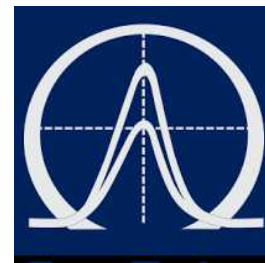


Dec 12, 2023

Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap



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Margaux Billen^{1,2}, Scott Denham², Joanna P Simpson², Natalie Homer²

¹KU Leuven; ²University of Edinburgh

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Margaux Billen

University of Edinburgh

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We use this protocol and it's working

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Abstract

In our lab we focus on targeted analysis and have found that acquiring LC-HRMS data using Xcalibur software and assessing the data using TraceFinder software is the most robust and flexible approach.

This protocol describes our approach to evaluating LC-HRMS data that has been collected in Xcalibur software, on LC-HRMS instruments including ThermoScientific Exploris mass spectrometers. It describes how to build a processing method in TraceFinder and using it to evaluate a full scan analysis data set acquired in Xcalibur software.

The LC-HRMS data set must contain a calibration curve and unknowns analysed as a batch using the same Acquisition method in Xcalibur. The results from TraceFinder are then transferred to Microsoft Excel to summarise the calculated amounts of the analytes of interest in the samples as a final result.

Guidelines

Use methods that have well defined calibration protocols, method details (masses, analyte retention times) and well defined calibration ranges and known units for the calibration ranges. TraceFinder is only successful if the calibration range is well defined, the calibration units are known and the volume of extracted sample is recorded. QCs used across multiple batches are best included in each batch, and give confidence to the results.



Materials

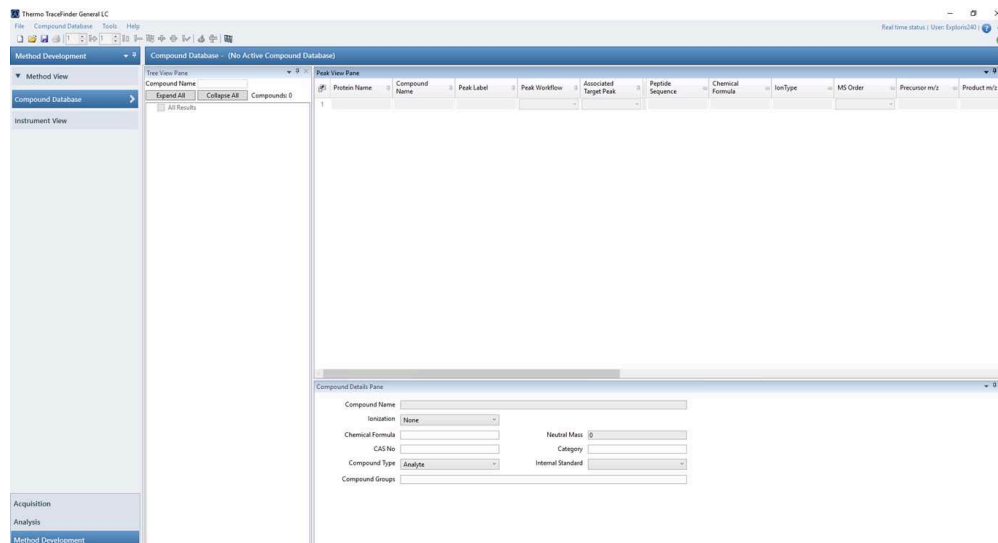
1. Xcalibur Data Acquisition and Interpretation Software 4.5 SP1 (ThermoScientific)
2. Tracefinder 5.1 software license (ThermoScientific)
3. Excel software package (Microsoft)
4. Details of the method used to acquire data in Xcalibur - including retention times, names of analytes, calibration ranges and calibration units and volume (or mass) of sample extracted.

