Alpha-, beta- and gamma-synuclein quantification in cerebrospinal fluid by multiple reaction monitoring reveals increased concentrations in Alzheimer´s and Creutzfeldt-Jakob disease but no alteration in synucleinopathies

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Supplement tables and figures

Supplement table 1. Composition and characteristics of calibration standards, QC samples and IS solution

Peptide sequence (position)	**Protein**	**Peptide/protein used for calibration/QCs (internal standard peptide/protein[a])**	**Quantification of stock solution**	**Concentration in calibration standards** (pM)	**Concentration in QC samples (pM)** (low,medium,high, CSF-QC)	**LOD[b]/LLOQ[c]** (pM)	**Concentration in IS solution** (pM)
MDVFMK (1-6) (1-6)	αSyn βSyn	Rec. αSyn protein Rec. βSyn protein (rec. ^{15}N-αSyn protein)	AAA MRM -	6.52/13.1/26.1/65.2/91.3/131/261/652/1040/1310	19.6/78.3/326/n.d.	1.00 / 6.52	- - 7500
Ac-MDVFMK (1-6) (1-6)	αSyn βSyn	Ac-MDVFMKGLSK (Ac-MDVFM**K**GLSK)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/25.3	1.50 / 5.00	- 1155
EGVVAAAEK (13-21) (13-21)	αSyn βSyn	Rec. αSyn protein Rec. βSyn protein (rec. ^{15}N-αSyn protein)	AAA MRM -	6.52/13.1/26.1/65.2/91.3/131/261/652/1040/1310	19.6/78.3/326/41.5	0.2 / 6.52	- - 7500
QGVAEAAGK (24-32)	αSyn	Rec. αSyn protein (rec. ^{15}N-αSyn protein)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/19.6	2.00 / 5.00	- 7500
EGVLYVGSK (35-43) (35-43)	αSyn βSyn	Rec. αSyn protein Rec. βSyn protein (rec. ^{15}N-αSyn protein)	AAA MRM -	6.52/13.1/26.1/65.2/91.3/131/261/652/1040/1310	19.6/78.3/326/42.2	0.5 / 6.52	- - 7500
EGVVHGVATVAEK (46-58)	αSyn	Rec. αSyn protein (rec. ^{15}N-αSyn protein)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/16.6	0.5 / 5.00	- 7500
EGVVHGVAT(Phospho)VAEK (46-58)	αSyn	- (TKEGVVHGVAT(Phospho)VAE**K**TK)	- -	-	-/-/-/n.d.	320[d] / -	- 32000
EGVVHGVAT(O-GlcNAc)VAEK (46-58)	αSyn	- (TKEGVVHGVAT(O-GlcNAc)VAE**K**TK)	- -	-	-/-/-/n.d.	38[d] / -	- 7600
EQVTNVGGAVVTGVTAVAQK (61-80)	αSyn	Rec. αSyn protein (rec. ^{15}N-αSyn protein)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/13.4	2.00 / 5.00	- 7500
TVEGAGSIAAATGFVK (81-96)	αSyn	Rec. αSyn protein (rec. ^{15}N-αSyn protein)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/13.1	1.00 / 5.00	- 7500
TVEGAGS(Phospho)IAAATGFVK (81-96)	αSyn	TVEGAGS(Phospho)IAAATGFVK (QKTVEGAGS(Phospho)I**AAA**TGFVKKD)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/n.d.	325[d] / -	- 2275
TVEGAGS(O-GlcNAc))IAAATGFVK (81-96)	αSyn	TVEGAGS(O-GlcNAc)IAAATGFVK (QKTVEGAGS(O-GlcNAc)I**AAA**TGFVKKD)	AAA -	5.00/10.0/20.0/50.0/70.0/100/200/500/800/1000	15.0/60.0/250/n.d.	2.50 / 5.00	- 1200
EGYQDYEPEA (103-112)	αSyn112	- (GKEGYQDYEP**E**A)	- -	-	-/-/-/n.d.	13[d] / -	- 14000
EGVLYVVAEK (35-44)	αSyn126	- (TKEGVLYVVAE**K**TK)	- -	-	-/-/-/n.d.	1.5[d] / -	- 875
EGVVQGVASVAEK (46-58)[e]	βSyn	Rec. βSyn protein (TREGVVQGVASVAE**K**TK)	MRM -	1.52/3.05/6.10/15.2/21.3/30.5/61.0/152/244/305	4.57/18.3/76.2/77.8	- / -[e]	- 6000
EQASHLGGAVFSGAGNIAAATGLVK (61-85)	βSyn	Rec. βSyn protein (TKEQASHLGGAVFSGAGNIAAATGLV**K**RE)	MRM -	1.52/3.05/6.10/15.2/21.3/30.5/61.0/152/244/305	4.57/18.3/76.2/13.9	0.5 / 1.52	- 1500
ENVVQSVTSVAEK (46-58)	γSyn	Rec. γSyn protein (TKENVVQSVTSVAE**K**TK)	- -	-/-/40.0/100/140/200/400/1000/1600/2000	-/120/500/286	10.0 / 40.0	- 2250
TVEEAENIAVTSGVVR (81-96)	γSyn	Rec. γSyn protein (TKTVEEAENIAVTSGVV**R**KE)	- -	-/-/40.0/100/140/200/400/1000/1600/2000	-/120/500/437	32 / 40.0	- 3720

