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IgG AND HSA ADSORPTION STUDY ONTO LAYERED DOUBLE HYDROXIDE

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ABSTRACT: In this work a non-calcined Mg-Al layered double hydroxide (Mg-Al LDH) with molar ratio of 3:1 was studied on the adsorption of IgG and HSA. Mg-Al LDH was synthesized using co–precipitation method. This material was characterized by X-ray diffraction (XRD), N_2 adsorption-desorption isotherm at 77K, pHzpc, particle size and FTIR. The effects of pH, kinetic and isotherm adsorption of high purity IgG and HSA were studied by batch adsorption. Adsorption of IgG and HSA shows a dependence of solution pH. IgG was more selective for pH higher than 6.5 while HSA was low at the range of pH. The adsorption isotherms were well fitted to Langmuir and Freundlich models. The maximum IgG adsorption, achieved using 25 mmol/L sodium phosphate buffer at pH 6.5, was 164.88 mg/g obtained by Langmuir model . The maximum adsorption capacity to HSA was five times less than IgG, around 31 mg/g also Langmuir model. Studies on fixed bed in better conditions for IgG adsorption was performance to evaluate the adsorption from both proteins. The results showed the high IgG adsorbed on Mg-Al LDH.

KEYWORDS: Layered double hydroxide, Protein adsorption, Immunoglobulin G, Human serum albumin.

1. INTRODUCTION

Layered double hydroxides (LDHs) are anionic nanostructured materials, natural or synthetic. The most commonly known natural LDH is the hydrotalcite with Mg and Al as metals divalent and trivalent, respectively. Layered double hydroxides are a class of inorganic materials with positively charged layers formed by octahedral clusters with divalent and trivalent metals cations. The octahedron vertices have hydroxyl groups and the interlayer space have water molecules and anion intercalated (Jin *et al*., 2012). The general formula to LDH is often written as $[M(II)_{1-x}M(III)_x(OH)_2]^{x+}[A^{n-}_{x/n}.]^{x-}.mH_2O$, were M(II) and M(III) are divalent (Mg, Zn, Ni,…) and trivalent (Al, Fe, Cr,…) metals respectively, A^{n-} is a anion of charge *n*, and *m* is the molar amount of cointercalated water (Bellezza, 2012; Jin *et al*., 2012). The selection of the adsorbent is an important consideration because it needs to be effective to adsorb the target molecule.

Few studies have are reported on the use of HDL as adsorbent for proteins. The most common use of stirred tanks system (Jin *et al*., 2012; Bellezza *et al*., 2012; Ralla *et al*., 2011)

There are some factors that influence on amount of adsorbed protein, is the properties of the protein (charge, size, amino acid composition) and of the solid surface and experimental conditions (Bellezza *et al*., 2012). The structural stability of protein is also important and there are proteins with a high internal stability (so-called “hard” proteins) and with low internal stability (so-called “soft” proteins). The hard protein adsorb onto hydrophilic surfaces only in the presence of electrostatic attraction and the soft protein, such as myoglobin and bovine serum albumin, normally to adsorb onto all surfaces (Bellezza, 2012).

The human plasma contain many proteins like human serum albumin (HSA) (50 wt%), immunoglobulins (12 wt%), fribinogen (4 wt%) and others proteins (32 wt%) (Travis *et al*., 1976). Immunoglobulin G (IgG) is one of the most important protein from human serum and is

indicated for the treatment for many diseases, like cancer, infectious disease, and selective deficiencies of antibody (Burnouf, 2007; Burnouf, 2001). IgG is a group of glycoproteins composed of four subclasses (IgG1, IgG2, IgG3 and IgG4) of antibodies having a isoelectric point between 6.3 to 9.0 and a molecular mass of 170 KDa (Bresolin *et al*., 2010).

There are few works with LDH to protein adsorption in literature, but no one to investigate the adsorption of IgG on LDH. This work it was important to understand if exist selectivity of this adsorbent to IgG due to your medicinal potential to combat some diseases. Thus, the aim of this work was to synthesized LDH by variable pH coprecipitation method, characterization of this adsorbent and studing the adsorption of standard protein IgG and HSA with batch and fixed bed adsorpiton experiments on Mg-Al LDH.

