Schiff base: A high affinity chemical agent to decrease the concentration of aflatoxin M$_1$ in raw milk contaminated artificially

Lejla Duraković[1]*, Alma Tudić[3], Frane Delaš[1], Katarina Huić-Babić[2]

[1]Faculty of Food Technology and Biotechnology, Department of Biochemical Engineering, Laboratory for General and Food Microbiology, University of Zagreb, Pierottijeva 6, Zagreb, Croatia
[2]Faculty of Agriculture, Department of Microbiology, University of Zagreb, Svetošimunska 25, Zagreb, Croatia
[3]Croatian Waters, Sector of Development and Water Economy, Vukovarska 220, Zagreb, Croatia

Summary

In the present study were conducted the effect of pH (5.5, 6.0 and 6.5) and concentration of new synthesized 3-/2-aminophenylimino-(p-toluoyl)/-4-hydroxy-6-(p-tolyl)-2H-pyrane-2-one (Schiff base) on decrease the concentration of aflatoxin M$_1$ (AFM$_1$) in raw milk contaminated with known concentration of this toxin. Experiments were carried out at temperature of 4 °C during 35 days. At pH 5.5 Schiff base concentration of 0.1 μmol/L was lessening the concentration of AFM$_1$ after 35 days by 55 %. However, at pH 6.5 the most effective concentration for lessening of AFM$_1$ was 0.5 μmol/L. Schiff base was not effective at pH value of 7 or higher. The ability of Schiff base to act as antimycotoxigenic agent provides new perspective for possibly using this compound to control AFM$_1$ contamination in milk and to extent shelf lives of this food. Detection of toxicity of investigated Schiff base was performed by using the brine shrimp (*Artemia salina*) larvae as an biological indicator to determine their sensitivity to this chemical agent.

Key words: milk, aflatoxin M$_1$, TLC, Schiff base, *Artemia salina*

Introduction

Since the early 1960's when the outbreak of "Turkey X" disease focused a lot of scientific attention to the recent, then rather neglected area of mycotoxins and mycotoxicosis, a wealth of information about mycotoxins has been produced. The problem of food and feed contamination with mycotoxins is of current concern, and has received a great deal of attention during the last five decades.

Exposure to mycotoxins through food is widely recognized as human health hazard (Bhat and Vasanthy, 1999; Trucksess, 1999; Duraković et al., 2008; Nemati et al., 2010; Duraković et al., 2011a, 2011b). Of all the mycotoxins, aflatoxins

(AFs) are considered to be the most toxic/carcinogenic compounds (IARC, 1993; Manetta et al., 2005). AFs are highly toxic, mutagenic, teratogenic, and carcinogenic compounds that have been implicated as causative agent in human hepatic and extrahepatic carcinogenesis (Dichter, 1984; Groopman et al., 1988; Massey et al., 1995, Nemati et al., 2010).

AFs are secondary metabolites produced by species of *Aspergillus*, especially *A. flavus* and *A. parasiticus*. AFs are dihydrofuran or tetrahydrofuran moieties fused to coumarin ring (D'Mello and MacDonald, 1997). Of all aflatoxins in naturally contaminated substrates, the aflatoxin B$_1$ (AFB$_1$)

*Corresponding author/Dopisni autor: Phone/Tel.: +385 1 4605 045; E-mail: lejla.durakovic@pbf.hr

occurs predominantly, the others being often present in small proportion only or even in some cases undetectable.

AFB$_1$ is biotransformed by hepatic microsomal cytochrome P$_{450}$ to aflatoxin M$_1$ (AFM$_1$) which possesses 10 times lower carcinogenic potential with respect to parent molecule (Lopez et al., 2003; Unusan, 2006). The toxicological concern with AFM$_1$ arises in principle from its close structure similarity to AFB$_1$ (Figure 1) (Duraković, 2007; Duraković et al., 2011a).

When diets contaminated with AFB$_1$ are fed to lactating animals, AFM$_1$ is secreted into milk (van Egmond and Paulsch, 1986; Škrinjar et al., 1995; Galvano et al., 1996a; Tratnik, 1998; Rastogi et al., 2004). There was a relationship between the amount of AFM$_1$ in milk and AFB$_1$ in feed consumed by the animals (Wood, 1991; Unusan, 2006). About 0.3-6.2 % of AFB$_1$ in animal feed is transformed to AFM$_1$ in liver and it is secreted into milk (Cathey et al., 1994; Jackson and Groopman, 1999; Creppy, 2002; Manetta et al., 2005; Rastogi et al., 2005). The amount excreted, as a percentage of AFB$_1$ in feed, is usually 1-3%, but values as high as 6 % have been reported (Pittet, 1998; Lopez et al., 2003; Diaz et al., 2004).

Like AFB$_1$, AFM$_1$ is toxic and carcinogenic, although toxicity of AFM$_1$ is somewhat lower than of AFB$_1$ (Cole and Cox, 1981; Rastogi et al., 2004;

Kamkar, 2006). However, AFM$_1$ is great concern because of the high consumption of milk and milk products by humans, especially children. International Agency for Research on Cancer (IARC) of World Health Organization (WHO) included AFB$_1$ as primary and AFM$_1$ as secondary groups of carcinogenic compounds (Cathey et al., 1994; Dragacci et al., 1995; Unusan, 2006).

Many countries have carried out various control and inspection programs of this subject fairly concerning about public health for many years. According to the results obtained, maximum aflatoxin levels were determined for food and feed. Regulatory limits throughout the world are influenced by consideration each country's conditions, and may vary from one country to another (van Egmond, 1989; Stahr et al., 1990; Stoloff et al., 1991; Duraković et al., 2011a). The European Community and Codex Alimentarius prescribe that the maximum level of AFM$_1$ in liquid milk and dried or processed milk products should not exceed 50 ng/kg (Codex Alimentarius Commission, 2001).

