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AFLATOXIN M(1) ANALYSIS: EFFECTS OF FORMALDEHYDE AND STORAGE CONDITIONS

The University of Arizona M.S. 1986

AFLATOXIN M_1 ANALYSIS:

EFFECTS OF FORMALDEHYDE AND STORAGE CONDITIONS

by

Susan Klara Heimbecher

A Thesis Submitted to the Faculty of the

DEPARTMENT OF NUTRITION
AND FOOD SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN FOOD SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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APPROVAL BY THESIS DIRECTOR

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Date

ACKNOWLEDGMENTS

I wish to express my gratitude and appreciation to my major professor, Dr. Ralph L. Price, for his assistance and guidance during the research and writing of this thesis. Sincere thanks are also due Karen Jorgensen for her valuable technical assistance. I would also like to thank Michael Billotte for his generous permission and assistance in the use of laboratory facilities at the United Dairymen of Arizona.

Special thanks to my husband, Eugene, for his love and encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Spray-dried milk, naturally-contaminated with aflatoxin M_1 (AFM_1), was added to either raw or pasteurized whole milk to a final concentration of 1.1 ng AFM_1/ml milk. Formalin (37% w/w) was added to these milk solutions to final concentrations of zero, .025, .05 and 0.1% formaldehyde and samples were stored in the dark at 21°C in either plastic or glass containers. Samples were analyzed for AFM_1 at 0, 1, 2, 3 and 4 weeks. This experiment was repeated using only raw milk and glass containers with AFM_1 analyses at 0, 1 and 2 weeks. Twenty-five percent less AFM_1 was detected in milk stored in plastic containers than in glass but milk type, i.e. raw or pasteurized, had no effect. Aflatoxin M_1 losses increased over time and with formaldehyde concentration. In both experiments, AFM_1 milk concentrations after two weeks were less than .05 ng/ml in samples containing 0.1% formaldehyde.

INTRODUCTION

Given a known initial concentration, the amount of aflatoxin M_1 (AFM_1) detected in milk varies with a number of factors including the analytical method used, lab experience, storage conditions and sample age (McKinney 1986). Both the length of time involved in shipping samples and the unrefrigerated storage conditions can lead to milk deterioration and reduced AFM_1 levels. For this reason, milk samples to be analyzed were previously preserved with sodium azide; however research conducted in our laboratory showed that this was undesirable as the amounts of AFM_1 detected were inconsistent (Price and Jorgensen 1982). Currently formaldehyde is being used in place of sodium azide for preservation of milk samples. In this study the effects of milk type (raw and pasteurized), container type (glass and plastic), formaldehyde level and time on AFM_1 levels were examined for the following reasons.

1) Milk Type (raw and pasteurized) - Aflatoxin M_1 may be broken down by enzymes occurring naturally in milk or from microbial souces. Therefore, due to the destruction of enzymes and bacteria at pasteurization temperatures, AFM_1 levels might differ between raw and pasteurized milk.

2) Container Type (glass and plastic) - Although milk samples may be shipped in either glass or plastic, plastic containers are more easily handled as the risk from breakage is reduced. However, variation could be introduced by the adherence of AFM_1 to glass or plastic or by

the migration of plasticizers into the milk. These plasticizers might lead to either AFM_1 breakdown or to its occlusion by milk proteins.

3) Formaldehyde Level - Formaldehyde, typically added as formalin, has previously been used for the preservation of analytical milk samples and is currently added to milk in the Smalley check sample series for AFM_1 at level of 0.03% (McKinney 1986). It has also been used to decrease aflatoxins in contaminated peanut meal (Codifer, Mann and Dollear 1976). The effect of this preservative on AFM_1 levels was therefore investigated in milk samples containing zero to 0.1% formaldehyde.

4) Time - The amount of AFM_1 detected in milk samples is known to decrease with sample age (Stoloff et al. 1975). This is of practical importance as milk samples may sit for several weeks before analysis due to problems in shipping or handling. For this reason AFM_1 levels in milk samples stored up to four weeks were examined.

Due to analytical problems and incomplete data collection at the starting point, the effects of formaldehyde level and time on AFM_1 levels were re-examined in a second experiment. Naturally-contaminated milk could not be obtained for these experiments and, therefore, naturally-contaminated spray-dried milk was added to fluid commercial milks to obtain the desired levels of AFM_1.

LITERATURE REVIEW

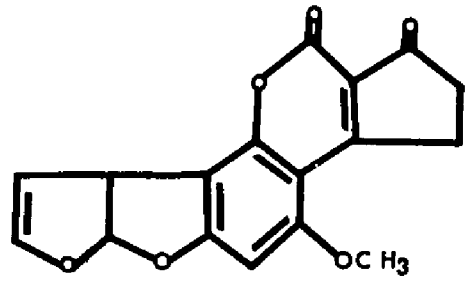
Although a variety of mycotoxins may contaminate agricultural commodities, aflatoxins are the most toxic and of greatest concern. Since their discovery in the 1960's, significant losses in the corn, cottonseed, peanut, dairy, cattle, swine and poultry industries have been attributed to aflatoxins (Brown 1982). Aflatoxin residues may be found in animal products and the need to quantitate AFM_1 in milk in the part per billion range has led to a number of analytical problems.

The Aflatoxin M_1 Problem

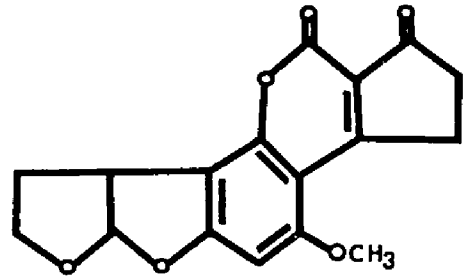
Background

Aflatoxins are a group of naturally-occurring hepetotoxic and carcinogenic metabolites of the molds *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are found co-existing in four forms; aflatoxin B_1, (AFB_1); aflatoxin B_2, (AFB_2); aflatoxin G_1, (AFG_1) and aflatoxin G_2, (AFG_2) (Figure 1). Of these, AFB_1, the most toxic, is found in the highest concentration in contaminated products. These mycotoxins were first discovered in 1960 after 100,000 young turkeys died in England of Turkey X Disease (Blount 1961). The source of the disease was traced to a shipment of Brazilian peanut meal heavily infected with *A. flavus* mycellium. The fluorescent, toxic compounds isolated from this meal were called aflatoxin.

