Analysis of Lipid Experiments (ALEX): a software framework for analysis of high-resolution shotgun lipidomics data

1. Introduction

ALEX (Analysis of Lipid Experiments) is a platform for processing, management and visualization of high-content shotgun lipidomics datasets acquired using high-resolution Orbitrap mass spectrometry. The platform supports automated identification and export of lipid species intensity directly from proprietary mass spectral data files and the integration of accessory lipid features and sample information into a single output structured in database table format. This design supports rapid data processing and lipidome visualization across large sample sizes using an auxiliary workflow powered by the database exploration tools: Orange and Tableau Software.

In this manual we first describe each module of the framework including the core modules of ALEX, folder structure, the complementary exploration tools Orange and Tableau, installation requirements and download instruction. In the second part we provide a step by step guide using a sample data set from a neurolipidomics study which is available for download.

2. Download and Installation requirements

Download: www.msLipidomics.info

ALEX software should be operated in Windows XP, SP2 or higher. Windows 7 users should use Windows XP Mode. The different components should be installed in the following order:

1. The **MSFileReader** 2.2 library (Thermo Scientific)
2. **Python 2.7**, **PySide** and **NumPy** as part of the **ALEX extractor** installer
3. Python modules **comtypes** and **SciPy**
4. **ALEX converter**
5. **ALEX target list generator**
6. **ALEX lipid database**
7. **ALEX lipid calculator** and **ALEX lipid database source files**
8. **ALEX unifier**
9. Additional tools **Orange version 2.6.1** and **Tableau version 7.0**

3. Workflow

The ALEX framework comprises of 6 core modules (grey colored boxes)

Figure 1). The output of the ALEX framework includes a data file with identified lipid species, intensities and accessory lipid features across all processed samples and FT MS scan ranges. The ALEX output is organized in database table format that can be accessed and processed by the auxiliary workflow using Orange and Tableau software. The auxiliary workflow is designed to integrate sample information, compute lipid molar abundance, implement quality control procedures and visualize lipidome data.

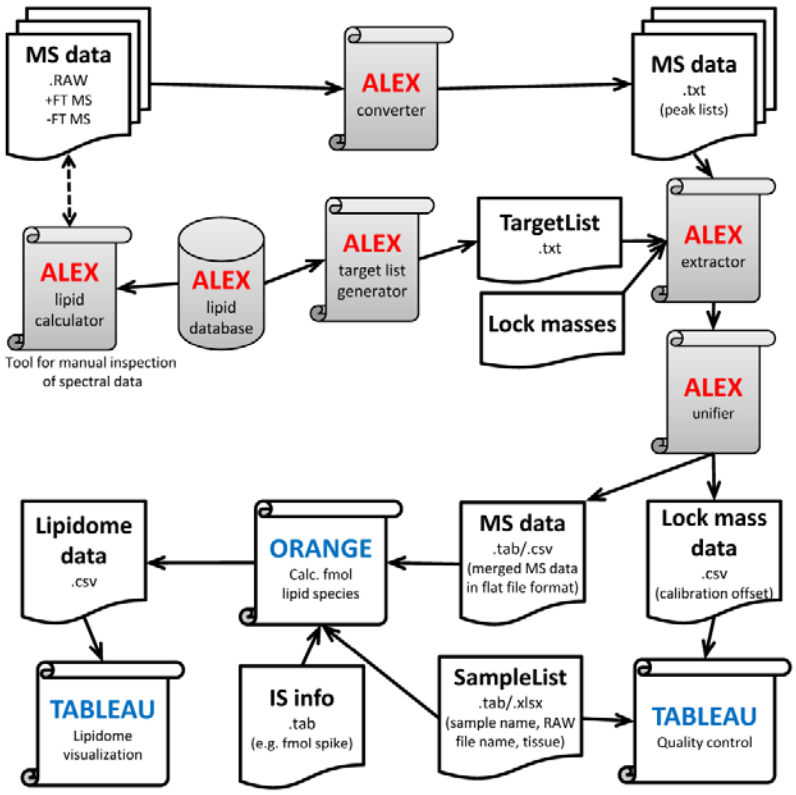
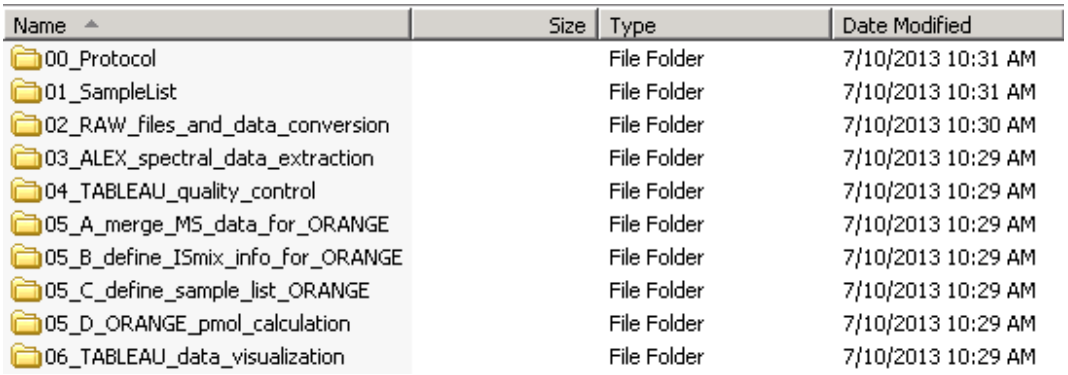


Figure 1. Overview of the ALEX software framework and auxiliary workflow

4. Folder Structure

It is recommended to create a file management structure with folders associated to each step of the framework

Figure 2). Numbering the folders (e.g. 00– 06) keeps the folders in the right order when sorting them by name.



Name	Size	Type	Date Modified
00_Protocol		File Folder	7/10/2013 10:31 AM
01_SampleList		File Folder	7/10/2013 10:31 AM
02_RAW_files_and_data_conversion		File Folder	7/10/2013 10:30 AM
03_ALEX_spectral_data_extraction		File Folder	7/10/2013 10:29 AM
04_TABLEAU_quality_control		File Folder	7/10/2013 10:29 AM
05_A_merge_MS_data_for_ORANGE		File Folder	7/10/2013 10:29 AM
05_B_define_ISmix_info_for_ORANGE		File Folder	7/10/2013 10:29 AM
05_C_define_sample_list_ORANGE		File Folder	7/10/2013 10:29 AM
05_D_ORANGE_pmol_calculation		File Folder	7/10/2013 10:29 AM
06_TABLEAU_data_visualization		File Folder	7/10/2013 10:29 AM

Figure 2. Recommended file managment structure

- **00_Protocol :** Folder for cataloging experimental protocols and details.

- **01_SampleList:** The folder contains the sample list in excel format. Information regarding file name, sample number, sample name, organism, tissue type etc. is entered into the sample list.

- **02_RAW_files_and_data_conversion:** This folder contains the ALEX converter (5.1) and all associated files. The “raw” subfolder contains the proprietary “.RAW” files which serve as an input for the ALEX converter. All acquired data files should be copied into this folder. The ALEX converter output folder is the “txt” subfolder.

- **03_ALEX_spectral_data_extraction:** This folder contains all related files for the ALEX target list generator (5.3) and ALEX extractor (5.4).

- **04_TABLEAU_quality_control:** This folder contains the results from the quality control using Tableau Software (7). Typically the intensity and m/z offset (offset between measured *m/z* vs. accurate *m/z*) of lock mass ions and internal standards (ISTD) are accessed and displayed for the complete experimental data set.

- **05_A_merge_MS_data_for_ORANGE:** This folder contains the ALEX unifier (5.5) and the resulting output file, a single CSV file created from multiple CSV from the ALEX extractor. It also produces an Orange-friendly ".tab" file (6) of the combined data.

- **05_B_define_ISmix_info_for_ORANGE:** This folder contains a file in tab format with information for the internal standards and their concentrations spiked into the sample for the use in Orange (6).

- **05_C_define_sample_list_ORANGE:** This folder contains the sample list for the use by Orange.

- **05_D_ORANGE_pmol_calculation:** This folder contains the Orange scheme used to calculate the molar abundance of lipid species (6) and the affiliated output file.

- **06_TABLEAU_data_visualization:** This folder contains the results from the Tableau data visualizing. The data can be sorted and visualized in different display formats (7) depending on the sample background and biological question.

5. ALEX software framework

5.1. ALEX converter

The ALEX converter interfaces with the proprietary dynamic-link library MSFileReader (Thermo Fisher) to export individual spectral peak lists in profile mode format, to average peak lists for specific FT MS scan ranges and to save these averaged peak lists in .txt format. The output consists of a directory with separate folders named according to each FT MS scan range containing corresponding ".txt" files named according to the originating ".RAW" files

(

Figure 3).

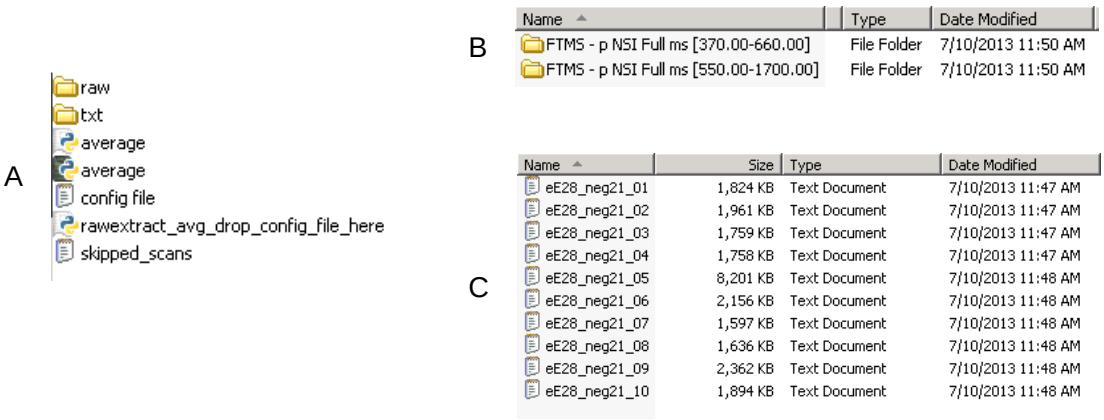


Figure 3. File management structure of the folder containing the Alex converter (A). The "raw" subfolder contains the original experimental data files. The "txt" subfolder contains the ALEX converter output files (C) sorted by folders named according to the scan.

The ALEX converter configuration file (config file.txt (
Figure 3 A)) is a simple txt file which can be opened using notepad and consists of following parameters (Figure 4):

- **input_dir/output_dir:** Set the input (“raw”) and output (“txt”) directory. These folders need to be in the same directory as the configuration file.

- **state_file** is a file recording the settings (skipped scans) that each file has been processed with. Files already been processed will not be processed again, if the settings haven’t changed. Delete the file “skipped_scans.txt”, if you want all files to be reprocessed.

- **skip_scans** specifies how many scans to skip from the beginning and the end of the analyses. A single global setting can be specified by: "skip_scans" : { "(default)" : [0,0] } (Figure 4 A) or for each individual file as : {“file name”: [2,2]} (Figure 4 B).

