



**Comprehensive targeted method
for global lipidomics analysis**



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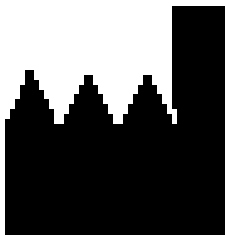
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1.0 Introduction

Aim of the method

Quantification of lipids in complex biological samples is challenging and often requires a detailed knowledge of lipids and lipids 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 quantification of a very large number of lipid species across multiple lipid classes using a targeted LC-MS method.

Here, a detailed LC-MRM method is provided that will enable comprehensive coverage of many of the multiple lipid classes present 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 to be a starting point for further method development by users. 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 polar stationary phase (SP) and 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 but it is believed that dipole interactions (via H-bonding) and ion-exchange often dominate the selectivity and retention; while in RPLC hydrophobic partitioning plays a more key role and the elution time can be adjusted by changing the mobile phase composition (B%). Like IEX, polar compounds tend to retain on 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.

Quantification strategy

The method described here can utilize different strategies for relative lipid quantification. 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 quantification and comparison between sample groups. A second approach uses the Lipidyzer platform standards kit, which contains multiple internal standards (up to 10) per lipid class, covering phospholipid classes PC/LPC, PE/LPE, PS/LPS, PI/LPI, PG/LPG, MG, DG, TG, CL, CE, CER, DCER, HexCER, LacCER and SM. This approach allows for a more accurate quantification, because endogenous lipid species are matched to the most closely resembling internal standard. User-selected internal standards can be added to the method for quantification of 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 for this variability, an Excel tool (sMRM Pro Builder) was developed to assist in acquisition method development. The workflow consists of an initial unscheduled method to determine very 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 information, a final acquisition method using the Scheduled MRM algorithm is developed, consisting of over 1900 lipid species. The advanced features including flexible retention tolerance and dwell time weighting is used to improve the resulting data quality. This iterative two-step process using this Excel tool will allow users to quickly adapt this method to their LC-MS setup. It is **strongly recommended** to use an internal standard (such as SPLASH or Lipidyzer kits) to ensure that the correct retention time is determined from the unscheduled acquisition.

Please review the method documentation completely before using this method. 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/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 μ L 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 2.0 or later
- Microsoft Excel template – [sMRM Pro Builder Template Mx Lx 1.4](#)
- [Master Assay List Global Lipid Method 1.6](#)

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 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. Assay result may deviate from this optimized method if using other reagents other than the ones suggested in this SOP.

Table 3-2. Lipid standards		
Supplier	Description	Part number
Avanti Polar Lipids, Inc. https://avantilipids.com/	SPLASH Lipidomix Mass Spec Standard	330707
	17:1 Lyso PG	858127C
	17:1 Lyso PS	858141C
	17:1 Lyso PI	850103P
SCIEX	Lipidyzer QC Spike	5040408
SCIEX	AA 45/32™ Phys Control Plasma	4386703
SCIEX https://sciex.com	Internal Standard Kit for Lipidyzer platform **Available as specific lipid classes as well**	5040156

The above listed standards are recommended to order to cover wide ranges of lipid classes during method development. They are not mandatory for the assay, depending on lipid classes required for study.

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 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 critical 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 impact on performance of the assay, and alterations of the procedure 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 towards 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 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 2 mL 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 2 mL ammonium acetate stock solution and mix vigorously.
 - b. Add ammonium hydroxide ($\text{NH}_3\%$ = 28-30%) to adjust pH of mobile phase B. The pH values of mobile phase B should be adjusted to pH 8.2.
4. **Needle rinse** (100% Isopropanol):
 - a. To a 1 L bottle, add 1 L of isopropanol.
5. **Lipids dilution buffer**:

- a. To a 50 mL bottle, add Buffer A.

NOTE:

- Retention time on an Amino column is sensitive to changes of mobile phase. A minor pH difference or organic composition change will result in retention time shifts of the analytes. To minimize retention time drift, try to prepare water/acetonitrile stock solvent in large volume 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 refrigerator up to 1 month.
- Mobile phases should be prepared fresh every two-three days to prevent changes in retention time that may shift the eluting analytes outside of the specified retention time windows.

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 μ L in 500 μ L MPA.
 - b. For Lipidizer kits, use 5 μ L in 500 μ L MPA.
3. **QC sample** (bovine heart extract with optional IS, or user supplied matrix):
 - a. For SPLASH mix, use a 1:40 dilution of extracted control plasma*, add 5 μ L SPLASH Mix into 995 μ L of diluted plasma.
 - b. For Lipidizer kits, use a 1:40 dilution of extracted control plasma, add 5 μ L of each lipid standard into 935 μ L of diluted plasma. Remember to record the lot number for the standard mix for use in downstream computations.

