Agilent MassHunter Workstation – Data Acquisition for 6400 Series Triple Quadrupole LC/MS

Familiarization Guide

Agilent Technologies

Use the exercises in this guide to learn how to use the Agilent 6400 Series Triple Quad LC/MS. You can do these exercises with the demo data files, SulfaDrugs, shipped with the system (in the **Data** folder of your Qualitative Analysis installation disk), or with data you acquire.

In Exercise 1, you learn how to determine the best acquisition settings for analyzing your compounds of interest. These instructions help you understand not only how to set up a worklist to optimize instrument parameters for best sensitivity in acquisition, but also how to use the Qualitative Analysis program to identify parameter values producing optimum signal response. You can also learn about the Qualitative Analysis program by using the *Qualitative Analysis Familiarization Guide* or the Qualitative Analysis online Help.

In Exercise 2, you learn how to use the Optimizer program. The Optimizer Software helps you optimize acquisition parameters. Specifically, it automates the selection of the best precursor ion and the fragmentor voltage for the most abundant precursor ion, selection of the best product ions, and optimization of collision energy values for each transition for a list of compounds you specify.

In Exercise 3, you learn how to use either an acquired data file or the Quantitative Analysis report results to update a dynamic MRM method. This method allows you to easily set up a dynamic MRM method.

NOTE See the *Concepts Guide* to learn more about how the triple quadrupole mass spectrometer works and why the fragmentor and collision energy voltages are important. For background information, see Chapter 3, "Agilent Triple Quad MS and Sensitivity", in the *Concepts Guide*. See the online Help for detailed information on how the program works.

Each task is presented in a table with three columns:

- Steps – Use these general instructions to proceed on your own to explore the program.
- Detailed Instructions – Use these if you need help or prefer to use a step-by-step learning process.
- Comments – Read these to learn tips and additional information about each step in the exercise.

Before you begin

Before you begin, you need to check that your system is ready. If you plan to acquire data, you also need to set up the instrument.

Prepare your system

1 Check that:

- The Data Acquisition program has been installed.
- The LC modules and the 6400 Series Triple Quad LC/MS have been configured.
- The performance has been verified.
- The system has been turned on.

If these actions have not yet been done, see the *Installation Guide* for your instrument.

2 Copy the data files to your PC.

Copy the folder named **SulfaDrugs** in the **Data** folder on your Qualitative Analysis installation disk to any location on your hard disk. This folder contains all the data files needed for this exercise.

NOTE Do not re-use the sulfa drug data files already on your system unless you know that you copied them from the originals on the disk and you are the only one using them.

Prepare to acquire data

If you do not intend to acquire data but want to learn how to use the Qualitative Analysis program for method development, you can skip this step, which tells you how to prepare the demo sample. You then do those tasks that show you how to use the Qualitative Analysis program with the sulfa drug data files shipped with the system.

Parts List The exercise in this guide uses this equipment and materials:

- Agilent 1200, 1260 Infinity or 1290 Infinity LC modules: well-plate sampler, binary pump, thermostatted column compartment, DAD
- Zorbax column (see Table 1 on page 4)
- A 10-µL sulfa mix sample (prepared in this step)

Table 1 Zorbax columns

Triple Quadrupole	Column Description	Film Thickness	Pore Size	Part Number
6410B, 6420, 6430, 6460 and 6490	SB-C18 2.1mm x 50mm	1.8 µm	80Å	822700-902

1 Prepare the LC solvent.

In 1-liter reservoirs of HPLC-grade water and acetonitrile (ACN), add 1 mL of 5M NH_4HCO_2 (Ammonium Formate) each to make 5mM NH_4HCO_2 in water and acetonitrile and use for the A and B channels, respectively.

2 Prepare the sample.

a Add 10 µL sulfa mix from one of the ampoules (500 µL) to 990 µL of solvent A in an Eppendorf vial so that the final concentration is 1 ng/µL.

b Place a sample vial containing an injectable amount of the prepared sample in the autosampler.

3 Set up the LC column.

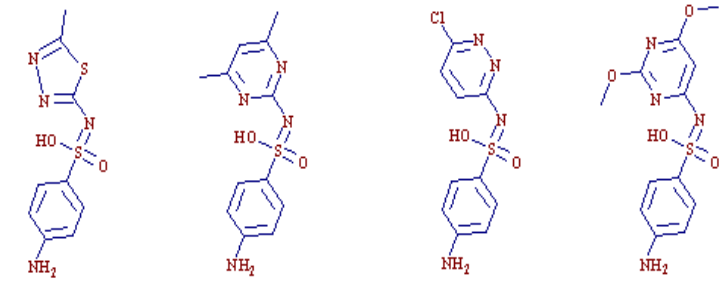
Use the appropriate Agilent column from Table 1.

4 Set the column temperature.

For the Agilent 6460 and 6490 with Agilent Jet Stream Technology, set the column temperature to 60°C.

For the Agilent 6420 and 6430 and for the Agilent 6460 and 6490 without Agilent Jet Stream Technology, set the column temperature to 40°C. This exercise can also run at room temperature.

The Electrospray LC Demo Sample (P/N 59987-20033) contains five ampoules with 100 ng/µL each of sulfamethizole $(M+H)^+ = 271$, sulfamethazine $(M+H)^+ = 279$, sulfachloropyridazine $(M+H)^+ = 285$, and sulfadimethoxine $(M+H)^+ = 311$.



NOTE Determining optimal parameter values for acquiring sample compound data requires that the Agilent Triple Quad instrument already be tuned on the Tuning Mix calibrant ions. Before proceeding with this exercise, make sure you have used Checktune or Autotune to verify that calibrant ions each have the proper mass assignment, peak width, and signal intensity.

See the *Quick Start Guide, Installation Guide* or online Help for instructions on tuning the instrument.

Exercise 1 – Develop an acquisition method for the 6400 Series

For this exercise you analyze a mixture of four sulfonamide compounds.

Task 1. Enter acquisition parameters and acquire data

In this exercise, you enter the conditions for the analysis of the sulfa drug mix.

Steps	Detailed Instructions	Comments
1 Enter LC parameters appropriate for sulfa drug mix. See Table 2.	**a** Double-click the **Data Acquisition** icon. **b** Make sure that Acquisition appears as the selection in the **Context** text box. If Tune is the selection, click **Acquisition** from the **Context** dropdown menu in the Combo bar. **c** Enter the LC parameters listed in the Table 2.	• The Data Acquisition window appears. See Figure 1.

Table 2 LC parameters for sulfa drug mix

Parameter	LC Parameter
PUMP	
• Flowrate	800 µL/min
• Solvent A	5 mM NH_4HCO_2 in H_2O
• Solvent B	5 mM NH_4HCO_2 in 90:10 acetonitrile:water
• Gradient (min - %B)	0 min - 13% 1.80 min - 60% 2 min - 60%
• Stop Time	2.0 min
• Post Time	2.0 min
INJECTOR	
• Inj. Vol.	2.0 µL
• Injection	Standard

Table 2 LC parameters for sulfa drug mix (continued)

Parameter	LC Parameter
• Draw Position	0.0 mm
UV DETECTOR	
• Ch A	254 nm (4 nm BW on DAD)
• REF A (DAD only)	400 nm (80 nm BW)
COL THERM	
• Temp	60 °C for the 6460 and 6490 with Agilent Jet Stream Technology 40 °C for other instruments

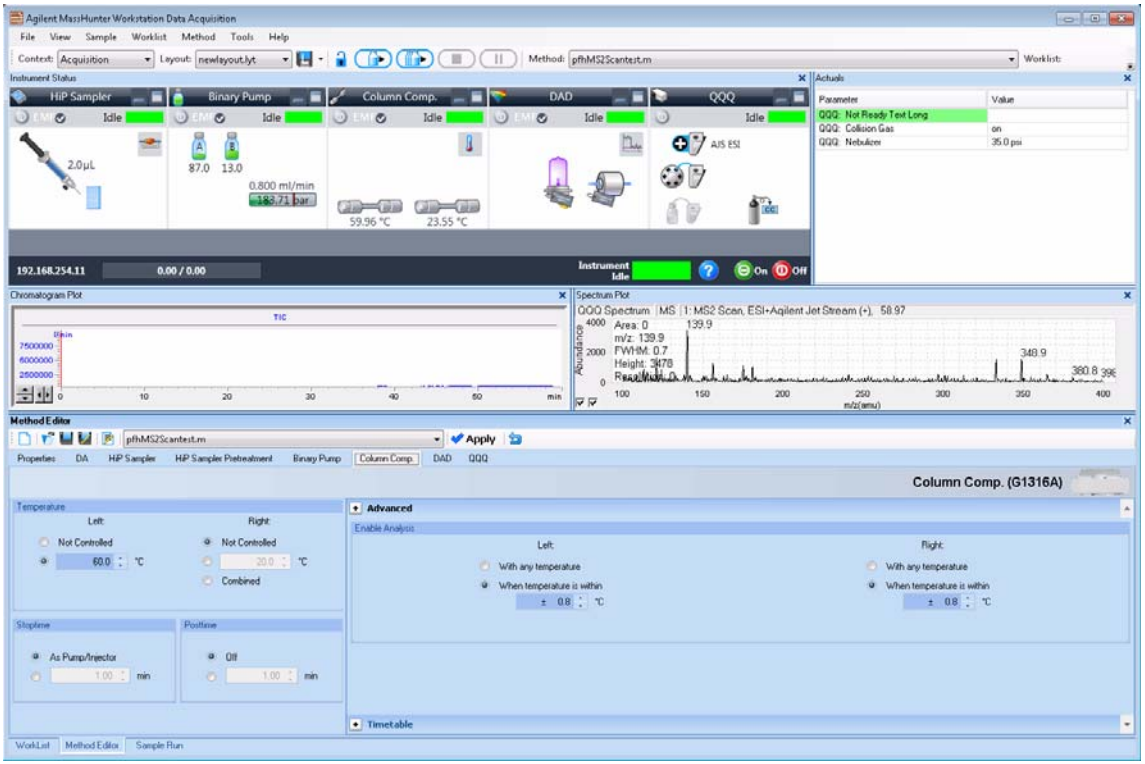


Figure 1 Agilent MassHunter Workstation Software – Data Acquisition window

Steps	Detailed Instructions	Comments
2 Enter MS parameters appropriate for sulfa drug mix and save the method as ***iii*MS2Scantest.m**, where ***iii*** are your initials. See Table 3.	**a** Click the **QQQ tab** in the **Method Editor** window. **b** Select **MS2Scan** from the **Scan Type** list in the Time Segments table. **c** Enter the other MS parameters as listed in Table 3. These parameters are in either the Acquisition or the Source tabs. **d** Save the method as ***iii*MS2Scantest.m**, where ***iii*** are your initials.	

