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Passamonti, M.

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Chapter 4

Influence of ion-pairing reagents on the separation of intact glycoproteins using hydrophilic-interaction liquid chromatography - high-resolution mass spectrometry

Abstract

Hydrophilic-interaction liquid chromatography (HILIC) of intact proteins offers high-resolution separations of glycoforms of glycoproteins differing in the number of (neutral) glycans. However, to obtain efficient separations it is essential that the positively charged sites of the proteins are shielded by acidic (negative) ion-pair reagents (IPRs), so as to enhance the contribution of the hydroxyl groups of the (neutral) sugars in the glycoprotein. Here, we studied the influence of various IPRs that differ in physico-chemical properties, such as hydrophobicity and acidity, on the capillary-scale HILIC separation of intact (glyco)proteins. We evaluated the use of fluoroacetic acid (MFA), difluoroacetic acid (DFA), trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA) as diluents for sample preparation, as solvents for sample loading on a reversed-phase trap prior to the HILIC separation, and as mobile-phase components for HILIC and HILIC-MS. To reduce the contribution of ion-exchange interaction with the (silica-based) stationary phase, we used an acrylamide-based monolithic column. We studied the influence of the different IPRs on each step of the separation of a mixture of proteins of different size and hydrophilicity and on the separation of the five glycoforms of ribonuclease B. The content of IPR in the sample was shown not to affect the separation and the MS detection. However, a low content of TFA and DFA in the mobile phase is favourable, as it reduces adduct formation and leads to a high signal intensity. The results of this study are successfully demonstrated by the analysis of horseradish peroxidase (HRP), as an example of a complex glycoprotein.

4.1 Introduction

Hydrophilic-interaction liquid chromatography (HILIC) is an approach for the separation of polar compounds based on hydrophilic partitioning, hydrogen bonding, dipole-dipole and ionic interactions[1]. HILIC in combination with mass spectrometry (HILIC-MS) is frequently used for the characterization of small polar molecules and metabolites[2,3] and, more recently, such methods have been extended to the analysis of intact proteins, particularly glycoproteins.[4,5] Glycosylation is a common post-translational modification (PTM), in which saccharide chains are attached to the protein[6]. These chains are referred to as glycans. Nowadays, intact-glycoprotein analysis by HILIC employs silica-based polar stationary phases (mostly with an amide chemical selector). Elution is performed using shallow linear gradients using mixtures of acetonitrile and (increasing concentrations of) water, with an ion-pair reagent (IPR) added. The use IPRs, such as strong acids, enhances the separation performance and reduces band broadening and peak tailing. Furuki *et al.*[7] systematically studied such effects in glycopeptide separations and concluded that negatively charged IPR bind to the basic and positively charged groups of the glycopeptide, reducing the interaction of the molecule with the stationary phase.

Trifluoroacetic acid (TFA) is the most widely used IPR for the separation of proteins and specifically for the separation of glycoforms of glycoproteins. It provides high separation efficiency, and little peak tailing, it facilitates protein solubilization, and improves protein recovery[8]. Nevertheless, the use of TFA has some drawbacks when coupling HILIC to MS. TFA inhibits the ionization of the analytes, causing a low signal, distributed over multiple protein-TFA adducts. Finally, in electrospray ionization (ESI) the presence of TFA influences the spray, due to its high conductivity and the high surface tension of the eluent.

One way to reduce the negative effects of TFA is to use high in-source collision energies (e.g. above 50 eV) during the MS analysis. However, this may lead to in-source fragmentation of proteins with labile structures, hampering their intact analysis. Several post-column approaches have been applied to eliminate TFA adducts. The most recent one was reported by Wouters *et al.*[9] and involved a multi-channel microfluidic device that selectively removed TFA ions, exchanging them for propionic or formic acid. In several studies, it was attempted to replace TFA by another IPR[10] or to reduce its concentration in the mobile phase. Tengattini *et al.*[11] found that at least 0.05% of TFA was needed to achieve a satisfactory separation of glycoforms. Zhang *et al.*[12] were able to lower the amount of TFA by adapting on stationary phase. A thick (1 nm to 17 nm) brush layer of polyacrylamide was used to modify silica particles, so as to minimize interactions between free silanol groups and the cationic groups on the protein. Recently, Lardeux *et al.*[13] concluded from a study on monoclonal antibodies that the use of TFA as IPR is unavoidable for HILIC-MS separations.

