Targeted profiling method for metabolomics

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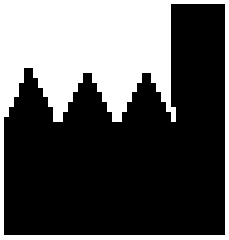

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1.0 Introduction

Aim of the method

Quantification of metabolites in complex biological samples can be difficult as confident identification of detected compounds requires significant informatic expertise. Targeted profiling strategies can greatly simplify data acquisition and processing in quantitative metabolomics when broad methods are available. Reasonable retention on column of polar metabolites as well as separation across the gradient of many other classes of metabolites is key to the development of a generic global profiling method. Due to broad structural diversity in metabolomics, having an MS platform that can analyze metabolites in both positive ion and negative ion mode in a single injection is also imperative.

Here, a detailed LC-MRM method is described that will enable targeted quantification of a wide range of metabolites in complex biological samples. Further, the method includes the MRM parameters for a panel of important metabolites. This method has not been fully validated and is intended for initial method development by users.

Separation of metabolites

Chromatography of metabolites can be difficult due to the broad range of properties across the compounds of interest. A reversed-phase method with Kinetex F5 column (Phenomenex) is used to retain both nonpolar and polar analytes to develop a broad targeted metabolite assay.

Quality control of chromatography

QReSS internal standard metabolomics kit (MSK-QReSS1-1 and MSK-QReSS2-1) consisting of eighteen isotopically labeled internal standard mixes (consisting of amino acids, vitamins, purine derivatives, amino acid degradation products and organic acids from Cambridge Isotope) is used for quality control of the chromatography. The mixture consists of several small molecules that elute at different retention times.

Method development using Scheduled MRM algorithm

While the chromatographic separation is very reproducible, a slight retention time variability due to different column batches, HPLC system configurations or mobile phase preparations is expected. This variability can be taken into account while building this targeted method, an Excel tool (sMRM Pro Builder) was developed to assist in the development of the MRM method. The workflow consists of an initial unscheduled method to determine expected retention time for the metabolites in the method. This is used to generate a first pass time-scheduled method, which will then be used to run LC-MRM replicates on a pooled sample of the biological matrix to be run in each study. Using peak area,

retention time and peak width information, a final acquisition method using the Scheduled MRM algorithm is developed. A data rejection step can also be applied to adapt the assay to the matrix being analyzed and remove MRM transitions of metabolites that are not observed. This iterative process using this Excel tool will allow users to quickly adapt this method to their LC-MS workflows in various matrices.

Please review the method documentation completely before using this method. Performance of this method is not guaranteed due to many different potential experimental variations, including instrument performance, tuning, and maintenance, chemical variability and procedures used, technical experience, and environmental conditions. The user must make adjustments to account for differences in equipment and/or materials as well as to validate the performance of this method for a given instrument. Note that a working knowledge of instrumentation and Analyst software or SCIEX OS software is required.

2.0 Instrumentation

This method has been created, developed and optimized for use with the following equipment:

- SCIEX Triple Quad 7500 system
- ExionLC system with the following components:
    - Controller, Autosampler, Pumps, and Column Oven
    - Solvent Mixer: 25 µL volume
    - 0.013 x 250 mm line from the injection port to the diverter valve
    - 0.005 inch red PEEK tubing from diverter valve to column
- SCIEX OS software version 2.0 or later
- Microsoft Excel Template – sMRM Pro Builder Template 1.4
- Master Assay List_Targeted metabolomics method

This method was optimized for the SCIEX 7500 system, but it is applicable on other SCIEX Triple Quad / QTRAP systems (3500, 4500, 5500, 6500 and 6500+). To achieve the best sensitivity on the other systems, MS parameters may need to be optimized. In addition, different LC systems could be used for this method but retention times may need more significant adaptation.

3.0 Analytes, reagents and assay materials

Details for ordering the appropriate materials for metabolomic analysis are provided in this section. To order materials; the supplier's name, contact information, and the part number for each reagent or piece of equipment required are indicated below:

- Chemicals and reagents (**Table 3-1**)
- Standards (**Table 3-2**)
- Recommended column (**Table 3-3**)

Table 3-1. Chemicals and reagents

Supplier	Description	Part Number
Sigma	Water-HPLC grade	34877-4L
Sigma	Methanol-HPLC grade	646377-4L
Sigma	Acetonitrile-HPLC grade	34998-4L
Thermo Fisher	Formic Acid-LCMS grade	85178
Equivalent reagents from other suppliers can also be used. Assay result might deviate from this optimized method if using reagents other than the ones suggested in this SOP.		

Table 3-2. Metabolite standards

Supplier	Description	Part Number
Cambridge Isotopes	METABOLOMICS QRESS STANDARD 1	MSK-QRESS1-1
	METABOLOMICS QRESS STANDARD 2	MSK-QRESS2-1
The above listed standards are recommended to order to cover wide ranges of metabolites during method development. They are not mandatory for the assay.		

Table 3-3. HPLC columns

Supplier	*Description	Part number
Phenomenex http://www.phenomenex.com/	Kinetex, 2.6µM F5 100 A (150x 2.1 mm)	00F-4723-ANY0
Equivalent equipment or instruments from other suppliers can also be used.		

4.0 Preparation of reagents and samples

Please note that the following sample preparation procedures are for reference purposes only and represent protocols created during development of this method. Proper preparation of samples and reagents is essential to ensure optimal assay performance. Since all of these materials can be obtained from a wide variety of sources, these sample preparation procedures are offered as examples only. All qualified users must be trained in the sample preparation procedures described here. End-users should verify performance parameters (such as, but not limited to, recovery, precision, linearity, and accuracy) for each procedure at the end-user laboratory location. Matrix choice will also have a significant effect on the assay performance, and alterations of the procedure might be necessary for successful sample preparation from selected matrices. SCIEX offers on-site training through purchase and inquiries regarding support services can be directed towards any local SCIEX sales representative.

