4-1-2015

Removing aflatoxin M1 from milk using activated carbon and its effects on protein concentration

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Removing Aflatoxin M_1 from Milk using Activated Carbon and its Effects on Protein Concentration

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A Thesis,

Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for *Cursus Honorum*

Mississippi State, Mississippi

April 2015

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CHAPTER

1. INTRODUCTION

1.1 History of Aflatoxins

Mycotoxins are natural toxins produced by fungi including molds. The name mycotoxin is translated from Greek as μύκης (mykes, mukos) "fungus" and τοξικόν (toxikon) "poison," with this term being introduced by British researchers in 1962. [10] Mycotoxins are known to grow on nuts, grains, and corn. There are three subcategories of mycotoxins: aflatoxins, fumonisins, and vomitoxins, all of which are regulated due to the danger to human and animal health they impose. Out of all mycotoxins, aflatoxins cause the greatest losses and highest management costs due to their extremely high toxicity on a unit basis and their long history of harsh regulation. [17] They are also known to be the most toxic/carcinogenic compounds of all the mycotoxins. [7]

Aflatoxins are produced by toxigenic strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and are found in feed as aflatoxin B_1, B_2, G_1, and G_2 and found in milk as aflatoxin metabolite M_1 and M_2. [2, 20] All are quite stable in many foods while also being fairly resistant to degradation. [2, 15] Because of the danger of these toxins, the maximum allowable aflatoxin concentration is regulated by the Food and Drug Administration (FDA) for the United States for feeds at 20 μg/kg and by the European Union (EU) for Europe at a much lower 2 μg/kg. [17, 20]

Aflatoxins were first discovered when an outbreak of "Turkey X Disease," now known to be aflatoxicosis, occurred over many turkey populations in England in the 1960s, which was traced to aflatoxin contaminated groundnut meal that was being used as feed for the turkeys. [6] From there, research was conducted that eventually led to the discovery of the four types of aflatoxin and the two aflatoxin metabolites as mentioned previously. The aflatoxins were named based on the physical characteristic of fluorescence at 395nm, with B_1 and B_2 fluorescing blue

and G_1 and G_2 fluorescing green. Both M_1 and M_2 were named instead for their presence in milk, rather than their fluorescence. [10]

1.2 Propagation of Aflatoxin

Fungal contamination and subsequent production of aflatoxin can occur in crops while growing in the field, at harvest, during postharvest operations, and in storage. [23] However, *Aspergillus* typically flourishes in grains stored in improper conditions. Hot, humid conditions, usually caused by un-aerated storerooms, are linked to increased aflatoxin contamination in stored feed. [8, 9] Animals given feed that has been in a storeroom exhibit higher aflatoxin levels than animals that rely on grass and shrubbery as their main food source. [8] Because of this, cold seasons usually yield animals with higher levels of aflatoxin due to the animals having to be fed supplemental feed. In contrast, spring and summer seasons show a drop in aflatoxin levels in animals due to the abundance of fresh grass and other edible greenery. [13]

1.3 Aflatoxin M_1

1.3.1 Structure and Formation of Aflatoxin M_1

Aflatoxin B_1, the most toxic and most frequent form found in contaminated food and feeds, is ingested by humans or animals and metabolized by attaching to macromolecules. [2] It is then transformed into different metabolites in the liver by the hepatic microsomal mixed function oxidase system and cytochrome P_{450}. [9, 13] The metabolites produced include aflatoxin B_2a, aflatoxin Q_1, aflatoxin P_1, aflatoxicol H_1, aflatoxin M_1 (AFM_1), AFB_1 aflatoxicol-M_1, and epoxide. Aflatoxin M_1 is the compound 4-hydroxy AFB_1. [9, 10] The biliary, or bile ducts, eliminates about 60% of these metabolites with aflatoxin B_1 exiting the body through urine. [9] About 0.3-6.2% of AFB_1 present in animal feed is metabolized to AFM_1 that is secreted through milk in the mammary glands of lactating humans and animals. [1, 10]

1.3.2 Aflatoxin M_1 in Milk and Milk Products

Aflatoxin M_1 binds to milk proteins such as casein and whey. [2] However, this binding to proteins is not homogeneous. Most researchers find that aflatoxin binds preferentially to casein. For example, a study by Grant and Carlson in 1971 showed that 80% of AFM_1 is found in the skim portion of milk, which is made of casein proteins, when the cream is separated due to AFM_1 binding to casein. In contrast, other researchers have found that aflatoxin binds preferentially to whey at 50%, 50%, 53-58%, 60%, and 66%. [13] Despite this discrepancy, it is now a commonly accepted fact that AFM_1 binds preferentially to casein based on the reports of many different researchers.

