Improvement of Electrospray Stability in Negative Ion Mode for nano-PGC-LC-MS Glycoanalysis via Post-Column Make-Up Flow

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Abbreviations:

ABC – ammonium bicarbonate; BPC – base peak chromatogram; DTT – dithiothreitol; ESI – electrospray ionization; EIC – extracted ion chromatogram; HCD – higher-energy collisional dissociation; HPLC – high-performance liquid chromatography; HILIC – hydrophilic interaction liquid chromatography; ID – inner diameter; IAA – iodoacetamide; LC-MS – liquid chromatography coupled to mass spectrometry; MS – mass spectrometry; C18 – octadecyl carbon chain; OD – outer diameter, PGC – porous graphitized carbon; PCMF – post-column make-up flow, KOH - potassium hydroxide; $NaBH_4$ - sodium borohydride; MS/MS – tandem mass spectrometry; UPLC – ultra performance liquid chromatography

Keywords:

Nano-electrospray, stability, post-column make-up flow, negative ion mode, glycans, glycomics, porous graphitized carbon, nano-liquid chromatography, Orbitrap, mass spectrometry

Abstract

Analysis of glycans via a porous graphitized carbon liquid chromatography (PGC-LC) coupled with electrospray ionization (tandem) mass spectrometry (ESI-MS(/MS)) is a powerful analytical method in the field of glycomics. Isobaric glycan structures can be identified reliably with the help of PGC-LC separation and subsequent identification by ESI-MS(/MS) in negative ion mode. In an effort to adapt PGC-LC-ESI-MS(/MS) to the nano-scale operation, spray instability along the nano-PGC-LC gradient was repeatedly observed on an LTQ Orbitrap Elite mass spectrometer equipped with a standard nano-electrospray ionization source. A stable electrospray was achieved with the implementation of a post-column make-up flow (PCMF). Thereby, acetonitrile was used to supplement the eluate from the nano-PGC-LC column. The improved spray stability enhanced detection and resolution of glycans during the analysis. This was in particular the case for smaller O-glycans which elute early in the high aqueous content regime of the nano-PGC-LC elution gradient. This study introduces PCMF as an easy-to-use instrumental adaptation to significantly improve spray stability in negative ion mode nano-PGC-LC-ESI-MS(/MS)-based analysis of glycans.

Introduction

The vital role glycans have in a large number of biotechnological and biological processes makes it ever more important to characterize and understand glycan structures in the context of such complex bio(techno)logical processes [1-3]. Glycosylation is a non-genomic-template driven co- and posttranslational modification of glyco-conjugates. The elaborate non-linear oligosaccharide structures confer a level of complexity that emphasizes the need to use high-resolution analytical techniques rather than referring to transcriptomes or genomes to effectively profile such molecules [4,5]. By definition, a glycomic investigation is aimed at characterizing the entire glycan population in a heterogeneous biological sample. This is not a trivial exercise as a glycomic profile can contain structures differing in linkages, branching and anomericity [1,6]. Furthermore, glycans can exist in either neutral or charged forms, for instance due to carrying a combination of sialic acids as well as further modifications such as sulfation and/or phosphorylation [1,7-10]. Such complexity means that with standard detection methods and without modifying the glycan through labelling or stabilizing sialic acid residues, a glycome cannot be analyzed efficiently with one analytic method [11,12].

Central to high-performance glycoanalysis are the advances made to ultra/high-performance liquid chromatography (U/HPLC) [13-15], capillary electrophoresis (CE) [16-20] and mass spectrometry (MS) [21-25], which now enable the extensive structural analysis of released glycans. Analytical techniques primarily employed include hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLR) [13,14], liquid chromatography coupled with electrospray (tandem) mass spectrometry (LC-ESI-MS(/MS)) [22,23,25,26], matrix assisted laser desorption/ionization with time-of-flight (tandem) mass spectrometry (MALDI-TOF(/TOF)-MS(/MS)) [24,27,28] and more frequently also multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF) [17,18,20,29-34]. To increase sensitivity or facilitate quantitation, glycans can undergo chemical modification such as

permethylation [28,35,36], esterification [24] or numerous fluorescent labelling like 2-aminobenzamide [37-39], 2-anthranilic acid [40], procainamide [41], RapiFluor-MS [42] and 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt [17,19,20]. Each of these techniques affects certain properties of glycans such as their hydrophilicity (HILIC), electrokinetic mobility (CE), composition (MS), as well as their fragmentation behavior (MS/MS). Complete characterization of co-eluting glycan structures or glycan isomerism requires orthogonal methods such as sequencing via exoglycosidase digestion [32-34,43,44].

Glycan analysis using porous graphitized carbon liquid chromatography (PGC-LC) coupled with negative ion mode MS detection and MS/MS fragmentation is gaining traction as a method for comprehensive MS-based glycoanalysis [22,23,45-53]. Very little modification of the native glycan is required other than reduction of the anomeric ring of the glycan's reducing end. The analysis produces information-rich data from chromatographic separation of even isobaric *N*- and *O*-glycans on the PGC column as well as from unique MS/MS fragmentation patterns due to the negative ion mode. Both neutral and negatively charged glycans can be detected using negative ion polarity MS [54-56]. Additional MS/MS fragmentation and detection in negative ion mode can yield diagnostic fragment ions of rich information owing to cross-ring fragments which can be used to confirm a glycan's topology, branching and monosaccharide linkages [23,54,55,57].

