Hydrophilic interaction liquid chromatography-mass spectrometry for the characterization of glycoproteins at the glycan, peptide, subunit, and intact level

Gargano, A.F.G.; Haselberg, R.; Somsen, G.W.

DOI
10.1016/B978-0-12-821447-3.00018-4

Publication date
2021

Document Version
Final published version

Published in
Carbohydrate Analysis by Modern Liquid Phase Separation Techniques

CHAPTER

5

Hydrophilic interaction liquid chromatography-mass spectrometry for the characterization of glycoproteins at the glycan, peptide, subunit, and intact level

Andrea F.G. Gargano[a,b,*], *Rob Haselberg*[a,c,*], *and Govert W. Somsen*[a,c]

[a]Centre for Analytical Sciences Amsterdam, Amsterdam, The Netherlands, [b]van't Hoff Institute for Molecular Science, University of Amsterdam, Amsterdam, The Netherlands, [c]Division of BioAnalytical Chemistry, Amsterdam Institute for Molecular and Life Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Abbreviations

2-AB	2-aminobenzamide
2D-LC	two-dimensional liquid chromatography
ACN	acetonitrile
ADC	antibody-drug conjugate
AGP	α-1-acid glycoprotein
Asn	asparagine
CID	collision-induced dissociation
DTT	dithiothreitol
ECD	electron capture dissociation
EndoH	endoglycosidase H

[*] Both authors contributed equally.

Carbohydrate Analysis by Modern Liquid Phase Separation Techniques
https://doi.org/10.1016/B978-0-12-821447-3.00018-4

EPO erythropoietin
ER endoplasmatic recticulum
ESI electrospray ionization
ETC electron transfer dissociation
F phenylalanine
FLD fluorescence detection
Fuc fucose
GalNAc *N*-acetylgalactosamine
GlcNAc *N*-acetylglucosamine
Hex hexose
HexNAc *N*-acetylhexosamine
HILIC hydrophilic interaction liquid chromatography
IdeS IgG-degrading enzyme of *Streptococcus pyogenes*
IgG immunoglobulin G
LC liquid chromatography
mAb monoclonal antibody
Man mannose
MS mass spectrometry
MS/MS tandem mass spectrometry
NPLC normal phase liquid chromatography
PNGaseF peptide *N*-glycosidase F
Pro proline
PTM posttranslational modification
RNase B ribonuclease B
RPLC reversed phase liquid chromatography
Ser serine
TFA trifluoroacetic acid
Thr threonine
Y tyrosine

1 Introduction

The liquid chromatographic (LC) separation of polar compounds on polar stationary phases using partly aqueous eluents was firstly published more than 45 years ago. Silica phases were used mostly for carbohydrate analysis. In the early 1990s, new stationary phases started to emerge, and the practice was coined hydrophilic interaction liquid chromatography (HILIC) by Alpert [1]. After the first exploring studies, the number of publications on HILIC increased substantially as of the early 2000s [2], promoted by the growing need to analyze (highly) polar compounds in very complex samples.

HILIC might be seen as a variant of normal phase LC (NPLC). Indeed, like NPLC it employs polar stationary phases such as traditional silica, and more recently, amino, amide, diol, and cyano bonded phases. However, in contrast to conventional NPLC, the mobile phase used in HILIC is partly aqueous and more similar to eluents employed in reversed-phase LC (RPLC). Consequently, the retention mechanism of HILIC can be quite versatile, involving various types of interactions, and many HILIC studies

indeed have shown multimodal separations. Analyte retention in HILIC may arise from (1) partitioning between the organic solvent-rich mobile phase and a water-enriched, polar layer on the stationary phase surface, (2) electrostatic (ionic) interactions (attractive or repulsive), (3) polar adsorptive interactions (hydrogen bonding, dipole-dipole interactions, and (4) (to some extent) hydrophobic interactions with the stationary phase [3–11]. These interactions may contribute concurrently to different extents and may be tuned during method development depending on the chemical nature of the compounds analyzed (e.g., pK_a, log P_{ow}) and the stationary and mobile phases applied.

Over time, HILIC has shown advantages over conventional RPLC and NPLC. For example, it offers good potential for the analysis of highly polar compounds, such as many metabolites that show no or very little retention in RPLC. Polar compounds often show poor solubility in the fully organic mobile phases used in NPLC, but mostly are readily soluble in aqueous HILIC eluents. Moreover, HILIC can be coupled to mass spectrometry (MS), especially when using electrospray ionization (ESI). The aqueous HILIC eluents that are typically rich in organic solvent (e.g., 90%–60% acetonitrile), are quite favorable to achieve highly efficient ESI of polar analytes. Over the years, HILIC has developed into the separation mode of choice for highly hydrophilic and amphiphilic compounds. These are normally too polar to be sufficiently retained in RPLC and lack the potential for ionic interactions needed for retention in ion-exchange chromatography.

Since its inception, HILIC has been successfully applied to the analysis of, for example, carbohydrates, metabolites, amino acids, peptides, and polar pharmaceuticals [2]. In the field of glycoproteins, HILIC has developed into an established technique for the analysis of released glycans. The specific polar and electrostatic interactions between glycan units (kind, number, and spatial arrangement) and the HILIC stationary phase has rendered HILIC as a high-resolution separation technique with unique glycan selectivities, making it highly suitable for the detailed study of protein glycosylation. Next to the separation of released glycans, HILIC has also shown particularly useful for resolving glycopeptides, subunits of glycoproteins, as well as intact glycoproteins based on their glycan content. With HILIC separation conditions being well compatible with ESI-MS, HILIC-MS has allowed the unambiguous assignment of glycosylated compounds and glycans [12], including resolution of highly similar or even isobaric species in some cases [13]. In order to delineate the present performance, potential and limitations of HILIC-MS in protein glycosylation analysis, this chapter will give a systematic literature overview outlining state-of-the-art and current practicalities in the field. The overview encompasses the use of HILIC-MS for the characterization of released glycans, glycopeptides, protein subunits, as well as intact

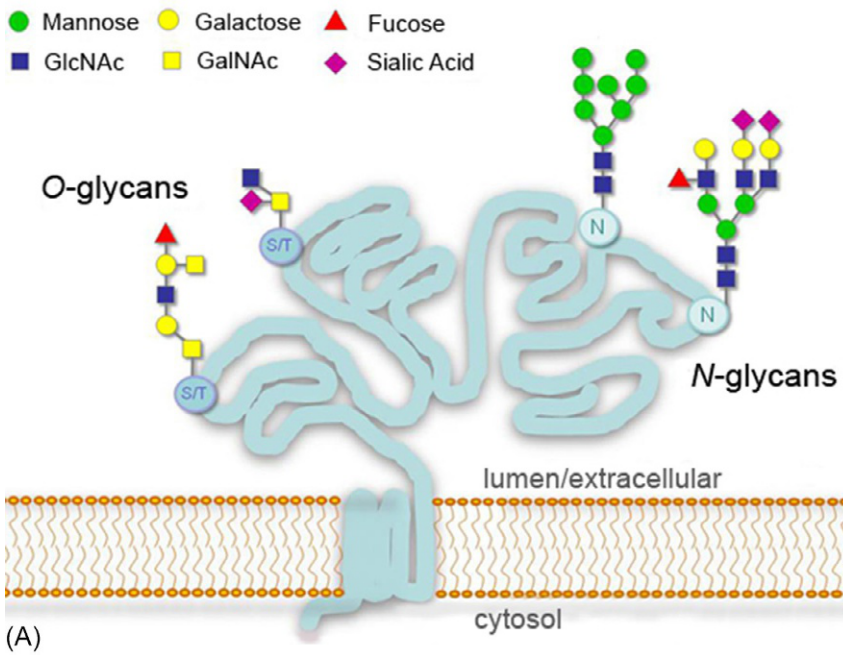
glycoproteins. HILIC conditions and practicalities regarding glycoprotein analysis are discussed. Before sketching these glycoprofiling approaches in more detail, the importance and nature of protein glycosylation will be briefly introduced.

2 Protein glycosylation

Glycosylation is an enzymatic modification that produces glycosidic linkages of saccharides to other saccharides, proteins, or lipids [14]. It is one of the most common posttranslational modifications (PTMs) of proteins, together with phosphorylation, sulfation, and acetylation and has shown to impact the biological function of proteins significantly [15–17]. For example, attachment of glycan moieties modifies the three-dimensional structure of proteins since they extend outward from their protein backbone. Glycosylation (and changes therein) can also significantly alter the overall charge and biological activity of a (glyco)protein [17]. Apart from structurally impacting proteins, glycans also fulfill protein-independent functions that mainly have to do with recognition by glycan binding proteins [16]. Glycans also play a role in a variety of biological processes, such as cell adhesion, cell proliferation, signal transduction, and immune response [16, 18–20]. They are sometimes contingent on the specific life cycle phase of the host, having different functions during different developmental stages [20]. In tumors, aberrant glycosylation of proteins can stimulate cancer development and progression, and metastasis [18, 21].

Two major types of glycans are *N*-glycans and *O*-glycans (Fig. 1a), which are linked to the amide nitrogen in asparagine (Asn) residues or the hydroxyl oxygen in threonine (Thr) or serine (Ser) residues, respectively. To be eligible for *N*-glycosylation, asparagine residues need to be localized in the specific amino acid sequence Asn-X-Ser/Thr, where X could be any amino acid, except proline (Pro) [14–17, 22–24]. The majority of proteins in human circulation are glycosylated [18, 25]. For example, the most common serum proteins immunoglobulin G (IgG) and α-1-acid glycoprotein (AGP) are *N*-glycosylated [24, 26].

All *N*-glycans have a common pentasaccharide core structure sugar sequence, which is a trimannosyl chitobiose: three mannose (Man) residues linked to a dimer of *N*-acetylglucosamine (GlcNAc) residues [16, 22, 23, 27]. In addition, the core structure is able to receive fucosylation as well as a bisecting GlcNAc residues [16]. *N*-Glycans can be divided into three main types: (1) high mannose or oligomannose, in which only Man residues are attached to the core; (2) complex, in which the core is extended by GlcNAc units (antennae) on both mannose arms; and (3) hybrid, in which only mannose residues are attached to one arm of the core, while the other arm contains one or two antennae, comparable to complex *N*-glycans (Fig. 1b). Antennae can be further extended by galactosylation,



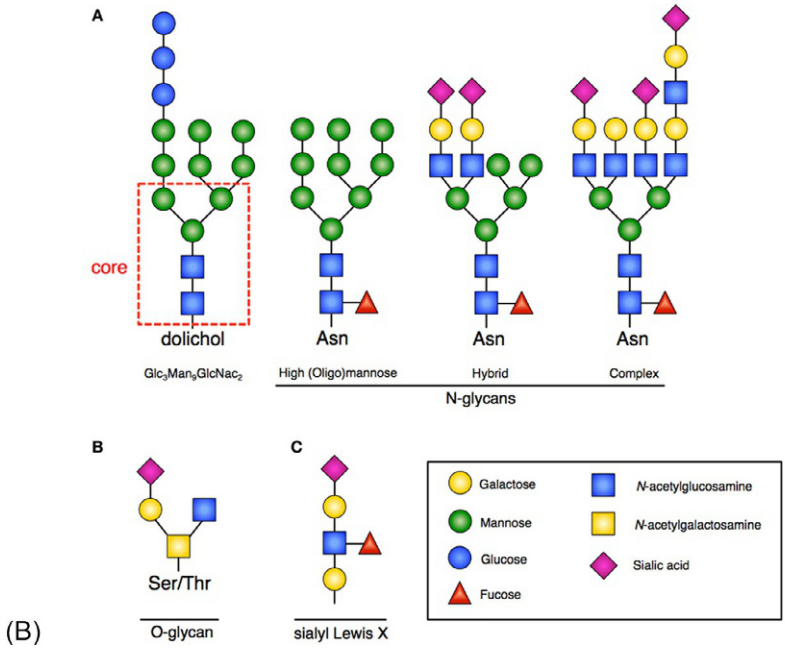


FIG. 1 (a) Schematic representation of typical *N*- and *O*-linked glycosylation of a protein backbone and (b) classes and nomenclature used to represent the different glycan structures. *(a) Reproduced with permission from P.E. Magnelli, A.M. Bielik, E.P. Guthrie, Identification and characterization of protein glycosylation using specific endo- and exoglycosidases, J. Vis. Exp. (2011) 8–12, doi: 10.3791/3749. (b) Modified from J.J. Lyons, J.D. Milner, S.D. Rosenzweig, Glycans instructing immunity: the emerging role of altered glycosylation in clinical immunology, Front. Pediatr. 3 (2015), doi:10.3389/fped.2015.00054.*

sialylation, and fucosylation [16, 22]. For *O*-glycan synthesis onto a protein, only *N*-acetylgalactosamine (GalNAc) is required to be present as a first monosaccharide. Many *O*-glycans are extended into long chains with variable termini that may be similar to the termini of *N*-glycans. However, *O*-glycans are generally less branched than most *N*-glycans and are commonly biantennary structures (Fig. 1b).

Glycosylation happens either post- or co-translationally and starts in the endoplasmatic reticulum (ER) lumen [17, 22]. This is a complex, highly efficient, and nontemplate process (in contrast to protein synthesis) [15, 17, 25]. *N*-Glycosylation is initiated with the synthesis of glycan precursors, before the other monosaccharides are added. Thereafter, the glycan is transferred to the eligible asparagine residue of a protein and further *N*-glycan remodeling into their mature structure happens in the ER and Golgi complex by a series of glycosyltransferases and glycosidases [22]. Overall, glycan structures are determined by the amino acid composition of their carrier protein, the availability of specific nucleotide sugar donors, and the availability of glycosyltransferases and glycosidases [24]. Protein glycosylation is a heterogeneous process and leads to very diverse glycans attached to a single site within a protein. Taking into account that a protein can have more than one potential glycosylation site and other posttranslational modifications (e.g., acetylation, phosphorylation, methylation) may be present on the protein sequence as well, glycoproteins are very heterogenous.

3 Glycoprofiling approaches

To study protein glycosylation and the attached glycan distribution, three different approaches have been developed over time. The first approach involves the release of glycans from the peptide backbone. This approach is used in glycomics, the comprehensive study of protein glycans that a cell or tissue produces. Peptide *N*-glycosidase F (PNGaseF) is commonly used to enzymatically remove *N*-glycans from the protein [28, 29]. This amidase cleaves between the glycan and the Asp residue, removes the intact *N*-linked glycan by hydrolyzation, and keeps the peptide backbone intact [30, 31]. Consequently, the Asp residue is converted into aspartic acid [32]. *N*-Glycans can also be released form the protein by the enzyme endoglycosidase H (EndoH). EndoH leaves a GlcNAc core at the peptide, which can provide information on the composition and location of the glycan [12, 14]. Usually, *O*-linked glycans are removed after the removal of *N*-linked glycans. *O*-Linked glycans can be released from the peptide using β-elimination in the presence of dithiothreitol (DTT) [28, 33]. This removal is carried out using a reducing agent, where the glycan is cleaved and released from the attached GalNAc [34].

Analysis at glycan level can result in detailed information regarding the structure and conformation of the glycans present on a protein. As only the released glycans are analyzed, this approach does not provide information on the protein the glycan was attached to, nor on the site of glycosylation. Approaches that include information on glycosylation site are used in glycoproteomics, where glycopeptides or glycan-carrying protein subunits resulting from enzymatic protein digestion are analyzed by LC-MS. Proteolytic digestion is commonly carried out using trypsin—which cleaves the protein backbone on the C-terminal side of lysine and arginine—resulting in a mixture of peptides. An enzyme frequently used for the analysis of immunoglobulins (IgGs) is the IgG-degrading enzyme of *Streptococcus pyogenes* (IdeS). IdeS specifically cleaves the IgG below the hinge resulting in F(ab)′2 and Fc/2 fragments (Fig. 2). Typically, the F(ab)′2 subunit is further reduced using DTT, generating the Fd′ fragment and light-chain subunits. MS-based analysis of the resulting protein species can reveal the presence, nature, and heterogeneity of multiple and co-occurring modifications (including glycosylation) on the specific subunits.

A disadvantage of cleaving the glycoprotein into parts (i.e., glycans, peptides, and/or subunits) prior to analysis is that information on co-occurring glycosylations, other PTMs, and sequence differences (and the distributions thereof) on the same proteoform is lost. Hence, accurate inference toward the full original protein structures is often not possible from the fragment information only. Characterization of individual proteoforms requires a top-down approach where the glycoprotein is analyzed intact.

For unambiguous assignment of glycans, peptides, or proteins separated by HILIC, hyphenation to MS often is crucial, in particular when

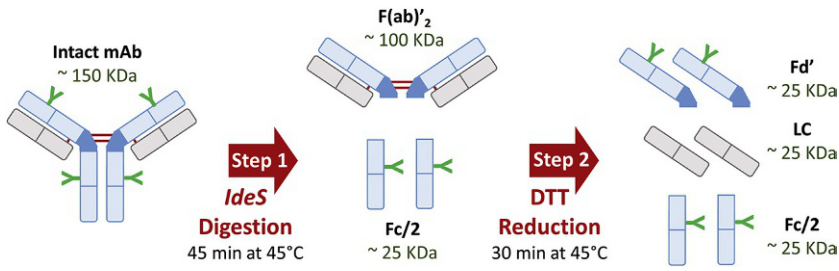


FIG. 2 Middle-up approach, showing the various antibody fragments and their molecular weight that are formed during the subsequent IdeS digestion and DTT reduction. This is a common subunit approach to get information of the glycosylation side and heterogeneity. *Reproduced with permission from V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Hydrophilic interaction chromatography hyphenated with mass spectrometry: a powerful analytical tool for the comparison of originator and biosimilar therapeutic monoclonal antibodies at the middle-up level of analysis, Anal. Chem. 89 (2017) 2086–2092, doi:10.1021/acs.analchem.6b04726.*

more complex samples and highly similar proteoforms are investigated. ESI-MS employing high-resolution mass analyzers, such as modern time-of-flight and Orbitrap instruments, enable the precise and accurate mass determination of glycans, peptides, protein subunits, and even intact proteins. As diverse glycans often have different molecular masses, MS can aid their discrimination and assignment, even when mass differences are relatively small. Tanden MS approaches (MS/MS) additionally promote identification capabilities. Mass spectra obtained after, for example, collision-induced dissociation (CID) may provide information on glycosylation sites and peptide and glycan sequences. The introduction of alternative fragmentation methodologies, such as electron transfer dissociation (ETD) or electron capture dissociation (ECD), have further extended identification possibilities. Still, protein species may comprise a large number of glycoforms, subtly differing in number and nature of the attached glycans (including isobaric ones), which may not be distinguished consistently by MS only. In these cases, separation prior to MS detection is essential to achieve reliable assignment, especially for low abundant glycoforms. Thus, the interplay of selective HILIC separations and high-resolution MS provides a very powerful platform for the analysis of protein glycosylation.

In the field of glycoprotein characterization, HILIC-MS indeed has shown to be a remarkably versatile analytical tool, potentially providing separation of released glycans, of glycopeptides, of subunits of glycoproteins, and of intact protein glycoforms. In Fig. 3 the number of studies reported in literature until September 2020 investigating and applying

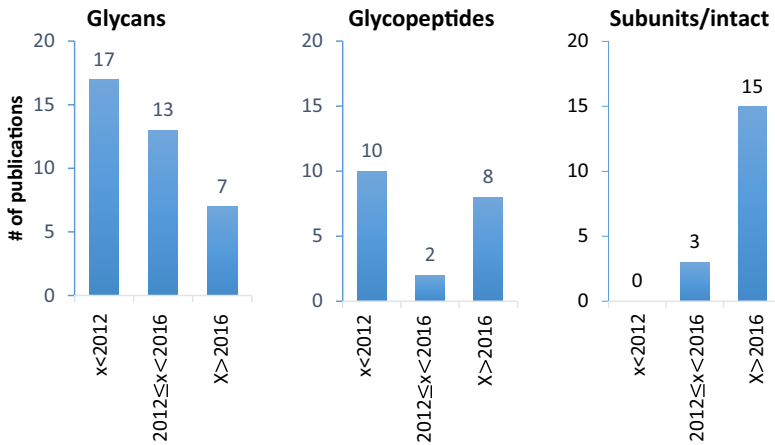


FIG. 3 Bar chart representation of the number of publications versus the year of publication of HILIC-MS glycoprotein studies at the glycan, glycopeptide, and subunit/intact protein level. Original data is tabulated in Tables 1–3. *Bar-chart created by the authors for this publication.*

HILIC-MS for the characterization of glycoproteins are sorted according to protein property examined (i.e., analysis at the glycan, glycopeptide, or protein subunit/intact protein level) and period of publication. More details on the respective studies are summarized in Tables 1–3. HILIC-MS has been introduced around 2009 [37] as an alternative orthogonal analytical tool with respect to RPLC-MS for the analysis of glycans and glycopeptides derived from glycoproteins. Today, HILIC is considered an established technique for the analysis of glycans. Most typical, glycan HILIC is performed in combination with fluorescence detection (FLD; not covered in this review) after glycan derivatization with a fluorescent label. HILIC-MS of glycans is often used as an approach to provide, support, and confirm identifications of glycan structures separated and detected by HILIC-FLD. Although less frequently than for glycans, HILIC-MS is also more or less routinely applied in glycoproteomics experiments for the analysis of tryptic glycopeptides. The analysis of glycoprotein subunits and intact glycoproteins by HILIC-MS is a relatively recent development, being described firstly around 2016 [101]. It has gathered significant attention since, as illustrated by the relatively high number of publications in this area published in the last 4 years.

4 HILIC-MS of glycoproteins: Conditions and practicalities

4.1 HILIC retention mechanism

HILIC can be considered a form of aqueous NPLC used to resolve (highly) polar species. HILIC employs polar stationary phases in combination with water-organic mobile phases, mostly mixtures of acetonitrile (ACN) and water. These mobile phases typically are compatible with ESI-MS [102]. In this section, first some general considerations when performing HILIC are discussed. Subsequently, practical implications for glycoprotein profiling will be treated.

HILIC selectivity can be tuned by selecting the appropriate mobile phase and stationary phase chemistries, which overall determine the types and strengths of molecular interactions that should lead to resolution of sample components. The most common mobile phases used in HILIC typically are ACN-water mixtures containing buffer salts (e.g., ammonium acetate) or acids (e.g., formic acid). The aprotic nature of ACN promotes the formation of a water layer on the surface of the stationary phase and enhances hydrogen-bond driven interactions between analyte and stationary phase. In HILIC, methanol tends to provide a stronger elution strength as compared to ACN, as methanol being both proton donor and acceptor decreases hydrophilic partitioning and reduces analyte interactions with polar stationary phases [3, 103].

TABLE 1 Overview of HILIC-MS characterization of glycoproteins at the glycan level.

