Electronic Supplementary Material (ESI) for Analyst.

Performance Evaluation of In-source Ion Activation Hardware and Collision-Induced Unfolding of Proteins and Protein Complexes on a Drift Tube Ion Mobility-Mass Spectrometer

Supplementary Information

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Table S1 – Protein Standards Used in This Work

Protein	**Product Number**	**Molecular Weight (kDa)**
SiLu Lite SigmaMAb Standard antibody	MSQC4	150
Bovine Serum Albumin	A2153	66.5
Streptavidin from *Streptomyces Avidinii*	S0677	52.8
Myoglobin from equine heart	M1182	17.6
Ubiquitin from bovine erythrocytes	U6253	8.6

Table S2 – Optimized Instrument Parameters for Native Conditions

Region	Setting Name	Default	Native Ubiquitin	Native Myoglobin, Streptavidin, BSA	Native IgG Production Grade
Source	Sample Infusion Rate (µL/min)	n/a	2-5	2-5	2-5
	Gas Temp (deg. C)	325	140	140	250
	Drying Gas (L/min)	5	5	5	5
	Nebulizer Pressure (psi)	20	35	20	20
	Sheath Gas Temp (deg. C)	275	140	140	140
	Sheath Gas Flow (L/min)	10	8	11	11
	VCap, Capillary Voltage (V)	4000	3000	2500-3000	3000
	Nozzle Voltage (V)	2000	2000	2000	2000
	Fragmentor (V)	400	400	400	400
Pressures	High Pressure Funnel (Torr)	4.00	4.80	4.80	4.10-4.30
	Trapping Funnel (Torr)	3.80	3.80	3.80	3.80
	Drift Tube (Torr)	3.95	3.95	3.95	3.95
Acquisition	Max Drift Time (ms)	n/a	90	90	90
	Trap Fill Time (µs)	20000	80000	80000	80000
	Trap Release Time (µs)	150	1000	1000	1000
Advanced Parameters	In-Source CE (V)	20	10 - 200	10 - 450	10-410
	High Pressure Funnel Delta (V)	150	50	150	180
	High Pressure Funnel Radio Frequency (Vpp)	150	50	150	200
	Trapping Funnel Delta (V)	180	160	164	164
	Trapping Funnel Radio Frequency (Vpp)	150	180	150	200

Table S3 – CIUSuite2 Data Processing Parameters

		Ubiquitin +6	Myoglobin +8	Streptavidin +11	BSA +16	SigmaMAb
Data Import	Smoothing Savitzky-Golay	Window: 5; Iteration: 1 (Default)	Window: 5; Iteration: 1 (Default)	Window: 5; Iteration: 1 (Default)	Window: 5; Iteration: 1 (Default)	Window: 7 Iteration: 1
	Crop	10-200 V 10-20 nm^2	10-240 V 15-35 nm^2	100-440 V 30-50 nm^2	10-410 V 40-70 nm^2	10-410 V 70-140 nm^2
	Plot Options	Default	Default	Default	Default	Default
Feature Fitting	Mode	Standard (Default)	Standard (Default)	Standard (Default)	Standard (Default)	Standard (Default)
	Minimum Feature Length [x-axis] (Data Points)	5 (Default)	2	4	2	4-6
	Feature Width [y-axis] Allowed (nm^2)	1	1	1	1.5	2-4
	Max CV Gap	0 (Default)	0 (Default)	0 (Default)	0 (Default)	0 (Default)
CIU50 Fitting	Mode	Standard (Default)	Standard (Default)	Standard (Default)	Standard (Default)	Standard (Default)
	Trans. Region Padding	15 (Default)	15 (Default)	15 (Default)	15 (Default)	15 (Default)
	Max CV Gap	0 (Default)	0 (Default)	0 (Default)	0 (Default)	0 (Default)

Table S4 – Inter-Laboratory RMSD Values for Ubiquitin, Myoglobin, Streptavidin, and BSA

