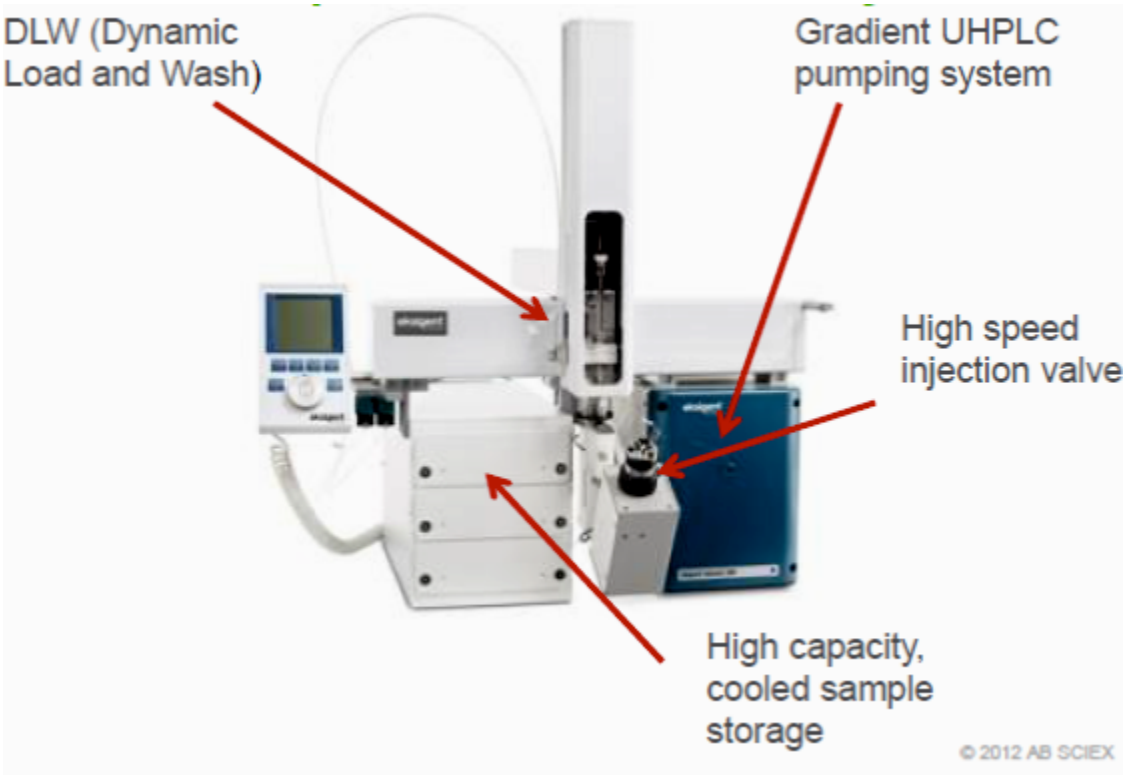
Development of an MRM method

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.

ekspert ™ microLC 200 system



Microflow refere to HPLC using columns whose inner diameter (ID) is ≤ 0.5 mm.

Typical flow rates: 4-50 uL/min.

System delay (void) volume: 1 – 3 uL

Solvent usage decreased from ~ 2-3 Liters/week to ~ 20 mls/week

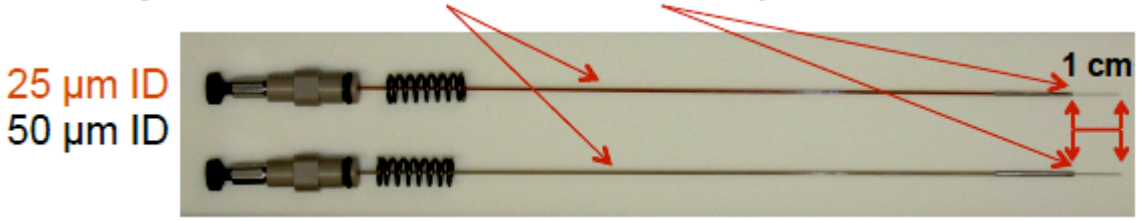
Microflow LC has several advantages over regular LC:

- **Improve throughput:** very low delay volume of system enable ultrafast gradient for LC/MS without compromising chromatography
- **Use less sample:** injection system enables sub 1 uL injections and minimal sample wasted
- **Improves sensitivity (LOQ) of LC/MS and detection limits:** improved ionization efficiency and improved chromatographic peak capacity translate into improved sensitivity & S/N
- **Improve LC and MS uptime:** lower flow rates & injection volumes provide reduced solvent load on MS
- **More cost effective on mobile phases:** lower mobile phase costs, including storage and disposal
- **Generates less waste**

