Flow Lectin Affinity Chromatography – A Model with Sambucus nigra Agglutinin

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Keywords: Lectin affinity chromatography; Sambucus nigra agglutinin; Automation; Flow system; Cancer biomarkers

Introduction

Never like today have proteomic and glycoproteomic procedures been used as valuable tools to obtain information and characterize general or specific proteomes such in disease states and to discover disease biomarkers that can help in the diagnosis, prognosis and treatment monitoring of several diseases, for instance in cancer.

Plasma and serum are the most informative samples for proteomic studies [1] but they are also the most difficult versions of the human proteome, due to the high heterogeneity they present [2]. The complexity of these samples is potentiated by plasma or serum proteolysis, the presence of protein and glycoprotein isoforms and post-translational modifications of proteins. Additionally, the dynamic range of protein abundance in these samples is very wide (in plasma the range of protein abundance or glycoproteins significantly complicates the discovery process by being covered by a single analytical technique without fractionation, depletion or concentration [3-5]. Furthermore, disease biomarkers occur in very low concentrations, at least in early stages, and the analytical techniques must present adequate sensitivity to detect low-abundant proteins or glycoproteins.

The dominance of highly abundant serum proteins and glycoproteins significantly complicates the discovery process by masking the presence and limiting the detection of low abundance species. Several techniques have been used to perform serum proteome partitioning such as affinity chromatography based on protein A and G, avian IgY and monoclonal antibodies that bind to albumin, immunoglobulins and other highly abundant proteins, with different affinities and specificities [6]. In all these techniques, the ligand reversibly captures proteins of high abundance thereby yielding an enriched pool of low abundance glycoproteins for glycoproteomic studies [4].

Lectins are proteins that have been used as recognition agents to specifically bind glycan structures present in complex samples like biological ones. They show differential affinity for a diversity of glycocojugate structures, binding to them in an antigen-antibody interaction way, although with lower specificity. An advantage over antibodies is that lectins detect glycan structures in glycoproteins, which is useful since many disease biomarkers are glycoproteins with aberrant glycans, namely in cancer [7-9]. Another benefit compared to antibodies is that the affinity of lectins to glycan is lower than the corresponding antibody-antigen interactions, so elution of bound...
glycoproteins is more efficient and higher recovery rates are achieved with lectins [10]. Other advantages over antibodies include the cost of the enrichment column, the life-time and the loading capacity of lectin columns versus antibody columns [10].

Several analytical techniques have been developed to detect glycan structures by using lectins, for example lectin microarrays [11-13] and lectin affinity chromatography (LAC) [14]. LAC is performed by using lectin columns, where the lectin is immobilized to agarose and other conventional biochemical matrices [14] or to magnetic beads, gold foils, silica materials and affinity membranes [15]. By performing the LAC procedure, the lectin bound fraction is enriched in the glycans of interest, and enables the detection of low-abundant glycoproteins.

In biochemical laboratories, LAC can be carried out by using commercial kits and columns customized for enrichment of specific classes of glycostructures. These columns and kits present high efficiency and selectivity in the enrichment process, and permit the downstream analysis of the enriched fractions, through 2D-gel electrophoresis or mass spectrometry. Also, they can be applied in fractionation of diverse biological samples, such as cell lysates and serum. In other cases, LAC is performed according to established procedures, not requiring commercial devices but needing special installations such as refrigerated rooms, which are not accessible to many laboratories.

Although there is a rising tendency to automate analytical procedures used in proteomic research through flow methodologies [10,16-19], usually LAC is carried out in batch conditions (using the commercial columns and kits) [20] or using sophisticated chromatographic equipments [21-23], which implies a long procedure time and high consumption of consumables, or capability of laboratories to purchase expensive apparatus. If the LAC procedure is performed under flow conditions, this allows automation of the process, especially important when several samples need to be run, and reduces solution and sample manipulation steps and resultant losses, ex-vivo proteolysis or chemical modifications during procedures [10]. Furthermore, lab-made flow systems are, usually, low-cost manifolds, enable a reduction in assay duration and increase reproducibility in results, compared to batch procedures.

