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Nota de aplicación

Amyloid Beta Peptides Quantification by SPE-LC-MS/MS with Automated Sample Preparation for Preclinical Research and Biomarker Discovery

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Abstract

This application note describes the optimization of the methodology to improve the speed, cost and useability for routine quantitative analysis of aβ peptides in hCSF.

Herein we demonstrate the suitability of the Xevo TQ-S micro, a cost-effective platform for biomarker quantification, for the accurate quantification of multiple aβ peptides (1–38, 1–40, 1–42) extracted from hCSF at reported concentrations of 0.1–10 ng/mL, while using a decreased sample volume (100 µL) with respect to previous application notes.

Benefits

- Fast, flexible platform for aβ peptide quantification adequate for their endogenous levels, achieving 0.1 ng/mL LLOQs
- Selective and simple sample preparation using Oasis PRiME MCX µElution Plates with a simplified SPE protocol
- Robust, high throughput automatable sample preparation (96 samples processed <90 min and 5 day LC auto-sampler stability)
- SPE-LC-MS/MS method for aβ peptide quantification meet FDA recommended bioanalytical method validation guidelines.[6]

Introduction

Amyloid beta (aβ) peptides, compounds involved in Alzheimer's disease pathogenesis, have been targeted as Alzheimer's biomarkers and almost exclusively quantified using immunoassay techniques.[1,2,3] These techniques are known to be time consuming, subject to cross-reactivity, and with high batch to batch variation. To overcome these challenges, Waters developed a fast and flexible Solid Phase Extraction-Liquid Chromatography method coupled to tandem Mass Spectrometry (SPE LC-MS/MS) for the quantification of multiple aβ peptides in human CSF (hCSF) to support preclinical research and biomarker discovery.[4,5] This application note describes the further optimization of the methodology to improve the speed, cost and useability for routine quantitative analysis of aβ peptides in hCSF.

Herein we demonstrate the suitability of the Xevo TQ-S micro, a cost-effective platform for biomarker quantification, for the accurate quantification of multiple aβ peptides (1–38, 1–40, 1–42) extracted from hCSF at reported concentrations of 0.1–10 ng/mL, while using a decreased sample volume (100 µL) with respect to previous application notes.[4,5] The method performance meets the FDA Bioanalytical Method Validation Guidelines for the accurate quantification of endogenous aβ peptides in hCSF.[6] Reproducible and accurate quantification of aβ 1–42 Certified Reference Material was achieved with this method.[7] The automation compatibility of the sample preparation workflow was also demonstrated using a Tecan automated pipetting platform.

Experimental

UPLC conditions

Recommended UPLC hardware components:	ACQUITY UPLC I-Class configured with fixed loop sample manager
Sample loop volume:	100 µL (p/n: 430004209)
Needle:	20 µL (p/n: 700005927)
Injection mode:	Partial loop with needle overfill
Binary solvent mixer:	100 µL (p/n: 205000854)
Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 × 150 mm (p/n 186003687)
Column temp.:	55 °C
Sample temp.:	15 °C
Injection volume:	40 µL

Flow rate:	0.2 mL/min
Mobile phase A:	0.3% NH_4OH in H_2O
Mobile phase B:	90:10 (v/v) ACN:Mobile phase A
Strong needle wash:	80:20 (v/v) ACN:IPA +10% NH_4OH (600 µL)
Weak needle wash:	95:5 (v/v) H_2O:ACN + 0.3% NH_4OH (600 µL)

Gradient:

Time(min)	Profile		Curve
	%A	%B	
0.0	90	10	6
1.0	90	10	6
6.5	55	45	6
6.7	55	45	6
7.0	90	10	6
9.0	90	10	6

MS conditions

Capillary voltage:	2.5 V
Desolvation temp.:	650 °C
Cone gas flow:	150 L/hr

Desolvation gas flow:	1000 L/hr
Collision cell pressure:	$2.6 \times 10^{(-3)}$ mbar
MRM transitions monitored:	ESI^+: See Table 1

Peptide name	Precursor ion (*m/z*)	Product ion (*m/z*)	Product ion identification	Cone voltage (V)*	Collision energy (eV)*
aβ 1–38	1033.5	1000.3	B 36	33	23
aβ 1–38 ^{15}N (IS)	1046	1012.5	–	30	22
aβ 1–40	1083	1053.6	B 39	33	25
aβ 1–40 ^{15}N (IS)	1096	1066.5	–	35	22
aβ 1–42	1129	1078.5	b 40	28	30
aβ 1–42 ^{15}N (IS)	1142.5	1091.5	–	35	28

Table 1. Multiple reaction monitoring (MRM) transitions and MS conditions for the aβ peptides and their corresponding ^{15}N labeled internal standards.

