Comprehensive targeted method for global lipidomics analysis

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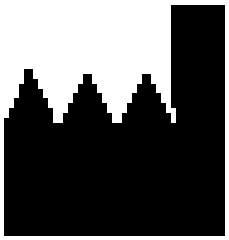

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1.0 Introduction

Aim of the Method

Quantifying lipids in complex biological samples is challenging and requires a detailed knowledge of lipids and lipid-analysis methods. The inherent complexity of the lipidome means that identifying and quantifying different lipid classes and isobaric species in a single analysis method is difficult and often requires a high degree of expertise and method development time. The lack of simplified methods and poor quality of quantitative data makes it more challenging to understand the role of lipids in biology. This method provides a means for the relative quantitation of many lipid species across multiple lipid classes using a targeted LC-MS method.

Here, a detailed LC-MRM method will provide comprehensive coverage of many lipid classes in complex biological samples. The method includes the MRM information for a panel of lipids. This method has not been fully validated and is intended as a starting point for users to develop further methods. The technique can be adapted to include additional lipid classes; however, maintaining sufficient points across the LC peaks for quantitative analyses must be monitored.

Lipid Class Separation

Hydrophilic interaction liquid chromatography (HILIC) is a variant of normal-phase liquid chromatography, which partly overlaps with other chromatographic techniques such as reversed-phase liquid chromatography (RPLC) and Ion-Exchange Chromatography (IEX). The ethylene-bridged hybrid (BEH) Amide columns have enabled a new level of stability and robustness while harnessing the power of HILIC chromatography. This specific column utilizes a trifunctionally bonded amide phase and BEH particle for stability and reproducibility of the assay. HILIC uses a polar stationary phase (SP) and a polar mobile phase (MP) as eluents, in contrast to RPLC, which uses non-polar SP and polar MP. The mechanism of HILIC is not well understood. Still, it is believed that dipole interactions (via H-bonding) and ion exchange often dominate selectivity and retention, while in RPLC, hydrophobic partitioning plays a more key role. The elution time can be adjusted by changing the mobile phase composition
(B%). Like IEX, polar compounds retain on the HILIC column, and analytes elute in the order of increasing polarity. Lipid molecules can be separated by lipid class using HILIC chromatography. Retention of lipids on HILIC is primarily determined by the dipole interactions (between head groups and the stationary phase) and is less affected by the fatty acid chain length.

Quantitation Strategy

The method described here can utilize different strategies for relative lipid quantitation. The most commonly used approach is to spike one single standard per lipid class into the sample, e.g., the SPLASH Lipidomix (Avanti Polar Lipids). This approach allows for the relative quantitation and comparison between sample groups. However, it should be noted that using more than one internal standard per lipid class will provide more accurate quantitation of individual lipids by enabling closer structure-matching of analytes to internal standards. User-selected internal standards can be added to the method to quantify further lipid classes.

Method Development using *Scheduled MRM Algorithm*

Although the chromatographic method has proven to be very reproducible, there may be retention time variations between column batches, HPLC systems or mobile phases. To accommodate this variability, an Excel tool (sMRM Pro Builder) was developed to assist in developing the acquisition method. The workflow consists of an initial unscheduled method to determine rough retention times. 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 (at 50% height) information, a final acquisition method using the Scheduled MRM Algorithm, consisting of over 1900 lipid species, is developed. The advanced features, which include flexible retention time tolerance and dwell time weighting, are used to improve the resulting data quality. This iterative two-step process using this Excel tool will allow users to adapt this method to their LC-MS setup quickly. It is **strongly recommended** that at least one internal standard (such as SPLASH or Lipidyzer kits) be used per lipid class to ensure the correct retention time is determined from the unscheduled acquisition.

Please review the method documentation entirely before using this method. The performance of this method is not guaranteed due to many different potential 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 materials and validate the performance of this method for a given instrument. 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 – QTRAP Ready
- 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 analytical column
    - 0.005 inch red PEEK tubing post column to divert valve (12 inches)
    - 0.005 inch red PEEK tubing post divert valve to source union (13 inches)
    - 0.005 inch red PEEK tubing post source ground to electrode (16 inches)
- SCIEX OS software version 3.3.1 or later
- Microsoft Excel Template – sMRM Pro Builder Template 1.4 with master assay list loaded
- Master Assay List_Global Lipid Method 1.5.1

This method was optimized for the SCIEX 7500 system but applied to other SCIEX Triple Quad / QTRAP systems (3500, 4500, 5500, 6500, 6500+, and 7500+). 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 require more significant adaptation.

3.0 Analytes, Reagents and Assay Materials

Details for ordering the appropriate materials for lipidomic 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**)
- Lipid standards (**Table 3-2**)
- Recommended column (**Table 3-3**)

Table 3-1. Chemicals and reagents

Supplier	Description	Part Number
Sigma-Aldrich http://www.Sigmaaldrich.com	Water	270733
	Acetonitrile	34967
	Methanol	34966
	Isopropanol	PX1834-1
	Ammonium Acetate	5330040050
	Dichloromethane	270997

Equivalent reagents from other suppliers can also be used. However, if other reagents are used than those suggested in this SOP, the assay result may deviate from this optimized method.

Table 3-2. Lipid standards

Supplier	Description	Part Number
Avanti Polar Lipids, Inc. https://avantilipids.com/	SPLASH Lipidomix Mass Spec Standard	330707
	UltimateSPLASH One	330820
	17:1 Lyso PG	858127C
	17:1 Lyso PS	858141C
	17:1 Lyso PI	850103P
SCIEX	AA 45/32™ Phys Control Plasma	4386703

The above-listed standards are recommended to cover a wide range of lipid classes during method development. Depending on the lipid classes required for the study, they are not mandatory for the assay.

Table 3-3. HPLC Columns

Supplier	*Description	Part number
Phenomenex http://www.phenomenex.com/	Luna® 3um NH2, 2 x 100 mm column	00D-4377-B0
	SecurityGuard Standard NH2	AJ0-4301
.		

4.0 Preparation of Reagents and Samples

Please note the following sample preparation procedures are for reference purposes only and represent protocols created during the development of this method. Proper preparation of samples and reagents is critical to ensure optimal assay performance. Since these materials can be obtained from various sources, 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 significantly impact the assay's performance, and procedure alterations may be necessary for successful sample preparation from selected matrices. SCIEX offers on-site training through purchase, and inquiries regarding support services may be directed to any local SCIEX sales representative.

Preparation of Reagents and Solutions

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

1. **Ammonium acetate stock solution** (2 M Ammonium Acetate in Methanol)
    a. Weigh 15.4165 grams of ammonium acetate and add it to a 100 mL glass bottle. To the same glass bottle, add 100 mL of methanol. Shake vigorously to ensure that all solids have dissolved before usage. A smaller volume can be prepared, but it is recommended that you use the same stock of ammonium acetate to make your mobile phases.
2. **Mobile Phase A** (2mM Ammonium Acetate in 7/93, Dichloromethane/Acetonitrile)
    a. Prepare 2 L of 7/93 dichloromethane/acetonitrile stock solvent by mixing 140 mL dichloromethane with 1860 mL acetonitrile. Add 2ml ammonium acetate stock solution. Mix vigorously.
3. **Mobile Phase B** (2 mM Ammonium Acetate in 50/50-Water/Acetonitrile):
    a. Prepare 2 L of 50/50 water/acetonitrile stock solvent by mixing 1 L water with 1 L acetonitrile. Add 2mL ammonium acetate stock solution and mix vigorously. This mobile phase can be modified using ammonium hydroxide to increase the pH to 8.2. This may be necessary to improve the signal strength for cardiolipins and BMP. However, the retention times of analytes will shift because of the change in pH. Car must be taken to adjust the pH carefully, and an unscheduled MRM experiment will be needed to verify analyte retention times. (See note).

