# Supplementary Material

## Micro-flow Size-Exclusion Chromatography for enhanced native Mass Spectrometry of proteins and protein complexes

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**Figure S1.** Schematic representation of the trap-and-elute micro-flow SEC-MS set-up. A 10-port-valve configuration was used with an ion-exchange trap and a 1.0-mm I.D. SEC column. The proteins were loaded onto the IEX trap with a low-salt-concentration mobile phase. Switching to a high salt-concentration eluent led to elution of the proteins from the trap onto the SEC column. The grounding union of the ESI was bypassed. Instead, the 10-port valve was grounded as indicated.

**Figure S2.** Total-ion current chromatograms (TIC) obtained with micro-flow SEC-MS of A) ovalbumin, B) transferrin and C) trastuzumab, varying the concentration of ammonium acetate in the mobile phase from 20 up to 400 mM. Flow rate, 15 µL/min.

**Figure S3.** Peak area of BSA (M), ovalbumin (M) and transferrin obtained using micro-flow SEC-MS (flowrate, 15 µL/min; red) and conventional SEC-MS (flowrate, 200 µL/min, blue). M, monomer. A four-fold lower protein concentration was injected using micro-flow SEC-MS than with conventional SEC-MS.

**Table S1.** List of proteins and uracil used for the calibration of the SEC columns and the development of the low-flow trap-SEC-MS set-up.

**Figure S4.** SEC calibration using reference proteins and various mobile phases (ionic strength, IS; type of salt). $Log_{10}MW$ is plotted against the protein elution volume using A) a benchmark PBS eluent of 600 mM IS; B-D ammonium-acetate solutions of B) 400 mM IS, C) 200 mM IS, D) 100 mM IS, and E) 50 mM IS. Graphs were constructed using thyroglobulin, L-asparaginase (ASNase) tetramer ($M_4$), bovine serum albumin (BSA) monomer (M), ovalbumin, RNAse A and uracil. The pH of the eluent was 6.8 at all examined conditions.

**Table S2.** Estimated amount of ammonium acetate directed to MS varying the flow rate and salt concentration of eluent.

**Figure S5.** Cumulative extracted-ion chromatograms (EICs) of pyruvate kinase tetramer ($M_4$) obtained with SEC-MS using A,C) the 4.6-mm I.D. SEC column at 200 µL/min; injected concentration 17 µM, and B,D) the 1-mm I.D. SEC column at 15 µL/min, injected concentration 4 µM. The EIC were constructed for the $[M+27H]^{27+}$, $[M+26H]^{26+}$, $[M+25H]^{25+}$, and $[M+24H]^{24+}$ ions of the PK monomer (blue; A, D) and for the $[M_4+35H]^{35+}$, $[M_4+36H]^{36+}$, $[M_4+37H]^{37+}$, $[M_4+38H]^{38+}$ ions of the tetramer (red; B,C).

**Figure S6.** Mass spectrums of the impurity found in the PK sample. A. the mass spectrum assigned to the impurity identified using the 1 mm-I.D. SEC column. B. the mass spectrum of the impurity peak from the analysis using the 4.6-mm I.D. SEC column.

