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นางสาวชลธิดา บรรเทากุล

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CARRY-OVER RATE OF AFLATOXIN M1 INTO COW MILK DURING EARLY LACTATION PERIOD

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การศึกษาครั้งนี้เป็นการศึกษาหา Carry-over rate of AFM1 ที่ถูกขับทางน้ำนมโดยทำการศึกษาในสัปดาห์ที่ 2 ถึงสัปดาห์ที่ 12 ของการให้นมของโคนมพันธุ์โฮลสไตล์ฟรีเซียนจำนวน 10 ตัวจากฟาร์มโคนมทั้งสิ้น 10 ฟาร์ม โคนมถูกเลี้ยงและกินอาหารตามปกติของแต่ละฟาร์ม เก็บตัวอย่างอาหารโคนมและตัวอย่างน้ำนมดิบจากโคนมแบบรายตัว โดยเก็บตัวอย่างอาหารล่วงหน้า 1 วันก่อนการเก็บน้ำนมดิบ ตัวอย่างน้ำนมดิบเก็บทั้งเช้าและบ่ายของการรีดนม สกัดและแยก aflatoxin จากตัวอย่างอาหารด้วยวิธี column chromatography, thin layer chromatography และวิเคราะห์หาปริมาณโดยใช้ densitometer ที่ความยาวคลื่น 366 นาโนเมตร ผลการวิเคราะห์พบว่าร้อยละ 83 (n=50/60) ของตัวอย่างอาหารข้นมีการปนเปื้อน AFB1 อยู่ในช่วง 0.16 – 42.54 ppb (Mean = 16.23 ppb, SD = 13.16 ppb) สำหรับตัวอย่างอาหารหยาบ ตรวจไม่พบ AFB1 ปนเปื้อนในฟางข้าว (n=0/60) แต่พบว่าร้อยละ 85 (n= 51/60) ของอาหารหยาบอันประกอบด้วยข้าวโพด ซังข้าวโพด เปลือกข้าวโพด แยมสัปปะรด และเปลือกสัปปะรดมีการปนเปื้อน AFB1 อยู่ในช่วง 0.19 – 58.73 ppb (Mean = 4 ppb, SD = 16.77 ppb) จากการศึกษาพบว่าปริมาณ AFB1 ที่ปนเปื้อนในอาหารโคนมซึ่งโคนมกินต่อวันอยู่ในช่วง 3.30 – 1530 ppb (Mean 213.45 ppb, SD = 428.35 ppb, n=60/60) ส่วนตัวอย่างน้ำนมดิบนำมาสกัดและแยก AFM1 ด้วยวิธี Chromatography และวิเคราะห์หาปริมาณโดย High performance liquid chromatography ผลการวิเคราะห์พบว่าร้อยละ 70 (n=42/60) ของตัวอย่างน้ำนมดิบมี AFM1 ปนเปื้อนอยู่ในช่วง 0.014 – 2.463 ppb (Mean =0.692, Median = 0.445 ppb)

การวิจัยครั้งนี้พบว่า อัตราการขับออกของ AFM1 ทางน้ำนมของแม่โคในช่วงแรกของการให้นมเท่ากับ 2.57 $\pm$ 0.47 % (n=4) (Median = 2.69 %).

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : CARRY-OVER RATE / AFLATOXIN B1 / AFLATOXIN M1 / MILK / COWS / EARLY LACTATION PERIOD

CHOLTHIDA BANTAOKUL: CARRY-OVER RATE OF AFLTOXIN M1 INTO COW MILK DURING EARLY LACTATION PERIOD. THESIS ADVISOR: ASST.PROF SUTHEP RUANGWISES, Ph.D., 85 pp.

This study is aimed to study carry-over rate (AFM1 excreted in milk/AFB1 ingested) of AFM1 excreted into cow milk during the 12-week of lactation period in ten Holstein Friesian cows from ten dairy farms. All dairy cows were fed with concentrate and roughage feed naturally given by the farm owners. All samples were collected individually. Feeds were collected one day earlier before the collection of milk samples. Milk samples were collected from both morning and afternoon milking. The feed samples were analyzed for AFB1 by using column chromatography, thin layer chromatography, and densitometer at the wavelength 366 nm. It was found that 83 % (50/60) of concentrate samples contain AFB1 in the range of 0.16 – 42.54 ppb (Mean = 16.23 ppb, SD = 13.16 ppb). All rice straw samples were not found to be contaminated with AFB1; however, approximately 85 % (51/60) of roughage feed samples were contaminated with AFB1 in range of 0.20 – 58.73 ppb (Mean = 4 ppb, SD = 16.77 ppb). The daily dairy feeds contaminated with AFB1 in the range of 3.30 – 1530 ppb (Mean 213.45 ppb, SD = 428.35 ppb, n=60/60). Milk samples were analyzed for AFM1 by using chromatography, and high performance liquid chromatography. We found that 70 % (n= 42/60) of milk samples contaminated with AFM1 in the range of 0.014 and 2.463 ppb (Mean =0.692 ppb, Median = 0.445 ppb).

The carry-over rate of AFM1 into cow milk during lactation period was found to be 2.57 $\pm$ 0.47 % (n=4) (Median = 2.69%).

Department : Veterinary Public Health Student's Signature .....

Field of Study : Veterinary Public Health Advisor's Signature .....

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LIST OF ABBREVIATIONS

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
° C	Degree Celsius
ng	Nanogram (s)
µg	Microgram (s)
mg	Milligram (s)
g	Gram (s)
kg	Kilogram (s)
ml	Milliliter (s)
µl	Microliter (s)
ppb	Part per billion, µg/µl
ppm	Part per million ; µg/ml; mg/ml
nm	Nanometer
cm	Centimeter
%	Percentage
R^2	Coefficient of determination
et al.	et alii, and other
i.e.	id est, such as
e.g.	example gratia, for example
TLC	Thin layer chromatography
HPLC	High Performance Liquid Chromatography
AOAC	Association of Official Analytical Chemists
USP	The United State Pharmacopoeia
Conc.	Concentrate
Std.	Standard
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulfate

EtOH	Ethanol
MeOH	Methanol
$CHCl_3$	Chloroform
CH_2Cl_2	Methylene chloride
UV	Ultra-violet
min	Minute
hr	Hour
rpm	Revolution per minute

CHAPTER I

INTRODUCTION

In 1960, there was an outbreak of mysterious epidemic “Turkey X disease” in the southeast of England. The disease caused the death of more than 100,000 young turkeys and thousands of ducklings (van Egmond, 1989). The outbreak of Turkey X disease led to a multidisciplinary investigation of the cause of the disease, which was later shown to be dietary not infections (Lancaster et al., 1961). Eventually, groundnut imported from Brazil was found to be the cause of the disease. An intensive investigation of the suspect peanut meal was undertaken and it was quickly found that this peanut meal was highly toxic to poultry and ducklings with symptoms typical of Turkey X disease. Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as *Aspergillus flavus* and the toxin was given the name aflatoxin by virtue of its origin (A.flavis--> Afla) (Sargeant et al., 1961).

Aflatoxins are a group of toxic mycotoxins, including some metabolites of aflatoxins, produced mainly by the molds *Aspergillus flavus* and *Aspergillus paraciticus.* These molds contaminate many commodities including, cereal grains, corn gluten, soybean products, peanuts, sunflower seeds, cotton seeds, and palm kernals. Four major forms of aflatoxins occuring in natural environment are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). Aflatoxins also include metabolites of the four natural aflatoxins; aflatoxin M1 (AFM1), aflatoxin M2 (AFM2), aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1) and aflatoxicol. The chemical structures of aflatoxins are illustrated in Figure 1.

AFB1 causes liver damage, liver tumor, and cancer in animals (Purkhiser, 1991). Poultry is considered susceptible to aflatoxin toxicity or aflatoxicosis. The clinical signs of aflatoxicosis in poultry are anorexia, weight loss, lower egg production, compromised egg quality, and other nervous symptoms. In addition, aflatoxicosis in poultry is known to

suppress immunity, increase susceptibility to environmental and microbial stressors, and induce formation of cancer cells (van Egmond, 1993). Aflatoxins can also decrease activities of digestive enzymes and cause malabsorption of nutrients in animals. In swine, it has been reported that aflatoxin toxicity is responsible for decrease in growth rate, poor feed utilization, immunosuppression, toxic hepatitis, nephrosis, and abortion (จักรกริศน์, 1997).

In general, ruminant animals are less susceptible to aflatoxins than non-ruminant animals. The clinical signs of ruminant aflatoxicosis are not as prominent as those of other mono-gastric animals. Nibbelink (1986) reported that dairy cows fed with long term low level of AFB1 contaminated feeds were appeared to loss appetite and reduce feed consumption, conditions which led to weight loss, decrease in growth rate, and decrease in milk production (Masri et al., 1969; Patterson and Anderson, 1982; Pier, 1992). Guthrie (1979) reported that the reproductive system was compromised in dairy cows fed with feeds contaminated with 120μg/L (ppb) of AFB1. In human, AFB1 is carcinogenic, hepatotoxic, immonotoxic and teratogenic agent and is categorized by the International Agency for Research on Cancer (IARC) as group 1 toxin (IARC, 2002).

Several studies proved that aflatoxins contaminated human and animal feeds. In Thailand, aflatoxin contamination in animal feed commodities has always been an inherent problem as Thailand is located in the tropical region. The tropical climate is suitable for the growth of aflatoxin producer; *Aspergillus spp*. Yaowaman et al. (2000) reported that high amounts of AFB1 in the domestically cultivated groundnuts ranging from 200 ppb to 1,500 ppb (เยาวมาลย์และคณะ, 2000). In addition to domestic commodities, aflatoxin contamination was reported in imported raw materials for production of animal feeds (ภัทนีย์, 1997).

For people, aflatoxins can spread into the body by either a direct consumption of agricultural products contaminated with aflatoxins such as peanuts and by an indirect consumption of animal products that contaminated with metabolites and their precursor such as eggs, meats, and milk. Allcroft et al. (1968) found that a dairy cow fed with AFB1 contaminated feeds produce milk contaminated with AFM1, a hydroxylated

metabolite of AFB1. Although, AFM1 toxicity is not as severe as AFB1, it was reported that animals fed with milk contaminated with AFM1 suffered from intestinal carcinoma (Cullen et al., 1987) and AFM1 can cause hepatocarcinoma in animal (Wogan and Paglialunga., 1974; Hsieh et al., 1984). AFM1 is considered to be a possible human carcinogen (group 2B) by IARC (1993). The concern of AFM1 contaminated milk is increasing because milk is a major source of protein for children including infants. Infants are considered more susceptible to AFM1's adverse effects and their capacity to biotransformation of toxic substances is generally slow than adults. Therefore, AFM1 is categorized by IARC (2002) as group 1 toxin, a carcinogenic to human.

AFM1 and it precursor, AFB1, are the most important aflatoxins in dairy industry from dairy cow husbandry to dairy product manufacturing. Several countries have established a maximum residue limits (MRLs) of AFM1 in cow milk and dairy products. For example: United States Food and Drug Administration (U.S.FDA) establishes the MRL of AFM1 in whole milk, skim milk and low fat milk at 0.5 ppb while the European United (E.U.) sets at 0.05 ppb. The Codex Alimentarius Commission tends to establish the MRL of AFM1 in cow milk at 0.5 ppb based on the research data presented by Joint FAO/WHO Export Committee on Food Additive (JECFA, 2001). In Thailand, the MRL of AFM1 in milk and dairy products are not established yet. To enhance the quality of milk and dairy products in Thailand, the regulation should be established to standardize milk quality. Thailand is required to maintain a quality of milk and to regulate the standardization of milk quality. Results from these measures will expand opportunities for Thailand's milk exports to neighboring countries, and reduce the risk of AFM1 contaminated milk consumption of people in the country and abroad.

Although several studies have been done on contamination of AFB1 in feed and AFM1 in cow milk in Thailand, repetition of those studies is useful to explain the effects of the climate change on the toxin contamination. This study was designed to update the recent situation of the carry-over rate (AFM1 excreted in milk/AFB1 ingested) of AFM1 into cow milk during early lactation period. In addition, the results from this study are

useful for Thai food safety authorities when they are required to set up the MRL of AFM1 in milk and milk products.

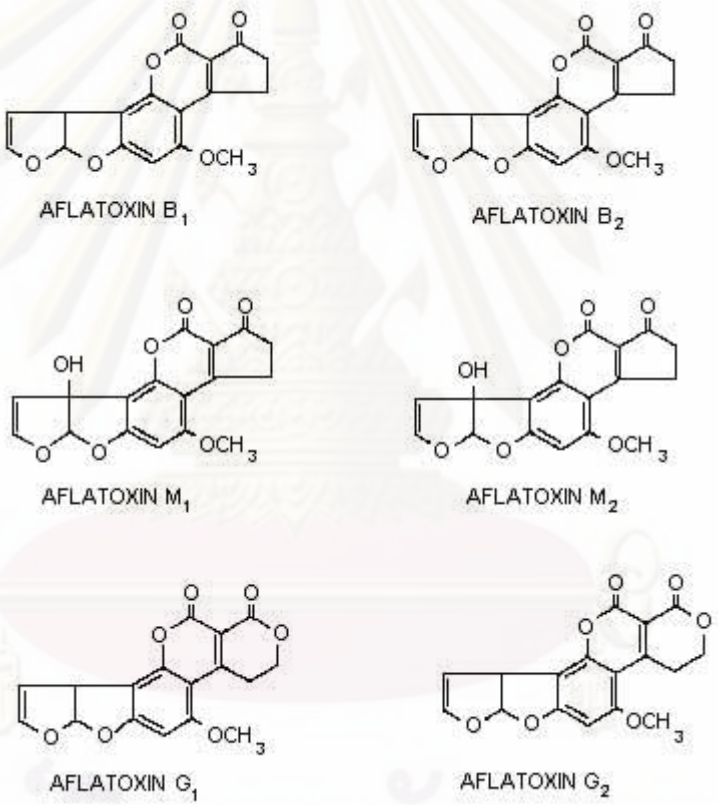


Figure 1 The chemical structures of aflatoxins
(Source:http://www.food-info.net/uk/tox/afla.htm)

CHAPTER II

LITERATURE REVIEWS

2.1 Aflatoxins

Mycotoxins are the toxic chemical substances produced by fungi when humidity and temperature are sufficient. One mold species may produce many different mycotoxins and one mycotoxin may be produced by different mold species. Aflatoxins are mycotoxins produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*. The temperature suitable for these fungi between 12°C and 48°C, but the optimal temperature is 30 to 38°C. Aflatoxin production occurs within 24 to 48 hours under temperature between 20°C and 30°C, with sufficient conditions including a humidity of more than 8%, a relative humidity of more than 65%, pH of 4-5, and availability of oxygen and nutrients (Yoshizawa, 1991; ภัทนีย์, 1997; Coppock et al., 2007; Mahanna, 1999). Because Thailand is located in the tropical region; therefore, Thailand's the tropical climate is suitable for aflatoxin production. The toxin has been found in many feedstuffs. Main sources of aflatoxins in commodities are peanut meal, maize, cottonseed meal, corn gluten, soybean products, sunflower seeds, cotton seeds and palm kernels. Aflatoxins and their metabolite are toxic carcinogenic substance existed in approximately 20 forms (Desphande, 2002) including AFB1, AFB2, AFB2a, AFG1, AFG2, AFM1, AFM2, AFM2a, AFP1, AFQ1, and aflatoxicol. AFM1 and its precursor; AFB1, are the most important aflatoxins in dairy industry.