A

```
{
  "input_dir" : "raw",
  "output_dir" : "txt",
  "state_file"  : "skipped_scans.txt",
  "skip_scans" : {
    "(default)" : [0,0]}}
```

B

```
{
  "input_dir" : "raw",
  "output_dir" : "txt",
  "state_file"  : "skipped_scans.txt",
  "skip_scans" : {
    "(default)" : [2,2],
    "pos_151" : {
      "aCE14_2_pos_151_run4_23.RAW" : [18,2],
      "aCE14_2_pos_151_run4_16.RAW" : [14,2],
      "aCE14_2_pos_151_run4_28.RAW" : [13,2],
      "aCE14_2_pos_151_run5_05.RAW" : [2,35]
    },
    "neg_151" : {
      "aCE14_2_neg_151_run2_01.RAW" : [27,2],
      "aCE14_2_neg_151_run4_23.RAW" : [2,14]
    },
    "neg_21" : {
      "aCE14_2_neg_21_run5_41_repeat.RAW" : [2,58]
    },
    "neg_sterol_sulfation" : {
      "aCE14_2_neg_sulf_run2_05.RAW" : [2,26],
      "aCE14_2_neg_sulf_run2_35.RAW" : [2,22],
      "aCE14_2_neg_sulf_run3_25.RAW" : [13,2]
    }
  }
}
```

Figure 4. ALEX converter configuration file. Scans at the beginning and end of the run can be skipped by using the skip_scan function. This setting can be by default for all data files (A) or defined for each individual data file.

The ALEX converter can be started by dragging the configuration file onto the icon of “ALEX_converter_drop_config_file_here.py”. Depending on data file size, this process may take several minutes. If needed, the process can be terminated using “ctrl+c”.

5.2. ALEX lipid database

The ALEX lipid database covers more than 20,000 lipid species from more than 85 lipid classes. Each lipid species in the database is annotated by sum composition and contains a range of accessory lipid features denoting its chemical formula, mono-isotopic mass, adduction in positive and negative ion mode, lipid category, lipid class, and the total number of C atoms, double bonds and hydroxyl group in the fatty acid and long chain base moieties (termed C index, db index and OH index, respectively). The database is used by the ALEX target list generator (5.3) and ALEX lipid calculator (5.6). The lipid database can be modified using the Microsoft Excel files as templates which should be copied to an .txt file as saved.

5.3. ALEX target list generator

The ALEX target list generator compiles target lists by querying the ALEX lipid database (5.2). A distinct target list consists of the appropriate lipid species with respective *m/z* values and lipid features derived from the ALEX lipid database. These features (chemical formula, lipid category, lipid class, total number of C atoms etc.) are also incorporated into the final output, and are used for processing and visualization by the auxiliary workflow (6,7). To compile a target list following parameters can be set in the ALEX target list generator (
Figure 5):

- **Output file:** Name for the output txt file

- **Lipid species:** Define an individual lipid species (e.g. PC 34:1) to be included into the target list

- **Lipid Class:** Define a complete Lipid class (e.g. TAG, PC, Cer) to be included into the target list.

- **Adduct:** Define possible adduct formation (e.g. $+NH_4^+$, $+H^+$, $+CHCOO^-$)

- **C index, even/odd:** Constrict the target list by defining the minimum and maximum number of carbon atoms in the fatty acid moieties and allow just even and/or odd number.

- **db index:** Constrict the target list by defining the total number of double bound in the fatty acid moiety. Set the minimum and maximum number of double bonds or set a linear regression depending on the C index.

- **OH index:** Constrict the target list by defining the minimum and maximum number of hydroxyl group in the long chain base moieties.

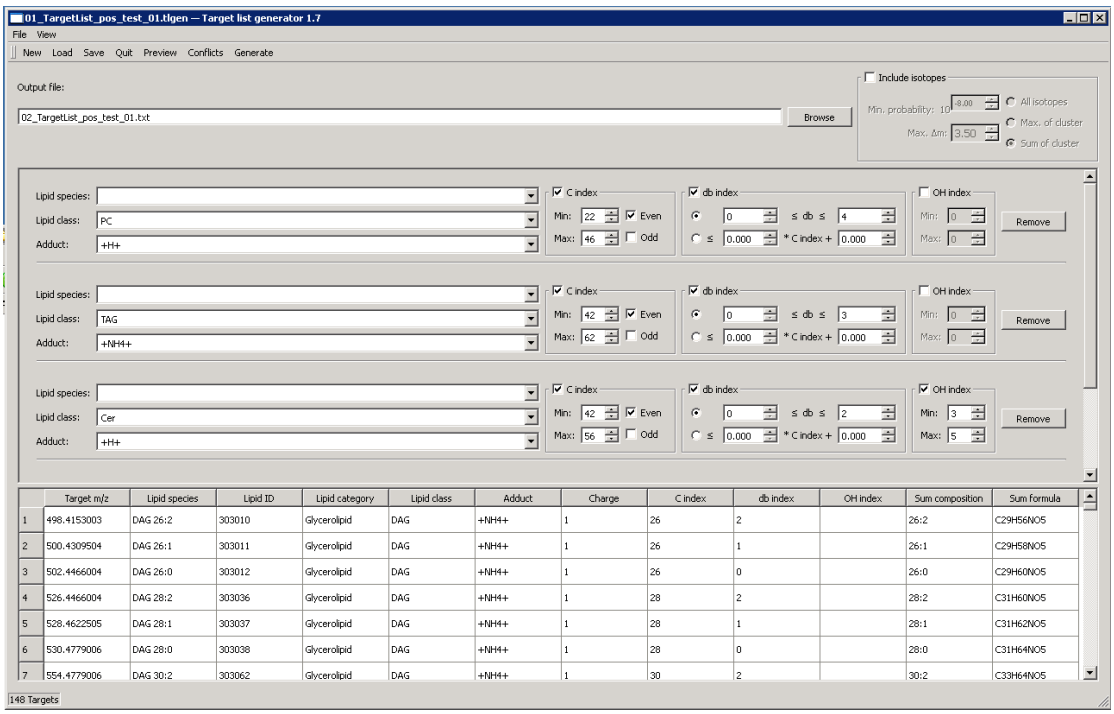


Figure 5. Screenshots of the ALEX target list generator, which allows users to select lipid classes and species to be identified using criteria such as lipid class, adduction, C index, db index and OH index. Individual lipid species including internal standards can also be selected. The ALEX target list generator output is a ".txt" file with a shortlist of selected lipid species, respective m/z values and accessory lipid features.

The target list will be generated by pressing the "Generate" button. A list of all possible Lipid species within the defined constrains is created and can be saved as the output file. The target list configuration can be saved by pressing the "Save" button. Isobaric species within a defined window can be displayed by pressing the "conflict" button (Figure 6).

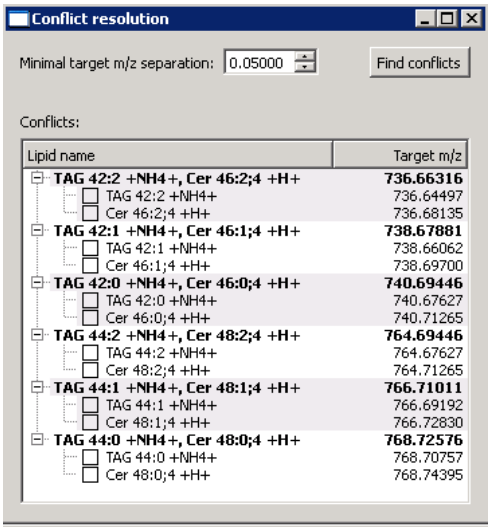


Figure 6. Screenshots of the ALEX target list generator conflict window. Isobaric species within a defined window of m/z 0.05 can be displayed.

5.4. ALEX extractor

The ALEX extractor identifies lipid species and exports intensities by querying the averaged peak lists produced by the ALEX converter (
Figure 7).

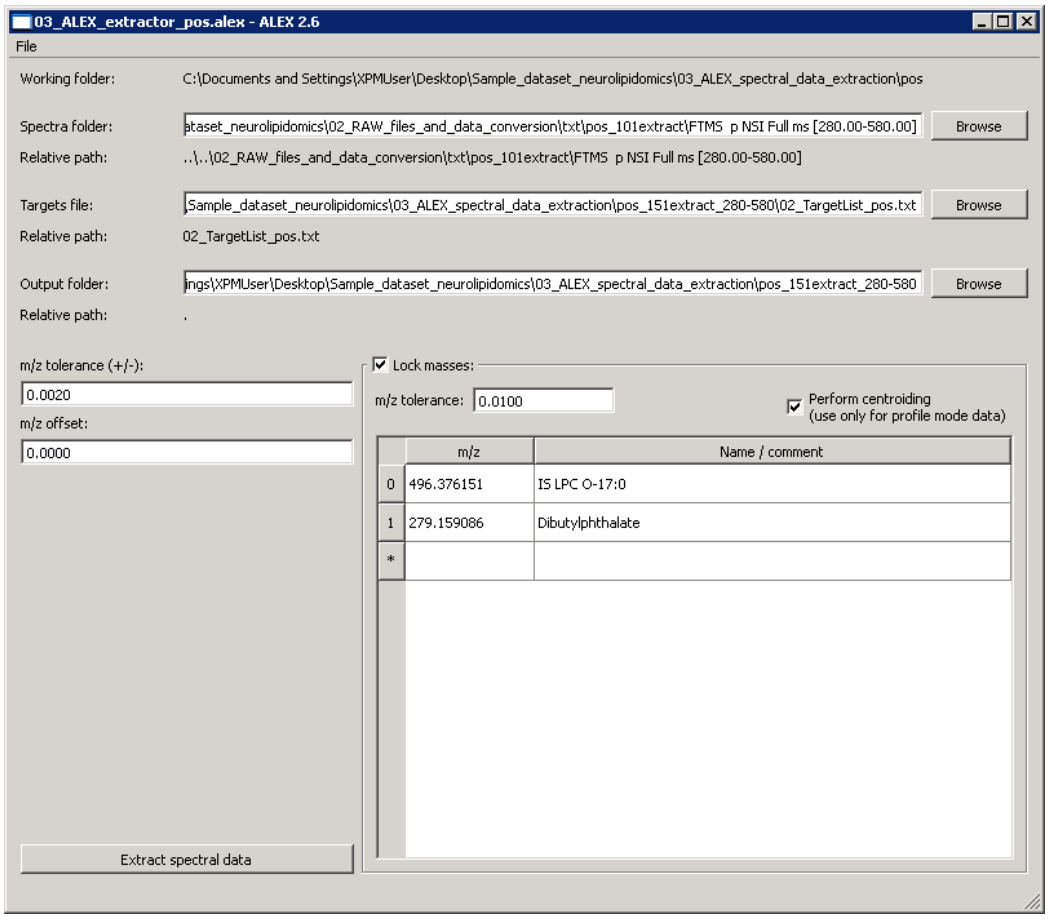


Figure 7. Screenshots of the ALEX extractor which identifies lipid species, exports intensity data and incorporates accessory lipid features.

- **Spectra folder:** Location containing averaged peak lists created by the ALEX converter (5.1)

- **Targets file:** Appropriate target list compiled by target list generator (5.3)

- **Output folder:** Destination folder to deposit output text files

- ***m/z* tolerance:** Tolerance window to identify lipid species dependent on instrumental mass resolution

- ***m/z* Offset:** Constant m/z offset to correct lipid searches for a constant FT MS calibration offset

- **Lock masses:** Automatic lock mass adjustment to correct the m/z values of targeted lipid species for calibration drifts. Requires specification of well-characterized and ubiquitous lock mass ions in order to estimate the FT MS calibration offset.

The ALEX extractor outputs, for each FT MS scan range, several comma-separated value ".csv" files with lipid species intensity data and calculated lock mass adjustments for all processed samples. Press "Extract spectral data" to start the extraction.

5.5. ALEX unifier

The ALEX unifier merges multiple ".csv" files from different FT MS ranges created by ALEX extractor (5.4) into a single file. It also produces an Orange-friendly ".tab" file (6) of the combined data.

5.6. ALEX lipid calculator

The ALEX lipid calculator complements manual inspection of FT MS spectra when using proprietary Xcalibur software. The calculator is available as executable program (Figure 8) and as an online application (Figure 9) at www.mslipidomics.info/lipid-calc.