However, according to US Regulation the level of AFM$_1$ in milk should not be higher than 500 ng/kg (Stoloff et al., 1991). In Austria and Switzerland, the maximum level is further reduced to 10 ng/kg for infant commodities (FAO, 1997). There are thus differences in maximum permissible limit of AFM$_1$ in milk and dairy products (van Egmond, 1989; Unusan, 2006; Nemati et al., 2010; Sefidgar et al., 2011). These findings, along with the demonstration of occurrence of AFM$_1$ in human and animal milk, cheese, yoghurt, infant formula and dried
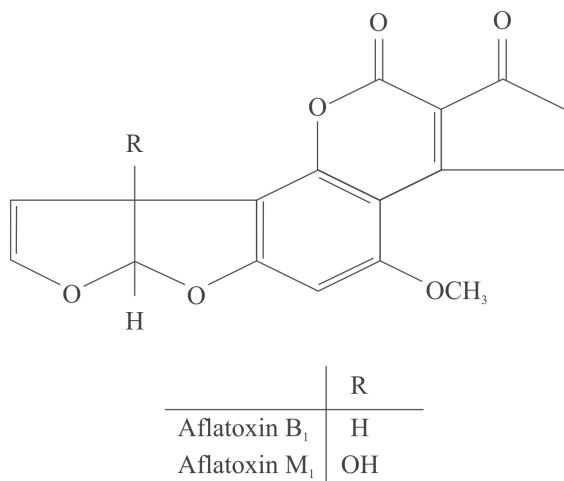

	R
Aflatoxin B$_1$	H
Aflatoxin M$_1$	OH

Figure 1. Chemical structures of aflatoxins B$_1$ and M$_1$ (Duraković, 2007; Duraković et al., 2011a)
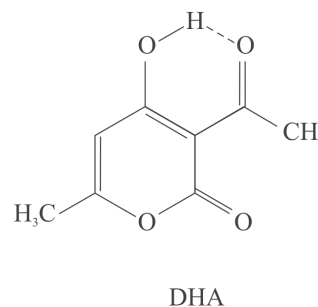

DHA

Figure 2. Chemical structure of dehydroacetic acid (Coleman and Wolf, 1947; Duraković et al., 1994, 2004, 2010b)

milk, maybe a great significance. This information requires that AFM$_1$ has to be given high priority for further investigation.

To prevent of AFM$_1$ formation in milk it is first necessary to prevent the growth of AFB$_1$-producing fungi. The use of chemical antifungal agents to control AFB$_1$ production has been intensively investigated (Diaz et al., 2003; Raman et al., 2003; Verma et al., 2004; Zhong et al., 2009; Alpsoy, 2010; Duraković et al., 2010b, 2010c). However, the use of certain antifungal agents must be viewed with reservation, because of ecological problems, which may develop later. In 1947, Coleman and Wolf discovered the antimicrobial action of dehydroacetic acid (DHA) (Figure 2).

This chemical compound appears to be effective even in the high pH range, but has never acquired great significance because of its relatively high toxicity. Since the use of DHA as a food preservative is not permitted in Europe, in the Laboratory of Organic Chemistry of the Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia, many DHA analogues and tetraketones were synthesized as potential antimicrobial agents. Some new synthesized analogues of DHA and tetraketones have been found to reduce chosen mycotoxins, especially aflatoxins accumulation (Hasan, 1993; Duraković et al., 1994, 1995, 2004, 2006; Prakash et al., 2007; Duraković et al., 2010a, 2010b, 2010c, 2011b).
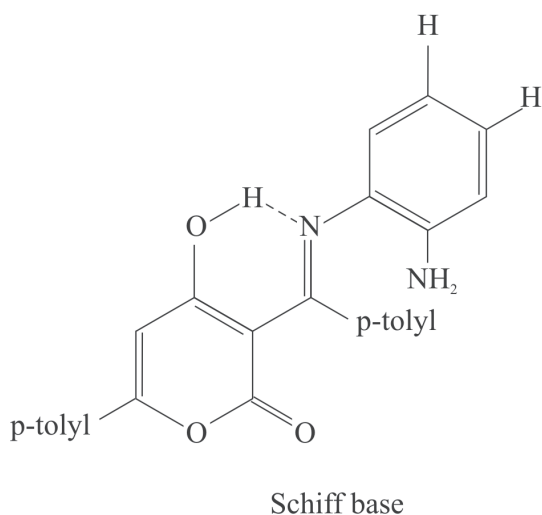
Methods for the synthesis of these compounds are described by Filipović-Marinić and Laćan (1982); Sušnik et al. (1992); Govori et al. (2002); Prakash et al. (2007) and Amanullah et al. (2011). Preliminary studies in the Laboratory of Microbiology of the same Faculty demonstrated that several of these new synthesized compounds strongly inhibit the growth of certain species of bacteria, yeasts, and moulds, including aflatoxin-producing fungi (Duraković et al., 1987, 1989 1994, 2004, 2006; Duraković, 2007; Duraković et al., 2010a, 2010b).

In experiments with toxigenous moulds *Aspergillus flavus*, *Aspergillus ochraceus*, and *Fusarium graminearum*, the authors (Duraković et al., 1987, 1989, 1994, 2004, 2006; Duraković, 2007; Duraković et al., 2010a, 2010b, 2011b) showed that some of the new synthesized DHA analogues strongly inhibit growth of these moulds and aflatoxin B$_1$, ochratoxin A, and vomitoxin accumulation in chosen cereals and in liquid media. One of these compounds with circumstantial evidence minimal inhibitory concentration (MIC) of 0.1 and 0.2 μg/mL used in these experiments was DHA analogue 3-/2-aminophenylimino-(p-toluoyl)/-4-hydroxy-6-(p-tolyl)-2H-pyrane-2-one (Schiff base). Its structure is presented in Figure 3.

The objective of this investigation was to assess the convenience of treating raw milk with new synthesized analogue of DHA (Schiff base), which was tested *a priory* by a novel *in vitro* assay for reduction of AFM$_1$ concentration in dairy cattle milk, and to gain indication of whether effective *in vitro* can predict *in vivo* efficacy.

Materials and methods

AFM$_1$ standard

Stock solution for AFM$_1$ (Immunolab GmbH, Kassel, Germany), was prepared in a methanol/chloroform mixture (81:19, v/v), 50 μg/mL concentration and is kept frozen at -20 °C, when using it was diluted in methanol/chloroform (1:1, v/v) at proper concentrations (final concentration became 0.1 μg/mL) (van Egmond, 1989).

Apparatus

Thin layer plates: Silica gel 60 (Merck, Darmstadt, Germany), dimensions 20x20 cm, layer thick-

Schiff base

Figure 3. Chemical structure of Schiff base (Duraković et al., 1994, 2010b, 2011b)

ness 0.2 mm. Ultraviolet lamp: An ultraviolet lamp (UV) lamp with 254 and 364 nm wavelength was obtained from Camag.

Primary AFM₁ standard solution

Ten milliliter of chloroform was added to 10 μg AFM₁ standard bottle that was obtained from Immunolab GmbH, Kassel (Germany). Concentration in the bottle became 1 μg/mL in chloroform. The solution bottle was sealed and wrapped with aluminum foil, and stored in cool (4 °C) dry place.

