

Development of an MRM method

Previously optimized parameters (i.e. CE, DP, CXP,...etc) are used to construct the MRM method for mass spec analysis. The LC system is as equally important to be optimized to yield better separation and resolution.

UPLC System (Acquity)

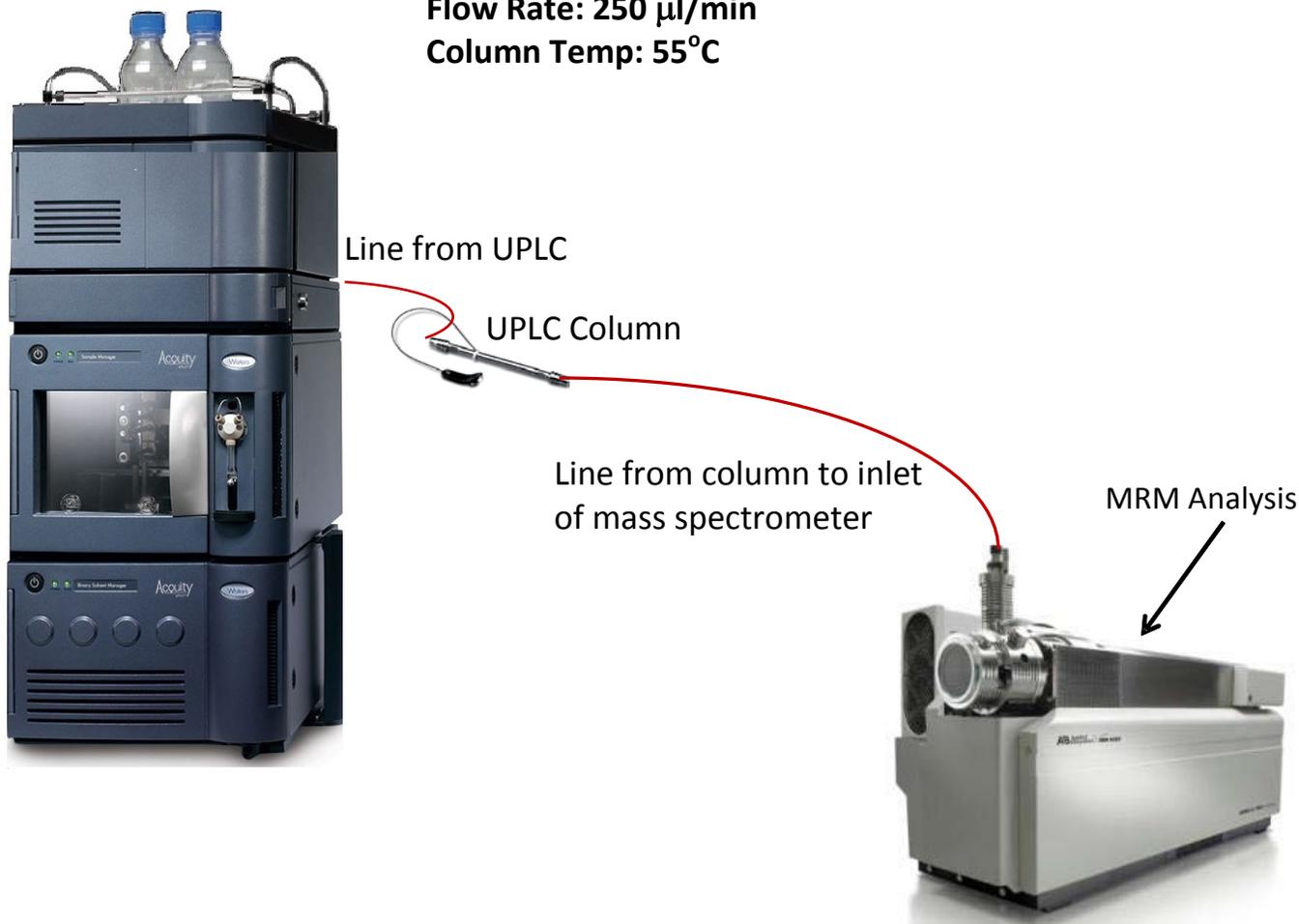
- Binary Solvent Manager
- Sample Manager
- Column Manager
- Detector