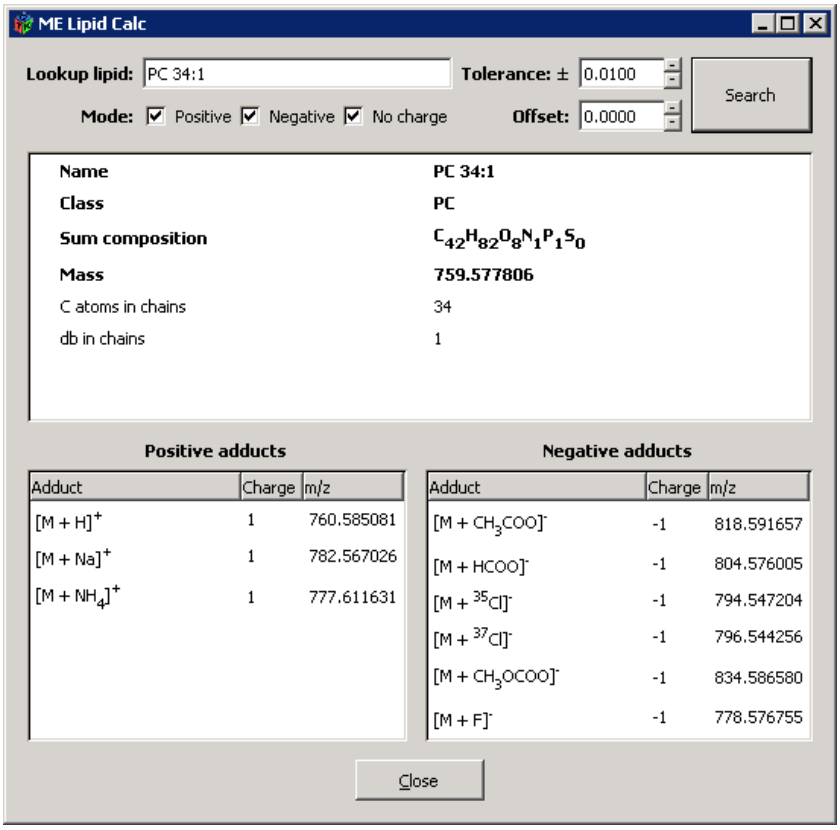
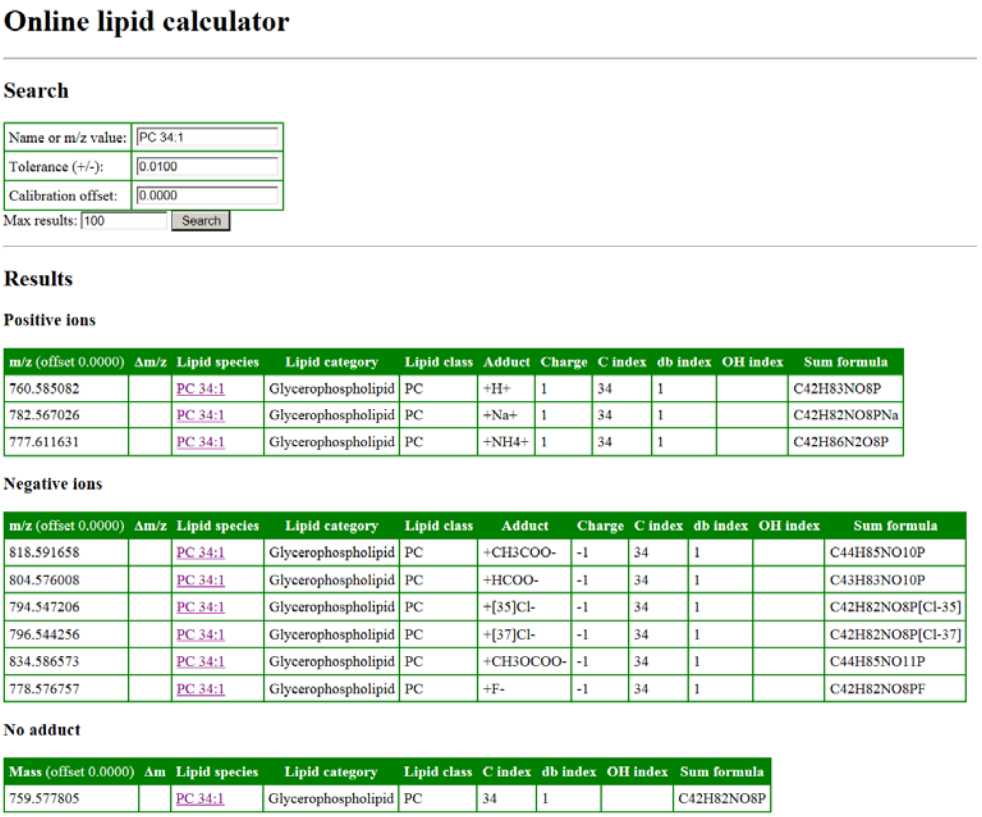


Figure 8. Screen shot of the ALEX Lipid calculator executable program

- **Lookup lipid:** Input lipid species to survey for. The sum composition is typed in in the following format: {lipid class} {total no. of C} : { total no. of double bonds for example PC 34:1.

- **Tolerance ±:** Tolerance window to identify lipid species dependent on instrumental mass resolution

- **Calibration Offset:** Constant m/z offset to correct lipid searches for a constant FT MS calibration offset



Online lipid calculator

Search

Name or m/z value:	PC 34:1
Tolerance (+/-):	0.0100
Calibration offset:	0.0000

Max results: 100 Search

Results

Positive ions

m/z (offset 0.0000)	Δm/z	Lipid species	Lipid category	Lipid class	Adduct	Charge	C index	db index	OH index	Sum formula
760.585082		PC 34:1	Glycerophospholipid	PC	+H+	1	34	1		C42H83NO8P
782.567026		PC 34:1	Glycerophospholipid	PC	+Na+	1	34	1		C42H82NO8PNa
777.611631		PC 34:1	Glycerophospholipid	PC	+NH4+	1	34	1		C42H86N2O8P

Negative ions

m/z (offset 0.0000)	Δm/z	Lipid species	Lipid category	Lipid class	Adduct	Charge	C index	db index	OH index	Sum formula
818.591658		PC 34:1	Glycerophospholipid	PC	+CH3COO-	-1	34	1		C44H85NO10P
804.576008		PC 34:1	Glycerophospholipid	PC	+HCOO-	-1	34	1		C43H83NO10P
794.547206		PC 34:1	Glycerophospholipid	PC	+[35]Cl-	-1	34	1		C42H82NO8P[Cl-35]
796.544256		PC 34:1	Glycerophospholipid	PC	+[37]Cl-	-1	34	1		C42H82NO8P[Cl-37]
834.586573		PC 34:1	Glycerophospholipid	PC	+CH3OCOO-	-1	34	1		C44H85NO11P
778.576757		PC 34:1	Glycerophospholipid	PC	+F-	-1	34	1		C42H82NO8PF

No adduct

Mass (offset 0.0000)	Δm	Lipid species	Lipid category	Lipid class	C index	db index	OH index	Sum formula
759.577805		PC 34:1	Glycerophospholipid	PC	34	1		C42H82NO8P

Figure 9. Screen shot of the ALEX Lipid calculator online program

6. Orange

Orange is an open source data visualization and analysis program (http://www.orange.biolab.si/). Data mining is performed through visual programming or Python scripting, packed with features for data analytics.

To compute the molar abundance of lipid species a sequence of processing steps has to be constructed (Figure 10).

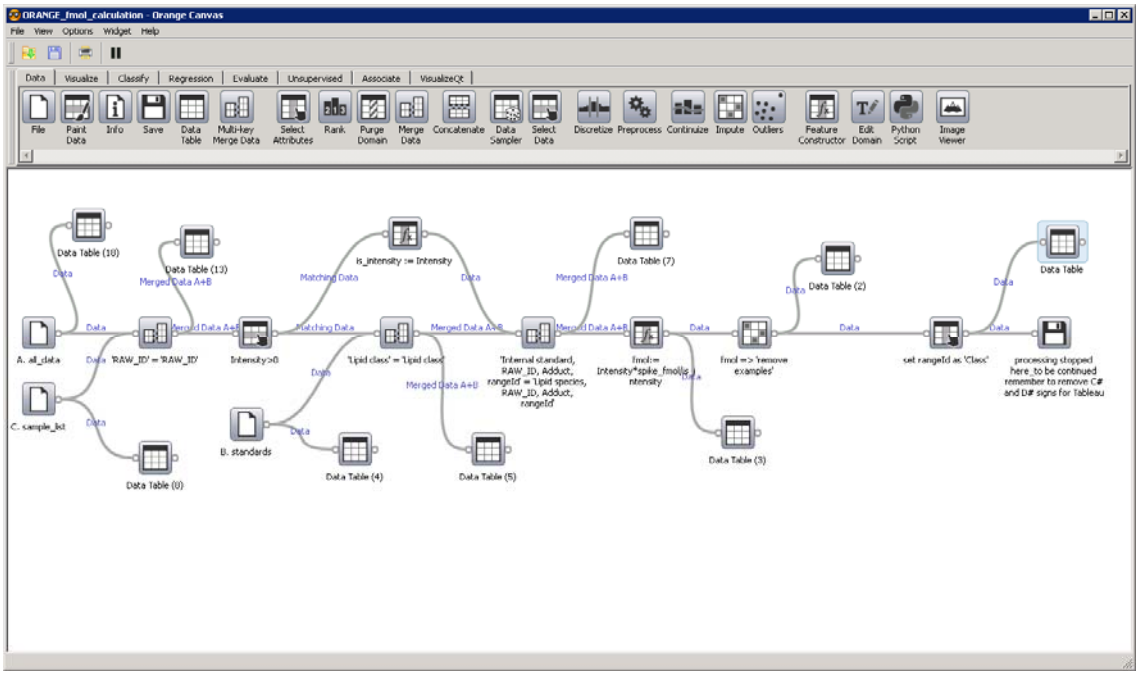


Figure 10. Screen shot of Orange scheme to calculate molar abundance of lipid species

- **A.all_data:** The lipid species intensity data generated by the ALEX framework is specified as input

- **C.sample_list:** Input for sample information like tissue type, genetic information, name of mice etc. defined previously in the sample list

- **Data Table (x):** Input data and derived data after each step can be accessed

- **'RAW_ID'='RAW_ID'**: Merged data by aligning the column "RAW_ID" of the intensity data with the column "RAW_ID" of the sample list

- **Intensity>0:** Intensity filter to remove intensities below a user-specified threshold.

- **B.standards:** Input specifying the spiked amount of internal standards

- **is_intensity:=Intensity:** Attribute is defined by specifying the intensities of internal standards as "Intensity" to later compute the molar abundance.

- **'Lipid class'='Lipid class':** Merged data by aligning the column "Lipid class" with the column "Lipid class" of the internal standards

- **'Internal standard, RAW_ID, Adduct, rangeId' = 'Lipid species, RAW_ID, Adduct, rangeId':** internal standards and corresponding intensities are defined for the subsequent calculation of molar lipid abundances.

- **fmol:= Intensity*spike_fmol/is_intensity:** Calculation of the molar lipid abundance using the attributes lipid species intensity, internal standard intensity and spiked amount of internal standard.

- **set rangeId as 'Class':**

- **processing stopped here_to be continued remember to remove C# and D# signs for Tableau:** Data is saved as a .csv output file (in database table format) that can be accessed for computation of mol% and lipidome visualization by Tableau Software (7).

7. Tableau

Tableau software (www.tableausoftware.com) is an interactive data visualization engine which can be dynamically linked to the Orange output files such that any modifications within the data processing procedure can be visualized interactively by updating the Orange output file and the link to Tableau. The data can be sorted and visualized in different display formats depending on the sample background and biological question (Figure 11).

