Published in final edited form as:

Anal Biochem. 2015 April 1; 474: 59–65. doi:10.1016/j.ab.2014.12.014.

Production of stable isotope-labeled acyl-coenzyme A thioesters by yeast stable isotope labeling by essential nutrients in cell culture

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Abstract

Acyl-coenzyme A (CoA) thioesters are key metabolites in numerous anabolic and catabolic pathways, including fatty acid biosynthesis and β -oxidation, the Krebs cycle, and cholesterol and isoprenoid biosynthesis. Stable isotope dilution-based methodology is the gold standard for quantitative analyses by mass spectrometry. However, chemical synthesis of families of stable isotope labeled metabolites such as acyl-coenzyme A thioesters is impractical. Previously, we biosynthetically generated a library of stable isotope internal standard analogs of acyl-CoA thioesters by exploiting the essential requirement in mammals and insects for pantothenic acid (vitamin B5) as a metabolic precursor for the CoA backbone. By replacing pantothenic acid in the cell media with commercially available [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenic acid, mammalian cells exclusively incorporated [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate into the biosynthesis of acyl-CoA and acyl-CoA thioesters. We have now developed a much more efficient method for generating stable isotope labeled CoA and acyl-CoAs from [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate using St_{able} Isotope Labeling by Essential nutrients in Cell culture (SILEC) in Pan6 deficient yeast cells. Efficiency and consistency of labeling were also increased, likely due to the stringently defined and reproducible conditions used for yeast culture. The yeast SILEC method greatly enhances the ease of use and accessibility of labeled CoA thioesters and also provides proof-of-concept for generating other labeled metabolites in yeast mutants.

Keywords

Yeast; mass spectrometry; stable isotope labeling; coenzyme A; Krebs cycle; acetyl-CoA

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Introduction

Liquid chromatography (LC)¹ coupled with selected reaction monitoring mass spectrometry (SRM/MS) is a highly sensitive and specific platform for quantifying a wide range of analytes from complex biological samples. To control for the effects of complex analytical workflows including, analyte stability, extraction efficiencies, and ionization suppression, internal standards are required [1]. The ideal internal standard is a stable isotope labeled analog that reproduces the exact chemical properties of the analyte of interest with no overlap in detection of the relevant analyte by MS. This methodology, which is termed stable isotope dilution LC-SRM/MS, represents the current gold standard for quantitation of a variety of endogenous analytical targets. However, with gains in instrument performance allowing increasingly multiplexed analyses, affordable and efficient generation of diverse sets of stable isotope labeled internal standards remains challenging.

Acyl-coenzyme A (CoA) thioesters are key metabolites in numerous anabolic and catabolic pathways, including fatty acid biosynthesis and β -oxidation, the Krebs cycle, and cholesterol and isoprenoid biosynthesis [2]. Previously, we biosynthetically generated a library of stable isotope internal standard analogs of acyl-CoA thioesters by exploiting the essential requirement in mammals and insects for pantothenic acid (vitamin B5) as a metabolic precursor for the CoA backbone [3] (Fig. 1). By replacing pantothenic acid in the cell media with commercially available [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenic acid, mammalian cells exclusively incorporated [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate into the biosynthesis of acyl-CoA and acyl-CoA thioesters (Fig. 2). We termed this methodology Stable Isotope Labeling by Essential nutrients in Cell culture (SILEC), after the similarity to Stable Isotope Labeling by Amino acids in Cell culture (SILAC) methods employed for proteins [4]. Since the labeling efficiency of CoA thioesters in this method was found to be over 98%, the resulting stable isotope analogs can be used as internal standards in LC-SRM/MS analysis while maintaining a low limit of detection and high analytical precision. Labeling efficiency is critical as this parameter scales inversely with the sensitivity of the LC-MS/MS method, as unlabeled contamination increases the signal in the blank and introduces an additional source of variability into the analysis.

