OCHRATOXIN A AND AFLATOXINS B1, B2, G1, G2 IN GRAPE JUICE: SIMULTANEOUS ADSORPTION WITH ACTIVATED CARBON

N.D. MARTÍNEZ, A.M. RODRÍGUEZ and D.A. GÓMEZ

Instituto de Ingeniería Química, Univ. Nac. de San Juan, 5400 San Juan, Argentina
noramart@unsj.edu.ar

***Abstract*— Ochratoxin A (OTA) and aflatoxins B1, B2, G1 and G2, are produced by fungi which develop in the vineyard and they appear in products obtained from contaminated grapes, such as wine, raisins and must. These toxins are considered harmful to living beings.**

In this work activated carbon (AC) was used as adsorbent material for OTA and aflatoxins jointly.

The adsorbent material tested was obtained from grape stalk. It was produced by carbonization and subsequent physical activation with steam.

In order to perform the adsorption tests a sample of sulphited must was seeded with OTA and aflatoxins. Three samples of 200 ml were taken. Each one of them was contacted with 25, 50 and 100 mg of activated carbon, with continuous agitation to 300 K. In each test the concentration of mycotoxins at 30, 60 and 120 minutes was determined by extraction with immunoaffinity columns and subsequent quantification by HPLC.

In all tests a concentration decrease of mycotoxins was obtained, according to the amount of coal used. The OTA had better adsorption (96%). The aflatoxins B1 and B2 appear to have higher affinity with the adsorbent (95% and 93% respectively). Aflatoxins G1 and G2 had 85% and 80% abatement respectively.

***Keywords*— Ochratoxin A, Aflatoxin, Activated carbon, Grape juice, HPLC.**

I. INTRODUCTION

Grape juice is the liquid obtained from grinding or pressing of fresh unfermented grape. It is widely used as a sugar replacement in the food industry. Sulphiting must is the grape juice conserved by addition of potassium metabisulphite or sulfur dioxide. Being neutral sugar, the juice is used in the pharmaceutical industry and in the manufacture of soft drinks, juices, baby food, sweets and sweeteners.

Argentina is the world's leading exporter of concentrated grape must. Its main destinations are USA, Canada, Japan and to a lesser extent Chile, Brazil, Venezuela and Ecuador. Nowadays there are 21 plants of concentrate must in the province of San Juan, including the largest in South America (MCyT, 2007). Exporting requires compliance with the standards set by destination countries. One of the most important requirements involves the concentrations of ochratoxin A (OTA) and aflatoxins B1, B2, G1 and G2.

These mycotoxins are produced by fungi that develop on the vineyard, such as Aspergillus flavus, A. parasiticus and A. nominus (Gimeno y Martins, 2003). They are toxic fungi, capable of generating a variety of substrates that can contaminate food when its conditions of cultivation, processing or storage favor their development. The growth of these microorganisms and the toxin production depends on many factors such as sustrate, acidity, temperature, environmental humidity and the presence of competing microflora (Ravelo Abreu *et al.*, 2011). Once generated, these mycotoxins are transferred to the products obtained from contaminated grapes as wine, raisin or grape juice. They are considered harmful to living beings, with carcinogenic, nephrotoxic and teratogenic action (López de Cerain and Soriano, 2007).

Figure 1 shows the chemical structure of OTA. Aflatoxins are very similar between them, differing in a double bond and a 5 or 6 carbons ring, as shown in Fig. 2.

In Regulation No. 1881/2006 on the European Community (2006) a maximum of 2 μg/l for OTA is specified in grape juice. The food code of Argentine Republic does not accept the presence of aflatoxins in meals for infant consumption (ANMAT, 1971).

In this work the abatement of OTA and aflatoxins B1, B2, G1 and G2 in grape juice was studied using ac-

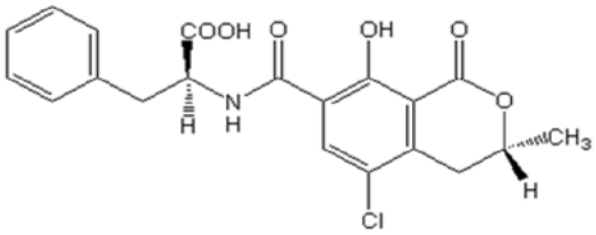

Fig. 1. Molecule of ochratoxin A.