Table 3 MS parameters for sulfa drug mix

Parameter	Value	
• Inlet	ESI (positive polarity)	ESI (positive polarity) with Agilent Jet Stream Technology
• Scan Type	MS2Scan	MS2Scan
• Delta EMV pos	400 V	200 V
• Mass Range	100 to 400	100 to 400
• Cell Acceleration Voltage	7 V	7 V
• Gas Temp	350 °C 250 °C for iFunnel on Agilent 6490	350 °C 250 °C for iFunnel on Agilent 6490
• Gas Flow	12 L/min	10 L/min
• Nebulizer	50 psi	35 psi (nitrogen)
• Sheath Gas Temperature	not applicable	400 °C
• Sheath Gas Flow	not applicable	12 L/min
• Nozzle Voltage	not applicable	0 V
• Capillary Voltage positive	4000 V	4000 V

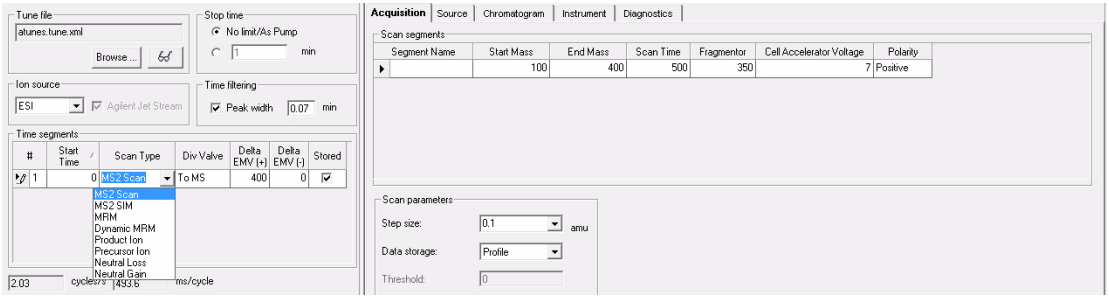
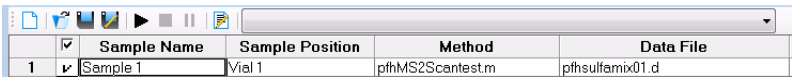


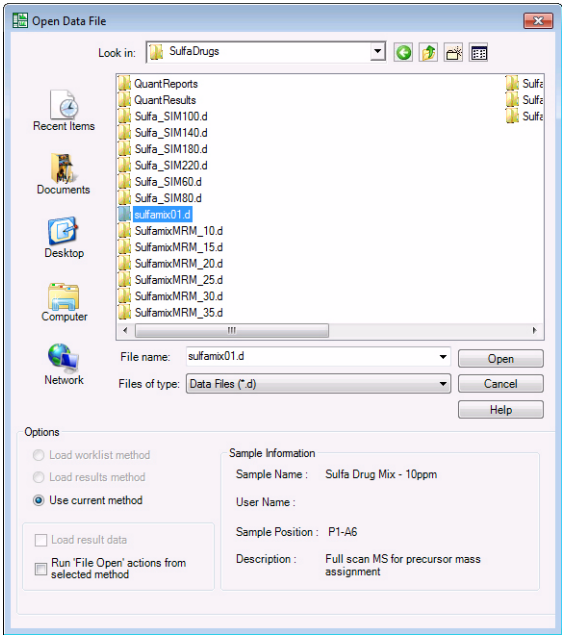
Figure 2 Select **Scan Type** of MS2 Scan in the QQQ tab

Steps	Detailed Instructions	Comments
3 Acquire data (optional). • Set up a one-line worklist with the method you just created. • Name the data file ***iii*sulfamix01.d**, where ***iii*** are your initials. • Designate a directory path to hold your data files and method.	**a** If necessary, click **View > Worklist** to display the Worklist window. **b** Click **Worklist > Worklist Run Parameters.** Verify that the parameters are set properly. Click **OK**. **c** Click **Worklist > Add Multiple Samples**. **d** Type `iiisulfamix01.d` as the data file name **e** `Select iiiMS2Scantest.m` as the method name. **f** Click the **Sample Position** tab. **g** Select the Autosampler, Well-plate or Vial Tray. **h** In the graphic, select a single position. Click **OK.** **i** In the Worklist window, mark the check box to the left of the sample as shown below.  **j** Click the **Start Worklist Run** icon in the main toolbar, the **Run Worklist** icon in the Worklist toolbar or click the **Worklist > Run** command.	• The Worklist window is tabbed with the Method Editor window by default. Click the **Worklist** tab to show the Worklist window. • The **Number of samples** is set to 1. • You have just acquired a full scan MS data file to see what ions are being formed from the sample. • This step is optional because you can perform the next step with an example data file that comes with the program. If you prefer, you can create your own data file as described in this step.

Task 2. Determine precursor ion masses

In this exercise, you determine the precursor ions for each of the sulfa drugs in the acquired data file.

Steps	Detailed Instructions	Comments
1 Open the acquired data file. • In the Qualitative Analysis program, open either the example file, **sulfamix01.d,** or the data file you created in "Task 1. Enter acquisition parameters and acquire data" on page 6.	**a** Double-click the **Qualitative Analysis** icon.  The program displays the "Open Data File" dialog box.	• When you open the sulfa drug directory after installation, the **Load result data** (lower left corner) check box is grayed out. • If you see the check box marked, this means that the data file(s) already contains results. ***Clear this check box before opening the file***.



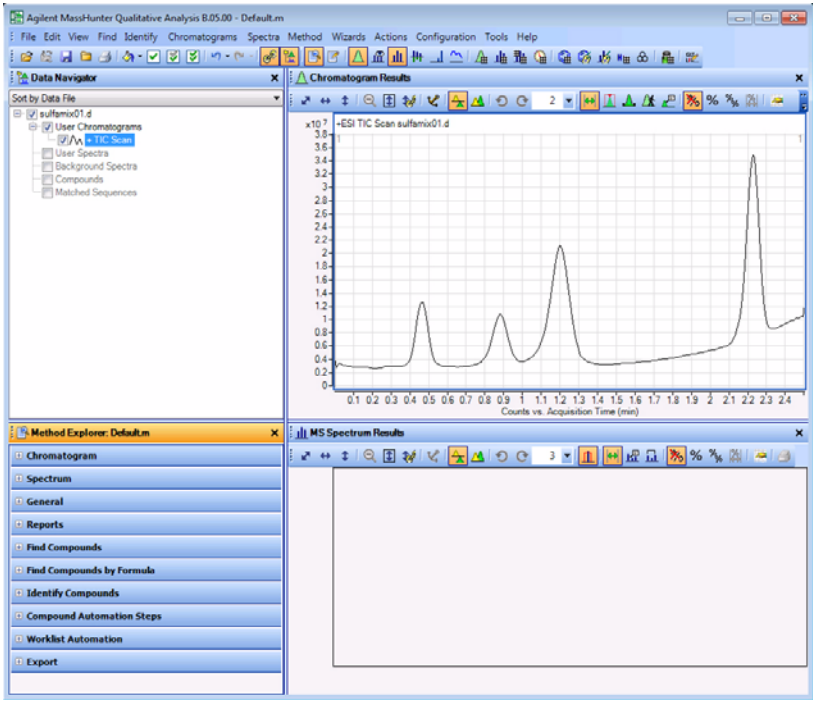
Steps	Detailed Instructions	Comments
	b Do one of the following: • Select the example data file **sulfamix01.d**, and click **Open**. • Select the data file you created in "Task 1. Enter acquisition parameters and acquire data" on page 6, and click **Open**. By default, the system displays the Total Ion Chromatogram (TIC).	• The figure below shows the default layout. • The Qualitative Analysis program displays a newly opened data file with the same layout and display settings used for the previous data file. Therefore, you MUST make sure to return to the default settings for this exercise.

Before you begin, make sure that all previous settings are returned to their default values:

- **Restore default layouts**
  - **Click Configuration > Window Layouts > Restore Default Layout.**
- **Make sure the method is default.m. (see title bar)**
  - **Click Method > Open.**
  - **Select default.m, and click Open.**
- **Return display options to default settings.**
  - **In the Configuration menu, click each of the Display Options commands.**
  - **Click Default, and then OK.**

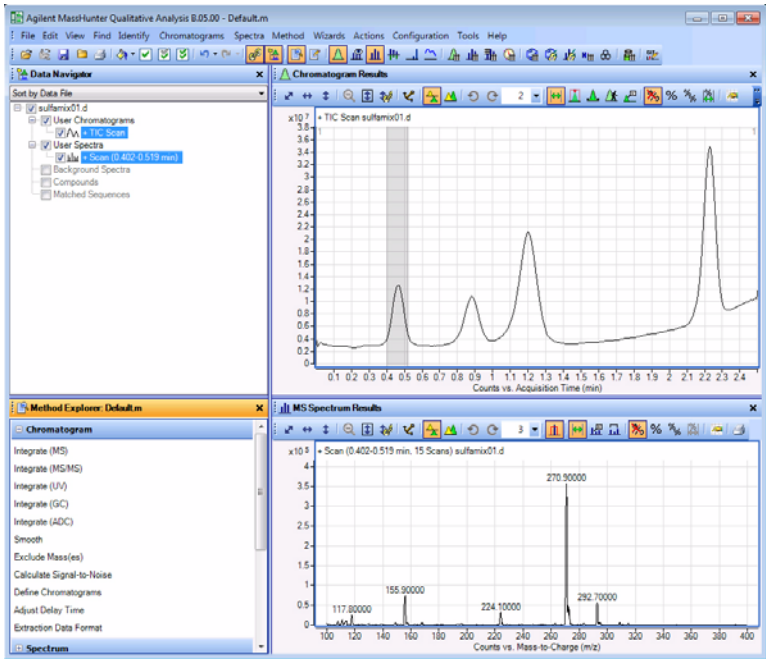
Or...

- **Restore the General layout.**
  - **Click Configuration > Configure for Workflow > General.**
  - **Click OK.**
  - **(optional) You may be asked to save method changes.**
- **Return display options to default settings.**
  - **In the Configuration menu, click each of the Display Options commands.**
  - **Click Default, and then OK.**



Steps	Detailed Instructions	Comments
2 Determine precursor ion masses for all four peaks. • You have determined them correctly if you find the values are similar to those shown in this table:	**a** In the Chromatogram Results window, make sure that the Range Select icon in the toolbar is on. **b** Click the left mouse button and drag the cursor across the first peak to produce a shaded region, as in the figure below. **c** Right-click the shaded area, and click **Extract MS Spectrum** from the shortcut menu.	• The system displays an averaged spectrum across the peak in the MS Spectrum Results window. • The precursor mass of the first compound, sulfamethizole, is determined to be m/z 270.9. • To obtain a single scan, double-click the apex of the peak.
• If you acquired the data file using the Agilent Jet Stream Technology, the retention times may be different. • The sulfamix01.d data file was acquired with a different column so your retention times are different. • Close the data file after finding the precursor ion masses.	**d** Repeat step a through step c for the other compounds. The precursor ion masses should match those in the table in step 2. **e** Click **File > Close Data File**. **f** When asked if you want to save the results, click **No**.	• Some compounds form sodium (Na) and/or potassium (K) adducts as well, corresponding to M + 23 and M + 39 masses respectively. Seeing these masses along with the M + H can make for an easy confirmation of which ion is the pseudo-molecular ion (M + H)+.

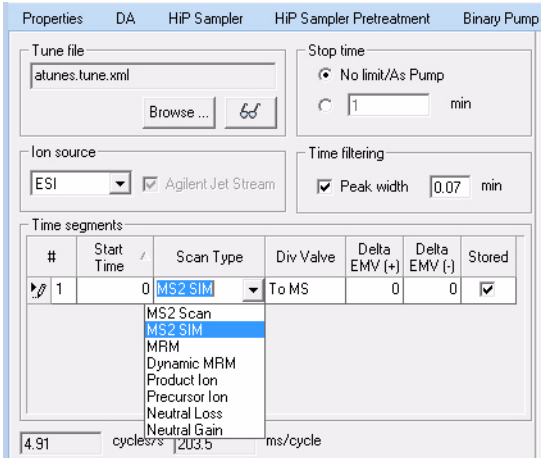
Compound	RT	*m/z*
Sulfamethizole	0.47	270.9
Sulfachloropyridazine	0.88	284.9
Sulfamethazine	1.20	279.0
Sulfadimethoxine	2.23	311.0



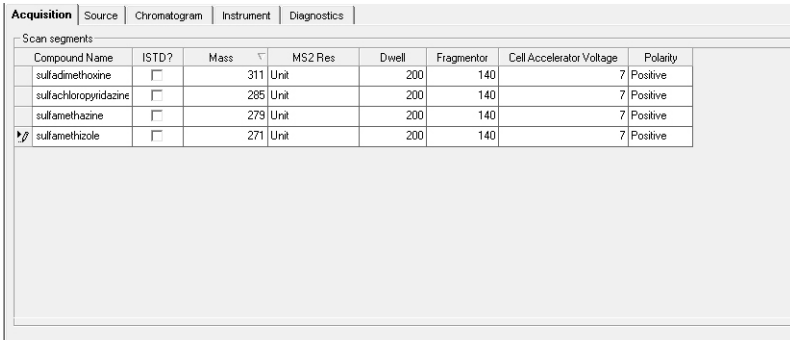
Task 3. Find optimum fragmentor voltage for maximum response

Task 3 shows you how to carry out the optimization for fragmentor voltage by creating selected ion-monitoring experiments for each compound within a method and setting up multiple methods with varying fragmentor voltages.