Preparation of reagents and solutions

The instructions for preparing each reagent/solution are provided below:

1. **Mobile phase A** (0.1% formic acid in water)
    a. Mix 1 mL of formic acid with 999 mL of water in a 1 L bottle.
2. **Mobile phase B** (0.1% formic acid in acetonitrile):
    a. Mix 1 mL of formic acid with 999 mL of methanol in a 1 L bottle.
3. **Needle rinse** (1:1:1:1 water/methanol/iso-propanol/acetonitrile):
    a. Mix 250 mL of water, 250 mL of methanol, 250 mL of isopropanol and 250 mL of acetonitrile in a 1 L bottle.

NOTE:

- Sensitivity of metabolomics analysis is affected largely by the purity of mobile phases which can contribute to high background via contaminants. Contamination of mobile phases with fungal and bacterial growth as well as general particulates accumulate over time especially in water and with exposure to light and air. To minimize high background and ionization interference, try to prepare fresh solvents weekly and in amber bottles.

Preparation of samples

The instructions for preparing the double blank, blank and QC samples are listed below:

1. **Internal standard stock solution**
    a. QReSS vial 1: Add 1 mL of 50% methanol via a needle to the stoppered vial to reconstitute (do not remove stopper prior to reconstitute).
    b. QReSS vial 2: Add 1 mL of 50% methanol via a needle to the stoppered vial to reconstitute (do not remove stopper prior to reconstitute).
    c. Take 10.25 µL of each QReSS vial 1 and vial 2 and add 979.5 µL of methanol.
2. **Double blank sample**:
    a. Pipet 1 mL 1:1 water/methanol into an autosampler vial.
3. **Blank sample:**
    a. Add 50 µL water to 390 µL methanol and 10 µL of internal standard stock solution.
4. **Pooled sample for method development:**
    a. Sample extracts containing internal standards (see Appendix A for examples) can be used.

5.0 Building an un-scheduled MRM method

This LC-MRM acquisition method utilizes a positive/negative polarity switching method to cover a broad range of metabolite classes. Due to the large numbers of metabolites in this method, the retention times for the specific molecules must be determined to allow for time-scheduled acquisition. This assay development strategy is a two-step process which is described in Section 9.0. While in section 5.0–7.0, the required acquisition methods will be built for use in the assay development process.

1. To create an acquisition method in SCIEX OS software, begin by double-clicking the SCIEX OS software icon to open software.
2. On the *Configuration* tab, click on hardware profile and enable the profile that correctly matches your LC-MS instrumentation.
3. On the *Acquisition* panel of software home screen (**Figure 5-1**), click *MS Method*. From the drop-down menu for scan type, select *MRM*.

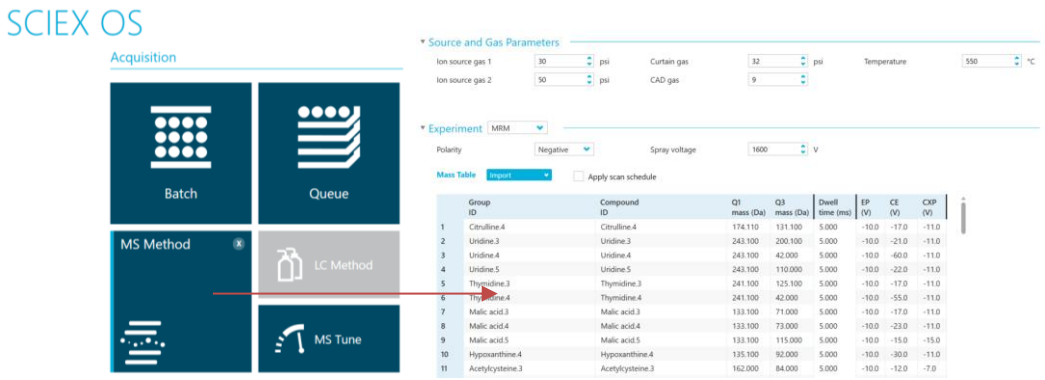

Figure 5-1: Building an acquisition method in SCIEX OS software.

4. Select Negative polarity for the first experiment as indicated in the orange box in **Figure 5-2**.

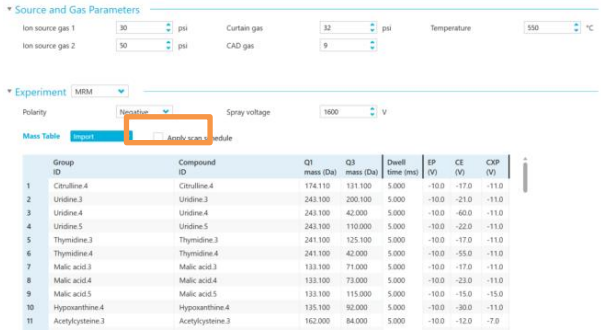

Figure 5-2: Defining the negative polarity information for Experiment 1.

5. Complete the *Source and Gas Parameters* in the acquisition method and enter the parameters outlined in **Table 5-1** for each of the polarities.
6. Repeat the process to build a second unscheduled method for the positive ion polarity.

Table 5-1. SCIEX 7500 system parameters for metabolite analysis

Source parameters	Positive polarity	Negative polarity
IS	1600	-1600
CUR	40 psi	40 psi
TEM	350 °C	350 °C
*GS1	30 psi	30 psi
*GS2	50 psi	50 psi
CAD	9	9
**These values may need to be optimized to obtain maximum sensitivity.*		
Compound parameters		
EP	10	-10
CXP	10	-10
MS		
Scan type	MRM	MRM
Duration	20 min	20 min
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

7. Paste the Master Assay List for the Global metabolomics method into the sMRM Pro Builder Template in the *Master Assay Table* tab, then click F9 to compute the template.
8. Copy all the populated columns from the tab called *OS_OUTPUT ASSAY (+) Initial* and paste into the positive polarity MRM table of the positive method.
9. Set the pause time and the dwell time to 2 msec.
10. Save the method as *Mx_MRM_Unscheduled_Pos_1*.
11. Next, copy all the populated from the tab called *OS_OUTPUT ASSAY (-) Initial* and paste into the negative polarity MRM table of the negative method.
12. Again, set the pause time and the dwell time to 2 msec
13. Save the method as *Mx_MRM_Unscheduled_Neg_1*.

