A review of aflatoxin M_1 in liquid milk

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ABSTRACT

Mycotoxins continue to pose a health concern via human exposure to contaminated food. Aflatoxin M1 (AFM1), the hydroxylated metabolite of aflatoxin B1 (AFB1), may be found in the milk of dairy cattle and other mammals. In humans, AFM1 is excreted through the feces, urine, and in the case of lactating mothers, also in breast milk after consumption of aflatoxin contaminated food. Concentration of AFM1 in milk is a function of several factors, namely: animal type, milking day, milk yield, season, feeding regime, geographic, and climatic conditions. A linear relationship has been established between the amount of AFM1 in milk and the amount of AFB1 in feed consumed by animals, emphasized at first on the reduction or removal of AFB1 from feedstuffs and then elimination of AFM1 from milk. This review aims to bring up to date the current global status of AFM1 contamination of liquid milk destined for human consumption and the effects of processing and reduction methods on the elimination of aflatoxins from liquid milk.

Key words: Liquid Milk, Aflatoxin M1, Occurrence, Reduction

INTRODUCTION

Aflatoxins are fungal toxins produced by certain species of *Aspergillus* especially *A. flavus, A. parasiticus* but rarely by *A. nomius* [1] which may grow on several kinds of agricultural products. The major types of naturally occurring AFs have been identified: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2). AFB1 represents the highest degree of toxicity; followed by AFM1, AFG1, AFB2 and AFG2 [2]. AFB1 is considered to be the most potent hepatocarcinogen, teratogen and mutagen of this group of mycotoxins. AFM1, the hydroxylated metabolite of AFB1, may be found in the milk, milk products and meat of dairy cattle and mammals that have ingested feedstuffs contaminated with AFB1 [3]. AFM1 can cause serious human disease, especially primary liver cancer, DNA damage and acute toxicity and carcinogenicity comparable with that of the parent molecule (AFB1); therefore it is now classified by the International Agency for research on cancer (IARC) as a group 1 human carcinogen [4]. Among human foods of animal origin, the rate of feed-to-tissue transfer of aflatoxin is highest for milk. Milk has the greatest demonstrated potential for introducing AFM1 into the human diet and exposure to AFM1 through milk products is a serious problem for public health [5]. Exposure of children, including infants, to AFM1 is of particular concern, because they have potentially greater vulnerability and sensitivity than adults and their capacity for biotransformation of carcinogens is generally slower than in adults [6]. Several countries have established regulatory limits for AFM1 in milk and dairy products, which vary from 0.05 µg/l in European countries to 0.5 µg/l in the USA.

The risk of contamination by AFM1 is an important food safety concern for milk. A number of research investigations have been conducted to study the occurrence of AFM1 in milk. Currently, attention is focused on the development of an efficient and sensitive method for the routine assay of AFM1 in milk and milk products. Indeed recent progress has been made toward the development of sensor devices for the rapid and in field determination of AFM1 in milk without highly skilled personnel [7]. Immunosensors detect a signal generated from antigen–antibody interaction and convert it into a measurable signal. With regard to evidence of the hazardous nature of AFM1, it is important to devise practical procedures to inactivate or remove the AFM1 present in milk. Various physical, chemical and biological agents have been studied to detoxify AFB1 and AFM1 from food and feed materials [8].

This review aims to bring up to date the current global status of AFM1 contamination of liquid milk destined for human consumption and the effects of processing and reduction methods on the elimination of aflatoxins from liquid milk.

METHOD

A total of 123 papers were studied. The collected papers had focused on different aspects of AFM1 including the occurrence in liquid milk, biotransformation and toxicity, human exposure, and determination methods. Furthermore, some papers related to AFM1 reduction in milk or aflatoxin B1 reduction in feed were discussed. The papers related to AFM1 in other dairy products such as cheese, butter, and yogurt was excluded. The worldwide occurrence of AFM1 in liquid milk from 2000 to 2014 has been studied but the only data summarized in the current review (Table 1) are the ones related to the countries that have published at least four studies about AFM1 occurrence in milk.

WORLDWIDE OCCURRENCE OF AFM_1 IN MILK

Natural occurrence of AFM1 in milk and milk products is increasingly reported. Worldwide occurrence of AFM1 in liquid milk from 2000 to 2014 has been summarized in Table 1(only the countries with at least four publications). In some samples, especially those obtained from Asian countries, AFM1 concentration was higher than the limit of 0.05 μg/l imposed by many countries [9]. In India 87.3% of a total of 87 liquid milk samples, were reported to show contamination of AFM1 ranging from 28–164 μg/l. Almost 99% of the contaminated samples exceeded the European Communities (EC) limit [10]. Similar high levels of AFM1 levels were detected in Indonesia, Philippines and Thailand [11]. However, in some European countries, relatively low levels of AFM1 were determined in milk samples [12]. These differences may be related to stringent regulations of aflatoxin B1 in complementary feedstuffs for dairy cattle in countries of Europe [13].

