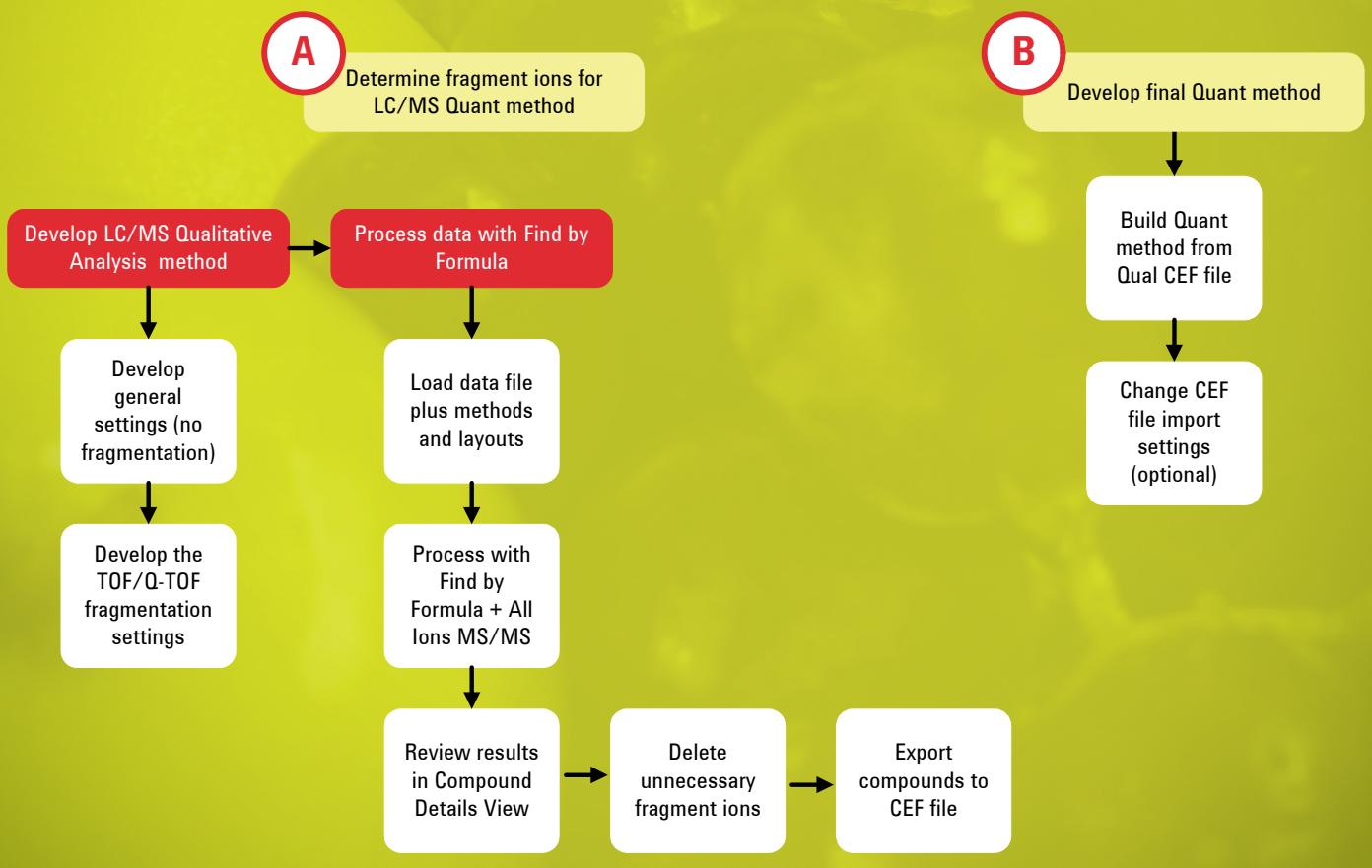
Agilent All Ions MS/MS

Workflow Guide



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Notices

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Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

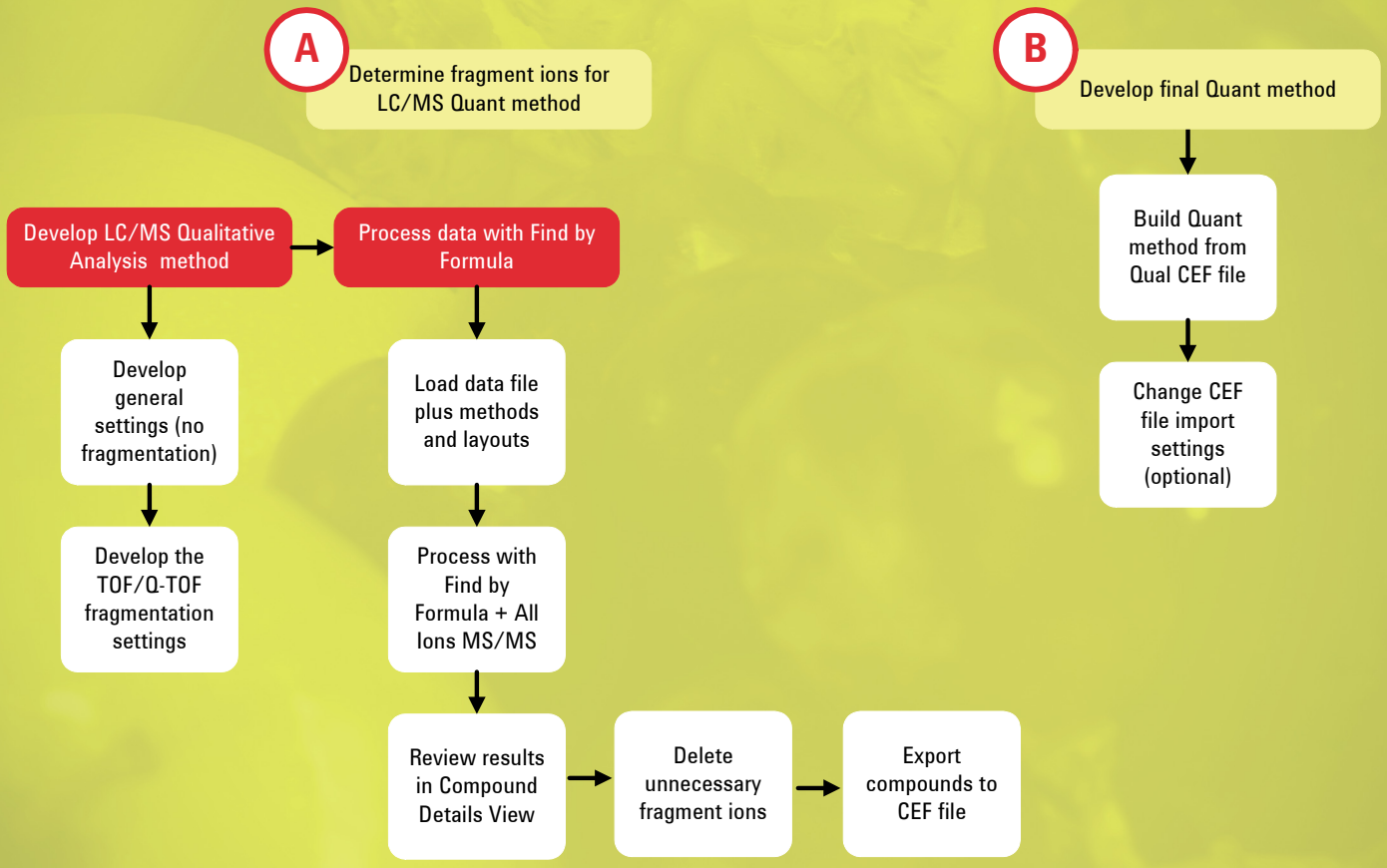
WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

Contents

Before You Begin

Make sure you read and understand the information in this chapter and have the necessary instrumentation, software, solvents, and lab supplies before you start the analysis.

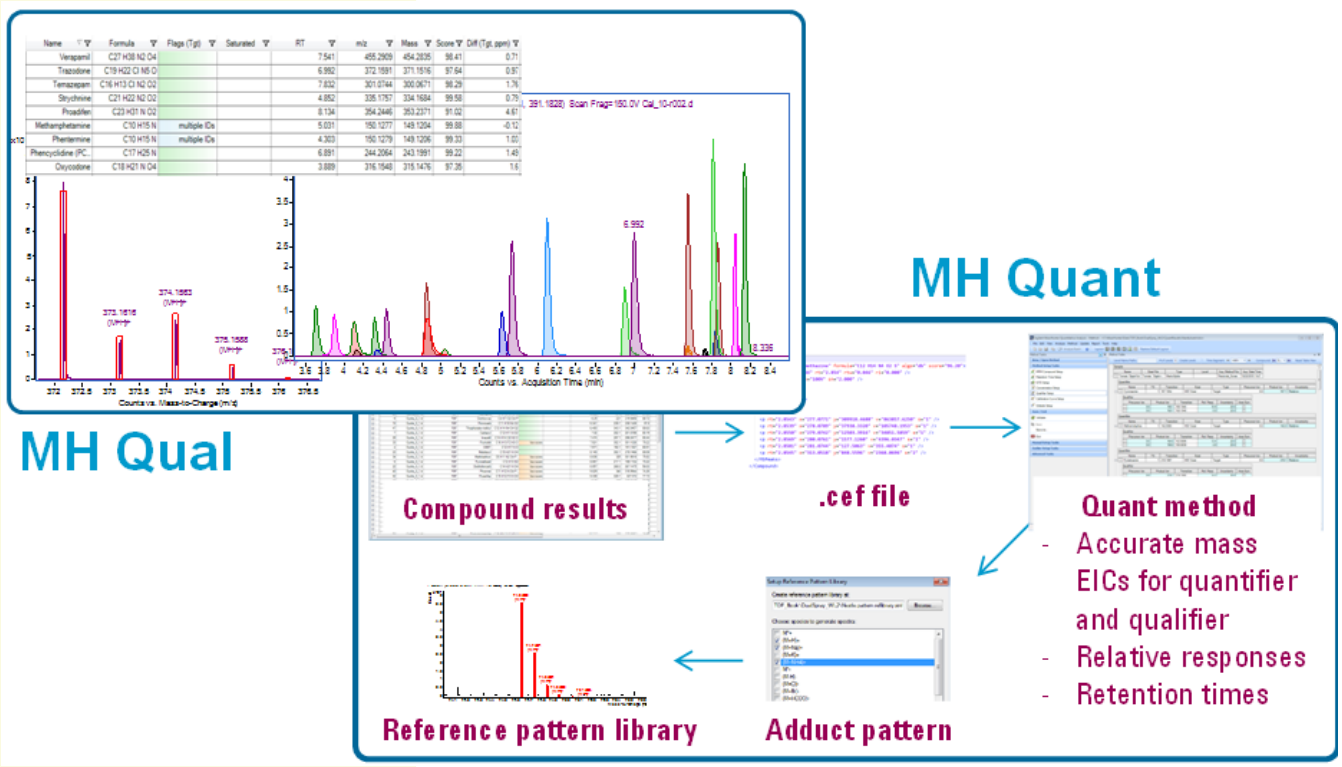


Introduction

A number of applications require broad-range LC/MS quantification of large numbers of analytes. These applications include analyses of pesticides in food and environmental samples, as well as forensics analyses. This manual describes how to rapidly set up a MassHunter Quantitative Analysis method for applications like these.

