Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information:

Complete identification of all 20 relevant epimeric peptides in β-amyloid: a new HPLC-MS based analytical strategy for Alzheimer's research

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1. Materials and sample preparation

All tryptic β-amyloid (Aβ) peptide standards were purchased from Peptide 2.0 (Chantilly, VA, USA) at > 98% purity. Ammonium formate and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-MS grade methanol and water were purchased from Sigma-Aldrich and ultrapure water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All the peptide standards were prepared in methonal and water (50:50) at the concentration of 1 mg/mL.

2. Optimization of tryptic Aβ peptide epimers separation by HPLC

2.1 Retention and separation characteristics of tryptic Aβ peptide epimers

Initial screening work for the separation and characterization of Aβ peptide standards was performed on a 1220 Infinity II HPLC instrument (Agilent Technologies, Santa Clara, CA, US). Among all the stationary phases screened, three chiral stationary phases have shown promising results: modified Q-Shell-1, modified Q-Shell-2, and NicoShell chiral stationary phases which were provided by AZYP, LLC (Arlington, TX, USA). Thus, the separation conditions of Aβ peptide epimers were further optimized on these chiral stationary phases.

2.1.1 Effect of organic modifier content on retention behavior of tryptic Aβ peptide epimers

Typical U-shaped retention curves were obtained for the tryptic Aβ peptide epimers (**Figs. S1-3**). Peptides retained long at the high aqueous and high organic modifier content. The lowest retention time was typically around 50/50 organic/aqueous condition, see **Figs. S1** & **S3**. For Group B, however, the lowest retention was achieved at 30% organic modifier content (**Fig.**

S2). In addition, higher than 60% organic modifier content caused solubility issue for Group B peptides. It has been studied that the solubility of peptides contributes to the longer retention at higher organic content [1,2].

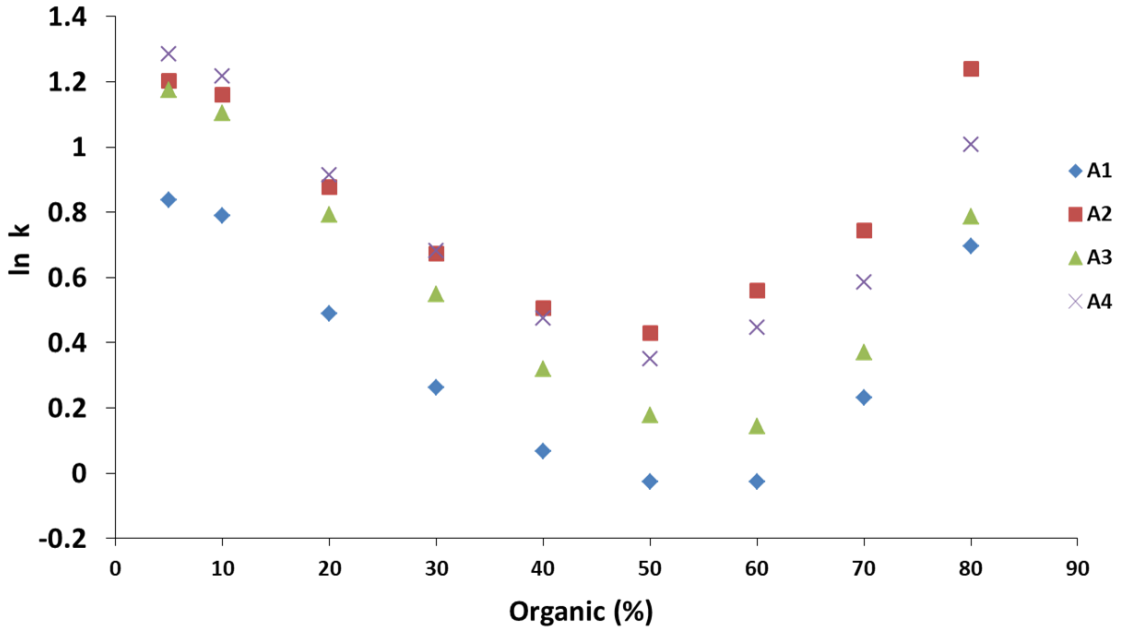


Fig S1. Retention behavior characteristics of Group A Aβ (1-5) peptides. Chromatographic conditions were as follows: modified Q-Shell-1 (3 x 150 mm), methanol/5 mM ammonium formate (pH 3.5), 0.3 mL/min, 25 °C, UV: 230 nm.