4. **Needle Rinse** (100% Isopropanol):
    a. To a 1 L bottle, add 1 L of isopropanol.
5. **Lipids Dilution Buffer**:
    a. To a 50mL bottle, add Buffer A.

NOTE:

- Retention time on an amino column is sensitive to changes in the mobile phase. A minor pH difference or organic composition change will result in retention time shifts of the analytes. To minimize retention time drift, prepare water/acetonitrile stock solvent in large volumes and use the same bottle of stock solvent for mobile phase preparation over the entire analysis.
- The mobile phases can be scaled up or down as needed.
- 2 M ammonium acetate stock solution can be stored in a refrigerator for up to 1 month.
- Mobile phases should be prepared fresh every 2-3 days to prevent changes in retention time that may shift the eluting analytes outside of the specified retention time windows in the scheduled MRM method.

Preparation of Samples

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

1. **Double blank sample** (100% Mobile Phase A [MPA]):
    a. Pipet 1 mL MPA to an autosampler vial.
2. **Blank sample** (100% MPA with IS spiked):
    a. For SPLASH Mix, use 5 µL in 500 µL MPA. Other I.S. can be added here as well, but be sure the final volume of the Blank sample with all I.S. is 1mL

3. **QC sample** (bovine heart extract with optional IS or user-supplied matrix):
    a. For the SPLASH mix, use a 1:40 dilution of extracted control plasma and add 5 µL SPLASH Mix to 995 µL of diluted plasma. Other I.S. can also be added here, but be sure the final volume of the QC sample with all I.S. is 1 mL.
4. **Pooled sample for method development** (with optional IS):
    b. Refer to Appendix A on suggested protocols for isolating lipids from various samples.
    c. Create a pooled sample of the biological matrix to be analyzed. It is recommended that samples from all sample types be included in the pooled sample so that most

lipids of interest are represented for method development. Dilute the matrix accordingly depending on the sample.

d. Add 5 µL SPLASH Mix to 995 µL of diluted pooled sample. Other I.S. can also be added here, but be sure the final volume of the pooled sample with all I.S. is 1 mL.

5. **Biological Samples**:
   a. Refer to Appendix A on suggested protocols for isolating lipids from various samples.
   b. Dilute the matrix accordingly depending on the sample.
   c. Add 5 µL SPLASH Mix to 995 µL of diluted pooled sample. Other I.S. can also be added here, but be sure the final volume of the pooled sample with all I.S. is 1 mL.

5.0 Building An Un-Scheduled MRM Method

This LC-sMRM acquisition method utilizes a positive/negative polarity switching method to cover a range of lipid classes in the final scheduled MRM method. However, if using the entire master assay list, the large number of lipid molecular species analyzed requires the retention times for the specific molecules to be reliably determined in separate polarities in respective unscheduled MRM methods first to allow for confident construction of a time-scheduled MRM (sMRM) acquisition method. This assay development strategy is a two-step process described in Section 9. Sections 5 – 7 will provide the required acquisition methods for the assay development process.

NOTE: If your interest lists consist of less than 1000 MRMs, then you can make one polarity-switching method for the unscheduled MRM method while keeping in mind the data quality for the unscheduled peaks may only have a few points per peak until data quality is improved in the scheduled MRM method.

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

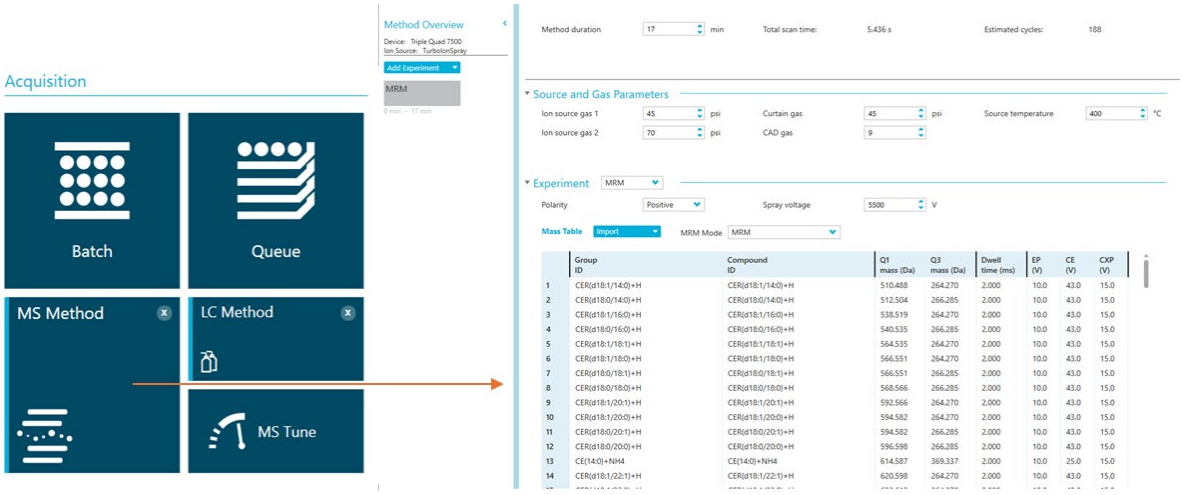


Figure 5-1: Building Separate Unscheduled MRM Acquisition Methods for Each Polarity in SCIEX OS Software.

4. Select positive polarity
5. Complete the *Source and Gas Parameters* in the acquisition method and enter the parameters outlined in Table 5-1.

Table 5-1. SCIEX Triple Quad 7500 LC-MS/MS System Parameters for Lipid Analysis

Source Parameters	Positive Polarity	Negative Polarity
IS	5200	4500
CUR	45 psi	45 psi
TEM	400 °C	400 °C
*GS1	45 psi	45 psi
*GS2	70 psi	70 psi
CAD	9 (Medium)	9 (Medium)
**These values may need to be optimized to obtain maximum sensitivity.*		
Compound Parameters		
EP	10	-10
CXP	15	-15
MS		
Scan type	MRM	MRM
Duration	17 min	17 min
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

6. 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.

7. Copy all the populated columns from the OS_OUTPUT ASSAY (+) Initial tab and paste them into the positive polarity unscheduled MRM table.
8. Change the dwell time for all MRMs in the table to 2 ms (you can change the first one and then right-click and select "***Fill Down***").
9. Click the **Advanced** tab at the top right corner and click "Show advanced parameters." Under the Advanced Experimental Settings, change the pause time to 2ms.
10. Double-check and save the method as *Lipid_Pos MRM_Unscheduled_1_date*.
11. Repeat the same steps above to create an unscheduled method with negative polarity and save the method as *Lipid_Neg MRM_Unscheduled_1_date.*
12. The next step will be constructing the LC method accompanying the MS acquisition.

NOTE: The maximum number of TOTAL MRMs in a single method (even with polarity switching) cannot exceed 1250 in SCIEX OS. If you can edit your master assay list to 1000 total MRMs or less, you can do one polarity-switching experiment for the unscheduled MRM acquisition.

6.0 HPLC System and Operating Parameters

Lipids from extracted samples are separated by HPLC mobile phases, an elution gradient on an NH2 column, and an oven, collectively outlined in Tables 6-1 and 6-2 below. Representative extracted ion chromatograms (XICs) of lipids from the acquisition method are shown in Figure 6-3, and a visual elution map of retention times for different lipid classes for each polarity can be seen in **Figure 6-4**.

