# Effective Enrichment of Glycopeptides Using iSPE®-HILIC HILIC Material

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Glycoproteomics aims at the concomitant identification of not only the glycans composition but also the sites of glycosylation and the identification of the protein attached with glycans. However, glycopeptides analysis is challenging as their microheterogeneity results in the reduced concentration of each individual glycopeptide molecule when compared to unmodified peptides even if obtained from the very same digest [1]. Also, glycopeptides exhibit poor ionisation efficiency compared to their non-glycosylated counterparts [2]. Therefore, selective and efficient glycopeptides enrichment by solid phase extraction (SPE) prior to mass spectrometry analysis is essential to allow their detection and identification [3-5]. Hydrophilic Interaction liquid chromatography (HILIC) SPE has extensively been applied due to its low bias towards different glycan types [6,7]. In contrast to the normal phase liquid chromatography, the HILIC retention mechanism is mainly a based on the hydrophilic partitioning of the analyte to the water enriched surface layer surrounding the polar stationary phase [8]. Ionic interaction and hydrogen bonding may also be involved in the separation depending on the sample and the character of stationary phase. Here, we demonstrated the glycopeptides enrichment efficiency of iSPE®-HILIC material for glycoproteomics application using a well characterised glycoprotein standard.

## Experimental

**Sample Preparation and HILIC SPE Enrichment:** Three microgram of standard glycoprotein (human IgG) was subjected to trypsin digestion, and glycopeptides were enriched using iSPE®-HILIC materials (50 µm, 60 Å, HILICON, Umeå, Sweden) by Drop HILIC approach as described earlier [6]. The dried samples containing the enriched glycopeptides was reconstituted in 100 µL of 0.1% TFA and an aliquot corresponding to 30 ng of the samples was used for the further MS analyses. Equal volumes of sample and matrix (20 mg/mL 2,5-dihydroxybenzoic acid, DHB, in 30% ACN/0.1% TFA)

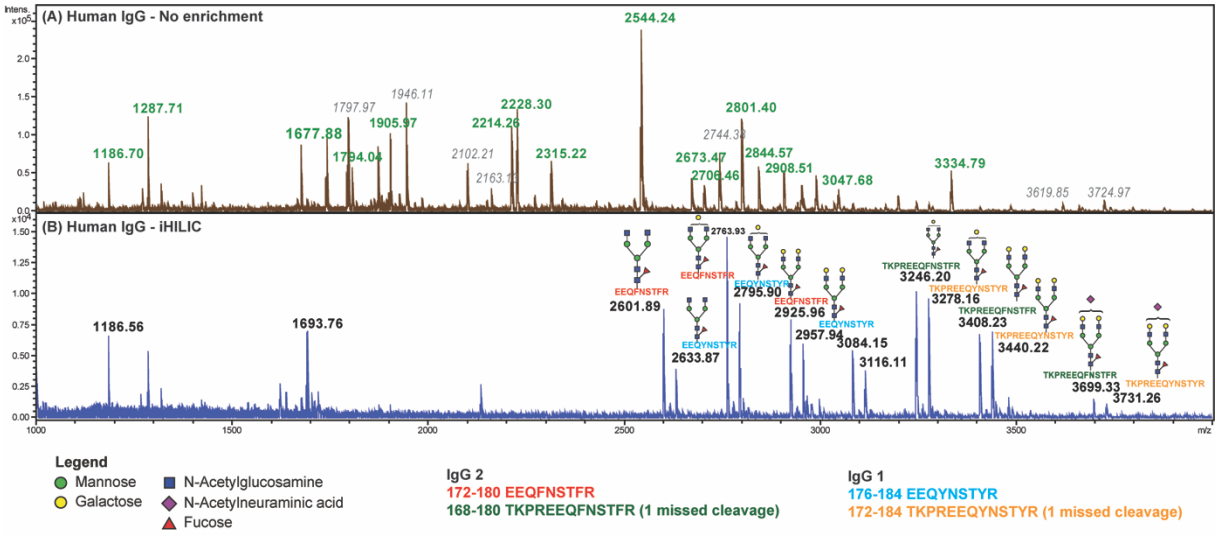


**Figure 1:** MALDI-TOF MS spectra of tryptic IgG (glyco)peptides co-crystallised with DHB and analysed in reflectron-positive ion mode. **(A)** No enrichment; *m/z* values highlighted in green correspond to the tryptic peptides derived from Human IgG 1-4 subclasses as listed in the Table 1. **(B)** The same sample analysed after enrichment using iSPE®-HILIC; Exemplary glycopeptides are annotated and the exhaustive list of glycopeptides with the most likely structures is shown in Table 2.

