A New Lipid Software Workflow for Processing Orbitrap-based Global Lipidomics Data in Translational and Systems Biology Research

David A Peake,[1] Yasuto Yokoi,[2] Junhua Wang,[1] and Yingying Huang[1]
[1]Thermo Fisher Scientific, San Jose, USA; [2]Mitsui Knowledge Industry, Tokyo, Japan

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Overview

Purpose: We present a new workflow for high-resolution Thermo Scientific™ Orbitrap™-based mass spectrometers for lipidomics using a model system consisting of a wild-type strain vs. knockout for Co-Q production in yeast[1].

Methods: Lipids in yeast mitochondria were analyzed by high resolution LC-MS and MS/MS. Lipid Search® software, an MS^2 based search using a comprehensive lipid database, was used to identify the lipid species and determine significant differences.

Results: The yeast lipidomics results obtained from the LC/MS data using Lipid Search are comparable to results obtained using infusion lipidomics. We also compared the lipids identified using metabolomics analysis of the same data set – component finding and molecular weight (MW) search for assignment of metabolites and lipids. Due to the complexity of lipid extracts we found that the comprehensive lipid database MS^2 search method is superior to the accurate mass based MW search for lipidomics.

Introduction

Application of lipidomics to disease phenotype analysis is a growing area in medical research. Identification of unique biomarkers to distinguish healthy humans compared to individuals with disease can have an impact on the early detection of diseases and personalized medicine.

The complexity of the lipidome (Table 1) includes 8 major categories of lipids, over 80 major classes, 300 sub-classes and thousands of lipid species[2] many with overlapping isomeric or isobaric molecular ions. Because of this complexity, MW searches alone are not sufficient to identify lipids in a complex biological extract.

Identification of lipids requires sophisticated software with an extensive database. The combination of ultra-high resolution MS and MS^n analysis should provide unambiguous and precise identification of lipids in biological samples. A robust algorithm for database searching of high-resolution data was developed by Professor Ryo Taguchi and co-workers[3] and was commercialized by MKI (Tokyo, JP) as described recently[4].

Methods

Phenotypes of WT (wild-type) and Knockout (KO) Yeast Strains (*S. Cerevisiae*)

WT yeast continue to grow after glucose is exhausted from the media (Diauxic shift point) whereas KO yeast have a defect in Coenzyme Q production and do not grow after the shift. Duplicate biological replicates of WT and KO yeast were collected post shift for metabolomic/lipidomic analyses and analyzed by LC-MS.

Sample Preparation

Yeast were treated with zymolase, homogenized and mitochondria were enriched by differential centrifugation. Mitochondrial protein levels were determined by BCA assay. Mitochondria (~0.25 mg) were extracted 3 times with 400 µL of IPA for 10 min at 4 °C. After centrifugation, supernatants were combined and vacuum dried. Samples were dissolved in 250 µL of 65:35:5 Acetonitrile, Isopropanol, Water with 5 µg/mL17:0 PG.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Thermo Scientific™ Accela™ 1250 chromatograph and Accela Open autosampler, 10 µL Injection. Column: 2.1 x 100 mm C18, 2.7µm was operated at 260 µL/min and 55 °C. The RP HPLC method[1] is described in S. Bird, et al., Anal. Chem. **2011**, *83*, 940-949, 6648-6657. A Thermo Scientific™ Q Exactive™ high-resolution Orbitrap mass spectrometer was operated at 70K resolution for electrospray ionization (ESI) pos. ion LC-MS and 35K for Top5 MS/MS (CE 35).

Data Analysis Software

Metabolomics -Thermo Scientific™ SIEVE™ software and Lipidomics – Lipid Search software (MKI).