6.0 HPLCs and operation parameters

Small molecule metabolites are separated by HPLC using the mobile phases and gradient conditions outlined in **Table 6-1**. Initial conditions, autosampler conditions, and oven settings are outlined in **Table 6-2,** below.

1. To create an LC method in SCIEX OS software, again go to the *Acquisition* panel of software home screen (**Figure 5-1**), and now click *LC Method*. From the drop-down menu for scan type, select *MRM*.
2. Fill out the details in each tab, using the LC gradient details in **Table 6-1 and 6-2**. Save the LC method as *Mx_F5_20 mins* after LC information has been added to each.
3. A representative XIC of analytes in the panel are shown in **Figure 6-1**.

Table 6-1. LC gradient and mobile phase composition

Total time (min)	Module	Event	Parameter (%)	Total flow (mL/ min)
0	Pumps	Pump B conc.	0	0.2
2.1	Pumps	Pump B conc.	0	0.2
14	Pumps	Pump B conc.	95	0.2
16	Pumps	Pump B conc.	95	0.2
16.1	Pumps	Pump B conc.	0	0.2
20	Pumps	Pump B conc.	0	0.2
**Mobile phase A: 0.1% formic acid in water*				
**Mobile phase B: 0.1% formic acid in acetonitrile*				

Table 6-2. Additional HPLC parameters and LC settings	
Pumps	**Parameters / settings**
Flow rate	0.2 mL/min
Pump B concentration	0%
Low pressure	0 psi
High pressure	15000 psi
Autosampler	
Use Autosampler	*Select AS*
Rinsing Solution	(1:1:1:1) water/methanol/isopropanol/acetonitrile
Rinse Type	External
Rinsing volume	500 µL
Needle stroke	52 mm
Rinsing speed	35 µL/sec
Sampling speed	5 µL/sec
Injection volume	1 µL
Purge time	25 min
Rinse dip time	3 Sec
Rinse mode	Before and After aspiration
Cooler Enabled	Yes
Cooler Temperature	6°C
Oven	
Temperature control	Enabled
Temperature	30 °C

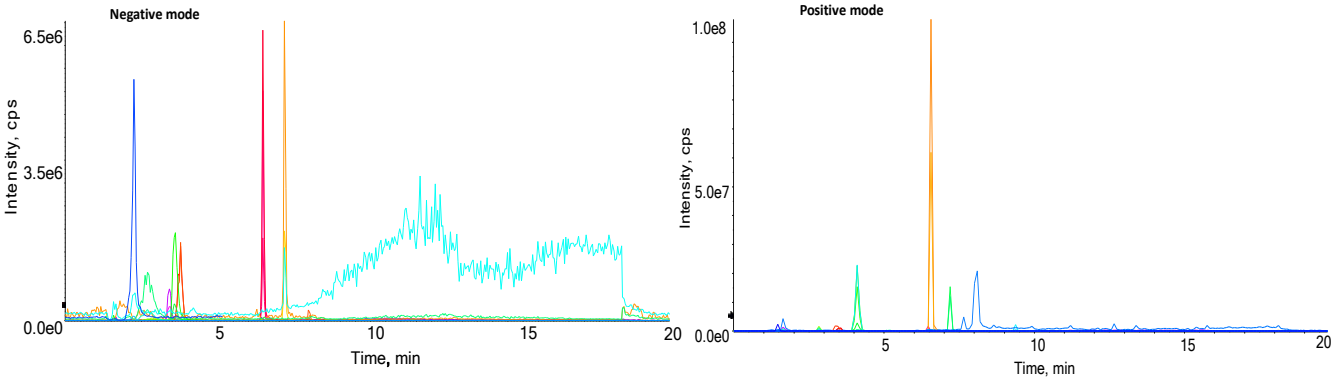

Figure 6-1. Representative XIC of the pooled sample with the QReSS standard mix added.

7.0 Building acquisition methods with Scheduled MRM algorithm

Two method types will be required during assay optimization, a non-scheduled method that was built in section 5.0 and 6.0 and also a method constructed using the Scheduled MRM algorithm in enhanced mode, described here.

1. Open the MRM acquisition method build in Section 5.0 to start.
2. Select the Scheduled MRM algorithm by checking the **Apply scan schedule** box shown in the orange box in **Figure 7.1**.
3. If on the first scheduled filtering step, enter 20 seconds into the Retention time tolerance column. This provides a detection window of 40 secs. Right-click and use the fill down option as shown by the red box.

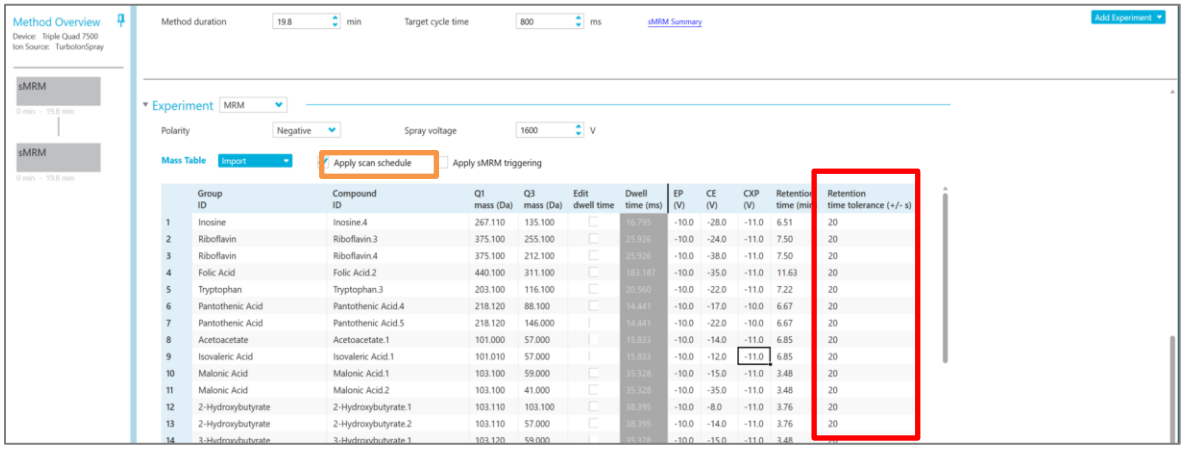

Figure 7-1: Select the *Apply scan schedule* check box.

