SHOTGUN LIPIDOMICS WITH DIFFERENTIAL ION MOBILITY SEPARATION:

A comprehensive targeted method

for flow injection analysis of lipids

Using the SCIEX 6500+ series systems or SCIEX 5500+ system and SelexION device

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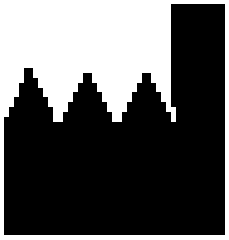

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1.0 Introduction

Aim of the method

Direct infusion or flow injection lipid analysis is an established workflow and has been utilized for many decades. Without additional separation, however, the amount of isobaric overlap within a lipid class and between lipid classes can lead to misrepresentation of lipid species or class changes. Traditionally, chromatographic solutions combined with MRM analysis were developed and optimized to reduce ambiguity in identification, but the authentic standards needed to confirm identifications and retention times using reverse phase analysis do not exist for a large breadth of analysis. Normal phase methods have been employed to confirm lipid class identifications but often suffer from poor reproducibility over large cohort studies.

Differential ion mobility spectrometry (DMS) provides more selectivity without adding chromatography to achieve higher confidence in identification. Here, a flow injection method containing over 1,650 lipid species was developed to profile the lipidome in a multitude of different matrices. This method requires 2 injections with a total runtime of 25.4 minutes. This method has not been fully validated and is intended to be a starting point for further method development by users. Additional lipid species and classes can also be included.

Separation of lipid classes using a SelexION device

Lipids as a class of compounds are inherently known for their extensive isobaric overlap across many molecular species. The SelexION device provides an orthogonal separation technique to separate lipids by class without LC separation. With the introduction of a chemical modifier in the gas phase, the dipole moment of these lipid molecules is induced as they fly through the planar geometry of the cell. There are 2 voltage fields in the cell. First is the separation voltage (SV) that separates the molecules based on their mobility through the high and low field portions of the radio frequency (RF). Second is the compensation voltage (COV), which is an offset voltage that corrects the trajectory of these ions as they pass through the cell to guide them into the MS for detection. The choice of chemical modifier is important. As the lipid molecules are moving through the SV field, the declustering of the modifier during the high field and the clustering of the modifier at the low field around the headgroup of the phospholipid will provide superior separation. In this assay, 1-propanol provides the best separation, however, 2-propanol will also give sufficient separation.

Quantification strategy

Quantitative lipid analysis has numerous challenges due to the complexity of the lipidome, including high structural diversity and the presence of many isobaric interferences. It has also been shown that the variety of fatty acid chain lengths and degree of unsaturation result in differential fragmentation efficiency, which impacts quantification. Traditionally, a single internal standard per lipid class has been used as a strategy to quantify lipid molecular species. With this approach, the diversity of fatty acid chain lengths and degree of unsaturation for molecular species that provide differential fragmentation efficiency and the impact that these factors have on quantification are unaccounted for. The Internal Standards kit for the Lipidyzer platform, presented here, contains more than 50 labeled molecular species across 13 lipid classes and can therefore neutralize the quantitative bias and allow for more accurate measurement. To cover the additional lipid classes added to the assay that are not covered with the Internal Standards kit for the Lipidyzer platform, the UltimateSPLASH standards from Avanti Polar Lipids were utilized.

Please note that every biological sample will have a different lipid profile depending on its source, experimental conditions and more. When performing this experiment, it is important to consider the amount of material you will extract and use as your final dilution volume. If too little material is used, low abundant classes might not be sampled robustly, as they are collected outside of the linear range of the instrument and method. Conversely, if too much material is used, saturation of the detector can occur or the instrument might become contaminated, requiring frequent cleaning. If needed, a linearity study can be performed in matrix to confirm that each lipid class is quantified within the linear response of the instrument and method.

Different quantitative strategies can be used with this method: relative quantitation, which requires a single internal standard per lipid class, and accurate quantitation, which requires multiple internal standards per lipid class, as provided by the Lipidyzer Internal Standards or UltimateSPLASH. For accurate quantitation, the appropriate internal standard must be matched with each analyte, based on the Q3 fatty acid fragment in the negative ion mode or the neutral loss of the ammoniated fatty acid in the positive ion mode. It is suggested you contact SCIEX technical support for specific questions regarding the internal standard strategies.

Quantitative methods

Three methods will be used to execute this protocol for FIA lipid profiling.

System suitability method – This test is designed to determine the correct COV values for the lipid classes and to ensure the DMS cell has the needed sensitivity for the assays (Section 7). The SST is used for quality control before running each study, but also can be run periodically within a study to track performance over the study.

When measuring each lipid extract from the study samples, two methods are used to get broad lipid class coverage (Section 9).

Method 1 – This method is for measuring the polar lipids and uses the DMS device for specificity.

Method 2 – This method does not use DMS but the device operates in transparent mode. This method is for monitoring the other lipid classes.

2.0 Instrumentation

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

- SCIEX 5500+ system or SCIEX 6500+ series systems
- SelexION or SelexION+ device
- ExionLC system with the following components:
    - Controller, autosampler and pumps
    - Solvent mixer: 25 µL volume
- Analyst software, version 1.7.1
- SCIEX OS software, version 2.0 or later
- Shotgun Lipidomics Assistant (SLA) software
- Microsoft Excel template
- Master assay table for flow injection analysis

This method was optimized for the SCIEX 6500+ series systems, but it is applicable on the SCIEX 5500 series systems and the SCIEX 5500+ system. To achieve the best sensitivity on the other systems, MS parameters might need to be optimized. Different LC systems could be used for this method, but please consider the high organics used as mobile phases when choosing your LC system.

