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Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis

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ABSTRACT

Hydrophilic-interaction liquid chromatography (HILIC), reversed-phase chromatography (RPC) and porous graphitic carbon (PGC) chromatography are typically applied for liquid chromatographic separations of protein N-glycans. Hence the performances of these chromatography modes for the separation of fluorescently labeled standard glycan samples (monoclonal antibody, fetuin, ribonuclease-B) covering high-mannose and a broad range of complex type glycans were investigated. In RPC the retention of sialylated glycans was enhanced by adding an ion-pairing agent to the mobile phase, resulting in improved peak shapes for sialylated glycans compared to methods recently reported in literature. For ion pairing RPC (IP-RPC) and HILIC ultra-high performance stationary phases were utilized to maximize the peak capacity and thus the resolution. But due to the shallow gradient in RPC the peak capacity was lower than on PGC. Retention times in HILIC and IP-RPC could be correlated to the monosaccharide compositions of the glycans by multiple linear regression, whereas no adequate model was obtained for PGC chromatography, indicating the significance of the three-dimensional structure of the analytes for retention in this method. Generally low correlations were observed between the chromatography methods, indicating their orthogonality. The high selectivities, as well as the commercial availability of ultra-high performance stationary phases render HILIC the chromatography method of choice for the analysis of glycans. Even though for complete characterization of complex glycan samples a combination of chromatography methods may be necessary.

1. Introduction

Protein glycosylation has implications for a variety of biological functions, e.g. cell–cell signaling, protein stability and solubility and affinity to target molecule. The glycan profile is particularly relevant for therapeutic proteins, because it impacts efficacy and safety of the product [1–3]. Thus, glycans of biopharmaceuticals must be characterized and also monitored during production, requiring both, in-depth characterization methods and fast profiling methods.

Chromatographic analysis of glycans is typically accomplished utilizing hydrophilic-interaction chromatography (HILIC), but may also be performed on conventional reversed-phase or porous graphitic carbon (PGC) stationary phases. The glycans are typically labeled with a fluorescence dye prior to their analysis. In reversed-phase chromatography (RPC) labeling with an aromatic tag is mandatory to generate retention of these highly hydrophilic analytes. In contrast PGC is typically employed also for reduced glycans, which are detected by mass spectrometry (MS). This fact is particularly remarkable, because interferences of the separation of glycans on PGC with the high-voltage of the electrospray ionization source were reported [4,5]. Nevertheless PGC is also capable of separating fluorescence labeled glycans. This aromatic label may then be utilized to introduce isotope tags for mass spectrometric detection [6].

In HILIC the fluorescent labeled glycans are retained according to the number and accessibility of polar groups. Several commercially available phases possess charged moieties additionally providing coulomb interactions [7]. Retention is generally correlated to size of the glycans with certain selectivities for isomers. Due to the high fraction of organic solvent in HILIC (usually acetonitrile) the column back pressure is comparably low, allowing for the use of ultra-high-performance liquid chromatography (UHPLC) columns with standard HPLC equipment at least at elevated temperature. Furthermore, remarkable resolution of a highly sialylated glycan sample was achieved on a weak anion-exchange column operated in HILIC mode, illustrating the potential of combinations of retention mechanisms [8].

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In RPC the glycans elute in groups according to their structure elements [9]. The elution order may depend on specific column properties, e.g. end-capping or silanol activity, because retention order varies between ODS columns of different brands [10]. Furthermore retentivity of fluorescently labeled glycans on conventional RP columns is quite low thus limiting the fraction of organic solvent in the mobile phase.

Porous graphitic carbon (PGC) is employed for glycan analysis mainly in the academic field. This stationary phase solely consists of graphite type carbon and offers remarkable selectivities for isomeric glycans and increased retention particularly for charged glycans [11,12]. The strong adsorption of polar analytes on PGC compared to conventional reversed-phases even allows for the analysis of native and reduced oligosaccharides [13,14]. In previous experiments we identified the nature of the organic modifier, the column temperature and the redox state of the PGC material as important parameters impacting retention of oligosaccharides [15,16].

The development of UHPLC equipment significantly increases the peak capacities achievable in liquid chromatography and enables for shorter analysis times. For several years the range of UHPLC columns was restricted to RPC, which have been investigated for high-throughput analysis of fluorescently labeled immunoglobulin G (IgG) glycans [10]. But recently a HILIC type UHPLC stationary phase dedicated to glycan analysis has become available, which was tested for RNase-B, fetuin and IgG glycans [17].