Working standard solution for thin layer chromatography (TLC)

Before preparing working dilutions, it is essential that 1 μg/mL stock solution, having previously been stored at a temperature below 4 °C, is after that stored at a temperature of 20 °C, before removing aliquots of the solution for subsequent dilution. By means of pipette, 0.5 mL of the 1 μg/mL AFM₁ stock solution was transferred to a 10 mL graduated conical tube and diluted to the 10 mL marked with chloroform. This solution contains 0.05 μg/mL of AFM₁. The solution was well sealed and stored in a dark place at room temperature, and was not used for longer than 14 days.

Milk

Raw milk was obtained randomly directly from the farms of continental parts of Croatia. The milk showed no detectable levels of AFM₁ (results not presented). The raw milk samples were stored frozen in the dark until used. The milk was dispensed in 50 mL aliquots in 350 mL Erlenmeyer flasks. The pH was adjusted to 5.5, 6.0, and 6.5 (0.2).

Milk contamination with AFM₁ and impact of new synthesized analogue

A solution of AFM₁ was prepared by adding to a 10 mL centrifuge tube, 1 mL of the AFM₁ solution (10 mg/mL). Content of the centrifuge tube was concentrated to dryness. The tube was then rinsed with a 1 % sodium bicarbonate solution; the rinsing combined and brought to a final volume of 50 mL, which was added to the raw milk used. The presence of AFM₁ was determined in milk before and after contamination. For the quantitative analysis of AFM₁, a series of dilutions were chromatographed from the sample extracts to determine the lowest dilution, at which AFM₁ can still be found.

From lowest limit of detection of AFM₁ (sensitivity on TLC plate) and the dilution factor, the appropriate quantity of AFM₁ was calculated. Each sample was determined in triplicate. The investigated Schiff base was dissolved in chloroform at concentrations of 0.5 and 1.0 mmol/L. The required amounts of their solutions were pipetted into test Erlenmeyer flasks to give 0.01, 0.05, 0.5, and 1.0 μmol/L in 50 mL of raw milk. Control flasks and duplicate test flasks containing the various concentrations of investigated compound, were stored at temperature of 4 °C during 35 days.

Methods

Each sample was analyzed for the determination of AFM₁ concentration every 7 days for a period of 35 days by the technique described by van Egmond and Wagstaffe (1987) and Škrinjar et al. (1995). AFM₁ was extracted from 50 mL of raw milk samples with 125 mL of chloroform in a laboratory shaker (50 cycles/min.) for one hour at room temperature. The extracts were then dried over anhydrous sodium sulphate and concentrated in a rotary evaporator (50 °C; 1.33 kPa) to a volume of 5 mL. Evaporated extracts are purified by silica gel column chromatography, and each purified extract was evaporated to a known volume. The solution was evaporated at a temperature of approximately 50 °C in a current of inert gas, and when the tube has cooled, added 100 mL of chloroform using an injection syringe. The contents were mixed by means of vortex mixer for 1 min.

AFM₁ is quantified by two-dimensional thin layer chromatography. The detection limit of the method used is 0.015 μg/L. The average recoveries of AFM₁ spiked in raw milk at concentrations of 0.01, 0.05, and 0.1 μg/L were found to be 83.8, 86.9, and 90.2 (n=5). The survey results were not corrected for recovery. In this method 20 μL of the sample extract was obtained, and 3, 6, 12, 24, and 48 μL of AFM₁ working solution (0.05 μg/mL) spotted on plates in the direction of first and second mobile phase, carried out in ether/methanol/water and chloroform/acetone/methanol mixture's, respectively.

The amount of AFM$_1$ was estimated visually by comparing the fluorescence intensity of the AFM$_1$ from the raw milk sample, with that of one or more amounts of AFM$_1$ standard. The identity of AFM$_1$ was confirmed by the formation on the plate of a derivative using trifluoroacetic acid and comparison of the thin layer chromatographic properties of the sample derivatives spot and the standard (van Egmond and Wagstaffe, 1987).

Calculation: The AFM$_1$ level in the sample of raw milk expressed is given by the formula:

$$\frac{V_{st} \times C_{st} \times V_{ext}}{V_m \times M \times V_f / 125}$$

in which is the volume, in μL, of the AFM$_1$ standard used, in interpolation was used the nearest spot intensity to the fluorescence intensity of the sample; the mass concentration, in μg/mL, of the AFM$_1$ standard; the volume, in μL, in which the extract was absorbed; the volume, in μL, of sample used; the volume of milk, in mL; the volume, in mL, of the filtrate obtained in extraction steps, and 125 is the amount, in mL, of chloroform, used during extraction. The AFM$_1$ levels in milk were expressed as μg/L (ppb).

Detection of toxicity of investigated new synthesized chemical compound

The toxicity of new synthesized Schiff base was evaluated by using the brine shrimp (*Artemia salina*) larvae as a screening system for the determination of its sensitivity to some chemical agents. The lethality test was performed by using the method of Harwing and Scott (1971) and McLaughlin et al. (1991). The brine shrimp dehydrated eggs were obtained by Hans Brustman, Düsseldorf (Germany) and were hatched in artificial seawater (35 g sea salt per liter of water), and after an average of 24 h from hatching, the shrimp larvae were used for experimental bioassay. For each experiment, 100 to 200 mg of brine shrimp was placed in a 500 mL Erlenmeyer flask and shaken as described by Favilla et al. (2006) and Duraković et al. (2011a). Hatching can occur in less than 24 h at 27 °C.

Results of 10 parallel experiments showed that concentration resulting in 50 % mortality of larvae exposed to investigated chemical for 24 h at 30 °C was (μg/mL): 28.5 and 27.9, respectively. The results suggest that the investigated Schiff base had a poor toxic effect in relation to *A. salina* larvae in selected parameters of cultivation, and was almost eight times less than toxicity of a parent molecule (dehydroacetic acid) (Duraković et al., 1986). The bioassay with brine shrimp larvae shows considerable promise as a screening test under field conditions, especially since very little sophisticated equipment is required.

Determination of reduction index

The reduction index, which is the ng of AFM$_1$ reduced per liter of milk per day, was calculated according to the formula (Duraković et al., 1994):

$$Reduction\ index\ (ng/L/day) = \frac{AFM_1\ at\ Lof\ milk\ sample}{Deposit\ time\ (days)}$$

Statistical analysis of the data

AFM$_1$ recoveries from the milk samples were performed by using the method recommended by AOAC (1995).