With the ever-increasing interest to develop methods with higher sensitivity, there is significant interest in transitioning MS-based glycomics analysis to nano-scale instrument architecture (nano-LC and nano-ESI). This is due to the significantly lower flow rate and sample dilution within nano-LC columns and the superior ionization and sampling rate of nano-ESI [58,59]. Unlike capillary flow-adapted source sprayers, the architecture of standard nano-source sprayers does not rely on a nebulizer to facilitate the desolvation of the liquid. Instead, electrospray efficiency is functionally dependent on the potential gradient between the electrospray tip and the orifice [58,59].

In this work, we describe an easy-to-use setup which enables the analysis of reduced native glycans via nano-PGC-LC-ESI-MS(/MS) in negative ion mode. From our observations, periodical droplet forming attended by intermittent corona discharge events occurred at the nano-electrospray needle. This was particularly the case during periods of high aqueous buffer content of the nano-PGC-LC elution gradient. In an effort to improve the spray, we introduced an organic post-column make-up flow (PCMF) to enhance the electrospray stability and to improve the overall performance of the analyses.

Materials and Methods

Chemicals and Buffers

Bovine fetuin (F2379-100MG), Peptide *N*-glycosidase F (PNGase F, P7367), Potassium hydroxide (KOH, 484016-1KG), dithiothreitol (DTT, D5545-5G), iodoacetamide (IAA, I1149-25G), ammonium bicarbonate (ABC, 09830-500G), sodium borohydride ($NaBH_4$, 71320-100G), acetic acid (49199-50ML-F), ammonium acetate (A-1542) and acetonitrile (ACN, LC-MS Grade ≥ 99.5%, 34967) were all purchased from Sigma-Aldrich (Germany). Urea was obtained from AppliChem (A1049, Germany). Methanol LC/MS-grade was purchased from Fisher Scientific (10031094, Germany). All buffers and solutions were prepared with deionized and purified water (dH_2O) using a Milli-Q water purification system (18.2 $M\Omega \cdot cm^{-1}$ at 25°C, total organic carbon of 3 ppb) from Merck Millipore (Germany). For LC-MS solvents, water was further purified using an LC-Pak Polisher from Merck Millipore.

Experimental

N- and *O*-linked glycan release for mass spectrometric analysis was adapted from methods previously described [22,25,60]. One hundred microgram (100 µg) of bovine fetuin were dissolved in 200 µL of 8 M urea buffer (0.05 M $ABC_{(aq)}$ pH 8.5; $urea_{(aq)}$). The denatured and dissolved sample was transferred to a filter unit (Nanosep® Omega™ with polyethersulfone membrane, molecular weight cut-off 10 kDa; PALL Life Sciences, USA) [61]. The sample was centrifuged at 14,000 x *g* for 10 min at standard room temperature (RT). Afterwards, 30 µL of 10 mM DTT (in $urea_{(aq)}$) was added to the sample and incubated at 56°C for 20 minutes using an Eppendorf ThermoMixer (Germany). The solution was then centrifuged (17,000 x *g*, 10 min, RT) before 30 µL of 55 mM IAA (in $urea_{(aq)}$) was added and incubated at RT for 20 minutes in the dark. The sample was then washed three times with 100 µL 50 mM $ABC_{(aq)}$ followed by centrifugation (17000 g, 10 min RT). The denatured, reduced and alkylated protein on top of the filter membrane was finally reconstituted in 50 µL of 50 mM $ABC_{(aq)}$.

The *N*-linked glycans were released by adding 1U of PNGase F reconstituted in PBS (pH 8.0) to the filter unit containing the reduced and alkylated fetuin protein. Samples were incubated overnight at 37°C using a temperature-controlled incubator (Titramax 1000 with Incubator 1000, Heidolph Instruments, Germany). The released *N*-glycans were harvested by washing and centrifuging the filter unit at 14,000 x *g* for 10 min at RT in three steps as followed: Centrifuging the supernatant, washing and centrifuging with 50 µL 50 mM $ABC_{(aq)}$ + 5% ACN (v/v) and washing and centrifuging with 50 µL 50 mM $ABC_{(aq)}$. The flow-through was collected and pooled. Glycosylamines were removed from the reducing terminus of the glycans by adding 10 µL of 100 mM ammonium acetate at pH 5 (final concentration 15 mM) for 60 min at RT. Approximately 150 µL flow-through was collected and dried in a vacuum centrifuge (RVC 2–33 CDplus, ALPHA 2–4 LDplus, Martin Christ, Germany). The *N*-glycans were reduced with 20 µL of 1 M $NaBH_4$ in 50 mM $KOH_{(aq)}$ at 50^{o}C for 3 h. The reduction was quenched with 1 µL acetic acid, and the *N*-linked glycan alditols were desalted as described below.

