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# DEVELOPMENT OF A NEW PROCESS FOR PURIFICATION OF CAPSULAR POLYSACCHARIDE FROM *Streptococcus pneumoniae* SEROTYPE 14

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**Abstract -** The main virulence factor of *Streptococcus pneumoniae* is the capsular polysaccharide (PS), which is the antigen of all current vaccines that are prepared with PS purified from serotypes prevalent in the population. In this work, three purification strategies were evaluated and a new process was developed for purification of serotype 14 PS (PS14), responsible for 39.8% of diseases in children of 0-6 years old in Brazil. The developed method consists of cell separation by tangential microfiltration, concentration of the microfiltrate by tangential ultrafiltration (50 kDa), diafiltration in the presence of sodium dodecyl sulfate using a 30 kDa ultrafiltration membrane, precipitation with 5% trichloroacetic acid, precipitation with 20% and 60% ethanol, and anion exchange chromatography. The required purity regarding nucleic acids (≤ 2%) and proteins (≤ 3%) was achieved, resulting in a relative purity of 439 mg PS14/mg nucleic acids and 146 mg PS14/mg proteins. The final polysaccharide recovery was 65%, which is higher than the recovery of the majority of processes described in the literature.
*Keywords*: Pneumococcal vaccine; Purification strategy; Sodium dodecyl sulfate; Trichloroacetic acid; Anion exchange chromatography.

## INTRODUCTION

*S. pneumoniae* or pneumococcus is an encapsulated Gram-positive bacterium that colonizes the human upper respiratory tract and can cause severe diseases, such as pneumonia, bacteremia, meningitis and sepsis, which are responsible for about half a million deaths of children above 5 years old worldwide (WHO, 2012). The capsular polysaccharide covers the pneumococcal external surface and is the main virulence factor of this microorganism, due to the ability of this molecule to inhibit phagocytosis. Polysaccharides are high molecular mass polymers formed by repeated subunits of oligosaccharides. Chemical differences in PS structure differentiate the pneumococci into more than 90 immunologically distinct serotypes, each one with different capability to provoke diseases (Yother, 2011). In Brazil, the serotype 14 is responsible for 39.8% of pneumococcal diseases in children between 0 and 6 years old and for 10.8% of diseases in 6-14-year-old children. This serotype is also the most prevalent in Latin America and presents a high level of antibiotic resistance (*Organização Pan-Americana de Saúde*, 2011); therefore, the PS from serotype 14 (PS14) is present in all commercially available pneumococcal vaccines.

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The classical purification processes of bacterial capsular PS used in vaccines include several selective precipitations with ethanol and/or cationic detergent, followed by continuous centrifugation and ultracentrifugation and deproteinization with phenol (Frasch, 1990). In our laboratory, purification processes were developed for purification of capsular polysaccharides of *Neisseria meningitidis* (Tanizaki *et al*., 1996)*, Streptococcus pneumoniae* (Gonçalves *et al*., 2003; Gonçalves *et al*., 2007) and *Haemophilus influenzae* type b (Takagi *et al*., 2008). In these processes, the number of ethanol precipitation steps and centrifugations were reduced and the use of phenol was completely eliminated, being replaced by treatment with endonucleases and proteases, followed by tangential ultrafiltration, wherein the low molecular weight hydrolysate material was removed while PS was retained in the concentrate. The tangential filtration in these processes replaced the ultracentrifugation, with the advantage of being less expensive.

Among the various publications that describe the purification methods to obtain PS from *S. pneumoniae*, Yavordios and Cousin (1983) described the inactivation of the fermented broth using phenol, alcoholic precipitations, enzymatic treatment and treatment with the cationic detergent cetyltrimethylammonium bromide (CTAB), followed by diafiltration. Cano *et al.* (1980) described the cell lysis with sodium deoxycholate (DOC), two alcoholic precipitations, responsible for removing large amounts of proteins and other contaminants, followed by treatment with CTAB, three alcoholic precipitations, treatment with activated charcoal, in addition to filtration and diafiltration. However, due to the large structural diversity, these two processes must be adapted according to the physicochemical properties of each pneumococcal PS.

