Comprehensive targeted method for lipid mediator analysis

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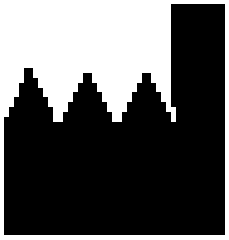

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1.0 Introduction

Aim of the method

Quantification of lipid mediators in complex biological samples is challenging and often requires a detailed knowledge of lipids, lipid mediators and lipid mediator analysis methods. The inherent complexity of lipid mediator profiling arises from the need to identify and quantify different lipid mediators that carry distinct and wide-ranging physiological functions while sharing a number of common structural features. Comprehensive analysis requires a combination of strategic chromatography and specific mass spectrometry methods in order to distinguish between numerous isobaric and epimeric species in a single analysis method. This can be difficult, and often requires a high degree of expertise and method development time. The lack of simplified and standardized methods for generating quantitative data makes it more challenging to understand the role of lipid mediators in biology. This targeted LC-MS method provides a means for the relative quantification of a very large number of lipid mediator species as well as their precursors, metabolites, and non-enzymatic breakdown products across multiple classes.

Here, a detailed LC-MRM method is provided that will enable comprehensive coverage of many of the multiple lipid mediator 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 mediator classes, however maintaining sufficient points across the LC peaks for quantitative analyses must be monitored.

Separation of lipid mediators, precursors, and enzymatic/non-enzymatic isomers

Lipids as a class of compounds are inherently known for their extensive isobaric overlap across many molecular species, and lipid mediators are not exempt. Separation of many of these epimers and structurally similar analytes is crucial to determine the physiological role they are playing in inflammation, disease, and resolution. A reverse phase approach is used in this method to retain both nonpolar and polar lipid mediators. Utilizing the Kinetix® Polar C18 column, the separation of several epimers and isobaric species is achieved to improve assay quality and confidence in proper identification of these molecules.

Quantification strategy

The method described herein can utilize different strategies for relative lipid mediator quantification. The most commonly used approach is to spike one single standard for most of the lipid mediator classes into the sample. This approach allows for the relative quantification and comparison between sample groups. A second approach uses multiple internal standards for most lipid mediator classes. This approach allows for more accurate quantification, because endogenous lipid mediator species are matched to the most structurally similar 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 can 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 132 lipid mediator, metabolite, isomer, and precursor 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 or reference standard 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 because of many 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 LC-MS/MS 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 2.0 or later
- Microsoft Excel template – sMRM Pro Builder Template 1.4
- Master Assay List_Lipid Mediators

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 might need to be optimized. In addition, different LC systems could be used for this method but retention times might 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 and 3-3**)
- Recommended column (**Table 3-4**)

Table 3-1. Chemicals and reagents		
Supplier	**Description**	**Part Number**
Fisher Scientific	Water	W64
	Methanol	A454SK-4
	Formic Acid	A11750
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. Lipid mediator standards (External Standards)		
Supplier	**Description**	**Part Number**
Cayman Chemicals (External Standards)	Primary COX and LOX LC-MS Mixture	19101
	SPM D-Series LC-MS Mixture	18702
	SPM E-series LC-MS Mixture	19417
	Lipoxin LC-MS Mixture	19412
	Polyunsaturated Fatty Acid LC-MS Mixture	17941
	LTB_4	20110
	20-OH-LTB_4	20180
	5,15-diHETE	35280
	5S,6R-diHETE	35200
	5,6 EET 8,9 EET	50211 50351
	11,12 EET	50511
	14,15 EET	50651
	5,6 DiHETrE	51211
	8,9 DiHETrE	51351
	11,12 DiHETrE	51511
	14,15 DiHETrE	51651
	11-HETE	34500
	20-HETE	90030
	8-iso-$PGF_{2\alpha}$	16350

Table 3-2 cont. Lipid mediator standards (External Standards)		
Supplier	**Description**	**Part Number**
Cayman Chemicals (External Standards)	LXA_5	10011453
	5-HEPE	32200
	11-HEPE	32500
	12-HEPE	32540
	15-HEPE	32700
	8,9-EpETE	10470
	11,12-EpETE	10462
	14,15-EpETE	10173
	17,18-EpETE	50861
	17(R)-RvD3	9002881
	RvD4	13835
	PD1	10010390
	PDX	10008128
	Maresin 1	10878
	Maresin 2	16369
	4-HDHA	33200
	7-HDHA	33300
	13-HDHA	33500
	14-HDHA	33550
	17-HDHA	33650
	7,8-EpDPA	10465
	10,11-EpDPA	10471
	13,14-EpDPA	10464
	16,17-EpDPA	10174
	19,20-EpDPA	10175
	9-HODE	38400
	13-HODE	38600
	10-Nitrolinoleate	10037
	LTC_4	20210
	LTD_4	20310
	LTE_4	20410
	MCTR1	17007
	MCTR2	17008
	MCTR3	19067
	PCTR1	19064
	PCTR2	19065
	PCTR3	19066
	PAF	60900
The above listed standards are recommended to order to cover wide ranges of lipid mediator classes during method development. They are not mandatory for the assay, depending on lipid classes required for study.		

Table 3-3. Lipid mediator standards (internal standards)		
Supplier	**Description**	**Part Number**
Cayman Chemicals (internal standards)	PGE_2-d4	314010
	15-deoxy-PGJ_2-d4	318570
	8-iso-$PGF_{2\alpha}$-d4	316350
	LTB_4-d4	320110
	LXA_4-d5	10007737
	11,12-EET-d8	10006413
	5-HETE-d8	334230
	12-HETE-d8	334720
	15-HETE-d8	334570
	RvE1-d4	10009854
	RvD2-d5	11184
	RvD3-d5	19512
	Maresin 1-d5	21823
	LTC_4-d5	10006198
	LTD_4-d5	10006199
	LTE_4-d5	10007858
The above listed standards are recommended to order to cover wide ranges of lipid mediator classes during method development. They are not mandatory for the assay, depending on lipid classes required for study.		