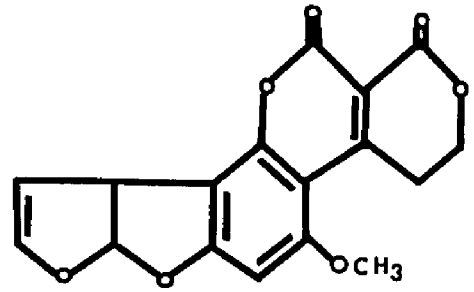
Aspergillus flavus and *A. parasiticus* spores are found in soil and air throughout most of the world and aflatoxin is most frequently



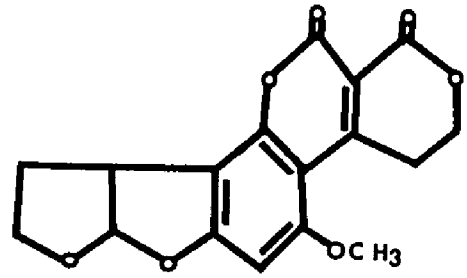
AFB_1



AFB_2



AFG_1

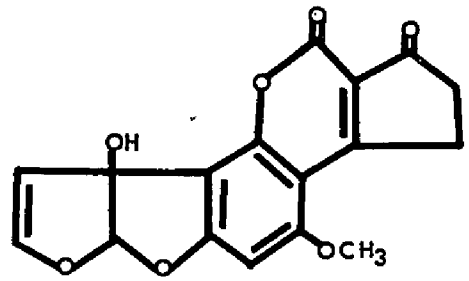


AFG_2

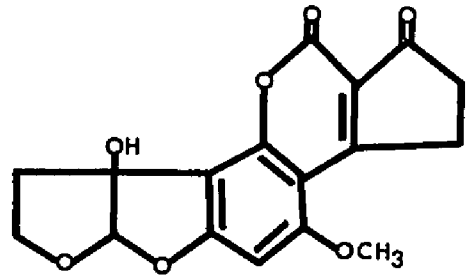
Figure 1. Aflatoxin Structures (AFB_1, AFB_2, AFG_1, AFG_2)

detected in agricultural commodities such as corn, peanuts, cottonseed and tree nuts (e. g. almonds and pistachios) (Council for Agricultural Science and Technology (CAST) 1979). Aflatoxin contamination was thought originally to be a storage or harvest problem; however, research has shown that it is more of a field problem which is influenced by the climatic environment (CAST 1979; Applebaum et al. 1982). Contamination is most extensive in regions with high temperatures and humidity such as southeastern and irrigated areas of southwestern United States and regions of Africa, India and southeastern Asia (CAST 1979).

Aflatoxin enters the milk supply when contaminated commodities such as corn, peanut meal or cottonseed are used as part of a dairy feed ration. The aflatoxin which is ingested by dairy cows is converted by the liver to less toxic forms for excretion from the body. About 1% of the original aflatoxin is excreted into the milk as AFM_1 which is the major aflatoxin found in milk (Stoloff 1980). Another aflatoxin form, aflatoxin M_2 (AFM_2), may also be excreted in milk but is of less concern as it has approximately one fourth the toxicity of AFM_1 (Kiermeier 1977) and its concentration in milk is much lower than that of AFM_1 (Figure 2). Because the young of many species, including humans, are more susceptible than adults to aflatoxin (Stoloff 1980) and in view of milk's role as a primary nutrient for infants and children, the Food and Drug Administration (FDA) established an action level of 0.5 ppb total aflatoxins in milk (FDA 1977). Although this Federal action level applies only to milk entering interstate commerce, many states have



AFM_1



AFM_2

Figure 2. Aflatoxin Structures (AFM_1, AFM_2)

adopted similar guidelines. Milk supplies are monitored to ensure this criteria is met.

Toxicity and Carcinogenicity

Aflatoxin toxicity studies have been based primarily on AFB_1, termed the most potent naturally occurring carcinogen known (CAST 1979). The daily dose of carcinogens which is required to cause a 50% tumor incidence in rats or mice over the course of their lifetime is 1 µg/kg body weight for AFB_1 while the dose for saccharin, one of the weakest carcinogens, is over a million fold greater (Maugh II 1978). Fewer studies have been conducted with AFM_1 than with AFB_1 due insufficient supplies of the former. However, for both AFB_1 and AFM_1 the liver is the target organ (Applebaum et al. 1982) and susceptible species include Fisher rats and rainbow trout (Wogan and Newberne 1967; Pong and Wogan 1971). Although AFM_1 is a metabolic product of AFB_1 their acute toxicities are similar. For instance, the LD_{50} value for AFB_1 or AFM_1 given as a single dose to one-day-old ducklings is 0.3-0.5 mg/kg body weight (Purchase 1967). However, the resistance of animal species to aflatoxins varies and in mice the LD_{50} for a single dose is 9 mg/kg body weight (Applebaum et al. 1982). The carcinogenic potential of AFM_1 relative to AFB_1 is only 2 to 10% in Fisher rats (Hsieh, Cullen and Ruebner 1984) and 30% in rainbow trout (Sinnhuber et al. 1970) while the comparative mutagenic potential by the Ames salmonella microsomal/ mammalian mutagenic test is 3.2% (Wong and Hsieh 1976). The young of many species are especially susceptible to aflatoxin (Stoloff 1980).

Occurrence in Milk

Aflatoxin M_1, "the milk toxin" is the major aflatoxin found in cow's milk (Applebaum et al. 1982). It is metabolically formed when the fourth carbon of AFB_1 is hydroxylated by the liver's microsomal mixed function oxidase system (Applebaum et al. 1982). Aflatoxin M_1 appears in cow's milk within 24 hours of ingesting AFB_1 contaminated feeds and falls to non-detectable levels within 4-5 days of removing AFB_1 from the diet (Stoloff 1980). The level of AFM_1 in milk increases linearly with AFB_1 intake (Purchase 1972) and the conversion rate or percentage of ingested AFB_1 excreted as AFM_1 in milk has been found to vary from 0.2 to 3.2%. Possible reasons for this variation include differences in the metabolic rate and stage of lactation of the dairy cows studied (Price et al. 1985).

Aflatoxin M_1 was first reported in commercial milk in 1968 from peanut producing areas of South Africa where 24% of the milk samples tested were positive. Since that time AFM_1 has been detected in commercial milks from a number of countries including West Germany, United States, Belgium and the Netherlands (Brown 1982). In West Germany, a postive relationship has been found between the occurrence of aflatoxin in milk and the use of imported oil seeds or other supplemental feeds amd aflatoxin positive milk samples in the United States show a geographic distribution related to known sources of contaminated feeds (Stoloff 1980).

The current Federal action level for dairy feeds is 20 ppb total aflatoxins (Labuza 1983). At an average conversion rate of about 1%

ingested AFB_1 to AFM_1 excreted, milk should contain no detectable aflatoxin if the federal regulations are met (Stoloff 1980). However, during 1977 corn grown in southeastern United States was unusually heavily contaminated with aflatoxin and in 63% of the milk surveyed from North Carolina, South Carolina, Georgia and Alabama AFM_1 was detected (Stoloff 1980). This prompted the FDA to establish an action level for milk of 0.5 ppb total aflatoxin (Brown 1982). The following year Arizona cottonseed was heavily contaminated and 40% of the milk samples surveyed in this state contained greater than 0.5 ppb AFM_1 and more than 100,000 L of milk were destroyed (Kobbe, Hsieh and Dunkley 1979). However, studies indicate that during years more typical of aflatoxin field contamination milk in the United States will not contain more than 0.1 ng/ml AFM_1 (Stoloff, Wood and Carter 1981).