Before start

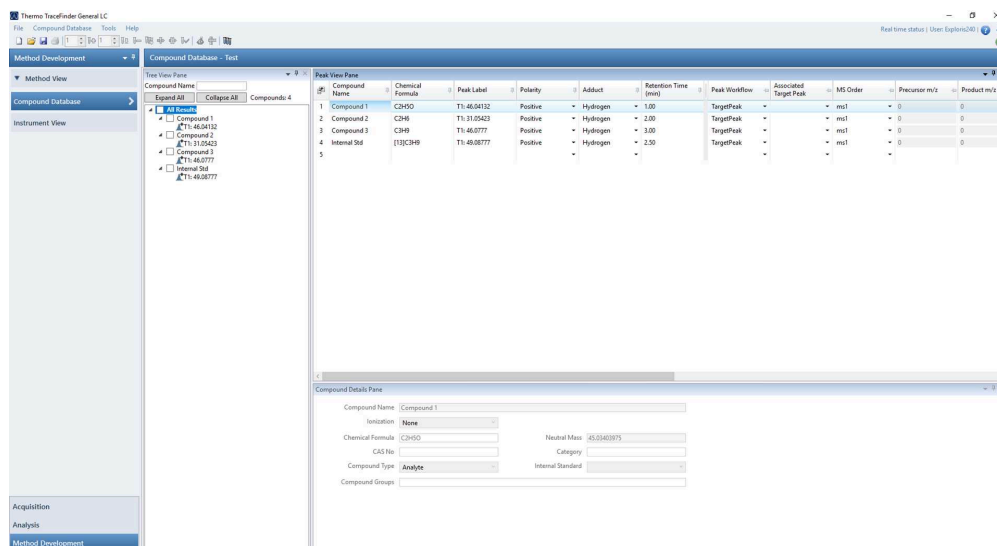
Acquire a dataset in Xcalibur and use the raw file in TraceFinder.

Creating a compound database in TraceFinder

- 1 In TraceFinder, navigate to the 'Method Development' tab in the bottom left corner of the screen.
- 2 Select 'Compound Database' on the left-hand side. The screen that pops up looks like this:

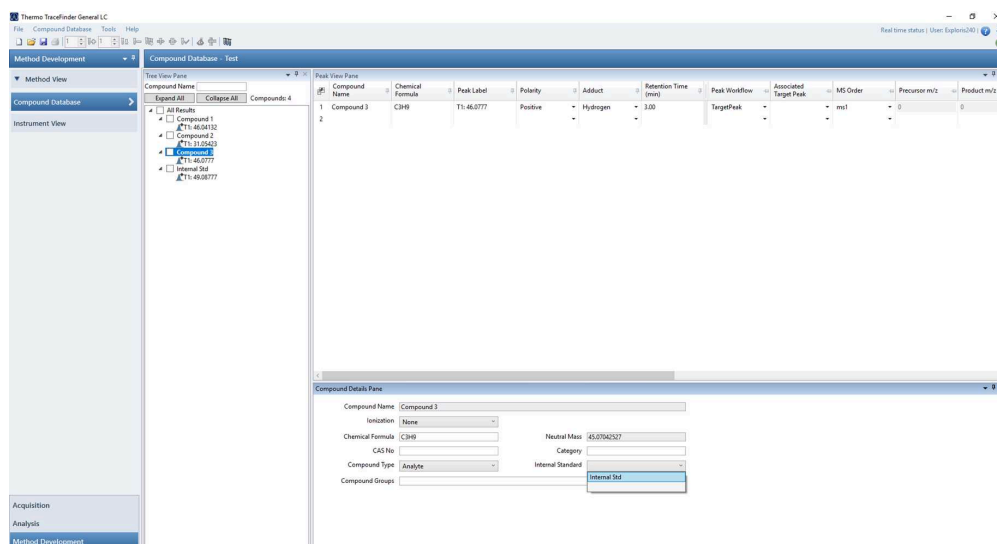


- 3 Go to File > New Small Molecule Compound Database
A new screen pops up where you can give a name to your new compound database.
- 4 Build your compound database as seen in this example:



- Enter each individual compound that should be analysed + all internal standards in the method and enter the chemical formula, the polarity, the adduct and the expected retention time for each of the compounds.

