Research Project on SSAO Extraction and Enzyme Kinetics

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# Preface

**Overview**

This is a research project carried out at the University of Hertfordshire in compliance with its regulations under the supervision of Dr. John Skamarauskas. The project was submitted in partial fulfillment of my Master’s in Drug Discovery and Toxicology. The original submission was prepared in Microsoft word, and the data analysis was carried performed using Graphpad prism.

This version of the project published as a quarto book is purely an exploratory effort by me. Same data from original research was used, and R was used to generate plots, models and tables. The results obtained from the R code was compared with the original results from Graphpad which were identical. I decided to do this to improve my working understanding of R and Quarto Markdown.

## **Abstract**

Rat brown adipose tissue (BAT) is recognized as a rich source of semi carbazide-sensitive amine oxidase (SSAO), yet no standardized methods for extracting SSAO from rat BAT have been published. This study aims to develop and optimize a robust methodology for SSAO extraction from rat BAT and to investigate its enzymatic kinetics using benzylamine as a substrate. The study involved comparing different extraction methods, quantifying the extracted protein using Bradford and BCA assays, and assessing SSAO activity through the Amplex® Red monoamine oxidase assay.

The kinetic parameters of SSAO were determined, with a Km value of 0.03193mM for benzylamine, closely aligning with literature under similar experimental conditions. Furthermore, the inhibitory effects of caffeine and simvastatin on SSAO activity were evaluated, although, the Ki values obtained were based on a single experiment, leading to wider error margins and lower confidence.

This research provides a detailed methodology for SSAO extraction from rat BAT, confirming that proteins can be successfully extracted and that SSAO activity remains quantifiable post-extraction. However, further studies with improved experimental controls are recommended to refine the inhibitory kinetics of caffeine and simvastatin on SSAO.

## **Abbreviations**

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| BAT - Brown Adipose Tissue |
| BCA - Bicinchoninic Acid |
| BSA - Bovine Serum Albumin |
| CAF - Caffeine |
| Ki - Inhibition Constant |
| Km - Michaelis Menten Constant |
| SIM - Simvastatin |
| SSAO - Semi Carbazide Sensitive Amine Oxidase |
| V0 - Initial Velocity |
| VAP-1 - Vascular Adhesion Protein 1 |
| Vmax - Maximum Velocity |
| HRP - Horseradish Peroxidase |
| EtOH - Ethanol |
| SEM - Standard Error in Mean |
| MAO A - Monoamine Oxidase A |
| MAO B - Monoamine Oxidase B |
| AOC - Amine Oxidase Copper-Containing |
| dH20 - Deionised Water |
| ReLi - Removal of Excess Lipids |
| CST - Cell Signalling Technologies – Commercial kit. |
| DMSO - Dimethyl Sulfoxide |

# 1. Introduction

## 1.1 **Amine Oxidases and substrates**

Amine oxidases are a common group of copper containing enzymes highly expressed in smooth muscle cells, vascular endothelial cells and adipocytes in humans and rats (Manasieva et al. 2022; Salmi, Tohka, and Jalkanen 2000; Salmi and Jalkanen 1992). The key enzymatic function of these proteins is oxidating primary amines into aldehydes . This also results in formation of byproducts such as ammonium (NH4+) and hydrogen peroxide(H2O2) in a two-step reaction [Figure 1.1](#fig-Deamination_of_primaryamine_SSAO) . (Salmi and Jalkanen 2019)

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| fig  Figure 1.1: Deamination of a primary amine by SSAO to form an aldehyde, ammonium, and hydrogen peroxide in a 2-step reaction. |

Semi carbazide sensitive amine oxidase (SSAO) also known as the vascular adhesion protein belongs to this class of enzymes and are primarily present on the epithelial cell membranes of the vascular surfaces. Some of the known forms of SSAO enzymes in humans are AOC1, AOC2, and AOC3. AOC1 is involved in regulating histaminase, whereas AOC2 and AOC3 (VAP-1) catalyse various monoamines. AOC2 has a large substrate channel compared to the AOC3. The preferred substrates for AOC2 are ethylamine, tyramine, and p-tyramine, whereas AOC3 or VAP 1 prefers methylamine (Salmi and Jalkanen 2019). Benzylamine is a xenobiotic substrate of AOC-3 or VAP1 and is more commonly used in invitro studies of SSAO kinetics and VAP 1 contributes to approximately 90% of cellular SSAO activity in mammals (Salmi and Jalkanen 2019) . Other synthetic substrates such as aminoacetone, allylamine, and methylamine are also used in invitro experiments (Maria Carmen Iglesias-Osma et al. 2005; Lyles 1995; Manasieva et al. 2023, 2022). This project studies the activity of SSAO using benzylamine as the only substrate as it is specific to SSAO and no other mono amine oxidases (MAO).

## 1.2 **SSAO/VAP1 Physiology and Pathophysiology**

The primary role of SSAO as an enzyme is catalysing primary amines that are present in the circulation into aldehydes and hydrogen peroxide. However, their expression and activity has been found to be upregulated by inflammatory and immune mediators such as IL-1, TNF-α, and other lipopolysaccharides in an organ culture study using human tonsillar tissue (Arvilommi, Salmi, and Jalkanen 1997). The study also reported that there is no significant difference in the characteristics of VAP-1 induced by inflammatory mediators and the naturally occurring VAP-1. Studies have also shown that VAP-1 directly regulates lymphocytes / leukocytes rolling under a defined laminar shear supporting the lymphocyte extravasation process (Salmi, Tohka, and Jalkanen 2000). This increases the understanding on VAP-1 expression and the outcomes of its enzymatic activity on physiology and pathophysiology. The resulting products of SSAO activity are aldehydes and hydrogen peroxides which are implicated in development of various pathologic complications that include atherosclerosis, diabetes, and obesity (Murata et al. 2017; Wang et al. 2018). The extravasation cascade of leukocytes influenced by VAP-1 is defined as a key point in development of various pathologies such as Fibrosis, inflammation, ischemia reperfusion injury, and even cancer (Salmi, Tohka, and Jalkanen 2000). Most of these effects were studied by blocking the activity of VAP-1 using SSAO inhibitors (Danielli et al. 2022; H. Li et al. 2021; Wang et al. 2018). VAP-1 is also found in circulation / serum with almost the same characteristics of the transmembrane bound VAP-1. Studies have reported that the levels of serum VAP-1 is increased with pathologies such as colorectal cancer, chronic liver disease, gastric cancer, inflammation, smoking, and aging (Kurkijarvi et al. 2000; Pannecoeck et al. 2015; Toiyama et al. 2009; Yasuda et al. 2011).

