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Evaluation of Jacalin lectin sorbents for the extraction of the human chorionic gonadotropin glycoforms prior to analysis by nano liquid chromatography-high resolution mass spectrometry

Authors

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Keywords

human chorionic gonadotropin; Jacalin; lectin; liquid chromatography; mass spectrometry; solid phase extraction

Abstract

Human chorionic gonadotropin (hCG) is a dimeric, highly glycosylated hormone with a total of 4 N- and 4 O-glycosylation sites in its two subunits, hCG α and hCG β . Recently, we developed a novel nano liquid chromatography coupled to high resolution mass spectrometry (nanoLC-HRMS) method for the analysis and thus the detection of the intact glycoforms of hCG. Here, a sorbent functionalized with the Jacalin lectin was evaluated in solid-phase extraction (SPE) for its potential to fractionate the hCG glycoforms prior to their nanoLC-HRMS analysis at the intact level, which may facilitate the detection of low-abundance glycoforms and may lead to a more detailed characterization of the hormone glycosylation. A commercial sorbent based on Jacalin immobilized on Sepharose and having a lectin density of 4.5 mg per ml of gel was selected to carry out SPE and its capacity was estimated to be of some tens of μg of hCG per ml of lectin sorbent. Next, the SPE protocol was modified to improve the extraction recoveries. Especially, it was noticed that an extensive pre-conditioning procedure prior to the first use of a cartridge was necessary to remove the residual non-grafted lectins. Indeed, if on-grafted lectins are not eliminated, they may bind a part of hCG glycoforms preventing their retention by the sorbent, leading to low extraction recoveries (around 10%). With the extensive pre-conditioning procedure, the average extraction recoveries for both hCG α and hCG β glycoforms were about 50%, with either recombinant or urinary hCG. Qualitatively, the fractionation of hCG glycoforms between the washing and elution fractions was achieved with the urinary hCG sample by determining the number of glycoforms detected in each fraction. It appears that 12 hCG α glycoforms have a low affinity (detected only in the washing fraction), 1 a low-medium affinity (detected in washing and elution 1 fractions), 16 a medium affinity (detected in washing, elution 1 and 2 fractions), and 12 a high affinity (detected only in elution 1 and 2 fractions). For the hCG β glycoforms, similarly, 3 have a low affinity and 12 a low-medium affinity. Additionally, the 3 hCG β glycoforms were detected better. A different behavior was observed with the recombinant hCG sample, which indicates glycosylation differences between the two hCG samples. This shows the potential of lectin-based affinity fractionation before nanoLC-HRMS analysis to better characterize the glycosylation state of hCG at the intact level.

1. Introduction

Human Chorionic Gonadotropin (hCG) is one of the most important hormones secreted during pregnancy, playing a crucial role in placental development and fetal growth [1]. This hetero-dimeric glycoprotein is composed of two subunits, hCG α and hCG β , that are non-covalently linked. hCG α has 92 amino acids and a molecular weight (MW) of 10,188 Da, while hCG β has 145 amino acids and a MW of 15,509 Da, without considering glycans. Indeed, hCG is a highly glycosylated protein: 2 N-glycosylation sites are present on hCG α (Asn-52 and Asn-78), while 2 N-glycosylation sites (Asn-13 and Asn-30) and also 4 O-glycosylation sites (Ser-121, Ser-127, Ser-132, and Ser-138) are present on hCG β [2,3]. It is established that glycosylation has a direct impact on the hormone functions while it can also be an indication of pathologies during pregnancy or of certain malignancies [4,5]. Moreover, since this hormone is used in assisted fertility treatments, as for example by the administration of recombinant hCG (rhCG), the characterization of the glycosylation state is also crucial for batch-to-batch consistency [6].

Nevertheless, the study of the hCG glycosylation, with a total of 8 glycosylation sites, remains challenging. Various studies have been performed, including bottom-up approaches that incorporate a digestion step before liquid chromatography-mass spectrometry (LC-MS) analysis of the generated glycopeptides [2,7,8]. Glycan release approaches have also been carried out as complementary to bottom-up applications [7,9,10]. Intact protein analysis with LC-MS [11,12] or capillary electrophoresis-MS [13,14] has offered additional information concerning the macro-heterogeneity of hCG and/or the possibility of a glycan profiling of the relative abundance of the glycoforms in a sample [15]. This is why, in our lab, an optimized nano liquid chromatography (nanoLC) with reversed phase mode combined with high resolution mass spectrometry (HRMS) method has been developed for the analysis of the hCG glycoforms at the intact level [16]. With this method, multiple glycoforms of both hCG α and hCG β of a rhCG-based drug were detected and characterized in a semi-quantitative manner. However, the glycoform mixture was so complex that the data treatment was time-consuming and it is questionable whether some glycoforms were not masked by LC co-elution or ionization defect at the spectrometer inlet.

However, another complementary tool that has contributed to the knowledge of the glycosylation state of hCG is lectin proteins. Lectins can bind specific carbohydrates of glycoproteins and as each lectin has an affinity for certain glycosylation pattern(s), they can selectively capture glycoforms according to their glycosylation [17]. Regarding hCG, lectin immunoassays have been used for the profiling of the hCG glycosylation in samples of

various origins [5,18-21]. Lectin affinity chromatography has been used for solid phase extraction (SPE). Briefly, the sample was percolated through a cartridge or column containing a sorbent functionalized with a lectin [22]. Afterwards, the non-retained fraction of the sample, which therefore does not contain the specific glycosylation motifs for which the lectin has an affinity, was removed from the sorbent with a washing solution. Then, the retained glycoforms were eluted with a buffer containing a competitive sugar. Among the most popular solid supports for lectin immobilization are agarose-based materials, such as Sepharose, since they have a relatively low cost and are macroporous and hydrophilic polymers with a stability over a wide range of pH [23]. Consequently, many companies propose sorbents based on lectins immobilized on Sepharose that can be used in SPE processes for glycoprotein fractionation.

Among the various lectins that have so far been applied for the study of hCG, Jacalin, which is derived from *Artocarpus integrifolia* (jackfruit seeds), has been used for the investigation of the O-glycosylation. Indeed, Jacalin binds to some of the most common O-glycosylation patterns. More specifically, it has affinity toward the Tn antigen (N-acetylgalactosamine (GalNAc) attached to the Ser or Thr amino acid), the T antigen (Core 1, the Tn antigen extended by the addition of a galactose (Gal β 1-3GalNAc α -Ser/Thr)), and to the sialyl T antigen (Neu5Ac α 2-3Gal β 1-3GalNAc α -Ser/Thr). It binds also to the less commonly encountered Core 3 (N-acetyl glycosamine (GlcNAc) in a β 1-3 position (GlcNAc β 1-3GalNAc α -Ser/Thr)). However, when the 6th carbon of the initial GalNAc sugar is occupied, the affinity is impeded and structures like the core 2 type O-glycans and the disialyl T antigen are not expected to have affinity [24]. Finally, Jacalin can also bind high mannose N-linked structures [25].

