SUPPLEMENTARY MATERIAL

Amicon-adapted enhanced FASP: an in-solution digestion-based alternative sample preparation method to FASP

David Pellerin,[1] Hugo Gagnon,[2] Jean Dubé,[1] and François Corbin[1*]

[1]Department of Biochemistry, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, QC, J1H 5N4, Canada

[2]PhenoSwitch Bioscience Inc., 3001, 12e Avenue Nord, Sherbrooke, QC, J1H 5N4, Canada

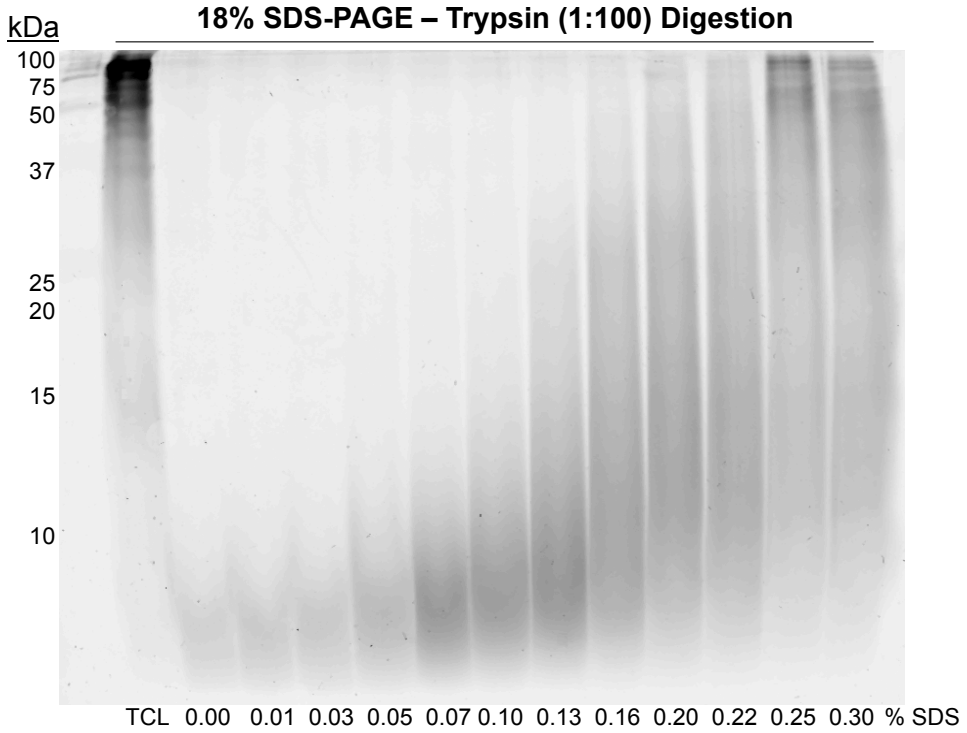
* Corresponding author: Tel: (1) 819-820-6868 ext. 15801. Fax: (1) 819-564-5430. E-mail: francois.corbin@usherbrooke.ca

Supplementary Figures

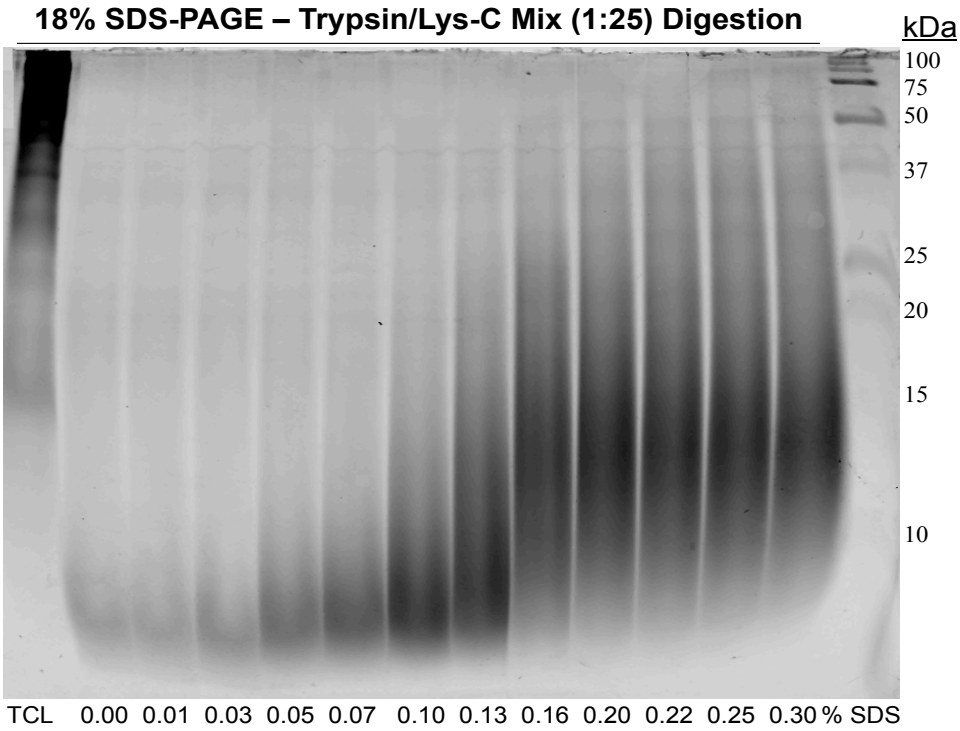
Supplementary Figure 1. Effect of increasing concentrations of SDS on FASP (trypsin) and Amicon-adapted eFASP (trypsin/Lys-C) overnight digestion efficacy

MEG-01 lysate aliquots were overnight digested in one step with (A) trypsin (enzyme-to-protein ratio 1:100 w/w) or (B) trypsin and Lys-C (ratio 1:25 w/w) in either ABC buffer alone or ABC buffer containing increasing concentrations of SDS ranging from 0.01% to 0.30 % w/v. Digested peptides were next loaded onto a 18% SDS-PAGE gel and Coomassie-stained bands visualized by an Odyssey® Imaging System (LI-COR Biosciences, Lincoln, NE). Total cell lysates (TCL) and Odyssey® protein molecular weight marker were also loaded on the gels.

A



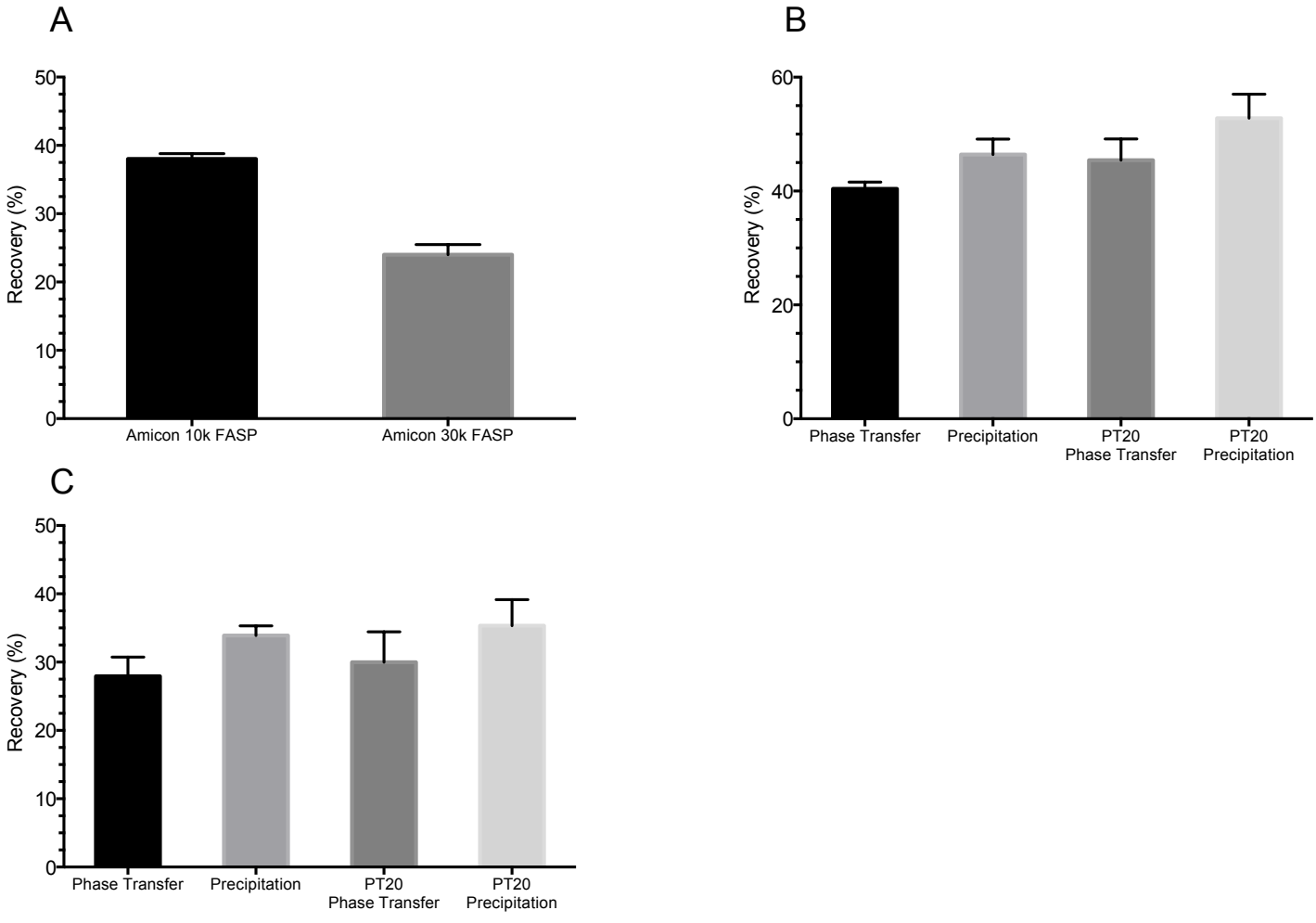
B


18% SDS-PAGE – Trypsin/Lys-C Mix (1:25) Digestion
kDa
100
75
50
37
25
20
15
10
TCL 0.00 0.01 0.03 0.05 0.07 0.10 0.13 0.16 0.20 0.22 0.25 0.30 % SDS

Supplementary Figure 2. Peptide yields

Displayed are the mean recoveries of (A) FASP, (B) Amicon-adapted eFASP Amicon 10k and (C) Amicon-adapted eFASP Amicon 30k protocols. Error bars are representative of the standard deviation of the mean associated with each replicate analysis. For FASP and Amicon-adapted eFASP without passivation experiments, data were summarized from triplicate experiments. For Amicon-adapted eFASP with passivation experiments, data were summarized from duplicate experiments.

PT20: 5% Tween-20 (v/v) overnight passivation of filter device.

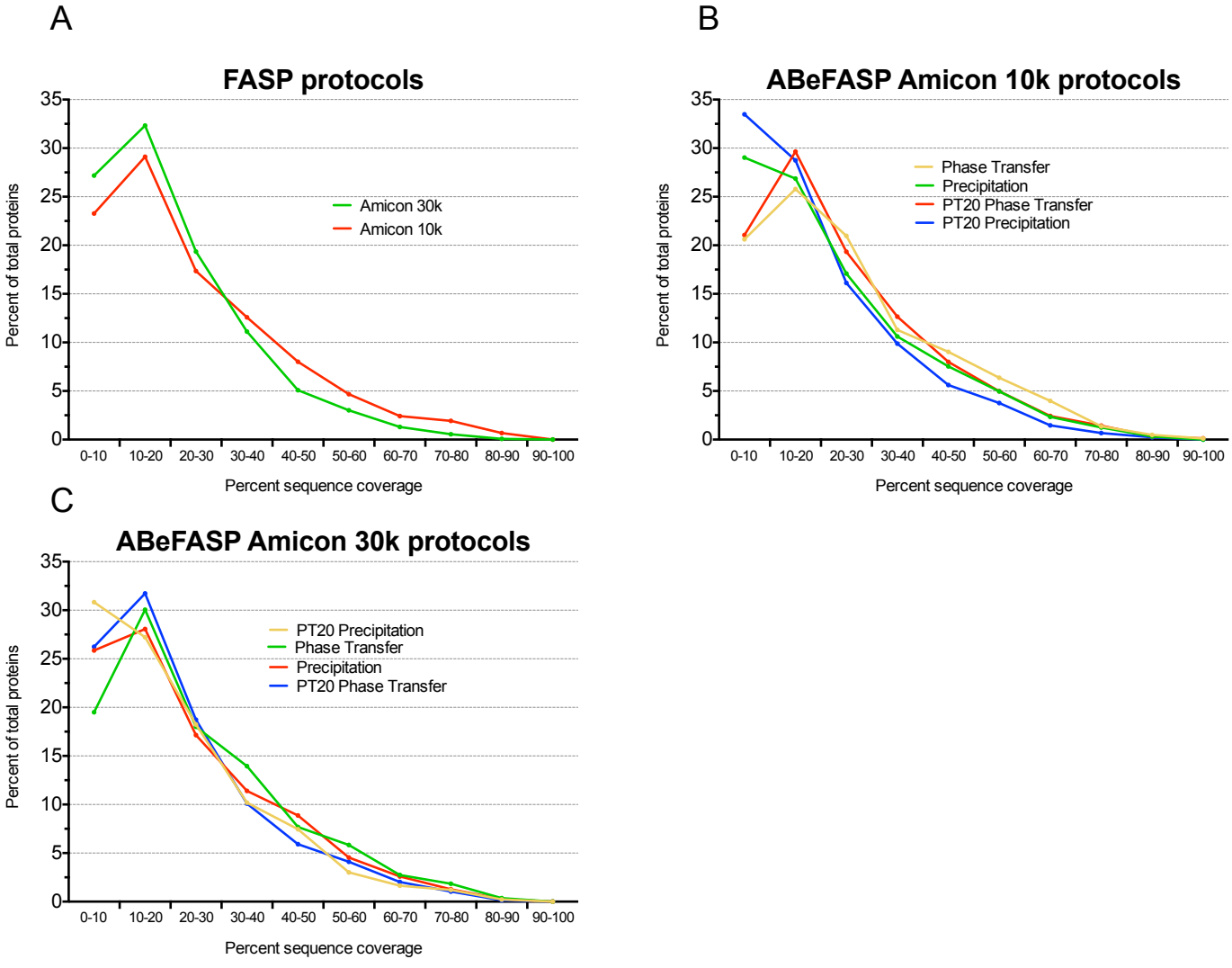


Supplementary Figure 3. Sequence coverage by intervals

The relative frequencies (%) of proteins identified by combined analysis of replicates in (A) FASP, (B) Amicon-adapted eFASP Amicon 10k and (C) Amicon-adapted eFASP Amicon 30k protocols were plotted against 10% wide sequence coverage intervals.

Replicates per protocol: n=3 for FASP and Amicon-adapted eFASP without passivation; n=2 for Amicon-adapted eFASP with passivation.

PT20: 5% Tween-20 (v/v) overnight passivation of filter device.

