A robust microflow LC-MS/MS system for biopharmaceutical analysis

Integration of the Waters ACQUITY UPLC M-Class system and SCIEX 7500 system

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Microflow LC has been shown to provide significant increases in sensitivity for the quantification of biopharmaceutical relevant analytes such as peptides[1], antibodies[2], and oligonucleotides[3]. It is frequently employed when faced with a limited amount of sample such as in drug metabolism studies with small test animals, or when the lowest possible limit of quantitation (LLOQ) is required. This technical note describes integrating the Waters ACQUITY UPLC M-Class Microflow LC system into the SCIEX OS software for use with the SCIEX 7500 system or ZenoTOF 7600 system.

An integrated system was evaluated for 1,000 consecutive trap-and-elute injections of an antibody signature peptide. No software or hardware interruptions were observed, and all runs were completed with excellent results. One set of Phenomenex microflow columns was used for the 1,000 injections. Very good peak area stability (RSD 2.5%) and retention time precision (RSD 0.5%) were observed for the signature peptide in 1,000 consecutive injections over seven days. Additionally, a standard quantification curve was measured with five replicate injections of each standard, showing a linear dynamic range (LDR) of 3.3 orders of magnitude and a LLOQ of 1.0 ng/mL with a precision of 7.8% for a 10 µL injection.

Key features of the Waters ACQUITY UPLC M-Class and SCIEX 7500 system for bioanalysis

- Comprehensive and robust integration of the ACQUITY UPLC M-Class system into the SCIEX OS software
- Trap-and-elute capability allows for large sample volume analysis without increasing method run time, resulting in high sample throughput
- Easily switch between analytical and microflow setup with no need for electrode position optimization, using the OptiFlow Pro ion source with E Lens probe
- SCIEX OS software increases productivity by providing a user-friendly interface and one single and compliance-ready platform for data acquisition, processing, and management

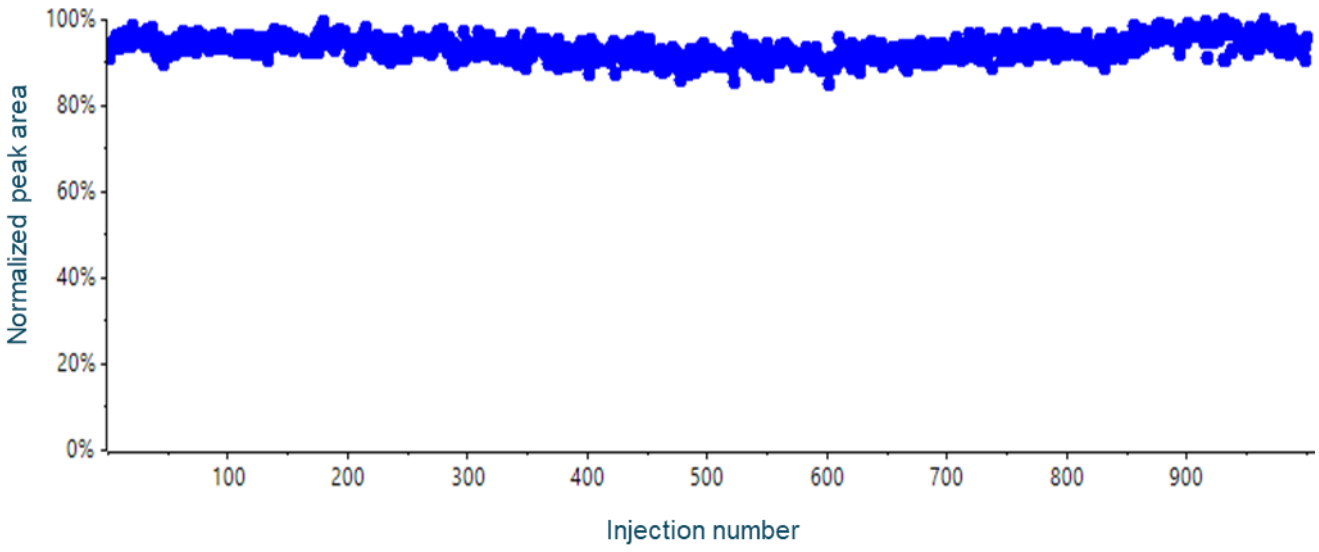

Figure 1. Normalized peak areas of the signature peptide FTISADTSK of trastuzumab emtansine for 1,000 consecutive trap-and-elute microflow LC-MS/MS analyses. The RSD was 2.5%.

