Vacuum Insulated Probe Heated ElectroSpray Ionization source (VIP-HESI) enhances micro flow rate chromatography signals in the Bruker timsTOF mass spectrometer

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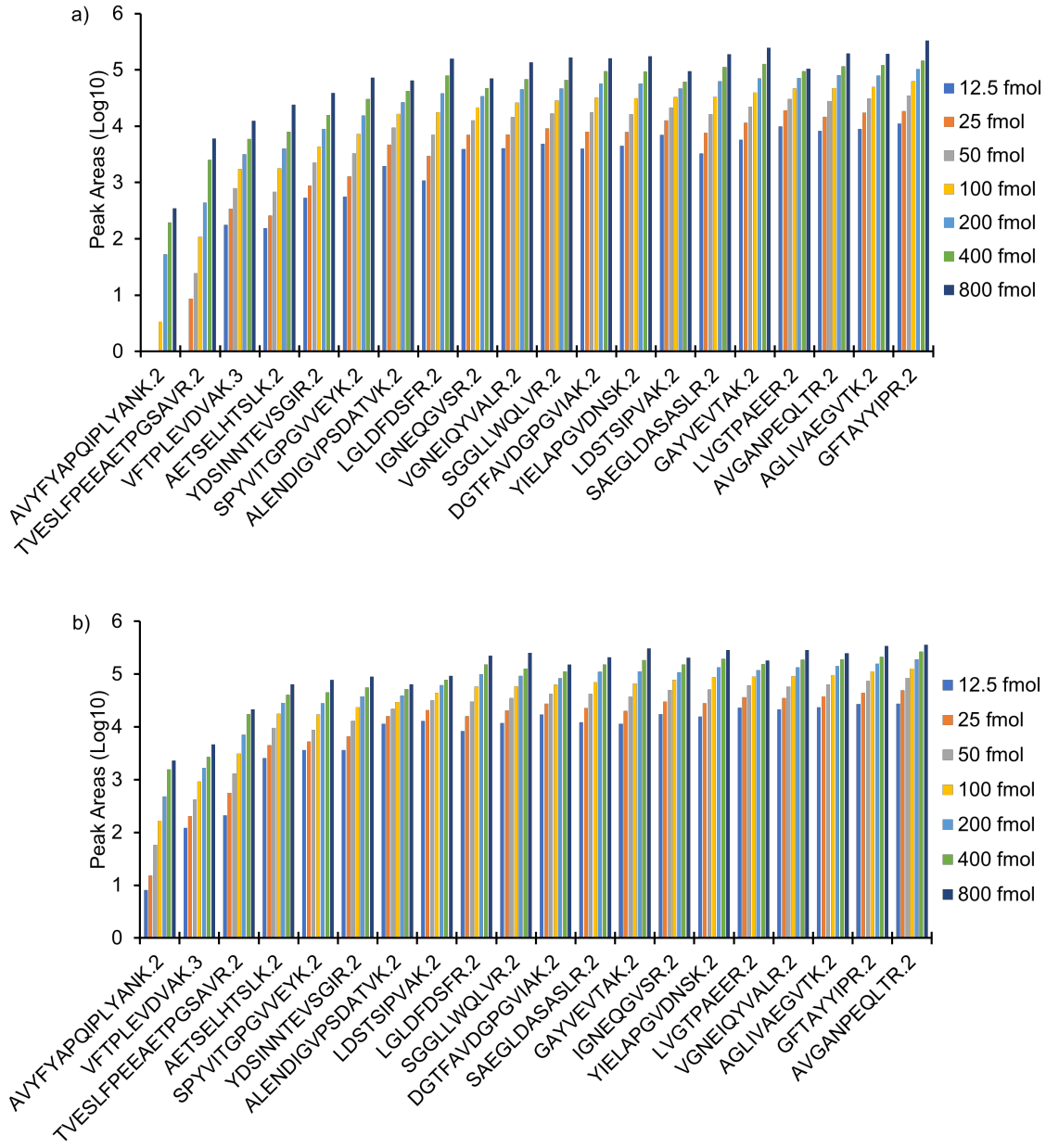
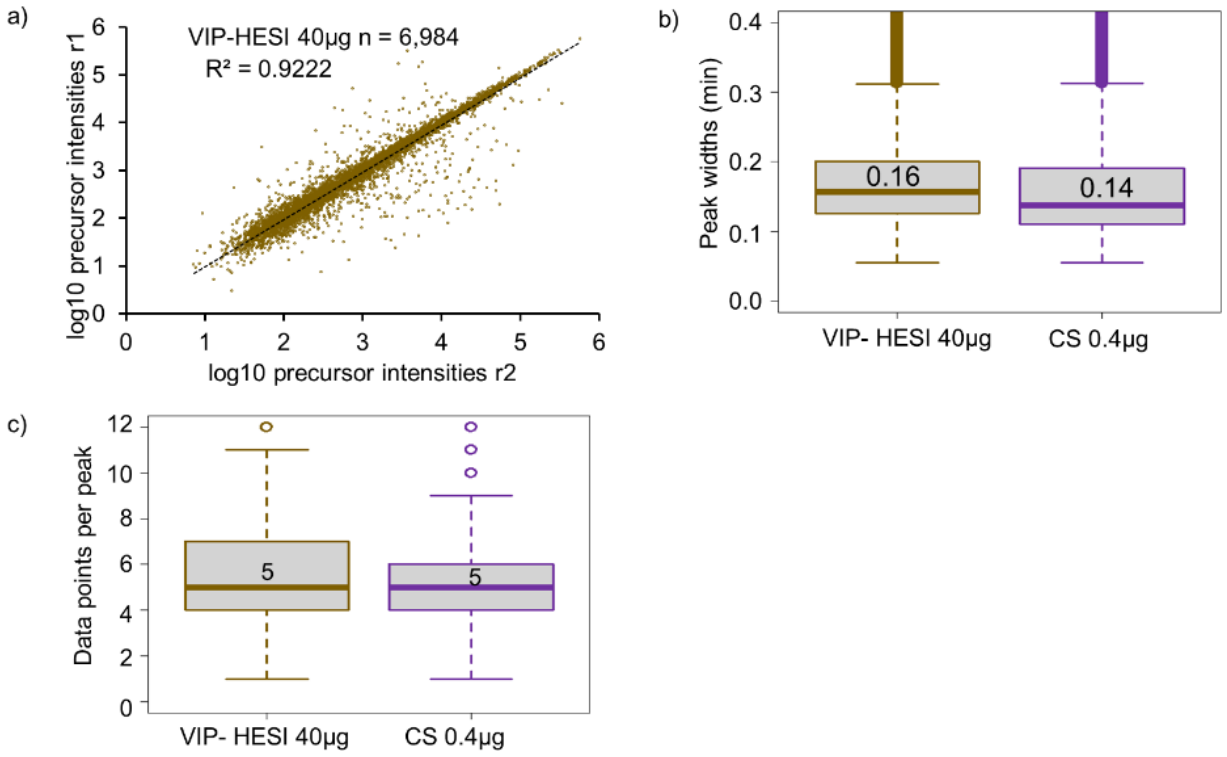
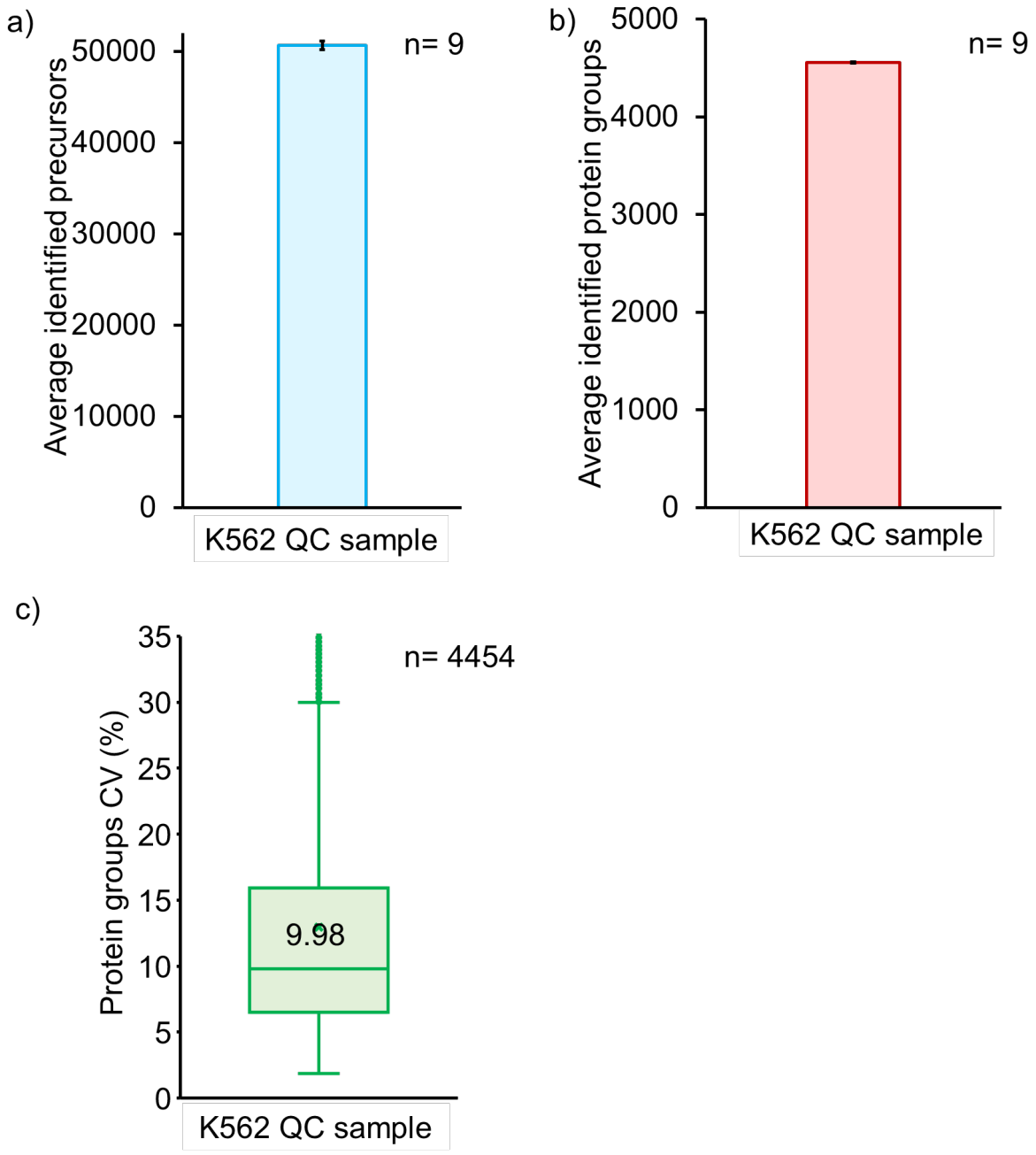


