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Removal of aflatoxin M_1 from artificially contaminated yoghurt by using of new synthesized dehydroacetic acid analogues

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Summary

Dehydroacetic acid (DHA) and its new synthesized analogues, 4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (DHT) and 5-Bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (BrDHT) were tested for removal of aflatoxin M_1 (AFM_1) from artificially contaminated yoghurt with known concentrations of this toxin to determine the possible use of these chemicals as a means of controlling AFM_1 accumulation. Yoghurt from cow's milk was artificially contaminated with AFM_1 at levels of 0.01 to 0.5 μg/L. Yoghurts were stored at 4 °C and 7 °C, respectively, for up to 28 days. Analysis of AFM_1 in yoghurt was carried out using two-dimensional thin-layer chromatography (TLC) - visual estimation. The limit of detection was 0.15 ng/L. The recoveries of AFM_1 from the samples spiked at levels of 10, 50, 100, and 500 ng/L were between 80.6 and 107.8 %, respectively. Concentrations of DHA and DHT of 0.01 and 0.03 μmol/L had non or little effect on AFM_1 content in experimentally contaminated yoghurt, whereas concentrations higher than 0.05 μmol/L, partially inhibited AFM_1 content. The percentage loss of the initial AFM_1 amount in yoghurt was estimated by about 15 and 25 %, and 22 to 45 % by the end of storage, respectively. In experiments with 0.01 and 0.05 μmol/L of BrDHT or higher, the concentration of AFM_1 was reduced after 28 days by 20 to 95 % or completely, respectively, depending on the time and temperature of deposit. Detection of toxicity of investigated analogues was evaluated by using the brine shrimp (*Artemia salina*) larvae as a screening system for the determination of their sensitivity to some chemicals.

Key words: aflatoxin M_1, yoghurt, dehydroacetic acid analogues, TLC, *Artemia salina*

Introduction

Mycotoxin is a general term used to describe compounds or metabolites, which are toxic or have other biological effects in living organisms (primarily animals and/or man), and which are produced by moulds. The term is derived from the Greek words "mykes" meaning fungus and "toxicum" meaning poison or toxin (Goldblatt, 1969). Thus, the term literally means fungus poison or fungus toxin. A number of the compounds which are today classed as mycotoxins were actually firstly studied as potential antibiotics in the early 1930's and 1940's, only to be discarded as being too toxic to higher life forms to be of value in treating diseases.

The outbreak of Turkey "X" disease in England in 1960 culminated in the discovery of aflatoxins and the realization that low levels of mould metabolites in foods and feed could cause disease in man and animals. This gave a great impetus of the study of mycotoxins. Mycotoxin-producing moulds are

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quite ubiquitous and frequently contaminate food and agricultural commodities. Fortunately, the more presence of a toxic mould in food does not automatically mean the presence of mycotoxins.

Mycotoxins currently receiving the most attention as potential hazards to human and animal health include aflatoxins, ochratoxin A, sterigmatocystin, patulin, penicillic acid, citrinin, zearalenone and the toxic trichothecenes. These compounds all cause some degree of acute toxicity when given in high amounts. In addition, aflatoxin, sterigmatocystin, patulin and penicillic acid are potential carcinogens (Duraković et al., 2008).

Mycotoxins may enter the food supply by direct contamination, resulting from mould growth on the food, or by indirect contamination with contaminated ingredient in processed foods (Duraković, 2007). Indirect exposure to mycotoxins can also result from consumption of animal products such as milk, which contain mycotoxin residues, caused by feeding mouldy feed to the food-producing animal. Commodities susceptible to direct contamination with mycotoxins include nuts, oilseeds, grains and to a limited extent, certain fruits. Residues of aflatoxins have been found in animal products such as fluid milk, non-infant dry milk, cottage cheeses, and imported cheeses. Refrigerated foods, such as cheeses, cured meats and certain flour-based products, subject to mould growth during storage, have been shown to be contaminated with a variety of potential mycotoxin-producing moulds.

Of all the mycotoxins, aflatoxin B_1 (AFB_1) is considered the most toxic/carcinogenic compound (IARC, 1993; Duraković, 2007). AFB_1 is metabolized to aflatoxin M_1 (AFM_1) and is secreted in milk at rate of 1-3 % of the ingested AFB_1, by animals that have been fed with contaminated feeds (Applebaum and Marth, 1982; Duraković et al., 2012a). AFM_1 constitutes the principal milk contaminant. The fact that carcinogenicity of AFM_1 is less than of AFB_1 (Diaz et al., 2004; Manetta et al., 2005; Unusan, 2006; Duraković et al., 2012a), although acute toxicity potentials are similar, should not decrease the need to control its presence in milk and other dairy products by reducing it to the minimal or zero levels. AFM_1 is relatively stable in raw and processed milk products, and is unaffected by pasteurization or processing into yoghurt (Stubblefield and Shannon, 1974; El-Deeb et al., 1992; Jasutiene et al., 2006).

