDA) recommends that 48 lb peanuts and 5 - 10 lb corn, milo, and other grain be collected for aflatoxin analysis (Whitaker *et al.*, 1979). The Food and Drug Administration (FDA) gives detailed descriptions of sampling sizes for various commodities and processed products (FDA, 1988). Samples from the same lot can be collected from 10 - 15 sites using different probe patterns or an automatic sampler (Whitaker *et al.*, 1979).

The entire primary sample must be ground and mixed so the analytical test portion has the same concentration of toxin as the original sample. A 2lb portion is sufficient when coarse or pelleted feed is tested as any toxins in the individual ingredients have been mixed throughout the feed. Whenever possible, the grain should be analyzed before it is processed into feed, because of the many other components of the feed that might interfere in the analysis. In general, 50g ground material is used for analysis. One study indicates that a 10g test portion of a sufficiently ground and blended sample produces an analyte variance statistically comparable with that of a 50g portion (Francis *et al.*, 1988b). An overview of sampling and analyte purification for the identification and quantitation of natural toxicants in foods and feeds offers information in these areas, is given by Park and Pohland (1989).

REGULATION OF MYCOTOXIN IN DIFFERENT COUNTRIES

Many countries have their own regulations for aflatoxins established on different principles. Earlier protection of food was mostly a local affair and municipal ordinances were promulgated for the purpose. Inspections were relatively simple as there were no auxiliary sciences. Later, as bacteriology, chemistry and microscopy developed, plans for statutory regulations were formed in many countries, leading at the beginning of the twentieth century, to the adoption of official food legislation. Food laws now not only prohibit the introduction, delivery or receipt in commerce of adulterated and misbranded food, but often they include specific legislation that imposes limits or tolerances on the concentrations of specific contaminants in food. Such contaminants may be of industrial or natural origin. Of the natural contaminants, the mycotoxins are the most recent to be considered. After the discovery of the aflatoxins in the early 1960s, specific mycotoxin legislation was developed in several countries, initially referring only to aflatoxins. Later regulations for other mycotoxins such as deoxynivalenol, ochratoxin A, patulin and zearalenone were also included in the food laws of some countries (Kamimura, 1993).

Obviously many developing countries, where mycotoxin problems may be severe, have no mycotoxin regulations. The priorities vary from country to country. In addition, industrialized countries with no domestic production of susceptible commodities generally have lower tolerances than countries where susceptible commodities are produced, especially when the imported foods may be classified as "luxury goods". The low tolerance limits in countries with high national incomes can have dramatic effects on the countries that are significantly dependent on exporting these susceptible foods. They have to establish export criteria that meet their customers requirements. This may lead to selection of the better crops for export and to local consumption of the more highly contaminated crops, with an increased risk of toxic effects in the local populations that often already have an unbalanced nutrition. Over fifty countries have now adopted legislative measures to

control aflatoxin levels in food, this has led to a need for rapid, reproducible, accurate and cost-effective methods of analysis (Van Egmond, 1989).

EFFECTS OF AFLATOXIN ON ANIMALS AND HUMANS

The presence of mycotoxins in food and feed products is harmful to health (Flannigan *et al.*, 1991). The economic impact of mycotoxicosis in animals was emphasized by the discovery that leukoencephalomalacia, a neurotoxic and fatal disease of horses, that is caused by fumonisins from *Fusarium moniliforme* present in corn. Direct toxic effects of mycotoxins in humans are only occasionally apparent. Epidemiological studies have linked aflatoxins to primary liver cancer, while other mycotoxins may also be associated with human diseases due to consumption of contaminated food.

Aflatoxins are acute toxins, which can also inflict long-term chronic effects (Bourgeois, 1975). AFB_1, the most abundant component of the group, is also the most acutely toxic. The descending order of potency is AFB_1, AFG_1, AFB_2 and AFG_2. Aflatoxins exhibit hepatotoxic (Wogan *et al.*, 1971), teratogenic (Elis and Di Paolo, 1967), mutagenic (Ong, 1975; Wong and Hsieh, 1976) and carcinogenic properties (Blunden *et al.*, 1991). The ingestion of aflatoxin-contaminated food has an adverse effect on the immune system in animals (Jakubowska *et al.*, 1984). Aflatoxins have been implicated in the high incidence of human liver cancer in Asia and Africa (Austwick, 1984). The toxins may interact with other agents to cause human hepatocellular carcinoma, the most plausible explanation in developing countries being an interaction between aflatoxins and the hepatitis B virus (Van Rensburg, 1977).

In animals, some of the characteristic features of the disease Kwashiorkor, such as hypoalbuminemia, fatty liver and immunosuppression, are among the pathological changes

caused by aflatoxin. A study in Sudanese children showed that aflatoxin occurred more frequently and at higher concentrations in serum from children with Kwashiorkor than in controls (Hendrickse *et al.*, 1991). Aflatoxins have been implicated in the etiology of several human diseases including Reyes syndrome, Kwashiorkor and hepatitis B (Pitt, 1986). The problems associated with human health and the ingestion of aflatoxins have been reviewed by Blunden *et al.*, (1991).

In view of their carcinogenicity, legal limits on levels of aflatoxins permitted within human food and animal feeds have been imposed in many countries. While varying from country to country, regulatory limits generally fall between 1 and 5 μg/kg for AFB_1 in human food and 5-20 μg/kg in animal feeds (Van Egmond, 1989).

EFFECTS OF AFLATOXIN ON BROILERS

In chickens, some of the most common mycotoxicosis symptoms included pale and enlarged livers, swollen kidneys, oral lesions, impaired immune functions, increased susceptibility to bruising, decreased egg production and lower egg weight, decreased bone strength, increased intestinal fragility, reduction in pigmentation, inhibition of nutrient absorption and reduced growth rates (Waldrop, 1997). The effect of aflatoxin levels on the relative performance of broilers in commercial production facility was reported by Jones *et al.*, (1982). Parameters on broiler performance; number of growers, number birds marketed, average weights (lbs), average feed conversion, % survival, condemnations (%), grower payments (cents/chick), aflatoxin positive feed and aflatoxin concentration (p.p.b.) were compared under three levels of growth classification (good, mediocre and poor), Feed samples within the animal houses were examined for mycotoxins and performance correlated to incidence and aflatoxin levels. Growers classified as "good" had a lower incidence of aflatoxin in their feed with a lower level of contamination than those classified as "mediocre" or "poor" even though receiving feed from the same mill. As a result, their

birds grew better and more efficiently with less condemnation at the processing plant and consequently, these growers received a higher payment for their chicks. This study also reported the aflatoxin contamination rates at different days of feeds (1-5, 6-10, 11-15 and 16-20 days). The average level of aflatoxin contamination increased with time from 7.9 to 27.9 ppb, and the percentage of samples showing positive results increased from 20.5 to 66.7 %.

According to several studies, the effects of aflatoxins on broilers were a significant decrease body weight and a variety of symptoms, such as enlarged liver, spleen and pancreas, repressed bursa and pale combs, shank and bone marrow (Smith and Hamilton, 1970; Tung *et. al.*, 1975; Huff, 1980). Aflatoxin inhibits fat digestion in broilers by decreasing enzyme levels and bile acids required for fat digestion. A high fat and protein diet made aflatoxicosis less severe in broilers (Osborne *et al.*, 1975). Aflatoxin is hepatotoxic, resulting in elevated liver lipid levels (Tung *et al.*, 1973) and disruption of hepatic protein synthesis (Tung *et al.*, 1975). Aflatoxin increases the susceptibility of young broiler chickens to bruising (Tung *et al.*, 1971). Doerr *et al.* (1983) showed that the abnormalities normally encountered in broilers fed moderate to high levels of aflatoxins can be produced with much lower levels of toxins (0.075-0.675 ppm) if they are continuously exposed to contaminated feed from one-day-old to market. The regime reduced growth, and pigmentation, and fatty livers were observed. The effects of aflatoxin on laying birds was reported by Garlich *et al.* (1973). Aflatoxin decreased egg production about 2-4 weeks after toxin administration. It decreased egg weight, but had no significant effect on shell thickness (Hamilton and Garlich 1971). These workers also suggested that dietary aflatoxin can cause fatty liver syndrome in laying hens. This was confirmed later, when 2 ppm aflatoxin in the feed decreased egg production and egg weight and increased the incidence of fatty livers in laying hens (Petterson, 1991). Residues of aflatoxin B_1 occur in eggs and in tissues from hens and broilers fed aflatoxin-contaminated rations (Jacobson and Wiseman, 1974). Aflatoxicol is the most toxic of the known B_1 metabolites, in eggs or meat. Trucksess *et al.* (1983) demonstrated that aflatoxin B_1 and aflatoxicol can be detected in eggs and edible tissues from hens given fed contaminated with 8 ppm aflatoxin B_1.

The influence of aflatoxin on immunity was reviewed by Richard *et al.* (1978). They suggested that aflatoxin affected the production of certain non-specific humoral substance, the activity of thymus-derived lymphocytes and the formation of antibodies. Aflatoxin increased the susceptibility to bacterial infection in chickens, with the exceptions of *Salmonella gallinarum* and *Candida albicans* (Pier, 1986). Aflatoxins affected the immune responsiveness of chicken through non-specific defense mechanisms. The effect of aflatoxin on gamma-globulin levels and antibody titers are less consistent than the effects on non-specific humoral substances. Consumption of moderate levels of aflatoxin does not decrease the levels of immunoglobulin. However, decreased levels of immunoglobulins IgA and IgG have been reported when relatively high doses (2.5-10 ppm) of aflatoxin were administered (Tung *et al.* 1975; Giambrone *et al.* 1978). Moreover, feeding aflatoxin to poultry resulted in a decrease in antibody and cell-mediated immune responses, resulting in severe disease outbreaks even after vaccination (Mohiuddin, 1992). Aflatoxicosis reduces the ability of chickens to synthesize protein, and thus their ability to synthesize antibodies is reduced. This results in very low antibody titers if aflatoxin has been consumed either prior to, during, or after antigen exposure. Aflatoxin ingestion causes atrophy of the bursa and the thymus resulting in deficiencies in both humoral and cell-mediated immunity.

Toxicity of aflatoxin to animals requires their activation in the biological system. Aflatoxin B_1 is activated to AFB_1 8, 9-epoxide primarily by cytochrome P450s. This metabolite is unstable and its diol products undergo base-catalyzed rearrangement to a dialdehyde that reacts with protein lysine residues. The epoxide also reacts with DNA to give an adduct with high yields (> 98 %). This epoxide can be conjugated by glutathione S- transferases (GSTs) to give more polar metabolites and hence is readily excreted. It was suggested that chemoprotective agents for aflatoxin toxicity act by both inhibiting cytochrome P450s and inducing GSTs (Guengerich *et al.*, 1998).

REMOVAL OR DESTRUCTION OF AFLATOXIN FROM CONTAMINATED SAMPLES

The technology to prevent mycotoxin contamination of crops during harvest and/or during storage is not yet available. Large economic losses may be incurred if producers or processors are required to destroy these commodities. Therefore, effective methods to separate, remove, isolate, or detoxify contaminated commodities are essential until effective technology for prevention becomes available (Kamimura, 1993).

1. Behaviour of Mycotoxin at High Temperature

Usually, heating processes such as boiling and frying are used for cooking. Mycotoxins cannot be decomposed or eliminated completely at temperatures and durations that are usually used for cooking.

2. Behaviour of Mycotoxin During Cooking

The majority (50-80 %) of mycotoxins remained in boiled food samples, and 10-50 % of mycotoxins can be detected in the water used for the boiling contaminated food. This indicates that boiling is almost ineffective for removing mycotoxins and that they can be transferred from the food into the water (Kamimura, 1993).

Mycotoxins also survived frying in oil and steaming, remaining in the cooked food. This indicates that domestic cooking processes do not remove mycotoxins.

3. Behaviour of Mycotoxin in Food Additives

Many additives and preservatives are used in various foods manufacturing processes. The stability of mycotoxins when they encounter acid and alkaline agents used in food manufacturing processes and bleaching agents such as sodium sulphite and sodium

hypochlorite was studied. Acidic agents, such as hydrochloric acid and sulphuric acid exert no effect on many mycotoxins, while aflatoxin B_1 is converted to B_{2a} and aflatoxin G_1 to G_{2a}. Alkaline agents, such as sodium hydroxide and sodium carbonate, deform mycotoxins almost completely. However, when the pH is reversed from alkaline to acidic, aflatoxin was recovered. This may be explained by a reversible chemical reaction, where a lactone ring is opened in the alkaline state and closed in the acid state to recover aflatoxin (Kamimura, 1993).

4. Removal of Mycotoxin During Manufacturing

Food manufacturing plants use heating, washing and other processes similar to those used in homes. However, manufacturing lines can incorporate other processes for removal of defective materials and food additives. Mycotoxins are resistant to heat and cannot be degraded easily during many manufacturing processes as stated previously.

5. Removal of Mycotoxin by Separation

Separation processes are divided into two types: mechanical separation by picking up defective grain, and hand-picking by workers based on visual observation. For visual separation, hand picking of grain is very effective since defective and normal grain can be differentiated, however variation between workers in visual inspection standards and the time this takes make it impractical for large scale processing. Mechanical separation involves procedures such as sieving-separation, gravity-separation, wind-separation, metal-separation and colour-separation. Wind and colour separation applied in the removal of defective grain use a series of sorting process comprising of sieve sorter and a gravity sorter, followed by a wind sorter to separate different sizes and weights of grain. Colour sorting involves different coloured grain being detected by a machine. The separation processes using wind and colour, in particular, are effective in removing mycotoxins, but still remain inadequate (Kamimura, 1993).

6. Weakening and Growing of Mycotoxin During Storage

Great improvements have been made in storage techniques and management of products (as reflected, for instance, in the spread of low-temperature warehouses) as more food products are transported and distributed in a frozen or refrigerated state. However, many of the material warehouses currently available are unsatisfactory in maintaining the quality of the products, because they are designed as mere containers. Mold can grow rapidly as relative humidity increases and where vapor forms easily and temperature changes rapidly (Kamimura, 1993).

Relative humidity is maintained at around 60 % in most material warehouses in Japan. Mycotoxins are not produced under such conditions although care should still be taken.

SURVEY OF MYCOTOXIN CONTAMINATION IN FOOD AND FEEDS

The Tokyo Metropolitan Government has surveyed various commercial food products since 1971 for mycotoxin contamination. Mycotoxins including aflatoxin and Fusarium toxins such as deoxynivalenol, nivalenol, fumonisin, moniliformin, zearalenone, as well as ochratoxin and citrinin have been detected in various foods as detailed in Table 2.

Aflatoxins have been detected in grain products such as buckwheat, adlay (Coix lacryma-jobi var, ma-yuen) and corn. Fusarium toxins have been found in wheat, barley, adlay, corn and popcorn. Ochratoxin and/or citrinin have been detected in adlay, buckwheat and rye. Mycotoxins including aflatoxin have also been found in other products including seeds, nuts, spices, beans and dairy products.

Table 2. Distribution of positive samples in Japanese mycotoxin surveys (after Kamimura, 1993).

Cereals	Wheat, Flour, Polished barley, Buckwheat, Corn, Corn meal, Popcorn adlay, Rye flour, Others.
Nuts and Seeds	Peanut, Peanut butter, Peanut oil, Pistachio nut, Sesame, Brazil nut.
Beans	Butter bean, Red bean, Soy bean, Bean jam.
Spices	Nut meg, White pepper, Red pepper, Paprika, Mixed spices.
Dairy Products	Natural cheese

Important commodities susceptible to aflatoxin contamination include: edible nuts (especially ground nuts), oil seed, cereals, and spices. Aflatoxins contamination of cereals and oil seed used as animal feed is an important public health issue, since aflatoxin B_1 fed to dairy cattle is partly metabolized to AFM_1, which is subsequently secreted in milk. AFB_1 and its metabolites have also been reported in eggs, meat and dairy products (Jelinek *et al.*, 1989).

AFLATOXIN CONTAMINATION IN ANIMAL FEEDS IN VARIOUS COUNTRIES

Early studies of aflatoxin contamination of feed from various countries have been reviewed by Jelinek *et al.* (1989). Poultry feed surveys in Egypt, India, Indonesia, Mexico, Nigeria, Pakistan, Saudi Arabia, South Africa, Turkey and USA showed that the incidence of aflatoxin in countries from hot regions varied from 18.9 to 94.4 % of samples. This exceeds the Food and Drug Administration and European Community permissible level of 20 p.p.b.. For mixed poultry feeds, a mycological survey was undertaken by Bragulat *et al.* (1995) who found: *Aspergillus flavus, A. candida, A. amstellodami, Penicillium chrysogenum and Fusarium moniliforme*. Shreff *et al.* (1998) found *Aspergillus* in commercial poultry mixed feeds in 82 % of the samples. *Penicillium* and *Fusarium* were found in 77 % and 57 % of the samples, respectively. In Argentina, the two dominant groups in commercial poultry feeds were *Aspergillus* (85 %) and the *Fusarium* (70 %) and *A. flavus* was the most common species (Dalcero *et al.*, 1998). *Aspergillus flavus* was predominant in poultry feed manufactured in Brazil (Oliveira *et al.*, 1998). In Cyprus, aflatoxins in locally produced and imported feed (nuts, cereals, oil seed, pulses etc) were monitored and controlled systematically and effectively from 1992-1996. The highest incidence of aflatoxin contamination was in peanut butter (56.7 %) and the highest level of AFB_1 was in peanuts. Twelve percent of samples had detectable levels of AFM_1 (Kakouri *et al.*, 1999). In Costa Rica, 3,000 samples of maize from various regions were evaluated the aflatoxin contamination. Contamination with *A. flavus* was frequent and about 80 % of all samples contained more than 20 ng aflatoxin g (-1) grain (Mora and Lacey, 1997). In Italy, surveys of aflatoxin M_1 in dairy products from supermarkets and drug stores by immunoaffinity column extraction and HPLC showed 86 % of the milk samples had 1-108.5 ng/l and 80 % of yogurt samples had 1-496.5 ng/l (Galvano *et al.*, 1998).

STATUS OF AFLATOXIN CONTAMINATION IN ANIMAL FEEDS IN THAILAND

In Thailand, there were reports on the contamination of aflatoxins in agricultural products marketed in the country resulting in serious economic problems (Asanuma and Vayuparn, 1985; Chu *et al.*, 1987.). Khajaroen *et al.* (1997) reported the contamination of aflatoxin found in corn, rice bran, local and imported soya bean, local and imported fishmeal, peanut oil and peanuts. Charoenwai (1999) studied the aflatoxin contamination in raw feed materials, swine feeds, and duckling feeds. Based on aflatoxin analysis by mini-columns, 200 p.p.b. aflatoxin contamination was found in fishmeal and 500 p.p.b. in peanut meal. Aflatoxin contamination was 80 p.p.b. in swine feed for weaning pigs and 30 p.p.b. in meat-based duckling feed. However, the mini-column method was not considered to be very sensitive and accurate.

RATIONALE OF THIS STUDY

Based on the preliminary survey of Department of Animal Science Khon Kaen University, the quality of broilers raised in the northeast during 1996-1997 indicated that there were severe problems with broiler rearing that needed to be resolved. The initial assumption, based on the characteristics of some of the broilers was that the source of the problem was likely to be high exposure to aflatoxin, possibly from contaminated broiler feeds. This thesis reports a pilot study at 8 broiler farms to collect data on broiler abnormality characteristics and establish the relationship between broiler abnormalities and aflatoxin exposure. At the inception of the study the methods used for aflatoxin quantification in Thailand for feed raw materials and some animal feeds were mainly mini-column and thin layer chromatography based. Suitable, sensitive and reliable methods for the determination of aflatoxins in complex animal feeds, especially in broiler mixed feeds, were not established. hence as part of this study appropriate methodologies for aflatoxin analysis in broiler mixed feeds had to be developed.

The rationale of this study was to work with the farmers involved in the pilot study to improve their animal husbandry techniques both in feed storage and usage. To check whether improvements that were suggested were correctly implemented and establish whether these reduced the number of broiler abnormalities appearing in the flocks. Then to test the new commercial clean-up columns used in the extraction of feed samples for their potential routine application for mixed feeds in Thailand. Prior to this study routine analysis of mycotoxins in Thailand were undertaken by mini-columns, TLC, HPLC and, in some limited cases ELISA. Most samples tested were raw materials such as corn, peanut meal, peanuts, rice, rice bran, soya bean meal, fish meal and peanut oil. Aflatoxin contamination in mixed animal feeds, especially mixed broiler feeds, has never been reported in Thailand. There were also no reports on the use of commercial clean-up columns in Thailand, either with the feed raw materials or any mixed feeds. Establishing routine reliable methods for aflatoxin measurements in Thai feed and broilers is important as a large export market has recently been established for this animal commodity.

CHAPTER 3

CHAPTER 3: MATERIALS AND METHODS

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Field collection sites

Eight farms were selected for experimental sampling. Fifty percent of these farmers were supplied with their feed by the Betagro company the remainder by the Charoen Pokphand (C.P.) company. Farms occur as a collective cluster. The four Betagro farms started raising broilers twice on August 20, 1998 and August 24, 2000 and completed rearing on September 30, 1998 and October 4, 2000, respectively in the Nongrour district of Khon Kaen province (see Figure 12). The four C.P. farms also started raising broilers twice on August 25, 1998 and August 28, 2000 and completed rearing on October 5, 1998 and October 8, 2000, respectively in the Ban Pai district of Khon Kaen province (see Figure 11 and 12). There was approximately 80 Km. between the two farming collectives.

Figure 11. Map of Thailand, showing Khon Kaen province with a red spot.

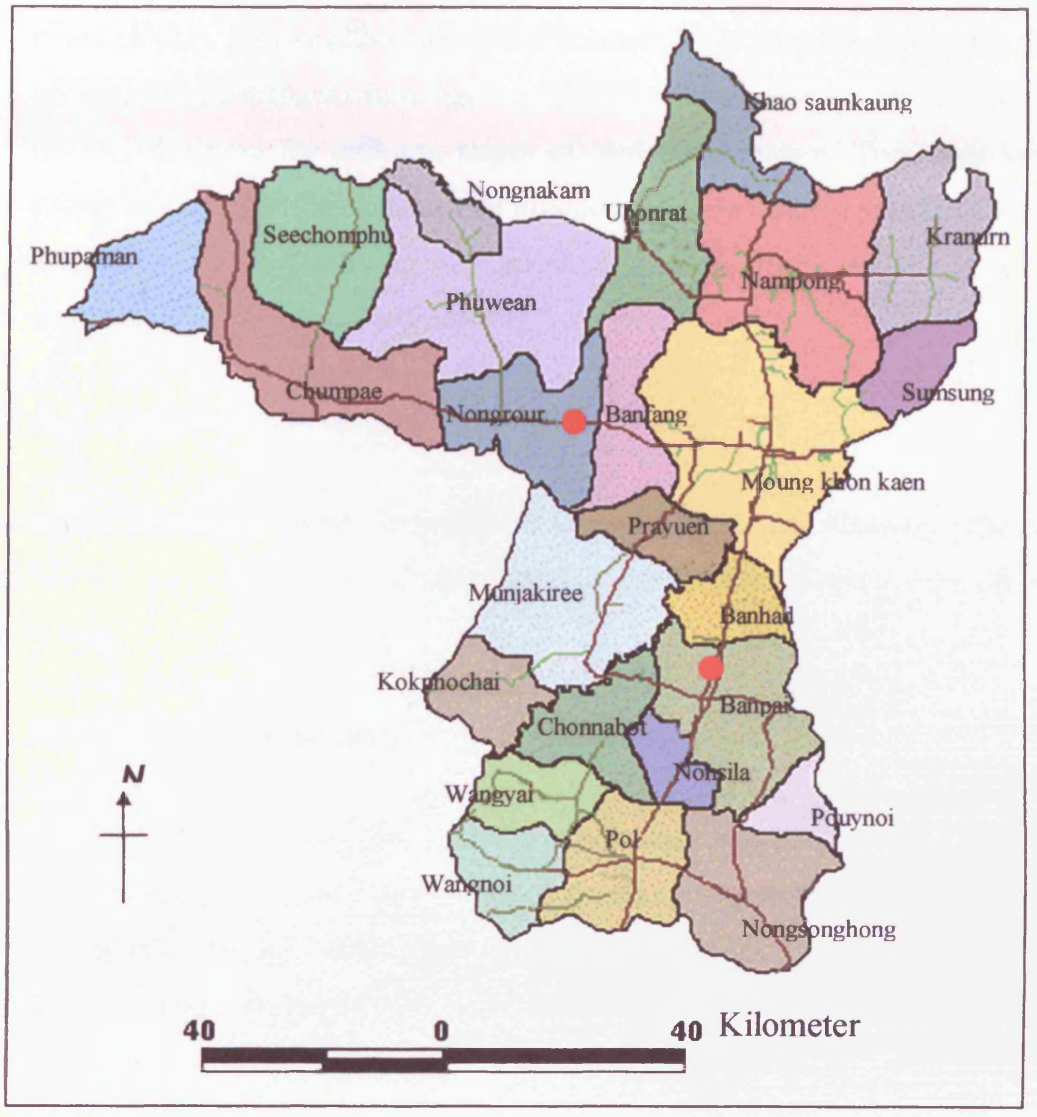


Figure 12. Map of Khon Kaen province, showing locations of C.P. (Ban pai district) and Betagro (Nongrour district) farms with red spots.

Feed collection periods.

Feeds were sampled from each of the farms during three broiler growth periods: starter (0-21 day old chicks), grower (22-35 days) and finisher (36-42 days) period. These three periods reflected three different formulations of broiler feed, supplied by the two feed companies and designed for different stages of bird development. The feeds varied predominantly in their protein content. The minimum protein content standards for the feeds were set by the Thai Ministry of Agriculture at 21%, 19 %, and 17 % for the starter, grower and finisher feeds, respectively.

Broiler feed used to evaluate column efficiency.

Feed samples for the laboratory experiments to evaluate column clean-up efficiency were collected from new batches of each brand of broiler feed, directly from the two manufacturers.

Broiler feed used for the field study.

Feed was supplied by the companies in sacks, which were then stacked at the farm and subsequently transferred to feed bins to be used. As aflatoxin contamination could occur at the point of manufacture, when stored on the farm in sacks or when stored on the farm in bins, a sampling regime was established to check the feed under all storage conditions.

Feed was sampled from sacks and storage bins on the farms.

a) *Sampling of new feed from sacks delivered to the farms:* Feed samples were collected from all four farms supplied with each brand of broiler feed, to examine rates of aflatoxin contamination generated during shipping.

b) *Sampling from feed bins:* Feed samples were collected from all four farms supplied with each brand of broiler feed, to examine rates of aflatoxin contamination during storage on the farm during the broiler raising period.

As part of this programme detailed discussions were also held with each farmer on good practise in feed handling storage. This was undertaken to improve the farmers animal husbandry and to ensure their co-operation with the study.

Storage of feed samples after sampling.