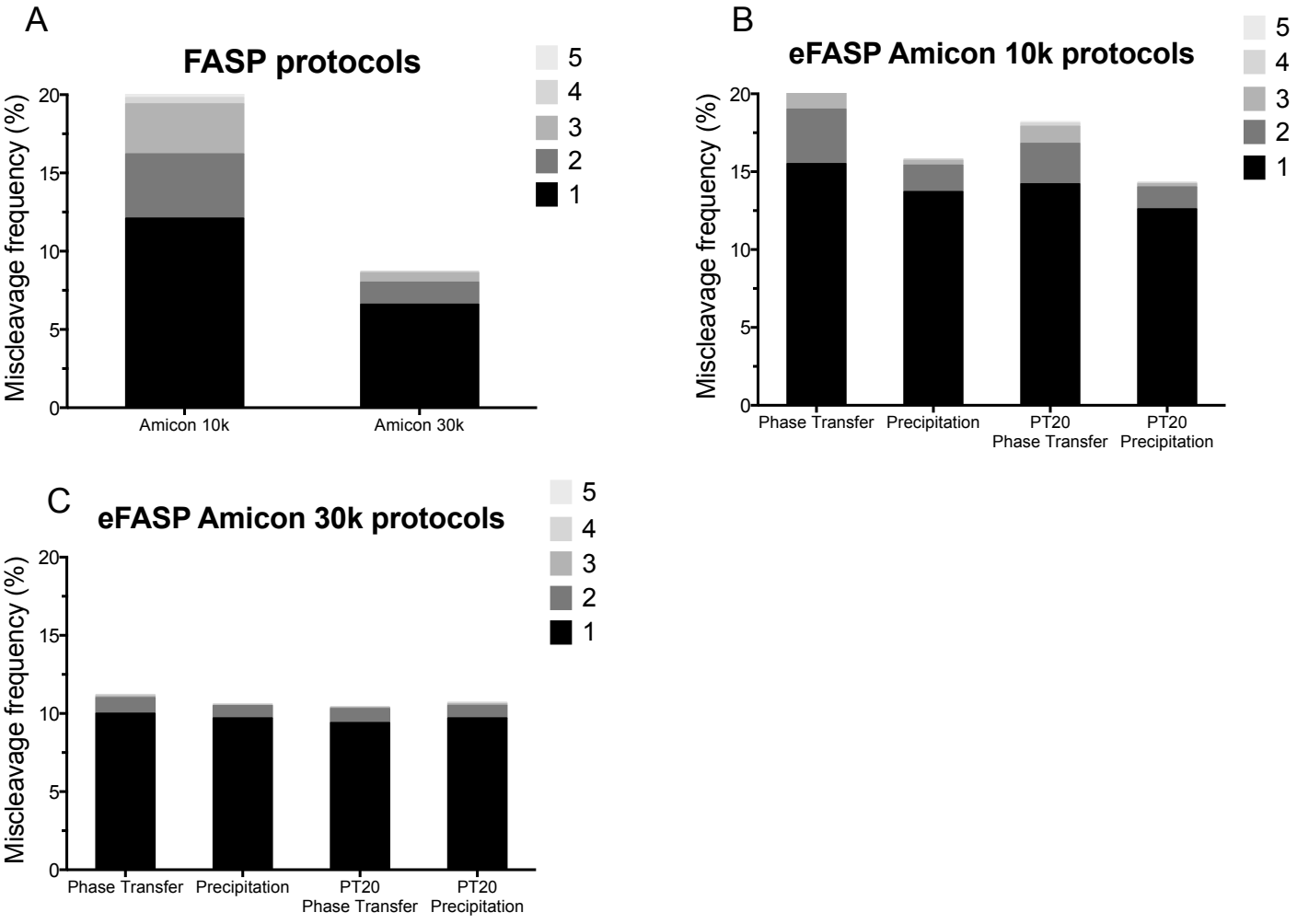


Supplementary Figure 4. Miscleavages frequencies

Displayed are the miscleavages (≥1 missed cleavage) frequencies of proteins identified by combined analysis of replicates in (A) FASP, (B) Amicon-based eFASP Amicon 10k and (C) Amicon-based eFASP Amicon 30k protocols. Non-missed cleaved proteins have been omitted for more clarity.

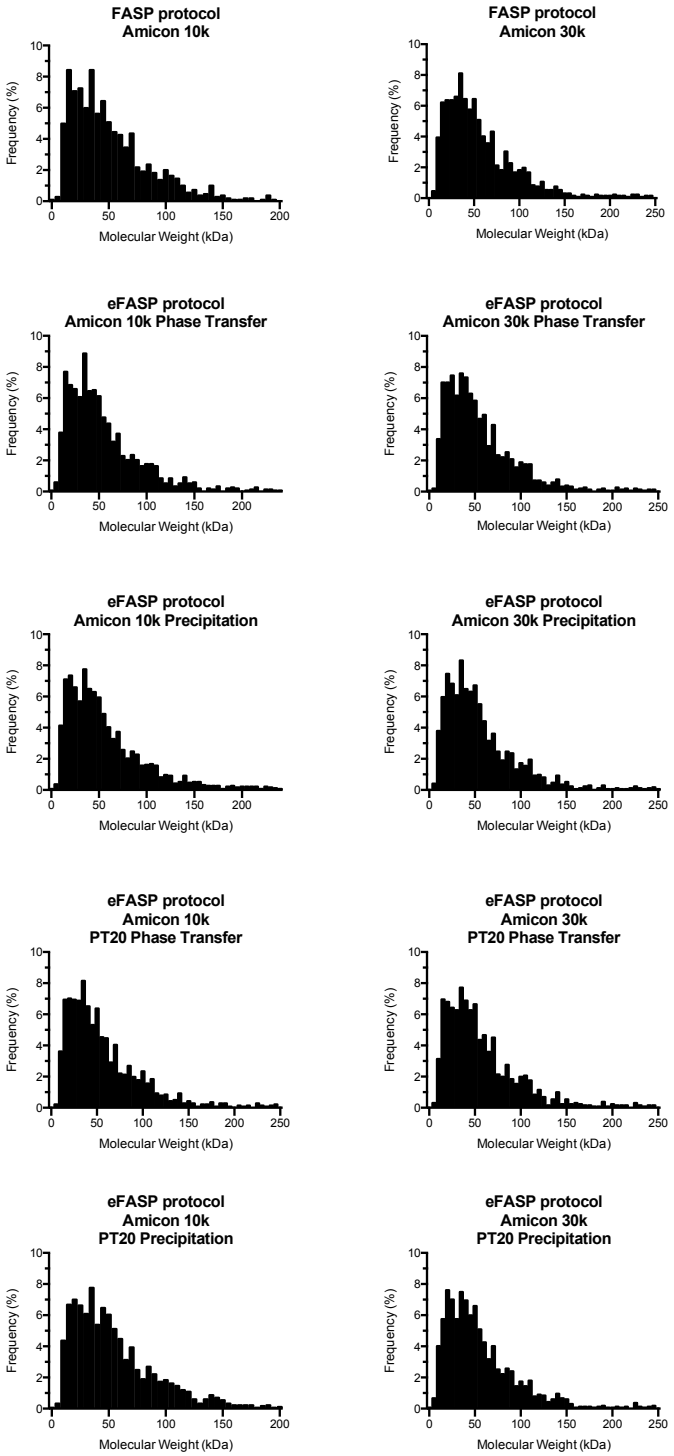
Replicates per protocol: n=3 for FASP and Amicon-adapted eFASP without passivation; n=2 for Amicon-adapted eFASP with passivation.

PT20: 5% Tween-20 (v/v) overnight passivation of filter device.



Supplementary Figure 5. Frequency of identified proteins' theoretical molecular weight (kDa) for each protocol investigated.

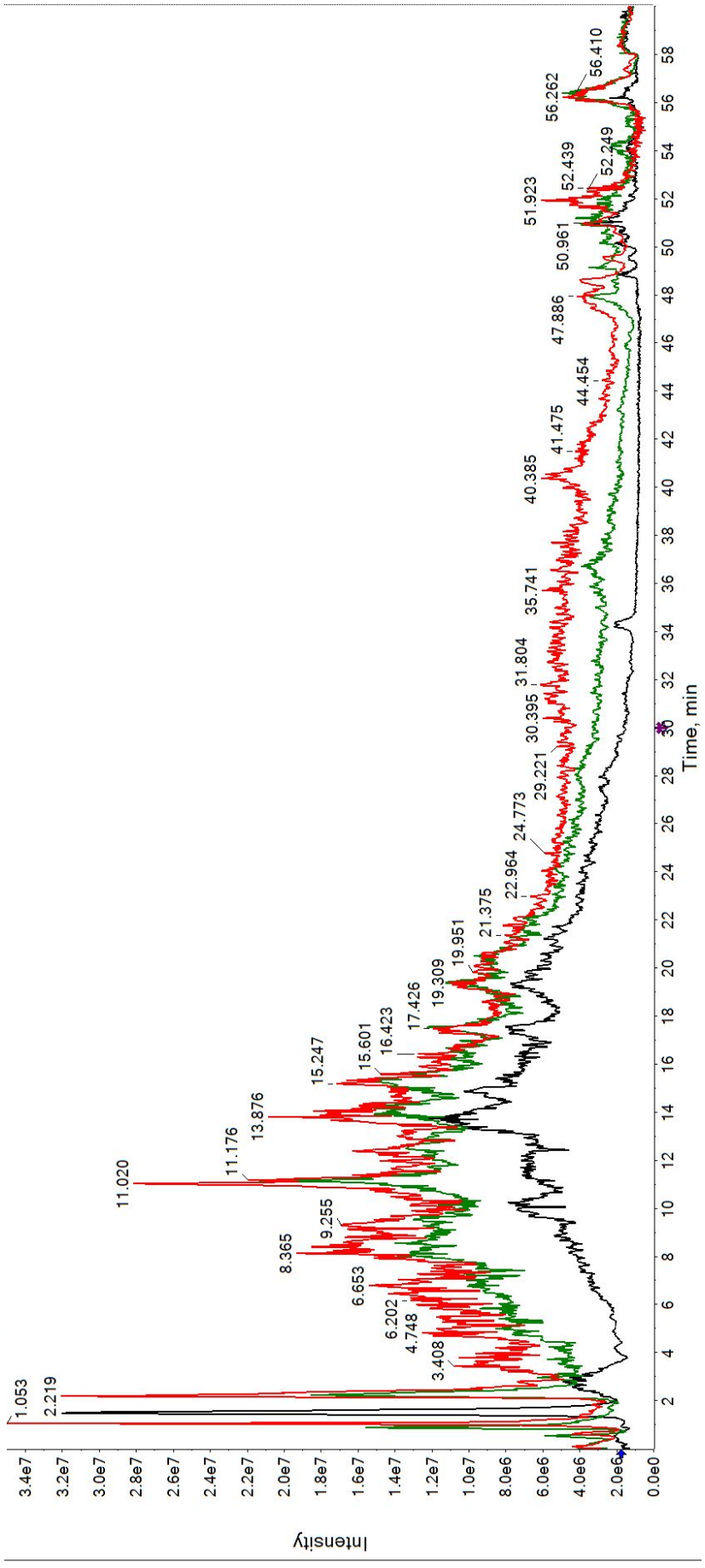
Proteins were identified by combined analysis of replicates.



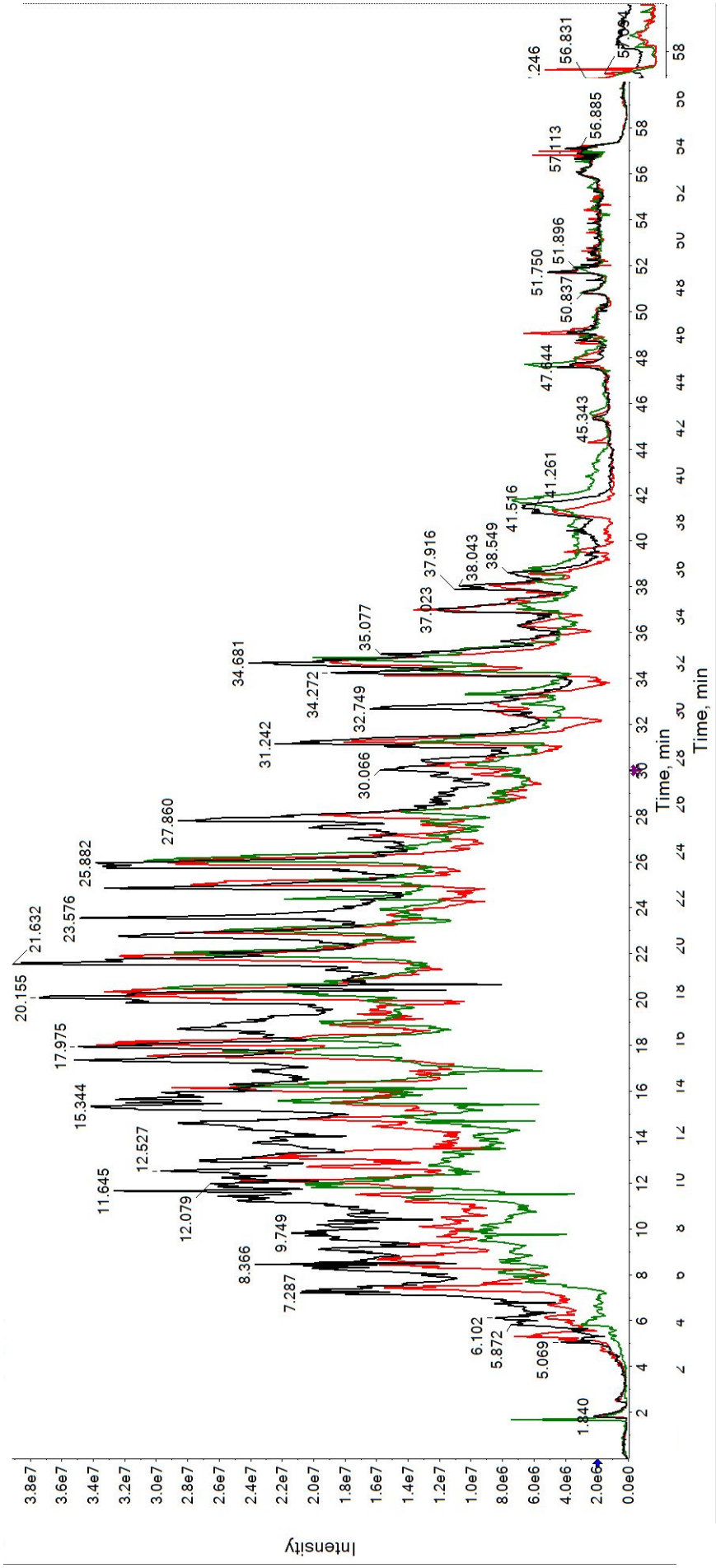
Supplementary Figure 6. Total ion current chromatograms

Displayed are the combined total ion current chromatograms of the replicates from FASP performed with: (A) Amicon 30k (FASP/A30k); and Amicon-adapted eFASP performed with: Amicon 10k and (B) phase transfer (eFASP/A10k/Phase), (C) passivation and phase transfer (eFASP/A10k/PT20/Phase), and (D) passivation and precipitation (eFASP/A10k/PT20/PPT); Amicon 30k and (E) phase transfer (eFASP/A30k/Phase), (F) precipitation (eFASP/A30k/PPT), (G) passivation and phase transfer (eFASP/a30k/PT20/Phase), and (H) passivation and precipitation (eFASP/A30k/PT20/PPT).