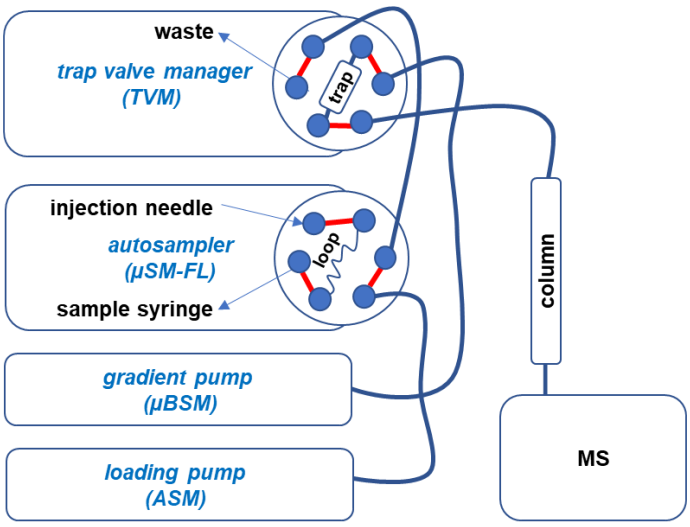

Figure 2. Trap-and-elute set-up on the ACQUITY UPLC M-Class system

Methods

Samples and reagents: The antibody trastuzumab emtansine was purchased from Myonex, and SILUmab (Sigma) was used as internal standard. For digestion, a trypsin/lys-C mixture (Promega) was used.

Sample preparation: For this study, the sample was similar to that of an immunoaffinity extracted antibody, which typically is free of interference and can be readily analyzed by microflow LC-MS/MS after digestion[2]. A 150 µL aliquot of a 20 µg/mL solution of either trastuzumab emtansine or SILUmab in 500 mM NH_4HCO_3/1 mM CaCl was first denatured at 90°C for 10 minutes, and then digested with 1 µg of trypsin/lys-C for 1 hour at 50°C. The digestion was stopped by adding 3 µL formic acid. Samples were prepared from the stock solutions by dilution with the same mixture of digestion buffer and formic acid as was used for the digestion. Finally, 2% acetonitrile and 5% acetic acid was added for increased sample stability. For the robustness test, the concentration of both the antibody trastuzumab emtansine and the internal standard (IS), SILUmab, was 100 ng/mL. The final concentration of the IS for the standard curves was also 100 ng/mL.

Chromatography: A Waters ACQUITY UPLC M-Class system, consisting of a micro binary solvent manager (µBSM), a micro sample manager with fixed loop (µSM-FL), an auxiliary solvent manager (ASM), and a trap valve manager (TVM) was used in trap-and-elute mode. Figure 2 shows the setup for the trap-and-elute mode.

Table 1. LC gradient method.

Time (min)	Analytical pump %B
0.0	*3*
4.0	*50*
4.5	*80*
5.5	*80*
6.0	*3*
7.0	*3*

Gradient and other chromatographic conditions and columns are summarized in Tables 1 and 2. A volume of 10 µL of sample was loaded onto the trap column for 1 minute with a loading flow rate of 35 µL/min. The analytical flow rate was 5 µL/min, and a 4 min gradient from 3 to 50 %B was used for the separation.

Table 2. Chromatographic conditions.

Parameter	Setting
Loading solvent	*Water with 0.1% formic acid*
Loading flow rate	*35 µL/min*
Loading time	*1 minute*
Mobile phase A	*Water with 0.1% formic acid*
Mobile phase B	*Acetonitrile with 0.1% formic acid*
Analytical flow rate	*5 µL/min*
Trap column	*0.3 x 10 mm, Phenomenex C18 5 µm 100 Å*
Analytical column	*0.3 x 50 mm, Phenomenex Kinetex XB-C18 2.6 µm, 100 Å*
Analytical column temperature	*40°C*
Injection volume	*10 µL*
Loop volume	*20 µL*

Mass spectrometry: A SCIEX 7500 system with an OptiFlow Pro ion source with E Lens probe and a micro (low) electrode was used in MRM mode. All source and MS parameters were optimized (Tables 3 and 4). The MS method included multiple MRM transitions of signature tryptic peptides for both trastuzumab emtansine and the IS.

Table 3. MRM transitions and MS parameters.