Jacalin SPE has been used for the study of the glycosylation patterns of hCG derived from urine of healthy pregnant women [26] and from urine of patients with benign endometrioma or Ia, IIa or advanced stages of cervical cancers with and without metastases [27]. In these studies, measurement of the hCG content in the collected fractions after lectin SPE was performed with radioimmunoassay or immunoassay, but no further structural characterization was performed. Interestingly, Jacalin SPE has also been used for the enrichment of the O-GalNAc glycopeptides obtained after tryptic digestion, N- deglycosylation and partial O-deglycosylation of a purified commercial hCG, with the lectin fractions analysed off-line with LC-MS/MS [28]. In this study, hCG was used as a model glycoprotein to confirm the potency of the Jacalin sorbent to isolate the glycopeptides from the overall mixture. To our

knowledge, this is the only study found in literature where lectin SPE and LC-MS analysis were coupled for the characterization of hCG, and it was done at the bottom-up level.

Regarding applications of lectin SPE coupled with LC-MS for the characterization of hCG at the intact level, there is only one study, which we have recently published [29]. It involved lectin SPE using a different lectin, Concanavalin A (Con A), coupled off-line with nanoLC-HRMS for the analysis of the fractions. It was the first demonstration that the combination of lectin SPE with intact protein analysis by nanoLC-HRMS can contribute to a more detailed glycosylation profiling of hCG since it allowed the detection of hCG α with two tetra-antennary N-glycans that couldn't be detected by direct analysis in nanoLC-HRMS without Con A SPE. However, no fractionation of hCG glycoforms according to differences in the hCG β subunit was observed. The main difference between the two hCG subunits is that hCG α may contain N-glycans whereas hCG β may contain N- and O-glycans. Therefore, a different lectin, Jacalin known for its affinity for some O-glycans, was selected for this new study and the same approach was used. First, the capacity, meaning the amount of hCG in μg that can be captured per ml of a commercial Jacalin-based sorbent was investigated. The SPE protocol was next modified to improve the extraction recoveries of the glycoforms present in two hCG samples (rhCG and a hCG of urinary origin (uhCG)). Lastly, the potential of Jacalin SPE was investigated for the fractionation of the hCG glycoforms.

2. Materials and methods

2.1 Reagents and analytes

LC-MS grade acetonitrile (ACN), LC-MS grade water and formic acid were obtained from Carlo Erba (Val-de Reuil, France). Trifluoroacetic acid (TFA), galactose (Gal), sodium chloride, Trizma® base, sodium acetate, sodium azide, CNBr-activated Sepharose 4B, bovine serum albumin (BSA), 1 M HCl and 0.1 M NaOH for pH adjustment, ammonium hexafluorophosphate, bichononic acid (BCA) protein assay reagent, disposable polypropylene cartridges (SPE tubes of 1 ml and 3 ml), polyethylene frits (20 μm porosity) and Amicon® Ultra Centrifugal Filters with 10 kDa molecular weight cut-off (MWCO) were all purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Potassium dihydrogen phosphate, di-sodium hydrogen phosphate dihydrate, sodium bicarbonate and boric acid were ordered from VWR (Fontenay-sous-Bois, France). *Artocarpus integrifolia* lectin (Jacalin) – Separopore® 4B with a density of 4.5 mg of Jacalin per ml of solid support was ordered from Coger (Paris, France). Ultra-pure water was produced by a Milli-Q purification system (Millipore, Molsheim, France).

The human chorionic gonadotropin from human urine (uhCG) was purchased as a lyophilized powder (10,000 International Units (IU)) from Sigma Aldrich. A final concentration at 1,000 IU ml⁻¹ was achieved by diluting the powder with 10 ml of LC-MS grade water as recommended by the provider. After this reconstitution the formulation also contained 0.1 M sodium phosphate buffer (pH approx. 7.2) and 10 mg ml⁻¹ of mannitol, as stated by the provider. Taking into account that 1 IU equals to 0.092 µg [30], the solubilized drug equals to a concentration of 92 µg ml⁻¹.

The recombinant human chorionic gonadotropin (rhCG)-based drug, Ovitrelle®, was purchased from Organon (Oss, The Netherlands). Before use, the additives and excipients of the drug formulation were removed by filtering with the Amicon® Ultra Centrifugal Filters with a 10 kDa MWCO. The filtration and two additional steps of washing with LC-MS grade water were performed with centrifugation at 13,300 rpm for 10 min. The drug was recovered by inverting the filter and performing a final step of centrifugation for 2 min at 10,000 rpm. The solution was further diluted with LC-MS grade water at a final concentration of 500 µg ml⁻¹ of rhCG.

2.2 Preparation of SPE cartridges

For the extraction of rhCG, 200 µl or 400 µl of the Jacalin sorbent were packed between two polyethylene frits in a 1 ml polypropylene cartridge, which was then stored in a 0.05 M Tris buffer pH 7.0 containing 0.15 M NaCl and 0.02% NaN₃ until usage. For the extraction of uhCG, 800 µl of the Jacalin sorbent were packed between two polyethylene frits in a 3 ml polypropylene cartridge, which was stored in the same storage buffer.

A control sorbent consisting of CNBr-activated Sepharose grafted with BSA at the same density as the Jacaline-functionalized sorbent was synthesized using an immobilization process previously developed in our laboratory. [29,31]. Briefly, the immobilization was performed by swelling 233 mg (about 400 µl) of Sepharose with 2 ml of 1 mM HCl under rotation (6 rpm for 15 min), which was then washed twice with 2 ml of a 0.1 M bicarbonate buffer pH 8.3 plus 0.5 M NaCl. Then, 900 µl of a 2 mg ml⁻¹ BSA solution in the bicarbonate buffer were added to the Sepharose and incubated for 18 h at 18°C under rotation (6 rpm). The control sorbent was placed between two polyethylene frits in a 1 ml cartridge and conditioned with 48 ml of 0.1 M Tris buffer pH 8.0 (solution S4), 8 ml of 0.1 M acetate buffer pH 4.0 plus 0.4 M NaCl (solution S5), and 8 ml of 0.1 M bicarbonate buffer pH 8.3 plus 0.5 M NaCl (solution S6). The supernatant after the immobilization was kept for the estimation of the grafting yield using a BCA assay [8,31] and plotting a calibration curve with 5

concentration points in a range of 0.1 to 2 mg ml⁻¹ of the BSA solution in the bicarbonate buffer and comparing the absorption at 562 nm of the standard curve with the one of the collected supernatant.

2.3 SPE protocols

First, the solution used for the percolation and washing steps was always the same and will be called percolation-washing solution in the manuscript. For all the procedures described below, the initial sample amount was diluted with the appropriate volume of the percolation-washing solution, to reach the final cartridge volume (V_c), *i.e.* 200 μ l, 400 μ l or 800 μ l. Unless stated otherwise, conditioning of the cartridge was performed with 10 V_c of the percolation-washing solution.

