Unraveling proteoform complexity by native liquid chromatography-mass spectrometry

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Dopant-enriched nitrogen gas to boost nano-electrospray ionization of glycoproteins analyzed under native conditions

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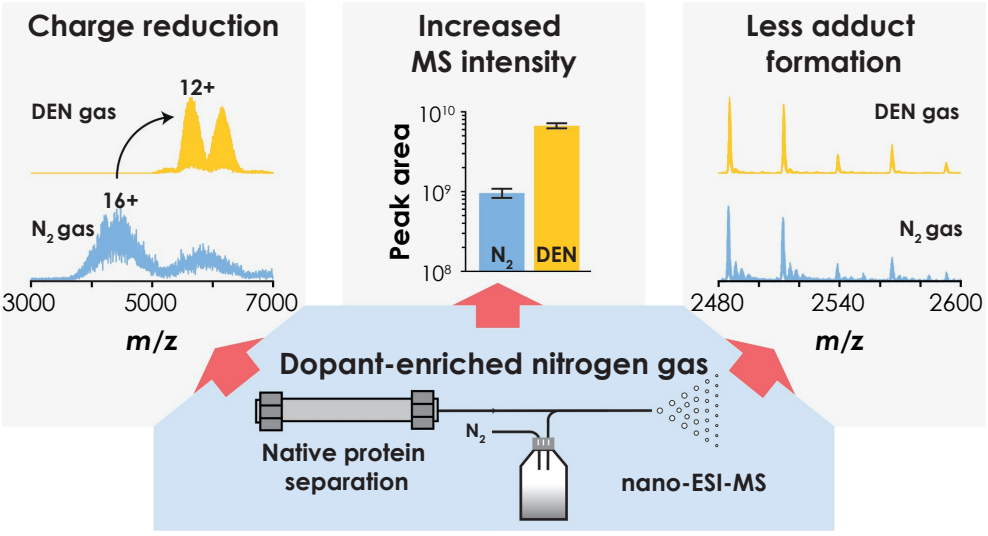
Proteins carry a plethora of post-translational modifications (PTMs), such as glycosylation or phosphorylation, which may affect stability and activity. Analytical strategies are needed to investigate these PTMs in their native state to determine the link between structure and function. The coupling of native separation techniques with mass spectrometry (MS) has emerged as a powerful tool for in-depth protein characterization. Yet obtaining high ionization efficiency still can be challenging. Here, we explored the potential of dopant-enriched nitrogen (DEN) gas to improve nano-electrospray ionization (nano-ESI)-MS of native proteins after anion exchange chromatography. The dopant gas was enriched with different dopants (acetonitrile, methanol, and isopropanol) and the effects were compared with the use of solely nitrogen gas for six proteins covering a wide range of physico-chemical properties. The use of DEN gas resulted generally in lower charge states, independent of the selected dopant. Moreover, less adduct formation was observed, particularly for the acetonitrile-enriched nitrogen gas. Importantly, striking differences in MS signal intensity and spectral quality were observed for extensively glycosylated proteins, where isopropanol- and methanol-enriched nitrogen appeared to be most beneficial. Altogether, the use of DEN gas improved nano-ESI of native glycoproteins and increased spectral quality for highly glycosylated proteins that normally suffer from low ionization efficiency.



3.1 Introduction

Separation techniques, such as liquid chromatography (LC), hyphenated with mass spectrometry (MS) have grown into the primary analytical approach to study intact proteins. LC addresses sample complexity by resolving different molecular entities that can then be identified by online MS. Many advances have been made to improve the hyphenation of these two techniques, yet some challenges remain regarding mobile phase incompatibility and/or ionization efficiency [119, 217].

