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Analysis of the Influence of Five Variables on an Established Immunoaffinity Chromatography Procedure to Purify a *Picchia pastoris* Yeast Derived-HBsAg for Pharmaceutical Use

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SUMMARY. Immunoaffinity chromatography (IAC) has a wide application in protein purification. The aim of the study was to investigate the influence of five operational factors on an IAC procedure to purify a yeast derived-Hepatitis B surface antigen (rHBsAg) for pharmaceutical use. The immunosorbent adsorption capacity of the rHBsAg is affected at 4 °C. The applied antigen concentration, 100-1000 μg mL^{-1}, does not have influence on this IAC efficiency. The residence time, applied antigen amount and column geometry have a significant influence on the adsorption and elution capacity, and recovery of the rHBsAg. There is a marked retention of the rHBsAg into the matrix, which is reduced at residence times higher than 2 h. A high height/diameter ratio of the column, 3.75, reduces the antigen adsorption to the matrix but increases the recovery and the productivity for this high molecular weight (multiple chemical forms) antigen and a high affinity constant ligand. The solution of the rHBsAg retention into the matrix could increase by 30% the active pharmaceutical ingredient purification and the Hepatitis B vaccine production.

INTRODUCCION

The envelope of the Hepatitis B virus (HBV) is a macromolecular structure composed by proteins, carbohydrates and host-derived lipids. The main protein of the HBV envelope is the Hepatitis B surface antigen (HBsAg) [1]. Due to its high immunogenicity, this protein is used as the active pharmaceutical ingredient of many recombinant vaccines for human use [2,3].

A large scale purification process for the isolation of the recombinant Hepatitis B surface antigen (rHBsAg) from *P. pastoris* yeast fermentation has been previously described [4]. This purification process employs as main purification step the immunoaffinity chromatography (IAC), a very specific method [5,6] which uses the exquisite specificity of the antibody's complementarity determining regions (CDRs) [7]. These highly selective regions on the antibody surface capture the antigen with high affinity and almost without interactions with impurities or contaminants.

The CB.Hep-1 monoclonal antibody (mAb) is a mouse mAb secreted by the hybridoma 48/1/5/4. This mAb was obtained by fusion of Sp2/0-Ag14 myeloma cells and spleen cells of a BALB/c mouse immunized with the HBsAg purified from a chronically infected Hepatitis B human serum [8]. The CB.Hep-1 is an IgG-2b kappa light chain mAb, specific for the "a" determinant of the HBsAg. This molecule specifically inter-

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acts with the Cys-Lys-Thr-Cyst-Thr-Thr sequence within the "a" determinant of the HBsAg and it is routinely used in the chromatographic purification, IAC, process of the rHBsAg for the production of the commercial available vaccine Heberbiovac™ HB [9,10].

Several papers have been published on the manufacture of this specific immunosorbent, but these papers only focused on the study of the ligand density, immunosorbent manufacture procedures and antigen elution conditions [11-14]. Nevertheless, the best behavior of a given immunosorbent also depends on other operational factors such as: applied antigen concentration, applied antigen amount, residence time, temperature and column geometry [6,15].

The aim of the present study was to investigate the influence of these operational factors on the adsorption capacity, elution capacity and antigen recovery of the CB.Hep-1 immunosorbent to make a contribution to the understanding of the purification of this pharmaceutical protein. An important variable that characterize the immunopurification, ligand leakage, was excluded from this study because we have not observed influence of the ligand leakage on the IAC behavior.

