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Utah State University

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COMPARATIVE PERFORMANCE OF FLUOROMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN THE DETECTION OF AFLATOXIN M_1 IN TWO COMMERCIAL CHEESES

by

Gustavo Peña

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Dairy Science

Approved:

Allen J. Young Major Professor	Jeffery O. Hall Committee Member
Jong-Su Eun Committee Member	Kenneth L. White Department Head

Byron R. Burnham
Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2010

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ABSTRACT

Comparative Performance of Fluorometry and High Performance Liquid Chromatography in the Detection of Aflatoxin

M_1 in Two Commercial Cheeses

by

Gustavo Peña, Master of Science

Utah State University, 2009

Major Professor: Dr. Allen Young
Department: Animal, Dairy and Veterinary Sciences

Aflatoxin M_1 (**AFM_1**) is frequently found in milk and dairy products. It is a metabolite formed in cows from aflatoxin B_1 (**AFB_1**), contained in animal feeds. In cheese production AFM_1 distributes between curds and whey. In this study, cows were fed 64 µg/AFB_1/d for the high treatment, and 5 µg/AFB_1/d for the low treatment, to obtain milk contaminated with AFM_1 over the 0.5 µg/L and under 0.05 µg/L restrictions, respectively. Cheese was manufactured with milk contaminated with AFM_1 at 0.8 and 0.03 µg/kg by the higher and lower treatment, respectively. Two commercial cheeses were elaborated: a hard-aged cheese (cheddar cheese) and soft high moisture cheese (fresco cheese) to evaluate whether the cheese type had any impact on AFM_1 analysis. AFM_1 was extracted from cheese using immunoaffinity columns. Analyses were carried out by using high pressure liquid chromatography (**HPLC**) as the reference method and fluorometry as a method of validation. Analysis was by 2-way fixed factor analyses. AFM_1 was detected in all samples by both methods of analysis. There were no detectable statistical

differences between cheese types ($P>0.05$). AFM_1 content was significantly different between the high and low concentration of AFB_1 used to make the cheese type ($P<0.01$). Our regression model shows a linear relationship between fluorometry and HPLC methods; $R^2 = 0.9141$ from cheddar cheese and $R^2 = 0.9141$ from fresco cheese. There were no statistical differences between methods of analysis ($P>0.05$). Carryover of AFM_1 in cheese detected by fluorometry in cheddar cheese was 163% and 80% for high and low treatments, respectively, and in fresco cheese was 119 and 133 for high and low treatments, respectively. These carryovers are below that reported in the literature. Results suggest that fluorometry is a simple and reliable AFM_1 detection method for screening samples of complex matrices such as cheese.

(58 pages)

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INTRODUCTION

The ubiquitous nature of fungi (Coulombe, 1993), creates a worldwide problem by contaminating food and feeds (Kamkar, 2005). Such a problem is exacerbated by change in climatic conditions, agricultural manufacturing, transportation and storage practices (CAST, 2003). Some varieties of fungi are capable of produce secondary metabolites called mycotoxins; today more than 100 molds are known to be responsible for producing approximately 400 mycotoxins with toxigenic potential (Kabak et al., 2006). In domestic animals, ingestion of this toxic compounds compromise economic and productive parameters (Binder et al., 2007). In addition, many mycotoxins represent a threat to human health (Coulombe, 1993).

Aflatoxins (AF) are secondary metabolites produced by five species of the fungi *Aspergillus: A. flavus, A.Parasiticus, A. nomius, A. tamari and A. pseudotamarii*. Only *A. flavus and A. parasiticus* produce high enough concentration to be economically important. Aflatoxins are produced by the fungi in response to changes in environmental conditions, contaminating the substrates on what they grow either pre-harvest or during post-harvest storage (Flannigan, 1991; Kozakiewicz and Smith, 1994).

Aspergillus mainly affects corn, peanuts, cotton, tree nuts and their by-products (CAST, 2003). In the US, the Food and Drug Administration regulates AFB_1 in feeds intended for dairy cattle consumption at 20 µg/kg and limits AFM_1 in milk for human consumption at 0.5 µg/L (Coulombe, 1993).

Aflatoxins are the most studied group of mycotoxins because of their natural carcinogenic properties (Hussain and Anwar, 2007). Aflatoxin B_1 (AFB1) ingested from

feed is metabolized to aflatoxin M_1 (AFM1) and excreted in milk and urine. According to the International Agency of Research on Cancer (IRCA), AFB_1 and AFM_1 have been classified as human carcinogens (Hussain and Anwar, 2007).

Quantitative carryover of AFB_1 to milk is around 3% of the concentration consumed and it is excreted into milk primarily as AFM_1 (Diaz et al., 2004). When contaminated milk is used to manufacture dairy products the toxins is transferred to the final product. The AFM_1 is unaffected by pasteurization or processing (Galvano et al., 2005). Studies have demonstrated that AFM_1 binds mainly with casein increasing the concentration in the curd during cheese manufacturing (Galvano et al., 2005).

Sensitive and reliable methods have been developed for detection in order to control the AFM_1 concentration in cheese. High Performance Liquid Chromatography (HPLC) analysis is one of the best choices when sensitivity, reliability and precision are required (Scudamore, 2005). More rapid methods like flourometry are being developed to determine AFM_1 and AFB_1 in foods, but there are not publications to investigate the use of fluorometry in complex foods like cheese.

In this study, the objective was to evaluate the efficiency of a quick analytical fluorometry procedure against the HPLC method at two different concentrations of AFM_1 residues in two kinds of cheeses (Fresco and Cheddar cheese), while studying the carryover of AFM_1 to cheese and the recovery AFM_1 using immunoaffinity columns as a clean-up step.

LITERATURE REVIEW

Mold Growth and Mycotoxin Formation

With a family of over 200 members, *Aspergillus* molds were first described in 1729 by an Italian priest, Micheli, who took the name from the shape of an aspergillum (holy water sprinkler) (Samson, 1992). Today those fungi are recognized as the most common genera in the world. *Aspergillus* was the first mold organism cultivated in artificial media for their biochemical properties. In Asian countries, *Aspergillus spp* have been used to ferment foods (Gervais and Bensoussan, 1994). They are known for the many contributions to biochemistry, molecular biology, serology and recently their potential as mycotoxin producers (Samson, 1992).

The genera *Aspergillus* is a soil born fungus important for its ability to break down vegetative material (CAST, 2003). However, a small group of *Aspergillus* species are mycotoxin producers. Species of the complex genera, *Aspergillus* has been adapted to a wide range of temperatures and habitats. They are most abundant in subtropical and warm climates (Krska, 1999; CAST, 2003). Various species are involved in producing AF on grains in the field or under storage conditions; in most parts of the world, *Aspergillus flavus* and *A. parasiticus* are the most representative agents although other species such as *A. nomius,* and *Penicilium* may be implicated (Agag, 2004).

Filament molds like *Aspergillus* are affected by physiobiochemical parameters such as water, temperature, pH and gas composition. Fungi cannot germinate without water but growth rate is controlled by temperature; such parameters are different for optimum mycotoxin formation than for optimal fungus growth (Kozakiewicz and Smith,

1994). (See Table 1.) Water activity (**a**^{w}) below 0.70 may affect germination and mycelial growth and higher that 0.98 increases the lag time before germination from a few days (Kozakiewicz and Smith, 1994). Production of AFB1 ranges from 12 to 41 °C with most favorable temperature occurs between 24 to 28 °C (Kozakiewicz and Smith, 1994; Agag, 2004). Growth pH is difficult to control because fungi change the pH in the substrate when growing. Some growth inhibitions have been observed by *Aspergillus* after O_2 concentration was below 1% (Kozakiewicz and Smith, 1994). However there are complex interactions between those parameters (Kozakiewicz and Smith, 1994).

Table 1. Comparison of Physiobiochemical Parameters for Fungus Growth and Aflatoxin Production of *Aspergillus flavus* and *A. parasiticus*[1]

	a_w[2]	Moisture %[3]	Temperature[4]	pH	CO_2 Gas	Stress
Growth	0.78	18 - 25	25 – 37 °C	4.0 – 6.5	79 %	Reduced
Toxin[5]	0.82		12 – 41 °C			Increased

[1] Modified from (Kozakiewicz and Smith, 1994; Agag, 2004).
[2] a_w = water activity for optimal growth and mycotoxin production
[3] Field = 22 – 25; Storage = 13 – 18; Decay 18
[4] Optimum temperatures for toxin production range from 25 – 32 °C
[5] Toxin production

Aflatoxins

The first described report of aflatoxicosis was in a British journal in 1960 where a strange disease, "Turkey "X" disease," was affecting and killing turkeys and other animals at an early age (Sargent et al., 1961). The disease was not cured by antibiotic treatment, and no bacterial, viral or pesticide agent was isolated (Carnaghan and Sargeant, 1961). The toxic compound was named aflatoxin from letters in the name *Aspergillus flavus* and the word toxin: "(a- (*aspergillus*) + fla-(*flavus*) + toxin)". The

fungus and the toxin were found in peanut meal coming from Brazil (Sargent et al., 1961).

