Application Note # LCMS-70

Detailed Structural Characterization of IgG *N*-glycans Using Porous Graphitic Carbon LC-ESI Ion Trap MS/MS

Introduction

Because minimal structural modifications can significantly influence IgG function, detailed characterization of IgG *N*-glycosylation is a subject of great interest in pharmaceutical research and development. For such characterizations, highly sensitive, robust and selective techniques are required that enable identification and differentiation of glycan-specific structural properties.

Due to its unique separation capacity, porous graphitic carbon (PGC) has become the most widely used chromatographic medium for reproducible and robust separation of non-derivatized oligosaccharides and isobaric glycans [1,2,3,4]. Separation using PGC LC enables MS instruments to acquire separate and specific tandem MS spectra for isobaric glycans that would otherwise co-elute. The combination of PGC LC and LC-ESI ion trap MS/MS provides a powerful platform for detailed glycomic characterization of individual proteins and complex mixtures.

MOPC-21 – a mouse IgG1 antibody produced in mouse myeloma cells – was used to demonstrate the unique capabilities of this approach.

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Keywords	Instrumentation and Software
Glycosylation	amaZon speed ETD
Glycans	ProteinScape
Negative mode	Ion Trap

Experimental

N-glycan release and PGC LC-ESI MS/MS analysis were performed according to Jensen et al. [5].

Sample Preparation

In short, 5 µg of the monoclonal antibody MOPC-21 was separated using 1D SDS-PAGE and subsequently electro-blotted onto PVDF membranes. The blot was stained using Direct Blue-71, and membrane pieces containing the heavy chain were cut from the blot and transferred to 96-well microplates filled with 100 µL 1% (w/v) polyvinylpyrrolidone.

After washing the membrane with water, *N*-glycans were released enzymatically using PNGase F, isolated and reduced using sodium borohydride before samples were desalted by cation exchange chromatography. Borate was removed by repeated evaporation with 100 µL of methanol. Subsequently, glycans were resolubilized in 10 µL of 10 mM ammonium bicarbonate buffer.

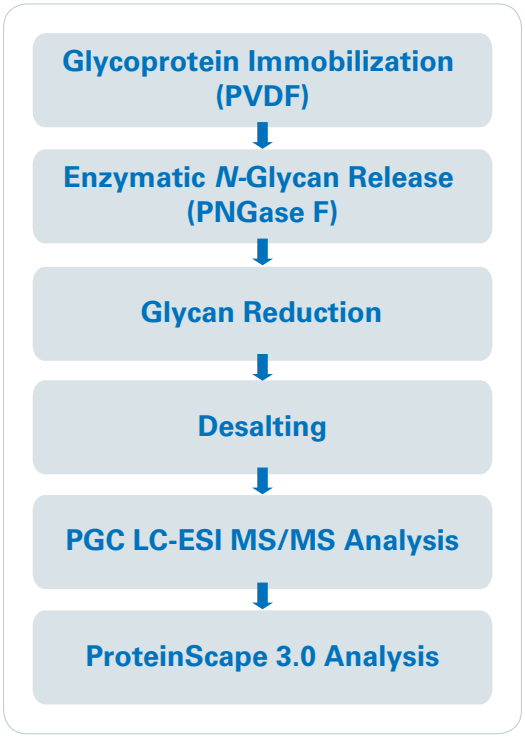

Figure 1: PGC LC-ESI MS/MS workflow for glycan analysis.

Table 1: Required materials for releasing *N*-glycans after immobilization on PVDF membranes.

