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ASPECTS CONCERNING AFLATOXINS INCIDENCE IN MILK AND MILK PRODUCTS

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Abstract: *Mycotoxins production was found most frequently in pre-harvest grains that are harvested under high temperatures, prolonged drought and high insect activity. Aflatoxine incidence in milk and milk products has become a typical issue for many countries, as there were detected amounts over legal limits in consume milk. Origins of aflatoxins in milk and dairy products are mainly resulted from feeds supplied to animals. This paper proposes a study on aflatoxin incidence in compound feed and milk - raw material in the dairy industry. For determinations was used Elisa enzyme- immunity-test. Records should be maintained for all feeds, feeding practices, milk contamination and animal health and performance for all cases of aflatoxin contamination of milk.*

Keywords: *safety, carcinogenicity, maximum residue limit, monitoring*

1. Introduction

Production and marketing of quality and health-safety foods are primary objectives for food industry. Recently, the aspect of aflatoxins contamination for milk producers was coming back, due to the Serbia milk contamination event.
Panic has been induced among consumers, and that was the moment when authorities had to recognize the overtaking level for aflatoxins, much higher than legal limits, in drinking milk.
In 2012, in United States, was found high level of aflatoxins, half of the corn harvest being compromised. In our country, National Sanitary Veterinary and Food Safety Authority has specified that in 2012, 81 samples of milk were analyzed for aflatoxins determination, all in conformity with regulations and also, that the country hadn't received contaminated milk from Serbia [1,2].
Last years were the most favorable for mycotoxins development, in whole Europe, including Romania. The mycotoxins European contamination level is about 20%, even in economic developed countries, the percent being much higher through less competitive agriculture conditions. In these countries has been estimated a harvest loss of 5-10%, because of molds'attack, that's why have been required special measures [3, 4, 5].
The mycotoxins analysis of feedstuffs or foodstuffs represents initial stadium of mycotoxicologic research. An ideal model should be analysis for each sample, in initial medium conditions. Unfortunately, the phenomenon of apparition, growth, developing and regression of mould species are not known completely. That is

why mycotoxins investigation has limited area and must be completed with mycotoxicological research for monitoring vegetables safety.

Hereinafter are presented the main conditions for molds developing and mycotoxins production: high values for temperature and moisture, favorable parameters for the substrate, water activity. The food contamination with aflatoxins producing molds could compromise the food safety and could affect the consumers' health, by producing intoxication, cancer, even death [6].

European and international researchers have found over 3500 molecules inside mycotoxins group. Trying to synthesize and classify these substances has encountered a problem with the fact that the same mould could synthesize more mycotoxins and, vice versa, the same mycotoxin could be obtained from different molds. All groups of mycotoxins are produced from 5 (five) genus of alteration molds: *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps.*

Milk and milk products could contain aflatoxins from biosynthesis by *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius.* These molds could get consumer illness by their development on different foods. In the case of cheese assortments, we could meet many types of mycotoxins, so derived from environment molds, as well as mold culture used in cheese fabrication [7,8].

High values were found in the case of cheese assortments with long time of preservation and advanced proteolysis degree. Aflatoxins could be present even in melted cheese, with lower incidence. Aflatoxins are among those most risky and carcinogenic substances, because their methabolisation through liver, toward intermediate substances, M1 or M2.

The highest concentration of aflatoxins is formed during cereal cultivation and storage, but also during their processing. Growth of aflatoxins in diet and increase in the rate of these toxins from the critical range (20 in diet and 0.5µg/kg in milk) cause decrease in fertility and reproductive ability in animals [9].

In 1987, World Health Organization classified Group 1 of aflatoxins as carcinogen, with a great impact on liver health and DNA destroyer agents, M1 aflatoxin being considered the most aggressive. The incidence of M1 aflatoxin in milk could be explained by molded feed (B1) consumed by animals.

Short-time ingestion of large quantities of aflatoxins can lead to acute poisoning, which can manifest through bleeding, acute liver failure and even death. Lethal dose depends on several factors, the amount of aflatoxin ingested, age, health and nutritional status of the individual. Eating a small amount, but for a long period of time may cause chronic poisoning. Effects and symptoms are often difficult to identify, both because of low intensity but mostly because of their non-specific character [10].