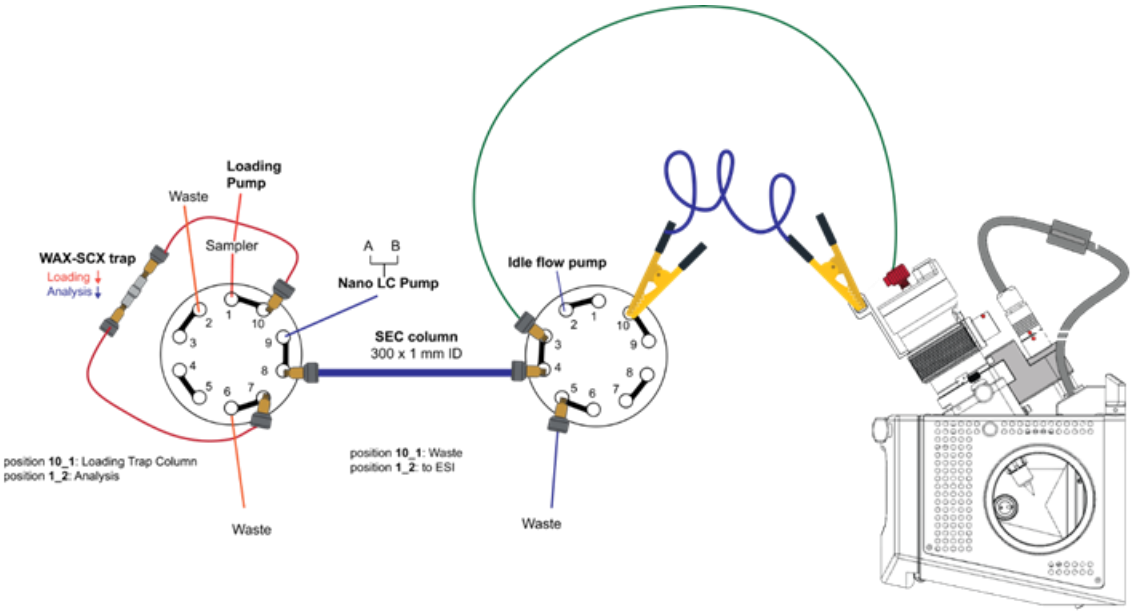
**Figure S7.** Microflow-SEC-UV of BSA using the benchmark PB eluent (pH 6.8) at a flow rate of 15 µL/min. A) Effect of increasing absolute injected amount at a constant injection volume of 1 µL; sample concentrations, 0.2 mg/mL (purple); 1 mg/mL (orange); 3 mg/mL (blue); 10 mg/mL (green); 20 mg/mL (red). B) Effect of injection volume at a constant injected amount of 5 µg; injection volume indicated varied from ca. 0.5% up to 10% of the column volume ($V_{col}$).

**Table S3.** Comparison of the chromatographic results for BSA monomer (M) and dimer ($M_2$) using loop and trap injections.

**Figure S8.** Micro-flow trap-SEC-UV of indicated proteins. Proteins were injected and trapped using 100 mM ammonium acetate (pH 6.8) and eluted using 400 mM ammonium acetate (pH 6.8).

**Figure S9.** Micro-flow trap-SEC-UV of indicated proteins. Proteins were injected and trapped using 100 mM ammonium acetate (pH 6.8) and eluted using 400 mM ammonium acetate (pH 6.8). The retention time of the protein species is indicated.

**Figure S10.** Micro-flow trap-SEC-MS of BSA (10 ng/µL; 0.15 µM) using an injection volume of 20 µL (0.2 pg BSA injected). A) The total-ion-chromatogram (TIC); B) the respective mass spectrum of BSA monomer (M) with the signal-to-noise (S/N) and peak width (W) indicated of the $[M+15H]^{15+}$ and $[M+16H]^{16+}$ ions.

