Cotton HILIC SPE microtips for micro-scale purification and enrichment of glycans and glycopeptides

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Abstract

Solid phase extraction microtips are important devices in modern bioanalytics, as they allow miniaturized sample preparation for mass spectrometric analysis. Here we introduce the use of cotton wool for the preparation of filter-free HILIC SPE microtips. To this end, pieces of cotton wool pads (approximately 500 μg) were packed into 10 μl pipette tips. The performance of the tips was evaluated for micro-scale purification of tryptic IgG Fc N-glycopeptides. Cotton wool HILIC SPE microtips allowed the removal of salts, most non-glycosylated peptides, and detergents such as SDS from glycoconjugate samples. MALDI-TOF-MS glycopeptide profiles were very repeatable with different pristine tips as well as reused tips, and very similar profiles were obtained with different brands of cotton wool pads. In addition, we used cotton HILIC microtips to purify N-glycans after PNGase-F treatment of IgG and transferrin followed by MALDI-TOF-MS detection. In conclusion, we establish cotton wool microtips for glycan and glycopeptide purification with subsequent mass spectrometric detection.

Introduction

Due to its speed, resolution and sensitivity, modern mass spectrometry provides great opportunities for detailed structural characterization of protein glycosylation including protein identification, determination of site-specific glycosylation profiles, and structural characterization of glycans at the level of glycopeptides and released glycans. For the efficient ionization and detection of glycopeptides and glycans, enrichment or purification steps are often required. Next to graphitized carbon solid phase extraction (SPE), hydrazine coupling, and lectin or antibody affinity chromatography, hydrophilic interaction liquid chromatography (HILIC) has been found to be very suitable for this purpose.[168-170] While the specificity of lectins and antibodies often only allows the isolation of a subset of glycans or glycopeptides from a pool, HILIC and graphitized carbon SPE can be employed for isolation of a broad range of glycoconjugates, making these adsorption chromatography methods applicable in a wide range of glycomics and glycoproteomics studies.[168-170]

HILIC SPE has been found to be particularly useful for the enrichment of tryptic N-glycopeptides. For this purpose, ZIC-HILIC stationary phases have been applied in ion-pairing mode using trifluoroacetic acid as a mobile phase additive.[138] Alternatively, carbohydrate-based stationary phases such as Sepharose and microcrystalline cellulose have been used for N-glycopeptide isolation.[102,148] An important feature of these carbohydrate-based stationary phases is that they are non-ionic, and HILIC adsorption is, therefore, dominated by hydrogen bonding of the glycan moieties with the stationary phase, while non-glycosylated peptides, lipids, salts and detergents tend to show low retention.[94,138,139] Retention of glycoconjugates is usually achieved with acetonitrile concentrations in the range of 80% while elution is performed with high water content.[139] The required elution conditions make HILIC well compatible with mass spectrometry, both in online and off-line mode.[92,138,139,171]

Immunoglobulin G (IgG) Fc N-glycosylation profiles can be analyzed in a reproducible and robust manner by MALDI-MS after HILIC SPE with Sepharose as well as microcrystalline cellulose performed in batch mode or at the 96-well plate format.[102,148] Here we introduce the use of cotton wool in microtips as versatile HILIC micro-SPE tool and demonstrate the reproducible application of these tips for isolation of tryptic IgG Fc N-glycopeptides from human plasma, followed by subclass-specific glycosylation profiling at the glycopeptide level. Moreover, the cotton wool microtips were successfully applied for the purification and enrichment of released N-glycans from detergent-containing glycoprotein samples treated with PNGase-F.

Experimental section

IgG purification. Polyclonal human IgGs were purified from plasma by affinity chromatography with immobilized protein A as described previously,[4] with minor

modifications. Briefly, rProtein A-Sepharose™ beads (GE Healthcare, Eindhoven, The Netherlands) were washed three times with PBS. To each well of a 96-well OF1100 filter plate (Orochem Technologies Inc., Lombard, IL) 50 µl PBS, 50 µl of slurry containing ca. 5 µl of beads and 2 µl of human plasma (containing ca. 20 µg of IgG) were applied. The plate was covered with a lid and incubated at room temperature with gentle agitation for 1 h. After incubation, beads were washed with 3x 200 µl PBS and 3x 200 µl of water on a vacuum manifold. Captured human polyclonal IgGs were released from protein A by 5 minutes incubation with 40 µl of 100 mM formic acid (Fluka, Steinheim, Germany), eluted into a polypropylene 96-well V-bottom plate (V96 microwell; NUNC, Roskilde, Denmark) by centrifugation (1 min at 18 g). After centrifugation, the eluates were neutralized (final pH > 7) with 20 µl of 300 mM ammonium bicarbonate. Alternatively, the eluates were dried by vacuum centrifugation for 2 hours.

Trypsin digestion. Tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (Sigma-Aldrich, Steinheim, Germany) was dissolved in ice-cold 20 mM acetic acid (Merck, Darmstadt, Germany) to a final concentration of 0.05 µg trypsin per µl and stored in aliquots at -80°C until use. To each of the neutralized IgG samples 8 µl of the trypsin stock (400 ng in total) and 12 µl of water were added. The samples were shaken for 3 min and incubated overnight at 37°C. Tryptic IgG digests were stored at -20°C until Sepharose or cotton HILIC desalting and purification. Bovine fetuin (1 mg; Sigma-Aldrich) was dissolved in 200 µl 50 mM ammonium bicarbonate containing 10 mM DTT and reduced at 60°C for 40 min. Cysteine alkylation was achieved by addition of 30 µl 100 mM iodoacetamide dissolved in 50 mM ammonium bicarbonate followed by 30 min incubation at room temperature in the dark. The alkylation reaction was stopped by putting the sample under a fluorescent lamp (gas discharge lamp) for 30 min. Trypsin digestion was achieved overnight at 37°C with 20 µg sequencing grade modified trypsin (Promega, Madison, WI).