Despite the utility of this method, mammalian cell culture is relatively time consuming, expensive, and requires appropriate cell culturing facilities. Additionally, mammalian cell culture requires either defined media or fetal bovine serum (FBS), which may introduce variability in labeling as well as residual unlabeled pantothenate. In our hands, various lots of charcoal stripped (cs) FBS have produced labeling efficiencies ranging from 68%-99%, and dialyzed (d) FBS can result in only 95% labeling [5]. Furthermore, mammalian cells require multiple media changes and passages to achieve a high labeling purity, as well as time consuming expansions to scale the culture to generate the required amount of internal standard.

¹**Abbreviations used:** AA, acetic acid; ACN, acetonitrile; CID, collision-induced dissociation; CoA, coenzyme A; csFBS, charcoal stripped FBS; dFBS, dialyzed FBS; ESI, electrospray ionization; IPA, 2-propanol; LC, liquid chromatography; MH^+ , protonated molecule; MS, mass spectrometry; MS/MS tandem mass spectrometry; SILEC, stable isotope labeling by essential nutrients in cell culture; SILAC, stable isotope labeling by amino acids in cell culture; SPE, solid phase extraction; SILEC, stable isotope labeling by essential nutrients in cell culture; SRM, selected reaction monitoring; SSA, 5-sulfosalicylic acid; TCA, trichloroacetic acid.

Materials and Methods

Materials

Biotin, folic acid, inositol, niacin, *p*-aminobenzoic acid, pyridoxine-HCl, riboflavin, dextrose, sodium propionate, sodium butyrate, sodium hexanoate, sodium octanoate, sodium decanoate, sodium dodecanoate, sodium myristate, sodium palmitate, trichloroacetic acid (TCA), 5-sulfosalicylic acid (SSA), ammonium formate, and potassium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO). FBS, csFBS, and dFBS were obtained from Gemini Bio-Products (West Sacramento, CA). Ammonium acetate and Optima LC-MS grade water, methanol, acetonitrile (ACN), 2-propanol (IPA), and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Thiamine HCl, Yeast Nitrogen Base w/o AA & w/o AS, w/o Vitamins, and Drop-out Mix Complete w/o Yeast Nitrogen Base were purchased from United States Biological (Salem, MA). Granulated agar was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Ammonium sulfate was purchased from Mallinckrodt Pharmaceuticals (St. Louis, MO). Nitrogen gas was purchased from Airgas (Radnor Township, PA). Oasis solid phase extraction (SPE) cartridges were purchased from Waters (Milford, MA). 2-(2-Pyridyl) ethyl silica gel solid phase extraction cartridges were purchased from Supelco (Bellefonte, PA). Calcium [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate was purchased from Isosciences (King of Prussia, PA). The *pan6* *S. cerevisiae* strain was obtained from the Yeast Knockout Collection library (GE Healthcare Bio-Sciences, Piscataway, NJ). Hepa 1c1c7 murine hepatoma cells were obtained from ATCC (Manassas, VA; # CRL-2026).

Yeast cell culture

Yeast growth media was prepared with 200 μg biotin, 200 μg folic acid, 200 mg inositol, 40 mg niacin, 20 mg *p*-aminobenzoic acid, 40 mg pyridoxine-HCl, 20 mg riboflavin, 40 mg thiamine HCl, 20 g dextrose, 400 μg [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate, 2.0 g Drop-out Mix Complete w/o Yeast Nitrogen Base, 1.7 g Yeast Nitrogen Base w/o AA & w/o AS, w/o Vitamins, and 5.0 g ammonium sulfate dissolved in 1.0 L of distilled H_2O . The vitamins and [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate were filter sterilized and the dextrose, drop-out mix, yeast nitrogen base mix, and ammonium sulfate were autoclaved. The *pan6* *S. cerevisiae* strain was confirmed by a pantothenate auxotrophy test. Agar plates were prepared, with one batch omitting pantothenate from the growth media. The plates were inoculated with *pan6* yeast, and then incubated at 37 °C for 24 h.