Table 1. Lipid Complexity from the LIPID MAPS Structure Database (LMSD) [2]

	Lipid Category	# Class	# Sub-Class	# Lipids
FA	Fatty acyls	14	36	5,787
GL	Glycerolipids	6	19	7,568
GP	Glycerophospholipids	21	120	8,001
SP	Sphingolipids	10	31	4,317
ST	Sterol lipids	6	38	2,678
PR	Prenol lipids	5	21	1,200
SL	Saccharolipids	6	7	1,293
PK	Polyketides	15	28	6,741
	Total	**83**	**300**	**37,585**

Results

High-Resolution LC-MS Data – Metabolomics Analysis

To characterize the yeast phenotypes we analyzed the sample extracts using an LC-MS method suited for analysis of both metabolites and lipids. The LC-MS chromatogram from WT yeast (Figure 1) shows the regions where lipid classes elute during the LC gradient. Metabolomics analysis using an accurate-mass search tentatively identified 160 metabolites and lipids were present. Principal component analysis (Figure 2) and t-Test statistics (Figure 3) show key metabolite differences.

FIGURE 1. LC-MS Chromatograms of Lipids from WT and KO Yeast

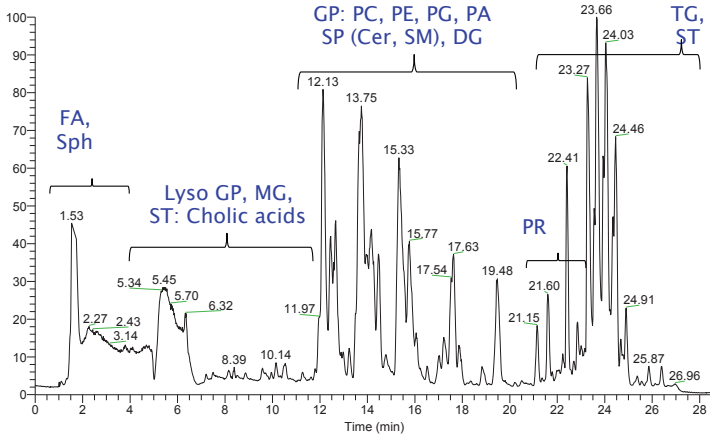


FIGURE 2. Principal Components Analysis of WT and KO Yeast Metabolites

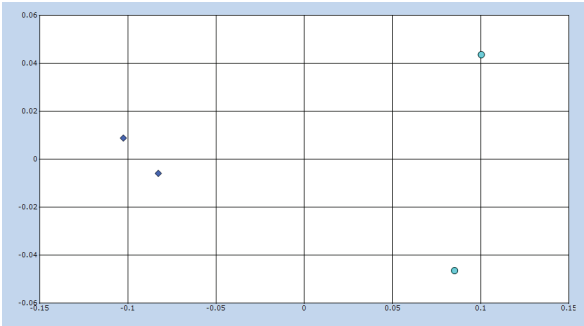
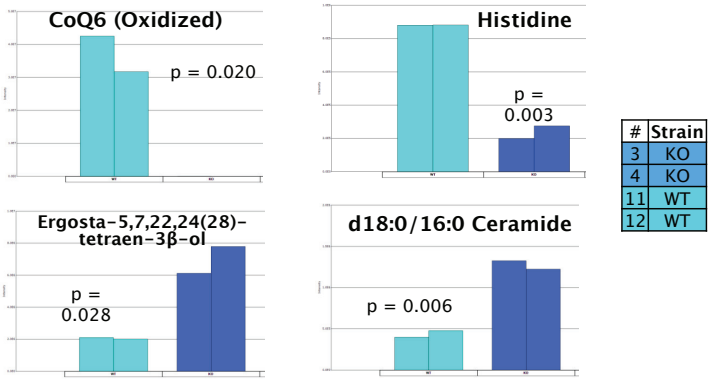


FIGURE 3. Significant Metabolite Differences Observed WT vs. KO Yeast



#	Strain
3	KO
4	KO
11	WT
12	WT

LC–MS/MS Data Processing Workflow using Lipid Search Software (Figure 4)

1) Peak Detection. Read raw files, MS^n and precursor ion accurate masses.