When fungi grows in cereal or feedstuff used to feed dairy cattle, there is a chance that mycotoxins will end up in milk and their carryover represents a latent threat to the health of the human population (Coffey et al., 2009). The production of AFB_1 and aflatoxin B_2 (AFB_2) is attributable to *A. flavus*, while *A. parasiticus* produces AFB_1, AFB_2, aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Agag, 2004). The letters B and G were from the fluorescense color produced under ultraviolet light (B for blue and G for green). Further metabolisms of AFB_1 and AFB_2 in animals produce the hydroxylated metabolites AFM_1 and aflatoxin M_2 (AFM_2), which are found in urine and milk. Although 18 types of AF are known, the most toxic are AFB_1 and AFM_1. The following metabolites are listed in order of decreased toxicity: AFG_1, AFM_2, AFB_2, AFG_2 (Gimeno and Martins, 2006).

Aflatoxin occurs as natural contaminants in diverse substrates. They are mainly found in cereals like corn, wheat and rice, cereal byproducts, peanuts, pistachio and other nuts, cottonseed, oilseed meals, cassava, dry fruits, sausage products, spices, wine, coffee, legumes, fruits and their juices, milk and dairy products (Flannigan, 1991). While the four naturally occurring AF (B_1, B_2, G_1, G_2) are common in the same foods, AFB_1 represents the highest percentage (60 to 80%) of the total aflatoxin content (Weidenborner, 2001).

Despite the fact that the liver is the target organ for AF, tumors in the respiratory systems have been associated with AF intoxication. The hepatotoxicity, immunodeficiency, acute and chronic toxicity will be discussed in later sections.

Worldwide Occurrence of Aflatoxin M_1

The problem of food contamination with AFM_1 is of great concern and has received enormous attention. The International Cancer Research Association classified AFB_1 as a Group 1 carcinogen (known carcinogen to humans) while AFM_1 is classified as a Group 2B carcinogen (possibly carcinogenic to humans) (IACR, 1997). Because of this, many countries today have been implementing controls and inspection programs to monitor the presence of AF in feeds and foods, resulting in maximum tolerance law for such compounds (FAO, 2004).

Milk and dairy products are the main commodities to introduce AF, primarily AFM_1 into the human diet. Many European countries have imposed a limit of 0.05 µg/L of AFM_1 in milk. However, in the US and other Latin American countries the maximum concentration is 0.5 µg/L. By 2003 AFM_1 regulation in food was undertaken by 98 countries (FAO, 2004). Data from a recent publication on the world wide occurrences of AFM_1 in cheese are summarized in Table 2. As expected there are more reports from regions where climate is favorable to AF production. In the U.S., the only report of milk contaminated by AFM_1 was in 1978 in Arizona where 413, 636 kg of milk were discarded (Park, 1993).

Biological Action of Aflatoxin

The biosynthesis of AF is the best characterized of any mycotoxin (CAST, 2003). Aflatoxin represents a group of difuranocoumarin compounds classified into two groups: the difurocoumarocyclopentenone which include AFB_1, AFB_2, aflatoxin B_{2a} (AFB_{2a}), AFM_1, AFM_2, aflatoxin M_{2a} (AFM_{2a}) and aflatoxicol (AFL). The other group,

Table 2. Data of the Occurrence on Aflatoxin M_1 in Cheese on Several Countries

Country	Sample	Positive	Method[3]	Range, ng/kg	Reference
Brazil	75	56[1] (75%)[2]	HPLC	20 - 6920	Prado, 2000
Libya	20	15[1] (75 %)[2]	HPLC	110 - 520	Elgerbi, 2004
Turkey	63	28[1] (44 %)[2]	ELISA	7 – 202	Gurses, 2004
Turkey	400	327[1] (82 %)[2]	ELISA	> 250	Sarlmehmetoglu, 2004

[1]Represents number of positive samples from all samples
[2]Represents the percentage of positive samples
[3]HPLC = high performance liquid chromatography; ELISA = Enzyme linked immunosorbent assay

difurocoumarolactone is represented by AFG_1, AFG_2, aflatoxin G_{2a} (AFG_{2a}), aflatoxin GM_1 ($AFGM_1$), aflatoxin GM_2 ($AFGM_2$), aflatoxin GM_{2a} ($AFGM_{2a}$), and aflatoxin B_3 (AFB_3) (Agag, 2004).

Following a rapid absorption from the gastrointestinal tract, AFB_1 tends to accumulate in the liver. Metabolism is mainly in the liver but other tissues are also involved (Agag, 2004). Several metabolites can be produced, some of which are highly reactive and can react with cellular macromolecules. The reactive metabolites attach to proteins and nucleic acids. Unbound metabolites are excreted in urine and milk, and conjugated aflatoxin is excreted in feces (Riley and Pestka, 2005).

Aflatoxins enter the cell and are metabolized via monooxygenases in the endoplasmatic reticulum to hydroxylated metabolites which are further metabolized to glucuronide and sulfate conjugates or oxidized to the reactive epoxide which undergoes spontaneous hydrolysis to AFB_1-8,9-dihydrodiol or bind to proteins, resulting in cytotoxicity. The epoxide can react with DNA or protein, or be detoxified by an inducible

GST to the GSH-conjugate. Both DNA and protein adducts have been proven to be useful as biomarkers in humans and laboratory animals to monitor AF exposure (Riley and Pestka, 2005).

Toxic effects of AF are related to their biotransformation pathway (Eaton and Gallagher, 1994). In the liver and other tissues, AFB_1 requires microsomal oxidation to the reactive AFB_1-8, 9-epoxide to produce its hepatoxicity and carcinogenic effects. The oxidation also produces other polar metabolites like AFM_1. During the epoxidation of AFB_1, microsomal cytochrome P450 (CYP450) generates a reactive epoxide responsible for nucleic acid alkylation. Even though CYP450 is the main pathway to form epoxide, independent pathways have also been demonstrated (Eaton and Gallagher, 1994).

Monooxygenase hydroxylation of AFB_1 produces several metabolites (aflatoxin Q_1 (AFQ_1), aflatoxin P_1 (AFP_1) and AFM_1) with lower carcinogenic potential than parent toxin. AFQ_1 is created via 3a-hydroxylation. Aflatoxin P_1 is formed by o-demethylation and AFM1 is formed by 9a-hydroxylation. AF can be reduced to AFL by reduction of the 1-keto-group through a cytosolic NADPH-dependent reductase. The formations of AFL may not be an excretion pathway because AFL rapidly converts back to AF by microsomal dehydrogenase. Another product of reduction is the AFB_1-8,9-aldehyde formed by hydrolysis of AFB_1-8,9-epoxide. This may be partially responsible for the acute toxic effects of AFB_1 (Eaton and Gallagher, 1994).

Detoxification of AF epoxide is mediated by glutation-S-transferase (GST) by conjugation with reduced glutathione (GSH) in the liver. Other conjugation pathways include glucoronidation and sulfations of hydroxylated metabolites are excreted in biliary

fluids. AFQ_1 and AFP_1 may conjugate with GSH to form detoxification products (Eaton and Gallagher, 1994).

Aflatoxin M_1 is a metabolite found in milk of animals consuming AF contaminated feed. According to Diaz et al. (2004), the AFM_1 hydroxilated metabolite of AFB_1 can be detectable during the first milking after animals consumed AF contaminated feed. It reached its' maximum concentration in three days and disappeared four days after the last feeding time of contaminated feed. AFM_1 has lower carcinogenic potency than AFB_1, with carcinogenic properties of AFM_1 being 30% less in trout than AFB_1 (Eaton and Gallagher, 1994). Nuryono et al. (2008) cited authors who found that AFM_1 is about 10 times less carcinogenic than AFB_1, but its genotoxicity is higher than the parent toxin. Even with lower toxicity, AFM_1 is hepatotoxic and carcinogenic (Coulombe, 1993).