4. Adjust the Target Cycle time to 800 msec as show in **Figure 5.2** indicated by the green box.
5. The pause time should be set to 3 msec.
6. Using the computed MRM table in the *sMRM Pro Builder.xlsx* workbook, paste the populated columns from the tab called *OS_Output Assay (-)* into the negative polarity experiment.
7. Click Add Experiment to add another experiment, and then select MRM and the positive polarity. Note that the settling time is set to 15 msec. Ensure that the pause time is set to 3 msec in this experiment as well.
8. Paste the populated columns from the tab called *OS_Output Assay (+)* into the positive polarity experiment.
9. Save the method as *Mx_sMRM_Opt_1.*

10. Use the sMRM summary for the method to check the resulting concurrency and dwell times for the method.
11. Save the experiment again with the name *Mx_sMRM_Final_1 as* the final time-scheduled method with polarity switching.
12. These two methods along with the method built in Section 5.0 (*Mx_MRM_Unscheduled_Pos_1 and Mx_MRM_Unscheduled_Neg_1*) will serve as template methods for the assay optimization steps described in Section 9.0.

8.0 Data analysis in SCIEX OS software

This section describes how to use SCIEX OS software version 2.0 or later during assay development, for determination of retention times, peak areas, etc.

1. Before starting data processing, ensure that the Integration Parameters are set correctly. For that, go to Project→ Project Default Settings → Integration defaults →integration algorithm, select AutoPeak as in **Figure 8-1**.

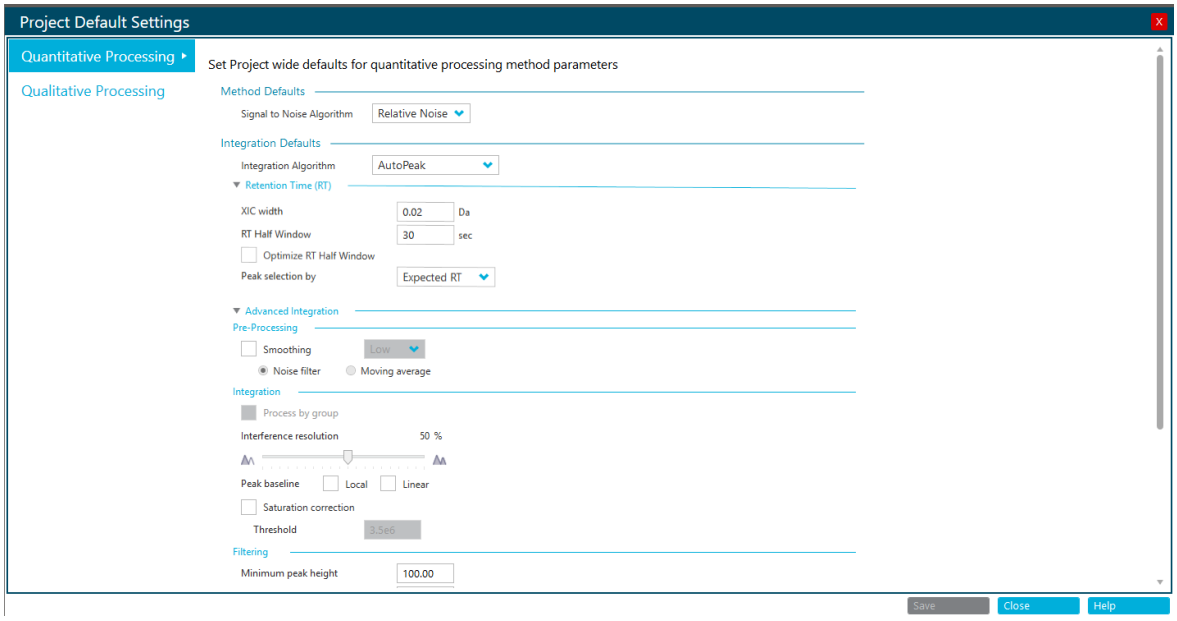

Figure 8-1. Setting the default settings for project.

2. Click Process Method then Edit Embedded Method. Click Integration in the left-hand panel. Set the integration defaults as shown in **Figure 8-2**. Ensure to select Peak Selection by Group as this will help with selecting the right set of peaks for each metabolite when multiple MRMs per metabolite are present.