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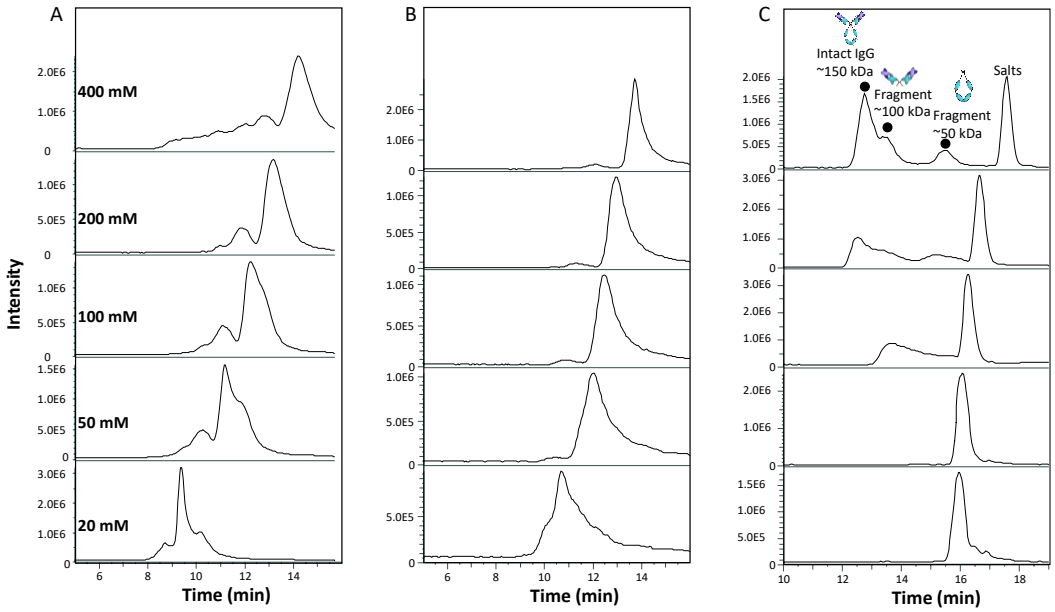


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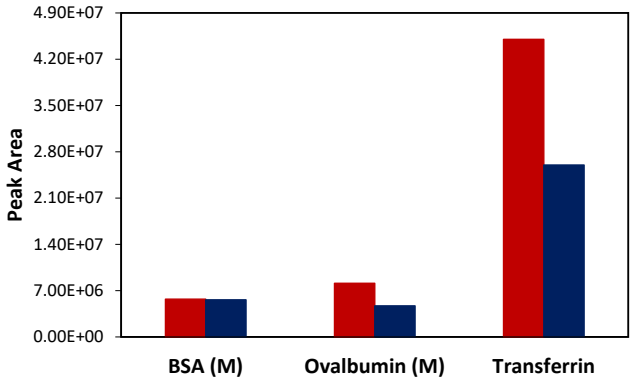


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Table S1. List of proteins and uracil used for the calibration of the SEC columns and the development of the low-flow trap-SEC-MS set-up

| **Analyte** | **Origin** | **MW (kDa)** | **pI** |
|---|---|---|---|
| Thyroglobulin | Bovine | 660 | 4.5 |
| Pyruvate kinase | Rabbit muscle | 237 | 5.2 |
| γ-Globulin | Bovine | 158 | 7.2 |
| Trastuzumab | - | 148 | 8.7 |
| L-Asparaginase | E.Coli | 140 | 8.6 |
| Transferrin | Human serum | 80 | 5.8 |
| Ovalbumin | Chicken | 44 | 4.6 |
| RNase A | Bovine pancreas | 13.7 | 8.6 |
| Uracil | - | 0.1 | - |