Effects of Aflatoxin in Ruminants

Detrimental effect of AF in animals by damage to vital organs causes serious economic impact (Coulombe, 1993; Whitlow, 2005). Aflatoxin is known to cause liver damage and immmunosuppresion, as well as having carcinogenic, teratogenic and mutagenic effects. But, vulnerability is influenced by breed, species, age, dose, length of exposure and nutritional status of the animals (Richard, 2007). Calves that consume AFM1 from milk are more susceptible than older animals (Whitlow, 2005).

Intoxication by AF in dairy calves is associated with decreased feed intake, body weight loss and death. Lesions in liver include fat accumulation, cellular necrosis, fibrosis, venous occlusion, disorganization of liver lobules, and biliary hyperplasia. Other changes include serum enzyme changes and reductions of vitamin A. Calves up to 6

months of age are most susceptible to the toxic effects of AF but true infections by *Aspergillus* has been linked in heifers and adult cattle to mycotic pneumonia and mycotic abortion (Lynch, 1971).

Adult ruminants have been considered to be less affected by AF because rumen flora could degrade and inactivate mycotoxins (Fink-Gremmels, 2008). However, research does not support this theory completely since biodegradation of AFB_1 in the rumen has shown ranges anywhere from 42% to no apparent degradation in several in vitro experiments (Jouany and Diaz, 2005). Yiannikouris and Jouany (2002) found that concentrations of AF ranging from 0.1 to 10 μg/ml are poorly degraded in the rumen; rumen bacteria are inhibited by AF which may disturb the metabolism and growth of the rumen micro-organisms.

Fink-Gremmels (2008) described that AFB_1 is changed in the rumen to AFL which can be transformed back to AFB_1. According to Coulombe (1993), AFL toxicity is equal to the parent toxin. A significant amount of the AFB_1 that escapes ruminal fermentation is converted in the liver into AFM_1, which is partially excreted into milk (Fink-Gremmels, 2008).

Chronic aflatoxicosis is characterized by poor feed conversion, reduced growth, jaundice, cirrhosis with bile duct proliferation and significant reduction in milk production (Applebaum et al., 1982). Lesions of aflatoxicosis in dairy cattle show liver cell injury, congestion and bleeding, and fatty liver syndrome (pale livers) (Agag, 2004; Fink-Gremmels, 2008).

Edrington et al. (1994) reported that lambs had lower daily feed intake and daily weight gain when 2.5 mg of AF was feed. Semen of buffalo bulls that consumed 2, 3, and

4 grams of rice contaminated with 15.6 mg/kg AF showed a 10 % reduction in live cells and more than 54 % abnormal cell (Hafez et al., 1982).

Aflatoxin in the rumen decreased cellulose digestion and volatile fatty acids production and decreased rumen motility. Cook et al. (1986) fed steers with 0.2, 0.4, 0.6, and 0.8 mg of AF per kilogram of body weight. They observed that the amplitude and frequency of rumen contractions were altered depending on the dose of AF received.

Immunosuppression caused by AF has been connected with disease epidemics in farm animals. A cell-mediated response is particularly sensitive, and phagocytic response is also decreased affecting innate immunity (CAST, 2003). Cordova et al. (2003) looked at the consumption of small quantities of AFB_1 on reproductive performance in dairy cattle and found that immunosuppressant and teratogenic effects of AFB_1 could compromise reproductive parameters. Calving percent in the herd of study was 62%, which is 20 to 30% below of that reported in the literature to be normal and calving intervals was 444 d, 70 d higher than optimal (350 -380 d optimal).

Effects of Aflatoxin on Humans

The impact of food and feed contaminated with mycotoxins, especially AF, is a concern for food organizations worldwide and is expected to receive more attention in coming years (Kamkar, 2005). The problem is more accentuated in developing countries where the food system, economics and infrastructure limits the management of AF contamination (Williams et al., 2004). An investigation by Jonsyn-Ellis (2001) in Sierra Leone showed an average of 96% exposure to AF based on 434 urine samples of school children.

Human exposure to AF is primarily from consumption of contaminated food. However, an inhaled contaminated particle represents another form of AF contact, especially for those workers interacting with grain dust. There are reports where AF was found in the lung of one textile worker, two agriculture workers, a Brazilian engineer who worked with contaminated peanuts and two agriculture employees whom died from pulmonary interstitial fibrosis (Selim et al., 1998).

Aflatoxicosis is the name given to the aflatoxin disease. There are two courses of the disease: acute and chronic. Acute aflatoxicosis results in deaths from hepatic necrosis and liver failure. Chronic aflatoxicosis in humans and animals are related to cancer, immune suppression, heptacellular carcinoma, reyes syndrome, cirrhosis and kwashiorkor (Stora et al., 1983; Bennett and Klich, 2003).

The symptoms of acute aflatoxicosis disease consists of hemorrhagic necrosis of the liver, bile duct proliferation, edema, lethargy and death (usually in children because human adults are more tolerant to the toxin) (Williams et al., 2004).

Chronic exposure has been related to nutritional and immunotoxicities. Lamplugh and Hendrickse (1982) reported an association between AF and Kwashiorkor. Nelson et al. (1980) and Stora et al. (1983) reported associated AF contamination with Reye's syndrome. Coulombe (1993) suggested that the carcinogenic and mutagenic effects of AF result from the binding of the reactive epoxide with DNA.

There are reports of more than 200,000 deaths annually in the Republic of China due to hepatocellular carcinoma (HCC) related to the consumption of AF (Wang et al., 2002). According to Kamkar (2005) AF is a concern because it may be the contributing agent to human hepatic and extra hepatic carcinogenesis.

Human exposure to AF can be made directly by consuming contaminated feeds like cereals, seeds, fruits, etc., or indirectly by eating food products and subproducts obtained from animals consuming contaminated feeds (Galvano et al., 2005). Milk is an important way to introduce AFM_1 in the human diet. Because milk is the main nutrient for infants and children, the presence of AFM_1 is a concern (Kamkar, 2005). Sadeghi (2009) suggests that exposure to AF prenatally can produce poor neonatal survival and growth retardation.

When dairy products are manufactured from milk contaminated with AFM_1, the toxins are transmitted to the final product because AFM_1 is not degraded when processed into cheese, yogurt, cream and butter. In some kinds of cheeses, AF can be produced by the addition of fungi during the ripening process (Galvano et al., 2005).

Aflatoxin Analysis

Diagnosis of aflatoxicosis in animals is usually performed after other causes have been discarded and, and long after the contaminated feed had been consumed. This reduces the possibilities to obtain samples of feed which caused the problem (CAST, 2003). On the other hand, the analysis of AF is important for enforcing legislature, maintaining the quality of material for feed industry and for estimating occurrence and consumer exposure. Methods of analysis for AF may be classified as fully quantitative, semi-quantitative, or simple yes/no answer, and the requirement for the method will depend on the reason for the analysis (Scudamore, 2005).

According to CAST (2003), major principles were described to ensure a mycotoxin quality assurance program. A good quality assurance program depends on proper implementation of each principle.

Sample Selection and Preparation. Proper sampling is important for decreasing variability in the overall testing. Different commodities have different mycotoxin distribution in the lot. Use of adequate devises like probes to sample bins, rail cars, or piles typically helps to obtain a better sample. When samples are taken from a transferred product, such as conveyor belt or other dynamic lot, small samples can be taken at different intervals of time. At least 100 small samples from the entire lot are recommended (CAST, 2003). Liquids are more homogeneous in the distribution of mycotoxin, but if they contain suspended material, mycotoxins could be unequally distributed (Scudamore, 2005). Sample preparation consists of grinding the sample from a solid phase to facilitate the extraction of mycotoxins (Scudamore, 2005).

Extraction. After grinding, the sample is mixed with a solvent to take out as much mycotoxin as possible. Acetronitrile and methanol are the most common solvents used, and the combination of solvent and water improved recovery better than solvent alone (Scudamore, 2005). Stroka et al. (1999) reported that the ratio between solvent and sample weight affected the final result obtained. Extraction uses two methods: The slow method consists of shaking the sample and solvent from 30 min up to 1-2 hours (CAST, 2003). The other method consists of blending the sample with the solvent for a few minutes. Blending is a more rapid process than shaking, but care must be taken to ensure that the solvent wets all particles (Scudamore, 2005).

