Occurrence of aflatoxin m1 in italian cheese: results of a survey conducted in 2010 and correlation with manufacturing, production season, milking animal, and maturation of cheese

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OCCURRENCE OF AFLATOXIN M_1 IN ITALIAN CHEESE: RESULTS OF A SURVEY CONDUCTED IN 2010 AND CORRELATION WITH MANUFACTURING, PRODUCTION SEASON, MILKING ANIMALS, AND MATURATION OF CHEESE

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ABSTRACT

Aflatoxin M_1 (AFM_1), a carcinogenic metabolite secreted into milk by animals fed with crops contaminated by aflatoxin B_1, can be found in dairy products because of its relative stability to treatments used to produce foodstuffs, and also to long-term storage. Maximum admissible limits of AFM_1 in milk have been set up worldwides; specific regulations regarding dairy products have also been established in some countries. Nevertheless, little and rather discordant data on the occurrence of such a contaminant in cheese and other dairy products is available, and mainly in those countries which are important producers and consumers of cheese, such as, for example, Italy. Therefore, a one-year survey was conducted by measuring AFM_1 contamination in cheese purchased on the Italian market. More than a hundred samples representing the highest variability in terms of type of cheese, origin, cheese-making process, and maturation were collected and analysed through a previously described ELISA method coupled to a very rapid, simple and solvent-free extraction. More than 83% of samples showed detectable levels of AFM_1 (>25 ng kg^{-1}); most of them were found to be contaminated at a level between 50 and 150 ng kg^{-1}. The measured AFM_1 concentration was correlated to four factors which were presumed to influence the contamination level: manufacturing, production season, milking animal, and maturation. Statistical analyses demonstrated that milking animals and manufacturing affect AFM_1 concentrations, as cheeses obtained from cows' milk and from artisanal production are more contaminated than cheeses produced with milk belonging to other animals and in industrial contexts. The others two factors showed statistically non-significant differences between groups.

KEYWORDS Aflatoxin M_1, cheese, ELISA, survey.

1. INTRODUCTION

Aflatoxins are secondary metabolites produced by fungi, mainly *Aspergillus flavus* and *A. parasiticus*. Until today, more than 300 aflatoxins have been identified, the most toxic and diffuse is the aflatoxin B_1 (AFB_1), which has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002). AFB1 contamination can affect a variety of crops, including nuts, cereals, oil seeds, dried fruits, legumes, potatoes (Lee et al., 2004), and crops used as feed for dairy cattle. Once ingested it is rapidly absorbed and transformed into a hydroxylated metabolite, which is secreted into the milk and has been designated as aflatoxin M_1 (AFM_1). The hepatotoxicity and carcinogenic effects of AFM_1 have also been demonstrated and IARC have included it in group I human carcinogens as well (IARC, 2002). Due to toxicity, most countries have set up maximum admissible levels of AFM_1 in milk, which varies from the 50 ng kg^{-1} established by the EU to the 500 ng kg^{-1} established by US FDA (European Commission, 2003; U.S. Food and Drug Administration, 2011). More restrictive MRLs have been decided by the EU for the presence of AFM_1 in baby food (European Commission, 2004).

Aflatoxin M_1 is relatively resistant to heat treatments such as pasteurization of milk and to treatments used during cheese production, like, for example, acidic conditions (Oruc et al., 2006; Deveci, 2007). In addition, it has been demonstrated that as AFM_1 is bound to milk proteins, thus it is unevenly distributed between whey and curd, with the highest concentration found in the curd (Govaris et al., 2001; Kamkar et al., 2008). Therefore, when contaminated milk is used in making cheese, AFM_1 is found in the dairy product at levels which are 3-8 fold higher than in the milk (Govaris et al., 2001; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009). Several survey studies regarding the levels of contamination of AFM_1 in dairy products have been recently reported. Most of them have been carried out in the Middle East or in Asia (Kim et al., 2000; Lin et al., 2004; Maqbool et al., 2009; Fallah et al., 2009; Dashti et al., 2009; Amer and Ibrahim, 2010), with the exception of some studies carried out in Brazil (Oliveira et al., 2011) and in some regions of Italy (Montagna et al., 2008; Virdis et al., 2008). These studies demonstrate that AFM_1 is frequently found in cheeses, with a significant incidence of highly contaminated samples. Nevertheless, an adequate regulation about admissible limits of AFM_1 in dairy products is still lacking in most countries. The strategy applied by the EU and the USA to assure food safety is based on the assumption that strict controls on milk would prevent contamination of derived products. On the other hand, a specific maximum admissible level for AFM_1 in cheese has been set in some countries (Dashti et al., 2009; Creppy, 2002) at 250 ng kg^{-1}, with the exception of Italy

which, in 2004, established a 450 ng kg^{-1} limit applicable to hard cheese to protect its parmesan production, which was generally highly contaminated in that year as the result of a random peak of AFB_1 contamination in feed.

