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Article

Decrease of Aflatoxin M_1 Concentration in Milk During Cholesterol Removal by B-Cyclodextrin Application

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Abstract: Approximately one-third of mankind is chronically exposed to the carcinogenic aflatoxin M_1 contained in milk and dairy products and there is no ready to use procedure for decontamination purposes applicable in milk technology. Since β-cyclodextrin is frequently used in food industry, its effect on aflatoxin M_1 concentration was investigated during cholesterol removal. So, milk samples were spiked with aflatoxin M_1 at the average level 0.89 μg/kg and cholesterol removal was carried out by 2.0% (w/w) β-cyclodextrin addition. As found, average cholesterol concentration decreased by 92.3% while aflatoxin M_1 concentration decreased to 0.53 μg/kg, i. e. by 39.1% after the treatment. The procedure itself is easy, inexpensive, and ready to use in milk processing technology on current production lines without any investments, thus fully applicable with a high potential of full aflatoxin M_1 milk decontamination efficiency and such way to strengthen considerably the food safety issues associated with milk and dairy products on global level.

Keywords: aflatoxin M_1; milk; dairy; cholesterol; β-cyclodextrin; food safety; global warming

1. Introduction

The prevalence and level of human exposure to aflatoxins (AF) on a global scale have revealed that 4.5 billion people living in developing countries are chronically exposed to largely uncontrolled amounts of the toxins [1]. Although hundreds of fungal toxins are known, a limited number of toxins are generally considered to play an important role in food [2]. The fungal toxins of greatest concern are produced by species within the genera of *Aspergillus*, *Fusarium*, and *Penicillium* that frequently occur in major food crops in the field and continue to contaminate them not only during storage but also during production of food products as well. One of the most dangerous mycotoxins is AFB_1, which is a common contaminant of foods, particularly in the staple diets of many developing countries. This toxin is produced by the action of *Aspergillus flavus* and *Aspergillus parasiticus* during production, harvest, storage, and food processing, and is considered by the US Food and Drug Administration (FDA) an unavoidable contaminant of foods. AFB_1 has various serious adverse health effects in humans, such as acute illness and death, liver cancer, nutritional interference [1], and immunologic suppression [3]. After consumption of contaminated food/feed, AFB_1 is metabolized in the liver to aflatoxin AFM_1 and subsequently excreted into the milk of lactating humans/animals [4]. *In vivo* genotoxic tests in *Drosophila melanogaster* revealed that AFM_1 is 3 times less dangerous compared to AFB_1 in relation to its ability to damage DNA, while its genotoxic effect is compatible with AFB_1 [5]. In the study of the cytotoxic effect of AFB_1 and AFM_1 on Caco-2 cells, it was found that both mycotoxins significantly inhibited undifferentiated and differentiated cell growth and caused genetic damage. However, AFB_1 exhibited more toxicity in both undifferentiated and differentiated cells compared to AFM_1 [6]. Due to these adverse effects, some countries have limited maximum acceptable limits for AFM_1 in milk, for example, the FDA set a limit of 0.5 μg/kg [7] valid in the USA, while the limit in the EU is 0.05 μg/kg for adults and 0.025 μg/kg for infants` foods [8]. Milk is a highly nutritious food that contains many macro and micronutrients that are essential for the growth and maintenance of human health, especially infants, children, and older adults [9]. According to the Food and Agricultural Organization report, the mean milk consumption per capita in the world is calculated at approximately 100 kg/year, however, it is variable from country to country [10]. Dairy is expected to be the fastest growing livestock sector in the next decade, with global milk production projected to increase by 22%. Increased dairy production will be driven by expanding yields due to optimization of milk production systems, improved animal health, better genetics and improved feeding efficiencies, and expansions in the inventory of milking animals. The increase in production will be largely supported by the consumer demand for fresh dairy products dominantly in Asian countries. India and Pakistan are expected to account for more than 30% of global milk production by 2030 [11].

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AFM_1 is a frequent task of scientific activity, as it is the subject of published articles. For example, during the last ten years 779 records in Web of Science Core Collection and 883 records in Scopus databases can be found when research has been conducted on all aspects of AFM_1 presence in milk and dairy products, such as findings, risk assessment, and mitigation strategy proposals [12-15]. For illustration, some findings of AFM_1 in milk around the world are briefly summarized in the Table 1.

Table 1. Findings of AFM_1 in milk in some countries round the world.