Select each individual compound in the Tree View Pane on the left hand side and fill in the 'Compound Details Pane' on the bottom:



- For the internal standard(s), select 'Internal Standard' for compound type in the lower tab and fill in the ISTD concentration as 1.
- For the analytes, select 'Analyte' for compound type in the lower tab.

5 Save the compound database.
Go to File > Save Compound Database.

Creating a processing method in TraceFinder

- 6 Go to the 'Method View' tab on the left hand side.
Select File > New > Master method...

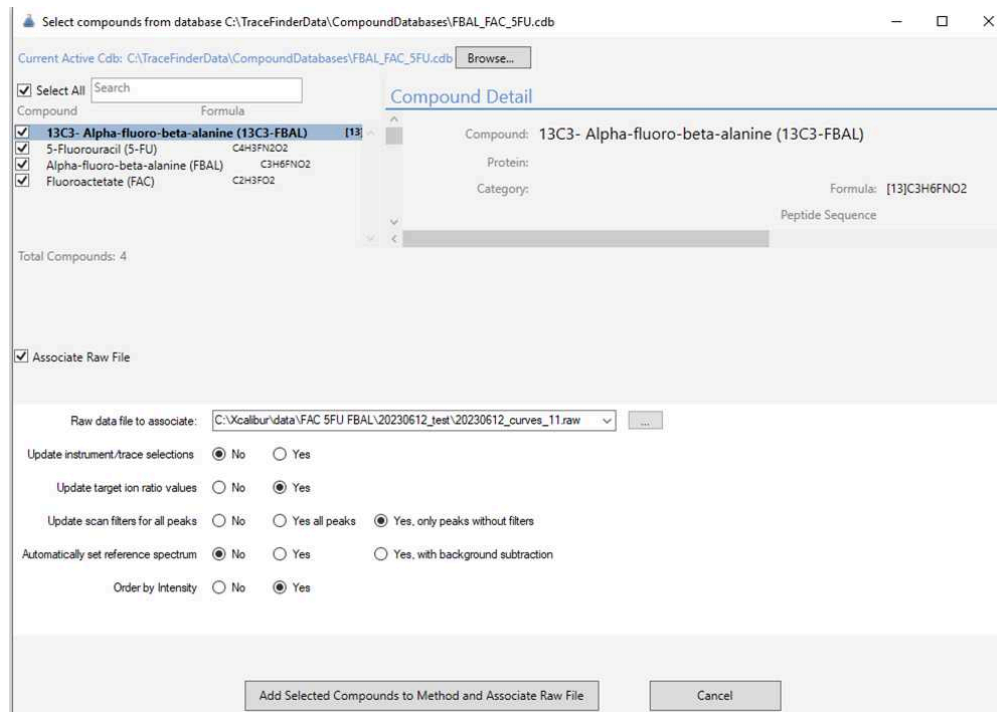
- 7 The following screen will pop up:

Select Workflow Template	
☐ Quan using Method Forge	Method Forge Performs peak detection against a raw data file. Performs library lookup if requested.
☐ Quan by importing an Xcalibur Processing Method	Import Xcalibur Processing Method Imports a previously created processing method, finding configured compounds and reference spectra.
☐ Quan - blank method	Create blank method Associate a raw data file and manually select peaks.
☒ Quan by Selecting compounds from CDB	Select Compounds from a compound database Creates a blank master method and displays the configured compound database, allowing compound selection.
☐ Target Screening method	Create a target screening method.
☐ Unknown Screening method - Only	Create an unknown screening method. This method will not have quan or target screening elements.

OK Cancel

Select the option 'Quan by Selecting compounds from CDB' and click on 'OK'.

