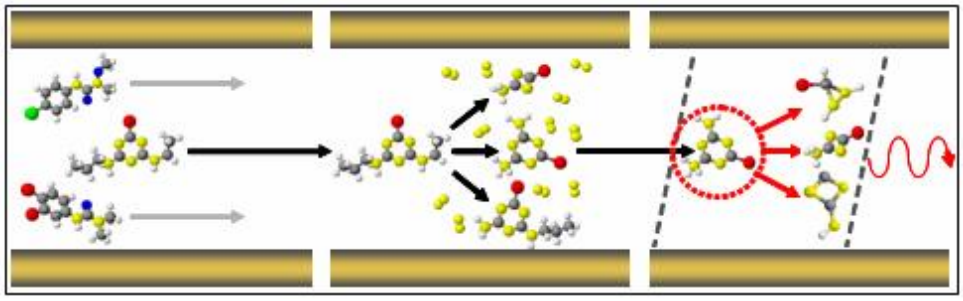
MRM^3 Optimization Script for MRM^3 Quantitation

1.0 Description

The use of MRM^3 for quantitation on the QTRAP® systems can provide increased specificity and therefore improved detection when quantifying analytes in complex matrices.



MRM^3 for Quantitative Analysis by LC-MS. Analyte ion is first selected in the Q1 quadrupole, then fragmented in Q2 collision cell. Fragment ions are trapped then the 2nd precursor is isolated in the linear ion trap. This 2nd precursor is then fragmented by excitation to generate second generation fragment ions in a second fragmentation step. Second generation product ions are scanned out to the detector.

The MRM^3 Optimization script is designed to simplify and automate the development of these MRM^3 acquisition methods. The script is designed to generate an optimal MRM^3 acquisition method on any QTRAP® system with any source and at any flow using infusion.

The script basically performs the following optimization steps:

- Confirm precursor mass.
- Optimize transmission to collision cell.
- Determine the major fragment ions.
- Optimize the Collision Energy (CE) for each fragment ions.
- Perform MS^3 scans on each fragment ion.
- Optimize Excitation Energy (AF2) for all MS^3 scans.
- Generate a report.
- Save all data and acquisition methods.

The script can also be used in qualitative applications to generate collections of MS/MS and MS^3 spectra for compounds in a semi-automated way (i.e. one compound at a time).

2.0 Installation Instructions

Before installing the script, Analyst® Software 1.5.1 or later must be installed.

MRM^3 Optimization for Analyst 1.5.1 Software.exe extracts three (3) files in the C:\MRM3 Optimization for Analyst 1.5.1 Software folder by default:

- MRM3 Optimization for Analyst 1.5.1 Software.msi
- UpdateInfo.exe
- HowToUseUpdateInfo.doc

Double click on **MRM^3 Optimization for Analyst 1.5.1 Software.msi** and follow the instructions.

1. The installer **MRM^3 Optimization for Analyst 1.5.1 Software.msi**:
    a. Replaces one component, OptMS3.exe, from the AnalystData\Projects\API Instrument\Processing Scripts directory with newer version
    b. Installs new MRM3 Optimization.dll into AnalystData\Projects\API Instrument\Processing Scripts
2. To uninstall the script, open "Control Panel"-> "Add/Remove Programs" and locate the item **MRM^3 Optimization for Analyst 1.5.1 Software**. Click the "Remove" button.

3.0 Using the MRM^3 Optimization Script

The MRM^3 Optimization script can be called from the "Script" menu in Analyst® Software. Before you start using the script, you must build a "Starter" acquisition method if one does not already exist. The "Starter' method should be a Q1 acquisition method created in Manual Tune which contains the source conditions desired for the tuning process since these are not optimized by the script. The method should be saved in the "Acquisition Methods" folder of the desired project in which all generated files will be saved.

Script Overview

The main window, shown in Figure 1, contains controls that allow the user to navigate through the script and it also displays the optimization results as they are generated. An overview of the various sections in this window is found below. More details about these window sections are found in the "Optimization In Progress" section.

- **Status Window** – When the script is first started, this window displays the current optimization settings that will be used for optimization. When the optimization is started, spectral information is displayed in this window.
- **Log File** – Displays the results found during optimization in text format. Each entry found in this section is also added to the generated *"Log.txt"* file.
- **Overall Progress** – This is a visual display of the overall optimization progress.
- **Main Controls** – Contains all of the main functions associated with the setting and execution of the optimization process.
    - § After the optimization is completed, a *"Results.txt"* file is automatically generated and saved. By selecting the "View Results" button, this file can be opened and reviewed with Microsoft Notepad. More details about this *"Result.txt"* file are provided in the "Optimization Complete" section.
    - § The "Settings" button opens a window where the user enters compound information required for the optimization process. Refer to "Setting the Preferences" for more details about this window.
    - § The "Start" button initiates the optimization process. During optimization, this button is renamed to "Abort" which allows the user to terminate the optimization process.