3.0 Analytes, reagents and assay materials

Details for ordering the appropriate materials for lipidomic analysis are provided in this section. To enable ordering these materials, the supplier 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 consumables (**Table 3-3**)
- Recommended software (**Table 3-4**)

Table 3-1. Chemicals and reagents

Supplier	Description	Part number
Honeywell https://www.honeywell.com	Water	LC365-4
	Methanol	LC230-4
	Dichloromethane	299-4
	1-propanol	34871

Equivalent reagents from other suppliers can be used. Assay results might deviate from this optimized method if using reagents other than the ones suggested in this SOP.

Table 3-2. Lipid standards

Supplier	Description	Part number
SCIEX https://sciex.com	Internal Standard Kit for the Lipidyzer platform **Available as specific lipid classes as well**	5040156
	QC Spike for the Lipidyzer platform	5040408
	System Suitability Mix for the Lipidyzer platform	5040407
	AA 45/32 Phys Control plasma	4386703
Avanti Polar Lipids, Inc. https://www.avantilipids.com/	EquiSPLASH LIPIDOMIX Quantitative Mass Spec Internal Standard	330731
	15:0-18:1-d7-PA	791642
	UltimateSPLASH PI	330830
	UltimateSPLASH PG	330827
	UltimateSPLASH PS	330828

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. Consumables		
Supplier	**Description**	**Part number**
SCIEX https://www.sciex.com	Tubing, PEEKsil, 50 µm ID, 1/32 inch OD, 10 cm	205-00069
Phenomenex https://phenomenex.com	SecurityLINK PEEKsil 50 µm ID, 300 mm length with 10-32 fittings	aj1-2251
	SecurityLINK PEEKsil 50 µm ID, 750 mm length with 10-32 fittings	aj1-2291
IDEX https://www.idex-hs.com	MicroTight Adapter PEEK 1/16" OD x 1/32” with fittings	P-881
Equivalent equipment or instruments from other suppliers can also be used.		

Table 3-4. Software		
Source	**Description**	**Link**
GitHub	A DMS Shotgun Lipidomics Workflow Application to Facilitate High-Throughput, Comprehensive Lipidomics	Shotgun Lipidomics Assistant

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 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 have a significant impact on performance of the assay and alterations to 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. **Sample running solution** (1:1 methanol/dichloromethane with 10 mM ammonium acetate)
    a. To a 1 L bottle, add 500 mL of methanol and 500 mL of dichloromethane
    b. Add 0.7708 g of ammonium acetate
    c. Sonicate for 15 minutes or until all ammonium acetate has been dissolved
2. **Needle rinse** (100% isopropanol):
    a. To a 1 L bottle, add HPLC-grade isopropanol
3. **Sample dilution buffer**:
    a. To a 50 mL bottle, add 50 mL of Sample running solution.

NOTE: Please bring all QC and SST vials to ambient room temperature before using. If needed, gently warm and vortex vial to redissolve lipids.

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 of 1:1 methanol/dichloromethane with 10mM ammonium acetate into an autosampler vial
2. **QC sample**:
    a. Add 5 µL of Internal Standard Mix and 5 µL of QC Spike Mix to 490 µL of 1:1 methanol/dichloromethane
3. **SelexION device tuning mix for the SCIEX 6500+ series systems**:
    a. Add 10 µL of the EquiSPLASH into a vial
    b. Add 490 µL of the Sample running solution
    c. Gently vortex
4. **SelexION device tuning mix for the SCIEX 5500 series systems or SCIEX 5500+ system:**
    a. Add 25 µL of the EquiSPLASH into a vial
    b. Add 490 µL of the Sample running solution
    c. Gently vortex
5. **Lower limit of detection (LOD) mix using System Suitability Mix:**
    a. Add 10 µL of the System Suitability Mix into a vial
    b. Add 10 µL of the EquiSPLASH
    c. Add 980 µL of the Sample running solution
    d. Gently vortex

5.0 Optimizing COV values for lipid classes

This direct infusion method utilizes the SelexION device for 1 of the 2 injections. To determine the compensation voltage distinct to each lipid class, a group of standards from each class must be run and the COV must be ramped. This assay development strategy is a 2-step process, which is described here.

To create an acquisition method in Analyst software:

1. Begin by double-clicking the Analyst software icon to open the software
2. On the *Configuration* tab, click on hardware profile and enable the profile that activates the mass spectrometer with the syringe pump enabled
3. On the *Acquisition* tab of the software home screen (**Figure 5-1**), click *Build Acquisition Method*. From the drop-down menu for scan type, select *MRM*.