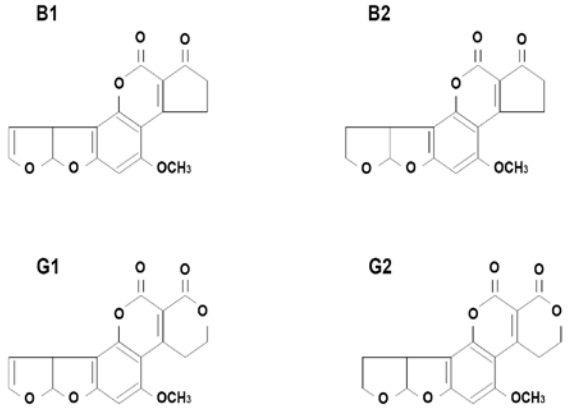

Fig. 2. Molecules of aflatoxins.

tivated carbon from grape stalk, residue of the wine industry. The Instituto Nacional de Vitivinicultura of Argentine Republic, set out in Resolution no. C-1/99 (INV, 1999) that a maximum dose of 1 g/l of activated carbon may be used to correct the color of white musts obtained from red grapes or those who, coming from white grapes, appear yellow or rusty, and 5 g/l in the treatment of musts having as exclusive export destination or not wine use in the domestic market.

Charcoal was obtained by charring the stalk in a retort with an inert atmosphere. This coal was subjected to leaching with hydrochloric acid and subsequent physical activation using steam (Martínez *et al*., 2015).

Adsorption assays were conducted using a sample of sulphited must expressly contaminated with OTA and the four mentioned aflatoxins, testing with different amounts of activated carbon, always below the limit allowed by law, with different contact times. All analyses from the trials were carried out by HPLC. The laboratory is accredited in a Standard IRAM 301-ISO/IEC 17025 for OTA in grape juice determination.

II. EXPERIMENTAL

A. Activated Carbon Production

The material used for producing charcoal was grape stalks, woody skeleton remaining after the grains are removed from the cluster. The preparation procedure of the activated carbon was described in previous papers (Tancredi *et al.*, 1996; Deiana *et al.*, 2002; Martínez *et al.*, 2009).

The characterization was focused on the measurement of textural parameters, point of zero charge (pH_{PZC}), pH, acidity, basicity and ash. The methodology for determining these parameters is described in detail in Martínez *et al.* (2015).

B. Adsorptions Trials

To perform the adsorption tests, sulphited must was seeded with OTA and total aflatoxins (B1, B2, G1 and G2), using Trilogy® mycotoxins standards.

From the seeded grape juice sample three aliquots of 200 ml were taken. Each one of them was contacted respectively with 25, 50 and 100 mg of activated carbon, with continuous agitation at an ambient temperature of 300 K. In each test, three samples of 30 ml at 30, 60 and 120 minutes were extracted.

At all times the pH of the solution was measured to test whether a change in it could change the adsorption behavior. A pHmeter ADWA 1000, interlaboratory tested with pH test in water was used to verify its behavior with a proficiency test.

Each one of the nine aliquots taken from the treated grape juice was centrifuged for 10 minutes to remove the carbon particles.

The supernatant was subjected to quantification of OTA and the four aflatoxins. The method of determination of OTA has been validated by the laboratory. The removal of toxins from the tested samples was performed by clean-up using immunoaffinity columns R-Biopharm Rone EASI-Extract® Aflatoxin and OCHRAPREP®. Finally toxins were quantified by HPLC, using a Perkin Elmer Series 200 chromatographer with fluorescence detector (FLD) and Kobra Cell electrochemical cell for derivatization of aflatoxins, prior to their detection by FLD.

The percentage of toxin abatement in samples treated with 25, 50 and 100 mg of activated carbon for all contact times is defined as:

$$\%abatment = \frac{(C_{i0} - C_i)}{C_{i0}} \times 100 \quad (1)$$

where C_{i0} is the mycotoxin initial concentration and C_i is the concentration at time *t*.