## 1.3 **SSAO in metabolic disorders**

Recent trends in metabolic health have shown that obesity, diabetes, and cardiovascular diseases are the leading causes of morbidity and loss of quality of life (Chew et al. 2023). This makes it one of the growing health concerns for every major health care organisation and insurance companies.

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| Figure 1.2: Global burden of metabolic diseases portraying various factors that lead to metabolic diseases causing increase in morbidity and disability adjusted life years (DALYs) |

[Figure 1.2](#fig-DALY) provides a comprehensive overview of the several factors leading to fatal metabolic disorders such as obesity, hyperlipidemia, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus, and hypertension.

Circulating SSAO is implicated in the pathogenesis of metabolic disorders such as obesity, atherosclerosis, and diabetes mellitus by promoting weight gain by producing an insulin like action (Zorzano et al. 2003). Higher levels of SSAO in humans have been reported in various metabolic and cardiovascular conditions like stroke, myocardial infarction, atherosclerosis, diabetes, and obesity (Boomsma et al. 2000; Bour et al. 2009; Sun et al. 2018; Unzeta et al. 2021). The list of studies looking at SSAO as a target for management of cardiovascular and metabolic diseases provides conclusive evidence of the role SSAO plays in their pathogenesis (Abella et al. 2003; Boomsma et al. 2000; Jarnicki et al. 2016; Obata 2006; Papukashvili, Rcheulishvili, and Deng 2020; Schilter et al. 2015; Tábi et al. 2013; Unzeta et al. 2021; Yan et al. 2023; P. H. Yu and Deng 1998).

## 1.4 **Adipose Tissue**

Adipose tissue is an essential part of the mammalian lipid metabolism which is used to store the excess lipids as triglycerides and aiding the body to use them as fatty acids in between meals (Börgeson, Boucher, and Hagberg 2022). In metabolic aberrations, the function of the adipose tissues is compromised resulting in excess circulating lipids and fat deposition in peripheral organs leading to metabolic diseases such as diabetes, atherosclerosis, obesity, and fatty liver disease (Belligoli et al. 2019; Chait and Hartigh 2020; Chusyd et al. 2016). White adipose tissue (WAT) and brown adipose tissue (BAT) are the two most common types of adipose tissues present in mammals. White adipose tissue primarily stores the excess lipids and releases when the body needs it. BAT, in addition to storing the excess lipids they are also involved in thermogenesis in mice, rats, and humans Börgeson, Boucher, and Hagberg (2022).

The adipose tissues express a wide range of proteins that include amine oxidases such as the SSAOs (BARRAND et al., 1984; Barrant & Callingham, 1984). Both WAT and BAT higher levels of SSAO which plays a key role in activation of glucose transport and prevention of lipolysis in both humans and rodents (Zorzano et al. 2003).

This project is aimed at successfully extracting SSAO from rat brown adipose tissue by a suitable methodology. A major barrier in using adipose tissue is the quantity of lipids present in the adipose tissues. Brown adipose tissue from rats is chosen to be the source of the enzyme for the following reasons.

1. Previous studies involving the use of adipose tissue for extraction of proteins have been reported in the literature. These reports also provide the protocol for a successful removal of fat and compares the results of protein extracted from WAT and BAT (Marin et al. 2019).
2. The chosen animal for this project is rat as they are more commonly used at the University of Hertfordshire. Since a wide range of tissues are being used for other experiments, use of adipose tissue from this animal will aid in the three Rs of animal research.
3. The quantity of BAT that can be extracted from rats are significantly more than mice.

## 1.5 **Caffeine and Simvastatin**

***Caffeine*** is an aromatic purine alkaloid from the methylxanthine class compounds. Commonly used to increase alertness, concentration, and improve energy levels. Additionally, caffeine is shown to have various other health benefits such as neuroprotective, hepatoprotective, weight loss, and physical performance improvements owing to its antioxidant properties. Caffeine exhibits its antioxidant properties by acting as a scavenger of free radicals and increasing the concentration of endogenous antioxidants such as glutathione (GSH) (Reddy et al. 2024).

Caffeine has gained some attention as it has been reported to aid in weight loss by stimulating lipolysis, thermogenesis, appetite suppression, and increased metabolic rate. These effects closely match with observations when SSAO is inhibited by known inhibitors in various studies (Che et al. 2012). This study aims to determine if caffeine possess a quantifiable inhibitory effect on SSAO.

***Simvastatin*** is one of the common medications prescribed to lower cholesterol levels and used as preventative therapeutic for various cardiovascular and cerebrovascular conditions. A study by Sun et al., reported that simvastatin blocks soluble SSAO/VAP1 release in rabbit animal models of cerebral ischemia. However, this study does not provide a more accurate estimate of in IC50 or the Ki for simvastatin on SSAO but only discusses the reduction in SSAO release into the blood plasma (Sun et al. 2018).

Studying the effects of caffeine and simvastatin on SSAO activity paves direction for considering commonly used molecules in par with some of the new chemical entities being studied for their inhibitory properties on SSAO/VAP- 1.

## 1.6 **SSAO/VAP-1 Inhibitors**

Some of the most widely studied compounds with an inhibitory effect on SSAO are small molecules that include hydrazines, benzylamides, vitamin B1 derivatives, oxime based primary amine oxidase inhibitors and peptides (Manasieva et al. 2022; Pannecoeck et al. 2015).