*Note: For the Bovine heart extract, the working solution should be 10 μ g/mL for the SCIEX 7500 system and 100 μ g/mL for the SCIEX 6500+ solution. Therefore, dilute 1:1e5 in 50:50 methanol:dichloromethane and then further dilute to 10 μ g/mL in mobile phase A.
4. **Pooled sample for method development** (with optional IS):
 - a. Refer to Appendix A on suggested protocols for the isolation of lipids from various samples.

- b. 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 such that most lipids are represented in the pooled sample for method development. Dilute matrix accordingly depending on sample.
- c. Add 5 μL SPLASH Mix into 995 μL of diluted pooled sample.
- d. Add 5 μL of each lipid standard into 935 μL of diluted pooled sample.

5. Biological samples:

- a. Refer to Appendix A on suggested protocols for the isolation of lipids from various samples.
- b. Dilute matrix accordingly depending on sample.
- c. Add 5 μL SPLASH Mix into 995 μL of diluted pooled sample.
- d. Add 5 μL of each lipid standard into 935 μL of diluted pooled sample.

Note: the sample preparation protocol has been optimized for a 5 μL injection volume

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 lipid classes. Due to the large numbers 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 multi-step process which is described in Section 9. Here is Section 5 – 7, 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*.

The screenshot displays the SCIEX OS software interface. On the left, the 'Acquisition' panel features buttons for 'Batch', 'Queue', 'MS Method', 'LC Method', and 'MS Tune'. A red arrow points from the 'MS Method' button to the right-hand configuration panel. The configuration panel includes a 'Method Overview' section at the top with fields for 'Method duration' (17 min), 'Total scan time' (0.450 s), and 'Estimated cycles' (2267). Below this is the 'Source and Gas Parameters' section with fields for 'Ion source gas 1' (45 psi), 'Ion source gas 2' (50 psi), 'Curtain gas' (45 psi), 'CAD gas' (9 psi), and 'Source temperature' (400 °C). The 'Experiment' section is set to 'MRM' with 'Polarity' set to 'Positive' and 'Spray voltage' at 5000 V. The 'Advanced Experiment Settings' section includes 'Setting time' (15 ms), 'Flower time' (5 ms), 'Q1 resolution' (Unit), and 'Q3 resolution' (Unit). At the bottom, the 'Mass Table' is displayed with columns for 'Group #', 'Compound ID', 'Q1 m/z', 'Q3 m/z', 'Dwell time', 'EP', 'CE', and 'CXP'. Two rows of data are visible:

Group #	Compound ID	Q1 m/z	Q3 m/z	Dwell time	EP	CE	CXP
1	SM - SM/PS 1/PS-H ₂ O_SPLASH IS	718.663	184.200	30.000	10.0	43.0	15.0
2	CE - CE/PS-NH ₄ OF_SPLASH IS	675.960	369.400	30.000	10.0	25.0	15.0

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

4. Select positive polarity for the first experiment as indicated in the orange box in **Figure 5-2**.
5. Create two experiments, one for positive and negative polarities for the unscheduled acquisition and run separate injections for scheduling each polarity.
6. Adjust the Target Cycle time to 1000 msec as show in **Figure 5.2** indicated by the green box.

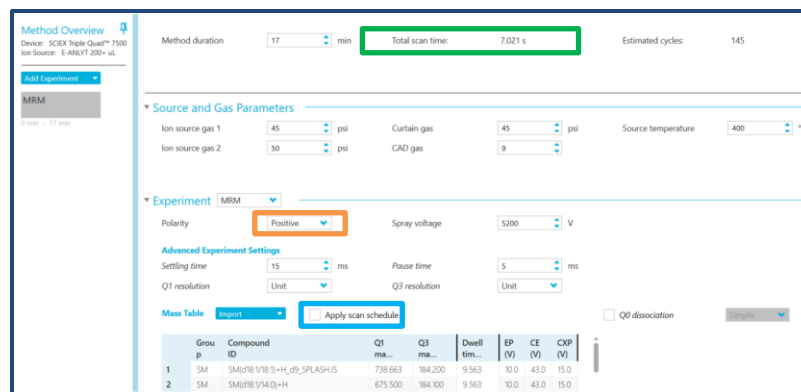


Figure 5-2: Defining the positive polarity information for experiment 1.

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.
8. Copy all the populated columns from the tab called *OS_OUTPUT ASSAY (+) Initial* and paste into the positive polarity MRM table and copy all the populated from the tab called *OS_OUTPUT ASSAY (-) Initial* and paste into the negative polarity MRM table.
9. Save the method as *LIPID_MRM_Unscheduled_1.dam*.
10. Next step will be to add the chromatography settings to this method in Section 6.

Note: if using large numbers of MRM transitions from the Master Assay List (>1000), build two separate methods, one for positive mode and one for negative mode for the unscheduled methods. Then after the retention times are optimized these can be combined into a single time-scheduled method in Section 7.

