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IN VITRO EFFICACY OF MYCOTOXINS' ADSORPTION BY NATURAL MINERAL ADSORBENTS

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Abstract: The paper describes *in vitro* model for evaluation of natural mineral adsorbents ability to adsorb mycotoxins. Bentonite, diatomite and zeolite were prepared in the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade. A total of six mycotoxins: aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), diacetoxyscirpenol (DAS) and T-2 toxin were tested *in vitro*. For adsorption experiments crude extracts of mycotoxins, produced in the Department of Microbiology of Bio-ecological Center in Zrenjanin, were used. The ability for binding mycotoxins was evaluated in the electrolyte 0.1 M K_2HPO_4 which pH value was adjusted to 3.0 and 6.9, respectively. Mass ratio of individual mycotoxin and natural mineral adsorbent was 1:5000. The experimental mixtures were incubated for 1 hour on a rotary shaker (185 rpm) at room temperature (22-25^oC). After incubation the extractions of unadsorbed mycotoxins from the filtrates were performed with organic solvents, and their quantifications were done by thin-layer chromatography (TLC). By the use of TLC method it was noted that bentonite, diatomite and zeolite binded more than 95% of applicated AFL. In the case of OTA only diatomite adsorbed that toxin - adsorption index was 66.67%. Binding of DON has been observed only at pH 3.0 of electrolyte. Its adsorption index varied from 25.00 to 50.00% depending on the type of mineral adsorbent. Effect of electrolyte pH value on the binding of ZON was not so expressed. Its adsorption index ranged from 12.20 to 37.00%. In the case of type A trichothecenes (DAS and T-2 toxin) bentonite, diatomite, and zeolite binded only T-2 toxin. The amount of adsorbed T-2 toxin ranged from 16.66 to 33.33%. The obtained results point out the need for activation or processing of natural mineral adsorbens, especially bentonite and zeolite, in order to increase the efficiency of adsorption of the wider spectrum of mycotoxins.

Key words: mycotoxins, mineral adsorbents, *in vitro*

Introduction

Considerable investigations had been undertaken at finding methods for prevention of toxic effects of mycotoxins. One approach includes detoxification and inactivation of these fungal metabolites by the use of mycotoxin binders. The aim of these non nutritive additives is to inhibit the uptake of mycotoxins by an animal *in vivo*. These adsorbent materials are intended to act as "chemical sponges" that bound mycotoxins in gastrointestinal tract thus preventing the uptake and subsequent distribution to target animal organs. The efficacy of adsorption process is depending on the chemical structure of binding agent and the mycotoxin, respectively. The most important characteristics for adsorption is the physical structure of the adsorbent (the size of pores, accessibility of surface area, total charge and charge distribution) and properties of adsorbed mycotoxin (polarity, solubility, shape and charge distribution) *(Kollosova et al., 2009)*. Various natural materials had the potential to bind mycotoxins in feed.

Bentonite (BEN) is a hydrated aluminum silicate of volcanic origin. It consists of minerals from the smectite group, mostly of montmorillonite (50-90%); hectorite, saponit, beidelit and nontronit may also be present. The crystal structure of this mineral adsorbent is built of SiO_2 tetrahedrons and Al_2O_3 octahedrons, which are interconnected and build a three-layer plates with a negative charge, while the edges of the lamellae have positive charge. BEN contains interchangeable cations Na, K, Ca, Mg. In the presence of water lamellae are separated and volume is increased. In feed industry BEN is used in the pelleting process of feed mixtures. This mineral adsorbent increases the hardness and strength of pellets. It adsorbs some mycotoxins. Particularly is pronounced BEN's affinity towards aflatoxins (90-95%) and less for zearalenone and ochratoxin *(Huwig et al., 2001)*. Besides mycotoxins it binds and radionuclides, toxic metals and ammonia *(Adamović et al., 2009)*. Positive effects of pelleting and sodium bentonite utilisation in concentrate mixtures on cattle performances was shown by *Stojanović et al. (2008)*.

