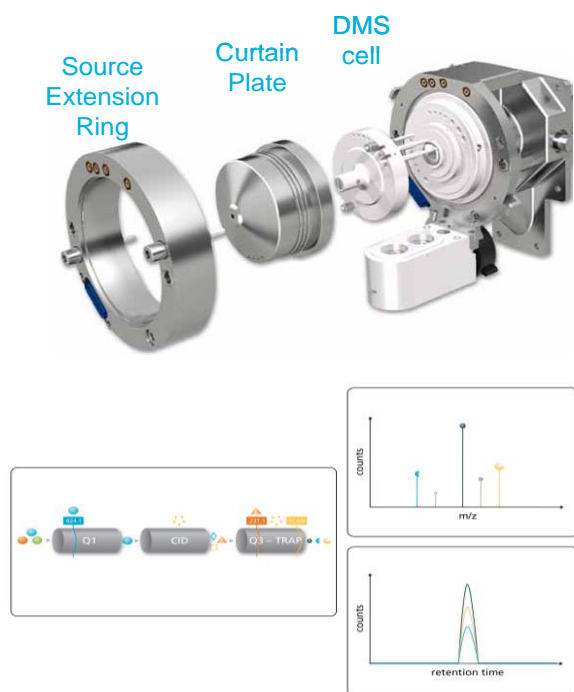


# Multiple Mass Spectrometric Strategies for High Selectivity Quantification of Proteins and Peptides

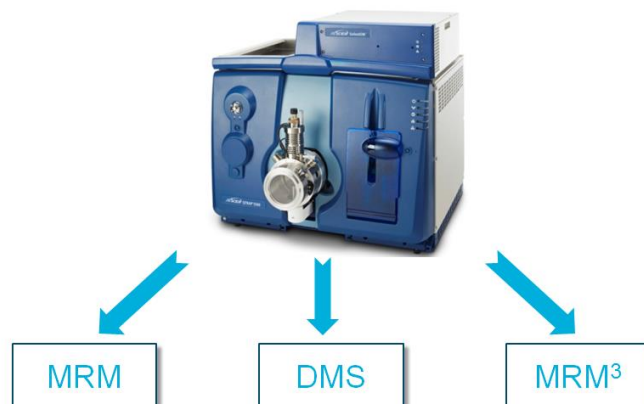
*Solve the Most Challenging Quantitative Problems with QTRAP® 5500 System*

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There has been an exponential increase in the number of potential protein biomarkers discovered; thus requiring the need for better quantification strategies to confirm or refute their ultimate utility for large scale validation studies. Also required is increased throughput which means reduced sample preparation and / or accelerated chromatography, both of which can increase the chance of interference that could confound robust quantification. The purpose of this study is to explore new MS methodologies on the QTRAP® 5500 System that enable higher selectivity for quantification. The different techniques rely on different properties of the molecule for specificity so their utility will depend to a large degree on the target molecules. In this study, we compare the quantification of Brain Natriuretic Peptide (BNP) using mass spectrometric methodologies including MRM, MRM3, and SIM with differential mobility separation.



**Figure 1. High Selectivity Quantification Methods on the QTRAP® 5500 System.** (Top) The SelexION® Technology is an easy to install differential mobility device that can be used to provide additional selectivity to any quantitative experiment. (Bottom) MRM<sup>3</sup> Workflow leverages the unique linear ion trap capability of the QTRAP system to provide additional specificity through additional fragmentation steps.



