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DETERMINATION OF AFLATOXIN M1 CONTAMINATION LEVELS IN MILK AND MILK PRODUCTS BY HPLC-FLD WITH POST – COLUMN DERIVATIZATION®

Oznaczanie poziomów zanieczyszczenia aflatoksyną M1 mleka i produktów mlecznych metodą HPLC-FLD z derywatyzacją pokolumnową®

This study was carried out as part of research project No. N N312 439837 co-financed by the Polish Ministry of Science and Higher Education in 2009–2011.

In the present study, 34 samples of pasteurised, ultra-high-temperature (UHT) treated milk and milk products (cheese, yoghurt and baby foods) in the city of Olsztyn, Poland, were analysed for aflatoxin M_1 (AFM_1). All samples were cleaned up using immunoaffinity column according to Romer Labs® procedure with minor modification. The Aflatoxin M_1 levels were investigated by high performance liquid chromatography with a fluorescence detection (LC-FLD) and post - column derivatization following sample clean-up using AflaStar™ M_1 immunoaffinity columns (Romer Labs®, Inc., America). The mean recovery of the method was 95 %. The standard curve was linear in the range of 0.01 – 0.25 µg/L with correlation coefficient of 0.9998. The limit of detection was 0.01 µg/L. Results showed 27 (79.4%) positive samples for AFM_1 at levels of 0.010-0.053 µg/L, which were below the tolerance limit of 0.500 µg/L as adopted for AFM_1 in this products by EU regulations. Mean levels of AFM_1 in pasteurized and UHT milk were 0.022±0,006 µg/L and 0.030±0,002 µg/L, respectively. However, only one sample among milk samples was contaminated at a level above the maximum permissible limit (0.050 µg/L) accepted by European Union and Poland for aflatoxin M_1 and six of seven samples of baby food were contaminated at a level above the maximum permissible limit (0.025 µg/L). It is concluded that the incidence of AFM_1 in milk traded in Olsztyn is high, but at levels that probably leads to a non-significant human exposure to AFM_1 by consumption of milk. Experimental results show that, in comparison to milk samples, AFM_1 contamination level was higher in samples of baby food. These data suggest that AFM_1 concentration in milk could be good predictor of its fate in milk products, especially for infants and babies. The results of this study imply that more emphasis should be given to the routine AFM_1 inspection of milk and milk products in Poland. Furthermore, both farmers and dairy companies should be informed on the

W niniejszej pracy analizie pod kątem zawartości aflatoksyny M_1 (AFM_1) poddano 34 próbki pasteryzowanego mleka po obróbce w ultra wysokiej temperaturze (UHT) i produktów mlecznych (ser, jogurt i żywność dla niemowląt) zakupionych w mieście Olsztyn, w Polsce. Wszystkie próbki oczyszczono przy użyciu kolumn immunoafinitywnych zgodnie z procedurą Romer Labs® z niewielkimi modyfikacjami. Poziomy aflatoksyny M_1 analizowano metodą wysokosprawnej chromatografii cieczowej z detekcją fluorescencyjną (LC-FLD) z derywatyzacją pokolumnową po oczyszczeniu próbki przy użyciu kolumn immunoafinitywnych AflaStar ™ M1 (Romer Labs®, Inc., Ameryka). Średni odzysk metody wyniósł 95%. Krzywa standardowa była liniowa w zakresie 0,01–0,25 µg/L ze współczynnikiem korelacji R^2 0,9998. Granica wykrywalności wynosiła 0,01 µg/L. Wyniki pokazały 27 (79,4%) pozytywnych próbek AFM_1 na poziomach 0,010-0,053 µg / l, które były poniżej granicy tolerancji 0,500 µg/L, przyjętej dla AFM_1 w tych produktach w przepisach UE. Średnie poziomy AFM_1 w mleku pasteryzowanym i UHT wynosiły odpowiednio 0,022 ± 0,006 µg/L i 0,030 ± 0,002 µg/L. Jednak tylko jedna próbka wśród próbek mleka była zanieczyszczona na poziomie powyżej maksymalnego dopuszczalnego limitu (0,050 µg/L) przyjętego przez Unię Europejską i Polskę dla aflatoksyny M_1, a sześć z siedmiu próbek żywności dla niemowląt było zanieczyszczonych na poziomie powyżej maksymalnego dopuszczalnego limitu (0,025 µg / l). Stwierdzono, że częstość występowania AFM_1 w mleku będącym przedmiotem obrotu w Olsztynie jest wysoka, ale na poziomach, które prawdopodobnie prowadzą do nieistotnego narażenia ludzi na AFM_1 w wyniku spożycia mleka. Wyniki eksperymentalne wskazują, że w porównaniu z próbkami mleka poziom zanieczyszczenia AFM_1 był wyższy w próbkach żywności dla niemowląt. Dane te sugerują, że stężenie AFM_1 w mleku może być dobrym wskaźnikiem jego losów w produktach mlecznych, zwłaszcza dla niemowląt