MATERIALS AND METHODS

CB.Hep-1 mAb manufacturing

Cells were cultivated in 1L spinner flasks, starting from $3x10^5$ cells mL^{-1}, using RPMI-1640 (GIBCO-BRL, Gaithersburg, USA) supplemented with 8% of foetal bovine serum (GIBCO-BRL, Gaithersburg, USA), 2 mM L-glutamine, 1 mM sodium piruvate and 17 mM sodium bicarbonate. Cells were always maintained at 37 °C in 5 % CO_2 atmosphere and the medium was changed every 48 h up to reach a maximal cell density. BALB/c males and females mice of 24 ± 2 g of weigh were used for ascites production. Animals were primed with 0.5 mL of mineral oil into the abdominal cavity 10 days before cell inoculation. The ascites was harvested under aseptic conditions inside of a sterile hood by abdominal paracentesis and centrifuged at 2000 rpm to separate cells from the liquid phase. The ascites was filtered and underwent to two ammonium sulfate precipitations. In both precipitations, the material was centrifuged at 4800 x g for 20 min at 4 °C. The dissolved pellet was desalted by size-exclusion chromatography in Sephadex G-25 coarse (Amersham-Biosciences, Uppsala, Sweden) using a BP113/60 column (Amersham-Biosciences, Uppsala, Sweden) at 130 cm h^{-1} of linear flow-rate and 150 mM phosphate buffered saline solution (PBS) pH 8.0 as mobil phase. Then the desalted material was purified by Protein A-Sepharose fast flow affinity chromatography using 150 mM PBS pH 8.0 adsorption buffer and 100 mM citric acid, pH 3.0 as elution buffer. The column used was a BPG100/50 (Amersham-Biosciences, Uppsala, Sweden) at a linear flow rate of 100 cm h^{-1}. Subsequently, incubation in 100 mM citric acid pH 3.0 at 4 °C was carried out as virus inactivation step. Immediately after, the buffer of the sample was exchanged to 20 mM Tris/150 mM NaCl, pH 7.6 by size-exclusion chromatography in Sephadex G-25 coarse using a BP113/60 column (Amersham-Biosciences, Uppsala, Sweden) at a linear flow rate of 130 cm h^{-1}. The SDS-PAGE purity of mAb CB.Hep-1 was always higher than 95% (Fig. 1).

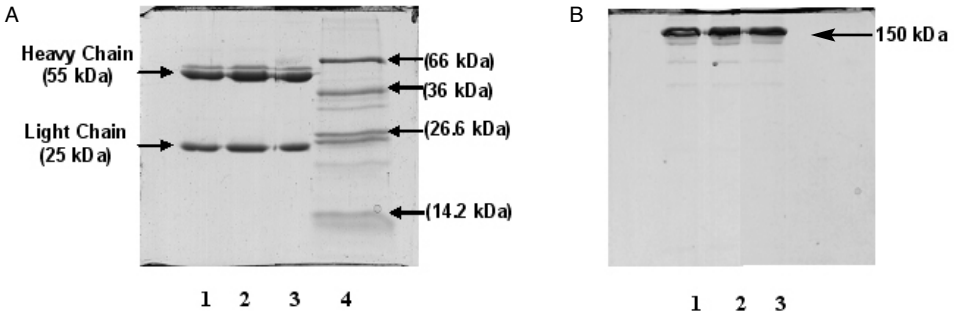

Figure 1. Coomassie blue stained SDS–PAGE under reducing conditions of the purified CB.Hep-1 mAb. (**A**) Lines 1 and 2, purified CB.Hep-1 mAb. Line 3, inner IgG control. Line 4, molecular weight markers. Selected proteins as molecular markers (Sigma-Aldrich ,St. Louis, MO, USA) were: Bovine serum albumin, 66 kDa; Glyceraldehyde-3-phosphate dehydrogenase, 36 kDa, Triosephosphate isomerase, 26.6 kDa, α-Lactalbumin, 14.2 kDa. (**B**) Coomassie blue stained SDS–PAGE under no reducing conditions of the purified CB.Hep-1 mAb. A. Lines 1 and 2, purified CB.Hep-1 mAb; Line 3 inner IgG control.

Source of the rHBsAg

The purified rHBsAg used as chromatographic column application material was obtained from a recombinant *P. pastoris* yeast strain cultivated in a bioreactor using a mixture of common inorganic salts such as: $(NH_4)2SO_4$, KH_2PO_4, and $MgSO_4$. The size- exclusion- high performance liquid chromatography purity of the rHBsAg was higher than 99% [16].