In general, the AFM1 concentration in milk is a function of following factors:

Animal type: Van Egmond [14] reported that excreted amount of AFM_1 in the milk of Animal type: Van Egmond [14] reported that excreted amount of AFM1 in the milk of dairy cow was 1-2% of ingested AFB1. However, the mean carry-over rates for mares were 0.04-0.05%, for ewes they ranged from 0.60 to 0.72% (with a maximum of 2.7%), and for goats they ranged from 2.5 to 2.7% [15]. For mares, calculated mean carry over, based on an estimated daily milk yield of 3 kg, was 0.04 - 0.05 %, which is some 10 times lower than that in dairy cattle suggesting a better ability of mares to degrade AFB1 [16].

Milking day [even from one milking to the next]: day after the AFB1- containing ration was fed and milk toxin failed to be detected five days after the feeding program was discontinued. AFM1 can be found in milk within 12–24 h after the first ingestion of AFB1 [16], and increased as soon as the first milking after animal ingestion with a pattern of increment up between 7th and 12th days of AFB1 ingestion [17]. In a related study, dairy cows in the early lactation stage (2-4 weeks) and in late lactation weeks (34-36 weeks) were fed with AFB1 contaminated feed. After 12 days, the carry-over of AFM1 in the milk was 6.2% in the early stage, but it declined to 1.8% in the late lactation stage [18].

Milk yield: Masoero has suggested that milk yield is one of the major factors affecting the total excretion of AFM1 [17]. High yielding dairy cows with a production of up to 40 liters of milk per day, showed a carry-over percentage as high as 6.2 % has been reported [18].

Season: A number of studies have shown that the mean contamination level of AFM1 in autumn and winter (cold seasons), was significantly higher than those of spring and summer [6, 9, 13, 19- 22], due to the fact that grass, pasture, weed, and rough feeds were found more commonly in spring and summer than in winter [23-24]. Torkar and Vengust [25] reported that in winter cows are fed with greater amounts of compound feeds that exceed the allowed AFB1 content. Highly contaminated samples, some containing up to 5 μg/kg, were found during the winters of 1978–1983.

Feeding regime: : Information from Italy has indicated that in two regions the prevalence of AFM1 in milk samples above the statutory limit was increased as a result of incorporating home-grown maize into the animal feed (6% of tested samples in the first half of 2003, rising to 7.8% in July/October 2003). In a related study, a ewes' milk sample contained AFM1 levels above the statutory limit. The Scientific Panel on Contaminants in the Food chain (CONTAM) noted that the tight restrictions on controlling AFB1 in dairy cattle feed may not be applied in the same way for feedstuffs intended for ewes [58]. Battacone [59] reported that the AFM1 concentration was linearly related to the AFB1 intake/ kg of BW.

Table 1: The world wide occurrence of aflatoxin M_1 in raw milk.