1 L of media was inoculated with *pan6* *S. cerevisiae* and incubated at 30 °C while agitating overnight with 500 mL in two 2 L Erlenmeyer flasks covered loosely with aluminum foil. Optional fatty acid induction for some batches was begun the following day. Induction consisted of adding the sodium salts of propionate, butyrate, hexanoate, octanoate, decanoate, dodecanoate, myristate, and palmitate to an equal final concentration in the media for 1 h prior to harvesting. After approximately 31 h from the onset of the culture, the yeast cells were removed from the incubator, divided into 50 mL aliquots, and pelleted at 500 $\times$ g. The cells were resuspended in ice-cold 10% TCA or ACN/IPA (3:1; v/v) for short-chain or mixed-chain extraction, respectively. The cells were pulse sonicated for 30 half-second pulses, on ice. Samples were spun at 16,000 $\times$ g for 10 min at 4°C to remove

unbroken cells and debris. The final supernatant was transferred to a separate tube and stored at -80°C.

Acyl-CoA thioester extraction

Extractions of acyl-CoA thioesters were performed using previously documented procedures [3, 5-7]. Briefly, after pelleting, supernatant was transferred to 1.5 mL microcentrifuge tubes, transferring 1 mL for short-chain or 0.75 mL for short-, medium- and long-chain (mixed-chain) acyl-CoA thioester extractions. 250 µL of 10% KH₂PO₄ was added to the mixed-chain acyl-CoA thioester extractions. Samples were centrifuged at 16000g for 10 min at 4 °C and supernatants were transferred to fresh tubes. 125 µL of glacial acetic acid was added to the mixed-chain acyl-CoA thioester supernatant. Samples were then purified using a solid-phase extraction. For short-chain acyl-CoA thioester samples, Oasis HLB 1cc (30 mg) SPE columns (Waters) were conditioned with 1 mL methanol and washed with 1 mL water. Supernatant was loaded, followed by a 1 mL wash with water, and eluted using 1 mL of 25 mM ammonium acetate in methanol. For mixed-chain acyl-CoA thioester samples, 2-(2-pyridyl)ethyl functionalized silica gel SPE tubes (Supelco) were conditioned with 1 mL of ACN/IPA/H₂O/AA (9:3:4:4; v/v/v/v). Supernatants were loaded, followed by a 2 mL wash with ACN/IPA/H₂O/AA (9:3:4:4; v/v/v/v), and then the SPE columns were eluted using 1 mL of 250 mM aqueous ammonium formate/methanol (1:4; v/v). Eluents were evaporated to dryness under nitrogen gas. Short-chain acyl-CoA thioester samples were resuspended in 100 µL of 5% 5-sulfosalicylic acid, while mixed-chain acyl-CoA thioester samples were resuspended in 100 µL of H₂O:ACN (7:3; v/v). Injections of 10 µL were made for analysis by LC- electrospray ionization (ESI)/SRM/MS.

LC-ESI/SRM/MS analysis

Analytes were separated using a reversed-phase Phenomenex HPLC Luna C18 column (2.0 mm × 150 mm, particle size 5 µm) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in acetonitrile/water (95:5. v/v) as solvent B, and acetonitrile/water/formic acid (80/20/0.1, v/v/v) as solvent C. Gradient conditions were as follows: 2% B for 1.5 min, increased to 25% over 3.5 min, increased to 100% B in 0.5 min and held for 8.5 min, washed with 100% C for 5 min, before equilibration for 5 min. The flow rate was 200 µL/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive ESI mode. Samples (10 µL) were injected using a Leap CTC auto- sampler (CTC Analytics, Switzerland) where they were maintained at 4 °C, and data was analyzed with Analyst 1.4.1 software. The column effluent was diverted to the mass spectrometer from 8 to 18 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), gas 2 (15 units), and collision-induced dissociation (CID) gas (5 units). The ESI probe temperature was 450 °C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. Isotopic labeling using [¹³C₃ ¹⁵N₁]-pantothenate resulted in labeled CoA and acyl-CoA thioesters with a 4 Da increase in mass.