Protein quantification and CB.Hep-1 mAb estimation by an enzyme-linked immunosorbent assay (ELISA)

Protein concentration was quantified according to Lowry *et al.* [17] using bovine serum albumin as standard material. The range of the calibration curve was 100-500 µg mL^{-1}. The CB.Hep-1 mAb concentration was estimated as follow. A polystyrene microplate (Costar, Cambridge, USA) was coated with 10 µg per well of the rHBsAg in 100 mM $NaHCO_3$ buffer for 20 min at 50 °C. Then, samples were added to the plate in 0.05 % Tween 20/150 mM PBS and incubated for 1 h at 37 °C. Several washes with 0.05 % Tween 20/150 mM PBS were done and subsequently plate was incubated for 1 h at 37 °C with a horseradish peroxidase conjugate (Sigma Chemical, St. Louis, USA). The reaction was then revealed using 100 µL per well of 0.05 % *o-phenylenediamine* and 0.015% H_2O_2 in citrate buffer; pH 5.0, and stopped with 50 µL per well of 1.25M H_2SO_4. Absorbance was measured by a Multiskan ELISA Reader (Labsystems, Helsinki, Finland) using a 492-nm filter [18]. The range of the calibration curve was 3.13-50 ng mL^{-1}. The inner standard mAb used was the IgG2B070305 supplied by the Center for Genetic Engineering and Biotechnology, Havana, Cuba. The eluted antigen concentration was determined by UV measurement [A280 (1 cm, 1mg mL^{-1}) = 5]. This molar absorption coefficient was determined by the Pace's procedure [19].

Immunosorbent manufacturing

The Sepharose CL-4B (Amersham-Biosciences, Uppsala, Sweden) was moderately activated with CNBr (Merck, Darmstadt, Germany) according to Wilcheck classification (6-12 µmol cyanate esters mL^{-1} of matrix) [20]. The activation reaction was made as Axen, Porath and Ernback´s work [21]. Concentration of cyanate esters was determined by a modified König reaction [22]. The CB.Hep-1 mAb coupling to the matrix was performed according to a previously described procedure [13] and the selected ligand density was 3.7 mg mL^{-1} of matrix. This ligand density value is within the parameter optimal range of the immunosorbent [13].

Immunoaffinity chromatography common aspects

The IAC was performed in PD-10 columns (Amersham-Bioscences, Uppsala, Sweden) packed with 12.1 mL of immunosorbents under hydrostatic pressure. Inmunosorbents were equilibrated with 60 mL of 20 mM Tris–3 mM EDTA–1M NaCl, pH 7.0. Columns were directly loaded with the purified rHBsAg in the same buffer. Columns were washed with the equilibrium buffer and the elution was performed using 20 mM Tris–3 mM EDTA–1M NaCl–3M KSCN, pH 7.0. The flow rate was the same for all steps and its value depended on the specific conditions for each experiment.

Chromatography conditions for control experiment

Values of residence time (1 h), applied antigen concentration (100 µg mL^{-1}), applied antigen amount (0.58 mg mL^{-1} of matrix), temperature (25 °C) and column geometry (3.75 h/d) used at the rHBsAg industrial-scale purification process served as control during experiments.

Temperature influence experiment

In order to know the influence of the temperature on the rHBsAg immunopurification capacity, three temperatures were studied: 4, 25 and 33 °C. Immunoaffinity systems were placed into an oven WTBbinder type 78532 (Tuttlingen, Germany) to guarantee the specific temperature in all parts of the systems and buffers.

Applied antigen concentration influence experiment

Experiments developed to study this variable were performed using the same control chromatography conditions except for the applied antigen concentration, which was also evaluated at 200, 400 and 1000 µg rHBsAg mL^{-1}.

Residence time influence experiment

The studied residence times were 1, 2 and 3 h. The rest of the chromatographic variables were similar to the control experiment.

Applied antigen amount influence experiment

This variable was explored at four levels, 0.58, 1.24, 1.65 and 2.31 mg rHBsAg mL^{-1} of

matrix, like other variables mentioned above. The rest of the chromatographic parameters were invariable respect to the control experiment.

Column geometry influence experiment

To evaluate the influence of the column geometry on IAC efficiency; a column with an unusual diameter, 2.5 cm, was used. The packed matrix volume was different to the control while the rest of the factors were kept constant. The evaluated height/diameter ratios were 0.48 (5.9 mL of matrix), 0.96 (11.8 mL of matrix) and 3.75 (control). The control experiment was performed in a PD-10 column.

Statistical analysis

The Statgraphics Plus version 5.0 (2000) from Statistical Graphics Corp. and Microsoft Excel programs were use as tools for the statistical analysis. The Kruskall-Wallis test was employed in all multiple sample comparisons for different analyzed variables. This statistical test is a non-parametric test recommended for independent samples [23,24]. The significance level used was 0.05 and the experimental groups were statistically compared in terms of adsorption capacity, elution capacity and antigen recovery. All experiments were made in triplicate.

