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Sensitive and Reproducible LC-MS Quantification of C-Reactive Protein in Plasma: A Potential Biomarker of Inflammation

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APPLICATION BENEFITS

High sensitivity quantification of a protein biomarker, demonstrated speed and reproducibility of a generic kit-based approach for protein quantification, high sensitivity of the Xevo® TQ-XS mass spectrometer, mixed-mode SPE specificity, no affinity purification required.

WATERS SOLUTIONS

ProteinWorks™ eXpress Direct Digest Kit

ProteinWorks µElution SPE Clean-Up Kit

ACQUITY UPLC® HSS T3 Column

ACQUITY UPLC System

Xevo TQ-XS Mass Spectrometer

MassLynx® Software

TargetLynx™ Application Manager

KEYWORDS

C-reactive protein, CRP, biomarker, protein digestion, tryptic peptides, protein quantification, ProteinWorks, eXpress Direct Digest, µElution SPE, Oasis® MCX, Xevo TQ-XS, Skyline

INTRODUCTION

C-Reactive Protein (CRP)[1] is naturally synthesized in the liver and released into the bloodstream in response to inflammation. There is, therefore, interest in measuring CRP both in cases of chronic inflammation such as Rheumatoid Arthritis (RA),[2,4] as well as to evaluate cardiovascular risk[3] and inflammatory cancerous processes.[4] Tissue injury or inflammation causes a > 100-fold[2] increase in plasma CRP levels. While endogenous plasma levels in healthy individuals are relatively low, generally between 0.1 and 3 µg/mL (4–120 nM),[2] levels present in diseased patients are so highly elevated, multiple ELISA or immunoturbidimetric[3] tests are required to achieve accurate CRP quantification. The need for multiple tests arises out of the limited linear dynamic range of ligand binding assays (LBA). This inherent short coming, as well as lack of standardization, possible cross-reactivity, and expensive, difficult to reproduce reagents are just a few of the reasons that the industry is moving towards LC-MS. Mass Spectrometry detection offers many benefits for protein quantification such as sensitivity, specificity, broad linear dynamic range, fast method development times, and the ability to multiplex. However, LC-MS protein quantification still presents challenges. There is no single standardized workflow and the various workflow options can be complex and laborious, making it difficult for a scientist to achieve success. In this application note, we describe a generic, kitted approach which requires only 2 hours for digestion vs. a standard 24 hour method described in the literature.[5] Digestion and subsequent peptide level purification are performed using the ProteinWorks eXpress Direct Digest (p/n: 176003688) and ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) for the accurate and reliable quantification of endogenous CRP in human plasma. The kits and their included protocols achieve LLOQs between 0.025-0.1 µg/mL (1-4 nM) from only 35 µL of plasma.

EXPERIMENTAL

To prepare calibration curve standards and quality control (QC) samples, CRP (human sequence) was spiked into rat or human plasma at various concentrations over the range of 0.025–100 µg/mL (1–3987 nM). CRP QC samples were prepared in one lot of rat plasma and four lots of human plasma. Calibration curve standards were prepared in duplicate, each QC level prepared in triplicate, and blank (non-spiked) plasma samples were prepared in quadruplicate. Plasma samples (35 µL) were digested for 2 hours using the ProteinWorks eXpress Direct Digestion Kit, specifically, the 3-Step (no reduction/alkylation) method included in the kit with an abbreviated denaturation time of 5 minutes (p/n: 176003688). Post digestion purification of signature peptides was done using the ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) and included protocol.

Method conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 50 mm (p/n: 186003538)
Temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phases:	A: 0.1% Formic acid in H_2O B: 0.1% Formic acid in ACN

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.300	100.0	0.0	6
1.0	0.300	100.0	0.0	6
5.0	0.300	70.0	30.0	6
5.5	0.300	10.0	90.0	6
6.3	0.300	10.0	90.0	6
6.4	0.300	100.0	0.0	6
7.5	0.300	100.0	0.0	6

MS conditions

MS system:	Xevo TQ-XS
Ionization mode:	ESI+
Capillary:	3.5 kV
Source offset (V):	60 V
Source temp.:	150 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	1000 L/Hr
Collision gas flow:	0.15 mL/Min
Nebulizer gas flow:	7 Bar
Data management:	MassLynx (v4.1)
Quantification software:	TargetLynx

RESULTS AND DISCUSSION

Early detection of elevated CRP levels could be imperative for proper treatment or prevention of RA or heart disease. Availability of a sensitive analytical method that can detect low levels and differentiate between small concentrations can facilitate early detection. Mass spectrometric detection, though not traditionally used for CRP quantification, has the sensitivity and reproducibility required for measuring both the low endogenous CRP levels in healthy individuals as well as elevated levels in diseased populations.

