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Research Note

Survey of the Occurrence of Aflatoxin M_1 in Dairy Products Marketed in Italy

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ABSTRACT

During 1995, 159 samples of milk, 97 samples of dry milk for infant formula, and 114 samples of yogurt were randomly collected in supermarkets and drug stores in four large Italian cities and checked for aflatoxin M_1 (AFM_1) by immunoaffinity column extraction and HPLC. AFM_1 was detected in 136 (86%) of the milk samples (in amounts ranging from <1 ng/liter to 108.5 ng/liter; mean level: 10.19 ng/liter), in 81 (84%) of the dry milk samples (in amounts ranging from <1 ng/kg to 101.3 ng/kg; mean level: 21.77 ng/kg), and in 91 (80%) of the yogurt samples (in amounts ranging from <1 ng/liter to 496.5 ng/liter; mean level: 18.08 ng/liter). Altogether, only two samples of milk, two samples of yogurt, and one sample of dry milk had levels of AFM_1 exceeding the Swiss legal limits, which are the most restrictive in the world. AFM_1 contamination levels in milk and yogurt samples collected in the period of November to April were ca. four times as high as those in samples collected in the period of May to October. It is concluded that during 1995, despite the widespread occurrence of AFM_1, the mean contamination levels in dairy products sold in Italy were not a serious human health hazard.

Aflatoxin M_1 (AFM_1) is the major hepatic carcinogenic metabolite of aflatoxin B_1 (AFB_1) excreted in milk when lactating animals are fed AFB_1-contaminated feeds. Evidence of potential hazardous human exposure to AFM_1 from dairy products arises from numerous studies on the occurrence of AFM_1 in dairy products *(6)*. Since milk has the greatest demonstrated potential for introducing aflatoxin (AF) residues from edible animal tissues into the human diet and is also the main nutrient for infants and children, occurrence of AFM_1 in commercially available milk and dairy products is a concern. Regulatory limits enacted in the world are influenced by economic considerations and are usually based on little or no scientific basis *(6)*. Consequently, their effectiveness in preventing AF-related chronic effects from continued exposure to subacute levels of AF is doubtful. According to Van Egmond and Dekker *(16)*, imposing a zero tolerance for AF in foods would be appropriate if these were not natural contaminants that can never completely be eliminated. Therefore data on the occurrence of AF in foods and feeds are needed to make exposure assessment possible and estimate the effects of regulatory limits. AFM_1 may or may not be present in dairy products in a particular year depending on the weather for that period. Hence, widespread and frequent monitoring surveys should be carried out. A large amount of literature data is available on the worldwide occurrence of AFM_1 in milk *(6)*, but the most recent survey in Italy was performed in 1989 *(5)*. Few literature data are available on the presence of AFM_1 in dry milk for infant formula *(6)*. Three surveys were conducted in Italy in the years 1985 to 1989 *(6)*, but no recent data are available. The consumption of yogurt in latter years in Italy, as well as in Europe, has increased greatly, and today it partially substitutes for milk in the human diet because of its positive influence on digestion. However, only one study in 1985 has been concerned with the occurrence of AFM_1 in yogurt, reporting from 36 to 334 ng/liter of AFM_1 in 6 out of 8 samples *(13)*.

The focus of this paper was to investigate the occurrence of AFM_1 in milk, dry milk for infant formula, and yogurt marketed in Italy during 1995.

MATERIALS AND METHODS

Samples. During 1995, 159 samples of milk, 114 samples of yogurt, and 97 samples of dry milk for infant formula were randomly collected in supermarkets and drug stores in four large Italian cities. Most of the milk (112 samples) was ultrahigh-temperature-treated (UHT) milk, produced and processed in Italy. The rest was pasteurized milk (24 samples) and sterilized milk (23 samples, exposed to 120°C for 20 min). Of the dry milk samples, 21 were produced in Italy, the rest were imported from Denmark (28), France (22), Germany (18), Spain (5), and Switzerland (3).

Most of the samples of yogurt were produced in Italy (90); the rest were imported from France (12) and Belgium (12).

In Italy the official regulatory limit for AF in foods and feeds

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is the one established by the directive of the Commission of the European Communities. However, we compared our data to the Swiss limits (50 and 10 ng of AFM_1 per liter of milk and per liter of diluted dry milk for infant formula, respectively) because they are the most restrictive in the world.

