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HUMAN IGG ADSORPTION USING DYE-LIGAND EPOXY CHITOSAN/ALGINATE AS ADSORBENT: INFLUENCE OF BUFFERS SOLUTIONS

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ABSTRACT: The aim of this work was to measure the ability of adsorbent epoxy chitosan / alginate immobilized with Cibacron Blue F3GA (E-Ch/Al-cibacron) on IgG (high purity) adsorption using different buffers: TRIS/HCl, MOPS, MES and HEPES. All these buffers solutions were used with ionic strength of 25mM. To elution step was used the buffer with NaCl 1.0 M. The pH that showed the best results was the pH 7.8 with TRIS/HCl buffer. The Langmuir-Freundlich model provides a better fit in adsorption isotherm. The IgG adsorption capacity onto E-Ch/Al-cibacron with TRIS/HCl (pH 7.8) was 110.9 mg/g. In fixed bed experiments with buffer TRIS/HCl the IgG adsorption was higher onto E-Ch/Al-cibacron with 13.4 mg/g representing 60.8% of IgG injected on system.

KEYWORDS: Imunoglobulins, Affinity choromatography, adsorptions, chitosan.

1. INTRODUCTION.

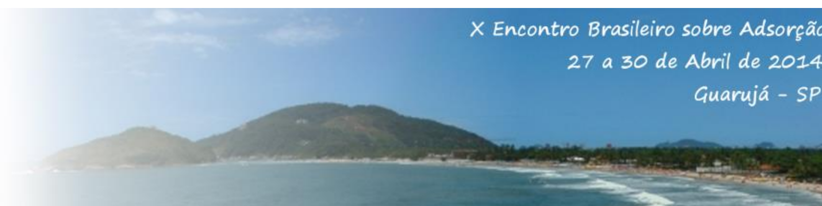
Antibodies belong to a class of molecules, the Immunoglobulins, which forms approximately 20% of the plasma proteins in human body. All the immunoglobulins have a common structure, independently of their functions, that consists in four polypeptide chains: two identical and heavy (H) and two identical light (L). The heavy and the light chais are connected by a dissulfide Bond (Amersham Biosciences, 2002).

The immunoglobulins are separated into five major classes according to the components of their heavy chains: IgG, IgA, IgM, IgD and IgE. Immunoglobulin G is divided into 4 subclasses: IgG_1, IgG_2, IgG_3 and IgG_4. The difference among these types is in the amino acid sequence within each class (Amersham Biosciences, 2002).

The high affinity of protein G for antibodies forms the basis for purification IgG, IgG fragments and subclasses. These proteins are indicated for treatment of cancer, infectious disease, antibodies selective deficiency, but this requires a slew quantity of protein with high purity (Burnouf and Radosevich 2001). There are many methods to purify IgG, mainly by precipitation and chromatography techniques including ion exchanges, hydrophobic interactions and affinity, besides gel filtration (Ozkara *et al*., 2004; Yavuz *et al*., 2006).

The affinity chromatography is an adsorption technique that explores the relation between an immobilized binder and the molecule which will be separated (Vijayalakshmi, 1989). The affinity chromatography has been used for more than 30 years and it has been considered the most powerful method to recuperate and purify proteins and enzymes (Wu *et al*., 2006; Denizli and Pişkin, 2001).

The pseudobiospeciphycal ligands used in affinity chromatography presents the ability to form binds by hydrophobic, electrostatic or coordination interactions with proteins and this technique has been study in large scale (Vijayalakshmi, 1989). Among these ligands the tiophilic ones, immobilized metallic ions, dyes and amino acids get more attention because of their low production costs, commercial availability,



easily immobilized, mainly in composes containing hydroxyl groups (Denizli and Piskin, 2001; Yavuz *et al*., 2006). Some examples of the dyes used in affinity chromatography are: Cibacron BlueF-3GA, Reactive Blue, Reactive Green, and they act as dye-ligand in separation process for affinity because they have attractive molecular structures (Wongchuplan *et al*., 2009). These immobilized dyes applied in different ways have been successfully used in affinity chromatography (Yavuz *et al*., 2006, Bayramoglu *et al*., 2007, Wongchuplan *et al*., 2009., Gondim *et al.,* 2012).

The mean of this work was to investigate the influence of four different buffers solutions in IgG adsorption on absorbent epoxy chitosan/alginate immobilized with Cibacron Blue F-3GA. Affinity chromatography was the technique and the assay was performance in batch and fixed bed column.