Occurrence in Manufactured Dairy Products

The AFM_1 concentration in milk products increases as water and fat are removed. For instance, in dried milk the AFM_1 concentration is increased 8 fold (Brown 1982) while in butter, due to AFM_1 insolubility in milk fat and adsorption to curd, the concentration is 10% that of the original milk (Stoloff et al. 1981). Due to the even distribution of AFM_1 between curd and whey in cheese making and the loss of water during curing, cheese should contain 3.5 - 5 times more AFM_1 than the milk from which it was made (Stoloff et al. 1981). However, concentration effects are offset by the mixing of milks from many different regions at the food processing plant. In 1979 the FDA surveyed dairy products produced in the United States. Of the 992 samples of nonfat dry milk, vanilla

ice cream, yogurt, Cheddar cheese and cottage cheese examined only one, a cottage cheese containing 0.3 ng/mg AFM_1, was positive (Stoloff et al. 1981).

The Loss of AFM_1 in Milk

A number of studies have shown AFM_1 levels in milk to decrease over time (McKinney et al. 1973; Stoloff et al. 1975; Kiermeier and Mashaley 1977). This loss may be explained by either AFM_1 degradation or occlusion.

Several naturally occurring components of milk could lead to AFM_1 degradation. Applebaum and Marth (1982) found that, in milk containing 1% H_2O_2, 11% of the AFM_1 was inactivated after thirty minutes at 63°C and that this effect was enhanced by the addition of riboflavin. Lactoperoxidase in the presence of H_2O_2 and chloride ions has been shown to degrade AFB_1 and AFG_1 (Doyle and Marth 1978) and would be expected to degrade AFM_1. Formaldehyde, which may occur naturally in milk (Kulshrestha and Marth 1970), has been shown to decrease levels of aflatoxins B_1, B_2, G_1 and G_2 (Codifer et al. 1976) and may lead to AFM_1 degradation.

Aflatoxin M_1 is generally thought to be associated with the protein fraction of milk (Allcroft and Carnaghan 1963; McKinney et al. 1973) and increased AFM_1 - protein complexation over time could lead to reduced recovery. Several researchers (Purchase and Steyn 1967; Stubblefield, Shannon and Shotwell 1973; Stubblefield 1979) have suggested that the lack of AFM_1 recovery from powdered milk following direct spiking with some AFM_1 standard solutions could be due to AFM_1 -

protein binding. The occlusion of AFM_1, versus its degradation, is also supported by the increased AFM_1 recoveries reported during some stages of cheese making (Brackett and Marth 1982).

Aflatoxins can also be either degraded or removed from solution by a number of yeasts, molds and bacteria (Dolye et al. 1982) and an increase in microbial populations during milk storage could lead to a loss of AFM_1. Lillehoj et al. (1971) found that AFM_1 could be removed from milk by inoculating samples with the bacteria Flavobacterium aurantiacum NRRL B-184. Whether this AFM_1 loss was due to AFM_1 degradation or to the irreversible binding of AFM_1 by bacteria cells has not been resolved. Mold peroxidases can lead to formation of free radicals, singlet oxygen and oxidizing agents which may result in aflatoxin degradation (Dolye et al. 1982).

Analytical Methods

Aflatoxin M_1 analyses were originally done using duckling bioassays with the severity of liver lesions being indicative of AFM_1 concentration in milk. This method was only semi-quantitative with a probable limit of detection of a total dose of 0.4 mg/kg body weight over 5 days (Purchase 1972) and has been replaced by physico-chemical assays which generally involve 1) extraction of AFM_1 with an organic solvent 2) clean up procedures with column chromatography to remove interfering substances and 3) separation/quantitation by TLC or HPLC with UV absorption or fluorescence detection (Applebaum et al.1982). Compared to bioassays, physico-chemical determinations are much more specific and sensitive. For routine analyses which utilize the fluorescent property of

AFM_1, the practical limit of detection is 0.1 ppb AFM_1 in milk (Stoloff 1980) while AFM_1 at the ppt level may be detected with newer methods such as ELISA and RIA (Scott 1986).

Variation in Aflatoxin M_1 Measurement

There are conflicting reports in the literature regarding AFM_1 levels in dairy products. For instance Kiermeier and Mashaley (1977), using artificially and naturally contaminated raw milk, noted AFM_1 losses of 11-25% after 1-3 days at 5°C. Stoloff et al. (1975) studied milk which was spiked with AFM_1 and then either pasteurized or stored raw and found no AFM_1 losses after 17 days at 4°C. Stoloff et al. (1975) also investigated AFM_1 losses in artificially contaminated milk samples stored at -18°C and found a detectable AFM_1 loss after 68 days and a loss of 45% after 120 days while McKinney et al. (1973) found that, under these same conditions, AFM_1 levels had decreased by 14% at 30 days and by 87% after 120 days. Furthermore, in a 1981-82 International Smalley Aflatoxin Check Sample Program of the American Oil Chemists' Society (AOCS) the between-laboratory coefficient of variation for the quantitation of AFM_1 in provided milk samples was 98% (McKinney 1984). Explanations for this variability include the use of artificially versus naturally contaminated milk, the use of different analytical methods or extraction solvents, instability of AFM_1 extracts or standard solutions, interfering substances and the low levels of AFM_1 measured. The loss of AFM_1 in milk over time, as discussed earlier, also increases the variation in results.

Artificially vs. Naturally Contaminated Milk. Aflatoxin M_1 contaminated milk is produced either artificially, by directly adding AFM_1, or naturally by feeding cows AFB_1 contaminated feed, and the amount of AFM_1 detected may vary depending on which milk type is used. Kiermeier and Mashaley (1977) found a greater AFM_1 loss in artificially versus naturally contaminated milk stored at -18°C after 7 days, while Applebaum and Marth (1982) found AFM_1 losses, following H_2O_2 treatment, of 40% in spiked raw milk samples and 57% in naturally contaminated samples. Stubblefield et al. (1973) investigated the extraction of AFM_1 from powdered milk and found that with the Purchase and Steyn (1967) extraction method, using acetone and water (70:30), only 65-75% of the AFM_1 was recovered in spiked milk samples versus more than 90% of the calculated amount in naturally-contaminated samples.

Analytical Methods/Extraction Solvents. The amount of AFM_1 found in milk samples varies with the type of extraction solvent used. Purchase and Steyn (1967) compared the ability of 25 solvents to extract AFM_1 from naturally contaminated powdered milk and found the AFM_1 concentrations of this powder to vary from zero, when chloroform was used as the extracting solvent, to .06 µg/g when acetone:chloroform:water (38:58:4) was used. Stubblefield et al. (1973) compared 3 methods of extracting AFM_1 from liquid milk and found the highest levels, 99-105% at an AFM_1 concentration of 1 µg/l, using the method of Jacobson, Harmeyer and Wiseman (1971) which utilizes a methanol water extraction solvent. However, no AFM_1 was detected at a concentration below 1 µg/l when the Fehr, Bernage and Vassilopoulos (1971) method, with methanol

extraction and hydrochloric acid precipitation of proteins, was used. McKinney and Cavanaghan (1977) found that AFM_1 levels in contaminated raw milk, which had been stored at -10°C for 25 months, had decreased by 80% when several AOAC extraction solvents were used while no decrease was seen when acetone:water:acetic acid (75:15:10) was used as the extracting solvent.