Clean-up. A clean-up step to eliminate impurities is recommended but not mandatory for all quantification methods, like ELISA (CAST, 2003). The most common method for clean-up is performed using a solid-phase extraction column which is made of porous silica modified to absorb the impurities or the mycotoxin. One example is the immunoaffinity columns for AFM_1 where solution is passed over the column at a rate of 2-3 ml/min. The precise antibodies are bound to AFM_1, and all other components from the matrix pass through and are washed off using water. The mycotoxin is recovered by washing the column with a solvent (Grosso et al., 2004).

Detection. This final step is classified into two groups according to the ability of the determination assay (see Table 3). Less meticulous methods of detection are called rapid method (CAST, 2003) in which its advantage is to reject or accept materials in rapid commercial decisions (Scudamore, 2005). The other methods of classification constitute quantification of the mycotoxin which is usually more accurate but more expensive (CAST, 2003).

According to Rodriguez et al. (2003) there are advantages of using an ELISA test, such as the reduction in assay time, a simple sample extraction, and specificity for the toxin. On their experiment with milk collected from farms of Leon, Spain, samples run by the ELISA test detected 95.4% positive samples, and the negative samples (4.6%) were run with the HPLC method to detect possible false negatives. All samples were negative to HPLC testing, confirming the efficiency of ELISA essays. However this contrasts with a study reported by Lin et al. (1998) who saw poor results using the ELISA method. Scudamore (2005) published a review of some advantages and disadvantages from methods available for mycotoxin analysis which is summarized in the Table 3.

Table 3. A Review of Methods Available for Mycotoxin Analysis

Method[1]	Advantages	Disadvantages
TLC	* Simple, cheap, rapid * Detect most mycotoxins * Sensitive to aflatoxin * Number of samples can run together	* Identification of spots may need confirmation * Insensitive to some toxins * Poor precision * Separation may require 2-dimentional approach
HPTLC	* Quantitative when used with densitometry * Number of samples can run together * Improved separation compared with TLC	* Insensitive to some toxins
ELISA	* Visual assessment possible * Plate reader assists	* Matrix interference may affect result * Limited to a few solvents * Antibodies may cross react
HPLC	* Sensitive, selective * Easy to automate	* Compounds must have UV absorption or fluoresce or require derivatisation
HPLC/MS	* Provides high level of confirmation * Multi-detection? * Very sensitive	* Expensive * Specialist expertise required
GC/MS	* Provides high level of confirmation * Very sensitive	* Expensive * Specialist expertise required * Compounds must be volatile
CEP	* Very low solvent use * Alternative separation technique * Rapid	* Not yet widely evaluated

[1] TLC = Thin layer chromatography; HPTLC = High pressure thin layer chromatography; ELISA = Enzyme linked immunosorbent assay; HPLC = High performance liquid chromatography; HPLC-MS = High performance liquid chromatography with mass spectrometry; GC/MS = Gas column with mass spectrometry; CEP = Capillary electrophoresis.

Fluorometry Analysis

Stokes fluorescence is the re-emission of longer wavelength (lower frequency) photons (energy) by a molecule that has absorbed photons of shorter wavelengths (higher frequency). Both absorption and radiation (emission) of energy are unique characteristics of a particular structure of the molecule during the fluorescence process. Light is absorbed by molecules which cause electrons to become excited to a higher electronic state.

The electrons remain in the excited state for a few seconds then, assuming all of the excess energy was not lost by collisions with other molecules, the electron returns to the ground state. Energy is emitted during the electrons return to their ground state. Emitted light is always a longer wavelength than the absorbed light due to limited energy loss by the molecule prior to emission (Turner Designs, 2008).

Based on the fluorometric technology, VICAM developed AflaTest® a quantitative fluorometric method for the detection of AF in many commodities. This process can be used in a wide variety of locations from the local farm to the government laboratory. It is quick, easy to perform and accurate (Vicam, 1999).

Cucci et al. (2007) felt that in order to ensure reproducibility of fluorometric measurement it is important to have a systematic protocol, because the major disadvantage of the fluorometry method is the variability of quantitative results influenced by very low AFM_1 concentration, sample preparation, presence of impurities, or, air bubbles in the liquid. The same group mentioned that the use of fluorescence

enhancer such as succunyl-β-CD, shows promising result as the most efficient enhancer for AFM_1.

High Pressure Liquid Chromatography

The HPLC uses fluorescence detection, and has been used as the reference technique for detecting AF (Gilbert, 2002; Van Eijkeren et al., 2006). HPLC works well to separate and quantify AF (Wilson et al., 1998). However, this method is complex, laborious, and time-consuming, and it has large requirements for solvents, and needs large investments in costly equipment (Peña et al., 2002). Separation of AFM_1 with HPLC is normally performed using normal or reverse phase methods with fluorimetric detection leading to specificity, high sensitivity, and simplicity of action (Muscarella et al., 2007). A compilation of official methods for AF analysis is presented by Gilbert (2002).

Aflatoxin in Milk

A current serious problem in food hygiene is the occurrence of AF in breast milk, commercial milk and milk products. Considering that milk is a key source of nutrients for humans, this is more important for infants and children whose diet is basically milk. In addition, they are more sensitive to the toxic effects (Galvano et al., 2005).

As the economy improves in developing countries, milk consumption also increases. According to Delgado (2003) a boost of milk consumption in developing countries has been surpassing the growth of milk consumption in the developed countries. The same author estimates that by 2020 milk consumption will be 177 million

metrical tons more than the consumption in 1998. Thus it will elevate the consumption of AF if milk is contaminated.

In North America, milk and other dairy products compose an important part of the diet. According to USDA, the trend in U.S. per capita consumption is downward; in 2001 it was 37 gallons of whole milk per person, eight gallons less than in 1941. But there was an increase in consumption of low fat milk, 15 gallons in 2001 compared with four in1945. However as the consumption of fluid milk in the U.S. dropped, consumption of cheese increased eight times compared with 1909. This is almost 30 pounds per person and convenience foods such as pizzas are primarily responsible for this increase (Putnam and Allhouse, 2003).

Many studies demonstrate the presence of AF in ingredients used to feed dairy cattle (Yiannikouris and Jouany, 2002; Binder et al., 2007; Driehuis et al., 2008). The conversion of the AF into AFM_1 and its subsequent excretion into milk varies from less than 1 to 3% (Applebaum et al., 1982), to 6% (Galvano et al., 2005) with a median transfer level of 1.7%, disregarding the change in the day to day operation (Jouany and Diaz, 2005).

The presence of AFM_1 in milk is proportional to the contamination of feedstuff. Concentrations of AFM_1 in milk and milk products are controlled in several countries (Boudra et al., 2007). As reported by Van Egmond and Jonker (2004), regulation for AFM_1 in 2002 was established in 98 countries, a growth of 30 %, compared to 1995. Currently the regulations of AFM_1 in milk have one standard applied to the European Union at 0.05 μg/kg and another limit of 0.5 μg/kg applied to the United States and several other European and Latin America countries. The calculation for liver cancer for

both levels is very low, and there is not a noticeable benefit when the limit is reduced from 0.5 to 0.05 µg/kg (Van Egmond and Jonker, 2004). Using the 1.7% carryover rate, Jouany and Diaz (2005) calculated that cows consuming 30 µg/kg AFB_1 in feed easily reach the 0.5 ppb of AFM_1 in milk. The European Union level of 0.05 ppb of AFM_1 will be reached when the diet contains 3 µg/kg of AFB_1.

Various studies have shown that AFM_1 in milk and cheese show a tendency towards lower AFM_1 concentration in the summer months. This is accredited to cows grazing in these months and receiving less concentrated feeds (Van Egmond and Dragacci, 2000; Hussain and Anwar, 2007).

Using the ELISA procedure Ghanem and Orfi (2008) found that local farms in Syria had the highest number of positive samples compared with milk powder which was mostly imported from the European Union, which imposes a strict upper limit for AFM_1. This result is in agreement with CAST (2003) which stated that AFM_1 is more abundant in tropical countries because the temperature and humidity favors the growth of the *Aspergillus* fungi.

The AFM_1 restrictions have used similar analytical methodology (Van Egmond and Dragacci, 2000). The ELISA (Ghanem and Orfi, 2008), TLC (Grosso et al., 2004; Kamkar, 2005), HPLC (Kim et al., 2000; Bognanno et al., 2006) and flourometric (Cucci et al., 2007; Hussain and Anwar, 2007) methods, all reported good, quality results. However, the most important addition to the analysis of AFM1 was the use of immunoaffinity columns to the clean up step (Van Egmond and Dragacci, 2000).