2. MATERIAL AND METHODS

2.1. Material

The Cibacron Blue F3GA immobilization into epoxide chitosan/alginate, referred to in this work as E-Ch/Al-cibacron.

Human IgG was acquired from Sigma (USA). Chitosan (Polymar, Brasil), sodium alginate (VETEC) and Cibacron Blue F3GA (Polysciences) were used to do the synthesis of the adsorbent. Morpholinoethane sulfonic acid (MES), morpholinopropane sulfonic acid (MOPS), hydroxyethylpiperazine ethanesulfonic acid (HEPES) and Trizma/HCl (TRIS/HCl) were adquired from Sigma (USA). All other chemicals were of analytical reagent grade. The water used for buffers and solution preparation was ultrapure (Milli-Q System, Millipore, USA).

2.2. Preparation particles of epoxy chitosan/alginate (E-Ch/Al)

Epoxide chitosan/alginate was prepared according to procedure described by Rodrigues *et al* . (2013). Chitosan was dissolved in 400 mL of acetic acid 5% (v/v). After that, it was added 40 mL of methanol and 4 mL of anhydride acetic. Sodium alginate was added to the mixture and kept under mechanical agitation for 1 h, so that, chitosan and alginate concentrations were 2.5% (m/m). The solution was coagulated into NaOH 0.1 mol/L for 4 h under moderate agitation. The gel was filtered and thoroughly rinsed with distilled water until it was neutral. Chemical modification of chitosan/alginate gel was carried out in a glass reactor. 10.0 g of gel and 100 mL of dimethylformamide were added into the reactor and maintained for 30 min at 60° C. Then, 0.8 g of KOH dissolved in 3 mL of isopropanol and 10 mL of epichlorohydrin were added the gel and maintained under agitation at 60°C overnight. After that, the material was washed again with distilled water until neutrality.

2.3 Cibacron blue F3GA dye immobilization in particles of epoxy chistosan/alginate

Cibacron Blue F3GA dye was immobilized into E-Ch/Al according to previously work (Gondim *et al.,* 2012) first mentioned by Ruckenstein and Zeng (1998). Firstly, it was added 1.0 g of E-Ch/Al to 100 mL of dye solution (5 mg/mL) at 60°C for 1h. After that, 5 mL of NaCl 20% (m/v) was added to the suspension aiming dye adsorption and the pH value was corrected to 10.5 with 2 mL of Na_2CO_3 (25% m/v) improving adsorption process. This suspension was kept for 4h at 60°C. The material containing dye was washed with distilled water, methanol, NaCl 2 mol/L, Urea 6 mol/L, Tween 80 (1 % m/v) and buffers solution 25 mmol/L.

2.4. Batch adsorption

In order to determine the effect pH of buffer system and isotherms adsorption of IgG onto E-Ch/Al-cibacron, several experiments were carried out (in duplicate) at 25ºC in batch adsorption system. For this aim, 15 mg of adsorbent was put in contact in acrylic tubes containing 3.0 mL of the IgG solution. The tubes were agitated end-over-end in orbital shaker (Tecnal TE-165, Brazil). To investigate the effect of buffers, the mixture was shaken during 2 h (IgG concentration equal to 1.0 mg/mL). For the measurement of adsorption isotherms, different initial concentrations (1.0 to 9.0 mg/mL for IgG) were shaken during time interval necessary to reach the equilibrium (3.0 h). In all these experimental procedures, the supernatant were collected, centrifuged at 10000 rpm for 10 min (refrigerated microcentrifuge Cientec CT – 15000R, USA) and the protein equilibrium concentration for the liquid phase (supernatant) was determined by absorbance at 280 nm (UV-Vis spectrophotometer Biomate 3, ThermoScientific, USA).

The mass of protein adsorbed per mass of adsorbent (mg/g) was calculated using a mass balance described equation (1):

$$q^* = \frac{V_{SOL}(C_0 - C_{eq})}{m_{ads}} \tag{1}$$

where C_0 and C_{eq} (mg/mL) are the initial and equilibrium protein concentration in liquid phase, q^* (mg/g) is the amount of protein adsorbed in solid phase, m_{ads} (g) is the mass adsorbent and V_{SOL} (mL) is the volume of the sample solution.

