Lipidomics study in liver metabolic diseases

Singh, M.

Chapter 4

Recent developments in the analytical approaches of acyl-CoAs to assess their role in mitochondrial fatty acid oxidation disorders

Based on

Recent developments in the analytical approaches of acyl-CoAs to assess their role in mitochondrial fatty acid oxidation disorders

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Abstract

Fatty acid oxidation disorders (FAOD) are inborn errors of metabolism that occur due to deficiency of specific enzyme activities and transporter proteins involved in the mitochondrial metabolism of fatty acids, causing a deficiency in ATP production. The identification of suitable biomarkers plays a crucial role in predicting the future risk of disease and monitoring responses to therapies. Acyl-CoAs are directly involved in the steps of fatty acid oxidation and are the primary biomarkers associated with FAOD. However, acyl-CoAs are not used as diagnostic biomarkers in hospitals and clinics as they are present intracellularly with low endogenous levels. Additionally, the analytical method development of acyl-CoAs is quite challenging due to diverse physicochemical properties and instability. Hence, secondary biomarkers such as acylcarnitines are used for the identification of FAOD. In this review, the focus is on the analytical techniques that have evolved over the years for the identification and quantitation of acyl-CoAs. Among these techniques, liquid chromatography-mass spectrometry clearly has an advantage in terms of sensitivity and selectivity. Stable isotope labeling by essential nutrients in cell culture (SILEC) enables the generation of labeled internal standards. Each acyl-CoA species has a distinct pattern of instability and degradation, and the use of appropriately matched internal standards can compensate for such issues. Although significant progress has been made in measuring acyl-CoAs, more efforts are needed for bringing these technical advancements to hospitals and clinics. This review also highlights the difficulties involved in the routine use of acyl-CoAs as a diagnostic biomarker and some of the measures that can be adopted by clinics and hospitals for overcoming these limitations.

Keywords

Acyl-CoA; Fatty acid oxidation disorders; Newborn screening; LC-MS; Biomarkers; SILEC

1. Introduction

Mitochondrial fatty acid oxidation disorders (FAOD) are inborn errors of metabolism resulting from defects in the mitochondrial fatty acid oxidation (FAO) pathway. These disorders are presented through various symptoms affecting the quality of life of patients and often lead to sudden death of infants. The diagnosis of FAOD is done through newborn screening and novel diagnostic biomarkers can help for the detection of the disorder at an early stage of life and subsequently provide a suitable personalized treatment. Acyl-Coenzyme A (Acyl-CoAs) are intermediate compounds formed during the process of FAO, and their profiles can be used as biomarkers for FAOD. Coenzyme A (CoASH, also called as free CoA) is synthesized naturally from pantothenic acid, also known as vitamin B5 or pantothenate [1,2]. Pantothenate is an essential nutrient present in foods such as eggs, milk, beef, vegetables and grains [3]. Pantothenate is used to synthesize CoASH through five enzymatic steps which further leads to the formation of acyl-CoA thioesters as mentioned in **Figure 1** [2,4]. Acyl-CoAs are generated from the actions of acyl-CoA synthetase or ketoacid dehydrogenase [4,5]

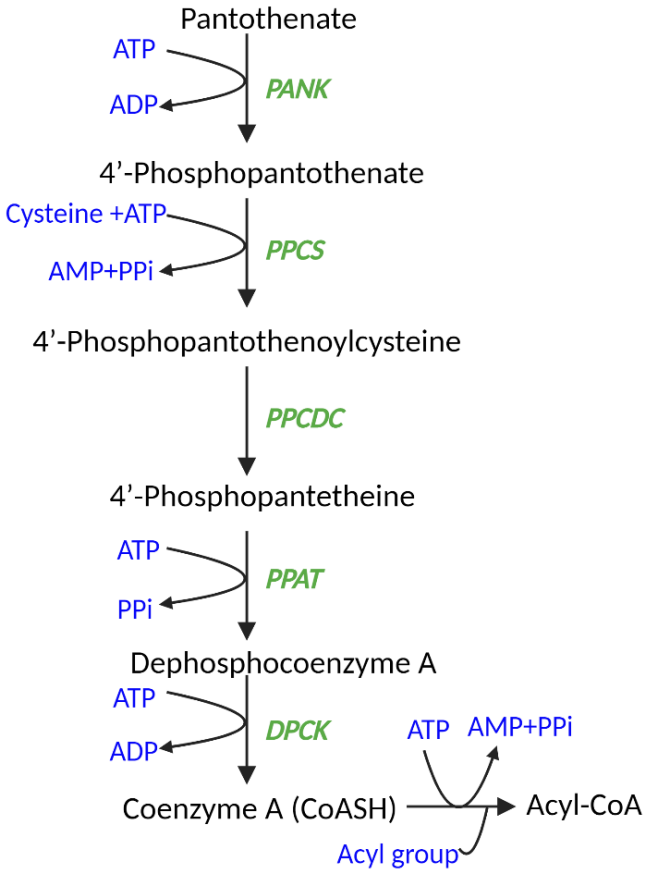


Figure 1. Biosynthetic pathway of CoASH and acyl-CoAs. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; PPi, pyrophosphate; PANK, pantothenate kinase; PPCS, phosphopantothenoylcysteine synthetase; PPCDC, phosphopantothenoylcysteine decarboxylase; PPAT, phosphopantetheine adenylyltransferase; DPCK, dephosphocoenzyme A kinase.

In FAOD, acyl-CoA molecules accumulate depending on the type of disorder and result in energy deficiency and other toxic effects. However, due to several analytical challenges associated with acyl-CoAs, acylcarnitines are commonly employed as diagnostic biomarkers for these disorders. Acyl-CoAs are quite diverse in their physicochemical structures due to varying fatty acyl chain length, unsaturation index and functional groups. They lack resonance stabilization of the energy-rich thioester bond which makes them prone to hydrolysis [6], hence they degrade in alkaline and strongly acidic solutions [7]. Also, these molecules have thiols in their structure which are prone to oxidation or other intra and extracellular reactions [8]. Apart from chemical instability, acyl-CoA species are also unstable in biological samples and often lower signals are reported as they can form anhydrides [9] or S-acyl glutathione [10,11] in tissue samples. Therefore, analysis of these molecules is quite challenging and requires careful sample handling and measurement strategies. Nevertheless, it is important to be able to measure concentrations of these molecules as CoASH and acyl-CoAs have numerous important roles in various biological processes. CoASH is an important co-factor in various cellular oxidative reactions and biosynthetic processes [4] and acts as an activator of carboxylic acids for biochemical transformation and carrier of acyl groups [12]. CoASH and acyl-CoAs are involved in fatty acid synthesis and complex lipid synthesis and also reported to have an important role in cancer [13,14], diabetes [15,16], xenobiotics metabolism [4,17], pantothenate kinase-associated neurodegeneration [18], etc.

This review considers the important role of acyl-CoAs as these are directly involved in the FAO pathway. Developing analytical techniques to enable the detection of acyl-CoAs is quite important to assess their roles in FAOD. The detection and measurement of acyl-CoAs, in addition to acylcarnitines, can enable researchers and clinicians to gain insight into defective enzymes responsible for FAOD and hence, provide for appropriate and timely treatments. In this review, the focus is on the analytical techniques that have evolved over the years for identification and quantitation of acyl-CoAs, limitations involved in the routine use of acyl-CoAs as the diagnostic biomarker for FAOD and efforts that can be taken to bridge the gap between technological advancement and routine diagnosis.

2. Role of CoASH (free CoA) and acyl-CoAs in FAO

2.1 Mechanism of mitochondrial FAO: an essential process for survival

During prolonged fasting and strenuous exercise, the blood glucose level decreases and the body needs compensatory mechanisms to replenish energy sources. In the first few hours of

fasting, the glycogen reserve is used to maintain the blood glucose level and after its depletion, fats are used as the energy source, where FAO is a key pathway for energy supply [19–21]. Peroxisomes and mitochondria are the two sites for FAO, where mitochondrial FAO pathway facilitates the oxidation of fatty acids (FA) with ≤20 carbons while peroxisomal FAO involves the oxidation of very long-chain FA with >20 carbons and branched-chain FA [22]. Peroxisomal beta-oxidation is involved in biosynthetic pathways while mitochondrial beta-oxidation is involved in the production of ATP [23]. Most tissues such as heart, skeletal muscles and liver, with the exception of the brain, can use FAO as an energy source and it has been reported that approximately 80% of the total requirement of energy during fasting is provided by FAO [21,24,25]. Brain, on the other hand, can use ketone bodies produced by the liver during FAO as the major source of energy during periods of fasting [26,27].

In the presence of insulin, excess glucose from the diet is stored in the form of glycogen, which acts as a reserve for glucose for brain and other tissues [28,29]. In the initial few hours of fasting, the insulin level will decrease, activating glycogenolysis in liver and muscles to maintain the normal blood glucose level [27,29]. As the fasting period continues, the hepatic glycogen stores deplete and at this stage gluconeogenesis and FAO play an important role in maintaining energy balance. Gluconeogenesis is a process of glucose synthesis from non-carbohydrate sources, occurring primarily in the liver and kidney [30]. As the main focus of this review is on disorders related to FAO, we will describe FAO pathway in a detailed way.

FA serves as substrate for FAO and can be taken up by cells through multiple pathways. These pathways include dietary intake, the synthesis of FA within the body (*de novo* synthesis), or the breakdown of triglycerides stored in adipose tissues, resulting in the release of free fatty acids [19,31–33]. The FAO pathway is driven by multiple enzymes involved at different stages of this pathway. The medium- (C6-C10) and short-chain (<C6) fatty acids can diffuse freely across the plasma and mitochondrial membrane and subsequently enter inside the mitochondrial matrix where they get activated to acyl-CoA esters [34]. The long-chain (C12-C20) fatty acids are transported across the plasma membrane by protein carriers such as fatty acid transport proteins, fatty acid translocase and fatty acid-binding proteins [21,34]. After entering inside the cytosol, the long-chain FA are converted to fatty acyl-CoA esters via a thioesterification reaction with the help of cofactor CoASH by the action of acyl-CoA synthetase enzymes [35]. The mitochondrial membrane is impermeable to acyl-CoA esters, hence the transport of long-chain fatty acyl-CoA esters into the mitochondria requires the carnitine cycle **(Figure 2A)**. The carnitine cycle is mediated through different membrane bound proteins. Carnitine transporter

(OCTN2) will facilitate the entry of carnitine to the cell. This carnitine will be taken up by the carnitine palmitoyltransferase 1 (CPT1) present in the mitochondrial outer membrane, which is responsible for converting acyl-CoAs into acylcarnitines [36]. CPT1 catalyzes the rate limiting step of FAO in liver [37]. During the fasting state, malonyl-CoA (an inhibitor of CPT1) decreases, thus activating CPT1 which subsequently enhances FAO, while the opposite happens during the fed state [38]. Carnitine-acylcarnitine translocase (CACT) promotes the transfer of these acylcarnitines into the mitochondrial matrix. Long-chain acylcarnitine molecules are then re-esterified into their corresponding acyl-CoA esters by carnitine palmitoyltransferase 2 (CPT2) present on the mitochondrial inner membrane. Inside the mitochondrial matrix, the activated fatty acids in the form of acyl-CoA thioesters will undergo FAO (**Figure 2A**), which consists of four steps with different enzymes as depicted in **Figure 2B.**

Step-1 Acyl-coenzyme A dehydrogenases (ACAD) are the first set of enzymes involved in the cyclic process of FAO. Depending on the fatty acid chain length, there are different members in this class of enzymes which include very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and short-chain acyl-CoA dehydrogenase (SCAD). These enzymes dehydrogenate the acyl-CoA ester to yield a trans-2-enoyl-CoA.

Step-2 The next enzyme involved in the process, 2-enoyl-CoA hydratase, is responsible for the hydration of the double bond, resulting in a 3-hydroxyacyl-CoA.

