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Development of an SPE-LC/MS/MS Assay for the Simultaneous Quantification of Amyloid Beta Peptides in Cerebrospinal Fluid in Support of Alzheimer's Research

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Waters Corporation

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Waters Europe

Background: Amyloid β Peptides

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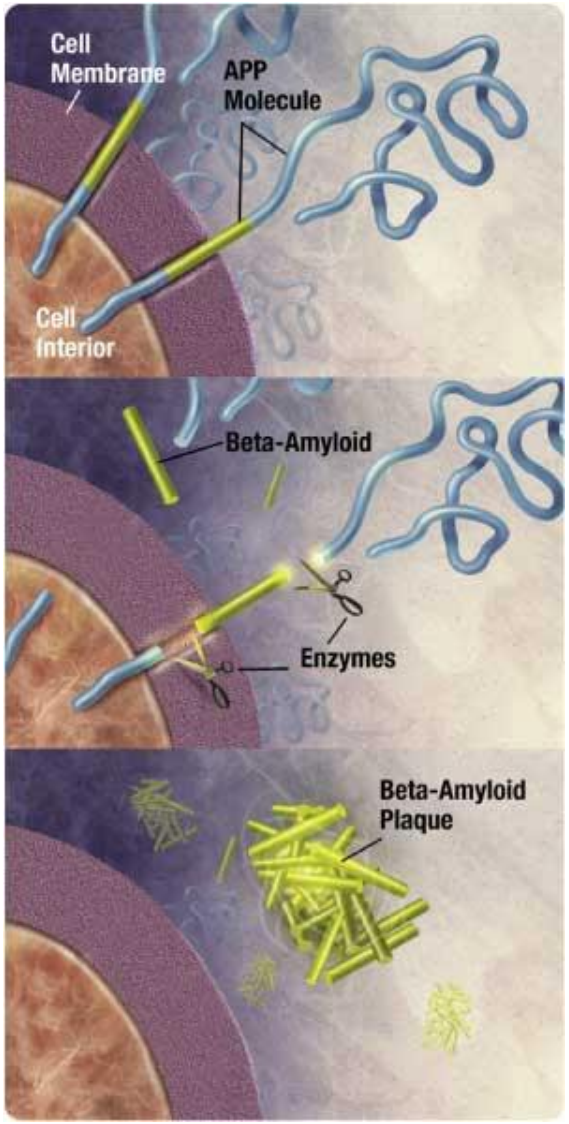
- Clinical significance of amyloid β peptides
  - Formation of aggregates/plaques in the brain is a critical event in Alzheimer's Disease
  - Drug development strategies aimed at lowering production of these peptides or enhancing their clearance
    - Inhibition/modulation of beta and gamma secretase enzyme
- Current analytical methodologies
  - ELISA- based assays
  - Immunoprecipitation (IP) followed by LCMSMS



Amyloid β Peptides for Quantification in CSF

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Amyloid β 1-38

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG

MW 4132, pI 5.2, HPLC index 96

Amyloid β 1-40

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

MW 4330, pI 5.2, HPLC index 103

Amyloid β 1-42

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

MW 4516, pI 5.2, HPLC index 117

Internal standards are N15 labeled versions of 1-38, 1-40, and 1-42

Waters Regulated Bioanalysis System Solution

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Sample Preparation Solutions

- Best in class

ACQUITY UPLC® I-Class

- The fastest and most resolving LC without compromise in robustness

ACQUITY UPLC Columns

- Different choices for best performance

® TQ-S

- Most sensitive MS

UNIFI

- Compliant, Interactive workflow-driven data platform

Service

- Installation, maintenance & training
- Compliance services

Outline

- Introduction
- Mass Spectrometry
- Liquid Chromatography
- Choice of Standard Curve Matrix
- Solid Phase Extraction
- Partial Validation

Why LCMSMS?

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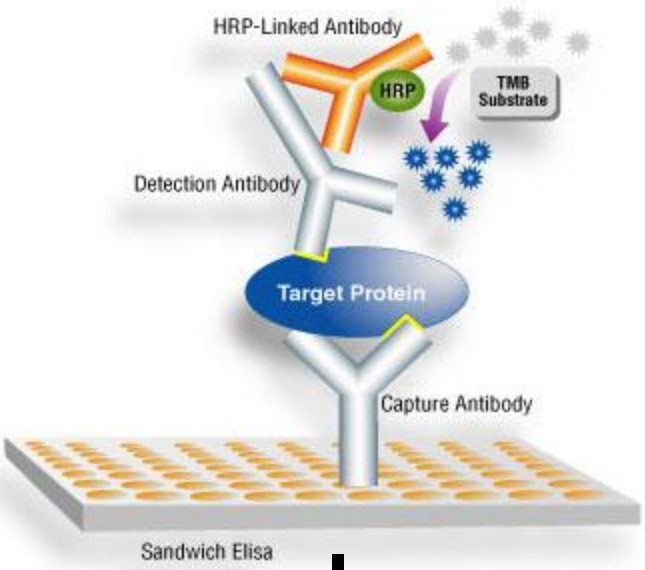
- Why an LCMSMS based assay?
  - ELISA assays not practical for discovery, no antibodies available yet
  - Challenges with ELISA assays
    - time consuming, expensive to develop
    - require separate assay for each peptide
    - limited linear dynamic range
    - Possible cross reactivity
- Benefits of LC/MS/MS for peptides
  - LCMSMS provides single assay for multiple amyloid peptides
  - Broad linear dynamic range
  - Accurate, precise
  - Universal
  - Faster, cheaper method development



