DExSI: Getting Started

Before you begin:

DExSI requires four key sets of data in order to automate the processing of stable isotope-labelled GC-MS data. These are as follows:

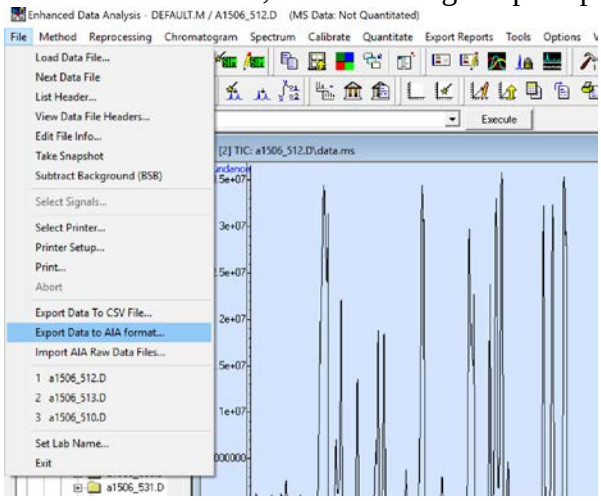
1) GC-MS data files converted to CDF format.
2) GC-MS data from a standards mixture, containing metabolites of interest at known quantities.
3) A customised library file, defining each metabolite of interest, and key parameters including retention time, isotopologue series (unlabelled to maximally labelled masses), chemical formula of the parental ion.
4) Define your sample replicates in groups by creating a 'groups' file.

These files are outlined in more detail below.

Further documentation is provided in the DExSI Help files, accessible from the Help menu.

Step 1: Generate CDF data files

For Agilent users, CDF files can be created in MSD ChemStation by selecting File->Export Data to AIA Format, and following the prompts.



A .CDF file will be created for each selected Agilent .D data file.

Step 2: Metabolite standards mixture

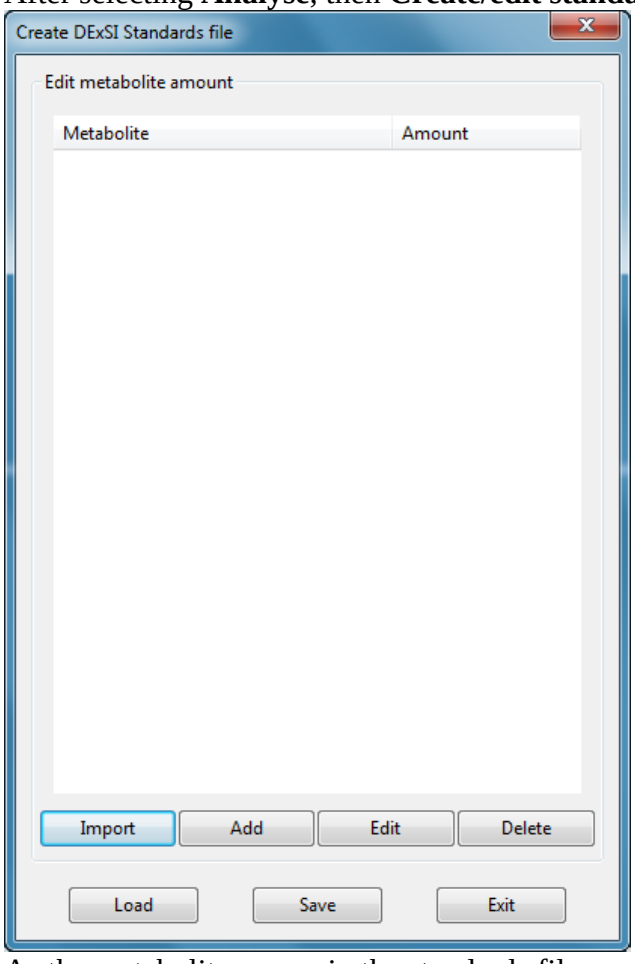
As part of your experiment, please include a single sample which contains known amounts of each metabolite of interest. This standards mix serves two important purposes: firstly, it allows you to identify the retention time and key quantifiable ions for each metabolite of interest, and secondly, this sample will be used in combination with the known amounts of each metabolite present to calculate a response faction when determining the absolute amount of metabolites present. Each standards sample included in the 'Standards' group must have identical

composition, containing the same amount of the same mixture of metabolites, as the response factor for each metabolite will be calculated from the average of **all** samples in the 'Standards' group. While this standards mixture is recommended and helpful for building a metabolite library, it is only and absolute requirement for the calculation of absolute metabolite abundance; it is not required for the calculation of isotopologue distribution or fractional labelling.
Note: This sample should be subjected to the same derivatization procedure as experimental samples.

In order to perform absolute quantitation, a standards file must be created in DExSI. This file contains the names (which must match the library entries) of each metabolite in the standards mixture, and the amount present (note that the units of quantity are entered during analysis, not in the standards file); only metabolites defined in the standards file and present in the authentic standards can be quantified in absolute terms.

