Electronic Supplementary Material (ESI) for Analyst.

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Online Protein Digestion in Membranes Between Capillary Electrophoresis and Mass Spectrometry

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Supporting Information

Table S1. Average intensities for a BSA peptide obtained from online digestion using a pepsin-containing membrane in the emitter tip. The emitter tip final diameter was varied between 15 and 60 µm, and 4 emitter tips were tested for each diameter range.

Tip Size	15-20 µm	30-35 µm	50-60 µm
Average Intensity x 10^8	1.9 ± 0.31	2.3 ± 0.063	0.47 ± 0.11

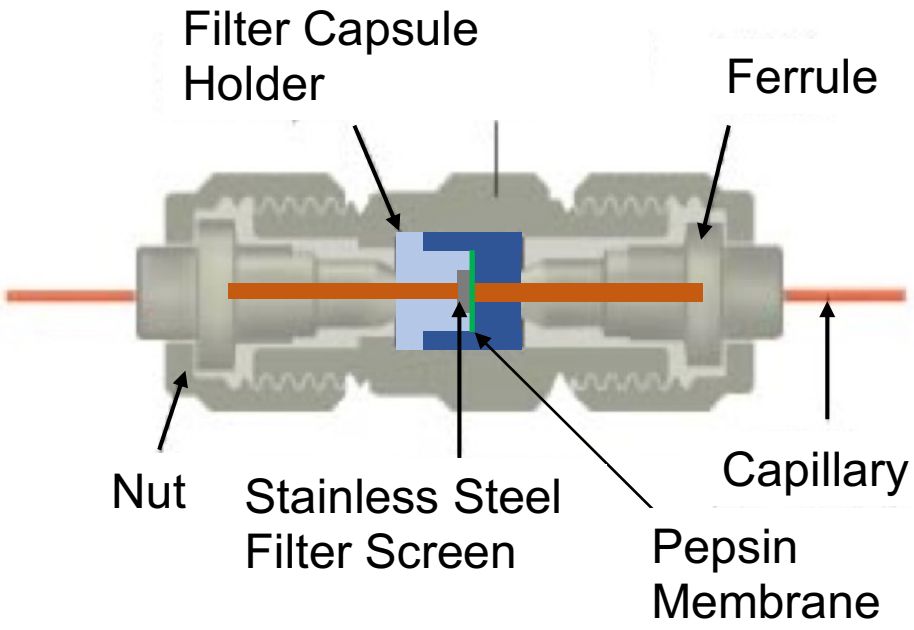

Fig. S1 IDEX M-533 mini microfilter assembly modified to contain a membrane in the filter capsule for integration into 360 µm od capillaries for CE separation. The figure is adapted from IDEX published sales literature.[49]

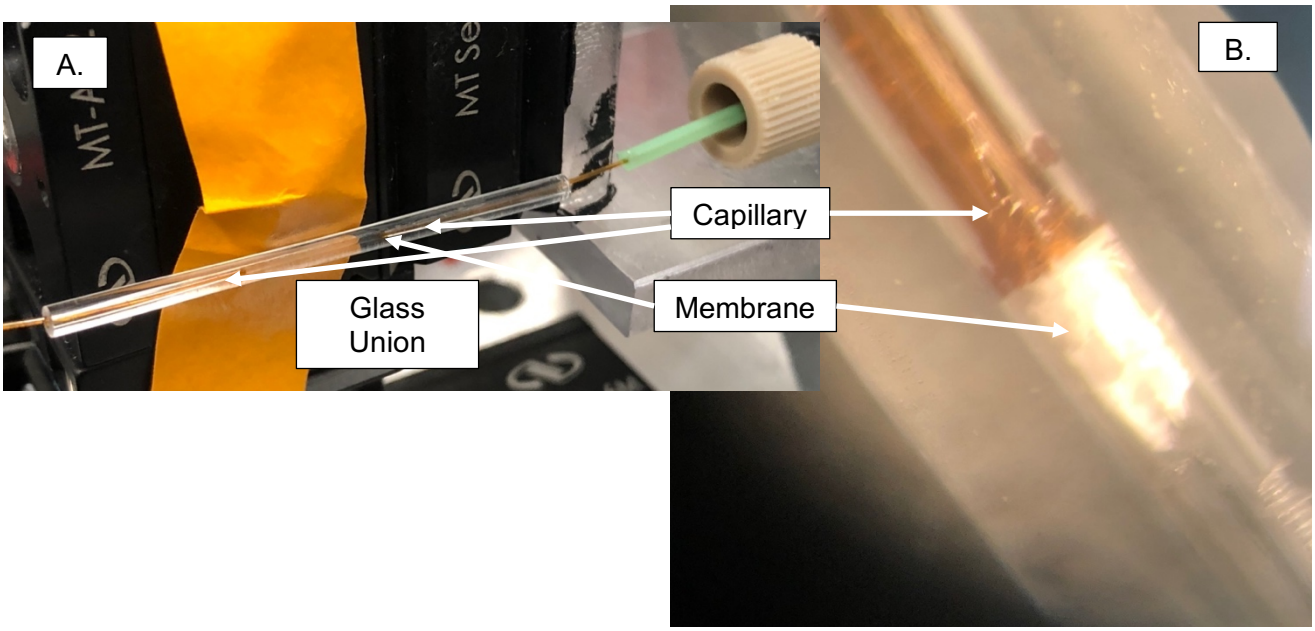

Fig. S2 A. Glass capillary union packed with a pepsin membrane to digest proteins in-line with a CE separation. This setup yielded an unacceptably high EOF. B. Close-up view of the membrane packed into the glass union. A second capillary farther down in the union links the membrane and digested proteins to the sheath-flow ESI source.

Table S2 Myoglobin sequence coverages and numbers of identified unique peptides for 20 repeat CE injections of intact equine myoglobin with all analyses using the same pepsin-containing emitter tip. A blank wash was run after the 20th injection. The table also shows the sequence coverage and number of identified peptides for pepsin, which may elute from the membrane in the emitter tip.