11. Complete the *Source and Gas Parameters* in the acquisition method and enter the parameters outlined in [Table 5-1](#) for each of the polarities.

Table 5-1. SCIEX Triple Quad 7500 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	50 psi	50 psi
CAD	9 or Medium	9 or Medium
<i>*These values may need to be optimized to obtain maximum sensitivity.</i>		
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

7.0 HPLC system and operation parameters

Lipids from extracted samples are separated by HPLC using the mobile phases and gradient conditions outlined in **Table 6-1**. Divert valve settings, initial conditions, autosampler conditions, and oven settings are outlined in **Table 6-2** below.

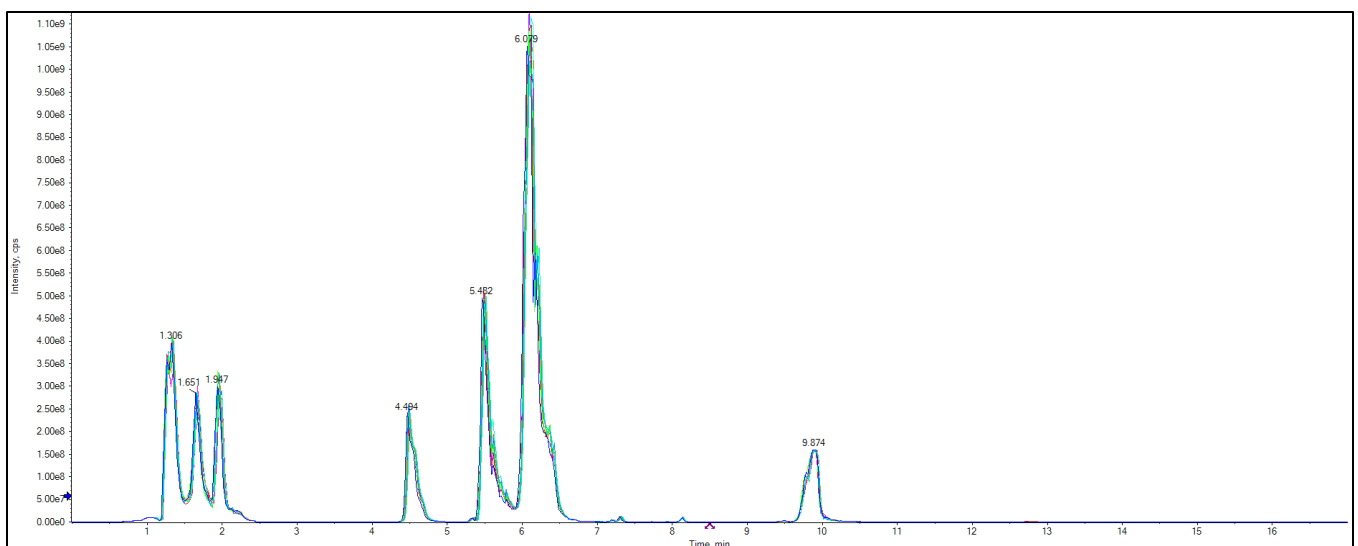
In the acquisition method that was saved in [Section 5](#), click on the ExionLC system in the left navigation panel of the method. Fill out the details in each tab, using the LC gradient details in **Table 6-1 and 6-2**. Save the method again as *LIPID_MRM_Unscheduled_1.dam* after LC information has been added.

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
2	Pumps	Pump B Conc.	0.0 (%)	0.2000
2.01	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
<i>*Mobile Phase A: 2 mM Ammonium Acetate in 7/93 DCM/Acetonitrile</i>				
<i>*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))</i>				

Table 6-2. Additional HPLC parameters and LC settings (ExionLC AC system)

Pumps	Parameters/settings
Flow rate	0.2 mL/min
Pump B concentration	0.0%
Low pressure	0 psi
High pressure	6000 psi
Autosampler	
Use Autosampler	Select AS
Rinsing Solution	100% Isopropanol
Rinse Type	External
Rinsing volume	500 µL
Needle stroke	52 mm
Rinsing speed	35 µL/sec
Sampling speed	5 µL/sec
Purge time	25 min
Rinse dip time	3 Sec
Rinse mode	After aspiration
Cooler Enabled	Yes
Cooler Temperature	15 °C
Oven	
Temperature control	Enabled
Temperature	35 °C

Note: these settings have been optimized for the ExionLC AC system, please adapt for the LC system being used. Key aspects are to ensure the isopropanol is used to rinse the lines, after aspiration.

