

## Reduction of Ochratoxin A Levels in Red Wine by Bentonite, Modified Bentonites, and Chitosan

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Adsorption method may play an important role to remove ochratoxin A (OTA) from wine by bentonite (B), nonylammonium bentonite (NB), dodecylammonium bentonite (DB), KSF-montmorillonite (KSF), and chitosan bead (CB). The optimum conditions of OTA adsorption from synthetic solutions were revealed at room temperature and pH 3.5. The adsorption equilibria of B and NB were almost established within 120 and 240 min, respectively. DB, KSF, and CB had about 90 min of equilibration time. The adsorption efficiency carried out in the synthetic OTA solution did not change remarkably when the amounts of adsorbents were 25 mg for bentonite, DB, and KSF and 100 mg for NB and CB. Furthermore, 25 mg of adsorbents was used at all adsorption studies in synthetic solution. The adsorption isotherm was fitted with mostly a Freundlich equation with respect to the correlation coefficients. The adsorption data were evaluated using Langmuir and Freundlich equations having  $K_f$  values ranging from 0.011 to 9.5 with respect to correlation coefficients ( $R^2 = 0.900\text{--}0.977$ ). DB and KSF have the highest adsorption capacity for OTA in synthetic solutions. In wine, the removal of OTA was succeeded at a percentage of 60–100 by KSF and CB. Furthermore, the highest adsorption capacity of OTA for red wine was obtained by using 250 mg of KSF, which caused less damage to the nature of wine and also low adsorption of polyphenols and anthocyanins.

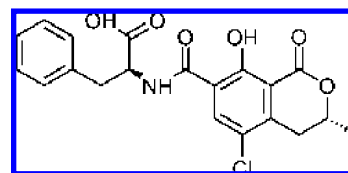
**KEYWORDS:** Ochratoxin A; red wine; adsorption; adsorbent

### INTRODUCTION

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families and are weak organic acids consisting of a derivative of an isocoumarin. The family of ochratoxins consists of three members, A, B, and C, which differ slightly from each other in chemical structures. However, ochratoxin A (OTA), *R-N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]phenylalanine, is the most abundant and hence the most commonly detected member but is also the most toxic of the three (Figure 1).

OTA can contaminate a wide variety of foods as a result of fungal infection in crops in the field during growth, at harvest, in storage, and in shipment under favorable environmental conditions, especially when they are not properly dried. OTA is especially found mainly in cereal and cereal products. Besides, OTA is also found in coffee, cocoa, wine, beer, pulses, spices, dried fruits, grape juice, pig kidney, and other meat and meat products of animals exposed to feedstuffs contaminated with this mycotoxin (1–6).

To remove mycotoxins from contaminated foods and feed, several technologies in food processing may play an important



**Figure 1.** Structure of OTA.

role for reducing the content of OTA in foods and beverages like physical, chemical, and microbiological methods, but few of these methods have practical applications (6–9). Huwig et al. discussed applications particularly concerning efficacy, specificity, and the mechanism of the adsorption process of activated charcoal, aluminosilicates, etc. for mycotoxin detoxification (10). The efficiencies of adsorbents having different origins like minerals such as zeolite, bentonite (B), and their modified ones, polymers such as chitin, chitosan, and their derivatives, and biological materials such as yeast have been investigated (11–17).

The efficacy of binding mycotoxins is dependent on the crystal structure and physical properties of the adsorbent as well as on the physical and chemical properties of the mycotoxins (11). In general, adsorption involves the accumulation of molecules from a solvent onto the exterior and interior (i.e., pore) surfaces of an adsorbent. The surface phenomenon is a manifestation of complex interactions (van der Waals, resonance,

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**Table 1.** Characterization of Clay Samples (22)

sample	BET surface area (m <sup>2</sup> /g)	d <sub>001</sub> (Å)	monolayer value (cm <sup>3</sup> /g)	% OC	% H	% N
B	73	10.030	16.67			
NB	50	13.690	11.54	6.99	1.25	0.58
DB	10	18.059	2.42	16.825	1.428	3.298

and electrostatic forces and hydrogen bonding) between the adsorbent, the adsorbate, and the solvent. To achieve adsorption, the interaction between OTA and adsorbent should be stronger than the one between OTA and solvent (12). OTA is a weak acid with a pK<sub>a</sub> value for the carboxyl group of the phenylalanine moiety of 4.4 (18). This demonstrates that OTA is partially dissociated at the pH of wine (ca. 3.5) and carries a negative charge that may interact with a positively charged surface. Additionally, OTA may also react by means of a phenol moiety and carboxylic group. The phenol group could be adsorbed onto a negatively charged surface through hydrogen bonding and/or charge transfer complexes (19). Moreover, adsorption of phenol onto a hydrophobic adsorbent (e.g., carbon) is the result of the interaction of two  $\pi$ -electron orbitals (20).