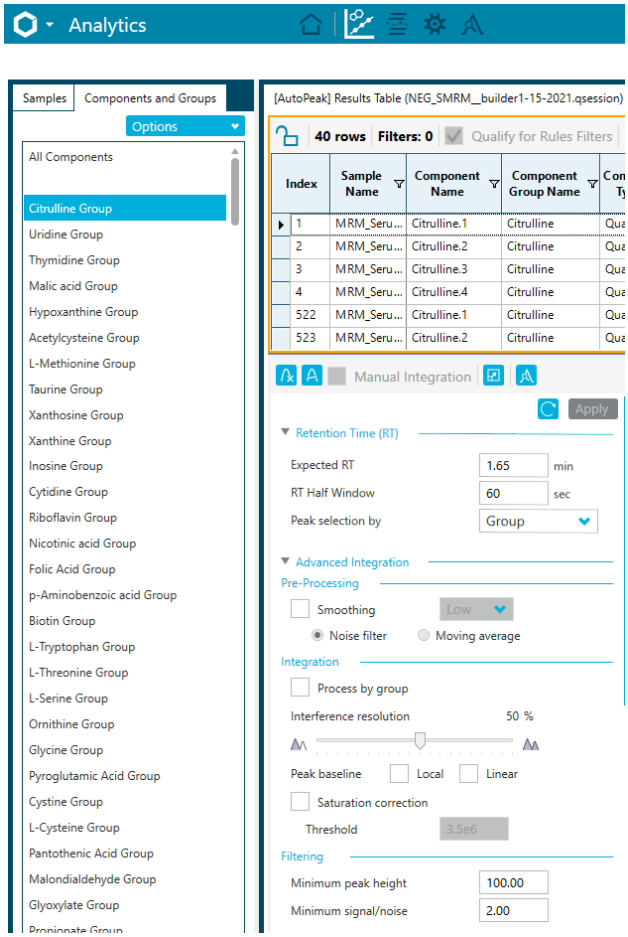

Figure 8-2. Integration defaults for data processing the global metabolomic data.

1. Go to the *Results* drop down menu in SCIEX OS software, and choose *New*.
2. Select the data files to be processed and double-click the data files to move them to the *Selected Pane* or select the data file and use the “=>” button to move the data files.
3. Select **Create New Method** and name it Mx MRM Method <date>. Click **Next**.
4. Select all the samples to obtain optimized integration parameters for the whole sample set.
5. On the Define Components Pane, the individual metabolites can be viewed. Click **Next**.
6. Integrations can be now reviewed for each individual metabolite. Review the integration of each metabolite species to ensure it is correctly integrated. Note that not all metabolites will be detected, depending on the biological matrix.
7. After all MRM transitions have been reviewed, click **Next**.
8. Click **Finish** on the next pane to complete.
9. Data will populate and the results table can now be saved.

9.0 Protocol for determining metabolite retention times

The retention times of metabolites are affected by multiple experimental factors including mobile phase preparation, HLPC mixer size and dead volume. Therefore, the retention time must be determined during initial method development. Once retention time is determined, it is typically stable throughout the biological study, as long as experimental factors remain constant, especially mobile phase preparation. It is recommended that assay development be performed on a pooled sample that is generated from the range of biological samples to be analyzed, such that most metabolites are represented in the pooled sample for the assay development step.

This section describes the iterative method development process for determining retention time and building a final highly optimized method for targeted metabolite profiling using the sMRM Pro Builder Template.xlsx.

First, the Master Assay List for the Global Mx Method is pasted into the *Master Assay Table* Tab in the sMRM Pro Builder template. This allows the first unscheduled methods to be built (Mx_MRM_Unscheduled_Pos_1 and Mx_MRM_Unscheduled_Neg_1). LC-MS analysis is performed on the sample matrix of interest to determine rough retention times. Using this data, the time-scheduled acquisition method is then built from this information (Mx_sMRM_Opt_1) and then used to acquire replicate data on a pooled biological sample. Replicate injections are now performed in this second iteration using the Scheduled MRM algorithm. Enough replicates should be performed to generate stable results (10 replicates are recommended). This data can then be used to generate a final optimized LC-MRM method (Mx_sMRM_Final_1) to use in the biological study. The data generated in each iteration will be processed by SCIEX OS software for peak integration, and the exported data will be analyzed using the sMRM pro Builder template in Excel. At each step, acquisition method values are generated that can be pasted back into Analyst software or SCIEX OS software for method building.

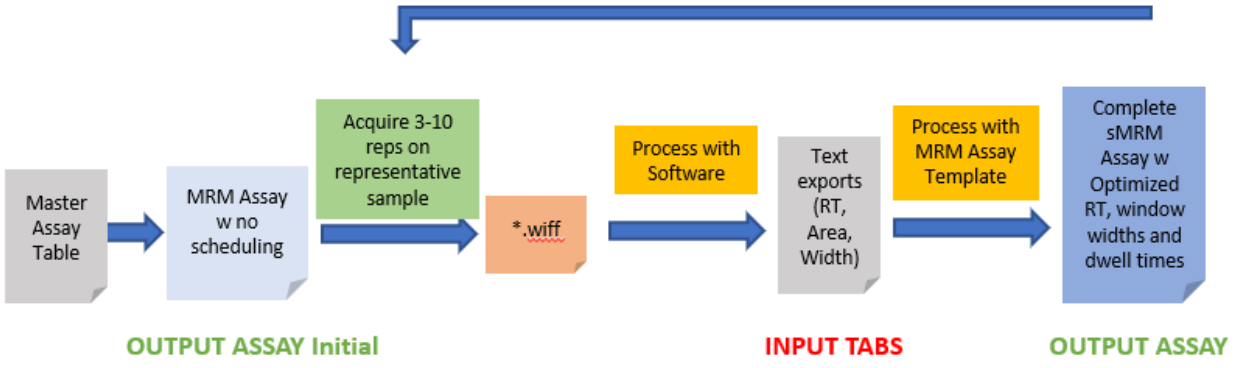

Figure 9.1. Method development workflow using sMRM Pro Builder template.

1. Create a pooled sample of the biological matrix to be used in the study (Section 4.0). It is recommended that this includes sample from the various sample types to be studied (ie. condition 1 vs condition 2) such that most metabolites will be represented in the pooled sample. Internal standards can also be included in the sample as described in Section 4.0.
2. Place the pooled samples into the autosampler.
3. Copy the populated columns from the tab called *OS_OUTPUT ASSAY (+) Initial* and paste into the positive polarity MRM method.
4. Save the method as *Mx_MRM_Unscheduled_Pos_1*.
5. Copy the populated columns from the tab called *OS_OUTPUT ASSAY (-) Initial* and paste into the negative polarity MRM method.
6. Save the method as *Mx_MRM_Unscheduled_Neg_1*.
7. Perform 3 injections of the Double Blank sample using the *Mx_MRM_Unscheduled_Neg_1* acquisition method, to equilibrate the LC-MS system.
8. Perform 3-10 injections of the pooled sample using *Mx_MRM_Unscheduled_Neg_1* acquisition method.
9. Process the data in SCIEX OS software (Section 8.0). At this point careful data review is recommended to ensure the right metabolites are being integrated. Once satisfied, export the results for analysis in the sMRM Pro Builder. Select Reporting → Export Results→ Results Table – Metric, then export the Area, Retention Time, Width at 50% as shown in **Figure 9.2**.