Ion	CIU Reproducibility RMSD (%)			
	UM	TAMU	VU	Inter-laboratory
Ubiquitin +6	3.8 ± 1.4	2.5 ± 0.6	1.9 ± 0.4	18 ± 2
Myoglobin +8	3.7 ± 0.6	2.9 ± 0.5	1.9 ± 0.2	37 ± 11
Streptavidin +11	1.0 ± 0.1	2.3 ± 0.4	4.4 ± 0.4	20 ± 6
BSA +16	2.2 ± 0.3	2.6 ± 0.3	3.0 ± 0.3	18 ± 4

Table S5 – $^{DT}CCS_{N2}$ Values from this study, and referenced literature

		$^{DT}CCS_{N2}$ values reported for each protein ($Å^2$) [a.]						
		[1]*2018 May et al.*	[2]*2020 Stiving et al.* [b.]	[3]*2020 Zheng et al.*	[4]*2020 Gadkari et al.* [c.]	*Lit. Avg.*	*This work* [d.]	*% Bias* [e.]
Ubiquitin (+6)	F1	1220±10 (3)	--	1210±10 (3)	1190±10 (3)	1210 (3)	1220±10 (4), 0.9%	*0.5%*
	F2	1470±20 (4)	--	--	1350±10 (3)	1410 (2)	1430±40 (4), 2.6%	*1.5%*
	F3	1630±10 (5)	--	--	1470±10 (3)	1550 (2)	1660±10 (3), 0.7%	*6.5%*
	F4	--	1730±10 (3)	--	--	1730 (1)	1730±10 (2), 0.3%	*0.0%*
Myoglobin (+8)	F1	1940±30 (5)	--	--	--	1940 (1)	1970±10 (4), 0.5%	1.6%
	F2	--	--	--	--	--	2110±50 (3), 2.4%	--
	F3	--	--	--	--	--	2730±30 (4), 1.0%	--
Streptavidin (+11)	F1	--	3760±40 (3)	--	--	3760 (1)	3680±10 (4), 0.2%	2.1%
	F2	--	--	--	--	--	3520±10 (3), 0.3%	--
	F3	--	--	--	--	--	4350±20 (4), 0.4%	--
BSA (+16)	F1	--	4510±40 (3)	--	4530±20 (3)	4520 (2)	4520±50 (4), 1.2%	0.0%
	F2	--	--	--	--	--	5360±40 (3), 0.7%	--
	F3	--	--	--	--	--	5760±40 (4), 0.7%	--
	F4	--	--	--	--	--	6150±70 (4), 1.1%	--
	F5	--	--	--	--	--	6410±60 (4), 0.9%	--
IgG1 (+26)	F1	--	--	--	--	--	7980±300 (4), 3.2%	--
	F2	--	--	--	--	--	9000±90 (2), 0.9%	--
	F3	--	--	--	--	--	10000±200 (4), 2.4%	--
	F4	--	--	--	--	--	10410±80 (4), 0.8%	--
	F5	--	--	--	--	--	10700±200 (3), 1.8%	--

a. Number of measurements are indicated in the parenthesis. Percent RSDs, when reported, are converted to standard deviations in $Å^2$.
b. The CCS values reported for ammonium acetate solution are used for ubiquitin, and BSA, and the value measured in TEAA solution is used for streptavidin. For ubiquitin, only one CCS value is reported for the +6 charge state and it is assumed this is the fully-extended conformer. The streptavidin +11 and BSA +16 values are assumed to be the lowest-energy states. CCS measurements were obtained with nano-ESI on a traveling wave ion mobility-mass spectrometer.
c. The CCS values for ubiquitin are assumed to correspond to F1, F2, and F3 in this study. CCS measurements were obtained with nano-ESI.
d. The interlaboratory relative standard deviations are also provided at the end of each entry. In some cases, not all features were observed across all laboratories/hardware configurations.
e. The percent bias is referenced against the CCS values obtained in this study.