Aflatoxin Determination in Cheese

As stipulated above, AF exposure in humans is mainly by the ingestion of milk and dairy products (Battacone et al., 2005). If dairy products are manufactured from milk tainted with AFM_1, the toxin is transferred to the final product. It is generally accepted that AFM_1 is unaffected by pasteurization or other milk process such cheese, yogurt, cream, and butter. Cheese may become contaminated with AFM_1 in one of the following two ways; AFM_1 is present in milk during cheese processing due to carryover or AF is produced by flora growing in cheese during maturity. Aflatoxin couples with casein causing about a 2.5 to 3.0 fold higher concentration of AFM_1 in soft cheeses and 3.9 to 5.8 fold higher in hard cheese curd than in milk used to make them (Yousef and Marth, 1989). Battacone et al. (2005) found concentration of AFM_1 in cheese curd twice as high as in milk. Kaniou-Grigoriadou et al. (2005) found low levels of AFM_1 in milk below the tolerance level. However, AFM_1 value increased 4.9 times in cheese curd when feta cheese was made.

Cheese may also be contaminated by the addition of desirable mycoflora during ripening, but its toxicological relevance remains unclear (Galvano et al., 2005). Several studies by Oruc et al. (2006) established that bacterial starter used in cheese production may inactivate the AF during the ripening. Bacteria that produce lactic acid such as bifidobacteria can bind AF and almost eliminate it from the milk.

Storage of cheese may not reduce the concentration of the AFM_1 in the curd. Oruc et al. (2006) determines the stability of AFM_1 during the processing and ripening of a traditional white pickled cheese, a traditional soft Turkish cheese. During a three-month ripening period, they did not observe any reduction in toxin concentrations. Govaris et al.

(2001), working with Telemes cheese, observed after 6 months of storage only a 30% reduction of AFM_1 compared to the initial cheese curd concentration. It should be noted that Talemes cheese is usually consumed after two months of ripening.

Control and Prevention of Aflatoxin M_1

The prevention of AFB_1 contamination in material used to feed dairy cattle has proved to be the most successful system of controlling AFM_1 in human food supply. Wu and Bhatnagar (2008) estimated losses related to AF in the United States were more than $550 million per year including cost of market rejection and animal health and management. Worldwide the cost is much higher.

There are various attempts to stop the production of aflatoxin under field conditions. Research on developing host resistance of plants shows some potential. Corn breeders have explored the corn genotype for genes related to resistance (Brown et al., 1998). Studies on Midwestern and Mexican corn lines show effective results. The genetic manipulation of pathways in plants to produce antifungal compounds and the use of biocompetitive agents are other areas of experimentation (Brown et al., 1998; Palumbo et al., 2008). Positive crop management practices such as proper fertilization, weed control, irrigation, and insect control reduce AF contamination in corn (Plasencia, 2004).

However, when attempts to stop aflatoxin in the field are not possible or fail, several approaches for post-harvest decontamination can be made, including physical, chemical and biological inactivation (Sinha, 1998). Aflatoxin production post-harvest could be minimized if grains are dried properly before storage, and implementing good management storage practices (CAST, 2003). Mechanical and hand separation has been

used with successful results in nut industries and with corn using flotation and density segregation. The principle is based on removing damaged kernels according to the variation in size, shape, color and visible moldy material (Sinha, 1998).

Chemical inactivation of AF using gaseous ammonia has been successful in animal feeds (Sinha, 1998; Park and Price, 2001). Biological methods, using molds, bacteria, and algae, have reduced AF by competing and excluding toxigenic strains (Sinha, 1998; Yin et al., 2008).

The use of inert absorbents is considered the most promising inactivation treatment. Absorbents like aluminosilicates, clays such as bentonite and zeolite, active carbons, glucomannans and mannanoligosaccharides, aluminosilicates mixed with clays, have been utilized for their ability to sequester AF and reduce their availability for absorption from the gastrointestinal tract (Diaz and Smith, 2005).

Several physical and chemical processes for eliminating or inactivating AFM_1 in milk have been investigated, because degradation of AFM_1 was not observed during industrial processing of milk. Pasteurization, acidification for cream and yogurt production, separation of butter and cheese ripening did not decrease the concentration of AFM_1 to acceptable concentrations (Galvano et al., 2005). Studies of chemicals that could be used for their ability to degrade AFM_1 are limited to those that are permitted as food additives such as sulfites, bisulfites, and hydrogen peroxide (Applebaum and Marth, 1982).

Other processes that have been explored to remove AFM_1 from milk include absorption and radiation. Applebaum and Marth (1982) reported that 5% bentonite in milk adsorbed 89% of AFM_1. In a study of the effects of ultra-violet radiation with and

without hydrogen peroxide, the concentration of AFM_1 was reduced by 3.6–100%, depending on the length of time the milk was exposed to radiation, the volume of treated milk, and the presence of hydrogen peroxide (Yousef and Marth, 1989).

Utilization of materials with chemo-absorption properties has possibilities. Attempts of direct chemi-absorption of AFM_1 in milk were made by Soha et al. (2006) who added bentonite and aluminosilicate at concentrations of 0, 0.05 and 2% to contaminated AFM_1 raw milk. Chemi-absorption compounds significantly reduce milk AFM_1 without affecting milk components.

The chemical and physical treatments described are not readily applicable in the dairy industry, at present, as little is known about the biological safety, or the nutritional value of the treated products. Moreover, the costs of the processes may be considerable and prohibitive for large-scale application (Sinha, 1998). If AFM_1 cannot be easily destroyed or removed from milk, the only way that can be excluded is by eliminating AFB_1 from the animal's diet (Diaz and Smith, 2005)

Developing Methods for Aflatoxin M_1 in Cheese

Advances in developing new methods to study mycotoxins should offer a benefit over existing methods (Scudamore, 2005). New methodology or changes to previous methods need to be supported by comparing results for an existing reference method. After the assay has been evaluated in one or more laboratories, it may go through the process to be established as a new method. Internal validation and collaborative testing provide strong information about method performance and possible problems (CAST, 2003). Methods for validation should take into consideration economical factors as well

as efficiency in time and materials (Gilbert, 2002). Because of frequent contamination of dairy products with AFM_1, the development of fast, accurate and economically viable methods is quite important.

HPLC and TLC methods are the gold standard to determine AFM_1 in milk. There are only a few methods that have been developed for cheese products due to high complexity of their food matrix (Monaci et al., 2007). Cucci et al. (2007) implemented a method to detect of AFM_1 in liquid solutions using a fluorometric process. Once the AFM_1 is extracted from cheese using organic solvent, this method offers several advantages over HPLC.Fluorometric measures of purified extract have been developed because it is a faster method (Stroka and Anklam, 2002) and allows for on-site determination of AF before the final product is made (Sibanda et al., 1999). Fluorometry values are similar to HPLC; however, the results of this method need to be validated with complex matrices such cheese (Stroka and Anklam, 2002).

The ubiquity of mycotoxins causes great economical losses worldwide in the animal production industry. The analysis of AFM_1 is a great concern due to its prevalence in milk and milk products. Consumption of contaminated products can lead to carcinogenic, teratogenic and other detrimental diseases in humans. Of the various methods for mycotoxins detection, HPLC appear to be more sensitive and with automation could be the preferred method. However, equipment is expensive. For this reason it is inaccessible for developing countries or farm analysis. New methods for testing mycotoxins need to be validated that are useful for those sectors.

Interestingly, only minimal or no research exists investigating and validating those new methods for complex matrices such as cheese or dairy products. The present

study attempts to demonstrate that the concentration of AFM_1 in cheese is higher than found in milk and investigate whether fluorometric analysis is comparable to HPLC and could be used to screening and predict AFM_1 from milk products.

MATERIAL AND METHODS

Contaminated Corn with Aflatoxin B_1

Strains of *Aspergillus spp* were planted in flasks containing sterile steam rolled corn. After the analysis, the strain which produced more AFB_1 was grown in a bigger scale. We mixed for 1½ h through a concrete mixer 4 kg of steam-rolled contaminated corn at 400 µg/kg to with 36 kg of steam rolled AF free corn to obtain corn with 40 µg/kg. Bags containing 1.6 kg and 0.125 kg, respectively, were weighed, marked, and stored until their use for feeding cows in high and low treatment groups.