- 8 The following screen will pop up:



- Using the browse function, select your prepared compound library.
- Tick the 'Select All' and 'Associate Raw Datafile' boxes.
- Select a datafile from a mid-high standard on the calibration curve.
- Use the settings as displayed in the screenshot above.
- Click on 'Add Selected Compounds to Method and Associate Raw File'

9 Save your Master Method by selecting File > Save as...

10 Navigate to the 'Compounds' tab on the left hand side. The screen should look like this:

The screenshot shows the Thermo TraceFinder software interface. The 'Acquisition List' tab is active, displaying a table of compounds. The table has columns for Compound Name, Peak Label, Peak Workflow, Associated Target Peak, Chemical Formula, MS Order, Precursor m/z, Product m/z, Adduct, and Polarity. A 'Compound Details Panel' is visible at the bottom right, showing information for '13C3-Alpha-fluoro-beta-alanine (13C3-FBAL)'.

You are currently in the 'Acquisition List' tab on top. Here:

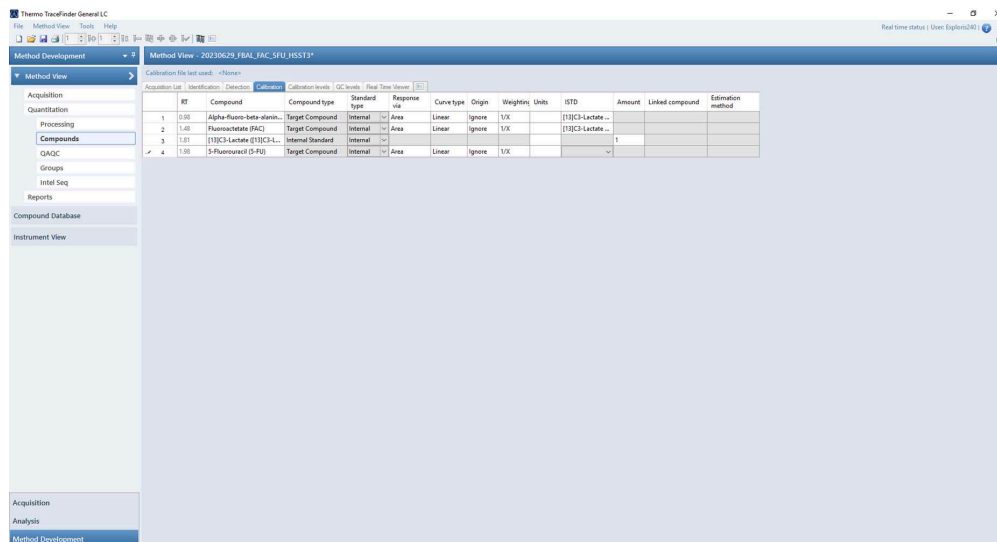
- Select the each individual compound and check that the chemical formula, the polarity, the adduct and the expected retention time are all correct.
- For the analytes, select which internal standard should be used for each individual analyte.

11 Navigate to the 'Detection' tab on top. The screen should look like this:

The screenshot shows the Thermo TraceFinder software interface with the 'Detection' tab selected. The 'Peak Settings' panel is visible, showing various parameters for peak detection. The 'Detection algorithm' is set to 'ICIS'. The 'Peak detection strategy' is set to 'Nearest RT'. The 'Peak threshold type' is set to 'Area'. The 'Area noise factor' is set to 1.0. The 'Peak noise factor' is set to 10. The 'Baseline window' is set to 40. The 'Peak height (m/z)' is set to 1.0. The 'Tailing factor' is set to 1.0. The 'Min peak height (m/z)' is set to 1.0. The 'Noise method' is set to 'Repetitive'. The 'Min peak width' is set to 3. The 'Multiple resolution' is set to 10. The 'Area tail extension' is set to 3. The 'Area scan window' is set to 0. The 'RMS' checkbox is unchecked.