Several methods for aflatoxin M_1 determination in dairy products have been developed, including high-performance liquid chromatography associated to fluorescence or mass spectrometric detection (Muscarella et al., 2007; Cavaliere et al., 2006). Immunochemical methods have also been described (Kim et al., 2000; Lopez et al., 2001; Pei et al., 2009) and are employed as screening methods in routine analysis, mainly because of their simplicity and rapidity. However, the analysis of dairy products still involves a time-consuming extraction of AFM_1, which, in fact, strongly limits its determination. Recently, we described a very simple and fast procedure for the extraction of AFM_1 from dairy products, which uses an aqueous extracting medium and which allows us to process several samples at the same time (Anfossi et al., 2008). The validity of the aqueous extraction followed by AFM_1 quantification by means of an ELISA protocol was verified on yogurt samples and on different types of cheese; fresh, creamy, soft, semi-hard, hard, blue, and elastic cheese. Validation of the described approach was also made by comparing results of naturally contaminated cheese with those obtained through a HPLC-FLD reference method. Using this approach for the quantification of AFM_1, the occurrence of the toxin in Italian cheese was investigated during a one-year monitoring program. More than a hundred samples, belonging to different kinds of cheeses, milking animals, manufacturing methods, and cheese maturation was collected and analysed. The first aim of the work was to assess the occurrence of the aflatoxin M1 in Italian cheese, given that Italy is a cheese producer of global importance and that Italians are high consumers of both national and imported cheeses (U. S. Department of Agriculture Foreign Agricultural Service, 2011). Besides this first purpose of snapshotting the amplitude of the risk associated with AFM_1 contamination in the Italian cheeses, the main objective of the work was the identification of correlations between levels of AFM_1 in cheese and some factors which were identified as potentially influencing the presence and the concentration of the toxin. For this purpose, samples were divided into four categories according to: the animal which supplied the milk used to produce the cheese, the type of manufacturing, the season of production, and the maturation of the cheese. Within each category, samples were further sub-divided into groups, which were compared with each other by statistical tests to highlight significant differences between groups. In fact, most published surveys on AFM_1 contamination in cheese merely showed results and the distribution of the contamination between levels of AFM_1 concentrations, without interpreting or generalising the data. An exception is the recent work of Fallah et al. (2011), who found significant correlation of AFM_1 contamination with the production season (which is

associated with animal feeding and, therefore, with the AFB_1 intake of animals) and with manufacturing. Reported data demonstrates a significantly higher contamination of samples produced during winter, compared to those produced in summer, and of samples produced in industrial manufacturing compared to those produced by small-scale manufacturing. In addition, cheese obtained from cows' milk showed higher levels of the target toxin, in accordance with previous observation of Hussain and co-workers (2010).

2. MATERIALS AND METHODS

2.1 Sample preparation

Samples classified as belonging to major brands were obtained from local supermarkets, while samples classified as belonging to small-scale producers were kindly provided by the Slow Food association and by Eataly Distribuzione srl (Cuneo, Italy). Hard and medium matured cheese samples were stored at -18°C until analysed. Fresh cheese samples were immediately analysed without freezing. All samples were analysed before their expiry dates.

A portion of sample (c. 100 g) was roughly cut and then thoroughly broken up and homogenized in a kitchen mixer. Aflatoxin M_1 extraction was performed as previously described (Anfossi et al., 2008). Briefly, 5 g of homogenised cheese sample was weighed in a 50-mL conic tube. 20 mL of the extraction solution provided by the ELISA kit manufacturer was added and the combination was maintained at 50°C for 15 min under vigorous stirring. The mixture was then centrifuged in a refrigerated centrifuge (25°C) for 15 min at 3200 x g. The fatty semi-solid upper layer was discarded and the liquid serum was withdrawn and directly analysed. Unless otherwise specified, samples were extracted singly and analysed in triplicate.

