In vitro SSAT Enzyme Activity Study Using Rat and Mouse Liver Tissue preparations

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, '*In vitr*o SSAT Enzyme Activity Study Using Rat and Mouse Liver Tissue preparations', contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Abbreviations

Spd: Spermidine

AcSpd: Acetylspermidine

Spm: Spermine

AcSpm: Acetylspermine

DAT: Diacetyltriethylenetetramine

TETA: Triethylenetetramine

MAT: Acetyltriethylenetetramine

Spd-D6: Spermidine-D6

AcSpd-D6: Acetylspermidine-D6

DES: N^{1},N^{12}-Diethylspermine

SSAT: Spermidine/spermine N^{1}-acetyltransferase

SSAT1: Spermidine/spermine N^{1}-acetyltransferase 1

SSAT2: Spermidine/spermine N^{1}-acetyltransferase 2

HDA: Hexamethylenediamine

APAO: Acetyl-polyamine oxidase

ODC: Ornithine decarboxylase

AMD1: Adenosylmethionine decarboxylase

CoA: Coenzyme A

AcCoA: Acetyl Coenzyme A

BSA: Bovine serum albumin

MCF: Methyl chloroformate

PBS: Phosphate-buffered saline

HCl: Hydrogen chloride

KCl: Potassium chloride

HFBA: Heptafluorobutyric acid

LC-MS: Liquid chromatography–mass spectrometry

DcAdoMet: Decarboxylated S-adenosylmethionine

AdoMetDC: S-adenosylmethionine decarboxylase

TCA: Tricarboxylic acid cycle

NADH: Nicotinamide adenine dinucleotide

TLAT: Thialysine N-ε-acetyltransferase

TSA: Trypticase soy agar

Abstract

Polyamines such as spermidine (Spd) and spermine (Spm) are the organic compounds which have two or more amino groups. It serves multiple physiological functions including cell proliferation, cell adhesion, specific signalling and the repairing of extracellular matrix. Spermidine/spermine N^1-acetyltransferase (SSAT) is the enzyme which acetylates Spd to acetylspermidine (AcSpd). SSAT is also capable of acetylating polyamine analogues such as triethylenetetramine (TETA) to acetyltriethylenetetramine (MAT). However, this process is debatable because while the overexpression of Spermidine/spermine N^1-acetyltransferase (SSAT1) increases the MAT level, the knockout of SSAT1 remains the same MAT level as the non-treatment groups. One of the theories explaining this is that another enzyme, Spermidine/spermine N^1-acetyltransferase 2 (SSAT2), is responsible for the acetylation of TETA because the knockout of SSAT2 significantly decreases the MAT level. Thus, this research was designed to establish a method to precisely measure the activity of SSAT via the quantification of the metabolites resulting from SSAT catalysis by using spermidine-D6 (Spd-D6), the stable isotope of Spd, to get rid of the disturbance from endogenous Spd. Through this method, it is possible to find out if SSAT2 is really active in acetylating TETA by comparing the data of Spd-D6 with TETA. It is required to be mentioned that although SSAT *in vitro* polyamine acetylation assay has been done by some studies already, the application of Spd-D6 is unprecedented.

This research first of all used differential centrifugation method and Ca^{2+} sediment centrifugation method to prepare mouse and rat liver microsome and cytosol, which are also considered as the enzyme sources of SSAT enzyme. Then Bradford Protein Assay was used to determine the protein concentrations of these liver preparations. After that, *in vitro* polyamine acetylation assay was applied to measure the SSAT enzyme activity by using Spd-D6 as the substrate. Finally, this research developed a liquid chromatography-mass spectrometry (LC-MS)

method to detect the metabolites, acetylspermidine-D6 (AcSpd-D6).

This research not only measured the acetylation activity of enzyme from different tissue origin with various polyamine substrates, but also compared them with the results of different non-enzymatic controls. This research also compared the standard curves of AcSpd-D6, AcSpd and MAT in different matrix including microsome, cytosol and water to find out their effects on LC-MS detection. The results presented that non-enzymatic controls were all showing polyamine acetylation activities, and such activities were similar to the enzymatic groups, which suggested the incapability of SSAT acetylating polyamines. Standard curves were not showing obvious differences, which means the enzyme sources were not influencing the LC-MS detection.

In conclusion, spermidine acetylation happens spontaneously without needing SSAT catalysis. The role of SSAT may be: 1) SSAT inhibits AcCoA from reacting with other substances. 2) SSAT accelerates the transportation of AcCoA from mitochondria to the cytoplasmic matrix. 3) SSAT is a carrier protein which transports AcCoA to other cellular locations.

Chapter 1 introduction

1.1 Background

Polyamines are the organic compounds which have two or more amino groups. These compounds have multiple cellular functions which affects cell growth, cancer and aging. Polyamine intracellular level need to be precisely adjusted, too high or too low of it could cause serious consequences. Spermidine is one of the major endogenous polyamines, and SSAT, one of the enzymes serves in polyamine catabolism pathways, acetylates Spd to AcSpd. Such function of SSAT fulfils two major purposes: to control the intracellular polyamine level, and to provide an intermediate product to catalyse spermidine back to putrescine (Madeo et al., 2018; Pegg, 2008; Wang and Casero, 2006). Although few studies reveal the direct relationship between SSAT enzymes and any specific diseases (except cancers), there are evidences of SSAT influencing glucose and fat metabolism, which are potentially related to diabetes and obesity (Jell et al., 2007; Tong et al., 2018). In addition, many theories of SSAT are still debatable currently, such as the real functions of SSAT1 and SSAT2 and SSAT subcellular distribution (Pegg, 2008; Ragione and Pegg, 1983; Holst et al., 2008).

TETA is an artificial polyamine analogue, which is also a potent Cu^{II}-selective chelator. TETA is clinically-applied in copper binding drugs such as penicillamine, and it is also a second-line treatment for Wilson's disease (Nurchi et al., 2013). As a polyamine analogue, TETA could also be acetylated by SSAT, and the metabolite is MAT. Thus, TETA is a normal selection for studying SSAT activity. However, there is a debatable problem in polyamine and SSAT enzyme studies: when using TETA as the substrate, although the SSAT1 overexpression mice show an increase in MAT level, the SSAT1 knockout mice show the same level of MAT as SSAT1 wildtype mice. One of the explanations is that SSAT2, an enzyme whose major substrate is thialysine

instead of polyamines, is acetylating this reaction (Hyvönen et al., 2013). Nonetheless, such phenomenon also exists in spermidine acetylation, and SSAT2 is proved to be not capable of acetylating polyamines, the explanation might be not persuasive enough. Moreover, endogenous polyamines in animal samples are also disturbing the results of the SSAT related assays using Spd and Spm as substrates. To clarify the functions of SSAT, this research uses Spd-D6, a stable isotope of Spd which could be distinguished by a Mass Spectrometer (MS), to avoid the disturbance of endogenous polyamines. Although some previous studies have already applied *in vitro* polyamine acetylation assays for SSAT, the application of Spd-D6 is unprecedented.

1.2 Research purpose, hypothesis and conclusion

This research measures the activity of SSAT by *in vitro* polyamine acetylation assays. The main substrate in this research is Spd-D6, and the SSAT enzyme sources are healthy mouse and rat liver microsome and cytosol. Spd and TETA are also used as substrates in this research. The original purpose of this research is to develop a method which could measure the SSAT activity under *in vitro* conditions, and the application of Spd-D6 allows more precise quantification of metabolite (AcSpd-D6) concentration by LC-MS detection. The original hypothesis is that by using Spd-D6, it is possible to better distinguish the functions of SSAT1 and SSAT2: if Spd-D6 presents lower activity than the assay using normal polyamines such as Spd and TETA, then SSAT2 may indeed have specific acetylation ability towards TETA, and SSAT1 also supports such ability.

However, this research finds that even if the SSAT enzyme sources are absent, the assays are still able to produce acetylated polyamines, and the rates of acetylated polyamine production from different *in vitro* polyamine acetylation assays are very similar, which is unexplainable because the energy barrier is considered to be way too high for spontaneous acetylation to happen under

the physiological conditions. Therefore, instead of comparing the Spd-d6 SSAT activity assays with other normal assays, this research gives multiple possibilities to explain why such phenomenon is happening.

1.3 Overview

This thesis contains six chapters.

Chapter 1 is an introduction of the whole thesis.

Chapter 2 is the literature review part, which gives detailed information about polyamines, a brief history of polyamine researches, the diseases which are potentially related to polyamines, polyamine physiological pathways including polyamine biosynthesis and catabolism. This chapter also describes information about SSAT enzyme itself including SAT gene, SSAT protein structure, SSAT catalytic mechanism, SSAT2 and subcellular distribution of SSAT enzyme. After that, some reviews about the experimental techniques are also included in this chapter for the better understanding of the methodology part. Some of the theories about polyamines and SSAT are debatable due to the different phenomena and conclusions made by different studies. But still, these theories are having high reference values.

Chapter 3 illustrates the methodology part of this research. The information of the animal samples, materials, chemicals, software, experimental equipment and technics are included in this chapter. This chapter also describes the experimental design and data analysis methods. The experimental design includes four major steps: the preparation of mouse and rat liver microsome and cytosol, protein concentration determination, *in vitro* polyamine acetylation assay and LC-MS detection. Two liver tissue preparation methods and two LC-MS detection methods were applied in this research.

Chapter 4 is the chapter which presents all the results from this research, including all the

standard curves, the velocity versus substrate concentration curves, K_m and V_{max} of enzymatic kinetic assays, protein concentration optimisation curves and the comparison tables. It should be mentioned that the data provided in this research is different from the hypothesis (especially the protein concentration optimisation and the comparison tables).

Chapter 5 discusses the results obtained by this research. Due to the surprising results, this chapter speculates some new presumptions of SSAT physiological functions to explain these results. This chapter also listed some previous studies to compare with the results provided by this research, and to support some of the presumptions in this chapter.

Chapter 6 is the conclusion part. It summarises the whole thesis and findings from this research.

Chapter 2 Literature review

2.1 General aspects of polyamines

2.1.1 Definition and physical property of the polyamines

The term “Polyamine” refers to the organic compounds which have two or more amino groups. They are small and positively charged polycations, which belong to a range of essential amines. Although these compounds exist in animal eukaryotic cells at millimolar concentration, they have multiple physiological functions including cell growth, immunity, development, gene regulation and differentiation. They also have multiple cellular functions which affect cell growth, cancer and aging (Madeo et al., 2018; Pegg, 2008; Wang and Casero, 2006, Yuan et al., 2018). Polyamine levels in cells are strictly controlled within a narrow range which avoids serious physiological consequences (Bae et al., 2018). Excess levels of intra-cellular polyamines become rapidly toxic, while levels which are too low generate toxic by-products (Kusano and Suzuki, 2015). Furthermore, polyamine deficiency could lead to negative effects on reproduction (Larqué et al., 2007). Low polyamine concentration is also considered as one of the causes which leads to aging and neurodegenerative diseases (Minois, 2014). High levels of polyamines have been proved to be a sign of cancer formation. Moreover, endogenous enzymatic polyamine production decreases with age, so it is suggested to increase polyamine intake during reproduction, memory loss and aging (Hussain et al., 2017). Polyamines also serve a predominant role in cell adhesion, as well as specific signalling and repairing the extracellular matrix (Moinard, Cynober and de Bandt, 2005). Some research also indicates that polyamines, especially Spd and Spm, have biological functions including regulating intracellular calcium, antioxidation, scavenging free radicals and suppressing the activating of transport pores on mitochondria (Das and Misra, 2004; Sava et al, 2006; Salvi and Toniello, 2004; Tong et al, 2018).

2.1.2 Different types of polyamines

Figure 1 demonstrates the chemical structures of three major polyamines in mammalian cells, namely putrescine, Spd and Spm respectively. These low molecular weight amines are water soluble and aliphatic, possessing pKa values between 8.3 and 10.9 (Bencini et al., 1999). Both primary and secondary amine groups of polyamines are completely protonated in cells at physiological conditions (Seiler, Delcros and Moulinoux, 1996). The polyamines and their derivatives share similar structures. One of the peculiarities of the amine group is that it can form primary, secondary and tertiary functional groups in organic compounds depending on how many non-hydrogen groups are bound to them. They also have a lone electric pair, which allows amines to bind to either cations or anions based on pH values in the system. Different amine groups in one polyamine molecule can also be individually protonated. Therefore, depending on how many amine groups are protonated, polyamines have different pKa values and multiple dissociation constants of protonation. Take TETA as an example, it has four dissociation constants (3.3, 6.7, 9.1, and 9.8 respectively) because TETA has four amine groups (Crisponi et al., 2010).

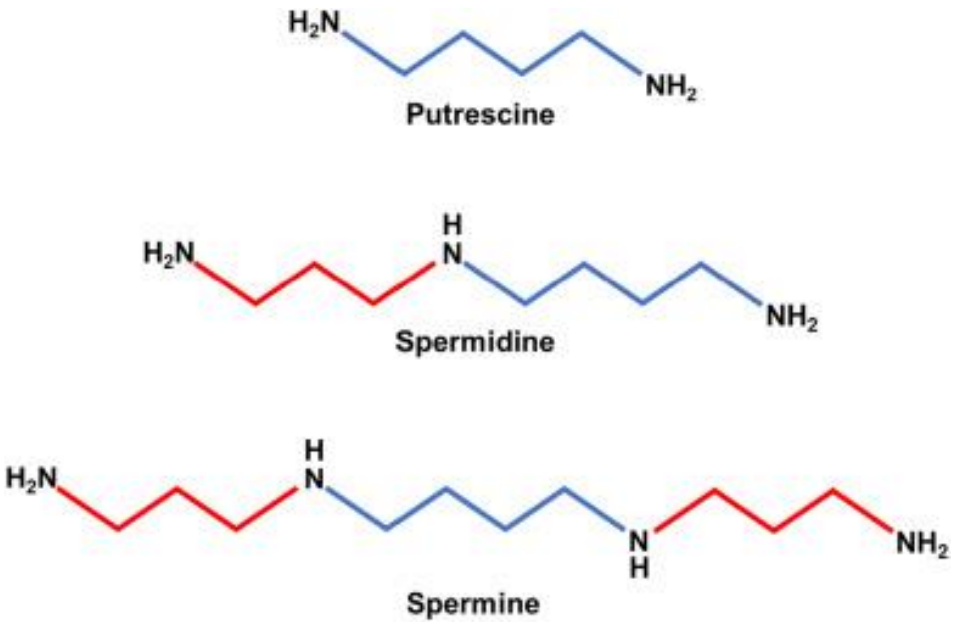

Figure 1 Three major polyamines: putrescine, spermidine and spermine (Bae et al., 2018).

TETA is a synthetic tetra-amine closely related in structure to the endogenous linear polyamines, Spd and Spm. It is also a potent Cu^{II}-selective chelator, whose absolute specificity for divalent (over univalent) copper distinguishes it from other clinically-applied copper-binding drugs such as penicillamine (Nurchi et al., 2013). Copper is an essential trace element in mammals, but becomes cytotoxic if its physiological regulation is impaired, as occurs in Wilson's disease and diabetes. Accordingly, TETA is used therapeutically as a second-line treatment for Wilson's disease, which is characterised by the accumulation of catalytically-active copper in several tissues, and which in the absence of treatment by Cu^{2+} chelation causes liver cirrhosis, neurological disease, and a number of other symptoms related to the defective transport and distribution of copper.

As polyamine analogues, TETA and its metabolites (MAT and DAT) show similar reactivity and usage as other polyamines. It was recently shown that TETA is not only a treatment for Wilson's disease, but also reverses diabetic heart failure (Lu, Chan, Poppitt & Cooper, 2007). In addition, a new TETA (and MAT) quantitative method by using LC-MS has been developed by Lu and his colleagues (2007), which is effective and reliable. Thus, both TETA and Spd are capable of being referential substances for measuring the activity of SSAT.

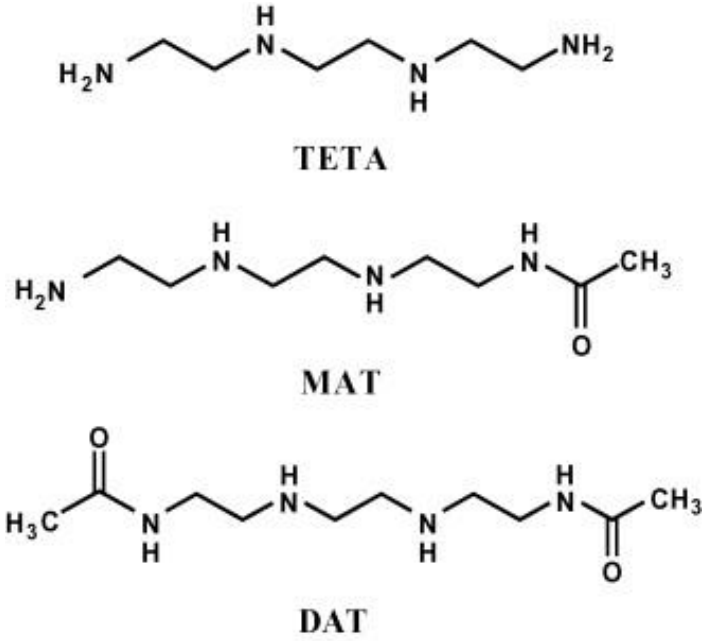

Figure 2 Structures of triethylenetetramine (TETA), N^1-acetyltriethylenetetramine (MAT) and N^1, N^{10}-diacetyltriethylenetetramine (DAT) (Lu, Chan, Poppitt & Cooper, 2007).

Another class of polyamines called macrocyclic polyamines (polyazacycloalkanes), refers to the polyamines which possess a cyclic structure (macrocycles). Unlike open-chain polyamines (such as Spd), their proton transfer properties are strictly related to physical properties such as the overall structure of the molecule, the existence of different donor atoms, and the number of amino groups in the molecular skeleton (Bencini et al., 1999). Due to the fact that these macrocyclic polyamines are not natural polyamines, no further discussion will be presented in this section.

2.1.3 History of polyamine studies and related diseases

Polyamines were first observed in 1678 by Antonie van Leeuwenhoek who isolated some "three-sided" crystals from human semen. The chemical structure of the crystal was not deduced until 1924 (Dudley, Rosenheim and Rosenheim, 1924), and it took two years to chemically synthesise the identified products after the structures were discovered (Dudley, Rosenheim and Starling, 1926). The names spermidine and spermine thus reflect their original discovery in 1678.

Putrescine (1,4-diaminobutane), was first isolated from *Vibrio cholerae*, and named as putrescine because of its large quantities in putrefying flesh (Stadthagen and Brieger, 1889). Polyamines are found in almost all living species except Methanobacteriales, Archaea and Halobacteriales. Such widespread presence is a strong evidence that they have very important functions in cells. However, it is also possible that the existence of polyamines is a drawback because of the lack of specific roles for the molecules (Heather, Alison and Alun, 2003).

The first two enzymes which recorded as polyamine-related enzymes are named diamine oxidase (DAO) (Zeller, 1938) and ruminant serum amine oxidase (SAO) (Hirsch, 1953). These two enzymes are catabolic enzymes, but polyamine metabolism is far more complicated than just degradation, and the physiological and biological roles of DAO and SAO in the whole polyamine metabolism spectrum are not fully understood. The study of polyamines was limited until a huge expansion resulted in 1983 due to research of a key enzyme called ornithine decarboxylase (ODC) (Pegg, 1986). In 1967, Siimes suggested that the pathway of putrescine (Put), spermidine (Spd) and spermine (Spm) biosynthesis can be reversed. In 1993, Casero and Pegg found a new enzyme, spermidine/spermine N^1-Acetyltransferase (SSAT), which is a participant in the first step of the polyamine catabolic pathway.

Almost every enzyme in this pathway has been identified, and selective inhibitors to each of these enzymes which regulate polyamine metabolism have also been developed (Wallace & Fraser, 2005; Casero & Marton, 2007). In addition, the functions of polyamines in gene regulation and the involvement of polyamines in cellular signal transduction has also been researched (Tabib & Bachrach, 1999; Bachrach, et al., 2001). Research has not only expanded the possibilities of using polyamines or polyamine-based drugs as chemoprevention and chemotherapy, but also established the foundation of other polyamine applications in areas such as diabetes and prion-related diseases (Lu et al., 2007; Supattapone et al., 2002).

Sufficient evidence indicates that higher levels of polyamines in liver, skeletal muscle and

white adipose tissue are able to upregulate energy consumption and provide resistance to obesity and fatty liver disease (Kraus et al., 2014; Bonhoure et al., 2015; Jose et al., 2019). Variations in polyamine metabolism regulation have been proven to influence glucose, lipid and energy homeostasis (Niiranen et al., 2006; Pirinen et al., 2007; Cerrada-Gimenez et al., 2012; Kraus et al., 2014; Bonhoure et al., 2015; Yuan et al., 2018; Jose et al., 2019). Moreover, multiple studies using obese animal models have indicated that the polyamine levels in liver, adipose tissue, pancreatic islets and urine are impaired (Jamdar et al., 1996; Yun et al., 2013; Kwak et al., 2015; Sjoholm et al., 2001; Pelantova et al., 2016). However, while these studies suggest the clinical benefits of increased polyamine levels, some studies have a contrary conclusion. For instance, polyamine levels in blood have been reported to be remarkably higher in obese individuals (Codonor-Franch et al, 2011). Research related to polyamine metabolism mostly relates to cancer, obesity and diabetes due to the fact that polyamines (especially spermidine and spermine) influence cell proliferation abilities, and also affect the AcCoA pools in cells, meaning fat metabolism and glucose oxidation are also influenced when intracellular polyamine level changes.

2.1.3.1 Polyamine chemotherapy development

Initially, most polyamine studies concentrated on cancer applications due to the fact that SSAT and ODC were found to be overexpressed in several cancers (Pegg, 2008; Casero and Pegg, 2009; Bernacki et al., 1995; Nowotarski et al., 2013; Yuan et al., 2018). Most of the studies that aim to discover the antiproliferative strategies using polyamine pathways try to decrease the polyamine pools by suppressing the biosynthesis of polyamines. Other studies investigate the methods to activate SSAT polyamine acetylation (Vujcjc et al., 2000). According to previous research, the SSAT overexpression in prostate adenocarcinoma cells suppressed cell growth and proliferation by decreasing the AcCoA pools (Kee et al., 2004; Jell et al., 2007). Overexpression

of SSAT causes the increase in polyamine biosynthesis. Meanwhile, SSAT also stabilises the metabolic flux via the catabolic and biosynthetic branches of the polyamine pathway (Kee et al., 2004; Tucker et al., 2005; Jell et al, 2007).

During the past several decades, studies of polyamine-based chemotherapy has taken the following pathways for investigation: 1) The inhibition of biosynthetic enzymes including ODC, adenosylmethionine decarboxylase (AdoMetDC), Spm synthase and Spd synthase, which are capable of influencing the polyamine pool in cells directly (William-Ashman and Schenone, 1972); 2) the inhibition of polyamine functions by the interruption of polyamine analogues (Seiler, 2003); 3) decreasing polyamine intake (a physiological method which prevents polyamines from being utilised by tumour cells) (Porter, Miller and Bergeron, 1984); 4) using the structural analogues of natural polyamines to alter the polyamine content in the polyamine pool, using these analogues as competitive substrates to disturb the feedback loop of the polyamine pathways (Seiler, 2003). Despite such a range of study points and discoveries, a polyamine-based anti-tumour drug has not yet been developed.

2.1.3.2 Polyamine obesity study

Obesity refers to a medical condition where excess body fat is accumulated. A person is categorised as overweight when the body mass index (BMI) is between 25 and 30 kg/m^2, while over 30 kg/m^2 is considered as obese (Rahman and Berenson, 2010). Polyamine metabolism has been implicated in adipogenesis, which means the increase of polyamine pools is related to obesity (Ishii et al., 2012; Hyvonen et al., 2013; Brenner et al., 2015; Jose et al., 2019). Due to the fact that polyamines influence fat metabolism, and the changes in intracellular polyamine pools may affect body fat accumulation, polyamine studies are justifiably focused on obesity.

Polyamine studies on SSAT transgenic and knockout mice suggest that the major function

of polyamine metabolism is in adjusting adiposity and energy expenditure (Jell et al., 2007; Liu et al., 2014; Pirinen et al., 2007; Yuan et al., 2018). Whole-body SSAT knock-out mice developed late-onset obesity, while the overexpression of systematic and adipose-specific SSAT was able to prevent obesity caused by diet disorder. With the knockdown or overexpression of SSAT, the PGC-1α expression and AMPK activities are changed in white adipose tissues (Jell et al., 2007; Liu et al., 2014; Pirinen et al., 2007; Yuan et al., 2018).

According to Jell and his colleagues (2007), AcCoA tissue levels in mouse white adipose tissues decreased in SSAT overexpression mice, whereas in SSAT knockout mice, AcCoA levels increased. Such changes also occurred in levels of malonyl-CoA, the downstream inhibitor and an allosteric inhibitor of carnitine palmitoyltransferase 1, which is considered as the transporter of fatty acids into mitochondria. In addition, the SSAT overexpression mice seemed to be slimmer, while SSAT knockout mice gained more weight compared to SSAT wild type mice (McGarry and Brown, 1997; Jell et al., 2007). Such phenomena are caused by the effects of SSAT on β-oxidation: When the levels of methyl-CoA are low, carnitine palmitoyltransferase 1 inhibition is relieved, which leads to the activating of β-oxidation of fatty acids in the mitochondria. In contrast, when the malonyl-CoA pools are exceeding normal levels, β-oxidation of newly synthesised fatty acids in mitochondria is prohibited by carnitine palmitoyltransferase 1 inhibition, a defensive system that avoids useless recycling of AcCoA moieties. Thus, SSAT overexpression mice are leaner than SSAT wild type mice, while the SSAT knockout mice are fatter.

It should be mentioned that such changes occurring in AcCoA and methyl-CoA are not observed in liver tissues. According to the previous studies, AcCoA and methyl-CoA pools were decreased in the prostate and white adipose tissue of SSAT overexpression mice, but such effects did not show in liver tissues. This finding is likely because liver tissues are freely permeable to glucose (Cadhill et al., 1958; Jell et al., 2007). Therefore, when intracellular AcCoA levels in liver tissues decrease, the up-regulation of glucose oxidation, which produces AcCoA, will be activated

as long as the serum is continuously suppling glucose (Attwood, 1995; Agius and Alberti, 1985; Jell, 2007). The better appetite of SSAT overexpression (lean) mice also suggests that the change in AcCoA levels is influencing glucose metabolism. In addition, lipid metabolism in liver tissues is also different from other tissues. Liver tissue plays a role as the centre of redistribution and recycling of lipids. It can absorb lipids from not only chylomicrons and very low-density lipoprotein via hepatic lipase, but also non-esterified fatty acids from plasma. Liver tissues are also the major position for *de novo* lipogenesis by consuming AcCoA and malonyl-CoA. In liver tissues, surplus lipids are re-esterified to triglycerides, the lipids are then wrapped into Apolipoprotein B and phospholipids as very low-density lipoproteins for export. The initial very low-density lipoproteins are then released for effective transport of lipids to peripheral tissues via the circulatory system (Robert and Justin, 2011; Shi and Burn, 2004).

2.1.3.3 Polyamine type-2 diabetes study

Type-2 diabetes refers to a chronic disorder of metabolism which has characteristic high blood sugar levels caused by insulin resistance and/or the lack of insulin. The symptoms of type-2 diabetes are able to be examined at several levels. At the molecular level, proteins and metabolites which are related to lipid and glucose metabolism are disturbed in biosynthesis, translation, degradation and transcription pathways. At the cellular level, mitochondria are damaged. At the organ level, different tissues such as the liver, kidneys and heart can all be influenced.

According to Bohm and his colleagues (2014), polyamine pools are increased in human adipose tissue of insulin-resistant obese subjects as compared to the insulin-sensitive controls. In addition, the serum putrescine levels of the type-2 diabetes patients are significantly higher than the non-diabetic groups, which means the activity of ODC enzyme is remarkably increased in

type-2 diabetes patients. It is also known that insulin is able to inhibit SSAT activity on heart ischemia and reperfusion injury in diabetes mellitus. These results indicate that insulin could suppress polyamine catabolism on heart ischemia and reperfusion injury on diabetes mellitus. According to previous research, heart ischemia and reperfusion injury are significant pathophysiological phenomena. Diabetes mellitus is associated with the deterioration observed in ischemic heart disease, reperfusion injuries and myocardial damage (Tong et al., 2018). Multiple studies have proven that insulin is capable of inducing ODC activity in many responsive cells (Potter et al., 1984; Rinehart and Canellakis, 1985; Tong et al., 2018). Additionally, ODC expression, Spd, Spm and total polyamine pools are reduced, while putrescine is increased in heart ischemia and reperfusion injury in diabetes mellitus. On the other hand, insulin stimulates ODC expression, which increases total polyamine pools levels, indicating that insulin is able to upregulate ODC polyamine in diabetes mellitus. Furthermore, the inhibition of ODC and other enzymes related to polyamine synthesis abolished the influence that insulin played in decreasing myocardial infarction size and apoptosis on heart ischemia and reperfusion injuries in diabetes mellitus. Endogenous polyamine therefore is seen to participate in insulin mediated cardioprotective effects on heart ischemia and reperfusion injury in diabetes mellitus (Tong et al., 2018).

2.1.4 Polyamine Synthesis and Catabolism

In most cases, polyamines tend to incorporate electrostatically into DNA, RNA and phospholipids, which are negatively charged molecules, instead of binding to other macromolecules. This characteristic gives them a strong ability to stimulate cell proliferation. Thus, homeostatic adjusting levels of polyamines in cells is significant in supporting cell proliferation. This is achieved by the synthesis and catabolism systems of polyamines that

regulate export, uptake, biosynthesis and degradation of these molecules (Jell et al., 2007).

The metabolism of polyamines is strongly controlled by multiple rate-limiting enzymes for synthesis and catabolism. Particularly, the synthesis of polyamines is regulated by adenosylmethionine decarboxylase (AMD1) and ornithine decarboxylase ODC, while SSAT1 acetylates Spd and Spm to N^1-AcSpd, N^1-AcSpm and N^1, N^{12}-diacetylspermine. The acetylated polyamines are then oxidised by acetyl-polyamine oxidase (APAO) or be expelled with urine. Such oxidations generate putrescine, spermidine and hydrogen peroxide (H_2O_2). The recovered polyamines are then returned to the polyamine metabolic cycle (Pegg, 2008; Casero and Pegg, 2009; Yuan et al., 2018).

Figure 3 shows the polyamine pathway in mammalian cells. Three types of enzymes (APAO, SSAT, SMO) are included in the Spd catabolic pathway. As Figure 3 shows, Spd and Spm could be transferred to N^1-acSpd and N^1-acSpm (and N^1 ,N^{12}- Diacetylspermine) respectively through SSAT (Casero & Pegg, 1993).

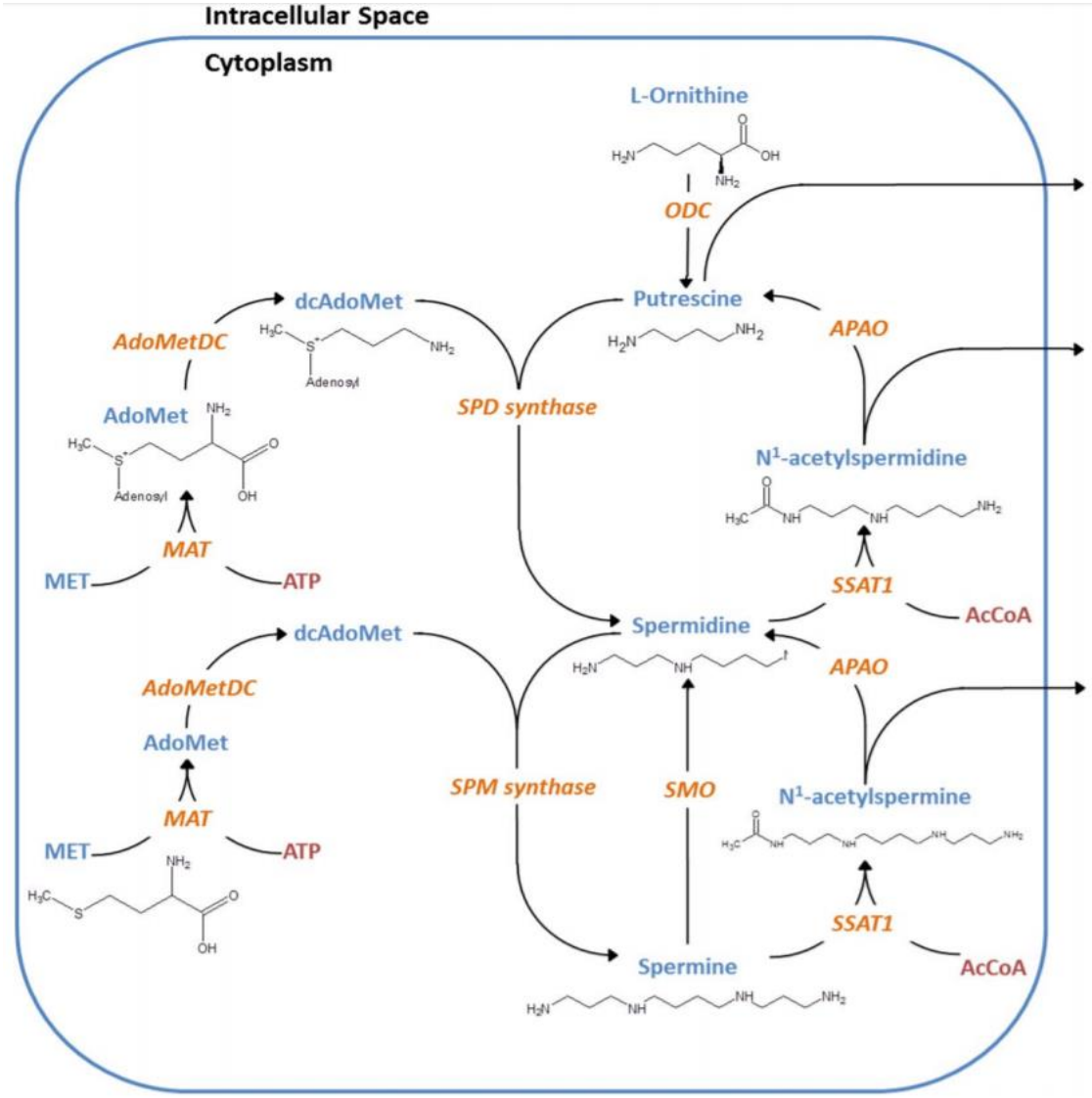

Figure 3 The mammalian polyamine catabolic pathway.

2.1.4.1 Polyamine Biosynthesis

Figure 4 (a) demonstrates the polyamine biosynthesis pathways. The red arrows stand for mammalian animals, and green and blue arrows represent plants and microorganisms (same as b). The starting materials are two amino acid precursor molecules, methionine and L-ornithine. According to figure 4 (a), arginase converts L-arginine to L-ornithine, and then L-ornithine is converted to putrescine by ODC, which is the first step of the polyamine synthesis pathway. In mammalian cells, the arginine decarboxylase pathway, which is catalysed by agmatine, is not

available. After putrescine is produced, Spd synthase and Spm synthase accomplish the synthesis of Spd and Spm in a stepwise fashion (Kusano, 2008). In addition, ornithine in mammalian cells can also be synthesised directly from proline by ODC (Wu et al. 2005). Bacteria and plants, are able to form putrescine effectively by the arginine decarboxylase pathway. Bacteria convert agmatine to putrescine by agmatinase directly, which is even more effective than the process in plants. Unlike plants and animals, bacteria are not capable of synthesising spermine because the bacterial genome does not encode spermine synthase (Wortham et al. 2007).

Decarboxylated S-adenosylmethionine (DcAdoMet) is produced by S-adenosylmethionine decarboxylase (AdoMetDC), and then it provides aminopropyl groups to putrescine (or Spd), forming Spd (or Spm) by Spd synthase and Spm synthase respectively (figure 3). DcAdoMet levels in human cells are also strictly controlled within translation, degradation and transcription processes by the concentrations of substrates or metabolites. Putrescine here initialises the processing of AdoMetDC proenzyme to functional AdoMetDC, and it activates the AdoMetDC pathway as well (Reguera et al., 2007). In addition, a small open reading frame (ORF) upstream of the AdoMetDC-mRNA controls translation, and downstream translation requires the peptide encoded by AdoMetDC-mRNA, which is also down-regulated based on increases in polyamines (Hanfrey et al., 2005). Ubiquitin-mediated degradation is prevented by decreases in Spd and Spm levels, while the increases of Spd and Spm levels are related to the up-regulated degradation, and down-regulated transcription of, AdoMetDC (Pegg, 2009). Moreover, DcAdoMet is produced by methionine and ATP in a reaction which is stimulated by methionine adenosyltransferase (Reguera et al., 2007).

2.1.4.2 Polyamine degradation

Figure 4 (b) shows the catabolism pathway of polyamines. In animals (red arrows),

polyamine catabolism initiates with SSAT (Casero and Pegg 1993; Seiler 2004), catalysing the acetylation of Spd and Spm by transferring the acetyl group of AcCoA to the N^1 position of Spd and Spm. After that, AcSpd and AcSpm are converted to putrescine and spermidine respectively by the peroxisomal and constitutive PAO (APAO) (Bolenius and Seiler 1981). The activity of APAO is only controlled by the existence of the N^1-acetyl polyamines (Pegg, 2009). The degradation will also produce 3-aceto-aminopropanal and H_2O_2. The aldehydes produced in the process can be further degraded to β-alanine by reactions of aldehyde dehydrogenase and N-acetyl-β-alanine deacetylase. According to Seiler, Duranton and Raul (2002), mice chronically treated with the N^1 ,N^4-bis(2,3-butadienyl)-1,4-butanediamine (PAO inhibitor MDL72527) die. Accumulation of spermine in blood means that there is a blockage of the polyamine catabolism pathway, especially the blockage of spermine degradation, which is lethal to these animals. In addition, Spm can also be transformed to Spd directly by SMO (figure 3). The reaction by-products are the same as the APAO reaction. Both the human and mouse active splice variants of SMO are found in the nucleus and cytosplasm, and SMO1 is most abundant in humans (Murray-Stewart et al., 2002; Murray-Stewart et al., 2008; Seiler 2004). Normally, SMO is regulated at the transcriptional level. However, it is also influenced by many other stimuli including polyamine analogues. Thus, SMO plays a significant role in ROS-mediated apoptosis (Devereux et al., 2003).

According to figure 4 (b), although microorganisms do not have a pathway containing Spm, they possess the same catabolic pathway as animals which converts Spd to putrescine. Moreover, Spd and Spm are catabolised by APAO and produce 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal respectively. Both Spd and Spm in this pathway are able to be converted to 1, 3-diaminopropane. Polyamine catabolism in plants is the same as in microorganisms except plants are not capable of converting Spd to putrescine, and plants can directly catabolise Spm to Spd by Spm oxidase. Furthermore, plant polyamines also serve as substrates for secondary metabolites (Kusano, 2008). For example, in the first step of polyamine catabolism, putrescine is methylated

by N-methyltransferase, and then oxidatively deaminated to 4-methylaminobutanal, which is catalysed by N-methylputrescine oxidase (Katoh et al., 2007; Heim et al., 2007; Hibi et al., 1994).

