Supporting information

Automated on-line isolation and fractionation system for nanosized biomacromolecules from human plasma

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Chemicals and reagents

Phosphate buffered saline (PBS) tablets, ethanolamine, and acetonitrile (gradient grade, purity ≥99.9%) were purchased from Sigma-Aldrich (St. Louis, USA). Ammonia (25%) was purchased from Riedel-de Haën (Seelze, Germany). $NaHCO_3$ and Na_2CO_3 anhydrous were purchased from Merck KGaA (Darmstadt, Germany). NaOH (1 M) and formic acid (99-100%) were purchased from VWR Chemicals (Fontenay-sous-Bois, France). Glycine-2,2-d_2 (Gly-d_2) (98 atom % D), L-Phenylalanine-3,3-d_2 (Phe- d_2) (98 atom % D), and L-Lysine-4,4,5,5-d_4 (Lys- d_4) (98 atom % D, 98% (CP)) were purchased from Sigma Aldrich (St. Louis, USA) and used as internal standards (ISTDs). D-Fructose-13C6 were purchased from Carbosynth Ltd (Berkshire, UK) and was use as ISTD as well. Methanol (LC-MS Chromasolv™, ≥ 99.9%) was purchased form Honeywell (Honeywell Riedel-de Haën, Germany). Amino acid standards, L-Alanine (Ala), γ-Aminobutyric acid (GABA), L-Arginine (Arg), L-Aspartic acid (Asp), L-Citrulline (Cit), L-Cysteine (Cys) HCl, L-Glutamic acid (Glu), L-Glutamine (Gln), Glycine (Gly), L-Histidine (His) HCl, L-Isoleucine (Ile), L-Leucine (Leu), L-Lysine (Lys) HCl, L-Methionine (Met), L-Phenylalanine (Phe), L-Proline (Pro), L-Threonine (Thr), L-Tryptophan (Trp), L-Valine (Val), and L-Ornithine (Orn) HCl, were purchased from Seikagaku Kogyo Co., Ltd (Tokyo, Japan). L-Serine (ca. 99%) was purchased from Ega-Chemie (Steinheim, Germany), L-Asparagine (Asn), and D(-)-Fructose was purchased from Merck KGaA (Darmstadt, Germany). D-(+)-Glucose (purity ≥99.9%) was purchased from Sigma-Aldrich (St. Louis, USA). MilliQ water was obtained from MilliQ system (Millipore, USA). CD9 Monoclonal Antibody (eBioSN4 (SN4 C3-3A2)), eBioscience™ was purchased from Thermo Fisher Scientific (USA), and Purified Mouse Anti-Human CD61 (clone VI-PL2) antibody was purchased from BD Biosciences (USA). Anti-apoB-100 monoclonal antibody (code Anti-h ApoB 2101 SPTN-5) was donated by Medix Biochemica Co. Inc (Helsinki, Finland). Human blood plasma was provided by the Finnish Red Cross Blood Service (Helsinki, Finland) with permission (Permission no. 37/2015).

Instrumentation

CIM® carbonyldiimidazole (CDI) disks (0.34 mL, pore size 1.3 µm) and housing cartridges were purchased from BIA Separations (Ljubljana, Slovenia). AsFlFFF system (Postnova Analytics, AF2000 system, Landsberg, Germany) consisted of a 350 µm spacer (Postnova AF2000 MF) and a 10 kDa cut off regenerated cellulose membrane (Postnova AF2000 MT series). The AsFlFFF channel was coupled with a UV (SPD-20A Prominence, Shimadzu, Japan), a multi-angle light scattering (Postnova PN3070 MALS detector), a diode array (DAD) (G1315A Agilent Technologies, Waldbronn, Germany), and a dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, UK) detectors. Fractions were collected using CBM-20A modular system controller (Shimadzu, Japan) and a fraction collector (FRC-10A, Shimadzu, Japan). Nanosep® centrifugal devices for pre-concentration of fractions with a 10K molecular weight cut-off membrane filters were purchased from Pall Corporation (New York, USA). Supor®-200 membrane filters (0.2 µm) were purchased from PALL Life Sciences (USA), and MILLEX® Low Protein Binding Hydrophilic LCR (PTFE) membrane filters (0.45 µm) were purchased from Millipore (USA).