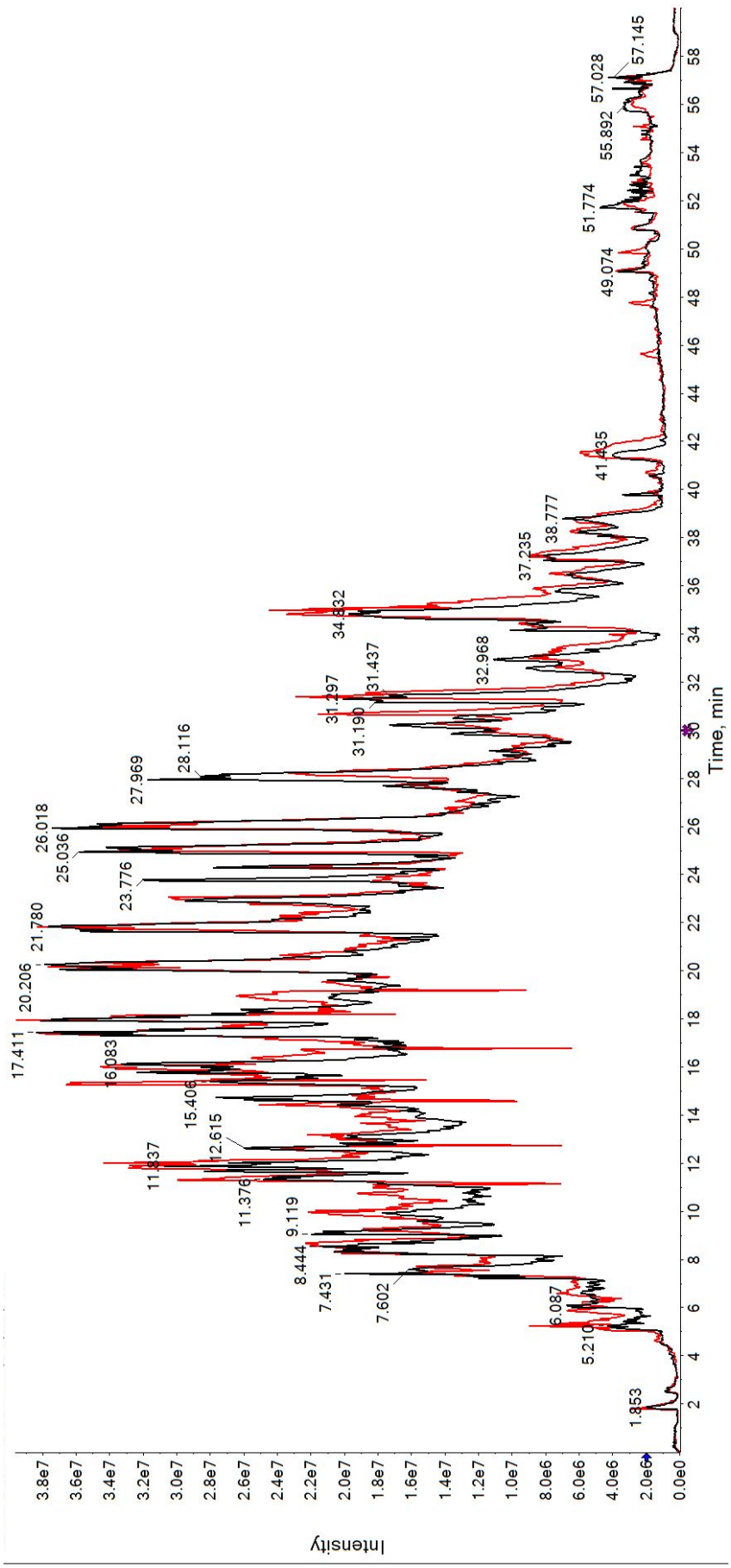
A


Intensity
Time, min
1.053
2.219
3.408
4.748
6.202
6.653
8.365
9.255
11.020
11.176
13.876
15.247
15.601
16.423
17.426
19.309
19.951
21.375
22.964
24.773
29.221
30.395
31.804
35.741
40.385
41.475
44.454
47.886
50.961
51.923
52.439
52.249
56.262
56.410

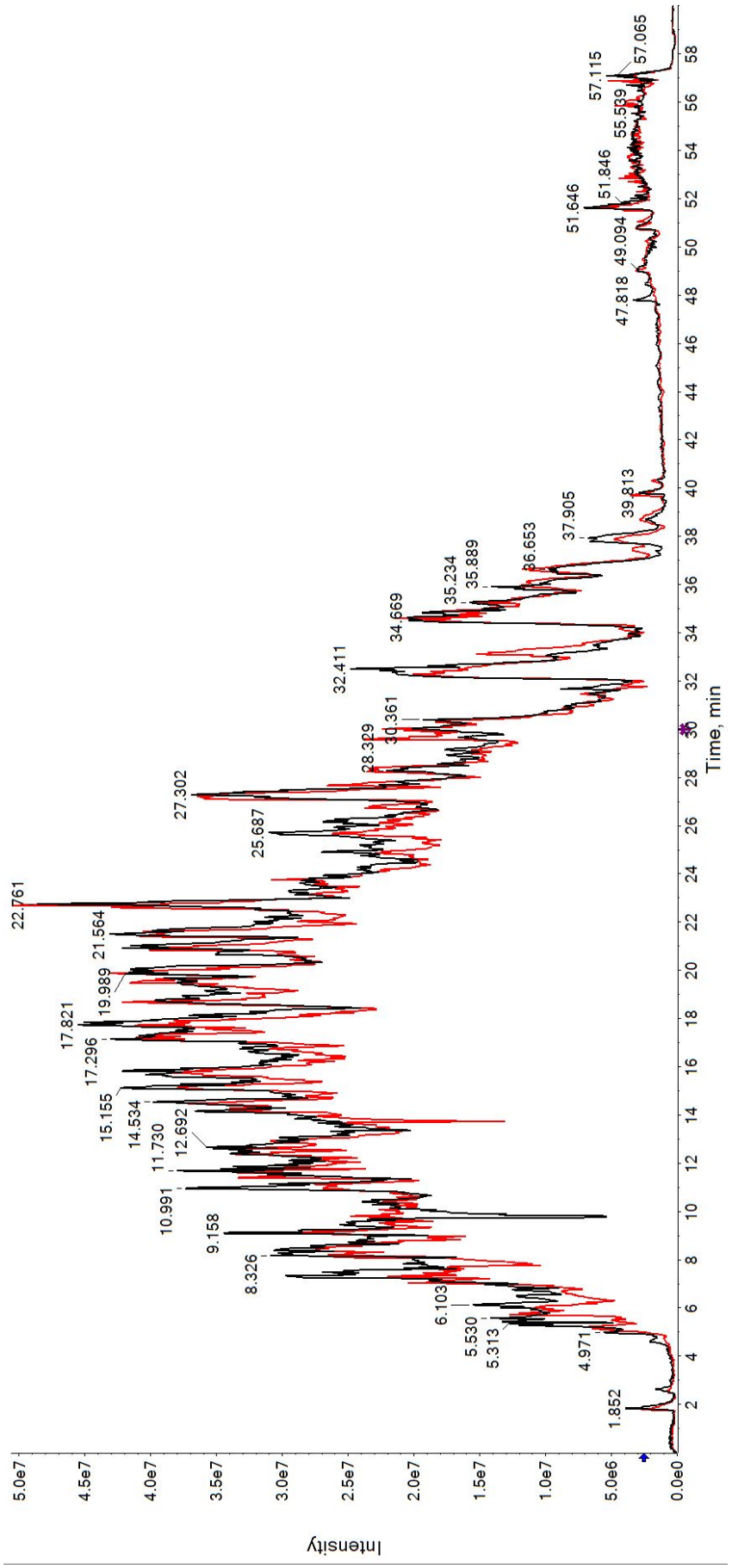
B


Intensity
Time, min
1.840
5.069
5.872
6.102
7.287
8.366
9.749
11.645
12.079
12.527
15.344
17.975
20.155
21.632
23.576
25.882
27.860
30.066
31.242
32.749
34.272
34.681
35.077
37.023
37.916
38.043
38.549
41.516
41.261
45.343
47.644
50.837
51.750
51.896
57.113
56.885
56.831

C


Intensity
Time, min
1.853
5.210
6.087
7.602
7.431
8.444
9.119
11.376
11.837
12.615
15.406
16.083
17.411
20.206
21.780
23.776
25.036
26.018
27.969
28.116
31.190
31.297
31.437
32.968
34.832
37.235
38.777
41.435
49.074
51.774
55.892
57.028
57.145

D


Intensity
5.0e7
4.5e7
4.0e7
3.5e7
3.0e7
2.5e7
2.0e7
1.5e7
1.0e7
5.0e6
0.0e0
Time, min
1.852
4.971
5.313
5.530
6.103
8.326
9.158
10.991
11.730
12.692
14.534
15.155
17.296
17.821
19.989
21.564
22.761
25.687
27.302
28.329
30.361
32.411
34.669
35.234
35.889
36.653
37.905
39.813
47.818
49.094
51.646
51.846
55.539
57.115
57.065

E

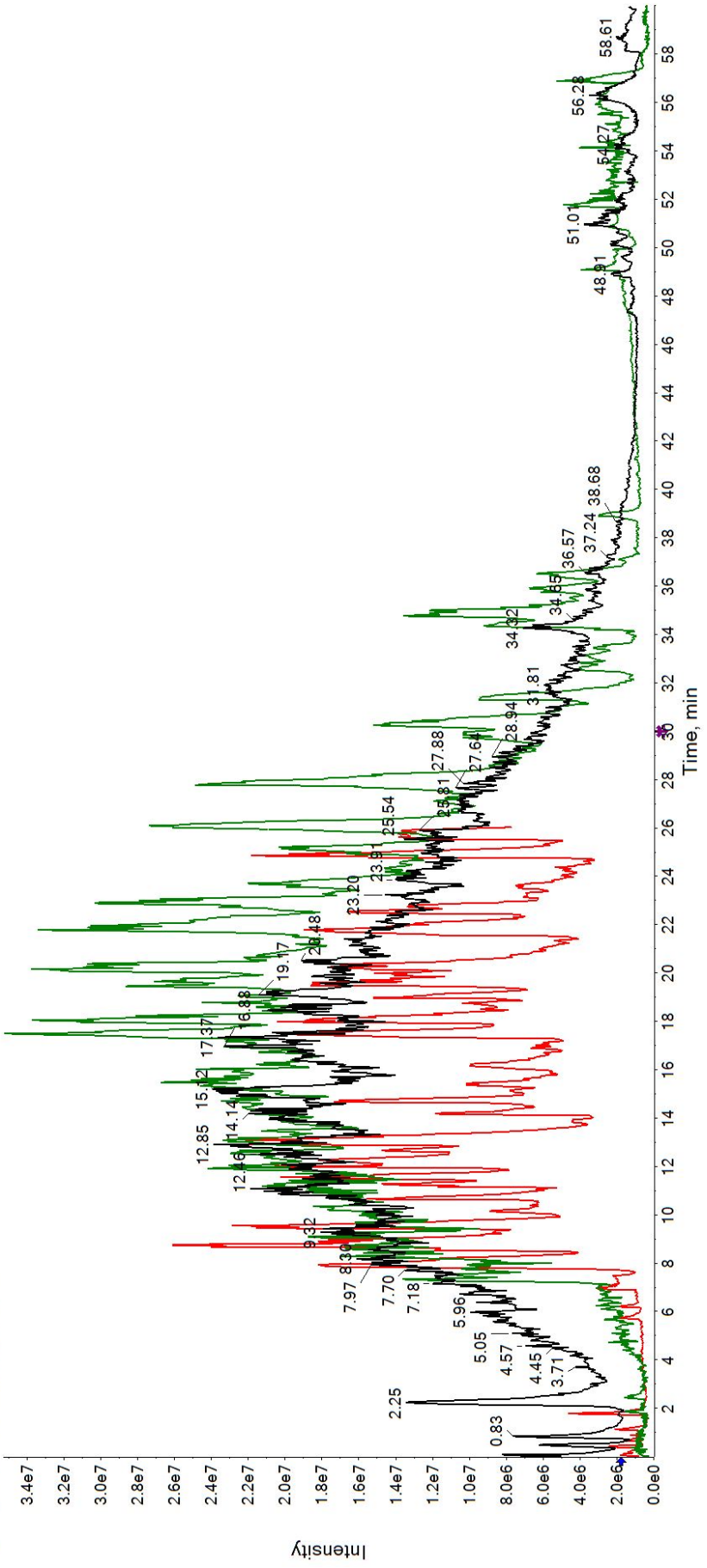
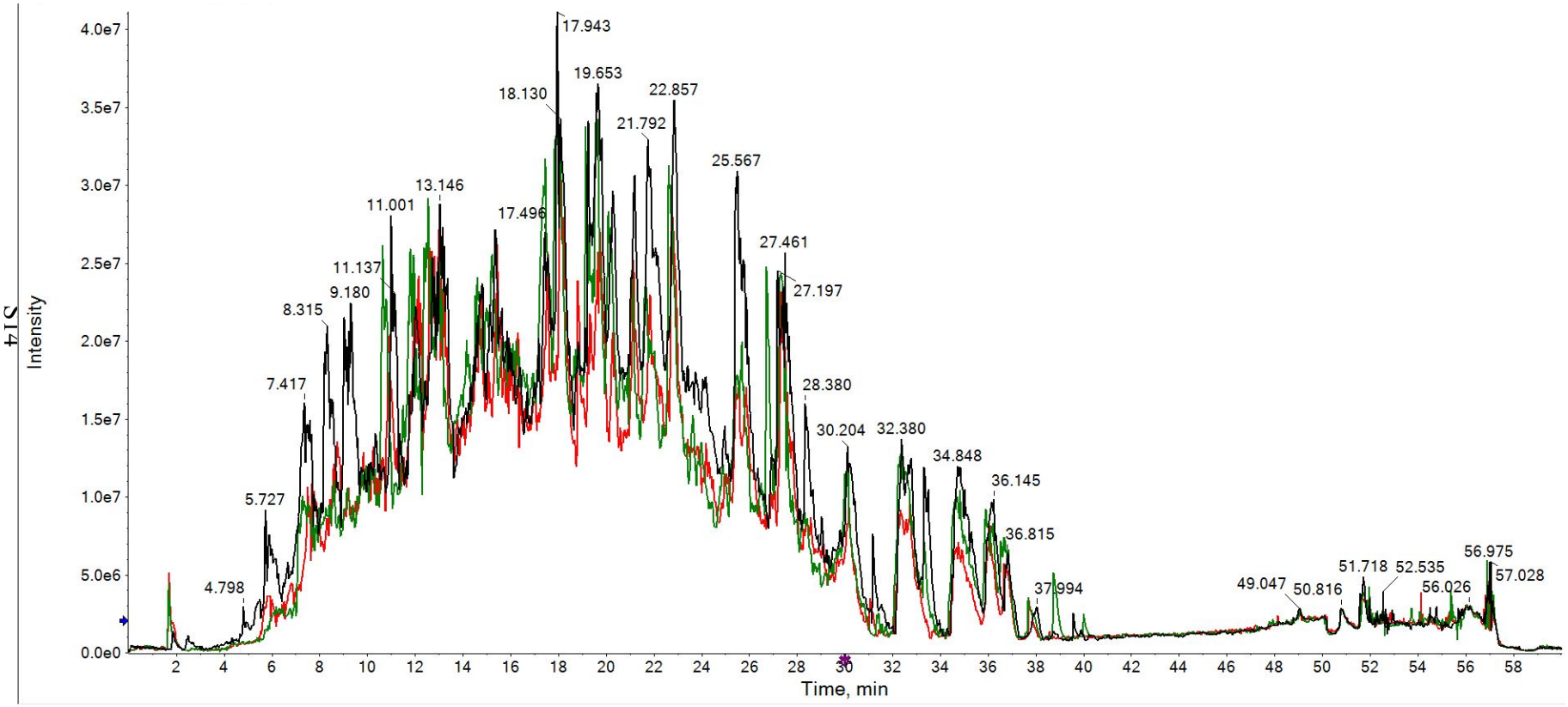
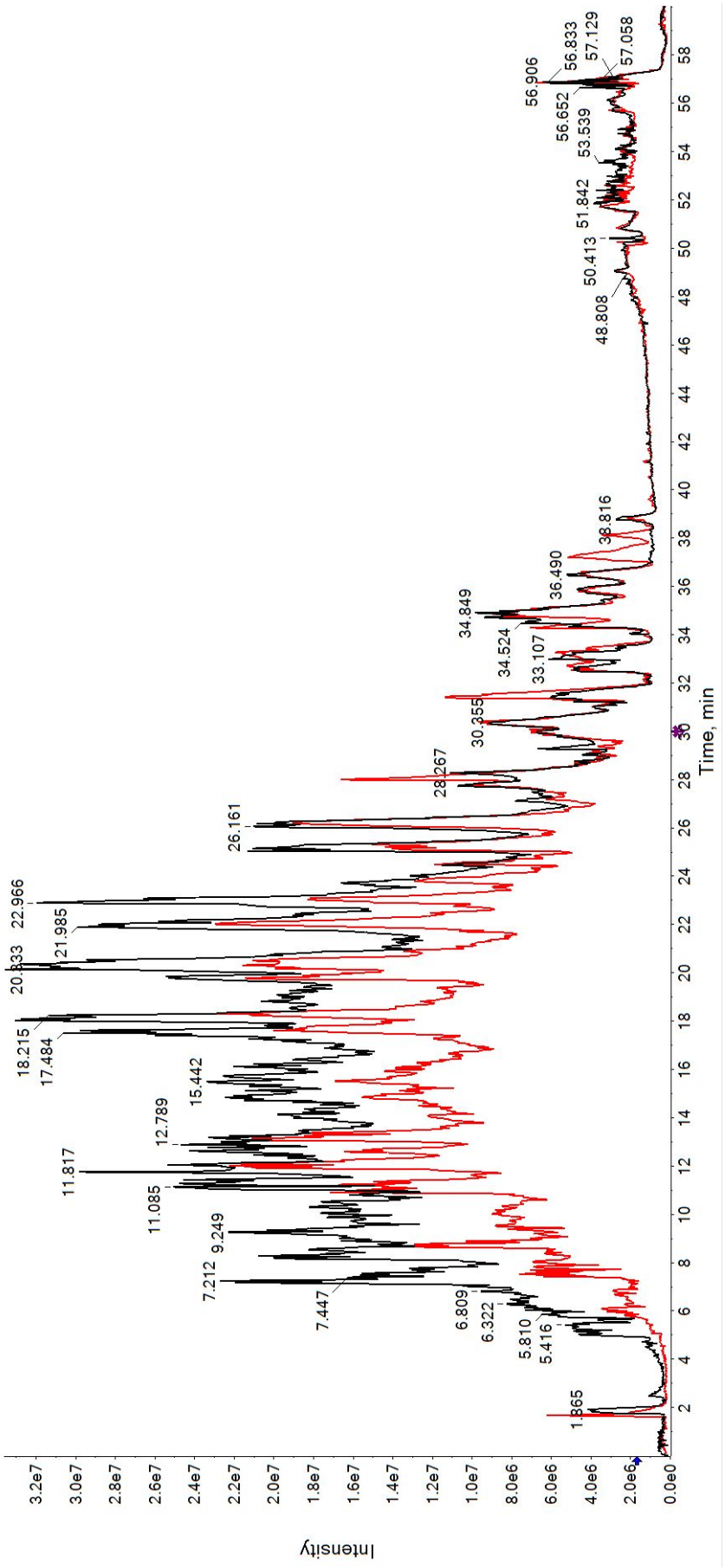

