Chapter 14

LC–MS-Based Lipidomics and Automated Identification of Lipids Using the LipidBlast *In-Silico* MS/MS Library

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Abstract

This protocol describes the analysis, specifically the identification, of blood plasma lipids. Plasma lipids are extracted using methyl *tert*-butyl ether (MTBE), methanol, and water followed by separation and data acquisition of isolated lipids using reversed-phase liquid chromatography coupled to quadrupole/time-of-flight mass spectrometry (RPLC–QTOFMS) operated in MS/MS mode. For lipid identification, acquired MS/MS spectra are converted to the mascot generic format (MGF) followed by library search using the *in-silico* MS/MS library LipidBlast. Using this approach, lipid classes, carbon-chain lengths, and degree of unsaturation of fatty-acid components are annotated.

Key words Liquid chromatography–mass spectrometry, Tandem mass spectrometry, Lipidomics, Lipids, Identification, LipidBlast

1 Introduction

Advances in mass spectrometry have had a big impact on overall lipidomics workflows over the last decade. Analytical protocols were streamlined and fast data acquisition mass spectrometers were introduced, enabling collecting multiple types of mass spectrometric data within a single run [1–3]. One of the key advantages of mass spectrometry is its ability to be used for quantification as well as molecule identification. By utilizing tandem mass spectrometry (MS/MS), the lipid class head group, the lengths of carbon-chains, and the degree of unsaturation of fatty-acid components of these acyl groups are annotated [4]. Licensed MS/MS repositories such as Metlin and NIST14, as well as public libraries such as MassBank, do not cover lipids well because these libraries are based on the acquisition of MS/MS spectra of pure chemical standards. For many lipids there are no commercially available lipid standards. Fortunately, many lipids break in an MS/MS experiment in a predictable manner. Fragmentation rules were compiled from the literature and from authentic standards for 29 lipid classes, and

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these rules were then applied to lipid structures that were generated using *in-silico* methods to yield a really comprehensive lipidomics library for compound annotations [5].

Generating an *in-silico* MS/MS library consists of the following steps: (1) selecting lipid classes of interest and defining structural boundaries to exclude biologically improbable compounds; (2) generating all possible structures *in-silico* within these structural boundaries; (3) interpreting experimental MS/MS spectra from literature, other libraries, and authentic standards; (4) generating rules based on characteristic fragmentations; (5) modeling MS/MS spectra including ion abundances for each *in-silico* molecular species; (6) validating the *in-silico* MS/MS spectra with additional compounds that were not included in the rule generation; (7) demonstrating the applicability of such library in proof-of-principle studies (Fig. 1) [5]. Besides the protocol presented here, different software solutions for lipid identification are available either from vendors (e.g., LipidView, Lipid Search, SimLipid) or as independent platforms (e.g., LipidBlast [5],LipidXplorer[6],LipidQA[7])foruntargetedLC–MS/MS-basedlipidomics. However, no thorough comparison of advantages and disadvantages of these programs has been published.

Here, we present a protocol for using the *in-silico* MS/MS library LipidBlast for the identification of blood plasma lipids separated using reversed-phase liquid chromatography coupled to high-resolution mass spectrometry with a quadrupole/time-of-flight mass analyzer (RPLC–QTOFMS) (Fig. 2). With slight modifications, the protocol can be used also with high-resolution mass analyzer such as a quadrupole/orbital ion trap, or unit resolution mass spectrometers such as an ion trap or a single quadrupole.

2 Materials

2.1 Equipment

1. Calibrated pipettes 1–10 μL, 10–200 μL, and 100–1000 μL.
2. Vortexer.
3. Orbital mixing chilling/heating plate.
4. Centrifuge.
5. Centrifugal vacuum concentrator.
6. Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) with a pump (G4220A), a column oven (G1316C), and an autosampler (G4226A).
7. Agilent 6550 iFunnel QTOFMS system (Agilent) with an electrospray ion source operated in positive and negative ion polarity.

2.2 Reagents

1. LC–MS-grade solvents: water, acetonitrile, isopropanol.
2. Mobile-phase modifiers: formic acid, ammonium formate, ammonium acetate.

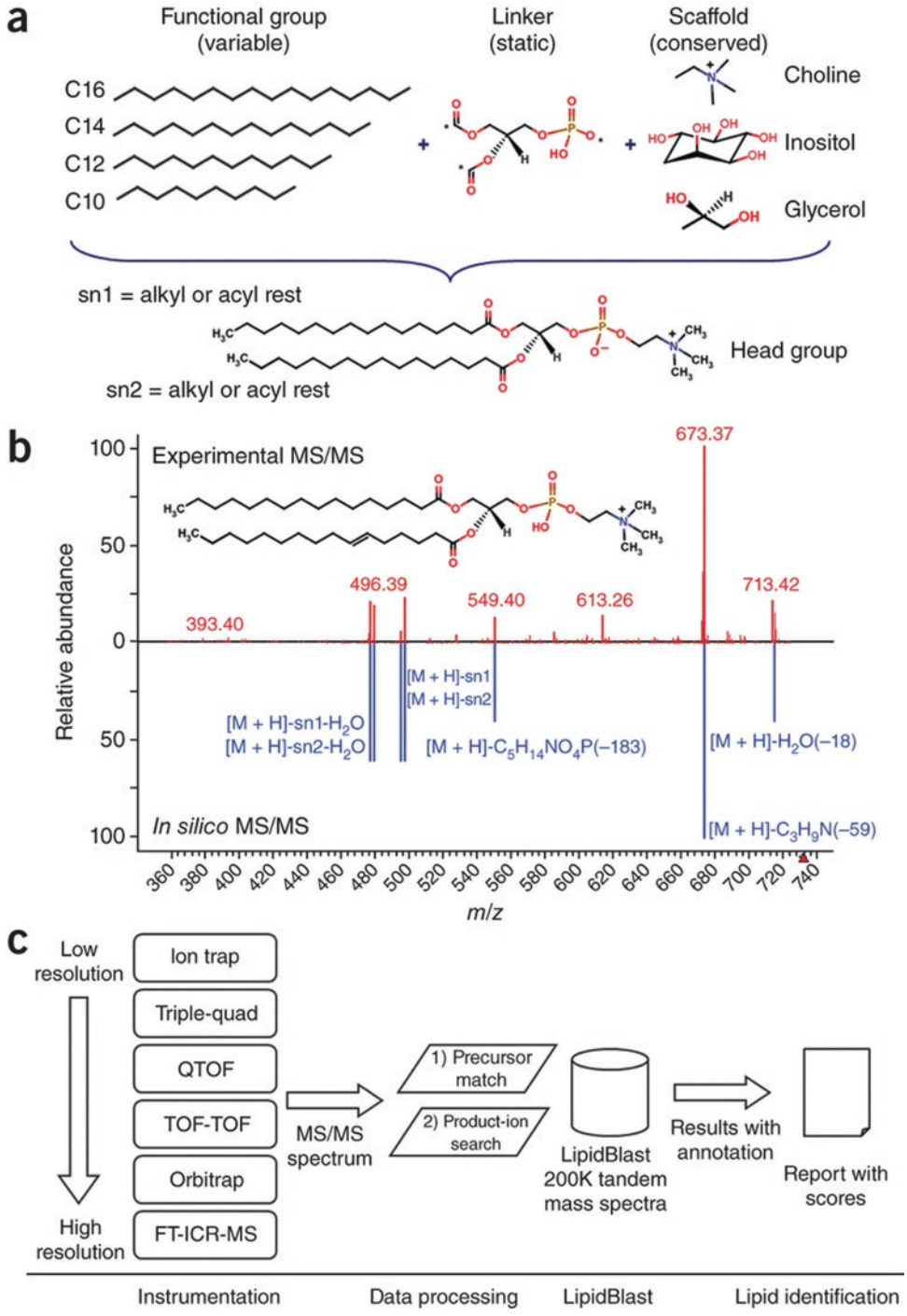

Fig. 1 Creating, validating, and applying *in-silico* generated MS/MS spectra in LipidBlast. (**a**) Lipid compound structures are generated using *in-silico* methods. Lipid core structure scaffolds are connected via a linker to fatty acyls with different chain lengths and different degrees of unsaturation. *Asterisks* denote connection points. (**b**) Reference tandem spectra (top) are used to simulate mass spectral fragmentations and ion abundances of the *in-silico* spectra (bottom). The compound shown is a phosphatidylcholine (PC), PC (16:0/16:1) at precursor *m/z* 732.55 $[M+H]^+$. (**c**) For lipid identification, MS/MS spectra obtained from LC–MS/MS experiments are submitted to LipidBlast. An *m/z* precursor-ion filter filters the data based on *m/z* precursors. If accurate mass data are used, usually 10 ppm precursor windows are used. Subsequently, experimental fragment ions are matched against the library spectra, generating a hit score that reflects the level of confidence for compound annotation. (Reproduced from [5] with permission from Nature Publishing Group)