Specific Challenges in Developing an LCMSMS Assay for Amyloid β Peptides

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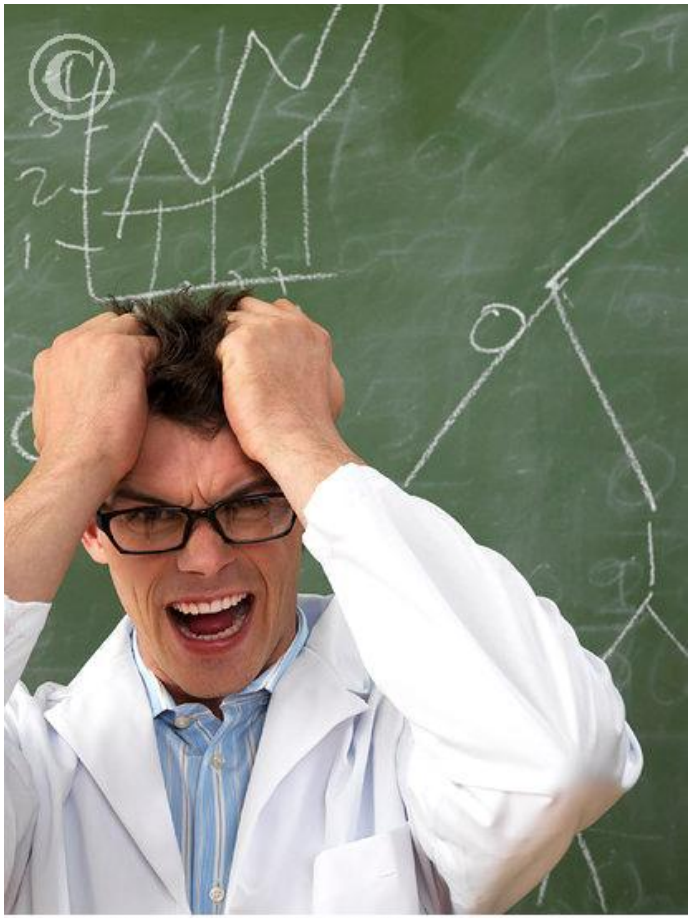
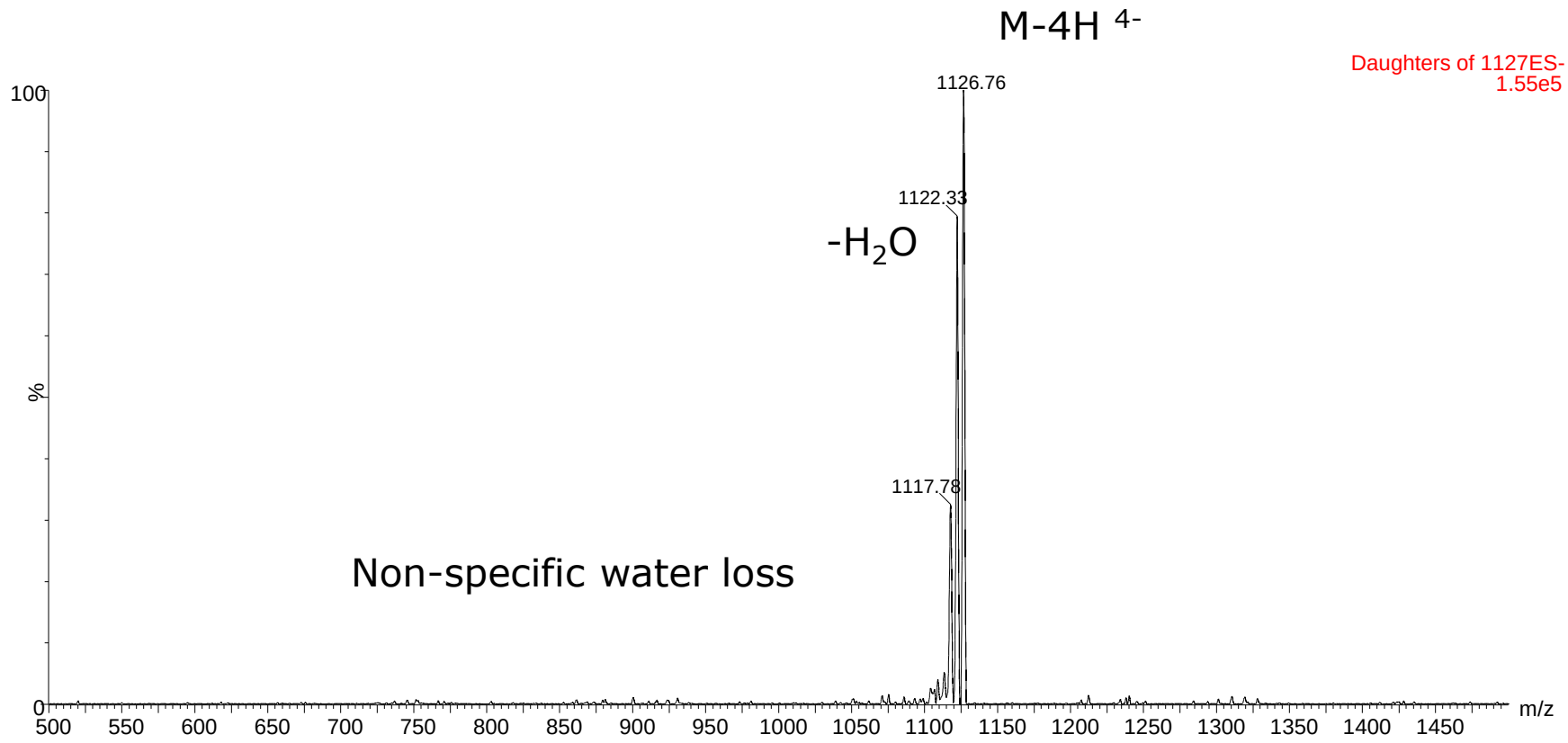
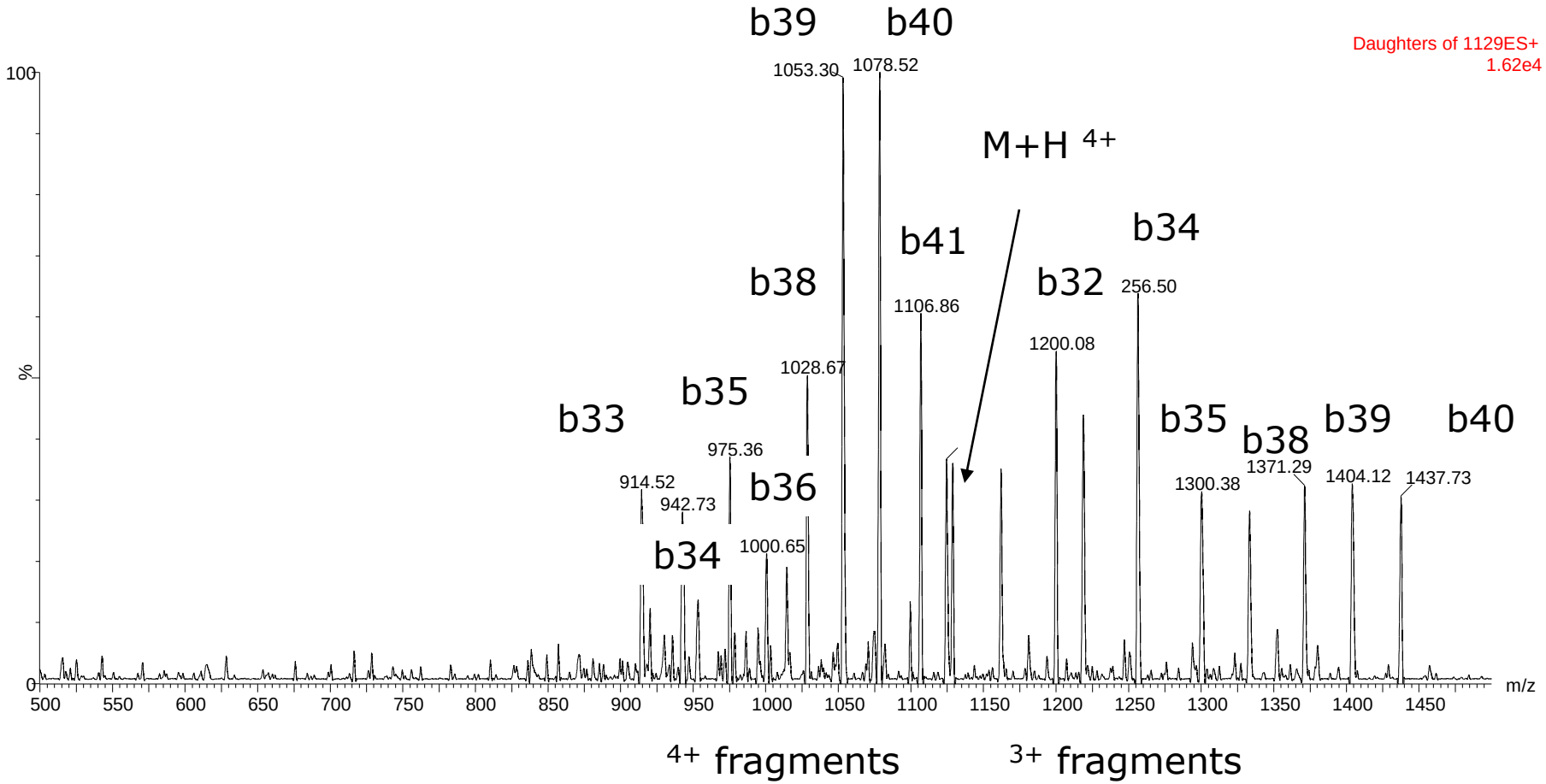
- Extremely hydrophobic
- Very poor peptide solubility
- High level of aggregation
- High level of non-specific binding (NSB)
- Low MS sensitivity
  - Very large and hydrophobic
  - Form many low abundance fragments
- Specificity in matrix



ESI- MSMS Spectra for Amyloid β 1-42



ESI+ MSMS Spectra for Amyloid β 1-42



Amyloid β Peptide 1-42: Identification of Fragments with BioLynx In Silico Fragmentation Model

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Proteins - B Amyloid 42.pep - [Sequence Ions]