Supplementary Figure 1. Bar graph of peak areas of individual 20 PepCalMix peptides to assess linearity measured at different sample amounts. **a)** ESI source, **b)** VIP-HESI source. In the ESI source measurements, AVYFYAPQIPLYANK.2 peptide ion was not detected in 12.5 fmol, 25 fmol, and 50 fmol samples, and TVESLFPEEAETPGSAVR.2 peptide ion was not detected in the 12.5 fmol sample. However, using the VIP-HESI source, all 20 PepCalMix peptides were detected for all sample amounts.



Supplementary Figure 2. Performance assessment of VIP-HESI and CS ion sources using mouse plasma samples. a) Pearson correlation of precursor intensity values obtained from 6,984 precursors that were quantified in both replicates using VIP-HESI 0.4 µg measurements of mouse plasma samples. **b)** Distribution of the base peak widths of precursors identified for VIP-HESI 40 µg and CS 0.4 µg measurements estimated by Spectronaut. A median value of 0.16 (9.6s) and 0.14 (8.4s) peak widths were observed for VIP-HESI 40 µg and CS 0.4 µg injections, respectively. **c)** Distribution of data points per elution peak for the VIP-HESI 40 µg and CS 0.4 µg measurements estimated by Spectronaut.



Supplementary Figure 3. Estimation of MS instrument variation across the 891 VIP-HESI injections using K562 QC samples. The average **a)** number of precursors and **b)** protein groups identified with nine technical replicates. **c)** Distribution of coefficient of variation (CV) proteins identified in all nine replicates at 1% protein FDR estimated by Spectronaut. The median CV of 10% correlates well with the signal stability achieved using the VIP-HESI source setup. The first and third quartile are marked by a box with a whisker marking a minimum/maximum value ranging to 10 interquartile and the median is depicted as a solid line.

Supplementary Table 1. Metadata of the mouse liver, kidney, gastrocnemius muscle tissues, and plasma samples.

Available via ProteomeXchange (PXD040497).

Supplementary Table 2. Acquisition and LC separation details used in this study.

S.No	Samples	Ion source used	LC gradient used (min)	LC used	Analytical column used	Flow rate	Injected amount	DIA mode	DIA tool	Number of raw folders (.d)	Name of folder
1	PepCalMix	ESI, CS, VIP-HESI	45	EasyLC nano, Vanquish Neo	C18, 15cm x 1mm x 1.7µm Kinetix column (Phenomenex), C18 UHP 15cm x 0.15mm x 1.5µm column (Bruker/PepSep)	40µL/min, 1µL/min	12.5, 25, 50, 100, 200, 400, 800 fm	dia-pasef	Spectronaut	27	PepCalMix-CS-ESI-VIP-HESI.zip