N-glycan release. N-glycans from protein A purified IgGs were released as described previously.[94] Briefly, the dried IgG samples were denatured with 2 µl SDS (2%) at 60°C for 10 min. Subsequently, 2 µl of a release mixture containing 2% NP-40, 2.5× PBS and 0.5 mU PNGase-F (Roche, Mannheim, Germany) was applied. The samples were incubated overnight at 37°C for N-glycan release. Human apo-transferrin (0.1 mg; Sigma-Aldrich) was dissolved in 21 µl 50 mM ammonium bicarbonate containing 10 mM DTT and reduced at 60°C for 40 min. Cysteine alkylation was achieved by adding 4 µl 200 mM iodoacetamide containing 50 mM ammonium bicarbonate followed by 30 min incubation at room temperature in the dark. The alkylation was stopped by putting the sample under a fluorescent lamp (gas discharge lamp) for 30 min. To the sample 6 mU PNGase-F was applied and incubated overnight at 37 °C for N-glycan release.

Sepharose HILIC SPE in 96-well plate format. Sepharose (45-165 µm; GE Healthcare, Uppsala, Sweden) HILIC SPE was performed as described previously.[148]

Preparing cotton HILIC SPE microtips. Cotton wool pads of three different brands (Da, Dynaretail, Leusden, The Netherlands; Etos, Etos bv, Beverwijk, the Netherlands; Bella, Groupe Lemoine; Paris, France) were purchased in local stores and used for the preparation of HILIC SPE microtips. According to the manufacturers the cotton wool pads consist of 100% pure cotton. A small piece of cotton wool with a weight of approximately 500 µg was taken from a cotton wool pad and pushed into the end part of a 10 µl pipette tip (Rainin, Tiel, The Netherlands) using a blunt needle. Microtips were stored in a closed box until use.

Cotton HILIC micro-SPE of tryptic IgG glycopeptides and N-glycans. The cotton HILIC SPE microtip was washed with 5 times 10 µl of water and conditioned with 3 times 83% of acetonitrile (Biosolve BV, Valkenswaard, The Netherlands) by aspirating and dispensing the solution. For less then 10% of the prepared tips the flow upon solvent aspiration was found to be slow and insufficient, and such tips were therefore discarded. For sample application to the cotton HILIC SPE microtip, 39 µl of acetonitrile was added to 8 µl of a tryptic IgG digest (corresponding to ca. 2 µg of IgG), a fetuin digest or an N-glycan release sample, and the mixture was pipetted up and down 20 times to allow glycoconjugate adsorption. The adsorbed glycoconjugates were washed 3 times with 10 µl of 83% acetonitrile containing 0.1% TFA and eluted directly onto a MALDI plate with 2 µl water.

Capacity and recovery of cotton HILIC micro-SPE. To evaluate the capacity and recovery of cotton HILIC micro-SPE, maltoheptaose (Sigma-Aldrich) was labeled with 2-aminobenzoic acid (2-AA; Sigma-Aldrich) and analyzed by HILIC-HPLC with fluorescence detection (HPLC-Flu) as described previously.[94] The labeled maltoheptaose stock was used to prepare a standard dilution series ranging from 0.98-1000 µg/ml. From each standard dilution 10 µl was diluted with 90 µl acetonitrile and applied to the cotton HILIC SPE microtip. The adsorbed maltoheptaose was washed 3 times with 10 µl of 90% acetonitrile containing 0.1% TFA and eluted into a 96-well storage plate with 10 µl water. The standard dilutions and cotton micro-SPE preparations were diluted 10 times with water, brought to 75% acetonitrile, and analyzed by HPLC-Flu (injection of 20 µl) in the order of increasing concentrations.

MALDI-TOF-MS. For IgG Fc N-glycopeptide profiling by reflectron positive mode MALDI-TOF-MS 2 µl of the Sepharose purified glycopeptides or glycopeptides eluted directly from cotton HILIC SPE microtips with 2 µl of water were spotted onto a polished stainless steel MALDI plate (Bruker Daltonics, Bremen, Germany) and allowed to air dry. Samples were overlaid with 2 µl α-cyano-4-hydroxycinnamic acid (CHCA, 5 mg/ml in 50% acetonitrile; Bruker Daltonics) and allowed to air dry. For glycosylation profiling in the linear positive mode, IgG glycopeptides were directly eluted onto an AnchorChip 600/384 MALDI plate (Bruker Daltonics) and allowed to air dry. Samples were overlaid with 1 µl dihydroxybenzoic acid (DHB, 5 mg/ml

in 50% acetonitrile with 0.1% TFA; Bruker Daltonics). The AnchorChip plate was covered with a pierced cap containing 5 holes of ca. 5 mm diameter, allowing the DHB matrix to air dry at room temperature in a controlled manner. Samples were analyzed on an Ultraflex II MALDI-TOF/TOF-MS (Bruker Daltonics), and mass spectra were processed with flexAnalysis software (Bruker Daltonics) as described previously.[148] Similarly, N-glycans were eluted from cotton HILIC SPE microtips, spotted directly onto an AnchorChip MALDI plate, allowed to air dry, and were overlaid with 1 µl DHB.

nanoLC-ESI-IT-MS(/MS) of fetuin. Tryptic fetuin digests (250 nl) were analyzed as described previously, with minor modifications.[172] Briefly, separation was achieved on a Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) equipped with a reverse phase guard (C18 PepMap, 300 µm × 5 mm; Dionex) and analytical column (C18 PepMap, 3 µm; 75 µm × 100 mm; Dionex). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 300 nl/min. After injection of the samples, a gradient was applied to 25% eluent B (95% acetonitrile, 5% water) in 15 min and 70% eluent B at 25 min followed by an isocratic elution with 70% eluent B for 5 min. The separation was monitored by UV absorption at 215 nm. The LC system was interfaced to an Esquire HCTultra ESI-IT-MS (Bruker Daltonics, Bremen, Germany) by a metal nano-ESI sprayer (Proxeon Biosystems, Odense, Denmark). The MS was operated in the positive-ion mode at 1250 V. The solvent was evaporated at 170°C with a nitrogen stream of 6 l/min. Ions from m/z 600-1600 were registered. Automatic fragment ion analysis was enabled, resulting in MS/MS spectra of the most abundant peaks.

