Application of Differential Ion Mobility Mass Spectrometry to Peptide Quantitation

Using SelexION™ Differential Mobility Separation Technology for better selectivity for peptides in complex mixtures on the AB SCIEX Triple Quad™ 5500 LC/MS/MS System

A Zerr, L Meunier, SW Wood, P Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

Key scientific challenges of peptide quant assays

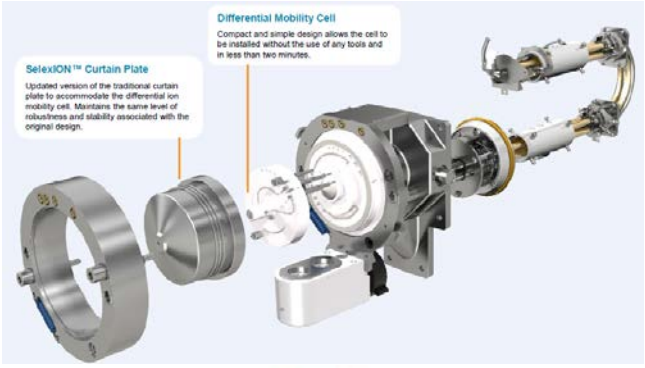
- **Reduced recovery, low sensitivity** – The adsorptive properties and/or polarity of peptides can compromise recovery, and interferences from biological matrices can negatively impact sensitivity and selectivity.
- **Limited quantitation range** – Poor MS/MS sensitivity combined with often poor selectivity can compromise the desired lower limits of quantitation (LLOQ).
- **Limited MRM selectivity** – MRM approaches and efficient UHPLC separations may not provide adequate signal-to-noise ratios at LLOQ due to isobaric interferences or high baseline noise.

Key benefits of differential mobility separation (DMS) for peptide quant assays

- **Background noise reduction enhances LLOQs** – For cases where background noise limits LOQ, DMS provides an additional level of selectivity, orthogonal to the mass spectrometer and LC system.
- **Better sensitivity even with less refined sample prep** – Selectivity gains from DMS permit less selective sample preparations, allowing for overall improvements in sensitivity due to more efficient extractions and better recovery.
- **Selectivity improvements overcome sensitivity losses** – DMS is often accompanied by a loss in absolute sensitivity, but the gains in selectivity improve the potential for real gains in LLOQ.

Key features of SelexION™ Differential Mobility Separation Technology for peptide quant assays

- **Separation of diverse species reduces baseline noise –** SelexION Technology separates isobaric species, and single and multiple charge state interferences to reduce background levels and achieve better selectivity and LOQs **–**while retaining compatibility with UHPLC/MRM speeds.
- **Simple installation and maintenance** – DMS is truly orthogonal to LC and MSMS; installs in minutes with no



tools required and no need to break vacuum. Device maintenance is minimal and very straightforward.

- **Shortened assay cycle** – SelexION™ Technology can potentially reduce chromatographic runtimes.
- **Efficient separation process** – Planar geometry results in short residence times, high speeds, and minimal diffusion losses for maximum sensitivity and UHPLC compatibility.
- **Chemical modifiers for further selectivity** – Introducing chemical modifiers to the homogenous fields of the SelexION™ Device cell allows for amplification of the separation capacity and adds a new dimension of selectivity.
- **Compatible with high-throughput, regulated environments** – SelexION™ Technology provides ruggedness and stability to enable high performance quantitative bioanalysis under GLP settings.

Introduction

The quantitative determination of therapeutic peptides to support pharmacokinetic and toxicokinetic studies can sometimes be challenging. Poor MS/MS sensitivity combined with poor selectivity fragments can compromise the desired lower limits of quantitation (LLOQ). In addition, the adsorptive properties and/or polarity of peptides can compromise recovery, and interferences from biological matrices can negatively impact sensitivity and selectivity. In such cases, MRM approaches—even when combined with efficient UPLC separations—may not be sufficient to provide adequate signal-to-noise ratios at LLOQ in the presence of isobaric interferences or high baseline noise.