	Myoglobin Matches		Pepsin Matches	
Run #	Sequence Coverage	# Peptides	Sequence Coverage	# Peptides
1	63%	7	22%	9
2	87%	11	13%	5
3	87%	12	11%	4
4	63%	9	5%	2
5	87%	21	5%	3
6	87%	18	5%	2
7	87%	20	8%	3
8	87%	21	5%	2
9	87%	21	5%	2
10	87%	23	5%	2
11	87%	21	5%	2
12	90%	25	5%	2
13	90%	23	5%	2
14	90%	26	5%	2
15	90%	26	5%	2
16	90%	28	5%	2
17	90%	26	5%	2
18	90%	26	5%	2
19	90%	28	5%	2
20	87%	25	5%	2
Wash	0%	0	5%	2

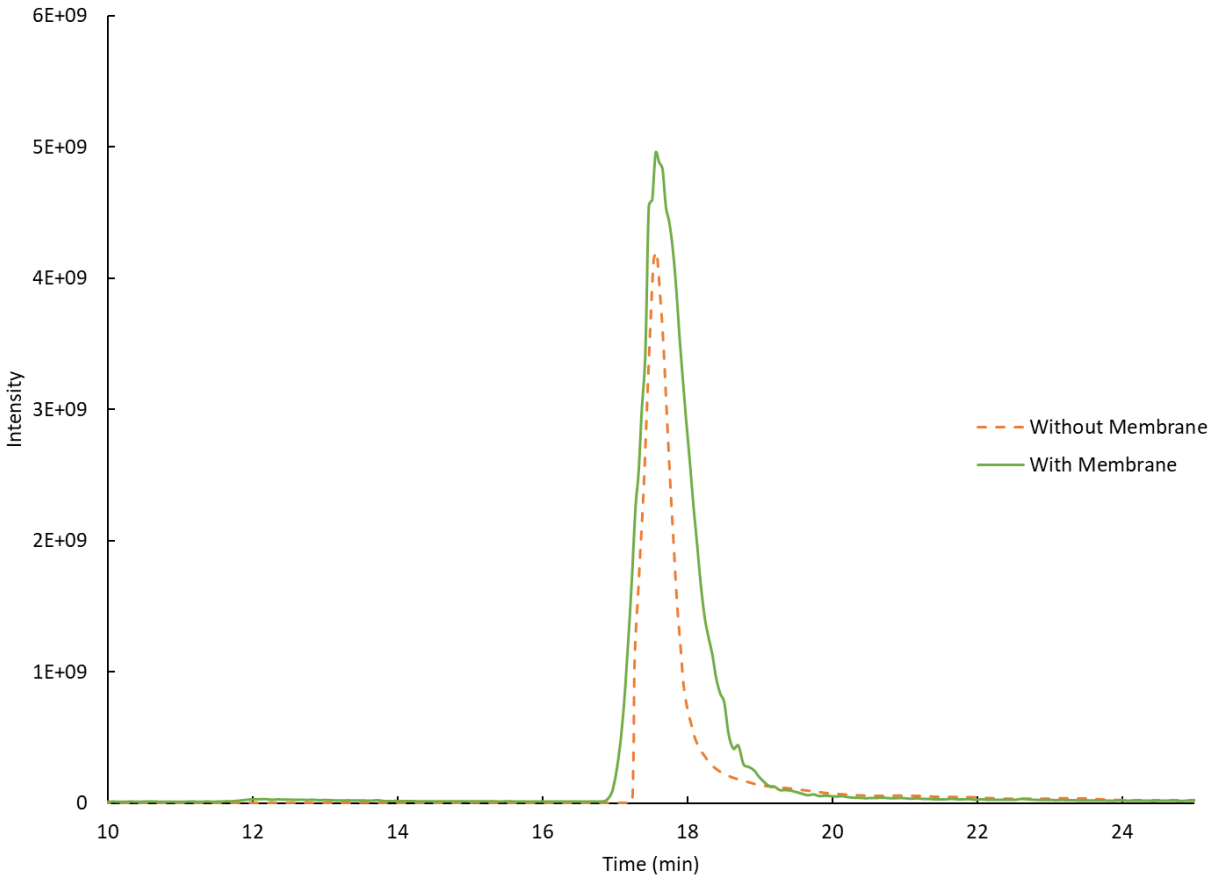

Fig. S3 BSA TIC electropherograms with and without a pepsin-containing membrane in the emitter tip. The figure shows limited broadening of the peak with the membrane in place.

Table S3 Sequence coverages and numbers of unique peptides for each individual emitter tip and replicate injections in the analysis of a six-protein mixture. *For Tip 4 replicates 1 and 2 were stopped at 30 minutes and only partially captured the migration of insulin, so these points were not included in the averages.

		Ribonuclease B		α-lactalbumin		Insulin		Serum Albumin		Carbonic Anhydrase II		Myoglobin	
		Peptides	Coverage	Peptides	Coverage	Peptides	Coverage	Peptides	Coverage	Peptides	Coverage	Peptides	Coverage
Tip 1	**Rep 1**	4	21%	8	51%	3	22%	5	14%	29	86%	17	99%
	Rep 2	2	11%	5	27%	2	19%	7	11%	9	56%	12	75%
	Rep 3	3	18%	7	56%	2	13%	7	14%	22	89%	22	99%
Tip 2	**Rep 1**	5	26%	6	32%	5	69%	8	22%	21	72%	28	99%
	Rep 2	4	21%	10	51%	7	59%	8	23%	29	94%	25	99%
	Rep 3	4	23%	8	58%	4	59%	13	43%	20	81%	25	99%
Tip 3	**Rep 1**	5	26%	5	40%	2	19%	9	24%	28	82%	21	99%
	Rep 2	3	18%	4	35%	1	10%	6	19%	18	70%	19	99%
	Rep 3	3	18%	3	35%	1	10%	2	7%	12	75%	13	99%
Tip 4	**Rep 1**	3	17%	8	38%	*	*	5	11%	25	62%	8	55%
	Rep 2	3	17%	7	27%	*	*	4	11%	30	70%	12	75%
	Rep 3	3	18%	5	22%	2	37%	6	13%	25	60%	15	75%
Combined		*5*	*26%*	*11*	*77%*	*14*	*69%*	*37*	*67%*	*63*	*96%*	*44*	*100%*
Median Number of Peptides and Average Sequence Coverage		3	20%	6.5	39%	2	32%	6.5	18%	23.5	75%	18	89%
Standard Deviation			4%		12%		22%		10%		12%		15%