This work describes the development of an automated flow LAC procedure, based on the use of Sambucus nigra agglutinin (SNA) immobilized on agarose beads, as an alternative to the conventional LAC batch procedures or commercial columns. The sample is introduced in the flow system and flows for a period of time with the SNA column, allowing the establishment of specific SNA-glycan interactions. Then, the column is washed, removing the unbound proteins and glycoproteins and, finally, the bound fraction is eluted from the column, liberating the glycoproteins trapped by SNA.

Materials and Methods

Chemicals and materials

Reagents of p.a. quality were used, without further purification. Deionised water purified by a Millipore Milli Q system (resistivity >18 MΩ cm) was used throughout.

Unless otherwise stated, solutions were prepared in phosphate buffer saline (PBS 1x) pH 7.4 containing 0.5 mmol L\(^{-1}\) of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\). Divalent metals must be present for carbohydrate binding as they are necessary for SNA-1 to have the active conformation to interact with the STn antigen [24].

Bovine submaxillary mucin (BSM) and transferrin (Trf), both from Sigma, were used as model glycoproteins in this study. Bovine serum albumin (BSA; Sigma) was used to assess interference of high abundant serum proteins on the column behaviour. AsialoBSM and asialofetuin were used as glycoproteins not presenting the STn antigen, to evaluate the selectivity of the SNA-I column towards different glycoproteins present in serum. The asialoforms were obtained though enzymatic desialylation of BSM and fetuin, respectively.

SNA-I bound to agarose beads (Vector Laboratories) was used in the proposed flow system and in the batch comparative method with no further processing. The beads had 45-165 μm size, a SNA-I concentration of 3.0 mg mL\(^{-1}\) of settled gel and were stable at pH between 3 and 11.

Desialylation of BSM and Fetuin

BSM and fetuin were enzymatically desialylated using neuraminidase from Clostridium perfringens type VI (Sigma). Briefly, 1.0 mg of each glycoprotein was incubated at 37°C during 24 h with 1.0 mL of neuraminidase solution at a concentration of 3 U mL\(^{-1}\), prepared in acetate buffer pH 5.5. After the incubation, desialylated glycoproteins were stored at -20°C until use.

Sample collection and processing

Human blood samples were obtained from healthy donors and from diagnosed cancer patients, from Centro Hospitalar São João (CHSJ) University of Porto Medical Faculty (Porto, Portugal) and Pachuca General Hospital (México), who gave their informed consent. Information about samples is provided in Table 1 (Supporting Information). Immediately after collection, the blood samples were left to clot in a vertical position for 1 h, then centrifuged at 3000 rpm for 10 min, at 4°C and serum was transferred to new tubes. To reduce inter-individual variability and enhance common characteristics to all samples (in this case cancer secreted STn antigens), aliquots of serum samples were pooled in two groups: controls and cases, with 25 samples each, and stored at -80°C until further use.

To reduce the dynamic concentration interval of serum proteins, equalizing the amounts of the more and less abundant proteins, and thus reducing the possible interference of high abundant proteins in the sensor measurements, a combinatorial peptide ligand library (CPLL, Proteominer, BioRad) [25] was used to process samples, according to the manufacturer’s recommendations. The processed sera were stored at -20°C until use. CPLL-processed samples were introduced in the flow system after dilution with PBS.

Protein concentration in glycoprotein solutions and in processed serum samples was determined by using the Pierce BCA Protein Assay Kit (Thermo), according to the manufacturer’s instructions. Since the eluting solutions used for the proposed method and for CPLL interfered in the quantification, all samples were precipitated with acetone before protein quantification, following the procedure recommended by Thermo.