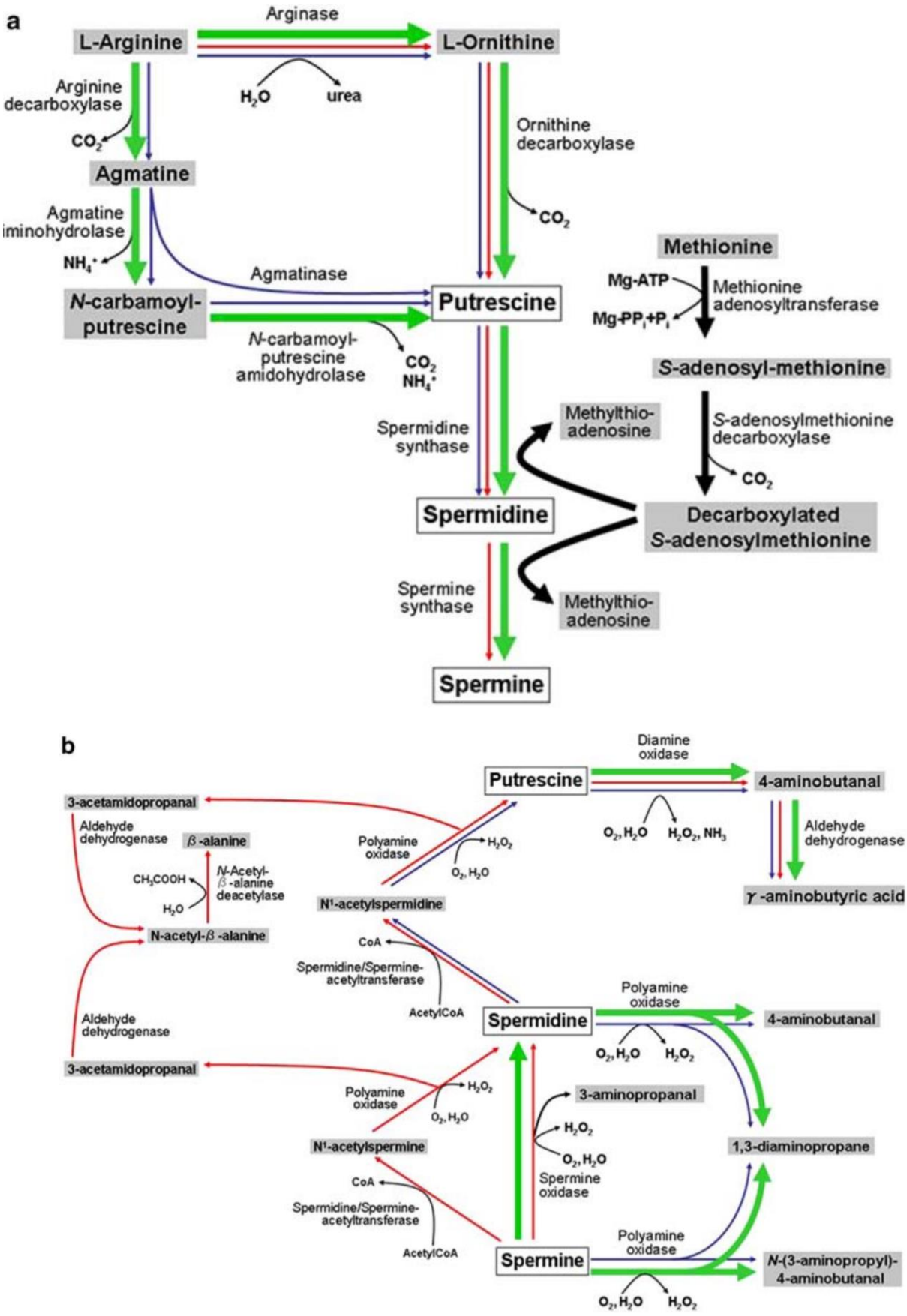

Figure 4 Polyamine biosynthesis (a) and degradation (b) (Kusano, 2008).

2.1.5 Polyamines metabolism in bacteria

Both prokaryotic and eukaryotic cells need polyamines to support cell proliferative functions, so microorganisms such as *Bacillus subtilis* and *Escherichia coli* also have polyamines (Tabor and Tabor, 1984). Some of these microorganisms even have polyamine acetyltransferase to adjust the polyamine level in cells (Woolridge et al., 1999). The *BltD* gene in *Bacillus subtilis* is the gene which controls the acetylation of Spd and Spm, and the sequence of it is similar to other bacterial and mammalian acetyltransferases (Woolridge et al., 1999). In bacteria, the most commonly existing polyamines are putrescine, Spd and cadaverine. Spm, however, is not present in most bacteria, unless Spm has been already applied to the growth medium, and the samples are pathogenic bacteria (such as *Escherichia coli*). It suggests that although Spm is not able to be synthesised in bacteria, it is possible for them to absorb Spm from the environment to fulfil some physiological functions. Another special biosynthetic pathway of Bacteria (and Archaea) is the arginine decarboxylase (ADC) pathway, which converts arginine into agmatine. Agmatine is eventually transformed to putrescine, and putrescine is then used for Spd synthesis. Mutations of these pathways are applied in studies investigating the role of polyamines in cells. The knockout of related genes causes a reduction of growth rate in bacteria including *Thermococcus kodakaraensis*, *Yersinia pestis*, *Salmonella typhimurium* and *Vibrio cholerae* (Gevrekci, 2017; Michael 2015). In addition, polyamines are extremely important in bacteria for their interaction with nucleic acids, and their functions in binding to DNA and RNA are notably different. For RNA, polyamines keep RNA soluble in order to stabilise the interacting ability of RNA with other molecules. For DNA, polyamines stabilise the double stranded structure, and enable DNA intermolecular interactions by binding to the DNA externally. Such stabilisation functions for nucleic acids is extremely important in some thermophilic microorganisms (which normally grow in temperatures between 50 ~ 60 °C, and grow even better around 80 °C). Multiple studies have suggested that polyamines may contribute to the observed heat resistance (Gevrekci, 2017; Katz

et al. 2017).

2.2 Spermidine/spermine N^1-acetyltransferase (SSAT)

SSAT is an enzyme which is highly regulated in cells. Due to the fact that SAT has lower basic activity and a shorter biological half-life (< 30 min), it is not stable in the interconversion cycle (the cycle of polyamine biosynthesis and degradation). The molecular weight of the SSAT enzyme is different between species. For example, the molecular weight of SSAT in chicken is 36000 Da, while mammalian is 65000-80000 Da. Although the constitutively expressed SSAT activity is low, the physiological stimulation provided by Spd, Spm, hormones, toxins, growth factors, specific drugs and antiproliferative stimuli are able to increase the SSAT activity dramatically (Fogel-Petrovic et al., 1997; Erwin and Pegg, 1986; Shappell et al., 1993).

Substrates of SSAT usually have the structure of R–NH–$(CH_2)_3$–NH_2. AcCoA is also required to provide acetyl groups. SSAT is catabolised by the ubiquitin-proteasomal pathway. The polyamine analogues such as N^1, N^{12}-bis(ethyl)spermine are able to greatly stabilise the enzyme because of conformational changes, and slow down polyubiquitination. The SSAT enzymes of cells that were modified to be resistant to polyamine analogues also lose some measurable enzymatic activities (lower V_{max}, and affinity for Spm and Spd) (Coleman and Pegg, 2001; McCloskey and Pegg, 2003; Seiler, 2004).

2.2.1 General aspects of SSAT and AcCoA

Acetylation of most drug molecules is catalysed by the acetyltransferases, N-acetyltransferase-1/2 (NAT1/2), whose actions are a prominent cause of drug-drug interactions. There are two functional NAT genes in humans: both are polymorphic, and they encode N-

acetyltransferase 1 (NAT1) and NAT2 respectively (Sim, Westwood and Fullman, 2007). Both are expressed mainly in the cytoplasm (Liu et al., 2006) and catalyse the acetylation of a range of arylamines by employing the acetyl moiety from AcCoA as the donor group. Their substrate specificities are overlapping but distinct (Kawamura et al., 2005). SSAT catalyses the reaction that transfers the acetyl groups from AcCoA to the terminal amines of polyamines, which allows them to be exported or back to the polyamine metabolism by the catabolism via APAO (Jell et al., 2007). The SSAT enzyme is very sensitive to the presence of regulatory interactions and short-lived, so it is highly regulated by intracellular polyamine pools. NAT 1/2 isoforms are easily induced by polyamine analogues and multiple cytotoxic substances (Seiler, 1987; Casero and Pegg, 1993; Jason et al., 2007).

There is another unrelated family of N-acetyltransferases whose range of physiological substrates includes linear polyamines and is different and more restricted than NAT1/2. This second family comprises SSAT1 and SSAT2, which have similar X-ray crystal structures but significant differences in the number/nature of their charged surface residues, and in the presumed substrate-binding residues at their active-site regions (Chen et al., 2003). There is evidence that both enzymes catalyse acetylation of TETA: the V_{max} of SSAT1 was 66% of that for SSAT2 in a notable study (Weisell et al., 2003). *In vivo* studies have indicated that SSAT1 knockout mice can acetylate TETA at a similar rate to wild-type mice (Cerrada-Gimenez et al., 2011).

2.2.1.1 SSAT gene

The DNA sequence of SSAT has been established. The human SSAT1 gene is abbreviated SAT1 (Xiao, et al., 1992). “The human SAT1 gene is located on the X chromosome at location Xp22.1, which has 6 exons. Exon 1 has one 5’-untranslated region (UTR) followed by first 22 codons, and exon 6 has the last 56 codons, the stop condo, which followed by a 3’-UTR” (Xiao

et al., 1992). The size of the SAT mRNAs is between 1.3 and 1.5 kb, and they are encoded by the 6 exons (Fogel-Petrovic et al., 1993a). The SSAT1 promoter is in the 5' flank of the SAT1 transcriptional region, and the size is about 2k bp. There are several proteins binding motifs within this region for different transcriptional factors such as CCAT/enhancer binding protein-β (C/EBPβ), peroxisome proliferator-activated proteins (PPARs), Sp-1 and AP-1, cAMP response element binding protein (CREB) and nuclear factor κ B (NF-κB) (Xiao et al., 1992; Babber et al., 2006; Choi et al., 2006; Fogel-Petrovic et al., 1993a). Moreover, the GC-box in the SAT gene sequence, located 42 to 51 base pairs upstream of the initiation codon seems to be significant to Spd transcription and binding (Tomitori et al., 2002).

The amino acid sequence of SSAT1 from other eukaryotic animals such as mouse, horse, pig, chicken, and frog are also well conserved, and the substrate binding residues are almost the same, except pig and frog have one and two different residues respectively (Montemayor & Hoffman, 2008). BALB-C mice possess an additional 110-bp exon between exons 3 and 4 (Fogel-Petrovic, 1993b). Compared to the human SAT1 gene, mouse SAT1 has a very similar size, with six exons and five introns (Xiao et al., 1991).

2.2.1.2 Acetyl coenzyme A (AcCoA)

AcCoA is a molecule that attends to various biomedical reactions and physiological functions in protein, lipid and carbohydrate metabolism. It is a central metabolic intermediate which plays critical roles in different cellular processes such as allosteric regulation of enzymatic activities, a precursor of anabolic reactions, and a fatal determinant of protein acetylation from archaebacteria to mammalians (Pietrocola et al., 2015; Nitschke and Russell, 2013; Choudhary et al., 2014).

AcCoA in mitochondria is normally metabolised in tricarboxylic acid (TCA) cycle to

produce Nicotinamide adenine dinucleotide (NADH), which is a main substrate of ATP synthesis through oxidative phosphorylation (Boroughs and DeBerardinis, 2015). Some cells (such as hepatocytes) can also use AcCoA as one of the ingredients to synthesise ketone bodies (Newman and Verdin, 2014). Ketone bodies in liver cells entering the TCA cycle are simply taken up by cardiomyocytes and neurons, utilising them to synthesise ATP for reconversion to AcCoA and participation in the TCA cycle (Cotter et al., 2013). On the other hand, cytosolic AcCoA is one of the precursors of several anabolic reactions, which is the base of the synthesis of steroids, fatty acids and special amino acids such as proline, glutamate and arginine (Pietrocola et al, 2015).

Polyamines (such as Spd) can compete with AcCoA for connecting with acetyltransferase, thus increasing the level of AcCoA required for optimal acetyltransferase activity (Pietrocola et al., 2015). Since polyamine concentrations are different between organelles (Casero and Marton, 2007), such phenomena may play a role in protein acetylation regulation.

The structure of AcCoA is a molecule composed of an acetyl group (CH_3CO) connected with coenzyme A (CoA), a derivative of vitamin B_5 and cysteine, by a thioester bond (Shi and Tu, 2015). Due to the fact that a thioester bond has very high energy, the AcCoA structure stimulates the transfer of CH_3CO to different kinds of acceptor molecules (such as amino groups) (Shi and Tu, 2015). Amino acid residues Arg101, Arg142 and Arg143 within the protein sequence, are fatal factors for AcCoA to bind to the substrate including 1,3-propanediamine, Spd, Spm and N^1 AcSpm (Lu et al., 1996; Coleman et al., 1996). The high energy bond also suggests that the acetylation reaction of AcCoA does not initiate spontaneously.

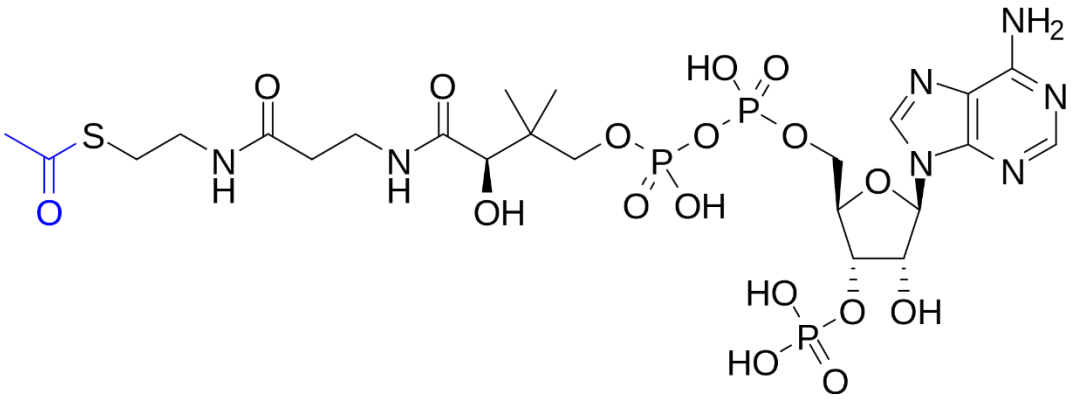

Figure 5 Chemical structure of AcCoA.

According to Kuhn (2014), the rate of non-enzymatic acetylation of AcCoA may rely on the highly reactive intermediate acetyl-phosphate, which functions as the donor of acetyl groups. Such reactions are highly affected by the physicochemical conditions of the microenvironment.

2.2.1.3 SSAT1 protein structure

The human SSAT1 crystal structure was first determined in 2006 (Bewley et al., 2006). It is a typical homodimer with α/β-construction which is found in GNAT superfamily. A barrel structure which formed through α-helices flanked by β-sheets is the core of SSAT1 dimer. In nature, there are two similar forms of SSAT1 dimers with slight conformational differences. These two forms of dimers are similar in amino acid sequences, but one of these is symmetric while the other one isn't. Both of them have two active sites on the surface of the opposite side of monomers. In addition, although both of the active sites in the symmetric form are able to transfer acetyl groups, only one of them is functional (Bewley et al., 2006). When substrates are absent, SSAT1 can also acetylate itself on Lys-26 by AcCoA. However, when Lys-26 has been already replaced by arginine, it will no longer be capable of being acetylated, but it is still able to acetylate substrates (Bewley et al., 2006). Bewley (2006) suggests that the self-acetylation reaction aims to protect SSAT1 from degradation. Figure 6 demonstrates the electron density map of the active

parts of SSAT1, and shows all residues involved in Spd/Spm acetylation.

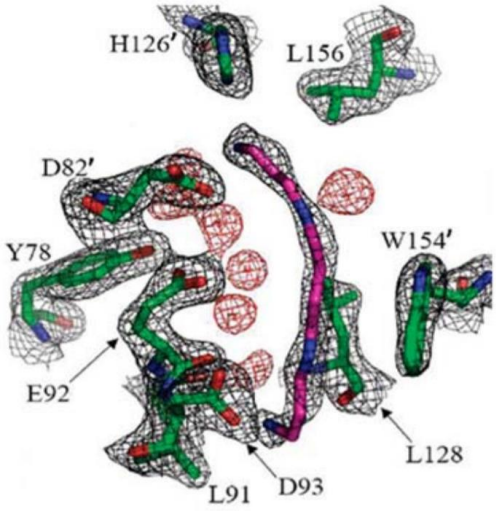

Figure 6 Electron density maps of SSAT1 (Montemayor and Hoffman, 2008).

Normally, proteins are produced to accomplish specific purposes in cells. Proteins that are biologically inactive (damaged proteins and normal proteins that have finished their jobs) will be degraded eventually. In eukaryotic cells, such protein degradation is called ubiquitin-mediated degradation. A ubiquitin tag is applied on targeted proteins, and then a subsequent "chopping" process will be carried out right after the proteases recognise the tag. The products of the degradation are peptide sections or single peptides (Sorokin et al., 2009).

SSAT1 is one of the enzymes that follows ubiquitin-mediated degradation, and it has a quick turnover time, which is 15 minutes in rats and 29 minutes in Chinese hamster ovary cells (Thrower et al., 2000; Zahedi et al., 2007). Once polyubiquitin moieties are labelled, SSAT1 will be subjected to protease-mediated degradation, with cleavage initiated at Lys141-Arg142-Arg143 by endoproteinase Lys-C or trypsin. The cleavage at Glu151-Glu152 by endoproteinase Glu-C also exists (Coleman et al., 1995). In addition, ubiquitination decreases SSAT1 activity at the cellular level (McCloskey, Coleman and Pegg, 1999). However, it is believed that polyamines and polyamine analogues are capable of stabilising SSAT1 and thereby prolong turnover time (Uimari

et al., 2009).

2.2.1.4 Catalytic Mechanism

It is suggested that SSAT1 catalyses acetylation by using a normal acid-base catalytic mechanism, which resembles the utilisation of other enzymes in the GNAT family. The existence of one catalytic base is necessary for the acetylation in a typical normal acid-base catalytic mechanism. In the SSAT1 catalytic mechanism, Tyr140 acts as the general acid protonating the sulphur on AcCoA, while Glu92 is the general base for deprotonating the N^1 amine of Spd by multiple water-mediated proton transfers. The mutations that occur on Tyr40 and Glu92 decrease the enzyme activity dramatically (Montemayor and Hoffman, 2008). The mutations on other residues also decrease the activity of SSAT1, with the influences far weaker than the mutations on Tyr40 and Glu92 (Hegde et al., 2007). Thus, the Glu92 and Tyr140 mediated acid-base mechanism is the most well-known mechanism for SSAT1 acetylation.

2.2.1.5 Spermidine/spermine N1-acetyltransferase 2

SSAT2 is a protein with 170 amino acids, and the predicted molecular mass is 20kDa based on the its homology to SSAT1. SSAT2 is 46% identical and 64% similar to SSAT1 (Chen et al., 2003). The crystal structures of human SSAT1 and SSAT2 are similar, but the presumed substrate-binding residues and the charged surface residues at the active site region are significantly different (Han et al., 2006). The function of SSAT1 in polyamine catabolism is to regulate intracellular pools of the polyamines including putrescine, Spd and Spm, and the overexpression of SSAT1 will cause the increase of acetylated polyamines such as N^1 AcSpd. Moreover, various studies indicate that although the overexpression of SSAT increases

acetylated polyamine levels, the knockout of SSAT1 does not alter observed levels, which suggests that SSAT2 may also participate in polyamine acetylation. It has been proven that TETA is one of the metabolites that SSAT2 is capable of acetylating (Hyvönen et al., 2013).

SSAT2 serves different functions than SSAT1 (Vogel, Boeke and Ashburner, 2006). For example, the overexpression of SSAT2 has no effect on the level of polyamines while the overexpression of SSAT1 influences levels considerably (Chen et al., 2003). The substrate affinity (K_{cat}/K_m) for Spd or Spm of SSAT2 is extremely low, which is lower than 0.001% of SSAT1's corresponding values. *In vitro* cell experiments made by Coleman and his colleagues (2004) have also illustrated that there is no obvious effect on cellular polyamine pools, while SSAT1 is able to decrease the polyamine substrate (Spd and Spm) concentrations by 30%. Nonetheless, SSAT2 has a much greater activity toward the ε-amino group of thialysine [S-(2-aminoethyl-L-cysteine)]. Thialysine is an analogue of lysine. The ε-amino group of lysine refers to the functional group's fifth carbon which is attached to the carboxyl group. Thus, SSAT2 is also called thialysine N-ε-acetyltransferase (TLAT). In addition, TETA is able to be acetylated by SSAT2 just as with SSAT1. Cerrada – Gimenez (2011) stated that the K_m and V_{max} of SSAT2 acetylating TETA is 2.5 ± 0.3 mM and 3.96 ± 0.15 μmol/min/mg respectively.

Moreover, the acetyltransferase activity of SSAT2 is lower than SSAT1, and the preferred substrate for SSAT2 is thialysine rather than polyamines, which suggests that SSAT2 may not be involved in polyamine metabolism (Coleman et al., 2004).

2.2.1.6 Subcellular distribution of SSAT enzyme

Polyamine acetyltransferase (later known as SSAT) was originally considered to be an enzyme distributed in the cell nucleus due to high putrescine, spermidine and spermine acetylation activity found in an *in vitro* polyamine acetylation assay which used rat liver

chromatin as the enzyme source (Blankenship and Walle, 1977). This idea was then rejected by a study which indicated that polyamine acetyltransferase is a cytosolic enzyme (Matsui and Pegg, 1980), and the enzyme was then purified from rat liver cytosolic preparations (Ragione and Pegg; 1983). However, recent findings continue to refine the subcellular distribution of SSAT via multiple experimental approaches including *in vitro* SSAT activity assay, western bolt analysis and immunogold labelling. SSAT responses are extremely high in mitochondrial fractions, notable in nuclear fractions, and far lower in cytosolic fractions (Holst et al., 2008). But according to Pegg (2008), the physiological significance of these findings is not clear enough to draw localisation conclusions. Thus, the correct localisation of the SSAT enzyme is still controversial.

2.3 Reviews of experimental techniques

2.3.1 Enzyme assay

An enzyme assay serves two different purposes: 1) to recognise a specific enzyme and to prove the existence or absence in an organism or tissue sample, and 2) to quantify the enzyme in the samples as accurately as possible. Unlike nucleic acids and other functional proteins which need direct detection, it is possible for enzymes to be identified by the reaction they catalyse. The metabolites of enzymes accumulate in amounts exceeding the original enzyme concentration. Nevertheless, the conclusions gained from an enzyme assay could be misleading due to the various pitfalls and difficulties in the procedures (Hans, 2014; Michaelis and Menten, 1913).

To study the polyamine acetylation catabolic pathway, a new *in vitro* enzymatic assay was applied, which uses TETA, Spd and Spd-D6 as substrates and LC-MS to detect metabolite concentrations. Specific parameters of the assay such as pH, temperature, substrate concentrations,

protein concentrations and reaction times were optimised by the Michaelis-Menten saturation curve. Additionally, the acetylation assay was carried out with different combinations of substrates and enzyme sources. The aim of this method is to measure the TETA, Spd and spermidine-d6 acetylation activities of tissue preparations (liver microsome and cytosol) *in vitro*.

2.3.1.1 Reaction conditions

The conditions for enzymatic reactions in enzyme assays have dramatic effects on the results. The main conditions that must be considered for an enzyme assay, are pH, temperature, ionic strength, cofactors and other parameters such as enzyme and substrate concentrations. Most enzymes, especially those from mammalian animals, have their greatest activity around a physiological pH of 7.5, and temperature of 37 °C (although normally the temperature of 25 °C is preferred due to the experimental reasons) (Hans, 2014).

2.3.1.1.1 pH value

The influence of pH value on enzyme activity normally follows a bell-shaped curve, which increases from zero in the strong acid region to neutral region and starts decreasing back to zero at the highly alkaline area (Figure 7). Such a phenomenon is basically caused by two conditions: 1) The state of protonation of functional groups in amino acids, and 2) the degree of damage of three-dimensional protein structure. The protonation of functional groups is reversible, but the damage of three-dimensional protein structures is irreversible. The simplest case of regulation is when the protonation of one functional group stimulates the enzyme's activity, and when another functional group is protonated, the activity is inhibited. However, the effect on protein three-dimensional structure can be affected by changes of ±2 pH units. The pH value of the highest

enzyme activity is the pH-optimum. Because enzymatic maximum activity (V_{max}) calculations occur in these conditions, pH-optimum is normally selected as the standard pH value for the assay of this enzyme. The pH-optimum is ~ pH 7.5, which is within the physiological range. The pH-optimum here is not very accurate because the optimum curve has a wider maximum (between 7-8), and so the physiological pH can be applied in such situations without remarkable decrease of the enzyme's activity (red curve in figure 7) (Hans, 2014). Some special enzymes are most active far from the normal physiological range. For instance, the pH-optimum of pepsin (the protease in the stomach) is pH 2; the pH-optimum of acid phosphate is pH 5.7; and the pH-optimum of alkaline phosphate is pH 10.5 (Brenda database).

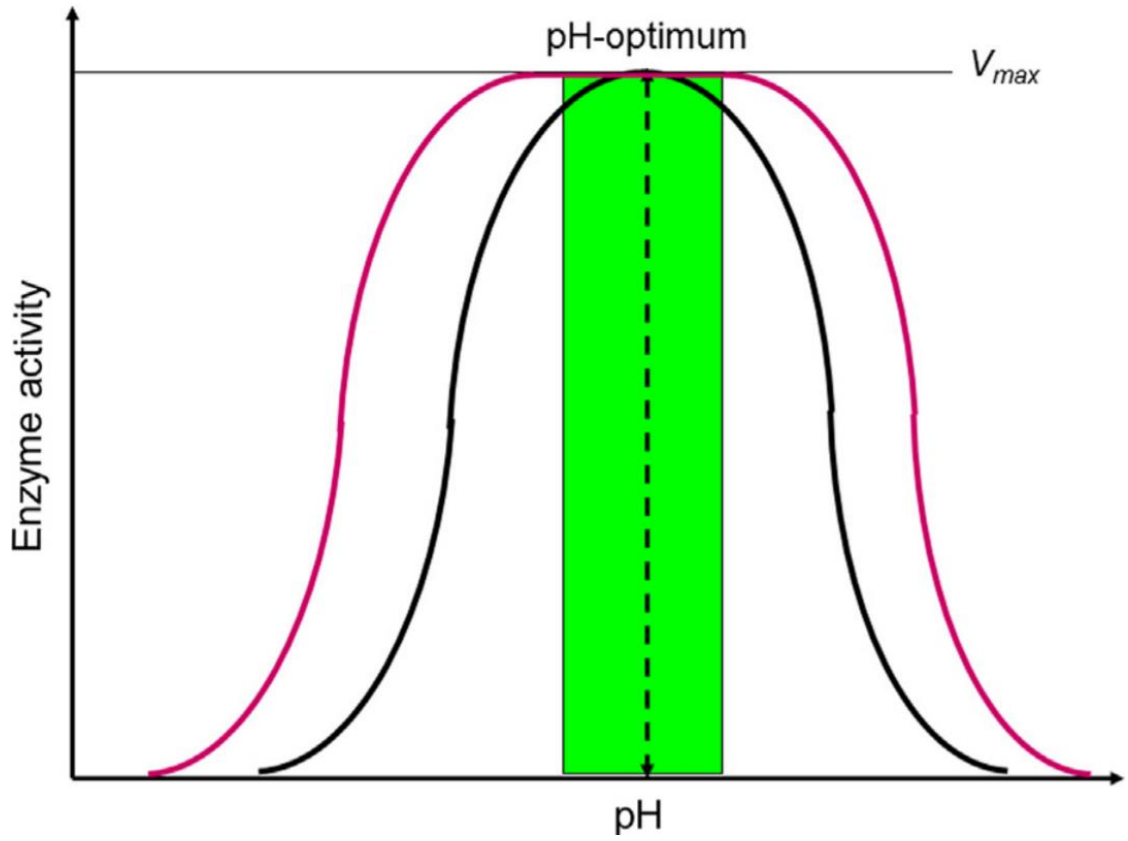

Figure 7 The influence of pH value on enzyme activity.

Generally, most enzymes are stable at their pH-optimum, and thus the pH condition is recommended not only for enzymatic assays, but also for storage. However, some special

enzymes such as trypsin, should be stored at a pH of 3.0 while its pH optimum is 9.5 (trypsin is able to tolerate extreme pH conditions); such procedures aim to suppress autolysis (Bisswanger, 2011). In such cases, it is necessary to account for the influence of the pH of the added aliquot, making sure that it does not influence the overall pH of the enzyme assay's mixture (Hans, 2014).

2.3.1.1.2 Temperature

The effect of temperature on enzyme activity is similar in some respect to the pH dependence: activity increases with rising temperature, and then decreases after reaching a maximum point. Such behaviour is normally considered as the optimal temperature, although the existence of an optimal temperature is not necessary because the velocity of all chemical reactions increases with temperature. Two aspects cause the different temperature dependence of enzymes: 1) The boiling of water limits its progression, and 2) a higher temperature environment can destroy the three-dimensional structure of enzymes, causing protein denaturation. Such denaturation depends on both time and temperature: the higher the temperature, the faster the denaturation will be. Thus, no fixed temperature can be applied to an enzyme assay for the maximum reaction speed. If the enzyme is put into a relatively higher temperature which causes protein denaturation in a short term, the enzyme activity will be significantly higher than if it is used in the same temperature for a long time period before the enzyme assay starts (Hans, 2014).

In an enzyme assay, such facts about specific temperature are usually taken in to account in the protocol, but with some special applications. For example, enzymes without a defined characterisation should be have temperatures applied in the enzyme assay that are within the known stability range. Some enzymes (such as alcohol dehydrogenase) denature slowly even around 37 °C. In a living cell, high protein concentrations stabilise the denaturation process, but the lifetime of an enzyme is still limited (Hinkson and Elias, 2011).

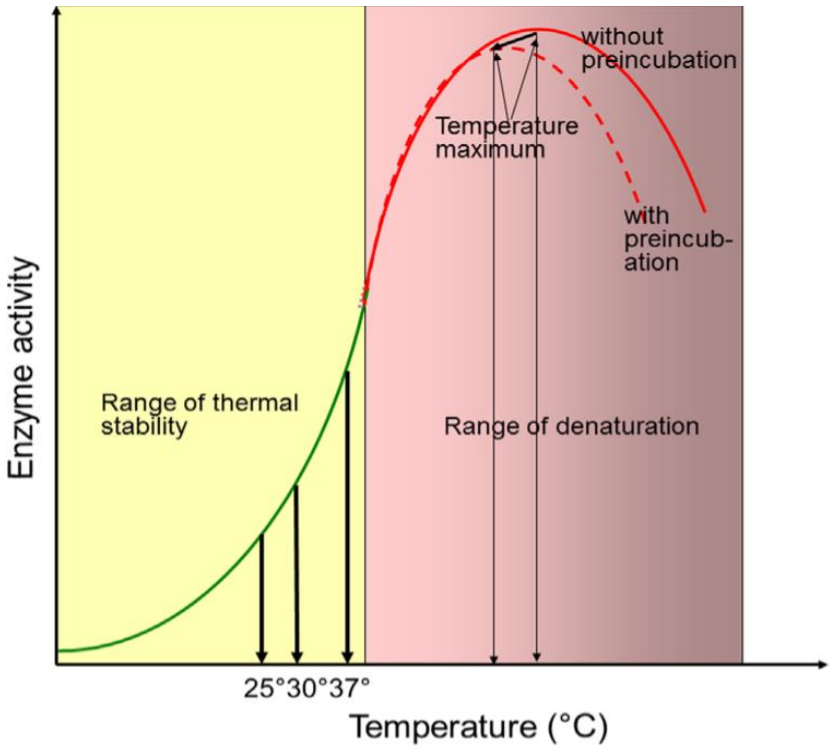

Figure 8 Typical temperature dependence of enzymes.

A general temperature standard for every enzyme assay cannot be defined. For most enzyme assays, especially the ones with mammalian enzymes, physiological temperature (37 °C) is applied. However, according to figure 8, this temperature is close to the denaturation range, which means accurate thermostat measurements are required. In addition, due to the fact that all enzymes are stored at low temperatures, a warm up process is required before starting any assay (Hans, 2014).

2.3.1.1.3 Substrates and cofactors

Alongside the enzyme itself, substrates and cofactors are the most important ingredients in an enzyme assay. The substrate conditions including state, purity and stability are required to be the highest quality. With regard to the substrates, known functionalities must be taken into account. Normally, it is accepted that the defined substrate of a certain enzyme is based on its physiological

function, for example, SSAT1 uses Spd and Spm to produce AcSpd and AcSpm respectively. Nevertheless, the substrates are not very exact in all enzyme reactions. A lot of enzymes show wide specificity, which means they also accept substances structurally similar to the predominant substrates, like SSAT1, which also catalyses the acetylation of TETA. Cofactors show a similar characteristic because preferred divalent cations can be substituted with other divalent cations as the necessary cofactors for many catalytic reactions. For example, glucose isomerase, a microbial enzyme, whose substrate is xylose, is isomerised to xylulose with Mn^{2+} in an assay environment, but the non-physiological reaction initiates faster with Co^{2+} rather than with Mn^{2+}. Thus, the change of the substrate for certain enzymes can also be associated with a change in the cofactor (Antrim et al., 1979; Lehmacher and Bisswanger, 1990). In some cases the physiological substrate has to be switched to an artificial analogue when the physiological substrate of the enzyme is not well defined, and it must then be considered as the genuine substrate (Hans, 2014).

2.3.1.1.4 Buffers and ions

Buffers in an enzyme assay serve the function of adjusting and stabilising the required pH in the assay. Buffers are normally composed of a strong basic component and a weak acid. The relationship between the pH and the buffer components is:

pH = pKa - log [HAc]/[Ac-]

In this formula, HAc and Ac- are the non-dissociated and the dissociated form of acid respectively; pH = log[H+] is the negative logarithm of the proton concentration; and pKa= log Ka is the negative logarithm of Ka, which is also called the dissociation constant of the buffer components. The pKa value indicates the pH value at which the buffer components are half-dissociated, which means that at this point the buffer is the strongest. An appropriate buffer for assays can be found from established tables in literature, many of which include the pH

optimum of specific enzymes. Various suppliers can provide reference standard buffers and prepared buffer solutions. Moreover, pH range is not the only condition a buffer provides. Conditions such as concentration, ionic strength and the nature of buffer ingredients are all required to be taken into account in order for assays to return results with greatest confidence (Hans, 2014).

The buffer system with a higher ionic concentration provides a better ability to stabilise its pH value. Nonetheless, most enzymes tolerate only medium ionic strength solutions, normally within 0.05 and 0.2 M. Only thermophilic and halophilic enzymes prefer more concentrated buffer systems (up to 1 M) (Vieille and Zeikus, 2001; Rainey and Oren, 2006; Gerday, 2007). On the other side, the buffer system with low ionic strength has deleterious effects on protein structures. Care must be taken with each addition of any substance which is capable of influencing the adjusted pH. For instance, if a component is added in an acid form (Such as Spd-2HCl) without previous neutralisation, the pH of the whole system will be influenced. Due to the fact that any deviation from the pH optimum decreases the enzyme activity dramatically, it is possible that the results of an assay are misleading relative to enzyme inhibition: the greater the acidic form of a component which is added, the worse the enzyme activity will be. Notably, some enzymes themselves can cause changes in pH, which leads to a continuous decrease of enzyme activity. If such shifts in pH are significant and the buffer system is not able to control it, only short initial reactions should be measured for this situation (Hans, 2014).

Ions influence enzymatic activity due to their ionic strength, and also by their nature. An enzyme can perform differently in two buffer systems, even if these two buffers are the same in pH and overall ionic concentrations. There are multiple reasons which could be responsible for such occurrences. In some cases, a certain kind of metal ions directly influence the enzymatic reaction (For example, heavy metal salt such as Cu^{2+} can denature the enzymes). Finally, because the dissociation of compounds is strongly related to the temperature, the pH changes

with the temperature must be taken into account for accurate pH adjustment in an enzyme assay (Hans, 2014).

2.3.1.1.5 Solvents

It is well accepted that the standard solvent for an enzyme assay is normally physiological balanced water. But in the cases of some enzymes, like those enzymes connected with the membrane (lipases), non-polar organic solvents are required. Most enzymes will be denatured in such solvents. Nevertheless, sometimes an organic solvent cannot be avoided. For example, when the substrate of the target enzyme is barely immiscible in water, it is necessary to use organic solvents such as DMSO, acetone or ethanol. In order to make the organic solvent concentration in the assay as small as possible, the volume of the aliquot should also be small and remain dissolved at its final concentration. To keep the weakly soluble compounds in the assay mixture from precipitation, either a lower concentration of weakly soluble compounds or a relatively higher organic solvent concentration should be applied in an enzyme assay. Moreover, temperature plays a significant role in solubility. Even if the compound is able to be totally dissolved at its assay temperature, it is still possible that the compound precipitates when the assay mixture is stored in cold conditions. Thus, the volume of organic solvent in an enzyme assay must be constant, even if the amount of weakly soluble substances is decreased (Hans, 2014).

2.3.1.2 Practical consideration

2.3.1.2.1 Preparation of the assay mixture

For most enzyme assays, multiple components such as substrates, activators, stabilising

reagents and cofactors are required. It is possible to add each component step by step, and use the final addition step to initiate the reaction of interest. However, such a way of proceeding with an assay has several disadvantages such as wasting time, inaccuracies in final conditions, labour consumption, and errors in pipetting steps. Thus, compared to adding different components separately, it is better to make an assay mixture before proceeding to the enzyme assay itself (Hans, 2014).

In the preparation of the assay mixture, the essential components are initially added to the assay mixture in the form of a concentrated stock solution. Although buffers are normally stable at the room temperature because they consist of inorganic salts, for long term storage, buffers need to be frozen to avoid microbial contamination. Some sensitive substrates (such as NADH) cannot endure the repeated freezing and thawing cycles associated with multiple uses, which means they should be divided into small portions for single-use thaw and discard aliquots. Finally, substrates and all other substance added into the assay mixture must be neutralised before addition if they exist in acidic or alkaline form (Hans, 2014).

A common issue in enzymatic assays is determining the substance to be used for initiating the assay. Due to the limited stability in dilute solution and possible interaction with other components in the assay mixture, enzymes are preferred as the initiating substance. However, sometimes substrates are the preferred initiators if they are not stable in aqueous solutions and must be added immediately before the enzymatic reaction. There are occasions where enzymes need to be preincubated and thereby activated by interaction with cofactors. In such a case, the cofactor, for example, must be used to start the reaction (Hans, 2014).