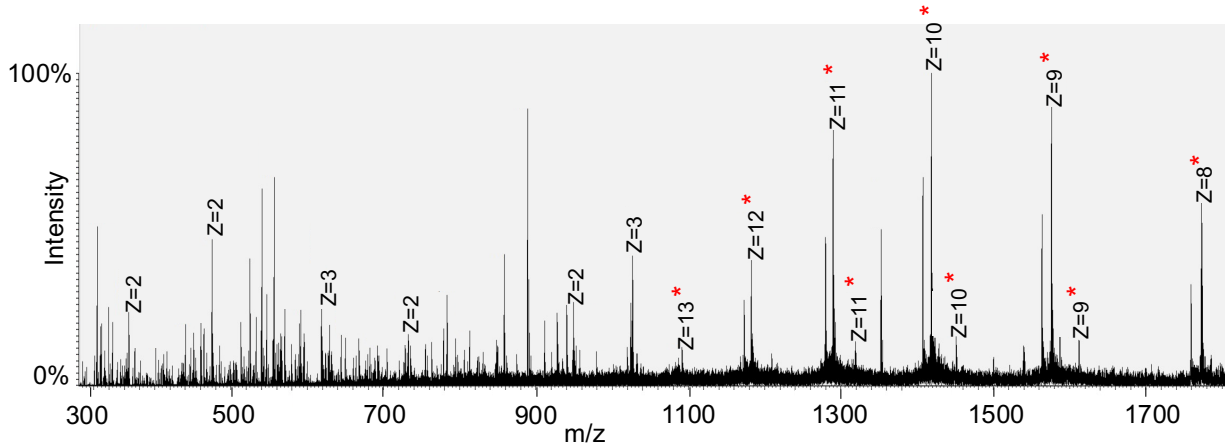

Fig. S4 Mass spectrum of α-lactalbumin after CE separation and online passage through a pepsin-containing membrane. Peaks labelled with asterisks stem from intact protein and exhibit high charge states. Peptides are present as well and show lower charge states (z = 2 or 3).

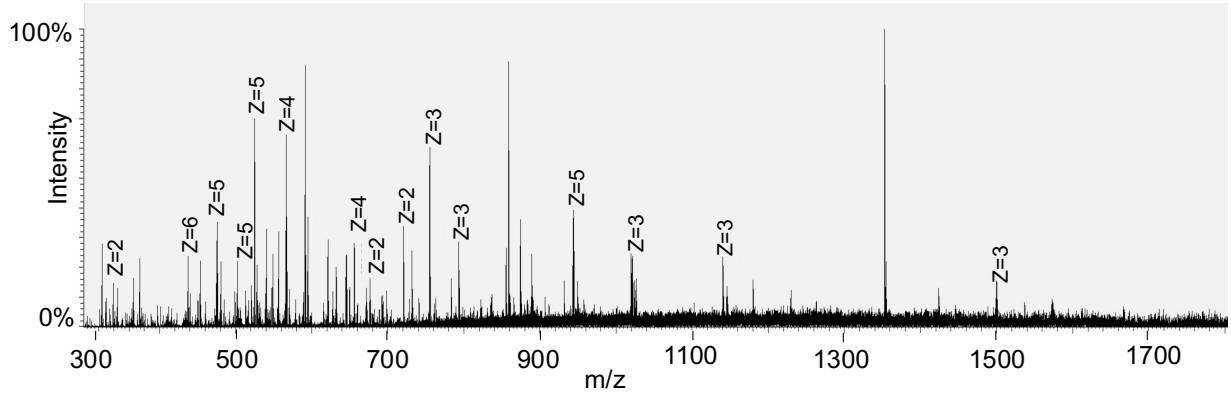

Fig. S5 Mass spectrum of myoglobin after CE separation and online passage through a pepsin-containing membrane. Intact protein is not present, and only peptides are apparent. Fig. S6 shows the expected spectrum if intact myoglobin was present.

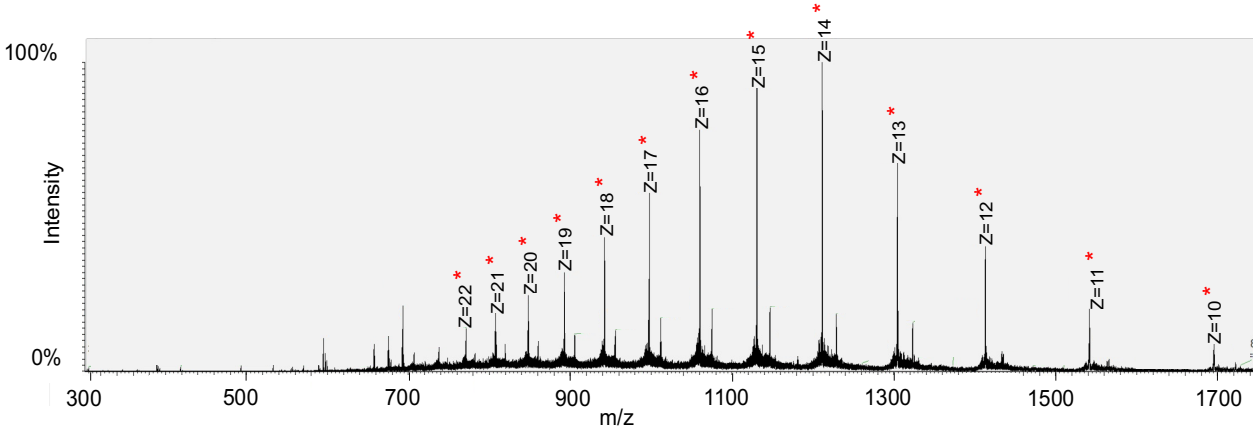

Fig. S6 Mass spectrum of myoglobin after CE separation without digestion.