Aflatoxin is dihydrofuran moieties fused with a coumarin ring resulting in a new chemical structure called a difuranocoumarin (D'Mello & MacDonald, 1997). Based on the chemical structure, aflatoxins are classified into two groups: the difurocoumarocyclopentanone series including AFB1, AFB2, AFB2a, AFM1, AFM2, AFM2a, and aflatoxicol; and the difurocoumarolactone series including AFG1 and AFG2. These two aflatoxin structures are shown in Figure 2.

Their chemical structures of aflatoxins determine the toxicity levels (Wogan, 1966). The difurocoumarocyclopentanone series is more toxic compared to the difurocoumarolactone series. Aflatoxin with double bonds at the first furan ring is more toxic than that with a single bond. The order of toxicity level is AFB1 > AFG1 > AFB2 > AFG2 (Wogan et al., 1974).

Aflatoxins were disintegrated gradually by sunlight, ultraviolent light, and gamma ray. A melting point of alflatoxins is high. Temperature below 250 □C including normal cooking temperature from boiling, baking, and steaming does not destroy aflatoxins (Ellis et al, 1991). Aflatoxins dissolve in organic solvent such as chloroform, benzene, acetone, ethanol, and methanol but merely dissolve in water. They do not dissolve in hexane, ether, and petroleum.

The fluorescence on Thin Layer Chromatography (TLC) at wavelength 365 - 366 nm ultraviolet light determines the groups of aflatoxins (Sargeant et al., 1961; Wogan, 1966; Deshphande, 2002). A blue fluorescence is the B group; including AFB1 and AFB2, and a Yellowish green fluorescence is the G group; including AFG1 and AFG2. The intensity of the fluorescence is direct proportion with aflatoxins concentration; therefore, the fluorescence is used to the screening test and the quantitative analysis of some aflatoxins.

The International Agency for Research on Cancer (IARC) of WHO classified all agents by evidence of carcinogenicity to 4 groups such as Group 1; the agent is carcinogenic to humans, Group 2A; the agent is probably carcinogenic to humans, Group 2B; the agent is possibly carcinogenic to humans, Group 3 ; the agent is not classifiable as to its carcinogenicity to humans, and Group 4 ; the agent is probably not carcinogenic to humans. The naturally occurring aflatoxins were declared as Group 1 human carcinogen in 1987 by IARC.

2.2 Aflatoxin B1 (AFB1)

A chemical structure of AFB1 is shown in Figure 2. Properties of AFB1 include a relative molecular mass of 312 DA, a melting point of 268°C - 269°C (Applebaum et al., 1982), and fluorescence in ultraviolet light (ca. 365 nm) of blue. AFB1 is a potent liver carcinogen and DNA-damaging agent. It is also hepatogenic, mutagenic and teratogenic and causes immunosuppression in animals and human. IARC concluded in 1993, that AFB1 is in group 1 toxin; the most toxic carcinogen in human and animals.

2.3 Aflatoxin M1 (AFM1)

AFM1 is a 4-hydroxy derivative of AFB1. The chemical structure of AFM1 is shown in Figure 1. AFM1 (CAS No. 6795-23-9), (a chemical formula- $C_{17}H_{12}O_7$), has a relative molecular mass of 328 DA and a melting point of 299°C (Henry et al., 2001). Several heating treatments cannot fully destroy AFM1. (Munksgaard et al., 1987). AFM1 can occurs in urine, tissue, and milk from animal consuming feed contaminated with AFB1 (Applebaum et al., 1982). In addition to, AFM1 is also found in milk of lactating mother that eating foodstuffs contaminated with AFB1 (Sadeghi et al., 2009). Human exposure to AFM1 occurs mainly through consumption of aflatoxin-contaminated milk and milk products including human breast milk is a serious problem for public health. Although, toxicity of AFM1 is less than AFB1, its cytotoxic, genotoxic, and carcinogenic effect is well demonstrated (I.F.H. Purchase, 1967; Green et al., 1982 and Neal et al., 1998). Hence, the IARC of WHO initially categorized AFM1 as a group 2B human possibly carcinogen (IARC, 1993), but IARC has transferred AFM1 as group 1 human carcinogen according to the recent investigations (IARC, 2002).

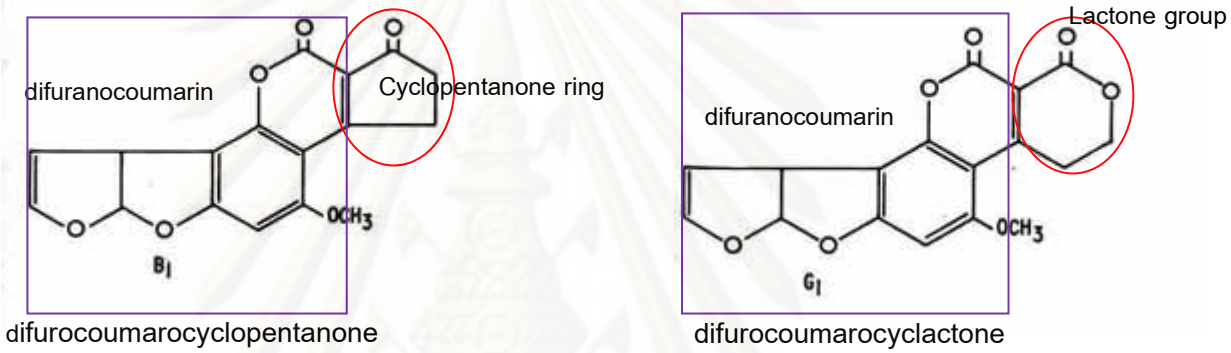


Figure 2 Structure of the difurocoumarocyclopentanone & difurocoumarolactone series of aflatoxins; Adapted from (IARC, 2002)

2.4 Toxicokinetics of aflatoxins in animals

Following ingestion of aflatoxins contaminated feeds; a part of AFB1 is degraded in rumen with less than 10%, resulting in the formation of aflatoxicol (Westlake et al., 1989; Jouany et al., 2009; review Fink-Gremmels, 2008). Formation of aflatoxicol has been observed (Auerbach et al., 1998; Jouany et al., 2009). Many ruminal bacteria are completely inhibited by concentrations AFB1 below 10μg/ml; therefore, digestive and fermentative functions of the rumen microbial ecosystem can be disturbed by aflatoxins (Jouany et al., 2009).

The remaining of aflatoxins is absorbed in the gastrointestinal tract by passive diffusion and is transferred from intestine to hepatic portal blood (Hsieh and Wong, 1994). Very little aflatoxins appear to be transported to the lymphatic systems (Kumagai, 1989).

An experiment that fed cattle with a single dose of aflatoxins in form of gelatin capsules and blood samples were collected from jugular vein periodically subsequent to aflatoxins administration revealed that AFB1 and AFM1 can be observed in the venous blood 30 minutes after dosing with maximum level of the toxins found 4 to 8 hours after dosing. These finding suggested that aflatoxins are rapidly absorbed in the rumen. AFM1 in blood samples reached to maximum level later than AFB1 while the maximum quantity of AFB1 was higher than AFM1 (Cook et al., 1986). Young animals are found to be absorbing aflatoxins more than older animals.

The most important organ for biotransformation of aflatoxins is the liver and it can also occur in the kidney and intestinal tract. The biotransformation of AFB1 involves two reaction phase. The first phase includes reductive, oxidative and hydrolytic reactions. Microsomal cytochrome P450 has a key role in the biotransformation of AFB1 to AFB1-8, 9-epoxide which adheres to DNA, RNA and protein, damages of some protein syntheses, and causes acute and chronic toxicity and hepatocarcinoma (Swenson et al., 1997; Ueno, 1983). The second phase involves conjugating reactions applied on the products of the first phase. These reactions decrease the toxicity of toxins and increase

their solubility in water for excretion out of body. Other metabolite of AFB1 include AFP1, AFM1, AFB2a, AFQ1, Aflatoxicol, Aflatoxicol M1, Aflatoxicol H1, AFM1-P1, AFB1-8,9-epoxide, AFB1-8,9-dihydrdiol (Hendrick, 1994) as shown in Figure 3.

Aflatoxins are excreted in milk, eggs, urine, semen, bile, and feces. AFB1 was excreted mostly as conjugated metabolites in bile to feces followed by urine (Shank and Wogan, 1965).AFM1 is conjugated to glucuronic acid and subsequently excreted via bile, or enters the systemic circulation. The circulating AFM1 can be excreted in the urine or appear in milk. AFM1 is detected in cow milk within 12 to 48 hours after ingestion; approximately 90% of AFM1 excretion occurred within 12 hours after consumption of AFB1 (Applebaum et al., 1982; Frobish et al., 1986; Bingham et al., 2004). AFM1 is either conjugated to glucoronic Coppock and Christian (2007) reported that aflatoxins in milk disappeared within 24 to 72 hours after all the aflatoxins had been removed from the diet (230µg AFB1/cow/day). The stage of lactation influences excretion of aflatoxins (Veldman et al., 1992). This is supported by findings that the percentage of aflaoxins excreted in milk is positively correlated with milk yield (Frobish et al., 1986) Aflatoxins are stable in milk and associated with in milk protein. Approximately 75% of aflatoxins in milk are found in the casein (protein) fraction and 25% in the whey fraction of milk (Govaris et al., 2002). Only 11% to 25% of aflatoxins are destroyed in raw milk after storage at 5˚C for 1 to 3 day (Govaris et al., 2002).

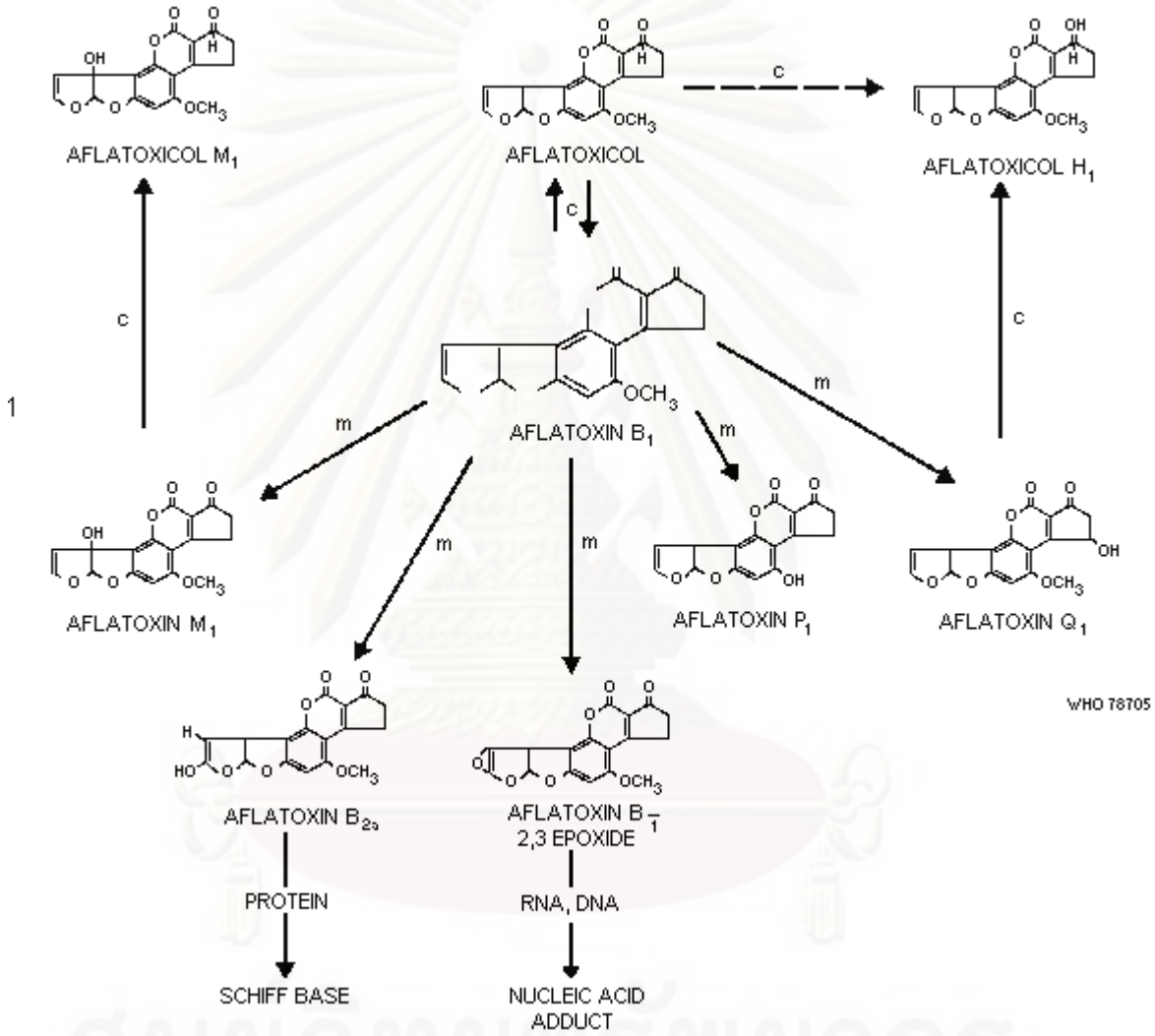


Figure 3 The AFB1 metabolism in liver (EHC11, 1979)

Source: http://www.inchem.org/documents/ehc/ehc/ehc011. htm

2.5 Carry-over rate of AFM1 into cow milk

The ratio between amount of AFM1 excreted into cow milk and amount of AFB1 that cow ingested per day is described as a “carry-over rate”. Patterson et al. (1980) reported that approximately 2.2% of ingested AFB1 appeared in the milk daily as a metabolite form, AFM1. Veldman et al. (1992) reported that the carry-over rate of aflatoxin was proportionately 0.062 and 0.018 for cows in early and late lactation respectively, and concluded that both milk yield and individual liver metabolism have an effect on the carry-over rate. Dragacci et al. (1995) reported that amount of AFM1 excreted into cow milk is a positively correlated with amount of AFB1 that cow ingested. Suthep and Benjamas (1996) found that the carry-over rate of AFM1 during the 2^{nd} to 4^{th} weeks of lactation was in range of 1.3 - 2.7% and decrease respectively and during the 34^{th} to 36^{th} weeks of lactation the carry-over rate of AFM1 was in range of 0.6 - 0.8 % of AFB1 ingested (สุเทพและเบญจมาศ, 1996). These results hold the conclusion that the stage of lactation influences to the carry-over rate (Munksgaard et al., 1987; Patterson et al., 1989; Veldman et al., 1992; สุเทพและเบญจมาศ., 1996). The average carry-over rate during the early lactation (2^{th} to 6^{th} week) of the ten cows was 2.00 ± 0.7 % (Suthatip, 1997). In dairy cows the amount of AFM1 excreted into milk could be up to 3% of the AFB1 intake (Diaz et al., 2004). JECFA (2001) reported that the carry-over rate was in a wild range of 0.3 – 6.2%.

