# Improving Sensitivity for Trastuzumab Emtansine Quantification using Trap-Elute MicroLC-MS with Large Volume Sample Loading

***Using SCIEX 6500+ QTRAP® System with OptiFlow™ Turbo V source and M5 MicroLC System***

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LC-MS based quantification of monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) has been routinely adopted in multiple stages of the biotherapeutic development, serving as an orthogonal technology to the traditional ligand binding assays (LBAs). For small animal PK analysis, the assay sensitivity and throughput are the key factors to evaluate the assay performance. Moreover, scientists have started to work on improving the quantification confidence by generating replicate data from multiple sets of sample preparation. Therefore, each individually prepared sample no longer need to be fractionated and injected multiple times into the LC-MS system. Instead, it can be fully injected at higher injection volumes, to improve the assay sensitivity. Herein, a trap-elute microLC-MS/MS workflow with large volume sample loading is demonstrated for ultra-sensitive quantification of Trastuzumab Emtansine in mouse plasma. Without affecting the throughput, a two-fold improvement on assay sensitivity with the LLOQ as low as 0.5 ng/mL is observed compared with previously published data[1].

## Key Feature of the SCIEX Immunoaffinity-MicroLC-MS/MS Solution

- M5 MicroLC system provides (Figure 1):
    - Microfluidic flow control for accurate flow rates down to 1 µL/min
    - Trap-elute option for fast and large volume sample loading
    - Flexibility to couple with any microLC column
- OptiFlow™ Turbo V Source on the QTRAP® 6500+ LC-MS/MS system provides (Figure 1):
    - Easy setup with no probe or electrode position optimization required
    - Robust performance and long electrode lifetime
- Optimized immunoaffinity sample preparation provides:
    - Decreased sample complexity and matrix interference
    - Desired assay linear dynamic range
    - Shortened sample preparation time

## Methods

***Immunocapture of Target Analyte:*** A streptavidin coated immunoaffinity magnetic bead slurry was aliquoted and washed with PBS Buffer (1x) three times. Biotinylated Goat Anti-Human IgG Antibody (0.5 mg/mL) was added to the beads and incubated at room temperature for 1 hour with shaking. The conjugated beads were washed three times and re-suspended in PBS Buffer (1x). (Figure 2) Calibration standard samples were prepared as 25 µL mouse plasma spiked with trastuzumab emtansine standard. All samples were prepared in triplicate and processed individually.  The concentrations of trastuzumab emtansine in plasma are 0.25, 0.5 1, 2, 10, 50, 100, 500, 1000 and 5000 ng/mL. SILuMab was used as the internal standard. To each calibration standard sample, 100 µL of PBS Buffer (1x), 50 µL conjugated bead slurry and internal standard were added and the mixtures were incubated at room temperature for 1 hour with shaking. The beads were accumulated by magnetic stand and washed sequentially with PBS Buffer (1x) and 10 mM ammonium bicarbonate. The target proteins were eluted by incubating the beads with 0.1% TFA in water with vortexing for 10 min.

***Protease Digestion of Immuno-Enriched Eluents:*** The eluents were transferred to 96 well plate wells and neutralized with 1 mM calcium chloride in 500 mM ammonium bicarbonate in water.

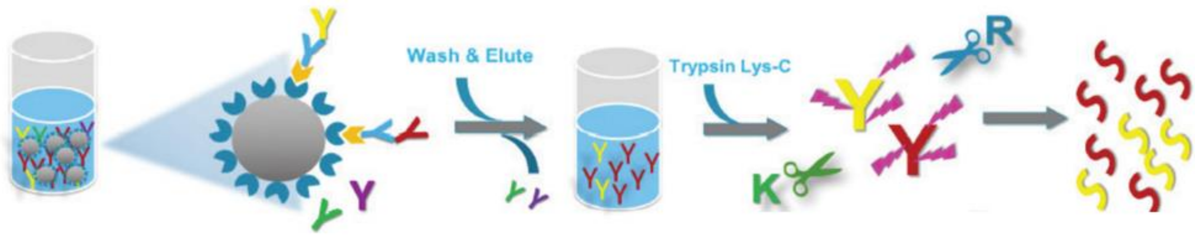


**Figure 2. Sample Preparation Workflow.**

The sample plate was placed into a deep well thermo-shaker and incubated at 95 °C for 10 mins with shaking. The plate was cooled to room temperature and 1 µg of trypsin was added to each sample. The trypsin digestion was conducted by incubating the samples for 1 hour at 50 °C with gentle shaking and aborted by adding formic acid. The supernatants from the samples were subjected to LC-MS/MS analysis.