Repeatability and recovery were determined by spiking 50 mL of raw milk with toxin standard solution at the levels 100 to 1000 μg/mL prior to the addition of solvent and extraction, and kept at room temperature. After 1 h, AFM$_1$ was extracted from spiked sample and quantified according to the protocol (van Egmond and Paulsch, 1986; Bakirci, 2001). The lowest detection limit of the method used is 0.01 ppb. Recovery rates of duplicate experiments were between 83.8 and 90.2, and standard

Table 1. Percent AFM$_1$ recoveries from spiked raw milk samples

Level spiked (μg/mL)	Recovery (%)	Average recovery (%) ± SD
0.1	74 96 94 70 117	90.2±6.6
0.5	83 80 79 85 92	83.8±12.8
1.0	76 72 87 93 106	86.9±8.3

SD = standard deviation

deviation (n=5) was between 6.6 and 12.8 (Table 1).

There was no significant difference in the mean percent recoveries. The results of toxin investigated were not corrected for recovery.

Results and discussion

The main emphasis of the study was on the determination of AFM_1 in raw milk after contamination with known concentration of raw milk samples with this toxin and impact of new synthesized analogue of dehydroacetic acid on lessening of AFM_1 concentration in selected parameters of storage in safekeeping. The content of AFM_1 in the raw milk samples before contamination was null.

Although the use of binding agents to remove or reduce of aflatoxins generally is not approved by the EU and FDA, respectively, for this purpose, some montmorrilonite clay products, some of which are known as hydrated sodium calcium aluminosilicate, bind AFB_1 *in vitro* and, apparently, *in vivo*. These products, which have been shown to bind AFM_1 in liquid milk (Harvey et al., 1991), reduce AFM_1 in milk obtained from cows consuming AFB_1-contaminated feed, and protect many species from the toxic effects of AFB_1 (Harvey et al., 1991; Galvano et al., 1996b; Ramos et al., 1996; Huwig et al., 2001). Activated carbon has also been effective experimentally in reducing milk AFM_1 in dairy cows consuming AFB_1-contaminated feed (Galvano et al., 1996b). Commercially available clay products are generally recognized as safe by the FDA, when used in feed manufacture as flow agents and pellet binders not exceeding 2 % on a dry weight basis (Code of Federal Regulations Part 582.2727 and 582.2729) (FDA's CVM, 1999).

A number of studies have shown that sequestering agents such as activated carbons effectively bind AFB_1 *in vitro* (Ramos and Hernandez, 1997; Galvano et al., 2001; Huwig et al., 2001; Diaz et al., 2003). In addition, there have been studies evaluating sequestering agents *in vivo* for their ability to protect animals from the effects of dietary AFB_1 and to prevent or reduce AFM_1 secretion in milk (Phillips et al., 1988; Kimaryamma et al., 1991; Galvano et al., 1996b). The best known of these are: activated carbon or charcoals, which have been variable in effectiveness (Galvano et al., 2001),

and NovaSil, a clay product often characterized as HSCAS, which have been extensively studied for its bind to AFB_1 and protection a number of species from its toxic, growth reducing effects (Ramos et al., 1996). NovaSil has also been shown to reduce the transmission of AFM_1 into milk in dairy cows (Harvey et al., 1991).

Many biological important Schiff bases ligands have been reported which possess antifungal, antimycotoxigenic, and antibacterial activity (Raman et al., 2003; Verma et al., 2004; Janos and Tamas, 2009; Zhong et al., 2010; Duraković et al., 2011b). Dehydroacetic acid (DHA) and its new synthesized analogues (Schiff bases, DHT and BrDHT) have also been shown to inhibition of growth and accumulation of some mycotoxins as aflatoxins, ochratoxin A, F-2 toxin and vomitoxin by moulds of the genera *Aspergillus* and *Fusarium* (*A. flavus*, *A. parasiticus*, *A. ochraceus* and *F. graminearum* (Duraković et al., 1989, 2004; Duraković, 2007; Duraković et al., 2008, 2010a, 2010b, 2010c, 2011a, 2011b).

A few studies in liquid media have focused on *Aspergillus* species, accumulation, and possible degradation of aflatoxins, but none has evaluated the impact of DHA analogues on AFM_1, which is contained in milk.

Few previous studies exist on antimycotoxigenicity of dehydroacetic acid and some of its analogues for chosen mycotoxins. Most of the existing studies in cereals and in liquid media refer to aflatoxin biosynthesis and biodegradation, but not any or very little information have about impact of DHA and its new synthesized analogues to degradation or decrease the concentration of AFM_1 that is contained in milk (Galvano et al., 1996b; Duraković et al., 2004, 2010b, 2011b).

Our results demonstrated clearly that investigated analogue (Schiff base) is lessening AFM_1 concentration in raw milk in respect to its concentration, pH value of milk and stored time. The effect of increasing concentrations of the investigated chemical on lessening concentration of AFM_1 was evaluated. The overall effect of Schiff base at pH 5.5, 6.0, and 6.5 is shown in Table 2 and in Figures 4a-4c. At pH 5.5, concentration of AFM_1 was lessened by 55 % with 0.1 μmol/L of Schiff base and lessened by 28 % with 0.5 μmol/L of Schiff base. At pH values of 6.0 and 6.5, Schiff base concentration of 0.1 μmol/L and 0.5 μmol/L decrease concentration of AFM_1 by

Table 2. Effect of investigated Schiff base on lessening AFM$_1$ concentration in raw milk during the deposit time of 35 days. pH values of milk were 5.5, 6.0, and 6.5 and AFM$_1$ concentration added was 100 ng/L

Deposit time (days)	Concentration of Schiff base (μmol/L)											
	(0.01)			(0.05)			(0.1)			(0.5)		
	pH value			pH value			pH value			pH value		
	5.5	6.0	6.5	5.5	6.0	6.5	5.5	6.0	6.5	5.5	6.0	6.5
14	85.9	88.0	86.0	66.4	79.6	76.5	60.5	75.2	68.5	80.5	73.5	79.0
21	89.0	82.9	98.2	75.8	91.3	90.4	50.7	68.8	86.5	72.0	96.2	80.5
28	102.5	105.4	105.2	96.5	104.8	103.0	56.5	85.9	86.8	109.6	88.5	86.0
35	106.2	112.7	112.5	111.4	96.5	102.8	45.0	72.6	90.5	112.7	84.7	100.5

31 % and 28 %, respectively, of that in control experiments (Table 2 and Figures 4b and 4c).