Intensity
Time, min
0.83
2.25
3.71
4.45
4.57
5.05
5.96
7.18
7.70
7.97
9.32
12.46
12.85
14.14
15.12
17.37
18.88
19.17
20.48
23.20
23.91
25.54
25.81
27.88
27.64
28.94
31.81
34.32
34.85
36.57
37.24
38.68
48.81
51.01
54.27
56.28
58.61

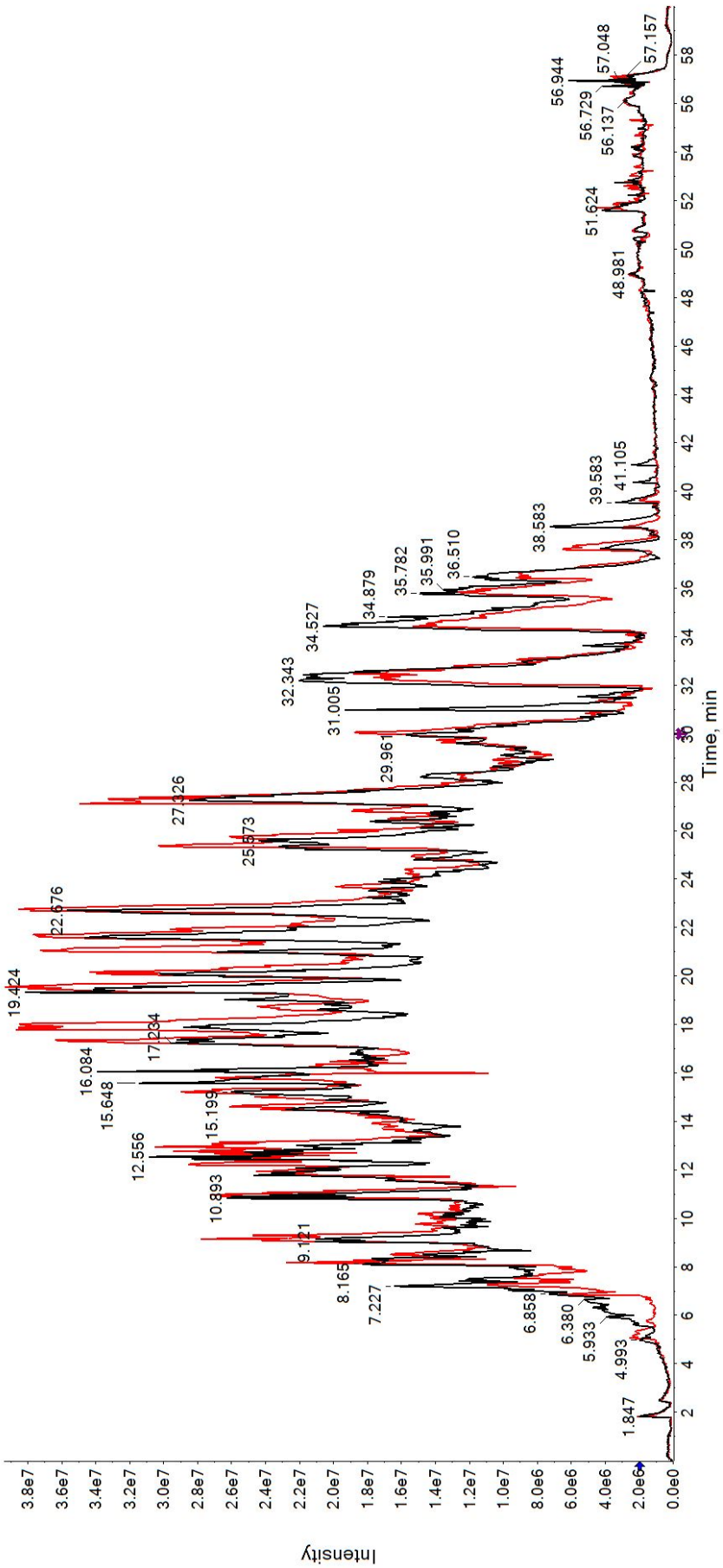
FIG. 14


Intensity
4.0e7
3.5e7
3.0e7
2.5e7
2.0e7
1.5e7
1.0e7
5.0e6
0.0e0
17.943
19.653
18.130
22.857
21.792
25.567
13.146
11.001
17.496
27.461
11.137
9.180
27.197
8.315
7.417
28.380
30.204
32.380
34.848
36.145
5.727
36.815
4.798
37.994
49.047
50.816
51.718
52.535
56.026
56.975
57.028
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
Time, min

G


Intensity
Time, min
1.865
5.416
5.810
6.322
6.809
7.212
7.447
9.249
11.085
11.817
12.789
15.442
17.484
18.215
20.833
21.985
22.966
26.161
28.267
30.355
33.107
34.524
34.849
36.490
38.816
48.808
50.413
51.842
53.539
56.652
56.906
56.833
57.129
57.058

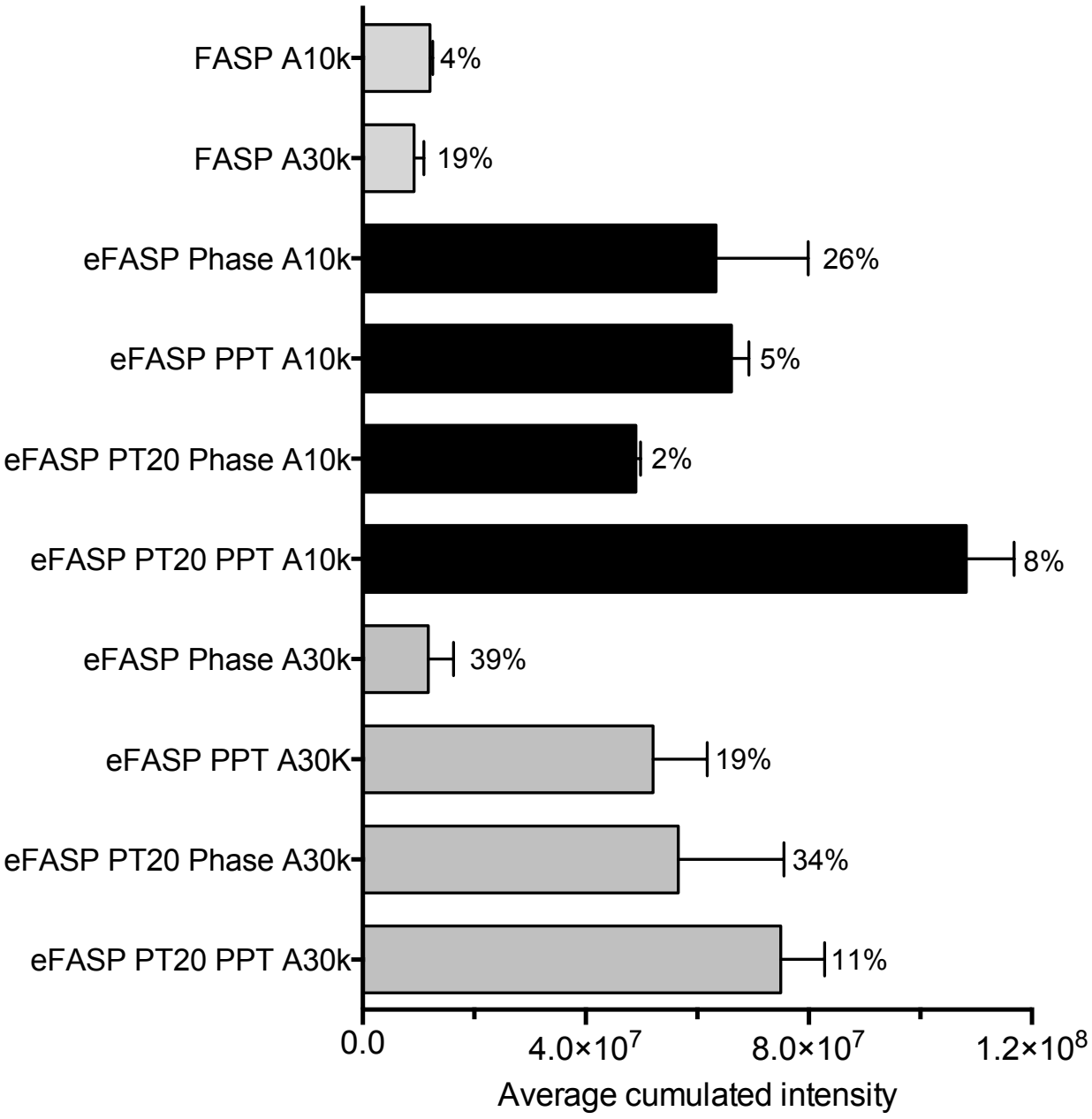
H


Intensity
Time, min
1.847
4.993
5.933
6.380
6.858
7.227
8.165
9.121
10.893
12.556
15.199
15.648
16.084
17.234
19.424
22.676
25.673
27.326
29.961
31.005
32.343
34.527
34.879
35.782
35.991
36.510
38.583
39.583
41.105
48.981
51.624
56.137
56.729
56.944
57.048
57.157

Supplementary Figure 7. Quantitative assessment of reproducibility: average cumulated signals intensity

Bars show the average cumulated MS signals intensities from each replicate for the five most intense peptides from the five most abundant identified proteins per protocol. Error bars are representative of the standard deviation of the mean associated with each replicate analysis. Data were summarized from triplicate experiments for FASP and Amicon-adapted eFASP without passivation and duplicate experiments for Amicon-adapted eFASP with passivation. Numbers to the right of error ars indicate the coefficient of variation in the average cumulated MS signal intensities.