**Mass positions, cone voltage, and collision energy optimized during instrument tuning.*

Sample preparation

Calibrators, quality controls (QC) and hCSF sample preparation and pretreatment

To prepare calibration standards, QCs and hCSF samples, the procedure described in Table 2 was followed. All standard and internal standard (IS) mixes were prepared in spiking solutions composed of 50:50:1 Acetonitrile:Water:NH_4OH containing 0.05% rat plasma. The standard mix solutions contained all 3 isoforms of aβ peptides at 25× the desired calibrator/QC concentration, while the IS mix contained all 3 isoforms of ^{15}N aβ peptides. Final concentrations of prepared standards ranged from 0.1 to 10 ng/ml in the case of standards and QCs for native aβ peptides, while IS peptide mix was prepared at 40 ng/mL. Samples were prepared and injected in triplicate, while standards and QCs were prepared and injected in duplicate.

Step 1	Add 100 µL of artificial CSF(aCSF) containing 4 g/L BSA (w/v) or hCSF into a low bind Eppendorf
Step 2	Add 5 µL of IS mix (40 ng/mL), to calibrators, QCs, and hCSF samples
Step 3	Add 4 µL of the 25x standard mix solution mix ONLY to calibrators and QCs samples
Step 4	Vortex for 10 seconds
Step 5	INCUBATION STEP: 30 min, room temperature
Step 6	Add 91 µL of 5M Guanidine-HCl solution for Standards and QCs or 95 µL in the case of hCSF
Step 7	INCUBATION STEP: 1 hour, 37 °C, 1200 rpm
Step 8	Add 100 µL of H_3PO_4 (4% v/v)
Step 9	Vortex for 10 seconds
Step 10	Samples ready for SPE purification

Table 2. Sample preparation and pretreatment procedure followed for calibrator standards, QCs, and hCSF samples.

SPE Sample Purification

The pretreated samples were extracted according to the protocol described in Figure 1 using Oasis PRiME MCX in the 96-well µElution format and performed on the Tecan Freedom EVO 100/4 automated pipetting platform. All solutions used for extraction were made up by volume.

