Removal of Ochratoxin A in Red Wines by Means of Adsorption Treatments with Commercial Fining Agents

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The presence in wine of the fungal metabolite, ochratoxin A (OTA), represents a serious risk for consumer health. A variety of fining agents, including activated carbon, silica gel, potassium caseinate, egg albumin, and gelatin, was evaluated in relation to their abilities to remove OTA in fortified wines. Freundlich adsorption isotherms were used to model the adsorption behavior between ochratoxin A and the fining agent. Potassium caseinate and activated carbon were found to be the best fining agents that could be used to remove OTA in wine. Potassium caseinate removed up to 82% of OTA when used at 150 g/hL, whereas activated carbon showed the highest specific adsorption capacity due to a high surface area per mass and low adsorption of total polyphenols.

Keywords: *Adsorption; fining; isotherm; ochratoxin A; red wine*

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin associated with outbreaks of renal toxic diseases, and it is classified as a *possible* human carcinogen.[1] Its chemical structure consists of a chlorine-containing dihydroisocoumarin linked through the 7-carboxyl group to l-*β*-phenylalanine (Figure 1). It is produced by several ubiquitous species of *Aspergillus* and *Penicillium* molds that contaminate agricultural commodities either before harvest or during storage.[2,3] OTA and some of its toxic hydroxy derivatives have been frequently found in human milk, blood serum, and plasma,[4−6] the major source of contamination coming from the consumption of contaminated cereals, coffee, beer, and wine.[7−17] In particular, commercial wines with a high level of contamination may increase the total daily intake of OTA, especially in high wine-consuming countries. On the basis of limited data, the Codex Alimentarius Commission[18] estimates that 15% of the total intake of OTA is due to wine consumption.

The use of appropriate technology in food processing may play an important role for reducing the content of OTA in food and beverages. Several physical, chemical, and microbiological methods have been proposed to remove mycotoxins from contaminated foods and feed, but few of these methods have practical application.[19−21] For example, the use of microorganisms may affect the sensorial quality of the wine. Fining, a common winery practice, involves the addition of an adsorptive compound to reduce levels of certain compounds from wine. Enological fining agents have been shown to reduce the OTA level in wine during the ordinary clarification practice.[22] Activated carbon has a good capacity to absorb OTA in model solution,[23] whereas bentonite has demonstrated a low affinity for OTA.[24]

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, and electrostatic forces and hydrogen bonding) between the adsorbent, adsorbate, and the solvent. To achieve adsorption, the interaction between OTA and adsorbent should be stronger than the one between OTA and solvent. The molecular size and the physicochemical properties of OTA clearly affect the efficiency of the binding action. OTA is a weak acid with a pK_a value for the carboxyl group of the phenylalanine moiety of 4.4.[25] This implies 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. In addition, OTA may also react by means of phenol moiety and carboxylic group. The phenol group could be adsorbed onto a negatively charged surface through hydrogen bonding and/or charge-transfer complexes.[26] Moreover, adsorption of phenol onto hydrophobic adsorbent (e.g., carbon) is the result of the interaction of two π-electron orbital.[27] Among many relationships used to characterize the solid−liquid adsorption systems, the Freundlich model[28] is purely empirical, and since it is widely used to describe monolayer adsorption thanks to its simplicity and versatility it assumes an infinite number of adsorption sites.

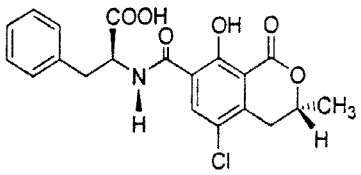

Figure 1. Structure of ochratoxin A (OTA).

This research aim fundamentally at examining the suitability of fining treatments to reduce the OTA levels of red wine. With this aim, two experiments were carried out: (a) a preliminary screening by using 18 enological fining agents tested at fixed dosage; (b) a fining agent concentration effect on removal of OTA from red wine by using six selected products. The

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Table 1. Relative Amount of OTA (%) Adsorbed from Wine by the Fining Agents

fining agent (charge)	dosage (g/L)	active area (m^2/g)	OTA adsorption (%) red wine #1 (OTA 3.78 ng/mL)	OTA adsorption (%) red wine #2 (OTA 1.50 ng/mL)
Inorganic				
1. bentonite (− +)[d]	1.00	≤50[a]	8	8
2. kaolinite	1.00		<1	<1
3. carbon A (hydrophobic)[1]	0.05	500−1500[b]	61	48
4. carbon B (hydrophobic)[2]	0.05	500−1500[b]	56	35
5. carbon C (hydrophobic)[3]	0.05	500−1500[b]	32	28
6. celite	1.00		<1	<1
7. KFeCN (−)[4]	0.10		7	8
8. PVPP (hydrophilic)[5e]	1.00		6	<1
9. silica gel A (+)[6]	1.00	175−450[c]	34	18
10. silica gel B (−)[7]	1.00	175−450[c]	7	11
11. silica gel C (−)[7]	1.00	175−450[c]	3	1
12. zeolite	1.00		2	5
Organic				
13. cellulose (−)[8]	1.00		<1	<1
14. carrageenan	1.00		5	9
15. egg albumin (+)	1.00		40	42
16. gelatin (+)[f]	1.00		30	17
17. pectin (−)[g]	1.00		3	9
18. potassium caseinate (+)[h]	1.00		35	24