MicroLC system uses new hybrid electrodes

- 25 µm ID - ideal for 0.3 mm columns (5-20 µL/min)
- 50 µm ID - ideal for 0.5 mm columns (20-100 µL/min)

Hybrid PEEKSIL/stainless steel tip electrodes

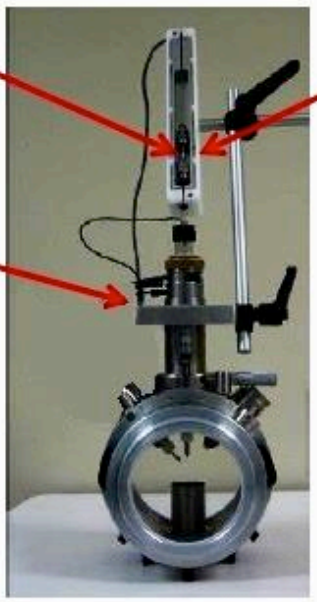


ekspert ™ microLC 200 interfacing with MS

Electrodes designed to work with a robust grounding kit with Grounding to clamp housing

Or

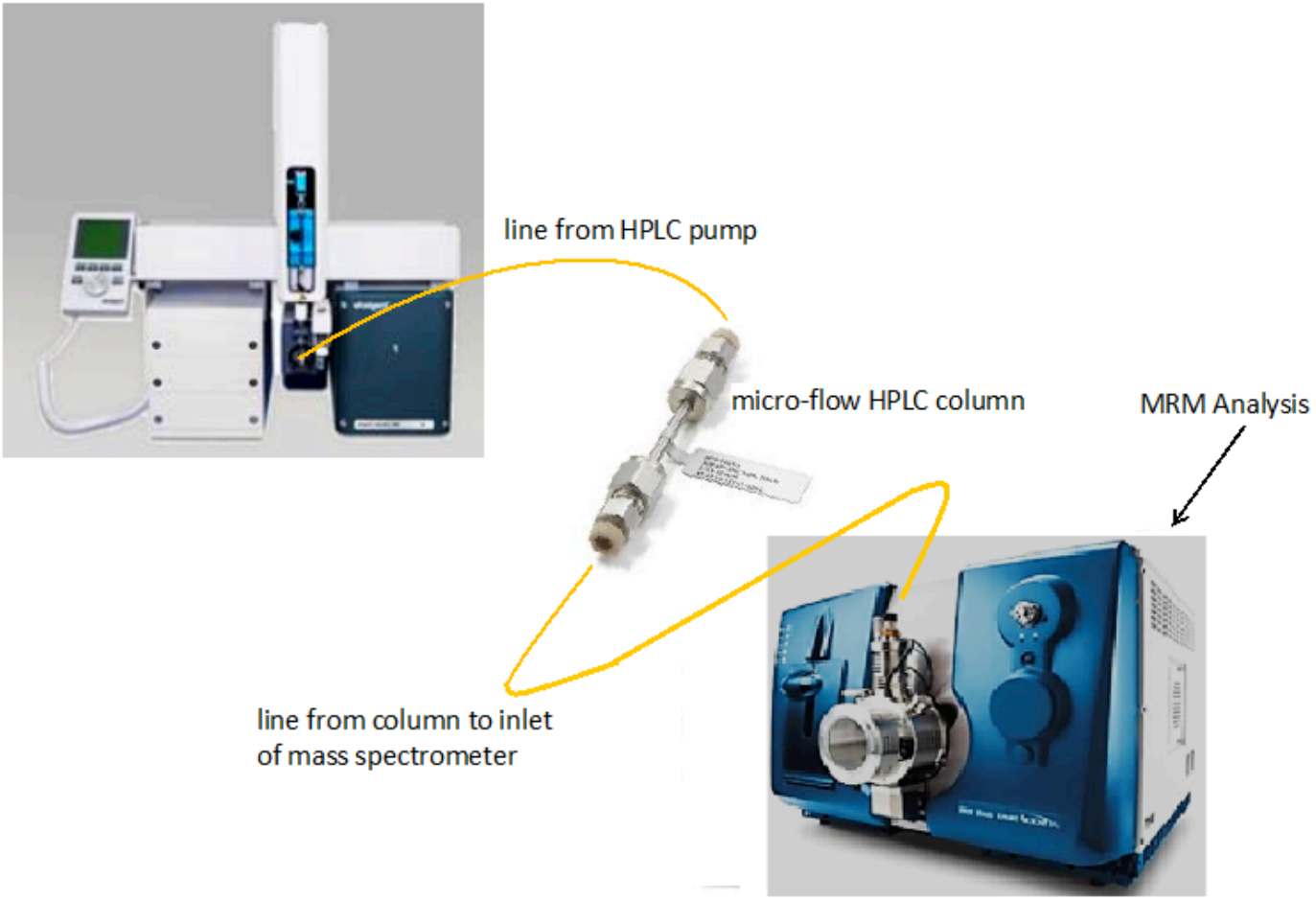
Grounding to source
Flexible mount for column oven allows for minimum connection volume from column to electrospray ionization source