**Figure 6-3.** Representative XIC of the NIST 1950 plasma replicates with selected lipid standards added.

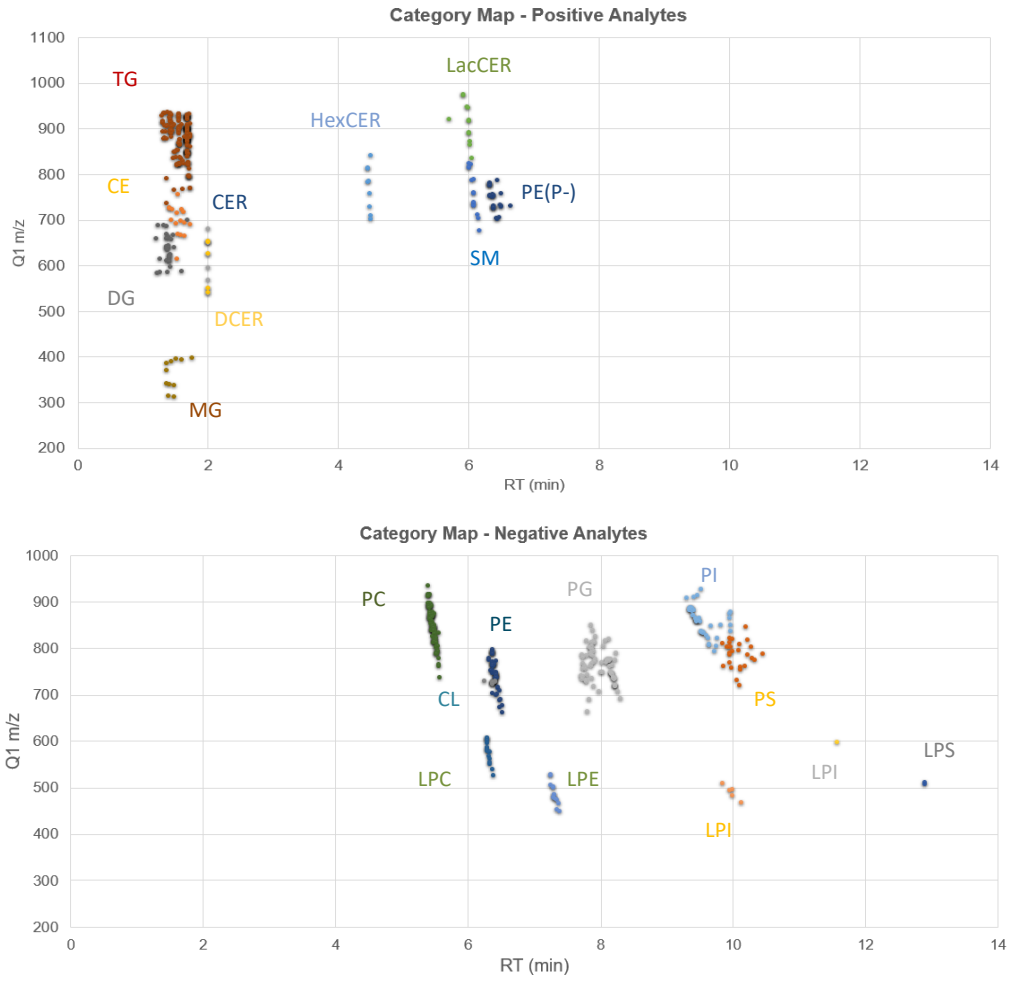


Figure 6-3. Elution maps for plasma with lipid standards added.

8.0 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 and 6 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 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, fill in 45 seconds into the Retention time tolerance column shown by the red box. Right click and use the fill down option.

▼ Experiment MRM

Polarity Positive Spray voltage 5200 V

Mass Table Import Apply scan schedule Apply sMRM triggering

Group ID	Compound ID	Q1 mass (Da)	Q3 mass (Da)	Edit Dwell Time	Dwell time (ms)	EP (V)	CE (V)	CXP (V)	Retention time (min)	Retention time tolerance (+/- s)
1	SM(18:1)+...	738.663	184.200		3,000	10.0	43.0	15.0	6.08	17
2	SM(14:0)+H	675.500	184.100		3,000	10.0	43.0	15.0	6.16	16
3	SM(16:0)+H	703.600	184.100		3,000	10.0	43.0	15.0	6.16	26
4	SM(18:0)+H	731.600	184.100		3,000	10.0	43.0	15.0	6.08	18
5	SM(18:1)+H	729.600	184.100		3,000	10.0	43.0	15.0	6.08	17
6	SM(20:0)+H	759.600	184.100		3,000	10.0	43.0	15.0	6.08	17
7	SM(20:1)+H	757.600	184.100		3,000	10.0	43.0	15.0	6.08	17

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

4. Adjust the Target Cycle time to 1000 msec as show in **Figure 5.2** indicated by the green box.
5. Using the computed MRM table in the *sMRM Pro Builder.xlsx* workbook, and paste the populated columns from the tab called *OS_Output Assay (+)* in to the positive polarity MRM table and the *OS_Output Assay (-)* into the negative polarity MRM table.
6. Save the method as *Lipid_sMRM_Opt_1.dam* or *Lipid_sMRM_Final_1.dam* depending on which method optimization step is being performed.
7. These two methods along with the method built in Section 5 (*LIPID_MRM_Unscheduled_1.dam*) will serve as template methods for the assay optimization steps described in Section 9.

