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Characterization of Concanavalin A-based lectin sorbents for the extraction of the human chorionic gonadotropin glycoforms prior to analysis by nano liquid chromatography-high resolution mass spectrometry

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Keywords

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

Declaration

The authors declare that there are no conflicts of interest.

Abstract

Human chorionic gonadotropin (hCG) is constituted of the hCG α and hCG β subunits and is a highly glycosylated protein. Affinity supports based on immobilized Concanavalin A (Con A) lectin were used in solid phase extraction (SPE) to fractionate the hCG glycoforms according to their glycosylation state. For the first time, the lectin SPE fractions were off-line analysed by a nano liquid chromatography - high-resolution mass spectrometry (nanoLC-HRMS) method keeping the glycoforms intact. For this, home-made Con A sorbents were prepared by immobilizing lectin on Sepharose with a mean grafting yield of 98.2% (relative standard deviation (RSD) of 3.5%, n=15). A capacity of about 100 μ g of purified urinary hCG (uhCG) per ml of sorbent, grafted with a density of 10 mg of Con A per ml, was estimated. Average extraction yields of around 60% for both hCG α and hCG β glycoforms were obtained after optimization of the extraction protocol. Intra- and inter-assay evaluation led to average RSD values of around 10%, indicating a repeatable extraction procedure. Similar results were obtained with commercial Con A-based sorbents but only after their 3rd use or after an extensive pre-conditioning step. Finally, the Con A SPE led to the fractionation of some glycoforms of uhCG, allowing the detection of an hCG α glycoform with two tetra-antennary N-glycans that couldn't be detected by direct analysis in nanoLC-HRMS without Con A SPE. Regarding a recombinant hCG, a fractionation was also observed leading to the detection of unretained hCG α glycoforms with tri-antennary N-glycans. Therefore, the combination of lectin SPE with intact protein analysis by nanoLC-HRMS can contribute to a more detailed glycosylation characterization of the hCG protein.

1. Introduction

The human chorionic gonadotropin (hCG) is one of the most important hormones produced during pregnancy, playing a well-established role in the initiation and preservation of pregnancy. hCG is a heterodimeric protein composed of two subunits, alpha (hCG α) and beta (hCG β), which are non-covalently linked. hCG α contains two N-glycosylation sites on two asparagine (Asn) amino acids, Asn-52 and Asn-78. hCG β has two N-glycosylation sites on Asn-13 and Asn-30 and four O-glycosylation sites on serine (Ser), Ser-121, Ser-127, Ser-132 and Ser-138.

It is well-known that the glycosylation state of a protein plays a role in its biological activity, half-life, folding process. The N-glycans of hCG identified in urine samples from normal pregnancies are predominately mono and bi-antennary structures with or without terminal sialic acids and core fucosylation [1-3]. Variations in the glycosylation state are observed during the different terms of a normally progressing gestation. In addition, some studies suggested that abnormal glycosylation patterns of hCG are indicative of pathologies during pregnancy. For example, hCG produced by the villous cytotrophoblast from women carrying foetuses with T21 (Down Syndrome) showed variations of its glycosylation state compared to control samples [4]. Abnormal glycosylation patterns of hCG can also be detected in patients with cancer [5]. Additionally, hCG is widely administrated for the induction of ovarian stimulation of infertile women. For this reason, injectable formulations containing purified or recombinant hCG are manufactured, such as Ovitrelle® which contains recombinant hCG (rhCG). For all these reasons, it is important to establish the glycosylation patterns of hCG.

The analysis of glycoproteins can be performed at three levels, providing complementary information. The most common approach is the bottom-up strategy, which consists in analysing glycopeptides after an enzymatic digestion [1, 3, 6]. Secondly, the glycan release allows the glycan profile of the protein to be determined [7, 8]. Finally, intact protein analysis can be performed [9]. In this case, the glycoforms are directly analysed without any prior pre-treatment steps. This approach can be advantageous for several reasons, including reduced sample handling and the possibility of obtaining information on the macro-heterogeneity of glycosylation. The use of reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC) or capillary electrophoresis (CE) hyphenated with mass spectrometry (MS) was implemented for the hCG analysis at the intact level [6, 10-13]. Moreover, native MS led to a detailed characterization of the glycosylation state of the dimeric hCG and of the individual subunits [14]. Recently, we have developed an analytical method based on nano liquid chromatography (nanoLC) in reversed phase mode combined with high-

resolution mass spectrometry (HRMS) [15]. This method allowed the detection and semi-relative quantification of a high number of glycoforms of both hCG α and hCG β . However, the large number of identified glycoforms leads to an increased complexity of the acquired MS spectra. In order to simplify the analysis, a sample pre-treatment step for the fractionation of the glycoforms with lectin affinity chromatography prior to the intact protein analysis can be advantageous.

Lectin affinity chromatography is mainly a solid phase extraction (SPE) process using lectins immobilized on a solid support that is usually an agarose gel. The affinity of the sample molecules with the lectin sorbent depends on their glycosylation state, since lectins can form chemical bonds with specific sugars of other complex biomolecules. Lectin-SPE can be combined with various analytical methods, like MS, either off- or on-line. In most cases, the bottom-up approach is used [16], the combination of lectin-SPE with intact protein analysis having been rarely reported [17].

Concanavalin A (Con A) is one of the most common lectins used for the study of the glycosylation state of proteins. This lectin preferably binds to the α -mannose (Man) sugar and has affinity towards high mannose or hybrid type N-glycans (firmly bound structures). It can also bind complex-type bi-antennary N-glycans (weakly bound structures). However, highly branched complex-type N-glycans like tri- and tetra-antennary structures are not retained by this lectin [16, 18]. Given its wide application, Con A sorbents with the lectin already immobilized on a solid support are available from different suppliers.

Multiple studies report the use of Con A in SPE to study the glycosylation of hCG [19, 20-23]. The aim of these studies was to estimate the predominant glycosylation pattern(s) of hCG in biological samples. Therefore, lectin-SPE was used as a “profiling” technique, to differentiate between the biological samples of normal pregnancies from “at-risk pregnancies” or gestational and non-gestational tumours. Concerning hCG in normal pregnancy urine, the affinity with the Con A sorbent has always been reported to be high [19, 20-23]. It is also important to note that the amount of hCG applied [22, 24] or the sample volume [20, 21, 23] is only mentioned with precision in a small number of these studies. Moreover, little information is provided on the performance characteristics of the lectin columns. Indeed, recovery values are only given in some cases [20-22], while the evaluation of sorbent capacity or the justification of the volume of sorbent used are not given. In a single study [22], the washing fraction from a first sorbent was applied through a second sorbent to verify that the Con A sorbent was not overloaded, and that its capacity was therefore not exceeded. Moreover, the methods used for the analysis of the fractions after Con A SPE concern only techniques like immunoassays to estimate the levels of

hCG in the different SPE fractions. Although lectin enrichment strategies have been extensively used in glycoproteomic studies using (LC)-MS analysis [16, 25], to our knowledge, characterisation of hCG glycosylation after Con A SPE has not yet been combined with MS alone or LC-MS. In this study, the analysis of the fractions from Con A SPE was carried out in nanoLC-HRMS keeping the glycoforms intact. Data treatment was carried out to improve the description of the hCG glycoforms present in the sample. In addition, a more detailed characterisation of the performance of the lectin cartridges, home-made or commercial, like the estimation of the sorbent capacity, the determination of the recoveries and inter- and intra-assay variations was performed.

2. Materials and methods

2.1 Reagents and analytes

LC-MS grade acetonitrile (ACN) and water and HPLC grade methanol were obtained from Carlo Erba (Val-de Reuil, France). Trifluoroacetic acid (TFA), methyl α -D-glucopyranoside (α -MG), methyl α -D-mannopyranoside (α -MM), sodium chloride, Trizma® base, magnesium chloride anhydrous, manganese (II) chloride tetrahydrate, calcium chloride dihydrate, sodium acetate, cyanogen bromide-activated Sepharose 4B, sodium azide, Con A Sepharose 4B, Con A from *Canavalia ensiformis* (Jack bean) lyophilized powder, Immunoglobulin G, 1 M HCl and 0.1 M NaOH for pH adjustment, ammonium hexafluorophosphate, bicinchoninic acid assay (BCA) reagent, disposable polypropylene cartridges (SPE tubes-1 ml), polyethylene frits (20 μ m porosity) and Amicon® Ultra Centrifugal Filters with 10 kDa molecular weight cut-off (MWCO) were all purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Sodium bicarbonate and boric acid were obtained from VWR (Fontenay-sous-Bois, France). Deionized water was obtained from a Milli-Q purification system (Millipore, Molsheim, France).

hCG from human urine (uhCG) was obtained as a lyophilized powder (10,000 International Units (IU)) from Sigma Aldrich. The powder was reconstituted with 10 ml of LC-MS grade water to a final concentration of 1,000 IU ml⁻¹. As stated by the provider, the diluted formulation also contained 0.1 M sodium phosphate buffer (pH approx. 7.2) and 10 mg ml⁻¹ of mannitol. Taking into consideration that 1 IU equals to 0.092 μ g [26], the solubilized drug corresponds to a concentration of 92 μ g ml⁻¹. The 10 ml stock was aliquoted accordingly and stored at -20 °C for further use.

The recombinant human chorionic gonadotropin (rhCG) drug Ovitrelle® from transfected Chinese hamster ovary (CHO) cells was provided from Organon (Oss, The Netherlands). A step of removal of the additives and excipients of the drug formulation was performed prior to

use, by filtering with the Amicon® Ultra Centrifugal Filters with a 10 kDa MWCO as previously described [15]. The concentrated solution after filtration was further diluted with LC-MS grade water at a concentration of 500 µg ml⁻¹ of rhCG.

2.2 Preparation of “home-made” and commercial Con A lectin-based sorbents and of the control sorbent

The immobilization of Con A on Sepharose was performed by following a modified procedure previously developed from our group for protein immobilization [3]. Briefly, 70 mg of Sepharose were swollen by the addition of 2 ml of 1 mM HCl under rotation in a carousel (6 rpm for 15 min). After swelling, the Sepharose was washed twice under rotation (6 rpm for 10 min) with 2 ml of a 0.1 M bicarbonate buffer pH 8.3 containing 0.5 M NaCl and 0.5 M α-MM. The addition of α-MM was performed to protect the binding sites of the lectin during immobilization [27]. Afterwards, 120 µg of Con A in the same bicarbonate buffer containing the protective sugar were incubated with the Sepharose for 18 h at 18 °C under rotation (6 rpm). The final volume of the swollen Sepharose with the grafted lectin was about 120 µl. A control sorbent using Immunoglobulin G (IgG) was prepared in the same manner. The lectin or control sorbent were then packed between two polyethylene frits in 1 ml polypropylene cartridges, and they were subsequently pre-conditioned at a flow rate of about 0.5 ml min⁻¹ with 48 ml of 0.1 M Tris buffer, pH 8.0 and then twice with 4 ml of 0.1 M acetate buffer pH 4.0 containing 0.4 M NaCl and 4 ml of 0.1 M bicarbonate buffer pH 8.3 containing 0.5 M NaCl. Finally, they were stored in 0.02 M Tris buffer pH 7.4 plus 0.5 M NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂ and NaN₃ 0.1% (w/v).

The supernatant obtained at the end of the immobilization process was preserved to estimate the grafting yield by using a BCA assay, as previously described [3]. Briefly 10 µl of five standards of either Con A or IgG in the concentration range between 0.1 to 2 mg ml⁻¹ and of the supernatant after immobilization was separately incubated with 100 µl of the working reagent (mix of bicinchoninic acid and Cu²⁺) in a 96-microwell plate; the absorbance was then measured at 562 nm with a UV-spectrophotometer (SpectraMax M2, Molecular Devices, St Gregoire, France). The grafted amount was determined by subtracting the amount of lectin or IgG estimated in the supernatant from initial amount added. The grafting yield was calculated by dividing the grafted amount by the initial amount.

The commercial Con A lectin cartridges were prepared by packing 120 µl of the commercially available Con A-Sepharose sorbent between two polyethylene frits in 1 ml polypropylene cartridges. The commercial sorbents were stored in a 0.1 M acetate buffer pH 6.0 containing 1

M NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂ and 20% of MeOH (v/v) as recommended by the supplier.

2.3 Solid-phase extraction on lectin-based sorbent

Unless stated otherwise, 5 ml of the conditioning solution (1 M NaCl, 5 mM MgCl₂, 5 mM MnCl₂ and 5 mM CaCl₂) were applied on either the “home-made” Con A, the commercial Con A or the control sorbent. Then, 136 µl of the 92 µg ml⁻¹ uhCG stock (*i.e.* 12.5 µg) or 10 µl of the 500 µg ml⁻¹ rhCG stock (*i.e.* 5 µg) diluted with 110 µl of the 0.02 M Tris pH 7.4 plus 0.5 M NaCl buffer were percolated by gravity (different amounts of uhCG and rhCG were used as the intensity signal resulting from the nanoLC-MS analysis was lower by a factor of around 2 for uhCG than for rhCG [11]). Then, by using a syringe pump (Gemini 88 plus dual rate syringe pump, KD Scientific Inc), the column was washed with 2 ml of the same Tris buffer at a flow rate of 0.4 ml min⁻¹. Elution was performed with 4 ml of elution buffer 1 (0.1 M acetate pH 4.5 plus 0.15 M NaCl and 0.5 M α-MM) at 0.4 ml min⁻¹. Finally, a second elution step was performed with 2 ml of elution buffer 2 (0.1 M acetate pH 4.5 plus 0.15 M NaCl, 0.5 M α-MM and 15% v/v of ACN) at 0.4 ml min⁻¹.

The percolation, washing (2 ml), elution 1 (4 ml) and elution 2 (2 ml) fractions were collected, filtered and concentrated through the Amicon® Ultra Centrifugal Filters. The final volume of about 50 µl was always precisely measured to calculate the expected final concentration. After extraction, the lectin sorbent was regenerated by applying twice 2 ml of 0.1 M acetate buffer pH 4.5 plus 0.5 M NaCl and 0.1 M borate buffer pH 8.5 plus 0.5 M NaCl.

2.4 NanoLC-HRMS analysis

2.4.1 LC-HRMS method

The analysis of the concentrated fractions resulting from Con A SPE was performed by implementing the nanoLC-HRMS method previously developed in our lab involving electrospray ionisation [15]. Briefly, a nanoLC system (NC 3500 RS, ThermoFisher Scientific, Courtaboeuf, France) was coupled to an Orbitrap mass analyser (Exactive plus, ThermoFisher Scientific) and the chromatographic separation was performed with an EASY-Spray C18 column (Acclaim PepMap300, 150 mm i.d. x 0.075 mm, 5 µm, 300 Å, ThermoFisher Scientific). To protect the LC column from any particles coming from the sample that could cause clogging, two minor modifications were made to the previously published method [15]. Firstly, an Opti Solv biocompatible nano filter (0.5 µm, ThermoFisher Scientific) was placed in the 10-port valve after the C18 pre-column (Acclaim PepMap300, 0.3 mm i.d. x 5 mm, 5

μm , 300 Å, ThermoFisher Scientific). Secondly, the sample was front-flush transferred instead of the back-flush mode of transferring in the original setup.