Table 3-4. HPLC columns		
Supplier	***Description**	**Part number**
Phenomenex http://www.phenomenex.com/	Polar C18 2.6 µm, 3.0 x 100 mm column	00D-4759-Y0
	Strata-X 33 µm Polymeric Reversed Phase, 100 mg/3mL	8B-S100-EBJ
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 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's laboratory location. Matrix choice will also have a significant impact on performance of the assay, 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. To a 1 L bottle, add 1 mL of formic acid to 999 mL of water
2. **Mobile phase B** (0.1% formic acid in methanol):
    a. To a 1 L bottle, add 1 mL of formic acid to 999 mL of methanol
3. **Needle rinse** (1:1:1 water/methanol/Iso-propanol):
    a. To a 1 L bottle, add 300 mL of water, 300 mL of methanol, and 300 mL of isopropanol
4. **Lipid mediator dilution buffer**:
    a. To a 50 mL bottle, add 25 mL of water and 25 mL of methanol

NOTE:

- Sensitivity of lipid mediator 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.
- If the project only requires the analysis of negative mode analytes, mobile phase A and mobile phase B should be prepared using 0.01% formic acid to maximize ionization

efficiency which can enhance overall sensitivity. Positive mode analytes, specifically the peptide conjugate species including LTC4, LTD4, and LTE4, require 0.1% formic acid for ideal peak shape.

Preparation of samples

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

1. **Double blank sample**:
    a. Pipet 1 mL 1:1 water/methanol to an autosampler vial.
2. **Blank sample**:
    a. Add 5 µL of Internal Standard Mix (200 pg/µL) to 495 µL of 1:1 water/methanol
3. **QC sample**:
    a. Add 5 µL of Internal Standard Mix (200 pg/µL) and 5 µL of External Standard Mix (200 pg/µL) to 490 µL of 1:1 water/methanol
4. **Pooled sample for method development**:
    a. Sample extracts containing internal standards (see Appendix A for examples) can be used. For initial method development, a standard mix (e.g. the QC sample) is recommended to ensure proper identification and scheduling of MRMs.

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. Because of the large numbers of lipid mediators 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 Sections 5 – 7, the required acquisition methods will be built for use in the assay development process.

To create an acquisition method in SCIEX OS Software:

1. Begin by double-clicking the SCIEX OS Software icon to open the 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 the software home screen (**Figure 5-1**), click *MS Method*. From the drop-down menu for scan type, select *MRM*.

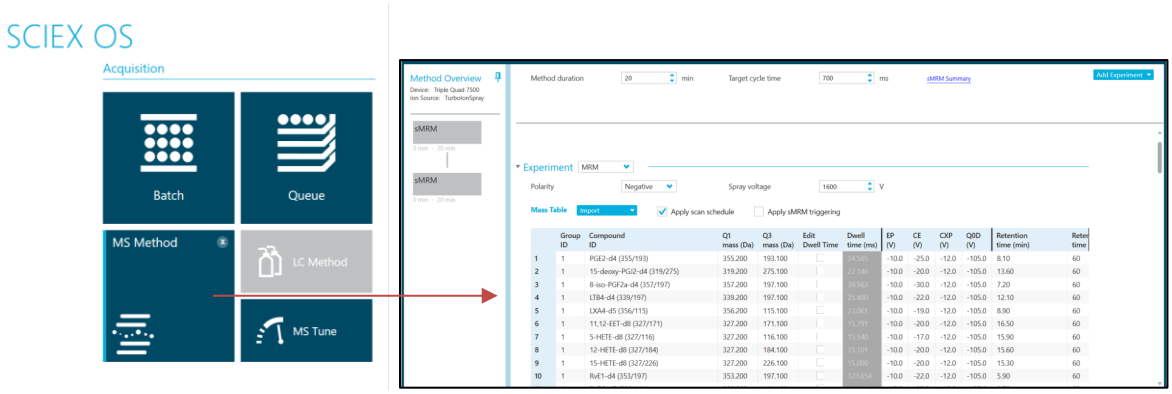


Figure 5-1: Building an acquisition method in SCIEX OS Software.

4. Select Negative polarity for the first experiment as indicated in the orange box in **Figure 5-2**.
5. Create two experiments, one each for positive and negative polarities for the unscheduled acquisition.
6. Adjust the Target Cycle time to 700 msec as show in **Figure 5-2** indicated by the green box.

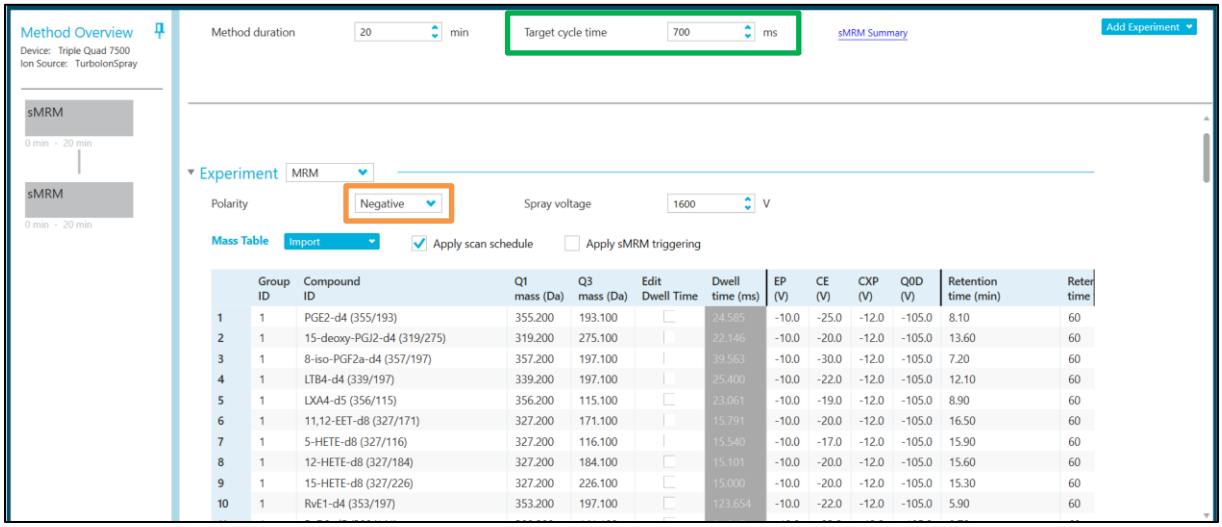


Figure 5-2: Defining the negative polarity information for Experiment 1.