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample (s)	Deglycosylation	*N*-/*O*-glycans	Labeling	Detection (MS)	References
Amide	GlycoSep N (Prozyme) Column: 1×150 mm dp: N/A Pore size: NR	ACN to water 50 mM NH_4HCO_2 pH 4.4 Temp NR	Reference proteins	Ovalbumin and human IgG	PNGaseF	*N*	2-AA	qTOF	[35]
	GlycoSep N (Prozyme) Column: 1×150 mm dp: N/A Pore size: NR	ACN to water 50 mM NH_4HCO_2 pH 4.4 30°C	Clinical	Human apolipoprotein(a)	Hydrazyne	*N*	2-AA	qTOF	[36]
	TSK Amide 80 (TOSOH) Column: 0.075×100 mm dp: 5 µm Pore size: 80 Å	ACN to water 50 mM $NH_4CH_3CO_2$ pH 4.5 Temp NR	Clinical	Human plasma	PNGaseF	*N*	No derivatization	FT-ICR	[37]
	BEH Amide (Waters) Column: 2.1×50 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 1–100 mM NH_4HCO_2 (pH 4.5) and 0.5% FA 40–60°C	Reference proteins	Fetuin, RNase B, and human IgG	PNGaseF	*N*	2-AB	qTOF	[38]
	TSK Amide 80 (TOSOH) Column: 2.0×150 mm dp: 5 µm Pore size: 80 Å	ACN to water 115 mM NH_4HCO_2 Temp NR	Biopharmaceutical	Recombinant mAb	PNGaseF	*N*	2-AB	LTQ-Orbitrap	[39]

	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 150 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins Biopharmaceutical	Fetuin, asialo-fetuin, RNase B, mAb	PNGaseF	*N*	2-AB	LTQ-Orbitrap	[40]
	TSK Amide 80 (TOSOH) Column: 0.075×100 mm dp: 5 µm Pore size: 80 Å	ACN to water 50 mM $NH_4CH_3CO_2$ pH 4.5 Temp NR	Clinical	Human plasma	PNGaseF	*N*	2-Phenylacetohydrazide, 3-phenylpropanehydrazide, 4-phe-nylbutanehydrazide, 5-phenylpentanehydrazide, 4-phenethylbenzohydrazide	FT-ICR	[41]
	TSK Amide 80 (TOSOH) Column: 0.075×100 mm dp: 5 µm Pore size: 80 Å	ACN to water 50 mM $NH_4CH_3CO_2$ pH 4.5 Temp NR	Clinical	Human plasma	PNGaseF	*N*	2-Phenylacetohydrazide	LTQ-Orbitrap	[42]
	GlycoSep N (Prozyme) Column: 0.075×250 mm dp: 5 µm Pore size: N/A	ACN to water 0.1% FA pH 4.4 Temp NR	Reference proteins	Haptoglobin	PNGaseF	*N*	2-AB, 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride	qTOF	[43]
	TSK Amide 80 (TOSOH) Column: 4.0×150 mm dp: 5 µm Pore size: 80 Å	ACN to water 100 mM AA Temp NR	Reference samples	Bovine serum and frog egg-jelly coat	Hydrazyne	*O*	3-methyl-5-pyrazolone	LTQ-Orbitrap	[44]
	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.5 40°C	Biopharmaceutical	Etanercept	PNGaseF, exoglycosidases	*N, O*	2-AB	qTOF	[45]

Continued

TABLE 1 Overview of HILIC-MS characterization of glycoproteins at the glycan level—cont'd

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample (s)	Deglycosylation	*N*-/*O*-glycans	Labeling	Detection (MS)	References
	BEH Amide (waters) Column: 2.1×50 and 150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins Biopharmaceutical	mAb, human IgG, bovine fetuin	PNGaseF	*N*	RapiFluor-MS	qTOF	[46]
	BEH Amide (Waters) Column: 2.1×100 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 100 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins Biopharmaceutical	mAb, human serum IgG	PNGaseF; endomannosidase	*N*	2-AB	LTQ-Orbitrap	[47]
	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.5 40°C	Clinical	Dromedary camels IgG	PNGaseF, exoglycosidase	*N*	2-AB	qTOF	[48]
	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins Clinical sample	Fetuin, human saliva	Hydrazyne	*O*	Proacainamide	Iontrap	[49]
	(1) Accucore (ThermoFisher) Column: 2.1×150 mm dp: 2.6 µm Pore size: 150 Å (core shell)	ACN to water 50 mM NH_4HCO_2 pH 4.4 40°C	Clinical	Polyclonal IgG	PNGaseF	*N*	2-AB	Orbitrap	[50]

	(2) BEH Amide (Waters) Column: 2.1×100 mm dp: 1.7 µm Pore size: 130 Å								
	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.4 40°C	Biopharmaceutical	Adalimumab, cetuximab, etanercept	PNGaseF, sialidase	*N*, *O*	RapiFluor-MS, 2-AB	qTOF	[51]
	BEH Amide (Waters) Column: 2.1×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins	Fetuin, IgG, lactoferrin, RNase B	PNGaseF	*N*	RapiFluor-MS	qTOF	[52]
	BEH Amide (Waters) Column: 2.1×100 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 50 mM NH_4HCO_2 pH 4.4 60°C	Biopharmaceutical	Recombinant erythropoietin	PNGaseF, sialidase A	*N*	2-AB	QE-Orbitrap	[53]
	TSK Amide 80 (TOSOH) Column: 4.0×150 mm dp: 5 µm Pore size: 80 Å	ACN to water 100 mM NH_4HCO_2 pH 6.0 20°C	Clinical	Asialo-fetuin, chicken ovomucin, human seminal plasma	NaOH, NH_3, dimethyl amine	*O*	3-methyl-5-pyrazolone	LTQ-Orbitrap	[54]
	PolyGLYCOPLEX A (PolyLC) Column: 0.075×300 mm dp: 3 µm Pore size: 300 Å	ACN to water 0.1% FA Temp NR	Reference proteins	Bovine thyroglobulin, bovine lactoferrin, RNase B, human serum protein mixture	PNGaseF	*N*	AminoxyTMT	Orbitrap	[55]

Continued

TABLE 1 Overview of HILIC-MS characterization of glycoproteins at the glycan level—cont'd

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample (s)	Deglycosylation	*N*-/*O*-glycans	Labeling	Detection (MS)	References
Amino	Ultramex NH2 (Phenomenex) Column: 4.6×250 mm dp: 5 µm Pore size: NR	ACN to water FA pH 3.5 Temp NR	Reference proteins	Ovalbumin	PNGaseF	*N*	1-Phenyl-3-methyl-5-pyrazolone, 2-aminonaphthalene trisulfone	QQQ	[56]
	Hypersil APS-2 Column: 2.1×250 mm dp: 3 µm Pore size: NR	ACN to water 10 mM NH_4HCO_2 pH8 60°C	Food analysis	Ovotransferrin	PNGaseF	*N*	Aniline	LTQ-Orbitrap	[57]
Diol	Penta-HILIC (HALO) Column: 2.1×150 mm dp: 2.7 µm Pore size: 90 Å (core shell)	ACN to water 50 mM NH_4HCO_2 pH 4.4 60°C	Reference proteins	Bovine fetuin, human serum	PNGaseF	*N*	Procainamide	qTRAP	[58]
Zwitterionic	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 100 mM $NH_4CH_3CO_2$ 40°C	Reference proteins	Human serum IgG	PNGaseF	*N*	2-AP	Ion trap	[59]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 100 mM $NH_4CH_3CO_2$ pH 6.8 40°C	Reference proteins	α-1-Acid glycoprotein	PNGaseF	*N*	2-AP	Ion trap	[60]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 10 mM $NH_4CH_3CO_2$ 40°C	Reference proteins	Human serum IgG	PNGaseF	*N*	2-AP	Ion trap	[61]

	ZIC-HILIC (SeQuant) Column: 0.075×150 mm dp: 5 µm Pore size: 200 Å	ACN to water 2 mM $NH_4CH_3CO_2$ pH 6.9 Temp NR	Reference proteins	RNase B, mAb	PNGaseF	*N*	No derivatization	qTOF	[62]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 34 mM AA pH 3.0 30°C	Reference proteins	RNase B, mAb	PNGaseF	*N*	2-AB	TOF	[63]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 0.1% AA pH 3.25 30°C	Biopharmaceuticals	mAb	PNGaseF	*N*	2-AP	qTOF	[64]
	ZIC-HILIC (SeQuant) Column: 0.3×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 10 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins	Ovalbumin, fetuin, bovine α-1-acid glycoprotein	PNGaseF	*N*	Aniline	qTOF	[65]
	ZIC-HILIC (SeQuant) Column: 0.3×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 10 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins Clinical sample (method development)	Human α-1-acid glycoprotein	PNGaseF Sialidase	*N*	Aniline	qTOF	[66]
	ZIC-HILIC (SeQuant) Column: 0.3×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 1 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins	Human α-1 acid glycoprotein	PNGaseF Sialidase Fucosidase	*N*	Aniline	qTOF	[67]
	ZIC-HILIC (SeQuant) Column: 0.3×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 1 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins Clinical (method development)	Human α-1 acid glycoprotein	PNGaseF Sialidase	*N*	Aniline	Ion trap Orbitrap	[68]

Continued

TABLE 1 Overview of HILIC-MS characterization of glycoproteins at the glycan level—cont'd

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample (s)	Deglycosylation	*N*-/*O*-glycans	Labeling	Detection (MS)	References
Multiple columns	**(1)** Xbridge amide (Waters) Column: 4.6×150 mm dp: 3.5 µm Pore size: NR **(2)** polySULFOETHYL A Column: 4.6×100 mm dp: 5 µm Pore size: NR	ACN to water 0.05%TFA 45°C	Biopharmaceutical	Recombinant mAb	PNGaseF, mannosidase, sialidase, galactosidase	*N*	2-AB	qTOF	[69]
2DLC	DEAE-5PW (TOSOH) Column: 2.0×50 mm dp: 5 µm Pore size: NR TSK Amide 80 (TOSOH) Column: 2.0×150 mm dp: 5 µm Pore size: 80 Å ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 5 µm Pore size: 200 Å	Multiple solvents ACN, water, 0.5 M $NH_4CH_3CO_2$ 40°C	Reference proteins	Human serum proteins	PNGaseF	*N*	2-AP	Ion trap	[70]

2-*AA*, 2-aminoacridone; 2-*AB*, 2-aminobenzamide; 2-*AP*, 2-amino pyridine; *AA*, acetic acid; *ACN*, acetonitrile; *dp*, particle size; *FA*, formic acid; *MS*, mass spectrometry; *NH4CH3CO2*, ammonium acetate; *NH4HCO2*, ammonium formate; *NH4HCO3*, ammonium hydrogen carbonate; *NR*, not reported; *QE*, Q-exactive; *QQQ*, triple quadrupole; *qTOF*, quadrupole time-of-flight; *RT*, room temperature; *TFA*, trifluoroacetic acid.

TABLE 2 Overview of HILIC-MS characterization of glycoproteins at the glycopeptide level.

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample(s)	Digestion	*N-/O-* glycopeptides	Detection (MS)	References
Amide	TSKgel Amide 80 (TOSOH) Column: 0.3×150 mm dp: 5 µm Pore size: 80 Å	ACN to water 0.05% TFA Temp NR	Clinical	H5N1 Influenza Hemagglutinin	Trypsin	*N*	qTOF	[71]
	TSKgel Amide 80 (TOSOH) Column: 0.075×180 mm dp: 3 µm Pore size: 80 Å	ACN/water to water 50 mM NH_4HCO_2 RT	Reference proteins	Fetuin, asialofetuin	Proteinase K	*N,O*	Ion trap	[72]
	TSKgel Amide 80 (TOSOH) Column: 0.15×800 mm dp: NR Pore size: 80 Å	ACN to water 0.5% FA Temp NR	Reference proteins	Bovine serum albumin, RNase B, horseradish peroxidase	Trypsin	*N*	qTOF	[73]
	BEH Glycan Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 130 Å	ACN/ water to water 100–10 mM NH_4HCO_2 pH 4.5 40°C	Reference proteins	Bovine fetuin, human α-1-acid glycoprotein, human transferrin, bovine RNase B	Trypsin	*N*	qTOF	[74]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 50 mM NH_4HCO_2 60°C	Reference proteins Biopharmaceuticals	Adalimumab, cetuximab, etanercept, α-1 acid glycoprotein, bovine fetuin, RNase B	Trypsin	*N*, *O* (labeled)	qTOF	[51]
	BEH Glycan Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 100 mM NH_4HCO_2 60°C	Biopharmaceuticals	NIST mAb	Trypsin	*N*	qTOF	[75]
	AdvanceBio Glycan Mapping (Agilent) Column: 2.1×100 mm dp: 1.8 µm Pore size: 300 Å	ACN to water 0.1%TFA 50°C	Biopharmaceuticals	Herceptin	Trypsin	*N*	qTOF	[12]

Continued

TABLE 2 Overview of HILIC-MS characterization of glycoproteins at the glycopeptide level—cont'd

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample(s)	Digestion	*N*-/*O*-glycopeptides	Detection (MS)	References
	XBridge Amide (Waters) Column: 4.6×150 mm dp: 3.5 µm Pore size: 135 Å	ACN/water to water 100–10 mM NH_4HCO_2 35°C	Food analysis	Royal jelly proteins	Trypsin	*N*	Ion trap	[76]
Zwitterionic	ZIC-HILIC (SeQuant) Column: 2.1×100 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 250 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins	α-1 acid glycoprotein	Trypsin	*N*	Ion trap	[60]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 100 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins	Human serum IgG	Trypsin	*N*	Ion trap	[59]
	ZIC-HILIC (SeQuant) Column: 0.075×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 10 mM $NH_4CH_3CO_2$ *T* = NR	Biopharmaceuticals	Recombinant erythropoietin	Glu-C	*N*	LIT TOF	[77]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 5 mM $NH_4CH_3CO_2$ Temp NR	Reference proteins	α-1-Acid glycoprotein	Trypsin	*N*	LIT TOF	[61]
	Monolithic ZIC-HILIC (Merk) Column: 0.1×300 mm dp: NR Pore size: NR	ACN to water 0.1% FA Temp NR	Reference proteins	Bovine α-1-acid glycoprotein, human serum IgG	Trypsin, Glu-C	*N*	qTOF	[78]

	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 0.05% FA Temp NR	Reference proteins	Bovine caseinomacropeptide	Trypsin,chymotrypsin	*O*	Ion trap	[79]
	ZIC-HILIC (SeQuant) Column: 2.1×150 mm dp: 3.5 µm Pore size: 200 Å	ACN to water 0.1% FA Temp NR	Reference proteins	Bovine Pancreatic RNase B, serum IgG	Lys-C	*N*	Orbitrap	[80]
Diol	Penta-HILIC (HALO) Chip: 0.075×150 mm dp: 2.7 µm Pore size: 90 Å (core shell)	ACN to water 100 mM NH_4HCO_2 60°C	Reference proteins Clinical	Bovine fetuin, serum IgGs	Trypsin	*N*	Ion trap	[81]
	Penta-HILIC (HALO) Column: 0.2×150 mm dp: 2.7 µm Pore size: 90 Å (core shell)	ACN to water 0.1% FA 50 mM NH_4HCO_2 Temp NR	Reference peptides	Synthetic peptides and glycopeptides	–	*O*	Ion trap	[82]
	Penta-HILIC (HALO) Chip: 0.075×150 mm dp: 2.7 µm Pore size: 90 Å (core shell)	ACN to water 0.1% FA 20 and 40 mM NH_4HCO_2 pH 4.0 and 6.5 20°C	Reference proteins	Hemoplexin	Trypsin	*N*	Ion trap	[83]
Bare silica	HILIC (HALO) Chip: 0.075×150 mm dp: 2.7 µm Pore size: 90 Å (core shell)	ACN to water 0.1% FA 30°C	Reference proteins	Hemoplexin	Trypsin	*N*	Ion trap	[84]

Continued

TABLE 2 Overview of HILIC-MS characterization of glycoproteins at the glycopeptide level—cont'd

Chemistry	Column characteristics	Mobile phase, additive, and temperature	Analyte class	Sample(s)	Digestion	*N-/O-* glycopeptides	Detection (MS)	References
Multiple columns	(1) PolyHYDROXYETHYL A (PolyLC) column: 2.1×100mm dp: 3μm Pore size: 300Å (2) XBridge Amide (Waters) column: 4.6×150mm dp: 3.5μm Pore size: 135Å (3) ZIC-HILIC (SeQuant) column: 2.1×150mm dp: 3.5μm Pore size: 200Å	ACN to water different additives: • 10 mM $NH_4CH_3CO_2$ • 5 mM $NH_4CH_3CO_2$ • 0.05% FA Temp NR	Reference proteins	Bovine caseinomacropeptide A and B	Trypsin, chymotrypsin	*O*	qTOF	[85]
	(1) BEH Amide (Waters) column: 2.0×150mm dp: 1.7μm Pore size: 300Å (2) ZIC-HILIC (SeQuant) column: 2.1×100mm dp: 3.5μm Pore size: 200Å (3) ZIC-pHILIC (SeQuant) column: 2.1×100mm dp: 3μm Pore size: 200Å	ACN to water Anionic ion-pairing agents and salts $T = 40°C$	Reference proteins Biopharmaceuticals	Trastuzumab, RNase B, human fibrinogen gamma chain, abatacept	Trypsin, Lys-C, IdeS	*N*	qTOF	[86]

ACN, acetonitrile; *dp*, particle size; *FA*, formic acid; *LIT*, linear ion trap; *NH4CH3CO2*, ammonium acetate; *NH4HCO2*, ammonium formate; *NR*, not reported; *qTOF*, quadrupole time-of-flight; *RT*, room temperature; *TFA*, trifluoroacetic acid.

TABLE 3 Overview of HILIC(-MS) characterization of glycoproteins at the intact level and subunit level.

Chemistry	Column characteristics	Mobile phase and temperature	Types of analyte	Protein analyzed	Approximate molecular weight	Detection (MS)	References
Amide	(1) Prototype polyacrylamide brush layer Column: 30×2.1 mm dp: 0.700 µm Pore size: non-porous silica (2) BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 130 Å	ACN to water 0.5% FA + 0.05% TFA or 0.1% TFA 30°C	Intact reference proteins	Lysozyme, cytochrome *c*, carbonic anhydrase, bovine serum albumin, RNase B	10–60 kDa, MS only on RNase B (15 kDa)	Ion trap	[13]
	TSKgel Amide 80 (TOSOH) Column: 2.0×150 mm dp: 3 µm Pore size: 80 Å	ACN to water 0.1% TFA 50°C	Intact reference proteins Intact biopharmaceutical	RNase B, neoglycoproteins	20 kDa	Ion trap	[87]
	Prototype Amide column Column: 2.0×150 mm 2× dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 80°C	Intact biopharmaceutical Subunits biopharmaceutical	Intact mAb, glycosylated and deglycosylated	150 kDa	qTOF	[46]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Intact reference proteins Subunit biopharmaceutical	RNase B, digested mAb	20–30 kDa	qTOF	[51]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Subunits biopharmaceutical	IdeS digested mAbs (Remicade, Herceptin, Erbitux)	25 kDa	qTOF	[88]
	TSKgel Amide 80 (TOSOH) Column: 2.0×150 mm dp: 3 µm Pore size: 80 Å	ACN to water 10 mM HClO4. 50°C	Intact biopharmaceuticals	Neo-glycoproteins	10–30 kDa	Ion trap	[89]

Continued

TABLE 3 Overview of HILIC(-MS) characterization of glycoproteins at the intact level and subunit level—cont'd

Chemistry	Column characteristics	Mobile phase and temperature	Types of analyte	Protein analyzed	Approximate molecular weight	Detection (MS)	References
	AdvanceBio Glycan Mapping (Agilent) Column: 2.1×100 mm dp: 1.8 µm Pore size: 300 Å	ACN to water 0.1% TFA 50°C	Subunits biopharmaceutical	IdeS digested mAb (Herceptin)	25 kDa	qTOF	[12]
	(1) TSKgel Amide 80 (TOSOH) Column: 2.0×150 mm dp: 3 µm Pore size: 80 Å (2) BEH Amide (Waters) Column: 3.0×150 mm dp: 2.5 µm Pore size: 130 Å (3) AdvanceBio glycan mapping (Agilent)Column: 2.1×150 mm dp: 2.7 µm Pore size: 130 Å (core shell)	ACN to water 0.05% TFA 50°C	Intact biopharmaceuticals	RNase B, selected antigens from *Mycobacterium tuberculosis*, glycoconjugates, TB10.4 and Ag85B	10 kDa	qTOF	[90]
	TSKgel Amide 80 (TOSOH) Column: 2.0×150 mm dp: 3 µm Pore size: 80 Å	ACN to water 0.1% TFA/DFA 50°C	Intact biopharmaceuticals	Interferon-β1a, erythopoietin	20–35 kDa	qTOF	[91]
	AdvanceBio glycan mapping (Agilent) Column: 2.1×150 mm (also 200 µm×200 mm) dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Intact reference proteins Clinical	Reference proteins, yeast lysate	10–600 kDa (MS up to 80 kDa)	qTOF	[92]

	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Intact biopharmaceuticals Subunits biopharmaceuticals	mAb, deglycosylated and partially reduced	20–150 kDa	Orbitrap	[93]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Intact reference proteins Subunits biopharmaceuticals	RNase B, ovalbumin and IdeS digested mAb	10–600 kDa (MS up to 40 kDa proteins)	qTOF	[94]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.08% TFA/0.02%FA 60°C	Subunits biopharmaceuticals	IdeS-digested and reduced mAb (daratumumab)	25 kDa	qTOF	[95]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 60°C	Subunits biopharmaceuticals	Subunits recombinant Fc fusion protein (Etanercept)	25–40 kDa	qTOF	[96]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 1% PA/0.025% TFA 60°C	Intact biotechnological products	Lipase enzyme	28–35 kDa	qTOF Orbitrap	[97]
	AdvanceBio glycan mapping (Agilent) Column: 200 µm×300 mm dp: 2.7 µm Pore size: 130 Å	ACN 0.1% TFA to water 0.1% TFA	Clinical	Fc subunits of serum IgGs	25 kDa	qTOF	[98]
Multidimensional LC	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% TFA 40°C	Subunits biopharmaceuticals	IdeS-digested and reduced mAb (atezolizumab, obinutuzumab and cetuximab). HILIC×RPLC-MS separation	25 kDa	qTOF	[99]
	BEH Amide (Waters) Column: 2.0×150 mm dp: 1.7 µm Pore size: 300 Å	ACN to water 0.1% FA/0.05% TFA 70°C	Subunits biopharmaceuticals	IdES digestion with IMER, RPLC reduction, HILIC separation	25 kDa	qTOF	[100]

ACN, acetonitrile; *DFA*, difluoroacetic acid; *dp*, particle size; *FA*, formic acid; *IMER*, immobilized enzyme reactor; *NR*, not reported; *PA*, propionic acid; *qTOF*, quadrupole time-of-flight; *TFA*, trifluoroacetic acid.

Generally, buffer salts are added to the mobile phase to regulate the ionic strength and control the pH. The pH of the buffer in the mobile phase can be used to manipulate the retention characteristics of ionizable compounds, enhancing or reducing electrostatic interactions [104, 105]. The addition of buffer salts affects analyte retention and separation in HILIC, and the addition of, for example, formic, acetic, or trifluoracetic acid to the mobile phase, can improve peak shapes, especially when basic analytes are analyzed (ion-pairing) [106]. Moreover, salts can enrich the aqueous layer, increasing the hydrophilicity of the stationary phase, and resulting in increased retention and/or reduced contribution of electrostatic interactions [104]. The selection of buffer salts and their concentration is limited by their solubility in the organic-solvent rich HILIC eluents [104, 106]. Buffer salts such as ammonium acetate or formate are often used because of their high solubility in ACN-water as well as their compatibility with MS.

HILIC mobile phase compositions for isocratic elution or at the start of a gradient are typically characterized by a high percentage of organic solvent (e.g., 70%–95% v/v of ACN). Under these conditions, a water-rich layer is formed on the surface of the polar stationary phase, inducing a partitioning mechanism for analytes between the stationary aqueous layer and the organic-solvent-rich mobile phase. The extent to which water is adsorbed on the stationary phase material depends on the mobile phase composition, the type of stationary phase, and the type and concentration of buffer [3]. For example, for bare silica materials in combination with eluents comprising 75%–90% v/v ACN, the pore volume occupied by a water-enriched layer is 4%–13% [107]. Changing the percentage of the organic solvent in the mobile phase causes variations in the volume of water adsorbed to the stationary phase. When the percentage of water in the mobile phase is increased, the distinction between the water-enriched layer and the bulk mobile phase decreases, reducing the contribution of partitioning to analyte retention in HILIC. Hence, for species of higher hydrophilicity requiring higher percentages of water (> 25% v/v) for their elution, partitioning becomes less important for their retention, and electrostatic interactions and hydrogen bonding become more dominant. In HILIC, analytes generally present a retention minimum between 70% and 40% v/v of ACN in the mobile phase. At water percentages above 60% v/v in the mobile phase, retention may start to be affected by reversed-phase mechanisms (i.e., involving hydrophobic interaction), giving rise to a typical U-shaped curve between the retention factor and the fraction of water in the eluent [108].

Several different types of HILIC stationary phase materials are commercially available. These are mostly based on silica particles and have polar surface chemistry. Common examples are bare silica, and the silica-bonded amide, diol, and zwitterionic (e.g., sulfoalkylbetaine/phosphocholine)

stationary phases. The chemistry of the column particles determines the type and strength of interactions between analyte and stationary phase [109]. In particular, diverse stationary phases may exhibit different capacities to adsorb water and, hence, affect the contribution of partitioning to retention. Dinh et al. [3, 9] studied the correlation between analyte retention and the thickness of the water-enriched layer on various polar stationary phases. Indeed, a lower water uptake by the stationary phase corresponded to weaker retention because of less partitioning and lower overall hydrophilicity of the stationary phase surface.

In order to achieve robust and efficient HILIC methods, some aspects need particular attention. Column equilibration times should be relatively long (i.e., corresponding to more than five column volumes) when performing gradient analysis. This is especially true for the analysis of compounds of relatively low polarity, which require high concentrations of ACN (>95% v/v) to obtain appreciable retention [110]. With respect to injection, the sample solvent should contain a relatively low percentage of water (i.e., the strong solvent in HILIC) in order to prevent peak distortion and analyte breakthrough [111]. This may, however, be challenging as some highly polar sample constituents and salts may not (fully) dissolve in, for example, an ACN-rich solvent. In the particular case of analyzing proteins, these may denature and precipitate during sample preparation in organic solvent-water mixtures. In general, the adverse effects of injecting in a relatively strong solvent can be circumvented by injection of small sample volumes (typically below 1% of the column volume), which, however, reduces the method sensitivity, potentially hindering the detection of low abundance compounds [11].

Interestingly, HILIC can provide retention for both polar and ionized sample constituents [11]. For this reason, HILIC is often used for the analysis of carbohydrates, oligonucleotides, amino acids, drugs, antibiotics, metabolites, peptides, and proteins [112]. HILIC and RPLC have shown distinct retention behavior differences for the same proteins rendering the two separation techniques orthogonal [113]. This is illustrated in Fig. 4A and B by the RPLC and HILIC separation of intact proteins using the same ACN-water mobile phase but applying opposite gradients. The elution order of the proteins is not only different but the protein retentions also clearly are uncorrelated (Fig. 4C). This characteristic has been exploited in several works where HILIC and RPLC were combined in an online or offline two-dimensional LC setup, yielding very high peak capacities (see e.g., Refs. [114–117]).