Column preparation

Sambucus nigra agglutinin type I, with affinity for NeuAc-α2,6 structures, such as the case of the STn antigen (NeuAc-α2,6-GalNAcα1-O-Ser/Thr) was used as biosensing agent. A lab-made acrylic column with 3.7 cm length and 1.8 mm inner tube diameter was packed with SNA-I agarose beads. The column volume was 140 μL. On each end of the column, filter disks with 10 μm pore size (Gilson) were placed in order to retain the beads in its interior. The column extremities were tightly screwed to the system manifold avoiding entrance of air bubbles. After each use, the SNA-I column was washed with PBS and...
stored at 4°C with the extremities sealed to prevent bead drying. The same column could be used for two analytical cycles without loss in binding activity.

**Flow system manifold**

In the developed flow system (Figure 1), a Gilson Minipuls 3 peristaltic pump equipped with Tygon pumping tubes propelled the solutions. Connection between the manifold components was made through Omnifit Teflon tubing (0.8 mm i.d.). Total manifold volume in the recirculation path was 150 μL. Figure 1 (Supporting Information) is a picture of the developed flow system.

**Analytical cycle**

Whenever new beads were used, the analytical cycle started with the column washing with PBS, for 10 min, as recommended by the SNA-agarose manufacturer, to remove sugar added to stabilize the lectin. Then, the analytical cycle was performed as follows:

1º - Binding: Flowing the sample or glycoprotein solution through the column for 10 min in order to fill the flow manifold and column with the sample and then close the recirculation path and let flowing for 10 h. After that time, the unbound fraction was collected for downstream analysis (for example, incubation with other lectins or other fractionation methods)—Figure 2a.

2º - Washing: Flowing PBS for 1 h (which corresponded to more than 10 times the column volume) to remove unbound glycoproteins—Figure 2b.

3º - Elution: Flowing the eluting solution, to separate the SNA-glycoprotein complexes, recovering the bound fraction. Bound fraction could then be further analyzed through 2D-gel electrophoresis and/or Western blotting—Figure 2c.

4º - Regeneration: Flowing PBS for 1 h to remove unbound glycoproteins and prepare the column for subsequent use—Figure 2b.

Incubation with samples or glycoprotein solutions was performed with the column on ice to favour binding. Elution and washing steps were performed at room temperature (20-25°C) to facilitate unbinding of the glycans. Incubation and washing steps were performed at a flow rate of 0.03 mL min⁻¹ and the elution was carried out at 0.01 mL min⁻¹.

**Comparative procedure: Batch lectin affinity chromatography**

As comparison method, a batch LAC procedure usually performed at our laboratory was performed as following: the eluates from CPLL were diluted 1:30 in PBS 1x and incubated overnight with 30 μL of SNA-I-agarose beads previously washed three times with 750 μL of washing buffer (20 mmol L⁻¹ Tris-HCl (pH 7.4)+150 mmol L⁻¹ NaCl+1.0 mol L⁻¹ urea+1.0 mmol L⁻¹ CaCl₂/MgCl₂/MnCl₂/ZnCl₂), at 4°C, under rotation. Afterwards, the samples were centrifuged for 5 min at 5000 g and at 4°C to remove the supernatant and the lectin-beads were washed three times with 750 μL of washing buffer, at the same centrifugation conditions. Bound glycoproteins were eluted by adding 100 μL of elution buffer (100 mmol L⁻¹ Tris-HCl (pH 7.5) and 1% SDS) to the lectin-beads and submitting them to extreme temperature conditions (95°C for 5 min with shaking). Three elution cycles were performed and 300 μL of eluate fraction were collected. The eluates were stored at -20°C for subsequent analysis.

**Results and Discussion**

**Formation of the SNA-I–glycoprotein complex at the SNA-1-agarose column**

There are different types of SNA namely I, II and III, with different specificity for glycan structures, and type I presents the...
highest selectivity for the STn epitope [26]. Therefore, SNA type-I bound to agarose beads was the recognizing agent immobilized in the column. Formation of the SNA I-glycoprotein complexes was evaluated by flowing BSM and Trf solutions through the column and determining the amount of glycoprotein present in the solution after the incubation time (unbound fraction). BSM is a highly glycosylated mucin, containing 65% w/w of carbohydrates, from which almost half are STn [27,28]. Human transferrin is a glycoprotein possessing 6% w/w of carbohydrates. One of its N-glycans terminates in the structure NeuAcα2,6-Gal-β1,4-Glc [29], for which SNA-I has shown affinity, in addition to the STn structure) [30,31]. Selection of BSM and Trf as models to assess the SNA column in this work was intended, due to their extremely different glycosylation pattern and enabled to simulate the variability of glycoproteins present in serum samples. During incubation time, the same solution flowed through the column by using a recirculation pathway and the column was kept on ice since binding is tighter at low temperatures [32].