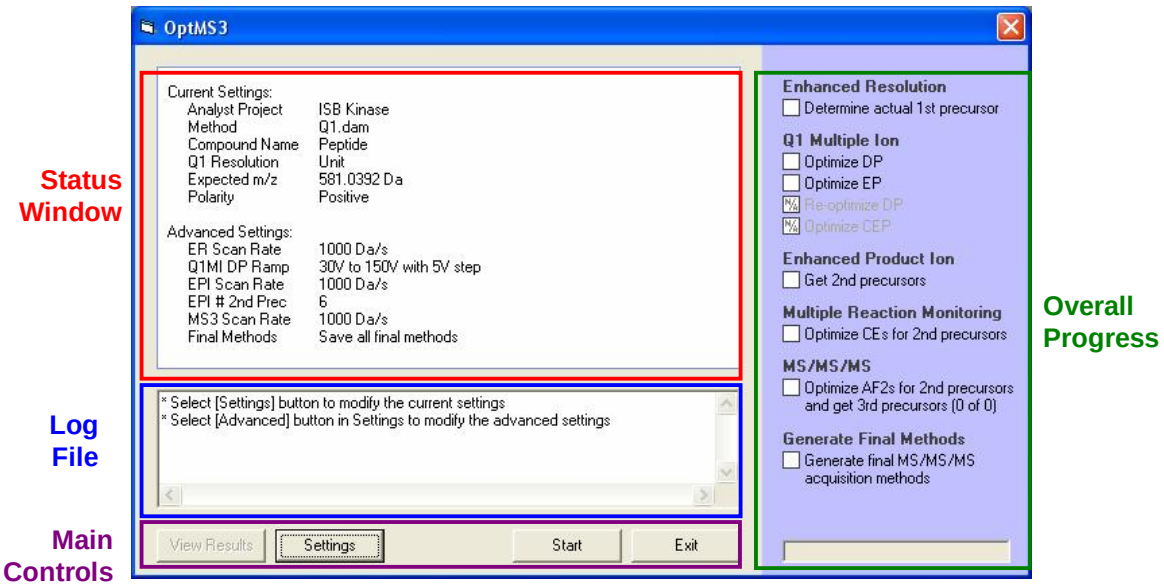


Figure 1: Main Window

Setting the Preferences

When the script is initiated for the first time, the Settings window (Figure 2) is automatically displayed. Otherwise, the "Status Window" displays the last values and information used for optimization. These values can be changed by selecting the "Settings" button which opens the Settings window (Figure 2). In this window, the user may:

- Select the desired "Starter" Acquisition Method using the "Browse…" button. This method is used predominantly to retrieve the desired source conditions to be used for the optimization.
- Specify a descriptive Compound Name. This name is used as a prefix to all of the acquisition methods and data files generated.
- Select a Q1 Resolution to be used for MS/MS and MS^3.
- Select a Polarity, which may differ from the starter method (note: the Both Polarity option is currently not supported).
- Specify the Expected m/z (amu). If you do not know the mass to charge ratio (m/z) of the compound then click on the "Calculate from chemical formula" button to calculate it from the chemical formula of the compound. Refer to the next section "Calculating m/z" for more details about this calculator.
- The "Advanced" button is used to modify some of the settings used by the optimization process. Refer to "Advanced Settings" for more information.
- Click the "OK" button to verify and use the updated settings.

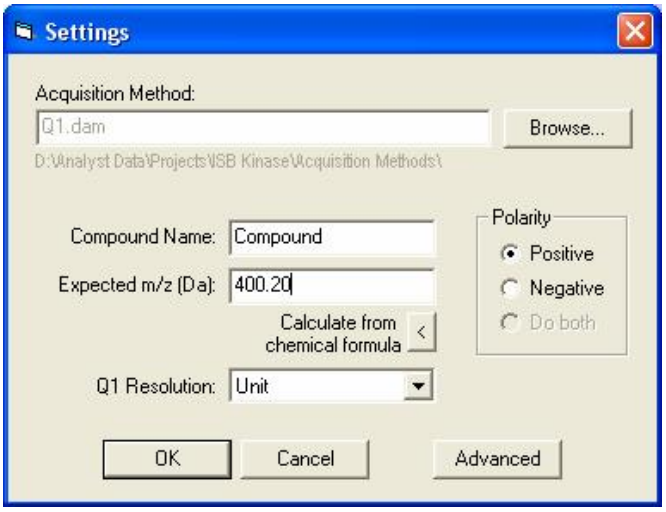


Figure 2: Settings Window

Calculating m/z

The m/z calculator, as seen in Figure 3, can be accessed through the Settings window by clicking on the "Calculate from chemical formula" button.

