Adsorption of endotoxins on \( \text{Ca}^{2+} \)-iminodiacetic acid by metal ion affinity chromatography

André Moreni LOPES\(^1 \), Jorge Sánchez ROMEO\(^{2*} \), Rolando Páez MEIRELES\(^2 \), Gabriel Marquez PERERA\(^2 \), Rolando Perdomo MORALES\(^3 \), Adalberto PESSOA Jr\(^2 \), Lourdes Zumalacárregui CÁRDENAS\(^4 \)

1. Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo (FCF/USP), 05508-000 São Paulo, Brazil; 2. Purification Development Department, Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba; 3. Center for Pharmaceuticals Research and Development (CIDEM), Havana, Cuba; 4. Higher Polytechnic Institute José Antonio Echeverría (CUJAE), Havana, Cuba

Abstract: Endotoxins (also known as lipopolysaccharides (LPS)) are undesirable by-products of recombinant proteins, purified from *Escherichia coli*. LPS can be considered stable under a wide range of temperature and pH, making their removal one of the most difficult tasks in downstream processes during protein purification. The inherent toxicity of LPS makes their removal an important step for the application of these proteins in several biological assays and for a safe parenteral administration. Immobilized metal affinity chromatography (IMAC) enables the affinity interactions between the metal ions (immobilized on the support through the chelating compound) and the target molecules, thus enabling high-efficiency separation of the target molecules from other components present in a mixture. Affinity chromatography is applied with \( \text{Ca}^{2+} \)-iminodiacetic acid (IDA) to remove most of the LPS contaminants from the end product (more than 90%). In this study, the adsorption of LPS on an IDA-Ca\(^2+\) was investigated. The adsorption Freundlich isotherm of LPS-IDA-Ca\(^2+\) provides a theoretical basis for LPS removal. It was found that LPS is bound mainly by interactions between the phosphate group in LPS and Ca\(^2+\) ligands on the beads. The factors such as pH (4.0 or 5.5) and ionic strength (1.0 mol/L) are essential to obtain effective removal of LPS for contaminant levels between endotoxin concentration values less than 100 EU/mL and 100 000 EU/mL. This new protocol represents a substantial advantage in time, effort, and production costs.

Key words: immobilized metal affinity chromatography (IMAC); lipopolysaccharides (LPS); endotoxin removal; recombinant proteins; isotherms; protein purification


Advances in biotechnology have opened up numerous possibilities for the large-scale production of many biomolecules that are important for research, pharmaceutical and industrial applications. These biomolecules may be expressed as soluble extracellular forms, soluble intracellular forms or inclusion bodies. In the last two cases, it is necessary to use cell disruption procedures for recovering the target biomolecules, which leads to the release of large quantities of endotoxins (also known as lipopolysaccharides (LPS)) [1,2]. LPS can be considered stable under a wide range of temperature and pH, making their removal one of the most difficult tasks in the downstream processes during protein purifications [3–5].

* Corresponding author: André Moreni LOPES. Tel: +55-11-3091-3862, Fax: +55-11-3815-6386, E-mail: andreml@usp.br

# This author and the first author contributed equally to this work.

Foundation item: This research was supported by grants from the Brazilian Agency Coordination of Graduate Level Training (CAPES, project 0366-09-9) and State of São Paulo Research Support Foundation (FAPESP-Brazil, project 2005/60159-7).

Received date: 2012-06-06
Many recombinant peptides and proteins are produced by *Escherichia coli* (*E. coli*), consequently LPS are the most common pyrogenic substances present in these biomolecules [6 - 8]. However, LPS need to be removed prior to the therapeutic application. Due to these adverse reactions, it is essential to remove LPS from the injectable drug preparations [5,9 - 11].

Upon administration into the blood stream, LPS cause inflammation and septic shock [5,12,13]. The general threshold level of LPS set by pharmacopoeias worldwide for intravenous applications is 5 endotoxins units (EU) per kg body weight per hour with 1 EU being equal to 100 pg LPS [14]. The amount of LPS allowed in pharmacological protein preparations depends on the intended use. For the proteins that are administered in relatively low doses, the regulatory demands are modest, for instance insulin and α-interferon could contain up to 10 and 100 EU/mg, respectively [15,16].