Country	Sample	No. of sample	No. of positive (%)	Range (µg/l)	No (%). of sample > 0.050 µg/l	References
Turkey	UHT milk	100	67	0.01-0.36	31 (31%)	26
	UHT whole	19	11 (57.9)	<0.01- >0.05	1 (5.3)	27
	UHT skimmed	5	3 (60)	<0.01 - 0.05	-	
	Pasteurized	3	2 (66.7)	<0.01 - 0.05	-	
	Raw milk	90	79 (87.8)	0.0125-0.1236	35 (44.3)	22
	UHT milk	129	58.1%	<0.010 – 0.5436	47.1%	28
	Pasteurized	85	88	0.127 (mean)		24
	Goat milk	110	85.45%	0.005-0.117	6.36%	29
	Milk	40	20%	0.04-0.076	2	30
	Buffalo	55	19 (34.5)	0.013±0.024	15.8%	31
Pakistan	Cow	40	15 (37.5)	0.024±0.022	20%	
	Goat	30	6 (20)	0.002±0.005	-	
	Sheep	24	4 (16.7)	0.002±0.004	-	
	Camel	20	Nd			
	Buffalo	360	153 (42.5)	0.002-0.205	13.9%	32
	Cow	120	63 (52.5)	0.004 – 0.263		
	UHT milk	79	11.3%	0.029- 0.103	7.59%	33
	Raw milk	68	68	0.69 to100	81%	34
	Milk	107	71%	0.004-0.845	58%	35
	Milk	232	76.3%	Ave: 0.252	75	36
Iran	Raw milk	111	85 (76.6)	0.015-0.28	40%	9
	pasteurized	624	624 (100)	0.045- 0.080	17.8%	37
	Cow	75	59	0.005 - >0.05	27	
	Water buffalo	75	29	0.005 - >0.05	6	1
	Camel	40	5	0.005 - >0.05	0	
	Sheep	51	19	0.005 - >0.05	2	
	Goat	60	19	0.005 - >0.05	4	
	UHT milk	30	30	-	20	38
	Pasteurized	48	48	0.01- >0.1	25	19
	UHT	48	48	0.031- >0.1	32	
	Pasteurized	140	117	<0.01- >0.1	36	
	Liquid milk	100	78%	0.052–0.113	78%	39
	Raw cow milk	40	40	0.004 - 0.352	56.7%	40
	Raw cow milk	122	122	0.004 – 0.112	14.75	13
Egypt	Buffalo	50	50	<0.010- >0.250	24	
	Cow	50	50	<0.010- >0.250	17	41
	Goat	50	50	<0.010- >0.250	13	
	Camel	50	50	<0.010- >0.250	5	
	Row cow milk	50	19	0.023-0.073	10	42
	Cow milk	15	3	Mean: 0.006		43
	Row cow milk	48	47 (97.9%)	0.63±0.32	53.19 %	44
China	Raw milk	12	12 (100)	0.16-0.5		45
	Pasteurized	104	66 (63)	>0-0.5		
	Raw milk	200	45(22.5%)	Ave: 0.015	-	46
	Raw milk	72	59.7%	0.001-0.42	-	47
	UHT milk	153	54.9%	0.006-0.160	20.3%	48
	Pasteurized	26	96.2%	0.023-0.154	65.4%	
Brazil	UHT milk	12	83.3%	0.010 - > 0.200	50%	49
	Raw	22	05 (22.8)	>LD- > 0.05	2 (9.0)	50
	Pasteurized	43	19 (44.2)		2 (7.2)	
	UHT	42	11 (26.2)		3 (7.1)	
	Pasteurized	10	7 (70)	0.01-0.02	-	51
	UHT	40	40 (100)	0.01-0.05	1 (2.5)	
	Raw milk	42	10	0.331- 1.975	3	52
	Raw milk	7	2 (28.6%)	Ave: 0.835	100	53
	Pasteurized	12	7 (58.3%)	Ave: 0.884	100	
	UHT milk	15	10 (66.75%)	Ave: 1.168	100	
	Concentrated	3	2 (66.7%)	Ave: 1.718	100	
	Powdered	3	0	nd	0	
Serbia	Cow milk	3	3	0.01-0.05	0	54
	Sheep milk	2	2	<0.01->0.01	0	
	Goat milk	18	18	<0.01->0.01	7 (30)	
	Raw milk	23	9	0.02-0.250	0	55
	Cow milk	150	98.7%	0.01-1.2	129	56
	Milk	50		Nd-1.44	38 (76%)	57

Geographical region: Data analysed from FDA surveys showed that 174 out of 380 milk samples from southern USA (1998–2000), 195 out of 225 raw and finished milk samples from USA (1995–2000), and 21 of 60 raw and pasteurized milk samples from Thailand (1990–1993) were contaminated with AFM1. In the European, Latin American, Middle Eastern, and African diets, the weighted mean value for AFM1 in milk was below the proposed maximum levels of 0.05 µg/kg and 0.5 µg/kg. Whereas in the Far Eastern region, the weighted mean value for AFM1 in milk (0.36 µg/kg) was greater than the proposed maximum level of 0.05 µg/kg but below 0.5 µg/kg. The data submitted by the EC showed that 96% of 7573 samples collected from, Portugal, Sweden and the United Kingdom collected in 1999 had AFM1 levels below the limit of detection (which varied between countries: 0.001–0.03 µg/kg) [60].

BIOTRANSFORMATION AND TOXICITY

As a rule, AFM1, aflatoxin Q1 (AFQ1), aflatoxin P1 (AFP1), aflatoxin M2 (AFM2), (the analogous metabolite of aflatoxin B2) and aflatoxin M4 (AFM4), the hydroxylated AFB1 metabolites, are poorer substrates for epoxidation and have reduced genotoxicity compared to AFB1, thus they are generally considered as detoxification products of AFB1. The cytochrome P450 (CYP450) enzymes, mixed-function monooxygenase system, are a family of haemoproteins that catalyze the metabolism of a large number of xenobiotics, including aflatoxins [61]. Of the CYP450 enzymes, CYP1A2 is responsible for formation of AFM1 preferentially, which is the major aflatoxin metabolite in humans. However in contrast with this idea Heinonen [21] reported that AFM1 is not strictly a detoxification product of AFB1 in biological responses, in which, cytotoxicity plays a significant role, such as immunotoxicity.

