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Rapid Digestion and Reproducible LC-MS Quantification of Cytochrome C: A Potential Biomarker for Apoptosis

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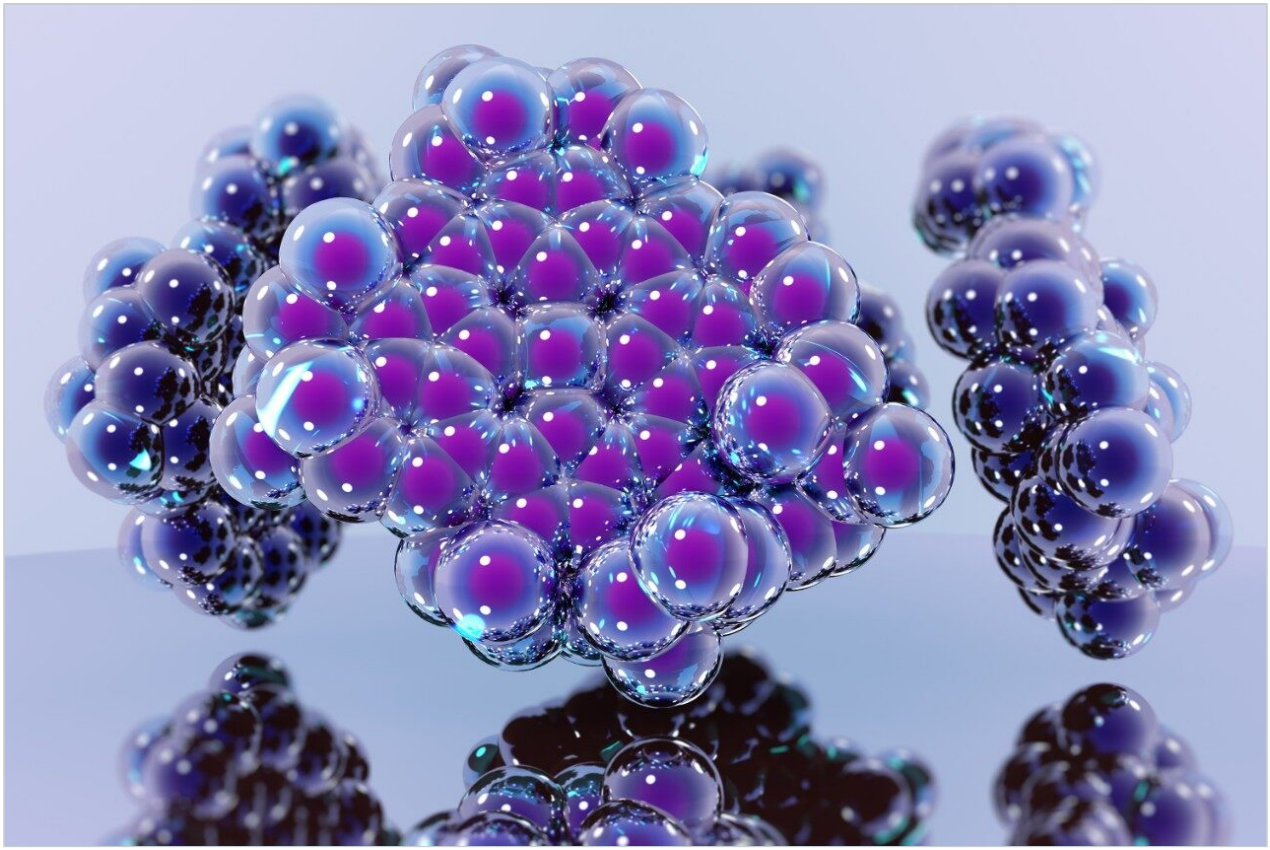

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Abstract

This application note demonstrates proof of concept for the rapid, accurate and precise quantification of the protein biomarker, cytochrome C.

Benefits

Rapid digestion and quantification of a protein biomarker, demonstrating the flexibility, speed, and reproducibility of a generic kit-based approach.