VNVDEVGGEALGR (19-31)	Hbb	- (VNVDEVGGEALG**R**)	- AAA	-	-	50[d]	- 31250

AAA: amino acid analysis, Hbb: hemoglobin beta subunit, IS: internal standard, LLOQ: lower limit of quantification, LOD: limit of detection, n.d. not detectable, QC: quality control sample

[a] stable-labeled amino acids are highlighted in bold and are A($^{13}C_3$; ^{15}N), E($^{13}C_5$; ^{15}N), K($^{13}C_6$; $^{15}N_2$) and R($^{13}C_6$; $^{15}N_4$)

[b] LOD is estimated from the LLOQ value applying an signal-to-noise ratio of 3

[c] the mentioned LLOQ value is the lowest value tested and does not mean that a lower LLOQ is not possible

[d] estimated from IS intensity

[e] this peptide was excluded from quantification, because data from method validation (see Results section) indicate interference.

Supplement table 2. LC gradient settings

Agilent 1260 (loading pump) Flow rate: 200µL/min[a]		Eksigent MicroLC200 Flow rate: 15µL/min[a]			
All fractions		Fractions 1, 3, 4		Fraction 2	
Time (min)	%B	Time (min)	%B	Time (min)	%B
0.0	1.0	0.0	5.0	0.0	1.0
0.5	1.0	0.25	Switching valve: Columns connected	0.25	Switching valve: Columns connected
2.0	30.0	10.0	Switching valve: Columns disconnected	10.0	Switching valve: Columns disconnected
10.2	30.0	10.1	30.0	10.1	30.0
11.0	80.0	10.3	98.0	10.3	98.0
12.0	80.0	11.5	98.0	11.5	98.0
12.1	1.0	11.6	5.0	11.6	1.0
13.5	1.0	13.5	5.0	13.5	1.0

[a] A pre-run equilibration time of 30s with initial flow rate settings was applied

Supplement table 3. MS parameters for scheduled MRM
(Ion source temperature was 175°C and CAD gas was set to “High” for all peptides)