Step-3 In the third step, 3-hydroxyacyl-CoA is dehydrogenated to 3-ketoacyl-CoA by the action of the enzyme 3-hydroxyacyl-CoA dehydrogenase.

Step-4 The final step of this cycle is thiolytic cleavage of the 3-ketoacyl-CoA by enzyme 3-ketoacyl-CoA thiolase resulting in one molecule of acetyl-CoA and a two carbon chain-shortened acyl-CoA [21].

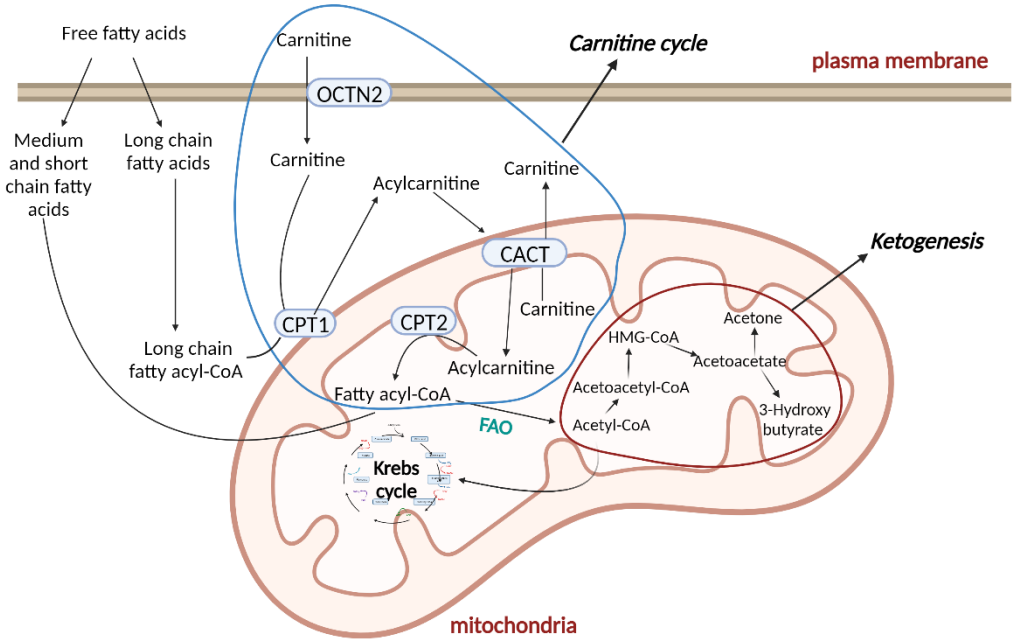
The acetyl-CoA formed at the end of this cycle will enter the Krebs cycle while the two carbon shortened acyl-CoA re-enters the FAO cycle for further oxidation. One round of mitochondrial beta-oxidation forms one $FADH_2$, one NADH, one acetyl-CoA and an acyl-CoA with two carbons less than the initial acyl-CoA [17]. The functions of 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase are executed by mitochondrial trifunctional protein (MTP), located in the mitochondrial inner membrane [39].

The odd chain fatty acids undergo the same steps of FAO but the last cycle with five carbon chain will form one molecule each of acetyl-CoA and propionyl-CoA. This propionyl-CoA is carboxylated to form D-methylmalonyl-CoA which further racemizes to form L-

methylmalonyl-CoA and undergoes isomerization to form succinyl-CoA, an intermediate for the Krebs cycle [40,41]. The unsaturated fatty acyl-CoA esters need additional auxiliary enzymes 2,4-dienoyl-CoA reductase and Δ^3-Δ^2-enoyl-CoA isomerase to act on the double bonds [42,43]. 2,4-dienoyl-CoA reductase reduces polyunsaturated fatty acid chains having double bonds at 2,4 positions (using NADPH) to form trans-3-enoyl-CoA, while Δ^3-Δ^2-enoyl-CoA isomerase catalyzes cis or trans double bonds at position 3 of fatty acyl-CoA to a trans double bond at position 2.

Acetyl-CoA, generated from FAO, combines with oxaloacetate in the Krebs cycle. In low glucose conditions, oxaloacetate is directed towards gluconeogenesis while acetyl-CoA is redirected towards the production of ketone bodies [44]. The ketogenesis in liver increases with an increase in FAO due to enhanced production of acetyl-CoA. In the mitochondrial matrix of liver cells, the acetyl-CoA molecules generated from the FAO pathway are used for ketogenesis (**Figure 2A**). Two acetyl-CoA molecules condense to form acetoacetyl-CoA by enzyme acetoacetyl-CoA thiolase. Further, mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase mediates the formation of HMG-CoA from acetoacetyl-CoA. HMG-CoA is further cleaved by HMG-CoA lyase to forms acetoacetate and is further reduced to 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase. The decarboxylation of acetoacetate will yield acetone. Acetoacetate and 3-hydroxybutyrate are transported to the brain and other extrahepatic tissues through the bloodstream where they are further converted back to acetyl-CoA for entering in the Krebs cycle to generate ATP [44].

(A)



(B)

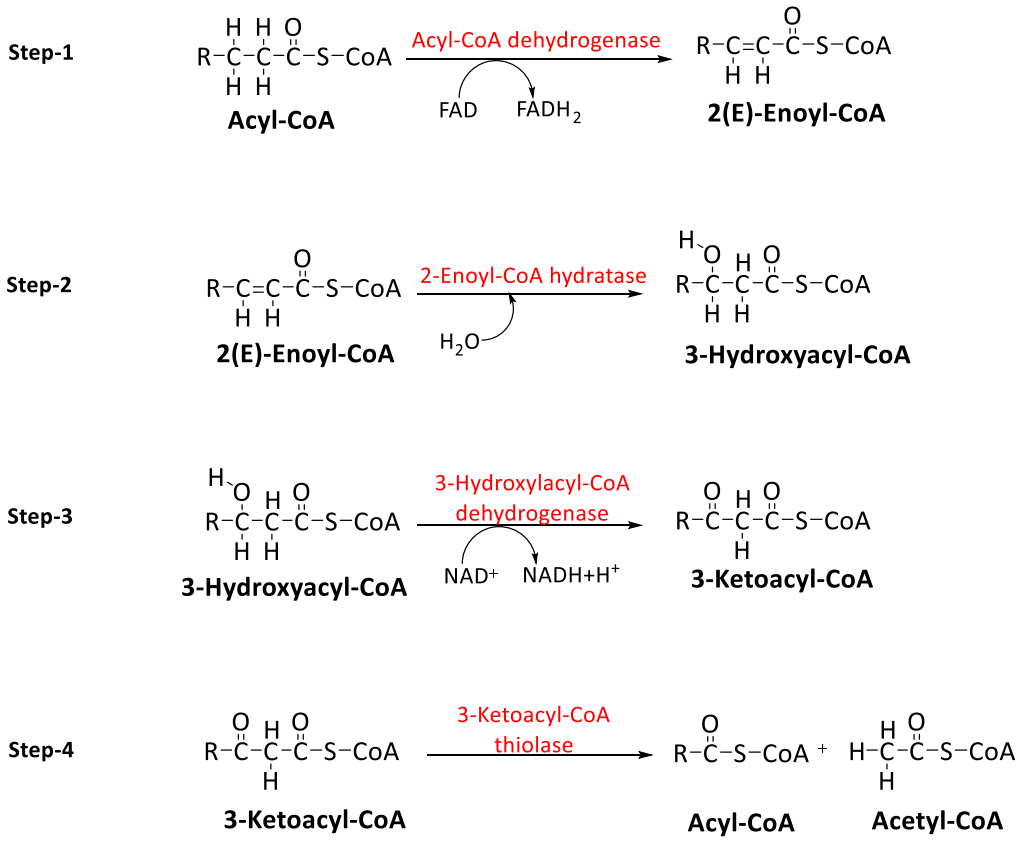


Figure 2. (A) Illustration of different stages involved in fatty acid oxidation cycle.; (B) Four key enzymatic steps of fatty acid oxidation. The first step is the uptake and activation of fatty acids followed by transport of fatty acids inside the mitochondria by the carnitine cycle. The activated fatty acids, in the form of acyl-CoA undergo fatty acid oxidation. OCTN2, carnitine transporter; CPT1, carnitine palmitoyltransferase 1; CACT, carnitine-acylcarnitine translocase; CPT2, carnitine palmitoyltransferase 2; FAD, flavin adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide**;** NADH, reduced nicotinamide adenine dinucleotide.

2.2 Disorders of fatty acid oxidation

FAOD are group of rare diseases that occur due to autosomal recessive inheritance that can lead to deficiency in energy. There are several types of FAO defects. Depending on the enzyme deficiency, they are divided in different categories [34,45,46] as shown in **Table 1**.

Table 1. Types of FAOD.

Categories of disorders	Enzyme Deficiency
Related to plasma membrane	Carnitine transporter (OCTN2)
	Long-chain fatty acid transporter protein (FATP1)
	Long-chain fatty acyl-CoA synthetase (LACS)
Related to transport through mitochondrial membrane	Carnitine palmitoyltransferase 1 (CPT1)
	Carnitine-acylcarnitine translocase (CACT)
	Carnitine palmitoyltransferase 2 (CPT2)
Beta-oxidation of long-chain inside mitochondria	Very long-chain acyl-CoA dehydrogenase (VLCAD)
	Long-chain acyl-CoA dehydrogenase (LCAD)
	Mitochondrial trifunctional protein (MTP)
	Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD)
	Long-chain 2-enoyl-CoA hydratase (LCEH)
	Long-chain 3-ketoacyl-CoA thiolase (LCKAT)
Beta-oxidation of medium-chain inside mitochondria	Medium-chain acyl-CoA dehydrogenase (MCAD)
	Medium-chain 3-hydroxyacyl-CoA dehydrogenase (MCHAD)
	Medium-chain 3-ketoacyl-CoA thiolase (MCKAT)
Beta-oxidation of short-chain inside mitochondria	Short-chain acyl-CoA dehydrogenase (SCAD)
	Short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD)
	Short-chain 2-enoyl-CoA hydratase (SCEH)
Related to deficiency of electron transfer flavoproteins	Multiple acyl-CoA dehydrogenase (MAD)

The prevalence of these disorders is approximately one in 5000-10000 births, and may cause severe life threatening complications in infants within a few hours of fasting and in adults after 48 h of fasting [46,47]. The clinical onset of symptoms is quite variable depending on the patient's age. In newborns and infants, the basal energy need is higher, their brains need more glucose, hence they are most susceptible for these disorders compared to older children and adults [48–50]. Hypoglycemia, hepatic steatosis and liver dysfunction are associated with all types of FAODs [20,33,39,46]. Several long-chain FAODs and disorders related to fatty acid transport across the mitochondrial membrane impact the cardiac muscle (symptoms include

cardiomyopathy, arrhythmia and sudden death) and skeletal muscles (myopathy and rhabdomyolysis). Other organs affected rarely include the retina in LCHAD and kidney in VLCADD. Due to defects in the FAO, the intracellular accumulation of fatty acyl-CoA species and corresponding carnitine or glycine conjugates will increase in liver, skeletal muscle and heart, leading to impaired functionality of these organs [51,52]. The impaired functioning of liver results in a decrease in the production of ketones, which are an important energy source for the brain in glucose deficit conditions. The combined effects of reduced glucose production and impaired hepatic ketogenesis result in depletion of available energy substrates, thus hampering the normal functioning of brain.