2. MATERIAL AND METHODS.

2.1. Material

Human IgG and HSA were purchased from Sigma (USA). All other chemicals were of analytical reagent grade. The water used for buffer and solution preparation was ultrapure (Milli-Q System, Millipore, USA).

2.2. Synthesis of LDH

LDH sample was prepared by co-precipitation method according to (Aguiar *et al.*, 2013). when solution of magnesium $Mg(NO_3)_2 \cdot 6H_2O$ (0.0996 mol) and aluminum $(Al(NO_3)_3 \cdot 9H_2O$ (0.0332 mol) nitrates were dissolved in 100 mL of distilled water) and added drop wise to 100 mL solution containing 0.249 mol/L $NaHCO_3$ at 60 °C, under vigorous stirring. The obtained precipitates was passed through a hydrothermal treatment 80 °C for 4 days and stirred at room temperature for 12 h. LDH with molar ratio Mg/Al = 3:1 was filtered and washed with deionized water until the filtered was at pH 7. The material was dried again at 80 °C for 24h and samples were characterized.

2.3. Characterizations

Particle size diameter was determined using the equipment MASTERSIZER 2000 (Malvern Instrments, U.K.) using water to disperse the particles. The average particle size was about 480 µm.

To determine the point of zero charge (pH_{ZPC}) of the Mg-Al LDH was used 0.01N NaCl solutions in different pH (among 2 and 12) in contact with Mg-Al LDH. For each 50.0 ml of NaCl solution was added 0.15 g of the material and allowed to stand for 48 h. Finally, the difference of $pH_{INICIAL}$ and pH_{FINAL} by $pH_{INICIAL}$ was plot. Similar procedures can be found in literature (Ip, Barford; Mckay, 2010).

The Mg-Al LDH samples were also characterized by XRD in a Philips X'Pert X-ray Diffraction System with a copper tube CuKα (λ = 0.1542 nm) operating at a voltage of 40 kV and a current of 40 mA.

The textural characterization was performed by adsorption-desorption of N_2 to 77 K (Micromeritics, ASAP 2000). The specific surface area was calculated by the B.E.T method and the pore size was calculated by the B.J.H method using the adsorption and desorption isotherms, respectively. The total pore volume was calculated from the maximum amount of nitrogen gas adsorption at partial pressure (P/P_0) = 0.999 (Rouquerol, Rouquerol; Sing, 1999).

FT-IR study was carried out using FTIR 8400S Shimadzu having a standard mid-IR DTGS detector. FTIR spectra were recorded, in the range of 400–4000 cm^{-1} with KBr pellets technique.

2.4.Experimental Adsorption Systems

2.4.1. Batch adsorption assays: In order to determine the effect of buffer system pH, the kinetics and isotherms adsorption of proteins (IgG and HSA) on Mg-Al LDH, 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 protein solution. The tubes were agitated end-over-end in orbital shaker (Tecnal TE-165, Brazil) with time interval necessary to reach the equilibrium. In order to evaluate the influence of pH buffer solution on amount adsorbed different IgG and HSA solutions (1.0 mg/mL) were prepared in 25 mM buffers at different pHs. For this aim, 15 mg of adsorbent was suspended in IgG or HSA solution (1.0 mg/mL) in different buffers ranging from pH 4.0 – 8.0 (pH 4.0 – 5.6 acetate buffer, 6.0 – 8.0 sodium phosphate buffer, 25 mM). For the kinetic

adsorption experiments, initial protein concentration equal to 1.0 mg/mL were prepared in sodium phosphate buffer solution with fixed pH (6.5). For the measurement of adsorption isotherms, different initial concentrations of IgG and HSA (0.5 to 8.0 mg/mL) with fixed pH and ionic strength were shaken during time interval necessary to establish the equilibrium. 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 dividing the 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 by equation 1:

$$q = \frac{V_{sol}(C_0 \quad C_{eq})}{m_{ads}} \qquad (1)$$

where V_{sol} is the volume solution (mL), C_0 is the initial liquid concentration (mg/mL), C_{eq} is the equilibrium liquid concentration (mg/mL) and mads is the mass adsorbent (g).