Results

IgG purification and tryptic cleavage. IgGs were affinity-captured from 2 µl of human plasma (containing ca. 20 µg IgG) in 96-well filter plates containing 5 µl of Protein A-Sepharose beads, followed by the elution of IgGs with 40 µl of 100 mM formic acid. While a previously described version of the protocol involves drying of the sample by vacuum centrifugation,[4,148] this procedure was replaced in the current protocol by a neutralization step with ammonium bicarbonate. Tryptic cleavage of IgGs was performed by an overnight incubation at 37°C either with 200 ng sequencing grade modified trypsin or 400 ng TPCK-treated trypsin. IgG glycopeptides were purified by Sepharose HILIC SPE in 96-well plate format[148] and analyzed by MALDI-TOF-MS in the reflectron positive mode. The IgG Fc N-glycopeptide profiles obtained for the neutralization procedure were very similar to those observed with the previously described vacuum centrifugation procedure[148] independent of the trypsin used (data not shown).

Glycopeptide purification using cotton HILIC microtips. Cotton was evaluated for its potential as a stationary phase in HILIC SPE of IgG glycopeptides. To this end, a small piece of a cotton wool pad (approximately 500 µg) was packed into the end of a pipette tip (Figure 1). MALDI-TOF-MS profiles obtained from blank elutions using cotton HILIC SPE microtips were evaluated and found to be virtually identical to matrix controls (only matrix spotted). Moreover, no cotton wool-related contaminant peaks were detected in the MALDI-TOF-MS profiles (data not shown).

Next, the obtained SPE microtips were tested for HILIC mode enrichment of IgG glycopeptides. Specifically, acetonitrile was added to an aliquot (8 µl) of a tryptic digest of human plasma IgG (corresponding to ca. 2 µg intact IgG), and glycopeptides were adsorbed to the HILIC micro-SPE stationary phase. After three washes with 10 µl of 83% acetonitrile containing 0.1% TFA, the retained glycopeptides were eluted directly onto a MALDI plate with 2 µl of water, followed by MALDI-TOF-MS profiling of IgG Fc N-glycopeptides. The obtained MALDI profiles represent the N-glycopeptide microheterogeneity of IgG1, 2 and 4 subclasses. Examples of the glycopeptide profiles registered by reflectron mode and linear mode MALDI-TOF-MS are shown in Figure 2. Structural assignment of the detected glycoforms was performed on the basis of

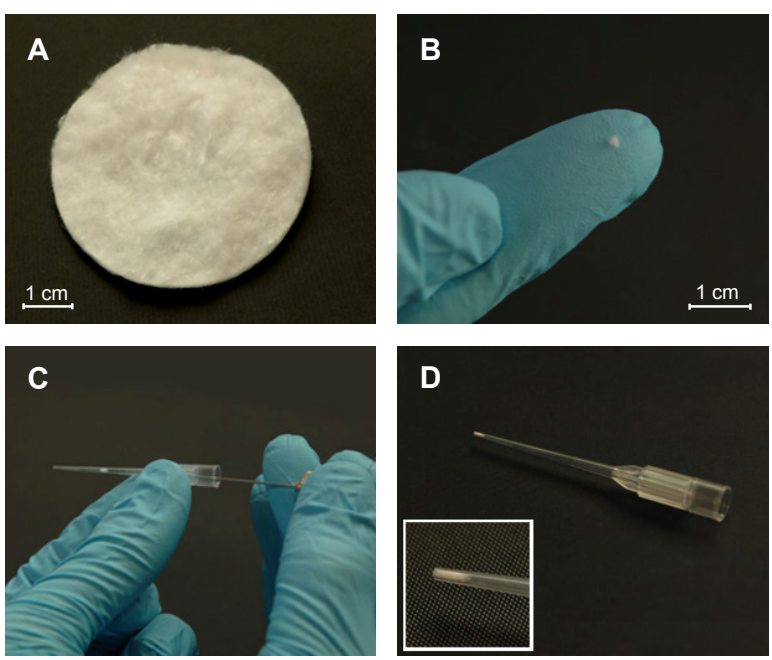


Figure 1. Preparation of a cotton HILIC SPE microtip. From a cotton wool pad (A), approximately 500 µg is taken (B) and pushed into a 10 µl pipette tip using a blunt metal needle (C). The cotton is pushed down into the end part of the tip (D).

literature knowledge of IgG N-glycosylation.[3,4,9,10,79,148] The most abundant signals were obtained for the glycopeptides of IgG2 followed by IgG1 and IgG4. While reflectron mode MALDI-TOF-MS analysis provided IgG glycopeptide spectra with isotopic resolution, only neutral glycoforms were observed. By contrast, linear mode MALDI-TOF-MS allowed low resolution detection of both sialylated glycopeptides and species with neutral glycoforms.

In addition to cotton we evaluated 1 and 3 mm Whatman cellulose chromatography paper for IgG glycopeptide enrichment. Glycopeptide profiles were obtained which were similar to those observed with cotton (data not shown). However, due to complications with inserting small pieces of paper into microtips without blocking the tip this approach was stopped.