3. Acyl-CoA as a diagnostic biomarker for FAOD

Newborn screening (NBS) is performed after birth for the diagnosis of FAOD. The current approach in hospitals and clinics is based on the routine profiling of secondary biomarkers such as acylcarnitines. Different types of FAOD can be distinguished based on the nature of acylcarnitines accumulated. Acyl-CoAs are directly involved in the steps of the FAO pathway and hence they are the primary biomarkers associated with these FAOD [53]. However, they are not routinely used, and instead acylcarnitines are the first choice as biomarkers for FAOD due to various reasons. Acylcarnitines can be analyzed from easily available matrices of patients such as plasma, blood, serum and urine, while acyl-CoAs are present only intracellularly, requiring tissue samples for their analysis which causes difficulties, especially in the case of newborns and infants. Additionally, acyl-CoAs are relatively unstable compounds, are present at low concentrations, and often require complicated chromatographic separations due to the wide polarity range between short- and long-chain species. Nevertheless, acyl-CoAs can provide more accurate insights as they are primarily located within the cellular matrix and are directly involved in the metabolic processes, making them more specific markers of cellular metabolic status. The changes in their levels can directly reflect changes in enzymatic activity and metabolic flux. Although acylcarnitines indicate changes in fatty acid metabolism, they are not central to the metabolic pathways like acyl-CoAs and can be influenced by factors such as carnitine availability. Hence, profiling of both acyl-CoA and acylcarnitines are quite important in interpreting the pathophysiological pathway associated with them. **Table 2** shows the reported biomarkers of acylcarnitines and acyl-CoAs for different types of FAOD. It is evident that more research and development has been done for analyzing acylcarnitines as biomarkers for FAOD while relatively fewer studies report the role of acyl-CoA compounds. The upcoming

sections in this review will focus on the analytical advances and strategies that have been adopted over the years for improving the detection and quantitation of acyl-CoA compounds.

Table 2. Diagnostic acylcarnitine and acyl-CoA biomarkers for FAOD.

Deficiency	Biomarker		Reference	
	Free carnitines (C0); Acylcarnitines (Plasma/Dried blood spot)[#]	**Acyl-CoA (matrix is defined in the corresponding rows)[#]**	**Acylcarnitines**	**Acyl-CoA**
CT	Decrease: C0, C2, C3, C16, C18, C18:1	NA	[34,39,54]	NA
CPT1	Increase: C0, C0/(C16 + C18) Decrease: C16, C18, C18:1, C18:2, (C16 + C18:1)/C2	**Matrix**: Human Fibroblasts Increase: C16	[34,39,46,55]	[56,57]
CACT	Increase: C14, C16, C16:1, C18, C18:1, C18:2, C14:1/C2, (C16 + C18:1)/C2 Decrease: C0, C2, C3/C16, C0/(C16 + C18)	**Matrix**: Human Fibroblasts Decrease: C16	[34,39,46,55]	[57]
CPT2	Increase: C12, C14, C16, C16:1, C18, C18:1, C18:2, C14:1/C2, (C16 + C18:1)/C2 Decrease: C0, C2, C3/C16, C0/(C16 + C18)	NA	[34,39,46,55]	NA
VLCAD	Increase: C12, C12:1, C14:2, C14:1, C14, C16:1 C16, C18, C18:1, C18:2 C14:1/C2, C14:1/C12:1, C14:1/C16 Decrease: C0	**Matrix**: Human Fibroblasts Increase: C16/C12 Decrease: C12	[34,39,46,55]	[56]
LCAD	NA	**Matrix**: Human Fibroblasts Increase: C16/C12 Decrease: C12	NA	[56]
MTP	Increase: C14, C14-OH, C14:1, C14:2, C16:1, C16-OH, C16:1-OH, C18-OH, C18:1-OH, C18:2-OH, C14:1/C2, C14:1/C16, C16-OH/C16, C18-OH/C18 Decrease: C0	**Matrix**: Human Fibroblasts Increase: C16:1, C16-OH Decrease: C12, C14	[25,34,35,46]	[57]

LCHAD	Increase: C14, C14:1, C14:2, C14-OH, C14:1-OH, C16:1, C16-OH, C16:1-OH, C18, C18:1, C18:2, C18-OH, C18:1-OH, C18:2-OH, C14:1/C2, C14:1/C16, C16-OH/C16, C18-OH/C18 Decrease: C0	**Matrix**: Human Fibroblasts Increase: C14:1, C16:1, C16-OH	[34,39,46,55]	[58]
LCEH	NA	NA	[34]	NA
LCKAT	Increase: C14:1, C16:1, C16-OH, C18-OH	NA	[34,39]	NA
MCAD	Increase: C6, C8, C10, C10:1, C8/C2, C8/C10, C8/C16, C8/C8:1 Decrease: C0, C2	**Matrix**: Human Fibroblasts Increase: C8, C10 Decrease: C12, C14	[34,39,46,55]	[57,58]
M/SCHAD*	Increase: C4-OH, C6-OH, C8-OH	NA	[25,34,35]	NA
MCKAT	NA	NA	[39]	NA
SCAD	Increase: C4, C4/C2, C4/C3, C4/C8 Decrease: C0	**Matrix**: Mouse liver Increase: C4, C6	[34,39,46,55]	[53]
SCHAD*	Increase: C4-OH	**Matrix**: Mouse liver Increase: C4-OH, C6, C6-OH, C8:1, C8 Decrease: C2	[34,39,46,55,59]	[53]
SCEH	NA	NA	[60]	NA
MAD	Increase: C4, C5, C6, C8, C10:1, C12, C14, C14:1, C16, C16:1, C18, C18:1, C16-OH, C16:1-OH, C18-OH, C18:1-OH, C4/C2, C4/C3, C5/C0, C5/C2, C5/C3, C8/C2, C14:1/C2, C14:1/C16, (C16 + C18:1)/C2 Decrease: C0	NA	[39,46]	NA

NA; not available.

*3-Hydroxyacyl-CoA dehydrogenase (HAD) deficiency term is now used to represent both short-chain hydroxyacyl-CoA dehydrogenase (SCHAD) deficiency and medium- & short-chain hydroxyacyl-CoA dehydrogenase (M/SCHAD), as this dehydrogenase enzyme encoded by single gene has chain length specificity for C4-C10 substrates [46,61].

Acylcarnitine biomarkers are reported in plasma or dried blood spots while acyl-CoA biomarkers are reported in different matrices as mentioned in the table.

4. Analytical advances for the measurement of Acyl-CoAs

4.1 Extraction of Acyl-CoAs from biological samples

Sample preparation is an important step for the quantitative determination of any compound and should be optimized to extract and recover the maximum quantity of compounds from the biological samples. As summarized by Rivera et al. [62], various methodologies such as protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been employed for the extraction of acyl-CoAs. Acyl-CoA species vary significantly in their polarity. Although soluble in polar solvents, long-chain acyl-CoAs can migrate into the non-polar organic layer during LLE which can result in the poor recovery of these compounds from the aqueous layer. The organic and aqueous layers can be combined to improve recovery, however this will introduce lipids in the sample and thus can cause matrix effects. For these reasons, researchers have also used the combination of LLE-SPE [63,64]. LLE followed by SPE is quite efficient in removing endogenous interferences such as phospholipids from the matrices and can provide better purification. On the other hand, these techniques can have a negative impact on recoveries, and are time-consuming and expensive. Hence, some studies completely eliminated LLE/SPE extraction and have used protein precipitation [65,66]. Fu et al. used 0.4 M perchloric acid containing 0.5 mM EGTA [65] while Pearce et al. used acidified methanol (5% acetic acid in cold methanol) [66] for extraction of acyl-CoAs. The selection of the extraction technique depends on the type of acyl-CoA species. Short-chain species are well-extracted at strongly acidic pH while long-chain species are not soluble under the same conditions [67]. Therefore, studies covering all short- to long-chain species employ weakly acidic solvents. **Table 3** summarizes various extraction strategies that have been used for acyl-CoAs since 2018.

Table 3. Sample preparation techniques for the extraction of acyl-CoAs from biological samples.

Extraction Techniques	Biological Samples	Extraction solvents	Recovery (%)	Reference
Protein precipitation	Mouse liver	0.4 M $HClO_4$ containing 0.5 mM EGTA followed by neutralization with 0.5 M K_2CO_3	84.44-112	[65]
	HepG2 cells, Bone marrow-derived macrophages from mice	2.5% (w/v) 5-sulfosalicylic acid solution	59-80	[2]
	Betaproteobacterium "Aromatoleum" sp. strain HxN1	2 mL MeOH, 0.5 mL of ice-cold 10 mM $HCOONH_4$ (pH 7)	NA	[68]
	Human liver adenocarcinoma cells (SK-HEP-1)	5% acetic acid in cold MeOH	79.5-133.1	[66]
	Rabbit heart, liver brain, spleen and kidney	80% aqueous MeOH (pre-cooled at -80 °C)	NA	[69]
	Rat liver, kidney, brain and heart	80% MeOH	NA	[70]
	Mouse liver	Ice-cold MeOH:H_2O (80:20)	NA	[71]
	Human whole blood	MeOH: 2.5 mM CH_3COONH_4 (80:20) (pH 6.5) (pre-cooled at -80 °C)	~100	[72]
	Saccharomyces cerevisiae, HEK-293T cells, single rice seed, mouse kidney glomeruli, single *Arabidopsis thaliana* seed	*Saccharomyces cerevisiae*, HEK-293T cells, single rice seed: 80% MeOH Mouse kidney glomeruli: 50 mM borate buffer solution (pH 7) Single *Arabidopsis thaliana* seed: 50 mM borate buffer solution (pH 7)	77.1-112.4	[73]
LLE	Drosophila larva and pupa	Extraction buffer (IPA, 50 mmol/L KH_2PO_4, glacial acetic acid and bovine serum albumin), petroleum ether, saturated ammonium sulfate and $CHCl_3$: MeOH (1:2)	86.4 ± 1.3	[74]
	Rat hepatic stellate cells (HSCs) T6; Human embryonic kidney cells 293T	Extraction buffer (IPA, 50 mmol/L KH_2PO_4, glacial acetic acid and bovine serum albumin), ethyl ether,$CHCl_3$: MeOH (1:2)	NA	[75]

Protein Precipitation-SPE	HepG2 cells	**Protein precipitation:** 1 mL of ice-cold 10% (w/v) trichloroacetic acid in H_2O **SPE:** *Cartridge:* Oasis HLB 1 cc (30 mg) SPE columns (Waters). *Pre-conditioning solvent:* 1 mL H_2O *Washing solvent:* 1 mL MeOH, *Elution solvent:* 1 mL MeOH containing 25 mM CH_3COONH_4	NA	[76]
LLE-SPE	Human cervical HeLa cells, Human platelet samples	**LLE:** ACN:IPA (3:1), KH_2PO_4 buffer (0.1 M, pH 6.7), acetic acid **SPE:** *Cartridge:* Oasis WAX (Waters, Milford, MA) *Pre-conditioning solvent:* MeOH:H_2O:acetic acid (3:1:1) *Washing solvent:* MeOH:H_2O:acetic acid (3:1:1); 50% MeOH *Elution solvent:* IPA:MeOH:3% ammonia (6:3:1)	43.4-76.1	[64]

$HClO_4$, perchloric acid; K_2CO_3, potassium carbonate; MeOH, methanol; $HCOONH_4$, ammonium formate; H_2O, (ultra-pure) water; CH_3COONH_4, ammonium acetate; IPA, isopropanol; $CHCl_3$, chloroform; KH_2PO_4, potassium phosphate monobasic; ACN, acetonitrile.

4.2 Methods for separation and detection of CoASH (free CoA) and acyl-CoA thioesters

There are various techniques employed for analyzing CoASH and acyl-CoA thioesters. The details of changes in the analytical techniques over the years are described in sections below.

4.2.1 Enzymatic methods

The two most common enzymatic assays that were employed for determination of CoASH and acyl-CoA compounds were: (i) endpoint assays: measurement of the end products of a reaction; (ii) recycling assays: multiple enzyme catalyzed reactions are involved for recycling of CoASH or acyl-CoAs and product is formed. The rate of formation of this product is proportional to the CoASH/acyl-CoA concentration. The detection for these enzymatic methods was through spectrophotometric, fluorometric and radioactive assays. **Table 4** shows the methods that were

commonly used to measure CoASH and acyl-CoAs. The details and principle of these assays are described comprehensively by Bieber et al. [77] and Tsuchiya et al. [67].

Table 4. Enzymatic methods for the determination of CoASH (free CoA) and acyl-CoAs.

