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APPLICATION NOTE 54

Targeted MRM Screening of U-^{13}C Lipid Yeast Extracts for Robust Lipidomics Applications

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Highlights

- Crude lipid yeast extracts (U-^{13}C and unlabeled) comprise 100s of fatty acids and lipids
- Extracts afford broad application potential (e.g., quality control, credentialing, quantification)
- Targeted methods, results, and applications for the U-^{13}C lipid yeast extract are described
- Methodologies are rapid (8-12 min) and simple to execute

Introduction

Fatty acids (FAs) and lipids are important biological compounds that are essential to the regulation and control of cellular functions (e.g., membrane structure, signal transduction, apoptosis) and metabolic pathways (e.g., fatty acid oxidation, ketone body synthesis, lipolysis).[1] These biomolecules are also functionally important to an organism's energy production and storage. Qualitative/quantitative analysis of these molecular species is a continual aim of lipidomics research. A driving factor is to better understand their underlying pathophysiology, as well as to identify new biomarkers (e.g., cardiovascular disease, diabetes, cancer) or diagnose existing ones toward improved precision/personalized medicine.[2-4]

Regardless of the research objective(s), a growing number of lipidomics methods utilize hyphenated analytical systems comprising a separation dimension (e.g., reversed-phase liquid chromatography, RPLC; hydrophilic interaction liquid chromatography, HILIC) and a mass spectrometer (operated in different acquisition modes e.g., multiple reaction monitoring or MRM).[5] Critical to the qualitative/quantitative analysis is the incorporation of stable isotope-labeled standards. Among the benefits, their use helps determine an assay's effectiveness (i.e., process efficiency), improve measurement precision, and enhance quantitative accuracy.

In this work, we qualified a condensed panel of stable isotope-labeled FAs/lipids in U-^{13}C crude lipid yeast extracts (CIL catalog no. L-ISO1) on robust, high-throughput, analytical platforms (i.e., RPLC- and HILIC-MRM/MS). The verification results of this extract analyses are presented here along with a discussion of its potential use in different types of MS lipidomics applications.

Materials and Methods

Chemicals and Reagents

All reagents were of the highest available grade, and solvents were LC-MS grade. The test samples were U-^{13}C lipid yeast extracts (~4 × 10^8 *Pichia pastoris* cells, ~3 mg dry weight) that were supplied by CIL (catalog no. **L-ISO1**) and originally manufactured by ISOtopic Solutions (Vienna, Austria). While not the focus of this article, unlabeled lipid yeast extracts (CIL catalog no. **L-ISO1-UNL**) were similarly supplied and analyzed. The U-^{13}C extracts have 99% chemical/isotopic purity and were stored in their dried-down form at -80°C (light protected) until use.

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Solution Preparation

The U-^{13}C lipid yeast extracts (n = 3) were reconstituted in 1 mL isopropanol (IPA) before a high-speed vortex and brief centrifuge. Note that alternate solvents (e.g., methyl *tert*-butyl ether) and volumes (e.g., 200 µL to 2 mL) for dissolution are possible. The solutions were diluted further in IPA to varying degrees yielding three concentration levels of standard mixes for each extract replicate. These were stored at 4°C until analysis.

Chromatographic Separation Methods

Analyte separations were performed by RPLC or HILIC using the Waters columns listed below:

- RPLC: ACQUITY™ Premier CSH™ C18 (100 × 2.1 mm, 1.7 µm, 130 Å)
- HILIC: ACQUITY Premier UPLC BEH Amide (100 × 2.1 mm, 1.7 µm, 130 Å)

These were contained within an ACQUITY Ultra Performance LC™ I-Class Plus system (Waters). The column was maintained at 55°C (for RPLC) or 45°C (for HILIC), while the autosampler was at 8°C. Following sample injection (2 µL for ESI+, 4 µL for ESI-), separations occurred over a solvent gradient (see **Tables 1** and **2**) at an analytical flow rate (0.4 mL/min for RPLC and 0.6 mL/min for HILIC).

Table 1. RPLC and HILIC gradients.

RPLC		HILIC	
Time (min)	% Eluent B	Time (min)	% Eluent B
0	50	0	0.1
0.5	53	2	20
4	55	5	80
7	65	5.1	0.1
7.5	80	8	0.1
10	99		
11	99		
12	50		

Table 2. Chromatographic mobile phase compositions.

