Data Dictionary Fiehn laboratory _NIH West Coast Metabolomics Center_09-2013

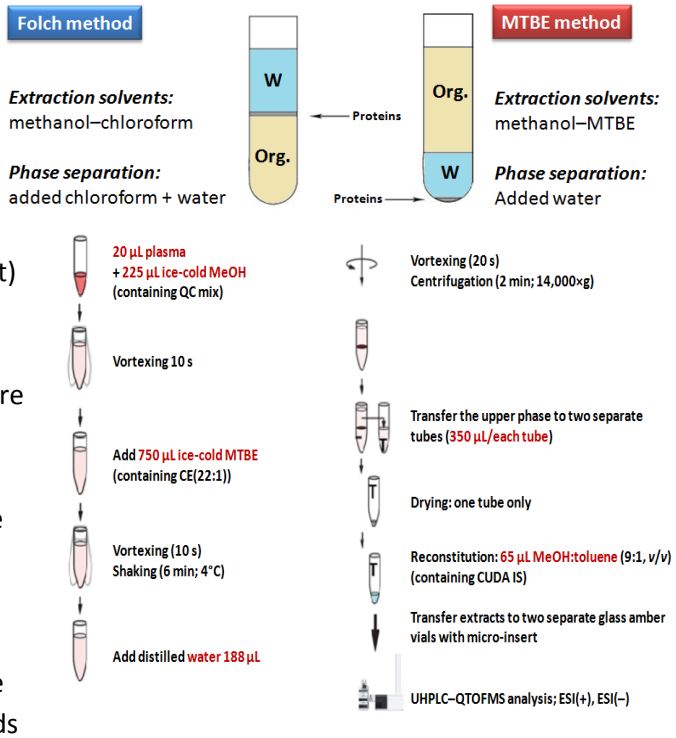
Lipidomics by CSH-ESI QTOF MS/MS

Glossary

CSH charged surface hybrid column. Waters corporation, a reversed phase UHPLC column.
UHPLC ultra high pressure liquid chromatography
ESI electrospray. The method uses both negative ESI and positive ESI for negatively charged and positively charged molecules.
QTOF quadrupole time of flight mass spectrometer
MS/MS tandem mass spectrometry. After soft ionization by electrospray, the precursor (intact) charged molecules are fragmented by collision with gas atoms, usually Helium. Fragments are then analyzed by time of flight mass spectrometry to obtain accurate mass information at high resolution.
Resolving power also called resolution. In MS, resolving power defines the ability to distinguish co-eluting masses that have the same nominal mass, but different accurate mass.
MTBE methyl-tertiary butyl ether
MeOH methanol
QC quality control
CE cholesteryl esters
PC phosphatidyl cholines (LPC is lyso-PC, see below)
PE phosphatidyl ethanolamines (LPE is lyso-PE, see below)
PS phosphatidyl serines
lyso- monoacylation of complex polar lipids at the sn1 position but not at the sn2 position
TAG triacylglycerols
DG diacylglycerols
MG monoacylglycerols
SM sphingomyelin
22:1 in lipidomic nomenclature the total number of acyl carbons (*here:* 22) and double bonds (1)
IS internal standards
CUDA N-cyclohexyl-N'-dodecanoic acid urea
v/v volumetric ratio
InChI International Chemical Identifier key. Denotes the exact stereochemical and atomic description of chemicals and used as universal identifier in chemical databases.
LIPIDMAPS Identifier used in the LIPIDMAPS database.
rt retention time (minutes)
mz also m/z, or mass-to-charge ratio. In metabolomics, ions are almost exclusively detected as singly charged species.
rt_mz identifier for individual metabolites in the MassHunter Quantification method consisting of the retention time and the m/z value of specific compounds.
IUPAC International Union of Pure and Applied Chemists
NIST National Institute of Standards and Technology
istd internal standard
PCA Principal Component Analysis

Extraction

Blood plasma or serum is extracted following the protocols first published in Matyash V. et al., *J. Lip. Res.* **49** (2008) 1137–1146. One of the major differences to the earlier protocols by Folch or Bligh-Dyer is that in the Matyash protocol, lipid extracts (labeled 'org' in the figure on the right) are separated from proteins and from polar hydrophilic small molecules (in the methanol/water phase, labeled 'W' in the figure on the right) in a way that the lipids are found in the top layer of liquid-liquid separations, rather than in the bottom layer. Decanting the top layer therefore ensures that extracts are not contaminated by proteins or polar compounds. The details of the extraction method are given in the panel to the right. We have optimized the choice of internal standards (see below) and chromatographic conditions, e.g. by using toluene in the reconstitution solvent mixture to ensure that very lipophilic components like CE and TAGs are efficiently transferred to the UHPLC column in the injection process.