Methods of analysis. AFM_1 in milk and dry milk was extracted using the immunoaffinity column method (Easi-Extract, Biocode Ltd, Heslington, York, UK) reported by Mortimer et al. *(10)*. Briefly, 100 ml of defatted milk was applied to the column which was previously washed with 20 ml of phosphate-buffered saline (pH 7.4). Then the column was washed with 20 ml of water, and the AFM_1 was slowly eluted with 1.5 ml of methanol. AFM_1 in yogurt was extracted using the method reported by Richard et al. *(15)* for dairy products. However, since this latter method was not specifically developed for yogurt, it was modified slightly with 100 g of yogurt and 150 ml $CHCl_3$ used for AFM_1 extraction. Before being placed on the Sep-pack silica chromatography column (Waters, Millipore, Milford, Mass.), the yogurt-$CHCl_3$-NaCl mixture was centrifuged (centrifuge model 972R, ALC, Milan, Italy) 10 min at 2,000 rpm or 6,000 rpm for yogurt obtained from skimmed or whole milk, respectively. AFM_1 was finally eluted with 30 ml of acetone-$CHCl_3$ (1:4).

The final extracts were evaporated under a nitrogen stream and finally redissolved in the mobile phase (500 μl) and filtered through a 0.45 μm filter; 50 μl of extract were usually injected.

Determinations of AFM_1 levels were carried out by HPLC using the following equipment. A Varian HPLC System 9010 (Varian, Analytical Instruments, San Fernando, Calif.) equipped with a Varian 9100 AutoSampler and HP3395 computing integrator (Hewlett-Packard, Palo Alto, Calif.), with a Varian 9070 fluorescence detector, excitation and emission wavelengths were 365 nm (slit 15 nm) and 440 nm (slit 20 nm), respectively. The stationary phase was LiChrosorb RP-18 (column dimensions 125 mm by 4 mm i.d.) 5 μm (Merck, Darmstadt, Germany), and the mobile phase was isocratic acetonitrile-water (28:72). The flow rate was 1.5 ml/min. The retention time for AFM_1 was 5.4 to 5.5 min. The detection limit was 1 ng/liter (signal to noise ratio >2). Samples with AFM_1 levels below 1 ng/liter were considered negative. Recoveries of AFM_1 added at concentrations of 25 ng/liter were 91 ± 11%, 90 ± 12%, and 85 ± 13% for milk, dry milk, and yogurt, respectively. The results were not corrected for percent recovery.

Statistics. In order to evaluate seasonal effects, data of samples collected in the period May to October were compared with those collected in the period November to April. Duncan's test was used to compare means when a significant variation was highlighted by analysis of variance. Differences were accepted as significant when $P < 0.05$.

RESULTS AND DISCUSSION

Few data from surveys performed in Italy on AFM_1 contamination in heat-treated milk are available *(1, 7, 13, 17)* whereas a lot of data on raw farm milk are available *(6)*. Nevertheless, since it is commonly accepted that heat treatments do not significantly reduce the AFM_1 content *(6)*, the latter data can be compared with our data. In the present study the incidence of AFM_1 contamination in milk samples was very high, in that 86% of samples were positive (Table 1). However, Table 1 shows that AFM_1 was not detected in 14% of the milk samples, detected at low levels (1 to 10 ng/liter) in 79% of the milk samples, and detected at levels above the legal limit in only two milk samples (1%). This supports the view that a high incidence of low-level AFM_1 contamination and infrequent high contamination levels in commercial milk is caused by only a few contaminated samples entering the bulk milk supply *(6, 12, 17)*. Nevertheless, the overall incidence of contamination was higher than in other surveys carried out in Italy *(1, 3, 7, 8, 11)*. According to Visconti et al. *(17)*, most of the negative results reported in the first studies performed in Italy should be attributed to the poor sensitivity of the analytical methods used. Today the increased efficiency of extraction procedures and the accuracy of analytical methods and equipment allow much lower limits of detection, thereby increasing the percentage of positive samples. The detection limit of the present study (1 ng/liter) was lower than detection limits of 10, 14, 25, and 100 ng/liter reported by Gelosa *(7)*, Davoli et al. *(4)*, De Natale et al. *(5)*, and Amodio et al. *(1)*, respectively. Any differences between our results and those of studies performed by similar extraction methods with similar analytical sensitivity *(2, 4, 11, 17)* may be attributed to variations in laboratory equipment, experimental conditions, variation in sample source, and environmental factors.

In our study the contamination levels in milk ranged from nondetectable (<1 ng of AFM_1 per liter) to 108.54 ng of AFM_1 per liter). This contamination range was lower than those observed in the other surveys performed in Italy in the years 1980 to 1987 *(6)*. This difference could be attributed to an increased attention to concerns about AFs at about that time in Italy and the subsequent establishment of more restrictive regulations and controls on AFs in animal feeds, milk, and dairy products.