Samples were transferred back to the laboratory in Khon Kaen on the day of collection and dried in an oven at 100 °C for two hours before storage and analysis. Calcium propionate 0.01 % was added to the feed samples to prevent de novo mold infection, and the sample were stored at 4 °C. Immediately before analysis the feed samples were equilibrated to room temperature.

Raising and collection of broilers.

Approximately 4,000-7,000 broilers were raised at each farm from which broiler feed samples were collected. Broilers were sampled from all 8 farms. All broilers were raised routinely by the personnel on each farm. The rearing format was identical in all farms with chicks being held in large, open-sided barns each with a large number of water and food stations. On the last day of rearing (42^{nd} day), twelve broilers were randomly collected from each farm according to their size (large, medium and small) for further investigation.

Examination of broiler performance.

All broilers from each farm were evaluated for their performance (broiler growth rate, food intake, feed conversion ratio (F.C.R.) mortality rate and feed cost/body weight). All broiler samples were evaluated for their carcass characteristics (body weight (g), % carcass, internal organ weight, liver colour, skin colour and carcass grade score) and their abnormality (feather score, leg pigmentation and leg deformity).

Commercial characteristics of the broilers were calculated by the following equations:

Mortality rate = Number of broilers dead from the first to the last day of raising.

$$\text{Percent broiler loss} = \frac{\text{Number of broilers dead}}{\text{Total No.of broilers}} \times 100$$

$$\text{Density of broilers (Broilers/m}^2\text{)} = \frac{\text{Number of broilers}}{\text{Farm area}}$$

$$\text{Average weight per broiler (Kg)} = \frac{\text{Total broilers weight}}{\text{No. of broilers}}$$

$$\text{Feed Conversion Ratio (F.C.R.)} = \frac{\text{Total feed intake (kg)}}{\text{Total broiler weight out (kg)}}$$

Feed cost/Body weight (Baht/Kg) = F.C.R. x Feed cost (Baht/kg)

Liver colour was graded on the degree of colour deviation from the normal colour with four categories: 1 = very pale, 2 = pale, 3 = red and 4 = dark red (normal).

Skin colour used the deviation from the normal colour (yellow) within four grades: 1 = very pale, 2 = pale, 3 = moderate and 4 = yellow

Carcass grade was categorized by varying degree of full breast, width and size. Three grades were used: A = 3, B = 2 and C = 1 (A was the highest grade).

Feather score: Quality of the broiler's feathers were categorized on the basis of feather growth and appearance. These were graded into 3 categories as follows: 1 = poor, 2 = moderate,
3 = good

Leg pigmentation score. Leg pigmentation was graded by the degree of colour in which yellow was regarded as normal. Three grades were used:
1 = pale, 2 = moderate, 3 = yellow (normal)

Leg deformity score: The abnormality of broiler legs were considered by direct observation and graded using the following criteria.

0 = normal.

1 = one leg slightly deformed.

2 = both legs slightly deformed.

3 = one leg slightly, another severely deformed.

4 = both legs severely deformed.

Feed Sampling Methodology.

a) Sampling of new feed from sacks.

1) Broiler feed was sampled immediately after it was received by the farmers, and thereafter every 3-5 days. When feed was stored in sacks, a small probe was used to penetrate half way through the sack from the top to the bottom. A 200 g sample of feed was then taken from 4 directions within the sack (see figure 13). One third of the sacks (i.e. 20 sacks) were randomly sampled from each farm.

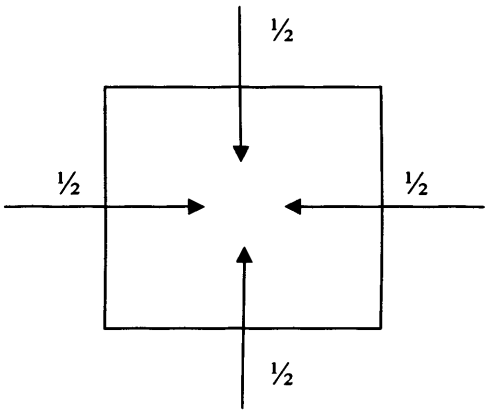
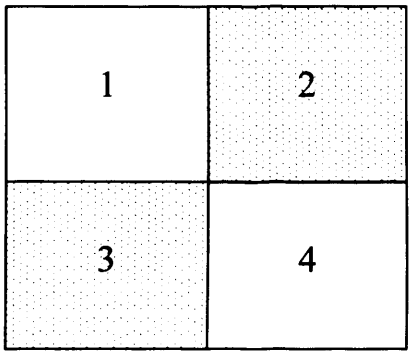


Figure 13. Demonstration of feed sampling from a sack using a small probe.

The 4,000 g samples of feed collected from each farm from this intensive sampling routine, were spread onto a clean sampling tray and reduce to about 2,000 g by placing a grid on the sampling tray and taking sectors 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 (see Figure 14). The combined feed from these sectors was re-sampled twice by the quartering method using feed from sectors 2 and 3 from the quartering tray to yield about 500 g of feed (Figure 14). These samples were ground in a beater mill until they would passes through a 20 mesh sieve.

1	2	3	4	5
6	7	8	9	10
11	12	13	14	15
16	17	18	19	20
21	22	23	24	25

Sampling tray 4 kg.



Quartering method 500 g

Figure 14. Demonstration of feed sampling by sampling tray and quartering method, which allowed a 4 kg field sample to be reduced to 500 g for analysis.

3) The feed for the same broiler growth period, i.e. starter, grower or finisher, from the same farm, was thoroughly mixed and sampled by further quartering to reduce the size of the sample to the 50 g required for analysis.

b) Sampling of feed from storage bins in the field.

1) Samples were collected with a 50 cm^3 cup from the top of the bin (50 g) and four points around the bottom (200 g) of the feed bin for bulk material giving a total sample of 250 g/bin. One third of feed bins (i.e. 32 feed bins) were randomly sampled from each farm daily for a period of 42 days.

2) The 8,000 g samples collected from the feed bins on each farm, were spread separately onto a clean sampling tray and reduce to 4,000 g by placing a grid on the sampling tray and taking sectors 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 (as in Figure 14). The combined feed from these sectors was re-sampled four times by the quartering method, with sectors 2 and 3 collected from each quartering tray, to give a daily yield of about 250 g of feed from each farm. Samples were ground in a beater mill until they passed through a 20-mesh sieve.

Mixing/blending

A beater mill (Retsch Mühle, type SK-1) with a 20 micron mesh, for grinding broiler feed samples was purchased from Fritsch. A high speed blender (model MX-T110 PN) with a 1litre jar was purchased from National. Glass syringe barrels (Rhône, manual type) (10 ml) and pump units were obtained from Rhône-Diagnostics Technologies Ltd.

Clean-up columns

Four different commercial clean-up columns for aflatoxin analysis were investigated:

a) MycoSep columns, No. 224, were purchased from ROMER Lab Inc.1301 Stylemaster Drive Union, Missouri 63084, U.S.A. (Multifunctional clean up column type, MFC).

b) PH Phenyl Bond Elute columns, 500 mg, were purchased from Varian Ltd. 23 Manor Road Walton-on-Thames Surrey KT12 2QF, England. (Phenyl bonded phase column type, PH).

c) Aflatest-P columns, the Vicam product, were purchased from ITS (Thailand) Co., Ltd. Monterey Tower Unit 606.6TH Floor. 2170 New Petchburi Road, Bangkapi Huay Kwang, Bangkok, Thailand. (Immunoaffinity column type, IAC).

d) AFLAPREP® columns were purchased from Rhône-Diagnostics Technologies Ltd. West of Scotland Science Park, Unit 3.06 Kelvin Campus, Maryhill Road, Glasglow Scotland G20 OSP, UK. (Immunoaffinity column type, IAC).

Reagents

Trifluoroacetic acid standards of aflatoxins B_1, B_2, G_1 and G_2 were purchased from Sigma Chemical Co. Ltd. Analytical grade methanol, chloroform and acetic acid were purchased from Merck. Liquid chromatography and reagent grade acetonitrile were from J. T. Baker, reagent grade acetone was from Carlo Erba and reagent grade lead acetate

from Fluka. Trifluoroacetic anhydride (TFAA) was purchased from Sigma Chemical Co. Ltd. Water was distilled and deionized.

High Performance Liquid Chromatography system

A Rheodyne 7010-090 autoinjector with a 100 µl sample loop, thermoseparation product model spectra system P-2000, scanning fluorometric detector (Thermoseparation product FL 2000) and data jet integrator (Thermoseparation product) were used. The detector was set at 360 nm for excitation and at 440 nm for emission with a range of 0.5 nm. A guard column was packed with µ Bondapack C_{18} and the main column was packed with Supelcosil LC-18, 25 cm x 4.6 mm, 5 µm.

Types of broiler feed.

Broiler feed samples were obtained from Betagro Co. Ltd. and Charoen Pokphand (C.P.) Co. Ltd., the two major feed manufacturers in Thailand. These two branded broiler feeds are distributed predominantly to the Thai market.

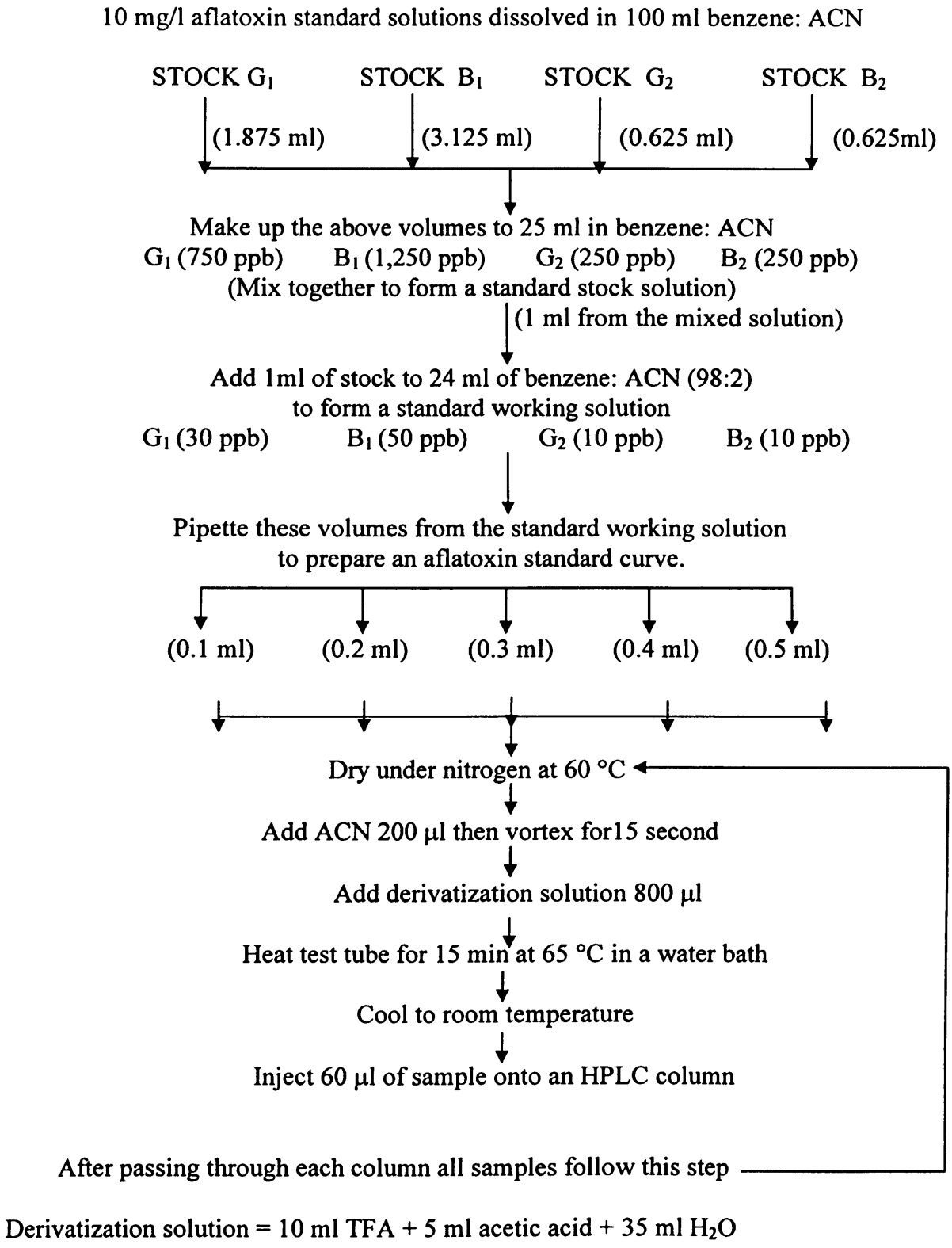
Preparation of a standard aflatoxin stock solution.

A standard aflatoxin stock solution was prepared by dissolving 1 mg authentic standards of aflatoxin B_1, B_2, G_1, and G_2 separately in 100 ml benzene-acetonitrile (98:2 v/v) to give stock concentrations of 10 mg/l.

To prepare the aflatoxin B_1 standard working solution 3.125 ml of aflatoxin B_1 stock solution was made up to 25 ml in benzene-acetonitrile (98: 2v/v), to give a 1,250 ppb(parts per billion) solution. Aflatoxin B_2 stock solution (0.625 ml) was diluted in 25 ml of benzene-acetonitrile to give a 250 ppb solution. To prepare a 750 ppb aflatoxin G_1 solution, 1.875 ml of aflatoxin G_1 stock solution was diluted to 25 ml with benzene-acetonitrile (98:2v/v), while 0.625 ml of aflatoxin G_2 stock solution was made up to 25 ml to give a 250 ppb solution. All these stock solutions were mixed together to produce

working standard solutions. Mixed aflatoxin working standard solutions were prepared from 1 ml of mixed stock solution and 24 ml of benzene-acetonitrile (98 : 2v/v), which gave aflatoxin concentrations of 50, 10, 30, and 10 ppb for B_1, B_2, G_1 and G_2, respectively (Figure 15).

Figure 15. Aflatoxins standard solution preparation and analysis.



Derivatization solution = 10 ml TFA + 5 ml acetic acid + 35 ml H_2O

Mixed aflatoxin standard solution concentrations (ng) containing different aflatoxin sub-types.

When the standards were set up as shown in Figure 15, the concentrations of aflatoxin for each point on the standard curves were as follows:

	G_1	B_1	G_2	B_2
Tube I with 0.1 ml of (SWS)	3	5	1	1
Tube II with 0.2 ml of (SWS)	6	10	2	2
Tube III with 0.3 ml of (SWS)	9	15	3	3
Tube IV with 0.4 ml of (SWS)	12	20	4	4
Tube V with 0.5 ml of (SWS)	15	25	5	5

* Standard Working Solution (SWS)

Preparation of aflatoxin-spiked broiler feed samples.

Fifty gram test samples of the C.P. and Betagro broiler feeds were spiked with aflatoxin to establish recovery rates. All spiked samples were prepared by adding the appropriate amounts of standard aflatoxin to 50 g of dry feed. Both C.P. and Betagro feed samples were spiked at concentrations of 10, 20, 30, 40 and 50 ng/g total aflatoxin. When 10 ng/g of aflatoxin was added, the ratio of aflatoxin was 5:1:3:1 for B_1:B_2:G_1:G_2, respectively. All test samples were mixed well before the aflatoxin was re-extracted to measure the efficiency of the clean-up procedures.

Extraction of feed samples.

Prior to the determination of aflatoxin, feed samples had to be extracted and cleaned-up. In this study, various types of solid-phase extraction (SPE) were applied and four different commercial columns were compared. The methods recommended for each column were initially followed and are described below.

a) For the Romer columns (MycoSep No. 224).

Fifty grams of weighed ground sample was placed into a blender jar. Extraction solvent (100 ml of acetonitrile: H_2O, 84: 16) was added and the mixture was blended at high speed for 3 minutes. The resultant homogenate was filtered through Whatmans No. 4 filter paper and 3 ml of the filtrate was placed into a 10 ml culture tube.

For the clean-up step, the MycoSep column was slowly pushed via its rubber flange end into the culture tube containing the filtrate. The rubber flange created a tight seal with glass wall of the culture tube. As the column was pushed further into the tube, the extract was forced through the frit via a 1-way valve and through the column packing material. Two millilitres of purified extract was then collected in the column reservoir and transferred to a derivatization vial.

b) For the Varian column (phenyl bonded-phase column)

Ground aliquots (50 g) of sample were extracted within 500 ml of acetone: water (85: 15) using a National, model MX-T110 PN, overhead mixer at high speed for 3 min. The mixture was filtered through Whatmans No. 1 filter paper and the filtrate was retained for clean-up.

In the clean-up step, 5 ml filtrate was made up to 60 ml with water: acetic acid: methanol (92.3:1:6.7) by volume and 3 ml of lead acetate solution was added. The lead acetate solution, was prepared as a 20 % w/v solution of lead acetate trihydrate in 3 % v/v acetic acid. This was added to precipitate colloidal material. The mixture was passed through a phenyl bonded column (PH, 500 mg) which had previously been washed with methanol (5 ml) and water (5 ml). The mixture was pulled through the column at a rate of approximately 10 ml/min under a vacuum. After washing the column with water, the aflatoxins were eluted with chloroform (7 ml). The water was subsequently removed by passing the sample through a column of anhydrous sodium sulphate (3 g) and the

chloroform removed at 45 °C under a stream of nitrogen, using a sample concentrator. The residue was retained for HPLC analysis.

c) For the Vicam column (Aflatest P)

Fifty grams of ground sample was mixed with 5 g NaCl in a blender jar. Aqueous methanol (80 % methanol in water) (100 ml) was added to the jar. The mixture was blended at high speed for 1 minute and the suspension poured onto fluted filter paper. The filtered extract (10 ml) was transferred into a clean vessel and diluted with 40 ml distilled water. The resultant sample was mixed well and filtered through a glass microfibre filter into a clean container.

In the clean-up step, the end caps from the Aflatest affinity column were removed, and the tip of the column cap was used as a coupling. The column was attached to a 10 ml reservoir outlet on a pump stand. Ten millilitres of the filtered extract (10 ml = 1 g sample equivalent) was passed through the Aflatest column at a flow rate of 1-2 drops per second. Two similar volumes of distilled water were then passed through the Aflatest column at the same flow rate. The aflatoxin was then eluted from the Aflatest column at a flow rate of approximately 1 drop per second with 1.0 ml HPLC grade methanol and collected in a clean glass cuvette. Distilled water (1 ml) was added to the eluate before it was injected onto the HPLC column.

d) For the Rhône AFLAPREP column

Two solvent systems, methanol and chloroform, were applied to this column, after poor results were obtained using methanol as recommended by the manufacturers.

With methanol as the solvent system. Fifty grams of well ground sample was combined with 4 g of sodium chloride and the mixture was placed in a solvent resistant blender jar. HPLC grade methanol: distilled water (250 ml, 60: 40 v/v) was added to the jar, and the contents blended for 1 minute at high speed. The extract was diluted with 250 ml of

distilled water. The solution was mixed well by swirling. Immediately after mixing, approximately 25-50 ml of sample was filtered through Whatmans No. 4 filter paper. Aliquots (10 ml) of filtrate (equivalent to 1 g of sample) were transferred into a glass syringe barrel for passage through the immunoaffinity column.

With chloroform as the solvent system. This extraction protocol provided better recoveries than methanol with certain commodities (e.g. animal feed and coffee). Ground sample (50 g) was added to 25 g celite, (also called diatomaceous earth, Sigma), in a 500 ml conical flask. Chloroform (250 ml) was added along with 25 ml distilled or deionised water and the mixture was shaken for 30 minutes. It was then filtered through Whatmans No. 4 filter paper and 20 ml of filtrate was collected. Ten millilitres of filtrate (equivalent to 2 g of sample) was evaporated to dryness in a rotary evaporator at 60 °C. The residue was redissolved in 5 ml of methanol and made up to 50 ml with distilled water before being passed through the immunoaffinity column at a flow rate of 2-3 ml/min. A slow steady pressure was essential to "capture" the aflatoxins with the antibody. The sample volume was 10 ml with the methanol extraction and 50 ml with the chloroform extraction method.

During the wash step, two aliquots of 10 ml of distilled water was added to the glass syringe barrel and passed through the immunoaffinity column at a rate of 5 ml per minute.

Elution. A vial was placed directly beneath the column, and 1 ml of HPLC grade methanol (i.e., eluant) was passed through the column at a flow rate of 1 drop per second. Back flushing (i.e. reversing the direction of flow) with the eluant 3 times was undertaken to ensure complete denaturation of the monoclonal antibody and the subsequent release of aflatoxins into the solution. Following methanol elution, 1 ml of distilled water was passed through the column and added to the methanol fraction to give a total volume of 2 ml.

Determination of aflatoxin levels from the column purified samples by HPLC.

Prior to HPLC analysis the aflatoxin samples had to be derivatized.

Aflatoxin derivatization: Derivatization solution (800 µl), consisting of 10 ml trifluoroacetic acetic acid, 5 ml acetic acid and 35 ml water, was added to 200 µl of working aflatoxin solution in CAN or semi-purified aflatoxin extract from feed samples in a derivatization vial. After capping and mixing, the vial was heated at 65 °C in a water bath for approximately 15 minutes before HPLC analysis.

HPLC System: The fluorometric detector (Thermoseparation product FL 2000) was set at 360 nm for excitation and at 440 nm for emission with a range of 0.5 nm. The guard column was packed with µ Bondapack C_{18} and the main column was packed with Supelcosil LC_{18}, 25 cm x 4.6 mm, 5 µm. Using a Rheodyne 7101-090 autoinjector, an injection volume of 60 µl and a flow rate of 1.0 ml/minute were applied in this study.

Proximate analysis of feed samples.

The broiler feed samples were used for proximate analysis by the procedures described by the AOAC (AOAC, 1990) to comply with the quality, standards recommended by the Thai Ministry of Agriculture.

Moisture content: Feed samples were finely ground and accurately weighed before being placed on an aluminium dish in an oven at 100 °C for 2 hours. They were then removed and allowed to equilibrate to room temperature. The samples were accurately weighed and then placed on the aluminium dish in a vacuum oven at 70 °C under 26-30 inch pressure for approximately 5 hours. The samples were removed, equilibrated to room temperature and weighed. The weight of the feed sample before and after treatment was used to calculate the moisture content.

Protein content: The protein content in feed samples was analyzed by the Kjeldahl method. One gram of feed sample was placed in a Kjeldahl digestion flask. The catalyst mixture, containing anhydrous sodium sulfate and anhydrous copper sulfate was added to the digestion flask followed by 25 ml of concentrated sulfuric acid. The flask was placed in a heating mantle and heated until a clear solution was obtained. It was then further heated for 1 hour until the reaction was completed. At this point, all proteinaceous nitrogen had reacted with sulfuric acid and the ammonium sulfated product was obtained. The solution was cooled and 500 ml of distilled water added and applied to the condensation step. Fifty millititres of 0.1 N sulfuric acid was added to the solution plus 4 drops of the pH indicator methyl red. Eighty millititres of 45 % sodium hydroxide was added to a Kjeldahl flask connected with the condensation equipment. During the condensation step, the ammonium sulfate reacted with sodium hydroxide to give ammonia which was condensed into the receiving flask. When the condensation was complete, or at least 150 ml of ammonia solution was obtained, heating was discontinued. The flask, containing an excess of acid, was then titrated with 0.1 N NaOH until the purple red end point colour was obtained. A similar procedure was conducted with a blank containing no feed sample. Protein estimations were calculated by the following equation:

$$\% \text{ Crude Protein} = \% \text{ Nitrogen x conversion factor.}$$

$$\% \text{ Nitrogen} = \frac{(B-S) \times 0.014 \times N \times 100}{W}$$

Where B is the amount (in ml) of NaOH required to titrate the blank, S is the amount (in ml) of NaOH required to titrate the samples, N is the normality of standard NaOH and W is the weight of feed. The appropriate factor for feed in general is 6.25.

Fat content estimation by ether extraction: Fat content was determined using extraction with ether in a Soxhlet apparatus. Two grams of moisture-free feed was placed in a thimble, which was closed with clean cotton and connected to the Soxhlet apparatus. The samples were extracted with 180 ml of petroleum ether at 40-60 °C for 6 hour. The container was heated until no ether remained and it was then heated at 100 °C for 4

hour. After cooling, it was accurately weighed. The fat content was then estimated using the equation:

$$\% \text{ Fat} = \frac{B-A}{W} \times 100$$

Where A is the weight of the dried flask, B is the weight of the flask plus fat after drying in the oven, W is the weight of feed sample placed in the thimble.

Ash content: A porcelain crucible was placed in a furnace at 550-600 °C for 2 hour and was then accurately weighed after cooling in a desiccator. Two grams of moisture-free feed was placed in the crucible and burned using a bunsen burner in a fume cupboard. Burning was completed in a muffle furnace at 550-600 °C until white or gray ash was obtained, after approximately 2 hours. The crucible containing the ash was cooled in the desiccator and accurately weighed. The amount of ash obtained allows an estimation of the percent ash in the feed sample. Ash content normally represents the inorganic constituents in the feed. A high value would reflect dilution or contamination.

Crude fibre content: Two grams of feed, which had been analyzed for fat content, were placed in a 600 ml beaker. Sulfuric acid (200 ml 1.25 %) was added and the beaker was connected to a fibre digestion equipment system and a condenser for 30 minutes. The solution was removed and filtered under reduced pressure in a Buchner funnel and washed with hot water until there was no acid remaining. The precipitate was filtered and added to 200 ml of previously boiled 1.25 % NaOH, before connecting with a fibre digestion equipment system and boiling for 30 minutes. The solution was removed and filtered. The precipitate was washed with NaOH and then with 20 ml methyl alcohol. The precipitate was transferred to a crucible which was placed in an oven at 100 °C for 2 hours, then cooled in a desiccator and weighed until the weight remained constant. It was then burned in a furnace at 600 °C for 30 minutes, cooled down in a desiccator and its weight recorded. The percent total fibre was calculated from the equation:

$$P = \frac{(A-B)}{W} \times 100$$

Where P is the percent of total fibre, A is the weight of the crucible containing fibre precipitate after drying, B is the weight of crucible containing ash after burning in the furnace and W is the weight of feed samples used in the experiment.

Data and statistical analysis

The raw data obtained from the detection of aflatoxins was calculated in parts per billion (ppb). The percentage recovery was calculated on the basis of actual amounts of aflatoxin recovered from known concentrations of spiked-aflatoxin samples detected by HPLC.

Statistically significant differences in recoveries between two or more variables was determined using SAS analysis in a Completely Randomized Design (CRD). For example, comparisons of aflatoxin recoveries were made between the Romer and Varian columns. In this case only one variable was compared, i.e. the source of the column. In other experiments where there were several variables, the SAS analysis for factorial in CRD was used. The following variables were compared:

1. Sources or type of columns (4): i.e. the Romer, Varian, Vicam and Rhône columns.

2. Brands of feed (2): i.e. the C.P. and Betagro feeds (using 2 x 4 factorial in CRD with 5 replicates).

3. Periods of broiler development (3) : i.e. the starter, the grower, the finisher

4. Sites of feed sample collection (2) : i.e. sacks or storage bins (using 2 x 3 factorial in CRD with 4 replicates)

The statistical analysis was conducted using the Proc GLM programme (SAS, 1988).