Differential ion mobility spectrometry (DMS) may provide a useful tool in such instances by providing an additional, orthogonal degree of selectivity. Although DMS analysis is often accompanied by a loss in absolute sensitivity, the gains in selectivity may be sufficient enough to realize real gains in the LLOQ. Alternatively, selectivity gains from DMS may permit a less selective sample preparation to be used, while still delivering overall improvements in sensitivity due to improved extraction recovery.

This poster presents two case studies were the SelexION™ Differential Mobility Separation Device was evaluated for the quantitation of therapeutic peptides. Selectivity, sensitivity and precision of peptide measurements obtained in experiments with and without DMS were compared. The potential benefits and limitations of the technique are discussed.

Materials and Methods

Sample Preparation

Celerion proprietary peptides, A and B, were used for all experiments

Chromatography

Peptide A-LC system:
Pumps: Series 200 Micro Pump from Perkin Elmer
Auto sampler: Pal CTC from CTC Analytics
Column: Onyx monolithic C18 100x3 mm
Column Temperature: Room Temperature
Injection: 20 uL
Flow Rate: 0.8 mL/min
Mobile Phase A: Methanol/Water/Formic acid 5:95:3 v/v/v
Mobile Phase B: Methanol/Water/Formic acid 95:5:0.2 v/v/v
Gradient

Time (min)	%A	%B
0	100	0
0.5.	100	0
1.0	35	65
2.0	25	75
4.0	10	90
7.0	0	100
7.5	100	0

Peptide B-LC system:
Pumps: Acquity Binary Solvent manager from Waters
Auto sampler: Acquity Sample Manager/Organizer from Waters
ColumnAscentis Peptide ES C18 50x2.1 mm 2.7 um

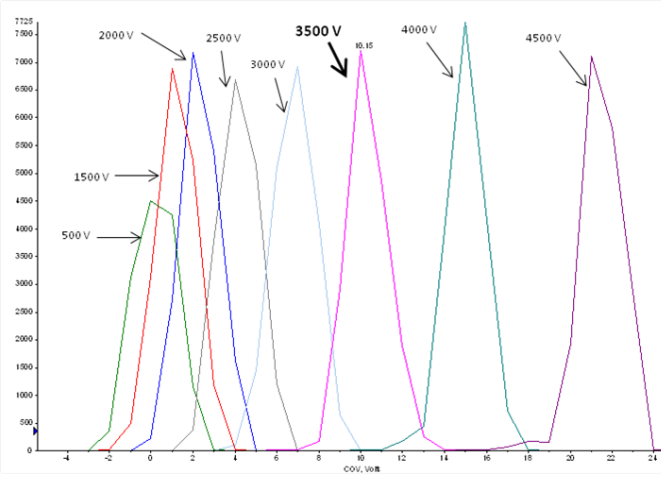


Figure 1 Optimization of separation voltage. The optimization of SV is performed by constant infusion at low analyte flow in solution whilst ramping the compensation voltage.

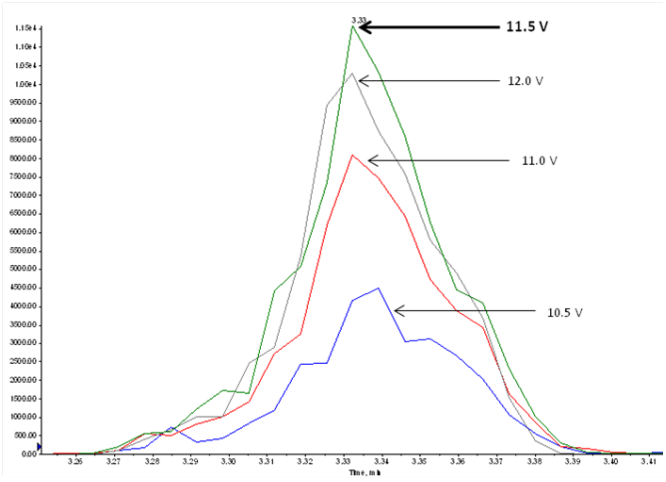


Figure 2 Optimization of compensation voltage. As compensation voltage (COV) is influenced by mobile phase and source conditions, on column COV optimization is performed by injection of analyte at a flow rate and mobile phase composition comparable to the intended LC conditions. In some instances, additional selectivity may be achieved by use of a modifier (*e.g.,* methanol, acetonitrile, isopropanol, acetone) introduced into the SelexION™ Device at low flow. No modifier was used in the case studies presented.