Table of inhibitors containing the experimental models they were tested on with key major effects and appropriate references. Table directly incorporated from (H. Li et al. 2021)

| Name of SSAO/VAP-1 Inhibitors | Off Target | Model | Species | Major effects | References |
| --- | --- | --- | --- | --- | --- |
| **Allyl-amines** |  |  |  |  |  |
| LJP-1586 | MAO-A/B | ICH, SAH, Atherosclerotic plaque | CD1 mice, Sprague-Dawley rats, LDLr-/- ApoB100/100 mice | Improved neurological scores. Improved neurological outcomes. | (Ma et al. 2011; Silvola et al. 2016; Xu et al. 2014) |
| MDL-72974A | MAO-B | Atherosclerosis, Obesity | KKAy mice | Reduction of weight gain and atherosclerotic lesions. Reduction of weight gain | (P. Yu et al. 2002; Peter H. Yu et al. 2004) |
| PXS-4728A | / | Atherosclerosis | New Zealand white rabbits, Apo E-/- mice | Reduction of weight gain and atherosclerotic plaques. Reduction of atheroma and oxidative stress. | (Wang et al. 2018) |
| **Hydra-zines** |  |  |  |  |  |
| Aminoguanidine | DAO | Atherosclerosis | KKAy mice | Reduction of weight gain and atherosclerotic lesions. | (P. Yu et al. 2002) |
| Phenyl hydrazine | MAO-B | Obesity | Zucker rat | Reduction of weight gain | (Carpéné et al. 2019) |
| SCZ | LO | Embolic stroke | Sprague-Dawley rats, LDLr-/- mice | Reduction of the infarct volume. Decreased macrophages/increased SMC in established lesions with/without lipid lowering. | (Hernandez-Guillamon et al. 2010; Ma et al. 2011; Peng et al. 2016; Yang et al. 2011; Zhang et al. 2016) |
|  |  | MI | Sprague-Dawley rats | Reduced infarction sizes | (Yang et al. 2011) |
|  |  | ICH | CD1 mice | Improved neurological scores | (Ma et al. 2011) |
| LJP-1207 | / | MI | Sprague-Dawley rats | Reduced infarction sizes | (Yang et al. 2011) |
| Hydralazine | MAO-A/B | MI | Sprague-Dawley rats | Reduced infarction sizes | (Yang et al. 2011) |
| **VAP-1 siRNA** |  | ICH | CD1 mice | Improved neurological scores | (Ma et al. 2011) |

*Note: All above inhibitors, except VAP-1 siRNA, are irreversible inhibitors that bind to the topaquinone (TPQ) cofactor of VAP-1. MAO-A: monoamine oxidase A; MAO-B: monoamine oxidase B; ICH: intracerebral haemorrhage; SAH: subarachnoid haemorrhage; MI: myocardial infarction; DAO: diamine oxidase; LO: Lysyl oxidase; SCZ: Semi carbazide.*

Two sides of SSAO action in adipocytes

1. Studies have shown that benzylamine administration promoted a higher SSAO activity in diabetic, obese and high fat diet fed mice and rabbits and increases glucose uptake and prevents lipolysis caused by an insulin mimicking action by the deamination activity of SSAO. This caused an improvement in glucose tolerance due to insulin mimetic action by benzylamine oxidation (María Carmen Iglesias-Osma et al. 2004; Zorzano et al. 2003).
2. The inhibition of the same deamination activity of SSAO has been demonstrated to reduce fat deposition, increase weight loss, and limit food composition. The study that reported this used semi carbazide, administered in oral form as the SSAO inhibitor to both obese and non-obese groups (Mercader et al. 2011).

Thus, investigating the inhibitory properties novel compounds on SSAO can prove to be effective in developing a pharmacologic agent for the management of obesity. Since the inhibition of SSAO on non-obese animal models prevents weight gain, any everyday consumption agent with SSAO inhibition property with higher safety profile can be used as prophylactic leading to a healthy weight and metabolic profile.

# 2. Aim and Objectives

Rat brown adipose tissue has been reported to be a rich source of SSAO and there have been no published methods in extracting SSAO from rat BAT. This project focuses on identifying and optimising methods for extracting SSAO and studying its kinetics with a known substrate – Benzylamine.

The primary aims of the project are to

1. Establish a robust and reproducible method to extract SSAO from rat brown adipose tissue.
2. Quantitative comparison of protein extracted from different methods.
3. Quantitative estimation of SSAO activity in extracts obtained from different methods.
4. Estimation of Vmax and Km values for benzylamine on SSAO activity.
5. Preliminary analysis of inhibitory activity of caffeine and simvastatin on SSAO activity.

The aim of this project is to identify a method that can be used to extract SSAO from rat brown adipose tissue extracted from male Wistar rats and optimise the method to yield a concentrated SSAO protein extract. The concentration of the protein from extracts obtained by different methods are to be estimated by Bradford and BCA assays.

Secondly, a quantitative estimation of SSAO activity using benzylamine as a substrate is to be done by the Amplex® red monoamine oxidase assay. Following a measurable SSAO activity, a kinetic experiment is to be conducted to assess the Km and Vmax of SSAO activity using Benzylaine as a substrate at different concentrations.

Thirdly, to study the effect of caffeine and simvastatin on SSAO kinetics using benzylamine as the substrate and measure the Ki inhibitory constant for the inhibitors.

# 3. Materials and Methods

## 3.1 Materials

100% Ethanol, 96 well plate (Flatbottom – Black), 96 well plate (Flatbottom – Clear), Amplex® red monoamine oxidase kit (Thermo-Fisher Scientific), BCA reagent, BMG plate reader (SPECTROstar Nano, CLARIOstar Plus), Bradford reagent, BSA, Caffeine-anhydrous, Centrifuge, Deionised water, DMSO, Eppendorf tubes (1ml and 2ml), Ethanol, Falcon tubes (25ml), Freeze dryer, Freeze-dryer, Gilson Pipettes – P5000, P1000, P200, P20, and P2, Peristaltic pump, pH meter, Potassium chloride, Potassium phosphate, Rat brown adipose tissue, Simvastatin, Sodium chloride, Sodium phosphate, and Tissue Homogeniser (Sartorius, Potter S). All materials were procured from Sigmund Aldrich unless specified.

**Animals used**: Male Wistar rats (*Rattus Norvegicus)* obtained from Envigo on a Teklad 2014 14% protein diet, weighing 260-274g approximately were used to obtain the brown adipose tissue from the scapular region. All animals used in this study were handled and treated in accordance with animal ethical guidelines provided by the University of Hertfordshire.

## 3.2 Methodology

### 3.2.1 **Tissue Collection**

The tissues were excised from the rats by animal technicians and was provided in a falcon tube. The falcon tube was weighed before and after adding the tissue to measure the weight of the tissue collected every day. After weighing the tissues, they were stored at -20℃ for processing later. The tissues were not processed on the same day due to less volume of the tissue, as only one animal was sacrificed each day for academic purposes. The tissue samples were collected every day and stored until adequate volume of tissue was available for further processing. Brown adipose tissue samples were specifically requested for the project as they were not being used for any other experiments carried out at the university. This enables the effective use of animals and animal tissues promoting refinement aspect of the three Rs (Replacement, reduction and refinement) of animal research.