Levels of aflatoxin in milk depend on several factors such as animal breed, lactation period, mammary infections etc., and can be detected 12-24 hours after AFB_1 ingestion, reaching a high level after a few days. If ingestion of AFB_1 is stopped, a period of 72 hours is required before AFM_1 is no longer detectable. [6] Urine can also be assessed for AFM_1 levels 24-48 hours after exposure. [11] However, it is a more common method to test milk because milk is sold as a product and urine is not. Experiments have been conducted with differing dairy cattle breeds to assess the effects of aflatoxin between breeds. Holstein cows were given rations of feed contaminated with 80, 86, 470, 557, 1089 and 1493 μg/kg of AFB_1 which resulted in AFM_1 concentrations of 0.245, 1.5, 13.7, 4.7, 12.4 and 20.2 mg/L, respectively. Brindle cows were given 540 μg/kg of AFB_1 resulting in 0.92 mg/L. [12] From these results, it can be concluded that Holstein milk harbors more aflatoxin M_1 per μg/kg of AFB_1 given than Brindle milk does. In other breeds, values of contamination range between 64 and 1799 μg/kg of AFB_1 giving some residues in milk between 0.35 and 14.2 mg/L of AFM_1. Therefore, with an intake of AFB_1 for 2-60 mg / cow / day, AFM_1 residues in milk can range between 1 and 50 μg/kg. [12]

In addition to being found in milk, AFM_1 is also found in milk products such as cheese and yogurt. Due to the process of concentrating milk to yield cheese, AFM_1 is three times higher in soft cheeses and five times higher in hard cheeses than the milk the cheese originated from. [21] It is important to note that the amount of AFB_1 ingested by animals does not have a 1:1 ratio to AFM_1 excretion in urine and milk. In fact, most of the aflatoxins ingested by ruminants is degraded by the flora in the rumen. [8] This leads to only a 1–7% excretion of aflatoxin M_1 of the total amount of aflatoxin B_1 ingested. [21]

1.3.3 Current Methods of Aflatoxin B_1 and Aflatoxin M_1 Extraction

Aflatoxin M_1 is categorized as a group 2B carcinogen (probable human carcinogen), which is in the same category as chloroform and diesel exhaust. [6] The hazard of ingesting this toxin has been combatted by research towards the extraction of aflatoxin B_1 from feed and aflatoxin M_1 milk. Extraction from feed has been successful, yielding many methods. Adsorbent compounds, such as NovaSil clay, can be directly mixed with animal feed and act as a high affinity and high capacity binder when in the GI tract for aflatoxins. Green tea polyphenols (GTPs) are another type of product that can be mixed with feed. These have been shown to inhibit the chemically-induced cancer that can result from AFB_1. Chlorophyllin, yet another feed component, prevents the absorption of aflatoxin within the digestive tract by sequestering it. [22] Although these methods are useful in preventing the formation of AFM_1 in the milk, the adding of compounds to feed can require expensive equipment and has been shown to reduce the nutritional quality of the feed. [20] Due to these complications, the search for a way to effectively extract aflatoxin directly from milk has been of recent interest.