To improve the low ionization efficiency of intact proteins, the use of dopant-enriched nitrogen (DEN) gas has been proposed [217]. This approach relies on the enrichment of nitrogen-containing nebulizing gas with solvent vapor. Subsequently, the enriched desolvation gas is introduced in the ion source around the spray emitter resulting in more efficient desolvation and ionization [218]. DEN gas has already been successfully applied for the analysis of released glycans [218, 219] and (glyco)peptides [220, 221]. Here, it was especially useful due to the less efficient ionization of carbohydrates and glycoconjugates compared to other analytes caused by their hydrophilic nature [218]. Most of these workflows used miniaturized separation dimensions, including nano/capillary-scale reversed-phase LC or capillary electrophoresis (CE), coupled to a nano-electrospray ionization (nano-ESI) source. For instance, acetonitrile (ACN)-enriched nitrogen gas in a CE-MS workflow resulted in up to 25-fold higher sensitivities for glycopeptide compared to non-glycosylated species [220]. For intact protein analysis, most applications currently focus on the use of DEN gas to minimize adduct formation caused by trifluoroacetic acid (TFA)-containing mobile phases [222-224]. The DEN gas counteracts ion suppression caused by TFA and thereby, allowing more efficient ionization of proteins. For example, Gargano et al. [224] showed a 400-fold increase in MS peak area of cytochrome C upon using ACN-enriched nitrogen gas as compared to solely nebulizer gas. Accompanied by reduced TFA adduct formation and increased signal intensity, the charge states were shifted towards lower *m/z* values [222-224]. Besides denaturing intact analysis, DEN gas might also be used to improve hyphenation of native LC and MS [89]. While these native LC-MS methodologies open possibilities to obtain additional layers of information on proteins' higher-order structures and functionality, often poor MS ionization efficiencies are encountered due to the need for (non-volatile) salts for the separation [217]. So far, the application of DEN gas after native separations of monoclonal antibodies (mAbs) was only shown to be beneficial for charge reduction [89].

Here, we aimed to systematically investigate the influence of DEN gas on

the ionization of a range of diverse (glyco)proteins under native conditions. For this purpose, we coupled anion exchange chromatography (AEX) online with nano-ESI via post-separation flow splitting. The effect of the dopants isopropanol (IPA), methanol (MeOH), and ACN was compared to solely nebulizing gas. We analyzed different glycoproteins varying in size (between 14 and 150 kDa) and PTM heterogeneity (i.e., different levels of glycosylation and phosphorylation).

3.2 Experimental section

3.2.1 Materials

Acetic acid (AA), ammonium acetate, ammonium formate, fetuin, and ovalbumin were purchased from Sigma Aldrich (Steinheim, Germany). ACN and MeOH (LC-MS grade) were obtained from Actu-All Chemicals (Oss, the Netherlands). Formic acid (FA) and IPA were supplied by Riedel-De Haen (Seelze, Germany). The milliQ water was obtained from a Purelab ultra system (ELGA Labwater, Ede, the Netherlands). The prolyl-alanyl-specific endoprotease (EndoPro) and lipase were provided by DSM (Delft, the Netherlands). The IgG4 molecule was provided by the Department of Human Genetics (LUMC, Leiden, the Netherlands). All proteins were dissolved in AEX mobile phase A with a final concentration of 5 mg/ml.

3.2.2 Anion exchange chromatography - mass spectrometry

AEX-MS measurements were performed using a biocompatible Ultimate 3000 instrument (Thermo Fischer Scientific; Landsmeer, The Netherlands) coupled to a 15T Solaris Fourier-transform ion cyclotron resonance (FT-ICR)-MS (Bruker Daltonics, Bremen, Germany). A ProPac SAX-10 column (2.0 x 250 mm, 10 µm) was used from Thermo Fisher Scientific. For EndoPro and fetuin, the mobile phases consisted of 50 mM ammonium formate at pH 5.5 (A) and 50 mM FA at pH 2.5 (B). For RNase B, ovalbumin, IgG4, and industrial lipase, mobile phases were 10 mM ammonium acetate and 10 mM ammonium formate at pH 6.8 (A) and 10 mM AA and 10 mM FA at pH 3.0 (B). After the gradients (**Fig. S1**), the column was cleaned (100% B for 5 min) and equilibrated to the start conditions (20 min). The flow rate, column temperature, and UV wavelength were set to 0.25 ml/min, 25 °C, and 280 nm, respectively. The AEX separation was coupled to a nano-ESI CaptiveSpray source via a flow splitter reducing the flow ~100 times prior to ionization. The source was operated in positive-ion mode. The nitrogen gas was enriched by filling the nanoBooster bottle (Bruker Daltonics) with solvent (ACN, MeOH, or IPA), where the total solvent volume was always above 500 ml. The DEN gas system was equilibrated for 1 hour prior to use. Details on the MS methods can be found in **Table S1**.