## High Selectivity Quantitation Strategies on the QTRAP® 5500 System

Three quantitative methodologies are available on the QTRAP® 5500 System to provide a solution to almost every quantitative problem:

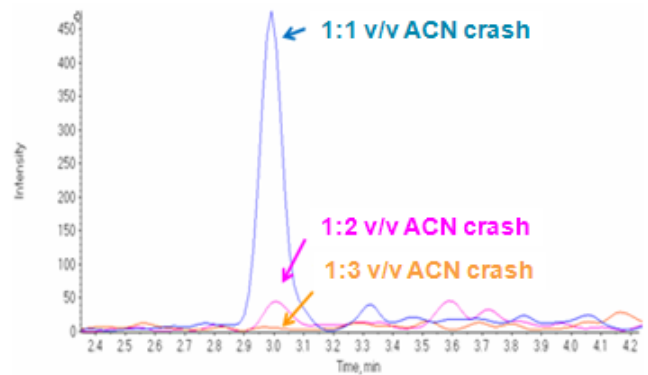
- **Multiple Reaction Monitoring (MRM)** using the *Scheduled MRM™* Algorithm will be the workhorse mode for many peptides in complex matrices. MRM is a very sensitive method which provides selectivity based on a specific precursor and MS/MS fragment masses. However, there will be cases where interferences or background can impact the ultimate sensitivity of the targeted assay and additional selectivity is required.
- **MRM<sup>3</sup> Workflow** is possible because of the hybrid triple quadrupole linear ion trap configuration of the QTRAP® system. The MRM<sup>3</sup> workflow adds selectivity to an assay through an additional fragmentation event and detection of secondary fragment ions.<sup>1</sup>
- **SelexION® Technology** is a planar differential mobility device (DMS) that separates peptides based on differences in their chemical properties, prior to entering Q1, providing an orthogonal level of separation and selectivity.<sup>2</sup>

## Methods

**Sample Preparation:** BNP peptide (0.5 mg, Sigma) was dissolved in 20% acetonitrile to make a stock of 5  $\mu\text{g}/\mu\text{L}$ . For matrix preparation, plasma was crashed using various concentrations of acetonitrile to test for endogenous peptide recovery. Plasma containing BNP was precipitated at 1:1, 1:2, and 1:3 ratios with plasma:acetonitrile and the resultant supernatant was diluted 1:1 with 0.1% formic acid (Figure 2). For determining sensitivity, the intact BNP was spiked into the crashed plasma matrix (0.25 equivalents of plasma on column) to create calibration curves.

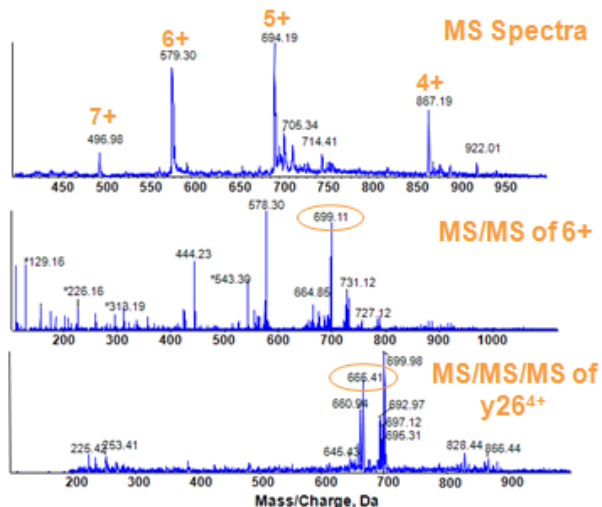
**HPLC Conditions:** A Shimadzu HPLC system with a Thermo Aquasil C18, 2.1mm x150mm column at 40 °C with a gradient of 2 to 70 % acetonitrile in 0.1% formic acid was used at a flow rate of 300  $\mu\text{L}/\text{min}$ . The injection volume was set to 10  $\mu\text{L}$ .

**MS/MS Conditions:** An SCIEX QTRAP® 5500 LC-MS/MS system equipped with Turbo V™ source was used. MRM transitions and MRM<sup>3</sup> experiments for BNP were developed and used to measure the calibration curves. For the differential mobility separations, a QTRAP 5500 system equipped with SelexION® Technology was used and BNP was analyzed in Single Ion Monitoring (SIM) mode (using a parent to parent MRM with no collision energy).