RPLC	
Eluent A:	acetonitrile:water (at 3:2 *v/v*) in 10 mM ammonium acetate
Eluent B:	isopropanol: acetonitrile (at 9:1 *v/v*) in 10 M ammonium acetate
HILIC	
Eluent A:	acetonitrile:water (at 9.5:.5 *v/v*) in 10 mM ammonium acetate
Eluent B:	acetonitrile:water (at 1:1 *v/v*) in 10 mM ammonium acetate

MS/MS Parameters and Conditions

The chromatographic column (RPLC or HILIC) was coupled to a Xevo™ TQ-XS mass spectrometer (Waters) via a UniSpray ion source. This source was operated in the positive and negative ionization mode, with data acquired by scheduled MRM. The general MS and specific MRM settings are outlined in **Tables 3** and **4**, respectively. Ultra-high-purity nitrogen served as the carrier gas throughout. Note that other instruments (e.g., Xevo G2-XS QTof, SYNAPT™ XS) and operative modes (e.g., data-dependent acquisition, parallel reaction monitoring) are possible. The consideration to bear is that the acquisition parameters should first be optimized. In practice, this should be empirically measured using a diluted yeast extract (U-^{13}C or unlabeled) as the MS infusion solution.

Table 3. General acquisition parameters for MRM analysis of the diluted extract samples on a Xevo TQ-XS.

Parameter	Value
Capillary voltage	2.8 kV (ESI+) and 1.9 kV (ESI-)
Source temperature	120°C
Desolvation temperature	500°C
Desolvation gas flow	1000 L/h
Cone gas flow	150 L/h
Nebulizer gas	7.0 bar
Collision gas flow	0.13 L/h
Ion guide offset 1	3.0 V
Ion guide offset 2	0.3 V
Q1/Q3 resolution	unit/unit

Table 4. Specific acquisition parameters for scheduled MRM analysis of a condensed set of FAs/lipids measured by targeted MRM/MS on a Xevo TQ-XS. The ions tabulated are U-^{13}C (unlabeled additionally shown for reference) with negative ESI highlighted blue. Abbreviations: CE – collision energy; CV – cone voltage.

Analyte (Fatty Acid Level)	Adduct	U-^{13}C		Unlabeled		CE (V)	CV (V)
		Precursor *m/z*	Product *m/z*	Precursor *m/z*	Product *m/z*		
AC 16:1	M+H	423.44	88.00	400.36	84.99	28	48
Cer d34:1 (d18:1/16:0)	M+H	572.61	282.40	538.50	264.30	30	20
Cer d36:1 (d18:1/18:0)	M+H	602.72	282.40	566.60	264.30	30	20
CL 72:6	M-H	1533.27	1533.27	1452.00	1452.00	10	20
DG 34:1 (16:0/18:1)	$M+NH_4$	649.72	332.30	612.60	313.30	20	50
DG 36:3 (18:1/18:2)	$M+NH_4$	675.73	355.40	636.60	337.30	20	50
FA 18:2	M-H	297.26	297.26	279.20	279.20	10	10
HexCer t36:2 (t18:1/18:1)	M+H	784.72	298.40	742.58	280.30	30	50
LPC 16:1	M+H	518.38	189.10	494.30	184.10	30	10
LPC 18:1	M+H	548.49	189.10	522.40	184.10	30	10
LPE 18:2	M+H	501.38	355.40	478.30	337.30	20	30
LPE 18:3	M+H	499.38	353.40	476.30	335.30	20	30
PA 34:1 (16:0/18:1)	M-H	710.62	271.20	673.50	255.30	40	20
PA 36:1 (18:0/18:1)	M-H	740.63	299.20	701.50	281.30	40	20
PC 34:1 (16:0/18:1)	M+H	802.69	189.10	760.55	184.10	25	40
PC 36:5 (18:2/18:3)	M+H	824.70	189.10	780.55	184.10	25	40
PE 34:1 (16:0/18:1)	M-H	755.63	299.20	716.50	281.30	31	50
PE 36:4 (18:1/18:3)	M-H	779.64	299.20	738.50	277.30	31	50
PG 34:1 (16:0/18:1)	M-H	787.63	299.20	747.50	281.30	40	30
PG 34:2 (16:1/18:1)	M-H	785.63	269.20	745.50	253.30	40	30
PI 34:2 (16:0/18:2)	M-H	876.64	297.30	833.50	279.30	50	50
PI 36:1 (18:0/18:1)	M-H	908.75	301.30	863.60	283.30	50	50
PS 36:1 (18:0/18:1)	M-H	830.64	301.30	788.50	283.30	42	60
PS 36:2 (18:1/18:1)	M-H	828.64	299.20	786.50	281.30	42	60
TG 50:1 (16:0/16:0/18:1)	$M+NH_4$	903.97	586.60	850.79	551.50	20	35
TG 54:1 (18:0/18:1/18:0)	$M+NH_4$	963.74	644.70	906.55	605.40	20	35