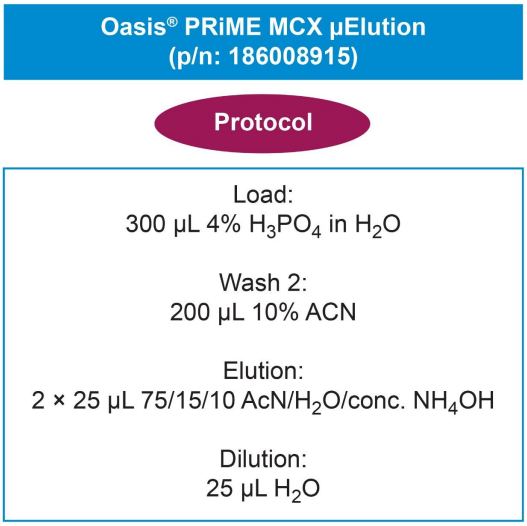
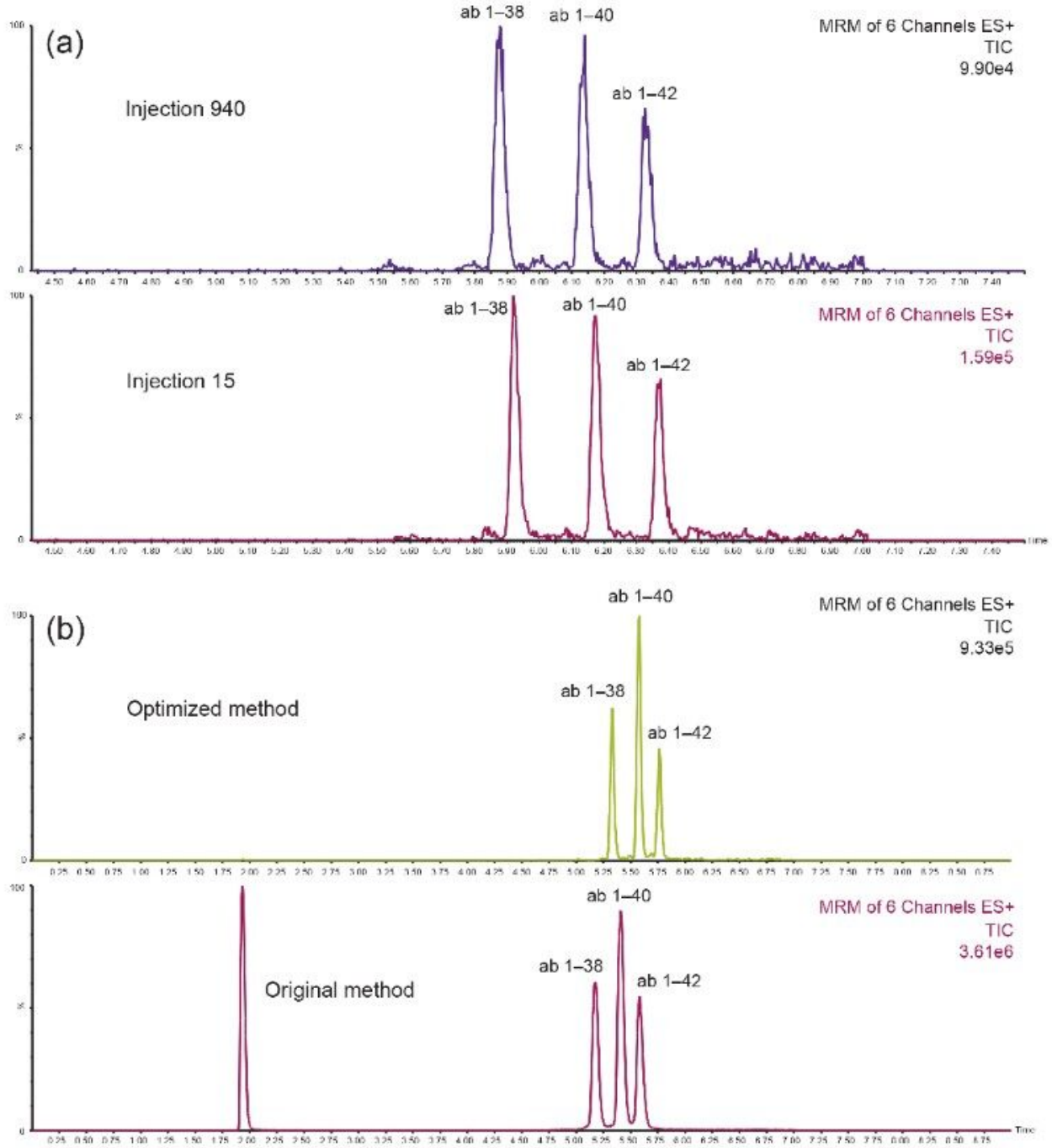


Figure 1. Oasis PRiME µElution MCX extraction protocol.

Results and Discussion

UPLC Separation

Chromatographic separation of the aβ peptides was achieved using an ACQUITY UPLC Peptide BEH C_{18} (300Å, 1.7 µm, 2.1 × 150 mm) Column with the ACQUITY UPLC I-Class System in the fixed loop sample configuration. A linear gradient using 10–45% mobile phase B over 5.5 minutes was employed.[4,5] A minor modification to the original application with the addition of a 2 min hold step using initial conditions (90% mobile phase A) at the end of the gradient increased the column life time up to 800 injections (Figure 2, Panel a).[4] Furthermore, the modification of the weak needle wash solvent (acetonitrile percentage decreased from 10% to 5% while maintaining NH_4OH at 0.3%) and strong wash solvent (decreasing IPA percentage from 40% to 20%while maintaining NH_4OH constant at 10%), resulted in the elimination of carryover (Figure 2, Panel b). Using this modified method, samples were shown to be stable for 5 days in the auto sampler, while mobile phase stability at room temperature was determined to be 3 days. In addition, when testing 150 injections in one run no appreciable loss of peptide signal was seen, further demonstrating the robustness of this developed method for high throughput analyses (Figure 2, Panel c). The improvements in LC optimization combined with Xevo TQ-S micro MS analysis enabled a lower limit of quantification for this assay of 0.1 ng/mL and the ability to readily detect and quantify endogenous aβ levels from hCSF (Figure 3).


(a)
Injection 940
ab 1–38
ab 1–40
ab 1–42
MRM of 6 Channels ES+
TIC
9.90e4
Injection 15
ab 1–38
ab 1–40
ab 1–42
MRM of 6 Channels ES+
TIC
1.59e5
(b)
Optimized method
ab 1–40
ab 1–38
ab 1–42
MRM of 6 Channels ES+
TIC
9.33e5
Original method
ab 1–40
ab 1–38
ab 1–42
MRM of 6 Channels ES+
TIC
3.61e6
(c)
Injection 124
ab 1–38
ab 1–40
ab 1–42
MRM of 6 Channels ES+
TIC
1.29e5
Injection 5
ab 1–38
ab 1–40
ab 1–42
MRM of 6 Channels ES+
TIC
1.32e5

isoforms showing (a) column robustness: comparison of injections 15 and 940 using the same column (injections correspondent to 1 ng/mL), (b) carryover: peak presence/absence before/after method optimization using a 5 ng/mL standard mix, (c) high throughput run: aβ peptides signal at beginning (injection 5) and after 124 injections for a 0.5 ng/mL standard mix demonstrating assay robustness.