Use the SCIEX OS Home icon tab or the SCIEX OS logo dropdown (top left corner) to navigate the **LC Method** workspace (see **Figure 5-1** for home tab reference). Fill out the details in each tab (binary gradient, autosampler, column oven) using the LC gradient details in **Tables 6-1 and 6-2**. Don't forget to select the appropriate injection volume. NOTE: The system controller tab is usually unnecessary unless expressly advised or set up by the service. Save the LC method as *Lipids_NH2_17 mins_Date*.

Table 6-1. LC Gradient and Mobile Phase Composition

Total Time (min)	Module	Event	Parameter	Total Flow
0	Pumps	Pump B Conc.	0.0[%]	0.2000

Table 6-1. LC Gradient and Mobile Phase Composition				
Total Time (min)	Module	Event	Parameter	Total Flow
2	Pumps	Pump B Conc.	0.0 (%)	0.2000
2.1	Pumps	Pump B Conc.	0.0 (%)	0.7000
11	Pumps	Pump B Conc.	50 (%)	0.7000
11.5	Pumps	Pump B Conc.	70 (%)	0.7000
12.5	Pumps	Pump B Conc.	100 (%)	0.7000
15	Pumps	Pump B Conc.	100 (%)	0.7000
15.1	Pumps	Pump B Conc.	0.0 (%)	0.2000
17	Pumps	Pump B Conc.	0.0 (%)	0.2000
**Mobile Phase A: 2 mM Ammonium Acetate in 7/93 DCM/Acetonitrile*				
Mobile Phase B: 2 mM Ammonium Acetate in 50/50 Water/Acetonitrile pH 8.2* *(make fresh every 3 days to ensure stable pH (or RT might shift)***				

Table 6-2. Additional HPLC Parameters and LC Settings	
Pumps	**Parameters/settings**
Flow rate	0.2 mL/min – 0.7 mL/min (see **Table 6-1**)
Pump B concentration	0.0%
Low pressure	0 psi
High pressure	9000 psi (confirm with LC hardware)
Autosampler	
Use Autosampler Check Box	Yes
Rinsing Solution	100% Isopropanol
Rinse Type	External
Rinsing volume	500 µL
Needle stroke	52 mm
Rinsing speed	35 µL/sec
Sampling speed	15 µL/sec
Purge time	25 min
Rinse dip time	3 Sec
Rinse mode	Before and After aspiration
Cooler Temperature Check Box	Yes
Cooler Temperature	15 °C
Oven	
Temperature control	Enabled
Temperature	35 °C

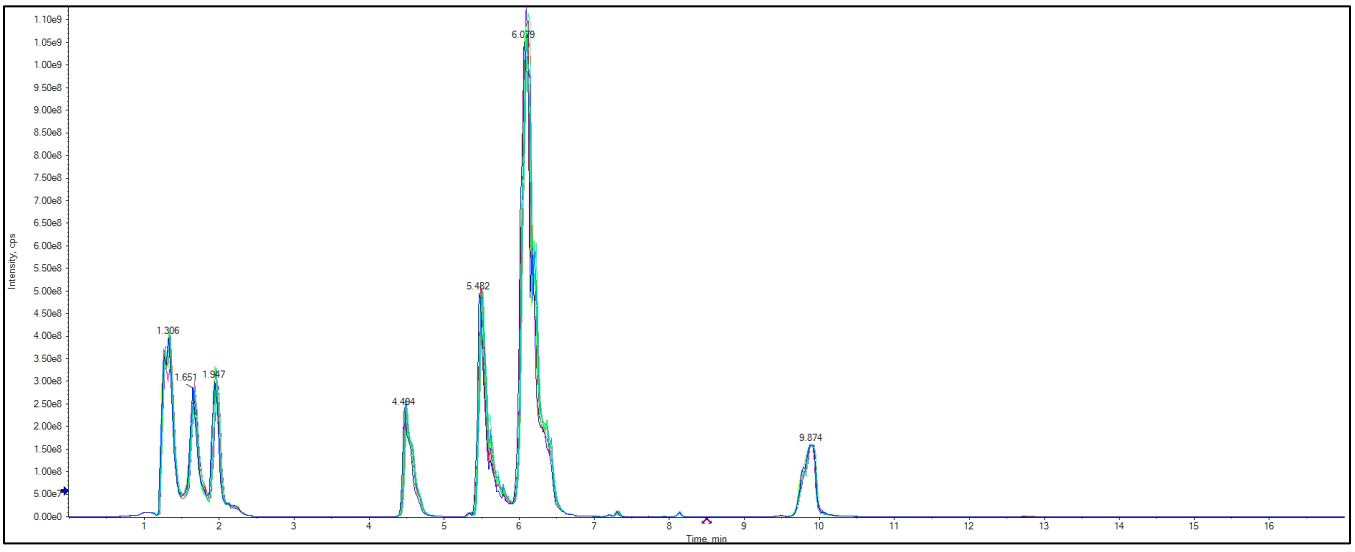


Figure 6-3. Representative XIC of the NIST 1950 plasma replicates with Selected Lipid Standards Added.