**LC-MS Conditions for Microflow Analysis:** The samples were analyzed by SCIEX QTRAP 6500+ mass spectrometer coupled with M5 MicroLC system at the trap-elute mode. Table 1 describes the chromatographic conditions for analyte trapping. Table 2 describes the chromatographic conditions for analyte separation. During sample loading, the analytes were trapped and desalted on the trap column. During analyte separation, the auxiliary valve was at "inject" position for the first 5 min to connect the trap column with analytical column. At 5 min, the auxiliary valve was switched to "load" position to connect the trap column with the loading pump for column washing (Figure 3).

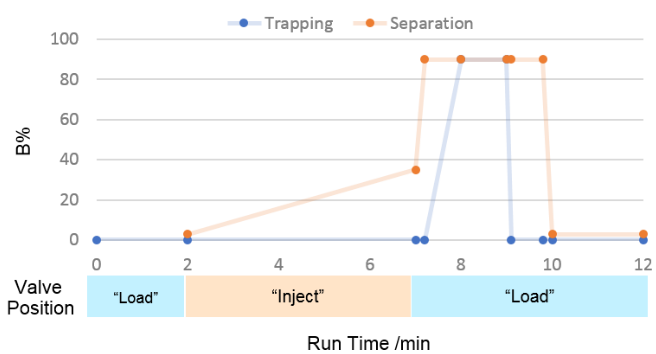


**Figure 3. LC Gradients for Trapping / Separation Pumps and Valve Positions.**

**Table 1: Chromatographic Conditions for Microflow Analysis: Analyte Trapping.**

| Parameter | Value |
|---|---|
| *Stationary phase* | *Phenomenex Luna 5 µm, C18 Trap Column, 20 x 0.3 mm* |
| *Mobile phase A* | *0.1% formic acid in water* |
| *Mobile phase B* | *0.1% formic acid in acetonitrile* |
| *Flow rate* | *50 µL/min* |
| *Column temperature* | *Room Temperature* |
| *Injection volume* | *50 µL* |

| Time | Flow Rate (µL/min) | %A | %B |
|---|---|---|---|
| *0* | *50* | *100* | *0* |
| *7* | *50* | *100* | *0* |
| *8* | *50* | *10* | *90* |
| *9* | *50* | *10* | *90* |
| *9.1* | *50* | *100* | *0* |
| *12* | *50* | *100* | *0* |

MS analysis was performed on a SCIEX QTRAP 6500+ system with OptiFlow™ Turbo V Source with a 25 µm SteadySpray™ probe and electrode. The OptiFlow Turbo V Source requires no physical adjustment of the probe or electrode positions. The optimized MS parameters are listed in Table 3. The data were processed using MultiQuant™ software 3.0.

**Table 2: Chromatographic Conditions for Microflow Analysis: Analyte Separation.**

| Parameter | Value |
|---|---|
| *Stationary phase* | *Phenomenex Kinetex 2.6 µm, XB-C18 Column, 50 x 0.3 mm* |
| *Mobile phase A* | *0.1% formic acid in water* |
| *Mobile phase B* | *0.1% formic acid in acetonitrile* |
| *Flow rate* | *5 µL/min* |
| *Column temperature* | *40 ºC* |
| *Injection volume* | *NA* |

| Time | Flow Rate (µL/min) | %A | %B | Comment |
|---|---|---|---|---|
| *0* | *5* | *97* | *3* | |
| *5* | *5* | *65* | *35* | *Valve Load* |
| *5.2* | *5* | *10* | *90* | |
| *7.8* | *5* | *10* | *90* | |
| *8* | *5* | *97* | *3* | |
| *10* | *5* | *97* | *3* | |

**Table 3. MS Conditions for Microflow Analysis.**

| Name | Q1 | Q3 | DP | CE | CXP |
|---|---|---|---|---|---|
| *IYPTNGYTR1*[1] | *542.8* | *808.4* | *50* | *24* | *12* |
| *IYPTNGYTR2* | *542.8* | *405.1* | *50* | *23* | *6* |
| *FTISADTSK1* | *485.2* | *721.3* | *60* | *21* | *15* |
| *FTISADTSK2* | *485.2* | *608.2* | *60* | *22* | *25* |
| *GPSVFPLAPSSK1* | *593.8* | *699.4* | *78* | *28* | *15* |
| *GPSVFPLAPSSK2* | *593.8* | *846.5* | *78* | *28* | *15* |
| *FNWYVDGVEVHNAK[H]*[2] | *562.9* | *713.3* | *60* | *23* | *15* |
| *GPSVFPLAPSSK[H]*[2] | *597.8* | *707.4* | *78* | *28* | *15* |

| Source/Gas Parameter | Value | Source/Gas Parameter | Value |
|---|---|---|---|
| *Curtain gas:* | *25* | *CAD gas:* | *High* |
| *Ion source gas 1:* | *20* | *Ion spray voltage:* | *5000* |
| *Ion source gas 2:* | *15* | *Source temperature:* | *150* |