The mobile phase compositions were based on (A) H₂O/ACN (98/2, v/v) and (B) H₂O /ACN (1/9, v/v), both with 0.05% TFA; a linear gradient of 10-65% of mobile phase (B) in 54 min (1.02%/min) was applied, followed by an isocratic 5 min step with 90% of mobile phase (B) before column equilibration for 15 min at the starting conditions. The temperature of the column was set at 60 °C, injection volume was 1 μl and the flow rate of the LC pump was 300 nl min^{-1} . A blank injection was performed between the analyses of the samples to avoid carry-over from one run to another.

The ionization parameters were: capillary voltage, 1.7 kV; capillary temperature, 280 °C; sheath, auxiliary, and sweep gas flows were set off. Analysis was performed in the positive full scan ion mode at a mass range from m/z 1000 to 4000 using a scan rate of 1 spectrum/s. On the tune page, the mass resolution was set at 140,000, the AGC target at 3e6 and the maximum injection time at 200 ms. Calibration of the orbitrap mass analyser was performed by using clusters of a salt of ammonium hexafluorophosphate in the mass range of the analysis (m/z 1000 to 4000). Control of the instrument and data acquisition were performed by using Excalibur 4 while data treatment was performed using the Freestyle 1.3 SP2 (ThermoFisher Scientific) software. The extracted ion chromatogram (XIC) of each peak present on the mass spectrum (reading m/z) was extracted with a tolerance window set at 30 mDa.

2.4.2 MS data analysis and recovery calculation

Compared to our previous method [15], MS data analysis was modified. For this study, it was performed by accurate mass measurements instead of spectra deconvolution followed by mass matching. Briefly, the calibration of the orbitrap mass analyser allows to perform accurate mass measurements determining thus the elemental composition of the detected protonated species. All the measurements were associated with an error expressed in ppm reflecting the accuracy of the experimental value compared to the theoretical one.

In the spectra, ions corresponding to the glycoforms of both hCG α and hCG β subunits were detected and identified. Ions corresponding to oxidized forms and some phosphorylated structures, as well as numerous TFA adducts of the identified glycoforms were not taken into account. The relative recovery calculation, meaning the relative distribution of the glycoforms in the SPE fractions was calculated by using the peak area of each individual glycoform from its corresponding XIC in each fraction divided with the sum of the areas of this glycoform in all fractions (washing and elution fractions 1 and 2). This process was performed for all the

glycoforms reported in **Tables S1-S4** (supplementary information) and the values were averaged.

The extraction recovery after the SPE procedure was calculated by using a filtered hCG sample, which was generated by using the same initial sample amount as for the SPE procedure and then performing the same filtration process described in section 2.3 without applying SPE. For each experiment, the extraction recovery of a glycoform was calculated by dividing its peak area measured on its XIC when analysing in nanoLC-HRMS the elution fraction from Con A SPE after their concentration with ultra-centrifugal filters until a volume of about 50 μ l with its peak area on its XIC obtained injecting the filtered hCG sample.

Finally, a semi-quantification of each glycoform was performed, as in our previous work [15]. It should be noted that the exact concentration of each glycoform is not known, a bias can be introduced in the analysis due to the electrospray ionization efficiency that may vary from one glycoforms to another. As a result, the glycoform corresponding to the most intense ion is not necessarily the one that predominates in the initial sample and absolute quantification is therefore not possible. Thus, the relative area of each hCG α glycoform was calculated by normalizing its peak area on its own XIC by the area of the hCG α glycoform with the highest area value (*i.e.* the glycoform visible on the mass spectrum with a charge state of 7 and corresponding to a monoisotopic mass of 13,941.90622 Da). For each hCG β glycoform, the same processing was performed, but with the hCG β glycoform with the highest area (*i.e.* the one visible on the mass spectrum with a charge state of 10 and corresponding to a monoisotopic mass of 23,636.52981 Da).

3. Results and discussion

3.1 Analysis by nanoLC-HRMS of rhCG and uhCG

The nanoLC-HRMS method used here was previously developed in our lab and the figures of merit determined [15]. Nevertheless, two modifications were made to protect the column from clogging: a nano filter was added and the sample was transferred in front-flush mode instead of back-flush mode. As it can be observed **Figure S1** (see Supporting information) when analysing rhCG, these changes did not affect the chromatographic profile, with hCG α and hCG β glycoforms still eluting as two distinct peaks in the base peak chromatogram (BPC). It can be noticed that under each peak, the different glycoforms of a given subunit are not well separated. Moreover, in our previous work, data treatment was based on spectra deconvolution. Unfortunately, the software used for deconvolution was not reliable as not all detected peaks belonging to the isotopic pattern were considered and mistakes were identified in the peak

selected as the monoisotopic peak used to determine the isotopic mass. Therefore, it was not possible to annotate all the hCG α glycoforms detected in the spectrum [15]. Additionally, none of the hCG β glycoforms were identified. This is why, to avoid wrong peak attribution and glycoform determination, in this study, the MS data treatment was performed manually with accurate mass measurements. For that, the experimental m/z value of the maximum isotopic peak has been deduced to the correspondent theoretical m/z value of the isotopic peak of the hCG α backbone at the same charge state (*i.e.* without sugars and disulfide bridges, $[C_{437}H_{672}N_{122}O_{134}S_{13+n}H]^{z+}$). Thus, the obtained value refers directly to the mass of the sugar combination divided by the charge. Knowing the mass of sugar residues (*i.e.* without 1 molecule of water) for hexose (Hex), N-Acetyl-Neuraminic Acid (NeuAc), N-Acetylhexosamine (HexNAc) etc., the elemental sugar combination can be determined. To validate the final elemental composition of the newly determined glycoform, its experimental m/z value at the maximum isotopic was then compared to its theoretical m/z value, and the error obtained between both values must be lower than 5 ppm. Most of the time, when one glycoform has been identified, a simple mass difference between peaks could reveal the sugar that is missing or added. This approach can lead to the identification of isobaric species even for the less intense ions present in the spectrum. It led to the identification of 29 hCG α and 22 hCG β glycoforms in the rhCG drug, presented in **Tables S1** and **S2** in Supporting information. A second hCG sample, based on hCG purified from human urine (uhCG), was analysed (BPC presented in **Figure S2**) and led to the identification of 29 hCG α and 12 hCG β glycoforms presented in **Tables S3** and **S4**. It should be highlighted that in those tables, the hexose (Hex) could correspond to mannose or galactose, while the N-Acetylhexosamine (HexNAc) could correspond to N-Acetylglucosamine or N-Acetylgalactosamine monosaccharides. Therefore, each of those identifications could correspond to more than one glycoform with the same overall glycan composition but with different glycans attached. Indeed, it has already been determined by computational annotations that numerous glycoforms can correspond to one identified combination [14]. However, this current study was mainly focused on the evaluation of the qualitative and quantitative data after Con A SPE; therefore, a full annotation of each possible glycoform for each identification was not within its scope.

3.2 Home-made Con A sorbents

3.2.1 Immobilization of Con A on Sepharose

Even if Con A-based commercial sorbents exist, home-made sorbents were synthesized in order to know precisely the amount of immobilized lectins. The lectin was grafted on Sepharose,

since it is a suitable solid support for the immobilization of proteins, with low non-specific interactions due to its hydrophilicity and resistance to pH variations [28]. The effect of the amount of lectin introduced on the grafting yield was first investigated, within a range corresponded to values given by suppliers of commercial cartridges. The amount varied between 3.3 and 17.6 mg of lectins per ml of Sepharose. Grafting yields of 97.5% and 100% were determined, respectively (Cartridges H1 and H2, see **Table S5**), showing that in the range studied, the lectin amount has no impact on the grafting yield. The repeatability of the grafting was next studied by grafting four times 10 mg of Con A per ml of Sepharose (Cartridges H3-H6, see **Table S5**). The average grafting yield was 99.0% with an relative standard deviation (RSD) of 1.0% (n=4), whereas the different cartridges were synthesized over a period of several months, thus demonstrating the repeatability of the grafting procedure.

3.2.2 Optimisation of the SPE protocol

After the successful grafting of the lectin on the solid support, the optimization of the SPE protocol was investigated. The initial protocol was chosen according to general recommendations concerning the use of Con A sorbents and conditions reported in literature for hCG extraction in particular (Protocol 1, **Table 1**). However, to avoid overloading the lectin cartridge by hCG, the experimental capacity was first investigated. First experiments were carried out on a 60 μl sorbent with a lectin content of 3.3 mg ml^{-1} (Cartridge H1). Given the hypothesis that one molecule of grafted lectin can bind one molecule of hCG, this sorbent should present a theoretical capacity of 75 μg . But, even with the introduction of three times less uhCG (25 μg), the capacity of this sorbent was exceeded, as most of the glycoforms were detected in the washing fraction (**Table S6**). Only when using a cartridge with a higher density (9.9 mg ml^{-1} , Cartridge H3) and a double volume of sorbent (120 μl) and introducing 12.5 μg of the sample, the sorbent capacity was reached. Therefore, the experimental capacity should be around 100 μg of hCG per ml of sorbent grafted with a density of about 10 mg of Con A per ml. The lower experimental capacity than the theoretical one (factor of about 35) could be explained by a low accessibility of the glycoforms to the binding sites of the immobilized lectins [29, 30]. Indeed, several parameters like the orientation of the lectin after immobilization and steric hindrance can reduce the accessibility to the binding sites and thus the capacity of the sorbent [31].

As seen in **Table 1**, the SPE protocol 1 resulted in average extraction recoveries below 50% for the hCG α and hCG β glycoforms. The missing hCG was not detected in the percolation or washing fractions. It could for example be still retained on the sorbent or lost during the SPE

procedure and the subsequent steps (fraction concentration, nanoLC-HRMS analysis) by precipitation or adsorption. An improvement of these values by varying some parameters of the SPE process was attempted. First, a longer interaction time of the sample with the lectin cartridge for 30 min before elution (protocol 2) was implemented, but this led to only a small increase in the extraction recoveries.

Table 1: Average extraction recoveries of hCG α and hCG β glycoforms after SPE on home-made Con A-based cartridges (120 μ l grafted with a density of about 10 mg of Con A per ml of gel) with different protocols (12.5 μ g of uhCG).

Proto-col	Flow rate (ml min ⁻¹)	Conditioning	Waiting time	Washing	Elution (E)		Average extraction recovery (%)	
							Elution 1	Elution 2
1	0.1	S1: 0.02 M Tris pH 7.4, 0.15 M NaCl, 1 mM MgCl ₂ , 1 mM MnCl ₂ , 1 mM CaCl ₂	30 min after percolation	S1	E1: S1 + 0.2 M α -MG E2: S1 + 0.2 M α -MM	hCG α hCG β	45.7 43.6	0.0 0.0
2	0.1	S1	30 min after percolation + 30 min after washing	S1	E1: S1 + 0.2 M α -MG E2: S1 + 0.2 M α -MM	hCG α hCG β	46.9 48.1	4.8 1.8
3	0.4 ¹	1 M NaCl, 5 mM MgCl ₂ , 5 mM MnCl ₂ , 5 mM CaCl ₂	none	0.02 M Tris pH 7.4 plus 0.5 M NaCl	E1: S2 :0.1 M acetate pH 4.5, 0.15 M NaCl, 0.5 M α -MM E2: S2 + 15% ACN	hCG α hCG β	60.6 55.6	9.6 1.2

In order to reduce the potential non-specific interactions of hCG with the Con A-grafted sorbent, a new conditioning buffer was chosen for the protocol 3. This buffer had a much higher ionic strength (1 M NaCl in the protocol 3 vs. 0.15 M NaCl in the protocols 1 and 2). It also contained in excess the metal ions necessary to bind the glycoforms to the lectin [32]. Additionally, the elution buffer was switched from a Tris buffer of pH 7.4 to an acetate buffer of pH 4.5, since it was reported that lower pH values may favour elution of glycosylated structures [33]. For the same reason, another sugar, α -MM, with a higher concentration (0.5 M) was selected, since it was suggested for the elution of firmly bound molecules [19, 21, 22]. Moreover, the metal ions were removed from the elution buffer, since it was reported that their absence can improve recovery [34]. Finally, to remove all strongly retained glycoforms from

the cartridge, a second elution buffer with the same composition as elution buffer 1 but with 15% v/v of ACN was applied. The organic eluent at this concentration meets the compatibility specifications for Sepharose and protein concentration filters, which are used after fraction collection and prior to nanoLC-HRMS analysis. In addition, as there was no clear indication of the need for waiting time before elution, it was decided to remove it. Similarly, if the sample was still percolated with gravity, a flow rate of 0.4 ml min⁻¹ was applied during the washing and elution process.

The BPCs obtained by applying the SPE protocol 3 and analysing the resulting concentrated fractions with the nanoLC-HRMS method are depicted in **Figure 1A** and the corresponding average recoveries for the glycoforms are given in **Table 1**. An improvement of the average extraction recoveries was observed, with values in the elution fraction 1 of 60.6% and 55.6% for the hCG α and hCG β glycoforms, respectively. The modifications also reduced the total extraction time from 75 to 25 min. Finally, a part of the sample was detected in the elution 2 fraction, showing a strong retention by the Con A-based sorbent. However, the organic solvent led to an increment of oxidation. Indeed, when uhCG prepared in a solution of the same composition as Elution 2 was analysed, an increase in the peaks of the oxidized variants in the BPC was observed (**Figure S3**, Supporting information). Therefore, it was preferred to keep the two-step elution with the buffer without and with ACN.

It is worthwhile to notice that on the chromatograms reported in **Figure 1A**, one or two peaks are present around 40 min, especially in the elution fractions of the home-made cartridge H4, which is not the elution area of hCG. The analysis of a Con A solution at three concentration levels with the same nanoLC-HRMS method led to peaks at about the same retention time (Supplementary information, **Figure S4**) and the same average MS spectrum. In the spectrum, the monomers of the tetrameric Con A lectin and some lower MW forms that could potentially be degradation products of the lectin or impurities were detected. Therefore, those peaks correspond to Con A. The presence of Con A could be due to some leakage from the home-made sorbent during its use, which can be attributed to the instability of the bond between the activated Sepharose and the amine ligand [35].