As LPS are an integral part of *E. coli*, there is always the risk of LPS co-purification with the target product. The LPS concentration in starting material varies since less than 100 EU/mL in cell culture supernatants to more than 2 000 000 EU/mL in supernatants after homogenization of high cell density bacterial cultivations [15]. Certain methods have been used for LPS removal from non-protein preparations [14]. Numerous methods have been studied for LPS removal from protein solutions [3,9,11,17 - 21], and affinity adsorption has proven to be the most effective technique [19,22 - 24].

Affinity ligands for LPS are reasonably effective for LPS removal from protein solutions with relatively high protein recoveries [19]. Immobilized poly-L-lysine-sepharose and diethylaminoethanol (DEAE)-sepharose supports [22] have also been shown to effectively remove LPS from protein solutions with clearance rates up to $1.50 \times 10^4$ EU/mL at a contamination level of 1 500 EU/mL. Both affinity and ion exchange chromatographies may only be used if contamination levels are below 1 500 EU/mL, in order to obtain acceptable final contamination levels less than 5 EU/mL [20]. Higher initial LPS concentrations and variations in protein isoelectric points (pI) and ionic strength (I. S.) lead to low protein recovery ratios, with insufficient LPS removal and extremely poor clearance rates [22].

The effects of pH and I. S. on the removal of LPS were investigated by some researchers [25,26]. Although the influence of I. S. on LPS removal was reported, Cu$^{2+}$-iminodiacetic acid (IDA), for instance, could be used for simultaneous removal of ribonucleic acid (RNA) and endotoxins directly from the alkaline cell lysate. Furthermore, the use of free metal ions for selective precipitation of plasmid deoxyribonucleic acid (DNA) or impurities from the alkaline cell lysate can be easily coupled with ion metal affinity chromatography (IMAC), contributing to increased IMAC binding capacity through the exclusion of impurities. At the same I. S., bivalent cations have much stronger influences on LPS aggregation state due to the “bridging effect” [15,23].

IMAC enables the affinity interactions between the metal ions (immobilized on the support through the chelating compound) and the target molecules, thus enabling high-efficiency separation of the target molecules from other components present in a mixture [27]. IMAC, a group-specific affinity separation technique, is based on specific and reversible coordinate interactions between various amino acid residues accessible on the surface of proteins and metal ions chelated to a multidentate ligand immobilized on a support material. A number of different amino acid residues can contribute to metal binding, but histidine residues are known to form strong complexes with transition metal ions [27 - 31].

The metal chelates have a high adsorption capacity, low cost, and high selectivity for the recombinant proteins fused with a poly(histidine) tag [29 - 32]. Various factors such as the nature of the chelating groups, the metal ion, the ligand density of the adsorbent, the surface amino acid composition of the protein, and the surrounding environment (e.g., pH, I. S., nature of buffer salts, and temperature) affect protein adsorption in
IMAC[33,34]. Thus, the adsorption selectivity and capacity of an immobilized metal ion affinity adsorbent for a target protein can be modified by varying these parameters[35–37]. LPS are known to have high affinity for bivalent metals such as calcium and magnesium[38] and they form aggregates with high stability. For that reason, the feasibility of using IMAC for LPS capture from pure LPS solutions was explored in this paper. The binding behavior of LPS solutions was investigated with metal ion (Ca$^{2+}$) immobilized on commercially available IMAC resins (i.e., IDA-Chelating Sepharose Fast Flow (CSFF)). The effects of pH and I. S. in the range used for histidine tagged proteins were explored for the removal of LPS; LPS-resin mechanisms of adsorption are discussed.

1 Experimental

1.1 Materials

The chromatographic matrix used was CSFF purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden), gel characteristics were bead size range of 45–165 μm and mean particle size approximately 90 μm[39]. NaCl, CaCl₂, Tris, ammonium chloride and sodium acetate were purchased from Sigma. All solution transfers were performed using LPS-free devices. Sterile, disposable plastic was used to prevent LPS contamination. The LPS concentration in the water for injection (WFI) was below 0.005 EU/mL, based on test results. All other reagents were of analytical grade.