All the tested protocols are summarized in supporting information (**Table S1**). The initial protocol (protocol 1) used the solution S1 (0.05 M Tris buffer pH 7.0 plus 0.15 M NaCl) for conditioning, percolation of the sample and washing. Elution was performed with the solution S1 plus 0.5 M Gal. This protocol was applied using a pressure to have an estimated flow rate of 1.2 ml min⁻¹ or under gravity (estimated flow rate of 0.1 ml min⁻¹). In protocol 2, PBS was used for conditioning, percolation, and washing and elution was attempted with 2 ml of 0.5 M formic acid, pH 2.2. The protocol 3 used the solution S2 (0.05 M Tris pH 8.0 plus 0.15 M NaCl, 0.2% L-arginine and 0.2% L-glutamic acid) for conditioning, percolation, and washing, and elution was performed with the solution S2 plus 0.5 M Gal plus 15% v/v ACN. In protocol 4, the extraction of rhCG was performed with 400 μ l of the Jacalin sorbent and under gravity, leading to a flow rate of 0.1 ml min⁻¹. The day before extraction, the sorbent was pre-conditioned with 48 ml of 0.1 M Tris buffer pH 8.0 (solution S4), then with 8 ml of 0.1 M acetate buffer pH 4.0 plus 0.4 M NaCl (Solution S5) and 8 ml of a 0.1 M bicarbonate buffer pH 8.3 plus 0.5 M NaCl (Solution S6) and stored in storage buffer. The day of the extraction, conditioning, sample percolation and washing was done with the Solution S3 (0.02 M Tris buffer pH 9.0 plus 0.15 M NaCl). A first elution step was performed with 2 ml of the Solution S3 plus 0.5 M Gal and a second elution step with 2 ml of the solution S3 plus 0.5 M Gal plus 15% v/v of ACN. A similar protocol was used for the extraction of 12.5 μ g of uhCG diluted in 800 μ l of Solution S3, but using a 800 μ l sorbent and an estimated flow rate of 1.8 ml min⁻¹. After each SPE, the sorbent was regenerated by applying 8 ml of 0.1 M acetate buffer pH 4.5 plus 0.5 M NaCl and 8 ml of 0.1 M borate buffer pH 8.5 plus 0.5 M NaCl before storage. Each collected SPE fraction was filtered through an Amicon® Ultra Centrifugal Filters for 30 min at 4000 rpm and then washed twice with LC-MS grade water for 30 min at 4000 rpm

followed by a final washing for 55 min at 4000 rpm. The final volume was about 50 μ l and it was measured each time accurately before the nanoLC-HRMS analysis. Each time, a hCG sample with a concentration corresponding to a recovery of 100% in a given fraction was also analyzed in nanoLC-HRMS to calculate the extraction recovery of the lectin SPE process. It was prepared by diluting hCG in 2 ml of the appropriate elution buffer used in SPE followed by a concentration with the Ultra Centrifugal Filter.

2.4 NanoLC-HRMS analysis

The nanoLC-HRMS method developed previously was used to analyze the hCG glycoforms at the intact level in the SPE fractions [16]. The nanoLC system (NC 3500 RS, ThermoFisher Scientific, Courtaboeuf, France) with a trapping C18 pre-column (Acclaim PepMap300, 0.3 mm i.d. x 5 mm, 5 μ m, 300 \AA , ThermoFisher Scientific) and an Opti Solv biocompatible nano filter (0.5 μ m, ThermoFisher Scientific) placed after the trapping column at the 10-port valve of the LC system, was connected to an EASY-Spray PepMap RSLC C18 column (Acclaim PepMap300, 150 mm i.d. x 0.075 mm, 5 μ m, 300 \AA , ThermoFisher Scientific). The injected sample was front-flush transferred to the EASY-Spray column for separation before detection with the Orbitrap mass analyser (Exactive plus, ThermoFisher Scientific). LC parameters were: mobile phase (A), H₂O/ACN (98/2; v/v) + 0.05% TFA; mobile phase (B), H₂O /ACN (1/9; v/v) + 0.05% TFA; linear gradient, 10-65% (B) in 54 min (1.02%/min); column temperature, 60 $^{\circ}$ C; injection volume; 1 μ l; flow rate, 300 nl min⁻¹. To avoid carry-over, a blank injection was performed between each run.

The ionization parameters were: capillary voltage, 1.7 kV; drying temperature, 280 $^{\circ}$ C; sheath, auxiliary, and sweep gas flows were not operated. Analyses were conducted at a positive full MS mode with a scan range from m/z 1000 to 4000 at a scan rate of 1 spectrum/s. The mass resolution was 140,000 at m/z 200, the AGC target 3e6 and the maximum injection time 200 ms. The MS system was calibrated using clusters of a salt of ammonium hexafluorophosphate in the mass range of the analysis (m/z 1000 to 4000). This salt was also used as external calibrant to determine the elemental composition of the detected species due to accurate mass measurements performed on the maximum isotopic of protonated glycoforms peaks. All the measurements were associated with an error expressed in ppm reflecting the accuracy of the experimental value compared to the theoretical value. The software Excalibur 4.1 (ThermoFisher Scientific) was used for the data acquisition and instrument control. Treatment of the data was carried out using the Freestyle 1.3 SP2 (ThermoFisher Scientific) software. The extracted ion chromatogram (XIC) of each peak of a

given glycoform was generated by the software by setting an error at 30 mDa. MS data analysis and recovery calculation were performed as previously reported [29].

3. Results and discussion

3.1 Analysis of uhCG and rhCG by nanoLC-HRMS

Before evaluating the potential of lectin SPE for the fractionation of hCG glycoforms, it was first necessary to determine the ones present in the two hCG samples used in this study by analyzing them in nanoLC-HRMS, using the method previously developed [16]. The resulting total ion chromatograms (TICs) obtained for uhCG and rhCG are depicted in **Figure S1** of the supplementary data. The glycoforms of the hCG α and hCG β subunits were eluted as two distinct chromatographic peaks, as already reported [16,29]. The signal intensity in the TIC of uhCG was lower than that of rhCG, although it is assumed that they were injected at similar concentrations. This observation was made also in past studies and could be the result of an incorrect conversion of concentration from IU to $\mu\text{g ml}^{-1}$ [14].

The detected forms in both preparations were identified as the non-modified glycoforms, the oxidized forms and adducts with TFA. The TFA adducts were not considered for the calculations described in the next sections, as they were induced by the presence of the additive in the mobile phase and their corresponding glycoforms are already considered. Similarly, only the non-oxidized glycoforms were considered to process the data because oxidation can occur before analysis, during separation or in the source during ionization.

Therefore, the identified glycoforms of hCG α and hCG β , whose elemental composition has been determined by accurate mass measurements, are reported in **Tables S2** and **S3** for uhCG and **Tables S4** and **S5** for rhCG in the supplementary data, respectively. These are the same forms as those mentioned in our previous work [29]. In these tables, the hexose (Hex) can correspond to either a mannose or a galactose, while the N-Acetyl hexosamine (HexNAc) could correspond to either a N-Acetylglucosamine or a N-Acetylgalactosamine monosaccharide.