7. 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 7500 System parameters for lipid analysis

Source parameters	Positive polarity	Negative polarity
IS	1600	-1600
CUR	40 psi	40 psi
TEM	350 °C	350 °C
*GS1	40 psi	40 psi
*GS2	70 psi	70 psi
CAD	12	12
**These values may need to be optimized to obtain maximum sensitivity.*		
Compound parameters		
EP	10	-10
CXP	13	-12
MS		
Scan type	MRM	MRM
Duration	20 min	20 min
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

8. 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.

9. Copy all the populated columns from the tab called *OS_OUTPUT ASSAY (+) Initial* and paste them into the positive polarity MRM table. Copy all the populated columns from the tab called *OS_OUTPUT ASSAY (-) Initial* and paste them into the negative polarity MRM table.
10. Save the method as *LM_MRM_Unscheduled_1.dam*.

The next step will be to add the chromatography settings to this method in Section 6.

6.0 HPLC system and operation parameters

Lipid mediators 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 *LM_MRM_Unscheduled_1.dam* after LC information has been added. A representative TIC 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
0	Pumps	Pump B Conc.	10	0.50
0.1	Pumps	Pump B Conc.	45	0.50
2.0	Pumps	Pump B Conc.	45	0.50
16.5	Pumps	Pump B Conc.	80	0.50
16.6	Pumps	Pump B Conc.	98	0.50
18.5	Pumps	Pump B Conc.	98	0.50
18.6	Pumps	Pump B Conc.	10	0.50
20.5	Pumps	Pump B Conc.		
**Mobile phase A: 0.1% formic acid in water*				
**Mobile phase B: 0.1% formic acid in methanol*				

Table 6-2. Additional HPLC parameters and LC settings	
Pumps	**Parameters/settings**
Flow rate	0.5 mL/min
Pump B concentration	10.0%
Low pressure	0 psi
High pressure	6000 psi
Autosampler	
Use Autosampler	*Select AS*
Rinsing Solution	(1:1:1) water : methanol : 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 Enabled	Yes
Cooler Temperature	8 °C
Oven	
Temperature control	Enabled
Temperature	50 °C

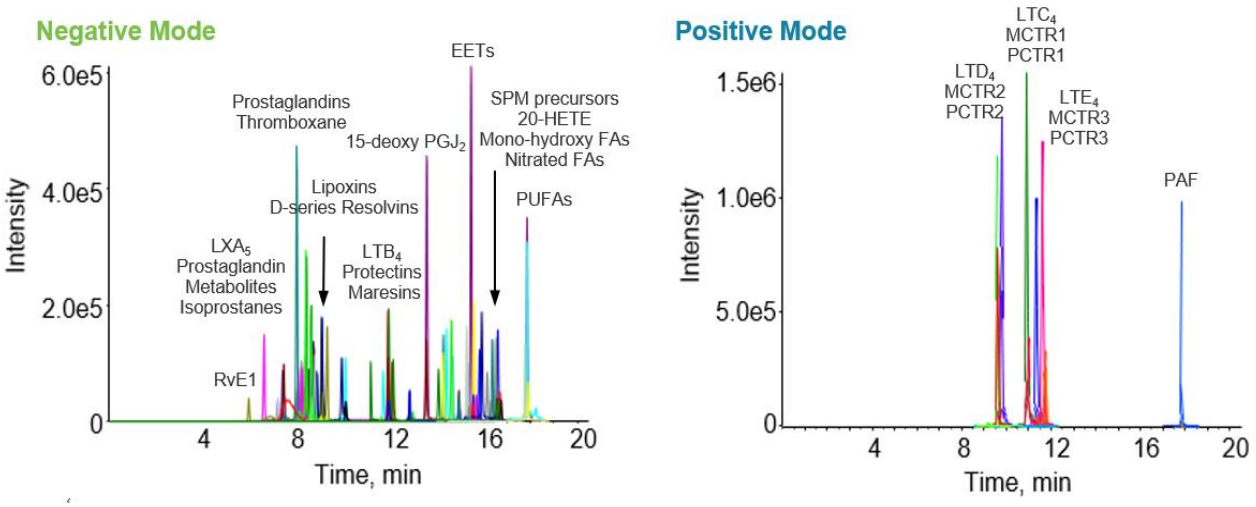


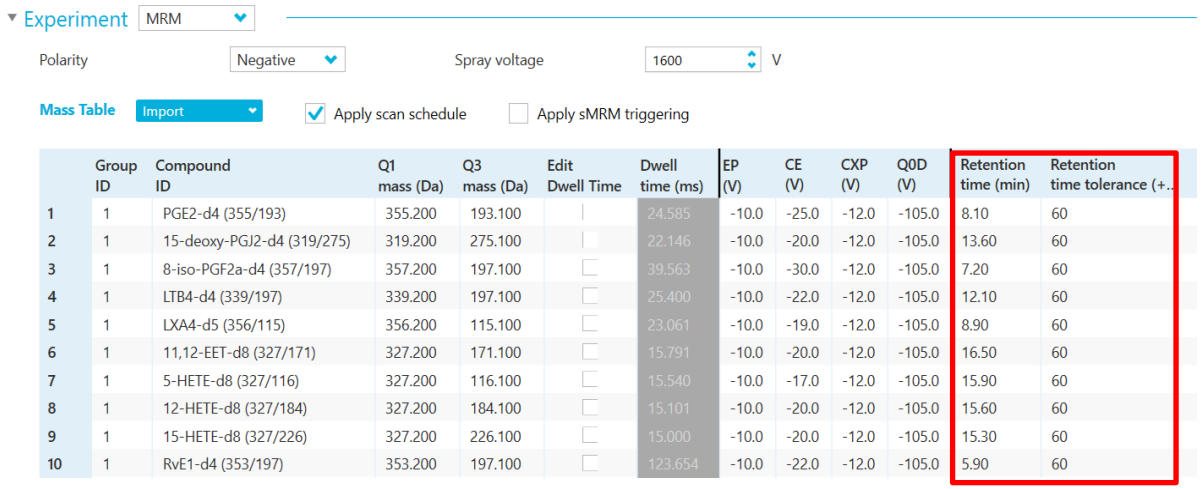
Figure 6-1. Representative XIC of the Standard Mix added.