As pH decreased from 6.5 to 5.5, the effect of Schiff base increased. Figures 4a-4c represent the reduction index curves of AFM$_1$ in respect to concentration of Schiff base, pH values of milk and deposit time. The results show that reduction index significantly decreases as concentration of Schiff base increased. At a concentration of Schiff base of 0.1 μmol/L and pH value of milk of 5.5 and 6.0, the reduction index is only 1.5 and 2.0 after 35 days in respect to values obtained after 14 days of deposit (Figures 4a and 4b).

It is suggested that specific concentrations of Schiff base and pH value of milk are able to lessen-

ing concentration of AFM$_1$. The results reveal that, depending on parameters of storage, the investigated Schiff base partly reduces the concentration of AFM$_1$. These findings suggest the ability of this chemical compound to partly decrease the concentration of this toxin and/or modify AFM$_1$ into compounds with differing chemical characteristics. The data obtained indicate that in milk and under certain conditions, Schiff base may provide some antiaflatoxigenic benefit, and thus health protection against AFM$_1$ accumulation. In general, although the overall efficacy was poor, investigated Schiff base was the good candidate to decrease AFM$_1$ concentration in raw milk. Further studies should include higher treatment, but without compromising organoleptic features.
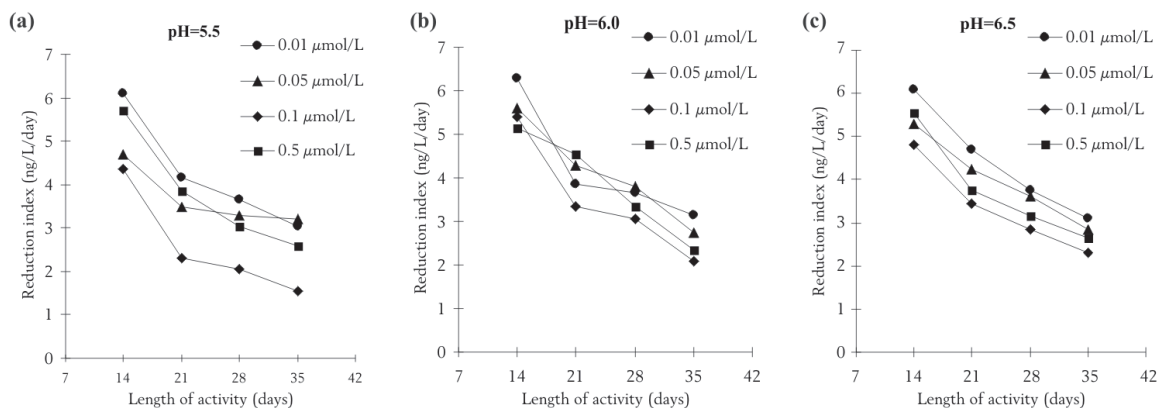

Figure 4. (a, b and c) Aflatoxin M$_1$ reduction index curves in raw milk containing various Schiff base concentrations of 0.01, 0.05, 0.1 and 0.5 μmol/L at pH values: (a) 5.5; (b) 6.0 and (c) 6.5 during 35 days of deposit

Sources of analytical difficulty

TLC method with visual estimation did not allow sufficient precision for quantification of AFM$_1$, and therefore that results must be used with reservation. Standard solutions of AFM$_1$ are a major source of error, as has been reported by Horwitz (1984).

Serious errors may also occur because of inappropriate handling and storage, e.g. concentration by evaporation from opened vials; influence of light, heat, or the laboratory atmosphere; poor dilution technique, and instability of some solvents. Where silica gel cleanup columns are used, particular attention must be paid to the instructions concerning the activity of the silica gel and the purity of the individual components used in the washing solvents.

Small deviation in the water content of the silica gel or the presence of impurities in some reagents and solvents (e.g., the water content in acetic acid) may lead to early elution and loss of AFM$_1$ during cleanup. The final determination of AFM$_1$ in milk in these investigations is generally based on thin layer chromatography. In this case, reliable quantification requires good separation of the AFM$_1$ spot from those coextracted components. It is therefore recommended that 2-dimensional TLC analysis should be used in preference to 1-dimensional TLC analysis to obtain reliable separation and quantification.

Conclusions

Among the contaminants of human's food, the residues of mycotoxins in the dairy products constitute a poorly studied problem. Residues of the diversified modes of synthesis, the mycotoxins have different properties and different rates of milk excretion. Only lipophilic compounds, which have escaped hydrolysis in the rumen, will undergo this phenomenon. Aflatoxin M$_1$, obtained from aflatoxin B$_1$, constitutes the principal milk contaminant. Because the origin of this contamination is known, and plans of monitoring are available, an improvement of the quality of the products is observed. Many mycotoxins are identified in food of the cattle, and may be responsible for accidents in breeding, without any information being available concerning their excretion in milk. Because these compounds may be carcinogenic, immunotoxic, poisons for the functions or reproduction, a level of zero contamination should be required.

Since a systematic decontamination of food is impossible, only an improvement of the quality of the raw materials is prove to guarantee the safety of the products obtained. The approach needs to be based on knowledge about the general mechanisms involved in the fungus contamination of food. It aims at preventing any risk for the consumer, and contribution to improve the image of the products. Bioassay may be useful in tracing sources of known mycotoxins. However, their use in the surveillance of food and foodstuffs for mycotoxins is minor importance.

In order to broaden the scale of investigations on Schiff bases, we have now synthesized, structurally characterized and determined antifungal and antimycotoxigenic activity of a number of Schiff bases, derived from various aromatic aldehydes and aromatic amines, and the results will be published.

Šifova baza: Kemijski agens visokog afiniteta za smanjenje koncentracije aflatoksina M$_1$ u umjetno kontaminiranom sirovom mlijeku

Sažetak

U navedenom istraživanju određivani su utjecaji pH (5,5, 6,0 i 6,5) i koncentracije novosintetiziranog spoja 3-/2-aminophenylimino-(p-toluoyl)/4-hydroxy-6-(p-tolyl)-2H-pyrane-2-one (šifove baze) na smanjenje koncentracije aflatoksina M$_1$ (AFM$_1$) u sirovom mlijeku, umjetno kontaminiranom s poznatom koncentracijom tog toksina. Istraživanja su provedena na temperaturi 4 °C u tijeku 35 dana. Pri pH 5,5, šifova baza koncentracije 0,1 μmol/L rezultirala je smanjenjem koncentracije AFM$_1$ za 55 % nakon 35 dana. Šifova baza nije bila djelotvorna pri pH 7 ili višim. Sposobnost šifove baze da djeluje kao antimikotoksigeni agens pruža novu perspektivu za moguću upotrebu navedenog kemijskog spoja u kontroli kontaminacije mlijeka s AFM$_1$ i produljenju trajnosti te namirnice. Određivanje toksičnosti istraživane šifove baze provedeno je pomoću larvi račića *Artemia salina* kao biologijskog indikatora za određivanje njihove osjetljivosti na ovaj kemijski agens.