In this paper, we report on a study aimed at minimizing the presence and the effects of TFA in HILIC-MS of intact (glyco-) proteins. We studied the influence of several IRPs with different hydrophobicity. We used a capillary setup with a trap-and-elute configuration[14,15] to load the sample on an acrylamide-based monolithic stationary phase.[16] The influence of the different IPRs was evaluated using two samples, *i.e.,* a protein mixture and the five main glycoforms of ribonuclease B (RnB). We took into account all the steps of the micro-LC separation, from the sample preparation to the MS analysis. Our

ultimate objective was to apply our method to the separation of a complex horseradish-peroxidase (HRP) glycoprotein, allowing us to monitor its seven main N-linked glycoforms.

4.2 Experimental section

4.2.1 Material

Acrylamide (AA, electrophoresis grade, 99%), *N,N'*-methylenebisacrylamide (MbA, 99%), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 98%), 2,2'-azobisisobutyronitrile (AIBN, 98%), 1-octanol (OctOH, 99%), dimethyl sulfoxide (DMSO >99.9%), toluene, sodium hydroxide (NaOH), carbonic anhydrase from bovine heart (CA, >90%), cytochrome C (CC), bovine serum albumin (BSA), ribonuclease B from bovine pancreas (RnB, ≥80%), transferrin from human serum, (Tf), trifluoroacetic acid (TFA, ≥99%), formic acid (FA, analytical grade >98%), difluoroacetic acid (DFA, 98%), heptafluorobutyric acid (HFBA, ≥98%), fluoroacetic acid (MFA, 95%), horse-radish peroxidase (HRP), sodium dihydrogen phosphate monohydrate (≥ 99%), sodium phosphate dibasic dihydrate (≥ 99%) and ammonium acetate (AmAc ≥ 98%) were purchased from Sigma Aldrich (St. Louis, MR, United States). Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid 37% (HCl) was obtained from Acros (Geel, Belgium). High-purity (HP) water (18.2 MΩcm) was produced by a Sartorius (Göttingen, Germany) Arium 611UV Ultrapure-Water System. Capillary tubing (0.20 mm ID, 0.36 mm OD) was purchased from CMScientific (Silsden, UK). All composition percentages in the text are by volume, unless otherwise specified.

Table 1. Properties of the different ion-pairing reagents used. *P* is the octanol-water distribution coefficient. All values were obtained from Sci-Finder[17].

	pKa	pH of 0.1% in H_2O	Log P	Molecular weight (g/mol)	Physical State	Boiling Point (°C)	Vapour pressure (×10^3 Pa)
MFA	2.62 ± 0.1	2.39	- 0.11 ± 0.26	78.04	Solid	165	110
DFA	1.32 ± 0.1	2.00	0.38 ± 0.35	96.03	Liquid	133	58.5
TFA	0.05 ± 0.1	2.04	1.35 ± 0.46	114.02	Liquid	73	128
HFBA	0.37 ± 0.1	1.74	3.39 ± 0.60	214.04	Liquid	121	13.1
FA	3.74 ± 0.1		-0.54 ± 0.19	46.03	Liquid	100.5	48.3

*pH was measured with the pH-meter in our laboratory

4.2.2 Preparation of poly(acrylamide-co-N,N'methylenebisacrylamide) monolithic columns

Poly(AA-*co*-MbA) monolithic columns were created in 200-*µ*m ID capillary tubing and used for the separations of intact (glyco)proteins. The capillaries were firstly etched and subsequently vynilized, as described by Passamonti *et al.*[16]. A polymerization mixture consisting of 13.75 wt% AA, 11.25 wt% MbA, 21.5 wt% OctOH, 53.5 wt% DMSO, and 1 wt% AIBN (with respect to the monomers) was prepared. The

capillaries were filled and sealed with rubber septa, before the polymerization took place in a water bath for 24 h at 60°C.

4.2.3 Sample preparation

Stock solutions of the single proteins were prepared by dissolving the protein standards in mixtures of 10 vol% ACN in MilliQ water. The analyzed samples consisted of a 0.1 mg/mL solution of four proteins, *viz.* CC, CA, BSA and Tf, a 0.5 mg/mL solution of RnB, and a 0.5 mg/mL solution of HRP with 1) no IPR, 2) 0.1% MBA, 3) 0.1% DFA, 4) 0.1% TFA, 5) 0.1% HFBA.