- Enter the chemical formula of the desired compound (use capital letters for the elements). For peptides, the chemical formula must also be entered in this window. This is easily attained by typing the peptide sequence into the "New Protein Sequence" Window in BioAnalyst™ Software.
- Specify the number of charges.
- Select the "Calculate" button to calculate the m/z for the entered chemical formula and charge.
- Click the "Use m/z" button to close the calculator and update the Expected m/z (amu) textbox in the Settings window with the calculated m/z.

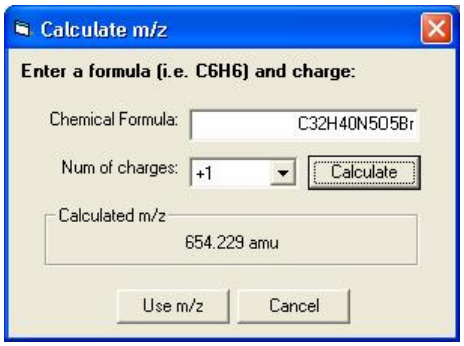


Figure 3: Calculate m/z Window

Advanced Settings

The Advanced Settings window is shown in Figure 4. In this window, a detailed description for each of the optimization steps is provided. The user can also modify some of the settings in order to customize the optimization.

- Specify a scan rate for ER, EPI and MS^3.
- Set the Declustering Potential (DP) range for optimization. The range is expressed in absolute values and the appropriate polarity is automatically applied based on the selection made in the "Settings" window.
- Specify the maximum number of 2^{nd} precursors (fragment ions) used for MS/MS/MS optimization. A number between 1 and 10 must be entered.
- Specify a mass range for the 2^{nd} precursors that will be selected for MS/MS/MS.
- Select a collision energy (CE) and a collision energy spread (CES) that should provide a good MS/MS spectrum from which to choose fragment ions.
- Select the "Save All Final Methods" option to generate all of the final MS^3 methods for each 2^{nd} precursor and the optimal MS^3 method for quantitation. In the optimal MS^3 method, the most intense secondary product ion is chosen and an MS^3 method is built with a limited scan range around that mass. Select the "Save Optimal Method Only" option to save only the optimal MS^3 method (most sensitive for quantitation).
- Click the "OK" button to accept the updated Advanced Settings.

