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

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#### **Overview**

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

**Methods:** Lipids in yeast mitochondria were analyzed by high resolution LC-MS and MS/MS. Lipid Search® software, an MS<sup>2</sup> 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<sup>2</sup> 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<sup>2</sup> 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<sup>n</sup> 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<sup>3</sup> and was commercialized by MKI (Tokyo, JP) as described recently<sup>4</sup>.

#### **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  $\mu$ L of IPA for 10 min at 4 °C. After centrifugation, supernatants were combined and vacuum dried. Samples were dissolved in 250  $\mu$ L of 65:35:5 Acetonitrile, Isopropanol, Water with 5  $\mu$ g/mL17:0 PG.

#### Liquid Chromatography-Mass Spectrometry (LC-MS)

Thermo Scientific<sup>TM</sup> Accela<sup>TM</sup> 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<sup>1</sup> is described in S. Bird, et al., <u>Anal. Chem.</u> **2011**, *83*, 940-949, 6648-6657. A Thermo Scientific<sup>TM</sup> Q Exactive<sup>TM</sup> 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<sup>™</sup> SIEVE<sup>™</sup> software and Lipidomics - Lipid Search software (MKI).

Table 1. Lipi	d Complexity	from tl	he LIPID	MAPS	Structure	Databa	ase
$(LMSD)^2$			" <b>C</b> L	# C. I.	<b>C</b> 1		_

"		Lipid Category	# Class	# Sub-Class	# Lipids
	FA	Fatty acyls	14	36	5,787
	GL	Glycerolipids	6	19	7,568
	GP	Glycerophospholipid	21	120	8,001
		S			
	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
	РК	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 accuratemass 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

0.06						
0.04						
0.02						
	•					
0						
		•				
0.02						
0.04					•	
0.06	15 -0	-0	.05	0.	05 0	.1 0.

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



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

1) Peak Detection. Read raw files, MS<sup>n</sup> 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	🗐 Туре	🗐 ЕхрТуре	Process	Result	🗐 🛛 Regist	🗐 Update
V	2013-05-25-21-54-24 🛈 🗋	4K0Post_1.raw	Product	LC	PIQ 🕹	8239 257	2013/05/25 21:54:24	2013/05/25 22:01:01
V	2013-05-25-21-54-24 🛈 🗎	3KOPost_1.raw	Product	LC	PIQ 🕲	8232 261	2013/05/25 21:54:24	2013/05/25 21:59:38
V	2013-05-25-21-54-24 🛈 🗋	12WTPost_1.raw	Product	LC	PIQ 🕹	8250 278	2013/05/25 21:54:24	2013/05/25 21:58:12
V	2013-05-25-21-54-24 🛈 🗎	11WTPost_1.raw	Product	LC	PIQ 🙂	<mark>®</mark> 245 <b>№</b> 269	2013/05/25 21:54:24	2013/05/25 21:56:58

#### FIGURE 5b. Alignment Results for Yeast Lipids

E.	Name 📑 T	ype 🗐	ExpType	Process		Result		Regist		Update
Yeast Lipids 🛈	Prod	uct	LC	м 🙂	<del>©</del> 38	80 35760	2013/05	5/25 22:06:18	2013/0	5/25 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<sup>2</sup> 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)



#### 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



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<sup>5</sup>.

