ASMS 2011

ThP 475

Using Thermal Gradient Focusing ESI to Develop Sensitive, High-Throughput Capillary Flow LC/MS/MS Peptide Quantitation Assays

Craig Love, Christine A. Miller, and Alex Mordehai

Agilent Technologies, Santa Clara, CA, USA

Introduction

Given the number of potential protein biomarkers discovered over the past several years, there is a compelling need for sensitive, high-throughput quantitative assays to address the large number of analyses needed to validate their use in clinical applications. Due to the complex nature of commonly used biological matrices, such as blood and plasma, the assay must also be robust. While assays based on capillary flow electrospray ionization offer superior flexibility and robustness, they typically lack the sensitivity of nanoelectrospray ionization based assays. In this study, we investigate the use of a dual funnel QQQ mass spectrometer with thermal gradient focusing electrospray ionization to develop sensitive, robust, high-throughput, capillary flow targeted multiple reaction monitoring (MRM) assays for protein biomarker quantitation.

Experimental

Sample Preparation:

A commercially available synthetic peptide mixture based on human serum albumin (HSA) tryptic peptides was used (Agilent). This HSA peptide standard contains equimolar amounts of AAFTECCQAADK, KVPQVSTPTLVEVSR, LVNEVTEFAK, RPCFSALEVDETYVPK, AVMDDFAAFVEK, YLYEIAR, and HPYFYAPELLFFAK was prepared neat or spiked into trypsinized serotransferrin (10 fmol/µL) at sample concentrations from 0.02 fmol/µL to 25000 fmol/µL.

Agilent 1200 Series Capillary LC:

Column: Agilent Pursuit C18, 0.32 x 150 mm, 3 µm Mobile phase: A= 0.1% Formic acid (FA) in water, B= 0.1% FA in 90% acetonitrile; Injection volume: 1 µL + 6 sec. needle wash; Flow rate: 15 µL/min; Column temp: 35 °C Gradient: 8%B 0 to 0.6 min, 25%B 1 to 4 min, 65%B 6 to 7 min, 90%B 7.1 to 8 min, 8%B at 9 min; Stop time: 9 min; Post time: 3 min

Agilent 1290 Infinity UHPLC:

Columns: Agilent Poroshell 120 2.1 x 150 mm column
Agilent Zorbax 300SB 2.1 x 150 mm column
Mobile phase: A= 0.1% Formic acid (FA) in water, B= 0.1% FA in 90% acetonitrile; Injection volume: 1 µL + 10 sec. needle wash; Injection volume: 1 µL; Column 1 flow: 0.5 mL/min; Column 2 flow: 0.3 mL/min; Column temp: 30 °C Gradient: 3%B at 0 min, 10%B at 0.01 min, 25%B at 3 min, 70%B 3.5 to 4 min, 3%B at 4.1 min; Stop time: 4.5 min; Post time: 3 min.

Experimental

Thermal Gradient Focusing ESI for Capillary LC Flows:

For capillary LC flows, the standard nebulizer of an extended flow thermal gradient focusing ESI source was replaced with a G1385A microflow LC/MS nebulizer. This nebulizer features a 50 µm I.D. needle to maintain chromatographic fidelity and produce smaller droplets, allowing Agilent Jet Stream technology to be used effectively at capillary LC flow rates. (Figure 1)

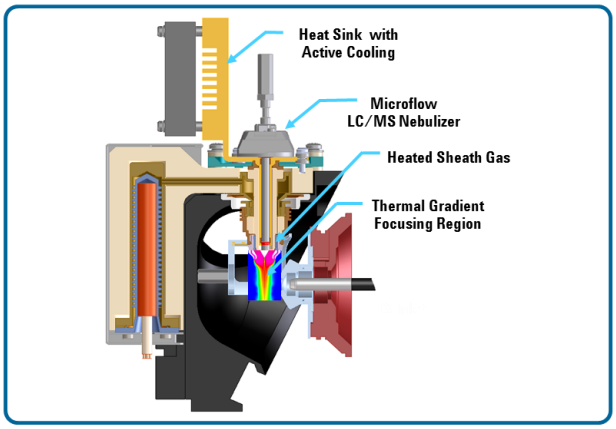

Figure 1: Extended Flow Agilent Jet Stream (AJS) Source

Dual Ion Funnel Triple Quadrupole MS:

An Agilent 6490 triple quadrupole mass spectrometer and MassHunter Optimizer software were was used to develop a targeted MRM assay for the HSA peptides standard. The 6490 QQQ incorporates iFunnel technology which includes the extended flow AJS source shown in Figure 1, coupled to a hexabore capillary array and dual ion funnel . (Figure 2)

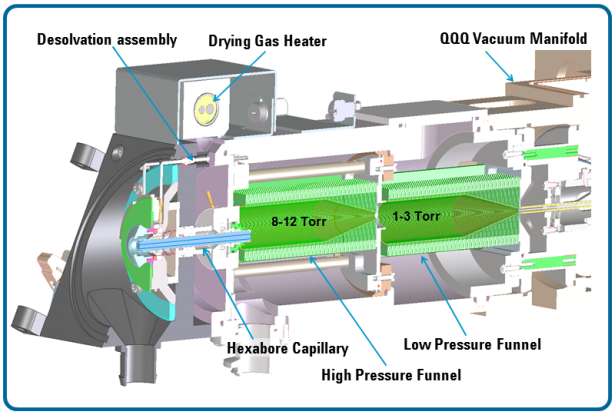

Figure 2: Agilent 6490 dual ion funnel assembly

Results and Discussion

Dual Ion Funnel Triple Quadrupole MS (continued):

MassHunter Optimizer software was used to determine optimal MRM parameters for each peptide using infusion or isocratic FIA injections with a restriction capillary. These results were then imported to develop targeted MRM methods for standard LC and capillary LC flows.

Scan type: MRM; Resolution: MS1/MS2: Unit/Unit (0.7 m/z); Polarity: Positive; Capillary voltage: 3500 V; Nozzle voltage: 300 V.

Source Parameters (1290 LC): Drying gas temp: 200°C, Drying gas flow: 20 L/min, Sheath gas temp: 250°C, Sheath gas flow: 11 L/min, Nebulizer pressure: 30 psig.

Source Parameters (1200 CapLC): Drying gas temp: 150°C, Drying gas flow: 11 L/min, Sheath gas temp: 300°C, Sheath gas flow: 11 L/min, Nebulizer pressure: 20 psig

Impact of Thermal Gradient Focusing on Sensitivity:

To determine the relative benefit of thermal gradient focusing ESI at capillary flow rates, the targeted MRM assay was run using both the extended flow AJS source and a standard ESI source with the following conditions:

Ionization mode:	Agilent Jet Stream	Electrospray
Capillary voltage	3500 V	4000 V
Nebulizer pressure	20 psi	20 psi
Drying gas temp.	150 °C	200 °C
Drying gas flow	11 L/min	14 L/min
Sheath gas temp.	300 °C	N/A
Sheath gas flow	11 L/min	N/A
Nozzle voltage	0 V	N/A

The resulting response normalized AJS vs. ESI MRM chromatograms for a 200 fmol/µL HSA peptides standard sample are shown in Figure 3. Note that the peak numbering in this figure indicates the elution order of the HSA peptides rather than the elution times. As the dashed line indicating the maximum ESI relative response confirms, thermal gradient focusing ESI does provide significant enhancement at capillary LC flows.

The rapid gradient control of the Agilent capillary LC provided separation of the seven peptides in under 7 minutes with a total cycle time of 12 minutes. With further gradient optimization, cycle times under 10 minutes should easily be attainable.