Diatomite (DIA) is a sediment formed in lacustrine and marine environments. It is composed of very small (from 0.01 to 0.4 mm) shells of silicon, unicellular algae (*Diatomeae*), whose number amounts to 10-30 millions in cm^3. In addition, these deposits contain the remains of sponges, *Radiolaria,* admixtures of clay, quartz etc. DIA has a small mass (0.5-0.8 g/cm^3) and high porosity. Due to the high content of silicon dioxide this mineral adsorbent has a large porosity, and thanks to that the high adsorption capacity. Among other things, DIA is used as a component for the production of certain mycotoxin adsorbents and for remediation of diarrhea in animals *(Whitlow, 2006; Živkovic et al. 2006)*.

Zeolite (ZEO) is a hydrated aluminosilicate of alkaline and alkaline earth metal ions, which possess an infinite three-dimensional crystal structure. Its main

ingredient is a mineral clinoptilolite (60-90%). ZEO is characterized by its ability to lose or receive water and change cations without major changes in the structure. Basic building unit of zeolite structure is a tetrahedron in whose center is an atom of silicon or aluminum, and at the top of tetrahedron there are oxygen atoms that are shared by two tetrahedra. Because of this mineral is rich in channels and cavities in one, two or three directions. Size of channels determine the possibility of sorption of different molecules. The cations placed in channels can be replaced with other metal ions. On that fact is based the ability of ZEO to, with greater or lesser success, adsorb specific mycotoxins on its negative charged surface *(Tomašević-Čanović et al. 2001)*. Besides that this mineral adsorbent can bind also radionuclides, toxic metals and ammonia *(Adamović et al., 2003; Vićentijević et al., 2006)* and its addittion has significant influence on all chemical parametrers of chemical composition and biochemical changes in whole maize plant silages *(Đorđević et al., 2006)*.

Materials and Methods

Mineral adsorbents. Bentonite was obtained from the site Šipovo, Republic of Bosnia and Herzegovina. Tested sample of diatomite originated from diatomite mine Kolubara - Lazarevac, location Baroševac, field "B", while zeolite was obtained from site Igroš, Kopaonik, Republic of Serbia. Samples of BEN and ZEO were prepared in semi-industrial conditions, while DIA in the laboratory conditions (the separation of impurities and grinding, without pretreatment) in the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade. Chemical composition of mineral adsorbents was determined by AAS Analyst device 300, and granulometric composition by Cyclosizer DM 10-0/44 device.

Production, quantification and isolation of mycotoxins. Aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZON) were produced employing solid state fermentation as per the methods of *Bočarov-Stančić et al. (2009a), Bočarov-Stančić et al. (2010)* and *Bočarov-Stančić et al. (2009b),* respectively. Type A trichothecenes (diacetoxyscirpenol - DAS and T-2 toxin) were biosynthesized by submerged fermentation in liquid medium *(Bočarov-Stančić et al., 2007)*. The respective fungal cultures used were: *Aspergillus flavus* GD-2 (leg. prof. dr G. Dimić, Technological Faculty, Novi Sad, R. Serbia), *A. ochraceus* CBS 108.08, *Fusarium graminearum* GZ-LES (leg. prof. dr J. Lević, Maize research institute, Belgrade-Zemun, R. Serbia), *F. graminearum* D2 (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, R. Serbia), *F. semitectum* SL-B (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, R. Serbia), and *F. sporotrichioides* ITM-391 (leg. dr A. Bottalico, Consiglio Nazionalle delle Richerce, Istituto Tossine a Micotossine da Parassiti Vegetali, Bari, Italy).

Isolations of mycotoxins and determinations of single mycotoxin content in solid substrates were done according to standard thin-layer chromatographic method for fodder analysis *(The Official Gazete of SFRJ, issue 15/87)*. Isolations of type A trichothecenes were done by ethyl acetate and their quantities were determined by thin-layer chromatographic (TLC) method according to *Rukmini and Bhat (1978)*. Isolated crude toxins were evaporated to dryness and dissolved in following solvents: ethanol (AFL, OTA, ZON), ethyl acetate (DAS, T-2) and methanol (DON). The final concentrations of stock mycotoxins' solutions were 0.1 μg/μl (AFL) and 1 μg/μl (OTA, DON, ZON, DAS and T-2), respectively.