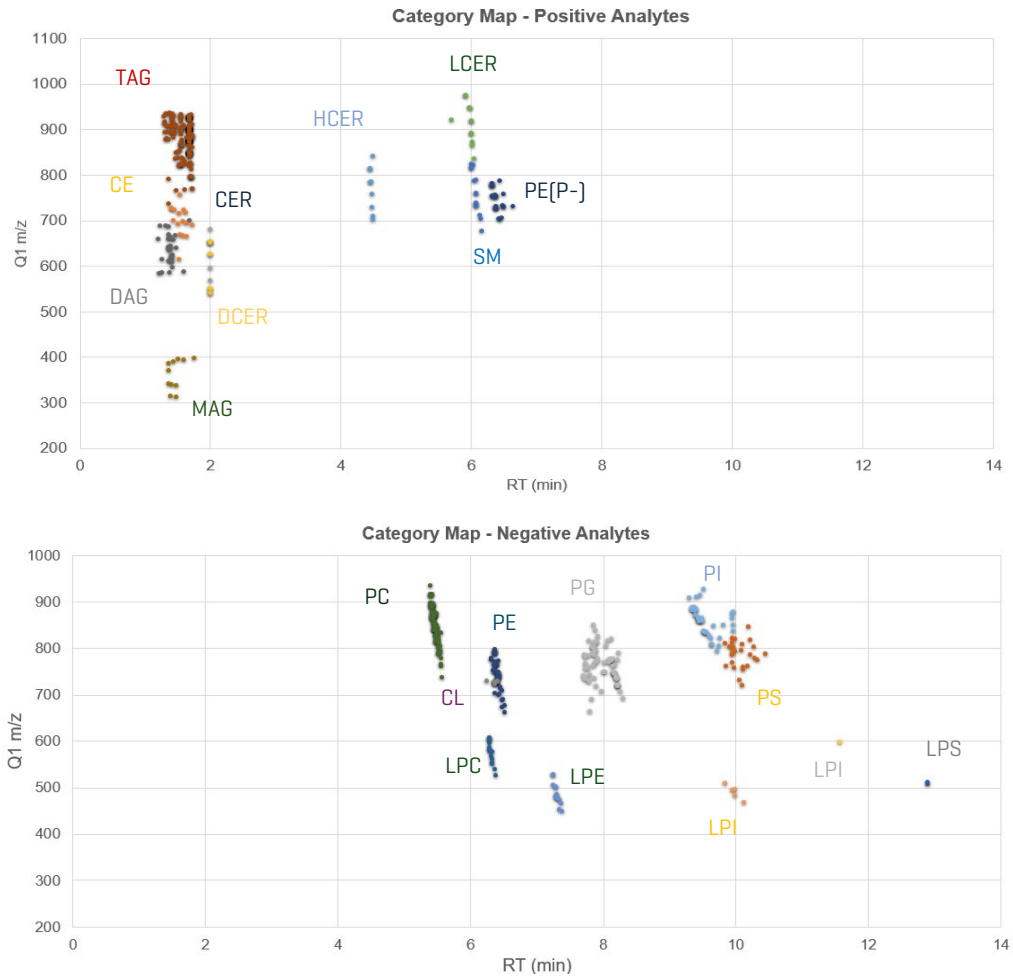


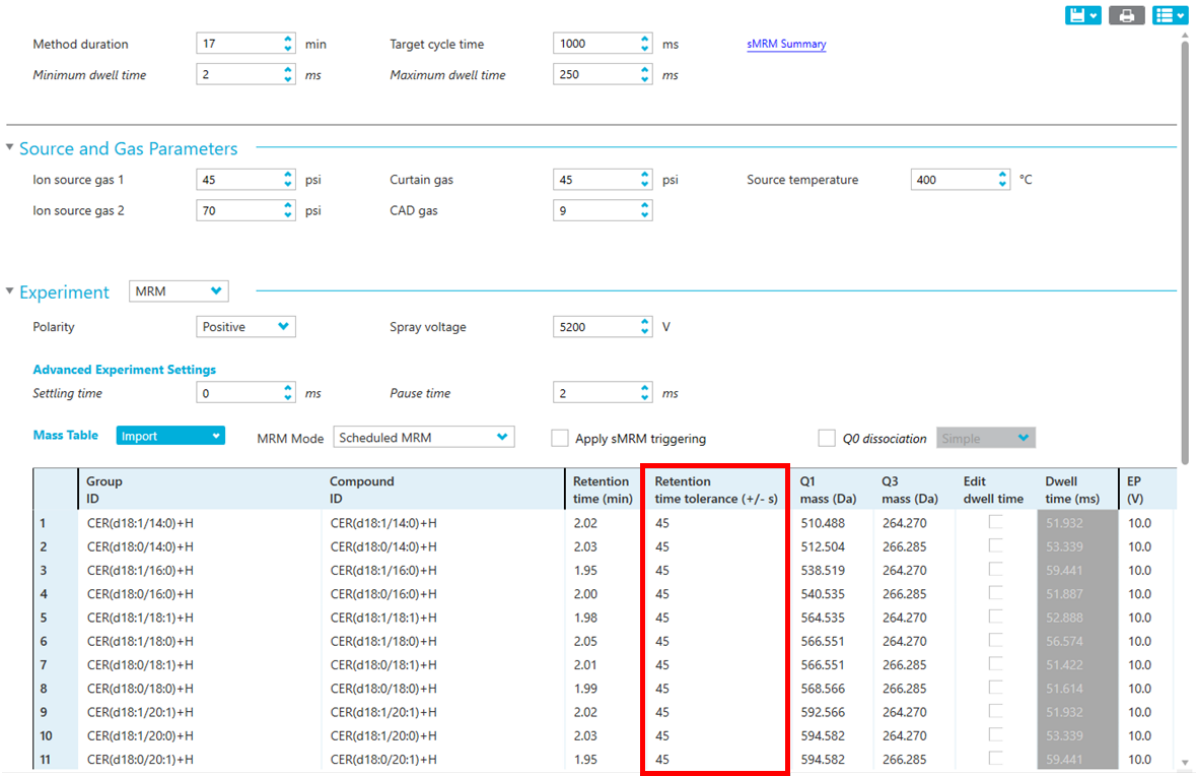
Figure 6-4. Elution Maps for Plasma with Lipid Standards Added. Top panel: lipid classes in positive mode. Bottom panel: lipid classes in negative mode.

Submit the Batch for Pooled Sample Injections: If following the protocol in order after completing section 6, the unscheduled MRM method(s) and the corresponding LC method have been constructed. These methods can be inputted in the batch, along with the other appropriate batch information that applies to your LCMS instrumentation. Save the data file (example: *Lipids_Pos Unscheduled_NH2_Date*) and submit the batch to inject replicates of the pooled sample. Tips for making data tables, determining retention times, and exporting table metrics to make the scheduled MRM method can be found in sections 8 and 9.

7. Building Acquisition Methods with the Scheduled MRM Algorithm

Two method types will be required during assay optimization: a non-scheduled method built in sections 5 and 6 and a method constructed using the Scheduled MRM Algorithm (sMRM), which is described here.

1. Open *Lipid_Pos MRM_Unscheduled_1 built* in Section 5 to start.
2. Save the method as *Lipid_sMRM_Opt_1.*
3. Clear the contents of all MRM transitions in the table.
4. Under Method Overview, select MRM from the **Add Experiment** dropdown menu and change the polarity of the newly added experiment to negative mode (notice the settling time will automatically adjust to 15ms).
5. 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 positive polarity MRM table and the *OS_Output Assay (-)* into the negative polarity MRM table.
6. Select **Scheduled MRM** from the **MRM Mode** dropdown in both MRM experiments (positive and negative). Use **Figure 7.1** to fill in the Target cycle time, Minimum dwell time and Maximum dwell time at the top of the overall method.
7. Enter 45 seconds into the retention time tolerance column for positive and negative mode MRMs (shown in the red box in Figure 7-1). Right-click and use the fill-down option.



	Group ID	Compound ID	Retention time (min)	Retention time tolerance (+/- s)	Q1 mass (Da)	Q3 mass (Da)	Edit dwell time	Dwell time (ms)	EP (V)
1	CER(d18:1/14:0)+H	CER(d18:1/14:0)+H	2.02	45	510.488	264.270		51.932	10.0
2	CER(d18:0/14:0)+H	CER(d18:0/14:0)+H	2.03	45	512.504	266.285		53.339	10.0
3	CER(d18:1/16:0)+H	CER(d18:1/16:0)+H	1.95	45	538.519	264.270		59.441	10.0
4	CER(d18:0/16:0)+H	CER(d18:0/16:0)+H	2.00	45	540.535	266.285		51.887	10.0
5	CER(d18:1/18:1)+H	CER(d18:1/18:1)+H	1.98	45	564.535	264.270		52.888	10.0
6	CER(d18:1/18:0)+H	CER(d18:1/18:0)+H	2.05	45	566.551	264.270		56.574	10.0
7	CER(d18:0/18:1)+H	CER(d18:0/18:1)+H	2.01	45	566.551	266.285		51.422	10.0
8	CER(d18:0/18:0)+H	CER(d18:0/18:0)+H	1.99	45	568.566	266.285		51.614	10.0
9	CER(d18:1/20:1)+H	CER(d18:1/20:1)+H	2.02	45	592.566	264.270		51.932	10.0
10	CER(d18:1/20:0)+H	CER(d18:1/20:0)+H	2.03	45	594.582	264.270		53.339	10.0
11	CER(d18:0/20:1)+H	CER(d18:0/20:1)+H	1.95	45	594.582	266.285		59.441	10.0

Figure 7-1: Setting Parameters for The Scheduled MRM Method.