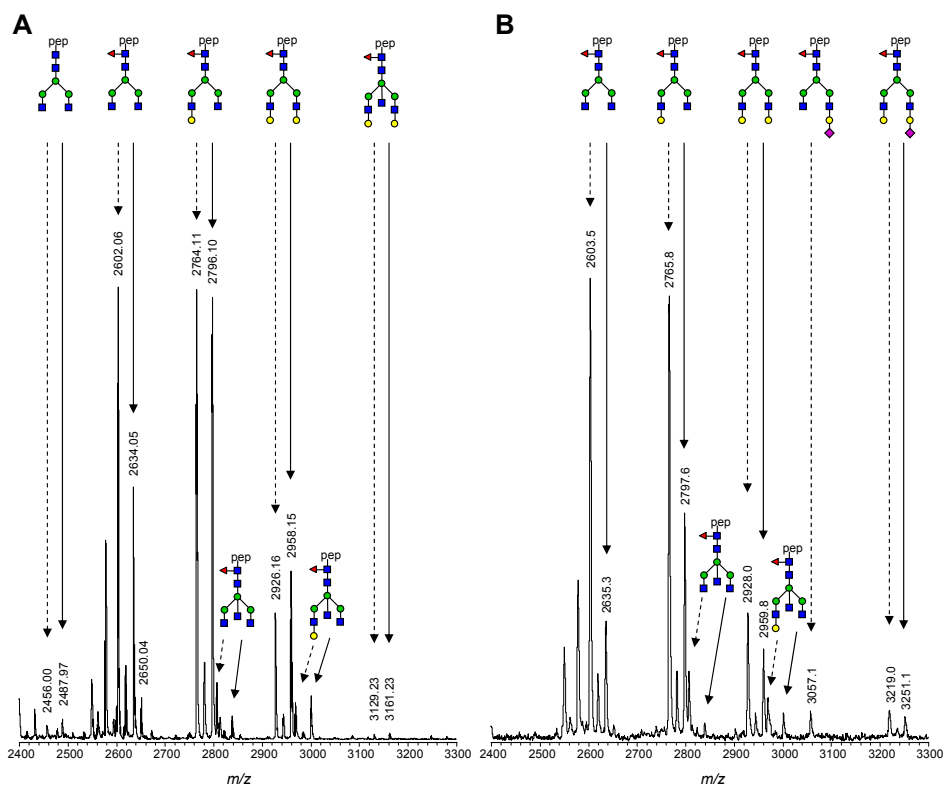


Figure 2. MALDI-TOF-mass spectrometric profiles of IgG glycopeptides prepared using cotton HILIC SPE microtips. Mass spectra were registered in reflectron positive mode using CHCA matrix (A) and in linear positive mode using DHB matrix (B). Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.

Validation of cotton HILIC micro-SPE. One cotton HILIC microtip was used 8 times for glycopeptide purification from a tryptic IgG digest pool followed by reflectron mode MALDI-TOF-MS of the eluted glycopeptides (Figure S-1 A and B). This experiment was repeated on four different days using each time a new cotton HILIC SPE tip. In another set of experiments, 8 different tips were used for IgG Fc N-glycosylation profiling from the above-mentioned tryptic IgG digest pool (Figure S-1 C and D). This experiment was also repeated on four different days using new tips for each experiment. Highly reproducible IgG Fc N-glycosylation profiles were obtained after cotton HILIC micro-SPE, independent of using the same tip repeatedly or using different tips for micro-scale purification.

Validation of the complete method. IgG Fc N-glycosylation profiling was performed in parallel on eight plasma aliquots of a control individual. This involved Protein A capturing, neutralization of the eluate, cleavage using TPCK-treated trypsin, cotton

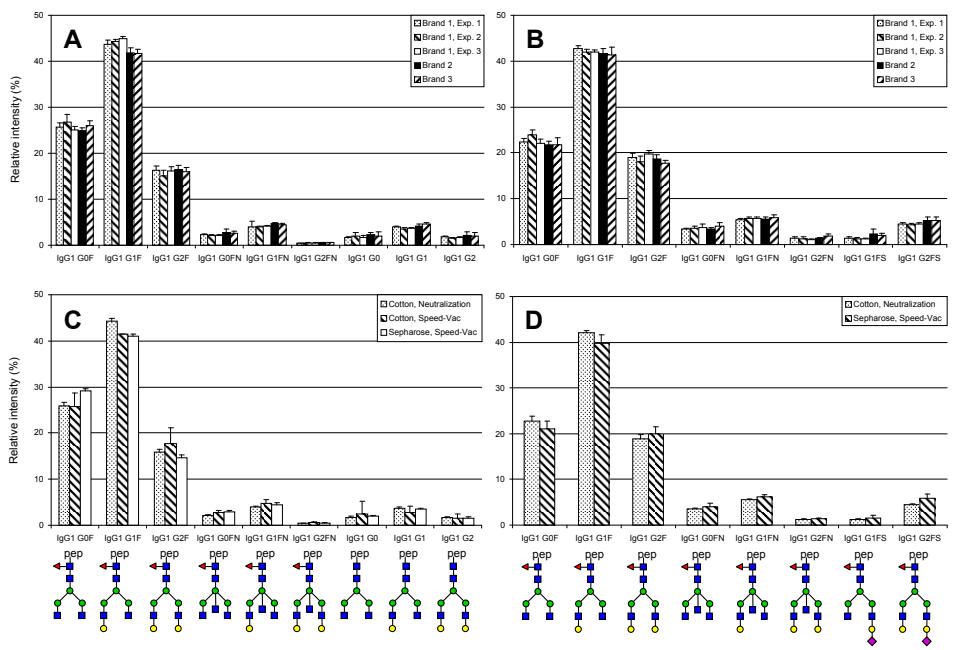
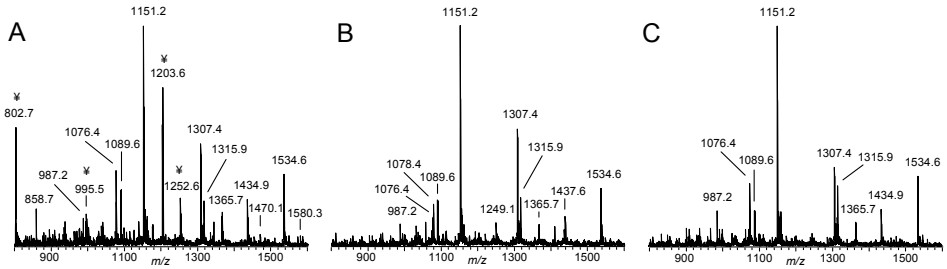


Figure 3. Repeatability of IgG1 glycopeptide profiling applying cotton HILIC SPE microtips. IgG1 glycopeptides were detected by MALDI-TOF-MS in reflectron positive mode using CHCA matrix (A, C) and in linear positive mode using DHB matrix (B, D). Samples were prepared after digestion using the neutralization method followed by desalting with cotton HILIC SPE microtips prepared from three different brands of cotton wool pads (A, B). The resulting profiles were compared with the pattern of glycoforms purified by Sepharose beads or cotton HILIC SPE microtips after drying by vacuum centrifugation, and digestion with sequencing grade trypsin (C, D). For each independent experiment, relative intensities and standard deviations (STD) were calculated from 8 replicates. Glycan code as in Figure 1 of Chapter 1.