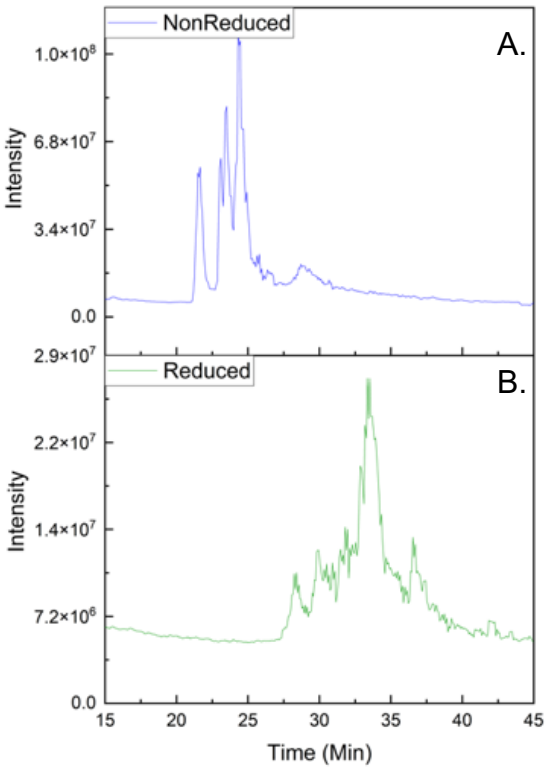

Fig. S7 TIC electropherograms of a six-protein mixture separated with CE. A. Separation of unreduced protein in 10 mM ABC. B. Separation of protein after acetic acid and TCEP-HCL denaturing and reduction. In B, the sample was dried after reduction and reconstituted in 10 mM ABC. The low resolution in B likely results from interaction of proteins with each other or the capillary wall. TCEP migrates much faster than proteins, so it should not interfere with the analyses.

Table S4 Sequence coverages for BSA at various injection volumes (different injection times) when using an aqueous sheath fluid with and without 1 mM TCEP. The sheath fluid contained 10% methanol, and 0.5% FA, and the experiments employed a pepsin-containing membrane in the emitter tip. As a reducing agent, TCEP might break disulfide bonds to aid in identifying proteolytic peptides. Higher concentrations of TCEP were not tested due to instrument fouling when constantly spraying nonvolatile salts. Peptide matching was performed using MaxQuant searched against a database of BSA only.

	Sequence Coverage	
Injection Time	**No TCEP**	**1mM TCEP**
5s	17.6%	17.3%
10s	26.4%	27.2%
20s	57.8%	47.1%