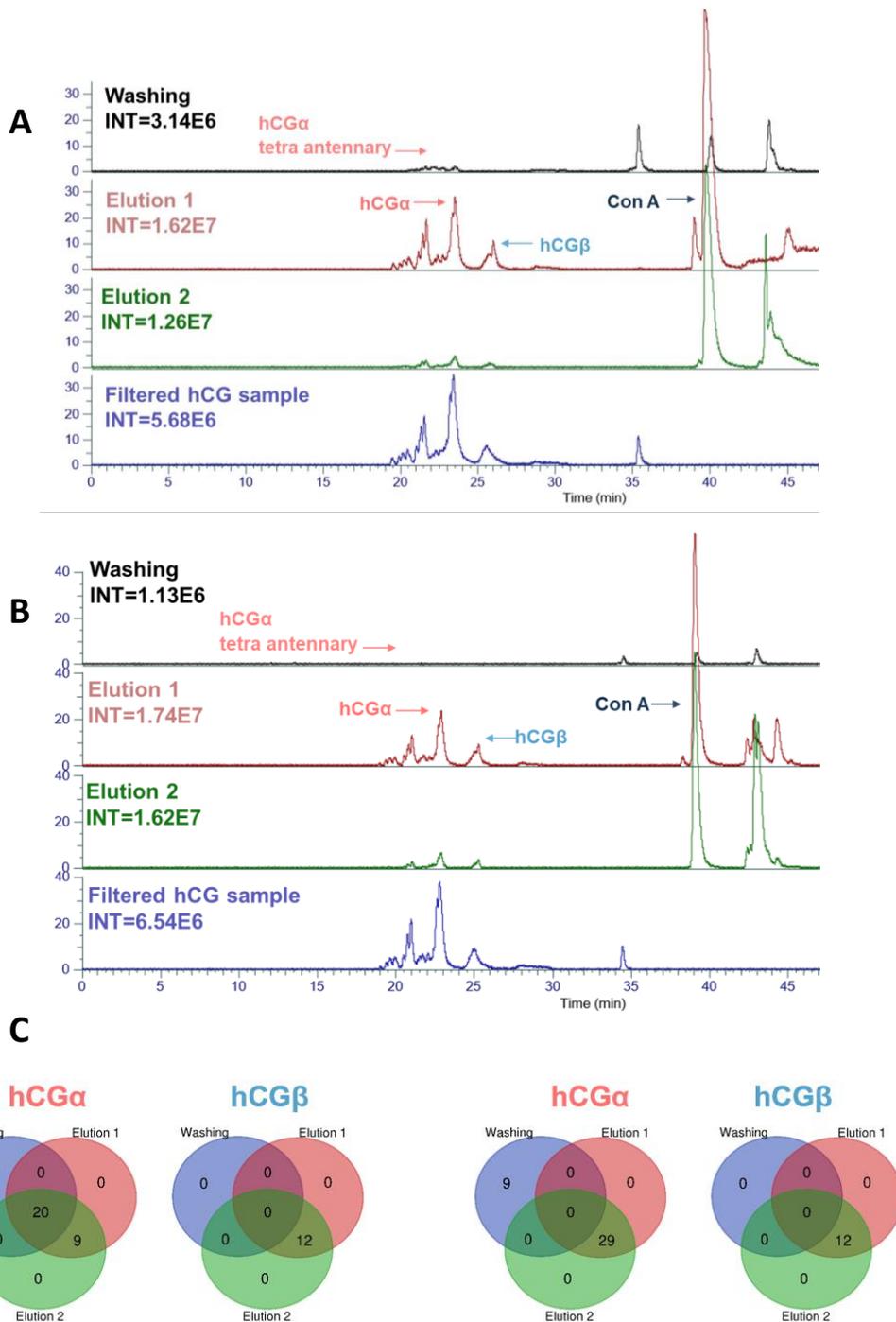


Figure 1: BPCs obtained by nanoLC-HRMS analysis of the concentrated Con A SPE fractions after percolation of 12.5 μg of uhCG on (A) the home-made cartridge H4 and (B) the commercial Cartridge C1 (protocol 3) and (C) the resulting Venn diagrams comparing the number of hCG α and hCG β glycoforms detected in each fraction (left, H4; right, C1). The BPC obtained by injecting the filtered hCG sample in nanoLC-HRMS at a concentration of 250 $\mu\text{g ml}^{-1}$ (corresponding to a recovery of 100% in a given fraction) is also represented. The intensities of the peaks in each SPE fraction and of the filtered hCG sample were normalized to the peak with the highest intensity. SPE conditions: see section 2.3. LC-MS parameters: see section 2.4.

3.3 Commercial Con A sorbents

To compare the performance of the home-made Con A sorbents to a commercial one, a cartridge containing the same volume (120 μl) of commercial Con A sorbent was prepared (cartridge C1). As the lectin content of the commercial sorbent was 13 mg ml^{-1} , the total amount of Con A in this cartridge was 1.56 mg, which was higher (30 %) than in the home-made cartridges (lectin density of 10 mg ml^{-1}). The specifications of the two Sepharose products are very close as both are based on 4% of agarose gel activated with cyanogen bromide with very similar particle sizes (45-165 and 40-165 μm for the commercial and home-made ones, respectively). Since the Con-A based sorbents are very similar, the improved SPE process (protocol 3) was used and 12.5 μg of uhCG were percolated. The collected fractions were concentrated and analysed by nanoLC-HRMS and the corresponding chromatograms are reported in **Figure 1B**. Similar observations were made for the commercial sorbent, since as with the home-made sorbent, the majority of glycoforms were detected in the elution fraction 1.

Moreover, the same peaks corresponding to Con A were observed in the chromatograms, showing also a Con A loss when the commercial cartridge is used. To estimate the amount of lectin leakage from the sorbent, a calibration curve was plotted with Con A diluted in the Elution 1 solution in the concentration range of 0.1-0.5 mg ml^{-1} and injected in nanoLC-HRMS (Supplementary information, **Figure S4**). As seen in **Figure 2**, the estimated amount of Con A leaked during the first use of the commercial cartridge C1 was almost double that calculated for the first use of the home-made sorbent (cartridge H4). Both sorbents showed a decrease in the lectin loss during their second use and the commercial sorbent exhibited a loss of 5.7 μg at the third use, which was further minimized by the fourth use. It should be noticed that, given the amount of lectin immobilized in the cartridge C1, *i.e.* 1.56 mg as previously mentioned, the loss of 12.4 μg on first use of the Cartridge C1 corresponds to less than 1% of the total amount, and is of the same order of magnitude for the home-made cartridge.

Nevertheless, it should also be noted that the average extraction yields are inversely correlated with the number of uses of the commercial sorbent and the estimated loss of Con A. Indeed, for the hCG α glycoforms, low average recoveries (below 20%) of the hCG α glycoforms were obtained in the elution fraction during the first and second use of the same cartridge and a high value of 58% was only obtained during the third use, which is equivalent to the extraction recovery determined with the home-made sorbent (61%). This behaviour was the same for the hCG β glycoforms: 7% during the first use, 16% during the second, and 62% during the third, which is close to the value measured with the home-made sorbent (56%). One hypothesis was that non-grafted Con A lectins were initially present in the packed cartridge and they may

interact with the hCG glycoforms, reducing their retention by the grafted Con A, and thus their extraction recoveries.

To test this hypothesis, an extensive pre-conditioning of the cartridge was implemented with the Tris, acetate, and bicarbonate buffers as it was usually performed with the home-made sorbents after the Con A grafting step (see section 2.2.) prior to their first use. A recovery of 52% and a loss of Con A of 5.4 μg were calculated during the first use of the extensively pre-conditioned Cartridge C2. This Con A loss value is similar to the one observed with the home-made cartridge during its first use. Without this extensive pre-conditioning, this value is reached only during the third use of the Cartridge C1. It is therefore concluded that good recoveries for the commercial sorbent can be achieved when residual non-grafted Con A lectins are efficiently removed from the sorbent, either by multiple uses of the same sorbent or by an extensive pre-conditioning procedure prior its first use. This phenomenon has never been reported in literature, as far as we know. This is because, more often than not, only qualitative and not quantitative studies are carried out.

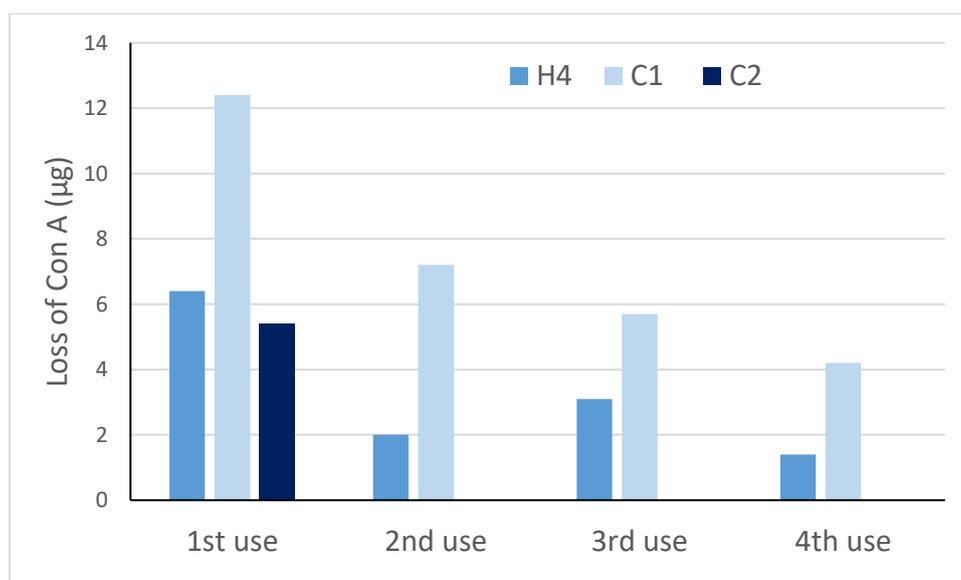


Figure 2: Histogram representing the loss of Con A from the home-made cartridge H4 (volume 120 μl , density 10 mg ml^{-1}) and the commercial cartridges C1 and extensively pre-conditioned C2 (volume 120 μl , density 13 mg ml^{-1}) as a function of the number of uses of the cartridge. SPE conditions: see section 2.3.

3.4 Intra- and inter-assay SPE repeatability

The intra-assay variation of the method was assessed by repeating the extraction of 12.5 μg of uhCG using protocol 3 on the same home-made Cartridge H4. All the data processing mentioned here were performed for the glycoforms detected in the elution fraction 1. **Table 2**

compiles the RSD ranges obtained for retention times, average extraction recoveries, and relative areas (section 2.4.2.). Regarding the intra-assay variation, the retention time RSD values are below 1.2% thus highlighting the repeatability of the nanoLC method, as already observed [15]. Regarding the relative areas, the lectin-based SPE, filtration, and nanoLC-MS steps should impact the repeatability. However, the RSD values of the relative areas did not exceed 31%, with an average of around 10%. Finally, the extraction recovery variation did not exceed 24% for both subunits and had an average value of 10.0% and 9.3% for the hCG α and hCG β , respectively. Overall, taking into account the complexity of the ionization process during analysis which can introduce many sources of variability as well as variations of the extraction process, the results were satisfactory. It should be noted that all glycoforms detected in the first experiment were also detected during the second and third repetition, giving a 100% overlap of the qualitative data.

The inter-assay variation of the method was evaluated in the elution fraction 1 by repeating the extraction on three cartridges (H4, H5 and H6) with the same lectin density and sorbent volume (Table S5). As seen in Table 2, even if the values of the inter-assay evaluation are, as expected, slightly higher than for the intra-assays, it can be concluded that inter-assay variation was satisfactory. The detailed list of the average extraction recovery of each hCG α glycoform with the corresponding RSD is presented in Table S7 (supplementary information). The average values of relative areas and their RSDs are presented in Table S8. Similarly, Table S9 and Table S10 contain the respecting information for the hCG β glycoforms. It is worthwhile to notice that regarding the individual extraction recovery values of the hCG α glycoforms and their corresponding RSD (n=3, Table S7), they are quite similar and close to the average extraction yield. This is why this simpler criterion was preferred for the discussion. A similar comment can be done for the extraction recoveries of the hCG β glycoforms (Table S9).

The same study was performed with the commercial sorbents, and the results are reported in **Table 2**. Regarding extraction recoveries and relative areas, the RSD values did not exceed 26% and 30%, respectively, for the intra-assays. The inter-assay variation was evaluated by repeating the extraction process on three different cartridges, two cartridges at their third use (Cartridges C1 and C3) and one cartridge after applying the extensive pre-conditioning process (Cartridge C2). RSD values below 23% and 29% were recorded for the relative areas and extraction recoveries, respectively. These results are quite satisfactory and similar to those obtained for home-made sorbents.

Table 2 : Intra- and inter-assay RSD of retention times, extraction recoveries, and relative areas obtained after Con A SPE and analysis of the concentrated fractions in nanoLC-HRMS on the same home-made Cartridge H4, on the same commercial Cartridge C1 or on three different home-made (H4, H5, and H6) or commercial (C1, C2, and C3) cartridges. SPE protocol 3 with 12.5 µg of uhCG (n=3).

		RSD range (average) of retention time (%)	RSD range (average) of relative area (%)	RSD range (average) of extraction recovery (%)
Home-made cartridges				
intra-assay	hCG α	0.20-0.90 (0.59)	2.8-30.2 (12.5)	1.7-18.8 (10.0)
	hCG β	0.32-1.19 (0.66)	4.1-22.8 (8.5)	2.4-23.9 (9.3)
inter-assay	hCG α	0.09-0.30 (0.21)	0.5-36.3 (11.2)	0.6-29.1 (9.5)
	hCG β	0.10-1.12 (0.41)	5.1-28.9 (13.8)	3.1-23.8 (15.7)
Commercial cartridges				
intra-assay	hCG α	0.12-0.35 (0.20)	0.8-22.1 (6.9)	1.0-25.4 (9.1)
	hCG β	0.31-1.59 (0.75)	1.8-21.7 (10.0)	5.0-25.6 (10.8)
inter-assay	hCG α	0.12-0.39 (0.25)	1.3-23.1 (10.3)	2.3-28.5 (11.3)
	hCG β	0.55-1.35 (0.79)	2.3-13.4 (8.3)	3.7-24.3 (13.6)

3.5 Study of specificity with a control sorbent

To verify the specificity of both the commercial and home-made Con A sorbents, a control sorbent was prepared with the immobilization of IgG on Sepharose. Indeed, the MW of IgG is about 150 kDa, whereas the MW of the tetramer of Con A is about 125 kDa. IgG is not expected to have affinity with hCG glycoforms and thus retain them. The grafting yield of IgG was estimated to be 90% and therefore a density of 9.0 mg ml⁻¹ was achieved by grafting 1.08 mg of the protein to 120 µl of the support.

The same SPE protocol 3 with uhCG was applied to this control sorbent. As observed in **Figure 3**, the uhCG glycoforms were mainly detected in the washing fraction, as expected. No glycoform was seen in the elution fraction 1, whereas around 11% of the glycoforms was found in elution fraction 2. This percentage should represent the retention of the sample by non-specific interactions with the solid support. However, the presence of most glycoforms in the washing fraction of the IgG-based sorbent, in contrast to the results obtained with the Con A sorbents for which most glycoforms were found in elution fraction 1, proves that the affinity of Con A sorbent towards hCG glycoforms results from specific lectin-glycoprotein interactions.

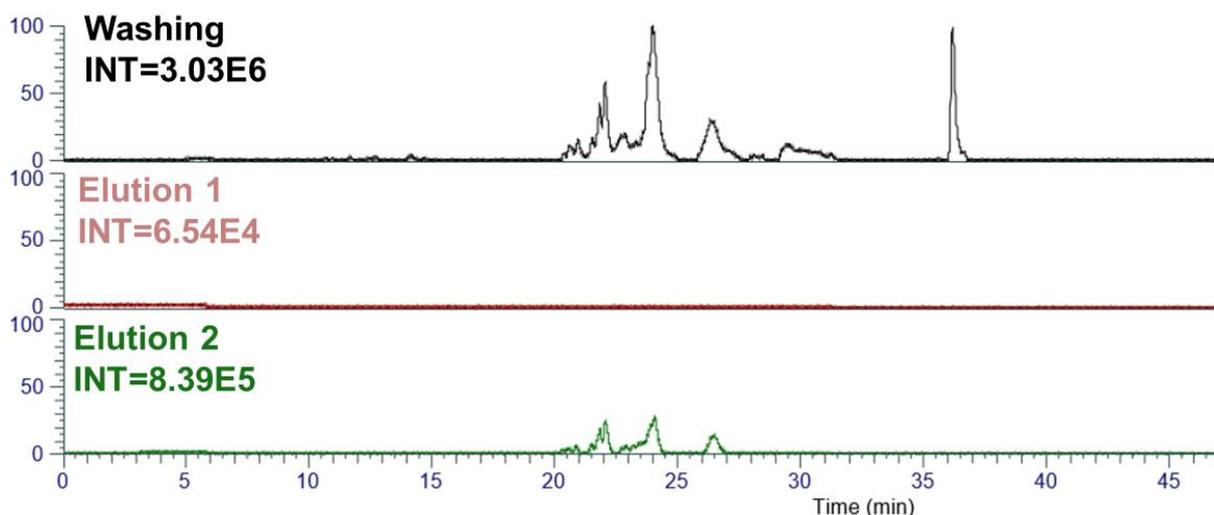


Figure 3: BPCs obtained by nanoLC-HRMS analysis of the concentrated washing fraction, elution fractions 1 and 2 from SPE with 12.5 μg of uhCG on the home-made IgG control sorbent (protocol 3). All peaks were normalized to the one with the highest intensity. SPE conditions: see section 2.3. LC-MS parameters: see section 2.4.