MASS SPECTROMETRY

Protein quantification by LC-MS is typically performed via the bottom up approach which uses enzymatic digestion (usually trypsin) and relies on analysis of resulting (tryptic) peptides. To identify an appropriate representative or surrogate peptide for human CRP (Figure 1), an in-silico digestion was performed using Skyline Software (MacCoss Labs, University of Washington).[6] In addition to identifying potential tryptic peptides, Skyline facilitated MS method development by selecting and optimizing precursors and collision induced fragments for MRM (multiple reaction monitoring) analysis. The full amino acid sequence of CRP[7] and the unique signature peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK (highlighted in blue), are illustrated in Figure 2. Optimized MS conditions and MRM transitions for the CRP tryptic peptides are listed in Table 1. Both primary and confirmatory (secondary) transitions are included for each peptide.

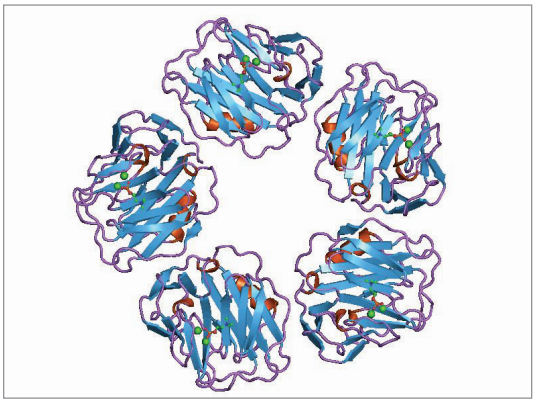

Figure 1. C-Reactive Protein (CRP) structure.[1]

MEKLLCFLVLTSLSHAFGQTDMSRKAFVFPKESDTSYVSLKAPLTKPLKAFTVCLHFYTELS
STRGYSIFSYATKRQDNEILIFWSKDIGYSFTVGGSEILFEVPEVTVAPVHICTSWESASGI
VEFWVDGKPRVRKSLKKGYTVGAEASIILGQEQDSFGGNFEGSQSLVGDIGNVNMWDF
VLSPDEINTIYLGGPFSPNVLNWRALKYEVQGEVFTKPQLWP

Figure 2. Amino acid sequence of human CRP; tryptic peptides used for quantification are highlighted in blue.

Peptide	Precursor charge state	MRM transition	Cone voltage (V)	Collision energy (eV)	Product ion identification
AFVFPK	$[M+2H]^{2+}$	354.71>244.17 (Primary)	35	9	[1H+]1/y2
	$[M+2H]^{2+}$	354.71>219.11 (Confirmatory)	35	3	[1H+]1/b2
ESDTSYVSLK	$[M+2H]^{2+}$	564.77>347.23 (Primary)	35	17	[1H+]1/y3
	$[M+2H]^{2+}$	564.77>696.39 (Confirmatory)	35	17	[1H+]1/y6
GYSIFSYATK	$[M+2H]^{2+}$	568.78>221.09 (Primary)	35	11	[1H+]1/b2
	$[M+2H]^{2+}$	568.78>716.36 (Confirmatory)	35	11	[1H+]1/y6

Table 1. Final MS conditions for CRP tryptic peptides, including precursor and fragment ions.

In an earlier study, CRP quantification was performed on a Xevo TQ-S MS. Quantification was subsequently transferred to a newer generation platform, a Xevo TQ-XS triple quadrupole mass spectrometer, which provided the benefit of improved sensitivity. Signal-to-noise (S/N) for the three CRP peptides used for quantification increased 2.5 X (AFV), 3.5 X (ESD) and 1.4 X (GYS) using the Xevo TQ-XS vs the older platform. This improvement in sensitivity for the AFV peptide is demonstrated in Figure 3. For the ESD, peptide (RT 3.63 minutes), the intensity for an interfering matrix peak (RT 3.72 minutes), present as a shoulder on the main peak, was decreased 7 X. This is shown in Figure 4. The reduced matrix interference facilitated easier peak integration and resulted in improved limits of detection.