Peptide sequence (position)	**Protein**	**Precursor mass** (labeled peptide)	**z**	**Product ion mass** (labeled peptide)	**Volt.** (V)	**CUR**	**GS1**	**GS2**	**DP**	**CE**	**CXP**	**MRM window** (s)	**Dwell weight** (labeled peptide)	**TST** (s)	**RT** (min)
MDVFMK (1-6) (1-6)	αSyn βSyn	385.7 (389.2)	2+	639.3 (645.3), y5 425.2 (429.2), y3	5100	30	20	30	80	19 23	34 26	40 40	1.0 (0.2) 1.0 (0.2)	0.3 0.3	5.1
Ac-MDVFMK (1-6) (1-6)	αSyn βSyn	812.4 (820.4)	1+	388.2 (388.2), b3 425.2 (433.2), y3	5500	30	35	30	100	49 47	23 23	80 80	1.0 (0.2) 1.0 (0.2)	0.35 0.35	9.2
EGVVAAAEK (13-21) (13-21)	αSyn βSyn	437.2 (442.2)	2+	489.3 (495.3), y5 588.3 (595.3), y6 347.2 (351.2), y3	4900	30	20	30	63.0	18 20 18	27 30 23	50 50 50	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.2 0.2 0.2	3.5
QGVAEAAGK (24-32)	αSyn	415.7 (421.2)	2+	346.2 (351.2), y6 546.3 (553.3), y4	4900	30	20	30	61.4	21 21	21 29	110 110	1.0 (0.2) 1.0 (0.2)	0.2 0.2	2.1
EGVLYVGSK (35-43) (35-43)	αSyn βSyn	476.3 (481.3)	2+	553.3 (559.3), y5 291.2 (295.2), y3	5500	30	40	30	75	20 21	27 18	120 120	1.0 (0.2) 1.0 (0.2)	0.4 0.4	4.4
EGVVHGVATVAEK (46-58)	αSyn	648.4 (656.3)	2+	774.4 (783.4), y8 911.5 (923.5), y9	5100	30	20	30	100	35 34	41 50	120 120	1.0 (0.2) 1.0 (0.2)	0.3 0.3	3.4
EGVVHGVAT(Phospho)VAEK (46-58)	αSyn	688.3 (692.3)	2+	522.3 (522.3), b5 854.4 (862.4), y8 446.3 (454.3), y4	5500	30	40	30	120	37 35 39	28 45 29	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.4 0.4 0.4	6.1
EGVVHGVAT(O-GlcNAc)VAEK (46-58)	αSyn	749.9 (753.9)	2+	522.3 (522.3), b5 678.4 (678.4), b7 977.5 (985.5), y8 204.1 (oxonium ion)	5500	30	40	30	110	38 46 35 38	28 31 50 25	40 40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.4 0.4 0.4 0.4	3.1
EQVTNVGGAVVTGVTAVAQK (61-80)	αSyn	964.5 (976.5)	2+	973.6 (985.5), y10 874.5 (885.5), y9 1072.7 (1085.6), y11	5500	30	35	30	120	44 44 44	50 50 53	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.35 0.35 0.35	7.9
TVEGAGSIAAATGFVK (81-96)	αSyn	739.9 (748.4)	2+	764.4 (773.4), y8 1021.6 (1033.5), y11 693.4 (701.4), y7	5500	30	40	30	125	34 34 34	40 55 38	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.4 0.4 0.4	6.7
TVEGAGS(Phospho)IAAATGFVK (81-96)	αSyn	779.9 (785.9)	2+	764.4 (776.5), Y8 693.4 (701.4), y7 679.8 (685.8), y14^{2+}	5500	30	35	30	90	34 34 29	42 36 39	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.35 0.35 0.35	7.8
TVEGAGS(O-GlcNAc))IAAATGFVK (81-96)	αSyn	841.4 (847.4)	2+	764.4 (776.5), y8 693.4 (701.4), y7 622.4 (626.4), y6 204.1 (oxonium ion)	5500	30	40	30	110	43 46 49 27	39 32 15 13	40 40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.4 0.4 0.4 0.4	6.3
EGYQDYEPEA (103-112)	αSyn112	1200.5 (1206.5)	1+	885.3 (885.3), b7 756.3 (756.3), b6 851.3 (857.4), y7	5500	30	35	30	110	52 52 59	43 47 43	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.35 0.35 0.35	5.3
EGVLYVVAEK (35-44)	αSyn126	553.8 (557.8)	2+	708.4 (716.4), y6 821.5 (829.5), y7 446.3 (454.3), y4	5500	30	40	30	80	23 25 28	41 38 28	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.4 0.4 0.4	6.0

Peptide sequence (position)	**Protein**	**Precursor mass** (labeled peptide)	**z**	**Product ion mass** (labeled peptide)	**Volt.** (V)	**CUR**	**GS1**	**GS2**	**DP**	**CE**	**CXP**	**MRM window** (s)	**Dwell weight** (labeled peptide)	**TST** (s)	**RT** (min)
EGVVQGVASVAEK (46-58)	βSyn	636.8 (640.9)	2+	760.4 (768.4), y8 888.5 (896.5), y9 987.5 (995.6), y10	5500	30	35	30	90	30 28 28	41 45 52	40 40 40	1.0 (0.2) 1.0 (0.2) 1.0 (0.2)	0.35 0.35 0.35	7.0
EQASHLGGAVFSGAGNIAAATGLVK (61-85)	βSyn	776.1 (778.8)	3+	730.5 (738.5), Y8 798.9 (798.9), b17^{2+}	5100	30	20	30	110	31 27	38 43	40 40	1.0 (0.2) 1.0 (0.2)	0.3 0.3	8.1
ENVVQSVTSVAEK (46-58)	γSyn	695.4 (699.4)	2+	820.4 (828.5), y8 1047.6 (1055.6), y10	5500	30	35	30	120	34 34	45 52	40 40	1.0 (0.2) 1.0 (0.2)	0.35 0.35	7.2
TVEEAENIAVTSGVVR (81-96)	γSyn	837.4 (842.4)	2+	788.5 (798.5), y8 717.4 (727.4), y7	5500	30	40	30	120	46 37	39 38	40 40	1.0 (0.2) 1.0 (0.2)	0.4 0.4	6.0
VNVDEVGGEALGR (19-31)	Hbb	657.8 (662.8)	2+	659.4 (669.4), y7 758.4 (768.4), y8	5500	30	35	30	90	30 31	34 41	40 40	0.1 0.1	0.35 0.35	6.9