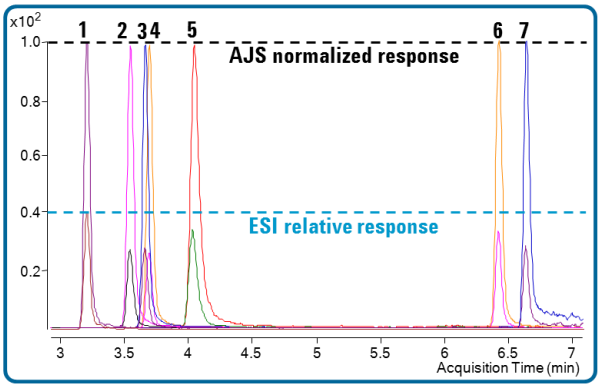

Figure 3: Normalized AJS vs. ESI MRM Chromatograms

The table below indicates the MRM parameters for the most intense transition determined by MassHunter Optimizer software as well as a comparison of the AJS vs. ESI response. A total of 21 transitions were monitored (3 per peptide), using a 10 msec dwell time.

Peak #	Compound Name	Precursor	Product	CE	Dwell	AJS vs. ESI
1	AAFTECCQAADK	686.29	852.3	20	10	2.5x
2	KVPQVSTPTLVEVSR	547.32	589.3	15	10	3.5x
3	YLYEIAR	464.25	651.3	12	10	3.4x
4	LVNEVTEFAK	575.31	937.5	16	10	3.7x
5	RPCFSALEVDETYVPK	637.65	851.4	18	10	3.0x
6	AVMDDFAAFVEK	671.82	1041.5	19	10	3.2x
7	HPYFYAPELLFFAK	581.64	779.4	16	10	3.5x

Impact of Dual Ion Funnel on Sensitivity

To evaluate the impact of iFunnel technology on sensitivity, a standard flow targeted MRM assay of the HSA peptide LVNEVTEFAK was run using an Agilent 6490 QQQ (Figure 4b) and compared with the same assay run using an Agilent 6460 QQQ (Figure 4a). This peptide should be a good model for a "generic" peptide MRM assay since co-eluting peaks are likely to be encountered with complex peptide samples.

Instrument: 1290 LC system + 6460 (AJS) or 6490 (AJS + iFunnel)
Column: Poroshell 120, 2.1 x 150 mm @ 0.5 mL/min
Sample: HSA peptides standard, LVNEVTEFAK (neat)

Although losses during sample preparation and dilution limited the ultimate sensitivity of the assay for both instruments, the Agilent 6490 QQQ with iFunnel technology provided a 10x improvement in sensitivity and greater than 5 orders of linear response.