ID	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Dwell time (ms)	CE (V)	CXP (V)	EP (V)
FTISADTSK, 2+y7	*485.2*	*721.3*	*5*	*22*	*10*	*10*
*FTISADTSK, 2+y6**	*485.2*	*608.2*	*5*	*23*	*10*	*10*
IYPTNGYTR, 2+y7	*542.8*	*808.4*	*5*	*24*	*10*	*10*
DTLMIS[R],heavy 1	*423.2*	*629.4*	*5*	*24*	*10*	*10*
*DTLMIS[R],heavy 2**	*423.2*	*516.3*	*5*	*22*	*10*	*10*

***Used for quantification.**

Table 4. Source conditions.

Parameter	Setting
Polarity	*Positive*
Curtain gas	*35 psi*
Gas 1	*50 psi*
Gas 2	*65 psi*
CAD gas	*12*
Ion spray voltage	*4500 V*
Source temperature	*100°C*

Data processing: MRM data was processed with SCIEX OS software 2.0 using the MQ4 integration algorithm. Linear regression with a 1/x weighting was used.

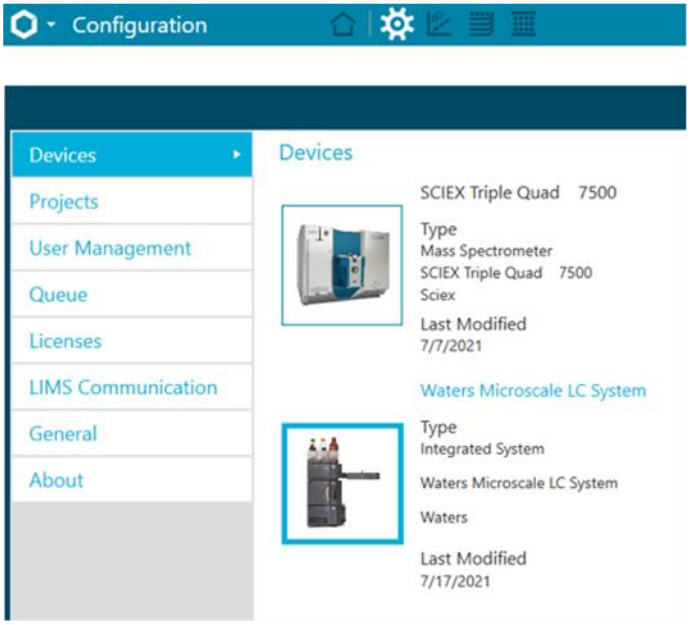

Figure 3. Configuration of the M-class system in SCIEX OS software.

Results and discussion

Software integration

Once the driver for the ACQUITY UPLC M-Class system is installed on the SCIEX 7500 system acquisition computer, the system can be added in SCIEX OS software configuration as an “Integrated System” (Figure 3). LC methods are generated in the “LC Method” section of SCIEX OS software, using the tabs for the various modules of the M-class system. As an example, Figure 4 shows the tab where the gradient method is entered. After the LC method is completed and saved, it can be used in the batch with the corresponding MS method.

Robustness test

One thousand consecutive injections were performed to demonstrate the robustness of the combined system. For every 200 injections, 10 sample vials were prepared from frozen aliquots of the sample prepared as described in the methods section. Figure 1 demonstrates the excellent stability of the entire system. The RSD based on peak area for the trastuzumab emtansine signature peptide used for quantification was 2.5% over the one thousand injections. The RSD based on the area ratio with the IS was 3.5%. Figure 5 shows the XICs for the signature peptides and IS peptide for the first and last injections. No significant changes in peak widths or tailing were observed. The RSD on retention time for the signature peptide used for quantification was 0.5% over all injections. Figure 6 shows an overlay of the analytical column pressure traces of the 1st and 1,000th injection; the pressure trace was comparable throughout

all runs. The trap column backpressure only increased by about 3% from 1,450 to 1,500 psi.

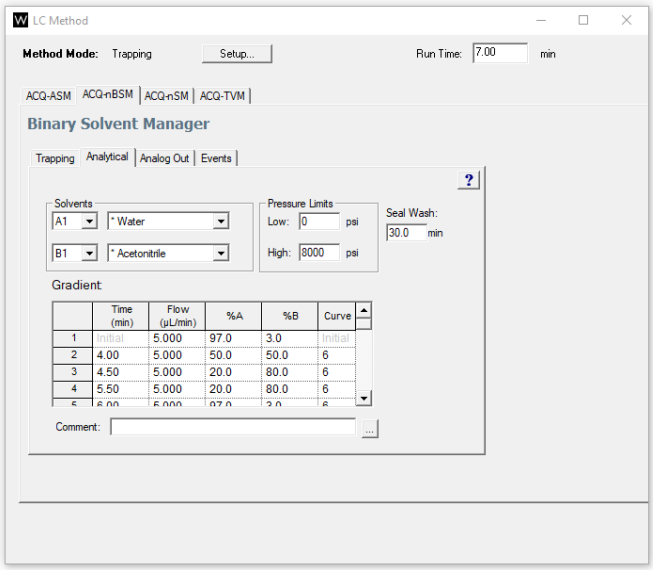

Figure 4. Tab in "LC Method" for entering the analytical gradient and setting LC parameters.