CE: collision energy, CUR: curtain gas, CXP: collision cell exit potential, DP: declustering potential, GS1: gas 1 (sheath gas), GS2: gas 2, RT: retention time, TST: target scan time, Volt.: voltage, z: charge state

Supplement table 4. Demographic and clinical characteristics of patients

	Con	**PD**	**PDD**	**LBD**	**PSP**	**CBS**	**AD**	**CJD**
n[a]	37	23	17	10	20	10	19	10
Gender (m/f)	18/19	19/4	13/4	6/4	10/10	8/2	7/12	3/7
Age[b] (years)	53.8 (32.0-69.9)	69.9 (55.0-74.4)	73.3[c] (70.5-76.9)	73.2[d] (65.2-74.4)	65.1 (61.0-69.3)	68.9 (65.5-73.3)	69.0 (61.0-73.0)	67.5 (65.3-71.0)
Hoehn & Yahr[e]	-	2.5 (1-4)	3 (2-5)	2.5 (1-4)	-	-	-	-
CSF αSyn[b] (pg/mL)	222 (161-260)	249 (185-339)	230 (182-300)	185 (153-279)	204 (165-235)	246 (156-337)	303 (247-323)	4083[f] (2211-9389)
CSF βSyn[b] (pg/mL)	254 (217-334)	264 (174-380)	304 (220-529)	291 (224-515)	256 (199-423)	314 (207-361)	422[g] (353-530)	9459[h] (4747-18039)
CSF γSyn[b] (ng/mL)	4.37 (3.05-6.12)	5.84 (4.34-7.89)	6.01 (4.77-10.8)	5.55 (4.48-10.9)	5.91 (4.75-7.79)	5.54 (4.96-9.36)	7.13[i] (6.49-8.02)	21.9[j] (11.7-32.0)
CSF tau[b] (pg/mL)	243 (148-446)	255 (172-308)	331 (188-423)	273 (208-507)	242 (173-309)	260 (242-421)	531[k] (419-947)	6055[l] (3755-12540)

AD: Alzheimer´s disease, CBS: corticobasal syndrome, CJD: Creutzfeldt-Jakob disease, Con: control, f: female, LBD: Lewy body dementia, m: male, PD: Parkinson´s disease, PDD: PD dementia, PSP: progressive supranuclear palsy

[a] Patients with hemoglobin >200ng/mL in CSF were excluded for αSyn and γSyn analysis