Recent studies were concerned with eliminating ultracentrifugation and replacing the use of phenol and enzymatic treatment for *S. pneumoniae* polysaccharide purification. Jung *et al.* (2011) described the purification process of PS from serotype 19A (PS19A) with the following steps: cell lysis with DOC and boiling, pH decrease, alcoholic precipitations and dialysis. Macha, *et al.* (2014) described the purification process of PS from serotypes 3, 6B, 14, 19F and 23F substituting phenol precipitation and enzymatic hydrolysis for adsorption on aluminum phosphate and ultrafiltration. Yuan *et al.* (2014) purified PS from serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F and 23F through the following steps: cell lysis with DOC, centrifugation, ultrafiltration/diafiltration, pH decrease and filtration of the precipitate using activated carbon, and another filtration / dialfiltration.

Despite the importance of PS14 for pneumococcal vaccines, there are only few publications specifically on its production and purification. PS14 is one of the few neutrally charged pneumococcal PS (Lindberg *et al*., 1977) and its repeat unit consists of a tetrasaccharide consisting of →6)-β-DGlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→, with monosaccharide side chains of a β-D-Galp(1→ linked to C4 of each N-acetylglucosamine residue (Kolkman *et al*., 1997). The PS14 purification method published by Institut Mérieux (1980) is based on cell lysis by DOC, selective precipitation with 15-35% and 50-70% ethanol, deproteinization with phenol and elimination of nucleic acids by activated charcoal, dialysis and another precipitation with 50-70% ethanol. Another purification method for PS14, described by Suàrez *et al*. (2001), eliminates the use of organic solvents by including an affinity chromatography in the process: after the cell lysis with DOC and centrifugation, the supernatant was concentrated by ultrafiltration, dialyzed and lyophilized, the sample is suspended in a sodium phosphate buffer, centrifuged and the supernatant is applied to a soy lecithin column, which has affinity for D-galactose and N-acetyl-D-glucosamine, therefore binding the residues of these sugars present in PS14, and the elution fraction is dialyzed against distillate water and lyophilized. The methods of Macha *et al*. (2014) and Yuan *et al*. (2014) were also applied to PS14 purification.

Since the purification process of a biological product is responsible for 20%-80% of the total production costs (Ansejo and Patrick, 1990), the development of new purification strategies is essential to reduce the production cost and allow the distribution of the vaccine for the entire population by the public health system. Hence, the objective of this work was to develop a robust purification process of PS14, obtained from different fermentation broths, in order to achieve the required purity regarding proteins (≤ 3% m/m) and nucleic acids (≤ 2% m/m) (WHO, 2005), independently of variations in the starting material.

## MATERIALS AND METHODS

### Materials

The cultivations were carried out in R'ALF Plus (Bioengineering, Wald, Swiss) and BioFlo2000 (New Brunswick Scientific, Enfield, CT, USA) bioreactors. The microfiltration membrane of 0.22 μm, 0.5 $m^2$ (Pellicon 2 Cassette Filter Module GVPP), the spiral mem-

brane of 50 kDa nominal cutoff, 0.54 m² (Prep/Scale PTQK 50 kD NMWL), the LabScale TFF system and ultrafiltration membrane cassettes Pellicon XL of 50 cm², PLCTK 30K, regenerated cellulose, 50 cm² and Biomax 50 K, polyethersulfone, were all purchased from Millipore (Bedford, MA, USA). The sodium dodecylsulfate was purchased from Dinâmica (Diadema, SP, Brazil). The tris hydroxymethylaminomethane (Tris) was purchased from Vetec (Duque de Caxias, RJ, Brazil). The trichloroacetic acid (TCA) and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or from Merck (Darmstadt, Germany). The anion exchange chromatography column HiTrap™ Q-Sepharose Fast Flow (1 mL) and Äkta Avant 150 chromatograph were all purchased from GE Lifescience. The rabbit serum against *S. pneumoniae* group 14 was obtained from Statens Serum Institut (Copenhagen, Denmark). Standard pneumococcal purified PS14 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All other reagents were of analytical grade.

**Microorganism and Cultivation**

The strain 5287 used in this work is a clinical isolate of *S. pneumoniae* serotype 14 deposited in the *Instituto Adolfo Lutz, Seção de Bacteriologia*, SP, Brazil. This strain was previously selected due to the high amount of PS14 produced (Gogola *et al*., 2012). The cultivations were carried out using different media and culture conditions.