Instability of AFM_1 Extracts and Standards. A number of storage solvents can lead to AFM_1 losses. Aflatoxin M_1 standard solutions in chloroform are unstable and form impurities (Stubblefield 1980). Wei and Chu (1973) demonstrated the photomodification of AFB_1 and AFG_1 when stored in water, methanol or ethanol and exposed to UV light; a similar reaction would by expected in AFM_1. Also, AFM_1 extracts are typically dried under nitrogen during AFM_1 analyses and the resulting dry film on glass containers may not completely redissolve when solvent is replaced (Stubblefield 1980). These factors may partly explain the apparent problems with AFM_1 standards seen in the 1980-81 AOAC collaborative study (McKinney 1984).

Interfering Substances. Some interfering substances are not removed by column chromatography or separated from AFM_1 during HPLC or TLC. Price, Jorgensen and Billotte (1981) found that the milk from cows fed dried citrus waste contained a compound which was not separated from AFM_1 by HPLC and gave a false positive test. Stubblefield (1979) suggested the occurrence of interfering substances in cheese extracts which enhance or quench the fluorescence seen on TLC plates in AFM_1 zones.

Level of AFM_1 Measured. Aflatoxin M_1 can currently be detected at the ppt level (Scott 1986). However, as the level decreases, variation among replicates increases. Collaborative studies conducted by the Association of Official Analytical Chemists (AOAC) on a number of different compounds indicate that analyses in the ppb range, typical for AFM_1, have a 50% between laboratory coefficient of variation (Horowitz 1982).

The Effect of Milk Preservatives on AFM_1 Levels

Milk preservation is important in AFM_1 analysis as bacterial growth may lead to AFM_1 degradation while milk coagulation results in non-representative samples and losses due to emulsions. However, several preservatives used in analytical milk samples may be expected to decrease AFM_1 levels.

Oxidizing Agents

Solutions of H_2O_2 added to milk maintain reduced bacterial counts (Zeehuizen, Soeratno and Monsjoer 1958) and have been traditionally used to preserve analytical milk samples (Kroger 1985). However, H_2O_2 has been shown to decrease aflatoxins in peanut meal (Screenivasamurthy et al. 1967) and AFM_1 in milk (Applebaum and Marth 1982). Potassium dichromate ($K_2Cr_2O_7$), added as a tablet, is currently the most frequently used preservative for analytical milk samples (Kvapilik and Suchanek 1974). Although the effect of this compound on AFM_1 has not been reported, it would be expected to react like H_2O_2, sodium hypochlorite (NaOCl) (Natarajon et al. 1975), potassium

permanganate (K_2MnO_4) (Trager and Stoloff 1967) and many other oxidizing agents and to degrade aflatoxins.

Sodium Azide

Sodium azide (NaN_3), added in tablet form (Buchberger and Kiermeier 1975) and as a mixture containing NaN_3, chloramine T and parabens (Jonas 1978) has been examined as a replacement for $K_2Cr_2O_7$ preservation of milk. Although this compound preserves milk adequately for fat and protein determinations, it is unacceptable due to the potential for explosive reactions between NaN_3 and metals in sewage systems (Kroger 1985). In our laboratory the amount of AFM_1 detected in milk preserved with an aqueous solution of NaN_3, with a final concentration of 60 mg NaN_3/l milk, was shown to be highly variable over time (Price and Jorgensen 1982).

Formaldehyde

Formalin solutions containing 37% formaldehyde and 15% methanol, to inhibit polymerization, have been used as preservatives in analytical milk samples (Schwartz, Gould and Harper 1956; Murthy 1969). Formaldehyde kills bacteria, fungi, molds and yeasts (Walker 1964, p. 569) and Kulrestha and Marth (1970), using a disk assay procedure, showed that formaldehyde inhibited all seven common bacterial species tested. Formaldehyde may also inhibit coagulation through stabilization of milk proteins. This could occur via formaldehyde reactions with casein amino groups which results in the formation of methylene cross links (Singh and Fox 1985). Pilkhane and Bhalerao (1971) found formaldehyde and

mercuric chloride to be the best preservatives of four types tested. Both preserved unrefrigerated milk samples for 28 days and refrigerated samples for 84 days. However, Kvapilik and Suchanek (1974) found formadehyde preservation was unsatisfactory for fat and protein reading.

Formaldehyde has been shown to reduce AFB_1, AFB_2, AFG_1 and AFG_2 levels and would therefore be expected to decrease AFM_1. Mann et al. (1970) showed that treatment of peanut meal containing AFB_1 and AFB_2 with a 2% formaldehyde solution at 100°C decreased total aflatoxin concentration from 110 ppb to a non-detectable level. Codifer et al. (1976) used a similar treatment on peanut meal containing AFB_1, AFB_2, AFG_1 and AFG_2 and found total aflatoxin levels reduced from 570 ppb to 7 ppb. Loss of AFM_1 in formaldehyde preserved milk might also occur through the action of xanthine oxidase. In the presence of oxygen, this enzyme oxidizes formaldehyde to H_2O_2 (Murthy and Campbell 1966) which, as discussed above, decreases AFM_1 levels. However, xanthine oxidase is denatured at temperatures above 60°C and therefore have no enzyme activity in pasteurized milk.

MATERIALS AND METHODS

Contaminated Spray-dried Milk

Non-fat spray-dried milk, which had been naturally-contaminated with AFM_1, was obtained from United Dairymen of Arizona. The original raw milk had been collected from Holstein dairy cows fed a 15% ration of naturally-contaminated cottonseed containing 2500-5000 mg total aflatoxins/kg. The contaminated milk was fed through a separator to remove the cream and then spray-dried with an inlet temperature of 440-410°F and an outlet temperature of 195-210°F. The resulting spray-dried milk was stored at -20°C for one year.

Sample Preparation

Experiment 1

Aflatoxin M_1 contaminated spray-dried milk was mixed with either raw milk (Beals) or pasteurized whole milk (A.J. Bayless) to a final concentration of approximately 1.1 ng AFM_1/ml. Formalin solution (37% w/w) was added to these milk solutions to final concentrations of zero, 0.25, 0.05 and 0.1% formaldehyde. Aliquots of 200 ml each of the contaminated milks were transferred to either glass or plastic containers, randomly coded to prevent bias and stored in the dark at 21°C for up to four weeks (Table I).