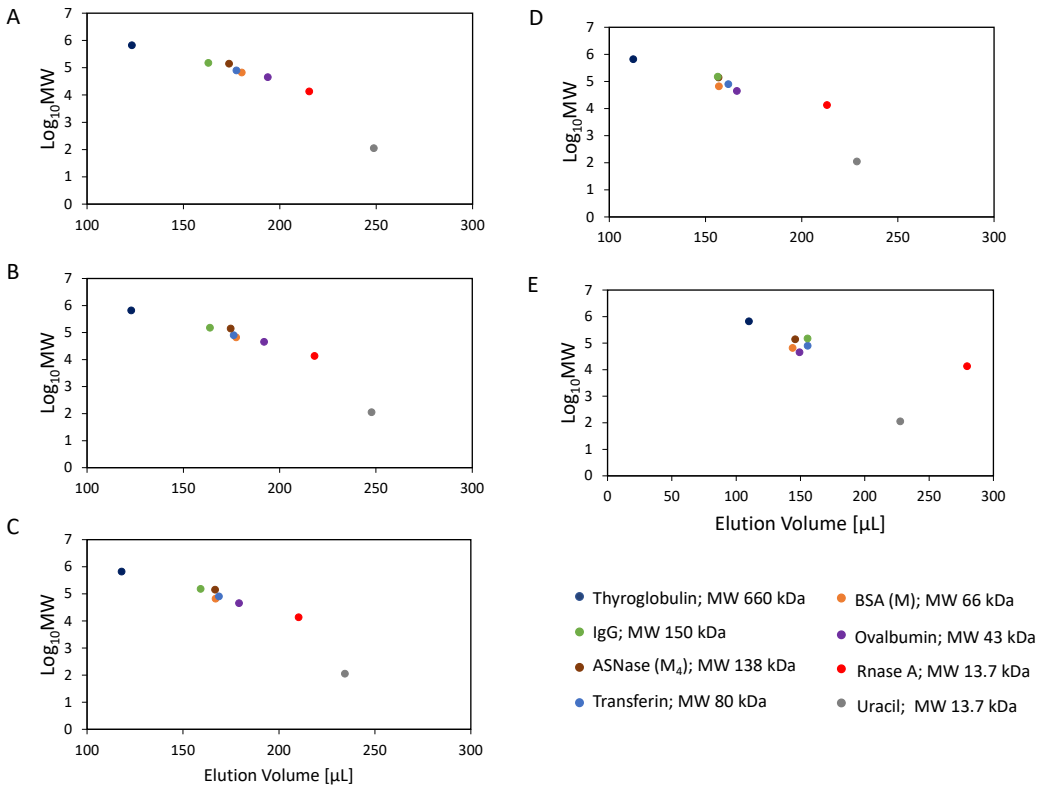


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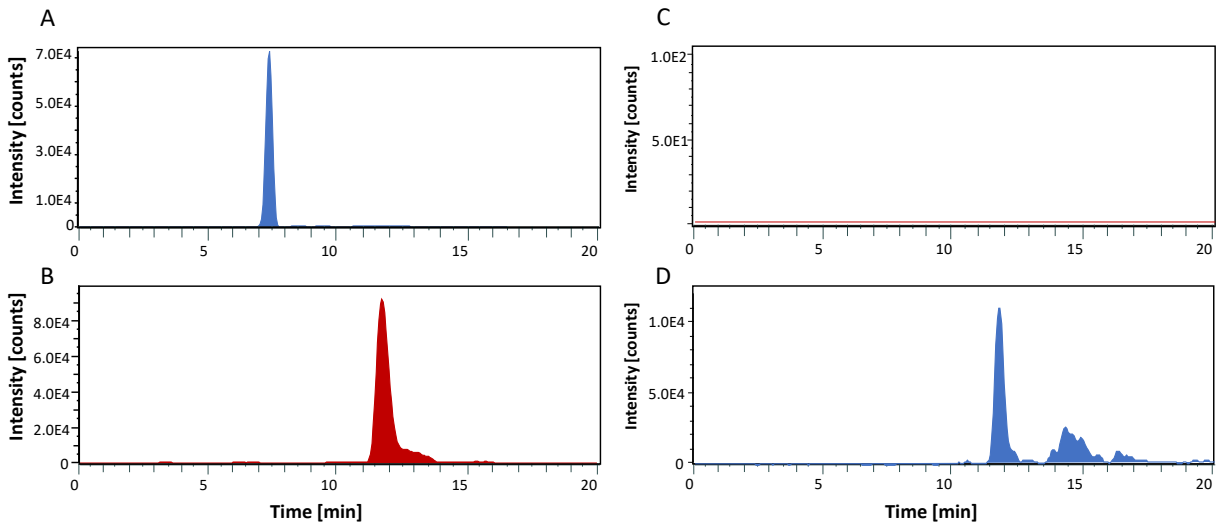
Table S2. Estimated amount of ammonium acetate directed to MS varying the flow rate and salt concentration of eluent.