were spotted onto a MTP 384 target plate ground steel (Bruker Daltonics, Bremen, Germany).

**MS System:** MALDI-TOF-MS analysis was performed on a RapifleX™ MALDI Tissuetyper™ TOF-TOF mass spectrometer equipped with Smartbeam™ 3D laser optics running at 5000 Hz and controlled by FlexControl 4.0 software (Bruker Daltonics, Bremen, Germany). MS analysis were performed in reflector-positive mode, and the spectra were acquired within the mass range of *m/z* 1000 to 4000. In total 20,000 shots were accumulated per spot, baseline corrected and smoothed using Gauss algorithm with *m/z* 0.2 width and 1 cycle.

## Results and Conclusion

The efficiency and selectivity of the iSPE®-HILIC material for glycopeptide enrichment was evaluated using a human Immunoglobulin G (IgG) mix. The tryptic (glyco)peptides mixture was analysed using MALDI-TOF MS before and after iSPE®-HILIC enrichment. In the non-enriched samples, no signals corresponding to the IgG glycopeptides could be detected (Figure 1A; Table 1). However, after glycopeptide enrichment using iSPE®-HILIC material, a total of 14 individual glycopeptides were identified from 30 ng of glycoprotein by MALDI-TOF MS (Figure 1B; Table 2), which could not be detected in the non-enriched sample at all. Next to these glycopeptides, a few unmodified polar peptides (in the lower *m/z* region as shown in Figure 1B) were also co-enriched, but they did not interfere with glycopeptides detection. Considering the fact that HILIC enriches glycopeptides based on their hydrophilicity, it does not come as a surprise other hydrophilic compounds are co-enriched [7]. Such an unavoidable co-enrichment of smaller hydrophilic peptides usually does not interfer the ionisation and detection of the target glycopeptides in the higher *m/z* range.

In summary, iSPE®-HILIC developed by HILICON offers a robust, reliable, and easily implementable solution for effective glycopeptides enrichment in glycoproteomics. In combination with optimised sample digestion protocols (e.g. salt removal), iSPE®-HILIC based glycopeptides enrichment opens a wide range of opportunities for site-specific and in-depth glycoproteomics.

**Table 1: List of identified peptides corresponding to the different IgG subclass by MALDI-TOF MS analysis in the non-enriched sample**