C. Adsorption isotherms

In order to obtain the adsorption isotherms corresponding to each of the studied toxins, adsorption tests were carried out with 14-hour contact to ensure the equilibrium state. Castellari *et al.* (2001) report that the equilibrium has been reached in 12 hours. These data were adjusted to an isotherm according to the Freunlich model because it appears as appropriate to represent the system behavior. Although it applies to low degrees of coating, good results have been obtained by applying this model (Castellari *et al., 2001*).

The proposed Freundlich equation is:

$$Q_{eq} = kC_{eq}^{1/n} \quad (2)$$

To make sure the fit of the proposed equation with the experimental data, Eq. (2) is linearized to be:

$$\log Q_{eq} = \log k + \frac{1}{n}\log C_{eq} \quad (3)$$

III. RESULT AND DISCUSSION

A. Activated Carbon

Textural parameters obtained from the data of the stalk activated carbon are described in Martínez *et al.* (2015). The obtained final values are: BET surface area 816.09 m^2/g, total pore volume 0.55 cm^3/g and micropore volume 0.42 cm^3/g. These values show that the activated carbon has properties as a highly microporous material, suitable for using as an adsorbent.

Also, it shows a pH = 10, acidity = 0.28 mmol/g and basicity = 1.30 mmol/g. The resulting point of zero charge (pH_{PZC}) was 11.30 with 10.2% in ash content. The high basicity presented for the carbon surface is consistent with the pH and pH_{PZC} results, which show the possible existence of basic groups, such as structures type chromene, quinone or γ□-pyrone and delocalized π electrons in the basal plane on the surface.

B. Adsorptions Tests

The pH of sulphited must, at all times and in all the tested aliquots was 3.4 units, whatever the amount of activated carbon added to any contact time. That is, the presence of such a small amount of coal did not change the natural acidity of the initial samples.

OTA initial concentration in the seeded sample was 0.95μg/l. In aflatoxins were: B1=0.72μg/l, B2=0.28μg/l, G1=0.99μg/l and G2=0.28 μg/l.

Table 1. Abatement percentages for all tested mycotoxins.

Activated Carbon (mg)	Time (min)	OTA	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2	Total Aflatoxins
		C_{i0} 0.95 µg/l	C_{i0} 0.72 µg/l	C_{i0} 0.28 µg/l	C_{i0} 0.99 µg/l	C_{i0} 0.28 µg/l	C_{i0} 2.27 µg/l
25	30	27.98	46.65	38.65	23.74	19.92	32.37
	60	55.92	58.01	51.85	30.79	29.05	41.81
	120	70.90	69.43	59.31	39.40	29.43	50.15
50	30	62.91	59.85	51.85	32.27	23.84	42.40
	60	84.57	77.01	75.06	53.73	52.77	63.63
	120	90.23	83.47	80.29	63.45	57.44	71.14
100	30	84.68	85.60	80.70	62.96	54.93	71.34
	60	84.75	91.24	87.50	75.41	68.75	81.10
	120	96.50	94.89	92.97	84.99	80.12	88.51

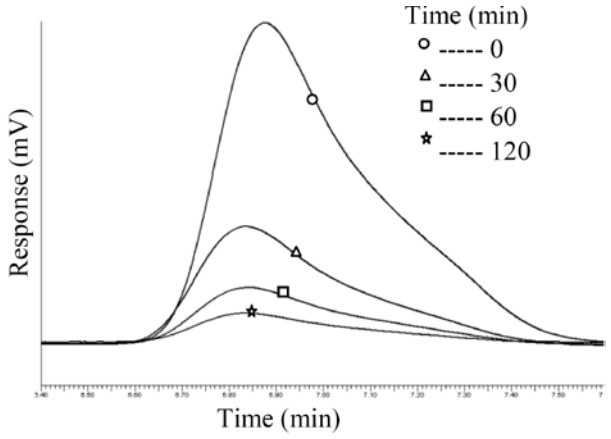

Fig. 3. OTA determination chromatograms for 50 mg of activated carbon test.