The objective of this research was to investigate the usability of selected adsorbents to reduce OTA levels in red wine. The selected adsorbents are natural B, nonylammonium bentonite (NB), dodecylammonium bentonite (DB), KSF-montmorillonite (KSF), and chitosan bead (CB). Our experiments were carried out in two parts. First, adsorption abilities for OTA using synthetic solutions were studied by controlling some parameters: screening contact times for each adsorbent at fixed dosages, effects of adsorbent amounts, and concentration effects from 1.5 to 500 ng/mL. The efficacy of adsorption was evaluated according to the Freundlich and Langmuir equations. Later, the reduction of OTA levels from red wine considering the effects of selected adsorbents was examined.

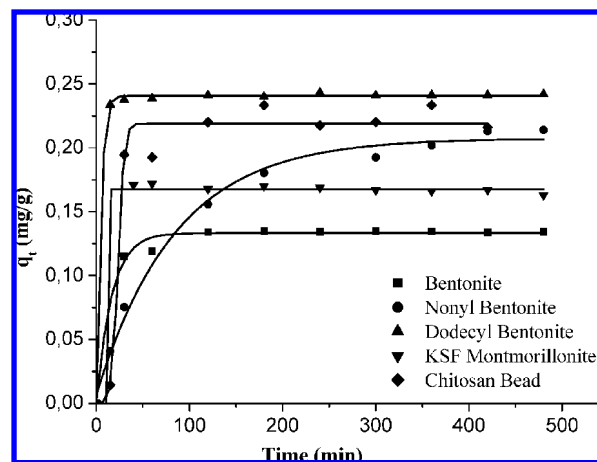
## MATERIALS AND METHODS

**Reagents.** The OTA standard was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were high-performance liquid chromatography (HPLC) grade. Methanol was from Panreac (Barcelona, Spain), acetic acid was from Carlo-Erba (Milano, Italy), acetonitrile was from Sigma-Aldrich, and ultrapure water was obtained from a Milli-Q apparatus (Millipore, Molsheim, France). OTA stock solution (1000 ng/mL) was prepared in methanol.

B was from a mining resource of Enez, Edirne that took place in the northwest part of the Marmara region in Turkey. The cation exchange capacity (CEC) equivalent of B was 97 mequiv/100 g clay. The BET surface area and pore size distribution of the samples were determined by gas phase adsorption of N<sub>2</sub> at 77 K. Adsorption data were evaluated according to the cited literature (21). The prepared and characterized modified clays by Seki (22), NB and DB, were used, and their characteristics are given in **Table 1**.

The chemical composition of KSF (Fluka, Buchs SG, Switzerland) was 55.0% SiO<sub>2</sub>, 18.0% Al<sub>2</sub>O<sub>3</sub>, 4.0% Fe<sub>2</sub>O<sub>3</sub>, 3.0% MgO, 3.0% CaO, <0.5% Na<sub>2</sub>O, 1.5% K<sub>2</sub>O, 5.0% sulfate, and 10.0% loss on ignition. KSF had a surface area of 20–40 m<sup>2</sup>/g. CBs were synthesized from low viscose chitosan (Fluka) as given in the literature (23). Red wine samples were purchased at a local supermarket and stored at +4 °C in the original bottles. The red wine samples were analyzed for their initial OTA content (2.71 ng/mL) and pH (3.52).

**Adsorption Studies for Synthetic OTA Solutions.** Twenty-five milliliters of synthetic solutions containing 1.5–500 ng OTA mL<sup>-1</sup> (pH 3.5, with 0.1 M HCl) was added to 25 mg of each adsorbent in 50 mL glass bottles. These test bottles were placed in a temperature-controlled shaker at room temperature until reaching equilibrium. The contact times of B, NB, DB, KSF, and CB were fixed at 120, 240, 90,



**Figure 2.** Effect of contact time for the adsorption of OTA in synthetic solution at pH 3.5 ( $c_{\text{int}} = 250 \text{ ng mL}^{-1}$ ).