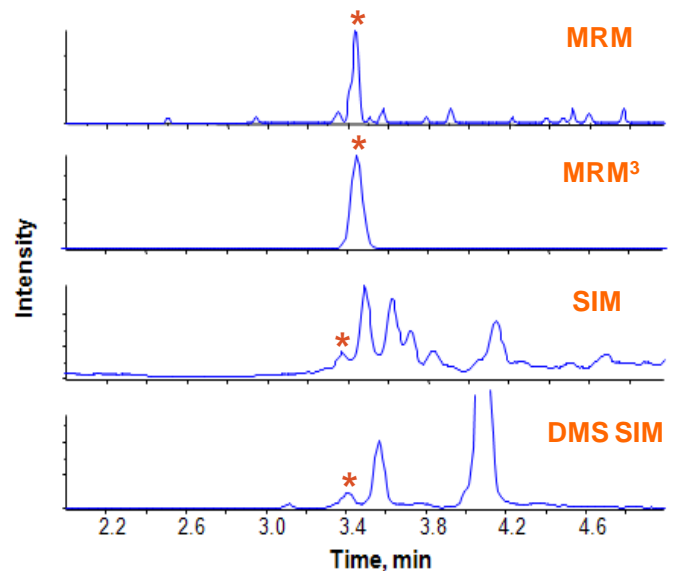


**Figure 2. Determination of Optimal BNP Recovery.** BNP (50 fmol) was spiked into plasma and various plasma precipitation techniques were tested to determine recovery of BNP during matrix preparation. Shown is the signal of the +6 peak of BNP after a) 1:1 v/v (plasma:acetonitrile) precipitation, b) 1:2 v/v (plasma:acetonitrile) and c) 1:3 (plasma:acetonitrile) precipitation. As can be observed, the 1:1 ratio provides the highest recovery of the BNP peptide and this matrix was used for the subsequent concentration curves.

**Data Processing:** Samples were analyzed in triplicate and data processed using MultiQuant™ Software to perform quantification and statistical analysis.



**Figure 3. Multiple Acquisition Modes Provide Enhanced Options for Peptide Quantification.** The top panel shows the detected charge state envelope of the BNP peptide. The middle panel shows the MS/MS spectrum of the 6+ m/z and good fragmentation was observed. The bottom panel shows the MS/MS/MS spectrum of the m/z 699+ product ion (y26). MRM<sup>3</sup> data generated by extracting the internal fragment at 665<sup>+</sup>. The corresponding quantitative data from these different spectral levels are highlighted in Figure 4.



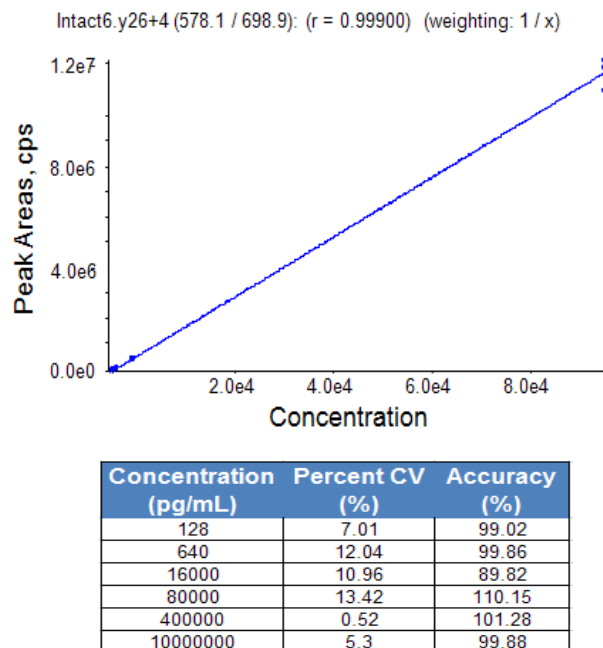
**Figure 4. Signal Observed for BNP Using Various Quantification Strategies.** The top panel shows the MRM signal of +6 charge state at 128 pg/mL on column in matrix. The second panel is the MRM<sup>3</sup> peak at the same concentration; the S/N is improved due to reduced background signal. The third panel is the trace from SIM, shown to be the least selective strategy, where the BNP peak is marked with an orange asterisk. However, when DMS is used to enhance selectivity of the SIM assay, the matrix interferences are significantly reduced, as shown in the bottom panel.