| Measured m/z | Theoretical Mass | Δmass (Dalton) | Peptide | Position | Modification | Missed Cleavages | IgG Subclass |
|---|---|---|---|---|---|---|---|
| **1186.69** | 1186.65 | -0.046 | (K)/GPSVFPLAPSSK/(S) | 5-16 | | 0 | IgG2 |
| **1287.70** | 1287.65 | -0.048 | (K)/GPSVFPLAPCSR/(S) | 5-16 | CYS_CAM | 0 | IgG1 |
| **1321.73** | 1321.68 | -0.047 | (K)/STSGGTAALGCLVK/(D) | 17-30 | CYS_CAM | 0 | IgG2 |
| **1423.77** | 1423.71 | -0.063 | (R)/STSESTAALGCLVK/(D) | 17-30 | CYS_CAM | 0 | IgG1 |
| **1677.86** | 1677.80 | -0.062 | (K)/FNWYVDGVEVHNAK/(T) | 158-171 | | 0 | IgG2 |
| **1794.05** | 1793.99 | -0.057 | (R)/VVSVLTVVHQDWLNGK/(E) | 181-196 | | 0 | IgG1 |
| **1797.96** | 1797.77 | -0.185 | (R)/CPEPKSCDTPPPCPR/(C) | 114-128 | CYS_CAM CYS_CAM CYS_CAM | 1 | IgG3 |
| **1808.06** | 1808.01 | -0.055 | (R)/VVSVLTVLHQDWLNGK/(E) | 185-200 | | 0 | IgG2 |
| **1873.99** | 1873.92 | -0.07 | (K)/TTPPVLDSDGSFFLYSK/(L) | 276-292 | | 0 | IgG2 |
| **1876.99** | 1876.90 | -0.094 | (R)/EPQVYTLPPSQEEMTK/(N) | 225-240 | | 0 | IgG4 |
| **1905.98** | 1905.89 | -0.081 | (K)/TTPPMLDSDGSFFLYSK/(L) | 272-288 | | 0 | IgG1 |
| **2151.21** | 2151.17 | -0.039 | (R)/CPAPELLGGPSVFLFPPKPK/(D) | 159-178 | CYS_CAM | 0 | IgG3 |
| **2214.27** | 2214.19 | -0.076 | (R)/VVSVLTVVHQDWLNGKEYK/(C) | 181-199 | | 1 | IgG1 |
| **2228.29** | 2228.21 | -0.078 | (R)/VVSVLTVLHQDWLNGKEYK/(C) | 185-203 | | 1 | IgG2 |
| **2315.21** | 2315.13 | -0.077 | (K)/GQPREPQVYTLPPSQEEMTK/(N) | 221-240 | | 1 | IgG4 |
| **2343.25** | 2343.18 | -0.071 | (K)/GQPREPQVYTLPPSREEMTK/(N) | 271-290 | | 2 | IgG3 |
| **2430.31** | 2430.24 | -0.063 | (K)/TTPPVLDSDGSFFLYSKLTV DK/(S) | 276-297 | | 1 | IgG2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **2462.29** | 2462.22 | -0.074 | (K)/TTPPMLDSDGSFFLYSKLTV DK/(S) | 272-293 | | 1 | IgG1 |
| **2544.22** | 2544.13 | -0.084 | (K)/GFYPSDIAVEWESNGQPENN YK/(T) | 254-275 | | 0 | IgG2 |
| **2673.46** | 2673.38 | -0.085 | (K)/TTPPVLDSDGSFFLYSKLTV DKSR/(W) | 276-299 | | 2 | IgG2 |
| **2705.44** | 2705.35 | -0.09 | (K)/TTPPMLDSDGSFFLYSKLTV DKSR/(W) | 272-295 | | 2 | IgG1 |
| **2801.38** | 2801.27 | -0.116 | (R)/WQQGNVFSCSVMHEALHNHY TQK/(S) | 300-322 | CYS_CAM | 0 | IgG2 |
| **2844.57** | 2844.46 | -0.112 | (K)/THTCPPCPAPELLGGPSVFL FPPKPK/(D) | 106-131 | CYS_CAM<br>CYS_CAM | 0 | IgG2 |
| **2908.52** | 2908.40 | -0.114 | (K)/CCVECPPCPAPPVAGPSVFL FPPKPK/(D) | 102-127 | CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>CYS_CAM | 0 | IgG1 |
| **2943.56** | 2943.52 | -0.036 | (K)/TISKAKGQPREPQVYTLPPS QEEMTK/(N) | 215-240 | | 3 | IgG4 |
| **3036.62** | 3036.50 | -0.119 | (R)/KCCVECPPCPAPPVAGPSVF LFPPKPK/(D) | 101-127 | CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>CYS_CAM | 1 | IgG1 |
| **3047.67** | 3047.55 | -0.12 | (R)/EPQVYTLPPSREEMTKNQVS LTCLVK/(G) | 224-249 | CYS_CAM | 2 | IgG1 |
| **3246.54** | 3246.57 | 0.024 | (K)/DTLMISRTPEVTCVVVDVSH EDPEVQFK/(W) | 179-206 | CYS_CAM<br>MSO | 1 | IgG3 |
| **3334.78** | 3334.64 | -0.136 | (K)/SCDKTHTCPPCPAPELLGGP SVFLFPPKPK/(D) | 102-131 | CYS_CAM<br>CYS_CAM<br>CYS_CAM | 1 | IgG2 |
| **3660.98** | 3660.87 | -0.105 | (K)/THTCPPCPAPELLGGPSVFL FPPKPKDTLMISR/(T) | 106-138 | CYS_CAM<br>CYS_CAM | 1 | IgG2 |
| **3724.97** | 3724.82 | -0.147 | (K)/CCVECPPCPAPPVAGPSVFL FPPKPKDTLMISR/(T) | 102-134 | CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>CYS_CAM | 1 | IgG1 |
| **3740.88** | 3740.81 | -0.062 | (K)/CCVECPPCPAPPVAGPSVFL FPPKPKDTLMISR/(T) | 102-134 | CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>MSO | 1 | IgG1 |
| **4151.15** | 4151.06 | -0.094 | (K)/SCDKTHTCPPCPAPELLGGP SVFLFPPKPKDTLMISR/(T) | 102-138 | CYS_CAM<br>CYS_CAM<br>CYS_CAM | 2 | IgG2 |
| **4151.15** | 4151.06 | -0.094 | (K)/SCDTPPPCPRCPAPELLGGP SVFLFPPKPKDTLMISR/(T) | 149-185 | CYS_CAM<br>CYS_CAM<br>CYS_CAM<br>MSO | 2 | IgG3 |