1.2 LPS measurement

Pure LPS samples were obtained from E. coli cultivation according to Westphal and Jann[40]. LPS concentrations in the samples were determined by the kinetic chromogenic method provided by the LAL Kinetic-QCL® kit. All sample dilutions and additions of lysate to microtiter plate wells were performed using a calibrated multichannel pipette with pyrogen-free tips. The plate-based photometric assay (λ = 405 nm) was incubated at 37 °C in ELISA microplate reader (ELx808cse — BIO-TEK Instruments). The positive control was contaminated with 5 EU/mL LPS, and the results for all tests were considered valid when the recovered LPS concentration was between 50% and 200% of this value[17].

1.3 Column preparation and operation

The CSFF was stored in 20% ethanol when purchased. The PD10-column (GE Healthcare Bio-Sciences) was used as a reservoir, convenient equilibration, sample application, and wash. The adsorption of LPS on an IDA-Ca$^{2+}$ was determined by batch. The empty PD10-column was loaded with 5 mL of CSFF previously washed with WFI. The column was then loaded with 10 mL of 0.5 mol/L NaOH solution and incubated for 1 hour to depyrogenize the material. Subsequently, the column was loaded with 5 mL of 0.1 mol/L HCl (37%) solution and washed with 15 mL of WFI. Later, the column was incubated for 1 hour with 2.5 mL of 0.1 mol/L CaCl₂ to bind Ca$^{2+}$ to the resin. The total concentration of Ca$^{2+}$ ions immobilized on IDA matrix was determined to be 30 mmol/mL of adsorbent. The value obtained is within the range specified by the manufacturer[39]. Finally, the column was washed with 5 mL of WFI to remove the unbound Ca$^{2+}$ from the column. The column was charged with metal ion and ready for use. The charged column was washed with 5 mL of 20 mmol/L sodium acetate buffer in 0.5 mol/L NaCl at pH 4.0 followed by 5 mL of 20 mmol/L sodium acetate buffer in 0.5 mol/L NH₄Cl at pH 4.0. Later, the column was equilibrated with 15 mL of starting buffer depending on the experimental design below. All the buffers were filtered through a 0.22 μm filter and degassed under vacuum for at least 25 minutes prior to being applied to the column. This study was carried with two replicates.

1.4 Batch adsorption study of LPS

A 100 μL of the resin was added to microtubes. The microtubes containing the resin were incubated with 30 times the volume of purified LPS solution (a 50 μL of the resin was incubated with 1.5 mL of the sample). Subsequently, the tubes were kept in a horizontal shaking plate (10 r/min), placed in a controlled temperature at 25 °C. After
the incubation, the microtubes were centrifuged at 16 110 × g for 30 s, and their supernatants were collected for the analysis. For LPS (with the concentration of 200 EU/mL) on IMAC-IDA-Ca\(^{2+}\) kinetic adsorption studies, the samples were collected at 0.008, 0.016, 0.032, 0.083, 0.16, 0.5, 1, 3, 6, 9, 12, 24, 48 and 72 h. The study was realized at pH 4.0 and 0.25 mol/L I. S. and three repetitions were done. For the adsorption isotherm experiment, purified LPS were applied to the resin. LPS concentrations used for isotherm construction were 20, 50, 100, 300, 500, 1 000, 3 000, 5 000, 10 000, 30 000, 50 000, 70 000 and 100 000 EU/mL. Contact time was 24 hours, obtained by adding 4 hours to the minimum time to reach a constant concentration during the kinetic experiments; temperature was maintained at 25 °C.

1.5 Experimental design
Statistical analysis was carried out evaluating the removal of pure LPS added to IMAC, using response surface methodology (RSM). The study characterized the effects of pH and I. S. (in mol/L) on two adsorption variables \(q_{\text{max}}\) (maximum adsorption capacity) and \(K\) (affinity constant between LPS and IDA-Ca\(^{2+}\)) at 25 °C. The entire experiment matrix can be seen in Table 1, showing a \(3^2\) design with three replicates at the central point. All the experiments were performed in duplicate in order to estimate the experimental variability that determines the statistical significance of effects. The statistical results were treated using Statgraphics Centurion XV (version 15.2.05) software.