The Langmuir and Freundlich models, described by equationns (2) and (3), respectively, were used to fitted to the experimental data by using Origin® software, Microcal, USA.

$$q^* = \frac{q_{max} c_{eq} k}{1 + k c_{eq}} \qquad (2)$$

$$q^* = k_f (C_{eq})^{1/n} \qquad (3)$$

where q_{max} (mg/g) is the maximum adsorption capacity, k (mL/mg) is defined as constant of Langmuir, C_{eq} (mg/mL) is the concentration of protein in solution at equilibrium, k_f and n are Freundlich adsorption constants related to feature of the adsorption system. k_f and n are indicators of the binding capacity and adsorption intensity, respectively.

2.4.2. Adsorption in Fixed Bed: The adsorption procedures (adsorption / washing / elution / Regeneration) in fixed bed system were carried out using an experimental apparatus composed of stainless steel column 0.4 I.D. x 12 cm, high performance liquid chromatographic pump (Varian ProStar 210) coupled to a fraction collector (C-660 Buchi, Swiss). The layered double hydroxide (0.68 g) was packed without compression into column to give a bed height of 12 cm.

Adsorption column experiments were carried in order to evaluate the effect of initial concentration of IgG and HSA (1.0, 2.0 and 3.0 mg/mL) on LDH and flow rate was 1.0 mL/min. The experimental runs were carried out at 25 oC. For this aim, the column was equilibrated with 25 mM sodium phosphate buffer at pH 6.5. After that, a volume of protein (IgG or HSA, 45 mL) diluted in sodium phosphate buffer (25 mM) solution at pH 6.5 was pumped to the column. For all experiments, after protein loading, the column was washed with the loading buffer (25 mM sodium phosphate at pH 6.5) 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 (adsorption/washing/elution/regeneration) the absorbance was monitored by UV/Vis detector (Thermo Scientific BioMate 3, USA) at 280 nm for all fractions collected. The amount of total mass protein was calculated by mass concentration and feeding of protein in the system over mass balance according procedure described by Gondim (Gondim *et al*., 2012).

3. RESULTS AND DISCUSSION

3.1. Characterizations

Figure 1 show the pH_{ZPC} from Mg-Al LDH. It is possible to notes that zone of net charge was very close to pH 8.0.

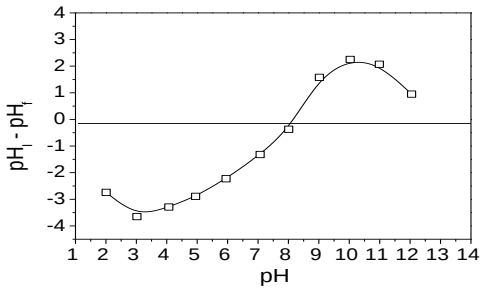

Figure 1. pH_{ZPC} from Mg/Al LDH.

Figure 2 shows the adsorption-desorption isotherm at 77 K of Mg-Al LDH. This resembles a type IV isotherm established by IUPAC (Gregg *et al.*, 1982). Type IV isotherms are found in many measures of adsorption. The turning point present in this isotherm corresponds to the occurrence formation of first adsorbed layer that covers the entire surface of the material. A steep slope for small values of P/Po indicates the presence of mesopores associated with micropores.

The presence of hysteresis is linked to the process of filling by capillary condensation. It is a typical characteristic of mesoporous materials (Gregg *et al.*, 1982). This type of hysteresis is characteristic of materials whose pores are regular, cylindrical and /or polyhedral with open ends.