Table I. Design of Experiment 1

Percent Formaldehyde \ Time	0 Time		1 Week		2 Weeks		3 Weeks		4 Weeks	
	Raw	Past.	Raw	Past.*	Raw	Past.*	Raw	Past.*	Raw	Past.*
0.000	glass	glass	glass	glass	glass	glass	glass	glass	glass	glass
			plastic	plastic	plastic	plastic	plastic	plastic	plastic	plastic
			Raw	Past.	Raw	Past.	Raw	Past.	Raw	Past.
0.025			glass	glass	glass	glass	glass	glass	glass	glass
			plastic	plastic	plastic	plastic	plastic	plastic	plastic	plastic
			Raw	Past.	Raw	Past.	Raw	Past.	Raw	Past.
0.050			glass	glass	glass	glass	glass	glass	glass	glass
			plastic	plastic	plastic	plastic	plastic	plastic	plastic	plastic
			Raw	Past.	Raw	Past.	Raw	Past.	Raw	Past.
0.100			glass	glass	glass	glass	glass	glass	glass	glass
			plastic	plastic	plastic	plastic	plastic	plastic	plastic	plastic

* Samples formed emulsions.

= Samples within double lines were not included in the 4-way analysis.

Experiment 2

Samples were prepared as in experiment 1 except that samples were held up to two weeks instead of four and only raw milk and glass containers were used.

Milk Analysis

Milk samples were analyzed according to an AOAC official method of AFM_1 analysis (AOAC 1984, 26.095-26.099). All chemicals used were AR grade. Methylene chloride was substituted for chloroform in these analyses. Extracts from experiment 1 were stored at -20°C in benzene: acetonitrile (9:1) for three months before AFM_1 quantitation. Depending on the AFM_1 concentration, high pressure liquid chromatography (HPLC) analysis was performed on 10-50 µl aliquots of the sample extracts. The HPLC system included a Waters HPLC modular unit, μC_{18} reverse phase column, M600A pump, Wisp 710A automatic injector, 420E fluorescence detector (365-400 nm), and a data modular integrator. The mobile phase was distilled water:methanol:acetonitrile (52:26:22), HPLC grade (Fisher Scientific) with a flow rate of 0.9 ml/min. and a pressure of 2000 psi for experiment 1 samples and 1500 psi for experiment 2 samples. The AFM_1 10 µg/ml standard was supplied by Eureka Laboratories, San Francisco, CA.

Statistical Analysis

Statistical analysis was performed with a computer using a program for a 4-way ANOVA for experiment 1 data and a 2-way ANOVA for experiment 2 data. Means were separated by least significant difference at $p<.05$.

RESULTS AND DISCUSSION

The effects of milk type, container type, time and formaldehyde level on the AFM_1 levels found in experiment 1 and of time and formaldehyde in experiment 2 are discussed in this section. Problems encountered with the analytical procedure are also discussed.

Experiment 1

Analytical Problems

Levels of AFM_1 Detected. The aflatoxin contaminated spray-dried milk powder was repeatedly analyzed and was determined to contain 7.5 ppb AFM_1 after a 1:10 dilution (w/v) with distilled water. The recovery from milk samples spiked with AFM_1 standard during this preliminary testing was 85-95%. The milk samples prepared from this powder were calculated to contain 1.1 ppb AFM_1 and, assuming this AFM_1 concentration, the average amount of AFM_1 detected ranged from 36%, for all samples extracted at zero time, to less than 5% for samples extracted after 4 weeks which contained .05 or 0.1% formaldehyde. The recovery from milk samples spiked with AFM_1 standard and extracted during these analyses averaged 42%. The low AFM_1 levels found for zero time samples and spiked samples may have been due to the analytical problems described below.

Standard. A decrease in the concentration of the AFM_1 standard used would explain the low spiked sample recoveries. However, this would not be expected as AFM_1 has been shown to be stable for 13 months

when stored in benzene:acetonitrile (9:1) at -20°C (Stubblefield 1980). It is possible, however, that the standard was contaminated which could lead to AFM_1 degradation.

Quantitation. Thin layer chromatography was used to quantitate extracts from samples stored for 0, 1 and 2 weeks, but some plates were unreadable due to excessive yellow streaking. This problem was resolved by water washing and filtering the methylene chloride used during analysis. However, samples already extracted had to be quantitated by another method and the solvent impurities they contained may have led to AFM_1 degradation and quantitative interferences. Furthermore, removal of methylene chloride impurities did not lead to improved spiked sample recoveries which averaged 42% for weeks 1-4. It was assumed that the Waters HPLC, used for the second method of quantitation, was sufficiently calibrated for the concentrations of AFM_1 expected. However, subsequent testing indicated that for extracts with high AFM_1 concentrations the instrument response was probably low. For which ever reason, AFM_1 levels were significantly less when extracts were re-quantitated by HPLC. For samples extracted at zero time the AFM_1 concentrations were 0.75 ppb (68% of 1.1 ppb) by TLC analysis versus 0.40 ppb (36% of 1.1 ppb) by HPLC.

Emulsions. Although the Stubblefield AOAC official method of analysis (AOAC 1984, 26.095-26.099) has greatly simplified the analytical procedures involved in the extraction of AFM_1 from milk, a continuing problem has been the formation of emulsions. Milk samples are normally mixed with an organic solvent in a separatory funnel to extract

the AFM_1. Following this procedure the aqueous and organic phases should again separate. However, milk is a complex liquid containing agents such as fatty acids and phospholipids which act to form emulsions. The higher total solids content of the prepared milk samples increases the tendency to coagulate and may increase emulsion formation. In addition, whole pasteurized milk is homogenized and contains a dispersion of fine fat droplets which further enhance organic and liquid phase interaction. The net result of these and other factors is often an emulsion. Several methods of breaking these emulsions have been described such as the addition of urea or sodium sulfate, heating, and centrifugation (Stubblefield 1979; AOAC 1984, 26.095-26.099).

Emulsions formed readily in samples stored for one week or longer and one third of the samples from the week one extractions were lost for this reason (additional milk in the sample containers allowed for the repeated extraction of some samples with the technique described below). These emulsions could not be broken with either sodium sulfate addition or centrifugation. Heat treatment was not attempted as, in our laboratory, cold milk samples have shown less tendency to form emulsions than those at room temperature. To avoid emulsions, several methods of shaking the separatory funnel during extractions were attempted. Some techniques, which involved less vigorous mixing of aqueous and organic phases, led to decreased AFM_1 recoveries. This problem was resolved for most milk samples by shaking the separatory funnel in a specific way during the extraction step. The separatory funnel was first inverted to release the gas and then held horizontally and shaken as hard and fast

as possible sixty times. The funnel was then immediately turned upright and the stopper removed. For most milk samples no emulsions were formed by this technique and layers separated within a few seconds.

Raw vs. Pasteurized Milk

In experiment 1 no significant difference in AFM_1 concentration was seen between raw and pasteurized milk and interactions with other variables - container type, time and formaldehyde level - were not apparent (Tables II-V).