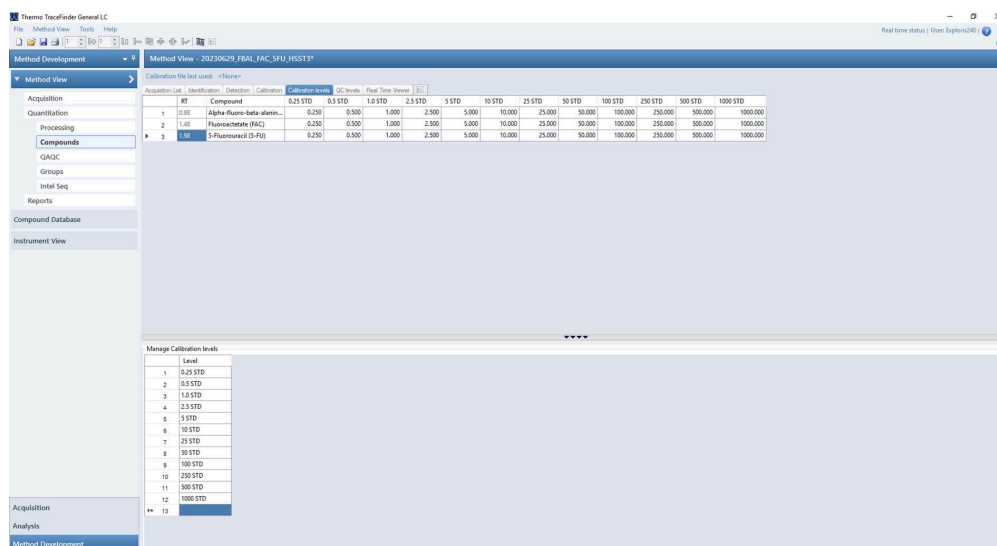
Here, you can alter the parameters for peak detection in your method, such as the expected retention times, the retention time window, the detection algorithm used and more.

12 Navigate to the 'Calibration' tab on top. The screen should look like this:



- Check that the information in this tab is correct.
- Select 'Linear' as the curve type and '1/X' as the weighting.
- Again, select the appropriate internal standard for each analyte.

13 Navigate to the 'Calibration levels' tab on top. The screen should look like this:



- Enter the names of all calibration levels in the 'Manage Calibration levels' tab at the bottom of the screen.
- For each individual analyte, fill in the concentration associated with each calibration level.

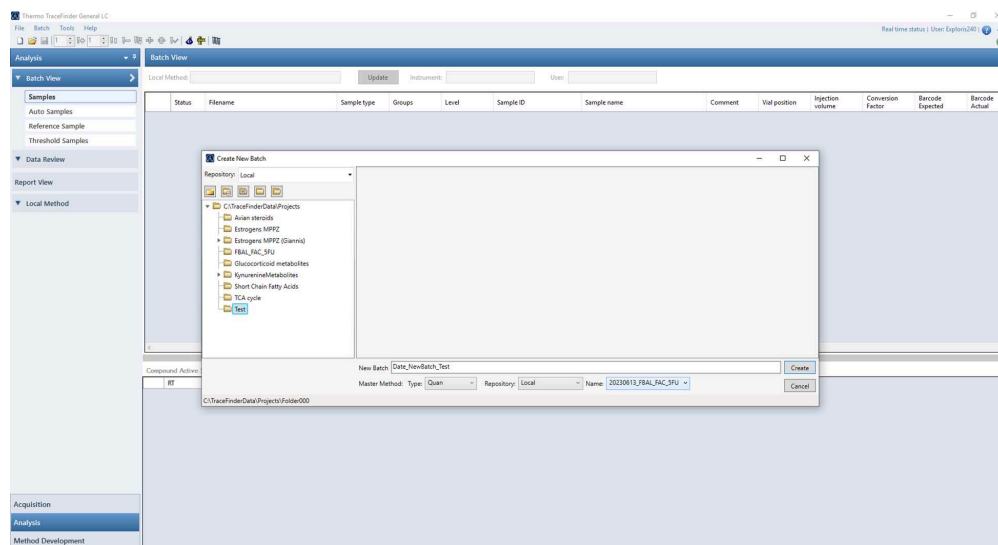
14 Save your Master Method by selecting File > Save.