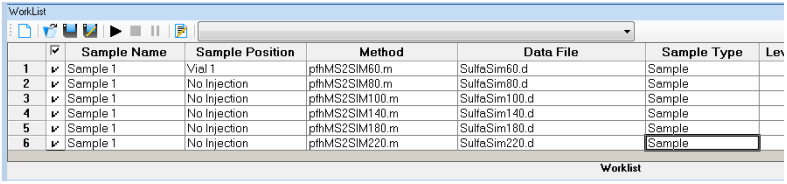
The Fragmentor Voltage for the 6490 is set automatically during Autotune, and it cannot be set in the Data Acquisition program. If your instrument is a 6490, skip to "Task 4. Determine product ion masses". You can do the Qualitative Analysis part of this task by using the data files that were shipped with the software.

Steps	Detailed Instructions	Comments
1 Set up six methods for six different fragmentor voltages. • Change to a SIM experiment. • Use 60, 80, 100, 140, 180 and 220 volts as the fragmentor voltages for the six methods. • Save the methods as ***iii*MS2SIM*xxx*.m**, where ***iii*** are your initials and ***xxx*** is the voltage.	**a** In the **Scan Type** dropdown list, click **MS2 SIM**. 	

Steps	Detailed Instructions	Comments
	b In the **Acquisition** tab, enter the **Compound Name** and **Mass** (precursor ion mass) for sulfadimethoxine. **c** Right-click anywhere in the Scan segments section, and click **Add Row**. **d** Type the **Compound Name** and the **Mass** for sulfachloropyridazine. **e** Repeat steps c and d for sulfamethazine and sulfamethizole. **f** **Save the method as *iii*MS2SIM140.m**, where ***iii*** are your initials**.** **g** Change the fragmentor voltage to 60, and save the method as ***iii*MS2SIM060**, where ***iii*** are your initials. **h** Repeat step g for voltages 80, 100, 180 and 220, saving the methods as ***iii*MS2SIM080, *iii*MS2SIM100**, ***iii*MS2SIM180 and *iii*MS2SIM220,** where ***iii*** are your initials.	• With the MS2SIM Scan Type set, a different set of columns appears in the Acquisition window. • The Instrument Control and Data Acquisition program creates a SIM experiment for each compound mass, starting with a default fragmentor voltage of 140. See the example below. • The Fragmentor column is grayed out if the instrument type is an Agilent 6490.

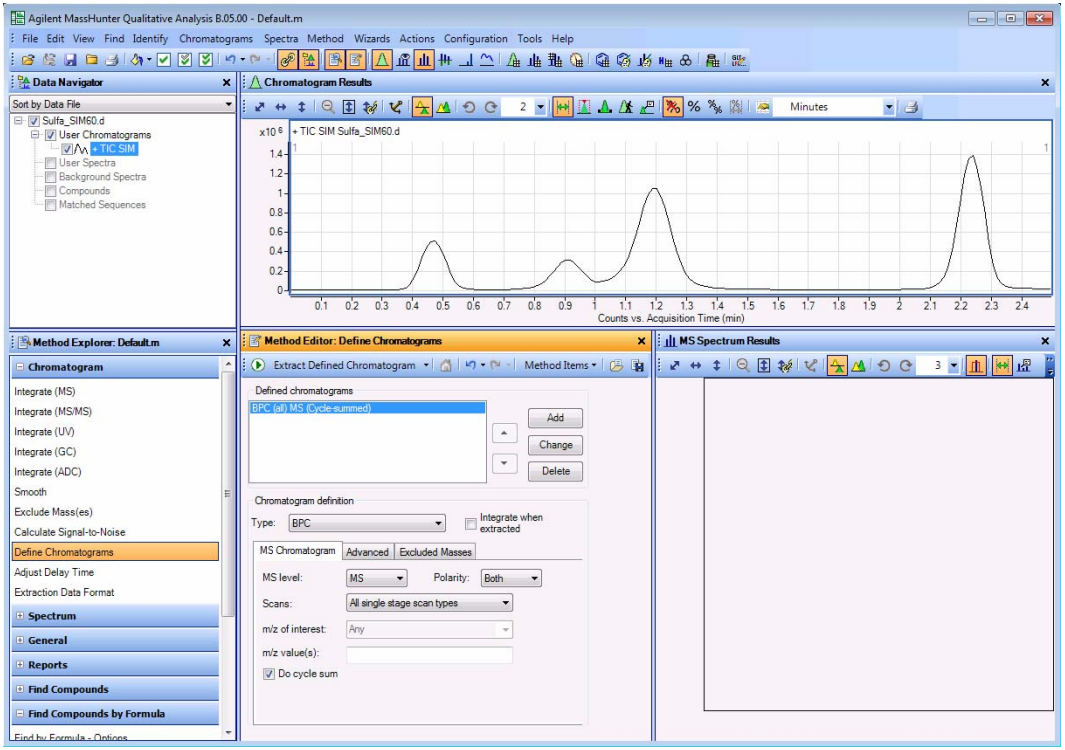


Compound Name	ISTD?	Mass	MS2 Res	Dwell	Fragmentor	Cell Accelerator Voltage	Polarity
sulfadimethoxine	☐	311	Unit	200	140	7	Positive
sulfachloropyridazine	☐	285	Unit	200	140	7	Positive
sulfamethazine	☐	279	Unit	200	140	7	Positive
sulfamethizole	☐	271	Unit	200	140	7	Positive

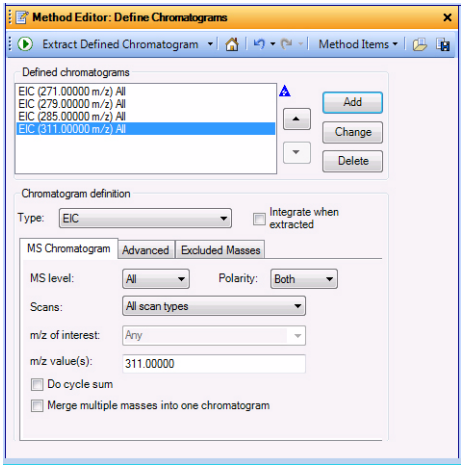
Steps	Detailed Instructions	Comments
2 Set up and run the worklist (optional). • Set up six samples with Sample Name SulfaDrugMix to inject 1ul from vials 1-6 or the ones you choose. • Specify the data files as ***iii*SulfaSIM*xxx*.d**, where ***iii*** are your initials and ***xxx*** is the voltage.	**a** Click the **Worklist** icon if necessary to make sure the worklist is visible. **b** Click **Worklist > New** to start a new worklist. You do not need to save the last worklist. **c** To set up the run, right-click the upper left corner of the worklist, and click **Worklist Run Parameters**. **d** Type the paths for the method and data files. **e** Type the information for the 60 voltage run. **f** Click **Worklist > Add Sample**. Another sample is added to the Worklist. Add five samples to the worklist for voltages 80-220. **g** Mark the checkbox to the left of the Sample Name for each of the six samples.	• This step is optional because you can use data files shipped with the system to perform many of the tasks in this exercise.
		
	h Start the worklist. • Click **Worklist > Run**. • Click the  icon in the main toolbar. • Click the  icon in the worklist toolbar.	• Note that the program only runs those samples that are marked with a checkmark. • You can also run the worklist in locked mode by clicking the  button in the main toolbar.

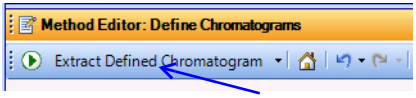
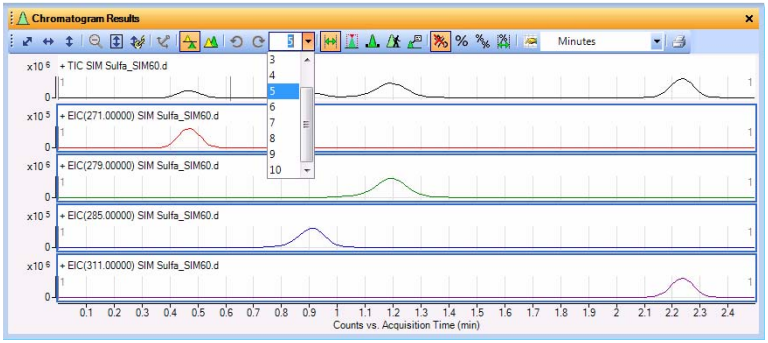
Sample Name	Sample Position	Method	Data File	Sample Type
1 Sample 1	Vial 1	pfhMS2SIM60.m	SulfaSim60.d	Sample
2 Sample 1	No Injection	pfhMS2SIM80.m	SulfaSim80.d	Sample
3 Sample 1	No Injection	pfhMS2SIM100.m	SulfaSim100.d	Sample
4 Sample 1	No Injection	pfhMS2SIM140.m	SulfaSim140.d	Sample
5 Sample 1	No Injection	pfhMS2SIM180.m	SulfaSim180.d	Sample
6 Sample 1	No Injection	pfhMS2SIM220.m	SulfaSim220.d	Sample

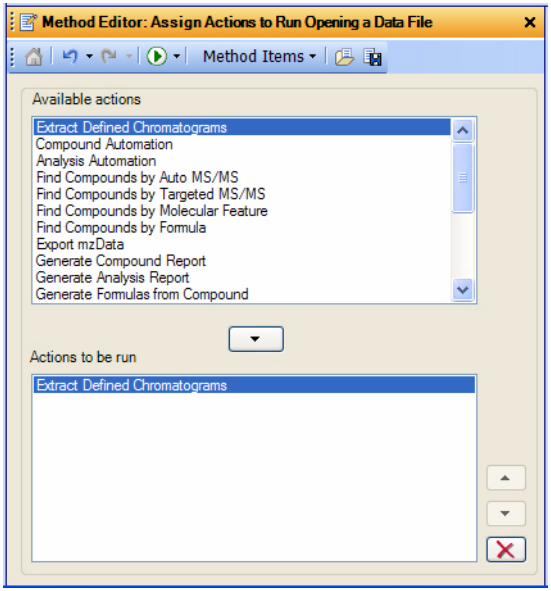
Steps	Detailed Instructions	Comments
3 Set up a qualitative method to view the EIC data automatically. • Open the data file **Sulfa_SIM60.d** or your own ***iii*Sulfa_SIM60.d**, where ***iii*** are your initials. • In the Method Editor, add in the EICs corresponding to the precursor ion masses of 271, 279, 285, and 311. • Save the method as *iii*Exercise1, where "*iii*" are your initials.	**a** Click **File > Open Data File**. The system displays the Open Data File dialog box **b** Select either **Sulfa_SIM60.d** or ***iii*Sulfa_SIM60.d**, and click **Open**. **c** Click **Method > Method Editor** or **View > Method Editor**. The system displays the Method Editor window.	• The Qualitative Analysis program should be open. If not, see "Double-click the Qualitative Analysis icon." on page 10.