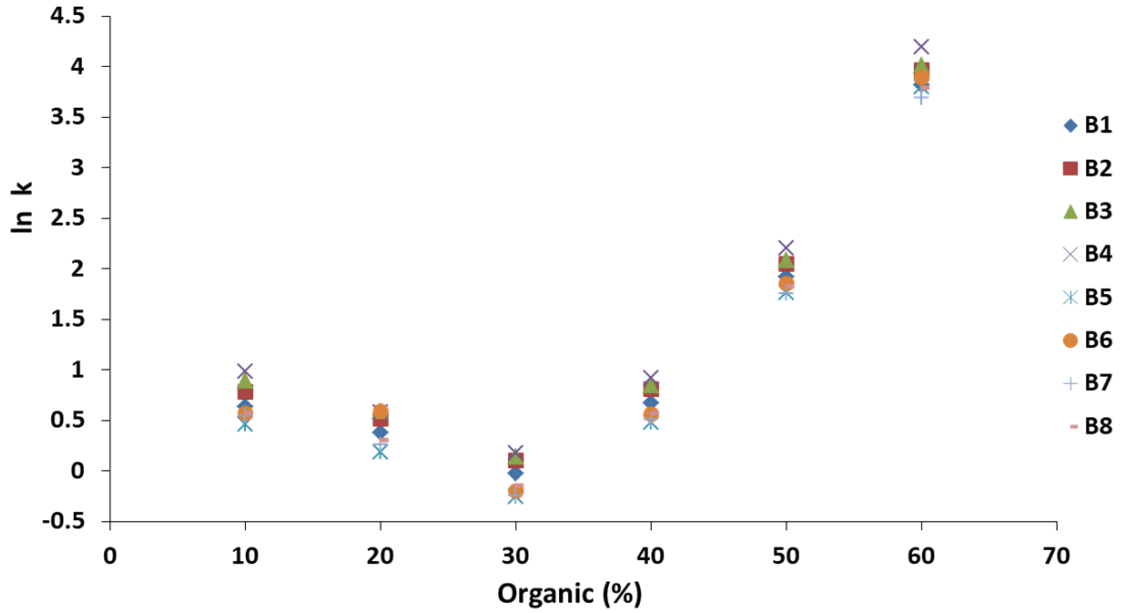


Fig S2. Retention behavior characteristics of Group B Aβ (6-16) peptides. Chromatographic conditions were as follows: NicoShell (2 x 100 mm), acetonitrile/50 mM ammonium formate (pH 3.5), 0.3 mL/min, 40 °C, UV: 230 nm

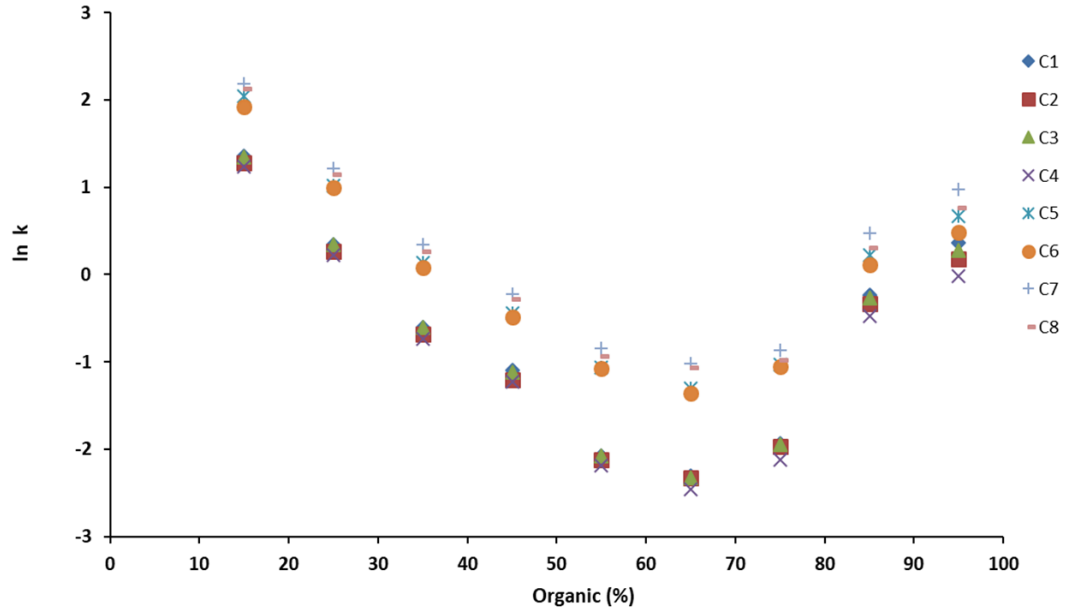


Fig S3. Retention behavior characteristics of Aβ (17-28) Group C peptides. Chromatographic conditions were as follows: NicoShell (3x 150 mm), methanol/5 mM ammonium formate (pH 3.0), 0.3 mL/min, 25 °C, UV: 230 nm

2.1.2 Effect of pH on retention behavior of tryptic Aβ peptide epimers

Tryptic Aβ peptides possess both basic and acidic amino acid residues, and their protonation and deprotonation contribute to the charge state of the peptides. In addition, unlike C18 columns, the chiral stationary phases used in this study are also sensitive to pH. The change of pH alters not only the charge state of peptides but also the stationary phases. Thus, the effect of pH on retention behaviors of Aβ peptides needs to be evaluated.

For Group A and C peptides on modified Q-Shell stationary phases, retention time increased with increasing pH (**Fig. S4** & **6**). As pH was increased from 3.0 to 5.0, the carboxylic acid on the peptides side chain changed from pronated to deprotonated. The interaction between the side chains of the peptides and the stationary phase increased, resulting in increased retention time. For Group B peptides on NicoShell stationary phase (**Fig. S5**), changing pH from 3.0 to 5.0 deprotonated the carboxylic acid on both the side chains of the peptides and the stationary phase. Thus, the increased retention observed for Group B peptides resulted from the charge states changing of both the analytes and the stationary phase.