Animals

Four Holstein cows in late lactation (>220 DIM), were used from the USU Caine Dairy Teaching and Research Center herd. All animal were allowed one week acclimation period to the tie stall facility. The cows were milked twice daily at 0530 and 1800 h and fed a TMR diet consisted of (corn silage (35%), alfalfa hay (25%), and energy-protein supplement (40%)) for ad libitum intake. Ration was formulated to meet the nutrient requirement of cows producing 30 ± 5 kg of 3.5% FCM/d according to NRC (2001) recommendation. Composition of the diet for each treatment is given in Table 4. Cows were feed twice daily (0600 h and 1730 h) ad libitum. The cows were divided in two groups. One group (high group) of two cows received 1.6 kg of contaminated corn and the other group (low group) received 0.125 kg of contaminated corn mixed with 1.475 kg of AF free steam rolled corn (to equal the matrix intake of the high treatment group) immediately after morning milking. To ensure cows consumed the contaminated grain, corn was put in a plastic container before the TMR feeding and consumption was

monitored. Fresh water was provided ad libitum. Animal care and procedures were approved and conducted under established standards of the Utah State University Institutional Animal Care and Use Committee.

Table 4. Ingredients and Chemical Composition of Diet

Composition[1]	
Ingredient	g/100 g of DM
Alfalfa hay	25.69
Corn silage	35.42
Steam rolled corn	12.63
Corn, hominy	9.55
Canola meal mechanically extracted	3.69
Whole-linted cottonseed	5.92
Soybean meal expeller	2.04
Blood meal, ring dried	1.00
Molasses, sugar beet	2.50
Minerals & vitamin mix	1.56
Chemical	
NE_L, Mcal/kg of DM	1.44
CP	16.6
NDF	32.8
ADF	21.9

[1]Extra 1.6 kg of steam rolled corn were fed to each cow. High AF treatment were given 1.6 kg of contaminated corn and low treatment were given 0.125 kg of contaminated steam rolled corn added to 1.475 kg of free AF steam rolled corn

Milk Collection and Processing

Cows were milked twice daily (0530 h and 1700 h) for 10 d each into vacuum-sealed milking cans. Milk used for processing into cheese was taken from the 5 and 7 d morning milking after AFB_1 addition in the diet. Milk from the high and low AF treatments was collected in 15 L buckets on the dairy farm. Milk was pooled and brought to the Richardson Dairy Products Laboratory at Utah State University (USU) and

transferred into a 15 L steel bucket. Milk not used for cheese production was disposed into the drain on the farm.

Cheese Production

The cheese production was during April of 2007. Two sets of cheeses, Cheddar and Fresco, with two AFM_1 concentrations, high and low were made for each concentration of AFM_1 in milk. The following are some characteristic describing each cheese batch. Cheddar cheese is hard-cooked, and long-matured cheese, it has a firm texture, pale yellow to orange color with different intensity of their sharp flavor going from mild to extra-sharp. Composition of Cheddar cheese consists of water content of 30 to 32%, 34 % protein and 29% fats. On other hand, Fresco cheese is bland high moisture, usually lower in fat and sodium than other cheeses. Composition of Fresco cheese is 59% water, 19% protein and 15% lipid content (Dr. Nicoletta Fuca, personal communication).

The 15 L buckets were placed in a small steam jacketed vat. Adequate water was added to the vat, without tipping over the containers. The milk buckets were heated to 65ºC for Cheddar cheese and 73ºC for Fresco cheese by heating the water and stirring the milk. When temperatures were reached, the water was drained and replaced with cold water. The milk buckets were cooled to the set point for cheese of 31ºC for Cheddar and 33ºC for Fresco cheese. This provided a pseudo-pasteurization heat treatment to facilitate cheese making. Twenty liters of milk from each treatment (AF high and AF low) were used to make each type of cheese.

Making of Cheddar Cheese

Milk from both treatments of AF (high vs. low) was converted into Cheddar cheese using a stirred curd method based upon typical manufacturing procedures at Richardson Dairy Products Laboratory at USU. Temperature for cheese production was set at 31ºC. After reaching that temperature 3 g of DVS 850 standard culture, 1.5 ml of $CaCl_2$, 1 ml of annatto colorant and 1 ml of coagulant were added. This was stirred for two min. and then left undisturbed to ripen until ready to cut. Curds were cut and left undisturbed for 5 min. The curds were stirred and slowly cooked until the temperature reached 49ºC. Whey was then drained, and curds were stirred vigorously to dry the curd. Salt was added (33 g) when the pH reached 5.3. Curds were pressed overnight in tubes at 20 psi to yield approximately 0.5 kg blocks of Cheddar cheese. Blocks were cut into 0.2 kg samples labeled, vacuum packaged and stored at 7ºC for 15 d.

Making of Fresco Cheese

Milk from cows administered high or low AF was converted into Fresco cheese based upon typical manufacturing procedures at Richardson Dairy Products Laboratory at USU as followed; milk temperature was set at 34ºC, and then 4 g of TC-20C *Strep. thermophilus* culture was added and the milk left undisturbed for 15 min, then, 1 ml of DS chymosin (aspartic acid enzyme) was added as rennet. Curds were cut after 30 min into 1.5 cm cubes and 8 L of the whey were drained and replaced with hot water. The curds were stirred for 30 min until they reached pH 5.7. Salt was added (70 g). Curds were filled into cloth lines tubes and pressed for 2 h at 5 psi. Blocks were cut into 0.2 kg samples, labeled, vacuum packaged, and stored at 3ºC for 15 d.

Detection and Quantification of Aflatoxin M_1 in Cheese

Chemicals, Standards and Laboratory. The fluorometer calibration standards Aflatest-M™, the immunoaffinity columns AflaM1™ and Developer were purchased from VICAM (Watertown, MA). All organic solvents were obtained from Fisher Scientific (Pittsburgh, PA). Deionization water used in the experiments was processed by a Barnstead water purification system (Barnstead PCS, Dubuque, IA). Microfiber filters (0.45µm) were obtained from Fisher Scientific. All other materials such as disposable pipets, filter paper, microfilters, disposable cubets and syringes were purchased at the chemist store at USU. Extraction and analysis of AF was made in a room protected from daylight and vials were of amber color or protected with foil paper, because AF are degraded by UV light.

Extraction of Aflatoxin M_1 from Cheese. Aflatoxin M_1 was extracted from cheese using the method described by Dragacci et al. (1995) with some modification. Cheese was brought to room temperature and then cut into small pieces and minced thoroughly. On a clean table minced cheese was distributed to form a square. Diagonals were traced on the square, and two of the opposite triangle sides were discarded. The remaining triangles went through the same process until the sample was around 20 g. A 10 g subsample of cheese was blended with 10 g of celite (Diatomaceous earth powder) 545 (Fisher Scientific Inc.), and 80 ml dichloromethane, for two min. at high speed (Waring blender, Model No 35BL 64, Merck, Poole, UK). After washing further with 40 ml dichloromethane, the mixture was filtered (filter paper #1 circles 24.0 cm; Whatman) and pressed to release maximum amount of filtrate. Each cheese was analyzed in triplicate.

The filtrate was evaporated at 40°C on a rotary flash evaporator (Buchler Instruments, Fort Lee, NJ) with a precision stainless steel water bath Model 183. The residue was dissolved in 1 ml methanol, 30 ml water (1:30, v/v) and 50 ml n-hexane, and then transferred to a separating funnel. The lower (water phase) layer was collected and then the hexane phase was washed twice with 10 ml water. The water phase was collected after each wash.

Bottles of the collected water fraction were wrapped in paper foil to avoid light contact and stored at 4º C until processed. The aqueous phase was passed through an AFM_1-HPLC immunoaffinity column (AFM_1 monoclonal antibody-based affinity chromatography of VICAM, Watertown, MA) at a rate of 2-3 ml/min. This process was performed in a solid phase extraction manifold (SPE Manifold**,** Waters, Watertown, MA). As the sample passed through the column, the antibodies selectively bound with AFM_1 forming a complex. Then the immunoaffinity column was washed twice with 10 ml of distilled water to eliminate impurities. The toxin was eluted using 2 ml methanol. Once the AF samples were eluted, the 2 ml liquid was divided into 0.5 ml vials for HPLC measurement and 1 ml glass tubes for fluorometric measurement.

Aflatoxin M_1 Determination

Fluorometric Determination. The analyses were performed by a VICAM Series-4ex Fluorometer optical system (VICAM, Watertown, MA) equipped with a high intensity pulsed Xenon lamp, together with selected fluorescence excitation and emission filters. Linear calibration of the instrument was developed by subsequently reading three quinine sulfate dehydrate reference standards (Aflatest-^{MTM}; VICAM). The first and the

second vials were used to set the higher and lower calibration points and the last vial was used to verify the instrument linearity, as referred to in the VICAM manual.