RESULTS AND DISCUSSION

The infection with HBV leads to the production of large virus particles of about 42-nm together with surface-antigen particles of 22-nm average size (HBsAg). For the production of this recombinant molecule as active pharmaceutical ingredient of the Hepatitis B vaccine, the HBsAg gene was integrated by homologous recombination to the genome of the *P. pastoris* strain MP-36 (his 3). The downstream process for purifying the rHBsAg was established first at laboratory scale [25-28] and further scaled-up to 500-fold [25]. However, the effectiveness of some key steps for the large-scale production of *P. pastoris*-derived HBsAg was described in details in the year 2000 [16].

This purification process employs as main purification step the IAC, using as ligand the CB.Hep-1 mAb. Therefore, the aim of the present study was to investigate the influence of several variables on the adsorption capacity, elution capacity and antigen recovery of the CB.Hep-1 mAb immunosorbent to make another contribution to the understanding of the purification of this pharmaceutical protein by IAC.

Temperature influence

The temperature has an important influence on the antigen adsorption, antigen dissociation and ligand stability [29-31]. Results demonstrated significant differences in the adsorption capacity at 4 °C, which was confirmed by the Kruskall-Wallis test ($p = 0.039$). This parameter was about 25% lesser than those values observed at 25 and 33 °C respectively (Table 1). Nevertheless, the elution capacity did not show relevant significant differences among assessed temperatures ($p=0.491$) and the rHBsAg retention into de matrix was similar: 30.2 (4 °C), 35.9 (25 °C), and 36.7 (37 °C). Therefore it suggests that the association probability in the loading buffer between the CB.Hep-1 mAb and the rHBsAg is higher at 25 and 33 °C and the antigen-antibody dissociation was not affected in the range of temperature. Thus, we confirmed that the only parameter affected by the temperature was the adsorption capacity of the antigen during this IAC.

Applied antigen concentration influence

The antigen concentration during the application step must be correctly selected. The application of diluted samples could increase the processing time without allowing an appreciable increment of the process efficiency of the ICA. True adsorption capacity values (Fig. 2) for each applied antigen concentration were not statistically different ($p = 0.103$). This result is notable because the chromatography time could be reduced up to 10 fold compared to the control conditions, 100 μ mL^{-1}, without any important antigen lost. The elution capacity showed a similar behavior to the adsorption capacity ($p = 0.237$, Fig. 2) and the average recovery for all experiments was 51.59%, which is within the

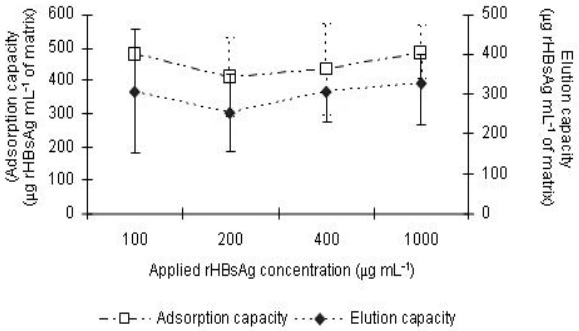

Figure 2. Results of the CB.Hep-1 mAb immunosorbent adsorption and elution capacity using different applied antigen concentrations. The figure shows the average and 95% confidence intervals for each experiment.

Temperature (°C)	Adsorption Capacity (µg rHBsAg mL^{-1} of matrix)	Elution Capacity (µg rHBsAg mL^{-1} of matrix)	Recovery (%)
4	358.69 ± 28.41	250.27 ± 32.49	43.26 ± 5.62
25	480.33 ± 4.64	308.07 ± 62.79	53.25 ± 10.85
33	440.63 ± 51.00	278.86 ± 23.39	48.20 ± 4.04

Table 1. Results of the influence of the temperature on the CB.Hep-1 mAb immunosorbent adsorption capacity, elution capacity and recovery. Reported values are averages and standard deviations.

Residence time (h)	Adsorption Capacity (µg rHBsAg mL^{-1} of matrix)	Elution Capacity (µg rHBsAg mL^{-1} of matrix)	Recovery (%)
1	480.33 ± 4.64	308.07 ± 62.79	53.25 ± 10.85
2	482.97 ± 218.79	427.00 ± 206.47	73.81 ± 12.15
3	475.21 ± 93.59	437.47 ± 74.09	75.61 ± 12.82

Table 2. Results of the influence of the residence time on the CB.Hep-1 mAb immunosorbent adsorption capacity, elution capacity and recovery. Reported values are averages and standard deviations.

range of values for other previously reports [32,33]. Concluding this set of experiments, the concentration of the applied antigen does not have influence on the recovery of the antigen.