Milk yield is the major factor affecting the total excretion of AFM1 (Patterson et al., 1989; Veldman et al., 1992; Suthatip, 1997). It is reported that the somatic cell count dose not relates to the AFM1 carry-over rate (Masoero et al, 2007). Other factors that affect carry-over rate of AFM1 into milk include species (Battacone et al., 2003), animal variability, nutritional and physiological factors (Van Egmond, 1989; Veldman et al., 1992; Fink-Gremmels J, 2008).

2.6 The legal regulations for AFM1 in milk and dairy products

Regulation of AFM1 in milk varies from country to country. United States Food and Drug Administration (US FDA) and Codex Alimentarius prescribe that the maximum level of AFM1 in milk and dairy products should not exceed 0.5 μg/L (ppb) (Van Egmond, 1989; Codex Alimentarius Commission, 2001), while AFM1 in liquid milk and dried or processed milk products should not be higher than 0.05 ppb for European Union regulation (European Commission, 2006). Furthermore in Austria and Switzerland, the maximum level for infant food commodities is reduced further to 0.01 ppb (FAO, 1997). Germany also sets the maximum level of AFM1 in milk not to exceed 0.01 ppb. In Asia, Korea Food and Drug administration prescribes that the maximum level for AFM1 in milk should not exceed 0.5 ppb (Korea Food and Drug administration, 2003). The maximum tolerance limit accepted by Turkish Food Codex is 0.05 ppb (Bakirci, 2001).

In 2001, JECFA reported that there were no statistic difference of the risk of cancer occurrence in human consuming milk contaminated AFM1 at level 0.05 ppb and 0.5 ppb. The Codex Alimentarius Commission adopts such findings that can be control to stay in this reality and also reduce the cost of control costs. Therefore, the Codex Alimentarius Commission establishes further the Maximum Residue Limits (MRL) of AFM1 in raw milk at level 0.5 ppb. However, the EU committees have against with this reason that AFM1 is carcinogenic agent when consuming will increases the risk of cancer. The EU committees believe that the level of AFM1 contamination should be minimized. However, many countries including Iran (Kamkar, 2005) and Thailand have no legal limits for AFM1 in milk and dairy products.

2.7 The legal regulations for AFB1 in feed

US FDA and EC regulation the AFB1 contaminated dairy feeds should not exceed 20 and 10 ppb respectively (Patterson, 1989). Germany regulates the AFB1 contaminated dairy feeds not to exceed 5 ppb (Eberhardt, 1991). The committee of Codex Alimentarius Commission in Food additive and Contaminants concludes that for

the developed countries the contamination of AFB1 in lactating animal feeds and feeds should not exceed 5 and 50 ppb, respectively. In Thailand, the contamination of AFB1 in feeds is limited as shown in Table 1.

Table 1 The contaminated AFB1 level in raw materials and feeds.

Type	Permissible AFB1 (µg/kg; ppb)
Soy bean meal	< 50
Pea nut meal	< 500
Rice bran and rice bran meal and rice bran related oil	< 50
Corn and corn meal	< 100
Base mixing feed in cattle	< 100
Pellet feed in cow age < 1 year	< 100
Pellet feed in cow age > 1 year	< 200

Source: กระทรวงเกษตรและสหกรณ์ (1995)

2.8 Analytical Procedure of aflatoxins

The method for analysis of aflatoxins should be suitable and reliable. The most popular and acceptable methods for analysis of aflatoxins were divided in 3 methods (Mary and Garnett, 1994) as follows;

1. Thin-Layer Chromatography (TLC)

Association of Official Analytical Chemists; AOAC (1990b) sets TLC as a standard method for quantifying AFB1, AFB2, AFG1 and AFG2. However this method is not appropriate to detect AFM1 in milk because of its low detection level of AFM1.

2. High Performance Liquid Chromatography (HPLC)

HPLC is a column chromatography technique used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column's stationary phase and a fluorescence detector that provides a characteristic retention time for the analyze. HPLC is the standard quantitative method for AFM1 at wavelength 365 – 455 nm because it has high accuracy and precision.

3. Immunochemical method

Principle of this method is the specific matching of Monoclonal or Polyclonal antibodies with antigen (AFM1). Three major types of immunochemical method include Radioimmunoassay (RIA), Enzyme – Like Immunosorbent Assay (ELISA) and Immunoaffinity Colum High Performance Liquid Chromatography (IAC-HPLC)

2.9 The relation of AFM1 excreted into cow milk and contaminated AFB1 feed ingested

Munksgaard et al (1987) reported relation by equation;

$$\text{AFM1 (ng/kg milk)} = 1.24\ \text{AFB1}^{0.834}\ \text{(ug/day)}$$

Veldman et al (1992) reported relation by equation;

$$\text{AFM1 (ng/kg milk)} = 1.19\ \text{AFB1 (ug/day)} - 1.9$$

Suthep and Benjamas (1996) reported relation by equation (สุเทพและเบญจมาศ, 1996);

$$\text{AFM1 (ug/kg milk)} = 0.02\text{AFB1 (ug/day)} \pm 0.007$$

And

$$\text{AFM1 (ug/kg milk)} = 0.007\text{AFB1 (ug/day)} \pm 0.001$$

CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Lactating dairy cows

Ten lactating Holstein Friesian cows, one cow from each of ten farms, were used in this study. Four farms located in Phetchaburi and six farms were in Ratchaburi. These cows calved during January to February 2009 and were fed and milked twice daily.

3.2 Raw milk samples

Raw milk samples were first collected at the second week postpartum and then every two week throughout the Week twelve postpartum durations. Milk samples of individual cow were collected in 180-ml bottle twice a day in during the routine morning and afternoon milking. Milk samples were freezed at refrigerator. During transportation, the milk samples were stored at 4 □C in the foam boxes with ice until they reached to the laboratory. The quantities of milk from individual cows were documented for each milking daily by dairy farmers.

3.3 Feed samples

Feed used by the ten dairy farms was classified into two types; feed concentrate and roughage. Eight out of ten farms used commercial concentrate pellets feeds purchased from a cooperative milk cow society while the remaining two farms used concentrate homemade mixed feeds. Roughage feeds varied from farm to farm and included pineapple flesh, pineapple shell, maize, shell corn, cob, grass and rice straw. Unlike other roughage feeds, rice straws were fed *ad libitum*. Therefore, in this study, rice straws were collected separately from other roughage feed samples. Raw material used in dairy cow feed ingredients in each farms are presented in Appendix A.

Five hundred grams of concentrate and roughage feed samples and rice straws were collected on a day ahead of milk sample collections. Feed samples were stored inside the refrigerator. During transportation, the samples were stored at 4 □C in the foam boxes with ice until they reached the laboratory. The amounts of concentrate and roughage feeds that cows ingested in each meal were recorded everyday by dairy farmers.

3.4 Milk samples preparation for analysis.

Both of the morning and afternoon milk samples for individual cows collected on the same day were combined in a proportion of morning milk yield to evening milk yield to represent a sample from a total of milk production in that day. Pooled milk samples were stored in a 180-ml bottle at – 20 □C until these samples were analyzed for AFM1

3.5 Feed samples preparation for analysis.

The concentrate feed samples for individual cows collected on the same day were combined and the roughage feed samples were mixed too. For rice straw samples were cut into small pieces. Three hundred grams of each pooled concentrate and roughage feed samples and one hundred grams of rice straw sample were stored in the zip-locked bags and were kept at – 20 □C until these samples were analyzed for AFB1

3.6 Chemicals and reagents

3.6.1 Standard AFB1

3.6.2 Standard AFM1

3.6.3 Silica gel

3.6.4 Celite, AR grade (Fluka chemika, Germay)

3.6.5 Sodium sulphate (Na_2SO_4), AR grade (Merck, USA.)

3.6.6 Trifluoroacetic acid (Sigma-aldrich, USA.)

3.6.7 Chloroform ($CHCl_3$), AR grade (Lab-scan, USA.)

3.6.8 Hexane (CH_3 $(CH_2)_4CH_3$), AR grade (Mallinkrodt, USA.)

3.6.9 Ether ($(C_2H_5)_2O$), purified (J.T.Baker, USA.)

3.6.10 Acetonitrile (CH_3CN), HPLC grade (J.T.Baker, USA. & Lab-scan, USA.)

3.6.11 Acetone (CH_3COCH_3), AR grade (Lab-scan, USA.)

3.6.12 Methanol (CH_3OH), AR & HPLC grade (Lab-scan, USA.)

3.6.13 Methanol, Proanalysi (Merck, USA.)

3.6.14 Benzene (C_6H_6), AR grade (AJAX laboratory chemical, USA.)

3.6.15 Methylene chloride, HPLC grade (Lab-scan, USA.)

3.6.16 Ethanol, HPLC grade (Lab-scan, USA.)

3.6.17 Isopropanol, HPLC grade (Lab-scan, USA.)

3.6.18 Water, Chromatography (Merck, USA.)

3.6.19 Distilled water

3.7 Apparatus

3.7.1 40,100,250 and 500 ml Beakers

3.7.1 20, 200 and 1000 µl Micropipette and micropipette tips

3.7.2 500 ml Erlenmeyer flask

3.7.3 25 and 100 ml Volumetric flasks

3.7.4 Filter paper (Whatman ® No.1)

3.7.5 Vacuum flask

3.7.6 10 and 50 ml Syringes

3.7.7 Glass funnel

3.7.8 Dropper

3.7.9 10 ml Polypropylene Column

3.7.10 15 ml Test tube

3.7.11 3 ml Vial tube

3.7.12 500 ml Duran flask

3.7.13 10,100 and 500 ml Cylinders

3.7.14 HPLC column: HiQSilC18w size 4.6 nm o *250mm; KYA Technologies Corporation, Japan

3.7.15 C18 Sep-Pak® Cartrilages, Water Corporation Milford, Mass U.S.A.

3.7.16 Target, Syringe filters; 17 mm Nylon 0.45 um, National Scientific Company

3.7.17 2 ml Clean Std open screw tread vial, National Scientific Company

3.8 Instruments

3.8.1 TLC applier (CAMAG Linomat5) : CAMAG, Switzerland

3.8.2 Densitometer (CAMAG TLC SCANNER3) : CAMAG, Switzerland

3.8.3 Long wave UV lamp (CAMAG UV-cabinet II) : CAMAG, Switzerland

3.8.4 TLC tank (CAMAG) : CAMAG, Switzerland

3.8.5 TLC plate silica gel 60 without fluorescence 20*20 cm pores 60 A: Merck, Germany. Cut plates measuring 10 * 10 cm from the TLC plates

3.8.6 Sonicator

3.8.7 Vortex Mixer

3.8.8 Water stream

3.8.9 A tank of Nitrogen gas

3.8.10 A waterbath

3.8.11 Dessicator

3.8.12 Electronic balance (Mettler PJ 3000 & Mettler AE 160) : Mettler, Switzerland

3.8.13 Suction pump (GAST, Model No. 1HAE25M104, Serial No.0494)

3.8.14 Shaker (Flask shaker SF1) : STUART scientific, Great Britain

3.8.15 Micropipette (Pipetman) : GILSON, France

3.8.16 Plunger pipette

3.8.17 High Performance Liquid Chromatography with Fluorescence detector composed of Shimadzu DGU-12A degasser, Shimadzu LC-10AD liquid chromatography Shimadzu RF-10Axl Fluorescence detector, Shimadzu CTO-10A Column oven, Shimadzu CBM-10A Communication's bus module and Shimadzu SIL-10A Auto injection

Methods

This experiment was divided into 3 parts;

Part 1: Analysis of AFB1 in concentrate and roughage feed was conducted using the AOAC Official Method 968.22.

Part 2: Analysis of AFM1 in raw milks was performed by using the AOAC Official Method 986.16.

Part 3: Calculation of the carry-over rate of AFM1 into cow milk.

Part 1 Analysis of AFB1 in concentrate and roughage feed

1. **Analysis of AFB1 in concentrated and roughage feed was conducted by using the AOAC Official Method 968.22** as shown briefly in Figure 4. The detail of the method was shown in Appendix B. Apparatus of extraction and purification of AFB1 in dairy cow feeds were shown in Figure 5. Apparatus of Thin Layer Liquid Chromatography (TLC) were shown in Figure 6.

2. **A quantitative analysis of AFB1 in feed sample by using the standard AFB1.**

   2.1 The area under the peak (AUP) and amount of standard AFB1 (ng) were plotted by using function ***fx*** = LINEST on Microsoft Excel® for the standard curve of AFB1 (ธีรศักดิ์ โรจนธาดา, 2008). The appropriate equation was Y = m X + b

   Where: Y = Area under the peak (AUP)
   X = amount of AFB1 (ng/spot)
   m = coefficient of linear regression
   b = point cut at axis Y

2.2 The AUP of feed sample was represented as the value Y in the equation of standard curve. The value X that calculated was the amount of AFB1 was detected by densitometer (ng).

2.3 The value X was represent in the value **b** in this equation; **a** = 60**b**,

Where: **a** = concentration of AFB1 was detected in feed sample (ppb)

b = amount of AFB1 was detected by densitometer calculated from STD curve (ng)

So, the concentration of AFB1 in feed sample was 60**b** ng/g (ppb). The principles of this calculation were shown in Appendix C.