COMPARISON OF THE EFFICACY OF FOUR DIFFERENT COMMERCIAL COLUMNS

The efficiency of clean-up columns for determination of aflatoxin contamination levels from feed samples may be dependent on a number of factors, for instance the complexity of the feed matrix, the solvent system used for aflatoxin extraction and the column material. To determine the relative importance of the variables in this analysis the following experiments were undertaken.

Experiment 1. Determination of aflatoxin concentrations from a standard aflatoxin solutions by HPLC.

Standard solutions of aflatoxin were prepared and derivatized as described previously. After derivatization, 60 µl of the different standard aflatoxin concentration solutions were injected onto the HPLC column. All HPLC runs produced four aflatoxin peaks, which demonstrated that the HPLC method gave a good separation of the standards. By using the data from 5 aflatoxin concentration standards, a standard curve was produced by linear regression (Table 3), from which further calculation of aflatoxin concentrations from experimental samples could be calculated.

Table 3. Concentration of the four aflatoxin sub-types in the five different aflatoxin standard solutions used to produce standard curves by HPLC.

Level / Sub-type	Total aflatoxin level (ppb)					
	0	10	20	30	40	50
B_1	0	5	10	15	20	25
B_2	0	1	2	3	4	5
G_1	0	3	6	9	12	15
G_2	0	1	2	3	4	5

Experiment 2. Determination of aflatoxin recoveries from the standard aflatoxin solutions using four different commercial columns.

Different standard total aflatoxin solutions (10, 20, 30, 40 and 50 ppb) were prepared, then extracted and cleaned-up following each column manufacturers' recommended instructions, as described previously. After clean-up via the column, the aflatoxins were detected and quantified by HPLC. Replicates using five separate samples of each column brand were undertaken to determine reproducibility.

Experiment 3. Determination of the ability of the commercial clean-up columns to retain aflatoxin from standard aflatoxin solutions extracted using the manufacturers recommended procedure.

For each brand of clean-up column, the 30 ppb total aflatoxin standard solution was used. The standard solution was extracted and cleaned-up following each manufacturers' recommended instructions, as described previously. After clean-up of the column, the aflatoxins were then detected and quantified by HPLC.

Experiment 4. Determination of the relative efficiencies of extraction with chloroform or dichloromethane as the extraction solvent for aflatoxin clean up using four commercial columns.

Generally, chloroform is the recommended solvent of choice for extractions of aflatoxins from feeds. However, there is pressure to replace chloroform with other solvents on environmental and toxicity grounds (Cole and Dorner, 1994). Dichloromethane, a solvent with a similar solubility profile to chloroform, could be used as a chloroform substitute. Therefore, comparisons between chloroform and dichloromethane as extraction solvents were made on aflatoxin extractions from spiked broiler mixed feeds.

Samples of both brands of broiler mixed feed were spiked with five aflatoxin concentrations (10, 20, 30, 40 and 50 ppb). These spiked mixed feeds were extracted with chloroform or dichloromethane as described previously. The extracts were then passed through one of four different commercial columns. The aflatoxin concentrations in the eluants were quantified by HPLC. Aflatoxin-spiked samples at each concentration were compared with blanks (non-spiked feed samples). Six replicate column extractions were compared for each feed type.

Experiment 5. Determination of aflatoxin concentrations from spiked broiler mixed feed using four different commercial columns.

Samples of broiler feed were spiked with five concentrations of aflatoxin solution. The method was tested with both feed brands and six columns from each manufacturer were tested to determine reproducibility, according to the schedule below.

Mixed aflatoxin standard + C.P. feed			Mixed aflatoxin standard + Betagro feed		
Romer	x	6 columns	Romer	x	6 columns
Vicam	x	6 columns	Vicam	x	6 columns
Varian	x	6 columns	Varian	x	6 columns
Rhône	x	6 columns	Rhône	x	6 columns

Experiment 6. Modification of extraction solvent for column clean-up.

A further experiment, similar to that described in experiment 4, was undertaken with 5 concentrations of spiked feed samples using the Rhône columns with chloroform substituted for methanol as the extraction solvent to determine whether this would improve aflatoxin recoveries.

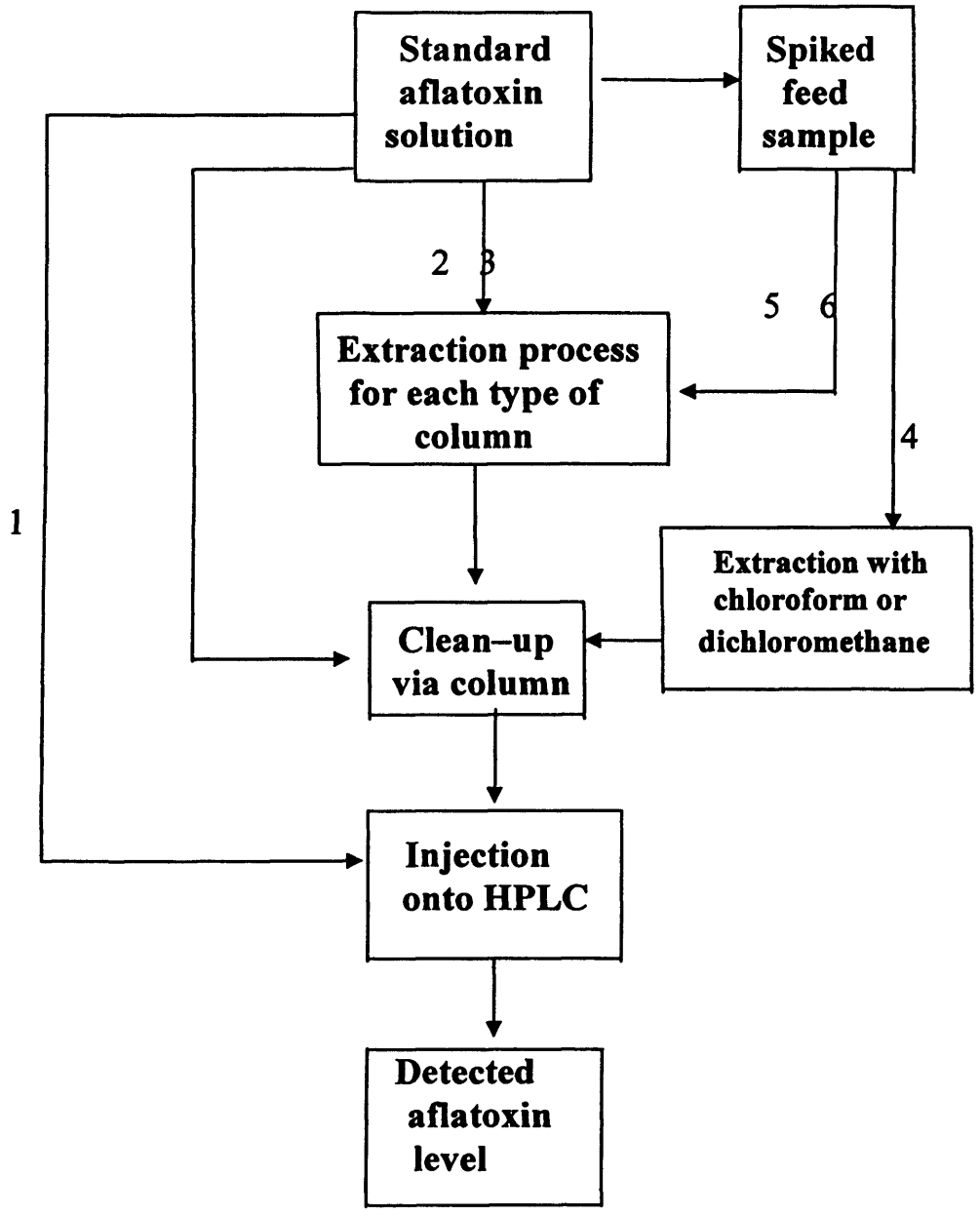


Figure 16. The steps involved in experiments 1 - 6 to determine the efficiency of each stage of the analysis on aflatoxin recovery and analysis. Numbers on the arrows indicate the route of analysis for each experiment.

DETERMINATION OF THE DEGREE OF AFLATOXIN CONTAMINATION IN BROILER MIXED FEED AND A SURVEY OF BROILER PERFORMANCE IN NORTHEAST THAILAND

The two major brands of broiler mixed feeds marketed in Thailand were investigated for the degree of natural contamination with aflatoxin. In addition, the performance and quality of chickens reared on these samples was examined. These experiments used only the Varian columns for extraction and clean-up.

Experiment 7. Determination of naturally contaminated aflatoxin in broiler mixed feeds: a field study undertaken in 2000.

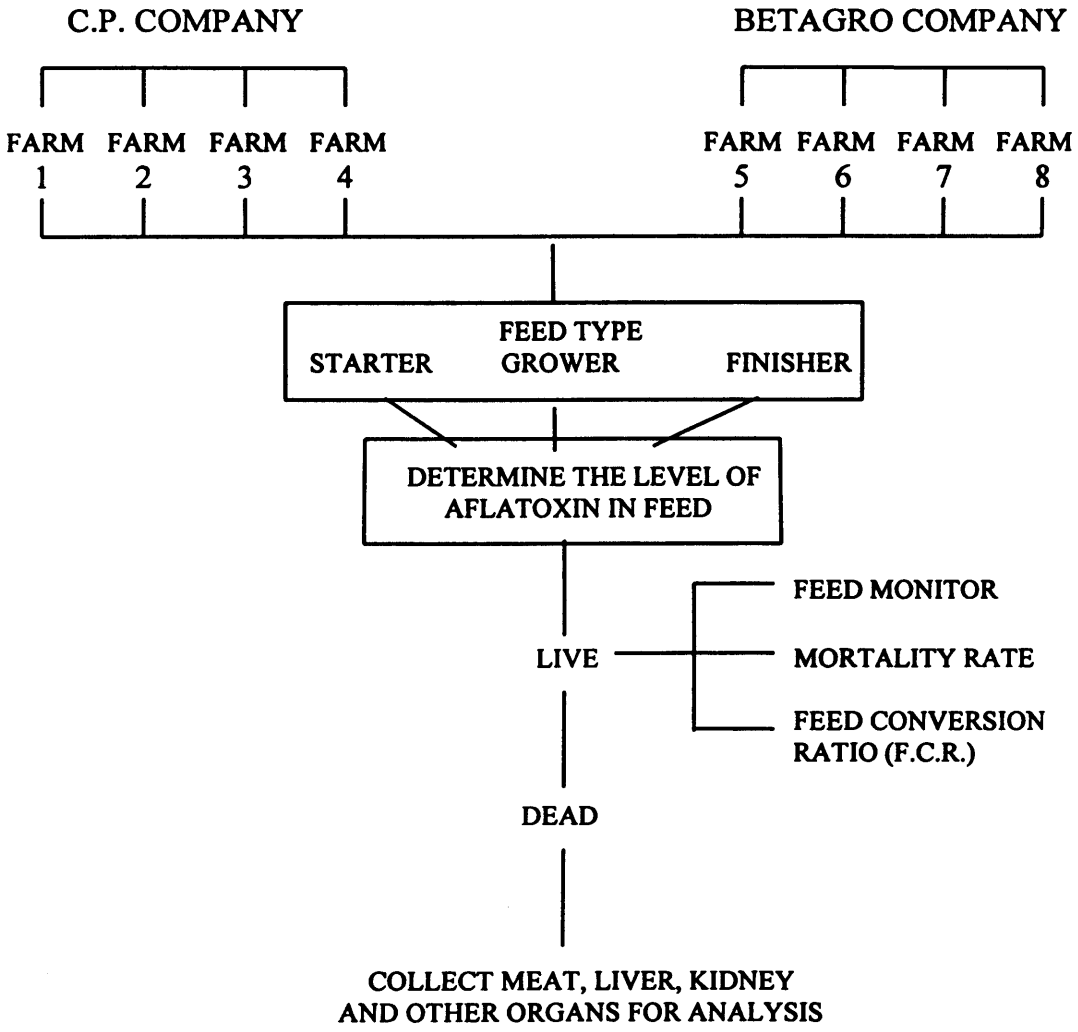
Broiler feed samples, from the C.P. and Betagro suppliers, were collected daily from 4 farms for a total of 42 days, which was equivalent to a complete broiler rearing cycle. All samples from the starter, grower and finisher periods were pooled and then extracted and cleaned-up using the Varian column. The isolates were then injected onto an HPLC to determine the amount of natural aflatoxin contamination in each feed sample. For each brand of feed, the samples were collected from two different sources: i.e. feed sacks and storage bins. Collection and sampling of feed were described previously.

Experiment 8. A survey of broilers performance when fed on broiler mixed feeds with known levels of alfatoxin contamination in Northeast Thailand (Khon Kaen) in 1998 and 2000.

Broilers raised at 4 farms using the C.P. company feed and 4 farms using the Betagro company feed, were raised for 42 days, after which they were examined for their performance indicators as illustrated in Figure 17. It was the original intention in this study to go on and examine the up-regulation of a range of different enzyme systems within the broilers in response to aflatoxin contamination during rearing. However problems with the column clean up procedures in the first year meant that it was year two of the PhD before routine broiler sampling could be undertaken and broiler contamination analysed. Working with the Thai farmers over the first year of the

program resulted in better feed handling and storage procedures, hence by late year two and year three of the PhD programme there was minimal aflatoxin contamination of the broilers and hence the planned quantitative PCR experiments on monooxygenases and glutathione S-transferases in the broilers were not undertaken.

Figure 17. The examination of performance indicators for broilers raised in Northeast Thailand using feed from two companies.



Experiment 9. Proximate analysis of broiler mixed feeds.

The Ministry of Agriculture, Thailand has established guidelines on the quality of animal feed, in particular the recommended levels of moisture, protein, fat, ash and total fibre content. To determine whether the feed samples used in this study were of acceptable national quality standards all these parameters were examined.

CHAPTER 4

CHAPTER 4: RESULTS AND DISCUSSION

IMPROVEMENT OF FEED STORAGE CONDITIONS ON THE FARMS.

Throughout the first year of the study regular visits were undertaken to all farms in the study area. The four farms in each cluster could be visited within one 12 hr day. During these visits sampling of the broilers and the feed was undertaken. Discussions were also held with the farmers on how they could improve their animal husbandry. All broilers were reared in large open sided sheds which were internally sectioned into smaller areas. This provided continuous shade for the broilers and shelter from rains while allowing ready circulation of air to ensure that temperatures did not rise above acceptable levels. Broilers were free range within the smaller areas and were not held under cramped or stressful conditions. Broilers were fed small amounts of food at regular intervals in small cylindrical feeders place on the ground in each of the feeding pens. Feed was eaten rapidly once placed in the feeders, hence there was little chance of aflatoxin contamination occurring once the feed was placed in the rearing pens.

The animal feed was delivered on a cyclical schedule to cover a single broiler rearing batch. Feed was delivered in sealed bags and was in a good condition on arrival at the farms. A small amount of the feed was then transferred to feeding bins for ease of access, the remainder was stored near to the broiler rearing sheds. Recommendations were made to the farmers that feed should be stored in the shade and should be placed on palettes to allow circulation of air underneath the feed stack. Feed sacks also needed to be kept in a dry area of the farm. During the first year of the study this advice was accepted by the farmers and an excellent collaborative working relationship was established with all farmers. Once the new storage conditions had been established the farmers realised the benefits of improving the storage on maintaining the quality of the feed and storage conditions remained good for the rest of the study.

It was also noted that the annual rainfall after the first year of the study was lower than average in the Khon Kaen area. This reduced the moisture content in the air generally and reduced the potential for aflatoxin growth in the feed. The improvements in the feed storage conditions coupled with the dry weather led to a noticeable reduction in the

number of broiler deaths and obvious deformities present in the flocks in all farms. This improvement was independent of the original source of the feed.

To establish the levels of aflatoxin contamination within the feed at delivery and then at various stages after its arrival at the farm a series of experiments were undertaken to optimise the aflatoxin analysis system and undertake the analysis.

DETERMINATION OF AFLATOXIN CONCENTRATIONS FROM A STANDARD AFLATOXIN SOLUTION BY HPLC

Standard aflatoxin solutions were prepared as described in Chapter 3, at concentrations of 10, 20, 30, 40 and 50 ppb, respectively. Each aflatoxin mixture contained the subtypes, G_1, B_1, G_2 and B_2 in the proportions 3: 5: 1: 1, respectively.

Table 4. Actual concentrations of aflatoxin subtypes in the different total aflatoxin mixtures used as standards for HPLC analysis.

Aflatoxin mixture concentration (ppb)	Actual aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
0	0.00	0.00	0.00	0.00	0.00
10	3.00	5.00	1.00	1.00	10.00
20	6.00	10.00	2.00	2.00	20.00
30	9.00	15.00	3.00	3.00	30.00
40	12.00	20.00	4.00	4.00	40.00
50	15.00	25.00	5.00	5.00	50.00

THE HPLC CHROMATOGRAMS OF AFLATOXIN

The aflatoxin standards were detected and quantified by HPLC, in the system described in Chapter 3. This methodology produced an HPLC chromatogram with 4 clear peaks, an example of which is given in Figure 18. The retention times for G_1, B_1, G_2 and B_2 in this system were 7.22, 9.78, 13.14 and 19.25 minutes, respectively.

Detection limits for the aflatoxins were 0.6, 0.7, 0.2 and 0.2 ppb for G_1, B_1, G_2 and B_2 respectively.

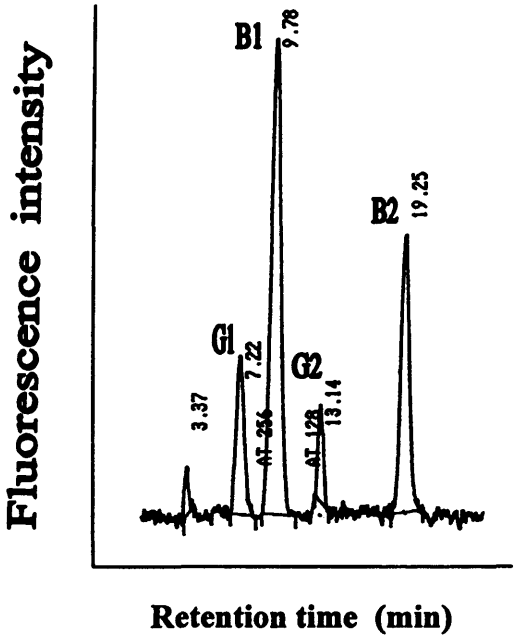


Figure 18. A representative HPLC chromatogram of aflatoxins using a 30 ppb aflatoxin standard solution.

The expected concentrations of each aflatoxin subtype were accurately calculated from HPLC analysis for the 5 standard solution concentrations. The results are shown in Table 5 and Figure 19.

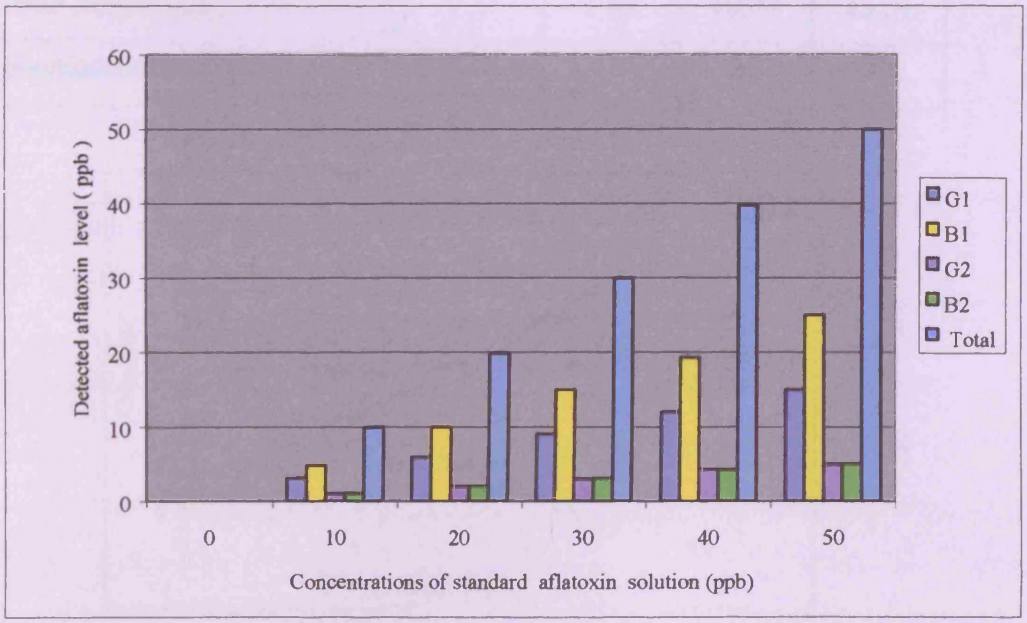
Replicate analysis of the 5 aflatoxin mixture standards was undertaken to generate standard curves for each aflatoxin subtype. The standard curves are shown in Figures

20-23. All standard curves corresponded to good fits to straight lines indicating that the concentrations of all standards were well within the viable detection ranges for the HPLC system used.

Table 5. The concentrations of aflatoxin subtypes in five concentrations of the standard aflatoxin solution as confirmed by HPLC.

Aflatoxin mixture (ppb)	Aflatoxin subtype concentration (ppb)					Percentage recovery
	G_1	B_1	G_2	B_2	Total	
0	0.00	0.00	0.00	0.00	0.00	0.00
10	3.08	4.79	0.98	1.04	9.89	98.90
20	5.93	9.90	1.94	2.03	19.80	99.00
30	8.94	14.89	2.98	3.12	29.93	99.77
40	11.95	19.23	4.28	4.26	39.72	99.30
50	14.95	24.95	4.98	5.02	49.90	99.80

Figure 19. The concentrations of aflatoxin subtypes, detected by HPLC from different concentrations of a standard aflatoxin solution.



Aflatoxin recovery rates from the column were from 98.9-99.8 % for the mixture standards for the concentration range 10-50 ppb. This indicated that the HPLC system and the analaysis methodology used were suitable for aflatoxin analysis.

Figure 20. Calibration curve for aflatoxin G_1.

Peak height (cm)	1.07	2.4	3.77	5.3	6.6
Concentration (ppb)	3	6	9	12	15

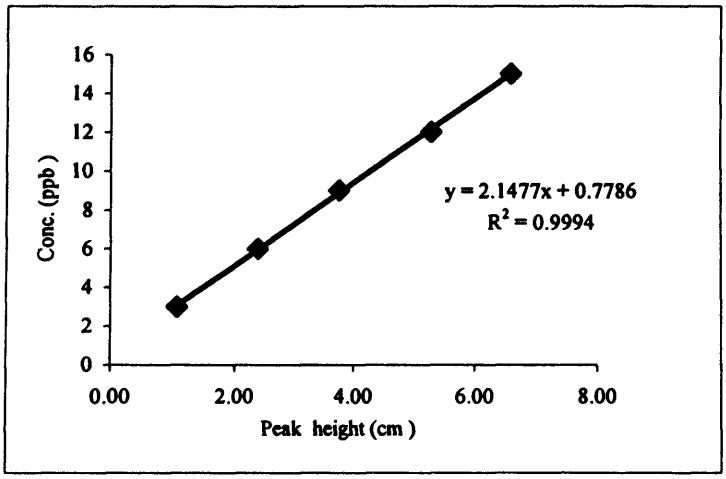


Figure 21. Calibration curve for aflatoxin B_1.

Peak height (cm)	2.33	5.14	7.88	10.79	13.51
Concentration (ppb)	5	10	15	20	25

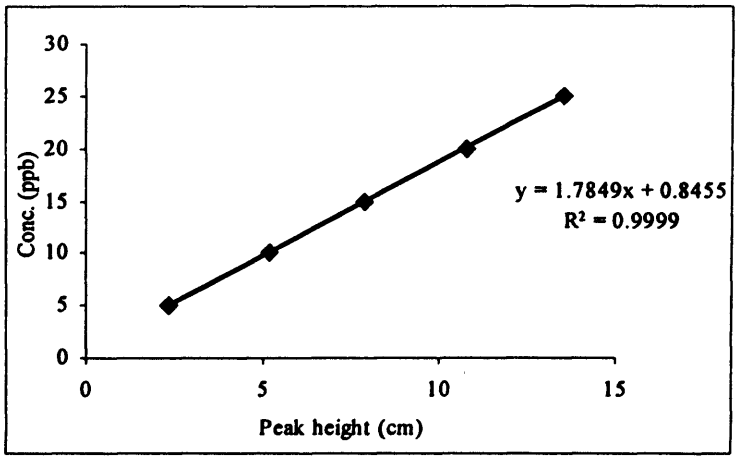


Figure 22. Calibration curve for aflatoxin G_2.

Peak height (cm)	0.85	1.7	2.72	3.57	4.47
Concentration (ppb)	1	2	3	4	5

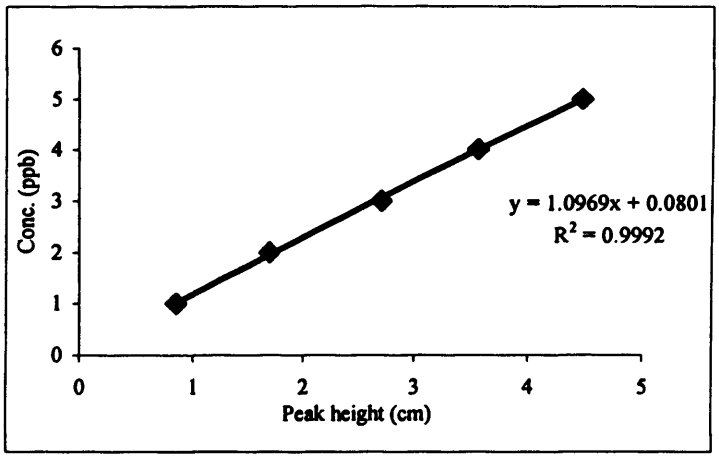
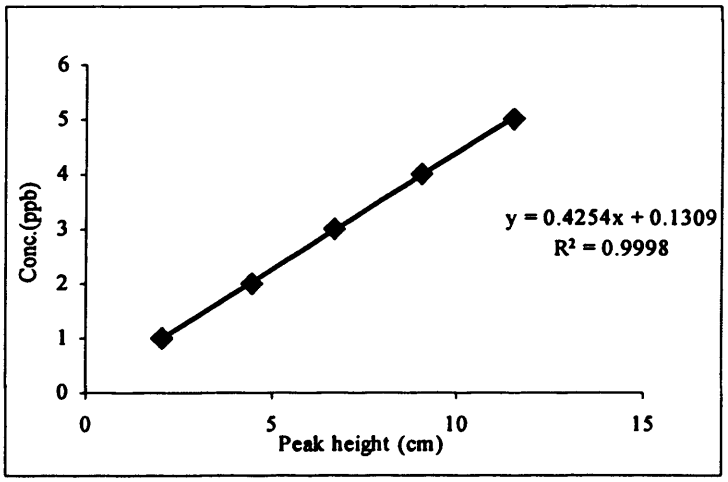


Figure 23. Calibration curve for aflatoxin B_2.