Workflow description

This *Workflow Guide* describes use of the "All Ions MS/MS" technique for compound confirmation and setup of a time-of-flight (TOF) or quadrupole time-of-flight (Q-TOF) quantitative method. The diagram below shows briefly how you use the All Ions MS/MS solution from MassHunter Qualitative Analysis (MH Qual) to set up a method in MassHunter Quantitative Analysis (MH Quant).



The workflow described in this guide is ideal for development of a quantitative method in food, environmental, and forensic labs, and you can apply the general concepts to other targeted analyses. The workflow uses two capabilities that are unique to Agilent:

- MS/MS spectral libraries to identify fragment ions
- Coelution score to confirm that fragments belong to a precursor ion.

The workflow features:

- Easy data acquisition method setup
- Targeted identification of product ions using Agilent MS/MS libraries
- Chromatographic confirmation of product ions (coelution score)
- Rapid development of a quantitative analysis method.

Method development is fast because after you perform a qualitative analysis, the software can automatically select high-quality product ions for quantification of each compound, which eliminates tedious manual steps.

This workflow enables screening that complies with SANCO 12495 and other regulations. SANCO 12495 procedures for analyses of pesticides in food and feed require at least two product ions and final confirmation with a reference compound.

Accurate-mass pesticide database and library

To accelerate pesticide analysis with its TOF and Q-TOF instruments, Agilent offers the MassHunter Personal Pesticide Database and Library. It contains exact monoisotopic masses for more than 1600 pesticides and related compounds. It also contains library searchable MS/MS spectra for more than 700 compounds. The database contains pesticide names, molecular formulas, structures, CAS registry numbers, and links to useful resources.

In addition, Agilent offers the most comprehensive accurate mass libraries for broad-range forensic screening, with 7500 compounds and accurate mass MS/MS spectra for more than 2500 compounds. Agilent also offers an accurate mass library for veterinary drug screening. It contains more than 1000 compounds, 600 of which have accurate mass MS/MS spectra.

More information

If you need a general introduction to the Agilent TOF or Q-TOF before you begin, see the *Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System Concepts Guide*.

Other useful references are:

- "Agilent TOF LC/MS and Q-TOF LC/MS Screening of Pesticides Workflow Guide." 5990-7072EN.
- "Agilent TOF LC/MS and Q-TOF LC/MS Screening of Pesticides Workflow Overview."5990-7069EN.
- "Agilent TOF LC/MS Drug Screening Workflow Guide."5991-1109EN.
- "Agilent TOF LC/MS Drug Screening Workflow Overview."5991-1110EN.
- "Agilent MassHunter Workstation Software Qualitative Analysis Familiarization Guide B.06.00." G3335-90156 (has section on analysis of All Ions MS/MS data).

NOTE

This manual gives links to references. If you have an electronic copy of this manual, you can easily download the documents from the Agilent literature library. Look for and click the blue hypertext; for example, you can click the library link in the previous sentence.

If you have a printed copy, go to the Agilent literature library at **www.agilent.com/chem/library** and type the publication number in the search box. Then click the icon for the magnifying glass.

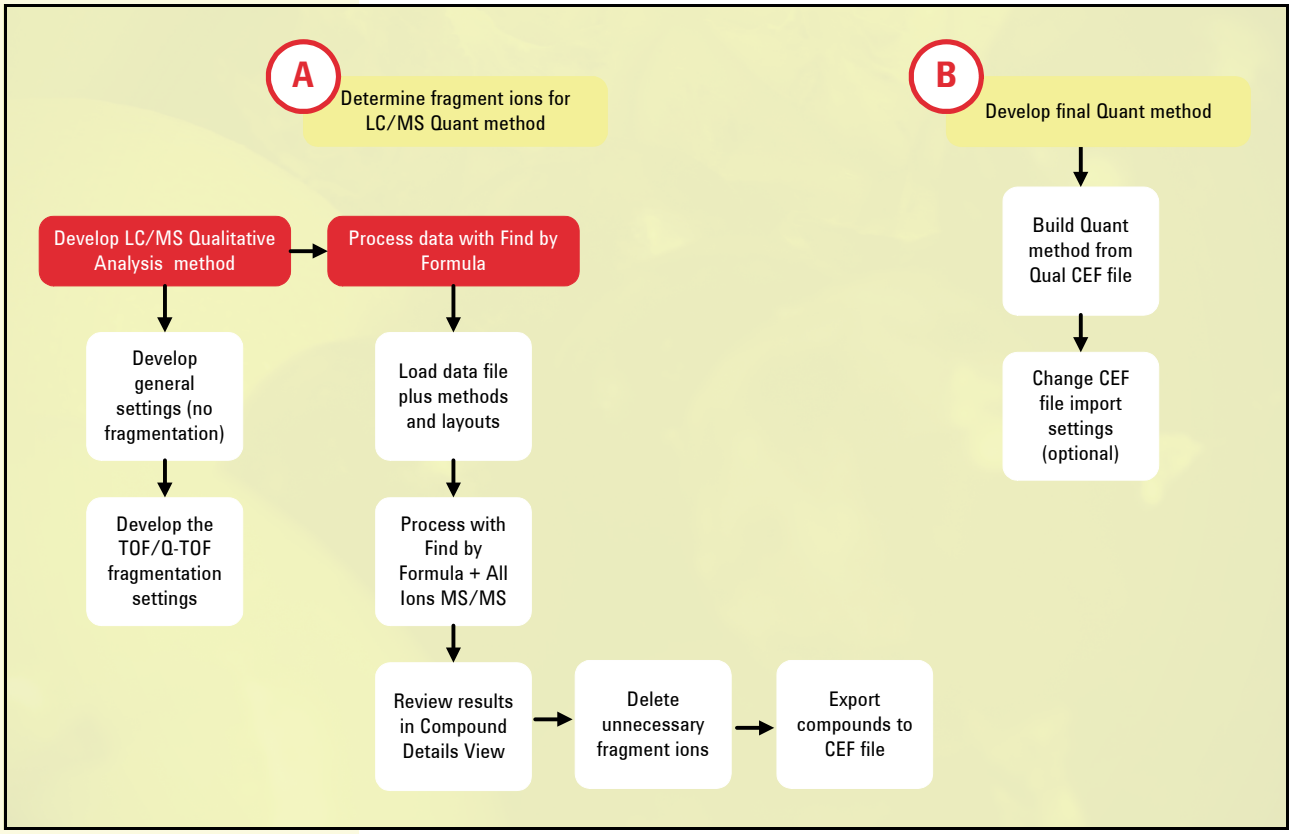
Overview of the workflow

This manual describes a rapid workflow to easily set up a method to do pesticide quantification with the Agilent MassHunter Workstation software and one of these instruments:

- Agilent 6200 Series Accurate-Mass TOF LC/MS System
- Agilent 6500 Series Accurate-Mass Q-TOF LC/MS System

While this *Workflow Guide* is written specifically for pesticides, the concepts apply to other types of compounds, such as drugs of abuse.

The workflow describes use of All Ions MS/MS for development of a MassHunter Quantitative Analysis method. With All Ions MS/MS, the MassHunter data acquisition method needs only to be set up with a "low-energy channel" (for example, no collision energy) and one or more "high-energy channels" (for example, collision energy = 20V). You do not need to type masses for every compound like you do in multiple reaction monitoring (MRM) on a triple quadrupole (QQQ) system. The result is much faster method setup.



Below is a summary of the process you use for All Ions MS/MS:

a Acquire data on an Agilent TOF LC/MS or Q-TOF LC/MS in MS mode with a "low-energy channel" and one or more "high-energy channels." With the "high-energy channels," you use either the fragmentor voltage or collision energy to produce fragmentation. The data acquisition method setup is extremely easy and does not require optimization like MRM on a QQQ.

b Load the data file into MassHunter Qualitative Analysis and run Find by Formula against the MassHunter Personal Pesticide Database and Library. Find by Formula returns possible precursor formulas found in the "low-energy channel," while the All Ions MS/MS algorithm uses the library to find the product ions to search.

Personal Compound Database and Libraries (PCDLs) contain library searchable MS/MS spectra. All Ions MS/MS uses the product ions in the spectra to confirm the identification of hits. The result is fewer false positives. The product ions are detected in the "high-energy channel."

Agilent has PCDLs for pesticides, veterinary drugs, forensics/toxicology, and metabolomics.

c Using the list of product ions, MassHunter Qualitative Analysis produces extracted ion chromatograms (EICs) from the "high-energy channel" and aligns them with an EIC of the precursor ion from the "low-energy channel." It calculates retention time difference, signal-to-noise ratio, and a coelution score, and qualifies each fragment ion that passes the user-set thresholds.

d Finally, you export the compounds as a Compound Export Format (.CEF) file and load them into MassHunter Quantitative Analysis. The program uses the list of precursors and product ions with their retention times (and collision energies or fragmentor voltages) to rapidly create a MassHunter Quantitative Analysis method with at least two qualifier ions.

Safety notes

WARNING **Always take proper precautions when you use and dispose of solvents, pesticides, and other chemicals. Read the material data safety sheets supplied by the vendors.**

WARNING **When you disconnect LC columns or fittings, solvents may leak. Use appropriate safety procedures (for example, goggles, safety gloves and protective clothing), especially when you use toxic or hazardous solvents. Read the material data safety sheets supplied by the solvent vendors.**

WARNING **Read, understand, and meet conditions of all warnings in the *Maintenance Guide* that you received with your TOF or Q-TOF instrument.**

CAUTION Read, understand, and meet conditions of all cautions in the *Maintenance Guide* that you received with your TOF or Q-TOF instrument.