Introduction

Cytochrome C (Figure 1),[1] is a mitochondrial protein (~13kDa) which plays important roles in oxidative phosphorylation and apoptosis, or programmed cell death.[2] Elevated plasma concentrations (~2 µg/mL) of circulating cytochrome C have been reported in patients with conditions associated with mitochondrial damage.[3] As a result, the ability to accurately quantify cytochrome C as a potential biomarker is of high interest. Historically, cytochrome C has been quantified using ligand binding assays (LBAs) or western blot analysis. However, use of LC-MS analysis for protein quantification has become more popular in the past few years due to the many benefits it offers (e.g., multiplexing, improved specificity, broader linear dynamic range, and faster method development times). For protein quantification by LC-MS, the bottom up approach using enzymatic digestion (usually trypsin) and analysis of resulting tryptic peptides is often employed. However, these protein digestion workflows are complex and time consuming, with enzymatic digestions often taking upwards of 24 hours to achieve sensitive and accurate quantification from complex biological matrices. Thus, there is a strong need for simpler, more standardized LC-MS workflows. In this application note, we describe a fast (10-minute) digestion using the ProteinWorks eXpress Direct Digest Kit and post digest peptide clean-up using ProteinWorks µElution SPE Clean-up Kit for the accurate quantification of cytochrome C in plasma.