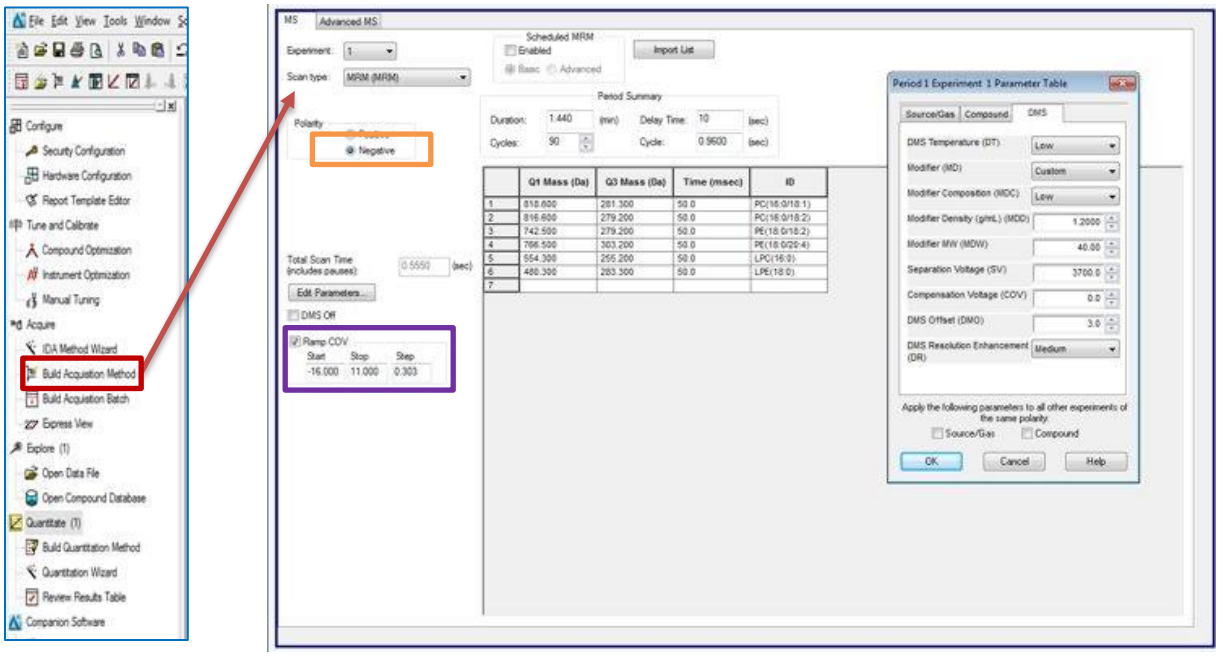


Figure 5-1. Building an acquisition method in Analyst Software.

4. Select Negative polarity for the first experiment, as indicated in the orange box in **Figure 5-1**
5. Copy and paste the transitions provided in **Table 5-1a** into the MRM table
6. Using **Table 5-2** as a reference, edit the Source, Gas and DMS experiment parameters for the negative polarity

Table 5-1a. SelexION device tuning MRM transitions for negative polarity							
Q1	**Q3**	**Time (ms)**	**ID**	**DP**	**EP**	**CE**	**CXP**
811.6	241.3	50	PC(15:0/18:1d7)	-80	-10	-50	-16
709.6	241.3	50	PE(15:0/18:1d7)	-80	-10	-50	-16
587.4	288.3	50	LPC(18:1d7)	-80	-10	-50	-16
485.3	288.3	50	LPE(18:1d7)	-80	-10	-50	-16
740.5	241.3	50	PG(15:0/18:1d7)	-80	-10	-50	-16
828.4	241.3	50	PI(15:0/18:1d7)	-80	-10	-60	-16
753.5	241.3	50	PS(15:0/18:1d7)	-80	-10	-50	-16

Table 5-1b. SelexION device tuning MRM transitions for positive polarity							
Q1	**Q3**	**Time (ms)**	**ID**	**DP**	**EP**	**CE**	**CXP**
739.6	184.1	100	SM(18:1d9)	80	10	43	16

Table 5-2. SCIEX 6500+ series systems parameters for lipid analysis: Methods 1 and 2		
Source parameters	**Positive polarity**	**Negative polarity**
IS	5200	-4100
CUR	25 psi	25 psi
*TEM	250°C	250°C
*GS1	25 psi	25 psi
*GS2	25 psi	25 psi
CAD	9	9
**These values may need to be optimized to obtain maximum sensitivity.*		
DMS parameters: Method 1 only		
DMS Temp	Low	Low
MD	Custom	Custom
MDD	0.803	0.803
MDW	60	60
SV	3500	3500
COV	-	-
DMO	-3	3
DR	5	5
Compound parameters: Methods 1 and 2		
EP	10	-10
CXP	16	-16
MS		
Scan type	MRM	MRM
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

7. Check the Ramp COV box, indicated in the purple box in Figure 5-1
8. Set the “Start” to -20 and the “Stop” to 10
9. Save the method as *Negative_Tuning_SV3500*
10. Repeat these steps for the positive polarity COV Ramp

a. The COV Ramp parameter should be adjusted to “Start” at -5 and “Stop” at 10

11. Use **Table 5-1b** to paste the MRM transitions
12. Fill a 1 mL syringe with the SelexION device tuning mix and place on syringe pump cradle
13. Create 2 batches within Analyst software to run triplicates of each polarity COV ramp
14. Ensure the line to the source is primed and submit the batch. Click Start.

6.0 Selecting COVs for lipid classes

Using the triplicate injections made in **Section 5**, follow the instructions on GitHub to use the SLA software to properly determine and update the MRM list that will be pasted in Analyst software. Examples of approximate COV values plotted by SLA software are shown in Figure 6-1.