Required hardware and software

Figure 1 *The workflow requires an Agilent LC and TOF or Q-TOF LC/MS System.*

To do this workflow, you need:

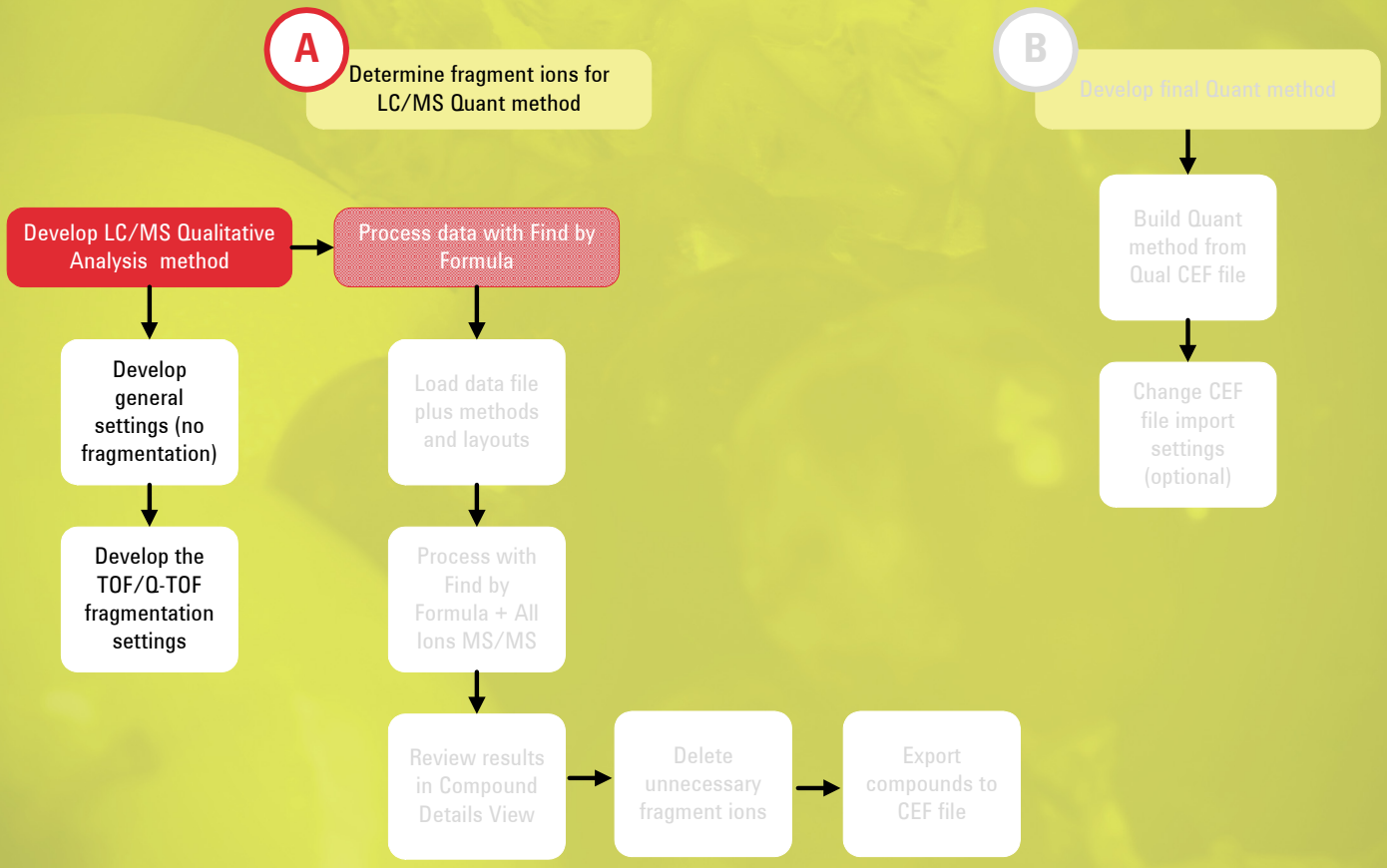
- One of the following LCs:
  - Agilent 1220 Infinity LC
  - Agilent 1260 Infinity LC
  - Agilent 1290 Infinity LC
- One of the following mass spectrometers:
  - Agilent 6200 Series Accurate-Mass TOF LC/MS System
  - Agilent 6500 Series Accurate-Mass Q-TOF LC/MS System
- Agilent MassHunter software:
  - Agilent MassHunter Data Acquisition software for TOF/Q-TOF version B.05.01 or later
  - Agilent MassHunter Qualitative Analysis software version B.06.00 or later
  - Agilent MassHunter Quantitative Analysis software version B.05.02 or later (requirement for new PCDLs)
  - Agilent MassHunter Personal Compound Database and Library version B.04.00 or later
  - Agilent MassHunter Personal Compound Database and Library (PCDL) Manager software version B.04.00 or later
- Microsoft Windows 7 Professional 64-bit

The instructions in this manual assume that:

- All instruments have already been installed and are working to specifications.
- You have some experience with pesticide analysis.
- You have been trained on the LC, TOF/Q-TOF instrumentation, and the MassHunter Workstation software. For example, you have taken an operator course at an Agilent training center or you have been trained on-site by an Agilent instructor (Application Engineer or consultant).

Developing the LC/MS Qualitative Analysis Method

These pages describe briefly how to set up a qualitative analysis method for pesticides with an Agilent TOF or Q-TOF LC/MS System and MassHunter Workstation software. You use information from this analysis to later set up the quantitative analysis method.



Develop the general LC/MS settings (no fragmentation)

The first analysis of this workflow is a qualitative analysis using the Agilent 6200 Series Accurate-Mass TOF LC/MS System or 6500 Series Accurate-Mass Q-TOF LC/MS System and MassHunter Workstation software. You use the data you acquire with this analysis to find the retention times and masses of your analytes. You later use that information to set up the quantitative analysis method.

This initial LC/MS method does not include the instrument settings you use to cause fragmentation (high fragmentor voltage on a TOF or high collision energy on a Q-TOF). You set up the fragmentation settings in the next subsection, which starts on page 13.

1. Choose or develop an initial LC/MS method for data acquisition.

- Do one of the following:
  - Choose one of the methods from the appendix of Agilent application note 5990-4251EN, "An Application Kit for Multi-Residue Screening of Pesticides using LC/TOF or Q-TOF with a Pesticide Personal Compound Database."
  - Choose one of the methods from *Agilent TOF LC/MS and Q-TOF LC/MS Screening of Pesticides Workflow Guide*, (Agilent publication 5990-7072EN, June 2012).
  - Choose an LC/MS method from another source, or create one yourself. Agilent recommends you still use the ion source settings from Appendix I of 5990-4251EN.

The screening method so far is MS-only and does not produce fragment ions.

The LC method may affect your choice of solvents for the standards.

2. Purchase any necessary standards and solvents, if you have not already done so.

a Determine which standards you need to purchase.

b Purchase the standards and solvents.

3. Prepare and refrigerate the standard solution.

Develop the fragmentation settings

This section describes development of the fragmentation settings for the Agilent TOF or Q-TOF data acquisition method. To acquire a data file for All Ions MS/MS, you must set up a method with time segments for two, three, or four fragmentation experiments. You will set up either a TOF MS-only method with multiple fragmentor voltages or a Q-TOF MS/MS method with multiple collision energies.

For example, you could have a method with two time segments and two collision energies.

- In the optional first 0.5 min time segment, you would divert the LC flow to waste. In the second time segment that starts at 0.5 min, the LC flow would go to the column.
- The second time segment could have two experiments. In the first experiment, the collision energy could be 0 V. In the second, the collision energy could be 20 V.

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1. Start with an appropriate data acquisition method.

- Start with a method that already has the correct settings, except for time segments for fragmentation. For example, start with the method you chose in the previous section, which starts on page 12.
- If you do not have a method, see the examples in *Agilent TOF LC/MS and Q-TOF LC/MS Screening of Pesticides Workflow Guide*, 5990-7072EN.

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2. Set the mode to MS.

a Click the **Acquisition** tab.

b Under Mode, click **MS (Seg)**.

- Because All Ions MS/MS is *not* an isolation experiment, you must set the mode to MS.

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3. Set the acquisition speed to 3 spectra/second.

a Click the **Acquisition** tab.

b Set **Rate** to **3 spectra/second**.

- This setting gives a good balance between spectral quality and generation of sufficient points across a chromatographic peak.

---

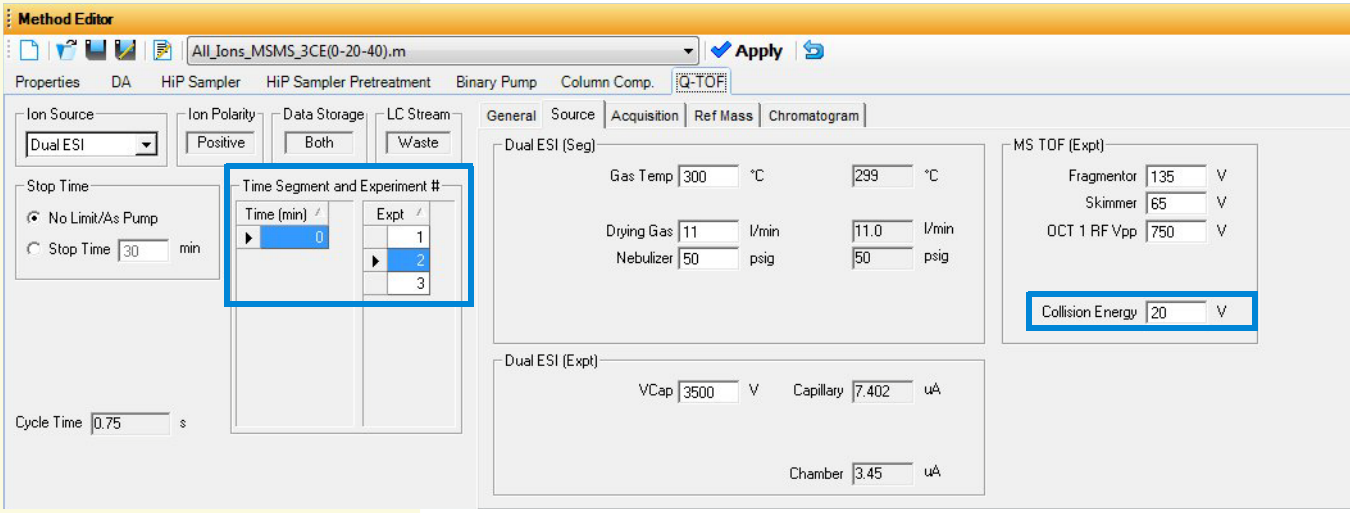
4. If you have a Q-TOF, set up experiments with three collision energies.

a Under **Time Segment and Experiment #,** for **Expt**, set collision energies to **0**, **20**, and **40 V**. Set them in increasing order.

b Set the fragmentor voltage to a low enough setting that no fragment ions are generated in the source, but high enough to transmit the precursor ions through the skimmer.

c Set up the collision energies in an increasing manner, with the lowest setting in the first experiment.

- The values of 0, 20, and 40 V give a good trade-off between spectral coverage and sensitivity.



- 10, 20, and 40 V are the collision energies that were used to acquire the accurate-mass MS/MS spectra in the Agilent MassHunter Pesticide Personal Compound Database and Library (PCDL) that MassHunter Qualitative Analysis uses to select fragment ions.
- Use of an experiment with two or three high-energy channels allows you to analyze target compounds across a wide range of stabilities.