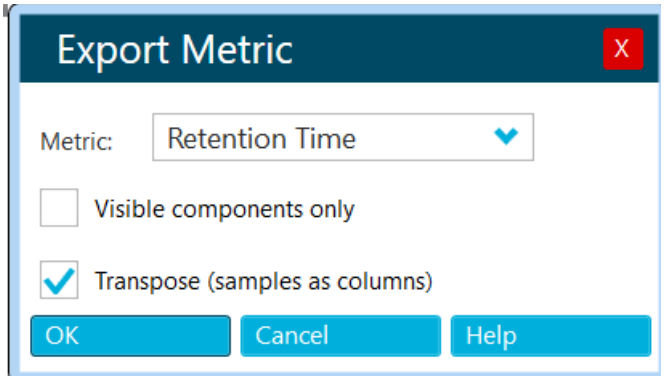

Figure 9.2. Exporting the results from SCIEX OS software for use in sMRM Pro Builder.

10. Repeat the process to collect the data for positive ion mode. Perform 3-10 injections of the pooled sample using *Mx_MRM_Unscheduled_Pos_1* acquisition method.

11. Process the data in SCIEX OS software (Section 8.0), again being careful to ensure correct peaks are being selected, then export the results for analysis in the sMRM Pro Builder. Select Reporting → Export Results→ Results Table – Metric, then export the Area, Retention Time, Width at 50% as text files.
12. Combine the exported results text files in a new blank workbook in Excel for each of the Area, Retention Time and Width at 50% data, pasting the positive ion mode data below the negative ion mode data. Remember to remove the header rows for the data pasted in the bottom. Ensure that the columns match as well for number of replicates. *Important note: Do not do this within the sMRM Pro Builder template as editing within the Input tabs will confound the formulas in the workbook and create errors.*
13. Paste the combined exported results into the Excel template (Input-RT, Input-Area, Input-Width tabs) and click Calculate (F9). Follow the instructions in the template for more information. Note if you want to save the intermediate assay development results, rename and save the template.
14. The template has the ability to filter out metabolites that are not detected by using the area, RT and peak width information. Typically, the filtering step is applied only for the last iteration and can be controlled through settings on the *INSTRUCTIONS AND CONTROLS* tab. At this point it is recommended that no metabolites are filtered out of the assay so remember to set the *Assay Subset for Output* set to **All** - **Figure 9.3**.

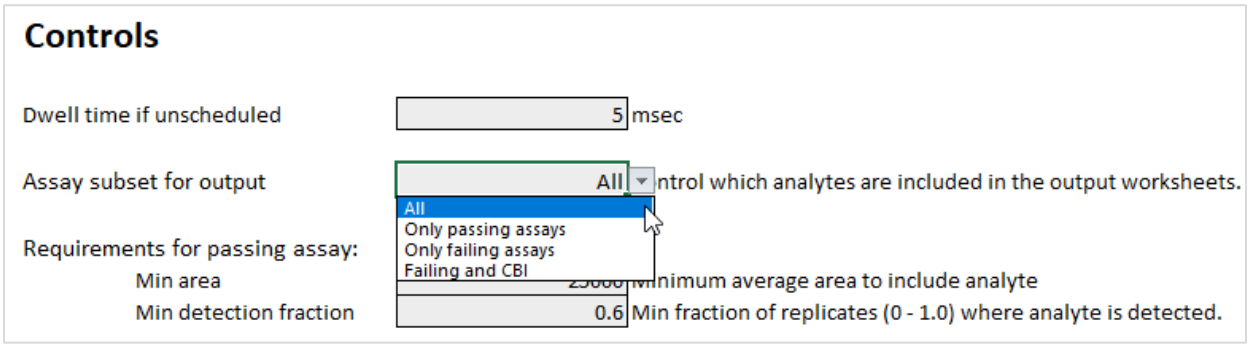

Figure 9.3. Filtering controls in the sMRM Pro Builder Template.

15. Paste the new MRM lists from the *OS_OUTPUT ASSAY (+)* and *OS_OUTPUT ASSAY (-)* tabs into the *Mx_sMRM_Opt_1* from Section 7.0 and rename. Note the assay now includes rough retention times for all metabolites. For the first round of scheduled method testing, no filtering should be applied.

16. Next perform 5-10 replicate injections of the Pooled Sample using this time-scheduled method. This data will be used to create the final highly optimized Scheduled MRM algorithm method.
17. Process the replicate data in SCIEX OS software, then export the results as described above in step 7. As the next round of analysis in the template will be used for rejecting metabolites that are not reliably detected, ensuring the input data is of good quality is important.
18. Paste the exports into the Excel template again (Input-RT, Input-Area, Input-Width tabs). To have the template automatically filter out the undetected metabolites, set the *Assay subset for output* setting to *Only Passing Assays* (**Figure 9.3**). Then click Calculate (F9). *Note that this could remove low intensity MRMs from some metabolites that have multiple MRMs.*
19. Paste the refined MRM lists from the *OS_OUTPUT ASSAY (+) and OS_OUTPUT ASSAY (-)* tabs into the *MX_sMRM_Final_1 from Section 7.0* and rename. This is now the final fully optimized method for targeted metabolite profiling on this biological matrix. Metabolites that are not reliably detected in this matrix have been removed.