File Edit Display Search Primary Secondary Window Help

a b c" x y" z

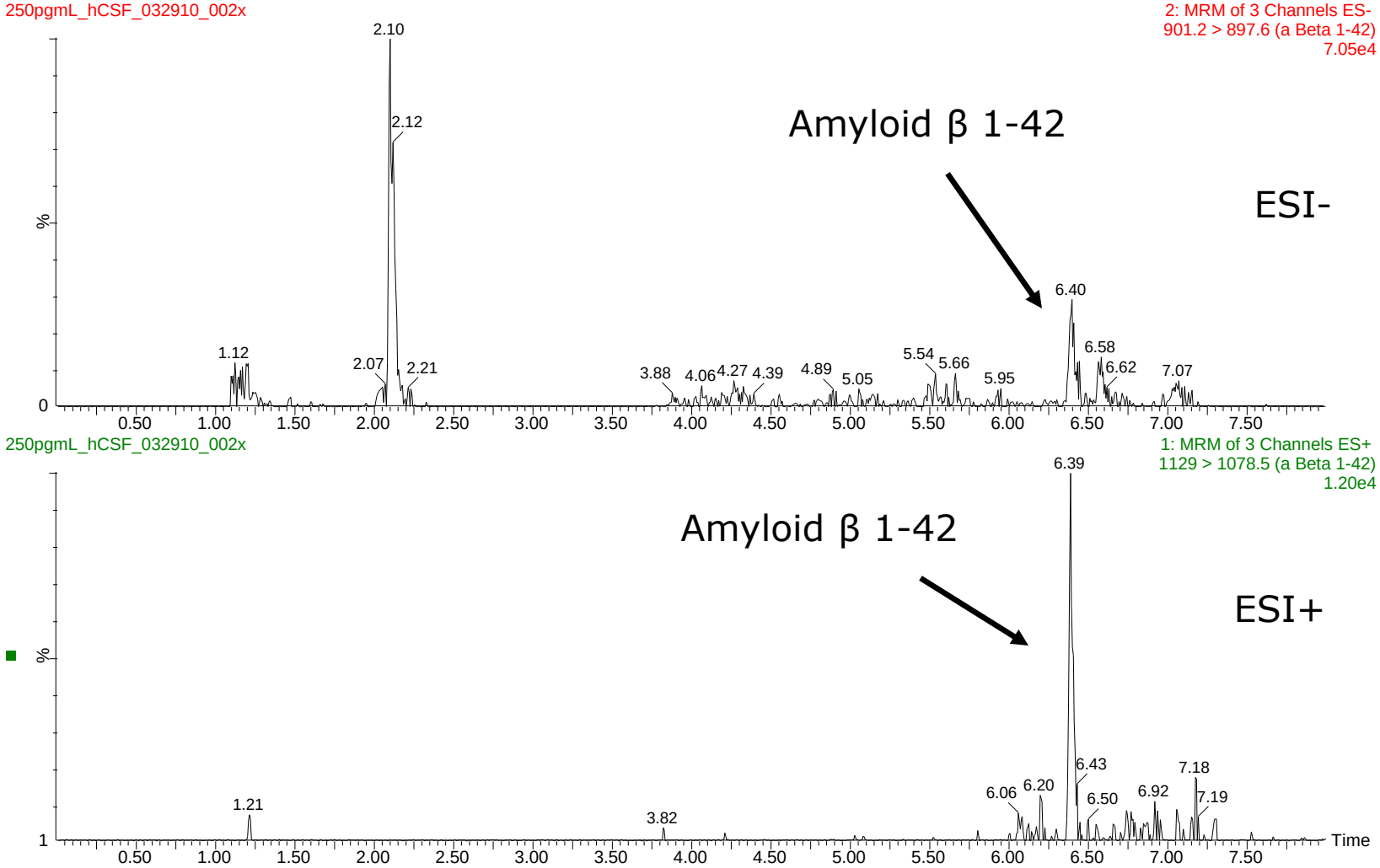
Fragment choice is 4+ b 40 ion

B Amyloid 42
Average Mass = 4513.1183, Monoisotopic Mass = 4510.2855
Residues: 1-42
N-Terminus = H, C-Terminus = NH2
Fragment ions: Monoisotopic/Average (1000) m/z ratios with 4 positive charge(s).

a	722.8	744.6	773.1	805.1	819.4	837.2	865.4	893.7	908.0	936.3	969.1	993.9	1008.1	1022.4	1047.2	1072.0	1100.3	-
b	729.8	751.6	780.1	812.1	826.4	844.2	872.5	900.7	915.0	943.3	976.1	1000.9	1015.1	1029.4	1054.2	1079.0	1107.3	-
c"	734.0	755.8	784.3	816.4	830.7	848.4	876.7	905.0	919.3	947.6	980.4	1005.1	1019.4	1033.7	1058.4	1083.2	1111.5	-
i	30.0	60.0	87.1	101.1	30.0	44.1	86.1	86.1	30.0	86.1	104.1	72.1	30.0	30.0	72.1	72.1	86.1	44.1
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	Met	Val	Gly	Gly	Val	Val	Ile	Ala
	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
x	421.3	407.0	385.2	356.7	324.7	310.4	292.6	264.3	235.9	221.6	193.4	160.6	135.8	121.6	107.3	82.6	57.8	29.5
y"	414.8	400.5	378.7	350.2	318.2	303.9	286.1	257.8	229.4	215.1	186.9	154.1	129.3	115.1	100.8	76.1	51.3	23.0
z	410.5	396.2	374.5	346.0	313.9	299.6	281.9	253.6	225.1	210.9	182.6	149.9	125.1	110.8	96.6	71.8	47.0	18.8

Chain 1 of 1

Positive Ion versus Negative Ion Detection: Specificity 1-42 in Human CSF



Positive Ion versus Negative Ion Detection: Specificity 1-38 in Human Plasma

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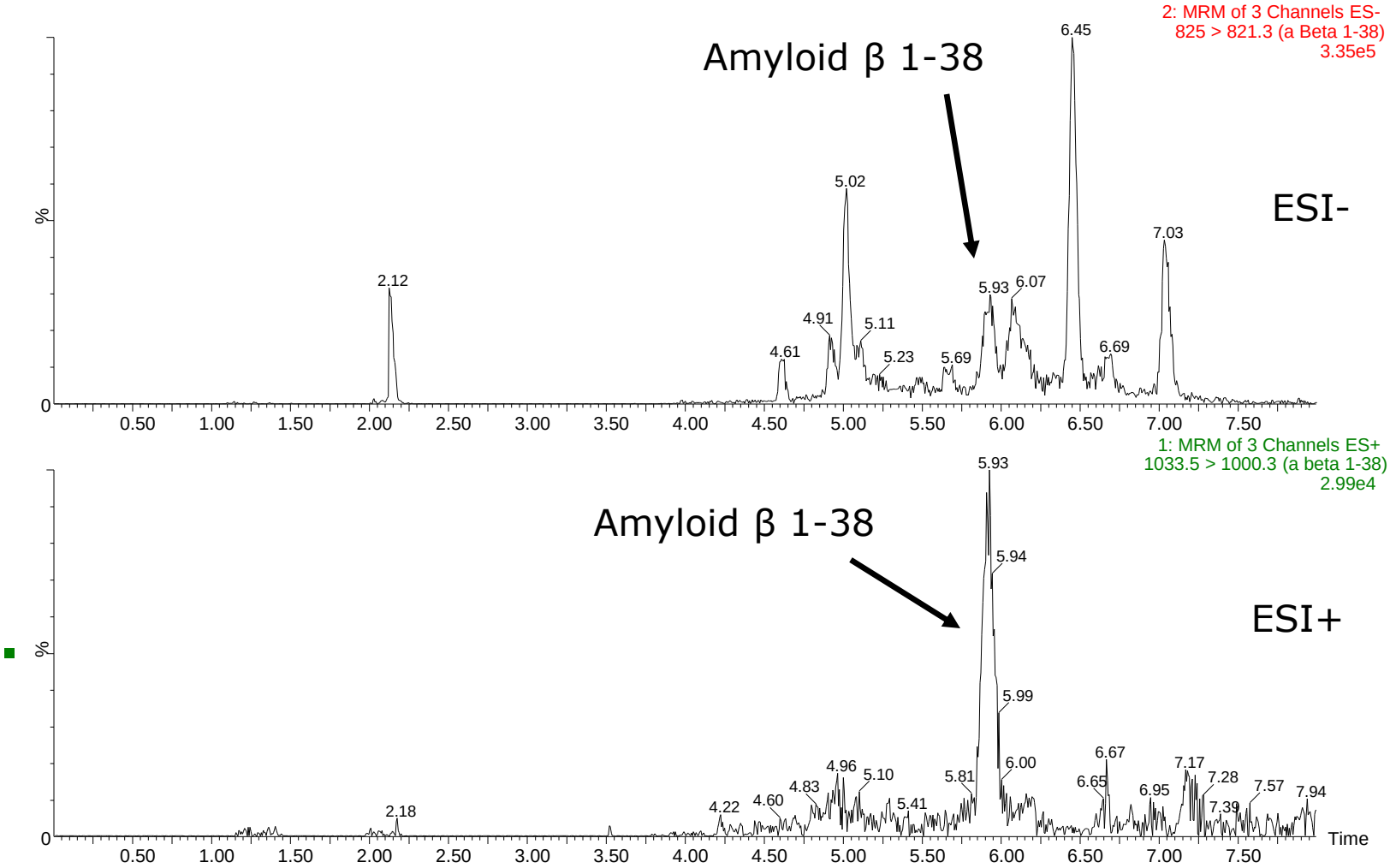
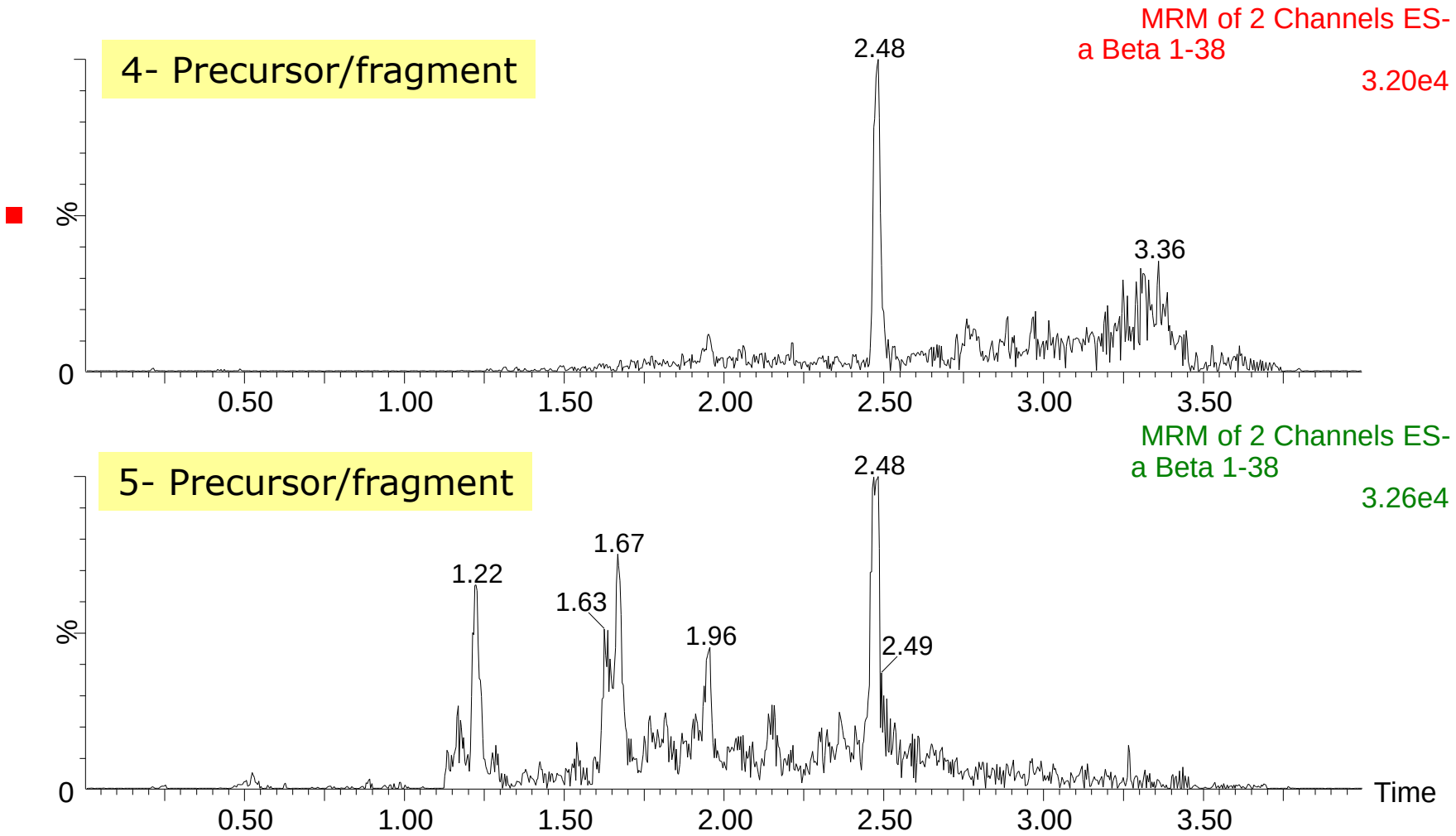