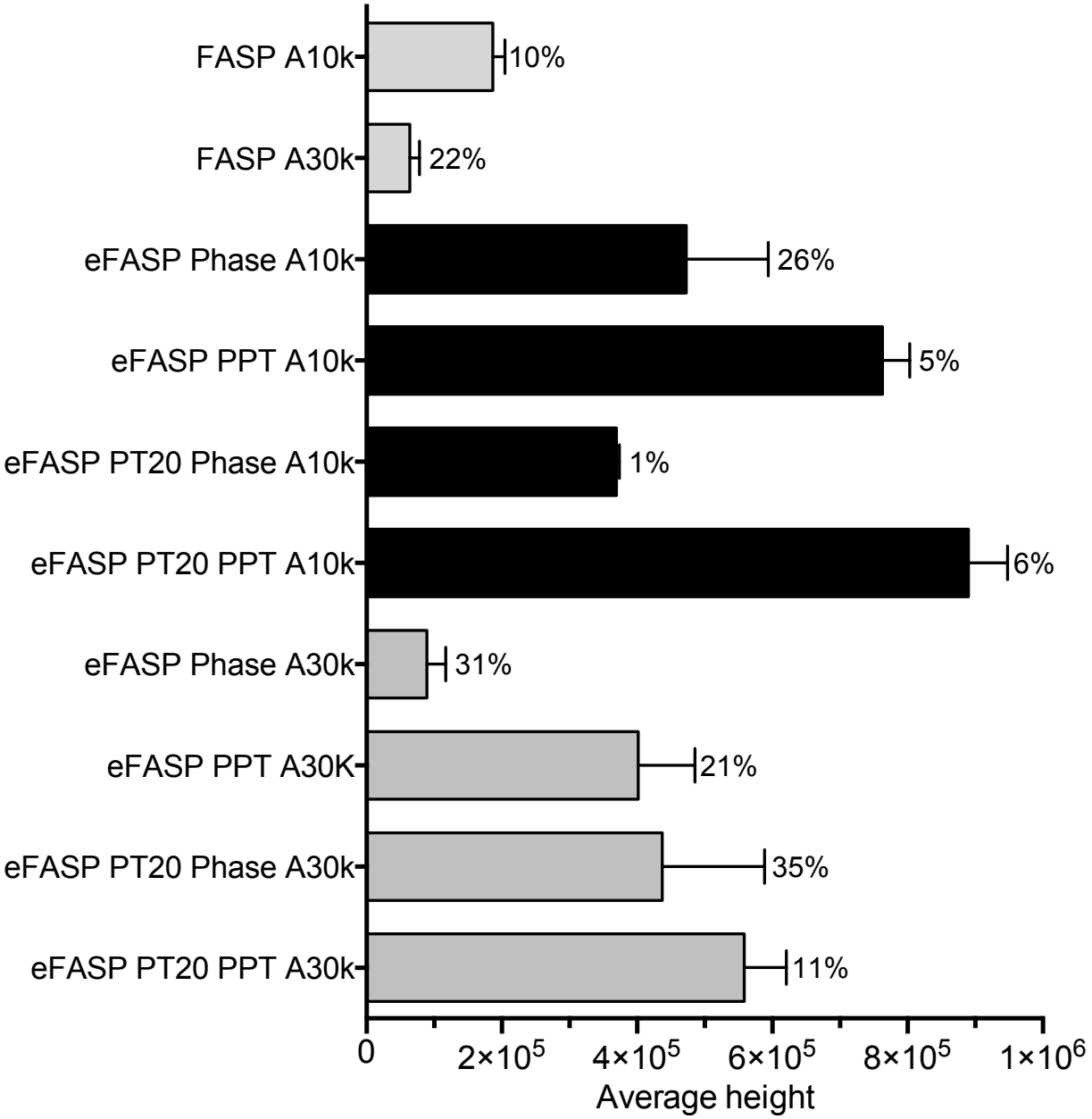
Phase: Phase transfer; PPT: Precipitation; PT20: 5% Tween-20 (v/v) overnight passivation of filter device.


FASP A10k
4%
FASP A30k
19%
eFASP Phase A10k
26%
eFASP PPT A10k
5%
eFASP PT20 Phase A10k
2%
eFASP PT20 PPT A10k
8%
eFASP Phase A30k
39%
eFASP PPT A30K
19%
eFASP PT20 Phase A30k
34%
eFASP PT20 PPT A30k
11%
0.0
4.0×10^7
8.0×10^7
1.2×10^8
Average cumulated intensity

Supplementary Figure 8. Quantitative assessment of reproducibility: average peaks height

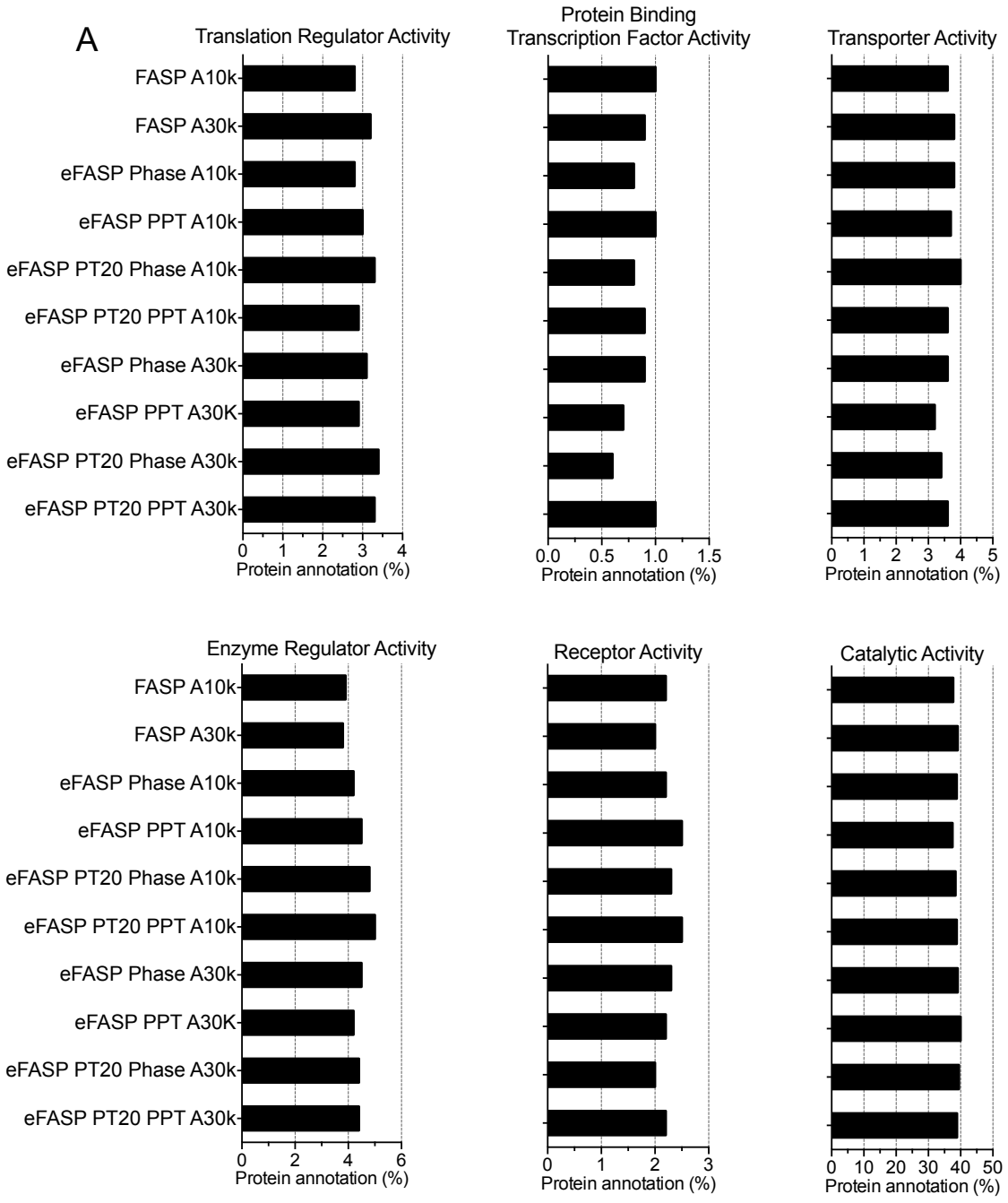
Bars show the average MS peaks intensities from each replicate for the five most intense peptides from the five most abundant identified proteins per protocol. The cumulated peaks height for each protein was calculated by adding up the peak height of each of each of its five most intense peptides and the global peak height was computed by averaging the cumulated peak height of the five proteins. Error bars are representative of the standard deviation of the mean associated with each replicate analysis. Data were summarized from triplicate experiments for FASP and Amicon-adapted eFASP without passivation and duplicate experiments for Amicon-adapted eFASP with passivation. Numbers to the right of error bars indicate the coefficient of variation in the average cumulated MS signal intensities.

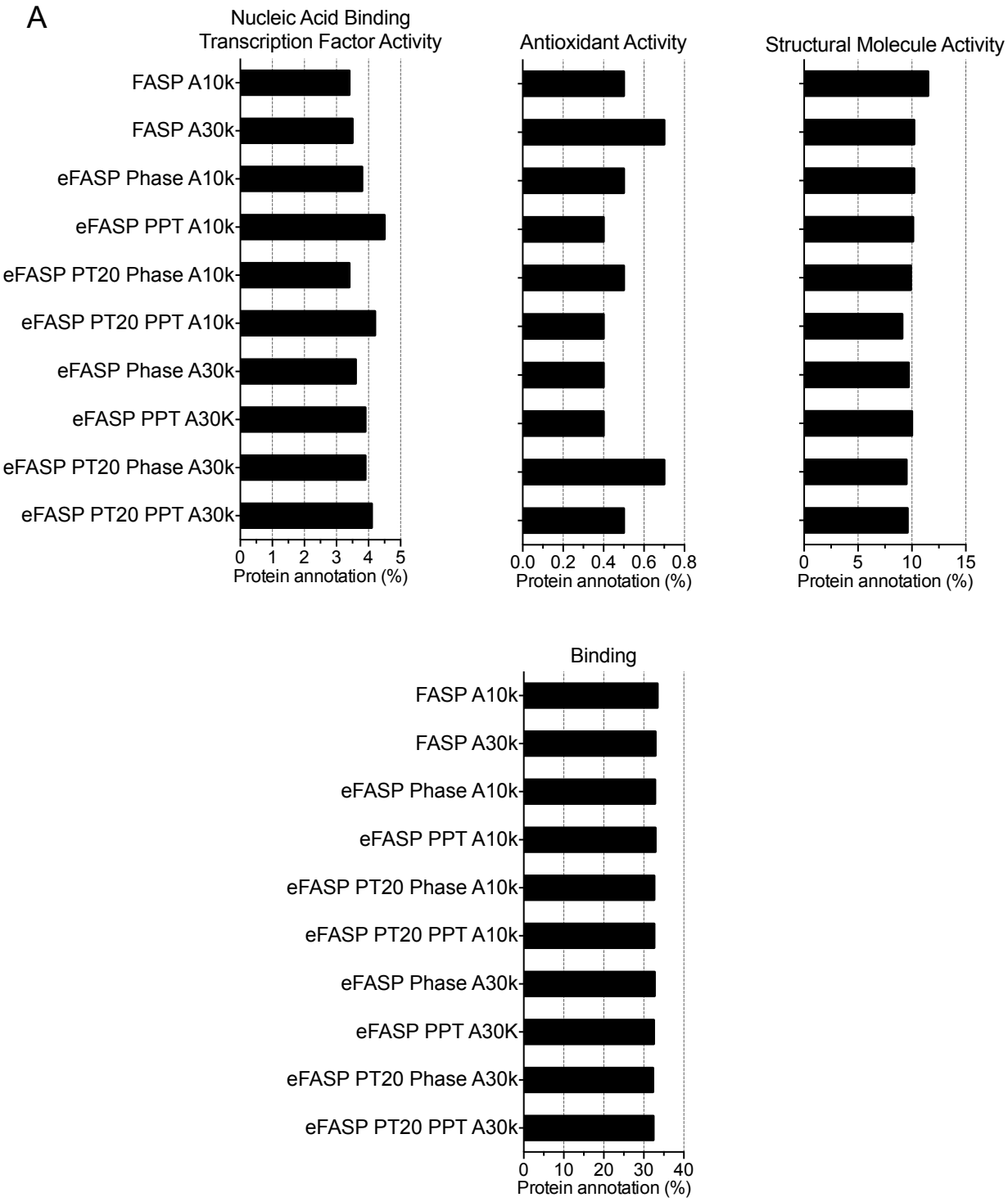
Phase: Phase transfer; PPT: Precipitation; PT20: 5% Tween-20 (v/v) overnight passivation of filter device.


FASP A10k
10%
FASP A30k
22%
eFASP Phase A10k
26%
eFASP PPT A10k
5%
eFASP PT20 Phase A10k
1%
eFASP PT20 PPT A10k
6%
eFASP Phase A30k
31%
eFASP PPT A30K
21%
eFASP PT20 Phase A30k
35%
eFASP PT20 PPT A30k
11%
0
2×10^5
4×10^5
6×10^5
8×10^5
1×10^6
Average height

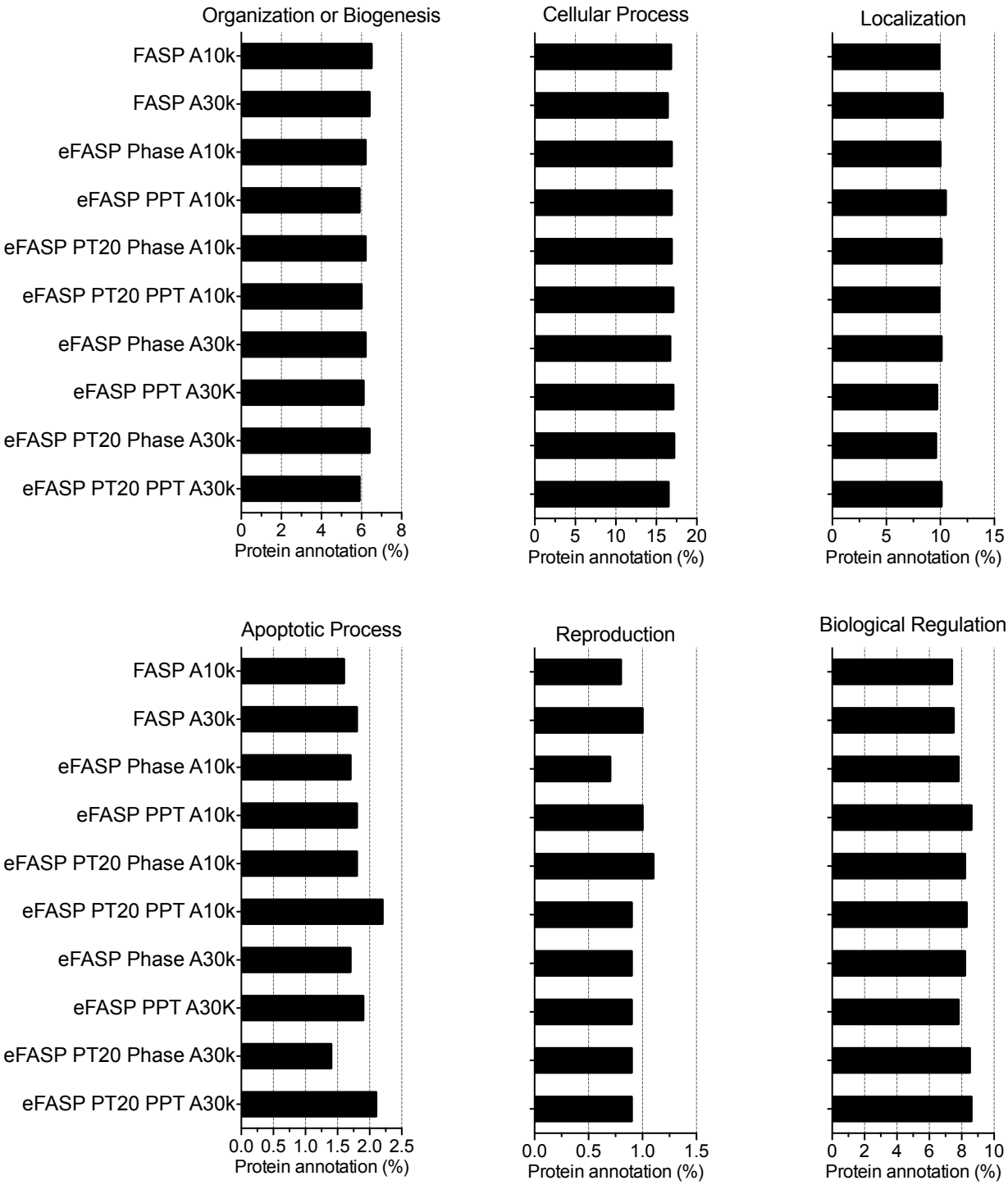
Supplementary Figure 9. Distribution of Gene Ontology (GO) annotations of human leukemic megakaryoblast (MEG-01) proteins identified by Amicon-adapted eFASP and FASP protocols (combined analysis of replicates)

(A) Molecular function, (B) Biological process, (C) Cellular component and (D) Cellular component, Organelle subclasses.