Following the *N*-glycan release, the *O*-linked glycans were released by aspirating the glycoproteins on top of the filter membrane and then conducting reductive β-elimination by incubating overnight with 20 µL of 0.5 M $NaBH_4$ in 50 mM $KOH_{(aq)}$ at 50^{o}C. Again, the reduction was quenched with 1 µL acetic acid, and also the *O*-linked glycan alditols were desalted as described below.

Desalting glycans

The separate fractions of *N*- and *O*-glycans were desalted using cation exchange columns comprising 30 µL of a methanolic slurry of AG50W-X8 cation-exchange resin (142-1431, BioRad, USA) packed on top of a 20 µL C18 StageTip Frit (SP201, Thermo Fisher Scientific, USA). Residual borate was removed by addition of 50 µL methanol followed by drying under vacuum. This was repeated 5 times.

Each separate *N*- and *O*-glycan fraction was dissolved in 25 µL dH_2O, respectively. Finally, both fractions were pooled. Ten microliters (10 µL) of released glycans (equivalent of 20 µg of bovine fetuin) were aliquoted (final concentration 2 µg/µL) and stored at -20°C.

Liquid chromatography coupled to mass spectrometry (LC-MS)

For LC separation an UltiMate 3000 RSLCnano system from Thermo Fisher Scientific (USA) was used. Five microliters (5 µL) of the pooled glycan sample were loaded isocratically through a nanoViper 20 µL FS/PEEK-sheathed sample loop on a Hypercarb PGC (Hypercarb Kappa, 320 µm x 3 cm, 35005-030315, Thermo Fisher Scientific, USA) trap column via 100% loading buffer A (10 mM $ABC_{(aq)}$) with a flow rate of 7 µL/min for 5 min. Afterwards, the loaded trap column was switched in-line with the analytical nano-column (Hypercarb Kappa, 75 µm x 10 cm, 35003-100065, Thermo Fisher Scientific, USA). The separation was performed at a flow of 0.9 µL/min by using a gradient of buffer A (10 mM $ABC_{(aq)}$) and buffer B (10 mM $ABC_{(aq)}$ + 90% ACN (v/v)). Specifically, separation was performed using the following multi-step binary gradient: 5-9% buffer B till 17 min, 9-56% buffer B till 65 min, 56-90% buffer B till 90 min and 5% buffer B 90-100 min.

To improve the electrospray stability, both the nano-PGC-LC gradient flow and the isocratic PCMF (100% ACN) were combined using a T-piece (SC901, Thermo Fisher Scientific, USA). The PCMF was pumped from a glass syringe (500 µL; Hamilton, USA) with 2 µL/min using the integrated syringe pump of the LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA). Both, the nano-LC flow and the PCMF were transferred to the T-piece, each via a NanoViper capillary (20 µm inner diameter (ID), 1/32" outer diameter (OD), 650 mm length, 6041.5275, Thermo Fisher Scientific, USA) and the resultant mixed flow was directed to a electropolished stainless steel spray needle (Nano-bore emitter, 30 µm ID, 1/32" OD, 40 mm length, ES542, Thermo Fisher Scientific, USA) via a NanoViper capillary (75 µm ID, 1/32" OD, 550

mm length, 6041.5760, Thermo Fisher Scientific, USA). The schematic of this architecture is illustrated in Figure 1.

The eluting glycans were measured on an LTQ Orbitrap Elite mass spectrometer from Thermo Fisher Scientific (USA) using a Nanospray Flex^{TM} source (Thermo Fisher Scientific, USA) in negative ion mode with a capillary voltage of 2.7 kV. Glycans were fragmented using higher-energy collisional dissociation (HCD) at a normalized collision energy of 75 with an activation time of 0.1 ms. The top 5 most intensive precursor ions with a charge state >1 were chosen for fragmentation. The mass range for MS and MS/MS was 150-2000 m/z. These settings were used in both setups, with and without PCMF.

Data analysis

Data files were analyzed manually using Xcalibur (Version 2.2, Qual Browser, Thermo Fisher Scientific, USA). Acquired MS scans were analyzed and a list of monoisotopic masses corresponding to glycans was sent to ExPASy GlycoMod (http://web.expasy.org/glycomod) to retrieve a list of possible matching glycan compositions. The compositions and glycan structures were confirmed by manually annotating the MS/MS fragment ion spectra of each glycan structure, also considering diagnostic ions that are characteristic for certain glycan epitopes.