It can be seen in Figure 2 a distribution with occurrence of micropores with values ranging from 24 to 51 Å. The distribution also showed that there are pores that are distributed over a range of 65 to 100 Å predominantly mesopores. Similar behavior was observed by (Jin et al, 2012; Zaghouane *et al*, 2012) found that pores of approximately 23 to 30 Å and classified as bimodal.

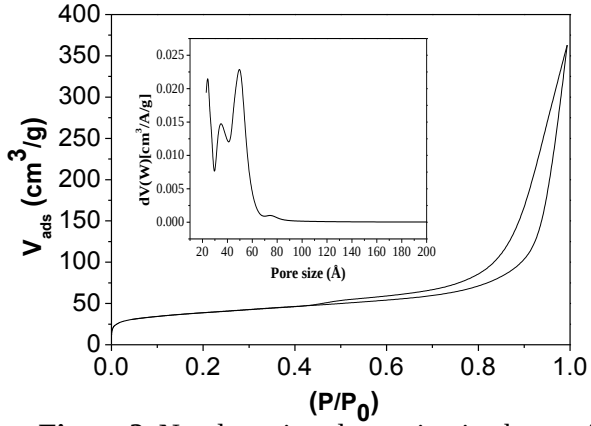

Figure 2. N_2 adsorption-desorption isotherm of Mg-Al LDH at 77 K.

Table 1 presented the textural properties of Mg-Al LDH obtained by BET analysis. This material showed a 74 m^2g^{-1} of surface area. These results are in agreement with literature (Aguiar *et al*., 2013; Ralla *et al*., 2011) which demonstrates a considerable area available for adsorption. For Mg–Al LDH containing simple anions such as carbonate, chloride, and nitrate, the surface area is mostly less than 100 m^2g^{-1} (Cavani *et al*., 1991).

Table 1 - Textural properties of LDH Mg/Al.

S_{BET} [m^2/g]	V_P [cm^3/g]	D_P [nm]	References
74	0.45	2.40	This work
105	0.56	17.9	(Aguiar *et al.*, 2013)
61.4	0.48	24.6	(Ralla *et al.*, 2011)
123	0.82	26.6	(Auxilio *et al.*, 2009)
100	0.50	30.0	(Lazaridis, Karapantsios and Georgantas, 2003)

Mg-Al LDH was also characterized by XRD and the result is shown in Figure 3.

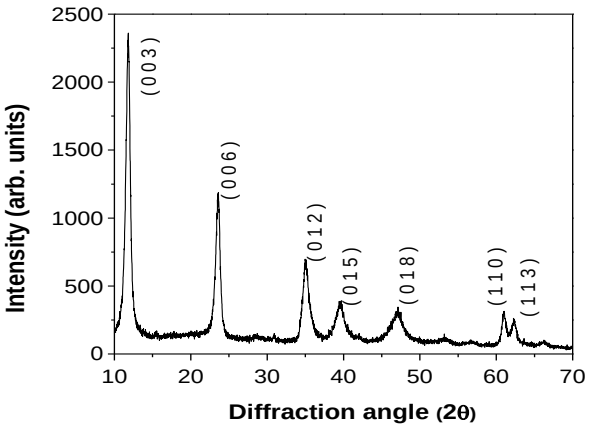

Figure 3. X-ray diffraction of Mg-Al LDH

The crystalline phase was identified using the International Center for Diffraction Data (ICSD) catalog. The diffraction pattern presents a crystal structure with very strong sharp and symmetrical reflections on both (003) and (006) planes, which is characterized as LDH structure (Cavani *et al.*, 1991). The High Score Plus software has identified the LDH pattern as hidrotalcite and after best Rietveld refinement it was obtained the following lattice parameters: a = b = 3.05 Å and c = 22.91 Å. This result is accordance with the standard available in ICSD-6296 (Mills *et al*., 2012; Allmann *et al*., 1969) that shows the same parameters a and b and c 22.81 Å for a lattice parameter.

Figure 4 show that FTIR spectroscopy of LDH non-calcined (Mg-AL) resembles those of other hydrotalcite-like phases.