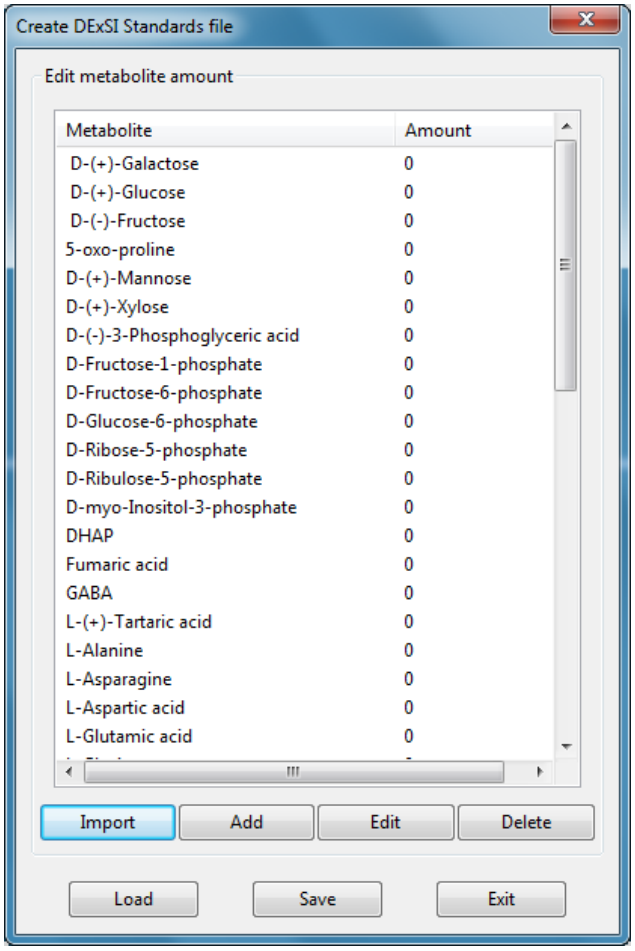
Creating a standards file in DExSI:

After selecting **Analyse**, then **Create/edit standards file...** you will see the following screen:



As the metabolite names in the standards file must match with names in the metabolite library in order for absolute abundance to be calculated, it is recommended that metabolite names be imported from you metabolite library. Alternately, metabolites can be added individually.

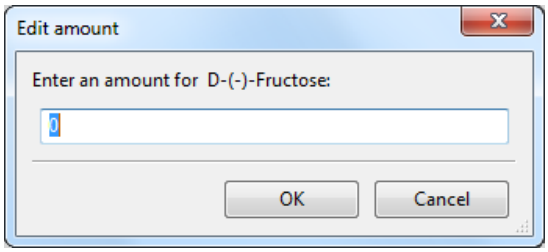
Importing metabolite names
1. Click on the **Import** button, and select the DExSI metabolite library containing your metabolites of interest.



Following the import, all metabolites will be listed and assigned a concentration of zero. Note that metabolites with a concentration of zero will not be saved, so it is not necessary that all irrelevant entries are deleted. The list can now be modified by adding, editing or deleting entries.

Edit metabolite amount

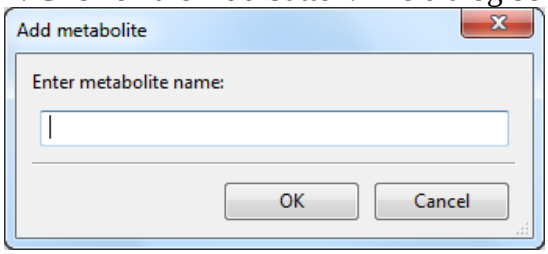
1. Select the metabolite for which you wish to edit or define an amount.
2. Next, click on the **Edit** button. A dialog similar to the one below will appear, requesting a amount for the selected metabolite.



3. Enter the amount of the metabolite (numbers only). Keep all entries in the same units (e.g. nmol, pmol, etc...) - you will need to specify the units when calculating absolute abundance.
4. Select **OK**. The amount will now appear listed with the metabolite.

Add a metabolite

1. Click on the **Add** button. The dialog below will appear.



2. Enter the metabolite name. Make sure that the name exactly matches the metabolite entry in the metabolite library. Click **OK**.
3. Next, enter the amount of the metabolite in your standard mix into the edit standards dialog, as below. The entry should be number only. Keep all entries in the same units (e.g. nmol, pmol, etc...) - you will need to specify the units when calculating absolute abundance.
4. Select **OK**. The amount will now appear listed with the metabolite.

Saving your standards file

Click on the **Save** button to save your standards file. Only metabolite entries with assigned standards greater than zero will be saved to the file.
Be sure to save your standards file before exiting. You will need to specify your standards file in order to calculate the absolute abundance of metabolites (the edited standards file will not be applied automatically).