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 and 6 and also a method constructed using the Scheduled MRM Algorithm in enhanced mode, described here.

To construct the method:

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, enter 60 seconds into the Retention time tolerance column then fill down as shown by the red box. Right click and use the fill down option.



	Group ID	Compound ID	Q1 mass (Da)	Q3 mass (Da)	Edit Dwell Time	Dwell time (ms)	EP (V)	CE (V)	CXP (V)	Q0D (V)	Retention time (min)	Retention time tolerance (+..
1	1	PGE2-d4 (355/193)	355.200	193.100		24.585	-10.0	-25.0	-12.0	-105.0	8.10	60
2	1	15-deoxy-PGJ2-d4 (319/275)	319.200	275.100		22.146	-10.0	-20.0	-12.0	-105.0	13.60	60
3	1	8-iso-PGF2a-d4 (357/197)	357.200	197.100		39.563	-10.0	-30.0	-12.0	-105.0	7.20	60
4	1	LTB4-d4 (339/197)	339.200	197.100		25.400	-10.0	-22.0	-12.0	-105.0	12.10	60
5	1	LXA4-d5 (356/115)	356.200	115.100		23.061	-10.0	-19.0	-12.0	-105.0	8.90	60
6	1	11,12-EET-d8 (327/171)	327.200	171.100		15.791	-10.0	-20.0	-12.0	-105.0	16.50	60
7	1	5-HETE-d8 (327/116)	327.200	116.100		15.540	-10.0	-17.0	-12.0	-105.0	15.90	60
8	1	12-HETE-d8 (327/184)	327.200	184.100		15.101	-10.0	-20.0	-12.0	-105.0	15.60	60
9	1	15-HETE-d8 (327/226)	327.200	226.100		15.000	-10.0	-20.0	-12.0	-105.0	15.30	60
10	1	RvE1-d4 (353/197)	353.200	197.100		123.654	-10.0	-22.0	-12.0	-105.0	5.90	60

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

4. Adjust the Target Cycle time to 1000 msec.
5. From the computed MRM table in the *sMRM Pro Builder.xlsx* workbook, paste the data from the populated columns on the tab called *OS_Output Assay (+)* into the positive polarity MRM table, and the data from the *OS_Output Assay (-)* tab into the negative polarity MRM table.
6. Save the method as *LM_sMRM_Opt_1.dam* or *LM_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 (LM_MRM_Unscheduled_1.dam) will serve as template methods for the assay optimization steps described in Section 9.

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 data such as retention times and peak areas.

To analyze data:

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**.

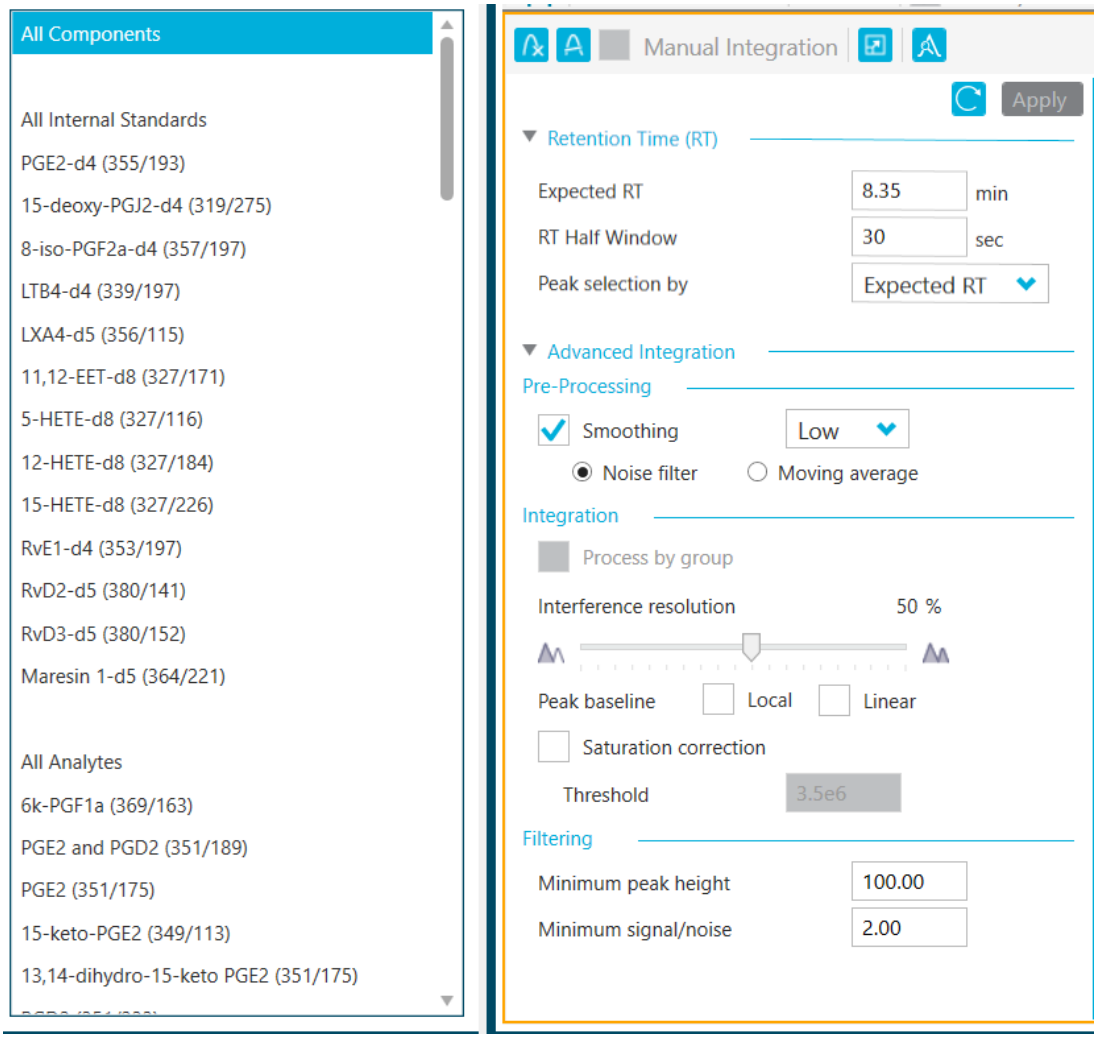


Figure 8-1. Integration defaults for data processing the targeted lipid mediator data.