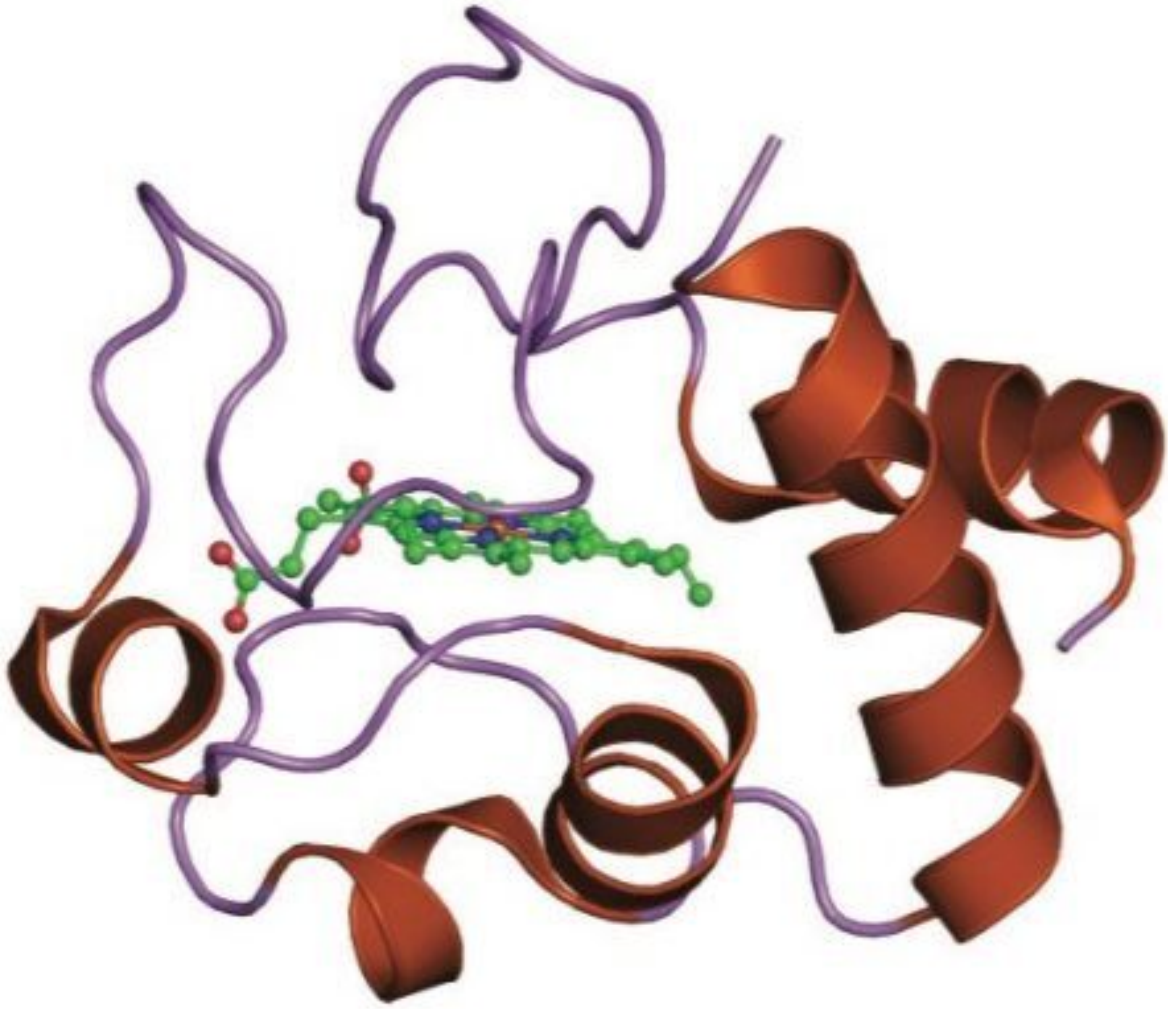

Figure 1. Cytochrome C protein structure.

Experimental

Sample description

To prepare standards and quality control (QC) samples, cytochrome C (derived from bovine heart) was spiked into plasma at various concentrations (0.5–250.0 µg/mL). Plasma samples (35 µL) were digested for 10 minutes using the ProteinWorks eXpress Direct Digest kit. Post digestion purification of signature peptides was completed using the ProteinWorks µElution SPE Clean-up Kit and supplied protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+
Column:	CORTECS UPLC C_{18+} Column, 1.6 µm, 2.1 mm x 50 mm
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	0.400	98.00	2.00	6
0.50	0.400	98.00	2.00	6
3.75	0.400	60.00	40.00	6
3.80	0.400	10.00	90.00	6
4.35	0.400	10.00	90.00	6
4.40	0.400	98.00	2.00	6

Time (min)	Flow rate (mL/min)	%A	%B	Curve
5.00	0.400	98.00	2.00	6

MS conditions

Capillary (kV):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/Hr):	150
Desolvation gas flow (L/hr):	1000
Collision gas flow (mL/min):	0.15
Nebulizer gas flow (Bar):	7

Results and Discussion

Mass spectrometry

Preliminary digestion experiments in buffer were performed to identify tryptic peptides and corresponding MRM transitions for cytochrome C quantification. Four unique signature tryptic peptides were identified: TGPNLHGLFGR, MIFAGIK, EDLIAYLK, and GITWGEETLMEYLENPKK. The amino acid sequence of

cytochrome C and unique signature peptides (highlighted in orange) can be seen in Figure 2.[4] With the exception of the GITW tryptic peptide, where the triply charged precursor was used, the doubly charged precursors were determined to be the most intense and generated highly specific y ion fragments. MS conditions are summarized in Table 1.

Peptide	MW	pI	Precursor charge state	MRM transition	Cone voltage (V)	Collision energy (eV)	Product ion identification
TGPNLHGLFGR	1168.32	10.2	$[M+2H]^{2+}$	584.82>505.90	30	15	[2H+]1/y9
MIFAGIK	779.01	9.4	$[M+2H]^{2+}$	390.23>534.60	30	10	[1H+]1/y5
EDLIAYLK	964.13	4.3	$[M+2H]^{2+}$	482.77>494.30	30	15	[1H+]1/y4
GITWGEETLMEYLENPKK	2138.43	4.5	$[M+3H]^{3+}$	713.35>840.50	30	15	[2H+]1/y14

Table 1. Final MS conditions for cytochrome C tryptic peptides, including precursor and fragment ions and other physiochemical properties (MWT and pI).

MGDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFSYTDANKN
KGITWGEETLMEYLENPKKYIPGTKMIFAGIKKKGEREDLIAYLKKATNE

Figure 2. Amino acid sequence and structure of cytochrome C (derived from bovine heart). Tryptic peptides used for quantification are highlighted in orange.