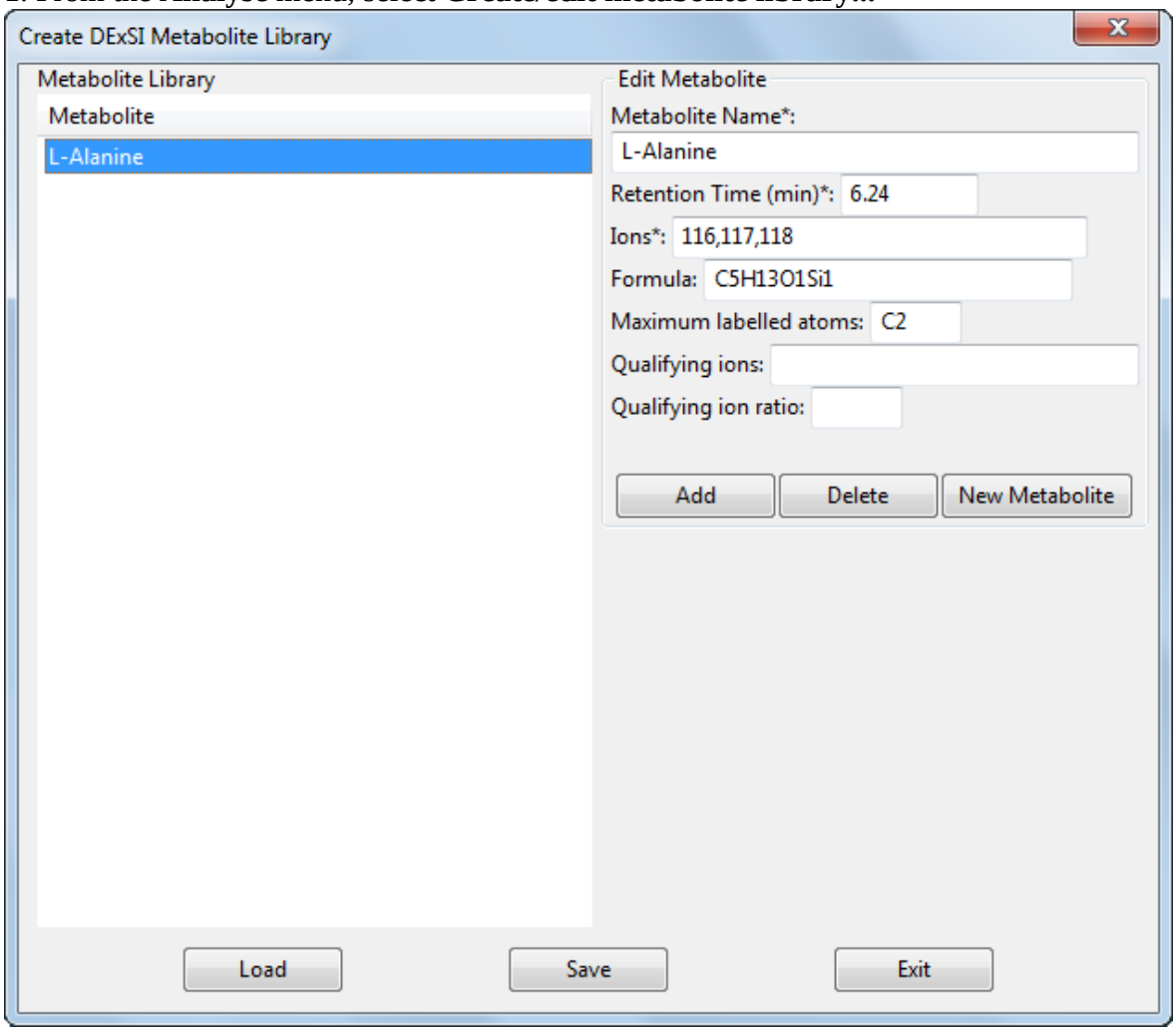
Step 3: Create a metabolite library

Each metabolite which is to be detected must be defined within the DExSI metabolite library. If retention time locking is used (this is highly recommended), metabolite libraries can generally be used unchanged for experiments which utilised the same derivatization and analysis conditions (including the same isotope label).
The minimal definition of a metabolite consists of a name (which must be unique), a retention time, and a series of ions (a sequential list from the parental ion through to the mass of the fully-labelled species). The inclusion of the formula for the parental ion, and maximal number of labelled atoms is required for correction for natural isotope abundance, which should be applied to isotopologue distributions and fractional labelling data.
Note: Selection of an ion series for quantitation should take account of: i) the abundance of the ions selected, ii) the uniqueness of these ions (i.e. they are absent from co-eluting or neighbouring species), and iii) the ion fragment will include and informative number of atoms potentially derived from the label of interest.
Metabolite libraries can be edited in DExSI once created.

A sample metabolite library is included within the 'Sample File' folder in the DExSI directory (i.e. C:\Program Files\DExSI). This can be used with the sample data files also provided, and was created for ^{13}C-glucose labelled samples derivatised with methoxyamine and 1% TMCS+BSTFA as per Saunders *et al. Methods Mol Biol.* (2015) 1201:281-96.

Creating a library in DExSI:

1. From the **Analyse** menu, select **Create/edit metabolite library...**



2. To edit an existing DExSI metabolite library, select **Load**. Otherwise, use the right panel to add entries to the blank metabolite table.
3. To enter a new metabolite, enter the text in the right panel and click **Add**. Metabolite name, retention time and ions for quantifying must be entered for each metabolite.
Fields are as follows:

Metabolite name
Description of your metabolite. Be sure to use standard nomenclature if you plan to map data onto pathways using VANTED - if your metabolite names do not match those in the pathways used by VANTED then they will not be mapped. Metabolite names must be unique - the same description can not be used twice.