Thus, if raw milk contains AFM_1, yoghurt made from such milk also contains AFM_1 (Blanco et al., 1988; Barbieri et al., 1994). Evidence of potential hazardous human exposure to AFM_1 from dairy products is available from many studies on the occurrence of AFM_1 in dairy products (Brown, 1982; Galvano et al., 1996a; Fallah, 2012). Therefore, humans are potentially exposed to these metabolites and it is generally assumed that neither storage nor processing provides a reduction of AFM_1 content (Unusan, 2006; Duraković et al., 2012a). It should be taken into account, that pasteurization processes (even those using UHT techniques), do not affect AFM_1 concentration because of its heat stability (Galvano et al., 1996a, 2001; Fallah et al., 2011), and because mycotoxin detoxification processes known for human diets, turn the foodstuffs inedible. Consequently, monitoring programs are now the main strategy to diminish exposure risk, both for animals and human beings.

Exposure of children, including infants, to AFM_1 is worrisome, because they are considered more susceptible to its adverse effects, and their capacity for biotransformation of carcinogenesis is generally slower than in adults. Unfortunately, in our country, in spite of the fact that the dairy industry has evolved a lot in the last years, in regards to production levels and technology, there are very few data about AFM_1 incidence in milk, milk powder and other dairy products.

Since a systematic decontamination of milk and dairy products is impossible, only an improvement of the quality of the raw milk is proven to guarantee the safety of the products obtained. The consumption of milk and dairy products by human population is quite high, particularly among infants and young children, thereby the risk of exposure to AFM_1 is increased. Since, milk is the major commodity for introduction of aflatoxins in human diet, the occurrence of AFM_1 in this product is of concern (Stoloff, 1980; Tabari et al., 2011). Evidence of hazardous human exposure to AFM_1 through dairy products has been shown by several investigators (Stoloff, 1980; Galvano et al., 1996b; Diaz et al., 2004; Bakirdere et al., 2012).

To prevent aflatoxin accumulation in agricultural commodities it is firstly necessary to prevent the growth of aflatoxin producing fungi (Duraković et al., 2008, 2012a, 2012b). This can be achieved by at

least three means: control of the environment, use of antifungal chemical agents and utilization of natural resistance in natural commodities (Coleman and Wolf, 1947; Webb, 1966; Stoloff, 1980; Buchanan and Fletcher, 1983; Duraković et al., 1989; Sušnik et al., 1992; Duraković et al., 2012b). 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 antimicrobial action of DHA. This compound appears to be effective even in the high pH range, but has never acquired great significance because of its relative toxicity. DHA has been used widely in USA as a food preservative, especially against moulds and is certainly one of the safest and most effective. This has stimulated extensive work to determine the minimal growth inhibitory concentrations for various microorganisms, some of the results of which are summarized in Table 1. Two things are immediately evident from this table. DHA is general and rather weak antimicrobial agent; it is of practical value because of its low toxicity. The DOW Chemical Company meanwhile had been studying the antimicrobial action of DHA and in June 1949 issued three patents for its use in food preservation.

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 in Zagreb, Croatia, many of new synthesized chemical compounds including DHA analogues were synthesized as potential antimicrobial agents (Sušnik et al., 1992). The Laboratory of General Microbiology and Food Microbiology of the same Faculty demonstrated that several DHA analogues inhibit growth of certain species of bacteria, yeasts and moulds (Duraković et al., 1986; Sušnik et al., 1992; Duraković et al., 1994; Duraković, 2007; Duraković et al., 2011, 2012b).

Methods for the synthesis of these chemicals are described by Filipović-Marinić and Laćan (1982), Sušac et al. (1989), Sušnik et al., (1992), Govori et al. (2004), Prakash et al. (2007) and Amanullah et al. (2011).

That was the basis of this study, which aimed at evaluating AFM_1 reduction in yoghurt contaminated artificially with known concentrations of this toxin in the presence of selected new synthesized DHA analogues.

Preliminary studies in the Laboratory of General Microbiology and Food Microbiology of the same Faculty have demonstrated that several of these new synthesized chemicals strongly inhibit the growth of certain species of bacteria, yeasts, and moulds, including aflatoxin producing and ochratoxin and trichothecenes producing fungi (Duraković et al., 1986, 1994, 2011, 2012b). Two of these compounds with circumstantial evidence minimal inhibitory concentrations (MIC) of 0.1 and 0.2 μg/mL used in these experiments were DHA analogues 4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (DHT) and 5-Bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (BrDHT). Their structures are presented in Figure 1.