HILIC-UV. LC analyses were performed on an UltiMate RSLCnano system (Thermo Fisher Scientific, Breda, The Netherlands) equipped with an autosampler (5-μL loop), thermostatted column compartment with a ten-port, two-position valve and a loading-pump system (NCS-3500RS), and a UV-vis detector (VWD-3400RS). Using a set-up similar to that described by Gargano *et al.*[14], a trap-column (5 mm × 0.3 mm ID; C4 stationary phase, 5 μm particle size, 300 Å pore size; Thermo Fisher Scientific) was used to inject samples from water-based solutions. The sample was loaded onto the trap-column using a mobile phase containing 2% ACN in MilliQ water with 1) no IPR, 2) 0.1% MFA, 3) 0.1% DFA, 4) 0.1% TFA and 5) 0.1% HFBA and two buffers 1) 10 mM ammonium acetate (AmAc) and 2) 10 mM phosphate-saline buffer (PB).
The valve was switched after 3 min from the injection and a linear gradient was applied, followed by three fast washing cycles and 10 min re-equilibration time. Mobile phases consisted of 98: 2 = MilliQ: ACN with additives (A) and 98:2 = ACN: MilliQ with additives (B) were used. All the analyses were carried at 45°C using a flow rate of 2 μL/min and the monitored UV wavelength was 214 nm. The influence of the IPR in the sample was investigated using a gradient from 94 to 81% B in 1 min and then down to 65% B in 26 min. Further information about the applied gradients can be found in the figure captions.
The recovery was calculated by dividing the average area (n=3) of the single protein obtained by the HILIC-UV separation by the area of the same protein obtained by direct injection.

HILIC-HRMS. The micro-LC system was coupled to a Q-Exactive Plus Biopharma (Thermo Fisher Scientific, Bremen, DE) MS. 1 μL of a 0.5 mg/mL solution consisting of 10% ACN in MilliQ water was injected in full-loop mode and chromatographic conditions were further optimized.
The MS was operated in positive-ion mode, using steel-based emitters, a nano-ESI source at a voltage of 2 kV, transfer-capillary temperature of 300°C, and S-lens of RF 70.
Acquisition parameters for the protein mixture and HRP were as follows: m/z range, 400 - 6000; resolution at 200 m/z, 17,500; 10 microscans; max IT (Injection Time), 200 ms, AGC (Automatic Gain Control), 10^6, in source CID (Collision-Induced Dissociation), 20 eV; HMR (High-Mass Range) activated (trapping gas 1.2).
Acquisition parameters for RnB: m/z range 400 – 3000; resolution, 14,000; 4 microscans; max IT 200 ms; AGC 10^6, in source CID 20 eV, intact-protein mode activated (trapping gas 0.2).

DATA ANALYSIS. The chromatograms with different IPRs in the mobile phase were obtained using different optimized gradients. To compare the different chromatograms, we plotted the observed signal intensity *vs*. the percentage of H_2O. The "raw" chromatograms (intensity *vs*. time) are reported in the Supplementary Information (SI). To transform the time in percentage of H_2O for the HILIC separation of the protein mixture, we used the following equations:

for 0.1% TFA, $\%H_2O = 0.69t + 14.2$ (4. 1)

for 0.1% DFA, $\%H_2O = 0.62t + 18.5$ (4. 2)

for 0.1% MFA, $\%H_2O = 0.77t + 17$ (4. 3)

for 0.1% HFBA, $\%H_2O = 0.62t + 7.5$ (4. 4)

The same strategy was applied for the separation of RnB and the following equations were used:

for 0.1% TFA and 0.1% DFA, $\%H_2O = 0.54t + 24$ (4. 5)

for 0.1% MFA, $\%H_2O = 0.73t + 15$ (4. 6)

for 0.1% HFBA, $\%H_2O = 0.81t + 8.8$ (4. 7)

The separation power of the columns was assessed by calculating the peak capacity (n_c) using the following equation (4.8):

$$n_C = \frac{t_G}{1.7 \times \underline{w}_{0.5}} + 1 \qquad (4.\ 8)$$

where t_G is the gradient time and $\underline{w}_{0.5}$ is the average peak width at half-height.

The asymmetry of the peaks (A_S) was calculated using Chromeleon software (Thermo Fisher Scientific) according to the following equation (3):

$$A_S = \frac{RW_{5\%} + LW_{5\%}}{2 \times LW_{5\%}} \qquad (4.\ 9)$$

Where $RW_{5\%}$ and $LW_{5\%}$ are right and left peak width at 5% of the peak height, respectively.

The resolution for the separation of the five RnB proteoforms was estimated using the peak-valley ratio (P) as suggested by Christophe[18]:

$$P_{v,n} = 1 - v/h_n \qquad (4.\ 10)$$

Where v is the signal intensity at the valley between two peaks, and h_n is the intensity of the peak taken into consideration.