Data Analysis

Acquisition and processing were performed with TargetLynx™ software (Ver. 4.2; Waters). Data visualization was conducted in Skyline (**https://skyline.ms/skyline.url**) where peak selections and integrations were validated manually in the absence of peak smoothing.

Results and Discussion

Through modification of the Neubauer et al.'s yeast production method,[6] U-^{13}C and unlabeled extracts of FAs/lipids were produced. **Table 5** lists the identities of an example set of high abundant FAs/lipids in the lipid yeast extracts.

Table 5. Examples of routinely identified FAs and lipids measured in the lipid yeast extracts from QC evaluations. All FAs and lipids in the U-^{13}C extracts were verified by comparison to ^{12}C standards and/or HRAM-MS data in combination with their fragmentation patterns. **Note**: This list is not finite, as alternate FAs/lipids (e.g., acylcarnitine C4-DC and C5-DC, lysophosphatidylethanolamine or LPE 20:5, monoglyceride or MG 16:1 and 18:0) have been observed.

Category	Class	Examples (Fatty Acid Level)		
Glycerolipids	Diglyceride (DG)	34:1 (16:0/18:1)	34:2 (16:1/18:1)	34:3 (16:1/18:2)
		34:4 (16:2/18:2)	36:1 (18:0/18:1)	36:2 (18:0/18:2)
		36:3 (18:1/18:2)	36:4 (18:1/18:3)	36:5 (18:2/18:3)
	Triglyceride (TG)	50:1 (16:0/16:0/18:1)	50:2 (16:0/18:2/16:0)	50:3 (16:1/16:1/18:1)
		50:4 (16:1/16:1/18:2)	50:5 (16:1/16:1/18:3)	52:1 (18:0/16:0/18:1)
		52:2 (16:0/18:1/18:1)	52:3 (16:1/18:1/18:1)	52:4 (18:2/18:2/16:0)
		52:5 (16:1/18:2/18:2)	52:6 (16:1/18:2/18:3)	52:7 (18:3/18:3/16:1)
		54:1 (18:0/18:1/18:0)	54:2 (18:0/18:1/18:1)	54:3 (18:1/18:1/18:1)
		54:4 (18:1/18:1/18:2)	54:5 (18:1/18:2/18:2)	54:6 (18:2/18:2/18:2)
		54:7 (18:1/18:3/18:3)	54:8 (18:3/18:2/18:3)	54:9 (18:3/18:3/18:3)
Glycerophospholipids	Phosphatidic acid (PA)	34:1 (16:0/18:1)	34:2 (16:0/18:2)	34:3 (16:0/18:3)
		36:1 (18:0/18:1)	36:2 (18:0/18:2)	36:3 (18:1/18:2)
	Phosphatidylcholine (PC)	34:1 (16:0/18:1)	34:2 (16:0/18:2)	34:3 (16:0/18:3)
		34:4 (16:1/18:3)	36:2 (18:0/18:2)	36:3 (18:1/18:2)
		36:4 (18:1/18:3)	36:5 (18:2/18:3)	36:6 (18:3/18:3)
	Phosphatidylethanolamine (PE)	34:1 (16:0/18:1)	34:2 (16:0/18:2)	34:3 (16:0/18:3)
		36:1 (18:0/18:1)	36:2 (18:0/18:2)	36:3 (18:1/18:2)
		36:4 (18:1/18:3)	36:5 (18:2/18:3)	36:6 (18:3/18:3)
	Phosphatidylglycerol (PG)	34:1 (16:0/18:1)	34:2 (16:1/18:1)	
	Phosphatidylinositol (PI)	34:1 (16:0/18:1)	34:2 (16:0/18:2)	36:1 (18:0/18:1)
		36:2 (18:1/18:1)		
	Phosphatidylserine (PS)	34:1 (16:0/18:1)	34:2 (16:0/18:2)	34:3 (16:0/18:3)
		36:1 (18:0/18:1)	36:2 (18:0/18:2)	36:3 (18:0/18:3)
Lysophospholipids	Lysophosphatidylcholine (LPC)	16:1 18:1 18:2 18:3		
	Lysophosphatidylethanolamine (LPE)	18:1 18:2 18:3		
Sphingolipids	Ceramide (Cer)	d34:1 (d18:1/16:0)	d36:1 (d18:1/18:0)	t34:1 (t18:1/16:0)
		t36:0 (t18:0/18:0)	t36:2 (t18:1/18:1)	
	Hexosyl ceramide (HexCer)	t36:2 (t18:1/18:1)		
Other	Acylcarnitines (AC)	16:1		
	Cardiolipin (CL)	72:2 72:6 72:8 72:9		
	Coenzyme (Co)	Q8		
	Fatty acid (FA)	18:2 18:3		