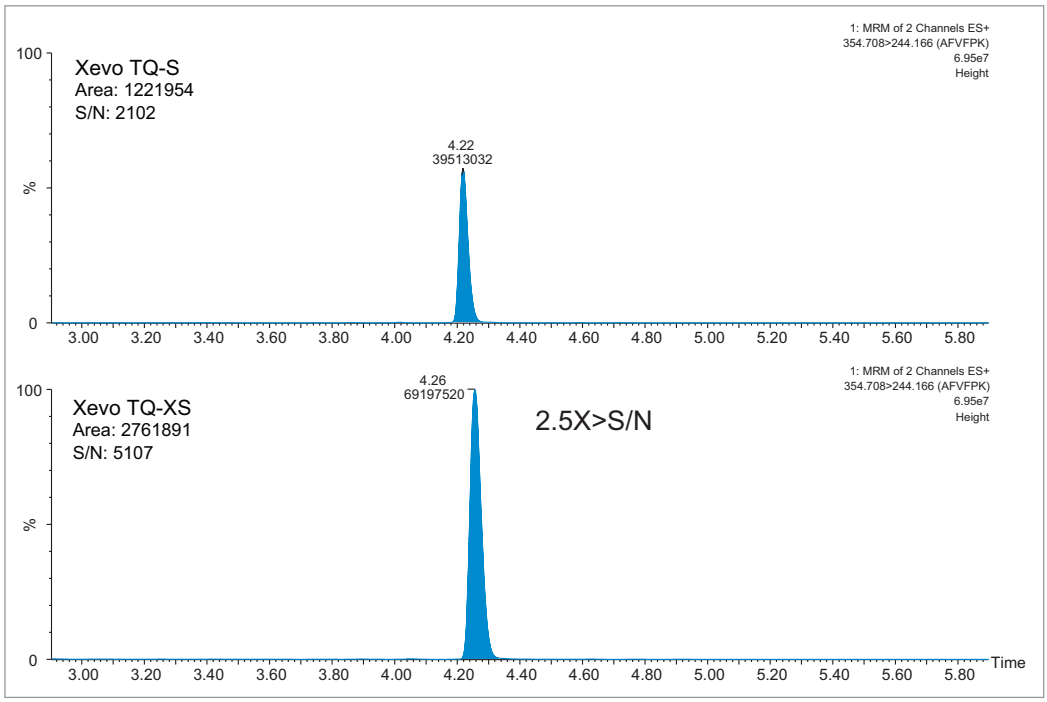

Figure 3. Representative chromatogram demonstrating improved sensitivity and S/N for the AFVFPK CRP peptide digested in rat plasma (100 µg/mL) and purified by SPE: Xevo TQ-S vs. Xevo TQ-XS.

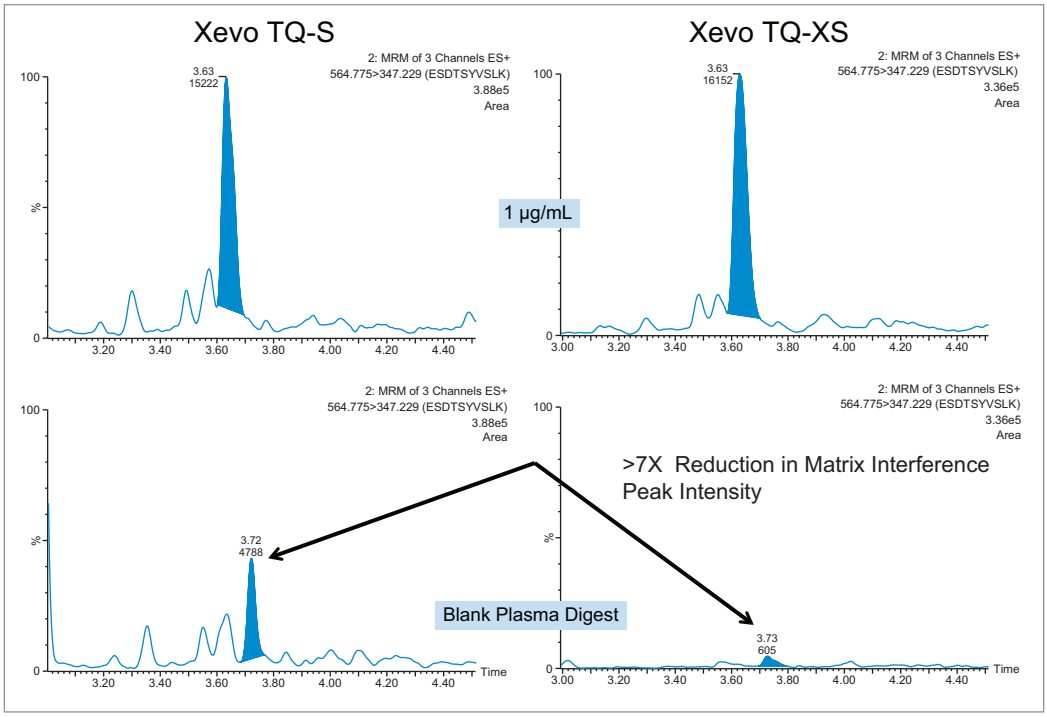

Figure 4. Representative chromatogram demonstrating reduced matrix interferences for the ESDTSYVSLK CRP peptide digested in rat plasma (blank and 1 µg/mL) and purified by SPE: Xevo TQ-S vs. Xevo TQ-XS.