Data acquisition

Data are acquired using the following chromatographic parameters:
Column: Waters Acquity UPLC CSH C18 (100 mm length x 2.1 mm internal diameter; 1.7 µm particles)
Mobile phase A: 60:40 acetonitrile:water + 10 mM ammonium formiate + 0.1% formic acid
Mobile phase B: 90:10 v/v isopropanol:acetonitrile + 10 mM ammonium formiate + 0.1% formic acid
Column temperature: 65°C
Flow-rate: 0.6 mL/min
Injection volume: 3 µL
Injection temperature: 4°C
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)

The analytical UHPLC column is protected by a short guard column (see left panel) which is replaced after 400 injections while the UHPLC column is replaced after 1,200 serum (or plasma) extract injections. We have validated that at this sequence of column replacements, no detrimental effects are detected with respect to peak shapes, absolute or relative lipid retention times or reproducibility of quantifications. This chromatography method yields excellent retention and separation of lipid classes (PC, lysoPC, PE, PS, TAG, ceramides) with narrow peak widths of 8–17 s and

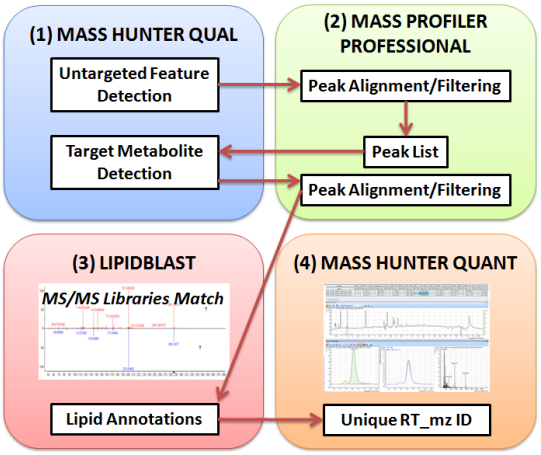
very good within-series retention time reproducibility of better than 6 s absolute deviation of retention times. We use automatic valve switching after each injection which we could show to reduce sample carryover for highly lipophilic compounds such as TAGs from 29% to 0.1%. This valve switching employs a dual solvent wash, first with a water/acetonitrile mixture (1:1, v/v) and subsequently with a 100% isopropanol wash.

Mass spectrometry parameters are used as follows: for positively charged lipids such as PC, lysoPC, PE, PS, an Agilent 6530 QTOF mass spectrometer is used with resolution R=10,000 while negatively charged lipids such as free fatty acids and phosphatidylinositols are analyzed using an Agilent 6550 QTOF mass spectrometer with resolution R=20,000.

Data processing

Data are analyzed in a four-stage process.

First, raw data are processed in an untargeted (qualitative) manner by Agilent's software MassHunter Qual to find peaks in up to 300 chromatograms. Peak features are then imported into MassProfilerProfessional for peak alignments to seek which peaks are present in multiple chromatograms, using exclusion criteria by the minimum percentage of chromatograms in which these peaks are positively detected. We usually use 30% as minimum criterion. In a tedious manual process, these peaks are then collated and constrained into a MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the LipidBlast library to identify lipids with manual confirmation of adduct ions and spectral scoring accuracy. MassHunter enables back-filling of quantifications for peaks that were missed in the primary peak finding process, hence yielding data sets without missing values. The procedure is given in the panel to the left as workflow diagram.