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	Cla	ass	Compound	RT min	Ratio	p-Value	Clas	s Compound	RT min	Ratio	p-Value
	Cer(d18:0/16:0)	16.73	2.92	0.006			Co(Q6)	15.30	0.00	0.021		DG(16:0/12:0)	15.45	1.20	0.027
	Cer(d18:0/16:1)	15.06	0.52	0.008	6		Co(Q7)	18.37	0.15	0.017		DG(16:1/15:0)	16.54	0.55	0.027
C	Cer(d18:0/18:0)	18.77	2.00	0.037		2	Co(Q8)	21.15	1.62	0.033		DG(16:1/15:1)	14.84	0.26	0.009
Cer	Cer(d18:0/28:6)	22.74	104.9	0.011			Co(Q9)	22.40	1.50	0.028		DG(16:1/18:3)	14.81	0.53	0.048
	Cer(d18:1/28:6)	22.49		0.002			PE(10:0/16:0)	10.51	1.83	0.034		DG(16:1/24:0)	22.65	0.35	0.012
	Cer(d18:2/18:1)	16.72		0.049			PE(10:0/17:1)	10.01	1.83	0.040		DG(18:0/18:1)	21.15	0.45	0.039
	So(d18:0)	3.03	0.28	0.026			PE(10:0/18:0)	12.55	0.11	0.019	DG	DG(18:1/18:1)	19.54	0.30	0.009
So	So(d20:0)	4.79	0.08	0.031			PE(12:0/14:0)	10.51	1.83	0.034	00	DG(18:1/18:3)	16.60	0.36	0.018
	So(d20:1)	4.97	0.20	0.003		PE	PE(16:0/12:0)	12.25	1.48	0.022		DG(26:0/14:0)	23.10	0.54	0.007
	PC(10:0/16:0)	10.02	6.06	0.000			PE(16:0/15:1)	13.49	1.29	0.018		DG(26:0/16:1)	23.12	0.46	0.023
	PC(12:0/18:2) 1	12.62	2.29	0.004			PE(16:0/16:1) 1	14.15	1.14	0.028		DG(26:0/18:1)	23.50	0.33	0.005
	PC(12:0/18:2) 2	12.88	3.50	0.003			PE(16:1/12:0) 1	10.62	1.83	0.003		DG(26:1/16:1)	22.60	0.06	0.005
	PC(15:0/18:2) 2	14.36	2.04	0.045	P		PE(16:1/12:0) 2	10.96	1.39	0.045		DG(26:1/18:1)	23.05	0.16	0.003
	PC(15:1/12:0)	9.58	2.75	0.023			PE(16:1/15:0)	13.49	1.29	0.018		DG(28:0/18:1)	23.86	0.13	0.000
	PC(16:0/12:0) 1	11.79	2.95	0.007			PE(16:1/16:1) 1	12.89	1.57	0.005		TG(10:0/12:0/16:0)	22.25	2.05	0.041
	PC(16:0/12:0) 2	12.29	3.25	0.024			PE(16:1/16:1) 2	13.18	1.49	0.021		TG(10:0/14:0/16:0)	22.83	2.52	0.036
	PC(16:0/17:1) 2	15.71	1.51	0.029			PE(16:1/18:1)	14.26	1.10	0.023		TG(10:0/14:0/16:1)	22.28	3.61	0.042
	PC(16:0/22:6)	12.70	0.23	0.015			PE(17:1/12:0)	11.80	1.54	0.029		TG(10:0/16:0/16:0)	23.28	3.87	0.005
	PC(16:0e/15:1)	18.37	0.11	0.021			PE(18:0/18:2) 1	15.97	0.25	0.000		TG(10:0/16:0/16:1)	22.84	3.91	0.020
	PC(16:1/12:0) 2	12.40	3.18	0.003			PE(18:1/14:0)	14.15	1.14	0.028		TG(10:0/16:0/17:1)	23.16	2.44	0.041
	PC(16:1/13:0)	11.31		0.003		_	PE(18:1/18:1)	15.91	0.35	0.003		TG(10:0/16:1/16:1)	22.30	3.54	0.032
	PC(16:1/14:0) 2	14.09	2.00	0.035			PG(16:0/17:1)	13.48	1.29	0.003		TG(12:0/12:0/14:0)	22.25	2.05	0.041
	PC(16:1/16:1) 1	14.24	1.60	0.002			PG(16:0/18:1)	13.97	0.95	0.037		TG(16:0/12:0/16:0)	23.68	2.90	0.002
PC	PC(16:1/18:2) 1	12.71		0.043			PG(16:0/18:2)	13.08	1.26	0.010		TG(16:0/12:0/16:1)	23.26	2.12	0.013
	PC(16:1/18:3)	11.85	2.47	0.002	P	G	PG(16:1/18:1) 2	12.85	1.28	0.047		TG(16:0/12:0/24:0)	25.39	2.09	0.032
	PC(16:1/20:4) 1	12.24	0.40	0.007			PG(17:1/17:1)	13.08	1.26	0.010	TG	TG(16:0/14:0/15:0)	23.86	1.37	0.027
	PC(16:1/20:5)	11.12	0.38	0.035			PG(17:1/18:1)	13.53	1.08	0.007	10	TG(16:0/14:0/16:0)	24.08	1.98	0.004
	PC(17:0/16:0e)	20.60	0.14	0.012			PG(17:1/19:1)	14.63	1.17	0.021		TG(16:0/14:0/16:1)	23.67	1.36	0.029
	PC(17:0/18:0p)	18.37	0.08	0.023			PI(10:0/16:0)	8.56	2.74	0.017		TG(16:0/15:0/16:0)	24.26	1.27	0.022
	PC(18:0/17:1)	17.56	1.56	0.007			PI(12:0/14:0)	8.56	2.74	0.017		TG(16:0/16:0/16:1)	24.07	1.61	0.025
	PC(18:0/18:1)	17.23	0.66	0.045	P		PI(15:0/18:1)	12.80	0.57	0.043		TG(16:0/16:0/17:0)	24.69	1.21	0.037
	PC(18:0/18:2)	15.48	0.48	0.006		•	PI(16:1/15:0)	11.23	0.64	0.022		TG(16:0/16:1/16:1)	23.67	1.22	0.046
	PC(18:0/24:2)	21.08	1.85	0.026			PI(16:1/17:0)	12.80	0.57	0.043		TG(16:1/12:0/15:0)	23.16	2.44	0.041
	PC(19:0/18:2) 1	16.71	0.45	0.003			PI(16:1/18:2)	10.94	0.59	0.047		TG(16:1/18:1/22:1)	24.83	0.62	0.046
	PC(20:0/18:2) 2	17.20	0.39	0.003	а	<u>،</u>	PS(16:1/16:1)	10.86	5.35	0.003		TG(17:1/18:1/18:1)	24.31	0.52	0.017
	PC(20:0/24:1)	22.76	0.51	0.011		~	PS(16:1/17:1)	11.94	2.96	0.010		TG(18:0/16:0/18:0)	25.39	2.09	0.032
	PC(8:0/18:1) 1	8.48	2.90	0.015								TG(18:1/18:1/18:1)	24.41	0.50	0.007
	PC(8:0/18:1) 2	8.80	5.38	0.025								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<sup>2</sup> spectra from <u>single</u> Orbitrap scans and 112 significant changes were found in the WT and KO yeast phenotypes.
- MS<sup>2</sup> 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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