

Untargeted and Targeted Lipidomics Using Ultra-High Resolution Qq-Time-Of-Flight Impact II™ Mass Spectrometer with SimLipid® Software

¹Ningombam Sanjib Meitei, ¹Himani Gupta, ²Arun Apte, ³Aiko Barsch, ⁴Anjali Alving
¹PREMIER Biosoft, Indore, India; ²PREMIER Biosoft, CA, USA; ³Bruker Daltonik GmbH, Bremen, Germany; ⁴Bruker Corporation, MA, USA

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Introduction

Lipids play a variety of important roles in the maintenance of nutrition and health. Their main biological functions include the storage of energy, acting as structural components of cell membranes and behaving as important signaling molecules. Abnormal levels of certain lipids, particularly cholesterol and trans fatty acids, are known risk factors for heart disease and other diseases. Lipids also play a key role in cell, tissue, and organ physiology for diseases that involve the disruption of metabolic enzyme pathways, such as cancer and diabetes.

Comparative studies of the complex lipid mixtures found in cells and tissues could potentially reveal lipid biomarkers since the presence of lipids in membranes, or as signaling molecules, is reflective of the physiological state of an organism at a given time. Targeted and non-targeted approaches based on chromatography and mass spectrometry have been employed for such studies and they both involve the identification and measurement of lipids in the sample.

The challenges with mass spectrometry based lipidomics lie in the chemical complexity and the large range of concentration of thousands of lipid species present in biological samples. In addition, the identification of lipids requires sophisticated software since automated interpretation of lipid MS/MS spectra is more challenging when compared to other biopolymers such as DNA, carbohydrates or peptides, since lipids show much less standardized fragment mass spectra. Each lipid class has its own fragmentation patterns as well as ionization efficiency [1].

In this study, we present a robust workflow (Figure 1) for the global lipid profiling of a plasma sample using an LC system coupled to a Bruker impact II™, an Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer (Bruker Daltonik, Bremen, Germany) with > 50,000 Full-Sensitivity Resolution and SimLipid® informatics software, (PREMIER Biosoft, USA).