In this contribution we report on the investigation of HILIC, ion pairing RPC (IP-RPC) and PGC chromatography for the analysis of 2-aminobenzamide (2-AB) labeled glycans. The retention times of 2-AB labeled glycans from fetuin, RNase-B and a mAb in HILIC, IP-RPC and PGC chromatography, respectively, were determined applying linear gradients. Based on this broad data set the retention properties of the stationary phases regarding protein N-glycans were correlated to the monosaccharide compositions by multiple linear regression (MLR). This evaluation allows to quantitatively assess the contributions of the different monosaccharides to the retention in each investigated chromatography mode. Furthermore the goodness of fit indicates whether retention is determined solely by the monosaccharide composition or it is impacted by further parameters.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were at least analytical grade. RNase-B was purchased from Worthington Biochemical (Lakewood, NJ, USA), fetuin was ordered from Sigma (Steinheim, Germany). The mAb was obtained from in-house development at Sandoz (Sandoz, Kundl, Austria). PNGase-F and neuraminidase were ordered from Roche (Roche Diagnostics, Vienna, Austria). Water was purified by a MilliQ® system (Millipore; Billerica, MA, USA). Gradient grade acetonitrile and methanol (p.a.) were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation

The glycans of a mAb were released by incubation with PNGase-F at 37 °C in 15 mM Tris/HCl pH 7.0 over night. Afterwards the glycans were separated from the proteins by ultrafiltration using Microcon® YM-30 centrifugal filter devices. The reducing end was labeled with 50 mg/mL 2-aminobenzamide and 63 mg/mL $Na[BH_3(CN)]$ in dimethylsulfoxide/acetic acid 7:3 at 37 °C over night. The excess label was depleted by gel filtration on PD MiniTrap™ Sephadex® G-10 column. Fetuin and RNase-B were denaturated in 6 M guanidine chloride at 80 °C for 2 h prior to the PNGase-F digest.

An aliquot of fetuin glycans was treated with neuraminidase, according to the instructions of the manufacturer, to remove the sialic acids. The resulting sample is termed “asialo fetuin” within this publication.

2.3. Instrumentation

Fluorescence chromatograms were recorded on an Agilent 1200 SL system, consisting of a binary pump, a vacuum degasser, an autosampler, a column thermostat and a fluorescence detector. The excitation wavelength was 250 nm and the emission was recorded at 428 nm.

2.4. Hydrophilic interaction chromatography

The glycan samples from fetuin, asialo fetuin and RNase-B were fractionated on a Waters Acquity UPLC® BEH Glycan column (2.1 mm i.d., 150 mm length) packed with 1.7 μm particles. The temperature of the column thermostat was set to 60 °C. Solvent A was acetonitrile and solvent B was 150 mM formic acid titrated to pH 4.4 with ammonia solution (25%). For fractionation of fetuin glycans a linear gradient starting with 37% B and reaching 42% B after 40 min was applied. Asialo fetuin and RNase-B glycans were fractionated utilizing a linear gradient from 30% B to 45% B within 40 min. The flow was 0.5 mL/min. Fractions were collected manually.

Retention times of the individual glycans were determined by re-injection on the same column. The method started with 30% B, which was increases by 0.5%/min. The column temperature was maintained at 60 °C. Retention times determined by re-injection were employed for data analysis.

The monosaccharide compositions of the glycans were determined by MS analysis using a LIT-Orbitrap® instrument (Thermo Electron). This type of mass spectrometer provides accuracies typically below 10 ppm, which allows for reliable determination of the monosaccharide composition.

For analysis by IP-RP and PGC chromatography the fractions were concentrated in a centrifugal evaporator. For re-chromatography the fractions were diluted with water 1:10. Samples exhibiting unsatisfactory peak shape when re-chromatographed were diluted with water 1:50.

2.5. Ion pairing reversed-phase chromatography

For the reversed-phase separation a Waters Acquity® UPLC® BEH300 C18 column (2.1 mm i.d., 100 mm length) packed with 1.7 μm particles was utilized. As for all chromatography methods, a linear gradient was developed to achieve elution of all glycans within approximately 30 min. Mobile phase A contained 20 mM diethylamine (DEA) as ion pairing agent and 50 mM formic acid in water. Mobile phase B was 20 mM DEA and 50 mM formic acid in 25% methanol and 75% water. The gradient started with 10% B, which was raised to 20% B within 40 min. The flow rate was 350 μL/min. The column was maintained at 60 °C.