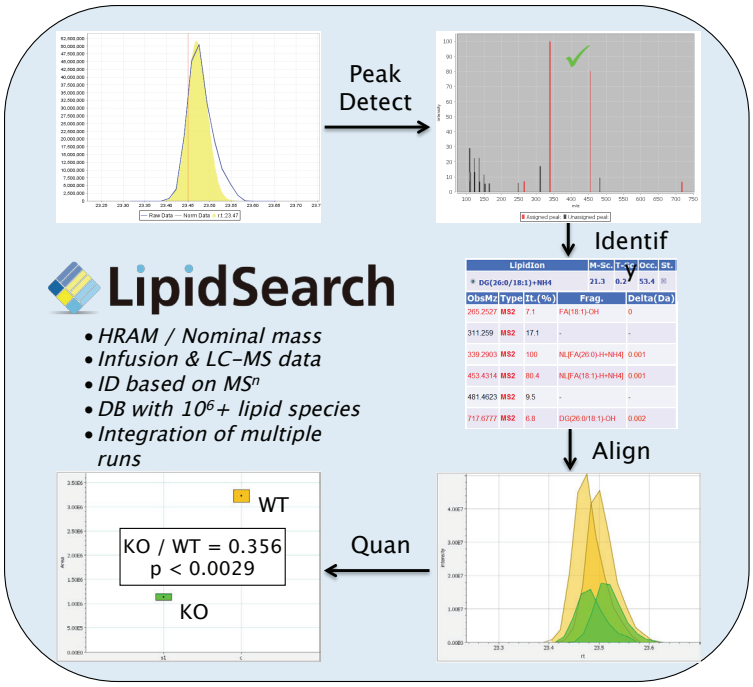
2) Identification. Candidate molecular species are identified by searching a large database > 1,000,000 entries of accurate masses (lipid precursor and fragment ions) predicted from each potential lipid structure and positive / negative ion adducts.

3) Alignment. The search results for each individual sample are aligned within a time window and the results are combined into a single report.

4) Quantification. The accurate-mass extracted ion chromatograms are integrated for each identified lipid precursor and the peak areas are obtained.

5) Statistical Analysis. t–Tests determine which lipid species are significantly different between sample vs. control groups, and results are displayed in a whisker plot.

FIGURE 4. Lipid Search Software LC–MS Workflow.



Submitting Data for Lipid Search Identification and Alignment

LC–MS raw data files containing full scan and data dependent–MS/MS were searched for PL, GL, SP and Co–enzyme lipid classes using a mass tolerance of 5 ppm for precursor ions and 10 ppm for product ions (Figure 5a).

The search results from the 4 samples were aligned using a 0.25 min tolerance window and a combined report was generated (Figure 5b).

FIGURE 5a. Search Results for Yeast Lipids

Name	RawData	Type	ExpType	Process	Result	Regist	Update
2013-05-25-21-54-24	4KOPost_1.raw	Product	LC		239 257	2013/05/25 21:54:24	2013/05/25 22:01:01
2013-05-25-21-54-24	3KOPost_1.raw	Product	LC		232 261	2013/05/25 21:54:24	2013/05/25 21:59:38
2013-05-25-21-54-24	12WTPost_1.raw	Product	LC		250 278	2013/05/25 21:54:24	2013/05/25 21:58:12
2013-05-25-21-54-24	11WTPost_1.raw	Product	LC		245 269	2013/05/25 21:54:24	2013/05/25 21:56:58

FIGURE 5b. Alignment Results for Yeast Lipids

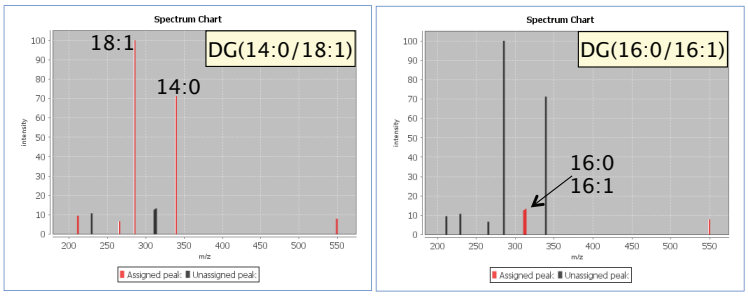
Name	Type	ExpType	Process	Result	Regist	Update
Yeast Lipids	Product	LC	M	380 760	2013/05/25 22:06:18	2013/05/25 22:07:32

Search results obtained in < 8 min with 64–bit laptop (MS Windows 7, 2.2 GHz, Intel i7 processor, 8GB RAM)

Identification Report (Figure 6)

For each MS^2 spectrum, search results are summarized for lipid species matching the predicted fragmentation pattern from the database with a score indicating the fit. If a mixture of lipids is found, the most abundant lipid is displayed. The fragment ions used to identify the lipid are highlighted in red when each of the species are selected.