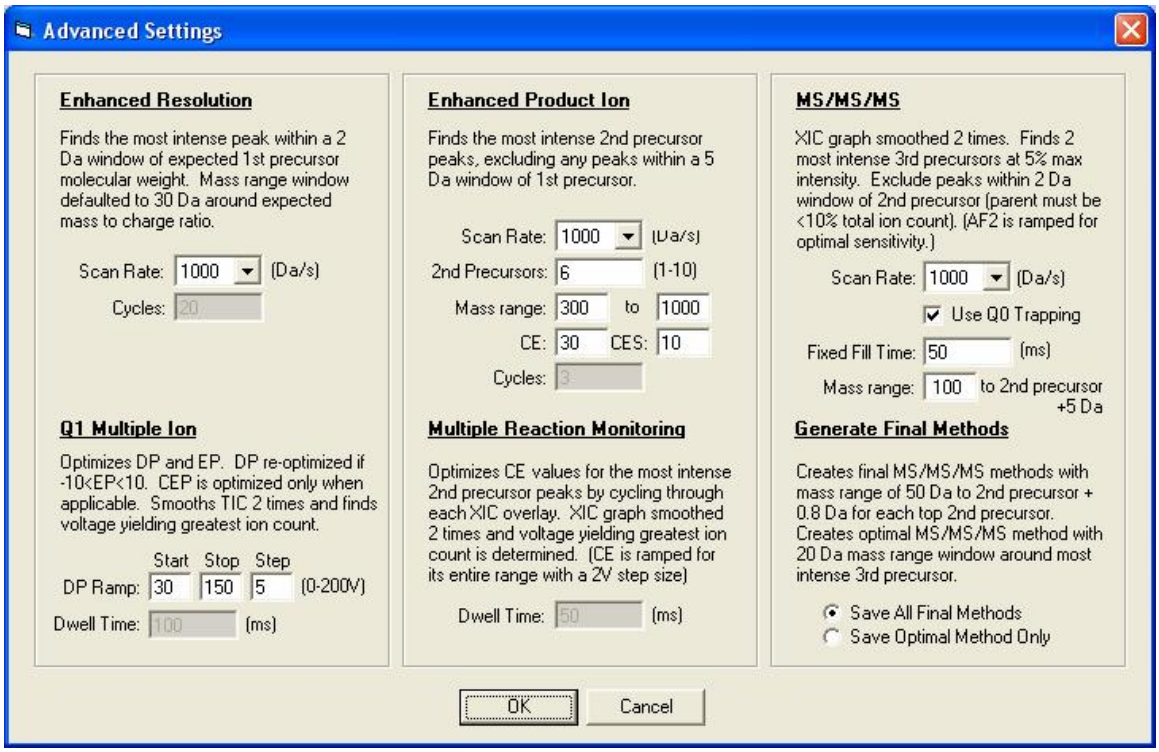


Figure 4: Advanced Settings Window

Optimization In Progress

When the optimization is started, Manual Tune in Analyst Software is automatically stopped. A Log.txt file is also updated as each part of the optimization procedure is completed. To abort the script at anytime, click on the "Abort" button. Examples of the script in progress are shown in Figures 5 and 6. In the Overall Progress section, the Checklist images and text fonts represent different statuses which are described below.

- ☐ Task not performed yet – text is black
  Task in progress – text is blue and italic
- ☒ Task will not be performed – text is grey
- ☑ Task completed (hyperlink) – text is blue and underlined
  Task completed (no link) – text is blue
  Part of task completed (hyperlink) – text is blue, underlined, and italic

When the text is underlined, you can select it like a web page hyperlink and the corresponding spectrum or chromatogram is displayed. The text found under "MS/MS/MS" also displays the MS^3 scan number that is being performed since it is possible to have between 1-10 scans. The Overall Progress section also includes a Message area. In this area, there is a progress bar that displays the current step progress. Above the progress bar, various messages are displayed such as the time and other statuses for the current optimization step.

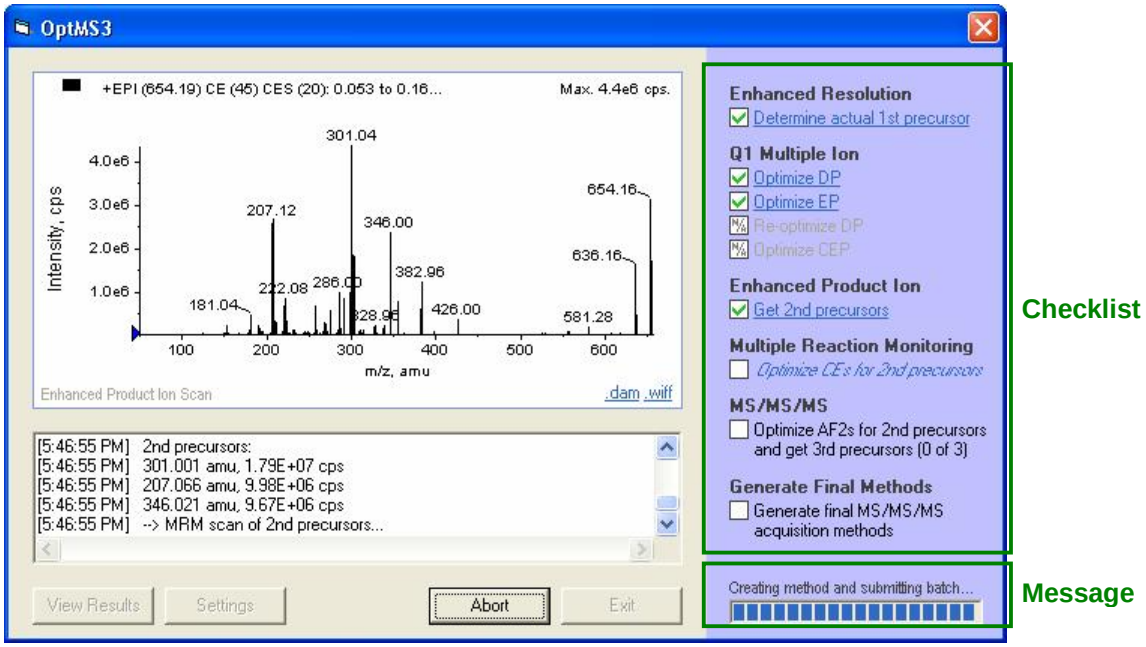


Figure 5: Main Window After EPI Scan

In the spectral status window, the previously generated spectrum or chromatogram is displayed. When one of the Checklist items is selected, the corresponding graph is shown instead. The scan type name, as seen in Figure 6, indicates which scan is currently being displayed. For each completed steps, it is possible to open the acquisition method (*.dam) or data file (*.wiff) associated with the graph displayed. If an "MS/MS/MS" scan is displayed, buttons will be visible which allow you to cycle through the different MS^3 scans.