Continuous cultivations using chemically defined medium (CDM) were carried out in a 500 mL R'ALF Plus bioreactor, using the conditions described by Ferri (2013). Batch cultivations using complex medium were carried out in a 5 L BioFlo2000 bioreactor, according to the conditions described by Gogola-Kolling *et al*. (2014).

**Purification Strategy**

After cultivation, the fermented broth was inactivated with 0.05% thimerosal. Cells were removed by tangential flow microfiltration and the cell-free microfiltrate was concentrated by tangential flow ultrafiltration in a spiral membrane. The concentrate was washed with saline, 0.9% (m/v) NaCl, using 10 times the concentrate volume. Particles and molecules smaller than the pore size were eliminated and the fraction called 50 kDa-concentrate-saline was stored at -20 °C for further purification. Three purification processes were evaluated, each one performed in duplicate (Figure 1). The starting material for purification strategies 1 and 3 was the fermented broth obtained in continuous cultivation with CDM. The starting material for the strategy 2 was obtained in bath fermentation with complex medium.

**Diafiltration with SDS**

The 50 kDa-concentrate-saline was thawed and diafiltrated in a *LabScale* system with three membranes of 30 kDa or 50 kDa. The PS14 was retained in the concentrate fraction, called 30 kDa-concentrate-SDS or 50 kDa-concentrate-SDS, depending on the membrane employed. Molecules smaller than the membrane pore were removed in the ultrafiltrate.

The following solutions were sequentially used for diafiltration: 0.5% (m/v) SDS in 25 mM Tris buffer containing 2 mM EDTA, 0.9% (m/v) NaCl in 25 mM Tris buffer and 25 mM Tris buffer, all solutions at pH 7.0. The concentrate was washed with 6 times the concentrate volume for each solution.

**Precipitation with TCA**

The water-dissolved fraction was precipitated with 5% (m/v) TCA in an ice bath for 30 min (Saeed, Thomassen, 1982). After, the suspension was centrifuged at 4 °C and 17.696 g for 1 h. The supernatant containing PS14 was neutralized with 5 M sodium hydroxide.

**Ethanol Precipitation Steps**

The ethanol precipitation steps were performed according to the Institut Merieux (1980), using 5% (m/v) sodium acetate and 20% (v/v) ethanol for the first precipitation and 8% (m/v) sodium acetate and 60% (v/v) ethanol for the second.

**Anion Exchange Chromatography**

The column volume (CV) was 1 mL and the flow rate was 1 mL/min for the anion exchange chromatography (AEX). The resin was equilibrated with 20 CV of 10 mM phosphate buffer, pH 7.5, and re-equilibrated with 20 CV of the same buffer after sample application. The PS14 was recovered in the non-adsorbed fraction.

**Analytical Procedures**

PS14 concentration was measured by the capture ELISA method (Gogola *et al*., 2012), using rabbit