Zeta potential measurements were done with Zetasizer Nano ZS (Malvern Instruments, UK). An Agilent 1260 Infinity HPLC system furnished with a SeQuant® ZIC®-cHILIC column (150 mm x 2.1 mm i.d., pore size 100 Å, 3 µm particle size) from Merck KGaA (Germany) coupled with an Agilent 6420 triple quadrupole mass spectrometer equipped with an electrospray ion source, was used for the individual isolation and quantitation of amino acids and sugars. The column was connected to an ultra HPLC in-line filter (2.0 µm KrudKatcher, Phenomenex, Torrance, CA, USA part number AF0-8497) and a SeQuant® ZIC®-cHILIC guard column (20 mm x 2.1 mm i.d., 200 Å particle size, particle size 5 µm, Merck KGaA, Germany). Scanning electron microscopy (SEM) images were taken with a Hitachi S-4800 field emission SEM (FESEM) (Hitachi, Japan).

Table S1. Optimized multiple reaction monitoring parameters for amino acids and sugar.

Compound name	Precursor ion	Product ion	Fragmentor voltage (V)	Collision energy (V)	Cell accelerator voltage (V)	Ionization mode
Alanine (Ala)	90.1	44.1	61	9	4	Positive
Arginine (Arg)	175.1	70.1	61	25	4	Positive
Asparagine (Asn)	133	74.1	61	17	4	Positive
Aspartic acid (Asp)	134	74.1	61	13	4	Positive
Citrulline (Cit)	176	159	70	11	2	Positive
γ-aminobutyric acid (GABA)	104	87	75	15	4	Positive
Fructose-13C6	185	92	75	5	0	Negative
Glucose*	203	203	121	0	4	Positive
Glutamic acid (Glu)	148.1	84.1	61	17	4	Positive
Glutamine (Gln)	147.1	84.1	61	17	4	Positive
Glycine (Gly)	76	30.1	61	9	4	Positive
Glycine-d_2 (Gly-d_2)	78.1	32.1	61	9	4	Positive
Histidine (His)	156	110	61	17	4	Positive
Isoleucine (Ile)	132.1	69.1	61	9	4	Positive
Leucine (Leu)	132.1	44	61	25	4	Positive
Lysine (Lys)	147.1	84.1	61	17	4	Positive
Lysine (Lys-d_4)	151.1	88.1	61	17	4	Positive
Methionine (Met)	150	56	61	17	4	Positive
Ornithine (Orn)	133	70	70	11	2	Positive
Phenylalanine (Phe)	166.1	120	61	13	4	Positive
Phenylalanine (Phe-d_2)	168.1	122	61	13	4	Positive
Proline (Pro)	116.1	70,1	61	17	4	Positive
Serine (Ser)	106.1	60.1	61	9	4	Positive
Threonine (Thr)	120.1	74.1	61	9	4	Positive
Tryptophan (Trp)	205	188	61	5	4	Positive
Tyrosine (Tyr)	182	136	61	13	4	Positive
Valine (Val)	118	72	61	9	4	Positive