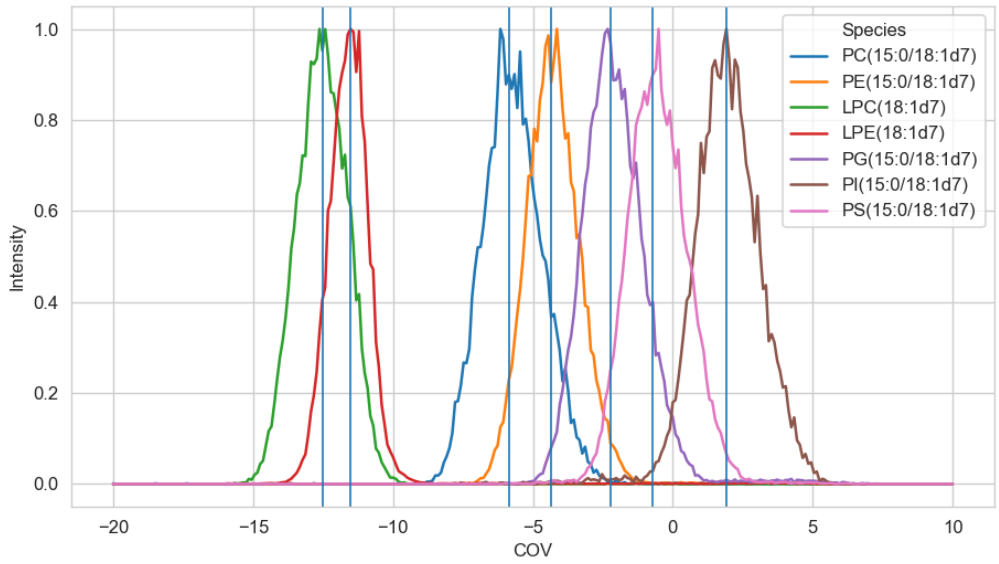


Figure 6-1. Representative XIC of the of the negative ion mode COV ramp.

7.0 System Suitability Test

This System Suitability Test (SST) is designed to ensure the COV values were properly picked and the DMS cell has the needed sensitivity before a run. It is not necessary to run this SST before every run but it is recommended to track the results of the SST over time to monitor performance.

To build the SST method in Analyst software:

1. On the Acquisition tab of the software home screen, click Build Acquisition Method. From the drop-down menu for scan type, select MRM.
2. Select Negative polarity for the first experiment
3. Add a second MRM experiment under the same Period. Select Positive for polarity.
4. Copy and paste the transitions provided in Table 7-1a into the MRM table for the Negative polarity experiment
5. Copy and paste the transitions provided in Table 7-1b into the MRM table for the Positive polarity experiment
6. Using Table 5-2, edit the Source, Gas and DMS experiment parameters for the SST method
7. In the Delay Time box, set the delay to 120 sec
8. In the Cycles box, set the number to 20 cycles
9. Using the HPLC total flow program described in **Section 8**, set up the FIA method
10. Save the method as SST
11. Place the LOD mix described in **Section 4** in the autosampler
12. Place a Sample running solution blank in the autosampler
13. Build a batch in Analyst software to run the blank sample, then the LOD sample
14. Submit and run the batch
15. For SST analysis, follow the instructions on the SLA software instructions page

Table 7-1a. SST MRM transitions for negative polarity							
Q1	**Q3**	**Time (ms)**	**ID**	**DP**	**EP**	**CE**	**CXP**
700	200	50	PCBLANK	-80	-10	-50	-16
790.6	253.2	50	PC(16:0/16:1)	-80	-10	-50	-16
818.6	281.2	50	PC(16:0/18:1)	-80	-10	-50	-16
816.6	279.2	50	PC(16:0/18:2)	-80	-10	-50	-16
814.6	277.2	50	PC(16:0/18:3)	-80	-10	-50	-16
842.6	305.2	50	PC(16:0/20:3)	-80	-10	-50	-16
840.6	303.2	50	PC(16:0/20:4)	-80	-10	-50	-16
838.6	301.2	50	PC(16:0/20:5)	-80	-10	-50	-16
868.6	331.3	50	PC(16:0/22:4)	-80	-10	-50	-16
866.6	329.2	50	PC(16:0/22:5)	-80	-10	-50	-16
864.6	327.2	50	PC(16:0/22:6)	-80	-10	-50	-16
600	200	50	PEBLANK	-80	-10	-50	-16
744.6	281.2	50	PE(18:0/18:1)	-80	-10	-50	-16
742.5	279.2	50	PE(18:0/18:2)	-80	-10	-50	-16
740.5	277.2	50	PE(18:0/18:3)	-80	-10	-50	-16
768.6	305.2	50	PE(18:0/20:3)	-80	-10	-50	-16
766.5	303.2	50	PE(18:0/20:4)	-80	-10	-50	-16
764.5	301.2	50	PE(18:0/20:5)	-80	-10	-50	-16
792.6	329.2	50	PE(18:0/22:5)	-80	-10	-50	-16
790.5	327.2	50	PE(18:0/22:6)	-80	-10	-50	-16
500	250	50	LPCBLANK	-80	-10	-50	-16
554.3	255.2	50	LPC(16:0)	-80	-10	-50	-16
350	200	50	LPEBLANK2	-80	-10	-50	-16
480.3	283.3	50	LPE(18:0)	-80	-10	-50	-16
766.5	200	50	PGBLANK	-80	-10	-50	-16
766.5	281.2	50	PG(d17:0/18:1)	-80	-10	-50	-16
854.5	200	50	PIBLANK	-80	-10	-50	-16
854.5	281.2	50	PI(d17:0/18:1)	-80	-10	-50	-16
779.5	200	50	PSBLANK	-80	-10	-50	-16
779.5	281.2	50	PS(d17:0/18:1)	-80	-10	-50	-16

Table 7-1b. SST MRM transitions for positive polarity							
Q1	**Q3**	**Time (ms)**	**ID**	**DP**	**EP**	**CE**	**CXP**
650	180	50	SMBLANK	80	10	43	16
703.6	184.1	50	SM(16:0)	80	10	43	16
729.6	184.1	50	SM(18:1)	80	10	43	16
815.7	184.1	50	SM(24:0)	80	10	43	16
813.7	184.1	50	SM(24:1)	80	10	43	16

8.0 HPLC system and operation parameters

In the acquisition method that was saved in **Section 7**, 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 8-1 and 8-4**. Save the method.