9.0 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.
2. Click Process Method then Edit Embedded Method. Click Integration in the left hand panel. Set the integration defaults as shown in **Figure 8-1**.

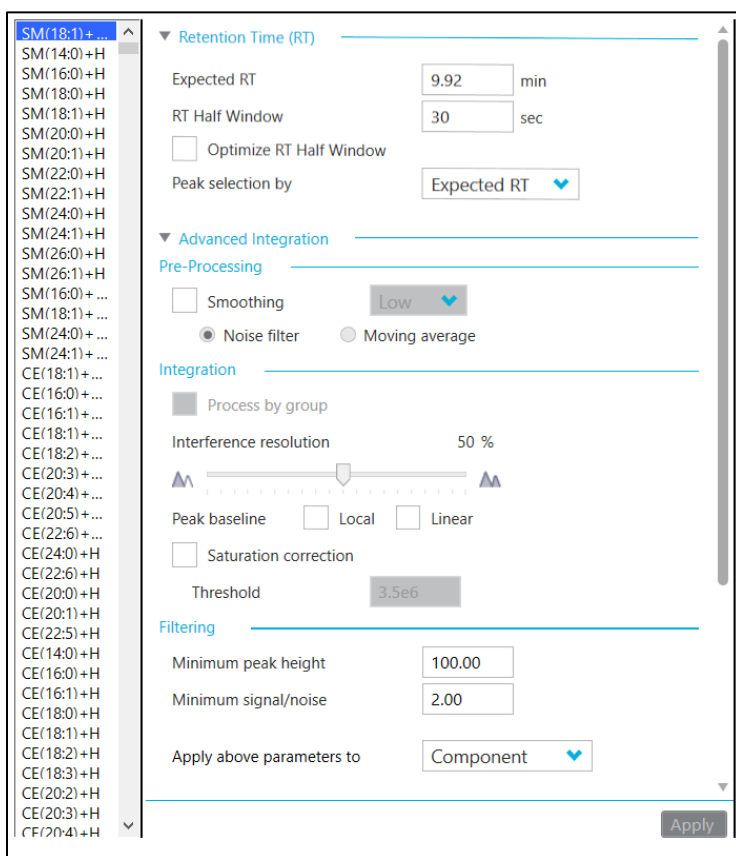


Figure 8-1. Integration defaults for data processing the targeted lipid profiling data.

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 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 Lipid MRM Method <date>. Click **Next**.
4. Select a representative injection from the data file upon which the quantification method will be optimized. Ensure good peaks are observed for all lipids.
5. On the Define Components Pane, the individual lipids can be viewed. Click **Next**.

6. Integrations can be now reviewed for each individual lipid. Review the integration of each lipid species to ensure it is correctly integrated. Note that not all lipid species 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.

10.0 Protocol for determining lipid class retention times

The retention times of lipids are affected by multiple experimental factors including mobile phase preparation, HPLC 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 is generated from the range of biological samples to be analyzed such that most lipids are represented in the 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 is pasted into the *Master Assay Table* Tab in the sMRM Pro Builder template. This allows the first unscheduled method to be built (LIPID_MRM_Unscheduled_1.dam). LC-MS analysis is performed on the sample matrix of interest to determine rough retention times. Note if very large numbers of MRM transitions are being analyzed at this point (>1000), it is recommended to run the positive and negative lipids in separate methods. Using this data, a second acquisition method is then built from this information (LIPID_sMRMPro_1.dam) and then used to acquire replicate data on a pooled biological sample. Replicate injections are now performed in a single method 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 (LIPID_sMRM_Final_1.dam) 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.

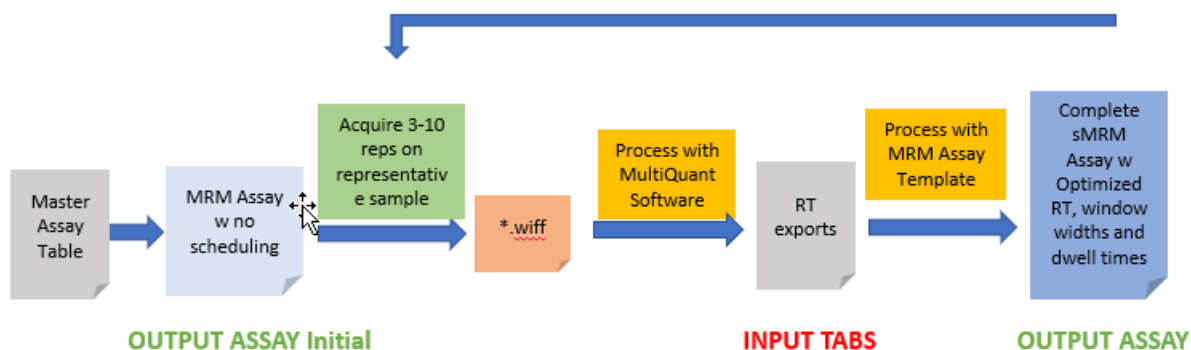


Figure 9.1. Method development workflow using sMRM pro Builder template.