Retention time
Retention time in minutes for metabolite peak.
Example: 6.23

Ions
List of ions (separated by commas) to be quantified for the metabolite.
Example: 116,117,118

Formula
Chemical formula of ion. Required for natural isotopic abundance correction. Must be specified with Maximum labelled atoms. Formula to be entered as a continuous string (no separating spaces or commas). Atoms do not need to be listed in a specific order.
Accepted atoms are: H, C, N, O, S, P and Si.
Example: C5H13O1Si1

Maximum labelled atoms
Enter the atom labelled and the maximum number of labelled atoms present in the ion of interest. Both this field and the ion formula field must be completed to perform natural isotopic abundance correction. Note that the number of ions listed (above) must greater than the number of maximum labelled atoms.
This parameter will need to be changed depending upon which stabile isotope is used for labelling - e.g. a different library will be required for ^{13}C labelling than for ^{2}H labelling.
Example: C2

Qualifying ions
A series of ions which must be present in the same peak as the quantifying ions in order to be identified as the best matched peak. This optional parameter can be used to improve peak identification in the event that multiple metabolites elute closely and produce the same ion fragments. A qualifying ion ratio must be provided if qualifying ions are listed.

Qualifying ion ratio
Approximate fraction of the integrated area of the qualifying ion series to the integrated area of the quantifying ion series.
Example: 1.5

Once a new metabolite entry has been completed, click on **Add**.

Saving your metabolite library
Once your library is complete, click on the **Save** button.
Be sure to save your library before exiting. You will need to specify your library before extracting GC-MS files (the edited library will not be applied automatically).

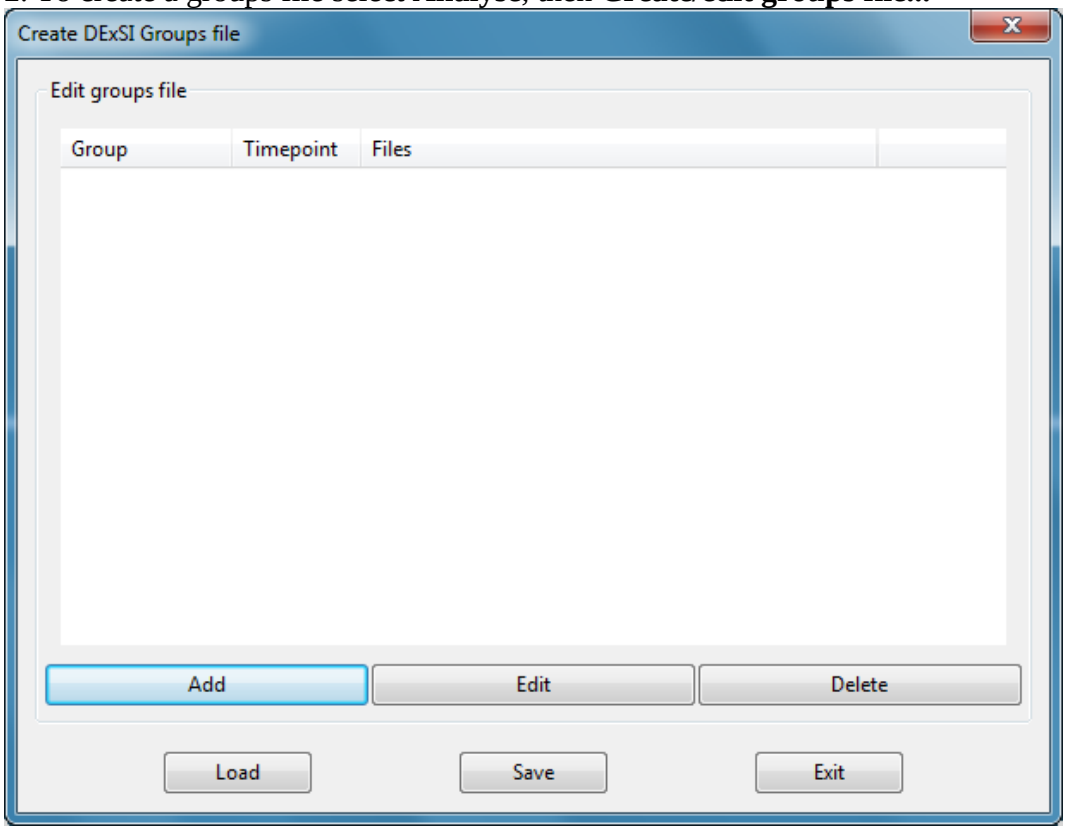
Step 4: Create a Groups file

The groups file defines the grouping of replicates in your experiment. Groups files are defined in a hierarchy with two levels: groups and timepoints. This allows you to have multiple groups (e.g. cell lines, strains, drug treatments) and compare them across multiple timepoints within the same experiment. Each CDF file must be assigned to a group and can only be included in a single group.
Two special groups, Standards and Blanks, can also be defined. The Standards group should contain file/s from runs of the authentic standards mixture. This group will be used calculate absolute abundance and data from these samples will be excluded from graphs and heatmaps. As noted above, each standards file must contain the same mixture of metabolites for quantitation as the response factor for each metabolite will be calculated from the average of **all** samples in the 'Standards' group. When three samples of standards mix are provided at different concentrations, DExSI can be calculate response factors based on the least-squares linear regression of these data points. This approach is recommended as it allows the linearity

of response for each metabolite to be confirmed over a given concentration range. Data files for blanks can be included in Blanks group. Data from files in the Standards group or Blanks group will not be included in graphs, heatmaps or in datasets exported for VANTED.