Stability of yeast SILEC-derived acyl-CoAs

SILEC derived acyl-CoAs were stored in 10 mL aliquots of 10% TCA at -80°C and an aliquot allowed to thaw to room temperature. The aliquot was then re-frozen and stored at -80 °C for 24 h. The freeze-thawing process was repeated for a total of five times and the amount of acetyl-CoA determined by LC-MS as described previously [3]. The amount of acetyl-CoA was then compared with the amount determined to be present in the originally prepared yeast SILEC CoA standard solution.

Variation in efficiency of acetyl-CoA labeling in mammalian cell culture

SILEC standards were prepared as described previously using Hepa 1c1c7 murine hepatoma cells and different batches of FBS. Incorporation of labeled pantothenate was determined as described above using high resolution LC-MS.

Results

Yeast cell culture

Due to the availability of genetic mutants and ability to rapid reproduce in inexpensive media, the yeast *Saccharomyces cerevisiae* provides an attractive alternative for the biosynthesis of a variety of endogenous metabolites. Unlike mammalian cells, yeast are capable of *de novo* biosynthesis of pantothenate (Fig. 1), meaning that replacement of only [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenic acid is insufficient for generating isotopically labeled acyl-CoAs with a high labeling efficiency [8]. In this method, we tested *pan6* yeast as a platform for acyl-CoA SILEC generation. *PAN6* encodes the enzyme pantothenate synthase, which combines pantoic acid and β -alanine to form pantothenate (Fig 1). *Pan6* cells are incapable of *de novo* pantothenate synthesis, and are auxotrophic for pantothenate [9]. Therefore, when the *pan6* yeast cells were cultured with exogenous [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenic acid as the sole source of pantothenate, CoA and all the acyl-CoA thioesters were isotopically labeled with the [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenate moiety (Fig. 2). Under these conditions, liters of *pan6* cells could be readily cultured (Fig. 3A) and stable isotope-labeled CoA thioesters extracted. *Pan6* cells grew only in media supplemented with exogenous pantothenate (Fig. 3B).

Optimization of the yeast SILEC method

[$^{13}\text{C}_3$ $^{15}\text{N}_1$]-pantothenic-labeled acyl-CoA species were observed with > 99 % incorporation of label after only 2 days of yeast SILEC culture. The yields of [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-acetyl-CoA were proportional to cell density, reaching a maximum as the culture approached stationary phase (Figs. 4A, 4B). Cell lysis by sonication or glass beads gave similar results (Fig. 4C) so sonication was used as the preferred method due to its simplicity. Acyl-CoA thioesters are stable in acidic conditions at -80 °C and so it was possible to produce and store large amounts stable isotope-labeled acyl-CoAs for future use as internal standards for LC-SRM/MS assays. The stable isotope labeled acyl-CoAs were stored in 10 mL aliquots of 10% TCA at -80°C, to avoid freeze-thaw cycles. We have successfully used batches of stable isotope labeled acyl-CoAs stored in this manner for over 6 months. The estimated

yield from 1 L of yeast culture was 405 μg of [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-acetyl-CoA, approximately the same amount of [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-HMG-CoA, and lesser amounts of other acyl-CoAs.

Abundance and labeling of CoA and acyl-CoA thioesters

The abundance of isotope-labeled acetyl-CoA, 3-hydroxy-3-methylglutaryl (HMG)-CoA (Fig. 5A), succinyl-CoA, CoA, and β -hydroxybutyryl (βHB)-CoA (Fig. 5B), increased with increasing cell density. Labeling efficiency was calculated for [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-CoASH (99.41%), [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-acetyl-CoA (99.88%), [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-succinyl-CoA (99.54%), and [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-HMG-CoA (99.82%). Constant neutral loss scans of 507 Da, a fragmentation specific for the CoA backbone, revealed a distribution of protonated molecules (MH^+) from acyl-CoAs in the Hepa 1c1c cells (Fig. 6A) that was somewhat different from the yeast SILEC extract (Fig. 6B). Signals corresponding to MH^+ were observed for [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-labeled CoA (m/z 772), acetyl-CoA (m/z 814), succinyl-CoA (m/z 872), and HMG-CoA (m/z 916). There was a significantly enriched abundance of HMG-CoA in the yeast extract (Fig. 5B) when compared with the Hepa 1c1c cell extract (Fig. 5A).