Results

The motivation of this work is based on the observation that during the periods of high aqueous content in any standard nano-PGC-LC elution gradient, the spray stability was compromised. As depicted in Figure 2, instances of inconsistent nano-ESI occurred whereby periodic droplets formed on the spray needle associated with intermittent corona discharge events. These droplets dissipated away from the orifice of the mass spectrometer, causing signal drop-outs in ionization- and sampling-rate, i.e. a breakdown of the ion current (see zoom-in Figure 3). As a result of the unstable nano-ESI, detection and fragmentation of the analytes was impaired. This is demonstrated in the base peak chromatogram (BPC) of pooled fetuin *N*- and *O*-glycans as displayed in Figure 3a. It is further emphasized in Figure 4a, where the extracted (glycan) ion chromatograms (EIC) of all selected (glycan) ion traces are repeatedly interrupted by such signal drop-outs. These signal drop-outs were in sync with the formation of the droplets. In particular, during the early periods of the nano-LC gradient (with high aqueous content) the signal was deleteriously affected. This is demonstrated during the elution of small *O*-glycans at the beginning of the gradient (Figure 4a, EIC 675.25^{1-} and EIC 966.34^{1-}). It should be emphasized that for both nano-PGC-LC-ESI-MS(/MS) setups (with and without PCMF), there was an efficient separation of the glycans under the given elution gradient, but without PCMF, the eluting glycans accumulated into the droplets growing at the needle tip. Thus, without PCMF, in addition to the spray instabilities, also the chromatographic separation was affected post-column. At lower extent, this occurred also during the later periods of the nano-LC gradient (with higher organic content; see Figure 4a, EIC 1111.39^{2-} and EIC 1439.51^{2-}).

To decrease the aqueous content throughout the analysis without compromising the chromatographic separation of the glycans, a PCMF was implemented in between column and sprayer, as explained in the Materials and Methods section. Three different capillary dimensions (20, 50 and 75 µm) were tested as

the capillary to deliver the flow from the T-piece to the electrosprayer. From our observation, using the 20 µm and 50 µm ID capillaries for the outlet (Figure 2) would still produce signal breakdowns caused by the formation of a droplet that would not desolvate properly (data not shown). A 75 µm ID capillary was the smallest capillary which would allow for a proper mixing of the column flow and the PCMF prior to electrospray. The slightly bigger ID of the electropolished stainless steel spray needle (30 µm, for capillary and tip) compared to the ID of common fused silica needles (20 µm for capillary and 10 µm at the tip) fitted better to the 3-fold increased flow-rate.

With the application of a PCMF of neat organic solvent (100% ACN), the spray was stable throughout the entire nano-LC run (see Figure 2b), i.e. from high aqueous to high organic content of the gradient (Figure 3b & zoom-in). With the application of the PCMF, a better signal stability and better peak shapes, and hence better BPC and EIC were achieved (see Figure 3b & 4b).

Table 1 reports the glycan compositions, their spectral copy numbers and retention times which were observed and confirmed via MS/MS interrogation with and without PCMF. Without PCMF not a single spectrum could be counted for *O*-glycans, eluting at early timepoints of the gradient when the aqueous content is high. For the *N*-glycans, eluting at later timepoints of the gradient with lower aqueous content, still a significantly lower spectral copy number was reached compared to the results achieved with PCMF. In fact, with PCMF, the spectral copy numbers of the glycans were improved by a factor of at least two for the majority of the fetuin glycans. Taken with the results in Table 1, one can summarize that at nano-flow, the detection of glycans, particularly those at low abundance, as well as the resolution of such peaks is severely compromised without using PCMF.

Discussion

The analysis of glycans using nano-PGC-LC-ESI-MS(/MS) with subsequent detection of the mass and fragmentation in negative ion mode is a powerful glycoanalytical tool. This is centered around the ability of the technique to not only allow to characterize both *N*- and *O*-glycans, but also to separate isobaric structures using PGC and to detect native sialylated and neutral structures in one run. Negative ion polarity detection of fragment ions is diagnostic for certain types of branching, linkage and thus central to identifying glyco-epitopes. Central to the detection of glycans in negative polarity is the use of basic buffers which deprotonate the analytes and increase their propensity for MS-detection in negative ion mode. This methodology for glycan analysis has already been described in detail, for analytical- [62] and capillary- [22,48,63-65], but not much for nano-scale PGC-LC-ESI-MS(/MS) [25].

For analytical- and capillary-scale LC-MS, a coaxial addition of sheath gas and/or liquid is standard, as the gas assists nebulization and evaporation/desolvation of the droplets and the added organic liquid reduces their surface tension[66-70]. For nano-scale LC-MS, usually flow-rates and spray-tip diameters are much smaller, hence no sheath gas/liquid is required to form a stable nano-electrospray[71-75].

For nano-PGC-LC, the only commercially available columns are from Thermo Fisher Scientific (see Materials and Methods section). Compared to common reversed-phase nano-LC columns with a typical maximum flow rate of 0.3 µL, the nano-PGC-LC columns are usually operated at about a three times higher flowrate. When coupling these columns at 0.9 µL/min to MS in negative ion mode with the nano-spray source from Thermo Fisher Scientific (see Materials and Methods section), we repeatedly observed spray instability, which is described to occur at higher propensity in negative ion mode [76,77]. As illustrated in the results section, the ion traces of the analytes are detrimentally compromised without augmentation of the spray with an appropriate organic solvent. Most significant improvement

of the electrospray was reached during the early gradient periods of high aqueous content (Figure 3 & 4).