## Comparing the Quantitative Workflows

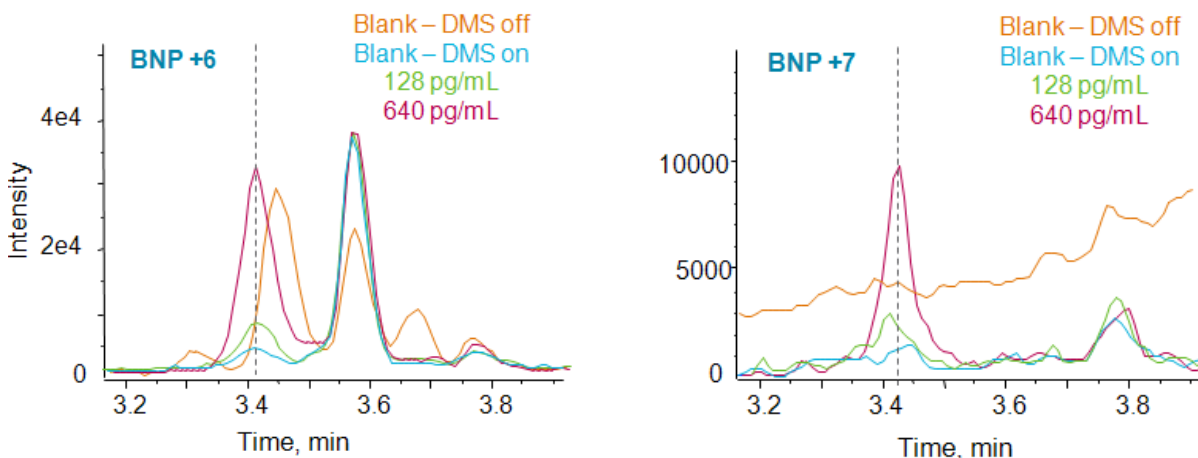
The recovery from the various extraction procedures was determined by monitoring the intensity of the 6+ ion of BNP. It was determined that plasma precipitated with a 1:1 ratio of plasma:acetonitrile provided the best relative recovery of BNP, and was therefore used as a matrix for all of the studies. (Figure 2) An in-depth investigation of sample preparation was not done and the reported lower limit of quantitation (LLOQ) in plasma could likely be improved with further optimization. However, the goal of this study was to explore the technology, not to develop an optimized BNP sample preparation protocol.

First, concentration curves using standard MRM and MRM<sup>3</sup> analyses were compared. Both techniques provided reasonably interference free traces at low BNP levels on column (Figure 4). The LLOQ for both the MRM and MRM<sup>3</sup> analyses were found to be 128 pg/mL on column. The LLOQ is expressed as peptide concentration per volume of matrix, and for these experiments matrix was 0.25 equivalents of plasma, therefore the effective detection limit of BNP in plasma is 512 pg/mL. (Figure 5).

To explore the utility of single ion monitoring (SIM) mode for analyzing large endogenous peptides that don't fragment well, SIM analysis with and without the differential mobility was performed. Here, the high selectivity provided by the mobility separation showed a significant reduction in background (Figure 6). The use of DMS resulted in a 25 fold improvement in the LLOQ achieved on the 6+ ion (Figure 7).



**Figure 5. Calibration Curve of BNP<sup>6+</sup> MRM in Plasma.** Calibration curve (top) and the statistics table (bottom) for BNP<sup>6+</sup> charge state in MRM mode is shown, and a 128 pg/mL LLOQ determined. The linear dynamic range is >4.5 orders and accuracy is between 80% and 120%, with no internal standard peptides utilized for analysis. The detection and quantitation limits for analysis in MRM<sup>3</sup> mode were found to be similar in this case, due to good peptide fragmentation and low background present in MRM.