Peak height (cm)	2.05	4.45	6.67	9.05	11.5
Concentration (ppb)	1	2	3	4	5



Once the HPLC methodology for the aflatoxin analysis had been established, a clean up methodology to extract the aflatoxins from the broiler feed without significant loss of the toxins needed to be established. The first step was to monitoring the aflatoxin subtype retention ability of the various commercially available columns.

THE ABILITY OF COLUMNS TO RETAIN AFLATOXIN FROM STANDARD AFLATOXIN SOLUTIONS WITH THE METHODOLOGY RECOMMENED BY EACH COLUMN MANUFACTURER WITH STANDARDISED BENZENE : ACETONITRILE DISSOLVED AFLATOXIN SAMPLES.

Initial HPLC analysis showed the efficacy of the analysis for samples of known concentration injected directly into the system. For experimental samples obtained from broilers or feed samples there will be some loss of aflatoxin attributable to the clean-up procedure. All the possible clean up procedures are multi-step processes (i.e. sample extraction followed by column separation prior to HPLC). To establish the extent of this loss, initially the different concentrations of aflatoxin solutions were passed directly through the different clean-up columns before being subjected to HPLC analysis.

Table 6. Aflatoxin subtype levels after passing standard aflatoxin solutions directly through one of four different clean-up columns.

Clean-up column	Aflatoxin level	Detected aflatoxin concentration (ppb)					
		G_1	B_1	G_2	B_2	Total	% recovery
Varian	10	NF	0.90	NF	NF	0.90	9.00
	20	NF	1.23	NF	0.32	1.55	7.75
	30	2.26	2.39	0.74	0.41	5.80	19.33
	40	3.54	4.49	0.95	0.71	9.69	24.23
	50	3.80	4.10	1.20	0.78	9.88	10.76
Romer	10	2.95	3.61	1.03	0.72	8.31	83.10
	20	6.71	10.18	1.76	1.54	20.19	100.95
	30	8.60	13.55	2.40	2.41	26.96	89.87
	40	10.05	15.54	3.56	3.07	32.22	80.55
	50	16.30	21.63	4.39	3.54	45.86	91.72
Vicam	10	NF	NF	NF	NF	NF	NF
	20	NF	0.62	NF	NF	0.62	3.10
	30	NF	1.52	NF	NF	1.52	5.07
	40	NF	0.18	NF	NF	0.18	0.45
	50	NF	0.73	NF	NF	0.73	1.46
Rhône	10	NF	NF	NF	NF	NF	NF
	20	NF	NF	NF	NF	NF	NF
	30	NF	NF	NF	NF	NF	NF
	40	NF	NF	NF	NF	NF	NF
	50	NF	NF	NF	NF	NF	NF

NF = not-found

The results in table 6 show that with the Varian and the Vicam columns, the low levels of aflatoxin detected compared to the amounts introduced to the clean-up columns indicate either a low initial retention of aflatoxin by these columns or a failure to elute some of the bound aflatoxins from the columns. Only aflatoxin B1 was recovered from the Vicam column, while a high proportion of the aflatoxins G1, G2 and B2 were lost from the Varian clean up. No aflatoxins were detected after clean up with the Rhône column. The Romer column gave the best aflatoxin recoveries for all the subtypes.

A further experiment, using a 30 ppb aflatoxin standard solution, was undertaken to determine to the site of aflatoxin loss during the clean-up. Solutions to be quantified by HPLC were divided into 3 fractions for the Vicam, Varian and Rhône columns. After passing the standard aflatoxin solution, in benzene-acetonitrile (98:2), into the columns, three consecutive fractions were collected. Fraction 1 was the solution that passed immediately through the column. The column was then washed twice with water, and the aqueous solution collected as fraction 2. The column was then washed with 1 ml methanol and 1 ml water for the Vicam and Rhône columns and 7 ml chloroform for the Varian column as per the manufacturers instructions, these solutions were collected as fraction 3. According to the multifunctional clean-up column procedure recommended by the manufacturer, the Romer column had only one fraction. The detection of aflatoxin in these fractions is illustrated in Table 7.

Table 7. The concentration of aflatoxin detected in three consecutive fractions collected after passing standard aflatoxin solution into four different columns.

Fraction	Aflatoxin subtype	Aflatoxin concentrations (ppb)			
		Cleaning-up column			
		Varian	Romer	Vicam	Rhône
Fraction 1	G_1	8.76	8.80	8.90	8.73
	B_1	15.52	13.55	12.78	12.89
	G_2	3.01	2.95	2.88	3.12
	B_2	2.89	2.88	3.01	2.98
Fraction 2	G_1	NF	-	NF	NF
	B_1	NF	-	NF	NF
	G_2	NF	-	NF	NF
	B_2	NF	-	NF	NF
Fraction 3	G_1	NF	-	NF	NF
	B_1	NF	-	NF	NF
	G_2	NF	-	NF	NF
	B_2	NF	-	NF	NF
Total		30.18	28.18	27.57	27.72
% recovery		100.60	93.93	91.90	92.40

NF = not found

The highest aflatoxin concentrations were detected in fraction 1 for all 4 columns and no aflatoxin was detected in fractions 2 and 3. This suggests that aflatoxin was not retained by the column materials of all 4 clean-up systems. This was the expected result for the Romer column but aflatoxin should have been retained on the other three columns.

While the manufacturers instructions for the columns had been followed the extraction procedure prior to the column clean up step had been standardised to maximize the extraction of aflatoxin from the broiler feed. To establish whether the poor performance

of the columns was influenced by the initial extraction step the exact extraction procedure recommended for the column samples was followed although this process was obviously designed for extraction from a simpler matrix than the broiler feeds.

DETERMINATION OF THE ABILITY OF THE COLUMNS TO RETAIN AFLATOXIN FROM THE STANDARD AFLATOXIN SOLUTIONS AFTER EXTRACTION PROCEDURES RECOMMENDED BY EACH COLUMN MANUFACTURER.

A 30 ppb aflatoxin standard solution was prepared in benzene: acetonitrile (98:2), evaporated to dryness and then resolubilised in the solvent system recommended by each column manufacturer. The recommended solvents and their concentrations are given in Table 8. The extracts were passed through the columns, the eluant was collected in 3 consecutive fractions, as described above, and each fraction was individually analyzed for the presence and quantity of aflatoxin by HPLC. The level of aflatoxin detected in each fraction is shown in Table 8.

In this experiment, there were three fractions collected from the Varian, Vicam, and Rhône column as detailed previously. Due to the extraction procedure with the Romer column, there was again only one fraction. With the Varian, Vicam and Rhône columns, aflatoxin was not detected in fractions 1 and 2. Aflatoxins were detected only in Fraction 3. This indicates that aflatoxin has been retained by these columns. The aflatoxin also behaved as expected by the Romer column material in that separation method. This indicates that the benzene: acetonitrile mixture used to make the original solutions was interfering with the column retention properties of three of the four columns. Once the aflatoxin subtypes were extracted into an alternative solvent to the benzene: acetonitrile used to make up the standard solutions, all columns were able to bind all 4 major aflatoxin subtypes as expected. Recovery rate for all columns was > 85 % (table 8).

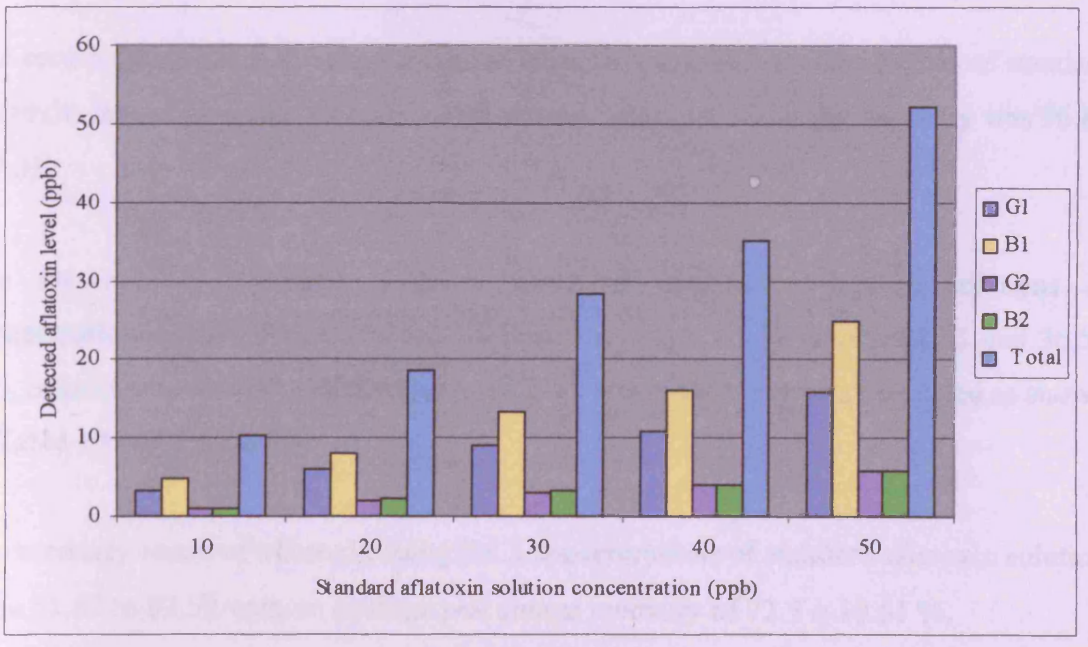
Table 8. Aflatoxin concentrations detected in three consecutive clean-up column fractions from a 30 ppb aflatoxin standard solution. The sample was solubilised in the solvent system recommended by the manufacturer before being applied to the column.

Column Fraction	Aflatoxin subtype	Aflatoxin concentrations (ppb)			
		Clean-up column			
		Varian	Romer	Vicam	Rhône
		Acetone+MeOH 10%	ACN 84%	MeOH 16%	MeOH 30%
Fraction 1	G_1	NF	8.56	NF	NF
	B_1	NF	12.55	NF	NF
	G_2	NF	3.15	NF	NF
	B_2	NF	2.88	NF	NF
Fraction 2	G_1	NF	-	NF	NF
	B_1	NF	-	NF	NF
	G_2	NF	-	NF	NF
	B_2	NF	-	NF	NF
Fraction 3	G_1	8.98	-	8.17	8.20
	B_1	14.52	-	11.78	11.89
	G_2	3.15	-	2.89	2.78
	B_2	3.10	-	2.88	2.69
Total		29.75	27.14	25.64	25.56
% Recovery		99.40	90.68	85.67	85.40

DETERMINATION OF AFLATOXIN FROM THE STANDARD AFLATOXIN SOLUTIONS USING FOUR DIFFERENT COMMERCIAL COLUMNS.

A standard aflatoxin solution was used to initially establish standard clean-up and extraction procedures that could be used for the larger study to avoid differences resulting from the feed samples and the complexity of matrix of the feed commodity. To compare the efficacy of the different columns, the aflatoxin standard solutions were dissolved in the manufacturers recommended solvent system prior to extraction procedures for each column and then passed through the clean-up column before being analyzed by HPLC. The aflatoxin levels detected and the percent aflatoxin recovery from the standard aflatoxin solution using four different columns are summarized in Tables 9-12.

Figure 24. The recovery of aflatoxin subtypes using the manufacturers recommended solvent systems to dissolve the standards and passing the aflatoxin standard solutions through a Varian column.



Using the Varian column, the average aflatoxin concentrations detected from the standard aflatoxin solutions of 10, 20, 30, 40 and 50 ppb were 10.21, 18.50, 28.37, 35.19 and 52.34 ppb, respectively. The percentage recovery at each aflatoxin concentration was calculated from the sum of the aflatoxin subtypes as shown in Table 9 and Figure 24.

Table 9. The recovery of aflatoxin subtypes after following the manufacturers extraction procedure and passing the aflatoxin standard solution through a Varian column.

Aflatoxin level(ppb)	Detected aflatoxin level (ppb)				
	G_1	B_1	G_2	B_2	Total
10	3.24	4.79	1.11	1.07	10.21
20	5.98	8.11	2.13	2.28	18.50
30	9.04	13.22	2.95	3.16	28.37
40	10.87	16.19	4.04	4.09	35.19
50	15.92	24.93	5.70	5.79	52.34

The recovery of aflatoxin with the Varian column from the 5 concentrations of standard aflatoxin ranged from 88.13 - 104.89 % and the average percentage recovery was 96.89 ± 7.02.

The amounts of aflatoxin detected from the standard aflatoxin solutions at concentrations of 10, 20, 30, 40 and 50 ppb were 8.26, 13.75, 23.41, 22.31 and 36.52 ppb, respectively with the Romer column. The percent recovery was estimated as shown in Table 10 and Figure 25.

The recovery range of aflatoxin using the 5 concentrations of standard aflatoxin solution were 55.87 to 83.52 with an average percentage recovery of 72.2 ± 10.51 %.

Figure 25. The recovery of aflatoxin after using the manufacturers recommended solvent system to dissolve the standards and passing the aflatoxin standard solution through a Romer column.

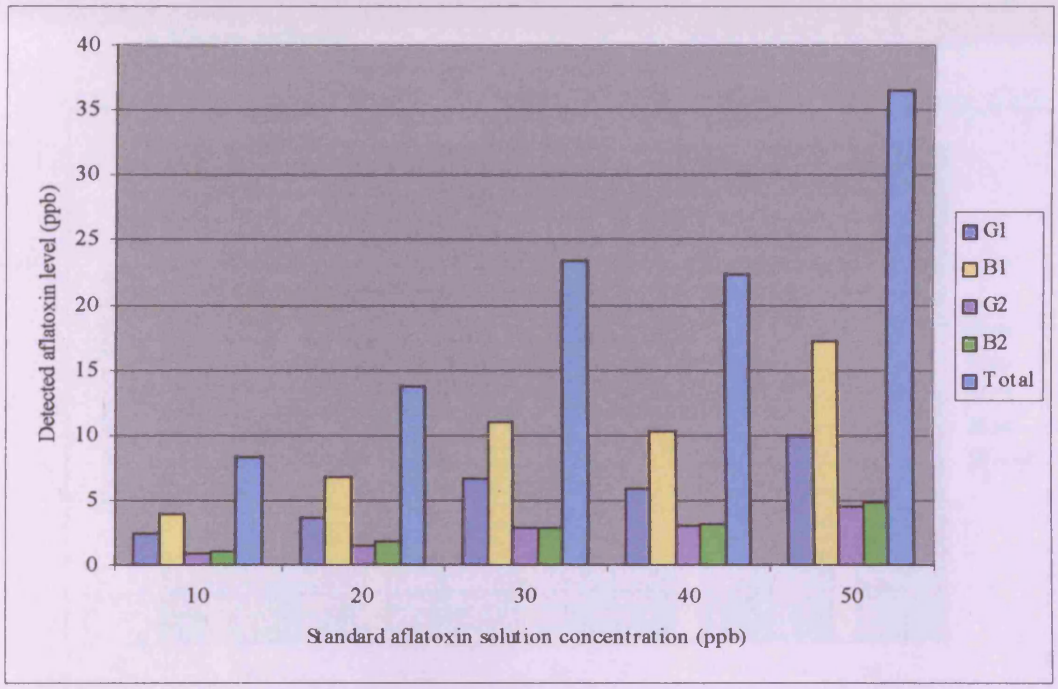


Table 10. The recovery of aflatoxin after following the manufacturers recommended extraction procedure and passing the standard aflatoxin solution through a Romer column.

Aflatoxin level (ppb)	Detected aflatoxin level (ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.38	3.89	0.97	1.02	8.26
20	3.67	6.72	1.48	1.88	13.75
30	6.57	11.06	2.84	2.94	23.41
40	5.92	10.22	3.00	3.17	22.31
50	9.90	17.26	4.52	4.84	36.52

The aflatoxin concentrations detected from the 5 different standard aflatoxin solutions after passing through the Vicam column were 8.93, 14, 23.54, 38.38 and 46 ppb. The recoveries are reported in Table 11 and Figure 26.

Figure 26. The recovery of aflatoxin after following the manufacturers recommended extraction procedure and passing the aflatoxin standard solutions through a Vicam column.

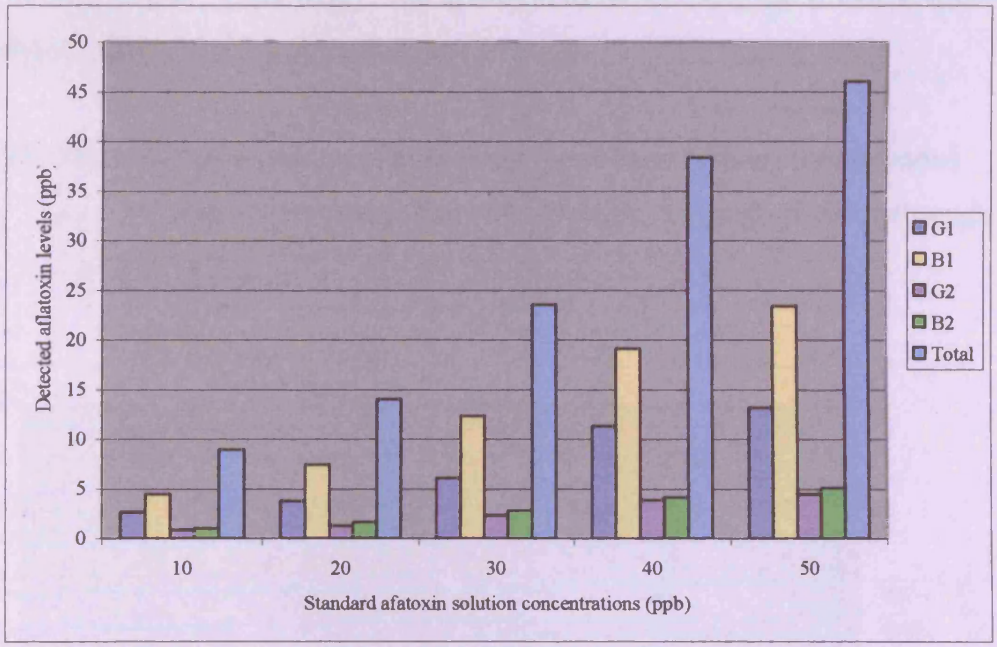


Table 11. The recovery of aflatoxin after following the manufacturers recommended extraction procedure and passing the aflatoxin standard solutions through a Vicam column.

Aflatoxin level (ppb)	Detected aflatoxin level(ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.64	4.44	0.84	1.01	8.93
20	3.72	7.41	1.24	1.63	14.00
30	6.09	12.33	2.31	2.81	23.54
40	11.30	19.12	3.85	4.11	38.38
50	13.13	23.41	4.41	5.05	46.00

The Vicam column gave good aflatoxin recovery rates from the standard aflatoxin solutions ranging from 70.7 to 96.1 % with an average percentage recovery of 85.6 ± 10.6 %.

The calculated levels of aflatoxin recovered from the Rhône column were 9.4, 14.6, 29.3, 32.2 and 39.3 ppb of aflatoxin from the standard aflatoxin concentrations of 10, 20, 30, 40 and 50 ppb, respectively. The aflatoxin recovery using the Rhône column was calculated as shown in Table 12 and Figure 27.

Figure 27. The recovery of aflatoxin after using the solvent system recommended by the manufacturer and passing the aflatoxin standard solutions through a Rhône column.

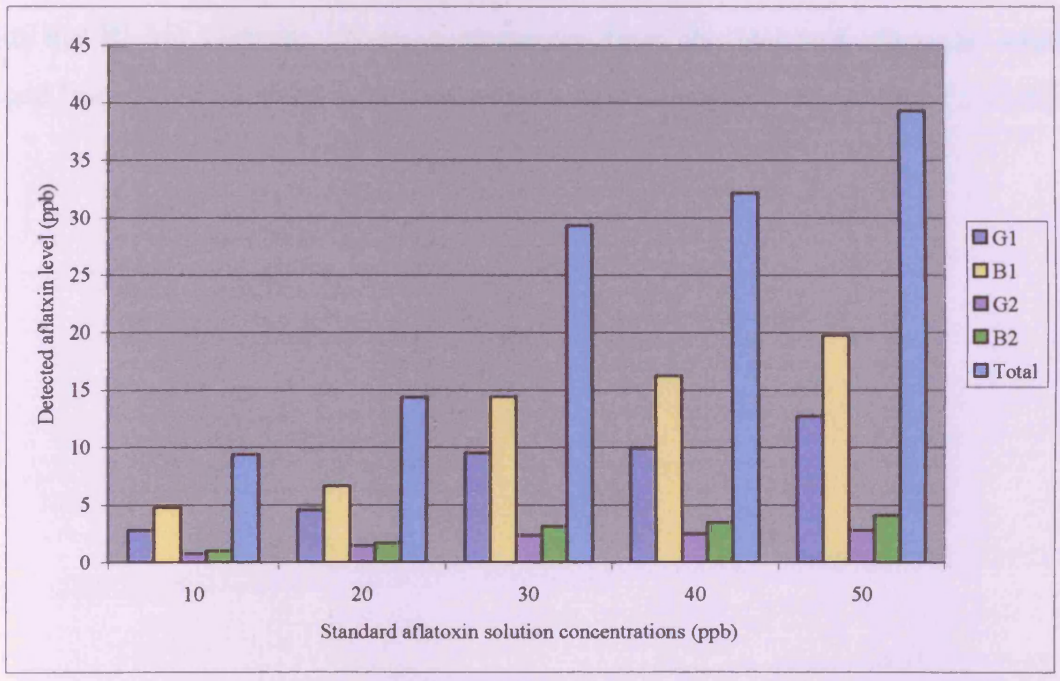


Table 12. The recovery of aflatoxin after following the manufacturers recommended extraction procedure and passing the aflatoxin standard solutions through a Rhône column.

Aflatoxin level (ppb)	Detected aflatoxin level(ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.81	4.76	0.79	1.04	9.4
20	4.53	6.64	1.48	1.70	14.35
30	9.53	14.37	2.33	3.11	29.34
40	9.96	16.24	2.49	3.47	32.16
50	12.70	19.72	2.79	4.09	39.30

With the Rhône column, aflatoxin recoveries from the standard aflatoxin solutions ranged from 72.47 to 98.03 % with an average percentage recovery of 84.97 ± 11.03 %.

THE COMPARATIVE RECOVERY OF AFLATOXINS AMONG THE FOUR COMMERCIAL CLEAN UP COLUMNS.

The aflatoxin clean-up and retention capabilities for the standard aflatoxin solutions for each column after HPLC are summarized in Table 13 and Figure 28.

Figure 28. The aflatoxin recovery rates from different aflatoxin standard solutions using four different commercial clean up columns.

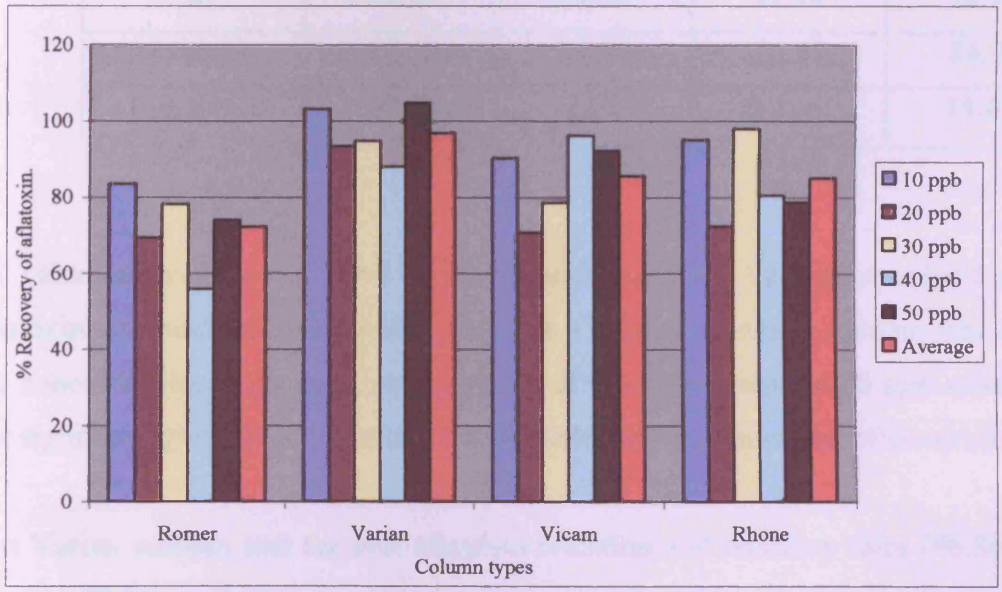


Table 13. The aflatoxin recovery rates from different aflatoxin standard solutions using four different commercial clean up columns.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin			
	Romer	Varian	Vicam	Rhône
10	83.52	103.23	90.29	95.04
20	69.44	93.43	70.71	72.47
30	78.20	94.79	78.65	98.03
40	55.87	88.13	96.12	80.54
50	73.99	104.89	92.18	78.76
Average	72.20	96.89	85.59	84.97
± SD	10.51	7.02	10.56	11.03

All columns gave recoveries >65 % (with the exception of the Romer column at 40 ppb aflatoxin). Concentration was obviously not a limiting factor in column retention over the concentration range used, as recoveries of aflatoxin from the 50 ppb solution were not significantly different from those with the lower concentrations of standards.

The Varian column had the best aflatoxin retention and recovery rates (96.86 %). The Vicam, Rhône and Romer columns gave a more moderate degree of retention and recovery, in the descending order these were 85.6 %, 84.9 % and 72.2 %, respectively.

Based on the ANOVA by Completely Randomize Design (CRD), the Varian column gave a significantly better aflatoxin recovery compared to the Romer column, but was not significantly better than the Vicam and Rhône columns. The statistical data for this analysis is shown in Table 14.

Table 14. Statistical SAS analysis of aflatoxin recovery rates from a range of aflatoxin standard solutions using 4 different commercially available clean up columns.

Analysis of Variance

Source	df	Sum of Squares	Mean Square	F-Value	Pr.> F
Model	3	1527.62196	509.20732	5.18	0.0108
Error	16	1571.33212	98.2082575		
Corrected Total	19	3098.95408			

R-Square = 0.492948 C.V. = 11.67064 Root MSE = 9.91000795

Recovery Mean = 84.914

Source	df	ANOVA SS	Mean Square	F-Value	Pr.> F
Model	3	1527.62196	509.20732	5.18	0.0108

Alpha = 0.05 df = 16 MSE = 98.20826

Number of Mean 2 3 4

Critical Range 13.29 13.93 14.34

Means with the same letter within the Duncan grouping are not significantly different.

Duncan grouping	Mean	N	Method
A	96.894	5	Varian
B A	85.590	5	Vicam
B A	84.968	5	Rhône
B	72.204	5	Romer

Once an accepted aflatoxin solvent system had been established to undertake the column clean up the next stage was to determine how well the aflatoxin could be recovered from the broiler feed matrix. To do this, samples of the two feed types were spiked with known concentrations of aflatoxin standards.