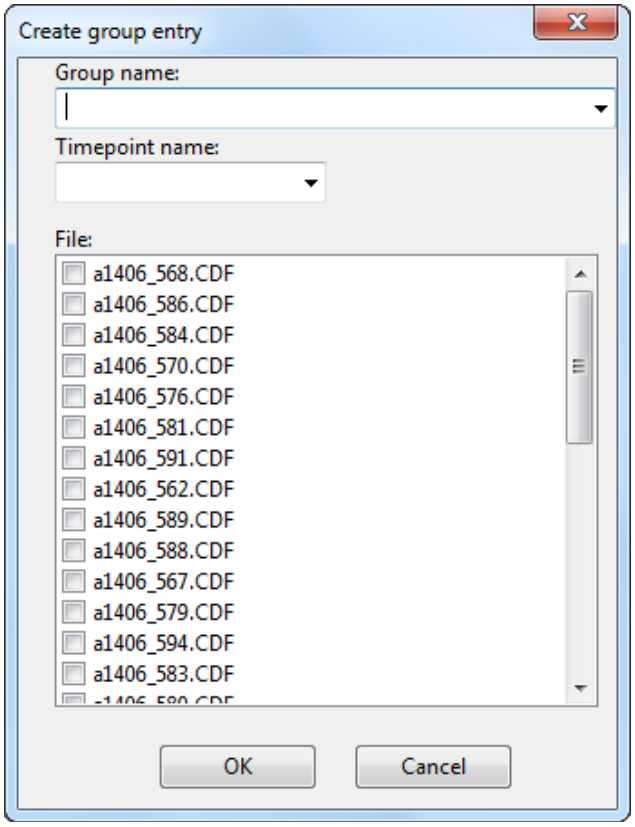
Creating a Groups file

1. To create a new groups file, first select files to include using the **Select files** option from the **Analyse** menu or load the extracted dataset of interest. *Groups can only be created using loaded CDF files.*
2. To create a groups file select **Analyse**, then **Create/edit groups file...**



Add a Groups file entry

1. Click on the **Add** button. A dialog box will appear with a list of all loaded files which have not been allocated to groups in the current groups file.



Group name

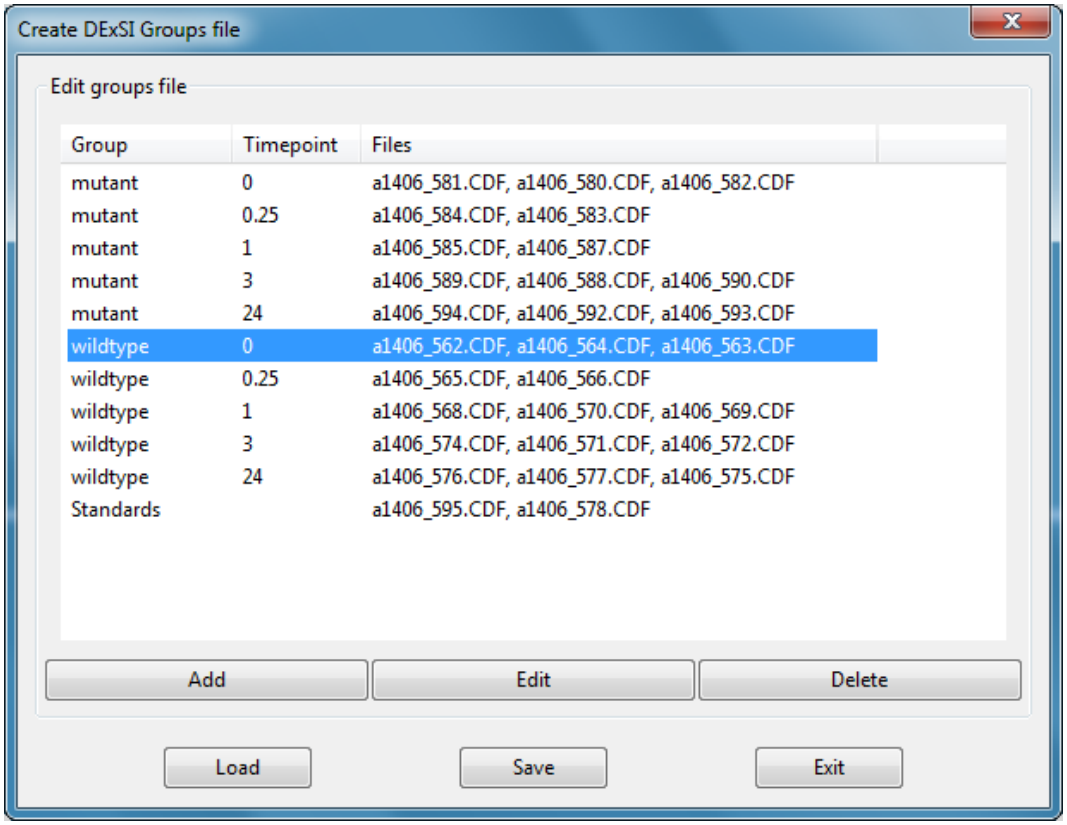
Enter a new group name, or select an existing group name from the drop-down list. Once a group name is added to the groups file, it can be selected from the drop-down list for subsequent entries. The special groups Standards and Blanks can only be defined once, and are described in detail above.