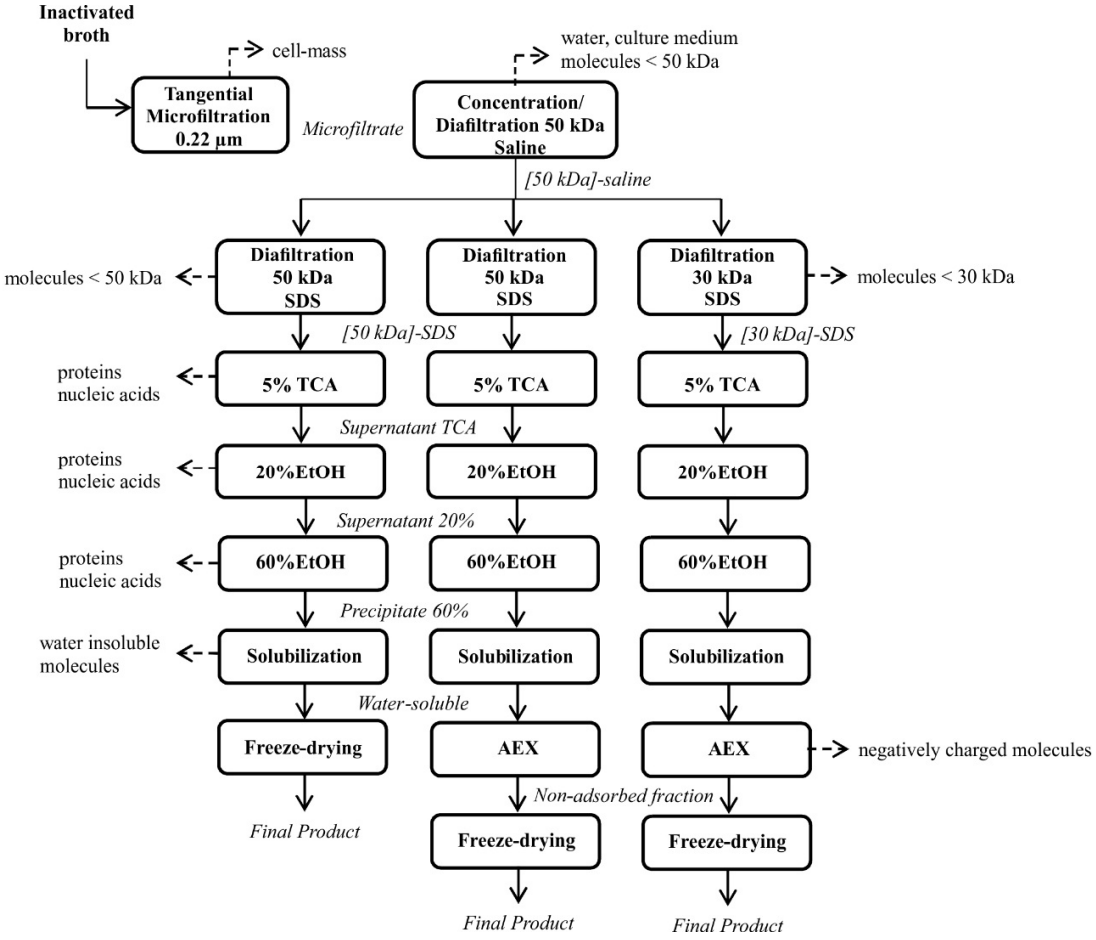


**Figure 1:** Flow diagram for purification of pneumococcal PS14. Fractions containing PS14 are in italics and unit operations in boxes. Dashed arrows indicate compounds eliminated in each step. [30 kDa] and [50 kDa] – concentrate fractions in 30 kDa or 50 kDa tangential ultrafiltration membrane, respectively. SDS – sodium dodecyl sulfate. TCA – trichloroacetic acid precipitation. EtOH – ethanol precipitation. AEX – anion exchange chromatography.

serum against *S. pneumoniae* group 14 as antibody for capture and purified PS14 from ATCC as standard. Protein was determined by the method of Lowry *et al*. (1951), using bovine serum albumin as standard. Nucleic acids were estimated by absorbance at 260 nm ($A_{260}$) and the amount was calculated assuming 1.0 $A_{260}$ = 50 µg/mL.

### Calculation of Yield and Relative Purity

The PS14 global yield was calculated as the percentage of PS14 recovered in the step in relation to the amount of PS14 present in the starting material.

The relative purity regarding nucleic acids was calculated as the ratio between PS14 and nucleic acids, and regarding protein, as the ratio between PS14 and proteins. Considering the required purity established by the World Health Organization (WHO, 2005), the relative purity should be ≥ 33 mg PS14/mg proteins and ≥ 50 mg PS14/ mg nucleic acids.

## RESULTS AND DISCUSSION

### Purification Strategy 1

The relative purity in the 50 kDa-concentrate-saline was 1.3 for nucleic acids and 0.5 for proteins. After the diafiltration step, these values increased to 2.1 and 0.3, respectively for nucleic acids and proteins. The decrease of relative purity regarding proteins could be due to low protein elimination (6%)

combined with high loss of PS14 (43%) in the ultrafiltrate fraction. In the 5% TCA precipitation, 65% of nucleic acids and 91% of proteins were removed, increasing the relative purity to 5.1 and 2.9, respectively. It is worthwhile to note that PS14 recovery in the 20% and 60% ethanol precipitation steps was 100%. The 60% ethanol precipitation removed 98% of nucleic acids and 92% of proteins, reaching the required relative purity (63 and 59, respectively). At the end of the purification process, 50% of the initial PS14 amount was recovered (Figure 2).