Ključne riječi: mlijeko, aflatoksin M$_1$,
TLC, šifova baza, *Artemia salina*

Acknowledgements

References

1. Alpsoy, L. (2010): Inhibitory effect of essential oil on aflatoxin activities. *African Journal of Biotechnology 9* (17), 2474-2481.

2. Amanullah, M., Sadozal, S.K., Rehman, W., Hassan, Z., Rauf, A., Iqbal, M. (2011): Cytotoxic, antibacterial activity and physico-chemical properties of some acid catalyzed Schiff bases. *African Journal of Biotechnology 10* (2), 209-213.

3. AOAC, Association of Official Analytical Chemists (1995): Natural toxins. *In: Official Methods of Analysis of AOAC International*, P., Cunnif (Ed.). AOAC, Gaithersburg, ML, pp: 45-46.

4. Bakirci, I. (2001): A study on the occurrence of aflatoxin M$_1$ in milk and milk products produced in Van province of Turkey. *Food Control 12*, 47-51.

5. Bhat, R.V., Vasanthy, S. (1999): Mycotoxin contamination of foods and feeds, Overview: occurrence and impact on food availability trade exposure of farm animals and related economic losses. *Presented in the 3rd Joint FAO / WHO / UNEP International Conference on Mycotoxins*, Tunis, Tunisia, 3-6 March, 1999.

6. Cathey, C.G., Nuang, Z.G., Sarr, A.B., Clement, B.A., Phillips, T.D. (1994): Development and evaluation of a minicolumn assay for the detection of aflatoxin M$_1$ in milk. *Journal of Dairy Science 77*, 1223-1231.

7. Cole, R.Y., Cox, R.H. (1981): *Handbook of Toxic Fungal Metabolites*. Academic Press, New York.

8. Coleman, G.H., Wolf, P.A. (1947): *In: US Patent. 2* (474), 228.

9. Codex Alimentarius Commission (2001): Comments submitted on the draft Maximum level for Aflatoxin M$_1$ in milk. *Codex committee on food additives and contaminants, 33rd session*, Hague, The Netherlands.

10. Creppy, E.E. (2002): Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters 127*, 19-28.

11. Diaz, D.E., Hagler, W.M., Hopkins, B.A., Whitlow, L.W. (2003): Aflatoxin Binder I: *In vitro* binding assay for aflatoxin B$_1$ by several potential sequestering agents. *Mycopathologia 156*, 223-226.

12. Diaz, D.E., Hagler, W.M., Blackwelder, J.T., Eve, J.A., Hopkins, B.A., Anderson, K.L., Jones, F.T., Whitlow, L.W. (2004): Aflatoxin Binders II: Reduction of aflatoxin M$_1$ in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia 8* (1), 1-8.

13. Dichter, C.R. (1984): Risk estimates of liver cancer due to aflatoxin exposure from peanuts and peanut products. *Food and Chemical Toxicology 22*, 431-437.

14. D'Mello, J.P.F., MacDonald, A.M.C. (1997): Mycotoxins. *Animal Feed Science and Technology 69*, 155-166.

15. Dragacci, S., Gleizes, E., Fremi, J.M., Candlish, A.A.G. (1995): Use of immunoaffinity chromatography as a purification step for the determination of aflatoxin M$_1$ in cheese. *Food Additives and Contaminants 12* (1), 59-65.

16. Duraković, S., Beritić, T., Duraković, Z., Pospišil, O., Delaš, F., Vorkapić-Furač, J. (1986): Application of dehydrated *Artemia salina* eggs in aflatoxins toxicity detection. *Hrana i ishrana 27* (4), 199-204.

17. Duraković, S., Filipović-Marinić, N., Šarac-Arneri, R., Radić, B., Delaš, F. (1987): New synthesized derivative of tetraketone as antifungal and antiaflatoxigenic agent. *Acta Biologica Iugoslavica (B Mikrobiologija) 23* (1), 27-32.

18. Duraković, S., Markov, K., Sušnik, I., Galić, K., Rajnović, P., Pospišil, O., Štilinović, L. (1989): Antifungal and antiochratoxigenic properties of new synthesized analogues of dehydroacetic acid. *Acta Biologica Iugoslavica (B Mikrobiologija) 26* (1), 2-13.

19. Duraković, S., Sušnik, I., Golem, F.V., Duraković, Z., Beritić, T., Radić, B., Filipović-Kovačević, Ž., Pavlaković, Z. (1994): Dehydroacetic Acid and the Newly Synthesized Schiff Base to Control Aflatoxin Accumulation. *Kemija u industriji 43* (1), 7-12.

20. Duraković, S., Pavlaković, Z., Filipović-Kovačević, Ž., Duraković, L., Šarac-Arneri, R., Lalić, Lj.M., Colić, I. (1995): Antifungal and Antimycotoxigenic Properties of Newly Synthesized Derivatives of Tetraketone. *In: Proceedings of the 8th European Conference on Food Chemistry, EURO FOOD CHEM VIII, Vol. 2.*, G., Sontag (Ed.). Vienna, Austria, pp: 320-323.

21. Duraković, L., Delaš, F., Markov, K., Duraković, S. (2004): Growth and Biosynthesis of Aflatoxin in the Presence of Newly Synthesized Analogues of Dehydroacetic Acid. *In: Proceedings of the 5th Croatian Congress of Food Technologists, Biotechnologists and Nutritionists, 2nd Central European Meeting*, D., Karlović (Ed.). Opatija, Croatia, pp: 366-370.

22. Duraković, S., Delaš, F., Govori, S., Kaljaj, V., Rapić, V., Kaljaj, L., Halt, M., Duraković, L. (2006): Antifungal and antimycotoxigenic properties of newly synthesized derivatives of coumarine. *In: Book of Abstracts of the 2nd International Scientific Congress Food Sciences and Food Biotechnology in Developing Countries*, N., Rocha-Guzman (Ed.). Saltillo, Mexico, p: 37.

23. Duraković, L. (2007): *The influence of chosen parameters on the growth of the mold Aspergillus flavus ATCC 26949 in mixed culture and biosynthesis of aflatoxins B$_1$ and G$_1$.* PhD Thesis. Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia.

24. Duraković, S., Duraković, L., Vahčić, N., Skelin, A., Duraković, Z. (2008): Affect of moulds growth in mixed cultures on production of aflatoxins on maize hybrid. *Cereal Research Communications 36* (Part 3, Supplement 5), 1615-1618.