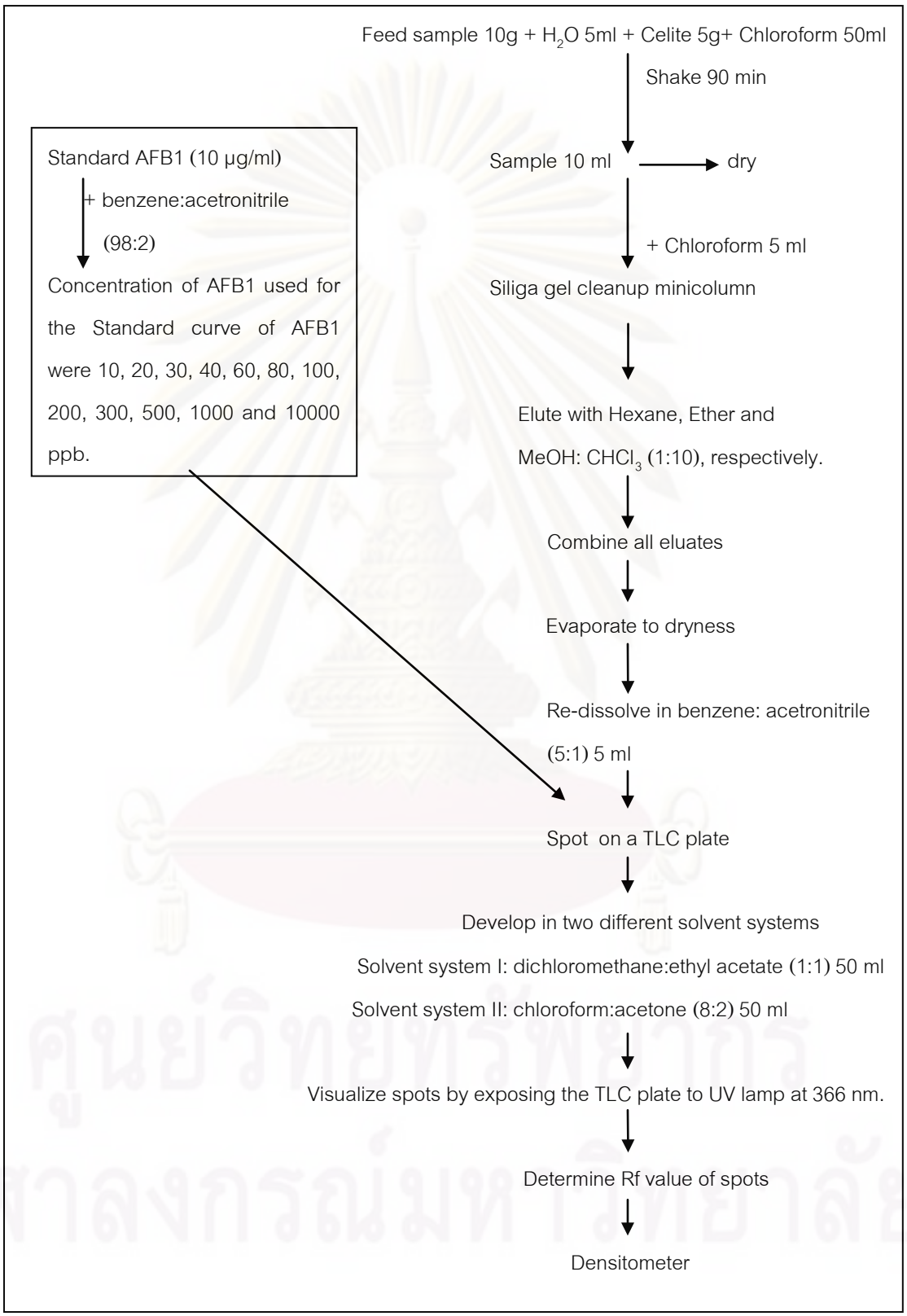

Feed sample 10g + H_2O 5ml + Celite 5g+ Chloroform 50ml
Shake 90 min
Sample 10 ml
dry
+ Chloroform 5 ml
Siliga gel cleanup minicolumn
Elute with Hexane, Ether and
MeOH: $CHCl_3$ (1:10), respectively.
Combine all eluates
Evaporate to dryness
Re-dissolve in benzene: acetronitrile
(5:1) 5 ml
Spot on a TLC plate
Develop in two different solvent systems
Solvent system I: dichloromethane:ethyl acetate (1:1) 50 ml
Solvent system II: chloroform:acetone (8:2) 50 ml
Visualize spots by exposing the TLC plate to UV lamp at 366 nm.
Determine Rf value of spots
Densitometer
Standard AFB1 (10 µg/ml)
+ benzene:acetronitrile
(98:2)
Concentration of AFB1 used for the Standard curve of AFB1 were 10, 20, 30, 40, 60, 80, 100, 200, 300, 500, 1000 and 10000 ppb.

Figure 4 Analysis of AFB1 in concentrate and roughage feed was conducted by using the AOAC Official Method 968.22.

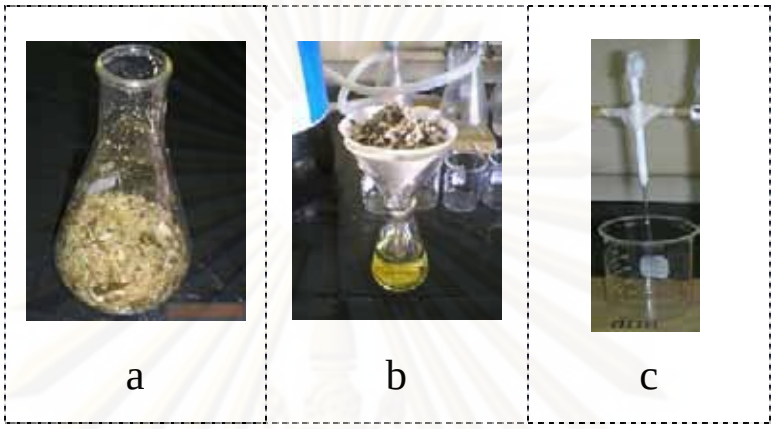


Figure 5 Apparatus of extraction and purification of AFB1 in dairy cow feed.
a. Feed sample b. Filtration of chloroform extract c. Purification of AFB1

Figure 6 Apparatus of Thin layer Liquid Chromatography (TLC)

Part 2 Analysis of AFM1 in raw milk

1. **Analysis of AFM1 in raw milk was performed by using the AOAC Official Method 986.16** as shown briefly in Figure 7. The detail of this analysis was shown in Appendix D. The apparatus of extraction of AFM1 in milk, purification and clean up of milk extracts and High Performance Liquid Chromatography (HPLC) were shown in Figure 8, 9, and 10 respectively.

2. **A quantitative analysis of AFB1 in feed sample by using the standard AFB1.**

   2.1 The AUP and amount of AFM1 derivatized with trifluoroacetic acid of the standard AFM1 were plotted for the standard curve. The standard curve of standard AFM1 was investigated linear relationship by using function ***fx*** = LINEST on Microsoft Excel®. The equation shows the relation is (linear regression); Y = m X +b,

   Where:

Y	=	Area under the peak (AUP)
X	=	amount of AFB1 (ng/spot)
m	=	coefficient of linear regression
b	=	point cut at axis Y

   2.2 The AUP of AFM1 derivatized with trifluoroacetic acid of extracted milk samples was represented as the value Y in the linear regression;

   Y = m X + b.

   2.3 The value X was the amount of derivatized AFM1 found in milk sample (ng/spot) and was used to compute AFM1 in a 20-ml milk sample by replacing **X** in the equation, **m** = 20 **n**, where:

   m = the amount of AFM1 in a 20-ml milk sample (ng)

   n = the amount of AFM1 was detected by HPLC.

This implies that the amount of AFM1 in a 1-ml sample was **n** ng/ml.

The principles of this calculation were shown in Appendix E.

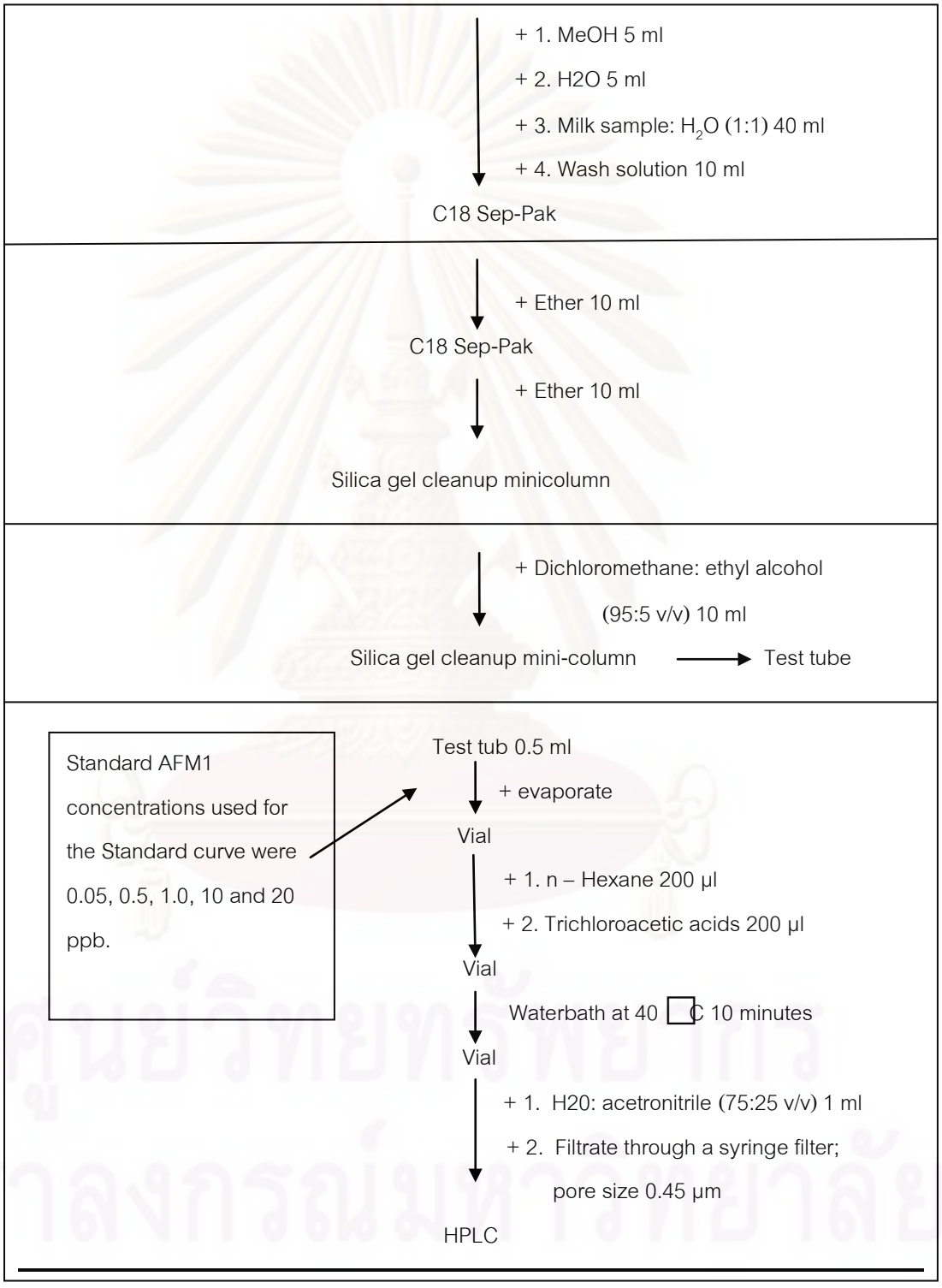


Figure 7 Analysis of AFM1 in milk sample was conducted by using

The AOAC Official Method 986.16

Figure 8 Apparatus for extraction of AFM1 in milk

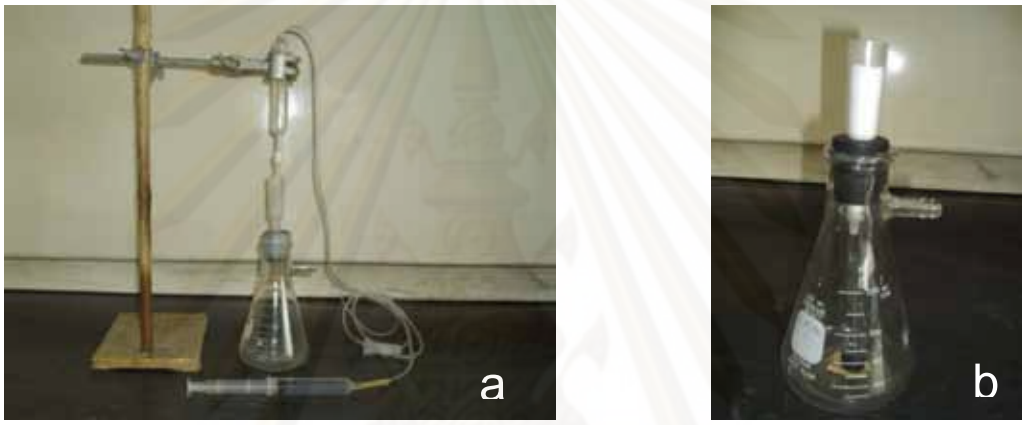


Figure 9 Apparatus for purification and cleanup of milk extracts
a. Purification of AFM1 b. Cleanup of milk extracts

Figure 10 High Performance Liquid Chromatography (HPLC)

Part 3 Calculation of Carry-over rate of AFM1 into milk

The carry-over rate was the ratio of the daily amount of AFM1 excreted into milk and the daily amount of AFB1 ingested. The carry-over rates were calculated by using the following equation (สุเทพและเบญจมาศ, 1996);

$$\text{Carry-over rate} = \frac{\{(Vm * Cmm) + (Va * Cma)\}}{\{(Fc * Cfc) + (Fr * Cfr)\}} * 100$$

In case of the morning milk samples were mixed with the afternoon milk samples, the carry-over rate were calculated using the following equation;

$$\text{Carry-over rate} = \frac{(Vm + Va) * Cm}{[(Fc * Cfc) + (Fr * Cfr)]} * 100$$

Where:

Vm	=	amount of morning cow milk (kg)
Va	=	amount of afternoon cow milk (kg)
Cm	=	concentration of AFM1 in daily cow milk (µg/kg)
Cmm	=	concentration of AFM1 in morning cow milk (µg/kg)
Cma	=	concentration of AFM1 in afternoon cow milk (µg/kg)
Fc	=	weight of concentrated feed that a cow intakes per day (kg)
Cfc	=	concentration of AFB1 in concentrated feed that a cow intakes per day (µg/kg)
Fr	=	weight of roughage feed that a cow intakes per day (kg)
Cfr	=	concentration of AFB1 in roughage feed that a cow intakes per day (µg/kg)

CHAPTER IV

RESULTS

4.1 Feeding for dairy cows

Feeding of dairy cows in each farm has similar concepts. Both of concentrate and roughage feeds were restricted. Even though the amount of concentrate feed was limited throughout the lactation period, it was increased as dairy cows produced high milk yield during early lactation period as shown in Figure 11. The average daily intake of concentrate feeds was 8.21 $\pm$ 3.89 kg (Median = 6.20 kg, Min. = 3 kg, Max. = 18 kg).

In order to maintain milk production, the amount of concentrate feeds was given to individual cows based on the milk production in the previous day, approximately 0.5 kg of concentrate feed per 1 kg of milk production. In this study, for a kilogram of milk yield, the average daily concentrate feeds intake of individual dairy cows was 0.47 $\pm$ 0.16 kg (median = 0.47 kg, mode = 0.54 kg, min = 0.21 kg and max =0.86 kg).

Roughage feeds consisted of pineapple flesh, pineapple shell, maize, shell corn, cob, grass and rice straw as shown in Figure 12. Unlike other roughage feeds that were fed limitedly, rice straws were fed *ad libitum.* Therefore, rice straw which cows ingested was not used to calculation and was collected separately from other roughage feed samples. The average intake of roughage feed was 38.20 $\pm$ 14.62 kg (Median = 40 kg, Mode = 20 kg, Maximum = 62 kg, Minimum = 20 kg). These roughage feeds of ten dairy cows fed were classified by using or un-using of a pineapple in roughage feed. Five dairy farms used roughage feeds composed of pineapple. The average roughage feed intakes were shown in Table 2. The dairy cow feed ingredients in each farms were shown in Appendix A.