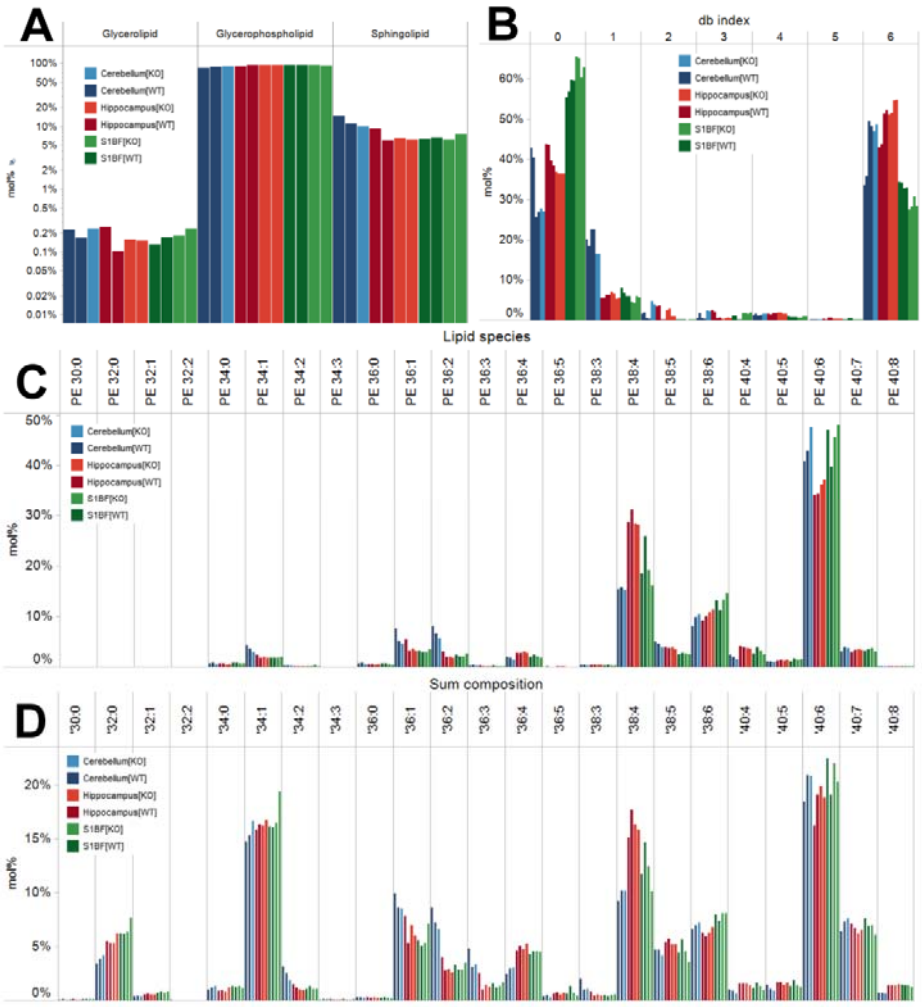


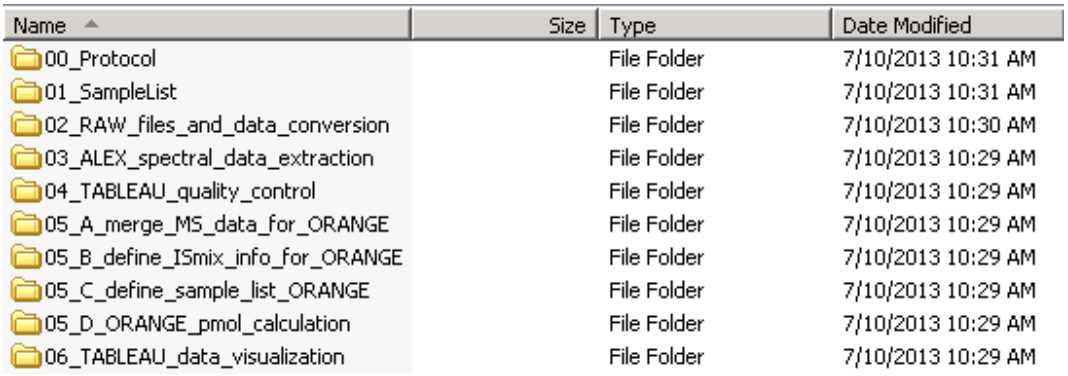
Figure 11.Tableau lipidome visualization using different display formats

8. Sample Data Set

This sample dataset from a neurolipidomics study is for testing the local installations of the ALEX software and the auxiliary workflow Orange and Tableau Software, and is available for download at http://mslipidomics.info .

The study involves the comparison of three mouse brain tissues (Cerebellum, Hippocampus and S1BF) from wild-type and PRG-1 knockout mice.

The file management structure consists of 6 folders associated to each step of the framework (Figure 12).



Name	Size	Type	Date Modified
00_Protocol		File Folder	7/10/2013 10:31 AM
01_SampleList		File Folder	7/10/2013 10:31 AM
02_RAW_files_and_data_conversion		File Folder	7/10/2013 10:30 AM
03_ALEX_spectral_data_extraction		File Folder	7/10/2013 10:29 AM
04_TABLEAU_quality_control		File Folder	7/10/2013 10:29 AM
05_A_merge_MS_data_for_ORANGE		File Folder	7/10/2013 10:29 AM
05_B_define_ISmix_info_for_ORANGE		File Folder	7/10/2013 10:29 AM
05_C_define_sample_list_ORANGE		File Folder	7/10/2013 10:29 AM
05_D_ORANGE_pmol_calculation		File Folder	7/10/2013 10:29 AM
06_TABLEAU_data_visualization		File Folder	7/10/2013 10:29 AM

Figure 12. File managment structure associated to each step of the framework

8.1. Protocol

All experimental protocols, lab book, sample prep details, background information etc. can be places into the protocol folder

8.2. Sample List

The sample list is created in excel and contains all the sample information like file name, sample number, sample name, organism, tissue type etc. (

Figure 13). In the neurolipidomics study three mouse brain tissues (Cerebellum, Hippocampus and S1BF) from wild-type (WT) and PRG-1 knockout (KO) mice have been first analyzed in pos. Ion mode (

Figure 13, row 4-17) followed by 4 blank runs and the analyses in neg. Ion mode (

Figure 13, row 32-55), followed by another 4 blank runs. We note that the sample list should include a “d” in each column of the second row in order to be processed correctly by Orange.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	RAW_ID	File_string	File_number	Sample_number	Sample_name1	Sample_name2	Sample_name3	Tissue	Strain	Mouse	Replica	Reinjection	Biological replica	Display order
2	d	d	d	d	d	d	d	d	d	d	d	d	d	d
3														
4	eE28_pos101_01	eE28_pos101_	01	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	Cerebellum	WT	A	A	no	A	A1
5	eE28_pos101_02	eE28_pos101_	02	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	Cerebellum	WT	A	B	no	A	A2
6	eE28_pos101_03	eE28_pos101_	03	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	Cerebellum	WT	B	A	no	B	A3
7	eE28_pos101_04	eE28_pos101_	04	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	Cerebellum	WT	B	B	no	B	A4
8	eE28_pos101_05	eE28_pos101_	05	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	Cerebellum	KO	C	A	no	A	A5
9	eE28_pos101_06	eE28_pos101_	06	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	Cerebellum	KO	C	B	no	A	A6
10	eE28_pos101_07	eE28_pos101_	07	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	Cerebellum	KO	D	A	no	B	A7
11	eE28_pos101_08	eE28_pos101_	08	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	Cerebellum	KO	D	B	no	B	A8
12	eE28_pos101_09	eE28_pos101_	09	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	WT	A	A	no	A	A9
13	eE28_pos101_10	eE28_pos101_	10	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	WT	A	B	no	A	A10
14	eE28_pos101_11	eE28_pos101_	11	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	WT	B	A	no	B	A11
15	eE28_pos101_12	eE28_pos101_	12	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	WT	B	B	no	B	A12
16	eE28_pos101_13	eE28_pos101_	13	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	KO	C	A	no	A	A13
17	eE28_pos101_14	eE28_pos101_	14	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	KO	C	B	no	A	A14
18	eE28_pos101_15	eE28_pos101_	15	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	KO	D	A	no	B	A15
19	eE28_pos101_16	eE28_pos101_	16	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	KO	D	B	no	B	A16
20	eE28_pos101_17	eE28_pos101_	17	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	S1BF	WT	A	A	no	A	A17
21	eE28_pos101_18	eE28_pos101_	18	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	S1BF	WT	A	B	no	A	A18
22	eE28_pos101_19	eE28_pos101_	19	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	S1BF	WT	B	A	no	B	A19
23	eE28_pos101_20	eE28_pos101_	20	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	S1BF	WT	B	B	no	B	A20
24	eE28_pos101_21	eE28_pos101_	21	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	S1BF	KO	C	A	no	A	A21
25	eE28_pos101_22	eE28_pos101_	22	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	S1BF	KO	C	B	no	A	A22
26	eE28_pos101_23	eE28_pos101_	23	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	S1BF	KO	D	A	no	B	A23
27	eE28_pos101_24	eE28_pos101_	24	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	S1BF	KO	D	B	no	B	A24
28	eE28_pos101_25	eE28_pos101_	25	13	blk	blk	blk	blk	blk	blk	A	no	blk	A25
29	eE28_pos101_26	eE28_pos101_	26	13	blk	blk	blk	blk	blk	blk	B	no	blk	A26
30	eE28_pos101_27	eE28_pos101_	27	14	blk	blk	blk	blk	blk	blk	A	no	blk	A27
31	eE28_pos101_28	eE28_pos101_	28	14	blk	blk	blk	blk	blk	blk	B	no	blk	A28
32	eE28_neg21_01	eE28_neg21_	01	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	Cerebellum	WT	A	A	yes	A	A1
33	eE28_neg21_02	eE28_neg21_	02	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	Cerebellum	WT	A	B	yes	A	A2
34	eE28_neg21_03	eE28_neg21_	03	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	Cerebellum	WT	B	A	yes	B	A3
35	eE28_neg21_04	eE28_neg21_	04	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	Cerebellum	WT	B	B	yes	B	A4
36	eE28_neg21_05	eE28_neg21_	05	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	Cerebellum	KO	C	A	yes	A	A5
37	eE28_neg21_06	eE28_neg21_	06	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	Cerebellum	KO	C	B	yes	A	A6
38	eE28_neg21_07	eE28_neg21_	07	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	Cerebellum	KO	D	A	yes	B	A7
39	eE28_neg21_08	eE28_neg21_	08	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	Cerebellum	KO	D	B	yes	B	A8
40	eE28_neg21_09	eE28_neg21_	09	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	WT	A	A	no	A	A9
41	eE28_neg21_10	eE28_neg21_	10	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	WT	A	B	no	A	A10
42	eE28_neg21_11	eE28_neg21_	11	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	WT	B	A	no	B	A11
43	eE28_neg21_12	eE28_neg21_	12	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	WT	B	B	no	B	A12
44	eE28_neg21_13	eE28_neg21_	13	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	KO	C	A	no	A	A13
45	eE28_neg21_14	eE28_neg21_	14	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	KO	C	B	no	A	A14
46	eE28_neg21_15	eE28_neg21_	15	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	KO	D	A	no	B	A15
47	eE28_neg21_16	eE28_neg21_	16	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	KO	D	B	no	B	A16
48	eE28_neg21_17	eE28_neg21_	17	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	S1BF	WT	A	A	no	A	A17
49	eE28_neg21_18	eE28_neg21_	18	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	S1BF	WT	A	B	no	A	A18
50	eE28_neg21_19	eE28_neg21_	19	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	S1BF	WT	B	A	yes	B	A19
51	eE28_neg21_20	eE28_neg21_	20	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	S1BF	WT	B	B	yes	B	A20
52	eE28_neg21_21	eE28_neg21_	21	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	S1BF	KO	C	A	no	A	A21
53	eE28_neg21_22	eE28_neg21_	22	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	S1BF	KO	C	B	no	A	A22
54	eE28_neg21_23	eE28_neg21_	23	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	S1BF	KO	D	A	no	B	A23
55	eE28_neg21_24	eE28_neg21_	24	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	S1BF	KO	D	B	no	B	A24
56	eE28_neg21_25	eE28_neg21_	25	13	blk	blk	blk	blk	blk	blk	A	no	blk	A25
57	eE28_neg21_26	eE28_neg21_	26	13	blk	blk	blk	blk	blk	blk	B	no	blk	A26
58	eE28_neg21_27	eE28_neg21_	27	14	blk	blk	blk	blk	blk	blk	A	yes	blk	A27
59	eE28_neg21_28	eE28_neg21_	28	14	blk	blk	blk	blk	blk	blk	B	yes	blk	A28
60														

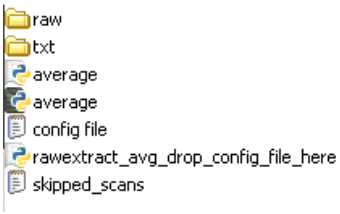
Figure 13. Sample list from a neurolipidomics study of three mouse brain tissues (Cerebellum, Hippocampus and S1BF) from wild-type and PRG-1 knockout mice.

