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High Resolution Orbitrap Mass Spectrometry for the Analysis of Deuterium-Labeled Lipids in *E. Coli*

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ABSTRACT

Employ ultra-high resolution at the MS/MS level for resolving deuterium from ^{13}C peak in deuterium labeling experiments

Utilize Thermo Scientific™ LipidSearch™ for lipid identification in *E.coli* lipid extracts

Chromatographic separation was performed with reverse-phase chromatography on a C30 column

Mass spectrometric analysis was performed on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer operated at a resolution of 120,000 to 500,000 (FWHM at *m/z* 200) for the MS/MS scans

Ultra-high resolution at the MS/MS scans allowed baseline separation of deuterium and ^{13}C isotopes in deuterium labeled lipids extracted from *E.coli*

INTRODUCTION

Labeling experiments are often used to estimate de novo biosynthesis rates in lipids. In D_2O-labeling experiments, the quantitation of absolute deuterium abundance is complicated by the natural abundance of ^{13}C, and, separation of deuterium from ^{13}C isotopes requires very high mass resolution. Here, we explore the feasibility of resolving deuterium from ^{13}C isotopes, in deuterium-labeled lipids extracted from *E.coli*, by utilizing ultra-high resolution. We have coupled reverse-phase chromatography, based on a C30 column separation, with the Orbitrap Fusion Lumos Tribrid mass spectrometer operated at a resolution of 120,000 to 500,000 (FWHM at *m/z* 200) for the MS/MS scans. Our data demonstrates that ultra-high resolution allows for deuterium and ^{13}C isotopes to be baseline resolved at the MS/MS level.

MATERIALS AND METHODS

Sample Preparation

E.coli K12 were grown in glucose M9 minimal medium with deuterium content in water varying from 0.015% to 4 % D_2O in separate cultures. Intact polar lipids were extracted from cell pellets with methyl tert-butyl ether.[1,2] Phosphatidylethanolamine (PE) (17:0/17:0), Phosphatidylglycerol (PG) (17:0/17:0) and 14:1(3)-15:1 cardiolipin were added as internal standards (Avanti Polar Lipids). Lipid extracts were dried under N_2, stored at -20 ° C and dissolved in 9:1 methanol:dichloromethane for LC-MS analysis.

Liquid Chromatography

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ UHPLC system using a Thermo Scientific™ Accucore™ C30 column (150 × 3.0 mm, 2.6µm). The LC-MS/MS analysis time was 31 min, including 6 min equilibration time. Mobile phases were (A) 60:40 acetonitrile:water with 10mM ammonium formate and 0.1% FA and (B) 90:10 isopropanol:acetonitrile with 10mM ammonium formate and 0.1% FA .The flow rate was 350 µl/min and the column temperature was set to 45 ° C. Injection volume was 3 µl.

Mass Spectrometry

Mass spectrometric analysis was performed on an Orbitrap Fusion Lumos mass spectrometer. For untargeted analysis, the Orbitrap Fusion Lumos MS was operated in a full MS scan mode (at a resolution of 120,000) followed by ddMS^2 (at a resolution of 30,000). The AGC target value was set at 4e5 and 5e4 for the MS and MS/MS scans, respectively. The maximum injection time was 50 ms for the full MS scan and 80 ms for the MS/MS scans. HCD was performed with a stepped collision energy of 30 ± 10 (%) for negative ion mode and 27 ± 3 (%) for positive ion mode.

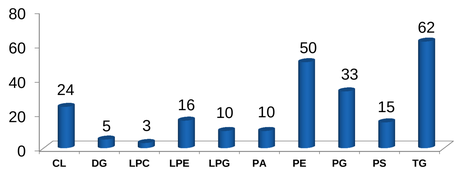
For deuterium-labeled samples, a targeted MS^2 approach was utilized with LC-MS/MS scans performed at 120,000, 240,000 or 500,000 resolution settings. The M+1, M+2 and M+3 isotopes of selected deuterium-labeled species were included in a targeted list and fragmented with HCD. The quadrupole isolation window was set at 1.0 Da to ensure single isotope isolation of precursor ions.

Data Analysis

Lipid identification was performed with the Thermo Scientific™ LipidSearch™ 4.1 SP1 software. The precursor and product ion mass tolerance was set to 3 ppm and 5 ppm, respectively.

RESULTS

Figure 1. Identified lipids in *E.coli*



Search results from three individual positive and negative ion mode raw files were aligned within a retention time window of ±0.12 min. The data was merged for each annotated lipid, following by data filtering to reduce false positive identifications.

