SILEC: a protocol for generating and using isotopically labeled coenzyme A mass spectrometry standards

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Abstract

Stable isotope labeling by essential nutrients in cell culture (SILEC) was recently developed to generate isotopically labeled coenzyme A (CoA) and short-chain acyl-CoA thioesters. This was accomplished by modifying the widely used technique of stable isotope labeling by amino acids in cell culture to include $[^{13}C_3{}^{15}N]$-pantothenate (vitamin B_5), a CoA precursor, instead of the isotopically labeled amino acids. The lack of a *de novo* pantothenate synthesis pathway allowed for efficient and near-complete labeling of the measured CoA species. This protocol provides a step-by-step approach for generating stable isotope-labeled short-chain acyl-CoA internal standards in mammalian and insect cells as well as instructions on how to use them in stable isotope dilution mass spectrometric-based analyses. Troubleshooting guidelines, as well as a list of unlabeled and labeled CoA species, are also included. This protocol represents a prototype for generating stable isotope internal standards from labeled essential nutrients such as pantothenate. The generation and use of SILEC standards takes approximately 2–3 weeks.

Introduction

High-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) provides a highly sensitive and specific platform to measure a broad range of analytes in a variety of complex biological matrices[1]. In addition, advancements in high-resolution MS[2,3] and the development of more dynamic software platforms[4–6] have provided scientists with the capability of measuring thousands of different analytes in a single run, making MS the optimal methodology for robust high-throughput metabolomic and proteomic analyses[7,8]. Nevertheless, appropriate internal standards are crucial for the success of any MS-based method, particularly when measuring endogenous metabolites in biological samples, because of matrix effects on analyte stability, extraction and ionization efficiency[9–14]. To account for these effects, an internal standard that faithfully reproduces the biological and chemical properties of the analyte of interest is needed[15].

Stable isotope analogs represent the best internal standards for MS-based analyses because they have the same biological and physicochemical properties as the analyte of interest, but they can still be distinguished by a mass spectrometer[15]. Difficulty in chemically synthesizing stable isotope analogs of complex biological molecules has spurred the

development of techniques to generate these standards in biological systems[16–18]. In the case of proteins, a method called stable isotope labeling by amino acids in cell culture (SILAC) has been developed[19]. In this method, cells are grown in the presence of a stable isotope-labeled essential amino acid such as lysine, with the unlabeled form specifically omitted. The inability of cells to synthesize lysine results in the exclusive uptake and incorporation of the labeled lysine into cellular proteins. After serial passages in this medium, greater than 99% of lysine residues are labeled. At this point, there are two possible modes by which these labeled cells can be used. In the 'classical' SILAC approach, an equal number of labeled and unlabeled cells are subjected to different stimuli, mixed, processed together and analyzed by MS[17]. Alternatively, the labeled cells can be lysed to produce a stable isotope-labeled proteome internal standard (SILAP)[20], which can then be spiked into different experimental samples to normalize the samples for relative quantification[21–23]. The advantage of this approach is the ability to apply these standards to both *in vitro* or *in vivo* samples; it also avoids any biological perturbations introduced by the labeled amino acids.

In addition to proteins, there are other endogenous molecules that have been difficult to chemically synthesize; consequently, labeled analogs for many of these compounds are not commercially available. One such compound is CoA, a ubiquitous fatty acyl carrier that has a crucial role in many metabolic and mitochondrial processes[24–26]. There are numerous CoA derivatives, including an acyl-CoA thioester for most individual fatty acids (Supplementary Table 1). MS/MS represents the optimal platform for developing CoA analysis. When subjected to collision-induced dissociation (CID), CoA derivatives show a characteristic fragmentation pattern (Fig. 1a), breaking at the ATP moiety, resulting in productions with a neutral loss of 507 atomic mass units (AMU)[27,28]. Typical parent protonated molecular ions observed for endogenous CoASH and its derivatives in a constant neutral loss/MS experiment are shown in Figure 1b. The use of this type of analysis not only facilitates LC–selected reaction monitoring (SRM)/MS method development, but also allows for more specific CoA discovery experiments. However, the lack of isotopically labeled CoA has limited the development of comprehensive stable isotope dilution mass spectral analysis of CoA compounds.

In this report, our focus is on generating stable isotope-labeled CoASH and short-chain acyl-CoA species, which would not only improve our ability to study metabolism[29–33] but also aid in the diagnosis of various metabolic diseases[34–38]. The use of labeled standards is particularly important, as cellular thiols can be rapidly oxidized or can react with both intracellular and extracellular molecules[39–41]. The purpose of this protocol is to provide a practical guide to the generation of stable isotope-labeled short-chain acyl-CoA thioesters for use as internal standards in quantitative stable isotope dilution LC-MS assays. An in-depth discussion of how to conduct the LC-MS experiments has not been included because there is a substantial literature describing such methodology[28,42–47]. As a guideline, however, the specific LC-MS parameters used by our laboratory to analyze CoA derivatives have been provided (Fig. 2).

To biosynthetically generate labeled CoA standards, we recently developed SILEC, a method similar to SILAC, except that a labeled vitamin, pantothenate, is used instead of labeled amino acids[18]. Unlike plants, fungi and most prokaryotes, animals are not capable of *de novo* pantothenate synthesis (Fig. 3)[48]. As a result, pantothenate is a dietary requirement and a necessary cell culture medium supplement. After uptake, pantothenate is converted to CoA through five enzymatic steps[24]. By growing cells in the presence of $[^{13}C_3{}^{15}N]$-pantothenate, we have developed a method for biosynthetically generating stable isotope-labeled CoASH and acyl-CoA thioesters using a single culture. These standards can then be extracted and subsequently applied to cells, tissues or biological fluids before processing,

and they can be used as internal standards for mass spectral analysis. To rigorously measure various short-chain acyl-CoA species using SILEC-labeled CoA standards, we have developed a stable isotope dilution LC-MS method (Fig. 2) on the basis of modifications of previously published procedures[28,42–47].

Although this protocol focuses on the generation and utilization of short-chain acyl-CoA species, LC-MS analysis of medium- and long-chain acyl-CoA species reported in the literature[27,45,49–53] could also be adapted to this SILEC approach and also used in the diagnosis of metabolic diseases[54–56]. Labeled CoASH that is generated by the SILEC method can also be used as a synthon to prepare isotope-labeled acyl-CoA species using conventional chemical synthesis methods[57–60]. A brief overview of the SILEC method, materials required, a step-by-step protocol and troubleshooting guidelines are provided below.

Overview

SILEC methodology can be used to label CoA in any cell system that does not contain a robust *de novo* pantothenate synthesis pathway, including mammalian and insect cell lines, both described here. Hepa 1c1c7 mouse hepatoma cells were used because of their rapid growth rates in SILEC medium, the abundance of short-chain acyl-CoA species of interest and relative ease in scalability. This method was also adapted to *Drosophila* S2 cells, which have even shorter doubling times and grow in suspension, facilitating larger-scale preparations of labeled standards.