**Optimization of the analytical cycle**

The flow system was designed in order to allow performing the LAC in an automated and easy way, with a reduced sample volume and with flexibility to enable its simple application to diverse fractionation methods, using other lectins or antibodies. That is why the flow system does not present a commutation or injection valve upstream the column since, even for reduced loop lengths, additional sample volume would be required and, frequently, the available volumes of biological samples are very small.

Initially, the column size was optimized by testing two columns with the same inner diameter but with different lengths, 9.0 and 3.7 cm. The larger column enabled to pack a higher amount of SNA-agarose beads, which increased the column capacity to bind glycoproteins. When the same Trf solution 0.1 mg mL⁻¹ was incubated on both columns for 5 h, at 0.03 mL min⁻¹, the % bound fraction was 64.9% for the larger column and 58.3% for the shorter one, as expected. On the other hand, the larger column generated a higher pressure inside the flow and consumes a higher amount of SNA-agarose, which is an expensive reagent. Considering the obtained binding results and the practical constraints of the flow system operation, the shorter column was selected to be used in the proposed methodology.

Following the selection of the column size, the influence of the flow rate on the column behaviour was studied. At first, a 0.2 mg mL⁻¹ Trf solution was incubated for 15 h at 0.0625 mL min⁻¹ and the % bound fraction was 27%. When the same solution was incubated for 5 h at 0.03 mL min⁻¹, the percentage of bound fraction increased to 55%, even though a 3-times shorter incubation period was used. Finally, the same experiment was performed at 0.01 mL min⁻¹ and a 52% bound fraction was obtained. From these results it was clear that lower flow rates favoured the formation of the SNA-glycoprotein complex, since the contact between molecules was carried out in slower conditions and interactions between the lectin and the glycans could be more easily established. Since the % bound fractions were similar for the lower flow rates assayed, further analyses were performed using both flow rates.

The incubation time (time during which samples flowed through the recirculation path) was optimized at 0.01 and 0.03 mL min⁻¹. For that, Trf solutions of 0.05, 0.1 and 0.2 mg mL⁻¹ were incubated for 5, 10 and 15 h, at 0.01 and 0.03 mL min⁻¹. The % bound fractions obtained are represented in Figure 3.

For the lower Trf concentration tested, higher % bound fractions were obtained for 0.01 mL min⁻¹ and 5 h of incubation were enough to achieve the highest binding percentage (100%). For the higher concentrations tested, better results were obtained for 0.03 mL min⁻¹ and 10 h of incubation time. Prolonging the incubation time to 15 h did not increase significantly the % bound fraction. Since the flow system was intended to be applied to a large concentration interval of glycoproteins in samples, the selected incubation time was 10 h, enabling to achieve higher % bound fractions for more concentrated samples. The optimum flow rate for incubation was set at 0.03 mL min⁻¹. The SNA column was submitted to these flow rate conditions, by flowing the PBS solution for 8 consecutive hours and no SNA was removed from the beads during that time, so the column was robust under the optimized flow conditions.

Plotting the % bound fraction versus Trf concentration (Figure 2-Supporting Information) revealed that the column behaviour was similar for 10 h and 15 h of recirculation time. For all incubation times, the % bound fraction decreased with the increase in glycoprotein concentration, as expected. For Trf concentrations of 0.1 and 0.2 mg mL⁻¹ the % bound fraction was similar, indicating that the lectin column saturated for Trf concentrations ≥ 0.1 mg mL⁻¹ (the binding capacity for Trf solutions of 0.05, 0.1 and 0.2 mg mL⁻¹ was 94%, 79% and 73%, respectively).