2.2 Indirect competitive ELISA

The AFM_1 quantification was carried out via a previously described ELISA protocol (Anfossi et al., 2008) as follows: 60 µL of AFM_1 standard solutions or sample extracts were added to the same amount of the diluted anti-AFM_1 antiserum and incubated in non-coated wells for 50 min. One hundred microliters of the mixture were transferred into coated wells (functionalised with an AFM_1-BSA conjugate) and incubated for 15 min. After washing, 100 µL of the diluted anti-rabbit antibody (conjugate with the peroxidase) was incubated in wells for 15 min. Colour development was obtained by a 20 min incubation with the TMB solution (100 µL per well), followed by the addition of 50 µL of a stop solution. Finally, absorbance was recorded at 450 nm.

Aflatoxin M_1 concentrations were determined by interpolation on a linear calibration curve. Linearization of the calibration curve was performed by logit-log transformation, by plotting the

logit of the ratio (as a percentage) between the absorbance at each concentration of analyte (B) and the absorbance in the absence of analyte (B_0) against the log of the analyte concentration. The best data fit was obtained by linear regression of the standard points. The optimized ELISA kit has a detection limit of 25 ng kg^{-1}, a dynamic range of 30-500 ng kg^{-1} and relative standard deviations lower than 20%.

Reproducibility (RSD% lower than 20%) and accuracy (recovery values comprising between 80 and 120%) of the assay were further assessed by measuring the AFM_1 content of six samples (belonging to different representative types of cheeses) on different days and comparing results to those of an HPLC-FLD reference method (Anfossi et al., 2008).

2.3 Analysis of data

Cheese samples were divided into sub-classes for each of the four categories, identified as conditions that could potentially influence the level of the AFM_1 contamination in cheese. The identified categories were: the length of cheese maturation, the origin and typology of production, the season of production, and the animal species that supplies the milk used to produce the cheese. A summary of samples analysed, together with sub-class division and numbering, is presented in Table 1.

The statistical analysis of data was carried out by SigmaPlot 11.0 software (Systat Software Inc., CA, USA). First, the Kolmogorov-Smirnov and Shapiro-Wilk tests to check the distribution of data were carried out. Statistical differences between groups were evaluated by means of the Wilcoxon-Mann-Withney Test on Ranks for the comparison between two groups. The extended test (Kruskall-Wallis ANOVA Test on Ranks) was used for the comparison between more than two groups. The decision level, expressed by the P value, was anyway set to 0.05. To be able to include undetectable samples (AFM_1 concentration below 25 ng kg^{-1}, which is the detection limit of the method) in the statistical analysis, an AFM_1 concentration value of 25 ng kg^{-1} was assigned to each of them, according to Zhang et al (2008), who demonstrated that if two right-censored samples have an identical censoring point (as in the case of comparison between groups of data obtained via the same analytical method and thus with the same LOD), non-parametric tests on ranks could be used, which considers the censored observations as observed at the censored point.

3. RESULTS AND DISCUSSION

During a one-year survey, 102 cheese samples were collected in various supermarkets as representative of major brands and were also kindly supplied by associations of small-scale producers . Samples were highly variable as regards the type (creamy, soft, semi-hard, hard, elastic,

blue), the process of cheese-making and the regional origin. National cheeses were mainly considered, except for a few samples, which were representative of those imported cheeses most frequently consumed in Italy. In addition, the variability of samples was pursued to ensure a statistical significance of results.

The AFM_1 content come out as undetectable in 17 of the 102 analysed cheeses, which means that more than 83% of analysed samples showed levels of toxin higher than 25 ng kg^{-1}. Forty-four samples were found to be contaminated at levels below 50 ng kg^{-1}, the current admissible limit of AFM_1 in milk (European Commission, 2003). One sample was contaminated at a level higher than 250 ng kg^{-1}; however, being a hard cheese, it was still complying with current Italian regulation (Montagna et al, 2008).

As shown in Table 1, for almost all identified sub-classes, the level of contamination between 50 and 150 AFM_1 ng kg^{-1} appears to be the most populated, with the notable exception of fresh cheeses and cheese made with goats' milk alone or mixed with other types of milk.