HILIC micro-SPE, and MALDI-TOF-MS analysis. This procedure was repeated on three different days. Mass spectrometric analyses were performed in reflectron mode and linear mode, demonstrating that both IgG1 (Figure 3A and B) and IgG2 (Figure S-2A and B) Fc N-glycosylation profiles could be registered with low intraday and interday variability. Next to the hitherto used cotton material, two other brands of cotton wool pads were used for HILIC SPE microtip preparation and IgG Fc N-glycosylation profiling. All three brands of cotton wool pads provided very similar results for both IgG1 (Figure 3A and B) and IgG2 (Figure S-2A and B). Moreover, the method was compared to a previously described approach.[148] The major differences compared to the previous method are that (1) drying of Protein A eluates by vacuum centrifugation was replaced for a neutralization step, (2) sequencing grade trypsin was replaced by TPCK-treated trypsin, and (3) cotton HILIC micro-SPE was performed instead of 96-well plate HILIC SPE using Sepharose beads. A third approach combined the previously described sample preparation[148] (i.e. protein A capturing, drying by vacuum centrifugation, and cleavage of IgG with sequencing grade trypsin) with cotton HILIC micro-SPE purification and reflectron mode MALDI-TOF-MS analysis. With all different protocols, very similar IgG1 (Figure 3C and D) and IgG2 (Figure S-2C and D) Fc N-glycosylation profiles were obtained by reflectron mode (Figure 3A and C and Figure S-2A and C) and linear mode MALDI-TOF-MS (Figure 3B and D and Figure S-2B and D).

In order to evaluate the suitability of cotton microtips for the purification of glycoconjugates with a high level of sialylation, we evaluated the performance of cotton HILIC micro-SPE and 96-well plate Sepharose HILIC SPE for glycopeptide extraction from a tryptic digest of bovine fetuin (5 μg). Reverse phase nanoLC-MS analyses of the HILIC micro-SPE (Figure 4B) and Sepharose (Figure 4C) eluates were dominated by glycopeptide signals and showed a glycopeptide pattern very similar to the one observed for the total fetuin tryptic digest (Figure 4A). Glycopeptides were identified and assigned based on MS and MS/MS data (Figure 4D). Non-glycosylated fetuin peptides were efficiently removed by both HILIC procedures: for example, fetuin peptides E_{29}PACDDPDTEQAALAAVDYINK$_{50}$ (at *m/z* 802.7 and 1203.6 in triple and double charged form; Figure 4A) and Q_{121}DGQFSVLFTK$_{131}$ (native at *m/z* 1269.7 and with pyroglutamate formation at *m/z* 1252.6, $[M+H]^+$; Figure 4A) were absent from the HILIC eluates (Figure 4B and C). Together with the results obtained for IgG, these data show that the two HILIC SPE methods employing Sepharose and cotton microtips are equally suitable for enriching sialylated as well as non-sialylated glycopeptides with both small and large peptide moieties.

Glycan purification using cotton HILIC SPE microtips. As another field of application we tested the use of cotton HILIC SPE microtips for sample preparation of PNGase-F released glycans for MALDI-TOF-MS. N-glycans were enzymatically released from human IgG and human transferrin samples containing detergents (SDS, NP-40) and salts (PBS). Both neutral and acidic (sialylated) N-glycans were detected by MALDI-TOF-MS



D

Fetuin peptide (P12763)*	Mass (Da)	Observed N-glycopeptides with charge states			
					
R_{72}-R_{103}	3671.8	1249.1$^{(5+)}$, 1561.2$^{(4+)}$	1089.6$^{(6+)}$, 1307.4$^{(5+)}$	1138.1$^{(6+)}$, 1365.7$^{(5+)}$	
V_{160}-R_{187}	3016.6	1176.3$^{(5+)}$, 1470.1$^{(4+)}$			
L_{145}-R_{159}	1740.8	1078.4$^{(4+)}$, 1437.6$^{(3+)}$	1534.6$^{(3+)}$, 1151.2$^{(4+)}$		987.2$^{(4+)}$, 1315.9$^{(3+)}$
V_{91}-R_{103}**	1441.7		1076.4$^{(4+)}$, 1434.9$^{(3+)}$		

Figure 4. Reverse phase nanoLC-MS analysis of a tryptic fetuin digest without purification (A) and after additional HILIC purification by cotton micro-SPE (B) or Sepharose (C). Tryptic glycopeptides covering all three N-glycosylation sites of fetuin were observed (D).[173,174] *, Fetuin SwissProt entry number; ¥, signals related from peptides; **, cleavage between H and V[175]; glycan code as in Figure 1 of Chapter 1.

after cotton HILIC micro-SPE purification whilst direct MALDI-TOF-MS analysis of the glycan release samples without SPE purification did not allow registration of N-glycans but was dominated by detergent clusters (Figure S-3). Hence, these experiments demonstrated the successful clean-up of N-glycan release samples for MALDI-TOF-MS analysis by removing detergents and salts with cotton HILIC SPE microtips.

Cotton HILIC SPE microtip capacity and recovery. To evaluate the capacity and recovery of the developed cotton SPE microtips, a dilution series of 2-AA labeled maltoheptaose was prepared with and without cotton micro-SPE and analyzed by HPLC-Flu. Initially, the 2-AA labeled maltoheptaose dilution series was prepared with the original cotton micro-SPE conditions using 83% acetonitrile. While this revealed a linear relation over the entire dilution curve (data not shown) recoveries were low (average recovery ~ 40%). By increasing the acetonitrile concentration to 90% the 2-AA labeled maltoheptaose recoveries could substantially be increased (average recovery 82% when employing 2-AA labeled maltoheptaose amounts of up to 2.5 µg (Figure S-4). When 10 µg of 2-AA labeled maltoheptaose were used, a recovery of approximately 50% was observed, indicating that the capacity is at least 5 µg 2-AA labeled maltoheptaose per 500 µg cotton. In addition, graphitized carbon nanoLC-MS[176] was employed to analyze the cotton HILIC micro-SPE efficacy of native (unlabeled) maltoheptaose resulting in an average recovery of 94% when applying up to 2.5 µg oligosaccharide (data not shown).

Discussion

We here established cotton wool HILIC microtips for the purification of glycopeptides with various degrees of sialylation and peptide moieties of different sizes. In addition, cotton HILIC micro-SPE was applied to purify neutral and sialylated N-glycans released by PNGase-F followed by mass spectrometric detection. Similar to Poly HEA and carbohydrate HILIC stationary phases such as Sepharose and microcrystalline cellulose, cotton wool is a non-ionic, neutral stationary phase, and HILIC retention is expected to be caused solely by hydrogen bonding.[139] By contrast, ionic interactions may contribute to HILIC retention on ZIC-HILIC as well as amine-based stationary phases, which may be modulated by the addition of salt and/or ion-pairing reagents.[138,139]

Cotton wool microtips are cheap and can easily and quickly be prepared in every lab. Microtips have been introduced with various stationary phases,[177,178] including ZIC-HILIC microtips.[179] In contrast to some other microtips,[178,179] the cotton stationary phase stays in position, both with liquid aspiration and dispension. Moreover, the stationary phase appeared to be compatible with acidic and high acetonitrile conditions.