2 Results and discussion
2.1 Kinetic study for LPS-IDA-Ca\(^{2+}\)
The kinetics for the adsorption process of LPS on IDA-Ca\(^{2+}\) matrix was investigated for 72 hours. The initial concentration of LPS was on the order of 200 EU/mL, and under these conditions the kinetics was unfavorable or slower. Fig. 1 shows \(C/C_0\) value versus time \((t)\) where \(C\) is the solute concentration in the liquid phase and \(C_0\) is the initial concentration of the sample. Based on the kinetic curve, the ratio of solute concentration in the liquid phase and the initial concentration of the sample decreased with time, attaining a practically constant value of 0.30.

![Fig. 1 Adsorption kinetics for LPS-IDA-Ca\(^{2+}\) system in stirred tank at different times at 25 °C](image)

Data were fitted by a mathematical model suggested by Bitton and Marshall[41]. This model considers the adsorbate diffusion through the liquid film surrounding the adsorbent particle as the controlling step for the adsorption kinetic. A multiple linear regression method was applied to obtain Daniel model’s parameters. Equation 1 (namely Daniel equation) represents the fitted model \((R^2 = 0.90,\) the standard error of estimates = 0.267, \(P\) value for analysis of variance (ANOVA) was 0 for 95% confidence).

\[
\ln\left( \frac{C}{C_0} \right) = 0.0269t - 0.3903t^{0.5}
\]  

(1)

Fig. 2 shows the fitness of the adjusted model to experimental data. Fig. 3 (a and b) represents the contribution of each terms for the logarithm of the dependent variable \((C/C_0)\). The goodness of

<table>
<thead>
<tr>
<th>Assay</th>
<th>pH</th>
<th>I. S. / (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>5.5</td>
<td>0.25</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>8.0</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10*</td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>11*</td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>12*</td>
<td>5.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Assays of the central point.
Daniel model can be considered as an indication of the mechanism that rules this kinetic adsorption, diffusion. From Daniel model, the minimum time for a constant $\ln(C/C_0)$ was obtained at 20 hours. It can be considered as the equilibrium value. The mass transfer in the adsorption process could be affected by the pH and I. S. due to the conformational change that could have the LPS of the monomers to micelles or vesicles.

As has been proposed in the literature, adsorption isotherms are used to represent the equilibrium between the concentrations of the solute adsorbed on the surface of the matrix ($q^*$) and the concentration of the solute in the solution ($c^*$). At a state of the equilibrium, the rate of the solute being adsorbed on the surface of the resin is equal to the rate that leave the surface [42]. The isotherm indicates the amount of adsorbent required and the sensitivity of the adsorption process on the concentration of the solute. The experimental data were fitted to two of the most common isotherms seen in the literature, Freundlich and Langmuir. The best regression parameters were obtained for Freundlich isotherm model.

### 2.3 Fitting to Freundlich isotherm

To obtain the parameters of the Freundlich model, which represents the adsorption of LPS-IDA-Ca$^{2+}$ system, the values of $q^*$ and $c^*$ from the experimental isotherms were linearized using the linear regression equation 2.

$$\ln q^* = \ln K + n \ln c^*$$  \hspace{1cm} (2)

Table 2 shows the estimated values for $K$ and $n$, the parameters that characterize the Freundlich isotherm at 25 °C for each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>I. S./mol/L</th>
<th>$K$</th>
<th>$n$</th>
<th>$R^2$ fitted/%</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.0</td>
<td>1.00</td>
<td>30.73</td>
<td>1.172</td>
<td>82.60</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>5097.47</td>
<td>0.477</td>
<td>74.70</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>105.23</td>
<td>0.866</td>
<td>78.70</td>
<td>1.90</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>1.00</td>
<td>59.90</td>
<td>1.071</td>
<td>93.40</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>464.32</td>
<td>0.681</td>
<td>72.60</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>53.57</td>
<td>0.962</td>
<td>95.10</td>
<td>0.68</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>1.00</td>
<td>42.68</td>
<td>0.900</td>
<td>95.10</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>53.57</td>
<td>0.824</td>
<td>85.50</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>44.51</td>
<td>0.782</td>
<td>87.80</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Values of $n$ represent linear ($n = 1$), convex ($n > 1$) or concave ($n < 1$) curves.