The following sections discuss the qualification results of FAs/lipids measured in U-^{13}C lipid yeast extracts by robust, high-throughput, MS-based methods (e.g., Waters library no. APNT135071759 for RPLC). The analysis was conducted by RPLC-MRM/MS and HILIC-MRM/MS on a broad panel of FAs/lipids. This included the 87 analytes listed in **Table 5**, along with additional ones from similar and dissimilar FA/lipid classes.

RPLC-MRM/MS Analysis

Using a previously developed RPLC-MS/MS method, MRM transitions for a broad panel of U-^{13}C FAs/lipids were theoretically determined. The results revealed the 87 targets in **Table 5** to be measured reproducibly across the extract replicates with commendable signal quality. Further observed was the measurement of additional FAs/lipids across several classes to the ones ascribed in **Table 5** as the QC panel. Note that equivalent results were found for the unlabeled lipid yeast extracts (data not shown). As an example of the identification results, **Figures 1** and **2** illustrate a representative set of extracted ion chromatograms (XICs) for DGs and PIs measured in the diluted U-^{13}C lipid yeast extracts by RPLC-MRM/MS.

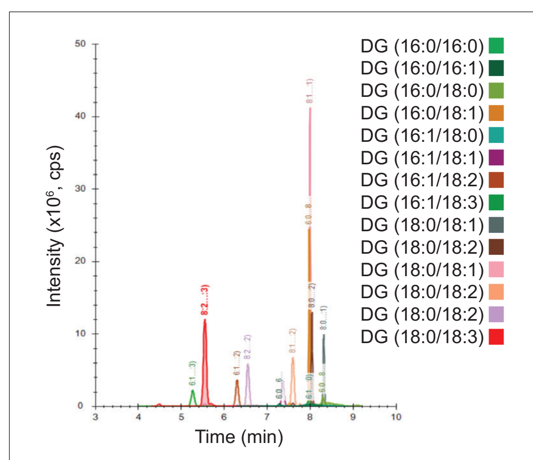

Figure 1. Representative XICs of example DGs measured in the diluted U-^{13}C lipid yeast extracts by RPLC-MRM/MS (ACQUITY Premier CSH C18, Xevo TQ-XS in ESI+). The examples comprise several DGs from the QC panel (e.g., 16:0/18:1, 16:1/18:2, 18:0/18:2), as well as additional DGs (e.g., 16:0/16:0, 16:1/18:3, 18:2/18:2).