5. If you have a TOF, set up experiments with three fragmentor voltages.

a Under **Time Segment and Experiment #,** for **Expt**, set fragmentor voltages to **120**, **270**, and **350 V**. (These are good voltages for pesticides; you may require other voltages for other types of compounds.)

b Set up the fragmentor voltages in increasing manner, with the lowest setting in the first experiment.

- Do not change both fragmentor voltage and collision energy in the same method, because results will be unpredictable.

6. Save the method.

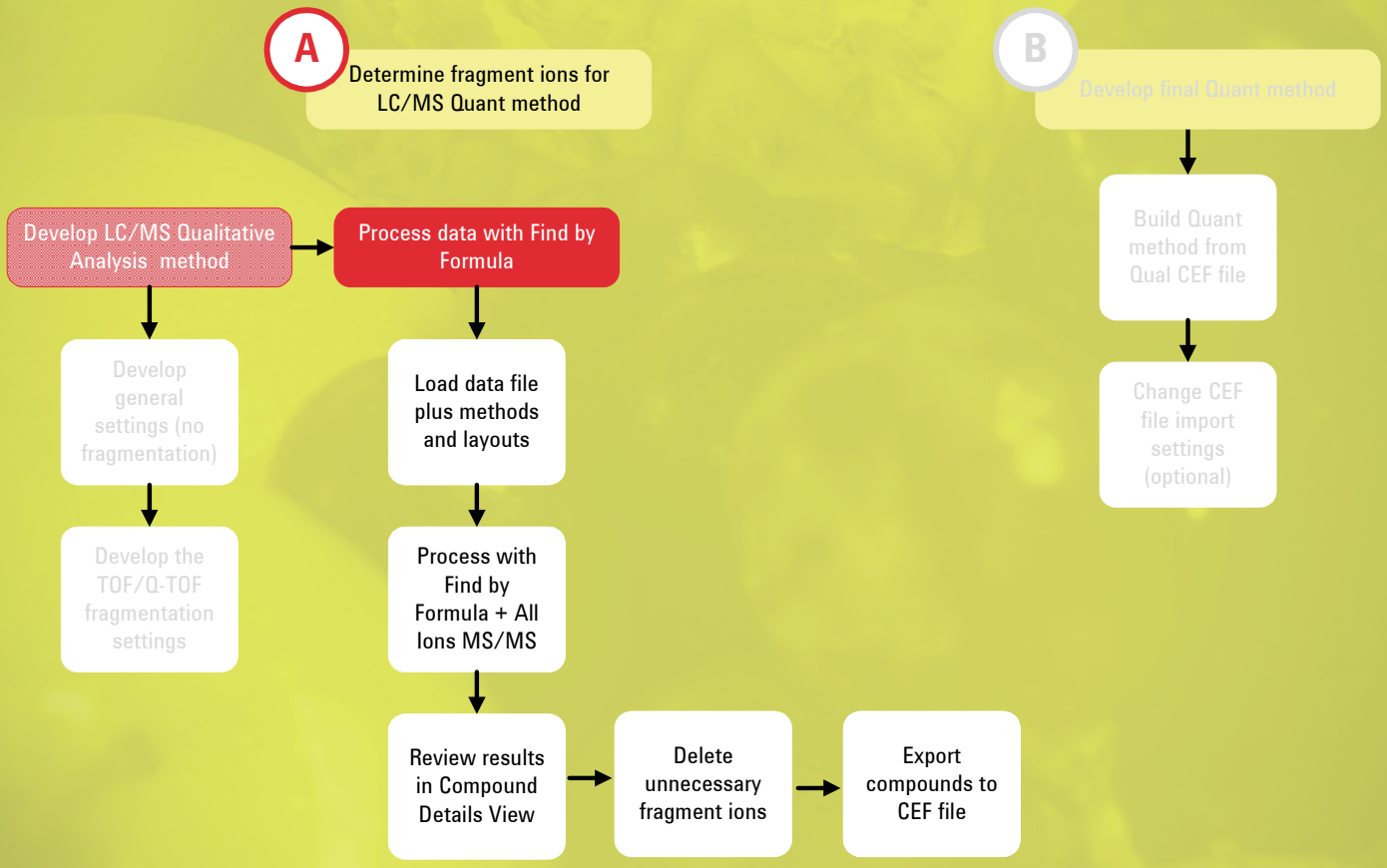
a Click **File > Save As > Method**.

b Give the method a name and click **OK**.

7. Analyze the standard solution with the method.

Processing Data with Find by Formula

This chapter describes the first major step to process data using the All Ions MS/MS technique. This step relies on the powerful Find by Formula capability in Agilent MassHunter Qualitative Analysis to find the compounds in an analysis of a standard. After you have found the compounds, you can very quickly set up a quantitative method, as described in the next chapter.



Overview

To quickly build a MassHunter Quantitative Analysis method, you follow these basic steps:

a Use MassHunter Qualitative Analysis to process data file with Find by Formula. In this step, you find all the target compounds in the file.

b Use MassHunter Qualitative Analysis to select fragment ions for target compounds.

c Build the MassHunter Quantitative Analysis method.

This chapter discusses steps a and b, while the next chapter describes step c. To illustrate the steps, the chapters use a data file that contains a mixture of 27 pesticides spiked into avocado. This sample was extracted using the QuEChERS technique and analyzed by MS/MS on an Agilent 6500 Series Accurate-Mass Q-TOF LC/MS System.

The highlighted portions of the workflow diagram on the previous page summarize the steps discussed in this chapter.

Process with Find by Formula

In this section, you use MassHunter Qualitative Analysis to process a data file with Find by Formula. In this step, you find all the target compounds in the file, so you can later use their masses and retention times to set up a quantitative analysis method.

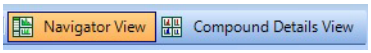
Load data file plus methods and layouts

For this exercise, you load a data file from your analysis of the analytes of interest. For demonstration, you may load the example data file **AIM_3CE(0-20-40).d.** The data file and method discussed here are on your MassHunter software disk. The layouts are installed with your software.

1. Make sure you are in the Navigator View.

a In MassHunter Qualitative Analysis, confirm that you see the buttons for the **Navigator View** and the **Compound Details View**.

b Click the **Navigator View** button.



- The Navigator View is ideal for browsing through multiple data files, while the Compound Details View is designed for browsing through a single data file compound-by-compound.
- The Compound Details View is a new Qualitative Analysis view for All Ions MS/MS. It was first available with MassHunter Qualitative Analysis B.06.00. You will use this view when you review the data.
- The program also retains the Navigator View that existed in previous versions of MassHunter Qualitative Analysis.

2. Use the Target Compound Screening Workflow.

a Click **Configuration > Configure for Workflow > MS Target Compound Screening**.

b Click **Load workflow's default method**.

c Click **Load workflow's default layout**.

d Click **OK**.

- These settings provide a good starting point for target compound screening.

3. Load the data file.

a Click **File > Open Data File**.

b Load a data file from an analysis of your analytes of interest.

- For demonstration, you may load the data file **AIM_3CE(0-20-40).d**.
- This data file was acquired by cycling through three collision energies: 0, 20, and 40 V. The 0 V channel provides the precursor ion, while the others provide fragment ions.
- You could instead do this experiment with only two collisions energies on a Q-TOF, or high/low fragmentor voltages on a TOF.

4. Load the method.

a Click **Method > Open**.

b Load the MassHunter Qualitative Analysis method.

- For demonstration, you may load the method **Pestmix_AIM_SP1.m**.
- If you do not see this method, copy it from your MassHunter software disk to the **MassHunter\Methods\B.06.00** folder.

5. Make sure your method points to the correct Personal Compound Database and Library (PCDL).

a In the lower left corner of the Qualitative Analysis window, click **Method Explorer: PestMix_AIM_SP1.m**.

b Click **+** to expand **Find Compounds by Formula**.

c Click **Find by Formula - Options**.

d Click the **Formula Source** tab.

e For **Database/Library**, make sure the method points to the PCDL you want to use for All Ions MS/MS. In the case of our example pesticide analysis, make sure it is set to **PestMix_AIM_PCDL_SP1.cdb**.

f If you change the database, save the method. (Click **Method > Save**.)

- If you do not see this database, or if a red exclamation point appears by its name, copy the database from your MassHunter software disk to the **MassHunter\PCDL** folder.

(Optional) To process a data file with Find by Formula *without* All Ions MS/MS

This demonstration shows that you can successfully confirm compounds using Find by Formula with the low-energy channel alone. However, the confirmation is not as reliable because you use only retention time and a single mass for the compound, rather than retention time and multiple masses (precursor and fragment ions). Therefore, this exercise is optional and is not shown in the workflow diagram.

1. Set up the Find Compounds by Formula options and find the compounds.

a For demonstration, load the example data file **AIM_3CE(0-20-40).d**.

b In the Find Compounds by Formula options, click the **Formula Source** tab and choose the **Database/Library**. For demonstration, choose the database **PestMix_AIM_PCDL_SP1.cdb**.

c Click the **Positive Ions** tab and choose charge carriers. To process the demo file, mark **+H** and **+Na**.

d Click the **Results** tab, mark **Extract EIC**, **Extract cleaned spectrum**, and **Include structure**.

e Click the **Fragment Confirmation** tab and clear the check box for **Confirm with Fragment Ions**. (For now, you do not confirm with fragment ions.)

f Click **Find Compounds by Formula**.

2. Verify the results.

a If you processed the demo data file **AIM_3CE(0-20-40).d**, which contains a mixture of 27 pesticides spiked into avocado, confirm that the Compound List shows 27 compounds and that results are similar to the ones shown just below. (To see the full list, right-click the **Compound List** title bar and click **Floating**.)

b Note that chlorpropham lacks confirmation because it has a poor response factor. For this compound, the Flags (Tgt) column is red. Propham and monolinuron have low scores and their Flags (Tgt) columns are orange. The other compounds have high scores, which shows successful identification using only the MS-level information from the low-energy channel.

c In the Formula column, confirm that the results include two sets of structural isomers (compounds with the same molecular formula). For these isomers, the Flags (Tgt) column is blue and shows multiple IDs. The first set contains three isomers, while the second contains two.

- The program assigns isomers by both mass and retention time, rather than by the mass alone. So the isomers in the demo data file are correctly assigned.