3.2.3 Data analysis

Data analysis was performed using Compass DataAnalysis (Bruker Daltonics). For the charge state deconvolution, the Maximum Entropy algorithm was employed. Base peak chromatograms (BPCs) and extracted ion chromatograms (EICs) were smoothed with the Gauss smoothing algorithm (1 cycle). For identification of PTMs, average masses were used, including fucose (Fuc, 146.14 Da), mannose/galactose (Man/Gal, 162.14 Da), N-acetylglucosamine/N-acetylgalactosamine (GlcNAc/GalNAc, 203.20 Da), sialic acid (Sia, 291.26 Da) and phosphorylation (Phos, 79.98 Da).

3.3 Results and discussion

DEN gas enrichment has already been applied for the charge reduction of mAbs in a native LC-MS workflow [89]. To show comparability with this previous experimental set-up, we examined the influence of DEN gas on ionization of a mAb (IgG4). Since mAbs have a high molecular weight (around 150 kDa) and rather distinct structural features, we also explored whether other proteins with different sizes and PTMs (**Table 1**) responded in a similar way to dopant gas enrichment. For this purpose, we selected (glyco)proteins ranging from relatively simple, such as RNase B with a mass below 15 kDa and one high mannose glycan, to more heterogenous proteins, such as fetuin and EndoPro. Fetuin contains several complex type N-glycans and O-glycans [225], whilst EndoPro has seven N-glycosylation sites containing large (partly phosphorylated) high mannose glycans [215]. Besides these three proteins, we also included a glycosylated industrial lipase of around 30 kDa with varying glycosylation site occupancy (no, one, two, or three high mannose glycans per enzyme) and ovalbumin of ~40 kDa that carries glycosylation (one glycan) and phosphorylation (up to two sites occupied).

For all these proteins, AEX methods were developed to resolve charge variants and thereby, allow comparison of the effects of DEN gas on the ionization of (separated) proteoforms. Elution was achieved using a pH gradient to minimize the salt concentrations entering the MS instrument. Two different mobile phases were used to ensure sufficient separation, where the proteins were grouped depending on their protein isoelectric point (pI). Fetuin and EndoPro have relatively low pI values (below 4.5) and therefore, required more acidic mobile phase B for proper separation. In this case, we found that 50 mM ammonium formate at pH 5.5 (A) and 50 mM FA at pH 2.5 (B) were optimal. The other proteins (IgG4, RNase B, ovalbumin, and lipase) have higher pI values (ranging from between 5.2 and 8.6) resulting in analysis with 10 mM ammonium formate and 10 mM ammonium acetate

at pH 6.8 (A) and 10 mM AA and 10 mM FA at pH 3.0 (B). After selecting the appropriate mobile phases, the gradients were finetuned to eventually obtain high-resolution AEX methods (**Fig. S1**).

Table 1 | Overview of the investigated proteins, including their amino acids backbone mass and possible PTMs. The grand average hydropathy (GRAVY) score was calculated by the summation of hydropathy values of all the amino acids, divided by the number of residues in the sequence [358]. The type (high mannose, complex, or hybrid) is indicated for the N-glycosylation and additional information is provided on the number of mannoses (Man) and the presence of sialic acids (Sia). Moreover, the ratio between the average number of monosaccharides and the backbone mass is provided. The effect of DEN gas on the ionization efficiency is provided per protein, where "-" indicate reduced intensity and "+" enhanced intensity. The MS peak areas of these proteins can be found in **Table S2**. In-depth proteoform characterization of the proteins can be found in the provided references.

Protein	Mass (kDa)	GRAVY	N-Glycosylation		Other PTMs	Ratio mono-saccharides to backbone mass	Effect of DEN gas compared to nitrogen gas			Ref
			Type of glycan	# of sites			IPA	MeOH	ACN	
IgG4	146	-0.298	Complex (without Sia)	2	-	0.1	--	--	--	226
Ova	42.8	-0.006	High mannose or hybrid (4-6 Man per glycan)	1	Phosphorylation	0.2	--	--	--	162, 226
RNase B	13.7	-0.663	High mannose (5-9 Man per glycan)	1	-	0.7	+	++	-	228
Lipase	28.4	-0.155	High mannose (4-14 Man per glycan)	3	Truncation	0.8	+	+	+/-	229
Fetuin	36.4	-0.085	Complex (with Sia)	3	O-glycosylation, phosphorylation	1.3	++	++	+	225
EndoPro	54.3	-0.377	High mannose (4-20 Man per glycan)	7	Phosphoglycan	1.5	++	++	+	215