Residence time influence

Usually the residence time is considered as the most important parameter in the affinity chromatography. In this study, three residence times were studied. Statistical differences were not observed among adsorption capacities ($p = 0.836$) and among elution capacities ($p = 0.061$). However, we observed an increment about 20 and 22 % in the elution capacity at 2 and 3 h of the residence time respectively (Table 2). Perhaps, these non-significant differences observed could be explained by value dispersions. The recovery increase could be produced by an augment in the chromatography radial diffusion coefficient at higher residence times. Thus, we recommend 2 h of residence time. Nevertheless, 3 h of residence time allowing for 2.3 fold more concentrated the eluted antigen, which could be convenient for further antigen processing. An additional interesting and important finding is that the rHBsAg retention into the matrix (percentage of the eluted antigen respect to the adsorbed antigen) decreased with the increment of the residence time (Table 2).

Applied antigen amount influence

The adsorption efficiency decreased with the increase of the applied antigen amount to the columns (Fig. 3a). However, significant differences were not observed from 0.58 to 1.65 mg rHBsAg mL^{-1} of matrix ($p = 0.193$). A drastic reduction in this parameter was detected when the applied amount of antigen was 2.31 mg rHBsAg mL^{-1} of matrix, especially during the second and the third purification cycles, where the adsorption capacity was about 27% of the first purification cycle. The best elution capacity was reached at 1.24 and 1.65 mg rHBsAg mL^{-1} of matrix while a substantial decrease was ob-

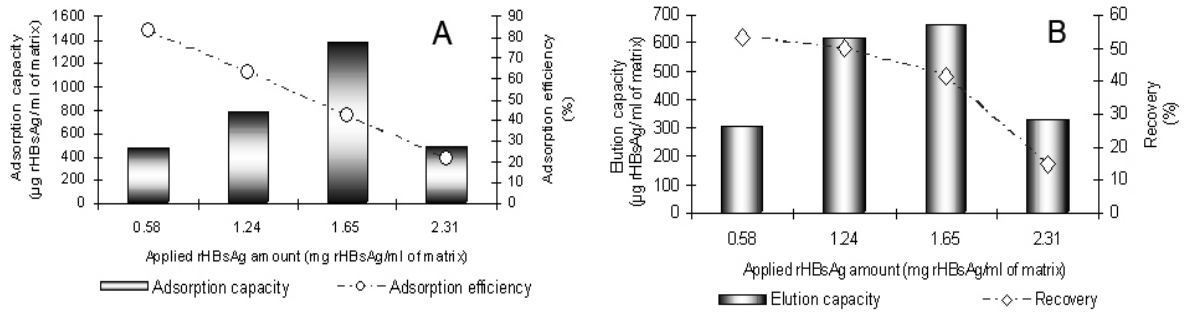

Figure 3. **A**) Results of the comparison of the CB.Hep-1 mAb immunosorbent adsorption capacity and efficiency using different applied antigen amounts. **B**) Results of the comparison of the CB.Hep-1 mAb immunosorbent elution capacity and recovery using different applied rHBsAg amounts.

Height/Diameter Ratio	Adsorption Capacity (μg rHBsAg mL^{-1} of matrix)	Elution Capacity (μg rHBsAg mL^{-1} of matrix)	Recovery (%)
0.48	996.67 ± 80.07	33.82 ± 5.69	5.80 ± 0.98
0.96	418.33 ± 71.26	53.50 ± 67.13	9.17 ± 11.51
3.75	480.33 ± 4.64	308.07 ± 62.79	53.25 ± 10.85

Table 3. Results of the influence of the column geometry on the CB.Hep-1 mAb immunosorbent adsorption capacity, elution capacity and recovery. Reported values are averages and standard deviations.

served at 0.58 and 2.31 mg rHBsAg mL^{-1} of matrix (Fig. 3b). The recovery was similar in the range between 0.58 to 1.65 mg rHBsAg mL^{-1} of matrix ($p = 0.430$). These results also allow the confirmation that the amount of the applied antigen 1.65 mg rHBsAg mL^{-1} of matrix is the best condition for this chromatography.