Type of assays	Detection technique	Species analyzed	Sensitivity (Limit of detection)	Reference
Endpoint	Fluorometric	CoASH; C2:0-CoA; long-chain fatty acyl-CoA	50 µmol	[78,79]
	Radioisotopic	Malonyl-CoA	10 pmol (approx.)	[80,81]
	Radioisotopic	CoASH; C2:0-CoA	10 pmol	[82]
Recycling	Spectrophotometric/ Fluorometric	CoASH; C2:0-CoA	<40 pmol per mL of tissue extract	[67,77,83]
	Spectrophotometric	C2:0-CoA	Not defined	[84]
	Fluorometric	CoASH; C2:0-CoA	0.04 pmol	[85]
	Spectrophotometric	C2:0-CoA; Malonyl-CoA	1 pmol	[86]

Although these enzymatic assays were very useful for determination of individual acyl-CoAs, their targets were mainly limited to CoASH, acetyl-CoA and malonyl-CoA. They lack the broad coverage and are considered less reliable due to interferences. Therefore, techniques that can resolve and identify multiple acyl-CoA species and are sensitive enough to detect these compounds in cells and tissues were needed.

Analytical methods coupling separation and detection techniques

To overcome the limitations associated with enzymatic methods, researchers started using methods that involve the combination of separation techniques such as liquid chromatography (LC) or gas chromatography (GC) with detection techniques such as ultraviolet (UV) absorption or mass spectrometry (MS).

4.2.2 Liquid chromatography-ultraviolet (LC-UV) detection methods

High-performance liquid chromatography (HPLC) started gaining popularity due to high speed and good resolution of separation. The first separation of acyl-CoA species was described by Baker and Schooley with the application of ion-pairing reversed-phase liquid chromatography (IPRP-LC) interfaced with a UV detector [87]. Ammonium-based counter ions were used as ion-pairing agents. Later, other studies employed reversed-phase chromatography with mobile phases containing (10-100) mM phosphate salts such as potassium phosphate monobasic (KH_2PO_4) [63,88–91] and sodium phosphate monobasic (NaH_2PO_4) at pH range of (4.5-5.3) [92]. Isocratic elution with 100 mM of NaH_2PO_4 had also been used for detection of CoASH

and acetyl-CoA species [93,94]**.** The most optimum wavelength for UV detection of acyl-CoA species is in the range of 254-260 nm [63,89,90,92–97]. The detection limit for long-chain acyl-CoA esters was reported to be 12 pmol [90] while that of CoASH and short-chain acyl-CoAs were reported over a wide range of 3-12000 pmol [91–94]. The major limitation associated with methods using LC-UV detection is their inability to resolve co-eluting peaks (specificity) [63]. There might be interference from other compounds which do not belong to the acyl-CoA species at the same absorbance and retention time. To ensure that peaks detected in the chromatogram belong to CoASH or acyl-CoA species, additional experiments had to be performed. Shurubor et al. spiked CoASH and acetyl-CoA standards in the deproteinized biological samples to match them with peaks appearing in experimental samples [94]. Additionally, biological samples were subjected to acidic and enzymatic treatments to breakdown CoASH and acetyl-CoA. Since these acidic and enzymatic treatments will specifically target CoASH and acetyl-CoA peaks [85], the disappearance of their peaks will confirm their identification [94]. These additional experiments required for confirmation of acyl-CoA species in LC-UV detection increase the time required for analysis. Apart from specificity, the sensitivities of these techniques were also lower which is critical considering the low endogenous levels of acyl-CoAs. These shortcomings prompted the use of other more sensitive and specific analytical techniques.

4.2.3 Gas chromatography (GC) methods

Gas chromatography has also been employed for quantitative determination of acyl-CoAs. In GC, typically a derivatization agent is used to convert the analytes to their volatile forms for their separation and detection. Some of the studies have used sodium borohydride to convert long-chain acyl-CoA esters to their alcohol forms [98,99]. Further, these alcohols are treated with t-butyldimethylchlorosilane to form t-butyldimethylsilyl ethers and are detected with flame ionization [99] or converted to pentafluorobenzoyl ester by treating the alcohol with pentafluorobenzoyl chloride and detected with negative ion chemical ionization mass spectrometry [98]. Tamvakopoulos et al. developed a method in which acyl-CoA esters are treated with glycine, the resulting N-acyl glycinates derivatized with pentafluorobenzyl bromide to form N-acylpentafluorobenzylglycinates, which are then quantified with negative ion chemical ionization mass spectrometry [100]. Long-chain acyl-CoA esters are also detected as methyl esters by treating them with a boron trifluoride methanol reagent [101]. In another study, propionyl-CoA was detected as pentafluorobenzyl derivative mediated via the formation of N-propionylsarcosine (reaction of propionyl-CoA with sarcosine), while methylmalonyl-

CoA and succinyl-CoA were detected as tert-butyl dimethylsilyl derivatives mediated by the formation of corresponding acids [102]. Kopka et al. derivatized acyl-CoA esters with n-butyl amine thus forming acyl butylamide derivatives and the detection was performed with electron ionization mass spectrometry [103]. It has been reported in several studies that the sensitivity for detection of acyl-CoAs can be increased by use of gas chromatography-mass spectrometry [98,102,103]. The lowest detection limits of acyl-CoA esters reported by this technique were in the range (0.4-500) fmol [98,100,103]. But gas chromatography requires derivatization of acyl-CoAs which makes the method lengthy and tedious [104]. There might also be a chance of cross reactions due to the reaction of acyl chains that do not belong to the acyl-CoA group (e.g.,fatty acids), which can in turn affect the accuracy of quantitation [105].

4.2.4 Liquid chromatography-mass spectrometry (LC-MS) methods

Compared to previously reported techniques for identification and quantitation of acyl-CoA, methods using LC-MS prove to be the most effective due to their better sensitivity, resolution and high coverage compared to enzymatic assays and LC-UV detection. Additionally, they do not need the derivatization steps required for gas chromatography. The first liquid-chromatography technique used for separation of acyl-CoAs was IPRP-LC [87]. Since then, numerous liquid chromatography techniques and methods have come into use which include normal phase, hydrophilic-interaction chromatography (HILIC), 2DLC, etc.

The detection by MS can be targeted or untargeted. In targeted mass spectrometry analysis, the information about the analytes is known and method parameters (such as mass and retention time) are set based on this available information whereas in untargeted analysis, instead of pre-defining a target mass or multiple reaction monitoring (MRM) transition, an entire mass range is acquired so that the information of both known and unknown analytes present in a sample can be collected. Triple quadrupole (QqQ) and quadrupole ion traps (QTRAP) are the common instruments employed in targeted analysis, while quadrupole time of flight (QTOF) and orbitrap instruments are mostly employed for untargeted analysis. The most common fragment ion for the identification and quantitation of acyl-CoAs is the neutral loss of 507 in positive ionization mode, occurring due to the loss of 3’-phosphonucleoside diphosphate from the precursor ion **(Figure 3).**

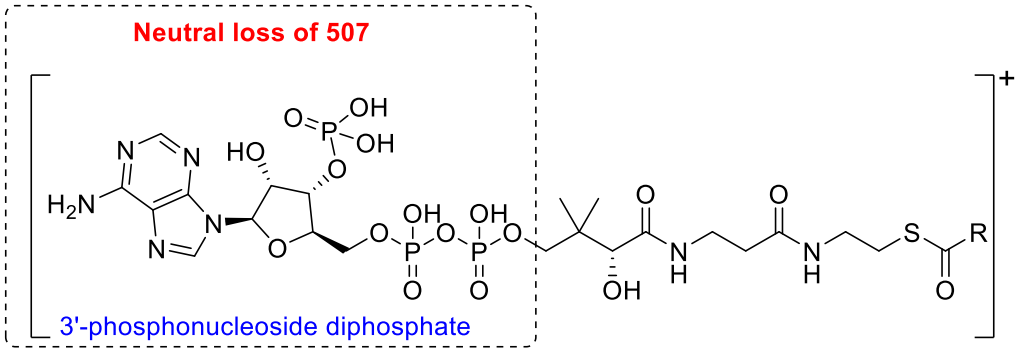


Figure 3. Neutral loss of 507 occurring due to the loss of 3'-phosphonucleoside diphosphate from precursor ion of acyl-CoAs.

Various methodologies have been used for the determination of acyl-CoAs using LC-MS. Reversed-phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS) [62,104–106] has been used more frequently and is suitable for long-chain compounds. The short-chain acyl-CoAs need slightly acidic mobile phases in RPLC conditions [11,107] while long-chain acyl-CoAs have serious peak tailing problems under these conditions. Therefore, an alkaline mobile phase was preferred for long-chain acyl-CoAs [106,108,109]. Rivera et al. has summarized comprehensively the major advances in the chromatographic techniques coupled with mass spectrometry of acyl-CoA species till 2018 [62]. The upcoming sections will explain the various approaches that have been used for improving the LC-MS analytical approaches of acyl-CoAs since 2018. These approaches include ion-pairing, derivatization, native chemical ligation as well as the efforts that have been taken to improve the current existing experimental databases of these compounds. **Table 5** lists the LC-MS parameters that have been employed for the detection of CoASH and acyl-CoA species.

Table 5. LC-MS techniques employed for measurement of acyl-CoAs.

Method	Chromatography	Instrument	Targets			Column	Mobile phase	Sensitivity	Reference
			CoA SH (free CoA)	Short-, medium-, long- and very long-chain[#]	Other species[*]				
Method **i**	RPLC-MS/MS	QTRAP 5500 (Sciex)	✓	Short to long	Malonyl; Acetoacetyl; 3-Hydroxybutyryl; Succinyl; Glutaryl; HMG; C12:1; C14:1; C16:1; C18:1; C18:2	Waters CSH C18 (50×2.1 mm, 1.8 µm)	***Aqueous Phase:*** 10 mM NH_4HCO_3 (pH 9) ***Organic Phase:*** ACN: H_2O (95:5)	LOD: (0.24-3.41) ng/mL LOQ: (5-10) ng/mL	[66]
Method **ii**	IPRP-LC-MS/MS	Exion UPLC (Sciex) coupled to QTRAP 6500+ (Sciex)	✓	Short to long	Malonyl; Succinyl; Glutaryl; HMG; Acetoacetyl; Crotonoyl; 3-Hydroxybutyryl; C14:1; C14:2; C15:0; C16:1; C16:2; C18:1; C18:2; C18:3; C20:1; C20:3; C20:4; C20:5; C22:6	Phenomenex Kinetex-EVO-C18 (100×2.1 mm, 1.7 µm)	***Aqueous Phase:*** 0.005% TEA in 2 mmol/L CH_3COONH_4 ***Organic Phase:*** 0.1% TEA in ACN:H_2O (4:1)	LOD: (0.15-62.5) pg LOQ: (0.5-10)[**] ng/mL	[74]
Method **iii**	IPRP-LC-MS; IPRP-LC-MS/MS	1290 UHPLC system (Agilent) coupled to 6530 QTOF mass spectrometer (Agilent); LC-30A system (Shimadzu) coupled to QTRAP 6500+ (Sciex)	✓	Short to medium	Crotonoyl; Tiglyl; Acetylacetyl; Malonyl; Succinyl; Glutaryl; 3-Hydroxybutyryl; HMG; Benzoyl; Phenylacetyl	Acquity BEH C18 (100×2.1 mm, 1.7 µm)	***Aqueous Phase:*** 5 mM hexylamine in H_2O (pH was adjusted to 10.2 using acetic acid) ***Organic Phase:*** MeOH containing 10% of 10 mM CH_3COONH_4 (pH 8.5)	NA	[69]