### 3.2.2 **Protein extraction**

Extracting proteins from adipose tissue is challenging due to a heavy fat contamination. These lipids are known to interfere in downstream assays which count affect the quality of results. Hence existing literature and methods that proposed a viable method were studied and parts of which were incorporated (An and Scherer 2020; Marin et al. 2019). Removing fat exclusively and retaining the proteins can be achieved by using an organic solvent such as acetone, ethanol, chloroform, or phenol to dissolve the lipids and precipitate the proteins in the sample (Dezse, Frank, and Baptista 2020). Acetone was the preferred choice of defatting agent; however, it was not used in this project due to limited availability. 100% Ethanol was used due to its availability. Since ethanol is known to denature proteins, care was taken to limit the duration of exposure to the adipose tissue, and tissues were dried to remove residual ethanol.

### 3.2.3 **Defatting the tissues**

The BAT tissues were weighed and transferred to a single falcon tube and 25ml of 100% ethanol was added to dissolve the fat. The falcon tube was shaken multiple times to enable even distribution of ethanol. The tissues were treated with ethanol for 10 minutes and the dirty ethanol containing the fat was decanted and 25 ml of fresh ethanol was added to the samples. This process was repeated for 3 times to remove maximum amount of fat from the tissue. The ethanol wash was limited to three times to prevent the denaturation of proteins by ethanol.

After ethanol wash, the tissues were dried under an airflow outlet produced using a peristaltic pump (3485 ml/min) for 2 hours [Figure 3.1](#fig-methodflow). The dried tissues were weighed to estimate the amount of fat removed by the ethanol wash.

#### 3.2.3.1 **Method A**

Adequate number of tissues were weighed for freeze drying to remove the water from the tissues for further processing with ethanol to remove the fat. The falcon tubes containing the tissues were removed from the freezer, the caps were removed and a layer of parafilm was applied, and tiny holes were made to allow the escape of vapour during the freeze-drying process. These falcon tubes were placed in the freeze dryer overnight, to completely remove any water from the tissues.

After 24 hours, the falcon tubes containing the samples were removed from the freeze dryer and weighed to estimate the loss of water. The defatting with ethanol was performed as per the steps mentioned in [Section 3.2.3](#sec-defatting-the-tissues) . This tissue was treated using PBS with a pH of 8.0 to dissolve the proteins. 10ml of cold PBS was added to the falcon tube containing defatted tissues on an ice bath. The samples were shaken thoroughly to ensure maximum dissolution of proteins into the buffer. After 30 minutes, the PBS was decanted into multiple 1.5ml Eppendorf tubes for storage and future experiments.

#### 3.2.3.2 **Method B**

Adequate amount of tissue was weighed into a falcon tube and 20ml of cold 100% ethanol was directly added to the tissue. The defatting with ethanol was performed as per the steps mentioned [Section 3.2.3](#sec-defatting-the-tissues) . The extracts were stored in 1.5ml Eppendorf tubes at -20 for future use.

#### 3.2.3.3 **Method C**

This method of removing fat from tissue was adapted from the ReLi protocol (Marin et al. 2019). The adaptations were made to accommodate reagent, and equipment availability. Adequate amount of tissue was weighed and chopped into tiny pieces using sterile forceps and scalpel. The tissues were then added to 25ml homogeniser tube and cold PBS (1ml/mg tissue) was added to the tissue. The homogeniser tube was attached to the Potter-Elvehjem homogeniser ensuring the bottom of the homogeniser tube was immersed in the ice bath. The tissue was mechanically homogenised at a constant speed of 500rpm with occasional stirring to ensure proper homogenisation. After the tissue was homogenised, the extract was transferred to 2ml Eppendorf tubes for centrifugation. The extracts were centrifuged at 12,000G for 15 minutes at 4℃. The fat cakes formed at the top of the Eppendorf was carefully removed and the pellet was resuspended and recentrifuged. This was repeated until there was no fat cake formation. The extracts were stored as aliquots for future use.

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| Figure 3.1: Flow diagram of steps involved in extracting SSAO from rat brown adipose tissue using Method A, Method B and Method C |

### 3.2.4 **Protein Quantification**

Bradford and BCA assays were performed to determine the concentration of proteins in each extract. Bradford assay quantifies the protein concentration by measuring the amino acid residues that binds to the Coomassie Brilliant Blue G-250 dye.

Proteins present in the sample bind to the dye resulting in bright blue appearance which can be measure at 595nm (Noble and Bailey 2009). Alternatively, Bicinchoninic acid (BCA) assay was also performed due to its high sensitivity and tolerance for interference. BCA measures the protein concentration in two steps, first (biuret reaction) the peptide bonds in the sample reduce the cupric ions in the reagent to cuprous ions and then one cuprous ion chelates with 2 molecules of BCA to produce an intense purple complex (Noble and Bailey 2009). This can be measured at 562nm, as per the manufacturer’s instructions.

Upon obtaining absorbances of undiluted stock samples that do not fit inside the standard curve, the stock samples were diluted and read again. The protein concentrations for the stock samples were calculated by correcting the concentration of the diluted sample with the dilution factor.

Known concentrations of Bovine Serum Albumin (BSA) is used to prepare a standard curve which was used to interpolate absorbances obtained from unknown samples (Noble & Bailey, 2009). 10 mg of BSA was weighed and dissolved in 10ml of deionised water to prepare a 1mg/ml stock BSA standard. The 1mg/ml stock was used to prepare further dilutions as per table 1.

BSA Standard dilution

| BSA Stock Volume | Deionised water volume (ml) | Final concentration (mg/ml) |
| --- | --- | --- |
| 0.8 | 0.2 | 0.8 |
| 0.6 | 0.4 | 0.6 |
| 0.4 | 0.6 | 0.4 |
| 0.2 | 0.8 | 0.2 |

#### 3.2.4.1 **Bradford assay**

10µl of each standard (2 replicates) and extracts (5 replicates) from each method was pipetted into each well of a 96 well plate with 200µl of Bradford reagent. Deionized water was used as blank for standards, and PBS was used as the blank for samples. 200µl of Bradford reagent was added to the wells containing the blanks. The plates were read using BMG Labtech CLARIOstar plate reader at 595nm. The stock sample from all methods were diluted 10-fold and the absorbance was corrected with the dilution factor. The absorbance readings were transferred to excel and then to GraphPad prism to plot standard curves and estimate unknown protein concentrations from the linear standard curve equation. However, for the purpose of displaying the results, same analysis are performed using R.