CHROMATOGRAPHY

Reversed-phase chromatographic retention can be challenging due to the relatively small size and polar nature of tryptic peptides. Use of an ACQUITY HSS T3 Column, 1.8 µm, 2.1 x 50 mm (p/n: 186003538) for the CRP peptides afforded improved retention as compared to a BEH C_{18} column and facilitated resolution from endogenous matrix interferences. Representative chromatograms of the three peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK are illustrated in Figure 5. Peak widths were <4.5 seconds wide.

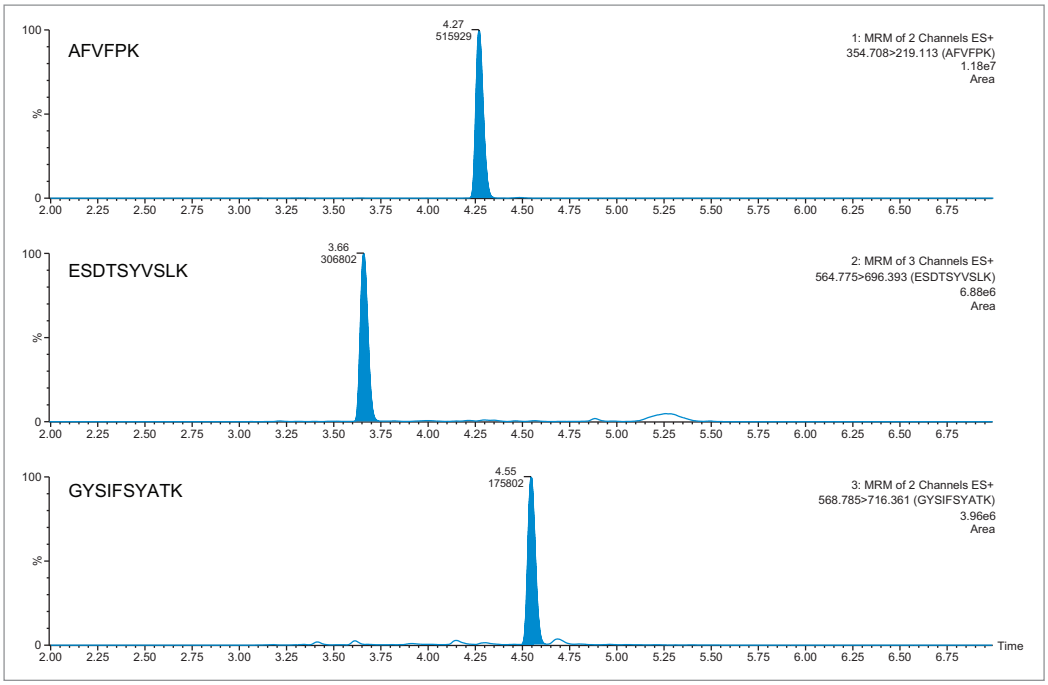

Figure 5. UPLC chromatographic separation of CRP tryptic peptides: AFVPFK, ESDTSYVSLK, and GYSIFSYATK, digested in rat plasma (50 µg/mL) using an ACQUITY HSS T3, 1.8 µm, 2.1 x 50 mm Column.

SAMPLE PREPARATION

Typical sample preparation workflows for protein quantification are often complex and laborious. In this application, we used the ProteinWorks eXpress Direct Digest Kit (p/n: 176003688) to directly digest plasma samples in only 2 hours. Subsequent purification of the peptides with the ProteinWorks µElution SPE Clean-Up Kit (p/n: 186008304) and protocol removed buffer salts, phospholipids, and excess digestion reagents post digestion. The SPE kit relies on a mixed-mode sorbent (Oasis® MCX, reversed-phase and ion-exchange) to provide enhanced specificity. Tryptic peptides are bound to the ion-exchange moiety of the SPE sorbent, thereby imparting orthogonality, and thus greater specificity, into the method as a whole. In addition, very polar peptides are more efficiently trapped by ion exchange than by traditional reversed-phased only sorbent. This was particularly important for the most polar peptide, ESD. Recoveries for all three CRP peptides were excellent, with greater than ≥90% recovery using the generic protocol provided.