4.2 Experimental parameters impacting glycoprofiling by HILIC-MS

The basis of the selectivity of HILIC for glycan and glycan-containing structures depends on three main factors: (1) the type and composition

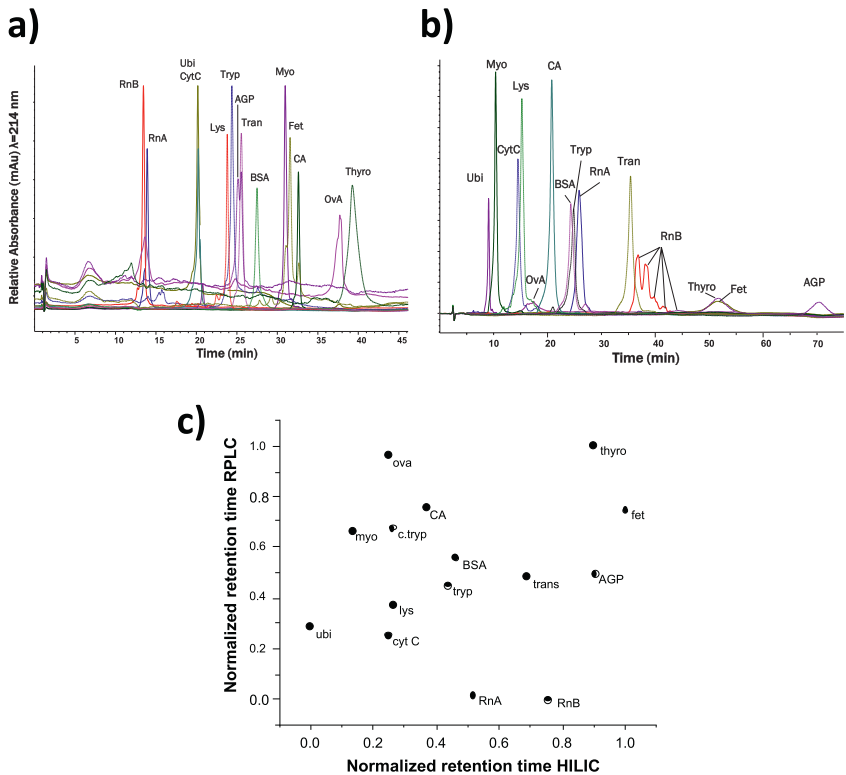


FIG. 4 Analysis of reference proteins using either (A) RPLC and (B) HILIC. (C) Normalized retention times of test proteins obtained during RPLC and HILIC using a C4 and amide-based column, respectively. The columns used were a (A) RPLC C8 column Agilent RRHD (50×2.1 mm, 300 Å, 1.8 µm) and (B) HILIC column, Agilent AdvanceBio glycan mapping (150×2.1 mm, 1.8 µm, 300 Å). The RPLC analysis (A) was performed using a linear gradient from 5% to 60% A in 45 min. HILIC analysis (B) started with 10% B for 1 min followed by a linear increase to 20% B in 1 min and then to 55% B in 90 min. Mobile phase A consisted of 98% ACN, 2% water, and 0.1% TFA. Mobile phase B contained 10% 2-propanol, 2% ACN, and 0.1% TFA. Flow rate, temperature, and UV absorbance detection for all runs were 0.2 mL/min, 60°C, and at 214 nm, respectively. Data for figure (C) were obtained using a Waters Acquity UPLC Protein BEH C4 column (50×2.1 mm, 1.7 µm, 300 Å) and a Waters Acquity UPLC glycoprotein Amide (50×2.1 mm, 1.7 µm, 300 Å) column. The analysis was performed applying a linear gradient from 5% to 60% A in 30 min, and for HILIC analysis the linear gradient was from 10% to 50% B in 30 min. Abbreviations used in panel (A) and (B): *Ubi*, ubiquitin; *Myo*, myoglobin; *CytC*, cytochrome C; *Lys*, lysozyme; *OvA*, ovalbumin from chicken egg; *CA*, carbonic anhydrase; *BSA*, bovine serum albumin; *Tran*, transferrin; *Fetm*, fetuin; *Tryp*, trypsinogen; *RnA*, ribonuclease A; *RnA B*, RNase B; *Thyro*, thyroglobulin; *AGP*, α-1-acidic glycoprotein. *Reproduced with permission from (A and B) A.F.G. Gargano, L.S. Roca, R.T. Fellers, M. Bocxe, E. Domínguez-Vega, G.W. Somsen, Capillary HILIC-MS: a new tool for sensitive top-down proteomics, Anal. Chem. 90 (2018) 6601–6609, doi;10.1021/acs.analchem.8b00382 and (C) G. van Schaick, B.W.J. Pirok, R. Haselberg, G.W. Somsen, A.F.G. Gargano, Computer-aided gradient optimization of hydrophilic interaction liquid chromatographic separations of intact proteins and protein glycoforms, J. Chromatogr. A. 1598 (2019) 67–76, doi:10.1016/j.chroma.2019.03.038.*

of glycans present and the relative glycan content of the analyte, (2) the chemistry of the applied stationary phase, and (3) the composition of the mobile phase used for the analysis. These, in combination with method conditions such as gradient steepness and time, injection solvent composition and volume, and temperature, mainly influence the HILIC selectivity and efficiency for glycoproteins. Some of these parameters also impact the ESI efficiency, and thus the MS detection sensitivity. This section discusses several of the above-mentioned factors in more detail.

4.2.1 Glycan composition and relative molecular weight

HILIC is selective toward the type of glycan present on the analyzed glycoproteins. In chromatography, this constitutes the so-called sample dimension, which determines the nature of potential interactions of the analyte with the stationary phase. Generally, glycosylation heterogeneity arises from a combination of neutral glycan building blocks (i.e., mannose, galactose, *N*-acetyl glucosamine, *N*-acetyl galactosamine, and fucose) and acidic units (i.e., *N*-acetyl neuraminic acid (or sialic acid) and *N*-glycolyl neuraminic acid) (Fig. 2B). HILIC studies at the glycan level have demonstrated that the retention of sugar structures depends on the number and accessibility of their polar groups. Although nonderivatized glycans can be separated by HILIC (as opposed to RPLC), most glycan HILIC studies use labeling to allow for sensitive fluorescence detection or enhance ESI. The presence of the label on the glycan structure usually has only modest influence on the HILIC selectivity. However, it usually encompasses a reduction of glycan retention as the derivatization reagents are typically lipophilic.

Melmer et al. [40] showed the contribution of the different monosaccharide building blocks on glycan retention by HILIC analysis of 2-aminobenzamide (2-AB) derivatized *N*-glycans containing fucose, *N*-acetylglucosamine, galactose, mannose, and/or sialic acid. A neutral amide-type HILIC stationary phase was used in combination with a gradient eluent of ACN and 150 mM ammonium formate (pH 4.4). The contribution to retention of fucose and *N*-acetylglucosamine appeared to be small, whereas mannose, galactose and sialic acid had a significant effect on retention. Interestingly, a high correlation was observed between HILIC retention and the number of hydroxyl groups present in the glycan (Fig. 5). *O*-Glycans are typically of smaller size than *N*-glycans, and thus carry a significantly smaller number of hydroxyl groups. Indeed, *O*-glycans and *O*-glycopeptides elute earlier than *N*-glycans and *N*-glycopeptides [72]. HILIC separations generally tend to be more sensitive toward differences in the number of neutral sugars present in glycans, and less to the presence and number of acidic glycan units. However, amino-based HILIC stationary phases provide a mixed-mode comprising weak anion-exchange mechanisms also allowing efficient separation of acidic glycans.

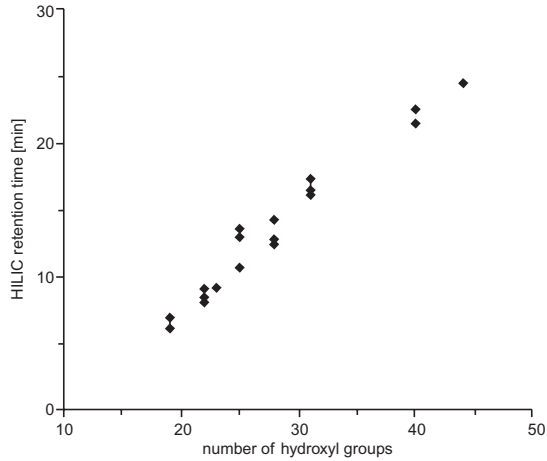


FIG. 5 HILIC retention times of 2-AB-labeled *N*-glycans from fetuin, asialo fetuin, RNAse B plotted against the number of hydroxyl groups per glycan. A Waters Acquity UPLC BEH Glycan column (150×2.1 mm, 1.7 µm) was used. Gradient elution was performed starting from 30% B, which was increases by 0.5%/min up to 45% B. Mobile phase A was acetonitrile and mobile phase B was 150 mM formic acid titrated to pH 4.4 with ammonia (25%). The flow was 0.5 mL/min. The temperature of the column thermostat was set to 60°C. *Reproduced with permission from M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis, J. Chromatogr. A. 1218 (2011) 118–123, doi:10.1016/j.chroma.2010.10.122.*

In glycoprotein analysis by HILIC, next to glycan character, the molecular weight of the peptide or protein moiety relative to the glycan is an important aspect affecting retention. This is exemplified by the analysis of glycans, glycopeptides, and subunits of monoclonal antibodies (mAbs) and intact mAbs using amide HILIC stationary phases (Fig. 6). HILIC of released and 2-AB labeled glycans showed a large peak capacity allowing resolution of subtle differences between glycan structures. For example, G1Fa and G1Fb glycans could be separated efficiently, although these only differ in the position of the galactose residue on either the α1–3 or α1–6 branch of the glycan (Fig. 6A [51]).

When analyzing enzymatic digests of glycoproteins by HILIC-MS, the resulting glycosylated peptides are more strongly retained and clearly separated from the nonglycosylated peptides (Fig. 6B [12]). This property also makes HILIC an interesting glycopeptide enrichment technique. For glycopeptides, the contribution of the glycan(s) to the overall size of the peptide is significant and can easily be more than 50% of its molecular weight. For example, the glycopeptide of trastuzumab resulting from tryptic digestion comprises a peptide (EEQYNSTYR) and a potential G2F glycan with molecular weights of 1189 and 1769 Da, respectively. The HILIC

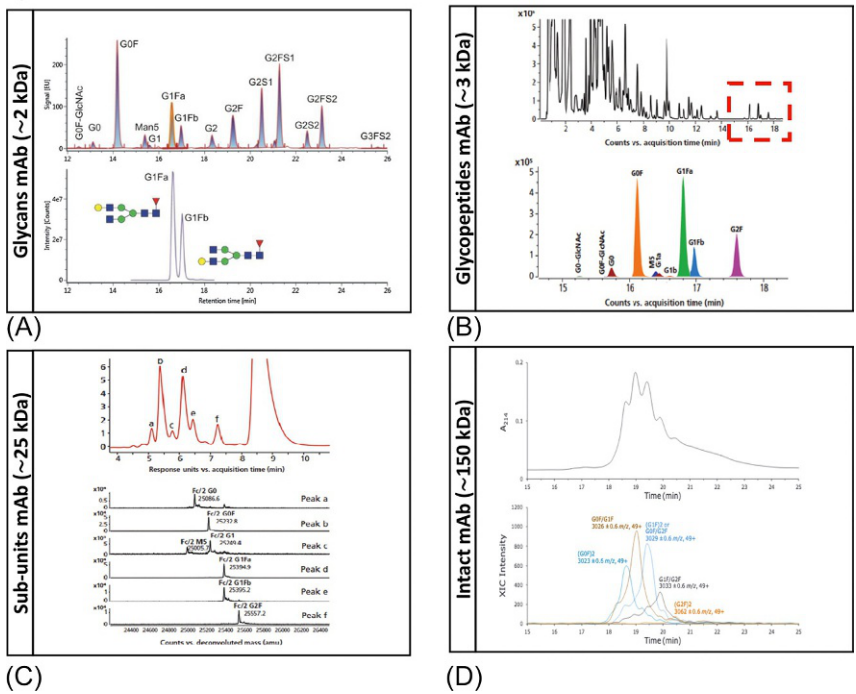


FIG. 6 HILIC-MS analysis of mAbs at the (A) glycan, (B) glycopeptide, (C) subunit, and (D) intact glycoprotein level. All the separation are performed using amide columns. Analyzed mAbs are (A) etanercept [51] and (B–D) trastuzumab [12, 118]. Chromatographic conditions for panel (A): A Waters Acquity UPLC BEH Glycan column (150×2.1 mm, 1.7 µm particles, 130 Å) was used. Mobile phase A was 50 mM ammonium formate (pH 4.4) and mobile phase B was acetonitrile. A gradient from 75% to 54% ACN over 35 min was applied. Flow rate and temperature were 400 µL/min and 60°C, respectively. Chromatographic conditions for panel (B and C): A Agilent Glycan Mapping column (100×2.1 mm, 1.8 µm, 300 Å) was used. Mobile phase A was water containing 0.1% TFA, whereas mobile phase B was ACN with 0.1% TFA. A gradient from 95% to 60% B in 20 min (B) and from 72% to 66% B in 14.8 min (C) were applied. Flow rate and temperature were 500 µL/min and 50°C, respectively. Chromatographic conditions for panel (D): A ACQUITY UPLC Glycoprotein BEH Amide column (150×2.1 mm, 1.7 µm particles, 300 Å) was used. Mobile phase A consisted of water with 0.1% TFA and mobile phase B was ACN with 0.1% TFA. A gradient from 70% to 63% B in 20 min was used. Flow rate and temperature were 200 µL/min and 30°C, respectively. *Modified from E. Largy, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel, Orthogonal liquid chromatography–mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level, J. Chromatogr. A. 1498 (2017) 128–146, https://doi.org/10.1016/j.chroma.2017.02.072; V. D'Atri, E. Dumont, I. van den Heede, D. Guillarme, P. Sandra, K. Sandra, Hydrophilic interaction chromatography for the characterization of therapeutic monoclonal antibodies at protein, peptide, and glycan levels, LCGC Eur. 30 (2017) 424–434; M.A. Lauber, S.M. Koza, Waters Application Notes – Mapping IgG Subunit Glycoforms Using HILIC and a Wide-Pore Amide Stationary Phase, (n.d.) 1–10.*

elution order of the glycopeptides is similar to the separation obtained for 2-AB labeled glycans, yet with lower chromatographic resolution, as exemplified for the G1Fa and G1Fb glycoforms [12].

Notably, glycopeptides exhibit a lower ESI efficiency relative to their nonglycosylated counterparts both in RPLC-MS and HILIC-MS. Moreover, as the ionization efficiency of glycopeptides is also dependent on the size of the glycan [119], direct quantification is not possible, as reference standards usually are not available. Interestingly, sialylated glycopeptides even show lower ESI efficiencies so that they often are underestimated relative to peptides with only neutral glycans. This is especially true for low abundant species [51]. For these reasons, for (relative) quantification of glycan levels, data from released glycan analysis with HILIC-FLD is typically preferred.

For glycosylated protein subunits (Fig. 6C [12]) and intact glycoproteins (Fig. 6D [118]), the glycoform resolving power of HILIC is smaller than for the corresponding glycans and glycopeptides. This is caused by the inherently lower chromatographic efficiency obtained for large molecules (due to low diffusion coefficients) and by the reduced relative contribution of the glycans to the overall molecular structure. For example, glycans represent about 7% of the molecular weight of the Fc/2 subunit of trastuzumab and about 2% of the total mAb molecular weight (i.e., 148,057 Da with two *N*-glycosylation sites). Still, as shown in Fig. 6C, at subunit level HILIC can resolve the six main glycoforms (G0, G0F, G1, G1Fa, G1Fb, G2F) of an antibody. However, for the intact antibody only a partial resolution of these glycoforms was obtained (Fig. 6D). The poorer glycoform separation at the intact level is a consequence of the relatively larger contribution of hydrophilic parts of the protein to the glycoprotein retention. Moreover, the *N*-glycans of mAbs are positioned between the two Fc chains partially hindering their interaction with the HILIC stationary phase. Indeed, HILIC-MS at the intact and subunit level has mostly been applied to proteins and subunits with molecular weights below 30 kDa (Table 1). Moreover, for most standard high-resolution mass spectrometers, efficient ion transmission for molecules above 30 kDa becomes problematic.

4.2.2 HILIC stationary phase

4.2.2.1 Stationary phase chemistry

The chemical nature of the stationary phase material used in HILIC obviously significantly determines the method's selectivity and retention of analytes [5, 120, 121]. Depending on the functional groups immobilized on the HILIC particles, hydrogen bonding, dipole-dipole, and/or electrostatic interactions can take place [103]. Hence, the types of interactions that certain materials promote [10, 122] as well as the build-up and thickness of a water layer on their surfaces (i.e., inducing partitioning) [3–5] can be quite different.

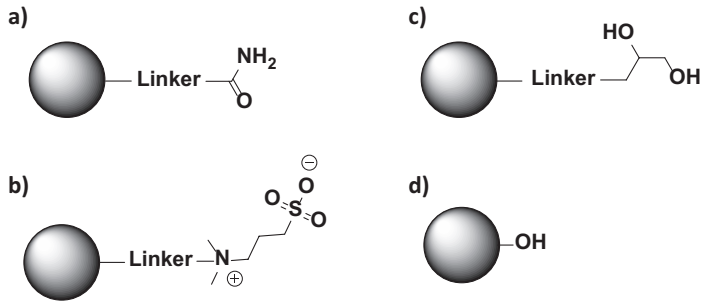


FIG. 7 Schematic representation of the functional groups of the most commonly adopted stationary phases for HILIC of glycoproteins; (A) amide, (B) sulfobetaine, (C) diol, and (D) bare silica.

HILIC columns materials are generally silica-based and can be classified according to functional group bound (i.e., chromatographic selector). The most commonly used materials for the analysis of glycoproteins are neutral, such as amide and diol bonded phases, or zwitterionic (e.g., sulfobetain). A schematic representation of the HILIC chemical selectors used for glycoprotein separations is given in Fig. 7. The silica surface can be functionalized by monolayers of the chemical selector (i.e., brush type or monomeric stationary phases) or by polymer selector structures (polymeric stationary phases). These two types of stationary phases may retain different amounts of water; in particular hydrophilic polymeric materials provide a thicker layer of water in comparison to the corresponding selector as brush type. This is also because of swelling phenomena that increase the surface of interaction with water [3].

Notably, polymeric phases have a less regular structure as a result of the heterogeneity of the polymer chains (e.g., molecular weight distribution) that composes them. This, together with swelling and shrinking that occurs when changing solvent composition, can translate in a reduced separation efficiency with respect to brush type phases. However, polymeric phases can withstand more harsh conditions, being stable under a broader pH range compared to monomeric phases. Moreover, polymeric selectors may reduce potential interaction of the analytes with (residual) silanols present on the silica surface. This can be used to prevent unwanted ionic interactions in neutral materials, such as diol and amide phases, as will be described later in this section.

Characteristics of the stationary phase materials commonly used in glycoprotein analysis by HILIC-MS are discussed later. The frequency of their use in the articles covered in this review is reported in Fig. 8A. Amide and zwitterionic functionalities have been described as most selective on revealing (partial) structural differences during analysis of small molecules [10, 122].

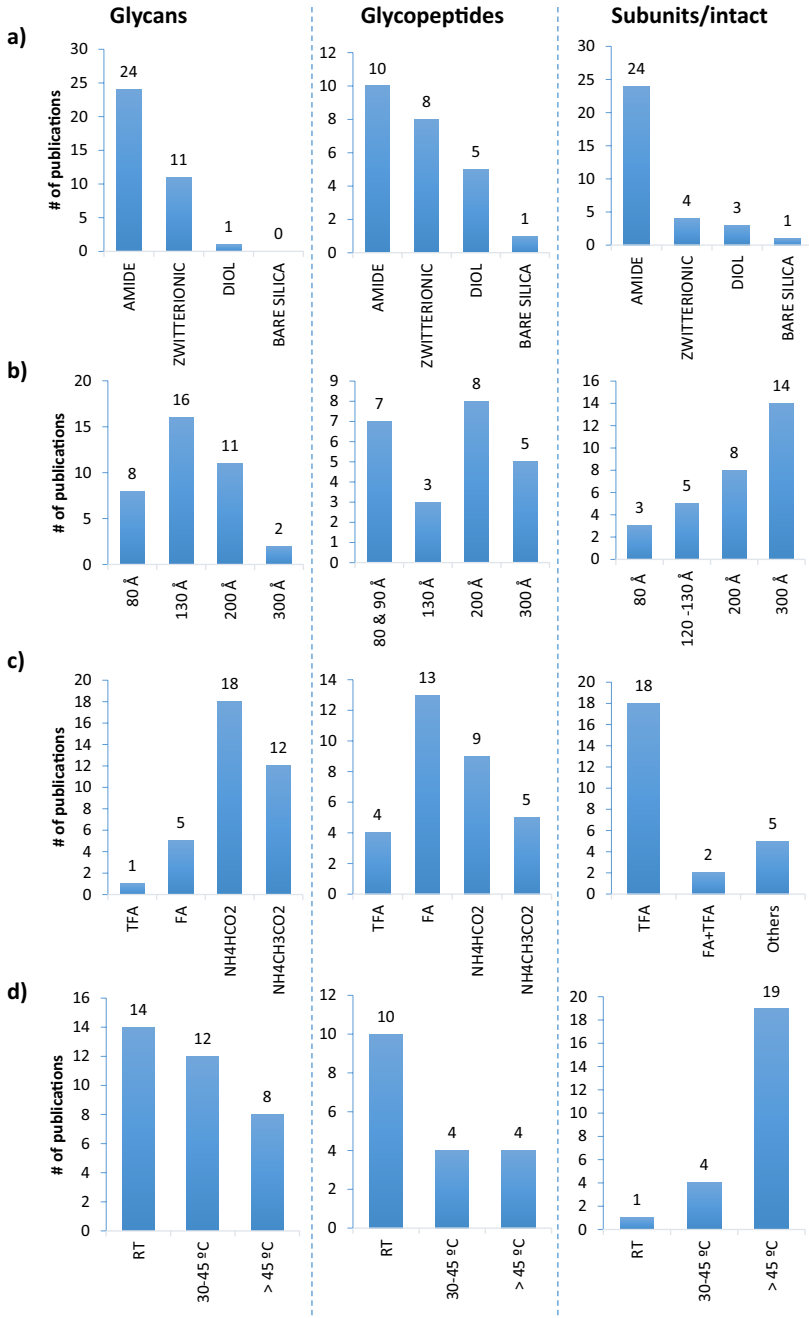


FIG. 8 Overview of trends in stationary phases for glycoprotein HILIC for (A) stationary phase material, (B) pore size, (C) mobile phase additives, and (D) column temperature, distinguished based on analysis at the glycan, glycopeptide, and subunit/intact protein level. All data is also tabulated in Tables 1–3.

Interestingly, their use in HILIC for glycoproteins reflects this finding, as they both chemistries have been used at all the glycoprotein analysis levels.

Amide-modified stationary phases (Fig. 7A) from TOSOH (TSKgel HILIC) or Waters (BEH-Amide) are the most commonly used materials for glycoprotein separations at all levels. Besides differences in porosity of the materials (80 Å for TSK vs 130 and 300 Å for BEH), also different strategies were used to immobilize amide groups on the surface of the materials. TOSOH columns feature carbamoyl groups that are covalently bound to the silica surface by a short aliphatic linker. Waters materials use ethylene bridged hybrid silica, allowing to reduce the number of residual (acidic) silanol groups. The BEH amide materials have a lower hydrophilicity. Yet, as glycans are very hydrophilic molecules, both materials are apt for glycoprofing.

For PolyLC materials the amide backbone (based on polysuccinimide) is further modified to introduce other functional groups resulting in, for example, PolyGlycoplex, PolyHydroxyethyl A, and PolySulfoethyl A materials. A novel type of amide material has recently been described making use of a polymeric coating on nonporous silica materials [13, 123, 124]. The coating allows covering the residual silanols of the silica to a larger extent, reducing unspecific ionic interactions.

The second most used HILIC column materials for glycoprotein analysis are zwitterionic modified stationary phases (Fig. 7B). These have been used in particular for the analysis of glycans and glycopeptides. The zwitterionic selectors (e.g., sulfobetaine) are typically immobilized via grafting polymerization on a wide-pore silica (200 Å) support. The polymeric character and the high hydrophilicity of the selector allow uptake of a relatively high amount of water. The most commonly used materials are branded as ZIC-HILIC (sulfobetaine selector) and ZIC-pHILIC (phosphocholine selector).

Diol-bonded phases (Fig. 7C) are preferred over bare silica, because of their reduced ion-exchange character [125], and are predominantly applied for glycopeptide analysis. Several commercial materials are available and made either as monomeric brush-type phases (e.g., using glycidoxypropyltrimethoxysilane and sequentially hydrolyzing it) or by forming a polymer layer by covalently cross-linking diol groups with an ether bridge. Mainly brush type diol phases are used for glycoprotein separations. One study reports the use a mixed-mode material, where a lipophilic linker is used to carry the diol functional group. This material was used to separate intact glycoproteins, but offered limited selectivity toward glycoforms [126].