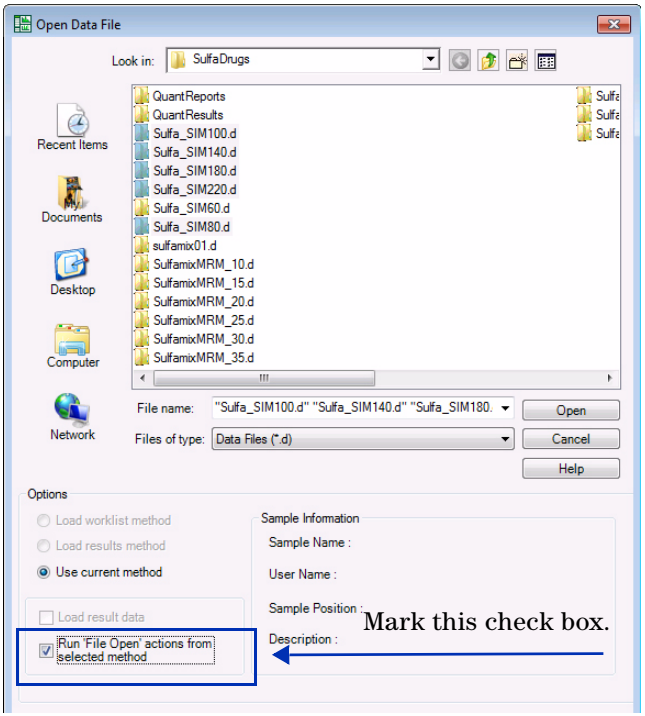
Steps	Detailed Instructions	Comments
	d If necessary, click **Define Chromatograms** in the Chromatogram section of the Method Explorer. **e** To delete the BPC chromatogram, click **Delete**. **f** Select **EIC** for the **Chromatogram Definition Type**, **g** In the MS Chromatogram tab, make sure **MS Level** is set to **All** and **Scans** is set to **All scan types**. **h** Clear the **Do cycle sum** check box. **i** Type `271` as the **m/z value**. **j** Click **Add**. **k** Repeat steps i and j for the other precursor ions, `279`, `285` and `311`. **l** Click **Method > Save As**. The system opens the Save As dialog box **m** Save the method as ***iii*Exercise 1.m**. **n** Click **Save**.	• The default Method Editor list selection after installation is **Integrate (MS)**. • You can also select Define Chromatograms from the Method Items list in the Method Editor window.



Steps	Detailed Instructions	Comments
4 Extract the chromatogram for the data file and view the results. • Make sure you can see all five chromatograms, the TIC and four EICs.	**a** Click the **Run** button on the Method Editor toolbar.  **b** To see the TIC and four EICs, click the arrow next to the Maximum Number of List Panes icon in the Chromatogram Results toolbar, as shown in the example below. **c** Select **5** to view five chromatograms simultaneously. The system displays chromatogram results as shown below.  	• You can also click the **Chromatograms > Extract Defined Chromatograms** command to extract the defined chromatograms.

Steps	Detailed Instructions	Comments
5 Extract the remaining ion chromatograms automatically. • Extract Defined Chromatograms should be the default action for Assign File Open Actions. • Open the remaining data files, Sulfa_SIM80.d through Sulfa_SIM220.d. • Close the Method Explorer.	**a** Select **File Open Actions** from the General section in the Method Explorer. **b** Make sure that **Actions to be run** list only contains **Extract Defined Chromatograms.**  **c** Click **File > Open Data File.** The system displays the Open Data File dialog box. **d** Select the data files to be opened, Sulfa_SIM80.d through Sulfa_SIM220.d. **e** Mark the **Run 'File Open' actions from selected method** check box. (lower left corner)	• The Qualitative Analysis Method Editor lets you define actions to be performed automatically upon opening a data file(s).

Steps	Detailed Instructions	Comments

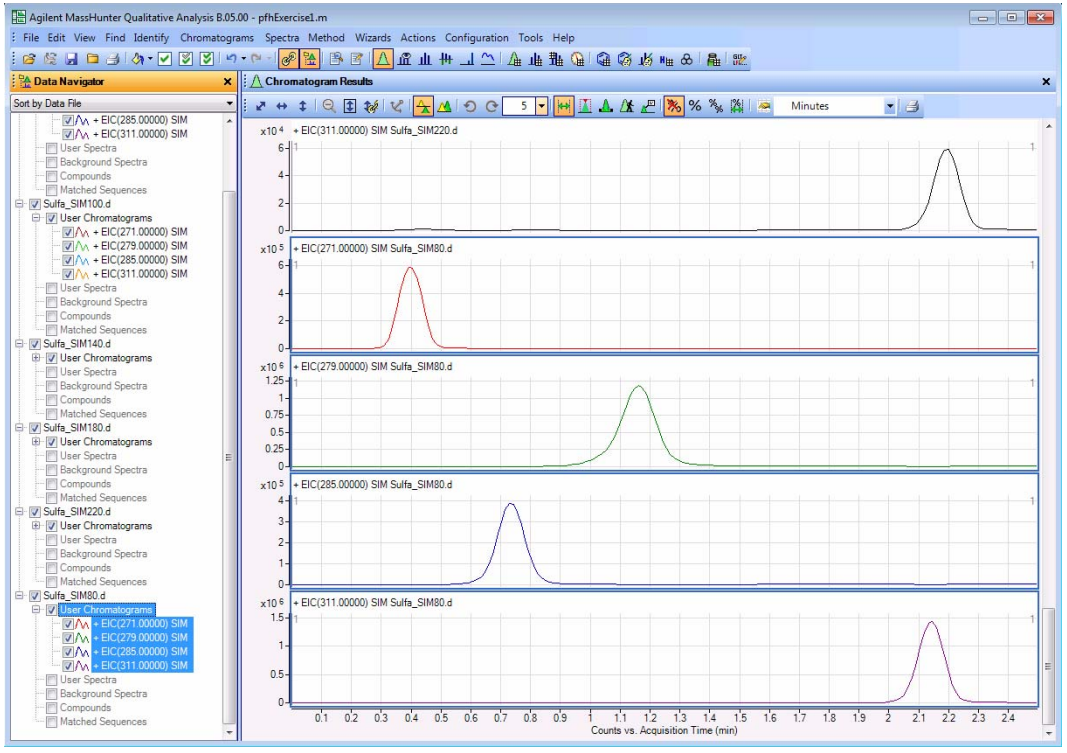


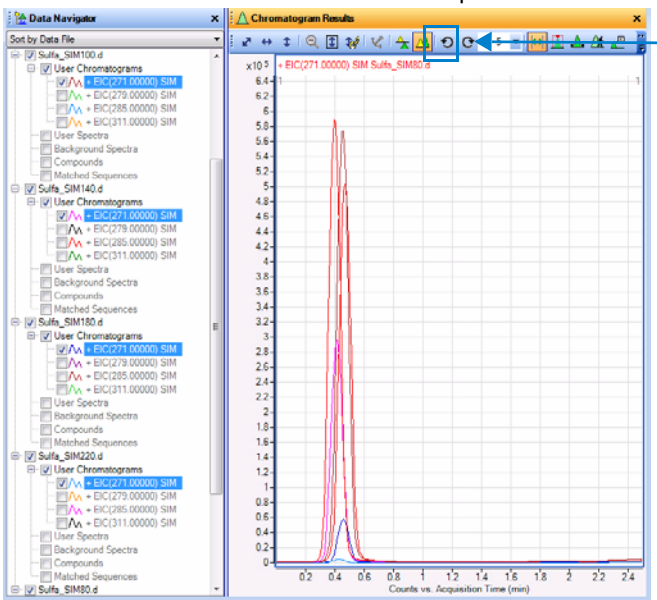
f Click **Open.**
The Qualitative Analysis program displays all the EICs for all the data files selected.

g To close the Method Explorer and Method Editor, click the **X** in the upper right corner of each window.

- You can also close the Method Explorer and Method Editor windows by clicking the **View > Method Explorer** command and the **View > Method Editor** command.

Steps	Detailed Instructions	Comments

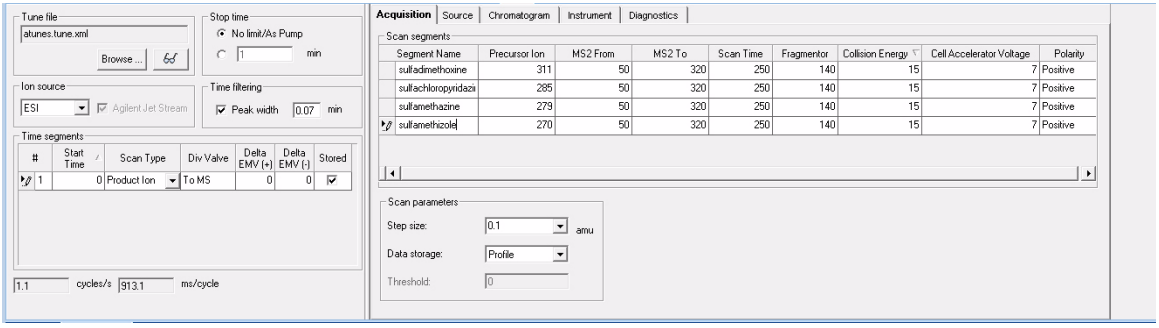


Steps	Detailed Instructions	Comments
6 Select the fragmentor voltage that produces the maximum response for each of the precursor ions. • Close the data files after you determine the optimum voltage.	**a** In the Data Navigator window, highlight the EICs for 271.0 *m/z*. **b** Click the **Show only the highlighted items** icon,  . Only the 271 m/z check boxes are now marked. **c** Look at the relative intensities of each peak to determine which fragmentor voltage setting will be best to use for the 271 precursor.	• You press the **Ctrl** key to be able to select multiple objects from the Data Navigator window. • You press the **Shift** key to be able to select a group of objects. • A fragmentor voltage of 100 should be sufficient for each precursor ion. • You can now determine the product ions that are available for the multiple-reaction monitoring experiments to maximize sensitivity
	 	**You can overlay the chromatograms by clicking**
	d Repeat step a through step c for the other three base peaks or precursor ions. **e** Click **File > Close Data File**. **f** Click **Close** when the Close Data File dialog box appears.	• Click the different EICs in the Data Navigator window to change which chromatogram is labeled in the Chromatogram Results window. When the color of the label of the chromatogram matches the color of the chromatogram that has the highest intensity, you use the fragmentor voltage that was used for that file.

Task 4. Determine product ion masses

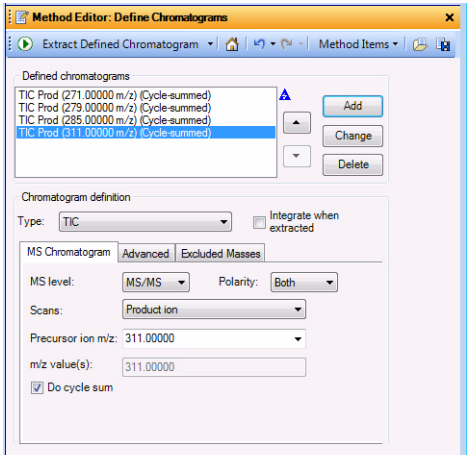
In this part of the method development, we will use three collision energies to determine the best fragment ions to use for the eventual Multiple Reaction Monitoring (MRMs) acquisition.