During recent years, a strong interest in derivatives of 3-aroyl-6-aryl-4-hydroxy-2H-pyrane-2-one

Table 1. Antimicrobial activity of dehydroacetic acid (Webb, 1966; Welling et al., 1985; Sušnik et al., 1992; Prakash et al., 2007)

Microorganism	Minimal inhibitory concentration (mmol/L)
Bacteria	
Alcaligenes faecalis	23.8
Bacillus anthracis	59.5
B. cereus	17.8
Corynebacterium diphteriae	3.0
Mycobacterium tuberculosis	5.9
Salmonella typhosa	12.0
Staphylococcus aureus	108.0
Moulds	
Aspergillus niger	3.0
Fusarium graminearum	0.47
Penicillium expansum	0.60
Rhizopus nigricans	2.4
Aspergillus parasiticus	12.50
Aspergillus ochraceus	8.40
Fusarium graminearum	6.25
Penicillium citrinum	0.30
Alternaria solani	0.12
Botrytis allii	0.30
Yeasts	
Candida albicans	1.9
Saccharomyces cerevisiae	5.9
Candida utilis	2.5
Trichosporon cutaneum	0.85

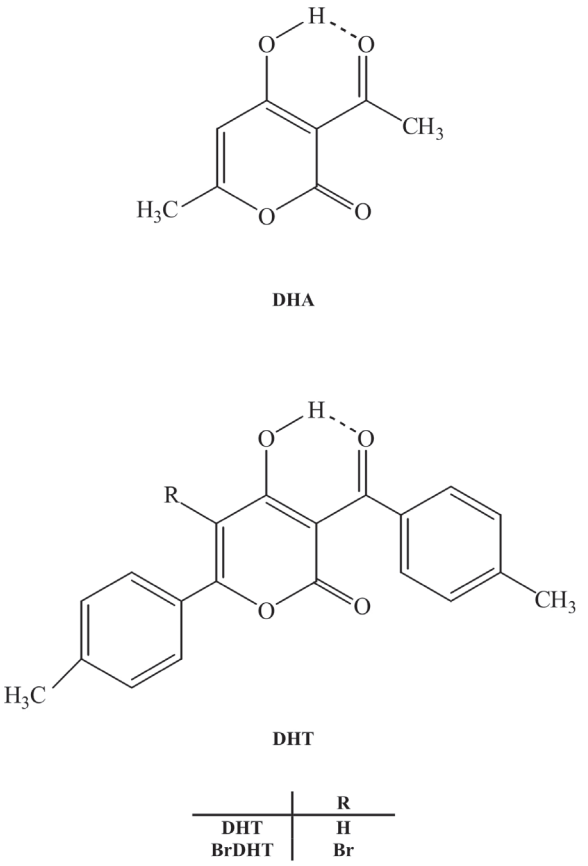

Figure 1. Chemical structures of DHA and its new synthesized analogues DHT and BrDHT (Duraković et al., 1989; Sušnik et al., 1992; Duraković et al., 1994, 2011)

with potential biological activity was maintained. Previous reports from these laboratories have described the effect of 4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one **(1.a)** and its 5-Bromo derivative **(1.c)** on bacteria, yeasts, and moulds. Compound **1.c** significantly inhibited growth of all tested microorganisms especially growth and aflatoxin accumulation of the aflatoxigenic mould

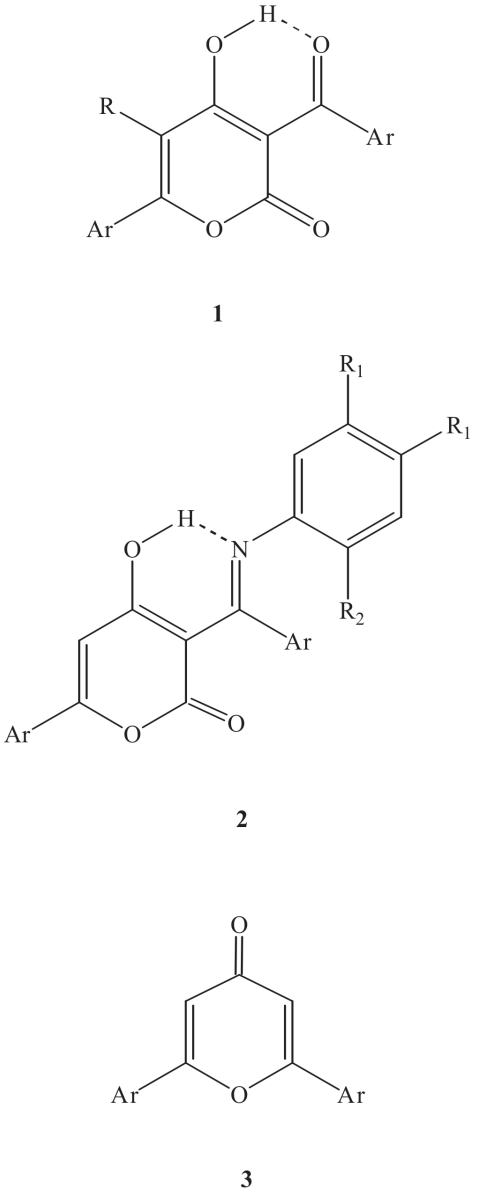

Figure 2. Chemical structures of a series of new synthesized DHA analogues (Sušnik et al., 1992; Duraković et al., 2011)

Table 2. The novel DHA analogues, which were tested for reduction of AFM_1 content

	R	Ar		R_1	R_2	Ar		Ar
1.a	H	p-tolyl	2.a	H	H	p-tolyl	3.a	p-tolyl
1.b	H	(1,1'-biphenyl)-4yl	2.b	H	NH_2	p-tolyl	3.b	(1,1'-biphenyl)-4-yl
1.c	H	p-tolyl	2.c	Cl	NH_3	p-tolyl	3.c	2-thienyl

Aspergillus parasiticus NRRL 2999 (Duraković et al., 1986, 1989).