8. Save the method and then also save it as Lipid_sMRM_Final_1. The final method is for fully optimized retention times only, and the optional method is for use on several injections of a pooled sample to ensure retention time accuracy and precision.
9. These two methods and the method(s) built in Section 5 will serve as template methods for the assay optimization steps described in Section 9.

8. Data Analyses in SCIEX OS Software

This section describes using SCIEX OS Software version 3.3.1 during assay development to determine retention times, peak areas, etc.

Before starting data processing, ensure that the Integration Parameters are set correctly: For that, from the Analytics workspace, go to Project → Project Default Settings → Under Integration defaults, in the integration Algorithm dropdown menu, select MQ4 and Apply as in **Figure 8-1**. Checking the box for "Use for new projects in current data root" is recommended unless you know you need a different integration algorithm for other workflows.

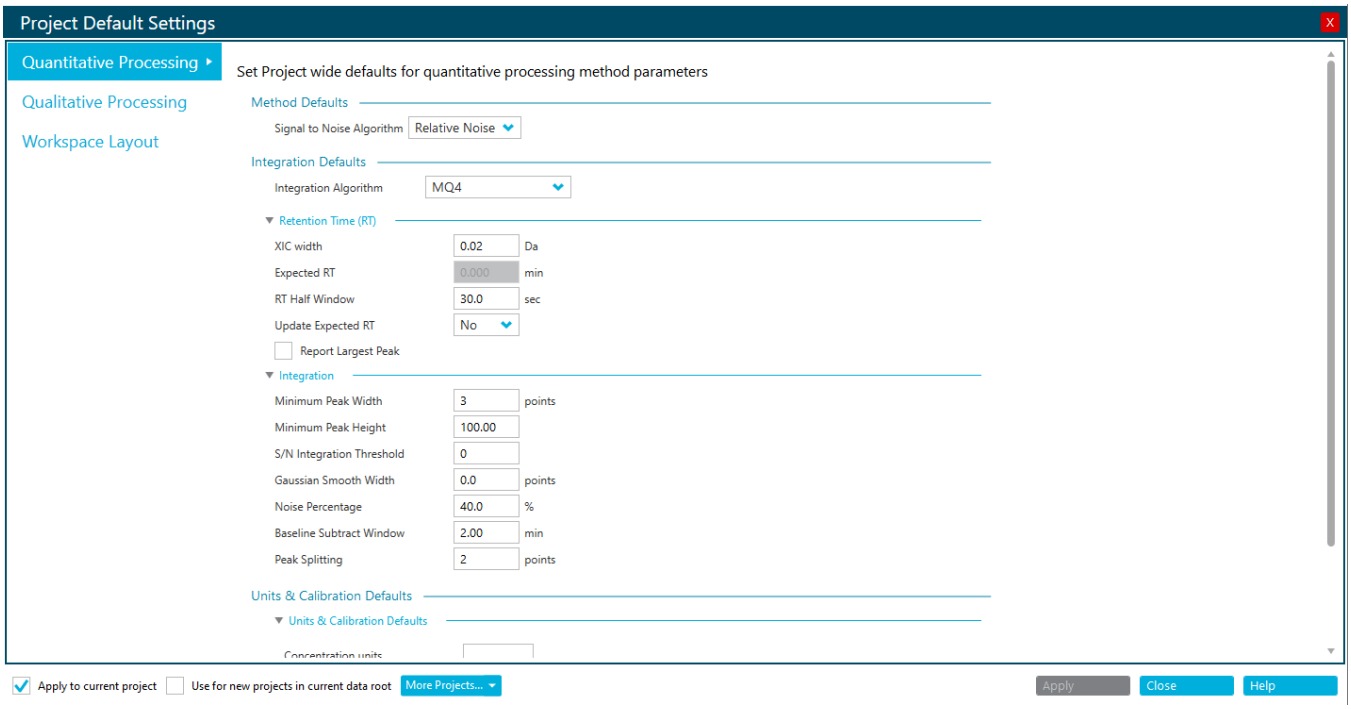


Figure 8-1. MQ4 Integration Defaults for data Processing the Targeted Lipid Profiling data.

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 them to move them to the Selected pane, or select the data file and use the "=>" button to move the data files.
3. Select **New** next to the box for "Select a processing method."

4. Select a representative injection from the data file upon which the quantitation method will be optimized. A pooled sample is suggested as a good reference. Check the box for "quantitation". The "quantitation and targeted identification" box can be unchecked unless you adapted your MRM method(s) to trigger MS2 spectra via the QTRAP.
5. The Components tab lets you view the individual lipid MRM transitions and define and assign internal standards.
6. In the Integration Tab, review the integration of each lipid species to ensure it is correctly integrated and the retention time makes sense based on the column chemistry and LC method. Depending on the biological matrix, not all lipid species will be detected.
7. Calculated columns and flagging rules can be added if desired but will not be gone over in this protocol.
8. After all MRM transitions have been reviewed, click **Save**. Example Name: Lipid_MRM_Processing_Date.
9. A previous workspace layout can be selected if desired, but this protocol will not discuss it. A comparison sample is not necessary for this analysis.
10. Click **Process** to complete.
11. Data will populate, and the results table will be saved. See section 9 for exporting table metrics (RTs, widths at 50%, and area).

9.0 Protocol for Determining Lipid Class Retention Times

The retention times of lipids are affected by multiple experimental factors, including mobile phase preparation, the matrix effect, 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 generated from the range of biological samples to be analyzed so that most lipids are represented in the pooled sample to be used in this assay development step. This section describes the iterative method development process for determining retention time and building a final highly optimized method for targeted lipid profiling using the sMRM Pro Builder Template.xlsx.

First, the Master Assay List for the Global Lipid Method should already be loaded into the Master Assay Table tab in the sMRM Pro Builder template, and class filters should be available for adjustment on the instruction tab. This allows the first unscheduled method to be built (LIPID_MRM_Unscheduled_1). LC-MS analysis is performed on the sample matrix of interest to determine rough retention times. Note: Running the positive and negative lipids in separate methods is recommended if very large numbers of MRM transitions are being analyzed at this point (>1000). Using this data, a second acquisition method (polarity switching if necessary) is built from this information (LIPID_sMRMPro_1) and then used to acquire replicate data on a pooled biological sample. Replicate injections are now performed using a single method in this second iteration using the LIPID_sMRMPro_1 method. Enough replicates should be performed to generate stable results (10 replicates are recommended). This data can then generate a final optimized LC-MRM method (LIPID_sMRM_Final_1) for the biological study. SCIEX OS Software will process the data generated in each iteration 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.

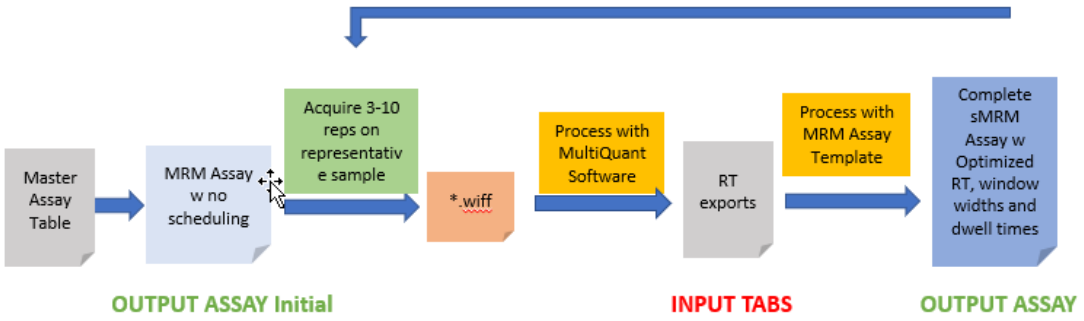


Figure 9.1. Method development workflow using sMRM pro-Builder template.