Time point name

Time points can be added to each group entry, with the exception of Blanks and Standards. Time points must be entered as numbers only (integers or decimals). As with the group names, once a time point is defined for one group entry, it can be selected from the drop-down list for future entries in the same file.

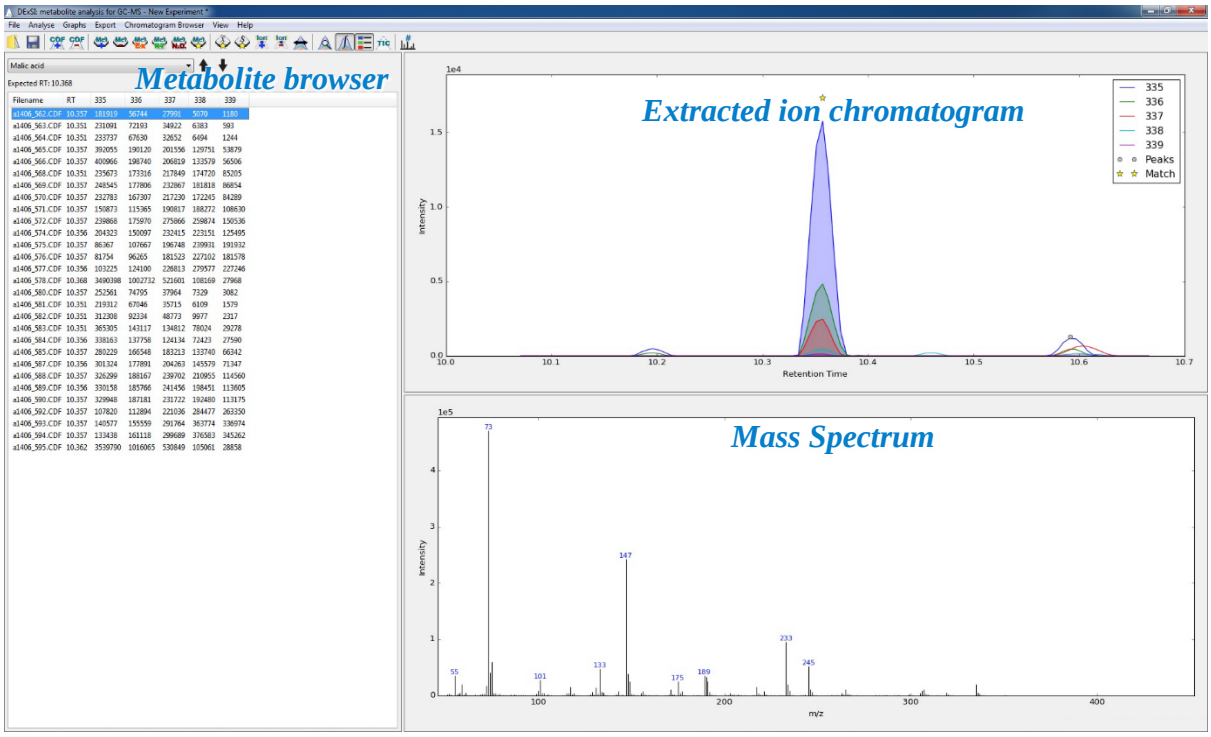
Files

Select files for each group by clicking on the checkboxes. Each file can only be allocated to a single group, and will be made unavailable for future group entries. If a file is wrongly allocated to a group it can be deselected by editing the groups entry.



Once your groups file is created, **be sure to save your groups file before exiting.** You can edit the groups file later if required. You will need to specify your groups file for most graphing and exporting functions (the edited groups file will not be applied automatically).

Step 5: Using *DExSI*:



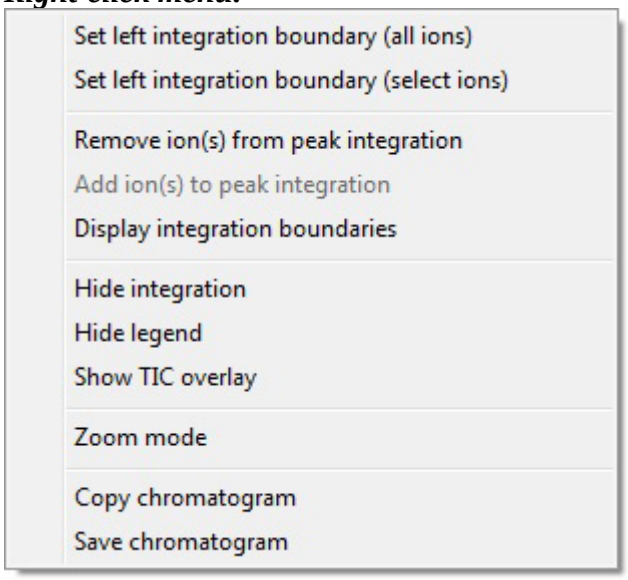
The DExSI main screen is split into three panels: extracted ion chromatogram *(top right),* mass spectrum *(bottom right), and the* metabolite browser *(left).*