Experimental design

Generation of labeled CoA—Biosynthetic generation of labeled CoA standards can be divided into four steps (Fig. 4). The objective of the first step is to label the majority of the intracellular CoA while still generating enough cellular material to accommodate the proposed experiment. To accomplish this, cells are passaged 3–5 times in medium containing 10% serum and 1 mg per liter $[^{13}C_3^{15}N]$-pantothenate, with unlabeled pantothenate specifically omitted. Unfortunately, undialyzed fetal bovine serum (uFBS) is often contaminated with numerous blood-borne hormones and nutrients, including pantothenate. Therefore, specialized serum such as dialyzed FBS (dFBS) or charcoal-dextran–stripped FBS (csFBS) must be used instead. In fact, we found that csFBS was optimal because it contained the least amount of residual unlabeled pantothenate[18]. By the third passage, approximately 98–99% of the CoA species in the cell are isotopically labeled. In the second step, the medium is replaced overnight with ultra-labeling medium containing a higher concentration of labeled pantothenate (2–3 mg per liter) and a lower concentration of serum (0–5%). This increases the concentration of labeled pantothenate in the medium while decreasing the level of the contaminating unlabeled pantothenate from the serum. Although serum-free culture could further decrease unlabeled pantothenate levels, such conditions can also affect growth, viability and metabolic characteristics of cells in culture[61–63]. Therefore, an optimal level of serum must be determined to balance the benefits of increased labeling while still maintaining cellular characteristics. For Hepa 1c1c7 cells, we found that using medium containing 3% csFBS and 3 mg per liter $[^{13}C_3^{15}N]$-pantothenate resulted in 99.5% labeling. At this point, the CoA species from a representative plate can be sampled to determine labeling efficiency and CoA profile using LC–neutral loss scan of $m/z = 507$. If the desired CoA species can be identified and measured, the third step is not necessary. However, if one or more of the labeled CoA species of interest are below a reproducibly quantifiable level, a customization step may be necessary. This can be accomplished in multiple ways. First, labeled cells can be supplemented with a particular fatty acid, which is then taken up by the labeled cells to generate a higher percentage of that fatty acyl-CoA. We have demonstrated this by adding

propionate to generate propionyl-CoA, but can be extended to other lower-abundance CoA species, such as by adding -hydroxybutyrate to generate -hydroxybutyryl-CoA (Fig. 5), an important intermediate in fatty acid oxidation and ketogenesis[64,65]. In addition, cells can be subjected to different biological perturbations or toxicological insults to increase and decrease particular CoA species or to generate xenobiotic-CoA adducts such as menadione-CoA (Fig. 6). The CoA species in these cells can then be extracted and pooled together to generate a SILEC reference metabolome containing a more appropriate or more global CoA profile for the experiment. Alternatively, the cellular lysate can be hydrolyzed with a strong base, and the resulting $[^{13}C_3^{15}N]$-CoASH can be generated. This can be further purified and used to synthesize a particular CoA derivative[57–60].

Application of SILEC standards for LC-MS analysis—The cellular extracts containing the SILEC-labeled CoA internal standards can be applied to biological samples and used to normalize for the CoA species of interest. These biological samples can include cultured cells, tissue samples or clinical samples of obtainable cells such as fibroblasts, lymphocytes or platelets. In each case, a constant amount of SILEC standard is spiked into the samples at an early point during sample processing: to scraped cells for *in vitro* experiments, to snap-frozen or lyophilized tissues for *in vivo* experiments or into purified cells such as lymphocytes or platelets for clinical studies. In addition to spiking the samples, the same amounts of SILEC-labeled CoA internal standards are also spiked into known quantities of unlabeled CoA standards to generate a standard curve. Both biological samples and unlabeled CoA internal standards can then be extracted using solid-phase extraction (SPE) and analyzed by LC-MS. The peak area of each unlabeled standard is divided by the peak area of the corresponding SILEC internal standard analog to generate a standard curve of standards to peak area ratio. The same analysis is then performed on each sample, and the peak area ratio is then used to calculate the amount of each of the CoA species in the sample. These amounts are then compared between experimental groups. This method is comparable to the SILAP or SILAC 'spike-in' methods that have been described previously[21,23]. The strength of using a stable isotope dilution MS method is not only in the specificity of CoA elution, but also that the labeled compounds will have the same stability as the unlabeled analyte of interest. Therefore, there would be a parallel degradation of the labeled internal standard that does not affect the ratio of unlabeled analyte to labeled internal standard.

Additional considerations—Limitations of the SILEC approach are similar to those encountered with SILAC labeling in that a biological system is used to generate stable isotope standards[17]. Although numerous labeled CoA species can be generated in a single biological system, batch-to-batch variability persists even under well-controlled conditions.

If you are using a starting material other than pantothenate, such as folates, it is important to use medium and the appropriate serum that does not contain that essential nutrient. Therefore, an omitted medium along with a modified serum (such as csFBS or dFBS) is crucial for generating an isotopically pure standard. Finally, finding the appropriate cell line is also necessary for optimization. As this is largely empirical, many cell lines likely need to be tested to optimize scale-up and production. A time course should be established in order to determine the optimal time for harvesting that particular cell line.

Materials

Reagents

- Cell line of choice. The protocol is written for Hepa 1c1c7 cells (ATCC, cat. no. CRL-2026) and *Drosophila* Schneider 2 cells (Invitrogen, cat. no. R690-07)

Medium

- Mammalian cells: custom RPMI 1640 medium, pantothenate omitted (AthenaES). This is a special request item (cat. no. 0500-19)

- Schneider's insect medium

- Hank's buffered salt solution (HBSS)

- Phosphate-buffered saline (PBS)

- $[^{13}C_3{}^{15}N]$-pantothenate (vitamin B_5-$[^{13}C_6{}^{15}N_2]$, Isosciences, cat. no. 5065)

- csFBS (Gemini Biosciences, cat. no. 100-119)

- Penicillin

- Streptomycin

- Glutamine

- Trichloroacetic acid (TCA)

- 5-Sulfosalicyilic acid (SSA)

- Potassium hydroxide (KOH)

- Oasis HLB 1cc (30 mg) SPE columns (Waters, cat. no. WAT094225)

- Conical glass centrifugation tubes, 10 ml

- Nitrogen gas

- Methanol

- Ammonium acetate

- Acetonitrile and water (mass spectrometry grade)

- Formic acid

- Propionic acid

- -Hydroxybutyric acid

- Menadione

Equipment

- Probe sonicator

- Tissue culture dishes, 10 cm

- Tissue culture flasks, 75 cm^2

- Cell scrapers

- Microcentrifuge

- Vacuum manifold

- Nitrogen evaporator

- Refrigerated autosampler

- HPLC system with three pumps

- Triple quadrupole mass spectrometer (API 4000 or instrument of similar sensitivity)

- Computer with mass spectral computational software

Reagent Setup

Heat-inactivated csFBS—Thaw csFBS at 37 °C and heat-inactivate the complement for 30 min at 55 °C. Aliquot into 50-ml fractions and store at − 20 °C. The heat-inactivated csFBS can be used for up to 1 year when stored at − 20 °C.