2.3.1.2.2 Pre-treatment of the enzyme

There are multiple ways to store enzymes: as a crystal suspension, frozen in solution, as a

precipitate, or lyophilised. In an enzyme assay, even if all the conditions are well-set before initiating the assay, various conditions can cause a loss of activity. Poisoning of thiol groups, oxidative processes and degradation by contaminating proteases are notable challenges to assays. Relatively higher temperature conditions also stimulate these processes, so enzyme solutions should be kept cold. To protect enzymes from oxidation, thiol reagents such as dithiothreitol and mercaptoethanol are utilised. To avoid contaminative degradation, high concentration components such as bovine serum albumin (BSA) is added to serve as a competitive protein to be degraded by microbial. Some protease inhibitors like phenylmethanesulfonylfluoride and macroglobulin can also perform the same function (Umezawa, 1976; Sottrup-Jensen, 1989). If the contaminating protease is a metallo-proteases, EDTA can be used as a protease inhibitor due to its ability to trap divalent metal ions. The enzyme stock solution should be freshly prepared instead of stored for longer time (Hans, 2014).

2.3.2 Liquid chromatography–mass spectrometry (LC-MS)

2.3.2.1 High-performance liquid chromatography (HPLC)

Chromatography aims to separate the single compounds of a mixture based on differences in physical characteristic differences, such as molecular size, charge and adsorption properties. The process of chromatography normally consists of a stationary phase, a chromatography bed, mobile phases, a delivery system and a detection system. The separation mechanism of a chromatography system is that the compounds which strongly interact with the stationary phase will be retarded in their movement through the material, while the compound which is not showing a strong interaction with the stationary phase will pass through the matrix with less delay, resulting in differences in elution times (Dean, 2017).

When using a liquid mobile phase, in order to achieve equal diffusion equilibration, the

dimensions for diffusion should be smaller. To promote timely movement of liquids through the narrow pathways which define stationary phase elements, greater pressures, and columns which are able to withstand these pressures, are required. An extremely thin layer of stationary phase is coating the small support particles of stationary phase and encourage retention (or exclusion) of the mobile phase accordingly. Finally, chromatography also requires a detector which can ascertain the analytes from small eluted peak volumes. The characteristics above are realised by creating an instrumental version of liquid chromatography (LC), which is also known as high-performance liquid chromatography (HPLC).

2.3.2.1.1 Mass spectrometry (MS)

Mass spectrometry (MS) is used to transfer ions from liquid-phase molecules into gaseous forms, separates the ions based on mass-to-charge ratio (m/z), and measures the ionic abundance. MS provides multiple functions of analysis for data interpretation, ranging from demonstrating the structure of complicated organic and biomolecules, to the quantification of specific molecules in the tested samples. All MS systems consist of a sample injector, an ionisation source, a mass analyser and a detector (Figure 9).

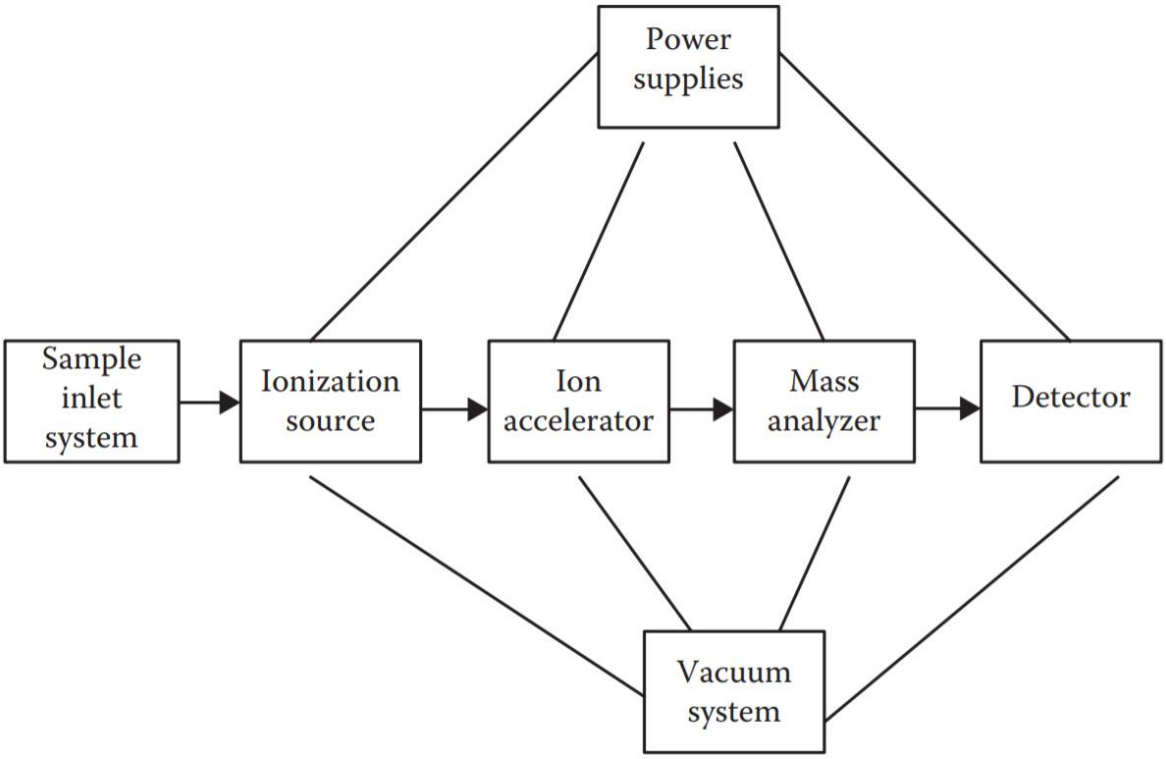

Figure 9 Flow chart of a mass spectrometer.

Ionisation Source

There are various ionisation sources for MS equipment. Electron ionisation sources, chemical ionisation sources, atmospheric pressure ionisation sources, desorption ionisation source are key to the methodology. Some ionisation sources specifically for inorganic MS such as GD sources, spark sources and ICP sources are also used in analytical runs (Robison et al., 2014).

Electron Ionisation (EI) and Chemical Ionisation (CI)

Electron ionisation sources are normally applied in organic gas chromatograph-MS (GC-MS). The sources have a small permanent magnet producing a magnetic field surrounding the electron beam, which leads electrons to follow a spiral path, including the target molecules encountered from test samples, even though only 0.0001% ~ 0.00001% of target molecules and atoms are ionised. As a result of the interaction with the beam, sample molecules are ionised,

becoming smaller pieces of their former structure. The EI source can be applied in both positive and negative mode. Due to the high energy given to the ions through an EI source, the fragmentation of organic molecules is significant. This aspect of the method gives EI sources very high efficiency and sensitivity. Such high energy ionisation sources are also called hard ionisation sources. EI sources demonstrate reproducibility which is also a benefit to the technique's objectives. However, the efficiency is so high that some molecules ions are cracked during the ionisation process, which means they are not easily distinguished from the background noise in the detection system, and the mass of the molecule is not able to be determined. EI sources are also mostly applied in GC-MS.

Contrary to EI sources, CI sources are called soft ionisation sources, which means less fragmentation efficiency and easier mass spectral identification. Such ionisation method produces more abundant molecular ions, so the determination of the molecular weights is more likely.

Atmospheric Pressure Ionisation Sources

There are two major types of atmospheric pressure ionisation sources, electrospray interface (ESI) and atmospheric pressure CI (APCI), which are normally applied in LC-MS. A standard LC-MS system requires a lower temperature for operations because they are applied to connect LC with MS for separation purposes to detect high molecular weight compounds. High molecular weight compounds may stick together and precipitate out of solution when the spray droplets evaporate. Only a small percent of molecules travel on the correct route through the dispersal region of the machinery to get into the collection duct, and only a part of them may enter as separate molecules.

In an ESI, instead of entering the spray chamber by heating the inlet capillary, additional positive or negative charges are achieved by transferring charges to droplets as they emerge from the sample's spray tip, which is electrically charged. A highly dried, heated and purified nitrogen

gas can accelerate the evaporation of the separated samples by focusing the gas at the emerging spray of charged droplets. This technique means that ionisation is completed under the liquid conditions. After samples are separated by HPLC and enter the ionisation source, the target molecules are vaporised by high-purity nitrogen. When these charged droplets shrink quickly, two things occur: Target molecules and solvent molecules bearing the additional charge polarity move to the chromatograph's surface under the effect of mutual repulsion. When these molecules are forced to stay together, conditions force the droplets to become even smaller. These smaller droplets have bigger total surface areas, and are capable of holding more charge. The process repeats at an extremely fast rate, producing huge amounts of smaller charged droplets. With the continuous accumulation of an electric field on the surface of droplets, target ions are expelled, embedding in a cluster of solvent molecules.

2.3.2.2 Methyl chloroformate (MCF) derivatisation

Methyl chloroformate (MCF) derivatisation is an alkylation reaction which consists of the reaction of carboxyl groups and primary and secondary amino groups of the target compounds. Almost 600 metabolites in a genome-wide metabolic model of MCF derivatisation have been documented (Förster et al., 2003), and nearly 40% of these are organic acids, amino acids and amines. These metabolites play important roles in both biosynthesis of amino acids and central carbon metabolism. In MCF derivatisation processes, amino and non-amino organic acids are transformed into volatile esters and carbamates respectively, and then detected by both LC-MS and GC-MS. The MCF compounds are extremely reactive, reacting with water, and producing ethanol and HCl. When alkaline conditions and pyridine are presented to the reaction conditions, target compounds tend to undergo a nucleophilic attack, with the chlorine acting as a leaving residue (Figure10 A). As for amino groups, the methyl carbonate retains its binding affinity to

nitrogen, producing a carbamate group due to loss of a proton (Figure 10 B). When the MCF derivatisation process is over, metabolites receive an addition of 14 mass units for each carbamate group and 58 mass units for each amino group derivatised. A limitation of MCF derivatisation is that it cannot always derivatise secondary amines on the target compounds (Smart et al., 2010).

Figure 10 shows the mechanism of methyl chloroformate derivatising amino acids in the presence of pyridine and methanol. After the direct reaction of methyl chloroformate with the carboxyl group, which produces the methoxycarbonyl residue, the methanol provides the methyl residues to the carboxyl groups of amino acids through an intermediate exchange reaction. Methanol is then formed by the recycled methyl chloroformate. The carboxyl methyl ester residue is usually lost while the compound is fragmented in the MS device, which results in producing a major fragment ion with the mass of $(M-59)+$ (Chen et al., 2003).

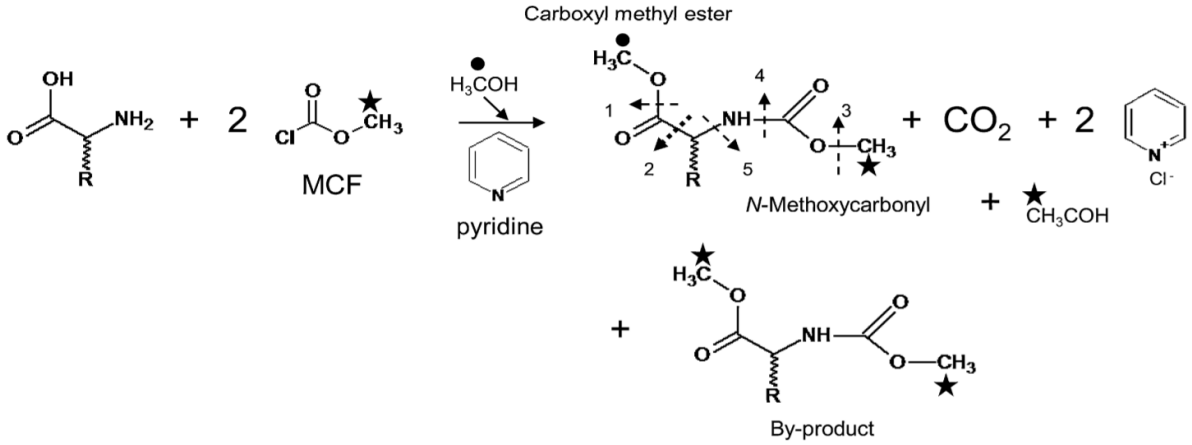

Figure 10. (A) MCF derivatisation of amino acid (Chen et al., 2003).

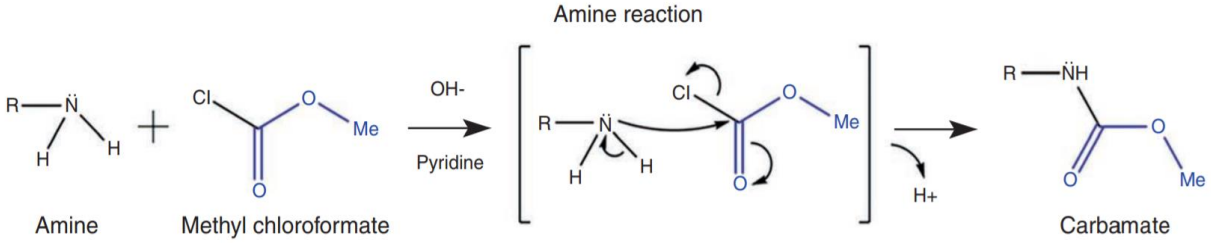

Figure 10 (B) MCF derivatisation of amine group (Smart et al., 2010).

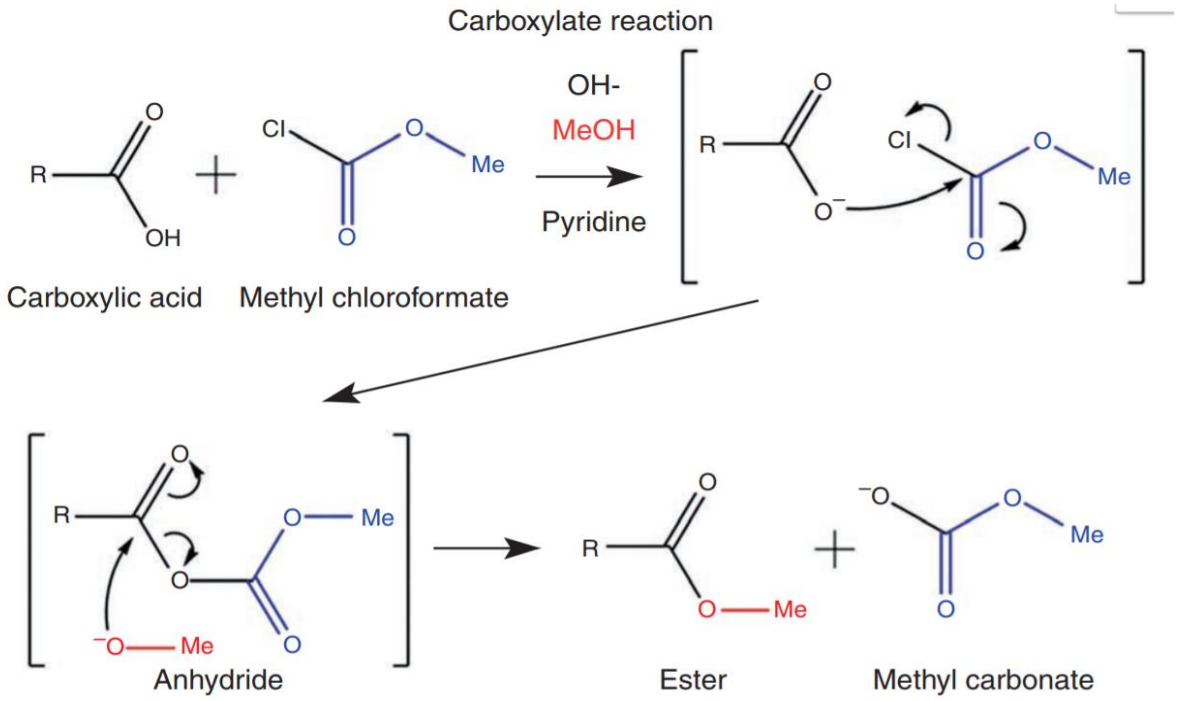

Figure 10 (C) MCF derivatisation of carboxyl group.

2.3.3 Microsomal and cytosolic preparation methods

Microsomes are reformed pieces of endoplasmic reticulum after the cells are broken apart under laboratory conditions. The cytosol is the liquid fraction inside the cells. Microsomal and cytosolic fractions can be separated from S9 fractionation by two different centrifugation methods.

The most commonly applied separation technique is differential centrifugation. For liver tissues, the whole process of centrifugation is typically performed at 600 g for 5 minutes (to remove whole cells, nuclei and debris), and the supernatant is transferred to a new tube. The samples are then spun at 9000 g for 10 minutes (to remove mitochondria), pelleted, with supernatant again transferred to appropriate tube, and ultimately spun at 105,000 g for an hour (to sediment microsomes). Due to the requirement of such high speed (100,500g) centrifugation, such a way is very expensive and not readily available (Hans, 2014).

An additional method for microsome separation is a Ca^{2+} sedimentation method. The principle underlying the Ca^{2+} sedimentation method is to precipitate microsomes at a lower centrifugation speed by the addition of calcium chloride ($CaCl_2$). It is more convenient because it only requires speeds of 30,000g to precipitate microsomes. The whole process begins with a 12,000g centrifugation step, removal of the supernatant to a new tube, and addition of $CaCl_2$ until the supernatant reaches 8mM. A final spin at 30,000g is performed to sediment microsomes. The sedimentation process can be repeated multiple times to get purer products. Although the Ca^{2+} sedimentation method is cheap and convenient, the method has not been accepted as a formal routine. In this research, the Ca^{2+} sedimentation method was applied to prepare microsomes. Additionally, the supernatant above the microsomal pellet was collected as liver supernatant, and is deemed the cytosolic fraction.

Chapter 3 Methodology

3.1 General materials and instruments

3.1.1 Materials

3.1.1.1 Rat and mouse livers

Liver tissues are from healthy SD rats and C57BL/6 mice, all animals were obtained from the Vernon Jansen Unit, University of Auckland, New Zealand. Healthy rat and mouse liver tissues were collected post-mortem from alternative studies' populations, therefore no ethical approvals were necessary.

3.1.1.2 Polyamines and other chemicals

1) Polyamines and their metabolites: Triethylenetetramine (TETA) dihydrochloride (CAS Number 38260-01-4, from Sigma Aldrich); N^1-Acetyl Triethylenetetramine Trihydrochloride (MAT) (CAS Number: 1429850-43-0, from Toronto Research Chemicals); Spermidine (Spd) trihydrochloride (CAS Number: 334-50-9, from Sigma Aldrich); N^8-acetylspermidine (AcSpd) dihydrochloride (CAS Number: 34450-15-2, from Sigma Aldrich); Spermidine-d6 (Spd-D6) Trihydrochloride (CAS Number: 124-20-9, from Toronto Research Chemicals), N^1-Acetylspermidine-d6 (AcSpd-D6) Dihydrochloride (CAS Number: 34450-16-3, from Toronto Research Chemicals).
2) *In vitro* acetylation: Acetyl coenzyme A (AcCoA) lithium salt (CAS Number: 32140-51-5, from Sigma Aldrich); Hydrochloric acid (provided by Auckland University of Technology).
3) BSA protein concentration determination: Bovine serum albumin (BSA) (Cat # SH30574D2,

from Thermo Fisher Scientific); Quick Start ™ Bradford 1x Dye Reagent (Cat # 500-0205, from BIO-RAD).

4) Microsome and cytosol preparation: Phosphate buffered saline tablet (MFCD00131855, from Sigma Aldrich); Calcium chloride ($CaCl_2$) (provided by Auckland University of Technology); Glycerol (provided by Auckland University of Technology).
5) Derivatisation: Pyridine (Provided by Auckland University of Technology); Methanol (Lot# 164296, from Optima Chemical); Methyl chloroformate (MCF) (Index-No: 607-019-00-9, from Merck KGaA); Sodium hydroxide (NaOH) (Provided by Auckland University of Technology); Formic acid (CAS Number: 64-18-6, from Fisher Chemical).
6) LC-MS: Acetic acid (Lot BCBS3388V, from Honeywell Fluka™); Acetonitrile (CAS Number: 75-05-8, from Scharlau Chemicals); Ammonia (Provided by Auckland University of Technology).

3.1.1.3 Instrument and materials

1) BSA protein concentration determination: Thermo Fisher Scientific Multiskan GO microplate spectrophotometer (REF 51119200); 96-well plates.
2) Microsome and cytosol preparation: Thermo Scientific Sorvall WX+ Ultracentrifuge (Cat # 46900); Thermo Scientific Fiberlite F50L-8x39 Rotor (Cat # 096-087051).
3) LC-MS: Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA), connected with an Agilent 6420 triple quadrupole mass spectrometer with multimode ionisation source (model G1978B); Vial screw 0.7mL PP Micro-Vial transparent (THC11191706) and 8mm screw thread cap, for 0.7mL PP Micro-Vial, black (THC08150293); XSELECT™ CSH™ C18 3.5uM 2,1x100mm column.

3.1.1.4 Software

1) The LC-MS qualitative software is Agilent MassHunter Qualitative Analysis B.07.00; The LC-MS quantitative software is Agilent MassHunter Quantitative Analysis B.07.01SP1.
2) The statistical software applied in this research is Microsoft® Excel® (16.0.11231.20164) and RStudio (1.0.136).

3.2 Tissue preparation

Healthy rat liver microsome and cytosol fractions were prepared from liver tissues of healthy SD rats and C57BL/6 mice. The rat tissues were previously frozen and stored at -80°C. The method for preparing liver microsome and cytosol in this study is slightly modified from an existing differential centrifugation method (Gill et al., 1995). Mouse (and rat) liver tissues are initially weighted, thawed, sliced and washed with ice-cold 1 x PBS. Livers are then immersed in 1 x PBS with 1.15% potassium chloride (KCl), and homogenised by using a glass cell homogeniser. The homogenisation process is repeated for three times to ensure that the mouse (and rat) livers are totally homogenised. In addition, the whole homogenisation process is operated on ice to minimise the activity loss of targeted enzymes (SSAT). A glass cell homogeniser is applied because it has a better homogenisation result, and produces no heat, which decreases the potential risk of denaturing the targeted enzymes. Mouse (and rat) liver homogenates are equally divided and transferred into two 10-mL ultracentrifuge tubes. Then, the ultracentrifuge tubes with liver homogenates are balanced and centrifuged at 12,500 g for 30 min in a Thermo Scientific Sorvall WX+ Ultracentrifuge and Thermo Scientific Fiberlite F50L-8x39 Rotor. This step aims to remove whole cells, nuclei, debris and mitochondria. It is also preferred to use a TissueLyser II (QIAGEN) to shake the samples at -30 °C before the centrifugation is carried out in order to achieve a better separation result. After the first

centrifugation, the sediment of the liver homogenate is discarded and the supernatant is collected. The supernatant collected in this step is defined as liver S9 fraction, a mixture of liver microsome and cytosol. The liver S9 fraction is then centrifuged at 105,000 g in the same ultracentrifuge and rotor for an hour. After this step, both supernatant and sediment are collected. The sediment of liver preparations is defined as the liver microsome, and the supernatant is defined as the liver cytosol. The liver cytosol is directly collected in a 10-mL centrifuge tube while the liver microsome is resuspended with 1 x PBS first. Mouse (and rat) liver microsomes and cytosol fractions are finally separated into multiple Eppendorf tubes, sealed and stored at -80 °C fridge to avoid activity loss due to the frequent freeze-thaw cycling of a single liver preparation.

Another method of preparing liver microsomes is also tried in this research. The rat liver tissues are initially sliced and washed in ice-cold 1x PBS to remove blood contaminants. Then, the rat liver tissues are immersed in 1.15% KCl 1 x PBS, in a volume equivalent to 3x the sample weight, preparatory for homogenisation. The homogenisation process proceeded in an ice-bath to keep the activity of enzymes from being influenced by the heat produced by the homogeniser (IKA Ultra-Turrax T25 SOP). The resulting liver homogenates are separated into several round-bottom ultracentrifuge tubes and centrifuged at 12,000 g to remove cells, nuclei, debris and mitochondria. Sediments are removed and 88mM $CaCl_2$ stock solution is added to the supernatant until the concentration reached 8mM. The addition of $CaCl_2$ aims to increase the molecular weight of proteins in microsome to make it easier to be separated by a relatively lower centrifuge speed. The supernatant with calcium is centrifuged at 30,000 g for 20 minutes. The supernatant and remaining pellet are both collected as cytosol and microsome fractions respectively. The 30,000 g centrifugation is repeated for several times until the supernatant no longer produce any sediment during the process. Microsomal products are resuspended in 1 mL 20% glycerol. Microsome and cytosol products are both stored at -80 °C fridge.

3.3 Bradford protein concentration determination

The protein concentrations of rat (and mouse) liver microsomes and cytosol samples were determined by Quick Start™ Bradford Protein Assay (BIO-RAD). Bovine serum albumin (BSA) standards was serial diluted (0 ~ 1,000 μg/mL) to set up a standard curve (Table 1). In addition, microsome and cytosol samples were diluted 100 and 1000 times to fit the standard curve. After BSA standard solutions and samples were placed on the microplate (5uL each well), 250 μL of 1x Dye Reagent was added. The microplate was incubated at room temperature for at least 5 min, the spectrophotometer was set to 595 nm, and the absorbance was measured.

Table 1 Dilution plan of BSA standard curve.

Dilution plan of BSA		
Number	Plan	Concentration (prepared)
a	0.2 mg BSA + 200 μL water	1,000 ug/mL
b	80ul a + 20 μL water	800 ug/mL
c	75ul b + 25 μL water	600 ug/mL
d	67ul c + 33 μL water	400 ug/mL
e	50ul d + 50 μL water	200 ug/mL
f	50ul e + 50 μL water	100 ug/mL
g	Water	0 ug/mL

3.4 *In vitro* polyamine acetylation

Almost all *in vitro* polyamine acetylation reactions have a similar method that includes four major procedures: 1) preparing the samples. 2) incubation (polyamine enzymatic reaction) 3) ceasing the reaction, and 4) LC-MS detection.

In the sample preparation step, polyamines are diluted with 0.5 x PBS to make an assay mixture. The pH value of the assay mixture was adjusted due to the fact that most of the polyamine standards are in acid form, and the buffer applied here is not able to keep the pH value constantly

within the physiological range. Due to the phosphate ions potential to cause blockage within the system, 0.5 x PBS is selected instead of 1 x PBS. Mouse (and rat) liver microsome or cytosol samples were mixed with the neutral assay mixture acting in the role of enzymes. The total volume of reaction mixture in this enzyme assay is 280 μL. It is important to calculate the concentration of polyamines and enzymes in the assay. The concentrations of substrates are: 500 μM, 400 μM, 300 μM, 200 μM, 100 μM, 50 μM and 25 μM. During the incubation process, the reaction mixtures are kept in Eppendorf tubes. AcCoA was used to initiate the reaction, and the final concentration of AcCoA was 167 μM. AcCoA was chosen to be the reaction initiator in that polyamines react with AcCoA spontaneously, and additionally, enzymes of interest won't perform any activity when the AcCoA is absent. After the acetylation reaction is activated, the Eppendorf tubes were immediately kept in a 37 °C water bath for 30 mins. The reaction mixtures are slightly vortexed to make sure all the components are well mixed. During the incubation, a slight agitation is preferred. Before the initiation of the acetylation reaction, a ten-minute preincubation was performed to encourage higher enzyme activity. After 30 mins, 100 μL of 37% HCl was added to terminate the reaction.

Following termination of the reaction mixtures, samples were centrifuged at 21,000g to remove denatured proteins and purify the samples to make sure they are sufficiently purified in order to be injected into the LC-MS for analysis.

3.5 LC-MS detection

3.5.1 MCF derivatisation

After centrifugation, the samples were derivatised before LC-MS detection. The protocol of MCF derivatisation for each sample in this research is as follows: Add 50 μL sample; 12 μL 10 mg/L HDA as internal standard; 27 μL of 10 M NaOH to provide alkaline environment; 100 μL

methanol and pyridine mixture (4:1); 20 μL MCF together, and then vortex the solution immediately (to avoid MCF completely reacting with water). Finally, 400 μL 2% formic acid was used to neutralise the samples. When making the polyamine standard curve, the amount of NaOH will be slightly decreased (20 μL) because the polyamine standards do not have as much acid present as the samples do (samples contain a higher concentration of HCl since the acid is used as the reaction terminator). The derivatisation is performed directly in the 0.7 mL PP Micro-Vials of the preparation steps.

3.5.2 Method development and Single polyamine detection

The LC-MS method development steps of this study contain a mixed standard which consists of TETA, MAT, Spd, AcSpd, Spd-D6, AcSpd-D6 and HDA (the internal standard). The concentration of polyamines is 8 μM, and the concentration of HDA is 1 μM. The mixed standard is then derivatised with MCF by the protocol described previously.

There are two purposes accomplished by polyamination: 1) identification of the m/z of fragments from the MCF-polyamines and subsequent determination of the highest response fragments, and 2) change in the retention time of each compound by adjusting the mobile phase components and gradient, which makes the peaks of these compounds better visualised in the final analysis, and not overlapping with each other.

3.5.3 Standard curve

LC-MS is only able to measure the absolute counts of the polyamine metabolites in the samples. To calculate the concentration of these polyamine metabolites, the counts must be compared to the standards of known amounts. The protocol of the standard curve is as follows: Add Spd 12 μL, AcSpd 15 μL, TETA 12 μL, MAT 15 μL, Spd-D6 12 μL and AcSpd 12 μL in a

0.7 mL PP Micro-Vial to make a polyamine standard mixture. The concentration of all these standards is 100 mg/L. The standard mixture is then serial diluted 10 times by mouse (or rat) liver microsome (or cytosol) blank, or water, which forms the serial concentrations of 8 μM, 4 μM, 2 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, 0.0625 μM, 0.0313 μM, 0.0156 μM and 0.0078 μM. After serial dilutions are completed, 12 μL 10 mg/L HDA aliquots are added in each 0.7 mL PP Micro-Vial as an internal standard. The purpose of using both liver tissue preparation blank and water to serve as the solvent of the standards is to find out if liver microsome and cytosol will influence the result of LC-MS detection by comparing the standard curves with different bases. Finally, the standard mixtures are derivatised by MCF and ready to be injected into LC-MS. In addition, the R^2 of these standard curves represents the validation of the LC-MS method.

3.5.4 Detection of *in vitro* polyamine acetylation

The LC-MS/MS system used in this research was an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA), connected with an Agilent 6420 triple quadrupole mass spectrometer with multimode ionisation source (model G1978B). The column is XSELECT™ CSH™ C18 3.5 μM 2.1x100mm column. This research also used a Luna 5μ CN 100A 150 x 3.00 mm column. The injection vial is a 0.7 mL PP Micro-Vial with an 8 mm screw thread black cap.

Mobile phase A was 0.1% formic and 10 mM ammonia in MilliQ water. Mobile phase B was 0.1% acetic acid with 10 mM ammonia in acetonitrile. The total run of one sample is 11 min. The gradient of mobile phase composition was 0.01 ~ 3.50 min, A: 90%, B: 10%; 3.50 ~ 4.20 min, A: 70%, B: 30%; 4.20 ~ 5.70 min, A: 20%, B: 80%; 5.70 ~ 11.00 min, A: 90%, B: 10%.

A multiple reaction monitoring (MRM) method was optimised by using Agilent MassHunter Acquisition software. MS-positive MRM was set at m/z values of 379.19 – 171, 363.19 – 257.1, 326.2 – 262.2, 320.2 – 256.1, 310.2 – 278.2, 304.2 – 272.2 and 233.2 – 200.5 Da, which represent

the MCF derivatives of TETA, MAT, Spd-D6, Spd, AcSpd-D6, AcSpd and HDA respectively.

Other LC-MS conditions include flow rate (0.04 mL/min), pressure limit (4.00 ~ 400.00 bar), draw speed (200 µL/min), ejection speed (200 µL/min), injection volume (5.00 µL), maximum flow gradient (4.000 mL/min^2), gas temperature (325 °C), APCI Heater (200 °C), gas flow (6 l/min), nebulizer (60 psi), capillary voltage (1800 V,) column temperature (25.00 °C.)

3.5.5 Additional LC-MS method

In this research, another additional LC-MS method was also utilised in order to assess acetylation methods and detections of sample fragments. Mobile phase A was 0.2% formic acid and 10mM ammonia in MilliQ water. Mobile phase B was 0.1% formic acid in acetonitrile. The column was a Luna 5µ CN 100A, size 150 x 3.00 mm. Selected ion Monitoring (SIM) was optimised by using Agilent MassHunter Acquisition software. MS-positive SIM was set at m/z values of 189.2, 146.2, 259.3 and 147.2 Da. Other MS conditions were: gas temperature 325 °C, APCI heater 200, gas flow (l/min) 6, flow rate 0.400 mL/min, pressure limit 5 ~ 350 bar, polarity positive. The total run time of each sample is 3.5 min.

The *in vitro* acetylation process of this method had some differences compared to the primary descriptions of the study. In the original method, HFBA is used to terminate the reaction instead of HCl. Unfortunately, HFBA and other ion-pairing reagents used in sample preparations were not able to run as part of the mobile phase in the study's LC-MS instrument. Samples were therefore vaporised by air to totally remove the HFBA, and then were resuspended with MilliQ water. DES was added as an internal standard immediately before the samples were injected. The samples were directly injected into the LC-MS without any derivatisation required.

Chapter 4 Results

4.1 Protein concentration determination

The relationship between BSA concentrations and OD values is presented in Figure 11. This standard curve is made for the protein concentration determinations of mouse liver preparations. The solvent of BSA solutions was water. The range of BSA concentration was 0 ~ 1000 μg/mL. The concentration range was based on the Quick Start™ Bradford Protein Assay (BIO-RAD) protocol. As figure 11 illustrates, the OD value of samples are all in the range of the standard curve, which means the results are reasonable. There were other standard curves specifically made for the protein concentration determinations of rat liver preparations and Ca^{2+} rat liver preparations. The relationships and R^2 are $y = 0.0006x + 0.4573$ and $y = 0.0007x + 0.3389$, 0.9617 and 0.9709 respectively.

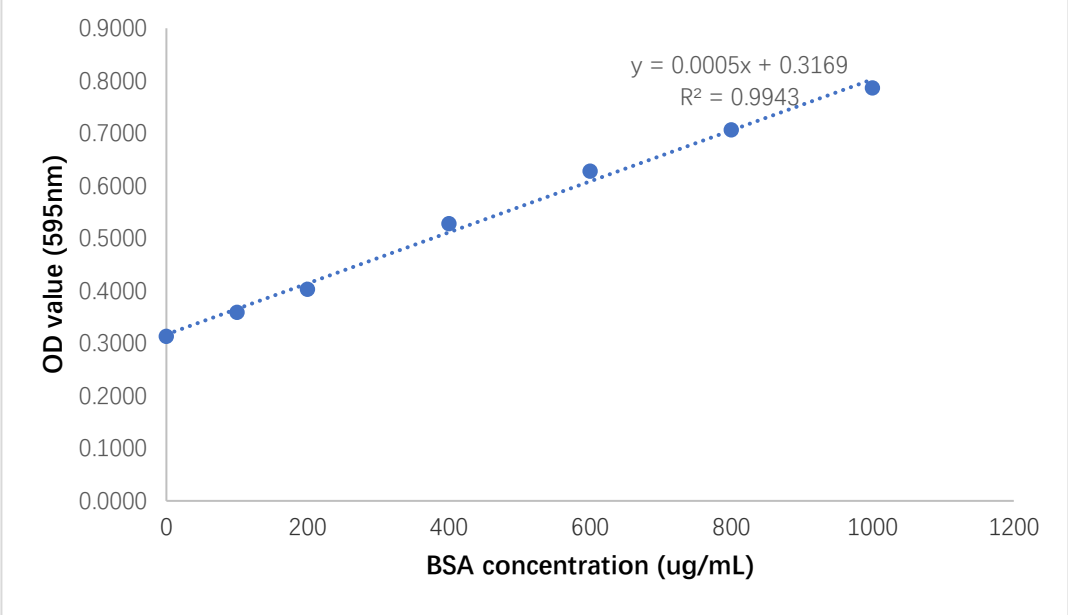

Figure 11. BSA protein concentration standard curve for mouse liver preparation (n = 3).

Table 2 shows the protein concentrations of six different liver preparations: mouse liver microsome, mouse liver cytosol, rat liver microsome and mouse liver cytosol: 31.0 mg/mL, 68.8 mg/mL, 58.3 mg/mL and 77.6 mg/mL respectively. The concentrations of the rat liver microsome and cytosol prepared by the Ca^{2+} precipitation method is also included: 31.2 mg/mL and 20.1 mg/mL respectively. As the table shows, the protein concentrations of liver cytosol are usually higher than liver microsome when the preparation method is differential centrifugation method, wherein the liver homogenates are processed at 105,000g for one hour. However, when it comes to the Ca^{2+} precipitate method, the situation is the opposite despite processing samples through exactly the same dilution procedure). It may suggest that Ca^{2+} precipitation has a better protein sedimentation ability, but the influences of Ca^{2+} towards the targeted enzymes need to be further discussed.

Table 2. Protein concentration of liver preparations

	Mouse liver microsome	Mouse liver cytosol	Rat liver microsome	Rat liver cytosol	Ca^{2+} rat liver microsome	Ca^{2+} rat liver cytosol
OD value (595nm)	0.3324	0.3513	0.8069	0.9228	0.5575	0.4796
Protein concentration	31.0 mg/mL	68.8 mg/mL	58.3 mg/mL	77.6 mg/mL	31.2 mg/mL	20.1 mg/mL

The appearances of these liver microsome and cytosol samples are markedly dissimilar. Mouse and rat liver cytosols are red and transparent, while mouse and rat liver microsomes are relatively sticky and have a pink colour (even though the protein concentration of the microsome is actually lower). It should be mentioned that the red colour of cytosol may be the result of the blood remaining in the liver tissues. It could also slightly influence the result of protein concentration determination.

4.2 Detection of seven polyamine standards

8 μM MCF-derivatised polyamine standards, including TETA, MAT, Spd, AcSpd, Spd-D6, AcSpd-D6 and HDA are presented in the following figures. Figures are generated and exported from Agilent MassHunter Qualification Analysis B.07.00 software. They show obvious peaks at the m/z of 379.19 – 171, 363.19 – 257.1, 320.2 – 256.1, 304.2 –272.2, 326.2 – 262.2, 310.2 – 278.2 and 233.2 – 200.5. The retention time of these polyamines are 3.248 min, 4.500 min, 6.274 min, 5.095 min, 6.233 min, 5.073 min and 6.378 min respectively, and a range of ± 1 min is added for the better polyamine quantification. Among these seven polyamines, TETA, Spd and Spd-D6 could be transformed to MAT, AcSpd and AcSpd-D6 respectively. Spd-D6 is the isotope of Spd (the same relationship between AcSpd and AcSpd-D6), and HDA is used as the internal standard. The retention times of isotopes and internal standards are not well separated, which is not a desired event. However, by using MRM, peaks are still able to be precisely distinguished.