Continuing this investigation, now the synthesis of compounds **1.a** and **1.c** as well as the synthesis of some novel derivatives of 4-hydroxy-3-(p-toluoyl)-6-(p-tolyl) - **(1.a)**, and 6-[(1,1'-biphenyl)-4-yl]-3-[(1,1'-biphenyl)-4-carbonyl]-4-hydroxy – **(1.b)** (Sušnik et al., 1992) (Figure 2 and Table 2) can be reported. Twelve new synthesized analogues previously tested in this Laboratory for reduction of AFM_1 content were evaluated in these experiments. These were (Table 2, Figure 2, and Figure 1): **1. (a-c)**, **2. (a-c)**, **3. (a-c)**, DHA, DHT and BrDHT.

Two of the twelve investigated analogues (DHT and BrDHT) significantly ($p<0.01$) reduced AFM_1 contamination of yoghurt, whereas parent molecule (DHA) had little and other analogues had no effect on AFM_1 content in yoghurt. Potential AFM_1 reduction agents should be evaluated experimentally to demonstrate efficacy. Obtained data show that investigated new synthesized DHA analogues, DHT and BrDHT can reduce AFM_1 content in yoghurt produced by cow's milk, and artificially contaminated with known concentrations of this toxin.

The consumption of dairy products especially yoghurt is widespread in Croatia. For this purpose, this study was designed to determine the abilities of several new synthesized DHA analogues that were tested *a priory* by a novel *in vitro* assay to reduce the content of AFM_1 in yoghurt produced by cow's milk and artificially contaminated with known concentrations of AFM_1 in controlling parameters of safe-keeping. The AFM_1 concentration was estimated by two-dimensional thin-layer chromatography - visual determination.

Materials and methods

Sample

A total of 25 yoghurt samples were taken from different markets located in the various districts of Zagreb and Karlovac, Croatia, and examined for the presence of AFM_1. The samples were transferred to the Laboratory in iceboxes at temperatures between 5 °C and 10 °C. All samples were collected between the period of July 2011 and August 2011.

AFM_1 standard solution

Stock solution for AFM_1 was obtained from Immunolab GmbH (Kassel, Germany). Ten millilitre of chloroform was added to 10 μg AFM_1 standard bottle that was obtained from Immunolab GmbH. Concentration in the bottle became 1 μg/mL in chloroform. The solution bottle was sealed and wrapped with aluminium foil, and stored in cool (4 °C) dry place.

Yoghurt contamination with AFM_1

Whole liquid yoghurt obtained from pasteurized milk was used. The yoghurt showed no detectable levels of AFM_1 (results not presented) and the toxin was added in different concentrations: 0.01, 0.02, 0.05, 0.1, and 0.5 μg/L of yoghurt. The samples were stored frozen in the dark until used. The presence of AFM_1 was determined in yoghurt before and after contamination. For the quantitative analysis of AFM_1, a series of dilutions were chromatographed from the sample extracts to determine the lowest dilution, at which AFM_1 can still be found. From lowest limit of detection of AFM_1 (sensitivity on TLC plate) and the dilution factor, the appropriate quantity of AFM_1 was calculated. Each sample was determined in triplicate.

The investigated DHA analogues (DHT and BrDHT) were dissolved in chloroform at concentrations of 0.1, 0.5, and 1.0 μmol/L. The required amounts of their solutions were added into test Erlenmeyer flasks to give 0.01, 0.05, 0.1, and 0.5 μmol/L in 50 mL of yoghurt. Control flasks and duplicate test flasks containing the various concentrations of investigated chemicals, were stored at temperatures of 4 °C and 7 °C during 28 days.

Determination of AFM_1 by thin-layer chromatography - visual estimation

The presence of AFM_1 was studied every 7 days for a period of 28 days by the technique described by Wiseman and Marth (1983), and Van Egmond and Wagstaffe (1987). AFM_1 is quantified by thin-layer chromatography - visual determination on the silica gel 60 (Merck, Darmstadt, Germany) thin-layer plates, dimensions 20 x 20 cm, layer thickness 0.2 mm. The amount of AFM_1 was estimated visually by comparing the fluorescence intensity of the AFM_1

from the yoghurt sample, with that of one or more amounts of AFM_1 standards. The detection limit of the method is 0.015 ng/mL.