[b] Values are median and interquartile range

[c] $p<0.001$ vs. Con; $p<0.05$ vs. PSP

[d] $p<0.05$ vs. Con

[e] values are median and range

[f] $p<0.05$ vs. PD, PDD; $p<0.01$ vs. LBD; $p<0.001$ vs. Con, PSP

[g] $p<0.05$ vs.Con

[h] $p<0.01$ vs. PDD, LBD, CBS; $p<0.001$ vs. Con, PD, PSP

[i] $p<0.05$ vs. Con

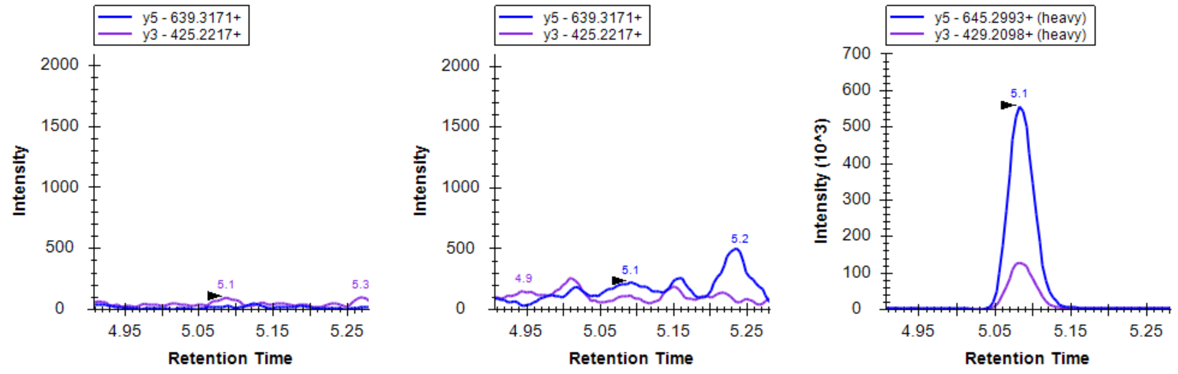
[j] $p<0.05$ vs. PD, PSP; $p<0.001$ vs.Con

[k] $p<0.01$ vs.Con, PSP; $p<0.001$ vs. PD

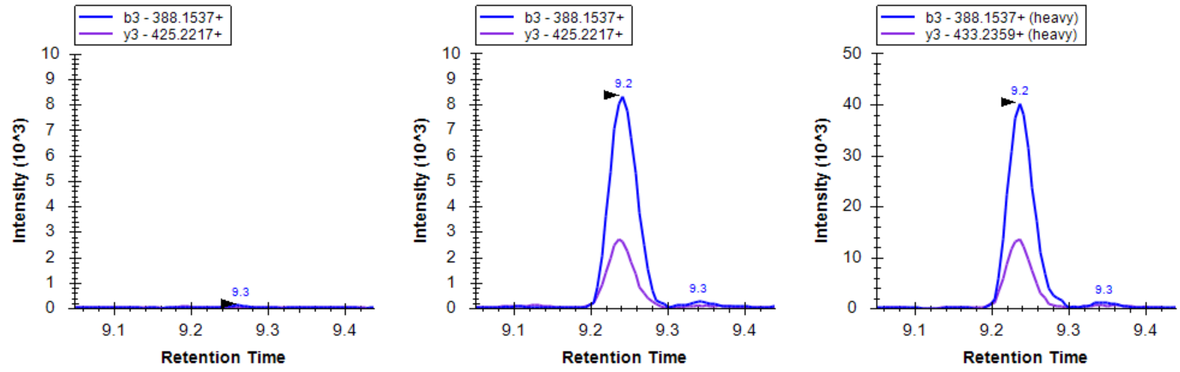
[l] $p<0.05$ vs. PDD; $p<0.01$ vs. Con; $p<0.001$ vs. PD, PSP

Supplement figure 1

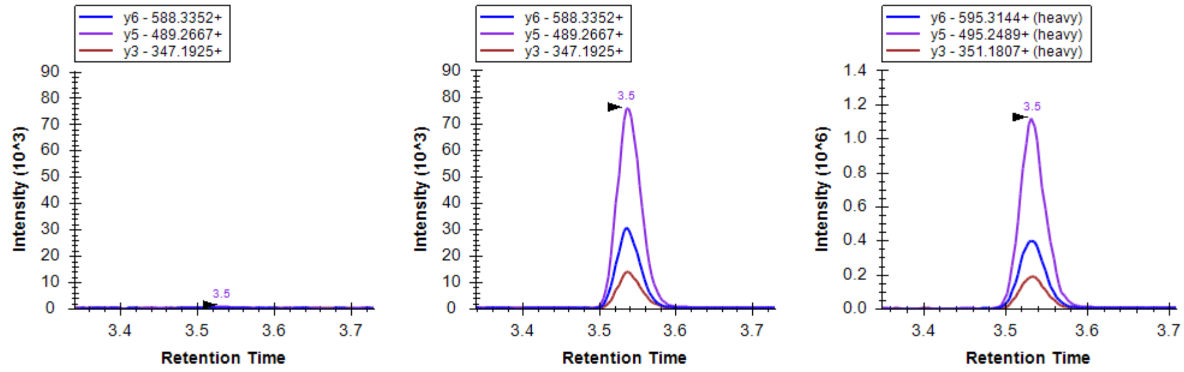
α/βSyn1-6 (MDVFMK)