The same experiment was carried out for BSM solutions and 10 h of recirculation time, and the results showed that the % bound fraction oscillated between 43% and 49% and no decrease tendency was observed, meaning that the SNA column saturated for all BSM concentrations analyzed. These results are justified by the differences in glycosylation of both glycoproteins. Trf is poorly glycosylated and, therefore, higher amounts can be introduced in the column without causing saturation. BSM is highly glycosylated, with a carbohydrate content corresponding to about 71% of total weight, and the majority

![Figure 3: Effect of incubation time on % bound fraction for Trf solutions of 0.05 (A), 0.1 (B) and 0.2 (□) mg mL⁻¹](image-url)
of the glycans are STn. So, for the same concentration of glycoprotein, the amount of SNA-binding glycans is much higher for BSM and that justifies the column saturation, even for the lower concentration tested.

An important aspect regarding glycan binding in lectin columns is displacement phenomena [33]. When a sample is put in contact with the lectin column, the binding of the various glycoproteins present depends on the amount and affinity of each glycan structure towards the lectin. In the same sample, with diverse glycoproteins in different amounts, the binding is competitive and it’s difficult to predict. If the amount of lectin in the column is enough to bind to all corresponding glycans present, no saturation occurs and no displacement phenomena are observed. On the contrary, if saturation occurs, the glycans that bind with more affinity displace the others, so at the end only the glycans or glycoproteins with higher affinity will bind. As a result, overloading columns will cause displacement of weakly bound species from the column and, therefore, the bound profile will be dependent of the sample loading, which affects reproducibility and accuracy of results. Furthermore, and worse, overloading the lectin column may lose disease biomarkers that are low-abundant and weakly-bound glycoproteins. So the rule is never overload lectin columns when they are used for analytical purposes [33].

In the developed flow system, saturation of the column could be detected by quantifying the protein amount in the original sample and on the unbound fraction. If the bound fraction was not 100%, it means that some saturation degree occurred. If that happened, the problem could be solved by introducing again the unbound fraction on the system and submitting it to one or more analytical cycles so that, at the end, all STn-carrying glycoproteins on the sample were trapped by the SNA-column.

After defining the incubation conditions, the focus was directed to elution conditions. A good eluting solution must provide high recovery rates of the bound fraction and should not affect the binding ability of the lectin, enabling the reuse of the same lectin beads. Several eluting solutions were evaluated namely PBS, ethylene glycol, glycine and lactose, at different concentrations. As reference, the Glycoprotein elution solution for sialic acids and renewal of the SNA-column without loss in binding ability. Results since a low standard deviation would be an indicator of complete elution deviations in two consecutive analysis of the same glycoprotein solution by comparing the % bound fractions and corresponding standard deviations.

Room temperature to facilitate the separation of the SNA-glycoprotein complex. Evaluation of the best eluting solution was performed by comparing the % bound fractions and corresponding standard deviations in two consecutive analysis of the same glycoprotein solution since a low standard deviation would be an indicator of complete elution and renewal of the SNA column without loss in binding ability. Results are shown in Table 2 – Supporting Information.

Most reproducible results were obtained for ethylene glycol 10% in PBS and lactose 0.5 mol L⁻¹ in PBS 1x/acetic acid, with comparable results to the commercial Glycoprotein elution solution for sialic acid-binding lectins, under the same eluting conditions. This was not surprising because, on one hand, lactose is the inhibiting sugar for SNA and, on the other, it is advised that elution of tightly bound proteins may be facilitated by adding 50% ethylene glycol to the elution sugar [32]. Additionally, the elution solution provided by the lectin supplier is composed of ethylene glycol 5-15%. Therefore, the use of an ethylene glycol solution is preferable to the commercial one, in terms of costs.