The identity of AFM_1 was confirmed by the formation on the TLC plate of a derivative using trifluoroacetic acid and comparison of the thin-layer chromatography properties of the sample derivatives spot and the standard (Van Egmond and Wagstaffe, 1987). The AFM_1 content in the sample of yoghurt expressed in micrograms per liter (ppb) is given by the formula:

$$\frac{V_{st} \times C_{st} \times V_{ext}}{V_m \times V \times V_f / 150}$$

where V_{st} is the volume, in μL, of the AFM_1 standard used, in the interpolation was used the nearest spot intensity to the fluorescence intensity of the sample; C_{st} is the mass concentration, in μg/mL, of the AFM_1 standard; V_{ext} is the volume, in μL, in which the sample extract is dissolved; V_m is the volume, in μL, of sample extract used; V_s is the volume of yoghurt, in mL; V_f is the volume, in mL, of the filtrate obtained in extraction steps. 150 is the amount, in mL, of chloroform, used during extraction.

Detection of toxicity of investigated analogues

The toxicity of new synthesized DHA analogues was evaluated by using brine shrimp (*Artemia salina*) larvae as a screening system for the determination of their 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). The brine shrimp eggs were hatched in artificial seawater (35 g of sea salt per liter of water) and, after an average of 24 hours from hatching, the brine shrimp larvae were used for experimental bioassay. For each experiment 100-200 mg of brine shrimp was placed in 100 mL of hatching medium contained in 500 mL Erlenmeyer flasks and shaken as described by Favilla et al. (2006) and Duraković et al. (2012b). Hatching can occur in less than 24 hours at 27 °C.

Results of 10 parallel experiments showed that concentration resulting in 50 % mortality of larvae exposed to investigated analogues for 24 h at 30 °C was (μg/mL): DHT, 9.5 and BrDHT, 21.5. The results suggest that both investigated analogues had a poor toxic effect in relation to *Artemia salina* larvae in selected parameters of cultivation, and was almost three and six times less than toxicity of a parent molecule (dehydroacetic acid) (Duraković et al., 1986).

The bioassay with brine shrimp (*Artemia salina*) 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/kg in yoghurt per day, was calculated according to the formula (Duraković et al., 1994):

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

The results show that reduction index significantly decreased as concentrations of DHT and BrDHT increased (Table 4). At a concentration of BrDHT of 0.1 μmol/L and 0.5 μmol/L, the reduction index is only 0.31-0.36, and 0.12-0.07, respectively, in respect to temperature and storage time.

Statistical analysis of the data

AFM_1 recoveries from the yoghurt samples were performed by using the method recommended by AOAC (1995).

Repeatability and recovery were determined by spiking 50 mL of whole liquid yoghurt with toxin standard solution at the levels of 0.01 to 1.0 ng/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.

The average recoveries of AFM_1 spiked in yoghurt at concentrations of 0.01, 0.02, 0.05, 0.1, 0.5, and 1.0 ng/mL were 80.6, 85.5, 101, 109.5, 80, and 107.8 % (n=6) (Table 3). Mean recoveries of duplicate experiments were between 80.6 and 107.8 %, and standard deviation (n=6) was between 3.7 and 9.6 (Table 3).

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

Table 3. Analytical parameters for the determined AFM_1 in spiked yoghurt using two-dimensional thin-layer chromatography - visual determination

Amount added (ng/L)	Mean amount recovered (ng/L)	Mean recoveries (%) ± SD	$R^{2\,a}$
10	14.6	80.6 ± 5.2	0.997
20	25.2	82.8 ± 9.6	0.993
50	44.7	86.9 ± 3.7	0.992
100	98.8	98.7 ± 9.2	0.994
500	512.5	107.8 ± 8.4	0.998

$R^{2\,a}$ = Regression coefficient

Results and discussion

As expected, AFM_1 was not detected (<0.001 ppb) in yoghurt samples purchased in markets. Several authors reported no influence of yoghurt manufacture on AFM_1 content (Stoloff, 1980; Wiseman and Marth, 1983; Van Egmond and Paulsch, 1986; Blanco et al., 1988; Van Egmond, 1989; Govaris et al., 2002; Martins and Martins, 2004; Atasever et al., 2011), in agreement with a study by Ismail et al. (1989) on AFM_1 behavior during kefir manufacture. On the contrary, Van Egmond and Wagstaffe (1987) observed variable increase of AFM_1 content in yoghurt related to the milk, as did Wiseman and Marth (1983).

Regarding AFM_1 stability during yoghurt storage, Van Egmond and Wagstaffe (1987) observed no reduction of AFM_1 in yoghurt stored for 7 days at 7 °C. Megalla and Hafez (1982) observed complete transformation of AFB_1 in its hydroxy derivative AFB_2A caused by the acid present in yoghurt, whereas Rašić et al. (1991) revealed a high reduction (up to 97 %) of AFM_1 in yoghurt and acidified milk. Maryamma et al. (1990) reported a high reduction of AFM_1 in fermented goat milk.