[$^{13}C_3$$^{15}N$]-Pantothenate stocks

Prepare in double-distilled water (ddH2O) at 1 mg ml $^{-1}$ and store at − 20 °C. The stocks are stable for 1 year if stored at − 20 °C

Unlabeled CoA standards

Prepare 5 mM stock solutions of acyl-CoA species in 5% (wt/vol) aqueous SSA. Store in 100-μl aliquots at − 80 °C. Thaw when needed and avoid repetitive freeze-thaw cycles. The aliquots are stable for 1 year when stored at − 80 °C.

SILEC expansion medium for mammalian cells

Prepare pantothenate-free medium with 10% (vol/vol) heat-inactivated csFBS, 2 mM L-glutamine, 100 IU ml $^{-1}$ penicillin, 100 μg ml $^{-1}$ streptomycin and 1 mg per liter [$^{13}C_3$$^{15}N$]-pantothenate.

SILEC expansion medium for insect cells (S2)

Prepare Schneider's medium with 10% (vol/vol) heat-inactivated csFBS, 2 mM L-glutamine, 100 IU ml $^{-1}$ penicillin, 100 μg ml $^{-1}$ streptomycin and 3 mg per liter [$^{13}C_3$$^{15}N$]-pantothenate.

SILEC ultra-labeling medium for mammalian cells

Prepare pantothenate-free medium with 3% (vol/vol) heat-inactivated csFBS, 2 mM L-glutamine, 100 IU ml $^{-1}$ penicillin, 100 μg ml $^{-1}$ streptomycin and 3 mg per liter [$^{13}C_3$$^{15}N$]-pantothenate.

SILEC ultra-labeling medium for insect cells (S2)

Prepare Schneider's medium with 3% (vol/vol) heat-inactivated csFBS, 2 mM L-glutamine, 100 IU ml $^{-1}$ penicillin, 100g ml $^{-1}$ streptomycin and 10 mg per liter [$^{13}C_3$$^{15}N$]-pantothenate.

Customization medium

Prepare HBSS medium supplemented with Ca^{2+}, Mg^{2+}, 1 g per liter D-glucose, 25 mM HEPES and the appropriate modifying compound (fatty acid, xenobiotic and so on) as shown in the table below.

All media should be filtered and can be stored at 4 °C for up to 3 months. Warm the media to 37 °C before cell treatment for mammalian cells and to room temperature (22–25 °C) for insect cells.

CoA extraction solution

Extraction solution is 10% (wt/vol) TCA in ddH$_2$O. Chill it on ice before the experiment.

CoA elution solution

CoA elution solution is 25 mM ammonium acetate in methanol.

CoA resuspension solution

Resuspension solution is 5% (wt/vol) SSA in ddH$_2$O.

CoA hydrolysis solution

If pure [^{13}C$_3$^{15}N]-CoASH is being generated, prepare 40% (wt/vol) KOH in ddH$_2$O.

LC-MS solvents

Prepare, sonicate and degas HPLC solvents before LC-MS analysis. Use solvents of mass spectrometry grade (Fisher Optima-grade solvents are used for our analysis). Solvent A is 5 mM ammonium acetate in water. Solvent B is 95:5 ACN:H2O and 5 mM ammonium acetate in water. Solvent C is 80:20:0.1 ACN:H2O:formic acid.

Procedure

Generating labeled CoA standards

1. Thaw frozen cells directly into the SILEC expansion medium.

2. For Hepa 1c1c7 (adherent) cells, allow cells to reach confluence (3-4 d), wash two times with PBS, trypsinize and split 1:5 into SILEC expansion medium. For S2 (suspension) cells, when cells have reached 1×10^7 cells per ml, split into a new flask containing SILEC expansion medium at a concentration of 2×10^6 cells.

3. Repeat Step 2, passaging cells in SILEC expansion medium three more times. At this point, CoA species should be greater than 98% labeled. If cell growth or morphological characteristics are considerably affected, see TROUBLESHOOTING.

   Pause Point Harvest and freeze down one plate of labeled cells in SILEC expansion medium containing 10% (vol/vol) DMSO. These cells can be thawed to seed future SILEC expansions. **?Troubleshooting**

4. After the fourth passage, allow cells to reach confluence or 1×10^7 cells per ml. Proceed to Step 6 if you do not want the ultimate incorporation of stable isotope label. Alternatively, perform Step 5.

5. (Optional) Wash cells with 10 ml of PBS, replace with SILEC ultra-labeling medium and incubate for 24 h. Perform this step if you want the highest level of stable isotope labeling, and then continue to Step 6. To produce purified [^{13}C3^{15}N]-CoASH, continue to Step 27.

Testing of SILEC labeling

6. *Short-chain acyl-CoA acid extraction (Steps 6-9).* Harvest one plate of cells by scraping gently into the medium and briefly spin down at 1,000*g*. The remaining plates should be kept until the first plate has been tested (Steps 7-20).

7. Resuspend cells in 1 ml of ice-cold extraction solution (10% (wt/vol) TCA).

8. Sonicate cells for 30 s on ice at a rate of 1 pulse per s.

9. Spin the sonicated cell lysate at 15,000*g* for 5 min at 4 °C to precipitate the protein pellet.

10. *SPE (Steps 10-16).* Using a vacuum manifold, condition Oasis HLB SPE columns with 1 ml of methanol.

11. Equilibrate columns with 1 ml of water.

12. Load acid-extracted lysate (supernatant) onto the columns.

13. Wash the columns with 1 ml of water.

14. Elute CoA compounds into fresh tubes by applying three subsequent applications of 0.5 ml of elution solution.

15. Dry the samples under nitrogen.

16. Resuspend in 50 μl of 5% (wt/vol) SSA.

17. *LC-MS analysis (Steps 17-20).* Using the LC-MS specifications in Figure 2, perform a CID neutral loss scan of 507 AMU, scanning a parent mass range of m/z = 750–1,200 AMU. Sample injections of 10 μl should be used for analysis.

18. *Characterizing the CoA SILEC Profile.* Generate a m/z list from the neutral-loss scan and compare them against a m/z list of CoA species (supplementary table 1); prepare a SRM experiment for each of these parent ions and their respective productions. The singly charged protonated molecule should be used for the parent CoA, whereas the associated product ion of 507 AMU lower than the parent protonated molecule should be used as the SRM product ion. For example, acetyl-CoA has a parent protonated molecule of m/z = 810.1 and a production at m/z = 303.1, whereas $[^{13}C_3{}^{15}N]$-acetyl-CoA has a parent protonated molecule of m/z = 814.1 and a product ion at m/z = 307.1.