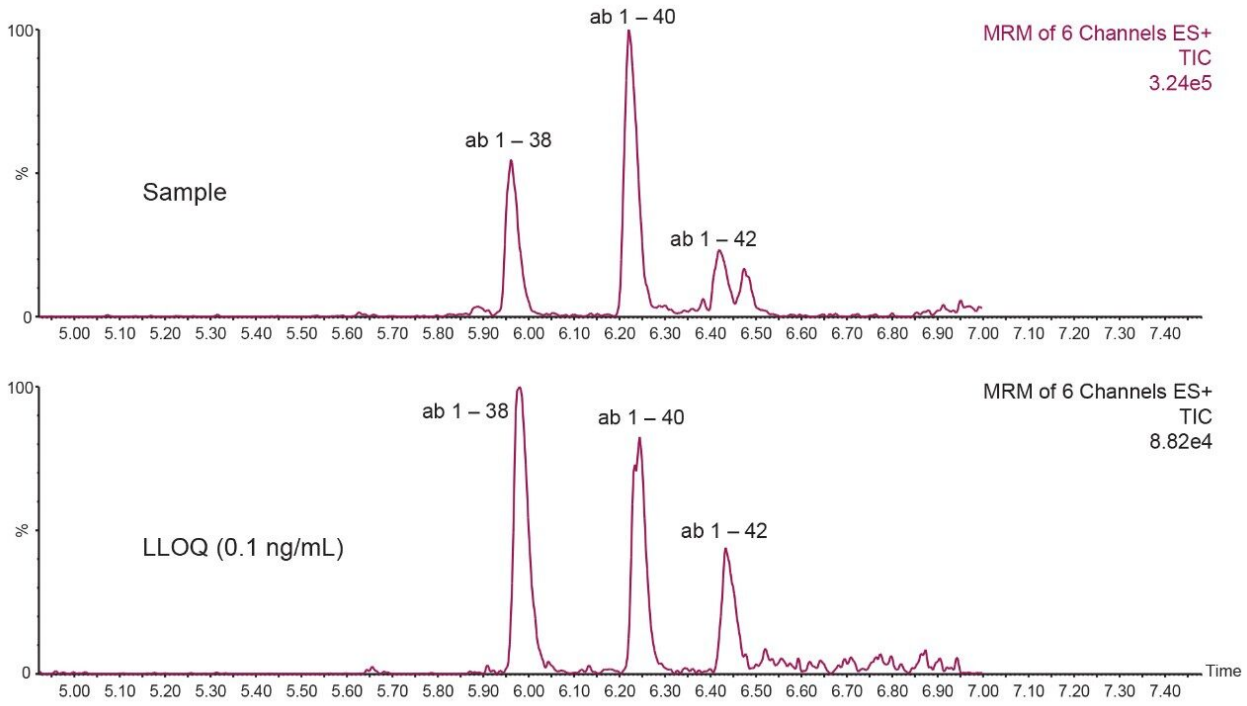


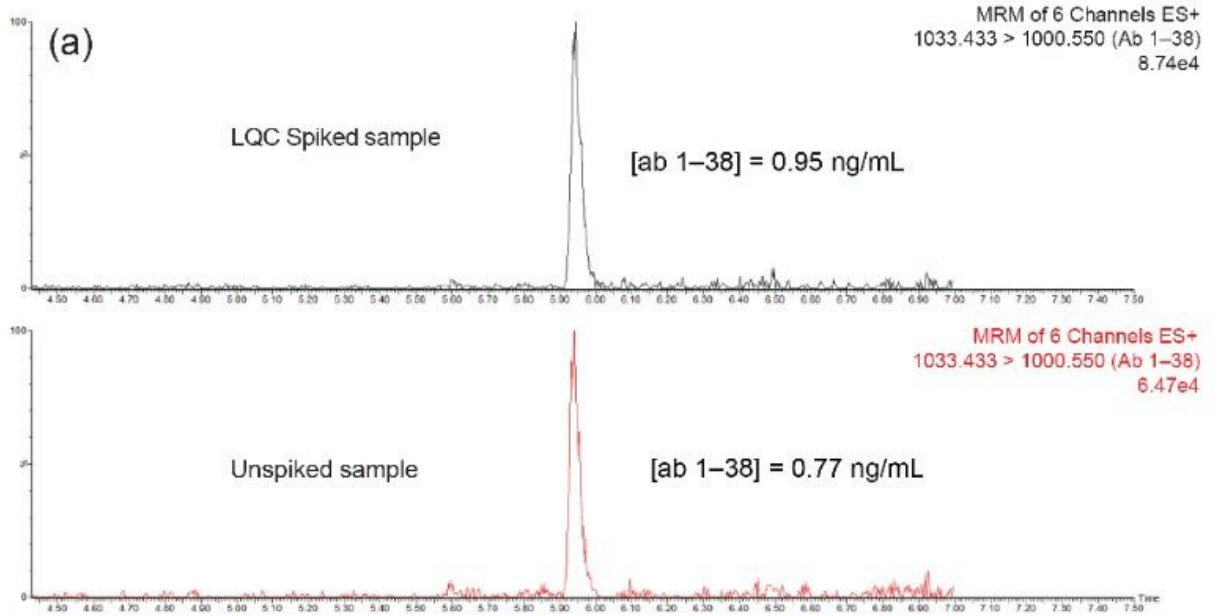
Figure 3. Representative UPLC-MS/MS TIC chromatograms of the 1–38, 1–40, and 1–42 aβ isoforms showing endogenous hCSF aβ levels vs. LLOQ standard mix (0.1 ng/mL).