Research on extracting AFM_1 from milk has mostly led to what doesn't extract AFM_1 from milk. Pasteurization, a heating process that milk undergoes to kill bacteria, and sterilization have little

effect on removing aflatoxin from milk. [8] A study by Choudhary et al. in 1998 reported that sterilization of milk at 121°C for 15 minutes only caused a 12.21% degradation of AFM_1, while boiling decreased AFM_1 by 14.5%. They suggested that an extended time period and increased temperatures might decrease AFM_1 by a greater amount. [13] Continued experiments involving heat have yielded similiarly disappointing results. Ultrafiltration with acidic or enzymatic treatments does not have an effect on aflatoxin M_1. [13] However, a combined method of low pH and heat was able to denature whey protein enough that they lost their affinity for aflatoxin M_1. [3] This combined method did not make much of difference, as aflatoxin is known to preferentially bind to casein. Other ineffective methods include using UV, light, and ionizing radiation. [20]

1.4 Aflatoxin's Effect on the Health of Humans and Animals

The reason aflatoxin is highly regulated rests highly on the impact it has on both human and animal health. Aflatoxin B_1 is categorized as a Group 1 carcinogen and is one of the most potent human chemical liver carcinogens known. Liver cancer flourishes in regions, such as South East Asia and Africa, without aflatoxin regulations on foodstuffs. [20] It is estimated that 26,000 Africans living south of the Sahara die annually of liver cancer associated with aflatoxin exposure. [21] Probably most concerning for humans is its indirect effect on children through milk, as children are more vulnerable to toxins and are known to ingest more milk when compared to adults. [21] Infants drinking AFM_1 contaminated milk exhibit immune suppression with higher rates of illness, stunted height, and stunted weight gain during the first year of life. [11, 21, 23] In addition to these negative effects on humans, animals also exhibit health, performance, and reproduction problems when given aflatoxin-contaminated feed. [15] Just as in humans, aflatoxins cause liver damage and immune suppression. Decreased milk and egg

production and embryo toxicity may also occur. [20] Feed conversion ratios are known to increase coupled with a decrease in average daily gain and general decrease in body weight. [21] Dairy animals are especially effected as, in addition to these health and reproduction problems, milk production also decreases. For example, a Gregorian dairy herd eating contaminated feed was found to produce 28% more milk after only three weeks of eating non-aflatoxin contaminated feed. [16] Because of these negative effects, regulatory limits for AFM_1 in milk are 0.5 μg/kg for milk in the US and 0.05 μg/kg for milk in Europe. [1, 9]

1.5 Aflatoxin's Effect on the Economy

All relevant studies to date indicate that there is a significant cost impact due to combating aflatoxins. For the United States, costs of biocontrol methods such as utilizing transgenic crops in the hopes of combating aflatoxin have an estimated cost of $42-79/hectacre. [22] Research costs for the year 2000 are known to be over $17.7 million. Sixty scientists were provided this amount for the primary focus of prevention of the fungus and toxin production in the crop. In addition to biocontrol methods and research costs, test costs add another $30-50 million worth of loss per year. The peanut industry suffers a $25 million loss per year from testing costs, market rejection, etc. For a particularly bad year (1999), south Texas alone exhibited estimated losses of $7 million due to aflatoxin-contaminated cottonseed. The tree nut industry is also affected. The total direct dollar market value loss of the walnut industry was $38,704,000 in 2000-2001. The almond market suffered a similar loss in 1995-2001 as the total direct dollar market value loss ranged from $23,265,000 to $47,310,000. [17]

However, the above numbers only represent the negative impacts to the United States. Other countries suffer even bigger losses due to warmer climates and lack of regulations. [4] For example, Roy reports that, due to regulations on African trade, the groundnut and cereal industry

suffers a loss of $750 million annually. [18] African trade in particular suffers from aflatoxin regulations due to the fact that Africa has not implemented aflatoxin regulations. When exporting foodstuffs with possible aflatoxin contamination, tests must be conducted in order to make sure the limitations other countries have set forth are upheld. Rejection and test costs are factored in to Roy's numbers. Lubulwa and Davis (1994) calculated aflatoxin's "social" costs—human liver cancer, animal diseases, and market rejection—in three Asian nations to be $1 billion annually.