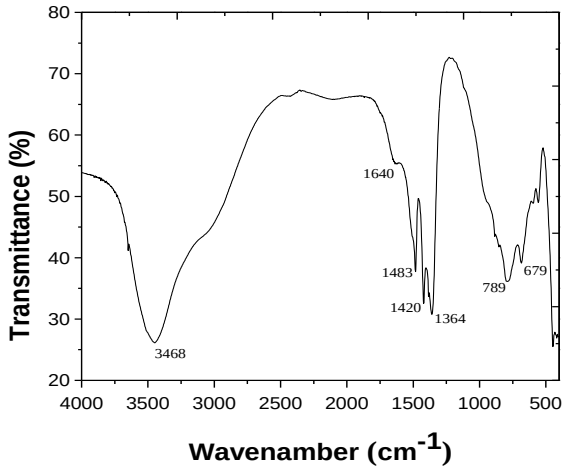

Figure 4. FTIR spectroscopy of Mg-Al LDH.

Typical for these spectra are the strong broad absorbance bands between 3600 and 3400 cm^{-1} associated with the stretching mode of hydrogen-bonded hydroxyl groups from both the hydroxide layers and interlayer water. A bending vibration band corresponding to a water deformation band is seen at 1640 cm^{-1}. It can be seen also the presence of the absorption bands arising from the carbonate anion observed at 1483–1420, 789 and 679 cm^{-1}. The two bands observed in the region 1360–1400 cm^{-1}, can be attributed to a lowering of the symmetry of the carbonate anions. The band of 1510 cm^{-1} and 1050 cm^{-1} also suggests a lowering of the symmetry of the carbonate ion. A series of bands are recorded at 579, 660 cm^{-1} ascribed to Mg-Al-OH translation, Al-OH translation and Al-OH deformation, respectively. These results are in agreement with others studies **(Jin *et al*., 2012; Lv *et al*., 2012)**.

3.2. Bath adsorption

The investigation of ideal pH is an important parameter in the protein capacity adsorption onto adsorbent because it induces changes in the net charges of the protein surface (Bellezza *et al*., 2012).

The adsorption data for IgG and HSA on LDH (Mg/Al) at two different buffers solution with pH values between 4.0 and 8.0 are shown in Figure 5. It can be observed the profile of IgG and HSA adsorption in all pH's. It also can see in Figure 5, when the pH was close to isoelectric point (pI) of HSA (4.9) the adsorption was high compared to IgG adsorption. However, when the pH moves away from pI of HSA and next to pI of IgG the HSA adsorption decrease substantially and IgG adsorption increase. This adsorption behavior can be explained by the electrostatic interactions and others forces, like hydrophobic interactions and/or hydrogen bounds may additionally be responsible (Ralla *et al*., 2011).

In contrast of our results Ralla (Ralla *et al*., 2011) studied adsorption of two proteins (bovine hemoglobin and human serum albumin) on a commercial hydrotalcite (Syntal 696), and they found a high adsorption for both proteins above the pI of each one. It is important to observe that in Ralla's work they did not study the adsorption on pI of proteins. They assumed that above pI of proteins are negatively charged and the hydrotalcite surface charge was positive so the electrostatic nature was predominant and the hydrotalcite behaves like an anion exchange material.

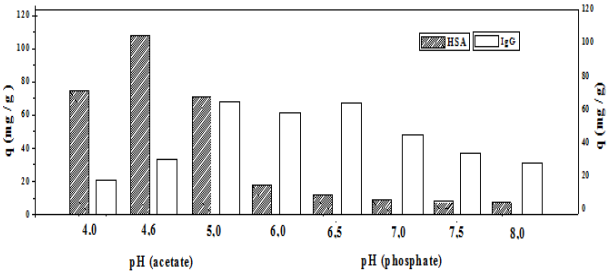

Figure 5. Efect of pH on protein (IgG and HSA) adsorption with Mg-Al LDH.

Kinetics and isotherm adsorption, was performed using sodium phosphate buffer at pH 6.5 to promote preferentially the IgG adsorption on Mg-Al LDH.