FIGURE 6. Search Results for *m/z* 584.5249, Rt = 17.3 min, DG(32:1)



Match Detail

LipidIon	M-Sc.	T-Sc.	Occ.	St.
DG(14:0/18:1)+NH4	30.2	0.2	60.3	
DG(18:1/14:0)+NH4	30.2	0.2	60.3	

ObsMz	Type	It.(%)	Frag.	Delta(Da)
211.2061	MS2	9.5	FA(14:0)-OH	0
229.2163	MS2	10.7	-	-
265.2531	MS2	6.7	FA(18:1)-OH	0
285.2427	MS2	100	NL[FA(18:1)-H+NH4]	0
311.2584	MS2	12.7	-	-
313.2739	MS2	13.3	-	-
339.2899	MS2	71.3	NL[FA(14:0)-H+NH4]	0.001
549.4881	MS2	7.9	DG(18:1/14:0)-OH	0

Match Detail

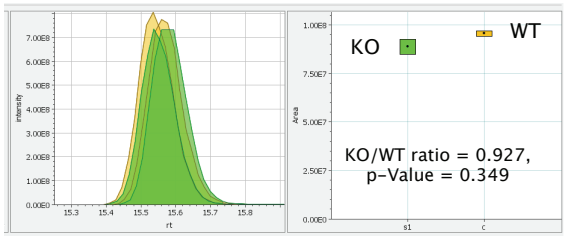
LipidIon	M-Sc.	T-Sc.	Occ.	St.
DG(16:0/16:1)+NH4	3.1	0.2	10.5	
DG(16:1/16:0)+NH4	3.1	0.2	10.5	

ObsMz	Type	It.(%)	Frag.	Delta(Da)
211.2061	MS2	9.5	-	-
229.2163	MS2	10.7	-	-
265.2531	MS2	6.7	-	-
285.2427	MS2	100	-	-
311.2584	MS2	12.7	NL[FA(16:0)-H+NH4]	0
313.2739	MS2	13.3	NL[FA(16:1)-H+NH4]	0
339.2899	MS2	71.3	-	-
549.4881	MS2	7.9	DG(16:1/16:0)-OH	0

Combined Report – Details (Figures 7 and 8)

Lipid species identified in each LC–MS data file were aligned across the dataset within a retention time tolerance. Quantification is performed on the relative amount of the precursor ion, which in some cases was identified as a mixture of isomers. For each lipid species in the aligned dataset, an interactive report allows review of the data. Relative amounts of each identified lipid were quantified by peak areas and significant differences were determined using t-Tests (Table 2) producing a heat map.

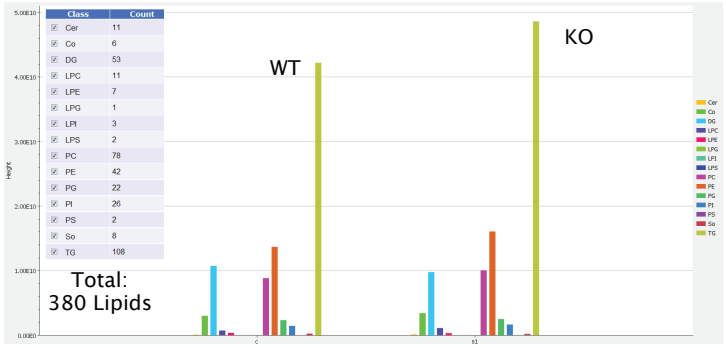
FIGURE 7. Combined Report Results for PG(17:0/17:0) Internal Standard



Sample	Area
3KO	36,510,595
4KO	32,134,023
11WT	37,382,803
12WT	36,640,219
Avg	**35,666,910**
CV	**6.69**

m/z 768.5749, Rt = 15.50 min

Figure 8. Combined Report Results – Total Lipid Profile



Yeast Lipidomics Results

The total number of lipids species identified in yeast WT and KO mitochondria (380) is comparable to the number of lipids quantified (250) by infusion lipidomics[5].