Total-ion-current (TIC) and extracted-ion-current (EIC) chromatograms and deconvoluted RnB masses were obtained using Freestyle software (Thermo Fisher Scientific), while deconvoluted masses of the other proteins were obtained using UniDec[19]. Charge range, mass range and sample-mass interval,

respectively, were 1-30, 12,000-13,000 Da, 5 Da for CC; 1-50, 28,000-29,500 Da, 1.0 Da for CA; 1-50, 66,000-68,000, 1.0 Da for BSA; 1-80, 79,000-83,000 Da, 1.0 Da for Tf.

EIC chromatograms were obtained by summing the intensities of different charge state for CC (883.8 , 951.7, 1030.9, 1124.4, 1236.8, 1374.1, 1545.7, 1766.2, 2060.5 m/z), CA (937.3, 968.4, 1001.9, 1037.6, 1076.6, 1117.3, 1161.9, 1210.4, 1262.8, 1320.3, 1383.0, 1452.3, 1528.5, 1613.4, 1708.2 m/z), BSA (1278.5, 1303.5, 1329.6, 1356.6, 1384.9, 1414.4, 1445.1, 1477.2, 1510.7, 1545.8, 1582.0, 1621.2, 1664.5, 1704.2 m/z) and Tf (2040.8, 2094.5, 2151.1, 2210.8, 2273.9, 2340.8, 2411.7, 2487.0, 2567.2, 2652.7, 2744.2 m/z).

EIC chromatograms of RnB (140,000 resolution) were obtained from 5 charge states. For RnB1 these were, in order of decreasing intensity, m/z 2129.3990, 1863.3501, 1656.4233, 1490.8819, 1242.5694; for RnB2 these were m/z 2152.5490, 1883.6070, 1674.4294, 1507.1871, 1256.0739; for RnB3 m/z 2175.6998, 1903.8632, 1692.4352, 1269.5785, 1523.2921; for RnB4 m/z 2198.8515, 1924.1203, 1710.5526, 1539.5978, 1283.1666; for RnB5 m/z 2222.0021, 1944.5020, 1728.5585, 1555.7030, 1296.6706. An extraction window of 0.2 m/z was used, and seven-point Gaussian smoothing was applied to all the chromatograms.

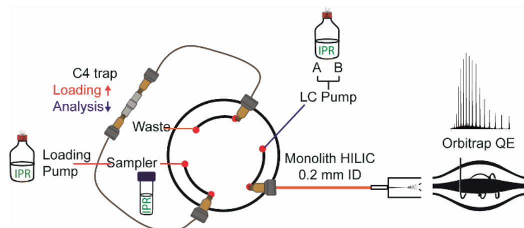

Figure 1. Schematic illustration of the HILIC-MS setup, indicating the three different areas where the influence of the ion pair reagent (IPR) was studied: sample, loading pump and mobile phase.

4.3 Results and discussions

4.3.1 Influence of ion-pairing reagents on chromatographic separations

We used an online trap-column to load and inject relatively large samples on a monolithic capillary column. The influence of IPR in the sample, in the loading pump and in the mobile phase was studied to assess the effects on the separation of (glyco)proteins.

IPR in the sample. Different IPRs, as described in Table 1, were used in the preparation of the samples. Using 0.1% TFA in the separation and in the loading solvent, we analyzed a 0.1 mg/mL solution containing CC, CA, BSA and Tf in triplicate. No variation in retention time was noticed (see SI, Figure S1). Small percentages of IPR in the sample (volume injection = 0.5 μL) were found not to influence the peak shape or the separation performance significantly. Therefore, we used 0.1% of TFA in the samples in all further experiments.

IPR in the loading solvent. To select the best IPR to load our sample onto the trap-column, we tested separations of the protein mixture using a gradient from 94% to 83% B in 1 min, then down to 65%B in 26 min at a flow rate of 2 μL/min. The mobile phases used for separations contained 0.1% of TFA, while in the loading solvent on the C4 trap column different IPRs were used *viz.* no IPR, acidic IPR (0.1% DFA, 0.1% TFA, 0.1% HFBA) and buffer systems (10 mM AmAc and 10 mM phosphate buffer (PB) at pH 7).