Table 1.MS/MS, MRM, and SelexION™ Device parameters for peptides A and B on the AB SCIEX QTRAP® 5500 System

MS/MS Settings		
	Peptide A	**Peptide B**
Ion source/polarity	ESI/Positive	ESI/Positive
CAD	High	High
CUR	30	30
TEM	700 °C	700 °C
Gas 1	70	50
Gas 2	50	60
Ion Spray Voltage	5500 V	5000 V

MRM Settings				
	Peptide A	**Labeled IS for A**	**Peptide B**	**Labeled IS for B**
Transitions	1029.3/136.0	1106.7/123.0	656.4/249.0	661.4/249.0
Dwell Time (msec)	150	100	100	100
Resolution Q1/Q2	*Unit*	*Unit*	*Unit*	*Unit*

SelexION™ Device Settings		
	Peptide A	**Peptide B**
DT (temperature)	*Low*	*High*
DR (throttle gas)	*Off*	*Off*
COV	*11.5*	*15.0*
DMO	*-3*	*-3*
SV	*3500*	*3500*

Column Temperature: Room Temperature
Injection: 20 uL
Flow Rate: 0.3 mL/min
Mobile Phase A: 0.02% Acetic acid aqueous
Mobile Phase B: Methanol
Gradient

Time (min)	%A	%B
0	90	10
1.0	90	10
3.0	5	95
3.1	90	10
4.0	90	10

SelexION™ Device Settings

The SelexION Device needs only a few minutes for installation onto the AB SCIEX Triple Quad™ 5500 LC/MS/MS System and can be accomplished without breaking the MS vacuum. For the best performance, equilibrating the electrode for 20–30 min at the desired temperature (low/med/high) is required.

Within the device, ions are separated by differential mobility due to an individual molecule's size and shape. An optimized combination of separation voltage (SV) and compensation voltage (COV) separates the analyte from background ions.

Optimization of these parameters is very simple and can be performed as part of instrument tuning. The optimization of SV is performed by constant infusion at low analyte flow in solution whilst ramping COV (**Figure 1**). The optimal combination of separation and compensation voltages gives the most separation whilst maintaining maximum peak intensity. Although optimal SV is usually obtained at around 4500 V, a lower value can be chosen to ensure system robustness and stability. As COV is influenced by mobile phase and source conditions, on column COV optimization is performed by injection of analyte at a flow rate and mobile phase composition comparable to the intended LC conditions (**Figure 2**).

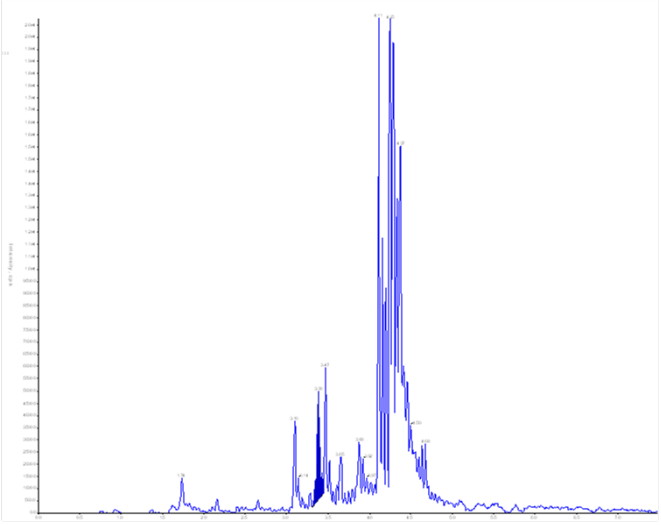


Figure 3. Peptide A at 20 ng/mL from a protein precipitation extract from human plasma without DMS.