Waters ACQUITY UPLC System

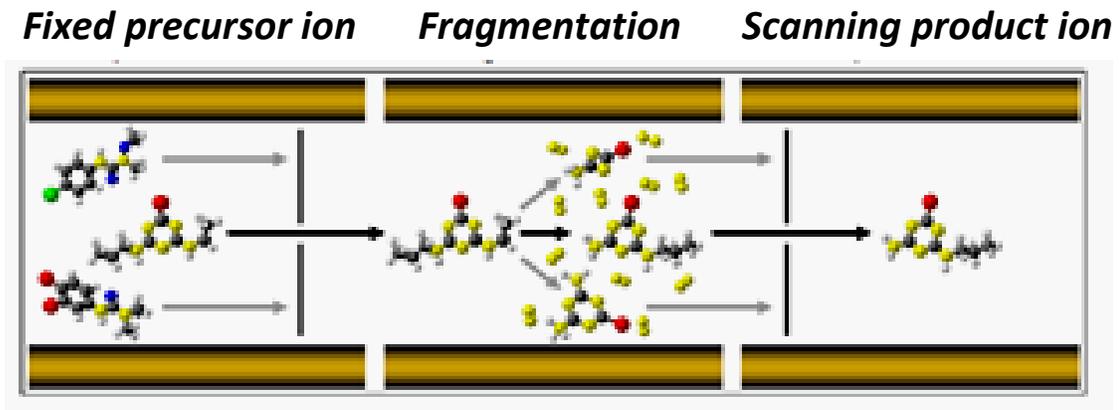


The determination of optimum LC (Liquid Chromatography) is an important step in the development of LC/MRM method. For this demonstration, LC conditions has already been optimized and included in the method. Reverse Phase Chromatography (RP-LC) will be used in which the analyte partitions between a hydrophobic stationary phase and a polar mobile phase. Typical stationary phases are based on C₁₈ hydrocarbon chains attached to silica particles through silyl-ether bonds: Si-O-CH₂-R. The particle sizes in this application are 1.8 μm and permit much higher chromatographic resolution (UPLC). The mobile phase usually consists of acetonitrile-water mixtures or methanol-water mixtures. Reverse phase chromatography is useful for a range of analytes from moderately polar to rather hydrophobic.

Column = Synergi hydro RP 2 X 250 mm
Mobile Phase A: 0.1% Formic Acid
Mobile Phase B: MeOH + 0.1 % Formic Acid
Gradient: 0 min = 5% B
8 min = 100% B
9 min = 5% B
12 min = Stop
Flow Rate: 250 μl/min
Column Temp: 55°C



LC/MS/MS Operated in Multiple Reaction Monitoring (MRM)



Features:

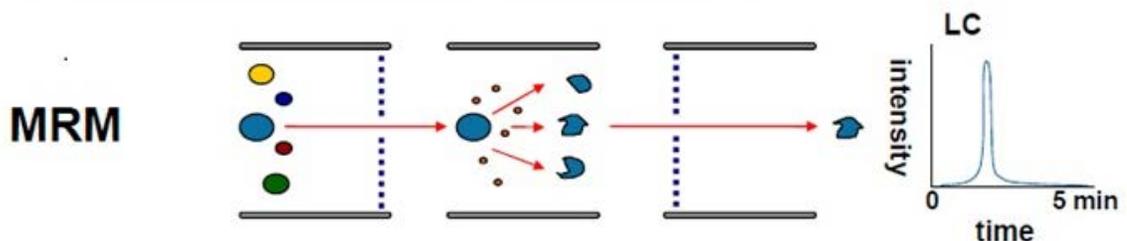
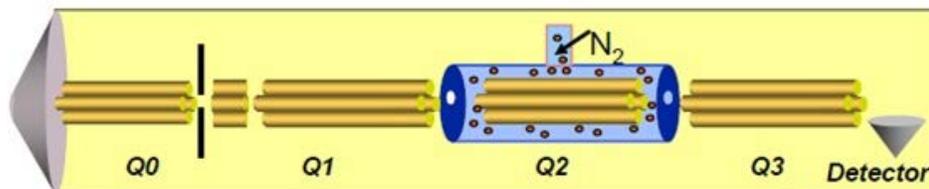
- Selectivity and sensitivity for quantitation of targeted compounds
- Simultaneous multiple compound identification

Selected Reaction Monitoring (SRM) for one compound

Scan type:

Polarity: Positive Negative

	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	ID
1	303.0	259	35	
2				



Key Parameters of Multiple Reaction Monitoring (MRM)

- **Dwell Time:** Dwell time is the time spent acquiring a specific MRM transition during each cycle. A very short dwell times can be used (5 ms or less). However, longer dwell times are always desirable for better signal/noise and sensitivity,
- **Duty Cycle:** Duty cycle is the total amount of time spent monitoring the chosen analytes. If chromatographic resolution requires a particular duty cycle time, then the dwell time for each analyte measured in the duty cycle is inversely related to the number of analytes. For example, if the duty cycle is 500 msec and there are 20 analytes to measure, the dwell time will be 25 msec.
- **Cycle Time:** The duty cycle time for an MRM assay must take into consideration chromatographic peak shape. Ideally, the peak must be sampled 8-10 times as it is eluted to get an accurate measurement of its area. So, if a peak is 10 sec wide, then the sample time would be every 1 sec. For 25 msec dwell times, then 40 MRM transitions can be monitored.

