ANALYTICAL METHODS FOR THE QUANTITATION OF XENOBIOTICS

by

LUIS GABRIEL RIVERA

(Under the Direction of Michael G. Bartlett)

ABSTRACT

To uncover information regarding the condition of a disease or ailment caused by dysregulation in biochemical pathways, it is imperative to utilize biomarkers to assess and quantitate changes in metabolite concentrations in response to atypical conditions. Analytical methods provide the means to quantitate analytes and uncover information for various studies including metabolic pathways, pharmacokinetics of a particular metabolite and its onset and duration of action. In Chapter 1, an introduction to liquid chromatography and mass spectrometry is presented. Chapter 2 is a literature review of the chromatographic methods used for the quantitation of acyl-CoAs with an emphasis on liquid chromatography tandem mass spectrometry methods.

INDEX WORDS: High-performance liquid chromatography, Mass spectrometry, LC-MS/MS analysis, Acyl-CoA, Quantitation.

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B.S., University of Georgia, 2018

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2019

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August 2019

DEDICATION

This thesis is dedicated to my loving and caring family: my mother Priscilla Pizarro-Rivera, my father Hector Luis Rivera, my brother Hector Daniel Rivera, and my sister Danelis Priscilla Rivera, for their unconditional love, inspiration, encouragement, and support.

ACKNOWLEDGEMENTS

Reaching this goal was possible because of those individual who believed in me and supported me throughout this journey. I express my sincere gratitude to Dr. Michael Bartlett, my advisor, for challenging me and supporting me throughout this process. I also express my gratitude to my committee members, Dr. Roberts and Dr. Cai for their guidance and support. Lastly, I would like to thank my lab mates for their assistance and advice.

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CHAPTER 1

INTRODUCTION

For pharmaceutical research and development, one of the critical steps in the process includes the discovery of a lead compound. If a compound exhibits a property likely to be therapeutically useful, it is often used as a starting point for drug design. During the early stages of drug development, analytical studies are conducted to assess the properties of the compound, including the structure of a compound. However, in order to identify the structure of a compound, the compound must first be isolated whether it is a product of a synthetic reaction that needs to be separated from its side products, or a compound from nature that needs to be isolated from its biological source. Afterwards, the isolated compound's structure must be determined; otherwise, researchers cannot identify how the compound's structure could fit into a specific target site and induce its therapeutic activity or how the compound's structure could be synthetically manufactured for mass production. Additionally, another core aspect of research and development of active pharmaceutical ingredients is assessing the correlation of the compound's presence in the body and how it affects normal biological processes and/or pathogenic processes *in vivo*. Specifically, biological markers, or biomarkers, are integral for pharmaceutical research because they are used to measure the effects of drugs in the body. According to the National Institute of Health (NIH), a biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention."[1] Essentially, when a drug is introduced into a biological system, the concentration of biomarker may be

altered, which may reveal safety issues related to the drug candidate or uncover a pharmacological effect that demonstrates the benefit of the treatment. Overall, a variety of techniques are employed to isolate and determine a compound's structure, or to quantitate the concentration of biomarkers after administering a drug compound. Nevertheless, one of the most robust and reliable techniques is liquid chromatography coupled with a mass spectrometer (LC-MS).[2,3]

The applicability of LC-MS is not limited to the isolation of analytes for structural confirmation. In other pharmaceutical applications, LC-MS provides a method for quality control (QC) of drugs by searching and confirming the presence of impurities in dosage forms that may have arose during the manufacturing process (i.e. active pharmaceutical ingredient/excipient interactions that may have formed a hazardous byproduct).[4] Similar to the first example, the applicability of LC-MS in proteomics in conjunction with drug discovery permits the development of analytical methods for structural confirmation and molecular weight determination of lead compounds.[5] However, the high sensitivity, specificity, and robustness of LC-MS highlights one of the most useful applications of this type of chromatographic assay: bioanalysis. The incorporation of LC-MS in a bioanalytical procedure permits the quantitation of drugs and their metabolites in biological matrices, pharmacokinetic studies,[6] and for pharmaceutical product analysis.[7]

The combination of both liquid chromatography and mass spectrometry provides the benefits of both analytical techniques while simultaneously mitigating the drawbacks of each technique if they were conducted on their own. Liquid chromatography is excellent for its capability to separate complex mixtures, but it is not an ideal method for elucidating structural information of the analyte or material, especially for compounds that lack a chromophore for a

UV/visible detector.[7] Mass spectrometry provides high sensitivity and selectivity for the quantitation of analytes and compounds based on their mass-to-charge ratio (*m/z*). However, MS alone cannot separate the desired analyte from a highly complex mixture, especially in biological matrices. Therefore, the combination of liquid chromatography and mass spectrometry alleviates the limitations of both instruments individually.

Liquid Chromatography

Liquid chromatography is an analytical technique that separates the analyte of interest from the other components of a solution or matrix for the purpose of the identification and quantification of specific components in the mixture. One of the most widely used liquid chromatographic equipment utilized in the industry and research is high-performance liquid chromatography (HPLC). In general, HPLC pumps a liquid mobile phase with a set amount of pressure through a stainless steel column to partition the analyte from the components of the liquid mobile phase based on the analyte's affinity to the stationary phase of the column. The instrumentation of a standard HPLC instrument consists of the following: the solvent reservoir, the pump, the mixer, the injector, and the stainless steel column. The solvent reservoir holds the mobile phase that elutes through the HPLC system. The pump generates a set amount of pressure to propel the mobile phase through the instrument at a specified flow rate. After the solvents are pumped, the mobile phases are mixed in the mixer in specific ratios; essentially, the mixer is programmed to have a fixed percentage of the mobile phase from each pump head and combines them before going to the column. The injector loads the sample into the flowing mobile phase that carries the sample into the column. The column performs the chromatographic procedure of the HPLC instrument. The stainless steel column is packed with chemically modified particles that will cause different components of the mobile phase to elute at different time intervals based

on the individual component's affinity to the packing material, or stationary phase, within the stainless steel column. However, the type of mobile phase varies depending on the type of column that is selected for liquid chromatography. Several types of columns are employed to retard and separate the analyte of interest from the mobile phase: normal phase chromatography,[7] reversed-phase chromatography,[8] hydrophilic interaction liquid chromatography (HILIC),[9,10] ion-pair chromatography,[11,12] affinity chromatography,[13,14] ion-exchange chromatography,[15-17] and size-exclusion chromatography.[18,19]

Normal phase columns are packed with unmodified, polar silica or alumina. Normal phase chromatography is ideal for the separation of compounds with very slight differences in their structures.[20] This type of chromatography is more suitable for retarding polar compounds from organic mobile phases (i.e. methanol, hexane, or chloroform). Reversed-phase columns, in contrast, are packed with octadecylsilyl (ODS) silica gels or similar reversed-phase packing that retard molecules based on their lipophilicity; this type of chromatography incorporates a combination of a buffered aqueous solvent and an organic phase. In addition, there is variation within reversed-phase packing material based on how the chemical modification of the silica gel is implemented (i.e. C8 versus C18 columns). Reversed-phase chromatography is the most popular chromatographic method implemented for liquid chromatography because of its myriad of applications and simplicity for LC operation.[21]

HILIC columns are packed with polar stationary phases such as silica, alumina, amino, or cyano; typically, bare silica is implemented, or silica gels modified with polar functional groups. Under HILIC, the more polar the compound, the more strongly it is retained in the column. Similar to reversed-phase chromatography, HILIC simultaneously employs both organic and aqueous solvents.[9,10]

Ion-pair chromatography is a type of reversed-phase chromatography that incorporates the same packing material used in a reversed-phase column except that the mobile phase used in ion-pair chromatography incorporates an amphipathic molecule called ion-paring reagent, to perform separation between organic and inorganic ionic compounds. The incorporation of the ion-pairing reagents affects the retention time and selectivity of ionic compounds. Examples of ion-pairing reagents include alkyl ammonium salts for acidic analytes and hexyl sulfonic acids for basic analytes.[11,12]

Affinity chromatography is based on a biochemical interaction of an interior component of the column that is specific to a target species from sample components. Affinity chromatography incorporates an affinity ligand or biologically active molecule that is covalently bonded to the solid interior of the HPLC column. As the mobile phase flows through the column, the affinity ligand will then selectively interact with the analyte of interest within a sample. The interaction between the affinity ligand and analyte forms a reversible complex, which can then be eluted by changing the mobile phase buffer conditions. This type of chromatography is more applicable in biological and biotechnology fields for quantitating molecular and cellular interactions.[13,14]

Ion-exchange chromatography separates ions and ionized-substances that are formed by pH manipulation or complex formation. Separations of ionized analytes are dependent on the differences in their affinity to the mobile phase ions and stationary phase ions. In ion-exchange chromatography, analytes interact with the stationary phase of the column by charge-charge interactions. Retention is dependent on the electrostatic attraction between the sample and eluent ions in the mobile phase and the ions of opposite charges in the stationary phase. Chromatographic separation corresponds on the charged analyte's affinity to the cationic or

anionic packing material inside the HPLC column, since molecules will differ from one another based on their surface charge properties. Oppositely charged analytes are attracted to the ionic groups of the ion-exchange media in the column. Afterwards, a gradient elution that changes the pH of the mobile phase will weaken the interaction between the analyte and the stationary phase, which will desorb and elute the analyte from the column. Charge type (i.e. anionic or cationic) and charge strength of analytes affects the retention of the analyte in the column; therefore, different types of column packing material are available. Ion-exchange chromatography columns can provide strong cation-exchange (SCX), weak cation-exchange (WCX), strong anion-exchange (SAX) and weak anion-exchange (WAX) depending on what kind of analyte is being selectively separated.[15-17]