The reading range was fixed at 0.01 – 22 μg/kg for AFM_1, with a precision of 0.5% and accuracy of 1%, as reported in the VICAM manual. Two ml of reagent (1ml methanol + 1mL developer) and 2mL of distilled water were consecutively read as blank samples. In the fluorometric method the AFM_1 elution was mixed with developer solution. Aflatoxin enhancer (VICAM developer), should be prepared daily. It is not recommended to use if color is lost or after six hours have elapsed. According to manufacture one part of concentrate developer is mixed with nine parts of distilled/deionized water. Then 1 ml of enhancer solution is used with 1 ml of eluted AFM_1 and read in the fluorometer. Analyses were performed at the same time as the HPLC was injected with the same sample (approximately every ten minutes). All cheese samples were run in triplicate.

High Pressure Liquid Chromatography. The HPLC analysis was performed using a Waters 2695 Separations Module equipped with a Waters 2996 PAD and a Waters 2475 multi λ fluorescence detector (Waters, Watertown, MA). The system and data acquisition were controlled by, Empower™ Chromatography PC software for the PDA detector (Waters, Watertown, MA). The AFM_1 was separated with a Simmetry® C18 LiChospher 100 column with 3.5 μm particle size (150 x 4.6 mm I.D.) at room temperature, with acetonitrile/water (25:75 v/v) as the mobile phase (flow rate one ml/min). The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths. Standard AFM_1 (Sigma Chemicals Co., St. Louis, MO) was used to prepare a series of working standards of 12, 120, 240, 360 and 480 μgAFM_1/L. Calibration

curves were arranged by plotting the peak area for each standard against the quantity of AFM_1 injected. All analyses were done in triplicate, injecting 100 µl into the HPLC.

Stability of the Method

Stability of the method was assessed by measurement AFM_1 from a cheese sample on four different days. The same extraction and analyses as above was performed.

Statistical Analyses

The design of the experiment was a 2-way Fixed Factor Analysis. Factor A concentration of AF (high and low) and factor B cheese type (fresco and cheddar) with 30 (cheese) repetitions. Data were analyzed using the PROC MIXED function in SAS (SAS, 2009). The dependent variables were methods of analysis and the independent variable was cheese type (Cheddar and Fresco), treatment (high and low AFM_1) and an interaction term. Least squares means were computed and means differences determinate by the Tukey's multiple means comparison with significance at $P < 0.05$. Pearson's correlation was run between the two analysis methods and the PROC REG function in SAS used to determine the linear relationship between the two analysis methods.

A linear relationship was computed and the correlation is represented by the equation ($y = mx + b$), where m = coefficient, x = fluorometer or hplc value and, b = intercept.

RESULTS AND DISCUSSION

Carryover of Aflatoxin M_1 Feeding Trail

A sample of milk for each treatment level was taken two days prior to cheese preparation and analyzed for AFM_1 in our laboratory by the HPLC technique (Dragacci et al., 1995). Another sample was sent to an external laboratory. Table 5 summarizes the concentration obtained for those samples.

Table 5. Concentration of Aflatoxin M_1 in Milk Prior to Cheese Preparation. Evaluation was made April 16, 2007 by the USU lab and February 12, 2009 by the external lab.

Treatment[1]	USU lab, µg/kg	External lab, µg/kg
High	0.8	0.7
Low	0.03	Trace

[1]Milk was pooled for both cows into one treatment

The experiment was not set up to measure AFM_1 carryover. However, based on the amount of grain fed to each group and the concentration of AFB_1 in grain, we retrospectively calculated the possible carryover. We calculated that the carryover of AFM_1 from the high treatments was approximately 1.3% and carryover for the low treatment of AFM_1 was 0.75%. The high AF group receive 1.6 kg of corn daily containing 40 µg/kg of AFB_1 (1.6 kg x 40 µg/kg = 64 µg/kg x 1.3% carryover = 0.8 µg/kg of AFM_1). The low group received the same amount of corn but only 0.125 kg were from contaminated corn at 40 µg/kg of AFB1 (0.125 kg x 40 µg/kg = 5 µg/kg x 0.75 % = 0.03 µg/kg). These concentrations represent the two universal restrictions for AFM_1, 0.03 µg/kg is below European regulatory value (0.05 µg/kg) and 0.8 µg/kg is over

American restriction (0.5 µg/kg). Those values are in accordance with Applebaum et al. (1982) and Jouany and Diaz (2005).

Detection of Aflatoxin M_1 in Cheese

To our knowledge, this is the first report using a fluorometric procedure to analyze AFM_1 in cheese. The two types of cheeses were chosen to represent a moist, soft cheese and a dry, hard cheese in order to evaluate whether the cheese type had any impact on AFM_1 analysis.

The main objective was to compare HPLC vs. fluorometry measurement of AFM_1 in cheese. The presence of AFM_1 was detected in all samples (100%) by both methods. Table 6 shows the statistical analysis for the cheese type, treatment, and analysis method.

Table 6. Means of Aflatoxin M_1 for High Performance Liquid Chromatography and Fluorometry Method From Different Cheese Types and Aflatoxin M_1 Fed Concentrations

Cheese	Treatment	Method	Mean	SD	Range	CV(%)
Cheddar	High	HPLC	1.03^a	0.41	0.22 - 1.98	39.52
		Fluorometry	1.36^a	1.48	0.29 - 8.06	108.9
	Low	HPLC	0.03^b	0.01	0.01 - 0.05	34.09
		Fluorometry	0.02^b	0.02	0.01 - 0.10	86.93
Fresco	High	HPLC	0.93^a	0.36	0.32 - 2.06	38.86
		Fluorometry	0.95^a	0.27	0.40 - 1.56	28.04
	Low	HPLC	0.04^b	0.03	0.01 - 0.19	81.94
		Fluorometry	0.05^b	0.02	0.01 - 0.08	44.60

$^{a-b}$ Means within a column with different superscripts differ ($P<0.01$)

There were no significant differences between cheese type ($P<0.05$). Amounts of AFM_1 in feed resulted in difference of AFM_1 in cheese regardless of its type ($P<0.01$). Interactions between concentration and cheese sample or cheese type were not statistical

significant at (P>0.05). There were no differences between methods of detection with treatment (P>0.05).

Yousef and Marth (1989) reported that the concentration of AFM_1 in cheese was about 3.0 (2.5 – 3.5) and 4.8 (3.9 – 5.8) fold higher for soft and hard cheese than that found in milk, respectively. In this study AFM_1 concentration in both cheeses was higher than they found in milk, except for low Cheddar cheese batch, but not as high as reported above by Yousef and Marth.

Carryover values of the AFM_1 (Table 7) were over 100% for the high concentration of AFM_1 in Cheddar and Fresco and for low concentration in Fresco cheese, respectively (average of 132%). Cheddar cheese low concentration of AFM_1 had lower carryover rate (89%) than other cheese batches. This may be due to limited casein proteolysis. Gürses et al. (2004) suggested that proteolysis of casein may increase the recovery of AFM_1 from cheese.

Table 7. Mean Carryover of Aflatoxin M_1 in Cheese as Compared to Milk Content

	Cheese[1]			
	Cheddar		Fresco	
	High	Low	High	Low
HPLC	128	97	115	133
Fluorometric	163	80	119	133

[1]Values are expressed in %

Based on the higher carryover for AFM_1 in cheese (Table 7), we also conclude that AF is heat stable. These results are in concordance with Barbiroli et al. (2007) who reported that AF was stable at temperatures used during cheese production.

Another difference found between methods of cheese production was the amount and kind of starter bacteria used to initiate the ripening process. In fresh cheese, 4 g of *Thermophilus* bacteria (TS-20CS) was used. In Cheddar cheese, 3 g of a starter culture (DVS 850) was used. Sinha (1998) reported that bacteria and fungus potentially degraded or modified AF.

A linear regression model was derived from data in Table 6. HPLC (µg/kg) = 0.303+ 0.344 (fluorometer value), $r^2 = 0.535$ for both methods, t = 8.04 ($P<0.01$). The regression analyses between HPLC and fluorometry (Figure 1 and Figure 2) shows that fluorometry is a good predictor to screen AFM_1 concentration in cheese.

Stability of the Analytical Method

Stability is a validated quantitative analytical procedure that can detect the changes with time of the active ingredient (CIPAC, 1999). Results of the stability for fluorometry and HPLC are summarized in Table 8. The fluorometry method showed good CV(%) for day one then increased by day 2 to slightly over 20%, and then increases again on day 3. For HPLC, the CV(%) stayed very close together for all 4 d. A possible recommendation for fluorometry is to use fresh samples during each determination to avoid variability on the detection of AFM_1 concentration in cheese.