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importance of AFM$_1$, and the consequences of the presence of the aflatoxin in dairy products.

Key words: aflatoxin M$_1$, milk, milk products, solid phase extraction, high performance liquid chromatography (HPLC).

i małych dzieci. Wyniki tego badania sugerują, że należy położyć większy nacisk na rutynową kontrolę AFM$_1$ mleka i przetworów mlecznych w Polsce. Ponadto zarówno rolnicy, jak i firmy mleczarskie powinni być informowani o znaczeniu AFM$_1$ i konsekwencjach obecności aflatoksyny w produktach mlecznych.

Słowa kluczowe: aflatoksyna M$_1$, mleko, prdukty mleczne, ekstarkcja do fazy stałej, wysokosprawna chromatografia cieczowa (HPLC).

INTRODUCTION

Humans are exposed to different chemicals including carcinogenic substances during their life. One of them are mycotoxins that have aroused significant public concern worldwide. The occurrence of mycotoxins in human, animal and milk products is one of the most serious problems of food hygiene since milk is important food for adults, and the unique nutrient for infant [23]. Aflatoxins are secondary metabolites produced by some moulds (mainly *Aspergillus flavus* and *Aspergillus parasiticus*) and are contaminants of animal feeds particularly in critical temperature and humidity conditions before or during harvest [23]. Contamination of milk with aflatoxin M$_1$ is considered as a potential risk for human health. Aflatoxin B$_1$ (AFB$_1$) has the highest toxicity [15]. Epidemiological studies have shown that with prolonged exposure to AFB$_1$ liver cancer may develop, especially in persons with hepatitis B antigens [5, 22, 27] Consequently, the World Health Organization (WHO) classifies AFB$_1$ as a human carcinogen and proposes no safe dose [3]. The major metabolite of AFB$_1$ is aflatoxin M$_1$ (AFM$_1$), which is detectable in the urine, blood, milk, and internal organs of animals ingesting AFB$_1$-containing feed [1]. Aflatoxin M$_1$ could be detected in milk 12–24 h after the first aflatoxin B$_1$ ingestion, reaching a high level after a few days. Aflatoxin M$_1$ is relatively stable during pasteurization, sterilization, preparation, and storage of various dairy products. Although AFM$_1$ is less carcinogenic than AFB$_1$ (2-10% of potency), it is also a health danger. It has comparable liver toxicity, can reduce the immunity of infants, and is

considered to be a possible human carcinogen (2B) by the International Agency for Research on Cancer (IARC) [7, 8, 12,14, 15, 16, 27]. The molecular structures of AFB$_1$ and AFM$_1$ are presented in Figure 1 [5].

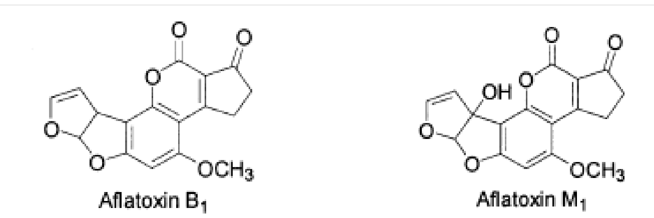

Fig 1. Molecular structures of aflatoxins B1 and M1 [5].
Rys. 1. Wzór strukturalny aflatoksyny B1 i M1 [5].

As milk is the main nutrient for infants and children who are considered to be more susceptible to adverse effects of mycotoxins, the presence of aflatoxin M$_1$ in milk is a concern [11, 13, 19, 20, 25, 26, 28]. But milk is not only consumed as liquid milk, but also utilized for the preparation of infant formulas, yogurt, cheese, and milk-based confectioneries. Therefore, it is important to determine aflatoxin M$_1$ levels in milk and dairy products in order to protect consumers in various age groups, from its potential hazards [1, 21, 24, 31].