The Langmuir, Freundlich and Langmuir-Freundlich models, described by equations (2), (3) and (4), respectively, were used to fitted to the experimental data by using the iterative Levenberg-Marquardt fitting method (Origin® software, Microcal, USA).

$$q = \frac{q_m C_{eq}}{K_D + C_{eq}} \tag{2}$$

$$q = K_f (C_{eq})^{1/a} \tag{3}$$

$$q = \frac{q_m (C_{eq})^n}{K_{DLF} + (C_{eq})^n} \tag{4}$$

where q_m (mg/g) is the maximum adsorption capacity, C_{eq} (mg/mL) is the concentration of protein in solution at equilibrium, K_f is the Freundlich adsorption constant, K_D and K_{DLF} are the dissociation and the apparent dissociation constants, respectively, which indicate the affinity between protein and adsorbent, a and n are the coefficients for Freundlich and Langmuir-Freundlich, respectively.

2.5. Fixed Bed adsorption

The chromatographic procedures were carried out using a peristaltic pump (Watson-Marlow, USA) and a fraction collector (C-660, Büchi, Swiss). The dye-affinity adsorbent (0.5 g) was suspended in water, degassed, and packed without compression into columns (10.0 cm x 1.0 cm I.D., GE Healthcare, USA) to give a bed height of 1.0 mL (representing 1.4 cm).

Adsorption column experiments were carried in order to evaluate the effect of initial concentration of IgG (1.0 mg/mL) and flow rate was 0.8 mL/min. The experimental runs were carried out at 25°C. For this aim, the column was equilibrated with 25 mM of buffer solution. After that, a volume of protein (IgG or human serum) diluted in buffer (25 mM) solution was pumped to the column. For all experiments, after protein loading, the column was washed with the loading buffer (25 mM) until the absorbance values at 280 nm at the outlet reached the baseline. Elution was performed with the loading buffer containing 1.0 mol/L NaCl. After each experiment, the column was regenerated with 25 mM NaOH, followed by Milli-Q water and the loading buffer to restore it to initial condition for a new experiment. During all chromatographic steps (A – adsorption / W – washing / E – elution / R – regeneration) the absorbance was monitored by UV/Vis detector (Thermo Scientific BioMate 3, USA) at 280 nm to measure IgG concentration. The amount of total mass protein was calculated by mass concentration and feeding of protein in the system over mass balance according to Gondim (Gondim *et al.*, 2012).

3. RESULTS AND DISCUSSION

The dye, Cibacron Blue F3GA, contain three sulfonic acid (SO^{3-}) that have affinity for biomolecules, like enzymes and serum proteins. This dye have used in many studies as affinity ligand in chromatography. This affinity can be attributed to both specific and non-specific interactions by cation exchange effects (probably due to the SO^{3-} groups) or hydrophobic and/or hydrogen bonding effects.

This material was mentioned and characterized in our previously work (Gondim *et al.*, 2012). The cibacron blue dye was immobilized onto epoxy chitosan/alginate (E-Ch/Al-cibacron) and any dye release during the experiments in batch and fixed bed with proteins.

3.1. Batch adsorptions

3.1.1. Effect of different buffers on the IgG adsorption: It can be observed in Figure 1 the influence of four different buffers in IgG adsorption onto E-Ch/Al-cibacron. All buffers have a different range of pH and this is important due to IgG is a protein that has an isoelectric point

(pI) variable between 6.3 and 9.0 (Bresolin *et al.*, **2010**).

The results in Figure 1 demonstrated that the IgG adsorption was influenced by pH and buffers. It also can be that IgG adsorption onto E-Ch/Al-cibacron at buffer HEPES in pH 6.8 has 76.56 mg/g and was superior to the others buffers, in the same conditions.

In our previously work (Gondim *et al.*, 2012) it was studied the IgG adsorption onto E-Ch/Al-cibacron with sodium phosphate buffer solution. The pH 6.0 presented the better results to IgG adsorption on sodium phosphate and the amount IgG adsorbed onto adsorbent was 43.34 mg /g.

When was used MES at buffer solution, the IgG adsorption onto E-Ch/Al-cibacron were lower than MOPS, HEPES and TRIS/HCl buffers, but was superior compared the results obtained for Gondim *et al.*, (2012). In this study, all buffers shown capacity to IgG adsorption superior to 43.34 mg/g.