Raw cotton is mainly composed of cellulose (over 90%).[180] Cotton is used in a variety of commercial products such as clothing, cotton swabs (q-tips) and cotton wool pads. For the manufacturing of cotton wool for pads and q-tips, the raw cotton is subjected to extensive bleaching after which the fibers are carded, randomized and treated with water at high pressure to cross and tie the fibers. During the manufacturing process traces of wax and proteins are removed from cotton, and as a result cotton wool pads are composed of virtually pure cellulose.

The cotton wool HILIC micro-SPE procedure was used for the extraction of Fc N-glycopeptides from human tryptic plasma IgG digests followed by MALDI-TOF-MS analysis. The method showed good repeatability and did not appear to depend on a specific brand or batch of cotton wool pads. The results were very similar to those obtained previously after 96-well plate sample preparation of IgG glycopeptides using either reverse phase-SPE desalting, or HILIC SPE purification with Sepharose and microcrystalline cellulose.[148] When analyzed in reflectron mode MALDI-TOF-MS, no registration of sialylated glycopeptides was achieved due to loss of sialic acids by in-source and metastable decay. By contrast, in linear mode MALDI-TOF-MS the lack of an extraction delay strongly reduced in-source decay, and metastable decay products are not resolved from their precursor ions, allowing detection of sialylated glycopeptides.

Next to establishing the HILIC SPE microtips, we introduce two additional modifications to a previously described IgG Fc N-glycosylation profiling protocol.[148] First, tryptic cleavage was performed using TPCK-treated trypsin instead of the more expensive sequencing-grade trypsin. Second, the rather laborious vacuum centrifugation step, which was applied to remove formic acid from the Protein A eluates, has been substituted by a simple neutralization, making the protocol easier and more suitable for automation.

The here described cotton wool microtips are convenient devices for simple and fast sample preparation of tryptic IgG digests, and allow the determination of IgG Fc N-glycosylation features such as galactosylation, sialylation, fucosylation, and incidence of bisecting N-acetylglucosamine by MALDI-MS analysis. IgG Fc N-glycosylation has an important biological role in modulating both antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.[181] Hence, IgG Fc N-glycosylation of biotechnologically produced IgGs is being designed in order to maximize the efficacy in e.g. anti-cancer therapy (next generation therapeutic antibodies). Cotton wool microtips are particularly convenient tools for the preparation of low amounts of samples for mass spectrometric analysis and may be applied in (1) IgG Fc N-glycosylation profiling of clinical samples which often show disease-associated IgG glycosylation changes,[15,22,24,67,147] and (2) analysis of recombinantly expressed IgG.

Carbohydrate-based stationary HILIC phases have previously been used for total plasma N-glycome analysis by mass spectrometry.[85,94] 2-AA labeled N-glycans are purified from the labeling mix which contains excess label, reducing agent, various salts, plasma lipids, and large amounts of detergents together with denatured proteins, followed by glycan detection using MS.[85,94] Notably, cotton wool microtips are likewise suitable for removal of salts and detergents after enzymatic N-glycan release which, together with the high capacity and favorable SPE elution conditions, resulted in efficient mass spectrometric detection of N-glycans (Figure S-3).

Cotton wool microtips may serve as sample clean-up devices in glycomics and proteomics applications in which denaturants and surfactants are often added to improve protein solubility and proteolytic cleavage.[143,182-186] Moreover, we expect that the tips could provide a valuable tool for the enrichment of other hydrophilic molecules as well. Minor modifications of the here presented protocol may, however, be required. While cotton wool HILIC microtips appear to be a good choice for processing low numbers of samples, they may be applicable to larger numbers of samples by using multi-channel pipettes or by transferring the SPE method to a robotic platform.

Acknowledgements

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Supporting information

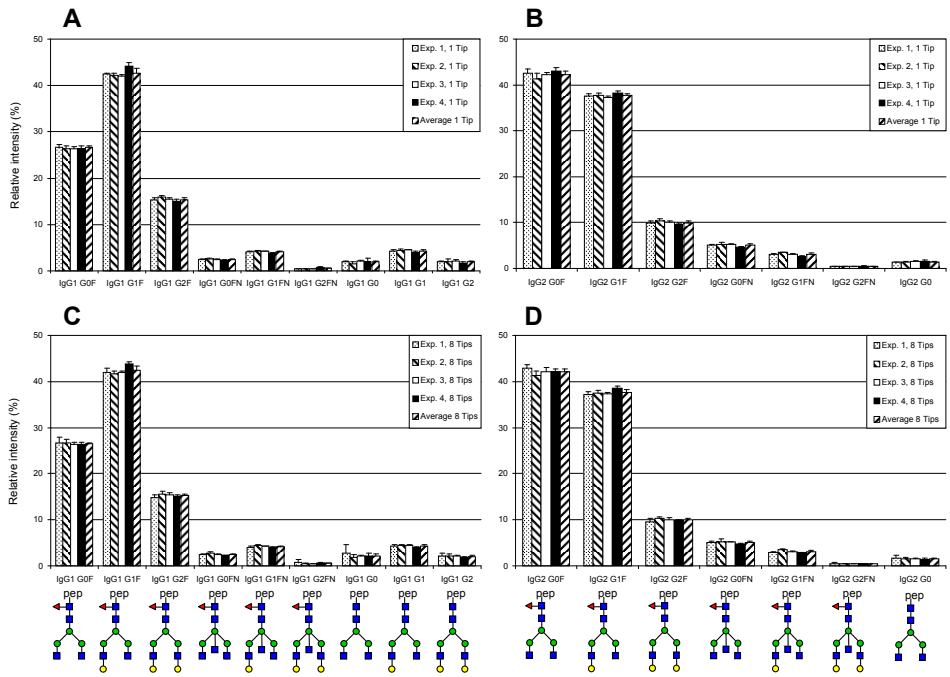

Figure S-1. Repeatability of cotton HILIC microtips for desalting and purification of IgG glycopeptides. Analysis was performed by reflectron mode MALDI-TOF-MS with CHCA matrix. A tryptic IgG digest pool was desalted 8 times either with one cotton HILIC microtip (A and B) or with eight different cotton HILIC microtips (C and D). The experiment was repeated on four different days. Glycan code as in Figure 1 of Chapter 1.