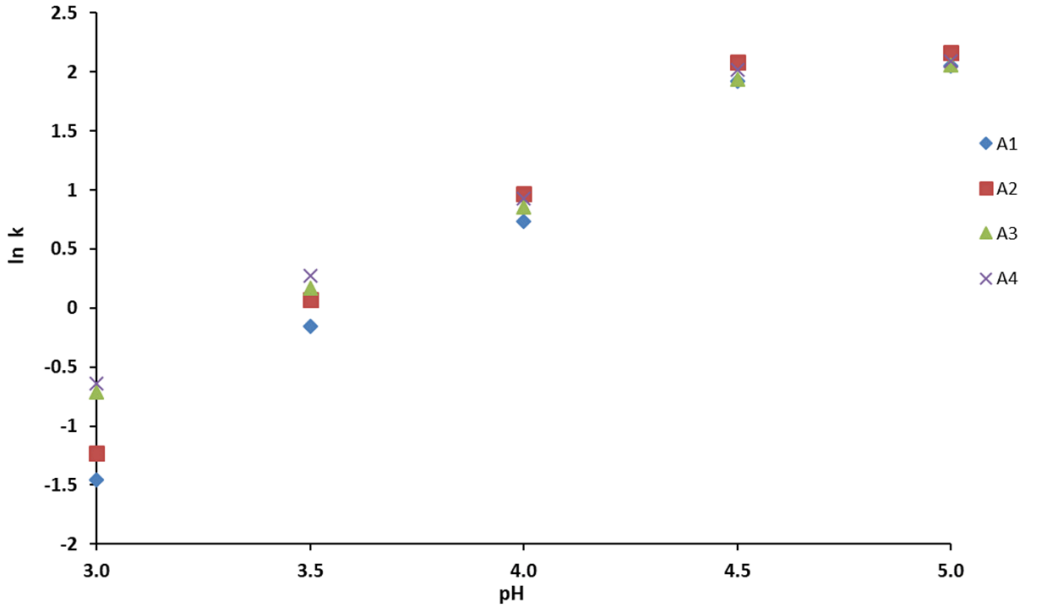


Fig S4. pH effect on retention and separation of Group A Aβ (1-5) peptides. Chromatographic conditions were as follows: modified Q-Shell-1 (3 x 150 mm), 10/90 methanol/25 mM ammonium formate, 0.3 mL/min, 25 °C, UV: 230 nm

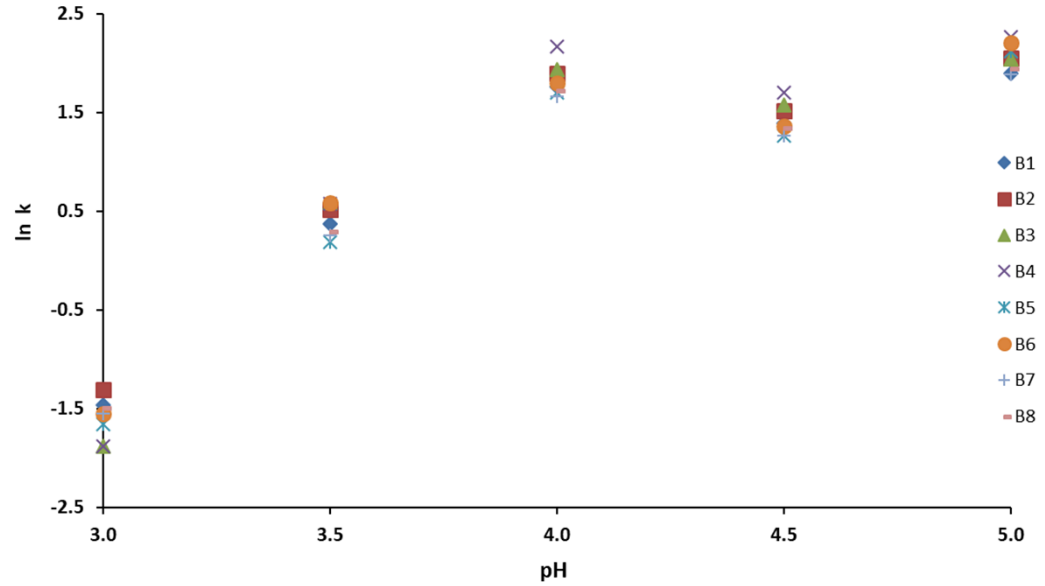


Fig S5. pH effect on retention and separation of Group B Aβ (6-16) peptides. Chromatographic conditions were as follows: NicoShell (2.1 x 100 mm), 20/80 acetonitrile/50 mM ammonium formate, 0.3 mL/min, 40°C, UV: 230 nm

