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Risk assessment scenarios of children's exposure to aflatoxin M1 residues in different milk types from the Greek market

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ABSTRACT

Occurrence of aflatoxin M1 (AFM1) was determined in 196 milk samples (conventional, organic and kids milk) from the Greek market during November 2009 to June 2010. AFM1 content was analyzed using an Enzyme-Linked Immunosorbent Assay (ELISA) commercial kit. Aflatoxin M1 was detected in 46.5% of the samples. 46.5% of the samples were found positive for AFM1. The most frequent range of detection was between 5 and 10 ng/l. Based on the EU regulation only 2 milk samples presented AFM1 levels higher than the maximum residue limits. Two different scenarios were used for the determination of hazard index: (a) scenario 1 using only positive (detected AFM1) samples and (b) scenario 2 when missing values where imputed with Limit of Detection (LOD) divided by 2. Significant statistical differences between different milk categories were presented only when the results were imputed with LOD/2 values. Exposure assessment scenarios were developed for ages 1, 3, 5, 7 and 12 and their respective estimated weights and daily milk consumption. Under the worst-case scenario all milk types presented a Hazard Index (HI) less than one. The highest HI values appear in the ages of 1–3.

1. Introduction

Quantitative exposure assessment is a methodology developed to analyze scientific information in order to evaluate the severity and probability of an adverse event. Risk assessment can be used to estimate human exposure to xenobiotics through the consumption of food and therefore to provide a link between possible hazards in the food chain and the risks reflected to human health. Risk assessment results may also offer the scientific grounds for risk management decisions and options. Two of the most important parameters affecting the risk assessment are the amount of food consumed during a specific period of time and the concentration of xenobiotic residues. As far as the evaluation of the toxicological properties of xenobiotic mixtures in foods is concerned, detailed information on the composition of the mixture and the mechanism of action of each specific xenobiotic are required (Reffstrup et al., 2010).

Milk is considered to be a perfect natural food for consumers of all age groups due to its high nutritional value. It is high in protein and a valuable source of calcium, vitamins and antioxidants (Zeluta et al., 2009). Additionally, certain milk lipids such as butyric acid are known to present anticarcinogenic properties (Michalski and Januel, 2006). Due to the central role milk plays in human diet it holds a great economical significance on a global level. According to the latest statistics from the European Commission (2011), milk production is a very important component of the agricultural economy as its total production share varies between 5.8% and 33.5% (http://ec.europa.eu/agriculture/publi/fact/milk/2007_en.pdf). Out of the 136.360.000 tonnes of total collected cow milk, 31.540.000 tonnes are consumed as dietary milk (Eurostat, 2011). Due to globalization, milk and its products may travel worldwide. This fact in combination with major food crisis situations (e.g. milk pollution with melamine) has rendered consumers increasingly conscious in terms of product safety and quality. Through recent bibliography it has been demonstrated that aflatoxin M1 (AFM1) is one of the most important xenobiotics affecting the two previously reported parameters.

Aflatoxins are a group of toxic chemical compounds produced by the species of *Aspergilus flavus*, *Aspergilus parasiticus* and rarely by *Aspergilus nomius*. B aflatoxins are primarily produced by *A. flavus*, while the other two species produce both B and G aflatoxins (Pei et al., 2009). Cereals and silage seem to be the most common

Abbreviations: AFB1, aflatoxin B1; AFM1, aflatoxin M1; ALARA, as low as reasonably achievable; EDI, estimated daily intake; ELISA, Enzyme-Linked Immunosorbent Assay; HI, hazard index; LOD, limit of detection; MRLs, maximum residues limits; TD_{50}, threshold dose; TDI, tolerable daily intake.

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feedstuffs susceptible to mycotoxin contamination (Scudamore et al., 1998). Aflatoxin B1 (AFB1) is the most toxic metabolite, demonstrating teratogenic, mutagenic and carcinogenic effects (Stagos et al., 2012). AFM1 is the main hydroxylated derivative of AFB1 formed in liver by means of P450 cytochrome enzymes and secreted into milk through the mammary gland of dairy cows. The AFM1 derivative can be detected in milk within 12–24 h after the first intake of AFB1, while its concentration decreases to an undetectable level 72 h after the initial intake is stopped (Fallah, 2010). AFM1 is very resistant to thermal treatments such as pasteurization and sterilization during dietary milk production (Galvano et al., 1996). Upon entering the body it may cause damage to the DNA ultimately leading to mutagenic and carcinogenic effects, whereas in the case of aflatoxicosis, liver is the main target organ (Fung and Clark, 2004). The International Agency for Research on Cancer has classified AFM1 as a group 2B carcinogen (possibly carcinogenic to humans) (Pei et al., 2009).

High milk consumption by all age groups and especially for children appears to be one of the most important exposure factors through diet for AFM1 (Rahimi et al., 2010; Prandini et al., 2009). The objective of the present study is to evaluate human exposure to AFM1 via dietary milk consumption and at the same time to evaluate the potential risk through different risk assessment scenarios.

2. Materials and methods

2.1. Sampling

A total of 196 samples of dietary cow milk from different market points were collected during November 2009 to June 2010. The characteristics of milk samples are presented in Table 1. Among 196 samples, 154 (78.57%) were conventional, 42 (21.4%) were organic and 30 (15.3%) of the milk samples were specific for children older than 12 months. Fresh milk samples represented 51% of the total samples while the rest were Ultra High Temperature (UHT) processed milk. The majority of samples were bottled in a Tetrapak® type package while the rest were bottled in plastic containers.