**Purification Strategy 2**

The purification strategy 2 followed the same steps of the purification 1 in order to evaluate the process reproducibility with a different starting material. However, the relative purity was not reached after 60% ethanol precipitation and an additional chromatographic step was necessary (Figure 1). This could be a result of the greater contamination of the starting material with intercellular impurities due to pneumococcal autolysis that occurs in batch, but is avoided in continuous cultivation (Gogola-Kolling *et al*., 2014). Furthermore, complex medium also leads to a starting material with higher concentration of contaminants than defined medium. Hence, the relative purity regarding nucleic acids and proteins was close to zero in the microfiltrate fraction, since there was high concentration of these contaminants in this sample, as explained above.

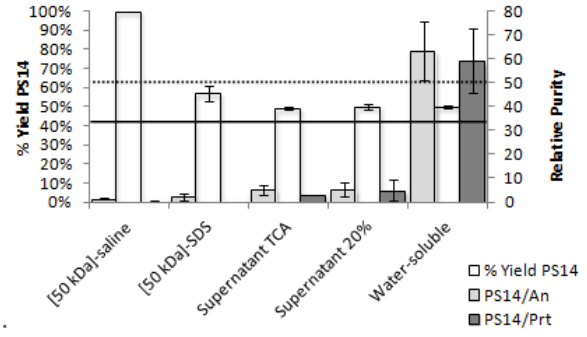


**Figure 2:** PS14 global yield and relative purity in the fractions of purification process 1. Average and deviation of two independent experiments. Horizontal lines represent the threshold of relative purity regarding proteins (solid line) and nucleic acids (dotted line).

After the diafiltration step, 83% of PS14 was recovered along with the elimination of 97% of nucleic acids and 84% of proteins. After 20% ethanol precipitation, 40% of nucleic acids and 91% of proteins were eliminated with loss of only 6% of PS14. The precipitation with 60% ethanol removed 99% of the remaining proteins, reaching the required purity for this contaminant (68 mg PS14/mg protein). However, the required purity for nucleic acids was not reached: it was only 18 mg PS14/mg nucleic acids. The AEX removed the remaining protein, increasing the relative purity to 154 mg PS14/mg protein. The AEX also removed 92% of nucleic acids, reaching the required purity (115 mg PS14/mg nucleic acids). PS14 global recovery at the end of this process was of 58% (Figure 3).

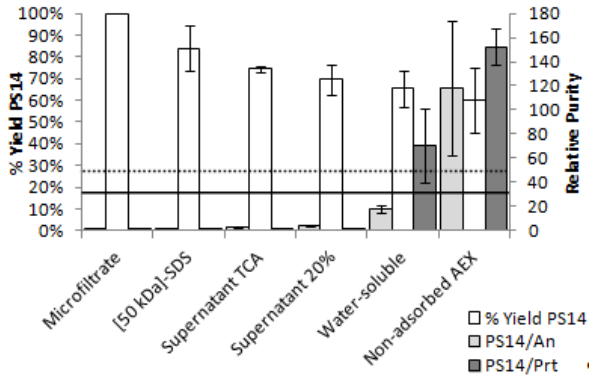


**Figure 3**: PS14 global yield and relative purity in the fractions of purification process 2. Average and deviation of two independent experiments. Horizontal lines represent the threshold of relative purity regarding proteins (solid line) and nucleic acids (dotted line).

**Purification Strategy 3**

In order to increase PS14 recovery, the 50 kDa ultrafiltration membrane was replaced by a 30 kDa membrane in order to reduce the loss of PS14 during the diafiltration step. Although the purification 3 was performed with the same starting material as purification 1, i.e., fermented broth from continuous cultivation with CDM, the decrease in the membrane pore reduced not only the loss of PS14, but also diminished the elimination of contaminants. Therefore, AEX was also necessary to guarantee the required purity.

The diafiltration step with SDS in the 30 kDa membrane was effective only for nucleic acid removal (69% removal), since 93% of proteins remained in the concentrate fraction. As expected, the recovery of PS14 in this step (85%) was higher than the recovery observed when the diafiltration was carried out using 50 kDa membranes in purification 1 (57%), which was performed with the same starting material. After 5% TCA precipitation, the relative