A standard MRM method looks for each mass transition throughout the entire analysis. In the following example, the mass spectrometer will measure the mass transition on line 1 for 35 msec and then advance to line 2. The instrument will measure each mass transition in the row for the specified amount of time (msec.) All of the data collection times added together will result in the **total scan time**. In the example below, the total scan time for a metabolomics experiment with 50 mass transitions is two seconds.

MS | Advanced MS

Experiment: 1 Scheduled MRM

Scan type: MRM (MRM)

Polarity: Positive Negative

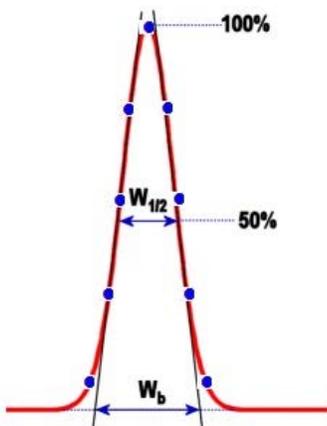
	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	ID
1	303.000	259.000	35	
2	319.000	115.000	35	
3	319.000	155.000	35	
4	319.000	151.000	35	
5	319.000	167.000	35	
6	319.000	179.000	35	
7	319.000	175.000	35	
8	353.000	193.000	35	
9	319.000	191.000	35	
10	319.000	151.000	35	
11	319.000	167.000	35	
12	319.000	175.000	35	

Total Scan Time (includes pauses): 2.0004 (sec)

Period Summary

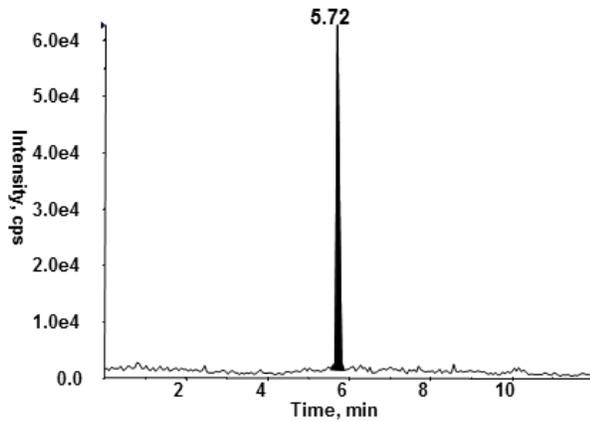
Duration: 30.005 (min) Delay Time: 0 (sec)

Cycles: 900 Cycle: 2.0004 (sec)



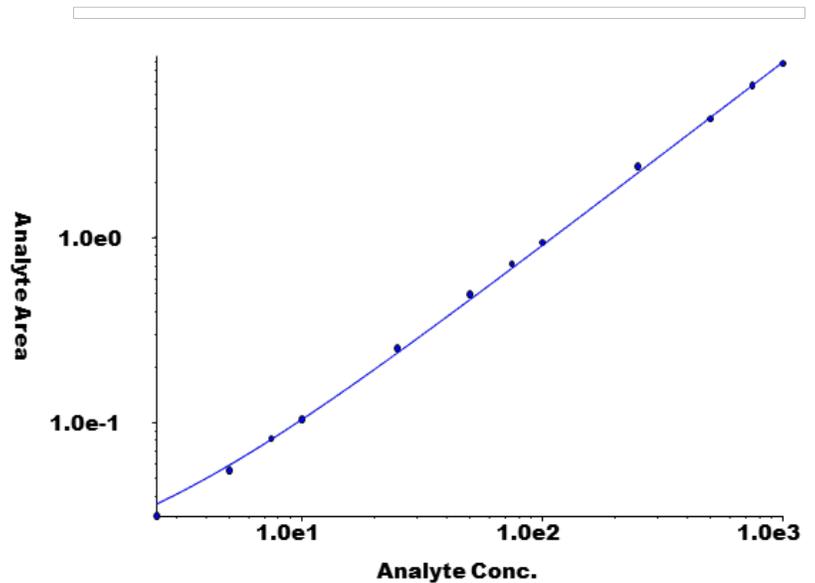
Ten to fifteen data points' collection across the peak is considered standard for MRM.