Peak capacity, retention time, peak area and asymmetry were the parameters used to evaluate the separation performance (see SI, Table S1). No significant changes or trends in retention times were noticed (variations were always within 2%). Similar asymmetry values (ranging between 1.2 and 1.8) were observed for the different IPRs, except for BSA and Tf, which showed some variation with the more hydrophobic IPRs (lower asymmetry values). Similar peak capacities were also found for the separations with DFA, TFA, HFBA and without IPR (ranging between 26 and 28). Lower peak capacity values were found for the separations with the buffers, 23 and 24 for the PBS and AmAc, respectively. While the separation with MFA gave a peak capacity of 20. Finally, the highest areas (see Figure 2) for all the proteins were observed when using no IPR or buffers in the loading pump, especially for the two largest proteins, BSA and Tf. Possibly, less-hydrophobic effluent causes a lower retention on the RP trap-column, resulting in some sample loss. Also, recovery values (see SI, Table S2) followed the same trend, with AmAc as loading pump solvent providing the highest values.

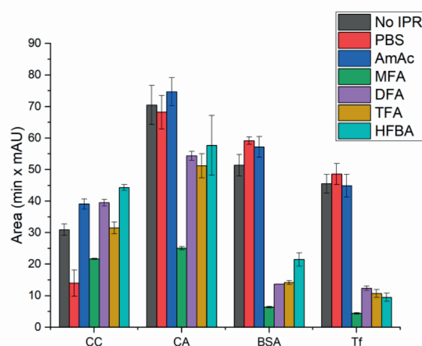

Figure 2. Area of the different proteins obtained using different loading pump solvents to load the samples onto the C4 trap column.

IPR in the mobile phase. The separations of the four standard proteins, *viz.* CC, CA, BSA and Tf (Figure 3), and of the five glycoforms of RnB (Figure 4) were performed using different IPRs in the mobile phases. Area, asymmetry value and (effective) peak capacity were the parameters taken into consideration in the evaluation. Depending on the IPR used, we adjusted the mobile-phase gradient, so as to spread the proteins over a time window of about 10 min, using the same monolithic column. TFA is generally believed to be the best IPR for HILIC separations of intact proteins. Indeed, the highest peak capacity (32) was achieved using 0.1% TFA in the mobile phase. Nevertheless, when the effective elution window is taken into consideration, the effective peak capacity[20] (n_c^{eff}) is similar for the

separations with different IPRs (see SI, Table S3). Lower performance (n_c^{eff} = 9) is found for the separation with 0.1% MFA, where CC and CA partially overlap, as do BSA and Tf (green line in Figure 3). This made it impossible to calculate the asymmetry values. We noticed a decrease in selectivity when using DFA or HFBA in the mobile phase (blue and black lines in Figure 3). The highest performance (n_c^{eff} = 13) was again found with 0.1% TFA, with all proteins well-resolved. Lower elution compositions (% H_2O) were recorded using more-hydrophobic IPRs in the mobile phase (Figure 3). In our previous work, shorter retention times were noticed with lower amounts of TFA and we hypothesized that this was due to the aqueous mobile phase becoming less hydrophilic[16]. When different IPRs are employed, also their acidity (pK_a) and partitioning coefficients (log *P*) have to be taken into account. HFBA is a strong acid, which binds with the basic group on the protein, forming a more-hydrophobic complex. The high log *P* of HFBA, leads to a relatively hydrophobic protein-HFBA complex, which elutes at high percentages of ACN, in contrast to the ore-hydrophilic protein-TFA complex. This is supported by separations performed at equal molar concentrations (0.1% TFA and 0.17% HFBA; see SI, Figure S2).

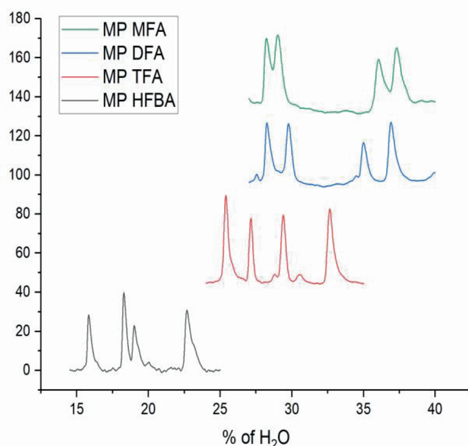

Figure 3. Comparison of HILIC-UV chromatograms of four intact proteins on the poly(AA-*co*-MbA) monolithic column (170 mm × 0.2 mm ID) using different IPRs in the mobile phase. The analyses were performed starting at 94% B, programmed down to initial conditions in 1 min. The gradients ranged from 80% to 60% of B in 26 min with 0.1% MFA (green), from 79% to 63% of B in 26 min with 0.1% DFA (blue), from 83% to 65% of B in 26 min with 0.1% TFA (red), and from 90% to 74% of B in 26 min with 0.1% HFBA (black). Protein elution order: CC, CA, BSA, Tf. Analysis temperature, flow rate and detection wavelength were 45°C, 2 μL/min and 214 nm, respectively.