Underivatized or bare silica (Fig. 7D) was mostly used in initial studies exploring the potential of HILIC for glycoprotein analysis at various levels. The acidic silanol groups on the silica surface (resulting from the condensation-polymerization of $Si(OH)_4$ with which spherical silica is

produced [127]) potentially offer ionic interactions that can be used to attract positively charged (basic) analytes or repulse for negative (acidic) compounds. However, at the glycopeptide and subunit/intact glycoprotein level, ionic interactions may overwhelm glycan interactions and therefore reduce the glycoselectivity of the method.

In order to obtain high-resolution HILIC separation of released glycans, the choice of glycan-derivatization reagent should depend on whether amide or zwitterionic stationary phases are adopted. In general, the amide column performs better for the separation of 2-aminobenzamide and 2-aminoacridone-labeled glycans, whereas 2-amino pyridine and aniline derivatized glycans are more commonly separated by zwitterionic stationary phases. Still, the glycan elution order typically is similar for the two methods [63].

Interestingly, despite differences in relative retention times, also glycopeptides generally present the same elution order on amide and zwitterionic stationary phases when using the same mobile phase systems. This was for example demonstrated by Furuki et al. who compared an ACQUITY UPLC Glycan BEH Amide and a ZIC-HILIC column for the analysis of the tryptic digest of RNase B (RNase B) [86]. On both column materials the unglycosylated peptides were well-resolved from the glycosylated peptide (SRNLTK). Moreover, the different glycopeptides arising from glycan heterogeneity were either partially or fully resolved on the ZIC and Amide column, respectively.

The HILIC separation of glycoforms of intact glycoproteins or protein subunits is almost exclusively performed using amide stationary phases (Fig. 8A). A possible explanation is that this type of chemistry largely reduces ion-exchange/electrostatic interactions. Ionic interactions tend to be stronger than hydrogen bonding [128]. Ionic interaction arise from sialic acid units in the glycans, but certainly also from acidic and basic amino acid residues present in the protein backbone. The latter strongly contribute to nonglycan specific interactions with the stationary phase and therefore will interfere with the attainable glycoselectivity.

Furiki et al. studied the HILIC analysis of intact RNase B on five stationary phase materials based on zwitterionic, diol, and amide chemistry. The resolution of the RNase B glycoforms was only observed for the amide stationary phases. The other materials gave a single protein peak and recovery issues were reported for the zwitterionic material. Similar observations were made for neoglycoproteins when analyzed by HILIC using various stationary phases [87]. Fig. 13 shows the HILIC analysis of the β-subunit of recombinant urinary human chorionic gonadotropin (r-hCG) [126], a highly heterogenous protein with 2*N*- and 4 *O*-glycosylation sites. The protein subunit was analyzed using three different HILIC materials: diol, anion exchange, and amide. For the diol and anion exchange phases only partial resolution of the different glycoforms was observed. The amide material provided better separation showing more detailed

proteoform profiles. In later work, the authors identified the first eluting peaks (1–8 in Fig. 9) as r-hCG glycoforms having 2 *N*-glycosylation sites occupied [129].

4.2.2.2 HILIC particle pore size

For small molecules (<1000 Da), pore sizes of the HILIC stationary phase particles typically are between 80 and 130 Å providing high specific surface areas for interaction (e.g., above 250 m^2/g for a 100-Å pore size particle). However, large molecules (such as intact glycoproteins) are (partially) excluded by narrow pores and hindered in their diffusion. Use of HILIC particles with wider pores (300–1000 Å) is indicated for macromolecules such as larger peptides, protein subunits, and intact proteins. For wide pore materials, the overall surface area is reduced (30–100 m^2/g) and sample loadability is lower. However, for large molecules the area of interaction per molecule is increased, still leading to an increase of retention time as compared to small molecules [130].

Glycans typically have molecular weights below 3 kDa and, as such, do not require the use of wide pore stationary phase particles. This is reflected by the type of pore size used in HILIC for the analysis of released glycans (Fig. 8B). The relatively frequent use of 200-Å materials is somewhat misleading as the numbers result from ZIC-HILIC stationary phases for which the pore size before polymerization of the selector on the surface is reported. The chemical modification significantly reduces the pore size of the original material. For glycopeptides with a molecular weight typically below 6 kDa, materials with various pore sizes have been used, including 300-Å materials. For protein subunits and intact proteins mostly wide pore materials are used, since higher separation efficiencies can be obtained, as indicated above.

4.2.3 Mobile phase

4.2.3.1 Mobile phase systems

In HILIC, ACN-water mixtures are typically used as mobile phase. Solvents such as tetrahydrofuran, acetone, and methanol in mixtures with water have also been applied as HILIC eluents, but these are not widely used, certainly not for the analysis of glycoproteins.

During method development, the mobile phase composition can be used to tune the type and strength of interactions between analytes and the stationary phase, and thus the analyte retention. Buffered solvents by their pH and ionic strength influence the ionization of the analyte, the thickness of the water layer adsorbed on the stationary phase, as well as the strength of the analyte interactions with the stationary phases. Ammonium acetate or formate are used almost exclusively as mobile phase buffer for glycopeptide and glycan analysis by HILIC-MS (Fig. 8C). Salt concentrations can vary between 10 and 200 mM, but are most often kept below 50 mM, and the pH is typically adjusted to 4.4. Notably, ammonium formate is preferred in

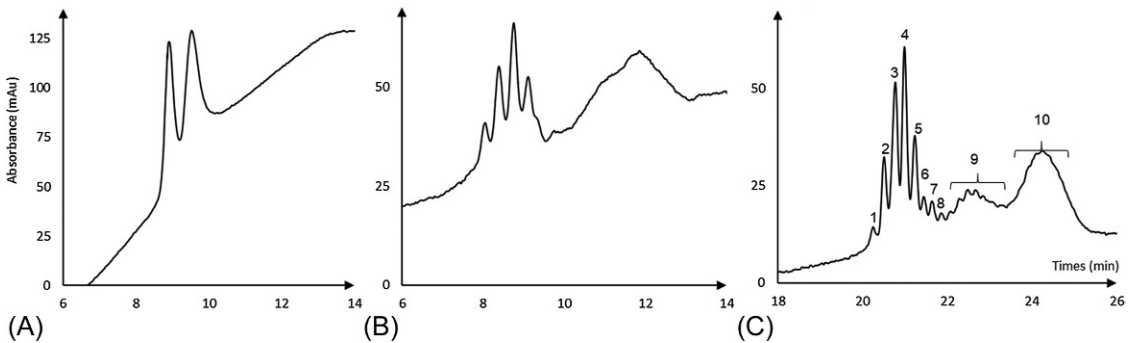


FIG. 9 HILIC of recombinant urinary human chorionic gonadotropin using stationary phases based on (A) diol, (B) anion exchange, and (C) amide chemistry. Chromatographic conditions: (A) Acclaim Mixed-Mode HILIC-1 column (150×2.1 mm, 3 µm, 120 Å). Gradient: 90% ACN for 3 min and subsequently from 90% to 60% ACN with a slope of 4.5%/min. (B) GlycanPac AXH-1 column (150×2.1 mm, 3 µm, 120 Å). Gradient from 85% to 40% of ACN with a slope of 1.5%/min. (C) Accucore Amide column (150×2.1 mm, 2.6 µm, 150 Å). Gradient: from 85% to 40% of ACN with a slope of 1.5%/min. In all cases mobile phase A was ACN with 0.1% TFA and mobile phase B was water with 0.1% TFA. *Reproduced with permission from J. Camperi, V. Pichon, T. Fournier, N. Delaunay, First profiling in hydrophilic interaction liquid chromatography of intact human chorionic gonadotropin isoforms, J. Pharm. Biomed. Anal. 174 (2019) 495–499, doi:10.1016/j.jpba.2019.06.014.*

combination with amide stationary phases for analyses at the glycan and glycopeptide level. Ammonium acetate is exclusively used with zwitterionic stationary phases in released glycan and glycopeptide analysis.

The work of Ahn et al. [38] clearly demonstrates the effect the buffer concentration can have on the HILIC retention time and selectivity of 2-AB labeled glycans (Fig. 10). For higher ammonium formate concentrations in the eluent, longer retention times of the glycans were observed. This was attributed to an enhanced water adsorption to the stationary phase causing an increased contribution of partitioning to retention. This is in accordance to what was described in other works [11, 131]. Interestingly, upon increasing the buffer concentration, the authors also observed a change in relative elution of glycans bearing sialic acids with respect to neutral glycans. This was explained by the reduction of the ionic repulsion between sialic-acid bearing glycans and silanols on the stationary phase at higher buffer concentration.

Mobile phases containing negatively charged ion-pairing agents, such as trifluoroacetic acid (TFA), are used in HILIC analysis of glycopeptides and, in particular, of intact glycoproteins and subunits thereof. (Fig. 8C). The shielding of positively charged protein sites by, for example, the TFA ions is essential to improve efficiency and enhance glycoform separation. Periat et al. [101] showed this for the HILIC analysis of RNase B using an amide stationary phase in combination with a mobile phase containing formic acid (0.5% v/v), ammonium formate (50 mM), and TFA (0.1% v/v) (Fig. 11). Use of formic acid and ammonium acetate in the eluent resulted in late elution and poor resolution of the glycoforms of RNase B.

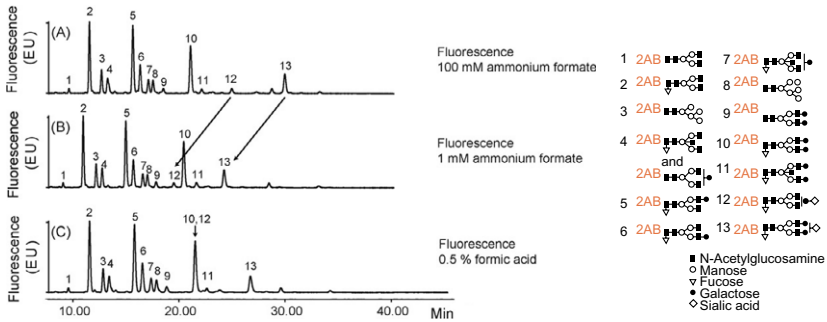


FIG. 10 HILIC-fluorescence analysis of 2-AB labeled IgG glycans using different concentration of ammonium formate (A, 100 mM; B, 1 mM at pH 4.5) and formic acid (C, 0.5% v/v) and an amide stationary phase. Chromatographic conditions: A Waters ACQUITY UPLC BEH Glycan column (150×2.1 mm, 1.7 µm, 130 Å) was used. A linear gradient from 72% to 62% ACN in 15 min was applied. Flow rate and temperature were 400 µL/min and 60°C, respectively. *Modified from J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 µm sorbent, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 403–408, doi:10.1016/j.jchromb.2009.12.013.*

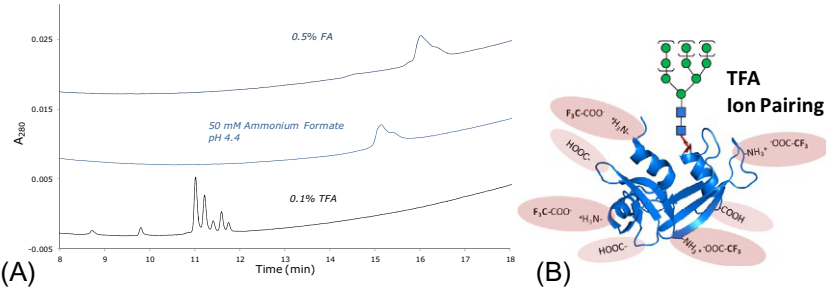


FIG. 11 (A) HILIC analysis of RNase B when using various mobile phases (FA, formic acid; TFA, trifluoroacetic acid) in combination with a column packed with 1.7 µm amide-bonded, wide pore (300 Å) silica particles. (B) Schematic representation of ion pairing of TFA with a glycoprotein inducing reduced hydrophilicity. Chromatographic conditions (A): linear gradient of 20 min gradient from 80% to 20% ACN using different mobile phase additives as indicated. Flow rate and temperature were 200 µL/min and 80°C, respectively. *Reproduced with permission from A. Periat, S. Fekete, A. Cusumano, J.-L.L. Veuthey, A. Beck, M. Lauber, D. Guillarme, Potential of hydrophilic interaction chromatography for the analytical characterization of protein biopharmaceuticals, J. Chromatogr. A. 1448 (2016) 81–92, doi:10.1016/j.chroma.2016.04.056.*

The combination of formic acid (0.1% v/v) and ammonium acetate (100 mM) in the mobile phase also did not provide glycoform separation [126]. However, when adding 0.1% v/v TFA to the eluent, the retention of intact RNase B significantly decreased and almost baseline resolution of its five main glycoforms was achieved. This could be attributed to the reduced overall hydrophilicity of the protein when binding TFA molecules (Fig. 11B). This observation is in line with other reports [86] on glycopeptide separations indicating that under ion-pairing conditions, the retention and glycoform resolution of glycoproteins on a HILIC column is primarily driven by the glycan composition of the protein.

Furuki et al. [86] reported a systematic investigation of the effects of mobile phase composition on the HILIC separation of glycopeptides from RNase B, trastuzumab, human fibrinogen, and CTLA4-lg. Various buffer salts and ion-pair agents (cationic and anionic) were tested in combination with both amide and zwitterionic HILIC stationary phases. In line with the observations made above for intact proteins, they concluded that anionic ion-pairing additives bind to the positively charged moieties of the basic amino acids of the glycopeptide, minimizing the nonglycospecific polar interactions of the peptide with the stationary phases. This results in a general decrease of peptide retention, but which is more pronounced for the nonglycosylated peptides. The retention decrease is larger when using more hydrophobic ion pairing agents, such as TFA, pentafluoropropionic acid, heptafluorobutyric acid, and nonafluorovaleric acid. The retention and resolution of glycopeptides seems hardly affected (only for the glycopeptides of CTLA4-Ig) by the concentration of ion pairing agents in the tested range of 5–20 mM.

Mobile phase conditions not only influence the chromatographic separation (retention and selectivity), but also may have a significant impact on the MS response of the analytes. For example, HILIC-MS employing TFA-containing eluents is less sensitive than RPLC-MS using formic acid as eluent additive, with fold differences up to 10 between the two techniques [129]. In particular, salts and ion pairing agents—which often are needed to achieve high separation performances—cause ionization suppression and/or adduct formation during electrospray ionization, reducing the sensitivity of the HILIC-MS methods [132]. Therefore, method optimization often leads to a compromise between conditions that either allow for enhanced chromatographic resolution or for high MS sensitivity. In the case of HILIC-MS of glycans and glycopeptides, mostly ammonium formate or acetate are used as buffer additives to the eluent. In order to prevent significant loss of sensitivity, the concentration of buffer in the mobile phase is minimized. When using amide HILIC phases, a volatile acid, such as formic acid, is normally added to the eluent. An example of the different responses obtained for released glycans is reported in Fig. 12A where exchanging ammonium formate for formic acid improved the MS signal response with a factor of 3–5 [38]. For the HILIC analysis of protein subunits and intact glycoproteins, the use of ion pairing agent is often essential to achieve good glycan-based selectivity. The most commonly adopted mobile phases for these analyses contain 0.1% v/v TFA, which still allows ESI-MS but at compromised sensitivity. Lowering the concentration of TFA can partly circumvent this loss of sensitivity, but at the expense of separation efficiency (Fig. 12B). Several strategies have been tested to reduce ion suppression in HILIC-MS by TFA. These include the use of a high in-source collision energy [90], the use of dopant gas during ESI [92] to enhance protein ionization and/or reduce TFA adduct, the (partial) substitution of TFA with difluroacetic acid (DFA) [90] (Fig. 12B) or propionic acid [97], or postcolumn exchange of TFA.

4.2.3.2 Gradient elution

In HILIC of glycans, glycopeptides, and intact glycoproteins, analyte elution is usually carried out with relatively shallow linear ACN-water gradients (gradient slope less than 1% v/v water per min). HILIC-MS methods for glycans and glycopeptides are often designed to resolve glycoforms of various glycoproteins at the same time, and therefore frequently employ relatively wide gradient windows. In bottom-up glycoproteomics methods a large portion of the separation window is also used to resolve nonglycosylated peptides, allowing to gain sequence information by tandem MS from the individual peptides composing the protein. The glycopeptides elute in the last portion of the HILIC gradient window, typically separated from the nonglycosylated peptides. For glycoform analysis of protein subunits and intact proteins relatively narrow and shallow gradients are

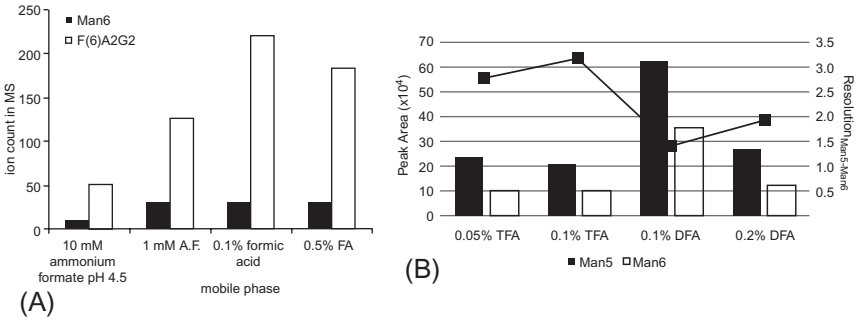


FIG. 12 (A) Comparison of the MS signal intensity of 2-AB labeled IgG glycans when using 100 mM ammonium formate, 1 mM ammonium formate, and 0.1% and 0.5% formic acid in the mobile phase. (B) Peak area (bars, left axis) and resolution (■, right axis) of the mannose-5 and mannose-6 glycoforms of RNase B obtained during HILIC-MS using 0.05%–0.1% TFA and 0.1%–0.2% DFA as mobile phase additive. *Reproduced with permission from (A) J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 403–408, doi:10.1016/j.jchromb.2009.12.013 and (B) E. Domínguez-Vega, S. Tengattini, C. Peintner, J. van Angeren, C. Temporini, R. Haselberg, G. Massolini, G.W. Somsen, High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry, Talanta 184 (2018) 375–381, doi:10.1016/j.talanta.2018.03.015.*

essential to obtain glycoform resolution. As the optimum gradient is glycoprotein dependent, method optimization has to be performed for each protein species. Van Schaick et al. demonstrated that computer-aided optimization can effectively facilitate this process [94].

An inventory of the various gradient profiles (width and slope) that have been used in mAb analysis for glycan, glycopeptide, mAb subunit, and intact mAb by HILIC using amide stationary phases is shown Fig. 13. These data confirm the statements made above. For the glycopeptide analysis, wider and more steep gradients are used, whereas for the analysis of released glycans, mAb subunits and intact mAbs, shallow gradients over narrower elution windows are used. Notably, when changing the gradient slope or initial solvent strength in the analysis of 2-AB labeled released glycans, the separation selectivity may change. Ahn et al. [38] attributed this to the "irregular sample" behavior of certain glycan structures. This effect has not been described for glycopeptide and glycoprotein analysis using gradient HILIC. When comparing gradient windows, it shows that released glycans need a higher percentage of water for elution compared to glycopeptides and subunits. This indeed points toward the effect that the increasing size of the protein backbone might have on the elution. The analysis of intact antibodies seems to deviate from the trend; however, the underlying cause is not well-described in literature.

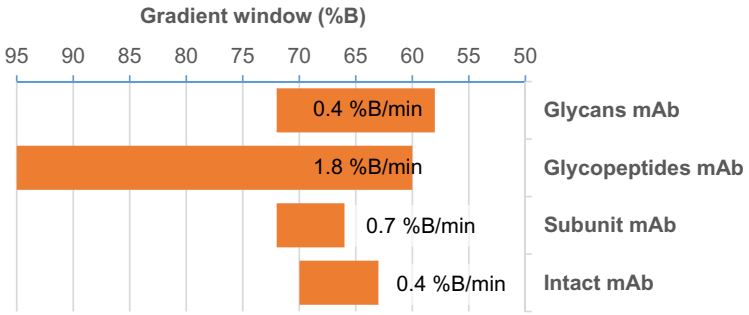


FIG. 13 Bar chart representing the gradient window and steepness used for the analysis of mAbs at the glycan, glycopeptide, subunit and intact level. Results are compiled from the analysis of mAbs using amide HILIC stationary phases (150×2.1 mm) in combination with mobile phases of water (solvent A) and ACN (solvent B) comprising 0.05% v/v for TFA for glycans [69] and 0.1% v/v TFA for glycopeptides [12], subunits [88], and intact protein [118]. The gradient time was 35, 20, 12, and 20 min for the analysis at the glycan, glycopeptide, subunits, and intact level, respectively.

4.2.4 Injection conditions

In order to avoid significant contribution of injection to band broadening, the sample should be dissolved in (starting) mobile phase or in an eluent-miscible solvent of weak elution strength. Hence, the injection solvent in HILIC should contain a relatively high percentage of ACN (i.e., a low percentage of water (strong solvent)). This is typically no problem in the analysis of labeled (e.g., with 2-AB) released glycans, where the derivatization agent increases the lipophilicity of the glycans and promoting their solubility in high percentages of ACN (e.g., 96% v/v [133]). However, for the complete dissolution of glycopeptides, glycoprotein subunits and intact glycoproteins, solvents comprising 20%–50% v/v water are often used.

When using too high percentages of water in the injection solvent in HILIC, there is a distinct danger that peak distortion and/or analyte breakthrough may occur (Fig. 14). This effect is even more prominent for larger injection volumes. Kozlik et al. described the effect of the injection solvent composition (60%–80% v/v ACN) on the analysis of a glycopeptide of hemopexin using low flow HILIC-MS. The injection of 500 nL sample in 60% v/v ACN on a 75 µm×150 mm column resulted in splitting of the glycopeptide peak (Fig. 14A) [84]. This splitting could be circumvented by injecting samples in 80% v/v ACN. Such ACN-rich solvent might work for (glyco)peptides, however, for protein subunits or intact proteins this is often not possible. Especially when keeping samples over a prolonged time (e.g., 1–2 days), high ACN percentages in the solvent may result in protein denaturation and precipitation, and therefore intact

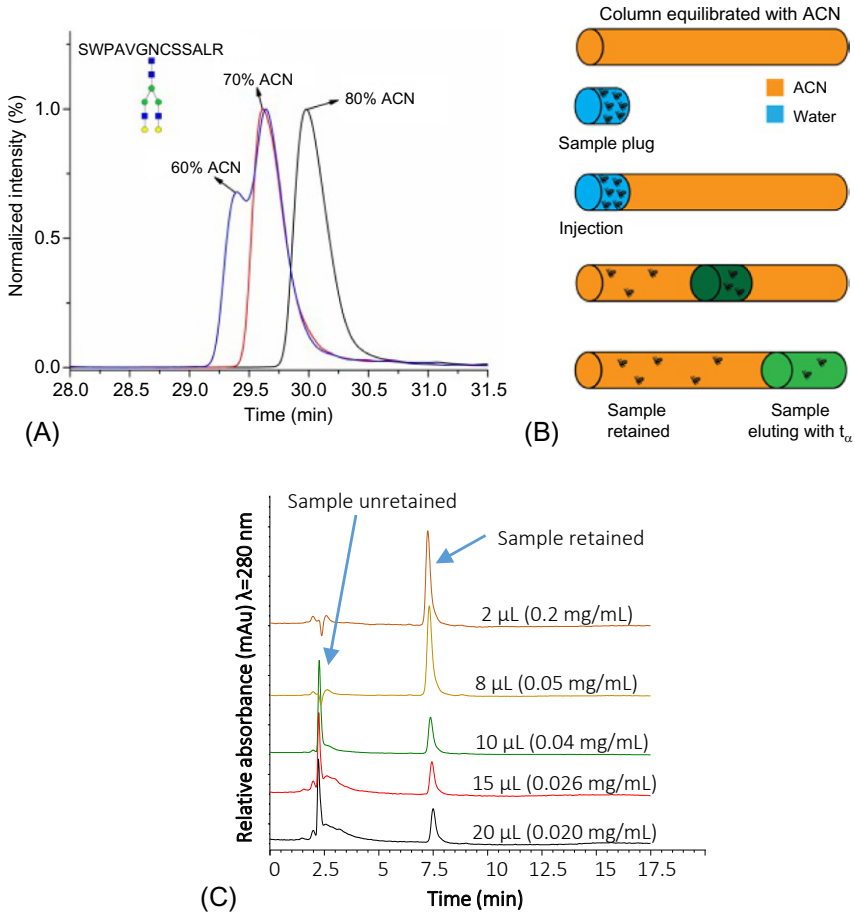


FIG. 14 (A) HILIC-MS chromatograms showing the influence of the ACN content of the sample solvent on chromatographic behavior of the A2G2 glycoform of the SWPAVGNCSSALR peptide of hemopexin. (B) Schematic representation of analyte breakthrough in HILIC. (C) HILIC chromatogram showing the effect of the injection volume—while keeping the injected same protein amount—on the breakthrough of intact transferrin. The protein was dissolved in 5% v/v ACN in water. Chromatographic conditions for panel (A): A Eksigent cHiPLC column HALO HILIC column (75 µm × 150 mm, 2.7 µm) was used. The optimized gradient program was [(min)/% B]: 0/85, 40/55, 45/20, 46/85, 60/85. The mobile phases consisted of 0.1% formic acid in 2% acetonitrile (mobile phase A) and 0.1% formic acid in 100% acetonitrile (mobile phase B). The flow rate of the mobile phase was maintained at 0.5 µL/min. The injection volume was 500 nL, samples were thermostated at 15°C and column temperature was 30°C. Chromatographic conditions for panel (B): A Waters Acquity UPLC glycoprotein Amide (150 × 2.1 mm, 1.7 µm, 300 A) was used. Mobile phase A was 98% ACN with 2% water and 0.1% TFA, whereas mobile phase B was 10% 2-propanol with 2% ACN and 0.1% TFA. A linear gradient of 15 min from 10% to 50% B was applied. Flow rate and temperature were 200 µL/min and 60°C, respectively. *(A) Reproduced with permission from P. Kozlik, M. Sanda, R. Goldman, Nano reversed phase versus nano hydrophilic interaction liquid chromatography on a chip in the analysis of hemopexin glycopeptides, J. Chromatogr. A. 1519 (2017) 152–155, doi:10.1016/j.chroma.2017.08.066. (B) Illustration created by the authors for this publication. (C) Reproduced with permission from A.F.G. Gargano, L.S. Roca, R.T. Fellers, M. Bocxe, E. Domínguez-Vega, G.W. Somsen, Capillary HILIC-MS: a new tool for sensitive top-down proteomics, Anal. Chem. 90 (2018) 6601–6609, doi:10.1021/acs.analchem.8b00382.*

protein samples are mostly dissolved in 80%–90% v/v water. As demonstrated by Gargano et al., for several test proteins using an injection solvent of 90% v/v water on an amide HILIC column, part of the protein elute unretained, indicating analyte breakthrough occurs (Fig. 14B and C) [92]. Analyte breakthrough could be circumvented by injecting small sample volumes (e.g., below 1% of the column volume). Another way to avoid injection of too large volumes of strong solvent is to involve a precolumn for trapping protein from highly aqueous samples prior to injection. In this approach a switching-valve setup is used to first concentrate the protein(s) on a small hydrophobic (e.g., C4) trap column, and subsequently, switching it online with the analytical column and desorb the protein using the largely organic HILIC mobile phase. This way, the analyte proteins can be dissolved in high percentages of water (e.g., 95%) and after desorption from the RPLC trap column with weak HILIC solvent, focusing on the HILIC column is attained [92]. This approach allows large sample volumes while it achieves very small actual injection volumes onto the HILIC column. As the approach is accompanied by a significant preconcentration and reduction of injection volume, it is particularly useful for analyzing low concentrations of glycoproteins using capillary HILIC-MS setups. This was recently demonstrated by Sénard et al. who studied the Fc *N*-glycosylation of polyclonal IgGs from human blood [98].