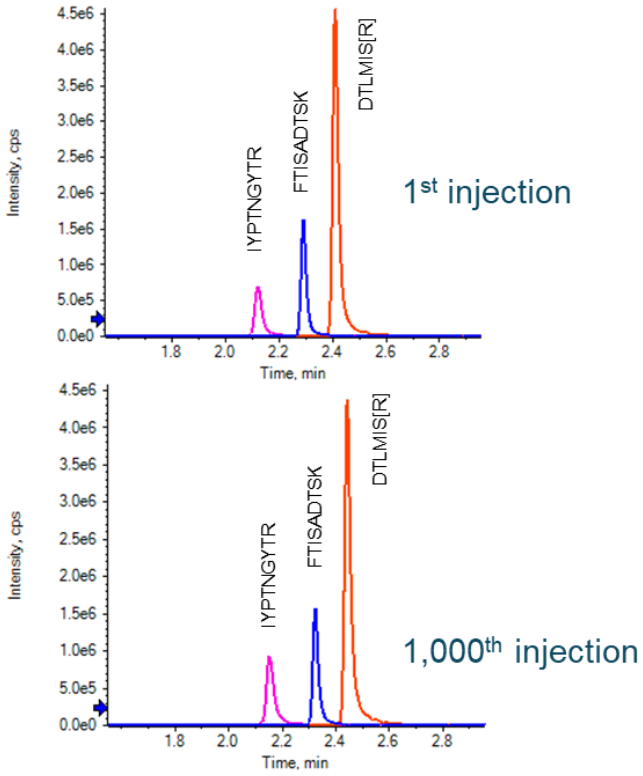

Figure 5. XICs for the first and 1,000th injection. Highly consistent chromatographic separation throughout all runs was observed.

Dynamic range and LLOQ

A standard calibration curve was measured from 1.0 ng/mL to 10,000 ng/mL, with 5 replicates for each concentration. Excellent accuracy and precision were achieved for all standards within this data set (Table 5). LLOQs and linear calibration curve range were determined based on the requirements that the %CV of the calculated mean of the concentration is below 20% at the LLOQ and below 15% for all higher concentrations, while the accuracy of the calculated mean is between 80% and 120% at the LLOQ and between 85% and 115% for the higher concentrations. The LLOQ was 1.0 ng/mL, and the LDR was 3.3 orders of magnitude. The precision was 7.8% at the LLOQ, and less than 3.6% at the higher concentrations. Accuracy was 90% at the LLOQ and between 93% and 102% above the LLOQ. Figure 7 shows the calibration curve, and Figure 8 the XIC at the LLOQ and blank.

Carryover was determined to be around 0.5% in the first blank after injecting of the 10,000 ng/mL sample. This will likely be lower after injection of the upper limit of quantification (ULOQ) of 3,000 ng/mL and could be further reduced by adding extended column and trap washes. Alternatively, a lower ULOQ can be declared.

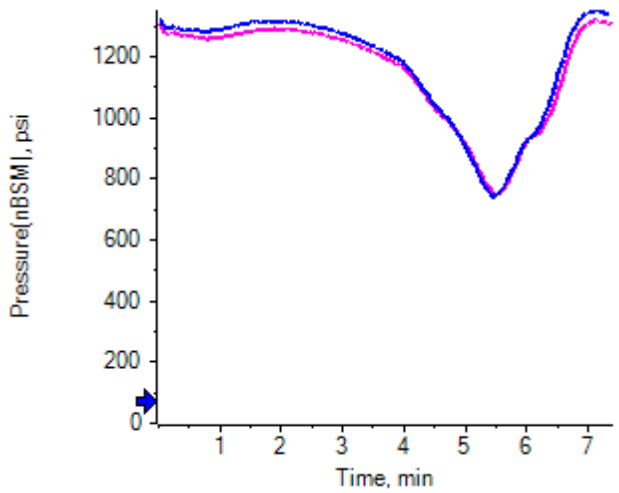

Figure 6. Overlay of the analytical pressure traces of the first and last injection of a thousand runs. The pressure traces were highly comparable across all runs.