Table 2. Summary of Differences between WT vs. KO Yeast Lipids. Analytes with p-Values < 0.05 for t-Test between WT and KO groups. Fold-change (KO vs. WT) indicated by Red (increase) or Green (decrease)

Class	Compound	RT min	Ratio	p-Value
Cer	Cer(d18:0/16:0)	16.73	2.92	0.006
	Cer(d18:0/16:1)	15.06	0.52	0.008
	Cer(d18:0/18:0)	18.77	2.00	0.037
	Cer(d18:0/28:6)	22.74	104.9	0.011
	Cer(d18:1/28:6)	22.49	5.93	0.002
	Cer(d18:2/18:1)	16.72	2.07	0.049
So	So(d18:0)	3.03	0.28	0.026
	So(d20:0)	4.79	0.08	0.031
	So(d20:1)	4.97	0.20	0.003
PC	PC(10:0/16:0)	10.02	6.06	0.000
	PC(12:0/18:2) 1	12.62	2.29	0.004
	PC(12:0/18:2) 2	12.88	3.50	0.003
	PC(15:0/18:2) 2	14.36	2.04	0.045
	PC(15:1/12:0)	9.58	2.75	0.023
	PC(16:0/12:0) 1	11.79	2.95	0.007
	PC(16:0/12:0) 2	12.29	3.25	0.024
	PC(16:0/17:1) 2	15.71	1.51	0.029
	PC(16:0/22:6)	12.70	0.23	0.015
	PC(16:0e/15:1)	18.37	0.11	0.021
	PC(16:1/12:0) 2	12.40	3.18	0.003
	PC(16:1/13:0)	11.31	1.72	0.003
	PC(16:1/14:0) 2	14.09	2.00	0.035
	PC(16:1/16:1) 1	14.24	1.60	0.002
	PC(16:1/18:2) 1	12.71	1.19	0.043
	PC(16:1/18:3)	11.85	2.47	0.002
	PC(16:1/20:4) 1	12.24	0.40	0.007
	PC(16:1/20:5)	11.12	0.38	0.035
	PC(17:0/16:0e)	20.60	0.14	0.012
	PC(17:0/18:0p)	18.37	0.08	0.023
	PC(18:0/17:1)	17.56	1.56	0.007
	PC(18:0/18:1)	17.23	0.66	0.045
	PC(18:0/18:2)	15.48	0.48	0.006
	PC(18:0/24:2)	21.08	1.85	0.026
	PC(19:0/18:2) 1	16.71	0.45	0.003
	PC(20:0/18:2) 2	17.20	0.39	0.003
	PC(20:0/24:1)	22.76	0.51	0.011
	PC(8:0/18:1) 1	8.48	2.90	0.015
	PC(8:0/18:1) 2	8.80	5.38	0.025

Class	Compound	RT min	Ratio	p-Value
CoQ	CoQ(Q6)	15.30	0.00	0.021
	CoQ(Q7)	18.37	0.15	0.017
	CoQ(Q8)	21.15	1.62	0.033
	CoQ(Q9)	22.40	1.56	0.028
PE	PE(10:0/16:0)	10.51	1.83	0.034
	PE(10:0/17:1)	10.01	1.83	0.040
	PE(10:0/18:0)	12.55	0.11	0.019
	PE(12:0/14:0)	10.51	1.83	0.034
	PE(16:0/12:0)	12.25	1.48	0.022
	PE(16:0/15:1)	13.49	1.29	0.018
	PE(16:0/16:1) 1	14.15	1.14	0.028
	PE(16:1/12:0) 1	10.62	1.83	0.003
	PE(16:1/12:0) 2	10.96	1.39	0.045
	PE(16:1/15:0)	13.49	1.29	0.018
	PE(16:1/16:1) 1	12.89	1.57	0.005
	PE(16:1/16:1) 2	13.18	1.49	0.021
	PE(16:1/18:1)	14.26	1.10	0.023
	PE(17:1/12:0)	11.80	1.54	0.029
	PE(18:0/18:2) 1	15.97	0.25	0.000
	PE(18:1/14:0)	14.15	1.14	0.028
	PE(18:1/18:1)	15.91	0.35	0.003
PG	PG(16:0/17:1)	13.48	1.29	0.003
	PG(16:0/18:1)	13.97	0.95	0.037
	PG(16:0/18:2)	13.08	1.26	0.010
	PG(16:1/18:1) 2	12.85	1.28	0.047
	PG(17:1/17:1)	13.08	1.25	0.010
	PG(17:1/18:1)	13.53	1.08	0.007
	PG(17:1/19:1)	14.63	1.17	0.021
PI	PI(10:0/16:0)	8.56	2.74	0.017
	PI(12:0/14:0)	8.56	2.74	0.017
	PI(15:0/18:1)	12.80	0.57	0.043
	PI(16:1/15:0)	11.23	0.64	0.022
	PI(16:1/17:0)	12.80	0.57	0.043
	PI(16:1/18:2)	10.94	0.59	0.047
PS	PS(16:1/16:1)	10.86	5.35	0.003
	PS(16:1/17:1)	11.94	2.96	0.010