90, and 90 min, respectively. Then, each test solution was centrifuged at 4000 rpm for 15 min. One milliliter of each supernatant was used for OTA analysis.

**Adsorption Studies for Red Wine.** To different amounts (250, 500, and 1000 mg) of each adsorbent, 25 mL of red wine sample was added. The adsorption procedure given before was applied completely. The supernatant of wine sample was analyzed for its OTA concentration, total polyphenol, and total anthocyan content. The percentage of removed OTA was calculated from the difference between the initial and the final concentration of OTA in the aqueous supernatant after equilibrium. Total polyphenol and total anthocyan contents were determined by using Shimadzu UV 1601 (Kyoto, Japan) series spectrophotometric system at 280 and 520 nm, respectively (24).

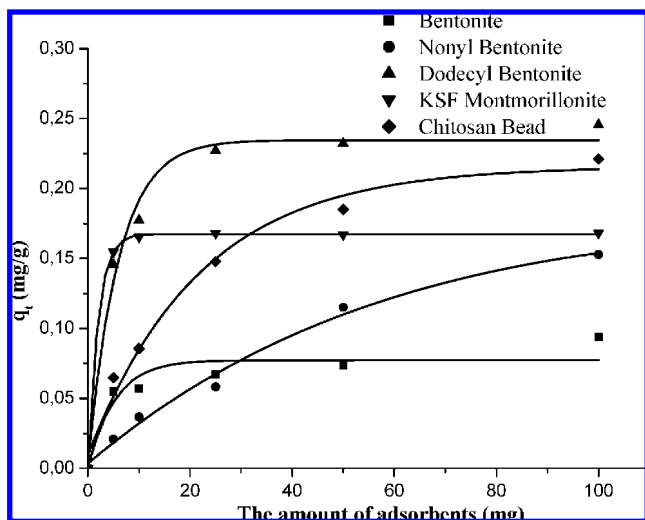
**OTA Analysis.** HPLC analyses were performed on an Agilent Technologies 1100 (Heilbron, Germany) series liquid chromatographic system equipped with a fluorescence at 333 (excitation) and 458 nm (emission), controlled by Chemstation 3D software. The chromatographic conditions were as follows: C<sub>18</sub> reverse phase (250 mm  $\times$  4 mm, 5  $\mu$ m) HPLC analytical column; volume of injection, 20  $\mu$ L loop; isocratic elution (water/acetonitrile/acetic acid, 48.5:50.5:1, v/v/v); flow rate, 1.5 mL/min; and temperature, 50 °C. An aliquot of the original OTA test solution was used as the HPLC standard.

In synthetic solutions, the calibration curve of OTA ranged from 1.0 to 10.0 ng/mL with an equation of  $y = 0.2152x + 0.0975$  with the regression coefficient  $R^2 = 0.9995$ . The direct injection method was applied to the analysis of the red wine sample (25). The supernatant containing OTA in adsorption experiments was collected in a vial, evaporated to dryness under an N<sub>2</sub> stream, and redissolved in the HPLC mobile phase. The limit of detection for OTA was 0.26 ng/mL; the limit of quantification was 1 ng/mL; the reproducibility of interday and intraday for 3.0 ng/mL was 3.44 and 5.22%, respectively.