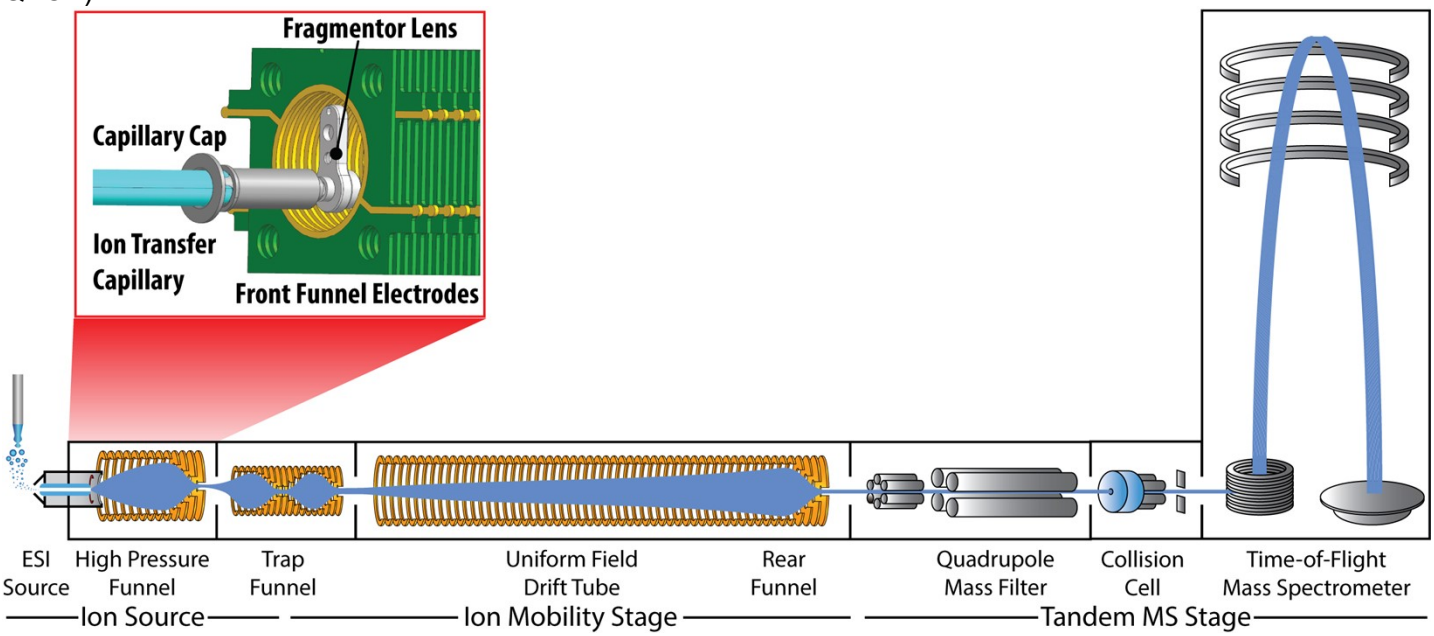
Table S6 – Production-grade Source RMSD, Feature and CIU50 Reproducibility of BSA

Production Grade RMSD Analysis	
Source Assembly	**RMSD (%)**
Production Grade 1 (PG1)	2.4 ± 0.1
Production Grade 2 (PG2)	2.3 ± 0.1
Production Grade 3 (PG3)	2.6 ± 0.1
Inter-Hardware Reproducibility	5 ± 1
Average Feature $^{DT}CCS_{N2}$ Measurements Across Production Grade Assemblies 3 Replicates: 3 Averaged CIU Fingerprints from PG1, PG2, PG3; R.S.D. in parentheses	
Feature #	**Average $^{DT}CCS_{N2}$ (nm^2)**
Feature 1	45.09 ± 0.02 (0.04%)
Feature 2	53.8 ± 0.1 (0.2%)
Feature 3	57.7 ± 0.1 (0.2%)
Feature 4	61.6 ± 0.2 (0.3%)
Feature 5	64.6 ± 0.2 (0.3%)
Average CIU50 Measurements Across Production Grade Assemblies 3 Replicates: 3 Averaged CIU Fingerprints from PG1, PG2, PG3; R.S.D. in parentheses	
Transition #	**Average CIU50 (V)**
CIU50-1	174 ± 5 (3%)
CIU50-2	200.0 ± 0.4 (0.2%)
CIU50-3	251 ± 7 (3%)
CIU50-4	348 ± 3 (1%)