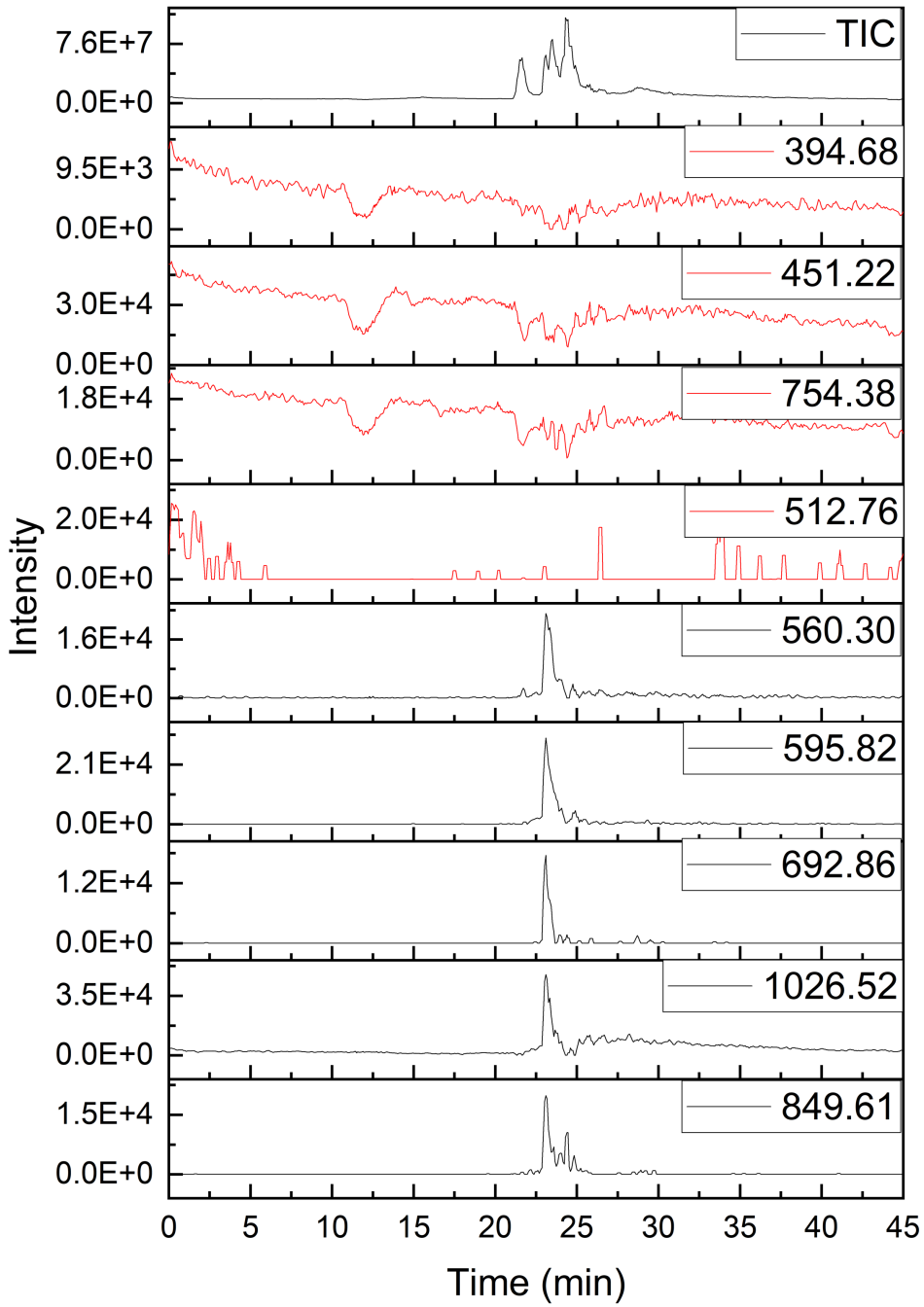

Fig. S8 XIEs for all peptides matching to myoglobin. Peptides outside the acceptable migration time window for myoglobin are highlighted in red. The TIC is shown at the top for reference. XIEs are labelled with their m/z values.

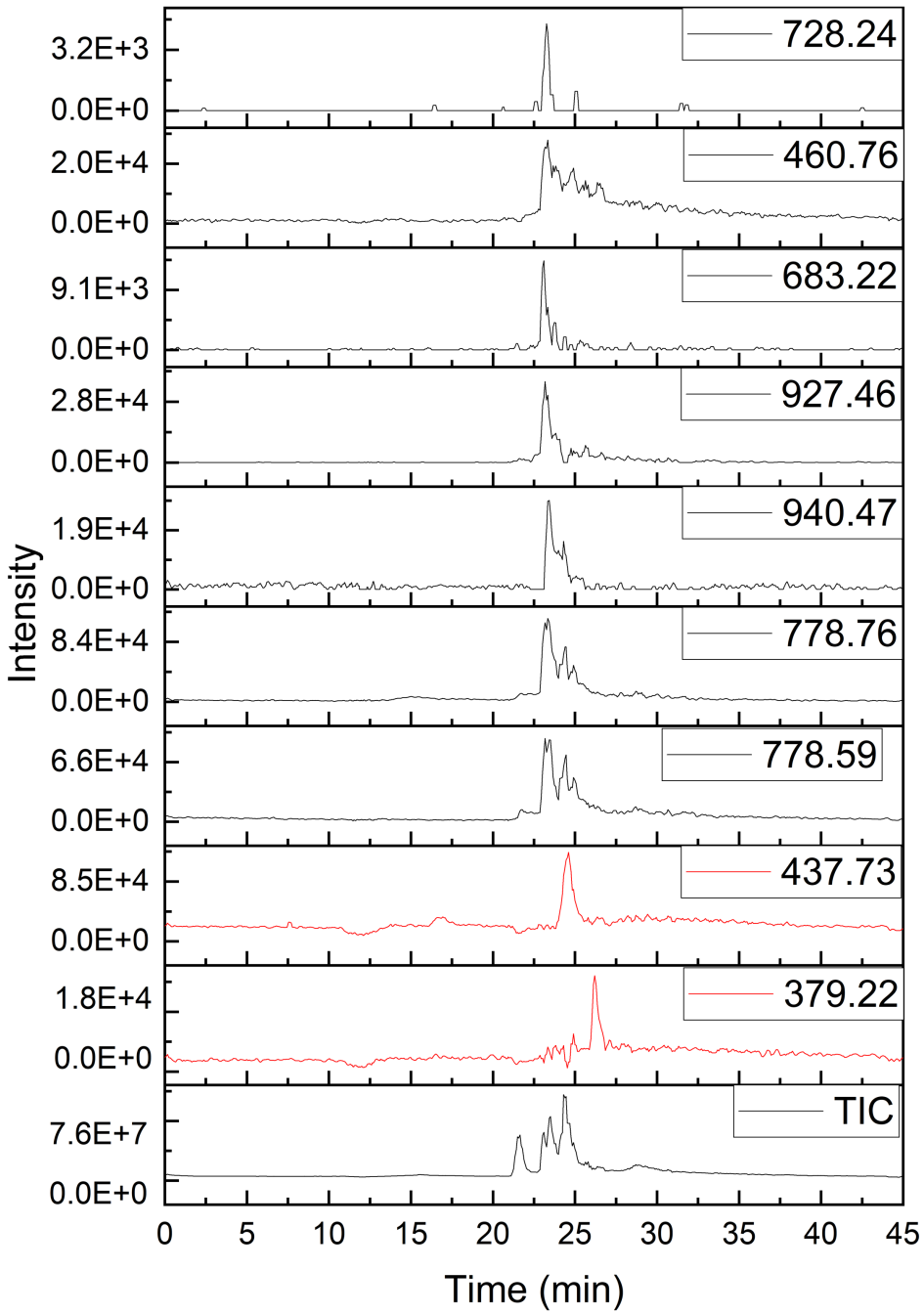

Fig. S8 (continued) XIEs for all peptides matching to myoglobin. Peptides outside the acceptable migration time window for myoglobin are highlighted in red. The TIC is shown at the bottom for reference. XIEs are labelled with their m/z values.

Table S5 Peptides identified from carbonic anhydrase II with sequences, matching scores (-10 log P), m/z values, migration times (MT), and intensities for each. Peptides with an inconsistent migration time are highlighted in red.