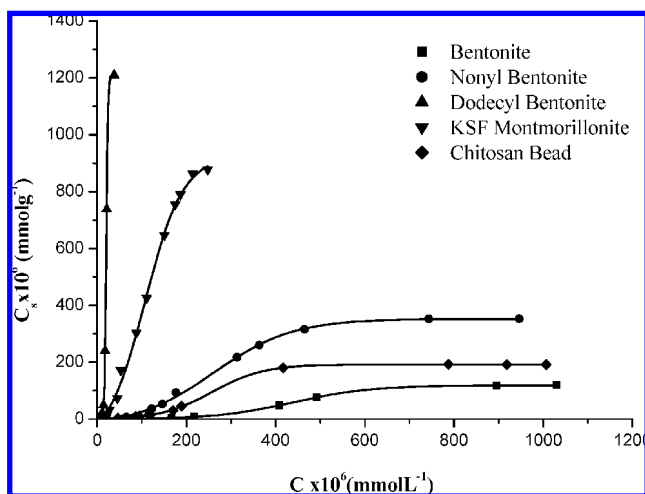
## RESULTS AND DISCUSSION

**Effect of Contact Time for OTA Adsorption onto Each Adsorbent.** By plotting the amount of OTA adsorbed ( $q_t$ , mg g<sup>-1</sup>) vs time (min), the optimum contact times for adsorption of OTA onto each adsorbent were determined (**Figure 2**). To determine the contact time of adsorbents, the adsorption experiments were performed with 250 ng/mL OTA synthetic solutions at various time intervals for 480 min. It indicates that the adsorption equilibria of B, NB, DB, KSF, and CB are almost established within 120, 240, 90, 90, and 90 min, respectively.

**Effect of Adsorbent Amount on the Adsorption of OTA.** The effect of adsorbent amount was shown by plotting the amount of OTA adsorbed ( $q_t$ , mg g<sup>-1</sup>) vs the amount of adsorbents (mg) (**Figure 3**). To get the optimum amount of adsorbent, 5–100 mg of each adsorbent was tested. The amount



**Figure 3.** Effect of adsorbent amount for the adsorption of OTA in synthetic solution at pH 3.5 ( $C_{\text{int}} = 250 \text{ ng mL}^{-1}$ ).



**Figure 4.** Adsorption isotherms of OTA in synthetic solution onto B, NB, DB, KSF, and CB at room temperature.

of OTA adsorbed by the adsorbents increased abruptly up to 25 mg of adsorbent. Above this, B, DB, and KSF clays showed little change in the amount of adsorption. The adsorption percentage of NB and CB clays increased by increasing the amount of adsorbent, but it could be said that they might reach a plateau around 100 mg of adsorbent.

**Adsorption Isotherms.** To reveal the effect of concentration of synthetic OTA working solutions onto adsorption, the concentrations were arranged as 1.5, 3, 5, 10, 25, 50, 100, 125, 250, and 500 ng/mL ( $3.7 \times 10^{-6}$  to  $123.8 \times 10^{-5}$  mmol/L), and these solutions were added to 25 mg of each adsorbent till contact times.

The adsorption isotherms of OTA for adsorbents were plotted as the amount adsorbed ( $C_s$ , mmol g $^{-1}$ ) as a function of equilibrium OTA concentration ( $C_e$ , mmol L $^{-1}$ ) and are shown in **Figure 4**. The level of OTA adsorbed increased with increasing dosage. To optimize the design of an adsorption OTA on each adsorbent, it is quite important to establish the most appropriate correlation for the equilibrium curves. Langmuir and Freundlich adsorption equilibrium models were used to describe the equilibria between OTA and each adsorbent,  $C_e$  at low ( $0.96 \times 10^{-5}$  to  $9.4 \times 10^{-5}$  mmol L $^{-1}$ ) and high ( $10.0 \times 10^{-5}$  to  $103 \times 10^{-5}$  mmol L $^{-1}$ ) concentration ranges.

The Langmuir isotherm can be written as  $C_e/C_s = 1/LC_m + C_e/C_m$ , where  $C_s$  is the amount of OTA adsorbed onto adsorbents (mmol g $^{-1}$ ) at equilibrium,  $C_e$  is the equilibrium OTA concentration in solution after adsorption,  $C_m$  is the maximal amount of OTA adsorbed per unit weight of adsorbents (mmol g $^{-1}$ ), and  $L$  is the Langmuir constant.  $C_m$  and  $L$  values were estimated by plotting the Langmuir equation (27). These values are summarized in **Table 2**. In low  $C_e$ , the Langmuir equation did not fit the experimental data because the monolayer adsorption capacities and Langmuir constants are negative values. However, for high  $C_e$ , the adsorbents showed a similar high correlation except for KSF and DB.