3.2 Estimation of the capacity of the Jacalin sorbent

After the determination of the hCG α and hCG β glycoforms that could be detected by a direct analysis of the sample in nanoLC-HRMS, the potential of lectin-based SPE for fractionation according to glycosylation before the analysis step was investigated. To reach this objective, a commercial sorbent, based on Jacalin immobilized on Sepharose was selected. The characterization of this sorbent in terms of affinity towards the hCG glycoforms requires the

percolation of an hCG amount that does not exceed the capacity of the sorbent, but which must also be quantifiable in the fractions analyzed by nanoLC-HRMS. Regarding the Jacalin sorbent, the supplier mentioned a density of 4.5 mg of lectin per ml of gel that could bind 1-3 mg of Immunoglobulin A (IgA) per ml of gel. By considering the molecular weight of the dimeric IgA, which is approximately 385 kDa, and converting the amount of this protein that can be retained by the sorbent from mg to mol, the range was 2.6-7.8 nmol per ml of gel. Then, by considering the molecular weight of hCG, which is at max 40 kDa for the hyperglycosylated forms, a theoretical capacity of 100-300 μg of hCG per ml of gel was estimated. A second parameter to consider for the subsequent SPE experiments is that the volume of the fractions from the lectin-SPE step is reduced, by passing through a filter, to a volume of approximately 50 μl (accurately measured after each filtration), and that these fractions must contain a final concentration of at least 12.5 $\mu\text{g ml}^{-1}$ of hCG, to be greater than the LOQ of the nanoLC-HRMS method [16].

The initial experiments for the estimation of the capacity were performed by using the protocol 1 described in section 2.3 and in supporting information (**Table S1**). The composition of the selected buffers, meaning a 0.05 M Tris buffer with a pH of 7.0 containing 0.15 M NaCl for percolation and washing and the same buffer plus 0.5 M Gal (competitive sugar having a high affinity with Jacalin) for elution were those recommended by the manufacturer. Using 200 μl of gel, a theoretical amount of 20-60 μg of hCG could be captured by the lectin. Therefore, 25 μg of rhCG were percolated, followed by a washing and an elution step using the protocol 1. Each SPE fraction was next concentrated and analyzed in nanoLC-HRMS. A relative recovery of each glycoform in each fraction was calculated by integrating the peak area of the glycoform in its corresponding XIC in the fraction and then divided it by the sum of the areas of this glycoform in all SPE fractions. For these preliminary investigations, data processing was only performed using the 5 hCG α and 5 hCG β glycoforms with the highest peak area on their own XIC (forms are highlighted in grey in the **Tables S2-S5**) and the values were averaged to calculate the average relative recovery, *i.e.* the distribution of either hCG α or hCG β glycoforms in each fraction. The results are presented in **Table 1**. It can be seen that when 25 μg of rhCG were percolated on a 200 μl cartridge at a flow rate of 1.2 ml min^{-1} , a great amount of the sample was detected in the percolation fraction. As a high flow rate was applied during percolation, this could be due to a kinetic limitation.

Repeating the SPE process but under gravity (flow rate of about 0.1 ml min^{-1}) resulted in not detecting the sample in the percolation fraction anymore, but mostly in the washing fraction,

which was not expected in view of the affinity of the Jacalin and the already published results related to its use for hCG [26]. Therefore, this lack of retention should be a consequence of an overloading of the Jacalin sorbent. The capacity of this sorbent was thus inferior to the theoretical calculated one, as it was already previously observed with Con A sorbents [29]. This may be due to a lack of accessibility to some interaction sites because of lectin orientation and steric hindrance. It was then decided to keep the percolation of the sample under gravity for all the rest of the study and to use a cartridge containing a double amount of sorbent (400 μ l, theoretical capacity of 40-120 μ g of hCG) and to reduce 5 times the hCG amount (5 μ g). In this case, 100% of the most intense 5 hCG α and 5 hCG β glycoforms were detected in the elution fraction. Thus, the experimental capacity was estimated to be between 12.5 and 100 μ g of hCG per ml of Jacalin sorbent. It is worthwhile to notice that in the rest of the paper the amount of hCG glycoforms detected in the percolation fractions is no longer mentioned, as it has always been equal to zero.

Table 1: Average relative recoveries of the most intense 5 hCG α and 5 hCG β glycoforms when percolating different amounts of rhCG on different volumes of Jacalin-based sorbents, applying the protocol 1 and analyzing the concentrated SPE fractions in nanoLC-HRMS.

| Cartridge volume (μ l) | hCG amount (μ g) | Flow rate (ml min^{-1}) | Average relative recoveries in the SPE fraction (%) | | | |
|-----------------------------|-----------------------|------------------------------------|---|-------------|---------|---------|
| | | | | Percolation | Washing | Elution |
| 200 | 25 | 1.2 | hCG α | 44 | 49 | 7 |
| | | | hCG β | 47 | 51 | 2 |
| 200 | 25 | 0.1 | hCG α | 0 | 90 | 10 |
| | | | hCG β | 0 | 86 | 14 |
| 400 | 5 | 0.1 | hCG α | 0 | 0 | 100 |
| | | | hCG β | 0 | 0 | 100 |

3.3 Improvement of the extraction recovery

Using the data obtained with the previously described experiments, the SPE extraction recoveries were next calculated. For each experiment, the recovery of a glycoform in a SPE fraction was calculated by dividing (i) its peak area measured on its XIC when analyzing in nanoLC-HRMS the fraction from Jacalin SPE after its concentration with an ultra-centrifugal

filter until a volume of about 50 μ l with (ii) its peak area on its XIC when analyzing directly hCG that has only been subjected to the same filtration process but without SPE (filtered hCG sample). It is worthwhile to notice that in this last case, the hCG sample was injected after its concentration with the filter to a value corresponding to a recovery of 100% in 50 μ l, i.e. a volume similar to the one of the concentrated SPE fractions.

The extraction recoveries were calculated for the most intense 5 hCG α and 5 hCG β glycoforms and the average value in the elution fraction was around 5%, indicating a problem in the SPE process. Therefore, an optimization of the extraction protocol was necessary. Starting from the hypothesis that low recoveries are related to insufficient conditioning of the cartridge, the volume used during conditioning was increased by applying 25 Vc (10 ml) instead of 10 Vc (4 ml). It is worthwhile to notice that the solution used for conditioning is also the percolation media and the washing one, which explained why it was used for the conditioning step. The results are depicted in **Table 2**. It was observed that the recovery remained similarly low.