Table 1. Elemental compositions and theoretical m/z values of the M, M+1, M+2 and M+3 isotopologues of the [M - H]$^-$ ions of phosphatidylglycerol PG (32:1)

Isotope	Precursor Ion	Precursor Ion Elemental Composition	Precursor Ion m/z
M	PG (32:1)	$C_{38}H_{73}O_{10}P_1$	719.4869
M+1	PG (32:1), D_1	$C_{38}H_{72}D_1O_{10}P_1$	720.4931
	PG (32:1), $^{13}C_1$	$^{13}C_1C_{37}H_{73}O_{10}P_1$	720.4902
M+2	PG (32:1), D_2	$C_{38}H_{71}D_2O_{10}P_1$	721.4994
	PG (32:1), $^{13}C_2$	$^{13}C_2C_{36}H_{73}O_{10}P_1$	721.4936
	PG (32:1), $^{13}C_1/D_1$	$^{13}C_1C_{37}H_{72}D_1O_{10}P_1$	721.4965
M+3	PG (32:1), D_3	$C_{38}H_{70}D_3O_{10}P_1$	722.5057
	PG (32:1), $^{13}C_3$	$^{13}C_3C_{35}H_{73}O_{10}P_1$	722.4969
	PG (32:1), $^{13}C_1/D_2$	$^{13}C_1C_{37}H_{71}D_2O_{10}P_1$	722.5028
	PG (32:1), $^{13}C_2/D_1$	$^{13}C_2C_{36}H_{72}D_1O_{10}P_1$	722.4998

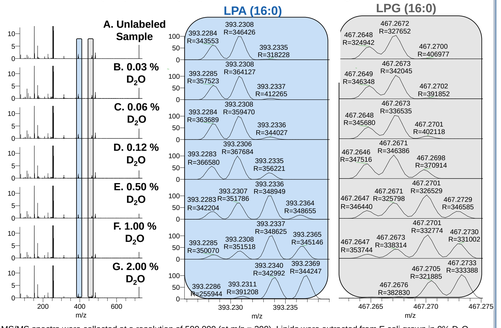
Table 2. Elemental compositions and theoretical m/z values of the isotopologues from LPA (16:0) product ion generated from fragmentation of the M+2 isotopologue of deuterated phosphatidylglycerol PG (32:1). MS/MS spectra are shown in Figure 2. The theoretical resolution for resolving the isotopologues is indicated in the table.

Isotope	Product Ion Elemental Composition	Product Ion m/z	Required Resolution
M+2	$C_{19}H_{36}O_6{}^{18}O_1P_1$	393.2286	314,500
	$^{13}C_2C_{17}H_{36}O_6P_1$	393.2311	
	$^{13}C_1C_{18}H_{35}D_1O_6P_1$	393.2340	262,100
	$C_{19}H_{34}D_2O_6P_1$	393.2370	

Table 3. Elemental compositions and theoretical m/z values of the isotopologues from the LPG (16:0) product ion generated from fragmentation of the M+2 isotopologue of deuterated phosphatidylglycerol PG (32:1). MS/MS spectra are shown in Figure 2. The theoretical resolution for resolving the isotopologues is indicated in the table.

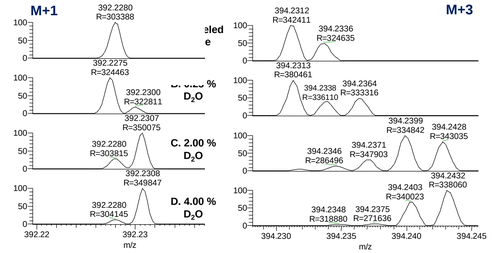
Isotope	Product Ion Elemental Composition	Product Ion m/z	Required Resolution
M+2	$^{13}C_2C_{20}H_{42}O_9P$	467.2654	373,800
	$^{13}C_2C_{20}H_{42}O_8P_1$	467.2679	
	$^{13}C_1C_{21}H_{41}D_1O_8P_1$	467.2708	322,200
	$C_{22}H_{40}D_2O_8P_1$	467.2737	

Figure 2. MS/MS spectra of the M+2 isotope of phosphatidylglycerol PG (32:1) [M - H]$^-$ anions. Product ions LPA (16:0) and LPG (16:0) are shown in the zoom-in regions.



MS/MS spectra were collected at a resolution of 500,000 (at m/z = 200). Lipids were extracted from E.coli grown in 0% D_2O (A), 0.03% D_2O (B), 0.06% D_2O (C), 0.12% D_2O (D), 0.50% D_2O (E), 1.00 % D_2O (F) and 2.00% D_2O (G). Assignments of isotopologues are shown in Tables 2 and 3.

Figure 3. Zoom-in region of the LPA (16:0) product ion generated from fragmentation of the M+1 and M+3 isotopologue of phosphatidylglycerol PG (32:1) [M - H]$^-$ anions.

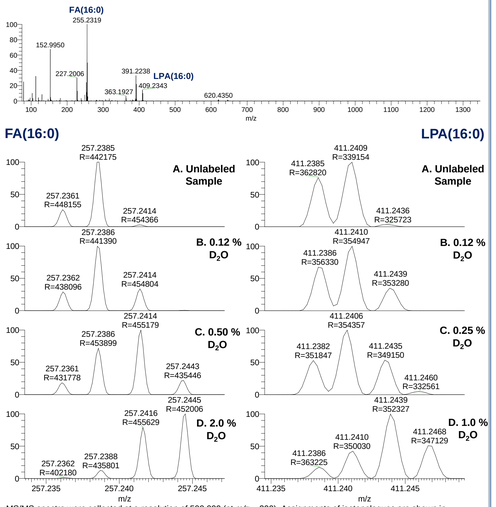


MS/MS spectra were collected at a resolution of 500,000 (at m/z = 200). Lipids were extracted from E.coli grown in 0% D_2O (A), 0.25% D_2O (B), 2.00% D_2O (C) and 4.00% D_2O (D). Assignments of isotopologues are shown in Table 4.