Except for a survey performed by Quintavalla and Casolari (1985), who found in 6 out of 8 samples with from 36 to 334 ng of AFM_1 per liter, and a study of Haydar et al. (1990) who reported 190 ng of AFM_1 per koshk, a blend of yoghurt and parboiled wheat, we are aware of no studies on the occurrence of AFM_1 in yoghurt. The general opinion is that surveys on aflatoxin occurrence in commercial marketed yoghurt and further studies on its stability should be carried out for the following reasons: (i) in recent years human consumption of yoghurt has greatly increased; (ii) there is contradictory data on AFM_1 stability during manufacture and storage in the literature; (iii) and the presence of aflatoxins in yoghurt could decrease or undo the nutritional benefits of its consumption.

In the literature, aflatoxin reduction by DHA and its analogues has been variable. Duraković et al. (1989, 1994); Duraković (2007); Duraković et al. (2011) investigated two DHA analogues (DHT and Schiff base) added on corn, soybeans, and YES medium. The authors speculate that the Schiff base was better candidate for decrease of the AFB_1 concentration in corn and soybeans samples. In the study of effect of Schiff base on reduction of AFM_1 content in artificially contaminated cow's milk the same authors show that Schiff base in concentration of 0.1 and 0.5 μmol/L reduced the AFM_1 content by 55 and 28 %, respectively, in respect to pH values and contact time (Duraković et al., 2012b).

The influence of DHA and its analogues DHT and BrDHT on AFM_1 in artificially contaminated yoghurt is shown in Figures 3-5 and Table 4. Investigations were observed at 4 °C and 7 °C, respectively, after contamination of yoghurt. In general, parent molecule (DHA) has little or no effect on AFM_1 content in investigated yoghurt.

The data presented in Figures 3-5 and in Table 4 show the effect of DHT and BrDHT analogues on AFM_1 content in yoghurt. The activities of these compounds were determined for the concentrations between 0.01 and 1.0 μmol/L. The effect of increasing concentrations of these chemicals on decrease of AFM_1 concentration was evaluated.

Figure 3 depicts the effect of DHT on AFM_1 content. The high rate of AFM_1 content decrease was observed after 28 days of storage.

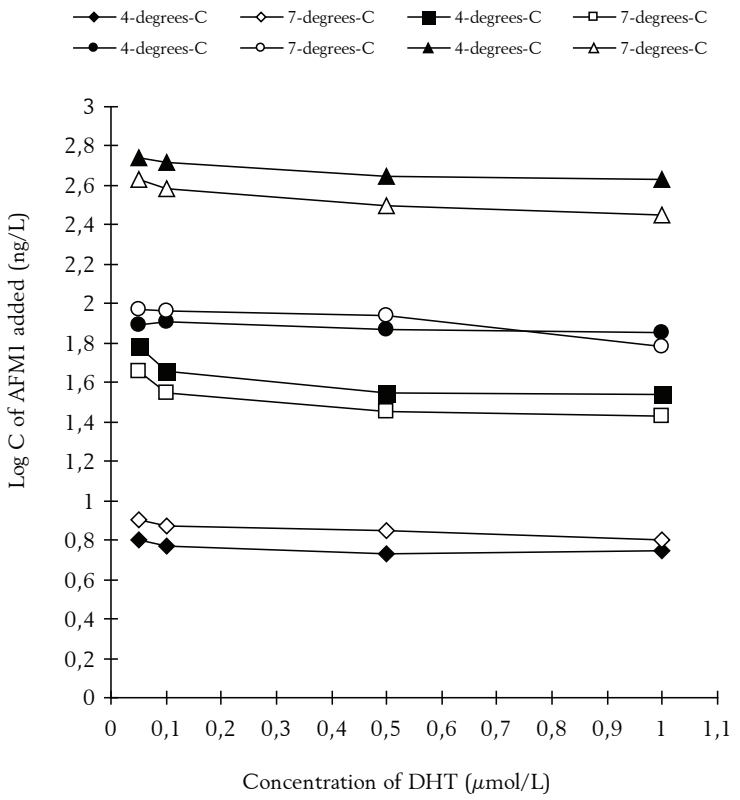

Figure 3. Effect of DHT on reduction of AFM_1 content in experimentally contaminated yoghurt at 4-degrees-C and 7-degrees-C after storage of 28 days. Concentrations of AFM_1 added were: 10, 50, 100, and 500 ng/L

Table 4. Effect of BrDHT on reduction of AFM_1 content in experimentally contaminated yoghurt at 4-degrees-C and 7-degrees-C after storage of 28 days. Concentrations of AFM_1 added were: 10, 50, 100, and 500 ng/L

BrDHT (μmol/L)	AFM_1 added (ng/L)	Reduction of AFM_1 content (%)		Reduction index for 100 ng/L/day	
		4 °C	7 °C	4 °C	7 °C
0.01	10	50	60	0.82	0.90
	50	20	10		
	100	15	35		
	500	10	30		
0.05	10	75	85	0.66	0.71
	50	25	50		
	100	40	30		
	500	20	95		
0.1	10	80	85	0.31	0.36
	50	25	65		
	100	80	60		
	500	60	25		
0.5	10	85	99	0.12	0.07
	50	75	80		
	100	65	55		
	500	70	45		