Lastly, size-exclusion chromatography, as the name implies, separates sample components from solution based on their size. The stationary phase of the size-exclusion column contains porous polymeric beads with a range of specified pore sizes. As the solution containing the sample components flows through the size-exclusion chromatographic column, smaller particles will retain within the pore of the column material and, consequently, will elute later than larger particles.[18,19,22]

Mass Spectrometry

Mass spectrometry is an analytical method that determines the molecular weight, molecular formula, and specific structural features of a compound.[23] The mass spectrometer, measures the mass to charge ratio (m/z) of molecules or molecular fragments with the application of either electrostatic or electromagnetic fields. Essentially, a mass spectrometer consists of three basic components: an ion source, which is used to generate ions; a mass analyzer, which is a high-vacuum region that separates the charged molecules based on their m/z

values; and lastly, a detector, such as an electron multiplier, measures the current in response to ions impacting the surface. Mass spectrometry, in conjunction with chromatographic techniques like HPLC, provides a highly sensitive and specific method for detecting different types of compounds, such as small inorganic molecules and large biomolecules.[24]

The ion source generates charged species before the analyte of interest enters the analyzer. With thorough progress and development of mass spectrometers, various ionization techniques are available for the production of charged compounds. Popular methods include electron ionization (EI) and matrix assisted laser desorption ionization (MALDI). Nevertheless, the most applicable and utilized ionization method is electrospray ionization (ESI). Electrospray ionization (ESI) produces gas phase ions from the solvent flow of the HPLC system and allows the ions to go through a heated capillary with the assistance of a heated inert gas, like nitrogen (N_2), for nebulization of the ions. A high electrical voltage is applied to the metal capillary tube to form charged droplets, which are then vaporized. As the droplets are being evaporated, the size of the droplet starts shrinking, and eventually the internal charge-charge repulsion forces, or Coulomb forces, on the surface of the droplets start to increase. Then, the droplets will become unstable as they approach their Rayleigh Limit, which is the theoretical limit of Coulomb forces necessary to overcome the surface tension of the droplet. Once internal charge-charge repulsion forces overcome the surface tension of the droplets, the droplets will undergo a Coulomb explosion, meaning that the droplets will deform and break up into smaller droplets. The ions desorb into the gas phase; then, the ions are attracted towards the mass spectrometer by an opposite charge that is applied to a capillary inlet.[24-28]

Next, the analyzer separates the ions according to their respective m/z values. Generally for mass spectrometers, ions are electrically driven to separation, although magnetic fields could

also be employed to separate ions. There are various ion separation techniques readily available, such as ion trap, time of flight (ToF), and orbitrap. However, one of the most robust and widely used mass analyzers is the quadrupole analyzer. The quadrupole instrument incorporates four cylindrical rods that are parallel to one another. The cylindrical rods utilize two electric fields to separate the ions: one field uses direct currents while the other field incorporates alternating radio-frequency potentials. By alternating the electric fields onto the rods, the quadrupole instrument will generate a resonance frequency that will correspond to the ideal *m/z* of the ion of interest. The ions that do not resonate with the resonance frequency will not pass through the path to reach the detector and will ultimately collide with the rods and be subject to ejection. However, the ions that do resonate with this frequency will traverse through the rods and reach the detector.[7,24]

Tandem Mass Spectrometry (MS/MS): Triple Quadrupole

With the advancements in bioanalytical methods, applications and instrumentations, researchers have further improved the sensitivity and specificity of mass spectrometry with the development of tandem mass spectrometry (MS/MS). When electrospray ionization is utilized in mass spectrometry, one of the few drawbacks is that very little fragmentation is induced in a molecule; consequently, structural identification can sometimes be compromised, meaning it would be difficult to derive the structure of a compound that undergoes little fragmentation.[7] Therefore, tandem mass spectrometry can overcome this drawback. Tandem mass spectrometry when coupled with liquid chromatography serves as an excellent technique in the pharmaceutical industry for the detection of trace impurities or the identification of a molecular structure of an analyte.[7] Tandem mass spectrometry provides analytical data that helps confirm the identity and structure of small molecules, xenobiotics, peptides, and proteins.[29,30] In addition to higher

sensitivity, tandem mass spectrometry permits higher Signal-to-Noise (S/N) ratios, which ultimately lead to the development of bioanalytical methods that can detect analytes at lower concentrations with better accuracy and reproducibility.[30]

As mentioned earlier, quadrupole instruments are one of the most widely used ion separation apparatuses, especially when they are configured into a triple quadrupole mass spectrometer. Tandem mass spectrometry consists of multiple separations of ions by incorporating a minimum of two mass spectrometric steps (MS1 and MS2); triple quadrupole instruments, as the name implies, contains three quadrupoles, where the first and third quadrupole perform ion separations. In the first mass spectrometric step, the first quadrupole selectively filters and isolates ions of a particular *m/z* value; the ion that is selectively filtered by the first quadruple is called the precursor ion. Afterwards, the second quadruple fulfills the role of a collision cell to further generate fragmentation of the ions that passed through the first quadrupole; the second quadrupole is filled with neutral inert gas in order to perform ion fragmentation. This process is usually performed by collision-induced dissociation (which is also called collision-activated dissociation). Thirdly, the ions are introduced into the third quadrupole where it performs an additional round of ion filtration before the ions of interest reach the detector; performing a second round of ion fragmentation and filtration yields higher selectivity and lower interference.[24,31,32]

Conclusion

The coupling of liquid chromatography and mass spectrometry provides a robust analytical method that has had a significant impact in the pharmaceutical industry and bioanalytical research. LC-MS provides a broad range of applicability, including impurity identification, metabolite identification, quantitative bioanalysis, and qualitative bioanalysis.

Also, the combination of LC-MS incorporates the benefits of both instruments. For LC, the different types of chromatographic columns that are incorporated for LC separation provide various methodologies to partition components from a complex mixture or matrices based on the component's affinity to the stationary phase and time retained inside the LC column. For MS, incorporation of an ion generation apparatus, ion separation apparatus, and detector permits for rapid identification and quantitation of trace impurities, metabolites, and biomarkers. In addition, liquid chromatography tandem mass spectrometry provides a large number of additional advantages over traditional LC-MS for concentration quantitation and qualitative bioanalysis, including higher sensitivity, lower LOQ and LOD values, better reproducibility, higher precision, and better accuracy at lower concentrations.

In summary, LC-MS assists in the quantitative measurements of analytes of interest from biological matrices. This type of analytical technique has played a pivotal role in bioanalysis as it highlights imperative information regarding the drug's disposition and metabolism from biological systems like plasma, whole blood, liver and brain tissue. Quantitating the concentrations of particular metabolites/analytes with the assistance of a calibration curve will yield results that may demonstrate a change in molecular or biochemical pathways.

In this thesis, a discussion on the impact of LC-MS methods for acyl-CoA quantitation is examined. Chapter 2 is a literature review discussing previous chromatographic methods (in particular LC-MS) sample preparation methods to effectively quantitate acyl-CoAs while simultaneously incorporating that information to identify between acyl-CoA concentrations and changes in metabolic behavior within the bioactive lipid pathway.

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CHAPTER 2

LITERATURE REVIEW

CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF ACYL-COAS

Luis G. Rivera and Michael G. Bartlett. Accepted by *Analytical Methods* 10, 5252-5264 (2018)
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Abstract

Acyl-CoAs have been identified in various metabolic pathways, such as β-oxidation, biosynthesis and/or remodeling of lipids, fatty acid modification, transcriptional regulation, protein post-translational modification, enzyme regulation, and participation in signaling pathways. Therefore, there is a growing interest in studying these metabolites to grasp a better understanding of how these fatty acid intermediates directly impact these metabolic pathways and induce diseases and cancer progression through their dysregulation. Liquid chromatography coupled with mass spectrometry provides the most robust and reproducible method for the analysis of acyl-CoAs. This review highlights chromatographic methods that have been validated for acyl-CoA determination; however, this review prioritizes LC-MS methods because this approach has recently been utilized more frequently for method development of acyl-CoAs analysis due to its robustness and quantitation applicability. Therefore, the review discusses how current LC-MS methods surpass previous analytical methods for acyl-CoA detection and quantitation, and summarizes the types of mass spectrometers used, preparation approaches for different biological samples, LC conditions for the separation of acyl-CoAs, and potential biomarker applications.

Introduction: Acyl-CoA

Fatty acids are aliphatic compounds with a terminal carboxyl group. These compounds are bioactive lipid species that derive from various endogenous sources, such as *de novo* fatty acid synthesis, and exogenous sources (i.e. dietary intake of fats).[1,2] Increased accumulation of fatty acids in the body as a consequence of a high-fat diet correlates with many diseases including obesity, type 2 diabetes, tumorigenesis that leads to the progression of cancer, and cardiovascular disease.[3-5] Before fatty acids can enter bioactive lipid networks and participate in several different cellular processes, they must be must be conjugated to a coenzyme A (CoA) to form acyl-CoAs.