Table 8-1. LC total flow program for SST

Total time (min)	Module	Event	Parameter (%B)	Total Flow
0.1	Pumps	Total Flow	50	0.008
7.0	Pumps	Total Flow	50	0.008
7.1	Pumps	Total Flow	50	0.030
9.0	Pumps	Total Flow	50	0.030
9.1	Pumps	Total Flow	50	0.008
10.0	Controller	Stop		

Table 8-2. LC total flow program for Method 1 (for section 9)

Total time (min)	Module	Event	Parameter (%B)	Total Flow
0.1	Pumps	Total Flow	50	0.008
9.4	Pumps	Total Flow	50	0.008
9.41	Pumps	Total Flow	50	0.030
11.4	Pumps	Total Flow	50	0.030
11.41	Pumps	Total Flow	50	0.008
12.4	Controller	Stop		

Table 8-3. LC total flow program for Method 2 (for section 9)

Total time (min)	Module	Event	Parameter (%B)	Total Flow
0.1	Pumps	Total Flow	50	0.008
7.9	Pumps	Total Flow	50	0.008
8.0	Pumps	Total Flow	50	0.030
12.0	Pumps	Total Flow	50	0.030
12.1	Pumps	Total Flow	50	0.008
13.0	Controller	Stop		

Table 8-4. Additional HPLC parameters and LC settings	
Pumps	**Parameters/settings**
Flow rate	0.008 mL/min
Pump B concentration	50.0%
Low pressure	0 psi
High pressure	6000 psi
Autosampler	
Use Autosampler	*Select AS*
Rinsing Solution	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	After aspiration
Cooler Enabled	Yes
Cooler Temperature	15°C
Oven	
Temperature control	Off

9.0 Building acquisition methods for flow injection analysis

Two methods that require 2 separate injections are required to be built for analysis of the lipidome. One requires that the DMS be “on” and the other requires the DMS to be in transparent mode or “off”.

To construct the methods:

1. Open a new MRM acquisition method with a Positive and Negative experiment under the same period
2. For the DMS on and off experiments, paste in the transitions from the dictionary file on GitHub in their respective polarity experiments
3. As shown in Figure 9-1, set the Delay Time to 120 sec, indicated by the red box
4. As shown in Figure 9-1, set the Cycles to 20, indicated by the purple box

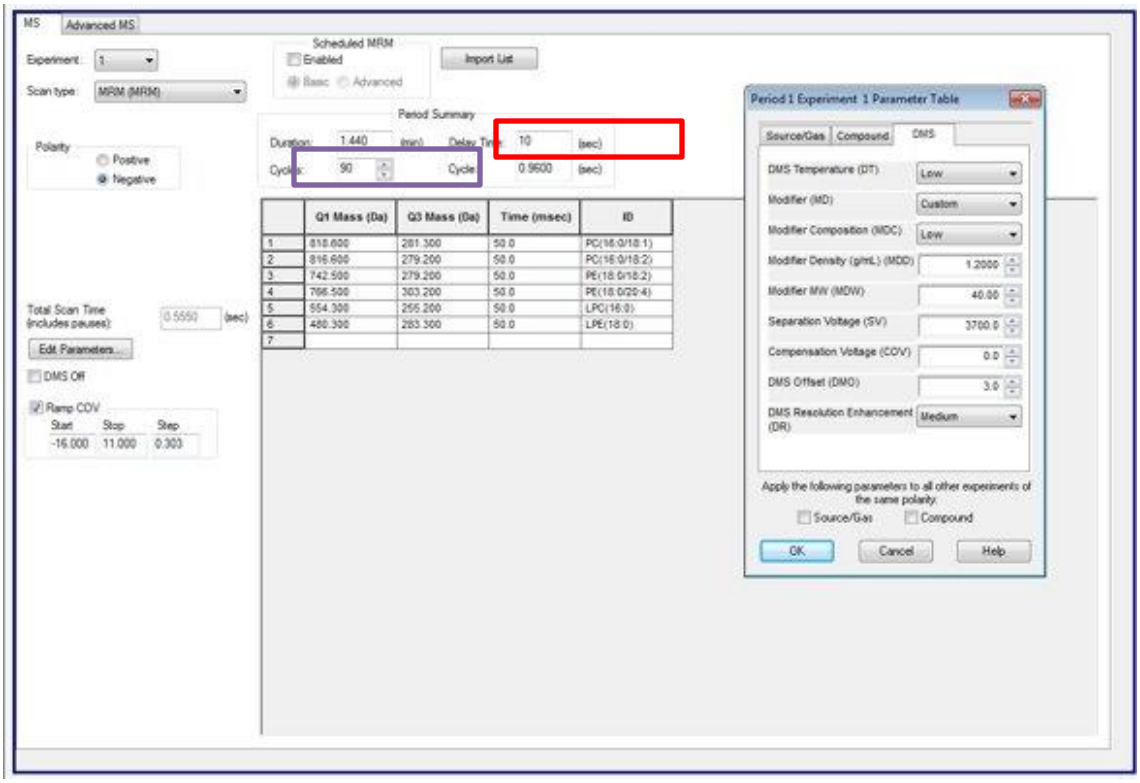


Figure 9-1. Setting delay time and cycles in Analyst software for SST, Method 1 and Method 2.