Column geometry influence

Previous papers have reported the poor mass transfer of rHBsAg into the Sepharose CL-4B matrix [14,28]. Experiments of this study demonstrated the highest antigen adsorption for the lowest height/diameter ratio, 0.48 (Table 3). These differences were statistically significant ($p = 0.027$) at 95% confidence level. Height/diameter ratios of 0.96 and 3.75 provoked a decrease by 58 and 48% respectively in the rHBsAg adsorption capacity. These results suggest better antigen diffusion inside of matrix bead during the loading stage at low height/diameter ratio. Conversely, the elution capacity increased in proportion with the increment of the height/diameter ratio. For instance, the elution capacity of the CB.Hep-1 mAb immunosorbents packed at 0.96 and 3.75 column geometry ratios were 1.58 and 9.11 fold higher respectively than the elution capacity obtained at 0.48 column geometry ratio ($p = 0.028$, Table 3). This phenomenon is the most interesting finding of this study and contradictory with the hypothesis that small height/diameter ratio is more convenient for affinity chromatography. Thus, an exceptional consideration should be done for a system, which involve an antigen with multiple chemical forms and high molecular weight such as: the rHBsAg (particle of ~$2x10^6$ Da), relative small pore size of the matrix (antigen diffusion problems) and a ligand with high association constant (10^8-10^9 M^{-1}). Because the deep penetration of the antigen inside the matrix during adsorption stage would make difficult the antigen releases during the elution stage. This generalization could be applied to any purification of antigens with high molecular weigh by IAC.

CONCLUSIONS

The immunosorbent adsorption capacity of the rHBsAg is affected at 4 °C. The applied antigen concentration, 100-1000 μg mL^{-1}, does not have influence on this IAC efficiency. The residence time, applied antigen amount and column geometry have a significant influence on the adsorption capacity, elution capacity and recovery of the rHBsAg. There is a marked retention of the rHBsAg into the matrix, which is reduced at residence times higher than 2 h. A high height/diameter ratio of the column reduces the antigen adsorption to the matrix but increases the recovery and the productivity for this high molecular weight antigens (multiple chemical forms) and a high affinity constant ligand. The solution of the rHBsAg retention into the matrix could increase by 30% the active pharmaceutical ingredient purification and the Hepatitis B vaccine production.

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REFERENCES

1. Gavilanes, F., J. M. González-Ros & D.L. Peterson (1982) *J. Biol. Chem.* **257**: 7770-7.
2. Ellis, R.W (1990) *"New Generation Vaccines",* (G.C. Woodrow & M.M. Levine ed.), Marcel Dekker, New York, p. 439.
3. Ellis, R.W & P.J. Keniskern, (1991) *"Molecular Biology of Hepatitis B Virus",* A. (McLachlan ed.), CRC Press, Boca Raton, Fda., p. 3077.
4. Pentón, E., L. Herrera, V. Muzio, V. Ramírez, A. García, C. Duarte, C. Ruiz, M. Izquierdo, L. Pérez, G. Fontirrochi, M. Gonzáles, M. Nazabal, A. Beldarraín, G. Padrón, J. García, G. de la Riva, A. Santiago, F. Ayan, R. Páez, A. Agraz, R. Díaz & Y. Quiñones (1992) *Eur. Pat. Apl.* **480**: 525.
5. Wilcheck, M. & T. Miron (1999) *React. Funct. Polym.* **41**: 263-8.
6. Yarmush, M. L., A.M. Weiss, K. P. Antonsen, D. J. Odde & D. M. Yarmush (1992) *Biotech. Adv.* **10**: 413-46.