Acyl-CoAs are derivatives of fatty acid molecules that are comprised of a coenzyme A moiety, a fatty acid of a certain length, and a thioester bond. The activation of fatty acids to acyl-CoAs is catalyzed by one of 26 acyl-CoA synthetase (ACS) isoforms, depending on the chain length of the fatty acids. For instance, short-chain acyl-CoA synthetases activate fatty acids of less than 6 carbons, while medium-chain acyl-CoA synthetases and long-chain acyl-CoA synthetases activate fatty acids of 6-10 carbons and 12-20 carbons respectively.[2] These acyl-CoAs have many metabolic fates such as cellular fatty acid metabolism via β-oxidation for energy production,[6] biosynthesis and/or remodeling of lipids (i.e. activated fatty acids can be converted into triacylglycerols or sterol esters when they are esterified with glycerol or sterol backbones respectively), fatty acid modification, membrane incorporation, transcriptional regulation, protein post-translational modification, regulating enzymes and participating in signaling pathways.[2,7,8] Nevertheless, the buildup of acyl-CoAs promotes adverse effects in the body. For example, acyl-CoA is an allosteric inhibitor of phosphofructokinase-1,[9] an allosteric enzyme that converts fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and is the

major control point for hepatic glucose metabolism; inhibition of this enzyme limits glycolysis, limiting hepatic uptake of dietary glucose and production of *de novo* lipogenic substrates.[10] Additionally, acyl-CoAs, specifically long-chain acyl-CoAs, are involved in cancer progression. An example of this is seen when the overexpression of acyl-CoA synthetase long-chain family member 4 (ACSL4) generates arachidonoyl-CoAs (20:4ω6-CoA), which may deplete the proapoptotic unesterified arachidonic acid (20:4ω6) and reduce the likelihood of apoptosis in colon cancer.[2,11] Additionally, 20:4ω6-CoA appears to promote tumorigenesis and cell proliferation by regulating signaling molecules such as protein kinase C, leading to androgen receptor reactivation in prostate cancer cells, or binding to a transcription factor like hepatic nuclear factor-4α, inhibiting its activity and promoting tumor growth in hepatocellular carcinoma.[2,12-14]

Considering the fundamental roles of acyl-CoA in physiological and pathological pathways and how the dysregulation of acyl-CoA metabolism leads to the progression of numerous diseases, analytical methods to identify and to quantitate acyl-CoAs would be beneficial. Among the many options, liquid chromatography tandem mass spectrometry (LC–MS/MS) has been shown to be the most effective analytical method for the detection and analysis of acyl-CoAs. Earlier methods for acyl-CoA quantitation utilized reversed-phase liquid chromatography interfaced with an ultraviolet (UV) detector.[15-20] However, LC-UV methods are less specific and may have difficulty with unresolved co-eluting peaks.[21] In addition, the sensitivity and selectivity of liquid chromatography combined with UV detectors are lower when compared to LC-MS/MS methods. Another method that has been utilized for the detection of acyl-CoAs is reversed-phased liquid chromatography coupled with fluorescent detection.[22-24] Although LC coupled with fluorometric detection is a more selective detection method, acyl-

CoAs are not very fluorescent compounds and typically chloroacetaldehyde is used to derivatize acyl-CoAs to increase their quantum yield following conversion to acyl etheno CoAs. Furthermore, this approach is less appealing due to the time involved in this derivatization reaction.

Another analytical method that has been utilized for the determination of acyl-CoA species is gas chromatography.[25] Gas chromatography is a versatile technique with several detector options such as flame ionization detection (GC-FID),[26] negative-ion chemical ionization mass spectrometry (GC/NICI-MS)[27,28], and electron ionization mass spectrometry (GC/EI-MS).[25,29] The selectivity of GC-MS is reduced due to the need to derivatize the acyl-CoA species into a volatile form. Side reactions and the chemicals used for the reaction increase the potential of forming interferants. According to Gao et al., the need for derivitization and hydrolysis of acyl-CoA compounds adds additional steps to the analysis and can potentially affect the accuracy of the quantitative measurements.[30] Additionally, Larson et al. reported that the cross-reaction of acyl chains not originating from acyl-CoAs, such as fatty acids, during the derivatization procedure reduced the selectivity of the results.[23] Therefore, not only does LC-MS tend to have better sensitivity than GC-MS, but the approach also has the benefit of not requiring derivatization.

Although this review briefly discusses several different chromatographic methods for the determination of acyl-CoA, this review will prioritize and highlight LC-MS methods because they surpass previous chromatographic methods in robustness, reproducibility, specificity and selectivity. Additionally, in recent years, more researchers have employed LC-MS techniques for the development of novel methods for the detection and quantitation of acyl-CoAs in different biological samples than preceding chromatographic methods.

Mechanisms of Mass Spectrometry to Detect Acyl-CoA

Mass spectrometry has been used in many different configurations for the determination of acyl-CoAs. This includes electron ionization and chemical ionization following separation using gas chromatography, matrix-assisted laser desorption ionization of isolated acyl-CoAs and electrospray ionization following separation using liquid chromatography. Each of these approaches has advantages and specific challenges.

Chemical Ionization

Chemical ionization consists of two different approaches: positive ion chemical ionization (PICI) and negative ion chemical ionization (NICI). For acyl-CoA analysis, NICI has been more widely used than PICI. Wolf et al. quantitated long-chain fatty alcohols in subfemtomole amounts utilizing GC/NICI-MS. The method of Wolf et al. was also applied to the measurement of long-chain acyl-CoA in femtomolar amounts by reducing them to fatty alcohols using sodium borohydride. Afterwards, the fatty alcohols were extracted and converted into a pentafluorobenzoyl ester for GC/NICI-MS analysis performed on a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5988 mass spectrometer. Analytes were separated with a Hewlett-Packard Ultraperformance capillary GC column (8 m × 0.31mm I.D., cross-linked methylsilicone, film thickness 0.17 μm).[26,27] Tamvakopoulos et al. also demonstrated an analytical method for the quantitation of acyl-CoAs by GC/NICI-MS. Glycine aminolysis and pentafluorobenzyl esterification of acyl-CoAs were implemented to generate N-acylpentafluorobenzyl glycinates for GC/NICI-MS detection.[28] Nevertheless, these derivitization processes add extra steps in the sample preparation and analysis, potentially affecting the accuracy of the quantitative measurements.[27,28]

Matrix-assisted Laser Desorption/Ionization

Matrix-assisted laser desorption/ionization (MALDI) ionizes samples using a laser before the ions are separated in the mass spectrometer.[31-33] The method of Hankin et al. incorporated MALDI with time-of-flight mass spectrometry to improve resolution and sensitivity. Prior to being irradiated with a 337 nm nitrogen laser, fatty acyl-CoAs were dissolved in 1:1 water-tetrahydrofuran (THF) at a concentration of 50 μM. Then, the samples were applied to a matrix plate following application of 65 mM of dihydroxybenzoic acid in 1:1 water-THF. The matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF) analyzed samples in negative and positive ion mode. For positive ion mode, trifluoroacetic acid was applied to the matrix plate. Nonetheless, Hankin et al. reported MALDI-TOF as having higher sensitivity than electrospray ionization but found that it was not as versatile as electrospray tandem mass spectrometry.[34]

Electrospray Ionization

Electrospray ionization (ESI) produces gas phase ions from the solvent flow of the LC system. Afterwards, the gas phase ions bleed into the high vacuum system prior to reaching a number of different mass analyzers.[35-37] Electrospray ionization for acyl-CoA determination has been accomplished in both positive[38-43] and negative ion modes.[39,43-46]

Acyl-CoAs form stable protonated species $[M+H]^+$ in positive ion mode. When undergoing collision-induced dissociation (CID), the most abundant fragment ion was produced following the neutral loss of 3'-phosphonucleoside diphosphate (507 daltons).[40,41,43,47-50] In negative ion mode, $[M–H]^-$ and $[M–2H]^{2-}$ have been observed.[39] Following CID, two abundant product ions have been observed corresponding to the 3'-phospho-AMP moiety (m/z 426) and then subsequent dehydration (m/z 408).[44,45,51] Nevertheless, ESI in positive ion mode is more

widely used because of its greater sensitivity and the ability to use the loss of the neutral phosphoadenosyl phosphate fragment for LC-MS/MS employing multiple reaction monitoring (MRM), which can be observed in Figure 2.1 from the paper of Yang et al. (Reprinted with permission from Yang, Ma, Li, Cai, and Bartlett. Copyright 2017 American Chemical Society).[40,41,43,47-50,52,53]

Sample Preparation and Extraction

Prior to mass spectrometric analysis, acyl-CoAs need to be extracted from biological samples. Acyl-CoAs can be extracted from plant tissues,[23,24,49,54,55] mammalian tissues,[16,30,33,39,41,42,45,47,48,52,56] and mammalian cells.[40,43,52,57-59] There are several reported methodologies for the extraction of fatty acyl-CoAs from biological samples. Although there are slight variations, the protocol of Mancha et al. for acyl-CoAs from plant tissue serves as the general basis for MS and MS/MS procedures for acyl-CoA extraction from biological samples.[54] The Mancha et al. method reports that the sample is first homogenized in an organic-aqueous solvent. Then, the sample undergoes liquid-liquid extraction (LLE) in order to separate polar and non-polar lipid species. Mancha et al. utilized isopropanol-water and a chloroform-methanol mixture to partition the acyl-CoA samples in the aqueous phase and to partition the lipids into the non-polar organic phase. Optionally, adding ammonium sulfate can cause protein precipitation of acyl-acyl carrier proteins or, in the method of Deutsch et al., to remove aqueous impurities from the extract.[21] Afterwards, solid-phase extraction (SPE) is implemented to concentrate and purify the acyl-CoAs samples. Mancha et al. incorporated neutral alumina gel in SPE to induce the final separation of acyl-CoAs from polar chloroform-methanol mixtures.[54]

Sample Preparation and Extraction from Mammalian Tissue

The preparation of mammalian biological samples varies depending on the type of sample being observed. Acyl-CoA samples have been extracted from different types of mammalian tissues. Studies have been conducted where the quantity of acyl-CoA has been assessed in human muscle and human adipose tissue,[60] rabbit muscle,[46] rat liver and hamster heart,[22,25] and bovine retina.[61] Nonetheless, the most common mammalian tissue that is prepared for acyl-CoA quantitation and determination are rodent tissues (i.e., rats and mice).