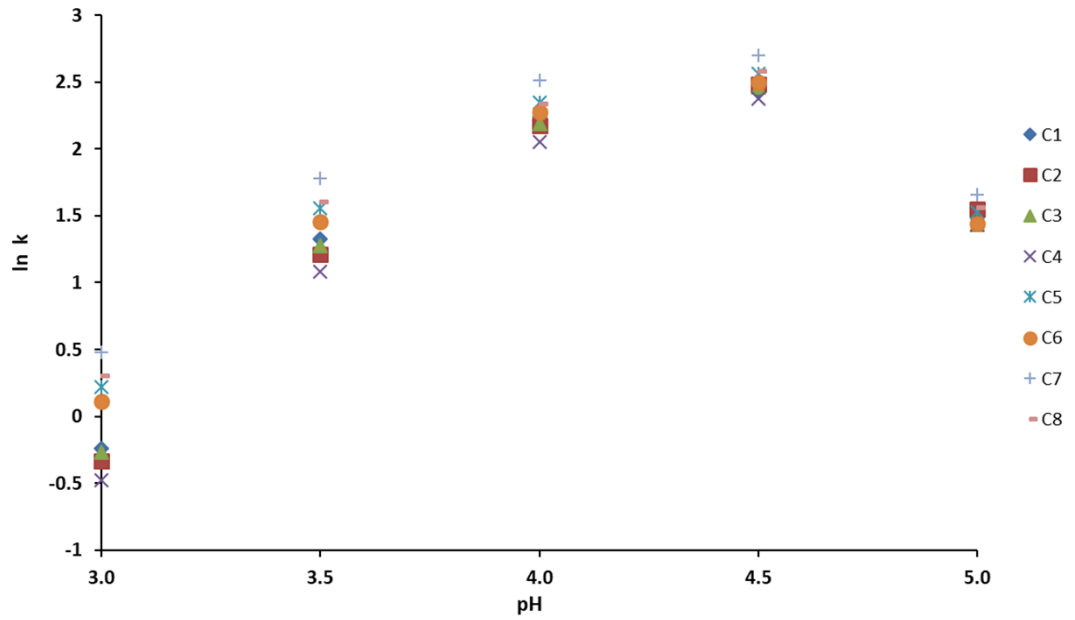


Fig S6. pH effect on retention and separation of Group C Aβ (17-28) peptides. Chromatographic conditions were as follows: modified Q-Shell-2 (3 x 150 mm), 85/15 methanol/5 mM ammonium formate, 0.3 mL/min, 25°C, UV: 230 nm

2.2 Optimized separation conditions

The optimized separation for Group A, B, and C peptide epimers are shown in **Figs.1-3**. For Group B and C peptide epimers, alternative separation methods were also developed, which have faster analysis time. Coeluting peaks, *i.e.,* B2 and B5, C1 and C3, can be easily separated on the same column at different mobile phase conditions. The alternative separation methods and conditions for Group B and C peptide epimers are shown in **Figs. S7** & **8**. Data (n=2) treated according to Wahab *et al.*[3]

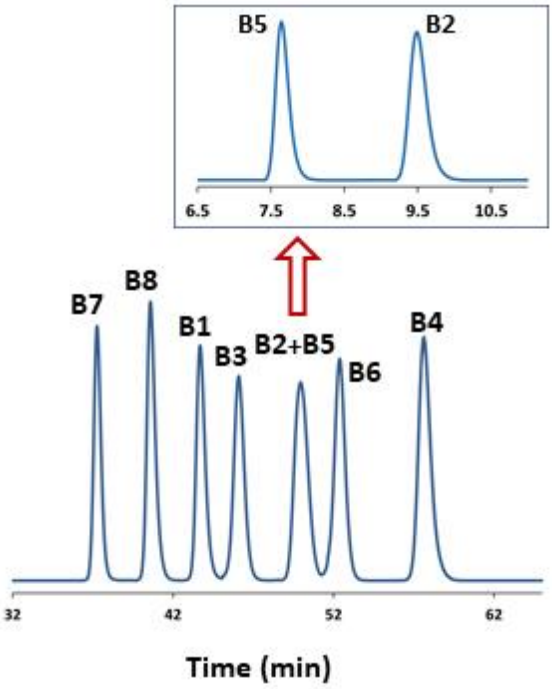


Fig. S7 Separation of Aβ (6-16) peptide epimers on NicoShell chiral stationary phase (3 x 150 mm, 2.7 µm). Condition: 35/65 acetonitrile/10 mM ammonium formate (pH 4.5), 0.4 mL/min, 40 °C. Separation condition for B2 and B5 on NicoShell chiral stationary phase: 25/75 methanol/20 mM ammonium formate (pH 3.5), 0.2 mL/min, 40 °C, UV:230 nm. The sequence of Aβ (6-16) is provided in Table 1.

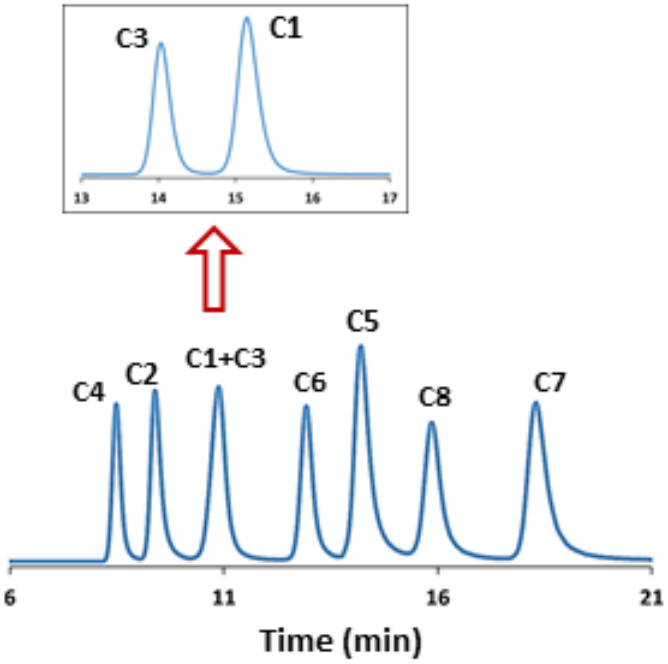


Fig. S8 Separation of Aβ (17-28) peptide epimers on modified Q-Shell chiral stationary phase (3 x 150 mm, 2.7 µm). Condition: 85/15 methanol/1 mM ammonium formate (pH 3.0), 0.2 mL/min, 10 °C. Separation condition for C1 and C3 on modified Q-Shell chiral stationary phase: 85/15 methanol/50 mM ammonium formate (pH 4.0), 0.3 mL/min, 10° C, UV: 230 nm. The sequence of Aβ (17-28) is provided in Table 1.