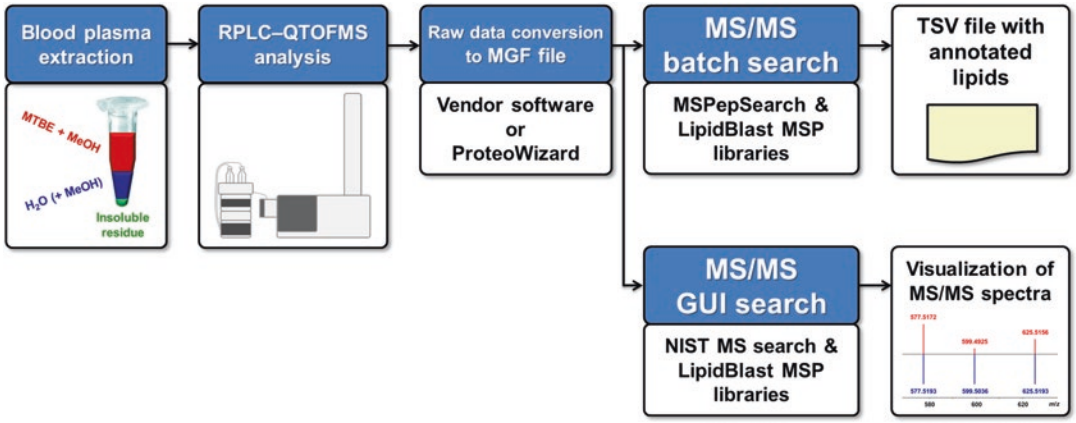

Fig. 2 Workflow of LC–MS-based lipidomics and automated identification of lipids using the LipidBlast *in-silico* MS/MS library. (*Legend: RPLC–QTOFMS* reversed-phase liquid chromatography coupled to quadrupole/time-of-flight mass spectrometry, *MGF* mascot generic format, *MSP* text files containing spectra in the NIST MS Search format, *TSV* tab-separated values, *GUI* graphical user interface)

3. Solvents for sample preparation: methanol, methyl *tert*-butyl ether, toluene, water.

2.3 Human Plasma

1. Disodium EDTA plasma, HMPLEDTA (Bioreclamation IVT, Westbury, NY, USA) stored at −80 °C prior to analysis.

2.4 Supplies

1. 1.5 mL Eppendorf tubes, uncolored.
2. Tips for organic solvents such as acetonitrile, methanol, and methyl *tert*-butyl ether.
3. Glass amber vials (2 mL volume).
4. Glass inserts for 2 mL standard opening vial (50 µL volume).
5. Screw caps for vials.
6. Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 µm) (Waters, Milford, MA, USA).
7. Acquity UPLC CSH C18 VanGuard pre-column (5 × 2.1 mm; 1.7 µm) (Waters).

3 Methods

Blood plasma lipids are extracted using a biphasic solvent system of methanol, methyl *tert*-butyl ether (MTBE), and water with some modification [8]. This extraction protocol extracts all main lipid classes in plasma with high recoveries, specifically phosphatidylcholines (PC), sphingomyelins (SM), phosphatidylethanolamines (PE), lysophosphatidylcholines (LPC), ceramides (Cer), cholesteryl esters (CholE), and triacylglycerols (TG). The plasma lipids are then separated using a short microbore column (100 × 2.1 mm

I.D.) with 1.7 μm particle size with C18 sorbent, which represents the currently preferred method in LC–MS-based lipidomics [1]. Using positive and negative electrospray ionization with different mobile-phase modifiers for each polarity increases the coverage of detected lipids [9]. Fig. 3 shows typical positive and negative electrospray ionization chromatograms of plasma lipids.

High-resolution mass spectrometry employing a quadrupole/time-of-flight mass analyzer is used for tandem MS/MS spectra collection. For the system used, different fixed collision energies (+20 eV and −40 eV for ESI(+) and ESI(−), respectively) are used to obtain information-rich MS/MS spectra. After data

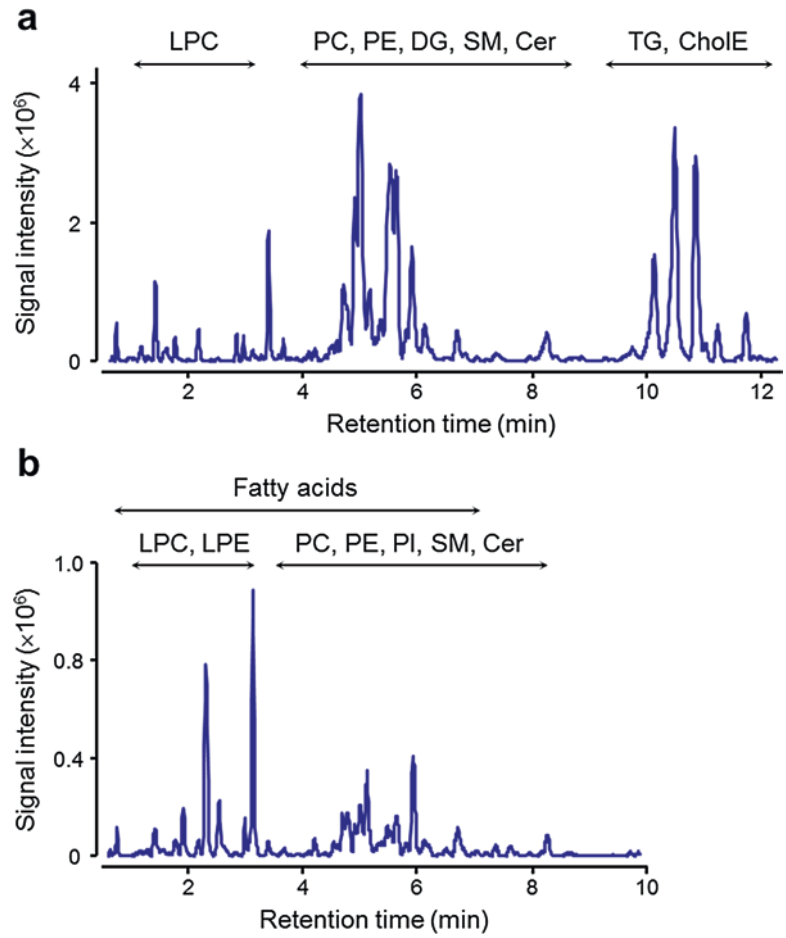

Fig. 3 Total ion chromatograms of plasma lipids, highlighting retention time ranges of particular lipid classes. (**a**) LC–ESI(+)-MS analysis; (**b**) LC–ESI(−)-MS analysis. (*Legend: Cer* ceramides, *CholE* cholesteryl esters, *DG* diacylglycerols, *LPC* lysophosphatidylcholines, *LPE* lysophosphatidylethanolamines, *PC* phosphatidylcholines, *PE* phosphatidylethanolamines, *PI* phosphatidylinositols, *SM* sphingomyelins, *TG* triacylglycerols)

acquisition, MS/MS spectra are converted to an MGF file. This MGF file is queried for lipid annotation using the *in-silico* MS/MS library LipidBlast [5].