Results and Discussion

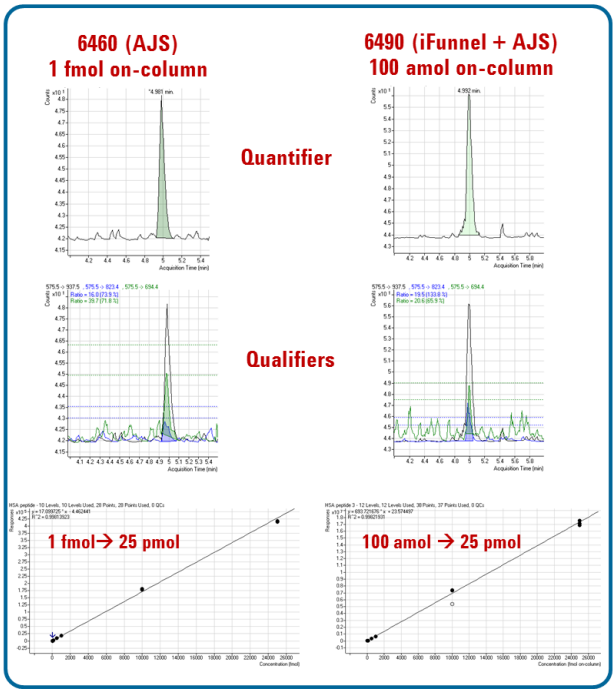

Figure 4: (a) 6460 vs. (b) 6490 Calibration Curves

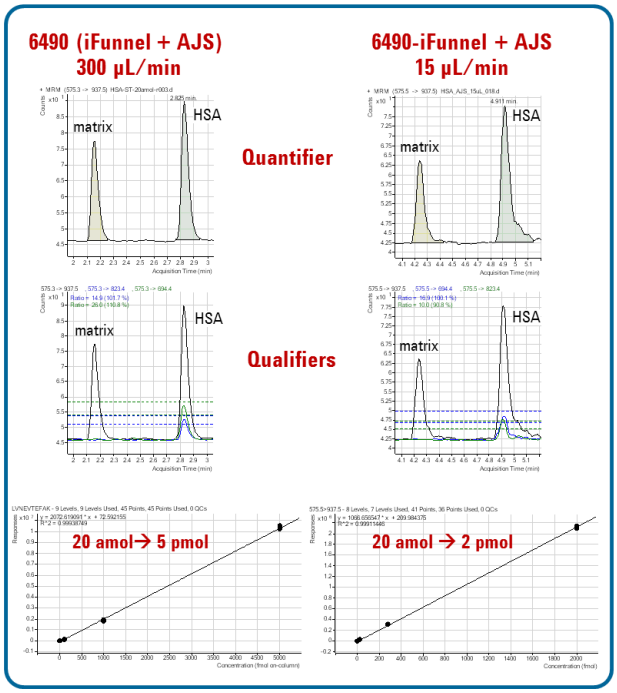

Figure 5: (a) Standard LC Flow vs. (b) Capillary Flow Calibration Curves

Optimizing

With the relative benefits of both Agilent Jet Stream (thermal gradient focusing ESI) and the Agilent 6490 QQQ (iFunnel technology) characterized, the next step was to improve the dilution procedure for the HSA peptides standard to minimize sample losses to active sites. It was found that this could be achieved by diluting the HSA peptides standard using a 10 fmol/µL trypsinized serotransferrin matrix.

Using the improved sample dilution approach, targeted MRM assays of the HSA peptide LVNEVTEFAK were run using an Agilent 6490 QQQ at both standard and capillary LC flows. Both the standard flow and capillary flow calibration curves (Figures 5a, 5b) demonstrate excellent linearity to 20 amol on column. These detection limits were previously achievable only with nanospray/HPLC-Chip based assays or by utilizing sample enrichment strategies.

Instrument: 1290 Infinity LC or 1200 CapLC & 6490 (AJS + iFunnel)
Column (Std. Flow): Zorbax 300SB-C18 2.1 x 150 mm at 0.3 mL/min
Column (Cap. Flow): Pursuit C18 0.32 x 150 mm at 15 µL/min
Sample: HSA peptides standard, LVNEVTEFAK in 10 fmol/µL
trypsinized serotransferrin

Conclusions

- Replacing the standard nebulizer with a microflow LC/MS nebulizer allows extended flow Agilent Jet Stream technology to be used effectively at Capillary LC flows.
- Thermal Gradient Focusing ESI provided greater than 3x signal enhancement vs. standard ESI at 15 µL/min.
- The Agilent 6490 QQQ with iFunnel technology provided over 5 orders of linear response with 10x greater sensitivity.
- Both the standard flow (300 µL/min) and capillary flow (µL/min) assays were able to achieve lower limits of quantitation (LLOQ) of 20 amol on column.
- Area % RSD's for the capillary flow MRM assay ranged from 9.7% at the LLOQ of 20 amol to 1.2% at 2 pmol on-column.
- With further gradient optimization, cycle times under 10 minutes for the seven HSA peptides are attainable for the capillary flow MRM assay.