DETERMINATION OF AFLATOXIN FROM THE AFLATOXIN-SPIKED BROILER MIXED FEEDS USING FOUR DIFFERENT COMMERCIAL COLUMNS

To establish recovery rates from actual feed samples, aflatoxin-spiked feed samples were used. Two types of broiler mixed feeds, the Betagro feed and the C.P. feed were used. The Betagro feed was spiked with a known amount of aflatoxin at 10, 20, 30, 40 and 50 ppb. Extractions were performed according to manufacturers recommendation, followed by clean-up with the 4 different commercial columns and analysis of eluants by HPLC. The results are summarized in Tables 15-18.

The aflatoxin concentrations calculated from the HPLC analysis were compared with the initial concentrations of aflatoxin that the samples had been spiked with and the percentage recoveries were calculated as shown in Table 15 and Figure 29.

Figure 29. Aflatoxin recoveries from spiked Betagro feed samples using a Varian column.

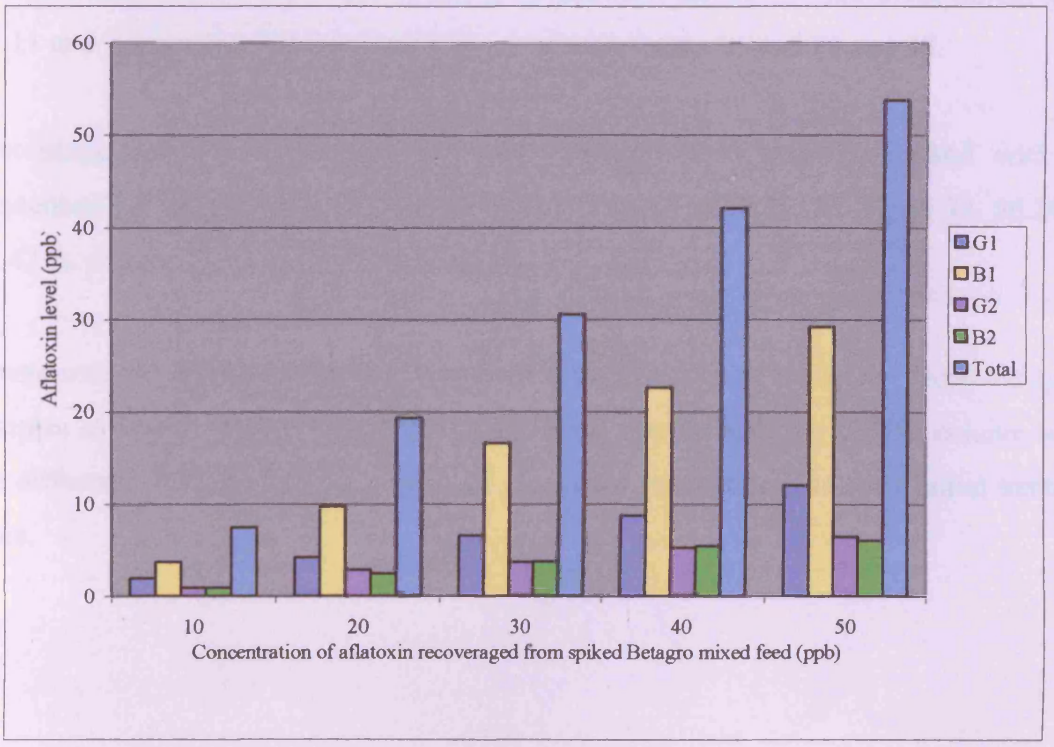


Table 15. Aflatoxins recovered from spiked Betagro feed samples using a Varian column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	1.94	3.70	0.92	0.86	7.42
20	4.22	9.79	2.87	2.45	19.33
30	6.58	16.62	3.67	3.74	30.61
40	8.78	22.61	5.28	5.41	42.08
50	12.06	29.28	6.42	6.02	53.78

The aflatoxin recovery rates from the Betagro feeds spiked with five different concentrations of aflatoxin ranged from 72.32 to 97.27 % with an average of 89.37 ± 9.77 %.

When the Romer column was used to clean up the aflatoxin extract from the Betagro spiked broiler mixed feed, the amounts of aflatoxin detected were 5.48, 12.18, 21.78, 27.11 and 33.15 ppb. The recoveries are shown in Table 16 and Figure 30.

Percentage recoveries of aflatoxin from Betagro feed samples spiked with five concentrations of aflatoxin using a Romer column were 48.79, 52.70, 61.33, 56.36 and 55.42 % with an average of 54.92 ± 4.63%.

It was notable with this column that the G_1 toxin was not recovered from the spiked samples at any treatment rate, in contrast to the results with the Varian column where the different aflatoxin subtypes were all recovered proportional to their initial treatment rates.

Figure 30. Aflatoxin recoveries from spiked Betagro feed samples using a Romer column.

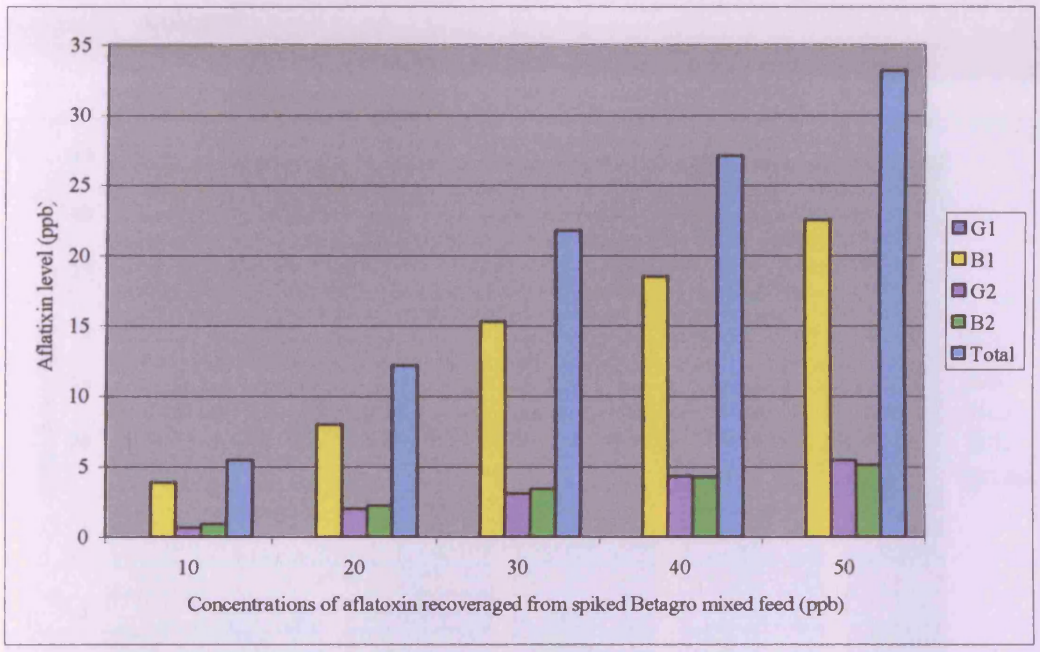


Table 16. Aflatoxin recoveries from spiked Betagro feed samples using a Romer column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	-	3.87	0.69	0.92	5.48
20	-	7.98	1.99	2.21	12.18
30	-	15.30	3.06	3.42	21.78
40	-	18.52	4.32	4.27	27.11
50	-	22.54	5.47	5.14	33.15

With the Vicam columns, aflatoxin concentrations recovered from the Betagro feed spiked with the five different aflatoxin concentrations were 6.08, 17.02, 26.63, 33.14

and 39.11 ppb for samples spiked with 10, 20, 30, 40 and 50 ppb respectively. The percent recovery of aflatoxin is reported in Table 17 and Figure 31.

Figure 31. Aflatoxin recoveries from spiked Betagro feed samples using a Vicam column.

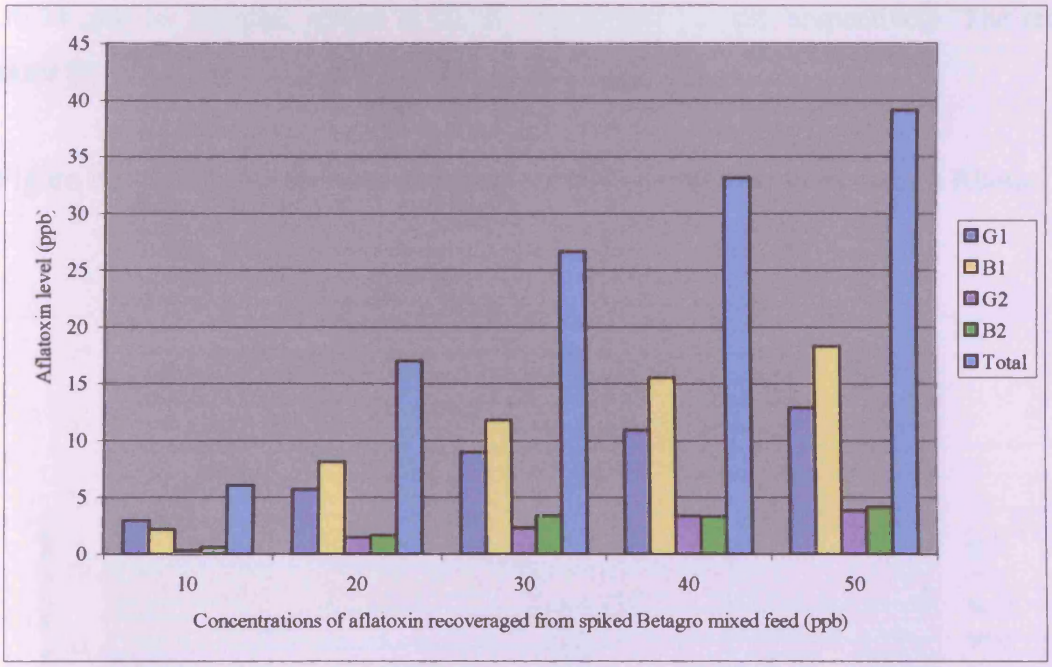


Table 17. Aflatoxin recoveries from spiked Betagro feed samples using a Vicam column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	3.00	2.14	0.34	0.60	6.08
20	5.74	8.14	1.50	1.64	17.02
30	9.01	11.84	2.34	3.44	26.63
40	10.92	15.54	3.36	3.32	33.14
50	12.90	18.26	3.79	4.16	39.11

The recovery of aflatoxin from the spiked Betagro feed ranged from 60.20 to 86.71 % with an average of 77.78 ± 10.80 %. Recoveries of the 4 aflatoxin subtypes were in proportion to the treatment rates, as with the Varian columns.

With the Rhône column, the aflatoxin concentrations recovered from the Betagro feed spiked with five different concentrations of aflatoxin were 5.81, 13.58, 17.99, 25.72 and 30.74 ppb for samples spiked at 10, 20, 30, 40 and 50 ppb, respectively. The recovery rates for aflatoxin are shown in Table 18 and Figure 32.

Figure 32. Aflatoxin recoveries from spiked Betagro feed samples using a Rhône column.

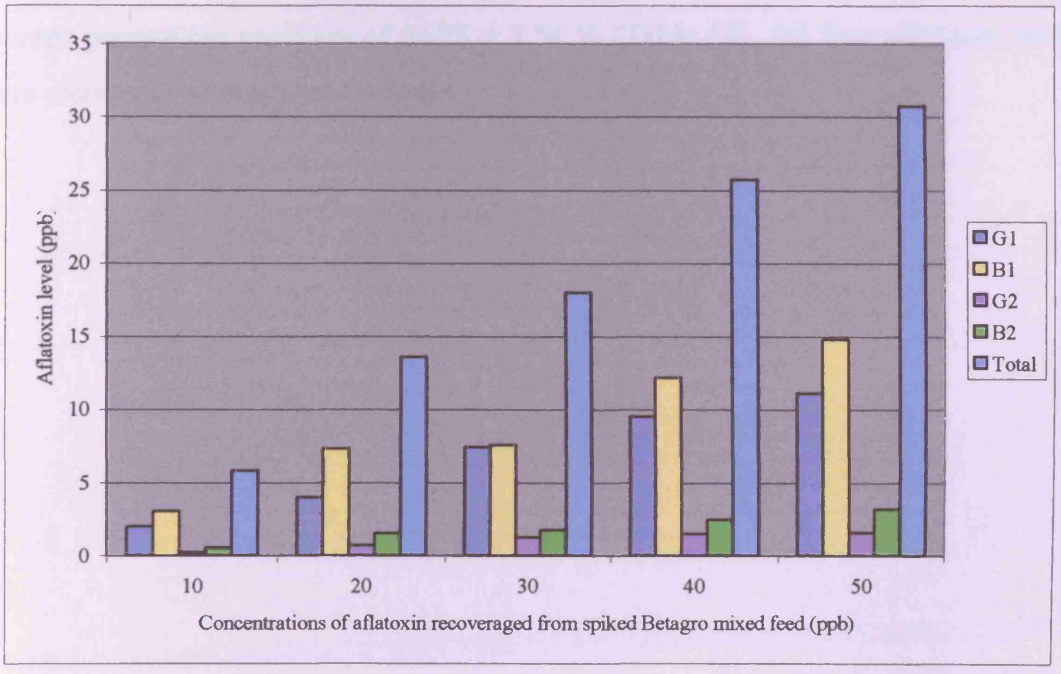


Table 18. Aflatoxin recoveries from spiked Betagro feed samples using a Rhône column.

Aflatoxin level (ppb)	Detected aflatoxin level from spiked feed samples				
	G_1	B_1	G_2	B_2	Total
10	2.01	3.04	0.20	0.56	5.81
20	3.99	7.32	0.73	1.54	13.58
30	7.42	7.57	1.26	1.74	17.99
40	9.54	12.18	1.49	2.51	25.72
50	11.14	14.81	1.61	3.18	30.74

The aflatoxin recoveries from the feed samples spiked with different levels of aflatoxin using a Rhône column were relatively low, ranging from 50.52 to 58.78 % with an average percentage recovery of 54.75 ± 3.54 % (Table 18). All four aflatoxin subtypes were recovered within these samples.

EFFICIENCY OF COLUMNS FOR THE DETERMINATION OF AFLATOXIN FROM THE SPIKED C.P. FEED.

The broiler C.P. mixed feed was spiked with different levels of aflatoxin in the same manner as the Betagro feed and the four different commercially available columns were used for the extraction and the purification processes prior to detection of aflatoxin by HPLC. The concentrations of aflatoxin detected using the four different columns are summarized in Tables 19-22.

At different aflatoxin spiked levels of C.P. broiler mixed feed, the Varian column produced 8.15, 17.36, 27.27, 36.29 and 43.90 ppb of aflatoxin. The aflatoxin recoveries are shown in Table 19 and Figure 33. The recoveries were 79.40-82.86 with an average percentage recovery of 80.73 ± 1.68 %.

Figure 33. Aflatoxin recoveries from spiked C.P. feed samples using a Varian column.

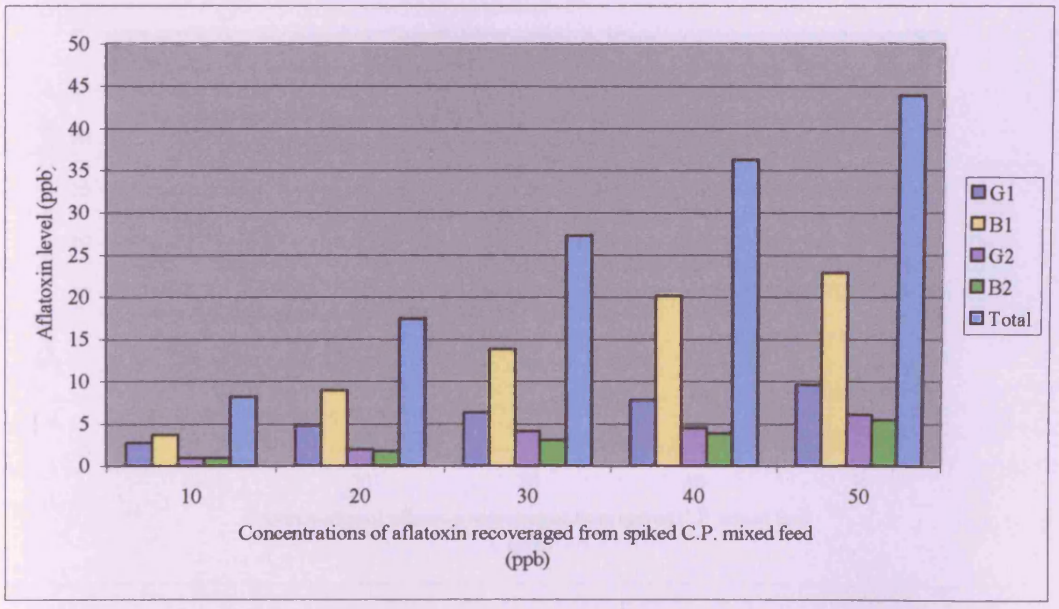


Table 19. Aflatoxin recoveries from spiked C.P. feed samples using a Varian column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.78	3.61	0.88	0.88	8.15
20	4.83	8.88	1.95	1.70	17.36
30	6.28	13.82	4.09	3.08	27.27
40	7.80	20.16	4.47	3.86	36.29
50	9.55	22.87	6.08	5.40	43.9

The Romer column was used for aflatoxin clean-up from spiked samples of C.P. feed, the concentrations of aflatoxin detected are shown in Table 20 and Figure 34.

Figure 34. Aflatoxin recoveries from spiked C.P. feed samples using a Romer column.

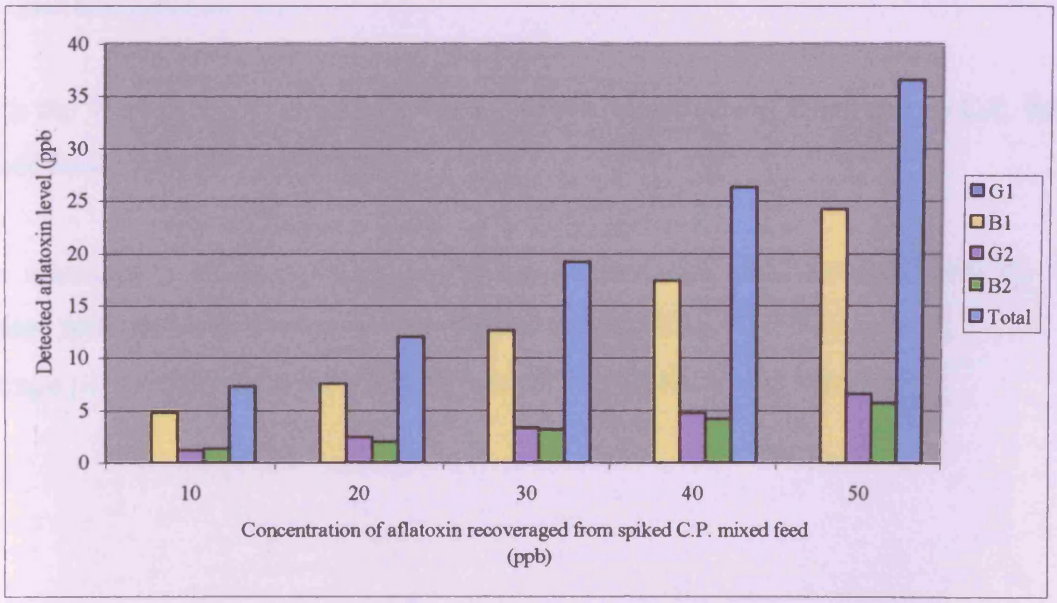


Table 20. Aflatoxin recoveries from spiked C.P. feed samples using a Romer column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	-	4.82	1.19	1.30	7.31
20	-	7.57	2.45	1.99	12.01
30	-	12.58	3.36	3.18	19.12
40	-	17.36	4.74	4.22	26.32
50	-	24.24	6.62	5.73	36.59

The total aflatoxin concentrations found in the spiked samples were 7.31, 12.01, 19.12, 26.32 and 36.59 ppb. Aflatoxin recoveries are shown in Table 20 and Figure 34. A range of 51.96 to 65.09 % with an average percentage recovery of 57.36 ± 5 .54 %, which is quite low, was obtained with the Romer column. Again with this feed brand the G_1 aflatoxin subtype was not recovered from the sample with this type of commercial column, suggesting that the Romer columns are unsuitable for G_1 aflatoxin analysis from mixed feeds.

With the Vicam column, the amounts of aflatoxin recovered from spiked C.P. broiler mixed feeds are shown in Table 21.

The aflatoxin recoveries when the Vicam column was used extracts from the C.P. broiler mixed feeds was relatively high, ranging from 71.74 to 105.35 % with an average percentage recovery of 90.62 ± 14.27 % (Table 21 and Figure 35)

Figure 35. Aflatoxin recoveries from spiked C.P. feed samples using a Vicam column.

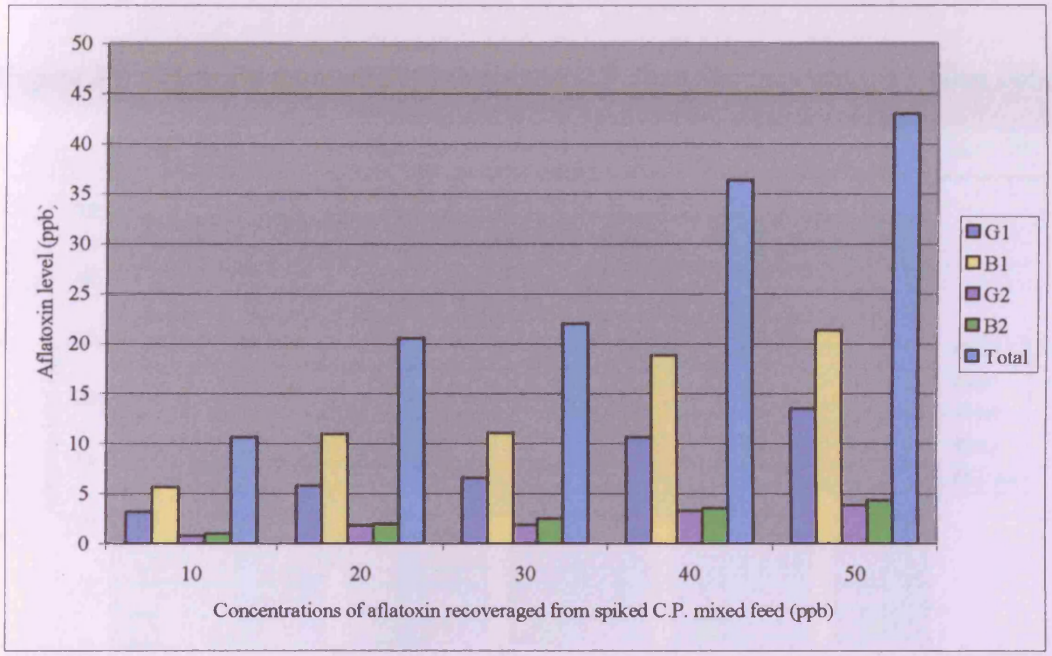


Table 21. Aflatoxin recoveries from spiked C.P. feed samples using a Vicam column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	3.16	5.60	0.84	1.02	10.64
20	5.82	10.95	1.80	1.98	20.55
30	6.58	11.04	1.88	2.53	22.03
40	10.62	18.84	3.33	3.56	36.35
50	13.50	21.32	3.86	4.32	43.00

With the Rhône column, the amount of aflatoxin recovered from the spiked C.P. broiler mixed feeds were 7.26, 15.66, 18.57, 23.70 and 29.94 ppb for the different spiked levels (Table 22 and Figure 36).

Figure 36. Aflatoxin recoveries from spiked C.P. feed samples using a Rhône column.

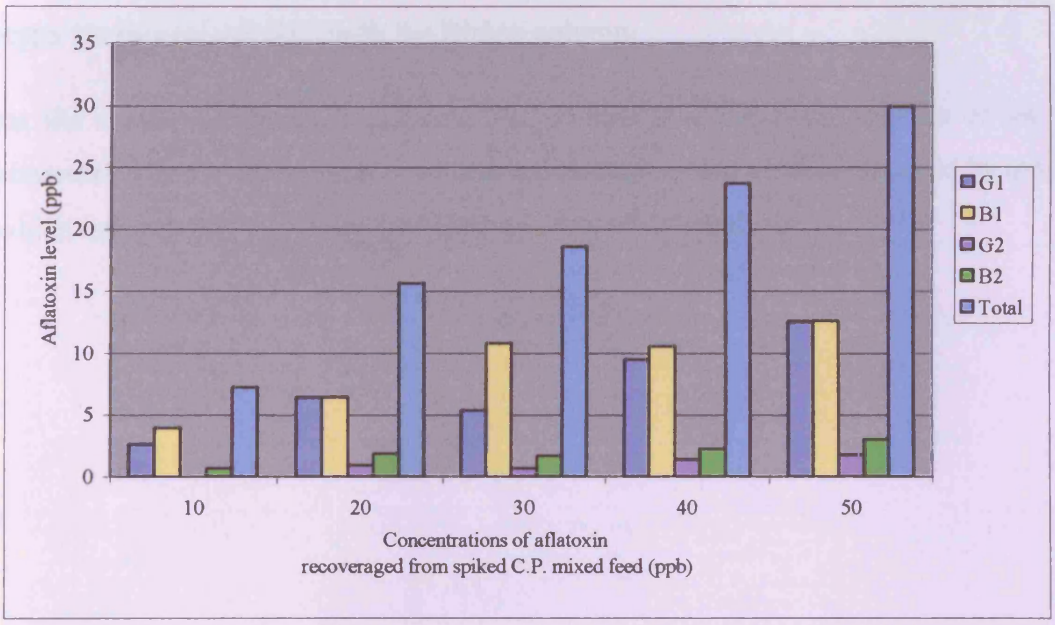


Table 22. Aflatoxin recoveries from a spiked C.P. feed sample using a Rhône column.

Aflatoxin concentration(ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.62	3.94	-	0.70	7.26
20	6.43	6.42	0.96	1.85	15.66
30	5.36	10.78	0.73	1.70	18.57
40	9.47	10.54	1.42	2.27	23.70
50	12.58	12.59	1.75	3.02	29.94

Similarly the percentage recoveries of aflatoxin from the spiked C.P. feed using the Rhône column were 63.13, 65.25, 53.22, 51.89 and 57.25 % for the spiked levels of 10, 20, 30, 40 and 50 ppb respectively with an average recovery of 58.15 %. This percentage is relatively low compared to some of the other columns. In the 10 ppb treatment no aflatoxin G_2 subtype was detected with this column, although it was detected in samples spiked with higher aflatoxin concentration, suggesting that the recovery of the G_2 subtype had fallen below the limit of HPLC detection for this subtype for this mixed feed with the Rhône column.

From the results of these experiments the comparative clean up abilities of the four commercially available columns could be calculated so that a decision could be made as to which column system to use for the larger scale field analysis.

COMPARATIVE CLEAN-UP ABILITIES AMONG FOUR COMMERCIAL COLUMNS USING THE SPIKED BROILER MIXED FEEDS.

The efficiencies of the 4 commercial columns were compared for Betagro (Table 23) and for the C.P. feed brands (Table 24).