3.1 Sample Preparation

1. Add 225 μL of cold methanol (*see* **Notes 1** and **2**) to a 10 μL blood plasma aliquot (*see* **Note 3**) in a 1.5 mL tube (*see* **Note 4**).
2. Vortex at maximum for 10 s.
3. Add 750 μL of cold MTBE (*see* **Note 1**).
4. Vortex for 10 s.
5. Shake for 6 min at 4 °C in the orbital mixer.
6. Add 188 μL of MS-grade water (*see* **Note 5**).
7. Vortex for 20 s.
8. Centrifuge the sample for 2 min at 14,000 × *g*.
9. Collect two 200 μL aliquots (*see* **Note 4**).
10. Evaporate the aliquots.
11. For LC–ESI(+)-QTOFMS analysis:
    (a) Resuspend dry extract using 150 μL of a methanol/toluene (9:1, v/v) mixture (*see* **Note 4**).
    (b) Vortex for 10 s.
    (c) Centrifuge the extract for 2 min at 14,000 × *g*.
    (d) Transfer 100 μL to a glass amber vial with a micro-insert.
    (e) Cap the vial.
    (f) Perform LC–ESI(+)-QTOFMS analysis.
12. For LC–ESI(−)-QTOFMS analysis:
    (a) Resuspend dry extract using 50 μL of a methanol/toluene (9:1, v/v) mixture (*see* **Note 4**).
    (b) Vortex for 10 s.
    (c) Centrifuge the extracts for 2 min at 14,000 × *g*.
    (d) Transfer 45 μL to a glass amber vial with a micro-insert.
    (e) Cap the vial.
    (f) Perform LC–ESI(−)-QTOFMS analysis.

3.2 LC–MS Analysis

3.2.1 Pre-Run Procedures

1. Prepare the tuning solution: 10 mL Agilent Low Concentration ESI Tuning mix (Agilent), 88.5 mL acetonitrile, 4.5 mL water, 3 μL 0.1 mM HP-0321. Make sure to add the components in the order listed to avoid precipitation of any components of the tuning mix.
2. Tune the instrument in both polarities using tuning solution.
3. Prepare the reference ion mass solution: 95 mL acetonitrile, 5 mL water, 100 μL 5 mM 921 Reference Ion, and 100 μL

10 mM Purine Reference Ion. This solution is used for mass correction during the analyses (lock mass).

(a) In ESI(+), check the intensity of ions *m/z* 121.0509 and *m/z* 922.0098, which should be between 5000 and 50,000 with 0.6 mL/min column flow rate. Adjust recipe to attain this intensity.

(b) In ESI(−), check the intensity of ions *m/z* 119.0363 and *m/z* 980.0164, which should be between 5000 and 50,000 with 0.6 mL/min column flow rate. Adjust recipe to attain this intensity.

4. Check the backpressure of the LC column. Backpressure should be within the range 500–580 bar at the beginning of each run [elution at 15% of the mobile phase (B)] and should not exceed the range 850–1000 bar [elution at 99% of the mobile phase (B)].
5. Use a new column after approximately 1000 sample injections. The LC column must be coupled to a VanGuard pre-column. The VanGuard pre-column is replaced after approximately 330 sample injections.

3.2.2 Analysis in ESI(+)

1. Prepare mobile phases: (A) 60:40 (v/v) acetonitrile:water with 10 mM ammonium formate and 0.1% formic acid; (B) 90:10 (v/v) isopropanol:acetonitrile with 10 mM ammonium formate and 0.1% formic acid (*see* **Note 6**).
2. LC gradient: 0 min 15% (B); 0–2 min 30% (B); 2–2.5 min 48% (B); 2.5–11 min 82% (B); 11–11.5 min 99% (B); 11.5–12 min 99% (B); 12–12.1 min 15% (B); 12.1–15 min 15% (B).
3. Column flow and temperature: 0.6 mL/min; 65 °C.
4. Injection volume: 3 μL (*see* **Note 4**).
5. Sample temperature: 4 °C.
6. MS conditions: MS^1 mass range, *m/z* 100–1700; MS/MS mass range, *m/z* 100–1700; collision energy, +20 eV; capillary voltage, +3.5 kV; nozzle voltage, +1 kV; gas temperature, 200 °C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min.
7. MS data acquisition: MS^1, 10 spectra/s (100 ms); MS/MS, 13 spectra/s (77 ms); total cycle time, 0.508 s; number of precursor ion per cycle, 4; mass range for selection of precursor ions, *m/z* 300–1200; isolation width, narrow (1.3 *m/z*); precursor threshold, 2000 counts; active exclusion, excluded after 3 spectra, released after 0.07 min.
8. Reference masses: *m/z* 121.0509, *m/z* 922.0098.

3.2.3 Analysis in ESI(−)

1. Prepare mobile phases: (A) 60:40 (v/v) acetonitrile:water with 10 mM ammonium acetate; (B) 90:10 (v/v) isopropanol:acetonitrile with 10 mM ammonium acetate (*see* **Note 6**).
2. LC gradient: 0 min 15% (B); 0–2 min 30% (B); 2–2.5 min 48% (B); 2.5–9.5 min 76% (B); 9.5–9.6 min 99% (B); 9.6–10.5 min 99% (B); 10.5–10.6 min 15% (B); 10.6–13.5 min 15% (B).
3. Column flow and temperature: 0.6 mL/min; 65 °C.
4. Injection volume: 5 μL (*see* **Note 4**).
5. Sample temperature: 4 °C.
6. MS conditions: MS^1 mass range, *m/z* 100–1700; MS/MS mass range, *m/z* 100–1700; collision energy, −40 eV; capillary voltage, −3.5 kV; nozzle voltage, −1 kV; gas temperature, 200 °C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min.
7. MS data acquisition: MS^1, 10 spectra/s (100 ms); MS/MS, 13 spectra/s (77 ms); total cycle time, 0.508 s; number of precursor ion per cycle, 4; mass range for selection of precursor ions, *m/z* 250–1100; isolation width, narrow (1.3 *m/z*); precursor threshold, 500 counts; active exclusion, excluded after 3 spectra, released after 0.07 min.
8. Reference masses: *m/z* 119.0360, *m/z* 980.0164 (acetate adducts).

3.2.4 Reducing Carryover between Sample Injections

Carryover between sample injections represents a critical point during LC–MS-based lipidomics analysis (*see* **Note 7**). For the LC system used we set up *Injector Cleaning* option in MassHunter Data Acquisition software.

1. Gradient for LC–ESI(+)-MS: Time 1: 0.1 min (bypass), Time 2: 11.6 min (mainpass/bypass), Time 3: 13.0 min (mainpass/bypass).
2. Gradient for LC–ESI(−)-MS: Time 1: 0.1 min (bypass), Time 2: 10.1 min (mainpass/bypass), Time 3: 11.5 min (mainpass/bypass).

3.3 Conversion of MS/MS Spectra to an MGF File

Mascot generic format (MGF) files are a common standard for MS/MS searches for small molecules. Each MS/MS scan is defined with the precursor ion (PEPMASS=), charge (CHARGE=) and *m/z*–abundance pairs (Fig. 4). Multiple product ion scans are usually combined into a single MGF file.

MGF files can be created either using vendor software or open software such as ProteoWizard.

1. Agilent .D format: Use MassHunter Qualitative Analysis software.

```
BEGIN IONS
PEPMASS=874.7866
CHARGE=1+
TITLE=CSH_pos_plasma_MSMS.d, MS/MS of 874.7866 1+ at 10.41 mins
RTINSECONDS=624.627
549.4851  416
575.5002  1693
576.1730  149
577.5222  347
578.5131  171
601.5133  440
603.5312  828
604.5497  104
857.7447  129
874.7862  750
875.7847  531

END IONS
```

Fig. 4 Example of a product ion scan in mascot generic format (MGF)

2. Thermo .raw format: Use extractMSn and MSFilereader plus dependency libraries.
3. SCIEX .wiff format: Use PeakView software and installation of MS Data Converter (downloadable at http://sciex.com/software-downloads-x2110).
4. Alternatively, ProteoWizard software can be used to create MGF files (Fig. 5).
   (a) Download and install the latest version of the ProteoWizard from the following website: http://proteowizard.sourceforge.net.
   (b) Run MSConvert.exe from ProteoWizard folder.
   (c) Browse and add files using MSConvert application.
   (d) Select output directory.
   (e) Select output format: mgf; binary encoding precision: 32-bit.
   (f) Select appropriate filters such as *MS Level* to work with MS/MS data only, *Peak Picking* to centroid data, and *Threshold Peak Filter* (count; most intense; 50).
   (g) Start conversion using *Start*, check the progress and create the MGF file.