Method **iv**	IPRP-LC-MS/MS	LC-20AD (Shimadzu) coupled to API 3200 triple-quadrupole mass spectrometer (Sciex)	×	Short: C2:0; C3:0	Malonyl; Succinyl	Waters xBridge C18 (150×2.1 mm, 3 µm)	***Aqueous Phase***: H_2O:MeOH (95:5) with 4 mM DBAA ***Organic Phase***: H_2O:ACN (25:75)	LOQ: (10-50)** ng/mL	[65]
Method **v**	IPRP-LC-MS/MS	1290 UHPLC (Agilent) coupled to 5500 QTRAP (Sciex)	✓	Short: C2:0; C3:0; C5:0	Malonyl; Succinyl	Phenomenex Kinetex UHPLC C18 (150×2.1 mm, 2.6 µm)	***Aqueous Phase:*** 5 mM CH_3COONH_4 with 2.5 mM DMBA (pH 5.6) ***Organic Phase:*** 95% ACN, 5% H_2O with 5 mM CH_3COONH_4	LOD: (1-3) pmol; LOQ: (3.7-7.4) pmol	[2]
Method **vi**	Derivatization-RPLC-MS/MS	1290 Infinity UHPLC system (Agilent) coupled to QTRAP 4500 (Sciex)	✓	Short to very long	Malonyl; C18:1; C18:2; C18:3; C20:4; C20:5; C22:6	Acquity UPLC CSH C18 (100× 2.1 mm, 1.7 µm)	***Aqueous Phase:*** 10 mM CH_3COONH_4 ***Organic Phase:*** MeOH	LOD: (1.2-4.6) nM; LOQ: (4.2-16.9) nM	[64]
Method **vii**	Derivatization-RPLC-MS/MS	LC-30AD UHPLC system (Shimadzu) coupled to MS-8050 mass spectrometer (Shimadzu)	×	Short: C2:0	Succinyl	Acquity UPLC BEH C18 (100×2.1 mm, 1.8 µm)	***Aqueous Phase:*** 2 mM NH_4HCO_3 ***Organic Phase:*** ACN	LOD: 36 pg/mL; LOQ: 100 pg/mL	[73]

Method **viii**	RPLC-MS/MS	Acquity UPLC I-Class system (Waters) coupled to Xevo TQ-S QqQ mass spectrometer (Waters)	×	Short to long; very long: C22:0	Malonyl; Succinyl; Glutaryl; HMG; 3-Hydroxybutyryl; C5:1; C6:1; C7:0; C7:1; C8:1; C9:0; C9:1; C10:1; C10:2; C11:0; C11:1; C12:1; C12:2; C13:0; C13:1; C14:1; C14:2; C15:0; C15:1; C16:1; C16:2; C16:3; C17:0; C17:1; C18:1; C18:2; C18:3; C18:4; C19:0; C19:1; C20:1; C20:2; C20:3; C20:4; C20:5; C21:0; C21:1; C22:1; C22:2; C22:3; C22:4; C22:5; C22:6	Acquity UPLC BEH C18 (50×1 mm, 1.7 µm)	***Aqueous Phase:*** 5% ACN with 0.1% HCOOH ***Organic Phase:*** 90% ACN with 0.1% HCOOH	LOD: (2-50) fmol of injected product	[70]
Method **ix**	RPLC-MS	Vanquish Flex UHPLC system (ThermoFisher Scientific) coupled to Orbitrap Fusion mass spectrometer (ThermoFisher Scientific)	×	Short to medium	3-Hydroxypropionyl; Malonyl; Succinyl; Methylmalonyl; Glutaryl; Methylsuccinyl; Ethylmalonyl; Acetoacetyl; 3-Hydroxybutyryl; HMG; Crotonoyl; 3-Oxohexanoyl; 3-Hydroxyhexanoyl; 2-Methylbutryl; Benzoyl; Phenylacetyl; Mesaconyl; 3-Methylmalyl; C6:1	Phenomenex Gemini C18 (150×2.0 mm, 3 µm)	***Aqueous Phase:*** 10 mM $HCOONH_4$ (pH 8.1) ***Organic Phase:*** ACN	LOD: (100-200) fM	[68]

Method **x**	RPLC-MS (MS/MS data was generated in data-dependent acquisition mode)	1290 UHPLC system (Agilent) coupled to 6550 iFunnel QTOF mass spectrometer (Agilent)	✓	Short to long	Glutathione; Succinyl; 3-Hydroxybutyryl; 3-Phenylpropanoyl; 2-Methylhexanoyl; Perillyl; C14:1; C16:1 ; C18:1; C18:2; C18:3; C20:3 C20:4; C22:6	Phenomenex Kinetex C18 (100×2.1 mm, 1.7 μm)	***Aqueous Phase*** 10 mM CH_3COONH_4 ***Organic Phase:*** ACN	NA	[71]
Method **xi**	RPLC-MS; RPLC-MS/HRMS	Ultimate 3000 Quaternary UHPLC (ThermoFisher Scientific) coupled to Q Exactive Plus (ThermoFisher Scientific)	×	×	Lactoyl	Waters XBridge C18 (150×2.1 mm, 3.5 μm)	***Aqueous Phase:*** 5 mM CH_3COONH_4 ***Organic Phase:*** ACN:H_2O (95:5) with 5 mM CH_3COONH_4	LOD: 0.2 fmol on column	[76,110]
Method **xii**	RPLC-MS/MS	Exion UPLC (Sciex) coupled to QTRAP 6500+ (Sciex)	×	Long	C14:1; C15:0; C15:1; C16:1; C16:2; C17:0; C18:1; C18:2; C18:3; C20:1; C20:2; C20:3; C20:4; C20:5; C22:1; C22:2; C22:3; C22:4; C22:5; C22:6	NA	NA	NA	[75]
Method **xiii**	RPLC-MS/MS	Acquity UPLC system (Waters) coupled to QTRAP 6500+ (Sciex)	×	Short: C2:0	×	Merck Chromolith Performance RP8e (100×4.6 mm)	***Aqueous Phase:*** 2.5 mM CH_3COONH_4 (pH 6.5) ***Organic Phase:*** ACN:IPA (98:2)	LOD: 1.95 ng/mL LOQ: 3.91 ng/mL	[72]

Method **xiv**	RPLC-MS/MS	Ultimate 3000 LC system (ThermoFisher Scientific) coupled to QqQ TSQ Quantum Ultra (ThermoFisher Scientific)	✓	Short: C2:0; C3:0	Succinyl; Methylmalonyl	Acquity Premier HSS T3 (50×2.1 mm, 1.8 µm)	***Aqueous Phase:*** 25 mM CH_3COONH_4 ***Organic Phase:*** ACN:H_2O (80:20) with 25 mM CH_3COONH_4	NA	[111]

✓- included in the target list; ×- not included in the target list.

[#] The common species in short-chain are C2:0-CoA; C3:0-CoA; C4:0-CoA; C5:0 CoA. Medium-chain includes C6:0-CoA; C8:0-CoA; C10:0-CoA, long-chain consists of C12:0-CoA; C14:0-CoA; C16:0-CoA; C18:0-CoA; C20:0-CoA and very-long includes C22:0-CoA and C24:0-CoA. If fewer species are observed within these chains, they are individually listed in the corresponding columns.

[*]Remaining acyl-CoA species as well as the unsaturated form of common short-, medium-, long- and very long-chain acyl-CoAs are mentioned under the column of other species.

[**]lowest value defined in the linear range of the method.

RPLC-MS/MS, reversed-phase liquid chromatography coupled to tandem mass spectrometry; IPRP-LC-MS/MS, ion-pairing reversed-phase liquid chromatography coupled to tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; NH_4HCO_3, ammonium bicarbonate; ACN, acetonitrile; H_2O, (ultra-pure) water; TEA, triethylamine; CH_3COONH_4, ammonium acetate; MeOH, methanol; DBAA, dibutylamine acetate; DMBA, N,N-dimethylbutylamine; HCOOH, formic acid; $HCOONH_4$, ammonium formate; IPA, isopropanol; NA, not available.

Acyl-CoAs have negatively charged phosphate groups in their structure which are responsible for peak tailing as they can bind to the stainless steel surface of HPLC columns and tubings in the instruments [112]. One of the considerations for improving the peak shape was the use of phosphate buffers [87,113,114], that can bind to these surfaces and thus decrease the interaction between the phosphate groups of acyl-CoA moiety with column and HPLC surfaces. But these phosphate buffers, on the other hand, are non-volatile and can contaminate the mass spectrometer. Most studies performing LC-MS have used buffers with ammonium as the counter ion (such as ammonium acetate and ammonium formate) in conjunction with other strategies, such as phosphoric acid wash or other ion-pairing agents. Pearce et al. reported a simple method using reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry (RPLC-ESI MS/MS) that involves a phosphoric acid wash **(Table 5, Method i)** to improve the peak shape and reduce the peak tailing and carryover problem commonly associated with acyl-CoAs [66]. The wash consists of acetonitrile: water: phosphoric acid (60:40:5 v/v/v) and was introduced during the column washing phase of each gradient cycle in 10 repeats of 50 µL injections. This method was able to semi-quantify 21 acyl-CoA species in human adenocarcinoma cells.

Ion-pairing

Lam et al. used a well-known ion-pairing reagent triethylamine (TEA) for the simultaneous quantitation of all short-, medium- and long-chain acyl-CoAs and acylcarnitines [74]. RPLC was used and TEA was incorporated in both organic and aqueous phases **(Table 5, Method ii)**. The extraction protocol of acyl-CoAs is described in **Table 3**. Acyl-CoAs were analyzed in both positive and negative mode while acylcarnitines were analyzed in positive mode. TEA maintains the pH as well as acts as an ion-pairing reagent to improve the peak shape of acyl-CoAs. This method was applied on wild-type Drosophila strain w^{1118} to estimate isotope incorporation into intra- and extra mitochondrial acyl-CoAs after being supplied with labeled fatty acids. The limits of detection were reported in the picogram (pg) range with medium- to long-chain acyl-CoAs in the range of 0.15 to 0.43 pg while short-chain acyl-CoAs were from 3.75 to 7.51 pg.

Another method using IPRP-LC coupled with mass spectrometry was reported by Hu et al. **(Table 5, Method iii)** [69]. Firstly, full scan analysis was performed on a QTOF instrument to identify retention times which were subsequently used for the construction of a quantitative structure-retention relationship model for the prediction of retention times. These measured and predicted retention times were then used to construct a scheduled multiple reaction monitoring

(sMRM) method on a QTRAP for the quantitation of metabolites. Hexylamine was used as the ion-pairing reagent. The pH of hexylamine greatly influences the MS responses and retention times of the metabolites. A pH of 10-10.3 was chosen as 80% of hexylamine is ionized at this range, which will help in the strong electrostatic interaction with the analyte. The phosphate groups on the coenzyme-A moiety are expected to be completely ionized at pH 10, resulting in the negative charge which facilitates the strong electrostatic interactions with hexylamine. Additionally, hydrophobic interactions also play a role as retention times of acyl-CoA esters increase with an increase in the acyl chain length.

Fu et al. used dibutylamine acetate (DBAA) as an ion-pairing reagent in an RPLC-ESI MS/MS method **(Table 5, Method iv)** [65]. This method was suitable to analyze short-chain acyl-CoAs such as acetyl-, malonyl-, succinyl- and propionyl-CoA simultaneously with nucleotides and pyridine dinucleotides in the positive ion mode. Various parameters such as concentration, effect on retention time and MS signal to choose the optimum concentration of DBAA were studied. Jones et al. proposed another IPRP-LC method with a simple sample preparation and simultaneous measurement of short-chain acyl-CoAs and precursors in the biosynthetic pathway of CoASH in one single run **(Table 5, Method v)** [2]. N,N-dimethylbutylamine (DMBA) as an ion-pairing reagent was added to ammonium acetate solution (pH adjusted to 5.6). 5-sulfosalicylic acid was used for sample deproteinization and extraction of acyl-CoAs as well as for their reconstitution. This eliminates the need of SPE, a well-known procedure for purification and enrichment of acyl-CoAs from mammalian cells and tissues [115–117]. Crotonoyl-CoA was found to be suitable as internal standard (IS) to control the variation during the extraction procedure.

Although ion-pairing reagents can improve the separation, they are well known for causing contamination in the mass spectrometer and deterioration of its performance over the long run. Often, dedicated instrumentation is required for ion-pairing chromatography.