**Table 2: List of tryptic IgG Fc *N*-glycopeptides detected in the samples after iSPE®-HILIC enrichment**

| S. No | Measured m/z | Theoretical glycopeptide mass | Glycoform Mass | Peptide Mass [M] | Δmass (Dalton) | Glycan Structure | Peptide Sequence |
|---|---|---|---|---|---|---|---|
| 1 | 2601.88 | 2602.06 | 1444.53 | 1156.52 | -0.17 | (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-180 EEQFNSTFR |
| 2 | 2763.92 | 2764.11 | 1606.59 | 1156.52 | -0.19 | (Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-180 EEQFNSTFR |
| 3 | 2925.95 | 2926.16 | 1768.64 | 1156.52 | -0.21 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-180 EEQFNSTFR |
| 4 | 3084.13 | 3084.35 | 1444.53 | 1638.81 | -0.22 | (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 168-180 TKPREEQFNSTFR (1 missed cleavage) |
| 5 | 3246.18 | 3246.41 | 1606.59 | 1638.81 | -0.22 | (Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 168-180 TKPREEQFNSTFR (1 missed cleavage) |
| 6 | 3408.23 | 3408.46 | 1768.64 | 1638.81 | -0.23 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 168-180 TKPREEQFNSTFR (1 missed cleavage) |
| 7 | 3699.33 | 3699.55 | 2059.74 | 1638.81 | -0.22 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3(GlcNAc)2 | 168-180 TKPREEQFNSTFR (1 missed cleavage) |
| 8 | 2633.87 | 2634.05 | 1444.53 | 1188.51 | -0.17 | (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 176-184 EEQYNSTYR |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9 | 2795.91 | 2796.10 | 1606.59 | 1188.51 | -0.19 | (Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 176-184 EEQYNSTYR |
| 10 | 2957.95 | 2958.15 | 1768.64 | 1188.51 | -0.20 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 176-184 EEQYNSTYR |
| 11 | 3116.11 | 3116.34 | 1444.53 | 1670.80 | -0.23 | (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-184 TKPREEQYNSTYR (1 missed cleavage) |
| 12 | 3278.17 | 3278.40 | 1606.59 | 1670.80 | -0.23 | (Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-184 TKPREEQYNSTYR (1 missed cleavage) |
| 13 | 3440.21 | 3440.45 | 1768.64 | 1670.80 | -0.24 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2 | 172-184 TKPREEQYNSTYR (1 missed cleavage) |
| 14 | 3731.26 | 3731.54 | 2059.74 | 1670.80 | -0.28 | (Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3(GlcNAc)2 | 172-184 TKPREEQYNSTYR (1 missed cleavage) |

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