Effect of Using Higher m/z Precursor/Fragment: plasma



Xevo™ TQ-S MSMS Conditions

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Xevo™ TQ-S MS operated in ESI+ mode
MRM Transitions:

Peptide Name	Precursor Ion 4+	Product Ion 4+	Product Ion ID	Cone voltage (V)	Collision energy (eV)
Amyloid β 1-38	1033.5	1000.3	b 36	33	23
Amyloid β 1-38 N15 IS	1046	1012.5		30	22
Amyloid β 1-40	1083	1053.6	b 39	33	25
Amyloid β 1-40 N15 IS	1096	1066.5		35	22
Amyloid β 1-42	1129	1078.5	b 40	28	30
Amyloid β 1-42 N15 IS	1142.5	1091.5		35	28

Impact of MS Platform Change

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Solvent Standard Comparison:
Area counts for 500 pg/mL standard

	Xevo TQ	Xevo TQ-S
amyloid β 1-38	108	1468
amyloid β 1-40	133	1741
amyloid β 1-42	54	1589

Xevo TQ-S
LOD: 12.5 pg/mL solvent standard

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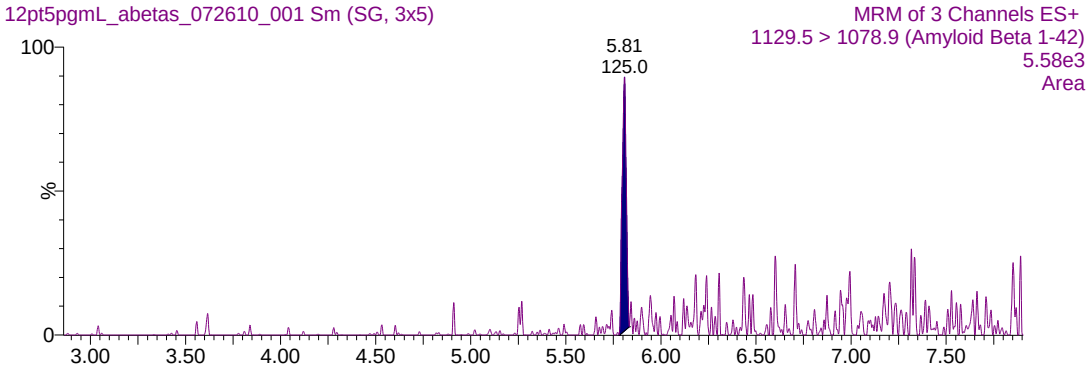
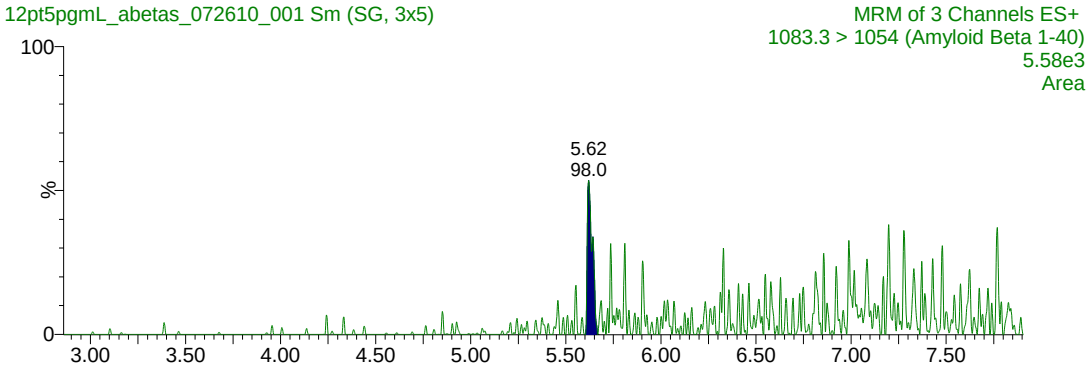
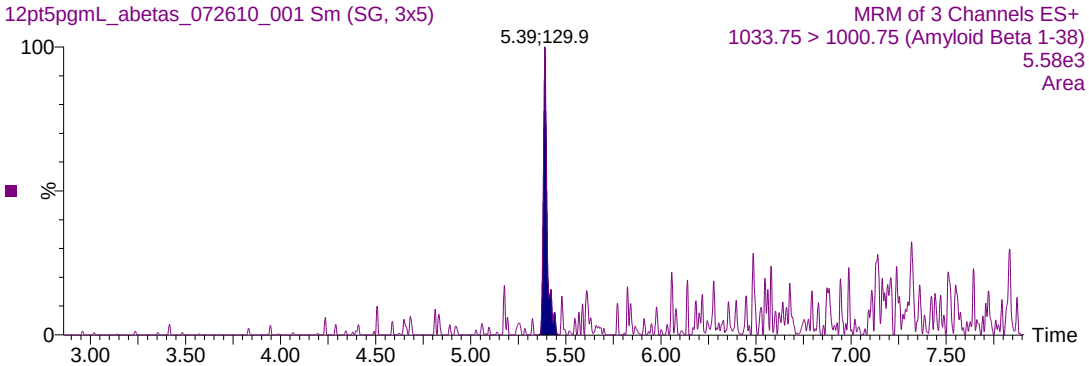
in 50/50 elution solvent/water



Amyloid β 1-42
Peak Area 125



Amyloid β 1-40
Peak Area 98



Amyloid β 1-38
Peak Area 130

10µL injection

Outline

- Introduction
- Mass Spectrometry
- Liquid Chromatography
- Choice of Standard Curve Matrix
- Solid Phase Extraction
- Partial Validation