2	Mouse Plasma control	CS, VIP-HESI	45	Vanquish Neo	C18, 15cm x 1mm x 1.7µm Kinetix column (Phenomenex), C18 UHP 15cm x 0.15mm x 1.5µm column (Bruker/PepSep)	40µL/min, 1µL/min	0.4, 2, 4, 10, 20, 40 µg	dia-pasef	Spectronaut	12	Mouse-Plasma-CS-VIP-HESI.zip
3	Mouse Plasma	VIP-HESI	45	Vanquish Neo	C18, 15cm x 1mm x 1.7µm Kinetix column (Phenomenex)	40µL/min	20 µg	dia-pasef	Spectronaut	284	Mouse-Plasma-284-VIP-HESI.zip
4	Mouse Liver	CS	30SPD	EvoSep One	C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep)	0.5µL/min	200 ng	dia-pasef	Spectronaut	291	Mouse-Liver-CS-291.zip
5	Mouse Kidney	CS	30SPD	EvoSep One	C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep)	0.5µL/min	200 ng	dia-pasef	Spectronaut	290	Mouse-Kidney-CS-290.zip
6	Mouse Gastrocnemius muscle	CS	30SPD	EvoSep One	C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep)	0.5µL/min	200 ng	dia-pasef	Spectronaut	290	Mouse-GastrocMuscle-CS-290.zip
7	K562	VIP-HESI	21	Vanquish Neo	C18, 15cm x 1mm x 1.7µm Kinetix column (Phenomenex)	40µL/min	2 µg	dia-pasef	Spectronaut	9	K562-VIP-HESI.zip
8	HeLa	VIP-HESI	21	Vanquish Neo	C18, 20cm x 0.5mm x 1.9µm column (Dr. Maisch GmbH)	20µL/min	10, 100, 1000 ng	slice-pasef	DIA-NN	18	HeLa-VIP-HESI.zip
9	HeLa (for spectral library generation)	CS	90	Nano Elute	C18, 25cm x 0.075mm x 1.6µm column (IonOptiks)	0.40 µL/min	35µg	dda-pasef	Spectronaut	24	HeLa-Library-CS.zip
			90	EasyLC nano	C18, 25cm x 0.075mm x 1.6µm column (IonOptiks)	0.40 µL/min	200 ng	dia-pasef	Spectronaut	2	
			88	EasyLC nano	C18, 40cm x 0.075mm x 1.9µm column (Bruker/PepSep)	0.30 µL/min	200 ng	dia-pasef	Spectronaut	4	
			200SPD	EvoSep One	C18, 8cm x 0.15mm x 1.5µm column (Bruker/PepSep)	2µL/min	200 ng	dia-pasef	Spectronaut	3	
			100SPD	EvoSep One	C18, 8cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.9µm column (Bruker/PepSep), C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep), C18, 25cm x 0.15mm x 1.9µm column (Bruker/PepSep)	1.5µL/min	200 ng	dia-pasef	Spectronaut	15	
			60SPD	EvoSep One	C18, 8cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.9µm column (Bruker/PepSep), C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep), C18, 25cm x 0.15mm x 1.9µm column (Bruker/PepSep)	1µL/min	200 ng	dia-pasef	Spectronaut	15	
			30SPD	EvoSep One	C18, 8cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.9µm column (Bruker/PepSep), C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep), C18, 25cm x 0.15mm x 1.9µm column (Bruker/PepSep)	0.5µL/min	200 ng	dia-pasef	Spectronaut	15	
			15SPD	EvoSep One	C18, 15cm x 0.15mm x 1.5µm column (Bruker/PepSep), C18, 15cm x 0.15mm x 1.9µm column (Bruker/PepSep), C18, 25cm x 0.2mm x 1.5µm column (Bruker/PepSep), C18, 25cm x 0.15mm x 1.9µm column (Bruker/PepSep)	0.22µL/min	200 ng	dia-pasef	Spectronaut	16	