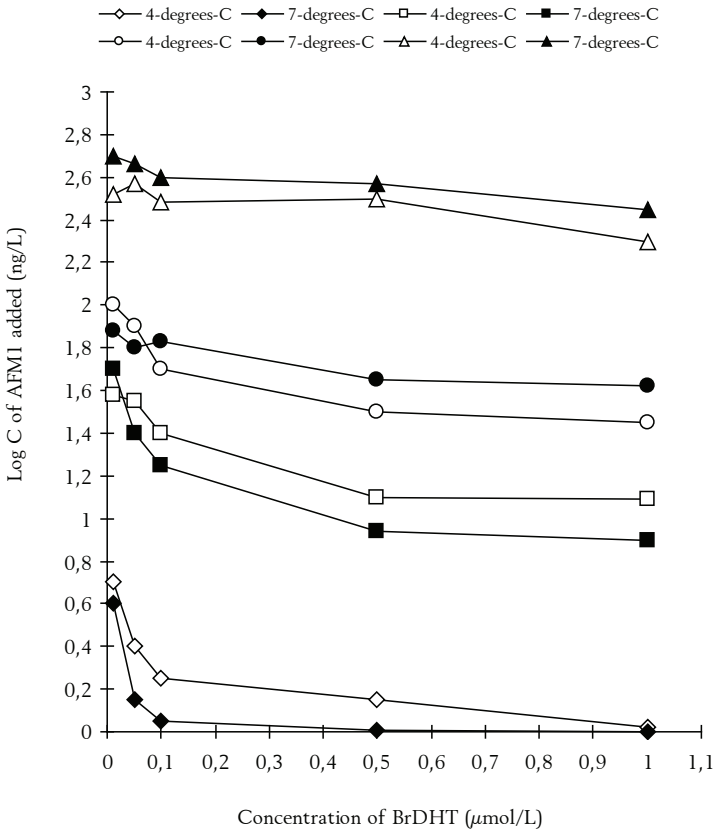

Figure 4. Effect of BrDHT on reduction of AFM_1 content in experimentally contaminated yoghurt at 4-degrees-C and 7-degrees-C after storage of 28 days. Concentrations of AFM_1 added were: 10, 50, 100, and 500 ng/L

Concentration of DHT of 0.01 and 0.03 μmol/L had little or no effect on AFM_1 content in investigated yoghurt, whereas DHT concentration higher than 0.05 μmol/L of this chemical, AFM_1 content decreased by 25 and 45 %, respectively, in respect to temperature and length of storage. The influence of BrDHT analogue on reduction of AFM_1 content in yoghurt is shown in Table 4 and Figures 4-5. The activity of this chemical was determined for the concentrations between 0.01 and 0.5 μmol/L.

In this study, BrDHT added at the lowest rate (0.01 μmol/L) did not significantly reduce AFM_1 in yoghurt (Table 4 and Figures 4-5). Concentrations from 0.05 to 0.5 μmol/L of this analogue reduced the content of AFM_1 to 25 and 99 %, respectively, at 4 °C and 7 °C in respect to the storage time. Table 4 represents the reduction index of AFM_1 in respect to concentration of BrDHT, storage temperature and deposit time. The results show that reduction index significantly decreases as concentration of investigated analogue increased. At a concentration of BrDHT of 0.1 and 0.5 μmol/L, respectively, the reduction index is only 0.31 and 0.36 and 0.12 and 0.07, respectively, after 28 days in respect to values obtained after 7 days of deposit.

These data indicated that in yoghurt contaminated with known concentrations of AFM_1 and under certain conditions, the investigated DHA analogues (DHT and BrDHT) might provide some anti-AFM_1 benefit and thus, health protection against possible AFM_1 accumulation.

These have often been tested for *in vitro* studies, but as presented data and those of others demonstrate, it is essential that DHA and its new synthesized analogues should be studied *in vivo* to establish actual effectiveness for animal use.