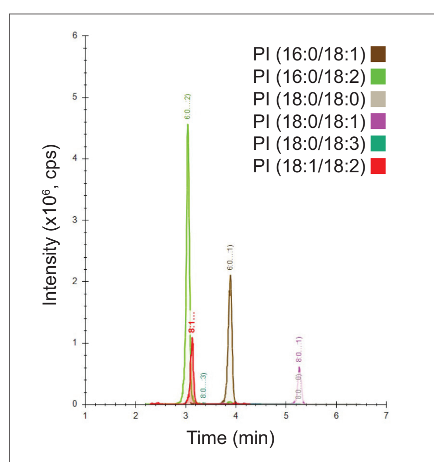

Figure 2. Representative XICs of example PIs measured in the diluted U-^{13}C lipid yeast extracts by RPLC-MRM/MS (ACQUITY Premier CSH C18, Xevo TQ-XS in ESI-). The examples comprise several PIs from the QC panel (e.g., 16:0/18:1, 16:0/18:2, 18:0/18:1) as well as additional PIs (e.g., 18:0/18:0, 18:0/18:3, 18:1/18:2).

As shown in **Figure 2**, excellent signal strength and resolution was detected for a collection of PIs that were and were not in the QC panel (**Table 5**). The expanded compound presence supports broad applications in MS lipidomics (e.g., identification, relative quantification). Alternate sample preparations and analytical platforms will likely provide further potential for application opportunities through the identification of additional FAs/lipids.

HILIC-MRM/MS Analysis

In a similar manner to the RPLC-MRM/MS analysis, the U-^{13}C lipid yeast extracts were measured by HILIC-MRM/MS. As was the case with the RPLC-MRM/MS data, FAs/lipids that were and were not in the QC panel (**Table 5**) were detected, with comparable results obtained for the unlabeled lipid yeast extracts (data not shown). **Figures 3** and **4** illustrate representative sets of XICs for the PCs and PEs measured in the diluted U-^{13}C lipid yeast extracts by HILIC-MRM/MS.

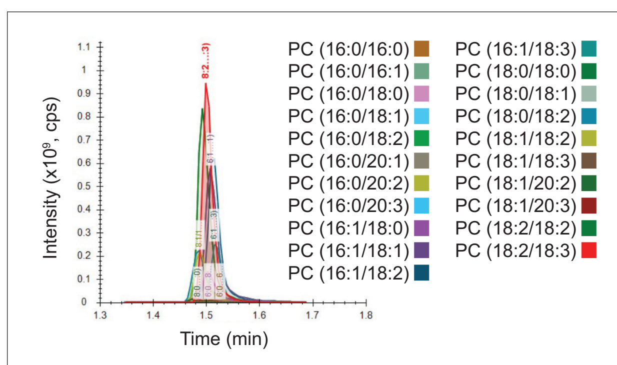

Figure 3. Representative XICs of example PCs measured in the diluted U-^{13}C lipid yeast extracts by HILIC-MRM/MS (ACQUITY Premier UPLC BEH Amide column, Xevo TQ-XS in ESI+). The examples comprise a collection of PCs from the QC panel as well as additional PCs (e.g., 16:1/18:1, 18:0/18:0, 18:1/18:1).

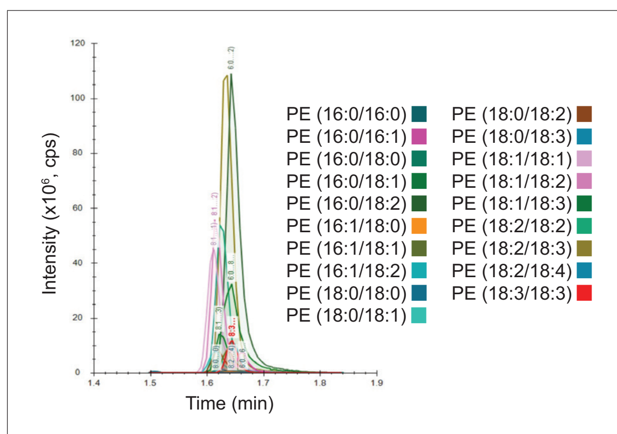

Figure 4. Representative XICs of example PEs measured in the diluted U-^{13}C lipid yeast extracts by HILIC-MRM/MS (ACQUITY Premier UPLC BEH Amide column, Xevo TQ-XS in ESI-). The examples comprise a collection of PEs from the QC panel as well as additional PEs (e.g., 18:1/18:1, 16:0/16:0, 18:2/18:4).