Wongchuphan *et al.*, (2009) studied the rabbit IgG adsorption in a resin *streamline* with dye ligands immobilized. They investigate a large range of pH (between 4.0 and 9.0, with tree different buffers) and found a better adsorption with phosphate buffer solution at pH 6.0, around 32.0 mg/mL. In other study with dye ligand affinity chromatography it was presented to Yavuz *et al.* (2006). They used as a dye ligand reactive green 5 immobilized on a polymer (poli(HPMA)) and found a better IgG adsorption at pH 7.0 (sodium phosphate buffer) with 71.1 mg/g of IgG adsorbed. Bayramoglu *et al.* (2007) studied the IgG adsorption onto a polymer with reactive green 5 immobilized in different buffers systems and found at pH 6.0 the best results.

MOPS (pH 6.5), HEPES (pH 6.8) and TRIS/HCl (pH 7.8) were chosen to investigate the adsorption isotherm and fixed bed adsorption with high purity IgG.

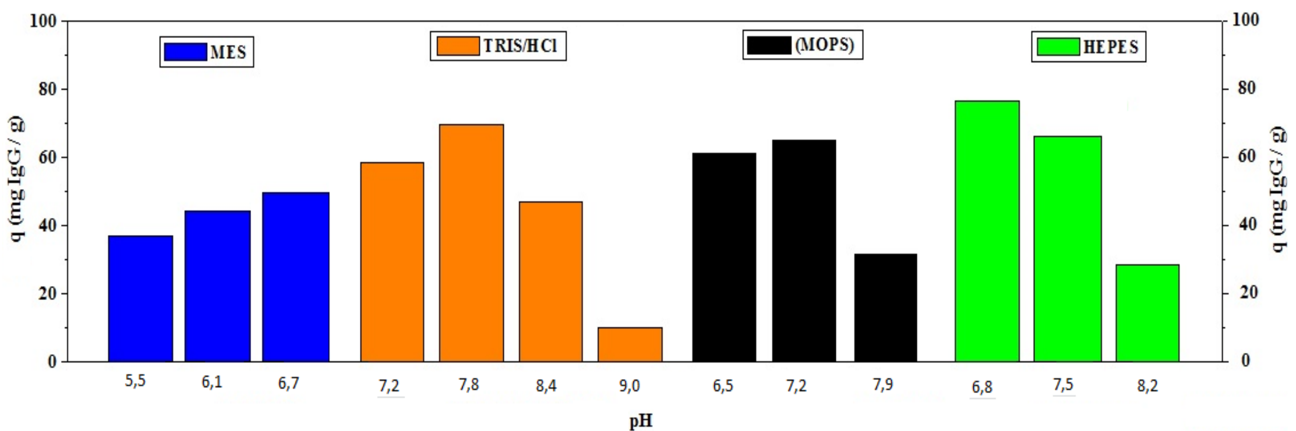
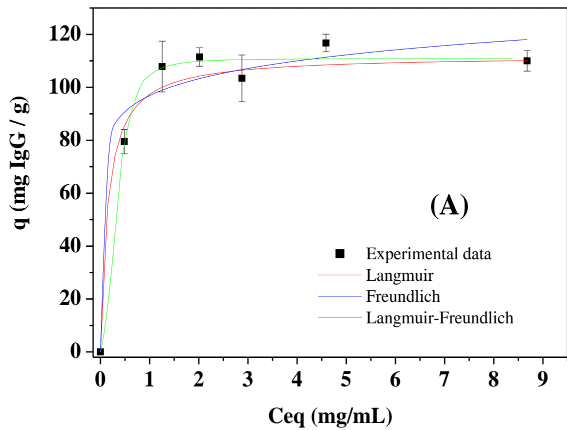


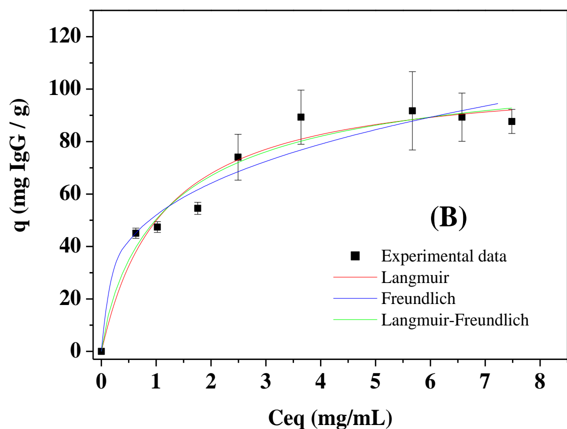
Figure 1.Effect of pH among different buffers solutions on IgG adsorption onto E-Ch/Al-cibacron.