The Freundlich isotherm has the general form in the equation as  $C_s = K_f C_e^{n_f}$  where  $K_f$  and  $n_f$  are related sorption capacity (mmol g $^{-1}$ ) and intensity of adsorption, respectively (28). The Freundlich adsorption model assumes that the adsorption occurs on the heterogeneous surfaces. The isotherm constants are very useful parameters for predicting sorption capacity and designing batch reactors. To calculate the Freundlich parameters, the linear form of the Freundlich equation ( $\ln C_s = \ln K_f + n_f \ln C_e$ ) is used. The constants of  $K_f$  and  $n_f$  were determined by plotting  $\ln C_s$  vs  $\ln C_e$ . Freundlich constants are shown in **Table 2** for high  $C_e$  and low  $C_e$  for only B. The Freundlich model fitted the experimental data of OTA adsorption by adsorbents at high  $C_e$  reasonably well. Additionally, B also has adsorption capacities for low  $C_e$ . The adsorption capacities of DB and KSF are higher than others. The affinity of adsorbents except NB showed similarity. At high  $C_e$ , the adsorption capacities of B and modified B clays, NB and DB, increased in the order given.  $K_f$  values increase in the order B, NB, CB, DB, and KSF. The Freundlich model is characterized by the heterogeneous surfaces with nonenergetically equivalent sites. At high  $C_e$ , correlation coefficient values ( $R^2$ ) of Langmuir equation were low as compared with  $R^2$  values of the Freundlich equation except for CB. It is concluded that the adsorption that occurred on the adsorbents is heterogeneous.

Additionally, the adsorption experiments were carried out for red wine. Initially, 0.025 g of each adsorbent was used, but the interference effect of the wine organic components was observed. Therefore, 0.25, 0.5, and 1.0 g of each adsorbent was tested (**Table 3**). It is clear that the higher amounts of adsorbents removed more OTA from the red wine. The adsorption percentage of OTA from wine increased in the order of B, DB, NB, CB, and KSF. Generally, it has been known that clay minerals have high adsorption capacities for organic and inorganic compounds. Therefore, higher amounts of adsorbents are required for wine having polyphenols and anthocyanins. Robert et al. (29) found that malic and tartaric acids are adsorbed at acidic pH by active carbon. Additionally, the polyphenols interfere with the adsorption of OTA by adsorbents (30–32).

From **Table 4**, 500 and 1000 mg of KSF and CB are of high adsorption performance for total polyphenols and total anthocyanins. Otherwise B and NB indicated high adsorption performance for only total anthocyan. DB has a low adsorption affinity for both total polyphenols and total anthocyan. It can be concluded that only KSF (250 mg) adsorbed a high amount of OTA without removing total polyphenols and total anthocyan from red wine. It is concluded that the best adsorbent is DB for synthetic OTA solutions and KSF for red wine.

Natural B is a layered aluminum silicate with a negatively charged surface. However, the removal of OTA is poor. The adsorption might be performed by means of positive terminal groups of B or cation exchange mechanism due to the exchangeable cations between the layers. For red wine, the

**Table 2.** Sorption Parameters of Langmuir and Freundlich Equations for OTA Adsorption from Synthetic Solutions onto Adsorbents

adsorbents	high $C_e$ (mmol L <sup>-1</sup> )					
	Langmuir parameters			Freundlich parameters		
	$R^2$	$C_m$ (mmol g <sup>-1</sup> )	$L$	$R^2$	$K_f$ (mmol g <sup>-1</sup> )	$n_f$
B (low)				0.960 ( $N=3$ )	0.36	1.29
B (high)	0.917 ( $N=3$ )	$2.66 \times 10^{-4}$	826	0.969 ( $N=3$ )	0.011	0.65
NB	0.960 ( $N=5$ )	$4.89 \times 10^{-4}$	3083	0.939 ( $N=4$ )	0.027	0.43
DB	0.797 ( $N=4$ )	$14.7 \times 10^{-4}$	122300	0.901 ( $N=4$ )	7.25	0.85
KSF	0.14 ( $N=6$ )	$63 \times 10^{-4}$	727	0.977 ( $N=5$ )	9.50	1.09
CB	0.999 ( $N=4$ )	$2.02 \times 10^{-4}$	1130	0.900 ( $N=4$ )	0.23	1.01