Beside the separation of reference proteins we tested the effects on glycoprotein separations using RnB as model. For the separation of the glycoforms of RnB, different gradients were used to spread the glycoforms of RnB across a time window of about 5 min. As observed in the experiment on reference proteins, lower elution compositions were noticed when using more-hydrophobic IPRs (especially HFBA, black line in Figure 4). However, pronounced differences were observed in separation performance The highest peak-to-valley ratios (see SI, Table S4) were obtained using 0.1% HFBA in the mobile phase. Nevertheless, Figure 4 shows how the glycoforms are almost equally resolved with

0.1% TFA (red line in Figure 4), while loss of selectivity is observable with 0.1% DFA (blue line) and, especially, with 0.1% MFA (green line).

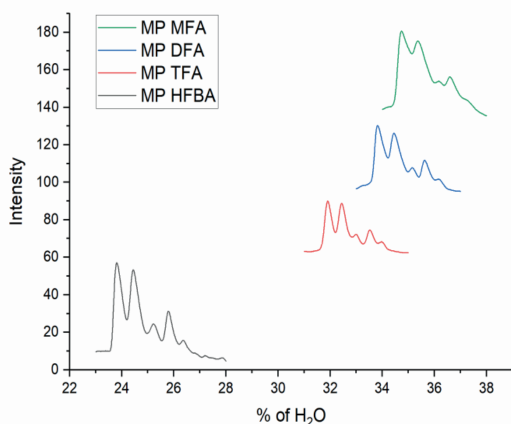

Figure 4. Comparison of HILIC separations of five glycoforms of RnB on the poly(AA-*co*-MbA) monolithic column (170 mm × 0.2 mm ID) using different IPRs in the mobile phases. The analyses were performed starting at 94% of B, programmed down to initial conditions in 1 min. The gradients were from 82% to 63% of B in 26 min with 0.1% FA (green), from 74% to 60% of B in 26 min with both 0.1% DFA (blue) or 0.1% TFA (red), and from 88% to 67% of B in 26 min with 0.1% HFBA (black). Analysis temperature, flow rate and detection wavelength were 45°C, 2 μL/min and 214 nm, respectively.

In this first part of the paper, our attention was focused on the role of the IPR in the sample, in the loading pump solvents and in the mobile phase. We can conclude that while the presence of IPR in the sample does not influence the chromatographic performance of the separation, its influence in the loading pump solvents is noticeable. Specifically, no IPR or buffers provide the highest areas and recovery. Finally, the use of 0.1% TFA in the mobile phase seems the best for the HILIC separation of intact proteins, while the glycoforms are better separated by HFBA. Nevertheless, a good separation of the glycoforms could be achieved also using TFA and DFA.

4.3.2 HILIC-MS of intact proteins and intact glycoforms of HRP

The HILIC-MS method was first applied to the mixture of proteins and, subsequently, to the HRP. Due to the poor chromatographic performance of the mobile phase containing 0.1% MFA, it was excluded from the MS study. When using 0.1% HFBA as IPR in the mobile phase, low and noisy signals were obtained. In the TIC chromatogram, the peaks are indistinguishable (see SI, Figure S5). Therefore, this IPR was also excluded from our pool.

Besides 0.1% TFA and 0.1% DFA as IRP, we also used a combination of 0.005% of TFA + 0.1% of FA, which showed better MS performance in our previous work[16]. The chromatographic performance of DFA was adequate (see above), but we need to consider the implications of its use in the MS detection. Although TFA is known for its ion-suppression behaviour, the highest intensities in this study were

obtained with 0.1% TFA and 0.005% TFA + 0.1% FA. However, 0.1% DFA yielded only slightly lower intensities for the four proteins slightly than 0.1% TFA.

DFA yielded fewer adducts compared to any other IPR used (see SI, Figure S6), but a noisier background. A shift to lower *m/z* is noticed, due to the formation of fewer adducts. The entire mass distribution shifted, so that also the most intense charge state was different depending on the IPR used. For 0.1% TFA, the most intense charge state was +27 with m/z 2947.384, for 0.1% DFA it was +31 at m/z 2567.189, and for 0.005% TFA + 0.1% FA it was +35 at m/z 2273.940. The highest charge state found for Tf was +39 (m/z = 2040.9163) for the separation with 0.005% TFA + 0.1% FA. The glycoforms of the RnB follow a similar trend (see SI, Table S6). The highest intensities were achieved using DFA and 0.005% TFA with 0.1% FA, while a fewer adducts was obtained using the latter.