Steps	Detailed Instructions	Comments
1 Set up three product ion acquisition methods and acquire data. • Use the MS parameters in the example below, but change the Fragmentor voltage to the optimum voltage you determined in the previous task. • Save methods as ***iii*Sulfamix PI_*xx*.m**, where ***iii*** are your initials and ***xx*** is the collision energy.	**a** Click the **MS QQQ** tab in the Method Editor pane. **b** Select **Product Ion** in the **Scan Type** combo box to scan each precursor ion for all its product ions. **c** Enter all MS parameters as listed in the example below, making sure the **Collision Energy** is set to `15` and the Fragmentor voltage is set to the optimum voltage determined in Task 3. **d** Save the method as ***iii*Sulfamix PI_15.m**. **e** Repeat step c and step d for collision energies of 30 and 45.	• When you change the **Scan Type** in the **Time Segments** table, the **Scan segments** table is reset. If you want to copy the **Scan segments** to the new **Scan segments** table, highlight all of the lines in the **Scan segments** table and then right-click the Scan segments table and click **Copy**. After you select a new Scan Type, right-click the Scan segments table and click Paste from Clipboard. • You cannot copy and paste the **Scan segments** table between all **Scan Types**.



2 Set up and run the worklist (optional). • Specify the data files as ***iii*Sulfamix PI_*xx*.d**, where ***iii*** are your initials and ***xx*** is the collision energy.	**a** Click the **Worklist** tab. **b** Add three samples to the worklist for collision energies 15, 30 and 45. **c** Mark the check box to the left of the Sample Name for each sample you are adding. **d** Click **Worklist > Run**.	• This step is optional because you can determine the product ion masses from the data files shipped with the system. • Use the instructions in Step 2 of Task 3 to set up the worklist.

Steps	Detailed Instructions	Comments
3 Set up a qualitative method to integrate and extract product ion spectra. • Use the data files **SulfamixPI_*xx*.d,** where *xx* is the collision energy, or your own data files, ***iii*SulfamixPI_*xx*.d**. • Open Method Explorer and Method Editor. • Use TICs set up for MS/MS, product ion and each of the precursor ions 271, 279, 285, 311. • Make sure the MS/MS integrator has been selected and the maximum number of peaks has been limited to the largest 100 peaks. • Add the ability to integrate and extract peak spectra to the file actions run upon data opening. • Save the changes to the current method.	**a** Click the **Open Data File** icon in the toolbar. **b** Select **SulfamixPI_15.d**. **c** Make sure that the **Run File Open Actions from Specified Method** check box is clear, and click **Open**. **d** Make sure the Method Explorer and the Method Editor windows are displayed; otherwise, click the **Method Explorer** and then **Method Editor** icons. **e** In the Chromatogram section in the Method Explorer window, select **Define Chromatograms.** **f** Delete any existing chromatograms in the **Defined Chromatograms** list. **g** Select **TIC** from the **Type** list in the **Define chromatograms** section. **h** For **MS level**, select **MS/MS**. **i** Mark the **Do cycle sum** check box. **j** For **Scans**, select **Product ion**. **k** For **Precursor ion m/z**, type `271`. **l** Click the **Add** button. **m** Repeat steps j and k for each ion.	• The Qualitative Analysis program should already be open and contain *iii*exercise 1.m as the method.



Steps	Detailed Instructions	Comments
	n From the Method Explorer in the Chromatogram section, click **Integrate (MS/MS)**. **o** Select **MS/MS** as the **Integrator selection**, if necessary.	• These data files contain MS/MS data, so you need to modify the parameters in the Integrate (MS/MS) section. If the data file contained only MS data, you would need to modify the parameters in the Integrate (MS) section.

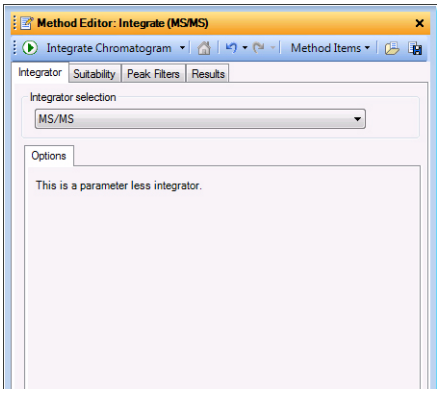


Figure 3 Integrate (MS/MS) > Integrator Tab

p Click the **Peak Filters** tab. Make sure that the **Limit (by height) to the largest** check box is marked and set to the value `100` as shown below.

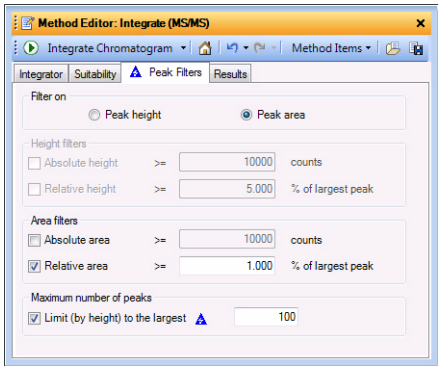
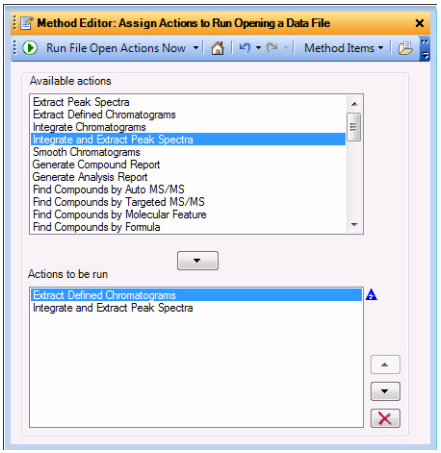


Figure 4 Integrate (MS/MS) > Peak Filters tab

Steps	Detailed Instructions	Comments
	q Click **General** in Method Explorer, and then click **File Open Actions**. **r** Select **Integrate and extract peak spectra** from the Available actions list and click  to add this to **Actions to be run**.	
	 **Figure 5** General > File Open Actions tab	
	s To apply the changes to the current method, *iii*exercise1.m, click the **Save Method** icon.  You can also click **Method > Save**.	
4 Run the qualitative method on the current data file.	• In the Method Editor toolbar, click the **Run** button,  . When the Assign Actions to Run Opening A Data File section is displayed, the **Actions to be run** list is executed.	• The program first extracts the product ion chromatograms for each precursor ion in the data file. • Next, it finds the largest peak in the total ion chromatograms, and integrates and extracts peak spectra from each integrated peak. • See Figure 6 on page 27.

Steps	Detailed Instructions	Comments

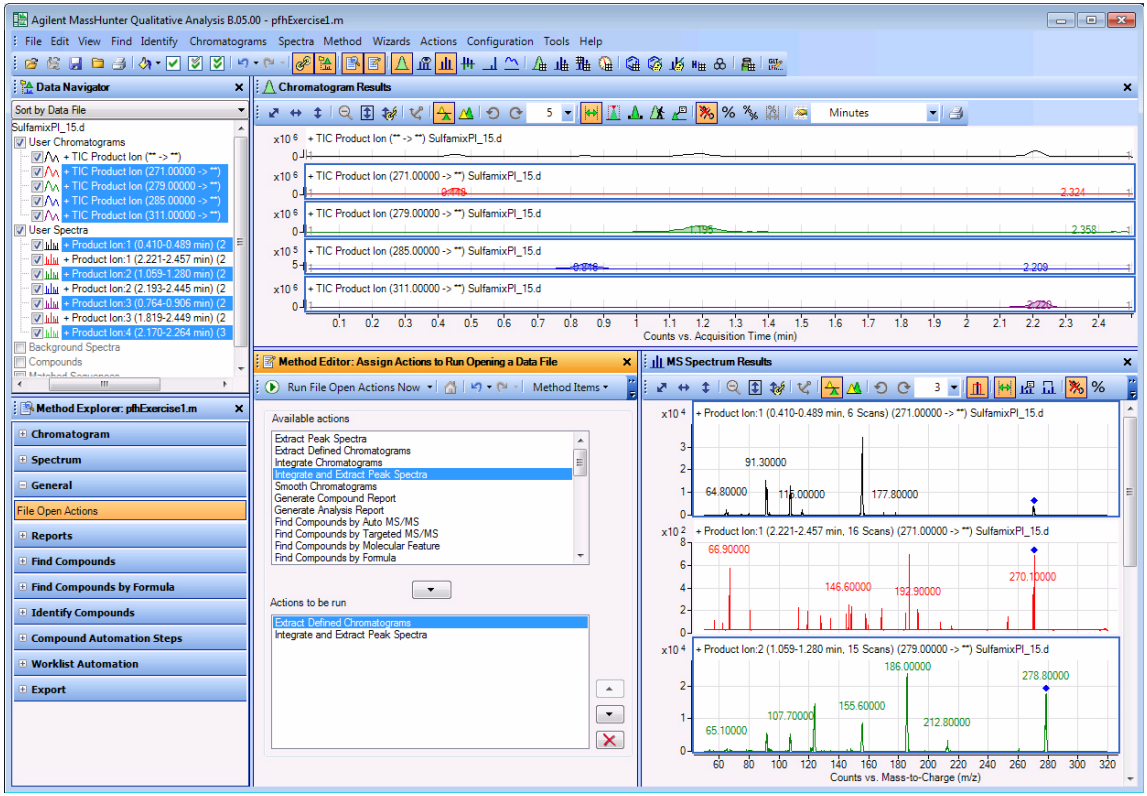


Figure 6 Results for integration and extraction of peak spectra.

5 Run the 'File Open' actions on the remaining product ion data files.

- Use either the example files, **Sulfamix PI_*xx*.d**, or the data files you acquired in step 2.

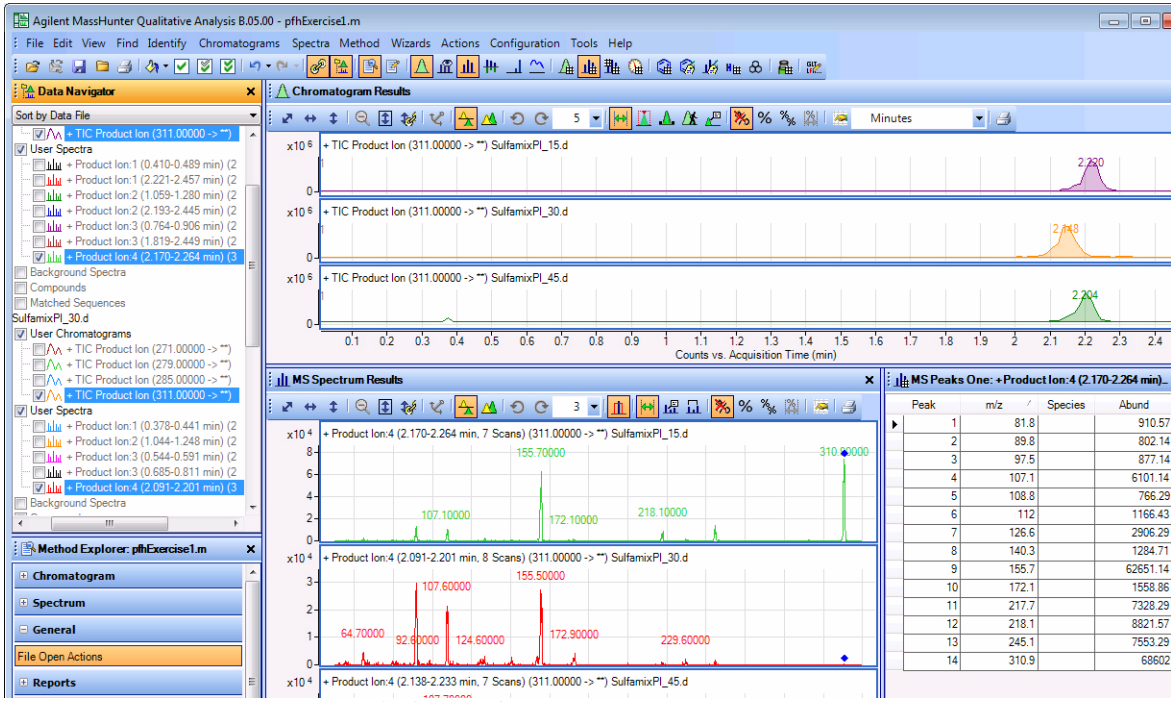
a Click **File > Open Data File.**
The system displays the Open Data File dialog box.

b Hold the **Ctrl** key and do one of these:

- Select the two data files **Sulfamix PI_30.d,** and **Sulfamix PI_45.d**.
- Select the data files you acquired in step 2.

c Mark the **Run 'File Open' actions from selected method** check box in the Open Data File dialog box, and click **Open**.