5. Using Table 5-2, set the parameters for Method 1
6. Using Table 9-1, set the parameters for Method 2

Table 9-1. SCIEX 6500+ series systems parameters for lipid analysis		
Source parameters	**Positive polarity**	**Negative polarity**
IS	5200	-4100
CUR	25 psi	25 psi
*TEM	250°C	250°C
*GS1	25 psi	25 psi
*GS2	25 psi	25 psi
CAD	9	9
**These values may need to be optimized to obtain maximum sensitivity.*		
DMS parameters		
DMS Temp	Low	Low
Modifier	Off	Off
MS		
Scan type	MRM	MRM
Advanced MS		
Q1 resolution	Unit	Unit
Q3 resolution	Unit	Unit

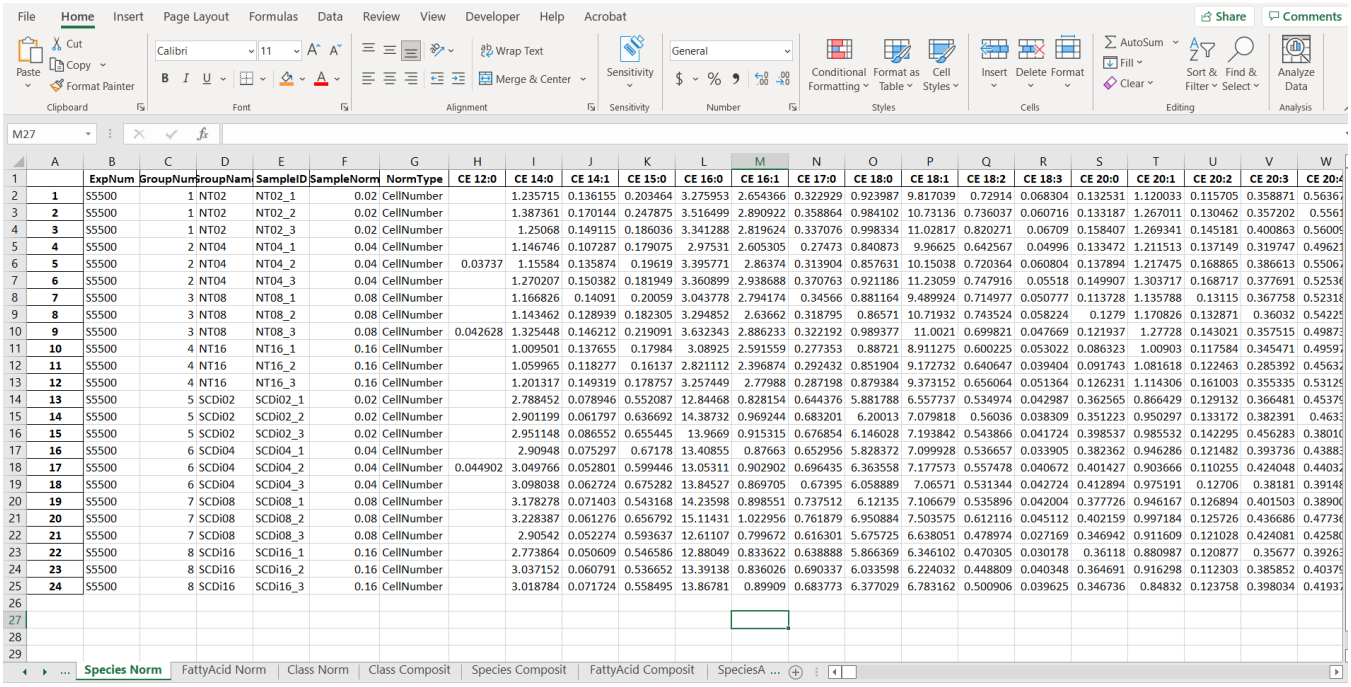
7. Save the methods as Method 1 or Method 2
8. Build a batch with the data file naming convention as “date - project name - 1” for Method 1 and “date - project name - 2” for Method 2. The date and project name can vary but if the SLA software will be used for analysis, the “-1” or “-2” is essential at the end of the data file name.

10.0 Data analysis in SLA software

To analyze data using SLA software, follow the instructions on GitHub.

First, the *.wiff files need to be converted to MZML format. For conversion, a link to the MSConvertGUI from Proteowizard can be found on the SLA software GitHub instructions page.

In brief, the SLA software will require a user-specified dictionary file containing a list of MRM transitions and assigned internal standards to create an output Excel file that contains the concentrations and compositions of lipid species, classes and fatty acids. These data can be normalized based on a user-defined input sheet that can, for example, normalize the data to tissue weight or cell count.