It is also known that parameters like the pH of the buffers used during extraction can affect the dissociation constants of the lectins complexed with glycoproteins [32]. Since a strong retention was next hypothesized to explain the low extraction recovery previously observed, acidic conditions with a considerably lower pH (2.2 instead of 7.0) were tested for elution (protocol 2) [33]. In this case, PBS was used for the cartridge conditioning, sample percolation and washing, and the elution was performed with 0.5 M formic acid. Still, as seen in **Table 2**, no great improvement in the average extraction recoveries was observed. Then, the protocol 3 was tested using the Solution S2 (0.05 M Tris, pH 8.0 plus 0.15 M NaCl, 0.2% L-arginine and 0.2% L-glutamic) during conditioning, percolation, and washing. Importantly, the elution buffer had the same composition as the washing solution plus 0.5 M Gal and also 15% v/v of ACN, to attempt to improve the elution of the glycoforms with this strong eluent and thus the extraction recovery if this hypothesis is correct. Indeed, an improvement of the extraction recoveries was observed, with average values of 37% for hCG α and 30% for hCG β (**Table 2**), which may result from a better elution due to the addition of the organic solvent and/or a slightly higher basic pH (a value of 8 here).

Figure 1 presents the TICs resulting from the nanoLC-HRMS analysis of the elution fractions obtained applying the protocol 1 or 3. Different peaks corresponding to hCG glycoforms appear between 18 and 25 min. Comparing the protocols 1 and 3, it is worthwhile to notice that the protocol 3 included 0.2% of L-arginine and L-glutamic acid in the used solutions to try to reduce protein aggregation and/or oxidation, especially with the presence of the organic

solvent in the elution buffer. Still, the oxidation rate was high, with some of the double and triple oxidized forms having a total extraction recovery of more than 100%, which may indicate that some oxidation occurred during the SPE process. Therefore, conditions that can result in lower oxidation rate were next investigated. However, also other very intense peaks were detected between 42 and 49 min, which were identified as Jacalin monomers and their oxidation products. This is an indication of some loss of the Jacalin lectin at each use of the commercialized sorbent. As seen in the TIC of the elution fraction containing 15% v/v of ACN, it seems that this loss was even more important when an organic solvent is present.

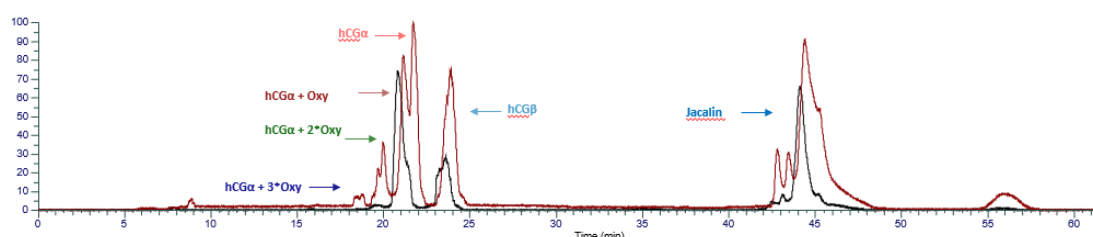


Figure 1: Overlay of the TICs resulting from the nanoLC-HRMS analysis of the SPE elution fractions carried out with 5 μg of rhCG and the protocol 1 (black) or 3 (red) using a Jacalin-sorbent (400 μl). Normalization was performed to the highest peak of both TICs. SPE conditions: see section 2.3. nanoLC-HRMS parameters: see section 2.4.

In our previous work dealing with home-made and commercial Con A-based sorbents, a lectin loss was also observed [29]. It corresponded on the one hand to ungrafted lectins that were not well removed after the immobilization step and on the other hand to a much lower loss of grafted lectins at each use of the support. It was observed that good recoveries for the commercial sorbent can be achieved when the non-grafted residual Con A were sufficiently removed from the sorbent, either by multiple uses of the same sorbent or by an extensive pre-conditioning procedure before its first use. When these non-grafted residual lectins are not removed, they can interact with the hCG glycoforms, reducing their retention by the grafted lectins on the sorbent, and thus their extraction recoveries.

Table 2: Average relative recoveries and average recoveries calculated for the hCG α and hCG β glycoforms measured in the percolation (P), washing (W) and elution (E) fractions concentrated and analyzed by nanoLC-HRMS applying different SPE protocols with 5 μ g of rhCG on 400 μ l of Jacalin sorbent used sometimes after an extensive pre-conditioning (Pre-C) step (n=3). Note that for the protocols 1 and 2, the calculations were performed only for the most intense 5 hCG α and 5 hCG β glycoforms.

| | N° | Pre-C + C | P + W | E | | Average relative recovery (%) | | | Average recovery (%) | | |
|---|---|-----------|--|--------------|----|-------------------------------|-----|----------------------|----------------------|----------------|--------------|
| | | | | | | W | E1 | E2 | W | E1 | E2 |
| | | | | | | SPE protocol | 1 | C: S1, 25 Vc (10 ml) | S1 | S1 + 0.5 M Gal | hCG α |
| | | | | hCG β | | | 100 | | | 12 | |
| 2 | C: PBS pH 7.4, 10 Vc (4 ml) | PBS | 0.5 M Formic acid pH 2.2 | hCG α | - | | 100 | N.A | - | 10 | N.A |
| | | | | hCG β | | | 100 | | | 8 | |
| 3 | C: S2, 10 Vc (4 ml) | S2 | S2 + 0.5 M Gal + 15% ACN | hCG α | 3 | | 97 | N.A | 1 | 37 | N.A |
| | | | | hCG β | 0 | | 100 | | 0 | 30 | |
| 4 | Pre-C: S4, 60 Vc (48 ml) + S5, 10 Vc (8 ml) + S6, 10 Vc (8 ml); C: S3, 10 Vc (4 ml) | S3 | E1: S3 + 0.5 M Gal E2: S3 + 0.5 M Gal + 15% ACN | hCG α | 12 | | 79 | 9 | 7 | 42 | 5 |
| | | | | hCG β | 16 | | 81 | 3 | 11 | 50 | 2 |
| | Pre-C: multiple cartridge uses; C: S3, 10 Vc (4 ml) | | | hCG α | 8 | 92 | 0 | 4 | 36 | 0 | |
| | | | | hCG β | 15 | 85 | 0 | 8 | 45 | 0 | |

E1/E2: successive elution steps; Gal: galactose; S1 (solution S1): 0.05 M Tris, pH 7.0 + 0.15 M NaCl; S2 (solution S2): 0.05 M Tris pH 8.0 + 0.15 M NaCl, 0.2% L-arginine and 0.2% L-glutamic acid; S3 (solution S3): 0.02 M Tris, pH 9.0 + 0.15 M NaCl; S4 (solution S4): 0.1 M Tris pH 8.0; S5 (solution S5): 0.1 M acetate pH 4.0 + 0.4 M NaCl; S6 (solution S6): 0.1 M bicarbonate pH 8.3 + 0.5 M NaCl. Vc: cartridge volume (400 μ l here).