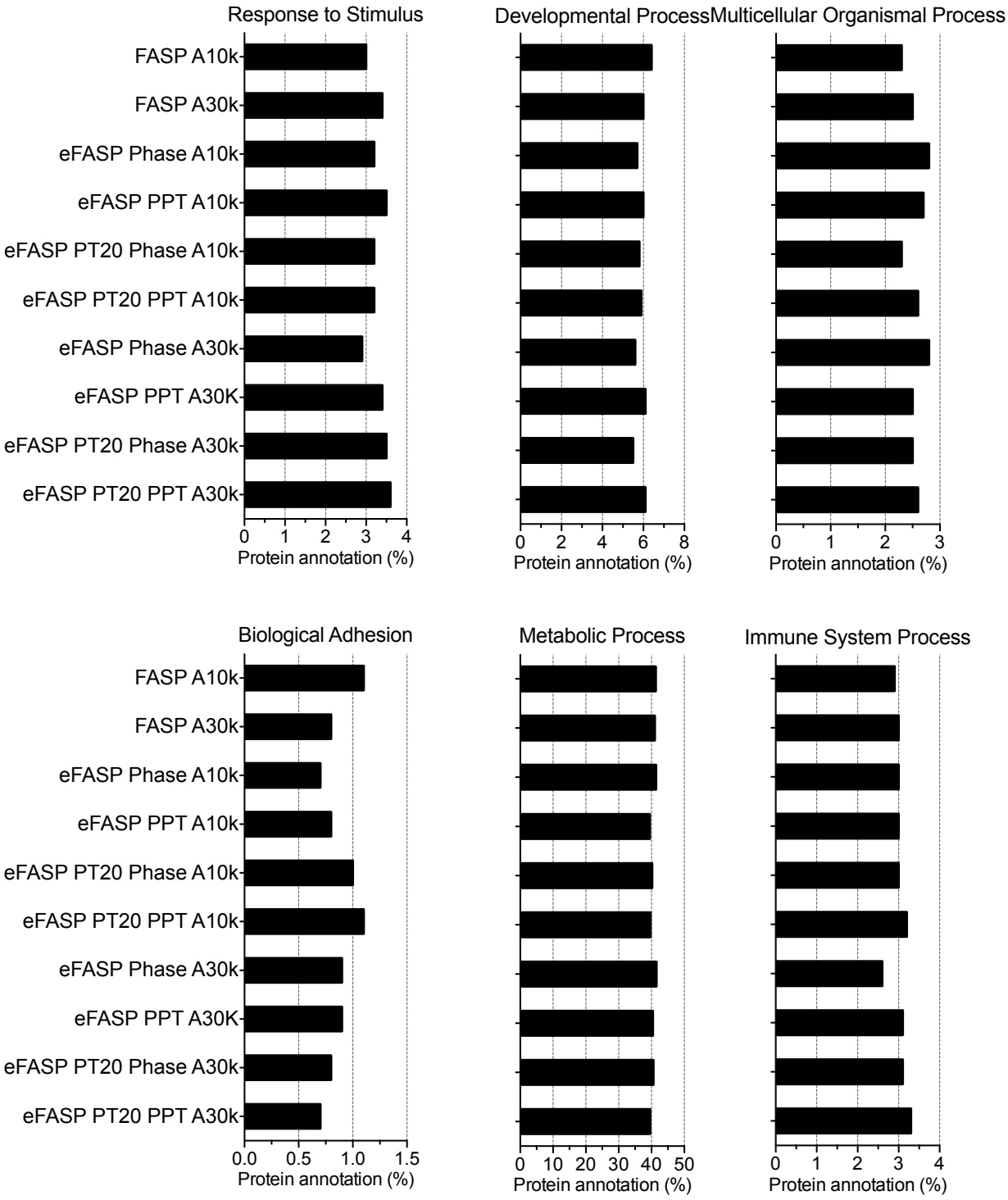



A
Nucleic Acid Binding
Transcription Factor Activity
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0 1 2 3 4 5
Protein annotation (%)
Antioxidant Activity
0.0 0.2 0.4 0.6 0.8
Protein annotation (%)
Structural Molecule Activity
0 5 10 15
Protein annotation (%)
Binding
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0 10 20 30 40
Protein annotation (%)

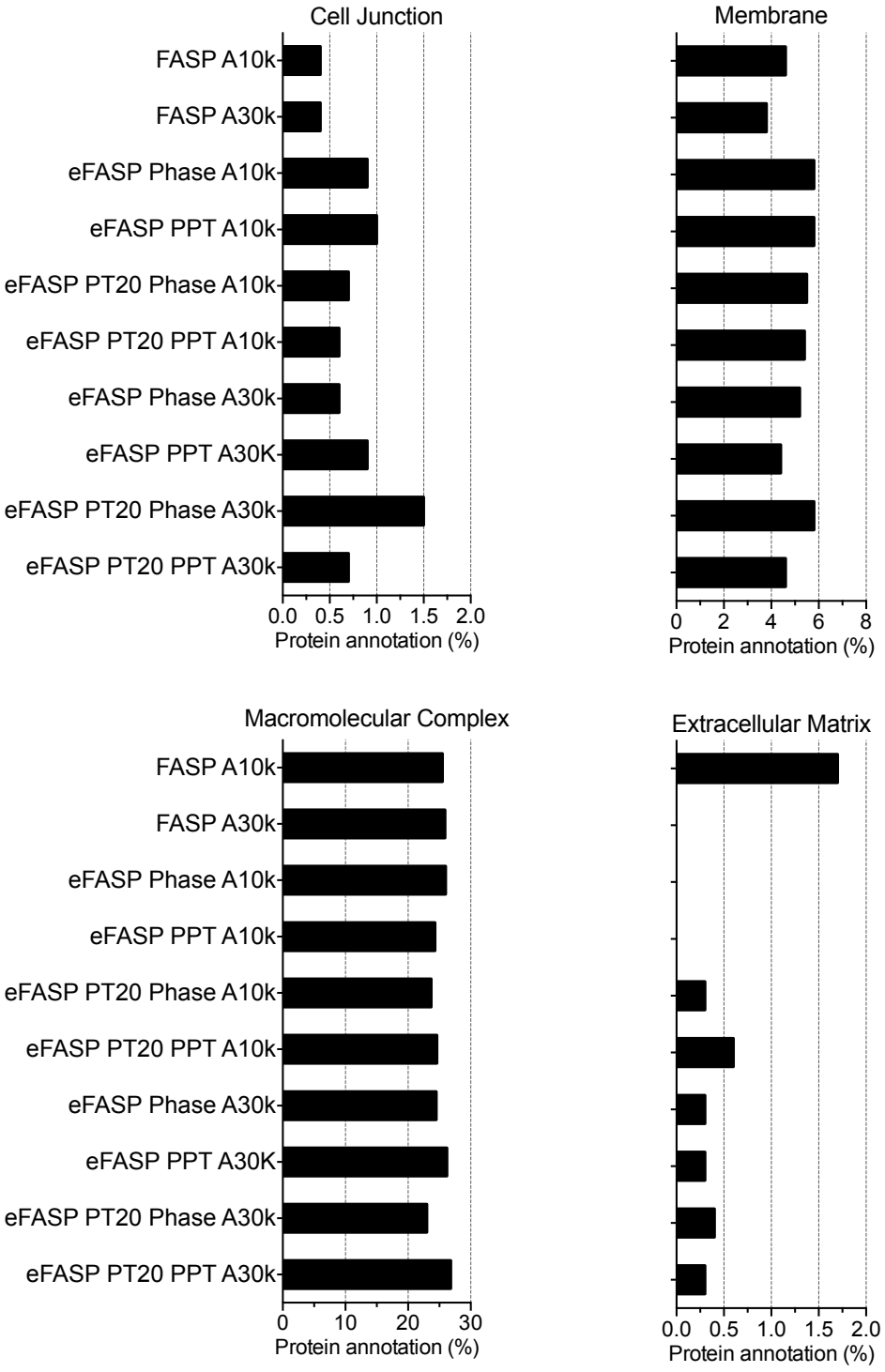
B


Organization or Biogenesis
Cellular Process
Localization
Apoptotic Process
Reproduction
Biological Regulation
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
Protein annotation (%)

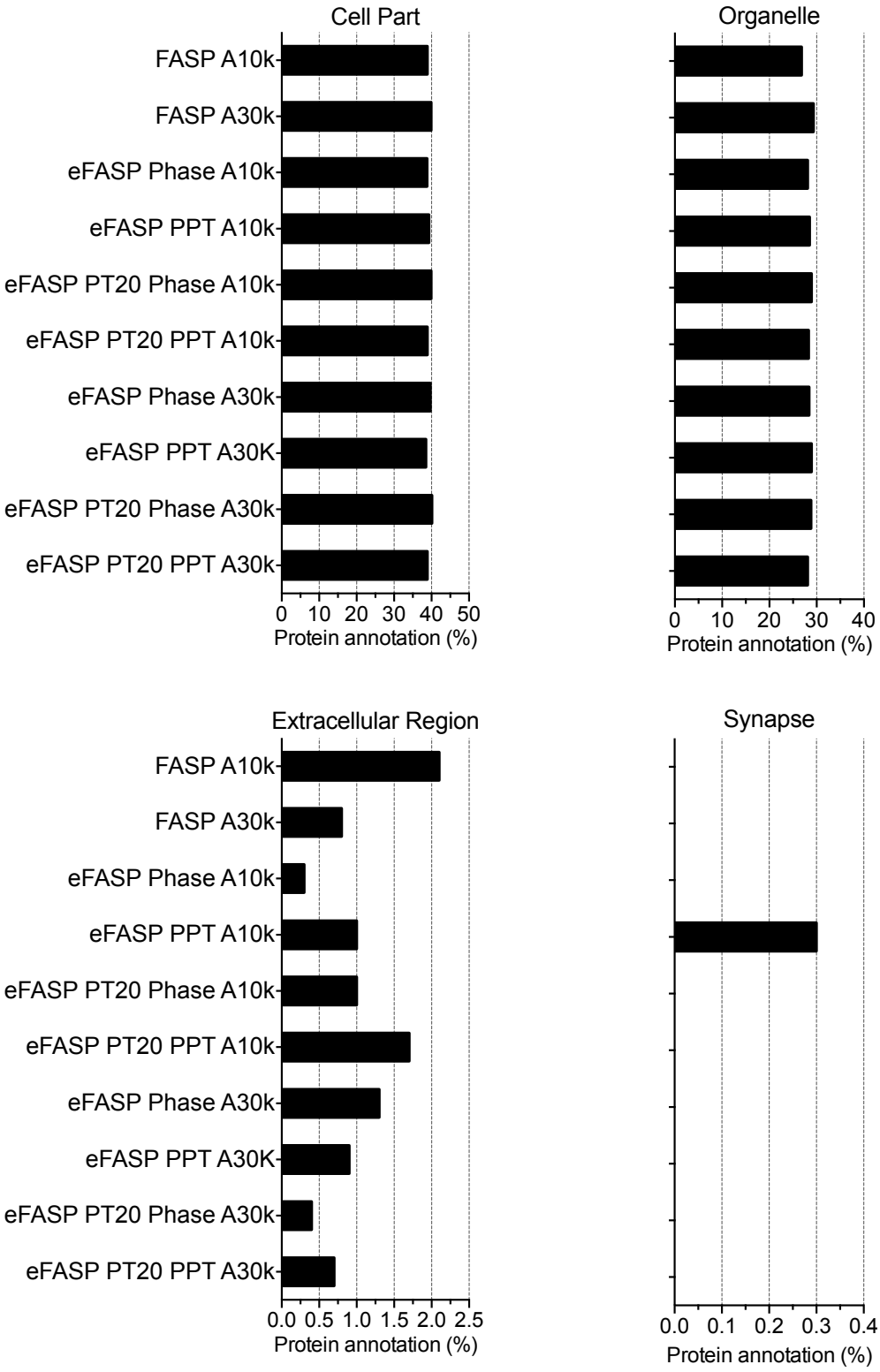
B


Response to Stimulus
Developmental Process
Multicellular Organismal Process
Biological Adhesion
Metabolic Process
Immune System Process
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
Protein annotation (%)

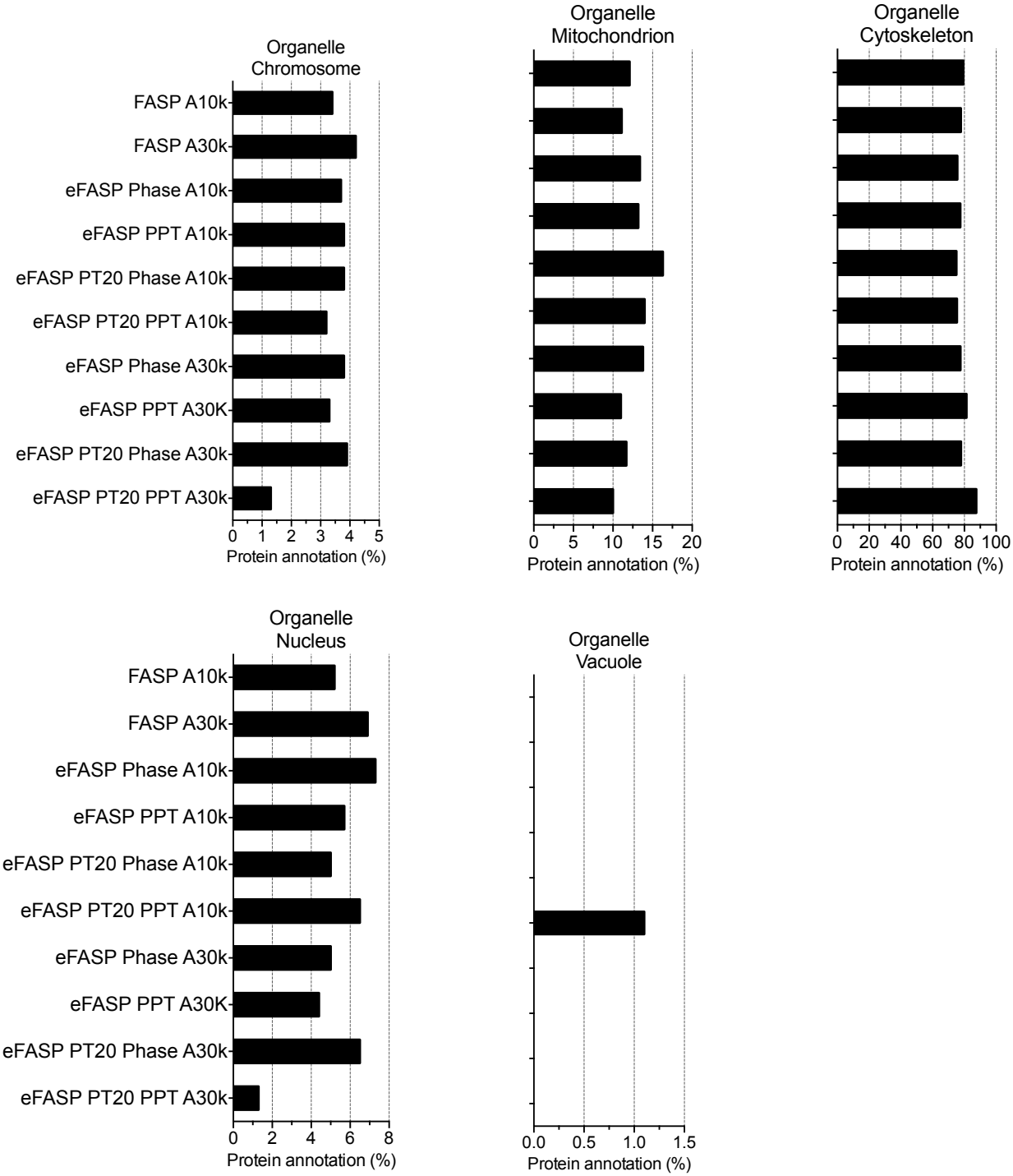
C


Cell Junction
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0.0 0.5 1.0 1.5 2.0
Protein annotation (%)
Membrane
0 2 4 6 8
Protein annotation (%)
Macromolecular Complex
0 10 20 30
Protein annotation (%)
Extracellular Matrix
0.0 0.5 1.0 1.5 2.0
Protein annotation (%)