Sample	No. of samples/No. of positive samples	Concentration range of AFM_1 [μg/kg]	Country	Source
Raw milk	290/145	nd* – 8.35	Mexico	[33]
Raw milk	100/45	0.02–0.08	South Korea	[34]
Fresh milk	44/42	0.22 – 6.90	Sudan	[35]
Raw milk	150/150	0.01-1.2	Serbia	[36]
Raw milk	1668/36	0.01 – 0.208	Italy	[24]
Fresh milk	52/21	0.01 3.385	Brazil	[37]
Fresh milk	107/76	0.004-0.845	Pakistan	[38]
Pasteurised and UHT milk	242/178	0.001-0.352	China	[39]
Bovine milk Buffalo milk	375/154 170/70	0.01 – 9.18 0.01-6.41	India	[40]
Raw milk Pasteurised milk UHT milk	105/75 15/15 15/15	0.005 – 0.198 0.017 – 0.187 0.012 – 0.146	Bangladesh	[41]
Bovine milk Goat milk	29/29 87/41	up to 0.081 up to 3.108	Nigeria	[42]

nd* - not detected

Hand in hand with the finding of AF in food products, a great effort has been devoted to elimination procedures of AF from food products. Considering that AF contamination of foods is a great threat to human health and national/international food trade, many studies have been carried out to find efficient detoxification methods. Until now, physical strategies (e. g. thermal decomposition, cold plasma, pulsed light), chemical methods (e. g. acid/alkali treatment, ozonation, ammoniation) and biological degradation (e. g. enzymatic degradation, biotransformation) are the three most important detoxification ways [16]. However, neither all AFs can be truly eliminated, nor all decontamination procedures are efficient enough, and, in final, some of them are even not applicable in a matrix due to labile and highly reactive compounds` content. In such cases, the effect of decontamination is strongly devalued by the formation of serious nutritional, organoleptic, and technological defects that considerably limit the acceptability of treated foods on the food market [17-18]. Since milk belongs to this kind of food matrix, removal of AFM_1 remains still open due to the lack of the 'fine and friendly' procedure that does not affect its nutritional, organoleptic, and technological parameters [19]. Seeing that β-cyclodextrin (β-CD) is approved food additive and it is frequently used in the food industry for various purposes [20] this study aimed to investigate the possibility of elimination of AM_1 from milk during cholesterol (CHO) removal using β-CD.

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2. Materials and Methods

2.1 Samples

Seven kinds of commercial milk (3.5% declared fat content in the samples 1; 2; 4; 5; 6; and 7, sample no. 3 - 4.0% declared fat content) were bought in a local market in Bratislava, Slovak Republic.

2.2 Chemicals

β-cyclodextrin was purchased from Wacker Chemie AG (Burghausen, Germany, ≥95.0%),
cholesterol of analytical standard grade and aflatoxin M_1 (analytical standard 0.5 µg/mL in acetonitrile) of analytical standard grade was purchased from Merck, KGaA (Darmstadt, Germany). Chloroform, n-hexane, ethanol 96% and anhydrous Na_2SO_4 p.a. grade were bought at Centralchem Ltd., (Bratislava, Slovak Republic). KOH p.a. grade was supplied by Mikrochem Ltd., Pezinok, Slovak Republic) and both methanol and acetonitrile of HPLC grade were provided by Fisher Chemical Ltd., (Loughborough, UK).

2.3 Instruments

HPLC system 1260 Infinity composed of vacuum degasser, quarterly pump, autosampler, UV-DAD detector, analytical column Zorbax Eclipse Plus C_{18}, 2.1x50 mm, 5 µm particle size, guard column Zorbax SB-C_{18}, 4.6x12.5 mm, 5 µm particle size, and PTFE filter with 0.2 µm membrane (Agilent, Santa Clara, CA, USA) were used. For sample preparation purposes, rotary vacuum evaporator (Heidolph, Germany), centrifuge (Hettich Zentrifugen, Germany), magnetic stirrer (Arex-6 Connect Pro, Velp Scientifica, Italy) and an analytical balance (Sartorius, Goettingen, Germany) were applied.

2.4 Experiments

The samples were divided into two groups. First one was analysed either for CHO or AFM_1 concentration, respectively. Second one was spiked with AFM_1 to obtain initial concentrations in milk as shown in Table 2 column F. Then, the samples were treated by β-CD to remove CHO and AFM_1. After treatment procedure, samples were analysed either for CHO or AFM_1 concentration.

2.5 Treatment of CHO and AFM_1 removal from milk

The samples were treated by [21] as described previously: 250 g of milk were placed in a beaker and 2.0% of β-CD were added. The mixture was stirred at 840 rpm using a magnetic stirrer for 10 min at 25 °C, then the treated milk was left static for 120 min at 4 °C and centrifuged at 130x*g* for 20 min. After centrifugation, the milk supernatant was analysed for CHO and AFM_1 concentration.