**Figure 6. Single Ion Monitoring (SIM) of BNP, With and Without Differential Mobility Separation.** (Left) Signals from single ion monitoring (SIM) of +6 charge state of BNP with and without DMS. DMS can be turned off and run in transparent mode by simply clicking a button in the acquisition method. When DMS is turned off (orange trace), a distinguishable BNP peak above the high matrix interference can be determined only above a concentration of 3.2 ng/mL of matrix. When DMS is enabled, the background drops (blue trace) and now quantifiable signal can be observed at 128 pg/mL (green trace). Ion mobility separates the interfering matrix peak away from the BNP peptide of interest thus providing a better S/N and a substantially improved LLOQ. (Right) Signals from the 7+ charge states of BNP with and without DMS. For this charge state, a high background level is observed in the blank matrix (orange trace), impacting the LLOQ. When DMS is turned on, the background drops significantly (blue trace) and now quantifiable peaks can be observed at 640 pg/mL (pink trace). Ion mobility reduces the high background such that the peak of interest can be quantified at lower signal level.

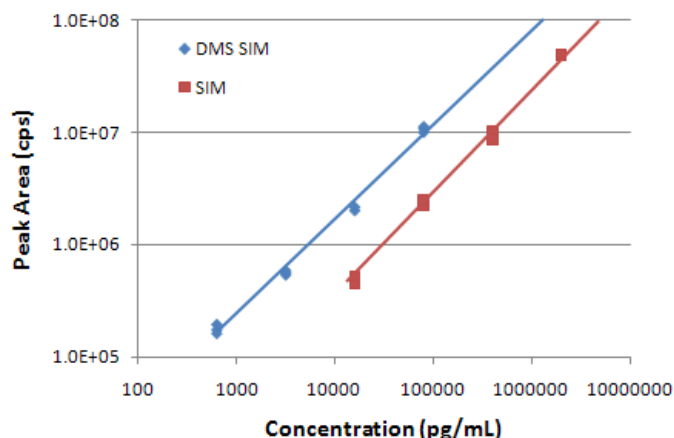
## Conclusions

Three quantitative methodologies can be used with the QTRAP® 5500 System:

- MRM provides selectivity based on the fragmentation of the peptide and monitoring of a specific product ion.
- When matrix interference is a problem with MRM, further selectivity can be achieved using MRM<sup>3</sup>, which provides a second level of selectivity based on monitoring a secondary product ion.
- Differential Mobility Separation (DMS) provides an orthogonal level of selectivity by separating components based on their chemical properties and mobility preceding the quadrupoles.

BNP peptide was chosen as a model analyte to investigate quantitation of large endogenous peptides. The BNP peptides provide good quality fragmentation for both MS/MS and MS<sup>3</sup>, thus good sensitivity was obtained using both the MRM and MRM<sup>3</sup> workflow. However, many large peptides do not fragment well, therefore monitoring the MS signal using SIM mode in combination with DMS was also explored. Application of DMS provided a 25x improvement in LLOQ over SIM alone, through substantial reduction in background.

This study demonstrates the effectiveness of these high selectivity workflows and the versatility of the QTRAP® 5500 System for the selective detection and quantitation of peptide analytes using MRM, MRM<sup>3</sup>, SIM, and SIM with DMS.



**Figure 7. Low End of the Concentration Curves for the SIM Experiments.** Shown are the lower points of the concentration curves for the SIM experiment on BNP 6+ charge state peptide, with DMS (blue trace) and without the DMS filtering (red trace). Due to the enhanced selectivity and near removal of a co-eluting interference, the LLOQ for the SIM with DMS on was substantially improved (~25 fold improvement) over the SIM only experiment.

## References

1. MRM<sup>3</sup> Quantitation for Highest Specificity in Complex Matrices. SCIEX Technical Note RUO-MKT-02-2739-A.
2. SCIEX SelexION® Technology: A New Solution to Selectivity Challenges in Quantitative Bioanalysis - Differential Mobility Separations Enhanced with Chemical Modifiers: A New Dimension in Selectivity. SCIEX Technical Note RUO-MKT-02-3251-A.

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