19. *Determining labeling efficacy.* Reinject and analyze the sample using the LC-SRM/MS method, monitoring both the labeled and unlabeled channels to determine the amount of unlabeled CoA remains for each acyl-CoA.

20. *Confirmation of acyl-CoA species.* To confirm the identity of the labeled CoA species, spike extracted SILEC internal standards with a mixture of the CoA thioesters of interest (1 pmol each) and repeat the LC-SRM/MS analysis. The labeled and unlabeled compounds should co-elute. This is particularly important when multiple peaks arise or if there are multiple isobaric CoA species with similar parent and productions.

Critical step If there are certain CoA species you are interested in that are not represented in the mixture, further steps may need to be taken (see *Generating customized SILEC standards,* Steps 23–36). In the case of incomplete labeling, systematic troubleshooting may be necessary (see TROUBLESHOOTING). **?
Troubleshooting**

Harvesting

21. If labeling is complete (> 99% labeling) and CoA profile is satisfactory, perform CoA extraction for all plates (Steps 6–9).

22. Pool together acid-soluble SILEC extracts, label the batch and freeze at − 80 °C in 10-ml aliquots.

Pause Point The pooled acid-soluble extracts contain the SILEC internal standards that will later be spiked into cell or tissue samples. These standards can be stored for up to 1 year at − 80 °C, but they should be spiked into a new standard curve for each analysis. Avoid repetitive freeze-thaw cycles.

Generating customized SILEC standards

23. (Continued from Step 21) Wash half of the plates twice with PBS.

24. For each of the washed plates, replace with 10 ml of customization medium (see REAGENT SETUP) and incubate for 1 h.

25. Test labeling using Steps 17–20.

26. After incubation, harvest and extract CoA from all plates (Steps 6-9) and pool extracts together. This pooled extract should contain a SILEC CoA profile with increased levels of the species of interest.

Producing purified [^{3}c$_3$^{15}n]-CoASH

27. (Continued from Step 5) After overnight incubation in ultra-labeling medium, perform Steps 6–9 for all plates and pool together acid-soluble lysates, which contain CoASH and short-chain acyl-CoAs.

28. Adjust the pH of the lysate to   13 with 40% (wt/vol) KOH. Add 500 μl of KOH to 5 ml of TCA extract and incubate at room temperature for 1 h.

29. Adjust the pH of the lysate to 6 with formic acid.

30. Purify the labeled CoA using SPE extraction (Steps 10–16).

31. Dry down the hydrolyzed extract, which contains purified [$^{13}C_3$$^{15}N$]-CoASH. This product can now be used to chemically synthesize a particular CoA derivative[57–60].

Using SILEC standards for CoA analysis

32. Thaw an adequate amount of previously generated acid-extracted SILEC CoA internal standard mixture. Generally, 1 ml of internal standard mixture is needed for every five samples. After thawing, vortex to ensure that the sample is mixed thoroughly and keep it on ice.

   Critical step It is important to determine the amount of labeled SILEC CoA standard needed before the experiment. If multiple batches are necessary for a single experiment, these batches must first be pooled together before application to samples/standard curve. Owing to the biosynthetic nature of SILEC generation, there can be substantial batch-to-batch variability; similarly, using different batches of labeled internal standards in different samples of the same experiment can lead to erroneous measurements.

33. To prepare standard curve samples, thaw and mix equal concentrations of unlabeled CoA standards of interest, creating a master mix, and perform twofold serial dilutions, from 1 μM down to   10 nM, in extraction solution (10% (wt/vol) TCA). Mix 50 μl of each dilution with 0.75 ml of extraction solution and 0.2 ml of thawed SILEC CoA internal standard extract (for a final volume of 1 ml) and vortex. This will generate a standard curve from approximately 100 fmol to 10 pmol of each CoA standard on the column.

34. To prepare experimental samples, add 0.8 ml of ice-cold TCA along with 0.2 ml of the same SILEC internal standard mixture to harvested cell pellets, snap-frozen or lyophilized tissues, or purified extracted cells (platelets, lymphocytes, fibroblasts and so on.)

35. For cell samples, sonicate for 30 s on ice in a pulsatile manner. For tissue samples, homogenize according to standard protocols. In either case, perform the same processing step for the standard curve samples as well.

36. Spin processed acid-extracted lysate at 15,000*g* for 5 min at 4 °C to precipitate the protein pellet.

37. Perform SPE extraction of CoA species as previously described (Steps 10–16).

38. Resuspend the samples in 50 μl of 5% (wt/vol) SSA.

   Pause Point The samples and standards can be immediately analyzed or frozen at −80 °C for future analysis. As the SILEC CoA internal standards are included, effects on sample stability should be reflected in the internal standards as well.

39. For analysis, inject 10 μl from each sample or standard mix and perform LC-SRM/MS analysis using the method developed previously.

40. From the raw data, develop a standard curve for each of the CoA species, with the *x* axis as the known amount of injected standard and the *y* axis as the ratio of unlabeled to labeled integrated peak areas.

41. Use the regression line from this standard curve to calculate the amount of each CoA thioester in the samples. **?Troubleshooting**

Perturbation of cell growth kinetics by SILEC medium (step 3)

Changes in cell medium components may affect a number of cell growth parameters, such as doubling times, cell viability, adherence and morphological characteristics. Although slightly slower cell growth is expected with SILEC medium, if there is severely deranged cell growth or morphological changes, medium components should be serially investigated.

Serum

Although serum is necessary for many cell lines, the lack of serum standardization and lot-to-lot variability makes it very challenging to reproducibly scale up many biosynthetically generated compounds. Charcoal stripping is commonly used to standardize serum and csFBS is the optimal serum for our purposes, as it markedly reduces unlabeled pantothenate in the serum, which will contaminate the labeled CoA pool. In addition to removing pantothenate, however, charcoal stripping also removes a number of other serum components including hormones, growth factors and other B vitamins, which can affect cell growth[66,67]. One method to overcome this is by either increasing the amount of csFBS or using dFBS during the expansion stage, which may be enough to overcome these effects. Although dFBS would slightly increase the level of unlabeled pantothenate, it still has considerably less pantothenate than undialyzed serum. This may improve cell growth during the expansion phase. If it is found, however, that dFBS is necessary during the ultra-labeling step, the concentration should be substantially reduced and the concentration of pantothenate should be increased to lower the percentage of unlabeled pantothenate in the serum. If both csFBS and dFBS are prohibitive to cell growth, and uFBS is necessary, a different cell line should be considered, as the high level of residual pantothenate in uFBS would be difficult to overcome by simply adding more labeled compound.

