ANALYSIS OF SARS-COV-2 USING LC-MS PEPTIDE ENRICHMENT FOR CLINICAL RESEARCH

Dominic Foley[1], Robert Wardle[1], Samantha Ferries[1], Rebecca Pattison[1], Jennifer Warren[2], Lisa J Calton[1]

[1]Waters Corporation, Stamford Avenue, Altrincham Road, Wilmslow, SK9 4AX, UK, [2]Waters Technologies Ireland Ltd, Wexford, Ireland

INTRODUCTION

Detection of SARS-CoV-2 is critically important in clinical research to understand the impact of SARS-CoV-2 infection. The detection of low levels of viral proteins may indicate viral replication and infection, and could enable investigation of long-term impact of the virus in longitudinal research studies. Polymerase Chain Reaction (PCR) is widely used to indirectly measure the virus through amplification of viral mRNA, whereas LC-MS/MS technology can be used to directly detect viral proteins through selective and analytically sensitive detection of a range of peptides liberated from viral proteins following tryptic digestion. SISCAPA® (Stable Isotope Standards with Capture of Anti-Peptide Antibodies) is a technique used in proteomics to improve the detection limits of LC-MS/MS peptide methods. In this instance, the analytical sensitivity of viral proteins is greatly improved, expanding the capability of the technique to detect SARS-CoV-2.

METHODS

Materials

- The Waters™ SARS-CoV-2 LC-MS Starter Kit (RUO) (Figure 1) was used to perform the sample preparation and analysis. The kit contains SARS-CoV-2 Nucleocapsid (NCAP) peptide calibrators; AYNVTQAFGR (AYN), ADETQALPQR (ADE) and NPANNAAIVLQLPQGTTLPK (NPA) peptide stable labelled internal standards, ammonium bicarbonate, Rapigest™ SF, trypsin, TLCK, CHAPS, PBS, magnetic bead bound anti-peptide antibodies, formic acid, the ACQUITY™ Premier Peptide BEH™ 300Å C18 Column and QuanRecovery™ 96-well plate with adhesive seals and silicone mat.
- Using the peptide calibrator, calibrators were prepared from 3 - 50000 amol/µL in Viral Transport Medium (VTM) (Liofilchem, Italy). Independent QCs were prepared using NCAP protein (R&D systems, USA) and NCAP peptides (Vivitide, USA) in VTM.

Figure 1. The SARS-CoV-2 LC-MS Starter Kit (RUO)

Methods

- The SARS-CoV-2 workflow (Figure 2) is described in the instructions for use (IFU)[1] accompanying the Kit. The Andrew Alliance™ Andrew+™ Pipetting Robot with OneLab™ software was used for sample preparation.
- DIGESTION: 180µL VTM samples were denatured for 15 minutes with Rapigest SF in ammonium bicarbonate.
- Trypsin was added, the well plate mixed, and samples digested for 30 minutes at 37°C. TLCK was then added to quench the digestion.
- The SIL NCAP peptides were added and samples were mixed.
- ENRICHMENT: SISCAPA ADE, AYN and NPA antibody magnetic beads were mixed together. An aliquot of the pool was added to each sample.
- The well plate was shaken at room temperature for 60 minutes.
- On completion, the well plate was placed on the sample magnetic array for 1 minute. The accessible liquid was discarded.
- The samples were mixed for 1 minute with CHAPS in PBS. The plate was transferred to the magnetic array and after 1 minute the entire liquid volume was discarded.
- The wash step was repeated prior to elution of the bound peptides.
- The samples were mixed for 5 minutes with formic acid in CHAPS. On completion the well plate was placed on the magnetic array and the eluate was transferred to a new QuanRecovery plate. This was sealed with the silicone mat prior to injection.
- ANALYSIS: Using a Waters ACQUITY UPLC™ I-Class System, samples were injected onto an ACQUITY Premier Peptide BEH 300Å C18 1.7 µm 2.1 x 30 mm Column, using a water/acetonitrile/formic acid gradient and analyzed with a Waters Xevo™ TQ-XS detector, using MRM (Table 1).
- The analysis time per sample was approximately 2.5 minutes injection to injection.