To evaluate if a similar phenomenon occurred here, an extensive pre-conditioning step was applied to a Jacalin cartridge (400 μ l) before its first use, using 48 ml of a 0.1 M Tris buffer pH 8.0, 8 ml of a 0.1 M acetate buffer pH 4.0 plus 0.4 M NaCl followed by 8 ml of a 0.1 M bicarbonate buffer pH 8.3 plus 0.5 M NaCl. It is important to note that these volumes have not been optimized yet and may be reduced. In addition, since it was observed that the basic pH of the Tris buffer could potentially lead to better recoveries, a more basic solution at pH 9.0 (Solution S3, 0.02 M Tris pH 9.0 plus 0.15 M NaCl) was selected for the conditioning, percolation, and washing. A first elution step with milder conditions using only the competitive sugar to avoid oxidation (Solution S3 plus 0.5 M Gal) was next carried out. The TICs of the SPE fractions after this experiment are represented in **Figure 2**. The average extraction recovery in the elution 1 fraction for the hCG α and hCG β glycoforms were 42% and 50%, respectively, which are improved values compared to those previously obtained. Finally, to investigate if the elution was total, a second elution step with the same elution solution used before but also containing 15% v/v of ACN was applied. A low sample amount was detected in this fraction (5% for the hCG α glycoforms and 2% for the hCG β). The detailed recovery values for all glycoforms are given in **Tables S6** and **S7** in supporting information.

The SPE protocol 4 was also applied to a cartridge that had been used multiple times (5th use) but was not subjected to the extensive pre-conditioning process. Results regarding the extraction recovery were again satisfactory, with values of 36% and 45% for the hCG α and hCG β glycoforms, respectively. Therefore, it seems consistent with our hypothesis, as the non-grafted lectins would have been eliminated during the repetitive uses of the same cartridge. Moreover, when the protocol 4 was carried out with a new cartridge without a preliminary extensive pre-conditioning, low extraction recoveries were again obtained (average extraction recovery of 13% for both hCG α and hCG β glycoforms), confirming our previous hypothesis that the removal of the non-grafted residual lectins is mandatory to get high extraction recoveries. In practice, even if multiple uses lead to similar results, it is recommended to carry out the extensive pre-conditioning of the cartridge before any first SPE use to get satisfactory extraction recovery values. It is worthwhile to notice that this is the second time that we observed this phenomenon, with lectin-based sorbents from two different suppliers. This may not have been reported in the literature until now, as most studies use a qualitative rather than quantitative approach, as is the case here. Therefore, this should be more carefully controlled by suppliers, by improving their process before releasing their

sorbents on the market. Users would save time and money if the extensive pre-conditioning required to remove residual non-grafted lectins were carried out by suppliers.

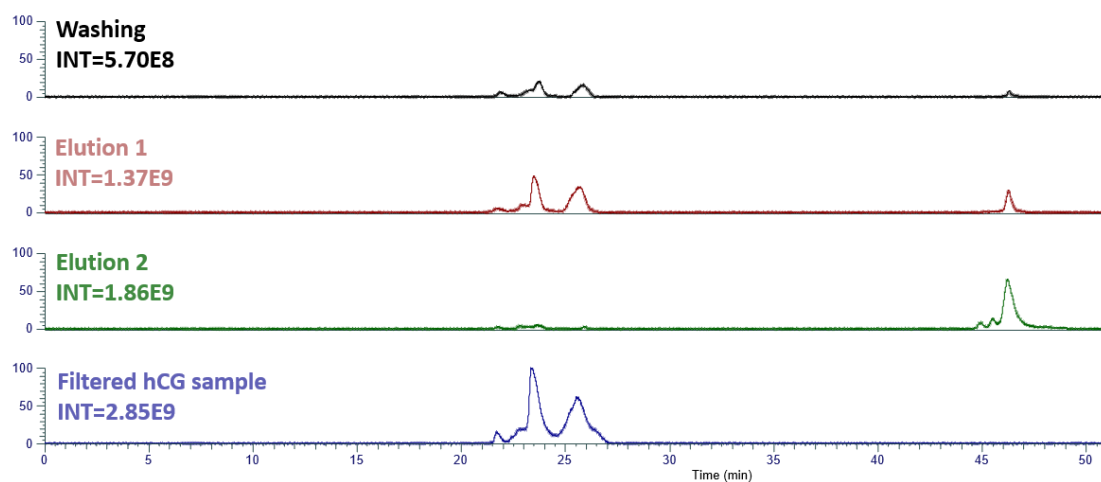


Figure 2: TICs resulting from the nanoLC-HRMS analysis of the concentrated SPE washing and elution fractions carried out with 5 μg of rhCG on a 400 μl sorbent with protocol 4 after the extensive pre-conditioning of the cartridge before its first use. The TIC of the filtered hCG sample corresponds to the analysis of the rhCG sample only with filtration process and was used to normalize all the other TICs. SPE conditions: see section 2.3. nanoLC-HRMS parameters: see section 2.4.

The extraction on the Jacalin sorbent was also carried out with a urinary based hCG (uhCG). As the MS signal of uhCG is lower than that of rhCG in similar theoretical concentrations (section 3.1), it was decided to use 12.5 μg of uhCG, as we did in our previous study [29]. Initially, the extraction protocol 1 was applied and 12.5 μg of uhCG were introduced onto a 400 μl cartridge. According to our previous estimation, the cartridge capacity should be between 5 and 40 μg of hCG. However, an overloading of the cartridge was observed with average relative recoveries of 66% and 59% for the hCG α and hCG β glycoforms, respectively, in the washing fraction. On the other hand, when reducing the sample at 5 μg , the glycoforms were no longer detected. To solve this issue, a higher volume of gel, 800 μl , was then used and the 12.5 μg of uhCG did not exceed the capacity of this cartridge in this case. Next, SPE was performed again but using the protocol 4 and after the extensive pre-conditioning of a new Jacalin sorbent (800 μl) before its first use. A low flow rate was kept, using gravity only, although this parameter could be further evaluated to try to save time for

future method validation. The resulting TICs of the nanoLC-HRMS analysis of the concentrated SPE fractions are represented in

Figure 3, where it is evident that most of the sample was detected in the elution 1 fraction.