For the preparation of the mammalian tissue samples derived from mice or rats, the rodents are euthanized and the tissues of interest are removed immediately. Then, the rodent tissues are freeze-clamped and stored for later use, powdered, or homogenized in an organic-aqueous solvent. For the quantitation of acyl-CoAs in mammalian tissues, the weights of the specimens are recorded prior to the homogenization of the samples. The units for acyl-CoAs in tissue are reported in nanomoles per gram of tissue (nmol/g).[16,19,21,25,62] Prior to homogenization, the tissue samples are spiked with a known concentration of an internal standard, such as heptadecanoyl-CoA, to assist the quantification of acyl-CoA and to recapitulate the instability of acyl-CoAs.[21,42,47,63,64]

LLE-SPE methods used in HPLC-UV methods, particularly the LLE-SPE method of Deutsch et al.[21] can also be used for LC-MS/MS methods for acyl-CoA extraction from mammalian homogenates. According to Deutsch et al. for LLE, the mammalian tissue was suspended and homogenized in 100 mM KH_2PO_4 buffer solution and 2-propanol to extract the acyl-CoA esters from the tissue.[21] Essentially, the tissue homogenate is centrifuged; then, the supernatant is extracted in an organic-aqueous solvent. Afterwards, the analyte is further separated from impurities through the use of SPE, which is similar to the method of Mancha et

al.[54] Different extraction efficiencies of acyl-CoAs occur depending on the type SPE cartridge and solvents used in the sample preparation. Table 2.1 lists the different SPE phases that have been implemented to purify mammalian acyl-CoAs with different chain lengths and analyte recoveries.

Nonetheless, modifications of the LLE-SPE procedure of Deutsch et al. have been implemented in other mammalian tissue studies. Mammalian tissues are complex biological matrixes that can induce matrix effects due to the presence of interferants and other unintended analytes in the sample, affecting analyte response. Therefore, Basu et al. suggested repeating SPE washing steps once or twice to separate the interfering substances and to increase the desired analyte in the sample.[66] Alternatively, some procedures eliminated SPE and only conducted LLE extraction to reduce the amount of time and chemicals involved in the procedure. Additionally, elimination of this step from the sample preparation may prevent possible analyte loss resulting from adherence to the SPE column.[39,46,71]

Sample Preparation and Extraction from Mammalian Cells

Various types of mammalian cells have been studied for acyl-CoA quantitation, including breast cells,[43,58] hepatic cells,[40,57,59,66,69] colon cells,[53,57] skeletal muscle cells,[69] and prostate cells.[40] For sample preparation, cells are maintained and grown in cell culture medium to provide amino acids and other essential nutrients to facilitate growth, metabolism, and to stimulate proliferation. Various mediums have been used for cell culture, and additional supplements have been incorporated to facilitate growth, metabolism, and to stimulate cell proliferation. A list of cell mediums and additional supplements can be found in Table 2.2. Cells were maintained in a humidified incubator at 37 ºC with 5% CO_2. Cells were grown until they reached 80-90% confluence. At this point, sample preparation and extraction can begin.

From a fundamental standpoint, acyl-CoA sample preparation occurs in a few basic steps: rinsing, cell detachment, quenching, and extraction. After the cell culture media was aspirated, the cells were washed, or rinsed, with buffer solutions or solvents prior to quenching. Next, cells need to be detached from the culture plate surface, whether they are detached enzymatically using trypin/EDTA or mechanically scraped.[40,52,72] Quenching was then conducted to halt or inactivate cell metabolism, minimizing the changes in metabolic profiles and reducing the potential for misleading results. Lastly, the acyl-CoA analytes are extracted from the supernatant.[72,73] These four basic steps are highlighted in Table 2.3.

Variations and alternative methods for acyl-CoA extraction from mammalian cells are available. Wang et al. incorporated LLE to further partition acyl-CoAs to the aqueous phase after adding 1 mL of methanol and scraping glioma cells from the culture plate to an Eppendorf tube.[52] Alternatively, Abrankó et al. reconstituted their samples in 4:1 methanol-water mixtures to achieve better recovery of longer-chain acyl-CoA compounds for their extraction protocol.[69] Additionally, an important parameter in the extraction of acyl-CoAs is their susceptibility to hydrolysis. Acyl-CoAs are prone to hydrolysis, meaning that they are unstable in aqueous solutions. Avoiding the hydrolysis of acyl-CoAs is imperative, because hydrolysis will lead to a poorer signal and lower recovery. Therefore, Yang et al. observed the stability of acyl-CoAs in various solutions (i.e. methanol, 50% methanol/50% 50 mM ammonium acetate (pH 7), water, 50 mM ammonium acetate (pH 7) and 50% methanol/50% 50 mM ammonium acetate (pH 3.5, adjusted with acetic acid)) to determine which solution would be suitable to reconstitute the dried samples after evaporation. They selected methanol to reconstitute the analyte because it provided the best stability for the acyl-CoA samples over the tested period of time.[40]

An additional parameter that could be implemented to improve the determination and quantitation of acyl-CoA is the incorporation of stable isotope analogues to generate isotopically-labeled acyl-CoA species. GC-MS studies have also utilized isotopic enrichments to determine the concentrations and mass isotopomer distribution of different acyl-CoAs in tissues.[74,75] However, GC-MS methods require more time-consuming derivatization steps to convert the acyl-CoA species to a volatile form. To illustrate, the method of Kasumov et al. converted propionyl-CoA to its pentafluorobenzyl derivative of *N*-propionylsarcosine by reacting propionyl-CoA with sarcosine to form an intermediate (*N*-propionylsarcosine) that reacts with pentafluorobenzyl bromide to form a volatile derivative, while methylmalonyl-CoA and succinyl-CoA are hydrolyzed to their acid forms and assayed as *tert*-butyl dimethylsilyl methylmalonate and succinate respectively.[75] In regards to stable-isotope labeling, LC-MS appears to have greater benefits for biomarker applications than GC-MS due to better detection of the analyte of interest from biological fluids and tissues as well as its ability to cover a wider range of biomarkers than GC-MS.

According to Ciccimaro et al., utilizing stable-isotope labeled tracers or internal standards in conjunction with LC-MS/MS enhanced the ability to verify and detect the analyte of interest and minimized matrix effects. Additionally, stable-isotope labeled internal standards exhibit similar biological and physicochemical properties as the analyte of interest, which allows the stable-isotope labeled internal standard to act as a carrier to reduce the loss of trace amounts of analyte during extraction and analysis.[76] Fundamentally, an isotopically labeled analogue of the target acyl-CoA species, which is usually a ^{13}C-isotopically labeled or ^{15}N-isotopically labeled tracer, is spiked into the sample. Examples of isotopically labeled tracers include U-^{13}C-palmitoyl-coenzyme A,[46] [$^{13}C_3{}^{15}N$]-propionyl-CoA,[50] and [$^{13}C_3{}^{15}N$]-pantothenate.[57,66] For

mammalian tissues, the method of Sun et al. infused 2 μmol/mL of U-^{13}C-palmitate acid into the rabbit at the rate of 50 mL/hr. Then, the samples were collected after 5 hours of infusion and kept at -80ºC. After completing the sample preparation to extract the acyl-CoA species from the rabbit tissues, the analytes underwent LC-MS analysis under ESI negative ion mode and selected ion monitoring (SIM) to measure the ^{13}C stable isotopic analogue of palmitoyl-CoA and the concentrations of five different long-chain acyl-CoA. Although the incorporation of ^{13}C stable isotopic enrichment produced sensitive and precise results, one drawback to this approach was that it was cost-prohibitive and was a complex process that involved several steps of sample purification and enrichment prior to LC-MS analysis.[46]

Acyl-CoA Determination in Different Biological Samples

According to Baker et al., the negatively charged phosphate moieties of the coenzyme A group need to be neutralized with a positively charged lipophilic ion-pairing reagent in order to partition the sample into the lipophilic stationary phase of the LC column for reversed-phase liquid chromatography (RPLC).[15] HPLC-UV techniques incorporated a phosphate buffer in the mobile phase to neutralize the negatively charged phosphate groups of the CoA molecule.