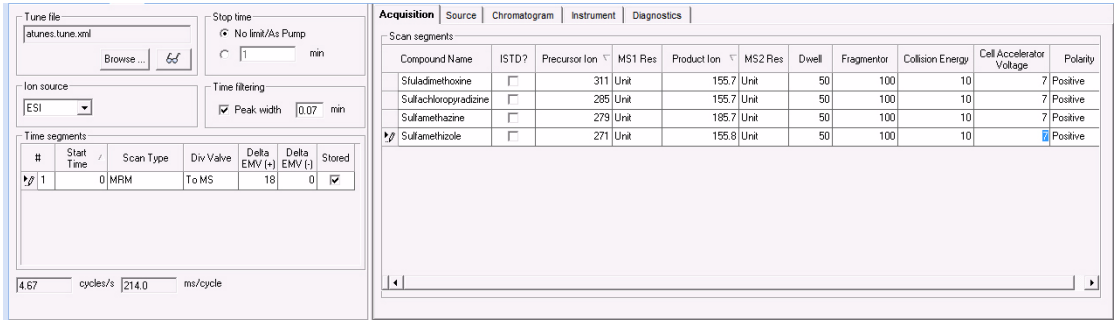
- After the data files open, the Qual method first extracts the product ion chromatograms for each precursor ion.
- Next, it finds the largest peak in the total ion chromatograms, and integrates and extracts peak spectra from each integrated peak.

Steps	Detailed Instructions	Comments
6 Identify product ions. • View each set of TICs and spectra individually (e.g., 271 m/z first). • Close the data files.	**a** In the Data Navigator, select the TICs and spectra for the 271 m/z precursor ion. **b** Click the **Show only the highlighted items** icon,  . **c** Click **View > MS Spectrum Peak List 1**. **d** Examine the spectra to see which fragment ions are produced at which collision energies. **e** Repeat steps a to d until all the product ions are identified.	• The m/z 155.7 product ion is the most abundant of any product ion and the highest signal is recorded at 15 V. This means that a good choice for the MRM for sulfamethizole would be 271.0 > 155.7 when the collision energy is around 15 V. • The peak may not be labeled if the peak is too wide.
		
	f Click the **Close Data File** icon in the main toolbar, and click **Close** when the dialog box containing the list of data files pops up.	• The product ions appear to be: Sulfamethizole-271.0 > 155.7 Sulfamethazine-279.0 > 185.8 Sulfachloropyridazine-285.0 > 155.7 Sulfadimethoxine-311.0 > 155.7

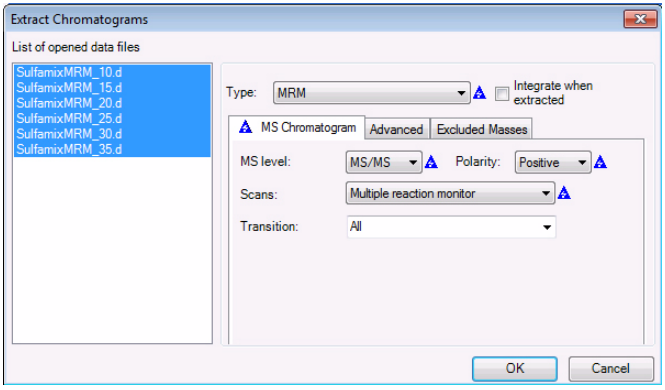
Task 5. Find optimum collision energy for MRM acquisition

In this task, you set up MRM acquisition methods for the sulfa drugs for different collision energies. By examining the spectra and comparing peak intensities, you determine the optimal collision energy settings for the compounds.

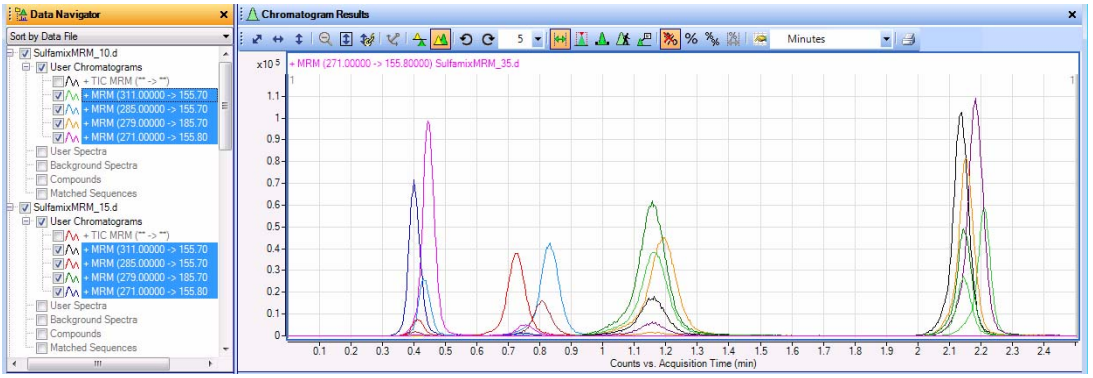
Steps	Detailed Instructions	Comments
1 Set up three MRM acquisition methods. • Use all the MS parameters in the example below except for the collision energy value. • Use collision energies of 10, 15 and 20. • Save methods as ***iii*Sulfamix MRM_*xx*.m**, where ***iii*** are your initials and ***xx*** is the collision energy.	**a** Click the **MS QQQ** tab. **b** Set **Scan Type** to **MRM**. **c** Enter all MS parameters shown in the example below except for the collision energy value. **d** In the collision energy column, type `10` for each compound. **e** Save the method as ***iii*Sulfamix MRM_10.m**. **f** Repeat step d and step e for collision energies of 15, 20, 25, 30 and 35 saving the methods as ***iii*Sulfamix MRM_*xx*.m**, where ***iii*** are your initials and ***xx*** is the collision energy.	• Because the largest peaks were produced with a collision energy of 15 in the previous exercise, you will look at only those collision energies to either side of 15.



2 Set up and run the worklist (optional). • Specify the data files as ***iii*Sulfamix MRM_*xx*.d**, where ***iii*** are your initials and ***xx*** is the collision energy.	**a** Click the **Worklist** tab to make the worklist visible. **b** Add six samples to the worklist for collision energies 10, 15, 20, 25, 30, 35. **c** Mark the checkbox to the left of the Sample Name for each of the three samples. **d** Click **Worklist > Run**.	• This step is optional because you can use the six example data files in the next step.

Steps	Detailed Instructions	Comments
3 Compare the compound transition intensities at different collision energies. • Open the MRM data files: SulfamixMRM_10.d SulfamixMRM_15.d SulfamixMRM_20.d SulfamixMRM_25.d SulfamixMRM_30.d SulfamixMRM_35.d • Set the MRM chromatogram extraction parameters as shown at right for all transitions. • Disable the TICs for clarity and examine the peak intensities. • Compare the intensities of each compound transition obtained at one collision energy with the same compound transition obtained at another collision energy. (Do this in Overlaid Mode with all the MRM chromatograms.) • Close the data files but don't save results. • Refer to Table 4 on page 31 for optimal method settings for each compound.	**a** Open the **Qualitative Analysis** program. **b** Clear the **Run 'File Open' actions...** check box. **c** Open the MRM data files in the Qualitative Analysis program. **d** Right-click the Chromatogram Results window, and click **Extract Chromatograms** from the shortcut menu. **e** To select all data files, click the last file while holding down the **Shift** key. **f** Enter the parameters as listed in the example below, and click **OK**. **g** Clear the TIC check boxes to make the MRM chromatograms easier to view. 	• Why a spectrum for MRM? It's a feature of the program to show spectra even for MRM experiments and can be quite handy for comparing relative intensities of product ions generated from the same precursor.
	h Click the **Overlaid Mode** icon,  . **i** Compare peak intensities for each compound transition in each data file in the Chromatogram Results window.	• Compare the colors shown in Chromatogram Results with the color next to the MRM transition name in the Data Navigator. • You can also right-click the Chromatogram Results window header and compare the colors of the chromatograms to the colors of the titles in the shortcut menu.

Steps	Detailed Instructions	Comments



Unless you decide to acquire MRMs at lower collision energies, you should find that the optimal method settings are as shown in Table 4.

j Click the **Close Data File** icon in the main toolbar, and click **Close** when the Close Data File dialog box appears.

- You now have all the information you need to do an MRM acquisition experiment of the sulfa drug mixture. Consider doing at least one more run with those settings.

Table 4 Compounds and Collision Energy

Compounds	MRM Transition	Fragmentor	Collision
Sulfamethizole	271.0 > 155.8	100 V	10
Sulfamethazine	279.0 > 185.7	100	15
Sulfachloropyradizine	285.0 > 155.7	100	10
Sulfadimethoxine	311.0 > 155.7	100	20

Exercise 2 – Optimize Acquisition parameters using Optimizer software

For this exercise you optimize a mixture of four sulfonamide compounds.

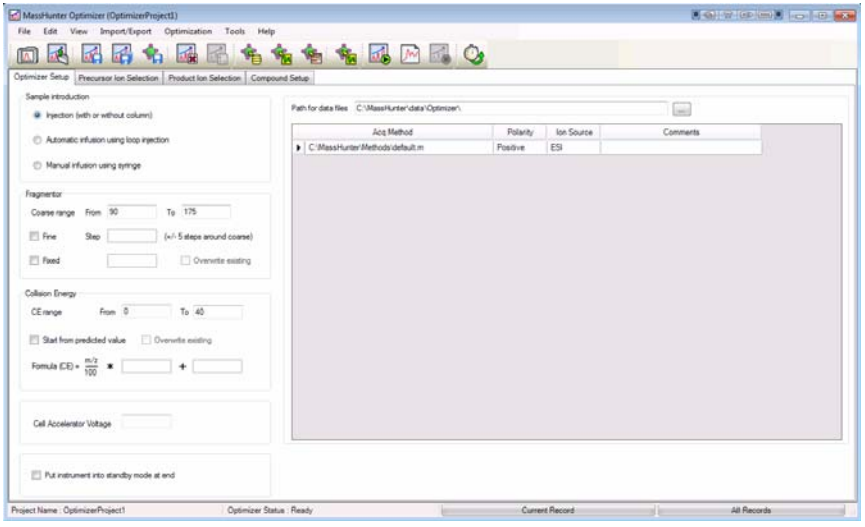
Task 1. Use the Optimizer Software to optimize acquisition parameters

The Optimizer Software helps you optimize acquisition parameters. Specifically, it automates the selection of the best precursor ions, the optimization of the fragmentor voltage for each precursor ion, selection of the best product ions, and optimization of collision energy values for each transition for a list of compounds you specify.

To do this task, you first need to create the method ***iii*Sulfamix MRM_10.m** in **"Task 5. Find optimum collision energy for MRM acquisition"** on page 29. You do not need to acquire the data file.