The aim of this study was to determine of aflatoxin M$_1$ contamination levels in milk and milk products (milk, cheese, yoghurt and baby foods) by HPLC – FLD with post - column derivatization.

EXPERIMENTAL

1. Chemicals

Acetonitrile (HPLC grade) of J. T. Baker was used. Immunoaffinity columns (IAC) AflaStar M$_1$™ (stored at 4°C until use) were acquired from Romer Labs® (**Romer Labs® Diagnostic GmbH**, Tulln, Austria). The water used during analysis was double distilled with Millipore water purification system (Milli Q, Millipore). Water was purified in a Milli-Q system on 18.2 MΩ/cm.

2. Instrumantal

An Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) consisted of an LC system equipped with a membrane degasser, a quaternary pump, an autosampler, a 100 µL loop, a termostated column compartment and a fluorescence detector set at 360 nm (emission) and 440 nm (excitation) was used for the analyses. The LC column was a ZORBAX Eclipse XDB-C18, 150 mm×4.6 mm i.d., particle size 3 µm, purchased from Agilent. The mobile phase consisted of water (A) and acetonitrile (B) (25:75, v/v) flowed at 0.8 mL/min. A Pickering Laboratories PCX 5200 series was used to post-column derivatives.

3. Preparation of standard solutions

Standard solution of aflatoxin M$_1$ (0,993 μg/mL in acetonitrile) was purchased from Romer Labs® (BCR-423) and stored with care in freezer. Each stock solution was diluted step by step with the combined solution (acetonitrile/water, 75/25, v/v) to prepare a sequence of working solutions which were stored in vials below 4 ºC.

4. Materials

The pasteurized (n=11) and UHT (n=4) milk, yoghurt (n=10), cheese (n=2) and baby food (n=7) samples were obtained from the local supermarket. Dairy samples were stored in freezer compartment inside a refrigerator until these were analyzed for AFM$_1$.

5. Preparation of samples

After warming at about 37 ºC in water bath, the samples were centrifuged at 2000 g to separate fat layer and then filtered. The prepared test portion of 50 mL was transferred into syringe barrel attached with AflaStar™ M_1 immunoaffinity columns (Romer Labs®, Inc., America) and passed at slow steady flow rate of 2–3 mL/min. The washing of column was done with 20 mL ultrapure water (Milli Q, Millipore) and then it was blown to dryness and afterwards aflatoxin M_1 was eluted with 4 mL pure acetonitrile by allowing it to be in contact with the column at least 60 s. The eluate was evaporated to dryness using gentle stream of nitrogen. The residue was solubilized in 500 µL of mobile phase and filtered by syrine driven filter unit Millex®-GN (0.2 µm, 13 mm, Millipore).

6. Peak identification

Identification and quantification of the AFM_1 residues was achieved by high performance liquid chromatography (HPLC). AFM_1 was identified on the base of retention time.

7. Statistical analysis

The experiment was comprised of three replications. The average value, standard deviation and the test of the significance were estimated using the Statistica 10.0 software. Results of the concentration of OTA in samples of beer and wine were analyzed by Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Milk and dairy products are highly nutritious foods containing many macro- andmicronutrients that are essential for the growth and maintenance of human health. The presence of aflatoxin M1 (AFM_1) in this products is an important issue, especially for developing countries [26]. Previous studies have shown that approximately 6.2% of AFB_1 ingested by livestock is metabolized into AFM_1 and excreted in milk. However, it mainly depends on the genetics of animals, milking process, seasonal variation and on the environmental conditions [17]. AFM_1 is very stable at high temperatures like other forms of AFs and the concentration of AFM_1 in milk is not affected significantly with the application of thermal processes i.e. pasteurization and ultra-high-temperature (UHT) treatments used in dairy industry [10, 17]. It was concluded that AFM_1 is 2–10% of less toxic than AFB_1 [2]. In our study, total 34 samples of milk and milk products were analyzed for the presence of aflatoxin M_1 (AFM_1) with the HPLC method equipped with fluorescence detector (Table 1).