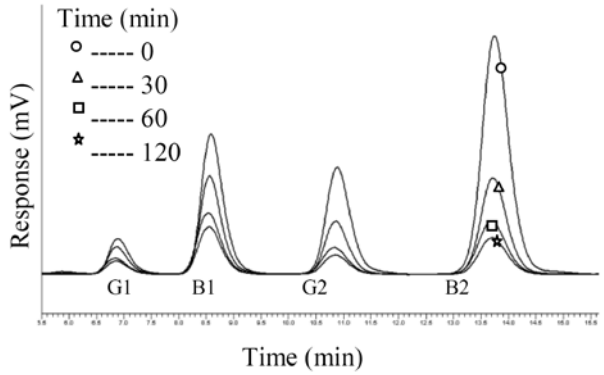

Fig. 4. Aflatoxins determination chromatograms for 50 mg of activated carbon test.

The fall in concentration of aflatoxins and ochratoxin A in all samples treated with activated carbon was very significant. As an example, Fig. 3 shows the chromatograms resulting from the measurements of the OTA in a simultaneous way for samples treated with 50 mg activated carbon at 0, 30, 60 and 120 minutes.

Figure 4 presents the chromatograms obtained in the quantification of the four studied aflatoxins. In these graphs all peaks behave as the latter, which is referenced with the contact time.

In Table 1 the percentages of all toxins abatement in samples treated with 25, 50 and 100 mg of activated carbon for all contact times are presented.

OTA and total aflatoxins abatement percentages calculated by Eq. (1) for different amount of adsorbent (AC) are shown in Fig. 5.

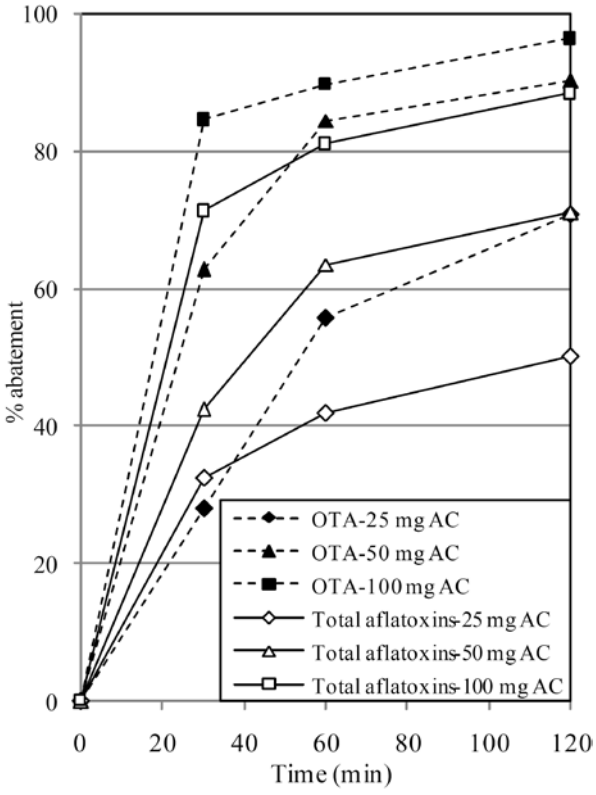

Fig. 5. Abatement of OTA and total aflatoxin vs. contact time for different amounts of activated carbon.

Based on the obtained experimental data, it is observed that the concentration of OTA and total aflatoxins decreases continuously from the initial value according to the amount of activated carbon used.

It should be remembered that % abatement is inversely proportional to the toxin concentration remaining in solution.

As expected, a higher amount of activated carbon leads to a greater adsorption, removing increasing amount of mycotoxins from the must. It is noted that the abatement of OTA is greater than that of aflatoxins for all toxin/charcoal ratio, reaching an abatement of 96% and 88% respectively for as much coal and maximum tested contact time.

Moreover, each of the aflatoxins showed different behaviors between them, according to the three comparative charts exposed in Fig.6. The percentages of four mycotoxins abatement are shown, corresponding to three different amounts of activated carbon, that is, 25,

50 and 100 mg respectively. The results in Table 1 show the behavior of abatement percentage, achieving up to 94.9% for B1, 93.0% for B2 85.0% for G1 and 80.1% for G2.

Total concentrations of aflatoxins decrease continuously from the initial value according to the amount of coal used. As expected, individual aflatoxins have the same behavior than OTA. A greater amount of activated carbon leads to a greater amount in removal.