LINEARITY, PRECISION, AND ACCURACY

Using only 35 µL of sample and the aforementioned ProteinWorks kits, quantification limits of 0.025 and 0.05 µg/mL (1–2 nM) were achieved in rat and human plasma, respectively. Calibration curves from the tryptic peptides, in both rat and human plasma, were linear with R^2 values >0.99 using 1/x or $1/x^2$ weighted regressions. A summary of standard curve performance in rat and human plasma is shown in Tables 2 and 3. Linearity, accuracy and precision data met typical method validation requirements. For both rat and human, standard curves were linear over 4 orders of magnitude with mean accuracies ranging from 94–105%. The precision and accuracy for the QC samples was excellent with mean % RSDs all <5% and % QC accuracy ranges of 94.3–106.2 (rat) and 89.4–103.5 (human). Rat QC performance is highlighted in Table 4. Human QC performance for the AFV and ESD peptides is shown in Table 5, Panels A and B, respectively. Demonstration of this performance is illustrated in Figure 6, Panels A and B.

Peptide	Curve (µg/mL)	Curve (nM)	Weighting	Linear Fit (R^2)	% Accuracy Range
AFVFPK	0.025–100	1.00–3987	$1/x^2$	0.999	95.4–103.2
ESDTSYVSLK	0.100–100	3.99–3987	1/x	0.997	92.9–105.1
GYSIFSYATK	0.050–100	1.99–3987	1/x	0.998	95.2–104.0

Table 2. Rat plasma: linear dynamic range and standard curve statistics for the CRP tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits

Peptide	Curve (µg/mL)	Curve (nM)	Weighting	Linear fit (R^2)	% Accuracy range
AFVFPK	0.050–100	1.99–3987	$1/x^2$	0.998	93.6–104.4
ESDTSYVSLK	0.050–100	1.99–3987	1/x	0.999	96.8–102.4

Table 3. Human plasma: linear dynamic range and standard curve statistics for the CRP tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits.

Peptide	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
AFVPFK	0.075	2.990	0.071	2.818	94.3	2.16
	0.750	29.904	0.763	30.422	101.7	3.18
	7.500	299.035	7.691	306.664	102.5	1.23
	75.000	2990.353	74.946	2988.213	99.9	3.49
ESDTSYVSLK	0.250	9.968	0.265	10.579	106.2	2.08
	0.750	29.904	0.738	29.425	98.4	0.72
	7.500	299.035	7.210	287.459	96.1	0.97
	75.000	2990.353	75.399	3006.262	100.6	3.77
GYSIFSYATK	0.075	2.990	0.078	3.097	104.0	2.68
	0.750	29.904	0.735	29.292	98.0	6.15
	7.500	299.035	7.394	294.822	98.6	1.98
	75.000	2990.353	74.918	2987.070	99.9	5.63

Table 4. Rat plasma: QC sample statistics for tryptic peptides used to quantify CRP.

Table 5A.

Peptide	CRP overspike concentration (µg/mL)	CRP overspike concentration (nM)	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
AFVPFK Lot #1	0.000	0.000	0.387	15.420	0.387	15.420	100.0	3.66
	0.075	2.990	0.462	18.421	0.481	19.191	104.3	0.60
	0.750	29.904	1.137	45.334	1.176	46.889	103.5	2.51
	7.500	299.035	7.887	314.466	7.941	316.605	100.7	2.23
	75.000	2990.353	75.387	3005.783	74.678	2977.515	99.1	1.25
AFVPFK Lot #2	0.000	0.000	1.162	46.331	1.162	46.331	100.0	4.55
	0.075	9.968	1.237	49.321	1.167	46.543	94.4	4.52
	0.750	29.904	1.912	76.234	1.750	69.775	91.5	1.15
	7.500	299.035	8.662	345.366	8.398	334.853	96.9	2.29
	75.000	2990.353	76.162	3036.684	72.141	2876.348	94.7	1.74
AFVPFK Lot #3	0.000	0.000	1.867	74.440	1.867	74.440	100.0	1.20
	0.075	9.968	1.942	77.430	1.787	71.250	92.0	3.92
	0.750	29.904	2.617	104.343	2.290	91.319	87.5	3.17
	7.500	299.035	9.367	373.475	8.527	339.983	91.0	1.29
	75.000	2990.353	76.867	3064.793	72.444	2888.429	94.2	13.31
AFVPFK Lot #4	0.000	0.000	18.128	722.778	18.128	722.778	100.0	2.26
	0.075	9.968	18.203	725.779	17.612	702.201	96.8	6.47
	0.750	29.904	18.878	752.692	17.586	701.178	93.2	1.72
	7.500	299.035	25.628	1021.824	23.988	956.421	93.6	5.59
	75.000	2990.353	93.128	3713.141	83.284	3320.634	89.4	1.37

Table 5B.