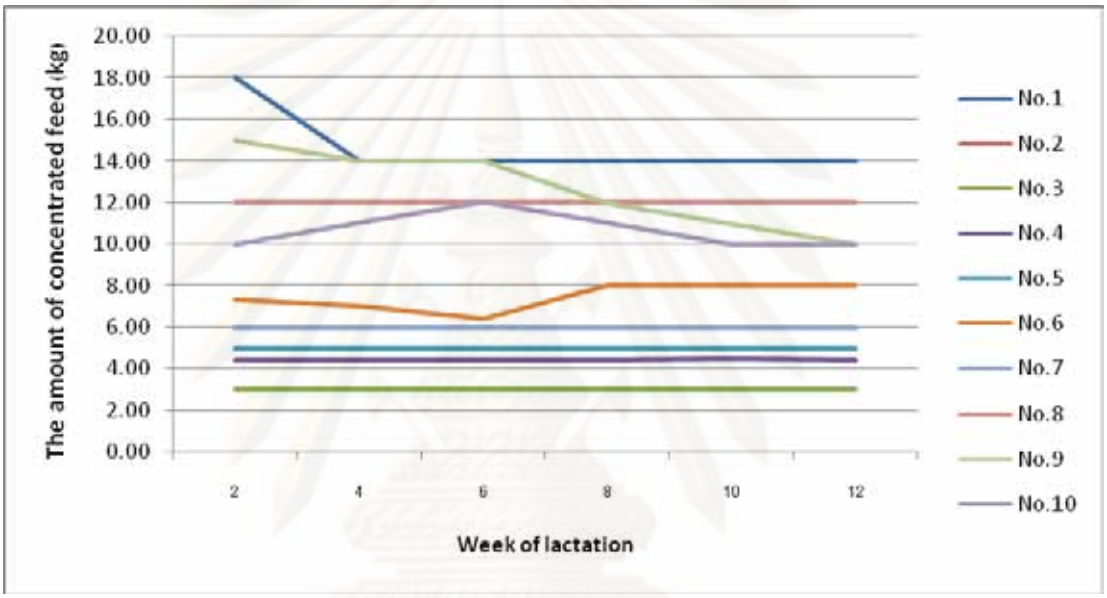


Figure11 The amount of concentrate feed was given to individual dairy cow during the 2th to 12th week of lactation

Table 2 The average daily intake of roughage feed

Statistic value	Roughage feed (kg) (n = 60)	Roughage feed (kg) (n = 60)	
		With pineapple (n = 30)	Without pineapple (n = 30)
Mean	38.20	26.00	50.40
Median	40.00	20.00	50.00
Mode	20.00	20.00	40.00
Max.	62.00	40.00	62.00
Min.	20.00	20.00	40.00
Standard deviation	14.62	8.14	7.79

Figure 12 Types of roughage feed that these dairy cows fed in this study a. pineapple flesh, b. pineapple shell, c. maize, d. shell corn, e. cob, f. grass and g. rice straws

4.2 Milk yield of dairy cows

The average of milk yield of total ten dairy cow is 17.17 $\pm$ 4.83 kg/day (median and mode = 16 kg). There are two patterns of milk yield observed in this study as shown in Figure 13. The first pattern (n=7) is the decrease in milk yield during the observation period (12 week after calving). The second pattern (n=3) is the increase in milk yield during the observation period. The milk yield of the first pattern averages 16.61 $\pm$ 4.97 kg (Mode= 15kg, Median=15.5kg), whereas the milk yield of the second pattern was 18.47 $\pm$ 4.34 kg (Mode= 18kg, Median=18kg),

4.3 Analysis of AFB1 in dairy cow feed

The recoveries of AFB1, as described by detected quantity of the known standard to actual quantity of such standard, in each study, were in a range of 60-75%, with an average of 70%. The standard AFB1 was compared against the extracts from feed samples in the developed TLC plate under the fluorescence and the retardation factor (Rf) using the long wave UV lamp at 365 - 366 nm ultraviolet light. If the extracts from feed samples were contaminated with AFB1, the blue fluorescence would appear at the same location on the TLC plate as shown in Figure 14. Subsequently, the TLC plate was scanned using the densitometer for quantitative analysis of AFB1. The TLC chromatograms of the standard AFB1 and AFB1 extracted from feed samples were shown in Figure 15. The Rf value of standard AFB1 from this study was approximately 0.38. Standard AFB1 concentrations used for standard curve were 10, 20, 30, 40, 60, 80, 100, 200, 300, 500, 1000 and 10000 ppb as shown in Figure 16. The least of amount of AFB1 fluorescence blue was 0.2 ng spot^{-1} as shown in Figure 17.

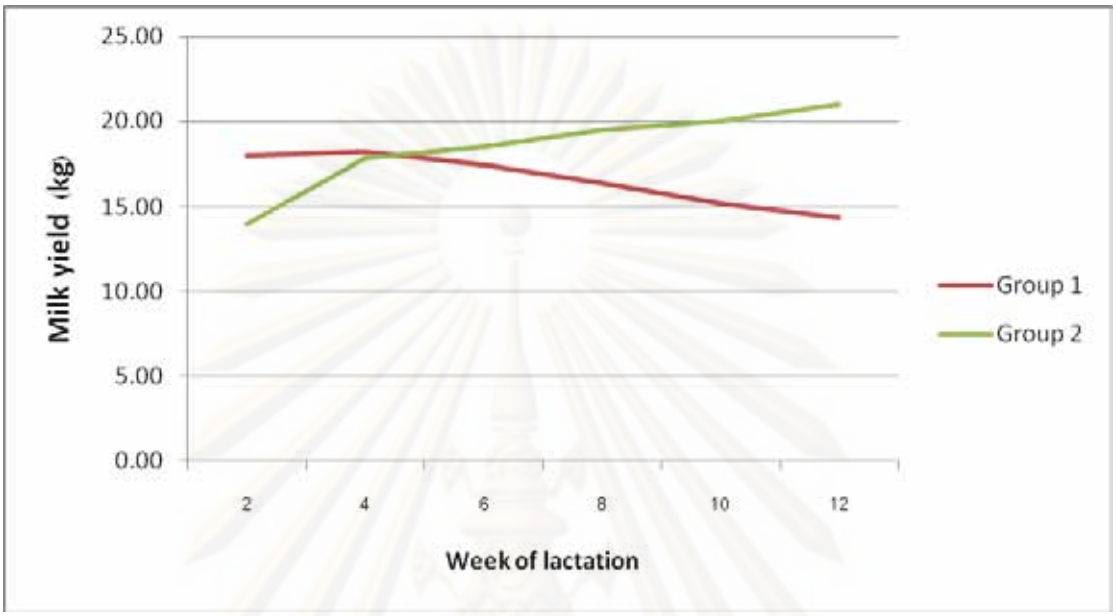
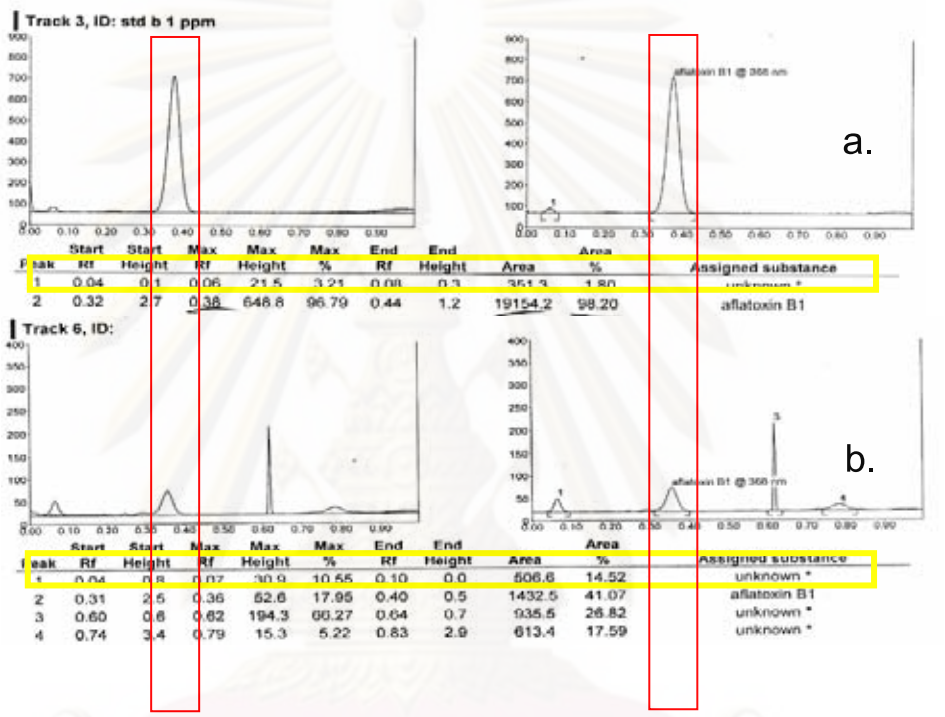


Figure 13 Milk yields of individual dairy cows during early lactation period

Figure 14 The fluorescence of standard AFB1 and AFB1extracted from samples on TLC plate at 365 – 366 nm ultraviolet light.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	%	Assigned substance
1	0.04	0.1	0.06	21.5	3.21	0.08	0.3	351.3	1.80	unknown *
2	0.32	2.7	0.38	648.8	96.79	0.44	1.2	19154.2	98.20	aflatoxin B1

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	%	Assigned substance
1	0.04	0.8	0.07	30.9	10.55	0.10	0.0	506.6	14.52	unknown *
2	0.31	2.5	0.36	52.6	17.95	0.40	0.5	1432.5	41.07	aflatoxin B1
3	0.60	0.6	0.62	194.3	66.27	0.64	0.7	935.5	26.82	unknown *
4	0.74	3.4	0.79	15.3	5.22	0.83	2.9	613.4	17.59	unknown *

Figure 15 The TLC chromatogram of standard AFB1 (a) and sample (b) The Rf values of the standard AFB1 and AFB1 in feed sample extracted were 0.38.

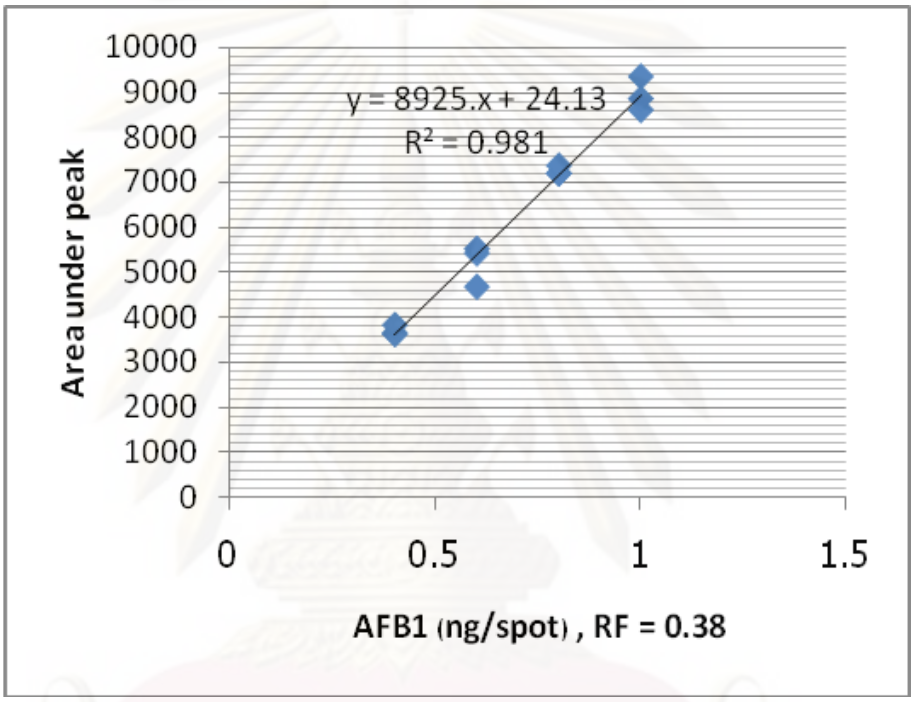


Figure 16 The standard curve shows the relation between the area under the peak and the amount of standard AFB1 by using function *fx* = LINEST on Microsoft Excel

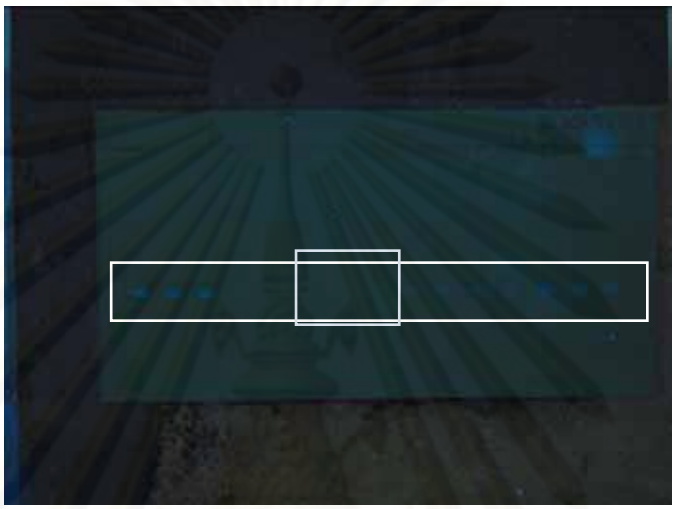


Figure 17 The fluorescence of standard AFB1 at 0.02 ng/spot at 365 – 366 nm ultraviolet light.

In this study, a total of 180 feed samples were analyzed. The samples were comprised of 60 concentrate feed samples, 60 roughage feed samples, and 60 rice straws samples. The blue fluorescent spots were found in 101 samples and their Rf values were equal to that of the standard AFB1. AFB1 was not detected in any rice straws samples. Approximately 85% (n = 51/ 60) of roughage feed samples were found to be contaminated with AFB1 in a range of 0.19 - 58.73 ppb. The average concentration of AFB1 found in roughage feeds was 13.48 $\pm$ 16.77 ppb with 50% of roughage feed samples were contaminated with AFB1 more than 4 ppb. Approximately 83% (n=50/60) of concentrate feed samples were contaminated with AFB1 in a range of 0.16 - 42.54 ppb with a median of 16.23 ppb, as shown in Table 3.

Table 3 Level of AFB1 contaminated in feed samples

Statistic value	Level of AFB1 (ppb)	
	Concentrate feed samples (n = 50/60)	Roughage feed samples (n = 51/60)
Mean	16.05	13.48
Median	16.23	4.00
Mode	1.60	51.02
Max.	42.54	58.73
Min.	0.16	0.19
Standard deviation	13.16	16.77

The participating dairy farms used different dairy cow concentrate and roughage feeds. Eight out of ten farms purchased commercial concentrate pellets feeds from a dairy cooperative. The remaining two farms used concentrate homemade mixed feed. The number of commercial concentrate pellets feed samples (n=48/60) contaminated with AFM1 was more than that of concentrate homemade mixed feeds (n=12/60). Five out of ten farms used pineapple for roughage feeds. Number of roughage feeds containing a pineapple was contaminated with AFB1 more than other roughage feeds as shown in Table 4.

Table 4 Contaminations of AFB1 were in a different dairy cow feed

Statistic value	AFB1 contaminated in concentrate feed (ppb)		AFB1 contaminated in roughage feed (ppb)	
	Commercial (n = 48)	Homemade (n=12)	With Pineapple (n = 30)	Without pineapple (n=30)
Mean	18.40	5.32	24.45	2.94
Median	18.09	0.23	20.65	1.78
Mode	1.60	N/A	51.02	N/A
Max.	42.54	18.10	58.73	19.19
Min.	0.30	0.16	0.98	0.19
Standard deviation	12.95	7.97	17.99	4.08

4.4 Analysis of AFM1 in milk

The recoveries of AFM1 in cow milk were in the range between 60 and 80 %; with an average of 70 %. Standard AFM1 concentrations used for standard curve were 20, 10, 5.0, 1.0, 0.5, 0.05 ppb as shown in Figure 18. The limit of AFM1 detection in raw milk used this study was 0.03 ppb as shown in Figure 19. The retention time of the standard AFM1 and AFM1 extracted from milk samples was derivatized with trifluoroacetic acid to form AFM2a under the conditions used in this study was approximately 5.50 minute as shown in Figure 20 and 21, respectively.