Chromatographic separation

Chromatographic separation of cytochrome C tryptic peptides was achieved using a CORTECS UPLC C_{18}+, 1.6 µm, 2.1 mm x 50 mm Column. CORTECS C_{18}+ Columns combine the benefits of solid-core particle technology and a low-level positive surface charge, which provides excellent peak shape, narrow peak widths (<3 secs at base), and resolution from matrix interferences. Representative chromatograms for the four cytochrome C tryptic peptides are shown in Figure 3. Here you can also see that the TGPN peptide (2.12 minutes) elutes immediately before the MIFA peptide (2.16 minutes).

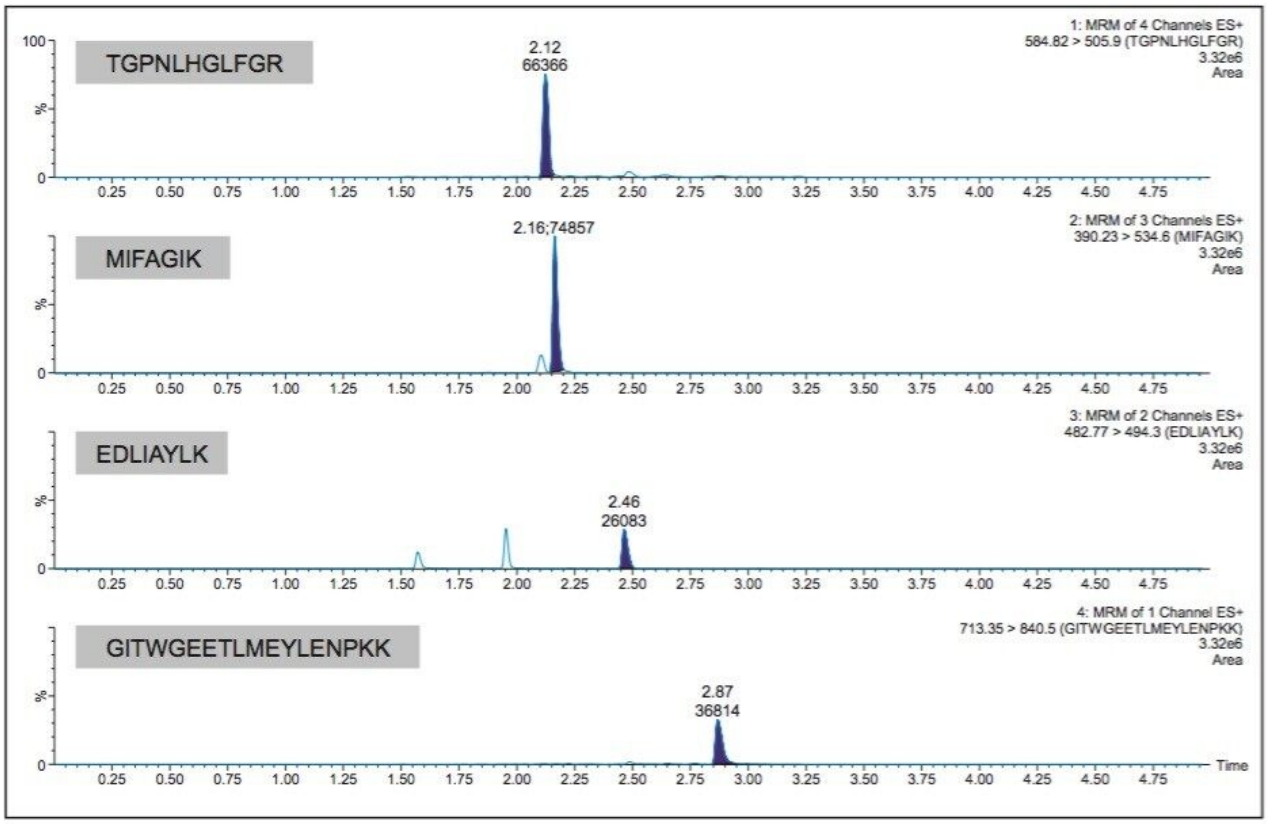

Figure 3. UPLC chromatographic separation of cytochrome C tryptic peptides (mid QC samples), using the CORTECS UPLC C_{18}+ 90Å, 1.6 µm, 2.1 mm x 50 mm Column.