This sheath/make-up flow ESI-setup is commonly used in analytical and capillary scale LC-MS solutions, yet to our knowledge has not been applied to a nano-PGC-LC-ESI-MS(/MS) approach, operated in negative ion mode. A similar solution to improve ESI for nano-scale LC-MS, is for example, the CaptiveSpray nanoBooster™ (Bruker Daltonics, Germany) that is also used for glycomic investigations with nano-PGC-LC-ESI-MS(/MS) in negative ion mode [25]. Staples et al., (2010) has described another solution, but for a nano-Chip-based format applied to HILIC-ESI-MS(/MS), analyzing glycosaminoglycans [78]. Ni et al., (2013) used a similar approach using fluorid-mediated negative ion-mode microchip PGC-LC-MS to analyze released N-glycans [79].

Our approach can be easily applied to any conventional nano-U/HPLC-ESI-MS(/MS) setup with an additional (syringe) pump, a T-piece and a respective spray needle, adjusted to the increased flowrate. The approach is modular and thus allows the user to adjust and replace any part as well as to individually optimize the setup to the given instrumentation. Based on this pilot study, further work is necessary to fully elaborate the robustness and sensitivity of the approach. Following the trend within the analytical community to increasingly move towards more sensitive nano-flow and nano-spray LC-MS technologies, the use of complementary organic solvents for PCMF might be useful to any analyte class that requires negative ion detection, for example (oligo-)nucleotides, metabolites or (glyco-)lipids.

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Etzler, M.E., Frank, M., Vliegenthart, J.F., Lutteke, T., Perez, S., Bolton, E., Rudd, P., Paulson, J., Kanehisa, M., Toukach, P., Aoki-Kinoshita, K.F., Dell, A., Narimatsu, H., York, W., Taniguchi, N., Kornfeld, S.: Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology **25**(12), 1323-1324 (2015). doi:10.1093/glycob/cwv091

TABLES AND FIGURES

TABLES

TABLE 1 *N*- and *O*-glycan compositions released from bovine fetuin detected via nano-PGC-LC-ESI-MS(/MS) with and without the use of a PCMF. Comparison of the spectral copy numbers (count of MS/MS fragment spectra of each precursor (glycan) ion $[M-2H]^{2-}$) corresponding to the glycan compositions found between the two analytical measurements. Note that there is no spectral copy number for the *O*-glycan 675.25^{1-}, because of the missing doubly charged precursor.

FIGURES

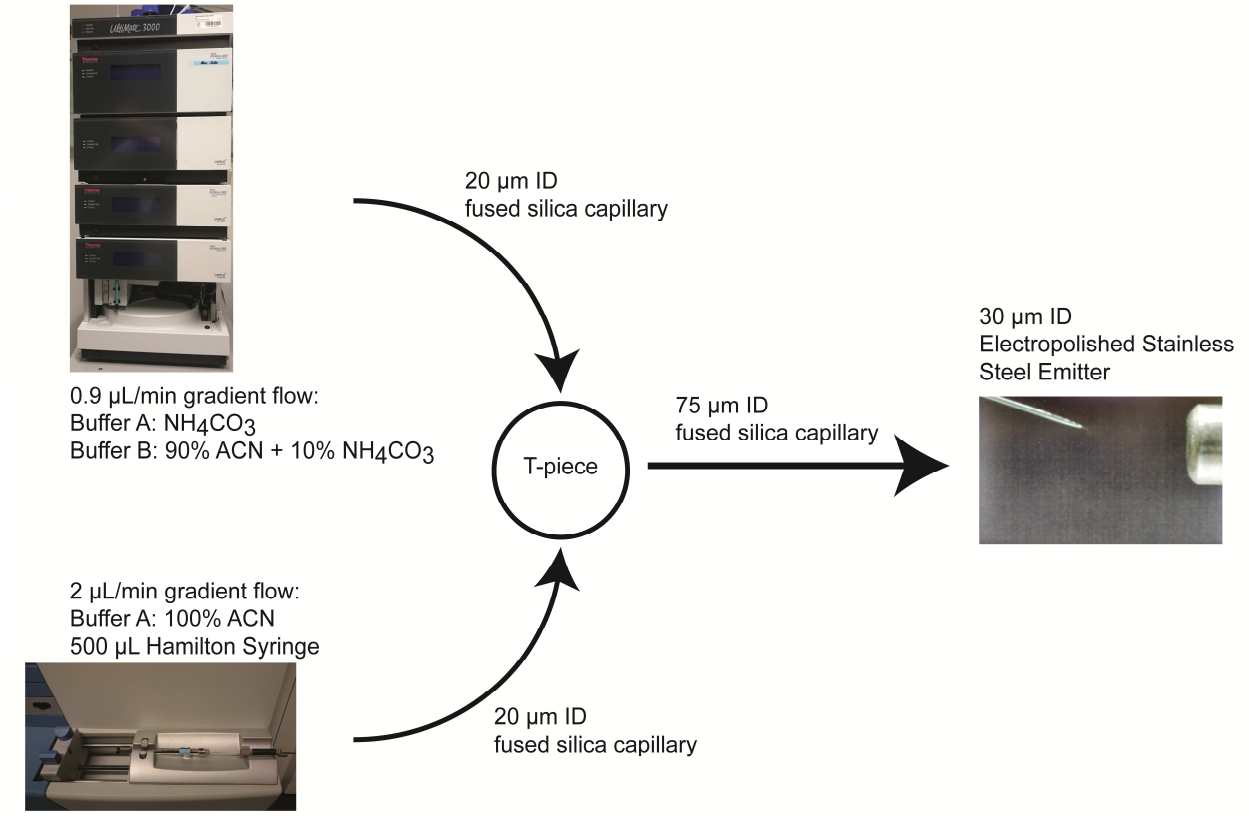
FIGURE 1 Schematic of the PCMF setup. The post-column nano-flow is supplemented with an additional capillary flow of 100% ACN via a T-piece. The combined flow is directed to a 30 µm ID stainless steel electrospray needle via a 75 µm ID fused silica capillary.