C


Cell Part
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0 10 20 30 40 50
Protein annotation (%)
Organelle
0 10 20 30 40
Protein annotation (%)
Extracellular Region
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0.0 0.5 1.0 1.5 2.0 2.5
Protein annotation (%)
Synapse
0.0 0.1 0.2 0.3 0.4
Protein annotation (%)

D

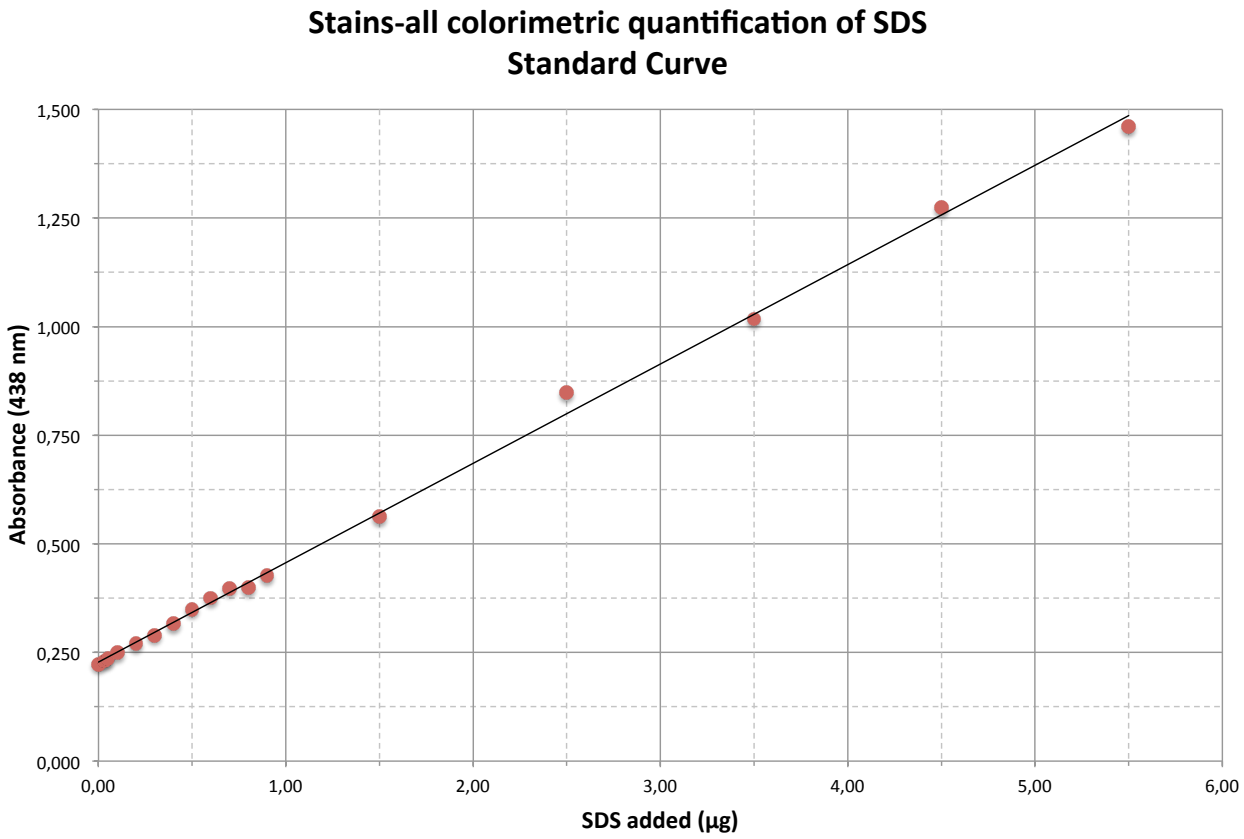

Organelle
Chromosome
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0 1 2 3 4 5
Protein annotation (%)
Organelle
Mitochondrion
0 5 10 15 20
Protein annotation (%)
Organelle
Cytoskeleton
0 20 40 60 80 100
Protein annotation (%)
Organelle
Nucleus
FASP A10k
FASP A30k
eFASP Phase A10k
eFASP PPT A10k
eFASP PT20 Phase A10k
eFASP PT20 PPT A10k
eFASP Phase A30k
eFASP PPT A30K
eFASP PT20 Phase A30k
eFASP PT20 PPT A30k
0 2 4 6 8
Protein annotation (%)
Organelle
Vacuole
0.0 0.5 1.0 1.5
Protein annotation (%)

Supplementary Tables

Supplementary Table 1. Spectrophotometric quantification of SDS with the stains-all colorimetric assay

Stains-all SDS standard curve				
	SDS added (µg)	Final [SDS] (µM)	% SDS	Absorbance 438 nm (OD)
1	0.000	0.000	0.000	0.223
2	0.030	0.104	0.003	0.230
3	0.050	0.173	0.005	0.236
4	0.100	0.346	0.010	0.249
5	0.200	0.692	0.020	0.270
6	0.300	1.037	0.030	0.290
7	0.400	1.382	0.040	0.316
8	0.500	1.725	0.050	0.350
9	0.600	2.068	0.060	0.375
10	0.700	2.411	0.070	0.397
11	0.800	2.752	0.080	0.400
12	0.900	3.093	0.090	0.427
13	1.500	5.194	0.148	0.563
14	2.500	8.648	0.247	0.848
15	3.500	12.095	0.346	1.017
16	4.500	15.535	0.444	1.274
17	5.500	18.968	0.542	1.460
$y = 0.2287x + 0.228$ $R^2 = 0.99831$				

OD: Optical density



SDS was quantified as described previously by Rusconi *et al.*[29]. The standard curve (presented in the above graph) was computed by linear regression of the optical density values obtained by spectrophotometric measurement of absorbance at 438 nm for the different amounts of SDS (µg) added to the stains-all dosage solution. SDS concentrations in micromoles per liter (µM) and percentage weight-volume (% w/v) corresponding to the various added amount of SDS (µg) are listed in columns two and three of the table. Standard curve equation and correlation coefficient are also presented at the bottom of the table.

Supplementary Table 2. Minimal effect of solutions and reagents used in FASP and Amicon-adapted eFASP protocols on stains-all dosage solution absorbance at 438 nm

Effect of FASP and ABeFASP buffers, proteins and peptides on stains-all dosage solution absorbance at 438 nm			
Compound	**Absorbance (438 nm) difference with stains-all dosage solution**	**Compound**	**Absorbance (438 nm) difference with stains-all dosage solution**
1 0.030% SDS	+ 0.067	14 NaCl 0.5 M	+ 0.003
2 BSA 2 mg/ml	+ 0.004	15 KCl 4 M	- 0.003
3 BSA 1 mg/ml	+ 0.003	16 Acetic Acid 50 mM	- 0.002
4 BSA 0.5 mg/ml	+ 0.003	17 Urea 8 M	- 0.001
5 BSA 0.25 mg/ml	+ 0.002	18 0.1% NaDoc in 0.1 M Tris-HCl pH 8	+ 0.005
6 "Non-SDS" Control Cell Lysate 2 mg/ml	+ 0.003	19 0.1% NaDoc in ABC	+ 0.003
7 "Non-SDS" Control Cell Lysate 1.8 mg/ml	+ 0.007	20 0.5% NaDoc in ABC	+ 0.014
8 "Non-SDS" Control Cell Lysate 1.5 mg/ml	- 0.001	21 4% NaDoc in 8 M Urea	+ 0.113
9 Peptide mixture 1 mg/ml	+ 0.003	22 2% Formic Acid	- 0.002
10 Peptide mixture 0.5 mg/ml	- 0.001	23 Ethyl Acetate	- 0.007
11 Peptide mixture 0.25 mg/ml	- 0.001	24 48% KCl 4 M: 48% ABC: 2% Formic Acid	- 0.001
12 ABC (50 mM NH_4HCO_3)	- 0.001	25 BSA 2 mg/ml : 0.1% NaDoc in ABC (50:50)	- 0.002
13 0.1 M Tris-HCl pH 8	- 0.003	26 Lysis Buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 0.5 mM PMSF	- 0.001

Most solutions, of whose 1 µl was added to the stains-all dosage solution and absorbance recorded at 438 nm, do not interfere with the colorimetric assay. Only high concentrations of sodium deoxycholate (NaDoc) readily react with stains-all, what causes a large increase in dosage solution's absorbance (438 nm). However, the

concentration of residual NaDoc at the end of the Amicon-adapted eFASP protocol was found sufficiently low to not interfere with the colorimetric assay; the absorbance of Amicon-adapted eFASP-processed 150-µg “non-SDS” control lysates, independently of filter unit, was not different from the one of reference dosage solution.

To further assess the specificity of the colorimetric assay, three different amounts (namely 150 µg, 100 µg and 50 µg) of “non-SDS” control cell lysates were processed and concentrated with Amicon-adapted eFASP and FASP protocols. Before digestion, 1 µl of the concentrated sample was withdrawn and added to the dosage solution, whose absorbance was recorded at 438 nm. Despite the theoretically high concentrations of proteins (2.4 mg/ml, 1.6 mg/ml, and 0.77 mg/ml, respectively) and nucleic acids, no sample modified the reference absorbance value, confirming the reliability and specificity of the stains-all assay to quantify SDS.

Supplementary Table 3. Summary of the 10 protocols compared in this study

Protocol no.	Replicates (n)	Experimental Conditions				
		Filter Unit	Filter Passivation	Purification	Cleanup	
1	3	Amicon 10k		FASP	SPE	
2	3	Amicon 30k				
3	3	Amicon 10k		Amicon-adapted eFASP	Precipitation	SPE
4	3				Phase Transfer	
5	2		5% Tween-20		Precipitation	
6	2		5% Tween-20		Phase Transfer	
7	3	Amicon 30k		Amicon-adapted eFASP	Precipitation	SPE
8	3				Phase Transfer	
9	2		5% Tween-20		Precipitation	
10	2		5% Tween-20		Phase Transfer	

SPE: Solid Phase Extraction; PT: Phase Transfer; PPT: Precipitation.

Supplementary Table 4. LC-MS/MS results of FASP and Amicon-adapted eFASP protocols

Results were either obtained by the combined analysis of replicates or by individual analysis of each replicate, presented as average (± S.D.). n = 3 for FASP and Amicon-adapted eFASP without passivation experiments. n = 2 for Amicon-adapted eFASP with passivation experiments.

ABC: 50 mM ammonium bicarbonate; Phase: phase transfer; PPT: precipitation; PT20: 5% Tween-20 v/v overnight passivation; S.D.: standard deviation; SPE: solid phase extraction.

Protocol		FASP		Amicon-adapted eFASP							
		Amicon 10k	Amicon 30k	Amicon 10k				Amicon 30k			
				Phase	PPT	P-T20/Phase	P-T20/PPT	Phase	PPT	P-T20/Phase	P-T20/PPT
	Total processing time (h)	18	15	16.5		30.5		15		28.5	
	Hands-on time (h)	6	3	4.5		6.5		3		4.5	
	Recovery (%) Mean (± SD)										
	Pre-SPE	38.01 (0.79)	24.01 (1.47)	40.39 (1.20)	46.42 (2.70)	45.42 (3.75)	52.80 (4.21)	27.93 (2.81)	33.91 (1.41)	29.99 (4.45)	35.34 (3.82)
	Post-SPE	25.05 (8.33)	11.93 (2.78)	27.35 (3.67)	23.46 (5.55)	24.83 (6.22)	18.86 (0.57)	20.55 (4.44)	19.23 (1.27)	25.42 (3.25)	22.10 (0.20)
	Remaining SDS (% w/v) Mean (± SD)	(n=3)	(n=3)	(n=6)		(n=4)		(n=6)		(n=4)	
	Digestion (40 µl)	0.186 (0.002)	0.061 (0.010)	0.148 (0.029)		0.124 (0.029)		0.037 (0.006)		0.034 (0.001)	
	Digestion (150 µl)			0.051 (0.013)		0.042 (0.012)		0.010 (0.003)		0.014 (0.001)	
	After Cleanup			< 0.003				< 0.003			
Peptide identifications	Total identifications (combined)	5,755	6,223	9,907	12,431	8,171	10,338	10,343	11,167	7,134	9,805
	Average (±SD)	3,080 (174.7)	3,299 (802.9)	5,153 (354.9)	6,490 (72.1)	5,262 (145.0)	6,716 (5.0)	4,313 (1,569.1)	6,007 (385.3)	4,643 (270.1)	6,395 (35.4)
Protein identifications	Total identifications (combined)	1,199	1,457	1,683	2,177	1,525	2,046	1,677	1,895	1,437	1,826
	Average (±SD)	851 (57.2)	1,010 (102.8)	1,214 (87.1)	1,598 (4.4)	1,208 (31.8)	1,680 (25.5)	1,075 (261.5)	1,406 (80.0)	1,131 (97.6)	1,508 (5.0)