25. Duraković, L., Delaš, F., Blažinkov, M., Šećerkadić, L., Frece, J., Redžepović, S., Bošnjak, M., Skelin, A., Duraković, Z., Duraković, S. (2010a): Aflatoxin Accumulation During the Growth of Mould *Aspergillus flavus* ATCC 26949 on Corn in Pure and Mixed Culture as Related to Various Temperature and Moisture Content. *Croatian Journal of Food Technology, Biotechnology and Nutrition 5* (1-2), 10-17.

26. Duraković, L., Blažinkov, M., Sikora, S., Delaš, F., Skelin, A., Tudić, A., Mrkonjić-Fuka, M., Bošnjak, M. (2010b): A Study of Antifungal and Antiaflatoxigenic Action of Newly Synthesized Analogues of Dehydroacetic Acid. *Croatian Journal of Food Technology, Biotechnology and Nutrition 5* (3-4), 127-135.

27. Duraković, L., Blažinkov, M., Šeol, B., Tudić, A., Delaš, F., Bošnjak, M., Sikora, S., Skelin, A., Huić-Babić, K., Duraković, Z. (2010c): Mould Growth and Aflatoxin Accumulation Affected by Newly Synthesized Derivative of Coumarine Treatment of Maize Hybrid. *Agriculturae Conspectus Scientificus 75* (4), 169-174.

28. Duraković, L., Blažinkov, M., Skelin, A., Sikora, S., Delaš, F., Mrkonjić-Fuka, M., Huić-Babić, K., Redžepović, S. (2011a): Temperature-dose relationships with aflatoxin M₁ in milk on the brine shrimp (*Artemia salina*) larvae. *Mljekarstvo 61* (2), 145-153.

29. Duraković, L., Skelin, A., Sikora, S., Delaš, F., Mrkonjić-Fuka, M., Huić-Babić, K., Blažinkov, M. (2011b): Impact of new synthesized analogues of dehydroacetic acid on growth rate and vomitoxin accumulation by *Fusarium graminearum* under different temperatures in maize hybrid. *African Journal of Biotechnology 10* (52), 10798-10810.

30. FAO - Food and Feed contaminants Publications (1997): Worldwide regulations for mycotoxins 1995. - A compendium. *FAO Food and Nutrition Papers (FAO)*, No. 64, FAO Rome (Italy), Food and Nutrition Div.; WHO, Geneva (Switzerland), 1997.

31. Favilla, M., Macchia, L., Gallo, A., Altomare, C. (2006): Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) intervertebrate bioassays. *Food and Chemical Toxicology 44*, 1922-1931.

32. Filipović-Marinić, N., Laćan, M. (1982): Contribution to the Chemistry of Tetraketones, I.-Synthesis of Tetrasubstituted 4,4'-Methylenediisoxazoles and 5-Aryl-5-hydroxy-2-[aryl(hydroxyimino)methyl]-4-[1-(hydroxyimino)ethyl]-1-cyclohexanones. *Liebigs Annual Chemistry 1982* (11), 2089-2092.

33. Food and Drug Administration's Center for Veterinary Medicine (FDA's CVM) (1999): CVM Position On Mycotoxin Binding Claims On Anticaking Agents. *21 Code of Federal Regulations Part 582.2727 and 582.2729*.

34. Galvano, F., Galofaro, V., Galvano, G. (1996a): Occurrence and stability of aflatoxin M₁ in milk and milk products: A worldwide review. *Journal of Food Protection 59* (10), 1079-1090.

35. Galvano, F., Pietri, A., Bertuzzi, T., Fusconi, G., Galvano, M., Piva, A., Piva, G. (1996b): Reduction of carryover of aflatoxin from cow feed to milk by addition of activated carbons. *Journal of Food Protection 59*, 551-554.

36. Galvano, F., Galofaro, V., Ritieni, A., Bognanno, M., De Angelis, A., Galvano, G. (2001): Survey of the occurrence of aflatoxin M₁ in dairy products marketed in Italy: second year of observation. *Food Additives and Contaminants 18* (7), 644-646.

37. Govori, S., Kaljaj, V., Rapić, V., Kaljaj, I., Đaković, S. (2002): Synthesis and structure of some 3,4-annelated coumarin systems. *Heterocyclic Communications 8* (2), 129-134.

38. Groopman, J.D., Cain, L.G., Kensler, T.W. (1988): Aflatoxin exposure in human populations: measurements and relationship to cancer. *Critical Reviews of Toxicology 19*, 113-146.

39. Harvey, R.B., Phillips, T.D., Ellis, J.A., Kubena, L.F., Huff, W.E., Petersen, H.D. (1991): Effects on aflatoxin M₁ residues in milk by addition of hydrated sodium calcium aluminosilicate to aflatoxin-contaminated diets of dairy cows. *American Journal of Veterinary Research 52*, 1556-1559.

40. Harwing, J., Scott, P.M. (1971): Brine shrimp (*Artemia salina* L.) Larvae as a Screening System for Fungal Toxins. *Applied Microbiology 21* (6), 1011-1016.

41. Hasan, H.A. (1993): Fungicide inhibition of aflatoxin, diacetoxiscirpenol and zearalenone production. *Folia Microbiologica 38*, 295-298.

42. Horwitz, W. (1984): *In: Proceedings of a Conference Challenges to Contemporary Dairy Analytical Techniques*, March, 28-30., University of Reading, England.

43. Huwig, A., Freimund, S., Kappeli, O., Dutler, H. (2001): Mycotoxin detoxification of animal feed by different adsorbents. *Toxicology Letters 122*, 179-188.

44. IARC (1993): Aflatoxins: naturally occurring aflatoxins (Group 1). Aflatoxin M₁ (Group 2B). *International Agency for Research on Cancer*. Lyon, France, *Vol. 56*, pp: 245-252.

45. Jackson, P.E., Groopman, J.D. (1999): Aflatoxin and liver cancer. *Baillieres Best Practice and Research in Clinical Gastroenterology 13* (4), 545-555.

46. Janos, G., Tamas, L. (2009): Macrophage Migration Inhibitory Factor (MIF) Tautomerase Inhibitors as Potential Novel Anti-Inflammatory Agents: Current Developments. *Current Medical Chemistry 18*, 1091-1114.

47. Kamkar, A. (2006): A study of the occurrence of aflatoxin M₁ in Iranian Feta cheese. *Food Control 17*, 768-775.

48. Kimaryamma, A.B., Rajan, B., Gangadharan, B., Manomohan, C.B. (1991): *In vitro* and *in vivo* studies on aflatoxin B₁ neutralization. *Indian Journal of Animal Science 61*, 58-60.