Lipidomics

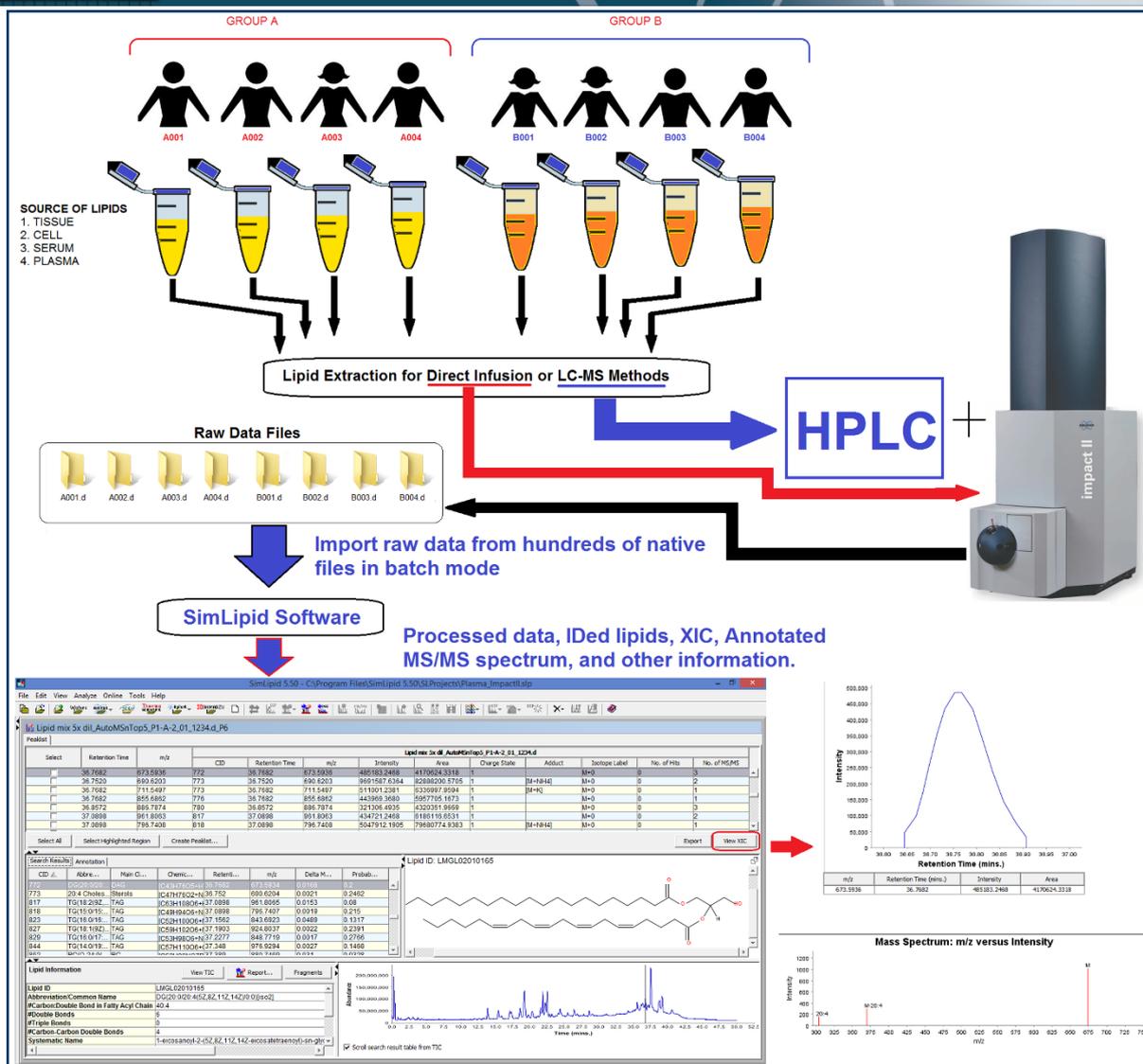


Figure 1: Schematic representation of workflow for global lipid profiling of plasma samples by employing a LC - /direct infusion-UHR-QqTOF-MS system with SimLipid software.

Experimental

Sample Preparation

Mixed lipids were extracted from human whole blood using Folch's extraction.

HPLC Instrumentation

- Agilent 1290 Infinity
- Buffer A – 60:40 acetonitrile: water + 10 mM ammonium formate + 0.1% formic acid
- Buffer B – 90:10 isopropanol: acetonitrile + 10mM ammonium formate + 0.1% formic acid
- Column – ACQUITY UPLC CSH C18 2.1 x 100 mm, 1.7 μm
- Injection volume – 1 μL
- Column Temperature – 45 °C

HPLC Gradient

RT (min)	Flow (ml/min)	%A	%B	Curve
0	0.26	68	32	0
0	0.26	68	32	6
1.5	0.26	55	45	6
4	0.26	50	50	6
8	0.26	45	55	6
18	0.26	40	60	6
20	0.26	30	70	6
35	0.26	5	95	6
40	0.26	5	95	6
45	0.26	68	32	6
47	0.26	68	32	6

MS Instrumentation

- Impact II QTOF (Bruker Daltonik, Bremen, Germany)
- Source – ESI positive mode
- Calibration – Calibration with sodium formate (m/z 90-1500 Da) using valve switching
- Capillary voltage – 4500V

Data Analysis Overview

SimLipid v. 5.50 software was used for lipid identification using the following steps:

1. Import raw data: Directly load data from multiple LC-MS, or ESI-MS experimental runs (using supported file formats namely, .baf, and .yep files) into a SimLipid project; the program, using its proprietary peak picking algorithm, assigns recalibrated precursor m/z values to MS/MS scans (see Figure 2).
2. Visualize whole data in single workbench using data table, chromatogram, and spectrum views (see Figure 3).
3. Direct Infusion methods: Identify lipids using MS and MS/MS database search in batch mode. Go to step 7.

4. LC-MS methods: Perform peak detection, peak deconvolution, molecular feature finding, and cluster MS/MS scans to LC-compounds (see Figure 6: (A), (B)).
5. Single biological group, go to step 7.
6. In case of comparative lipidomics analysis across biological groups, model experimental design by grouping peak lists into biological groups and their technical replicates (see Figure 7: (A)); following this, LC-compounds are aligned based on retention times. Furthermore, fold changes as well as p-values based on ANOVA are calculated for these compounds (see Figure 7: (B)).
7. Identify lipid using exact mass search and MS/MS database search. In case of an untargeted approach, use the SimLipid database while in case of a targeted approach; select only the custom database containing curated information (see Figure 5: (B), (C)).
8. Export identified lipids along with related information into MS excel files for further downstream data analysis.