The unsaturated bond in the terminal furan ring carbons 8 and 9 in AFB1 and AFM1 (atoms are numbered according to instructions given by the International Union of Pure and Applied Chemists, IUPAC) is the site at which its bio-activation forms a highly reactive epoxide structure. AFB1 epoxide has been shown to exist as two stereoisomeric endo- and exo-epoxides; the exo-epoxide being the DNA-reactive form. A similar situation may apply to AFM1 epoxide. AFM1 can be further activated to form an AFM1-8,9-epoxide that binds to DNA and is excreted into urine in the form of AFM1-N7-guanine [62].

Biomarkers of AFB1 exposure include urinary aflatoxin metabolites, such as AFB1-N7-guanine and AFM1, serum AF-albumin [63], and AFM1 in milk [64]. AFM1 is the primary aflatoxin metabolite in animals and human milk, comprising 95 % of the total amount of aflatoxins excreted in milk. It has been estimated that 0.09– 0.43% of dietary intake is excreted in human milk as AFM1 [65]. AFB1 and its metabolites (AFM1, AFQ1, and AFP1) are excreted through the feces, urine, and in the case of lactating mothers, also in breast milk after consumption of aflatoxin contaminated food [62]. In several animal species (rats, sheep, pigs, cows), AFM1 is the main non-conjugated AFB1 metabolite in the urine and accounts for 2-9% of the dose [66]. A number of studies have emphasised the presence of AFM1 and other metabolites in urine. Zhu [67] analyzed 252 urine samples from 32 households in China and reported a good correlation between total dietary AFB1 intake and AFM1 excretion. Between 1.2 and 2.2% of dietary AFB1 was present as AFM1 in the urine. In a related study Groopman and co-workers [68] confirmed the presence of AFM1 and also AFB1, AFQ1, AFP1 and AFB1-N7-guanine in urine. The percentage of AFB1 excreted as the above metabolites was 4.4% in women and 7.6% in men.

While a number of studies have focused on the extent of conversion of AFB1 to AFM1, the factors affecting the excretion of AFM1 in human milk have yet to be studied in detail.

AFM1 was found to be a DNA-damaging agent, with an activity of about one-third that of AFB1. In the wing spot test, the genotoxicity of AFM1 and AFB1 was compared by Shibahara [69]. The authors concluded that AFM1 is genotoxic in mammalian systems in vivo.

The relative carcinogenicity of AFB1 and AFM1 has also been the subject of several studies. Sinhuber [70] reported similar carcinogenic effect for both aflatoxins B1 and M1 in trout liver. They observed that AFM1 has high genotoxic activity, although it was found that AFM1 was about 10 times less carcinogenic than AFB1. In a related study, over 7200 trout fry with an average initial body weight of 1.2 g were used to investigate carcinogen dose response curves for both AFB1 and AFM1 and an estimate of the DNA binding index after a single dose. The results showed, the relative tumorigenic potencies were 1.0 for AFB1 and 0.086 for AFM1 [71].

Acute toxicity results in direct liver damage and subsequent illness or death. The acute toxicity of AFM1 was reviewed by van Egmond [64]. In ducklings and rats, the toxicity of AFM1 is similar or slightly less than that of AFB1 [72]. The LD 50 for one-day-old ducklings is 0.24 mg of AFB1 kg/1 and 0.32 mg of AFM1 kg/1, respectively [73]. Studies on the acute toxicity of aflatoxins in 1-day-old ducklings

suggest that AFM1 and AFB1 act by a similar mechanism in causing acute toxicity and subcellular alterations, such as changes in liver parenchymal cells, dissociation of ribosomes from the rough endoplasmic reticulum, and proliferation of the smooth endoplasmic reticulum, and that only the naturally occurring isomer of each aflatoxin is biologically active. Naturally contaminated milk showed fewer lesions than artificially contaminated milk, suggesting differences in the bioavailability of naturally and artificially occurring AFM1 [74].