*Product ion

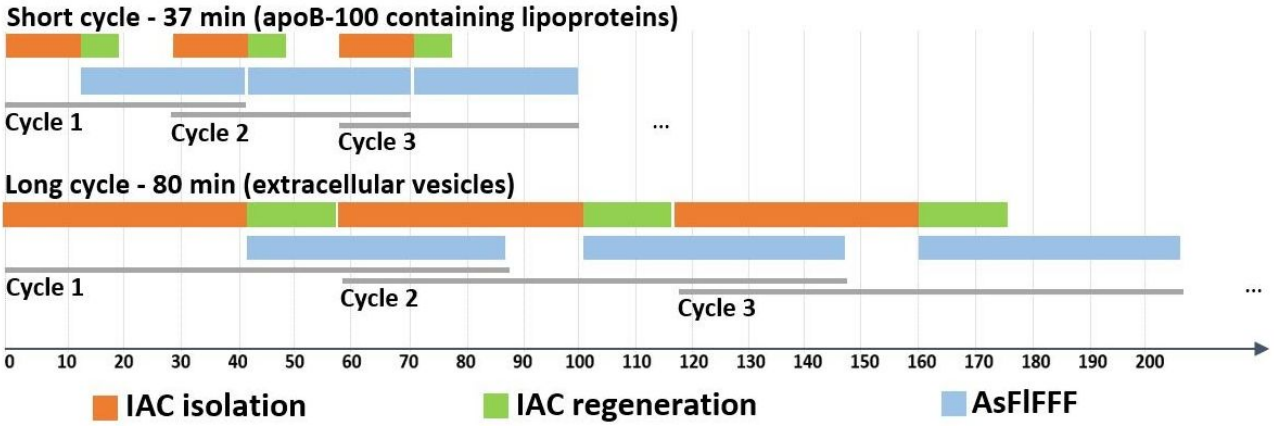

Figure S1. Repeated IAC- AsFlFFF analysis cycles. Short cycle was used for isolation and fractionation of apoB-100 containing lipoproteins and long cycle for extracellular vesicles (including exosomes and exomeres). An automated injection to the AsFlFFF was done when eluate from IAC was fully transferred to the sample loop of six port valve.

Table S2. Process cycles used in the IAC.

Isolation program for apoB-100 containing lipoproteins				**EV isolation program**			
	mL	mL/min	min		mL	mL/min	min
Sample loading	1	6	0.167	**Sample loading**	5	6	0.833
Sample injection	1	0.5	2	**Sample injection**	5	0.25	20
PBS loading	3	6	0.5	**PBS loading**	3	6	0.5
PBS injection	3	0.5	6	**PBS injection**	3	0.25	12
Ammonium hydroxide loading	2	6	0.333	**Carbonate-bicarbonate loading**	2	6	0.333
Ammonium hydroxide injection	2	0.5	4	**Carbonate-bicarbonate injection**	2	0.25	8
PBS loading	3	6	0.5	**PBS loading**	3	6	0.5
PBS injection	3	1	3	**PBS injection**	3	1	3
total			16.5	**Ammonium hydroxide loading**	2	6	0.333
				Ammonium hydroxide injection	2	1	2
				PBS final loading	3	6	0.5
				PBS final injection	3	1	3
				total			51

Table S3. Optimal AsFlFFF conditions for fractionation of apoB-100 containing lipoproteins and EVs.

Mobile phase	PBS, pH 7.4	Sigma-Aldrich	
Channel thickness	350 µm	Postnova AF2000 MT spacer	
Membrane	Regenerated cellulose, 10 kDa mass cut-off	Postnova AF2000 MT series Membrane	
Injection loop	500 µL	**ApoB-100 containing lipoproteins**	**Extracellular vesicles**
Injection step	Time Flow	5 min 0.1 mL/min	5 min 0.1 mL/min
Detector	Flow	0.5 mL/min	0.5 mL/min
Transition time	Time Cross flow	1 min 3 mL/min	1 min 3 mL/min
Separation	Time	2 min	5 min
	Cross-flow	Linear decay 3.0 mL/min to 0.5 mL/min	Linear decay 3.0 mL/min to 1 mL/min
	Time	1 min	15 min
	Cross-flow	Linear decay 0.5 mL/min to 0 mL/min	Linear decay 0.5 mL/min to 0 mL/min
	Time	15 min	14 min
	Cross-flow	0 mL/min	0 mL/min