Stability of yeast SILEC-derived acyl-CoAs

Multiple freeze-thaw cycles resulted in some degradation of the acyl-CoA content of the prepared batches with some degradation after the second freeze-thaw and a significant loss after the fifth cycle. This is reflected in data for acetyl-CoA where there was a loss of 16 % after five freeze-thaw cycles (Fig. 5C). This means that appropriate analytical technique should include standard curves run from the same aliquot of the same batch for absolute quantitation and comparisons across the same aliquot of the same batch for relative quantitation.

Customization of the yeast SILEC method

The composition of isotope-labeled acyl-CoA thioesters produced by *pan6* cells could be modified by both the extraction used [6] and fortification of the medium with specific precursors [7]. Extraction of acyl-CoAs using a procedure to capture acyl-CoAs of mixed length resulted in a wider library of [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-acyl-CoA internal standards as reflected by the MH^+ signals that were detected from both Hepa1c1c7 SILEC (Fig. 6C) as well as yeast SILEC (Fig. 6D) in the constant neutral loss mass spectra. Supplementation of the yeast SILEC media with 1 mM sodium propionate, sodium butyrate, sodium hexanoate, sodium octanoate, sodium decanoate, sodium laurate (dodecanoate), sodium myristate (tetradecanoate), and sodium palmitate for 1 h before the acyl-CoA thioester extraction yielded a distribution of isotopically labeled products enriched in medium- and long-chain acyl-CoAs. Intense peaks corresponding to MH^+ from [$^{13}\text{C}_4$ $^{15}\text{N}_1$]-labeled CoA (m/z 772), acetyl-CoA (m/z 814) decanoyl-CoA (m/z 926), lauroyl-CoA (m/z 954), myristoyl-CoA (m/z 982), and palmitoleoyl-CoA (m/z 1008) were observed in the constant neutral loss mass spectrum (Fig. 6E).

Variation in efficiency of acetyl-CoA labeling in mammalian cell culture

The efficiency of isotopic purity in mammalian culture differed between types and batches of FBS (Table 1). This is due to presence of residual amounts of unlabeled pantothenate in

the serum. Therefore, labeling efficiency was significantly higher in the yeast SILEC system (typically 99.9 %) where FBS was not required.

Discussion

In order to establish the physiological relevance of altered levels of acyl-CoAs, the bioanalytical methodology that is employed must unequivocally determine the specific concentrations of these structurally diverse molecules. The variety of individual chemical entities making up the acyl-CoA family poses a significant challenge to the analyst. In addition, the energy-rich thioester bond in acyl-CoAs lack of resonance stabilization and so they are destabilized relative to the corresponding esters, making them more susceptible to hydrolysis [7]. This means that acyl-CoA thioesters readily decompose in aqueous solutions and they are particularly labile under basic conditions. The use of stable isotope analogs as internal standards can correct for such losses [1]. Furthermore, when using LC-ESI/MS methodology suppression of the analyte signal can occur from substances that are present in the biological matrix that is being analyzed. Stable isotope analog internal standards overcome this problem by adjusting for selective suppression of the ESI signal [1].

Currently, seven high quality individual stable isotope labeled acyl-CoA derivatives are commercially available from Sigma for use as internal standards in LC-MS assays - [$^{13}\text{C}_4$]-acetoacetyl-CoA, [$^{13}\text{C}_2$]-acetyl-CoA, [$^{13}\text{C}_3$]-malonyl-CoA, [$^{13}\text{C}_4$]-octanoyl-CoA, [$^{13}\text{C}_{18}$]-oleoyl-CoA, [$^{13}\text{C}_{16}$]-palmitoyl-CoA, and [$^{13}\text{C}_{18}$]-stearoyl-CoA. However, considering the wide variety and interconnected metabolism of acyl-CoAs, these available products are completely inadequate to conduct comprehensive metabolic profiling. Searching the term “CoA” on Human Metabolome Database [10] currently produces 268 acyl-CoA species and this value is likely an underestimate of the true variety of acyl-CoA intermediates considering the diversity of metabolic pathways featuring a critical acyl-CoA intermediate. The same term searched on MetaCyc [11] revealed some 524 structurally distinct acyl-CoAs. Chemical synthesis of one or two isotopically labeled acyl-CoA analogs is practical; but generating a library of relevant CoA species through synthesis is impractical for most researchers. In contrast, this is a relatively easy task for the SILEC method when appropriate precursors are added to the media.