Analyte	ESI Mode	MRM Transition (m/z)	Cone Voltage (V)	CE (eV)
ADE	+	564.8 > 400.2 (584.4)	35	19 (20)
ADE SIL	+	569.8> 410.2	35	19
AYN	+	563.8 > 679.4 (892.5)	35	19
AYN SIL	+	568.8 > 689.4	35	19
NPA	+	687.4 > 841.5 (865..5)	35	18 (23)
NPA SIL	+	690.4 > 849.5	35	18

Table 1. MRM parameters for the analysis of the NCAP peptides ADE, AYN and NPA and their stable labelled internal standards. Qualifier ion parameters are in parentheses

RESULTS

Analytical Sensitivity

- The lower limit of quantification (LLoQ) was determined to be 3 amol/µL for AYN, ADE and NPA, which provide a concentration with precision <20%, bias within ±20% and S/N >10:1 (PtP) (Figure 3).

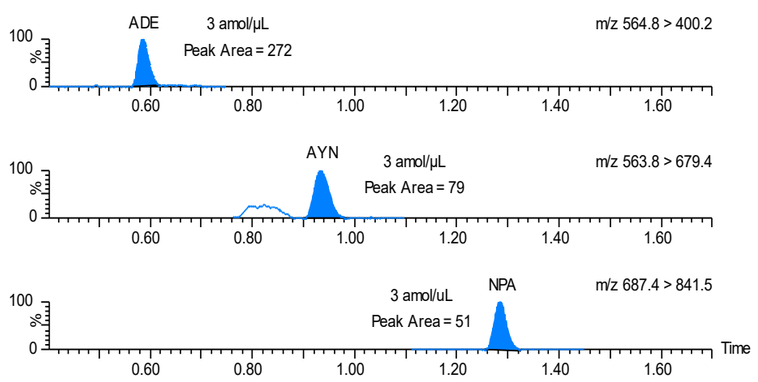

Figure 3. Chromatogram of the ADE, AYN and NPA peptides at 3 amol/µL in VTM after antibody enrichment

Linearity

- Peptide calibration lines prepared from 3 – 50000 amol/µL in VTM were linear with correlation coefficients (r2) >0.99.

Carryover, Autosampler Stability and Matrix Effects

- No significant autosampler carryover was noted in blank samples following injection of 50000 amol/µL.
- Extracted samples were stable on the autosampler for 48 hours.
- Matrix effects in PBS and two types of VTM ranged from 94-97% for ADE, 102 -107% for AYN and 103-108% for NPA.

Precision

- The precision (%CV) of the method was evaluated at 10, 400 and 25000 amol/µL for synthetic peptides and NCAP protein spiked into VTM.
- The inter- and intra-day precision of the method was shown to be ≤12.4% for synthetic peptides and ≤18.8% for NCAP protein derived peptides over five runs (n=25) (Table 2).

Peptide	Intra-Day Precision			Inter-Day Precision		
	10	400	25000	10	400	25000
Spiked NCAP Peptide						
ADE	5.0%	2.0%	2.9%	8.4%	2.6%	3.3%
AYN	6.8%	2.4%	3.0%	10.2%	6.8%	4.7%
NPA	12.4%	3.0%	4.5%	10.9%	5.2%	3.6%
Spiked NCAP Protein						
ADE	7.8%	2.5%	3.1%	16.8%	18.8%	11.8%
AYN	10.2%	2.4%	2.9%	17.6%	18.5%	11.1%
NPA	10.8%	4.1%	3.8%	15.4%	18.2%	13.3%

Table 2. Intra-day and Inter-day precision of the spiked NCAP peptides and spiked NCAP protein enriched from VTM

Identification of the Delta Variant

- Following the rise of the B.1.617.2 (Delta) variant, it was discovered there was an amino acid substitution in the ADE peptide (D377Y, AYETQALPQR) that differentiated it from previous Variants of Concern.
- Through the addition of the AYE MRM transition, it was demonstrated that the ADE SISCAPA could still capture the mutated AYE peptide, which could then be chromatographically separated from the ADE peptide.