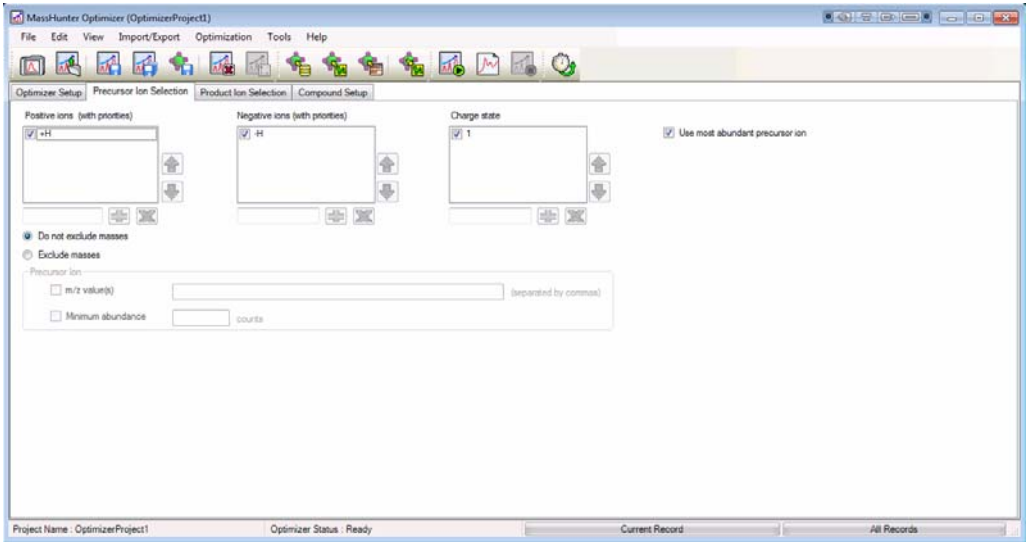
The Fragmentor Voltage for the 6490 is set automatically during Autotune. The Fragmentor voltage for a 6490 is not optimized. The Fragmentor parameters and results will not be displayed for a 6490 instrument.

Steps	Detailed Instructions	Comments
1 Start the MassHunter Optimizer software.	• Double-click the **Optimizer** icon.  .	• If you are optimizing peptides, use the **Optimizer for Peptides** program.

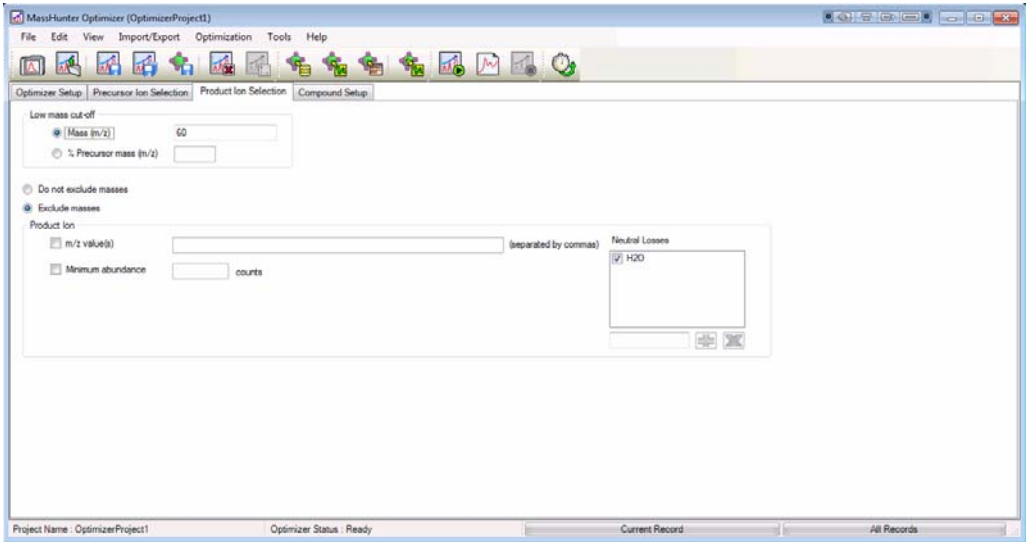


Steps	Detailed Instructions	Comments
2 Set the optimization parameters.	**a** Click the **Optimizer Setup** tab. **b** Set the **Sample introduction** method to **Injection**. **c** Set the Fragmentor ramp parameters as follows: • Set the range for ramping the **Fragmentor** values from 90 to 135. • Clear the **Fragmentor Fine** check box. **d** Set the range for ramping the Collision Energy from 0 to 40 V. **e** Select a Path for data files to store the optimization run data. **f** Right-click the table on the right and select **Add Method** from the shortcut menu. **g** Click the button on the right side of the Acq Method cell to open the Open Method dialog box. **h** Select the method created in the previous exercise ***iii*Sulfamix MRM_10.m** and click **OK**. The Polarity and Ion Source will be filled in from the values set in the selected method. **i** Check to make sure that the Ion Source from the method matches the physical configuration of your instrument. **j** Repeat step f to step i to select additional methods.	• Fine optimization refines the coarse ramping values and provides better optimization but takes longer to run. • The data can be displayed later with Agilent MassHunter Qualitative Analysis software. • The Fragmentor Voltage is not optimized for an Agilent 6490 Triple Quadrupole. It is set automatically when you Autotune. The Fragmentor parameters and results for a 6490 are not shown in the Optimizer program.

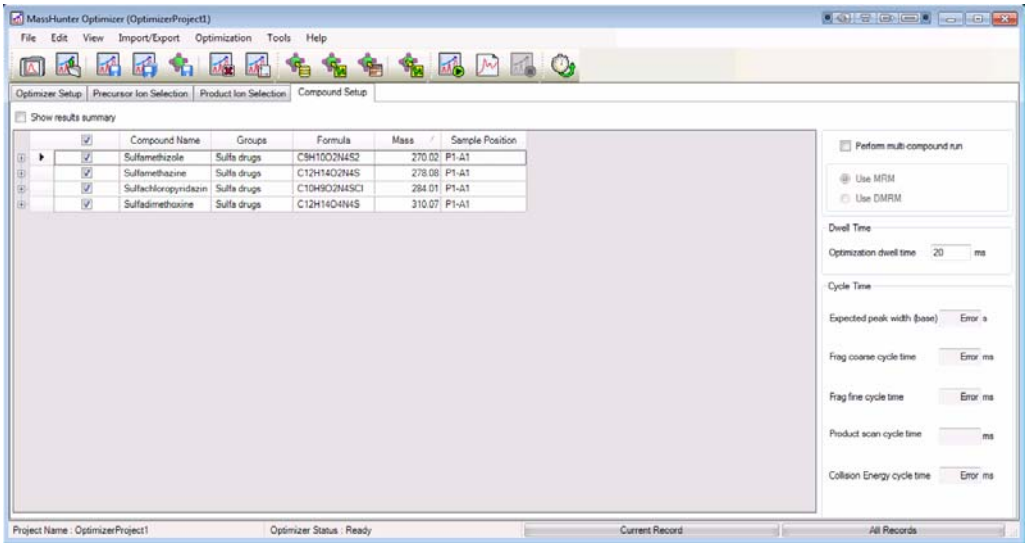
Steps	Detailed Instructions	Comments
3 Select the precursor ions	**a** Click the **Precursor Ion Selection** tab. **b** Select the **Positive ions** +H adduct. **c** Select the **Charge state** of **1**. **d** Set the search priority of the precursor ions. **e** (optional) To exclude certain masses from consideration, click **Exclude masses** at the bottom of the screen. Enter the **m/z Values** to exclude separated by commas and/or enter a **Minimum abundance value** in counts.	• Mark the **Use most abundant precursor ion** check box to use the most abundant precursor ion. • Clear the **Use most abundant precursor ion** check box and use the **Up** and **Down** arrow buttons to set the search order (ions at the top of the list are given more priority). • You can also enter Neutral Losses to exclude (for example H_2O).



Steps	Detailed Instructions	Comments
4 Select the product ions	**a** Click the **Product Ion Selection** tab. **b** Enter a Low mass cut-off value. Select Mass (m/z) of 60 *m/z*. **c** To exclude certain masses from consideration, click **Exclude masses** option at the bottom of the screen. Enter the **m/z Values** to exclude separated by commas and/or enter a **Minimum abundance value** in counts. **d** If desired, you can also enter **Neutral Losses** to exclude, for example H_20. Enter a formula in the box and click the button to add it to the list.	



Steps	Detailed Instructions	Comments
5 Set up a compound list. The formula for the four Sulfa Drugs are: • Sulfamethizole (SMT) $C_9H_{10}O_2N_4S_2$ • Sulfamethazine (SMZ) $C_{12}H_{14}O_2N_4S$ • Sulfachloropyridazine (SCP) $C_{10}H_9O_2N_4SCl$ • Sulfadimethoxine (SDM) $C_{12}H_{14}O_4N_4S$	**a** Click the **Compound Setup** tab. **b** Clear the **Show results summary** check box above the table while you set up the compound list. **c** Right-click the table and select **Add Compound** from the shortcut menu to add a row to the end of the table. **d** Enter `Sulfamethizole` as the **Compound Name**. **e** Enter `Sulfa drugs` as the group name in the **Groups** column. **f** Enter `C9H10O2N4S2` as the **Formula** of the compound. The mass is calculated. **g** Enter the **Sample Position** for the new compound. **h** (optional) Enter an **Optimization dwell time** value to set longer or shorter cycle times. **i** Repeat the steps above to add the other three sulfa drugs to the table. **j** Mark the **Select** columns for the compounds (rows) to use for optimization. **k** Save the compound list to the database or to the current project.	• Compounds are global to all projects. Compound information such as name, group, formula, and mass in one project will be reflected in the entire database. • If no methods or ions are specified here, then optimization for the compound uses the methods from the Optimizer Setup tab and information from the Precursor Ion Selection and Product Ion Selection tabs to generate the ions. • You can also enter the monoisotopic mass in the **Mass** column instead of the **Formula**.

Steps	Detailed Instructions	Comments
		**The Fragmentor parameters and results are not displayed**
6 Start the optimization process.	• Click the Start Optimization button ( ) on the toolbar or • Click the **Ion Breakdown Profile** button ( ) on the toolbar.	•
7 Review results.	**a** Click the **Compound Setup** tab. **b** Mark the **Show results summary** check box above the table. **c** Review the following values for each transition ion in the Compound Table: • Fragmentor • Collision Energy **d** Review the printed optimization report.	• (optional) Use the Agilent MassHunter Workstation Qualitative Analysis program to look at the data. • See the online Help for the Optimizer program or the Optimizer Quick Start Guide to learn how to import optimization results to acquisition for MRM time segments.

Exercise 3 – Develop a Dynamic MRM acquisition method from an MRM acquisition data file or an MRM method

The purpose of this exercise is to create a Dynamic MRM method from an acquired MRM data file for sulfamix_MRM data files with the correct retention times for Dynamic MRM using the Quantitative Analysis program. All transitions in the MRM method must have the same polarity.

For this exercise, you have three main tasks:

- "Task 1. Create a batch file from an existing MRM data file" on page 38
- "Task 2. Print a report in the Quantitative Analysis program" on page 41
- "Task 3. Create a Dynamic MRM method using Update dMRM feature" on page 42

You can easily create a Dynamic MRM method from an existing MRM method.

- "Task 4. Create a Dynamic MRM method from an MRM method" on page 44

Task 1. Create a batch file from an existing MRM data file

In this exercise, you create a batch and a method from an existing MRM data file.