As DEN gas different organic modifiers (IPA, MeOH, and ACN) were evaluated in terms of charge state distribution (CSD), declustering power, and sensitivity. The CSD of all proteins showed a consistent shift to lower charge states after enrichment of the nitrogen gas with an organic modifier, which was in line with previously reported mass spectra of mAbs [89]. Noteworthy, this effect is the opposite compared to DEN gas usage for denaturing protein analysis, where the CSD shifts towards higher charges [224]. This shift towards higher m/z regions in native analysis comes with several benefits, including enhanced spatial resolution between adjacent charge states (i.e., smaller risk of overlapping species) and generation of more native-like protein ions that experience lower Coulomb repulsion (particularly useful for labile proteins) [89, 109]. The extent to which the CSDs shifted was dependent on the protein size. Small

proteins showed only minor reduction, whereas large proteins revealed more drastic shifts. The most abundant charge state of the small protein RNase B decreased from 7 charges for nitrogen gas to 6 charges for the different DEN gases (**Fig. 1**), whereas the mass spectra of the mAb showed a decrease from 27 charges for the non-doped nitrogen gas to 20 charges for IPA, 19 charges for MeOH and 18 charges for ACN (**Fig. S2a**). The obtained CSDs of the mAb are highly comparable with previous reports [89, 109]. A similar charge reduction of EndoPro was observed from 16 charges for solely nebulizer gas to 12 charges for IPA, 11 charges for MeOH, and 10 charges for ACN (**Fig. 2**).

Besides charge reduction, another previously described effect of DEN gas was reduced adduct formation [89]. While for the measured mAb no difference in adduct formation was observed with or without DEN gas (**Fig. S2**), other proteins revealed fewer adducts with DEN gas leading to cleaner spectra and improving the identification of proteoforms. RNase B showed reduced adduct formation using ACN-enriched nitrogen gas compared to the other conditions (**Fig. 1**).

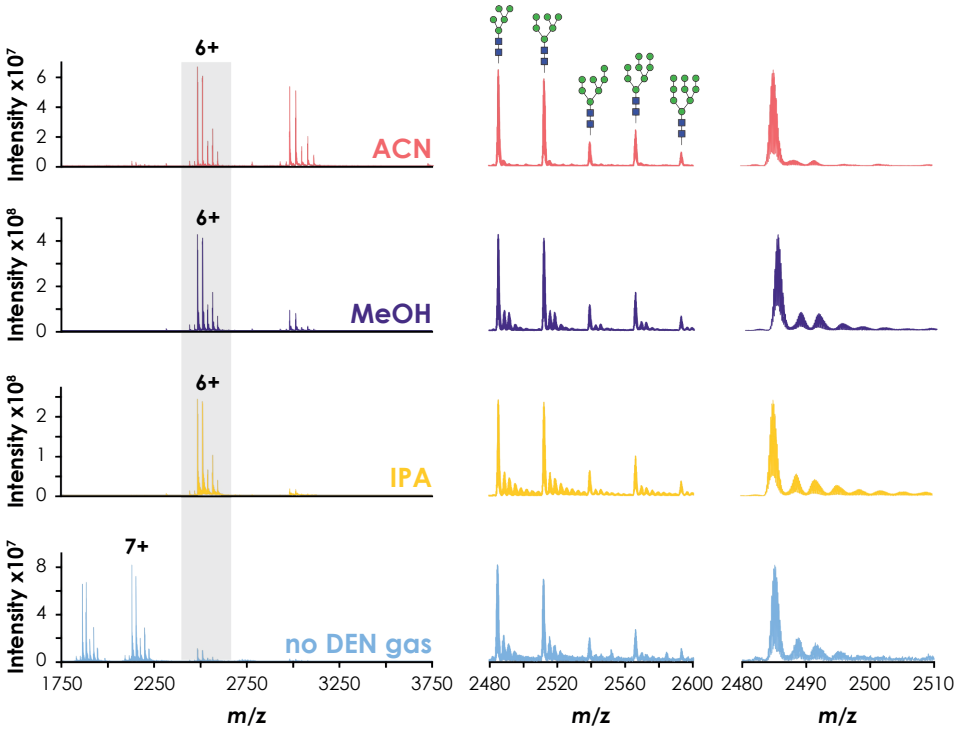


Figure 1 | Mass spectra of RNase B obtained using nitrogen gas enriched with IPA (yellow), MeOH (purple) and ACN (red), as compared to solely nitrogen gas (blue). Left: the most abundant charge state is labeled for each condition. Middle: zoom of the 6+ charge state including assignment of the glycoforms. Right: zoom of the H5N2 glycoform of the 6+ charge state showing isotopic resolution under all conditions and decreased adduct formation for ACN-enriched dopant gas.