7. Iwahashi M., D. Milenic, E. Padlan, R. Bei, J. Schlom & S. Kashmiri (1999) *Mol. Immunol.* **36**: 1079-91.
8. Fontirrochi, G., M. Dueñas, M.E. Fernández de Cossio, P. Fuentes, M. Pérez, D. Mainet, M. Ayala, J.V. Gavilondo & C.A. Duarte (1993) *Biotechnol. Appl.* **10**: 24-30.
9. Fernández de Cossío, M.E., T. Díaz, A. Galván, R. Valdés, E. González, M. Ayala, J. Díaz, M. Bestagno, O. Burrote & J. Gavilondo (1997) *J. Biotechnol.* **56**: 69-80.
10. Geada, D., A. Sánchez, V. Besada, R. Valdés, L. Betacourtt, Y. Ramos, Y. Támbara, L. González, S. Padilla, W. Ferro, L. Gómez, A. Tamayo, O. Mendoza & C. Borroto (2007) *Bioprocessing J.* **6**: 8-13.
11. Hernández, R., E. Chong, R. Morales, E. Pérez, Y. Amador, J. R. Zubiaurrez, R. Valdés, A. Figueroa, A. Agraz & L. Herrera (2001) *J. Chromatogr. B* **754**: 77-83.
12. Gómez, L., R. Hernández, N. Ibarra, R. Valdés, Y. Campos, A. Tamayo, D. Fernández, A. Figueroa, T. Álvarez & J. A. Montero (2002) *J. Biochem. Biophys. Meth.* **52**: 151-9.
13. Hernández, R., L. Plana, L. Gómez., N. Expósito, J. Valdés, R. Páez, E. Martínez & A. Beldarraín (2005) *J. Chromatogr. B* **816**: 1-6.
14. Ibarra, N., A. Caballero, E. González & R. Valdés (1999) *J. Chromatogr. B* **735**: 271-7
15. Chase, H.A. (1984) *Chem. Eng. Sci.* **39**: 1099-125.
16. Hardy, E., E. Martínez, D. Diago, R. Díaz, D. González & L. Herrera (2000) *J. Biotechnol.* **77**: 157-67.
17. Lowry, D.H., N.J. Rosembrough, A.L. Farr & R.J. Randal (1951) *J. Biol. Chem.* **193**: 265-9.
18. Valdés, R., T. Alvarez, A. Tamayo, E. Fernandez, J. Montero, D. Geada, W. Ferro, Y. Medina, Y. Guevara, S. Padilla, D. Cecilia, L. Dorta, M. González, R. Sosa & T. González (2008) *Chromatographia* DOI: 10.1365/s10337-008-0607-5.
19. Pace, C.N., F. Vajdos, L. Fee, G. Grimsley & T. Gray (1995) *Protein Sci.* **4**: 2411-23.
20. Wilchek, M., T. Miron & J. Kohn (1984) *Methods Enzymol.* **104**: 3-55.
21. Axen, R., J. Porath & S. Ernback (1967) *Nature* **214**: 1302-4.
22. Kohn, J. & M. Wilchek (1978) *Biochem. Biophys. Res. Commun.* **84**: 7-14.
23. Gómez, M., C.D. Banck & L. Vega (2003) Rev. Mex. Pediatría 70: 91-9.
24. Fernández, E., M. Fernández, R. Oliveira, B. Bermudez, I. Pérez, Y. Morón, V. García, I. Perdomo & N. Pérez (2008) *Lat. Am. J. Pharm.* **27**: 1-11.
25. Agraz, A., Y. Quinónes, N. Expósito, F. Breña, Y. Madruga, E. Pentón & L. Herrera (1993) *Biotech. Bioeng.* **42**: 1238–44.
26. Páez, R., A. Agraz & L. Herrera (1993) *Acta Biotechnol.* **13**: 117–22.
27. Agraz, A., C. Duarte, L. Costa, L. Pérez, R. Páez, V. Pujol & G. Fontirrochi (1994) *J. Chromatogr. A.* **672**: 25–33.
28. Pérez, L., S. López, A. Beldarraín, D. Arenal & E. Pentón (1994) *Adv. Bioprocess Engin.* 527-34.
29. Roy, P., C.M. Roth, M.N. Margolies & M.L. Yarmush (1999) *Mol. Immunol.* **36**: 1149-58.
30. Ohlson, S., M. Bergström, P. Påhlsson & A. Lundblad (1997) *J. Chromatogr. A.* **758**: 199-208.
31. Zheng, J.Y. & L.J. Janis (2006) *Int. J. Pharm.* **308**: 46-51.
32. Liitti, S., M.T. Matikainen, M. Scheinin, T. Glumoff & A. Goldman (2001) *Protein Expres. Purif.* **22**: 1-10.
33. Van Sommeren, A.P.G., P.A.G. Machielsen, W.J.G. Schielen, H. P. J. Bloemers & T.C.J. Gribnau (1997) *J. Virol. Meth.* **63**: 37-46.