Approximately 70% (n = 42/60) of milk samples were found to contain AFM1 at a level between 0.014 – 2.463 ppb (Mean = 0.731 $\pm$ 0.672 ppb, Mode = 0.453 ppb), as shown in Table 5.

Figure 18 The standard curve showing the relation between the area under the peak and the amount of standard AFM1 by using function ***fx*** = LINEST on Microsoft Excel®

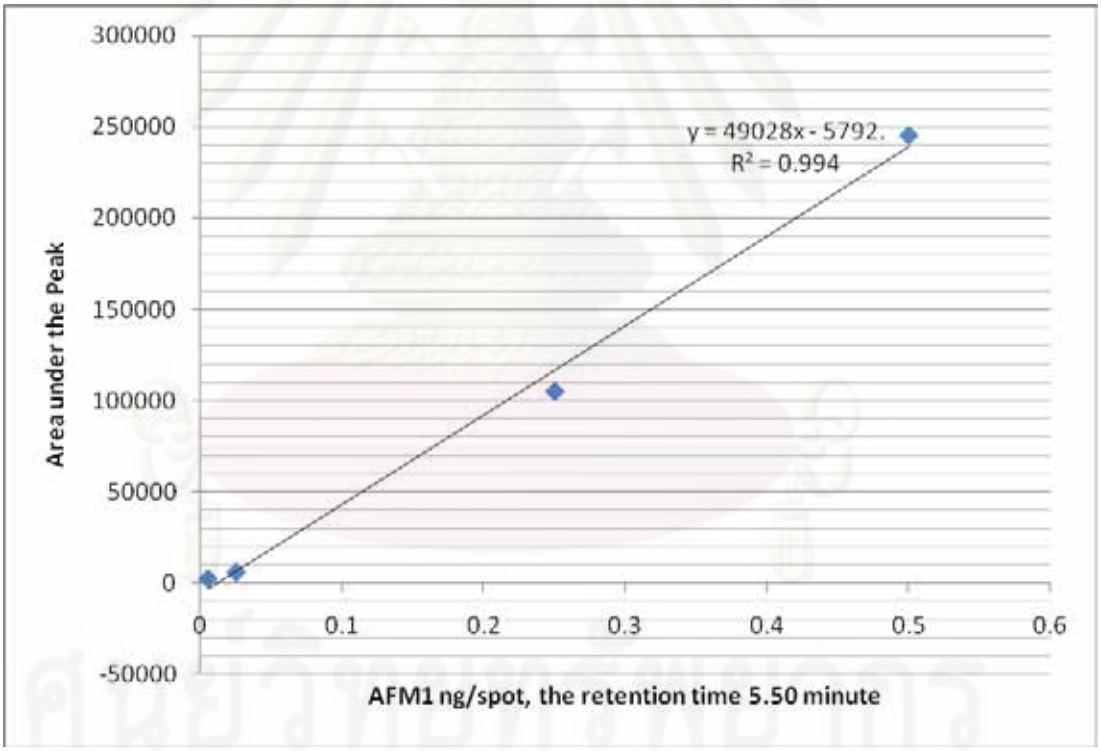


Figure 19 HPLC chromatogram showing the limit of detection of standard AFM1 was derivatized with trifluoroacetic acid at 0.03 ppb, the retention time 5.527 minute

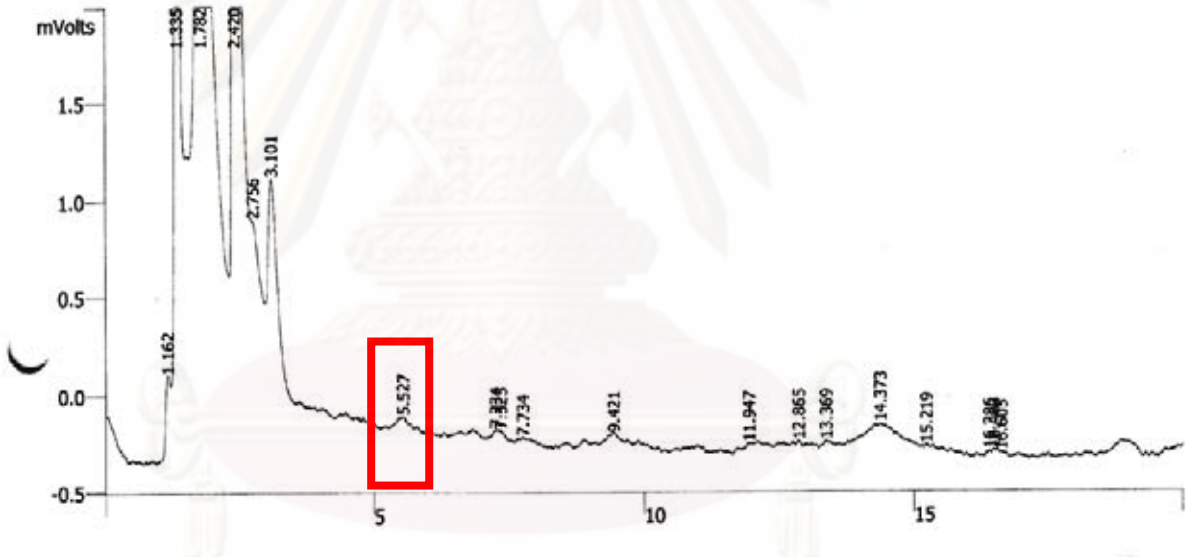
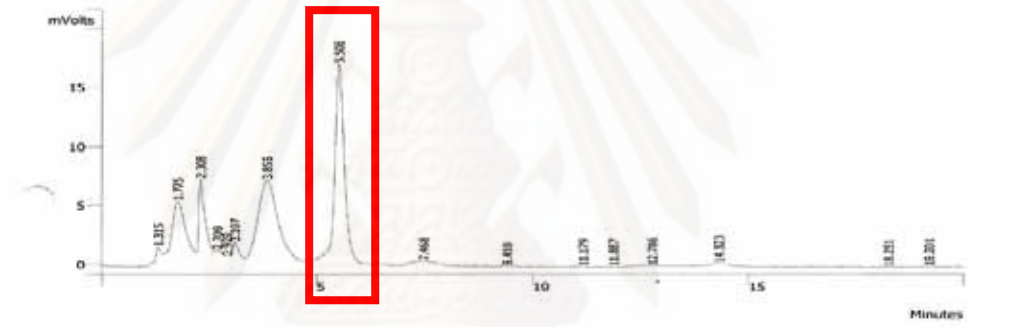
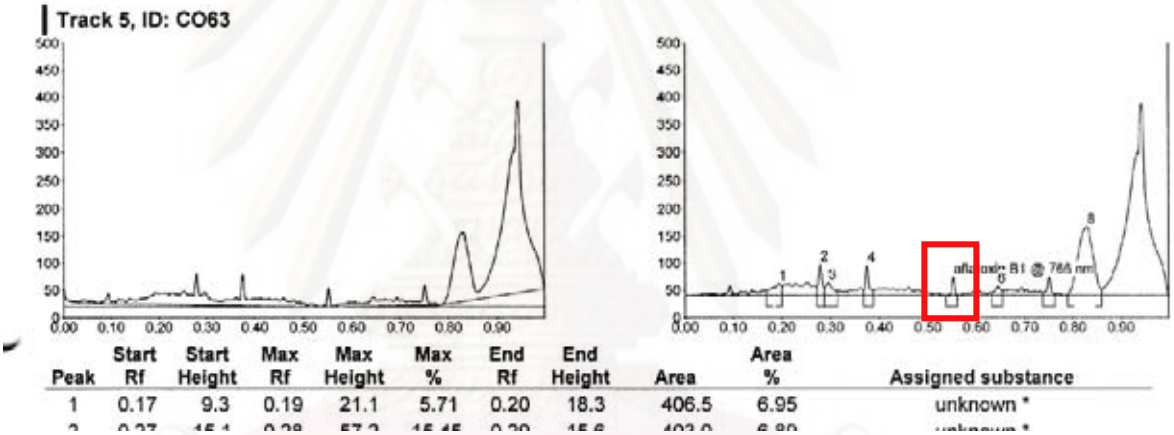


Figure 20 The HPLC chromatogram of standard AFM1 derivatized with trifluoroacetic acid to form AFM2a showing a single peak with retention time of approximately 5.50 minute



Peak No	Peak Name	Result (%w/v)	Ret. Time (min)	Time Offset (min)	Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes	Group
1		2.1953	1.315	0.000	17384	0.00	BV	6.9		0
2		16.5004	1.775	0.000	130667	0.00	VV	21.8		0
3		10.7372	2.308	0.000	85028	0.00	VV	10.1		0
4		2.1943	2.709	0.000	17377	0.00	VV	0.0		0
5		0.3402	2.929	0.000	2694	0.00	VV	0.0		0
6		2.7163	3.107	0.000	21510	0.00	VV	11.7		0
7		28.5580	3.855	0.000	226151	0.00	VB	31.7		0
8		32.3343	5.506	0.000	256056	0.00	BB	12.7		0
9		2.0328	7.468	0.000	16098	0.00	BB	37.9		0
10		0.2142	9.410	0.000	1696	0.00	BB	17.2		0
11		0.1905	11.179	0.000	1509	0.00	BB	30.2		0
12		0.0195	11.887	0.000	155	0.00	BP	8.9		0
13		0.5745	12.786	0.000	4550	0.00	PB	0.0		0
14		1.0114	14.323	0.000	8009	0.00	BB	25.4		0
15		0.0250	18.251	0.000	198	0.00	BV	6.8		0
16		0.3563	19.201	0.000	2821	0.00	VB	23.7		0
	Totals	100.0002		0.000	791903					

Figure 21 The HPLC chromatogram of AFM1 extracted from milk sample and derivatized with trifluoroacetic acid to form AFM2a showing a peak at the retention time of approximately 5.50 minute



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.17	9.3	0.19	21.1	5.71	0.20	18.3	406.5	6.95	unknown *

Table 5 Level of AFM1 contaminated in milk samples

Statistic value	Level of AFM1 (ppb) in milk samples (n = 42/60)
Mean	0.692
Median	0.445
Mode	N/A
Max.	2.463
Min.	0.014
Standard deviation	0.0656

4.5 Carry-over rates of AFB1 in dairy cow feeds to AFM1 into milk

The data on morning and afternoon of milk yield of individual dairy cows, the AFM1 concentrations in milk samples, daily feed consumption, the AFB1 concentrations in feed samples and carry-over rate of AFB1 to AFM1 of the ten individual dairy cows are shown Appendix F. In this study, the carry-over rate was calculated using the following equation;

$$\text{Carry-over rate} = \frac{(Vm + Va)* Cm}{[(Fc * Cfc) + (Fr * Cfr)]} * 100$$

Where:

Vm = amount of morning cow milk (kg)

Va = amount of afternoon cow milk (kg)

Cm = concentration of AFM1 in daily cow milk (μg/kg)

Fc = weight of concentrated feed that a cow intakes per day (kg)

Cfc = concentration of AFB1 in concentrated feed that a cow intakes per day (μg/kg)

Fr = weight of roughage feed that a cow intakes per day (kg)

Cfr = concentration of AFB1 in roughage feed that a cow intakes per day (μg/kg)

The carry-over rates are the ratio of the daily amount of AFM1 excreted into milk to the daily amount of AFB1 ingested. The daily amounts of AFB1 dairy cows ingested are the sum of amounts of AFB1 in concentrate and roughage feeds.

In this study, AFB1 was detected in 101 feed samples out of the 120 feed samples. The average of concentration of AFB1 in dairy cow feeds was 423.61 $\pm$ 428.35 ppb, with over 213.46 ppb of AFB1 found in approximately 50 % of the samples(Maximum = 1530.60 ppb, Minimum = 3.30 ppb). AFM1 was detected in 42 milk samples out of 60 milk samples. Thus, 42 carry-over rates were calculated in this study.

These carry-over rates of AFM1 were in the range of 2.47 $\pm$ 0.49 kg (Median = 2.53 kg, Maximum = 3.16 kg, Minimum = 1.60 kg). The example computation of the carry-over rate of AFM1 of cow No.2 was as follow;

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (µg/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	7.50	6.50	14.0	0.356	4.984	4.40	31.00	45	0.47	157.55	3.16
	Vm	Va		Cm		Fc	Cfc	Fr	Cfc		

The Carry-over rate = {[(Vm + Va) * Cm] / [(Fc * Cfc) + (Fr * Cfr)]} * 100

= {[(7.50 + 6.50)* 0.356] / [(4.40*31.0) + (45*0.47)]} * 100

= [(14.0)*0.356] / [(136.40) + (21.15)]} * 100

= {[4.984] / [157.55]} * 100

= (0.0316) * 100

= 3.16 %

Therefore, the carry-over rate of AFM1 of cow No. 2 in the second week of lactation was 3.16 %.

For this study, the average carry over rate of during early lactation (weeks 2, 4, 6, 8, 10 and 12) of each cow represented the carry-over rate of cow. The carry-over rate of individual cows (n = 4) decreases from the 2nd of lactation as shown in the Figure 22. The average carry-over rate of individual cows (n = 4) was 2.57 $\pm$ 0.47% (Median = 2.685 %, Maximum = 3.16 % and Minimum = 1.65 %).

Due to the result of the published carry-over rate of individual cow was the average of carry-over rates in the early (2, 4 and 6 weeks) lactation period accordingly the carry-over rate from this study (n=4) was calculated in the same condition was

2.62 ± 0.40 % (Median = 2.57 %, Maximum = 3.16 % and Minimum = 1.96 %) as shown in Table 6.

Figure 22 The carry-over rates of individual cows (n = 4) decrease from the 2nd to the 12th of lactation.