Next, we evaluated the influence of DEN on the desolvation process, by monitoring the MS signal intensity. For the mAb, we found that the highest MS signal intensity was obtained using only nitrogen gas (**Fig. S2b**), which was in line with a previous report [89]. From the DEN gases, the decrease in MS intensity was 7- to 10-fold for IPA and MeOH and 100-fold for ACN (**Table S2**). Similar results were obtained for ovalbumin, where the intensity of the species in the main peak (elution at 21.4 min) decreased 10- to 29-fold upon DEN gas usage (**Fig. S3**; **Table S2**). In contrast, the other investigated proteins (i.e., RNase B, lipase, fetuin, and EndoPro) benefited from nitrogen gas enriched with (particularly alcohol-based) solvents resulting in substantially increased signal. The mass spectra of RNase B revealed a 1.5- or 3.2-fold increase in peak areas upon enrichment with IPA or MeOH compared to solely nitrogen gas, while peak areas obtained with ACN-enriched nitrogen gas decreased (**Fig. 1**; **Table S2**). The MS signal intensity obtained with ACN-enriched nitrogen gas was similar to results obtained with solely nitrogen gas. Similarly, the ionization of the lipase was slightly improved when employing IPA- or MeOH-enriched nitrogen gas, where ~1.5-fold increase was observed for the main species with one glycan containing between 4 and 14 mannoses (elution at 25.3 min) (**Fig. S4**). Notably, the ionization of proteoforms without glycans was not favored by the application of DEN gas, indicating that glycosylation is probably (partly) responsible for the gain in intensity upon the use of DEN gas. Even though ovalbumin and the mAb are also glycosylated, the contribution of the glycans to the molecular weight of RNase B and the lipase was substantially higher (**Table 1**) and higher levels of glycosylation substantially increase the overall hydrophilicity of proteins. Therefore, we hypothesize that the advantageous effect of the DEN gas is related to the hydrophilicity of the proteins. Moreover, it was already shown that more hydrophilic molecules tend to be less efficiently ionized by ESI [230] (without DEN gas). Of note, there was no clear correlation between the hydrophilicity of the protein backbone (presented in **Table 1** as the GRAVY score, where lower values correspond to more hydrophilic proteins [358]) and the positive influence of DEN gas on ionization.

While for RNase B and lipase the DEN gas effect was still minor, a substantial improvement was observed for the heterogeneous glycoproteins fetuin and EndoPro (**Fig. 2** and **S5**). The three N- and two O-glycosylation sites of fetuin complicate the PTM profile. For the assignment of this heterogenous pool of glycoforms, sufficient MS intensity and spectral quality are essential. Using DEN gas, both factors were greatly improved leading to the detection of more proteoforms, as illustrated by the deconvoluted mass spectra of the last eluting peak (at 32.2 min) (**Fig. S5b**). Although the main variant could be

detected under all conditions, lower abundant species were solely observed with DEN gas. For EndoPro (with seven occupied glycosylation sites), the MS peak areas increased ~7-fold for the proteoforms without phosphoglycans (elution at 11.5 min) with IPA as dopant compared to nitrogen gas only (**Table S2**). Similar results were obtained with MeOH as dopant and slightly lower efficiency was observed for ACN. Enhanced MS intensity came along with higher spectral quality for the IPA-enriched nitrogen gas allowing the assignment of glycoforms varying from 51 to 75 mannoses per protein (**Fig. 2**). In contrast, without DEN gas spectral quality was too poor for any proteoform assignment (**Fig. S6**).