Experimental procedure. For adsorption experiments stock solution of AFL was diluted to 0.2 μg/ml, of ZON to 0.8 μg/ml, and of all other mycotoxins to 2.0 μg/ml with electrolyte (0.1M K_2HPO_4). pH value of electrolyte was adjusted with 0.1M HCl or 0.1 NaOH to 3.0 and 6.9, respectively.The *in vitro* binding ability of BEN, DIA and ZEO was tested as follows: aliquots (50 ml) of test solutions were added to Erlenmayer flasks (250 ml) containing 500 mg of single adsorbent in the case of OTA, DON, DAS and T-2 toxin, 200 mg in the case of ZON, and 50 mg in the case of AFL. Controls were prepared by adding of 50 ml of test solutions without mineral adsorbent. The flasks were stoppered, incubated for 1 hour on rotary sheaker (185 rpm) at room temperature (22-25 ^oC) and then filtered. Mycotoxins' concentrations in 25 ml aliquots of electrolyte with adsorbent (C) and without it (C_o) were determined after extraction with 2 x 15 ml of organic solvents: benzene (ZON), benzene-acetonitrile (AFL), and ethyl acetate (OTA, DON, DAS and T-2) respectively by TLC methods *(The Official Gazette of SFRJ, issue 15/87*; *Rukmini and Bhat, 1978)*. All analysis were were performed in three replications. The adsorption index of individual mycotoxin in percentages was calculated by the following formula:

$$Adsorption\ index = [\frac{C_0 - C}{C_0}]\ x\ 100$$

Results and Discussion

The chemical composition and cation exchange capacity (CEC) of the employed mineral adsorbents is presented in Table 1. All three used adsorbents had a high content of SiO_2. (48.48 to 79.79%). The highest content of SiO_2 was found in DIA (derived from the shells of silicon unicellular *Diatomeae* algae) the adsorbent significantly different from the BEN and ZEO. On the other hand DIA had significantly lower content of other components, mainly Al_2O_3 CaO and MgO, and other cations, which has impacted on its' cation exchange capacity.

Table 1. The chemical composition of mineral adsorbens

Parameter (%)	Bentonite	Diatomite	Zeolite
SiO_2	48.48	79.79	65.69
Al_2O	22.39	9.41	14.03
CaO	5.86	0.63	3.57
Fe_2O_3	4.73	1.11	2.34
MgO	1.71	0.14	1.09
K_2O	0.40	0.79	1.39
Na_2O	0.07	0.08	1.41
TiO_2	0.34	0.21	0.17
Loss by ignition	16.02	7.84	10.29
CEC mEq/100 g	141.23	42.75	142.24

CEC: *cation exhance capacity*

Despite differences in chemical composition the common property of all investigated mineral adsorbents was their porosity, due to the existence of cavities and canals that spread in different directions, allowing them to exchange cations and thus to adsorb certain mycotoxins. The highest cation exchange capacity had ZEO (142.24 mEq/100 g), slightly lower BEN (14.23 mEq/100 g) and the lowest DIA (42.75 mEq/100 g).

Table 2. Grading composition of mineral adsorbents (particle size)

Bentonite		Diatomite		Zeolite	
Diameter (μm)	M (%)	Diameter (μm)	M (%)	Diameter (μm)	M (%)
63-61	12.60	63-48	1.30	63-53	3.50
61-46	2.0	48-36	0.90	53-40	6.80
46-32	3.50	36-25	0.65	40-28	11.83
32-21	2.70	25-16	0.55	28-18	13.63
21-15	3.50	16-12	1.15	18-13	15.20
<15	75.00	<12	95.45	<13	49.04
∑	100.00	∑	100.00	∑	100.00

M = mass of particles in % in the specified size range

The particle size of tested adsorbents (100%) was below 63 μm (Table 2). Smallest particles were found in DIA - 95.45% of mass was below 12 μm. The BEN had 75.00% of particle mass below 15 μm, while ZEO had 49.04 %f particle mass mass smaller than 13 μm. These data indicate that there exist, to some extent, the difference of distribution of particle sizes. In our following tests we are going to have in mind this detail and try to equalize the size of the particles of studied natural mineral adsorbents.