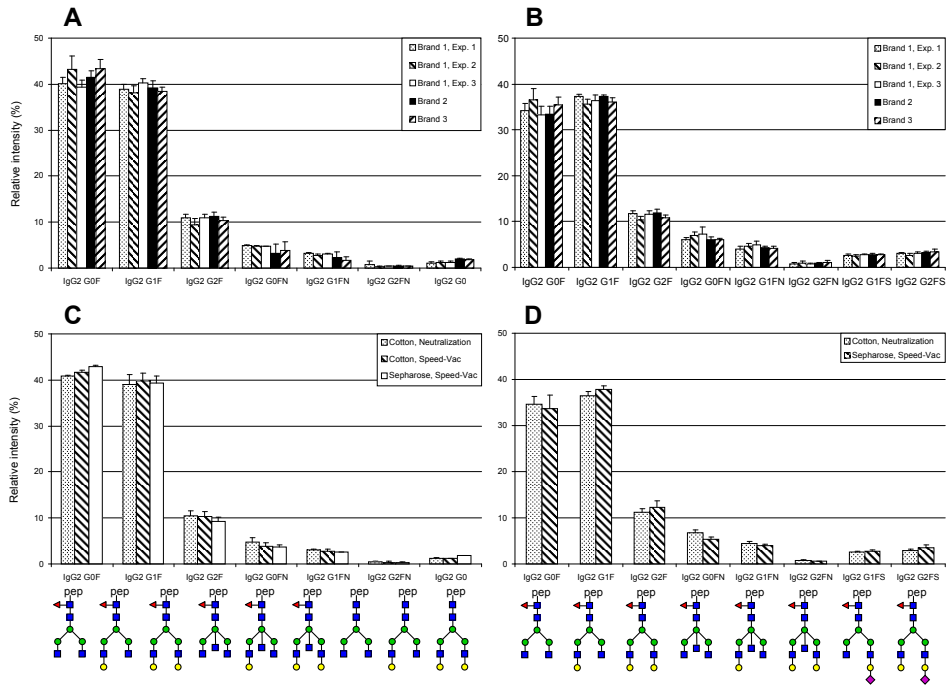

Figure S-2. Repeatability of IgG2 glycopeptide profiling applying cotton HILIC SPE microtips. IgG2 glycopeptides were detected by MALDI-TOF-MS in reflectron mode using CHCA matrix (A, C) and in linear mode using DHB matrix (B, D). Samples were prepared after digestion using the neutralization method followed by desalting with cotton HILIC SPE microtips prepared from three different brands of cotton wool pads (A, B). The resulting profiles were compared with the pattern of glycoforms purified by Sepharose beads or cotton HILIC SPE microtips after drying by vacuum centrifugation, and digestion with sequencing grade trypsin (C, D). For each independent experiment relative intensities and STDs were calculated from 8 replicates. Glycan code as in Figure 1 of Chapter 1.