	ExpNum	GroupNum	GroupNam	SampleID	SampleNorm	NormType	CE 12:0	CE 14:0	CE 14:1	CE 15:0	CE 16:0	CE 16:1	CE 17:0	CE 18:0	CE 18:1	CE 18:2	CE 18:3	CE 20:0	CE 20:1	CE 20:2	CE 20:3	CE 20:4
1	1	S5500	1	NT02	NT02_1	0.02	CellNumber		1.235715	0.136155	0.203464	3.275953	2.654366	0.322929	0.923987	9.817039	0.72914	0.068304	0.132531	1.120033	0.115705	0.358871	0.56367
2	2	S5500	1	NT02	NT02_2	0.02	CellNumber		1.387361	0.170144	0.247875	3.516499	2.890922	0.358864	0.984102	10.73136	0.736037	0.060716	0.133187	1.267011	0.130462	0.357202	0.5561
3	3	S5500	1	NT02	NT02_3	0.02	CellNumber		1.25068	0.149115	0.186036	3.341288	2.819624	0.337076	0.998334	11.02817	0.820271	0.06709	0.158407	1.269341	0.145181	0.400863	0.56009
4	4	S5500	2	NT04	NT04_1	0.04	CellNumber		1.146746	0.107287	0.179075	2.97531	2.605305	0.27473	0.840873	9.96625	0.642567	0.04996	0.133472	1.211513	0.137149	0.319747	0.49621
5	5	S5500	2	NT04	NT04_2	0.04	CellNumber	0.03737	1.15584	0.135874	0.19619	3.395771	2.86374	0.313904	0.857631	10.15038	0.720364	0.060804	0.137894	1.217475	0.168865	0.386613	0.55067
6	6	S5500	2	NT04	NT04_3	0.04	CellNumber		1.270207	0.150382	0.181949	3.360899	2.938688	0.370763	0.921186	11.23059	0.747916	0.05518	0.149907	1.303717	0.168717	0.377691	0.52536
7	7	S5500	3	NT08	NT08_1	0.08	CellNumber		1.166826	0.14091	0.20059	3.043778	2.794174	0.34566	0.881164	9.489924	0.714977	0.050777	0.113728	1.135788	0.13115	0.367758	0.52318
8	8	S5500	3	NT08	NT08_2	0.08	CellNumber		1.143462	0.128939	0.182305	3.294852	2.63662	0.318795	0.86571	10.71932	0.743524	0.058224	0.1279	1.170826	0.132871	0.36032	0.54225
9	9	S5500	3	NT08	NT08_3	0.08	CellNumber	0.042628	1.325448	0.146212	0.219091	3.632343	2.886233	0.322192	0.989377	11.0021	0.699821	0.047669	0.121937	1.27728	0.143021	0.357515	0.49873
10	10	S5500	4	NT16	NT16_1	0.16	CellNumber		1.009501	0.137655	0.17984	3.08925	2.591559	0.277353	0.88721	8.911275	0.600225	0.053022	0.086323	1.00903	0.117584	0.345471	0.49597
11	11	S5500	4	NT16	NT16_2	0.16	CellNumber		1.059965	0.118277	0.16137	2.821112	2.396874	0.292432	0.851904	9.172732	0.640647	0.039404	0.091743	1.081618	0.122463	0.285392	0.45632
12	12	S5500	4	NT16	NT16_3	0.16	CellNumber		1.201317	0.149319	0.178757	3.257449	2.77988	0.287198	0.879384	9.373152	0.656064	0.051364	0.126231	1.114306	0.161003	0.355335	0.53125
13	13	S5500	5	SCDi02	SCDi02_1	0.02	CellNumber		2.788452	0.078946	0.552087	12.84468	0.828154	0.644376	5.881788	6.557737	0.534974	0.042987	0.362565	0.866429	0.129132	0.366481	0.45375
14	14	S5500	5	SCDi02	SCDi02_2	0.02	CellNumber		2.901199	0.061797	0.636692	14.38732	0.969244	0.683201	6.20013	7.079818	0.56036	0.038309	0.351223	0.950297	0.133172	0.382391	0.4633
15	15	S5500	5	SCDi02	SCDi02_3	0.02	CellNumber		2.951148	0.086552	0.655445	13.9669	0.915315	0.676854	6.146028	7.193842	0.543866	0.041724	0.398537	0.985532	0.142295	0.456283	0.38010
16	16	S5500	6	SCDi04	SCDi04_1	0.04	CellNumber		2.90948	0.075297	0.67178	13.40855	0.87663	0.652956	5.828372	7.099928	0.536657	0.033905	0.382362	0.946286	0.121482	0.393736	0.43883
17	17	S5500	6	SCDi04	SCDi04_2	0.04	CellNumber	0.044902	3.049766	0.052801	0.599446	13.05311	0.902902	0.696435	6.363558	7.177573	0.557478	0.040672	0.401427	0.903666	0.110255	0.424048	0.44032
18	18	S5500	6	SCDi04	SCDi04_3	0.04	CellNumber		3.098038	0.062724	0.675282	13.84527	0.869705	0.67395	6.058889	7.06571	0.531344	0.042724	0.412894	0.975191	0.12706	0.38181	0.39148
19	19	S5500	7	SCDi08	SCDi08_1	0.08	CellNumber		3.178278	0.071403	0.543168	14.23598	0.898551	0.737512	6.12135	7.106679	0.535896	0.042004	0.377726	0.946167	0.126894	0.401503	0.38900
20	20	S5500	7	SCDi08	SCDi08_2	0.08	CellNumber		3.228387	0.061276	0.656792	15.11431	1.022956	0.761879	6.950884	7.503575	0.612116	0.045112	0.402159	0.997184	0.125726	0.436686	0.47736
21	21	S5500	7	SCDi08	SCDi08_3	0.08	CellNumber		2.90542	0.052274	0.593637	12.61107	0.799672	0.616301	5.675725	6.638051	0.478974	0.027169	0.346942	0.911609	0.121028	0.424081	0.42580
22	22	S5500	8	SCDi16	SCDi16_1	0.16	CellNumber		2.773864	0.050609	0.546586	12.88049	0.833622	0.638888	5.866369	6.346102	0.470305	0.030178	0.36118	0.880987	0.120877	0.35677	0.39263
23	23	S5500	8	SCDi16	SCDi16_2	0.16	CellNumber		3.037152	0.060791	0.536652	13.39138	0.836026	0.690337	6.033598	6.224032	0.448809	0.040348	0.364691	0.916298	0.112303	0.385852	0.40375
24	24	S5500	8	SCDi16	SCDi16_3	0.16	CellNumber		3.018784	0.071724	0.558495	13.86781	0.89909	0.683773	6.377029	6.783162	0.500906	0.039625	0.346736	0.84832	0.123758	0.398034	0.41937