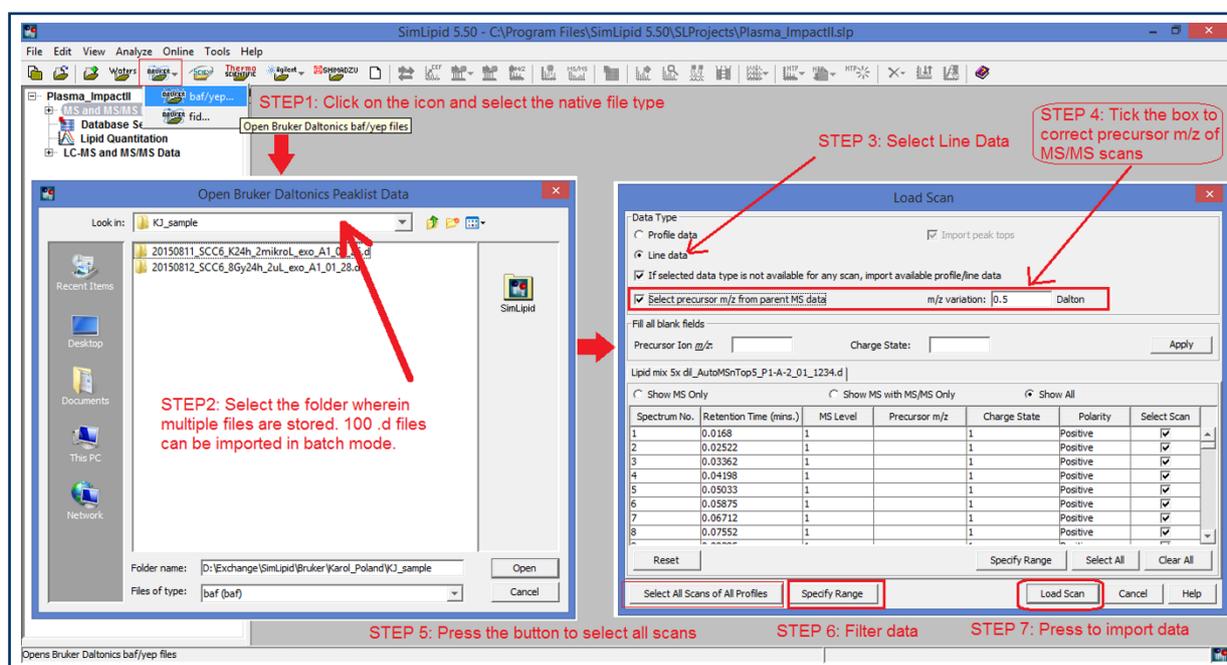


Figure 2: Typical SimLipid software graphical user interfaces (GUIs) showing interactive steps of reading raw data from Bruker's native files.

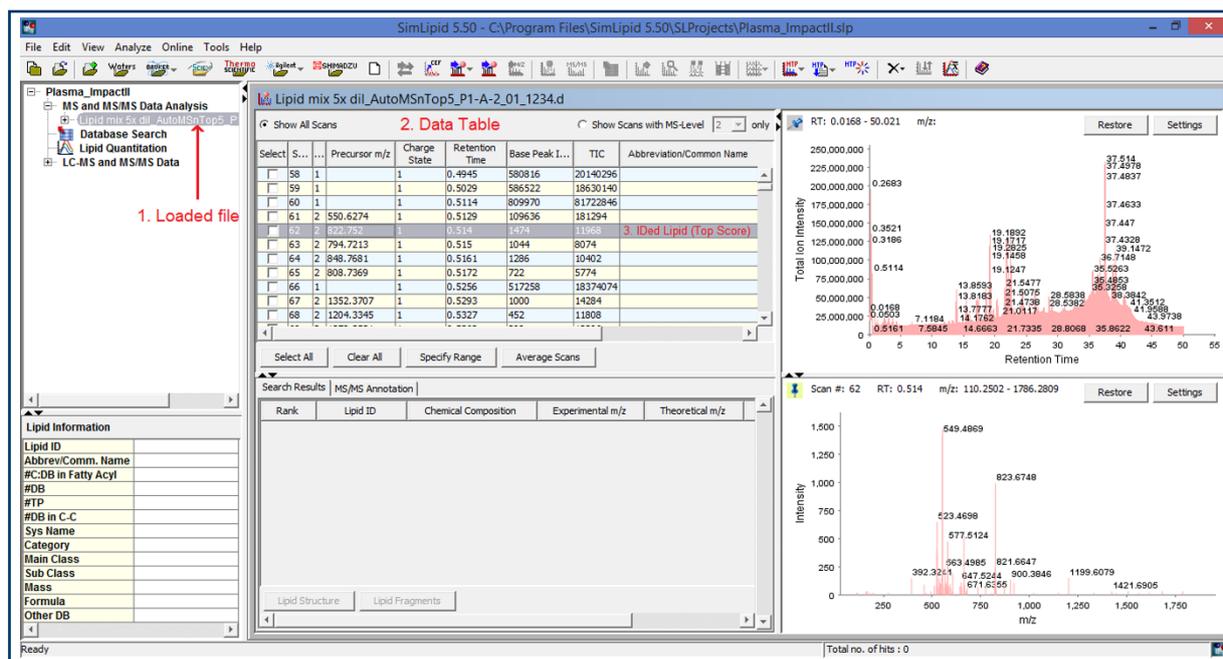


Figure 3: Typical SimLipid software GUI facilitating single workbench data- and result-visualization of a loaded data file.