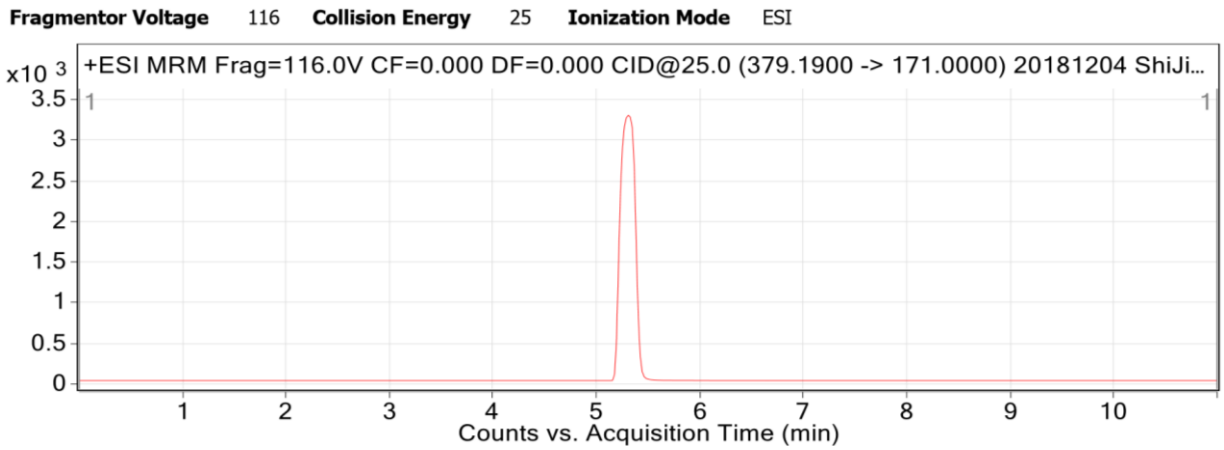

Figure 12 (A) MCF – TETA 8 μM.

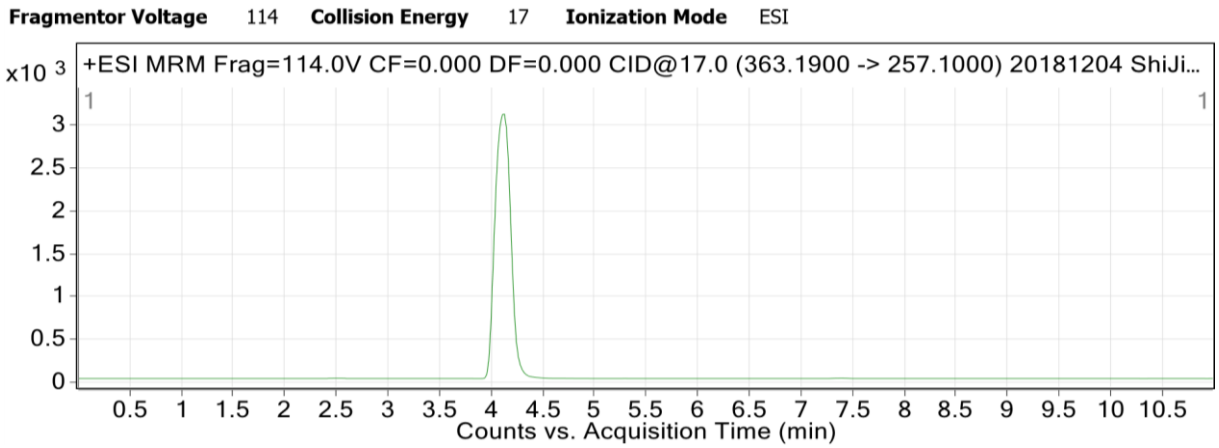

Figure 12 (B) MCF – MAT 8 µM.

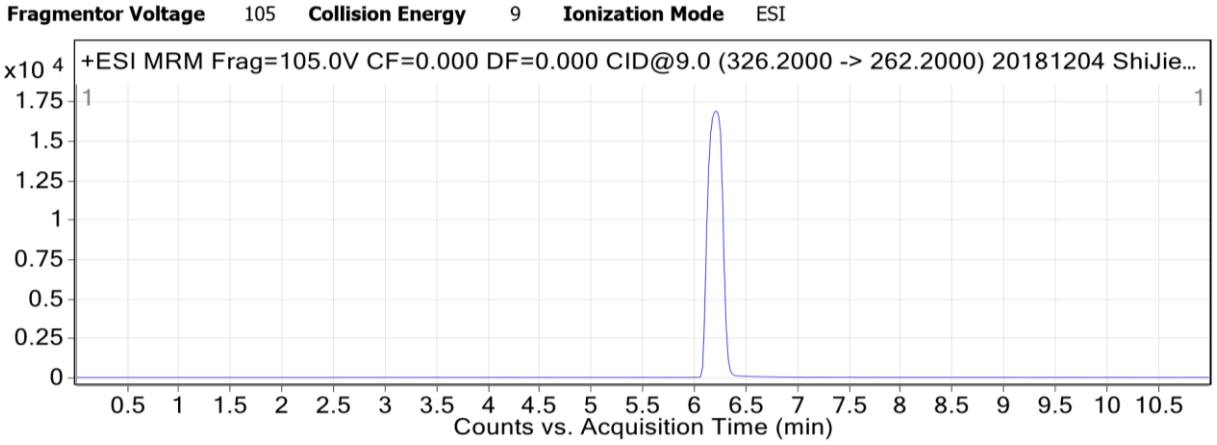

Figure 12 (C) MCF – Spd-D6 8 µM.

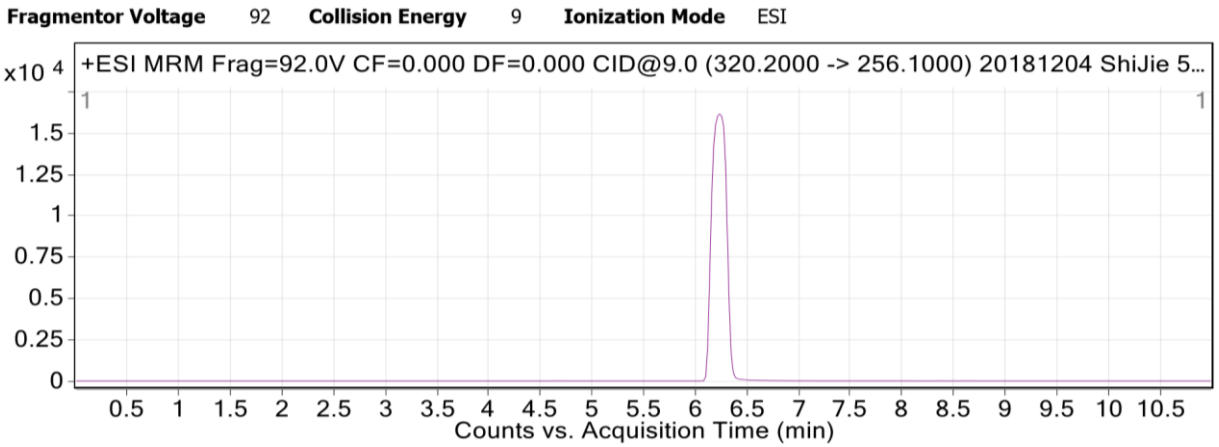

Figure 12 (D) MCF – Spd 8 µM.

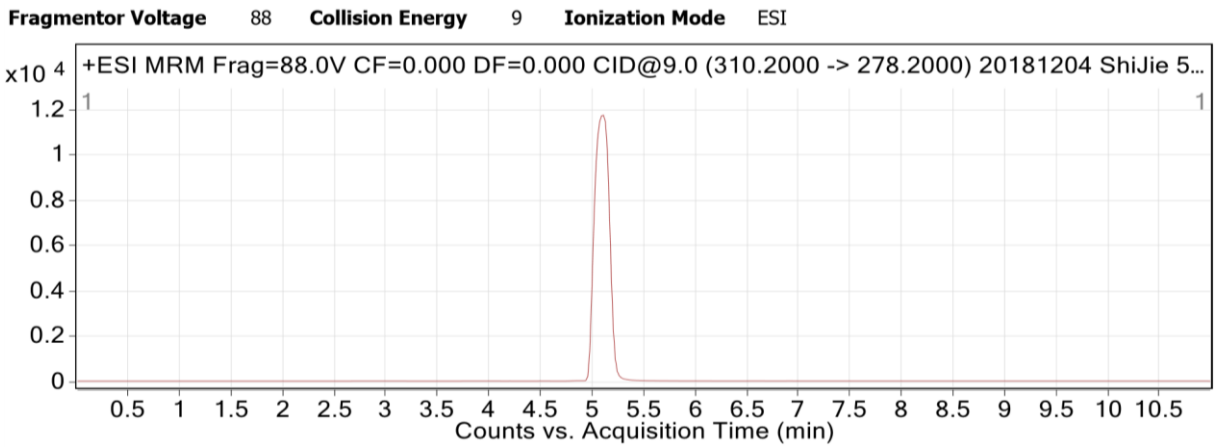

Figure 12 (E) MCF – AcSpd-D6 8uM.

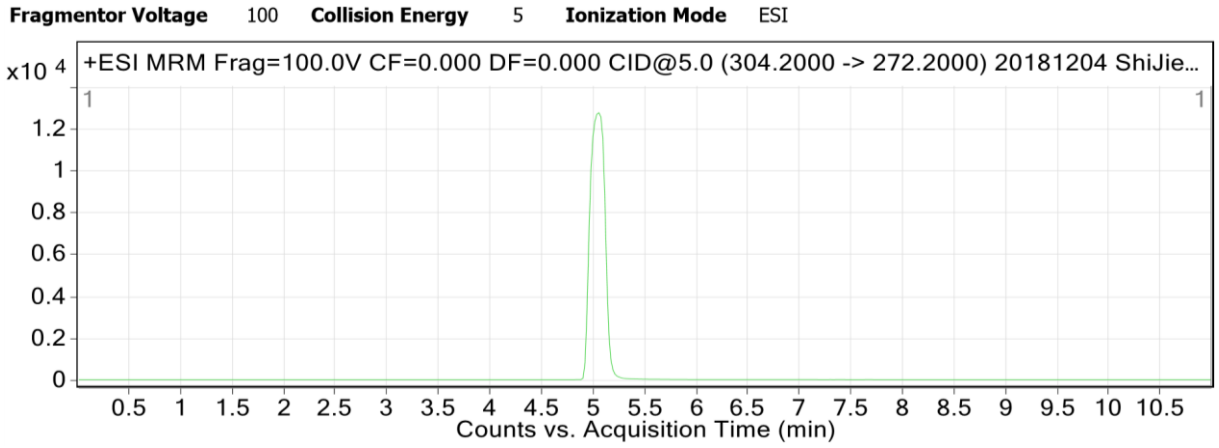

Figure 12 (F) MCF – AcSpd 8uM.

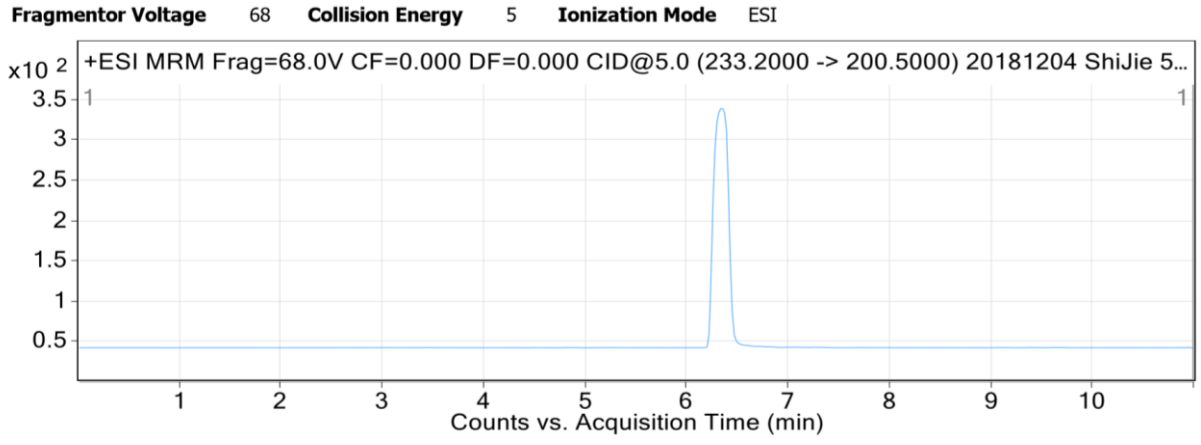

Figure 12 (G) MCF – HDA 2uM.

4.3 Comparison of polyamine standard curves in different substances

4.3.1 MAT standard curves

Figure 13 (A), (B), (C), (D) and (E) demonstrate the standard curves of MAT in mouse liver microsome, mouse liver cytosol, rat liver microsome, rat liver cytosol and water. The intercepts of all standard curves are set at 0 in order to better quantify the metabolites at lower concentrations. The standard curves of MAT in different substances show some differences. The great R^2 (0.9983, 0.9993, 0.997, 0.999 and 0.9888 respectively) also suggest the good validation of the LC-MS assay.

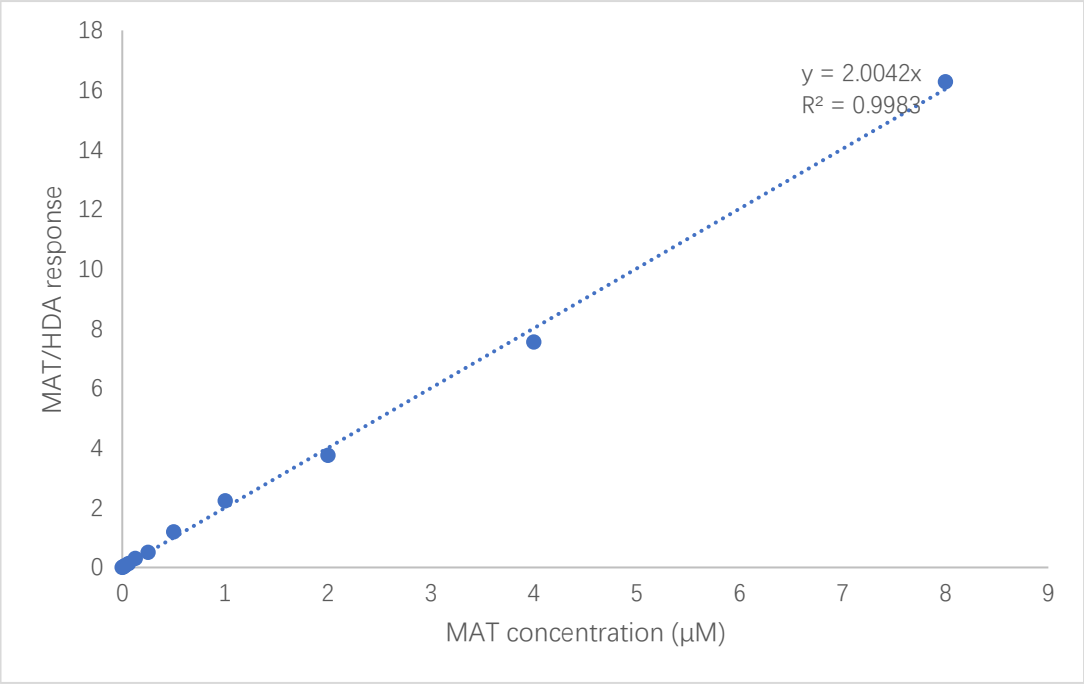

Figure 13 (A) MCF – MAT standard curve with mouse liver microsome.

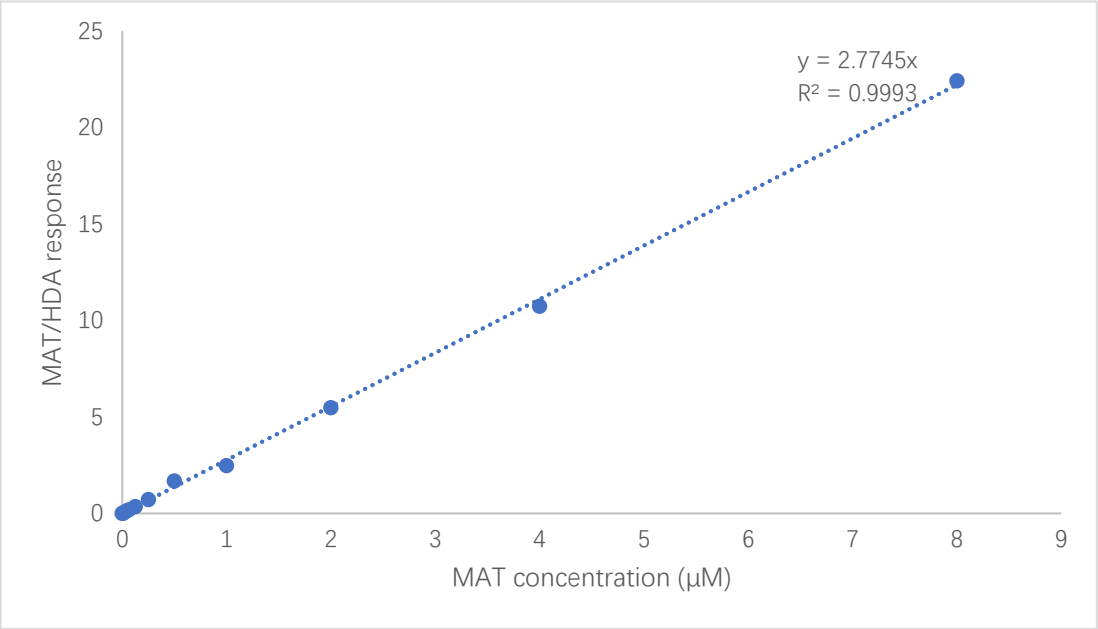

Figure 13 (B) MCF – MAT standard curve with mouse liver cytosol.

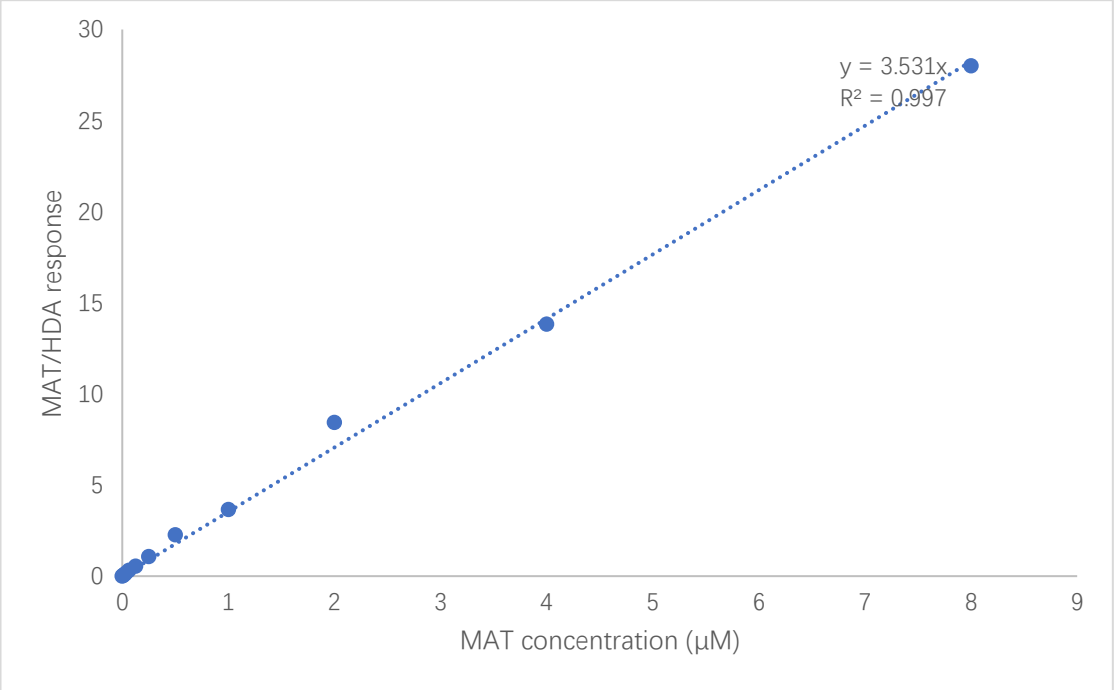

Figure 13 (C) MCF – MAT standard curve with rat liver microsome.

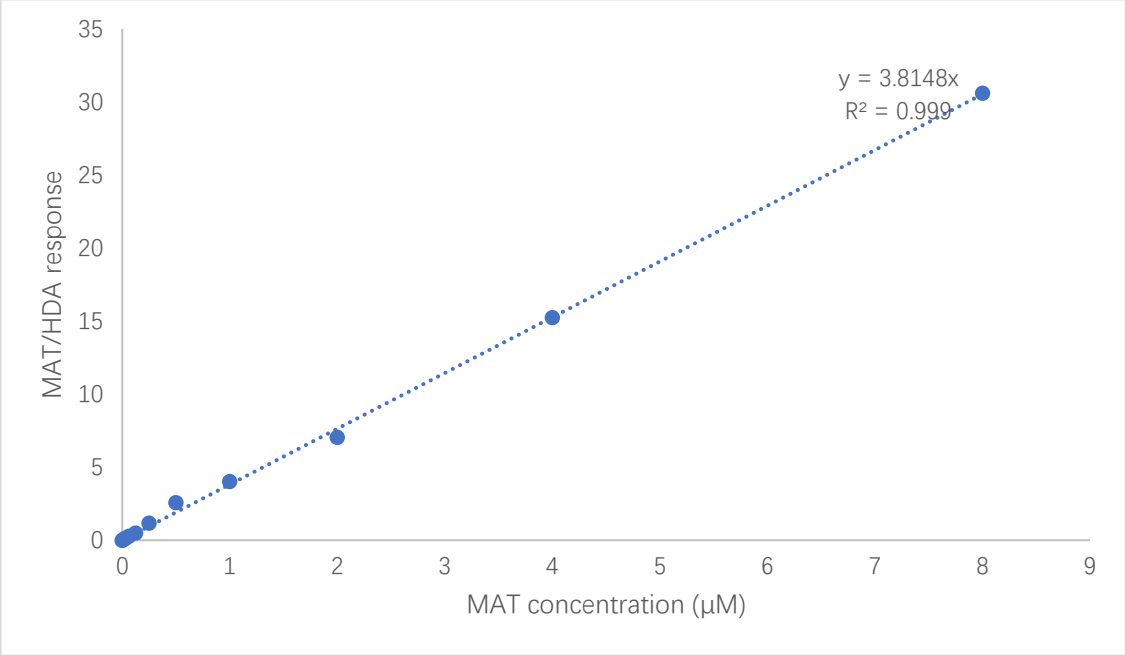

Figure 13 (D) MCF – MAT standard curve with rat liver cytosol.

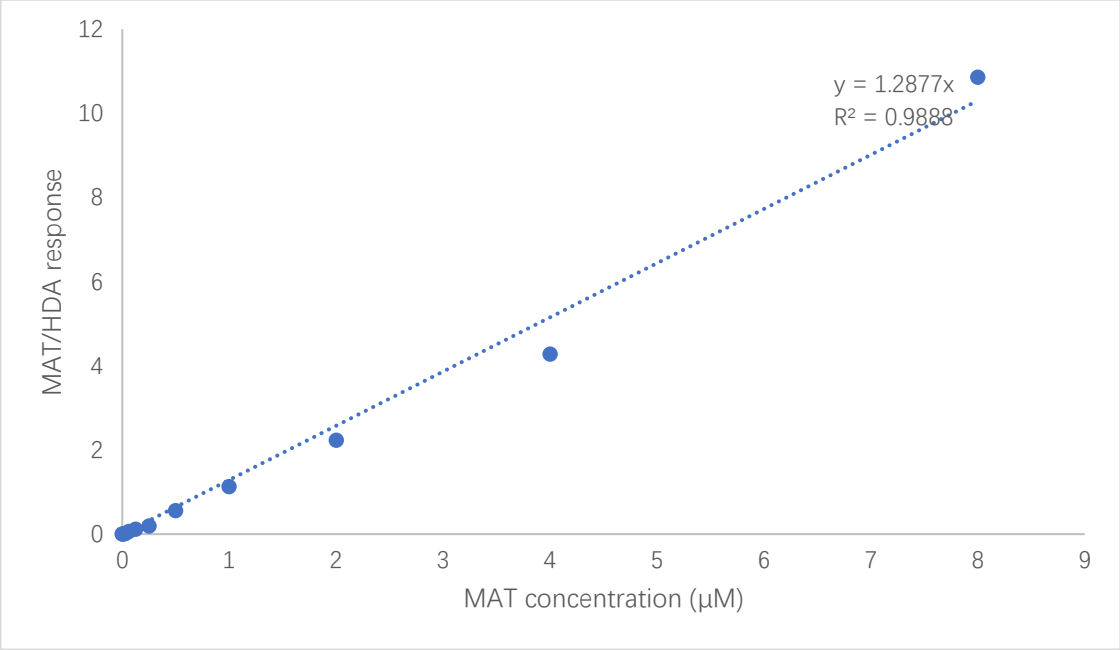

Figure 13 (E) MCF – MAT standard curve in water.

4.3.2 AcSpd standard curves

Figure 14 (A), (B), (C), (D) and (E) demonstrate the standard curves of AcSpd in mouse liver microsome, mouse liver cytosol, rat liver microsome, rat liver cytosol and water. The great R^2 (0.999, 0.9973, 0.9876, 0.9959 and 0.9991 respectively) also suggest the good

validation of the LC-MS assay.

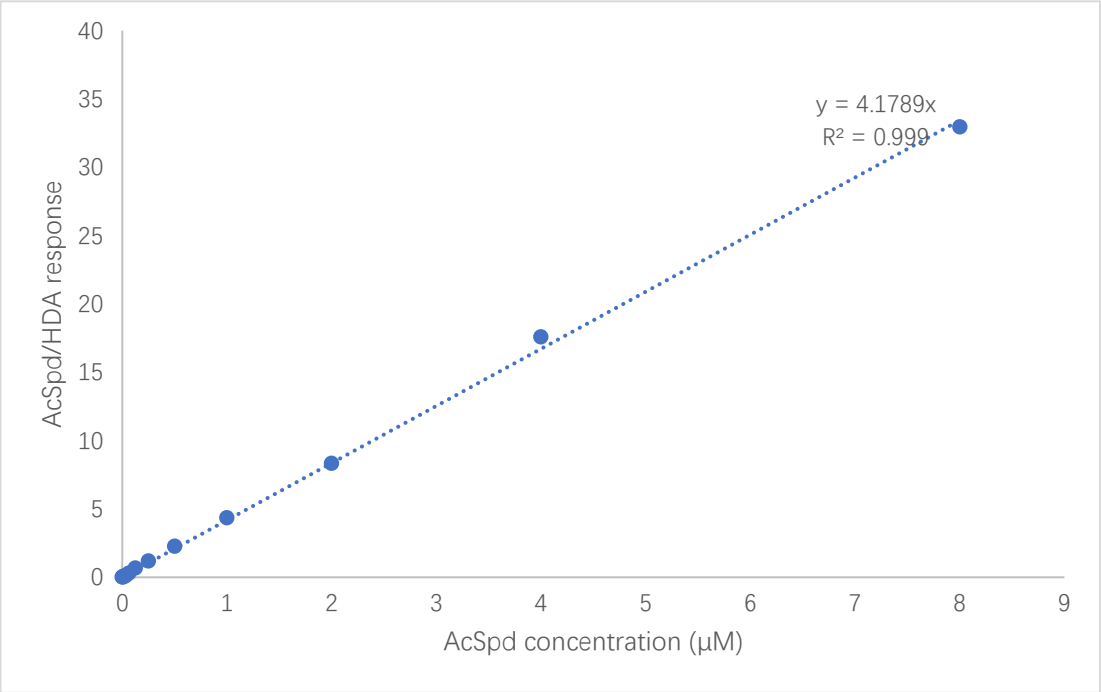

Figure 14 (A) MCF – AcSpd standard curve with mouse liver microsome.

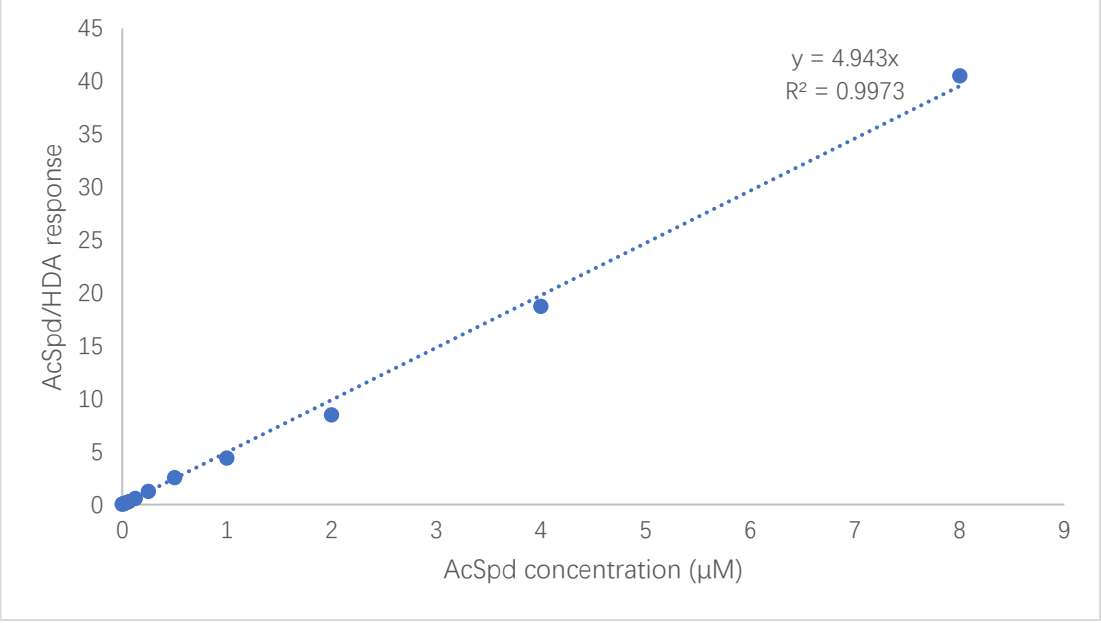

Figure 14 (B) MCF – AcSpd standard curve with mouse liver cytosol.

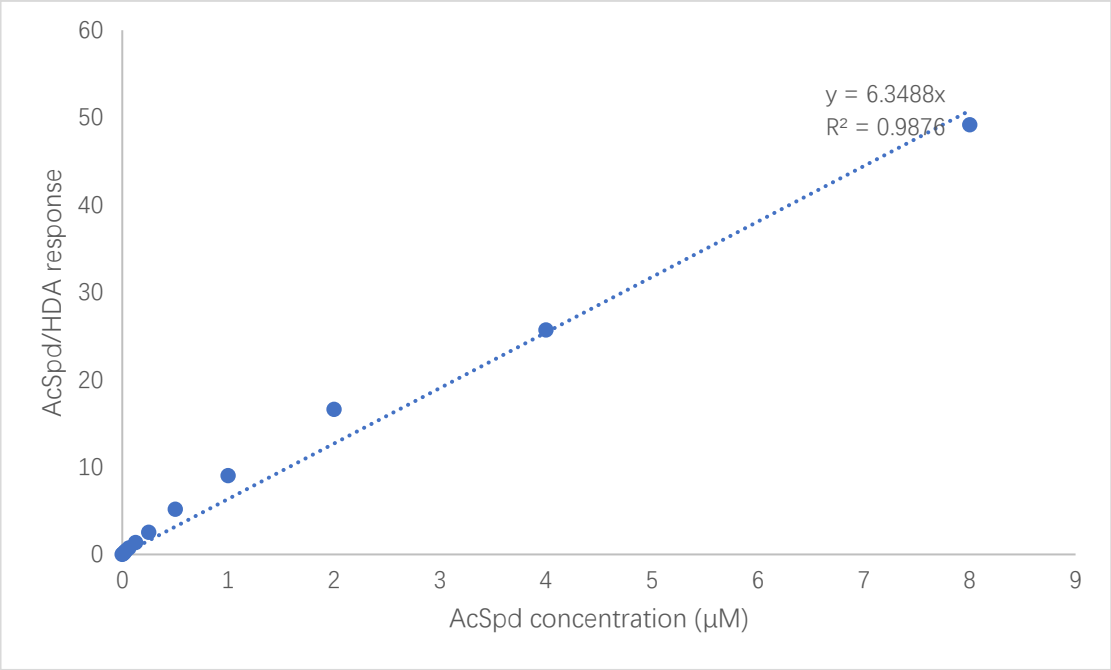

Figure 14 (C) MCF – AcSpd standard curve with rat liver microsome.

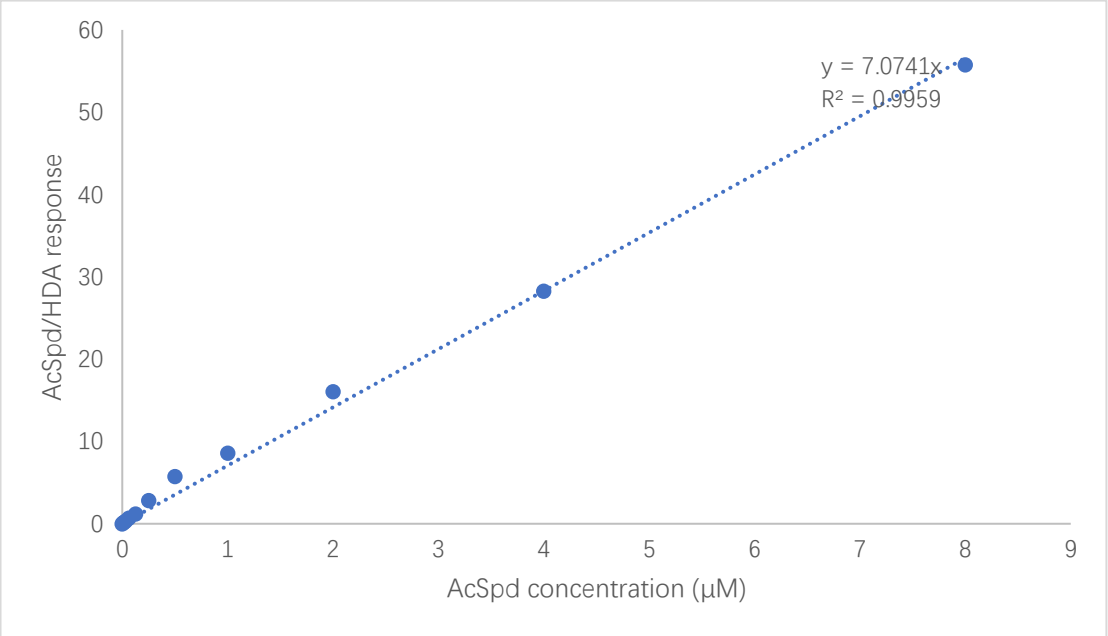

Figure 14 (D) MCF – AcSpd standard curve with rat liver cytosol.

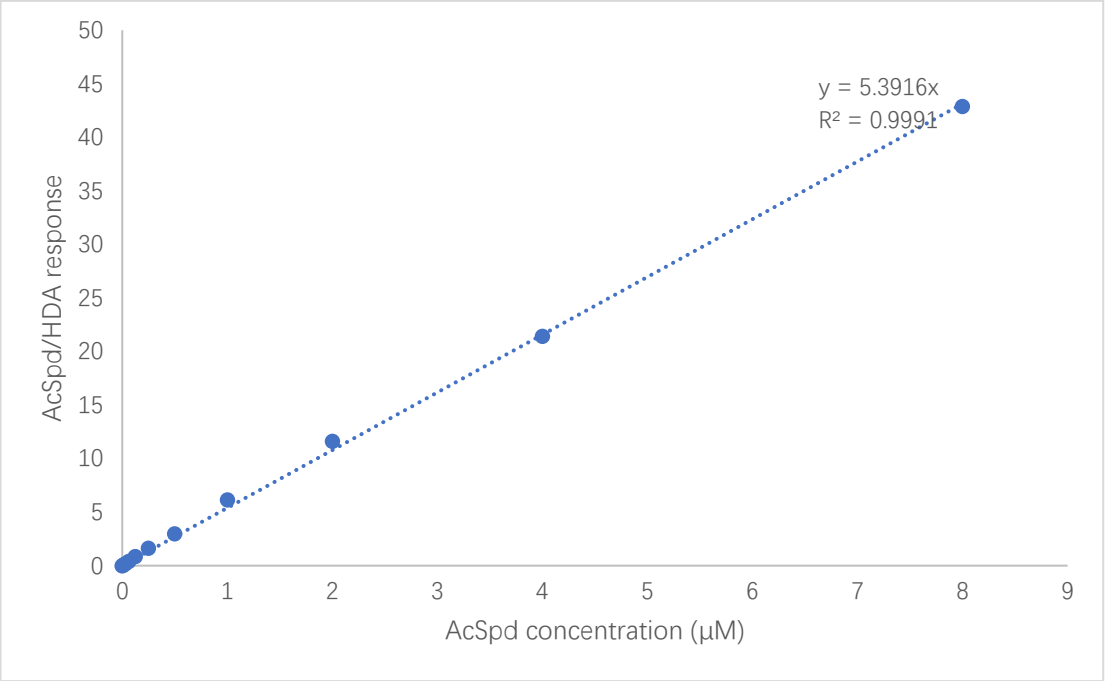

Figure 14 (E) MCF – AcSpd standard curve in water.

4.3.3 AcSpd-D6 standard curves

Figure 15 (A), (B), (C), (D) and (E) demonstrate the standard curves of AcSpd-D6 in mouse liver microsome, mouse liver cytosol, rat liver microsome, rat liver cytosol and water. The great R^2 (0.9982, 0.9977, 0.9823, 0.9937 and 0.9988 respectively) also suggest the good validation of the LC-MS assay.

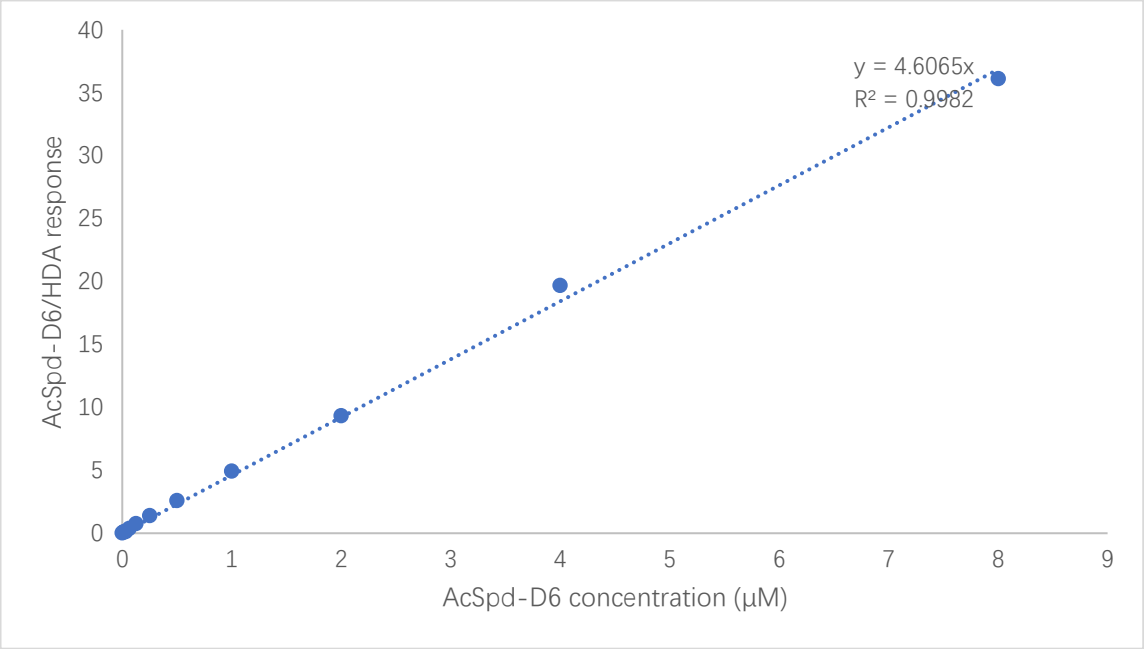

Figure 15 (A) MCF – AcSpd-D6 standard curve with mouse liver microsome.