3.4 LipidBlast in-Silico MS/MS Library

3.4.1 Installation

1. Download the latest version of the LipidBlast from the following website: http://fiehnlab.ucdavis.edu/projects/LipidBlast (section *Download LipidBlast*)
2. Unzip the file. The LipidBlast folder contains several sub-folders:

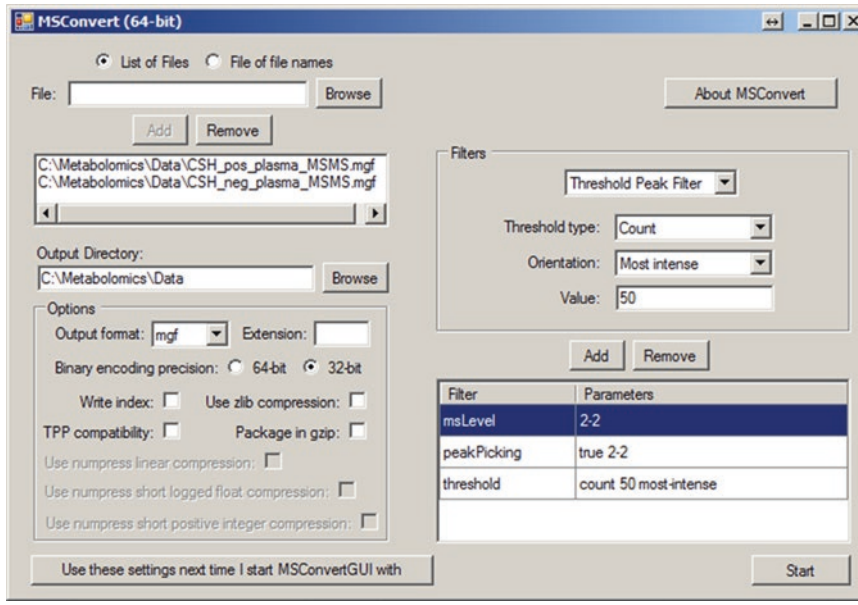

Fig. 5 MSConvert (ProteoWizard) settings for converting raw MS/MS data files to MGF files

(a) LipidBlast-ASCII-spectra.
(b) LipidBlast-Development.
(c) LipidBlast-Examples.
(d) LipidBlast-HandBook.
(e) LipidBlast-MSSearch.
(f) LipidBlast-mz-lookup.
(g) LipidBlast-Paper.
(h) LipidBlast-Validation.

3. Download MS PepSearch software from this website: http://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:mspepsearch.
4. Unzip 2013_11_14_MSPepSearch_x32.zip file to \\LipidBlast-Full-Release-3\ 2013_06_04_MSPepSearch_x32 folder.

3.4.2 MS/MS Batch Search

1. Run MSPepSearchGUI_x64.exe or MSPepSearchGUI_x32.exe from \\LipidBlast-Full-Release-3\2013_06_04_MSPepSearch_x32 and follow settings in Figs. 6 and 7 based on polarity used.
2. Upload MGF file: Input → ⊙ File → Open → Select MGF file.
3. Set up Output directory path.
4. Upload MS/MS libraries to search → Select → Select MS/MS library in MSP format from folder \\LipidBlast-Full-Release-3\ LipidBlast-MSSearch. Name of MSP libraries, ionization mode, lipid class, and molecular species covered are listed in

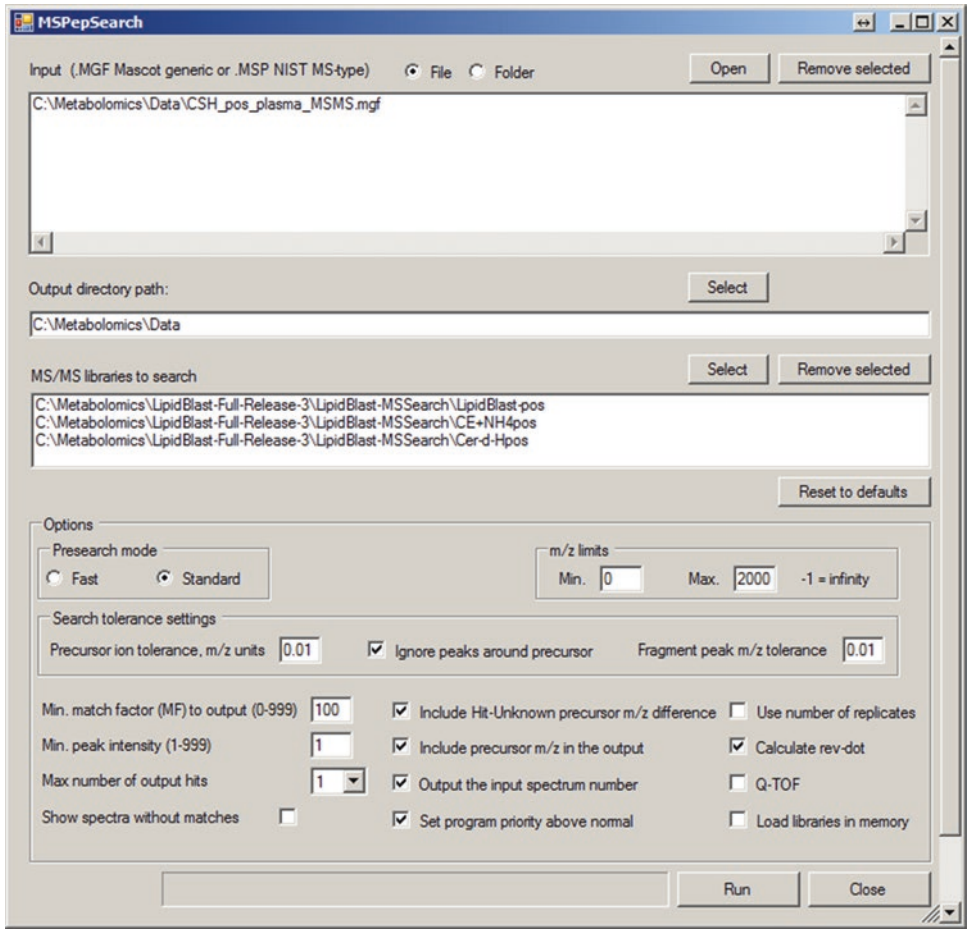

Fig. 6 General settings for MSPepSearch for ESI(+)

Table 1. A maximum of four MSP libraries can be uploaded for one search (*see* **Note 8**).

5. Change Options for Presearch mode from Fast to Standard.
6. Adjust Search tolerance settings:
   (a) Precursor ion tolerance, *m/z* units: 0.01 (*see* **Note 9**).
   (b) Fragment peak *m/z* tolerance: 0.01 (*see* **Note 9**).
   (c) Ignore peaks around precursor: keep checked.
7. Adjust other Options parameters:
   (a) Match factor (MF) to output (0–999): 100.
   (b) Min. peak intensity: 1.
   (c) Max. number of output hits: 1.
   (d) Load libraries in memory: unchecked.
8. Click Run bottom to start MS/MS library search.