	Protocol	FASP		Amicon-adapted eFASP							
				Amicon 10k				Amicon 30k			
		Amicon 10k	Amicon 30k	Phase	PPT	P-T20/Phase	P-T20/PPT	Phase	PPT	P-T20/Phase	P-T20/PPT
Average sequence coverage (%)	Combined Analysis	23.96	20.38	25.84	22.22	24.15	19.68	25.01	23.10	21.39	20.71
	Individual analysis (±SD)	18.83 (0.22)	15.27 (1.87)	20.64 (1.29)	18.29 (0.24)	19.74 (0.79)	17.58 (0.21)	16.94 (2.78)	18.47 (0.19)	17.83 (0.57)	18.25 (0.04)
Molecular Weight (kDa)	Average (±SD)	62.43 (127.70)	61.62 (57.23)	58.61 (54.32)	61.24 (56.49)	60.63 (57.13)	64.02 (105.60)	60.30 (59.72)	59.89 (55.93)	60.41 (54.20)	59.88 (54.95)
	Median	43.90	46.99	44.76	46.42	46.39	46.90	45.49	46.41	46.87	46.42
Ratio peptides-to-protein	Combined Analysis	4.8	4.3	5.9	5.7	5.4	5.1	6.2	5.9	5.0	5.4
	Average	3.6	3.2	4.2	4.1	4.4	4.0	3.9	4.3	4.1	4.2
Miscleavage frequency (%)	0 Missed Cleavage	79.9	91.3	78.8	84.1	81.7	85.7	88.9	89.3	89.6	89.3
	≥1 Missed Cleavage	20.1	8.7	21.2	15.9	18.3	14.3	11.1	10.7	10.4	10.7
Peptide Type (%)	Tryptic peptides	96.4	94.9	95.5	95.1	95.7	95.9	94.9	93.7	94.5	94.1
	Semi-tryptic peptides	3.5	5.0	4.4	4.9	4.2	4.1	5.1	6.2	5.5	5.8
	Non-specific peptides	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.0	0.1
Tryptic C-Terminal frequencies (%)	Lysine	56.8	62.7	56.3	58.3	55.9	56.5	56.6	57.4	56.2	57.0
	Arginine	41.9	35.4	42.4	40.0	42.6	41.9	41.7	40.6	42.0	41.2
	Other amino acid	1.3	1.8	1.4	1.7	1.5	1.6	1.7	2.1	1.8	1.8

Supplementary Table 5. Concentration of residual SDS in 0.2% deoxycholic acid-based enhanced FASP (eFASP)-processed MEG-01 whole cell lysates

eFASP Sample	% SDS (w/v) remaining
eFASP 5% PT20 150 *µ*g (1)	0.035
eFASP 5% PT20 150 *µ*g (2)	0.041
eFASP 5% PT20 200 *µ*g (1)	0.040
eFASP 5% PT20 200 *µ*g (2)	0.052

Low-volumes of MEG-01 lysates – 150 µg (18.75 µl) and 200 µg (25 µl) – were prepared and concentrated according to the eFASP standard protocol described by Erde *et al*. [18]. After depositing 40 µl of ABC onto the 30k filter, 1 µl of each resuspended sample was withdrawn from filter unit and added to the stains-all dosage solution for colorimetric SDS quantification. Despite the fact that the volume used in the 150-µg eFASP experiments was 2.5 times lower than the one of our Amicon-adapted eFASP experiments, the former method was less efficient to remove SDS from proteins.

PT20: 5% Tween-20 (v/v) overnight passivation of filter device.

Supplementary Table 6. Volume processing capacity of Amicon-adapted eFASP

	Processing capacity: Volume (µl)	
	Amicon 10k	Amicon 30k
Sample ID: starting volume	% SDS in sample	% SDS in sample
30 µl (40)	0.019	0.002
30 µl (150)	0.007	0.007
50 µl (40)	0.050	0.007
50 µl (!50)	**0.018**	0.008
75 µl (40)	0.215	0.014
75 µl (150)	0.067	0.005
100 µl (40)	0.297	0.019
100 µl (150)	0.082	0.007
125 µl (40)	0.469	0.043
125 µl (150)	0.246	**0.016**
150 µl (40)	0.469	0.126
150 µl (150)	0.290	0.046
200 µl (40)	0.469	0.388
200 µl (150)	0.403	0.106
250 µl (40)	0.490	0.722
250 µl (150)	0.394	0.317
300 µl (40)	0.454	0.741
300 µl (150)	0.355	0.348

Varying volumes of protein extracts (~50 µg) were processed in parallel either with Amicon 10k or 30k according to the Amicon-adapted eFASP method and the remaining SDS concentration (% w/v) was quantified with the stains-all colorimetric assay before digestion.

Numbers in parentheses indicate the dilution volume (either 40 µl or 150 µl) of the sample in which SDS quantification was conducted. The 40-µl volume served as the dilution benchmark for comparisons with FASP SDS removal efficiency.

Data were summarized from duplicate experiments.

Supplementary Table 7. Protein processing capacity of Amicon-adapted eFASP

	Processing capacity: Protein (µg)	
	Amicon 10k	Amicon 30k
Sample ID: starting amount of protein	% SDS in sample	% SDS in sample
100 µg (40)	0.019	0.002
100 µg (150)	0.008	0.002
150 µg (40)	0.046	0.010
150 µg (150)	0.011	0.004
200 µg (40)	0.043	0.015
200 µg (150)	0.018	0.006
250 µg (40)	0.055	0.028
250 µg (150)	0.023	0.011
300 µg (40)	0.060	0.037
300 µg (150)	0.025	0.014
350 µg (40)	0.109	0.056
350 µg (150)	0.037	0.019
400 µg (40)	0.115	0.068
400 µg (150)	**0.041**	0.027
500 µg (40)	0.188	0.096
500 µg (150)	0.071	0.030
600 µg (40)	0.280	0.169
600 µg (150)	0.111	**0.048**

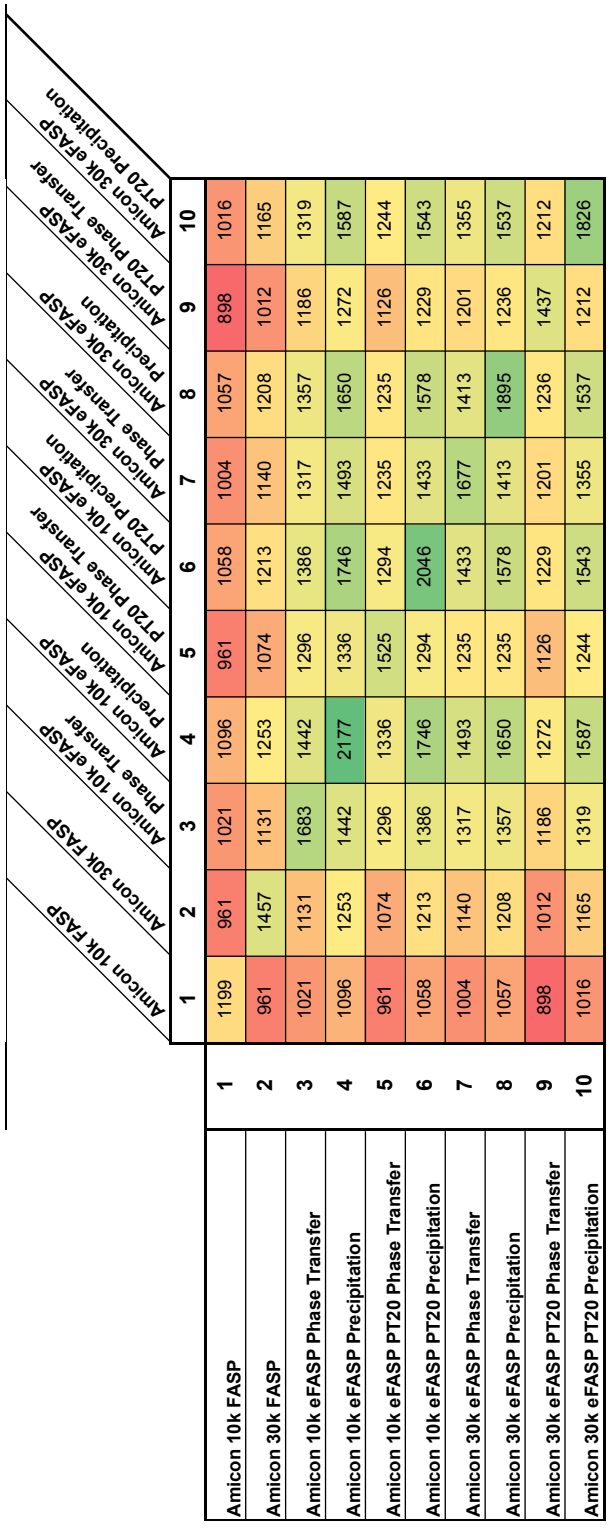
Varying amounts of protein extracts (solubilized in <60 µl lysis buffer) were processed in parallel either with Amicon 10k or 30k according to the Amicon-adapted eFASP method and the remaining SDS concentration (% w/v) was quantified with the stains-all colorimetric assay before digestion.

Numbers in parentheses indicate the dilution volume (either 40 µl or 150 µl) of the sample in which SDS quantification was conducted. The 40-µl volume served as the dilution benchmark for comparisons with FASP SDS removal efficiency.

Data were summarized from duplicate experiments.

Supplementary Table 8. Protein identifications overlap

Results computed by the Protein Pilot Protein Alignment Template Tool.



		1	2	3	4	5	6	7	8	9	10
Amicon 10k FASP	1	1199	961	1021	1096	961	1058	1004	1057	898	1016
Amicon 30k FASP	2	961	1457	1131	1253	1074	1213	1140	1208	1012	1165
Amicon 10k eFASP Phase Transfer	3	1021	1131	1683	1442	1296	1386	1317	1357	1186	1319
Amicon 10k eFASP Precipitation	4	1096	1253	1442	2177	1336	1746	1493	1650	1272	1587
Amicon 10k eFASP PT20 Phase Transfer	5	961	1074	1296	1336	1525	1294	1235	1235	1126	1244
Amicon 10k eFASP PT20 Precipitation	6	1058	1213	1386	1746	1294	2046	1433	1578	1229	1543
Amicon 30k eFASP Phase Transfer	7	1004	1140	1317	1493	1235	1433	1677	1413	1201	1355
Amicon 30k eFASP Precipitation	8	1057	1208	1357	1650	1235	1578	1413	1895	1236	1537
Amicon 30k eFASP PT20 Phase Transfer	9	898	1012	1186	1272	1126	1229	1201	1236	1437	1212
Amicon 30k eFASP PT20 Precipitation	10	1016	1165	1319	1587	1244	1543	1355	1537	1212	1826

Supplementary Protocol

Amicon-adapted enhanced Filter-Aided Sample Preparation (eFASP)

Selection of the appropriate molecular weight cut-off (MWCO) filter depends on the goals and the purposes of experiments. However, the 10k filter is to be preferred for purification of protein extracts in shotgun proteomic analyses, especially for biomarker discovery experiments.

1. Materials

Reagents

Ammonium bicarbonate (Sigma-Aldrich: A6141)

DL-Dithiothreitol (Fisher BioReagents: BP172)

Formic acid, Optima^{TM} LC/MS grade (Fisher chemical: A117)

Iodoacetamide (Sigma-Aldrich: I1149)

Potassium Chloride (Sigma-Aldrich: P9541)

Sodium deoxycholate (Sigma-Aldrich: 30970)

Sodium dodecyl sulphate (Sigma-Aldrich: L6026)

Trizma base (Sigma-Aldrich: 93362)

Trypsine/LyC Mix, Mass Spec Grade (Promega: V5073)

Tween-20 (Fisher BioReagents: BP337)

Urea (Sigma-Aldrich: U5128)

Water, Optima^{TM} LC/MS grade (Fisher chemical: W6-4)

Solutions

Lysis buffer: 2% (w/v) SDS, 0.1 M Tris-HCl pH 8, 10 mM DTT

Passivation solution: 5% (v/v) Tween-20

Buffer A: 8 M urea in 0.1 M Tris-HCl pH 8.5. Prepare 2.0 ml per sample.

Buffer B: 4% (w/v) sodium deoxycholate in buffer A. Prepare 0.5 ml per sample.

Alkylation solution: 500 mM iodoacetamide in buffer A. Prepare 0.05 ml per sample.

ABC: 50 mM NH_4HCO_3 in water.

Trypsin/Lys-C Mix, Mass Spec Grade. Stock 1 µg/µl (-80°C).

4 M KCl in water

Note

Buffers A and B, and alkylation solution must be freshly prepared.

Alkylation solution must be kept in the dark.

Equipment

Amicon Ultra-0.5 ml Centrifugal Filter Unit 10k (Millipore: UFC501096)

Eppendorf Protein LoBind microcentrifuge tubes (Eppendorf: 022431081)

Wet chamber with a rack for Eppendorf tubes

Bench-top microcentrifuge, temperature set to 20°C

2. Methods

2.1 Sample lysis

Cultured cells are pelleted by centrifugation at 1,500 rpm for 5 min (4°C) and resuspended in ice-cold 0.9% NaCl. Washed cell pellet is harvested by centrifugation at

1,500 rpm for 5 min (4°C), lysed with the 2% SDS buffer, and heated for 7 min at 98°C. Nucleic acids are sheared by on-ice sonication consisting in three 10-sec cycles with 50% output. Unbroken cells and debris are cleared by centrifugation at 16,000 g for 10 min. Sonication and centrifugation are repeated once. The final supernatant is carefully removed and its protein concentration quantified by the BCA protein assay.

2.2 Sample processing

Filter passivation

1. Fill filter reservoir and retentate collection vial with passivation solution (5% (v/v) Tween-20 in water), cap the device and soak overnight at room temperature
2. Discard passivation solution, and immerse filter and collection vial in water for 10 min with low-speed shaking. Repeat immersion once in clean water for another 10 min.
3. Add 500 µL of water to device and spin at 14,000 g/25 min. Repeat this step once.
4. Insert filter unit in non-passivated filtrate collection vial and reserve retentate collection vial for final recovery of proteins.

Amicon-adapted enhanced Filter-Aided Sample Preparation (eFASP)

Following each centrifugation run, the flow-through is systematically discarded.

1. Alkylation, 50 mM iodoacetamide: mix 50 µl of alkylation solution and up to 50 µl or 400 µg of already-reduced sample. Adjust volume to 500 µl with buffer A. Mix well. Incubate 30 min at room temperature, in the dark.

2. Transfer the entire volume into filter unit and concentrate sample by spinning at 14,000 g for 30 min.
3. Add 500 µl of buffer B, mix the content well by inverting the device thrice and spin at 14,000 g for 45 min.
4. Fill device to completeness with buffer A, mix the content well by inverting the device and spin at 14,000 g for 30 min. Repeat this step once.
5. Fill device to completeness with ABC, mix the content well by inverting the device and spin at 14,000 g for 30 min. Repeat this step once.
6. Recover concentrated proteins by inverting filter unit in passivated collection tube and spin at 1,000 g for 5 min.
7. Transfer the collected volume into a LoBind tube.
8. Adjust theoretical protein concentration to 2-1 µg/µl with ABC and add trypsin/Lys-C Mix (enzyme-to-protein ratio 1:25 to 1:50 w/w)
9. Gently mix the solution and incubate 9-14h in a wet chamber at 37°C.
10. Clean sample from residual sodium deoxycholate and SDS by precipitation
    a. Add an equivalent volume of 4M KCl
    b. Acidify with 2% (v/v) formic acid (final)
    c. Vortex for 30 sec
    d. Incubate 5 min at room temperature to form precipitates
    e. Pellet KDS and deoxycholate by spinning at 15,700 g for 15 min
    f. Carefully remove supernatant and transfer to new tube
11. Desalt and clean the filtrate with solid phase extraction (SPE).