purity regarding nucleic acids increased from 4.1 to 8.9 mg PS14/mg nucleic acids and regarding proteins doubled, from 0.3 to 0.6 mg PS14/mg protein. This step also recovered most of the PS14 from the 30 kDa diafiltration (Figure 4). The 20% ethanol precipitation recovered 77% of PS14 and increased relative purity to 12 mg PS14/mg nucleic acids and 1.7 mg PS14/mg protein. As in the previous processes, precipitation with 60% ethanol eliminated great amounts of impurities (87% of nucleic acids and 95% of proteins). After this step, the required purity for nucleic acids and for proteins was attained (89 mg PS14/mg nucleic acids and 37 mg PS14/mg protein), but since the purity for proteins was still near the minimum threshold, the AEX step was included to assure that the required purity would always be reached, even if starting materials with higher amounts of contaminants would be processed. The AEX eliminated almost 100% of the remaining proteins, increasing the relative purity to 146 mg PS14/mg protein; the relative purity for nucleic acids reached 439 mg PS14/mg nucleic acids and the final recovery of PS14 was 65% (Figure 4), the greatest recovery reached so far.

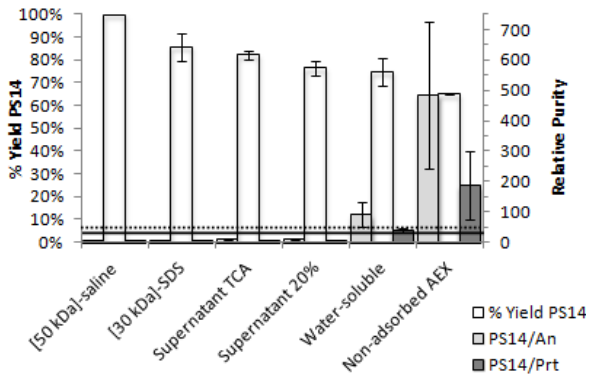


**Figure 4:** PS14 global yield and relative purity during the purification process 3. Average and deviation of two independent experiments. Horizontal lines represent the threshold of relative purity regarding proteins (solid line) and nucleic acids (dotted line).

## Comparison of Purification Strategies

The main difference between processes 1 and 3 is the size of the diafiltration membrane pore and between processes 2 and 3 is the huge amount of nucleic acids and proteins in the starting material. Although an additional step was included in the purification 3, the PS14 recovery was higher than in purification 1 and 2. In addition, the final content of nucleic acids and proteins was lower in purification 3 than in purification 1, due to the inclusion of AEX. The AEX was also essential to remove nucleic acids and proteins and achieve the required purity when the initial fraction presented very high amounts of these compounds, as is the case of purification 2 (Table 1).

The traditional processes for PS14 purification described in the literature have disadvantages such as the use of phenol, toxic and corrosive reagent, and a great number of steps (Institut Mérieux, 1980; Yavordios and Cousin, 1983). Besides, Institut Mérieux and several other processes perform cell lysis with DOC (Cano *et al*., 1980; Suarez *et al*., 2001; Jung *et al*., 2011; Macha *et al*., 2014; Yuan *et al*., 2014) (Table 2). This detergent lysis step breaks down cell membranes, releasing large amounts of intracellular contaminants and causing a negative impact on the purification process similar to that observed in purification 2.

Tanizaki *et al*. (1996) eliminated the use of phenol, replacing it by enzymatic treatment and ultrafiltration for the *Neisseria meningitides* serogroup C capsular PS. This process also proved to be efficient for capsular polysaccharide purification of other microorganisms (Gonçalves *et al*., 2003; Gonçalves *et al*., 2007; Takagi *et al*., 2008). However, after the enzymatic treatment, it is necessary to remove the hydrolyzed material and the enzymes, which may also cause loss of the product of interest. Furthermore, regulatory agencies have restricted the use of animal enzymes in products for humans because of the risk of contamination with prions (Mackay and Kriz, 2010).

**Table 1: Comparison of the three purification strategies.**

| | PS14 (mg) | | Nucleic acids (mg) | | Proteins (mg) | |
|---|---|---|---|---|---|---|
| | **Initial** | **Final** | **Initial** | **Final** | **Initial** | **Final** |
| Purification strategy 1 | 177±25 | 89±11 | 138±3 | 1.4±0.1 | 379±96 | 1.5±0.5 |
| Purification strategy 2 | 356±50 | 116±43 | 15647±2213 | 1±0 | 20357±2879 | 0.7±0.2 |
| Purification strategy 3 | 196±26 | 118±18 | 131±2 | 0.3±0.1 | 565±137 | 0.8±0.6 |