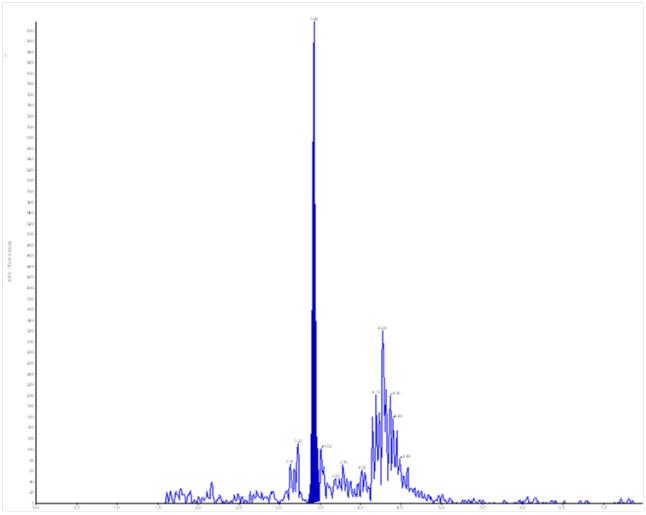


Figure 4. Peptide A at 20 ng/mL from a protein precipitation extract from human plasma with DMS.

Data Processing

Samples were acquired with the Analyst® 1.5.2 Software. Quantification was completed with MultiQuant™Software.

Results and Discussion

Case study 1

In this case study, a proprietary peptide (peptide A, MW 4,113.7 g/mol) was evaluated. This peptide is known to exhibit adsorptive characteristics, and solid phase extraction (SPE) cleanup (reverse phase or mixed mode) from human plasma results in very low recoveries. As a consequence, protein precipitation with methanol was the only feasible extraction approach from human plasma. Additionally, due to poor fragmentation, a wide selection of fragments was not available for quantitation, and fragment m/z 136 was the only suitable fragment displaying adequate sensitivity. Under these conditions, the resulting LLOQ is severely compromised by the lack of selectivity, despite separation using a 6 min chromatographic gradient (**Figure 3**). Without DMS, using MRM with +ESI (**Table 1**), an LLOQ of only 50 ng/mL is achievable from human plasma. An elevated baseline and a number of closely-eluting peaks were observed for spectra obtained for peptide A, which required careful set-up of peak integration parameters.

Using the same protein precipitation procedure and LC gradient conditions, DMS was added to the workflow (optimized parameters, **Table 1**), and peptide A spectra were evaluated for any improvement in selectivity. A significant improvement was observed. Despite a loss of absolute signal (approximately a factor of 5), a reduction of background interference of approximately a factor of 20 was observed. This resulted in an overall gain in S/N of an approximate factor of 4-5 (**Figure 4**). This facilitated a lowering of the feasible LLOQ from 50 to 8 ng/mL (**Table 2**) without changing extraction or gradient LC conditions.

Table 2. Lower limit of quantitation (LLOQ) data for peptide A

	Spiked level of peptide A (ng/mL)			
	8	**20**	**50**	**100**
Precision (CV%) without SelexION™ Device	N/AP	25.0	*13.7*	*6.0*
Precision (CV%) with SelexION™ Device	6.3	*7.8*	*8.1*	*6.3*
n	*6*	*6*	*6*	*6*

Case study 2

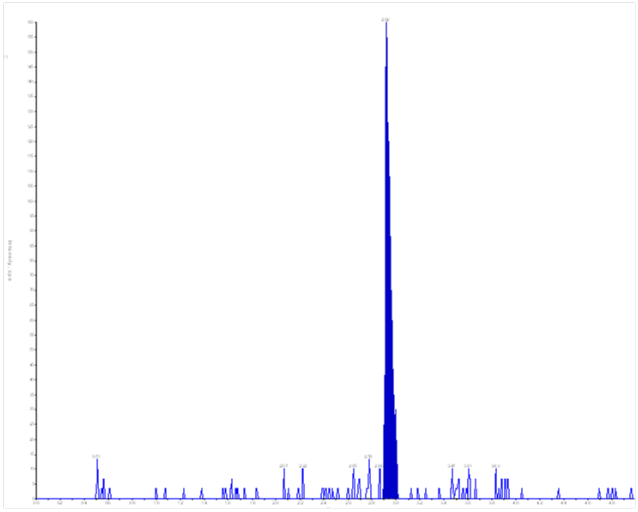


Figure 6 Peptide B at 0.04 ng/mL extracted with solid phase extraction with DMS.