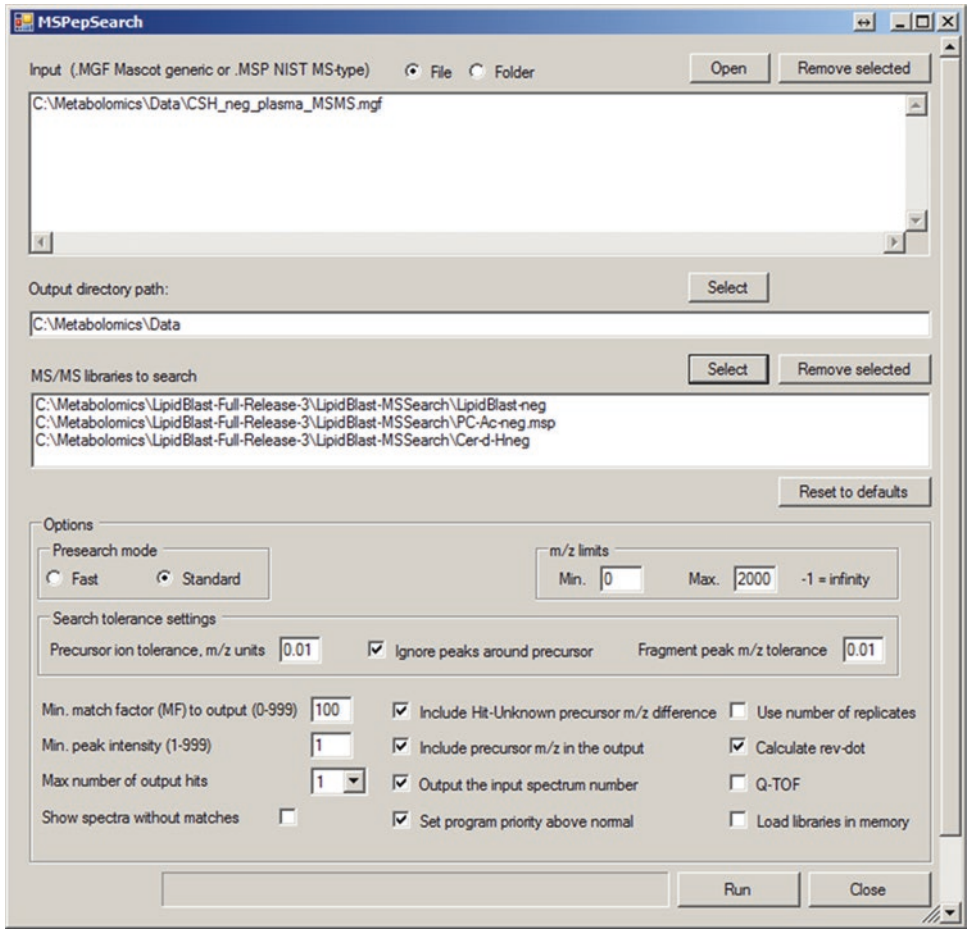

Fig. 7 General settings for MSPepSearch for ESI(−) (*see* **Note 8** for selecting MSP libraries)

9. For selected MGF file the MSPepSearch program will create TSV (tab-separated values) file containing results of MS/MS search.
10. Open TSV file in Excel for manual inspection (Fig. 8). Use *Dot Product* and *Rev.-Dot* scores for further filtering of data set.
    (a) Spectral dot product (*Dot Product*) calculation uses the cosine of the angle between the unknown and library spectral vectors.
    (b) Reverse dot product (*Rev-Dot*) scores are calculated in a similar manner but ignore all ions that are present in the sample spectrum but absent from the library spectrum.

As a general guide for reverse dot product scores, a match >900 is an excellent match; scores between 800 and 900 are good matches; scores between 700 and 800 are fair matches. Scores less than 600 are regarded as very poor matches. MS/MS spectra with many peaks will tend to yield lower spectral dot products

Table 1
Lipid classes covered by the LipidBlast *in-silico* MS/MS library

MSP library	Ionization mode	Lipid class	Abbreviation in LipidBlast	Molecular species covered
LipidBlast-pos	(+)	Ceramide-1-phosphates	CerP	$[M+H]^+$
		Diacylglycerols	DG	$[M+NH_4]^+$, $[M+Li]^+$
		Digalactosyldiacylglycerols	DGDG	$[M+Na]^+$
		Lysophosphatidylcholines	lysoPC	$[M+H]^+$, $[M+Na]^+$
		Lysophosphatidylethanolamines	lysoPE	$[M+H]^+$, $[M+Na]^+$
		Monoacylglycerols	MG	$[M+NH_4]^+$, $[M+Li]^+$
		Monogalactosyldiacylglycerols	MGDG	$[M+NH_4]^+$, $[M+Na]^+$, $[M+NH_4–CO]^+$
		Phosphatidic acids	PA	$[M+Na_2–H]^+$, $[M+Na]^+$
		Phosphatidylcholines	PC	$[M+H]^+$, $[M+Na]^+$
		Phosphatidylethanolamines	PE	$[M+H]^+$, $[M+Na]^+$
		Plasmenylphosphatidylcholines	plasmenyl-PC	$[M+H]^+$, $[M+Na]^+$
		Plasmenylphosphatidylethanolamines	plasmenyl-PE	$[M+H]^+$, $[M+Na]^+$
		Phosphatidylserines	PS	$[M+H]^+$, $[M+Na]^+$
		Sphingomyelines	SM	$[M]^+$, $[M+Na]^+$
		Triacylglycerols	TG	$[M+NH_4]^+$, $[M+Na]^+$, $[M+Li]^+$
CE + NH4pos	(+)	Cholesteryl esters	CE	$[M+NH_4]^+$
Cer-d-Hpos	(+)	*N*-Acylsphingosines (ceramides)	Cer-d	$[M+H]^+$
custom-lysoPC + Hpos	(+)	Lysophosphatidylcholines	lysoPC	$[M+H]^+$
CustomPC + Hpos.msp	(+)	Phosphatidylcholines	PC	$[M+H]^+$
CustomPC + Napos.msp	(+)	Phosphatidylcholines	PC	$[M+Na]^+$

(continued)

Table 1
(continued)

MSP library	Ionization mode	Lipid class	Abbreviation in LipidBlast	Molecular species covered
custom-SM + Hpos	(+)	Sphingomyelines	SM	$[M]^+$
lysoPA + Hpos.msp	(+)	Lysophosphatidic acids	LPA	$[M+H]^+$
LipidBlast-neg	(−)	Diacylated phosphatidylinositol monomannoside	Ac2PIM1	$[M-H]^-$
		Diacylated phosphatidylinositol dimannoside	Ac2PIM2	$[M-H]^-$
		Triacylated phosphatidylinositol dimannoside	Ac3PIM2	$[M-H]^-$
		Tetraacylated phosphatidylinositol dimannoside	Ac4PIM2	$[M-H]^-$
		Ceramide-1-phosphates	CerP	$[M-H]^-$
		Cardiolipins	CL	$[M-H]^-$, $[M-2H]^{2-}$
		Digalactosyldiacylglycerols	DGDG	$[M-H]^-$
		Gangliosides	[glycan]-Cer	$[M-H]^-$
		Diphosphorylated hexaacyl Lipid A	LipidA-PP	$[M-H]^-$
		Lysophosphatidylethanolamines	lysoPE	$[M-H]^-$
		Monogalactosyldiacylglycerols	MGDG	$[M-H]^-$
		Phosphatidic acids	PA	$[M-H]^-$
		Phosphatidylethanolamines	PE	$[M-H]^-$
		Phosphatidylglycerols	PG	$[M-H]^-$
		Phosphatidylinositols	PI	$[M-H]^-$
		Phosphatidylserines	PS	$[M-H]^-$
		Sulfoquinovosyldiacylglycerols	SQDG	$[M-H]^-$
		Sulfatides	ST	$[M-H]^-$

Cer-d-Hneg	(−)	*N*-Acylsphingosines (ceramides)	Cer-d	$[M–H]^-$
PC-Ac-neg.msp	(−)	Phosphatidylcholines	PC	$[M+CH_3COO]^-$
PC-Form-neg.msp	(−)	Phosphatidylcholines	PC	$[M+HCOO]^-$
PC-Me-neg.msp	(−)	Phosphatidylcholines	PC	$[M–CH_3]^-$

> MSPepSearch ver. 0.91 build Jun 3 2013 19:11:46. Command line:

> "C:\Metabolomics\2013_06_04_MSPepSearch_x64\MSPepSearch.exe" /INP "C:\Metabolomics\Data\CSH_pos_plasma_MSMS.mgf" /OUTTAB "C:\Metabolomics\Data\CSH_pos_plasma_MSMS.mgf.tsv" /LIB "C:\Metabolomics\LipidBlast-Full-Release-3\LipidBlast-MSSearch\Lipid

(1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11)