3.6 Glycoform fractionation of uhCG by Con A SPE

Con A sorbents can serve as tools for the fractionation of hCG glycoforms that exhibit affinity towards the lectin and the ones that do not. Thanks to the high performance of our analytical method, the data were contemplated from this point of view and led to Venn diagrams showing the number of hCG α and hCG β glycoforms detected in the different SPE fractions (**Figure 1C**). Using a home-made or commercial Con A sorbent, 9 hCG α glycoforms were present only in the washing fractions. The average mass spectrum corresponding to the nanoLC-HMRS analysis of the concentrated washing fraction from the home-made Con A SPE cartridge is reported in **Figure S5** and a similar average mass spectrum was obtained for the analysis of the same fraction from the commercial sorbent (data not shown). They could correspond to glycoforms with N-glycans having tetra-antennary structures and two phosphorylated structures as seen in **Table S11** (supplementary information), *i.e.* glycans known to have no affinity with Con A, even if the low intensity of their isotopes and bad peak shape (isobaric species that could not be separated by chromatography) did not allow their unequivocal identification, as their identification by accurate mass measurement gave an error of about 16 ppm. More importantly, they were not detected by analysing uhCG in nanoLC-HRMS without Con A SPE. Indeed, the identification of the detected glycoforms without prior Con A SPE indicated that no tri- or tetra-antennary glycans in both glycosylation sites could be attributed to any of the hCG α detected glycoforms (**Table S1**). However, recent studies concerning the glycosylation of hCG have described the presence of tetra-antennary structures in pregnancy urine, but in a much lower

abundance compared to bi-antennary structures or mono-antennary and oligo mannose structures [7]. Analysis of cancer samples, e.g. choriocarcinoma, in which glycans with higher antennarity have been reported, could also support this hypothesis [1]. Further validation of the nature of these glycoforms could be performed by MS/MS and/or glycan release approach and/or enzymatic digestion followed by LC-MS/MS.

In the chromatogram of the washing fraction, some other peaks were also detected with retention time ranges of roughly 27-28 min, 29-30.5 min, 35-35.8 min (supplementary information, **Table S12**). However, it was not possible to identify these structures due to low intensity and bad mass peak shape resulting from the chromatographic co-elution of peaks with very similar m/z. Further complementary studies are needed to identify them. Nevertheless, the Con A SPE using both the home-made and the commercial sorbent exhibited potency to differentiate those structures from the main uhCG glycoforms.

3.7 Glycoform fractionation of rhCG by Con A SPE

To investigate the ability of the lectin-based sorbents to fractionate the hCG glycoforms in different preparations, the SPE protocol 3 was applied to an hCG of different origin, *i.e.* rhCG. The BPCs of the SPE fractions obtained when using a home-made and a commercial Con A sorbent are reported in **Figure 4A** and **4B** respectively. In both cases, most glycoforms were detected in the elution fraction 1.

Qualitatively, the glycoform hCG α + HexNAc(10)Hex(12)NeuAc(5), which could correspond to two tri-antennary N-glycans with 5 NeuAc overall, was only detected in the washing fraction obtained with the commercial sorbent (see **Figure 4C**, left). This is consistent with the selectivity of the lectin, which has no affinity with the high antennarity forms. In addition, no unique glycoforms were detected in elution fraction 2 compared to elution fraction 1, meaning that these glycoforms eluted in fraction 2 were retained by non-specific interactions. Average recoveries were 41% and 42% for the hCG α and hCG β glycoforms, respectively, in elution fraction 1 and approximately 10% in elution fraction 2 (detailed extraction recoveries are presented in **Tables S13** and **S14**).

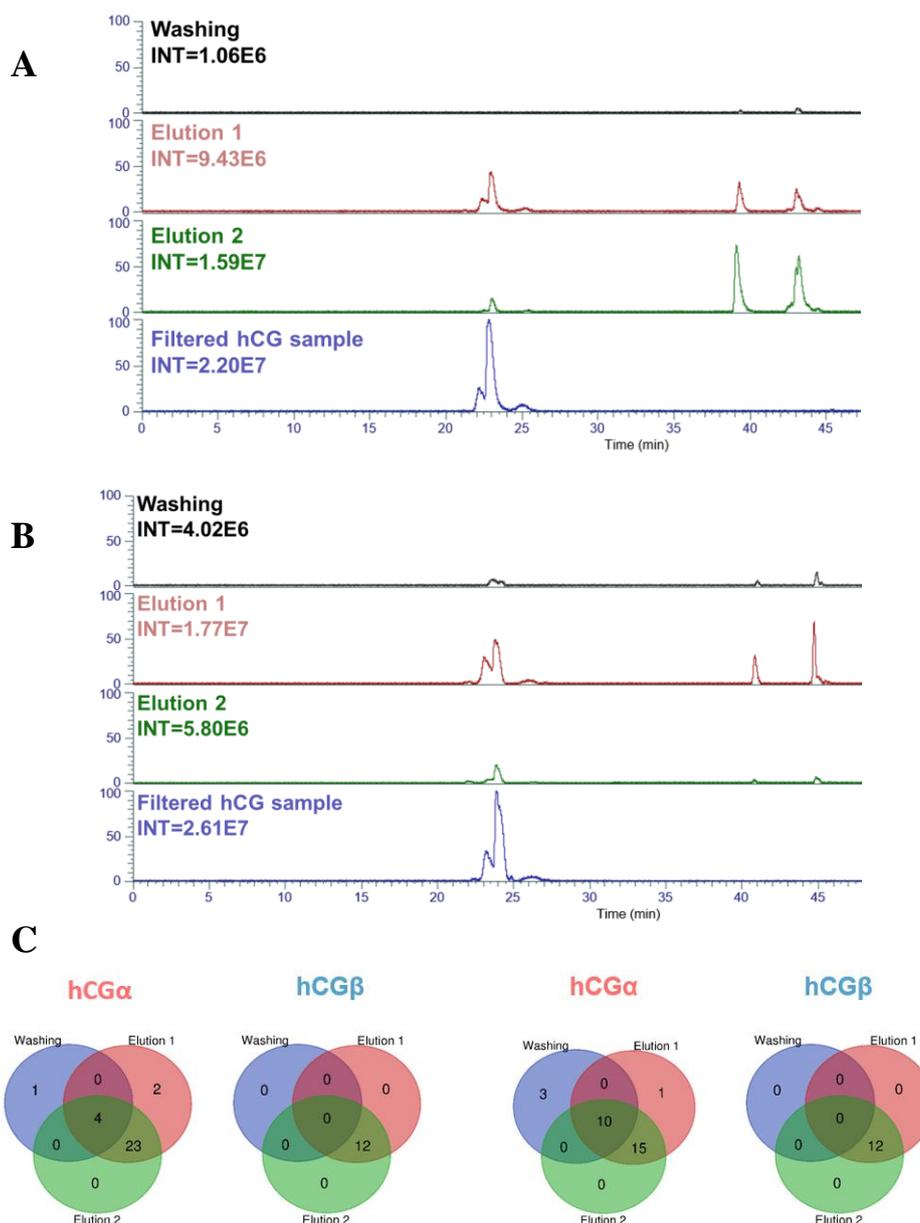


Figure 4: BPCs obtained by nanoLC-HRMS analysis of the concentrated Con A SPE fractions with 5 µg of rhCG on (A) the home-made Cartridge H4 and (B) the commercial Cartridge C1 (protocol 3) and (C) the resulting Venn diagrams comparing the number of hCGα and hCGβ glycoforms detected in each fraction (left, C1; right, H4). The BPC obtained by injecting the filtered hCG sample in nanoLC-HRMS at a concentration of 100 µg ml⁻¹ is also represented and its intensity was used to normalize the SPE fraction intensities. SPE conditions: see section 2.3. LC-MS parameters: see section 2.4.

Regarding the results obtained with the home-made Con A sorbent, and more especially the Venn diagrams in Figure 4C, 3 hCGα glycoforms were detected only in the washing fraction, including the one previously detected with the commercial sorbent: hCGα + HexNAc(10)Hex(12)NeuAc(5), plus hCGα + HexNAc(10)Hex(12)NeuAc(4), and hCGα

+HexNAc(10)Hex(12)NeuAc(3). Therefore, it seems that the home-made sorbent led here to a better fractionation according to glycosylation, isolating 2 more hCG α glycoforms with tri-antennary N-glycans, known for having no affinity with Con A. However, it is worthwhile to notice that only one replicate was performed, which may explain this difference in behavior between the home-made and commercial cartridges, especially as the glycoforms in question have low signal intensity, making their detection even more difficult. The average extraction recovery was 45% in elution fraction 1 for the hCG α glycoforms, when excluding the 3 hCG α glycoforms with the tri-antennary N-glycans. It was 10% in the elution fraction 2, as previously observed with the commercial sorbent and also with the uhCG sample. Concerning average extraction recoveries of the hCG β glycoforms, values of 50% and 10% in the elution fractions 1 and 2 were obtained, respectively. Once again, the detailed extraction recoveries are presented in **Tables S13** and **S14**. These results obtained for both subunits are similar to those obtained with the commercial cartridge.

4. Conclusions

In this study, an analysis of hCG with a nanoLC–HRMS method after SPE with lectin Con A sorbents was evaluated. Sorbents with a wide range of grafting densities were prepared with high and repeatable grafting rates, the general advantage of homemade sorbents being to adapt the ligand density to the requirements of each study. Moreover, the capacity of the sorbents was estimated, to use as little sample and sorbent amount as possible and still be within the detection and quantification limits of the analytical method. In past studies the actual amount of hCG applied on Con A lectin cartridges was rarely clearly defined [22, 24]. In this current study however, with the investigation of the capacity, we demonstrated that as little as 120 μ l of sorbent were adequate to analyse 12.5 μ g of uhCG and 5 μ g of rhCG. Moreover, with the capacity investigation, we verified that any glycoforms detected in the washing fraction would be due to a lack of affinity with the lectins and not from overloading the sorbent. In addition, optimization of the extraction parameters led to a minimized analysis time and improvement of the recovery (up to 70%). A comparison with the corresponding commercial lectin sorbents showed that repeatable extraction recoveries could only be achieved after multiple usage or extensive pre-conditioning of the sorbent to remove the residual non-grafted lectins that could potentially bind a part of hCG and prevent its retention by the lectin sorbent. Moreover, the inter- and intra-repeatability of the extraction process was evaluated with satisfactory results for both the home-made and commercial sorbents (after an adapted conditioning step of this lasts). This is the first time that a detailed characterization of lectin sorbents for the specific

extraction of hCG glycoforms is performed and then combined with a high-performance analytical method such as nanoLC-HRMS. Finally, a potency for fractionation of the sample was observed, which may enable better detection of low-abundance forms. Especially for the uhCG sample, hCG α glycoforms that are hypothesized to have two tetra-antennary N-glycans and which could not be detected without this fractionation process were revealed. This is important, as more complex glycosylated structures are potential biomarkers for various pathologies. Overall, Con A sorbents can improve the glycosylation profiling of hCG in combination with more sophisticated analytical techniques like the nanoLC–HRMS for intact protein analysis. To our knowledge, this is the first study combining lectin fractionation protocols for the investigation of the glycosylation of hCG with such an analytical technique. This study focused on fairly pure hCG samples and has the prospect of characterizing hCG glycoforms in biological fluids, particularly those of pregnant women, in order to further investigate the link between hCG glycosylation and certain pathologies of pregnancy. This will require a study of the potential influence of other compounds, including other glycoproteins present in biological samples, on the extraction yields of hCG glycoforms for example.

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Author contribution

Anastasia Goumenou: data analysis and interpretation, drafting the article

Christophe Chendo: MS 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, critical revision of the article,

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

Conflict of interest

The authors declare no conflict of interest.

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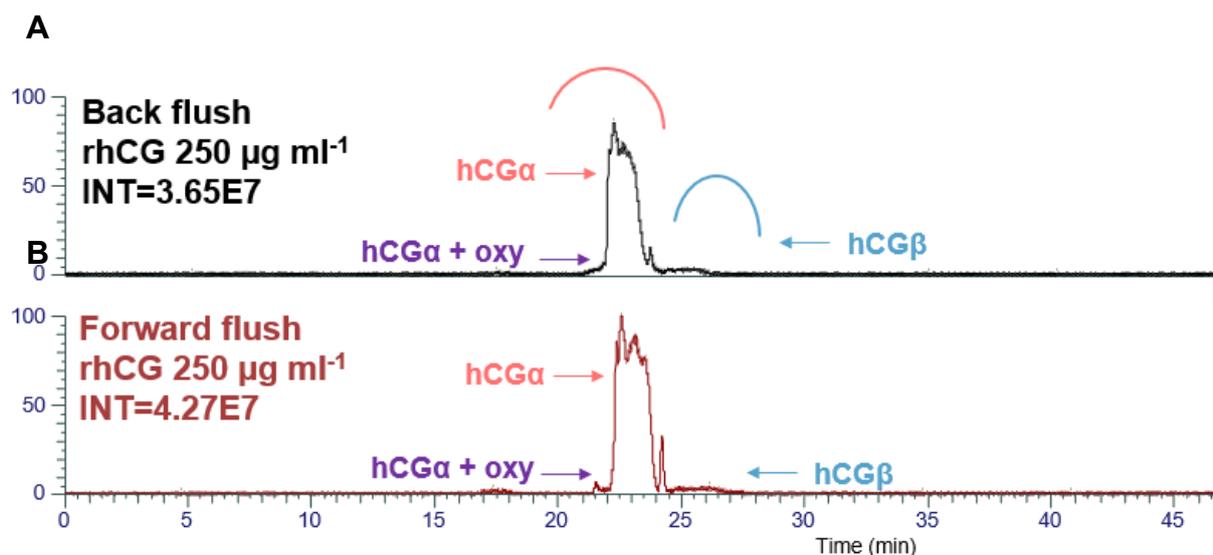


Figure S1: Base Peak Chromatogram (BPC) obtained by analysing rhCG (250 µg ml⁻¹) by nanoLC-HRMS (A) in back flush or (B) in forward flush with the Opti Solv biocompatible nano filter. All peaks were normalized to the most intense peak. In the Figure, oxy represents the oxidized variants of the subunit. LC-MS parameters: see section 2.4.