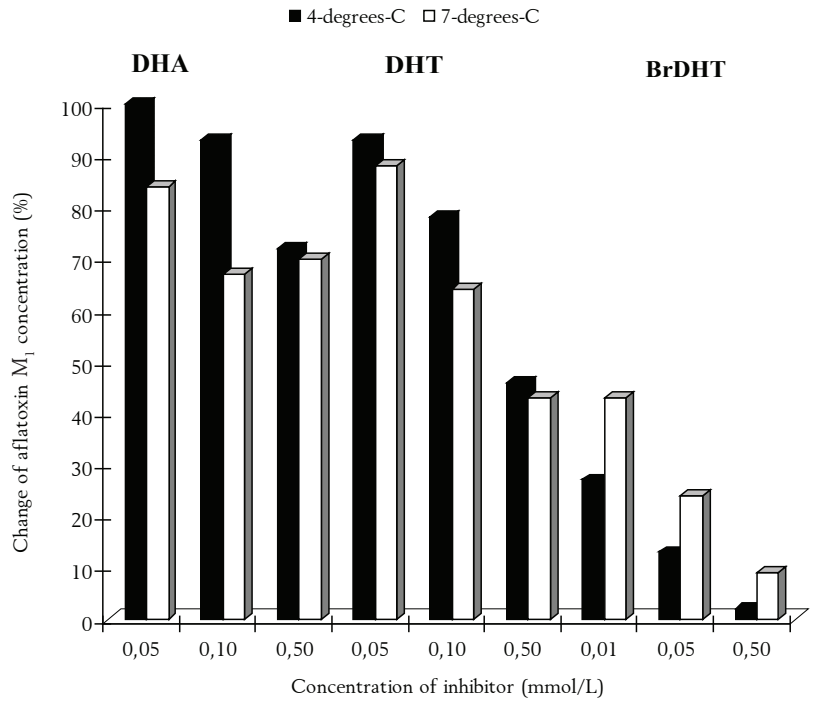

Figure 5. Comparative presentation of concentration change of aflatoxin M_1 in investigated yoghurt with respect to concentration of DHA and its analogues DHT and BrDHT (measured after 28 days in relation to temperature of storage)

Conclusions

The occurrence of AFM_1 in cow's milk and dairy products is widespread, although, considering the current scientific fund of information, contamination levels do not seem to be a serious health hazard. Wide and frequent monitoring programs performed by accurate and reliable analytical technique remain the primary strategy to provide protection for milk and dairy products consumer. For this reason, in many countries an appropriate attention should be used to control milk and dairy products other than those from cows, as well foods and feeds.

The increased interest in biopreservation of food systems has recently led to the development of the new synthesized antimicrobial compounds having different origin. Among them, dehydroacetic acid and its new synthesized analogues are effective antifungal and antimycotoxigenic agents.

The purpose of this study was to examine the effectiveness of the new synthesized analogues of DHA (DHT and BrDHT) for reduction of AFM_1 concentration in yoghurt contaminated artificially with this toxin in controlling parameters of storage.

Using different concentrations of DHT and BrDHT, it was found that BrDHT was the better candidate for the reduction of AFM_1 concentration in investigated yoghurt. AFM_1 levels in yoghurt samples showed a significant decrease ($p<0.01$) compared with those initially added to yoghurt. Application of BrDHT prior storage may be a potential means of AFM_1 accumulation in yoghurt contaminated with AFM_1. Studies are now being conducted to evaluate the BrDHT analogue as anti-AFM_1 agent.

Uklanjanje aflatoksina M_1 iz umjetno kontaminiranog jogurta upotrebom novosintetiziranih analoga dehidracetne kiseline

Sažetak

Dehidracetna kiselina (DHA) i njeni novosintetizirani analozi 4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (DHT) i 5-Bromo-4-hydroxy-3-(p-toluoyl)-6-(p-tolyl)-2H-pyrane-2-one (BrDHT) testirani su radi uklanjanja aflatoksina M_1 (AFM_1)

iz umjetno kontaminiranog jogurta s poznatim koncentracijama tog toksina, za određivanje moguće upotrebe tih kemijskih spojeva zbog kontrole nakupljanja AFM_1. Jogurt proizveden od kravljeg mlijeka umjetno je kontaminiran s AFM_1 koncentracije od 0,01 do 0,5 μg/L. Jogurti su skladišteni na 4 stupnja celzijusa, odnosno na 7 stupnjeva celzijusa tijekom 28 dana. Analiza AFM_1 u jogurtu načinjena je s pomoću dvodimenzionalne tankoslojne kromatografije (TLC) - vizualnom metodom. Granica detekcije iznosila je 0,15 ng/L. Dobivene vrijednosti AFM_1 iz uzoraka uzetih u količinama od 10, 50, 100 i 500 ng/L iznosile su između 80,6 odnosno 107,8 %. Koncentracije DHA i DHT od 0,01 do 0,03 μmol/L nisu nimalo ili su vrlo malo utjecale na sadržaj AFM_1 u eksperimentalno kontaminiranom jogurtu, dok su koncentracije veće od 0,05 μmol/L djelomično smanjile sadržaj AFM_1. Postotak gubitka početne količine AFM_1 u jogurtu, procijenjen je u iznosu od oko 15 i 25%, odnosno 22 do 45 % na kraju skladištenja. U pokusima s BrDHT u koncentracijama 0,01 i 0,05 μmol/L ili višim, koncentracija AFM_1 smanjena je, nakon 28 dana za 20 do 95 %, odnosno u potpunosti, u ovisnosti o vremenu i temperaturi skladištenja. Određivanje toksičnosti istraživanih analoga provedeno je s pomoću larvi račića *Artemia salina*, kao biološkog indikatora za određivanje njihove osjetljivosti na odabrane kemijske spojeve.

Ključne riječi: aflatoksin M_1, jogurt, analozi dehidracetne kiseline, TLC, *Artemia salina*

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