Materials

Reagents/Equipment	Manufacturer	Ordering Number
Ethanol	Carl Roth GmbH	9065.3
Methanol	Fluka Analytical	34966 (1 L)
Glacial acetic acid	Sigma Aldrich	695084 (1 L)
Direct Blue 71	Aldrich Chemistry	212407 (50 g)
Polyvinylpyrrolidone (PVP40)	Sigma Aldrich	PVP40 (50 g)
PNGase F (*Flavobacterium meningosepticum* recomb. in *E.coli*)	Roche	11365177001
Ammonia solution (≥25% NH_3 in H_2O)	Sigma Aldrich	221228
$NaBH_4$	Aldrich Chemistry	71320
KOH	Sigma Aldrich	221473
HCl	Carl Roth GmbH	P074.3
ZipTip® C18 columns	Millipore Corporation	ZTC18S960
Cation exchange resign	Bio-Rad	142-1451
NH_4HCO_3	Carl Roth GmbH	T871.1
Acetonitrile	Fluka Analytical	34967 (2.5 L)
PVDF membrane	Millipore Corporation	IPVH20200
KIMTECH SCIENCE KIMWIPES® tissue	Kimberly Clark®Professional	05511

LC Separation

A Hypercarb™ column (see Table 2) was used to separate glycans.

MS Data Acquisition

MS experiments were carried out using an amaZon speed ETD ion trap system equipped with an ESI source. CID fragmentation experiments were performed in autoMS^n mode using the Enhanced resolution mode for MS and MS/MS acquisition.

Because tandem MS spectra of negatively ionized glycans are known to produce more specific cross-ring cleavage signals, data was recorded in negative ion mode. Experimental details are given in Table 3.

Table 2: Experimental LC conditions used for separation of released and reduced *N*-glycans.

LC settings	
System	Dionex RSLCnano
Analytical column	Hypercarb™ KAPPA Capillary Column 180 µm x 100 mm. 5 µm
Solvent A	10 mM ammonium bicarbonate
Solvent B	90% acetonitrile in 10 mM ammonium bicarbonate
Flow rate	3 µL/min
Gradient:	
0-16.9 min	1% B
17 min	8% B
19 min	10% B
65 min	25% B
70 min	98% B
75 min	98% B
77 min	1% B
85 min	1% B
Loading buffer	1% B

Table 3: Acquisition parameters used for the amaZon speed ETD ion trap in negative ion mode.

MS settings	
Source	ESI source
MS conditions	Enhanced resolution mode (8100 m/z s^{-1})
	350–1800 m/z scan range
	80000 ICC target
	Negative ion mode
	5 Spectra averages (2 Rolling Averaging)
	Target Mass 900 m/z (CID)
MS/MS conditions	Enhanced resolution mode (8100 m/z s^{-1})
	100–2500 m/z scan range
	3 precursors
	Fragmentation amplitude: 70% (SmartFrag active)

Data Processing and Glycan Identification

MS data were processed using DataAnalysis 4.1. Mass annotation settings were adapted to the intensities of the MS/MS signals. Deconvoluted MS/MS signals were exported as singly charged masses.

Glycan spectra were imported into ProteinScape 3.0, where the negative charge of the signals was detected automatically. Spectra were searched against the Glycome DB database (www.glycome-db.org) using the search engine GlycoQuest, which is integrated in ProteinScape. The search parameters used are listed in Table 4.

Results

PGC chromatography is able to separate isobaric glycans that differ in structure but have the same mass. This means that even glycans with subtle differences in linkage – for example, neuraminic acid [α 2,3 or α 2,6], galactose [α 1,4 or α 1,3] and the two G1 isomers that are frequently found on IgG *N*-glycans – can be separated ([2,3] and Figure 2).

Table 4: Description of the GlycoQuest search parameters for glycans acquired in negative ion mode.