Table 23. Aflatoxin recoveries from spiked Betagro feed samples using four different clean-up columns.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin from spiked feed samples			
	Betagro feed			
	Romer	Varian	Vicam	Rhône
10	48.79	72.32	60.20	50.52
20	52.70	91.78	86.44	56.58
30	61.33	93.01	86.71	51.56
40	56.36	92.46	76.59	56.32
50	55.42	97.27	78.95	58.78
Average	54.92	89.37	77.78	54.75
± SD	4.63	9.77	10.80	3.54

Figure 37. Aflatoxin recoveries from spiked Betagro feed using four different clean-up columns.

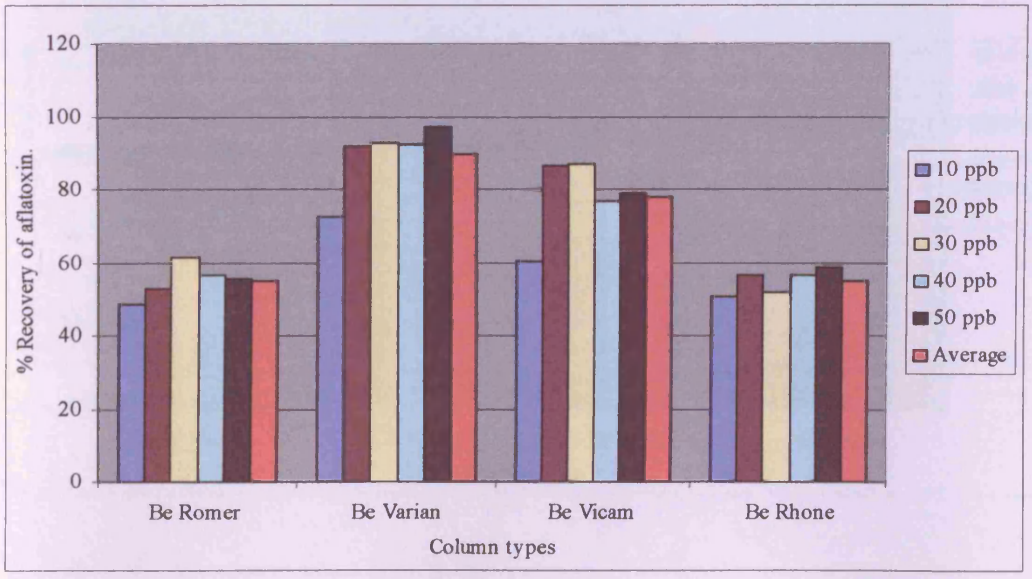
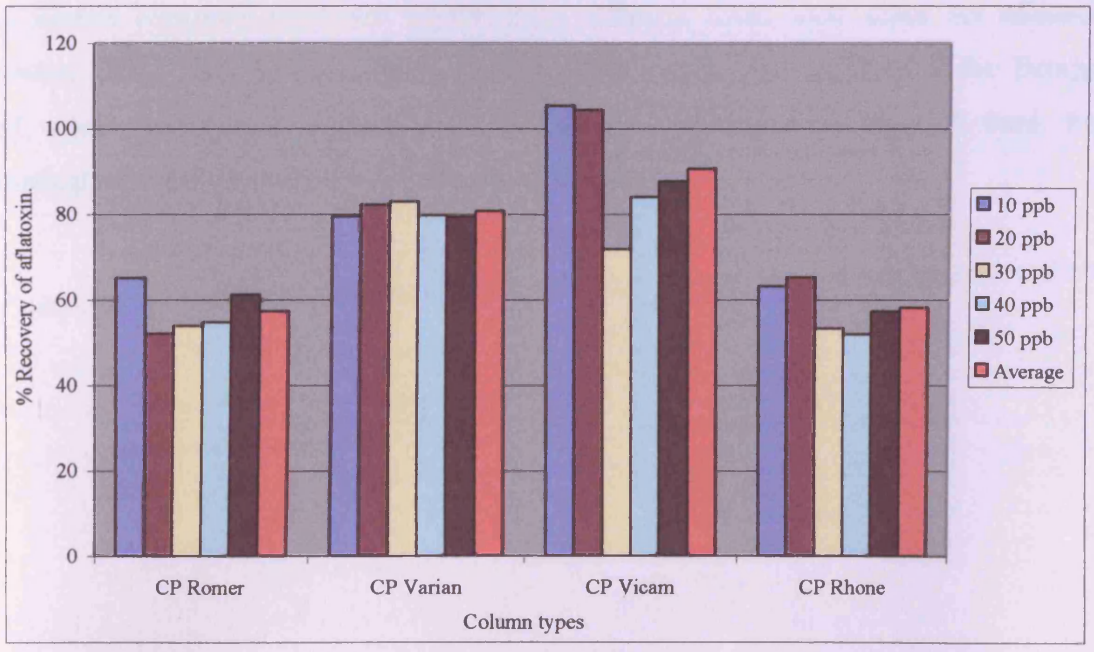


Table 24. Aflatoxin recoveries from spiked C.P. feed samples using four different clean-up columns.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin from spiked feed samples			
	C.P. feed			
	Romer	Varian	Vicam	Rhône
10	65.09	79.43	105.35	63.13
20	51.96	82.24	104.36	65.25
30	53.84	82.86	71.74	53.22
40	54.72	79.74	84.01	51.89
50	61.18	79.40	87.66	57.25
Average	57.36	80.73	90.62	58.15
± SD	5.54	1.68	14.27	12.30

Figure 38. Aflatoxin recoveries from spiked C.P. feed samples using four different clean-up columns.



The Varian column produced the highest average percentage recovery of aflatoxin with the Betagro broiler mixed feed. The lowest percentage recovery was with the Rhône column. The recoveries of aflatoxin in descending order among the four different columns was Varian (89.37 %), Vicam (77.78 %), Romer (54.92 %) and Rhône (54.75 %), respectively, although the Romer column failed to extract the G_1 aflatoxin from both mixed feed brands. HPLC traces from this clean-up procedure also had a number of contaminants which had carried through and co-chromatographed with the HPLC aflatoxin peaks making this method a poor choice.

The Vicam column had the highest recovery of aflatoxin (90.62 %) with the C.P. broiler mixed feed and the Romer column again gave one of the lowest recoveries (57.36 %). The Varian column and the Rhône column gave aflatoxin recoveries of 80.73 % and 58.15 %, respectively.

An ANOVA test using the factorial in CRD design showed that there was no significant difference between the Betagro and C.P. mixed feeds for column efficiency. There was a significant difference in efficiencies between columns with both feed brands. Both the Varian and Vicam columns were significantly better than the Rhône and Romer columns at recovering aflatoxin. The Varian versus the Vicam columns and the Rhône and Romer columns were not significantly different from each other for aflatoxin recovery rates. The Varian column gave the best results when applied to the Betagro feed, while the Vicam column provided the best recovery with the C.P. feed. The statistical data for this analysis are shown in Table 25.

Table 25. Statistical SAS analysis of aflatoxin recoveries from spiked Betagro and C.P. feeds using four different commercially available clean up columns.

Concentration (ppb)	% Recovery of aflatoxin							
	Romer		Varian		Vicam		Rhône	
	Betagro	C.P.	Betagro	C.P.	Betagro	C.P.	Betagro	C.P.
10	48.79	65.09	72.32	79.43	60.20	105.35	50.52	63.13
20	52.70	51.96	91.78	82.24	86.44	104.36	56.58	65.25
30	61.33	53.84	93.01	82.86	86.71	71.74	51.56	53.22
40	56.36	54.72	92.46	79.74	76.59	84.01	56.32	51.89
50	55.42	61.18	97.27	79.40	78.95	87.66	58.78	57.25
Average	54.92	57.36	89.37	80.73	77.78	90.62	54.75	58.15
± SD	4.63	5.54	9.77	1.68	10.80	14.27	3.54	12.30

Analysis of Variance

Source	df	Sum of Squares	Mean Square	F-Value	Pr.> F
Model	7	8673.4408575	1239.06297964	19.13	0.0001
Error	32	2072.64344	64.7701075		
Corrected Total	39	10746.0842975			

R-Square = 0.807126 C.V. = 11.42203 Root MSE = 8.04798779

Recovery Mean = 70.46025

Source	df	Anova ss	Mean Square	F-Value	Pr.> F
Method	3	8030.8350275	2676.94500917	41.33	0.0001
Company	1	63.0763225	63.0763225	0.97	0.3311
Method*Company	3	579.5295075	193.17650250	2.98	0.0458

Alpha = 0.05 df = 32 MSE = 64.77011

Number of Mean 2 3 4

Critical Range 7.331 7.705 7.949

Means with the same letter are not significantly different.

Duncan grouping	Mean	N	Method
A	85.051	10	Varian
A	84.201	10	Vicam
B	56.450	10	Rhône
B	56.139	10	Romer

Alpha = 0.05 df = 32 MSE = 64.77011

Number of Mean 2

Critical Range 5.184

Means with the same letter are not significantly different

Duncan grouping	Mean	N	Company
A	71.716	20	C.P.
A	69.205	20	Betagro

Level of method	Level of company	N	% Recovery	
			Mean	± SD
Rhône	Betagro	5	54.752	3.539904
Rhône	C.P.	5	58.148	5.9057785
Romer	Betagro	5	54.92	4.6345712
Romer	C.P.	5	57.358	5.5422306
Varian	Betagro	5	89.368	9.768806
Varian	C.P.	5	80.734	1.6774922
Vicam	Betagro	5	77.778	10.8002532
Vicam	C.P.	5	90.624	14.2711993

The Rhône and Romer columns gave relatively low aflatoxin recoveries with both brands of broiler mixed feeds, whereas the Vicam and Varian columns had relatively high levels of aflatoxin recoveries with both brands.

The four different columns also exhibited variability in their ability to extract and recover G_1 aflatoxin as shown in Table 26. Interference occurred due to the existence of other peaks co-chromatographing with the G_1 aflatoxin, which interfered with the accurate determination of this aflatoxin concentration. The aflatoxin HPLC peaks in chromatograms obtained from experiments using Vicam and Rhône columns showed no interference from contaminants whereas, with the Varian and Romer columns, aflatoxin determination was adversely affected. This interference may depend on the nature of each feed sample, but similar results were obtained with both brands of mixed feed. This result also indicated the need to establish better extraction solvent systems to eliminate contaminants.

Table 26. The level of interference on aflatoxin G_1 determination by contaminant peaks after various column clean-up procedures.

Columns	Interference
Varian	+
Romer	+++
Vicam	-
Rhône	-

The manufacturers recommended solvent systems were obviously developed for use with simple feed systems. To establish whether an alternative solvent system would improve the performance of the aflatoxin extraction and recovery a number of alternative solvent systems were tried.

MODIFICATION OF THE EXTRACTION SOLVENT USED FOR THE COLUMN CLEAN UP

The recommended extraction procedure with the Rhône column uses methanol and water. Chloroform was used to replace methanol, with aflatoxin spiked Betagro broiler mixed feed samples to see whether this would improve aflatoxin recoveries. The aflatoxin recoveries are summarized in Table 27. A similar protocol was applied to the C.P. mixed feed with results summarized in Table 28.

Table 27. Aflatoxin recoveries from spiked Betagro feed using chloroform as the extraction solvent instead of methanol with a Rhône column.

Aflatoxin concentration(ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	3.08	4.58	0.88	0.96	9.50
20	6.02	9.51	1.78	1.74	19.05
30	9.08	15.28	3.18	3.20	30.74
40	12.01	18.90	3.75	3.80	38.46
50	15.40	25.08	5.38	5.08	50.94

Table 28. Aflatoxin recoveries from spiked C.P. feed using chloroform as the extraction solvent instead of methanol with a Rhône column.

Aflatoxin concentration (ppb)	Detected aflatoxin concentration (ppb)				
	G_1	B_1	G_2	B_2	Total
10	2.99	4.39	0.92	0.92	9.22
20	5.82	9.67	2.03	1.92	19.44
30	9.38	14.67	2.90	2.81	31.71
40	13.40	19.71	4.54	3.82	41.47
50	14.72	25.66	5.44	5.20	51.02

Comparison of aflatoxin recoveries with the Rhône columns using either aqueous methanol or choloroform as the extraction solvent is summarized in Table 29 (Betagro feed brand) and Table 30 (C.P. feed brand). When chloroform was used in the extraction procedure with the Rhône column, it gave a higher recovery rates for the different aflatoxins for both brands of broiler feeds than the methanol : water solvent system.

Table 29. Aflatoxin recoveries from spiked Betagro broiler mixed feed using an aqueous 60 % methanol solvent compared to a chloroform extraction.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin	
	Methanol	Chloroform
10	50.52	98.65
20	56.58	94.68
30	51.56	100.56
40	56.32	95.77
50	58.78	102.97
Average	54.75	98.53
± SD	3.54	3.40

Table 30. Aflatoxin recoveries from spiked C.P. broiler mixed feed using an aqueous 60 % methanol solution compared to a chloroform extraction.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin	
	Methanol	Chloroform
10	63.13	93.70
20	65.25	97.30
30	53.22	103.66
40	51.89	95.85
50	57.25	104.98
Average	58.15	99.09
± SD	12.30	4.95

A higher aflatoxin recovery from both spiked brands at all spiking concentrations was observed with the chloroform extraction compared to the aqueous methanol solvent. Average percentage recoveries of 99.09 % versus 58.15 % for the C.P. feed and 98.53 % versus 54.75 % for the Betagro feed were obtained.

Using the factorial CRD design, an ANOVA showed that there was no significant difference in aflatoxin recoveries between the C.P. and Betagro feeds when the same solvent system was used. There was no interaction between solvent and feed. The aflatoxin recoveries obtained with chloroform were significantly higher than with methanol : water.

Table 31. Aflatoxin recoveries from Betagro and C.P. broiler mixed feeds using an aqueous 60 % methanol extraction compared to a chloroform extraction process.

Aflatoxin level (ppb)	% Recovery of aflatoxin			
	Methanol		Chloroform	
	C.P. feed	Betagro feed	C.P. feed	Betagro feed
10	63.13	50.52	93.70	98.65
20	65.25	56.58	97.30	94.68
30	53.22	51.56	103.66	100.56
40	51.89	56.32	95.85	95.77
50	57.25	58.78	104.98	102.97
Average	58.15	54.75	99.09	98.53
± SD	12.30	3.54	4.95	3.40

Once the optimum solvent system was established for all columns comparison of the different column efficiencies was calculated.

COMPARATIVE EXTRACTION EFFICIENCY OF EACH COLUMN FOR DIFFERENT TYPES OF FEED SAMPLE.

If samples are subjected to an appropriate extraction procedure the majority of aflatoxins should be extracted from the feed matrix. The loss of aflatoxin due to extraction from feed is summarized in Tables 32-35.

Table 32. Comparison of aflatoxin recoveries using an aflatoxin standard solutions and aflatoxin-spiked feed samples passing through a Romer column.

Aflatoxin concentration (ppb)	% Recovery of aflatoxin (Romer column)				
	Aflatoxin standard	Spiked Betagro feed	% Loss	Spiked C.P. feed	% Loss
10	83.52	48.79	34.73	65.09	18.43
20	69.44	52.70	16.74	51.96	17.48
30	78.20	62.33	16.87	53.84	24.36
40	55.87	56.36	-0.49	54.72	1.15
50	73.99	55.42	18.57	61.18	12.81
Average	72.20	54.92	17.28	57.36	14.84
± SD	10.51	4.99	12.47	5.54	8.69

The difference in aflatoxin recovery between the standard solutions and the spiked feed samples indicates the efficiency of the extraction procedure for each column method. On average, the Romer column clean up system resulted in 14.84 % versus 17.28 % loss of aflatoxin with the C.P. and Betagro feeds respectively (Table 32).

Table 33. Comparison of aflatoxin recoveries using an aflatoxin standard solutions and aflatoxin-spiked feed samples passing through a Varian column.

Aflatoxin level (ppb)	% Recovery of aflatoxin (Varian column)				
	Aflatoxin standard	Spiked Betagro feed	% Loss	Spiked C.P. feed	% Loss
10	103.23	73.32	30.91	79.43	23.80
20	93.43	91.78	1.65	82.24	11.19
30	94.79	93.01	1.78	82.86	11.93
40	88.13	92.46	- 4.33	79.74	8.39
50	104.89	97.27	7.62	79.40	25.49
Average	96.89	89.37	7.52	80.73	16.16
± SD	7.02	9.33	13.74	1.68	7.88

On average, the Varian column clean up procedure resulted in aflatoxin losses of 16.16 % and 7.52 % for the C.P. and Betagro feeds respectively (Table 33). However, after Romer column clean up of C.P. feeds there was a lower loss of aflatoxins than with the Varian column (14.84% vs 16.16%) while with Betagro feeds, the Romer column gave a higher loss than the Varian column (17.28% vs 7.52%).

Table 34. Comparison of aflatoxin recoveries using an aflatoxin standard solutions and aflatoxin-spiked feed samples passing through a Vicam column.

Aflatoxin level(ppb)	% Recovery of aflatoxin (Vicam column)				
	Aflatoxin standard	Spiked Betagro feed	% Loss	Spiked C.P. feed	% Loss
10	90.29	60.20	30.09	105.35	-15.06
20	70.71	86.44	-15.73	104.36	-33.65
30	78.65	86.71	-8.06	71.74	6.91
40	96.12	76.59	19.53	84.01	12.11
50	92.18	78.95	13.23	87.66	4.52
Average	85.59	77.78	7.81	90.62	- 5.03
± SD	10.56	10.80	19.16	14.27	19.02

On average, the Vicam column gave aflatoxin losses of 7.81 % for the Betagro feeds whereas there was no loss of aflatoxin for the C.P. feeds. In the latter case, aflatoxin recoveries were higher for the spiked C.P. feeds than for the aflatoxin standard solutions (Table 34).

Table 35. Comparison of aflatoxin recoveries using aflatoxin standard solutions and aflatoxin-spiked feed samples passed through a Rhône column using methanol as the extraction solvent.

Aflatoxin level (ppb)	% Recovery of aflatoxin (Rhône column)				
	Aflatoxin standard	Spiked Betagro feed	% Loss	Spiked C.P. feed	% Loss
10	95.04	50.52	44.52	63.13	31.91
20	72.47	56.58	15.89	77.37	-4.90
30	98.03	51.56	46.47	44.88	53.15
40	80.54	56.32	24.22	51.89	28.65
50	78.76	58.78	19.98	57.25	21.51
Average	84.97	54.75	30.22	58.90	26.07
± SD	11.03	3.54	14.27	12.32	20.94

The Rhône column gave an average aflatoxin loss of 30.22 % for the Betagro feeds and 26.07 % for the C.P. feeds. The difference in percentage recovery (30.22 % vs 26.07 %) between the two feeds may reflect the better extraction from C.P. feed compared to the Betagro feed (Table 35).

For the Betagro mixed feed, losses of aflatoxin in ascending order were 7.52, 7.81, 17.28 and 30.22 % for the Varian, Vicam, Romer and Rhône columns, respectively. For the C.P. mixed feed, losses of aflatoxin in ascending order were –5.03, 14.84, 16.16 and 26.08 % for the Vicam, Romer, Varian and Rhône columns, respectively.

The results of this analysis combined with a cost analysis suggested that the Varian column was the most appropriate system to use for field sampling.

Development of the methodology for aflatoxin extraction and clean up took much longer than was originally anticipated due to the poor recovery rates using standard methodologies and the co-extraction of a number of impurities from the complex mixed feeds that interfered with the HPLC analysis. A standard methodology was established as described above by the end of the first year of the PhD programme. However, the extended period of method development meant that the extensive set of samples of both broilers and feed collected during the first year of study could not be properly analysed due to the difficulty of maintaining samples in a format where the aflatoxin concentrations would not increase with storage. Hence field samples for the entire first year had to be discarded.

Further problems were encountered at this stage due to the difficult economic conditions in Thailand. There was a currency crisis which resulted in a devaluation of the Thai Baht by around 80%. This meant that the budget to cover the imported manufacturers columns was no longer sufficient to cover the cost of the number of columns that were needed to support the sampling analysis that was originally developed to give a measure of the position of the aflatoxin contamination within the feed bins as well as an overall measure of the aflatoxin contaminant levels within the feed. As these problems were completely outside the control of the research programme and further funding was unavailable due to the difficult economic conditions facing the country due to the currency devaluation a reduced analysis had to be undertaken on a series of new samples that were collected during 2000.

DETERMINATION OF NATURALLY CONTAMINATED AFLATOXIN IN BROILER MIXED FEED: A FIELD STUDY UNDERTAKEN IN 2000.

Broiler feed samples were collected from the four selected study farms for each feed type at different time points throughout the broiler rearing cycle. Three types of feed samples, the starter, grower and finisher feeds, from each manufacturer were sampled. The samples were extracted and cleaned-up by the Varian column method prior to being quantified by HPLC. The Varian column was chosen as it provided a good rate of aflatoxin recovery and the cost of columns and related laboratory expenses were relatively low, making this a potentially useful system for routine monitoring of aflatoxins by the poultry industry in Thailand. The aflatoxin levels detected in various grades of broiled feed (starter, grower and finisher) are shown in Table 36.

Both C.P. and Betagro feed samples collected from the bins at the farms over a one year time period had no detectable contamination with aflatoxin G_2 for at all three feed types (starter, grower and finisher) with the exception of one C.P. farm in the grower feed.. For both feed brands, more aflatoxin B_1 was found than G_1 and B_2. The total aflatoxin contamination levels in the C.P. feed brand were similar for all three feed types; (averages of 20.39, 18.49 and 20.15 ppb for the starter, grower and finisher period, respectively). For the Betagro feed samples a similar result was found, with average aflatoxin contamination levels of 17.30, 20.67 and 17.56 ppb for the starter, the grower and the finisher feed types, respectively.

Feed sample collections were also made from the newly opened sacks of feed at the time they were delivered to the farms. The results of the aflatoxin contamination levels from these samples are reported in Table 37.

Table 36. Average aflatoxin levels detected from broiler feed samples collected from the bins at the fields of four C.P. farms (A, B, C and D) and four Betagro farms (A, B, C and D).

A.F. / Feed	Aflatoxin concentrations (ppb)									
	C.P. farms					Betagro farms				
	G_1	B_1	G_2	B_2	Total	G_1	B_1	G_2	B_2	Total
A-Starter	0.00	20.40	0.00	2.16	22.56	6.09	17.19	0.00	1.47	24.75
B-Starter	2.13	17.31	0.00	1.47	20.91	4.29	9.90	0.00	0.00	14.19
C-Starter	0.00	12.09	0.00	0.90	12.99	4.56	9.90	0.00	0.00	14.46
D-Starter	13.74	11.37	0.00	0.00	25.11	8.13	7.68	0.00	0.00	15.81
Average	3.97	15.29	0.00	1.13	20.39	5.77	11.17	0.00	0.37	17.30
± SD	6.59	4.31	0.00	0.91	5.23	1.76	4.15	0.00	0.74	5.02
A-Grower	5.94	19.32	2.01	1.95	29.22	4.80	18.18	0.00	1.14	24.12
B-Grower	0.00	8.70	0.00	0.00	8.70	3.78	13.86	0.00	1.05	18.69
C-Grower	0.00	12.09	0.00	0.72	12.81	4.80	10.41	0.00	0.00	15.21
D-Grower	12.00	9.93	0.00	1.32	23.25	6.60	17.19	0.00	0.87	24.66
Average	4.49	12.51	0.50	1.00	18.50	5.00	14.91	0.00	0.77	20.67
± SD	5.74	4.75	1.01	0.83	9.41	1.17	3.52	0.00	0.52	4.53
A-Finisher	7.74	16.50	0.00	0.00	24.24	6.00	10.65	0.00	0.00	16.65
B-Finisher	5.76	11.37	0.00	0.72	17.85	5.07	14.31	0.00	0.00	19.38
C-Finisher	6.75	7.98	0.00	0.00	14.73	4.56	12.39	0.00	0.00	16.95
D-Finisher	9.99	12.81	0.00	0.99	23.79	8.13	9.15	0.00	0.00	17.28
Average	7.56	12.17	0.00	0.43	20.15	5.94	11.63	0.00	0.00	17.57
± SD	1.81	3.53	0.00	0.51	4.64	1.58	2.23	0.00	0.00	1.24

Table 37. Aflatoxin levels detected from sacks of feed at four C.P. and Betagro farms.

A.F. / Feed	Aflatoxin concentrations (ppb)									
	C.P. farm					Betagro farm				
	G_1	B_1	G_2	B_2	Total	G_1	B_1	G_2	B_2	Total
A-Starter	0.00	19.23	0.00	2.10	21.33	5.07	6.69	0.00	0.00	11.76
B-Starter	0.00	18.39	0.00	1.89	20.28	5.07	6.69	0.00	0.00	11.76
C-Starter	0.00	7.26	0.00	0.99	8.25	4.14	4.71	0.00	0.00	8.85
D-Starter	0.00	15.48	0.00	1.71	17.19	4.80	8.16	0.00	0.00	12.96
Average	0.00	15.09	0.00	1.67	16.76	4.77	6.56	0.00	0.00	11.33
± SD	0.00	5.46	0.00	0.48	5.94	0.44	1.42	0.00	0.00	1.75
A-Grower	0.00	17.34	0.00	2.10	19.44	5.07	11.88	0.00	0.72	17.67
B-Grower	0.00	8.13	0.00	0.00	8.13	6.45	12.87	0.00	0.00	19.32
C-Grower	0.00	15.24	0.00	1.32	16.56	4.80	10.41	0.00	0.00	15.21
D-Grower	6.00	7.50	0.00	0.00	13.50	4.56	15.00	0.00	0.96	20.52
Average	1.50	12.05	0.00	0.86	14.41	5.22	12.54	0.00	0.42	18.18
± SD	3.00	4.97	0.00	1.04	4.84	0.85	1.93	0.00	0.49	2.30
A-Finisher	0.00	23.01	0.00	1.80	24.81	4.95	9.15	0.00	0.00	14.10
B-Finisher	0.00	17.43	0.00	1.14	18.57	6.33	10.65	0.00	0.00	16.98
C-Finisher	0.00	12.33	0.00	0.00	12.33	5.58	9.42	0.00	0.00	15.00
D-Finisher	8.25	9.66	0.00	0.00	17.91	5.82	16.86	0.00	1.05	23.73
Average	2.06	15.61	0.00	0.74	18.41	5.67	11.52	0.00	0.26	17.45
± SD	4.13	5.89	0.00	0.89	5.11	0.57	3.62	0.00	0.53	4.35

There was no detectable level of aflatoxin G_2 contamination for all three types of feed samples (Table 37). For the C.P. feed, the total aflatoxin levels found were, on average 16.76, 14.41 and 18.4 ppb for the 3 feed types (starter, grower and finisher, respectively). The predominant aflatoxin contaminant was B_1 and there were very low levels of aflatoxins B_2 and G_1. For the Betagro feed, the total levels of aflatoxin were, on average 11.33, 18.18 and 17.45 ppb for the 3 feed types (starter, grower and finisher, respectively) with the predominant aflatoxin contaminants being B_1 and G_1. There was a very low level of aflatoxin B_2.