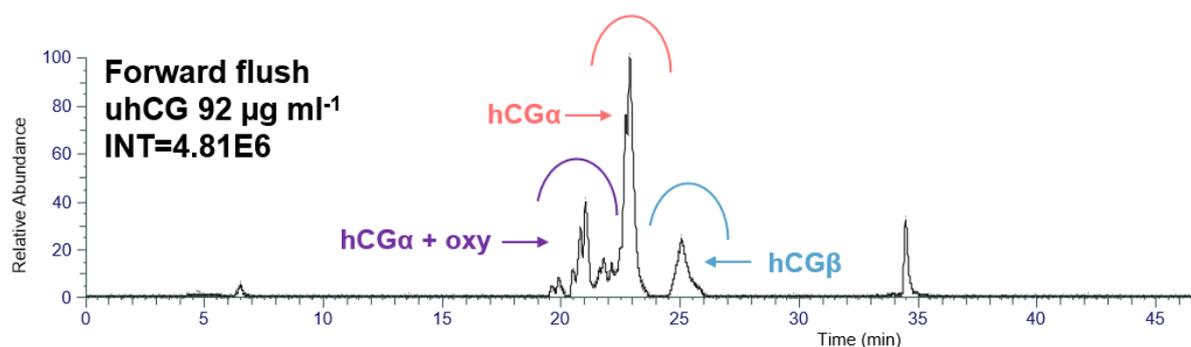


Figure S2: BPC obtained by analysing uhCG (92 µg ml⁻¹) by nanoLC-HRMS in forward flush with the Opti Solv biocompatible nano filter. In the Figure, oxy represents the oxidized variants of the subunit. All peaks were normalized to the most intense peak. LC-MS parameters: see section 2.4.

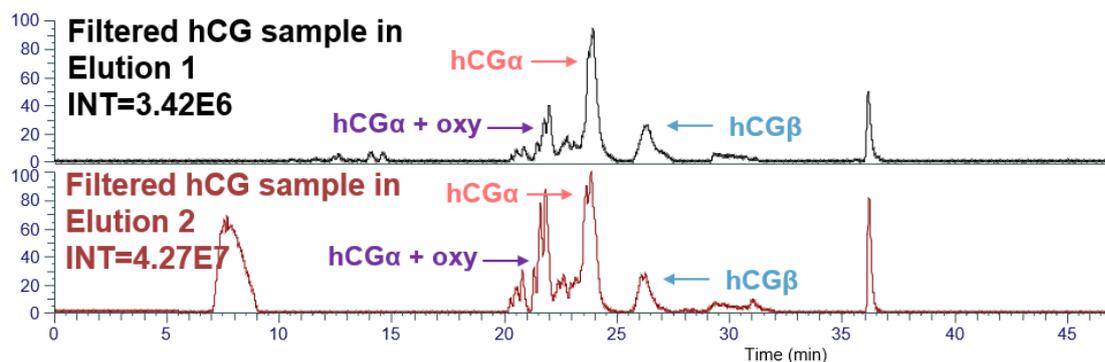


Figure S3: BPC corresponding to the analysis by nanoLC-HRMS of filtered uhCG spiked in the Elution 1 or Elution 2 fractions. All peaks were normalized to the peak with the highest intensity. In the Figure, oxy represents the oxidized variants of the subunit. LC-MS parameters: see section 2.4.

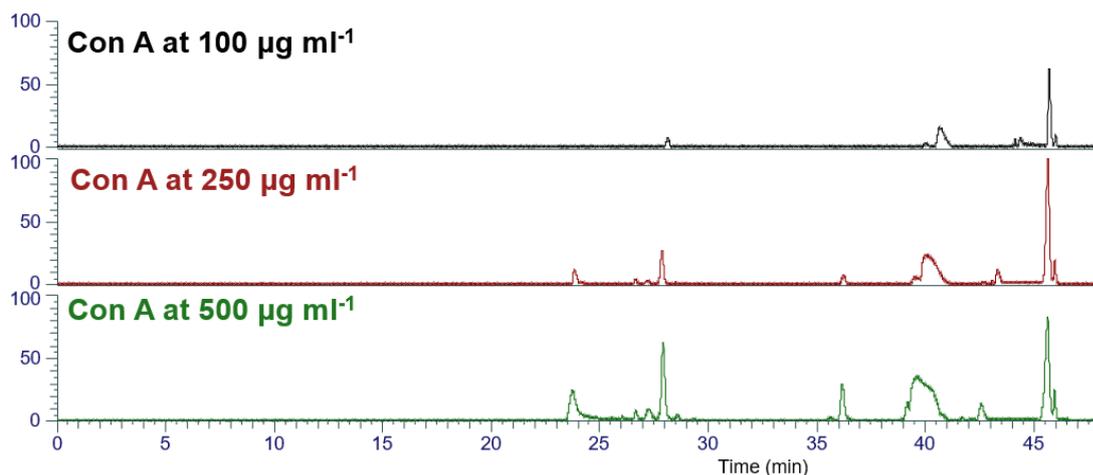


Figure S4: BPC corresponding to the analysis by nanoLC-HRMS of Con A at three concentration levels. All peaks were normalized to the peak with the highest intensity. LC-MS parameters: see section 2.4.

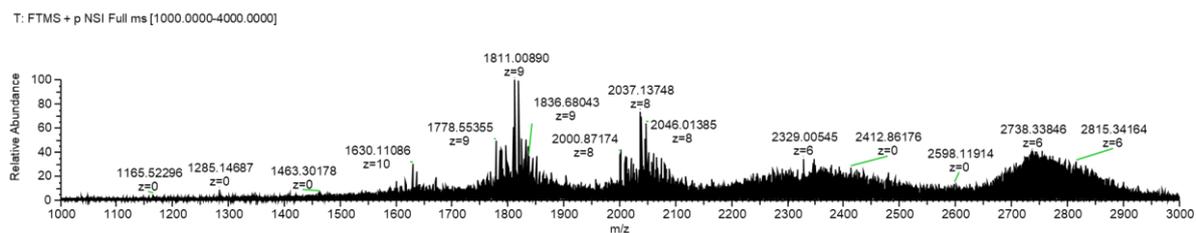


Figure S5: Average mass spectrum for the retention time range 21.9-22.1 min of the chromatogram resulting from the analysis in nanoLC-HMRS of the concentrated Con A SPE washing fraction (12.5 µg of uhCG, protocol 3, home-made cartridge H4) corresponding to the possible hCGα with N-glycans having tetra-antennary structures. SPE conditions: see section .2.3. LC-MS parameters: see section 2.4.

Table S1: Compilation of the detected hCG α glycoforms when analyzing the rhCG preparation at a concentration of 100 $\mu\text{g ml}^{-1}$ by nanoLC-HRMS detected in the average MS spectrum with a retention time range of 22.7-23.0 min. The error (in ppm) was calculated after accurate mass measurements performed at the maximum isotopic peak of the $[\text{M}+7\text{H}]^{7+}$ species.

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	m/z theo max iso (7 ⁺)	error (ppm)
C ₆₄₄ H ₁₀₀₇ N ₁₃₇ O ₂₈₄ S ₁₃	HexNAc(10)Hex(12)NeuAc(5)	15619.49365	2233.65230	0.6
C ₆₃₃ H ₉₉₀ N ₁₃₆ O ₂₇₆ S ₁₃	HexNAc(10)Hex(12)NeuAc(4)	15328.39823	2192.06721	1.8
C ₆₃₀ H ₉₈₄ N ₁₃₆ O ₂₇₄ S ₁₃	HexNAc(9)Hex(11)NeuAc(5)	15254.36145	2181.49052	3.6
C ₆₂₂ H ₉₇₃ N ₁₃₅ O ₂₆₈ S ₁₃	HexNAc(10)Hex(12)NeuAc(3)	15037.30281	2150.48213	1.4
C ₆₁₉ H ₉₆₇ N ₁₃₅ O ₂₆₆ S ₁₃	HexNAc(9)Hex(11)NeuAc(4)	14963.26603	2139.90544	3.7
C ₆₀₈ H ₉₅₀ N ₁₃₄ O ₂₅₈ S ₁₃	HexNAc(9)Hex(11)NeuAc(3)	14672.17062	2098.32036	4.0
C ₆₀₅ H ₉₄₄ N ₁₃₄ O ₂₅₆ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)	14598.13384	2087.74367	3.6
C ₆₀₀ H ₉₃₇ N ₁₃₃ O ₂₅₃ S ₁₃	HexNAc(8)Hex(11)NeuAc(3)	14469.09124	2069.30900	-3.6
C ₅₉₇ H ₉₃₃ N ₁₃₃ O ₂₅₀ S ₁₃	HexNAc(9)Hex(11)NeuAc(2)	14381.07520	2056.73528	3.7
C ₅₉₆ H ₉₃₀ N ₁₃₄ O ₂₄₈ S ₁₃	HexNAc(9)Hex(9)NeuAc(3)	14348.06497	2052.01952	2.2
C ₅₉₄ H ₉₂₇ N ₁₃₃ O ₂₄₈ S ₁₃	HexNAc(8)Hex(10)NeuAc(3)	14307.03842	2046.15859	4.1
C ₅₉₁ H ₉₂₃ N ₁₃₃ O ₂₄₅ S ₁₃	HexNAc(9)Hex(10)NeuAc(2)	14219.02238	2033.58486	0.1
C ₅₈₆ H ₉₁₄ N ₁₃₂ O ₂₄₃ S ₁₃	HexNAc(7)Hex(10)NeuAc(3)	14103.95905	2017.14723	4.7
C ₅₈₆ H ₉₁₆ N ₁₃₂ O ₂₄₂ S ₁₃	HexNAc(9)Hex(11)NeuAc(1)	14089.97978	2015.15019	2.7
C ₅₈₃ H ₉₁₀ N ₁₃₂ O ₂₄₀ S ₁₃	HexNAc(8)Hex(10)NeuAc(2)	14015.94300	2004.57350	4.4
C ₅₈₃ H ₉₁₀ N ₁₃₂ O ₂₃₉ S ₁₃	HexNAc(8)Hex(9)NeuAc(2)Fuc(1)	13999.94809	2002.28851	1.2
C ₅₈₁ H ₉₀₇ N ₁₃₁ O ₂₄₀ S ₁₃	HexNAc(7)Hex(11)NeuAc(2)	13974.91645	1998.71256	2.4
C ₅₈₀ H ₉₀₄ N ₁₃₂ O ₂₃₈ S ₁₃	HexNAc(7)Hex(9)NeuAc(3)	13941.90622	1993.99681	3.7
C ₅₇₇ H ₉₀₀ N ₁₃₂ O ₂₃₅ S ₁₃	HexNAc(8)Hex(9)NeuAc(2)	13853.89018	1981.27996	2.3
C ₅₇₄ H ₈₉₆ N ₁₃₂ O ₂₃₂ S ₁₃	HexNAc(9)Hex(9)NeuAc(1)	13765.87413	1968.70623	-0.3
C ₅₇₅ H ₈₉₇ N ₁₃₁ O ₂₃₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(2)	13812.86363	1975.41902	4.0
C ₅₇₂ H ₈₉₃ N ₁₃₁ O ₂₃₂ S ₁₃	HexNAc(8)Hex(10)NeuAc(1)	13724.84759	1962.84529	4.3
C ₅₆₉ H ₈₈₇ N ₁₃₁ O ₂₃₀ S ₁₃	HexNAc(7)Hex(9)NeuAc(2)	13650.81081	1952.26860	4.6
C ₅₆₉ H ₈₈₉ N ₁₃₁ O ₂₂₉ S ₁₃	HexNAc(9)Hex(10)NeuAc(0)	13636.83154	1950.27157	-0.7
C ₅₆₇ H ₈₈₄ N ₁₃₀ O ₂₃₀ S ₁₃	HexNAc(6)Hex(10)NeuAc(2)	13609.78426	1946.40767	4.7
C ₅₆₆ H ₈₈₃ N ₁₃₁ O ₂₂₇ S ₁₃	HexNAc(8)Hex(9)NeuAc(1)	13562.79476	1939.69488	2.1
C ₅₆₄ H ₈₈₀ N ₁₃₀ O ₂₂₇ S ₁₃	HexNAc(7)Hex(10)NeuAc(1)	13521.76821	1933.83394	2.9
C ₅₆₁ H ₈₇₆ N ₁₃₀ O ₂₂₄ S ₁₃	HexNAc(8)Hex(10)NeuAc(0)	13433.75217	1921.26021	3.0
C ₅₅₈ H ₈₇₀ N ₁₃₀ O ₂₂₂ S ₁₃	HexNAc(7)Hex(9)NeuAc(1)	13359.71539	1910.68352	2.1

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetylhexosamine (N-Acetylglucosamine or N-Acetylgalactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S2: Compilation of the detected hCG β glycoforms when analyzing the rhCG preparation at a concentration of 100 $\mu\text{g ml}^{-1}$ by nanoLC-HRMS detected in the average MS spectrum with a retention time range of 25.0-25.3 min and 25.6-25.9 min. The error (in ppm) was calculated after accurate mass measurements performed at the maximum isotopic peak of the $[\text{M}+10\text{H}]^{10+}$ species.

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	m/z theo max iso (10^+)	error (ppm)
C ₉₉₅ H ₁₆₁₃ N ₂₁₇ O ₄₄₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)Fuc(1)	24073.68314	2409.77913	0.1
C ₉₈₉ H ₁₆₀₃ N ₂₁₇ O ₄₃₇ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)	23927.62523	2395.17333	3.5
C ₉₈₄ H ₁₅₉₆ N ₂₁₆ O ₄₃₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)Fuc(1)	23782.58772	2380.66958	0.6
C ₉₇₈ H ₁₅₈₆ N ₂₁₆ O ₄₂₉ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)	23636.52981	2366.06378	4.3
C ₉₇₃ H ₁₅₇₉ N ₂₁₅ O ₄₂₅ S ₁₃	HexNAc(18)Hex(24)NeuAc(1) Fuc(1)	23491.49231	2351.56002	-1.6
C ₉₆₇ H ₁₅₆₉ N ₂₁₅ O ₄₂₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(1)	23345.43440	2336.95422	3.3
C ₉₅₈ H ₁₅₅₀ N ₂₁₈ O ₄₀₈ S ₁₃	HexNAc(18)Hex(15)NeuAc(4)Fuc(2)	23052.36105	2307.64687	0.4
C ₉₄₇ H ₁₅₃₃ N ₂₁₇ O ₄₀₀ S ₁₃	HexNAc(18)Hex(15)NeuAc(3)Fuc(2)	22761.26564	2278.53732	-1.1
C ₉₃₉ H ₁₅₂₃ N ₂₁₃ O ₄₀₁ S ₁₃	HexNAc(16)Hex(22)NeuAc(1)	22615.17000	2263.92775	21.8
C ₉₃₅ H ₁₅₁₆ N ₂₁₄ O ₃₉₆ S ₁₃	HexNAc(17)Hex(20)NeuAc(1)	22494.14373	2251.82511	-1.1
C ₉₁₉ H ₁₄₉₀ N ₂₁₂ O ₃₈₆ S ₁₃	HexNAc(15)Hex(20)NeuAc(1)	22087.98498	2211.10901	-2.8
C ₉₁₆ H ₁₄₈₄ N ₂₁₂ O ₃₈₄ S ₁₃	HexNAc(14)Hex(19)NeuAc(2)	22013.94820	2203.70532	4.0
C ₉₁₁ H ₁₄₇₇ N ₂₁₁ O ₃₈₁ S ₁₃	HexNAc(14)Hex(20)NeuAc(1)	21884.90561	2190.80106	1.1
C ₉₁₀ H ₁₄₇₆ N ₂₁₂ O ₃₇₇ S ₁₃	HexNAc(16)Hex(18)NeuAc(0)Fuc(1)	21821.92120	2184.50262	1.6
C ₉₀₅ H ₁₄₆₇ N ₂₁₁ O ₃₇₆ S ₁₃	HexNAc(14)Hex(19)NeuAc(1)	21722.85279	2174.59577	5.0
C ₉₀₀ H ₁₄₆₀ N ₂₁₀ O ₃₇₃ S ₁₃	HexNAc(14)Hex(20)NeuAc(0)	21593.81019	2161.69150	2.3
C ₈₉₇ H ₁₄₅₂ N ₂₁₀ O ₃₇₂ S ₁₃	HexNAc(11)Hex(18)NeuAc(3)	21533.75268	2155.68575	4.7
C ₈₉₆ H ₁₄₄₉ N ₂₁₁ O ₃₆₉ S ₁₃	HexNAc(11)Hex(14)NeuAc(4)Fuc(1)	21484.74753	2150.78523	2.3
C ₈₉₄ H ₁₄₅₀ N ₂₁₀ O ₃₆₈ S ₁₃	HexNAc(14)Hex(19)NeuAc(0)	21431.75737	2145.48621	5.7
C ₈₉₃ H ₁₄₄₄ N ₂₁₄ O ₃₆₁ S ₁₃	HexNAc(15)Hex(11)NeuAc(3)Fuc(1)	21357.75831	2138.08630	-6.9
C ₈₉₁ H ₁₄₄₃ N ₂₁₃ O ₃₆₀ S ₁₃	HexNAc(16)Hex(13)NeuAc(1) Fuc(1)	21302.75250	2132.58572	6.6
C ₈₈₅ H ₁₄₃₃ N ₂₁₃ O ₃₅₅ S ₁₃	HexNAc(16)Hex(12)NeuAc(1) Fuc(1)	21140.69968	2116.38043	4.2

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S3: Compilation of the detected hCG α glycoforms when analyzing the uhCG preparation at a concentration of 92 $\mu\text{g ml}^{-1}$ by nanoLC-HRMS detected in the average MS spectrum with a retention time range of 23.3-23.6 min. The error (in ppm) was calculated after accurate mass measurements performed at the maximum isotopic peak of the $[\text{M}+7\text{H}]^{7+}$ species.