2.6. Porous graphitic carbon chromatography

A Thermo Hypercarb™ column (3 mm i.d., 100 mm length) packed with 3 μm particles was used for PGC chromatography. Mobile phase A contained 100 mM trifluoracetic acid (TFA) and 50 mM ammonia in water. Mobile phase B contained 100 mM TFA and 50 mM formic acid in 55.6% acetonitrile and 44.4% water. A linear gradient from 45% to 100% B within 30 min was applied. The

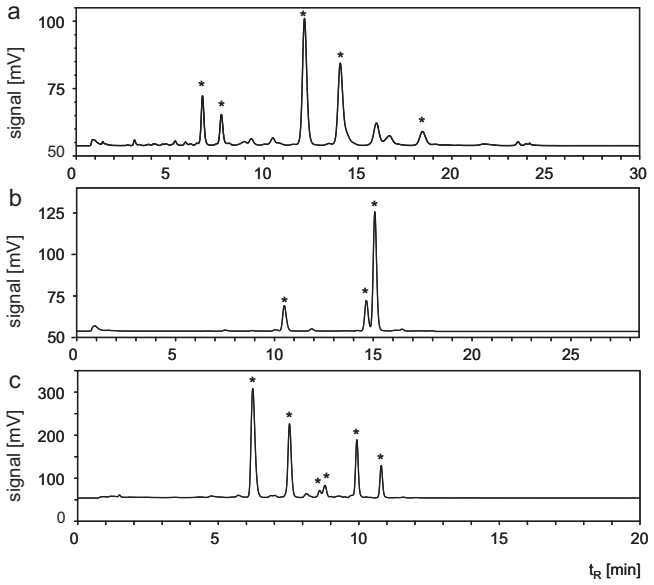

Fig. 1. HILIC chromatograms of (a) fetuin glycans, (b) asialo fetuin glycans, and (c) RNase-N glycans used for fractionation of these samples. Fractionated peaks are indicated by asterisks.

flow was 500 μL/min. The column compartment temperature was set to 75 °C.

2.7. Multiple linear regression analysis of retention data

The retention data obtained by HILIC, IP-RPC and PGC chromatography were analyzed by multiple linear regression (MLR) using the Microsoft Excel® *Data Analysis* add-in. For each method the retention times were correlated to the numbers of fucose (Fuc), N-acetylglucosamine (GlcNAc), galactose (Gal), mannose (Man) and sialic acid (N-acetylneuraminic acid – NeuNAc), respectively, of the glycans, according to Eq. (1). The MLR provides the parameters a_n for each chromatography method by means of maximizing the variance explained by the model. By comparison of these parameters the impact of different types of monosaccharides could be quantitatively evaluated. High correlation is obtained for chromatography methods where retention is primarily determined by the monosaccharide composition, whereas low correlation indicates that significant factors, e.g. the three-dimensional structure, were not included.

Equation used for modelling of the retention times of the glycans according to their respective monosaccharide composition:

$$t_R = a_0 + a_1 \times \mathrm{Fuc} + a_2 \times \mathrm{Man} + a_3 \times \mathrm{Gal} + a_4 \times \mathrm{GlcNAc} + a_5 \times \mathrm{NeuNAc} \quad (1)$$

3. Results and discussion

For the unambiguous correlation of retention times of glycans on different columns mass spectrometric detection is insufficient, because isomeric glycans cannot be differentiated by their mass. Hence, the glycan samples containing isomers in comparable amounts (fetuin, asialo fetuin, Rnase-B) were fractionated by HILIC, which is the standard chromatography method for fluorescently labeled glycans. Due to the high content of organic solvent in the running buffer, as well as the elevated column temperature the back pressure was comparably low allowing for the use of an ultra-high performance column with our HPLC system with a pressure limit of 600 bar.