Table II. Experiment 1 - AFM_1 Concentration vs. Milk Type

Milk Type	Means (ppb)
Raw	0.093
Pasteurized	0.100

LSD (.05) = 0.020
STD. ERROR = 0.007 (N = 72.00)
NON-SIGNIFICANT

Table III. Experiment 1 - AFM_1 Concentration vs. Milk Type and Container Type

Milk Type		Means (ppb)
Raw	- glass	0.099
	- plastic	0.088
Pasteurized	- glass	0.122
	- plastic	0.078

LSD (.05) = 0.028
STD. ERROR = 0.010 (N = 36.00)
NON-SIGNIFICANT

Table IV. Experiment 1 - AFM_1 Concentration vs. Milk Type and Formaldehyde Level

Milk Type		Means (ppb)
Raw	- .025%	0.151
	- .050%	0.093
	- .100%	0.036
Pasteurized	- .025%	0.142
	- .050%	0.099
	- .100%	0.059

LSD (.05) = 0.034
STD. ERROR = 0.012 (N = 24.00)
NON- SIGNIFICANT

Table V. Experiment 1 - AFM_1 Concentration vs. Milk Type and Time

Milk Type		Means (ppb)
Raw	- 1 week	0.156
	- 2 week	0.093
	- 3 week	0.076
	- 4 week	0.049
Pasteurized	- 1 week	0.182
	- 2 week	0.075
	- 3 week	0.088
	- 4 week	0.057

LSD (.05) = 0.040
STD. ERROR = 0.014 (N = 18.00)
NON-SIGNIFICANT

Although the higher concentration of enzymes and bacteria present in raw milk might be expected to increase AFM_1 degradation, the addition of formaldehyde would minimize these effects by inhibiting bacteria and/or enzymes (Shwartz, Gould and Harper 1956; Kulshrestha and Marth 1970). The levels of AFM_1 in pasteurized and raw milk samples without formaldehyde could not be compared due to an emulsion problem described below.

All pasteurized samples which did not contain formaldehyde and were kept for one week or longer formed emulsions during extraction and could not be analyzed. Although both milk types with zero formaldehyde were coagulated after one week, the raw milk samples did not form emulsions. This variation was probably due to homogenization of the whole pasteurized milk and the distribution of small lipid micelles. An

increase in the micelles free fatty acid content over time would lead to increased interaction with the extracting solvent, methylene chloride, and interfere with separation of the organic and aqueous phases.

Glass vs. Plastic Containers

The aflatoxin M_1 concentration of milk kept in plastic containers was 25% less than from milk stored in glass containers (Table VI). An interaction occurred between container type and formaldehyde level such that the loss of AFM_1 for samples with 0.025% formaldehyde was greater in milk from plastic than from glass containers while no difference was seen between container types for samples with 0.05% and 0.1% formaldehyde (Table VII). Also, no interaction was seen between container type and time (Table VIII).

Table VI. Experiment 1 - AFM_1 Concentration vs. Container Type

Container Type	Means (ppb)
Glass	0.110 a
Plastic	0.083 b

LSD (.05) = 0.020
STD. ERROR = 0.007 (N = 72.00)

Table VII. Experiment 1 - AFM_1 Concentration vs. Container Type and Formaldehyde Level

Container Type		Means (ppb)
Glass	- .025%	0.177 a
	- .050%	0.093 bc
	- .100%	0.061 cd
Plastic	- .025%	0.116 b
	- .050%	0.099 b
	- .100%	0.033 d

LSD (.05) = 0.034
STD. ERROR = 0.012 (N = 24.00)

Table VIII. Experiment 1 - AFM_1 Concentration vs. Container Type and Time

Container Type		Means (ppb)
Glass	- 1 week	0.188
	- 2 week	0.088
	- 3 week	0.089
	- 4 week	0.077
Plastic	- 1 week	0.150
	- 2 week	0.079
	- 3 week	0.074
	- 4 week	0.028

LSD (.05) = 0.040
STD. ERROR = 0.014 (N = 18.00)
NON-SIGNIFICANT

The lower AFM_1 level found in milk stored in plastic containers was unexpected. Aflatoxin M_1 loss due to glass adherence is known to occur but has not been studied in plastic. Components of polyethylene containers are known to migrate into food stuffs (Crompton 1979, pp. 1-19) and could play a role in AFM_1 breakdown. However, these substances consist of antioxidents, metals, triglcerides and short chain fatty acids (Crompton 1979, pp. 1-19) - none of which have been associated with AFM_1 degradation although transition metals would aid in oxidative reactions. On the other hand, formaldehyde interaction with plastic components may lead to products which degrade or trap AFM_1. Although losses were significantly greater in milk from plastic containers with 0.025% formaldehyde, the interaction between formaldehyde and container type is mot clear. No trend could be established as many samples containing zero formaldehyde formed emulsions and could not be compared.

Aflatoxin M_1 Concentration Over Time

Aflatoxin M_1 concentration decreased over time for all treatment groups with averages of 0.17 ppb at 1 week to 0.05 ppb at 4 weeks (Table IX), and an interaction was seen between time and formaldehyde (Figure 3).

Table IX. Experiment 1 - AFM_1 Concentration vs.Time

Time	Means (ppb)
1 week	0.169 a
2 week	0.084 b
3 week	0.082 b
4 week	0.053 c

LSD (.05) = 0.028
STD. ERROR = 0.010 (N = 36.00)