UPLC® Conditions: Final Method

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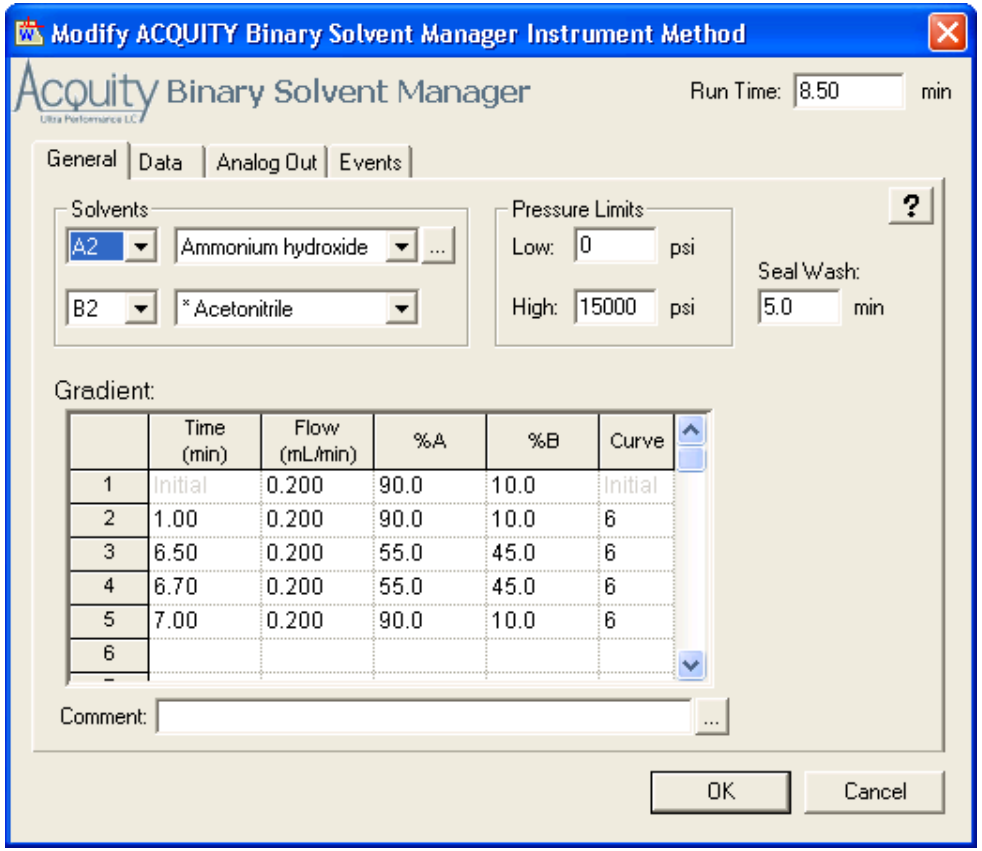
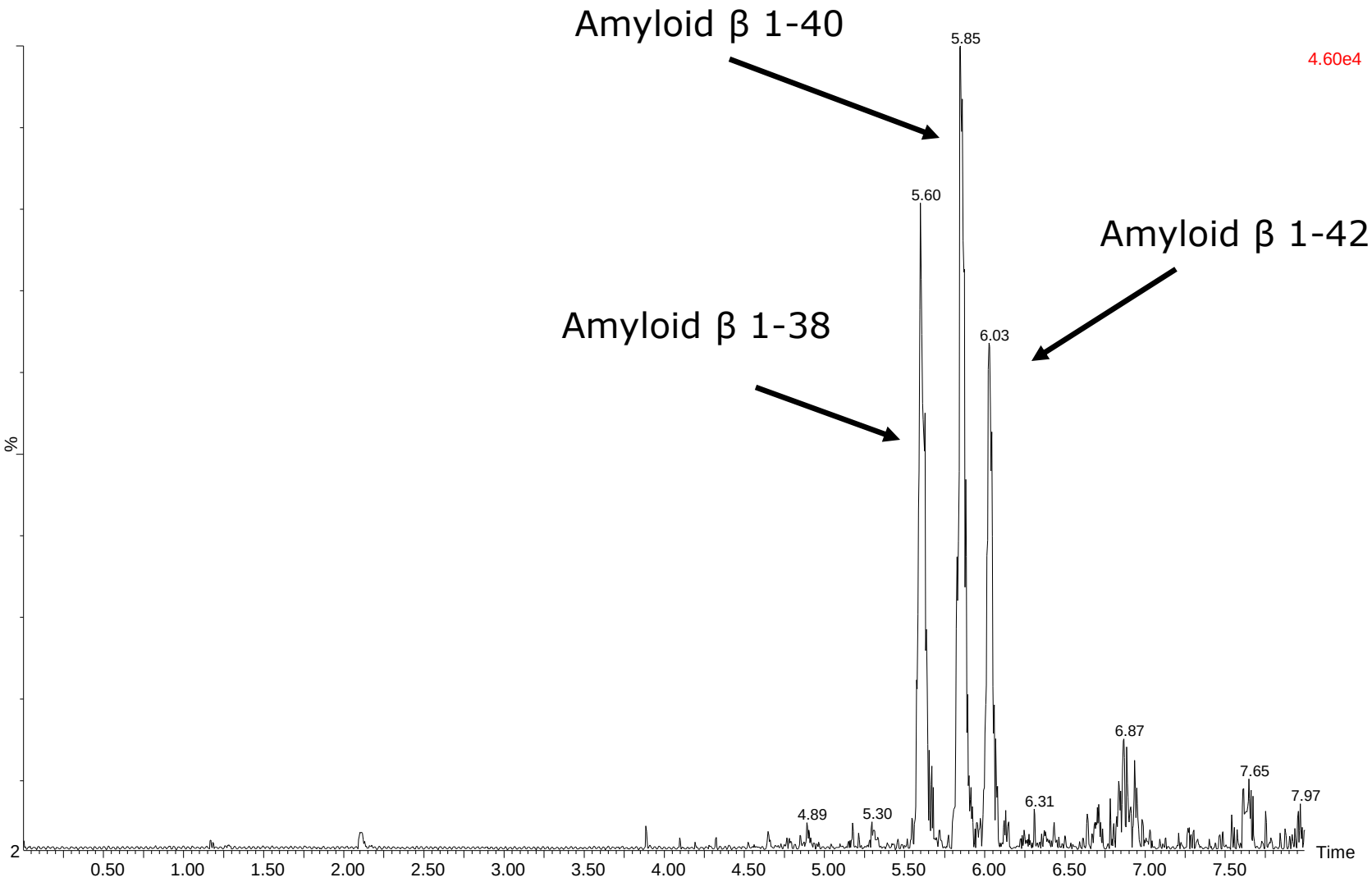
Column: 2.1 X 150mm, ACQUITY BEH C18 300Å, 1.7µm
Mobile phase A: 0.3% NH_4OH by volume, or 0.1% absolute
Mobile Phase B: 90/10 ACN/mobile phase A
Temperature: 50° C
SNW: 65/25/10 ACN/water/NH_4OH
SNW volume: 600 µL
WNW: 90/10 water/ACN + 0.3% NH_4OH
WNW volume: 600 µL
Flow rate: 0.2 mL/min
Injection mode: partial loop
Injection Volume: 10 µL
Injection Solvent for standards:
SPE elution solvent diluted with water;
SPE elution solvent =
75% ACN, 15% water, 10% NH_4OH

Gradient Table



	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.200	90.0	10.0	Initial
2	1.00	0.200	90.0	10.0	6
3	6.50	0.200	55.0	45.0	6
4	6.70	0.200	55.0	45.0	6
5	7.00	0.200	90.0	10.0	6
6					