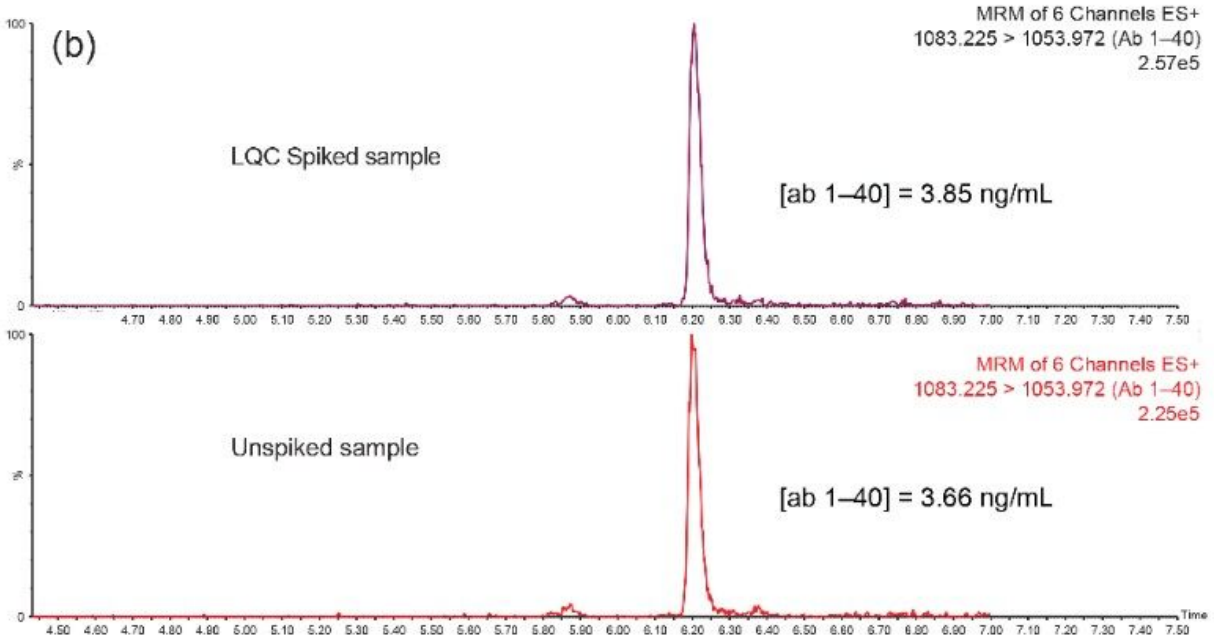
Sample Preparation

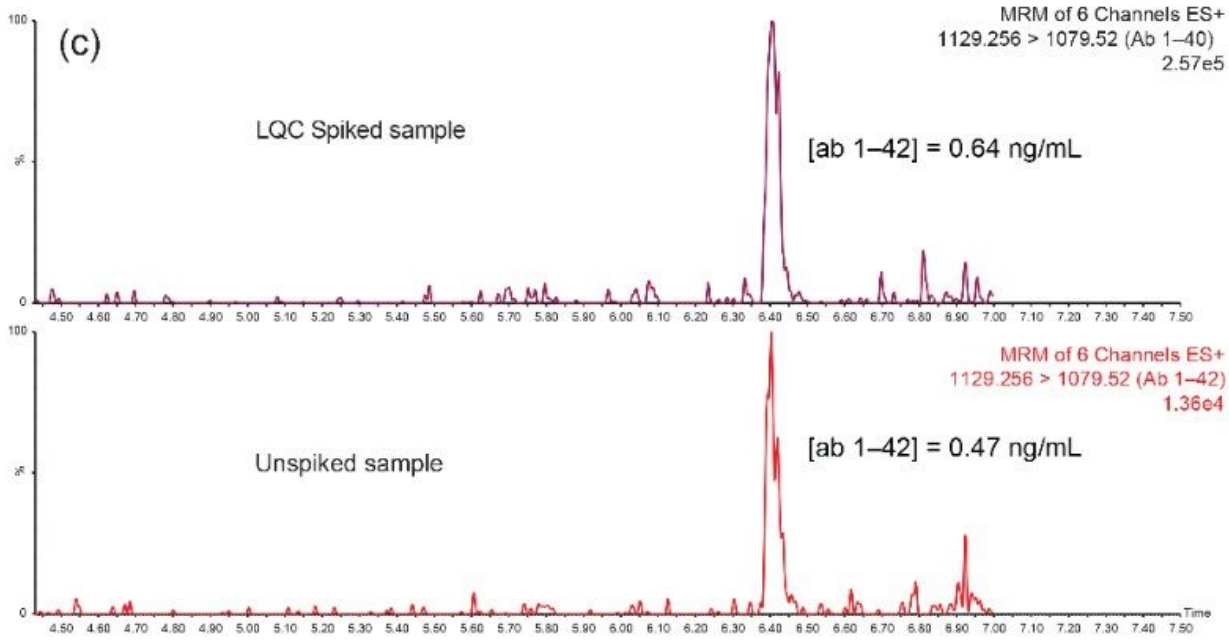
Oasis PRiME MCX SPE

SPE extraction of the aβ peptides was achieved using Oasis PRiME MCX, a mixed-mode sorbent, in the µ Elution format using the extraction procedure shown in Figure 1. For peptides, SPE sample preparation in the micro elution format is ideal. It provides rapid sample cleanup, high recovery, sample concentration without the need for sample evaporation, and helps ensure peptide solubility throughout the extraction process. Due to the water wettable nature of the Oasis PRiME sorbents, we were able to eliminate the conditioning and equilibration steps, reducing time and number of steps. In addition, Oasis PRiME MCX is designed to yield highly consistent flows across cartridges and plates, making processing time exceptionally reproducible.

Use of the Oasis PRiME MCX SPE sorbent and the described protocol provided excellent recovery and selectivity for the extraction of the aβ peptides from hCSF, eliminating other high abundance endogenous polypeptides and matrix interferences (Figure 4). A summary of sample recoveries and quantitative performance, for unspiked, low-level