OCCURRENCE of AFM_1 IN HUMAN MILK

Maternal to child exposure of AFM1 in breast milk is an evaluated risk factor from dietary exposure to AFB1. Bhat and Vasanthi, [75] emphasized that children exposed to aflatoxins may become stunted, are underweight, and more susceptible to infectious diseases in childhood and later life. For example, study results showed that AFs have been directly related to underweight status in children in Benin and Togo [76] and to the protein-energy malnutrition condition of kwashiorkor [77]. A number of studies have highlighted the presence of AFM1 in human milk (Table 2). The reporting of high AFM1 levels in relatively few individual mothers suggests that individual dietary habits may result in the exposure of their infants even after weaning. In a study conducted to determine the prevalence of AFMl in breast milk of women from low-exposure areas (Victoria, Australia) and women living in assumed high-exposure areas (Thailand), AFM1 was detected in 11 samples from Victoria and five samples from Thailand ranging from 28-1031 pg/ml and 39-1736 pg/ml, respectively [78]. In a related study 6 out of 54 samples collected from women in rural villages in Zimbabwe, were found to be contaminated with AFM1 at levels up to 50 pg/ml, however, no positive samples were detected out of 42 milk samples obtained from women in France [79]. In the United Arab Emirates (UAE) 92% of breast milk samples (n = 140) contained AFM1 in the range of 5-3400 pg/ml [80].

Table 2: Worldwide occurrence of AFM_1 in human milk

Country	No. of sample	No. of positive sample	Range µg/l	Reference
Turkey	50	33 (66%)	0.038- 0.0943	81
Turkey	61	8 (13.1%)	0.0051- 0.0069	82
Turkey	75	75	60.90-299.99	83
Turkey	73	18 (24.6%)	0.001-0.006	84
United Arab Emirates	140	92%		80
Zimbabwe	54	6 (11%)	> 0.05	79
France	42	None	-	79
Kuwait	12	5	0.0883- 0.0152	85
Brazile	50	1	0.024	86
Brazile	100	2	0.03-0.08	87
Australia	73	11 (15%)	0.028-1.031	78
Thiland	11	5	0.039-1.736	78
Iran	160	157 (98.1%)	0.0003-0.0267	88
Iran	182	11%	0.0051-0.008	89
Egypt	388	36%	0.0103-0.022	90
Egypt	443	248 (56%)	0.006.3-0.497	91

In one report on five lactating women in the Gambia [65], the percentage of aflatoxin in the diet excreted as AFM1 in milk ranged from 0.09 to 0.43%. In a recent study AFM1 was detected in 157 samples from Iran ranging from 0.3–26.7 ng/kg. The concentration of AFM1 in one sample was higher than the maximum tolerance limit accepted by the European Union and USA (25 ng/kg), but in 55 samples was higher than the maximum concentration recommended by Australia and Switzerland (10 ng/kg) [88]. Among 445 donors of breast milk, 99.5% of samples contained AFM1 at concentrations ranging from 2–3 µg/L. The mothers were drawn from a wide range of nationalities, ages and health status; no correlation was observed between these factors and AFM1 content of the milk [92]. AFM1 was measured in 40 mothers of low income status by Martinez and his colleague 2009 where 40% of milk samples and 72% of urine samples had AFM1.

Since breast milk is a major nutrient for infants and feeding of infants with safe milk is essential, monitoring and determination of AFM1 in human breast milk, especially in developing and underdeveloped countries is of great concern and training programs should be conducted to aware mothers from importance of their food habits.

DETERMINATION OF AFM1

In order to comply with the maximum levels established by the European Commission (and other

countries), it is essential to control the sources of contamination using rapid, sensitive, reliable and cost effective methodologies. A number of methods for determination of AFM1 have been developed which can be classified as two main groups: chromatographic methods and immunochemical methods. As a general rule, aflatoxins are low molecular weight compounds, which possess significant UV absorption and fluorescence properties. For this reason, liquid chromatographic techniques have predominated in their analysis, initially TLC [9], and subsequently HPLC [15].

The Association of Official Analytical Chemists (AOAC International) and the European Standardization Committee (CEN), the European equivalent of the International Standards Organization (ISO), have standardized and validated some methods of analysis for AFM1 liquid milk. Since 1990, TLC has been considered as an AOAC official method to identify and quantify AFs at levels as low as 1 ng/g. ISO 14674 (2005) and ISO 14501 (2007), described methods for determination of AFM1 in milk and milk powder by TLC and HPLC respectively.

Although the number of publications on TLC has declined in recent years, this method is still used for determination of AFM1 in milk and dairy products. Shundo and Sabino [50] demonstrated a satisfactory correlation between results obtained from TLC and HPLC for determination of AFM1 in milk. In general most of the chromatographic methods used for determination of AFM1 in liquid milk are based on solid phase extraction (SPE) or immunoaffinity chromatography clean-up in combination with reversed-phase HPLC and fluorescence detection with or without derivatisation. Pre-column and post column derivatisation with a suitable fluorophore is used to enhance natural fluorescence of AFM1 and improves its detection ability. Pre-column derivatisation relies on the formation of the corresponding hemiacetals with trifluoroacetic acid (TFA) [93], while post-column derivatization uses either bromination via an electrochemical cell (Kobra Cell) or addition of bromide or pyridinium hydrobromide perbromide (PBPB) to the mobile phase [93]. Generally chromatographic methods require extensive sample preparation steps and well trained personnel; therefore they are usually used for confirmation of the results obtained from rapid tests that have been used for screening of mycotoxins or for accurate quantitative determination of mycotoxins.