The elution solution selected as best was ethylene glycol 10% instead of lactose 0.5 mol L⁻¹ because, when elution is performed with a sugar with high affinity for the lectin, differential elution may occur, depending on the relative affinity of each glycoprotein to the lectin and the affinity of the eluting sugar [33]. By using ethylene glycol as eluting solution it is expected that all glycoproteins elute equally, independently of their affinity for the lectin, since ethylene glycol is one of the most useful protein salting-out agents, disrupting the lectin-glycoprotein complexes [34].

The eluting conditions chosen were 5 h at 0.01 mL min⁻¹. Longer eluting times did not improve the results and the lower flow rate tested originated better reproducibility for both Trf and BSM.

On the optimized flow system, the sample volume necessary to fill the flow path (manifold+column) was 130 μL. For each new analysis, samples were let to flow through the system for 10 min at 0.03 mL min⁻¹ before closing the recirculation path in order to completely fill the manifold and the column. This totalized a sample volume requirement of 300 μL which is an adequate and reduced volume considering the availability of serum for glycoproteomic analysis.

Selectivity studies

Highly abundant proteins may interfere in the lectin enrichment process, through nonspecific interactions with the lectin, negatively affecting the lectin binding capacity. Human serum albumin is the major protein in serum and is known as a nonspecific binding protein due to its biological role as a carrier protein [1]. Therefore, BSA was used as a model to simulate the behaviour of HSA present in serum samples after CPLT-treatment at the expected concentration (5 mg mL⁻¹). Other glycoproteins were also incubated with the SNA-column, namely asialofetuin (aFet) and asialoBSM (aBSM), obtained by enzymatic desialylation of fetuin and BSM, respectively. Fetuin is a glycoprotein which is reported to have no STn glycans [35] but presents other sialylated glycans that can bind to SNA. The asialoforms of fetuin and BSM represented serum glycoproteins not carrying the STn epitope. These asialoforms were incubated in the maximum concentration used for the model glycoproteins (BSM and Trf) which was 0.2 mg mL⁻¹. Incubation was performed under the optimized experimental conditions. Results are presented in Figure 4.

Figure 4: Selectivity of the SNA-column for several proteins/glycoproteins. Trf, BSM, aFet and aBSM solutions were 0.2 mg mL⁻¹. BSA was 5.0 mg mL⁻¹. Incubation for 10 h at 0.03 mL min⁻¹, elution with ethylene glycol 10%. Error bars correspond to standard deviation of duplicate measurements.
The % bound fractions were 72.6% for Trf, 52.0% for BSM, 9.7% for BSA and 0% for aFet and aBSM. It is common that nonglycosylated proteins are associated with other proteins through biospecific or nonspecific protein–protein interactions [Cho]. So, it’s not surprising the response of the SNA-column to a BSA solution 5 mg mL⁻¹, which is the approximate concentration of HSA in serum samples after CPLL-treatment. As for the desialylated glycoproteins, the SNA-column showed very high selectivity. Altogether, these results demonstrate the feasibility of using the SNA-column in serum analysis.

### Analytical features

<table>
<thead>
<tr>
<th>Provider</th>
<th>Product</th>
<th>Serum amount per column 50 µL serum</th>
<th>Amount bound</th>
<th>% binding</th>
<th>Reuse</th>
<th>Price per column, euros</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qiagen</td>
<td>Oproteome sialic glycoprotein kit - SNA spin column</td>
<td>50 µL serum</td>
<td>30 µL proteins in pooled eluate</td>
<td>0.86</td>
<td>no</td>
<td>~500</td>
</tr>
<tr>
<td>TCS Biosciences (in association with EY Laboratories)</td>
<td>Sambucus nigra Gel -SNA- Immobilized Lectin, pre-packed in column</td>
<td>not indicated</td>
<td>the amount of glycoprotein being purified depends on the molecular weight of the glycoprotein</td>
<td>yes</td>
<td>~400</td>
<td></td>
</tr>
<tr>
<td>bioWORLD</td>
<td>Sambucus nigra (Elderberry) lectin (SNA1)-OnePASS TH Separaprep® Columns (Fast Flow)</td>
<td>not indicated</td>
<td>~1.5 mg fetuin/mL of gel</td>
<td>not indicated</td>
<td>170-360 (depending on the column size)</td>
<td></td>
</tr>
<tr>
<td>GALAB Technologies</td>
<td>AffiSep® HPLC SNA column</td>
<td>depends on the glycoprotein concentration in serum</td>
<td>≥ 50 µg glycoproteins/0.6 ml adsorbent (for 75 µL of applied serum the bound protein is 480 µg)</td>
<td>yes</td>
<td>370-840 (depending on the column size)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 1: Comparison of analytical features of the flow and the batch methods.