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 LM 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 now be 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.

9.0 Protocol for determining lipid class retention times

The retention times of lipid mediators 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 that 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 LM Method is pasted into the *Master Assay Table* Tab in the sMRM Pro Builder template. This allows the first unscheduled method to be built (LM_MRM_Unscheduled_1.dam). LC-MS analysis is performed on the sample matrix of interest to determine rough retention times. Using this data, a second acquisition method is then built from this information (LM_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 (LM_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.

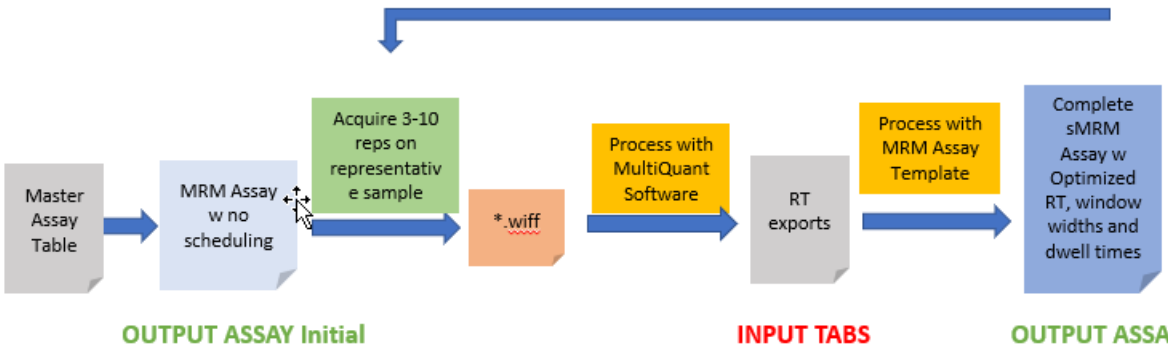


Figure 9-1. Method development workflow using sMRM Pro Builder template.

The process is as follows:

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 entries from the populated columns on the tab called *OS_OUTPUT ASSAY (+) Initial* and paste them into the positive polarity MRM table. Copy the entries from the populated columns on the tab called *OS_OUTPUT ASSAY (-) Initial* and paste them into the negative polarity MRM table.
4. Save the method as *LM_MRM_Unscheduled_1.dam*.
5. Perform 3 injections of the Double Blank sample using the *LM_MRM_Unscheduled_1.dam* acquisition method, to equilibrate the LC-MS system.
6. Perform 3 injections of the pooled sample using *LM_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**.

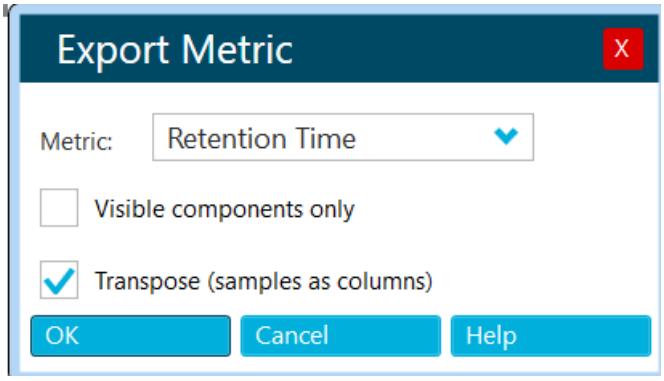


Figure 9-2. Exporting the results from SCIEX OS Software for use in sMRM Pro Builder.

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 that if you want to save the intermediate assay development results, you must 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**).

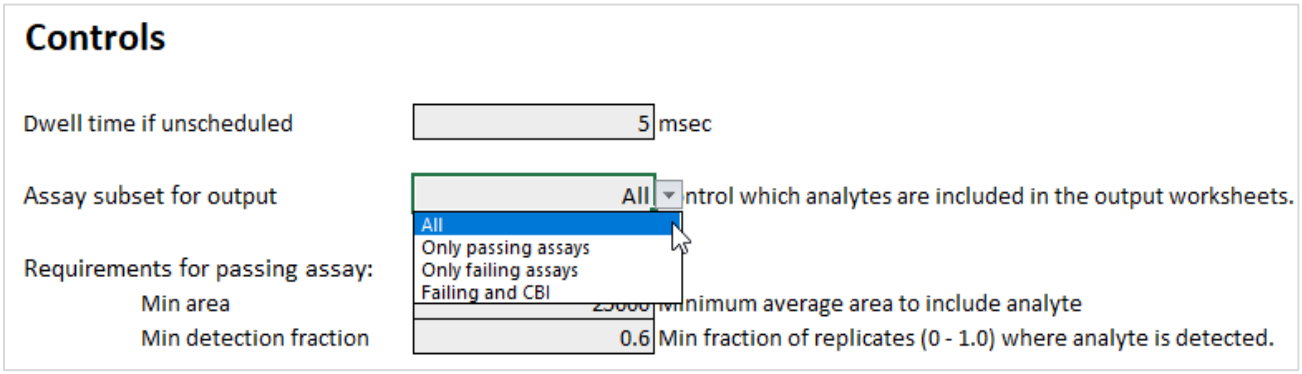


Figure 9-3. Filtering controls in the sMRM Pro Builder Template.

10. Paste the new MRM lists from the *OS_OUTPUT ASSAY (+)* and *OS_OUTPUT ASSAY (-)* tabs into the *LM_sMRM_Opt_1.dam* file from Section 7 and rename it. 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 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 *LM_sMRM_Final_1.dam* file from Section 7 and rename it. 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 were 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 to limit conjugated double bond isomerization).