Immunochemical methods are used for rapid screening of aflatoxins in various samples. These techniques are based on using specific antibodies with good sensitivity. A number of immunochemical approaches: enzyme-linked immunosorbent assay (ELISA), immunoaffinity column assays (ICA), sequential injection immunoassay (SIIA) and radioimmunoassay (RIA), have been developed for the determination of AFM1 in milk. Standardized rapid methods are available with sensitivity (limit of detection) as low as 3 ng/l, while, these may be difficult to achieve in all circumstances. However, while ELISA based techniques are used for rapid mycotoxins screening [94], they suffer from the disadvantage of false-positive results and, on occasion, unacceptable quantification accuracy, therefore confirmatory analysis is required [95]. ELISA kits also are not feasible for on-site detection because of the long incubation time and numerous washing steps.

REDUCTION OF AFM_1

Aflatoxin reduction in feed

A linear relationship between the amount of AFM1 in milk and AFB1 in feed consumed by the animals has been reported [22]; therefore the emphasis has been on reduction or removal of AFB1 from feedstuffs with consequent elimination of AFM1 from milk.

Since the most effective way to control AFM1 in liquid milk and dairy products is to prevent the initial contamination of feeds consumed by dairy cattle, specific regulations for feed exist in many countries [96]. Practical programs are also applied; e.g. a code of practice for reducing AFB1 in raw materials developed by the Codex Committee on Food Additives and Contaminants [64], Good manufacturing practices (GMP) and good storage practices which reduce AFB1 in feed stuffs through the prevention of mould growth. If such methods fail to reduce AFB1 formation in agricultural commodities intended for use as animal feeds, other chemical, physical or biological methods may be applied. Ammoniation (0.5–2.0%) at high temperature (80–1000 °C) for 20–60 min, commonly called the “high-pressure, high-temperature method”, was shown to eliminate AFB1 from feed and consequently AFM1 residues in milk. Although the processing and the treatment of products are largely accepted by the dairy industry, it has been shown in the USA to increase non-protein nitrogen in animal feeds [74]. Alkaline heat treatment, or nixtamalization, which is used traditionally in the treatment of maize for the manufacture of tortillas, significantly reduced the concentration of aflatoxins in feed. However much of the original aflatoxin can be re-formed after treatment with acids [97]. Physical methods such as irradiation, extraction by alkaline solvents, absorption by sequestering agents and heat treatments showed different levels of aflatoxins reduction in feedstuffs. Removal of aflatoxins from

animal feed onto bentonite and hydrated sodium calcium alumino silicate (HSCAS) has been used in the feed industry [98]. A number of studies, evaluating sequestering agents in vivo, showed their ability to protect animals from the effects of dietary AFB1 and to prevent or reduce AFM1 secretion into milk. Activated carbons and montmorillonite clays effectively bind AFB1 in vitro [99-100]. Feeds that have higher concentrations of AFB1 may be blended with feed that has lower concentrations, however, it is not permitted to mix feed for dilution purposes within the European countries. Biological methods can also be used to eliminate aflatoxins. For example, procedures have been developed to degrade aflatoxins in feedstuffs by exposing them to the bacterium *Flavobacterium auranthiacum* [74]. However, despite the use of many prophylactic measures against fungal growth, contamination of feed and thus of milk is sometimes unavoidable, therefore the last means of avoiding or reducing the occurrence of AFM1 in milk is to eliminate at least part of the toxins.

AFM1 reduction in milk

Apart from the method introduced for reduction of AFB1 in feed, other types of chemical, physical and biological methods have a role to play in preventing and reducing the concentrations of AFM1 in milk. In a research conducted by Diaz [101] activated carbon, sodium bentonite and esterified glucomannan were shown to reduce transfer of AFM1 into milk (31-65%). It was reported that supplementation of sodium bentonite and activated charcoal by 1% for early lactating goats resulted in significant reduction of AFM1 content of milk and carryover of aflatoxin from feed to milk without causing any change in the composition of milk [102].