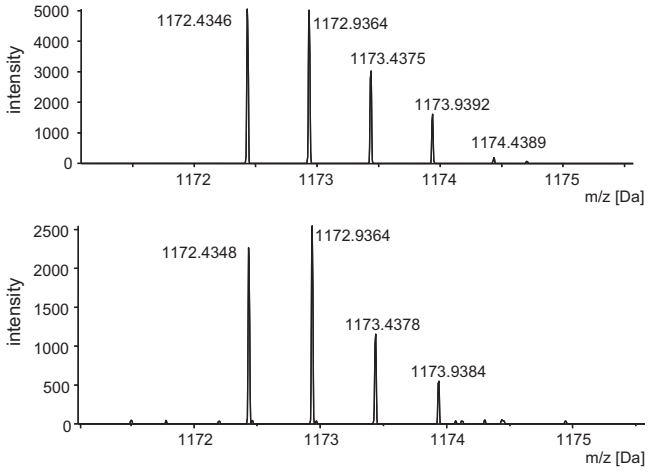

Fig. 2. MS spectra of isomeric fetuin glycans with the composition $HexNAc_4Hex_5NeuAc_2$.

Fig. 1 shows representative HILIC chromatograms, which were used for fractionation of the fetuin, the asialo fetuin, and the RNase-B samples. The monosaccharide compositions of the fractionated glycans were determined by MS on an Orbitrap instrument. As an example Fig. 2 shows the MS spectra of the $HexNAc_4Hex_5NeuNAc_2$ isomers found in the fetuin glycan sample. The deviation from the theoretical *m*/*z*-value (1172.4337 Da for the $[M+2H]^+$ ion) are remarkably small, corresponding to −0.8 ppm and −0.9 ppm, respectively.

The glycans of the mAb sample are well characterized [18]. The main glycans exhibit the compositions $HexNAc_4Hex_3dHex_1$, $HexNAc_4Hex_4dHex_1$, and $HexNAc_4Hex_5dHex_1$. The corresponding peaks could be assigned due to their characteristic profile in all chromatography methods employed, as demonstrated by the chromatograms in Fig. 3. Solely in the IP-RPC method the $HexNAc_4Hex_4dHex_1$ isomers were not resolved, i.e. they exhibited the same retention time. Hence, this sample needs not to be fractionated.

To attain comparability between the retention data of the three chromatography methods linear gradients were applied achieving

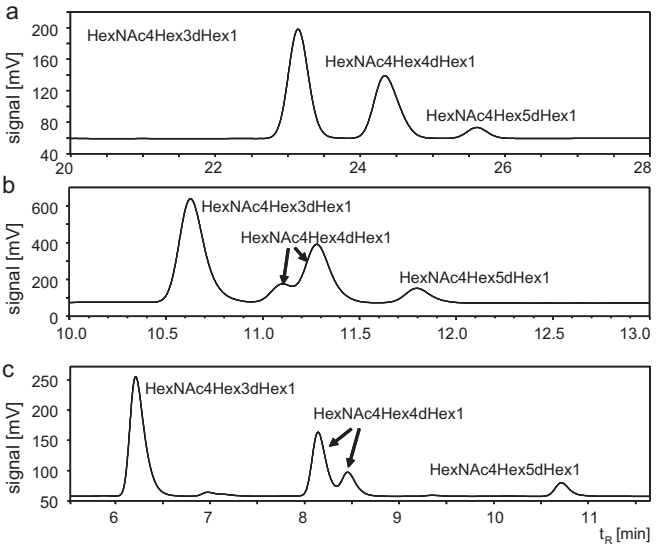

Fig. 3. Chromatograms of the mAb glycan sample obtained by (a) reversed-phase, (b) PGC, and (c) HILIC showing the peak pattern of the main glycan variants $HexNAc_4Hex_3dHex_1$, $HexNAc_4Hex_4dHex_1$ (two isomers), and $HexNAc_4Hex_5dHex_1$. Note that in RPC mode the $HexNAc_4Hex_4dHex_1$ isomers are not separated.

Table 1
Retention times of 2-AB-labeled N-glycans from different sources on HILIC, IP-RPC and PGC, respectively. For all methods a linear gradient was applied to achieve elution of all glycans within approximately 25 min. The monosaccharide composition was assigned based on exact mass. Isomeric glycans are indicated by the shading of the area.