Our previous SILEC methodology employed mammalian cell culture to generate a library of CoA standards [3]. The new yeast SILEC method is much more efficient because the time required to produce a batch of labeled material is significantly reduced due to the growth rates of yeast and ease of scalability (Fig.1). In addition, the efficiency of isotopic labeling is increased versus mammalian culture, which results of the ability to omit any form of serum (Table 1). Considerable variability in SILEC labeling efficiency exists when using different types and even batches of serum in mammalian cell culture (Table 1). Sensitivity and precision of the stable isotope dilution LC-MS method relies heavily on highly efficient labeling. The yeast SILEC method is therefore an important improvement because the high labeling efficiency (> 99.5 %) makes it possible to maximize sensitivity and specificity of CoA analysis by LC-MS. The uniform [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-labeled standards generated by the yeast SILEC method provides a consistent label across all acyl-CoAs that imparts a 4 Da increase in mass resulting in no isotopic overlap, permitting rapid method development coupled with

high sensitivity and precision. The introduction of a [^{15}N]-label also allows neutron encoded isotope analysis on high-resolution mass spectrometers [12]. In addition, the genetic yeast SILEC method will enable studies of pantothenate synthesis tracing in both normal and mutant yeast cells to be conducted. Furthermore, the method can be elaborated to other metabolites and organisms. Finally, the composition of the internal standard stocks can be quickly modified by providing appropriate metabolic precursors to increase the abundance of specific acyl-CoAs.

The availability of a diverse set of stable isotope analogs for use as internal standards is important because the biological roles of individual acyl-CoA species are distinct. Acetyl-CoA and succinyl-CoA are important components of the Krebs cycle. Additionally, acetyl-CoA is used as a metabolically sensitive rheostat in acetylation reactions, such as in post-translational modifications of histone proteins [13]. β HB-CoA is a key intermediate in both fatty acid oxidation and ketone body metabolism. HMG-CoA is critical in cholesterol metabolism (or ergosterol for yeast). The capacity to produce medium- and long-chain acyl-CoA standards such as octanoyl-, decanoyl-, lauroyl-, myristoyl-, and palmitoyl-CoA greatly adds to the utility of this method, as these metabolites are conserved metabolic intermediates and precursors for protein post-translational modifications. The ability to measure panels of acyl-CoAs is crucial because this multiplexing allows dissection of compensatory metabolic responses, as was demonstrated for the complex I inhibitor rotenone [14, 15].

The capacity to generate high quality internal standards for a panel of acyl-CoA species using a yeast auxotrophic mutant should be applicable to other biosynthetic pathways. Stable isotope labeling incorporating [^{13}C]- and [^{15}N]-labeled standards is superior to the use of [^2H]-labeled standards due to the potential for hydrogen exchange and differences in chromatographic retention times with [^2H]-analogs when compared with the corresponding protium forms. Thus, other synthetic intermediates that incorporate carbon or nitrogen backbones efficiently from a single metabolic precursor could be amenable to the SILEC approach. Yeast mutant strains that confer auxotrophy of key metabolites are available. In these cases, growth in defined media is well-established, and the only limiting factor may be obtaining a stable isotope analog of the key metabolite, which can alleviate the auxotrophy.

In summary, we have reported a simple method for producing relatively large quantities of stable isotope-labeled acyl-CoA internal standards in yeast for use as internal standards in LC-SRM/MS assay. These stable isotope analogs could be incorporated into other published methods for targeted [16-19] or untargeted quantitative analyses [20, 21]. The yeast SILEC method is simpler, less expensive, and more rapid than our previous approaches. Efficiency and consistency of labeling were also increased, likely due to the stringently defined and reproducible conditions used for yeast culture. This method greatly enhances the ease of use and accessibility of the SILEC method for the preparation of stable isotope labeled acyl-CoA thioesters for use as internal standards in quantitative LC-MS assays. Furthermore, it provides proof of principle that yeast can be used for the preparation of stable isotope analogs of other cellular metabolites.