The summarised data is reported in Figure 1, which shows the tailed profile of the distribution. The normality assumption test carried out on all data pointed out that the distribution is not Gaussian, with a skewness of 1.645, a kurtosis value of 4.762 and a Kolmogorov-Smirnov distance of 0.120. Experimental data could be modelled by log-normal distribution; the logarithmic transformed data passed normality test at a significance level of 0.05, and a good correlation (r^2 = 0.9918) was observed between raw data and a 3-parameter log-normal equation (Harris and De Mets, 1972). The normality assumption test was separately repeated on the categories, which demonstrated that data was not normally distributed within each category either. Therefore, the differences between groups were evaluated via non-parametric or distribution-free statistical tests, which, in addition, enabled the comparison between unequally populated groups and also for those groups composed of few elements. Table 2 summarises the descriptive statistics of data regrouped into a single class (total) and of data divided into categories and sub-classes. The total distribution has a median value of 60.4 ng kg^{-1} and a range of 281.1 ng kg^{-1}. Although median values highlight some differences between groups within the various categories, ranges are generally large for all groups, which mean that results are spread and occasional high contaminations have been found in samples belonging to each of the identified categories and groups. Considering the distribution within the classes of the highest values of contamination (above 150 ng kg^{-1}), it would seem that there is a greater incidence of high AFM_1 concentration in long matured cheese, in cheese produced on a small-scale and in those obtained from cows' milk. Nevertheless, statistical data analysis brought to light that the only factor which determined significant differences among groups was the origin of the milk (Figure 2).

More specifically, cheese made with cows' milk showed itself to be more contaminated than cheese made with goat or sheep (or mixed goat/sheep, mixed goat/cow and sheep/cow) milk.

This result is in agreement with those reported in the literature about the different production and excretion of AFM_1 in milk (Barbiroli et al. 2007). It was shown that milk from goats and sheep is less contaminated than cows' milk, both because of the different digestive apparatuses and mechanism of AFB_1 assimilation of animals, and for the different feeding, provided that cattle fodders are more likely to be contaminated by AFB_1 than those used to feed sheep and goats. This finding also confirms previous observations of other authors, who also reported that cow's cheeses are more contaminated than others (Montagna et al., 2008; Fallah et al., 2011; Hussain et al. 2010).

As a consequence of this first observation, samples made with cows' milk (82 samples) were isolated from the rest and the statistical analysis was repeated on them for the other three identified categories: manufacturing, cheese maturation, and production season (Table 2 and Figure 2).

In contrast to the findings recently reported by Fallah et al. (2011), industrial scale products were confirmed to be less contaminated than small-scale products. However, a lower contamination of major brand products may be due to the fact that checks conducted on milk to be used in cheese production are more stringent in industrial scale production than in artisanal contexts. In addition, artisans often make use of only one milk source, which can occasionally be contaminated with high AFM_1 levels (although within the legal limit) thus determining a peak of contamination which of the derived cheese as well. Industrial production uses a combination of milk from various sources, therefore the risk of the occurrence of contamination peaks is lower. Actually, excluding samples obtained in periods of the year in which milk-producing animals were grazing (and therefore supposedly not subject to the ingestion of AFB_1), the median of AFM1 concentration in cheeses produced on a small-scale increased and the distance respect to those obtained from industrial producers augments (data not shown).

Contrary to what appears first from the data shown in Table 1, maturation does not influence AFM_1 content in cheese. Several other authors observed that maturation does not significantly alter the AFM_1 concentration. A decrease of aflatoxin M_1 concentration during maturation could be assumed, because of degradation of the toxin with time. Nevertheless, this degradation has not been pointed out in any previous works aimed at assessing the fate of the toxin (Oruc et al., 2006; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009).

The production season (and consequently, or partially consequently, the animal feeding) is also irrelevant according to statistical analysis, which could be partially explained by the fact that aflatoxin producing fungi also affects crops pre-harvest (Bankole and Mabekoje, 2004). Nevertheless, the main limitation in making this analysis was the uncertainty of the attribution of

samples. In fact, some samples were accompanied by exhaustive information (period of production, animal feeding), however for most of them information was incomplete or unavailable. In these cases, attribution to groups was assumed on the basis of generic information regarding the type of cheese, the expiry date and the similarity to other samples. Therefore, results on this factor cannot be considered as conclusive and would need further investigation.