library(tidyverse)  
# Data: Standard Concentration and Absorbance values  
bradford\_sc <- tibble(  
 standard\_Concentration = c(1.0, 0.8, 0.6, 0.4, 0.2, 0.0),  
 absorbance\_1 = c(0.682, 0.592, 0.499, 0.334, 0.138, 0),  
 absorbance\_2 = c(0.725, 0.61, 0.493, 0.324, 0.161, 0)  
)  
  
# Calculate the mean absorbance  
bradford\_sc$mean\_absorbance <- rowMeans(bradford\_sc[, c("absorbance\_1", "absorbance\_2")])  
  
# Fit a linear model  
brad\_model <- lm(mean\_absorbance ~ standard\_Concentration, data = bradford\_sc)  
  
# Extract the equation and R-squared value  
coefficients <- coef(brad\_model)  
intercept <- round(coefficients[1], 4)  
slope <- round(coefficients[2], 4)  
r\_squared <- round(summary(brad\_model)$r.squared, 4)  
equation <- paste0("y = ", slope, "x + ", intercept)  
  
# Plotting the standard curve  
ggplot(bradford\_sc, aes(x = standard\_Concentration, y = mean\_absorbance)) +  
 geom\_point() +  
 geom\_smooth(method = "lm", se = FALSE, color = "blue") +  
 labs(  
 title = "Bradford Assay - BSA Standard Curve",  
 x = "BSA Standard Concentration (mg/mL)",  
 y = "Mean Absorbance"  
 ) +  
 annotate("text", x = 0.75, y = 0.1, label = paste(equation, "\nR² = ", r\_squared), color = "blue") +  
 theme\_classic()

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| Figure 3.2: Standard Curve for BSA protein concentration by Bradford assay. The absorbance was measured at 595nm using BMG Labtech SPECTROstar plate reader. |

#### 3.2.4.2 **BCA assay**

BCA assay was performed to estimate the protein concentration in the extracts obtained from Method A, Method B, and Method C. 25µl of standard and sample were pipetted into each well of a clear flatbottom 96 well plate with 200µl of BCA reagent. The plates were incubated at 37℃ for 30 minutes and absorbance was measured at 562nm using the BMG Labtech CLARIOstar plate reader. The stock sample from all methods were diluted 10-fold and the absorbance was corrected with the dilution factor.

library(tidyverse)  
# Data: Standard Concentration and Absorbance values  
bca\_sc <- tibble(  
 standard\_Concentration = c(1.0, 0.8, 0.6, 0.4, 0.2, 0.0),  
 absorbance\_1 = c(1.263, 0.976, 0.749, 0.602, 0.291, 0),  
 absorbance\_2 = c(1.162, 0.943, 0.663, 0.498, 0.266, 0)  
)  
  
bca\_sc$mean\_absorbance <- rowMeans(bca\_sc[, c("absorbance\_1", "absorbance\_2")])  
  
# Fit a linear model  
bca\_model <- lm(mean\_absorbance ~ standard\_Concentration, data = bca\_sc)  
  
# Extract the equation and R-squared value  
coefficients <- coef(bca\_model)  
intercept <- round(coefficients[1], 4)  
slope <- round(coefficients[2], 4)  
r\_squared <- round(summary(bca\_model)$r.squared, 4)  
equation <- paste0("y = ", slope, "x + ", intercept)  
  
# Plotting the standard curve  
ggplot(bca\_sc, aes(x = standard\_Concentration, y = mean\_absorbance)) +  
 geom\_point() +  
 geom\_smooth(method = "lm", se = FALSE, color = "blue") +  
 labs(  
 title = "BCA Assay - BSA Standard Curve",  
 x = "BSA Standard Concentration (mg/mL)",  
 y = "Mean Absorbance"  
 ) +  
 annotate("text", x = 0.75, y = 0.1, label = paste(equation, "\nR² = ", r\_squared), color = "blue") +  
 theme\_classic()

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| Figure 3.3: Standard Curve for BSA protein concentration by BCA assay. The abosorbance was measured at 562nm using BMG labtech SPECTROstar plate reader |

### 3.2.5 **Measurement of SSAO activity.**

SSAO activity is estimated by measuring the levels of hydrogen peroxide (H2O2) produced in the samples. is produced when benzylamine is catalysed by SSAO. Concentration of H2O2 produced in the reaction directly corresponds to SSAO activity levels. Amplex® red assay is a highly specific fluorescence-based assay that can be used to measure levels in the samples.

#### 3.2.5.1 **Amplex**® **red monoamine oxidase assay**

Amplex® red is a colourless, non-fluorescent compound that converts into resorufin in presence of H2O2 and Horseradish peroxidase. Resorufin is a highly fluorescent product which has an absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Cai et al. 2022; Karakuzu et al. 2019). Amplex® red monoamine oxidase kit was obtained from Thermo-Fisher scientific to measure the SSAO activity in the samples prepared from various methods. The stock solutions for Amplex® red, HRP, H2O2, Benzylamine, and Resorufin were prepared as per manufacturer instructions

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Table 3.1: Stock solution preparation for Amplex® red monoamine oxidase assay   | Stock solution | Quantity/Volume | Diluent Volume | Stock Conc. | | --- | --- | --- | --- | | Amplex® red | 1Vial | 250µl DMSO | 20mM | | 5X reaction buffer | 5ml | 20ml dH2O | 1X buffer | | HRP | 1Vial | 1ml 1X buffer | 200U/ml | | Benzylamine | 1Vial | 1.2ml dH2O | 100mM | | Resorufin | 1Vial | 1ml dH2O | 2mM | |

The stock solutions were used to prepare a reaction mixture containing 400µM Amplex® red, 2U/ml HRP, and appropriate substrate concentration. For example, 2000 µl of the reaction mixture was prepared by combining 40µl Amplex® red stock(20mM), 20µl HRP stock(200U/ml), 1900µl sodium phosphate buffer (pH 7.4) (1X reaction buffer), and 40µl of benzylamine solution of the desired concentration ([Table 3.2](#tbl-substrate-solution-prep)) . A reaction mixture without substrate was prepared by replacing the substrate with same volume of 1X reaction buffer. For measuring the inhibitory activity of caffeine and simvastatin on SSAO, 10µL of each inhibitor at 0 .1, 1, and 10mM concentration were added to the samples and incubated for 15 minutes prior to adding the reaction mixture containing the substrates triggering the enzyme reaction.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Table 3.2: Benzylamine substrate dilution for preparation of Amplex® red monoamine oxidase assay reaction mixture.   | Benzylamine Dilution | Stock volume | dH2O - Diluent Volume | Final concentration | | --- | --- | --- | --- | | Dilution 1 | 20µl of 100mM Benz | 1980µl | 1 mM | | Dilution 2 | 1000µl of Dilution 1 | 1000µl | 0.5 mM | | Dilution 3 | 1000µl of Dilution 2 | 1000µl | 0.25 mM | | Dilution 4 | 1000µl of Dilution 3 | 1000µl | 0.125 mM | | Dilution 5 | 1000µl of Dilution 4 | 1000µl | 0.0625 mM | |