2.6 Preparation of milk for CHO analysis

The samples were prepared by [22] as follows: 5.0 g of milk was refluxed with 15 mL of 1 mol/L methanolic solution of KOH during 15 min. Then, the cooled matter was extracted twice with a mixture of n-hexane and chloroform (1:1, v/v) to obtain 15 mL of total extract. For an increase in the polarity of the saponifiable residue, 10 mL of deionized water was added. To avoid the formation of emulsion during extraction, 1 mL of ethanol (96%) was added to the saponified matter. Then, the extract was filtrated through anhydrous Na_2SO_4, and evaporated using rotary vacuum evaporator until dryness; residue was dissolved in 3 mL of methanol, filtered using syringe PTFE filter with 0.2 µm membrane and analysed by HPLC.

2.7 HPLC determination of CHO concentration

HPLC was performed after [22] using isocratic elution at a flow rate of 0.5 mL/min mobile phase composed of acetonitrile/methanol 60:40 (v/v). The injection volume was 10µL and the temperature was set at 30 °C. At these conditions, CHO eluted in 2.2 min of the analysis and detector operated at 205 nm. Data were recorded and treated using the OpenLab CDS software, ChemStation Edition for LC, and LC/MS systems (product version A.01.08.108). All determinations were carried out in duplicate.

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2.8 Preparation of milk for AFM_1 HPLC analysis

Sample treatment, based on the AOAC method [23] and modified by [24] was carried out as follows: 50 g of milk, previously skimmed by centrifugation at 3700 *g* for 15 min was loaded on immuno-affinity chromatography column, and washed with 50 mL water. Then, the analyte was eluted with 2 mL acetonitrile-methanol mixture (60:40 v/v), eluent evaporated to near dryness, residue dissolved with 200 µL acetonitrile-methanol mixture (60:40, v/v) plus 200 µL water and, in final, filtered on a 0.2 µm membrane filter.

2.9 HPLC determination of AFM_1 concentration

HPLC was performed after [24] using isocratic elution at a flow rate of 1 mL/min mobile phase composed of water-acetonitrile-methanol mixture (65:15:20, v/v/v). The injection volume was 10µL and the temperature was set at 30 °C. At these conditions, AFM_1 eluted in 4.5 min of the analysis and fluorescence detector operated at excitation wavelength 360 nm and excitation wavelength 430 nm. Data were recorded and treated using the OpenLab CDS software, ChemStation Edition for LC, and LC/MS systems (product version A.01.08.108). All determinations were carried out in duplicate.

2.10 Statistical Analysis

Results were expressed as mean ±standard deviation. Statistical analysis was performed using the XLSTAT tool of Microsoft Excel 365 (version 2012, Microsoft, USA). The obtained data were subjected to one-way analysis of variance (ANOVA) and the Tukey's comparison test, and the values were considered significantly different when $p < 0.05$.

2.11. Validation of analytical procedures

Method for determination of CHO concentration was validated in *in house* regime using reference materials previously [22]. Method for determination of AFM_1 concentration met validation criteria (LOD, LOQ, recovery, ruggedness, repeatability, linearity) set by European Commission [25].

3. Results and discussion

β-CD has special properties, resulting in the ability of the so-called inclusion complex formation. It means that the core of its blunted cone structure forms a dimensionally stable hydrophobic cavity that can trap or encapsulate predominantly nonpolar molecules, to which CHO and AFM_1 belong to. The scheme of inclusion complex formation is shown in Figure 1.

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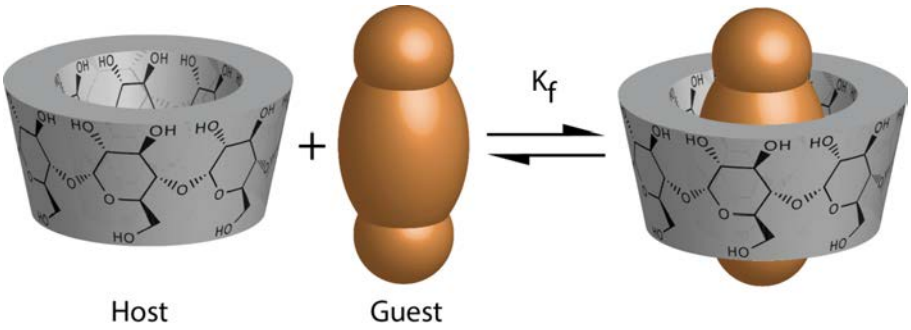

Figure 1. Schematic illustration of the association of free β-CD ('host') and substrate ('guest') to form substrate−β-CD inclusion complex. Reprinted with permission from Crini, G. (2014). A history of cyclodextrins. *Chemical Reviews, 114,* 10940-10975. Copyright © (2014) American Chemical Society.