1. Create a pooled sample of the biological matrix for the study (Section 4). Including a sample from the various sample types to be studied (i.e., condition 1 vs. condition 2) is recommended so that most lipids will be represented in the pooled sample. The sample can also include internal standards, as described in Section 4. It is strongly recommended that internal standards be used to determine the approximate retention time for each lipid class.
2. Place the pooled samples into the autosampler in known vial positions.
3. Copy the populated columns from the tab called *OS_OUTPUT ASSAY [+] Initial* and paste into the positive polarity MRM table and copy the populated columns from the tab called *OS_OUTPUT ASSAY [-] Initial* and paste into the negative polarity MRM table. If very large numbers of MRMs are to be tested (> 1000), running this step as two separate methods, one for positive and one for negative, is recommended.
4. Save the method as *LIPID_<Polarity> MRM_Unscheduled_1*.
5. To equilibrate the LC-MS system, perform 3 injections of the Double Blank sample using the LIPID_MRM_Unscheduled_1.dam acquisition method.
6. Perform 3 injections of the pooled sample using the *LIPID_MRM_Unscheduled_1* acquisition method.
7. Process the data in SCIEX OS Software (Section 8), then export the results for analysis in the sMRM Pro Builder. Select Reporting → Export Results→ Results Table – Metric, then export the Area, Retention Time, and Width at 50%, as shown in **Figure 9.2**.

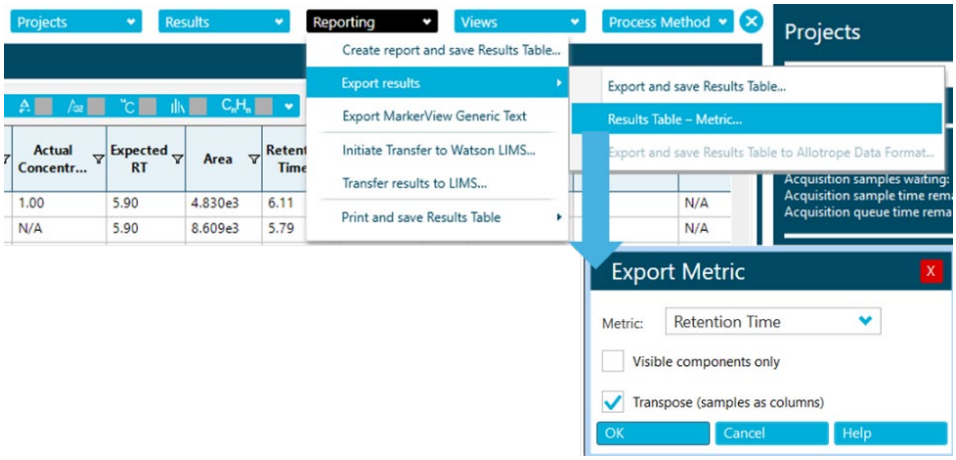


Figure 9.2. Exporting the Results Table Metrics from SCIEX OS Software for use in sMRM Pro Builder.

8. Paste the 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.
9. The template can filter out lipids that are not detected 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 lipids be filtered out of the assay, so remember to set the Assay Subset for Output to All—**Figure 9.3**.

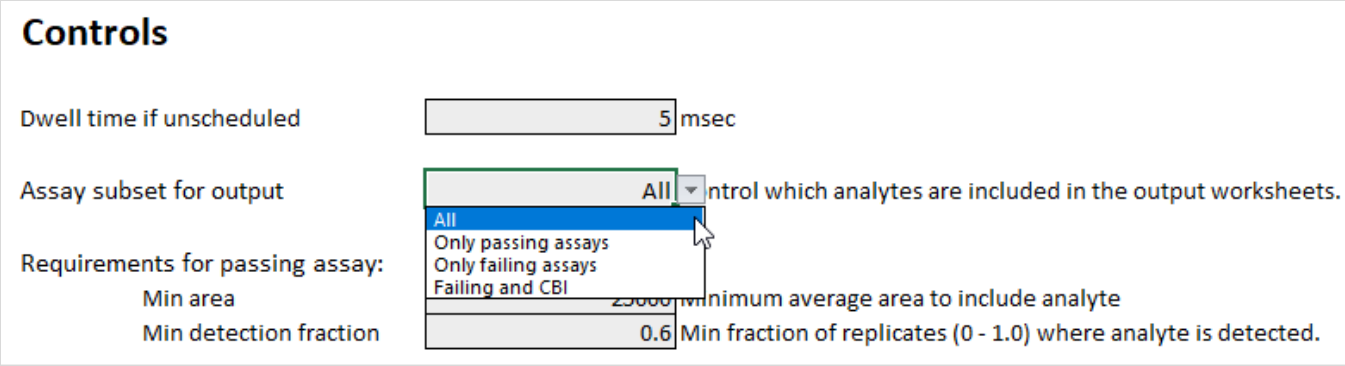


Figure 9.3. Filtering Controls in the sMRM Pro Builder Template.

10. 10. Paste the new MRM lists from the OS_OUTPUT ASSAY (+) and OS_OUTPUT ASSAY (-) tabs into the Lipid_sMRM_Opt_1 from Section 7 and rename. Note that the assay now includes rough retention times for all lipids. Lipids that were not reliably detected are assigned the retention time of their lipid class.
11. Next, use this time-scheduled method to perform 5-10 replicate injections of the Pooled Sample. This data will create the final highly optimized Scheduled MRM Algorithm.
12. Process the replicate data in SCIEX OS Software, then export the results described above in step 7. At this point, careful data review is recommended to ensure the correct lipids are being integrated. As the next round of analysis in the template will be used to reject lipids that are not reliably detected, ensuring the input data is of good quality is essential.
13. Paste the exports into the Excel template again (Input-RT, Input-Area, Input-Width tabs). To have the template automatically filter out the undetected lipids, set the *Assay subset for output* setting to *Only Passing Assays* (**Figure 9.3**). Then click Calculate (F9).
14. Paste the refined MRM lists from the *OS_OUTPUT ASSAY (+) and OS_OUTPUT ASSAY (-)* tabs into the *LIPID_sMRM_Final_1 from Section 7* and rename. This is the final, fully

optimized method for targeted lipid profiling on this biological matrix. Lipids 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 were determined using the following suggested sample extractions.

A1. Extraction Protocol for Plasma

1. Use 13 x 100 mm new glass screw-capped tubes. Do not use washed tubes, as you may extract detergent residue.
2. To 25 µL plasma, add 975 µL H_2O; let sit on ice for 10 min.
3. Add 2.0 mL MeOH
4. Add 0.9 ml of Dichloromethane (CH_2Cl_2).
5. Vortex
6. Make sure there is a monophase at this stage. If two distinct phases are observed, add 50 µL MeOH and vortex and check whether the solution is a single phase. If not, repeat the addition of 50 µL MeOH and vortex.
7. Add internal standard and vortex and let mixture sit for 30 min at room temperature.
8. Add 1 ml H_2O
9. Add 0.9 ml CH_2Cl_2
10. Invert tubes 10 times. DO NOT VORTEX, or an emulsion will be formed.
11. Centrifuge at 1200 rpm for 10 min.
12. Collect the lower layer and put it into a fresh glass tube.
13. Add 2 mL CH_2Cl_2 to the remains in the extraction tube.
14. Mix, centrifuge, collect the lower layer and add to the first extract.
15. Evaporate solvent under a stream of nitrogen.
16. Re-suspend lipids in injection solvent.