Representative Chromatogram: Extracted Spiked Artificial CSF



Representative Chromatography: Extracted Human CSF Sample

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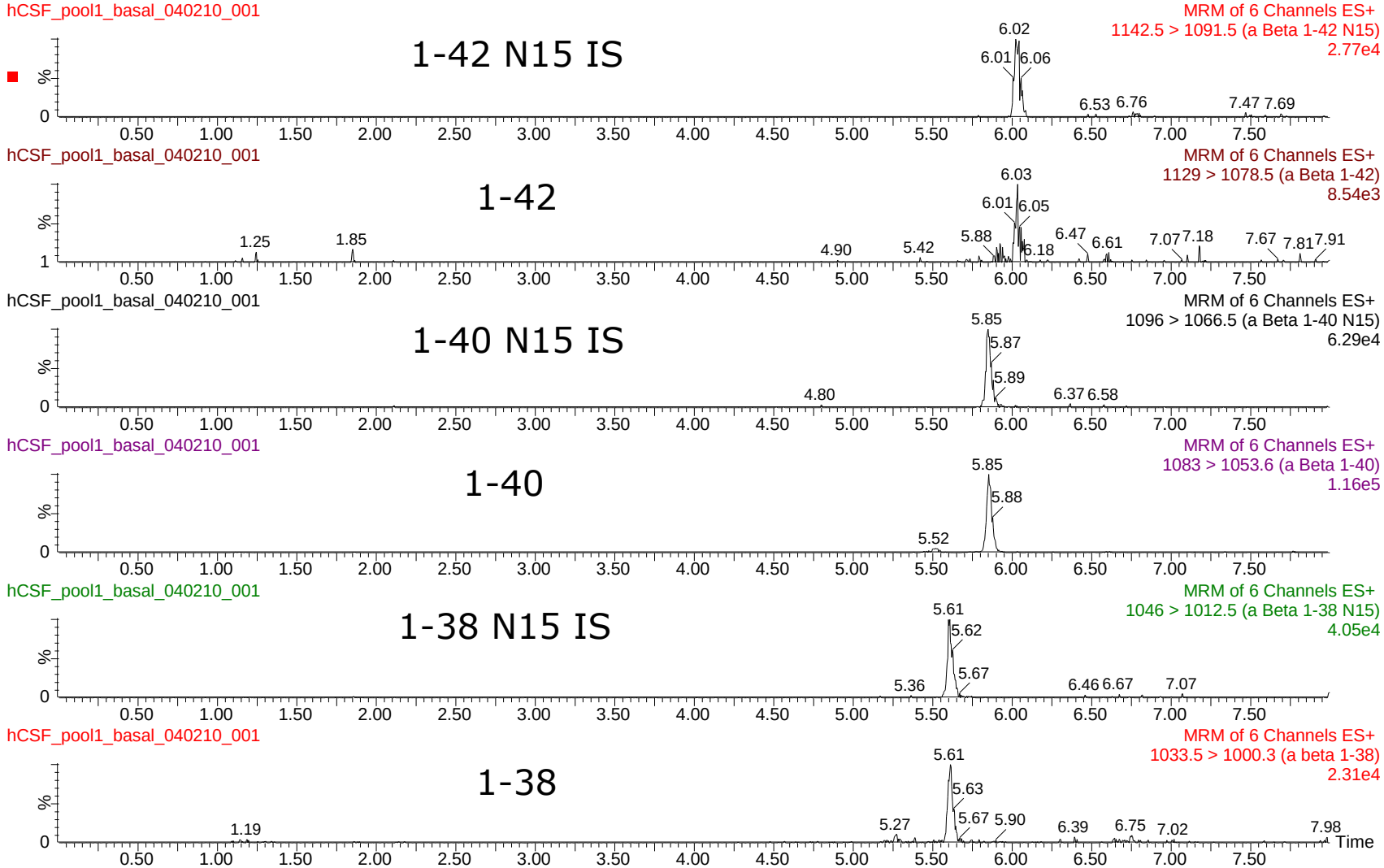
Basal Levels in Human CSF



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Average Basal Levels in Human CSF: Comparison of Results with Different Standard Curve Preparations

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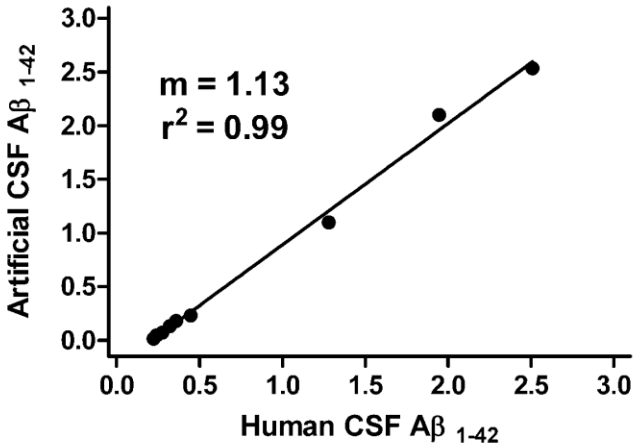
Standard curves prepared from either artificial CSF or by over-spiking human CSF

Amyloid β 1-38	Conc.(ng/mL) Source 1	Conc. (ng/mL) Source 2
Conc from curve in art CSF	0.82	0.826
Conc from curve in human CSF	0.679	n/a
average	0.750	
std deviation	0.100	
RSD	13.302	

Amyloid β 1-40	Conc.(ng/mL) Source 1	Conc. (ng/mL) Source 2
Conc from curve in art CSF	3.713	3.136
Conc from curve in human CSF	4.335	n/a
average	4.024	
std deviation	0.440	
RSD	10.930	

Amyloid β 1-42	Conc.(ng/mL) Source 1	Conc. (ng/mL) Source 2
Conc from curve in art CSF	1.105	0.763
Conc from curve in human CSF	1.189	0.804
average	1.147	0.784
std deviation	0.059	0.029
RSD	5.178	3.700



*Human CSF corrected for basal level

Outline

- Introduction
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- Liquid Chromatography
- Choice of Standard Curve Matrix
- → Solid Phase Extraction
- Partial Validation

Sample Extraction: Pretreatment

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Sample Pretreatment

Spike human or artificial CSF + 5% rat plasma samples, mix

Equilibrate samples at room temperature for 30 minutes

Remove 50 µL spiked artificial CSF (to which 5% rat plasma was added) for standard curves, or human/monkey CSF (basal level or over-spike QC's)

Add 50µL 5M guanidine HCl

Shake samples at room temperature for 45 minutes

Add 50µL 4% H_3PO_4 in water, mix

Note: 4X less sample is used than TQ MS method: 50 µL instead of 200 µL

Sample Extraction: Final Method

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Oasis® MCX µElution Plate

Amyloid β Peptide	% SPE Recovery
1-38	94%
1-40	92%
1-42	90%

Condition: 200 µL MeOH

Equilibrate: 200 µL 4% H_3PO_4

Load: 150 µL diluted sample (pretreated sample: 50 µL human CSF, 50µL 5M guanidine HCl, 50µL 4% H_3PO_4 in water)

Wash 1: 200 µL 4% H_3PO_4

Wash 2: 200 µL 10% ACN in water (by volume)

Elute: 2 X 25 µL 75/10/15 ACN/conc. NH_4OH/water (by volume)

Dilute: 25 µL water

Inject: 10 µL

Key Attributes for High Extraction Recovery

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- Wash with no more than 10% ACN
  - Removes polar interferences
  - Does not impact recovery of 1-38 (earliest eluting/least hydrophobic of these 3 amyloid β peptides)
- Elute with no less than 75% ACN
  - Provides required elutropic strength for 1-40 and 1-42 (the more hydrophobic of these 3 peptides)
- Elute with no less than 10% NH_4OH
  - Provides required *solubility* to fully elute 1-42 (least soluble/most hydrophobic of these 3 peptides)
- Addition of rat plasma to artificial CSF to eliminate non-specific binding increases SPE recovery in this matrix from 60% on average to >90%
- Guanidine HCl denaturation
  - Improves reproducibility of method; eliminates aggregation and protein binding

Impact of MS Platform: Standard Curve and QC Range Comparison

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	200 µL sample	50 µL sample
	Xevo TQ	**Xevo TQ-S**
Standard Curve Range	0.1 to 10 ng/mL	0.025 or 0.05 to 10 ng/mL
QC Range	0.2 to 6 ng/mL	0.04 to 6 ng/mL

Outline

- Introduction
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Summary of Samples Extracted

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- Standard curves prepared in artificial CSF + 5% Rat plasma
  - Calibration points are 0.025, 0.05, 0.1, 0.25, 0.35, 0.5, 0.75, 1, 5, 7.5, and 10 ng/mL
- Basal levels in human CSF
  - 3 individual sources pooled human CSF samples, 1 source cynomalgous monkey
  - 6 replicates from each source
- QC samples
  - Prepared in each of the human CSF matrices
  - 7 concentrations: 0.04, 0.075, 0.15, 0.2, 0.8, 2 and 6 ng/mL over-spike
  - N=3 for each concentration, from each source of CSF

Representative Chromatogram: Basal Levels of Amyloid β 1-38 in Human and Monkey CSF