The adsorption kinetics profiles of IgG and HSA on Mg-Al LDH with three different concentrations (1.0, 2.0 and 3.0 mg/mL) were shown in Figure 6. According to Figure 6, the equilibrium time was found near to 180 min. The reduction of IgG concentration in liquid phase was slowly and probably due the hard diffusional mechanisms, but in comparison to HSA adsorption it is observed that IgG was more adsorbed.

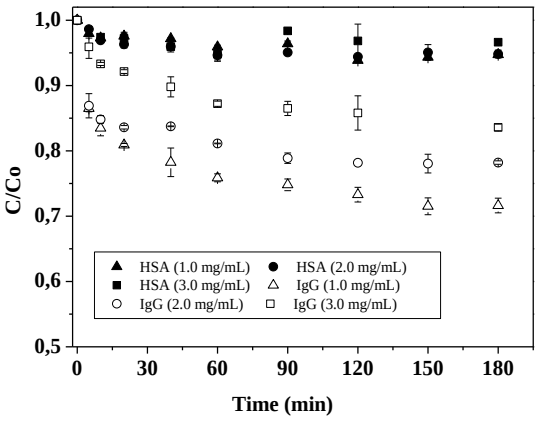

Figure 6. Kinetic profile for IgG and HSA adsorption with Mg-Al LDH in three different concentration (1.0, 2.0, 3.0 mg . mL^{-1}).

The adsorption isotherm of IgG and HSA was presented in Figure 7 and were performed at pH 6.5 in 25mM sodium phosphate buffer. It was observed that the amount of IgG adsorbed on Mg-Al LDH increased by increasing IgG concentration in buffer solution reaching the equilibrium at high concentration, next to 8.0 mg/mL. The HSA adsorption profile was different of the IgG because in low concentrations the maximum adsorption capacity was reached. In comparison between both proteins is evident that IgG was more adsorbed and have more selectivity on adsorbent surface.

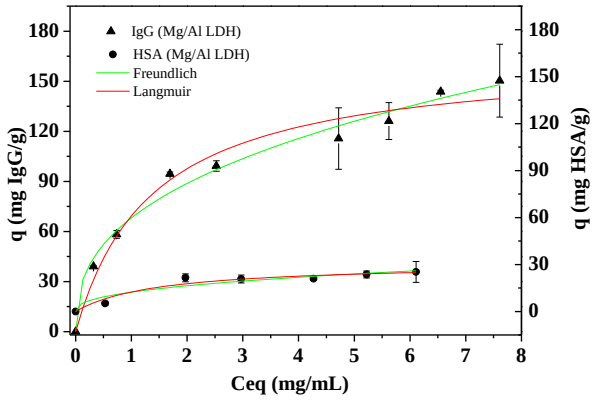

Figure 7. Adsorption isotherm of IgG and HSA on Mg-AL LDH. Theoretical profile: the line correspond to fitting (nonlinear regression) of experimental data according to Langmuir and Freundlich models.

These isotherms were adjusted to Langmuir and Freundlich models. The Langmuir model can generally be used to describe the protein adsorption, but has some particular assumptions: the adsorbate is adsorbed in a monolayer, all surface sites are energetically equivalent, and that the surface is homogeneous and without interaction between adjacent sites (Langmuir, 1918).

The adsorption model parameters to IgG and HSA are shown in Table 2.

Table 2. Equilibrium adsorption parameters according to Langmuir (L) and Freundlich (F) models to protein adsorption.