2. HYPOTHESIS AND OBJECTIVE

AFM_1 binds to milk proteins such as casein, whey, and especially curds (which are made out of acidified and concentrated casein proteins). During the heat treatment of milk, whey proteins begin to denature and completely denature during fermentation. Whey proteins lose their aflatoxin binding ability when denatured. Concurrently, casein is the protein that aflatoxin mainly binds to via casein's hydrophobic sites. [2] Only the combined action of heat and low pH is able to denature whey proteins to a point where they lose their AFM_1 binding ability. [3]

The purpose of this study is to determine the effect that aflatoxin M_1 has on casein and whey protein concentration in milk. The ability of activated carbon to remove AFM_1 after interaction with added milk proteins will also be measured.

3. MATERIALS AND METHODS

2.1 Protein Determination in Milk

Raw milk was obtained from the Mississippi State Dairy Farm and separated into two 2000 mL volumetric flasks. AFM_1 was obtained from Sigma Aldrich and used to spike raw milk. One 2000 mL volumetric flask served as the control (raw milk only), while the other was spiked with 1 ppb AFM_1. Casein was obtained from Fisher Science Education (Nazareth, PA), and spray-dried whey from bovine milk (concentration 11%) was obtained from Sigma Aldrich, both for the use of increasing protein concentration in milk. Activated carbon (DARCO 12x20 LI) was obtained from Norit (Marshall, TX) for use in binding AFM_1 from milk. Acetonitrile was obtained from Optima for use in performing salting out with QuEChERS.

A 2^4 (4 factors each at 2 levels) factorial arrangement of treatments was performed yielding 16 treatments of samples with different additions of AFM_1 (0 or 1 ppb), casein (0 or 2%), whey (0 or 1%), and activated carbon (0 or 1%). Milk was measured out into 50 mL centrifugation tubes and the appropriate amount of casein and whey was added to each sample. Sodium hydroxide (0.1g) was added to each sample tube to assist in dissolving the protein with milk. Each sample was also inverted, then stirred slowly for 5 minutes using a stir bar to ensure complete mixing without breakdown of protein. Activated carbon was then added to respective samples and allowed a 15 minute contact time with gentle shaking via Burrell Wrist-Action Shaker. Each sample was run in 3 reps using LECO in order to determine protein concentration.

2.2 Determination of AFM_1 in Milk

A volume of 10 mL acetonitrile was added to each 15 mL milk sample for the extraction of AFM_1 from milk samples. Samples were allowed to shake in a GenoGrinder 2010 Spex

Sampleprep for 1 min at 1000 strokes/min. QuEChERS extraction salts (AOAC method), obtained from Agilent Technologies, were then added to each sample (1 packet per sample). The samples were allowed to shake in the GenoGrinder again for 1 min at 1000 strokes/min. Samples were centrifuged (IEC HN-SII centrifuge) for 5 min at 3500 rpm. A volume of 1.5 mL of the supernatant was collected from each sample and pipetted through PTFE syringe filters into 2 mL auto sampler vials. An Agilent 1260 Infinity LC Triple Quadrupole Mass Spectrometry was used to analyze samples for residual AFM_1 quantification.

4. RESULTS/DISCUSSION

Average percent protein results proved that activated carbon does not significantly affect protein concentration. Other experiments have also proved that activated carbon allows the preservation of chlorides and organic acids in addition to preserving proteins. [14] A comparison between sample one 3.89±0.07% (the control) and sample three 3.83±0.03% which contains 1% activated carbon can be made. The average percent protein differs only by 0.06% (Table 1). Therefore, average percent protein is not significantly affected by activated carbon.

Also as expected, upon the addition of casein or whey, average percent protein increased, regardless of the presence of activated carbon. Methods from Damin et al. suggest that milk proteins can be agitated for 10 minutes at 800rpm without denaturing significantly. [5] Our method of gentle shaking for 15 minutes ensured that added casein and whey proteins were not denatured, which is reflected in the results as an increase in average percent protein upon addition of casein and/or whey.

Table 1. Average Percent Protein and AFM_1 Levels after Binding. Average percent protein between 3 reps upon addition of differing amounts of AFM_1, activated carbon, casein, and whey to raw milk. Amount of AFM_1 remaining in samples after extraction using activated carbon is also shown. Bolded numbers indicate unexpected results in relation to casein.