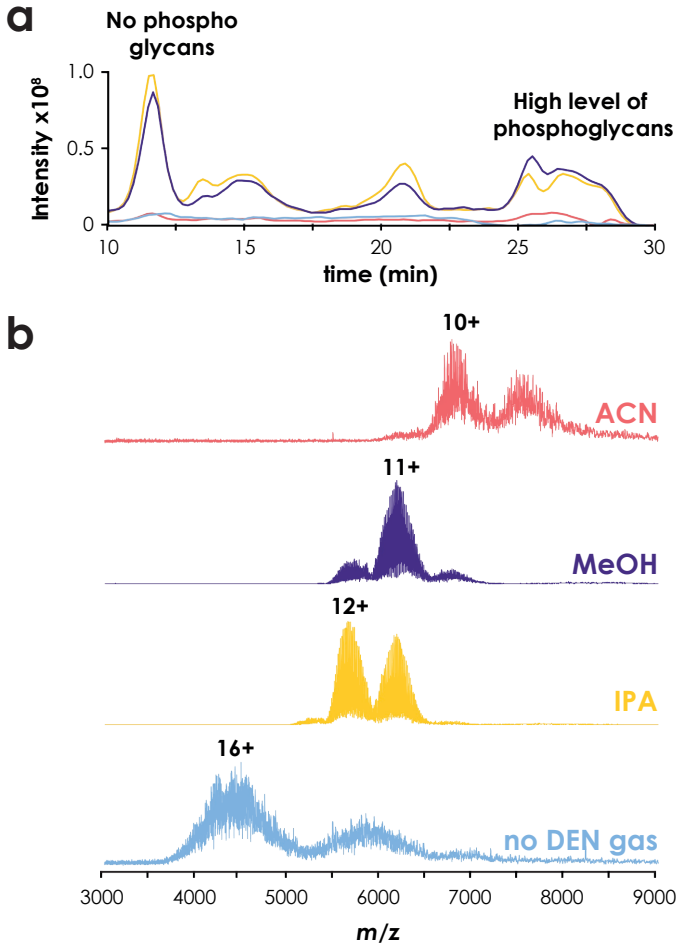


Figure 2 | AEX-MS analysis of EndoPro with or without dopant gas enrichment, including with IPA (yellow), MeOH (purple) and ACN (red), or solely nitrogen gas (blue). (**a**) BPCs of EndoPro measured with different dopant gases. (**b**) Obtained mass spectra of the first eluting peak without phosphoglycans. The highest charge state is labeled for each condition.

Interestingly, a later eluting peak containing high levels of phosphorylated glycans (at 26.6 min) benefited even more from the use of DEN gas with a ~11-fold increase in signal intensity after using IPA as dopant gas. A (minor) contribution of phosphorylation to the effect of DEN gas was also observed for ovalbumin. Whilst the ionization efficiency of ovalbumin was the highest for the experimental set-up without dopant enrichment, the extent of phosphorylation appeared to affect the ionization with DEN gas (**Fig. S3b**). The MS peak areas of the non-phosphorylated and two-times-phosphorylated variants were similar for solely nitrogen gas, but the latter peak area was increased after the application of DEN gas (**Fig. S3d**; **Table S2**). Altogether, the ionization of negatively charged and/or heterogenous proteins mostly benefited from the use of DEN gas.

3.4 Conclusion

In this study, we investigated whether DEN gas could create more favorable nano-ESI conditions for glycoproteins after native separations. For this purpose, we analyzed six proteins with varying physico-chemical properties with AEX-MS. All proteins exhibited a shift in CSD to lower charges upon the usage of DEN gas. Furthermore, proteins with a large glycan content particularly benefited when DEN gas was applied in the analytical workflow. Alcohol-based solvents as dopants (IPA and MeOH) resulted in the largest increase in sensitivity. The complex, highly glycosylated proteins EndoPro and fetuin could only be properly analyzed with DEN gas, where the use of DEN gas resulted in increased MS intensity together with improved mass spectral quality. The exploration of DEN gas may be a decisive factor in establishing native LC-MS methods for the characterization of complex and heterogenous proteins, in particular highly glycosylated proteins.

Acknowledgments

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Supplementary information

The following supplementary files are available on request:

AEX-UV chromatograms (**Fig. S1**); Influence DEN gas on mAb ionization (**Fig. S2**); Effect of DEN gas on ionization of ovalbumin (**Fig. S3**); Influence of DEN gas on ionization of lipase (**Fig. S4**); AEX separation and deconvoluted mass spectra of fetuin (**Fig. S5**); Deconvoluted mass spectra of EndoPro (**Fig. S6**); MS methods of the proteins (**Table S1**); MS Intensity of assigned proteoforms using the different DEN gases (**Table S2**).