1. Create a pooled sample of the biological matrix to be used in the study (Section 4). It is recommended that this includes sample from the various sample types to be studied (ie. condition 1 vs condition 2) such that most lipids will be represented in the pooled sample. Internal standards can also be included in the sample as described in Section 4. It is strongly recommended to use the internal standard for determination of approximate retention time for each lipid class.
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 table, copy the populated columns from the tab called *OS_OUTPUT ASSAY (-) Initial* and paste into the negative polarity MRM table. Note if very large numbers of MRMs are to be tested at this points (> 1000), it is recommended to run this step as two separate methods, one for positive and one for negative mode.
4. Save the method as *LIPID_MRM_Unscheduled_1.dam*.
5. Perform 3 injections of the Double Blank sample using the *LIPID_MRM_Unscheduled_1.dam* acquisition method, to equilibrate the LC-MS system.
6. Perform 3 injections of the pooled sample using *LIPID_MRM_Unscheduled_1.dam* 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, Width at 50% as shown in **Figure 9.2**.

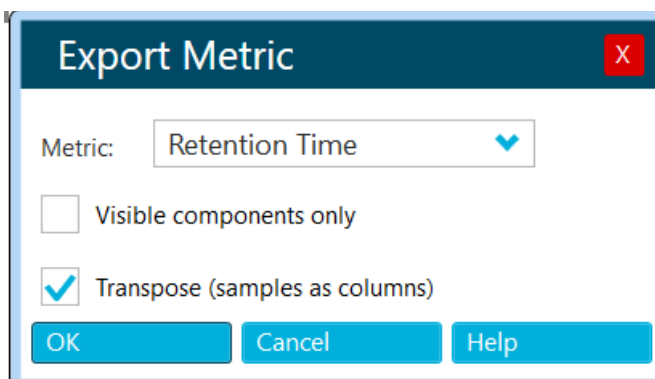


Figure 9.2. Exporting the Results from SCIEX OS software for use in sMRM Pro Builder.

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 has the ability to filter out lipids 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 lipids are filtered out of the assay so remember to set the *Assay Subset for Output* set to **All** - **Figure 9.3**.

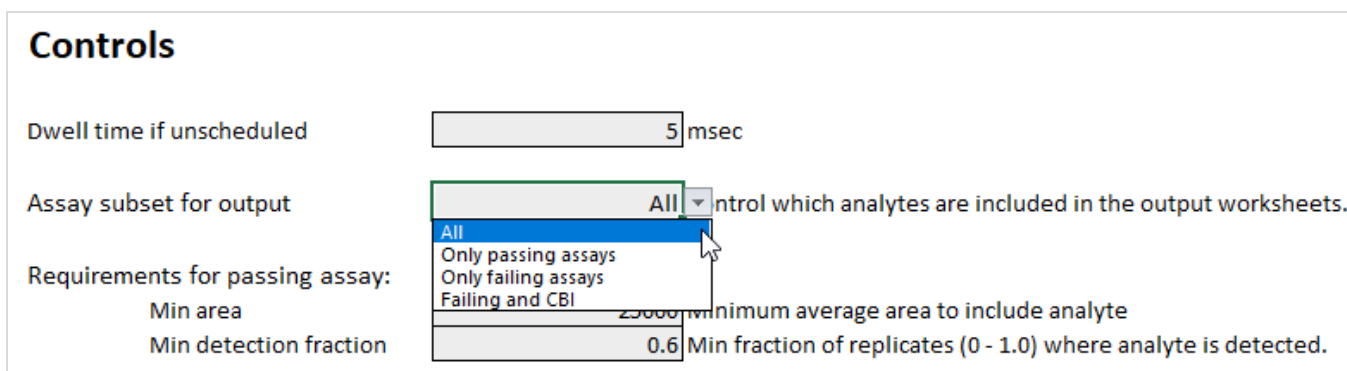


Figure 9.3. Filtering controls in the sMRM Pro Builder template.