Search parameters

Parameter	Value
Submitted to search	All spectra
Glycan type	*N*-glycan
Taxonomy	No restriction
Database	GlycomeDB
Composition restriction	Hex < 7; HexNAc < 5; NeuAc < 3; Fuc < 1; NeuGc < 3
Derivatization	Underivatized
Reducing End	Reduced end
Ions	H^+ up to 2; charge permutation for negative mode 1 to 2
MS tolerance	0.3 Da
MS/MS tolerance	0.35 Da
#^{13}C	1
Fragmentation	A, B, C, X, Y, Z, max. 3 deavages, max. 1 cross-ring cleavage

Isobaric structures

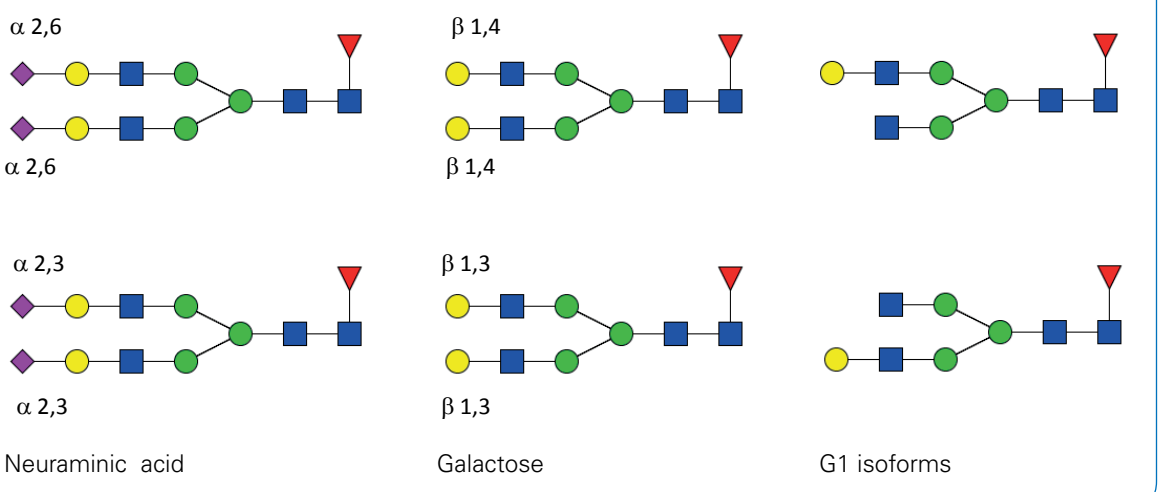

Figure 2: Examples of isobaric structures that can easily be separated and identified using PGC LC-ESI ion trap MS [2,3,5].

Online coupling of PGC chromatography to MS allows acquisition of MS/MS spectra of the separated isobaric compounds. Whereas in positive ion mode the vast majority of signals can be attributed to glycosidic cleavages, fragmentation of oligosaccharides in negative ion mode has been shown to result in a larger number of specific cross-ring cleavages [6]. In this way, particular glycan structural features can be deduced from a tandem MS spectrum that otherwise might not be obtained during a data-dependent LC-MS/MS analysis. Furthermore – due to their chemical nature – neutral and acidic oligosaccharides are comparatively easy to ionize in negative mode. Figure 3 shows an example of the MS and MS/MS characterization of two *N*-glycans that were separated by PGC chromatography.