3. Improved detection sensitivity for Aβ peptides using HPLC-MS/MS

Separation method was successfully transferred from HPLC-UV to HPLC-MS/MS to improve the detection sensitivity. HPLC-MS/MS analysis was performed on LCMS-8060 (Shimadzu Scientific Instruments, Columbia, MD, USA), triple quadrupole spectrometer with electrospray ionization (ESI). The drying gas and nebulizing gas flow rate were 10 L/min and 2 L/min, respectively; the desolvation line temperature and heat block temperature were 275 °C and 400 °C, respectively. HPLC-MS/MS was operated in multiple reaction monitoring (MRM) mode with positive ESI source. Peptide fragmentation ions were further confirmed on a high-resolution MS, LCMS-9030 Q-TOF (Shimadzu Scientific Instruments), quadruple time-of-flight mass spectrometer with ESI. MS/MS spectrum for all 20 peptides were shown in **Figs. S9-11**. Collision energies and MRM transitions were optimized for each peptide group on LCMS-8060, results are summarized in **Table S1**. Limit of detections (LODs) were determined for the most sensitive fragment at S/N =3, and a second fragment was used to confirm peak identity, shown in **Table S1**. Shimadzu LabSolution software was used for data acquisition.

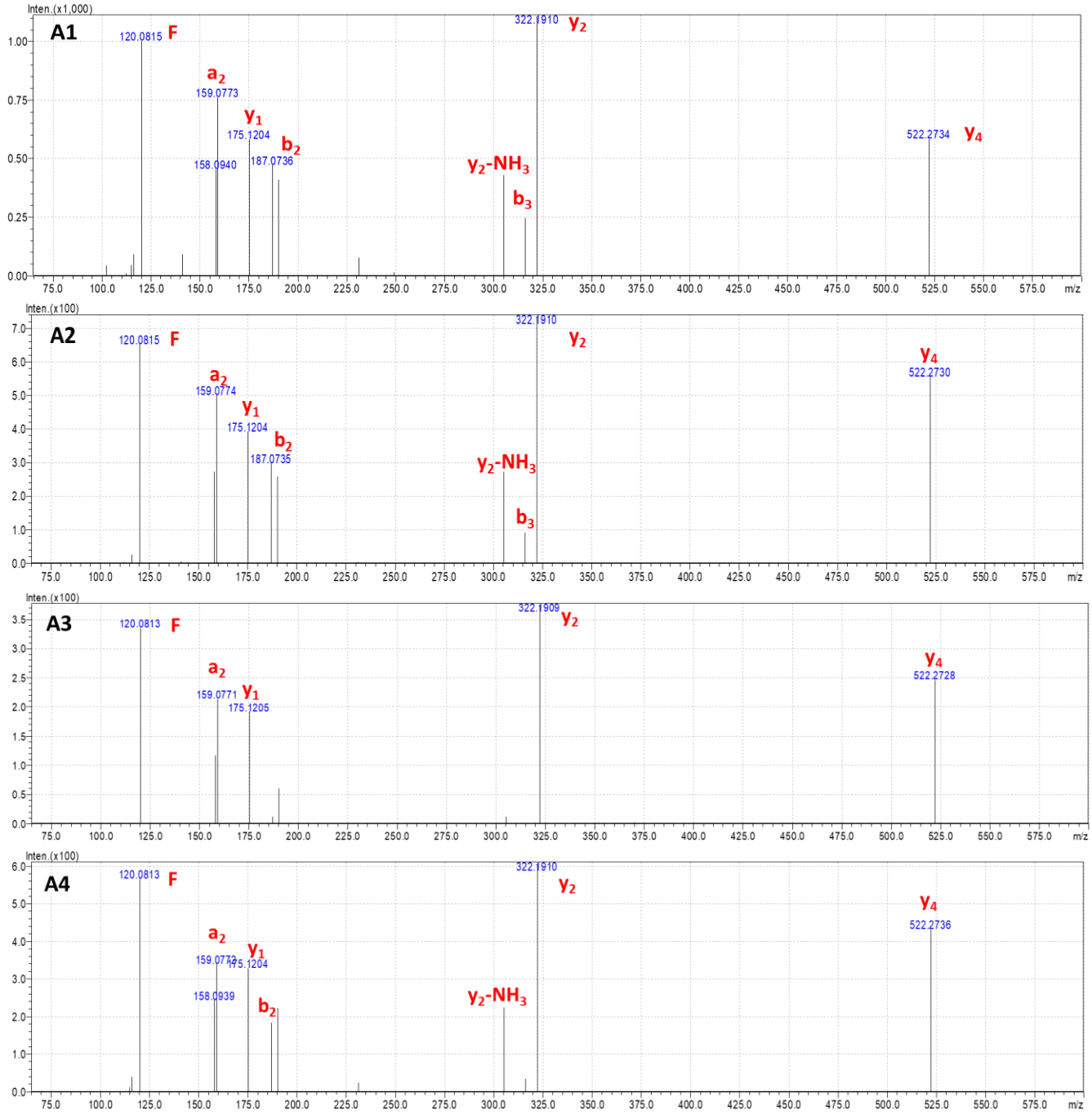
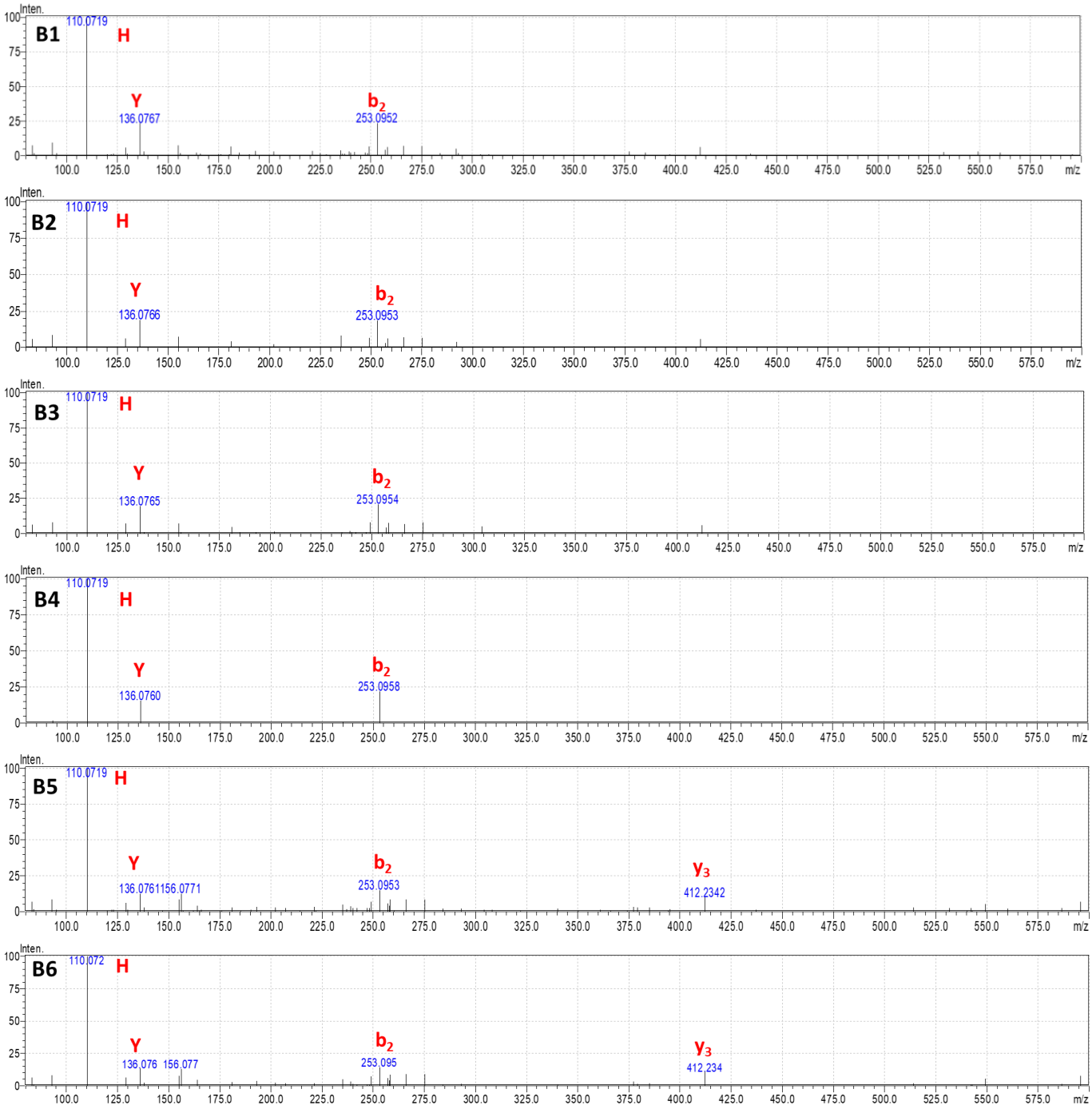


Fig. S9 MS/MS spectra for Group A peptides (A1-A4) obtained from LCMS-9030 Q-TOF to confirm the exact mass of each fragment and peak identity.


Inten.
B1
110.0719
H
Y
136.0767
b2
253.0952
B2
110.0719
H
Y
136.0766
b2
253.0953
B3
110.0719
H
Y
136.0765
b2
253.0954
B4
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b2
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b2
253.095
y3
412.234
m/z