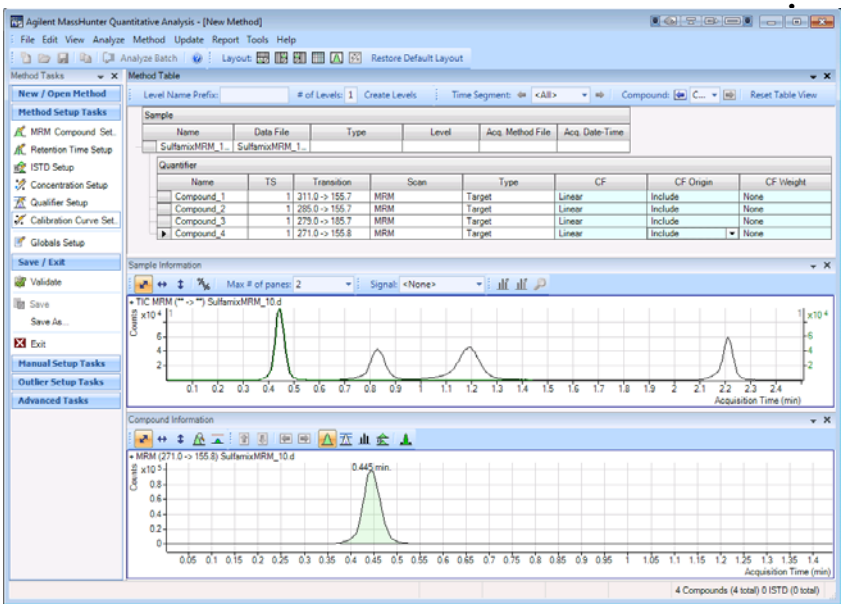
Steps	Detailed Instructions	Comments
1 Open the Quantitative Analysis program and create a batch file with one sample file, SulfamixMRM_35.d. • Copy the data file SulfamixMRM_35.d from the installation disk to the **\MassHunter\Data\MRM_to_DMRM** folder.	**a** Double-click the **QQQ Quantitative Analysis** icon. **b** Click **File > New Batch**. **c** Navigate to the **\MassHunter\Data\MRM_to_DMRM** folder. **d** Type `MRM_to_DMRM` in the **File Name** text box. **e** Click **Open**. **f** Click **File > Add Samples**. **g** Select the file **SulfamixMRM_35.d**. **h** Click **OK**.	• The file **SulfamixMRM_35.d** is on the installation disk in the **\Support\Data** folder. Copy this entire folder to the **\MassHunter\Data\MRM_to_DMRM** folder.

Steps	Detailed Instructions	Comments
2 Create a method for that batch using MRM data.	**a** Click **Method > New > New Method from Acquired MRM data**. **b** Select the **SulfamixMRM_35.d** data file. **c** Click **Open**.	
3 Set the Concentration Setup, Qualifier Setup, and Calibration Curve Setup. • Add calibration level 1 with a concentration of 10000. • Set the **Uncertainty** to Relative for all qualifiers. • Set the **Curve Fit** to Linear. • Set the **Curve Fit Origin** to Include. • Set the **Curve Fit Weight** to None.	**a** Select **Concentration Setup** in the Manual Setup Tasks section in the Method Tasks pane. **b** Select the first compound in the table. **c** Right-click the compound row and click **New Calibration Level** from the shortcut menu. **d** Enter `1` in the **Level** column and `10` in the **Conc.** column. **e** Right-click in the Level box and click **Copy Calibration Levels To**. **f** Click **Select All**. Click **OK**. **g** Select **Qualifier Setup** in the Manual Setup Tasks section in the Method Tasks pane. **h** Verify that the **Uncertainty** is Relative. **i** Select **Calibration Curve Setup** in the Manual Setup Tasks section in the Method Tasks pane. **j** Set **Curve Fit** to **Linear** for all compounds. **k** Set **CF Origin** to **Include** for all compounds. **l** Set **CF Weight** to **None** for all compounds.	• Refer to the online Help in the Quantitative Analysis program for additional help on these tasks.

Exercise 3 – Develop a Dynamic MRM acquisition method from an MRM acquisition data file or an MRM method

Task 1. Create a batch file from an existing MRM data file

Steps	Detailed Instructions	Comments



4 Verify method and then save the method and apply the method to the batch.	**a** Click **Method > Validate.** **b** Click OK on the message box. Fix any errors, if necessary. **c** Click **Method > Save As**. **d** Enter `MRM_to_DMRM`. **e** Click the **Save** button. **f** Click **Method > Exit**. **g** Click **Yes** to apply the method to the batch.	
5 Analyze and save the batch.	**a** Click **Analyze > Analyze Batch.** **b** Click **File > Save Batch**.	

Task 2. Print a report in the Quantitative Analysis program

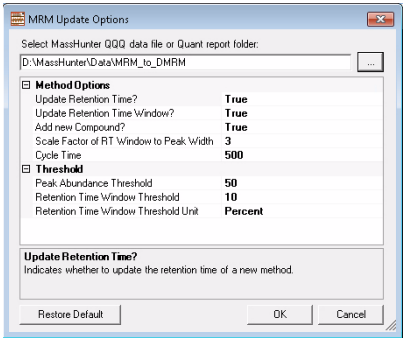
In this task, you print a report using any template.

You can update a Dynamic MRM method using either a data file or a quantitation report folder, so this task creates the quantitation report folder.

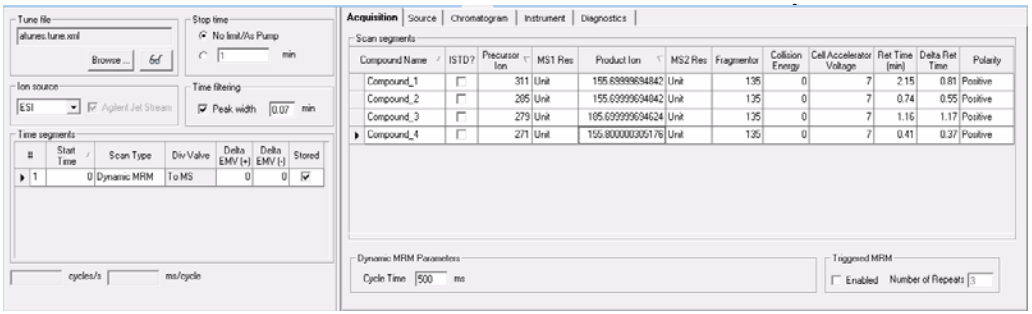
Steps	Detailed Instructions	Comments
1 Print a report using the template MRM_to_DMRM.xltx.	**a** Click **File > Save**. **b** Click **Report > Generate**. The system displays the Report dialog box. **c** Select the **Template** file. **d** Select the **Report** folder. This folder name will be used in the next task. **e** Click **OK**.	• Copy the **MRM_to_DMRM.xltx** template from the **\Support\Data** folder on the installation disk. • For this report, you do not need to print the report. You need to click Advanced to select a different printer. If you don't want to print this report, click **Advanced** instead.
2 Check the status of the report using the Queue Viewer program.	**a** Click **Report > Queue Viewer**. **b** Wait for the report to finish printing. **c** Close the **Task Queue Viewer** program.	

Task 3. Create a Dynamic MRM method using Update dMRM feature

You can create a Dynamic MRM method from an MRM data file or a Quantitative Analysis method. You first set the **Scan Type** to Dynamic MRM, and then you use the Update MRM Method dialog box.

Steps	Detailed Instructions	Comments
1 Open the method *iii*Sulfamix MRM_10.m and save it to a new name with the format ***iii*Sulfamix dMRM.m**, where ***iii*** are your initials.	**a** Click **File > Open > Method**. **b** Select the *iii*Sulfamix MRM_10.m method. **c** Click **OK**. **d** Click **Method > Save As**. **e** Type the new method name with the format ***iii*Sulfamix_dMRM.m.**	• In this example, the batch is in the **\MassHunter\Data\ MRM_to_DMRM** folder.
2 Change the method to a dynamic MRM method with the same compounds. You can either use a data file or the report that was generated in the last task.	**a** Click the **Acquisition** tab in the QQQ tab in the Method Editor window. **b** Right-click the **Scan segments** table and click **Update MRM Method**. The Update MRM Editor dialog box is opened. **c** Select the folder containing the *report.results.xml* file or the data file ***iiiSulfamix MRM_10.d***. **d** Select **True** for **Update Retention Time?**. **e** Select **True** for **Add new Compound**. **f** Click **OK**.	• The Update MRM Method tool automatically sets the Scan type to **Dynamic MRM**. • You can select either a data file that was acquired with a **Scan Type** of **MRM** or a Quant Report folder as the input to this dialog box. The Scan segments are created from one of these two input sources.
	**You can update the compounds in the Scan segments table by using a QQQ data file or a Quantitative analysis report folder.**	

Steps	Detailed Instructions	Comments



The compounds from the data file or quantitation report are automatically added to the Scan segments table. If you use a data file and you didn't enter the Compound Name in the Scan Segments table before the data file was acquired, then the Compound Name is not added automatically.

g Select the original compound in the Scan segments table.

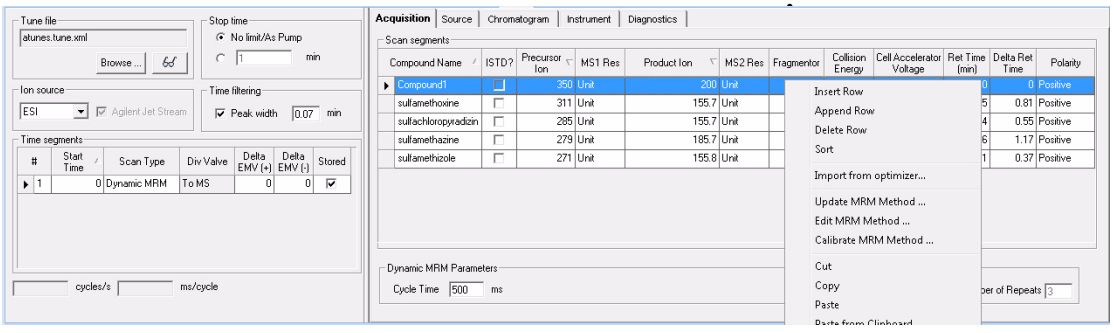
h Right-click the row and click **Delete Row**.

i Click **Method > Save**.

Task 4. Create a Dynamic MRM method from an MRM method

You can create a Dynamic MRM method directly from an MRM method by using the Paste from Clipboard command from the shortcut menu.

Steps	Detailed Instructions	Comments
1 Open the method *iii*Sulfamix MRM_10.m and save it to a new name with the format ***iii*Sulfamix dMRM_Easy.m**, where ***iii*** are your initials.	**a** Click **File > Open > Method**. **b** Select the *iii*Sulfamix MRM_10.m method. **c** Click **OK**. **d** Click **Method > Save As**. **e** Type the new method name with the format ***iii*Sulfamix_dMRM2.m.** **f** Click the **Save** button.	
2 Copy all compounds from the Scan segments table in the MRM method.	**a** Click the **Acquisition** tab in the QQQ tab in the Method Editor. **b** Select all of the rows in the Scan segments table. **c** Right-click the Scan segments table and click **Copy**.	• To select all of the rows in the Scan segments table, you select the first row in the table, Then, you scroll to the last row in the Scan segments table. Press the **Shift** key and select the last row in the table.
3 Change the Scan Type to Dynamic MRM and paste the rows into the new Scan segments table.	**a** Select **Dynamic MRM** as the Scan Type. **b** Right-click the Scan segments table and click **Paste from Clipboard**. **c** Select the original compound in the Scan segments table. **d** Right-click and click **Delete Row**. **e** Click **Method > Save**.	• To combine multiple Time Segments into one Dynamic MRM Time Segment, you paste the Scan segments into Excel and create one long list. When all of the scan segments have been pasted into Excel, then copy all of the Scan segments in Excel.



Exercise 3 – Develop a Dynamic MRM acquisition method from an MRM acquisition data file or an MRM method

Task 4. Create a Dynamic MRM method from an MRM method

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In This Book

This exercise helps you use the Agilent 6400 Series Triple Quadrupole LC/MS system. In this guide, you acquire data and then analyze the results using the Qualitative Analysis program to learn how to develop an acquisition method.

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