Sample Number	AFM_1 (ppb)	Activated Carbon (%)	Casein (%)	Whey (%)	Average Protein (%)	AFM_1 detected after binding (ppb)
1	0	0	0	0	3.89±0.07	0.0000
2	1	0	0	0	3.91±0.02	0.3232
3	0	1	0	0	3.83±0.03	0.0000
4	1	1	0	0	**4.03±0.07**	0.3768
5	0	0	2	0	**7.25±0.05**	0.0000
6	1	0	2	0	**5.44±0.03**	0.5521
7	0	1	2	0	**7.42±0.06**	0.0000
8	1	1	2	0	**5.26±0.09**	0.4240
9	0	0	0	1	5.44±0.18	0.0000
10	1	0	0	1	5.62±0.06	0.8777
11	0	1	0	1	5.62±0.07	0.0000
12	1	1	0	1	5.47±0.09	0.9206
13	0	0	2	1	**5.19±0.15**	0.0000
14	1	0	2	1	**7.24±0.10**	0.9218
15	0	1	2	1	**5.46±0.03**	0.0000
16	1	1	2	1	**6.99±0.12**	not determined

However, some unexpected results were also identified. Upon addition of casein coupled with AFM_1, average percent protein decreased by 1.81% (samples 5 and 6). With the addition of 1% activated carbon to casein and AFM_1, the average percent protein decreased again by 2.16% (samples 7 and 8). This suggests that AFM_1 interacts with casein in a way that affects protein percentage in a negative manner. Because casein is known to form micelles (10-300nm), it can be assumed that AFM_1 is taken up by these micelles instead of being extracted by the activated carbon. [14] In an experiment performed by Sandra and Dalgleish, ultra-high pressure

homogenization (186±7 MPa) plus heat treatment (85±1°C for 10 min) was found to decrease the average diameter of casein micelles. [19] This method may be used in future experiments to lessen the amount of AFM_1 binding to casein micelles and allow for easier extraction of AFM_1 from milk proteins. Samples containing only whey and AFM_1 did not show this same pattern, which suggests that AFM_1 does not interact with whey in a way that decreases protein concentration.

Another unexpected result was identified in samples thirteen-sixteen. In samples thirteen and fifteen, average percent protein concentration did not increase as it should have upon the addition of 2% casein and 1% whey, only reaching an increase of 1.57% from the control. In samples fourteen and sixteen, average percent protein increased abnormally, from 5.19% to 7.24% and from 5.46% to 6.99%, considering the fact that AFM_1 was shown to decrease average percent protein when coupled with casein. These results suggest that too much protein oversaturated the milk samples. The combination of 2% casein and 1% whey added to the 3.89% protein that already existed in the raw milk likely caused oversaturation of the protein causing skewed results. To resolve this, percent protein added can be decreased or milk can be made to undergo ultra-high pressure homogenization, as mentioned above, which has been found to make casein to become more soluble. [19]

The results obtained from the HPLC indicated that little, if any, AFM_1 was bound from the samples (Table 1). Techniques using HPLC coupled with mass spectrometry have been proven to successfully quantify aflatoxins, including AFM_1, in bovine milk and other milk products. [24] This can be attributed to the fact that the samples were not sieved to remove activated carbon and were only allowed a 15 minute contact time, rather than sieving the activated carbon out with a 200-US mesh sieve and allowing a one hour contact time, as done in previous experiments. [14]

5. CONCLUSIONS

The results show that activated carbon does not affect percent protein concentration. However, AFM_1 may interact with casein in a manner that decreases protein concentration. We hypothesize that AFM_1 binds strongly to casein even when activated carbon is added. Future experimentation sieving out activated carbon and allowing a longer contact time for the purpose of extracting AFM_1 might be able to show whether or not AFM_1 will stay bound to casein or be extracted as normal. Ultra-high pressure homogenization coupled with a heat treatment may be used to make casein proteins more soluble. The effect on protein concentration after extraction of AFM_1 will also be important in order to determine if AFM_1 extraction by use of activated carbon results in decreased protein concentration in milk.

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