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identifier	name	formula	comment	LipidMAPS	InChI key	internal stan	batch_mz	batch_rt			
0.78_341.28	CUDA	C19H36N2O3	[M+H]+	no entry	HPTJABJPZM	istd	341.2799	0.78	209276	193114	208345
1.04_286.28	Sphingosine (	C17H35NO2	[M+H]+	LMSP010400	RBEJCQPPFC	istd	286.2752	1.05	107806	86635	87168
1.34_466.29	LPE 17:1	C22H44NO7I	[M+H]+	LMGP02050(	LNJNONCNAS	istd	466.2925	1.35	69154	55579	55731
1.82_510.36	LPC 17:0	C25H52NO7I	[M+H]+	LMGP01050(	SRRQPVVYXI	istd	510.3551	1.84	707687	577154	547244
3.03_345.30	MG 17:0/0:0,	C20H40O4	[M+H]+	LMGL01010(	SVUQHVRAG	istd	345.2999	3.09	74532	67939	64973
3.17_421.29	DG 18:1/2:0/	C23H42O5	[M+NH4]+	no entry	PWTCCMJTP	istd	421.2925	3.23	464696	434328	421719
3.49_636.46	PC 12:0/13:0	C33H66NO8I	[M+H]+	LMGP01010(	FCTBVSCBB\	istd	636.4596	3.56	66807	55981	55165
4.26_479.37	DG 12:0/12:(	C27H52O5	[M+NH4]+	LMGL02010:	OQQOAWVK'	istd	479.3707	4.36	123139	116038	119977
4.81_376.40	Cholesterol d	C27H39D7O	[M-H2O+H]+	LMST01010(	HVYWMOML	istd	376.3955	4.93	61179	52093	59266
5.06_717.59	SM 17:0	C40H81N2O(	[M+H]+	LMSP030100	YMQZQHIES(	istd	717.5914	5.19	180663	131169	154248
5.95_552.54	Ceramide C1:	C35H69NO3	[M+H]+	LMSP020100	ICWGMOFDL	istd	552.5350	6.11	212902	175209	161458
6.23_720.56	PE 17:0/17:0	C39H78NO8I	[M+H]+	LMGP02011:	YSFFAUPDXI	istd	720.5561	6.41	308869	247214	234193
10.98_869.83	TG d5 17:0/1	C54H97D5O(	[M+NH4]+	LMGL03010(	OWYYELCHN	istd	869.8329	11.17	358760	286446	222391
11.71_724.70	CE 22:1	C49H86O2	[M+NH4]+	LMST01020(	SQHUGNAFK	istd	724.6966	11.86	142338	123855	118649
1.23_520.34	LPC 18:2	C26H50NO7I	[M+H]+	LMGP01050(	SPJFYYJXNPE		520.3395	1.24	450668	998939	449344
1.47_496.34	LPC 16:0	C24H50NO7I	[M+H]+	LMGP01050(	ASWBNKHCZ		496.3395	1.48	3836583	1863185	1402174
1.62_522.36	LPC 18:1	C26H52NO7I	[M+H]+	LMGP01050(	YAMUFBLW(		522.3551	1.63	581444	377007	279551
1.79_548.37	LPC 20:2	C28H54NO7I	[M+H]+	LMGP01050:	YYQVCMMXF		548.3708	1.81	7746	5023	3730
2.23_524.37	LPC 18:0	C26H54NO7I	[M+H]+	LMGP01050(	IHNKQIMGV		524.3708	2.26	1512584	654412	530437
2.37_550.39	LPC 20:1	C28H56NO7I	[M+H]+	LMGP01050(	GJTDRNFWII		550.3864	2.41	15438	5691	5096
3.05_552.40	LPC 20:0	C28H58NO7I	[M+H]+	LMGP01050(	UATOAILWG		552.4021	3.11	19133	8781	6632

Data reporting

Data are reported including metadata, see previous page as example.

The **'identifier column'** denotes the unique identifier for the technology platform, given as rt_mz. This identifier is set for a given method and does not change over time. It is given for both identified and unidentified metabolites in the same manner.
The **'name'** denotes the name of the metabolite, if the peak has been identified. A chemical name is not a unique identifier. We use names recognized by biologists instead of IUPAC nomenclature.
The **'elemental composition'** denotes the formula of the metabolite, if the peak has been identified.
The **'comment'** denotes comments. Most regularly, we use the comment field to clarify which ion species (metabolite charged adduct) was used for quantification.
The **'LIPIDMAPS'** identifier gives the unique identifier associated with an identified lipid in the community database LIPIDMAPS.
The **'InChI key'** identifier gives the unique chemical identifier defined by the IUPAC and NIST consortia.
The **'internal standard'** column clarifies if a specific metabolite has been added into the extraction solvent as internal standard. These internal standards serve as retention time alignment markers, for quality control purposes and for absolute quantifications.
The **'batch mz'** column details the m/z value that was detected in a specific data processing sequence of chromatograms. This value may be slightly different from the mz value given in the 'identifier column'.
The **'batch rt'** column details the retention time that was detected in a specific data processing sequence of chromatograms. This value may be slightly different from the rt value given in the 'identifier column'.

The **'comments'** row gives comments about the platform and type of sample. A sample is given as "sample" in comparison to e.g. a quality control or a blank injection.
The '**Acq.Date-Time'** row details the acquisition date and time when the data acquisition was completed.
The **'Data File Name'** row denotes the name of the raw data file. Raw data files are secured at the NIH Metabolomics database, www.metabolomicsworkbench.org

The **actual data** are given as peak heights for the quantification ion (mz value) at the specific retention time (rt value). We give peak heights instead of peak areas because peak heights are more precise for low abundant metabolites than peak areas, due to the larger influence of baseline determinations on areas compared to peak heights. Also, overlapping (co-eluting) ions or peaks are harder to deconvolute in terms of precise determinations of peak areas than peak heights. Such data files are then called 'raw results data' in comparison to the raw data file produced during data acquisition (see 'data file name'). The worksheets are called 'Height'.