8.3. Raw files and data conversation

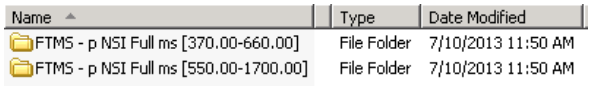
The experimental data files are copied into the “raw” folder (

Figure 14 A). Two subfolders were created for the 2 data sets. A 2:1 ($CHCl_3$:MeOH) lipid extraction was performed and acquired in neg. Ion mode and a 10:1 ($CHCl_3$:MeOH) lipid extraction was acquired in pos. Ion mode.

A



B



C

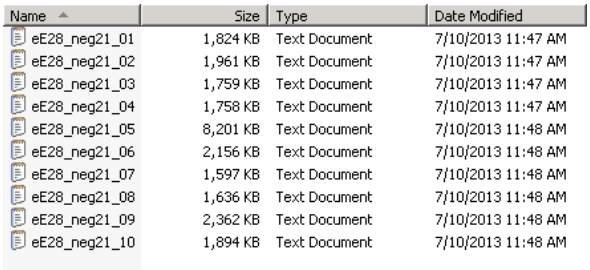


Figure 14. File management structure of the folder containing the Alex converter (A). The ”raw” subfolder contains the original experimental data files. The ”txt” subfolder contains the ALEX converter output files (C) sorted by folders named according to the scan.

In the ALEX converter configuration file, the input (“raw”) and output (“txt”) directory are defined. Note that these folders need to be in the same directory as the configuration file. Specific scans can be ignored for processing using the "skip_scans" command and this information is saved in the skipped_scans.txt file. In this data set the first and last 2 scans from each data file are ignored for processing (
Figure 15).

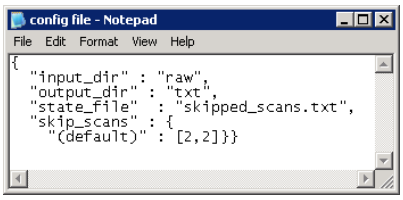


Figure 15. ALEX converter configuration file

The ALEX converter can be started by dragging the configuration file onto the icon of “rawextract.py”. Depending on data file size, this process may take several minutes. If needed, the process can be terminated using “ctrl+c”.The processing is finished with the message “Extraction successful” (Figure 16).

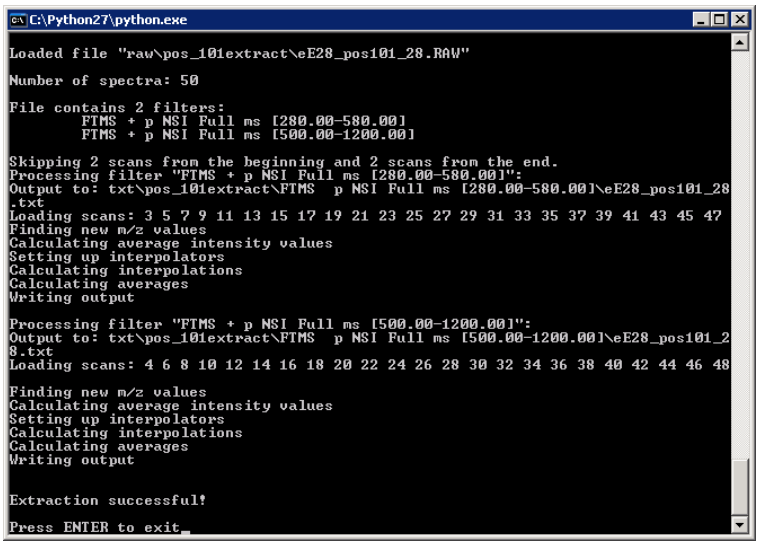


Figure 16. Screenshot from the ALEX converter processing

The output files (Figure 14 C) are saved in the directory according to each FT MS scan range (Figure 14 B). This experiment consists of 2 scan ranges (m/z 280-580 and m/z 500-1200) in pos. Ion mode, and 2 scan ranges (m/z 370-660 and m/z 550-1700) in neg. Ion mode, therefore 4 folders were created by the ALEX converter.

8.4. Alex spectral data extraction

8.4.1. ALEX target list generator

The ALEX target list generator compiles a target list which consists of the appropriate lipid species with respective m/z values and lipid features (chemical formula, lipid category, lipid class, total number of C atoms etc.) derived from the ALEX lipid database. In this study a target list was created specifically for each scan range and saved in the specific folder together with the Target List definition file (Figure 17). We note that the folders have to be created manually.

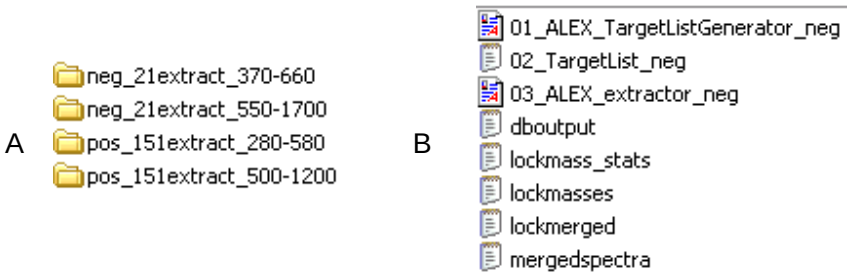


Figure 17. Screenshot of the folders created manually (A). Each folder contains the TargetList definition file, the created target list, the ALEX extractor parameter file and the output files (B)

The target list for the scan window m/z 280-580 in pos. mode consists of the internal standard (IS) Lipid species IS LPC O-17:0 and the Lipid classes LPC, LPE, LPC-O and LPE-O (Figure 18) with carbon atoms in the fatty acid moieties (C index) ranging from 14-22. Only an even carbon atom number in the fatty acid moiety is allowed. The number of double bounds in the fatty acid moiety is calculated by the relation:

Double Bound (DB) = 0.750* C index + (-10.500)

For example a fatty acid moiety with 14 carbon atoms will not have any double bound where one with 22 carbon atoms up to 6 double bounds.
These parameters are saved as “01_ALEX_TargetListGenerator_pos” in the corresponding folder.

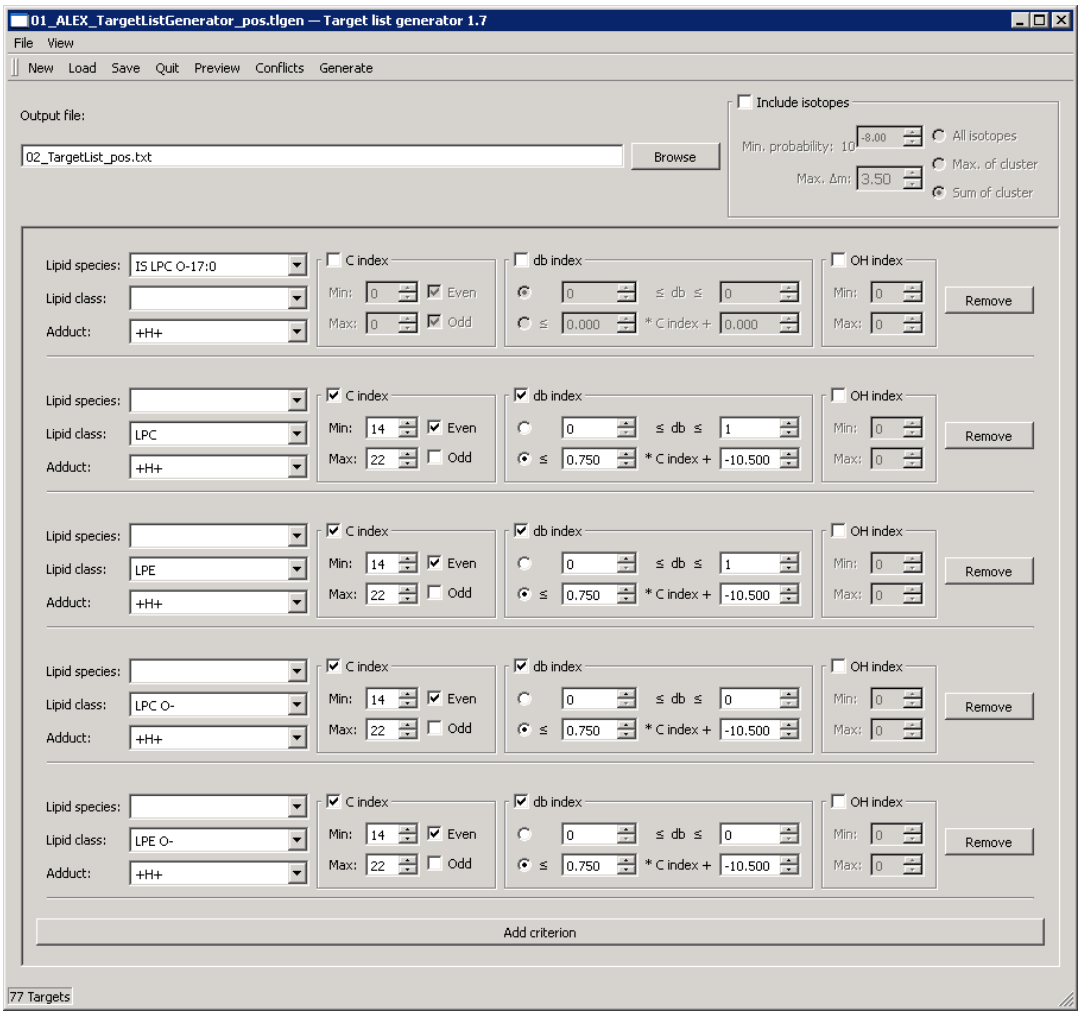


Figure 18. Screenshots from the ALEX target list generator with settings used to create a target list for the *m/z* range 280-580 in pos.mode

The target list is created by pressing the “Generate” button and saved as “02_TargetList_pos.txt” defined in the Output file txt box (Figure 18).