A summary of aflatoxin contamination levels from the feed samples collected from two different storage containers for the two different feed brands is given in Table 38.

Table 38. Aflatoxin levels detected in feed collected from bins and sacks at four C.P. and Betagro farms.

Feed type	Detected aflatoxin level (ppb)			
	From sacks		From bins	
	C.P. Total	Be Total	C.P. Total	Be Total
A-Starter	21.33	11.76	22.56	24.75
B-Starter	20.28	11.76	20.91	14.19
C-Starter	8.25	8.85	12.99	14.46
D-Starter	17.19	12.96	25.11	15.81
Average	16.76	11.33	20.39	17.30
± SD	5.94	1.75	5.23	5.01
A-Grower	19.44	17.67	29.22	24.12
B-Grower	8.13	19.32	8.70	18.69
C-Grower	16.56	15.21	12.81	15.21
D-Grower	13.50	20.52	23.25	24.66
Average	14.41	18.18	18.49	20.67
± SD	4.84	2.30	9.41	4.53
A-Finisher	24.81	14.10	24.24	16.65
B-Finisher	18.57	16.98	17.85	19.38
C-Finisher	12.33	15.00	14.73	16.95
D-Finisher	17.91	23.73	23.79	17.28
Average	18.40	17.45	20.15	17.56
± SD	5.10	4.35	4.64	1.24

The factorial in a CRD analysis showed that there was no significant difference in the level of aflatoxin contamination between the C.P. and the Betagro feed brands for all three raising periods (the starter, the grower and the finisher) from both collection sources (bins and sacks). Comparing the aflatoxin contamination levels between bins and sacks for the same feed brands, there was a significant difference, with aflatoxin contamination higher when the feed was collected from the bins (19.09 versus 16.09 ppb). Hence the storage conditions on the farm had allowed a small amount of aflatoxins growth even though the storage conditions on the farms had been improved from the storage conditions that the farmers had employed in the first year of the programme.

The first element of this PhD programme was to establish the levels of aflatoxin contamination in the feed prior to its deliver at the farms and the effect of storage on the farm on aflatoxin levels. The second element was to determine whether the detected levels of aflatoxin in the broiler feed produced any evident effect on the broilers that would affect their marketability either for the local or the export market. If contaminantion levels were detected in the feed that produced adverse effects in the broilers further work would be undertaken to determine whether molecular assays for enzymes induced by the aflatoxins in the broilers could be used as markers to monitor the broiler effects.

A SURVEY OF BROILERS PERFORMANCE FED ON BROILER MIXED FEEDS WITH KNOWN LEVELS OF AFLATOXIN CONTAMINATION IN NORTHEAST THAILAND (KHON KAEN) IN 1998 AND 2000.

Broilers raised on the C.P. and Betagro farms were examined for their commercial characteristics and performance throughout the rearing period. The numbers of broilers going into the rearing process, the numbers of broilers that successfully completed the rearing process as marketable birds, mortality rate, percentage losses, farm size, rearing starting date, chick density, total broiler weight, total feed intake, average weight per

broiler, F.C.R. and feed cost per kg. of body weight were measured for all four farms for each feed type.

These studies were undertaken in 1998 and in 2000.The results obtained from the field study for Betagro farms in 1998 and in 2000 are given in Table 39 and Table 40, respectively. Those of C.P. farms in 1998 and 2000 are given in Table 41 and 42, respectively. Samples taken in 1998 corresponded to the sampling period in which aflatoxin contamination levels in the feed could not be measured due to problems with the analysis method. Samples in 2000 were correlated with the aflatoxin levels measured in the feed.

Table 39. The commercial characteristics of broilers at the Betagro farms (1998).

Characteristics and Performance	Farm A	Farm B	Farm C	Farm D	Average	± SD
Feeding period (days)	42	42	42	42	42.00	0.00
Number of broiler in	4,984	4,849	4,832	4,882	4,886.75	68.08
Number of broiler out	4,761	4,643	4,539	4,685	4,657.00	92.59
Mortality rate	223	206	293	197	229.75	43.52
Percent loss (%)	4.47	4.25	6.06	4.03	4.70	0.92
Farm size (m^2)	620	620	630	615	621.25	6.29
Starting date	98/8/20	98/8/20	98/8/20	98/8/20	-	-
Density (Broilers/ m^2)	8.04	7.82	7.67	7.94	7.87	0.16
Total broiler weight out (kg)	8,231.34	8,035.80	7,787.34	8,319.72	8,093.55	236.11
Total feed intake (kg)	17,532.75	16,794.82	16,742.78	17,138.62	17,052.24	365.32
Average weight per broiler (kg)	1.73	1.73	1.72	1.78	1.74	0.02
F.C.R.	2.13	2.09	2.15	2.06	2.11	0.04
Feed cost/Body weight (Baht/kg)	19.17	18.81	19.35	18.54	18.97	0.36

*Feed cost = 9 Baht/kg

Table 40. The commercial characteristics of broilers at the Betagro farms (2000).

Characteristics and Performance	Farm A	Farm B	Farm C	Farm D	Average	± SD
Feeding period (days)	42	42	42	42	42.00	0.00
Number of broiler in	4,884	4,753	4,857	4,748	4,810.50	70.18
Number of broiler out	4,758	4,540	4,554	4,674	4,631.50	103.58
Mortality rate	126	213	303	74	179.00	100.61
Percent loss (%)	2.58	4.48	6.24	1.56	3.72	2.07
Farm size (m^2)	625	608	621	607	615.25	9.11
Starting date	00/8/24	00/8/24	00/8/24	00/8/24	-	-
Density (Broilers/ m^2)	7.81	7.81	7.82	7.82	7.815	0.01
Total broiler weight out (kg)	8,904.49	8,348.57	8,370.81	9,377.52	8,750.35	490.77
Total feed intake (kg)	17,948.57	17,254.28	17,718.75	19,034.04	17,988.91	754.23
Average weight per broiler (kg)	1.87	1.83	1.83	2.00	1.88	0.08
F.C.R.	2.02	2.07	2.12	2.03	2.06	0.05
Feed cost/Body weight (Baht/kg)	19.19	19.66	20.14	19.28	19.57	0.43

*Feed cost = 9.50 Baht/kg

When broiler characteristics and performance were compared for Betagro farms in 1998 and 2000 all characteristics and performance were similar. The data suggests that the levels of aflatoxin contamination in the feed in 1998 was insufficient to result in high rates of broiler motality or poor feed : weight conversion rates compared to 2000.

Table 41. The commercial characteristics of broilers at the C.P. farms (1998).

Characteristics and Performance	Farm A	Farm B	Farm C	Farm D	Average	± SD
Feeding period (days)	42	42	42	42	42.00	0.00
Number of broiler in	4,968	4,895	4,784	4,887	4,883.50	75.69
Number of broiler out	4,673	4,574	4,522	4,612	4,595.25	63.62
Mortality rate	295	321	262	275	288.25	25.70
Percent loss (%)	5.94	6.56	5.48	5.63	5.90	0.48
Farm size (m^2)	640	620	610	620	622.50	12.58
Starting date	98/8/25	98/8/25	98/8/25	98/8/25	-	-
Density (Broilers/ m^2)	7.76	7.89	7.84	7.88	7.84	0.06
Total broiler weight out (kg)	8,504.86	8,141.72	8,320.48	8,255.48	8,305.64	151.98
Total feed intake (kg)	18,030.30	17,667.53	17,389.80	17,749.28	17,709.23	263.60
Average weight per broiler (kg)	1.82	1.78	1.84	1.79	1.81	0.03
F.C.R.	2.12	2.17	2.09	2.15	2.13	0.04
Feed cost/Body weight (Baht/kg)	19.08	19.53	18.81	19.35	19.19	0.31

*Feed cost = 9 Baht/kg

Table 42. The commercial characteristics of broilers at the C.P. farms (2000).

Characteristics and Performance	Farm A	Farm B	Farm C	Farm D	Average	± SD
Feeding period (days)	42	42	42	42	42	0
Number of broiler in	6,808	6,600	6,602	6,095	6,526.25	303.61
Number of broiler out	6,411	6,233	6,268	5,827	6,184.75	250.62
Mortality rate	397	367	334	268	341.50	54.34
Percent loss (%)	5.83	5.56	5.06	4.40	5.21	0.63
Farm size (m^2)	700	648	640	616	651.00	35.38
Starting date	00/8/28	00/8/28	00/8/28	00/8/28	-	-
Density (Broilers/ m^2)	9.73	10.18	10.31	9.89	10.03	0.26
Total broiler weight out (kg)	13,430.71	13,211.55	14,125.13	12,702.60	13,367.50	590.03
Total feed intake (kg)	24,735.79	25,419.13	26,492.31	25,560.00	25,551.81	722.98
Average weight per broiler (kg)	2.09	2.12	2.25	2.18	2.16	0.07
F.C.R.	1.84	1.92	1.87	2.01	1.91	0.07
Feed cost/Body weight (Baht/kg)	17.5	18.28	17.82	19.11	18.18	0.70

*Feed cost = 9.50 Baht/kg

Table 43. Comparison of commercial characteristics of broilers at the C.P. and Betagro farms in 1998 and 2000.

Characteristics and Performance	Betagro 1998	Betagro 2000	C.P. 1998	C.P. 2000
Feeding period (days)	42	42	42	42
Number of broiler in	4,886.75	4,810.50	4,883.50	6,526.25
Number of broiler out	4,657.00	4,631.50	4,595.25	6,184.75
Mortality rate	229.75	179.00	288.25	341.50
Percent loss (%)	4.70	3.72	5.90	5.21
Farm size (m^2)	621.25	615.25	622.50	651.00
Starting date	20/ 8/98	24/8/00	25/8/00	28/8/00
Density (Broilers/ m^2)	7.87	7.82	7.84	10.03
Total broiler weight out (kg)	8,093.55	8,750.35	8,305.88	13,367.50
Total feed intake (kg)	17,052.24	17,988.91	17,709.23	25,551.81
Average weight per broiler (kg)	1.74	1.88	1.81	2.16
F.C.R.	2.11	2.06	2.13	1.91
Feed cost/Body weight (Baht/kg)	18.97	19.57	19.19	18.18

Between 1998 and 2000 the C.P. farms expanded the number of birds reared in line with the growing export market for broilers by increasing the density of rearing. Percentage losses were marginally lower even though numbers had been expanded and the average weight of the broilers was increased. These results were achieved by the farmers despite rearing the broilers at higher densities. There was no obvious signs of over-crowding stress from the higher density reared broilers. When results for C.P. farms were compared between 1998 and 2000 most broiler characteristics and performance were similar.

The data in Tables 44 can be used as a baseline to indicate the magnitude and economy of scale of the developing broiler business in Thailand. In 1998, direct observation on the external appearance of farm reared broilers indicated a relatively high abnormality rate in broilers, such as feather growth, leg deformity and leg pigmentation (Table 44), which may be related to the aflatoxin exposure from the feed intake. These abnormalities may have reflected a relatively high exposure to aflatoxin. A subsequent

examination of the same characteristics was undertaken in 2000 and the results are given in Table 46. In contrast to the data for 1998, in 2000 there were no signs of leg deformities or abnormal feather growth even though broilers were reared at higher densities in the C.P. farms.

Table 44. Feather score, leg pigmentation score and leg deformity score from broilers at C.P. and Betagro farm in 1998.

Farm	Feather Score	Leg pigmentation score	Leg deformity Score
Be A	2.12	1.94	0.25
Be B	2.28	2.17	0.52
Be C	2.33	2.08	0.37
Be D	2.58	2.36	0.42
Average	2.33	2.14	0.39
±SD	0.19	0.18	0.11
C.P. A	2.23	2.14	0.14
C.P. B	2.17	2.40	0.08
C.P. C	2.24	2.45	0.15
C.P. D	2.02	1.84	0.53
Average	2.17	2.21	0.22
±SD	0.10	0.28	0.21

Be = Betagro farm C.P. = Charoen Pokphan farm

Feather score:

1 = poor, 2 = moderate, 3 = good

Leg pigmentation score:

1 = pale, 2 = moderate, 3 = yellow

Leg deformity score:

0 = normal,

1 = one leg slightly deformed,

2 = both legs slightly deformed

3 = one leg slightly, another severely deformed

4 = both legs severely deformed

The levels of abnormal feather growth, leg pigmentation and leg deformities in field broilers in 1998, may be symptomatic of aflatoxin toxicity in the broilers, but this still needs to be confirmed due to the problems that occurred in establishing an aflatoxin analysis method in a time period that would have allowed the feed samples collected in the farms at the same time as the broiler observations were made.

Carcass characteristics were examined in both 1998 and 2000. After approximately 42 days of rearing, 12 broilers from each of the four farms, (a total of 48 broilers), were randomly selected and examined for their body weight and then sacrificed. Internal organs, carcass, liver, heart, gizzard, spleen, and proventiculus, were weighed. The colour of the broiler livers was compared and ranked on a 4-point scale (see Table 45 and 46). The body skin colour was examined and similarly ranked. The quality of the carcass was graded on a 3-point scale. The results are given in Table 45 and 46.

Table 45. Carcass characteristics of 42 day-old broilers at Betagro and C.P. farms in 1998.

Commercial carcass Characteristics	Average of Betagro farm	± SD	Average of C.P. farm	± SD
Body Wt. (g)	1,519.34	43.57	1,592.30	58.02
Carcass wt. %	77.81	0.73	77.22	1.20
liver wt. (g)	2.54	0.07	2.63	0.12
Heart wt. (g)	0.49	0.04	0.47	0.03
Gizzard wt. (g)	2.02	0.25	1.99	0.32
Spleen wt. (g)	0.23	0.04	0.25	0.06
Proventiculus wt. (g)	0.46	0.08	0.47	0.06
Liver colour (Score*)	2.48	0.09	2.32	0.08
Skin colour (Score**)	2.93	0.13	2.88	0.21
Carcass grade(Score***)	2.51	0.14	2.58	0.18

Table 46. Carcass characteristics of 42 day - old broilers at Betagro and C.P. farms in 2000.

Commercial carcass Characteristics	Average of Betagro farm	± SD	Average of C.P. farm	± SD
Body wt. (g)	1,521.75	32.61	1,664.00	64.60
Carcass wt. %	80.67	0.69	78.66	1.00
liver wt. (g)	2.24	0.08	2.33	0.19
Heart wt. (g)	0.48	0.06	0.48	0.02
Gizzard wt. (g)	2.05	0.22	1.69	0.22
Spleen wt. (g)	0.18	0.03	0.24	0.08
Proventiculus wt. (g)	0.45	0.09	0.45	0.09
Liver colour (Score*)	3.08	0.07	3.17	0.07
Skin colour (Score**)	3.13	0.11	3.19	0.04
Carcass grade(Score***)	2.73	0.11	2.71	0.20

* Liver colour: 1 = very pale; 2 = pale; 3 = red; 4 = dark red

** Skin colour: 1 = very pale; 2 = pale; 3 = moderate; 4 = yellow

*** Carcass grade: A = 3, B = 2, C = 1 which A is the highest grade.

The higher percentage losses, F.C.R. ratios, feather growth abnormalities, skin palour and leg deformity found in farm raised chicks in 1998 indicated that there was a problem with broiler rearing at that time. These abnormalities were probably associated with aflatoxin contamination of the broiler feeds, but this could not be confirmed, as the aflatoxin clean up method had not yet been fully developed. The broiler qualities in 2000 were more acceptable and all the broilers were released to and acceptable for the international markets. In 2000 there was no evidence to indicate that the amounts of aflatoxin found in the broiler feed samples were adversely affecting the development or quality of the broilers.

The improvement in the broilers between 1998 and 2000 may have been due to a combination of better animal husbandry by the farmers and unusually dry weather conditions in central Thailand. The farmers were taking more care handling and storing there feed as a result of the interactions established during the initial year of this PhD programme. The real test of how well the new storage conditions reduced the potential for aflatoxin growth required a wet broiler growing season.

During 2001 the abnormal weather conditions continued and the quality of the broilers remained consistently high, hence the planned studies on enzyme families that should have been induced by aflatoxin exposure were not carried out. Reasons for this were two fold. First the continued good health of the broiler flocks meant that meaningful results were unlikely to be obtained within the timeframe of the PhD programme. Second the initial economic problems in Thailand that affected the sampling methodology reducing the number of columns that could be used also affected the time available for further studies and the ability to fund the molecular part of this study that was originally planned to be carried out in Cardiff.

Over the 2001 period a more detailed analysis of the temperature and humidity conditions on the farms during broiler rearing was carried out along with a detailed analysis of feed quality.

THE TEMPERATURE AT C.P. AND BETAGRO FARMS.

Temperature and humidity are major determinants affecting the rate of aflatoxin proliferation in feed samples. Temperature fluctuations were measured throughout a 42-day broiler rearing cycle at all 8 farms. The environmental conditions, in particular the farm rearing shed temperatures, where the broiler feed samples were stored was recorded daily at 08.00 am, 01.00 and 05.00 pm in all 8 farms. The average temperatures from the 4 farms are shown in Figure 39 for the Betagro farms and in Figure 40 for the C.P. farms. The temperature pattern for the C.P. farm was relatively stable during the whole rearing cycle (42 days), whereas on the Betagro farms, the temperature fluctuate more, particularly during the 6^{th} to18^{th} days. However, in general, the variability in the temperature levels at which all feed samples were kept was low.

Figure 39. The average temperature at 4 Betagro farms at 08.00 am, 1.00 pm. and 5.00 pm. (°C) over a broiler rearing cycle.

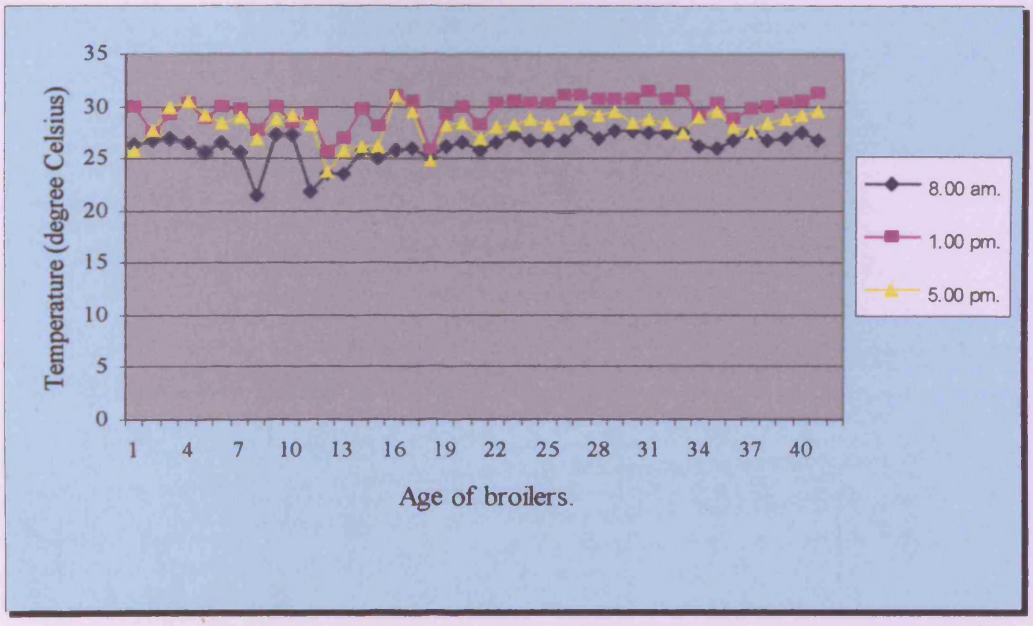
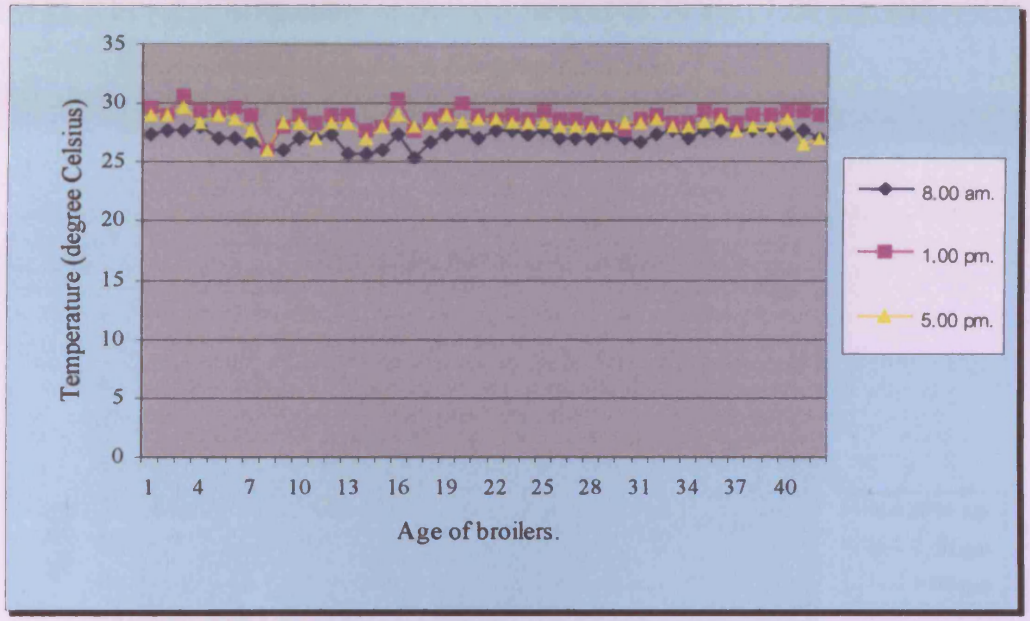


Figure 40. The average temperature at 4 C.P. farms at 08.00 am., 1.00 pm. and 5.00 pm. (°C) over a broiler rearing cycle.



The average humidity at the sites, where feed samples were stored, was also determined and is shown in Figure 41 for the Betagro farms and in Figure 42 for the C.P. farms.

Figure 41. The relative humidity at Betagro farm at 08.00 am., 1.00 pm. and 5.00 pm. (% RH) over a broiler rearing cycle.

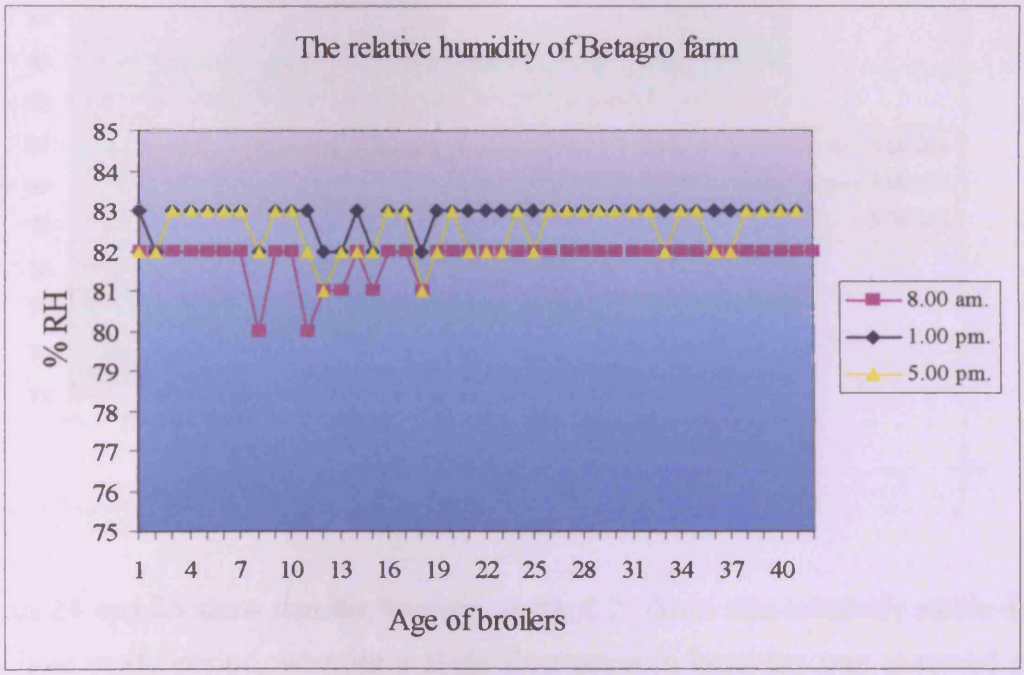
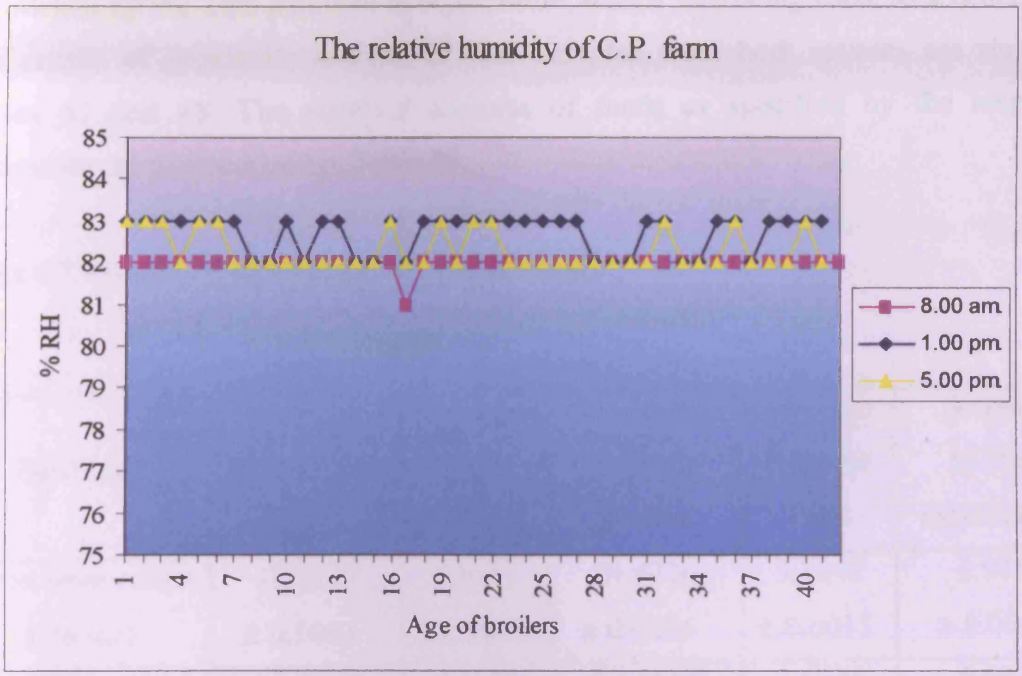


Figure 42. The relative humidity at C.P. farms at 08.00 am., 1.00 pm. and 5.00 pm. (% RH) over a broiler rearing cycle.



Figures 24 and 25 show that the humidity at the C.P. farms was relatively stable during the whole study period, whereas a slight fluctuation in humidity was observed at the Betagro farm between the 7^{th} - 13^{th} rearing days. In general, the relative humidity at both farms was between 82-83 %. This level of humidity is quite high compared with other parts of Thailand and may be an environmental characteristic that poultry farmers in the Khon Kaen region need to be aware of, as higher humidity conditions will favour aflatoxin proliferation if feed storage conditions are poor.