With the knowledge gathered, we decided to proceed with the characterization of the glycoforms of HRP. HRP is a glycoprotein used in biomedical research and diagnostic assays. It is a metalloenzyme with many isoforms, It has nine N-glycosylation sites, eight of which present N-linked oligosaccharides of the $H_xN_yF_zP_w$ group (H= mannose, N= N-acetylglucosamine, F= fucose, P= xylose) or N-linked disaccharides comprising one F and one N. Since it is highly heterogeneous, it is used here as model glycoprotein.

We applied a gradient from 90 to 50% of B, using three different IPRs *viz.* 0.1% TFA, 0.1% DFA and 0.005% TFA + 0.1% FA (Figure 5). The retention time of the glycoforms increased when using a less hydrophobic IPR, as demonstrated with the protein mixture and RnB. Overall, the retention of the glycoforms increased with the size of the glycans. Sharp peaks are obtained with these three IPRs. DFA gave the narrowest peaks on average (see SI, Table S7), but provided less selectivity. The elution window of the HRP glycoforms is different for the three IPRs (7 min for 0.1% TFA, 5 min for 0.1 DFA and 8 min for 0.005% TFA + 0.1% FA). The three different elution windows of the HRP were divided into 1-min intervals and deconvoluted mass spectra were obtained. A table with the observed masses, theoretical masses and assigned formulas can be found for the separations obtained using different IPR in the SI (Table S7). The majority of proteoforms could be identified using a high content of TFA. DFA also allowed the identification of the main glycoforms. Unfortunately, the lowest coverage was obtained with the mobile phase containing 0.005% TFA + 0.1% FA, probably due to the substantial tailing of the peaks.

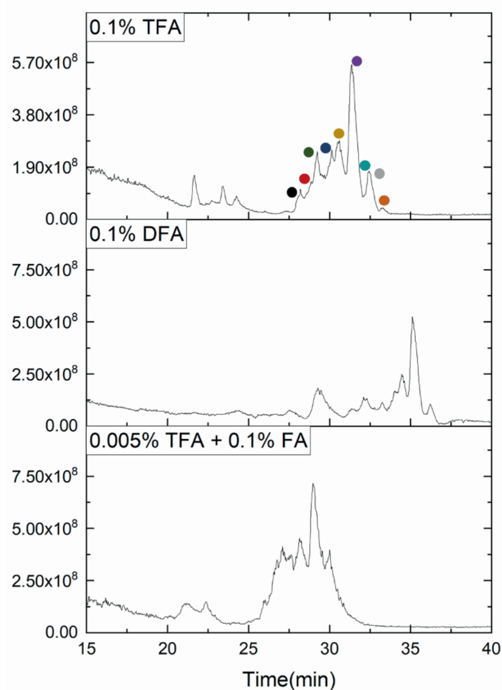

Figure 5. Comparison of TIC chromatograms of HRP on the poly(AA-*co*-MbA) monolithic column (170 mm × 0.2 mm ID) using different IPRs in the mobile phase. The analyses were performed using a gradient from 94 to 90% (v/v) of B (95/5 ACN/water) in 1 min and then down to 50% B in 34 min, with 0.1% TFA, 0.1% DFA and 0.005% TFA + 0.1% FA in the mobile phases. All the analyses were carried out at 45°C using a flow rate of 1.5 μL/min. The dots represent the glycoforms highlighted in Figure 6.

Nine glycoforms spread across the elution windows of the three methods were used to compare the mobile-phase conditions in terms of peak area, peak intensity, peak width, and retention time. For each glycoform, an extracted ion-current (EIC) chromatogram was obtained using three mass ranges (Figure 6, right-hand side). To obtain the mass of a glycoform from that of the intact protein, the summed mass (protein plus glycan) needs to be corrected for the breaking of four disulfide bonds (-8.1 Da), the formation of pyroglutamate (-17.1 Da) and the loss of serine (-87 Da)[21].

The deconvoluted mass of the most intense peak (HRP6) in the three TIC chromatograms of Fig.5 corresponds to a mass of 43174 Da, which could be assigned to the protein backbone with 8 $H_3N_2F_1P_1$ (see SI, Table S8).