4.2.5 Column temperature

In HILIC, the change of column temperature has a limited effect on the selectivity. High temperatures usually decrease retention as a result of reduced analyte-stationary phase interactions (e.g., hydrogen bonding, polar interactions) and differences in cohesive energy. Still, increasing the separation temperature (e.g., above 40°C) has a favorable effect on chromatographic efficiency because of a reduction of the mobile-phase viscosity and increase of analyte diffusion [102, 121]. Higher column temperatures (>45°C) have been used for the analysis of glycans and glycopeptides, however, most HILIC studies of these compounds employ room temperature (Fig. 8D). In contrast, the analysis of protein subunits and intact glycoproteins has been almost exclusively reported using high column temperatures. Next to achieving better plate numbers, high temperature can promote protein desolvation and reduce adsorption, providing narrower, more symmetrical peaks, and improving the recovery of sample proteins. Bobály et al. [134] studied the effect of column temperature on HILIC separations of IdeS-digested antibodies and antibody-drug conjugates (ADCs). As shown in Fig. 15A, the highest column temperature tested (i.e., 90°C) significantly increased the recovery of the N-terminal heavy chain and light chain of the ADC bentuximab vedotin. For some subunits, recoveries even increased from 0% at 40°C to nearly 100% at 90°C (Fig. 15B).

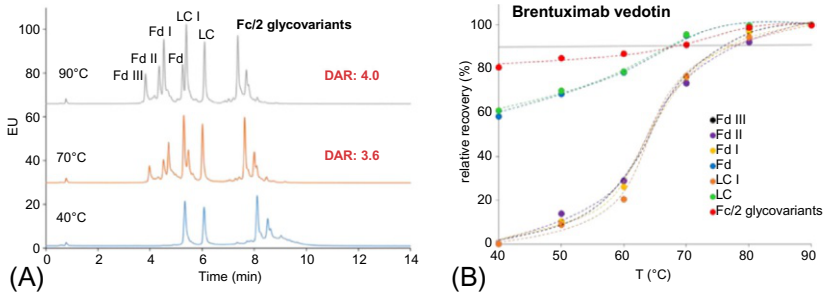


FIG. 15 (A) HILIC chromatograms obtained during the analysis of the antibody-drug conjugate brentuximab vedotin at column temperatures of 40°C, 70°C, and 90°C. (B) Relative recovery of subunits as a function of column temperature indicate that highest recoveries are obtained at the highest temperatures. Chromatographic conditions: A Waters Acquity UPLC Glycoprotein BEH Amide column (150 mm × 2.1 mm, 1.7 µm, 300 Å) was used. Mobile phase A was water with 0.1% TFA and mobile phase B was ACN with 0.1% TFA. A linear gradient from 75% to 60% B in 15 min was applied. A flow rate of 450 µL/min was used. *Modified from B. Bobály, V. D'Atri, A. Beck, D. Guillarme, S. Fekete, Analysis of recombinant monoclonal antibodies in hydrophilic interaction chromatography: a generic method development approach, J. Pharm. Biomed. Anal. 145 (2017) 24–32, doi:10.1016/j.jpba.2017.06.016.*

5 Applications of HILIC-MS for glycoprotein characterization

Fig. 16 shows the application fields of HILIC-MS subdivided by the level of glycoprotein analysis. During initial studies, mostly standard glycoproteins were used to develop methods and find out how to tune/optimize selectivity and detection sensitivity. Typical standard proteins are (followed by molecular weight of protein backbone and glycan type): RNase B (~14 kDa; singly *N*-glycosylated, high mannose), ovalbumin (~43 kDa; singly *N*-glycosylated, high mannose and hybrid (up to 3 kDa)), α-1 acid glycoprotein (~23 kDa, 5–6 *N*-glycosylation sites, complex (up to 20 kDa)). Many glycan analysis methods have also been developed for clinical samples (Fig. 16). The list of tabulated publications (Table 1) shows that most studies focus on profiling of *N*-glycans, which probably is related to the ease of enzymatically releasing *N*-glycans from proteins. This happens almost exclusively by employing PNGaseF. In almost all glycan studies, labeling with 2-AB is applied as a way to increase detection sensitivity.

Glycopeptide, protein subunit, and intact glycoprotein analysis by HILIC-MS has become an established method for biopharmaceutical characterization (Fig. 16), with mAbs being the main type of analytes studied. As mAbs carry *N*-linked glycans, it is not surprising that the majority of glycopeptide studies by HILIC-MS have focused on *N*-glycosylation (Table 2). Only few studies have delved into *O*-glycopeptide characterization. For generation of peptides from glycoproteins, trypsin is generally

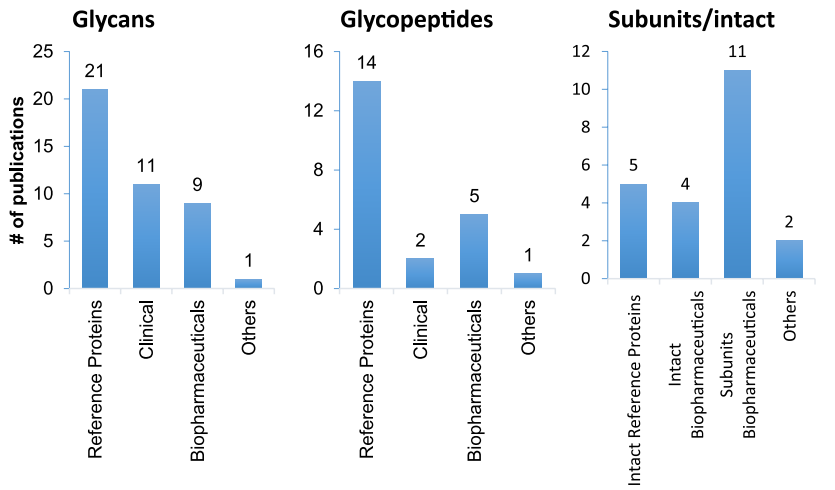


FIG. 16 Bar chart representation of the number of publications versus the application fields for HILIC-MS glycoprotein analysis at the glycan, glycopeptide, and protein subunit/intact protein level. More data on the cited studies is tabulated in Tables 1–3. The number of publications per category can contain multiple sample types (e.g., standard proteins were used for system optimization and a specific target protein was then analyzed) and, therefore, the total score does not resemble the total number of articles analyzed.

used. In cases where generated peptides are too large to analyze or when more protein sequence coverage is needed, other enzymes or enzyme combinations are used. For the generation of subunits from mAbs, the enzyme IdeS has been used exclusively over the last years (Table 3).

This section highlights the application of HILIC-MS for glycoprotein analysis by outlining selected examples from the clinical and biopharmaceutical field. Where possible, the interplay between the analytical levels of glycoprotein characterization will be illustrated. In the final part, some applications where HILIC was used in multidimensional LC of glycoproteins will be treated. A complete overview of recent applications of glycoprotein analysis by HILIC-MS is provided in Tables 1–3. More related information can be found in a few reviews [33, 135], which include protein HILIC studies employing UV detection, glycoprotein enrichment, and comparisons of stationary phases that are not covered in this chapter.

5.1 Comprehensive glycoprotein characterization

So far only few studies have been reported on the comprehensive analysis of glycoprotein in which HILIC-MS was used for glycoprofiling on each level, that is, glycan, glycopeptide, subunit and intact. Largy et al. [51] studied the therapeutic proteins adalimumab, cetuximab, and etanercept. The first two are mAbs bearing merely *N*-glycosylation, whereas the latter

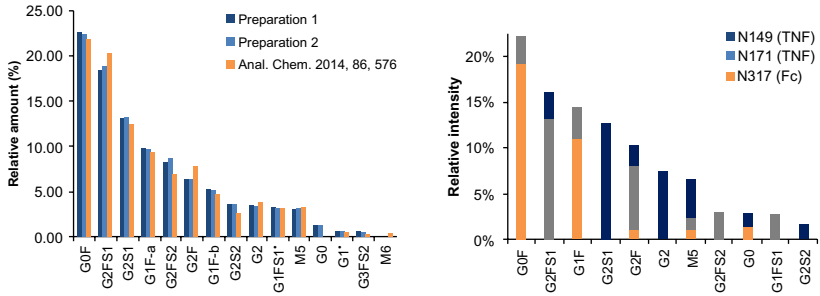


FIG. 17 Relative quantitation by HILIC-MS of (A) *N*-glycans and (B) *N*-glycopeptides of etanercept. In panel (A) two independent sample preparations are compared to published results obtained on a different batch. *Modified from E. Largy, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel, Orthogonal liquid chromatography–mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level, J. Chromatogr. A. 1498 (2017) 128–146, doi:10.1016/j.chroma.2017.02.072.*

is a fusion protein that exhibits both *N*- and *O*-glycosylation. Released *N*-glycan profiling by was achieved with high confidence by HILIC-FLD/MS following 2-AB or RapiFluor-MS labeling (Fig. 17A). Additional information on sialylation was obtained by using a mixed-mode HILIC material, where an anion-exchange component fractionated *N*-glycans. For identification of the *N*-glycosylation sites on the protein backbone a wide pore HILIC amide column was used after tryptic digestion. This approach enabled a complete separation of glycosylated from nonglycosylated peptides, as well as an excellent resolution of the various glycoforms from a given peptide. Fragmentation data from tandem MS provided further confirmation on the presence and identity of glycans. The data obtained from glycopeptide analysis (Fig. 17B) nicely matched and complemented data inferred from released-glycan analysis. Relative quantitation was very consistent for high-mannose or typical mAb glycosylation, however, the authors report their approach was less accurate for sialylated glycans.

The same authors also used HILIC-MS (amide column) in the discovery of the *O*-glycosylation sites of etanercept. For this, the protein was first *N*-deglycosylated and desialylated, in order to generate short glycopeptides carrying desialylated *O*-glycans exclusively. Targeted fragmentation in tandem MS was subsequently performed to determine the *O*-glycosylation sites at the amino acid level. Using this approach, 13 *O*-glycosylation sites of etanercept were identified. The same amide column was subsequently used to identify *O*-glycosylation after separation of protein subunits. Crucial was the addition of *N*-deglycosylation and reduction steps prior to HILIC-MS analysis. The *O*-glycosylated subunit was well-separated from other subunits allowing the unequivocal assignment of 31 different *O*-glycoforms.

A similar characterization study by HILIC-MS, but limited to trastuzumab, was published by D'Atri et al. [12]. As this protein only carries *N*-glycosylation on one specific site on the heavy chains, less complicated sample preparation was required. *N*-Glycans were released using PNGaseF and labeled with either 2-AB or procainamide. While reductive amination with 2-AB is well adapted in many laboratories, it suffers from providing limited MS sensitivity. It was shown that by using procainamide, a slightly higher fluorescence (factor 1.5) and a 50-fold better ESI efficiency was obtained. The latter could be explained by the high proton affinity of the tertiary amine moiety of procainamide. Glycopeptides and mAb subunits were generated using trypsin and IdeS, respectively. Interestingly, the amide HILIC column used for glycan profiling also yielded good separations of glycoforms on both the peptide and subunit level. This was achieved by finetuning the eluents and gradient slope and width. Finally, they showed that the glycoprofiles obtained on the different analytical levels were corresponding well and could be successfully employed to compare originator and biosimilar mAb products, to evaluate batch-to-batch consistency, and for mAb production process optimization.

5.2 Clinical applications

Glycan profiling by HILIC-MS is most established for clinical samples. In general, the focus in these studies is on disclosing the total glycosylation of serum proteins or, more specifically on IgG glycosylation in plasma or serum samples (Table 1). Initial studies in this field focused on the release of glycans from proteins [37] and on glycan labeling compatible with MS detection [41, 42, 54], paving the way for the application of HILIC-MS. Besides standard proteins, these studies comprised clinical samples for which over 40 different glycan structures could be easily identified [37]. These studies mainly dealt with *N*-glycans; only one study did present a workflow for the simultaneous release and labeling of *O*- and *N*-glycans, allowing for rapid glycomic analysis by HILIC-MS [54]. For this, glycoproteins were incubated with 1-phenyl-3-methyl-5-pyrazolone (PMP) in aqueous ammonium hydroxide, releasing *O*-glycans from protein backbones by β-elimination, and *N*-glycans were liberated by alkaline hydrolysis. The released glycans were promptly in situ derivatized with PMP, preventing glycan peeling degradation almost entirely.

Optimized *N*-glycomic HILIC-MS platforms have been used to study clinical conditions, such as, pathogenesis of plasma cell disorders [50] and pregnancy-associated changes [48]. These studies often employed a comprehensive approach, combining several analytical techniques. For the study of plasma cell disorders, the authors used HILIC, exoglycosidase digestions, weak anion-exchange chromatography, and MS to obtain

in-depth polyclonal IgG *N*-glycosylation profiles from 35 patients. This allowed mapping of glycan structures in distinct disease phases of multiple myeloma. Over 50 glycans were structurally identified and quantified using this combined approach. Using principal component analysis (PCA), glycotraits that distinguish disease subgroups could be identified, suggesting that these glycans could act as markers of disease progression and early relapse. A similar approach enabled the detection of pregnancy-associated changes of IgG and serum *N*-glycosylation in camels [48]. *N*-Glycans were released by PNGaseF, labeled with 2-AB, and analyzed by HILIC-FLD/MS. Complex multipeak chromatograms were obtained with several overlapping structures (Fig. 18A) that could only be structurally identified using exoglycosidase sequencing. In serum, IgG *N*-glycans dominated the glycan profile and presented a mixture of core- and noncore-fucosylated *N*-glycans containing either *N*-glycolylneuraminic or *N*-acetylneuraminic acid. The heat maps of the derivatized glycosylation traits summarize the distinct and breed-independent glycosylation differences between pregnant and nonpregnant camels (Fig. 18B). Hierarchical clustering of the samples reinforced the potential of glycans to be used as pregnancy biomarkers, as two clusters, one composed of pregnant and one composed of nonpregnant camels, was obtained.

Variation in the human salivary *O*-glycome has been studied in depth by Kozak et al. [49]. Saliva is an interesting biofluid for potential glycan biomarker discovery, as its collection is easy and noninvasive. Saliva is rich in glycoproteins which are secreted from the bloodstream or produced by salivary glands. Mucins, which are highly *O*-glycosylated proteins, are particularly abundant in human saliva. The authors analyzed and compared *O*-glycans released from whole human mouth saliva collected three times a day from a healthy individual over a 5 days period. *O*-Linked glycans were released by hydrazinolysis, labeled with procainamide and analyzed by HILIC-MS. The sample preparation method showed good reproducibility allowing them to identify up to 27 different *O*-glycans. Furthermore, the data demonstrated that *O*-glycosylation in human saliva changes significantly during the day. The authors advice to take this observation into account when evaluating salivary *O*-glycosylation, for example, for its diagnostic biomarker potential.

In all discussed applications so far, the occurrence of glycan isomers was not considered. Gimenez et al. included isomer selectivity in their study design by selecting a ZIC-HILIC column for probing AGP *N*-glycans [66]. They could distinguish at least two isomers for AGP released glycans carrying sialic acids. These isomers most probably arise from co-occurring 2,3- and 2,6-linkage of the terminal sialic acids (Fig. 19). In two follow-up studies, the authors implemented exoglycosidase treatment [67] as well as analysis of ion fragments with higher diagnostic value obtained by tandem MS [68] as ways to unequivocally assign glycan structures released

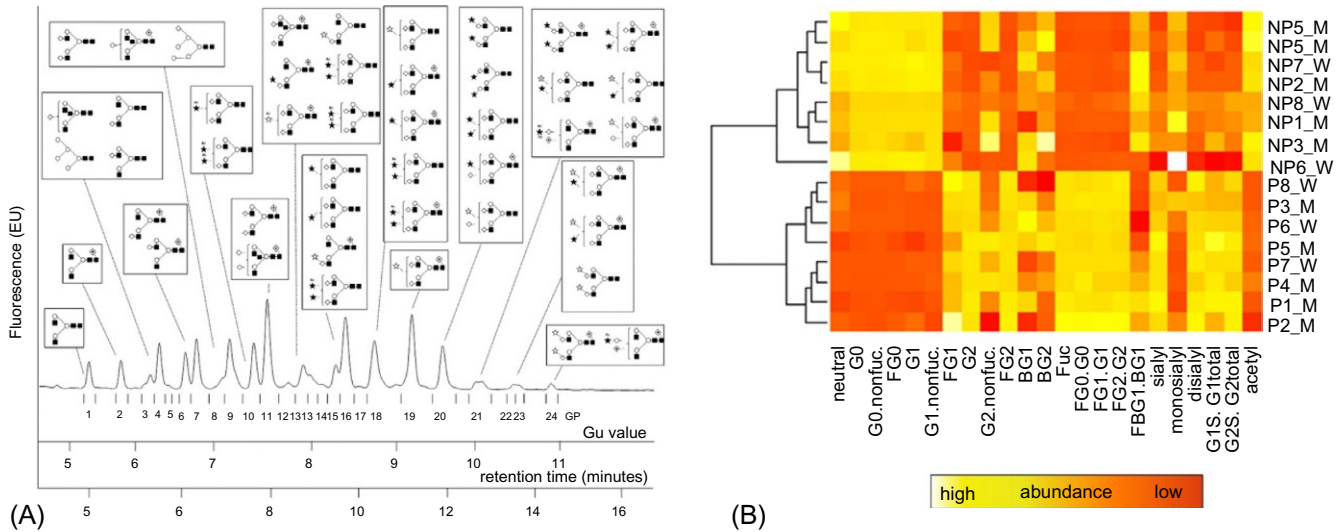


FIG. 18 (A) HILIC-fluorescence detection separation obtained upon the analysis of *N*-linked glycans released from camel serum. Glycan structures are indicated at the chromatographic peak. (B) Heat map of derivatized glycan traits from camel serum, including hierarchical clustering of samples from pregnant (P; $n=8$) and nonpregnant (NP; $n=8$) camels of the Majaheem (M; $n=10$) and Wadha (W; $n=6$) breed. *Light color*, relative high abundance of derivatized glycan trait; *dark color*, relative low abundance of derivatized glycan trait. Chromatographic conditions for panel (A): A Waters BEH Glycan column (150×1 mm, 1.7 μm) was used. Mobile phase A composed of a 50 mM ammonium formate buffer pH 4.4 and mobile phase B was aCN. The method used a 30 min a linear gradient of 24.81 min between 70% and 53% of mobile phase B. A flow rate and temperature of 560 μL/min and 60°C were used, respectively.*Modified from B. Adamczyk, S. Albrecht, H. Stöckmann, I.M. Ghoneim, M. Al-Eknah, K.A.S. Al-Busadah, N.G. Karlsson, S.D. Carrington, P.M. Rudd, Pregnancy-associated changes of IgG and serum N-glycosylation in camel (Camelus dromedarius), J. Proteome Res. 15 (2016) 3255–3265, doi:10.1021/acs.jproteome.6b00439.*

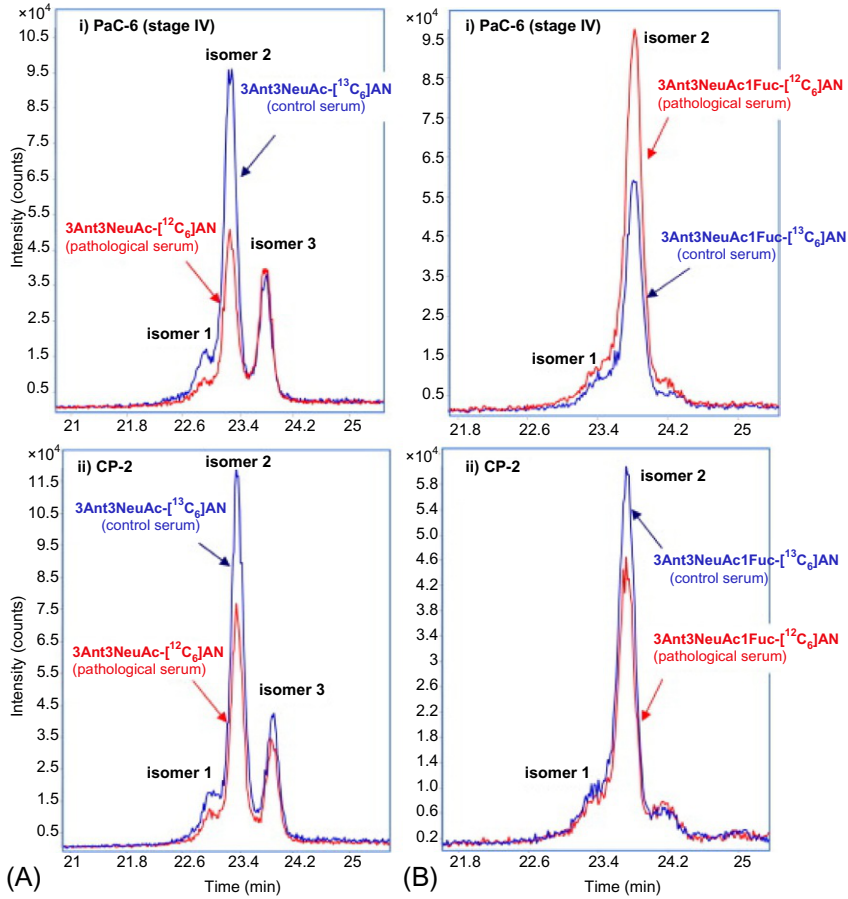


FIG. 19 ZIC-HILIC-MS analysis of a mixture aniline-labeled *N*-glycans released from control AGP and pathological hAGP. Traces represent extracted-ion chromatograms obtained for the (A) 3Ant3Neu5Ac glycan and (B) 3Ant3Neu5Ac1Fuc glycan. Control serum is indicated in *blue* (*dark gray* in print version), AGP from pathological serum in *red* (*light gray* in print version). (i) Sample with pancreatic cancer with stage IV (PaC-6) and (ii) sample with chronic pancreatitis (CP-2) as depicted. Chromatographic conditions: A SeQuant ZIC-HILIC column (150×0.3 mm, 3.5 µm) was used. Mobile phase A consisted of 10 mM NH_4Ac solution and mobile phase B was ACN. Gradient conditions were used: solvent B from 90% to 50% within 40 min as linear gradient, followed by cleaning and equilibration steps of B: 50%→0% (within 5 min), 0% (over 10 min), 0→90% (within 5 min) and 90% (over 10 min). A flow rate and temperature of 4 µL/min and 25°C were used, respectively. *Modified from E. Giménez, M. Balmaña, J. Figueras, E. Fort, C. de Bolós, V. Sanz-Nebot, R. Peracaula, A. Rizzi, Quantitative analysis of N-glycans from human alfa-acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis, Anal. Chim. Acta. 866 (2015) 59–68, doi:10.1016/j.aca.2015.02.008.*

from AGP. Indeed, the linkage-specific attachment of sialic acids as well as fucoses was confirmed. This resulted from an initial assignment of about 25 glycans (including isomers) to over 50 different glycan structures identified. Interestingly, the authors also applied their method to probe AGP glycan profiles as a tool for pancreatic disease diagnosis. Their analyses showed that pancreatic cancer samples had a clear increase in AGP fucosylated glycans (Fig. 19B). Moreover, the difference in certain glycan isomers was occasionally evident (Fig. 19A).