LC Separation details		
Stepwise 45-minute gradient		
Time (min)	**%A**	**%B**
0	99	1
1	97	3
37	75	25
45	65	35
46	20	80
48	20	80
Linear 21-minute gradient		
Time (min)	**%A**	**%B**
0	99	1
3	97	3
21	65	35
22	20	80
24	20	80
Linear 90-minute gradient		
Time (min)	**%A**	**%B**
0	95	5
90	65	35
110	20	95
120	20	95
121	97	5
130	97	5

Supplementary Table 3. DIALib-QC report of spectral assay libraries "HeLa_timsTOF_DDA_DIA_Library" and “Mouse_tissue_plasma_DDA_DIA_Library"

DIALib-QC parameters	HeLa_timsTOF_DDA_DIA_Library	Mouse_tissue_plasma_DDA_DIA_Library	Parameter definations
format	Spectronaut	Spectronaut	Library format, one of OpenSWATH, Peakview, or Spectronaut
pepions	226138	468099	Number of peptide ions (i.e. precursor, sequence + modifications + charge)
fragments	4299713	9163950	Number of fragment (fragment ions) in library
ptp_percent	100	100	Percentage of proteotypic pepions (not shared)
shared_percent	0	0	Percentage of shared peptide ions (pepions)
shared_pepions	0	0	Number of shared pepions
peptides	160917	180681	Number of distinct peptide sequences
mod_peps	180516	335758	Number of distinct modified peptides (sequences + modifications)
mod_percent	30.9	58.6	percentage of distinct modified peptides with a mass modification
total_mods	71605	349020	Number of mass modified amino acids
chg_2	57	26.2	Percentage of charge 2 precursors
chg_3	34.1	39.8	Percentage of charge 3 precursors
precursor_min	312.9	306.2	Minimum precursor m/z (mass/charge) in library
precursor_max	1673.8	1692.9	Maximum precursor m/z in library
fragment_min	199.1	199.1	Minimum fragment m/z in library
fragment_max	1700	1699.9	Maximum fragment m/z in library
avg_len	15.4	17.7	Average peptide Length
avg_num_frags	19.01	19.58	Average number of fragment per assay (precursor)
avg_frag_len	1.23	1.11	Average fragment sequence length
short_perc	100	100	Percentage of assays with 5 or fewer transitions
fragment_above_precursor	0.462	0.58	Percentage of fragment m/z above precursor m/z
y_perc	61.2	62.7	Percentage of y ions
b_perc	38.8	37.3	Percentage of b ions
t6_y_perc	59.6	63.8	Percentage of y ions considering only the top 6 fragments per assay
avg_intensity	0	0	Average Intensity
rt_min	-91	-1953.7	Minimum RT (retention time) in library
rt_max	368.9	404.5	Maximum RT in library
rt_med	36.1	40.4	Median RT in library
rt_rsq	1	1	r-squared value of fit between RT of +2 and +3 charge states for the same modified peptide
rt_five	100	100	Percentage of +2/+3 charge pairs of the same mod pep within 5 RT units of each other
n_irt	11	11	Number of iRT peptides in library
irt_cnt	301	582	Number of iRT assays (precursor + fragment)
low_nr	0	0	Number of fragment annotated as y1,y2,b1, or b2
db_peps	0	0	Peptides in reference library, often 7-50 AA
seen_peps	0	0	Number of library peptides seen
mc_peps	27571	70416	Number of Missed cleavage peptides
db_prots	0	0	Number of Proteins in reference library
lib_prots	10258	10530	Number of proteins with at least one assay in ion library
seen_prots	N/A	N/A	Number of library proteins seen
ux_prots	N/A	N/A	Unexplained (not in reference library) proteins
decoy_pct	0	0	Percentage of decoy (optionally includes "alternative", non-db decoys) assays
mixed_pct	0	0	Percentage of mixed decoy/target (has both decoy and no-decoy annotations) assays
fwd_pct	100	100	Percentage of target (non-decoy) assays
med_ppp	14	20	Median number of pepions per protein
mean_ppp	22	44.5	Mean number of pepions per protein
stddev_ppp	28.8	102.1	Standard deviation of the number of pepions per protein
3_sigma_ppp	143	103	number of pepions per protein more than 3 standard deviations from the mean
max_intensity_idx	1	1	Average index of most intense fragment
precursor_ok	226138	468099	Number of assays where precursor is within 5 PPM (parts per million m/z) of theoretical
precursor_bad	0	0	Number of assays where precursor is more than 5 PPM from theoretical
fragment_ok	4299713	9163950	Number of assays where fragment is within 1 PPM of theoretical
fragment_bad	0	0	Number of assays where fragment is more than 1 PPM from theoretical
fragment_na	0	0	Number of assays where peak annotation not found in expected b/y series
fragment_avg_mdiff	0	0	Average m/z difference between reported and theoretical fragment
problem_assays	0	0	Assays whose precursor or any fragment m/z values do not match SWATHs file or differ significantly from theoretical values
im_min	0.677	0.5757	Minimum ion mobility reported in library
im_max	1.558	1.544	Maximum ion mobility reported in library