4. CONCLUSIONS

The reported findings of the study conducted in a one-year survey on various types of cheese in Italy and their correlation to some of the factors which could influence aflatoxin M_1 presence in cheese allowed the identification of some relevant factors (milk origin, manufacturing type) and to rationalise the results of the study and also those of preceding observations. The statistical approach is promising; however, further investigations on factors which have been already identified, together with attempts to widen the number of considered factors, would occur. From the point of view of the risk to consumers posed by AFM_1 intake with cheese, the assumption seems verified that control strategies to limit AFB_1 in feed and AFM_1 in milk are an adequate protection for consumer health. Despite the high incidence of AFM_1 at detectable concentrations, all samples were contaminated beyond the admissible limit (250 ng kg^{-1}), except for 1 hard cheese, which still complied with Italian legal limits (450 ng kg^{-1}).

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FIGURE CAPTIONS

Figure 1. Distribution of 102 analysed samples between levels of AFM_1 contamination. Data was interpolated by a 3-parameter log-normal equation (solid line), $r^2 = 0.9918$

Figure 2. Box plots for the statistical comparison of groups within each of the four categories considered. Grey boxes represent the 25^{th}-75^{th} percentiles, vertical bars the 5^{th} and 95^{th} percentiles, dots the outliners, and the horizontal line the median value. The first plot represents all analysed samples (n=102), whereas the other three represent the statistical evaluation of samples made with cow's milk (n=82). Significant differences can be highlighted in the first (milk type) and in the second plot (manufacturing). The third and fourth plots (production season and maturation) show no significant differences between groups.

TABLES

Table 1. Number of cheese samples analysed for the various groups identified as potentially influencing the level of AFM1 contamination, and distribution of samples between these groups as a function of the level of AFM1 contamination.

Category	Group	Total of analysed samples	N of samples contaminated at a level (ng kg^{-1}) / total of analysed samples (%)			
			<50	50-150	150-250	>250
Maturation	Long (>3 months)	29	31.0	62.1	3.4	3.4
	Medium (>45 days; <3 months)	46	43.5	54.3	2.2	0.0
	Fresh (<45 days)	27	55.6	40.8	3.7	0.0
Manufacturing	Big brands	38	47.4	52.6	0.0	0.0
	Small-scale	64	40.6	53.1	4.7	1.6
Production season	Winter-spring	65	38.4	56.9	3.1	1.5
	Summer-autumn	37	51.4	45.9	2.7	0.0
Milk type	Cow	82	39.0	56.1	3.7	1.2
	Sheep	6	50.0	50.0	0.0	0.0
	Goat	6	83.3	16.7	0.0	0.0
	Buffalo	3	33.3	66.7	0.0	0.0
	Mix [a]	5	60.0	40.0	0.0	0.0
TOTAL		102	43.1	52.9	2.9	1.0

[a] goat, sheep and mixed goat/sheep, goat/cow, sheep/cow

Table 2. Descriptive statistics for data on AFM1 concentrations (ng kg^{-1}) found in analysed cheese samples. Cheeses were differently regrouped within each of the four categories, both considering all analysed samples and only samples produced from cows' milk.

Category	Group	All samples				Cows' milk samples			
		N	Median	Range	Percentiles (25^{th}-75^{th})	N	Median	Range	Percentiles (25^{th}-75^{th})
TOTAL		102	60.4	281.1	39.7-89.9				
Maturation	Long	29	64.5	257.4	46.0-97.0	23	74.8	257.4	46.2-100.5
	Medium	46	61.1	193.5	42.4-76.8	43	63.8	193.5	43.3-87.6
	Fresh	27	44.6	130.0	25.8-88.0	20	45.8	130.0	28.8-92.4
Manufacturing	Big brands	38	54.8	116.3	36.7-74.2	36	56.2	116.3	40.4-74.2
	Small-scale	64	64.8	257.8	40.6-95.5	46	73.3	257.8	45.3-103.2
Production season	Winter-spring	65	63.8	257.8	42.5-89.8	56	65.1	257.8	45.3-90.2
	Summer-autumn	37	47.1	130.0	35.7-89.9	32	52.8	130.0	39.7-102.2
Milk type	Cow	82	64.0	257.8	43.1-95.0				
	Others [a]	17	30.0	112.6	25.0-63.3				

[a] goat, sheep and mixed goat/sheep, goat/cow, sheep/cow

FIGURES

Figure 1.