[1]*Most suitable transition for quantification*
[2]*Internal standard transitions*

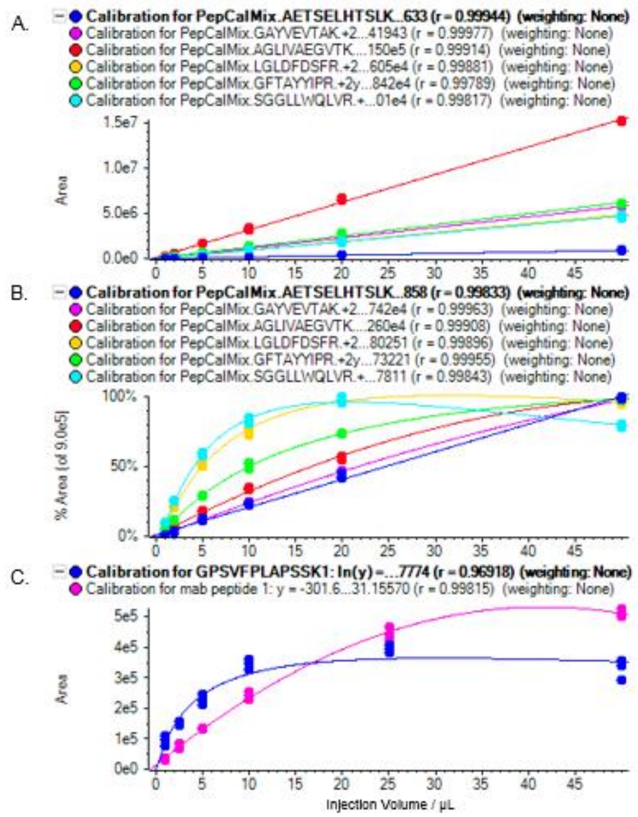


**Figure 4. Testing Injection Volumes.** The correlation curve between injection volumes and peak areas for A) sample A, B) sample B, C) sample C. Please note these are NOT peak area – concentration calibration curves.

## Results and Discussion

To identify the relation between the sample loading volume and the assay sensitivity (MRM peak areas), a few peptide samples with difference matrix complexities were prepared for a various-injection-volume analysis. The samples include:

1. A mixture of peptide standards diluted with 5/3/92 (V/V/V) acetic acid / acetonitrile / water (sample A),
2. A mixture of the same peptide standards diluted with 5/3/92 (V/V/V) acetic acid / acetonitrile / water containing 10 ng/mL β-galactosidase digest (sample B),
3. A mAb digest prepared by immunoaffinity approach from mouse plasma matrix (sample C).

Each sample were subjected to LC-MRM analysis with various injection volumes, including 1, 2, 5, 10, 20 and 50 µL.

As shown in Figure 4A, the MRM peak areas of the six peptide standards in sample A are increasing proportionally with the increased sample injection volumes. Whereas, the same six peptides in sample B don't show similar responses as sample A (Figure 4B). While two out of six peptides still have relative linear responses, the other four peptides show quadratic or ragner fittings between the MRM peak areas and the injection volume. This non-linear response is caused by the matrix suppression at the specific retention time for each peptide. This finding suggests that the optimal sample injection volume needs to be determined case by case for each target peptide in the biological assay.

The similar test was performed for sample C to determine the optimal injection volume for the trastuzumab emtansine mouse plasma samples. As shown in Figure 4.C, the 50 µL injection volume provides the largest peak area for the quantifier peptide (pink trace) comparing with the other injection volumes. Therefore, 50 µL was set as the injection volume for the following experiments.