Consumption of food contaminated with aflatoxins can have serious consequences such as cirrhosis and hepatocarcinoma, a particular form of liver cancer. Epidemiological studies developed in India and some African countries have shown an association between the consumption of food contaminated with aflatoxin and increased incidence of liver cancer.

In humans, aflatoxins have caused a number of sub-acute and chronically effects. These effects mainly include liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis caused by repeated ingestion of small quantities of aflatoxins.

This paper proposes a study of aflatoxin contamination in milk and dairy products compared with their presence in feed.

2. Materials and methods

In present, the immununologic tests consists time one of the rapid methods for aflatoxin and other micotoxin detection in foodstuffs. Immunological tests were succesful used since 1970 *(Pestka et al., 1995)*, for mycotoxins detection. For qualitative or semi-quantitative analyses of majority food matrix it can be used mycrotitration boards or Elisa membranes with polyclonal or monoclonal antibodies.
However, interference matrix or the presence of mycotoxins could interfere with conjugated antibodies that could leads to errors for quantitative results of mycotoxins. Elisa kit is used for routine analyses for majority of food matrix.

The principle of method
The kit board consists of 12 stripes with 8 places, coated with rabbit antibody and anti IgG mouse. In the first step, are added specific antibody (mouse anti aflatoxine), aflatoxine marqued with enzyme conjugated and AFM1 standards and also the samples to be analysed. Specific antibody were binded by rabbit antibody strike-bound, and, in the same time, free aflatoxin(unbinded), from standards or samples and marqued aflatoxin are in competition for binding situs of specific antibody, that is immunoenzyme competitive test.
After one hour, the reagents (marqued with enzyme) unbinded, were washed. The enzyme quantity that is binded was revealed by adding chromogen substrate (tetramethylbenzidine, TMB). This will turn chromogen in a coloured one. The reaction was stopped by adding 100 µL H_2SO_4 1M solution. The colour intensity was measured by spectrophotometry, at 450 nm, with ELISA reader system, taking account that the aflatoxine M1 concentration is in inverse ratio with sample colour intensity. In the same time was established a calibrating curve with standard solutions of M1 aflatoxine: 5(S1); 10(S2); 25(S3); 50(S4); 100(S5) şi 200(S6) ng/L.

The sample preparation
The samples were prepared in compliance with technical informations of R-Biopharm AG, Ridascreen kit for AFM1.
Milk samples were centrifugated for fat carrying-off for 10 min / 3500 g and after centrifugation, the Kit contain the following:
- microtitration board (96 places), 12 stripes with 8 places, coated with mouse antibody anti-IgG;
- 6 aflatoxine M1 flasks (1,3 mL) with concentration 0 ng/L (zero standard), 5 ng/L , 10 ng/L , 25 ng/L , 50 ng/L , 100 ng/L, 200 ng/L;
- 1 conjugate solution flask (aflatoxine marqued with peroxidase) manifold drying antibody anti- M1 (manifold drying 1,3 mL);
- chromogen substrate solution flask (tetramethylbenzidine) , (10 mL);
- buffer solution flask pH 7, (20 mL);
- stopping solution flask, H_2SO_4 1N (14 mL);
- washing buffer solution flask(30 mL).

Reagents and solutions
With kits indicated for ELISA method could be effectuated 96 determinations, including standards.
Reagents that are used, out of kit:
- methanol
- n-heptane
- diclormethan
- buffer solution, pH 7.2: (0.55 g NaH_2PO_4 x H_2O + 2.85 g Na_2HPO_4 x 2 H_2O + 9 g NaCl în 1000 ml distilled water)

Equipments and materials:
- ELISA Microstrip Reader 303
- Centrifuge EBA-20
- Micropipete de 20-200 µl şi respectiv 200-1000 µl

- Pasteur pipette
- Graduate pipette
- Gloves

Method proceeding

100 µL buffer solution from standard dilution consists as blank (places A1 and A2). Than, was putting 50 µL buffer dilution, S0 standard (places B1 and B2) and 50 µl standard dilution in downward disposal for concentration, duplicate form (places S1 and S6), and for sample analyses we had put 50 µl in other places (P1, P2, etc.)