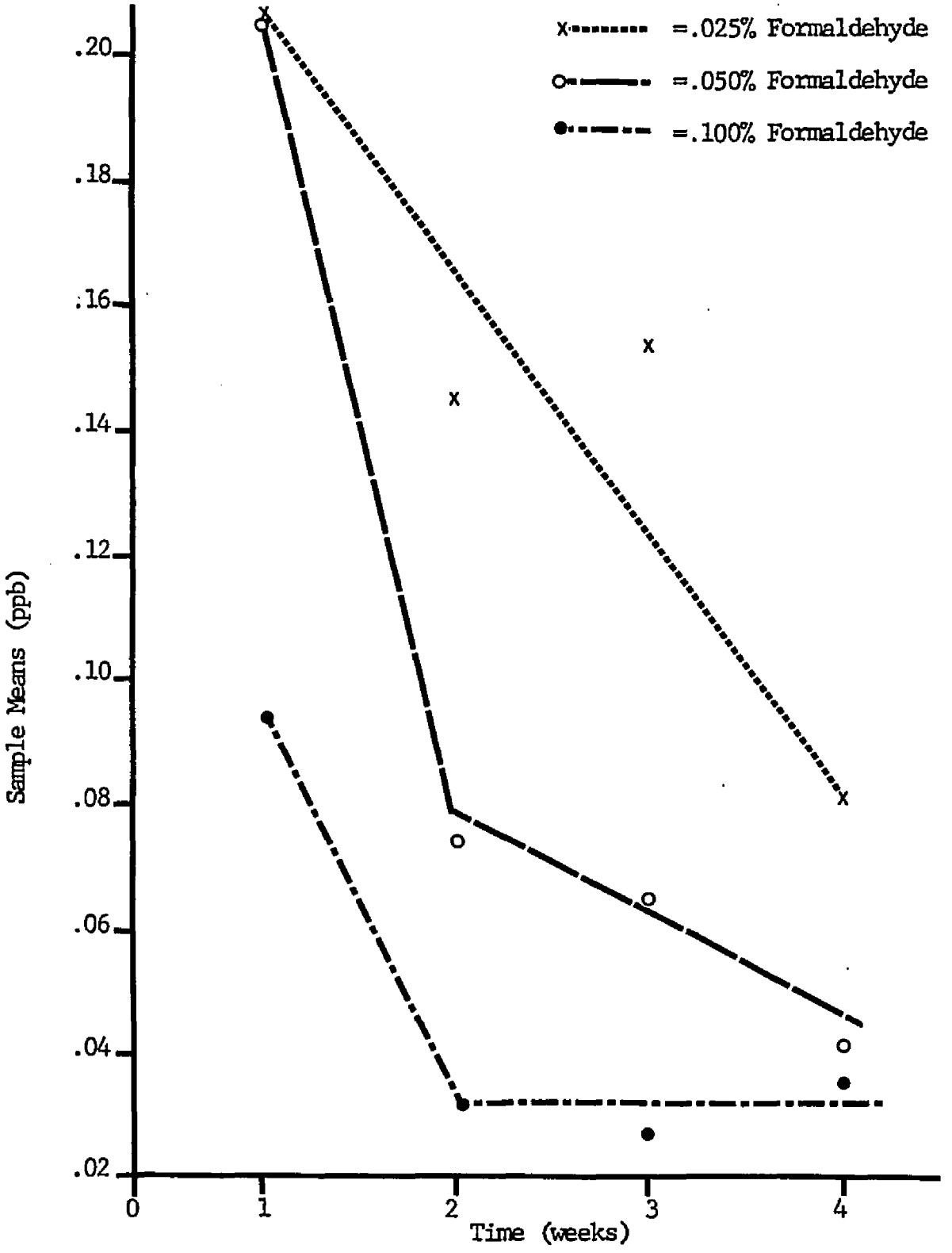


Figure 1. Experiment 1 - AFM_1 Concentration vs. Time and Formaldehyde Level

No extractions were done at zero time for samples containing .025% - 0.1% formaldehyde as it was expected that formaldehyde level would have no effect on AFM_1 recovery at zero time. Although this assumption was confirmed by measurements performed in experiment 2, the data for zero time samples was incomplete and therefore could not be included in the 4-way ANOVA.

Aflatoxin M_1 levels in raw milk samples not containing formaldehyde decreased by 38% over a period of 4 weeks (pasteurized milk samples with zero formaldehyde formed emulsions). Previous studies have also shown AFM_1 levels to decrease with time. Stoloff et al. (1975) found a 45% decrease in AFM_1 levels in samples stored at -18°C after 120 days while McKinney et al. (1973) noted an 87% reduction for this same time and temperature. Kiermeier and Mashaley (1977) found losses of 11-25% after 1-3 days at 5°C.

Two changes which occurred in zero formaldehyde samples after one week were coagulation and a decrease in pH. After 4 weeks the pH of samples had decreased from 6.5 to 5. This increase in acidity or milk souring is one of the first effects of milk spoilage. Although AFM_1 is degraded by strongly acidic solutions, it should be stable at a pH of 5 (Doyle and Marth 1978) and loss of AFM_1 due to increased acidity is probably minimal. However, coagulation generally accompanies a drop in pH and might decrease AFM_1 levels in the following manner. Studies of aflatoxin association with casein milk protein (Shantha and Screenivasamurthy 1981) and AFM_1 distribution during cheese making (Allcroft and Carnaghan 1963; McKinney et al. 1973) indicate that AFM_1

associates with the protein fraction of milk. The agglomeration of protein micelles during coagulation could entrap AFM_1 and thereby lead to decreased recovery. Enzymes occurring in milk naturally or from microbial sources might also be expected to influence AFM_1 levels. Dolye and Marth (1978) found that lactoperoxidase, which occurs naturally in milk, decreased AFB_1 and AFG_1.

Samples containing formaldehyde were not coagulated and showed only a minimal pH decrease after four weeks. Also, formaldehyde might be expected to minimize enzymatic losses of AFM_1 through its bacteriocidal effects or by direct enzyme inhibition. However, these samples showed greater AFM_1 loss over time and will be discussed under formaldehyde treatment.

Formaldehyde Treatment

Aflatoxin M_1 concentrations decreased from 0.15 ppb to 0.05 ppb as formaldehyde levels increased from 0.025% to 0.1% (Table X).

Table X. Experiment 1 - AFM_1 Concentration vs. Formaldehyde Level

Formaldehyde Level	Means (ppb)
0.025%	0.146 a
0.050%	0.096 b
0.100%	0.047 c

LSD (.05) = 0.024
STD. ERROR = 0.009 (N = 48.00)

The decrease seen in AFM_1 concentration with increasing formaldehyde level may be due to either AFM_1 degradation or loss of AFM_1 extractability or to a combination of the two. Codifer et al. (1976) found that 80% of the AFB_1 in contaminated peanuts was destroyed by formaldehyde treatment. It is likely that AFM_1 would also be broken down by this treatment as AFB_1 and AFM_1 have similar structures and both are degraded by H_2O_2 (Doyle et al. 1982). The acidity of formalin solutions, pH ~ 4, might also be expected to play a role in AFM_1 degradation. However, milk is a buffer and the pH of samples was not affected by formaldehyde additions. An attempt was made to see whether formaldehyde can directly break down AFM_1, but there were a number of complications such as - the acidic formalin solution must be buffered, AFM_1 and formaldehyde have different solubilities in organic/aqueous solutions and, as the formalin solution is evaporated for quantitation of AFM_1, a polymerized product forms - and the results from this investigation were inconclusive.

Formaldehyde, a cross linking agent, is known to react with casein proteins and its effect on the microenvironment of milk could lead to entrapment of AFM_1 and reduced recovery. Studies indicate that AFM_1 is associated with the protein fraction of milk. Changes in AFM_1 extractability were proposed by Brackett and Marth (1982) to explain the decrease and subsequent increase in AFM_1 levels seen during stages of cheese making. The re-formation of AFM_1 could also explain variable AFM_1 levels. Parker and Melnick (1966) and Price and Jorgensen (1985)

found that AFB_1, partially broken down by calcium hydroxide ($CaOH_2$) treatment, reformed upon acidification.

Either AFM_1 degradation or loss of AFM_1 extractability would explain the decrease in AFM_1 levels which was seen over time. Also, a plateau may be occurring during weeks 2-3 for samples with .025% - .05% formaldehyde although the reason for this is not apparent.

Experiment 2

Due to the analytical problems and lack of data at the zero time point in experiment 1, the effect of formaldehyde and time on AFM_1 levels was re-examined in a second experiment. Samples containing 0, .025, .05 and 0.1% formaldehyde were analyzed at 0, 1 and 2 weeks. Because there was no significant difference between milk types in experiment 1, this parameter was eliminated as a variable in experiment 2. All experiment 2 samples utilized raw milk due to its greater resistance to formation of emulsions. In order to further reduce the number of samples only glass containers, which showed less of a negative effect on AFM_1 levels, were used in experiment 2.