However, the non-volatile phosphate buffers can contaminate the MS or impair the ion optics of the MS for LC-MS procedures. Nonetheless, various LC-MS methods have incorporated an ammonium counter-ion in the mobile phase to replace the phosphate buffer. Examples of the ammonium counter-ions include ammonium formate,[38,47,55] ammonium acetate,[39,40,71,77,78] and ammonium hydroxide.[41,48,79]

Additionally, the polarity of the acyl-CoA species must be taken into consideration during separation in the LC column. The polarity of acyl-CoA varies depending on the chain length of the hydrophobic tail; the longer the hydrocarbon tail, the more hydrophobic the acyl-

CoA species is. Consequently, different chain lengths of acyl-CoAs require different LC conditions to obtain ideal separation. Generally, slightly acidic mobile phases are utilized for short-chain to medium-chain acyl-CoAs,[53,78,80] while alkaline mobile phases are utilized for medium-chain to long-chain acyl-CoAs.[41,42,48,53] Incorporating acidic mobile phases for short-chain acyl-CoA species improves retention by reducing the tendency of the analyte to elute in the solvent front of the LC. Under similar conditions, long-chain acyl-CoAs have a longer retention time, resulting in severely tailed or distorted peaks.[57] Replacing acidic mobile phase conditions with alkaline mobile phase conditions for long-chain acyl-CoAs enhances peak shape and resolution while simultaneously achieving weaker retention, which reduces peak tailing.[41,42,79]

For LC instrumentation, researchers have utilized either high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC).[69] For studies incorporating HPLC for the separation of the components in the analyte, many methods incorporated Agilent 1100 series HPLC systems,[40,46,55,57] Agilent 1200 series HPLC systems,[81] or UltiMate-System HPLC.[47,48,53,58,79] Acyl-CoAs must be thoroughly separated during the chromatographic process to avoid ion suppression due to the competition for ionization between the coeluting analytes of interest and other endogenous species in the sample that have not been removed from the sample matrix during sample preparation and acyl-CoA extraction. Next, selecting UHPLC over HPLC provides several advantages. UHPLC produces narrower peaks than HPLC, which indicates that UHPLC methods can achieve lower limits of detection (LOD) than HPLC methods. Additionally, UHPLC columns achieve comparable separations to HPLC columns but with a much shorter run time. For the studies incorporating UHPLC, the types of UHPLC systems used were Thermo Fisher Scientific Vanquish Binary UHPLC systems[69] and

Waters Acquity UHPLC systems.[41,42,49] The types of columns utilized in the LC-MS analysis for HPLC or UHPLC are listed under Table 2.4.

In various acyl-CoA quantitation papers, different types of mass analyzers have been implemented in various LC-MS methods for the separation of ions. Examples of ion separation techniques include quadrupoles, quadrupole time of flights (QTOF), ion traps, and orbitraps. One of the most widely used mass analyzers for detecting acyl-CoAs in biological samples is the quadrupole. For example, Kasuya et al. incorporated a single quadrupole mass spectrometer (a Micromass ZQ mass spectrometer) for their LC-MS analysis of medium-chain acyl-CoA esters in mouse liver, brain, heart, and brain tissues using selected ion monitoring.[39]

For LC-MS/MS methods, triple quadrupole mass spectrometers have been used for MRM of acyl-CoAs due to the precursor ions fragmenting to the specific product ion via neutral loss of 3'-phosphonucleoside diphosphate $[M–507]^+$.[40,42,48-50,58] Applications using triple quadrupole instruments for the quantitation of acyl-CoAs include the measurement of medium and long-chain acyl-CoAs,[40] determination of short-chain acyl CoAs to investigate the metabolism of propionic acid to acyl-CoA intermediates,[50] quantitation of long-chain acyl-CoAs in tissue samples[42,48] or short-chain acyl-CoAs in plant species,[49] and the determination of coenzyme-A derivatives, such as malonyl-CoA, in various biological samples.[58] Another trait quadrupole instruments have is that they can be coupled with other mass analyzers to improve resolution and sensitivity, such as TOF or an orbitrap mass analyzer.[52,53] For example, the method of Cabruja et al. incorporates a QTOF instrument to perform a comprehensive scan of the entire acyl-CoA profile for specific actinomycetes in a single analytical run.[81] Additionally, Wang et al. incorporated a quadrupole-orbitrap mass spectrometer along with two-dimensional liquid

chromatography coupled with high-resolution mass spectrometry (2D LC/HRMS) to scan the entire acyl-CoA profile in mouse liver and malignant glioma cells.[52]

The method of Mauriala et al. incorporated an ion trap mass analyzer to determine long-chain acyl-CoAs in rat liver samples. The instrument used was equipped with an ESI source utilizing negative ion mode. Negative ion mass spectral data was obtained by scanning the mass range from *m/z* 200 to 1200. Tandem mass spectrometry was used to quantitatively determine acyl-CoAs from rat liver samples.[45]

Another type of trapping mass spectrometer that has been implemented in acyl-CoA quantitation is an orbitrap. The method of Wang et al. incorporated a quadrupole-orbitrap hybrid mass spectrometer to scan the full range from 600 *m/z* to 1500 *m/z*. Wang et al. conducted a tandem mass spectrometry procedure to detect the short-chain, medium-chain, and long-chain acyl-CoAs coupled with a heated electrospray ionization (HESI) ion source. The advantage of utilizing an orbitrap mass analyzer, compared to the ion-trap analyzed used by Mauriala et al., is that you can achieve higher mass resolution and improve the separation of acyl-CoA peaks that differ slightly in their *m/z* values.[45,52]

Comprehensive Analysis of Acyl-CoA

As mentioned previously, acyl-CoAs vary in different chain lengths or the number of saturations. The variation in these properties affects the retention time of each acyl-CoA in the chromatogram, the type of mobile phase needed for different acyl-CoAs, and the type of column. According to the observations reported by Yang et al., the retention time increased as the length of the fatty acid chain of the acyl-CoAs increased and decreased as the number of double bonds in the fatty acid chain increased. [40] Therefore, different LC mobile phases were used to enhance the retention of acyl-CoAs on reversed-phase columns. Slightly acidic mobile phases were

utilized to elute short-chain to medium-chain acyl-CoAs at reasonable retention times,[53,78,80] while alkaline mobile phases have been utilized for medium-chain to long-chain acyl-CoAs.[41,42,48,53] To illustrate this, the method of Liu et al. performed two binary gradients: one binary gradient of water with 5 mM ammonium acetate (at pH 6.8) and methanol to analyze short-chain to medium-chain acyl-CoAs, while another binary gradient of water with 10 mM ammonium acetate (at pH 8.5 after adjustment with ammonium hydroxide) and acetonitrile was utilized to lower the retention time of medium-chain to long-chain acyl-CoAs.[53]

As a consequence of the variations in chain lengths and saturation of acyl-CoAs, many LC-MS methods can only cover a limited range of acyl-CoA species in one analytical run. Although the LC-MS method of Liu et al. can cover short-chain, medium-chain, and long-chain acyl-CoAs, a minimum of two analytical runs must be performed to conduct a comprehensive analysis. Recently, Wang et al. designed a 2D LC-HRMS to simultaneously cover short-chain, medium-chain, and long-chain acyl-CoAs from mouse liver tissues and malignant glioma cells in a single analytical run. In the first dimensional prefractionation, the acyl-CoA species were separated by a BEH C18 pre-column (2.1 × 5 mm, 1.7 μm) into two fractions based on their hydrophobicities: 1) short-chain acyl CoAs or 2) medium-chain and long-chain acyl-CoAs. The short-chain acyl-CoAs (ranging from C2:0-CoA to C6:0-CoA) were eluted out of the pre-column after 5.5 minutes and transferred into an HSS T3 column (2.1 × 50 mm, 1.7 μm) for chromatographic separation with a gradient elution method consisting of the following mobile phases from pump 1: 10 mM ammonium acetate (A1) and 0.05% ammonium hydroxide (B1) at a rate of 0.3 mL/min. Afterwards, a change in valve positioning coupled the pre-column to a BEH C18 column (100 × 2.1 mm, 1.7μm), where the retained medium-chain and long-chain acyl-CoAs were transferred to the BEH C18 column and chromatographically separated with a

gradient elution method with the following mobile phases from pump 2: water containing 0.5% ammonium hydroxide (A2) and acetonitrile containing 0.5% ammonium hydroxide (B2) at a rate of 0.3 mL/min. After chromatographic separation, the separated acyl-CoA components were detected and analyzed by a quadrupole-orbitrap hybrid mass spectrometer. The analyzer was scanned from 600 to 1500 *m/z* with a very high resolution value of 60,000. The 2D-LC-HRMS method obtained high resolution and good peak shapes for all acyl-CoAs, shown in Figure 2.2 (Reprinted with permission from S. Wang, Z. Wang, Zhou, Shi, Xu. Copyright 2017 American Chemical Society).[52]

In 2018, another comprehensive analytical method for short-chain, medium-chain, and long-chain acyl-CoAs was developed. Abrankó et al. developed a UHPLC–ESI-MS/MS method that combined reversed-phase and hydrophilic interaction liquid chromatography (HILIC) separations in series in an automated manner to detect acyl-CoA samples from mouse liver tissue, human hepatocellular carcinoma cells and human skeletal muscle cells. A binary UHPLC system was used; a BEH C18 column (50 × 2.1 mm, 1.7 μm) and a BEH HILIC column (50 × 2.1 mm, 1.7 μm) were used to separate medium-chain to long-chain acyl-CoAs and short-chain acyl-CoAs, respectively. A switching valve was in operation to change the mobile phase between the reversed-phase column and the HILIC column. First, reversed-phase chromatography separation of the more hydrophobic acyl-CoAs occurred under the following gradient elution mobile phase: 10 mM ammonium acetate at a pH of 8.5 (A1) and acetonitrile (B1). Between each injection, an acidic wash (40:60:0.1 mixture of water-acetonitrile-phosphoric acid) and a neutral wash (50:50 mixture of water-acetonitrile) were introduced to clean the system. Once the system was equilibrated after reversed-phase separation, a switching valve was activated to divert the mobile phase to the BEH HILIC column for HILIC separation of the more polar acyl-