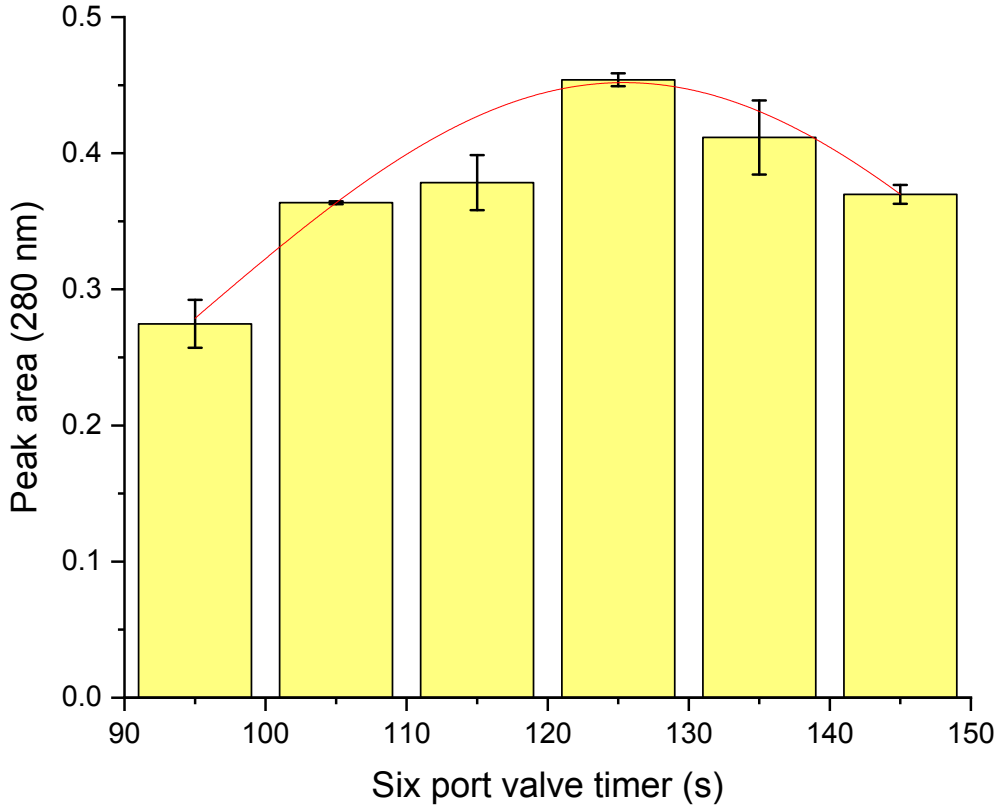

Figure S2. Optimal time (125 s) for six port valve timer of IAC isolated 100 µg/mL LDL eluted in AsFlFFF based on UV 280 nm peak areas (n=25).

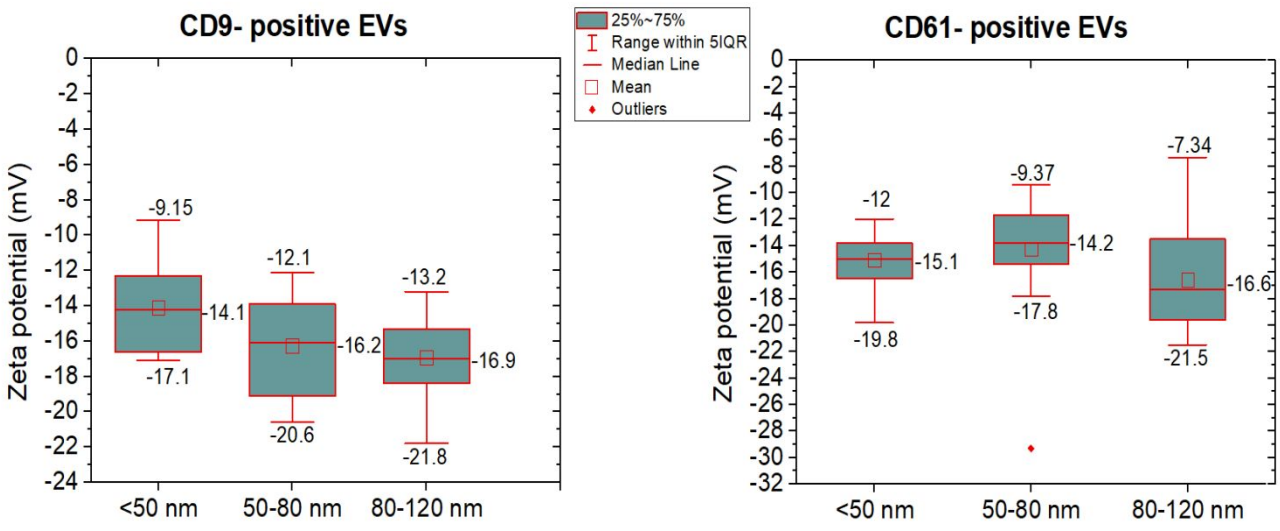

Figure S3. Zeta potential of the $CD9^+$ and $CD61^+$ EV subpopulations.