Appendix A – Sample preparation protocols for biological samples

All sample preparation techniques listed in the protocol are only suggested sample preparation methodologies and have not been validated. The retention times and compound sensitivity shown in this protocol was determined using the following suggested sample extractions.

Note: each step should be followed while avoiding sample exposure to UV light (for example, avoid leaving samples exposed to sunlight by keeping samples covered or in a closed refrigerator or freezer).

A1. Extraction protocol for plasma

1. 50 µL of plasma sample is taken in 1.5 mL centrifuge tube. Make sure the plasma is settled in the bottom of the tube but not on the walls of the tube
2. 400 µL of methanol (390 µL of clean methanol + 10 µL of QReSS IS)
3. Vortex the sample/ solvent mixture for 10 sec
4. The sample/ solvent mixture is centrifuged at 15000 rcf for 10 min
5. Filter the mixture with cellulose acetate spin filter
6. The filtrate is kept in 4° C for 2 hrs for any further precipitation of proteins (This step is essential if microflow systems are used for chromatography)
7. The sample/solvent mixture is centrifuged again at 15000 rcf for 10 min using a centrifuge filter. The sample extract is transferred to vial.
8. 1 µL of the supernatant is injected for LC-MS analysis.

Note: be aware of the potential for precipitated proteins to remain in the sample. Sometimes it is necessary to quickly centrifuge sample before placing in the autosampler vial, or adjust the needle draw height on the autosampler to ensure no precipitate is transferred onto the analytical column.

Appendix B - Sample processing using Analyst and MultiQuant software

Building an un-scheduled MRM method on the QTRAP 6500+ system or other platforms using Analyst software

This LC-MRM acquisition method utilizes a positive/negative polarity switching method to cover a broad range of metabolite classes. Due to the large numbers of metabolites analyzed in this method, the retention times for the specific molecules must be first determined to allow for time scheduled acquisition. This assay development strategy is a two-step process which is described in Section 9.0. Here in Section 5.0–7.0, the required acquisition methods will be built for use in the assay development process.

1. To create an acquisition method in Analyst software 1.6.3 or later, begin by double-clicking the Analyst software icon to open software.
2. On the left panel under *Configure*, click on hardware profile and enable the profile that correctly matches your LC-MS instrumentation.
3. On the left panel under *Acquire* (**Figure B-1**), double click *Build Acquisition Method*. From the drop-down menu for scan type, select *MRM*.

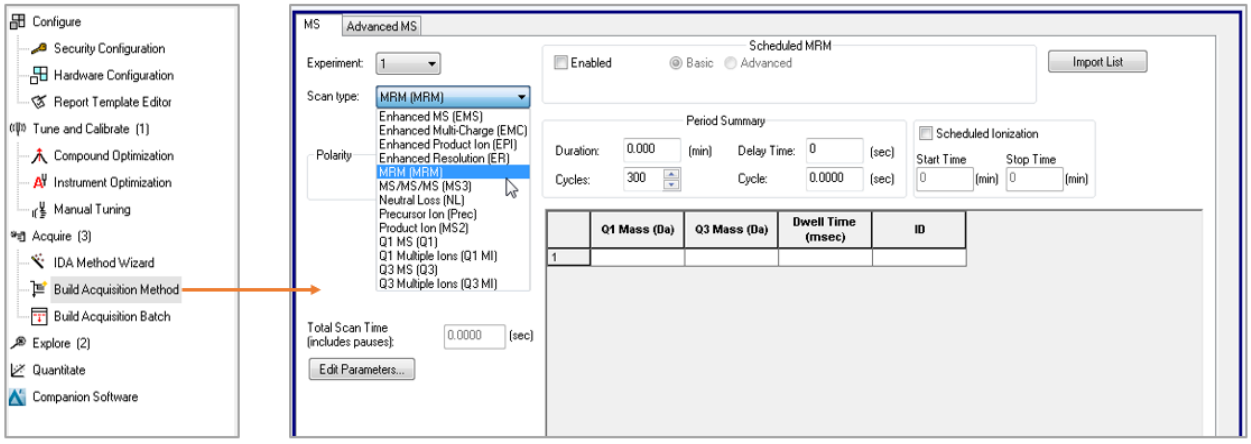

Figure B-1: Building an acquisition method in Analyst software.

4. Select positive polarity for the first method as indicated in the orange box in **Figure B-2**. Move the mouse to where the blue box is indicated and right click to add the Collision Energy (CE) to the MRM table.

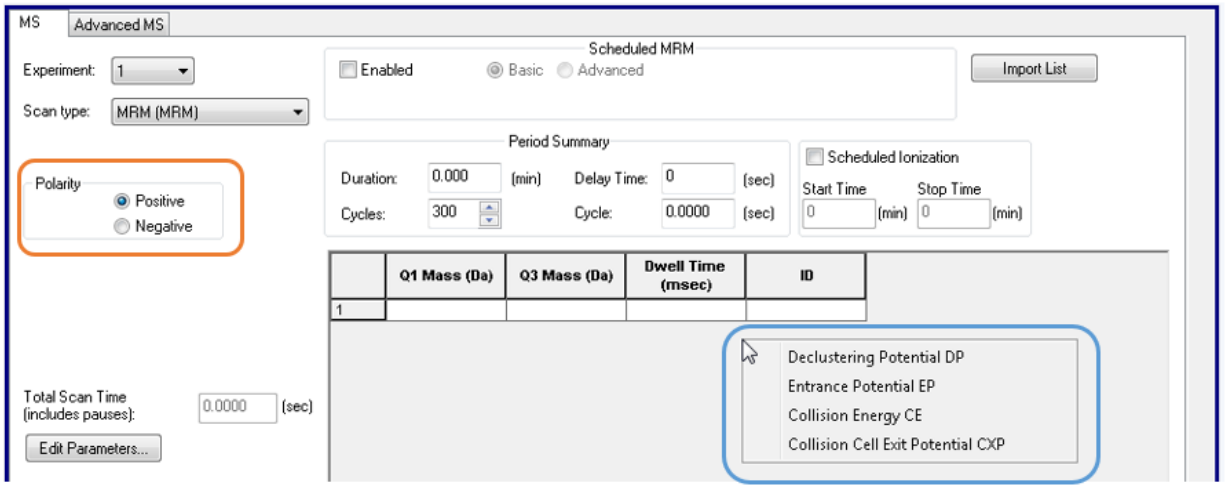

Figure B-2: Defining the positive polarity information for method 1.