Num	Unknown	Precursor m/z	Rank	Library	Id	Mass	Delta(m/z)	Lib Precursor m/z	Score	Dot Product	Prob(%)	Rev-Dot	Peptide
134	CSH_pos_plasma_MSMS.d, MS/MS of 544.3387909 1+ at 1.1364 mins RT:1.13640	544.3388	1	LipidBlast-pos	9231	544	0.0015	544.3403	128	516	33.3	937	lysoPC 20:4; [M+H]+; PC(20:4(5E,8E,11E,14E)/0:0)
135	CSH_pos_plasma_MSMS.d, MS/MS of 544.3387909 1+ at 1.14191666666667 mins RT:1.14192	544.3388	1	LipidBlast-pos	9231	544	0.0015	544.3403	356	616	33.3	883	lysoPC 20:4; [M+H]+; PC(20:4(5E,8E,11E,14E)/0:0)
154	CSH_pos_plasma_MSMS.d, MS/MS of 520.3391647 1+ at 1.21858333333333 mins RT:1.21858	520.3392	1	LipidBlast-pos	9209	520	0.0012	520.3403	157	592	16.7	835	lysoPC 18:2; [M+H]+; PC(18:2(2E,4E)/0:0)
182	CSH_pos_plasma_MSMS.d, MS/MS of 466.2942047 1+ at 1.33558333333333 mins RT:1.33558	466.2942	1	LipidBlast-pos	9355	466	-0.0008	466.2934	113	458	100	671	lysoPE 17:1; [M+H]+; PE(17:1(9Z)/0:0)
187	CSH_pos_plasma_MSMS.d, MS/MS of 546.3538086 1+ at 1.3549 mins RT:1.35490	546.3538	1	LipidBlast-pos	9229	546	0.0022	546.356	397	971	50	971	lysoPC 20:3; [M+H]+; PC(20:3(5Z,8Z,11Z)/0:0)
192	CSH_pos_plasma_MSMS.d, MS/MS of 518.3228048 0 at 1.40496666666667 mins RT:1.40497	518.3228	1	LipidBlast-pos	9269	518	-0.0005	518.3223	110	840	100	995	lysoPC 16:0; [M+Na]+; PC(16:0/0:0)
193	CSH_pos_plasma_MSMS.d, MS/MS of 518.3228048 1+ at 1.41048333333333 mins RT:1.41048	518.3228	1	LipidBlast-pos	9269	518	-0.0005	518.3223	265	635	100	995	lysoPC 16:0; [M+Na]+; PC(16:0/0:0)
194	CSH_pos_plasma_MSMS.d, MS/MS of 496.3396423 1+ at 1.41601666666667 mins RT:1.41602	496.3396	1	LipidBlast-pos	9189	496	0.0007	496.3403	188	630	100	874	lysoPC 16:0; [M+H]+; PC(16:0/0:0)
198	CSH_pos_plasma_MSMS.d, MS/MS of 496.3396423 1+ at 1.42563333333333 mins RT:1.42563	496.3396	1	LipidBlast-pos	9189	496	0.0007	496.3403	201	639	100	886	lysoPC 16:0; [M+H]+; PC(16:0/0:0)
210	CSH_pos_plasma_MSMS.d, MS/MS of 518.3228048 1+ at 1.47418333333333 mins RT:1.47418	518.3228	1	LipidBlast-pos	9269	518	-0.0005	518.3223	266	633	100	995	lysoPC 16:0; [M+Na]+; PC(16:0/0:0)
212	CSH_pos_plasma_MSMS.d, MS/MS of 496.3396423 1+ at 1.4869 mins RT:1.48690	496.3396	1	LipidBlast-pos	9189	496	0.0007	496.3403	200	622	100	873	lysoPC 16:0; [M+H]+; PC(16:0/0:0)
2890	CSH_pos_plasma_MSMS.d, MS/MS of 898.7847222 0 at 10.1069333333333 mins RT:10.10693	898.7847	1	LipidBlast-pos	77189	898	0.0012	898.7859	478	735	97.7	953	TG 54:5; [M+NH4]+; TG(18:1/18:2/18:2)
2898	CSH_pos_plasma_MSMS.d, MS/MS of 872.7698181 0 at 10.1261666666667 mins RT:10.1262	872.7698	1	LipidBlast-pos	75855	872	0.0004	872.7702	412	679	96.4	949	TG 52:4; [M+NH4]+; TG(16:0/18:2/18:2)
2902	CSH_pos_plasma_MSMS.d, MS/MS of 872.7698181 1+ at 10.1357833333333 mins RT:10.13578	872.7698	1	LipidBlast-pos	75855	872	0.0004	872.7702	414	678	96.5	949	TG 52:4; [M+NH4]+; TG(16:0/18:2/18:2)
2910	CSH_pos_plasma_MSMS.d, MS/MS of 846.7548218 1+ at 10.1550166666667 mins RT:10.15502	846.7548	1	LipidBlast-pos	75737	846	-0.0002	846.7546	535	758	98.9	956	TG 50:3; [M+NH4]+; TG(16:0/16:1/18:2)
2914	CSH_pos_plasma_MSMS.d, MS/MS of 846.7548218 1+ at 10.1646333333333 mins RT:10.16463	846.7548	1	LipidBlast-pos	75715	846	-0.0002	846.7546	359	575	61.1	938	TG 50:3; [M+NH4]+; TG(16:0/16:0/18:3)
2915	CSH_pos_plasma_MSMS.d, MS/MS of 690.6188354 1+ at 10.166 mins RT:10.1660	690.6188	1	CE+NH4pos	25	690.619	0.0001	690.6189	314	706	100	849	CE(20:4); [M+NH4]+; 20:4 Cholesteryl ester
2916	CSH_pos_plasma_MSMS.d, MS/MS of 820.7402242 1+ at 10.16735 mins RT:10.16735	820.7402	1	LipidBlast-pos	75731	820	-0.0013	820.739	284	611	99	953	TG 48:2; [M+NH4]+; TG(16:0/16:1/16:1)
2918	CSH_pos_plasma_MSMS.d, MS/MS of 898.7847222 1+ at 10.1742333333333 mins RT:10.17423	898.7847	1	LipidBlast-pos	77189	898	0.0012	898.7859	457	696	98.1	953	TG 54:5; [M+NH4]+; TG(18:1/18:2/18:2)
3588	CSH_pos_plasma_MSMS.d, MS/MS of 964.9240112 1+ at 12.05845 mins RT:12.05845	964.924	1	LipidBlast-pos	75972	964	0.0026	964.9267	171	355	100	674	TG 58:0; [M+NH4]+; TG(16:0/21:0/21:0)
3612	CSH_pos_plasma_MSMS.d, MS/MS of 992.9559631 0 at 12.1288666666667 mins RT:12.12887	992.956	1	LipidBlast-pos	77640	992	0.002	992.9579	119	381	77.3	674	TG 60:0; [M+NH4]+; TG(19:0/19:0/22:0)
3617	CSH_pos_plasma_MSMS.d, MS/MS of 992.9559631 1+ at 12.1415 mins RT:12.1415	992.956	1	LipidBlast-pos	77752	992	0.002	992.9579	179	555	86.4	913	TG 60:0; [M+NH4]+; TG(20:0/20:0/20:0)

> Completed. 3790 spectra in 1.209 sec, process time = 1.211 sec (read 0.062s print 0.012s init pio 0.002s aux 0.008s sp 0.002s, search only: 0.639s+0.487s=1.126s, 3365.80 spec/s) 3134.44 spec/s Compared 17430 spec, No spectra compared 3023, peaks/spec 3.63

Fig. 8 Example of TSV file containing an overview of identified lipids based on MS/MS and LipidBlast search. Legend: (**1**) scan number in MGF file; (**2**) name of data file processed with particular precursor ion and retention time; (**3**) precursor *m/z*; (**4**) rank; (**5**) MSP library name; (**6**) identification number in particular MSP library; (**7**) difference between theoretical (Lib Precursor *m/z*) and experimental (Precursor *m/z*) masses; (**8**) theoretical mass of precursor ion; (**9**) spectral dot product; (**10**) reverse dot product; (**11**) annotated lipid

(*Dot Product*) than similar spectra with fewer peaks. This can be the case when using higher collision energy leading to extensive fragmentation of precursor ion (Fig. 9).

11. If lipid annotations occur multiple times, there can be two reasons: (1) a true separation of isomers, as in Fig. 10, where two triacylglycerols have the same precursor mass and the same number of acyl carbons and double bonds, however, differ in acyl chain lengths. (2) If MS/MS spectra are annotated with identical isomeric structures, peaks should be at least one peak width apart from each other to exclude erroneous MS/MS selection of the same peak.
12. Some lipids may have lower dot product score because their MS/MS spectra represent a mixture of two or even more lipid isomers having the same number of carbon and double bonds but differ in acyl chain lengths (Fig. 11). Such coelutions unavoidably occur during LC–MS-based lipidomic profiling of complex samples. In this case, the particular lipid is annotated using both species (e.g., TG 52:3; TG 16:1_18:1_18:1 and TG 16:0_18:1_18:2). To get these annotations in TSV file change the *Max number of output hits* parameter from 1 to 2 or 3 in the MSPepSearch (Figs. 6 and 7).