_N_-glycan characterization after PGC separation

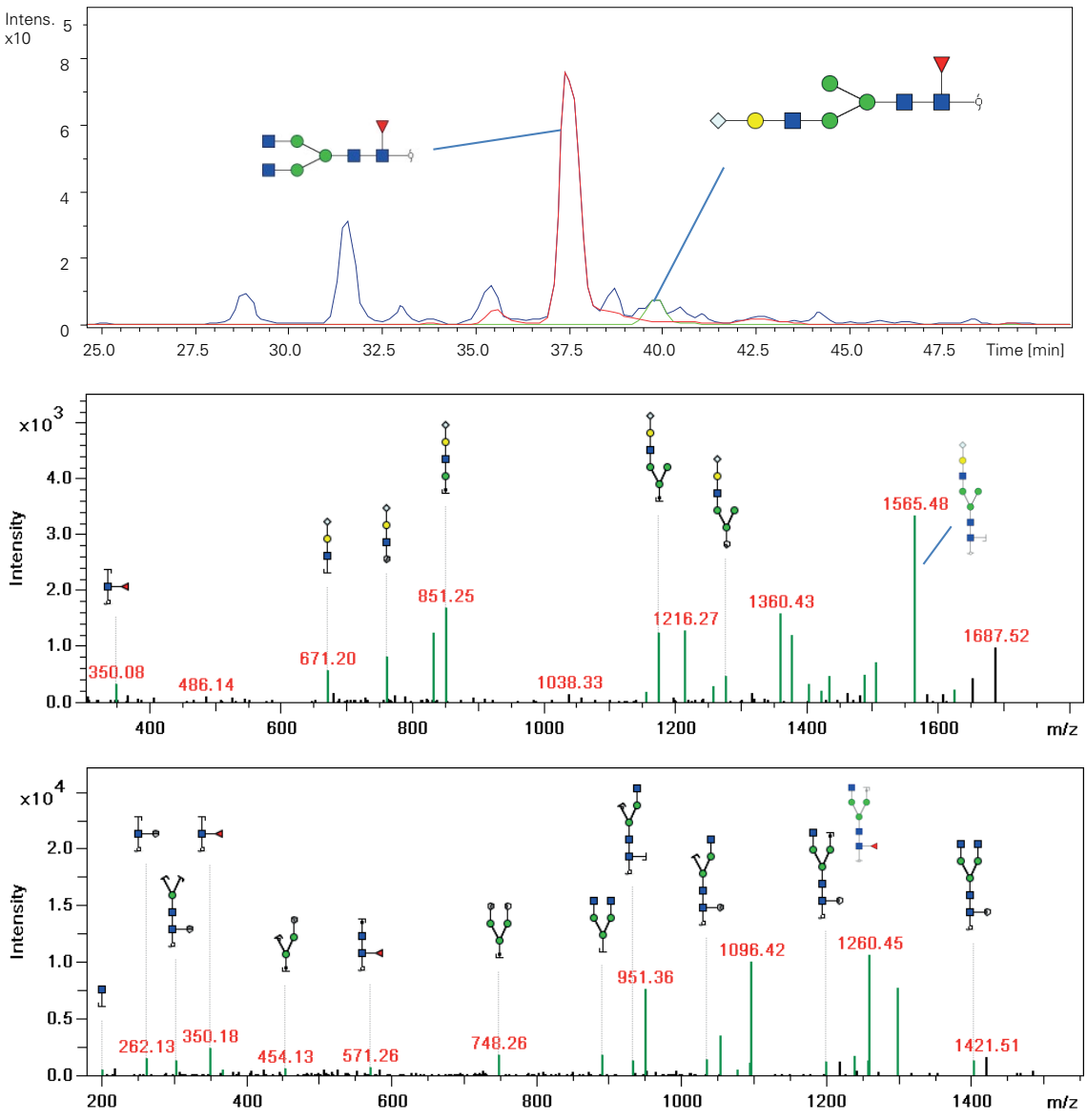

Figure 3: Examples for *N*-glycan characterization after PGC separation and MS detection. Top: Chromatogram view of Base Peak chromatogram (blue) overlaid with extracted ion chromatograms for m/z 864.3 (green) and 731.1 (red, see also Table 5). Middle & bottom: ProteinScape spectrum viewer with ion trap MS/MS spectra of 864.3 (2-) at 39.7 and of 731.3 (2-) at 37.8 minutes. Glycans and fragments are annotated in CFG-style [8].

For the mAB MOPC-21, ProteinScape was able to successfully identify 21 different *N*-glycan compositions from as little as 5 µg (33 pmol) of protein initially immobilized on the PVDF membrane (Table 5). A manual examination of the data performed in parallel resulted in identification of 22 compositions. It should however be noted that two of these structures (Hex5HexNAc3 and Hex3HexNAc2Fuc) were identified based on their mass alone, because their respective MS/MS spectra were insufficient for reliable interpretation. Interestingly, ProteinScape successfully picked up a structure overlooked during the manual examination (Hex7HexNAc4Fuc).