Compound List

Automatically Show Columns

Cpd	Name	Formula	m/z	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Score (Tgt)	Flags (Tgt)
1	Atrazine-desethyl-desisopropyl	C3 H4 Cl N5	146.0221	145.015	145.0155	-3.66	92.62	Qualified
2	Fenuron	C9 H12 N2 O	165.1023	164.095	164.095	0.48	99.93	Qualified
3	Crimidine	C7 H10 Cl N3	172.0636	171.0564	171.0563	0.14	99.61	Qualified
4	Propham	C10 H13 N O2	202.0858	179.0945	179.0946	-0.93	72.71	low score; No H adduct;Qualified
5	Atrazine-desethyl	C6 H10 Cl N5	188.07	187.0627	187.0625	1.02	99.83	Qualified
6	Simazine	C7 H12 Cl N5	202.0858	201.0785	201.0781	1.86	99.45	Qualified
7	Metamitron	C10 H10 N4 O	203.0932	202.0859	202.0855	2.11	99.29	Qualified
8	Isoproturon	C12 H18 N2 O	207.1498	206.1425	206.1419	2.76	98.9	Qualified
9	Chlorotoluron	C10 H13 Cl N2 O	235.061	212.0724	212.0716	3.71	97.3	Qualified
10	<Chlorpropham>	C10 H12 Cl N O2	214.0663	213.059	213.0557	15.57	43.24	m/z tolerance
11	Monolinuron	C9 H11 Cl N2 O2	237.0408	214.0515	214.0509	2.84	71.27	low score;Qualified
12	Metribuzin	C8 H14 N4 O S	215.0967	214.0894	214.0888	2.78	98.6	Qualified
13	Atrazine	C8 H14 Cl N5	216.1016	215.0942	215.0938	1.96	98.08	Qualified
14	Methabenzthiazuron	C10 H11 N3 O S	222.0702	221.0629	221.0623	2.62	98.65	Qualified
15	Metoxuron	C10 H13 Cl N2 O2	229.0744	228.0671	228.0666	2.35	99.05	Qualified
16	Sebuthylazine	C9 H16 Cl N5	230.1174	229.1101	229.1094	3.03	98.47	multiple IDs;Qualified
17	Terbuthylazin	C9 H16 Cl N5	230.1179	229.1106	229.1094	5.14	96.08	multiple IDs;Qualified
18	Propazine	C9 H16 Cl N5	230.1173	229.11	229.1094	2.47	98.88	multiple IDs;Qualified
19	Diuron	C9 H10 Cl2 N2 O	233.0248	232.0175	232.017	1.88	99.23	Qualified
20	Cyanazine	C9 H13 Cl N6	263.0784	240.0896	240.089	2.47	98.15	Qualified
21	Terbutryn	C10 H19 N5 S	242.1441	241.1368	241.1361	3	98.3	multiple IDs;Qualified
22	Prometryn	C10 H19 N5 S	242.1442	241.137	241.1361	3.51	97.74	multiple IDs;Qualified
23	Linuron	C9 H10 Cl2 N2 O2	249.0199	248.0126	248.0119	2.78	98.56	Qualified
24	Metobromuron	C9 H11 Br N2 O2	259.0082	258.0008	258.0004	1.76	99.39	Qualified
25	Metazachlor	C14 H16 Cl N3 O	300.0877	277.099	277.0982	2.95	99.5	Qualified
26	Metolachlor	C15 H22 Cl N O2	306.1235	283.1348	283.1339	2.99	98.22	Qualified
27	Chloroxuron	C15 H15 Cl N2 O2	291.0905	290.0832	290.0822	3.49	97.5	Qualified

(Optional) To use Find by Formula with multiple structural isomers

This topic illustrates how Find by Formula processes multiple structural isomers in a target compound database. This optional topic is not shown in the workflow diagram.

Without further information (such as MS/MS information), the program lists structural isomers in alphabetical order. If you add retention times to the database, the program readjusts the ranking and the correct hit appears at the top of the list.

If your analysis includes structural isomers:

1. Increase the number of isomers for Find by Formula.

a In the Formula Source tab of Find by Formula - Options, under **Matches per formula**, mark **Automatically increase for isomeric compounds**.

b Set **Maximum number of matches** to **1**.

- The method as supplied already uses these settings.
- This step does the following: If the program finds multiple hits with same formula in the database, it increases the number of hits to match the number of isomers in the database.

2. Add retention times for isomers to the PCDL.

- This step allows you to distinguish the structural isomers.
- For details, see the MassHunter Personal Compound Database and Library Manager Quick Start Guide, G3336-90014.
- If desired, you can also include in your method any ions that unambiguously distinguish the structural isomers.

3. Find the compounds.

a In the Formula Source tab, under **Values to match**, click **Mass and retention time (retention time optional**).

b Find the compounds.

4. View the results.

- If you processed the example data file (**AIM_3CE(0-20-40).d**), confirm that you see two sets of isomers.

Process a data file with Find by Formula *with* All Ions MS/MS confirmation

These instructions describe how to find compounds using Find by Formula with spectra from the low-energy channel and then confirm them with fragment ions from the high-energy channels. In this section, you search a data file for target compounds using Find by Formula, and you confirm them with the coelution profile of fragment ions. The following overview outlines the process that the algorithm uses to do this task:

a Select known fragment ions for each of the target compounds from their MS/MS spectra in the database. (In our example, the database is **PestMix_AIM_PCDL_SP1.cdb**.)

If the database does not have the compound spectra, the algorithm selects a few most abundant ions from the spectrum from the high-energy channel.

b Extract and integrate the extracted ion chromatogram (EIC) for each fragment ion of the compound across all the high-energy channels.

c Qualify each fragment ion based on:
- Retention time difference between the precursor ion peak and the fragment ion peak
- Signal-to-noise ratio
- Coelution profile, which is measured by the coelution score.

To process a data file with Find by Formula and All Ions MS/MS confirmation, do the following:

1. Set up the Find Compounds by Formula options and find the compounds.

a Click the **Formula Source** tab and choose a **Database/Library**. For demonstration, choose the database **PestMix_AIM_PCDL_SP1.cdb**.

b Click the **Positive Ions** tab and choose charge carriers. To process the demo file, mark **+H** and **+Na**.

c Click the **Results** tab, mark **Extract EIC**, **Extract clean spectrum**, and **Include structure**.

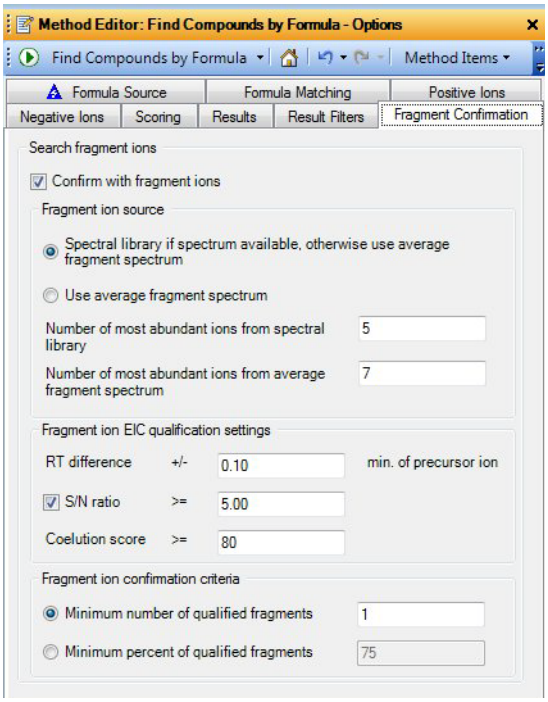
d Click the **Fragment Confirmation** tab and mark the check box for **Confirm with fragment ions**.
   - This step is very important, so be sure to do it.

e Under Fragment ion source, click **Spectral library if spectrum available, otherwise use average fragment spectrum**.

f Use default values for the other settings, as shown in the figure below.

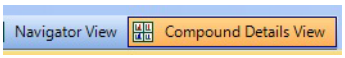
g Click **Find Compounds by Formula**.

h Observe that the program finds the fragment ions and confirms them with signal-to-noise, retention time difference, and coelution score.



2. Make sure you are in the Compound Details View.

a Locate these two views:



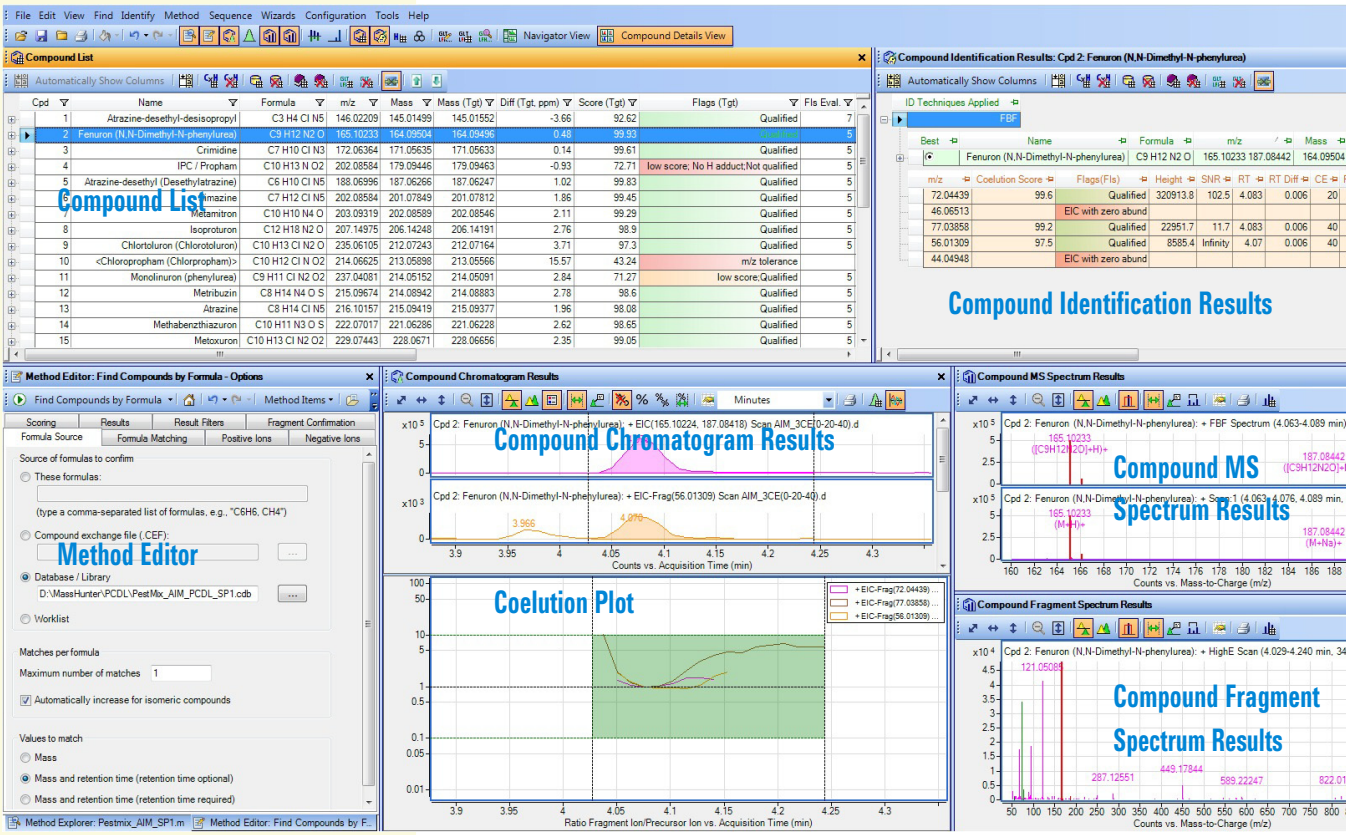
b Click the **Compound Details View** button.