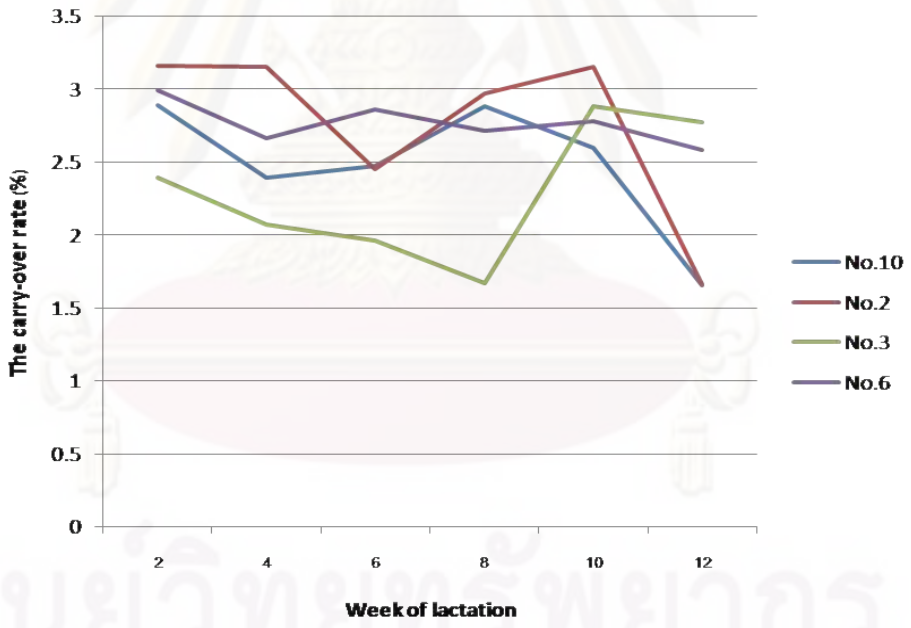


Table 6 Comparison of the average carry-over rate of individual cows

Statistic value	The average carry-over rate		
	Of all samples (n = 42/60)	(2,4,6,8,10 and 12 week of lactation) (n =4)	(2,4, and 6 week of lactation) (n=4)
Mean	2.47	2.57	2.62
Median	2.53	2.69	2.57
Mode	2.39	2.39	2.39
Max.	3.16	3.16	3.16
Min.	1.60	1.65	1.96
Standard deviation	0.49	0.47	0.40
Variance	0.24	0.16	0.22

CHAPTER V

CONCLUSION DISCUSSION AND SUGGESTION

5.1 DISCUSSION

Thailand is divided into four regions depending on livestock areas; North, Central, Northeast and South. The Central region has the highest raw milk production. Ratchaburi and Petchaburi provinces are classified in this region. In 2008, raw milk production of the Central region made up 65 % of the total of Thailand (กลุ่มสารสนเทศและข้อมูลสถิติ, 2008).

Thin layer chromatography (TLC), also known as flat bed chromatography or planar chromatography, is one of the most widely used separation techniques in aflatoxin analysis. Since 1990, TLC has considered the AOAC official method and the method of choice to identify and quantify aflatoxin at level as low as 1 ng/g. The TLC method is used to verify findings by newer, more rapid techniques and also provides the basis for extremely sensitive analytical method. The use of silica gel coated TLC plates for the resolution of the AFB1 was introduced by DE longh et al. (1964) who used chloroform: methanol for development. The ability to segregate aflatoxin from other interfering compounds, on TLC plate, imparts a reasonable level of selectivity and sensitivity to TLC quantification method.

The development of highly automated HPLC systems has afforded very precise, selective and sensitive quantification techniques for aflatoxin analysis. HPLC methods had been developed by using both normal and reverse phase systems in conjunction with UV adsorption and fluorescence detection techniques. Reverse phase HPLC separation of aflatoxin are more widely used than normal-phase separation. AFM1 in milk samples were extracted and purified by using two separate columns; C18 Sep-Pak and Silica gel minicolumn. The extracted AFM1 was derivertized with trifluoroacetic acid to form AFM2a to increase the sensitivity of detection by high performance liquid

chromatography (HPLC). The HPLC chromatograms of AFM2a show separate from to others, with the retention time about 5.50 minute as shown in Figure 17.

For dairy cows, AFM1 are detected in cow milk within 12 to 48 hours after ingestion and approximately 90% of AFM1 excretion in milk occurs within 12 hours after consumption of AFB1 (Applebaum et al., 1982; Frobish et al., 1986; Bingham et al., 2004)). Coppock and Christian (2007) reported aflatoxin in milk disappear within 24 to 72 hours after all aflatoxin (230µg AFB1/cow/day) was removed from the diet. Considering the results of the above studies, feed samples in this study were collected one day prior to collection of milk samples.

In Thailand, there are three seasons, composed of summer (March to June), rainy season (June to October) and winter (November to February). Each season is approximately four months. In this study, ten dairy cows calved in January to February, 2009. Most samples were collected in summer. The rest were collected in winter. These seasons had less humidity and relative humidity and high temperature. Therefore, none of the rice straws were found contaminated with AFB1 so in this study, the rice straws samples were excluded from the calculation of roughage feed. Approximately 85% (n=51/60) of roughage feed samples were contaminated with AFB1 between the range of 0.19 – 58.73 ppb. Most of roughage feed samples (74.51%; n=38/51) were less than 20 ppb. These roughage feed samples with pineapple were contaminated with AFB1 higher than others. This might be due to the storage location and methods were inappropriate.

Two types of concentrate feed samples in this study were commercial pellet and homemade mixed feed. Concentration of AFB1 found in commercial pellet concentrated feeds was higher than homemade mixed feed. This could be due to the quality and type of raw materials, cycle of feed production, the storage period, the storage locations and the storage methods. AFB1 was detected in 60 concentrated feed samples (83%; n=50/60) ranging from 0.16 – 42.54 ppb. Most of the concentrated feed samples (64%; n=32/50) passed the USFDA regulation at < 20 ppb. and 30% (n=15/50) passed the EU regulation at < 5 ppb. All of them passed the Thai Ministry of Agriculture and

Cooperative regulation at 100 ppb. AFB1 concentrations of concentrated feed samples in this study were higher than the previous study (นพดล และเพชรรัตน์, 2006).

Approximately 70% (n=42/60) of milk samples, were detected AFM1 between 0.014 – 2.463 ppb. Approximately 47.62% (n=20/42) of them contaminated with AFM1 higher than the USFDA regulation of 0.5 ppb. The previous study reported only 9.6% (n=13/136), exceed the USFDA regulation (ลักษณ์กนก และคณะ, 2005). It could due to milk samples in this study were collected from individual cows.

Relation between AFB1 contaminated feed samples and AFM1 contaminated milk samples in this study are divided in three groups. In group 1 (n=38/60), cows fed high AFB1 contaminated feeds; AFM1 excreted into milk was high. In group 2 (n=3/60), cows fed low AFB1 contaminated feeds; AFB1 excreted into milk was low. Conversely, in group 3 (n=19/60), cows fed high AFB1 contaminated feed; AFM1 excreted into milk was low. This might be due to animal variability (van Egmond, 1989; Veldman et al, 1992).

In this study, the carry-over rate of AFM1 into cow milk was studied during early lactation period. Because of the AFM1 excreted in milk is correlated with milk yields (Frobish et al., 1986) and milk yields in the early lactation is highest during lactation period. The average of the carry-over rate was studied in the 2^{nd}, 4^{th}, 6^{th}, 10^{th}, and 12^{th} of lactation period due to the day in peak (DIP) of dairy cattle in Central of Thailand is during 8 – 73 day (อามีนา และศกร, 2008). From this study, the average carry-over rate of individual cows (n=4) during the 12^{th} of lactation was 2.57 $\pm$ 0.47%. In previous study in Thailand, Suthatip (1997) reported the average carry-over rate during early lactation (2^{nd} to 12^{th} week) at 2.00 $\pm$ 0.7 % and the average carry-over rate of AFM1 was between 1.3 – 2.7 % (สุเทพและเบญจมาศ, 1996). Although the average carry-over rate in this study was higher than the previous study, it was exceed 3% that correlated with the study of Diaz et al (2004).

5.2 CONCLUSION

Analysis of dairy concentrated feed samples from 10 dairy farms showed that 85% of samples were contaminated with AFB1 between 0.16 – 42.54 µg/kg (ppb) (n = 51/60). All rice straw samples were not contaminated with AFB1. Of 60 roughage feed samples, 51 samples were contaminated with AFB1 in the range of 0.20 - 58.73 ppb.

Seventy percentages of milk samples (n = 42/60) were contaminated with AFM1. Concentration of AFM1 contaminated in milk was in the range of 0.014 - 2.463 µg/kg (ppb).

In this study, the average carry-over rate of AFM1 excreted into cow milk during early lactation period (the 2^{nd} to 12^{th} week) was 2.57 $\pm$ 0.47 % which was in the acceptable range between 0.47 and 3.29%.

5.3 SUGGESTION

The carry-over rate of obtained in this study may be applied to establish a limit of AFB1 concentration in dairy cow feeds which would give a safety level of AFM1 in dairy cow milk.

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APPENDICES

APPENDIX A

The raw materials used as ingredients in dairy concentrate feed and roughage feed in these ten dairy cows fed.

1. No.1 Cow's name was Dum. Calving date was 02/02/2552, >4th lactation. Concentrate feed used a commercial concentrate pellets feed from one company. It was composed of fish meal, soybean meal or peanut meal or sunflower meal or kapok meal or cotton meal, corn, wheat rice bran or rice bran or rice bran oil relate, cassava root, leucaena leaf meal, coconut meal, brewer's grain, molasses, malt rice, oil palm meal or palm meal, calcium carbonate or bone meal or oyster shell meal, salt, vitamin, mineral and feed additive. Roughage feed was composed of maize excluding cob and TMR (Total mixed ration) (25 kg/meal/head), rice straw (*ad libitum*).

2. No.2 Cow's name was Boonperm. Calving date was 09/02/2552, >4th lactation. Concentrate feed used a commercial concentrate pellets feed from one company. It was composed of fish meal, soybean meal or green bean meal or black bean meal or peanut meal or sunflower meal or sesame meal or rapeseed meal or oil palm meal or coconut meal, corn, rice or rice or cassava root or molasses, dicalciumphosphate or calcium carbonate, salt or bone meal or oyster shell meal, salt, vitamin, mineral, preservatives and feed additive. Roughage feed was composed of grass (20kg /head/meal), maize excluding cob (5 kg/head/morning) and rice straw (*ad libitum*).

3. No.3 Cow's name is 74. Calving date was 09/02/2552, 1st lactation. Concentrate feed used a commercial concentrate pellets feed from one company. It was composed of fish meal, soybean meal or green bean meal or black bean meal or peanut meal or sunflower meal or sesame meal or rapeseed meal or oil palm meal or coconut meal, corn, rice broken or paddy rice or sorghum meal or cassava root or molasses, dicalciumphosphate or calcium carbonate or bone meal, salt, vitamin, mineral, preservatives and feed additive. Roughage feed was composed of maize

(30 kg/head/morning, 20 kg/head/afternoon), hair of corn (5 kg/head/afternoon) and rice straw *(ad libitum*).

4. No.4 Cow's name was Junggum. Calving date was 09/02/2552, 1st lactation. Concentrate feed used a commercial concentrate pellets feed from one company. It was composed of fish meal, dried milk or whey, soybean meal or black bean meal or green bean meal or peanut meal or sunflower meal or repseed meal or oil palm meal or coconut meal, corn meal or rice broken or paddy rice or sorghum meal or cassava meal or molasses, dicalciumphosphate or calcium carbonate or bone meal, salt, vitamin, mineral, preservatives and feed additive. Roughage feed was composed of pineapple meal (10 kg/head/meal) maize or grass (10 kg/head/meal) and rice straw (*ad libitum*)

5. No.5 Cow's name was Pingky. Calving date was 16/02/2552, 1st lactation. Concentrate feed used a commercial concentrate pellets feed from 2 company in ratio 1:1. It was composed of fish meal, soybean meal or peanut meal or sunflower meal or kapok meal or cotton meal, corn, wheat rice bran or rice bran or rice bran oil relate, cassava root, leucaena leaf meal, coconut meal, brewer's grain, molasses, malt rice, oil palm meal or palm meal, calcium carbonate or bone meal or oyster shell meal, salt, vitamin and mineral. Roughage feed was composed of pineapple shell and flesh (10 kg/meal/head) rice straw (3 kg/head/meal).

6. No.6 Cow's name was Baipai. Calving date was 17/02/2552, 4th lactation. Concentrate feed used a concentrate homemade mixed feed. It was composed of rice barn, rice bran oil relate, soybean meal and shell, green bean meal, oil palm meal, cassava root, corn and coconut meal and minerals and calcium together. Concentrated feed added brewer's grain (3 kg/meal/head). Roughage feed was composed of pineapple shell (15 kg/meal/head), rice straw (*ad libitum*).

7. No.7 Cow's name was Coke. Calving date was 05/02/2552, 3rd lactation. Concentrate feed used a commercial concentrate pellets feed from 2 company in ratio 1:1. It was composed of fish meal, soybean meal or peanut meal or sunflower meal or kapok meal or cotton meal, corn, wheat rice bran or rice bran or rice bran oil relate, cassava root, leucaena leaf meal, coconut meal, brewer's grain, molasses, malt rice, oil

palm meal or palm meal, calcium carbonate or bone meal or oyster shell meal, salt, vitamin and mineral. Roughage feed was composed of pineapple shell and flesh (10 kg/meal/head) rice straw (30 kg/9 lactated cows/meal).

8. No.8 Cow's name was Mai. Calving date was 30/01/2552, 1st lactation. Concentrate feed used a commercial concentrate feed from one company. It was composed of fish meal, soybean meal or peanut meal or sunflower meal or kapok meal or cotton meal, corn, wheat rice bran or rice bran or rice bran oil relate, cassava root, leucaena leaf meal, coconut meal, brewer's grain, molasses, malt rice, oil palm meal or palm meal, calcium carbonate or bone meal or oyster shell meal, salt, vitamin, mineral and feed additive. Roughage feed was composed of maize, cob (25 kg/head/meal), grass (6 kg/head/meal) and rice straw (*ad libitum*).

9. No.9 Cow's name was Ning. Calving date was 18/02/2552, 1st lactation. Concentrate feed used a concentrate homemade mixed feed. It was composed of rice barn, rice bran oil relate, soybean meal and shell, green bean meal, oil palm meal, cassava root, corn and coconut meal and minerals and calcium together. Concentrated feed added brewer's grain (3 kg/meal/head). Roughage feed was composed of pineapple shell (10 kg/meal/head), rice straw (3.5kg/head/meal).

10. No.10 Cow's name was Ruay. Calving date was 12/02/2552, >4th lactation. Concentrate feed used a commercial concentrate pellets feed from two formula from a company, ratio 2:1. It was composed of fish meal, soybean meal or green bean meal or black bean meal or peanut meal or sunflower meal or sesame meal or rapeseed meal or oil palm meal or coconut meal, corn, rice or rice or cassava root or molasses, dicalcium phosphate or calcium carbonate, salt or bone meal or oyster shell meal, salt, vitamin, mineral, preservatives and feed additive. Roughage feed was composed of grass (20 kg/ head /meal) and rice straw (*ad libitum*).

APPENDIX B

Analysis of AFB1 in concentrated and roughage feed was conducted by using the AOAC Official Method 968.22.

25.1 A feed sample was crushed and the sample was weighed 10g into a glass Erlenmeyer flask as shown in Figure 5a.

25.2 About 5 g of Celites, 5 ml of distilled water and 50 ml of chloroform were added into the flask and were shaken on a wrist-action shaker for 90 min. The chloroform extract was filtered through a filter paper No1 as shown in Figure 5b.

25.3 A fraction of 10 ml chloroform filtrate was evaporated on a waterbath and dissolved residue with 5 ml chloroform.

25.4 About 5 ml of solution was added to silica gel minicolumn cleanup and allowed to pass through the column by gravity as shown in Figure 5c.