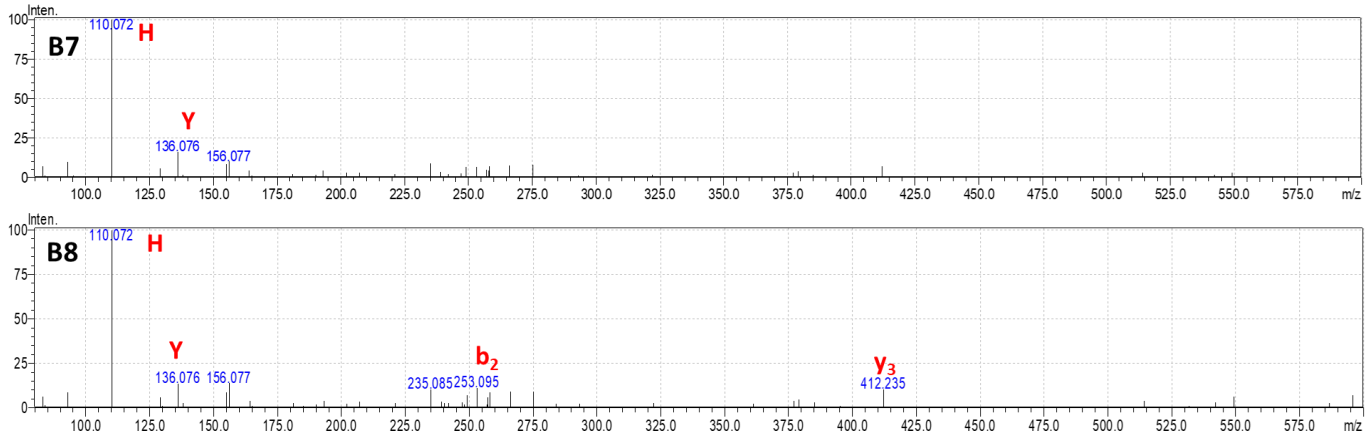
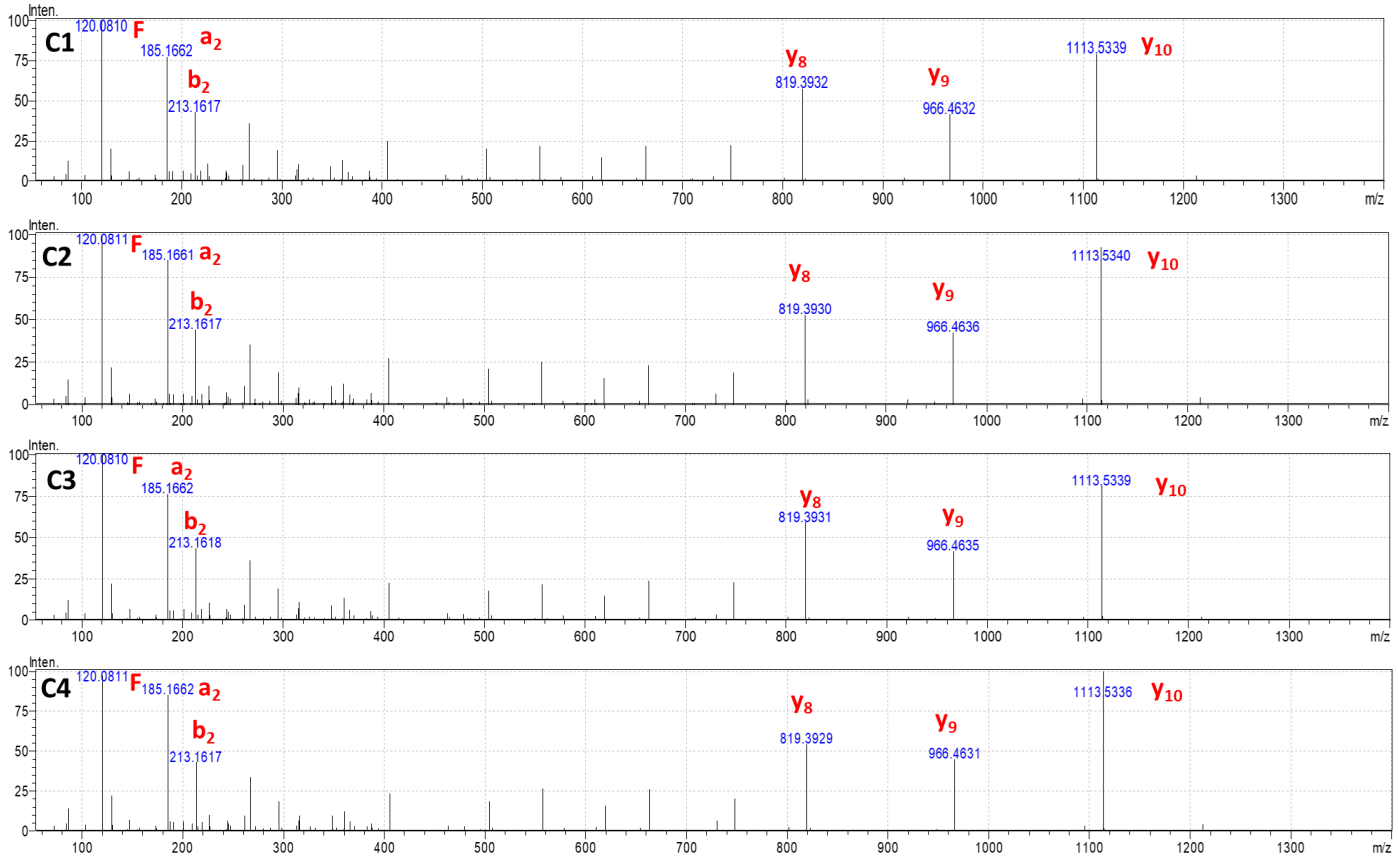


Fig. S10 MS/MS spectra for Group B peptides (B1-B8) obtained from LCMS-9030 Q-TOF to confirm the exact mass of each fragment and peak identity.