| **Flow rate [µL/min]** | **Concentration ammonium acetate [mM]** | **Ammonium acetate amount directed to MS [µg / min]** |
|---|---|---|
| 200 | 50 | 771 |
| 15 | 50 | 58 |
| 15 | 200 | 231 |
| 15 | 400 | 462 |

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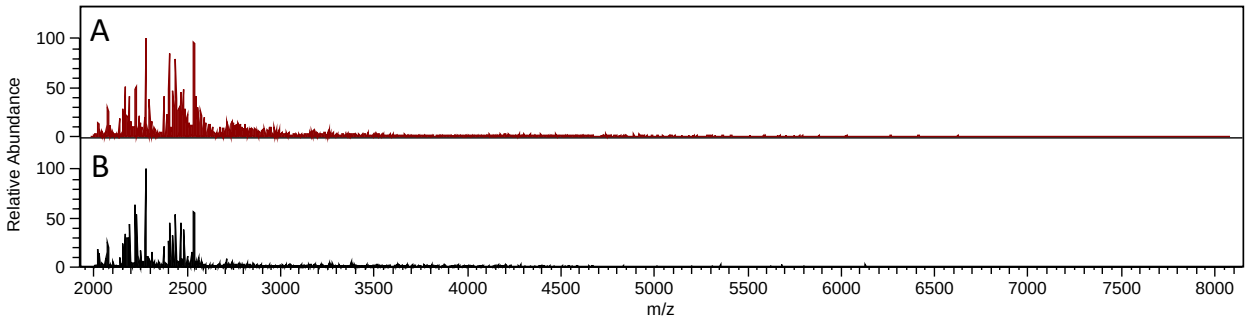


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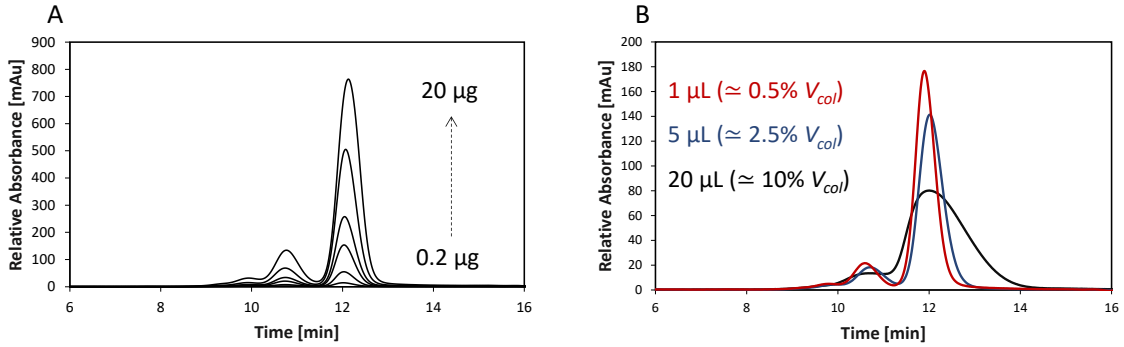