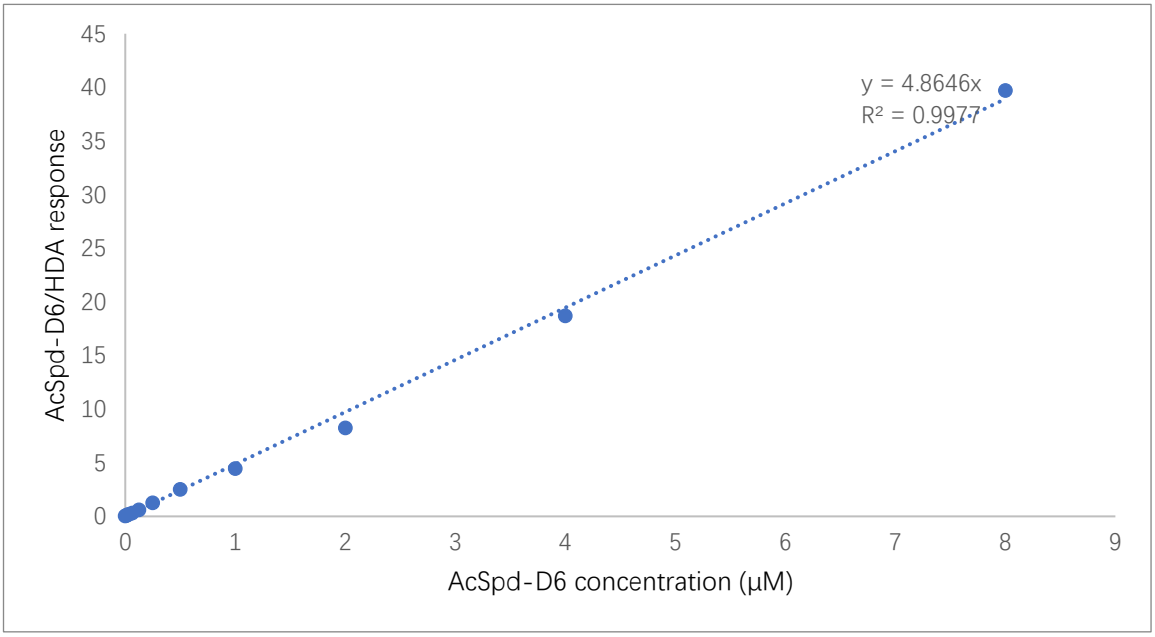

Figure 15 (B) MCF – AcSpd-D6 standard curve with mouse liver cytosol.

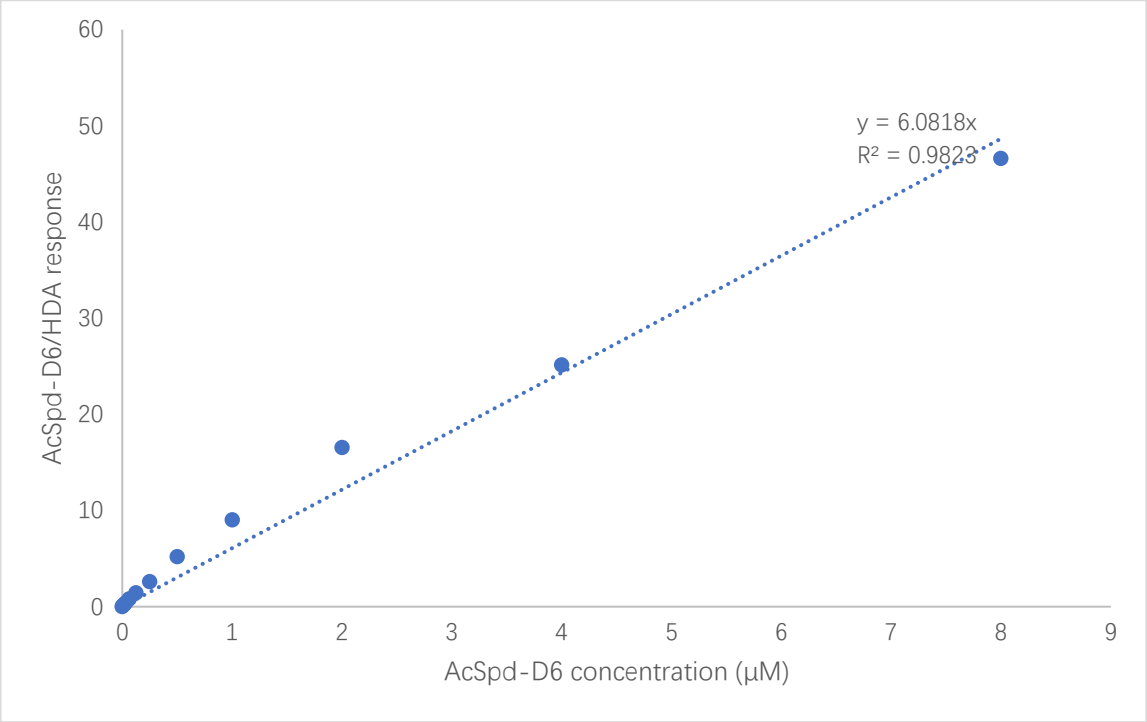

Figure 15 (C) MCF – AcSpd-D6 standard curve with rat liver microsome.

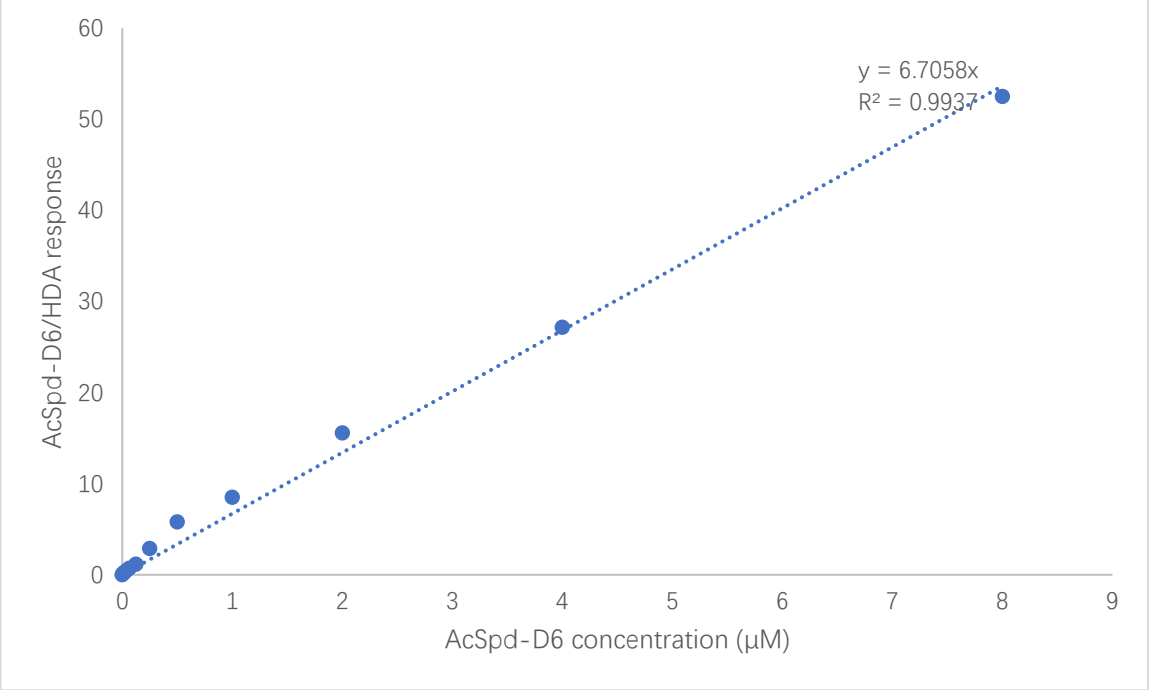

Figure 15 (D) MCF – AcSpd-D6 standard curve with rat liver cytosol.

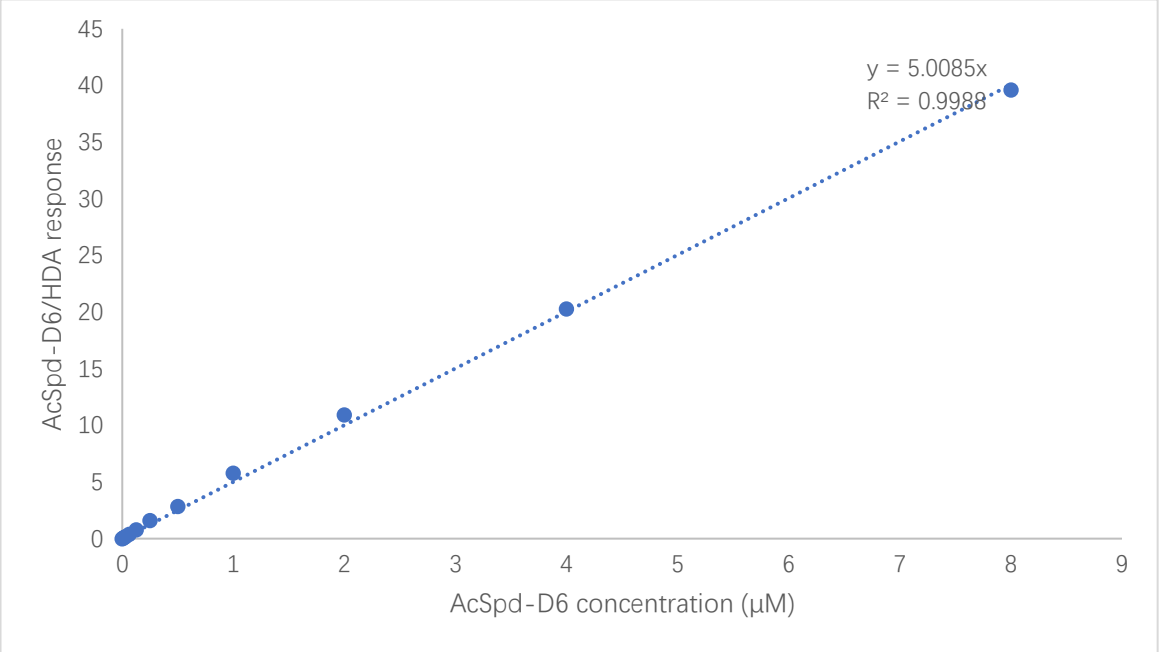

Figure 15 (E) MCF – AcSpd-D6 standard curve in water.

4.4 Polyamine acetylation in mouse or rat liver preparation

Michaelis-Menten kinetic curves of *in vitro* acetylation assays are indicated in Figure 16. The vertical axis is the acetylation velocity (pmol/min/mg), while the horizonal axis refers to substrate concentration (μM). The substrates used here are TETA, Spd and Spd-D6, and the metabolites are MAT, AcSpd and AcSpd-D6 respectively. Most of the data have three or more replicates to make sure the curves are finely fitted, and that all data sets show obvious Michaelis-Menten kinetic trends.

Four kinds of controls are used in this research. Water is the control to ensure that the *in vitro* acetylation reaction occurs without the existence of mouse (and rat) liver preparations. The second and third controls are liver preparation controls, which add liver microsome (or cytosol) after *in vitro* acetylation is ceased by acid. The concentration of liver microsome (or cytosol) to the same protein concentration as the *in vitro* acetylation assays. Due to the extremely low pH in the system, mouse (and rat) liver microsome (or cytosol) are expected to denature instantly. The fourth control is a BSA control, which replaces the liver preparations in the acetylation assay. The reason for

these different controls is that protein (especially from tissue preparations) will absorb some of the metabolites, which causes a decrease in assay response results. It is also possible that APAO or other enzymes transfer acetylated polyamines back to normal polyamines, which in turn causes potential escape of accumulated metabolites. In this research, liver preparation controls are able to determine how much acetylated polyamines liver preparations absorb after proteins are denatured by acid. The BSA control measured amounts of metabolites that normal protein absorbed during the whole *in vitro* acetylation assay process. Interestingly, although the groups with enzymes are supposed to have higher metabolite concentrations, the controls normally show greater or similar responses in this research.

Due to the fact that mouse (or rat) liver microsome (or cytosol) absence is controlled, AcCoA still reacts with polyamine substrates to form acetylated polyamine, and argues that AcCoA is not a cofactor, but also one of the substrates. In this case, the enzymatic kinetics of SSAT may not follow the Michaelis-Menten modelled kinetics because the acetylation reaction may behave as a reaction with multiple substrates. In addition, all of the controls (*in vitro* acetylation assay without liver preparations in the system) show similar responses. Such findings suggest that the existence of SSAT may not be a necessary condition for *in vitro* acetylation. This phenomenon will be further discussed in Chapter 5. Detailed raw data for this finding are presented in Table 3.

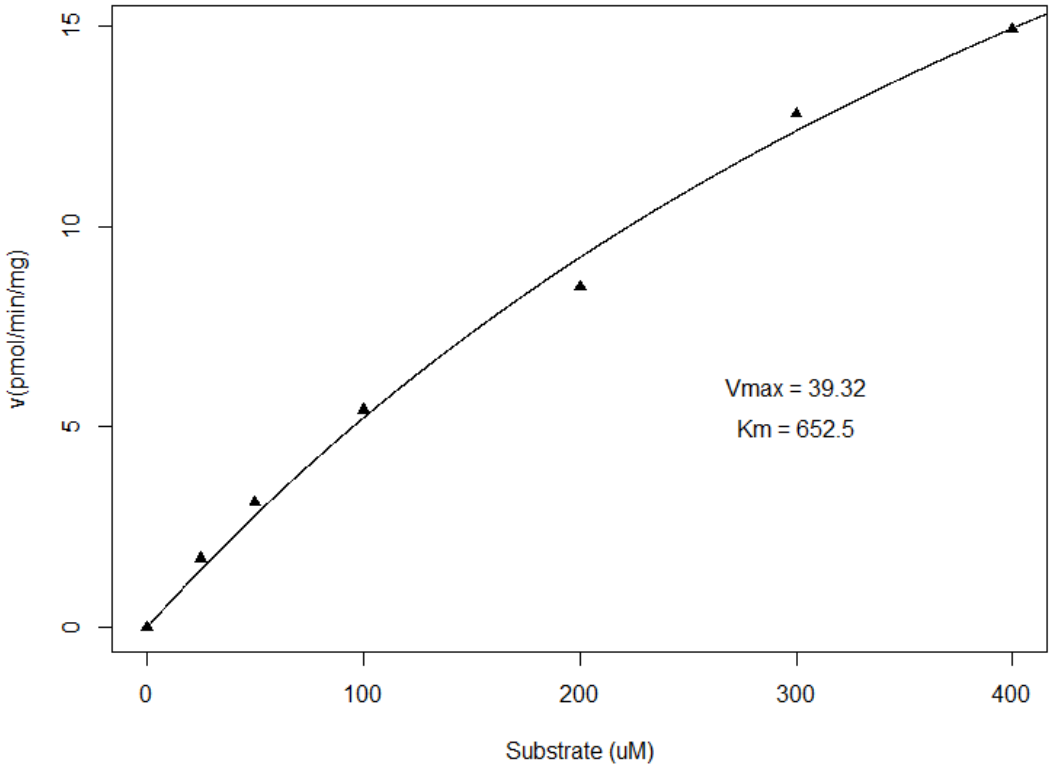

Figure 16 (A) *In vitro* acetylation assay of Spd-D6 with mouse liver microsome.

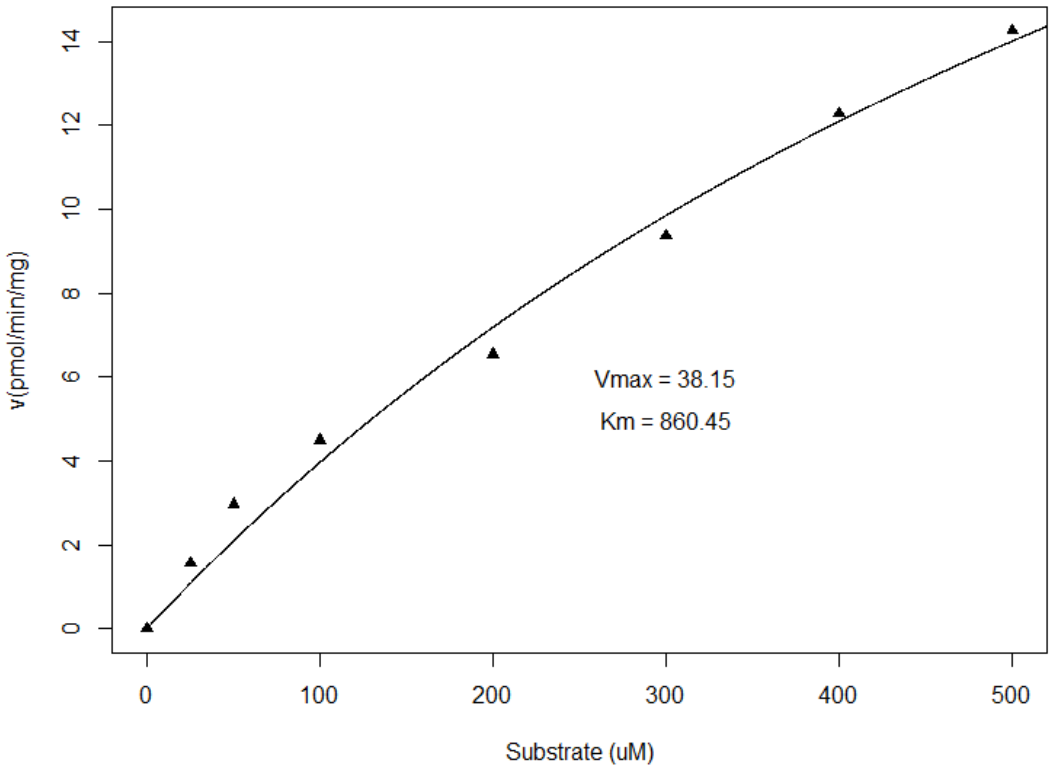

Figure 16 (B) *In vitro* Spd-D6 acetylation assay mouse liver microsome best negative control.

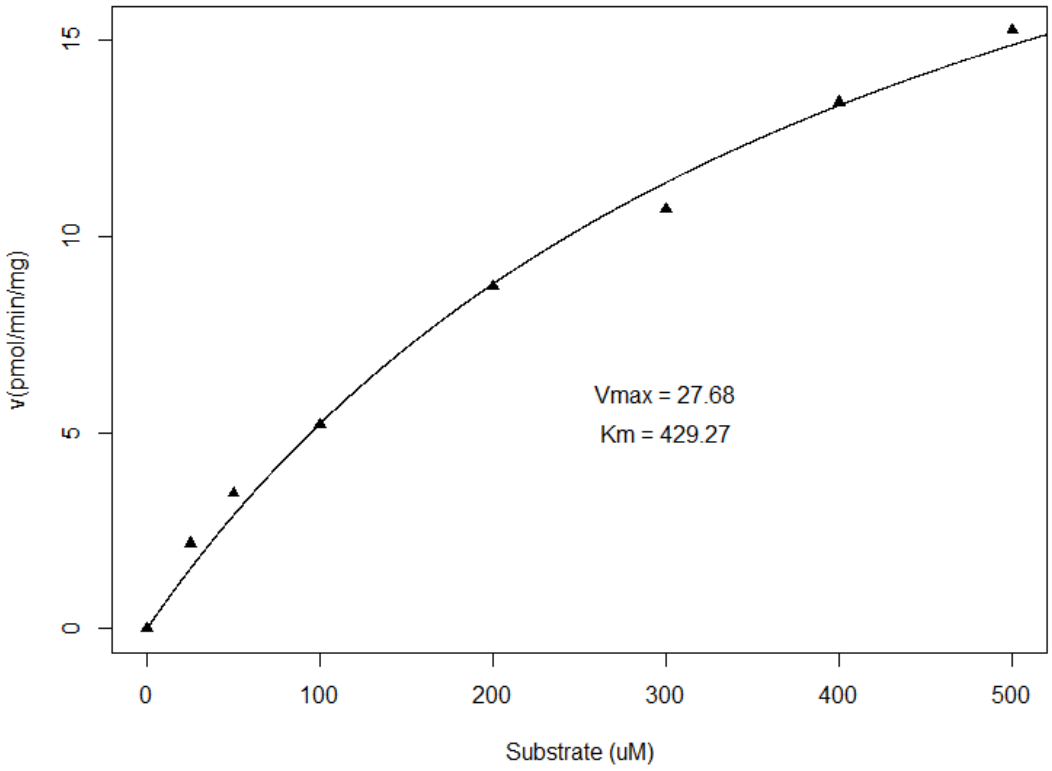

Figure 16 (C) *In vitro* acetylation assay of Spd-D6 with mouse liver cytosol.

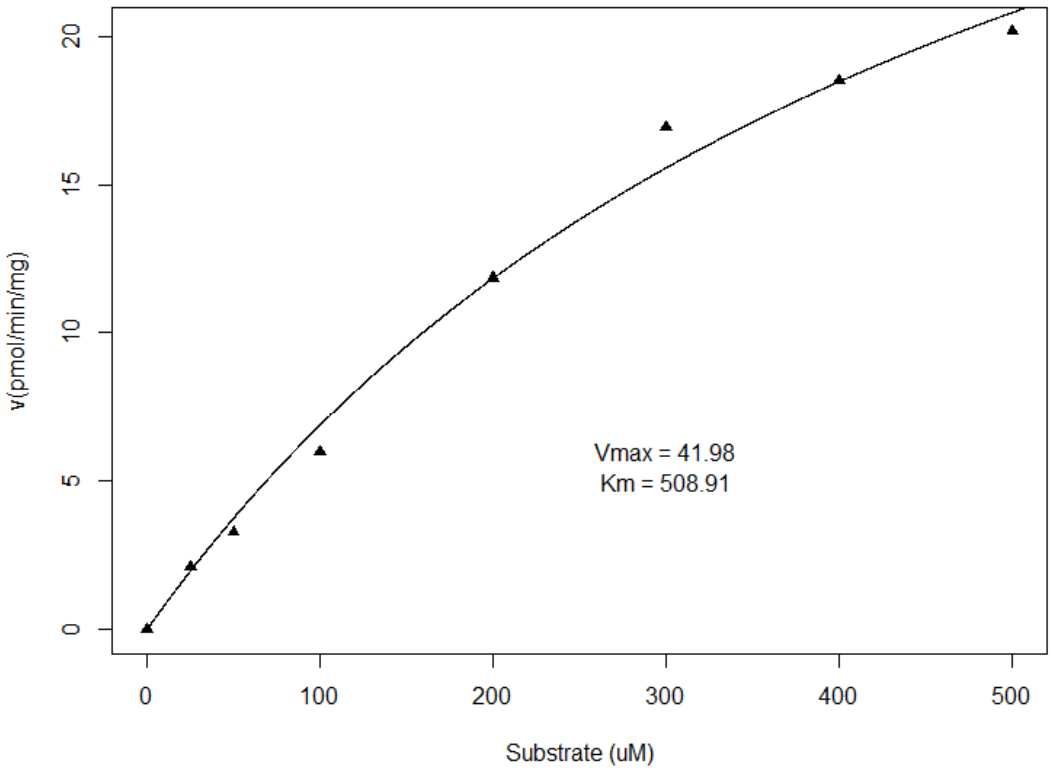

Figure 16 (D) *In vitro* acetylation assay Spd-D6 mouse liver cytosol best negative control.

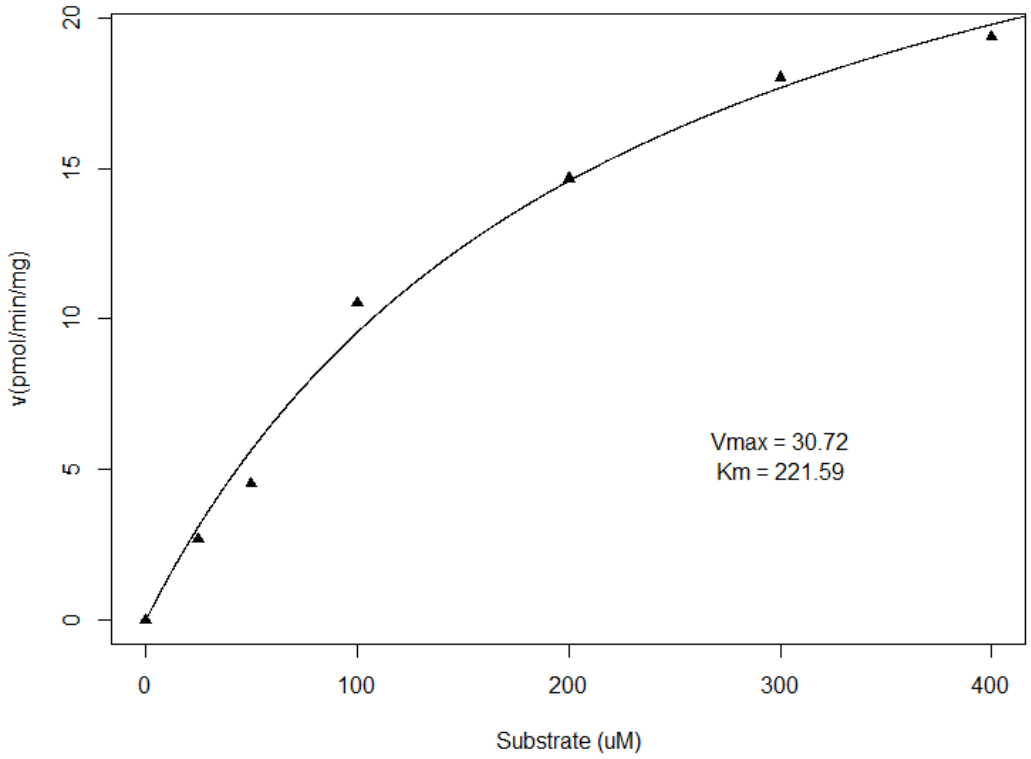

Figure 16 (E) *In vitro* acetylation of Spd-D6 BSA control.

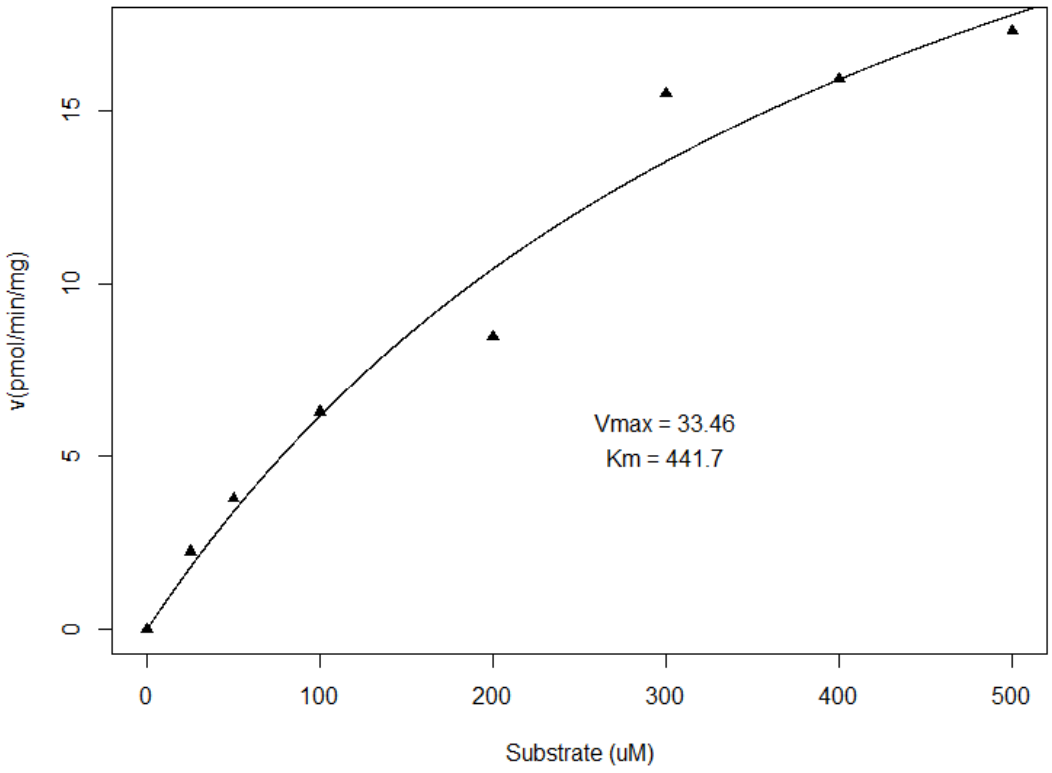

Figure 16 (F) *In vitro* acetylation of Spd-D6 water control.

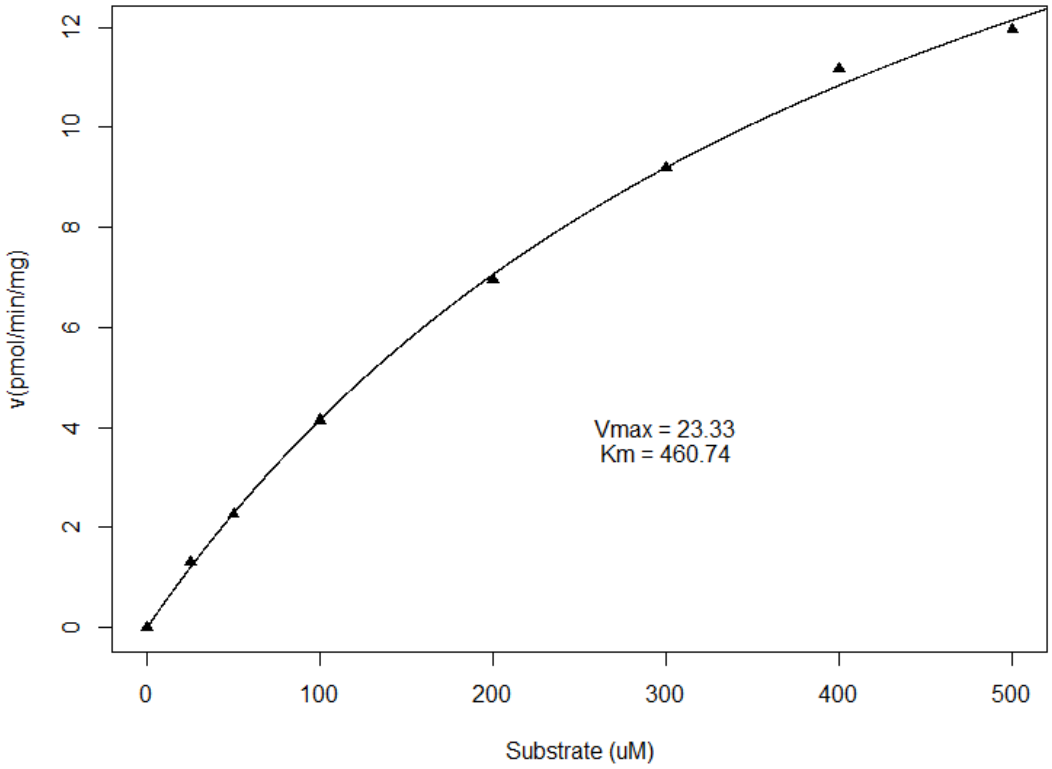

Figure 16 (G) *In vitro* acetylation of Spd with mouse liver microsome.

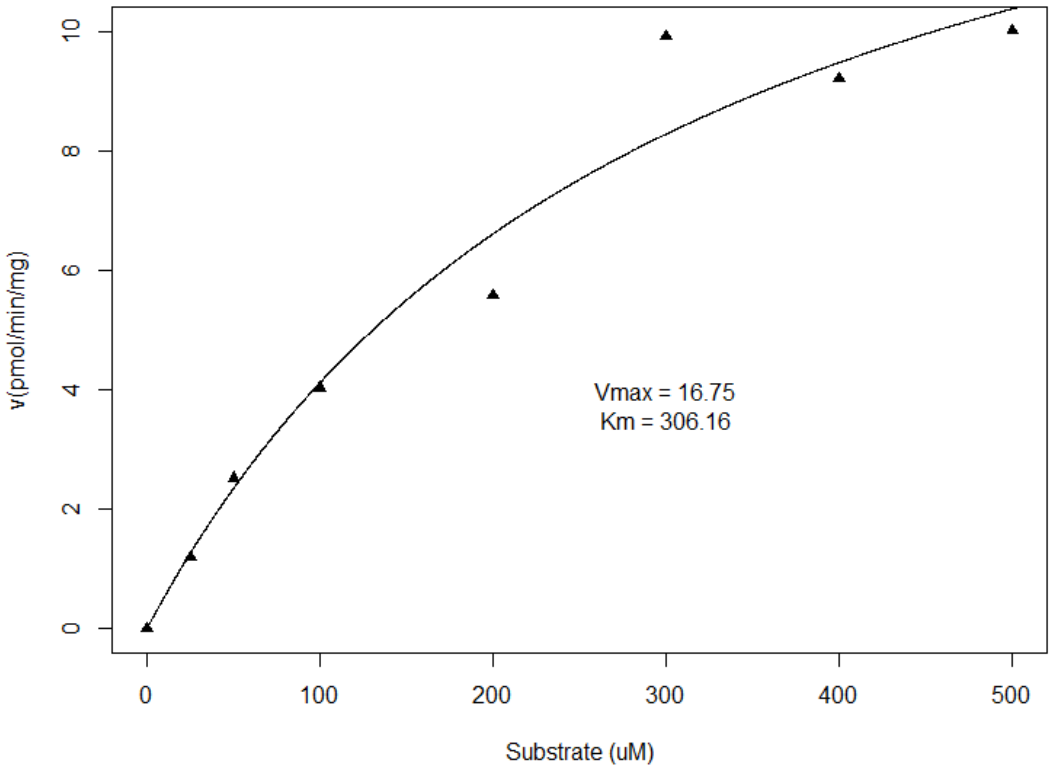

Figure 16 (H) *In vitro* acetylation assay of Spd with mouse liver cytosol.

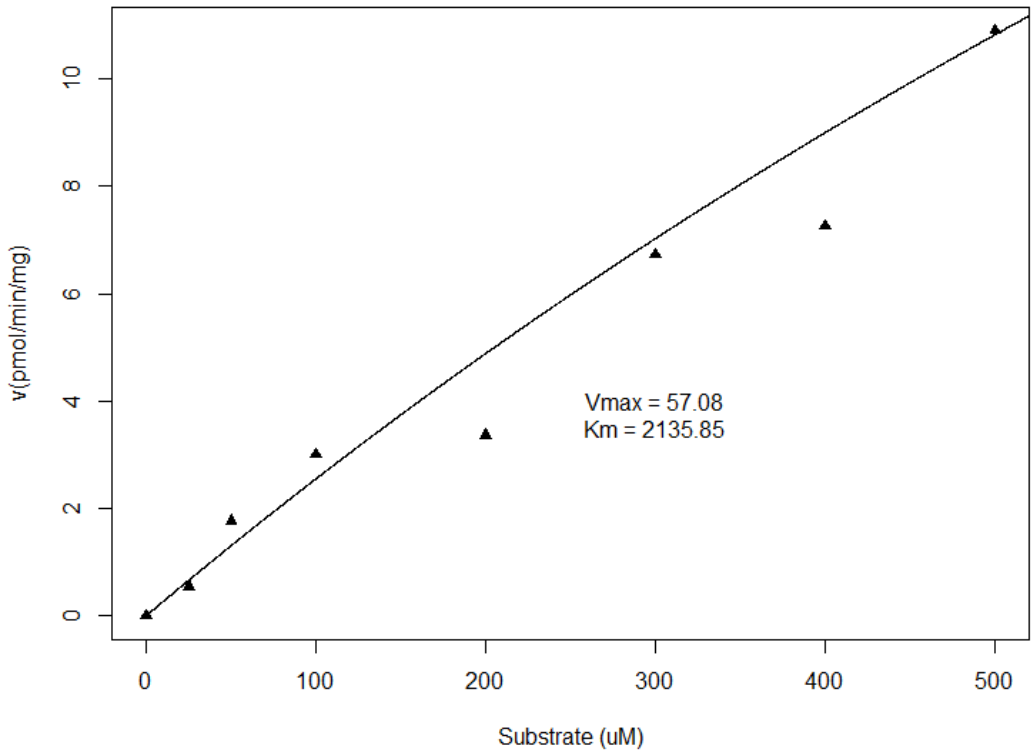

Figure 16 (I) *In vitro* acetylation assay of TETA BSA control.

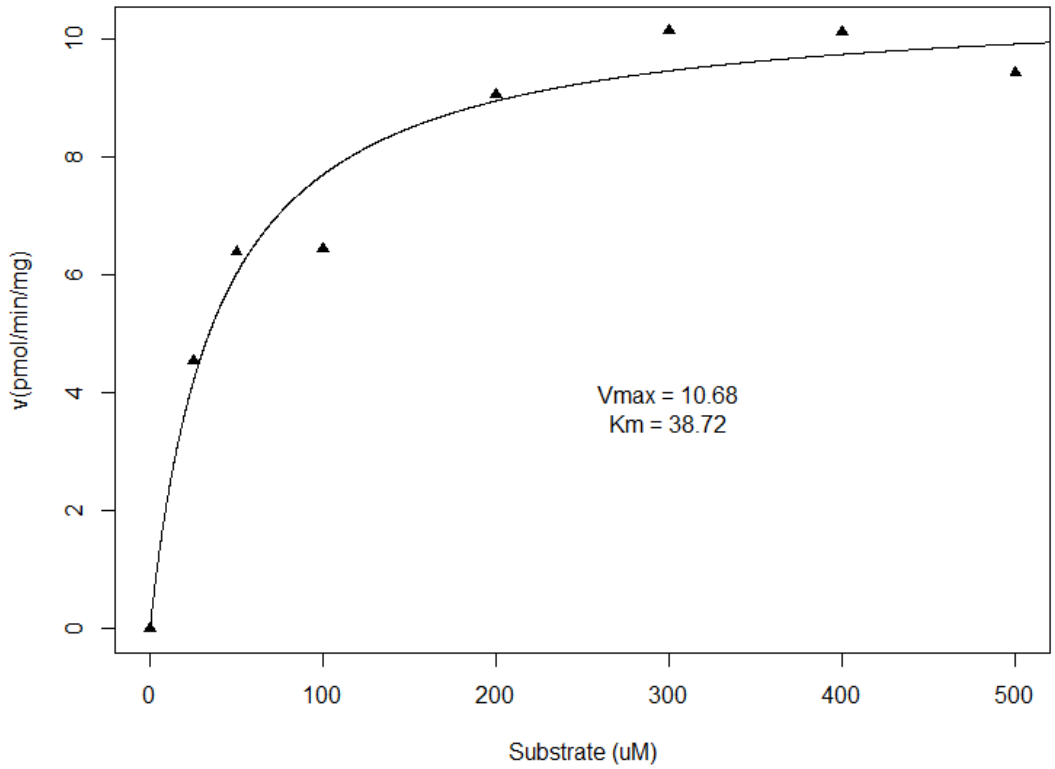

Figure 16 (J) *In vitro* acetylation assay of TETA with mouse liver microsome.