The obtained curves are regular and their shape suggests that, for a given amount of activated carbon, adsorption tends to an asymptote. This final value could be determined by the amount of active sites on the carbon surface or the availability of free entry micropores, considering that coal has lots of micropores smaller than 2 nm.

Obviously, a higher amount of activated carbon added involves a greater number of active sites available for adsorption, yielding better abatement percentage for a given time.

When an activated carbon with a lot of basic surface groups is in an acid medium, its surface charge is predominantly positive, equivalent to the loss of hydrogen with its electron in the chromene group and the loss of an electron of oxygen basal (Martínez *et al*., 2015). Probably these positive charges act as links (active sites) with the molecules of toxins studied.

Between aflatoxins, it was observed to have greater affinity for the activated carbon, those of lower molecular weight, these being the following: aflatoxin B1, 312 Da; aflatoxin B2, 314 Da; aflatoxin G1, 328 Da; aflatoxin G2, 330 Da. This behavior is probably related to the size of each one of them and the ease of entry into the pores of smaller molecules, since they all have the same molecular structure, with very small differences that are not in the functional groups.

IV. ADSORPTION ISOTHERMS

In order to predict the adsorbed quantities using different amounts of activated carbon the behavior of adsorption isotherms was also studied. It was considered that the system reaches equilibrium at 14 hours of contact. The results refer only to the temperature considered in this work.

The initial concentration of OTA was 0.5 μg/l. Initial concentrations of aflatoxins were: B1 = 0.72 μg/l, B2 = 0.28 μg/l, G1 = 0.99 μg/l and G2 = 0.28 μg/l. The amounts of activated carbon in each case were the same as those used in the adsorption tests.

The amount of mycotoxin (μg) in contaminated 200 ml of each solution was calculated for each trial. This value was subtracted from the weight of toxin remaining in the 200 ml of solution, considering the final concentration (C_{eq}) determined by HPLC after 12 hours in contact.

The obtained value (in μg) is the whole amount of toxin adsorbed on the activated carbon. This value is linked with the amount of coal used in each test.

Calculated values are presented in Table 2. Figures 7 and 8 show the adsorption isotherm for all mycotoxins. These graphics show the amount of adsorbed toxin in micrograms per gram of activated carbon *vs.* the amount of toxin remaining in solution.

Figures 9 and 10 show the adjustment of Freundlich model to the experimental data. Table 2 shows both the value of the proportionality constants k of Freundlich equations obtained from intercept origin in fit line and the exponent ($1/n$) obtained from the slope of the straight line.

Also, in Table 2, the regression coefficient (R^2) is shown. Overall, the fit is good enough, so it is considered that the Eq. (2) represents the system behavior reliably.

V. CONCLUSIONS

The used grape stalk activated carbon proved to be a good adsorbent material for ochratoxin A and aflatoxins B1, B2, G1 and G2, showing activity below regulatory limits on the amount of activated carbon which can be used in the process of production.

Its behavior was expected according to the amount of carbon used and the contact time in tests.

Ochratoxin A showed higher affinity for adsorbent than aflatoxins.

Between aflatoxins, affinity was related to its molecular weight, that is, greater affinity for lower molecular weight: B1> B2> G1> G2.

Adsorption isotherms for all tested mycotoxins can be modeled according to the Freunlich model.