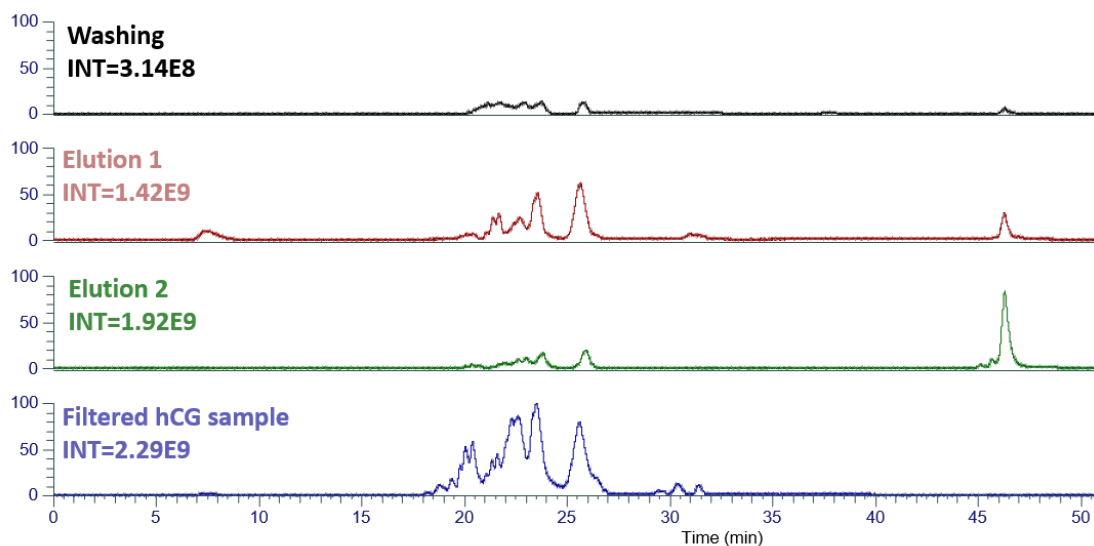


Figure 3: TICs resulting from the nanoLC-HRMS analysis of the concentrated SPE washing, elution 1 and elution 2 fractions carried out with 12.5 μg of uhCG on a 800 μl cartridge using protocol 4 after the extensive pre-conditioning of the cartridge before its first use. The TIC of the filtered uhCG corresponds to the analysis of the rhCG sample only with filtration process and was used to normalize all the other TICs. SPE conditions: see section 2.3. nanoLC-HRMS parameters: see section 2.4.

Data processing showed that the relative average recovery of the hCG α and hCG β glycoforms in the elution 1 fraction was 81% and 80%, respectively (**Table 3**). Moreover, the average recovery for the hCG α glycoforms was 48% in the elution 1 fraction and 9% in the elution 2 fraction, leading to an overall value of 57%. For the hCG β glycoforms, the average recovery was 59% and 12% in the elution 1 and elution 2 fractions, respectively, leading to an overall value of 71%. These values were thus satisfying. The recovery values of all the hCG α and hCG β glycoforms can be found in **Table S8** and **Table S9**, respectively. In the TICs of the SPE process (**Figure 3**), it is worthwhile to notice that some loss of the Jacalin from the sorbent was again observed (peak at retention time of about 46 min), but mainly in the elution 2 fraction, when 15% of ACN were used. This Jacalin loss did not prevent from a high retention of the hCG glycoforms and should correspond to some grafted ones, since the removal of the non-grafted residual lectins was done before the first use of the cartridge.

Table 3: Average relative recoveries and average recoveries for the hCG α and hCG β glycoforms measured in the concentrated washing (W) and elution (E) fractions by nanoLC-HRMS applying the protocol 4 with 12.5 μ g of uhCG on a 800 μ l Jacalin sorbent after the extensive pre-conditioning before its first use (n=3).

| | Average relative recovery | | | Average recovery | | |
|--------------|---------------------------|----|----|------------------|----|----|
| | W | E1 | E2 | W | E1 | E2 |
| hCG α | 3 | 81 | 16 | 2 | 48 | 9 |
| hCG β | 4 | 80 | 16 | 3 | 59 | 12 |

3.4 Study of the Jacalin sorbent specificity with a control sorbent

To ensure that the retention of the hCG glycoforms on the Jacalin sorbent previously observed was due to specific interactions with Jacalin rather than by non-specific interactions, a home-made control sorbent was synthesized. The BSA protein was selected as the control protein since it has a MW of 66.5 kDa, which is similar to that of the tetrameric two-chain structure of Jacalin (66 kDa). BSA was then covalently grafted on CNBr-activated Sepharose, as the one used in the commercial sorbent, with a procedure often used by our group [29,31,33]. The BSA amount to be immobilized was calculated by considering the density value of 4.5 mg of Jacalin per ml of sorbent given by the supplier in order to get a control sorbent as close as possible to that of Jacalin in terms of protein density. The grafting yield of the BSA immobilization was estimated to be 90% with a BCA assay, which was considered as satisfactory.

The SPE protocol 4 was then applied on this control sorbent, using 5 μ g of rhCG. As illustrated on **Figure 4**, the sample was mainly detected in the washing fraction and not in the elution 1 fraction with the control sorbent, confirming the specificity of the retention on the lectin sorbent as rhCG was mainly detected in the elution 1 fraction. A small part was detected in the elution 2 fraction, coming from the non-specific interactions with the control sorbent, as no unique glycoforms were detected in this fraction as compared to the washing fraction.

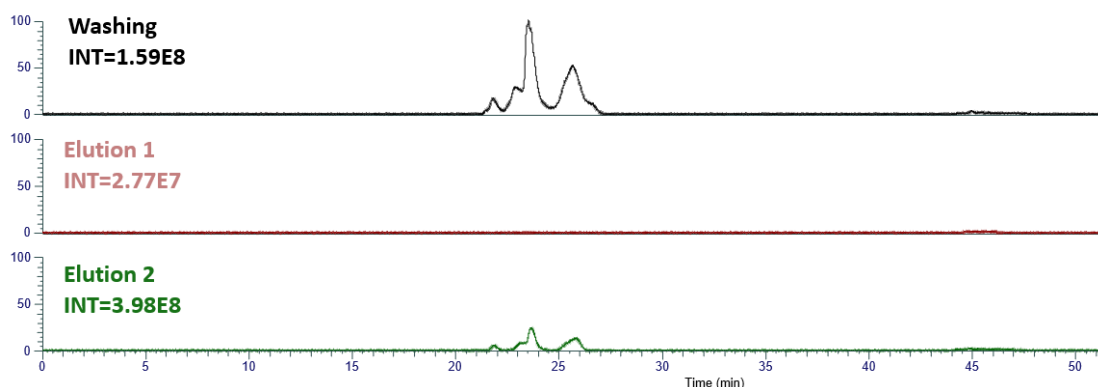


Figure 4: Total ion chromatograms (TICs) resulting from the nanoLC-HRMS analysis of the concentrated SPE washing and elution fractions obtained with 5 μg of rhCG on a control BSA-cartridge by application of protocol 4 (**Table S1**). SPE conditions: see section 2.3. nanoLC-HRMS parameters: see section 2.4.

3.5 Fractionation of hCG glycoforms

It was previously demonstrated that the developed SPE procedure (protocol 4 after an extensive pre-conditioning of the cartridge before its first use) led to good extraction recoveries without overloading the cartridge and that retention was specific. Therefore, it was next possible to explore whether the lectin sorbent can be used for the fractionation of the hCG glycoforms. A treatment of the data from this point of view was thus performed. Regarding rhCG, unfortunately, none of the glycoforms was uniquely detected in the washing fraction as seen in the Venn diagrams in **Figure 5A**. In addition, no new glycoform was revealed due to the lectin SPE pre-treatment.