Class	Compound	RT min	Ratio	p-Value
DG	DG(16:0/12:0)	15.45	1.26	0.027
	DG(16:1/15:0)	16.54	0.55	0.027
	DG(16:1/15:1)	14.84	0.26	0.009
	DG(16:1/18:3)	14.81	0.53	0.048
	DG(16:1/24:0)	22.65	0.35	0.012
	DG(18:0/18:1)	21.15	0.45	0.039
	DG(18:1/18:1)	19.54	0.30	0.009
	DG(18:1/18:3)	16.60	0.36	0.018
	DG(26:0/14:0)	23.10	0.54	0.007
	DG(26:0/16:1)	23.12	0.46	0.023
	DG(26:0/18:1)	23.50	0.33	0.005
	DG(26:1/16:1)	22.60	0.06	0.005
	DG(26:1/18:1)	23.05	0.16	0.003
	DG(28:0/18:1)	23.86	0.13	0.000
TG	TG(10:0/12:0/16:0)	22.25	2.05	0.041
	TG(10:0/14:0/16:0)	22.83	2.52	0.036
	TG(10:0/14:0/16:1)	22.28	3.61	0.042
	TG(10:0/16:0/16:0)	23.28	3.87	0.005
	TG(10:0/16:0/16:1)	22.84	3.91	0.020
	TG(10:0/16:0/17:1)	23.16	2.44	0.041
	TG(10:0/16:1/16:1)	22.30	3.54	0.032
	TG(12:0/12:0/14:0)	22.25	2.05	0.041
	TG(16:0/12:0/16:0)	23.68	2.90	0.002
	TG(16:0/12:0/16:1)	23.26	2.12	0.013
	TG(16:0/12:0/24:0)	25.39	2.09	0.032
	TG(16:0/14:0/15:0)	23.86	1.37	0.027
	TG(16:0/14:0/16:0)	24.08	1.98	0.004
	TG(16:0/14:0/16:1)	23.67	1.36	0.029
	TG(16:0/15:0/16:0)	24.26	1.27	0.022
	TG(16:0/16:0/16:1)	24.07	1.61	0.025
	TG(16:0/16:0/17:0)	24.69	1.21	0.037
	TG(16:0/16:1/16:1)	23.67	1.22	0.046
	TG(16:1/12:0/15:0)	23.16	2.44	0.041
	TG(16:1/18:1/22:1)	24.83	0.62	0.046
	TG(17:1/18:1/18:1)	24.31	0.52	0.017
	TG(18:0/16:0/18:0)	25.39	2.09	0.032
	TG(18:1/18:1/18:1)	24.41	0.50	0.007
	TG(18:1/18:1/18:3)	23.80	0.54	0.019

Conclusions

- Lipid Search provides an automated workflow for high quality Orbitrap LC-MS/MS lipidomics data and enables reliable and comprehensive lipid identification.
- Lipid Search identified 380 lipids in MS^2 spectra from single Orbitrap scans and 112 significant changes were found in the WT and KO yeast phenotypes.
- MS^2 searching using Lipid Search is a more efficient approach than component finding and MW search for lipid identification.
- Lipid Search reliably identifies product ion mixtures from two or more lipids.
- Data analysis time was dramatically reduced from hours to a few minutes.

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