The addition of an $H_3N_2F_1P_1$ unit or a core-fucose residue (N_1F_1) increased the retention time. A one-minute shift resulted the addition of the bigger units (*e.g.*, $H_3N_2F_1P_1$, $H_3N_2P_1$), while smaller units produced a smaller shift in retention time. The highest peak areas and peak intensities were obtained using 0.005% TFA + 0.1% FA. The charge distribution of the separations carried with 0.1% DFA and with 0.005% TFA + 0.1% FA were shifted to lower m/z (see SI, Figure S7). Also, more adducts were visible in the charge distribution obtained with 0.1% TFA, mostly at high m/z.

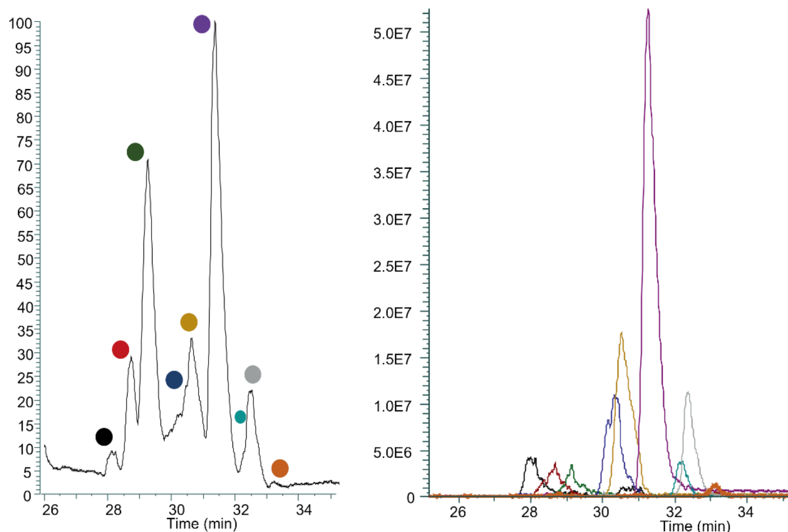

Figure 6. Base-peak (left-hand side, black trace) and EIC chromatograms from a HILIC-MS method with 0.1% TFA, carried out using a gradient from 94 to 90% (v/v) of B (95/5 ACN/water) in 2 min and then down to 50% in 35 min. All the analyses were carried at 45°C using a flow rate of 1.5 µL/min. The EIC chromatograms (overlaid on the right-hand side) have the same colour for the lines as the dots in the base-peak chromatogram. EIC signals were obtained by summing the intensities of different charge states, *i.e.,* HRP1 (black; 2668.4, 2858.8, 2501.6 m/z), HRP2 (red; 2691.6, 2883.8, 2526.4 m/z), HRP3 (green; 2402.9, 2723.1, 2553.0 m/z), HRP4 (blue; 2471.8, 2626.1, 2801.1 m/z), HRP5 (yellow; 2492.3, 2648.0, 2824.4 m/z), HRP6 (purple; 2399.6, 2540.6, 2699.3 m/z), HRP7 (light blue; 2456.6, 2600.9, 2763.4 m/z), HRP8 (grey; 2464.7, 2609.5, 2772.5 m/z) and HRP9 (orange; 2388.9, 2669.8, 2836.6 m/z). All the EIC are +/- 0.2 m/z.

4.4 Conclusions

Here we report a study into the influence of several ion-pairing reagents (IPRs) in a HILIC-MS separation of intact (glyco)proteins. The study was conducted taking into account every step from the sample loading, separation and MS detection. On the chromatographic side we concluded that IPRs had no influence on the sample preparation, possibly because we used a complex trap-and-elute method to load the sample,. The use of a mixture of water-acetonitrile with no IPR or buffers as loading pump solvent seems to yield higher peak areas when loading the sample on the reversed-phase trap column. As far as the use of IPR in the mobile phase is concerned, the use of TFA or HFBA is strongly advisable for the separation of intact proteins and/or glycoforms. DFA remains a viable, but less-selective option. When using MS detection HFBA is an unsuitable IPR, due to the noisy and low-intensity spectra produced. DFA, on the other side, provides clean and intense spectra due to less adduct formation. A mobile phase with 0.005% TFA + 0.1% FA as IPR was also tested. The latter provides the worst chromatography performance (broad and tailing peaks), but the best results in terms of MS detection (i.e., high intensity and low adduct formation). These results were also confirmed by studying HRP, a larger (*ca.* 40 kDa) reference glycoprotein. Nine N-glycoforms with a basic glycan structure of $H_3N_2F_1P_1$ were identified and the peaks were tentatively assigned.

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