SimLipid Database Overview

The SimLipid database contains 40,234 structures and 1,487,728 structure-specific *in-silico* MS/MS characteristic ions. The ions were generated based on sub-class specific fragmentation rules reported in the literature [3, 5-8] and the 529 experimental MS/MS spectra of standard lipids listed in LIPID MAPS [4].

This database is recommended for discovery lipidomics analysis.

Custom Databases (Used in Targeted Analysis)

For a targeted lipid analysis workflow the SimLipid software enables to create custom databases.

The program enables the user to add curated lipids into either an existing or a new database by importing lipid structures in .SDF or .Mol format data from text files. Figure 4 shows the typical GUIs of the web-based module of the SimLipid database software system.

Alternatively, users can create custom databases by browsing through a list of target lipids from the main SimLipid database. The main database can be filtered by multiple criteria and target lipids copied to a new or existing custom database.

SimLipid also enables to add/edit retention time information which is stored along with a lipid structure in a curated database (Figure 5: (A)). These retention times, along with the exact mass of the lipids in the curated databases, can be used as an LC-MS lipid template for distinguishing isomeric lipids structures. The user, simply by ticking the box and specifying a retention time tolerance in minutes (Figure 5: (B)), can instruct the program to use retention time as one of the database search predicates for lipid identification in addition to the typical predicates such as precursor m/z, fragment ions, and other taxonomy-based filters. Search of candidate lipids can be limited to only the selected curated databases (Figure 5: (C)).