10. Paste the new MRM lists from the *OS_OUTPUT ASSAY (+)* and *OS_OUTPUT ASSAY (-)* tabs into the *Lipid_sMRM_Opt_1.dam* from Section 7 and rename. Note 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 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.
12. Process the replicate data in SCIEX OS software, then export the results as 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 for rejecting lipids that are not reliably detected, ensuring the input data is of good quality is important.
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.dam* from Section 7 and rename. This is now 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 was 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 μ L plasma, add 975 μ L water; let sit on ice for 10 min.
3. Add 2.0 mL methanol
4. Add 0.9 mL of dichloromethane (CH_2Cl_2).
5. Vortex
6. Make sure there is a mono-phase at this stage. If two distinct phases are observed, add 50 μ L methanol and vortex, check to see if solution is a single phase. If not, repeat addition of 50 μ L methanol and vortex.
7. Add Internal standard, vortex and let mixture sit for 30 min at room temperature.
8. Add 1 mL water
9. Add 0.9 mL dichloromethane
10. Invert tubes 10 times. DO NOT VORTEX or an emulsion will be formed.
11. Centrifuge at 1200 rpm for 10 min.
12. Collect lower layer and put into a fresh glass tube.
13. Add 2 mL dichloromethane to remains in extraction tube.
14. Mix, centrifuge, collect lower layer and add to 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 cell culture medium.
 - b. For cells in suspension, centrifuge, discard saline and add 1 mL water. Vortex and transfer to glass tube for extraction. Allow to rest on ice for 10 min. Ensure final volume is 1 mL—adjust if necessary.
 - c. For adhered cells, wash cells with non-buffered saline. Add 1 mL water to lyse cells and scrap. Collect cell lysate and transfer to glass tube for extraction. Allow to rest on ice for 10 min. Ensure final volume is 1 mL—adjust if necessary.
3. Add 2.0 mL methanol
4. Add 0.9 mL dichloromethane
5. Vortex
6. Make sure there is a mono-phase at this stage. If two distinct phases are observed, add 50 μ L methanol and vortex, check to see if solution is a single phase. If not, repeat addition of 50 μ L methanol and vortex.
7. Add Internal standard, vortex and let mixture sit for 30 min at room temperature.
8. Add 1 mL water
9. Add 0.9 mL dichloromethane
10. Vortex
11. Centrifuge at 1200 rpm for 10 min.
12. Collect lower layer and put into a fresh glass tube.
13. Add 2 mL dichloromethane to remains in extraction tube.
14. Mix, centrifuge, collect lower layer and add to 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 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 tissue so that total water volume = 1 mL
 - a. E.g., 100 mg Brain corresponds to 60 μ L water—add 940 μ L water
3. Homogenize tissue using a bead mill or equivalent
4. Transfer homogenate into 13 x 100 mm glass screw top tube.
5. Add 2.0 mL methanol
6. Add 0.9 mL dichloromethane
7. Vortex
8. Make sure there is a mono-phase at this stage. If two distinct phases are observed, add 50 μ L methanol and vortex, check to see if solution is a single phase. If not, repeat addition of 50 μ L methanol and vortex.
9. Add Internal standard, vortex and let mixture sit for 30 min at room temperature.
10. Add 1 mL water
11. Add 0.9 mL dichloromethane
12. Vortex
13. Centrifuge at 1200 rpm for 10 min.
14. Collect lower layer and put into a fresh glass tube.
15. Add 2 mL dichloromethane to remains in extraction tube.
16. Mix, centrifuge, collect lower layer and add to 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 a broad range of lipid classes. Due to the large numbers 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 which is described in Section 9. Here in Section 5 – 7, 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*.

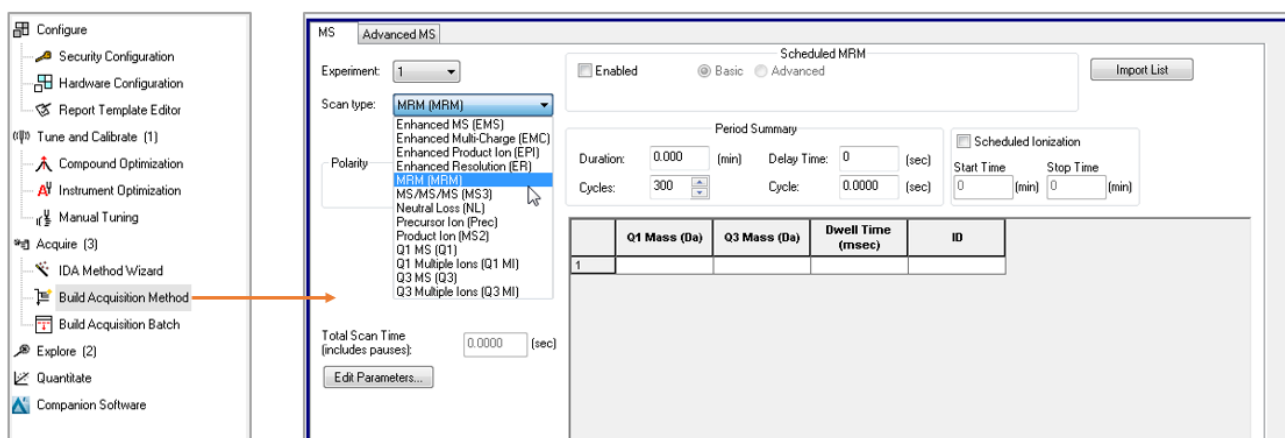


Figure B-1: Building an acquisition method in Analyst software.