In general, the formation of inclusion complexes includes five elementary steps[26]: (I) the substrate approaches the β-CD molecule; water molecules escape from the β-CD cavity and acquire a new energy level, corresponding to that of the gaseous state; the van der Waals interactions and the number of hydrogen bonds decrease, while the degrees of freedom of translation and rotation of the freed water molecules increase; (II) the guest molecule becomes released from the layer of water that envelopes it and also acquires a different state; the layer of water becomes dispersed and rearranges; (III) the guest molecule, considered to be in a perfect gas state, enters the cavity and the complex formed is stabilized by van der Waals forces and/or hydrogen bonds; (IV) the expelled water molecules are rearranged and form hydrogen bonds between each other; and (V) the structure of the water is restored around the part of the substrate that remains in contact with the solvent and that is integrated into the hydration shell around the β-CD. In the final, the intermolecular and intramolecular hydrogen bonds cause conformational changes that lead to overall thermodynamic stabilization of the inclusion complexes [27]. One of the most frequent practical applications of inclusion complex formation is the removal of CHO from milk when the formation of the CHO-β-CD inclusion complex provides a fundamental basis to produce functional low cholesterol food products to protect consumers` health against long lasting high daily intake of CHO from milk and dairy products [28]. The same situation was observed also in these experiments, as shown in columns A, B, and C of Table 2, when CHO concentrations were effectively decreased in all treated samples and the average CHO decrease was 92.3%.

Table 2. Effect of β-CD treatment on the concentration of CHO and AFM_1 in milk.

No.	A	B	C	D	E	F	G	H	I
1	129.04±2.13	10.36±2.11	92.0	11.46	<LOQ*	0.20	0.13±0.06	35	0.54
2	135.78±6.01	6.47±1.59	95.2	19.99	<LOQ*	0.40	0.25±0.02	38	0.60
3	150.39±0.64	5.25±0.03	96.5	27.65	<LOQ*	0.60	0.36±0.02	40	0.67
4	113.32±6.30	8.92±0.02	92.1	11.70	<LOQ*	0.80	0.47±0.03	41	0.70
5	123.01±2.21	1.43±0.63	98.8	85.02	<LOQ*	1.00	0.55±0.04	45	0.82
6	103.92±0.43	9.47±0.21	90.9	9.97	<LOQ*	1.20	0.80±0.04	33	0.50
7	122.33±1.45	23.49±1.50	80.8	4.21	<LOQ*	2.00	1.16±0.06	42	0.72
Average	125.40	9.34	92.3	12.43	<LOQ*	0.89	0.53	39.1	0.68

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Legend to the Tab. 2
A – Initial concentration of cholesterol [mg/kg]
B – Concentration of cholesterol after its removal [mg/kg]
C - Measure of cholesterol removal [%]
D - Distribution coefficient δ_{CHO}
E – Initial concentration of AFM_1 [μg/kg]
F - Concentration of AFM_1 after spiking [μg/kg]
G – Concentration of AFM_1 after its removal [μg/kg]
H - Measure of AFM_1 removal [%]
I - Distribution coefficient δ_{AFM1}
LOQ* = 0.013 μg/kg

However, much more interesting are the data related to the removal of AFM_1, when the average concentration of AFM_1 also decreased during the experiments in all samples and the average AFM_1 decrease was 39.1%, as follows from the columns E, F, G and H of Table 2. In general, a measure of removal of a contaminant can be expressed by distribution coefficient δ given by ratio [1]