- The new Compound Details View is best when you want to navigate and review compounds in a single data file.
  - It shows both MS and MS/MS level results.
  - Because it shows data from a single data file, no Navigation Tree is necessary.

3. Verify the results.

a Load the **Demo_AIM_CDV.xml** layout.

b Float the Compound List. (Right-click the **Compound List** header bar and click **Floating**.) This step allows you to see the entire Compound List at once.



c View the **Flags (Tgt)** column in the Compound List.

Flags (Tgt) - combines Find by Formula MS-level information (mass accuracy of main isotope, isotope abundance, isotope spacing, retention time) with confirmation by fragment ions from the high-energy channel (either MS/MS spectra or MS spectra acquired at a high fragmentor voltage).

Review results of Find by Formula in Compound Details View

These instructions show you how to review results of Find by Formula with All Ions MS/MS in the Compound Details View (CDV). This view was designed to accelerate data review for All Ions MS/MS.

While the steps below give the procedure, a video provides additional helpful information. The title of the video is **AIM6: Reviewing Results of FbF with AIM in the Compound Details View(CDV).wrf**. You can find it on your MassHunter Qualitative Analysis version B.06.00 or later software disk.

1. Become familiar with the basic components in the Compound Details View.

a If necessary, click the **Compound Details View** button.

b Use the labels in the following graphic to identify the components of the Compound Details View.

c Observe that this view has no navigation tree because you only want to look at results from one data file at a time.

This view is perfect for All Ions MS/MS, where you want to build a quantitative method from a data file you processed in MassHunter Qualitative Analysis. You use the Compound Details View to observe both the MS information from Find by Formula and the MS/MS information from the All Ions MS/MS functionality.

d Click each of the components to review your data.
- Compound List (top left) - allows you to navigate from compound to compound
- Compound Identification Results (top right) - looks at detailed results for the selected compound. This pane contains the fragment ion confirmation table, which shows the fragment ions evaluated and whether they qualified.
- Compound MS Spectrum Results (middle right) - displays the spectrum from low-energy channel
- Compound Chromatogram Results (bottom middle) - shows individual ion traces from the high-energy channel and the EIC of the precursor ion from the low-energy channel. The Coelution Plot in the bottom pane shows the quality of coelution between fragment ions and the precursor ion. Coelution plots are only created for the qualified fragment ions.
- Compound Fragment Spectrum Results (bottom right) - displays the average spectrum across all high-energy channels

2. Review the Compound List (top left).

- Open any node in the Compound List (click + on left) to view detailed results for each fragment ion that is associated with the precursor ion. The program gets these fragment ions from the library spectra or from the spectra in the data file.
- Alternatively, you can more conveniently see these results in the Compound Identification Results table, as discussed next.

3. Review the Compound Identification Results (top right).

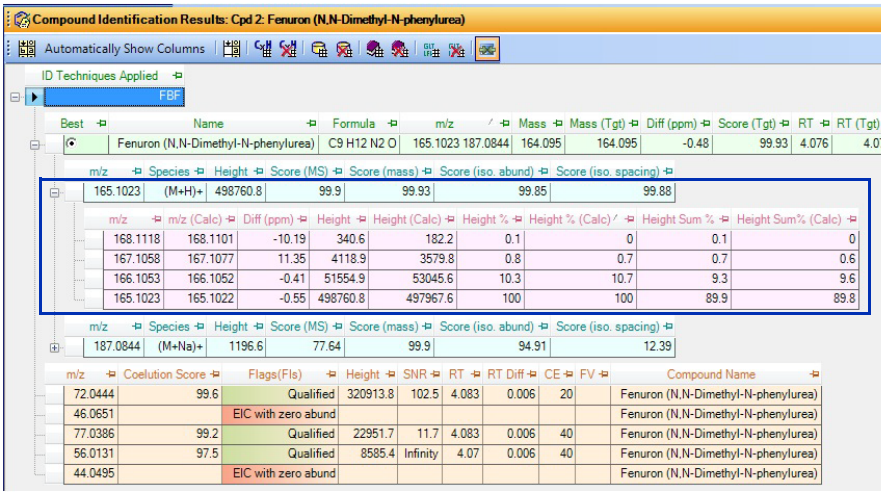
a Click + to open a node.

b Observe the fragment ions that originate in various high-energy spectra.

c Note that the Compound List with MassHunter Qualitative Analysis B.06.00 or later has three columns that are especially useful for this type of analysis:
- FIs Eval. - number of fragment ions evaluated
- FIs Conf. - number of fragment ions confirmed
- FIs Conf. % - percentage of fragment ions confirmed

Flags (Tgt)	FIs Eval.	FIs Conf.	FIs Conf. %
Qualified	7	4	57.1
Qualified	5	3	60
Qualified	5	4	80
low score; No H adduct;Qualified	5	5	100
Qualified	5	5	100
Qualified	5	5	100
Qualified	5	4	80
Qualified	5	3	60
Qualified	5	1	20
m/z tolerance			
low score;Qualified	5	3	60
Qualified	5	5	100
Qualified	5	4	80
Qualified	5	5	100
Qualified	5	5	100
multiple IDs;Qualified	7	6	85.7
multiple IDs;Qualified	7	4	57.1
multiple IDs;Qualified	7	6	85.7
Qualified	5	2	40
Qualified	5	5	100
multiple IDs;Qualified	9	7	77.8
multiple IDs;Qualified	9	6	66.7
Qualified	5	5	100
Qualified	5	5	100
Qualified	5	5	100
Qualified	5	5	100
Qualified	5	5	100

d Click a sub-node to see the isotope results, as shown in the highlighted portion of the image below.



- This data display above shows fragment ions that originate at various collision energies. Some are formed at low collision energy, while others require greater energy.
- The algorithm finds the optimal collision energy or fragmentor voltage for each fragment ion.
- The coelution score takes into account full peak information, not just retention time. Deviations in retention time, peak width, and peak symmetry all affect the coelution score.

4. Review the Compound MS Spectrum Results (middle right).

- View the clean Find by Formula spectrum at the top and the raw spectrum at the bottom. If you do not see the raw spectrum, you probably did not extract it. If desired, modify your method to add extraction of the raw spectrum.

- The spectrum shows only ions that have been associated with the compound.
- The spectrum is annotated with formulas, and the adduct ions are overlaid with rectangles that show the isotopic abundances.

5. View the Compound Fragment Spectrum Results (bottom right).

- View the fragment ion spectrum.

- Qualified fragment ions that belong to the compound (as shown by coelution) are green. Sometimes, you must zoom in to see them. You can see the same fragment ions in the Compound Identification Results.
- When a qualified fragment ion has very low intensity, due to the MS/MS peak filter settings, the fragment ion may not be shown in the fragment spectrum. In this case, the algorithm finds the closest *m/z* to the fragment ion in the spectrum and colors it green.

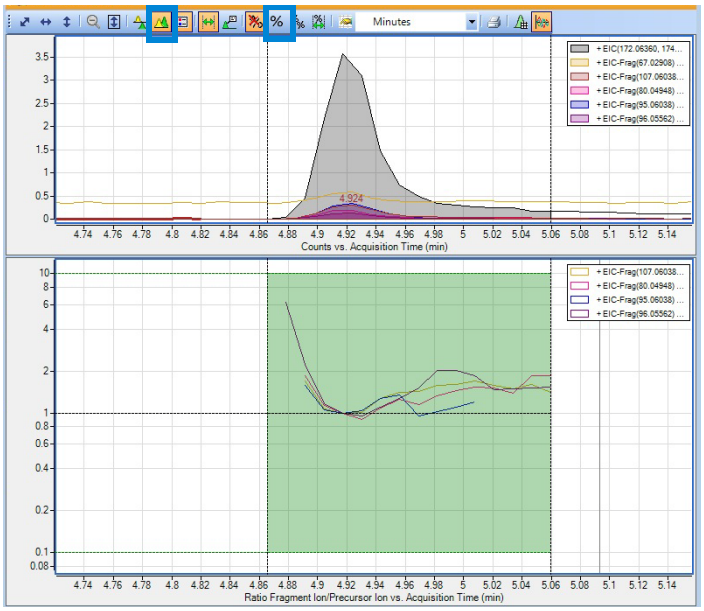
6. View the Compound Chromatogram Results (middle middle).

a View the results.

b Click the icon for **Overlaid mode**. It is the first icon highlighted with the blue square in the figure just below.

c If you want to click the icon to **Scale to the Largest in All Chromatograms**, it is the second icon highlighted with the blue square in the figure just below.

- These results show fragment ion EICs extracted from all the collision energies. The EIC for the precursor ion is also shown.
- The Overlaid mode shows the overlay of precursor and fragment ions.



7. View the Coelution Plot (bottom middle).

a View the coelution between fragment ion peaks and precursor ion peak. If the coelution plot is not visible, click the coelution plot icon at the end of the toolbar.

b Verify that the ion ratio across the majority of the chromatographic peak is approximately 1.

- Typically, in the main portion of the peak, ion ratios do not deviate by more than a factor of 5 or 10 from 1. These ratios indicate good coelution.
- Another indicator of good coelution is green shading, as shown prominently in the figure above.
- The program generates the coelution plot using this procedure:
  - Each fragment ion chromatographic peak is normalized to the precursor ion chromatographic peak.
  - The program plots the ratios of normalized ion intensity against the ion intensity for the precursor ion.
- In the coelution plot:
  - The ion ratio for two identical chromatographic peaks is 1.
  - The noise is exaggerated at the front and back of the peak, so the ratio deviates from 1 in those locations.

(Optional) To select fragment ions without MS/MS spectra

This subsection discusses how to set up an All Ions MS/MS method for target compounds for which you have no MS/MS spectra in the Personal Compound Database and Library (PCDL). For demonstration, you may apply these instructions to the data file **AIM_3CE(0-20-40).d**.

1. Find compounds without the use of library spectra.

a In the Method Editor for **Find Compounds by Formula - Options**, click the **Fragment Confirmation** tab.

b Mark the **Confirm with fragment ions** check box.

c Under Fragment Ion Source, click **Use average fragment spectrum**.

This setting tells the algorithm to choose fragment ions from the high-energy channels across the time range where the precursor ion elutes. The algorithm does this instead of looking at MS/MS spectra in the PCDL.

d Set **Number of most abundant ions from average fragment spectrum** to **10**.