CoAs. The mobile phase used for the HILIC separation was the same as the mobile phase used for reversed-phase, except the gradient elution was different (the HILIC gradient started at 95% B1 and decreased linearly over time to 50% to elute the more polar acyl-CoAs, while the reversed-phase gradient started at 5% B1 and increased linearly until it reached 100% B1). After chromatographic separation, the separated acyl-CoA components were detected and analyzed by a triple quadrupole MS/MS instrument equipped with a HESI. The 507 ion from the 3'-phosphonucleoside diphosphate moiety was followed using the precursor mass range of 800–1100 *m/z*. According to the results, the acyl-CoAs ranging from C4 to C20 were resolved and detected with the reversed-phase method, while the highly polar acyl-CoAs (i.e., C2 and malonyl CoA), despite the poor separation by the reversed-phase method, were resolved and detected using HILIC. The method not only comprehensively analyzed a large range of acyl-CoAs, but also achieved results with a much lower LOD than previous methods.[69]

Application of Acyl-CoA Analysis as Biomarkers for Toxicities

With the advancement of LC-MS methods to determine and quantitate acyl-CoAs in different biological samples, the possibility of incorporating acyl-CoAs in lipidomic and metabolomic approaches for biomarker profiling is becoming clearer. As mentioned earlier, acyl-CoAs are involved in various metabolic pathways. They are substrates that affect posttranslational protein modifications, enzyme regulation, and energy production of fatty acids.[8] Although methods of quantitating how acyl-CoAs directly induce metabolic dysregulation have not been yet identified, there are methods that measure endogenous levels of acyl-CoA compounds and metabolites to suggest how acyl-CoA could affect metabolic pathways. Many analytical methods have incorporated hierarchical clustering and heat maps to remodel differential acyl-CoA profiles across various biological samples.[40,47,52,53] Interpretations of the

heat maps suggest how acyl-CoAs correlate with metabolic pathways and potentially impact metabolic dysregulation. For example, Yang et al. remodeled acyl-CoA profiles in the following cell lines after treatment with various fatty acids: PNT2 (normal prostate cells), DU145 (prostate cancer cells), HepG2 (normal hepatic cells), and Hep3B (hepatic tumorigenic cells), which are shown in Figure 2.3 (Reprinted with permission from Yang, Ma, Li, Cai, and Bartlett. Copyright 2017 American Chemical Society). Their hierarchal clustering of acyl-CoA changes reflected metabolic differences between normal and tumorigenic cells, suggesting that the metabolic alterations were correlated with the concentration of acyl-CoAs in each cell.[40]

Acyl-CoAs could play a role in biomarker profiling for certain drugs. As mentioned earlier, acyl-CoAs are derived from fatty acids, which are carboxylic acids with a long aliphatic chain and a terminal carboxyl group. However, they can also be derived from most carboxylic-containing metabolites and carboxylic acid-containing drugs. With the application of acyl-CoA detection and quantitation methods, the biotransformation of drugs to acyl-CoA metabolites may provide additional insight into the metabolic fate of the drug. For example, the carboxylic acid drugs zomepirac and tolmetin were detected *in vitro* and *in vivo* after they were biotransformed into acyl-CoA thioesters.[82,83] In addition, current acyl-CoA detection methods can be utilized to determine if these carboxylic acid-containing drugs induce toxicities in living systems, especially when they are bioactivated to acyl-CoA conjugates.[84-87]

Conclusion

LC-MS surpasses previous methods due to its better sensitivity and resolution while simultaneously having minimal interference from other unintended components in a biological sample, avoiding time-consuming derivatization procedures, and having shorter analytical run times. LC-MS methods are able to quantitate acyl-CoA concentrations in various biological

samples and demonstrate differences in acyl-CoA profiling to indicate fatty-acid patterns, pathways, and enzymes that are involved in acyl-CoA synthesis and metabolism. Although current methods of quantitating how acyl-CoAs directly induce metabolic dysregulation have not been yet identified, remodeling of acyl-CoA profiles demonstrated individual acyl-CoAs that differ between normal and tumorigenic cells, indicating metabolic deficiencies that could be associated with enzyme deficiency or dysregulation.

Analytical advancement over the years has provided methodologies to quantitate and determine acyl-CoA concentrations *in vitro* and *in vivo*. Recent studies have observed changes in acyl-CoA concentrations between normal biological samples and dietary-altered samples or tumorigenic samples that correlate to acyl-CoA concentrations and metabolic alterations. While current LC-MS methods have superseded LC-UV methods in terms of robustness, reproducibility, specificity and selectivity, these methods are unable to determine if changes in cellular acyl-CoA levels are the result of a change in energy production or epigenetics. As mentioned previously, acyl-CoAs are intermediates and regulators of metabolism. They are involved in fatty acid synthesis, fatty acid degradation, fatty acid modification, and post-translational modifications of proteins.[2] Dysregulation of metabolic pathways induced by changes in acyl-CoA levels is either caused by changes in energy production or epigenetics, but it is currently not feasible to determine how much of the acyl-CoA attributes to either one of these conditions. With the results generated from current LC-MS methods, is the data meaningful enough to predict that acyl-CoA, an intermediate involved in almost all pathways associated with metabolomics, is a key attributor to disease or cancer progression? Further studies should consider understanding other metabolites that may also contribute to diseases and

tumorigenic conditions that correlate with changes in their metabolite levels similar to that of acyl-CoA alterations.

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Table 2.1 SPE cartridges and solvents used for the Acyl-CoA Extraction from Mammalian Samples.

SPE Cartridge	Biological Sample	Conditioning Solvent	Wash	Elution Solvent	Type of Acyl-CoA	Extraction Efficiencies
Oligonucleotide purification cartridges[16,21,44,63] (16, 21, 44, 63)	Rat Hepatic Tissue,[44,63] Rat Heart Tissue[63] Rat Kidney Tissue[63] Rat Muscle Tissue[63] Rat Brain Tissue[16,21]	H_2O[63] ACN[16,21,44,63] 25 mM KH_2PO_4[21,44,63]	25 mM KH_2PO_4[16,21,44]	4% 2-propanol in 25 mM KH_2PO_4 containing 16% MeOH[16] 60% ACN in 100 mM KH_2PO_4[21] 75% 2-propanol containing 1 mM glacial acetic acid[63] 0.4 ml of 60% ACN in 100 mM KH_2PO_4[44]	Short-Chain[16] (16) Long-chain[21,44,63]	70-90%[16,21,44,63]
Sep-Pak C18[65]	Rat Hepatic Tissue[65]	Acidic H_2O (pH 3 by HCl) [65]	Petroleum ether, chloroform, and MeOH[65]	65:35 ethanol/H_2O containing 0.1 M ammonium acetate[65]	Short-Chain[65]	60-70%[65]
Varian C18[44]	Rat Hepatic Cell[44]	50 mM Tris-HCl[44]	H_2O[44]	MeOH[44]	Long-Chain[44]	Not reported[44]
Oasis HLB[48,66-68]	Mouse Liver Cells[66,67] Rat Liver Tissue[48] Rat Brain Tissue[68]	ACN[48] 25 mM KH_2PO_4[48] MeOH[66-68] H_2O[66,67]	H_2O[48,66-68]	40:60 ACN/H_2O solution containing 15 mM NH_4OH[48] MeOH[68] 25 mM of ammonium acetate in MeOH[66,67]	Short-Chain[66-68] Long-Chain[48]	5%[68] 60%[48]
2-(2-pyridyl)ethyl-functionalized silica sorbent[69,70]	Rat Liver Tissue[71] Mouse Liver Tissue[70] Human Hepatocellular Carcinoma Cells[69] Human Skeletal Muscle Cells[70]	ACN/isopropanol/H_2O/acetic acid (9+3+4+4, v+v+v+v)[69,70]	ACN/isopropanol/H_2O/acetic acid (9+3+4+4, v+v+v+v)[69,70]	2 ml of MeOH/250 mM ammonium formate (4+1, v+v)[69,70]	Short-, Medium-, and Long-Chain[69,70]	83-90%[71]

Table 2.2 List of Cell Media and Additional Supplements Used for the Preparation of Cells for Subsequent Acyl-CoA Analysis

Cell Media	• **Dulbecco's Modified Eagle's Medium or Dulbecco's minimum essential modified (DMEM) medium**[43,52,57,58,69] • **Hank's Bank Salt Solution (HBSS) medium**[66] • **F-12K medium**[57] • **Roswell Park Memorial Institute (RPMI) medium**[53,57] • **Krebs bicarbonate medium**[17]
Additional Supplements	• Fetal bovine serum (FBS)[40,43,57,58] • Penicillin/streptomycin[40,43,57] • Cord blood serum[52]

Table 2.3 Examples of rinsing, cell detachment, quenching, and extraction prior to LC-MS analysis.