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 C_{18} hydrocarbon chains attached to silica particles through silyl-ether bonds: Si-O-CH_2-R. The particle sizes in this application are 3 μm and permit much higher chromatographic resolution. 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.

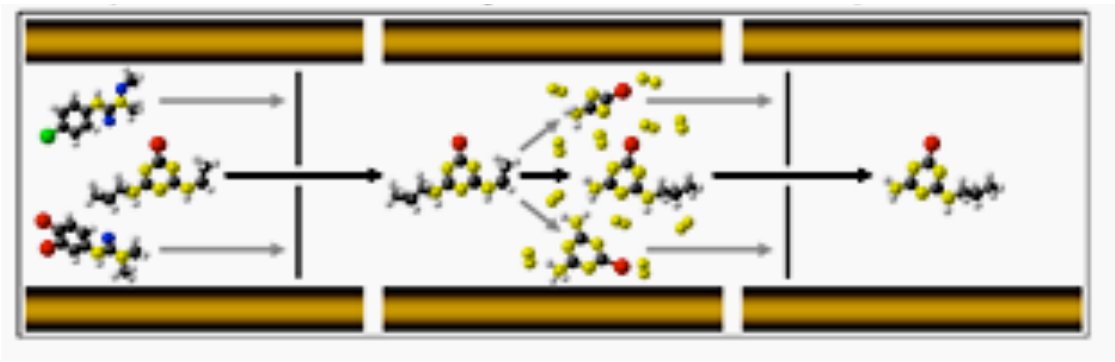
The LC (liquid chromatography) part of an MRM method has to be determined for each compound. For this demonstration, these parameters have already been determined and entered into the method.

Column = Eksigent Halo C18 0.5 x 50 mm
Mobile Phase A: 0.1% Formic Acid
Mobile Phase B: Acetonitrile + 0.1 Formic Acid
Gradient: 25%B to 100%B over one minute.
Flow Rate: 40 µl/min
Column Temp: 55° C



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

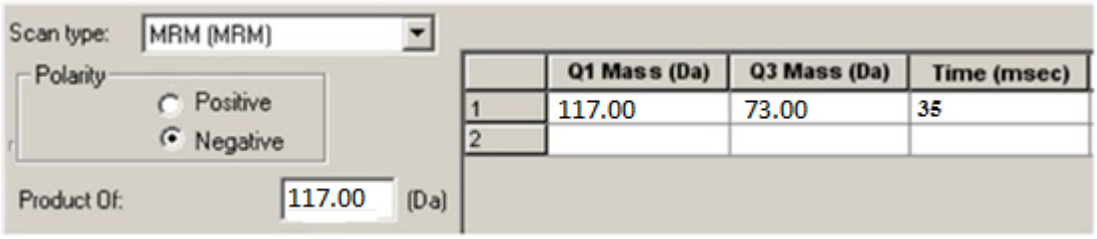
Fixed precursor ion ***Fragmentation*** ***Scanning product ion***

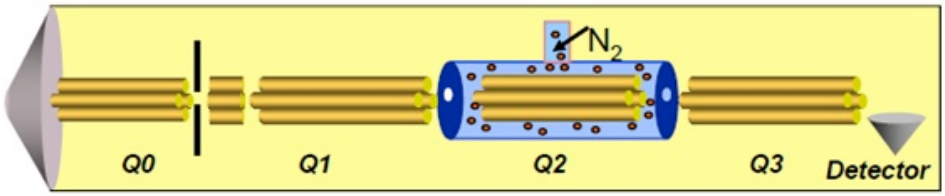


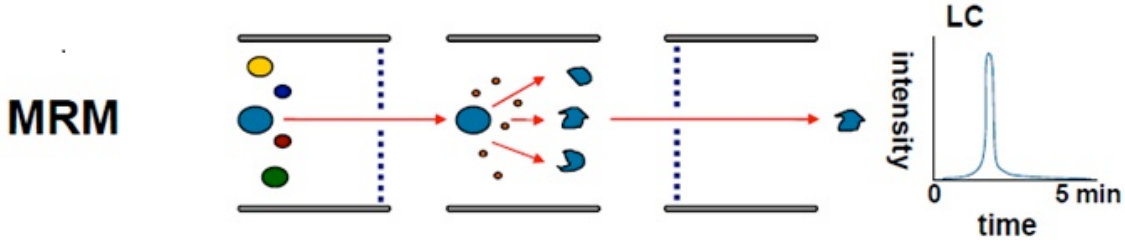
Features:

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

Selected Reaction Monitoring (SRM) for one compound







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 | Import List

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	

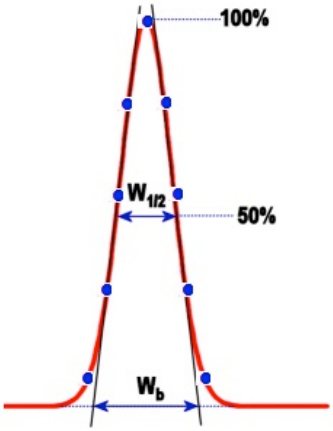
Total Scan Time (includes pauses): 2.0004 (sec)

Edit Parameters...

Period Summary

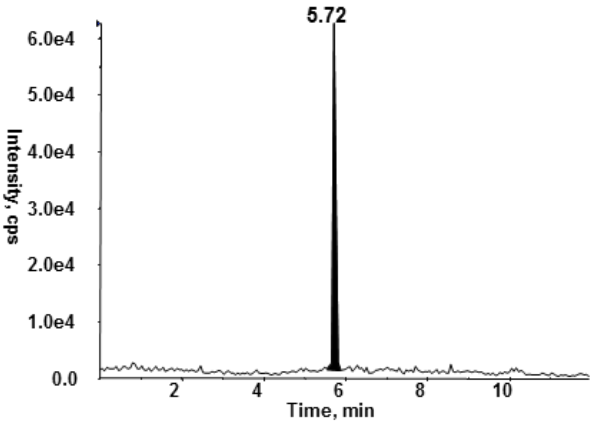
Duration: 30.005 (min) Delay Time: 0 (sec)

Cycles: 900 Cycle: 2.0004 (sec)



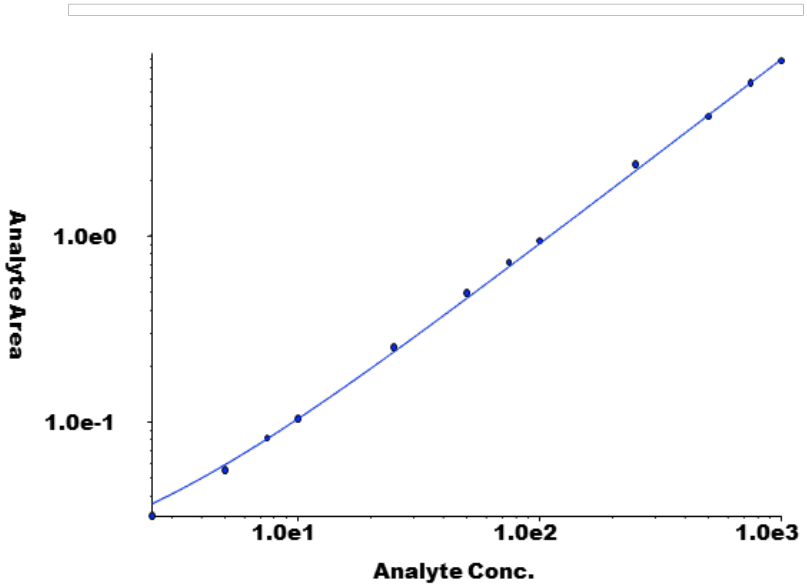
Ten to fifteen data points' collection across the peak is considered standard for MRM.

A large number of mass transitions can decrease sensitivity.



The shaded area represents the integrated peak area for succinate.

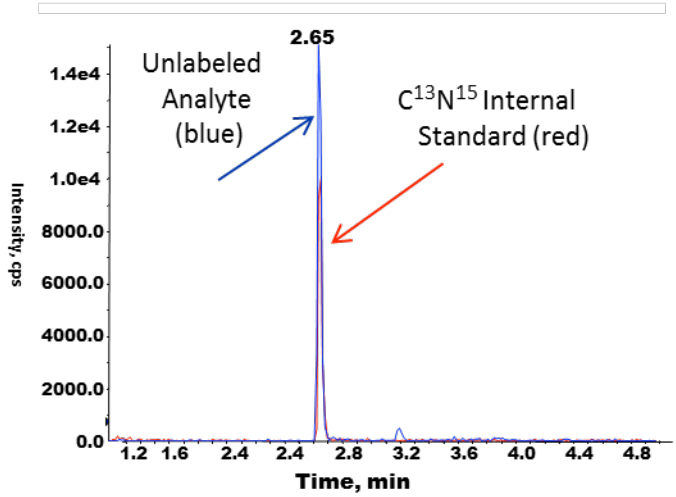
The standard curve is generated by plotting the area of the integrated peak (y-axis) as a function of concentration (x-axis).