Based on the results, it was observed that to obtain a high value of $q^*$ corresponding to the highest amount of adsorbate per unit volume of mol/L NaCl). Based on the results, the best pH and I. S. to obtain the highest adsorption capacity ($q^*$) for LPS-IDA-Ca$^{2+}$, and to determine the affinity constant ($K$) for adsorbate-adsorbent were obtained.
adsorbent, in other words, higher adsorption, it is desirable to have the highest values of $K$ and $n$. On the other hand, for the studies of chromatographic columns, $n < 1$ is recommended. In this case, it is considered a favorable isotherm, because high loads can be obtained at low concentration in the liquid phase [43]. From Table 2, it is concluded that the highest values of $K$ were for pH 4.0 and 5.5 with I. S. at 0.5 mol/L. For these conditions of higher $K$, lower values of $n$ were obtained.

Endotoxin concentrations involved in biotechnological processes greatly depend upon the source of the product. They range from less than 100 EU/mL in cell culture supernatants to more than 1,000,000 EU/mL in the supernatants of high cell density bacterial cultivations [15]. In order to compare the influences of pH and I. S. on $q^*$, the adsorption isotherm models were represented for different $c^*$. Fig. 4 shows those results, divided in three concentration intervals, low (a), medium (b) and high (c). pH 4.0 and 5.5 with I. S. at 1.0 mol/L showed the highest values of $q^*$ with 2,677 061 and 2,534 314 EU/mL respectively or equivalent to 26.7 and 25.3 μmol/mL gel, at high concentration values, as the exponent $n$ for these conditions is higher than one ($n > 1$), and therefore, the isotherm does not tend to stabilize at a maximum, as occurs if $n$ is less than one.

The adsorption behavior observed at pH 4.0 and 5.5 with I. S. at 1.0 mol/L, where $n$ is greater than 1, is the typical of systems where the solvent is strongly adsorbed, or that there is a strong intermolecular attraction within the adsorbed layer [44].

According to the obtained data, depending on the concentration of LPS present in the medium, pH and I. S. could be evaluated separately. For instance, at low concentrations of LPS, less than 1,000 EU/mL, the highest adsorption was achieved at pH 4.0 and I. S. of 0.5 mol/L (Fig. 4a), which shows a concave adsorption curve from the axis of concentration in the liquid phase, which tends to stabilize at a maximum. At medium and high concentrations of LPS (Figs. 4b and 4c), the maximum adsorption was achieved at pH 4.0 and I. S. of 1.0 mol/L, demonstrating that for the lower pH and higher I. S., more molecules were adsorbed on the LPS-IDA-Ca\(^{2+}\) matrix. Over the concentration range of LPS studied (low, medium or high), pH 8.0 at any I. S. showed the lower values of adsorption.
Common purification protocols, including several chromatographic steps, may provide sufficient endotoxins clearance. Generally, the high concentrations of endotoxins at the beginning of purification procedures can be reduced to about 100 EU/mL without special treatment \[15\]. High endotoxin removal can be obtained beginning with an initial concentration of 100 EU/mL. It can be demonstrated by combining the isotherm (equation 2) and the mass balance equations (equation 3). The intersection of these two curves is the final operating condition for a batch process. The results show that in the whole range of I.S. and pH between 4 and 5.5, the LPS removal is above 91%. In the case of pH 8.0 the removal of LPS is between 76% and 87%. For all these cases the load in the gel was 3000 EU/mL gel. Further studies are in progress to evaluate the LPS removal and recovery in molecules with different pI. Unpublished results by our group demonstrated that for an antibody fragment with pI 8.8 high protein recovery and endotoxin removal can be achieved when operated both at acidic and basic pH, under the working conditions selected for LPS.

\[
q^* = \frac{(v + V)(C_0 - c^*)}{v}
\]  

(3)

where \(V\) is the sample volume and \(v\) is the resin volume.