As indicated earlier, glycans can have many isomeric species. Huang et al. evaluated the potential of HILIC-MS to resolve glycan isomers by analyzing glycopeptides rather than released glycans [81]. Using digested fetuin as model system, they observed that the glycopeptides were retained longer on the applied HILIC column than the corresponding labeled glycans (Fig. 20A). However, the presence of the peptide backbone did not significantly alter the relative retention of the different glycoforms with respect to the order obtained for the released glycans, as shown by a strong similarity of the HILIC profiles of the glycopeptides and the glycans. The achieved glycopeptide resolution was more than sufficient to distinguish isomeric *N*-glycan structures, such as sialylated *N*-glycan isomers differing in α2–3 and α2–6 linkages. For unambiguous isomer assignment, their separation prior to MS detection is required. The discovery of the ability of HILIC to resolve glycopeptides with different glycan linkages led to the investigation of tryptic digests of human serum IgGs, which occur in four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4). As IgGs only have one *N*-glycosylation site, a targeted analysis of the glycopeptide was performed. Interestingly, the amino acid sequence of the glycopeptide is distinct for IgG2/3 (EEQFNSTFR), IgG4 (EEQFNSTYR), and IgG1 (EEQYNSTYR); the difference only being a phenylalanine (F) to tyrosine (Y) substitution at one or two locations. As Y is more hydrophilic than F, IgG2/3 has the more hydrophobic amino acid sequence, IgG4 has the intermediate hydrophobicity, and IgG1 is the most hydrophilic, explaining their HILIC elution order (Fig. 20B). Within one IgG subclass, the HILIC separation was driven by the glycan portion with larger glycans eluting last. Remarkably, also here a separation of isomeric glycoforms was obtained. This is clearly illustrated by the presence of two peaks detected for the glycopeptide comprising the G1 (Hex4-HexNAc4Fuc1) glycan. Overall, the method allowed to detect at least 10 different glycoforms on each individual IgG subtype within a run time of 30 min (Fig. 20C).

In the only HILIC-MS report so far dealing with subunit/intact protein characterization in a clinical setting, Sénard et al. characterized the concomitant presence of sequence variance (allotype distributions) and glycoforms in the Fc portion of serum IgG [98]. Human plasma IgG was isolated using Fc-specific beads followed by an on-bead digestion with the enzyme IdeS. The obtained mixture of Fc subunits was analyzed by

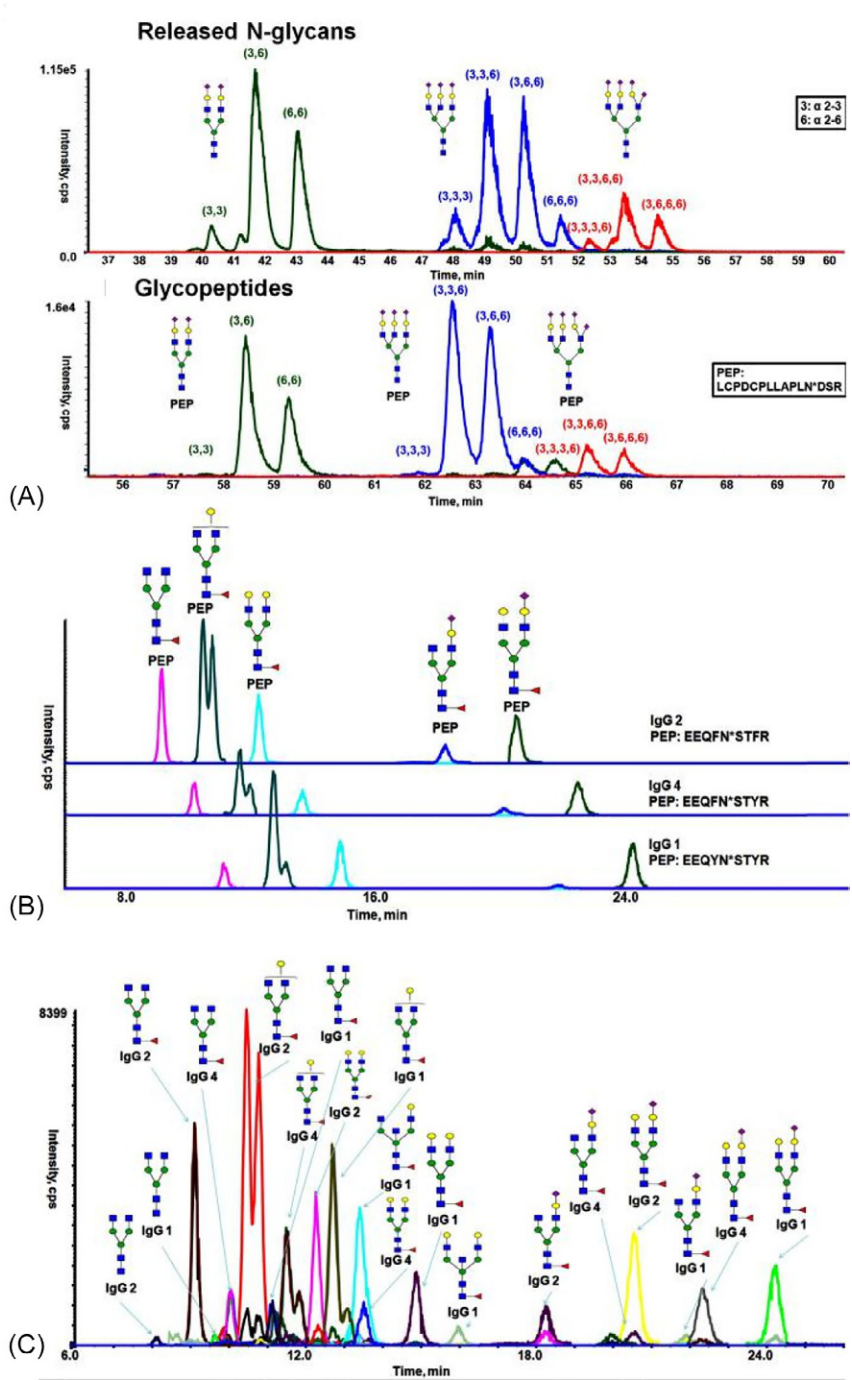


FIG. 20 (A) Comparison of the isomeric separation obtained from released *N*-glycans and glycopeptides of fetuin by HILIC. Numbers indicate the glycan linkage; 3, α2,3 linked; 6, α2–6 linked. (B) HILIC separation of major *N*-glycopeptides in IgG_1, IgG_2, and IgG_4.

(Continued)

capillary electrophoresis (CE) and HILIC hyphenated with MS. CE-MS provided separation of different IgG-subclasses and allotypes, while HILIC-MS allowed resolution of the different glycoforms and their oxidized variants. The orthogonality of these techniques was key to reliably assign Fc allotypes. Five individual donors were analyzed using this approach. Heterozygosis was observed in all the analyzed donors resulting in a total of 12 allotypes identified. The assignments were further confirmed using recombinant monoclonal IgG allotypes as standards. While the glycosylation patterns were similar within allotypes of the same subclass, clear differences were observed between IgG subclasses and donors, highlighting the relevance of the proposed approach.

5.3 Biopharmaceutical characterization

Over the past 20 years, a large number of new biopharmaceuticals have been introduced for the treatment of serious diseases. Many biopharmaceuticals are glycoproteins, carrying oligosaccharides that are attached posttranslationally. Consequently, glycosylation is a major source of protein drug heterogeneity. Recently, quite some studies have reported the characterization of glycosylated biopharmaceuticals using HILIC-MS. These studies mainly focused on mAbs, analyzing either at the glycan or subunit/intact protein level.

Most commonly, mAb subunit analysis after IdeS-digestion is used to obtain comprehensive glycoprofiles. Addition of a reducing agent after IdeS-digestion results in the formation of the so-called Fc/2, light chain, and Fd fragments. All these units are approximately 25 kDa and can be efficiently separated by HILIC-MS. Hence, a complete picture of the modifications on the different parts of the mAb can be derived. This approach has been used for the characterization of, for example, daratumumab [95], etanercept [96], and infliximab, cetuximab and trastuzumab [88]. These studies aimed to probe either overall glycosylation patterns, differences after stress conditions, or biosimilarity.

FIG. 20, CONT'D (C) Application of HILIC-MS for the analysis of human serum IgGs demonstrating the ability to resolve isomeric glycopeptide. Below the glycan structures the IgG variant is indicated. Chromatographic conditions: A Halo Penta-HILIC columns (150×2.1 mm, 2.7 µm) was used. Mobile phase A consisted of 95% water/ACN with 50 mM ammonium formate (adjusted to pH 4.4 with formic acid) and mobile phase B was ACN. For glycopeptides of fetuin, a linear gradient of 85% mobile phase B to 48% mobile phase B in 75 min was used. For glycopeptides of IgGs in human serum, the following segmented linear gradients were used: (1) 62% mobile phase B to 61.2% mobile phase B in 9 min, (2) 61.2% mobile phase B to 60.2% mobile phase B in 10 min, and (3) 60.2% mobile phase B to 58% mobile phase B in 11 min. In each case, the column was flushed at 25% mobile phase for 5 min before returning to the starting mobile phase composition. A flow rate and temperature of 400 µL/min and 60°C were used, respectively. *Reprinted from Y. Huang, Y. Nie, B. Boyes, R. Orlando, Resolving isomeric glycopeptide glycoforms with hydrophilic interaction chromatography (HILIC), J. Biomol. Tech. 27(3) (2016) 98–104, doi:10.7171/jbt.16-2703-003. Copyright (2016), with permission from Association of Biomolecular Resource Facilities.*

Focusing on the latter, D'Atri et al. demonstrated the utility of HILIC-MS for the qualitative profiling of mAb glycosylation patterns by comparing three originators and biosimilars products using a middle-up (i.e., forming protein subunits prior to analysis) approach [88]. The analysis of cetuximab provides a striking example of the power of HILIC-MS in this field. In contrast to most other therapeutic mAbs which only show Fc *N*-glycosylation, cetuximab exhibits a second *N*-glycosylation site on the variable portion of its heavy chain. Subunit analysis by RPLC-MS showed little to no glycoform separation (Fig. 21A and B) with the MS data revealing the masses of the main glycoforms only. On the other hand, HILIC provides efficient glycoselectivity, revealing a plethora of glycoforms on both the Fc/2 and Fd portions of the mAb (Fig. 21C and D). These detailed profiles allow making useful distinctions among originator and biosimilar products based on comparison of the peak occurrence and intensities of major and minor glycoforms.

Intact mAb analysis by HILIC-MS was used to study product-related impurities other than glycoforms [93]. After PNGaseF removal of *N*-linked glycans, the HILIC method separated mAb-related impurities. Subsequent mass measurements using a high-resolution accurate mass spectrometer allowed direct and unambiguous identification of the low-abundance impurities within a single run. The authors identified free light chain, half antibody, antibody possessing a single light chain, and protein backbone-truncated species. Moreover, they were able to elucidate truncation sites and associated PTMs.

The characterization of mAb glycosylation on the glycan level usually follows a rather standard approach. *N*-glycans are enzymatically released, purified, labeled with 2-AB, and analyzed by HILIC-MS [39, 46, 47, 64]. As mAbs have quite predictable glycoprofiles, the obtained data can be interpreted straightforwardly when the focus is on assigning main glycoforms. However, when unknown glycan structures are present, additional efforts are needed. Zhao et al. [47] elucidated unknowns using MS^n fragmentation in negative ion mode followed by identification of signature D ions. These are typical fragments that result from two glycosidic cleavages and can help in the assignment of both monosaccharide sequence and linkage specificity. The developed method was used successfully to characterize structural isomers of A1G1F (assigned as terminal sialic acid attached in the 1,6 branch at 2,3 position), and A1G1F′ (assigned as terminal sialic acid attached in the 1,3 branch at 2,3 position). Moreover, using the same approach, previously unknown low-abundant species could be identified unambiguously. For example, a glycan previously assigned as a Man8 structure was found to be a glycan comprising seven mannoses and one glucose unit(s). This assignment was confirmed by exoglycosidase treatment to achieve the cleavage of α-1,3 linked terminal disaccharides. Using the developed method, different lots and productions of mAbs were examined. It was found that the newly identified

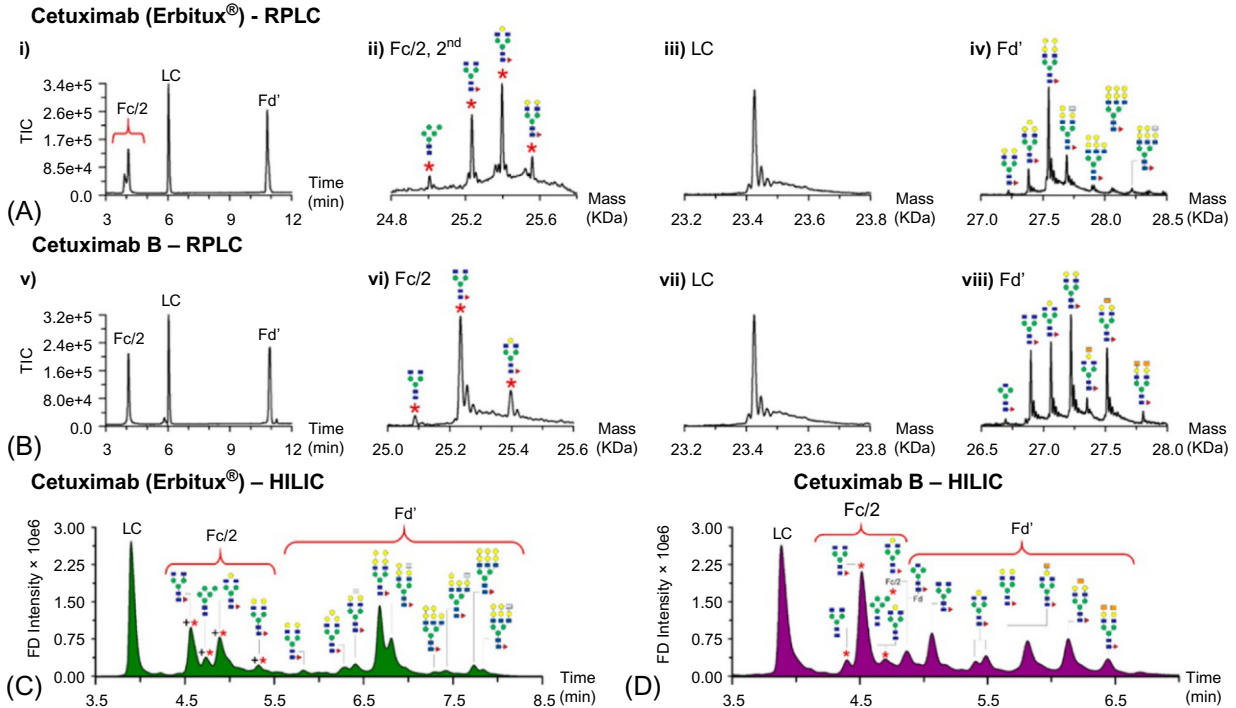


FIG. 21 Middle-up analysis of cetuximab and its biosimilar cetuximab B by RPLC-MS and HILIC-MS. For the RPLC-MS analysis of (A) cetuximab and its (B) biosimilar the total ion chromatograms (i, v) and associated deconvoluted mass spectra of Fc/2 (ii, vi), light chain (iii, vii), and Fd′ (iv, viii) fragments are shown. HILIC-MS analysis of (C) cetuximab and (D) its biosimilar are provided with glycan assignments based on obtained deconvoluted mass spectra. Fc/2 fragment assignments are accompanied by "+" or a *red asterisk* (*dark gray* in print version) depending on whether or not the eluting species contains the C-terminal lysine residue, respectively. Chromatographic conditions: An ACQUITY UPLC glycoprotein amide column (150×2.1 mm, 1.7 µm, 300 Å) was used. Mobile phase A consisted of 0.1% of TFA (v/v) in water, whereas mobile phase B was 0.1% TFA (v/v) in ACN. The gradient went from 85% to 73% B in 0.2 min, then from 73% to 65% B in 10. A flow rate and temperature of 450 µL/min and 80°C were used, respectively. *Reproduced with permission from V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Hydrophilic interaction chromatography hyphenated with mass spectrometry: a powerful analytical tool for the comparison of originator and biosimilar therapeutic monoclonal antibodies at the middle-up level of analysis, Anal. Chem. 89 (2017) 2086–2092, doi:10.1021/acs.analchem.6b04726.*

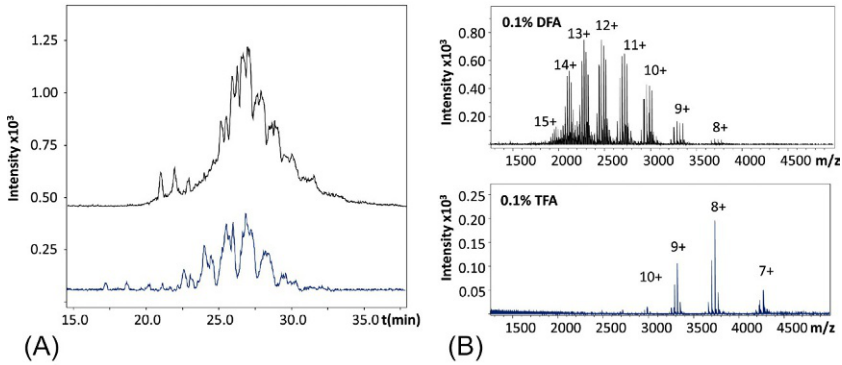
glycan (i.e., Man7 + glucose) was also present in earlier batches and in some commercially available therapeutic mAbs.

Only few HILIC-MS studies have looked into other biopharmaceuticals than mAbs. Erythropoietin (EPO) [91], interferon-β1a [91], and neoglycoproteins [89, 90] have been analyzed by HILIC-MS, mainly on the intact protein level. Neoglycoproteins are an interesting class of compounds, as synthetic saccharides are chemically conjugated to the protein via the lysine residues in a controlled fashion with the goal to be able to define the efficacy of a protein drug by tuning its physicochemical and pharmacological properties. Moreover, these proteins allow systematic study of the effect of glycan type and size on HILIC retention. Tengattini et al. prepared glycoconjugates of the recombinant tuberculosis antigens TB10.4 and Ag85B, and analyzed these by HILIC(-MS) [89, 90]. Proper selection of HILIC column chemistry and optimization of eluent composition and gradient was performed, leading to excellent resolution of the intact glycoconjugates and minimal ionization suppression. The results very clearly indicated that the obtained HILIC selectivity toward protein glycoforms is predominantly governed by the size of the conjugated glycans and/or number of glycans attached. In addition, HILIC-MS/MS allowed assignment of several side products, including conjugation of TB10.4 at its N-terminus.

The biopharmaceutical EPO is strongly and very heterogeneously glycosylated; it comprises three *N*-glycosylation and one *O*-glycosylation site. Recently, HILIC-MS analysis of rhEPO has shown useful for intact glycoprofiling providing a wide pattern of partially resolved peaks (Fig. 22A) [91]. Mass spectra recorded throughout the entire peak profile allowed determination of the masses of the detected glycoforms. Overall, when using TFA as a mobile phase additive, 51 distinct glycoform compositions could be assigned that differed in number of HexHexNAc or sialic acid residues (Fig. 22C). Moreover, due to the efficient HILIC separation, low-abundance glycoforms with only two *N*-glycosylation sites occupied could be discerned. As TFA is known to impair detection sensitivity, the authors also tested DFA as ion pairing agent in the eluent. This resulted in a partial loss of glycoform resolution, but also in a gain in detection sensitivity, overall providing the detection of 21 extra glycoforms (Fig. 22C).

5.4 Multidimensional separations

Over the past couple of years, there has been increasing activity in the development of two-dimensional (2D-)LC methods to improve the performance of protein analyses. HILIC has occasionally been included in 2D-LC workflows probing either released glycans [70] or subunits of mAbs [99, 100]. Deguchi et al. [70] employed HILIC-HILIC-MS to characterize *N*-glycans from human serum proteins. They used a HILIC column with an anion-exchange stationary phase in the first dimension



(C)

	SiA_7	SiA_8	SiA_9	SiA_{10}	SiA_{11}	SiA_{12}	SiA_{13}	SiA_{14}	SiA_{15}
$Hex_{15}HexNAc_{13}Fuc_2$	*25641.4*	25933.3	26223.6	26515.3					
$Hex_{16}HexNAc_{14}Fuc_2$		26297.1	26588.6	26880.6					
$Hex_{17}HexNAc_{15}Fuc_2$		26663.9	26954.9	27245.9					
$Hex_{18}HexNAc_{16}Fuc_2$			*27320.2*	*27611.2*					
$Hex_{18}HexNAc_{15}Fuc_3$	*26681.2*	26972.2	27263.2	27554.2					
$Hex_{19}HexNAc_{16}Fuc_3$	*27046.0*	27337.0	27628.0	27919.0	28210.0				
$Hex_{20}HexNAc_{17}Fuc_3$		27702.0	27993.0	28284.0	28575.0	28866.0	29157.0		
$Hex_{21}HexNAc_{18}Fuc_3$		*28068.0*	28358.7	28649.7	28940.7	29232.0	29523.0		
$Hex_{22}HexNAc_{19}Fuc_3$		*28433.4*	28724.4	29014.7	29305.7	29596.7	29887.7	30179.2	30469.7
$Hex_{23}HexNAc_{20}Fuc_3$			*29089.2*	29380.2	29671.3	29962.4	30253.5	30544.5	
$Hex_{24}HexNAc_{21}Fuc_3$			*29453.7*	29745.5	30036.5	30327.5	30618.7	30909.6	
$Hex_{25}HexNAc_{22}Fuc_3$			*29819.7*	*30109.7*	30401.7	30693.0	30984.0	31275.0	
$Hex_{26}HexNAc_{23}Fuc_3$			*30185.6*	*30476.6*	30767.6	31058.6	31349.6	31640.6	
$Hex_{27}HexNAc_{24}Fuc_3$					*31132.9*	*31423.9*	*31714.9*	*32005.9*	
$Hex_{28}HexNAc_{25}Fuc_3$					*31496.7*	*31789.2*	*32080.2*	*32371.2*	

FIG. 22 HILIC-MS of rhEPO. (A) Base-peak chromatograms and (B) mass spectra recorded in the main peak using 0.1% TFA (bottom traces) or 0.1% DFA (top traces) as mobile phase additive. (C) Molecular mass and assigned glycan composition of EPO glycoforms observed with HILIC-MS employing TFA or DFA as mobile phase additive. Shaded masses are observed using TFA or DFA as additive. Masses printed italic were only observed using DFA. Chromatographic conditions: An Agilent Technologies AdvanceBio Glycan Map column (150×2.1 mm, 2.7 µm) was used. The mobile phases were composed of ACN (mobile phase A) and water (mobile phase B) both containing 0.1% (v/v) TFA or 0.1% DFA (v/v). The gradient program used was: 28%–33% B in 7 min and from 33% to 40% in 28 min followed by 40% B for 10 min. A flow rate and temperature of 500 µL/min and 40°C were used, respectively. *Modified from E. Domínguez-Vega, S. Tengattini, C. Peintner, J. van Angeren, C. Temporini, R. Haselberg, G. Massolini, G.W. Somsen, High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry, Talanta 184 (2018) 375–381, doi:10.1016/j.talanta.2018.03.015.*

and a ZIC-HILIC material in the second dimension, respectively. The first dimension separated released glycans based on sialic acid content, whereas in the second dimension glycans bearing the same number of sialic acids were separated based on neutral glycan differences. Glycan structures carrying up to four sialic acids could be discerned efficiently,

demonstrating the potential usefulness of a 2D-HILIC approach for glycan profiling of complex samples such as human serum.