FIGURE 2 Comparison of the spray stability during elution when using nano-PGC-LC-ESI-MS(/MS) in negative ion mode. A) Image of the needle and the nano-source. A droplet forms and is released periodically. B) Due to the supplementation with a PCMF of 100% ACN, no droplet is forming anymore, and a stable electrospray is achieved.

FIGURE 3 Comparison of the BPC of eluted *N*- and *O*-glycans released from bovine fetuin before (A) and after (B) PCMF supplementation. The recovery of smaller alditols such as *O*-glycans (like, $HexNAc_1Hex_1NeuAc_1$ and $HexNAc_1Hex_1NeuAc_2$) can be observed, while still the larger *N*-glycans (like, $HexNAc_4Hex_5NeuAc_2$, $HexNAc_5Hex_6NeuAc_3$, $HexNAc_5Hex_6NeuAc_4$) are recovered after the PCMF implementation, as well. Using PCMF, no breakdown of the BPC ion trace occurs anymore (zoom-in). Symbolic representations of *N*-glycan structures were drawn using GlycoWorkbench Version 1.1, following the guideline of the Consortium for Functional Glycomics[80].

FIGURE 4 Comparison of the EIC of selected masses corresponding to *N*- and *O*-glycans released from bovine fetuin before (A) and after (B) PCMF supplementation. With PCMF, the EIC ion traces of the different *N*- (m/z 1439.51^{2-} and 1111.39^{2-}) and *O*-glycans (966.34^{1-} and 675.25^{1-}) are significantly improved and the nano-PGC-LC separation of their isoforms can nicely be seen