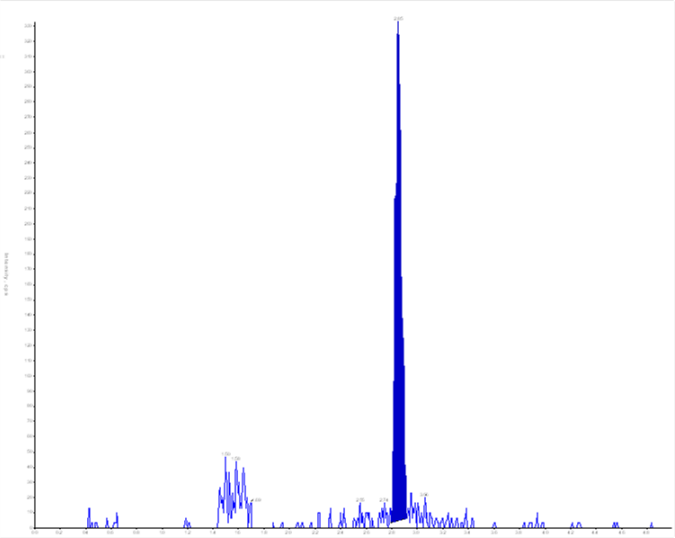


Figure 8 Peptide B at 0.08 ng/mL extracted with protein precipitation with DMS.

In this case study the quantitation of a therapeutic peptide (peptide B, MW 1,311 g/mol) in rat plasma was evaluated. This peptide was extracted from rat plasma using polymer-based, reversed-phase SPE. A recovery of 65% was achieved. Samples were chromatographed on a fused-core peptide column using a methanol/water gradient with formic acid as the acid modifier. Using +ESI-MRM (**Table 1**), a range of 0.04–10 ng/mL could be routinely achieved. At LLOQ a S/N of 10 (analyte intensity 1,000 counts, background 100 counts) resulted in a precision of 7.4% (**Figure 5**). Applying DMS to this method resulted in a 10-fold decrease in background with absolute analyte sensitivity exibiting a 6-fold decrease. Whilst S/N improved to 16 at LLOQ (0.04 ng/mL), there was no marked improvement in precision at this level (**Table 3**). A lowering of LLOQ could also not be facilitated under these conditions. Background, however, was almost completely eliminated allowing for easier and more consistent peak integration (**Figure 6**).

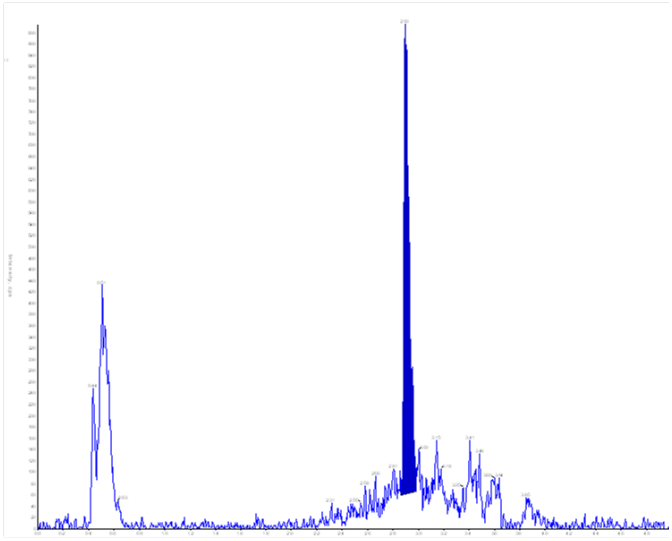


Figure 5 Peptide B at 0.04 ng/mL extracted with solid phase extraction without DMS.