Table 8. Stability of the Analytical Method for Aflatoxin M_1 Detected by Fluorometry and HPLC

	Fluor				HPLC			
Day	1	2	3	4	1	2	3	4
Mean	0.09	0.11	0.09	0.09	1.24	1.28	1.08	1.28
SD	0.01	0.02	0.02	0.03	0.03	0.02	0.02	0.07
CV%	8.27	20.11	29.21	28.24	2.10	1.68	1.72	2.81

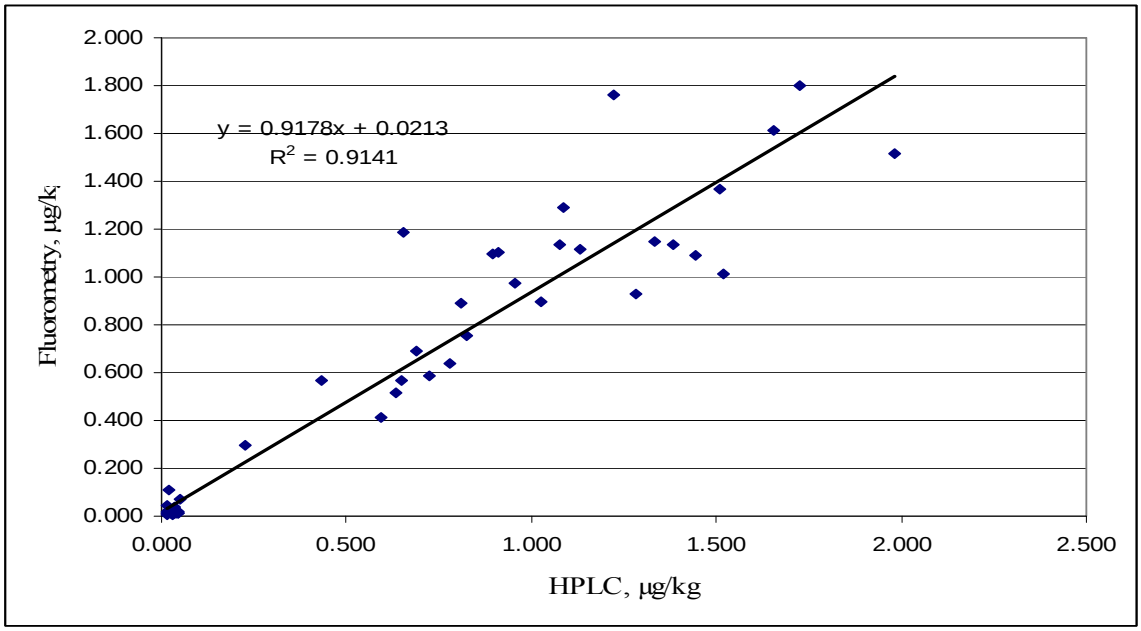

Figure 1. Relationship of Aflatoxin M_1 High and Low Concentration Between HPLC and Fluorometry Method From Cheddar Cheese

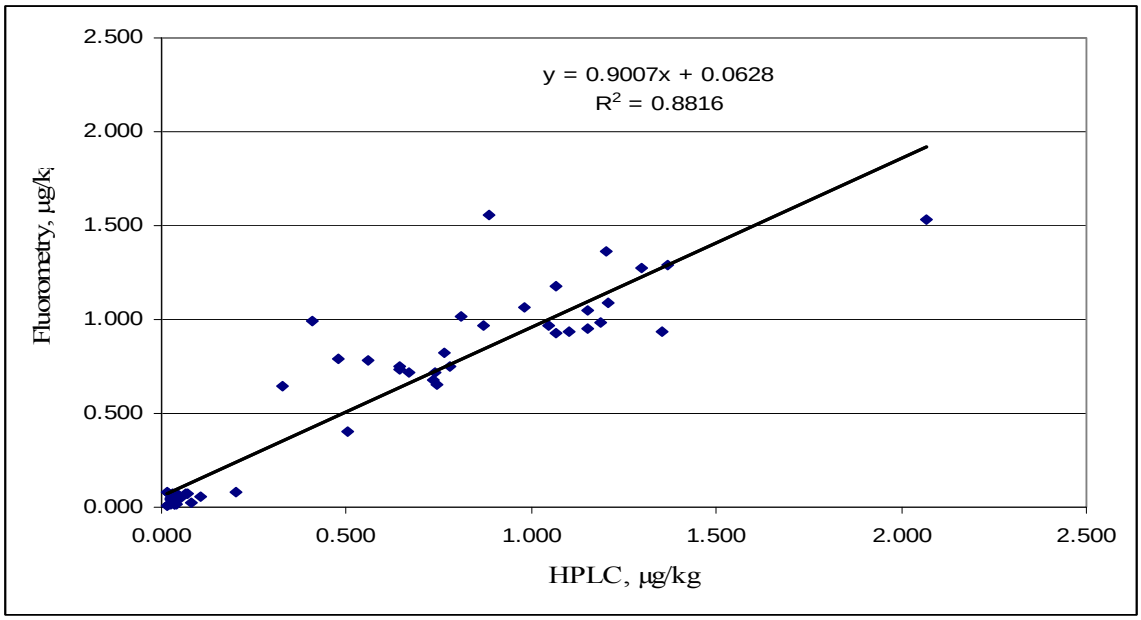

Figure 2. Relationship of Aflatoxin M_1 High and Low Concentration Between HPLC and Fluorometry From Fresco Cheese

Fluorometry Cross-Validation

Several properties are required to make a laboratory assay suitable for assimilation into commercial protocol, properties such as sensitivity, specificity, and accuracy (Shah, 2007). A cross validation is the comparison of two or more methods to generate data for the same study. In our study we use HPLC as a reference method and fluorometry as the method to be validated. We follow the criteria published by CIPAC 3870 (CIPAC, 1999).

Estimation of the Precision. Data obtained from calibration and readings by AFM1 blank dilution were correct and indicated that repeatability was acceptable.

Demonstration of Accuracy. In the present study, AFM_1 used to calibrate the fluorometer was purchased with certification of authenticity and handled as recommended. To avoid the interference from excipients, AFM_1 was extracted the same way for both methods. The results were found to be not significantly different ($P<0.01$) between fluorometry and HPLC. In all cases we found a lack of interference.

Comparison of the Results Obtained by the Two Methods. Each cheese batch was compared; results were the same for both methods of analysis. The fluorometry method is validated for the detection of AFM_1 in cheese.

According to Gilbert (2002), methods for validation should take into consideration economical factors as well as efficiency in time and materials. We evaluate the cost of the fluorometry compared to the HPLC method, respectively. Table 9 shows comparison between those two methods.

Comparing the cost of the equipment between the fluorometer and HPLC, the HPLC is almost 20-fold greater than the fluorometer. Considering the variable time, the

HPLC took longer per sample. Although an automatic injection was use, time average for each sample is 10 min. Taking into consideration the variable cost of analysis, material for running the HPLC include HPLC quality solvents which are more expensed than regular solvents.

Table 9. Comparison of Variables From Fluorometry Against High Pressure Liquid Chromatography

	Methods	
	HPLC	Fluorometry
Equipment cost	$78,000.00	$4.000.00
Time[1]	20[2]	2
Difficult	Very difficult[3]	Easy[4]
Variable cost[5]	$480.00	$273.00

[1]Time calculated by 120 samples
[2]Did not include time to warmed the machine, washed columns time, respectively
[3]Include standards calibration and curves determination
[4]Print read-out containing the results in ppb (µg/kg)
[5]Did not include labor fees

CONCLUSIONS

The reduced time to run a sample, the lower cost of the fluorometer machine and the lower variable costs of analysis make the fluorometry method more useful than the HPLC method for screening samples. A printable readout with the amount of AFM_1 can be obtained from fluorometer and eliminate some of variability due to determination at the beginning and end of HPLC curves.

The fluorometric method is simple and reliable. Although there were slight numerical differences between the HPLC and fluorometry methods in detecting AFM_1 in different types of cheeses, those were not statistically different.

This study also indicates that the AFM_1 immunoaffinity column developed for milk, plus the different solvents can be used for AFM_1 detection in cheese. Carryover rate, compared to original milk content, of the AFM_1 in cheeses differs from those previously reported in the literature. Those differences may be attributed to cheese preparation. It will be interesting to compare the protocol used in this study at other cheese factories.

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