8.4.2. Alex extractor

The ALEX extractor identifies the lipid species and exports intensities by querying the averaged peak lists produced by the ALEX converter. For the data set acquired from *m/z* 280-580 in pos. Ion mode following parameters were set (Figure 19):

Spectra folder: Folder containing the “txt.” files created by the ALEX converter

Target file: Target List created by the ALEX target list generator

Output folder: Folder of the resulting output file

***m/z* tolerance:** the tolerance window for the search was set to m/z ± 0.020

***m/z* offset:** The m/z offset was set to 0

Lock mass: The internal standard LPC-O 17:0 was used as lock mass

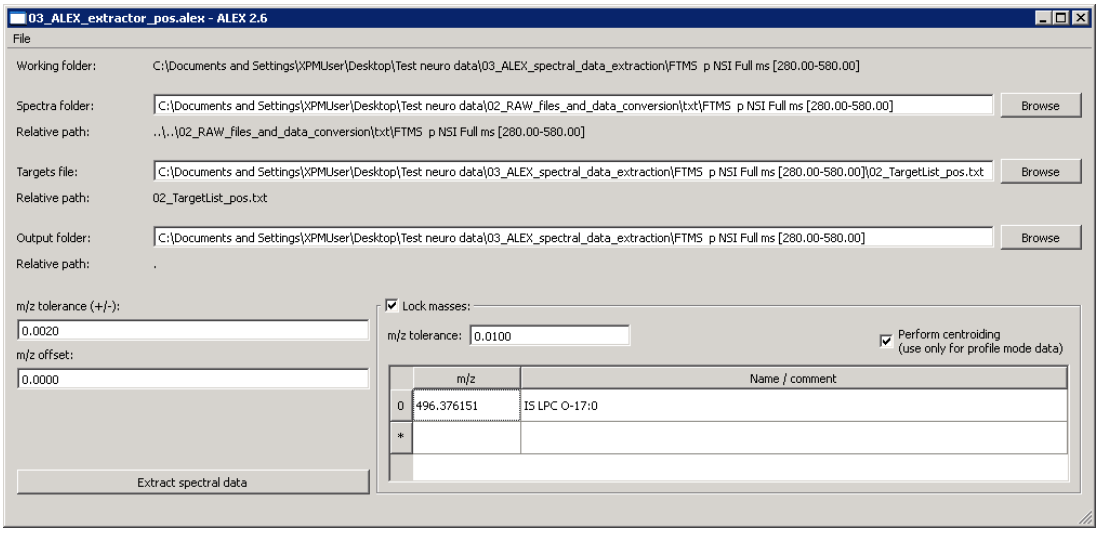


Figure 19. Screenshots of the ALEX extractor with the parameters used for the mass range *m/z* 280- 580 acquired in pos. Ion mode.

The ALEX extractor outputs 5 files (Figure 17 B):

lockmass_stats.txt: This file contains information for the applied offset from the lock mass ion (LPC-O 17:0) for each processed data file

lockmasses.csv: This file contains the measured offset and intensity for the lock mass ion (LPC-O 17:0) for each processed data file. This file will be used for the quality control (8.5).

lockmerged.csv: This file contains the average measured *m/z* with offset of the lock mass ion and their intensities from the processed data file.

dboutput.csv: This file contains all identified lipid species with respective *m/z* values and lipid features (chemical formula, lipid category, lipid class, total number of C atoms etc.) for all processed data files.

mergedspectra.csv: This file organizes data in .xls spreadsheet format. This option might be convenient for those who would like to use Microsoft Excel for data processing.

The complete process described here for the data extraction (8.4) for the mass range *m/z* 280- 580 acquired in pos. Ion mode has to be repeated for the other 3 mass ranges in this data set, therefore a total of 4 dboutput.csv files were created by the ALEX extractor. These 4 data outputs will be merged later by the ALEX unifier (8.6) for further processing using Orange, but before a quality control can be performed by comparing the signal intensity and m/z offset (offset between measured *m/z* vs. accurate *m/z*) for the used internal standards in the complete data set.

8.5. Tableau quality control

In the quality control using Tableau software for visualization, the measured offset and the measured intensity for the internal standard is compared for the experimental data set using the “lockmass.csv” file created by ALEX extractor for the particular *m/z* range and polarity (Figure 20). By default, Tableau treats all relational fields containing text or date values as dimensions and all relational fields containing numbers as measures (Figure 20 left hand side). A histogram can easily be created by dragging the fields onto the Columns or Rows shelf. For this data set the “Raw_ID” (Data file name) and “Name” (ISTD Name) have been placed into the Columns shelf and “Measured offset” and “Intensity” into the Rows shelf. For more information on how to use Tableau visit www.tableausoftware.com and the online help at http://onlinehelp.tableausoftware.com/v7.0/pro/online/en-us/help.htm.

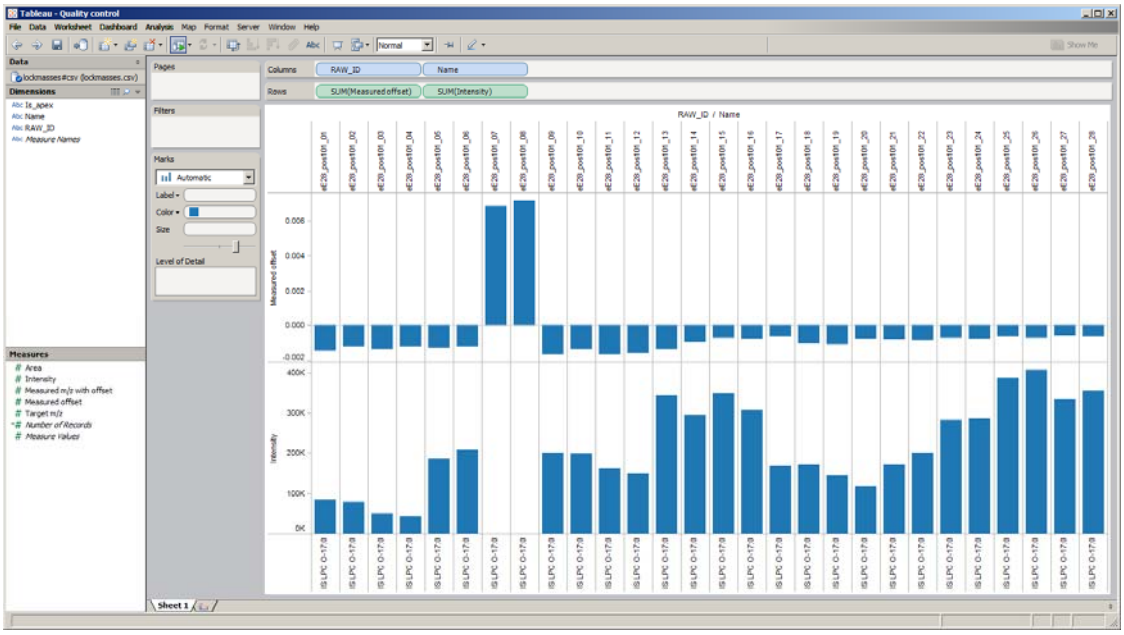
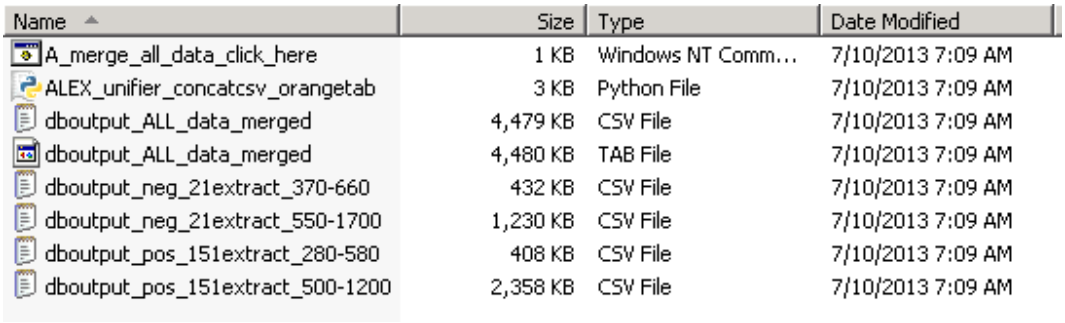


Figure 20. Quality control analysis using Tableau for visualisation by monitoring the lock mass offset and intensity for the internal standard LPC-O 17:0 as function of sample injection

We note that the lock mass and internal standard LPC-O 17:0 is not detected in injection 07 and 08. Manual inspection of FT MS spectra revealed that the particular sample had not been spiked with internal standards. The quality control is repeated for the 3 other mass ranges.

8.6. A merge MS data for Orange

The 4 different CSV files from the different FT MS ranges created by the ALEX extractor can be merged into a single file using the ALEX unifier located in the "A merge MS data for Orange" folder (Figure 21). The unifier is started by executing the "A_merge_all_data_click_here". The output files are copied into the folder and renamed according to the command line from the ALEX unifier (
Figure 22).



Name	Size	Type	Date Modified
A_merge_all_data_click_here	1 KB	Windows NT Comm...	7/10/2013 7:09 AM
ALEX_unifier_concatcsv_orangetab	3 KB	Python File	7/10/2013 7:09 AM
dboutput_ALL_data_merged	4,479 KB	CSV File	7/10/2013 7:09 AM
dboutput_ALL_data_merged	4,480 KB	TAB File	7/10/2013 7:09 AM
dboutput_neg_21extract_370-660	432 KB	CSV File	7/10/2013 7:09 AM
dboutput_neg_21extract_550-1700	1,230 KB	CSV File	7/10/2013 7:09 AM
dboutput_pos_151extract_280-580	408 KB	CSV File	7/10/2013 7:09 AM
dboutput_pos_151extract_500-1200	2,358 KB	CSV File	7/10/2013 7:09 AM

Figure 21. Screenshot from the " A merge MS data for Orange" folder containing the ALEX unifier, the 4 CSV files created previously by the ALEX extractor and the single merged CSV file and Orange friendly Tab file

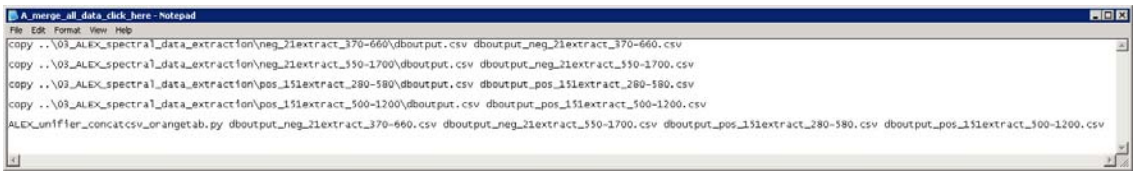


Figure 22. Screenshot from the Alex Unifier Command line

We note that that the command lines have to be changed if the folder names change in order to find the correct path of the output files.

8.7. B ISmix info for Orange

Orange is used to calculate the molar abundance of lipid species by applying a sequence of processing steps to the data set. The workflow requires 3 data inputs:

1. The merged data file created by the ALEX unifier in ".tab" format (8.6)
2. The sample list (Figure 24) created with Excel and saved as tab deliminated file
3. The spiked internal standard list (Figure 23) created with Excel and saved as tab delimitated file.

	A	B	C
1	Internal standard	fmol spike	Lipid class
2	d	c	d
3			
4	IS cholesterolD7	140247.8594	sterol
5	IS CE 19:0	54495.04297	CE
6	IS TAG 17:1/17:1/17:1	34738.92969	TAG
7	IS DAG 19:0/19:0	34798.41406	DAG
8	IS LPA O-16:0	25719.5918	LPA
9	IS PA 17:0/14:1	34675.14844	PA
10	IS LPS 17:1	24868.25586	LPS
11	IS PS 17:0/20:4	13352.54688	PS
12	IS PE O-20:0/O-20:0	49670.83203	PE
13	IS LPC O-17:0	30001.87109	LPC
14	IS PC 18:3/18:3	136568.7344	PC
15	IS PI 17:0/20:4	34696.10156	PI
16	IS PG 17:0/17:0	29925.87109	PG
17	IS Cer 18:1;2/17:0;0	54646.02734	Cer
18	IS SM 18:1;2/17:0;0	69476.07813	SM
19	IS HexCer 18:1;2/12:0;0	49108.55469	HexCer
20	IS SHexCer 18:1;2/12:0;0	27649.51758	SHexCer
21	IS MAG 15:0	31645.73633	MAG
22	IS HexCer 18:1;2/12:0;0	49108.55469	Hex2Cer
23	IS HexCer 18:1;2/12:0;0	49108.55469	Hex3Cer
24	IS PC 18:3/18:3	136568.7344	PC O-
25	IS PE O-20:0/O-20:0	49670.83203	PE O-
26	IS LPC O-17:0	30001.87109	LPC O-
27	IS LPC O-17:0	30001.87109	LPE
28	IS LPC O-17:0	30001.87109	LPE O-

Figure 23. Information on internal standards spiked to the neurolipidomics sample set

We note that the sample list and internal standard list requires in each column of the second row a "d" to be treated as text or "c" to be treated as numerical value. These files also need to be renamed to .tab to be recognized by Orange.