**Table 3.** Comparison of Adsorption Efficiency between Red Wine and Synthetic OTA Samples

type of adsorbent	active area (m <sup>2</sup> /g)	adsorption (%)							
		amount of adsorbent (mg)							
		red wine (2.57 ng/mL)				synthetic OTA (250 ng/mL)			
		250	500	1000	5	10	25	50	100
B	73	0	26	44	19	18	22	24	31
NB	50	22	69	74	20	19	14	60	79
DB	10	14	42	43	90	96	98	97	98
KSF	20–40 (26)	67	82	100	52	63	80	79	87
CB		59	78	100	30	41	48	51	72

**Table 4.** Decrease of Wine Parameters in OTA Adsorption for the Studied Adsorbents

type of adsorbent	adsorption (%)					
	amount of adsorbent (mg)					
	total polyphenols, 280 nm			total anthocyan, 320 nm		
	250	500	1000	250	500	1000
B	1	5	9	24	100	100
NB	12	22	30	32	100	100
DB	18	31	46	24	24	24
KSF	4	100	100	10	100	100
CB	63	100	100	69	100	100

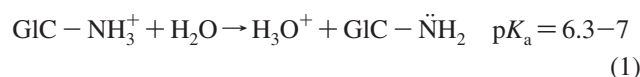
adsorption of OTA onto B is affected by the interference of other components of wine for the sorption. The removal of OTA from wine is nearly 26% at 0.5 g of B.

Modification of clay with nonylammonium chloride and dodecylammonium chloride has an advantage to increase the hydrophobic interactions on adsorption of OTA from aqueous solution. By increasing the amount of carbon for modified Bs, the adsorption performance for removing OTA was improved. With respect to  $K_f$  values obtained from the Freundlich equation, the adsorption capacities of DB are higher than NB. The adsorption of OTA from red wine is influenced by the interference of polyphenols, anthocyanins, and organic acids. Hence, NB might be a better adsorbent for the removal of OTA from the red wine sample than DB in this present study.

Montmorillonite is the main constituent of B, which is a layered aluminum silicate mineral with a negatively charged surface and which has a high surface area that supplies great sorption capacity. Montmorillonite KSF is acid-activated clay that has a pH value of 3.31 in an aqueous solution (33). Because the protonated form of clay demonstrates a partially positive charge, the negative moiety of OTA might be pulled by protonated regions and also the interaction might be possible between the  $\pi$ -electron orbital of OH moiety of OTA and the partially negative surface of KSF. For synthetic solutions, KSF has the highest affinity for the adsorption of OTA according to

the Freundlich results. The removing percentage of OTA for red wine is nearly 67% at 0.25 g of KSF in which interference effects of polyphenols and anthocyan were minimal. As apparently seen, KSF is a fine adsorbent for the removal of OTA from both synthetic OTA solutions and red wine.

Chitosan contains  $-OH$  and  $-NH_2$  groups that can give rise to hydrogen bonding (34). Chitosan has a positive value in acidic conditions.



The amino groups of chitosan are protonated at low pH values. OTA adsorbed onto Chitosan through the negative charge on the carboxyl group of its phenylalanine moiety. It is clearly known that it is impossible the complete removal of any matter using any adsorbents but in this study the removal percentage of OTA from red wine was about 100% for 1000 mg of KSF and CB. Results showed that the functional groups and the different shapes of OTA could have influence on the adsorption of organically modified clays, as well as chitosan.

$K_f$  values of adsorbents increase in the order of B, NB, CB, DB, and KSF. The  $K_f$  values of Bs increased depending on the modification with organic groups. KSF has the highest value for  $K_f$ , and it shows the highest affinity for OTA for synthetic solutions (Table 2). Removal of OTA by adsorbents in red wine increased in the order of B, DB, NB, CB, and KSF. In red wine, the higher amount of adsorbent was required due to the adsorption of total polyphenols and anthocyanins.

In a literature survey,  $K_f$  values of DB and KSF were higher than the yeast samples used by Ringot et al. (14). The reduction level of OTA in this work was similar with the work done by Belajova et al. using active carbon and modified silica gel from beer (13) and by Tomasevic-Canovic et al. and Dakovic et al. using organozeolites (11, 15). In real wine, the decreased amount of OTA was approximately same with the obtained by active carbon (12) and B (17). This study will give route to new researches interested in the removal of mycotoxins from contaminated foods, feed, and drinks using organo-modified mineral and organic polymer adsorbents.

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Received for review November 22, 2007. Revised manuscript received January 24, 2008. Accepted February 6, 2008.

JF073419I