3.1.2. Isotherm adsorptions: The equilibrium time it was reached in 3 h of contact between IgG and E-Ch/Al-cibacron. The adsorption isotherm of IgG is presented in Figure 2 (A, B and C). It was observed that the amount of IgG adsorbed onto E-Ch/Al-cibacron increased by increasing IgG concentration in all buffers solution (MOPS, HEPES and TRIS/HCl) reaching the equilibrium at high concentration.

It can be noted in Figure 2 (A) an isotherm with high adsorption capacity at low IgG concentrations. This behavior is probably one of the reasons for owning the best fit. The shapes of the isotherms are a typical rectangular form and could be described by Langmuir, Freundlich and Langmuir-Freundlich (continuous line) equations. These models get adjusted to the experimental data.

According to Figure 2 (B and C) it can be observed the same performance to adsorption isotherm in both cases. The capacity to IgG adsorption were very similar, around 100.0 mg/g.





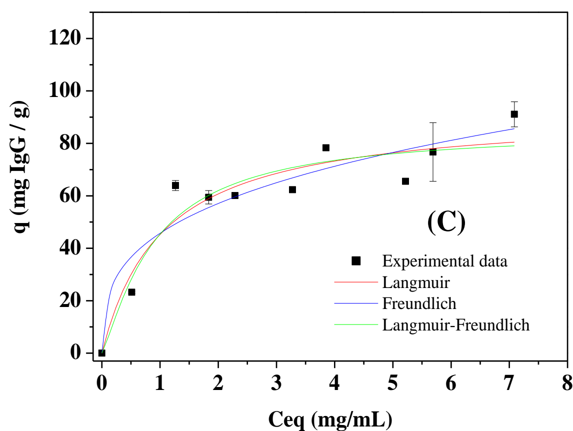


Figure 2. Adsorption isotherm of IgG onto E-Ch/Al-cibacron at buffers solutions (TRIS/HCl (A), MOPS (B) and HEPES (C)). Theoretical profile: The lines correspond to fitting (nonlinear regression) of experimental data according to Langmuir, Langmuir-Freundlich and Freundlich models.

The adsorption model parameters are shown in Table 1 and can be noted that IgG adsorption was close to all buffers systems. However with TRIS/HCl (pH 7.8) buffer it presented the maximum amount of IgG adsorbed (117.5 mg /g of adsorbent), among all buffers.

An important parameter that establishes a relationship between the protein and adsorbent are the constants K_D, K_f and K_{DLF}. These constants when the values are between 10^{-4} to 10^{-8} M demonstrate that the adsorbent could be considered as dye-ligand affinity chromatography. How smaller the constants values more intensity the interaction protein-adsorbent. According to the values in Table 1 for different buffers systems to IgG adsorption, the TRIS/HCl buffer presented the best values to the constants (K_D, K_f and K_{DLF}). In this buffer the adsorbent (E-Ch/Al-cibacron) have more intensity interaction protein-adsorbent. Probably this is one more reason to explain the high IgG adsorption in this system (TRIS/HCl buffer) and the better adjust from the models. The results on adsorption with buffer HEPES show lower values to maximum capacity to IgG adsorption (all models) and had a poorer fit to all models.

3.2. Fixed bed adsorptions

All experiments in fixed bed were conducted with high purity IgG at the same buffers system used in isotherm adsorption. Figure 3 show the chromatograms of IgG with 10 mL of injected volume.

Table 1. Parameters of adjusted for Langmuir (L), Freunlich (F) and Langmuir-Freundlich (LF) to IgG adsorption onto E-Ch/Al-cibacron at buffers MOPS (pH 6.5), HEPES (6.8) and TRIS/HCl (pH 7.8) 25 mM.