Peptide	CRP overspike concentration (µg/mL)	CRP overspike concentration (nM)	CRP QC concentration (µg/mL)	CRP QC concentration (nM)	Mean (N=3) calculated concentration (µg/mL)	Mean (N=3) calculated concentration (nM)	Mean (N=3) % accuracy	%RSD
ESDTSYVSLK Lot #1	0.000	0.000	0.439	17.514	0.439	17.514	100.0	5.21
	0.075	2.990	0.514	20.494	0.507	20.215	98.7	1.61
	0.750	29.904	1.189	47.407	1.196	47.686	100.5	5.37
	7.500	299.035	7.939	316.539	7.781	310.252	98.0	0.73
	75.000	2990.353	75.439	3007.857	73.159	2916.937	97.0	1.19
ESDTSYVSLK Lot #2	0.000	0.000	1.188	47.347	1.188	47.347	100.0	2.36
	0.075	9.968	1.263	50.358	1.269	50.597	100.5	2.99
	0.750	29.904	1.938	77.271	1.894	75.503	97.7	1.26
	7.500	299.035	8.688	346.403	8.295	330.746	95.5	1.40
	75.000	2990.353	76.188	3037.720	74.171	2957.300	97.3	1.36
ESDTSYVSLK Lot #3	0.000	0.000	1.867	74.440	1.867	74.440	100.0	1.20
	0.075	9.968	1.942	77.430	1.787	71.250	92.0	3.92
	0.750	29.904	2.617	104.343	2.290	91.319	87.5	3.17
	7.500	299.035	9.367	373.475	8.527	339.983	91.0	1.29
	75.000	2990.353	76.867	3064.793	72.444	2888.429	94.2	13.31
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	0.075	9.968	18.203	725.779	17.612	702.201	96.8	6.47
	0.750	29.904	18.878	752.692	17.586	701.178	93.2	1.72
	7.500	299.035	25.628	1021.824	23.988	956.421	93.6	5.59
	75.000	2990.353	93.128	3713.141	83.284	3320.634	89.4	1.37

Table 5. Human plasma: QC sample statistics for tryptic peptides, AFVPFK (A) and ESDTSYVSLK (B), used to quantify CRP in four lots of human plasma.