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	m/z theo max iso (7 ⁺)	error (ppm)
C ₆₁₁ H ₉₅₄ N ₁₃₄ O ₂₆₀ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)Fuc(1)	14744.19175	2108.60910	1.3
C ₆₀₅ H ₉₄₄ N ₁₃₄ O ₂₅₆ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)	14598.13384	2087.74367	2.5
C ₅₉₈ H ₉₃₄ N ₁₃₂ O ₂₅₅ S ₁₃	HexNAc(7)Hex(12)NeuAc(3)	14428.06469	2063.44807	-1.0
C ₅₉₅ H ₉₂₇ N ₁₃₅ O ₂₄₆ S ₁₃	HexNAc(9)Hex(7)NeuAc(4)	14315.05474	2047.30377	-2.1
C ₅₉₄ H ₉₂₇ N ₁₃₃ O ₂₄₈ S ₁₃	HexNAc(8)Hex(10)NeuAc(3)	14307.03842	2046.15859	3.3
C ₅₉₂ H ₉₂₄ N ₁₃₂ O ₂₄₈ S ₁₃	HexNAc(7)Hex(11)NeuAc(3)	14266.01187	2040.29765	1.8
C ₅₈₆ H ₉₁₄ N ₁₃₂ O ₂₄₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(3)	14103.95905	2017.14723	2.1
C ₅₈₄ H ₉₁₀ N ₁₃₄ O ₂₃₈ S ₁₃	HexNAc(9)Hex(7)NeuAc(3)	14023.95932	2005.71869	-1.0
C ₅₈₂ H ₉₀₇ N ₁₃₃ O ₂₃₈ S ₁₃	HexNAc(8)Hex(8)NeuAc(3)	13982.93277	1999.85775	-0.3
C ₅₈₁ H ₉₀₇ N ₁₃₁ O ₂₄₀ S ₁₃	HexNAc(7)Hex(11)NeuAc(2)	13974.91645	1998.71256	1.3
C ₅₈₀ H ₉₀₄ N ₁₃₂ O ₂₃₈ S ₁₃	HexNAc(7)Hex(9)NeuAc(3)	13941.90622	1993.99681	2.7
C ₅₇₆ H ₈₉₇ N ₁₃₃ O ₂₃₅ S ₁₃	HexNAc(8)Hex(7)NeuAc(3)	13820.87995	1976.56421	0.2
C ₅₇₅ H ₈₉₇ N ₁₃₁ O ₂₃₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(2)	13812.86363	1975.41902	2.7
C ₅₇₃ H ₈₉₄ N ₁₃₀ O ₂₃₅ S ₁₃	HexNAc(6)Hex(11)NeuAc(2)	13771.83708	1969.55808	0.8
C ₅₇₁ H ₈₉₀ N ₁₃₂ O ₂₃₀ S ₁₃	HexNAc(8)Hex(8)NeuAc(2)	13691.83736	1958.12954	-2.4
C ₅₇₀ H ₈₈₇ N ₁₃₃ O ₂₂₈ S ₁₃	HexNAc(8)Hex(6)NeuAc(3)	13658.82712	1953.41379	0.6
C ₅₆₉ H ₈₈₇ N ₁₃₁ O ₂₃₀ S ₁₃	HexNAc(7)Hex(9)NeuAc(2)	13650.81081	1952.26860	4.6
C ₅₆₇ H ₈₈₄ N ₁₃₀ O ₂₃₀ S ₁₃	HexNAc(6)Hex(10)NeuAc(2)	13609.78426	1946.40767	2.8
C ₅₆₅ H ₈₈₀ N ₁₃₂ O ₂₂₅ S ₁₃	HexNAc(8)Hex(7)NeuAc(2)	13529.78453	1934.97912	-1.2
C ₅₆₁ H ₈₇₄ N ₁₃₀ O ₂₂₅ S ₁₃	HexNAc(6)Hex(9)NeuAc(2)	13447.73143	1923.25725	2.3
C ₅₅₉ H ₈₇₀ N ₁₃₂ O ₂₂₀ S ₁₃	HexNAc(8)Hex(6)NeuAc(2)	13367.73171	1911.82871	-1.6
C ₅₅₇ H ₈₆₇ N ₁₃₁ O ₂₂₀ S ₁₃	HexNAc(7)Hex(7)NeuAc(2)	13326.65234	1905.96777	0.2
C ₅₅₆ H ₈₆₇ N ₁₂₉ O ₂₂₂ S ₁₃	HexNAc(6)Hex(10)NeuAc(1)	13318.68884	1904.82258	2.3
C ₅₅₅ H ₈₆₄ N ₁₃₀ O ₂₂₀ S ₁₃	HexNAc(6)Hex(8)NeuAc(2)	13285.67861	1900.10683	2.5
C ₅₅₁ H ₈₅₇ N ₁₃₁ O ₂₁₅ S ₁₃	HexNAc(7)Hex(6)NeuAc(2)	13164.72160	1882.81735	0.0
C ₅₄₅ H ₈₄₇ N ₁₃₁ O ₂₁₀ S ₁₃	HexNAc(7)Hex(5)NeuAc(2)	13002.59951	1859.66693	-0.4
C ₅₄₄ H ₈₄₇ N ₁₂₉ O ₂₁₂ S ₁₃	HexNAc(6)Hex(8)NeuAc(1)	12994.58319	1858.52175	2.9
C ₅₄₂ H ₈₄₄ N ₁₂₈ O ₂₁₂ S ₁₃	HexNAc(5)Hex(9)NeuAc(1)	12953.55664	1852.66081	2.7
C ₅₃₂ H ₈₂₇ N ₁₂₉ O ₂₀₂ S ₁₃	HexNAc(6)Hex(6)NeuAc(1)	12670.47755	1812.22091	-1.3

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S4: Compilation of the detected hCG β glycoforms when analysing the uhCG preparation at a concentration of 92 $\mu\text{g ml}^{-1}$ by nanoLC-HRMS detected in the average MS spectrum with a retention time range of 25.5-25.8 min. The error (in ppm) was calculated after accurate mass measurements performed at the maximum isotopic peak of the $[\text{M}+10\text{H}]^{10+}$ species.

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	m/z theo max iso (10^+)	error (ppm)
C ₉₈₉ H ₁₆₀₃ N ₂₁₇ O ₄₃₇ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)	23927.62523	2395.17333	0.1
C ₉₈₄ H ₁₅₉₆ N ₂₁₆ O ₄₃₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(2) Fuc(1)	23782.58772	2380.66958	-4.9
C ₉₇₈ H ₁₅₈₆ N ₂₁₆ O ₄₂₉ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)	23636.52981	2366.06378	0.5
C ₉₇₇ H ₁₅₈₀ N ₂₂₀ O ₄₂₃ S ₁₃	HexNAc(19)Hex(17)NeuAc(5)	23578.52567	2360.26335	-3.5
C ₉₇₀ H ₁₅₇₃ N ₂₁₅ O ₄₂₃ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)Fuc(1)	23417.45553	2344.15634	0.2
C ₉₆₇ H ₁₅₆₉ N ₂₁₅ O ₄₂₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(1)	23345.43440	2336.95422	0.2
C ₉₆₄ H ₁₅₆₃ N ₂₁₅ O ₄₁₉ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)	23271.39762	2329.55054	-0.2
C ₉₅₉ H ₁₅₅₆ N ₂₁₄ O ₄₁₅ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)Fuc(1)	23126.36011	2315.04679	-1.9
C ₉₅₆ H ₁₅₅₂ N ₂₁₄ O ₄₁₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(0)	23054.33898	2307.84467	-0.4
C ₉₅₃ H ₁₅₄₆ N ₂₁₄ O ₄₁₁ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)	22980.30220	2300.44099	-0.2
C ₉₄₁ H ₁₅₂₃ N ₂₁₁ O ₄₀₆ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(2)	22691.13843	2271.52460	3.0
C ₉₃₅ H ₁₅₁₃ N ₂₁₁ O ₄₀₂ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(1)	22545.08052	2256.91880	1.8

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetylhexosamine (N-Acetylglucosamine or N-Acetylgalactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S5: List of the synthesized Con A-Sepharose sorbents with the measured grafting yields and their corresponding lectin density.

cartridge number	Amount of Sepharose (μl)	Amount of lectin added (mg)	Amount of lectin grafted (mg)	Grafting yield (%)	Lectin density (mg ml^{-1})
Cartridge H1	60	0.20	0.195	97.5	3.3
Cartridge H2	34	0.60	0.600	100.0	17.6
Cartridge H3	120	1.20	1.192	99.3	9.9
Cartridge H4	120	1.20	1.200	100.0	10.0
Cartridge H5	120	1.20	1.172	97.7	9.8
Cartridge H6	120	1.20	1.187	98.9	9.9

Table S6: Theoretical capacity values and relative recovery (distribution) of the hCG α and hCG β glycoforms between the washing and elution fractions by applying different amounts of the uhCG sample on cartridges of different volumes and lectin densities. SPE protocol conditions are those applied before the optimisation of extraction.

Flow rate	Cartridge conditioning	sample interaction	Washing conditions	Elution conditions	sorbent: volume (μ l), lectin density (mg/ml)	Percolated uhCG (μ g)	Theoretical capacity (μ g/cartridge)		Average relative recovery in the SPE fractions (%)		
									Washing	Elution 1	Elution 2
0.1 ml min ⁻¹	S1: 0.02 M Tris buffer pH 7.4 plus 0.15 M NaCl, 1 mM MgCl ₂ , 1 mM MnCl ₂ , and 1 mM CaCl ₂	30 min after percolation	S1	Elution 1: S1 + 0.2 M α -MG	H1: 60, 3.3	25.0	75.0	hCG α	81.4	18.6	0.0
				Elution 2: S1 + 0.2 M α -MM	H3: 120, 9.9	12.5	458.5	hCG α	3.1	96.9	0.0
								hCG β	78.4	21.6	0.0
								hCG β	0.0	100.0	0.0

Table S7: Compilation of the detected hCG α glycoforms in the concentrated elution 1 fraction analysed by nanoLC-HRMS after Con A SPE following protocol 3 with 12.5 μ g of uhCG and their average extraction recovery (<extraction recovery>) with their corresponding RSD values on three different home-made cartridges (H4, H5, H6) or commercial cartridges (C1, C2, C3).