Other medium components

Commercial services are available for the production of specialty medium such as pantothenate-omitted RPMI 1640 medium, which is used in our protocol. However, should you choose to prepare your own SILEC medium, ensure that all components are dissolved and that your cells show similar growth characteristics when this medium is supplemented with standard FBS and the same concentration of pantothenate. As certain medium components can also degrade over time, prepared medium should be kept at − 20 °C for long-term storage. Finally, as with any cell culture system, contamination with mycoplasma

may result in decreased cell growth. Therefore, thaw fresh cells or perform simple tests for mycoplasma to eliminate this possibility.

Incomplete labeling (step 20)

There are three underlying causes for incomplete labeling: (i) contaminating unlabeled pantothenate in the medium, (ii) unlabeled pantothenate in the $[^{13}C_3{}^{15}N]$-pantothenate stock and (iii) insuffcient turnover of pantothenate or CoA pool within the cell line.

First, determine whether there is contaminating unlabeled pantothenate in the medium, as this is the most likely cause of the incomplete labeling. For mammalian cell culture, if the pantothenate-omitted medium was commercially prepared, verify that pantothenate is not in the formulation. If this medium was prepared in-house, verify that the base nutrient mix you are using does not contain any pantothenate. For insect cell culture, Schneider's medium contains yeast extract, which contains a variable (although marked) amount of different B vitamins, including pantothenate. For this reason, a higher concentration of labeled compound is needed for insect SILEC preparation.

If there is incomplete labeling in these cells, it could very well be coming from increased amounts of pantothenate in the yeast extract (yeastolate). To overcome this, the ultra-labeling step can be performed overnight with HBSS medium containing 4 mg per liter labeled pantothenate, which should increase labeling to greater than 99%. However, it is more likely that the unlabeled pantothenate is originating from the csFBS. Although there should be < 0.1 mg per liter of pantothenate remaining in the csFBS[18] (resulting in an effective 0.01 mg per liter in medium containing 10% serum), it is possible that a particular lot may have more, or that a higher concentration of serum is needed for a particular cell line. Adding additional labeled pantothenate to the medium should overcome this problem. If this is a newly occurring problem, check the lot of the csFBS, as lot-to-lot variability can exist for serum. If a particular lot of csFBS has worked in the past, it may be worth ordering several bottles of this for future use. The serum can be frozen and stored at −20 °C for long-term storage.

The second possibility is that the stock itself is contaminated with unlabeled pantothenate. This can be assessed by performing mass spectral analysis of the labeled stock. To do this, reconstitute a small amount of the labeled pantothenate stock and using flow-infused syringe injection and SRM/MS analysis. The relative ratio of labeled to unlabeled pantothenate represents the maximal purity that the CoA can be labeled. If this is not acceptable, another source of labeled pantothenate should be sought. For our experiments, we found that the isotopic purity of the stock was 99.5%.

Finally, the last problem that can arise, particularly with suspension culture, is that a certain amount of the unlabeled compound is not turning over and remains in the cellular pool because of cell death or senescence. This is more of a problem with SILAC protocols because different proteins turn over at various frequencies, and salvage pathways of particular amino acids may cause these unlabeled species to continue to propagate in culture. In addition, unlabeled albumin may also be taken up by cells. For CoA species, this turnover is greater and more than 50% of the CoA can turn over in the cell in 24 h (ref. 18), although different CoA species may not turn over as rapidly. To overcome this problem, simply passage the cells two or three more times in the expansion medium. If the cells are being split into 1:3, < 0.5% of the original cell mass will remain after five passages. Although this may add an additional week to the expansion phase, it would replace at least 99.5% of the original cell mass and, likewise, of the original unlabeled CoA.

Problems in sample preparation and LC-Ms

Solid-phase extraction

Oasis HLB columns have been found to be optimal for short-chain acyl-CoA extraction, although other methods such as liquid-liquid extraction could be used as well[18,47]. However, when the experiment involves more complex biological matrix such as in tissue samples, or if a larger amount of biological material is being used (to measure lower abundance analytes), a greater matrix effect may be observed. In this case, repeating the SPE washing step once or twice may help. In addition, using a molecular weight cutoff filter before SPE extraction may also reduce the matrix effect, although this will increase processing time. The use of methanol containing concentrated ammonia may improve elution[47].

HPLC

Although acyl-CoA analysis is generally robust, repeated injections of cell and tissue extracts can lead to buildup of biological materials on the column, which can lead to distortions in peak shape[27]. For this reason, we have added solvent C in a column wash step. If peak distortion is occurring, increasing the volume of the wash step, as well as slowing down the ramp time for washing, can improve column performance, although this will also increase run times. In addition, lowering the pH of this wash can also improve the ruggedness of the method.

Mass spectrometry

In the case of inadequate sensitivity, use acetonitrile instead of methanol in the mobile phase whenever possible, and decrease formic acid concentration during MS analysis. Moreover, if LC-SRM/MS analysis is increased to include a large number of species, a proper number of analytical points may not be achieved across each peak and may lead to incorrect quantification, even with internal standards. To overcome this, SRM dwell time should be reduced so as to increase the number of points across the peak. For example, if eight CoA species along with their internal standards are to be measured (16 analytes in total), using dwell times of 80–100 ms may be acceptable. However, if the number of analyzed CoA species is increased to 20–30 species (40–60 total), shorter dwell times (20–30 ms) may be necessary to achieve enough points across the peak. In addition, dividing up the analysis into shorter segments can allow for longer dwell times.

● Timing

Typical times used in our laboratory for the various steps are as follows:

SILEC labeling—Expansion: 12–16 d

SILEC labeling—Ultra-labeling: 1 d

SILEC labeling—Testing of labeling: 3 h

SILEC labeling—Customization: 1–12 h

SILEC labeling—Harvesting and extraction: 30 min

Application of internal standards—Sample preparation: 1 h

Application of internal standards—Analysis: 30 min per sample, 1 d per experiment (total time: 15–20 d)

Anticipated Results

The SILEC approach presented in this study can be used to generate labeled short-chain acyl-CoA MS standards to improve specificity and precision in mass spectral analysis. As previously mentioned, a crucial aspect of the biosynthetic generation of labeled standards is minimizing the unlabeled fraction. Typical LC-SRM/MS chromatograms of reduced CoA (CoASH) harvested from [$^{13}C_3$$^{15}N$]-pantothenate-labeled cells are provided in Figure 7. Figure 7a shows an example of insufficient labeling, with > 5% of the CoASH unlabeled, whereas Figure 7b depicts CoASH from a SILEC batch with more complete labeling (< 1% unlabeled). In addition, cumulative spectra from a LC-constant neutral loss/MS scan analysis of CoA (m/z = 507) are presented throughout this report to illustrate the labeling and customization of various CoA reference metabolomes. Unlabeled cells are shown in Figure 1b, whereas examples of SILEC-labeled CoA reference metabolomes customized with fatty acids or xenobiotics are shown in Figures 5 and 6a, respectively. Furthermore, a representative chromatogram of menadione-CoA is presented in Figure 6b. It should be noted that using exogenous biological and toxicological stimuli to change the CoA profile in a given cell type is largely empirical and needs to be optimized for different cell systems and conditions. Before scaling up, optimization can be performed in unlabeled cells, although it should be confirmed in labeled cells as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HPLC parameters