These two peptides share a common precursor and fragment pair (390.23>534.6) derived from different charge states and fragment ions. This common MRM pair corresponds to the 3+ precursor and [2H+]1/y10 fragment for the TGPN peptide and the 2+ precursor and [1H+]1/y5 fragment for the MIFA peptide. Use of the CORTECS UPLC C_{18}+ Column ensured chromatographic separation of these peaks that was not obtainable using a BEH C_{18} 300Å (also 2.1 x 50 mm) Column. This is illustrated in Figure 4.

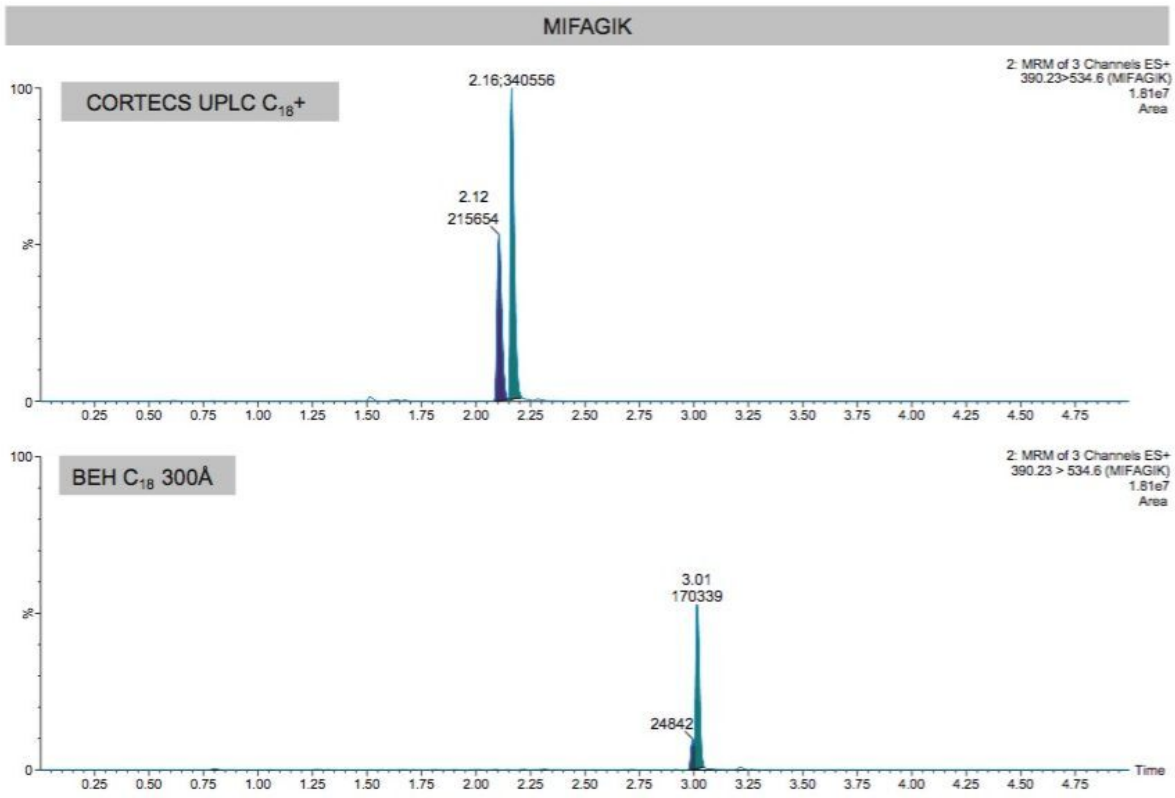

Figure 4. Representative chromatograms demonstrating improved specificity and resolution from interference (RT 2.12) using the CORTECS UPLC C_{18}+ for the MIFAGIK (RT 2.16) cytochrome C tryptic peptide.