Comprehensive 2D-LC was applied for the characterization of mAb subunit glycosylation by Stoll et al. [99]. A HILIC separation was performed in the first dimension, whereas RPLC was selected for the second dimension. Next to achieving overall enhanced analyte resolution, this approach also facilitated more sensitive ESI-MS detection by circumventing protein ionization suppression by TFA. The 2D scheme encompassed a switch from the TFA-containing HILIC eluent to the more MS-friendly RPLC eluent containing only formic acid as additive. In order to mitigate the adverse effect of the ACN-rich HILIC mobile phase on the RPLC separation, active solvent modulation (i.e., lowering the elution strength the first dimension effluent has on the second dimension stationary phase) appeared essential and efficient 2D-LC results could be obtained. The 2D-LC approach was applied to the glycoprofiling of IdeS-digested and subsequently reduced obinutuzumab and cetuximab (Fig. 23). The LC and Fd subunits of obinutuzumab are only partially separated by HILIC (Fig. 23A), but the RPLC in second dimension increase their separation. The Fc/2 subunit of obinutuzumab shows a highly resolved profile of glycoforms which, however, have the same retention in RPLC (Fig. 23A and B). The added value of 2D-LC is more apparent for cetuximab, of which both the Fc/2 and Fd subunits are glycosylated. The HILIC glycoform profiles obtained for the two subunits show some overlap (Fig. 23C), but the involved FC/2 and Fd glycoforms could be nicely separated by RPLC. MS detection further enabled the assignment of the separated subunits and glycoforms thereof. In total, over 40 proteoforms could be discerned for cetuximab.

Another multidimensional approach for mAb analysis involving HILIC encompasses a hyphenation of protein digestion and subunit separation [100]. A small column with immobilized IdeS was used in a first dimension to digest mAbs in less than 10 min. Following online reduction on a RPLC column (second dimension), the resulting proteolytic fragments of 25 kDa were analyzed using HILIC-MS (third and fourth dimension). This analytical workflow demonstrated the ability to accurately profile glycosylated variants within a total run time of 95 min. It was subsequently applied for the glycoprofiling of two mAbs. Results were similar to those obtained following a stepwise offline approach, demonstrating the proof-of-concept of the set-up.

6 Conclusion

This chapter provided an overview of the background, practical aspects, current status, and application of HILIC-MS for the analysis of glycoproteins. The combination of the unique selectivity and high separation efficiency of HILIC with mass-selective and sensitive detection has demonstrated to be particularly useful for achieving unsurpassed resolution of

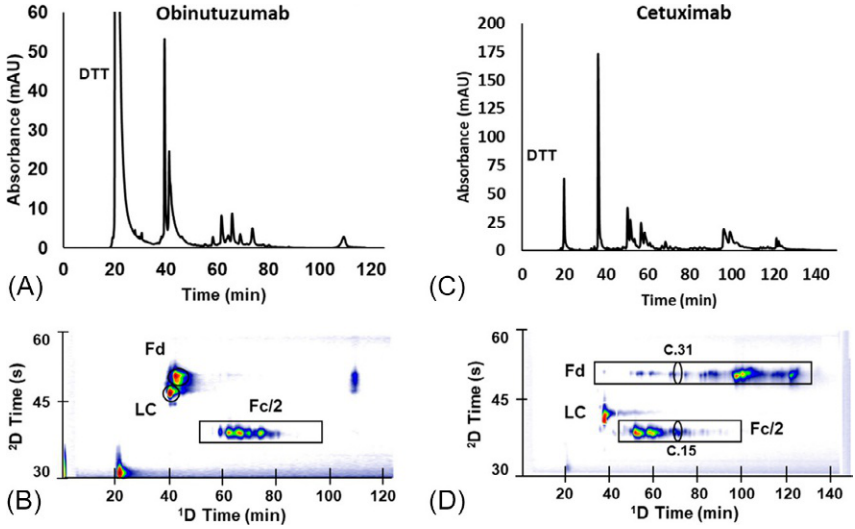


FIG. 23 One dimensional HILIC (A, C) and two-dimensional HILIC×RP (B, D) chromatograms of IdeS-digested and reduced obinutuzumab (A, B) and cetuximab (C, D). *Black rectangles* comprise the peaks of glycoforms observed for the indicated mAb subunit. Chromatographic conditions: First dimension HILIC separations were performed using two Waters Acquity UPLC Glycoprotein Amide columns (150×2.1 mm, 1.7 µm, 300 Å) connected in series. Gradient elution was used in the first dimension, where mobile phase A contained 0.3% (v/v) trifluoroacetic acid in water, and mobile phase B was ACN. The HILIC gradient used was as follows: 69–65.5–65.5–61–61–69–69%B from 0–10–70–112.5–120–120.01–150 min. The column temperature was 40°C, and the flow rate was 40 µL/min. Second dimension reversed-phase separations were performed using an Agilent Technologies column (30×2.1 mm, 5 µm, 1000 Å). Mobile phase A was 0.1% (v/v) formic acid in water, and solvent B was ACN. The column temperature was 75°C, and the flow rate was 1.0 mL/min. In each ^{2}D separation cycle, 40 µL of ^{1}D column effluent was diluted 1:2 (1 part effluent, 2 parts ^{2}D mobile phase) using the in-active solvent modulation interface valve, and the modulation time was 60 s. The ^{2}D gradient elution program was from 2–2–25–37–50–2–2%B from 0–0.41–0.42–0.84–0.92–0.93–1.0 min, where the hold at 2% B for the first 24 s is required for in-line dilution of the injected sample. *Modified from D.R. Stoll, D.C. Harmes, G.O. Staples, O.G. Potter, C.T. Dammann, D. Guillarme, A. Beck, Development of comprehensive online two-dimensional liquid chromatography/mass spectrometry using hydrophilic interaction and reversed-phase separations for rapid and deep profiling of therapeutic antibodies, Anal. Chem. 90 (2018) 5923–5929, doi:10.1021/acs.analchem.8b00776.*

glycomolecules, whether released glycans, glycopeptides, glycosylated protein subunits, or even intact glycoproteins. HILIC-MS has developed into a valuable option in the field of glycoprofiling, where it takes a complementary position with respect to RPLC-MS, in particular for released glycan and protein subunit/intact protein analysis. The field is still in a status of development, a maturing process where both methodological (i.e., more fundamental) studies and application-driven works are being performed. Many studies have focused on condition optimization and on potential pitfalls and good practices of HILIC-MS for all analytical levels

of glycoprotein characterization. The diversity of available HILIC column chemistries in principle provides good possibilities to find suitable approaches for many glycosylation-related queries. For now, it seems that in many cases amide- and zwitterionic-based columns are the preferred starting point for method development.

From an application point of view, it appears that HILIC-MS so far has been used mostly for the characterization of therapeutic glycoproteins and to study protein glycosylation in biological matrices for glycomics purposes. Biopharmaceuticals were mainly analyzed at the protein subunit or intact protein level, whereas glycomics studies mostly involved analysis of released glycans. Interestingly, several studies have shown that both purposes can be served with a single HILIC column chemistry while only modifying the mobile phase conditions. Although separation of glycopeptides is well possible with HILIC-MS, the field of glycoproteomics has not been touched too much by HILIC-MS with regards to real-life applications. Probably the added value of HILIC-MS in gaining glycopeptide information is still insufficient with respect to the routine and robust RPLC-MS-based workflows.

When it comes to future developments, further improvements can be expected in protein HILIC and MS, multidimensional separations, and data analysis. So far, subunit/intact glycoprotein analysis with HILIC-MS could conveniently be applied to proteins up to about 30 kDa molecular weight. The ongoing commercial efforts (driven by biopharma) to adapt column particle porosities and chemistries to larger protein requirements, and the constant advancements made in ionization and MS of high-molecular-weight compounds, will undoubtedly further enhance HILIC-MS performances for proteins. The developments in equipment and strategies for multidimensional LC will further facilitate the incorporation of HILIC in multimodal separations, also improving MS compatibility. Until now, in glycan and glycopeptide characterization often automated data analysis is still lacking, analysts are hampered by tedious and time-consuming manual data handling and structural annotations. The same issues arise when interpretating complex MS data of larger proteins or when multidimensional chromatograms have to be visualized and integrated. It is expected that in the coming years, protein HILIC-MS workflows will benefit from the currently strong developments in data analysis. Dedicated algorithms for data deconvolution and interpretation will facilitate the annotation of complex mass spectra originating from the analysis of large heterogeneous proteins as well as complex biological matrices.

Overall, we conclude that HILIC-MS in all of its facets is a very useful technique for glycoprotein analysis, potentially providing essential information on glycan position, isomerization, heterogeneity, and quantity. Compared to other current glycoprotein methodologies, HILIC-MS is beneficial in cases where separation and identification of highly similar glycan species or complex mixtures of glycoforms is paramount.

References

[1] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, J. Chromatogr. A 499 (1990) 177–196, https://doi.org/10.1016/S0021-9673(00)96972-3.

[2] P. Hemström, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 1784–1821, https://doi.org/10.1002/jssc.200600199.

[3] N.P. Dinh, T. Jonsson, K. Irgum, Water uptake on polar stationary phases under conditions for hydrophilic interaction chromatography and its relation to solute retention, J. Chromatogr. A 1320 (2013) 33–47, https://doi.org/10.1016/j.chroma.2013.09.061.

[4] J. Soukup, P. Jandera, Adsorption of water from aqueous acetonitrile on silica-based stationary phases in aqueous normal-phase liquid chromatography, J. Chromatogr. A 1374 (2014) 102–111, https://doi.org/10.1016/j.chroma.2014.11.028.

[5] P. Jandera, P. Janás, Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography. A review, Anal. Chim. Acta 967 (2017) 12–32, https://doi.org/10.1016/j.aca.2017.01.060.

[6] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231–247, https://doi.org/10.1007/s00216-011-5308-5.

[7] D.V. Mccalley, Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionized solutes in hydrophilic interaction chromatography, J. Chromatogr. A 1217 (2010) 3408–3417, https://doi.org/10.1016/j.chroma.2010.03.011.

[8] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, A revisit to the retention mechanism of hydrophilic interaction liquid chromatography using model organic compounds, J. Chromatogr. A 1218 (2011) 2871–2879.

[9] N.P. Dinh, T. Jonsson, K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 5880–5891.

[10] G. Schuster, W. Lindner, Comparative characterization of hydrophilic interaction liquid chromatography columns by linear solvation energy relationships, J. Chromatogr. A 1273 (2013) 73–94, https://doi.org/10.1016/j.chroma.2012.11.075.

[11] D.V. McCalley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1523 (2017) 49–71, https://doi.org/10.1016/j.chroma.2017.06.026.

[12] V. D'Atri, E. Dumont, I. van den Heede, D. Guillarme, P. Sandra, K. Sandra, Hydrophilic interaction chromatography for the characterization of therapeutic monoclonal antibodies at protein, peptide, and glycan levels, LCGC Eur. 30 (2017) 424–434.

[13] Z. Zhang, Z. Wu, M.J. Wirth, Polyacrylamide brush layer for hydrophilic interaction liquid chromatography of intact glycoproteins, J. Chromatogr. A 1301 (2013) 156–161, https://doi.org/10.1016/j.chroma.2013.05.076.

[14] S.S. Pinho, C.A. Reis, Glycosylation in cancer: mechanisms and clinical implications, Nat. Rev. Cancer 15 (2015) 540–555, https://doi.org/10.1038/nrc3982.

[15] M.J. Kailemia, D. Park, C.B. Lebrilla, Glycans and glycoproteins as specific biomarkers for cancer, Anal. Bioanal. Chem. 409 (2017) 395–410, https://doi.org/10.1007/s00216-016-9880-6.

[16] M. Sethi, S. Fanayan, M.K. Sethi, S. Fanayan, Mass spectrometry-based *N*-glycomics of colorectal cancer, Int. J. Mol. Sci. 16 (2015) 29278–29304, https://doi.org/10.3390/ijms161226165.

[17] S.R. Stowell, T. Ju, R.D. Cummings, Protein glycosylation in cancer, Annu. Rev. Pathol. Mech. Dis. 10 (2015) 473–510, https://doi.org/10.1146/annurev-pathol-012414-040438.

[18] M. Wang, J. Zhu, D.M. Lubman, C. Gao, Aberrant glycosylation and cancer biomarker discovery: a promising and thorny journey, Clin. Chem. Lab. Med. (2018) 407–416, https://doi.org/10.1515/cclm-2018-0379.

[19] K. Ohtsubo, J.D. Marth, Glycosylation in cellular mechanisms of health and disease, Cell 126 (2006) 855–867, https://doi.org/10.1016/J.CELL.2006.08.019.
[20] A. Varki, Biological roles of oligosaccharides: all of the theories are correct, Glycobiology 3 (1993) 97–130, https://doi.org/10.1093/glycob/3.2.97.
[21] S. Holst, M. Wuhrer, Y. Rombouts, Glycosylation characteristics of colorectal cancer, Adv. Cancer Res. 126 (2015) 203–237. Academic Press.
[22] P. Stanley, H. Schachter, N. Taniguchi, *N*-Glycans, in: A. Varki, R. Cummings, J. Esko (Eds.), Essentials of Glycobiology, second Ed., Cold Spring Harbor Laboratory Press, 2009.
[23] J.M. Berg, J.L. Tymoczko, G.J. Gatto, L. Stryer, Carbohydrates, in: Biochemistry, eighth ed., Kate Ahr Parker, New York, 2015, pp. 315–340.
[24] A. Silsirivanit, Glycosylation markers in cancer, Adv. Clin. Chem. 89 (2019) 189–207.
[25] A. Kirwan, M. Utratna, M.E. O'Dwyer, L. Joshi, M. Kilcoyne, Glycosylation-based serum biomarkers for cancer diagnostics and prognostics, Biomed. Res. Int. 2015 (2015) 1–16, https://doi.org/10.1155/2015/490531.
[26] F. Clerc, K.R. Reiding, B.C. Jansen, G.S.M. Kammeijer, A. Bondt, M. Wuhrer, Human plasma protein *N*-glycosylation, Glycoconj. J. 33 (2016) 309–343, https://doi.org/10.1007/s10719-015-9626-2.
[27] J.N. Arnold, R. Saldova, U.M.A. Hamid, P.M. Rudd, Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation, Proteomics 8 (2008) 3284–3293, https://doi.org/10.1002/pmic.200800163.
[28] A. Shajahan, C. Heiss, M. Ishihara, P. Azadi, Glycomic and glycoproteomic analysis of glycoproteins—a tutorial, Anal. Bioanal. Chem. 409 (2017) 4483–4505, https://doi.org/10.1007/s00216-017-0406-7.
[29] K. Mariño, J. Bones, J.J. Kattla, P.M. Rudd, A systematic approach to protein glycosylation analysis: a path through the maze, Nat. Chem. Biol. 6 (2010) 713–723, https://doi.org/10.1038/nchembio.437.
[30] M.L.A. De Leoz, D.L. Duewer, A. Fung, L. Liu, H.K. Yau, O. Potter, G.O. Staples, K. Furuki, R. Frenkel, Y. Hu, Z. Sosic, P. Zhang, F. Altmann, C. Gruber, C. Shao, J. Zaia, W. Evers, S. Pangelley, D. Suckau, A. Wiechmann, A. Resemann, W. Jabs, A. Beck, J.W. Froehlich, C. Huang, Y. Li, Y. Liu, S. Sun, Y. Wang, Y. Seo, H.J. An, N.-C. Reichardt, J.E. Ruiz, S. Archer-Hartmann, P. Azadi, L. Bell, Z. Lakos, Y. An, J.F. Cipollo, M. Pučić-Baković, J. Štambuk, G. Lauc, X. Li, P.G. Wang, A. Bock, R. Hennig, E. Rapp, M. Creskey, T. Cyr, M. Nakano, T. Sugiyama, P.A. Leung, P. Link-Lenczowski, J. Jaworek, S.J. Yang, H. Zhang, T. Kelly, S. Klapoetke, R. Cao, J.Y. Kim, H.K. Lee, J. Lee, J.S. Yoo, S. Kim, S. Suh, N. de Haan, D. Falck, G.S.M. Lageveen-Kammeijer, M. Wuhrer, R.J. Emery, R.P. Kozak, L.P. Liew, L. Royle, P.A. Urbanowicz, N. Packer, X. Song, A. Everest-Dass, E. Lattová, S. Cajic, K. Alagesan, D. Kolarich, T. Kasali, V. Lindo, Y. Chen, K. Goswami, B. Gau, R. Amunugama, R. Jones, C.J.M. Stroop, K. Kato, H. Yagi, S. Kondo, C. Yuen, A. Harazono, X. Shi, P. Magnelli, B.T. Kasper, L.K. Mahal, D.J. Harvey, R.M. O'Flaherty, P. Rudd, R. Saldova, E.S. Hecht, D.C. Muddiman, J. Kang, P. Bhoskar, D. Menard, A. Saati, C. Merle, S. Mast, S. Tep, J. Truong, T. Nishikaze, S. Sekiya, A. Shafer, S. Funaoka, M. Toyoda, P. de Vreugd, C. Caron, P. Pradhan, N.C. Tan, Y. Mechref, S. Patil, J.S. Rohrer, R. Chakrabarti, D. Dadke, M. Lahori, C. Zou, C.W. Cairo, B. Reiz, R.M. Whittal, C. Lebrilla, L.D. Wu, A. Guttman, M. Szigeti, B.G. Kremkow, K. Lee, C. Sihlbom, B. Adamczyk, C. Jin, N.G. Karlsson, J. Örnros, G. Larson, J. Nilsson, B. Meyer, A. Wiegandt, E. Komatsu, H. Perreault, E.D. Bodnar, N. Said, Y. Francois, E. Leize-Wagner, S. Maier, A. Zeck, A.J.R. Heck, Y. Yang, R. Haselberg, Y.Q. Yu, W. Alley, J.W. Leone, H. Yuan, S.E. Stein, NIST interlaboratory study on glycosylation analysis of monoclonal antibodies: comparison of results from diverse analytical methods, Mol. Cell. Proteomics (2019), https://doi.org/10.1074/mcp.ra119.001677. mcp.RA119.001677.

[31] D.A. Fischler, R. Orlando, N-linked glycan release efficiency: a quantitative comparison between NaOCl and PNGase F release protocols, J. Biomol. Tech. 30 (2019) 1–6, https://doi.org/10.7171/jbt.19-3004-001.
[32] X. Yang, M.G. Bartlett, Glycan analysis for protein therapeutics, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1120 (2019) 29–40, https://doi.org/10.1016/j.jchromb.2019.04.031.
[33] T. Ikegami, Hydrophilic interaction chromatography for the analysis of biopharmaceutical drugs and therapeutic peptides: a review based on the separation characteristics of the hydrophilic interaction chromatography phases, J. Sep. Sci. 42 (2019) 130–213, https://doi.org/10.1002/jssc.201801074.
[34] M. Fukuda, β-Elimination for release of O-GalNAc-linked oligosaccharides from glycoproteins and glycopeptides, Curr. Protoc. Mol. Biol. (2001) 4–9, https://doi.org/10.1002/0471142727.mb1715bs31.
[35] J. Charlwood, H. Birrell, E.S.P. Bouvier, J. Langridge, P. Camilleri, Analysis of oligosaccharides by microbore high-performance liquid chromatography, Anal. Chem. 72 (2000) 1469–1474, https://doi.org/10.1021/ac991267n.
[36] B. Garner, A.H. Merry, L. Royle, D.J. Harvey, P.M. Rudd, J. Thillet, Structural elucidation of the N- and *O*-glycans of human apolipoprotein(a). Role of *O*-glycans in conferring protease resistance, J. Biol. Chem. 276 (2001) 22200–22208, https://doi.org/10.1074/jbc.M102150200.
[37] M.S. Bereman, D.D. Young, A. Deiters, D.C. Muddiman, Development of a robust and high throughput method for profiling N-linked glycans derived from plasma glycoproteins by nanoLC-FTICR mass spectrometry, J. Proteome Res. 8 (2009) 3764–3770, https://doi.org/10.1021/pr9002323.
[38] J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 403–408, https://doi.org/10.1016/j.jchromb.2009.12.013.
[39] M. Melmer, T. Stangler, M. Schiefermeier, W. Brunner, H. Toll, A. Rupprechter, W. Lindner, A. Premstaller, HILIC analysis of fluorescence-labeled *N*-glycans from recombinant biopharmaceuticals, Anal. Bioanal. Chem. 398 (2010) 905–914, https://doi.org/10.1007/s00216-010-3988-x.
[40] M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis, J. Chromatogr. A 1218 (2011) 118–123, https://doi.org/10.1016/j.chroma.2010.10.122.
[41] S.H. Walker, L.M. Lilley, M.F. Enamorado, D.L. Comins, D.C. Muddiman, Hydrophobic derivatization of N-linked glycans for increased ion abundance in electrospray ionization mass spectrometry, J. Am. Soc. Mass Spectrom. 22 (2011) 1309–1317, https://doi.org/10.1007/s13361-011-0140-x.
[42] S.H. Walker, J. Budhathoki-Uprety, B.M. Novak, D.C. Muddiman, Stable-isotope labeled hydrophobic hydrazide reagents for the relative quantification of n-linked glycans by electrospray ionization mass spectrometry, Anal. Chem. 83 (2011) 6738–6745, https://doi.org/10.1021/ac201376q.
[43] F. Tousi, J. Bones, W.S. Hancock, M. Hincapie, Differential chemical derivatization integrated with chromatographic separation for analysis of isomeric sialylated N-glycans: a nano-hydrophilic interaction liquid chromatography-MS platform, Anal. Chem. 85 (2013) 8421–8428, https://doi.org/10.1021/ac4018007.
[44] C. Wang, J. Yuan, Z. Wang, L. Huang, Separation of one-pot procedure released *O*-glycans as 1-phenyl-3-methyl-5-pyrazolone derivatives by hydrophilic interaction and reversed-phase liquid chromatography followed by identification using electrospray mass spectrometry and tandem mass spectromet, J. Chromatogr. A 1274 (2013) 107–117, https://doi.org/10.1016/j.chroma.2012.12.005.

[45] S. Houel, M. Hilliard, Y.Q. Yu, N. McLoughlin, S.M. Martin, P.M. Rudd, J.P. Williams, W. Chen, N- and *O*-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-flight mass spectrometry equipped with electron-transfer dissociation functionality, Anal. Chem. 86 (2014) 576–584, https://doi.org/10.1021/ac402726h.

[46] M.A. Lauber, Y.Q. Yu, D.W. Brousmiche, Z. Hua, S.M. Koza, P. Magnelli, E. Guthrie, C.H. Taron, K.J. Fountain, Rapid preparation of released N-glycans for HILIC analysis using a labeling reagent that facilitates sensitive fluorescence and ESI-MS detection, Anal. Chem. 87 (2015) 5401–5409, https://doi.org/10.1021/acs.analchem.5b00758.

[47] J. Zhao, S. Li, C. Li, S.L. Wu, W. Xu, Y. Chen, M. Shameem, D. Richardson, H. Li, Identification of low abundant isomeric *N*-glycan structures in biological therapeutics by LC/MS, Anal. Chem. 88 (2016) 7049–7059, https://doi.org/10.1021/acs.analchem.6b00636.

[48] B. Adamczyk, S. Albrecht, H. Stöckmann, I.M. Ghoneim, M. Al-Eknah, K.A.S. Al-Busadah, N.G. Karlsson, S.D. Carrington, P.M. Rudd, Pregnancy-associated changes of IgG and serum *N*-glycosylation in camel (Camelus dromedarius), J. Proteome Res. 15 (2016) 3255–3265, https://doi.org/10.1021/acs.jproteome.6b00439.

[49] R.P. Kozak, P.A. Urbanowicz, C. Punyadeera, K.R. Reiding, B.C. Jansen, L. Royle, D.I. Spencer, D.L. Fernandes, M. Wuhrer, Variation of human salivary *O*-glycome, PLoS One 11 (2016) 1–15, https://doi.org/10.1371/journal.pone.0162824.

[50] S. Mittermayr, G.N. Lê, C. Clarke, S. Millán Martín, A.M. Larkin, P. O'Gorman, J. Bones, Polyclonal immunoglobulin G *N*-glycosylation in the pathogenesis of plasma cell disorders, J. Proteome Res. 16 (2017) 748–762, https://doi.org/10.1021/acs.jproteome.6b00768.

[51] E. Largy, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel, Orthogonal liquid chromatography–mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level, J. Chromatogr. A 1498 (2017) 128–146, https://doi.org/10.1016/j.chroma.2017.02.072.

[52] X. Sun, L. Tao, L. Yi, Y. Ouyang, N. Xu, D. Li, R.J. Linhardt, Z. Zhang, *N*-Glycans released from glycoproteins using a commercial kit and comprehensively analyzed with a hypothetical database, J. Pharm. Anal. 7 (2017) 87–94, https://doi.org/10.1016/j.jpha.2017.01.004.

[53] Z. Szabo, J.R. Thayer, D. Reusch, Y. Agroskin, R. Viner, J. Rohrer, S.P. Patil, M. Krawitzky, A. Huhmer, N. Avdalovic, S.H. Khan, Y. Liu, C. Pohl, High performance anion exchange and hydrophilic interaction liquid chromatography approaches for comprehensive mass spectrometry-based characterization of the *N*-glycome of a recombinant human erythropoietin, J. Proteome Res. 17 (2018) 1559–1574, https://doi.org/10.1021/acs.jproteome.7b00862.

[54] C. Wang, Y. Lu, J. Han, W. Jin, L. Li, Y. Zhang, X. Song, L. Huang, Z. Wang, Simultaneous release and labeling of O- and *N*-glycans allowing for rapid glycomic analysis by online LC-UV-ESI-MS/MS, J. Proteome Res. 17 (2018) 2345–2357, https://doi.org/10.1021/acs.jproteome.8b00038.