Of these 21 detected compositions, 18 were also successfully identified by separate glycopeptide analysis of the same sample [7]. To reduce the number of resulting glycans, it is essential to use available information (such as glycan type = *N*-glycan) for database searches. Nevertheless, due to known limitations in current databases entries, results must carefully reviewed and validated.

Table 5: Overview on N-glycans identified on MOPC-21.

***N*-glycans identifications**

Composition	Accession	m/z meas.	z	m/z calc.	Mr calc.	Δ MH^+ [Da]	Rt [min]	Score	rel. abund. [%]
Hex3HexNAc3	1014	1114.50	-1	1114.42	1115.42	0.08	28.9	32.6	4.8
	11498	1114.49			1115.42	0.07	36.0	4.2	
Hex5HexNAc2	2256	1235.53	-1	1235.44	1236.45	0.09	37.2	34.1	2.6
Hex3HexNAc3dHex1	1228	1260.53	-1	1260.47	1261.48	0.06	35.6	14.1	7.0
	1021	1260.52				0.04	44.4	14	
Hex4HexNAc3	1016	1276.52	-1	1276.47	1277.48	0.05	33.1	20	2.6
Hex3HexNAc4	1857	1317.71	-1	1317.49	1318.50	0.21	31.5	35.7	17.8
	1017	658.25	-2	658.24		0.02	32.0	30.9	
Hex4HexNAc3dHex1	36373	1422.58	-1	1422.53	1423.53	0.05	38.5	33.4	4.3
Hex3HexNAc4dHex1	888	1463.62	-1	1463.55	1464.56	0.07	37.6	27.9	46.2
	2620	1463.23				-0.32	42.5	12.7	
	888	731.29	-2	731.27	1464.56	0.04	37.8	45.5	
Hex4HexNAc4	1018	739.29	-2	739.27	1480.56	0.03	33.9	19.9	0.8
	1019	739.29				0.03	33.9	19.9	
Hex4HexNAc3NeuGc1	29035	791.34	-2	791.28	1584.57	0.13	34.7	20.1	0.4
Hex5HexNAc3dHex1	28682	791.81	-2	791.79	1585.59	0.05	43.3	26.3	0.5
Hex4HexNAc4dHex1	36233	812.31	-2	812.30	1626.61	0.02	40.0	43.3	3.6
	1050	812.36				0.12	39.2	35.1	
	1026	812.34				0.08	40.8	25.1	
Hex4HexNAc3NeuGc1dHex1	29031	864.34	-2	864.30	1730.62	0.07	39.7	27.6	3.5
Hex5HexNAc3NeuGc1	36336	872.36	-2	872.30	1746.62	0.11	33.7	20	0.4
Hex5HexNAc4dHex1	32883	893.38	-2	893.33	1788.67	0.11	45.3	11.1	0.2
Hex6HexNAc3NeuGc1	36318	953.39	-2	953.33	1908.67	0.12	36.5	22.4	0.8
Hex4HexNAc4NeuGc1dHex1	36234	965.87	-2	965.84	1933.70	0.06	41.0	23.4	1.6
Hex6HexNAc4dHex1	1446	974.39	-2	974.35	1950.72	0.07	45.8	8.4	0.2
Hex5HexNAc4NeuGc1dHex1	9279	1046.93	-2	1046.87	2095.76	0.11	42.4	18.1	1.1
Hex7HexNAc4dHex1	20023	1055.39	-2	1055.38	2112.77	0.02	49.4	11.5	0.2
Hex6HexNAc4NeuGc1dHex1	31817	1127.92	-2	1127.90	2257.81	0.04	45.5	19.7	0.6
Hex5HexNAc4NeuGc2dHex1	28851	1200.42	-2	1200.42	2402.85	0.01	42.6	7.8	0.7

Based on peak intensities of the detected signals, relative distribution information was obtained for the detected structures. Hex3HexNAc4Fuc (46%) and Hex3HexNAc4 (18%) were identified as the main *N*-glycan structures attached to the monoclonal antibody MOPC-21 (Table 5, Figure 4). The remaining compositions were found as minor constituents of the sample. NeuGc was the only sialic acid found and was attached to approximately 9% of the identified *N*-glycans.