[a] $ZnCl_2$ activated. [b] H_3PO_4 activated. [c] Steam activation. [d] Potassium-ferrocyanide. [e] Polyvinylpolypyrrolidone. [f] $AlCl_3$ activated. [g] OH. [h] Microcrystalline. [i] Manfredini (*43*). [j] Manfredini (*44*). [k] Manfredini (*45*). [l] Main and Morris (*35*). [m] Doner et al. (*46*). [n] Paetzold and Glories (*47*). [o] Rinaudo (*48*). [p] Manfredini (*49*).

efficacy of absorption was evaluated according to the Freundlich equation, and the effect of fining treatments on the red wines composition is discussed.

MATERIALS AND METHODS

Wines. Two unclarified red wines were provided by the Experimental Winemaking Station of the University of Bologna (Italy). The wines were analyzed for their initial OTA content (3.78 and 1.50 ng/mL), pH (3.47 and 3.56), total polyphenols (2.87 and 3.29 g/L), pigment polymeric content (3.41 and 4.12), and optical density at 420 nm (3.10 and 3.91) and 520 nm (4.75 and 5.70).

Fining Agents and Clarification Experiments. Eighteen enological fining agents (Table 1) were purchased in specialized stores and added with bidistilled water as recommended by the producer. Two clarification experiments were performed: (a) a preliminary screening by using all the fining agents at fixed dosage and (b) the evaluation of selected fining agents at various dosages (adsorption isotherm). The single fining agents were tested at dosages commonly used in the current winemaking practice. Aliquots of each red wine (50 mL) were poured in cap-vials and added with a single fining agent. Wines were mixed, kept in the dark at +4 °C for 12 h, and then centrifuged at 2900g × 15 min. The supernatant was collected and analyzed for its OTA concentration, total polyphenols,[29] pigment polymeric content, and optical density at 420 and 520 nm.[30] The percentage of removed OTA was calculated both on the basis of the initial OTA concentration and accounting for the dilution effect caused by the addition of the fining agents.

OTA Analysis. Sample cleanup with immunoaffinity column (OchraTest, Vicam Science Technology, MA) and OTA quantification in wines were carried out as previously described.[13] Briefly, the immunoaffinity column was washed with 5 mL of phosphate-buffered saline at pH 7.4, then 4 mL of wine, adjusted to pH 7.8 using 2 M NaOH, were diluted with 10 mL of buffer and directly loaded to the column. After washing with 10 mL of PBS, the column was dried under a gentle stream of air. OTA was eluted by passing 2 mL of methanol through the column. The eluate containing OTA was collected and mixed with 2 mL of mobile phase before the HPLC analysis by using a Jasco system (Tokyo, Japan). The chromatographic conditions were as follows: column Inertsil RP-ODS-2 (GL Science, Tokyo, Japan); isocratic elution (water/acetonitrile/acetic acid, 49:49:2, v/v/v); flow rate 0.75 mL/min (Jasco PU-980); temperature 35 °C; volume of injection 100 μL loop (valve 7725, Rheodyne, Cotati, CA); fluorescence detection with $\lambda_{ex} = 333$ nm and $\lambda_{em} = 460$ nm (Jasco FP-1520). Data acquisition and handling were made with the Borwin 1.5 software (JMBS Developments, Grenoble, France). The recovery of purification was 90%, and the precision of the method obtained on five replicates was 2.0%.

Statistical Analysis. The adsorption isotherms describe the relationships between the equilibrium concentration of OTA in the adsorbent and in red wine by varying the adsorbent/wine ratios. The Freundlich adsorption isotherm[28] may be written as: $x/m = K_F C^{(1/n)}$, where K_F represents the adsorptive capacity of the adsorbent, 1/n indicates the intensity of adsorption, x/m is the amount of solute that is adsorbed per mass of adsorbent (mg/g), and C is the concentration of the solute in the solution that is in equilibrium with the adsorbent (mg/L). The constant K_F and (1/n) were determined from a log−log plot of (x/m) versus C using linear regression (Statistica 5.1, StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

Preliminary Screening. Red wine may contain a high level of OTA ranging between 0.01 and 7.60 ng/mL.[31] Table 1 shows the result of the preliminary screening obtained by using all the fining agents at fixed dosages. Hydrophobic and positively charged fining agents as activated carbons, silica gel positively charged, egg albumin, gelatin, and potassium caseinate were the most effective in reducing the OTA content in red wines. On the basis of these observations, it is postulated that OTA was mainly adsorbed onto the fining agents by means of the negative charge existing on the carboxyl group of its phenylalanine moiety.