Table S7 – Production Production-grade Source RMSD, Feature and CIU50 Reproducibility of SigmaMAb

Production Grade RMSD Analysis					
Source Assembly	**RMSD (%)**				
	+26	**+27**	**+28**	**+29**	**+30**
Production Grade 1 (PG1)	4.70 ± 0.30	2.22 ± 0.02	1.92 ± 0.09	2.20 ± 0.10	3.10 ± 0.30
Production Grade 2 (PG2)	4.60 ± 0.10	2.80 ± 0.10	1.80 ± 0.20	2.00 ± 0.20	3.40 ± 0.40
Production Grade 3 (PG3)	5.00 ± 0.40	2.70 ± 0.10	1.87 ± 0.09	2.20 ± 0.10	3.20 ± 0.10
Inter-Hardware Reproducibility	4.40 ± 0.50	3.30 ± 0.70	2.60 ± 0.90	2.70 ± 0.40	3.50 ± 0.60
Average Feature $^{DT}CCS_{N2}$ Measurements Across Production Grade Assemblies 3 Replicates: 3 Averaged CIU Fingerprints from PG1, PG2, PG3; R.S.D. in parentheses					
Feature #	**Average $^{DT}CCS_{N2}$ (nm^2)**				
	+26	**+27**	**+28**	**+29**	**+30**
Feature 1	81.90 ± 0.40 (0.5%)	83.60 ± 0.40 (0.5%)	85.17 ± 0.04 (0.05%)	87.23 ± 0.02 (0.05%)	90.29 ± 0.04 (0.04%)
Feature 2	97.60 ± 0.40 (0.4%)	110.20 ± 0.40 (0.4%)	111.91 ± 0.01 (0.01%)	116.83 ± 0.01 (0.01%)	119.60 ± 0.50 (0.4%)
Feature 3	107.00 ± 0.40 (0.4%)		115.47 ± 0.01 (0.01%)		126.90 ± 0.50 (0.4%)
Average CIU50 Measurements Across Production Grade Assemblies 3 Replicates: 3 Averaged CIU Fingerprints from PG1, PG2, PG3; R.S.D. in parentheses					
Transition #	**Average CIU50 (V)**				
	+26	**+27**	**+28**	**+29**	**+30**
CIU50-1	201.6 ± 0.6 (0.3%)	211 ± 4 (2%)	203 ± 3 (1%)	200 ± 3 (2%)	191 ± 3 (2%)
CIU50-2	260 ± 8 (3%)		335 ± 3 (1%)		373 ± 4 (1%)

Figure S1 – Modified Agilent 6560 Drift Tube Ion Mobility Quadrupole Time-of-Flight Mass Spectrometer (DTIM-QTOF)



Full instrument diagram of the Modified Agilent 6560 DTIM-MS, now commercially referred to as the Agilent 6560C (diagram adapted from[4]). The capillary/high pressure funnel (4.8 torr) is modified to enable high-energy in-source activation of biomolecules for collision induced unfolding (CIU), as described in detail in the main text. After ion activation, the ions are accumulated in the trap funnel (3.80 torr) for a fixed amount of time (Trap Fill Time), and then released into the drift tube for mobility separation. The drift tube is operated at ambient temperature, 3.95 torr, and with an electric field of ~18 V/cm. After mobility separation, ions traverse the tandem MS stage of the instrument where they can be isolated by the quadrupole mass filter, further activated in the collision cell, and/or detected by the time-of-flight mass spectrometer. The firmware of this instrument is upgraded to extend the mass range of the time-of-flight mass spectrometer to 20,000 m/z.