Aflatoxin B1. By the use of TLC method it was noted that the BEN, DIA and ZEO adsorbed more than 95% of applicated AFL (Table 3). Obtained result is

not surprising because it is known that surface of alumosilicate adsorbens (BEN and ZEO) when saturated with water atract polar functional groups of AFL and other polar mycotoxins *(Tomašević-Čanović et al., 2001; Kollosova et al., 2009)*. Unlike the results of *Thimm et al. (2001)* no influence of pH value of electrolyte on the binding of this mycotoxin was observed during present investigation.

Ochratoxin A. In the case of OTA only DIA adsorbed that toxin - adsorption index was 66.67% at pH 3.0 (Table 3). That most mineral adsorbents show higher adsorption indexes of OTA at pH 3.0 than at pH 6.5 was noted and by other authors *(Thimm et al., 2001)*. According to literature data *(Whitlow, 2006; Manafi et al., 2009)*, besides binding AFL and OTA, diatemaceous earth has the potential to adsorb *in vitro* and other mycotoxins (ZON, T-2 toxin and sterigmatosystin).

Deoxynivalenol. Adsorption index of DON varied from 25.00 to 50.00% depending on the type of mineral adsorbent (Table 3). *In vitro* binding of this fusariotoxin was observed only at pH 3.0. Contrary to our results other authors *(Döll et al., 2004; Sabater-Vilar et al., 2004)* found that most of the commercialy available mineral adsorbents were not able to bind DON in a appriciable percentage, while activated carbon was the best adsobent of this mycotoxin.

Zearalenone. Effect of electrolyte pH value on the adsorption of ZON was not so expressed (Table 3). Binding capacity of this mycotoxin ranged from 12.20 to 37.00%. And other investigators *(Bueno et al., 2005)* observed that bentonite can bind zearalenone to some extent, although ZON adsorption rates up to 100% have only been observed with organophylic bentonites *(Dakovic et al., 2001; Thimm et al., 2001; Stojšić et al., 2004)*.

Diacetoxyscirpenol. In tested *in vitro* conditions, natural mineral adsorbents (BEN, DIA, and ZEO) did not bind DAS. That there is litle or no beneficial effect against this type A trichothecene also showed the results of other authors *(Devegowda and Aravind, 2002)*.

Table 3. Adsorption indexes of tested natural mineral adsorbents at different pH values

Adsorbent	pH	Adsorption index of individual mycotoxins (%)					
		AFL	OTA	DON	ZON	DAS	T-2
Bentonite	3.0	96.90	0	50.00	37.00	0	25.00
	6.9	96.90	0	0	25.00	0	33.33
Diatomite	3.0	95.00	66.67	25.00	25.00	0	33.33
	6.9	95.00	0	0	25.00	0	33.33
Zeolite	3.0	95.50	0	50.00	12.20	0	0
	6.9	95.50	0	0	12.20	0	16.7

T-2 toxin. The amount of adsorbed T-2 toxin ranged from 16.66 to 33.33% (Table 3). With the exception of DIA, higher mycotoxin adsorption indexes were recorded at pH 6.9 of tested electrolyte. Simirar *in vitro* binding capacties were observed with commercial adsorbents – 33.3% Minazel and 31.3 % Minazel Plus *(Bočarov-Stančić et al., 2000a i b; Nešić et al., 2007).* In the case of three natural mineral adsorbents *Stojanović et al. (2008)* found that hectorite showed much higher adsorption index (95%) then BEN i ZEO.