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	Home-made cartridges		Commercial cartridges	
			<extraction recovery>	RSD	<extraction recovery>	RSD
C ₆₁₁ H ₉₅₄ N ₁₃₄ O ₂₆₀ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)Fuc(1)	14744.19175	63.3%	5.4%	56.2%	28.5%
C ₆₀₅ H ₉₄₄ N ₁₃₄ O ₂₅₆ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)	14598.13384	66.0%	9.8%	54.8%	11.5%
C ₅₉₈ H ₉₃₄ N ₁₃₂ O ₂₅₃ S ₁₃	HexNAc(7)Hex(12)NeuAc(3)	14428.06469	57.7%	29.1%	62.2%	15.4%
C ₅₉₅ H ₉₂₇ N ₁₃₅ O ₂₄₆ S ₁₃	HexNAc(9)Hex(7)NeuAc(4)	14315.05474	57.1%	7.4%	57.6%	10.4%
C ₅₉₄ H ₉₂₇ N ₁₃₃ O ₂₄₈ S ₁₃	HexNAc(8)Hex(10)NeuAc(3)	14307.03842	57.8%	7.9%	55.1%	6.7%
C ₅₉₂ H ₉₂₄ N ₁₃₂ O ₂₄₈ S ₁₃	HexNAc(7)Hex(11)NeuAc(3)	14266.01187	62.9%	6.7%	55.0%	5.0%
C ₅₈₆ H ₉₁₄ N ₁₃₂ O ₂₄₃ S ₁₃	HexNAc(7)Hex(10)NeuAc(3)	14103.95905	54.6%	2.7%	53.2%	2.3%
C ₅₈₄ H ₉₁₀ N ₁₃₄ O ₂₃₈ S ₁₃	HexNAc(9)Hex(7)NeuAc(3)	14023.95932	70.9%	6.5%	62.6%	26.8%
C ₅₈₂ H ₉₀₇ N ₁₃₃ O ₂₃₈ S ₁₃	HexNAc(8)Hex(8)NeuAc(3)	13982.93277	71.4%	3.4%	60.9%	13.2%
C ₅₈₁ H ₉₀₇ N ₁₃₁ O ₂₄₀ S ₁₃	HexNAc(7)Hex(11)NeuAc(2)	13974.91645	63.9%	10.3%	57.4%	21.2%
C ₅₈₀ H ₉₀₄ N ₁₃₂ O ₂₃₈ S ₁₃	HexNAc(7)Hex(9)NeuAc(3)	13941.90622	61.8%	7.1%	54.1%	2.4%
C ₅₇₆ H ₈₉₇ N ₁₃₃ O ₂₃₃ S ₁₃	HexNAc(8)Hex(7)NeuAc(3)	13820.87995	53.9%	5.1%	49.0%	7.9%
C ₅₇₅ H ₈₉₇ N ₁₃₁ O ₂₃₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(2)	13812.86363	58.9%	17.3%	62.9%	14.3%
C ₅₇₃ H ₈₉₄ N ₁₃₀ O ₂₃₅ S ₁₃	HexNAc(6)Hex(11)NeuAc(2)	13771.83708	68.7%	0.6%	55.4%	12.0%
C ₅₇₁ H ₈₉₀ N ₁₃₂ O ₂₃₀ S ₁₃	HexNAc(8)Hex(8)NeuAc(2)	13691.83736	53.5%	21.5%	61.7%	11.8%
C ₅₇₀ H ₈₈₇ N ₁₃₃ O ₂₂₈ S ₁₃	HexNAc(8)Hex(6)NeuAc(3)	13666.88973	61.2%	5.8%	55.3%	6.1%
C ₅₆₉ H ₈₈₇ N ₁₃₁ O ₂₃₀ S ₁₃	HexNAc(7)Hex(9)NeuAc(2)	13650.81081	62.6%	9.1%	59.1%	9.1%
C ₅₆₇ H ₈₈₄ N ₁₃₀ O ₂₃₀ S ₁₃	HexNAc(6)Hex(10)NeuAc(2)	13609.78426	70.4%	23.4%	59.4%	6.3%
C ₅₆₅ H ₈₈₀ N ₁₃₂ O ₂₂₅ S ₁₃	HexNAc(8)Hex(7)NeuAc(2)	13529.78453	58.5%	21.3%	59.1%	19.4%
C ₅₆₁ H ₈₇₄ N ₁₃₀ O ₂₂₅ S ₁₃	HexNAc(6)Hex(9)NeuAc(2)	13447.73143	59.6%	6.9%	54.8%	5.1%
C ₅₅₉ H ₈₇₀ N ₁₃₂ O ₂₂₀ S ₁₃	HexNAc(8)Hex(6)NeuAc(2)	13367.73171	51.2%	13.3%	53.8%	9.4%
C ₅₅₇ H ₈₆₇ N ₁₃₁ O ₂₂₀ S ₁₃	HexNAc(7)Hex(7)NeuAc(2)	13326.65234	66.2%	6.8%	58.9%	15.1%
C ₅₅₆ H ₈₆₇ N ₁₂₉ O ₂₂₂ S ₁₃	HexNAc(6)Hex(10)NeuAc(1)	13318.68884	56.5%	5.0%	73.0%	16.9%
C ₅₅₅ H ₈₆₄ N ₁₃₀ O ₂₂₀ S ₁₃	HexNAc(6)Hex(8)NeuAc(2)	13285.67861	58.3%	5.3%	50.8%	4.2%
C ₅₅₁ H ₈₅₇ N ₁₃₁ O ₂₁₅ S ₁₃	HexNAc(7)Hex(6)NeuAc(2)	13164.72160	64.4%	3.1%	55.0%	6.6%
C ₅₄₅ H ₈₄₇ N ₁₃₁ O ₂₁₀ S ₁₃	HexNAc(7)Hex(5)NeuAc(2)	13002.59951	58.3%	2.8%	53.3%	3.6%
C ₅₄₄ H ₈₇₆ N ₁₂₉ O ₂₁₂ S ₁₃	HexNAc(6)Hex(8)NeuAc(1)	12994.58319	55.0%	18.2%	52.1%	15.4%
C ₅₄₂ H ₈₄₄ N ₁₂₈ O ₂₁₂ S ₁₃	HexNAc(5)Hex(9)NeuAc(1)	12953.55664	62.3%	7.1%	53.9%	9.9%
C ₅₃₂ H ₈₂₇ N ₁₂₉ O ₂₀₂ S ₁₃	HexNAc(6)Hex(6)NeuAc(1)	12670.47755	61.4%	6.4%	59.6%	14.5%

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S8: Compilation of the detected hCG α glycoforms in the concentrated elution 1 fraction analysed by nanoLC-HRMS after Con A SPE following protocol 3 with 12.5 μ g of uhCG and their average relative area (<relative area>) measured on the XIC with their corresponding RSD on three different home-made cartridges (H4, H5, H6) or commercial cartridges (C1, C2, C3). Semi-quantification was performed by the normalization to the highest area value highlighted in grey.

Elemental composition	Proposed glycan structure	Theoretical MM (Da)	Home-made cartridges		Commercial cartridges	
			<relative area>	RSD	<relative area>	RSD
C ₆₁₁ H ₉₅₄ N ₁₃₄ O ₂₆₀ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)Fuc(1)	14744.19175	6.2%	16.2%	6.6%	2.2%
C ₆₀₅ H ₉₄₄ N ₁₃₄ O ₂₅₆ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)	14598.13384	20.8%	13.6%	25.8%	23.1%
C ₅₉₈ H ₉₃₄ N ₁₃₂ O ₂₅₃ S ₁₃	HexNAc(7)Hex(12)NeuAc(3)	14428.06469	13.9%	14.2%	14.9%	5.4%
C ₅₉₅ H ₉₂₇ N ₁₃₅ O ₂₄₆ S ₁₃	HexNAc(9)Hex(7)NeuAc(4)	14315.05474	22.8%	12.8%	25.3%	11.9%
C ₅₉₄ H ₉₂₇ N ₁₃₃ O ₂₄₈ S ₁₃	HexNAc(8)Hex(10)NeuAc(3)	14307.03842	16.6%	5.0%	19.7%	19.0%
C ₅₉₂ H ₉₂₄ N ₁₃₂ O ₂₄₈ S ₁₃	HexNAc(7)Hex(11)NeuAc(3)	14266.01187	36.3%	10.5%	35.2%	5.2%
C ₅₈₆ H ₉₁₄ N ₁₃₂ O ₂₄₃ S ₁₃	HexNAc(7)Hex(10)NeuAc(3)	14103.95905	62.0%	7.5%	67.4%	6.3%
C ₅₈₄ H ₉₁₀ N ₁₃₄ O ₂₃₈ S ₁₃	HexNAc(9)Hex(7)NeuAc(3)	14023.95932	9.5%	36.3%	11.3%	21.3%
C ₅₈₂ H ₉₀₇ N ₁₃₃ O ₂₃₈ S ₁₃	HexNAc(8)Hex(8)NeuAc(3)	13982.93277	37.7%	6.3%	39.4%	10.5%
C ₅₈₁ H ₉₀₇ N ₁₃₁ O ₂₄₀ S ₁₃	HexNAc(7)Hex(11)NeuAc(2)	13974.91645	12.9%	16.9%	17.3%	20.2%
C ₅₈₀ H ₉₀₄ N ₁₃₂ O ₂₃₈ S ₁₃	HexNAc(7)Hex(9)NeuAc(3)	13941.90622	100.0%	0.0%	100.0%	0.0%
C ₅₇₆ H ₈₉₇ N ₁₃₃ O ₂₃₃ S ₁₃	HexNAc(8)Hex(7)NeuAc(3)	13820.87995	36.0%	5.7%	38.9%	17.2%
C ₅₇₅ H ₈₉₇ N ₁₃₁ O ₂₃₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(2)	13812.86363	18.0%	2.7%	21.2%	5.5%
C ₅₇₃ H ₈₉₄ N ₁₃₀ O ₂₃₅ S ₁₃	HexNAc(6)Hex(11)NeuAc(2)	13771.83708	42.9%	5.3%	42.8%	12.0%
C ₅₇₁ H ₈₉₀ N ₁₃₂ O ₂₃₀ S ₁₃	HexNAc(8)Hex(8)NeuAc(2)	13691.83736	17.4%	24.6%	22.8%	2.9%
C ₅₇₀ H ₈₈₇ N ₁₃₃ O ₂₂₈ S ₁₃	HexNAc(8)Hex(6)NeuAc(3)	13658.82712	60.0%	4.3%	62.8%	11.9%
C ₅₆₉ H ₈₈₇ N ₁₃₁ O ₂₃₀ S ₁₃	HexNAc(7)Hex(9)NeuAc(2)	13650.81081	27.4%	7.3%	27.7%	2.5%
C ₅₆₇ H ₈₈₄ N ₁₃₀ O ₂₃₀ S ₁₃	HexNAc(6)Hex(10)NeuAc(2)	13609.78426	95.7%	5.7%	97.5%	1.3%
C ₅₆₅ H ₈₈₀ N ₁₃₂ O ₂₂₅ S ₁₃	HexNAc(8)Hex(7)NeuAc(2)	13529.78453	17.5%	21.3%	18.7%	2.2%
C ₅₆₁ H ₈₇₄ N ₁₃₀ O ₂₂₅ S ₁₃	HexNAc(6)Hex(9)NeuAc(2)	13447.73143	63.6%	9.8%	65.1%	6.8%
C ₅₅₉ H ₈₇₀ N ₁₃₂ O ₂₂₀ S ₁₃	HexNAc(8)Hex(6)NeuAc(2)	13367.73171	15.7%	17.0%	18.7%	8.5%
C ₅₅₇ H ₈₆₇ N ₁₃₁ O ₂₂₀ S ₁₃	HexNAc(7)Hex(7)NeuAc(2)	13326.65234	60.3%	6.3%	67.5%	4.7%
C ₅₅₆ H ₈₆₇ N ₁₂₉ O ₂₂₂ S ₁₃	HexNAc(6)Hex(10)NeuAc(1)	13318.68884	19.4%	7.9%	27.1%	19.0%
C ₅₅₅ H ₈₆₄ N ₁₃₀ O ₂₂₀ S ₁₃	HexNAc(6)Hex(8)NeuAc(2)	13285.67861	60.5%	0.5%	58.5%	10.6%
C ₅₅₁ H ₈₅₇ N ₁₃₁ O ₂₁₅ S ₁₃	HexNAc(7)Hex(6)NeuAc(2)	13164.72160	39.6%	5.9%	41.8%	12.0%
C ₅₄₅ H ₈₄₇ N ₁₃₁ O ₂₁₀ S ₁₃	HexNAc(7)Hex(5)NeuAc(2)	13002.59951	36.5%	4.3%	41.2%	13.6%
C ₅₄₄ H ₈₄₆ N ₁₂₉ O ₂₁₂ S ₁₃	HexNAc(6)Hex(8)NeuAc(1)	12994.58319	8.3%	31.6%	10.5%	7.8%
C ₅₄₂ H ₈₄₄ N ₁₂₈ O ₂₁₂ S ₁₃	HexNAc(5)Hex(9)NeuAc(1)	12953.55664	23.1%	8.6%	26.4%	13.8%
C ₅₃₂ H ₈₂₇ N ₁₂₉ O ₂₀₂ S ₁₃	HexNAc(6)Hex(6)NeuAc(1)	12670.47755	13.6%	5.0%	16.8%	12.1%

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S9: Compilation of the detected hCG β glycoforms in the concentrated elution 1 fraction analysed by nanoLC-HRMS after Con A SPE following protocol 3 with 12.5 μ g of uhCG and their average extraction recovery (<extraction recovery>) with their corresponding RSD values on three different home-made cartridges (H4, H5, H6) or commercial cartridges (C1, C2, C3).

Elemental composition	Proposed glycan structure	MM (Da) theoretical	Home-made cartridges		Commercial cartridges	
			<extraction recovery>	RSD	<extraction recovery>	RSD
C ₉₈₉ H ₁₆₀₃ N ₂₁₇ O ₄₃₇ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)	23927.62523	66.2%	3.1%	55.9%	15.6%
C ₉₈₄ H ₁₅₉₆ N ₂₁₆ O ₄₃₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(2) Fuc(1)	23782.58772	58.0%	15.4%	54.4%	14.3%
C ₉₇₈ H ₁₅₈₆ N ₂₁₆ O ₄₂₉ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)	23636.52981	57.2%	12.6%	58.7%	10.1%
C ₉₇₇ H ₁₅₈₀ N ₂₂₀ O ₄₂₃ S ₁₃	HexNAc(19)Hex(17)NeuAc(5)	23578.52567	57.8%	12.6%	56.2%	20.0%
C ₉₇₀ H ₁₅₇₃ N ₂₁₅ O ₄₂₃ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)Fuc(1)	23417.45553	65.1%	21.6%	55.0%	9.1%
C ₉₆₇ H ₁₅₆₉ N ₂₁₅ O ₄₂₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(1)	23345.43440	59.6%	12.9%	56.8%	14.5%
C ₉₆₄ H ₁₅₆₃ N ₂₁₅ O ₄₁₉ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)	23271.39762	55.5%	18.8%	52.5%	10.9%
C ₉₅₉ H ₁₅₅₆ N ₂₁₄ O ₄₁₅ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)Fuc(1)	23126.36011	58.1%	17.0%	55.5%	7.4%
C ₉₅₆ H ₁₅₅₂ N ₂₁₄ O ₄₁₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(0)	23054.33898	53.8%	23.8%	52.6%	17.9%
C ₉₅₃ H ₁₅₄₆ N ₂₁₄ O ₄₁₁ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)	22980.30220	50.0%	18.7%	46.6%	24.3%
C ₉₄₁ H ₁₅₂₃ N ₂₁₁ O ₄₀₆ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(2)	22691.13843	57.7%	17.8%	53.4%	15.7%
C ₉₃₅ H ₁₅₁₃ N ₂₁₁ O ₄₀₂ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(1)	22545.08052	58.9%	14.4%	60.8%	3.7%

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S10: Compilation of the detected hCG β glycoforms in the concentrated elution 1 fraction analysed by nanoLC-HRMS after Con A SPE following protocol 3 with 12.5 ug of u-hCG and their average relative area (<relative area>) measured on the XIC with their corresponding RSD values on three different home-made cartridges (H4, H5, H6) or commercial cartridges (C1, C2, C3). Semi-quantification was performed by the normalization to the highest area value highlighted in grey.

Elemental composition	Proposed glycan structure	MM (Da) theoretical	Home-made cartridges		Commercial cartridges	
			<relative area>	RSD	<relative area>	RSD
C ₉₈₉ H ₁₆₀₃ N ₂₁₇ O ₄₃₇ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)	23927.62523	65.0%	12.8%	65.4%	9.7%
C ₉₈₄ H ₁₅₉₆ N ₂₁₆ O ₄₃₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(2) Fuc(1)	23782.58772	61.9%	28.9%	58.7%	2.3%
C ₉₇₈ H ₁₅₈₆ N ₂₁₆ O ₄₂₉ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)	23636.52981	100.0%	0.0%	100.0%	0.0%
C ₉₇₇ H ₁₅₈₀ N ₂₂₀ O ₄₂₃ S ₁₃	HexNAc(19)Hex(17)NeuAc(5)	23578.52567	80.7%	14.2%	62.1%	13.4%
C ₉₇₀ H ₁₅₇₃ N ₂₁₅ O ₄₂₃ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)Fuc(1)	23417.45553	70.6%	10.9%	90.7%	5.2%
C ₉₆₇ H ₁₅₆₉ N ₂₁₅ O ₄₂₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(1)	23345.43440	88.5%	8.4%	79.3%	8.1%
C ₉₆₄ H ₁₅₆₃ N ₂₁₅ O ₄₁₉ S ₁₃	HexNAc(17)Hex(23)NeuAc(2)	23271.39762	76.3%	9.3%	66.9%	11.6%
C ₉₅₉ H ₁₅₅₆ N ₂₁₄ O ₄₁₅ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)Fuc(1)	23126.36011	74.4%	5.5%	56.7%	4.2%
C ₉₅₆ H ₁₅₅₂ N ₂₁₄ O ₄₁₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(0)	23054.33898	61.0%	14.7%	83.4%	6.1%
C ₉₅₃ H ₁₅₄₆ N ₂₁₄ O ₄₁₁ S ₁₃	HexNAc(17)Hex(23)NeuAc(1)	22980.30220	90.3%	5.1%	76.0%	9.3%
C ₉₄₁ H ₁₅₂₃ N ₂₁₁ O ₄₀₆ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(2)	22691.13843	53.9%	27.3%	43.0%	13.0%
C ₉₃₅ H ₁₅₁₃ N ₂₁₁ O ₄₀₂ S ₁₃	HexNAc(10)Hex(21)NeuAc(5)Fuc(1)	22545.08052	36.1%	15.0%	67.1%	8.1%

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNac: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid

Table S11: Suggested overall composition of the glycans occupying the detected isoforms of hCG α present only in the washing fraction at the retention times between 21.9 and 22.1 min after Con A SPE (12.5 μ g of uhCG, protocol 3) with either a home-made (H4) or commercial cartridge (C1). Those forms correspond to two tetra-antennary glycans with various degrees of sialylation or phosphorylated structures. The values of the experimental m/z are retrieved from the experiment on the H4 cartridge.