Sample	Monosaccharide composition	Number of hydroxyl groups	t_R(HILIC) [min]	t_R(IP-RPC) [min]	t_R(PGC) [min]
Fetuin	$HexNAc_4Hex_5NeuAc_2$	31	16.12	16.06	15.76
	$HexNAc_4Hex_5NeuAc_2$	31	17.34	15.20	12.31
	$HexNAc_5Hex_6NeuAc_3$	40	21.50	21.84	24.45
	$HexNAc_5Hex_6NeuAc_3$	40	22.57	21.07	19.42
	$HexNAc_5Hex_6NeuAc_3$	40	22.57	21.07	19.65
	$HexNAc_5Hex_6NeuAc_4$	44	24.55	22.35	21.53
Asialo fetuin	$HexNAc_4Hex_5$	23	9.14	12.05	8.92
	$HexNAc_5Hex_6$	28	12.47	16.20	11.04
	$HexNAc_5Hex_6$	28	12.84	16.21	11.85
mAb	$HexNAc_4Hex_3dHex_1$	19	6.22	23.14	10.63
	$HexNAc_4Hex_4dHex_1$	22	8.46	24.34	11.10
	$HexNAc_4Hex_4dHex_1$	22	8.15	24.34	11.28
	$HexNAc_4Hex_5dHex_1$	25	10.72	25.62	11.80
RNase-B	$HexNAc_2Hex_5$	19	6.81	6.10	11.85
	$HexNAc_2Hex_6$	22	9.06	4.91	6.86
	$HexNAc_2Hex_7$	25	13.01	5.23	6.76
	$HexNAc_2Hex_7$	25	13.58	4.18	6.04
	$HexNAc_2Hex_7$	25	13.58	4.62	6.43
	$HexNAc_2Hex_8$	28	14.34	3.96	5.71
	$HexNAc_2Hex_9$	31	16.50	3.97	5.73

elution within approximately 25 min. The mobile phases were composed of an aqueous buffer and an organic solvent. The determined retention times are shown in Table 1. The reported peak capacities were calculated from the chromatographic data of the latest eluting glycan in the respective method.

3.1. HILIC

HILIC has become the method of choice for the analysis of oligosaccharides due to the high retentivity and selectivity for these hydrophilic compounds. The amide-based ligand of the stationary phase used in this study exhibited typical HILIC selectivities, i.e. glycans are retained according to their hydrophilicity. Hence, the retention times correlate to the number of hydroxyl groups of the compound (correlation coefficient 0.981) as illustrated in Fig. 4.

The peaks are evenly distributed within the elution range, thus minimizing the number of overlapping peaks. Since the glycans used in this study significantly differ in their polarity a steep gradient was applied to achieve elution within about 25 min. This steep gradient resulted in narrow peaks. The now available UHPLC material additionally provides superior peak capacity, which was determined to be 92 based on the last eluting peak ($HexNAc_5Hex_6NeuNAc_4$, t_R = 24.55 min).

Since every monosaccharide contributes to the hydrophilicity of a glycan one may anticipate that retention times in HILIC-mode are correlated to all types of monosaccharides. But the results of the MLR analysis as shown in Table 2 indicate, that the contribution of Fuc and GlcNAc are insignificant ($P > 0.05$), which may be explained by the comparably low hydrophilicity of these monosaccharides. A regression including the remaining monosaccharides produced a

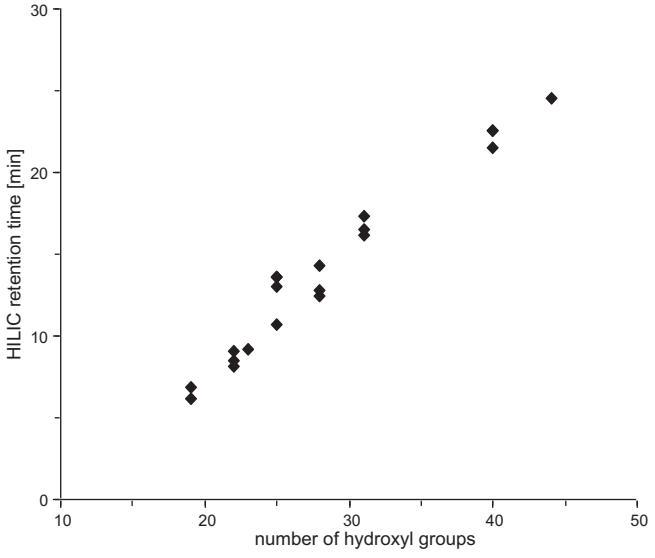

Fig. 4. HILIC retention times of glycans plotted against the number of hydroxyl groups, which are given in Table 1.