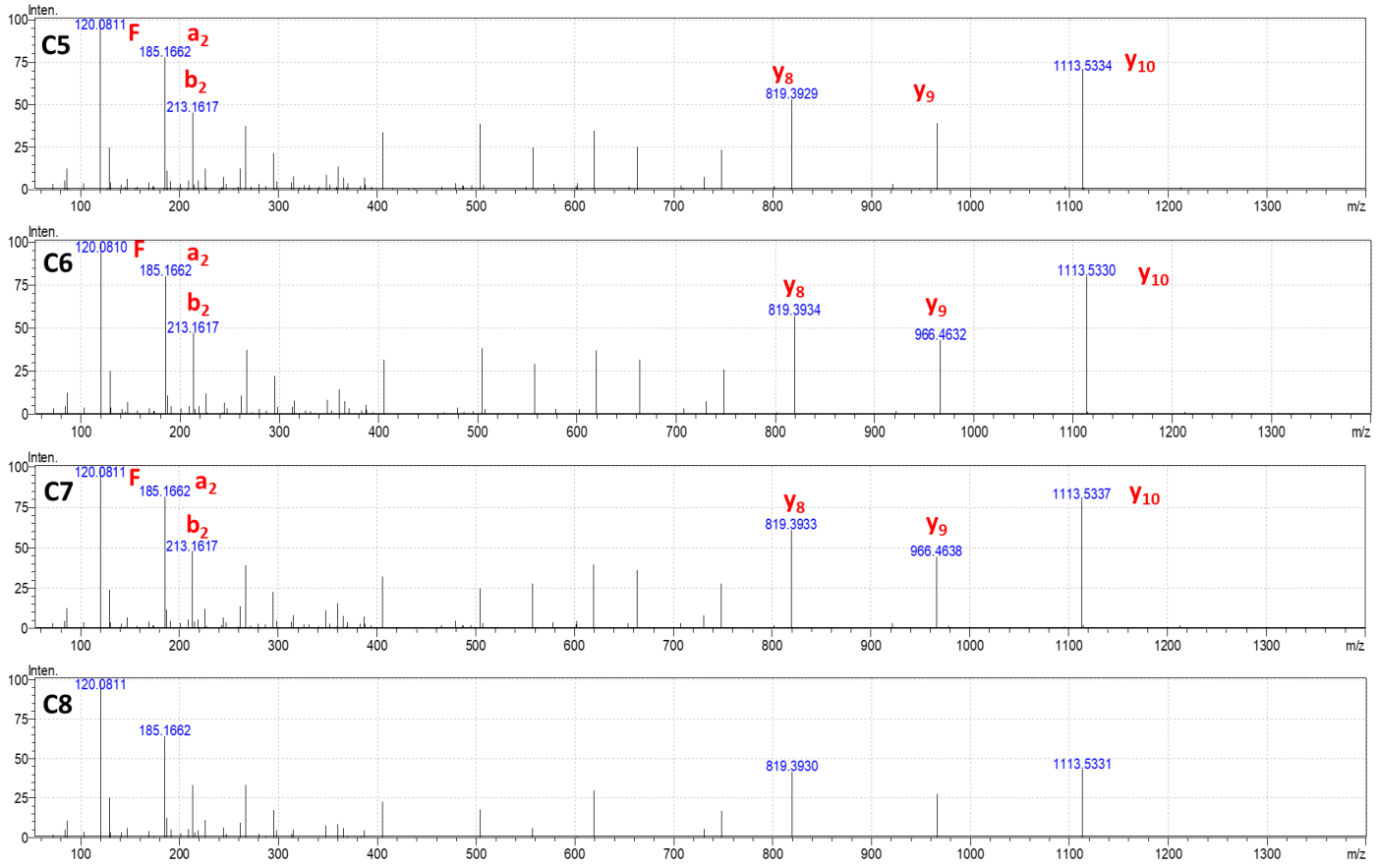


Fig. S11 MS/MS spectra for Group C peptides (C1-C8) obtained from LCMS-9030 Q-TOF to confirm the exact mass of each fragment and peak identity.

Table S1. Results of MRM optimization and LODs for tryptic Aβ peptides on Shimadzu LCMS-8060

	Precursor (m/z)	Product (m/z)	Q1 (V)	CE	Q3 (V)	LODs (pg)
Group A Aβ (1-5)	637.5 (M^{+1})	322.2(y_2 ion)	-32	32	-15	40
	637.5 (M^{+1})	522.0 (y_4 ion)	-32	31	-26	-
Group B Aβ (6-16)	669 (M^{+2})	110.2 (H ion)	-32	48	-19	250
	669 (M^{+2})	253. 3 (b_2 ion)	-32	27	-24	-
Group C Aβ (17-28)	663.5 (M^{+2})	1113.3 (y_{10} ion)	-32	24	-32	55
	663.5 (M^{+2})	185.4 (a_2 ion)	-32	23	-18	-

REFERENCES:

1. R. S. Blanquet, K. H. Bui and D. W. Armstrong, *J. Liq. Chromatogr.*, 1986, **9**, 1933-1949.
2. D. W. Armstrong and R. E. Boehm, *J. Chromatogr. Sci.*, 1984, **22**, 378-385.
3. M. F. Wahab, F. Gritti, T. C. O'Haver, G. Hellinghausen and D. W. Armstrong, *Chromatographia*, 2019, **82**, 211-220.