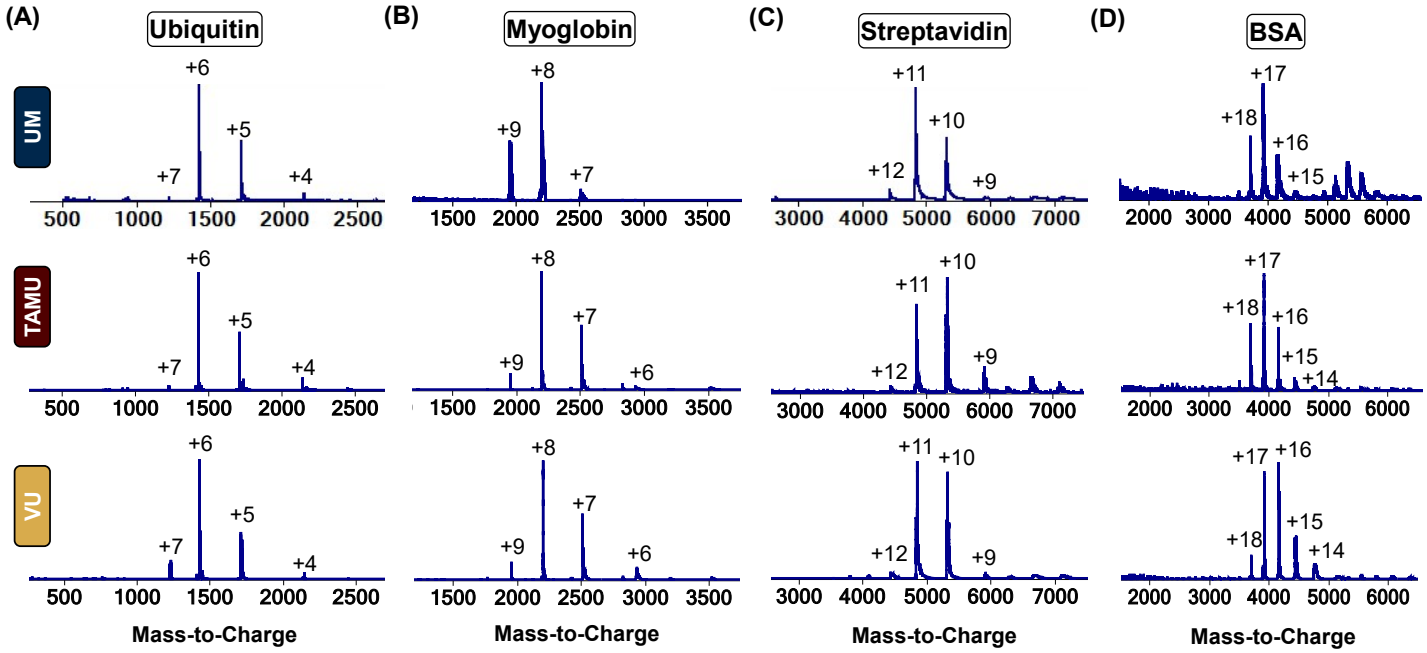


Figure S2 – Native Mass Spectra of **(A)** Ubiquitin, **(B)** Myoglobin, **(C)** Streptavidin, and **(D)** Bovine Serum Albumin