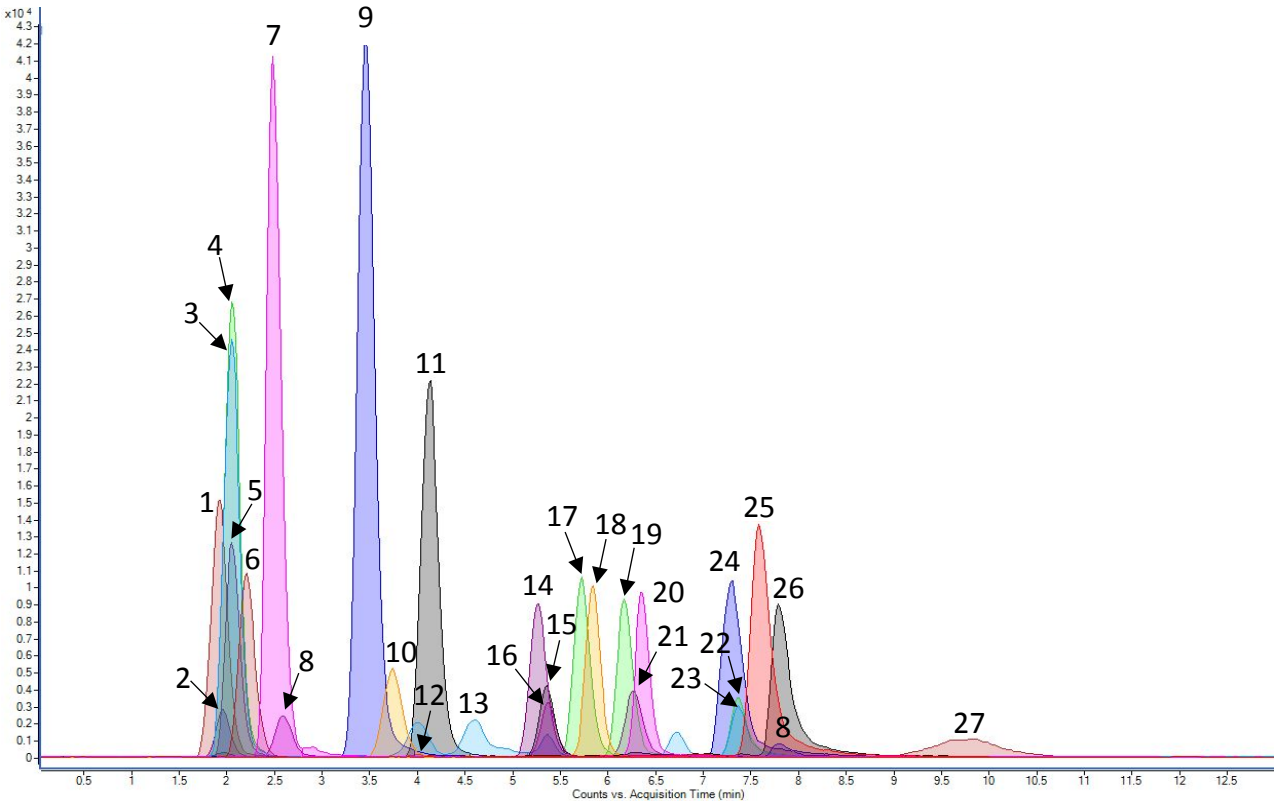

Figure S4. Extracted ion chromatograms (EICs) based on MRM of a standard mixture containing amino acids, glucose, and internal standards (ISTDs) (Gly-d_2, Lys-d_4, Phe-d_2, and Fructose-13C6) listed in Table S3. The concentration was 1 µg/mL for all standards and amino acid ISTDs and 5 µg/mL for Fructose-13C6. Peak identification: 1. Leu, 2. Ile, 3. Phe-d_2, 4. Phe, 5. GABA, 6. Trp, 7. Val, 8. Met, 9. Pro, 10. Tyr, 11. Ala, 12. Fructose-13C6, 13. Glucose, 14. Thr, 15. Gly-d_2, 16. Gly, 17. Gln, 18. Glu, 19. Cit, 20. Ser, 21. Asp, 22. Lys, 23. Lys-d_4, 24. Arg, 25. His, 26. Orn, and 27. Asn