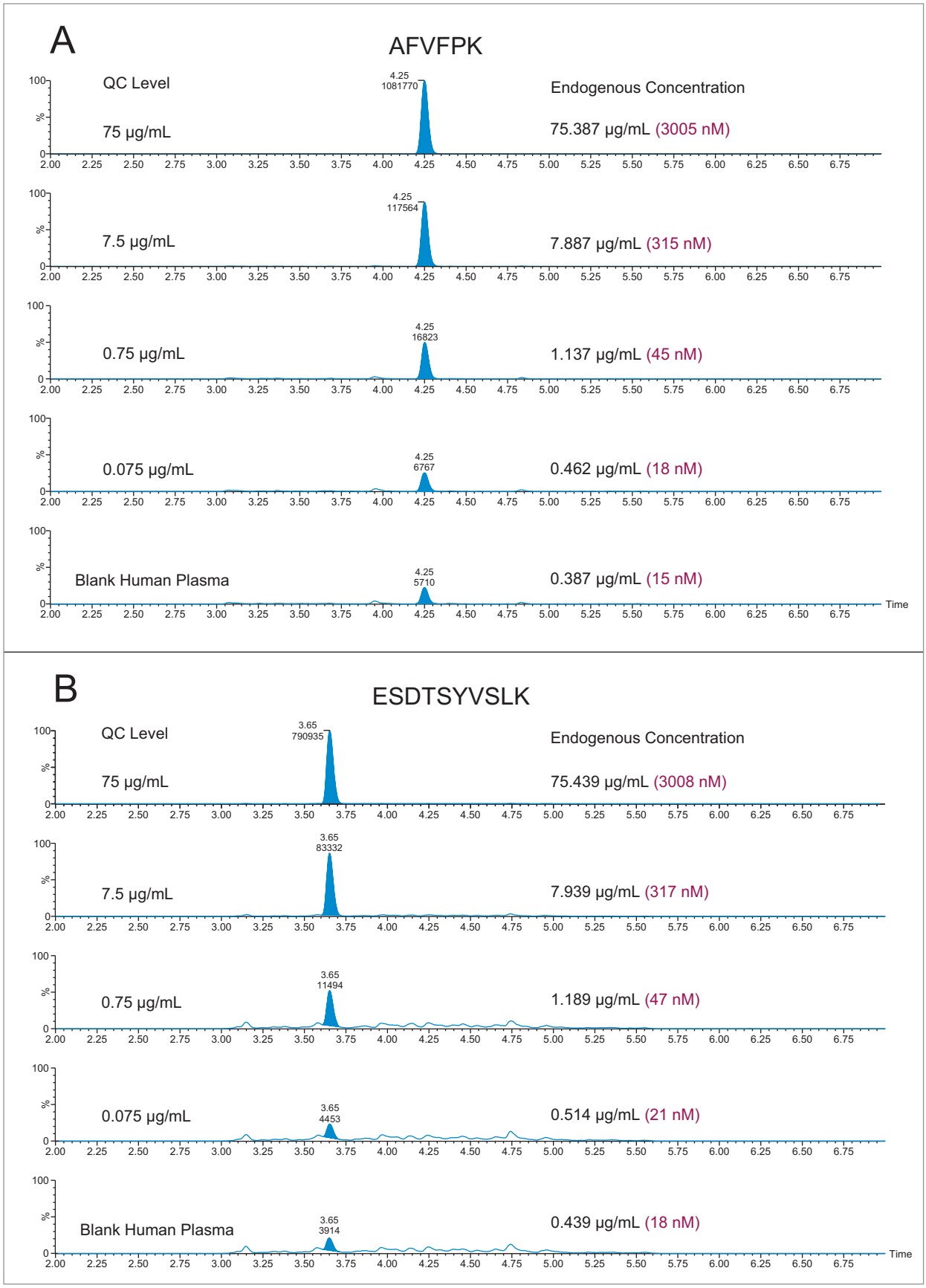

Figure 6. QC chromatograms for AFVPFK (A) and ESDTSYVSLK (B) in human plasma (Lot #1). QC concentrations (overspike concentration+endogenous concentration) can be seen to the right of the QC level.

Endogenous human CRP concentrations were accurately quantified in a total of four lots of human plasma. The lowest level of CRP that could be accurately and precisely quantified above the endogenous level in human plasma was 0.05 µg/mL (2 nM). For the blank human plasma digest (Figure 6), there is a strong signal from the human CRP peptide, due to endogenous CRP concentrations in the range of 0.4–0.666 µg/mL. In rat plasma blank digest (Figure 4), human CRP peptide is not detected. This is expected as the rat CRP amino acid sequence is different than human CRP sequence. Calculated endogenous CRP levels for the four lots of human plasma are summarized Table 6 and illustrated in Figure 7, panels A (AFV peptide) and B (ESD peptide). Within each plasma lot, the calculated endogenous CRP concentrations derived from either the AFV or ESD tryptic peptides were within 10% agreement. Confirmatory transitions for each peptide were used to verify endogenous concentrations of each plasma lot. With the exception of Lot 1, the concentrations calculated from the primary and confirmatory transitions were within 10% of each other. Endogenous CRP concentrations calculated from the GYS peptide did not correlate with those calculated by the AFV and ESD peptides. It is speculated that an underlying, co-eluting interference impeded accurate quantification using the GYS peptide.