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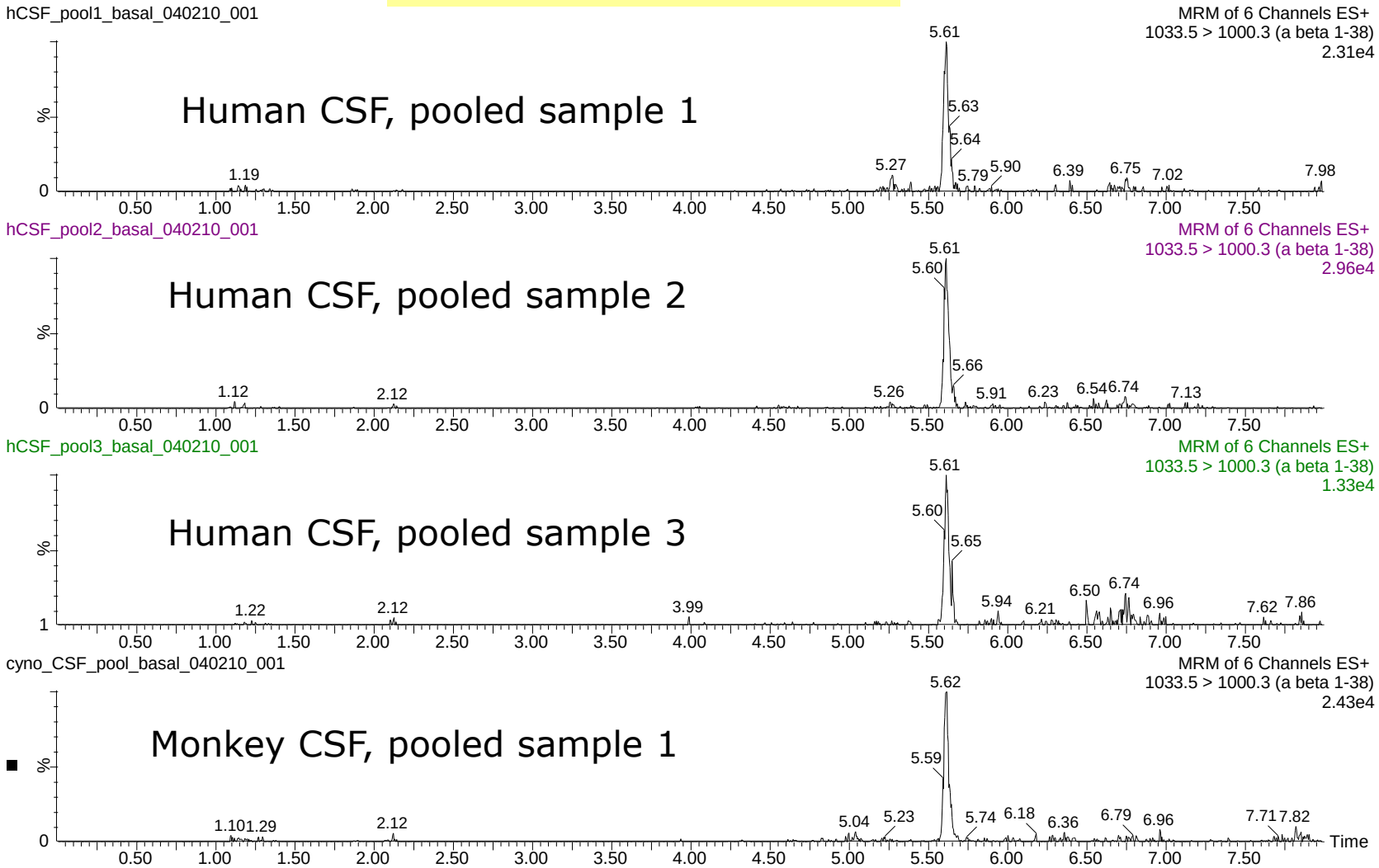
Basal Level Amyloid β 1-38



Representative Chromatogram: Basal Levels of Amyloid β 1-40 in Human and Monkey CSF

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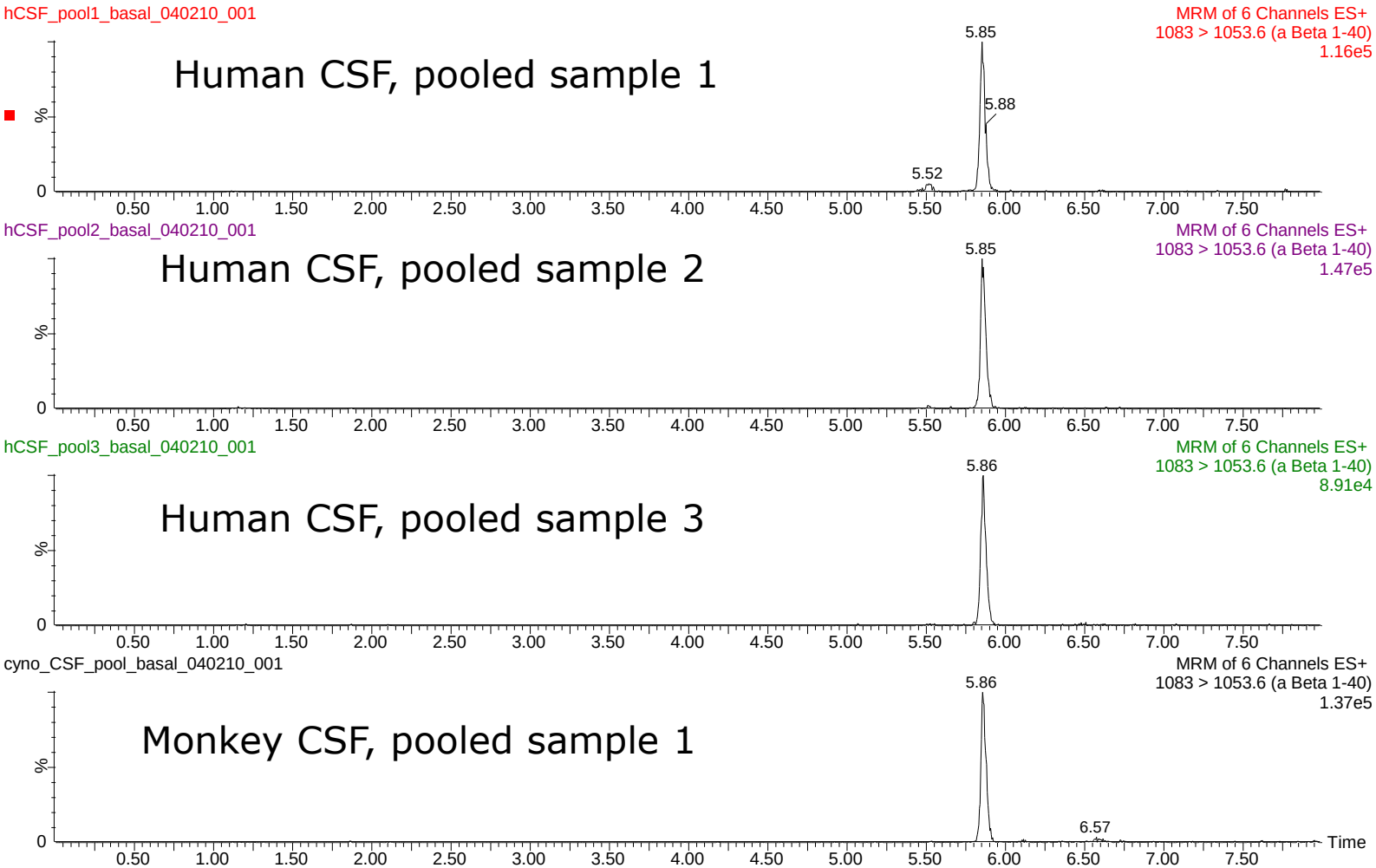
Basal Level Amyloid β 1-40



Representative Chromatogram: Basal Levels of Amyloid β 1-42 in Human and Monkey CSF

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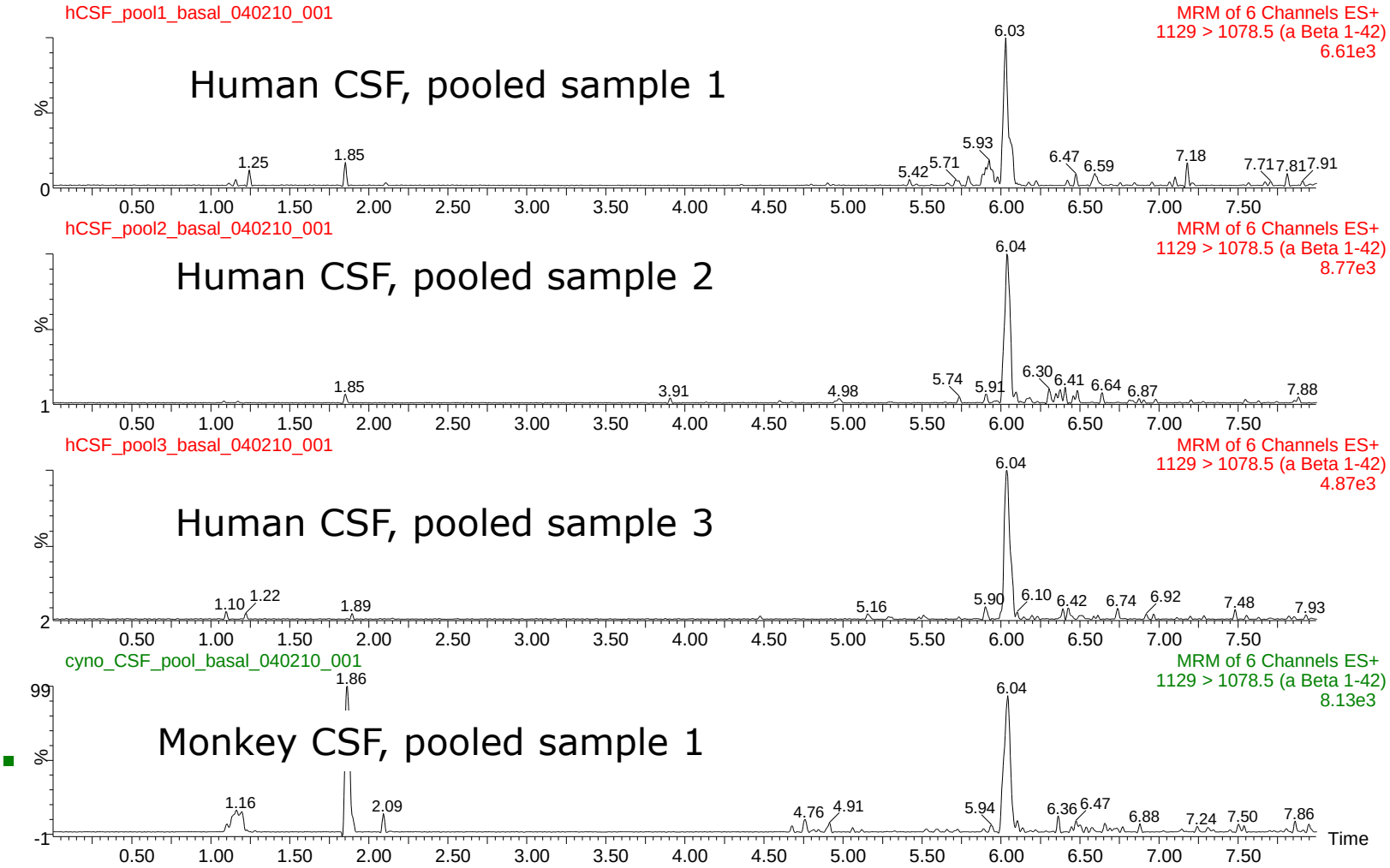
Basal Level Amyloid β 1-42