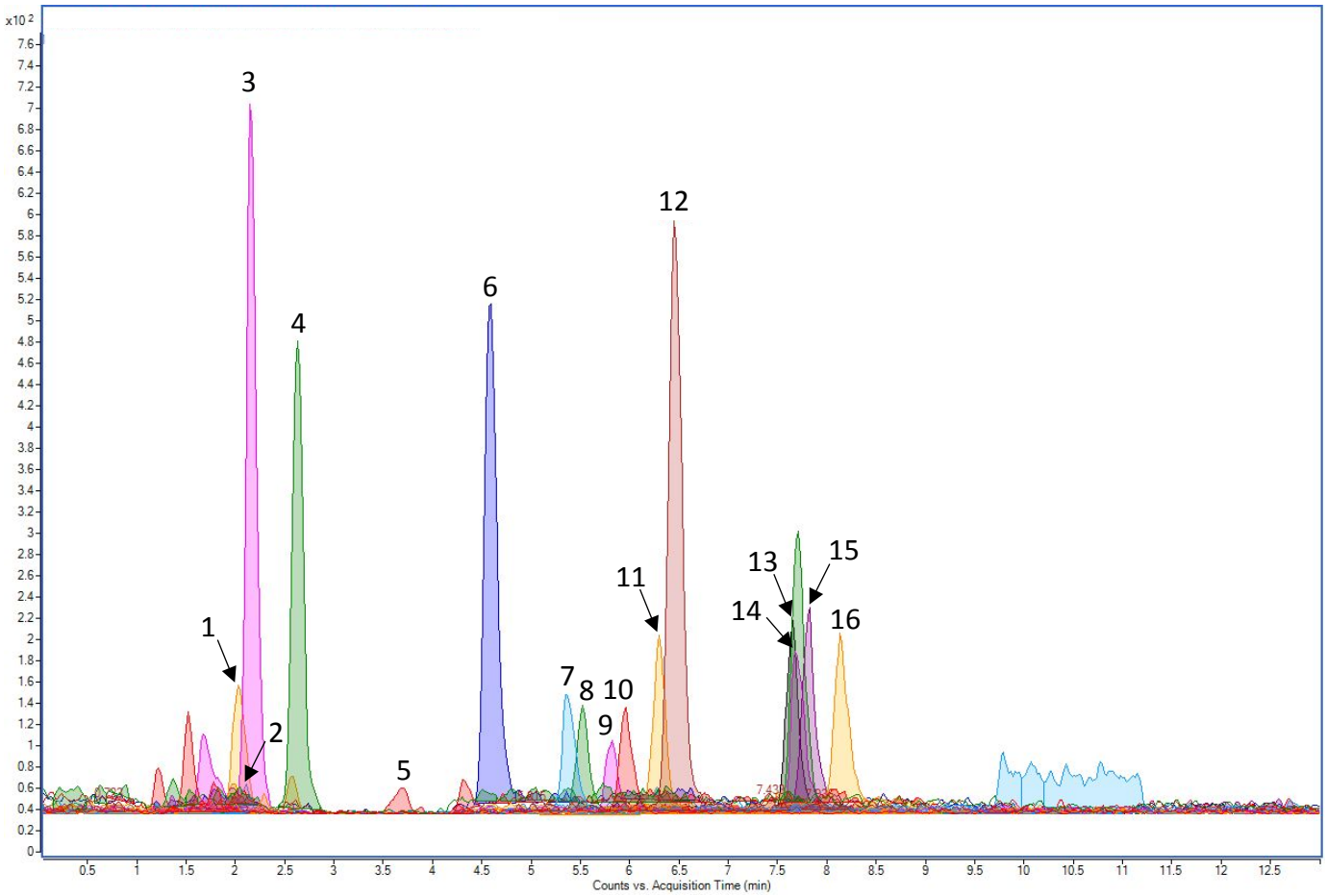

Figure S5. Extracted ion chromatograms (EICs) based on MRM of a 50-80 nm CD61+ EV subpopulation. Peak identification: 1. Leu, 2. Ile, 3. Phe, 4. Val, 5. Pro, 6. Ala, 7. Thr, 8. Gly, 9. Gln, 10. Glu, 11. Cit, 12. Ser, 13. Arg, 14. Lys, 15. His, and 16. Orn