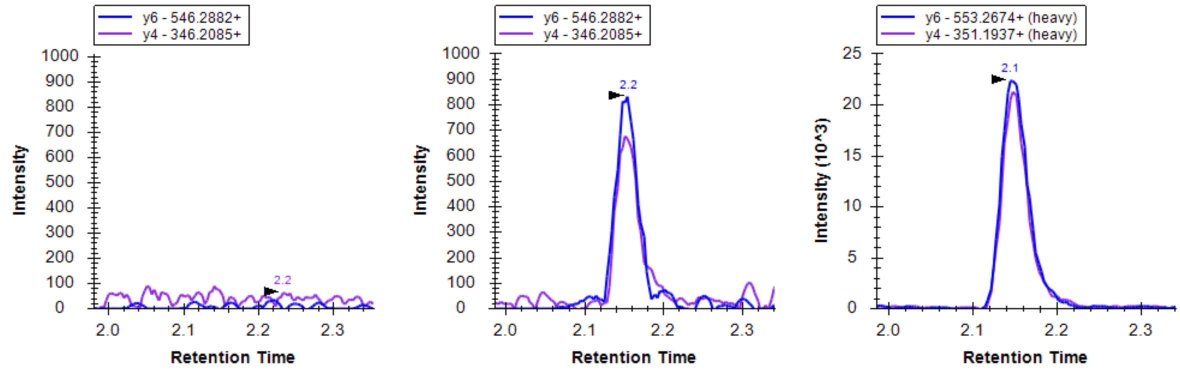
α/βSyn1-6 (Ac-MDVFMK)



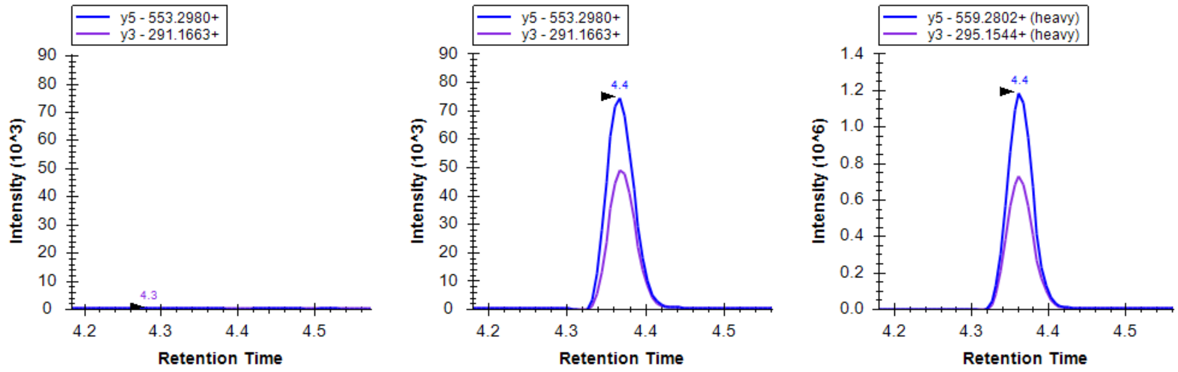
α/βSyn13-21 (EGVVAAAEK)



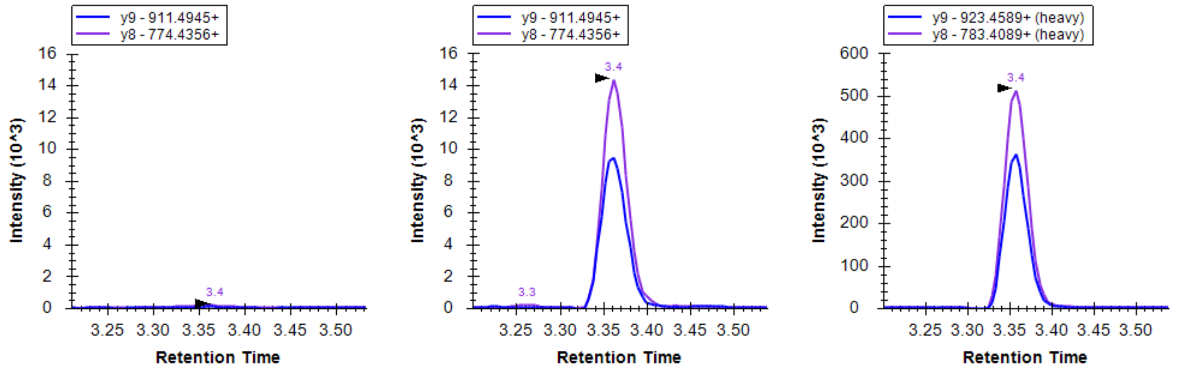
αSyn24-32 (QGVAEAAGK)