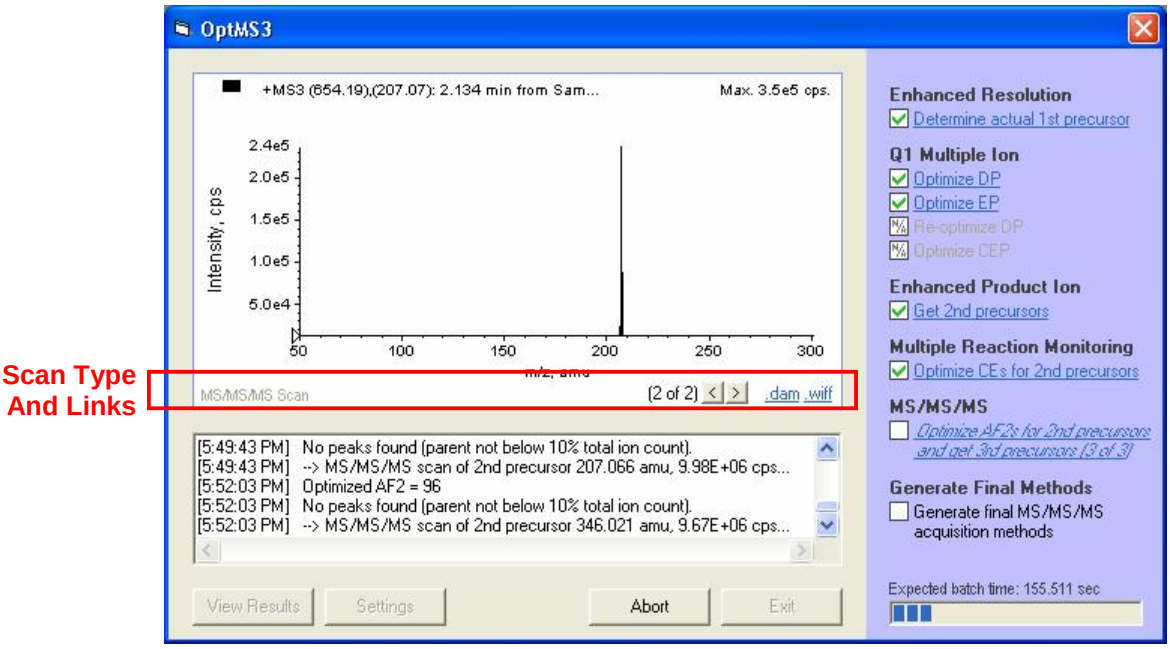


Figure 6: Main Window During MS^3 Scan

Optimization Complete

When the quantitative optimization for MS^3 is completed or aborted, a *"Results.txt"* file similar to the one in Figure 7 is generated. This file is automatically opened in Microsoft Notepad by the script. The file can also be viewed by clicking on the "View Results" button from the main window.

The various parts of the *"Results.txt"* file are described below.

- **Time and Duration** – Date and time duration of optimization.
- **User Starting Conditions** –Settings and Advanced Settings are listed in this section.
- **Optimization Conditions Found** – Displays the optimal conditions found during the ER and Q1MI scans.
- **MS^3 Fragments Found and Associated Losses** – Displays the fragments, optimal conditions (collision energy and excitation energy) as well as associated losses found for the EPI scan and MS^3