A.1 Extraction protocol for plasma

1. Use 15 mL Falcon (polypropylene) screw-capped tubes. Do not use washed tubes as you might extract detergent residue.
2. To 100 µL plasma, add 1 mL methanol containing 100 pg/mL internal standard mix. Cap the tube and vortex (5 seconds). Then let it sit on ice for 10 min.
3. Add 9 mL water (or enough water to make total methanol composition 10% or less). Cap the tube and vortex (5 seconds).
4. Centrifuge the sample at 2000 rpm for 10 min.
5. Pre-condition solid phase extraction (SPE) cartridge by eluting 1 mL methanol followed by 1 mL water.
6. Carefully decant or pipet sample supernatant into SPE cartridge. Make sure not to take any sample pellet to the cartridge. Slowly elute sample to waste dropwise (not an uninterrupted flow of liquid which is too fast and can affect analyte recovery). Before sample liquid completely elutes from cartridge, refill cartridge with more sample and repeat process until all of the sample has been eluted.
7. Wash sample cartridge by loading 1 mL of 10% methanol and carefully elute (dropwise) to waste. Make sure to elute as much liquid as possible. Increase the vacuum, if possible, to aid in removing liquid from stationary phase and dry the cartridge surfaces using a Kimwipe if drops are visible. Removing as much water as possible is critical during the evaporation step (see below) which can be significantly longer if substantial amounts of residual water remain in sample fraction.
8. Place a new 13 x 100 mm glass screw-capped tube in the sample collection position (directly beneath SPE cartridge). Do not use washed tubes as you might extract detergent residue.
9. Add 1 mL methanol to SPE cartridge.

10. Slowly elute methanol (dropwise) into the glass sample collection tube.
11. Take sample to a solvent evaporation chamber/system.
12. Evaporate sample completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37 °C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.
13. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds, and then centrifuge at 2000 rpm for 30 seconds.
14. Transfer sample supernatant to a 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min. Repeat this step until pellet is no longer visible.
15. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitates to the insert. A gel-loading tip works ideally for this transfer step. Place insert into autosampler vial and then close the vial with an autosampler cap.
16. Load sample vial into autosampler maintained at 8 °C. Note: if samples are not to be analyzed immediately, they can be stored at 4 °C for ~1-2 days. If longer storage is required, samples should be stored at -20 °C.

A.2 Extraction protocol for cell culture

1. Use 15 mL Falcon (polypropylene) screw-capped tubes. Do not use washed tubes as you might extract detergent residue.
2. To 1 mL cell culture sample (it is recommended to keep cells and media together) add 1 mL methanol containing 100 pg/mL internal standard mix. Cap the tube and vortex (5 seconds). Then let it sit on ice for 10 min. If cell culture sample is in a tissue culture well/dish, add methanol with internal standards directly to dish, gently swirl/mix, and let sit for 10 min on ice (covered from UV/sunlight) before transferring to a 15 mL Falcon tube.
3. Add 8 mL water (enough water to make total methanol composition 10% or less). Cap the tube and vortex (5 seconds).
4. Centrifuge sample at 2000 rpm for 10 min.
5. Pre-condition solid phase extraction (SPE) cartridge by eluting 1 mL methanol followed by 1 mL water.
6. Carefully decant or pipet sample supernatant into SPE cartridge. Make sure to not take any sample pellet to the cartridge. Slowly elute sample to waste in a steady stream of drops (not a stream of liquid which is too fast and can affect analyte recovery). Before sample liquid completely elutes from cartridge, refill cartridge with more sample and repeat process until all of the sample has been eluted.
7. Wash sample cartridge by loading 1 mL of 10% methanol and carefully elute (dropwise) to waste. Make sure to elute as much liquid as possible. Increase vacuum, if possible, to aid in removing liquid from stationary phase and dry the cartridge surfaces using a Kimwipe if drops are visible. Removing as much water as possible is critical during the evaporation step (see below) which can be significantly longer if substantial amounts of residual water remain in sample fraction.
8. Place 13 x 100 mm new glass screw-capped tube in the sample collection position (directly beneath SPE cartridge). Do not use washed tubes as you might extract detergent residue.
9. Add 1 mL methanol to SPE cartridge.
10. Slowly elute methanol (dropwise) into the glass sample collection tube.
11. Take sample to a solvent evaporation chamber/system.
12. Evaporate sample completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37 °C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.

13. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds and then centrifuge at 2000 rpm for 30 seconds.
14. Transfer sample supernatant to a 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min.
15. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitates to the insert. A gel-loading tip works ideally for this transfer step. Place insert into autosampler vial and close the vial with an autosampler cap.
16. Load sample vial into an autosampler maintained at 8°C. Note: if samples are not to be analyzed immediately, they can be stored at 4°C for ~1-2 days. If longer storage is required, samples should be stored at -20°C.

A.3 Extraction protocol for solid tissue

1. Use 15 mL Falcon (polypropylene) screw capped tubes. Do not use washed tubes as you can extract detergent residue.
2. To 10-100 mg tissue, add 1 mL methanol containing 100 pg/mL internal standard mix.
3. Using a ground glass pestle tissue grinder, gently disperse tissue by pressing tissue against the bottom and sides of tube (for ~10 sec). Tissue might not fully homogenize but solution should appear cloudy. Cap the tube and vortex (5 seconds). Then let it sit on ice for 10 min.
4. Add 9 mL water (or enough water to make total methanol composition 10% or less). Cap the tube and vortex (5 seconds).
5. Centrifuge sample at 2000 rpm for 10 min.
6. Pre-condition solid phase extraction (SPE) cartridge by eluting 1 mL methanol followed by 1 mL water.
7. Carefully decant or pipet sample supernatant into SPE cartridge. Make sure not to take any sample pellet to the cartridge. Slowly elute sample liquid to waste in a steady stream of drops (not a stream of liquid which is too fast and might affect analyte recovery). Before sample liquid completely elutes from cartridge, refill cartridge with more sample and repeat process until all of the sample liquid has been eluted.
8. Wash sample cartridge by loading 1 mL of 10% methanol and carefully elute (dropwise) to waste. Make sure to elute as much liquid as possible. Increase the vacuum, if possible, to aid in removing liquid from stationary phase and dry the cartridge surfaces using a Kimwipe if any liquid residue is visible. Removing as much water as possible is critical for the evaporation step (see below) which can be significantly longer if substantial amounts of residual water remain in sample fraction.
9. Place 13 x 100 mm new glass screw-capped tube in the sample collection position (directly beneath SPE cartridge). Do not use washed tubes as you might retain detergent residue.
10. Add 1 mL methanol to SPE cartridge.
11. Slowly elute methanol (dropwise) into the glass sample collection tube.
12. Take sample to a solvent evaporation chamber/system.
13. Evaporate sample completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37°C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.

14. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds, and then centrifuge at 2000 rpm for 30 seconds.
15. Transfer sample supernatant to a 1.5 or 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min.
16. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitates to the insert. A gel-loading tip works ideally for this transfer step. Place insert into autosampler vial and close vial with an autosampler cap.
17. Load the sample vial into an autosampler maintained at 8°C. Note: if samples are not to be analyzed immediately, they can be stored at 4°C for ~1-2 days. If longer storage is required, samples should be stored at -20 °C. Re-suspend lipids in injection solvent.

Appendix B – Secondary sample preparation protocol options

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 to limit conjugated double bond isomerization). The following protocols provide an alternative, simplified method to sample preparation when solid phase extraction is not feasible. Using these protocols are not recommended for large sample quantities (e.g. >50 mg of tissue or >100 µL plasma/serum), or for long term, high-throughput analysis. Careful attention to peak shape and MS contamination and overall performance is advised.

B.1 Extraction protocol for plasma

1. Use 15 mL Falcon (polypropylene) screw-capped tubes. Do not use washed tubes as you might extract detergent residue.
2. To 100 µl plasma, add 1 mL methanol containing 100 pg/mL internal standard mix. Cap tube and vortex (5 seconds). Then let it sit on ice for 10 min.
3. Centrifuge sample at 2000 rpm for 10 min.
4. Carefully decant or pipet sample supernatant into a 13 x 100 mm glass screw-capped tube. Make sure to avoid taking pellet. Do not use washed tubes as you might extract detergent residue.
5. Take sample to a solvent evaporation chamber/system.
6. Evaporate sample solvent completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37 °C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.
7. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds and then centrifuge at 2000 rpm for 30 seconds.
8. Transfer sample supernatant to a 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min. Repeat this step until pellet is no longer visible.

9. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitates to the insert. A gel-loading tip works ideally for this transfer step. Place insert into an autosampler vial and close vial with an autosampler cap.
10. Load sample vial into autosampler maintained at 8 °C. Note: if samples are not to be analyzed immediately, they can be stored at 4 °C for ~1-2 days. If longer storage is required, samples should be stored at -20 °C.

B.2 Extraction protocol for cell culture

1. Use 15 mL Falcon (polypropylene) screw-capped tubes. Do not use washed tubes as you might extract detergent residue.
2. To 1 mL cell culture sample (it is recommended to keep cells and media together) add 1 mL methanol containing 100 pg/mL internal standard mix and add 8 mL methanol (without internal standard mix). Cap the tube and vortex (5 seconds). Then let it sit on ice for 10 min. If cell culture sample is in a tissue culture well/dish, add methanol with internal standards directly to the dish. Gently swirl/mix, and then let it sit for 10 min on ice (covered from UV/sunlight) before transferring it to a 15 mL Falcon tube.
3. Centrifuge sample at 2000 rpm for 10 min.
4. Evaporate sample solvent completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37 °C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.
5. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds and then centrifuge at 2000 rpm for 30 seconds.
6. Transfer sample supernatant to a 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min. Repeat this step until pellet is no longer visible.
7. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitates to the insert. A gel-loading tip works ideally for this transfer step. Place insert into an autosampler vial and close the vial with an autosampler cap.
8. Load sample vial into an autosampler maintained at 8 °C. Note: if samples are not to be analyzed immediately, they can be stored at 4 °C for ~1-2 days. If longer storage is required, samples should be stored at -20 °C.

B.3 Extraction protocol for solid tissues

1. Use 15 mL Falcon (polypropylene) screw-capped tubes. Do not use washed tubes as you might extract detergent residue.
2. To 10-100 mg tissue, add 1 mL methanol containing 100 pg/mL internal standard mix.
3. Using a ground glass pestle tissue grinder, gently disperse tissue by pressing tissue against the bottom and sides of tube (for ~10 sec). Tissue might not fully homogenize but solution should appear cloudy. Cap and vortex (5 seconds) and let sit on ice for 10 min.
4. Centrifuge sample at 2000 rpm for 10 min.
5. Evaporate sample solvent completely under a gentle stream of nitrogen. The samples can be heated (but do not exceed 37 °C) during this step to speed up the evaporation process. The intensity of the nitrogen stream can be increased as the sample volume decreases. Make sure sample liquid droplets do not get dispersed from the bottom of the tube.
6. Add 50 µL of 1:1 water/methanol to sample tube. Vortex for 5 seconds and centrifuge at 2000 rpm for 30 seconds.
7. Transfer sample supernatant to a 1.7 mL microcentrifuge (Eppendorf) polypropylene tube. Centrifuge at max rpm (~14,000 rpm) for 1 min. Repeat this step until pellet is no longer visible.
8. Carefully, slowly transfer sample supernatant to a plastic or glass autosampler insert. Avoid taking pellet or precipitate to the insert. A gel-loading tip works ideally for this transfer step. Place insert into autosampler vial and close vial with an autosampler cap.
9. Load sample vial into an autosampler maintained at 8 °C. Note: if samples are not to be analyzed immediately, they can be stored at 4 °C for ~1-2 days. If longer storage is required, samples should be stored at -20 °C.

Appendix C – Sample processing using Analyst® and MultiQuant™ Software

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 Appendix C, the required acquisition methods will be built for use in the assay development process.