Regarding uhCG, some differences already appeared in the chromatograms of the SPE fractions (

Figure 3). Firstly, at the retention time range of 19.7-19.9 min, a low intensity peak in the chromatogram of the washing fraction was observed. In the average corresponding MS spectrum (**Figure S2**), some hCG α glycoforms were detected. In **Table S10**, their suggested glycan composition and elemental composition can be found. Those glycoforms were not detected in the average spectrum at the same retention time range analyzing the elution 1 and 2 fractions but were detected in the direct nanoLC-HRMS analysis of the uhCG sample. Moreover, at the retention time range of 22.9-23.0 min analyzing the washing fraction, the average spectrum (**Figure S3**) shows three glycoforms that could potentially be hCG β glycoforms. Their suggested overall glycan composition and elemental composition as determined by accurate mass measurements are presented in **Table S11**. Those glycoforms,

even if present, were not well ionized when analyzing uhCG without Jacalin SPE, which may be due to the presence of other co-eluting glycoforms and therefore there were not considered for the calculations in the previous sections. Finally, at the retention time range of 24.0-24.3 min, where the majority of the hCG α glycoforms elute, two glycoforms that correspond to phosphorylated or sulphated hCG α were only detected in the washing fraction (**Table S10**). Thus, it seems that some fractionation occurred.

After the full data treatment, the number of hCG α and hCG β glycoforms of uhCG detected in the different SPE fractions are summarized in the Venn diagram (**Figure 5B**). A clear fractionation appears for hCG α glycoforms with 12 having a low affinity (poorly retained and thus only detected in the washing fraction), 1 with a low-medium affinity (detected in washing and elution 1 fraction), 16 with a medium affinity (detected in washing and elution 1 and 2 fractions), and 12 with a high affinity (detected only in elution 1 and 2 fractions). For the hCG β glycoforms, similarly, 3 have a low affinity (poorly retained and thus only detected in the washing fraction) and 12 a low-medium affinity (detected in washing and elution 1 fraction). It is the first time that fractionation of hCG glycoforms according to differences in the hCG β subunit is reported. Therefore, it was observed that for uhCG, Jacalin SPE enabled fractionation of hCG glycoforms according to their affinity for the lectin, facilitating detection of some low-abundance glycoforms.

The differences observed in the Venn diagrams presented **Figure 5** reflect a difference in the glycosylation profiles of the two tested hCG samples. Since Jacalin has affinity towards some O-glycans but also towards the mannose sugar in the N-glycans [22], the interpretation of these results according to the nature of the glycans potentially present on a given glycoform can be complex. Moreover, the accessibility of some glycans towards the binding sites of the immobilized lectin on the solid support might not be optimal. Therefore, it would be particularly interesting to extract separately the dissociated hCG α and hCG β subunits, before and after an additional PNGase treatment to release the N-glycans before SPE. Considering that Jacalin has affinity for core 1 but not for complex Core 2 O-glycans [21], this additional pre-treatment could also lead to a better fractionation according to O-glycosylation. Finally, since some studies also suggest the existence of O-glycosylation sites on the hCG α and free α subunit [27,28,34,35], the same application could reveal some masked glycoforms of this subunit as well.

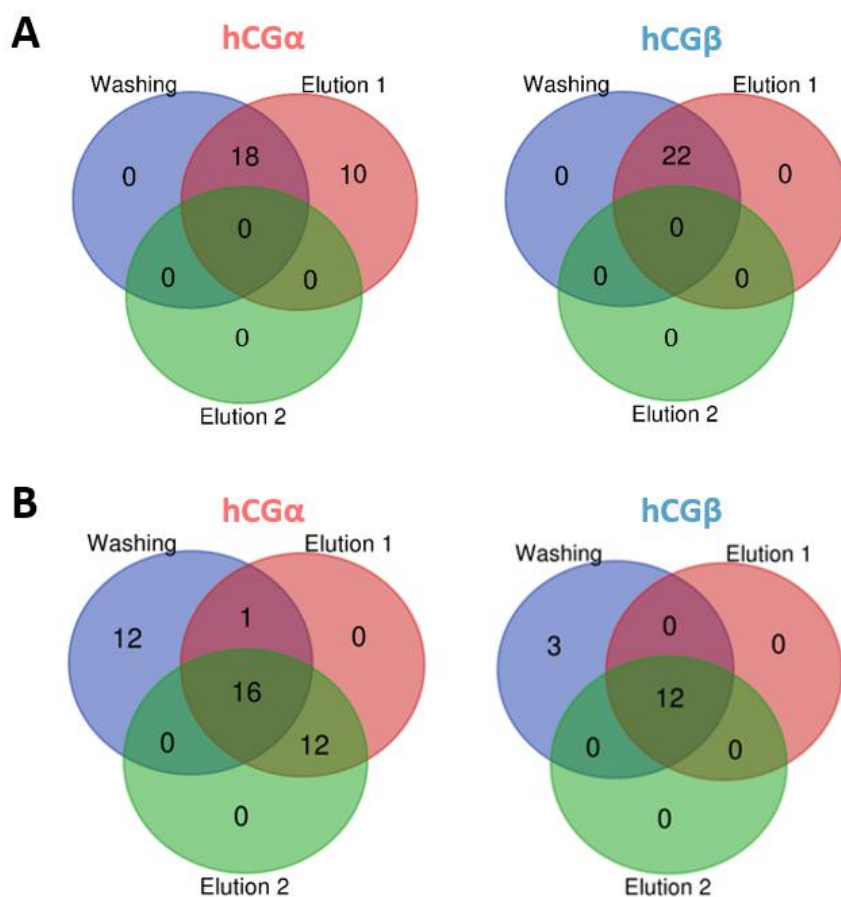


Figure 5: Venn diagrams of the number of hCG α and hCG β glycoforms detected in the concentrated SPE fractions after their nanoLC-HRMS analysis by applying (A) 5 μ g of rhCG on a 400 μ l cartridge or (B) 12.5 μ g of uhCG on a 800 μ l cartridge (protocol 4). SPE conditions: section 2.3. nano-LC HRMS conditions: section 2.4.

4. Conclusion

In this work, a more detailed evaluation as compared to the studies found in the literature of the Jacalin lectin sorbents applied to the extraction of hCG of different origins was performed. As a first step, to prevent overloading of the lectin-SPE sorbent, capacity was estimated. Secondly, it was again observed that the elimination of the residual non-grafted lectins from the sorbent by an extensive pre-conditioning before its first use was necessary to ensure good recoveries. Moreover, for the first time, SPE with Jacalin sorbents was coupled off-line with a nanoLC-HRMS for intact protein analysis. A fractionation was achieved for the uhCG sample, which led to a better detectability of some low-abundance hCG β glycoforms. This fractionation was based on differences in hCG α subunit but also in hCG β subunit, which is one of the novelty of this study. In conclusion, the use of lectin sorbents can expand the

knowledge of the hCG glycosylation hyphenated with an analysis at the intact level. This would be particularly interesting when applying lectin SPE protocols to fractionate biological samples before their analysis with the nanoLC-HRMS method to reveal glycoforms of low abundance.

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Author contribution

Anastasia Goumenou: data analysis and interpretation, drafting the article

Christophe Chendo: data analysis and interpretation

Audrey Combès: critical revision of the article

Thierry Fournier: critical revision of the article

Valérie Pichon: conception or design of the work, data interpretation, drafting the article

Nathalie Delaunay: conception or design of the work, data interpretation, drafting the article, final approval of the version to be published

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