Overall, the HILIC-MRM/MS results demonstrated the analyte signals to generally have good intensity and resolution. This demonstrates the extracts' utility, with additional FAs/lipids potentially measurable in untargeted MS lipidomics applications using different sample preparations and analytical platforms.

Utility and Prospective Applications

Applications for the U-^{13}C lipid yeast extract are extensive. One example could be in system suitability testing to determine if an analytical platform is fit-for-purpose. Here, performance could be tracked using a subset of the reproducibly identified, U-^{13}C FAs and lipids (see **Table 5**) measured in a matrix-free QC sample. In LC-MS (or -MS/MS) experiments, metrics to monitor include those from the chromatographic (e.g., retention time, peak width and symmetry, capacity factor) and mass spectrometric (e.g., mass accuracy, signal intensity, fragmentation efficiency) systems.[7] Through longitudinal QC monitoring, deviations in data quality relating to deficits (e.g., signal drift or offset, peak tailing or splitting) or issues (e.g., column integrity, mass calibration, fragmentation efficiency) in the LC and/or MS system can be revealed, with corrective action(s) then queued.

Other example applications include the qualification, relative quantification, and credentialing of the lipidome.[9] Qualification and quantification of FAs/lipids in sample measurements can be aided with this labeled extract because the analytes are ^{13}C-enriched. The ^{13}C isotopes confer exceptional chemical/isotopic stability and analytical reliability. Since the isotopes are not susceptible to back-exchange and the compounds have identical physicochemical properties to their unlabeled counterparts (i.e., natural or endogenous), the labeled/unlabeled partners will coelute during chromatographic separation aiding enhanced identification confidence and improved quantitative

accuracy. In credentialing, the U-^{13}C and unlabeled yeast extracts are to be mixed at discrete ratios (e.g., 1:1 and 1:2) before LC-MS analysis. Please refer to Wang et al.'s methods article for details on executing the credentialing workflow with sample extracts (*E. coli* used as an example there).[10] This unique credentialing protocol helps pinpoint the features that are derived from true biological analytes, and in turn helps to benchmark analytical parameters and methods in method development.

The U-^{13}C lipid yeast extract can also be applied to alternate analytical methods. An example involves ion mobility (IM)-MS, which is a technique lauded for its ability to separate isomers, isobars, and structurally similar analogs. IM-MS is orthogonal to traditional separation techniques, such as RPLC and HILIC; thus, it conceivably provides an effective means to improve the resolving power and specificity of analytes when operated alone or in a two-dimensional manner (e.g., RPLC-IM-MS). The use of IM-MS, therefore, represents an additional area to which the U-^{13}C lipid extracts can be potentially applied to enhance the identification/quantification depth and breadth.

Conclusions

Given the need for improved standard tools in MS lipidomics, U-^{13}C and unlabeled crude lipid yeast extracts were manufactured under novel, but strict, batch processes. After QC, these commercially available products were disseminated for verification as part of an inter-lab study. Here, the extracts were analyzed by RPLC- and HILIC-MRM/MS methods. The purpose of this analysis was to qualitatively verify the QC panel of FAs/lipids and determine if additional FAs/lipids could be readily measured. The results demonstrated complete verification and broader measurement capability. Overall, applications for these crude lipid yeast extracts can arise in the areas of quality control, credentialing, identification, and relative quantification. It is the hope that the utilities described here can be capitalized on in the future by researchers in the MS lipidomics field toward better achieving application objectives.

References

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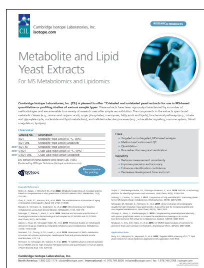
Related Products

Catalog No.	Description	Unit Size	Metabolites/Vial
ISO1	Metabolite Yeast Extract (U-^{13}C, 98%)	1 kit	100s
ISO1-UNL	Metabolite Yeast Extract (unlabeled)	1 kit	100s

Note: ISO1-KIT comprises a tube of both ISO1 and ISO1-UNL. Also, all kits are supplied with a document package (i.e., user manual, CoA, SDS, product flyer), which is accessible via a QR code on the product box.

Please click on the flyer for product-related application information and **isotope.com** for pricing.



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