N-glycan compositions	spectra copy numbers		[M-H]-	MS/MS confirmed	RT [min] (with PCMF)
	NO PCMF	PCMF			
(Hex)3 (HexNAc)3 (NeuAc)3 + (Man)3(GlcNAc)2	57	299	2880.0205	YES	38.59, 39.53, 42.35, 43.67
(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3(GlcNAc)2	52	156	2223.7788	YES	34.57, 37.99, 40.73
(Hex)3 (HexNAc)3 (NeuAc)2 + (Man)3(GlcNAc)2	33	70	2588.91724	YES	36.52, 38.24, 39.88
(Hex)2 (HexNAc)2 (NeuAc)3 + (Man)3(GlcNAc)2	20	41	2514.86639	YES	35.48, 36.92, 38.54, 42.03
(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3(GlcNAc)2	18	33	2734.97094	YES	36.27, 37.63, 38.98, 40.51
(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3(GlcNAc)2	15	19	2369.83618	YES	34.54, 36.47, 39.83, 45.93
(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3(GlcNAc)2	13	28	1932.68396	YES	32.84, 36.42, 39.36, 42.11
(Hex)2 (HexNAc)3 (NeuAc)2 + (Man)3(GlcNAc)2	6	5	2426.86084	YES	37.8
(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3(GlcNAc)2	3	2	2078.73754	YES	32.32, 34.99, 38.26
(Hex)4 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2	2	5	2111.7461	YES	36.9, 41.75, 45.02
(Hex)3 (HexNAc)3 (NeuAc)4 + (Man)3(GlcNAc)2	1	232	3171.07884	YES	41.72, 44.6, 46.64
(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 + (Man)3(GlcNAc)2	-	31	3026.06762	YES	40.98, 43.97
(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3(GlcNAc)2	-	8	2281.73242	YES	34.46, 37.96, 44.8, 51.27
(Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2	-	7	1625.59606	YES	33.13