Peptide	-10LgP	m/z	MT	Intensity
F.LKVGDAN.P	25.69	358.7006	0.17	5.25E+05
D.PALKPLAL.V	20.36	411.7768	0.17	1.31E+07
G.ERQSPVD.I	30.28	415.7043	0.17	9.38E+05
L.LM(+15.99)LANWRPAQPLKNRQVRGFPK	49.96	528.1028	24.39	5.15E+05
A.NWRPAQPLKNRQVRGFPK	65	548.8151	24.46	4.32E+06
L.ANWRPAQPLKNRQVRGFPK	102.95	566.5759	24.46	1.86E+08
M.LANWRPAQPLKNRQVRGFPK	80.77	594.847	24.46	8.99E+07
L.MLANWRPAQPLKNRQVRGFPK	62.85	502.2871	24.46	3.66E+07
W.IVLKEPISVSSQQM(+15.99)LKFRTLNF.N	27.33	649.3695	24.53	2.77E+05
L.LMLANWRPAQPLKNRQVRGFPK	68.64	524.9043	24.53	2.05E+08
W.IVLKEPISVSSQQMLKFRTLNFNAEGEPEL.L	70.34	1139.947	24.61	6.74E+05
Q.PLKNRQVRGFPK	59.73	720.4365	24.68	4.44E+06
W.IVLKEPISVSSQQMLKFRTLNF.N	80.5	645.3687	24.68	6.65E+07
F.LKVGDANPALQKVLDALDSIKTKGKSTDFPNFDPGSLLPNVLDY.W	90.57	943.71	24.68	1.87E+08
T.KAVVQDPALKPLAL.V	68.57	731.9557	24.91	3.95E+05
F.LKVGDANPALQKVLDAL.D	69.17	883.0175	24.91	2.41E+07
L.VYGEATSRRMVNNGHSFNVEYDDSQDKAVLKDGPLTGT.Y	97.01	835.0029	24.91	3.11E+07
A.VVQDPALKPLAL.V	61.4	632.3885	25.8	1.18E+08
W.IVLKEPISVSSQQ.M	70.5	714.4097	25.8	1.12E+07
F.NVEYDDSQDKAVLKDGPLTGT.Y	77.14	1133.05	25.8	1.49E+06
F.NVEYDDSQDKAVL.K	52.74	748.35	26.53	3.76E+06
F.LKVGDANPAL.Q	47.81	499.2888	28.23	6.84E+07
W.IVLKEPISV.S	35.23	499.3183	29.28	1.92E+07
L.QKVLDAL.D	19.1	393.74	30.67	2.86E+05
L.KDGPLTGT.Y	52.21	394.7113	30.67	1.34E+07
W.IVLKEPIS.V	41.58	449.7848	30.67	1.53E+07
E.YDDSQDKAVL.K	59.48	1153.539	30.67	1.24E+06