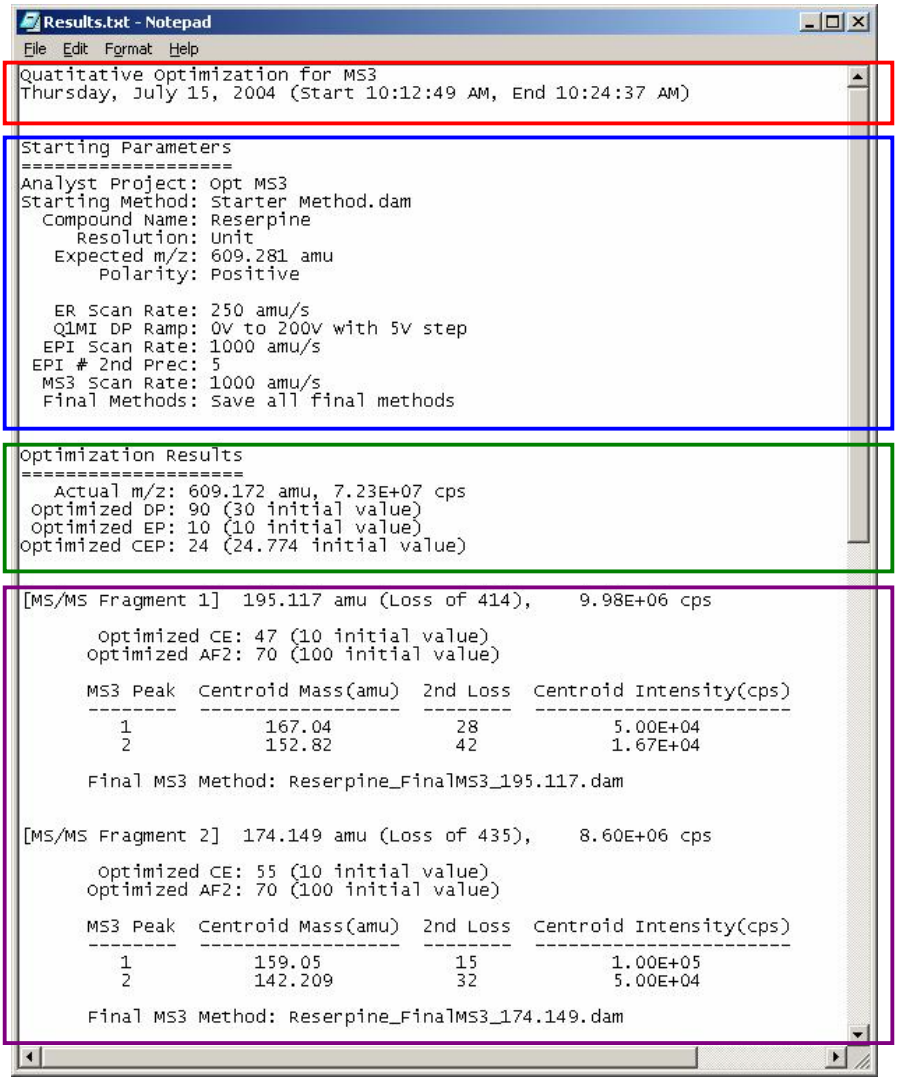


Figure 7: Optimization Report

All of the generated acquisition methods have a descriptive file name in the form of “[supplied compound name] + [scan type] + [m/z] + .dam”. These methods are saved in the same folder as the “starter” acquisition method as seen in Figure 8.

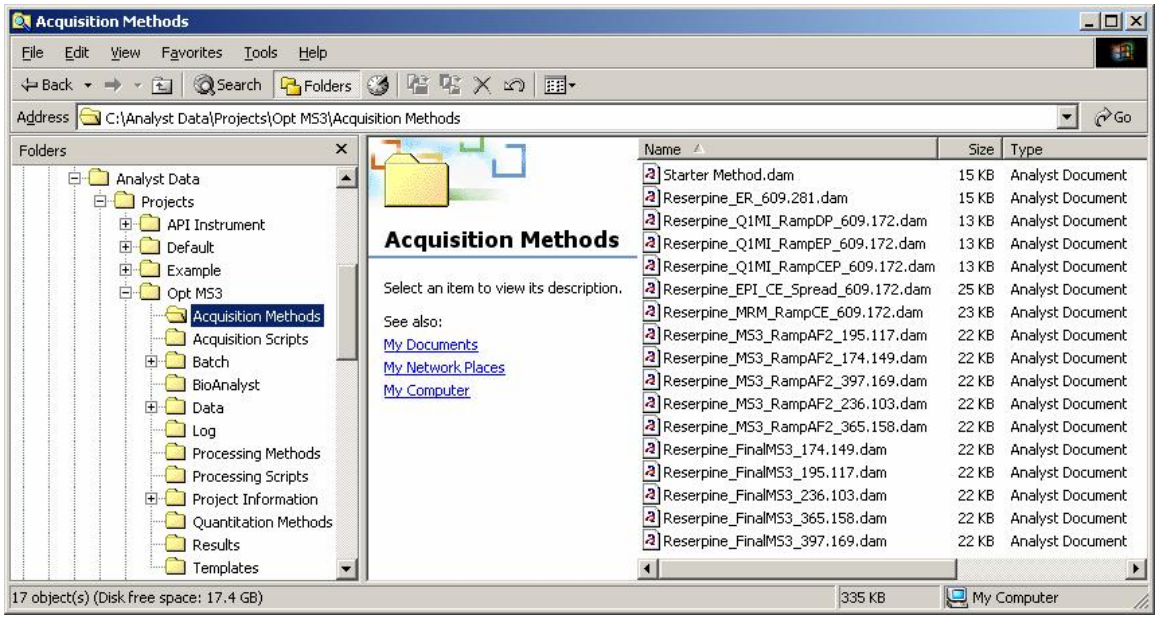


Figure 8: Acquisition Methods

All of the data, Log.txt and Results.txt files are saved into a Data sub-folder that is created in the same project as the “starter” acquisition method. The sub-folder has the format “[supplied compound name] + OptMS3 + ([date], [time])”. The data files have the format “[supplied compound name] + [scan type] + [m/z] + .wiff”. An example of the Data sub-folder and its contents is shown in Figure 9.