Table 4. Elemental compositions and theoretical m/z values of the isotopologues from LPA (16:0) product ion generated from fragmentation of the M+1 and M+3 isotopologues of deuterated phosphatidylglycerol PG (32:1). MS/MS spectra are shown in Figure 3.

Isotope	Product Ion Elemental Composition	Product Ion m/z	Required Resolution
M+1	$^{13}C_1C_{18}H_{36}O_6P_1$	392.2278	270,500
	$C_{19}H_{35}D_1O_6P_1$	392.2307	
M+3	$^{13}C_1C_{18}H_{36}O_5{}^{18}O_1P_1$	394.2320	315,400
	$^{13}C_3C_{16}H_{36}O_6P_1$	394.2345	
	$^{13}C_2C_{18}H_{35}D_1O_6P_1$	394.2374	271,900
	$^{13}C_1C_{18}H_{34}D_2O_6P_1$	394.2403	
	$C_{19}H_{33}D_3O_6P_1$	394.2432	

Figure 4. MS/MS spectrum of the M+2 isotope of unlabeled cardiolipin CL(62:1) [M - H]$^-$ anions and zoom-in regions of the FA (16:0) and LPA (16:0) product ions in unlabeled and labeled samples.



MS/MS spectra were collected at a resolution of 500,000 (at *m/z* = 200). Assignments of isotopologues are shown in Tables 5 and 6.

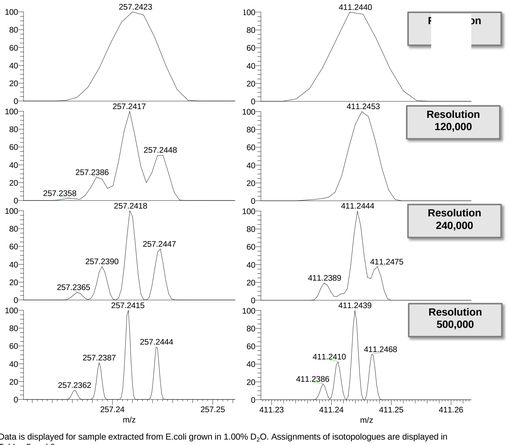
Table 5. Elemental compositions and theoretical m/z values of the isotopologues from the 16:0 fatty acid product ion generated from fragmentation of the M+2 isotope of deuterated cardiolipin CL (62:1). MS/MS spectra are shown in Figure 4. The theoretical resolution for resolving the isotopologues is indicated in the table.

Product Ion Elemental Composition	Product Ion m/z	Required Resolution
$C_{16}H_{31}O_1{}^{18}O_1$	257.2361	205,800
$^{13}C_2C_{14}H_{31}O_2$	257.2386	
$^{13}C_1C_{15}H_{30}D_1O_2$	257.2415	177,410
$C_{16}H_{29}D_2O_2$	257.2444	

Table 6. Elemental compositions and theoretical m/z values of the isotopologues from the LPA (16:0) product ion generated from fragmentation of the M+2 isotope of deuterated cardiolipin CL (62:1). MS/MS spectra are shown in Figure 4. The theoretical resolution for resolving the isotopologues is indicated in the table.

Product Ion Elemental Composition	Product Ion m/z	Required Resolution
$C_{19}H_{38}O_5{}^{18}O_1P_1$	411.2392	329,000
$^{13}C_2C_{17}H_{38}O_7P_1$	411.2417	
$^{13}C_1C_{18}H_{37}D_1O_7P_1$	411.2446	283,600
$C_{19}H_{36}D_2O_7P_1$	411.2475	

Figure 5. Effect of resolution settings on resolving deuterium and ^{13}C isotopes. Examples are shown for the product ions displayed in Figure 4.



Data is displayed for sample extracted from E.coli grown in 1.00% D_2O. Assignments of isotopologues are displayed in Tables 5 and 6.

CONCLUSIONS

- Lipid identification in *E.coli* extracts was performed with the LipidSearch software which provides an easy and automated solution for data analysis
- The ultra high resolution of the Orbitrap Fusion Lumos MS is vital for stable isotope labeling experiments as it allows separation of isotopologues and, therefore, accurate analysis of labeled species
- Our data clearly demonstrates that deuterium and ^{13}C isotopes can be successfully baseline resolved on the Orbitrap Fusion Lumos MS by employing ultra-high resolution at the MS/MS level

REFERENCES

1. Matyash, V., G. Liebisch, T. V. Kurzchalia, A. Shevchenko, and D. Schwudke. 2008. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res.* **49** : 1137–1146.
2. Neubauer C, Sessions AL, Booth IR, Bowen BP, Kopf SH, Newman DK, Dalleska NF. D_2O-labeling lipidomics enables a robust ratiometric readout of microbial lipid biosynthesis. *Submitted*

TRADEMARKS/LICENSING