Derivatization

Derivatization is another approach which assists in the separation of a broad range of acyl-CoAs without impairing the mass spectrometer's efficiency and has the following advantages while developing an acyl-CoA method:

1. Neutralizing the negative charge present on the phosphate moiety of acyl-CoAs,
2. One condition optimal for the elution of all chain length,
3. Improving the peak shape,

4. No carryover or instrument contaminations, which eliminates the need of extra washing steps after multiple injections.

Li et al. introduced the derivatization of acyl-CoAs that involves the methylation of their phosphate groups **(Figure 4)** [64]. After extraction of acyl-CoAs from biological samples **(Table 3)** the dried extracts were reconstituted in methanol followed by derivatization using tert-butyl methyl ether/methanol/water (10:3:2.5, v/v/v) and trimethylsilyl diazomethane (TMS-DM, 2 M in hexane). The methanol acts as the proton donor for the formation of *in situ* diazomethane (CH_2N_2) and CH_3OTMS **(Figure 4).**

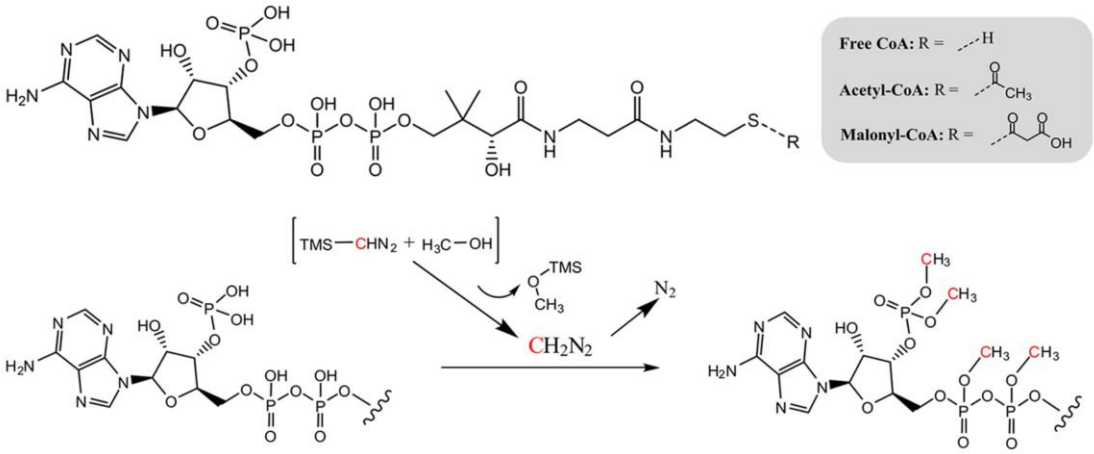


Figure 4. Phosphate methylation mechanism of acyl-CoAs using trimethylsilyl diazomethane. $TMS\text{-}CHN_2$ (TMS-DM), trimethylsilyl diazomethane; CH_3OH, methanol; CH_2N_2, diazomethane; N_2, nitrogen gas. Reprinted with permission from, Li et al. Targeted Profiling of Short-, Medium-, and Long-Chain Fatty Acyl-Coenzyme As in Biological Samples by Phosphate Methylation Coupled to Liquid Chromatography–Tandem Mass Spectrometry, Anal. Chem. 93 (2021) 4342–4350 [64], Copyright (2021).

The methylation of phosphate groups eliminates the negative charge. Different derivatization times were optimized and 30 min at ambient temperature was found to be the most suitable. After this derivatization step, the acyl-CoAs were separated by RPLC-MS/MS conditions in one single analytical run **(Table 5, Method vi)**. These methylated acyl-CoAs were stable for 48 h at 4 °C in the autosampler. Neutral loss of 507 Da is the most abundant fragment present for underivatized acyl-CoA species **(Figure 3),** but for this study a neutral loss of 563 Da was considered as all the phosphate groups of CoASH moiety were methylated (**Supplementary Figure S1**).

Another derivatization strategy was proposed with the use of 2-(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide [73]. This derivatization reaction was based on simple acid-base interactions without any requirement of catalysts. In this study, a pair of light and heavy isotope labeled, 2-

(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide and d_5-2-(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide were used. 2-(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide can derivatize the endogenous compounds while its d_5-isotope can produce the corresponding isotopic IS for accurate quantitation. The synthetic pathway of these derivatizing agents are defined by Liu et al. [118]. The derivatized internal standards were produced by treating the target standards with borate buffer and d_5-2-(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide. After the extraction of biological samples as specified in **Table 3**, the derivatization of endogenous compounds was performed by adding 50 mM borate buffer (pH 7) and 2-(diazo-methyl)-*N*-methyl-*N*-phenyl-benzamide in *Saccharomyces cerevisiae*, HEK-293T cells, and single rice seed while, in mouse kidney glomeruli and single *Arabidopsis thaliana* seed, this agent was directly added to the extracted samples. The reaction solution for all the matrices was vortexed and kept for the reaction for 1 h at 30 °C. The LC-MS/MS analysis was performed according to the conditions specified in **Table 5, Method vii.**

Derivatization can be quite beneficial for enhancing the sensitivity and detection of acyl-CoA compounds. But the limitations associated with it such as specificity, stability, loss of analyte, time, formation of byproduct or interference with the detection of target should be considered while developing the derivatization protocol for acyl-CoAs [119].

Increasing the acyl-CoA stability for its detection

Native chemical ligation of acyl-CoAs is a process that can spontaneously occur, acyl-CoAs can form anhydrides [9] or S-acyl glutathiones [10,11]. James et al. [70] adapted this process to stabilize and augment detection of acyl-CoAs. For this purpose, the authors developed a molecular probe CysTPP ([5-(2-amino-3-mercaptopropanoylamino)pentyl]triphenyl phosphonium) which changes acyl-CoA masses and fragmentation patterns. Incubating samples with CysTPP will cause a thioester exchange reaction generating S-acyl CysTPP intermediate, which undergoes further intramolecular rearrangement to form N-acyl CysTPP. The remaining S-acyl intermediates were removed by adding dithiothreitol (DTT) and free thiols of CysTPP and N-acyl-CysTPP were blocked by subsequent derivatization with iodoacetamide (IAM). This step will form N-acylated and S-carbamidomethylated (CAM) (N-acyl-CysTPP-CAM) as the final product. The species thus formed were considered to be stable for quantitation. With this strategy, the acyl moiety of acyl-CoAs attached to the N-terminal amine of cysteine residues were used to detect acyl-CoA thioesters **(Figure 5).** The positive charge on the TPP cation is known to enhance the MS detection [120,121] which enables the

quantitation of N-acylated CysTPP in the femtomole range. The diagnostic fragment in this case will be the neutral loss of 91 Da, arising due to the fragmentation of the relatively weak C-S bond to generate a dehydroalanine derivative. Approximately, 60 acyl-CoA species were identified *in vivo* (liver, brain, kidney and heart) with the application of this native chemical ligation technique **(Table 5, Method viii)**.

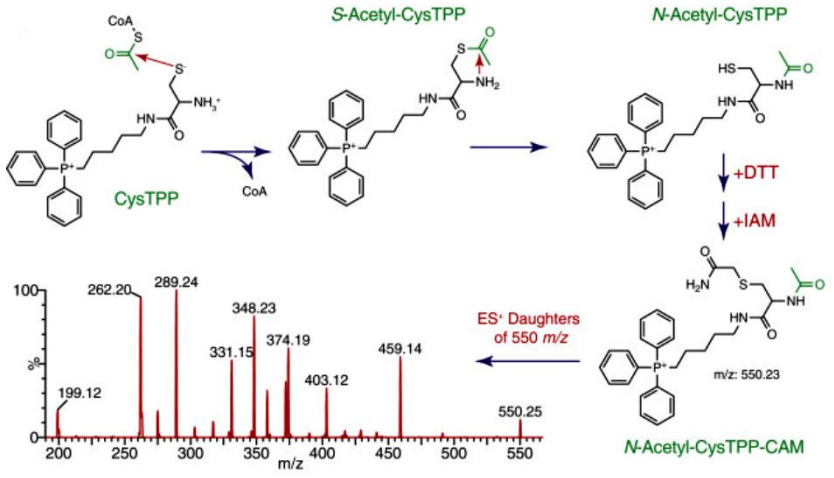


Figure 5. Detection of acetyl-CoA by CysTPP. CysTPP, [5-(2-amino-3-mercaptopropanoyl amino)pentyl]triphenylphosphonium; DTT, dithiothreitol; IAM, iodoacetamide; CAM, carbamidomethylated. Reprinted from, James et al. Native chemical ligation approach to sensitively probe tissue acyl-CoA pools, Cell Chem Biol. 29 (2022) 1232-1244.e5 [70], under the terms of the Creative Commons CC-BY license.

Expansion of current database of acyl-CoA species

Limited experimental acyl-CoA data is available in libraries such as NIST and MoNA, while ≥2000 are reported throughout the literature. These acyl-CoAs originate from conventional synthesis but also from enzyme promiscuity targeting yet unknown or unexpected carboxylic acid group containing compounds including pharmaceuticals [71]. Novel approaches are ongoing to find and identify these different acyl-CoAs to extend the experimental coverage beyond the existing databases.

Cakić et al. proposed a screening method using chromatography able to separate the isomeric species of short-chain acyl-CoAs **(Table 5, Method ix)**, including thioesters of linear and branched alkanoic acids and dicarboxylic acids **(Supplementary Figure S2)** [68]. Two detection strategies employed were based on in-source fragmentation (ISF) and data-dependent MS/MS experiments (ddMS^2) performed on a high resolution Orbitrap Fusion Tribrid mass spectrometer. For the ISF method, a full scan method was optimized to include the precursor and two major fragments, m/z 428.0365 and neutral loss of 506.9952. The precursor ions of

(unknown) acyl-CoAs with these two fragments were further evaluated on following criteria: a targeted mass trigger at m/z 428.0365, intensity filter to avoid false-positive selection and ensure MS^2 spectral quality, dynamic exclusion filter to narrow ion detection time range and limit scan repetitions, and finally targeted mass difference to confirm the neutral loss fragment. The molecular ions fulfilling all these conditions were sent for $ddMS^2$ scanning to (re)affirm the fragmentation pattern and accurate mass. The acyl-CoA species obtained from this screening process were incorporated in the inclusion list for targeted analysis. In the $ddMS^2$ method, the fragments originated from the high energy collisional dissociations scan instead of ISF. The selection criteria of molecular ions in this method were similar to the settings of the ISF experiment. Finally, the ISF method was preferred for its improved detection at lower concentrations and a list was compiled for targeted analysis. This targeted method was applied to detect targets in the degradation pathway of hexanoic acid to acetyl-CoA in betaproteobacterium “Aromatoleum” sp. strain HxN1. The extraction process of acyl-CoAs for these samples are defined in **Table 3**. In these bacterial cells, 35 species were identified, out of which eleven were confirmed by commercially available standards, thirteen by in-house synthesized standards, and eleven could not be confirmed.