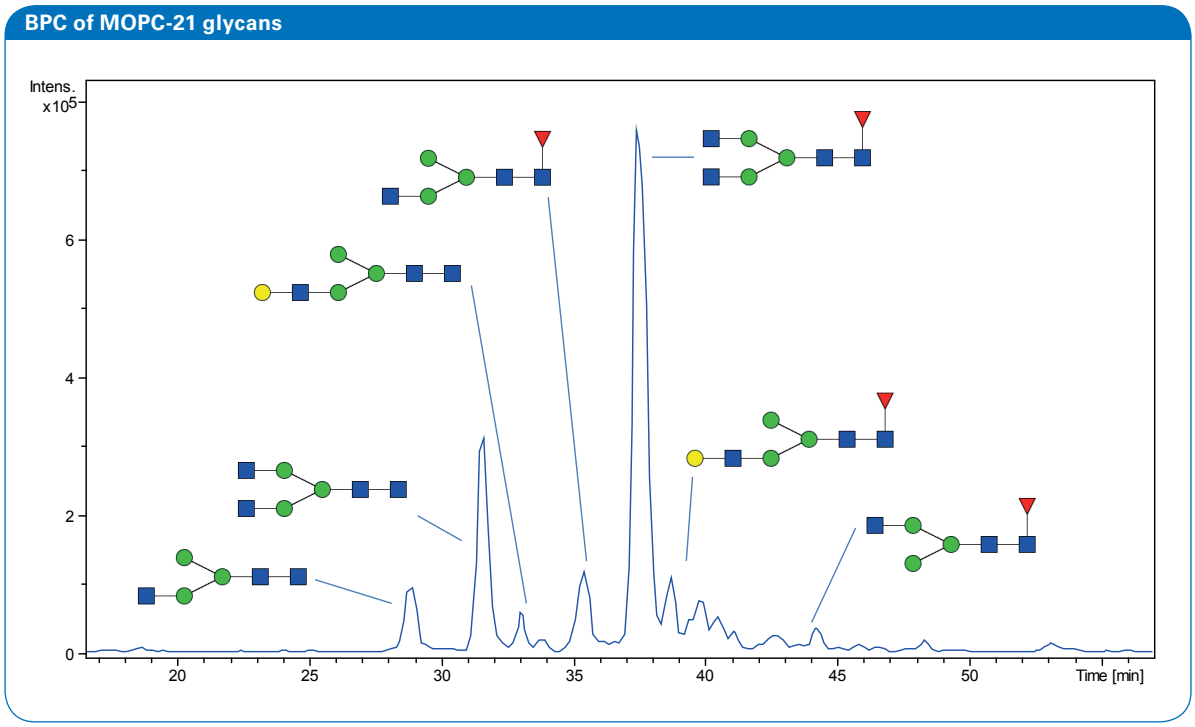

Figure 4: *N*-glycans separated and analyzed by PGC LC-ESI Ion Trap MS/MS. The major structures identified are indicated. The PGC separation behavior of these isobaric structures had been previously elucidated [2,5].

Conclusions

PGC LC-ESI ion trap MS in combination with ProteinScape-supported data evaluation is a powerful tool for LC-ESI based glycomics approaches. It enables sensitive and detailed structural characterization of *N*-glycans released from low-microgram amounts of proteins.

In combination with ion trap fragmentation, this approach provides a reliable and reproducible platform for negative ion mode analysis of released glycans. Isobaric glycan compounds that are otherwise hardly distinguishable by conventional tandem MS are separated by LC.

This means that separate signature tandem MS spectra of isobaric compounds can be obtained and used for improved structure differentiation.

References

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