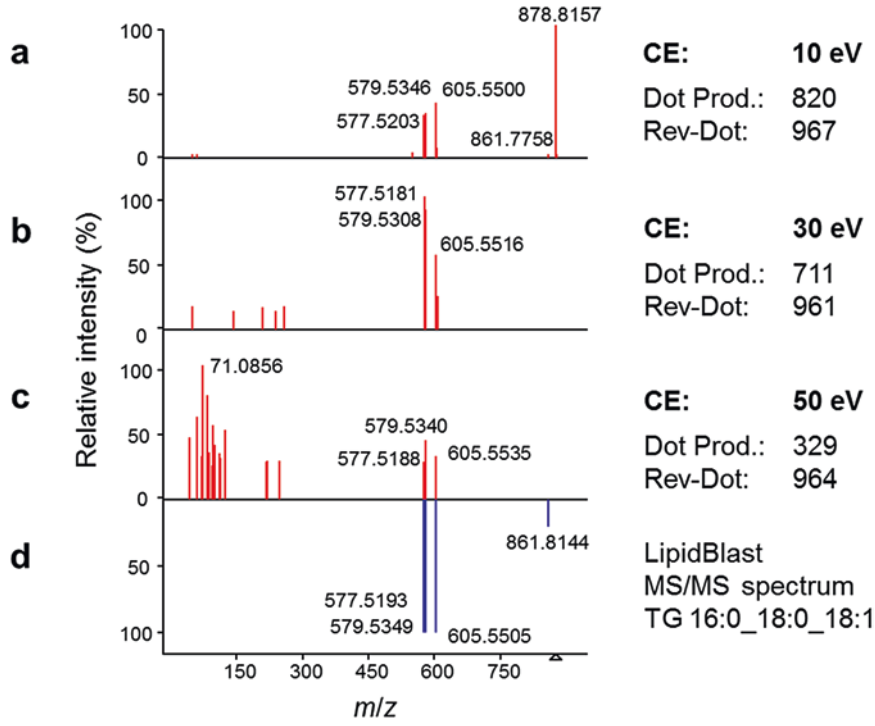

Fig. 9 Experimental MS/MS spectra of TG 16:0_18:0_18:1 (**a**, **b**, **c**) with a precursor ion *m/z* 878.8172 $[M+NH_4]^+$) and LipidBlast MS/MS spectrum (**d**). Using different collision energies (CE) yields more fragment ions and thus impacts calculating spectral dot products (Dot Prod.) and reversed dot products (Rev-Dot)

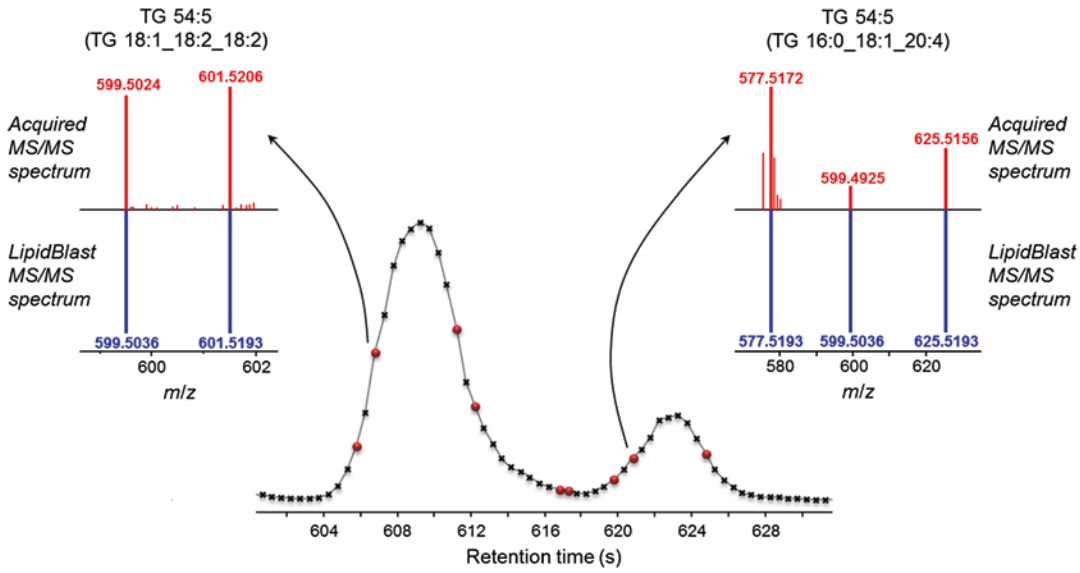

Fig. 10 Extracted ion chromatograms of TG 54:5, *m/z* 898.7859 $[M+NH_4]^+$ with marked data points per peak: ● MS/MS spectrum acquired, ✖ no MS/MS data acquired

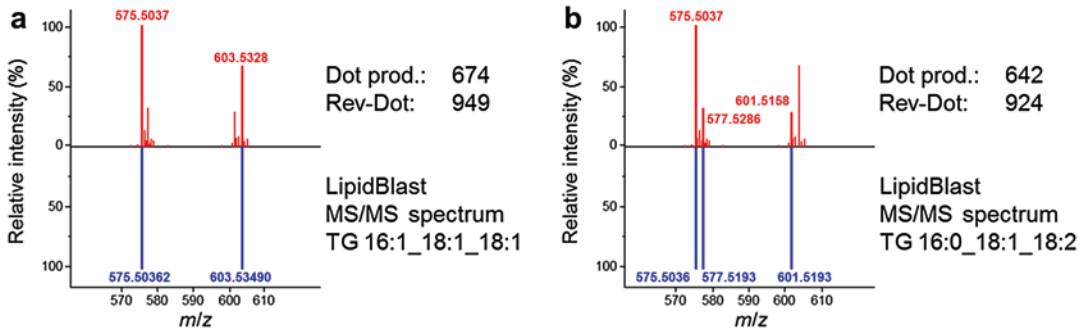

Fig. 11 MS/MS spectrum of TG 52:3 (precursor ion *m/z* 874.7859 $[M+NH_4]^+$) with two different MS/MS annotations from the LipidBlast library

13. Each identified lipid can be manually inspected based on scan number using *MS/MS Graphical User Interface (GUI)-based search.*

3.4.3 MS/MS Graphical User Interface (GUI)-Based Search

1. Run nistms.exe from \\LipidBlast-Full-Release-3\LipidBlast-MSSearch folder.
2. Upload MGF file: File → Open → Choose file for spectra/structures import with files of type MGF.