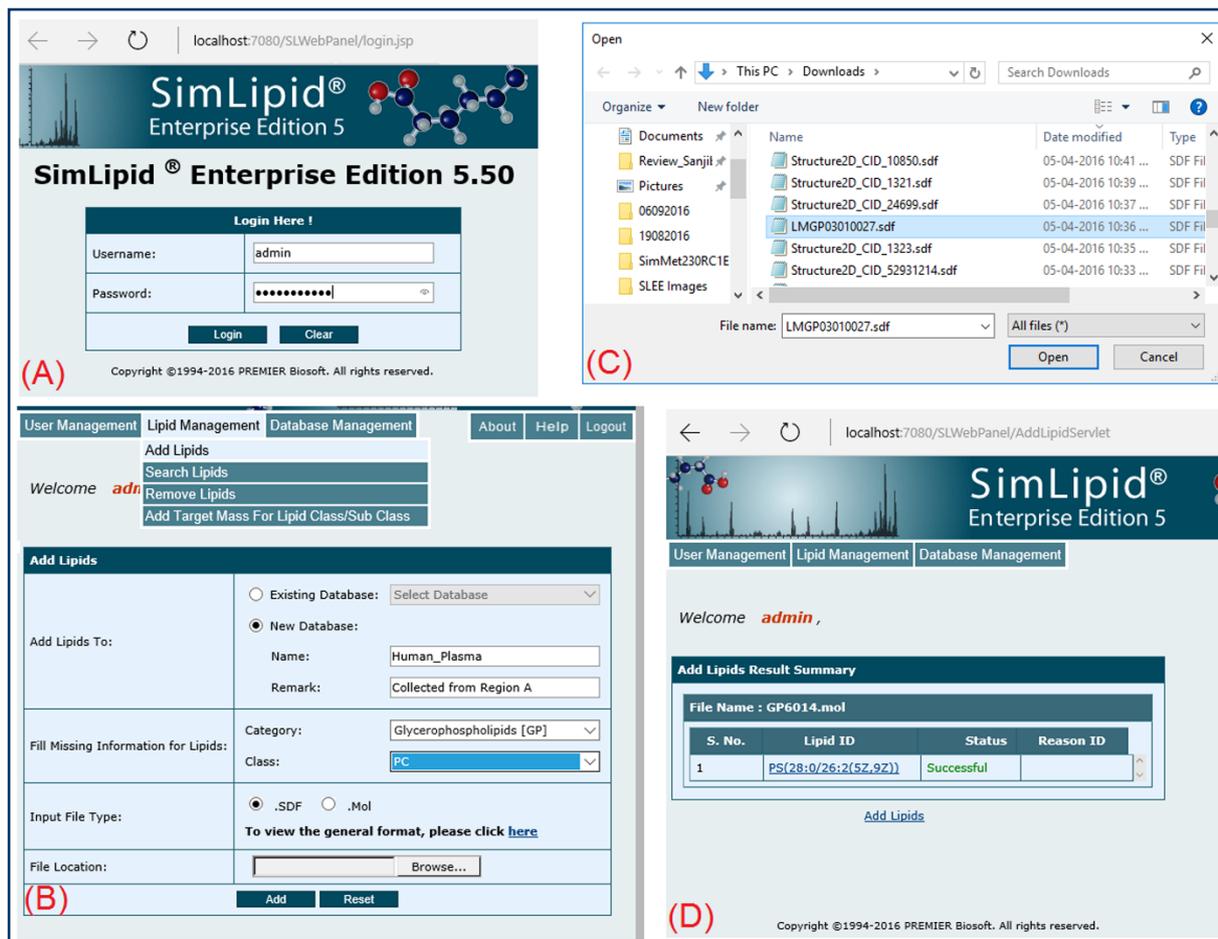


Figure 4: Typical GUIs of the web-based module of SimLipid software: (A) Login page; (B) Add curated lipids into either an existing or a new database; (C) Select the text files containing lipid structures in .SDF or .Mol format data; (D) List of lipids that have been successfully added into the database.

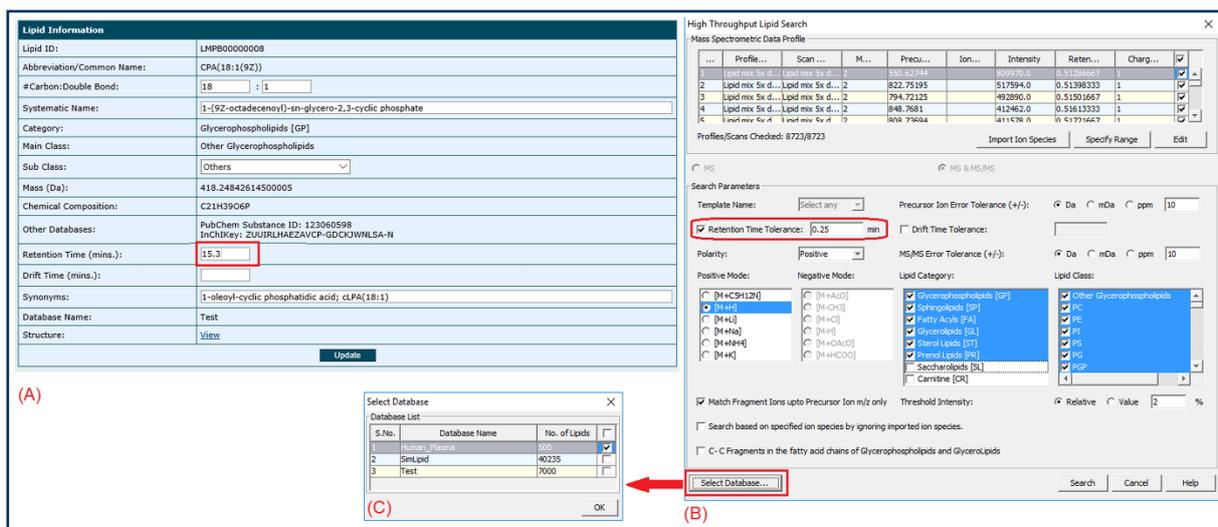


Figure 5: LC-MS lipid template: Typical GUIs of SimLipid software showing (A) web-based module to add/edit retention time stored along with the structure in a custom database; (B) Option to specify retention time tolerance; and (C) The list of existing custom databases in the SimLipid database software system.

Results

LC-MS data containing MS and data dependent MS2 spectra were processed for LC-peak detection, molecular feature finding and association of MS/MS scans to LC-compounds (see Table 1 for a summary of the results).

1	#LC-Peaks	7743
2	#LC-Compounds after molecular feature finding algorithm clusters LC-peaks based on retention time and delta masses of adducts	5239
3	#LC-compounds with identified ion species i.e., Adduct, and charge states	990
4	Total # MS/MS scans associated with LC-compounds	272

Table 1: Summary of the result generated by LC-MS data processing module of SimLipid software

The LC-compounds were then searched in the SimLipid database using the parameters shown in Figure 6: (C). First a search was performed with H as the primary adduct i.e., +H is assigned as the adduct of the LC-compounds with unknown ion species. Subsequently a second search with NH₄⁺ as the primary adduct was conducted.

In our example the searches were restricted to four main classes; Acidic glycosphingolipids, Neutral glycosphingolipids, Sphingoid bases, Sterols, and TAG. Table 2 shows a class-wise frequency distribution of lipids identified by the SimLipid software in an untargeted approach.