QC (LQC), mid-level QC (MQC), and high-level QC (HQC) is highlighted in Table 3. Quantitative performance was excellent with mean (N=3) recovery and accuracies values between 86.3–100.6% and 95.5–100.5%, respectively and precision values (CVs) ≤10%. In addition to QC performance, analysis of aβ concentrations was performed from two sources of pooled hCSF, as well as for the concentration determination for the ERM reference material (CRM) of aβ 1–42.


(a)
MRM of 6 Channels ES+
1033.433 > 1000.550 (Ab 1–38)
8.74e4
LQC Spiked sample
[ab 1–38] = 0.95 ng/mL
MRM of 6 Channels ES+
1033.433 > 1000.550 (Ab 1–38)
6.47e4
Unspiked sample
[ab 1–38] = 0.77 ng/mL


(b)
MRM of 6 Channels ES+
1083.225 > 1053.972 (Ab 1–40)
2.57e5
LQC Spiked sample
[ab 1–40] = 3.85 ng/mL
MRM of 6 Channels ES+
1083.225 > 1053.972 (Ab 1–40)
2.25e5
Unspiked sample
[ab 1–40] = 3.66 ng/mL


(c)
MRM of 6 Channels ES+
1129.256 > 1079.52 (Ab 1–40)
2.57e5
LQC Spiked sample
[ab 1–42] = 0.64 ng/mL
MRM of 6 Channels ES+
1129.256 > 1079.52 (Ab 1–42)
1.36e4
Unspiked sample
[ab 1–42] = 0.47 ng/mL

isoforms comparing endogenous level (unspiked hCSF) and hCSF spiked with low level QC (0.2 ng/mL): (a) a β 1–38, (b) aβ 1–40, (c) aβ 1–42.