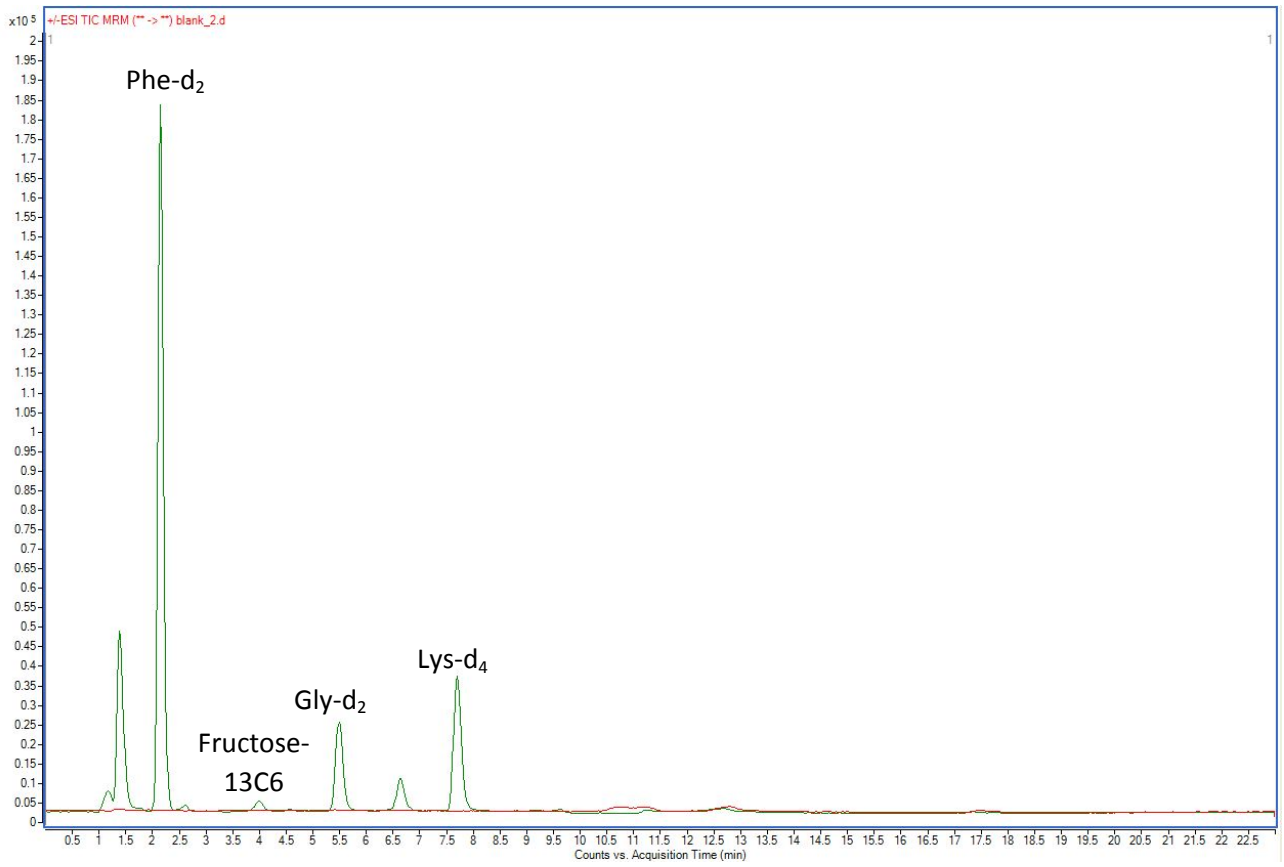

Figure S6. Total ion chromatograms (TICs) of blank containing mobile phase 1 and 2 (1:1 v/v) (red) and 50-80 nm $CD61^+$ EV subpopulation with identified ISTDs (green).