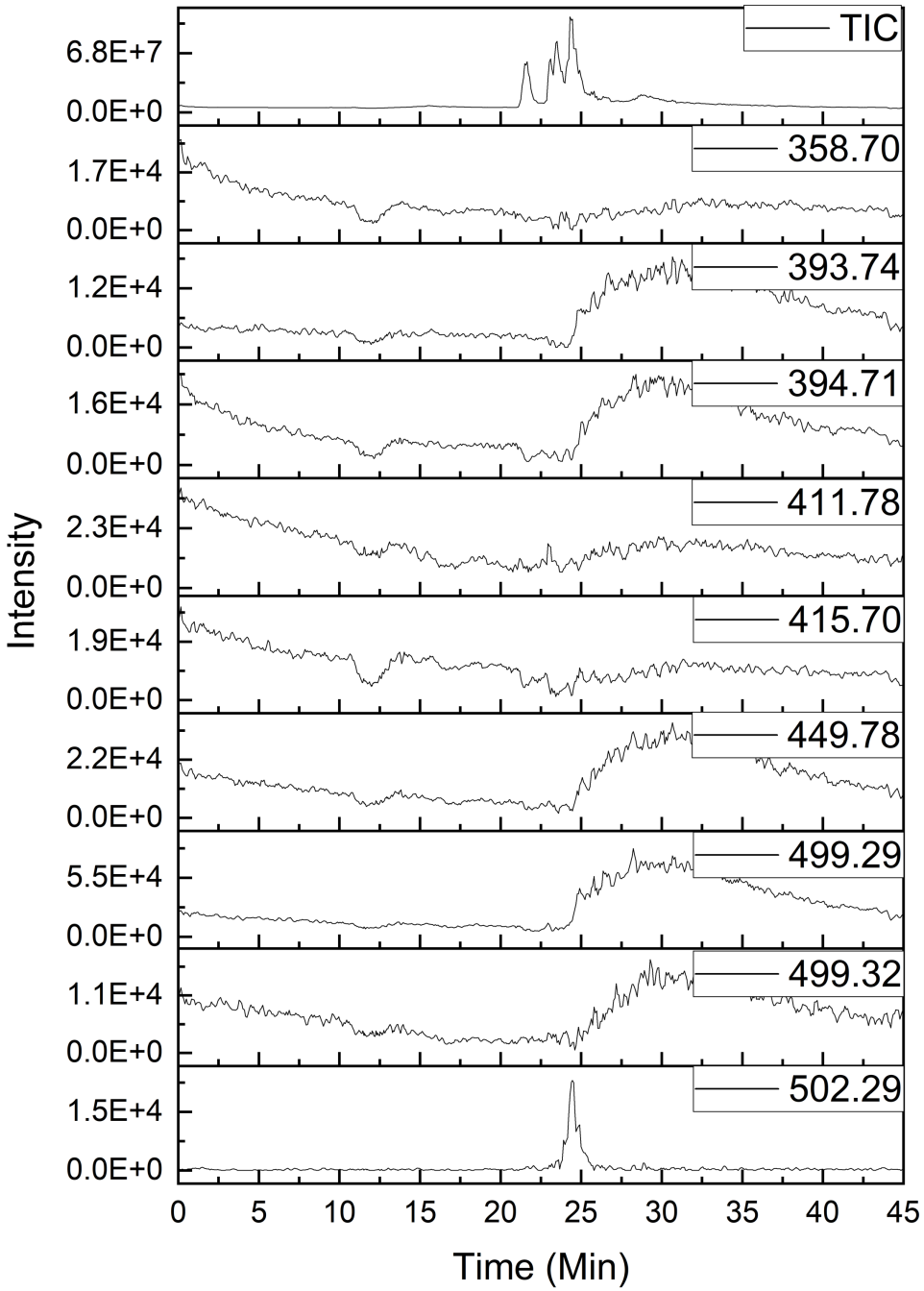

Fig. S9 XIEs for all identified peptides for carbonic anhydrase II. The TIC is shown at the top for reference. XIEs are labelled with their m/z values.

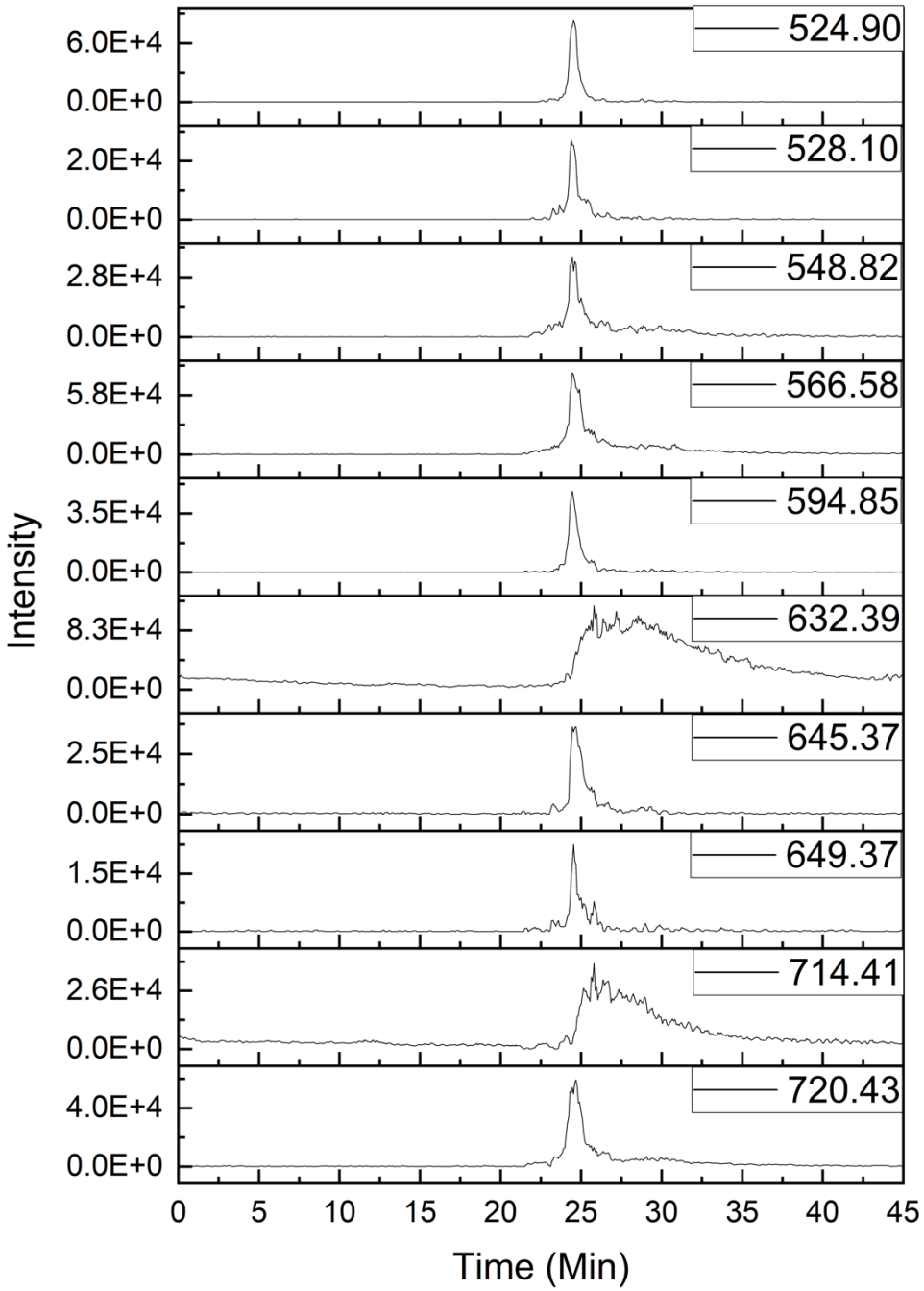

Fig. S9 (continued) XIEs for all identified peptides for carbonic anhydrase II. XIEs are labelled with their m/z values.