Table 2
Results of the multiple linear regression analysis of the retention times of the glycans in HILIC, IP-RPC and PGC chromatography. For each parameter (a_0 to a_5) the confidence interval (95%) is given. Furthermore the regression coefficient R^2, the significance value (F-value) and the number of data points (n) are reported for each model.

HILIC	Intercept	a_0	−5.4 ± 6.3
	Fucose	a_1	1.1 ± 2.1
	Mannose	a_2	2.3 ± 0.55
	Galactose	a_3	2.3 ± 1.5
	N-acetylglucosamine	a_4	0.87 ± 1.7
	N-acetylneuraminic acid	a_5	3.2 ± 0.5
	R^2		0.983
	F		167
	n		20
IP-RPC	Intercept	a_0	2.8 ± 3.6
	Fucose	a_1	12.9 ± 1.2
	Mannose	a_2	−0.34 ± 0.32
	Galactose	a_3	1.2 ± 0.85
	N-acetylglucosamine	a_4	2.1 ± 1.0
	N-acetylneuraminic acid	a_5	1.7 ± 0.29
	R^2		0.997
	F		1117
	n		20
PGC	Intercept	a_0	11± 15
	Fucose	a_1	1.4 ± 5.1
	Mannose	a_2	−0.57 ± 1.4
	Galactose	a_3	0.58 ± 3.6
	N-acetylglucosamine	a_4	0.11 ± 4.3
	N-acetylneuraminic acid	a_5	2.9 ± 1.2
	R^2		0.901
	F		25
	n		20

model with a regression coefficient of 0.987, which is comparable to the correlation of the retention times with the number of hydroxyl groups. Since the latter model bases upon only two parameters it offers a higher significance ($F = 167$). Nonetheless the number of hydroxyl groups provides a similar correlation applying a simple linear regression and thus the highest significance ($F = 449$) and is thus the best model for HILIC.

The two types of hexoses found in the glycans, mannose and galactose, both increased the retention time by 2.3 min (see Table 2). Both fucose, a deoxyhexose, and N-acetyl glucosamine caused lower increases in retention time, while the effect of N-acetylneuraminic acid was stronger, even though these differences were not significant at a 95% confidence level. HILIC also offers certain selectivities for isomeric glycans, as illustrated, e.g. in Fig. 3c by the separation of $HexNAc_4Hex_4dHex_1$ isomers.

3.2. IP-RPC

In reversed-phase chromatography (RPC) structurally related glycans elute in groups, whereas high-mannose type glycans exhibit the lowest retention. Man9 and Man8 eluted first with no significant difference in their retention times, which was already reported [10]. The chromatograms of both Man7 fractions separated on HILIC exhibited an additional peak, with a retention time between these two isomers. Deducing from the retention times on HILIC and on IP-RPC this peak was assigned to an additional Man7 isomer. Interestingly Man6 showed lower retention than the latest eluting Man7 isomer. Apart from that the retention of high-mannose glycans was inversely correlated to their size, as reported in literature [9,10].

The mAb glycans $HexNAc_4Hex_3dHex_1$, $HexNAc_4Hex_4dHex_1$, $HexNAc_4Hex_5dHex_1$ were baseline separated, whereas the method offered no selectivity for the $HexNAc_4Hex_4dHex_1$ isomers, as illustrated in Fig. 3a. For complex glycans the core fucose exhibited the most decisive effect on the retention. While the fucosylated $HexNAc_4Hex_5dHex_1$ offered the highest retention of all glycans the corresponding non-fucosylated glycan was the first eluting complex glycan. An additional antenna, as well as galactosylation resulted in increased retention times.

Sialylated glycans were well separated from corresponding neutral glycans by the addition of the ion pairing agent. In contrast to RP methods applying unbuffered acetic acid as additive in the mobile phase, peak tailing was not observed [9,10].

Despite the use of a UHPLC column the peak capacity of 50 calculated for the last eluting peak ($HexNAc_4Hex_5dHex_1$, $t_R = 25.62$ min) was the lowest for the tested methods, due to the shallow gradient applied. This may be a drawback for complex glycan samples, which necessitate a high peak capacity for resolution of all compounds for in-depth characterization. Nevertheless, due to the grouping of the glycans the IP-RPC method may be useful if only quality parameters, e.g. the degree of galactosylation, sialylation, fucosylation and mannosylation are required [19].