	IgG		**HSA**	
	L	**F**	**L**	**F**
q_{max} (mg/g)	164.88	-	30.95	-
K(mol /L)	$4.8x10^{-6}$	$1.7x10^{-5}$	$1.0x10^{-5}$	$1.9x10^{-4}$
Chi^2	69.10	38.49	5.69	10.28
R^2	0.976	0.987	0,952	0.913
n	-	2.62	-	2.42

L - Langmuir parameters

F – Freundlich parameters

The Langmuir parameters for IgG adsorption, q_{max} and K were found to be 164.88 mg/g and $4.8x10^{-6}$ mol/L, respectively. The maximum adsorption capacity to HSA was more the five times less than IgG. The dissociation constant (K) was very significant and the values were $4.8x10^{-6}$ and $1.0x10^{-5}$ mol/L, respectively to IgG and HSA adsorption onto LDH by Langmuir parameters. According to literature K measures how the adsorbate is attached onto adsorbent. To higher K value the adsorbate will stand more weakly bound onto adsorbent (Bresolin *et al.*, 2010). For this reason the biomolecule IgG have more intensity onto adsorbent promoting a higher adsorption.

Figure 8 show three different concentrations (1.0, 2.0 and 3.0 mg/mL) profiles for both proteins IgG (A) and HSA (B) on fixed bed.

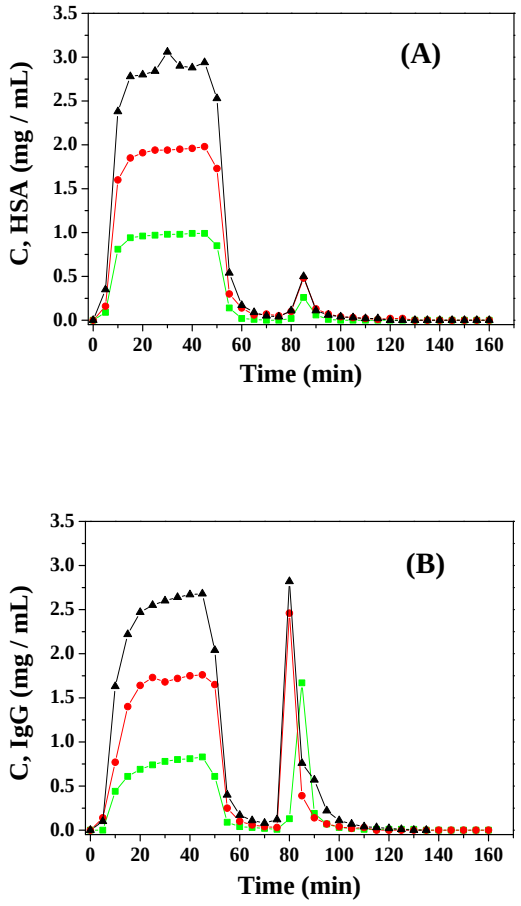

Figure 8. Fixed bed experiments with Mg-Al LDH: *Flow rate* = 1.0 ml/min, *Temp*.: 25 °C, *Injection volume*: 45.0 mL (HSA - **A** and IgG - **B**), m_{ads}: 0.68 g, *Elution (E):* NaCl 1.0 M in Buffer solution 25 mM at pH 6.5. Concentrations (mg/mL): 1,0 (■), 2,0 (●) and 3,0 (▲).

It was observed that all assays reached the saturation (firs peak) and the second peak represent the elution of proteins. It can be see in elution that HSA adsorption was lower than IgG adsorption. These results are in accordance with the previously discussed in this work in isotherm adsorption for both proteins when the IgG adsorption superior to HSA.

It is important to know that the material showed good reproducibility and stability on fixed bed and new tests will be carried with a solution containing IgG and HSA. This new results will be more conclusive if one of these proteins is being preferentially adsorbed.

4. CONCLUSION

The Mg-Al LDH show the surface diameter and volume pore close to literature. The adsorbent show adsorption capacity for standard proteins, however was more specific for IgG. The study of pH it was important to confirm that adsorption on both protein were more intensity at the pI and was chosen pH 6.5 to promote preferentially the IgG adsorption onto Mg/AL LDH. The adsorption isotherms were well fitted to Langmuir and Freundlich model and the maximum adsorption capacity to HSA was five times less than IgG.

The results on fixed bed experiments, with standard proteins, showed the high IgG adsorbed on Mg-Al LDH and more test need to be performed to conclude that the adsorbent will be effective to purify IgG from human serum.

5. REFERENCE

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