$$\delta = \frac{c_0 - c_\infty}{c_\infty} \qquad [1]$$

where c_0 is the initial concentration and c_∞ is equilibrium (final) concentration of a contaminant, respectively [29]. In this case, the higher the δ value, the greater part of AFM_1 was removed from milk by the formation of the inclusion complex AFM_1-β-CD. The values of δ_{AFM1} are listed in column I of Table 2. When comparing the average value of δ_{AFM1} with the average value of δ_{CHO} (column D of Table 2) it is clear that CHO was removed more than 18 times efficiently compared to AFM_1.This reality could be assigned to the fact that experimental conditions (amount of β-CD addition, time and speed of mixing, temperature, settling time, speed of centrifugation) were never optimized for removal of AFM_1. On the other hand, the obtained results are a promising base for further adjustment of the AFM_1 removal procedure parameters in to reach complete AFM_1 removal. The application of the procedure itself is easy, safe, effective, low cost and labour, thus fully realizable on current technological production lines without the need of additional investments and negative effects on nutritional, organoleptic or technological parameters of milk or dairy products. However, the greatest advantage of the procedure is its current usage in dairy industry, albeit for different purpose, i. e. CHO removal [28]. Also, the application of the procedure can prevent effectively to economic losses associated with findings of over limited AFM_1 concentrations in traded dairy products [30]. Moreover, in the last 15 years, several hot and dry seasons led to severe *Aspergillus flavus* infections of crop in several countries in south part of Europe, including Italy, Romania, Serbia, and Spain. As a result of the very dry weather in those years, *Aspergillus flavus* has become a significant problem as a dominant pathogen in crop [31]. Based on the predictive model developed for *Aspergillus flavus* growth and AFB_1 contamination, it was assessed that there is a high probability of its elevated incidence in agricultural products (i. e. feed) in the future due to the trend of in climate changes, as shown in Figure 2. In the +2 °C climate change scenario there is a clear increase in AF risk also in other, central, or even more norward areas of Europe [32]. Due to the global character of warming, it is reasonable to assume the same prognosis for the American, Australian, and Asian latitude areas. Therefore, the need for applicable method of AFM_1 milk decontamination is highly urgent because it could solve all the above-mentioned problems and considerably improve food safety issues associated with the increasing probability of the presence of AFB_1 in feed, thus the presence of AFM_1 in milk.

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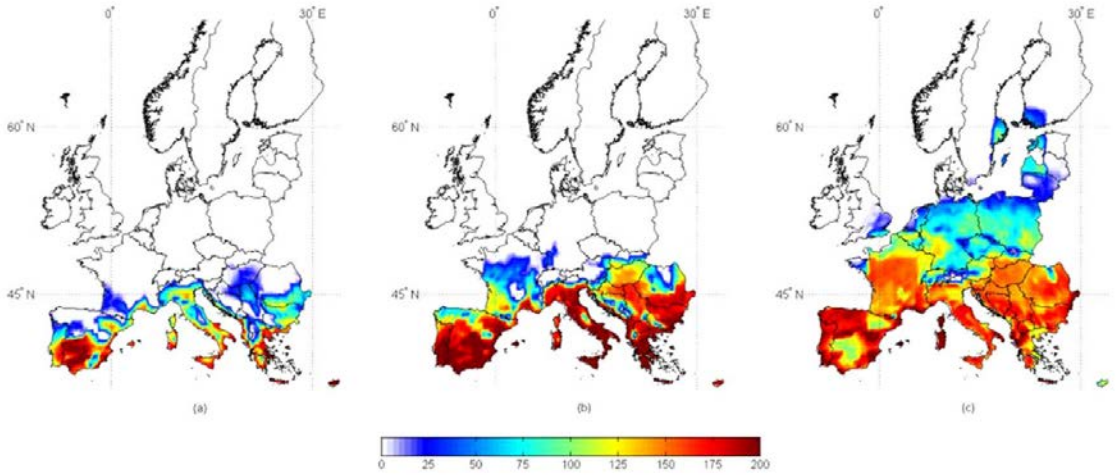

Figure 2. Risk maps for aflatoxin contamination in maize at harvest in 3 different climate scenarios, present, +2°C, +5 °C. Mean daily data used as input result from 100-year run of the predictive model AFLA-maize in 2254 geo-referenced points throughout Europe, in the 3 scenarios. The scale 0–200 refers to the aflatoxin risk index (AFI), output from the predictive model; increasing the (present (a), +2°C (b), +5°C (c)) number, the risk of contamination increases. Source: Battilani, P., Toscano, P., Van der Fels-Klerx, H. J., Moretti, A., Leggieri, M. C., Brera, C., & Robinson, T. (2016). Aflatoxin B_1 contamination in maize in Europe increases due to climate change. *Scientific Reports*, *6*, 1-7.

4. Conlusion

On the base of experimental data it can be postulated individual items as follows:

a) During procedure of CHO removal by physico-chemical interaction with β-CD, interaction of β-CD with AFM_1 is also going on
b) Although the formation of inclusion complex of AFM_1 with β-CD at given conditions is less effective in comparison to CHO with β-CD, average removal efficiency at level of 39.1% of was observed
c) The procedure is ready-to-use since β-CD is the compound allowed and frequently used in food technology for various purposes and its application does not affect organoleptic profiles of treated milk
d) Also, the procedure is applicable on existing production lines without any extra investment
e) Therefore, the procedure could help the health problems associated with the chronical presence of AFM_1 in milk and dairy products around the world and problems associated with over-limited concentrations in traded dairy products

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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