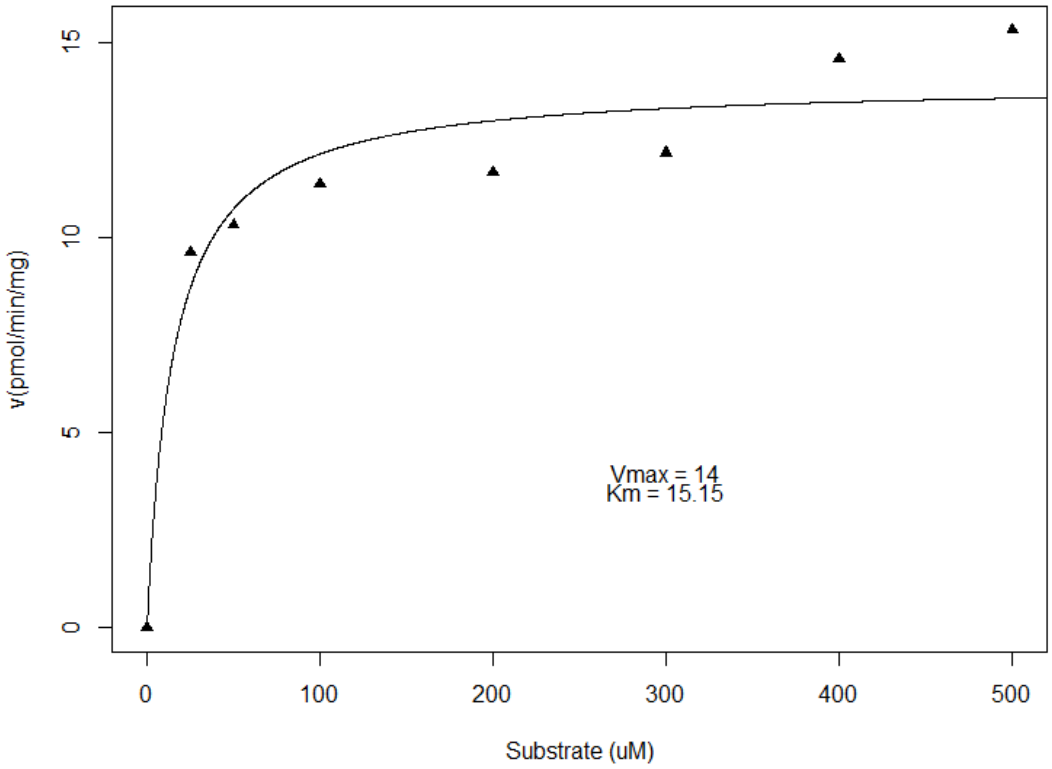

Figure 16 (K) *In vitro* acetylation assay of TETA with mouse liver cytosol.

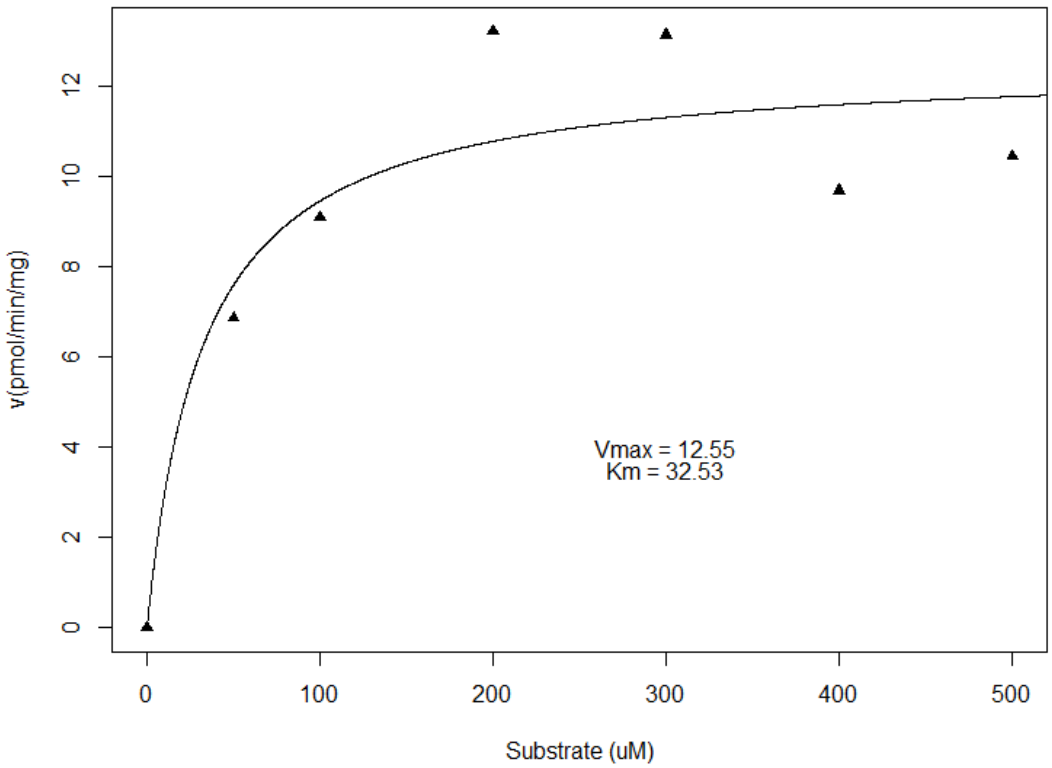

Figure 16 (L) *In vitro* acetylation assay of TETA with rat liver microsome.

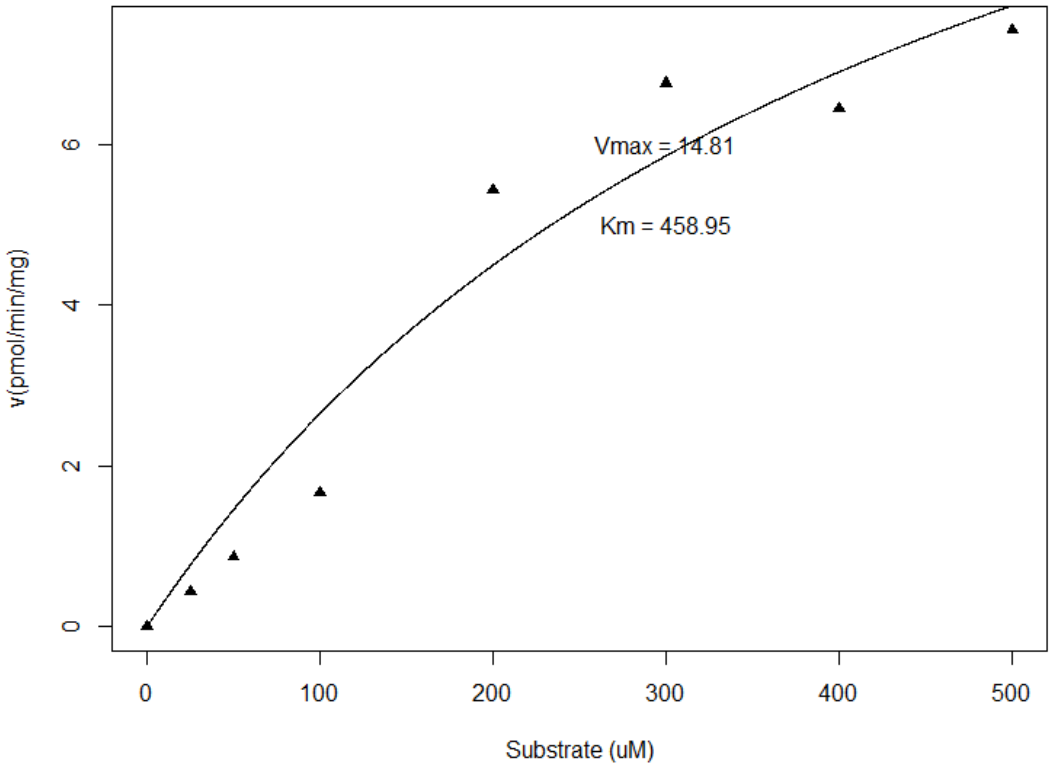

Figure 16 (M) *In vitro* acetylation assay of spermidine with rat liver microsome.

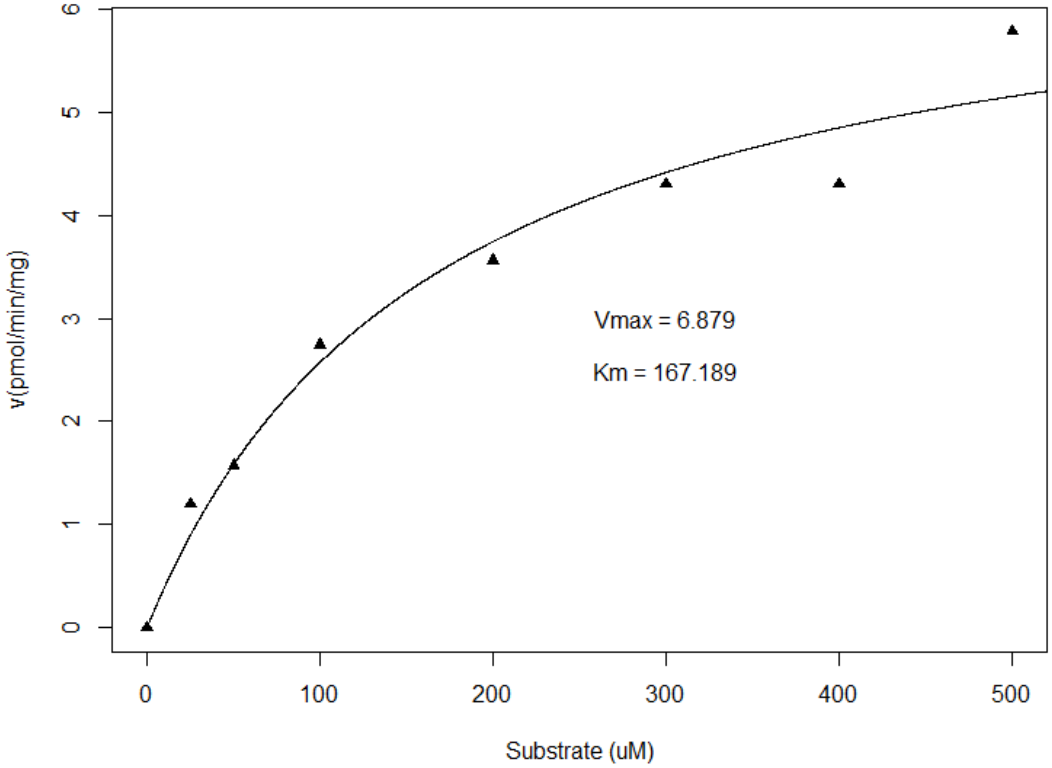

Figure 16 (N) *In vitro* acetylation assay of spermidine rat liver microsome best negative control.

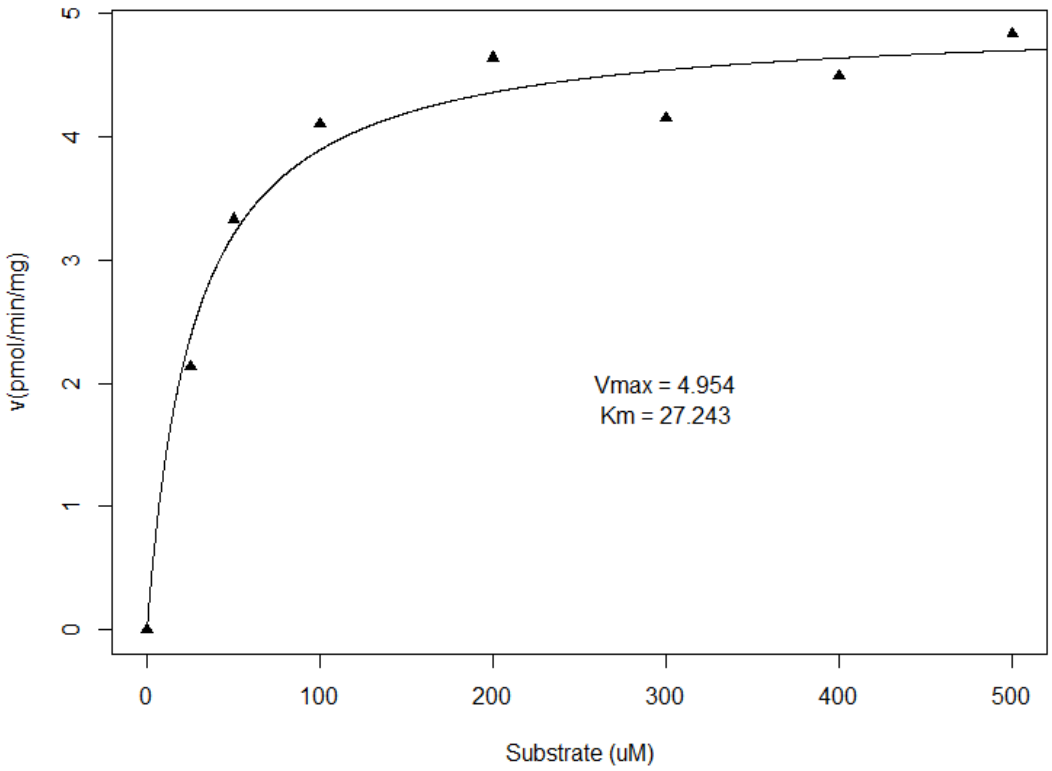

Figure 16 (O) *In vitro* acetylation assay of TETA with rat liver cytosol.

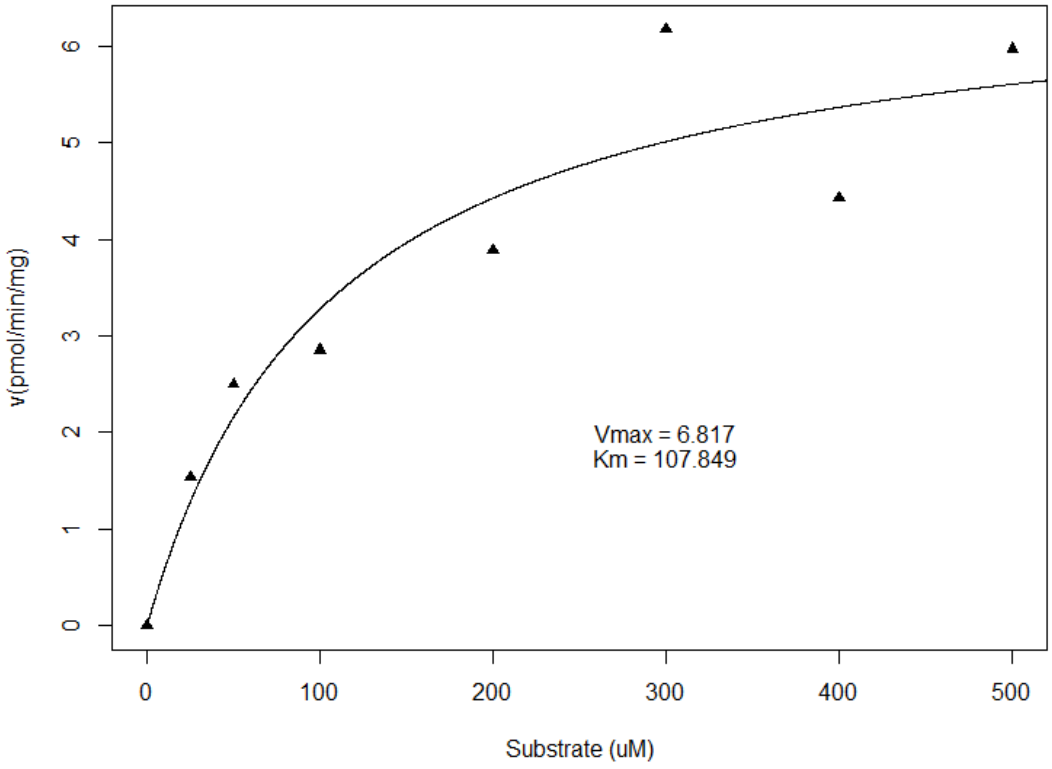

Figure 16 (P) *In vitro* acetylation assay of Spd with rat liver cytosol.

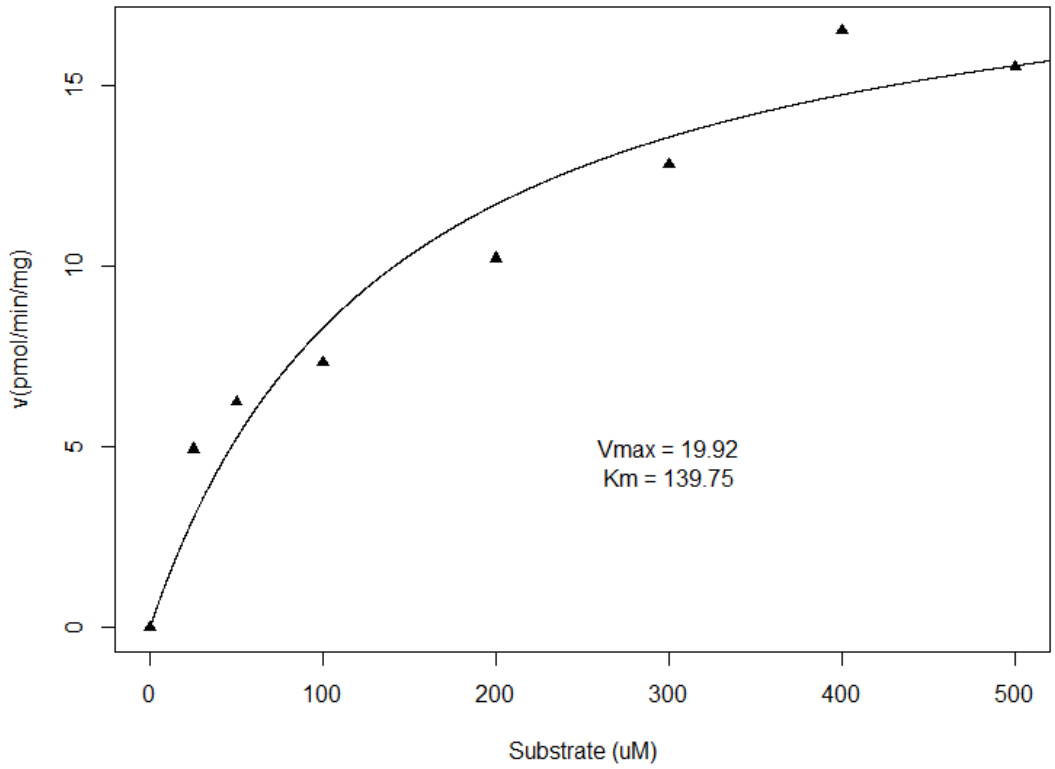

Figure 16 (Q) *In vitro* acetylation assay of Spd-D6 with rat liver cytosol.

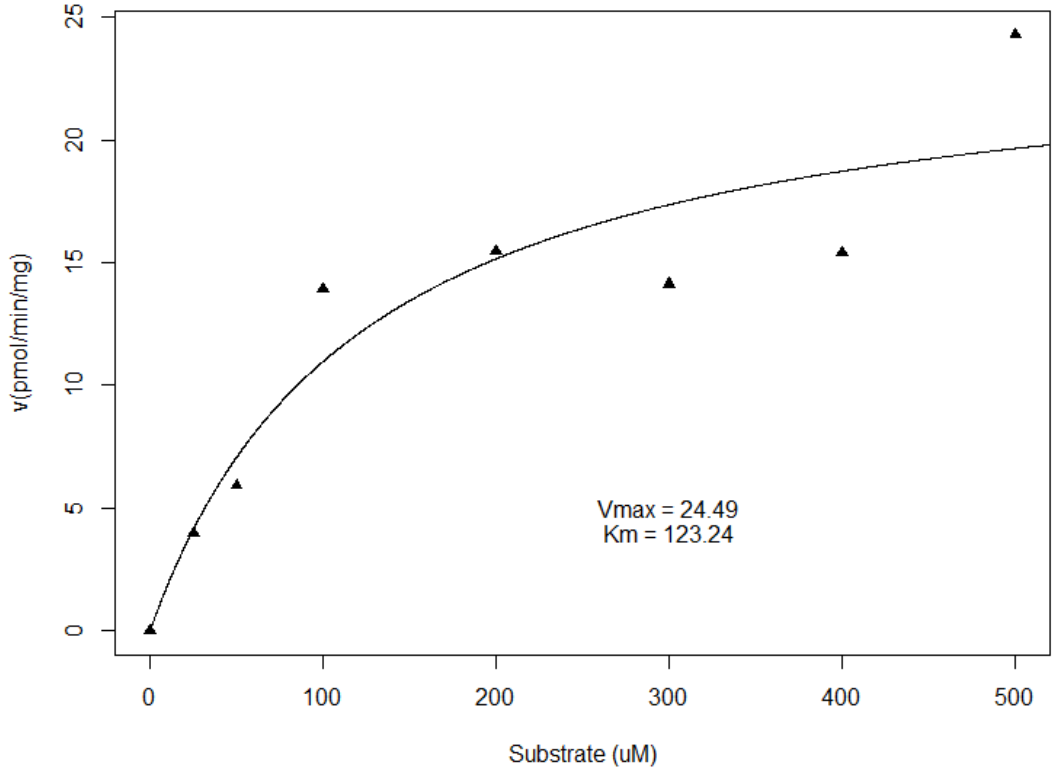

Figure 16 (R) *In vitro* acetylation assay of Spd rat liver microsome best negative control.

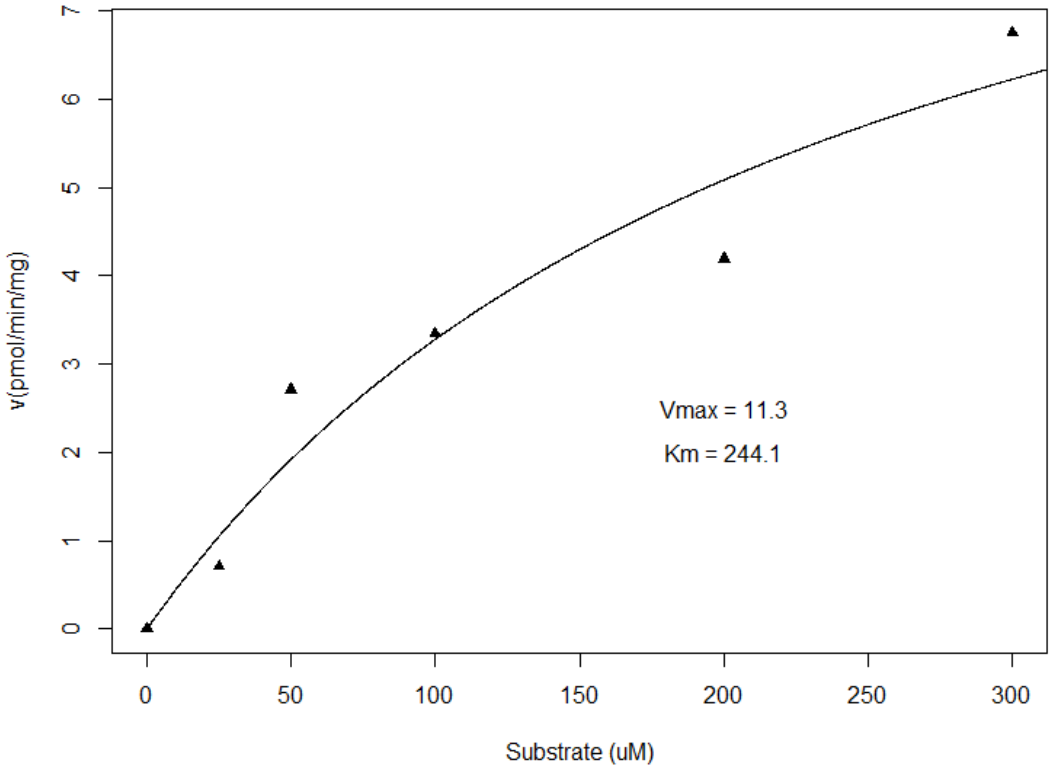

Figure 16 (S) *In vitro* acetylation assay of Spd-D6 with rat liver microsome.

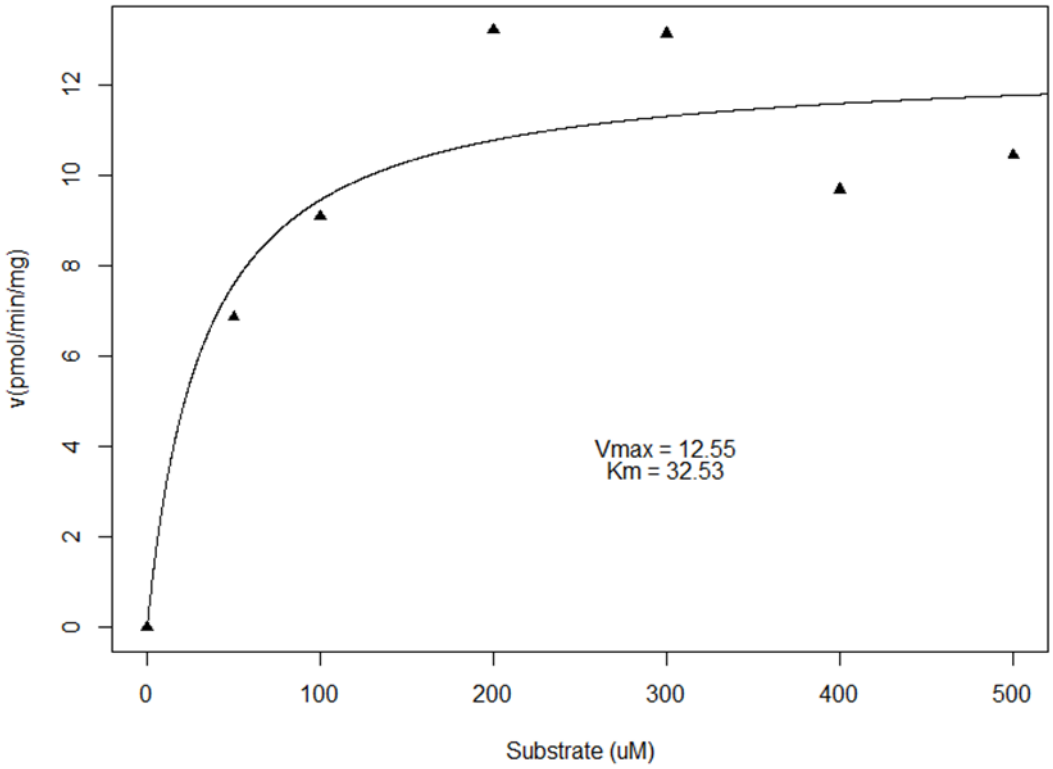

Figure 16 (T) *In vitro* acetylation assay of TETA with rat liver microsome.

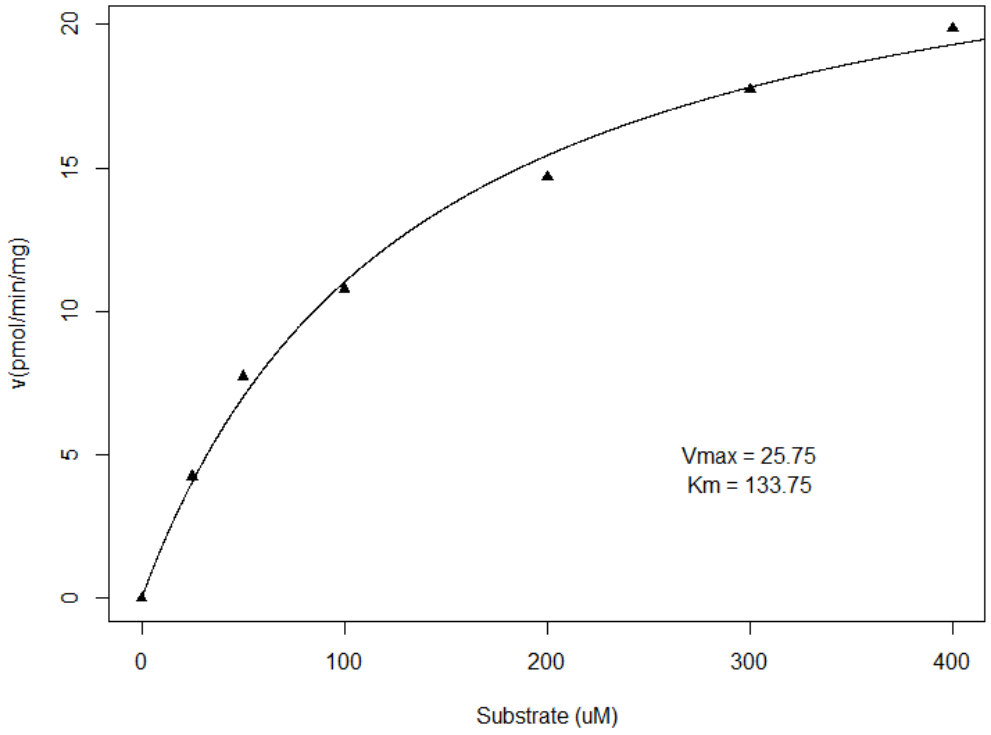

Figure 16 (U) *In vitro* acetylation assay of Spd-D6 rat liver cytosol best negative control.

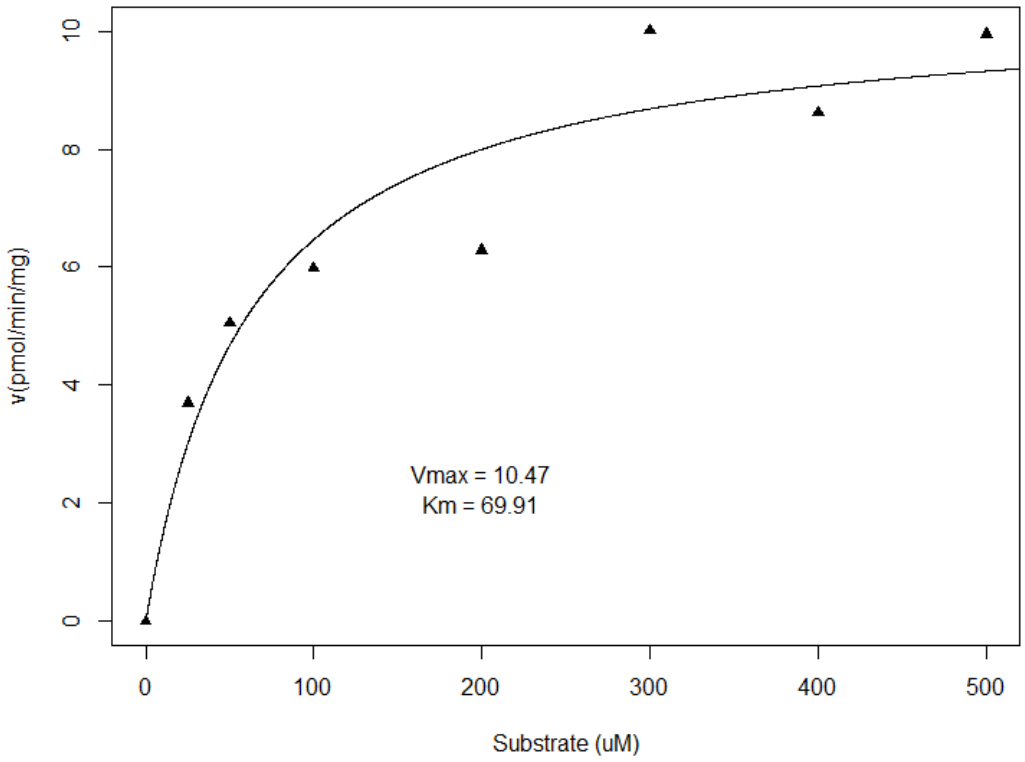

Figure 16 (V) *In vitro* acetylation assay of Spd-D6 rat liver microsome best negative control.

Table 3 Original data table of polyamine *in vitro* acetylation assay

No.	Substrate	Enzyme source	25uM	50uM	100uM	200uM	300uM	400uM	500uM
A	Spd-d6	Mouse liver microsome	1.7 ± 0.3	3.1 ± 0.4	5.4 ± 1.1	8.5 ± 1.6	12.8 ± 2.4	14.9 ± 2.6	20.6 ± 3.8
B	Spd-d6 (Control)	Mouse liver microsome	1.6 ± 0.6	3.0 ± 1.6	4.5 ± 2.0	6.5 ± 1.4	9.4 ± 1.8	12.3 ± 0.6	14.3 ± 7.9
C	Spd-d6	Mouse liver cytosol	2.2 ± 0.3	3.4 ± 0.3	5.1 ± 0.7	8.7 ± 0.4	10.7 ± 1.4	13.4 ± 1.5	15.3 ± 2.7
D	Spd-d6 (Control)	Mouse liver cytosol	1.8 ± 0.5	2.7 ± 0.8	5.0 ± 1.4	11.5 ± 0.9	12.8 ± 4.4	16.9 ± 3.0	20.2 ± 3.8
E	Spd-d6 BSA control	No enzyme source	2.7 ± 0.5	4.5 ± 1.5	10.5 ± 3.0	14.7 ± 4.9	18.0 ± 2.6	19.3 ± 5.0	24.4 ± 5.9
F	Spd-d6 water control	No enzyme source	2.2 ± 0.5	3.8 ± 0.2	6.3 ± 0.5	8.5 ± 0.7	15.5 ± 2.5	15.9 ± 3.3	17.3 ± 0.9
G	Spd	Mouse liver microsome	1.3 ± 0.3	2.3 ± 0.5	4.1 ± 1.1	6.9 ± 2.7	9.2 ± 3.3	11.2 ± 1.0	11.9 ± 0.3
H	Spd	Mouse liver cytosol	1.2 ± 0.1	2.5 ± 0.1	4.0 ± 0.04	5.6 ± 0.5	9.9 ± 2.2	9.2 ± 1.5	10.0 ± 1.3
I	TETA	Mouse liver microsome	4.5 ± 0.6	6.4 ± 0.6	6.4 ± 0.9	9.0 ± 1.4	10.1 ± 1.4	10.1 ± 1.4	9.4 ± 0.9
J	TETA	Mouse liver cytosol	9.9 ± 1.1	10.2 ± 1.5	11.7 ± 1.3	11.8 ± 2.3	12.4 ± 1.7	14.5 ± 2.3	15.4 ± 2.7
K	TETA BSA control	No enzyme source	0.5 ± 0.4	1.8 ± 0.8	3.0 ± 1.4	3.4 ± 0.5	6.7 ± 1.7	7.3 ± 2.0	10.9 ± 0.5
L	Spd-d6	Rat liver microsome	2.8 ± 1.4	3.6 ± 1.4	5.1 ± 1.8	6.1 ± 3.5	8.1 ± 3.5	5.4 ± 3.8	5.0 ± 3.0
M	Spd-d6 (control)	Rat liver microsome	3.7 ± 3.6	5.0 ± 4.9	6.0 ± 3.7	6.3 ± 3.3	10.0 ± 4.2	8.6 ± 5.0	9.9 ± 5.8
N	Spd-d6	Rat liver cytosol	4.9 ± 1.5	6.2 ± 1.6	7.3 ± 0.7	10.2 ± 2.7	12.8 ± 4.1	16.5 ± 3.6	15.5 ± 2.2
O	Spd-d6 (control)	Rat liver cytosol	3.8 ± 1.5	6.5 ± 1.6	8.7 ± 4.2	12.5 ± 6.3	14.3 ± 5.0	16.1 ± 6.3	18.7 ± 9.7
P	Spd	Rat liver microsome	0.4 ± 0.1	0.9 ± 0.3	1.7 ± 0.5	5.4 ± 2.1	6.8 ± 2.4	6.4 ± 2.0	7.4 ± 2.4
Q	Spd	Rat liver cytosol	1.5 ± 0.1	2.5 ± 0.2	2.8 ± 0.2	3.9 ± 0.3	6.2 ± 0.6	4.4 ± 0.5	6.0 ± 1.1

R	Spd (control)	Rat liver microsome	1.2 ± 0.8	1.6 ± 0.8	2.7 ± 0.9	3.6 ± 0.9	4.3 ± 1.3	4.3 ± 1.8	5.8 ± 1.1
S	TETA	Rat liver microsome	Nah	6.9	9.0	13.2	13.2	9.7	10.5
T	TETA (control)	Rat liver microsome	Nah	1.9	2.7	3.1	4.1	4.2	11.7

In this table, 25 ~ 500uM represent substrate concentration, and corresponding data are polyamine *in vitro* acetylation velocity (pmol/min/mg).

Table 4 illustrates the enzymatic and non-enzymatic kinetic information of *in vitro* acetylation assays including substrate, enzyme sources, K_m, V_{max} and P-value data. According to Table 4, most of the assay results show optimal curve fitting of Michaelis-Menten kinetics ($p < 0.05$). The rest of the *in vitro* acetylation assays do not have enough replicates to optimise the curve fitting. However, since almost all *in vitro* acetylation assays related with Spd-D6 have reliable data, it is still feasible to draw conclusions through following the kinetic information. In addition to this, TETA's BSA control shows extremely high response of the assessed metabolite (MAT), far higher in fact than the response of the enzymatic *in vitro* acetylation assay. Moreover, the groups using Spd as substrate normally show a lower response of metabolites than the groups using Spd-D6 as substrates. It is also an interesting phenomenon because theoretically the Spd study group should have higher responses due to the existence of Spd and AcSpd in mouse (and rat) liver microsome (or cytosol) fractions.

Table 4 Summary table of enzymatic and non-enzymatic kinetics of polyamine *in vitro* acetylation assay

No.	Substrate	Enzyme source	K_m (uM)	V_{max} (pmol/min/mg)	P-value
A	Spd-D6	Mouse liver microsome	652.5	39.32	9.52e-06 ***
B	Spd-D6 (control)	Mouse liver microsome	860.45	38.15	0.000115 ***
C	Spd-D6	Mouse liver cytosol	429.27	27.68	9.44e-10 ***
D	Spd-D6 (control)	Mouse liver cytosol	508.91	41.98	6.91e-08 ***
E	Spd-D6 BSA control	No enzyme source	344.73	32.51	0.00123 **
F	Spd-D6 water control	No enzyme source	441.7	33.46	9.6e-07 ***
G	Spd	Mouse liver microsome	460.74	23.33	0.000202 ***
H	Spd	Mouse liver cytosol	306.16	16.75	7.33e-06 ***
I	TETA	Mouse liver microsome	38.72	10.68	0.0013 **
J	TETA	Mouse liver cytosol	15.5	14	
K	TETA BSA control	No enzyme source	2135.85	57.08	3.11e-05 ***
L	Spd-D6	Rat liver microsome	244.1	11.3	
M	Spd-D6 (control)	Rat liver microsome	69.91	10.47	
N	Spd-D6	Rat liver cytosol	139.75	19.92	0.0103 *
O	Spd-D6 (control)	Rat liver cytosol	133.75	25.75	
P	Spd	Rat liver microsome	458.95	14.81	0.0037 **
Q	Spd	Rat liver cytosol	107.849	6.817	
R	Spd (control)	Rat liver microsome	123.24	24.49	

Table 5 illustrates the differences of the results between each group of enzymatic and non-enzymatic *in vitro* acetylation assay utilising Spd-D6 as the substrate. According to Table 5, except for the velocities of mouse liver microsome control and mouse liver cytosol control groups, none of them has obvious statistical difference ($p < 0.05$) from each other.