3. Results and discussion

Regulation nr. 1881/2006 establishes maximum levels for different types of contaminants in food products. In table 1 are presented the maximum limits accepted for aflatoxin in foods.

They were processed 10 samples of raw-milk, from many collecting centers in Bacau county. The results are presented in Table 2.

Regulation nr. 574/2011 establishes maximum levels for different types of additives and/or contaminants in feed products and also suggested the prohibition of those feeds that contains forbidden substances in overtaken quantities. In table 3 are presented the maximum limits accepted for aflatoxin in feeds.

Table 1

Maximum admitted level for micotoxins in food products

Type of micotoxin	Measure unit	Limit value
aflatoxine B1	µg/kg[ppb]	0.10
aflatoxine M1	µg/kg[ppb]	0.025
ochratoxine A	µg/kg[ppb]	0.50
patuline	µg/kg[ppb]	10
deoxinivalenol	µg/kg[ppb]	200
zearalenone	µg/kg[ppb]	20
fumonisine	µg/kg[ppb]	200

Table 2

Determination of aflatoxin M1 in milk

Sample	Provenience	Aflatoxin M1 µg/kg[ppb]	Conclusion
1	Serbesti	0.002	Comply with regulation
2	Gheorghe Doja	Less than LOD	Comply with regulation
3	Negri	0.003	Comply with regulation
4	Sascut	0.003	Comply with regulation
5	Onesti	Less than LOD	Comply with regulation
6	Targu Ocna	0.004	Comply with regulation
7	Filipesti	Less than LOD	Comply with regulation
8	Racaciuni	0.002	Comply with regulation
9	Valea Seaca	0.001	Comply with regulation
10	Nicolae Balcescu	Less than LOD	Comply with regulation

Table 3

Maximum level for micotoxines in feeds

Type of feed	Measure unit	Maximum level for feeds with moisture content 12%
B1 Aflatoxine		
Raw feeds	mg/kg[ppm]	0.02
Supplementary and complete feeds	mg/kg[ppm]	0.01
Combined feeds for milk cattle and calfs, milk ovine and lambs, milk goats and billy goats, piglets and young poultries	mg/kg[ppm]	0.005
Combined feeds for bovine(exception milk bovine), ovine (except milk ovine), poultries (except young poultries), swine(except piglets)	mg/kg[ppm]	0.02

Table 4

Determination of B1 aflatoxin in feeds

Sample	Type of feed	Aflatoxin B1 [mg/kg]	Conclusion
1	Lucerne	0.005	Comply with regulation
2	Hay1	Less than LOD	Comply with regulation
3	Complete feed for goats	0.004	Comply with regulation
4	Complete feed for adult bovine	Less than LOD	Comply with regulation
5	Complete feed for calfs	Less than LOD	Comply with regulation
6	Hay2	0.003	Comply with regulation
7	Complete feed for milk ovine	Less than LOD	Comply with regulation
8	Alfalfa	0.005	Comply with regulation
9	Complete feed for milk goats	Less than LOD	Comply with regulation
10	Lucerne	0.004	Comply with regulation

For aflatoxin B1 determinations in feeds were analyzed various types of animal feeds, also from Bacau county farmers, as well as, complete feeds and raw feeds (table 4).

4. Conclusions

Our country has implemented the most important regulations in many fields of activity, since 2005, especially in those domains with great impact in our trade collaboration with UE members or non-UE members.

Monitoring of mycotoxins content in foods is a constant concern of the authorities, and mainly for food processors in this industry.

Milk and dairy products are foods susceptible to contamination with aflatoxins, reasons why preventive measures should be taken, such as: ensuring optimal feed storage, monitoring of mycotoxin levels in feeds, risk assessment in accordance with the requirements of applicable law and achieve traceability to prevent aflatoxins contamination of milk and dairy products.

It is also necessary to develop procedures and guidelines of good management practices to prevent, reduce and / or eliminate contamination with aflatoxin B1 in feed, respectively aflatoxin M1 in milk and dairy products.

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