To bridge the current gap between available experimental and theoretical acyl-CoA fragmentation data, Keshet et al. generated and validated an *in silico* library containing the predicted MS/MS data of unknown/hypothetical acyl-CoAs. They termed it as *in silico* CoA-Blast tandem mass spectral library and made it publicly available (github.com/urikeshet/CoA-Blast) as an online resource for annotating features in untargeted workflows [71]. The construction of the comprehensive list of *in silico* acyl-CoA compounds is described in this section. Eighteen hundred forty acyl-CoA compound structures were generated by compiling and curating targets from PubChem which contained the CoASH substructure, incorporating carboxylic acid-containing drugs to account for hypothetical acyl-CoAs and adding the odd chain fatty acyl-CoAs. Fragmentation rules were formulated using the limited existing data combined with fragmentation prediction software packages. These rules were used to model MS/MS spectra and generate predicted fragmentation patterns for these compounds in both MS polarities. The CoA-Blast MS/MS library was validated by matching spectra with the NIST MS/MS experimental library. Caution was given for the isomers as they can generate similar *in silico* MS/MS spectra. The library was further validated by acquiring MS/MS data from acyl-CoA synthetic standards in both positive and negative mode using a LC-QTOF. Apart from accurate precursor mass and MS/MS spectra, the additional dimension of retention time was

added to further strengthen the confidence of species identification. A linear regression model was developed based on the distribution coefficient (logD) of the chemical structure of the compounds. With the help of this CoA blast library, 23 acyl-CoAs (including 8 novel species) were identified in the mouse liver and annotated. **(Table 5, Method x).**

4.3 Importance of correct internal standards for absolute quantitation of acyl-CoAs

The use of an internal standard (IS) is obligatory for unstable compounds like acyl-CoAs for precise quantitation. Fu et al. highlighted the importance of using correct IS for quantitation as they can compensate for any analyte-related instability and degradation [65]. They observed degradation of >20% when neutralized extracts of acyl-CoAs were stored at room temperature for a day, degraded at -20 °C within a few days of storage or within 2 weeks of storage of acidified extracts in -80 °C. This study used labeled IS such as [1,2-$^{13}C_2$] acetyl-CoA for the quantitation of acetyl-CoA and [1,2,3-$^{13}C_3$] malonyl-CoA for the quantitation of malonyl, succinyl and propionyl-CoA. The ratio between analyte and IS shows that acetyl-CoA and malonyl-CoA can be quantified upto 28 days, as the rate of degradation was similar when IS used are the same as the analyte, while succinyl and propionyl-CoA show a higher variability, hence demonstrating the use of correct IS is important to compensate for degradation and to increase the useful time window for analysis **(Supplementary Figure S3)**.

Stable isotope analogs are the most suitable IS for LC-MS analysis because they have the same chemical properties as the target analytes and can be distinguished by their masses. But the chemical synthesis of labeled standards can be laborious and challenging, as it is not easy to incorporate labels at the desired site. Due to the commercial unavailability of most of the required labeled standards, structurally similar analytes are most commonly used for routine analysis. However, these IS can lead to over- or under-estimation due to different ionization efficiency in the ESI source, matrix effect, or different stabilities of the analyte and IS during the sample preparation process. Therefore, the use of isotope labeled IS is quite important for accurate estimation of these CoASH and acyl-CoA species. A fully and uniformly labeled ^{13}C(U-^{13}C) cell extract has been used by some studies as a potential source of IS for the quantitation of acyl-CoAs [64,107,122]. For the generation of labeled IS using this method, yeast *Pichia pastoris* was cultured on fully ^{13}C labeled glucose (sole carbon source in fed batch cultivation). This labeling strategy was not specifically targeted for CoASH and acyl-CoA thioesters, but it aimed at multiple intracellular metabolites such as organic acids, amino acids,

nucleotides, etc. As mentioned previously, acyl-CoAs are unstable molecules, hence a dedicated technique which can provide high isotopic purity for their IS is crucial.

4.3.1 SILEC for absolute quantitation of acyl-CoAs

Basu et al. developed stable isotope labeling by essential nutrients in cell culture (SILEC) for the generation of stable isotope labels for CoASH and short-chain acyl-CoA species [115,123]. SILEC leads to the generation of labeled CoASH and acyl-CoAs IS which are chemically identical, have the same stability to their corresponding endogenous CoASH and acyl-CoAs and enables their accurate quantitation as there will be parallel degradation of both unlabeled and labeled species. The approach of SILEC is based on the concept of SILAC (stable isotope labeling by amino acids in cell culture) [124], where labeled protein IS were formed by incubating cells in presence of isotope labeled essential amino acids.

Pantothenate is an essential nutrient that cannot be *de novo* synthesized by mammals and is one of the co-factors required in the cell culture medium. The biosynthetic generation of stable isotope labeled CoASH and acyl-CoAs occurs by replacing unlabeled pantothenate with labeled pantothenate ([$^{13}C_3{}^{15}N$]-pantothenate) in the cell culture medium **(Figure 6)**. The murine hepatocytes (Hepa1c1c7) were cultured in the RPMI media containing 10% serum and [$^{13}C_3{}^{15}N$]-pantothenate. The serum is the major source of contamination of unlabeled pantothenate, therefore to minimize the contamination, specialized serum such as dialyzed fetal bovine serum (dFBS) or charcoal-dextran–stripped fetal bovine serum (csFBS) should be used. The labeling efficiency (ratio of unlabeled to labeled CoASH) was monitored for five passages at 0,1,3,6,12,18 and 24 h. The optimal condition for the labeling was found with a medium containing 3% csFBS and 3 mg/L [$^{13}C_3{}^{15}N$]-pantothenate after three passages resulting in $\geq$99.5% of the CoASH species being labeled, with labeling plateau achieved at 12 h. The labeling percentage was determined by equation 1.

$$\%Lmax = L/(L+U) \times 100 \qquad (1)$$

where %Lmax = fraction of labeled CoASH; L = labeled supplemented pantothenate; U = unlabeled pantothenate. The concentrations of endogenous acyl-CoA species were determined from calibration standard curves by serially diluting unlabeled CoASH and acyl-CoA species. The same concentrations of SILEC-labeled IS were spiked in the calibration curves and experimental biological samples, followed by extraction and LC-MS analysis. The area ratios (peak area of unlabeled standard to peak area of respective SILEC IS) were used to calculate the amount of each of the CoASH and acyl-CoA species in the sample.

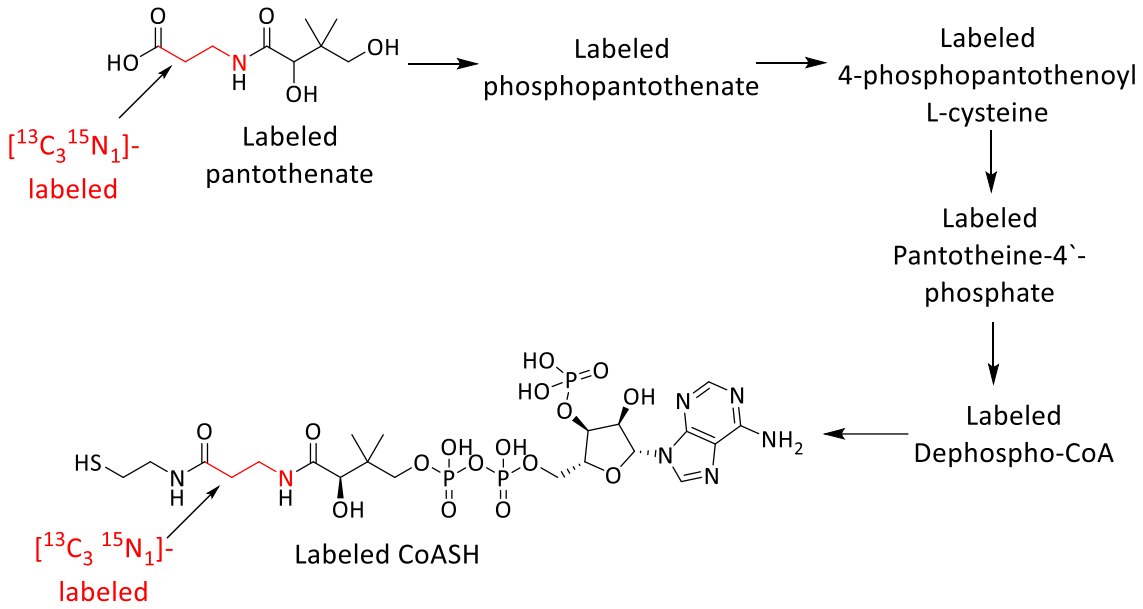


Figure 6. Metabolic pathway for biosynthesis of labeled CoASH using labeled pantothenate. The atoms marked in red are [^{13}C] and [^{15}N].
Figure is adapted from Basu et al. Stable Isotope Labeling by Essential Nutrients in Cell Culture for Preparation of Labeled Coenzyme A and Its Thioesters, Anal Chem. 83 (2011) 1363–1369. https://doi.org/10.1021/ac1027353 [115].

This assay was further expanded for the generation of stable isotope labeled medium- and long-chain acyl-CoA thioesters in Hepa1c1c7 cells by passaging cells for seven times in labeling media with 10% csFBS [6]. After establishing the labeled cell line, a 24 h passage was performed for ultra-labeling by completely removing csFBS to have the optimal acyl-CoA stable isotope labeling. Afterwards, Pan-6 deficient yeast cells were used to generate isotope labeled CoASH and acyl-CoA thioesters [125]. This yeast SILEC method was more efficient, less time consuming and more consistent as compared to mammalian SILEC method. Yeast cells can perform *de novo* biosynthesis of pantothenate. Hence, pan6 (gene responsible for synthesis of pantothenate in yeast) deficient yeast cells were used for SILEC generation. These pan6 deficient cells were cultured with [$^{13}C_3{}^{15}N_1$] pantothenic acid and was the only source for isotopically labeled pantothenate, CoASH and all the acyl-CoA thioesters. This yeast cell culture typically does not require any serum which further increases its labeling efficiency. With this technique, just after 2 days of yeast SILEC culture, ≥99% of labels were incorporated. This was a major improvement over mammalian cell culture as they have a slower growth rate compared to the yeast cells. The freeze-thaw stability of these isotope labeled standards was also tested for five cycles (each cycle was for 24 h). It was found that there was some degradation after the second cycle but after the fifth cycle, a significant degradation was

observed. This shows that calibration standard curves from the same aliquot of same batch should be considered for absolute quantitation. Many researchers have highlighted the importance of using SILEC technology for generating CoASH/acyl-CoA thioester IS [2,76,110] and have adopted this technique for carrying out the absolute quantitation in their studies [110]. The development of SILEC technology over the years has been shown in **Supplementary Figure S4.**

4.3.2 SILEC-SF: Quantitation of acyl-CoA species in subcellular compartments

Although the SILEC technology has a huge potential for defining the absolute quantities of acyl-CoAs in the biological samples, it is also important to understand the distribution of metabolites in the subcellular fractions (SF) to have deeper insight about metabolism. Trefely et al. developed another technique, SILEC-SF for quantitation of acyl-CoA thioesters in subcellular compartments [126]. SILEC has the ability to correct for many parameters such as loss of analyte, processing variation, ion-suppression and inefficient extraction. SILEC-SF technology has an added advantage for compensation of metabolic disruptions and sample losses that occur during the cell fractionation process. Using the SILEC-SF technique, the IS was included in the subcellular compartments which resulted in accurate relative quantitation of these metabolites. With this strategy, the IS was present in whole cells as well as in all the subcellular compartments after fractionation. Very distinctive profiles of acyl-CoAs were observed in the mitochondria and cytosol of cultured adipocytes, fibroblasts, mouse liver and human heart with the application of this technology.

SILEC-SF can be helpful in evaluating the acyl-CoA profiles in metabolic disorders such as FAOD at a subcellular level and thereby can assist in understanding the underlying pathophysiology by establishing a relationship between acyl-CoA supply and outcome of its metabolism.