8.8. C define sample list for Orange

The sample list (Figure 24) is created and can be manipulated using Excel, but has to be saved as tab delaminated txt file and renamed to ".tab" to be used by Orange.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	RAW_ID	File_string	File_number	Sample_number	Sample_name1	Sample_name2	Sample_name3	Tissue	Strain	Mouse	Replica	Reinjection	Biological replica	Display order
2	d	d	d	d	d	d	d	d	d	d	d	d	d	d
3														
4	eE28_post01_01	eE28_post01_	01	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	**Cerebellum**	**WT**	A	A	no	A	A1
5	eE28_post01_02	eE28_post01_	02	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	**Cerebellum**	**WT**	A	B	no	A	A2
6	eE28_post01_03	eE28_post01_	03	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	**Cerebellum**	**WT**	B	A	no	B	A3
7	eE28_post01_04	eE28_post01_	04	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	**Cerebellum**	**WT**	B	B	no	B	A4
8	eE28_post01_05	eE28_post01_	05	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	**Cerebellum**	**KO**	C	A	no	A	A5
9	eE28_post01_06	eE28_post01_	06	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	**Cerebellum**	**KO**	C	B	no	A	A6
10	eE28_post01_07	eE28_post01_	07	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	**Cerebellum**	**KO**	D	A	no	B	A7
11	eE28_post01_08	eE28_post01_	08	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	**Cerebellum**	**KO**	D	B	no	B	A8
12	eE28_post01_09	eE28_post01_	09	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	**WT**	A	A	no	A	A9
13	eE28_post01_10	eE28_post01_	10	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	**WT**	A	B	no	A	A10
14	eE28_post01_11	eE28_post01_	11	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	**WT**	B	A	no	B	A11
15	eE28_post01_12	eE28_post01_	12	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	**WT**	B	B	no	B	A12
16	eE28_post01_13	eE28_post01_	13	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	**KO**	C	A	no	A	A13
17	eE28_post01_14	eE28_post01_	14	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	**KO**	C	B	no	A	A14
18	eE28_post01_15	eE28_post01_	15	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	**KO**	D	A	no	B	A15
19	eE28_post01_16	eE28_post01_	16	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	**KO**	D	B	no	B	A16
20	eE28_post01_17	eE28_post01_	17	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	**S1BF**	**WT**	A	A	no	A	A17
21	eE28_post01_18	eE28_post01_	18	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	**S1BF**	**WT**	A	B	no	A	A18
22	eE28_post01_19	eE28_post01_	19	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	**S1BF**	**WT**	B	A	no	B	A19
23	eE28_post01_20	eE28_post01_	20	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	**S1BF**	**WT**	B	B	no	B	A20
24	eE28_post01_21	eE28_post01_	21	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	**S1BF**	**KO**	C	A	no	A	A21
25	eE28_post01_22	eE28_post01_	22	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	**S1BF**	**KO**	C	B	no	A	A22
26	eE28_post01_23	eE28_post01_	23	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	**S1BF**	**KO**	D	A	no	B	A23
27	eE28_post01_24	eE28_post01_	24	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	**S1BF**	**KO**	D	B	no	B	A24
28	eE28_post01_25	eE28_post01_	25	13	blk	blk	blk	blk	blk	blk	A	no	blk	A25
29	eE28_post01_26	eE28_post01_	26	13	blk	blk	blk	blk	blk	blk	B	no	blk	A26
30	eE28_post01_27	eE28_post01_	27	14	blk	blk	blk	blk	blk	blk	A	no	blk	A27
31	eE28_post01_28	eE28_post01_	28	14	blk	blk	blk	blk	blk	blk	B	no	blk	A28
32	eE28_neg21_01	eE28_neg21_	01	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	**Cerebellum**	**WT**	A	A	**yes**	A	A1
33	eE28_neg21_02	eE28_neg21_	02	1	1. Cerebellum[WT-A]	Cerebellum[WT-A]	Cerebellum[WT]	**Cerebellum**	**WT**	A	B	**yes**	A	A2
34	eE28_neg21_03	eE28_neg21_	03	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	**Cerebellum**	**WT**	B	A	**yes**	B	A3
35	eE28_neg21_04	eE28_neg21_	04	2	2. Cerebellum[WT-B]	Cerebellum[WT-B]	Cerebellum[WT]	**Cerebellum**	**WT**	B	B	**yes**	B	A4
36	eE28_neg21_05	eE28_neg21_	05	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	**Cerebellum**	**KO**	C	A	**yes**	A	A5
37	eE28_neg21_06	eE28_neg21_	06	3	3. Cerebellum[KO-A]	Cerebellum[KO-A]	Cerebellum[KO]	**Cerebellum**	**KO**	C	B	**yes**	A	A6
38	eE28_neg21_07	eE28_neg21_	07	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	**Cerebellum**	**KO**	D	A	**yes**	B	A7
39	eE28_neg21_08	eE28_neg21_	08	4	4. Cerebellum[KO-B]	Cerebellum[KO-B]	Cerebellum[KO]	**Cerebellum**	**KO**	D	B	**yes**	B	A8
40	eE28_neg21_09	eE28_neg21_	09	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	**WT**	A	A	no	A	A9
41	eE28_neg21_10	eE28_neg21_	10	5	5. Hippocampus[WT-A]	Hippocampus[WT-A]	Hippocampus[WT]	Hippocampus	**WT**	A	B	no	A	A10
42	eE28_neg21_11	eE28_neg21_	11	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	**WT**	B	A	no	B	A11
43	eE28_neg21_12	eE28_neg21_	12	6	6. Hippocampus[WT-B]	Hippocampus[WT-B]	Hippocampus[WT]	Hippocampus	**WT**	B	B	no	B	A12
44	eE28_neg21_13	eE28_neg21_	13	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	**KO**	C	A	no	A	A13
45	eE28_neg21_14	eE28_neg21_	14	7	7. Hippocampus[KO-A]	Hippocampus[KO-A]	Hippocampus[KO]	Hippocampus	**KO**	C	B	no	A	A14
46	eE28_neg21_15	eE28_neg21_	15	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	**KO**	D	A	no	B	A15
47	eE28_neg21_16	eE28_neg21_	16	8	8. Hippocampus[KO-B]	Hippocampus[KO-B]	Hippocampus[KO]	Hippocampus	**KO**	D	B	no	B	A16
48	eE28_neg21_17	eE28_neg21_	17	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	**S1BF**	**WT**	A	A	no	A	A17
49	eE28_neg21_18	eE28_neg21_	18	9	9. S1BF[WT-A]	S1BF[WT-A]	S1BF[WT]	**S1BF**	**WT**	A	B	no	A	A18
50	eE28_neg21_19	eE28_neg21_	19	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	**S1BF**	**WT**	B	A	**yes**	B	A19
51	eE28_neg21_20	eE28_neg21_	20	10	10. S1BF[WT-B]	S1BF[WT-B]	S1BF[WT]	**S1BF**	**WT**	B	B	**yes**	B	A20
52	eE28_neg21_21	eE28_neg21_	21	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	**S1BF**	**KO**	C	A	no	A	A21
53	eE28_neg21_22	eE28_neg21_	22	11	11. S1BF[KO-A]	S1BF[KO-A]	S1BF[KO]	**S1BF**	**KO**	C	B	no	A	A22
54	eE28_neg21_23	eE28_neg21_	23	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	**S1BF**	**KO**	D	A	no	B	A23
55	eE28_neg21_24	eE28_neg21_	24	12	12. S1BF[KO-B]	S1BF[KO-B]	S1BF[KO]	**S1BF**	**KO**	D	B	no	B	A24
56	eE28_neg21_25	eE28_neg21_	25	13	blk	blk	blk	blk	blk	blk	A	no	blk	A25
57	eE28_neg21_26	eE28_neg21_	26	13	blk	blk	blk	blk	blk	blk	B	no	blk	A26
58	eE28_neg21_27	eE28_neg21_	27	14	blk	blk	blk	blk	blk	blk	A	**yes**	blk	A27
59	eE28_neg21_28	eE28_neg21_	28	14	blk	blk	blk	blk	blk	blk	B	**yes**	blk	A28
60														

Figure 24. Sample list from a neurolipidomics study of three mouse brain tissues (Cerebellum, Hippocampus and S1BF) from wild-type and PRG-1 knockout mice.

8.9. D Orange pmol calculation

To calculate the molar abundance of lipid species a sequence of processing steps is applied to the data using Orange (Figure 25).

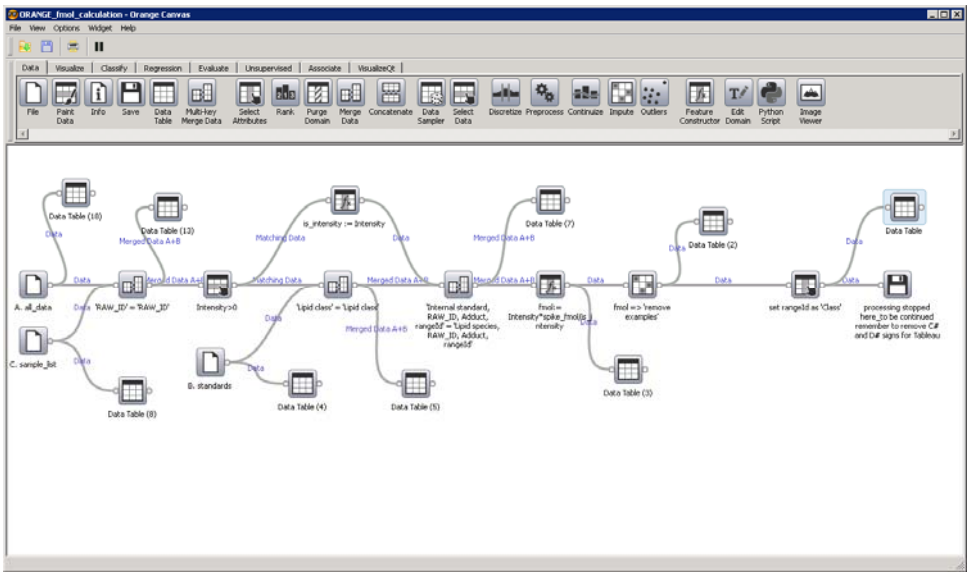


Figure 25. Screen shot of Orange scheme to calculate molar abundance of lipid species

Input the merged data file created by the ALEX unifier, the sample list created with Excel and saved as tab delimitated file and the spiked internal standard list created with Excel and saved as tab delimitated file into “A. all_data, B. standards, C. sample_list” by clicking on the respective Icon to select the file (Figure 26).