All samples were transported at 2–4 °C in dry ice to the laboratory and were analyzed with ELISA on the same day of sampling, while sub-samples were kept in the deep-freeze for pesticide residue analysis (performed in less than 5 days).

Sampling was completed according to the principles of European Commission Regulation No. 401/2006 and European Commission Directive No. 63/2002.

2.2. AFM1 residue determination analysis

2.2.1. Sample Preparation for AFM1 analysis

Ten ml of the milk samples were centrifuged at 3500*g* for 10 min at 10 °C. The upper creamy layer was removed by a Pasteur pipette and from the lower phase (defatted supernatant) 100 μl per well was used in the test plate.

Table 1
Characteristics of milk samples collected form Greek market during 2009–2010 and analyzed for AFM1.

	N	%
Organic	42	21.4
Kid	30	15.3
Milk fat		
Full (>3.0% fat)	112	57.1
Semi-skimed (2.0–3.0% fat)	28	14.3
Light (<2.0% fat)	56	28.6
Type of milk		
Fresh (storage life up to 4 days)	100	51.0
Long Life (storage life >4 days)	96	49.0
Type of bottle		
Tetrapack	135	89.1
Plastic	61	31.1

N: Number of samples.
%: Percentage of total samples.

2.2.2. ELISA test procedure

The quantitative analysis of AFM1 was performed by competitive ELISA using RIDASCREEN® Aflatoxin M1 30/15 test kit. One hundred micro litre of standard solutions and prepared samples were added into separate microtitre wells and incubated for 60 min at room temperature (22–25 °C) in the dark. The liquid was then poured out and the wells were washed with washing buffer (250 μl) twice. In the following stage, 100 μl of the diluted enzyme conjugate was added to the wells, mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Again, the wells were washed twice with washing buffer. Afterwards, 100 μl of substrate/chromogen was added, mixed gently and incubated in the dark at room temperature for 15 min. Finally, 100 μl of the stop reagent was added into the wells and the absorbance was measured at $\lambda = 450$ nm in ELISA plate reader, against air blank within 15 min. According to RIDASCREEN kit recommendations, the lowest detection limit is 5 ng/l for milk.

2.3. Statistical analysis and exposure estimation to AFM1

Levels of AFM1 were expressed in the form of mean and 95% confidence interval (mean 95%CI) and percentiles (25th, 50th, 75th and 80th). Differences in concentrations between two groups and more than two groups were assessed by Mann–Whitney and Kruskal–Wallis tests respectively. IBM SPSS Statistics 20.0 and EXCEL 2007 were used for statistical analysis and plots.

The estimation of Hazard Index (HI) is based on proposal of Kuiper-Goodman, 1990 which reported also in Shundo et al. (2009). In more details, estimated daily intake (EDI) was computed using 80th percentile of AFM1 residues in positive samples (scenario 1) and 80th percentiles of AFM1 residues when LOD/2 was imputed in the missing values (scenario 2). The consumption of milk was roughly estimated at 250 ml for the age of 1, 400 ml for the ages of 3, 5 and 7 and 800 ml for the age of 12 (according to municipal day care centers, elementary schools and pediatric clinics).

The exposure assessment scenarios were developed for the ages of 1, 3, 5, 7 and 12 and body weights of 10, 14, 19, 24 and 37 kg respectively (based on Greek pediatric development normograms).

As a denominator for hazard index, a TD_{50} (threshold dose per body weight which divided by 5000) was used.

3. Results and discussion

Out of 196 samples, 91 (46.5%) were positive for AFM1 with a mean value of 10 ng/l. Only 2 conventional milk samples presented levels of AFM1 residues higher than the Maximum Residues Levels (MRLs) according to European Commision Regulation EC 1881/2006 (European Commission, 2010). Table 2 presents the descriptive statistics of AFM1 residues (ng/l) in selected milk samples under scenario 1 and 2 assumptions. The most common range of detection was 5–10 ng/l as supported from median and quartile values for milk categories presented in Table 2.

In the first scenario AFM1 residues were detected at levels that bear no significant statistical difference between the different types of milk (see Table 2 for *p* values). However, the levels of AFM1 residues in milk samples from Tetrapak® type packaging were significantly higher than the corresponding ones from plastic bottles ($p = 0.003$). Although the current literature lacks of details about the specific issue, difference may explained by the life span of milk (fresh and long storage). In more details 51 of the 61 (77.3%) of the milk samples packaged in Tetrapak® were fresh (have a life span up to 4 days) while 15 of the 30 (60.0%) packaged in plastic bottles are long life ($p < 0.001$). Therefore, the difference in packaging could be explained by the higher AFM1 levels and the higher prevalence of fresh milk in Tetrapack®.

In the second scenario, when the AFM1 residues were imputed with LOD/2, the following significant differences were apparent. (1) The levels of AFM1 residues in semi-skimmed milk were significantly lower than the corresponding ones in light and full fat milk ($p = 0.003$). (2) The levels of AFM1 residues in children's milk were significantly lower than the corresponding ones in normal milk ($p < 0.001$). (3) The levels of AFM1 residues in long life milk were significantly lower than the corresponding ones in fresh milk ($p < 0.001$). (4) The levels of AFM1 residues in plastic bottles were significantly lower than the corresponding ones in Tetrapak® ($p = 0.050$). In both scenarios no significant statistical difference