Table 5 Comparison table of K_m and V_{max} of spermidine-d6 *in vitro* acetylation with different kinds of controls

Comparison groups	P-value for Vmax	P-value for K_m
A-B	0.116	0.202
A-C	0.335	0.271
A-D	0.986	0.999
A-E	0.752	0.087
A-F	0.518	0.220
B-C	0.946	0.999
B-D	0.027 *	0.226
B-E	0.702	0.995
B-F	0.897	0.999
C-D	0.087	0.304
C-E	0.983	0.937
C-F	0.999	0.999
D-E	0.349	0.093
D-F	0.184	0.247
E-F	0.998	0.992
L-M	0.996	0.695
L-N	0.595	0.946
L-O	0.814	0.999
L-E	0.206	0.768
L-F	0.356	0.368
N-O	0.998	0.977
M-N	0.848	0.990
M-O	0.970	0.784
M-E	0.396	0.999
M-F	0.609	0.990
N-E	0.953	0.997
N-F	0.997	0.837
O-E	0.809	0.847
O-F	0.951	0.450

4.5 Data from another method

This research also used another LC-MS method which uses a Luna 5μ CN 100A, size 150 x 3.00 mm column, with a Selected ion Monitoring (SIM) mode. The liver preparation used in this method is made by Ca^{2+} sediment method. Due to the lack of reproducibility of the internal standard (DES) and the column collapse during the study, this LC-MS methodology was been abandoned before completion of the entire data collection. Fortunately, data from this method still provides applicable reference results.

4.5.1 LC-MS detection of four polyamine standards

32 μM of polyamine standards including MAT, spermidine, DES and TETA are presented in the figures of this section. Figures show obvious peaks at the m/z values of 189.2, 146.2, 259.3 and 147.2. Among these four polyamines identified, TETA could be transferred to MAT during *in vitro* acetylation processing. DES was used as an internal standard in this research. In addition, spermidine could be transferred to AcSpd by an enzymatic reaction with SSAT.

The retention times of the polyamines in this study were not well separated. However, by using selected-ion monitoring chromatogram (SIM) methods, peaks were individually distinguished from one another. Moreover, analysis of the method revealed that all polyamine peaks in this study have an uncharacterised tailing peak. It is possible that the mobile phase is too weak for this analysis, and this is reflected in the observed tail of retained material. A previous study from our group, using this method, required not only HFBA in the mobile phase, but also needed HFBA in the samples to both cancel the acetylation reaction and to act as the ion-pairing reagent to stabilise target polyamines from being absorbed by other substances. However, this study did not use HFBA and other ion-pairing reagents because of the LC-MS requirements. This study was run in negative mode, and ion-paring reagents such as HFBA will permanently

influence the result of if used. Thus, the acetylation reaction terminator was switched to 37% HCl, and the HFBA previously used in the mobile phases was replaced by formic acid. It is possible that this change in chemistry could be the potential cause of tailing peaks observed in this method.

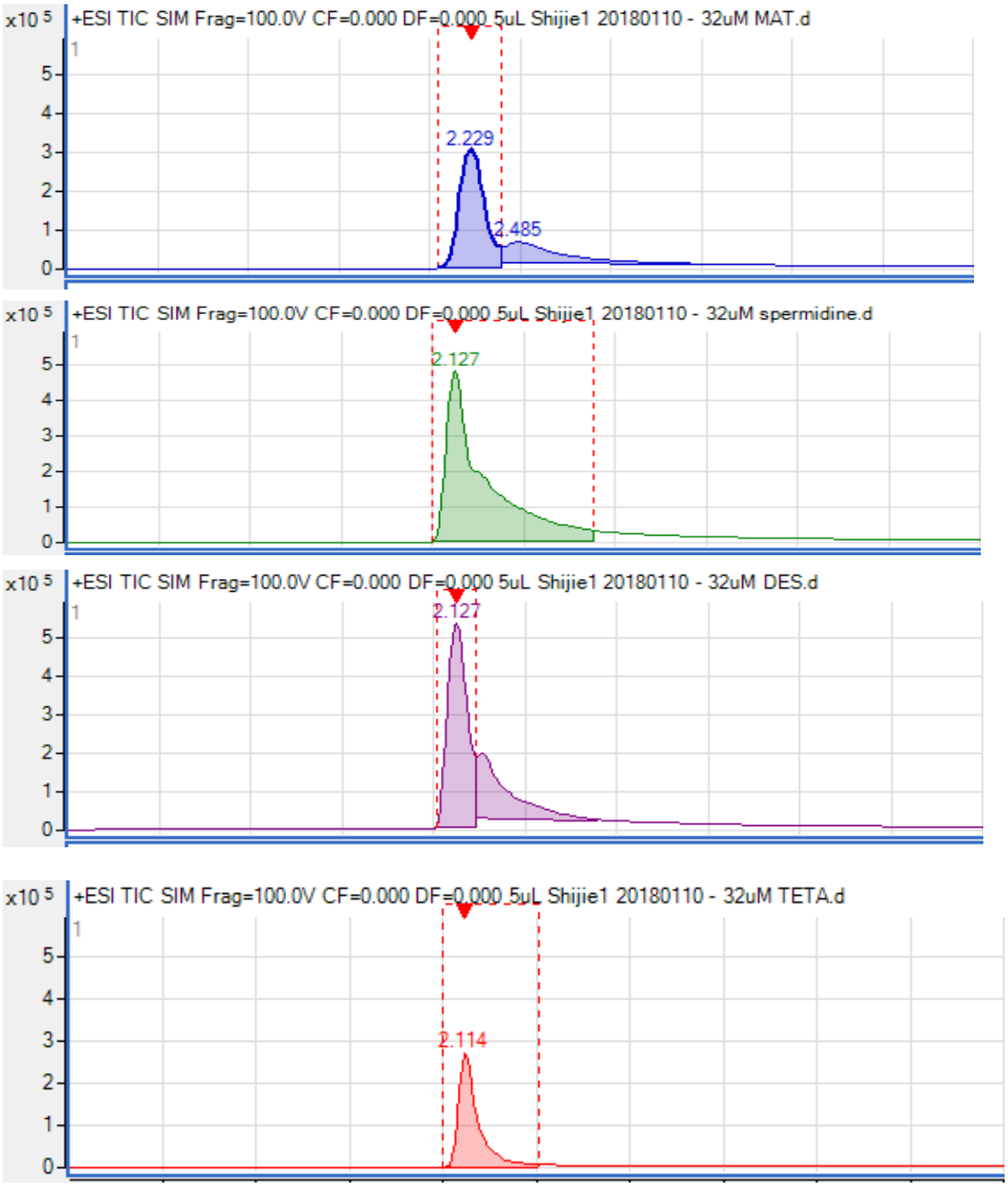

Figure 17 The LC-MS result of four polyamines (MAT, Spd, DES, TETA).

4.5.2 Protein concentration optimisation

Figure 18 (A) and (B) show the relationship between protein concentrations of rat liver preparations and MS responses of metabolites (MAT). Due to the lack of reproducibility of the study's internal standard (DES), the y-axes represent just the MAT response of LC-MS instead of a MAT/DES ratio. This method was also abandoned prior to completion of the study, with only rat liver microsome and cytosol acetylating TETA having been recorded. The concentration of TETA here is 2 mM, and the concentration of AcCoA is 1.67 mM, which are both higher than the MRM method's concentrations previously mentioned.

Interestingly, the MAT responses of both rat liver microsome and cytosol samples decrease with the increase of protein concentrations. This is not reasonable nor expected because normally, the increase of enzyme concentration leads to the increase of metabolite concentration, especially in the situation where the substrate concentration and cofactor concentration are far higher than the minimum requirement in an enzymatic assay, unless the enzymatic presence reaches a certain level that substrates are no longer enough for the increase of reaction velocity. Results however suggest that the liver preparation decreases the concentration of MAT in this study.

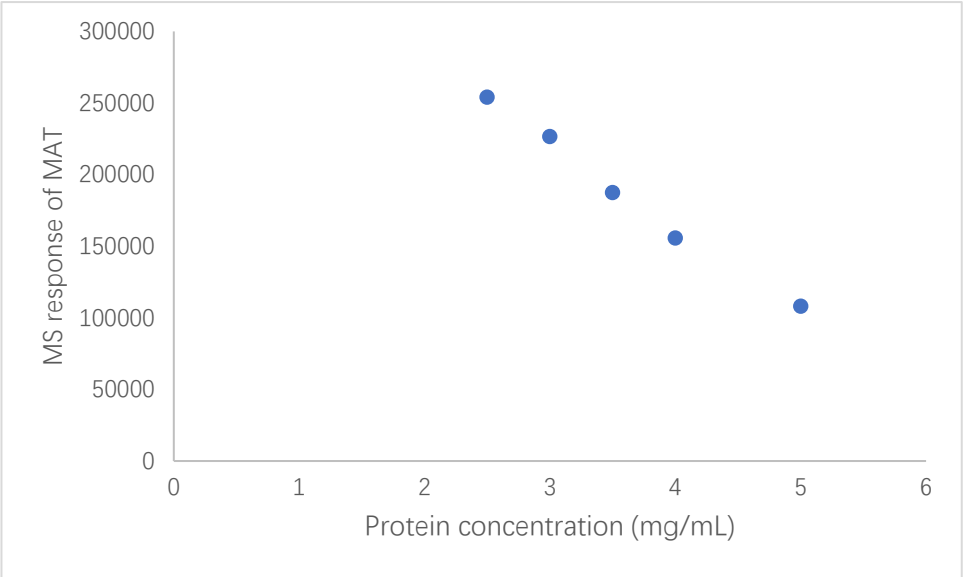

Figure 18 (A) Rat liver microsome protein concentration optimisation using TETA as substrate.

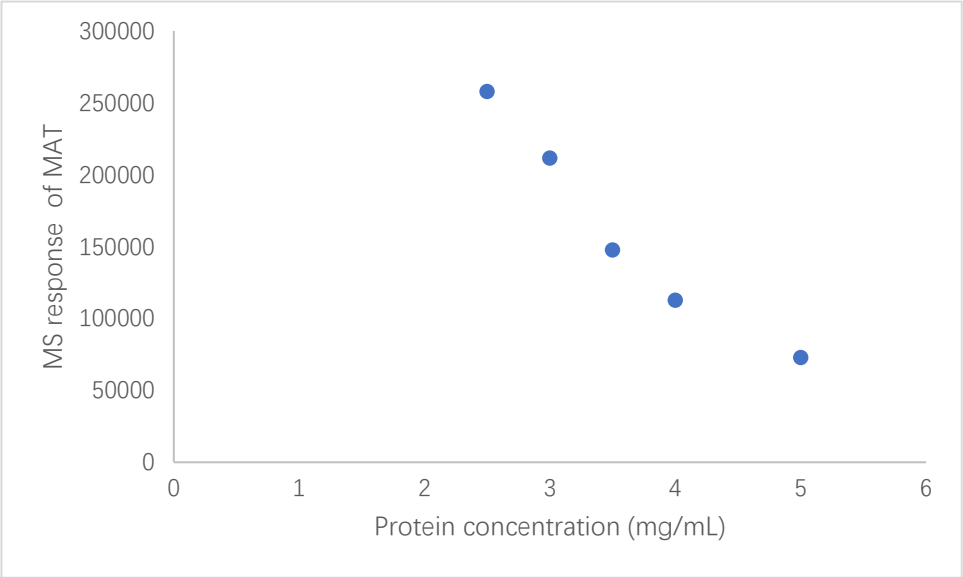

Figure 18 (B) Rat liver cytosol protein concentration optimisation using TETA as substrate.

4.5.3 Standard curves using Ca^{2+} rat liver microsome, cytosol

External standard methods were used to quantify the amounts of TETA and MAT in the rat liver microsome samples. This section presents the differences between TETA and MAT standard curves in the different substances analysed. Figure 19 (A) (B) and (C) are MAT standard curves in water, assessing preparations of microsome and cytosol fractions respectively. Figure 19 (E),

(F) and (G) are TETA standard curves in different substances (microsome, cytosol and water). Notably, microsome and cytosol preparations were made by using the Ca^{2+} sedimentation method, with the effect of Ca^{2+} in the system currently undefined.

Table 6 shows the p-values between standard curves, which reflect points of distinction. As Table 6 shows, only MAT (rat liver microsome) standard curve and MAT (water) standard curve show significant differences, with a p-value of 0.02694 < 0.05.

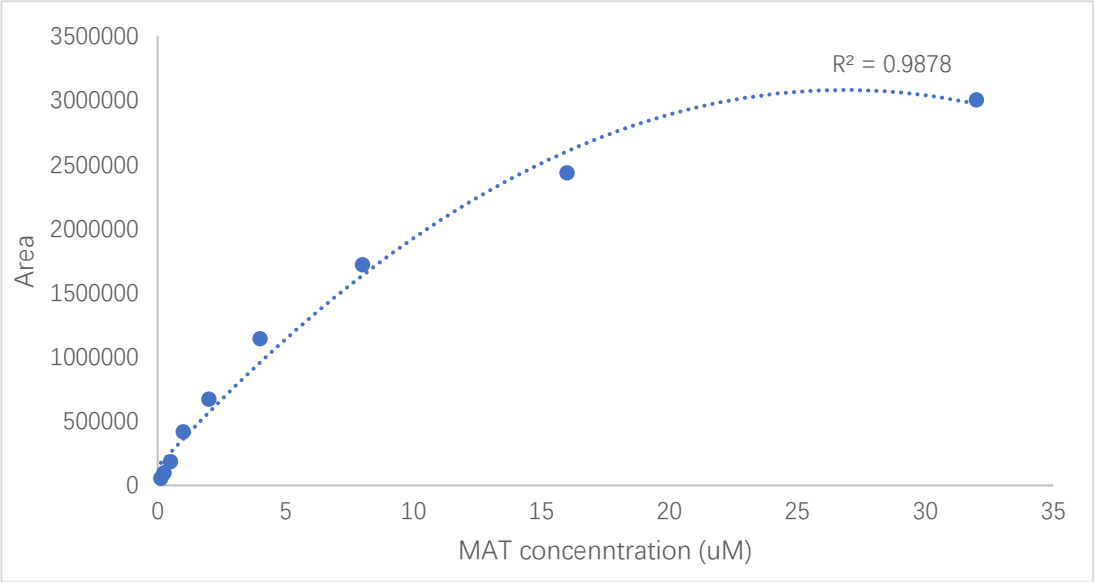

Figure 19 (A) MAT standard curve in water.

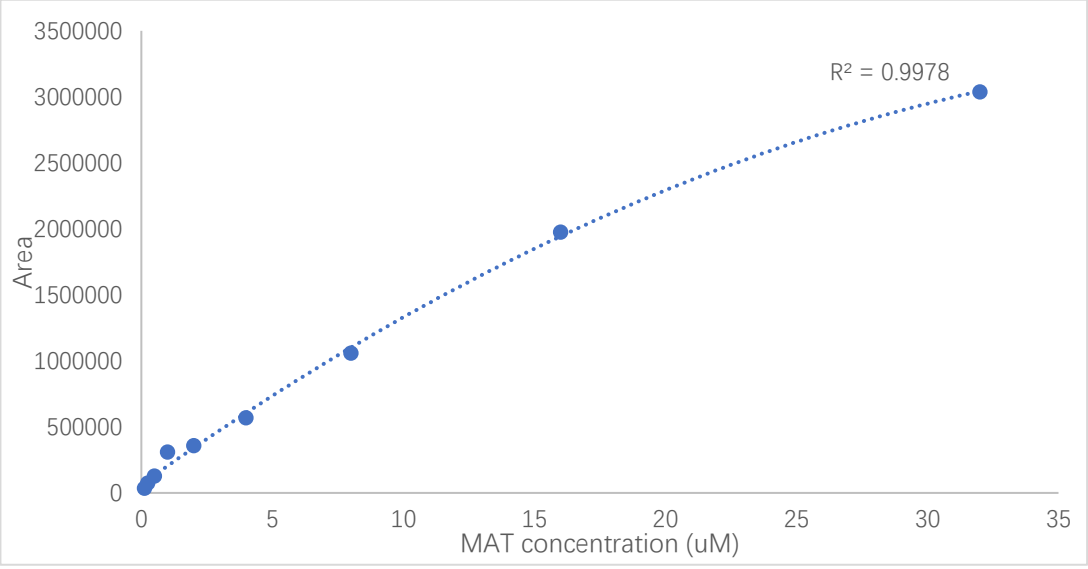

Figure 19 (B) MAT standard curve in Ca^{2+} rat liver microsome.

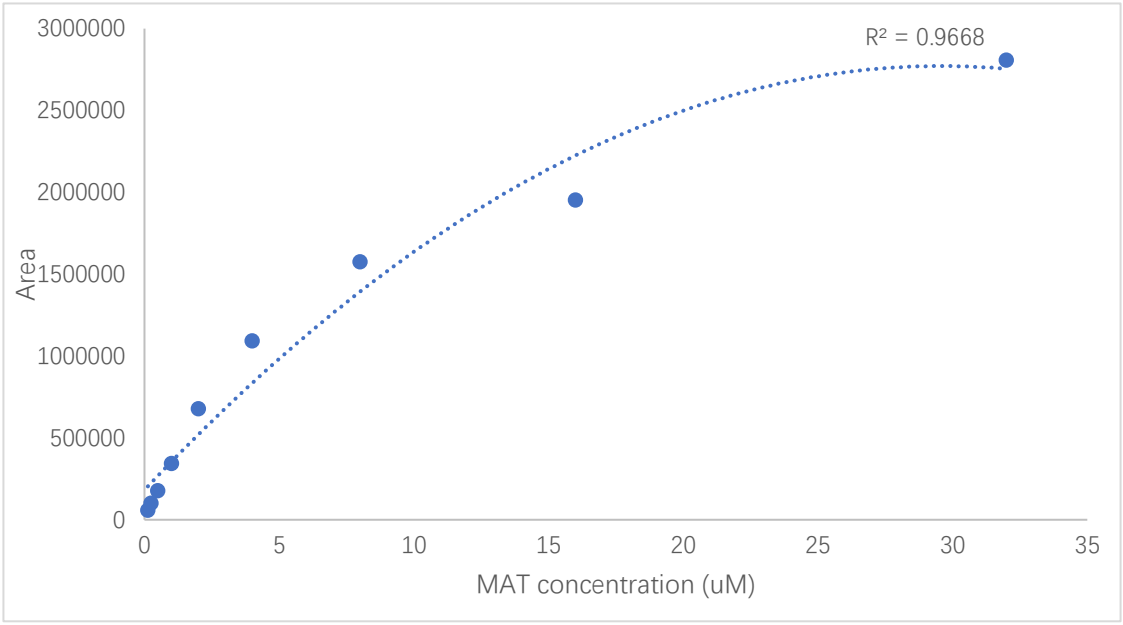

Figure 19 (C) MAT standard curve in Ca^{2+} rat liver cytosol.

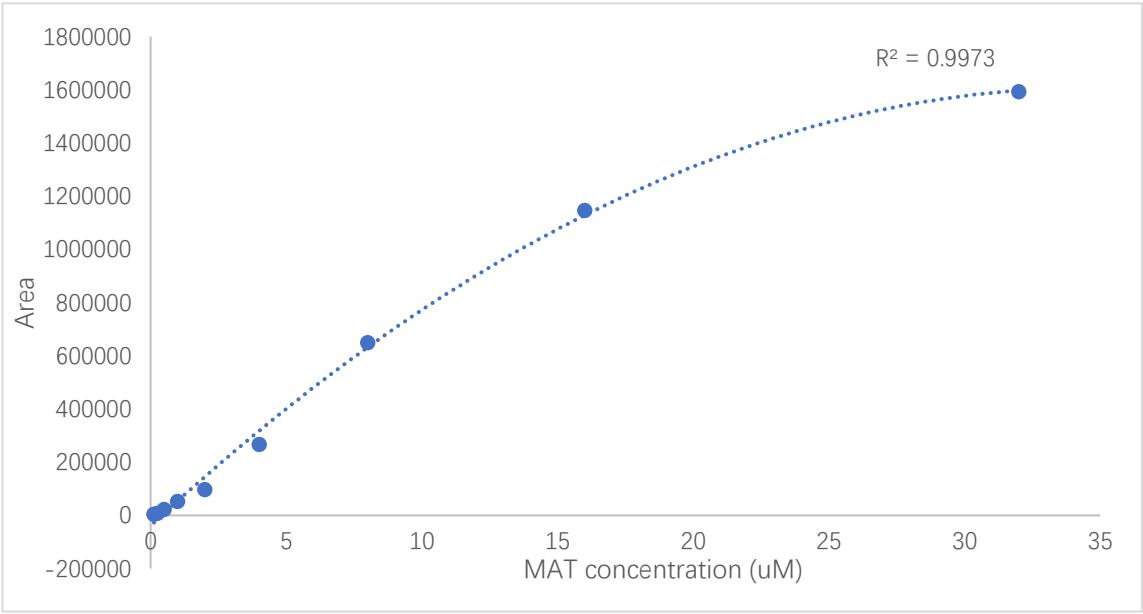

Figure 19 (D) TETA standard curve in water.

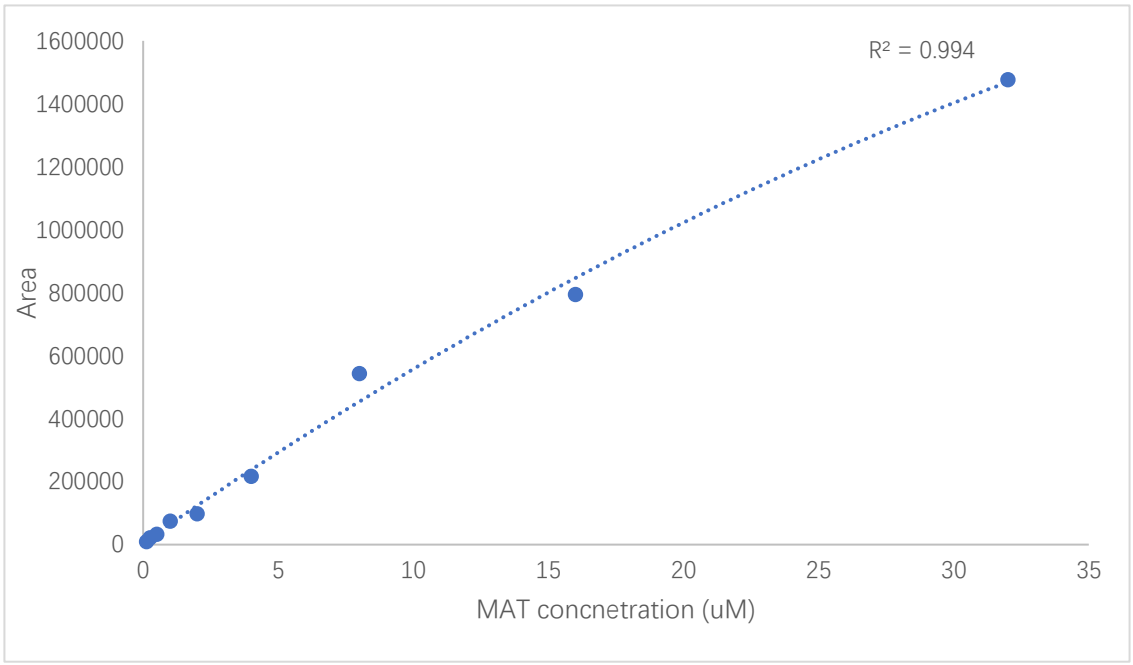

Figure 19 (E) TETA standard curve in Ca^{2+} rat liver cytosol microsome.

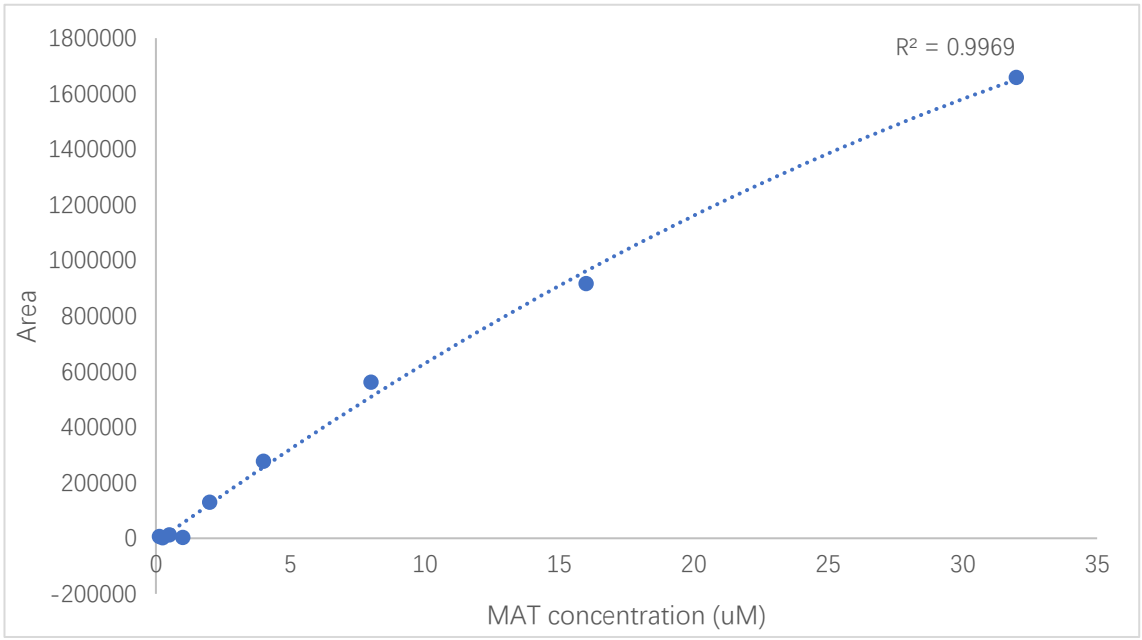

Figure 19 (F) TETA standard curve in Ca^{2+} rat liver cytosol.

Table 6 P-values between standard curves of MAT and TETA (* means $p < 0.05$)

Groups	P-value
MAT microsome ~ MAT cytosol	0.1471
MAT microsome ~ MAT water	0.02694 *
MAT cytosol ~ MAT water	0.08331
TETA microsome ~ TETA cytosol	0.2314
TETA microsome ~ TETA water	0.1525
TETA cytosol ~ TETA water	0.3283

4.6 A brief description of the assay validation for the LC-MS assays

In the first LC-MS assay, the MCF derivatisation provides not only good stability for different polyamines, but also well-shaped LC-MS peaks (figures showed in 4.2). Moreover, the R^2 of different standard curves are reliable enough to quantify the concentration of metabolites (figures showed in 4.3). The p-values in table 4 also suggests the good reproducibility of this assay. Thus, the overall validation of the first LC-MS assay is decent. The validation of the second LC-MS method mentioned in this study is not as good as the first one due to the tailing of the LC-MS peaks and the lack of an internal standard. However, the decreasing trends showed in figure 18 are obvious, which means the assay still has a great reference value.

Chapter 5 Discussion

5.1 Multiple possibilities to explain the lack of variability between enzymatic *in vitro* acetylation assays and systemic controls

As figure 16 (F) shows, without any protein source, Spd-d6 is still able to be acetylated by only AcCoA in the systems tested. This is surprising because the energy barrier is considered to be too high for spontaneous acetylation to occur under physiological conditions. Thus, the acetylation of polyamines is not supposed to occur in the absence of dedicated enzymes. Nonetheless, every negative control (preparations without any enzyme source during the reaction) showed a production of acetylated polyamines. Such phenomena require finding the most appropriate negative control and then defining it as the "best negative control". The metabolite concentration of each substrate concentration point on an enzymatic *in vitro* acetylation curve will then be subtracted from the corresponding metabolite concentration on the non-enzymatic *in vitro* acetylation curve to calculate "the real metabolite concentration". This calculated difference is the metabolite concentration acetylated only by SSAT (Figure 20 visualises the original plan of the "best negative control"). The hypothesis which led this reasoning was that after the subtraction, the results will still follow Michaelis-Menten kinetics, allowing the calculation of K_m and V_{max}. However, according to Table 5, K_m and V_{max} of all enzymatic and non-enzymatic *in vitro* acetylation are not significantly different from each other, which means that after the "control" subtraction, the "real metabolite concentration" will no longer exist. Multiple possibilities are taken into account for the dilemmas generated by this study. The loss of SSAT activity, environmental issues such as temperature and pH values in the assay protocols, the negative effects of metal ions toward SSAT in the buffer system, the (poor) quality of enzyme preparations, the method of producing liver microsome and cytosol fractions, the effect of the HFBA in the

original method, the protein concentrations used in the assay formulations, the influence of microbial contaminants, and the influence of liver microsome and cytosol fractions toward the metabolite have all been considered. Overall, these possibilities have been categorised into enzymatic problems, assay condition problems, and methodology problems. Some of these possibilities have been excluded by verification tests, while others remain to be explained or eliminated from consideration.

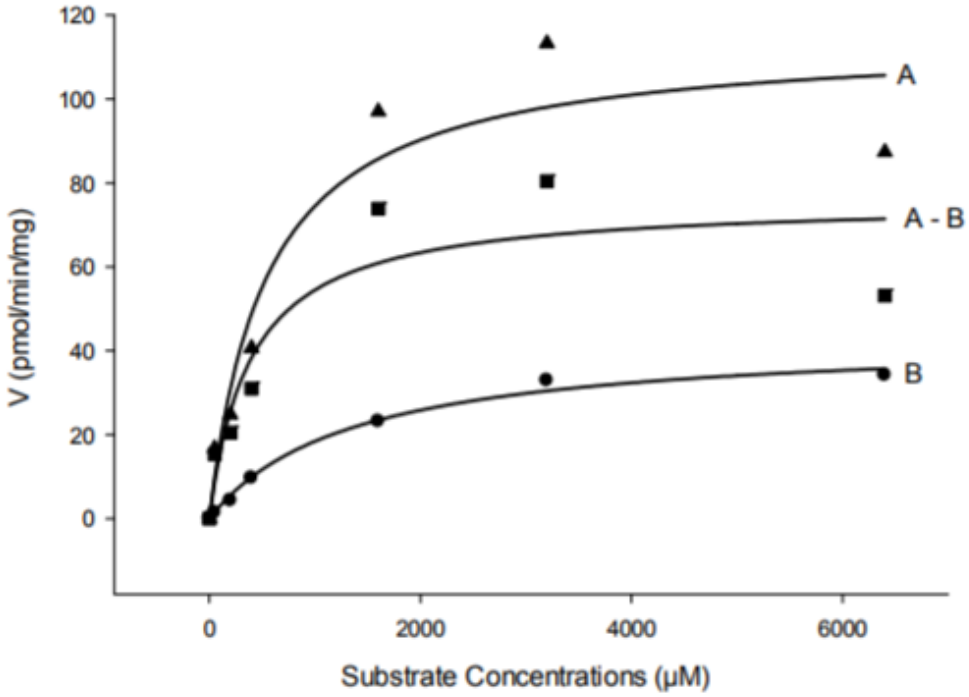

Figure 20 The original plan of setting “best negative control”.

A is the Michaelis-Menten curve for enzymatic polyamine *in vitro* acetylation assay, while B is the Michaelis-Menten curve for the “best negative control”. A - B is the curve formed after the subtraction. This figure is just the hypothesis of the research plan, not the final result or the method applied.

Beyond the wealth of reasons to explain aberrant, non-specific results, it is also extremely possible that the recorded data is just following the polyamine acetylation mechanism. The acetylation of polyamines may not require the existence of SSAT, which means SSAT may serve a different role than originally expected. Perhaps the enzyme influences the intracellular polyamine pools by other pathways outside of the traditional theory that SSAT directly acetylates

polyamines.

5.1.1 Discussion of SSAT activity from previous studies

Various studies regarding the activity of SSAT have been performed over the past few years. The methods for testing the SSAT activity that was applied in these studies are not only *in vitro* polyamine acetylation assays with liver preparations. Indeed, the methods were also quantifying the acetylated polyamines in tissues from SSAT transgenic mice and *in vitro* polyamine acetylation assays with purified SSAT.

5.1.1.1 An *in vivo* study comparing SSAT overexpression and knockout mice with SSAT wild type mice

Table 7 illustrates the comparison of SSAT-expression mice and SSAT-knockout mice with SSAT-wild type mice (Jell et al., 2007). As Table 7 shows, although SSAT-overexpression mice show significant upregulation in polyamine biosynthesis and urinary acetylated polyamines, SSAT knockout mice do not show any significant decrease in both of these two indicators, especially the urinary acetylated polyamine levels.

The urinary acetylated polyamines in this study may be a potential future indicator for SSAT activity. According to Jell and his colleagues (2017), the lack of urinary metabolites level changes in SSAT knockout mice might be caused by the low basal enzyme activities of SSAT and ODC (the basal acetylated polyamine level is too low for HPLC to detect the tiny decrease in the samples). However, it is also possible that SSAT is not acetylating polyamines in this study's samples, and the increase in urinary acetylated polyamines in SSAT-overexpression mice could be resulting from other pathways. The tissue sample preparation here (table 7) was a total

pulverisation method, with the results expected to reflect the presence of every component in a cell for the given fraction preparations. In addition, the same result was presented with TETA as the assay's metabolite: although the acetylation of TETA was significantly increased in SSAT1-overexpression mice compared with wild-type mice, the SSAT1- knockout mice showed the same levels of TETA acetylation as wild type mice (Hyvönen et al., 2013). One of the theories for this finding is that another enzyme is also participating in TETA acetylation, namely SSAT2. Since Spd also showed the same result, SSAT may have other physiological functions which support polyamine acetylation by other enzymes rather than being the only participant in acetylating polyamines. It should be mentioned that the overexpression of SSAT2 does not upregulate acetylated polyamine levels (Chen et al., 2003), which also supports this speculation.

Table 7 Summary of SSAT expression mice (SSAT-tg) and SSAT knockout mice (SSAT-ko) compared with SSAT wild type mice (SSAT-wt) (Jell et al., 2007)

Parameter	Change relative to SSAT-wt	
	SSAT-tg	SSAT-ko
Polyamine biosynthesis	↑	(↓)[a]
Urinary acetylated polyamines	↑	NQ[b]
Fat pad weight	↓	↑
Serum leptin	↓	↑
WAT acetyl-CoA and malonyl-CoA	↓	↑
WAT glucose oxidation	↑	↓
WAT palmitate oxidation	↑	(↓)
Metabolic flux	↑	(↓)
High fat diet response (weight gain)	↓	↑

(↓)[a] means that the evidence is convincing but the result is not statistically significant.
(NQ)[b] means that due to the low basal enzymatic activity of SSAT, it is difficult to quantify the acetylated polyamines by HPLC.

5.1.1.2 A SSAT *in vitro* polyamine acetylation assay using SSAT-overexpressing rat liver cytosolic fractions

Figure 21 indicates the curve generated by *in vitro* spermidine acetylation assays with SSAT transgenic mice liver cytosolic supernatant. The curve follows Michaelis-Menten kinetics, and the K_m and V_{max} of SSAT are 267 ± 46 μM and 9 ± 2 pmol/min/mg respectively (Sitar et al., 2004). These data seem to be similar to (or even lower than) the K_m and V_{max} illustrated in Table 4. Interestingly, the study that obtained figure 21 used the liver tissues from SSAT transgenic mice wherein SSAT enzymes are overexpressed. The mouse and rat livers used in this research are wild type rats and mice, which means that the overexpression of SSAT is not reflected in superior activity. It is worth mentioning that unlike the first study mentioned in this section, the assay was applied under *in vitro* conditions, indicating that the phenotype of SSAT overexpression is probably different between *in vitro* and *in vivo* conditions. Such a difference in fact supports the possibility of a different SSAT pathway, one which could be an answer for the question why under *in vivo* conditions, the urinary acetylated polyamine levels from SSAT-overexpression mice were increased, while the urinary acetylated polyamine levels from SSAT-knockout mice were unchanged. In addition, the liver cytosol fraction samples from mice without SSAT overexpression showed no evidence of any polyamine acetylation.

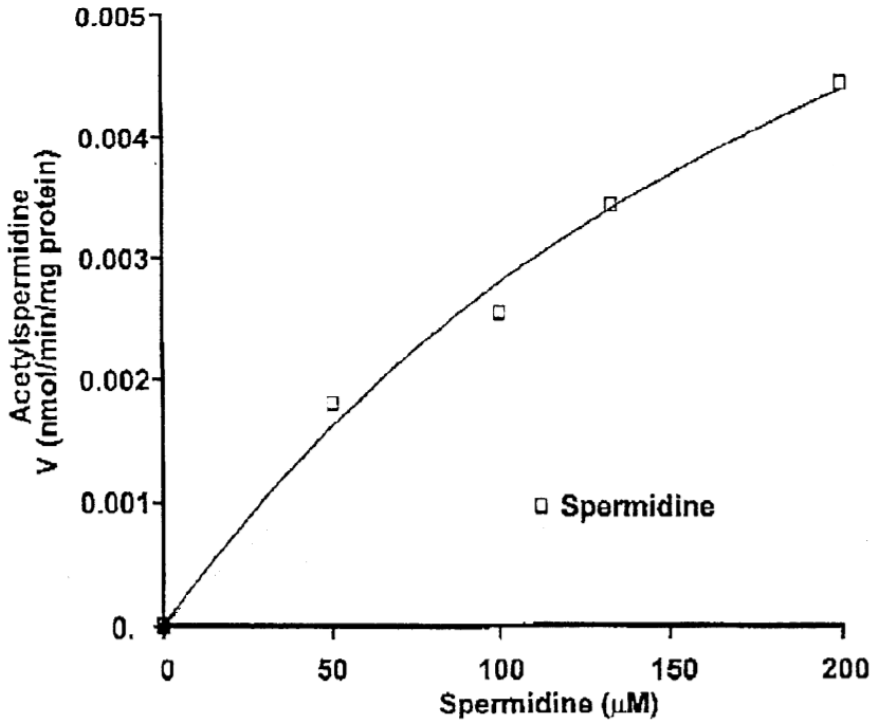

Figure 21 *In vitro* spermidine acetylation assay with transgenic mouse liver cytosol (Bras et al., 2001).

5.1.1.3 A SSAT *in vitro* polyamine acetylation assay using purified SSAT from mice liver microsome fractions

Ragione and Pegg (1983) conducted an applicable *in vitro* polyamine acetylation assay with purified SSAT enzymes. In their study, the relationship between substrate (spermidine) concentration and acetylation velocity followed Michaelis-Menten kinetics because the K_m and V_{max} values were provided (130uM and 1.3 umol/min/mg respectively). The V_{max} in their research was far higher than other studies, but the protein concentration in the formulations was extremely low (0.08ug/mL). The protein levels were seen to have a huge effect on the final result of the velocity. Application of their findings, assuming that purified SSAT concentrations in their work are similar to the mouse (and rat) liver microsome and cytosol concentrations in this research (2.68 mg/mL), the V_{max} of this study will decrease to 39 pmol/min/mg, a velocity similar to the V_{max} shown in Table 4. It turns out that with no matter what kind of enzyme sources, the SSAT

enzyme activities are still performing the same.

5.1.1.4 A SSAT *in vitro* polyamine acetylation assay using normal rat liver chromatin preparations

Blankenship and Walle (1977) stated that compared with the groups of *in vitro* polyamine acetylation assays without an enzyme source (or the groups with heat denatured enzyme sources), the acetylated polyamine levels of the groups incubated with rat liver (and kidney) chromatin were significantly higher. Due to the fact that cellular polyamine pools are highly related to cell proliferation, it was possible for chromatin to provide a better environment for a polyamine such as Spd and Spm to be acetylated by AcCoA. Moreover, despite of the significantly higher acetylation activity in assay groups with rat liver (and kidney) chromatin, the negative controls (without enzyme sources) are still showing some acetylation activity, unlike the study mentioned in 5.1.1.2, which stated that the negative controls (mice liver cytosol without SSAT overexpression) were not showing any acetylation activity. This lack of acetylation activity was similar to the data provided in this study, which found that non-enzymatic negative controls were still producing acetylated polyamines as a function of AcCoA.