Peptide	Spiked concentration (ng/mL)	Expected concentration (ng/mL)	Measured concentration (n=3) (ng/mL)	Accuracy (%)	Precision (%CV)	Recovery (%)
aβ 1–38	0.00	0.77	0.77	N/A	2.6	N/A
	0.20	0.97	0.95	97.9	2.1	87.8
	1.00	1.77	1.68	94.1	0.6	90.9
	7.50	8.27	8.31	100.5	4.2	100.6
aβ 1–40	0.00	3.66	3.66	N/A	1.9	N/A
	0.20	3.86	3.85	99.7	0.3	94.8
	1.00	4.66	4.64	99.6	1.3	97.9
	7.50	11.16	10.83	97.0	3.7	94.2
aβ 1–42	0.00	0.47	0.47	N/A	6.4	N/A
	0.20	0.67	0.64	95.5	1.6	86.3
	1.00	1.47	1.41	95.9	9.9	94.2
	7.50	7.97	7.78	97.6	1.3	97.5

Table 3. aβ % recovery and quantitative results for LLQC, MQC, and HQC levels extracted from hCSF using the optimized Oasis MCX SPE protocol analyzed on the Xevo TQ-S micro MS. All experiments were carried out in triplicate.

Results from this analysis were in good agreement with reported theoretical values (Table 4). Representative extracted ion chromatograms (EIC) of the 1–38, 1–40, and 1–42 ab isoforms comparing endogenous level (unspiked hCSF) and hCSF spiked with low level QC (0.2 ng/mL) are shown in Figure 4, Panels a–c. Remarkably, decreasing sample size two-fold did not have any effect on the SPE extraction or analytical parameters compared to previous works.[4,5]

Peptide	hCSF pool	Theoretical endogenous concentration (ng/mL)	Mean calculated endogenous concentrations (ng/mL)	Number of replicates	%CV
aβ 1–38	1	0.50–2.00	1.02	5	6.9
	2		0.77	4	3.5
aβ 1–40	1	2.00–4.00	3.77	5	4.8
	2		3.66	4	1.8
aβ 1–42	1	0.35–0.70	0.44	5	10.2
	2		0.47	4	6.2
aβ 1–42 Certified reference material	N/A	0.61–0.83	0.63	3	4.8

Table 4. Theoretical and experimental levels of aβ peptides extracted from 2 sources of pooled hCSF and the ERM CRM aβ 1–42 reference standard.

Mitigation of Non- Specific Binding

During initial method development, a high degree of non-specific binding (NSB) was observed when aβ peptides were extracted from artificial CSF. Thus, 5% rat plasma was added as a carrier protein to eliminate the NSB.[4,5] To simplify the analysis, reduce cost, increase assay robustness and eliminate lot variations in rat plasma, bovine serum albumin (BSA) was assessed as an alternative carrier protein. For this assessment LQC, MQC, and HQC samples were prepared in aCSF containing BSA (4 g/L) and were extracted using the extraction protocol (Figure 1). Mean accuracies of these results were excellent, from 94.1–100.5%. In addition these resulted correlated well with the original application note 720003682en, (highlighted in Table 5). This performance and correlation with the original work demonstrates that artificial CSF containing BSA effectively eliminated NSB with performance comparable to those using 5% rat plasma as carrier protein and is a suitable alternate carrier protein for this assay.

Peptide	Rat plasma (720003682)		Bovine serum albumin (BSA)	
	Spike level (ng/mL)	Mean accuracy (%)	Spike level (ng/mL)	Mean accuracy (%)
aβ 1–38	0.8	91.2	0.2	97.9
	2.0	99.9	1.0	94.1
	6.0	105.6	7.5	100.5
aβ 1–40	0.8	88.2	0.2	99.7
	2.0	99.9	1.0	99.6
	6.0	105.6	7.5	97.0
aβ 1–42	0.8	90.7	0.2	95.5
	2.0	99.9	1.0	95.9
	6.0	105.6	7.5	97.6

Table 5. Mean accuracy comparison using 0.5% rat plasma or bovine serum albumin (BSA) as protein carrier using the simplified extraction protocol.