Calibration curve was determined using a series of calibration solutions of AFM_1 in acetonitrile (range 0.01-0.25 µg/L, r^2 0.9998). The retention time for aflatoxin M_1 was 3.88 min. The obtained detection values (LOD – µg/L), quantification (LOQ – µg/L) and recovery (%) methods were respectively for aflatoxin M_1: 0.01/0.012/95. Recovery test were performed by spiking aflatoxin M_1-free milk, yoghurt, cheese and baby food samples with known amounts of AFM_1 and revealed mean recovery rates of 95% and mean relative standard deviations was < 8%.The obtained values of LOD, LOQ and recovery showed enough sensitivity for the detection of AFM_1 in all analyzed samples. The occurrence and levels of AFM_1 obtained are presented in Table 1. Analysis of 34 samples showed that 27 (79.4%) samples contaminated AFM_1 and in 6 (37.5%) baby food samples were found to be higher than the maximum acceptable limits for this type of milk products (above 0.025 µg/L) [6]. The levels of AFM_1 in UHT milk should be controlled and monitored continuously. Therefore, it is important to monitor the level of AFB_1 in feedstuffs of dairy animals, it is recommended that AFM_1 analysis and control must be taken seriously by the dairy industry in Poland to reduce AFM_1 contamination and improve the quality of milk and milk products. Among analyzed dairy

Table 1. The occurrence and mean concentration of AFM_1 in milk and milk products samples

Tabela 1. Występowanie i średnie stężenie AFM_1 w próbkach mleka i produktów mlecznych

Dairy samples	Production	Samples analyzed	Positive samples n (%)	Mean±SD* (µg/L or µg/kg)	Range (µg/L or µg/kg)
Pasteurized milk	Regional	3	0 (0)	< LOD	< LOD
Pasteurized milk	Ecological	2	0 (0)	< LOD	< LOD
Pasteurized milk	Commercial	6	6 (100)	0.022±0,006[a]	0.012-0.028
UHT milk	Commercial	4	4 (100)	0.030±0,002[b]	0.019-0.053
Cheese	Traditional	2	0 (0)	< LOD	< LOD
Yoghurt	Ecological	5	5 (100)	0.012±0,004[c]	0.010-0.015
Yoghurt	Commercial	5	5 (100)	0.022±0,008[a]	0.011-0.034
Baby food	Commercial	7	7 (100)	0.064±0,002[d]	0.013-0.097

The data in parenthesis represents the parentage of samples to total samples analyzed.

* EU limits (0.05 µg/L) for AFM_1 in pasteurized milk, UHT milk, yogurt, (0.02 µg/kg) for AFM_1 in cheese and (0.025 µg /L) for AFM_1 in baby food [26]

The English letter with different words represents the significant difference ($p \leq 0.05$).

< LOD – below limit of detection AFM_1 LOD= 0.01 µg/L

Dane w nawiasach przedstawiają udział procentowy próbek wobec wszystkich analizowanych próbek.

* Limity UE (0,05 µg/L) dla AFM_1 w mleku pasteryzowanym, UHT, jogurcie, (0,02 µg/kg) dla AFM_1 w serze i (0,025 µg/L) dla AFM_1 w żywności dla niemowląt [6]

Angielska litera z różnymi słowami przedstawia znaczącą różnicę ($p \leq 0,05$).

<LOD – poniżej granicy wykrywalności AFM_1 LOD = 0,01 µg/L

Source: The own study

Źródło: Badania własne

products, only in regional and ecological pasteurized milk not were detected of AFM_1 levels (< LOD).

The results of Iqbal et al. [17] orevealed that from winter season almost 45% samples of milk and milk products were found to be contaminated with AFM_1 (i.e. 51% of UHT milk, 40% of raw milk, 37% of yogurt, 60% of butter and 43% of ice cream samples and 24, 27, 25, 34 and 17% of samples were found above the recommended limit for AFM_1, respectively). However, from summer season 32% samples of milk and milk products were found to be contaminated (i.e. 36% of raw milk, 31% of UHT milk, 29% of yogurt, 40% of butter and 24% of ice cream and 23, 23, 18, 20 and 5% of samples were found above the permissible limit for AFM_1, respectively). The levels of contamination in winter milk and milk product samples were significantly higher ($p \leq 0.05$) than in summer season. In this study, the occurrence of AFM_1 in milk and milk products were higher, demanding to implement strict regulations and also urged the need for continuous monitoring of milk and milk products in order to minimize the health hazards [6]. In study de Oliveira et al. [4, 9] aflatoxin M_1 was determined in 75 samples of ultra-high-temperature (UHT)-treated fluid commercial milk from Brazil, in 2009. AFM_1 determinations were carried out by HPLC. Results showed that 23 positive samples for AFM_1 (30.7%) at levels of 1000-4100 ng/L, which were above the tolerance limit for AFM_1 in milk as adopted by Brazilian regulations [9].