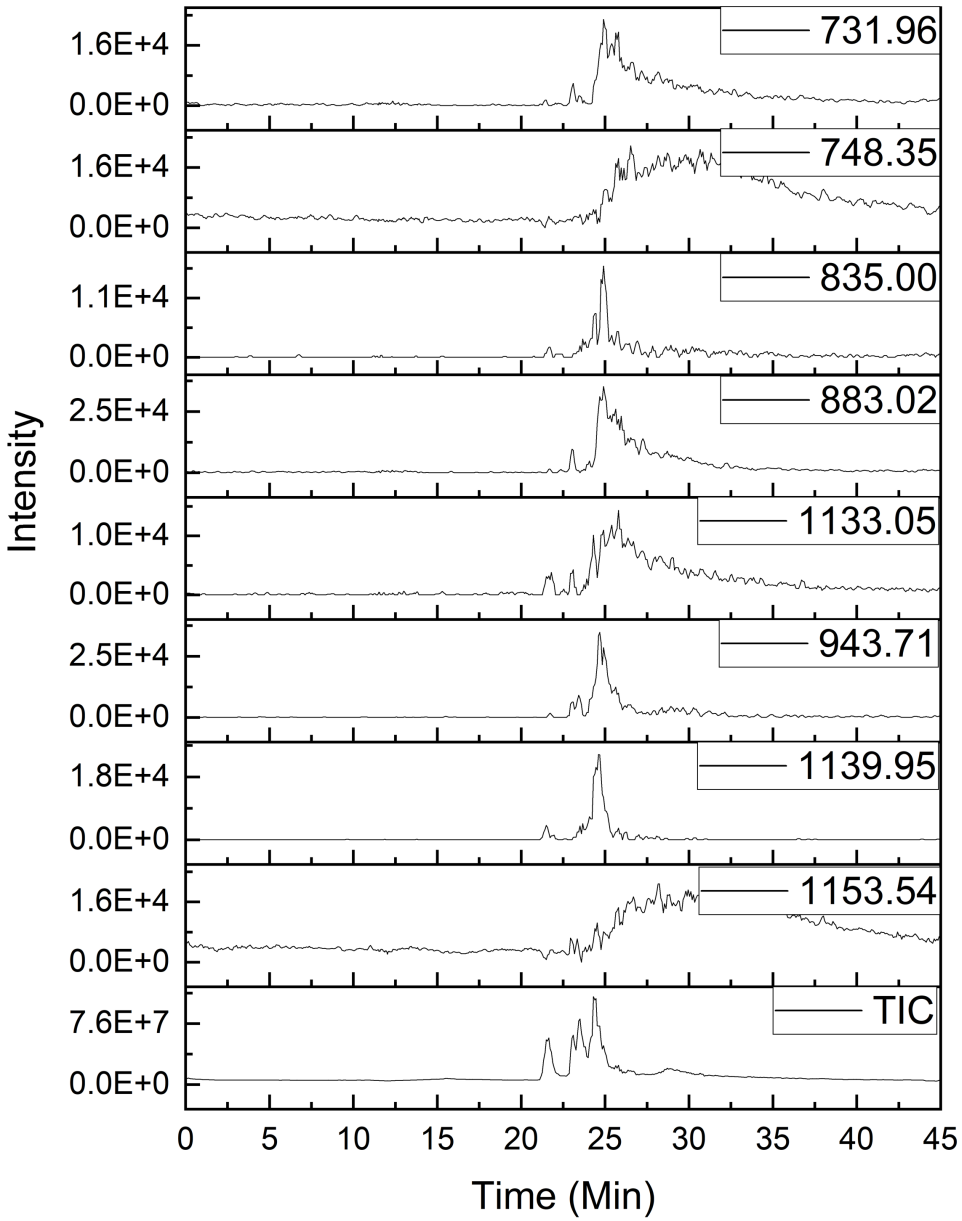

Fig. S9 (continued) XIEs for all identified peptides for carbonic anhydrase II. The TIC is shown at the bottom for reference. XIEs are labelled with their m/z values.

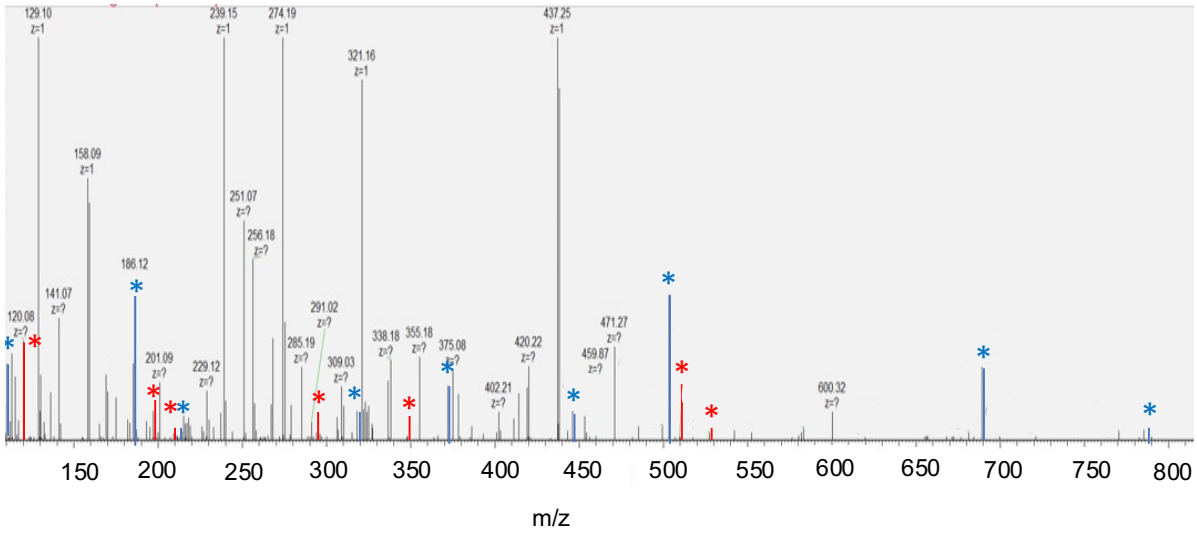

Fig. S10 MS/MS spectrum of m/z= 437.73 labelled with matching b and y ions highlighted in blue for myoglobin (determined by Peaks) and red for carbonic anhydrase II (determined by hand). The possible peptide sequences are NAWGKVEA for myoglobin and QSPVDIDT for carbonic anhydrase II.

Data File Submission

Raw data files were submitted to the MassIVE database. Private access can be found using the URL ftp://MSV000091085@massive.ucsd.edu and password; Membrane. Upon public submission the URL will change to ftp://massive.ucsd.edu/MSV000091085/.