5. Limitations in employing analytical advances for routine use of acyl-CoAs as diagnostic biomarkers

The developments of electrospray ionization and tandem mass spectrometry in 1980s and 1990s have led to rapid developments in the diagnosis of inborn errors of metabolism through the NBS program. Over the past decades, many classes of inborn errors have been included in newborn screening, as with a single injection, approximately 45 different disorders can be diagnosed [127]. Flow injection analysis-MS/MS is the most common technique that has been used for NBS. Some laboratories have started incorporating LC coupled to triple-quadrupole

mass spectrometer for expansion of the NBS as is summarized by Gelb et al. [128]. There are several methods reported for diagnosing FAOD by using LC-MS/MS, as it enables to better distinguish between compounds thus increasing the selectivity of the assays. High resolution mass spectrometers such as orbitrap, QTOF, etc. can increase the biomarker coverage as these instruments can detect a wide range of analytes and may also lead to the detection of novel metabolites. Yang and Herold summarized the current developments and advancements in the field of clinical mass spectrometry [129]**.** In the context of FAOD, acylcarnitines [130–133] are most commonly used as biomarkers followed by organic acids [134], acylglycines [135] and fatty acids [136,137]. The diagnosis of these FAOD at an early stage is crucial for a timely and effective therapy. Although acyl-CoAs are directly involved in the process of FAO, they are not routinely analyzed in clinics and hospitals. There are several techniques reported in literature related to LC-MS/MS for detecting and quantifying acyl-CoAs, but the problems associated with the analysis, like low endogenous level, requirement of tissue samples, instability and extreme variation in the physicochemical properties of different species, restricts their application as a biomarker for diagnosing FAOD. Measuring acyl-CoAs requires invasive sampling procedures to collect tissues or biopsies for cell culture. Few studies have reported the analysis of acyl-CoA species in human whole blood [72], platelets [138] and skin fibroblasts [56,58] but these have not been studied extensively. Another major concern associated in working with acyl-CoAs is the instability. Mostly they need a dedicated time-consuming sample preparation method and their extraction and analysis from the tissue samples should be done as soon as possible. In clinics, there is always a huge workload for sample screening and if analysis of these compounds is not performed carefully and timely, it may lead to faulty diagnosis. The well-known biomarkers of FAOD such as acylcarnitines, organic acid, acylglycines can be measured and integrated in one single method which also enables the diagnosis of other inherited metabolic disorders [139], while multiple methods or additional parameters such as ion-pairing are required to cover all acyl-CoA species, thus making the analysis more complicated.

Since analytical technologies are continuously evolving, there is a hope that in the near future, the current problems in the analysis of acyl-CoAs can be resolved. Moreover, the use of LC-MS/MS techniques enables the analysis of molecules in picomolar or even femtomolar range, which is a major advantage considering the low endogenous level of acyl-CoAs. There have been rapid and continuous advances in the analytical instrumentation and many vendors are trying their best to improve the technologies to make them user friendly.

6. Future perspectives and conclusion

Current strategies for diagnosing FAOD are based on the analysis of secondary biomarkers. Acyl-CoAs are more directly involved in the FAO pathway and are true indicators of the pathophysiology behind these disorders. Since these compounds are located intracellularly, most methods use cell or tissue samples. More research has to be performed especially on human samples to have alternative easily available matrices such as whole blood and fibroblasts, as these are easy to sample and can be more readily used for therapy monitoring. Determining the plasma levels of acylcarnitines are a routine way of diagnosing FAOD in the hospitals but the intracellular and extracellular level of metabolites may vary considerably during stress conditions. The inclusion of acyl-CoAs with the acylcarnitines in diagnosing FAOD will be of higher significance as the co-relation in profile of these compounds can help in understanding the pathophysiology behind FAOD.

A reference range has to be established from healthy individuals and should include representative subjects with a range of body mass index, age, gender, lifestyles, etc. as well as different metabolic stages such as fed and fasted state. LC-MS is clearly the most appropriate technique for sensitivity and selectivity. More efforts can be done in improving the chromatographic approaches like employing HILIC chromatography instead of using an ion-pairing reagent (can cause contamination in the mass spectrometers and subsequently diminish their performance) or two types of chromatography (lengthy and tedious approach). HILIC chromatography is more appropriate for polar compounds like acyl-CoAs. Although some of the earlier reported studies have used combinations of HILIC and reversed-phase chromatography for the coverage of a wide range of chain lengths of acyl-CoA compounds, future developments should be in the direction of using a single column and one method for comprehensively covering all side chains of acyl-CoAs. Zwitterionic stationary phase HILIC columns can be tried for their efficiency in analyzing acyl-CoA compounds. Chemical ligation is another interesting way to analyze these compounds. Implementation of SILEC in assays can further strengthen the absolute quantitation for acyl-CoAs. Creation of new libraries can help to extend databases for identification and confirmation of new species arising due to enzyme promiscuity. Once a standard protocol is established for routine analysis of these compounds, then clinicians and researchers should come together for the full integration of these techniques in the routine clinical practice. Developing a deeper understanding about the acyl-CoAs as biomarkers of FAOD can be of significant importance and has a huge potential in characterizing these disorders.

In this review, we have highlighted the analytical techniques that have been developed in the past few years for analyzing acyl-CoAs. Clearly, a lot of progress has been made, however more studies are required particularly focusing on ways to increase the stability and simplifying the analytical strategies of these compounds. These methodologies can not only be beneficial for FAOD but for other metabolic diseases, cancer, etc. The correlation of acyl-CoAs with other metabolites can also be helpful in predicting the different metabolic pathways involved in various other disorders.

Author contributions

Madhulika Singh: Conceptualization, Writing-original draft and editing; Hyung L. Elfrink: Writing-Review & editing; Amy C. Harms: Supervision, Review & editing; Thomas Hankemeier: Funding acquisition, Supervision, Review & editing. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Supplementary Material

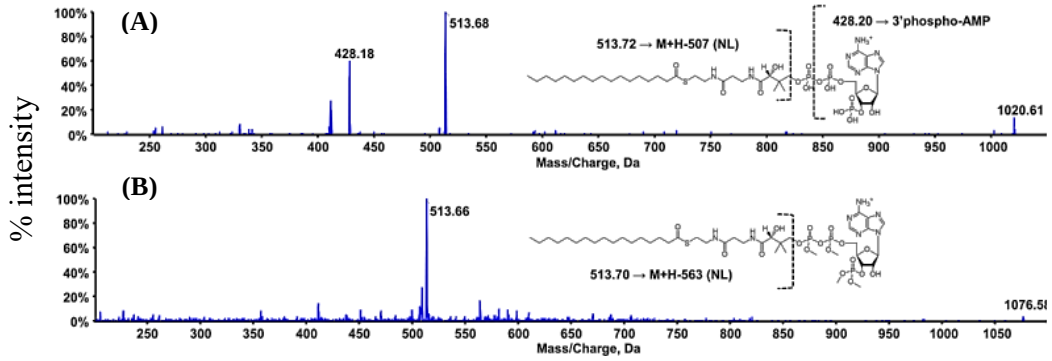

Figure S1. Product ion spectrum of (A) C17:0 CoA, (B) methylated C17:0 CoA. Phosphate methylation of CoA moiety will methylate all the phosphate groups of CoA leading to the neutral loss of 563 Da.
Reprinted with permission from, Li et al. Targeted Profiling of Short-, Medium-, and Long-Chain Fatty Acyl-Coenzyme As in Biological Samples by Phosphate Methylation Coupled to Liquid Chromatography–Tandem Mass Spectrometry, Anal. Chem. 93 (2021) 4342–4350, Copyright (2021) [1].

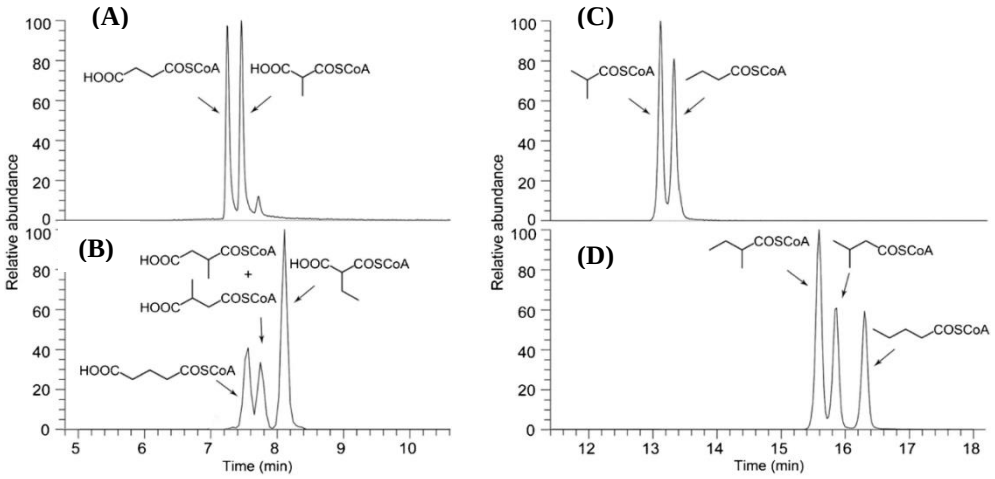

Figure S2. Separation of acyl-CoA thioesters isomers. XIC of acyl-CoA standards (A) Succinyl-CoA and methylmalonyl-CoA (m/z 361.1428); (B) Glutaryl-CoA, ethylmalonyl-CoA and a mixture of 2- and 3-methylsuccinyl-CoA (m/z 375.1584); (C) Butyryl- and isobutyryl-CoA (m/z 331.1686); (D) 2-methylbutyryl-CoA, isopentanoyl-CoA and pentanoyl-CoA (m/z 345.1844).
Reprinted from, Cakić et al. Suspect screening and targeted analysis of acyl-coenzyme A thioesters in bacterial cultures using a high-resolution tribrid mass spectrometer, Anal Bioanal Chem. 413 (2021) 3599–3610, under the terms of the Creative Commons CC-BY license [2].

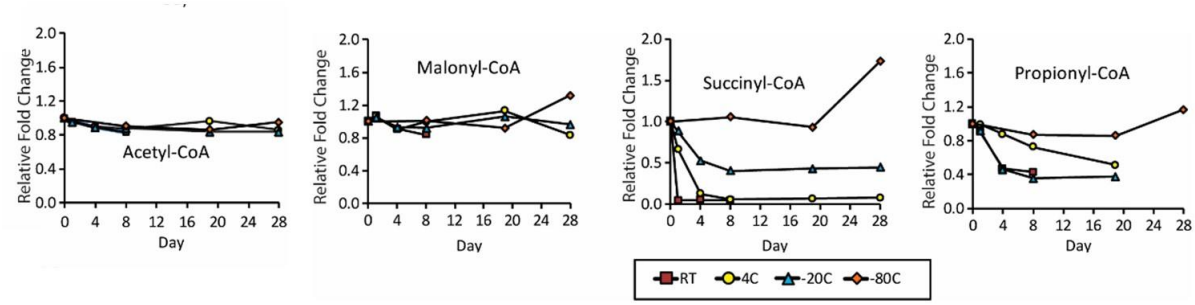

Figure S3. Assay stability using stable isotope labeled ISs at room temperature (RT), 4 °C, −20 °C, and −80 °C. Reprinted with permission from Fu et al. Targeted Determination of Tissue Energy Status by LC-MS/MS, Anal. Chem. 91 (2019) 5881–5887 [3], Copyright (2019), (https://pubs.acs.org/doi/10.1021/acs.analchem.9b00217) under the terms of ACS AuthorChoice License. Further permission related to the material excerpted should be directed to the ACS [3].

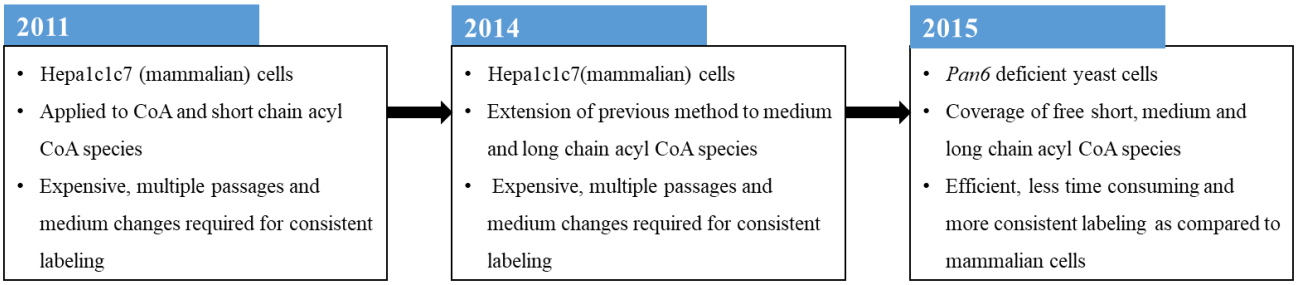

Figure S4. Development of SILEC technology over the years.

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