Two methods for removing AFM1 from naturally contaminated raw whole milk were examined. One involved chemical elimination using potassium sulphite (K_2SO_3), the other physical adsorption of the toxin using bentonite. Maximum elimination of AFM1 (percent of total eliminated) for the chemical method was 45 % using 0.05 M K_2SO_3 (25 °C, 5 h) and 89% by using 0.4 g of bentonite per 20 ml of raw milk (25 °C, 1 h) (103). Possible mechanisms responsible for degradation of AFM1 by K_2SO_3 may involve reactions between the toxin and the bisulphite radical (HSO_3^-).

Use of hydrogen peroxide (H_2O_2) under various conditions of temperature and time to inactivate AFM1 in naturally or artificially contaminated milk has been examined. Aman [104] reported that slight inactivation (4.3%) was obtained in milk boiled for 5 min without H_2O_2. By using 1% H_2O_2 followed by heat treatment at 36 °C for 30 min, 75 °C for 15 sec and boiling for 5 min, maximum inactivation (27.8%, 28.8% and 45.1%) were obtained respectively, whereas no change was observed in the content of AFM1 in milk contained H_2O_2 after 24 hours. It has been reported that AFM1 was decreased by 89.1% in milk containing 0.05% H_2O_2 as compared with 60.7% for H_2O_2-free milk when both were exposed to ultraviolet irradiation [105]. In a related study, the use of H_2O_2 plus riboflavin (30 °C, 30 min) followed by heating at 63 °C for 30 min resulted in 98% reduction of the AFM1 present in naturally contaminated raw milk.

Degradation of AFM1 has also been attempted by combined treatments, such as ultra-violet radiation followed by ultra filtration. Understanding the mechanisms of AFM1 detoxification by physical, chemical and microbiological methods will enable establishment of combined treatment procedures to effectively decontaminate contaminated foods and feeds. However, this method requires further research to ensure that the treated product is biologically safe and retains its nutritional and functional properties [103].

AFM_1 reduction in milk during processing

There are conflicting results from the effect of thermal treatments on AFM1 reduction in milk. Some studies indicate that heat does not cause an appreciable change in the amount of AFM1 in milk [106-107] whereas others report different levels of decontamination [108]. For example, pasteurization of milk at 62°C for 30 min was observed to reduce the AFM1 content in milk by 32% [109], whereas a similar treatment did not reduce the toxin content, as concluded by Stoloff [106]. Kiermeier and Mashaley [108] reported that heating of milk, depending on the conditions, caused a decrease of the aflatoxin-content of between 12 and 35%. They concluded that destruction of AFM1 depends on time and temperature combination of the heat treatment applied. In a related study, pasteurization caused a decrease in the level of AFM1 at the rate of 7.62% [22]. Recently Deveci [109] revealed that pasteurization can partially reduce AFM1 in liquid milk. He reported that sterilization of milk at 121 °C for 15 min caused 12.21% degradation of AFM1, whereas boiling decreased AFM1 by 14.50%. In contrast with this idea, Deshpandeh [110] reported that AFM1 is resistant to thermal inactivation and pasteurization. Autoclaving and other varieties of food processing procedures are not effective in the complete reduction of this toxin. Badea [111] also reported that AFM1 is relatively stable during milk pasteurization, storage and during the preparation of various dairy products.

Studies on the stability of AFM1 in milk during frozen and cold storage have also given variable results. About 40% and 80% of AFM1 in naturally

contaminated milk disappeared after storage at 0°C for 4 and 6 days respectively. In a related study a reduction ranging from 18.8% to 24.2% in AFM1 content in milk upon storage at 5°C for l-3 days was reported [108]. However Prandini [112] reported storage of frozen contaminated milk and other dairy products for a few months does not appear to affect their content of AFM1. Because of the variability of results, a final conclusion cannot be made from results of these studies. These differences in results are related to specific factors such as poor stability of AFM1 in milk; differences in the initial level of contamination; accuracy and precision of determination methods; and status of contamination, i.e. whether the treated milk was naturally or artificially contaminated [113]. In naturally contaminated systems, AFM1 is bound to the sample matrix, representing the real situation whereas in artificially contaminated milk, AFM1 could be present partially unbound and in the free form and undergoes different metabolic pathways [114] therefore it is easier to inactivate aflatoxins in artificially contaminated substrates than in those that are contaminated naturally.