Method	Rinsing	Cell Detachment	Quenching	Extraction
Haynes et al.[43]	Thoroughly rinsed MCF7 cells and RAW264.7 cells with PBS and H_2O to efficiently remove the culture media and FBS from the culture plate to facilitate trypsinization.	A rubber policeman was used to scrape the cells into a corner of the dish; the cells were mixed with water to form a uniform aliquot.	Placed the aliquot in a Teflon lined cap on ice and added 500 µL of EDTA in methanol to the aliquot to promote quenching.	Extracted the analytes from the aqueous phase of chloroform-water after sonication and reextracted twice from the interface and organic layer by adding a water-methanol-chloroform mixture (45:50:5) after centrifuging.
Yang et al.[40]	Human prostate cells and hepatic cells were rinsed twice with PBS to maintain constant pH and to match the osmolarity of the body.	The cell lysate was scraped down from the culture plate, transferred to a 5 mL Eppendorf tube, and centrifuged at 15,000 g at 5 ºC for 5 minutes. The pellet formed at the end of centrifuging the cell lystate was not resuspended. Next, the supernatant was transferred to a glass tube, mixed with 1 mL of acetonitrile, and evaporated in a vacuum concentrator at 55 ºC for 1.5 hours.	Before the cells were scraped down from the culture plate, the cells were incubated in one millliter of methanol at –80 ºC to ensure that cell metabolism has halted. In this step, an acyl-CoA internal standard was added to the incubated culture plate to facilitate quantification of the acyl-CoAs	Acetonitrile was added and mixed with the supernatant prior to being placed in the vacuum concentrator to facilitate evaporation and to help reduce the hydrolysis of acyl-CoAs due to residual water. After evaporation, 150 µL of methanol was added to the glass tube to reconstitute the sample, and the mixture was vortexed and centrifuged at 15,000 g at 5 ºC for 10 minutes to obtain an aliquot for LC-MS/MS analysis.

Table 2.4 List of columns used in HPLC or *UHPLC. UHPLC columns* are italicized.

Short-Chain Acyl-CoA	Medium-Chain Acyl-CoA	Long-Chain Acyl-CoA
○ Atlantis T3 100 Å-column (150 × 2.1 mm, 3 µm)[78] ○ Aqua C18 125 Å Column (50 × 1.0 mm, 5 µm)[30] ○ *High Strength Silica (HSS T3 Column (50 × 2.1 mm, 1.7 µm)[52]* ○ Hypersil GOLD C_{18} column (150 × 2.1 mm, 3 µm)[81] ○ Luna C18 column (100 × 2.0 mm, 3 µm)[53] ○ Luna C18 Column (150 × 2.0 mm, 3 µm)[66] ○ Luna C18 column (150 × 2.0 mm, 5 µm)[50,67] ○ Luna C18(2) (50 × 2 mm, 5 µm)[68] ○ XBridge BEH Amide (100 × 2.1 mm, 2.5 µm)[58] ○ XBridge C18 (150 × 2.1 mm, 3.5 µm)[80] ○ Hypersil BDS C18 (100 × 4 mm, 5µm)[56] ○ BEH HILIC column (50 × 2.1 mm, 1.7 µm)[69]	○ *BEH C18 column (50 × 2.1 mm, 1.7 µm)[69]* ○ *BEH C18 column (100 × 2.1 mm, 1.7 µm)[52]* ○ Luna C18 column (100 × 2.0 mm, 3 µm)[53] ○ Cosmosil C8-MS reversed-phase column (150 × 4.6 mm, 5 µm)[39] ○ XBridge C18 column (150 × 2.1 mm, 3 µm)[57] ○ Capcell Pak C18, UG120 (150 × 2.0 (I.D) mm)[38]	○ Zorbax 300SB-C8 column (100 × 2.1 mm, 5 µm)[47] ○ Zorbax 300 Extend C18 column (150 × 2.1 mm, 3.5 µm)[48] ○ Zorbax Extend-C18 (150 × 2.1 mm, 3 µm)[79] ○ Zorbax Extend-C18 (150 × 2.1 mm, 5 µm)[46] ○ Nova-Pak 60Å-C_{18} column (150 × 3.9 mm, 4 µm)[61] ○ Luna C18 column (100 × 2.0 mm, 3 µm)[53] ○ Luna C18(2) 100-Å column (100 × 2.0 mm, 3 µm)[40] ○ *BEH C18 column (50 × 2.1 mm, 1.7 µm)[69]* ○ *BEH C18 column (100 × 2.1 mm, 1.7 µm)[52]* ○ *BEH C8 column (100 × 2.1 mm, 1.7µm)[41]* ○ *BEH C8 column (150 × 2.1 mm, 1.7 µm)[42]* ○ Genesis C4 column (50 x 2.1 mm, 4 µm)[45] ○ Hypersil GOLD C_{18} column (150 × 2.1 mm, 3 µm)[81] ○ Gemini C18 column (150 × 2.0 mm, 5 µm)[43] ○ XBridge C18 column (150 × 2.1, 3 µm)[57]

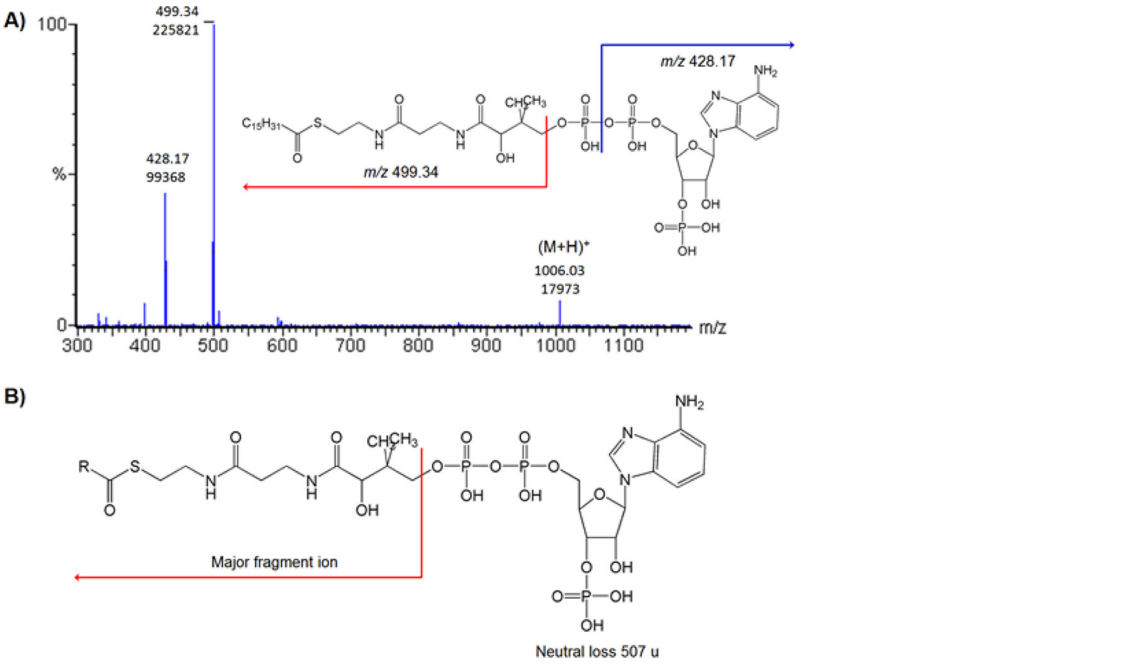

Figure 2.1 Fragment ions of acyl-CoAs. (A) Fragment ions of C16:0 CoA. (B) Major fragmentation mechanism for acyl-CoAs: a neutral loss of 507.

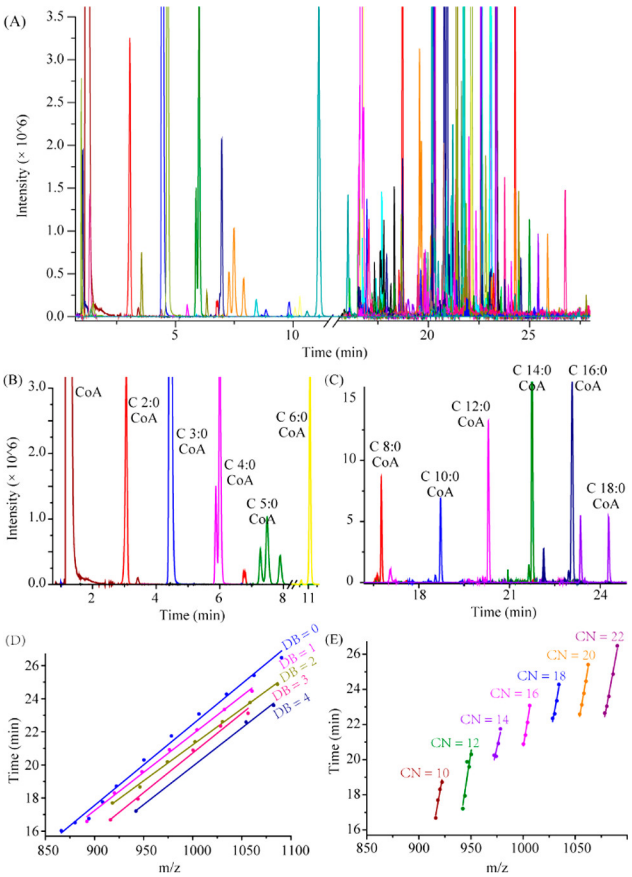

Figure 2.2 EIC chromatograms of (A) all identified acyl-CoAs; (B) saturated short-chain acyl-CoAs; (C) saturated medium- and long-chain acyl-CoAs in liver extracts. The correlation between the retention times of acyl-CoAs and (D) carbon number or (E) double bond number of acyl chains. DB: double bond number, CN: carbon number.