Sample preparation

For tryptic peptide purification, use of a mixed-mode sorbent (reversed-phase and ion-exchange retention) provided enhanced specificity, while the µElution plate format minimized peptide loss (eliminating evaporation and reconstitution). This combination provided excellent recoveries (≥90%) for all four cytochrome C tryptic peptides. A comparison of raw area counts for the MIFA (Panel A) and GITW (Panel B) cytochrome C tryptic peptides with and without SPE clean up is shown in Figure 5. For both peptides, the SPE samples exhibited a peptide area increase of more than 2X, without any form of concentration (SPE sample load volume of 100 µL and final SPE eluate volume of 100 µL). This increase could be the result of eliminating salts or phospholipids not seen in the specific transition.

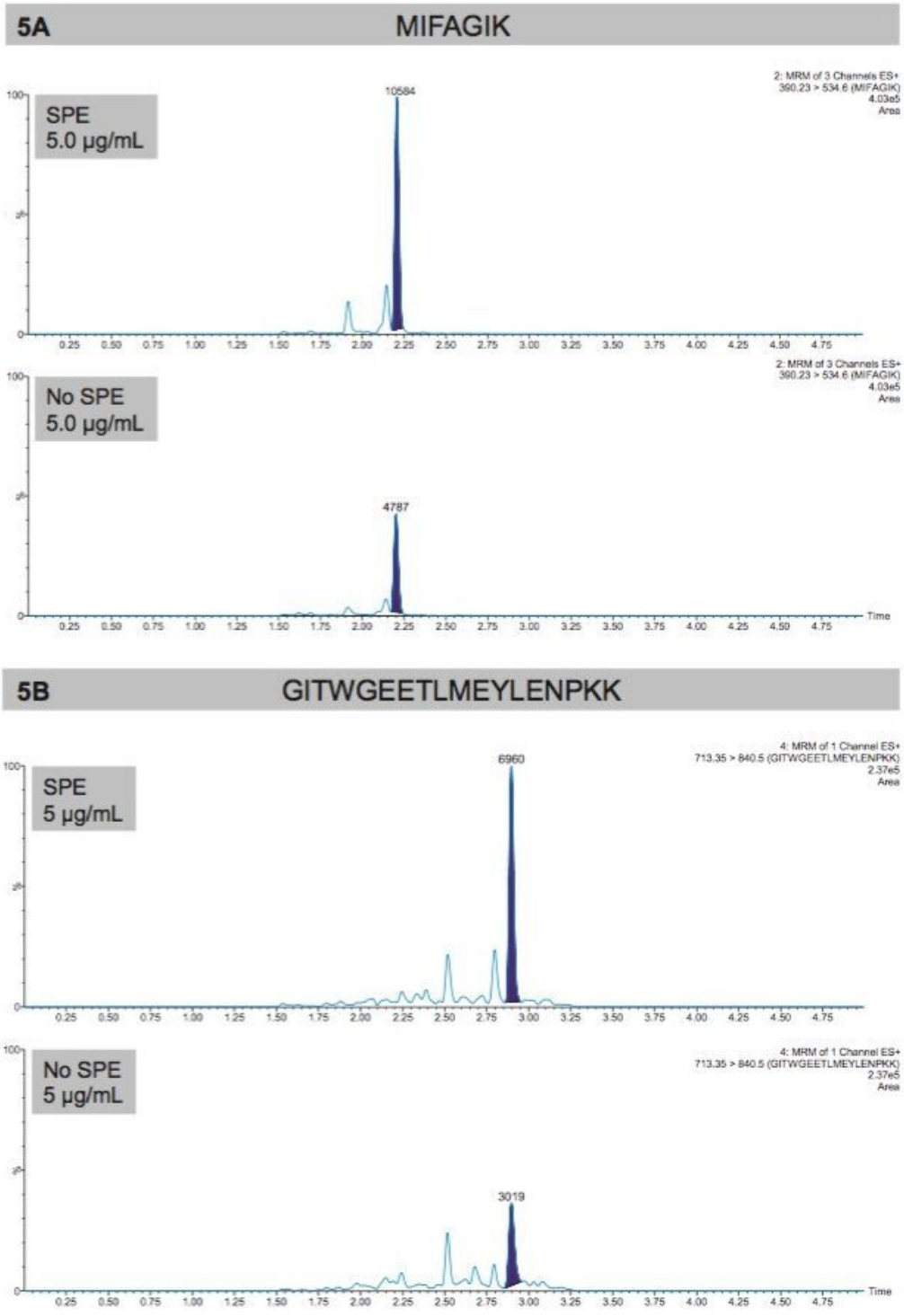

Figure 5. Chromatograms showing the improved sensitivity of the MIFA (A) and GITW (B) peptides following purification with µElution SPE Kit.

Linearity, precision, and accuracy

Following digestion of 35 µL of plasma and subsequent SPE clean-up, excellent linearity and single digit RSDs (relative standard deviation) for duplicate calibration curves were readily achieved. Using 1/x2 regression, the curves from all 4 tryptic peptides were linear with R^2 values >0.99. A summary of standard curve performance is shown in Table 2. Results from QC analysis are shown in Table 3. For all four peptides, at all QC levels, samples demonstrated excellent accuracy and precision with an average %CV of 2.5%. Figure 6, panels A and B, demonstrate sensitivity for plasma samples at 0.5 and 2.0 µg/mL compared to the blank plasma for the EDLI and GITW peptides. Generally, the limit of quantification (LOQ) and limit of detection (LOD) are