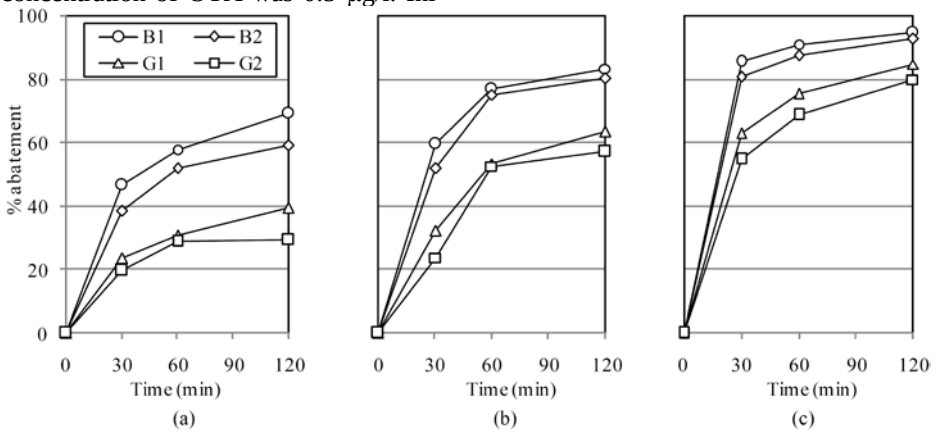

Fig. 6. Abatement of aflatoxins: a) 25 mg of activated carbon; b) 50 mg of activated carbon; c) 100 mg of activated carbon.

Table 2. Experimental results and Freundlich coeficients.

Toxin	C_0 (□µg/l)	Activated carbon (mg)	Q_{eq} (µg/g)	C_{eq} (□µg/l)	log Q_{eq}	log C_{eq}	k	1/n	R^2
OTA	0.50	25	0.00310	0.113	-2.5092	-0.9469	0.0164	0.778	0.995
		50	0.00176	0.060	-2.7546	-1.2208			
		100	0.00095	0.025	-3.0223	-1.6021			
		-	0.00000	0.000	-	-			
Total Aflatoxins	2.27	25	0.01434	0.457	-1.8433	-0.3401	0.0203	0.470	0.997
		50	0.00835	0.163	-2.0784	-0.7878			
		100	0.00442	0.0380	-2.3542	-1.4202			
		-	0.00000	0.000	-	-			
Aflatoxin B1	0.72	25	0.00522	0.047	-2.2820	-1.3279	0.6427	1.574	0.999
		50	0.00268	0.031	-2.5725	-1.5086			
		100	0.00136	0.020	-2.8665	-1.6990			
		-	0.00000	0.000	-	-			
Aflatoxin B2	0.28	25	0.03500	0.035	-2.6737	-1.4559	0.0155	0.581	0.985
		50	0.01000	0.010	-2.9355	-2.0000			
		100	0.00400	0.004	-3.2277	-2.3979			
		-	0.00000	0.000	-	-			
Aflatoxin G1	0.99	25	0.00594	0.258	-2.2265	-0.5884	0.0091	0.365	0.974
		50	0.00359	0.103	-2.4451	-0.9872			
		100	0.00197	0.014	-2.7051	-1.8539			
		-	0.00000	0.000	-	-			
Aflatoxin G2	0.28	25	0.00146	0.117	-2.8345	-0.9318	0.0023	0.190	0.993
		50	0.00112	0.020	-2.9508	-1.6990			
		100	0.00060	0.001	-3.2233	-3.0000			
		-	0.00000	0.000	-	-			

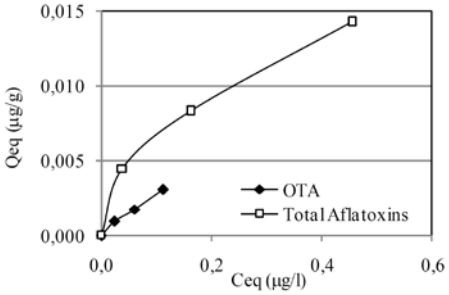

Fig. 7. OTA and total aflatoxins adsorption isotherms.

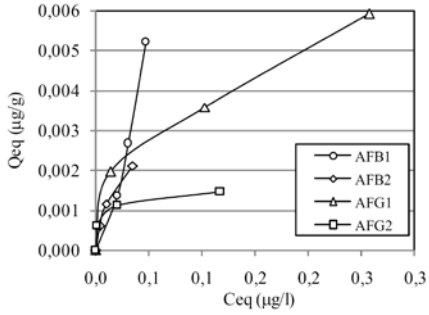

Fig. 8. Aflatoxins B1, B2, G1 and G2 adsorption isotherms.