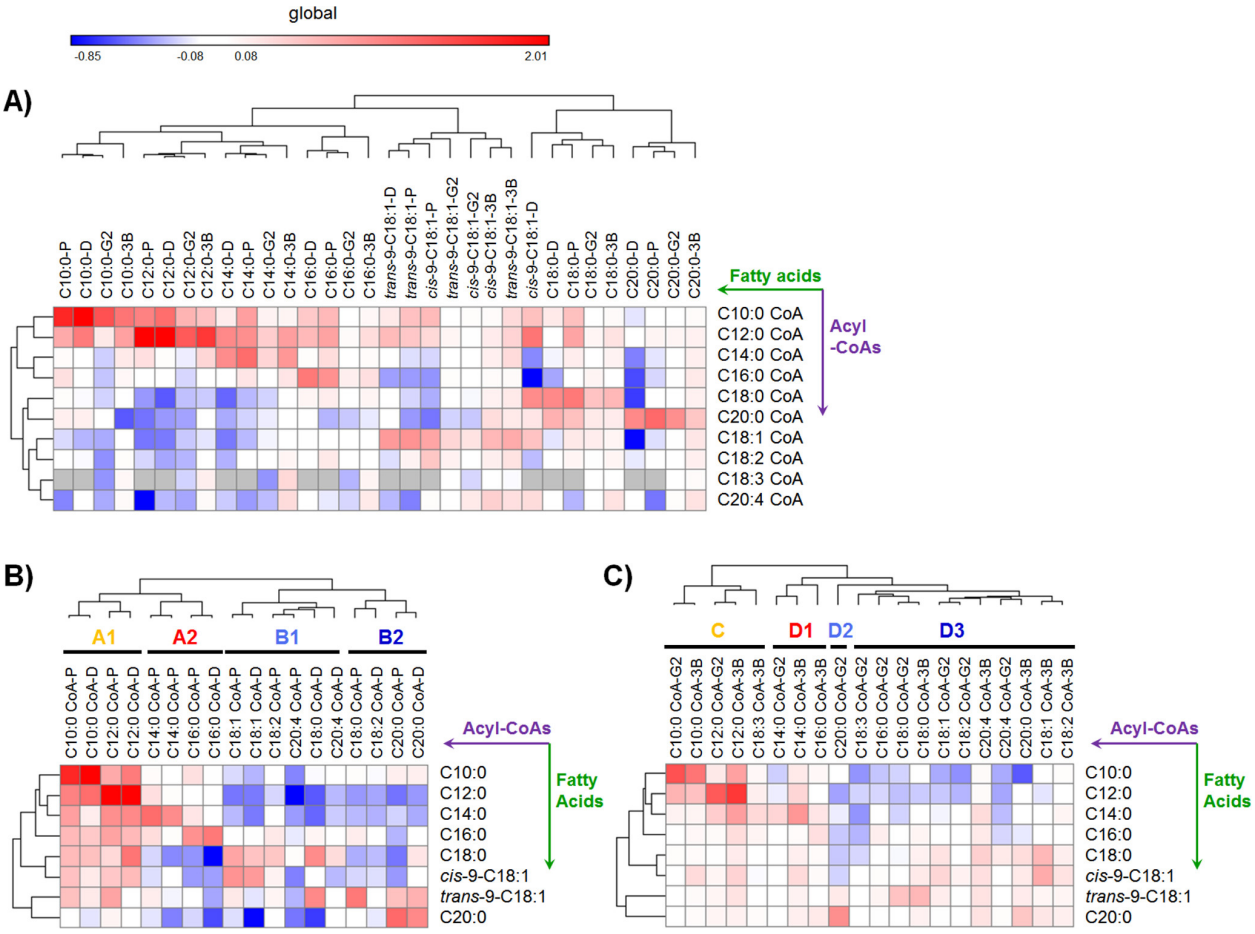

Figure 2.3 Remodeling of the acyl-CoA profile across cell lines after treatment with various fatty acids. (A) Hierarchical clustering of cells with a variety of fatty acid treatments based on remodeling of the acyl-CoA profile. (B–C) Hierarchical clustering of acyl-CoA changes (log ratio) in (B) prostate cells and (C) hepatic cells in response to fatty acids. The log ratio of the measure is defined as the average amount of an acyl-CoA in a treatment group over the amount in a control group. The color code for log ratio 0.08 indicates a log ratio $\geqslant$0.08 (log 1.2) and for -0.08 indicates log ratio $\leqslant -0.08$. (P: PNT2 cells, D: DU145 cells, G2: HepG2 cells, and 3B: Hep3B cells).

CHAPTER 3

CONCLUSIONS

In the pharmaceutical industry, researchers work extensively for the development of new drugs and the discovery of a correlation between a potential therapeutic and how it impacts specific metabolic pathways *in vivo*. The rigorous amount of time, effort, and cost spent for analytical research demand high-performance analytical instruments to achieve accurate and reproducible data to reject or fail to reject potential therapeutics/hypotheses about metabolic pathways in response to a concentration change for a specific xenobiotic. Analytical instruments are integral for various aspects in pharmaceutical research and development, especially for drug discovery of a lead compound and bioanalysis. For drug discovery, an analytical instrument must be capable of detecting potential therapeutic compounds in complex biological matrices to assess pharmacokinetic parameters and metabolic pathways of the compound. The ideal instrument must be applicable to the bioanalytical method to ensure the detection and quantitation of a new drug entity, its metabolites, and/or biomarkers.

To reiterate, pharmaceutical research and development requires analytical equipment for the quantitation of xenobiotics. For the determination and quantitation of xenobiotics, analytical equipment must achieve high selectivity, high sensitivity, and high robustness during its analytical run. The analytical technique that achieves all these necessary attributes is a liquid chromatographic instrument coupled with a mass spectrometer (LC-MS).

Overall, LC-MS has proven to be a critical asset for pharmaceutical research and development, quality control, and bioanalysis. It surpasses other analytical techniques for its

capability of separating the desired analytes from a complex measure and its ability of detecting and measuring the analyte of interest. Chapter 1 highlighted the benefits of the analytical technique while simultaneously discussing how the mechanism of each component in the instrument works. Liquid chromatography, especially high-performance liquid chromatography, helps to partition the analyte of interest from complex matrices based on the analyte's affinity to adsorb or adhere to the stationary phase of the column and its ability to be eluted by a flowing mobile phase at a set flow rate. Mass spectrometry elucidates the structural features, molecular weight, and molecular formula of an analyte by generating ionized analytes via the ion source (i.e. electrospray ionization), fragmenting and separating the molecular fragment ions of interest from the other ions in the mass analyzer (i.e. quadrupole), and assessing the intensity of the molecular fragment ions in the detector. Tandem mass spectrometry supersedes mass spectrometry in regards to its enhanced specificity for the molecular fragment ion of interest, resulting in higher sensitivity and higher Signal-to-Noise ratios. For bioanalysis and biomarker quantitation, LC-MS, especially, liquid chromatography tandem mass spectrometry (LC-MS/MS), permits the quantitation of xenobiotics in complex biological matrices with the incorporation of a calibration curve to uncover the concentration of analytes in real samples. This application applies to various exogenous or endogenous metabolites, including acyl-CoAs.

According to the literature review in chapter 2, several analytical methods have been utilized for the detection and quantitation of acyl-CoA metabolites to assess the role of that particular metabolite in different metabolic pathways or observe the concentration change of the metabolite in response to an alteration in a biochemical/metabolic pathway. Nonetheless, the analytical technique that has been implemented in most analytical methods for the quantitation of acyl-CoAs in biological fluids is LC-MS. LC-MS supersedes the other analytical methods (i.e.

GC-MS, LC-UV) in terms of its robustness, reproducibility, specificity and selectivity. In addition, the sample preparation for LC-MS is more cost-effective and less labor intensive compared to other analytical methods, like GC-MS methods, which require acyl-CoAs to be converted to a more volatile derivative. In addition, the second chapter highlighted the types of columns and mobile phases that have been utilized in the bioanalytical methods for acyl-CoA quantitation. In addition, the literature review highlighted two noteworthy methods that permit a comprehensive quantitative analysis of acyl-CoA quantitation in a single analytical run with high resolution, signal intensity, and excellent retention times for metabolites with different alkyl chain composition. However, one question was addressed in the literature review: how can the data provide meaningful clarity to confirm the extent to which acyl-CoAs are involved in the myriad of metabolic pathways (i.e. biosynthesis, lipid modification, myristoylation, post-translational modification)? Are they the key instigator or just a response to disease/cancer progression? For future development of acyl-CoA quantitation methods, the bioanalytical field should consider the other metabolites that are involved in the same biochemical pathways as acyl-CoAs during disease progression or metabolic dysregulation. Assessing the other metabolites will more fully elucidate whether or not they are directly or indirectly influenced by the changes in the metabolic levels of acyl-CoAs due to an increase of an exogenous xenobiotic or disruption in homeostatic pathways.