the concentrations which yield peak area counts ≥5X and 3X of the blank matrix sample, respectively. In the case of the EDLI and GITW peptides at 2.0 µg/mL, the reported LOQ peptide peak areas were 25X and 31X that of the blank plasma sample. The lowest concentration assessed was 0.5 µg/mL and was reported as the LOD. At this concentration the peptide peak areas were 7.5X and 8.3X that of the blank plasma sample. If one were to extrapolate based on the aforementioned criteria, the approximate LOD would be ~0.2 µg/mL.

Peptide	Std. curve range (µg/mL)	Limit of detection (LoD)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
TGPNLHGLFGR	2.0-250	2.0	1/x2	0.996	100.01
MIFAGIK	2.0-250	0.5	1/x2	0.998	100.86
EDLIAYLK	2.0-250	0.5	1/x2	0.999	99.99
GITWGEETLMEYLENPKK	2.0-250	0.5	1/x2	0.999	100.00

Table 2. Linear dynamic range and standard curve statistics for the cytochrome C tryptic peptides used for quantification. Plasma samples were digested and extracted using ProteinWorks eXpress Direct Digest and µElution SPE Clean-Up Kits.

Peptide	QC conc. (µg/mL)	Mean QC conc. (µg/mL)	Std. dev.	%CV	Mean accuracy
TGPNLHGLFGR	3.000	2.850	0.058	2.03	93.93
	15.000	15.800	0.503	3.19	105.60
	75.000	79.500	1.457	1.83	104.83
	200.000	186.200	2.858	1.54	93.90
MIFAGIK	3.000	2.900	0.058	1.99	95.50
	15.000	15.150	1.136	7.50	102.63
	75.000	81.450	1.513	1.86	108.30
	200.000	194.050	6.058	3.12	98.03
EDLIAYLK	3.000	2.700	0.000	0.00	90.00
	15.000	14.400	0.503	3.50	97.70
	75.000	76.150	0.874	1.15	101.37
	200.000	184.300	6.306	3.42	93.83
GITWGEETLMEYLENPKK	3.000	2.750	0.058	2.10	91.67
	15.000	14.500	0.346	2.39	98.03
	75.000	77.200	1.069	1.39	102.47
	200.000	187.300	6.080	3.25	95.27

Table 3. QC sample statistics for cytochrome C tryptic peptides used to quantify cytochrome C in plasma.