Autosampler:	Leap CTC Autosampler (CTC Analytics)
HPLC system:	Agilent 1100 Series (Agilent Technologies)
Column:	Luna C18 (2 mm × 150 mm, 3 μm pore) (Phenomenex, Torrance, CA)
Solvents:	A: 5 mM NH_4OAc in H_2O
	B: 5 mM NH_4OAc in 95/5 ACN/H_2O (vol/vol)
	C: 80/20/0.1 ACN/H_2O/formic acid (vol/vol/vol)

Time (min)	Flow rate (μl min⁻¹)	A (%)	B (%)	C (%)	Valco valve
0.0	200	98	2	0	
1.5	200	98	2	0	To waste
5.0	200	75	25	0	
5.5	200	0	100	0	
8.0	200	0	100	0	To MS
13.0	200	0	100	0	
14.0	200	0	100	0	
16.0	200	0	0	100	
20.0	200	0	0	100	To waste
26.0	200	98	2	0	
32.0	200	98	2	0	

Mass spectrometer:	API 4000 triple quadrupole mass spectrometer (Applied Biosystems)
Mode:	positive electrospray ionization (ESI)

Mass spectrometry parameters

Flow-dependent parameters		Compound-dependent parameters	
Ion spray voltage:	5,000 V	Declustering potential:	105 V
Curtain gas:	15 units	Entrance potential:	10 V
Ion source gas 1:	8 units	Collision energy:	45 eV
Ion source gas 2:	15 units	Collision exit potential:	15 V
CAD gas:	5 units	Dwell time (MRM):	30–80 ms
Probe temperature:	450 °C	Dwell time (NL scan):	1 s