The occurrence of AFM_1 in European milk and dairy products has been reported in Turkey, France, Italy, Spain, Croatia and from Greece [18, 30]. Ardic et al. (2009) found a mean AFM_1 level of 0.284 µg/kg in white brined cheese with the concentration ranging from 0.052 to 0.860 µg/kg. In another report, Tekinsen and Eked [29] analyzed 100 milk and 132 cheese samples and reported that 67 and 83% of these milk and cheese samples, respectively, were contaminated with AFM_1. The levels of AFM_1 in milk and cheese ranged from 0.010 to 0.630 µg/L and from 0.05 to 0.690 µg/kg, respectively. The range of AFM_1 levels from Turkey, followed by Croatia, is considerably higher compared to other countries. Generally, the levels and incidence of AFM_1 in milk and dairy products from Europe seems less than the South Asian countries, which may be the result of strict regulations on these mycotoxins in feed and milk products and from the adoption of good storage practices [18]. As descibed Iqbal et al. [28], in many parts of the world dairy livestock breeding has become increasingly difficult as the global temperature continues to rise. Elevated temperatures and extreme weather events, such as droughts and floods, may also indirectly influence milk production and its quality as a consequence of shifts in the availability and quality of feed and water [18]. Moreover, extremely high milk prices caused a decrease in the demand of milk by consumers, especially in countries where consumers could not pay the high prices. Aflatoxin M_1 in milk and dairy products could be a risk to human and animal health. High contamination in feed may result in a significant AFM_1 level in milk from animals which fed with highly contaminated foodstuffs. It is important the continuous aflatoxin level monitoring in animal feed and the implementation of strict regulations for mycotoxins in these countries [18].

CONCLUSION

The presence of AFM_1 in milk and milk products is a serious issue, since these products are regularly consumed by each age group in their daily diet [6]. This study is the continuous part of studies, to regularly monitor the contamination level of AFM_1 in milk and milk products. The results have revealed that 37.5% analyzed baby food samples were found to be above the EU limits for AFM_1. The recommendations includes that, there should be more studies on AFM_1 contamination in milk focusing on feeding practices in order to investigate the main factors that are responsible for high occurrence of AFM_1 contamination, especially in baby food. But contamination of milk and milk products (cheese, yoghurt) with aflatoxin M_1 does not appear to be a serious public health problem in the city of Olsztyn (Poland) at the moment. In short, adopting good harvesting practices, improving analytical facilities, and implementing strict regulations would avoid or reduce these natural contaminants in milk and ensure the safety of milk and milk products as human food.

PODSUMOWANIE

Obecność AFM_1 w mleku i produktach mlecznych jest poważnym problemem, ponieważ produkty te są regularnie spożywane przez każdą grupę wiekową w codziennej diecie [6]. Niniejsze badanie jest kontynuacją badań, mającą na celu regularne monitorowanie poziomu zanieczyszczenia AFM_1 w mleku i produktach mlecznych. Wyniki ujawniły, że 37,5% przeanalizowanych próbek żywności dla niemowląt przekraczało limity UE dla AFM_1. Zgodnie z zaleceniami należy przeprowadzić więcej badań dotyczących zanieczyszczenia AFM_1 w mleku, koncentrując się na praktykach żywieniowych w celu zbadania głównych czynników odpowiedzialnych za częste występowanie zanieczyszczenia AFM_1, zwłaszcza w żywności dla niemowląt. Jednak zanieczyszczenie mleka i produktów mlecznych (ser, jogurt) aflatoksyną M_1 nie wydaje się obecnie stanowić poważnego problemu zdrowotnego w Olsztynie (Polska). Krótko mówiąc, przyjęcie dobrych praktyk zbioru, ulepszenie zaplecza analitycznego i wdrożenie surowych przepisów pozwoliłoby uniknąć lub ograniczyć te naturalne zanieczyszczenie w mleku i zapewnić bezpieczeństwo mleka oraz produktów mlecznych jako żywności dla ludzi.

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