Figure 10-1. Example output of SLA software. The SLA software contains 6 tabs that denote the concentrations and compositions of lipid species, classes and fatty acids.

Appendix A – Sample preparation protocols for biological samples

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

A1. Extraction protocol for plasma

1. Use 13 x 100 mm new glass screw-capped tubes. Do not use washed tubes, as you may extract detergent residue.
2. To 25 µL of plasma, add 975 µL of water. Let sit on ice for 10 min.
3. Add 2 mL of methanol
4. Add 0.9 mL of dichloromethane
5. Vortex
6. Make sure there is a mono-phase at this stage. If 2 distinct phases are observed, add 50 µL of methanol and vortex, then check to see if the solution is a single phase. If not, repeat the addition of 50 µL of methanol and vortex.
7. Add internal standard, vortex and let mixture sit for 30 min at room temperature
8. Add 1 mL of water
9. Add 0.9 mL of 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 new glass tube
13. Add 2 mL of dichloromethane to remaining materials 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 the final volume is 1 mL, adjusting if necessary.
    c. For adhered cells: Wash cells with non-buffered saline. Add 1 mL water to lyse cells and scrape. Collect cell lysate and transfer to glass tube for extraction. Allow to rest on ice for 10 min. Ensure the final volume is 1 mL, adjusting if necessary.
3. Add 2 mL of methanol
4. Add 0.9 mL of dichloromethane
5. Vortex
6. Make sure there is a mono-phase at this stage. If 2 distinct phases are observed, add 50 µL of methanol and vortex, then check to see if the solution is a single phase. If not, repeat the addition of 50 µL of methanol and vortex.
7. Add internal standard, vortex and let mixture sit for 30 min at room temperature
8. Add 1 mL of water
9. Add 0.9 mL of dichloromethane
10. Vortex
11. Centrifuge at 1200 rpm for 10 min
12. Collect lower layer and put into a new glass tube
13. Add 2 mL of dichloromethane to remaining materials 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. Approximately 50–100 mg of tissue is sufficient. Calculate the 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, 65%
    e. Testes, 18%
2. Add water to tissue so that the total water volume is 1 mL
    a. For example, a 100 mg sample of brain contains 60 µL of water. To reach 1 mL total water volume, add 940 µL of water.
3. Homogenize tissue using a bead mill or equivalent
4. Transfer homogenate into a 13 x 100 mm glass screw-top tube
5. Add 2 mL of methanol
6. Add 0.9 mL of dichloromethane
7. Vortex
8. Make sure there is a mono-phase at this stage. If 2 distinct phases are observed, add 50 µL of methanol and vortex, then check to see if the solution is a single phase. If not, repeat the addition of 50 µL of methanol and vortex.
9. Add internal standard, vortex and let mixture sit for 30 min at room temperature
10. Add 1 mL of water
11. Add 0.9 mL of dichloromethane
12. Vortex
13. Centrifuge at 1200 rpm for 10 min
14. Collect lower layer and put into a new glass tube
15. Add 2 mL of dichloromethane to remaining materials 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.

A4. Internal standard preparation

1. Part numbers are provided in Section 3 of this SOP
2. Using Table A1, add the volume that is recommended for each lipid class to each sample
   a. Note that this volume is based on a single sample. You can scale this volume to yield a homogenous mixture that can be added to a batch of samples.
3. Please note: These volumes are the recommended starting volumes for each lipid class. They might need to be reoptimized based on your matrix of interest.

Table-A1. Recommended volumes for internal standard addition. The part number for each internal standard class is listed with the number of standards that comprise that subclass. Concentrations indicated are for the lot used and might need to be adjusted for future lots. The far-right column provides the recommended volume of internal standards to add to each sample.

Subclass	Product part number	Number of standards	Concentration (mg/mL)	µL volume used
CE	SCIEX 5040147	8	3	2.50
CER	SCIEX 5040146	1	0.02	2.50
DAG (DG)	SCIEX 5040148	8	0.025	2,57
DCER	SCIEX 5040387	1	0.004	2.50
FFA	SCIEX 5040149	2	0.1	2.50
HCER (HexCER)	SCIEX 5040388	1	0.03	2.50
LCER (LacCer)	SCIEX 5040389	1	0.03	2.50
LPC	SCIEX 5040150	1	0.1	2.50
LPE	SCIEX 5040151	1	0.05	2.50
PC	SCIEX 5040152	10	1.25	2.50
PE	SCIEX 5040153	8	0.05	2.50
SM	SCIEX 5040154	4	0.4	2.50
TAG (TG)	SCIEX 5040155	8	0.7	2.52
PG	Avanti 330827	5	0.225	1.00
PI	Avanti 330830	5	0.225	2.50
PS	Avanti 330828	5	0.225	2.50
PA	Avanti 791642	1	1	0.10

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