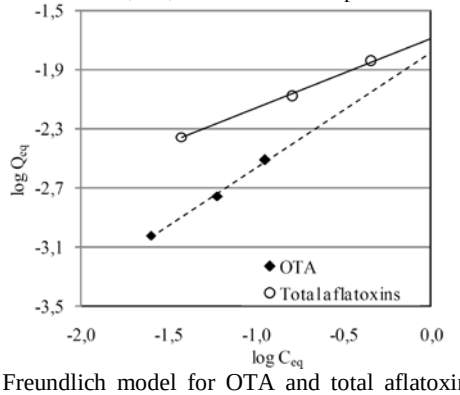

Fig. 9. Freundlich model for OTA and total aflatoxinas adsorption isotherms.

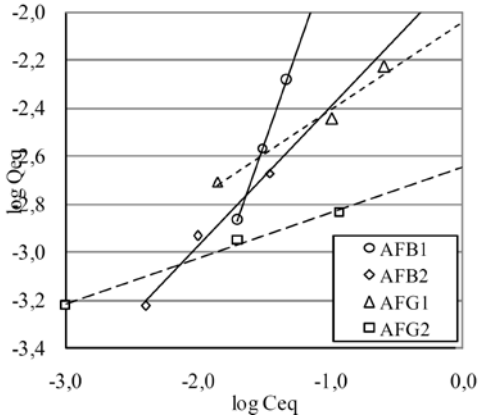

Fig. 10. Freundlich model for aflatoxins B1, B2, G1 and G2 adsorption isotherms.

REFERENCES

ANMAT, *Código Alimentario Argentino*, Decreto Nº 2126/71 (Reglamentario de la Ley 18.284), Capítulo 3, Artículo 156bis, http://www.anmat.gov.ar/alimentos/normativas_alimentos_caa. asp, Argentina (1971)

Castellari, M., A. Versari, A. Fabiani, G.P. Parpinello and S. Galassi, "Removal of ochratoxin a in red wines by means of adsorption treatments with commercial fining agents," *J. Agric. Food Chem.*, **49**, 3917–3921 (2001).

Deiana, A.C., L.M. Petkovic, H.S. Silva, E.W. Aguilar, M. F. Sardella and R.B. Venturini, "Residuos y subproductos para la obtención de briquetas de Carbón Activado," *VI Curso-Taller Iberoamericano de Adsorbentes y Catalizadores para la Protección Ambiental*, Redes Temáticas VC y VC – CYTED, Venezuela (2002).

European Commission, "Commission Regulation (EC) Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs," *Official Journal of the European Union*, **364**, 5-24 (2006).

Gimeno, A. and M. Martins, *Micotoxinas y Micotoxicosis en Animales y Humanos*, Special Nutrients Inc., USA (2003).

INV, *Resolución Nro. C-1/99*, http://infoleg.mecon.gov.ar/ infolegInternet/ anexos/ 55000- 59999/ 55835/norma.htm, Instituto Nacional de Vitivinicultura, Argentina (1999).

López de Cerain, A. and J. Soriano, "Ocratoxina A," *Micotoxinas en Alimentos*, Ediciones Díaz de Santos, España, 201-218 (2007).

Martínez, N.D., R.B. Venturini, H.S. Silva, J.E. González and A.M. Rodríguez, "Copper on Activated Carbon for Catalytic Wet Air Oxidation," *Materials Research*, **12**, 45-50 (2009).

Martínez, N.D., A.M. Rodríguez, R.B. Venturini, A.R. Gutiérrez and D.L. Granados, "Abatement of Ochratoxin A from Contaminated Wine and Grape Juice by Activated Carbon Adsorption," *Latin American Applied Research*, **45**, 33-38 (2015).

MCyT, *Debilidades y desafíos tecnológicos del sector productivo vitivinícola*, Ministerio de Ciencia y Tecnología de la República Argentina, COFECYT, http://www.cofecyt.mincyt.gov.ar/pcias_pdfs/rio_negro/UIA_vitivinicola_08.pdf, Argentina (2007).

Ravelo Abreu, A., C. Rubio Armendáriz, A.J. Gutiérrez Fernández and A. Hardisson de la Torre, "La ocratoxina A en alimentos de consumo humano: revisión," *Nutr Hosp.*, **26**, 1215-1226 (2011).

Tancredi, N., T. Cordero, J. Rodríguez-Mirasol and J. Rodríguez, "Activated carbon from uruguayan eucalyptus wood," *Fuel*, **75**, 1701-1706 (1996).

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