The affinity between the molecules of LPS immobilized on the metallic matrix may be related to the electrostatic interaction or coordination bond between the electron donor groups present in the lipid A portion of LPS (phosphate groups) and the electron acceptor groups in the matrix (Ca\(^{2+}\)), where this first interaction is unfavorable in the presence of sodium chloride \[45\]. This interaction is responsible for the adsorption phenomenon between LPS and IDA-Ca\(^{2+}\) and is the basis for IMAC affinity. This coordination bond is related to the pK values of LPS, pK\(_1\) = 1.3 and pK\(_2\) = 8.2 \[46\] and pK of the calcium-IDA complex \[47\]. Thus, it can be considered that at pH 8.0 LPS molecules are more negatively charged and calcium ions less protonated, which would explain the lower adsorption on the positively charged matrix (Ca\(^{2+}\)). Otherwise, for the lowest pH investigated, 4.0 and 5.5, the density of negative charges in the endotoxin molecules decreases slightly in comparison to basic pH, while calcium ions are highly protonated. This would increase the affinity to the matrix, and consequently increase the adsorption capacity.

The effect of I.S. may be associated with the observation of Tan et al. \[23\] who studied the adsorption of LPS on IDA-Fe\(^{3+}\). The authors reported that the increase of I.S. in the adsorption process can influence the increase of aggregate LPS molecules. The comparison of endotoxins removal efficiency by the immobilized metal ions in Tris-NaCl and Tris buffers revealed that endotoxins removal was facilitated at high I.S. This is probably due to the interaction of salt cations (e.g., Na\(^+\)) with negatively charged endotoxins and/or increase in the hydrophobicity of endotoxins with salt addition, compressing the endotoxin molecules and increasing accessibility of endotoxin molecules to the immobilized metal ions. In addition, the screening effect of Na\(^+\) on endotoxins-negative surface charge repulsion and Tris-immobilized metal ion attraction increases availability of metal ions to interact with endotoxins\[23\]. That is, more monomers were added to LPS, growing the micelle structure, reflecting an increase of the adsorption capacity of LPS aggregates with the bound metal, and consequently more LPS molecules were adsorbed on the matrix. This discussion can be corroborated with the results obtained in this study; higher values of I.S. (0.5 and mainly 1.0 mol/L) produced higher LPS adsorption capacities.

The possibility of LPS molecules to form vesicles could explain the lower adsorption capacities obtained at low I.S. In this case, the lipid A portion was being acquired by these structures, reducing the interaction of phosphate groups with the matrix. Thus, the adsorption capacity would be lower at low I.S. as a result of possible steric effects. This effect can be explained because under these conditions (low I.S.) the molecules associated with LPS in vesicular structures would...
be too large to penetrate the pores of the adsorbent. Micelle aggregates of LPS are approximately 10 to 70 nm in size, while the LPS subunits are smaller. The pores of the adsorbent should be large enough to allow free diffusion of particles; for LPS the pores should be larger than 1.4 μm. The manufacturer reported the pore size of about 100 nm for the IDA matrix. Therefore, under these conditions of low I. S. there would be no free distribution of LPS molecules in the pores of this matrix, and thus there would be no interaction with the immobilized affinity ligand.

To find the simultaneous influence of pH and I. S. on the concentration of adsorbed LPS, the results were fitted by statistical models applying a multiple regression (Tables 3 and 4, respectively), according to equation 4.

\[
\ln q = 0.8725 \ln c + 1.4761 \text{pH} - 0.1559 \text{pH}^2 + 5.6339 \text{I. S.} - 3.6641 \text{I. S.}^2
\]  (4)