Extracted ion chromatogram

The upper-right window shows the extracted ion chromatogram for the quantifying ions of the selected metabolite. s

- The best matching peak (based on metabolite parameters specified in the library file) is marked with a yellow star. Other peaks are marked with a grey circle. To re-assign the best matching peak, double-click on another marked peak. The y-axis will scale to the height of the best-matched peak.
- If the metabolite peak of interest not identified with a grey circle, then the extraction parameters may need to be relaxed: in the **Peak filtering** options in the Extract window, reduce the minimum number of ions per peak or the minimum intensity cut-off. All files, or a single file, can be re-extracted for a single metabolite using the icon on the toolbar.
  - If some ions within the quantifying ion series are identified as a separate peak, the peak extraction parameters may need to be refined: in the **Peak detection** options in the Extract window, increase the number of scans. This can be performed on a single file by selecting '**Re-extract current file and metabolite**' from the menu which appears when your right-click on a file name in the extracted data list, or by clicking on the icon on the toolbar and selecting 'Current file'. Alternately, all files can be re-extracted for a single metabolite using new parameters by clicking on the icon from the toolbar and selecting ‘All files’.

Right-click menu:



A pop-up menu will appear when right-clicking on the ion chromatogram:

Integration modification:

If the integration boundaries are displayed, it is possible to modify the left/right integration boundaries. The new integration boundary will be set to the location of the cursor when the right mouse button was pressed: if it is left of the best peak apex, the left boundary will be modified, if it is right of the best peak apex, the right boundary will be modified.

- The new left/right boundary for the best peak can be set for all ions by clicking on '**Set left/right integration boundary (all ions)**'.
- ***Shortcut:*** *Hold '**Shift**' and **right-click** on the ion chromatogram.*
- To set the boundary for selected ions, click on '**Set left/right integration boundary (select ions)**'. A dialog will appear allowing the selection of ions for integration boundary modification.
- ***Shortcut:*** *Hold '**Control**' and **right-click** on the ion chromatogram.*
- Ions can be removed from the best-matched peak (i.e. in the event that a contaminating ion peak or background signal is integrated). Select '**Remove ion(s) from peak integration**' and then from the dialog select the ions to remove. The integrated area for these ions will be set to zero.
- Alternately, if an ion peak needs to be added to the best-matched peak (i.e. was excluded during automatic detection due to the peak shape or signal below threshold), select '**Add ion(s) to peak integration**' and then from the dialog select the ions add. The integration boundaries will be set to the average of the other ion peak boundaries in the best-matched peak. This option will only be active when one or more ions are not detected for a given metabolite. *This feature should only be used on rare occasions - generally, if an ion peak is excluded due to poor shape or low signal then it is often similar to background should not be analysed further.*

Note: ***Manual integration options should be used sparingly and with care.***

In most cases, the automatic integration is accurate and consistent - minor adjustments to integration boundaries are unlikely to significantly impact data analysis. Avoid manually integrating weak signals and background noise - the minimum intensity cut-off setting in the extraction parameters is used to avoid this problem for automatic peak detection. Adding 'ion

peaks' which are weak, very broad, or are background noise, may adversely impact on the calculation of fractional labelling.

- '**Display integration boundaries**' will display a table of left and right integration boundaries for each ion in the best matched peak. This data can be copied to the clipboard.
- '**Show integration**' / '**Hide integration**' toggles the display of integration boundaries for the best matched peak. Integration boundaries must be visible to enable modification.
- '**Show legend**' / '**Hide legend**' toggles the display of legend.
- '**Copy chromatogram**' copies the chromatogram to the clipboard.
- '**Save chromatogram**' saves the chromatogram to a 'PNG' file.

Mass spectrum

The lower-right window shows the mass spectrum at the peak apex of the best-matched peak displayed in the extracted ion chromatogram panel.
Ions with the highest intensities are labelled.
- To **zoom-in** on the mass spectrum, left-click and drag a box around the area of interest.
- To **zoom-out** of the mass spectrum, double-click to restore the original view.

Right-click menu:
A pop-up menu will appear when right-clicking on the mass spectrum panel:

- The number of ions labelled in the mass spectrum can be toggled by selecting '**More ion labels**' or '**Fewer ion labels**'.
- '**Copy mass spectrum**' copies the mass spectrum to the clipboard.
- '**Save mass spectrum**' saves the mass spectrum to a 'PNG' file.

Metabolite browser

The metabolite browser is located in the left panel. Use this panel to select the metabolite and file to be displayed in the right panels. The toolbar provides functions for working with the data.

Toolbar:

 **Open DExSI File**

 **Save DExSI File**

 **Add CDF File:** Adds CDF file to the current data set. The new file will be extracted and searched for all metabolites currently defined in the DExSI file.

 **Remove CDF:** Removes CDF file from the current data set. All metabolite data from the file will be removed.

 **Add Metabolite:** Adds a metabolite to the current data set. All data files will be searched for the newly-added metabolite.