49. Lopez, C.E., Ramos, I.L., Ramadan, S.S., Bulacio, L.C. (2003): Presence of aflatoxin M₁ in milk for human consumption in Argentina. *Food Control 14* (1), 31-34.

50. Manetta, A.C., Di Giuseppe, L., Giammarco, M., Fusaro, J., Simonella, A., Gramenzi, A., Formigoni, A. (2005): High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M₁ in milk and cheese. *Journal of Chromatography, A. 1083*, 219-222.

51. Massey, T.E., Stewart, R.K., Daiels, J., Ling, L. (1995): Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B$_1$ carcinogenicity. *Proceedings of the Society for Experimental Biology and Medicine 208*, 213-227.

52. McLaughlin, J.L., Chang, C.J., Smith, D.L. (1991): Bench-top bioassays for the discovery of bioactivity natural products. *In: Studies in Natural Products Chemistry*, A.U., Rahman (Ed.). Elsevier, Amsterdam, pp: 201-214.

53. Nemati, M., Mehran, M.A., Hamed, P.K., Masoud, A. (2010): A survey on the occurrence of aflatoxin M$_1$ in milk samples in Ardabil, Iran. *Food Control 21*, 1022-1024.

54. Phillips, T.D., Kubena, L.F., Harvey, R.B., Taylor, D.R., Heidelbaugh, N.D. (1988): Hydrated sodium calcium aluminosilicate: A high affinity sorbent for aflatoxin. *Poultry Science 67*, 243-247.

55. Pittet, A. (1998): Natural occurrence of mycotoxins in foods and feeds-an updated review. *Revue de Médecine Vétérinaire 6*, 479-492.

56. Prakash, R., Kumar, A., Singh, S.P., Aggarway, R., Prakash, O. (2007): Dehydroacetic acid and its derivatives in organic synthesis: Synthesis of some new 2-substituted -4-(5-Bromo-4-hydroxy-6-methyl-2H-pyrane-2-one-3-yl) thiazoles. *Indian Journal of Chemistry 66* (10), 1713-1715.

57. Raman, N., Kulandaisamy, A., Thangaraja, C., Jeyasubramanian, K. (2003): Redox and antimicrobial studies of transition metal (II) tetradentate Schiff base complexes. *Transition Metal Chemistry (N.Y.) 28*, 29-36.

58. Ramos, A.J., Fink, G.J., Hernandez, E. (1996): Prevention of toxic effects of mycotoxins by means of nonnutritive adsorbent compounds. *Journal of Food Protection 59*, 631-641.

59. Ramos, A.J., Hernandez, E. (1997): Prevention of aflatoxicosis in farm animals by means of hydrated sodium calcium aluminosilicate addition to feedstuffs: A review. *Animal Feed Science and Technology 65*, 197-206.

60. Rastogi, S., Dwivedi, D.P., Khanna, K.S., Das, M. (2004): Detection of aflatoxin M$_1$ contamination in milk and infant milk products from Indian markets by ELISA. *Food Control 15*, 287-290.

61. Sefidgar, S.A.A., Mirzae, M., Assmar, M. (2011): Aflatoxin M$_1$ in Pasteurized Milk in Babol city, Mazandaran Province, Iran. *Iranian Journal of Public Health 40* (1), 115-118.

62. Shi, L., Fang, R.Q., Zhu, Z.W., Yang, Y., Cheng, K., Zhong, W.Q., Zhu, H.L. (2010): Design and synthesis of potent inhibitors of beta-ketoacyl-acyl carrier protein synthase III (FabH) as potential antibacterial agents. *European Journal of Medical Chemistry 45* (9), 4358-4364.

63. Stahr, H.M., Pfeiffer, R.L., Imerman, P.J., Bork, B., Hurburg, C. (1990): Aflatoxin-the 1998 outbreak. *Dairy Food and Environmental Station 10* (1), 15-17.

64. Stoloff, L., van Egmond, H.P., Park, D.L. (1991): Rationales for establishment of limits and regulations for mycotoxins. *Food Additives and Contaminants 8*, 213-222.

65. Sušnik, I., Vorkapić-Furač, J., Duraković, S., Koprivanac, S., Lasinger, J. (1992): Synthesis of some Schiff Bases of 3-Aroyl-6-Aryl-4-Hydroxy-2H-pyran-2-ones. *Monatshefte für Chemie 123* (2-3), 817-822.

66. Škrinjar, M., Danev, M., Dimić, G. (1995): Investigations on the presence of toxigenic fungi and aflatoxins in raw milk. *Acta Alimentaria 24* (4), 395-402.

67. Tratnik, Lj. (1998): *Mlijeko-tehnologija, biokemija i mikrobiologija*, V., Volarić (Ed.). Hrvatska mljekarska udruga, Zagreb.

68. Trucksess, M.W. (1999): Mycotoxins. *Journal of AOAC International 82*, 488-495.

69. Unusan, N. (2006): Occurrence of aflatoxin M$_1$ in UHT milk in Turkey. *Food Chemistry and Toxicology 44*, 1897-1900.

70. van Egmond, H.P., Paulsch, W.E. (1986): Mycotoxins in milk and milk products. *Netherlands Milk and Dairy Journal 40*, 175-188.

71. van Egmond, H.P., Wagstaffe, P.J. (1987): Development of Milk Powder Reference Materials Certified for Aflatoxin M$_1$ Content (Part I). *Journal of the Association of Official Analytical Chemists 70* (4), 605-610.

72. van Egmond, H.P. (1989): Aflatoxin M$_1$: occurrence, toxicity, regulation. *In: Mycotoxins in Dairy Products*, H.P., van Egmond (Ed.). Elsevier Applied Science, London, pp: 11-55.

73. Verma, M., Pandeya, S.N., Singh, K.N., Stables, J.P. (2004): Anticonvulsant activity of Schiff bases of isatin derivatives. *Acta Pharmaceutica (Bandung, Indonesia) 54*, 49-56.

74. Wood, G.E. (1991): Aflatoxin M$_1$. *In: Mycotoxins and Phytoalexins*, R.P. Sharma and D.K. Salunkhe (Eds.). CRC Press, Boca Raton, pp: 145-164.

75. Zhong, Z., Li, P., Xing, R., Chen, X., Liu, S. (2009): Preparation, characterization and antifungal properties of 2-(alpha-arylamino phosphonate)-chitosan. *International Journal of Biological Macromolecules 45* (3), 255-259.