In a complex matrix, the chance is high that some ions in the measured spectrum do not belong to the target compound. This setting allows the program to look at additional fragment ions to see which ones have the correct coelution with the precursor ion.

e Click **Find Compounds by Formula**.

2. Examine the results.

If you processed the data file **AIM_3CE(0-20-40).d**, do the following:

a Click the **Compound Details View** button.

b In the Compound Chromatogram Results, view the coelution plot. If you look across the middle of the precursor ion peak, values for the ions that belong to the compound do not vary by more than a factor of 5 or 10 from 1.

c In the Compound Fragment Spectrum Results, expand the spectrum and observe that the fragment ions that the program selected are colored green. These are the ions the program picked from the measured high-energy spectra.

If you processed another data file, do similar steps to manually review the results.

(Optional) To observe how the program selects fragment ions in case of multiple structural isomers

This subsection describes how the All Ions MS/MS algorithm selects fragment ions in general and how the algorithm processes multiple structural isomers in specific. It uses results from the example data file **AIM_3CE(0-20-40).d**, as processed in the previous subsection.

In case of structural isomers, the algorithm evaluates the combined list of the five most intense ions for each compound.

1. Observe the number of ions the program evaluated for structural isomers.

- In the Compound List, look at the second group of structural isomers: terbutryn and prometryn. (Click + to expand each node.) The program evaluated ten ions for terbutryn and confirmed seven. For prometryn, it evaluated ten ions and qualified (confirmed) eight.

2. If possible, find fragment ions that are unique for each structural isomer.

- Observe that two fragment ions (about 68 and 200) exist in the PCDL only for prometryn (but not for terbutryn). Include these two ions in your method, to unambiguously distinguish these two compounds.

  A designation such as **<Terbutryn>** means the ion exists in the spectrum, but was not evaluated because it was not among the five most intense ions.

Delete unnecessary fragment ions

This section shows how to delete some fragment ions prior to export to a CEF file. For example, you may want to delete ions you do not want to use for confirmation in the Quantitative Analysis method.

To delete fragment ions:

a In the Compound Chromatogram Results window, click a legend to select an ion.

b Right-click the ion's colored bar in the legend and click **Disqualify Fragment**.
- If you accidentally remove the wrong fragment ion, click the undo arrow near the top left of the Qualitative Analysis window.

c If desired, confirm that the ion has been removed from the analysis:
- In the coelution plot, confirm that the trace for the ion is removed.
- In the Compound Identification Results window, in the Flags (FIs) column, confirm that the ion has the label **Manually disqualified**.
- In the Compound Fragment Spectrum Results window, confirm that the spectral line for the ion is no longer green.

d After you finish reviewing all the results, export the compounds to a Compound Exchange Format (CEF) format, as described next.

Export compounds to a CEF file

This section illustrates how to export results from Find by Formula with All Ions MS/MS confirmation for import into a Quantitative Analysis method. You export a CEF file so you can build a Quantitative Analysis method without the need to type any masses. This CEF file contains information about precursor ions and qualified fragment ions for all the target compounds. CEF is the acronym for Compound Exchange Format.

To export a CEF file:

a Click **File > Export > as CEF**.

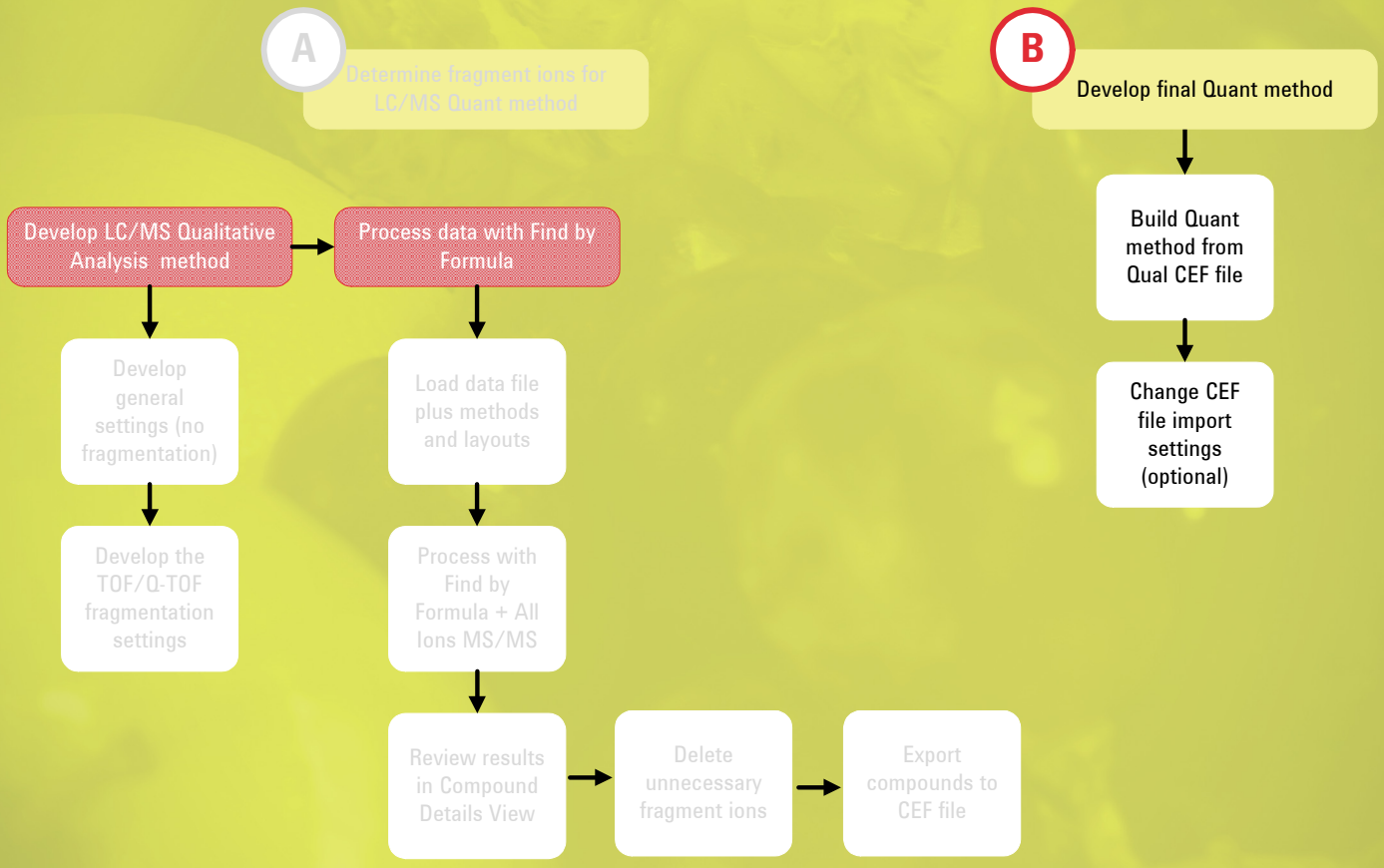
b Keep the default settings. (The CEF file is written in the same folder as the data file.)

c Click **OK**.

d Browse to the location of the CEF file and confirm its presence. (In the case of the example data, the file is **AIM_3CE(0-20-40).cef**.)

Developing the LC/MS Quantitative Analysis Method

This chapter shows you how to very quickly set up a MassHunter Quantitative Analysis method that takes advantage of the CEF file you exported in the previous chapter.



Build a Quant method from the Qual CEF file

This section illustrates how to rapidly build a MassHunter Quantitative Analysis method using a CEF file that you exported from MassHunter Qualitative Analysis after you did the All Ions MS/MS data processing. The CEF file contains the masses you need to create the method, so you do *not* need to do laborious transcription and typing.

Before you start, you may need to change the CEF file import settings. To learn more, see "Change the CEF file import settings (optional)" on page 34.

1. Create a batch and add samples.

   a In MassHunter *TOF* Quantitative Analysis, create a new batch. (Click **File > New Batch**.) Note that for this exercise, even though the example is a Q-TOF data file, you work in the TOF software rather than the Q-TOF software. This is because All Ions MS/MS is a full-scan technique.

   b Give the file a name, and then click **Open**. The file gets the extension **.batch.bin**.

   c Add samples to the batch. (Click **File > Add Samples**.) In the case of our example, just add the single data file **AIM_3CE(0-20-40).d**. For this illustration, the calibration curve will use this single data point, along with the origin.

2. Start to build a new Quant method from the CEF file.

   a Click **Method > New > New Method from File**.

   b From the drop-down menu in the lower right portion of the window, click **CEF files**.

   c Click the name of the CEF file you just exported from MassHunter Qualitative Analysis.

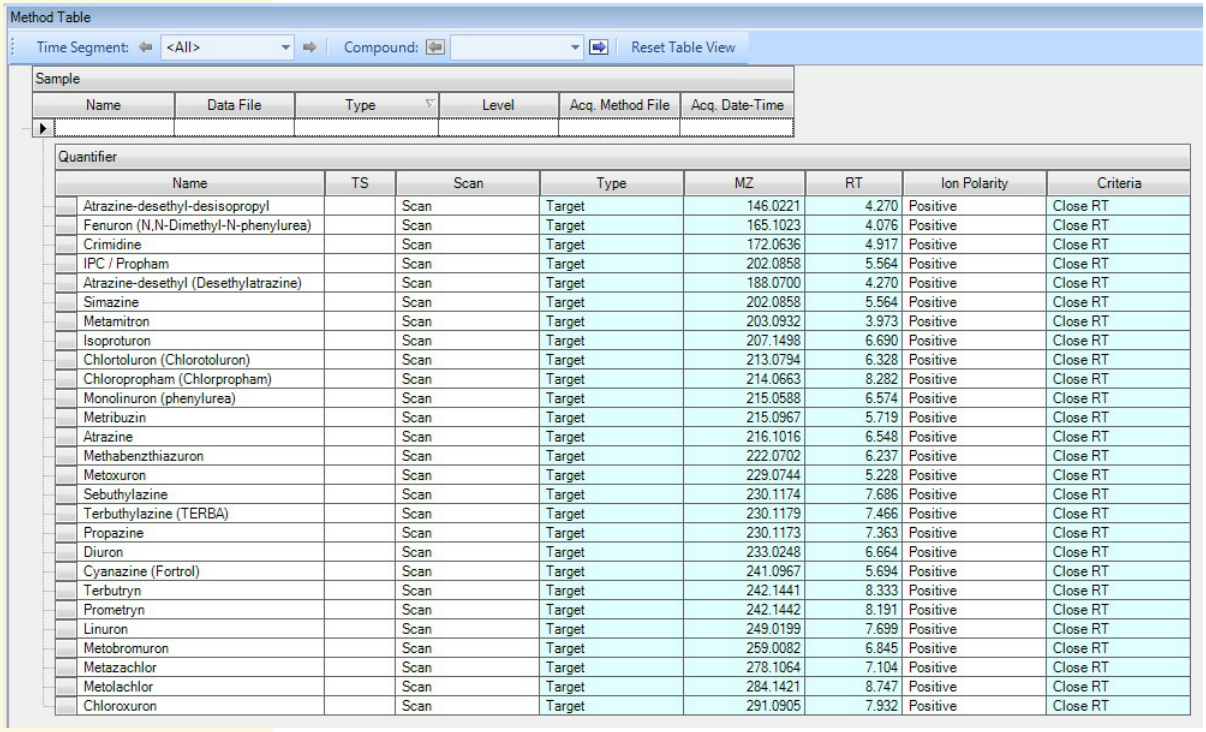
   d Click **Open**.