Supplementary Table 4. Performance of ESI and VIP-HESI sources with different sample amounts of PepCalMix peptides. a) Assessing linearity using total abundance of 20 PepCalMix peptides detected in three replicates at seven different sample amounts using ESI and VIP-HESI sources. **b)** Linearity assessment of ESI and VIP-HESI source using individual PepCalMix peptides across eight injected sample amounts. **c)** Sensitivity gains in abundance of 20 PepCalMix peptides using VIP-HESI compared to ESI source at different sample amounts. Fold change was estimated by taking the ratio of average abundance for each PepCalMix peptide at seven sample amounts. The gradient of colors in the visualization denotes the degree of change in fold increase to decrease. Dark green denotes higher gains, light blue indicates lower gains, and dark blue indicates no observed gains with VIP-HESI. NA represents missing abundance values in ESI source. **d)** Impact of different flow rates on the PepCalMix peptides abundances using ESI and VIP-HESI sources. **e)** Comparative performance of VIP-HESI with standard ESI and CS ion sources using 20 synthetic PepCalMix peptides.

a)

Sample amounts	ESI PepCalMix peptides abundances	VIP-HESI PepCalMix peptides abundances
12.5fmol.R1	2813.87	6302.291
12.5fmol.R2	2859.867	6424.048
12.5fmol.R3	2688.512	5869.221
25fmol.R1	5267.807	10959.11
25fmol.R2	5132.729	10284.6
25fmol.R3	5270.54	10376.88
50fmol.R1	10550.43	19400.86
50fmol.R2	10496.75	19092.57
50fmol.R3	10654.36	20386.2
100fmol.R1	20136.4	35183.6
100fmol.R2	19370.99	35694.14
100fmol.R3	19873.7	35103.41
200fmol.R1	36960.71	61478.5
200fmol.R2	36136.28	61519.64
200fmol.R3	34841.57	59875.65
400fmol.R1	63625.14	94031.12
400fmol.R2	62723.77	95216.07
400fmol.R3	63719.58	94905.24
800fmol.R1	129383.9	153838.7
800fmol.R2	129319.1	158268.3
800fmol.R3	132619.8	155157.8

b)

PepCalMix 20 peptide ions	ESI R^2	VIP-HESI R^2
AVYFYAPQIPLYANK.2	0.905	0.989
TVESLFPEEAETPGSAVR.2	0.994	0.986
VFTPLEVDVAK.3	0.998	0.994
AETSELHTSLK.2	0.995	0.986
YDSINNTEVSGIR.2	0.996	0.991
SPYVITGPGVVEYK.2	0.998	0.997
ALENDIGVPSDATVK.2	0.982	0.996
LGLDFDSFR.2	0.996	0.990
IGNEQGVSR.2	0.989	0.987
VGNEIQYVALR.2	0.996	0.995
SGGLLWQLVR.2	0.991	0.995
DGTFAVDGPGVIAK.2	0.993	0.990
YIELAPGVDNSK.2	0.998	0.990
LDSTSIPVAK.2	0.985	0.977
SAEGLDASASLR.2	0.994	0.982
GAYVEVTAK.2	0.999	0.999
LVGTPAEER.2	0.963	0.969
AVGANPEQLTR.2	0.995	0.986
AGLIVAEGVTK.2	0.994	0.987
GFTAYYIPR.2	0.993	0.993

c)