Activated carbon was an effective adsorbent having a high surface area per unit mass and its adsorption ability varied depending on the activation process. The chemically activated carbon has an irregular surface as compared to the steam activated carbon.[32] Therefore, the former carbon has highest adsorption surface to bind compounds from the media. The OTA-to-carbon ratio in wine 1 and 2 was 70 and 30 ng of OTA/mg of carbon,

respectively. Rotter et al.[33] using 3 μg of OTA/mg of carbon, reported 90% adsorption ability. This confirms the high affinity between carbon and OTA and suggests the presence of interference compounds in red wine. It is well-known that carbon also removes anthocyanins and other colored polyphenols from wine. Moreover, Robert et al.[34] found that malic and tartaric acids are adsorbed at acidic pH by active carbon, the adsorbed amount being between the range 30−290 μg of acid/mg of carbon.

As expected the silica gel positively charged showed a good affinity versus OTA, whereas the silica gel negatively charged was less effective. In fact, OTA is a weak acid partially dissociated at the pH of wine and carries a negative charge. Silica gels and sols are commonly used to remove haze-active proteins in wine and beer.

Bentonite, a layered aluminum silicate with a negative charged surface showed a relative efficiency (0.16% OTA adsorbed/m^2/g) as compared to carbons (61/1500 = 0.04% OTA adsorbed/m^2/g) and silica gel (34/450 = 0.07% OTA adsorbed/m^2/g). However, the total OTA removed was poor (8% adsorption) when compared to the most active fining agents. As bentonite carries also polar terminal regions which are locally positive,[35] molecules with negative charge may be adsorbed by a hydrogen bonding mechanism. Moreover, we formulated the hypothesis that OTA is adsorbed within the interlaminar spaces of bentonite by a cation exchange mechanism. As wine proteins are usually adsorbed by bentonite, they may interfere with the removal of OTA from wine.

Egg albumin (isoelectric point, pI 4.9), gelatin (pI > 7.4), and potassium caseinate (pI 4.5), which are proteins positively charged at the pH of wine, showed a good affinity for OTA. In particular, gelatin was able to remove up to 30% OTA from wine. According to previous findings[36,37] and in agreement with the literature,[38,39,49] we formulated the hypothesis that wine polyphenols interfere with the adsorption of OTA by fining agents. In particular, gelatin may interact with the negatively charged polymers (e.g., tannins) through hydrogen and ionic bonding, and also through hydrophobic interactions.[40,41] These findings suggest that the interaction between OTA and fining agents is regulated by complexities that for the time being have been only partially considered. Further studies are needed to clarify the mechanism of the binding process and to improve the adsorption performance for removing OTA.

Adsorption Isotherms. To study the adsorption isotherm of OTA from wine, the most active fining agents were selected, including carbons activated by H_3PO_4 and by $ZnCl_2$, silica gel positively charged, egg albumin, gelatin, and potassium caseinate. Activated carbons were tested in the range 1−10 g/hL, whereas the other fining agents in the range 25−150 g/hL. Figure 2 shows the percentage of OTA remaining in wine as a function of the fining agent dosage. The OTA level of wine decreased with the increase dosage, the silica gel being able to remove up to 82% of OTA. Activated carbons confirmed their great affinity for OTA. The exponential relationship for the adsorption of OTA from wine suggested the presence of specific binding on definite type of site and their gradual saturation, the complete removal of OTA from wine being theoretically impossible to achieve. Dumeau and Trioné[22] have recently been able to remove up to 91%

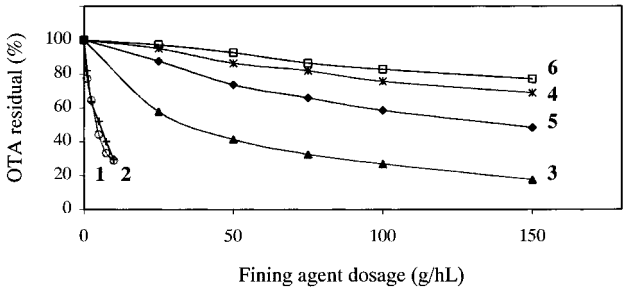

Figure 2. Influence of fining agent dosages on the adsorption of OTA from red wine. Legend: (1) carbon activated by H_3PO_4; (2) carbon activated by $ZnCl_2$; (3) silica gel positively charged; (4) potassium caseinate; (5) egg albumin; and (6) gelatin.