Table 3 Analysis of variance for the variable \( \ln q \), evaluating effects of LPS concentration, \( \text{pH} \) and ionic strength (mol/L)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression coefficient</th>
<th>Standard deviation</th>
<th>( t )-Value</th>
<th>( p )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \ln c )</td>
<td>0.8725</td>
<td>0.0314</td>
<td>27.7832</td>
<td>0.0000</td>
</tr>
<tr>
<td>( \text{pH} )</td>
<td>1.4761</td>
<td>0.2240</td>
<td>6.5886</td>
<td>0.0000</td>
</tr>
<tr>
<td>I. S.</td>
<td>5.6339</td>
<td>2.3109</td>
<td>2.4379</td>
<td>0.0157</td>
</tr>
<tr>
<td>( \text{pH}^2 )</td>
<td>-0.1559</td>
<td>0.0198</td>
<td>-7.8609</td>
<td>0.0000</td>
</tr>
<tr>
<td>I. S.(^2)</td>
<td>-3.6641</td>
<td>1.7669</td>
<td>-2.0738</td>
<td>0.0394</td>
</tr>
</tbody>
</table>

Table 4 Analysis of variance for multiple regression model for \( \ln q \)

<table>
<thead>
<tr>
<th>Data</th>
<th>Sum of square</th>
<th>DF</th>
<th>Mean square</th>
<th>( F )-Square</th>
<th>( p )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>25278.80</td>
<td>5</td>
<td>5055.76</td>
<td>2524.97</td>
<td>0.0000</td>
</tr>
<tr>
<td>Error</td>
<td>394.45</td>
<td>197</td>
<td>2.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25673.25</td>
<td>202</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the statistical analysis, we can infer that the factors with less influence are I. S. and I. S.\(^2\), given the lower \( t \) values and higher \( p \) values (Table 3). \( \text{pH} \) has an influence on two terms, \( \text{pH} \) and \( \text{pH}^2 \), one positive and one negative, respectively. As \( \text{pH} \) increases, the difference between the two terms becomes smaller, so \( \text{pH} \), when reaching 8.0, will have its smallest impact on \( \ln q \). Also noteworthy is that this effect for \( \text{pH} 4.0 \) and 5.5 is similar. In the case of I. S. the terms of I. S. and I. S.\(^2\) are opposites, but with the highest I. S. the difference is greater, so that increases adsorption. For I. S. values at 0.5 and 1.0 mol/L, the effects on \( \ln q \) are also similar. This model can be used to predict the expected \( q \) for the desired values of \( \text{pH} \), I. S. and \( c \).

3 Conclusion

The method described in this paper is a one-step protocol which is effective in removing LPS from protein histidine tag. Metal chelating is suggested as the interaction mechanism. The adsorption of LPS to the IDA-Ca\(^{2+}\) IMAC column may be due to a coordination bond established with the phosphate group of lipid A, a key part of LPS, and the hydrophobic interaction between LPS molecules. Those interactions result in multi-layer adsorption of LPS on IDA-Ca\(^{2+}\) columns. The highest adsorption capacity obtained was 2 677 061 EU/mL. The IMAC-IDA-Ca\(^{2+}\) is recommended for the removal of contaminating LPS present in different steps of a purification process at concentrations between values less than 100 EU/mL and 100 000 EU/mL, operated at low \( \text{pH} \) (4.0 or 5.5) and the highest ionic strength tested (1.0 mol/L) with the removal of LPS higher than 90%.

Acknowledgment: André Moreni LOPES is grateful for his study at the Biotechnology Development Unit-Laboratory Purification 3, Center for Genetic Engineering and Biotechnology (CIGB, Cuba).

References:

[16] Brito L A, Singh M. J Pharm Sci, 2011, 100; 34
[22] Ansphach F B. J Biochem Biophys Methods, 2001, 49; 661
[27] Porath J. Trends Anal Chem, 1988, 7; 254
[29] Eje N, Lacey E, Codd R. RSC Adv, 2012, 2; 333
[33] Shao T M, Liu Y X, Shao C P. Chinese Journal of Chromatography, 1996, 14(3); 218
[34] Porath J. Protein Express Purif, 1992, 3; 263
[38] Wang Y, Hollingsworth R I. Biochemistry, 1996, 35; 5647
[47] Sulkowski E. Makromol Chem-Makromol Symp, 1988, 17; 334