Using TraceFinder to process Xcalibur acquired LC-MS data

15 In TraceFinder, navigate to the 'Analysis' tab in the bottom left corner of the screen.

16 Select File > New > Batch

A screen pops up where you can save your new batch.



Select a folder, give the new batch a name, select the Master Method you designed and select 'Create'.

17 Go to Batch > Import samples



- Browse to the SLD file of the data set that you want to process.
- All samples in the SLD file will be imported into Tracefinder.
- Delete the topmost sample which is labeled 'Unknown' by right mouse click > remove selected samples.
- Select the correct calibration level for each of the standards.
- At this point of processing, 'sample status' should be blue.

18

The screenshot shows the 'Batch View' window in the Analyst software. A right-click context menu is open over the sample at row 12. The menu options include: 'Add sample', 'Insert sample', 'Insert copy sample', 'Reassign selected samples', 'Remove selected samples', 'Import samples...', 'Browse in raw file (Move) ...', 'Browse in raw file (Copy) ...', 'Map raw files to samples...', 'Renumber vial positions', 'Convert to Chromatogram Vial Format', 'Modify columns...', 'Show Sample Weight Calculation', 'Copy', 'Copy with headers', 'Paste', 'Export to CSV file', and 'Edit instrument method'. The 'Map raw files to samples...' option is highlighted.

Status	Filename	Sample type	Groups	Level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conversion Factor	Barcode Expected	Barcode Actual
1	20230612_curve_01	Main Blank						GH12	1.000	1.000		
2	20230612_curve_02	Main Blank						GH12	1.000	1.000		
3	20230612_curve_03	Main Blank						GH12	1.000	1.000		
4	20230612_curve_04	Cal Std		0 STD	0	0 STD (FAC)		GB1	1.000	1.000		
5	20230612_curve_05	Main Blank						GB1	1.000	1.000		
6	20230612_curve_06	Cal Std		0.25 STD	0.25	0.250 STD (FAC)		GB1	1.000	1.000		
7	20230612_curve_07	Cal Std		0.5 STD	0.5	0.500 STD (FAC)		GB1	1.000	1.000		
8	20230612_curve_08	Cal Std		1 STD	1	1.000 STD (FAC)		GB1	1.000	1.000		
9	20230612_curve_09	Cal Std		2.5 STD	2.5	2.500 STD (FAC)		GB1	1.000	1.000		
10	20230612_curve_10	Cal Std		5 STD	5	5.000 STD (FAC)		GB1	1.000	1.000		
11	20230612_curve_11	Cal Std		10 STD	10	10.0 STD (FAC)		GB1	1.000	1.000		
12	20230612_curve_12	Cal Std		25 STD	25	25.0 STD (FAC)		GB2	1.000	1.000		
13	20230612_curve_13	Cal Std		50 STD	50	50.0 STD (FAC)		GB2	1.000	1.000		
14	20230612_curve_14	Cal Std		100 STD	100	100.0 STD (FAC)		GB2	1.000	1.000		
15	20230612_curve_15	Cal Std		250 STD	250	250.0 STD (FAC)		GB2	1.000	1.000		
16	20230612_curve_16	Cal Std		500 STD	500	500.0 STD (FAC)		GB2	1.000	1.000		
17	20230612_curve_17	Double Blank						GB2	1.000	1.000		
18	20230612_curve_18	Double Blank						GB2	1.000	1.000		
19	20230612_curve_19	Double Blank						GB2	1.000	1.000		
20	20230612_curve_20	Double Blank						GB2	1.000	1.000		

- Right click on one of the samples and select 'Map raw files to samples...'
- Select all raw files which appear in the windows file explorer window that pops up.
- The raw files should now be linked to each individual sample in the batch.