TABLE 1. *Occurrence of AFM_1 in dairy products*

Dairy product	Samples tested	Samples positive	Frequency distribution of samples by AFM_1 contamination level per liter (milk and yogurt) or per kg (dry milk)				AFM_1 contamination level, ng per liter (milk and yogurt) or per kg (dry milk)	
			<1 ng	1–10 ng	>10–50 ng	>50 ng		
	n	n (%)	n (%)	n (%)	n (%)	n (%)	Range[a]	Average[b]
Milk	159	136 (86)	23 (14)	125 (79)	9 (6)	2 (1)	<1–108.51	10.19 ± 1.36
Dry milk	97	81 (84)	16 (16)	24 (25)	47 (48)	10 (11)	<1–101.25	21.77 ± 2.56
Yoghurt	114	91 (80)	23 (20)	61 (53)	28 (25)	2 (2)	<1–496.47	18.08 ± 5.72

[a] Minimum–maximum.

[b] Mean for positive samples ± standard error of the mean.

We noted a clear seasonal effect on the level of contamination. Samples collected in the period November to April showed a contamination level ca. four times as high as samples collected in the period May to October (difference significant at $P < 0.05$; Table 2). Furthermore, all the samples collected in the hottest month (August) were negative for AFM_1. Our observations with those of Vittani *(18)*, who in milk samples collected in the spring and summer period detected AFM_1 contamination levels lower by a factor of 1.5 to 3.7 than those in the winter period. Heeschen et al. *(9)* indicated late summer as the period of lowest AFM_1 contamination. Seasonal effects on occurrence of AFM_1 in milk are likely to be related to feed type, since in winter cows are fed on greater amounts of compound feeds, whereas during spring and summer forage, roughage, and pasture are widely available. However, since climatic conditions, agricultural systems, and feeding conditions are closely interrelated, it is not easy to distinguish their roles in influencing AFM_1 incidence and contamination levels. Observations over long periods and surveys on the occurrence of AFB_1 in feeds should provide additional information.

The incidence of AFM_1 contamination in dry milk was very high, with 84% of samples testing positive (Table 1). Table 1 shows that the AFM_1 frequency distribution in dry milk samples was different from those of milk and yogurt, in that the overall frequency distribution for dry milk samples was weighted more toward the higher contamination ranges. In fact, 48% of the samples showed contaminations in the range of >10 to 50 ng of AFM_1 per kg, and 11% showed contaminations higher than 50 ng of AFM_1 per kg. The differences in water content of milk, yogurt, and dry milk clearly affected the frequency distributions of the samples. However, after taking into account that dry milk is commonly diluted with water at a 1:8 ratio before consumption, only one sample had an AFM_1 level above the legal limit. The frequency of contamination was higher and the level of AFM_1 lower in the present study compared with those observed in a study performed in Italy by Riberzani et al. *(14)*. Again, the changed awareness regarding AFs and the greater sensitivity of analytical methods can explain the different results. No statistically significant seasonal effect was observed for dry milk samples, although samples collected in the period November to April showed a slightly higher level of contamination than samples collected in the period May to October (Table 2).

The incidence of AFM_1 contamination in yogurt was quite high also, with 80% of samples testing positive (Table 1). However, Table 1 shows that most of the samples (53%) were in the range of 1 to 10 ng of AFM_1 per liter and only two samples (2%) had AFM_1 levels above the legal limit (Table 1). A clear seasonal effect on the level of contamination in yogurt was noted, in that samples collected in the period November to April showed a level of contamination ca. 4.5 times as high as samples collected in the period May to October (difference significant at $P < 0.05$; Table 2).

In conclusion, although extreme care must be used in making deductions from the results of a survey based on limited sampling, it is possible to point out that (i) according to the current toxicological knowledge and despite a high incidence, AFM_1 contamination levels in dairy products sold in Italy during 1995 should not be regarded as human health risk; (ii) a seasonal effect in the occurrence of AFM_1 was confirmed; (iii) in comparing literature data it should be considered that agricultural systems, feeding and climatic conditions, laboratory equipment, and experimental conditions are in some cases widely different and vary over time; (iv) in latter years attention to concerns about AFs in feeds, milk, and dairy products has increased in Italy, and the current regulatory limits for AFs in feeds appear to be effective. However, surveillance must be continuous and widespread, since it is known that AFB_1 contamination in feeds follows recurrent patterns over long periods of time based on the overall climate, and it may or may not be present in a particular year depending on the weather for that period.

TABLE 2. *Levels of AFM_1 contamination in dairy products: comparison between samples collected in the period May to October and November to April*

	Number of samples and AFM_1 contamination levels by calendar period			
	May to October		November to April	
	Number of samples	AFM_1 contamination level, ng per liter (milk and yogurt) or per kg (dry milk)[a]	Number of samples	AFM_1 contamination level, ng per liter (milk and yogurt) or per kg (dry milk)[a]
Milk	74	4.19 ± 0.52 B[b]	62	16.84 ± 2.59 A
Dry milk	37	18.74 ± 2.56	44	25.38 ± 3.28
Yogurt	46	6.57 ± 1.28 B	45	29.84 ± 2.62 A

[a] Mean for positive samples ± standard error of the mean.
[b] Values in the same row with different letters are significantly different ($P < 0.05$).

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