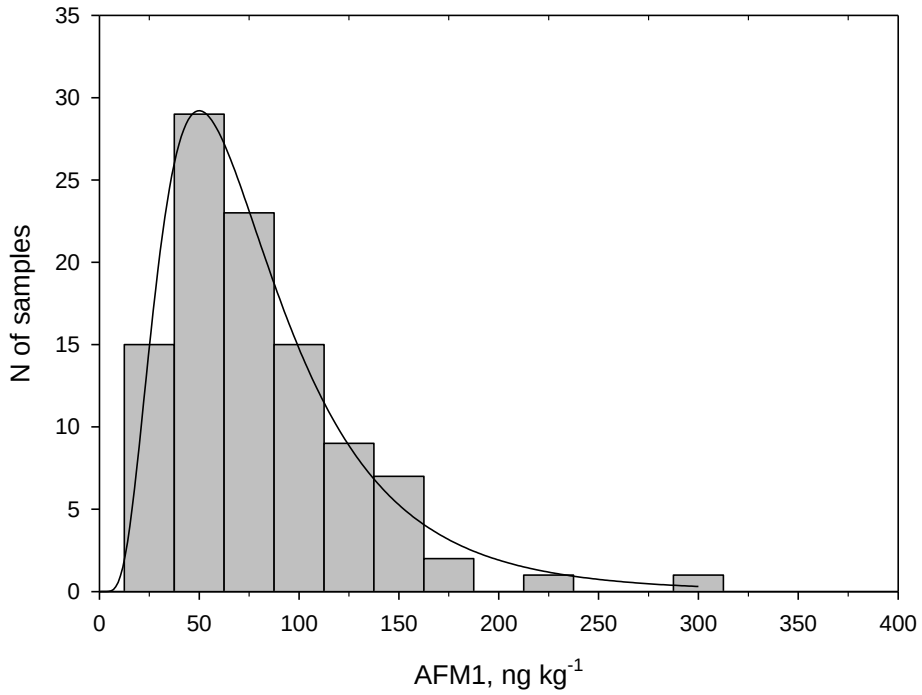
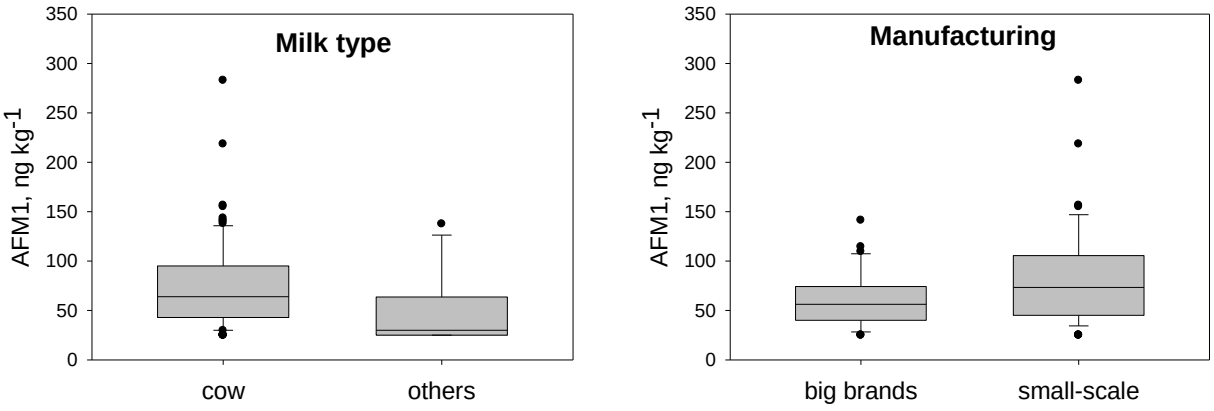


Figure 2.


Milk type
AFM1, ng kg-1
350
300
250
200
150
100
50
0
cow
others
Manufacturing
AFM1, ng kg-1
350
300
250
200
150
100
50
0
big brands
small-scale