Parameters	E-Ch/Al-cibacron								
	TRIS/HCl pH 7.8			MOPS pH 6.5			HEPES pH 6.8		
	L	LF	F	L	LF	F	L	LF	F
q_m (mg/g)	117.5	110.9	-	105.9	114.3	-	92.3	85.4	-
K_D (x 10^6)	3.4	-	-	5.9	-	-	6.4	-	-
K_F (x 10^4)	-	-	6.5	-	-	3.5	-	-	3.0
K_{DLF} (x 10^6)	-	0.38	-	-	8.5	-	-	5.9	-
R^2	0.98	0.99	0.92	0.97	0.97	0.95	0.91	0.91	0.89
Chi^2	31.9	22.2	75.4	37.5	42.6	49.2	72.2	81.6	85.7
a			10.9			3.3			3.1
n	-	2.7	-	-	0.84	-	-	1.2	-

Langmuir – L
Langmuir-Freundlich – LF
Freundlich - F

It can be observed in Figure 3 the profile of the IgG concentration at different buffers systems (MOPS, HEPES and TRIS/HCl). It is noted that almost IgG injected on system were adsorbed onto E-Ch/Al-cibacron at all buffers (at around 60% of mass injected). The elution peak of IgG adsorbed with TRIS/HCl buffer it was superior to the others systems. The others two profiles for IgG adsorption were very similar.

Table 2 presented the mass balance for IgG adsorption at the buffers systems. The amount of IgG recovered after the elution steps were 13.4, 10.2 and 10.8 mg / g, respectively with TRIS/HCl, MOPS and HEPES buffers. When used TRIS/HCl buffer the mass recovered of IgG was 60.8% of the mass inection on the system and was slightly higher than the other buffers. In our previously work (Gondim *et al.*, 2012) it was found a similar behavior for IgG adsorption and 10.6 mg/g, was adsorbed using phosphate buffers solution.

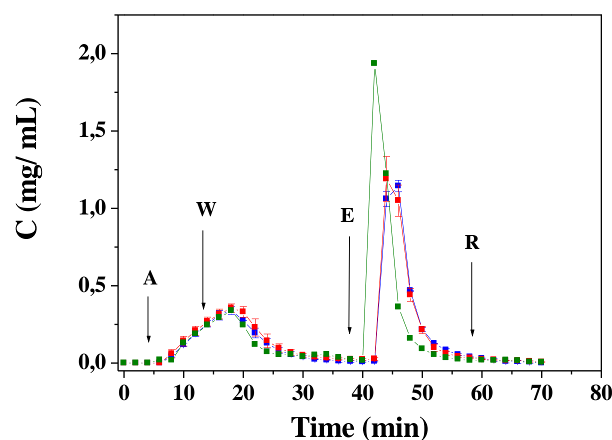


Figure 3. Fixed bed adsorption with high purity IgG (1.0 mg/mL) at MOPS (■), HEPES (▲) and TRIS/HCl (●) buffers solutions with 25 mM. Steps: A (Adsorption); W (Wash); E (Elution) and R (Regeneration).

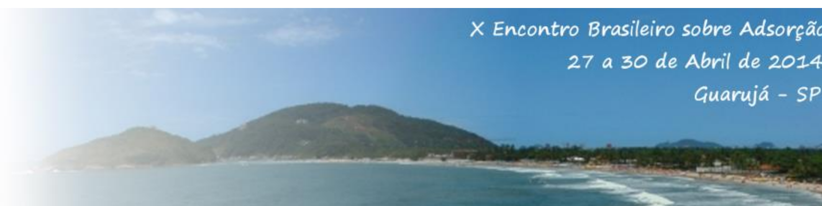


Table 2. Mass balance (MB) to IgG adsorption onto E-Ch/Al-cibacron (0.50 g) for each step: Injection (I), Adsorption (A), Wash (W), Elution (E) and Regeneration (R)

STEPS	TRIS/HCl		MOPS		HEPES	
	TP (mg)	%	TP (mg)	%	TP (mg)	%
I	10.7	100	9.2	100	8,9	100
A	1.5	13.9	1.0	11.0	1.1	12.3
W	1.8	17.21	2.5	26.6	2.7	30.8
E	6.5	60.8	5.1	54.8	5.3	59.4
R	0.2	2.1	0.4	4.5	0.1	0.4
MB	10.0	94.0	8.9	96.9	9.2	102.9

Total Protein (TP)to IgG at 280nm

4. CONCLUSION

The maximum IgG adsorbed was 117.5 mg/g onto E-Ch/Al-cibacron with TRIS/HCl buffer solution at pH 7.8. All adjusted models well fitted the experiments in bath systems. The dissociation constants proved that the adsorbent can be used as affinity dye-ligand for chromatography experiments. The IgG adsorption on fixed bed showed that high quantity IgG were adsorbed with all buffers, representing around 60% of mass injected.

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