AFM1 seems to be predominantly associated with the protein fraction of milk, and casein in particular, so that cheese curd made from contaminated milk contains a higher concentration than whey [112]. The distribution of AFM1 has been investigated in samples of whey, curd and a typical hard and long maturing cheese such as Grana Padano [ripened for twelve months], produced with naturally contaminated milk in a range of 30–98 ng/kg AFM1 [114]. Results showed in comparison to milk, AFM1 concentration levels increased both in curd (3-fold) and in long maturing cheese (4.5-fold), while AFM1 occurrence in whey decreased by 40%. However in a related study, artificially contaminated milk was used by Lopez [97] to produce home-made cheese. The authors reported that the greatest proportion (60%) was detected in whey while 40% AFM1 remained in cheese. In contrast with this idea Purchase and his colleagues [73] reported that following the preparation of cottage cheese from the contaminated milk, AFM1was found in the whey but not in the cheese itself. Elgerbi reported that the concentrations of AFM1 were lower in the cheese products than in the raw milk samples [115]. Some researchers demonstrated that cheese ripening and proteolysis of casein increase the recovery of AFM1 from naturally contaminated milk; proteolysis may affect hydrophobic regions on casein associated molecules releasing AFM1 [112]. The partitioning of AFM1 between whey and curd depends upon which kind of cheese-making procedure is used and the degree of milk contamination [110]. As a rule an appreciable amount of the toxin present in milk is concentrated in whey, but the final cheese produced shows a higher concentration of AFM1 than the milk from which it is made [114].

Concentration, evaporation and different drying methods lead to a concentration of milk solids and contaminants such as AFM1. Large losses of AFM1 were reported in some studies, whereas in other studies milk concentration did not affect the AFM1 content. Naturally contaminated milk was processed in a number of ways, and freeze dried, evaporated, roller-dried and spray-dried milk samples were produced. Chemical analysis showed that the processing of the milk reduced its AFM1 content, and that the higher the temperatures used, the smaller the amount of aflatoxin present. An actual reduction in the levels of AFM1 occurred in the freeze-dried and spray-dried milk was demonstrated by a reduction in the toxicity of such milk for ducklings [73]. AFM1 is mainly soluble in the aqueous phase of milk or adsorbed to casein particles therefore a small proportion of AFM1 in milk is transferred to cream, and yet a smaller proportion to butter [112]. When making butter from naturally contaminated cream 23% (18-28%) of the AFM1 appeared in the butter, whereas the butter milk contained the major amount of AFM1 [108].

Cultured dairy products such as kefir and yoghurt are manufactured by heating milk and adding a starter culture. Prandini reported that there was a significant decrease in the AFM1 content of cultured dairy products [112]. Some strains of lactic acid bacteria (LAB) have been reported to be effective in removing AFM1 from contaminated liquid milk [90, 116- 120]. In a related study conducted by El Khoury, the binding ability of AFM1 *by L. bulgaricus* and and *S. thermophilus* was investigated during the making of yogurt. Results showed both of them were effective in reducing the extent of free AFM1 Therefore, LAB seems to play a crucial role in AFM1 removal and has been proposed for use as a biological agent for AFM1 reduction [121].

Although the mechanism of aflatoxins removal by LAB is not clear, it has been suggested that aflatoxins molecules are bound to bacterial cell wall components rather than metabolically degraded [122]. Haskard [123] suggested that AFB1 is bound to bacteria through weak noncovalent bonds such as association with hydrophobic pockets on the bacterial surface. El Khoury and coworkers [121] reported that the differences in AFM1 binding by the bacterial strains are probably due to different bacterial cell wall and structures.

CONCLUSION

The aim of this review paper was to discuss the worldwide occurrence, international legislation, biotransformation and toxicity, human exposure, determination and reduction of AFM1 in liquid milk.

- The current review revealed that there is wide variation in AFM1 levels among different countries which may be related to geographic area, climate condition and differences in dairy cattle feeding system. Therefore to achieve a low level of AFM1 in liquid milk, both milk and animal feed should be evaluated and controlled continuously, for aflatoxin.
- Since the best strategy to avoid AFM1 in milk is its prevention, integrated control programs are required in order to control of risks associated with aflatoxin contamination of feeds.
- Simple, rapid, robust and low cost analytical methods are required to achieve low limits of AFM1 in milk and more research is required to validate rapid methods.
- The occurrence and identification of factors effect on the presence of AFM1 in human breast milk is of great concern.
- Extensive and periodic surveys should be performed to prevent serious health hazards to the mother, foetus, and infant.
- Further scientific evidence is also required for the possible adverse effects resulting from long-term exposure to low levels of AFM1.
- Despite the research did on the effect of milk processing on AFM1 reduction, the effect of heat treatments and the relationship between time and temperature during thermal processing is not clear.
- Further research should be conducted in the development of decontamination processes.
- However, national regulations establishing limits for AFM1 in milk have been intended, harmonization of regulatory limits worldwide should be promoted to overcome problems in the trade of some milk and dairy products.

ETHICAL ISSUES

Ethical issues have been completely observed by the authors.

COMPETING INTEREST

The authors declare that they have no competing interests.

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