Baseline Levels of Amyloid β in Pooled Human and Monkey CSF

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Amyloid Beta 1-38

Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	1.585	2.354	1.014	1.713
2	1.650	2.103	1.371	1.605
3	1.614	2.464	0.950	1.947
4	1.657	1.939	1.608	1.541
5	1.820	2.158	1.471	1.675
6	1.486	1.995	1.167	1.644
Mean	**1.635**	**2.169**	**1.264**	**1.688**
Std. Deviation	0.110	0.204	0.262	0.140
% CV	**6.7**	**9.4**	**20.7**	**8.3**

Amyloid Beta 1-42

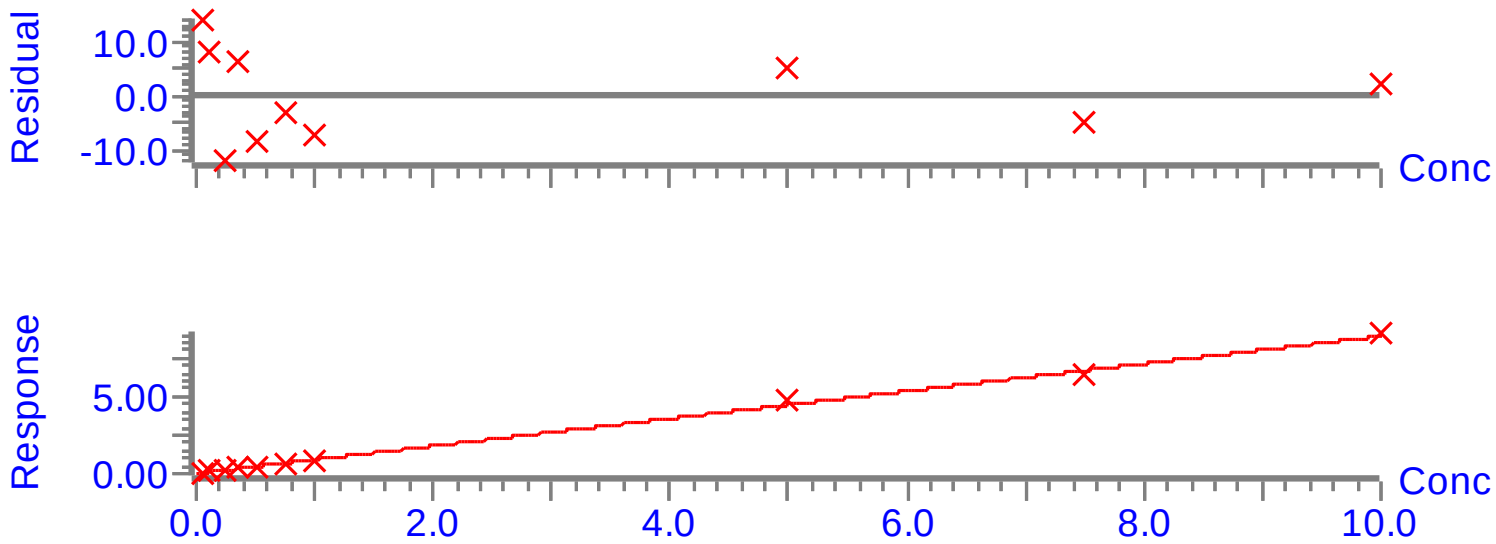
Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	0.519	0.616	0.421	0.675
2	0.421	0.656	0.481	0.621
3	0.542	0.644	0.534	0.623
4	0.471	0.567	0.348	0.659
5	0.476	0.573	0.487	0.700
6	0.561	0.713	0.510	0.688
Mean	**0.498**	**0.628**	**0.463**	**0.661**
Std. Deviation	0.052	0.055	0.068	0.033
% CV	**10.4**	**8.7**	**14.7**	**5.1**

Amyloid Beta 1-40

Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	3.083	4.031	2.541	3.699
2	3.391	3.776	2.593	3.989
3	3.292	3.598	2.580	3.525
4	2.884	3.533	2.612	3.956
5	3.131	3.230	2.508	3.284
6	3.656	3.619	2.490	3.595
Mean	**3.240**	**3.631**	**2.554**	**3.675**
Std. Deviation	0.269	0.266	0.049	0.268
% CV	**8.3**	**7.3**	**1.9**	**7.3**

Representative Standard Curve: Amyloid β 1-42

Compound name: Amyloid Beta 1-42
Correlation coefficient: r = 0.998799, r^2 = 0.997600
Calibration curve: 0.887923 * x + 0.0130161
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Representative Standard Curve: Amyloid β 1-42

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Name	Type	Std. Conc	RT	Area	IS Area	Response	Conc.	%Dev
blank artificial CSF			5.73	19.7	7.0			
50 pg/mL artificial CSF	Standard	0.05	5.71	230.4	3620.5	0.064	0.057	14
100 pg/mL artificial CSF	Standard	0.1	5.71	390.8	3585.1	0.109	0.108	8.1
250 pg/mL artificial CSF	Standard	0.25	5.71	778.3	3737.3	0.208	0.220	-12
350 pg/mL artificial CSF	Standard	0.35	5.71	1267.3	3693.8	0.343	0.372	6.2
500 pg/mL artificial CSF	Standard	0.5	5.71	1494.7	3566.8	0.419	0.457	-8.5
750 pg/mL artificial CSF	Standard	0.75	5.71	2733.5	4152.0	0.658	0.727	-3.1
1 ng/mL artificial CSF	Standard	1	5.71	3166.8	3792.5	0.835	0.926	-7.4
5 ng/mL artificial CSF	Standard	5	5.72	14773.9	3148.3	4.693	5.270	5.4
7.5 ng/mL artificial CSF	Standard	7.5	5.72	24576.9	3877.0	6.339	7.125	-5
10 ng/mL artificial CSF	Standard	10	5.72	33343.3	3662.5	9.104	10.238	2.4

Average Deviation Values for all Overspike QC Samples

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	QC 0.04 ng/mL	QC 0.075 ng/mL	QC 0.15 ng/mL	QC 0.2 ng/mL	QC 0.8ng/mL	QC 2 ng/mL	QC 6 ng/mL
Amyloid β 1-38 Human CSF 1 and 2	2.3	5.8	-3.2	7.3	14.8	5.1	13.1
Amyloid β 1-40 Human CSF 1 and 2	-0.8	-3.2	-1.9	2.5	-2.6	-4.2	-3.8
Amyloid β 1-42 Human CSF 1 and 2	1.3	13.4	-3.6	5.6	2.0	-0.6	-0.2

Conclusions

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- Single flexible LC/MS/MS platform developed for simultaneous quantification of multiple amyloid peptides
- Highly selective sample preparation based on mixed-mode SPE
- Improved MS selectivity using positive ion mode and sequence ion fragments
- High sensitivity using new MS platform
- Highly reproducible, accurate, and precise
- Sample pretreatment and choice of SPE and LC solutions eliminate handling (NSB, losses, etc.) problems
- One method for multiple Aβ peptides, advantage over ELISA which requires individual assays for each peptide
- Fast, simple sample prep

Total Solution for Regulated Bioanalysis - Bioanalysis of Peptides

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Sample Prep

- Kits and protocols
- µElution plate
- No evaporation & reconstitution
- 15x concentration

UNIFI

- IntelliStart and BioLynx functionalities
- Multiple charge precursor ions

ACQUITY UPLC I Class & Columns

- Lower dispersion
- Dedicated PST columns
- Longer columns if needed
- Lowest carryover

Xevo TQ-S

- Stepwave
- Mass range
- Minimal dwell time
- RADAR
- PIC

SERVICE

- Application support and troubleshooting

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