### Table 2: Comparison of analytical features of the flow and the batch methods.

### Sample analysis

Differences in the glycoprotein concentration in samples favour the binding of the more abundant ones over the less-abundant [33]. Therefore, samples were pre-treated with a combinatorial peptide ligand library (CPLL) in order to remove the highly abundant proteins and, thus, reduce the dynamic range of protein concentrations in serum, enabling the detection and binding of low-abundant glycoproteins and putative cancer biomarkers. Combination of abundant protein depletion and affinity enrichment allows a deeper mining of the serum glycoproteome [10]. Depletion procedures or lectin affinity protocols alone are not sufficient to detect low-abundant proteins or glycoproteins due to the complexity of serum and, therefore, deeper mining can be obtained by conjugating both strategies; depletion and enrichment [10].

Since BSA was partially and nonspecifically bound to the SNA-column in the concentrations expected for the CPLL-eluted samples, a previous study of the adequate dilution degree of CPLL-eluates was carried out. Eluates of Ctrl samples were incubated with dilutions of 1:3, 1:10, 1:20 and 1:30 with PBS 1x. Lower binding percentages for Ctrl samples were obtained for 1:30 dilution, demonstrating the absence of nonspecific interactions of highly abundant proteins or interference of CPLL-eluent on the sample incubation.
The flow method was validated through analysis of serum samples from healthy individuals (controls) and from cancer patients (cases). Individual and pooled samples were analyzed using the developed system and the batch method and the results are indicated in Table 3.

The agreement between the results obtained by the proposed method and by the reference method was evaluated through the Student t-test for paired samples, in which the calculated t value (0.47) was lower than the critical t value (2.07, two tail), for a confidence level of 95 % (n=9).

Conclusion

This work proposes an automated flow LAC procedure, based on the use of *Sambucus nigra* agglutinin for the selective fractionation of serum samples and isolation of STn-carrying glycoproteins, associated with carcinoma states, as an alternative to the conventional batch LAC procedure and the SNA commercial columns. The flow system allows automation of the process, especially important when several samples need to be run and reduces solution and sample manipulation. Due to the continuous and slow passage of flow through the column, samples are put in intimate contact with the lectin, favouring the formation of glycan-lectin complexes. Also, during elution, the flow movement facilitates separation of the previously formed complex and the recovery of the bound fraction with high yields. This way, regeneration of the lectin column is favoured and the same column can be used at least twice, reducing the cost of the assay (in opposition to what occurs with the conventional LAC methods) where the immobilized lectin can be used only once.

Moreover, the flow system can be applied to diverse applications and samples, requiring only the change of the column packing. The use of a flow system for LAC could further respond to the need for analyte monitoring, allows sample pre-treatments performed on-line and hyphenation of analytical techniques. The high reproducibility of the flow system functioning minimizes variability in sample processing, thus positively affecting the quality of results. Its simple design, ease of operation, miniaturization and low cost implementation favours its use in any analytical or biochemical laboratory, especially in those with limited budgets or where expensive equipments or specific installations are missing. The easy and cheap implementation of the proposed flow system would promote and generalize the execution of lectin affinity chromatography in any analytical or biochemical laboratory, and follow the current trends of automation and reduction of sample manual handling.

The paper must be seen as a guide on how to implement a simple flow system to perform LAC in an inexpensive and reproducible way, for those researchers who are not experienced with flow techniques, in order to simplify their routine work.

Acknowledgement

The authors thank the participating General Hospitals for providing the human blood samples.

References