While aflatoxin levels are major determinants on the suitability of the resulting broilers for the export market there are also minimum standards that the broiler feeds need to be manufactured to, to provide the nutritional requirements for carcasses designed for the export market. To establish whether the Betagro and C.P. feeds where being manufactured to international standards further analysis of the feeds was undertaken.

PROXIMATE ANALYSIS OF BROILER MIXED FEEDS

Feed samples from each company were examined for their essential contents, to determine whether the feed samples conformed with the quality and standard established by the Thai Ministry of Agriculture, which was being used, as a benchmark. The results of proximate analysis of feed samples from both sources are shown in Tables 47 and 48. The standard contents of feeds as specified by the respective companies are summarized in Table 48.

Table 47. Proximate analysis of C.P. broiler feeds.

Feed type	Average % moisture	Average % ash	Average % crude protein	Average % crude fiber	Average % ether extract (Fat)
Feed from sacks (starter)	12.2237 ± 0.0061	6.8417 ± 0.0039	24.4226 ± 0.0086	3.1342 ± 0.0011	4.9419 ± 0.0018
Feed from sacks (grower)	11.2340 ± 0.0039	6.6828 ± 0.0024	22.3417 ± 0.0087	3.5721 ± 0.0013	5.9635 ± 0.0021
Feed from sacks (finisher)	11.5307 ± 0.0040	6.4930 ± 0.0023	19.1028 ± 0.0067	3.8322 ± 0.0014	9.1215 ± 0.0032
Feed from bins (starter)	15.3037 ± 0.0054	8.3105 ± 0.0029	23.1344 ± 0.0081	3.0523 ± 0.0011	5.3342 ± 0.0019
Feed from bins (grower)	12.8325 ± 0.0045	6.9242 ± 0.0029	22.5230 ± 0.0081	3.5617 ± 0.0011	7.1629 ± 0.0019
Feed from bins (finisher)	14.0644 ± 0.0049	7.0132 ± 0.0025	20.6142 ± 0.0072	3.5308 ± 0.0013	8.3126 ± 0.0029

Table 48. Proximate analysis of Betagro broiler feed.

Feed type	Average % moisture	Average % ash	Average % crude protein	Average % crude fiber	Average % ether extract (Fat)
Feed from sack (starter)	13.3320 ± 0.0066	5.9608 ± 0.0030	23.0043 ± 0.0115	2.3614 ± 0.0011	5.3923 ± 0.0027
Feed from sack (grower)	12.0711 ± 0.0059	5.5924 ± 0.0027	22.6201 ± 0.0112	2.2030 ± 0.0011	7.4421 ± 0.0037
Feed from sack (finisher)	11.7932 ± 0.0058	5.4806 ± 0.0027	20.0535 ± 0.0099	2.2343 ± 0.0011	7.1519 ± 0.0035
Feed from bins (starter)	14.6342 ± 0.0072	7.1237 ± 0.0035	22.3041 ± 0.0110	2.7233 ± 0.0014	5.1227 ± 0.0025
Feed from bins (grower)	12.9411 ± 0.0064	5.9028 ± 0.0030	20.1540 ± 0.0100	2.3210 ± 0.0011	7.0922 ± 0.0034
Feed from bins (finisher)	14.1225 ± 0.0069	5.4803 ± 0.0027	19.0344 ± 0.0095	2.2331 ± 0.0015	6.0239 ± 0.0030

The Ministry of Agriculture in Thailand has established recommended guideline for the standard of feeds, which details their essential contents as shown in Table 49.

Table 49. Standard contents for broiler feeds produced by the C.P. and the Betagro companies as specified by the Ministry of Agriculture, Thailand.

Period	Standard content of broiler feed			
	Protein	Fat	Fibre	Moisture
Starter	> 21 %	> 4 %	< 5 %	< 13 %
Grower	> 19 %	> 4 %	< 5 %	< 13 %
Finisher	> 17 %	> 4 %	< 5 %	< 13 %

When the result of the proximate analysis was compared with the standards specified for feeds by the Thai Ministry of Agriculture, it was found that protein, fibre and fat contents of all samples of both brands satisfied the minimum levels specified. The moisture content of C.P. feeds met the standard for all samples except when samples were collected from the bins at the starter and finisher periods, when moisture content was slightly higher than the maximum levels specified (15.30 and 14.06 % versus 13.00 %). For the Betagro feed samples collected from the sacks the moisture content was lower than 13 %, but for the samples collected from the bins at the starter and finisher periods the moisture content was slightly higher. While the differences in moisture content were not significant the increase in moisture content reflects the high humidity conditions in which the feed had to be stored and again emphasises the care that the poultry farmers need to take to ensure that this increase in moisture is not accompanied by a proliferation of aflatoxins. Over the 2000 rearing season however, the aflatoxin contamination levels found in the feed samples was not directly linked to the levels of moisture content.

GENERAL DISCUSSION

The physical indicators of aflatoxin toxicity that occurred in broiler samples, taken from Thai farms in 1998, suggested that there was a need to determine the level of aflatoxin contamination in feeds. At that time accurate procedures for the determination of aflatoxin in mixed feed had not been established for the complex matrix of mixed feeds that were used for broiler feeding.

Development of a methodology for extraction of aflatoxins should have been relatively straightforward, but several problems were encountered. For example, at the beginning of the study the laboratory in the agricultural division of Khon Kaen University did not have the relevant HPLC equipment or staff expertise. The experimental work was moved to the Faculty of Pharmaceutical Science where all the necessary equipment for this analytical work were available. While the expertise to run the HPLC machines was available in this Faculty there was little expertise in column clean up techniques. Hence the problems encountered prior to putting the samples onto the column had to be tackled from first principles of chemistry. The delay in method development however meant that all the field samples that were collected in 1998 were no longer suitable for direct aflatoxin analysis due to the proliferation that occurs with most aflatoxins even under refrigeration.

There were several factors that contributed to the variability of efficiency of recovery with the different commercial clean-up columns. Different kinds of feed samples with different compositions and matrices affect differently the extraction and the purification steps involved in clean up. A standard aflatoxin solution was used in initial experiments to ensure that all the columns efficiently bound all the standard aflatoxin subtypes. All four commercial columns gave high aflatoxin recoveries, with the Varian column performing the best whereas the Vicam, Rhône and Romer columns gave more moderate recoveries (85.59 %, 84.97 % and 72.20 %, respectively)

When aflatoxin was extracted from broiler feed samples, after spiking with different levels of aflatoxin standards, good recoveries were obtained from the feed matrix. With

both commercial brands (C.P. and Betagro) of broiler mixed feeds, the complexity and the matrix of the samples differentially affected the efficiency of extraction and clean up. The efficiency of aflatoxin recoveries from spiked Betagro feed samples was in descending order obtained from the Varian, Vicam, Romer and Rhône columns, respectively. For the C.P. mixed feeds, the Vicam column had the highest aflatoxin recoveries followed by the Varian, Rhône and Romer columns. Different extraction and clean-up methods differentially influenced the results with each broiler feed. When factors of reagent cost and the time taken to undertake routine analysis through the extraction and clean-up processes were determined, it was established that the Varian column provided the most cost effective and economic means of establishing a routine analysis system. It had the lowest reagent costs, although it took the longest time to complete the clean up. In a low wage economy, such as that which currently operates in Thailand, this column is therefore the most viable method for large scale testing and quality assurance of feed samples.

When the data were analysed for the recovery of individual aflatoxin subtypes, variability was also found. Different aflatoxin subtypes were differentially recovered from the four different column types. The difference in feed source also influenced the recovery of aflatoxin subtypes.

The extraction solvent system was modified with the Rhône column from that originally recommended by the manufacturer, as poor recoveries resulted from the manufacturer's recommended method. When chloroform replaced methanol : water as the extraction solvent, the aflatoxin recoveries increased significantly in all tested broilers mixed feeds. The higher lipid solubility of chloroform may account for the better extraction of aflatoxin from these complex feed samples.

After comparisons of column efficiency, cost and analysis time were made, the Varian columns were selected for use in the determination of aflatoxin contamination levels in broiler mixed feeds in the field. The feed samples were collected from four C.P. and four Betagro frams. The levels of aflatoxin contamination ranged from 18.49-20.39 ppb for the C.P. feeds and from of 17.30-20.67 ppb for the Betagro feeds from samples

collected over the second year of the study. Therefore, the approximate level of natural contamination of aflatoxin from both sources of feed samples in this study was <21 ppb.

The maximum tolerated levels of aflatoxin have been reported in many countries for monitoring and quality control of both feeds and products. Kamimura (1993) lists the maximum tolerated levels of aflatoxin in many countries. The tolerated levels vary from country to country. The industrialized countries with no domestic production of commodities susceptible to aflatoxin contamination generally have lower tolerance than countries where susceptible commodities are produced. However, there is no published indication of the maximum tolerated level of aflatoxin from mixed animal feeds, although the levels reported here are in line with those required for developed countries for other feed sources. However, as there was no data to directly compare with these results, more studies to correlate broiler quality with feed quality and recommend the maximum contamination levels for aflatoxin from mixed animal feeds, especially broiler mixed feeds, are needed.

The maximum tolerated levels of aflatoxin for human food in the United States and the United Kingdom are 20 ppb specified for all foods in the U.S., and 10 ppb, specified for nuts and nut products in the U.K. (Kamimura, 1993). For animal feeds it is likely that higher tolerance levels will be applied. Therefore, there is a need to examine the maximum possible levels of aflatoxin allowed to contaminate feeds and for the Thai Ministry of Agriculture to adopt these as national standards for the growing broiler export market.

A proximate analysis of two Thai brands of broiler mixed feeds was conducted. The results confirmed that both feed brands were within the current government specifications, which indicated that the feed samples were of an acceptable nutritional quality for broiler rearing. The data on the broiler characteristics and their performance suggested that all tested broilers were also normal in 2000. The temperature and the relative humidity data during the study period were relatively stable. Moreover, aflatoxin in the broilers was not detectable or quantifiable. The relationship between aflatoxin in the feed, broiler aflatoxin contamination and any changes in the broilers general condition or inducible enzyme leveles could not, therefore, be determined.

At the time these experiments were planned, the most recently developed commercial columns for aflatoxin extraction were chosen for testing from four sources. Since this study was completed, Romer has marketed a Mycosep column (model No. 226) which can be used for mixed feeds. This should be compared for cost efficiency and extraction efficacy to the earlier columns and considered for the monitoring programme in Thailand. The Varian column used in this study, was recommended by Dr. Martin Negler from the Food Security Department, in the UK. During this study, a new model of the PH column, No. 1211-3010 or 1211-3036, has been marketed for use with mixed feeds. It is therefore suggested that this new column should also be examined for the efficiency of its extraction and clean up of broiler aflatoxins contaminating mixed feeds in the future.

COMPARISON AMONG FOUR DIFFERENT COMMERCIALLY AVAILABLE COLUMNS ON COSTS AND EXTRACTION ANDCLEAN-UP TIMES.

Table 50 shows the costs for each column type and classifies the costs for the columns and chemicals/reagents utilized for the extraction and clean-up processes. The cost of the HPLC analysis subsequent to column clean up for all four columns was constant and therefore is excluded from the reported costs.

Table 50. Comparison on costs of extraction and clean up among four different commercial columns.

Cost of extraction and clean up	Column source			
	Romer	Vicam	Rhône	Varian
1. Column cost per sample (GBP)	7.14	7.90	7.06	1.94
2. Chemicals and reagents per sample (GBP)	3.87	1.10	1.72	3.39
Total (GBP)	11.01	9.00	8.78	5.33

From Table 50, it is evident that the lowest costs for the extraction and clean up were with the Varian columns (£ 5.33 per sample). The ascending cost order was the Rhône, Vicam, and Romer columns, respectively when both the columns and chemical costs

were considered. The lower cost of the Varian column based method is due to the lower unit cost for the column itself.

Table 51 presents the time needed in the extraction/clean-up processes with the four different columns. Based on the steps involved, the time for extraction, dilution/filtration, adsorption/elution and evaporation by nitrogen was calculated. The longest clean up procedure took 61-82 minutes for the Varian column. The fastest clean up procedure, with the Romer column, took approximately half as long as with the Vicam and Rhône procedures. The clean up times for the latter two columns were comparable and significantly faster than the clean-up procedures for the Varian column (Table 51).

Table 51. Comparison of extraction and clean-up times for four different commercial columns.

Procedure / Time(min)	Column source			
	Romer	Vicam	Rhône	Varian
Extraction	1 – 2	1 – 2	1 – 2	1 – 2
Dilution and filtration	4 – 6	10 – 15	5 – 10	5 - 10
Adsorption / Solution	-	14 – 18	14 – 18	30 - 40
Evaporation by nitrogen	6 – 12	5 – 10	5 – 10	25 - 30
Total	11 – 20	30 – 45	25 – 40	61 - 82

Considering the cost and time needed for the clean-up of mixed feeds by the commercial columns, the Varian column was recommended for further field work due to its high accuary and relatrively low unit cost. Although the technical time needed for the analysis with this column is slightly longer than for the other columns, for developing countries, such as Thailand the import costs are more of a concern than the labour costs. With the immunoaffinity columns from Vicam and Rhône, although the time used for the analysis was relatively short, the column costs were much higher and the percentage of aflatoxin recovered was lower than for the bonded elute phase of the Varian column.

CHAPTER 5

CHAPTER 5: CONCLUSIONS

1. A method for determination of aflatoxin sub-types by HPLC was established. After samples were cleaned-up and derivatized by trifluoroacetic acid, they were injected onto an HPLC set at 360 nm for excitation and at 440 nm for emission using the scanning fluorometric detector. A guard column was packed with μ Bondapack C_{18} and the main column was packed with Supelcosil LC-$_{18}$, 25 cm x 4.6 μm. Injection volume was 60 μl and the flow rate was 1.0 ml/minute. The HPLC chromatogram showed separate aflatoxin B_1, B_2, G_1, and G_2 peaks which were identified by their retention times. The retention times of aflatoxin B_1, B_2, G_1, and G_2 were 9.78, 19.25, 7.22 and 13.14 minutes, respectively.

2. Chloroform and dichloromethane were used as extraction solvents for both brands of spiked mixed feeds and had a similar degree of solvent capability. However both solvents gave relatively low aflatoxin recoveries and they were, therefore, not suitable for the extraction of aflatoxin from the spiked broiler mixed feeds.

3. The relative efficiencies of 4 commercial columns was reflected by the aflatoxin recoveries from standard aflatoxin solutions. The Varian column had the best aflatoxin recoveries (96.86 %) while the Vicam, Rhône and Romer columns gave more moderate recoveries in the descending order 85.59 %, 84.97 % and 72.20 %, respectively. Based on SAS analysis by CRD, the Varian column gave a significantly better recovery of aflatoxin than the Romer column, but results were not significantly different between the Vicam and Rhône columns.

4. The efficiency of the columns when the HPLC sample source was from aflatoxin-spiked broiler feed samples was lower than with the standard aflatoxin solutions.

   For the Betagro broiler mixed feed the aflatoxin recovery order from the different types of columns was:

Varian >Vicam >Romer > Rhône.
(89.37%) (77.78%) (54.92%) (54.75%)

For the C.P. broiler mixed feed the aflatoxin recovery order from the different commercial columns was:

Vicam >Varian >Rhône >Romer.
(90.62%) (80.73%) (58.15%) (57.36%)

There was an overlap of the aflatoxin G_1 peak with other contaminating peaks with the Varian and Romer columns, but no interference occurred with the Vicam and Rhône columns for this aflatoxin subtype.

An SAS analysis by the factorial in CRD showed that there was no difference between Betagro and C.P. feed for column efficiency. Among the 4 different columns, there was a significant difference in column efficiency. The Varian and Vicam columns were more efficient than the Rhône and Romer columns. The Varian versus the Vicam columns and the Rhône and Romer colums showed no significant differences in aflatoxin recoveries. There was a significant interaction between two columns i.e. the Varian column gave a better result when applied with the Betagro feed while the Vicam column provided the better recovery with the C.P. feed.

5. Comparison of the four different columns on costs and extraction/ clean-up time was made. The costs of the extraction/clean-up process was the lowest with the Varian column (£ 5.33 per sample). The costs in ascending order were £ 8.78, £ 9.00 and £ 11.01 for the Rhône, Vicam, and Romer columns, respectively. The extraction and clean-up times were longest for the Varian column at 61-82 minutes. The times for the other column procedures were 30-45, 25-40 and 11-20 minutes for the Vicam, the Rhône and the Romer columns.

6. For the Rhône column, higher aflatoxin recoveries from both brands of broiler mixed feed were obtained when methanol was replaced by chloroform in the extraction process. The aflatoxin recoveries were improved from 54.75 % to 98.53

% for the Betagro feed and from 58.15 % to 99.09 % for the C.P. feed. Based on a statistical analysis by factorial CRD design, there was no significant difference in the levels of aflatoxin recoveries between the C.P. feed and the Betagro feed using the same solvent. There was no interaction between solvent and feed.

7. Extraction efficiencies of a particular column with different types of feed samples were calculated as the difference between the aflatoxin recoveries from the spiked feed samples and that of the standard aflatoxin solution. The extraction efficiency for one feed brand was superior to another for the same column as follows:

   The Romer column: C.P feed > Betagro feed
   The Varian column: Betagro feed > C.P. feed
   The Vicam column: C.P. feed > Betagro feed
   The Rhône column : C.P. feed > Betagro feed.

8. The degree of aflatoxin contamination in newly opened sacks of broiler mixed feeds from the manufacturing plant was higher for the C.P. brand than for the Betagro feed. The range of aflatoxin contamination levels were 2.06 to 3.28 ppb for the Betagro feed brand and 11.38 to 15.83 ppb for the C.P. feed brand. B_1 and B_2 aflatoxin were found, but no G_1 and G_2 aflatoxin were present.

   In a field study, the degree of aflatoxin contamination in broiler feeds collected from the sacks and from the feed storage bins at four C.P. farms and four Betagro farms was 14.41 to 18.40 ppb in sacks on C.P.farms and 18.49 to 20.39 ppb in bins on C.P farms. For the Betagro farms, ranges of 11.33-18.18 and 17.30-20.67 ppb of aflatoxin were detected from sacks and bins respectively.

   A CRD in factorial analysis showed no significant difference in the concentration of aflatoxin detected in the C.P.and the Betagro feed brands for all three broiler raising periods (the starter, the grower and the finisher). For both collection sources (bins and sacks) there was a significant higher aflatoxin contamination when the feed was collected from the bins (19.09 versus 16.09 ppb).

9. A proximate analysis on the study feed samples indicated that the protein, fiber and fat content of all samples of both brands satisfied the minimum levels specified by Thai Ministry of Agriculture. This data confirmed the quality of the feed samples. From the present study, there was no obvious link between environmental or feed moisture content and aflatoxin contamination level. Other factors may account for the different levels of aflatoxin contamination observed in different years in feed.

10. The higher percentage losses, feed conversion ratios (F.C.R), feather growth abnormalities, skin pale indices and leg deformities were found in broilers from all farms in 1998 compared to 2000. These abnormalities were probably associated with aflatoxin contamination of the broiler feeds. However, the broilers were much healthier in 2000 and were all acceptable for release to the markets. By 2000, there was no indication that the amounts of aflatoxin found in the broiler feed samples were adversely affecting the development or quality of the broilers.

11. The rapid improvement in the broilers at the farms may have been achieved by the improvements in animal husbandry at these farms during three years of the study or may have been influenced by the drier than usual weather conditions in 2000 in central Thailand.

12. The lack of obvious effects in the broilers and the lack of funding for overseas experimental work made an experimental analysis of enzyme induction in aflatoxin contaminated broilers impractical.

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APPENDIX

Table 51. SAS analysis of aflatoxin recoveries from the aflatoxin standard solution with four different types of commercially available columns.

Analysis of Variance

Source	df	Sum of Squares	Mean Square	F-Value	Pr.> F
Model	3	1527.62196	509.20732	5.18	0.0108
Error	16	1571.33212	98.2082575		
Corrected Total	19	3098.95408			

R-Square = 0.492948 C.V. = 11.67064 Root MSE = 9.91000795

Recovery Mean = 84.914

Source	df	ANOVA SS	Mean Square	F-Value	Pr.> F
Model	3	1527.62196	509.20732	5.18	0.0108

Alpha = 0.05 df = 16 MSE = 98.20826

Number of Mean	2	3	4
Critical Range	13.29	13.93	14.34

Means with the same letter within the Duncan grouping are not significantly different.

Duncan grouping	Mean	N	Method
A	96.894	5	Varian
B A	85.590	5	Vicam
B A	84.968	5	Rhône
B	72.204	5	Romer

Table 52. SAS analysis of aflatoxin recoveries from spiked Betagro and C.P. feed using four different columns.

concentration (p.p.b.)	% Recovery of aflatoxin							
	Romer		Varian		Vicam		Rhône	
	Betagro	C.P.	Betagro	C.P.	Betagro	C.P.	Betagro	C.P.
10	48.79	65.09	72.32	79.43	60.20	105.35	50.52	63.13
20	52.70	51.96	91.78	82.24	86.44	104.36	56.58	65.25
30	61.33	53.84	93.01	82.86	86.71	71.74	51.56	53.22
40	56.36	54.72	92.46	79.74	76.59	84.01	56.32	51.89
50	55.42	61.18	97.27	79.40	78.95	87.66	58.78	57.25
Average	54.92	57.36	89.37	80.73	77.78	90.62	54.75	58.15
± SD	4.63	5.54	9.77	1.68	10.80	14.27	3.54	12.30

Analysis of Variance

Source	df	Sum of Squares	Mean Square	F-Value	Pr.> F
Model	7	8673.4408575	1239.06297964	19.13	0.0001
Error	32	2072.64344	64.7701075		
Corrected Total	39	10746.0842975			

R-Square = 0.807126 C.V. = 11.42203 Root MSE = 8.04798779

Recovery Mean = 70.46025

Source	df	Anova SS	Mean Square	F-Value	Pr.> F
Method	3	8030.8350275	2676.94500917	41.33	0.0001
Company	1	63.0763225	63.0763225	0.97	0.3311
Method*Company	3	579.5295075	193.17650250	2.98	0.0458

Alpha = 0.05 df = 32 MSE = 64.77011

Number of Mean	2	3	4
Critical Range	7.331	7.705	7.949

Means with the same letter are not significantly different.

Duncan grouping	Mean	N	Method
A	85.051	10	Varian
A	84.201	10	Vicam
B	56.450	10	Rhône
B	56.139	10	Romer

Alpha = 0.05 df = 32 MSE = 64.77011

Number of Mean 2

Critical Range 5.184

Means with the same letter are not significantly different

Duncan grouping	Mean	N	Company
A	71.716	20	C.P.
A	69.205	20	Betagro

Level of method	Level of company	N	% Recovery	
			Mean	± SD
Rhône	Betagro	5	54.752	3.539904
Rhône	C.P.	5	58.148	5.9057785
Romer	Betagro	5	54.92	4.6345712
Romer	C.P.	5	57.358	5.5422306
Varian	Betagro	5	89.368	9.768806
Varian	C.P.	5	80.734	1.6774922
Vicam	Betagro	5	77.778	10.8002532
Vicam	C.P.	5	90.624	14.2711993

Table 53. SAS analysis of aflatoxin recoveries from Betagro and C.P.broiler mixed feeds using an aqueous 60 % methanol extraction compared to a chloroform extraction process.

Aflatoxin level (ppb)	% Recovery of aflatoxin			
	Methanol		Chloroform	
	C.P. feed	Betagro feed	C.P. feed	Betagro feed
10	63.13	50.52	93.70	98.65
20	65.25	56.58	97.30	94.68
30	53.22	51.56	103.66	100.56
40	51.89	56.32	95.85	95.77
50	57.25	58.78	104.98	102.97
Average	58.15	54.75	99.09	98.53
± SD	12.30	3.54	4.95	3.40

Analysis of Variance

Source	df	Sum of Squares	Mean Squares	F Value	Pr > F
Model	3	9002.34522	3000.78174	143.64	0.0001
Error	16	334.25596	20.8909975		
Corrected Total	19	9336.60118			

R-Square = 0.964199 C.V. = 5.887683 Root MSE = 4.57066707

Recovery Means = 77.631

Source	df	Anova SS	Mean Squares	F Value	Pr > F
Method	1	8972.69522	8972.69522	429.5	0.0001
Company	1	19.68128	19.68128	0.94	0.3462
Method * Company	1	9.96872	9.96872	0.48	0.4996

Alpha = 0.05 df = 16 MSE = 20.891

Number of Means 2

Critical Range 4.333

Means with the same letter within the same Duncan grouping are not significantly different.

Duncan grouping	Mean	N	Method
A	98.812	10	Chloroform
B	56.450	10	Methanol

Alpha = 0.05 df = 16 MSE = 20.891
Number of Means 2
Critical Range 4.333

Means with the same letter within the same Duncan grouping are not significantly differrent.

Duncan grouping	Mean	N	Company
A	78.623	10	C.P.
A	76.639	10	Betagro

Level of method	Level of company	N	% Recovery	
			Mean	± SD
Chloroform	Betagro	5	98.526	3.40179805
Chloroform	C.P.	5	99.098	4.95808632
Methanol	Betagro	5	54.752	3.53990395
Methanol	C.P.	5	58.148	5.90577853

Table 54. SAS analysis of aflatoxin levels in feed collected from sacks and bins at C.P.and Betagro farms.

Analysis of Variance

Source	df	Sum of Squares	Mean Square	F-Value	Pr.> F
Model	11	303.23840625	27.56712784	1.11	0.3786
Error	36	890.354025	24.73205625		
Corrected Total	47	1193.59243125			

R-Square = 0.254055 C.V. = 28.26748 Root MSE = 4.97313344

Detected Mean = 17.593125

Source	df	ANOVA SS	Mean Square	F-Value	Pr.> F
Period	2	33.1597125	16.57985625	0.67	0.5178
Type	1	108.45046875	108.45046875	4.39	0.0434
Company	1	12.45421875	12.45421875	0.50	0.4825
Period*Type	2	30.4326375	15.21631875	0.62	0.5461
Period*Company	2	108.0405375	54.02026875	2.18	0.1273
Type*Company	1	0.26551875	0.26551875	0.01	0.9181
Per.*Type*Com.	2	10.4353125	5.21765625	0.21	0.8108

Alpha = 0.05 df = 36 MSE = 24.73206

Number of Mean 2 3

Critical Range 3.566 3.749

Means with the same letter are not significantly different.

Duncan grouping	Mean	N	Period
A	18.394	16	Finisher
A	17.938	16	Grower
A	16.448	16	Starter

Alpha = 0.05 df = 36 MSE = 24.73206
Number of Mean 2
Critical Range 2.912

Duncan grouping	Mean	N	Type
A	19.096	24	bins
B	16.090	24	sacks

Alpha = 0.05 df = 36 MSE = 24.73206
Number of Mean 2
Critical Range 2.912

Duncan grouping	Mean	N	Company
A	18.103	24	C.P.
A	17.084	24	Betagro