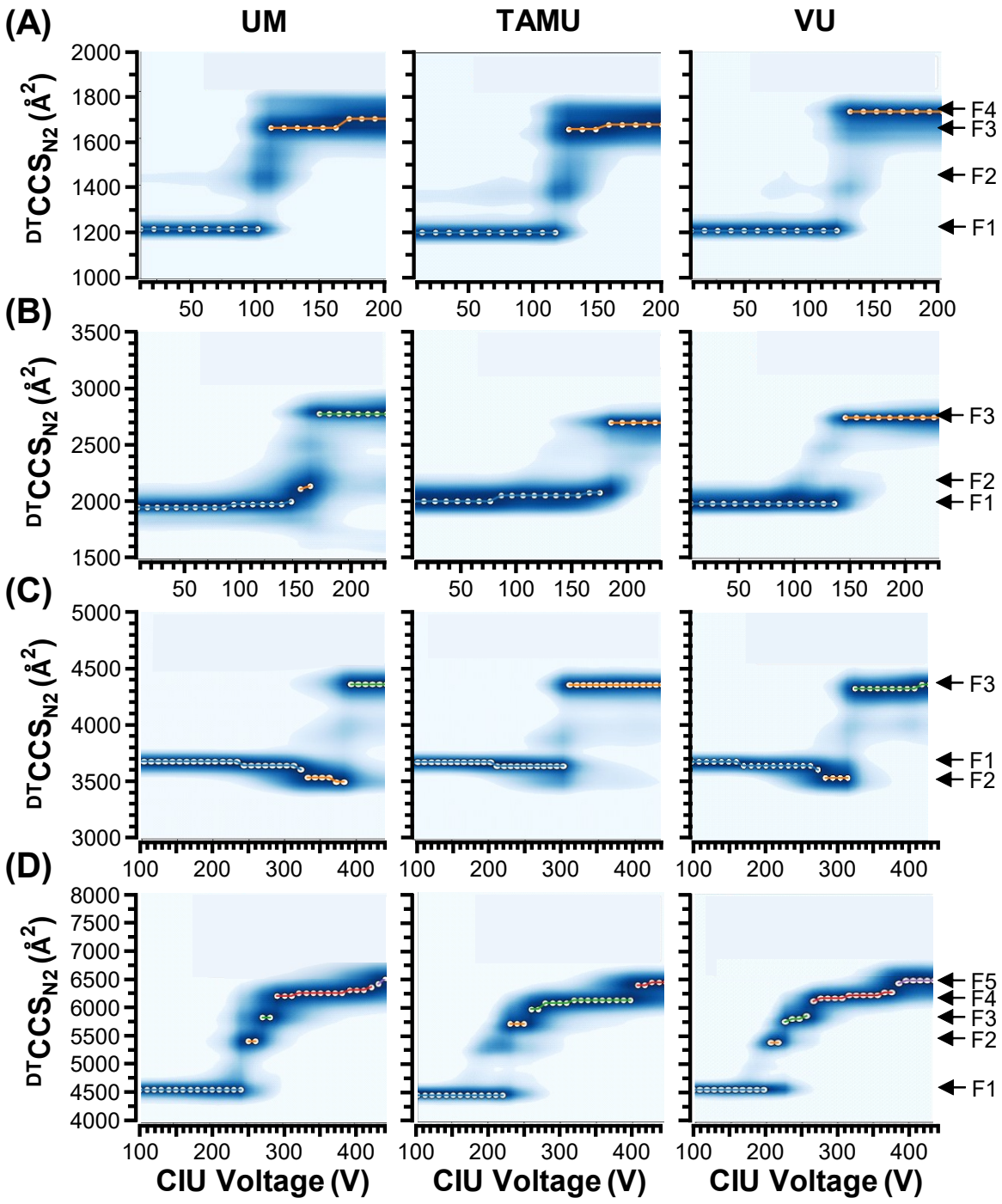


Figure S3 – CIU feature maps. Here, the average map generated from three replicate runs is used for feature annotation, with "significant features" (F1, F2, etc.) determined using specific constraints for the CIU step size and allowable CCS tolerance. These are (A) ubiquitin (+6), 5 steps minimum, 1 nm^2 width tolerance; (B) myoglobin (+8), 2 steps, 1 nm^2; (C) streptavidin (+11), 4 steps 1 nm^2; (D) BSA (+16), 2 steps, 1.5 nm^2.