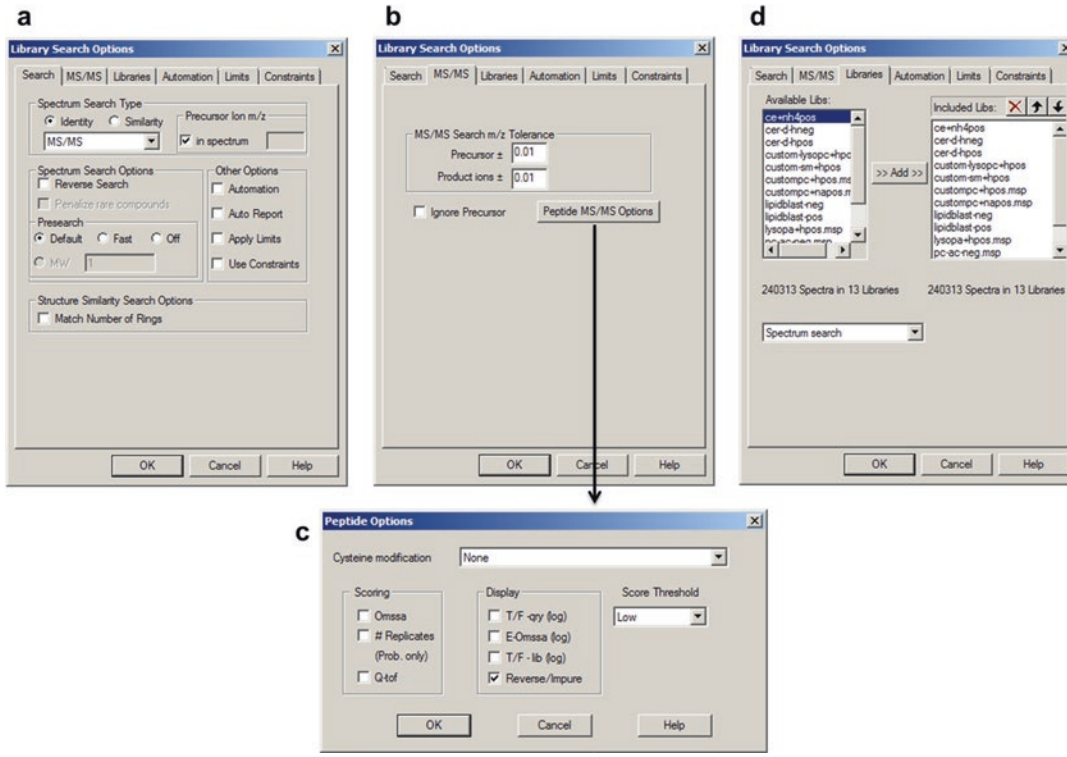

Fig. 12 Settings for MS/MS searches in the NIST MS graphic user interface

3. Check or change LipidBlast libraries including the settings and precursor and product ion window (Fig. 12):
   (a) Options → Library Search Options → Search → Identity (MS/MS).
   (b) Options → Library Search Options → MS/MS → MS/MS Search *m/z* Tolerance → Precursor ±0.01; Product ions ±0.01.
   (c) Options → Library Search Options → MS/MS → Peptide MS/MS Options → Cysteine modification: None; Display: Reverse/Impure; Score Threshold: Low.
   (d) Options → Library Search Options → MS/MS → Libraries → Add all libraries from Available Libs to Included Libs.
4. Double click on *m/z* value at a particular retention time to check if there is an MS/MS match with the LipidBlast MS/MS library (Fig. 13).

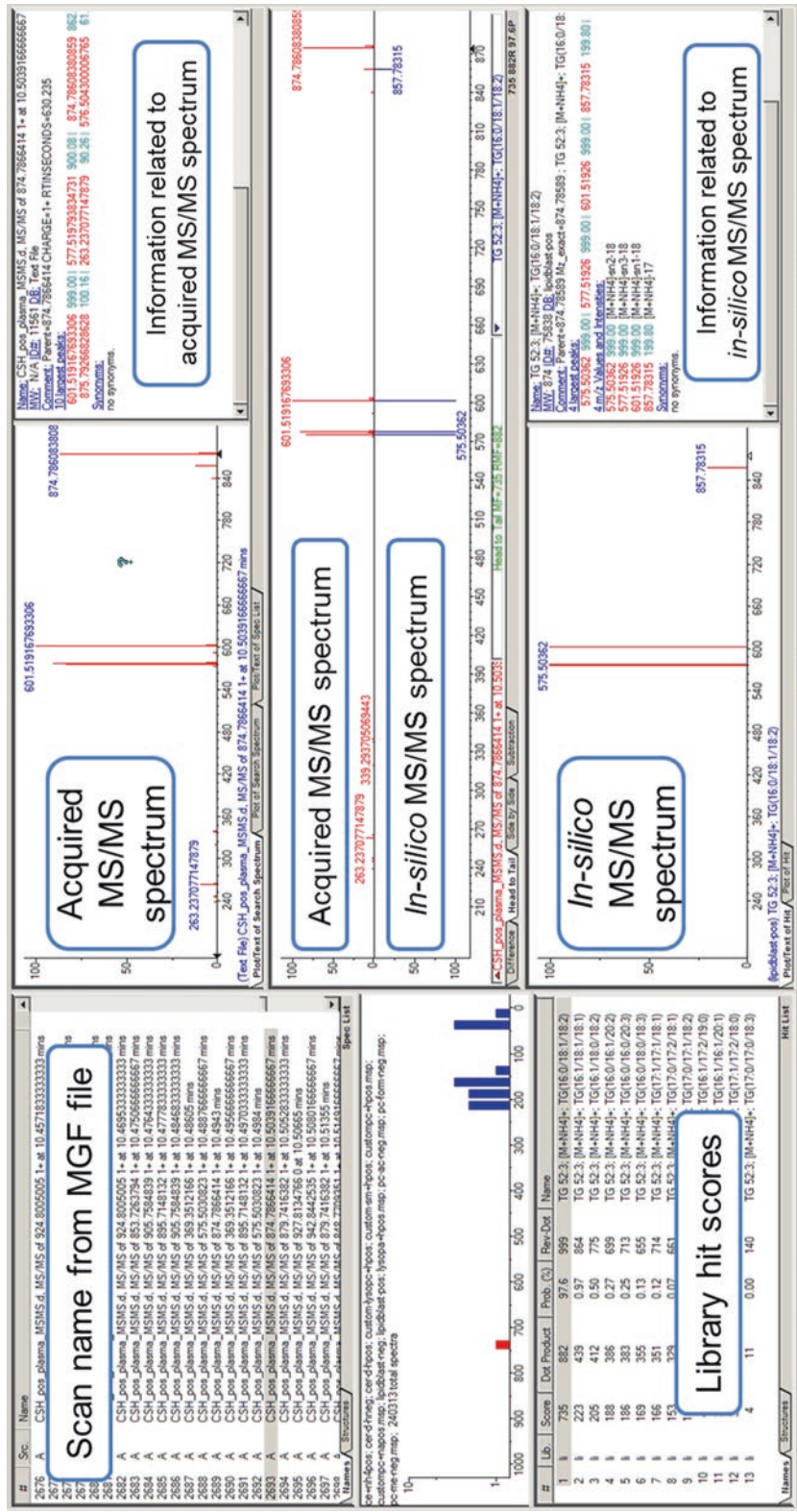

Fig. 13 Visualization of library search in NIST MS GUI

4 Notes

1. Store solvents (methanol, MTBE) in the −20 °C freezer to prechill.
2. To prevent contamination disposable material is used. To prevent inhalation of organic solvent vapor, use fume hood during lipid extraction.
3. Thaw each plasma sample on ice. During the extraction keep the Eppendorf tubes either on ice or use prechilled (−20 °C) block.
4. This extraction procedure has been optimized for the Agilent 6550 iFunnel QTOFMS system. For other LC–MS systems differing in their sensitivity the protocol can be modified including (1) volume of blood plasma for analysis (10–30 μL), (2) aliquot taken during the extraction (up to ~700 μL MTBE/methanol layer), (3) volume of resuspension solvent, and (4) volume injected. To avoid band broadening during LC–MS analysis, we recommend a maximum volume of 5 μL during injection.
5. Store water in the +4 °C fridge to prechill.
6. To enhance solubilization of ammonium formate and ammonium acetate after their addition in the mobile phase, dissolve these salts in a small volume of water before their addition in the mobile phases (0.631 g ammonium formate or 0.771 g ammonium acetate/1 mL water/1 L mobile phase). Mix each mobile phase with modifiers, sonicate them for 15 min to achieve complete dissolving of modifiers (salts), mix them again, and then sonicate for another 15 min.
7. Run plasma extract followed by injection of solvent mixture (methanol:toluene, 9:1, v/v) for checking the carryover. The carryover effect is observed mainly for triacylglycerols. Check the most intensive species such as TG 52:2 (*m/z* 876.802 $[M+NH_4]^+$) and TG 52:3 (*m/z* 874.786 $[M+NH_4]^+$) to evaluate the carryover. Carryover <0.2% for these most abundant lipid species is acceptable. If possible use different wash solvents during the sample injections, for instance, weak wash solvent: acetonitrile/water (50:50, v/v) followed by a strong wash solvent: isopropanol. If only one solvent for washing is available, use isopropanol and also check a software option to clean the injector using valve switching (e.g., Agilent) at the different composition of mobile phase to release adsorbed lipids.
8. $[M+Li]^+$ ions are observed if a base such as LiOH is used as a mobile-phase modifier or coinjected during ionization. For phosphatidylcholines, the formation of $[M+HCOO]^-$ and $[M+CH_3COO]^-$ adducts depends on mobile-phase modifier used. Use *PC-Ac-neg.msp* library if using ammonium acetate

(i.e., acetate adducts) and *PC-Form-neg.msp* library if using ammonium formate (i.e., formate adducts) mobile phase modifier.

9. Precursor and fragment ion tolerance depend on the mass accuracy of the particular instrument. Keep in mind that fixed mass tolerance in mDa has different values in ppm. For instance, using 0.01 Da mass tolerance this corresponds to 33, 20, 12, and 10 ppm mass tolerance for precursor ions *m/z* 300, 500, 800, and 1000, respectively. A wider ion tolerance (0.02–0.05 Da) can be used if precursor ions are very abundant, because mass accuracy is biased if the mass detector is saturated. However, default values should use narrower precursor window tolerances in order to limit false-positive peak detections.

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