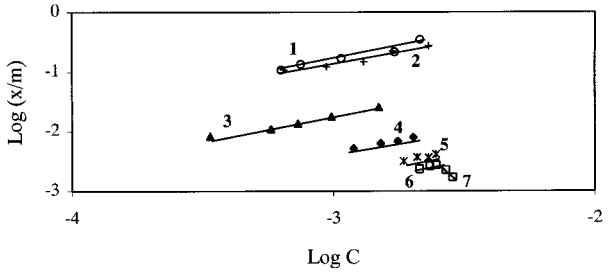

Figure 3. Freundlich isotherm for the adsorption of OTA from red wine by selected fining agents. Legend as reported in Table 2.

Table 2. Freundlich Isotherm Constants for the Adsorption of OTA from Red Wine by Selected Fining Agents

	Freundlich constant di ($x/m = K_F C^{1/n}$)		
fining agent	K_F	$1/n$	r^2
1. carbon H_3PO_4	126	1.03	0.944 ($N^a = 5$)
2. carbon $ZnCl_2$	76	0.98	0.937 ($N = 5$)
3. silica gel A (+)	8.7	0.96	0.983 ($N = 5$)
4. potassium caseinate	2.7	1.13	0.859 ($N = 4$)
5. egg albumin	2.5	0.98	0.977 ($N = 4$)
6. gelatin (1)	6.3	1.35	0.992 ($N = 3$)
7. gelatin (2)		−3.43	0.990 ($N = 3$)

[a] N= number of data points.

OTA from red wine by using a combination of gelatin, silica gel, and activated carbon.

The Freundlich model fitted the experimental data of OTA adsorption by fining agents reasonably well ($r^2 \geq 0.859$), the only exception being gelatin (Figure 3). To explain the inflection found in the isotherm of gelatin which indicated a decrease of OTA adsorption at high gelatin dosage, two hypotheses were formulated: (i) a *surcollage* of gelatin at high concentration; and (ii) different adsorption mechanisms (e.g., physical, chemical, multilayer) depend on the adsorbent concentration. The adsorption constants of gelatin showed a high experimental error due to the limited number of points ($N = 3$). Active carbon was the most efficient adsorbent (Table 2) with a high specific adsorption capacity (K_F value). This result confirmed the preliminary findings and may be attributed both to the high adsorption surface of carbon and to the limited interference from the wine compounds. Despite their different adsorption capacities, the fining agents showed a similar affinity for OTA as indicated by the values of the isotherm slope ($1/n$).

The hypotheses previously formulated of the interference of the red wine components on the OTA adsorption were verified by measuring the following wine param-

Table 3. Correlation between Adsorption of OTA and Decrease of Wine Parameters for the Selected Fining Agents

	wine parameters				
	total polyphenol	polymeric pigment	D.O. 420	D.O. 520	fining agent
OTA	−0.19	0.98	0.98	0.99	carbon H_3PO_4
OTA	−0.17	0.98	0.97	0.96	carbon $ZnCl_2$
OTA	0.58	0.58	0.84	−0.26	silica gel A (+)
OTA	0.94	0.97	0.98	0.98	potassium caseinate
OTA	0.97	1.00	0.97	0.99	egg albumin
OTA	1.00	0.98	0.98	0.99	gelatin

eters: total and colored polyphenols, optical density at 420 and 520 nm. Table 3 shows the results of the correlation between OTA adsorption and decrease of selected wine parameters for each type of fining agent. Correlation values close to the unit ($r^2 = 1.00$) indicate concurrent removal of OTA and wine components by the fining agents. Activated carbons adsorbed high amount of OTA without removing total polyphenols from red wine ($r^2 = -0.18$). This finding suggests that wine polyphenols are the major interfering compound for the removal of OTA with fining agents. The colored polyphenols were also removed by all the fining agents with the exception of the silica solution positively charged ($r^2 = -0.58$). According to the literature, potassium caseinate reduces total and polymeric phenols,[38] whereas gelatins preferentially removes high molecular weight galloylated proanthocyanidols.[42]

The results of this study clearly indicate that several enological fining agents were able to remove substantial amount of the mycotoxin OTA from red wine. In particular, activated carbon and potassium caseinate removed highest amount of OTA from red wine when used at level of 10 and 150 g/hL, respectively. Because of low adsorption of total polyphenols, the activated carbon used at dosage higher than 10 g/hL (in case combined with other fining agents) might remove completely the OTA from red wine. Therefore, the winemaking process plays an important role in reducing human risk of exposure to this toxin.

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