A protein-precipitation approach was also evaluated for peptide B. As absolute recovery was compromised using SPE, it was anticipated that a lowering of the LLOQ by a factor of 2 could be achieved by combining recovery gains of protein precipitation with selectivity gains from DMS. Without the benefits of SPE cleanup in this instance, an LLOQ of only 0.08 ng/mL could be achieved without DMS due to high background and closely eluting isobaric interferences (**Figure 7**). With DMS, all background interferences were removed (**Figure 8**). However, due to the inherent loss of absolute signal associated with DMS, the resulting LLOQ achieved was limited to the LLOQ demonstrated for SPE extraction.

Table 3. Linearity, accuracy, and precision for peptide B peak area measurements with and without DMS

	Accuracy (%)		Precision (%)	
Concentration (ng/mL)	+ DMS	- DMS	+ DMS	- DMS
0.04	100	100	10.2	7.4
0.08	101	101	6.0	4.8
0.2	97.7	97.6	4.7	2.2
0.8	97.0	97.2	2.4	0.9
1.6	102	102	2.2	0.6
10	103	103	1.3	1.2
Linear regression r value	0.9976	0.9987		

Intra-run replicates, n=6, for SPE extracts with and without DMS. Concentration curves were analyzed with linear fit and a $1/x^2$ weighting.

Conclusions

Differential ion mobility spectrometry provides a useful additional or orthogonal selectivity during the quantitation of peptides (and conventional small molecules). For applications involving peptide quantitation in particular, selectivity gains may be significant as separation of multiply-charged analyte precursors from singly-charged background interference. The true gain in sensitivity as a function of absolute sensitivity and selectivity will be analyte dependant and will also be influenced by choice of MRM transition, chromatographic separation and extract cleanliness

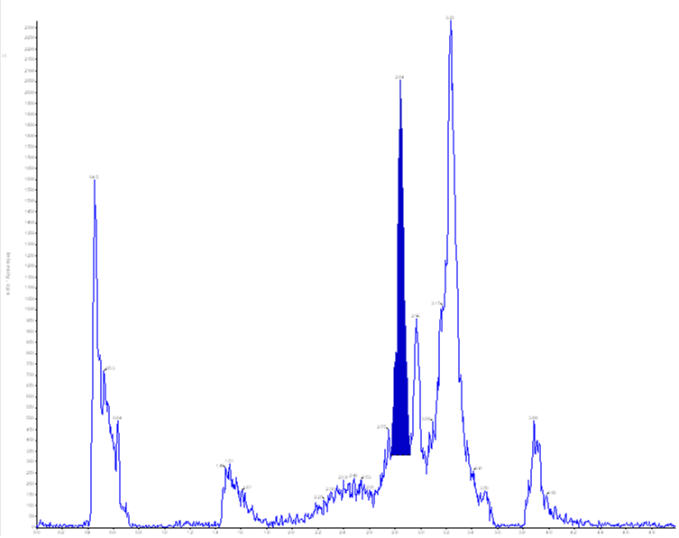


Figure 7 Peptide B at 0.08 ng/mL extracted with protein precipitation without DMS.

Often for peptides sensitivity is already compromised by a number of factors including low bioavailability, adsorption to surfaces, formation of multiple charge states and poor or non selective fragmentation. In these instances additional tools to aid lowering of LLOQs are to be welcomed. In some cases, true gains in sensitivity may not be realised or required, but improvements in selectivity may bring other benefits—namely, simpler extraction methods, shorter chromatographic runs, or improved peak integration.

References

1. A.Zerr, L.Meunier, S.Wood, P.Struwe. Application of Differential Ion Mobility Mass Spectrometry to Peptide Quantiation. Nov 14–16 2012. Celerion Poster Presentation. EBF 2012 Open Symposium, Barcelona.

Publication number: 9560314-01

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
www.absciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
www.absciex.com/offices