Table 3 summarizes the number of lipids from different Lipid classes identified by (i) our method, in comparison to published results from (ii) Sales *et al* (2016), and (iii) Quehenberger *et al* (2010).

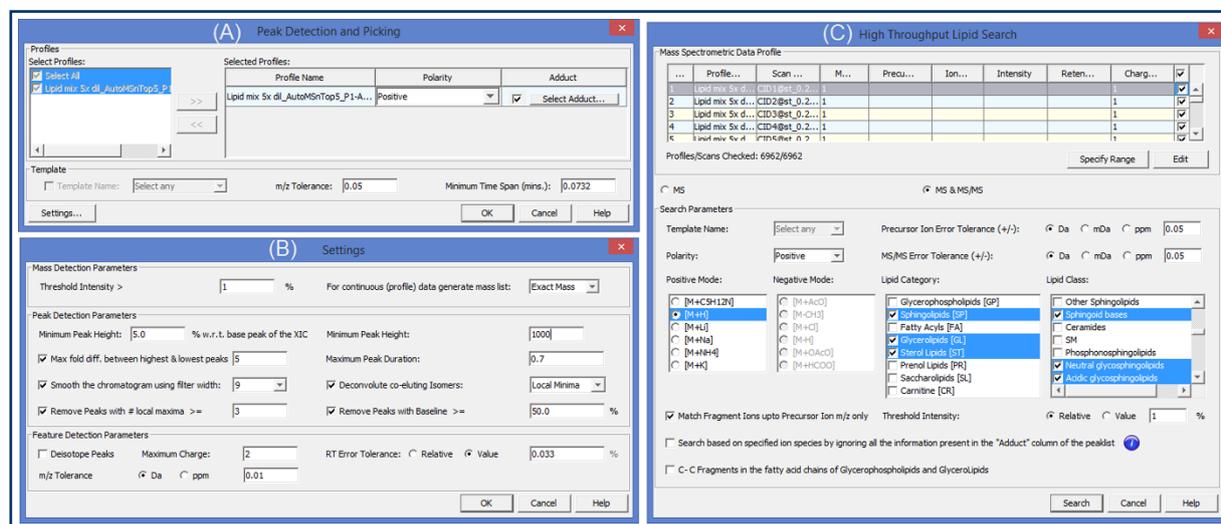


Figure 6: Typical SimLipid software GUIs depicting the parameter settings for LC-MS data pre-processing (Figures 6, A-B)), and MS and MS/MS database search of the LC-compounds (Figure 6, C).

Class (Sub class)	Frequency
TAG	477 (249*)
Neutral glycosphingolipids (Simple Glc series)	66
Sterols (Cholesterol and derivatives)	57
Sterols (Steryl esters)	44
Sterols (Ergosterols and C24-methyl derivatives)	31
Sterols (Stigmasterols and C24-ethyl derivatives)	20
Sphingoid bases (Sphingoid base analogs)	16
Acidic glycosphingolipids (Sulfoglycosphingolipids (sulfatides))	7
Sphingoid bases (Sphingoid base 1-phosphates)	3
Neutral glycosphingolipids (Galbeta1-4GlcNAc beta1-3Galbeta1-4Glc- (Neolacto series))	2
Other Sterols	24
Total	747 (519*)

Table 2: Class-wise frequency distribution of lipids identified by SimLipid software in an untargeted approach. * denotes the number of lipids after ignoring isomeric structures.