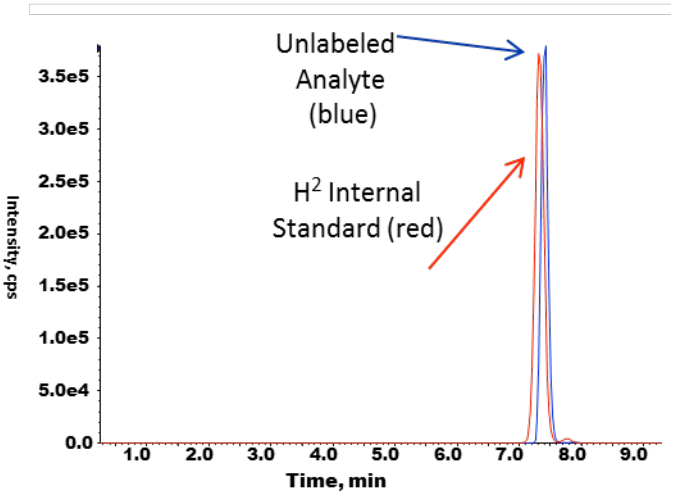
The simplest standard curve is based on the linear regression **y = mx + b**. The concentration of an unknown sample can be calculated by replacing y with the integrated peak area and solving for x. A non-linear standard curve can be fitted to the quadratic equation $\mathbf{ax^2 + bx + c}$.

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

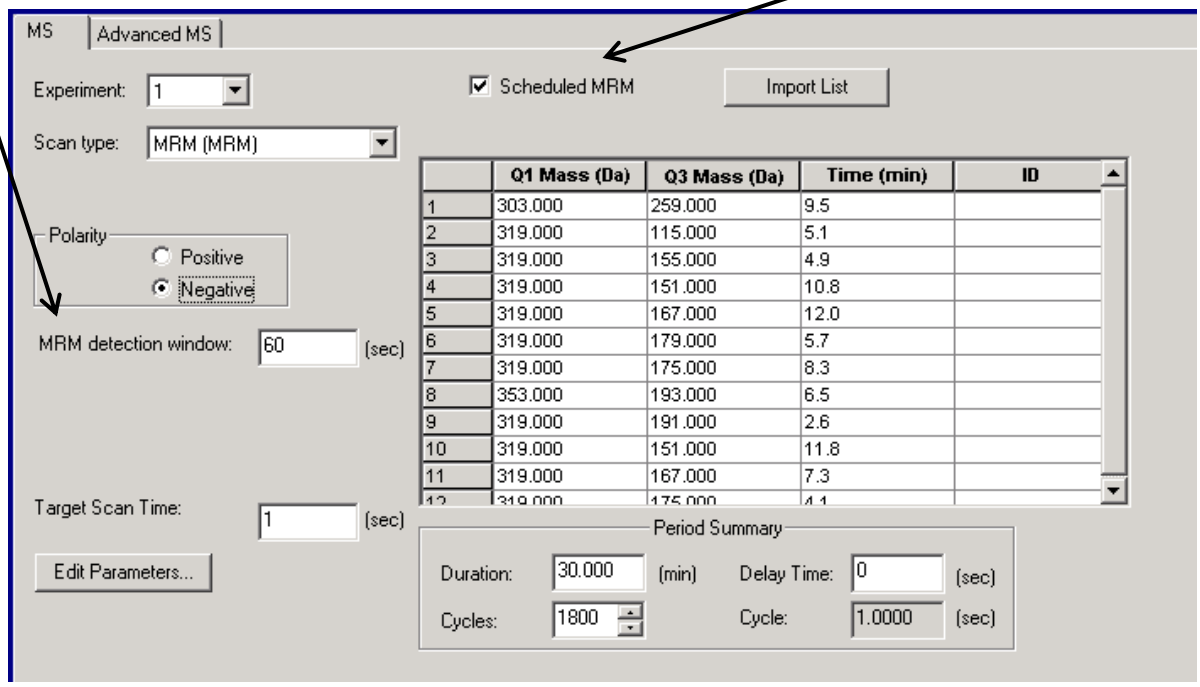


Example of H^2 Internal Standard

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.



	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	

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.