Blankenship & Walle's study also provided the relationship between protein concentrations of chromatin and radioactivity (which represented the spermidine acetylation ability in this current study) Figure 22 shows a clear increasing linear trend totally opposite to that presented in figure 18 (A) and figure 18 (B) from this research. Interestingly, the increasing trend was not slowing down when the protein concentration reached its highest point. The linear trend could be caused by the low concentration choices of enzymes. However, the results also suggest that chromatin was providing a better environment for polyamine acetylation rather than just acting as an enzyme source.

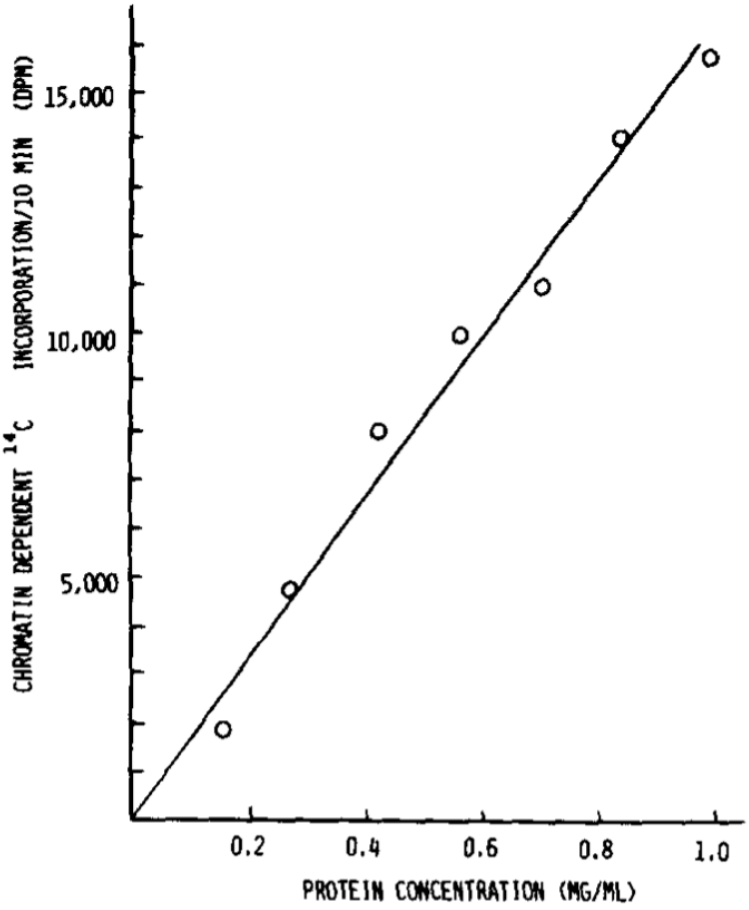

Figure 22 Chromatin protein concentration dependence of spermidine acetylation ability (Blankenship and Walle, 1977).

In short, three studies are mentioned in this section: an *in vivo* study with SSAT overexpression mice and SSAT knockout mice, an *in vitro* study with liver tissue cytosol from SSAT-overexpression mice and an *in vitro* study with purified SSAT from liver tissues of normal mice. The *in vivo* study showed that SSAT-knockout mice are not different compared to SSAT-wild type mice in urinary acetylated polyamine levels, while two *in vitro* studies show the diversity in enzyme sources is not affecting assay performance. The results of these three studies are all similar to data provided in Table 4. Moreover, according to Table 5, there is no significant differences between negative controls (assays without SSAT enzymes) and enzymatic assay groups, which means SSAT is not performing statistically significant measurable activities under

in vitro conditions. Thus, this study speculates that the acetylation of polyamines may not require the presence of SSAT. The increase of acetylated polyamine levels in SSAT overexpression may be caused by other pathways rather than the direct catalysis of SSAT.

5.1.2 Multiple potentially novel SSAT pathways

Combining the data obtained from this research and the studies mentioned above, results could be summarised into the following points:

1) There is no difference between different *in vitro* polyamine acetylation assays. Including the studies mentioned in section 5.1.1.2 and 5.1.1.3, K_m and V_{max} are all similar to each other.
2) It should be mentioned that in the study presented in section 5.1.1.1, although SSAT-overexpression mice showed obvious increases in urinary acetylated polyamine levels, SSAT-knockout mice didn't show any significant decrease, which could be a persuasive evidence for the incapability of SSAT acetylating polyamines.
3) In this research, various kinds of controls (*in vitro* polyamine assays without enzyme sources or with denatured enzyme sources) are applied to formulations to find out the effects of enzyme sources on the results of acetylated polyamine levels. However, no difference was found. Moreover, nonenzymatic controls are producing equivalent levels of acetylated polyamines.
4) The Ca^{2+} rat liver microsome and cytosol concentrations and the acetylated polyamine response surprisingly present a negative correlation. Nevertheless, the study mentioned in section 5.1.1.4 represented a totally opposite protein concentration optimisation result, where acetylated polyamine responses sharply increased with rat liver chromatin concentrations (Figure 22).
5) In the study mentioned in section 5.1.1.4, it was clearly presented that the groups with

enzymatic sources (rat liver chromatin) showed obvious higher levels of acetylated polyamines compared to non-enzymatic controls, which is different from this study (the enzyme sources are liver microsome and cytosol fractions).

Since this research speculates that SSAT is not capable of acetylating polyamines, and polyamines are able to react with AcCoA directly, the investigation of new pathways should be focused on AcCoA metabolism because it is the only factor that could influence acetylated polyamine levels under *in vitro* conditions tested. Figure 23 represents multiple AcCoA metabolic pathways in mitochondria and the cytosol. As figure 23 shows, AcCoA is a central metabolite that can not only provide materials for synthesis of fatty acids in the cytoplasmic matrix, but also support the oxidation of carbohydrates and fat in mitochondria (Pietrocola et al., 2015).

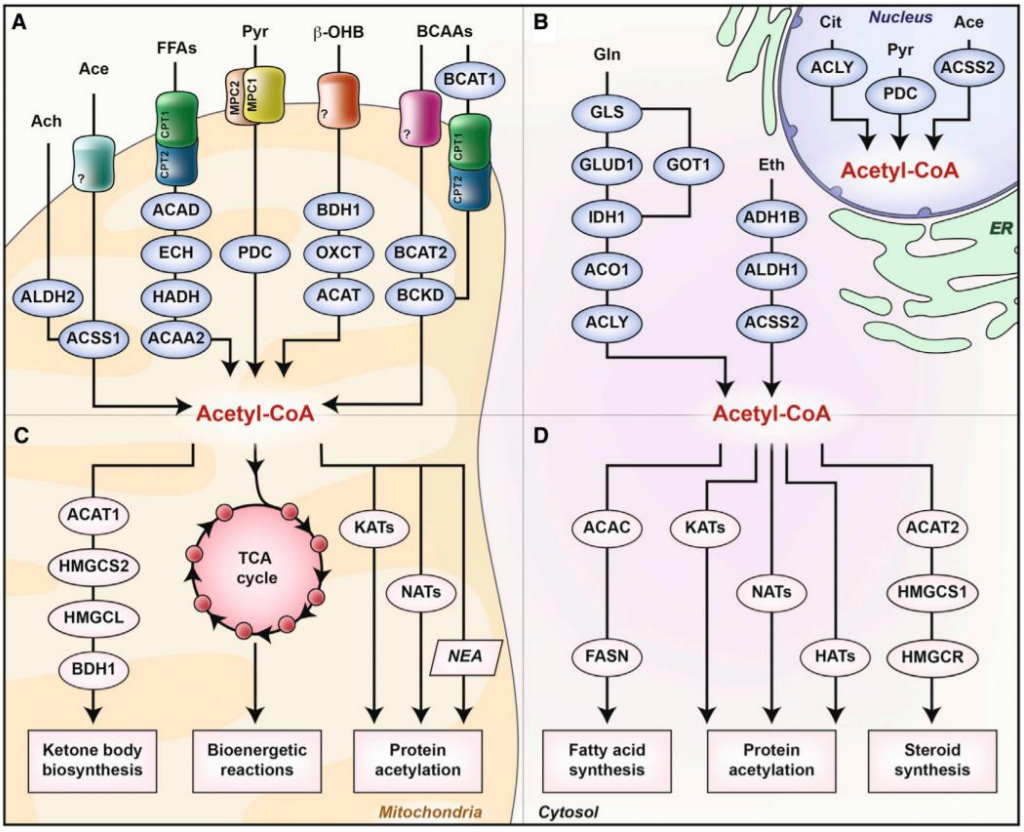

Figure 23 The metabolism of AcCoA in mitochondria and cytosol in mammalian cells (Pietrocola et al., 2015).

Abbreviation in this figure: Ach, acetaldehyde; Ace, acetate; FFAs, free fatty acids; Pyr, pyruvate; β-OHB, D-β-hydroxybutyrate; BCAAs, branched-chain amino acids; BCAT1, branched chain amino-acid transaminase 1; ALDH2, aldehyde dehydrogenase 2 family; ACSS1, acyl-CoA synthetase short-chain family, member 1; CPT1, carnitine palmitoyltransferase 1; MPC2, mitochondrial pyruvate carrier 2; MPC1, mitochondrial pyruvate carrier 1; ACAD, acyl-CoA dehydrogenase; ECH, enoyl-CoA hydratase; HADH, hydroxyacyl-CoA dehydrogenase; ACAA2, acetyl-CoA acyltransferase 2; PDC, pyruvate dehydrogenase complex; BDH1, 3-hydroxybutyrate dehydrogenase, type 1; OXCT, 3-oxoacid CoA transferase; ACAT, acetyl-CoA carboxylase; BCAT2, branched chain amino-acid transaminase 2; BCKD, branched-chain a-ketoacid dehydrogenase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGCL, 3-hydroxymethyl-3-methylglutaryl-CoA lyase; BDH1, 3-hydroxybutyrate dehydrogenase, type 1; TCA, tricarboxylic acid; KATs, lysine acetyltransferases; NAT, N^{α} acetyltransferase; NEA, non-enzymatic acetylation; Gln, glutamine; GLS, glutaminase; GLUD1, glutamate dehydrogenase 1; ACO1, aconitase 1; ACLY, ATP citrate lyase; GOT1, glutamic-oxaloacetic transaminase 1; ADH1B, alcohol dehydrogenase IB; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; ACSS2, acyl- CoA synthetase short-chain family, member 2; ACAC, acetyl-CoA carboxylase; FASN, fatty acid synthase; HATs, histone acetyltransferases; ACLY, ATP citrate lyase.

The effects caused by SSAT overexpression, summarised from different studies, are presented in figure 24. The overexpression of SSAT leads to such effects as an increase in glucose tolerance and insulin sensitivity, and a decrease in lipid accumulation. These effects are categorised into different causes including reduced polyamines and increased oxidation damage, decreased AcCoA and ATP levels, increased polyamine acetylation and other molecular level changes (Pegg, 2008). However, with the reconsiderations of SSAT functions in this research, these effects caused by SSAT overexpression may have new explanations.

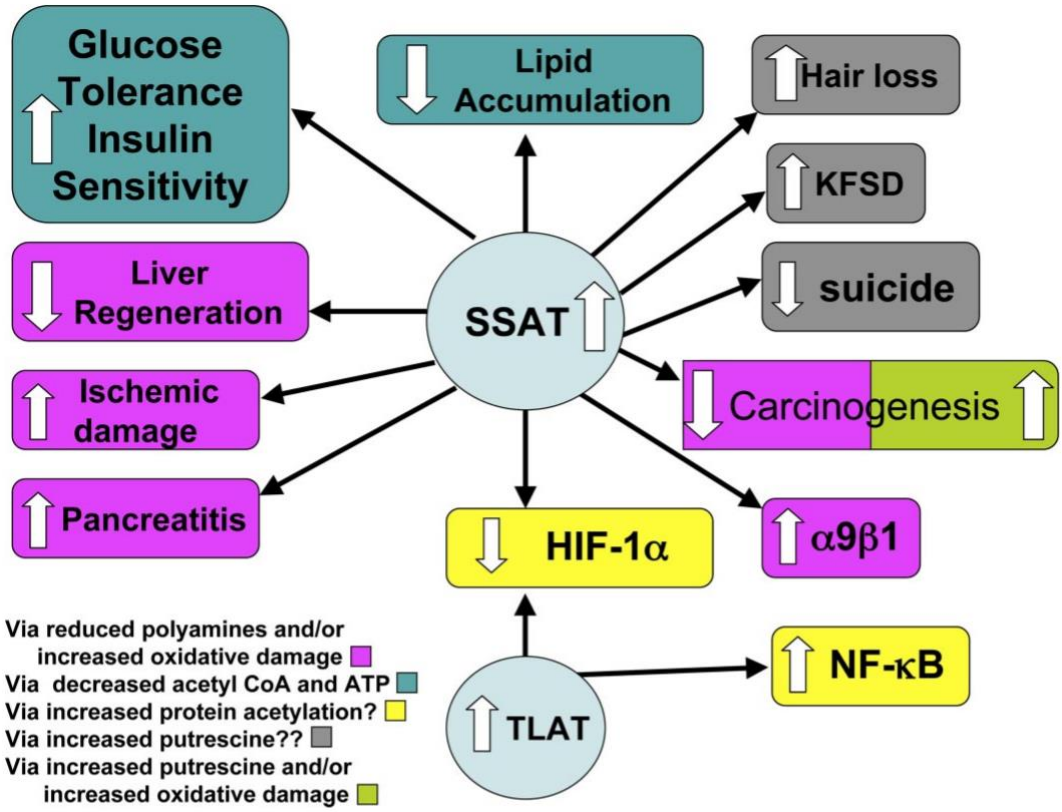

Figure 24 Summary of the effect caused by SSAT overexpression (Pegg, 2008).

Several possible pathways based on the data in this research (and previous studies) are discussed further. Reasonable possibilities for the pathways' abilities to explain uncharacterised observations will also be discussed.

5.1.2.1 Possible pathway 1: SSAT inhibits AcCoA from reacting with other substances

Due to the fact that polyamines are able to directly react with AcCoA, and the presence of SSAT does not affect the production of acetylated polyamines under *in vitro* conditions (Table 4), this research speculates that SSAT may inhibit other AcCoA-dependent reactions in order to increase the competitiveness of polyamines reacting with AcCoA. Theoretically, such competitiveness could only take place under *in vivo* conditions because no other AcCoA-dependent pathway exists under *in vitro* conditions. One of the most likely pathways is fatty acid synthesis due to the leaner phenotype of SSAT-overexpression mice (and the fatter phenotype of SSAT-knockout mice) (Jell et al., 2007). This proposed pathway can also explain why acetylated polyamine levels were increased in SSAT-overexpression mice, while no significant changes occurred in acetylated polyamine levels of SSAT-knockout mice — because the increase of acetylated polyamine levels is not caused by the acetylating ability of SSAT, but by the side effects of SSAT functions: inhibiting other AcCoA dependent pathways to increase the priority of AcCoA reacting with polyamines.

Other AcCoA dependent pathways, such as TCA and protein acetylation, are also potential target pathways that SSAT may have inhibitory effects on. Protein acetylation was considered as a modification process of intracellular proteins to regulate the cellular response toward AcCoA (or AcCoA/CoA ratios), which adjusts several biological pathways (James et al., 2018). However, after the discovery of an extremely low lysine N-acetylation *in vivo*, such consideration has been changed (Rardin et al., 2013). Similar to the data provided in this research, the lysines on mitochondrial proteins are able to spontaneously react with AcCoA *in vitro* and can also be acetylated *in vivo* without any known enzyme presence. It turns out that AcCoA and other acyl-CoAs (such as malonyl-CoA and succinyl-CoA) may have a “carbon stress”, which means long-

term exposure to AcCoA leads to cell damage related to aging and degenerative diseases (Trub and Hirschey, 2018; James et al., 2018). Such phenomena also support the conclusion that SSAT is not participating in polyamine acetylation: the acetylated polyamine levels depend on AcCoA availability, and the “carbon stress” may also be functional in polyamine acetylation.

5.1.2.2 Potential pathway 2: SSAT accelerates the transportation of AcCoA from mitochondria to the cytoplasmic matrix

The majority of AcCoA in a mammalian cell is normally generated in mitochondria because multiple pathways that synthesise AcCoA are also taking place in mitochondria (such as fatty acid β-oxidation, pyruvate decarboxylation and amino acid catabolism) (Pietrocola et al., 2015). AcCoA are then transported into the cytoplasmic matrix since the reactions only occur in the cytosol. Fatty acid and steroid synthesis are examples of this type of reaction. Since this research indicates that the acetylation of polyamines is not dependent on SSAT but only on AcCoA, and while the SSAT-overexpression mice have higher urinary acetylated polyamine levels (Jell et al., 2007), SSAT may have functions which support the transportation of AcCoA from mitochondria to the cytosol, because according to Pegg (2008), SSAT is basically a cytosolic enzyme.

This potential pathway is supported by findings established by Holst and his colleagues (2008), which localised the subcellular distribution of SSAT. After L56Br-C1 cells were previously treated with N^1 ,N^{11}-diethylnorspermine (10uM for 24h), SSAT was found in mitochondrial fraction with very high responses in both SSAT activity and western bolt analyses. SSAT was also found in nuclear fractions and cytosolic fractions, but with a lower response in activity. It suggests that SSAT indeed exists in mitochondria, especially when the cells are suffering from cytotoxic insults. SSAT performs the same as Pegg (2008) described: the enzyme is strongly induced by polyamines, polyamine analogues and some toxic reagents. The

predominant existence of SSAT in mitochondria is convincible evidence for the conjecture that SSAT supports and accelerates the transportation of AcCoA from mitochondria to the cytosol, although the physiological significance of these results remains unclear. It should be mentioned that the controls (the L56Br-C1 cell groups without previous treatment of N^1 ,N^{11}-diethylnorspermine only performed with low SSAT activity in mitochondrial fractions, while two other enzyme sources (nuclear fractions and cytosolic fractions) did not show any SSAT activity. These results are similar to the research mentioned in section 5.1.1.2 and the data obtained from this research. It also suggests that SSAT may not have any direct catalysis function on polyamines.

This possible pathway may give many explanations for why SSAT overexpression leads to the increase of glucose tolerance and insulin sensitivity. In respiratory actions, TCA is one of the biological pathways which utilises AcCoA in mitochondria, and is created by pyruvate decarboxylation. Glucose is initially degraded through glycolysis, forming two molecules of pyruvate. Pyruvates are then decarboxylated, which produces two molecules of AcCoA. Finally, AcCoA is involved in TCA pathways and thereafter consumed (Figure 25). In this process, if AcCoA molecules produced by pyruvate decarboxylation are transported out of the mitochondria by the accelerating effect of SSAT on citrates, pyruvate decarboxylation will be accelerated to fulfil the AcCoA requirement of TCA, which leads to the higher demand of pyruvate. In addition, the lack of ATP is also forcing the reaction to be accelerated. It consequently increases the consumption of glucose, reflected as an increase in glucose tolerance. This is potential evidence to support the existence of this putative pathway, namely that SSAT may not serve as an acetyltransferase, but a supporting enzyme to accelerate the transportation of AcCoA from mitochondria to the cytoplasmic matrix.

(A) $Glucose + 2\ NAD^+ + 2\ ADP + 2\ P_i \rightarrow 2\ Pyruvate + 2\ CO_2 + 2\ ATP + 2\ H_2O$

(B) $2\ Pyruvate + 2\ NAD^+ + 2\ CoA \rightarrow 2\ AcCoA\downarrow + 2\ CO_2 + 2\ NADH + 2\ H^+$

(C) $2\ AcCoA + 6\ NAD^+ + 2\ FAD + 2\ GDP + 2\ P_i + 4\ H_2O \rightarrow 4\ CO_2 + 6\ NADH + 6H^+ + 2\ FADH_2 + 2GTP + 2\ CoA$

Figure 25 (A) Glycolysis (B) Pyruvate decarboxylation (C) TCA

This pathway is also an exciting explanation for decreased fat accumulation: the low AcCoA levels in mitochondria of SSAT-overexpression cells stimulate fatty acid β-oxidation, consuming more fat than normal cells. As Table 7 shows, the overexpression of SSAT leads to a total increase in metabolic flux and glucose, while in SSAT-knockout mice, a clearly opposing result can also be provided, which means the pathway that SSAT accelerates, the transportation of AcCoA from mitochondria to the cytoplasmic matrix, is relatively more reliable.

5.1.2.3 Possible pathway 3: SSAT is a carrier protein which transports AcCoA to other cellular locations

The research mentioned in section 5.1.1.4 indicated that rat liver and kidney chromatin have strong polyamine acetylation activity, and a clear comparison between enzymatic and non-enzymatic assays was provided. It illustrated that in the presence of rat liver chromatin, polyamines are acetylated at levels far higher than the non-enzymatic assay groups, which are different from the data provided by this research. It was originally concluded that the acetyltransferase enzyme (which was later identified as SSAT by Matsui, Wiegand and Pegg in 1981) that acetylating the polyamines exists in chromatin (Blankenship and Walle, 1977). However, according to Pegg (2008), SSAT is a cytosolic enzyme, and although the enzymatic *in vitro* polyamine acetylation assays with liver cytosolic preparations in this research (and the study

mentioned in section 5.1.1.2) are not able to provide a better enzymatic activity result, recent studies also localised SSAT on both mitochondrial and nuclear pellet membrane preparations. Moreover, according to Holst and his colleagues (2008), the western blot analysis presented a remarkable increase in SSAT location to nuclear membranes (although such a result was not replicated in SSAT detection by immune-gold labelling in the same study). Various research indicates that nuclear preparations (such as chromatin and nuclear fractions) show better polyamine acetylation activity than cytosolic preparations (Blankenship and Walle, 1977; Holst et al., 2008). Due to the polycationic nature of polyamines, polyamines tend to bind to intracellular anions, especially nucleic acids. Take *Escherichia coli* as an example: 90% of intracellular spermidine is found in a form bound to RNA. It was also reported in recent years that polyamines are capable of binding to DNA, stabilising DNA double stranded structure, and enabling intermolecular interactions (Gevrekci, 2017; Katz et al. 2017). Therefore, this research speculates that the presence of liver chromatin in an *in vitro* assay was not serving as an enzyme source, but providing a better environment for non-enzymatic polyamine acetylation. SSAT, therefore could be a carrier protein which transports AcCoA into the cell nucleus instead of acting as an acetyltransferase which directly acetylates polyamines.

Owing to the non-enzymatic controls which were obviously lower than the chromatin groups provided by Blankenship and Walle (1977), it is hard to prove the incapability of SSAT to acetylate polyamines. Nevertheless, the polyamine acetylation activity in nuclear fractions was not the same response as SSAT detection by western bolt analysis revealed. As figure 26 shows, the western bolt analysis detection of mitochondrial and nuclear fractions is at a similar level. Meanwhile, as mentioned previously, the polyamine acetylation activity in nuclear fraction groups was far lower than mitochondrial fractions (Holst et al., 2008). The same amount of SSAT enzymes were not performing a similar polyamine acetylation activity, which also suggests a different pathway of SSAT for polyamine acetylation in cells. The conjecture that SSAT is a carrier

protein to transport AcCoA from mitochondria to different subcellular locations such as the nucleus, to fulfil some specific cellular functions, is one possible pathway. In addition, this conjecture seems to be reliable because one of the functions that polyamine acetylation serves is to regulate the polyamine level in cells due to the cellular effects of polyamines on cell proliferation -- which is strictly relevant to nuclear activity (Bae et al., 2018).

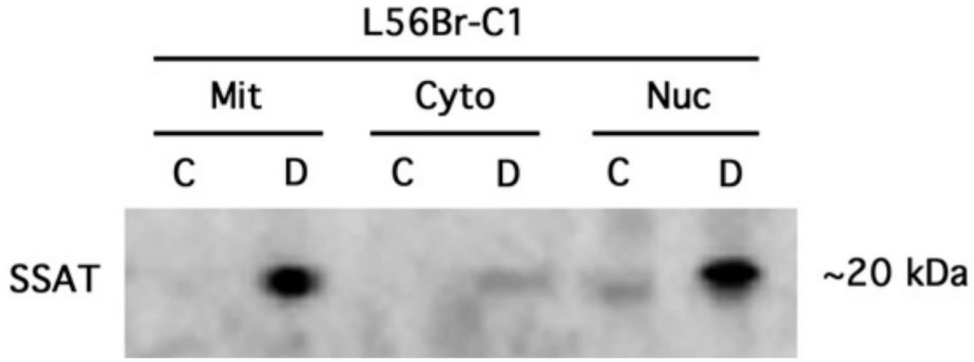

Figure 26 The western blot analysis of different subcellular fractions of N^1 ,N^{11}-diethylnorspermine treated L56Br-C1 cells (Holst et al., 2008). Mit: Mitochondria fractions; Cytos: Cytosolic fractions; Nuc: Nuclear fractions.

In addition, very small amounts of SSAT were detected in western blot analysis after cells were treated with N^1, N^{11}-diethylnorspermine, which is different from other earlier SSAT studies (Holst et al., 2008). It is possible that after the cytotoxic induction of N^1, N^{11}-diethylnorspermine, SSAT activity was activated and accelerated, transporting AcCoA to other locations as a result. The most possible pathway could be from mitochondrial to nuclear locations, which passes through the cytoplasm. This conjecture could explain the distribution of SSAT in L56Br-C1 cells, and also supports the pathway which proposes that SSAT is a carrier protein which transports AcCoA to other cellular locations.

5.1.3 General discussion of all three possible pathways

Three possible new pathways have been discussed in previous sections. All of these pathways aim to explain why SSAT is not performing any acetylation activity *in vitro* in this research (as well as in some previous studies), while the overexpression of SSAT indeed increases the acetylated polyamine levels. Three possible new pathways are all established based on the fact that SSAT is not able to directly catalyse polyamine acetylation reactions, while AcCoA is able to acetylate polyamines spontaneously. The question arises regarding what kind of functions are SSAT enzymes performing in cells. The pathway 1 proposal is the inhibition of other AcCoA dependent pathways such as fatty acid synthesis. This pathway is supported by various changes in phenotypes of SSAT overexpression mice and SSAT knockout mice (Jell et al., 2007). The second pathway is the accelerative function for transporting AcCoA from mitochondria to the cytoplasm, and pathway 3 presents the idea that SSAT may be a carrier protein which transports AcCoA to other subcellular locations. Pathways 2 and 3 are very similar, and both of them are supported by the SSAT subcellular distribution study (Holst et al., 2008) as well as metabolic changes in SSAT overexpression phenotypes (Jell et al., 2007). It is possible for all three putative pathways to be tenable at the same time because none of them are conflicting with each other.

Since all of these pathways are based on the incapability of SSAT acetylating polyamines and the spontaneous acetylation of polyamines with AcCoA, they still have some fallacies remaining to be discussed. Some studies show that only SSAT-overexpression tissue preparations are able to provide a good acetylated polyamine response (Bras et al., 2001), while other older studies had presented obvious acetylation activity of SSAT without any previous treatment (Ragione and Pegg, 1983; Blankenship and Walle, 1977). In addition, some studies were not able to provide any polyamine acetylation responses in some situations (Bras et al., 2001; Holst et al., 2008). These phenomena are all denying the conclusions of this research, which are also the basis of the three possible pathways presented herein. Even so, it is still necessary to reconsider the

SSAT functions in cells due to the various conflicting results and conclusions of this research and previous studies.

5.2 The possible mistakes made by this research

Although multiple convincible new pathways have been discussed in former sections based on the data obtained from this research and previous studies, the possibilities of mistakes caused by experimental conditions and operations cannot be ignored since the results provided in this research are totally different from the hypothesis, and the conclusions are rejecting the original theories. Therefore, this section will discuss the potential mistakes made by this research, and what kind of errant conclusions might arise. How to fix identified errors for improved future studies will also be taken into account.

5.2.1 The lack of SSAT enzyme in liver preparations

According to Pegg (2008), SSAT activity is only detectable with some pre-treatments including polyamines, polyamine analogues, cytotoxic insults, SSAT purification and SSAT overexpression. Multiple research studies support that SSAT activity can only be detected after the tissues or cells are previously treated by cytotoxic insults or polyamine analogues (Pegg, 2008). In addition, as the study in section 5.1.1.2 illustrated, no acetylation activity was detected even in the presence of rat liver cytosol without SSAT overexpression (Bras et al., 2001). Furthermore, SSAT protein levels are not detectable in all three subcellular fractions (mitochondrial, cytosolic, and nuclear fractions) by western blot analysis if the cells are not previously treated by cytotoxic substances (Holst et al., 2008). All these phenomena are suggesting that SSAT may not even exist in cells which are not previously induced. However, in this research, liver tissues are from healthy

SD rats and C57BL/6 mice, both of which are tissues without any SSAT-inductive pre-treatment or SSAT overexpression. Meanwhile, this research is not able to provide a direct enzyme detection such as western blot analysis, which means it is possible that there was no SSAT enzyme in the mouse (and rat) liver microsome and cytosol fractions in this research. If the mistake truly exists, the conclusion that SSAT is not performing any polyamine acetylation activity might be unreasonable. In order to exclude this potential mistake, a western blot result needs to be provided, and a pre-treatment of tissue preparations is also required.

5.2.2 The loss of SSAT enzyme activity

Multiple studies have indicated that SSAT is a very sensitive enzyme, and its life-span is also very short. After a specific antibody for SSAT was identified, rapid turnover and half-life analyses of SSAT were confirmed to be less than 15 min in thioacetamide-treated rats (Persson and Pegg, 1984). Thus, the possible SSAT enzymatic activity loss during this research should be taken into account. To obtain the mouse (and rat) liver microsome and cytosolic fractions, two methods were applied: the differential centrifugation method and the Ca^{2+} sedimentation centrifugation method.

Due to the fact that differential centrifugation requires 105,000 g rcf to separate microsomes and cytosolic fractions from liver tissue S9 fractions, a qualified ultracentrifuge is required to reach that speed. Unfortunately, the Thermo Scientific Sorvall WX+ Ultracentrifuge (Cat # 46900) and Thermo Scientific Fiberlite F50L-8x39 Rotor (Cat # 096-087051) used in this research for liver S9 fraction centrifugation were located at another institution (Auckland University), and the mouse (and rat) liver microsome and cytosolic fractions could not be immediately stored back at -80 °C after centrifugation was completed. Instead, the liver preparations were stored in a box of ice during the time period for transporting them. Such delay might be responsible for the loss of SSAT activity. In addressing the liver preparations made by the Ca^{2+} sedimentation centrifugation

method, it was possible to store samples at -80 °C immediately after the preparations were completed. However, due to equipment limitations, the centrifuge's lowest temperature is between 4 ~ 7 °C instead of 0 ~ 4 °C. The disparate temperature settings may have had potentially negative effects on SSAT enzyme activity. To avoid such adverse conditions, ways must be found to shorten the time period of transporting samples and decreasing the centrifuge temperature capability.

5.2.3 The overwhelmingly high spontaneous polyamine acetylation

Theoretically, non-enzymatic polyamine acetylation should not occur without any enzyme source because of the high energy barrier for such spontaneous reactions under physiological conditions. In addition, Pegg (2018) stated that SSAT activity under *in vivo* conditions is extremely low, so the results of assays which aim to measure SSAT activity by quantifying acetylated polyamine levels may be overestimates due to the non-enzymatic acetylation and acetylation carried out by other proteins. Although this research is carried out under *in vitro* condition, such effects may also exist. However, this research still presents equally high spontaneous polyamine acetylation responses consistent with all other enzymatic assays. Moreover, as previously mentioned, the K_m and V_{max} presented in this research are similar to former SSAT studies (Ragione and Pegg, 1983, Bras et al., 2001). It suggests that the spontaneous polyamine acetylation in this research may be abnormally higher than other studies (despite the lack of a clear non-enzymatic control).

One of the most likely reasons for the observed highly spontaneous reaction levels in this study could be microbial contamination. Both prokaryotic and eukaryotic cells require polyamines for cell proliferation (Tabor and Tabor, 1984), and bacteria also have enzymes which are capable of acetylating polyamines. For example, in *Bacillus subtilis*, the overexpression of

BltD gene leads to the acetylation of spermidine and spermine (the sequence of *BltD* is similar to several bacterial and mammalian acetyltransferase) (Woolridge et al., 1999). Although equipment and materials such as Eppendorf tubes, buffers and buffer containers may contain some bacteria, they could be easily sterilised via autoclaving. However, chemicals including TETA, Spd and AcCoA are also potential contaminant carriers, and it is relatively difficult for them to be sterilised due to the chemical stability issues. Thus, in this research, substrates are treated with HFBA and then dried by air before use to decrease the influence of microbial, and such methods made the response of spontaneous acetylation slightly decreased (data not shown). In addition, this step causes a dramatic pH drop in the final assay mixture (from 7.4 to 3.0). Although the pH of the assay mixture is readjusted to 7.4 before the *in vitro* polyamine acetylation assays were applied, the effects of such HFBA treatment on *in vitro* polyamine acetylation assays and polyamines in general are not clear. Therefore, the direct cause of this slight decrease could not be firmly confirmed as a result of the sterilising effects from HFBA.

It should be mentioned that a plate count for each chemical via trypticase soy agar (TSA) medium was also applied in this research to find out if these chemicals were contaminated. As the results showed, spermidine used in this research seemed to be contaminated (with numerous yellow bacterial colonies on the TSA plates), while TETA was totally free from contamination (data not shown).

5.2.4 The escape of acetylated polyamines from the total accumulation

Due to the fact that this research used mouse (and rat) liver tissue preparations as enzyme sources for the *in vitro* polyamine acetylation assays, SSAT is not the only enzyme existing in the prepared systems. Multiple enzymes related to polyamine synthesis and catabolism such as APAO, ODC and SMO are also included in the liver tissue preparations. Moreover, in an enzyme activity

assay which aims to quantify the metabolites as the result, the final response is the accumulation of acetylated polyamines instead of the total production of them. According to Pegg (2018), as long as SSAT is overexpressed or finely induced, the nonenzymatic acetylation should not be a problem because it is just a very small part of the total acetylation. Since this research did not use any induction or overexpression method for SSAT, even a tiny escape of acetylated polyamines in the system could influence the result dramatically. Thus, the escape of acetylated polyamines from the total accumulation must be taken into account. The possibility that the tiny SSAT activity is covered by the other enzyme activities which support the escape of acetylated polyamines still exists. Such possibility could be supported by figure 18 (A) and figure 18 (B) in this research. As figure 18 (A) and figure 18 (B) illustrate, with the increase of Ca^{2+} rat liver tissue preparation concentrations, the MAT response decreased. If SSAT was very functional in this research, the MAT response was supposed to be increased with the protein concentrations of Ca^{2+} rat liver tissue preparations. It suggests that the acetylated polyamines may escape from the total accumulation due to other components in Ca^{2+} rat liver preparations.

Various enzymes are potentially included in the escape of acetylated polyamines. For instance, it is possible for acetylated polyamines to be transformed back to normal polyamines by APAO, an enzyme that participates the catabolism of polyamines. It is also possible that protein in the system absorbs the acetylated polyamines, which leads to the loss of total acetylated polyamine accumulation. If this conjecture is true, these effects on acetylated polyamines escaping must be higher than SSAT activity. To avoid these effects, using APAO inhibitors could be an appropriate method. However, the rat liver microsome and cytosol fractions illustrated in figure 18 (A) and figure 18 (B) were prepared by Ca^{2+} sedimentation centrifugation method, and the effects of Ca^{2+} on SSAT and other polyamine-related enzymes remains unknown. It is hard to draw the conclusion that such a decrease is caused by APAO or a potential absorbing effect of proteins.

5.2.5 General discussion of all possible mistakes

This section discussed several possible mistakes made by this research: 1) the activity of SSAT was absent, 2) the SSAT enzyme itself was absent, 3) the spontaneous acetylation is too high, and 4) the target metabolites are escaping. It is necessary to emphasise that these are not mistakes which have been made. Instead, these are conjectures based on speculations of this research: SSAT does have activity, but for some reason, the enzyme was not present in the preparations used. Thus, these possible mistakes are totally irrelevant to the proposed new SSAT pathways mentioned.

In addition, this research uses Michaelis-Menten kinetics to measure the SSAT enzyme activity and velocity. However, due to the *in vitro* condition of polyamine acetylation assays, AcCoA is more likely to be a substrate instead of a cofactor because the *in vitro* environment is not able to recover CoA back to AcCoA. This fact leads to a problem that Spd (or TETA) acetylation is a reaction with multiple substrates, and Michaelis-Menten kinetics may not be an appropriate tool to measure the activity of SSAT.

This research used the liver tissues from healthy mice and rats, and it is possible that SSAT enzyme or the SAT gene was not activated in such specimens. Instead of SSAT overexpression or other treatments on rats and mice, it could be feasible to use animals with diabetes or obesity to find out if diseases are influencing the activity of SSAT.

Chapter 6 Conclusion

6.1 Overall conclusion

This research investigated the SSAT enzyme activity via *in vitro* polyamine acetylation assays using Michaelis-Menten kinetics. The enzyme sources in this research were healthy mouse (and rat) liver microsome (or cytosol) fractions prepared by a differential centrifugation method and a Ca^{2+} sedimentation centrifugation method. The substrates were Spd, Spd-D6 and TETA respectively. The original purpose of this research was to establish a method which uses Spd-D6 to precisely measure the activity of SSAT. By using Spd-D6, the endogenous Spd and AcSpd could be ignored, which means the results are able to be compared with the results of TETA, an exogenous polyamine that could be acetylated by SSAT2. However, the results presented in this research were totally different from the hypothesis: *in vitro* SSAT activity assay produced the same level of acetylated polyamines as nonenzymatic *in vitro* polyamine acetylation assays (which were also considered as nonenzymatic controls in this research). Therefore, multiple conjectures were established by this research to explain such phenomena. Basically, there are two major categories of conjectures: one is the incapability of SSAT to acetylate polyamines since according to this research, the presence of SSAT was not a compulsory requirement for the polyamine acetylation reaction. Another possibility is that SSAT is able to acetylate polyamines, but for some reason, such acetylating ability was not present in this research. The first category includes three new possible pathways: 1) SSAT inhibits AcCoA from reacting with other substances, 2) SSAT accelerates the transportation of AcCoA from mitochondria to the cytoplasmic matrix, and 3) SSAT is a carrier protein which transports AcCoA to other subcellular locations. The second category speculated four possible reasons: 1) the lack of SSAT enzyme in liver preparations, 2) the loss of SSAT enzyme activity, 3) the overwhelmingly high spontaneous

polyamine acetylation, and 4) the escape of acetylated polyamines from the total accumulation. These conjectures are supported by previous studies, but there is still a need for experimental verification.

6.2 Future directions

To prove the veracity of the new possible pathways described in this study, the potential mistakes made by this research should first be excluded. An appropriate direct protein detection such as Western blot analysis should also be applied to confirm the existence of SSAT protein in liver preparations. Moreover, it is also necessary to purify the SSAT enzyme from liver tissues to exclude the escape of metabolites under *in vitro* conditions, because the liver preparations in this research may contain other enzymes which are potentially able to consume metabolites. It is also recommended to replace the liver tissue to hepatic cells, so that SSAT could be easily induced by cytotoxic substances since multiple studies reported that SSAT need to be activated before being applied in an enzyme assay. Finally, it is also feasible to use animals with diabetes or obesity to find out if diseases are influencing the activity of SSAT.

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