A large number of mass transitions can decrease sensitivity.



The shaded area represents the integrated peak area for succinate.

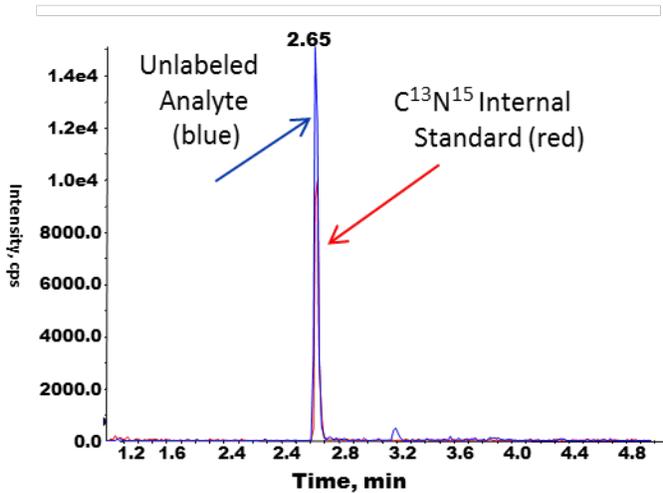
The standard curve is generated by plotting the area of the integrated peak (y-axis) as a function of concentration (x-axis).



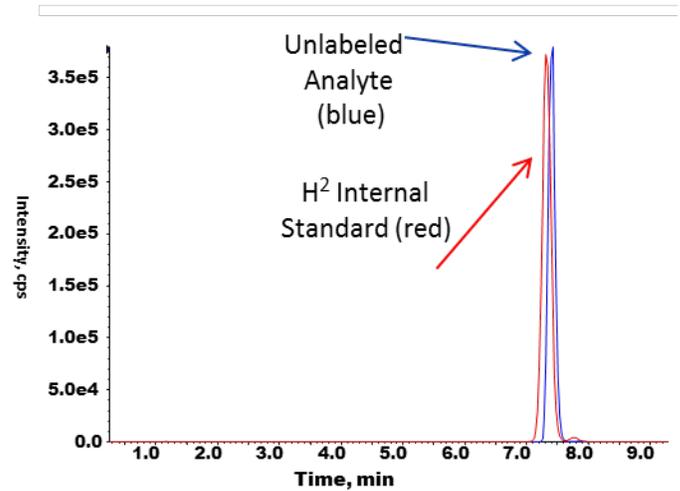
The simplest standard curve is based on the linear regression $y = mx + b$. The concentration of an unknown sample can be calculated by replacing y with the integrated peak area and solving for x . A non-linear standard curve can be fitted to the quadratic equation $ax^2 + bx + c$.

The accuracy of an LCMS/MRM analysis can be improved with the addition of an internal standard.

- An internal standard helps to normalize variations introduced by sample extraction
- The best choice of an internal standard is the C^{13} or $C^{13}N^{15}$ isotopes of the measured analyte.
- Deuterated standards are very common but have slightly different retention times than the unlabeled analyte. They might also be subject to deuterium-hydrogen exchange.



Example of $C^{13}N^{15}$ Internal Standard



Example of H^2 Internal Standard

If you have a large number of mass transitions for peaks that elute at different times, it is possible to use Scheduled Mass Transitions, or **Scheduled MRM**. This type of MRM analysis searches for a specific mass transition at a specific time. A different time can be entered for each mass transition. Scheduled MRM lowers the cycle time and increases sensitivity.

Scheduled MRM selected

The detection window specifies the number of seconds that a particular mass transition will be measured.

MS Advanced MS

Experiment: 1 Scheduled MRM Import List

Scan type: MRM (MRM)

Polarity: Positive Negative

MRM detection window: 60 (sec)

Target Scan Time: 1 (sec)

Edit Parameters...

	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	ID
1	303.000	259.000	9.5	
2	319.000	115.000	5.1	
3	319.000	155.000	4.9	
4	319.000	151.000	10.8	
5	319.000	167.000	12.0	
6	319.000	179.000	5.7	
7	319.000	175.000	8.3	
8	353.000	193.000	6.5	
9	319.000	191.000	2.6	
10	319.000	151.000	11.8	
11	319.000	167.000	7.3	
12	319.000	175.000	4.1	

Period Summary

Duration: 30.000 (min) Delay Time: 0 (sec)

Cycles: 1800 Cycle: 1.0000 (sec)

During Scheduled MRM, the mass transitions are measured only during the time that the corresponding molecule elutes from the column and not throughout the entire analysis.