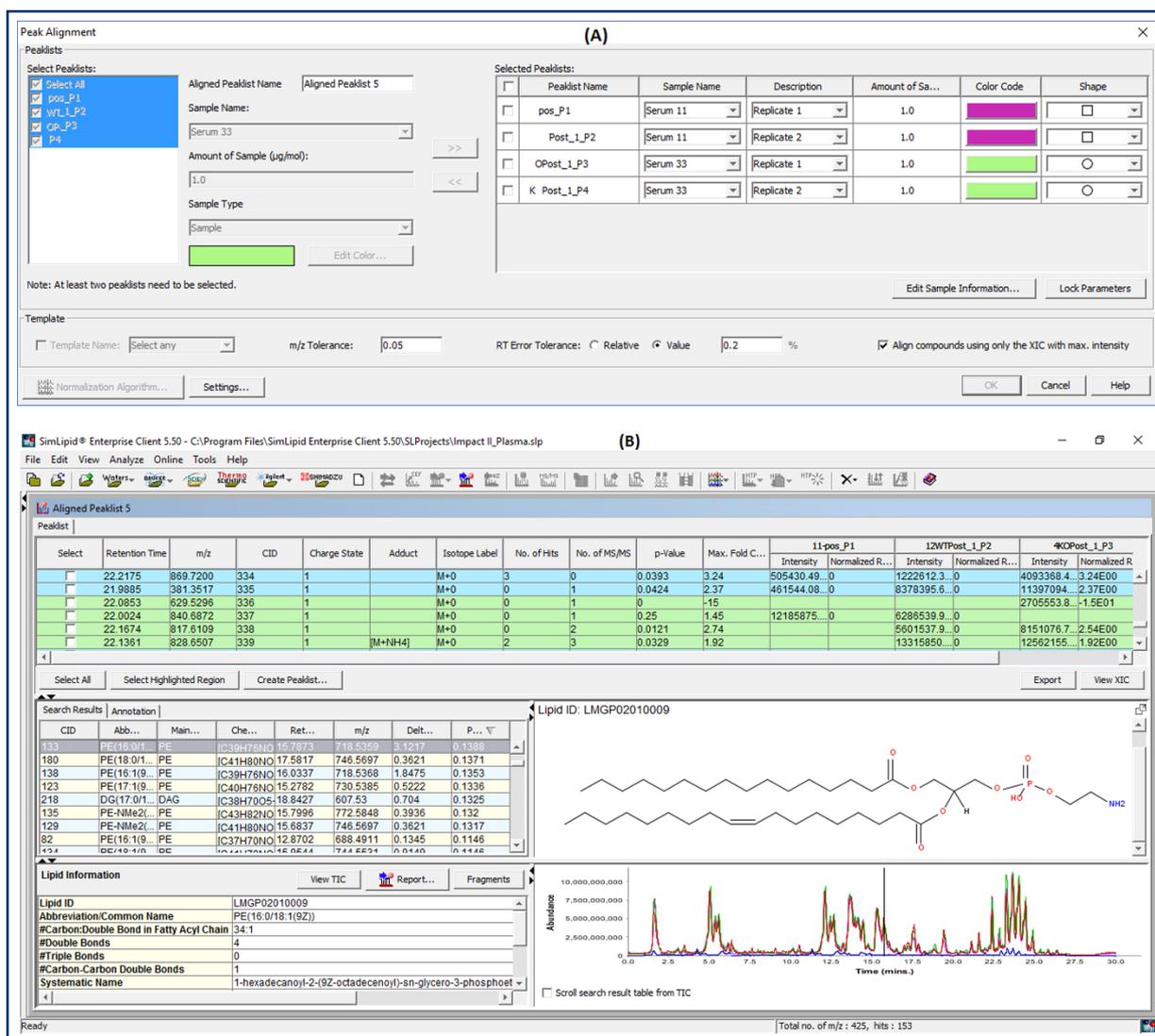


Figure 7: Typical SimLipid software GUIs (A) allowing users to model experimental design by grouping peaklists into different samples and their technical replicates, and (B) visualizing aligned samples based on LC-compounds; and corresponding fold change and p-values based on ANOVA for the assigned sample groups.

Identified by method	Cholesteryl Ester	TAG	LacCer	HexCer
(i) LC- Impact II™ + SimLipid software	28	249	21	6
(ii) Sales et al (2016) [9]	14	47	9	8
(iii) Quehenberger et al (2010) [10]	24	18	25	0

Table 3: Numbers of lipids identified from several selected lipid classes in our study compared to results from literature.

The SimLipid software provides a single workbench view of the LC-MS peaklist; a list of identified lipids in the experimental LC-MS runs; the structure of the identified lipid at a retention time, associated information of the identified lipid in the database, and the total ion chromatogram with a vertical

bar at the retention time of the identified lipid (see Figure 8: (A), (C), and (E)). Figure 8: (E) shows the same as in (A), and (C), except that the middle table shows the MS/MS spectrum list annotated with matched fragment ions of the identified lipid structure.