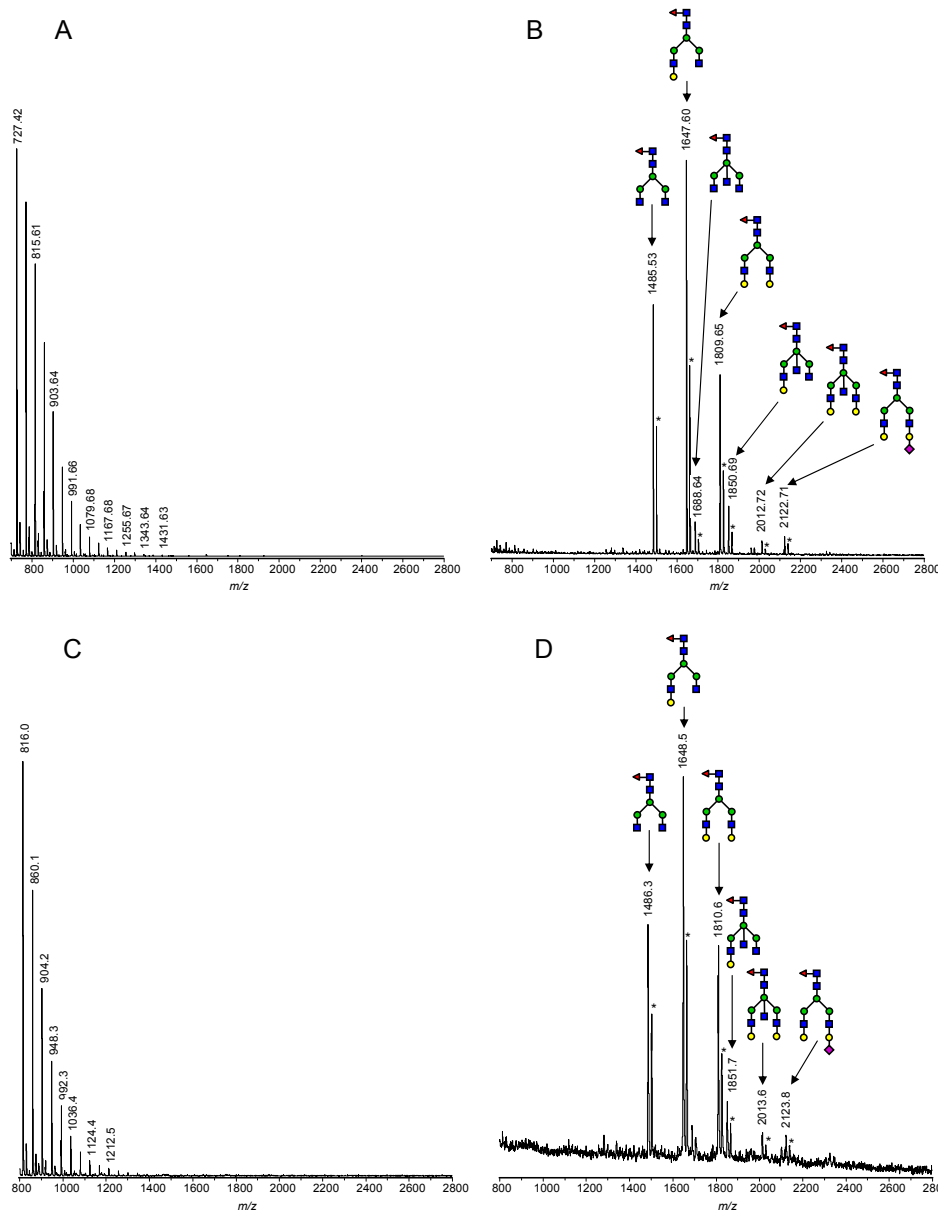

Figure S-3. MALDI-TOF-MS of N-glycans after cotton HILIC micro-SPE purification. N-glycans enzymatically released from IgG (A-F) and human transferrin (G, H) were analyzed by MALDI-TOF-MS in the reflectron positive mode (A, B), linear positive mode (C, D), and linear negative mode (E-H) without (left panels: A, C, E, G) and with (right panels: B, D, F, H) purification by cotton HILIC micro-SPE. Glycans were registered as sodium adducts in positive-ion mode and as deprotonated species in negative-ion mode. *, Potassium adducts; glycan code as in Figure 1 of Chapter 1.

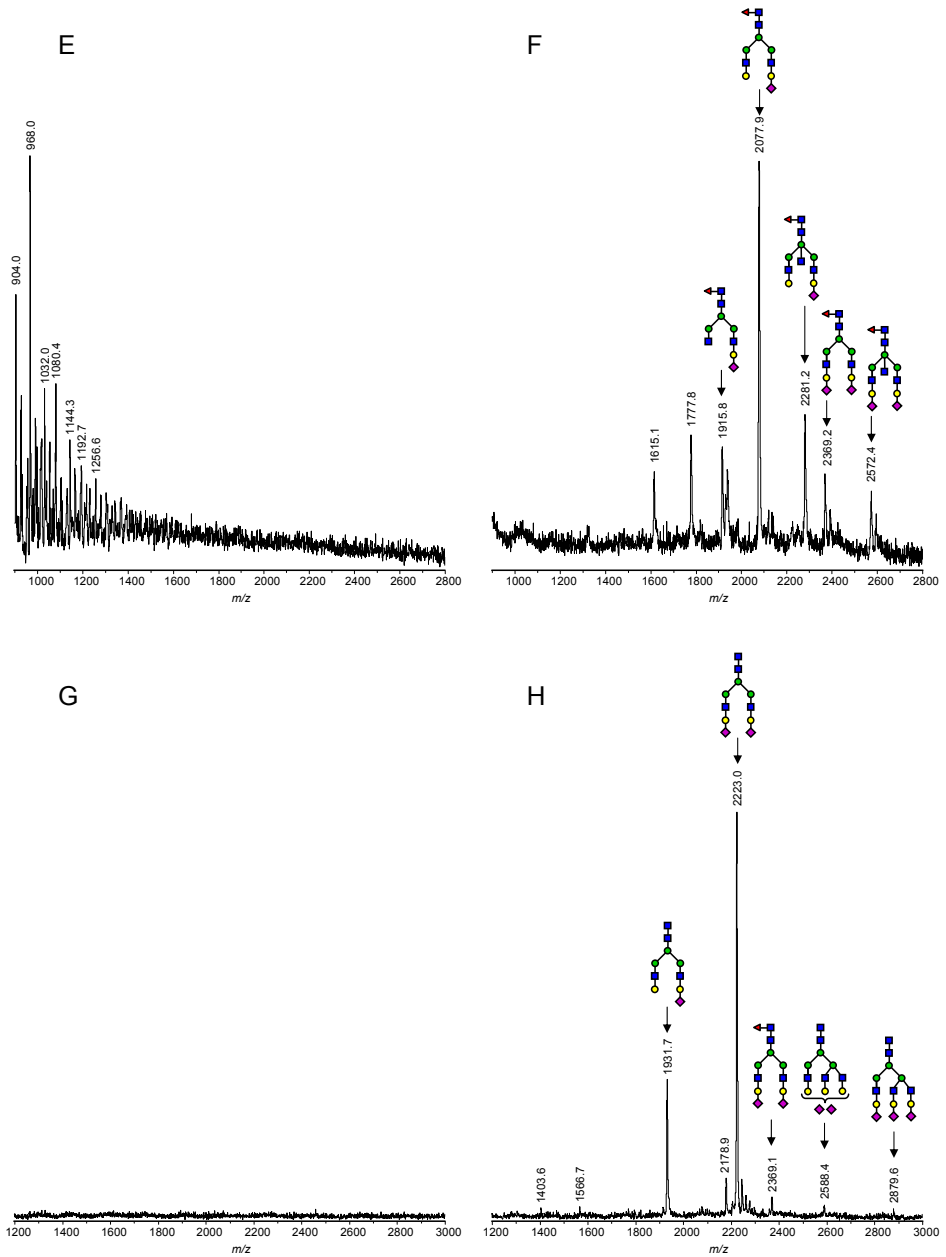

Figure S-3. Continued.

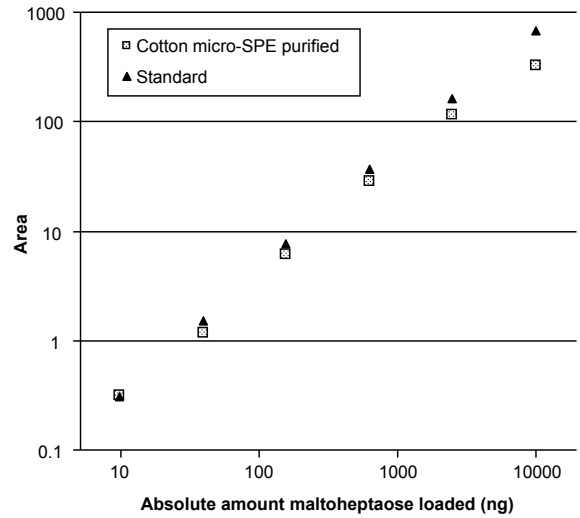

Figure S-4. Cotton HILIC microtip capacity and recovery. HILIC-Flu analysis of a 2-AA labeled maltoheptaose standard dilution series performed with (squares) and without (triangles) cotton SPE.