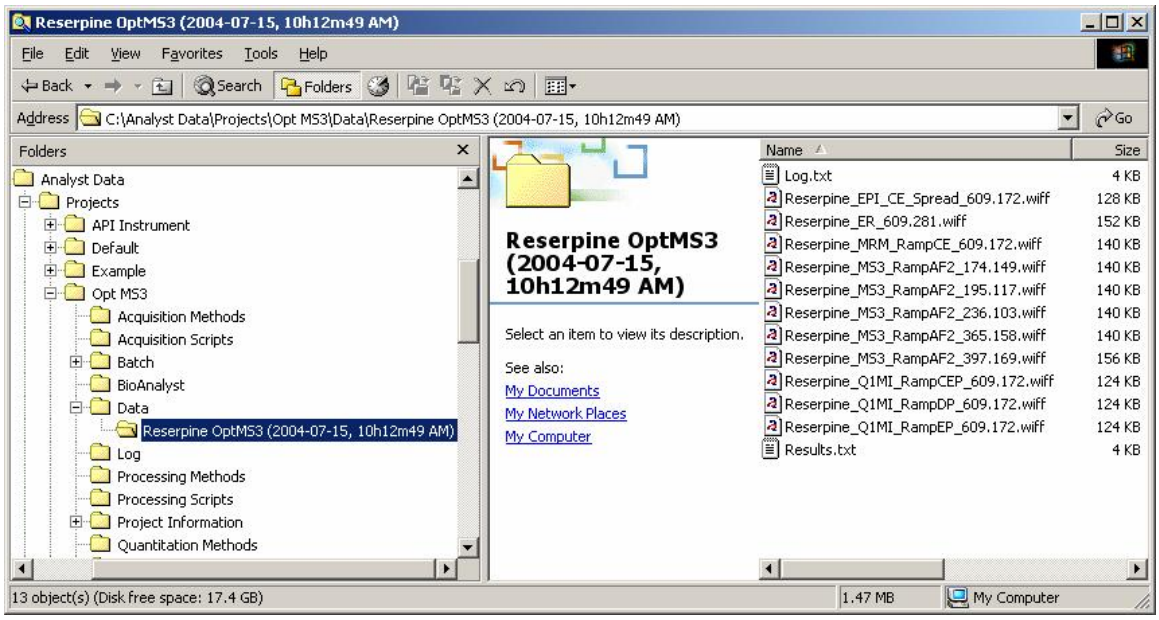


Figure 9: Data Files

4.0 Detailed Description of Script Logic

In order to get a better understanding of how this automated script works, each phase of the optimization process is described in more details in this section. All scans are performed with the "Number of scans to sum" set to 3.

Initialization

Before performing any optimization scans, the script first performs some initialization steps. If an error occurs during any of these steps, the script will not continue to perform the optimization.

- Check that Analyst Software is running.
- Load the "Starter" method to see if it is valid and check the device type.
- Create a new Data sub-folder to store the *.wiff files.
- Create the Log.txt file.

Enhanced Resolution Scan

This step essentially confirms the mass of the ion used for optimization. The ER scan is performed for 20 cycles at the specified scan rate. The most intense peak within ±1 amu of the expected 1st precursor m/z is then selected. By default similar to Analyst Software, this scan is performed with a 30 amu mass range around the specified m/z. For multiply charged species, the C12 ion will be determined in this step.

Q1 Multiple Ion Scan

This step essentially optimizes transmission of the ion of interest up to the collision cell. This is performed using a Q1 MI scan. The script first optimizes the DP parameter by performing the scan at the specified DP ramp. The EP parameter is then optimized by ramping it from 1V to 12V (-12V to –1V for negative mode) with 0.5V step. If the optimal EP is less than 10V (greater than –10V for negative mode), then DP is re-optimized. If not a 4000 QTRAP® system, the CEP parameter is also optimized by ramping from 0V to 100V (-100V to 0V for negative mode) with 2V step. In determining the optimal voltage, graphs are smoothed 2 times and the voltage yielding the greatest ion count is used. Dwell Time for each scan is set to 100 ms.

Enhanced Product Ion Scan

This step essentially selects the fragment ions that will be used for MS^3 optimization. This is performed using an EPI scan for 3 cycles at the selected scan rate. The user can specify an optimal CE for the compound to be analyzed, if this is unknown, then a Collision Energy Spread (CES) can also be specified such that a range of CE settings are used. The most intense 2nd precursor peaks are then found, excluding any peaks within ±2.5 amu window of 1st precursor. The number of 2nd precursors to use is selected in the Advanced Settings. The mass range is from from which the 2nd precursors are selected is specified by the user.