4. Select positive polarity for the first 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 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 indicated and right click to add the Collision Energy (CE) to the MRM table.

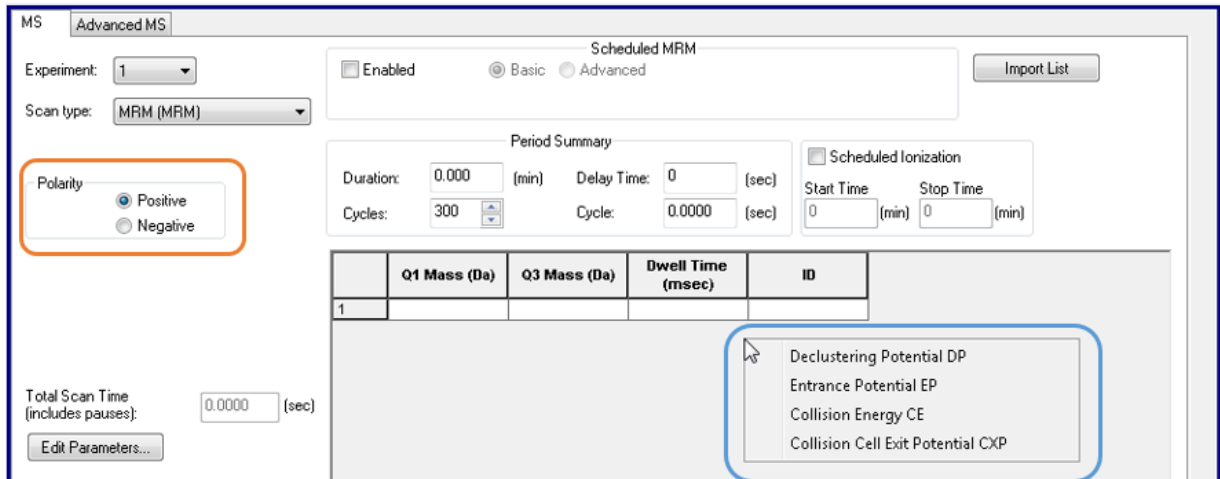


Figure B-2: Defining the positive polarity Information for experiment 1.

12. 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 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
<i>*These values may need to be optimized to obtain maximum sensitivity.</i>		
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

13. 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.
14. Save the method as *LIPID_MRM_Unscheduled_1.dam*.
15. Next step will be to add the 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 that was built in section 5 and 6 and also 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 shown in **Figure B.3**. Use the Advanced Mode.
3. Fill in the Scheduled MRM algorithm parameters as shown in **Table B.2**.

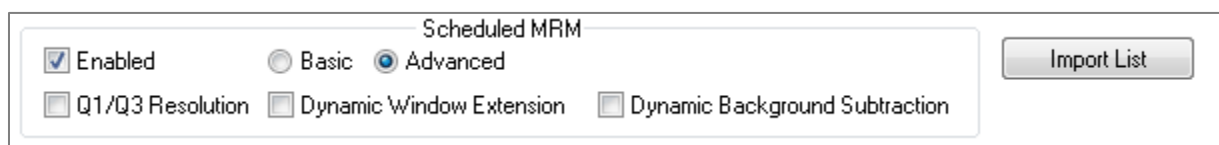


Figure B-3: Select the Scheduled MRM algorithm – enhanced mode.

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)	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 along with 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 how to use MultiQuant software version 3.0.2 or later during assay development, for determination of retention times, peak areas, etc.

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

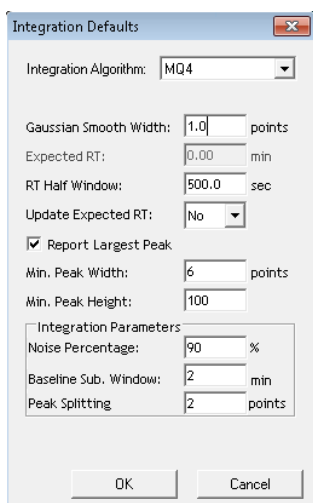


Figure B-4. Integration defaults for data processing the targeted lipid profiling data.

10. Go to the *File* drop down menu in MultiQuant software, then choose *New Results Table*.
11. 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.
12. Select **Create New Method** and name it Lipid MRM Method <date>. Click **Next**.
13. Select a representative injection from the data file upon which the quantification method will be optimized. Ensure good peaks are observed for all lipids.
14. On the Define Components Pane, the individual lipids can be viewed. Click **Next**.

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

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