(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3(GlcNAc)2	-	6	1567.55468	YES	31.97, 35.43
(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2	-	6	1787.64942	YES	33.97
(HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2	-	5	1463.54456	YES	30.95, 32.25
(Hex)1 (HexNAc)2 (NeuAc)1 + (Man)3(GlcNAc)2	-	2	1770.63586	YES	32.26, 35.67
(Hex)3 (HexNAc)2 (Deoxyhexose)1 + (Man)3(GlcNAc)2	-	2	1949.70092	YES	32.87, 35.45
(Hex)2 (HexNAc)2 + (Man)3(GlcNAc)2	-	1	1641.5879	YES	31.7
O-glycan compositions	spectra copy numbers		[M-H]-	MS/MS confirmed	RT [min] (with PCMF)
	NO PMCF	PMCF			
(Hex)1 (HexNAc)1 (NeuAc)2	-	17	966.35	YES	27.93
(Hex)1 (HexNac)1 (NeuAc)1	-	-	675.25	NO	24.12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Buffer B: 90% ACN + 10% NH_4CO_3
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Steel Emitter
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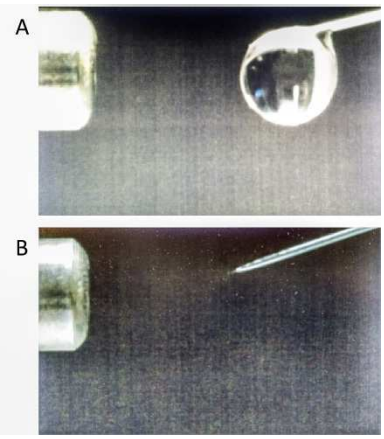
fused silica capillary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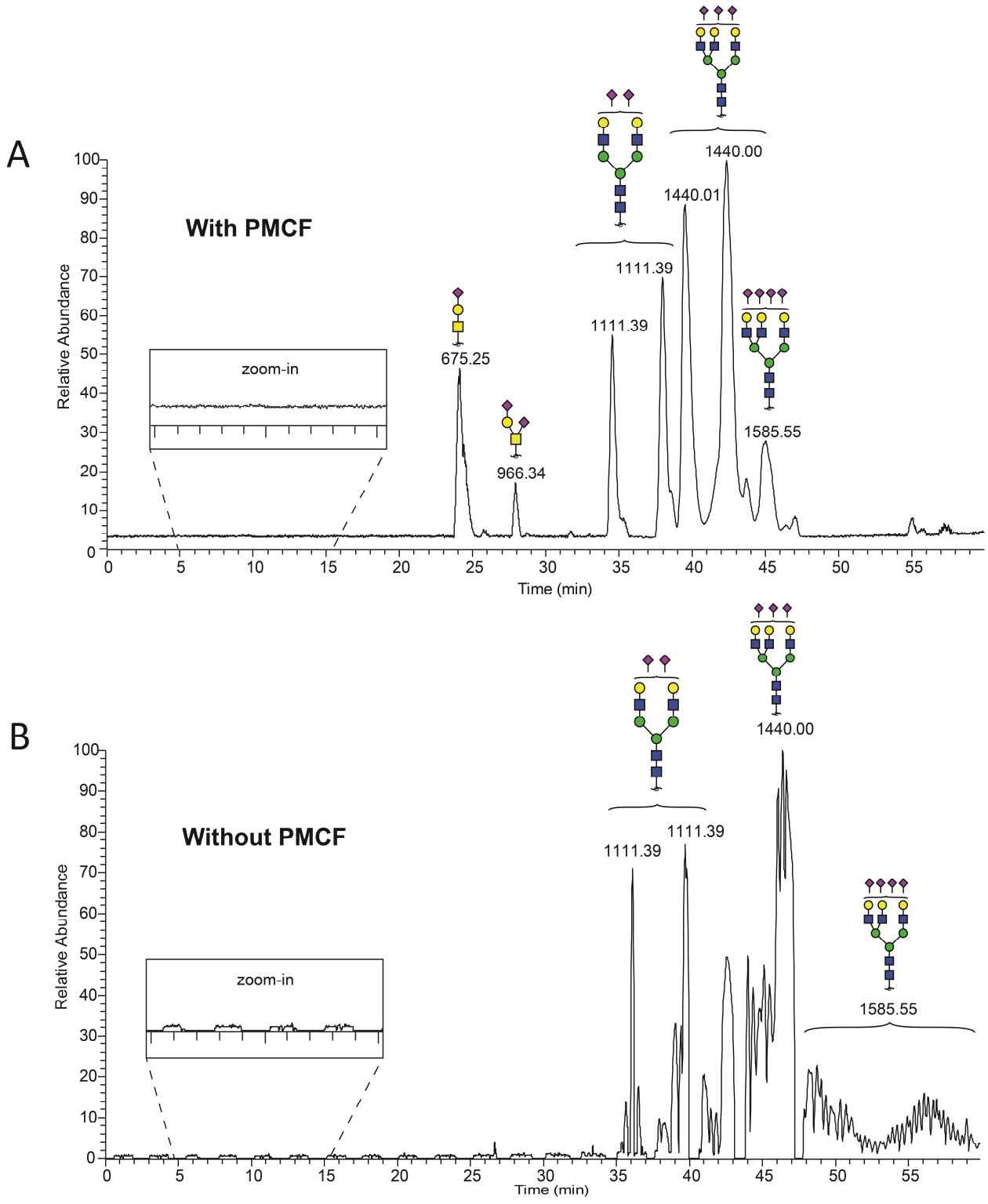
A
With PMCF
Relative Abundance
Time (min)
zoom-in
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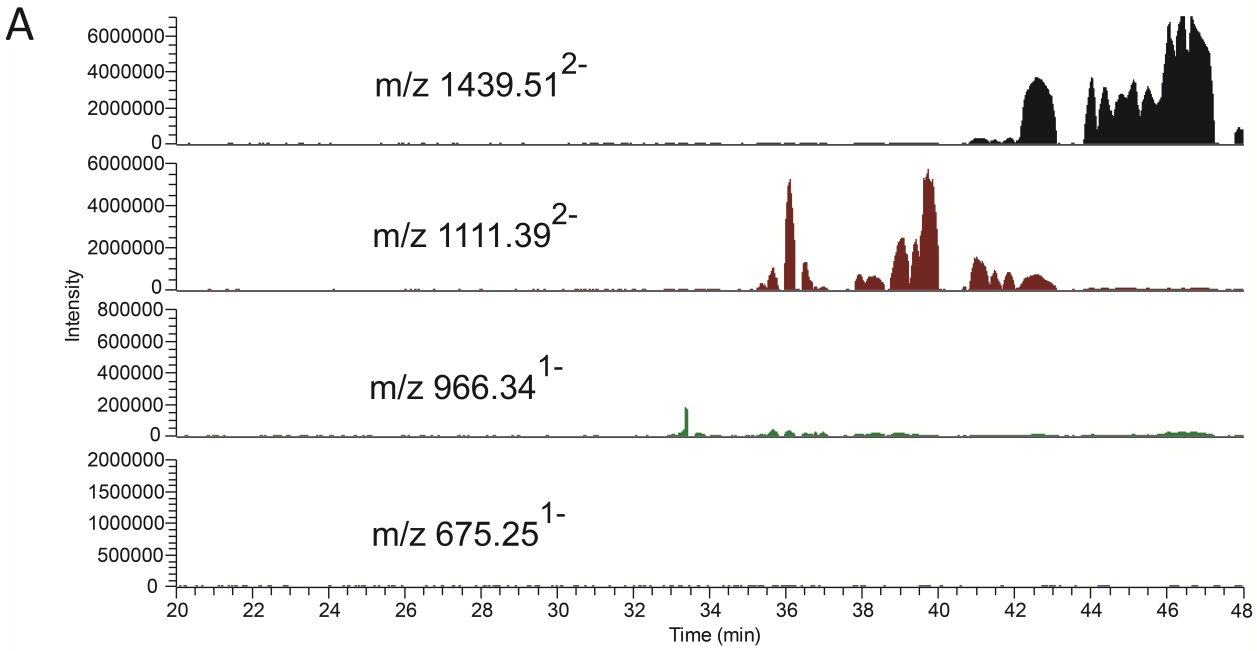
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m/z 966.34 1-
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Intensity
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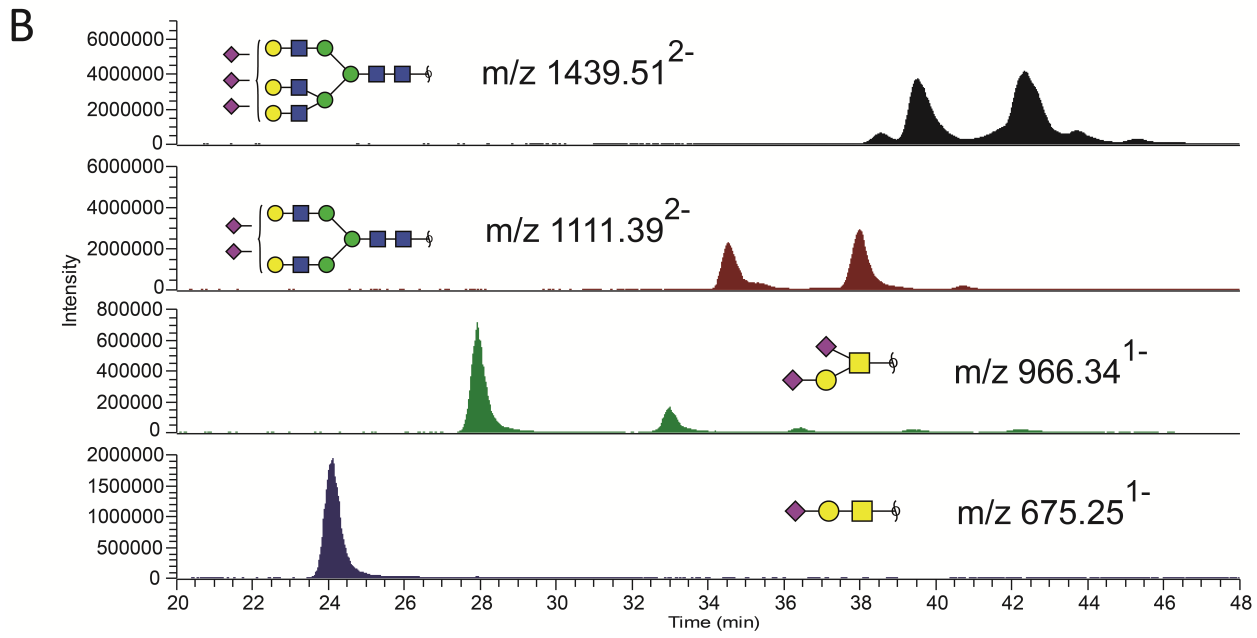
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m/z 966.34 1-
m/z 675.25 1-
Intensity
Time (min)