At this point of processing, 'sample status' should turn yellow.

The screenshot shows the 'Batch View' window after processing. The sample status icons (circles) are now yellow, indicating that the samples are ready for processing. The 'Map raw files to samples...' option is still visible in the context menu.

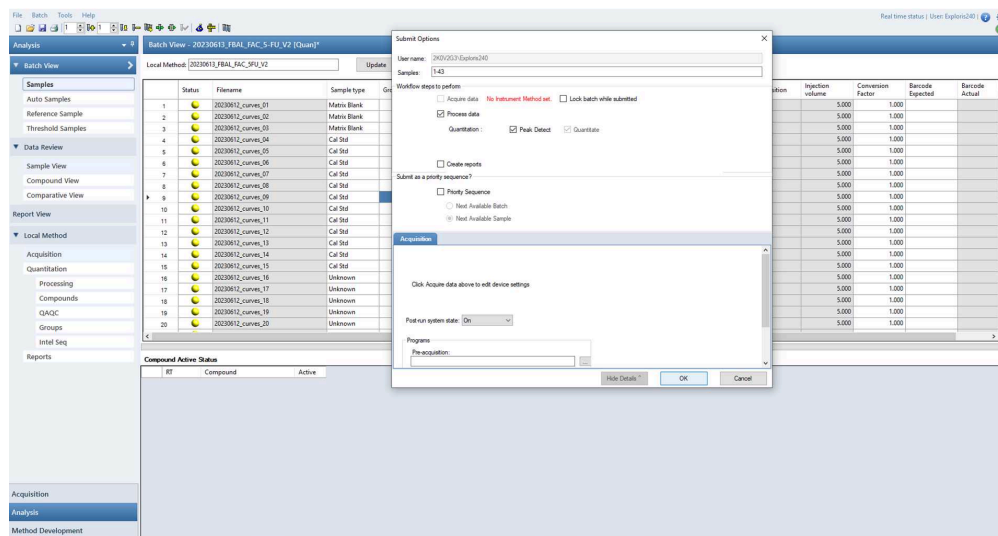
Status	Filename	Sample type	Groups	Level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conversion Factor	Barcode Expected	Barcode Actual
1	20230612_curve_01	Main Blank						GH12	5.000	1.000		
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5	20230612_curve_05	Main Blank						GB1	5.000	1.000		
6	20230612_curve_06	Cal Std		0.25 STD	0.25	0.250 STD (FAC)		GB1	5.000	1.000		
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8	20230612_curve_08	Cal Std		1 STD	1	1.000 STD (FAC)		GB1	5.000	1.000		
9	20230612_curve_09	Cal Std		2.5 STD	2.5	2.500 STD (FAC)		GB1	5.000	1.000		
10	20230612_curve_10	Cal Std		5 STD	5	5.000 STD (FAC)		GB1	5.000	1.000		
11	20230612_curve_11	Cal Std		10 STD	10	10.0 STD (FAC)		GB1	5.000	1.000		
12	20230612_curve_12	Cal Std		25 STD	25	25.0 STD (FAC)		GB2	5.000	1.000		
13	20230612_curve_13	Cal Std		50 STD	50	50.0 STD (FAC)		GB2	5.000	1.000		
14	20230612_curve_14	Cal Std		100 STD	100	100.0 STD (FAC)		GB2	5.000	1.000		
15	20230612_curve_15	Cal Std		250 STD	250	250.0 STD (FAC)		GB2	5.000	1.000		
16	20230612_curve_16	Cal Std		500 STD	500	500.0 STD (FAC)		GB2	5.000	1.000		
17	20230612_curve_17	Double Blank						GB2	5.000	1.000		
18	20230612_curve_18	Double Blank						GB2	5.000	1.000		
19	20230612_curve_19	Double Blank						GB2	5.000	1.000		
20	20230612_curve_20	Double Blank						GB2	5.000	1.000		

19

Submit your batch for processing by clicking on the following icon at the top of the screen:



The following screen should pop up:



- Click on 'OK' and all data should now be processed according to your master method.
- At this point of processing, 'sample status' should turn green.