Known concentrations of resorufin were prepared to produce a standard curve. This standard curve is used to measure the moles of resorufin produced as a result of SSAO activity in the samples. 120µl of each resorufin standard dilution was pipetted in duplicates in individual wells of a 96 opaque flatbottom well plate for the standard curve. For samples, 60µl of sample prepared from method A, method B, and method C were pipetted in individual wells and 60µl of reaction mixture containing 10mM benzylamine substrate was added to the wells containing the sample. The plates were incubated at 37℃ for 30 minutes prior to measuring the fluorescence using BMG Labtech CLARIOstar plus plate reader.

For continuous measurement of SSAO activity, the plates containing SSAO without the reaction mixture were incubated at 37℃, and the incubator attached to BMG Labtech CLARIOstar plate reader was also set at 37℃ during the analysis. For inhibitor kinetics measurements, the inhibitors at different concentrations were added to the sample and were incubated at 37℃ for 15 minutes without the reaction mixture.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Table 3.3: Resorufin dilution preparation for Amplex® red monoamine oxidase assay standard curve.   | Resorufin Stock | dH2O (ml) | Final Concentration (µM) | | --- | --- | --- | | Stock 2mM – 20µl | 1980 µl | 20 µM | | Stock 20 µM – 100 µl | 100 µl | 10 µM | | Stock 20 µM – 80 µl | 120 µl | 8 µM | | Stock 20 µM – 60 µl | 140 µl | 6 µM | | Stock 20 µM – 40 µl | 160 µl | 4 µM | | Stock 20 µM – 20 µl | 180 µl | 2 µM | |

library(tidyverse)  
# Data: Standard Concentration and Absorbance values  
reso\_sc <- tibble(  
 standard\_Concentration = c(1.0, 0.8, 0.6, 0.4, 0.2, 0.0),  
 absorbance\_1 = c(233718, 173498, 136768, 90371, 49091, 0),  
 absorbance\_2 = c(231342, 178368, 141037, 91900, 48934, 0)  
)  
  
reso\_sc$mean\_absorbance <- rowMeans(reso\_sc[, c("absorbance\_1", "absorbance\_2")])  
  
# Fit a linear model  
reso\_model <- lm(mean\_absorbance ~ standard\_Concentration, data = reso\_sc)  
  
# Extract the equation and R-squared value  
coefficients <- coef(reso\_model)  
intercept <- round(coefficients[1], 0)  
slope <- round(coefficients[2], 0)  
r\_squared <- round(summary(reso\_model)$r.squared, 4)  
equation <- paste0("y = ", slope, "x + ", intercept)  
  
# Plotting the standard curve  
ggplot(reso\_sc, aes(x = standard\_Concentration, y = mean\_absorbance)) +  
 geom\_point() +  
 geom\_smooth(method = "lm", se = FALSE, color = "blue") +  
 labs(  
 title = "Amplex Red Assay - Resorufin Standard Curve",  
 x = "Resorufin Standard Concentration (µM)",  
 y = "Corrected RFU"  
 ) +  
 annotate("text", x = 0.75, y = 5000, label = paste(equation, "\nR² = ", r\_squared), color = "blue") +  
 theme\_classic()

|  |
| --- |
| Figure 3.4: Standard Curve of Resorufin by Amplex® red monoamine oxidase assay. RFU measured at 585mM emission maxima using BMG Labtech CLARIOstar plus fluorescence plate reader. |

***Instrument settings for BMG Labtech CLARIOstar plus plate reader.***

A new protocol was created in the instrument for Amplex® red monoamine oxidase assay in plate mode and endpoint mode. The protocols were set to measure fluorescence at an excitation range of 540±20 and emission range of 590±20 as resorufin has fluorescence emission maxima of approximately 585 nm. Gain settings and focal length for the assays were set based on the wells containing 10 µM resorufin as they are optimal for highest level of fluorescence.  Gain settings for plate mode were set at 90% with a gain of 963 and a focal length of 7.6 to yield consistent fluorescent across multiple readings. These initial values were standardised from 10 µM resorufin fluorescence readings. For a continuous kinetic assessment, plate mode with the following settings were used. These settings were used to read the fluorescence of each well every 30s for 10 minutes. The RFUs were used to calculate the initial velocity of the enzymatic reaction.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Table 3.4: *BMG Labtech CLARIOstar settings for plate mode measurements of continuous kinetic activity.*   | Setting | Value | | --- | --- | | Optic | Top optic | | Settling time | 0.5 | | Measurement Start time | 0.0s | | Number of intervals | 20 | | Number of flashes | 20 | | Interval time | 30s | |

### 3.2.6 **Statistical and data analysis:**

For the purposes of creating this Quarto book, data analysis was performed using R. The results produced from the R code presented in this manuscript were verified to match the results obtained from graphad analysis.

All the data obtained from were recorded and formatted in Microsoft excel. Further analysis such as generating a standard curve, interpolating, and extrapolating values from standard curve for unknown samples, and regression analysis was performed in GraphPad prism 10. Slopes and intercepts represented in all graphs were calculated using linear regression. Km, Ki, and Vmax values were calculated using nonlinear regression using preset functions such as Michaelis Menten equation and competitive inhibition in GraphPad prism. No parametric or non-parametric analysis was performed in this project, as the number of experiments does not meet the minimum sample size required for such statistical analysis.