MRM transitions used for quantification are summarized in Table 1. Using the optimized SPE-LC-MS/MS method and the low cost TQ-S micro tandem quadrupole MS, highly robust and accurate quantification was achieved, demonstrating suitability for quantification of aβ peptides at the expected physiological levels found in hCSF. Xevo TQ-S micro MS quantification performance for the quantification of aβ peptides, compared to Xevo TQ and Xevo TQ-S MS Systems, is highlighted in Table 6. As expected, the Xevo TQ-S micro platform resulted in an analytical sensitivity decrease compared to the Xevo TQ-S, but similar to that described for the TQ. Using the optimized SPE-LC-MS/MS method with a 2-fold decrease in starting sample volume (100 vs. 200 µL), the low cost Xevo TQ-S micro Mass Spectrometer demonstrated highly robust and accurate quantification.

Peptide name	200 µL Sample Xevo TQ	50 µL Sample Xevo TQ-S	100 µL Sample Xevo TQ-S micro
Linearity	0.1–10 ng/mL	0.025–10 ng/mL	0.1–10 ng/mL
QC range	0.2–6 ng/mL	0.04–6 ng/mL	0.2–8 ng/mL
LOQ	0.1 ng/mL	0.025 ng/mL	0.1 ng/mL
Accuracy (%)	85.0–106.6	88.2–105.6	92.1–106.5

Table 6. Comparison of Xevo TQ-S micro MS quantification performance vs. Xevo TQ and Xevo TQ-S MS for the 3 aβ peptide isoforms (1–38, 1–40, 1–42) extracted from hCSF.

Automated Sample Preparation

The introduction of the automated liquid handler has enabled enhanced speed, workflow standardization and hands-free highthroughput sample preparation, maximizing productivity and method robustness. Automation compatibility for the developed aβ SPE protocols (sample pre-treatment – Table 2, and SPE – Figure 1) was successfully demonstrated on the Tecan Freedom EVO 100/4. An example of automated vs. manual performance is demonstrated in Figure 5 comparing peak area's for aβ 1–42. Results demonstrated no significant difference ($p < 0.05$) between the aβ 1–42 peak areas obtained for manually prepared samples vs. those samples prepared using a Tecan script. Additionally, there were no indications of loss of recovery in the automated sample preparation indicating that development of a validated automated pipetting script for the workflow is technically feasible.

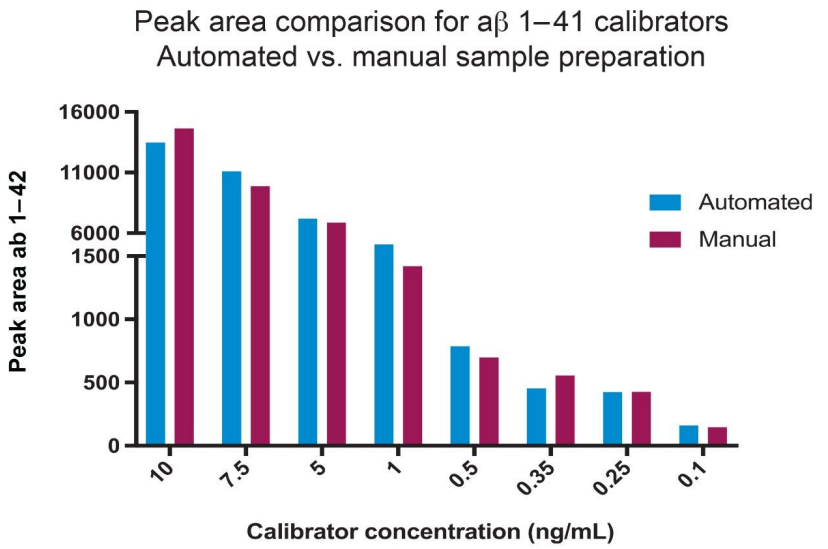


Figure 5. Peak area aβ 1–42 for comparison of samples prepared manually vs. Tecan automation.
Note: All calibrator responses are normalized with internal standard – data not shown.

Conclusion

- An improved SPE-UPLC-MS/MS bioanalytical method was developed and validated for the simultaneous

quantitation of multiple amyloid β peptides (1–38, 1–40, 1–42) extracted from human CSF.

- Use of the highly selective sample preparation with Oasis PRiME MCX SPE µElution Plate increased the analysis throughput.
- Use of the Tecan liquid handler for sample preparation simplified and ensured method performance, maximizing productivity, reduced errors, and ensured analytical performance.
- Fast sample preparation and analysis (96 samples can be extracted and ready for injection in <90 min) with a 9 minute LC-MS analysis time.
- Analysis with the Xevo TQ-S micro Tandem Quadrupole Mass Spectrometer, achieved a LLOQ of 0.1 ng/mL from 100 µL of sample. The excellent quantification performance of this method allows the reliable measurement of low levels of amyloid β peptides from human CSF, demonstrating its utility in support drug discovery and clinical research studies.

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