Analytical Problems

The milk samples were prepared to contain 1.1 ppb AFM_1. Assuming this level of AFM_1 concentration, the amount of AFM_1 detected ranged from 91% of the original, for zero time samples, to non-detectable levels for samples extracted at 2 weeks which contained 0.1% formaldehyde. The high AFM_1 levels detected at zero time indicate that most AFM_1 losses were due to treatment effects, as opposed to analytical

procedures. This result was not surprising as the problems with methylene chloride, quantitation and emulsions were resolved by experiment 2. However, the recovery of AFM_1 from milk spiked with AFM_1 standard and extracted during these analyses averaged only 13%. When the standard used to spike these samples was later quantitated by TLC, the AFM_1 concentration was found to be at 20% of its initial value. This suggests that the poor recovery from these spiked samples was due to unknown problems with the AFM_1 standard. The standard used for spiking was not the same as the standard used for comparison during analyses.

Aflatoxin M_1 Concentration Over Time

Aflatoxin M_1 concentration decreased from 1.0 ppb at zero time to 0.24 ppb at 2 weeks (Table XI) and a positive interaction occurred between time and formaldehyde level (Figure 4).

Table XI. Experiment 2 - AFM_1 Concentration vs. Time

Time	Means (ppb)
0 week	0.999 a
1 week	0.487 b
2 week	0.239 c

LSD (0.05) = .102657

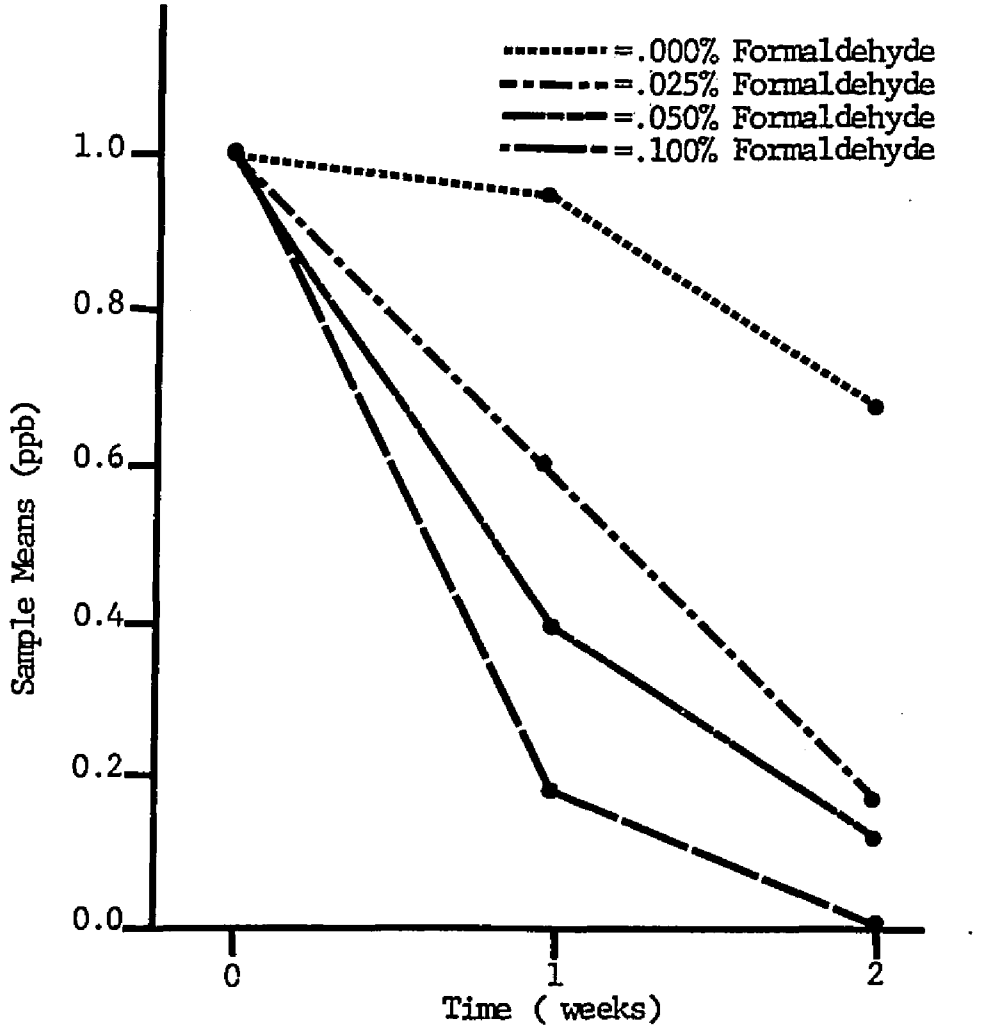


Figure 4. Experiment 2 - AFM_1 Concentration vs. Time and Formaldehyde Level

Aflatoxin M_1 levels in milk samples containing zero formaldehyde decreased by 34% over two weeks and AFM_1 losses increased with increasing formaldehyde concentration. The greatest losses, in samples containing formaldehyde, occurred between zero time and week 1 and may have resulted from the high initial concentrations of chemical reactants. A similar trend of AFM_1 loss with time and formaldehyde concentration was seen in experiment 1 milk samples. However, lower AFM_1 levels were found for all treatment groups in experiment 1 due to the analytical problems described earlier.

Formaldehyde Treatment

Aflatoxin M_1 levels decreased from 0.87 ppb to 0.39 ppb as formaldehyde levels increased from zero to 0.1% (Table XII).

Table XII. Experiment 2 - AFM_1 Concentration vs. Formaldehyde Level

Formaldehyde	Means (ppb)
0.000%	0.869 a
0.025%	0.592 b
0.050%	0.434 c
0.100%	0.386 c

LSD (0.05) = .118538

Although formaldehyde had no immediate effect on AFM_1 levels, samples extracted at weeks 1 and 2 showed increasing AFM_1 losses with increasing formaldehyde concentration, as was seen in experment 1.

CONCLUSIONS

There was no difference in the amount of AFM_1 detected in raw and pasteurized milk samples which contained formaldehyde. Because all the pasteurized milk samples with zero formaldehyde formed emulsions, the non-preserved samples could not be compared.

Aflatoxin M_1 losses were greater in samples stored in plastic than in glass containers. This effect was significant for samples containing 0.025% formaldehyde but not for samples containing 0.05% or 0.1% formaldehyde. Because zero formaldehyde samples could not be included in this analysis, the nature of this interaction - whether formaldehyde increases or decreases AFM_1 losses in plastic containers - is not clear.

The effects of formaldehyde on AFM_1 are not immediate and no differences were seen between samples extracted at zero time. However, AFM_1 levels did decrease with both time and formaldehyde level. The greatest reduction in AFM_1 was seen between zero time and one week and these losses increased with formaldehyde concentration.

This data indicates that AFM_1 levels in milk samples preserved with 0.025% to 0.1% formaldehyde and stored at room temperature may be expected to decrease by 45-85% after one week. Formaldehyde decreased emulsions in pasteurized milk and reduced coagulation in both raw and pasteurized milk. However, its negative effect on AFM_1 levels outweighs these benefits, and it should not be used to preserve milk samples which will be analysed for AFM_1

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