Multiple Reaction Monitoring Scan

This step essentially optimizes the collision energy for each of the fragment ions selected from the EPI scan. This is performed using MRM scans. CE ramps of 5V to 130V (-130V to –5V in negative mode) with 2V step and Dwell Time of 50 ms are used. Each overlaid graph is then smoothed 2 times and the voltages yielding the greatest ion count are used as the optimal CE values.

MS/MS/MS Scan

The script performs an MS^3 scan for each chosen 2nd precursor at the specified scan rate and with an AF2 ramp of 0 to 100 mV with 2 mV step (or 0 to 0.4 V with 0.01 V step on QTRAP® 5500 system) for both polarities. The fill time of the scan is set and Q0Trapping can be turned on for maximum sensitivity if required. The lower limit of the mass range for the MS/MS/MS scan can be specified and the upper limit is 2nd precursor + 5 amu.

The generated graphs are smoothed 2 times and the optimal AF2, as seen in Figure 9, is obtained when the residual intensity of the 2nd precursor (based on XIC) is at 5% of its maximum intensity. The spectrum at this AF2 value is then used to find the 2 most intense second generation fragment ions, excluding peaks within ±1 amu of the 2nd precursor. If the 2nd precursor m/z is greater than 10% of the total ion count, no fragments from that spectrum will be used. This condition exists because if the 2nd precursor m/z is greater than 10%, there is insufficient fragmentation.

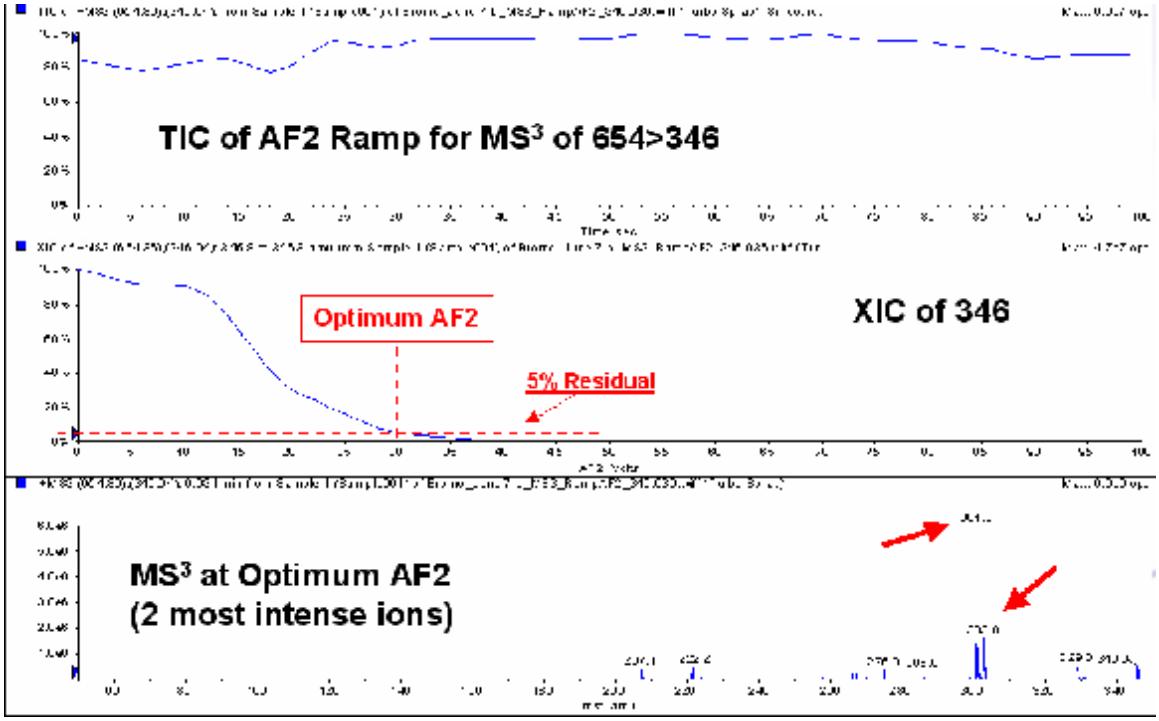


Figure 9: How AF2 is Determined

Generate Final Methods

After the optimization scans have been performed, the script then generates the final MS^3 methods. If the option to “Save Optimal Method Only” is selected in the Advanced Settings, only an optimal MS^3 method with ±10 amu around the most intense second generation fragment ion is created. If the option to “Save All Final Methods” is chosen then the optimal method as well as an MS^3 method for each of the top 2^{nd} precursors are created using a mass range from the user defined lower limit to an upper limit of (2^{nd} precursor + 5) amu.

Headquarters
353 Hatch Drive Foster City CA 94404 USA
Phone 650-638-5800
www.absciex.com

International Sales
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www.absciex.com/offices