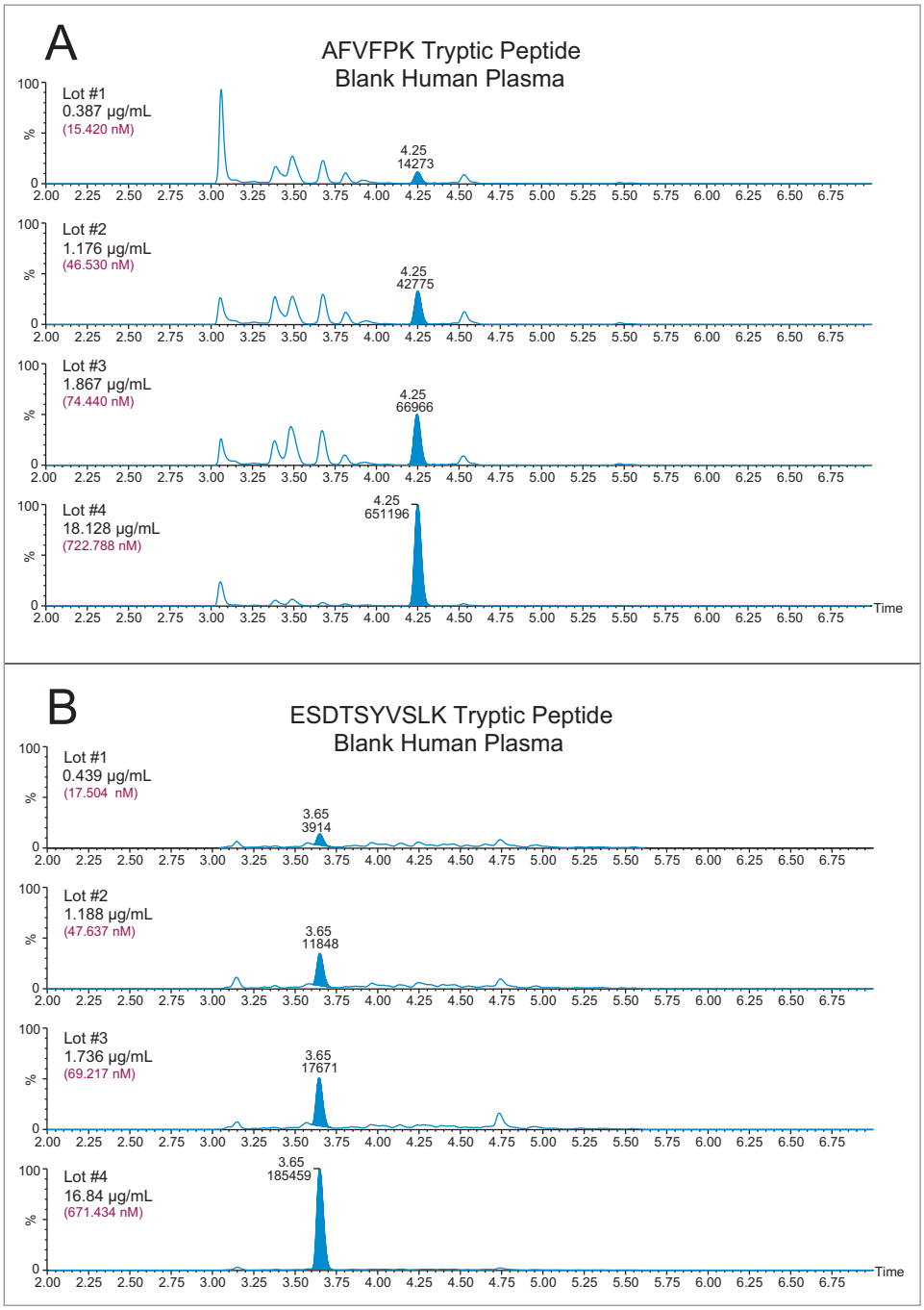

Figure 7. Representative chromatograms highlighting endogenous CRP concentrations in four lots of human plasma represented by the AFVPFK (A) and ESDTSYVSLK (B) tryptic peptides of CRP.

Peptide	Plasma	Mean (N=3) Calculated Endogenous Concentration (µg/mL) Primary MRM	Mean (N=3) Calculated Endogenous Concentration (µg/mL) Confirmatory MRM	Mean (N=3) Calculated Endogenous Concentration (nM) Primary MRM	Mean (N=3) Calculated Endogenous Concentration (nM) Confirmatory MRM
		354>244	**354>219**	**354>244**	**354>219**
AFVPFK	Lot #1	0.387	0.381	15.420	15.191
	Lot #2	1.167	1.145	46.331	45.653
	Lot #3	1.867	1.89	74.440	75.357
	Lot #4	18.128	18.273	722.778	728.570
		564>347	**564>696**	**564>347**	**564>696**
ESDTSYVSLK	Lot #1	0.439	0.666	17.514	26.554
	Lot #2	1.188	1.145	47.347	45.653
	Lot #3	1.736	1.952	69.197	77.829
	Lot #4	16.84	17.015	671.414	678.411

Table 6. Calculated endogenous CRP concentrations in four lots of human plasma using the AFVPFK (A) and ESDTSYVSLK (B) tryptic peptides of CRP.

CONCLUSION

Endogenous CRP was reliably quantified down to 0.025 µg/mL (1 nM) using commercially available digestion and purification kits. Plasma (35 µL) was directly digested (no affinity purification necessary) and subsequent tryptic peptides purified using the generic protocols provided in the kits. The combination of mixed-mode SPE and protocol resulted in >90% recovery for the three peptides. Total sample preparation time, including SPE, was <3 hours. Updating the MS platform, Xevo TQ-S to Xevo TQ-XS, improved sensitivity and S/N, consequently improved the LOQ. This method demonstrates the accurate, reproducible quantification of endogenous CRP levels in plasma using a generic kit-based approach. The broad dynamic range and specificity of LC-MS reliably measures low endogenous levels and the elevated levels expected in diseased patients.

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