3. Set up the compounds.

   a In the Method Editor, under Method Setup Tasks, click **Compound Setup**.

   b In the Method Table, verify that all the compounds from MassHunter Qualitative Analysis were automatically imported into the Quant method.

   • If you set up compounds from **AIM_3CE(0-20-40).d**, see the figure below.



Name	TS	Scan	Type	MZ	RT	Ion Polarity	Criteria
Atrazine-desethyl-desisopropyl		Scan	Target	146.0221	4.270	Positive	Close RT
Fenuron (N,N-Dimethyl-N-phenylurea)		Scan	Target	165.1023	4.076	Positive	Close RT
Crimidine		Scan	Target	172.0636	4.917	Positive	Close RT
IPC / Propham		Scan	Target	202.0858	5.564	Positive	Close RT
Atrazine-desethyl (Desethylatrazine)		Scan	Target	188.0700	4.270	Positive	Close RT
Simazine		Scan	Target	202.0858	5.564	Positive	Close RT
Metamitron		Scan	Target	203.0932	3.973	Positive	Close RT
Isoproturon		Scan	Target	207.1498	6.690	Positive	Close RT
Chlortoluron (Chlorotoluron)		Scan	Target	213.0794	6.328	Positive	Close RT
Chloropropham (Chlorpropham)		Scan	Target	214.0663	8.282	Positive	Close RT
Monolinuron (phenylurea)		Scan	Target	215.0588	6.574	Positive	Close RT
Metribuzin		Scan	Target	215.0967	5.719	Positive	Close RT
Atrazine		Scan	Target	216.1016	6.548	Positive	Close RT
Methabenzthiazuron		Scan	Target	222.0702	6.237	Positive	Close RT
Metoxuron		Scan	Target	229.0744	5.228	Positive	Close RT
Sebuthylazine		Scan	Target	230.1174	7.686	Positive	Close RT
Terbuthylazine (TERBA)		Scan	Target	230.1179	7.466	Positive	Close RT
Propazine		Scan	Target	230.1173	7.363	Positive	Close RT
Diuron		Scan	Target	233.0248	6.664	Positive	Close RT
Cyanazine (Fortrol)		Scan	Target	241.0967	5.694	Positive	Close RT
Terbutryn		Scan	Target	242.1441	8.333	Positive	Close RT
Prometryn		Scan	Target	242.1442	8.191	Positive	Close RT
Linuron		Scan	Target	249.0199	7.699	Positive	Close RT
Metobromuron		Scan	Target	259.0082	6.845	Positive	Close RT
Metazachlor		Scan	Target	278.1064	7.104	Positive	Close RT
Metolachlor		Scan	Target	284.1421	8.747	Positive	Close RT
Chloroxuron		Scan	Target	291.0905	7.932	Positive	Close RT

4. Set up the qualifier ions.

a Under Method Setup Tasks, click **Qualifier Setup**.

b Observe that for each fragment ion that has qualifier ions, the qualifier ions are automatically imported.

- The default is that two fragment ions are chosen as qualifier ions.
- The qualifier ions may come from different high-energy channels (different collision energies or fragmentor voltages).
- It is also possible to use a single fragment ion (from a single collision energy or fragmentor voltage).

5. Set up concentrations.

a In the Method Setup Tasks, click **Concentration Setup.** To process the example data file, you will set up a one-point calibration.

b Right-click any of the compounds and click **New Calibration Level**.

c To process the example data file, set **Level** to **1** and **Conc.** to **100**.

d Right-click the compound name, click **Copy Calibration Levels To**, and copy these calibration levels to all other compounds. (Click **Select All**, then **OK**.)

6. Add the origin to the calibration curve.

a Under Method Setup Tasks, click **Calibration Curve Setup**.

b In the Method Table, click one of the compounds. Then, in the **CF Origin** column, click the down-arrow, and then click **Include**.

c Right-click **Include** and click **Fill Down**.

7. Validate the method.

a Under Save/Exit, click **Validate** to verify that the Quant method is complete.

b Confirm that you see a message that says, "Method validated. No errors or warnings found."

8. Exit the Method Editor.

a Under Save/Exit, click **Exit**.

b When you see "Would you like to apply this method to the batch?", click **Yes.**

9. Define that the sample is a calibration sample and assign the level for the method.

a In the Type column for the Batch Table, click the down-arrow, then click **Cal**.

b In the Level column, type the default of **1**.

10. Analyze the batch and examine the analysis results.

a Click **Analyze Batch**.

b To see one compound at a time, right-click the sample table and click **Single Compound/Sample View**. Then click the arrows to browse through the compounds to confirm both the quantifier ions and the qualifier ions.

c To see the fragment ions, click the button for **Show/Hide Qualifiers**.

d In the Qualifier Ratio column, observe that the qualifier ratios are not correct. (They are colored blue.) This is because if you build a Quart method from a CEF file, the selection of the data file before you enter the Method Editor is not considered.

11. If necessary, fix the problem with the qualifier ratios.

a Click to select the data file again, then go back to the Method Editor (**Method > Edit**).

b Click **Update > Update Qualifier Ratios**.

c Click **Select All**, then **OK**.

d If you are processing the example data file, when you see a message that says, "Qualifier ratios will not be updated for compounds (Propham)", click **Yes**. (The response for this compound was poor.)

12. Exit and analyze the batch again.

a Under Save/Exit, click **Exit.**

b When you see "Would you like to apply this method to the batch?", click **Yes**.

c Click **Analyze Batch**.

d Confirm that the accuracy is now correct. (For any compound, verify that **Final Conc.** is **100**.)

13. To make it easier to review results, set all ion traces to 100% of full scale.

a Right-click the Compound Information window.

b Click **Properties**.

c Click the **Compound Information (2)** tab.

d Mark the check box for **Normalize qualifiers**.

e Click **OK**.

f Confirm that all ions are now displayed full-scale.

Change the CEF file import settings (optional)

When you create a Quant method from a CEF file for an All Ions MS/MS experiment, you can choose to configure the import settings for the CEF file to include as qualifier ions:

- Up to two isotopes in the precursor ion cluster (because if matrix suppression occurs, the isotope ion ratios may be more stable than those of other fragment ions)
- Up to three fragment ions from a high-energy channel

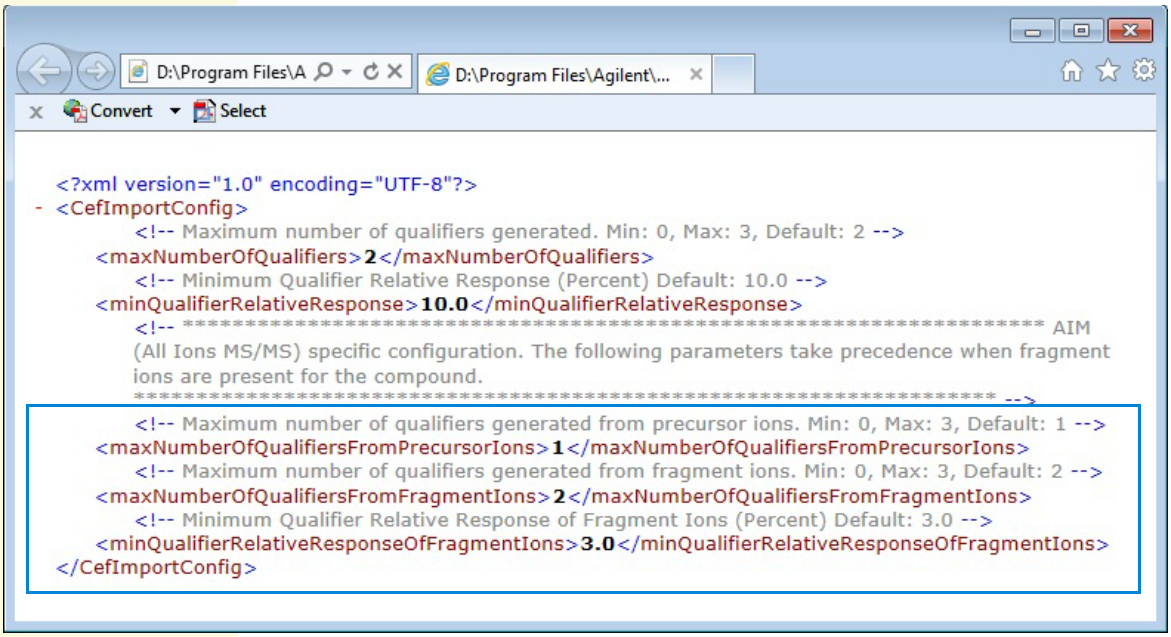
The file **CEFImportConfig.xml** controls these import settings. This section describes how to edit this file.

1. Open the configuration file for editing.

   a Navigate to **C:\Program Files\Agilent\MassHunter\Workstation\Quant\bin**.

   b Right-click the file **CefImportConfig.xml** and click **Edit**.

   c Observe the features of this file, as described below.



- The first lines of the file (above the gray note about "All Ions MS/MS specific configuration") apply when no fragment ions are available in the CEF file. Then the algorithm picks up to two qualifier ions from isotopes of the precursor ion.
- If there are fragment ions in the CEF file, then the last lines apply (below the note about "All Ions MS/MS specific configuration").

2. If desired, modify the All Ions MS/MS settings near the bottom of the file.

The lines below the note about "All Ions MS/MS specific configuration" control:

- Maximum number of qualifiers generated from isotopes of precursor ions
- Maximum number of qualifiers generated from fragment ions
- Minimum qualifier relative response of fragment ions (percent)

These lines are enclosed in the blue rectangle in the screen capture above.

3. If you make changes, save the file.

4. Go back to Quant and recreate the method from the CEF file.

   a Click **Method > New > New Method from File**.

   b From the drop-down menu in the lower right portion of the window, click **CEF files**.

   c Click the name of the CEF file you just exported from MassHunter Qualitative Analysis.

   d Click **Open**.

   - You can get more details about the method setup in "Build a Quant method from the Qual CEF file" on page 30.

5. Verify that the method is properly set up.

   a In the Method Setup Tasks, click **Qualifier Setup**.

   b For one of the compounds, verify that the qualifier ions are correct.

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