Table S4. Information on calibration curves and estimated limit of quantification (LOQ).

Compound	ISTD	Calibration curve slope	Calibration curve range (ng/mL)	R^2	LOQ (pg/mL)*
Ala	Gly-d_2	6.2	5-750	1.000	3.3
Arg	Lys-d_4	4.2	5-1000	0.996	3.7
Asn	Gly-d_2	1.1	5-750	1.000	7.1
Asp	Gly-d_2	1.0	25-1000	0.998	27.7
Cit	Gly-d_2	2.2	5-750	0.998	8.4
GABA	Phe-d_2	0.5	5-750	1.000	1.1
Gln	Gly-d_2	2.4	5-750	0.998	5.7
Glu	Gly-d_2	2.3	5-750	1.000	0.7
Glucose**	Fructose 13C6	100.7	5-250	0.995	9.1
Gly	Gly-d_2	0.8	5-750	1.000	13.3
His	Lys-d_4	5.4	5-750	0.999	20.4
Ile	Phe-d_2	0.1	5-750	0.999	8.1
Leu	Phe-d_2	0.7	5-750	1.000	0.9
Lys	Gly-d_2	1.2	5-750	0.998	29.6
Met	Phe-d_2	0.1	5-750	0.999	7.6
Orn	Lys-d_4	3.4	5-1000	1.000	16.3
Phe	Phe-d_2	1.1	5-750	1.000	1.0
Pro	Phe-d_2	1.9	5-1000	1.000	2.7
Ser	Gly-d_2	2.1	5-750	1.000	7.9
Thr	Gly-d_2	2.4	5-750	1.000	2.5
Trp	Phe-d_2	0.5	5-750	1.000	2.4
Tyr	Phe-d_2	0.2	5-750	0.999	0.3
Val	Phe-d_2	1.6	5-750	0.999	1.5

*Calculated from LOQ = 10*standard deviation of the lowest calibration point/slope of the calibration curve

**Product ion

Table S5. Recovery percentage of target amino acids and glucose in EV subpopulations based on standard addition analyses.

Compound	CD61+ < 50 nm	CD61+ 50-80 nm	CD61+ 80-120 nm	CD9+ < 50 nm	CD9+ 50-80 nm	CD9+ 80-120 nm
Ala	72-104%	93-101%	99-100%	40-96%	81-103%	72-104%
Arg	101 %	99 %	98-120%	83-104%	96-101%	99 %
Asn	93-98%	70 %	95-99%	87-117%	82-101%	-
Asp	104-105%	89-131%	55-105%	136-140%	63-98%	-
Cit	96-102%	78-115%	97-125%	-	97-99%	90-110%
GABA	95 %	101 %	99 %	90-92%	93-97%	91 %
Glucose*	42-52%	18-68%	54-72%	92-107%	-	56-88%
Glu	82-94%	83-113%	68-92%	-	92-102%	-
Gln	90-95%	80-121%	90-108%	53-65%	95-98%	85 %
Gly	88-98%	89-91%	85-86%	86-103%	93-94%	83-84%
His	87-101%	97-100%	87-111%	89-102%	98-101%	99 %
Ile	102-104%	86-87%	98-99%	103-104%	93-97%	87-107%
Leu	100-101%	102 %	77-90%	92-93%	94-101%	84-102%
Lys	97-99%	97-98%	89-93%	86-99%	95-101%	69-105%
Met	128-149%	72-80%	96-101%	77-79%	82-88%	80-92%
Orn	96-101%	93-102%	80-85%	78-95%	98-100%	-
Phe	102 %	96-97%	95-99%	95-99%	80-93%	94-96%
Pro	93-99%	95-98%	92-96%	86-95%	85-102%	100 %
Ser	84-102%	100-110%	97-101%	-	94-100%	-
Thr	90-100%	99-101%	98-99%	97-99%	92-100%	94-95%
Trp	84-93%	109-110%	89-92%	89-94%	95-108%	89-97%
Tyr	99%	99 %	95-101%	74-77%	83-86%	97 %
Val	92-100%	99%	100%	96-98%	93-100%	108%

*Product ion