C.1 Data processing using Analyst® Software

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

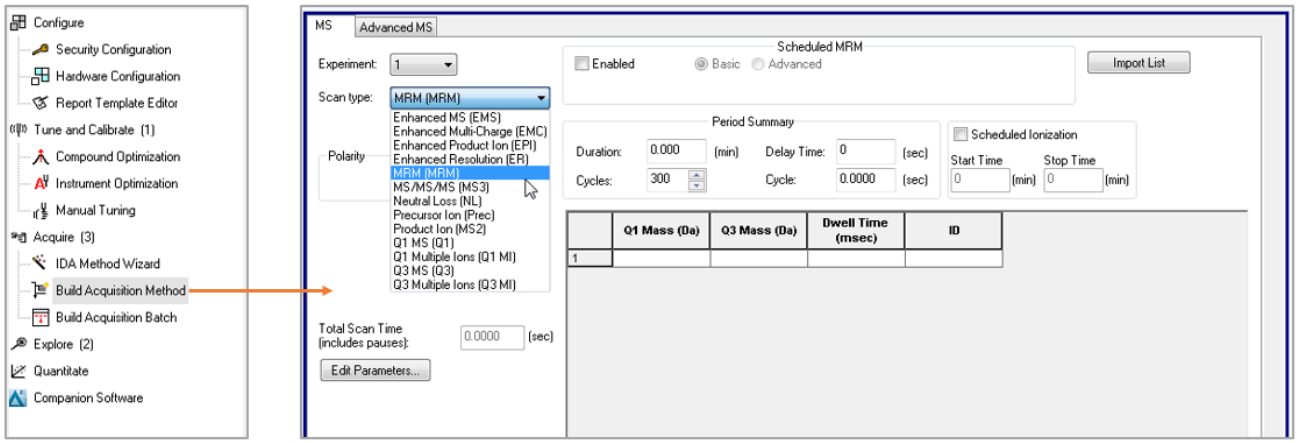


Figure C-1: Building an acquisition method in Analyst® Software.

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

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

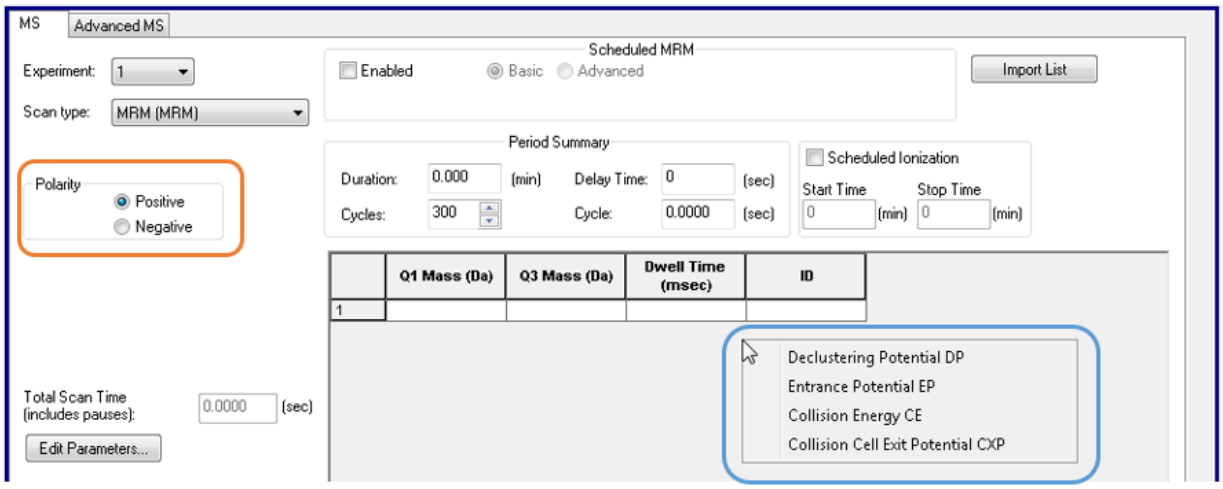


Figure C-2: Defining the positive polarity information for experiment 1.

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

Table C-1. QTRAP System 6500+ LC-MS/MS System parameters for lipid analysis		
Source parameters	**Positive polarity**	**Negative polarity**
IS	5500	-4200
CUR	30 psi	30 psi
TEM	520 °C	520 °C
*GS1	85 psi	85 psi
*GS2	50 psi	50 psi
CAD	12 or Medium	12 or Medium
**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

12. 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 them into the positive polarity MRM table. Copy **Columns A - E** from the tab called *Analyst_OUTPUT ASSAY (-) Initial* and paste them into the negative polarity MRM table.
13. Save the method as *LM_MRM_Unscheduled_1.dam*.
14. The next step will be to add the chromatography settings to this method.

C.2 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 built in Section 5 to start.
2. Select the Scheduled MRM Algorithm by checking the **Enable** box shown in **Figure C-3**.
3. Fill in the Scheduled MRM Algorithm Parameters as shown in **Table C-2**.

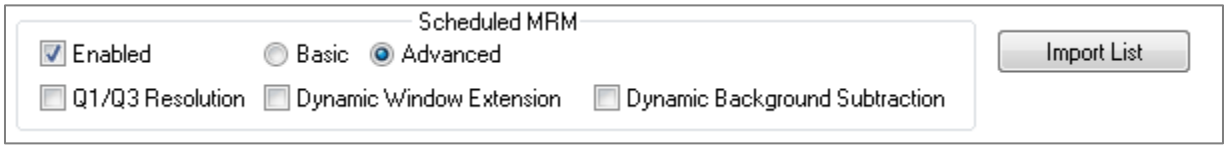


Figure C-3: Select the Scheduled MRM Algorithm – enhanced mode.

Table C-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 and save the method as *LM_sMRMPro_1.dam.*
5. Next select Advanced and save the method as LM_sMRM_Final_1.dam.
6. These two methods along with the method built in Section 6 (LM_MRM_Unscheduled_1.dam) will serve as template methods for the assay optimization steps described in Section 9.

C.3 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 data such as retention times and peak areas.

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 C-4**.

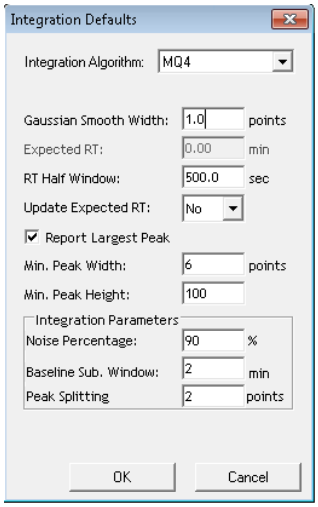


Figure C-4. Integration defaults for data processing the targeted lipid profiling data.

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

8. 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.
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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