**Table 2: Comparison of the process developed in this work with previously published processes.**

| Author | Main characteristics | | | | Number of steps | Yield (%) |
|---|---|---|---|---|---|---|
| Institut Mérieux (1980) | cell lysis with DOC | phenol | activated charcoal | ultracentrifugation | 11 | 4%* |
| Cano *et al.* (1980) | cell lysis with DOC | CTAB | activated charcoal | | 11 | n.a. |
| Yuan *et al.* (2014) | cell lysis with DOC | pH reduction | activated Charcoal | | 7 | 55% |
| Suárez *et al.* (2001) | cell lysis with DOC | | affinity chromatography | | 9 | 2.3%* |
| Macha *et al.* (2014) | cell lysis with DOC | | aluminum phosphate | | 10 | 81% |
| This work | SDS | TCA | AEX | | 7 | 65% |

n.a. – not available; * values calculated using incomplete information supplied by the authors

Recent publications addressed the problem of using phenol or enzymatic treatment for pneumococcal polysaccharide purification. Macha *et al*. (2014) described a purification process based on precipitation with aluminum phosphate and tangential ultrafiltration, while Yuan *et al.* (2014) used activated charcoal followed by tangential ultrafiltration. In the process proposed in this work (purification 3), part of the proteins and nucleic acids were eliminated by diafiltration with SDS and TCA precipitation. The subsequent alcoholic precipitation steps contributed to remove TCA and SDS along with the contaminants still present in the sample, leading to the achievement of pure polysaccharide with 65% yield (Table 2).

Strong detergents such as SDS and CTAB disrupt membranes and irreversibly denature proteins and nucleic acids. In the developed process, after the diafiltration with SDS, 7% of proteins and 68% of the nucleic acids were eliminated from the previous step. Cano *et al.* (1980) reported similar values of nucleic acid elimination after treatment with CTAB: 69%, but protein elimination was 55%. However, the method developed by Cano *et al*. (1980) consists of 11 steps: 5 steps of alcoholic precipitation, treatment with activated charcoal and dialysis, and therefore it is a more complex process than the one developed in this work (Table 2).

Most proteins in aqueous solution can be precipitated by the addition of certain acids, such as perchloric acid and TCA (Saeed and Thomassen, 1982). The use of TCA eliminated 51% of the proteins and also 56% of nucleic acids, a property that, as far as we know, has not been described before.

The purification method proposed by Suárez *et al.* (2001) is simpler and more practical than traditional methods because it eliminates the use of solvents and does not employ enzymatic treatment. However, the soybean lectin affinity resin used to adsorb the polysaccharide has low capacity (1.1 mg PS per ml of resin) and high cost, making it difficult to be used for large-scale purification. In addition, the yield of this process was very low (Table 2). Unlike the method described by Suárez *et al*. (2001), our process uses negative chromatography, where the contaminants adsorbed in Q-Sepharose and the PS was collected in the non-adsorbed fraction. This resin has high capacity (120 mg protein per 1 mL of resin) and low cost, which favors its use in industrial processes.

Finally, the yield of the PS14 purification process was higher than the purification method described by Yuan *et al.* (2014), which achieved 55% of yield, but lower than that described by Macha *et al*. (2014), whose recovery was 81% (Table 2). The process established in this work and the process of Macha *et al*. (2014) are both based on microfiltration, ultrafiltration and alcoholic precipitations, but Macha's process consists of cell lysis using DOC, filtration and precipitation with aluminum phosphate and filtration on activated charcoal, whereas the process of this work includes TCA precipitation and anion exchange chromatography. Unfortunately, it was not possible to compare the yield of the polysaccharide and removal of contaminants at each step of the process, as Macha *et al*. (2014) did not show the complete table of purification, presenting only the initial and final values.

## CONCLUSION

A new process for purification of the capsular polysaccharide of *S. pneumoniae* serotype 14 was developed. This process is composed of the following steps: tangential microfiltration, concentration in a 50 kDa ultrafiltration membrane, diafiltration in a

30 kDa membrane in the presence of SDS, precipitation with 5% TCA, two alcoholic precipitations with 20% and 60% ethanol and an anion exchange chromatography using Q-Sepharose FF resin. This process achieved the purity required by the WHO for the use in human vaccine preparation, with a lower number of steps and higher yield than the majority of processes described in the literature.

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