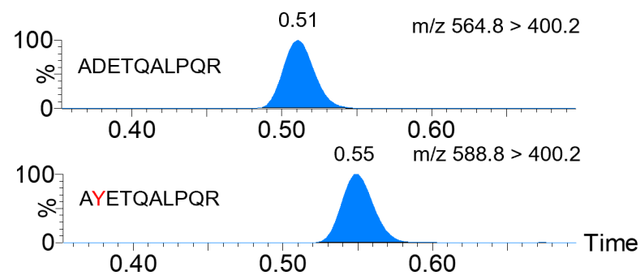

Figure 4. Chromatographic separation and MRM detection of the ADE and AYE peptides following capture with SISCAPA

CONCLUSION

- **The SARS-Cov-2 LC-MS Kit (RUO) was demonstrated to be able to quantify NCAP peptides over the range 3 – 50000 amol/µL, with a precision performance of ≤18.8% CV and a lower limit of quantification of 3 amol/µL.**
- **Direct protein analysis of the virus with quantitative measurement can be used to compare and harmonize results across LC-MS systems and across research labs.**
- **LC-MS analysis offers the ability to detect and measure more than one analyte at a time, meaning the system and Kit could be used to directly detect and quantify SARS-CoV-2 and monitor biomarkers for disease severity or progression for research studies in a single analysis.**

References

1. **https://www.waters.com/webassets/cms/support/docs/720007265en.pdf**

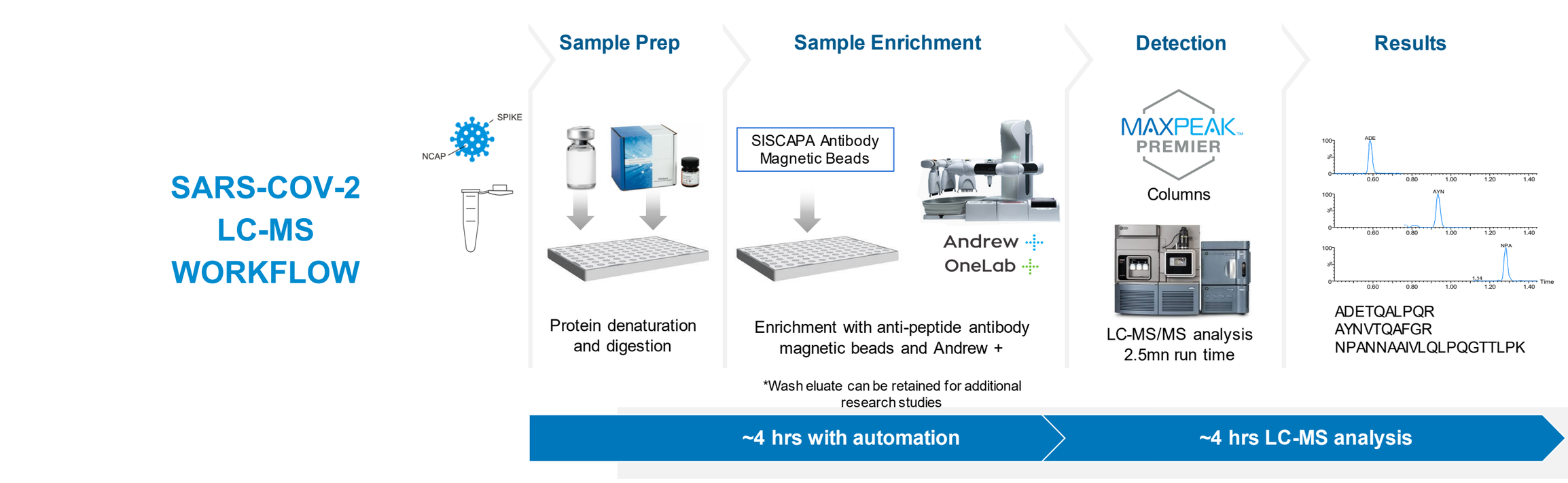

Figure 2. The SARS-CoV-2 LC-MS Kit (RUO) Workflow, utilizing tryptic digestion and SISCAPA automated on the Andrew+ pipetting robot, followed by analysis on the ACQUITY UPLC I-Class Plus and Xevo TQ-XS mass spectrometer. The times are representative of the preparation and analysis of 96 samples.

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