[55] B. Chen, X. Zhong, Y. Feng, S. Snovida, M. Xu, J. Rogers, L. Li, Targeted MultiNotch MS3 approach for relative quantification of *N*-glycans using multiplexed carbonyl-reactive isobaric tags, Anal. Chem. 90 (2018) 1129–1135, https://doi.org/10.1021/acs.analchem.7b03289.

[56] J.A. Saba, X. Shen, J.C. Jamieson, H. Perreault, Investigation of different combinations of derivatization, separation methods and electrospray ionization mass spectrometry for standard oligosaccharides and glycans from ovalbumin, J. Mass Spectrom. 36 (2001) 563–574, https://doi.org/10.1002/jms.158.

[57] K. Jiang, C. Wang, Y. Sun, Y. Liu, Y. Zhang, L. Huang, Z. Wang, Comparison of chicken and pheasant ovotransferrin *N*-glycoforms via electrospray ionization mass spectrometry and liquid chromatography coupled with mass spectrometry, J. Agric. Food Chem. 62 (2014) 7245–7254, https://doi.org/10.1021/jf501352j.

[58] S. Tao, Y. Huang, B.E. Boyes, R. Orlando, Liquid chromatography-selected reaction monitoring (LC-SRM) approach for the separation and quantitation of sialylated *N*-glycans linkage isomers, Anal. Chem. 86 (2014) 10584–10590, https://doi.org/10.1021/ac5020996.

[59] Y. Takegawa, K. Deguchi, T. Keira, H. Ito, Separation of isomeric 2-aminopyridine derivatized *N*-glycans and *N*-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography, J. Chromatogr. A 1113 (2006) 177–181, https://doi.org/10.1016/j.chroma.2006.02.010.

[60] Y. Takegawa, K. Deguchi, T. Keira, H. Ito, T. Keira, H. Nakagawa, S.I. Nishimura, Simple separation of isometric sialylated *N*-glycopeptides by a zwitterionic type of hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 2533–2540, https://doi.org/10.1002/jssc.200600133.

[61] Y. Takegawa, M. Hato, K. Deguchi, H. Nakagawa, S.I. Nishimura, Chromatographic deuterium isotope effects of derivatized *N*-glycans and *N*-glycopeptides in a zwitterionic type of hydrophilic interaction chromatography, J. Sep. Sci. 31 (2008) 1594–1597, https://doi.org/10.1002/jssc.200700659.

[62] L. Mauko, M. Pelzing, S. Dolman, A. Nordborg, N.A. Lacher, P.R. Haddad, E.F. Hilder, Zwitterionic-type hydrophilic interaction nano-liquid chromatography of complex and high mannose glycans coupled with electrospray ionisation high resolution time of flight mass spectrometry, J. Chromatogr. A 1218 (2011) 6419–6425, https://doi.org/10.1016/j.chroma.2011.07.006.

[63] L. Mauko, A. Nordborg, J.P. Hutchinson, N.A. Lacher, E.F. Hilder, P.R. Haddad, Glycan profiling of monoclonal antibodies using zwitterionic-type hydrophilic interaction chromatography coupled with electrospray ionization mass spectrometry detection, Anal. Biochem. 408 (2011) 235–241, https://doi.org/10.1016/j.ab.2010.09.035.

[64] L. Mauko, N.A. Lacher, M. Pelzing, A. Nordborg, P.R. Haddad, E.F. Hilder, Comparison of ZIC-HILIC and graphitized carbon-based analytical approaches combined with exoglycosidase digestions for analysis of glycans from monoclonal antibodies, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 911 (2012) 93–104, https://doi.org/10.1016/j.jchromb.2012.10.043.

[65] E. Giménez, V. Sanz-Nebot, A. Rizzi, Relative quantitation of glycosylation variants by stable isotope labeling of enzymatically released *N*-glycans using [12C]/[13C] aniline and ZIC-HILIC-ESI-TOF-MS, Anal. Bioanal. Chem. 405 (2013) 7307–7319, https://doi.org/10.1007/s00216-013-7178-5.

[66] E. Giménez, M. Balmaña, J. Figueras, E. Fort, C. de Bolós, V. Sanz-Nebot, R. Peracaula, A. Rizzi, Quantitative analysis of *N*-glycans from human alfa-acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis, Anal. Chim. Acta 866 (2015) 59–68, https://doi.org/10.1016/j.aca.2015.02.008.

[67] M. Mancera-Arteu, E. Giménez, J. Barbosa, V. Sanz-Nebot, Identification and characterization of isomeric *N*-glycans of human alfa-acid-glycoprotein by stable isotope labelling and ZIC-HILIC-MS in combination with exoglycosidase digestion, Anal. Chim. Acta 940 (2016) 92–103, https://doi.org/10.1016/j.aca.2016.07.043.

[68] M. Mancera-Arteu, E. Giménez, J. Barbosa, R. Peracaula, V. Sanz-Nebot, Zwitterionic-hydrophilic interaction capillary liquid chromatography coupled to tandem mass spectrometry for the characterization of human alpha-acid-glycoprotein *N*-glycan isomers, Anal. Chim. Acta 991 (2017) 76–88, https://doi.org/10.1016/j.aca.2017.07.068.

[69] T.Q. Shang, A. Saati, K.N. Toler, J. Mo, H. Li, T. Matlosz, X. Lin, J. Schenk, C.K. Ng, T. Duffy, T.J. Porter, J.C. Rouse, Development and application of a robust *N*-glycan profiling method for heightened characterization of monoclonal antibodies and related glycoproteins, J. Pharm. Sci. 103 (2014) 1967–1978, https://doi.org/10.1002/jps.24004.

[70] K. Deguchi, T. Keira, K. Yamada, H. Ito, Y. Takegawa, H. Nakagawa, S.I. Nishimura, Two-dimensional hydrophilic interaction chromatography coupling anion-exchange and hydrophilic interaction columns for separation of 2-pyridylamino derivatives of neutral and sialylated *N*-glycans, J. Chromatogr. A 1189 (2008) 169–174, https://doi.org/10.1016/j.chroma.2007.09.028.

[71] T.A. Blake, T.L. Williams, J.L. Pirkle, J.R. Barr, Targeted N-linked glycosylation analysis of H5N1 influenza hemagglutinin by selective sample preparation and liquid chromatography/tandem mass spectrometry, Anal. Chem. 81 (2009) 3109–3118, https://doi.org/10.1021/ac900095h.

[72] G. Zauner, C.A.M. Koeleman, A.M. Deelder, M. Wuhrer, Protein glycosylation analysis by HILIC-LC-MS of proteinase K-generated N- and O-glycopeptides, J. Sep. Sci. 33 (2010) 903–910, https://doi.org/10.1002/jssc.200900850.

[73] M.P.Y. Lam, S.O. Siu, E. Lau, X. Mao, H.Z. Sun, P.C.N. Chiu, W.S.B. Yeung, D.M. Cox, I.K. Chu, Online coupling of reversed-phase and hydrophilic interaction liquid chromatography for protein and glycoprotein characterization, Anal. Bioanal. Chem. 398 (2010) 791–804, https://doi.org/10.1007/s00216-010-3991-2.

[74] M. Gilar, Y.Q. Yu, J. Ahn, H. Xie, H. Han, W. Ying, X. Qian, Characterization of glycoprotein digests with hydrophilic interaction chromatography and mass spectrometry, Anal. Biochem. 417 (2011) 80–88, https://doi.org/10.1016/j.ab.2011.05.028.

[75] M.S. Han, J.T. Simpson, Method for mapping glycosylation sites in proteins, J. Biomol. Tech. 28 (2017) 142–149, https://doi.org/10.7171/jbt.17-2804-001.

[76] N. Lin, J. Li, R. Shao, H. Zhang, Site-specific analysis of N-linked glycosylation heterogeneity from royal jelly glycoproteins, J. Agric. Food Chem. 67 (2019) 9411–9422, https://doi.org/10.1021/acs.jafc.9b03080.

[77] Y. Takegawa, H. Ito, T. Keira, K. Deguchi, H. Nakagawa, S.I. Nishimura, Profiling of *N*- and *O*-glycopeptides of erythropoietin by capillary zwitterionic type of hydrophilic interaction chromatography/electrospray ionization mass spectrometry, J. Sep. Sci. 31 (2008) 1585–1593, https://doi.org/10.1002/jssc.200700679.

[78] J. Wohlgemuth, M. Karas, W. Jiang, R. Hendriks, S. Andrecht, Enhanced glycoprofiling by specific glycopeptide enrichment and complementary monolithic nano-LC (ZIC-HILIC/RP18e)/ESI-MS analysis, J. Sep. Sci. 33 (2010) 880–890, https://doi.org/10.1002/jssc.200900771.

[79] O. Hernandez-Hernandez, R. Lebron-Aguilar, J.E. Quintanilla-Lopez, M.L. Sanz, F.J. Moreno, Development of a new method using HILIC-tandem mass spectrometry for the characterization of O-sialoglycopeptides from proteolytically digested caseinomacropeptide, Proteomics 10 (2010) 3699–3711, https://doi.org/10.1002/pmic.201000156.

[80] C. Singh, C.G. Zampronio, A.J. Creese, H.J. Cooper, Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of *N*-linked glycoproteins, J. Proteome Res. 11 (2012) 4517–4525, https://doi.org/10.1021/pr300257c.

[81] Y. Huang, Y. Nie, B. Boyes, R. Orlando, Resolving isomeric glycopeptide glycoforms with hydrophilic interaction chromatography (HILIC), J. Biomol. Tech. 27(3) (2016) 98–104, https://doi.org/10.7171/jbt.16-2703-003.

[82] M.J. Badgett, B. Boyes, R. Orlando, Predicting the retention behavior of specific *O*-linked glycopeptides, J. Biomol. Tech. 28 (2017) 122–126, https://doi.org/10.7171/jbt.17-2803-003.

[83] P. Kozlik, R. Goldman, M. Sanda, Hydrophilic interaction liquid chromatography in the separation of glycopeptides and their isomers, Anal. Bioanal. Chem. 410 (2018) 5001–5008, https://doi.org/10.1007/s00216-018-1150-3.

[84] P. Kozlik, M. Sanda, R. Goldman, Nano reversed phase versus nano hydrophilic interaction liquid chromatography on a chip in the analysis of hemopexin glycopeptides, J. Chromatogr. A 1519 (2017) 152–155, https://doi.org/10.1016/j.chroma.2017.08.066.

[85] O. Hernandez-Hernandez, J.E. Quintanilla-Lopez, R. Lebron-Aguilar, M.L. Sanz, F.J. Moreno, Characterization of post-translationally modified peptides by hydrophilic interaction and reverse phase liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry, J. Chromatogr. A 1428 (2016) 202–211, https://doi.org/10.1016/j.chroma.2015.07.096.

[86] K. Furuki, T. Toyo'oka, Retention of glycopeptides analyzed using hydrophilic interaction chromatography is influenced by charge and carbon chain length of ion-pairing reagent for mobile phase, Biomed. Chromatogr. 31 (2017), https://doi.org/10.1002/bmc.3988.

[87] A. Pedrali, S. Tengattini, G. Marrubini, T. Bavaro, P. Hemström, G. Massolini, M. Terreni, C. Temporini, Characterization of intact neo-glycoproteins by hydrophilic interaction liquid chromatography, Molecules 19 (2014) 9070–9088, https://doi.org/10.3390/molecules19079070.

[88] V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Hydrophilic interaction chromatography hyphenated with mass spectrometry: a powerful analytical tool for the comparison of originator and biosimilar therapeutic monoclonal antibodies at the middle-up level of analysis, Anal. Chem. 89 (2017) 2086–2092, https://doi.org/10.1021/acs.analchem.6b04726.

[89] F. Rinaldi, S. Tengattini, E. Calleri, T. Bavaro, L. Piubelli, L. Pollegioni, G. Massolini, C. Temporini, Application of a rapid HILIC-UV method for synthesis optimization and stability studies of immunogenic neo-glycoconjugates, J. Pharm. Biomed. Anal. 144 (2017) 252–262, https://doi.org/10.1016/j.jpba.2017.03.052.

[90] S. Tengattini, E. Domínguez-Vega, C. Temporini, T. Bavaro, F. Rinaldi, L. Piubelli, L. Pollegioni, G. Massolini, G.W. Somsen, Hydrophilic interaction liquid chromatography-mass spectrometry as a new tool for the characterization of intact semi-synthetic glycoproteins, Anal. Chim. Acta 981 (2017) 94–105, https://doi.org/10.1016/j.aca.2017.05.020.

[91] E. Domínguez-Vega, S. Tengattini, C. Peintner, J. van Angeren, C. Temporini, R. Haselberg, G. Massolini, G.W. Somsen, High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry, Talanta 184 (2018) 375–381, https://doi.org/10.1016/j.talanta.2018.03.015.

[92] A.F.G. Gargano, L.S. Roca, R.T. Fellers, M. Bocxe, E. Domínguez-Vega, G.W. Somsen, Capillary HILIC-MS: a new tool for sensitive top-down proteomics, Anal. Chem. 90 (2018) 6601–6609, https://doi.org/10.1021/acs.analchem.8b00382.

[93] S. Wang, A.P. Liu, Y. Yan, T.J. Daly, N. Li, Characterization of product-related low molecular weight impurities in therapeutic monoclonal antibodies using hydrophilic interaction chromatography coupled with mass spectrometry, J. Pharm. Biomed. Anal. 154 (2018) 468–475, https://doi.org/10.1016/j.jpba.2018.03.034.

[94] G. van Schaick, B.W.J. Pirok, R. Haselberg, G.W. Somsen, A.F.G. Gargano, Computer-aided gradient optimization of hydrophilic interaction liquid chromatographic separations of intact proteins and protein glycoforms, J. Chromatogr. A 1598 (2019) 67–76, https://doi.org/10.1016/j.chroma.2019.03.038.

[95] B.L. Duivelshof, S. Fekete, D. Guillarme, V. D'Atri, A generic workflow for the characterization of therapeutic monoclonal antibodies—application to daratumumab, Anal. Bioanal. Chem. 411 (2019) 4615–4627, https://doi.org/10.1007/s00216-018-1561-1.

[96] V. D'Atri, L. Nováková, S. Fekete, D. Stoll, M. Lauber, A. Beck, D. Guillarme, Orthogonal middle-up approaches for characterization of the glycan heterogeneity of etanercept by hydrophilic interaction chromatography coupled to high-resolution mass spectrometry, Anal. Chem. 91 (2019) 873–880, https://doi.org/10.1021/acs.analchem.8b03584.

[97] A.F.G. Gargano, O. Schouten, G. van Schaick, L.S. Roca, J.H. van den Berg-Verleg, R. Haselberg, M. Akeroyd, N. Abello, G.W. Somsen, Profiling of a high mannose-type *N*-glycosylated lipase using hydrophilic interaction chromatography-mass spectrometry, Anal. Chim. Acta 1109 (2020) 69–77, https://doi.org/10.1016/j.aca.2020.02.042.

[98] T. Sénard, A.F.G. Gargano, D. Falck, S.W. de Taeye, T. Rispens, G. Vidarsson, M. Wuhrer, G.W. Somsen, E. Domínguez-Vega, MS-based allotype-specific analysis of polyclonal IgG-fc *N*-glycosylation, Front. Immunol. 11 (2020) 1–13, https://doi.org/10.3389/fimmu.2020.02049.

[99] D.R. Stoll, D.C. Harmes, G.O. Staples, O.G. Potter, C.T. Dammann, D. Guillarme, A. Beck, Development of comprehensive online two-dimensional liquid chromatography/mass spectrometry using hydrophilic interaction and reversed-phase separations for rapid and deep profiling of therapeutic antibodies, Anal. Chem. 90 (2018) 5923–5929, https://doi.org/10.1021/acs.analchem.8b00776.

[100] J. Camperi, L. Dai, D. Guillarme, C. Stella, Development of a 3D-LC/MS workflow for fast, automated, and effective characterization of glycosylation patterns of biotherapeutic products, Anal. Chem. 92 (2020) 4357–4363, https://doi.org/10.1021/acs.analchem.9b05193.

[101] A. Periat, S. Fekete, A. Cusumano, J.-L.L. Veuthey, A. Beck, M. Lauber, D. Guillarme, Potential of hydrophilic interaction chromatography for the analytical characterization of protein biopharmaceuticals, J. Chromatogr. A 1448 (2016) 81–92, https://doi.org/10.1016/j.chroma.2016.04.056.

[102] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 5920–5938, https://doi.org/10.1016/j.chroma.2011.06.052.

[103] K. Alagesan, S.K. Khilji, D. Kolarich, It is all about the solvent : on the importance of the mobile phase for ZIC-HILIC glycopeptide enrichment, Anal. Bioanal. Chem. 409 (2017) 529–538, https://doi.org/10.1007/s00216-016-0051-6.

[104] P.J. Boersema, S. Mohammed, A.J.R. Heck, Hydrophilic interaction liquid chromatography (HILIC) in proteomics, Anal. Bioanal. Chem. 391 (2008) 151–159, https://doi.org/10.1007/s00216-008-1865-7.

[105] E.S. Grumbach, M. Diane, R. Jeffrey, B. Alden, P.C. Iraneta, E.S. Grumbach, Hydrophilic interaction chromatography using silica columns for the retention of polar analytes and enhanced ESI-MS sensitivity, LCGC N. Am. 22 (2004) 1010–1023.

[106] D.V. Mccalley, Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range, J. Chromatogr. A 1411 (2015) 41–49, https://doi.org/10.1016/j.chroma.2015.07.092.

[107] D.V. McCalley, U.D. Neue, Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography, J. Chromatogr. A 1192 (2008) 225–229, https://doi.org/10.1016/j.chroma.2008.03.049.

[108] P. Jandera, T. Hájek, V. Škeříková, J. Soukup, Dual hydrophilic interaction-RP retention mechanism on polar columns: structural correlations and implementation for 2-D separations on a single column, J. Sep. Sci. 33 (2010) 841–852, https://doi.org/10.1002/jssc.200900678.

[109] A. Periat, I.S. Krull, D. Guillarme, Applications of hydrophilic interaction chromatography to amino acids, peptides, and proteins, J. Sep. Sci. 38 (2015) 357–367, https://doi.org/10.1002/jssc.201400969.

[110] D.V. Mccalley, Managing the column equilibration time in hydrophilic interaction chromatography, J. Chromatogr. A (2019), https://doi.org/10.1016/j.chroma.2019.460655.

[111] N.E. Hoffman, S.-L. Pan, A.M. Rustum, Injection of eluites in solvents stronger than the mobile phase in reversed-phase liquid chromatography, J. Chromatogr. A 465 (1989) 189–200, https://doi.org/10.1016/S0021-9673(01)92657-3.

[112] Q. Zhang, F.Q. Yang, L. Ge, Y.J. Hu, Z.N. Xia, Recent applications of hydrophilic interaction liquid chromatography in pharmaceutical analysis, J. Sep. Sci. 40 (2017) 49–80, https://doi.org/10.1002/jssc.201600843.

[113] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Orthogonality of separation in two-dimensional liquid chromatography, Anal. Chem. 77 (2005) 6426–6434, https://doi.org/10.1021/ac050923i.

[114] A.F.G. Gargano, J.B. Shaw, M. Zhou, C.S. Wilkins, T.L. Fillmore, R.J. Moore, G.W. Somsen, L. Paša-Tolić, Increasing the separation capacity of intact histone proteoforms chromatography coupling online weak cation exchange-HILIC to reversed phase LC UVPD-HRMS, J. Proteome Res. 17 (2018), https://doi.org/10.1021/acs.jproteome.8b00458. acs.jproteome.8b00458.

[115] A.F.G. Gargano, M. Duffin, P. Navarro, P.J. Schoenmakers, Reducing dilution and analysis time in online comprehensive two-dimensional liquid chromatography by active modulation, Anal. Chem. 1 (2016) 1–16, https://doi.org/10.1021/acs.analchem.5b04051.

[116] S. Chapel, F. Rouvière, S. Heinisch, Pushing the limits of resolving power and analysis time in on-line comprehensive hydrophilic interaction x reversed phase liquid chromatography for the analysis of complex peptide samples, J. Chromatogr. A 1615 (2020), https://doi.org/10.1016/j.chroma.2019.460753, 460753.

[117] Q. Li, F. Lynen, J. Wang, H. Li, G. Xu, P. Sandra, Comprehensive hydrophilic interaction and ion-pair reversed-phase liquid chromatography for analysis of di- to deca-oligonucleotides, J. Chromatogr. A 1255 (2012) 237–243, https://doi.org/10.1016/j.chroma.2011.11.062.

[118] M.A. Lauber, S.M. Koza, Waters Application Notes – Mapping IgG Subunit Glycoforms Using HILIC and a Wide-Pore Amide Stationary Phase, (n.d.) 1–10.

[119] S. Sinha, G. Pipes, E.M. Topp, P.V. Bondarenko, M.J. Treuheit, H.S. Gadgil, Comparison of LC and LC/MS methods for quantifying *N*-glycosylation in recombinant IgGs, J. Am. Soc. Mass Spectrom. 19 (2008) 1643–1654, https://doi.org/10.1016/j.jasms.2008.07.004.

[120] G. Schuster, W. Lindner, Development of Polar Chromatography Sorbents and Attempts to Describe Retention Mechanisms of Hydrophilic Interaction Chromatography Systems, University of Vienna, 2013, https://doi.org/10.25365/thesis.27444.

[121] P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review, Anal. Chim. Acta 692 (2011) 1–25, https://doi.org/10.1016/j.aca.2011.02.047.

[122] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: hydrophilicity, charge effects, structural selectivity, and separation efficiency, J. Chromatogr. A 1218 (2011) 5903–5919, https://doi.org/10.1016/j.chroma.2011.06.048.

[123] A.G. Huckabee, C. Yerneni, R.E. Jacobson, E.J. Alzate, T.H. Chen, M.J. Wirth, In-column bonded phase polymerization for improved packing uniformity, J. Sep. Sci. 40 (2017) 2170–2177, https://doi.org/10.1002/jssc.201601376.

[124] M.J. Wirth, R.E. Jacobson, E. Jhovany, A. Rodriguez, The detailed characterization of post-translational protein modifications relies on performance improvements in both HPLC and MS. But how do we strike a balance between LC resolution and MS sensitivity? Anal. Sci. (2018). https://theanalyticalscientist.com/techniques-tools/tricks-of-the-trade-off.

[125] J.Y. Wu, W. Bicker, W. Lindner, Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode, J. Sep. Sci. 31 (2008) 1492–1503, https://doi.org/10.1002/jssc.200800017.

[126] J. Camperi, V. Pichon, T. Fournier, N. Delaunay, First profiling in hydrophilic interaction liquid chromatography of intact human chorionic gonadotropin isoforms, J. Pharm. Biomed. Anal. 174 (2019) 495–499, https://doi.org/10.1016/j.jpba.2019.06.014.

[127] M.L. Jackson, The colloid chemistry of silica and silicates, Soil Sci. Soc. Am. J. 19 (1955) 390, https://doi.org/10.2136/sssaj1955.03615995001900030035x.

[128] R. Kaliszan, QSRR: quantitative structure-(chromatographic) retention relationships, Chem. Rev. 107 (2007) 3212–3246, https://doi.org/10.1021/cr068412z.

[129] J. Camperi, A. Combès, T. Fournier, V. Pichon, N. Delaunay, Analysis of the human chorionic gonadotropin protein at the intact level by HILIC-MS and comparison with RPLC-MS, Anal. Bioanal. Chem. 412 (2020) 4423–4432, https://doi.org/10.1007/s00216-020-02684-8.

[130] L.R. Snyder, J.J. Kirkland, J.W. Dolan, T.D. Lee, Introduction to Modern Liquid Chromatography, third ed., John Wiley & Sons, Inc., Hoboken, NJ, 2011.

[131] A.J. Alpert, Effect of salts on retention in hydrophilic interaction chromatography, J. Chromatogr. A 1538 (2018) 45–53, https://doi.org/10.1016/j.chroma.2018.01.038.

[132] B. Bobály, E. Tóth, L. Drahos, F. Zsila, J. Visy, J. Fekete, K. Vékey, Influence of acid-induced conformational variability on protein separation in reversed phase high performance liquid chromatography, J. Chromatogr. A 1325 (2014) 155–162, https://doi.org/10.1016/j.chroma.2013.12.022.

[133] D. Reusch, M. Haberger, B. Maier, M. Maier, R. Kloseck, B. Zimmermann, M. Hook, Z. Szabo, S. Tep, J. Wegstein, N. Alt, P. Bulau, M. Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles – part 1: separation-based methods, MAbs 7 (2015) 167–179, https://doi.org/10.4161/19420862.2014.986000.

[134] B. Bobály, V. D'Atri, A. Beck, D. Guillarme, S. Fekete, Analysis of recombinant monoclonal antibodies in hydrophilic interaction chromatography: a generic method development approach, J. Pharm. Biomed. Anal. 145 (2017) 24–32, https://doi.org/10.1016/j.jpba.2017.06.016.

[135] B.Y. Huang, C.K. Yang, C.P. Liu, C.Y. Liu, Stationary phases for the enrichment of glycoproteins and glycopeptides, Electrophoresis 35 (2014) 2091–2107, https://doi.org/10.1002/elps.201400034.