# 4. Results

The extracts obtained from each method were quantified using Bradford and BCA assays. These samples were diluted 10-fold to fit the absorbances obtained into the standard curve. The protein concentrations of the dilutes samples were then calculated using the standard curve. These results were corrected with the dilution factor to calculate the estimated protein concentration of undiluted samples. The results are tabulated and presented as mean [Table 4.2](#tbl-proteinestimate) and compared in [Figure 4.1](#fig-proteinesti). Amplex® red monoamine oxidase assay was used to determine the SSAO activity in the samples from each method after treating the sample with the Amplex® red reaction mixture with 10mM benzylamine as substrate and incubating at 37 ℃ for 30 minutes before measuring the fluorescence. The results of SSAO activity from each sample is presented in [Table 4.3](#tbl-ssaoactivity) and [Figure 4.2](#fig-ssaoactivity) .

## 4.1 Method A

The tissues collected for a period of 4 days were weighed and freeze dried using a freeze dryer to dehydrate the tissue. 11.1g of tissue were freeze dried resulting in a loss of 71.1% of weight(water) and 3.1g of dehydrated tissue was obtained. Defatting of the dehydrated tissue caused a further loss of 1g of fat weight, resulting in 2.1g of tissue. Bradford assay and BCA assay were performed to estimate the protein concentration in the extract obtained from this method A. Only one independent experiment was performed using Method A (refer to 4.2.1) due to unavailability of the freeze dryer during the project. The estimated protein concentration of the extract obtained from method A is 4.2 mg/ml (estimated by Bradford assay) and 4.5 mg/ml (estimated by the BCA assay). The SSAO activity was measured with 10mM Benzylamine as substrate and was found to be 2.02 µmol H2O2 /mg protein.

## 4.2 Method B

Three independent experiments were performed by method B [Section 3.2.3.2](#sec-method-b) for extracting SSAO from rat BAT. Frozen tissues collected over 4 days were used for each experiment. Tissue weights are presented in [Table 4.1](#tbl-defat) and the percentage of fat lost was calculated from the dried tissue weight after treatment with 100%EtOH. Bradford and BCA assays were performed to estimate the protein concentrations in each extract and the results are presented and compared as mean in [Table 4.2](#tbl-proteinestimate) and [Figure 4.1](#fig-proteinesti) . The average amount of estimated protein is 6.07 and 6.27 mg/ml by Bradford and BCA assays respectively. The average SSAO activity was measured with 10mM Benzylamine was found to be 1.64 µmol H2O2 /mg protein [Table 4.3](#tbl-ssaoactivity) .

library(tidyverse)  
library(knitr)  
library(patchwork)  
defat\_results<- tibble(  
 Experiment\_ID = c("Experiment 1", "Experiment 2", "Experiment 3"),  
 Frozen\_weight\_g =as.numeric(c(10.81, 9.64, 10.25)),  
 Defatted\_dried\_weight\_g = as.numeric(c(4.62, 5.91, 7.31)),  
) |>   
 mutate(  
 percentage\_fat\_lost = 100\*(Frozen\_weight\_g - Defatted\_dried\_weight\_g)/Frozen\_weight\_g  
 )  
kable(defat\_results, align = "c")

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Experiment\_ID | Frozen\_weight\_g | Defatted\_dried\_weight\_g | percentage\_fat\_lost | | --- | --- | --- | --- | | Experiment 1 | 10.81 | 4.62 | 57.26179 | | Experiment 2 | 9.64 | 5.91 | 38.69295 | | Experiment 3 | 10.25 | 7.31 | 28.68293 |   Table 4.1: Weights of rat BAT used in method B for protein extraction. |

## 4.3 **Method C**

Three independent experiments were performed by Method C [Section 3.2.3.3](#sec-method-c) . The tissues were weighed before cutting into tiny pieces for homogenisation. Since there was no ideal way to measure the amount of fat removed by centrifugation, percentage of fat lost was not calculated for extracts obtained from method C. The average estimated protein concentration for extracts obtained by method C is 5.27 and 5.43 mg/ml by Bradford and BCA assays respectively. The average SSAO activity from samples obtained by method C was 0.02 µmol H2O2 /mg protein.

library(tidyverse)  
library(knitr)  
bradford\_BCA<- tibble(  
 Experiment\_ID = c("Experiment 1", "Experiment 2", "Experiment 3",   
 "Experiment 1", "Experiment 2", "Experiment 3"),  
 Method\_A = as.numeric(c(4.2, NA, NA, 4.5, NA, NA)),  
 Method\_B = c(6.4, 5.8, 6.0, 6.5, 6.1, 6.2),  
 Method\_C = c(5.1, 4.9, 5.8, 5.4, 5, 5.9),  
 Assay = c("Bradford", "Bradford", "Bradford", "BCA", "BCA", "BCA")  
)  
kable(bradford\_BCA)

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
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| | Experiment\_ID | Method\_A | Method\_B | Method\_C | Assay | | --- | --- | --- | --- | --- | | Experiment 1 | 4.2 | 6.4 | 5.1 | Bradford | | Experiment 2 | NA | 5.8 | 4.9 | Bradford | | Experiment 3 | NA | 6.0 | 5.8 | Bradford | | Experiment 1 | 4.5 | 6.5 | 5.4 | BCA | | Experiment 2 | NA | 6.1 | 5.0 | BCA | | Experiment 3 | NA | 6.2 | 5.9 | BCA |   Table 4.2: Estimated protein concentration (mg/ml) by Bradford and BCA assay for samples obtained by Method A (One independent experiment), and Method B and Method C (3 independent experiments) |

mean\_data <-  
 bradford\_BCA |>   
 group\_by(Assay) |>   
 summarise(across(starts\_with("method"), mean, na.rm = TRUE)) |>   
 pivot\_longer(cols = starts\_with("method"),  
 names\_to = "Method",  
 values\_to = "Mean")  
  
ggplot(mean\_data, aes(x = Method, y = Mean, fill = Assay)) +  
 geom\_bar(stat = "identity", position = position\_dodge(width = 0.9))+   
 scale\_y\_continuous(limits = c(0, 7))+  
 labs(  
 title = "Protein estimates by Bradford assay (mg/ml)",   
 x = NULL,   
 y = "Average protein\nconcentration estimate (mg/ml)"  
 ) +  
 theme\_minimal() +  
 scale\_fill\_manual(values = c("Bradford" = "black", "BCA" = "grey")) +  
 theme(  
 plot.title = element\_text(size = 14, hjust = 0.5),  
 axis.title.y = element\_text(size = 12, margin = margin(t = 0, r = 15, b = 0, l = 0)),  
 axis.text.x = element\_text(size = 10),  
 axis.text.y = element\_text(size = 10),  
 legend.title = element\_text(size = 10),  
 legend.text = element\_text(size = 9),  
 legend.position = "right",  
 plot.margin = margin(10, 10, 10, 10)  
 ) +  
 geom\_text(  
 aes(label = round(Mean, 2)),  
 vjust = -0.5,  
 position = position\_dodge(width = 0.9),  
 size = 3  
 )+ theme\_classic()