Figure 1.
LC-MS analysis of acyl-CoA thioesters. (**a**) Generalized CoA structure, showing the CID-induced neutral loss of the ATP moiety (m/z = 507). The substituent (R group) can include any thioester or derivative of CoA arising from endogenous or exogenous sources (see supplementary table 1 for a list of typical endogenous CoA species). (**b**) LC-constant neutral loss/MS scans (m/z = 507) of short-chain acyl-CoA species extracted from unlabeled Hepa 1c1c7 mouse hepatoma cells. The extraction was performed as described in the accompanying protocol using the specific LC-MS parameters provided in Figure 2. The precursor parent molecules of various short-chain acyl-CoA species are annotated in the spectrum. c.p.s., counts per second.
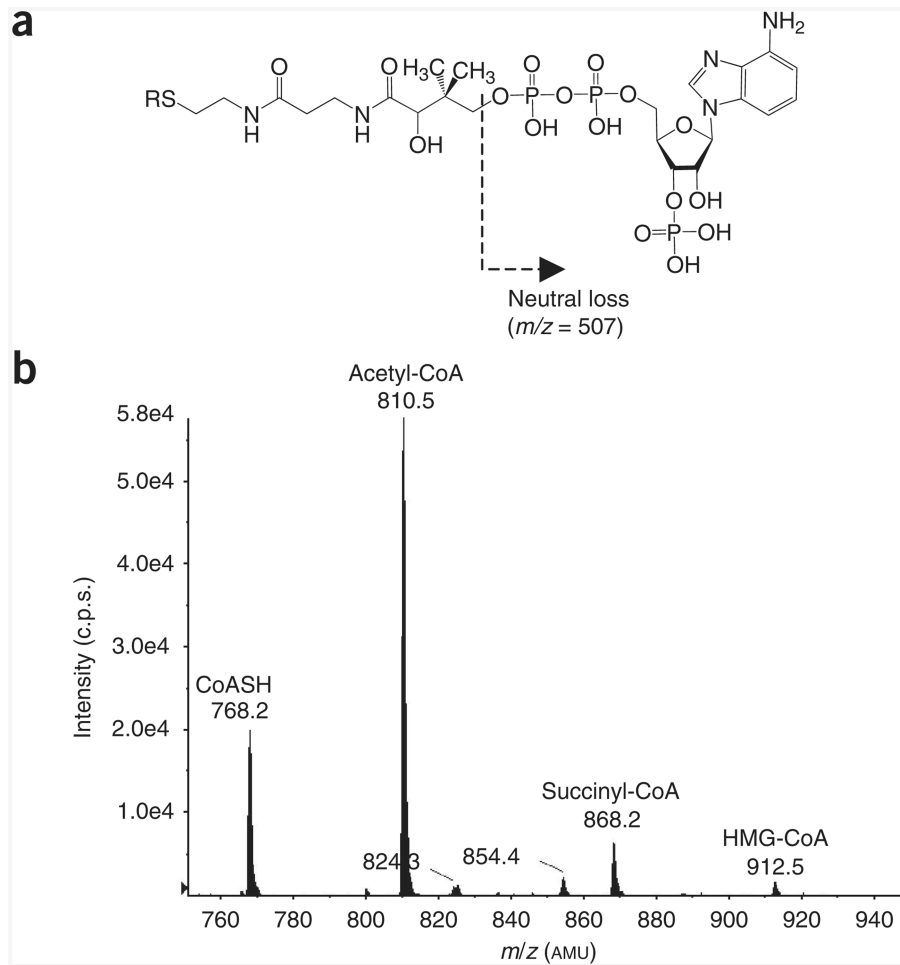
Figure 2.
LC-MS parameters used in our laboratory for the analysis of short-chain acyl-CoA thioesters.
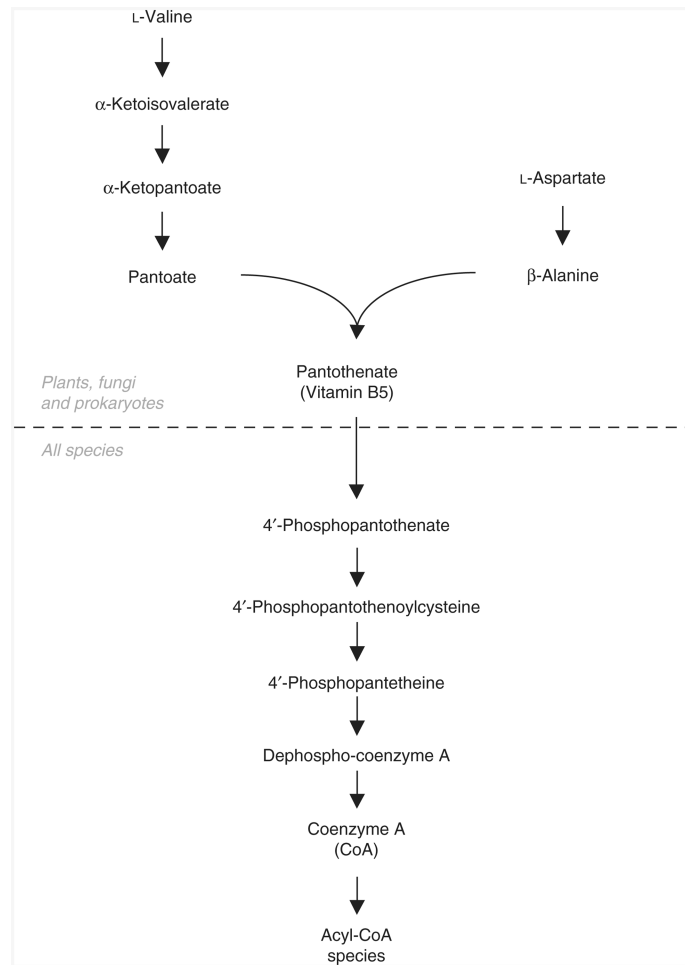
Figure 3.
Pantothenate and coenzyme A biosynthesis. Plants, fungi and many prokaryotes are capable of *de novo* pantothenate synthesis from valine and aspartate, whereas animals require it as part of their diet.
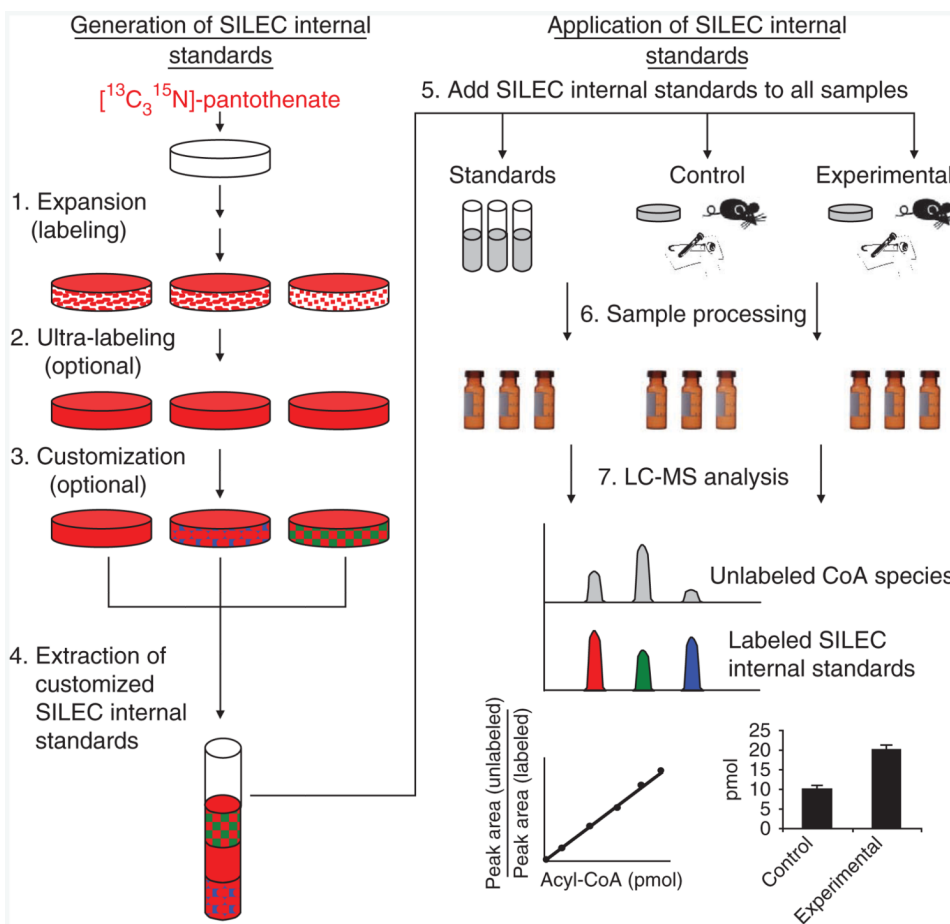
Figure 4.
General scheme for stable isotope labeling by essential nutrients in cell culture (SILEC). (1) Expansion: cells are serially passaged and expanded in the presence of labeled pantothenate. (2) Ultra-labeling: medium containing less serum and more labeled pantothenate is used to increase purity of labeled CoA. (3) Customization: labeled cells are subjected to different biological, pharmaceutical or toxicological exposures to modify the CoA derivative profile within the cells. (4) Extraction: cells are lysed and pooled together to generate a SILEC CoA internal standard mix with a more global CoA profile. (5) SILEC spike-in: equal amounts of SILEC internal standards are spiked into CoA standards, as well as *in vitro*, *in vivo* or into clinical experimental samples. (6) Sample processing: standards and samples are subjected to the same sample extraction procedures. (7) LC-MS analysis: CoA species are separated and analyzed by LC-SRM/MS. Co-eluting SILEC internal standards are used to confirm the identity and normalize for the different CoA species.
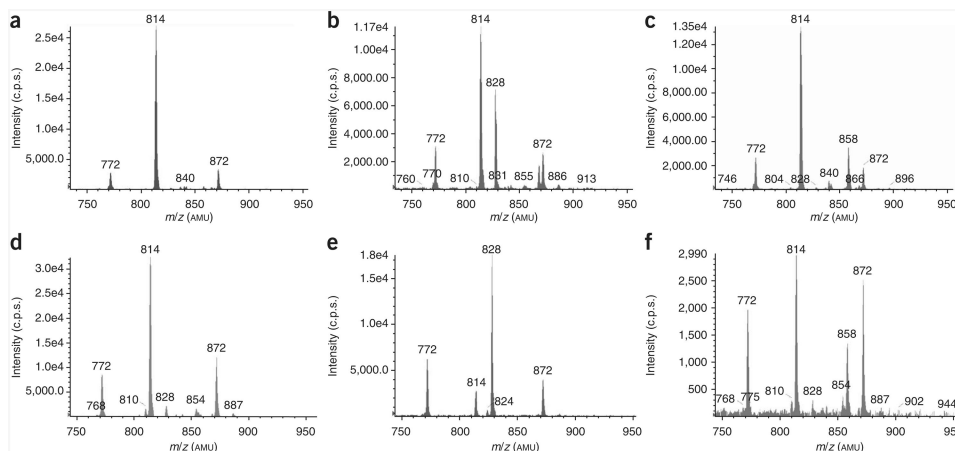
Figure 5.