The MLR analysis indicated statistical significance for all monosaccharides at the 95% confidence level. The resulting model exhibited an excellent approximation with a R^2 of 0.997. The significance of the model could be further enhanced by summing up Gal, GlcNAc and NeuNAc monosaccharide units giving a single parameter ($F = 1911$), because these monosaccharides increased retention times in a comparable magnitude. Thereby the number of parameters was reduced to four, while the correlation was not significantly affected. Hence the measured retention times of 2-AB labeled glycans in IP-RPC could be described quite precisely by their monosaccharide composition.

3.3. PGC chromatography

PGC offers increased retention of hydrophilic analytes due to the polar retention effect on graphite (PREG) [20] compared to conventional reversed phases. Even reduced glycans are sufficiently retarded to allow direct analysis, while the rigid surface provides alternative selectivities.

Similar to the IP-RPC method, high-mannose glycans elute first with the bigger glycans exhibiting lower retention. Man5 is considerably stronger retarded on PGC, eluting in the range of fucosylated biantennary glycans. Core fucosylation and additional antenna result in stronger retention, but this increase is significantly lower than in IP-RPC. Sialic acids cause a substantial increase in retention with the highly sialylated glycans exhibiting the highest retention times on PGC. This remarkable selectivity for sialylation indicates extensive interactions of PGC with charged moieties. Hence, for chromatography of oligosaccharides PGC can be considered as highly retentive reversed-phase type stationary phase with additional Coulomb type interactions with acidic moieties. To enhance the elution of highly sialylated glycans TFA was used in the mobile phase, which offers high elution strength for anionic species [21].

The peak capacity was 64 for the last eluting peak ($HexNAc_5Hex_6NeuNAc_3$; $t_R = 24.45$ min) outperforming the UHPLC ODS column used for IP-RPC. This was accomplished by the much steeper gradient, which could be applied due to higher selectivities on PGC.

The retention times on PGC could not be satisfactorily correlated to the monosaccharide compositions by MLR analysis ($R^2 = 0.901$). Solely the number of sialic acids exhibited a significant effect ($P = 0.000039$), which again demonstrates the impact of Coulomb type interactions on the retention. A simple regression of the retention times with the number of sialic acids moderately improved the significance of the model ($F = 65$).

Even though hydrophobic interactions contribute to retention of oligosaccharides on PGC the retention mechanism is quite distinct from conventional reversed-phase chromatography. PGC offered major selectivities for isomeric glycans, which generally cannot be predicted by the monosaccharide composition MLR model. Furthermore the effect on the retention resulting from the addition of a single monosaccharide unit differs for individual glycans, e.g. addition of a mannose moiety to Man8 has no effect on the retention, whereas addition to Man5 results in a significant decrease of the retention. Thus structural implications of attached monosaccharides need to be considered for estimation of the retention on PGC.

3.4. Comparison of chromatography systems

In the diagrams in Fig. 5 the retention times in the respective methods are plotted against one another. Thus selectivities for structural elements, e.g. core-fucosylation or sialylation, as well as correlations between the methods become directly apparent.

The low correlation between HILIC and IP-RPC (correlation coefficient 0.17) demonstrates the orthogonality of the retention mechanisms. Selectivities for isomeric glycans were quite low in both, HILIC and IP-RPC limiting the usefulness of this combination for detailed characterization of complex glycan samples.

In both, HILIC and PGC sialylated glycans are strongly retained due to their high hydrophilicity and their negative charges, respectively. Thus a certain correlation between these methods was observed, indicated by a correlation coefficient of 0.67. For neutral glycans the correlation coefficient was significantly lower (0.38).