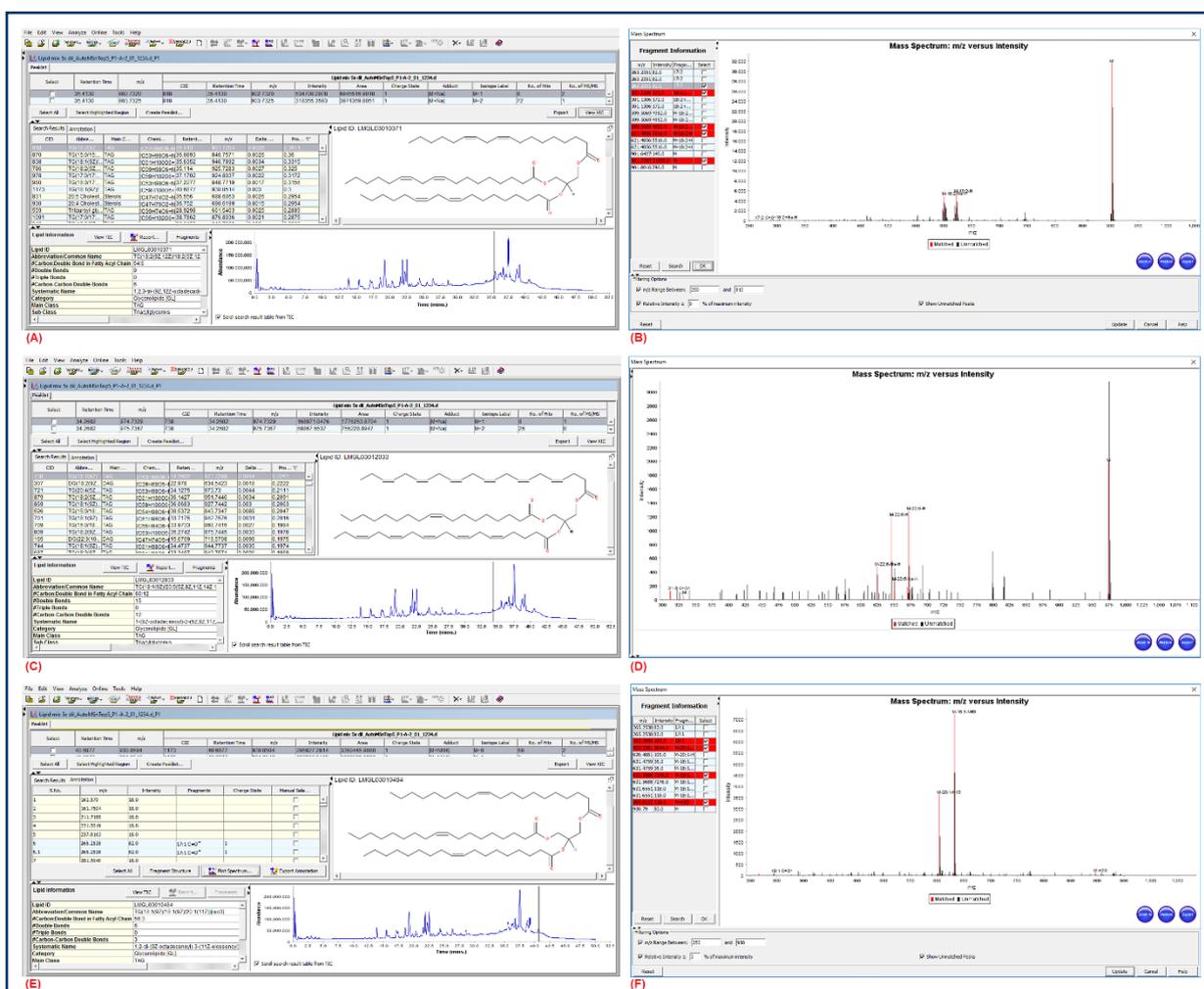


Figure 8: Typical GUIs of SimLipid software showing a single workbench view of LC-MS peaklist, results, structure, lipid information, annotated MS/MS spectrum list, and total ion chromatogram: (A) The lipid TG 54:6 and (B) the MS/MS spectrum with peaks annotated with fragment ions of TG 54:6. Similarly, the identified lipids TG 60:12, and TG 56:3 are shown in (C) -(D) and (E) -(F) respectively.

The TG 54:6 lipid species (Figure 8: (A) and (B)) was identified by our methods, as well as in [9, 10]. The TG 60:12 lipid species (Figure 8: (C), and (D)) was identified in this study but not reported in [9, 10]; and the TG 56:3 lipid species was identified here as well as in [9] but not reported in [10].

Conclusion

Improvements in analytical methods, especially liquid chromatography and mass spectrometry, have driven the recent advances in the development of lipid profiling methods. We have presented a robust workflow for the global lipid profiling of a plasma sample using an LC system coupled to an Impact II™ Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer (Bruker Daltonik, Bremen, Germany) with > 50,000 Full-Sensitivity Resolution and SimLipid® informatics software (PREMIER Biosoft, USA).

It should be noted that in addition to global lipidomics profiling presented here, targeted methods can provide complementary insights. This use of e.g., multiple shotgun-, LC-, MALDI- MS and MS/MS lipidomics methods for targeted and non-targeted approaches necessitates the multiple software workflows to automate data analysis. SimLipid software supports

automated data analysis for all the above-mentioned methods. The program allows its users to view the raw data; review the results; automatically annotate thousands of MS and MS/MS spectra; and export the results into MS excel files.

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