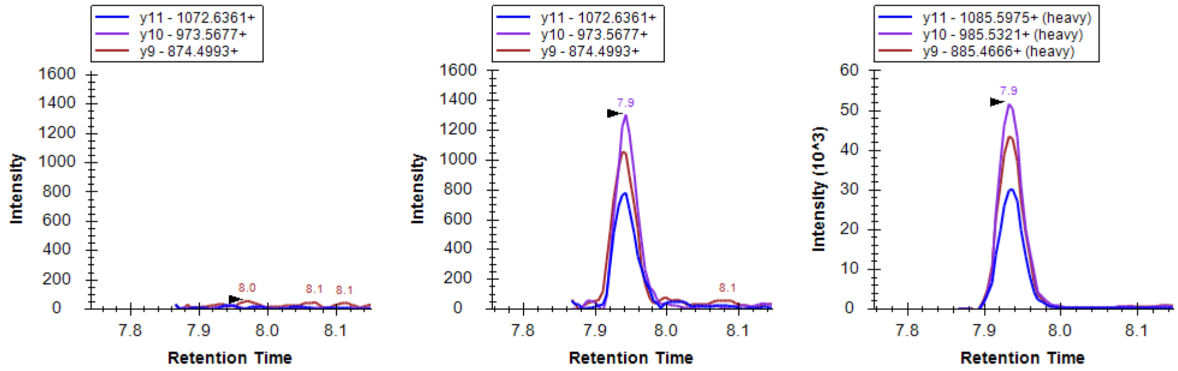
α/βSyn35-43 (EGVLYVGSK)



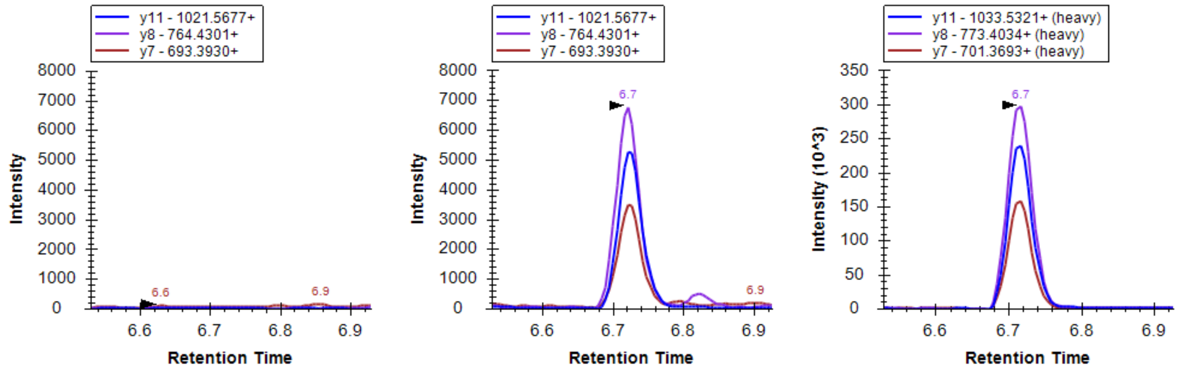
αSyn46-58 (EGVVHGVATVAEK)



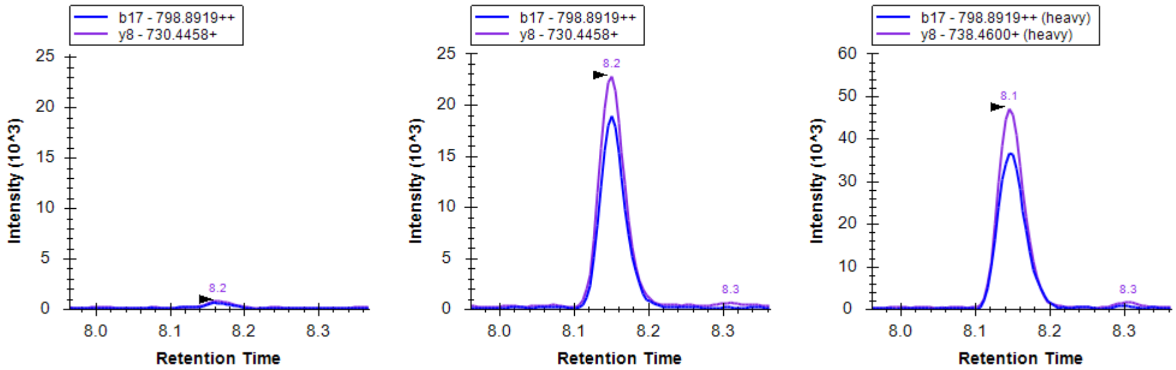
αSyn61-80 (EQVTNVGGAVVTGVTAVAQK)