Since PGC can be regarded as a reversed-phase with additional ionic interaction properties certain correlation to IP-RPC may be expected. Based on the whole glycan sample set the correlation coefficient of the retention times was 0.69 indicating moderate

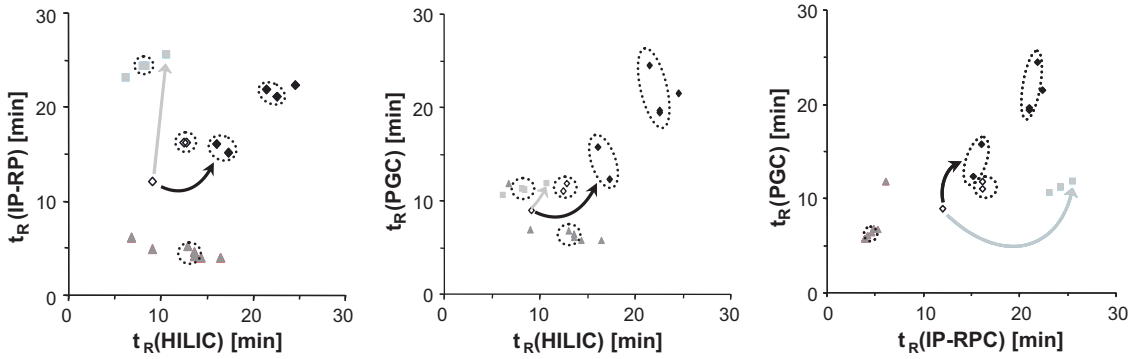

Fig. 5. Retention times of high-mannose (▲), fucosylated complex (■), non-fucosylated complex (◊) and non-fucosylated complex sialo glycans (♦) in HILIC, IP-RPC and PGC plotted against one another, respectively. Light grey arrows indicate the effect of the addition of a core-fucose. The black arrows indicate the effect of the addition of two sialic acids. Isomers are marked by dotted ellipses.

correlation of these chromatography methods. The selectivities for high-mannose glycans are similar on RPC and on PGC. In both methods three Man7 isomers were separated, while Man8 and Man9 could not be resolved on any of these columns. The increase of retention time caused by the core fucose was more prominent on RPC. Sialic acids exhibit a more prominent increase on the retention on PGC than in IP-RPC, demonstrating the impact of ionic interactions on PGC.

4. Conclusion

HILIC offered high selectivities for the tested glycans, thus allowing for a steep gradient to be applied. This fact together with the availability of a UHPLC stationary phase provided a superior peak capacity. Due to the high content of acetonitrile in the mobile phase the column backpressure is comparably low allowing for the use of a long column on a HPLC system with a pressure limit of 600 bar. Thus HILIC is particularly useful for detailed analysis of complex glycan samples.

In RPC fluorescently labeled glycans interact with the stationary phase primarily via the aromatic label. Thus selectivities for glycans in RPC were generally low and only a shallow gradient could be applied, which significantly reduced the peak capacity. DEA as an ion pairing agent increased the retention of sialylated glycans and enabled the separation of isomeric sialylated glycans by IP-RPC. Furthermore the peak shapes of sialylated glycans were improved compared to recently published methods employing un-buffered mobile phases. Due to the grouping of the glycans and the availability of UHPLC materials, reversed-phase chromatography may be a suitable method for quality control purposes, e.g. for rapid determination of the degree of galactosylation, sialylation, fucosylation and mannosylation of mAb glycan samples, respectively.

PGC interacts with hydrophilic analytes, e.g. with glycans, via induced dipoles, thus increasing retention compared to conventional reversed-phases. The polar retention effect accounts for increased selectivities, particularly for sialylated glycans. Furthermore the steeper gradient results in superior peak capacities compared to conventional reversed-phases. Hence PGC is an interesting alternative to HILIC for the separation of isomeric, sialylated N-glycans. On the other hand the reproducibility and robustness of operation of the PGC column are somewhat limited [4,22] hampering its application for routine analysis. The monosaccharide composition was found insufficient to explain the retention behavior of the glycans indicating the impact of the three-dimensional structure of the glycans on their retention on PGC.

Each of these chromatography methods may be suitable for specific questions, e.g. the demonstration of batch-to-batch consistency. But for the comprehensive characterization of complex glycan samples a combination of these three chromatography techniques and hyphenation to MS is recommended. The conventional reversed-phase column offered a high degree of orthogonality compared to HILIC. This combination will probably resolve the highest number of glycans in a complex sample, but due to the incompatibility of the mobile phases IP-RPC fractions must be diluted with acetonitrile prior to re-chromatography in the HILIC mode. On the other hand IP-RPC fractions can be directly injected on a PGC column minimizing sample handling. The high selectivity of PGC for isomeric, sialylated glycans renders this combination an interesting alternative for heterogeneous glycan samples containing highly sialylated glycans.

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