 **Remove Metabolite:** Removes a metabolite from the current data set.

 **Re-extract current metabolite:** All files in the current data set will be searched for the current metabolite using new extraction parameters. Use this feature when the retention time window, peak detection or peak filtering parameters need to be adjusted.

 **Set best peak to retention time:** The best peak assignment for the current metabolite will be set to the peak nearest to the specified retention time for all files in the data set.

 **Metabolite not detected:** Sets the best peak retention time and the integrated area for the peak to zero for each ion in the current metabolite for the selected file. For use when there are no peaks which correspond to the metabolite of interest. This can be over-ridden by double-clicking on a peak on the extracted ion chromatogram.

 **Revert to best peak:** Resets the best peak retention time to the best-matched peak as determined by the DExSI algorithm. For use to revert to the software designated peak and peak boundaries following manual modification.

 **Set best peak to median RT:** The best peak assignment for the current metabolite will be set to the peak nearest to the median retention time of the designated best peak in all samples. For use to correct mis-assigned peaks within a dataset.

 **Set best peak to median RT of standards:** The best peak assignment for the current metabolite will be set to the peak nearest to the median retention time of the designated best peak in all samples assigned to the 'Standards' group. For use to correct mis-assigned peaks within a dataset.

 **Add ion to peak:** Add a missing ion to the best peak integration. The integration boundaries will be set to the average of the other ion peak boundaries in the best-matched peak. This option will only be active when one or more ions are not detected for a given metabolite.

 **Remove ions from peak:** Remove an ion from the best peak integration.

 **View integration boundaries:** Displays a table of the integration boundaries of the best peak.

 **Zoom chromatogram:** Toggles the zoom tool in the main extracted ion chromatogram. Left-click and drag to zoom, double-click to revert to default zoom level. Best peak selection is disabled in zoom mode.

 **Show/Hide integration boundaries:** Toggles the display of integration boundaries for the best matched peak. Integration boundaries can be modified manually, but must be visible before the right-click menu functions for integration modification will be active.

 **Show/Hide Legend:** Toggles the display of the legend on the ion chromatogram. This function can also be accessed by right-clicking on the ion chromatogram and selecting '**Show legend**' or '**Hide legend**'.

 **Show/Hide TIC:** Toggles the display of the total ion chromatogram (TIC) overlayed on the extracted ion chromatogram. This function can also be accessed by right-clicking on the ion chromatogram and selecting '**Show TIC overlay**' or '**Hide TIC overlay**'.

 **Show/Hide more ion labels:** Toggles the display of additional ion labels on the mass spectrum. This function can also be accessed by right-clicking on the mass spectrum and selecting '**More ion labels**' or '**Fewer ion labels**'.

Metabolite List:

The **drop-down metabolite list** at the top of the metabolite browser allows you to select the metabolite data to be displayed in the extracted data list below. All metabolites present in the library used for extraction will be listed.

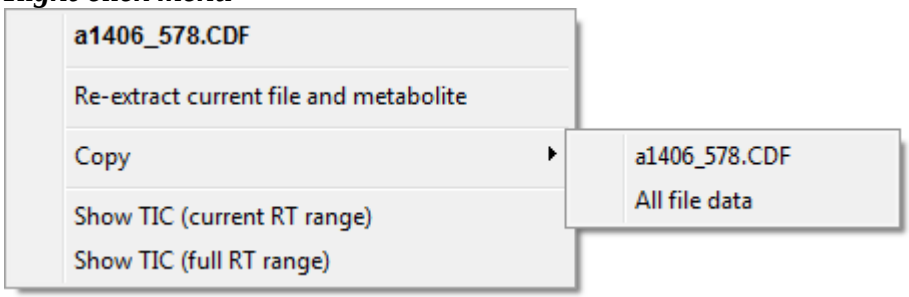
Below the drop-down metabolite list is the metabolite **expected retention time**. The value displayed is taken from the metabolite library used for data extraction.

Extracted data list

The **extracted data list** displays the names of each file, and for the selected metabolite will show the retention time of the best-matched peak and the integrated areas for each ion within the peak. If no peaks are detected, the retention time and ions will be zero, and no graphs will be displayed in the right panels.

- Click on a file within the **extracted data list** to display the extracted ion chromatogram and mass spectrum for the best-matched peak. The **extracted data list** will update if the best-matched peak is reassigned.
- The raw integrated areas for each metabolite can be exported to a CSV spreadsheet using the export menu.

Right-click menu



A pop-up menu will appear when right-clicking on a file name:

- If a groups file has been loaded, the **group name** associated with the file will appear as the first entry in the menu. If a groups file is not specified then the file name will appear.
- The current file can be re-extracted for the current metabolite using new parameters by selecting '**Re-extract current file and metabolite**'.
- The extracted data table for the current metabolite (filename, retention time and raw integrated areas) can be copied to the clipboard for either the current file (by selecting the file name), or for all files by selecting '**All file data**'.
- The total ion chromatogram (TIC) for the selected file can be displayed in a new window by selecting either '**Show TIC (current RT range)...**' or '**Show TIC (full RT range)...**'.