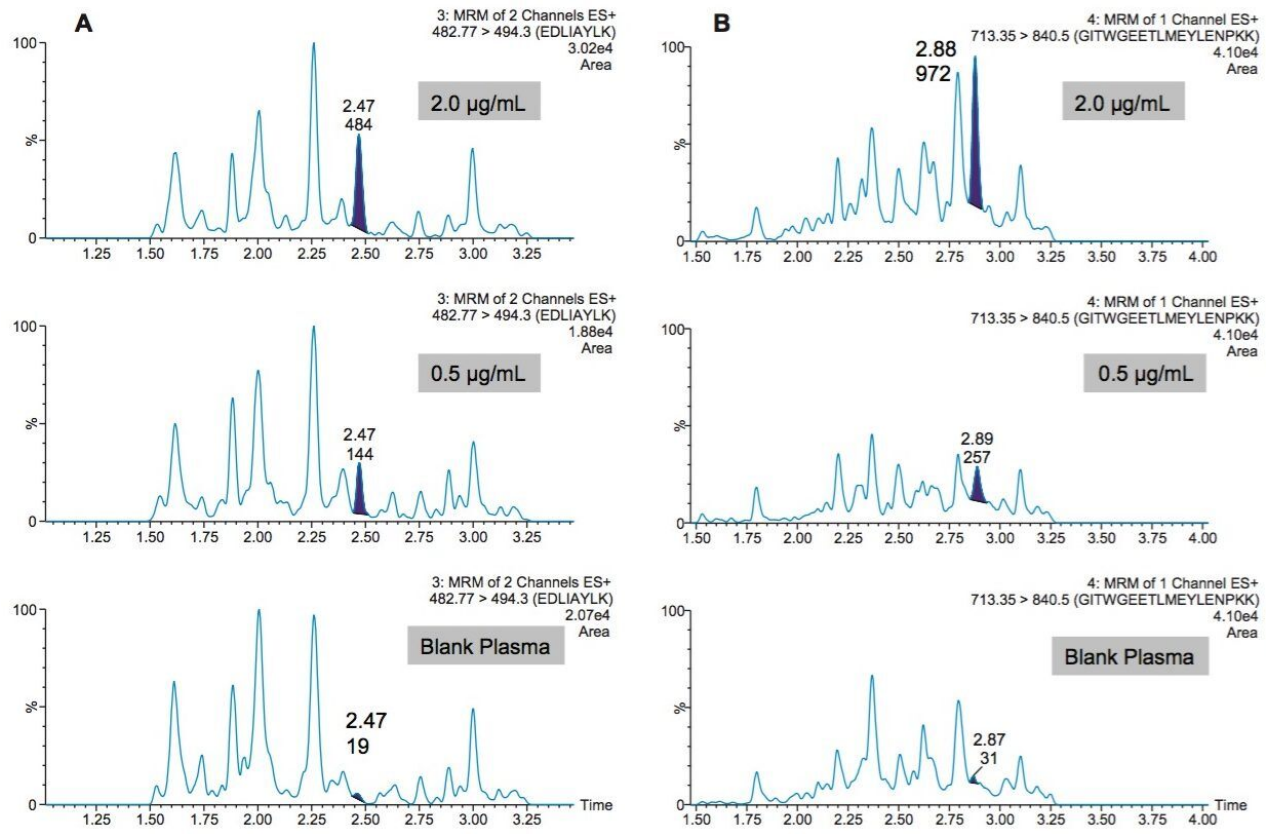

Figure 6. Chromatograms demonstrating sensitivity of the MIFA (A) and GITW (B) peptides, at the LOQ (2.0 µg/mL) and LOD (0.5 µg/mL), respectively.

Conclusion

This work demonstrates proof of concept for the rapid, accurate and precise quantification of the protein biomarker, cytochrome C. The combination of the ProteinWorks eXpress Digest Kit (10 minute sample digestion) and µElution SPE Clean-up Kit (15 minute SPE) allows sample preparation to be completed in ~40 minutes, while a fast, 5 minute LC-MS method allows analysis of a full 96-well plate in 8 hours. Using this kitted approach for protein quantification eliminates the need for method development and allows both inexperienced and experienced bioanalytical labs to quickly generate robust, accurate and precise data while also achieving low, single digit reproducibility.

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