A2. Extraction Protocol for Cell Culture

1. Use 13 x 100 mm new glass screw-capped tubes. Do not use washed tubes, as you may extract detergent residue.
2. Collect cells:
    a. Wash cells with non-buffered saline to remove the cell culture medium.
    b. For cells in suspension, centrifuge, discard saline and add 1 mL H_2O. Vortex and transfer to a glass tube for extraction. Allow to rest on ice for 10 min. Ensure the final volume is 1 mL—adjust if necessary.
    c. c. For adhered cells, wash them with non-buffered saline. Add 1 mL H2O to lyse them and scrap. Collect the cell lysate and transfer it to a glass tube for extraction. Allow it to rest on ice for 10 min. Ensure the final volume is 1 mL—adjust if necessary.
3. Add 2.0 mL MeOH
4. Add 0.9 ml CH_2Cl_2
5. Vortex
6. Make sure there is a monophase at this stage. If two distinct phases are observed, add 50 µL MeOH and vortex and check whether the solution is a single phase. If not, repeat the addition of 50 µL MeOH and vortex.
7. Add internal standard and vortex and let mixture sit for 30 min at room temperature.
8. Add 1 ml H_2O
9. Add 0.9 ml CH_2Cl_2
10. Vortex
11. Centrifuge at 1200 rpm for 10 min.
12. Collect the lower layer and put it into a fresh glass tube.
13. Add 2 mL CH_2Cl_2 to the remains in the extraction tube.
14. Mix, centrifuge, collect the lower layer and add to the first extract.
15. Evaporate solvent under a stream of nitrogen.
16. Re-suspend lipids in injection solvent.

A3. Extraction Protocol for Solid Tissue

1. Weigh the tissue to be extracted; ~50-100 mg is sufficient. Calculate water content in tissue:
    a. Adipose 18%
    b. Brain 60%
    c. Bone 44%
    d. Average value for Liver, Kidney, lung, heart, spleen, intestines and stomach is 65%
    e. Testes 18%
2. Add water to the tissue so that total water volume = 1 mL
    a. E.g., 100 mg Brain corresponds to 60 µL water—add 940 µL water
3. Homogenize tissue using a bead mill or equivalent
4. Transfer homogenate into a 13 x 100 mm glass screw top tube.
5. Add 2.0 mL MeOH
6. Add 0.9 ml CH_2Cl_2
7. Vortex
8. Make sure there is a mono-phase at this stage. If two distinct phases are observed, add 50 µL MeOH and vortex, and check whether the solution is a single phase. If not, repeat the addition of 50 µL MeOH and vortex.
9. Add internal standard and vortex and let mixture sit for 30 min at room temperature.
10. Add 1 ml H_2O
11. Add 0.9 ml CH_2Cl_2
12. Vortex
13. Centrifuge at 1200 rpm for 10 min.
14. Collect the lower layer and put it into a fresh glass tube.
15. Add 2 mL CH_2Cl_2 to the remains in the extraction tube.
16. Mix, centrifuge, collect the lower layer and add to the first extract.
17. Evaporate solvent under a stream of nitrogen.
18. Re-suspend lipids in injection solvent.

Appendix B – Sample Processing using Analyst and MultiQuant Software

Building An Un-Scheduled MRM Method

This LC-MRM acquisition method utilizes a positive/negative polarity switching method to cover various lipid classes. Due to the large number of lipid molecular species analyzed 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 described in Section 9. In Sections 5 – 7, the required acquisition methods will be built for the assay development process.

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

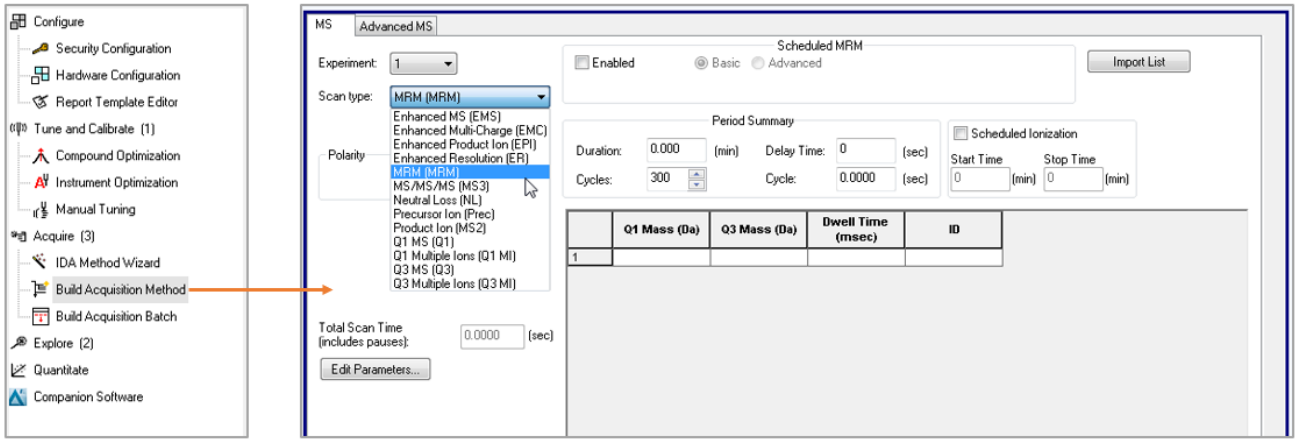


Figure B-1: Building an Acquisition Method in Analyst Software.

4. Select positive polarity for the first experiment 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.
5. In the left-hand navigation bar of the method, right-click on Period to add a new experiment. Select negative polarity for the second experiment as indicated in the red box in **Figure B-2**.

Move the mouse to where the blue box is shown and right-click to add the Collision Energy (CE) to the MRM table.

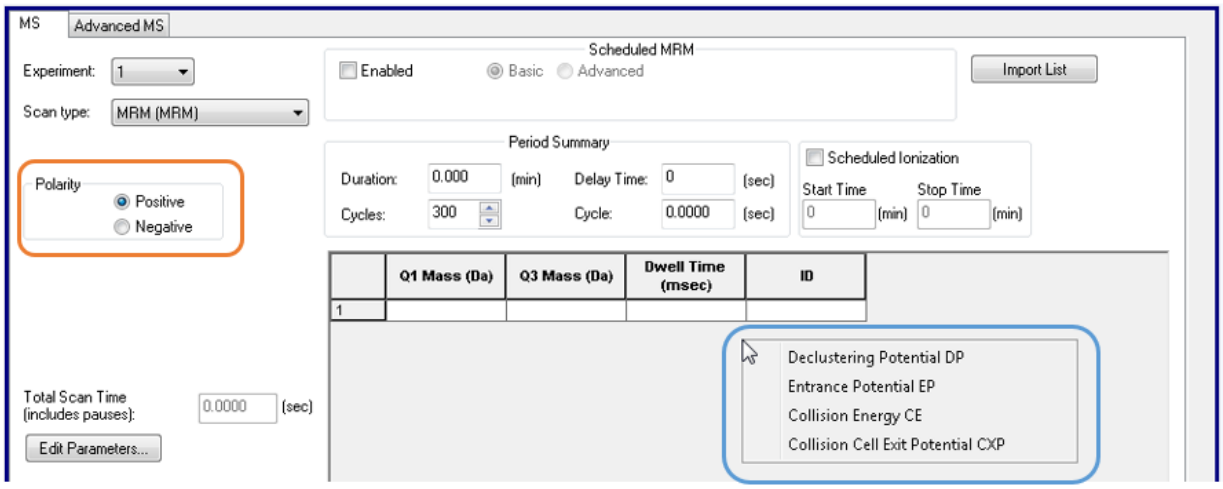


Figure B-2: Defining the Positive Polarity Information for Experiment 1.