Raw results data need to be normalized to reduce the impact of between-series drifts of instrument sensitivity, caused by machine maintenance, aging and tuning parameters. Such normalization data sets are called 'norm data' worksheets.

There are many different type of normalizations in the scientific literature. We usually provide first a variant of a 'vector normalization' in which we calculate the sum of all peak heights for all identified metabolites (but not the unknowns!) for each sample. We call such peak-sums "mTIC" in analogy to the term TIC used in mass spectrometry (for 'total ion chromatogram'), but with the notification "mTIC" to indicate that we only use genuine metabolites (identified compounds) in order to avoid using potential non-biological artifacts for the biological normalizations, such as column bleed, plasticizers or other contaminants.

Subsequently, we determine if the mTIC averages are significantly different between treatment groups or cohorts. If these averages indeed are different by $p<0.05$, data will be normalized to the average mTIC of each group. If averages between treatment groups or cohorts are not different, or if treatment relations to groups are kept blinded, data will be normalized to the total average mTIC.

Following equation is then used for normalizations for metabolite *i* of sample *j*:

$$\text{metabolite}_{ij,\text{normalized}} = \text{metabolite}_{ij,\text{raw}} / \text{mTIC}_{j} * \text{mTIC}_{\text{average}}$$

The worksheet is then called **'norm mTIC'**. Data are 'relative semi-quantifications', meaning they are normalized peak heights. Because the average mTIC will be different between series of analyses that are weeks or months apart (due to differences in machine sensitivity, tuning, maintenance status and other parameters), **additional normalizations** need to be performed. For this purpose, identical samples ('QC samples') must be analyzed multiple times in all series of data acquisitions. In fact, one must not exclude the possibility that even within a series of data acquisitions, a sensitivity shift or drift might occur. Hence, the following statistical analyses are suggested: (a) compute univariate statistics for mTIC values in batches within-series and between-series of data injections, using time/date stamps to find potential breaks during which machine downtime may have occurred. If there are no mTIC differences between such time/date stamp batches, calculate an overall mTIC covering all samples. (b) compute multivariate PCA plots for the , marking the potentially different samples of individual time/date stamp batches using different colors. If there is no apparent separation between PCA clusters of different colors, there is no large between-series effect and these PCA clusters can be treated as indistinguishable. If there is suspicion of hidden features that might be masked by overall variance analysis in PCA, supervised statistics by Partial Least Square regression models can unravel such between-series differences.
Once different clusters (i.e. series of undistinguishable QC samples) have been identified, correction factor models need to be developed that correct differences between those QC samples. Subsequently, these correction factors can be applied to the actual analytical samples to remove overt quantification differences that are not related to biological causes but solely due to analytical errors.
Such correction factor models can be computed in different ways, e.g. by unit-variance mean centering or by calculating simple offset vectors for each individual metabolite. The best way of such types of normalizations are being explored in the Fiehn laboratory. However, in any case, such correction models can only be developed if a sufficient number of QC samples have been included in the analytical sequences. For that reason, the Fiehn laboratory uses a suitable QC sample for every 11^{th} injection. Such QC samples need to be as similar to the actual biological specimen as possible, e.g. generated by pool samples during extractions or by obtaining typical community standard samples (e.g. the NIST standard blood plasma, or commercial serum or plasma samples as needed).

If the internal standards are used for absolute quantifications, the following equation is used for peak height normalizations for metabolite *i* of sample *j* and internal standard *k*

$$\text{metabolite}_{ij,\text{normalized}} = \text{metabolite}_{ij,\text{raw}} / \text{istd}_{k} * \text{concentration istd}_{k}$$

The worksheet is then called **'norm istd'**. Data are 'absolute quantifications', meaning they are normalized to the best suited internal standard for which we know the absolute concentration that we used in the spiking process. The best suited internal standard is defined as the internal standard that belongs to the same lipid class as the metabolite that needs to be normalized. For example, all phosphatidylcholine lipids are normalized to our internal standard PC (12:0/13:0). For unidentified

lipids, we do not know the exact lipid class. However, because chromatography roughly separates the different lipid classes in different retention time groups, we can use the closest eluting internal standard for normalizing unidentified metabolites in order to get a rough estimate of a likely absolute concentration.

The benefit of absolute quantifications is that these normalized values should be not dependent on between-series drifts or shifts in machine sensitivity. The drawback, however, is that the quantification relies on the accuracy of the internal standard addition (pipetting), peak finding and the quantification of a single internal standard. Quantification errors of a single peak (internal standards) are necessarily larger than errors of sum parameters (like the mTIC values). We are currently evaluating the benefits or disadvantages between both types of normalization strategies (**norm mTIC** versus **norm istd**). This evaluation has not been completed yet in the Fiehn laboratory.