Acknowledgments

We acknowledge the support of Navitor Pharmaceuticals (D.S.G.) and National Institutes of Health grants P42ES023720, P30ES013508, and T32ES019851.

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Figure 1. Pantothenate is an essential nutrient in organisms lacking pantothenate synthetase, encoded for by the PAN6 gene in *S. cerevisiae*

Enzymes are shown in bold: Valine-pyruvate transaminase (VPT); ketopantoate hydroxymethyl transferase (KPHMT); ketopantoate reductase (KPR); aspartate alpha-decarboxylase (ADC); pantothenate synthetase (PS); pantothenate kinase (PanK); phosphopantothenoylcysteine synthetase (PPCS); phosphopantothenoylcysteine decarboxylase (PPCDC); phosphopantetheine adenylyltransferase (PPAT); dephospho-CoA kinase (DPCK). (B) Incorporation of [$^{13}\text{C}_3$ $^{15}\text{N}_1$]-isotopic label into CoA via biosynthesis of CoA from pantothenate.

Figure 2. Incorporation of $^{13}\text{C}_3$ $^{15}\text{N}_1$ -isotopic label into CoA via biosynthesis of CoA from pantothenate

Chemical structures for conversion of pantothenate to 4'-phosphopantothenate (1), (R)-4'-phosphopantothenoyl-L-cysteine (2), 4'-phosphopantotheine (3), 3'-dephosphocoenzyme A (4), and coenzyme A (5).

Figure 3. Biosynthesis and incorporation of isotopic labels from pantothenate (vitamin B5) into Coenzyme A

(A) Comparative workflow for SILEC with mammalian versus yeast culture. **(B)**

Confirmation of pan6⁻ yeast auxotrophy for vitamin B5 (VB5) by growth on agar with (+) or without (-) pantothenate.

Figure 4. Yeast SILEC rapidly produces efficiently labeled [¹³C₃ ¹⁵N₁]-acetyl-CoAs from [¹³C₃ ¹⁵N₁]-pantothenate Pan6 yeast incubated over 31 h at 30°C show an increase in (A) optical density and (B) amount of isotopically labeled acetyl-CoA. (C) Lysis by sonication (black fill) or with glass beads (white fill) did not significantly alter yields. Maximum yield of [¹³C₃ ¹⁵N₁]-acetyl-CoA was obtained at 26-29 h, corresponding to the end of log phase growth.

Figure 5. Formation and stability of labeled acyl-CoAs by yeast SILEC

Pan6⁺ yeast was incubated over 31 h at 30°C and extracted using the short-chain acyl-CoA method. (A) Levels of [¹³C₃ ¹⁵N₁]-labeled acetyl-CoA and HMG-CoA. (B) Levels of [¹³C₃ ¹⁵N₁]-labeled succinyl-CoA, CoA, and βHB-CoA. (C) Stability of yeast SILEC-derived acetyl-CoA through five freeze-thaw cycles.

Figure 6. MH^+ signals from constant neutral loss scans of 507 Da for the acyl-CoA extract from (A) Hepal1c1c7 SILEC (B) yeast SILEC, and mixed chain acyl-CoA extract from (C) Hepal1c1c7 SILEC (D) yeast SILEC (E) yeast SILEC induced with fatty acid treatment.

Table 1

Variation in efficiency of acetyl-CoA labeling using different lots of csFBS or dFBS for 7 passages in Hepa 1c1c7 murine hepatoma cells.

FBS	Average labeling of acetyl-CoA
csFBS lot A47	99.63%
csFBS lot A64	82.76%
csFBS lot A65	40.15%
csFBS lot A81	98.80%
csFBS lot 63B	99.43%
dFBS	92.34%