6. In the acquisition method, click Edit Parameters and enter the parameters outlined in Table B1-1 for each polarity.

Table B-1. QTRAP System 6500+ LC-MS/MS System Parameters for Lipid Analysis		
Source Parameters	**Positive Polarity**	**Negative Polarity**
IS	5500	-4500
CUR	35 psi	35 psi
TEM	550 °C	550 °C
*GS1	50 psi	50 psi
*GS2	60 psi	60 psi
CAD	8 or Medium	8 or Medium
**These values may need to be optimized to obtain maximum sensitivity.*		
Compound Parameters		
DP	60	-80
EP	10	-10
CXP	15	-15
MS		
Scan type	MRM	MRM
Duration	17.5 min	17.5 min
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

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 table and copy **Columns A – E** from the tab called *Analyst_OUTPUT ASSAY [-] Initial* and paste into the negative polarity MRM table.
8. Save the method as *LIPID_MRM_Unscheduled_1.dam*.
9. The next step will be adding chromatography settings to this method in Section 6.

Building Acquisition Methods with Scheduled MRM Algorithm Pro

Two method types will be required during assay optimization: a non-scheduled method built in sections 5 and 6 and a method constructed using the *Scheduled* MRM Algorithm Pro in enhanced mode, described here.

1. Open the MRM acquisition method build in Section 5 to start.
2. Select the Scheduled MRM Algorithm by checking the **Enable** box in **Figure B.3**. Use the Advanced Mode.
3. Fill in the Scheduled MRM Algorithm Parameters as shown in **Table B.2**.

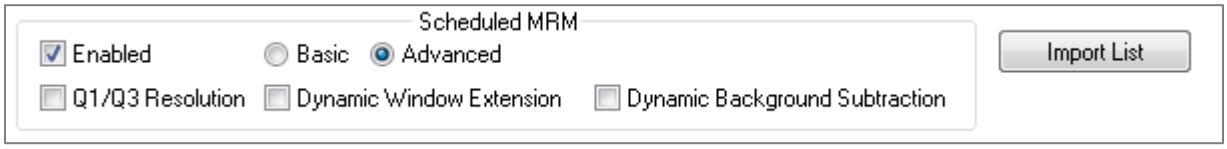


Figure B-3: Select the Scheduled MRM Algorithm – Enhanced mode.

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)	90	90
Target Scan Time (sec)	0.5	0.5

4. Select Basic, as shown in Figure B-3, and save the method as *LIPID_sMRMPro_1.dam.*

5. Next, select Advanced and save the method as *LIPID_sMRM_Final_1.dam.*
6. These two methods and the method built in Section 6 (*LIPID_MRM_Unscheduled_1.dam*) will serve as template methods for the assay optimization steps described in Section 9.

Data Analysis in MultiQuant Software

This section describes using MultiQuant Software version 3.0.2 or later during assay development to determine retention times, peak areas, etc.

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

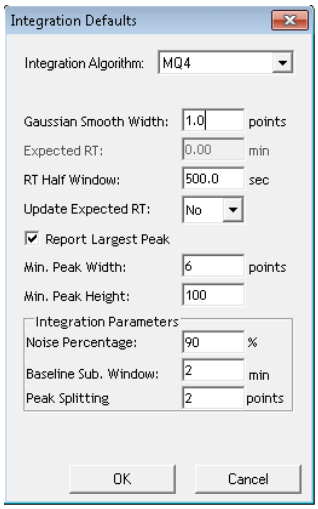


Figure B-4. Integration Defaults for data Processing the Targeted Lipid Profiling data.

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 them 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 Lipid 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 lipids.
7. the individual lipids can be viewed on the Define Components Pane. Click **Next**.

8. Integrations can now be reviewed for each lipid. Review the integration of each lipid species to ensure it is correctly integrated. Depending on the biological matrix, not all lipid species will be detected.
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 be saved and reviewed.

Appendix C – Making Methods Without Using The sMRM Pro Builder

Although the sMRM Pro Builder worksheet is a helpful tool for creating unscheduled and scheduled MRM methods and providing valuable statistics for detection, using the sMRM Pro Builder to create a scheduled MRM method is unnecessary. This protocol will walk users through creating unscheduled and scheduled MRM methods without using the sMRM Pro Builder.

1. Open a new MRM experiment from the MS Method tile on the home screen of SCIEX OS (**Figure 5-1**), select the desired polarity from the dropdown, and adjust the source and gas parameters to be the same as seen in **Table 5-1**.
2. Copy and paste any desired transitions from the Master Assay List into the mass table of the MRM method and be sure to match the column format of the MRM method. Also, make sure the correct polarity is used for each MRM transition.
3. It is suggested to use a pause time of 3ms and a dwell time of 3ms, but for large lists of transitions, the pause time and dwell time can be set to 2ms each for the unscheduled MRM to find retention times.
4. Save the method with a name that denotes the types of analytes, the polarity of the experiment(s), and that it is unscheduled. For example, *Lipid_Pos MRM_Unscheduled_Date.*
5. Repeat steps 1-4 for any transitions of the opposite polarity chosen in step 1, make sure the correct polarity is selected from the dropdown, and follow the applicable source conditions. Save the method (Example: *Lipid_Neg_MRM_Unscheduled_Date*).
    - ***NOTE:*** If your TOTAL number of MRMs is less than 1000 transitions, you can setup a single, polarity-switching experiment by adding an MRM experiment to a previous existing MRM experiment (the settling time should automatically change to 15ms after the polarity of the new experiment is selected to be different from the pre-existing one). Save the method (Example: *Lipid_Pos Neg MRM_Unscheduled_Date*).
6. Construct the same LC method as instructed in section 6 and follow the same elution gradient seen in **Table 6-1**.
7. Create a pooled sample (outlined in more detail in section 9) containing different treatment types of the same biological matrix used in the study (e.g. WT, KO, Control, etc.). These samples should have essentially all analytes of interest present.
8. Perform replicate injections of the pooled sample using the above MS and LC methods. If you require more information, follow section 9 for instructions on doing replicate injections of the pooled sample.

9. Use the Analytics section of SCIEX OS to construct a data table of MRMs detected in the pooled sample. Export the retention times from the data table. Follow section 9 for instructions on exporting retention times.
10. Open the exported retention times in Excel and make sure all "N/A" are changed to "0". For this, you can use the "Find & Replace" option in Excel.
11. Open the previously saved unscheduled MRM method and save as (example: *Lipid_Polarity sMRM _Date)*. Add a new MRM experiment of the opposite polarity if the unscheduled MRM method(s) were separated due to scanning for > 1000 Total MRMs.
12. Select "Scheduled MRM" in the MRM Mode dropdown menu (do this for both positive and negative polarity experiments if applicable).
13. Copy/paste the retention times for MRMs from the export file into each respective MRM experiment (positive and negative polarities).
14. A retention time tolerance of 45 sec is suggested for each MRM, but this can be adjusted if needed.
15. Remove any transitions that have a retention time of "0.00".
16. Save the method.
17. This method should now be used to acquire replicate injections of the pooled sample to ensure stability of peak shape and retention times of MRM transitions. If any edits need to be made to the sMRM method, do so and save the method as *Lipid_Polarity sMRM _Final_Date.*

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