In vivo experiments of *Carson and Smith (1983)* showed that 5% bentonite added to diet of laboratory animals most successfully overcame growth depression and feed refusal caused by T-2 toxin. Results of *Jaćević et al. (2007)* indicated that immune protection, in addition to previously established gut- and hepatic protection, significantly contributed to successful application against T-2 toxin sub acute intoxication in laboratory rats.

Conclusion

All tested mineral adsorbents showed the highest adsorption index *in vitro* for aflatoxin B1 (95.50-96.90%).

Only diatomite was efficient in binding ochratoxin A (66.67% at pH 3.0).

Deoxynivalenol was adsorbed only at pH value 3.0 by bentonite, diatomite and zeolite, respectively (50%, 25% and 50%, respectively).

Effect of electrolyte pH value on the binding of zearalenone was not much expressed (37%-12.20%).

None of the investigated natural mineral adsorbents bounded diacetoxyscirpenol.

The amount of adsorbed T-2 toxin ranged from 16.66 to 33.33%.

In tested laboratory conditiond the best mineral adsorbent was shown to be diatomite because it binded *in vitro* five (AFL, OTA, DON, ZON and T-2) out of six tested mycotoxins.

The obtained results point out the need for activation or processing of natural mineral adsorbents, especially bentonite and zeolite, in order to increase the efficiency of adsorption of the wider spectrum of mycotoxins.

Nonetheless *in vivo* experiments are indispensable to proof the efficacy of investigated natural mineral adsorbenrs (BEN, DIA and ZEO).

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In vitro efikasnost prirodnih mineralnih adsorbenata mikotoksina

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Rezime

U radu je prikazan *in vitro* model za evaluaciju sposobnosti za adsorpciju mikotoksina prirodnih mineralnih adsorbenata: bentonita, diatomita i zeolita pripremljenih u Institutu za tehnologiju nuklearnih i drugih mineralnih sirovina, Beograd. Testirano je ukupno šest mikotoksina: aflatoksin B1 (AFL), ohratoksin A (OTA), dezoksinivalenol (DON), zearalenon (ZON), diacetoksiscirpenol (DAS) i T-2 toksin. Za eksperimente adsorpcije su korišćeni sirovi ekstrakti mikotoksina, proizvedeni u Odeljenju mikrobiologije Bio-ekološkog centra, Zrenjanin.

Sposobnost za *in vitro* vezivanje mikotoksina je ocenjivana u elektrolitu 0,1 M K_2HPO_4 čija je pH vrednost podešena na 3,0 odnosno 6,9. Maseni odnos pojedinačnih mikotoksina i prirodnih mineralnih adsorbenasa je iznosio 1:5000. Eksperimentalne smeše su inkubirane tokom 1 sata na rotacionoj tresilici (185 o/min) i sobnoj temperaturi (22-25^{o}C). Nakon inkubacije vršena je ekstrakcija neadsorbovanih mikotoksina iz filtrata organskim rastvaračima i kvantifikacija istih metodom tankoslojne hromatografije.

Korišćenom metodom je konstatovano da su bentonit, diatomit i zeolit vezali više od 95% aplicirane količine AFL. U slučaju OTA samo je diatomit adsorbovao ovaj mikotoksin – indeks adsorpcije je bio 66,67%. Vezivanje DON-a je konstatovano samo pri pH vrednosti 3,0 elektrolita. Njegov indeks adsorpcije je varirao od 25,00-50,00% u zavisnosti od vrste mineralnog adsorbenta. Uticaj pH vrednosti elektrolita na vezivanje ZON-a nije bio izražen. Indeks adsorpcije ovog mikotoksina je iznosio od 12,20-37,00%. Od trihotecena tipa A (DAS i T-2 toksin) bentonit, diatomit i zeolit su vezivali samo T-2 toksin. Količina adsorbovanog T-2 toksina je bila od 16,66 do 33,33%. Dobijeni rezultati ukazuju na potrebu aktivacije ili oplemenjivanja prirodnih mineralnih adsorbenata, posebno bentonita i zeolita, u cilju povećanja efikasnosti adsorpcije šireg spektra mikotoksina.

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