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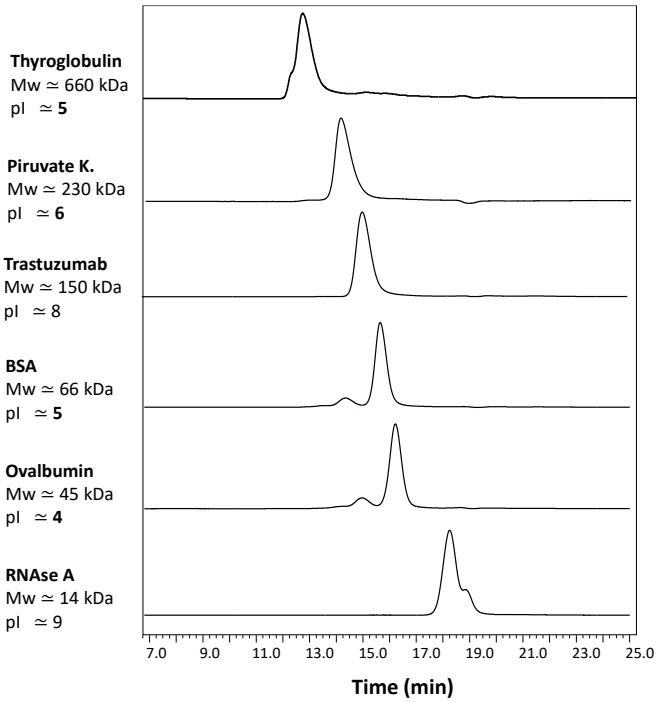
|---|---|---|---|---|
| | **Peak width (M) [min]** | **Peak width ($M_2$) [min]** | **Resolution (M-$M_2$)** | **Recovery** |
| Loop injection | 1.75 | 1.45 | 0.80 | - |
| Trap injection | 0.80 | 0.80 | 1.50 | 97% |

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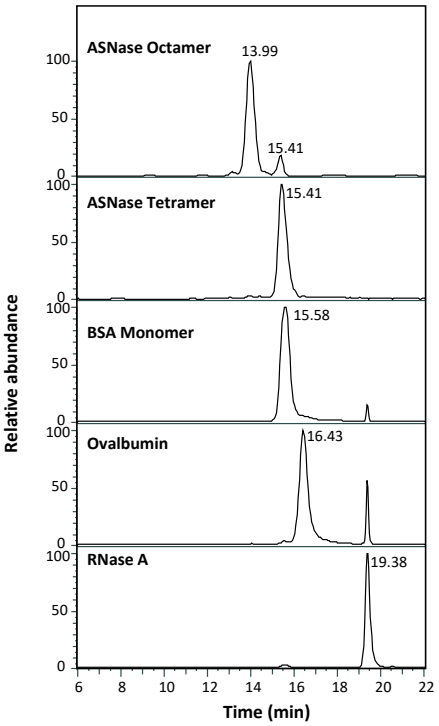


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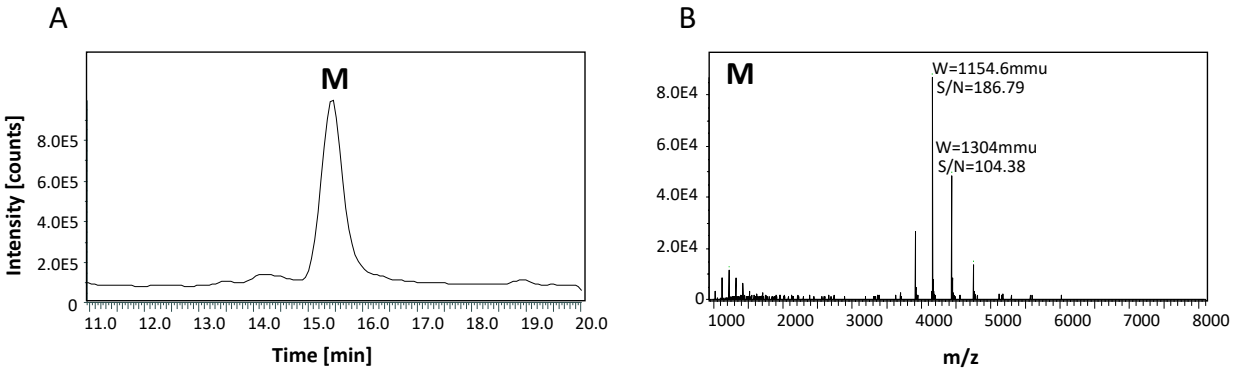


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