4. Click on *Edit Parameters* in the acquisition method and enter the parameters outlined in **Table B1-1** for each of the polarities.

Table B-1. QTRAP 6500+ system parameters for metabolite analysis		
Source parameters	**Positive polarity**	**Negative polarity**
IS	5500	-4500
CUR	30 psi	30 psi
*TEM	350 °C	350 °C
*GS1	80 psi	80 psi
*GS2	80 psi	80 psi
CAD	12 or High	12 or High
**These values may need to be optimized to obtain maximum sensitivity.*		
Compound parameters		
DP	40	-40
EP	10	-10
CXP	15	-15
MS		
Scan type	MRM	MRM
Duration	20 min	20 min
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

5. Ensure the pause time is set to 2 msec on the Advanced MS tab and the dwell time is set to 2 msec.
6. Repeat the process above to build a method for the negative polarity method.

7. Paste the Master Assay List for the Global Lipid Method into the sMRM Pro Builder Template in the *Master Assay Table* tab, then click F9 to compute the template. Copy the **Columns A - E** from the tab called *Analyst_OUTPUT ASSAY (+) Initial* and paste into the positive polarity MRM method and copy **Columns A - E** from the tab called *Analyst_OUTPUT ASSAY (-) Initial* and paste into the negative polarity MRM method.
8. Save the method as *Mx_MRM_Unscheduled_Pos_1 and Mx_MRM_Unscheduled_Neg_1*.
9. The Scheduled MRM Concurrency calculator can be used to compute the MRM concurrency and the dwell times resulting in the time-scheduled method.
10. Next step will be to add the chromatography settings to this method in Section 6.0.

Building acquisition methods with Scheduled MRM algorithm pro

Two method types will be required during assay optimization, a non-scheduled method that was built in section 5.0 and 6.0 and also a method constructed using the Scheduled MRM algorithm pro in Advanced mode, described here.

1. Open the MRM acquisition method build in Section 5.0 to start.
2. Select the Scheduled MRM algorithm by checking the **Enabled** box shown in **Figure B.3**. Use the Advanced Mode.
3. Fill in the Scheduled MRM algorithm Parameters as shown in **Table B.2**.

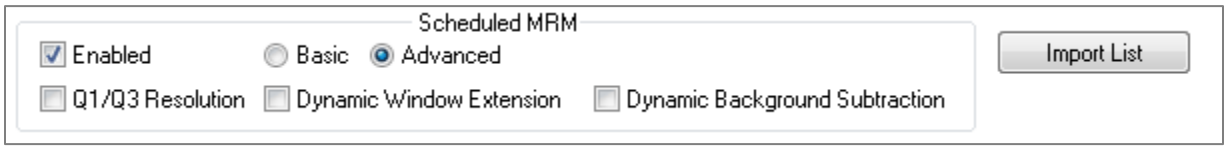

Figure B-3: Select the Scheduled MRM algorithm – enhanced mode.

Table B-2. Scheduled MRM algorithm parameters for positive and negative mode		
Parameter	**Positive polarity**	**Negative polarity**
Duration (min)	20	20
MRM Detection Window (sec)	60	60
Target Cycle Time (sec)	0.5	0.5

4. Set the pause time to 3 msec on the Advanced MS tab. Note the settling time will default to 50 msec.
5. Select Basic as shown in Figure B-3 and save the method as *Mx_sMRMPro_1.*

6. Next select Advanced and save the method as *Mx_sMRM_Final_1.*
7. These two methods along with the methods built in Section 6.0 (*Mx_MRM_Unscheduled_1*) will serve as template methods for the assay optimization steps described in Section 9.0.

Data analysis in MultiQuant software

This section describes how to use MultiQuant software 3.0.2 or later during assay development, for determination of retention times, peak areas, etc.

1. Before starting data processing, ensure that the Integration Parameters are set correctly.
2. Click Edit then Project Integration Defaults. Set the integration defaults as shown in **Figure B-4**.

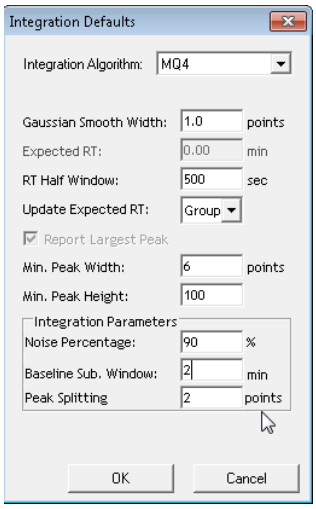

Figure B-4. Integration defaults for data processing the global metabolomics data.

3. Go to the *File* drop down menu in MultiQuant software, then choose *New Results Table*.
4. Select the data files to be processed and double click the data files to move them to the *Selected Pane* or select the data file and use the "=>" button to move the data files.
5. Select **Create New Method** and name it Mx MRM Method <date>. Click **Next**.
6. Select a representative injection from the data file upon which the quantitation method will be optimized. Ensure good peaks are observed for all metabolites.
7. On the Define Components Pane, the individual metabolites can be viewed. Click **Next**.

8. Integrations can be now reviewed for each individual metabolite. Review the integration of each lipid species to ensure it is correctly integrated. Note that not all metabolites will be detected, depending on the biological matrix.
9. After all MRM transitions have been reviewed, click **Next**.
10. Click **Finish** on the next pane to complete.
11. Data will be populated, and the results table can now be saved and reviewed.

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