25.5 The minicolumn was eluted with 1.5 ml n-hexane, 1.5 ml ether and 1.5 ml MeOH: $CHCl_3$ (1:10) respectively.

25.6 These eluates were mixed and were evaporated on the waterbath.

25.7 The residue was dissolved in 1ml of benzene: acetonitrile mixture (1 ml: 200 µl) for quantitative analysis of AFB1 by Thin Layer Chromatography (TLC). The apparatus of TLC was shown in Figure 6.

25.8 The TLC plate was labeled the final end of mobile phase stop.

25.9 Standard AFB1 and samples were spotted on the same TLC plate in volume 10 µl by CAMAG Linomate5.

25.10 Standard AFB1 concentrations used for standard curve were 10, 20, 30, 40, 60, 80, 100, 200, 300, 500, 1000 and 10000 ppb.

25.11 One of two TLC tanks was saturated with 50 ml of solvents in system I (dichloroform: ethyl acetate = 1:1) and the other was saturated with 50 ml of solvents in system II (chloroform: acetone = 8:2) within 90 minute.

25.12 The TLC plate was developed in the saturated tank until the mobile phase arrived at the mark.

25.13 When the TLC plate was evaporated to dry, it was taken to watch the fluorescence by the long wave UV lamp at 366 nm.

25.14 The TLC plate that standard AFB1 and sample have fluoresced blue in the same Rf was taken to densitometry by using densitometer CAMAG TLC Scanner3 at 366 nm and then was evaluated by program winCATS. The result shows in graph called “densitometric chromatogram” or “densitogram” or “TLC chromatogram”.

APPENDIX C

The computation sequences of AFB1 concentration in the samples

The steps of AFB1 concentration calculation were as follows:

Step	Procedure	Calculation	Quantity of AFB1 (ng) in the solution
1	Weight 10 grams of feed sample		a
2	Dissolve the sample in 60 ml of solvent	a/60	a/60
3	Purify only 10 ml of the solution in step1	(a/60) * 10	a/6
4	Use only 10 µl of the purified solution in step 3 to quantify AFB1	(a/6) * (0.010)	a/600
5	Assume that the densitometer detects **b** ng of AFB1		b

AFB1 of **(a/600)** ng is represented by **b**. To compute the amount of AFB1 in a 10-gram sample, we need to solve for **a**: in which **a** equals **(b * 600)**. The AFB1 of **a** ng in a 10-gram sample is then divided by **10** to arrive at a concentration of AFB1 in **ppb**. It can also be assumed that the concentration of AFB1 in **ppb** equals **(b * 600)/10** or **(b * 60)**.

APPENDIX D

Analysis of AFM1 in raw milk was performed by using the AOAC Official Method 986.16.

1.1 The inlet stem of C18 cartridge, luer tip of 50 ml syringe, syringe, cartridge and vacuum flask were assembled as shown in Figure 8.

1.2 About 5 ml of methanol and 5 ml of water were added into syringe to prime the cartridge.

1.3 The solvent was pulled through the cartridge in fast drop wise manner; approximately 10 ml/min. Vacuum pump was stopped when a small amount of water was left in syringe to prevent loss of prime.

1.4 A bottle of pooled raw milk samples was warmed at 40°C in a waterbath and was inverted gently 10 times to distribute cream in non-homogenized sample.

1.5 Pooled raw milk sample; about 20 ml, was transferred to a beaker containing 20 ml hot water; approximately 80°C.

1.6 The diluted milk sample was poured entire 40 ml into syringe and sample was pulled gently through cartridge at a flow rate 10 ml/min by using the vacuum pump.

1.7 About 10 ml of wash solution (water: acetonitrile 95:5 v/v) was added into syringe and pulled through.

1.8 The cartridge was removed from the extraction system and inside of both stems was dried with tissue paper to eliminate any remaining wash solution.

1.9 The cartridge was assembled as shown in Figure 9a. About 150 µl of acetonitrile was added into syringe to reprime the cartridge. The solvent was soaking into packing for 30 second.

1.10 Silica gel cleanup column was washed with 5 ml ether. About 10 ml of ether was added to syringe–cartridge positioned above silica gel cleanup column and was forced through the cartridge at a flow rate approximately 5 ml/min by using the vacuum pump.

1.11 Silica gel cleanup column was removed and then was inserted into 250 ml vacuum flask that a test tube was placed to catch the eluate from column reservoir as shown in Figure 9b.

1.12 About 10 ml of the elution solution (dichloromethane: ethyl alcohol; 95:5 v/v) was added into column reservoir and the solvent was pulled through column with vacuum at 1 ml/min flow rate by using vacuum pump.

1.13 Vacuum was stopped and the test tube was removed from assembly.

1.14 The eluate was evaporated to 0.5 ml under nitrogen gas and transferred to glass vial. The solvent was evaporated to dryness under nitrogen gas.

1.15 About 200µl n-hexane was added immediately into the vial tube to dissolve the residue.

1.16 About of 200 µl trifluoroacetic acid was added and was mixed on vortex mixer at 5 second. AFM1 must be derivatized with trifluoroacetic acid to form aflatoxin M2a to increase the sensitivity of the fluorescent detection to analysis by high performance liquid chromatography (HPLC).

1.17 The mixture was incubated at 40°C for 10 minute in a waterbath.

1.18 After incubation, the mixture was evaporated to dryness under nitrogen gas.

1.19 The residue was dissolved with 1 ml of water: acetonitrile (75:25 v/v) mixture into vial and the mixture was shaken well in vortex mixer and was filtered through a syringe filter; pore size 0.45 µm. into a HPLC vial.

1.20 Standard AFM1 concentrations used for standard curve were 0.05, 0.5, 1.0, 10 and 20 ppb, respectively. About 0.5 ml of the standard AFM1 was added into a vial. About 200 µl n-hexane and 200 µl trifluoroacetic acid

were added into vial and was mixed. The standard AFM1 was treated as described for sample derivative.

1.21 The filtrate was analyzed by using HPLC.

1.22 The HPLC system used for analysis of AFM1 consists of HPLC trademark Varian™; model Prostar, system control – Varian Star #1. Analytical column was a reverse phase C18; HiQsil C18 W 4.6*250 nm. Detectors with fluorescence, the excitation and emission wavelengths were 365 and 455 nm, respectively. The mobile phase was HPLC grade of water: acetronitrile: iso-propranol (80:12:8) and the flow rate of mobile phase was 1.0 ml/min. the injection volume was 50 µl. The apparatus of HPLC is shown in Figure 10.

1.23 The result shows in graph called “HPLC chromatogram”.

APPENDIX E

The computation sequences of AFM1 concentration in the samples

The steps of AFM1 concentration calculation were as follows:

Step	Procedure	Calculation	Quantity of AFB1 (ng) in the solution
1	Weight 20 ml of milk sample		m
2	Dissolve the residue with 1000 µl H20: acetonitrile for preparation for HPLC	m/1000	m/1000
3	Apply only 50 µl per spot in HPLC	(m/1000) * 50	m/20
4	Assume that the HPLC detects **n** ng of AFM1		n

AFM1 of **(m/20)** ng is represented by **n**. To compute the amount of AFM1 in a 20-ml sample, we need to solve for **m**: in which **m** equals **(m * 20)**. The AFM1 of **m** ng in a 20-ml sample is then divided by **20** to arrive at a concentration of AFB1 in **ppb**. It can also be assumed that the concentration of AFM1 in **ppb** equals **(n * 20)/20** or **(n)**.

APPENDIX F

Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12-weeks experimental periods of cow No. 1 – 10. Milk and feed samples were collected every other week started at week 2 of lactation.

Table 1 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No. 1.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	8.50	6.50	15.0	0.000	0.000	6.00	9.56	50	4.44	279.36	0.00
4	8.60	8.10	16.7	0.000	0.000	6.00	13.93	50	0	83.58	0.00
6	9.00	8.10	17.1	0.000	0.000	6.00	12.57	50	3.53	251.92	0.00
8	8.00	7.50	15.5	0.528	8.184	6.00	37.98	50	2.63	359.38	2.28
10	7.50	7.20	14.7	0.453	6.659	6.00	47.86	50	1.03	338.66	1.97
12	7.50	7.10	14.6	0.000	0.000	6.00	27.74	25	1.42	201.94	0.00

Table 2 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.2.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	7.50	6.50	14.0	0.356	4.984	4.40	31.00	45	0.47	157.55	3.16
4	7.00	6.50	13.5	1.071	14.463	4.40	40.94	45	6.20	459.136	3.15
6	7.50	7.20	14.7	0.445	6.546	4.40	31.67	45	2.83	266.698	2.45
8	7.30	7.00	14.3	0.675	9.651	4.40	33.03	45	4.00	325.332	2.97
10	6.50	7.00	13.5	2.463	33.245	4.50	42.54	45	19.19	1054.98	3.15
12	6.90	6.70	13.6	0.137	1.866	4.40	23.54	45	0.20	112.576	1.66

Table 3 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.3.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	9.80	7.60	17.4	0.315	5.484	5.00	27.7	55	1.65	229.25	2.39
4	9.50	7.70	17.2	0.354	6.091	5.00	26.83	55	2.92	294.75	2.07
6	9.00	7.00	16.0	0.125	2.000	5.00	20.43	55	0	102.15	1.96
8	8.50	6.50	15.0	0.133	1.990	5.00	23.78	55	0	118.9	1.67
10	8.00	6.00	14.0	1.473	20.616	5.00	24.97	55	10.76	716.65	2.88
12	7.50	6.00	13.5	0.232	3.131	5.00	22.63	55	0	113.15	2.77

Table 4 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.4.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	8.60	7.00	15.6	0.000	0.000	7.30	19.79	40	14.58	727.667	0.00
4	10.70	8.00	18.7	0.000	0.000	7.00	18.06	40	12.16	612.82	0.00
6	9.80	7.60	17.4	0.000	0.000	6.40	18.09	40	21.29	967.376	0.00
8	8.50	7.00	15.5	1.508	23.366	8.00	18.78	40	20.65	976.24	2.39
10	8.00	7.00	15.0	0.000	0.000	8.00	12.58	40	19.45	878.64	0.00
12	7.20	6.40	13.6	1.501	20.410	8.00	14.37	40	19.91	911.36	2.24

Table 5 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the12 week experimental period of cow No.5.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	10.00	6.00	16.0	1.471	23.536	10.00	0.00	20	38.6	772	3.05
4	13.50	8.50	22.0	0.000	0.000	11.00	0.00	20	28.29	565.8	0.00
6	14.50	9.00	23.5	0.000	0.000	12.00	0.00	20	26.67	533.4	0.00
8	14.50	10.00	24.5	1.504	36.848	11.00	4.59	20	58.73	1225.09	3.01
10	12.50	7.50	20.0	0.000	0.000	10.00	9.58	20	54.55	1186.8	0.00
12	13.50	7.50	21.0	1.463	30.719	10.00	1.60	20	51.02	1036.4	2.96

Table 6 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.6.

Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over Rate (%)
	Morning	Afternoon	Total								
2	8.00	6.00	14.0	1.549	21.686	12	10.52	30	19.95	724.74	2.99
4	10.00	7.00	17.0	1.464	24.890	12	18.01	30	23.94	934.32	2.66
6	12.00	7.00	19.0	2.178	41.376	12	0	30	48.3	1449	2.86
8	12.00	8.00	20.0	1.505	30.100	12	0	30	37.03	1110.9	2.71
10	14.00	10.00	24.0	1.460	35.040	12	18.10	30	34.8	1261.2	2.78
12	15.00	11.00	26.0	1.516	39.416	12	0	30	51.02	1530.6	2.58

Table 7 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.7.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over Rate (%)
	Morning	Afternoon	Total								
2	18.00	12.00	30.0	0.000	0.000	15.00	4.59	20	0	68.85	0.00
4	15.50	13.00	28.5	0.240	6.846	14.00	16.07	20	0	224.98	3.04
6	13.50	11.50	25.0	0.000	0.000	14.00	28.74	20	0	402.36	0.00
8	14.00	10.00	24.0	0.635	15.228	12.00	41.85	20	8.26	667.4	2.28
10	13.00	10.50	23.5	0.000	0.000	11.00	0.30	20	0	3.3	0.00
12	11.00	8.00	19.0	0.000	0.000	10.00	1.60	20	0	16	0.00

Table 8 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No. 8.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	7.00	5.00	12.0	0.000	0.000	3.00	0.00	62	1.01	62.62	0.00
4	8.00	6.50	14.5	0.056	0.806	3.00	0.00	62	0.56	34.72	2.32
6	7.00	6.00	13.0	0.070	0.913	3.00	0.32	62	0.69	43.74	2.09
8	8.00	6.00	14.0	0.072	1.008	3.00	0.48	62	0.99	62.82	1.60
10	9.00	7.00	16.0	0.014	0.224	3.30	0.55	62	0.19	13.595	1.65
12	9.00	7.00	16.0	0.000	0.000	3.80	0.00	62	0.35	21.7	0.00

Table 9 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No. 9.
Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Milk Yield (kg)			AFM1 Conc. (ppb)	Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc. (ppb)	Total AFB1 Consumed (µg/day)	Carry-over rate (%)
	Morning	Afternoon	Total								
2	14.0	9.0	23.0	0.000	0.000	18	0.16	20	5.10	104.88	0.00
4	13.0	9.0	22.0	0.028	0.6204	14	0.23	20	0.98	22.82	2.72
6	13.0	9.0	22.0	0.076	1.6742	14	0.32	20	3.48	74.08	2.26
8	13.0	8.0	21.0	0.047	0.987	14	0.18	20	2.65	55.52	1.78
10	11.0	6.0	17.0	0.040	0.6834	14	0.21	20	1.91	41.14	1.66
12	12.0	6.5	18.5	0.165	3.0451	14	0.17	20	8.00	162.38	1.88

Table 10 Daily milk production, aflatoxin M1 (AFM1) concentration, daily feed consumption, aflatoxin B1 (AFB1) concentration and carry-over rate of AFB1 to AFM1 during the 12 week experimental period of cow No.10.

Milk and feed samples were collected every other week started at week 2 of lactation.

Week of Lactation	Morning			Afternoon			Total AFM1 (ug/day)	Con. Feed Consumed (kg/day)	AFB1 Conc. (ppb)	Roughage Feed (kg/day)	AFB1 Conc (ppb)	Total AFB1 consumed (µg/day)	Carry-over rate (%)
	Milk Yield (kg)	Conc. AFM1 (ppb)	AFM1 (ug/day)	Milk Yield (kg)	Conc. AFM1 (ppb)	AFM1 (ug/day)							
2	6.00	0.000	0.00	5.00	0.403	2.01	2.014	6.00	1.60	40	1.50	69.6	2.89
4	6.00	0.235	1.41	5.00	0.290	1.45	2.858	6.00	7.30	40	1.90	119.8	2.39
6	4.80	0.351	1.68	5.30	0.588	3.11	4.799	6.00	22.02	40	1.55	194.12	2.47
8	4.60	0.529	2.43	4.80	0.569	2.73	5.163	6.00	16.38	40	2.02	179.08	2.88
10	4.30	0.000	0.00	4.30	0.683	2.94	2.937	6.00	3.38	40	2.32	113.08	2.60
12	3.60	0.000	0.00	4.30	0.301	1.30	1.296	6.00	0.00	40	1.96	78.4	1.65

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WORK EXPERIENCE

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Pet Clinic, Bangkok.

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