PepCalMix 20 peptide ions	12.5fmol	25fmol	50fmol	100fmol	200fmol	400fmol	800fmol
AETSELHTSLK.2	16.5	17.3	14.1	9.9	7.1	5.1	2.7
AGLIVAEGVTK.2	2.6	2.2	2.1	1.9	1.8	1.6	1.3
ALENDIGVPSDATVK.2	5.9	3.4	2.3	1.8	1.5	1.2	1.0
AVGANPEQLTR.2	3.3	3.3	3.0	2.7	2.4	2.3	1.9
AVYFYAPQIPLYANK.2	NA	NA	NA	49.7	9.1	8.0	6.7
DGTFAVDGPGVIAK.2	4.3	3.5	2.4	2.0	1.5	1.2	1.0
GAYVEVTAK.2	2.0	1.7	1.7	1.7	1.6	1.5	1.3
GFTAYYIPR.2	2.5	2.4	2.1	1.8	1.5	1.5	1.0
IGNEQGVSR.2	4.5	4.3	3.9	3.6	3.2	3.2	2.9
LDSTSIPVAK.2	1.9	1.7	1.5	1.3	1.3	1.2	1.0
LGLDFDSFR.2	7.7	5.4	4.3	3.3	2.6	1.9	1.4
LVGTPAEER.2	2.3	1.9	2.0	1.9	1.7	1.6	1.7
SAEGLDASASLR.2	3.7	3.0	2.6	2.1	1.8	1.4	1.1
SGGLLWQLVR.2	2.4	2.3	2.1	2.1	2.0	2.0	1.5
SPYVITGPGVVEYK.2	6.5	4.1	2.7	2.4	1.8	1.5	1.1
TVESLFPEEAETPGSAVR.2	NA	64.8	54.1	28.8	16.2	7.0	3.6
VFTPLEVDVAK.3	0.7	0.6	0.5	0.5	0.5	0.4	0.4
VGNEIQYVALR.2	5.3	4.9	4.0	3.5	3.0	2.8	2.1
YDSINNTEVSGIR.2	6.8	7.5	5.7	5.4	4.2	3.6	2.3
YIELAPGVDNSK.2	3.5	3.6	3.1	2.8	2.4	2.1	1.6

d)

Flow rates	Total abundance of 20 PepCalMix peptides	
	ESI-25 fmol	VIP-HESI-25 fmol
5µL.R1	23370.6	19235.0
5µL.R2	23583.0	20407.6
5µL.R3	18964.4	20405.6
10µL.R1	14601.4	20411.7
10µL.R2	14665.6	20376.8
10µL.R3	12707.4	19992.0
20µL.R1	8566.8	14285.9
20µL.R2	8175.4	15095.8
20µL.R3	7331.2	13825.6
40µL.R1	6503.8	12155.6
40µL.R2	6898.8	12050.0
40µL.R3	6443.5	12877.4
80µL.R1	3445.8	7911.2
80µL.R2	3314.9	8510.0
80µL.R3	3234.9	8279.1

e)

Sample amounts	Total abundance of 20 PepCalMix peptides at 40 µL/min
ESI-25fmol.R1	11409.95
ESI-25fmol.R2	11430.98
ESI-25fmol.R3	11326.41
CSI-25fmol.R1	149027.6
CSI-25fmol.R2	167811.5
CSI-25fmol.R3	156399
HESI-25fmol.R1	17558.73
HESI-25fmol.R2	16381.71
HESI-25fmol.R3	16760.01
HESI-800fmol.R1	189436
HESI-800fmol.R2	195298.2
HESI-800fmol.R3	197407.6

Supplementary Table 5. Comparative performance of the VIP-HESI and CS ion sources using mouse plasma sample by dia-PASEF analysis. a) VIP-HESI Mouse plasma measurements. **b)** CS Mouse plasma measurements.

Supplementary Table 6. Quantitative analysis of hundreds of plasma proteomes by VIP-HESI source coupled with micro-flow liquid chromatography. a) Mouse Plasma Precursor matrix. **b)** Mouse Plasma Protein matrix. **c)** Mouse Plasma RT-Chart Data.

Supplementary Table 7. Assessment of MS instrument variation across the 891 VIP-HESI injections using K562 QC samples analyzed by Spectronaut. a) Protein groups and **b)** Precursor sample matrices.

Supplementary Table 8. Comparative performance of VIP-HESI microflow setup using Slice-PASEF and dia-PASEF modes. a) dia-PASEF-10ng, **b)** dia-PASEF-100ng, **c)** dia-PASEF-1000ng, **d)** Slice-PASEF-10ng, **e)** Slice-PASEF-100ng, **f)** Slice-PASEF-1000ng.

Supplementary Table 5-8 are available via ProteomeXchange (PXD040497).