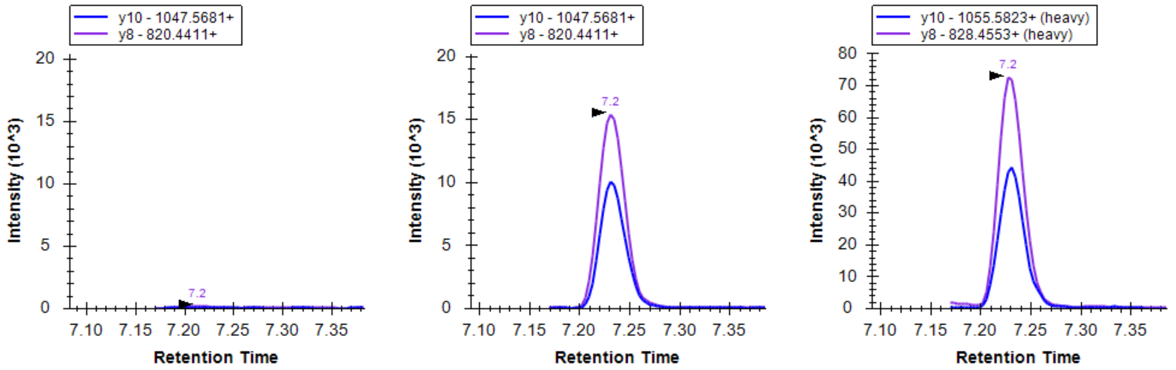
αSyn81-96 (TVEGAGSIAAATGFVK)



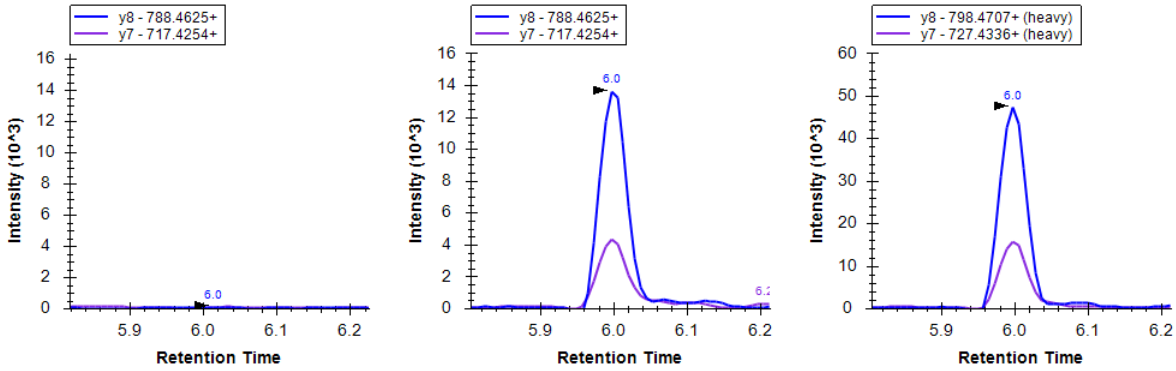
βSyn61-85 (EQASHLGGAVFSGAGNIAAATGLVK)



γSyn46-58 (ENVVQSVTSVAEK)



γSyn81-96 (TVEEAENIAVTSGVVR)



Supplement figure 1: Representative chromatograms of synuclein peptides. Chromatograms of peptides that were quantified in CSF in the study are shown. Peptides below the LOD in CSF are not included. The three chromatograms for each peptide are (1) blank sample (aCSF+HSA), (2) CSF sample and (3) heavy-labeled peptide spiked into CSF sample. Chromatograms were extracted from Skyline software 3.1.