Table 5. Calculated concentration, precision and accuracy for the signature peptide FTISADTSK of trastuzumab emtansine.

Actual (ng/mL)	Mean (ng/mL)	CV (%)	Accuracy (%)
1	0.9021	7.76	90.2
3	2.803	2.02	93.5
10	9.607	3.61	96.1
30	30.03	2.67	100
100	96.92	2.01	96.9
300	305.5	1.40	102
1,000	1,019	1.32	102
3,000	2,980	2.12	99.3

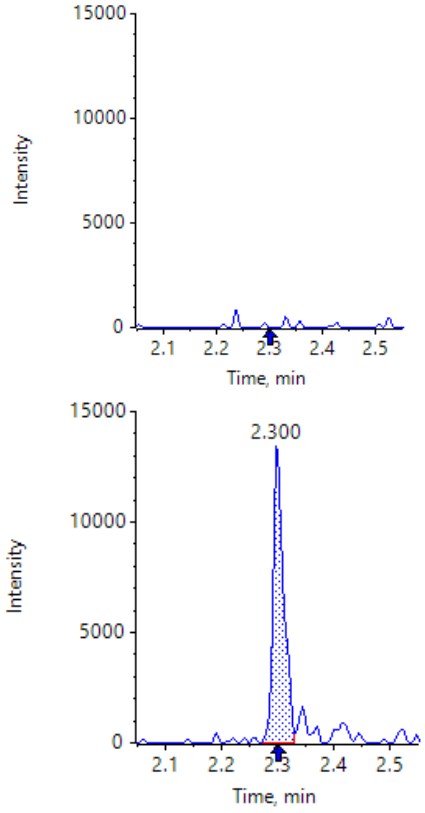

Figure 8. XICs for the signature peptide used for quantification (FTISADTSK) for the blank (top) and at the LLOQ of 1.0 ng/mL (bottom).

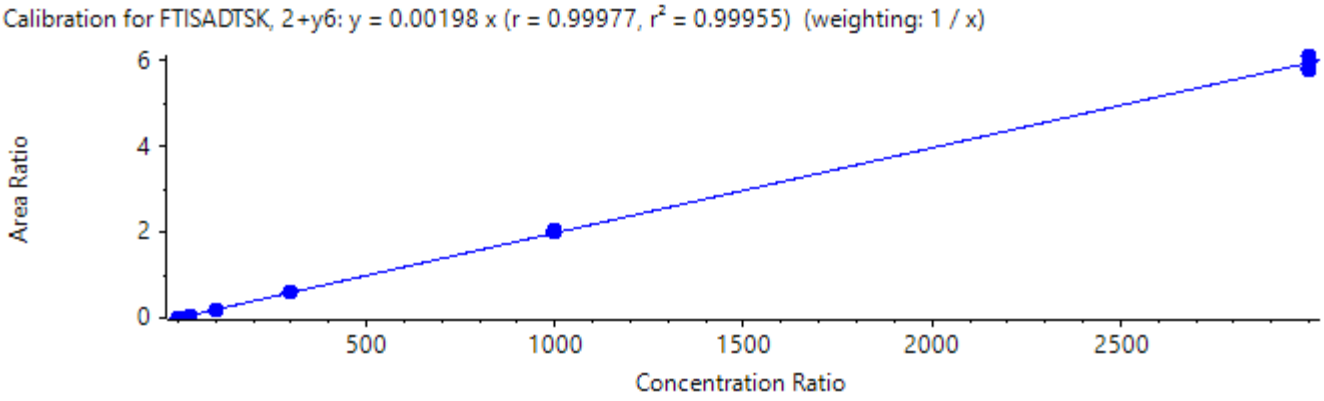

Figure 7. Calibration curve for the FTISADTSK peptide. The LDR was 3.3 orders of magnitude.

Conclusions

- Reliable integration of the ACQUITY UPLC M-Class system with the SCIEX OS software was demonstrated; the system ran without any issue for one thousand injections consecutively, plus another 100 for the calibration curve
- A low LLOQ of 1.0 ng/mL was achieved for a signature peptide of the antibody trastuzumab emtansine with an LDR of 3.3 orders of magnitude
- Trap-and-elute capability allows for large sample volume analysis without increasing method run time, resulting in high sample throughput
- Easily switch between analytical and microflow setup with no need for electrode position optimization, using the OptiFlow Pro ion source with E Lens probe
- SCIEX OS software increases productivity by providing a user-friendly interface and one single and compliance-ready platform for data acquisition, processing and management

References

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