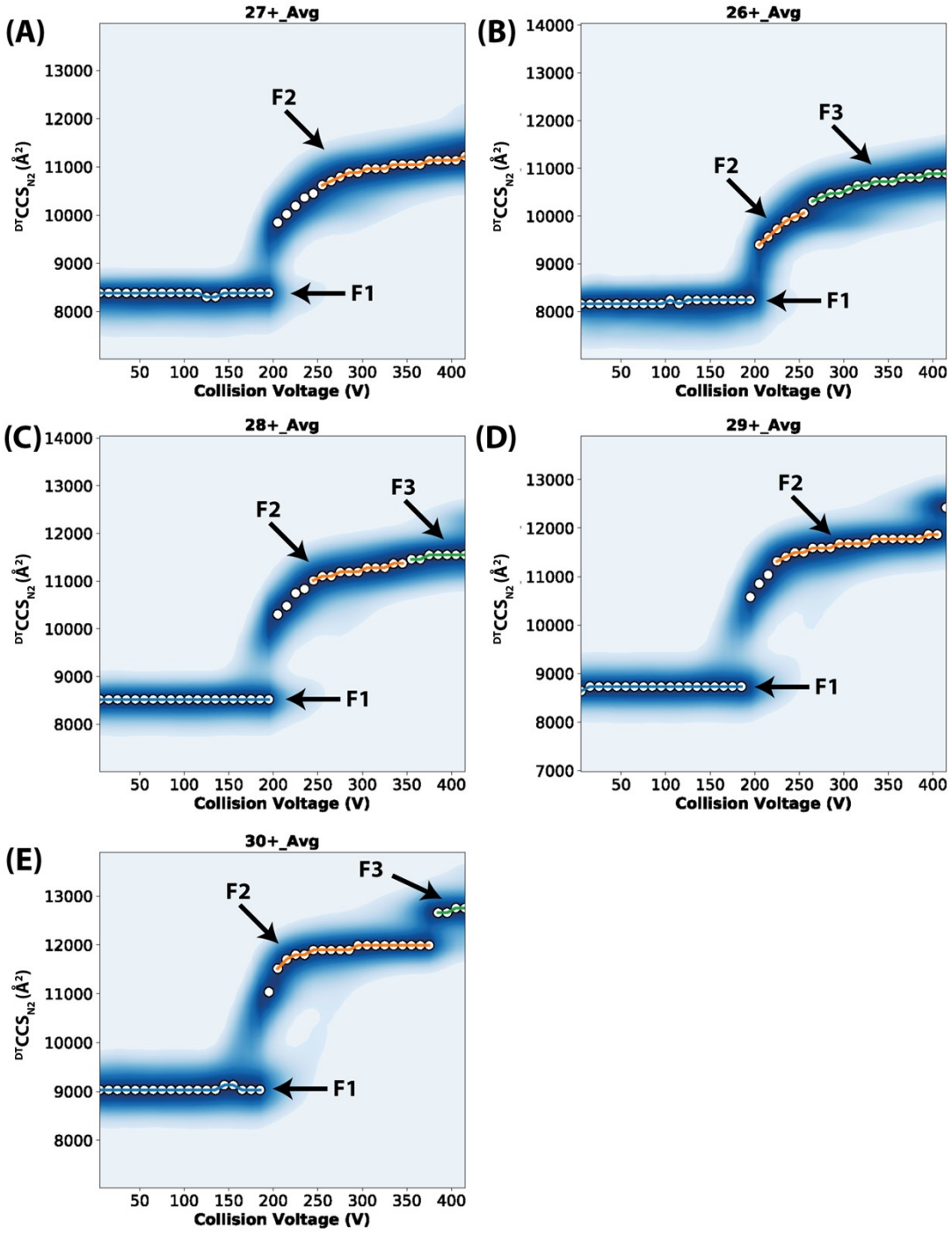


Figure S4 – CIU feature Plots of SigmaMAb. The average CIU fingerprint generated from three replicate runs is used for feature annotation, with "significant features" (F1, F2, etc.) determined using specific constraints for the CIU step size and allowable CCS tolerance. These are **(A)** 26+, 6 steps minimum, 3.5 nm^2 width tolerance; **(B)** 27+, 6 steps minimum, 3.5 nm^2 width tolerance; **(C)** 28+, 4 steps minimum, 2 nm^2 width tolerance; **(D)** 29+, 4 steps minimum, 2 nm^2 width tolerance; **(E)** 30+, 4 steps minimum, 2 nm^2 width tolerance;

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