A B C

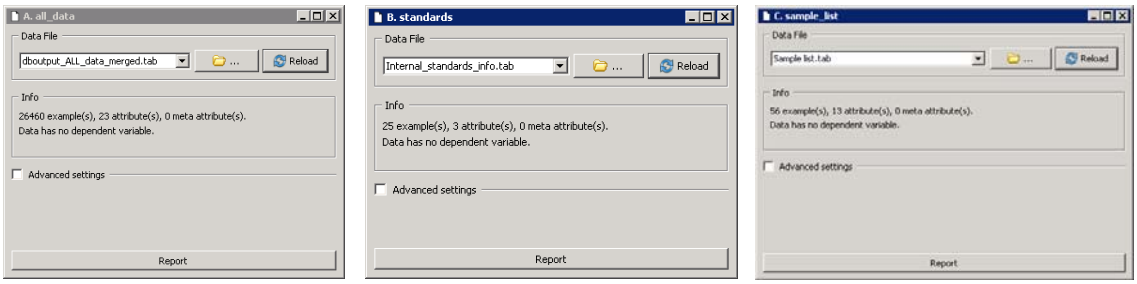


Figure 26. Orange input to calculate the abundance of lipid species

A detailed description of each step in the sequence can be found in this manual (6. Orange). After each step the data can be accessed by opening the corresponding Data Table (Figure 25). The data set can be saved by clicking on the last icon “processing stopped here_to be continued remember to remove C# and D# signs for Tableau”. The data are saved as a Comma-separated values (.csv) file (Figure 27) and can be visualized using Tableau. It is recommended to remove the C# and D# from the first row in the data set using Excel (Figure 28).

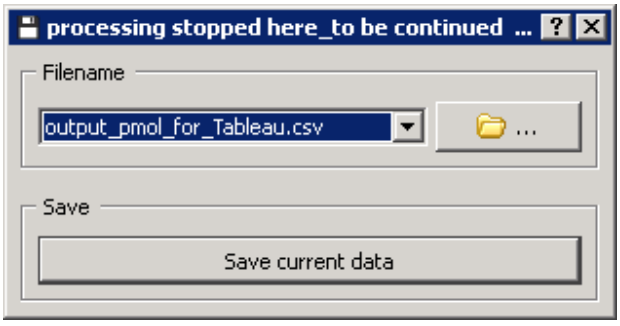


Figure 27. Saved output file as Comma-separated values formate in Orange

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	RAW_ID	Target m/z	Lipid species	D#Lipid ID	Lipid category	Lipid class	Adduct	Conflicts	Isotope cluster	Isotope probability	Isotope	Charge	D#C index	D#db index	OH index	Sum composition	Sum formula
2	eE28_neg21_01	381.2047729	LPA 14:0	101034	Glycerophospholipid	LPA	-H+	?	0	0.81560828	?	-1	14	0	?	'14:0	C17H34O7P
3	eE28_neg21_01	407.2204285	LPA 16:1	101047	Glycerophospholipid	LPA	-H+	?	0	0.798064055	?	-1	16	1	?	'16:1	C19H36O7P
4	eE28_neg21_01	409.2360535	LPA 16:0	101048	Glycerophospholipid	LPA	-H+	?	0	0.797880511	?	-1	16	0	?	'16:0	C19H38O7P
5	eE28_neg21_01	431.2204285	LPA 18:3	101059	Glycerophospholipid	LPA	-H+	?	0	0.781076855	?	-1	18	3	?	'18:3	C21H36O7P
6	eE28_neg21_01	433.2360535	LPA 18:2	101060	Glycerophospholipid	LPA	-H+	?	0	0.780897217	?	-1	18	2	?	'18:2	C21H38O7P
7	eE28_neg21_01	435.251709	LPA 18:1	101061	Glycerophospholipid	LPA	-H+	?	0	0.780717621	?	-1	18	1	?	'18:1	C21H40O7P
8	eE28_neg21_01	437.2673645	LPA 18:0	101062	Glycerophospholipid	LPA	-H+	?	0	0.780538067	?	-1	18	0	?	'18:0	C21H42O7P
9	eE28_neg21_01	457.2360535	LPA 20:4	101072	Glycerophospholipid	LPA	-H+	?	0	0.764275422	?	-1	20	4	?	'20:4	C23H38O7P
10	eE28_neg21_01	459.251709	LPA 20:3	101073	Glycerophospholipid	LPA	-H+	?	0	0.764099649	?	-1	20	3	?	'20:3	C23H40O7P

Figure 28. Orange output data file in Excel. The D# and C# should be removed from the first row before vizualising the data set with Tableau software

8.10. Tableau data visualization

The Orange output files are linked with Tableau (www.tableausoftware.com) used for data visualization. By default, Tableau treats all relational fields from the output file containing text or date values as dimensions and all relational fields containing numbers as measures. The data can be sorted and visualized in different display formats by dragging the fields onto the Columns or Rows shelf. Following are some examples how the lipidome data can be in interrogated:

- **Lipid category composition in mol% (**Figure 29**)**
  Columns: Lipid category, Sample_name1; **Rows:** fmol; Calculation Type: % of Total over Lipid category

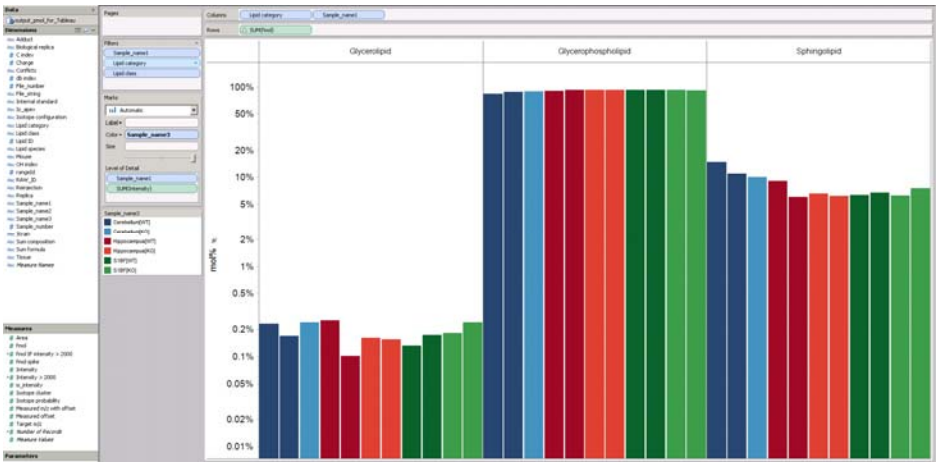


Figure 29. Lipidome visualization : mol% lipid category. Notice that the y-axis is logarithmic. Data is displayed as the average of the two technical replicates per sample.

- **Double bound index in mol% of LPS species (Figure 30)**

  Columns: db index, Sample_name1, Replica; **Rows:** fmol; Calculation Type: % of Total over Lipid category; **Filter:** LPS

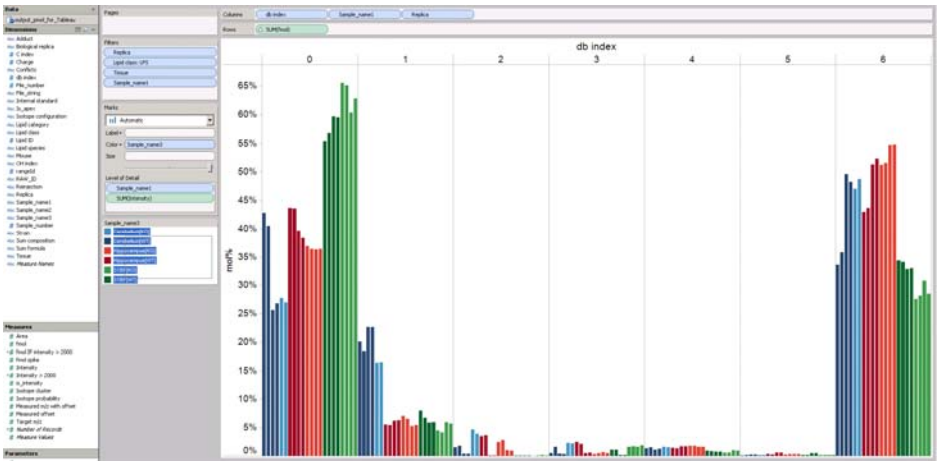


Figure 30. Lipidome visualization : mol% of db index of LPS species. Note that histogram include plot for both technical replicates.

- **PE species in mol% (Figure 31)**

  Columns: Lipid species, Sample_name1, **Rows:** fmol; Calculation Type: % of Total over Lipid category; **Filter:** PE

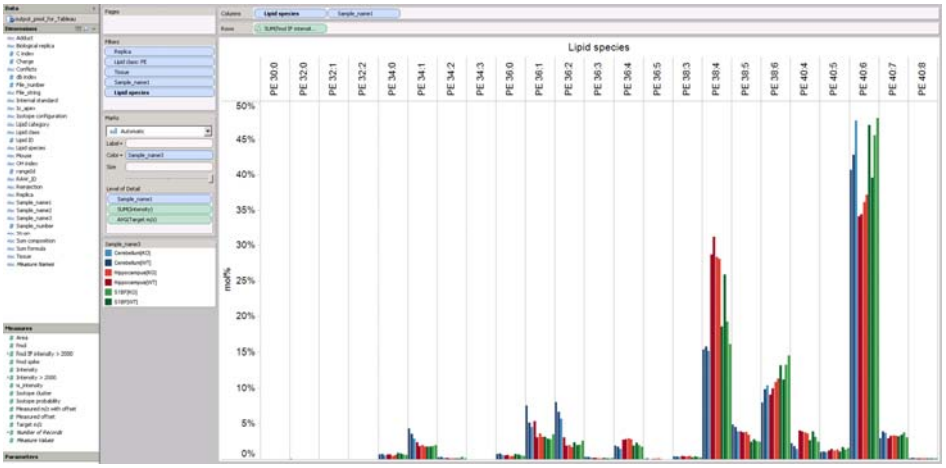


Figure 31. Lipidome visualization: mol% of PE species. Data is displayed as the average of the two technical replicates per sample.

- **All GPL species in mol% (Figure 32)**

  Columns: Sum composition, Sample_name1, **Rows:** fmol; Calculation Type: % of Total over Lipid category; **Filter:** Lipid category Glycerophospholipids

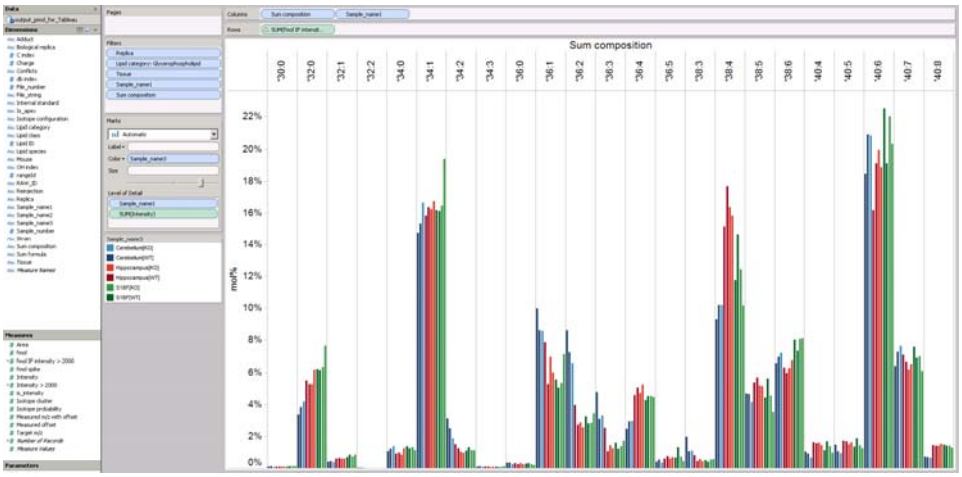


Figure 32. Lipidome visualization: mol% of all GPL species. Data is displayed as the average of the two technical replicates per sample.

For more information on how to use Tableau visit www.tableausoftware.com and the online help at

http://onlinehelp.tableausoftware.com/v7.0/pro/online/en-us/help.htm