Using TraceFinder to evaluate Xcalibur acquired LC-MS data

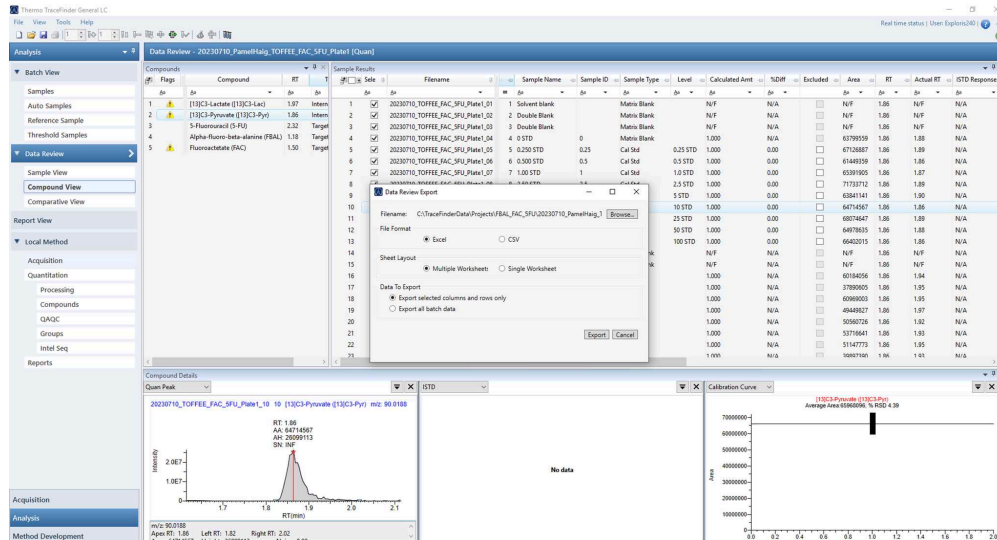
- 20 In TraceFinder, go to the 'Compound View' tab on the left hand side.
- 21 Here, each analyte can be evaluated in each sample.
 - Check that all peaks have integrated and that there are peak areas for each analyte - reassign retention time if it is not quite picking the peak and visually assess peak area and retention time to ensure that the correct peaks have been integrated.
 - Check the calibration curves for accuracy (<20%) and exclude those points that are outwith. Ensure there are a minimum of 6 points in each calibration curve and that they have a regression coefficient $R > 0.99$.

- 22 Once you are satisfied with all calibration curves and the data of each sample, go to File > Save... to save your results.

Transferring TraceFinder alphanumeric data into Excel to summarise results

- 23 Go to File > Export data to > CSV or Excel...

- 24 A screen pops up:



- Select 'Excel' as the file format.
- Select 'Multiple Worksheets' as the sheet layout.
- Select 'Export selected columns and rows only' as data to export.

- 25 Open the exported Excel file. All analytes should be sorted in individual tabs. Create a new tab and call it 'Summary'.
- 26 Copy the sample names from one of the analyte tabs, and paste into the summary tab. Then return to the Analyte tab and copy the '[Analyte name] ng' column. Repeat this copying of the [Analyte name] ng column until all analytes in the excel file, have been copied. This results in a Summary table that is the major results of the analysis in terms of concentration.
- 27 If the calibration curve units are ng and the sample extracted has been a volume, add a column ahead of all analyte concentrations, include the volume extracted for each sample and then use this column to calculate the ng/mL amount by dividing the



'calculated amount' by the volume (uL) and multiply by 1000 to give the ng/mL amount of each analyte.

28 Save the excel file in the appropriate folder.