With the increased sample loading amount, (50 µL of the total 60 µL sample is injected into LC-MS system), the presented microLC-MS/MS assay achieved a LLOQ of 0.5 ng/mL and a LOD of 0.25 ng/mL for trastuzumab emtansine quantification in 25 µL mouse plasma (Figure 5). As summarized in Table 4, the accuracies are between 80-120% for HLOQ, and between 85-115% for all other samples. The CV%s of the independently prepared triplicate samples are all below 15%. The calibration curve covers 4 orders of magnitude (0.5-5000 ng/mL) (Figure 6) and displays a regression coefficient (r) of 0.992 using a weighting of $1/x^2$. It is worth noting that the assay throughput is maintained as high as analytical flow assays, by utilizing the microLC trap-elute function and its capability for high flow rate sample loading.

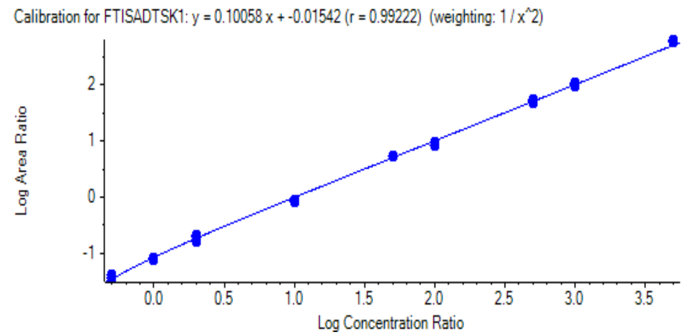


**Figure 6. Calibration Curve.** Log Concentration Ratio is plotted vs Log Area Ratio for quantitation of trastuzumab emtansine in mouse plasma (0.5 ng/mL to 5000 ng/mL).

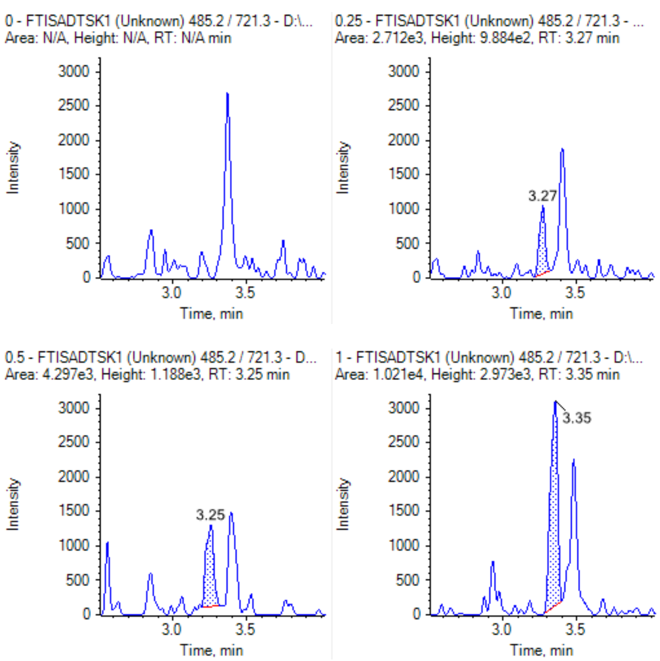


**Figure 5. Lower Limits of Quantitation.** Extracted ion chromatograms (XICs) of selected MRM for trastuzumab emtansine samples: blank; 0.25 ng/mL; 0.5 ng/mL; 1 ng/mL.

**Table 4: Quantitation Summary.**

| Actual Conc. (ng/mL) | Calculated Conc. (ng/mL) | Accuracy (%) | CV (%) |
|---|---|---|---|
| *0.5* | *0.518* | *103.5* | *8.2* |
| *1* | *0.938* | *93.8* | *4.6* |
| *2* | *2.015* | *100.7* | *12.8* |
| *10* | *8,778* | *87.8* | *3.6* |
| *50* | *53.40* | *106.8* | *0.6* |
| *100* | *86.22* | *86.2* | *6.54* |
| *500* | *506.0* | *101.2* | *5.1* |
| *1000* | *1020* | *102.0* | *5.8* |
| *5000* | *5896* | *117.9* | *4.5* |

## Conclusions

An improved immunoaffinity-microLC-MS/MS workflow for quantifying transtuzumab emtansine in mouse plasma was demonstrated. By taking advantages of the trap-elute function and the capability for high flow rate sample loading from the M5 microLC system, the QTRAP 6500+ LC MS/MS system with OptiFlow™ Source provides reliable ADC quantitation at 0.5 ng/mL level with high reproducibility, wide dynamic range, high throughput and minimum source optimization requirement.

## References

1. Ultra-Sensitive Quantification of Trastuzumab Emtansine in Mouse Plasma using Trap-Elute MicroLC-MS Method, SCIEX technical note RUO-MKT-02-8288-A.



Document number: RUO-MKT-02-8589-A

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