SILEC labeling and 'customization' of CoA metabolome. (**a**–**c**) LC–neutral loss/MS scans ($m/z = 507$) of acid-extracted acyl-CoA species in SILEC-labeled Hepa 1c1c7 cells that were untreated (**a**), treated with 10 mM propionate for 1 h (**b**) or treated with 10 mM -hydroxybutyrate for 1 h (**c**). (**d**–**f**) LC–neutral loss/MS scans ($m/z = 507$) of acid-extracted acyl-CoA species in *Drosophila* S2 cells that were untreated (**d**), treated with 10 mM propionate for 1 h (**e**) or treated with 10 mM -hydroxybutyrate for 1 h (**f**). The precursor ion masses of various labeled short-chain acyl-CoA species can be identified: $[^{13}C_3{}^{15}N]$-acetyl-CoA ($m/z = 814$), $[^{13}C_3{}^{15}N]$-succinyl-CoA ($m/z = 872$), $[^{13}C_3{}^{15}N]$-CoASH ($m/z = 772$), $[^{13}C_3{}^{15}N]$-propionyl-CoA ($m/z = 828$) and $[^{13}C_3{}^{15}N]$- -hydroxybutyryl-CoA ($m/z = 858$). These 'customized' CoA extracts can be pooled together to generate a more comprehensive CoA profile. Extraction was performed as specified in the protocol (Fig. 2).
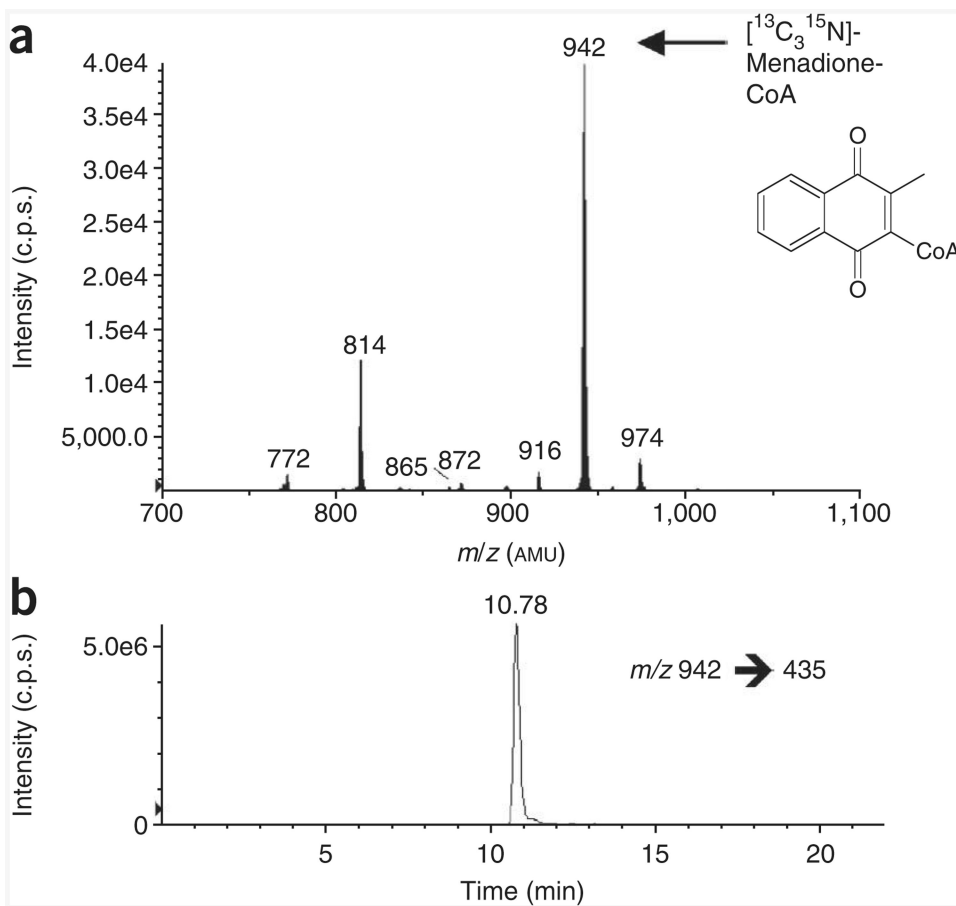
Figure 6.
Biosynthetic generation of isotopically labeled menadione-CoA. (**a**) LC–neutral loss/MS scan ($m/z = 507$) of acid-extracted CoA species from Hepa 1c1c7 cells treated with 20 µM menadione for 1 h. (**b**) LC-SRM/MS chromatogram of [$^{13}C_3$$^{15}N$]-menadione-CoA ($m/z = 942 \quad 435$) derived from the same extract. Extraction and analysis were performed as specified. By spiking this extract into experimental samples, this isotopically labeled analyte can be used as an internal standard for quantifying unlabeled menadione-CoA.
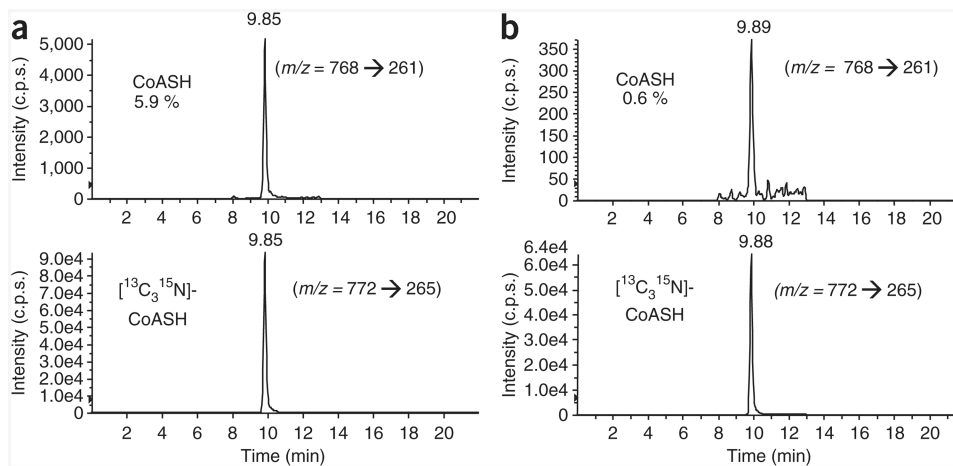
Figure 7.
LC-SRM/MS chromatograms of CoASH harvested from [^{13}C$_3$^{15}N]-pantothenate-labeled cells. Both unlabeled CoASH (m/z = 768 → 261) and [^{13}C$_3$^{15}N]-CoASH (m/z = 772 → 265) chromatograms are presented to illustrate the labeling efficiency. (**a**) Suboptimal labeling: the amount of unlabeled CoASH was 5.7%. (**b**) Near-complete labeling: the amount of unlabeled CoASH was 0.5%. Extraction and analysis were performed as specified in the protocol.

Desired acyl-CoA-thioester	Modifying compound (concentration)
Propionyl-CoA	Propionic acid (10 mM)
-Hydroxybutyryl-CoA	-Hydroxybutyric acid (10 mM)
Menadione-CoA	Menadione (20 μM)