Elemental composition	Proposed glycan structure	MM (Da) theoretical	m/z theo max iso (9 ⁺)	m/z exp max (9 ⁺)	error (ppm)
C ₆₈₄ H ₁₀₇₃ N ₁₃₉ O ₃₁₂ S ₁₃	HexNAc(12)Hex(14)NeuAc(5)Fuc(2)	16641.87386	1851.21816	1851.24781	16.0
C ₆₇₉ H ₁₀₆₆ N ₁₃₈ O ₃₀₉ S ₁₃	HexNAc(12)Hex(15)NeuAc(4)Fuc(2)	16512.83126	1836.88008	1836.79451	-46.6
C ₆₇₃ H ₁₀₅₆ N ₁₃₈ O ₃₀₄ S ₁₃	HexNAc(12)Hex(14)NeuAc(4)Fuc(2)	16350.77844	1818.87420	1818.90328	16.0
C ₆₆₆ H ₁₀₄₉ N ₁₃₆ O ₃₀₅ PS ₁₃	HexNAc(13)Hex(19)NeuAc(1)HPO3	16278.68619	1810.86395	1810.89972	19.7
C ₆₆₅ H ₁₀₄₃ N ₁₃₇ O ₂₉₉ S ₁₃	HexNAc(11)Hex(14)NeuAc(4)Fuc(2)	16147.69907	1796.30982	1796.33910	16.3
C ₆₆₂ H ₁₀₃₉ N ₁₃₇ O ₂₉₈ S ₁₃	HexNAc(12)Hex(14)NeuAc(3)Fuc(2) + 2*oxy	16091.67285	1790.08468	1790.11245	15.5
C ₆₆₂ H ₁₀₃₉ N ₁₃₇ O ₂₉₇ S ₁₃	HexNAc(12)Hex(14)NeuAc(3)Fuc(2) + oxy	16075.67794	1788.30746	1788.33335	14.5
C ₆₆₂ H ₁₀₃₉ N ₁₃₇ O ₂₉₆ S ₁₃	HexNAc(12)Hex(14)NeuAc(3)Fuc(2)	16059.68302	1786.53025	1786.56007	16.7
C ₆₅₅ H ₁₀₃₂ N ₁₃₅ O ₂₉₇ PS ₁₃	HexNAc(13)Hex(19)NeuAc(0)HPO3	15987.59078	1778.51999	1778.55355	18.8

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; NeuAc: sialic acid; oxy: oxidation

Table S12: Additional structures detected in the washing fraction after Con A SPE for the extraction of 12.5 µg of the uhCG on either the home-made (H6, H7, H8) or commercial cartridges (C1, C2, C3). Those structures are present in other parts of the TIC apart from the main elution areas of hCGα and hCGβ. Their identification by accurate mass measurement with the mass matching approach could not be performed. SPE conditions: see section 8.2.3. LC-MS parameters: see section 8.2.4.

Number of isoforms	M.W. range (Da)	Elution time range (min)	Fraction
32	15,300- 16,200	27 – 28	Washing
13	16,000 - 17,700	29 – 30.5	Washing
6	10,940 – 11,500	35 – 35.8	Washing

Table S13: Compilation of the detected hCGα glycoforms in the elution 1 fraction analysed by nanoLC-HRMS after the percolation of 5 ug of rhCG on home-made cartridge H5 and on commercial cartridge C1 Con A SPE cartridge following protocol 3.

Elemental composition	Proposed glycan structure	MM (Da) theoretical	Extraction recovery	
			Home-made cartridge	Commercial cartridge
C ₆₄₄ H ₁₀₀₇ N ₁₃₇ O ₂₈₄ S ₁₃	HexNAc(10)Hex(12)NeuAc(5)	15619.49365	N.D	N.D
C ₆₃₃ H ₉₉₀ N ₁₃₆ O ₂₇₆ S ₁₃	HexNAc(10)Hex(12)NeuAc(4)	15328.39823	N.D	33.1%
C ₆₃₀ H ₉₈₄ N ₁₃₆ O ₂₇₄ S ₁₃	HexNAc(9)Hex(11)NeuAc(5)	15254.36145	33.2%	34.2%
C ₆₂₂ H ₉₇₃ N ₁₃₅ O ₂₆₈ S ₁₃	HexNAc(10)Hex(12)NeuAc(3)	15037.30281	N.D	43.4%
C ₆₁₉ H ₉₆₇ N ₁₃₅ O ₂₆₆ S ₁₃	HexNAc(9)Hex(11)NeuAc(4)	14963.26603	34.6%	39.7%
C ₆₀₈ H ₉₅₀ N ₁₃₄ O ₂₅₈ S ₁₃	HexNAc(9)Hex(11)NeuAc(3)	14672.17062	37.1%	39.0%
C ₆₀₅ H ₉₄₄ N ₁₃₄ O ₂₅₆ S ₁₃	HexNAc(8)Hex(10)NeuAc(4)	14598.13384	39.1%	35.4%
C ₆₀₀ H ₉₃₇ N ₁₃₃ O ₂₅₅ S ₁₃	HexNAc(8)Hex(11)NeuAc(3)	14469.09124	49.4%	45.1%
C ₅₉₇ H ₉₃₃ N ₁₃₃ O ₂₅₀ S ₁₃	HexNAc(9)Hex(11)NeuAc(2)	14381.07520	41.7%	40.4%
C ₅₉₆ H ₉₃₀ N ₁₃₄ O ₂₄₈ S ₁₃	HexNAc(9)Hex(9)NeuAc(3)	14348.06497	50.5%	50.4%
C ₅₉₄ H ₉₂₇ N ₁₃₃ O ₂₄₈ S ₁₃	HexNAc(8)Hex(10)NeuAc(3)	14307.03842	40.3%	36.9%
C ₅₉₁ H ₉₂₃ N ₁₃₃ O ₂₄₅ S ₁₃	HexNAc(9)Hex(10)NeuAc(2)	14219.02238	45.9%	41.4%
C ₅₈₆ H ₉₁₄ N ₁₃₂ O ₂₄₃ S ₁₃	HexNAc(9)Hex(10)NeuAc(3)	14103.95905	44.9%	37.5%
C ₅₈₆ H ₉₁₆ N ₁₃₂ O ₂₄₃ S ₁₃	HexNAc(9)Hex(11)NeuAc(1)	14089.97978	37.0%	56.1%
C ₅₈₃ H ₉₁₀ N ₁₃₂ O ₂₄₀ S ₁₃	HexNAc(8)Hex(10)NeuAc(2)	14015.94300	40.7%	35.3%
C ₅₈₃ H ₉₁₀ N ₁₃₂ O ₂₃₉ S ₁₃	HexNAc(8)Hex(9)NeuAc(2)Fuc(1)	13999.94809	50.2%	39.9%
C ₅₈₁ H ₉₀₇ N ₁₃₁ O ₂₄₀ S ₁₃	HexNAc(7)Hex(11)NeuAc(2)	13974.91645	44.4%	35.3%
C ₅₈₀ H ₉₀₄ N ₁₃₂ O ₂₃₈ S ₁₃	HexNAc(7)Hex(9)NeuAc(3)	13941.90622	63.1%	62.0%
C ₅₇₇ H ₉₀₀ N ₁₃₂ O ₂₃₅ S ₁₃	HexNAc(8)Hex(9)NeuAc(2)	13853.89018	56.8%	42.1%
C ₅₇₄ H ₈₉₆ N ₁₃₂ O ₂₃₂ S ₁₃	HexNAc(9)Hex(9)NeuAc(1)	13765.87413	34.7%	46.1%
C ₅₇₃ H ₈₉₇ N ₁₃₁ O ₂₃₅ S ₁₃	HexNAc(7)Hex(10)NeuAc(2)	13812.86363	44.0%	50.6%
C ₅₇₂ H ₈₉₃ N ₁₃₁ O ₂₃₂ S ₁₃	HexNAc(8)Hex(10)NeuAc(1)	13724.84759	41.2%	37.7%
C ₅₆₉ H ₈₈₇ N ₁₃₁ O ₂₃₀ S ₁₃	HexNAc(7)Hex(9)NeuAc(2)	13650.81081	46.7%	50.6%
C ₅₆₉ H ₈₈₉ N ₁₃₁ O ₂₂₉ S ₁₃	HexNAc(9)Hex(10)NeuAc(0)	13636.83154	39.9%	42.5%
C ₅₆₇ H ₈₈₄ N ₁₃₀ O ₂₃₀ S ₁₃	HexNAc(6)Hex(10)NeuAc(2)	13609.78426	73.0%	50.3%
C ₅₆₆ H ₈₈₃ N ₁₃₁ O ₂₂₇ S ₁₃	HexNAc(8)Hex(9)NeuAc(1)	13562.79476	46.4%	28.4%
C ₅₆₄ H ₈₈₀ N ₁₃₀ O ₂₂₇ S ₁₃	HexNAc(7)Hex(10)NeuAc(1)	13521.76821	46.0%	30.6%
C ₅₆₁ H ₈₇₆ N ₁₃₀ O ₂₂₄ S ₁₃	HexNAc(8)Hex(10)NeuAc(0)	13433.75217	38.6%	28.4%
C ₅₅₈ H ₈₇₀ N ₁₃₀ O ₂₂₂ S ₁₃	HexNAc(7)Hex(9)NeuAc(1)	13359.71539	51.0%	40.3%

Notes: Fuc: Fucose; Hex: hexose (mannose or galactose); HexNAc: N-Acetyl hexosamine (N-Acetyl glucosamine or N-Acetyl galactosamine); MM: monoisotopic mass; N.D: not detected; NeuAc: sialic acid

Table S14: Compilation of the detected hCGβ glycoforms in the elution 1 fraction analysed by nanoLC-HRMS after the percolation of 5 ug of rhCG on home-made and on a commercial Con A SPE cartridge following protocol 3.

Elemental composition	Proposed glycan structure	MM (Da) theoretical	Extraction recovery	
			Home-made cartridge	Commercial cartridge
C ₉₉₅ H ₁₆₁₃ N ₂₁₇ O ₄₄₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)Fuc(1)	24073.68314	49.2%	39.6%
C ₉₈₉ H ₁₆₀₃ N ₂₁₇ O ₄₃₇ S ₁₃	HexNAc(18)Hex(24)NeuAc(3)	23927.62523	58.5%	39.2%
C ₉₈₄ H ₁₅₉₆ N ₂₁₆ O ₄₃₃ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)Fuc(1)	23782.58772	63.2%	43.7%
C ₉₇₈ H ₁₅₈₆ N ₂₁₆ O ₄₂₉ S ₁₃	HexNAc(18)Hex(24)NeuAc(2)	23636.52981	51.1%	43.0%
C ₉₇₃ H ₁₅₇₉ N ₂₁₅ O ₄₂₅ S ₁₃	HexNAc(18)Hex(24)NeuAc(1) Fuc(1)	23491.49231	46.2%	35.4%
C ₉₆₇ H ₁₅₆₉ N ₂₁₅ O ₄₂₁ S ₁₃	HexNAc(18)Hex(24)NeuAc(1)	23345.43440	55.0%	42.9%
C ₉₅₈ H ₁₅₅₀ N ₂₁₈ O ₄₀₈ S ₁₃	HexNAc(18)Hex(15)NeuAc(4)Fuc(2)	23052.36105	56.1%	44.1%
C ₉₄₇ H ₁₅₃₃ N ₂₁₇ O ₄₀₀ S ₁₃	HexNAc(18)Hex(15)NeuAc(3)Fuc(2)	22761.26564	59.6%	41.0%
C ₉₃₉ H ₁₅₂₃ N ₂₁₃ O ₄₀₁ S ₁₃	HexNAc(16)Hex(22)NeuAc(1)	22615.17000	45.7%	47.5%
C ₉₃₅ H ₁₅₁₆ N ₂₁₄ O ₃₉₆ S ₁₃	HexNAc(17)Hex(20)NeuAc(1)	22494.14373	48.6%	42.3%
C ₉₁₉ H ₁₄₉₀ N ₂₁₂ O ₃₈₆ S ₁₃	HexNAc(15)Hex(20)NeuAc(1)	22087.98498	50.6%	47.3%
C ₉₁₆ H ₁₄₈₄ N ₂₁₂ O ₃₈₄ S ₁₃	HexNAc(14)Hex(19)NeuAc(2)	22013.94820	45.2%	45.3%
C ₉₁₁ H ₁₄₇₇ N ₂₁₁ O ₃₈₁ S ₁₃	HexNAc(14)Hex(20)NeuAc(1)	21884.90561	43.7%	47.1%
C ₉₁₀ H ₁₄₇₆ N ₂₁₂ O ₃₇₇ S ₁₃	HexNAc(16)Hex(18)NeuAc(0)Fuc(1)	21821.92120	51.2%	41.2%
C ₉₀₅ H ₁₄₆₇ N ₂₁₁ O ₃₇₆ S ₁₃	HexNAc(14)Hex(19)NeuAc(1)	21722.85279	42.9%	37.7%
C ₉₀₀ H ₁₄₆₀ N ₂₁₀ O ₃₇₃ S ₁₃	HexNAc(14)Hex(20)NeuAc(0)	21593.81019	47.2%	40.9%
C ₈₉₇ H ₁₄₅₂ N ₂₁₀ O ₃₇₂ S ₁₃	HexNAc(11)Hex(18)NeuAc(3)	21533.75268	42.0%	52.1%
C ₈₉₆ H ₁₄₄₉ N ₂₁₁ O ₃₆₉ S ₁₃	HexNAc(11)Hex(14)NeuAc(4)Fuc(1)	21484.74753	46.2%	41.4%
C ₈₉₄ H ₁₄₅₀ N ₂₁₀ O ₃₆₈ S ₁₃	HexNAc(14)Hex(19)NeuAc(0)	21431.75737	47.3%	38.2%
C ₈₉₃ H ₁₄₄₄ N ₂₁₄ O ₃₆₁ S ₁₃	HexNAc(15)Hex(11)NeuAc(3)Fuc(1)	21357.75831	44.7%	53.6%
C ₈₉₁ H ₁₄₄₃ N ₂₁₃ O ₃₆₀ S ₁₃	HexNAc(16)Hex(13)NeuAc(1) Fuc(1)	21302.75250	52.4%	35.4%
C ₈₈₅ H ₁₄₃₃ N ₂₁₃ O ₃₅₅ S ₁₃	HexNAc(16)Hex(12)NeuAc(1) Fuc(1)	21140.69968	43.2%	33.0%