|  |
| --- |
| Figure 4.1: Comparision of average protein concentration estimate in extracts obtained from Method\_A, Method\_B, and Method\_C by BCA and Bradford assays. |

library(tidyverse)  
library(knitr)  
ssao\_activity<- tibble(  
 Experiment\_ID = c("Experiment 1", "Experiment 2", "Experiment 3"),  
 Method\_A = as.numeric(c(2.018182, NA, NA)),  
 Method\_B = c(1.553474, 1.72336, 1.649069),  
 Method\_C = c(0.024903, 0.013653, 0.011098),  
)  
  
means\_row <- tibble(  
 Experiment\_ID = "Mean",  
 Method\_A = mean(ssao\_activity$Method\_A, na.rm = TRUE),  
 Method\_B = mean(ssao\_activity$Method\_B, na.rm = TRUE),  
 Method\_C = mean(ssao\_activity$Method\_C, na.rm = TRUE)  
)  
  
ssao\_activity\_with\_means <- bind\_rows(ssao\_activity, means\_row)  
kable(ssao\_activity\_with\_means, align = "c")

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
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| | Experiment\_ID | Method\_A | Method\_B | Method\_C | | --- | --- | --- | --- | | Experiment 1 | 2.018182 | 1.553474 | 0.0249030 | | Experiment 2 | NA | 1.723360 | 0.0136530 | | Experiment 3 | NA | 1.649069 | 0.0110980 | | Mean | 2.018182 | 1.641968 | 0.0165513 |   Table 4.3: SSAO activity corrected to protein concentration from respective BCA results for Method A (One independent experiment), and Method B and Method C (3 independent experiments) |

means\_row |> pivot\_longer(cols = starts\_with("Method"),  
 names\_to = "Method",  
 values\_to = "Mean") |>   
 ggplot(aes(x = Method, y = Mean))+  
 geom\_bar(stat = "identity", fill = "black", width = 0.5  
 )+ theme\_classic()+  
 coord\_cartesian(ylim = c(0, 2.5))+  
 geom\_text(aes(label = round(Mean, 2)),  
 vjust = -0.5,  
 position = position\_dodge(width = 0.9),  
 size = 3)+  
 labs(title = "SSAO activity",  
 x = NULL,  
 y = "SSAO activity\n µmol H2O2 /mg protein ")

|  |
| --- |
| Figure 4.2: Comparison SSAO activity corrected to protein concentration from respective BCA results for Method A (One independent experiment), and Method B and Method C (3 independent experiments). |

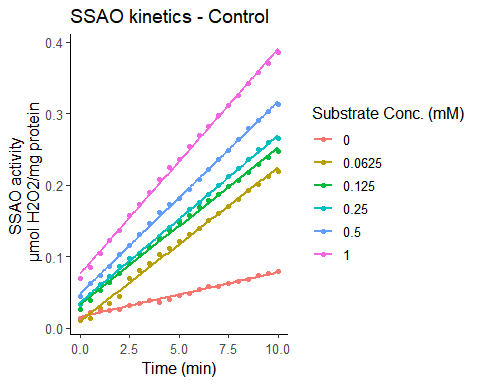
## 4.4 Enzyme Kinetics

The extract obtained from Experiment 1 of method B is used to measure the kinetic activity of SSAO by Amplex® red monoamine oxidase assay. The SSAO activity was measured as described in [Section 3.2.5](#sec-measureSSAO) . For measuring the continuous kinetic activity of SSAO, the instrument was setup in plate mode which measured the fluorescence every 30 seconds for 10 minutes. The fluorescence measurements resulting from the reaction mixture were used as blanks and all the values were corrected accordingly. The gain settings and focal length were maintained constant to obtain consistent readings.

Resorufin at different concentrations was used to establish a standard curve to determine the slope and offset using a linear regression curve. The fluorescence readings were exported into excel and formatted to ensure a consistent data format for processing. The mean of the replicate readings was converted into equivalent H2O2 concentrations using the resorufin standard curve equation. These values were plotted against time in minutes to calculate the change in H2O2 concentration over time using linear regression.

Individual curves were plotted for values obtained from each benzylamine concentration.

library(broom)  
library(ggpubr)  
  
# Importing the data from a csv file  
kinetic\_data <- read\_csv("E:/LearningR/rforlearn/GIT/academic\_works/kinetic\_data.csv")  
  
# Pivoting the data to fit the visualisation needs  
  
kinetic\_data\_pivot <- kinetic\_data |>   
 pivot\_longer(  
 cols = !(starts\_with("Time")),  
 names\_to = c("test\_group", "inhibitor\_concn", "substrate\_concn"),  
 names\_sep = "\_",  
 values\_to = "ssao\_activity"  
 ) |> mutate(inhibitor\_concn = parse\_number(inhibitor\_concn),  
 substrate\_concn = parse\_number(substrate\_concn))  
  
# Isolating the ssao activity for control group for plot  
  
kinetic\_data\_pivot |>   
 group\_by(test\_group) |>   
 filter(test\_group == "Control") |> mutate(substrate\_concn = factor(substrate\_concn)) |>   
 ggplot(aes(x = `Time (min)`,   
 y = ssao\_activity,   
 color = substrate\_concn,   
 group = substrate\_concn))+   
 geom\_point() +   
 geom\_smooth(  
 method = "lm",  
 formula = y ~ x,  
 se = FALSE,  
 )+  
 labs(  
 title = "SSAO kinetics - Control",  
 y = "SSAO activity \n µmol H2O2/mg protein", color = "Substrate Conc. (mM)"  
 )+ theme\_classic2()



SSAO activity measured over 10 minutes using benzylamine as substrate

The slope of these curves represents the initial velocity of each enzyme reaction at different substrate concentrations and reported as V0 (µmol H2O2/min/mg protein).

#|  
# Creating a separate df for the control group for deriving initial velocity  
  
kinetic\_data\_pivot\_control <- kinetic\_data\_pivot |> filter(test\_group == "Control")  
  
  
# lm modeling to generate slope for each substrate concentration  
  
lm\_results <- kinetic\_data\_pivot\_control |>   
 group\_by(substrate\_concn) |>   
 do(model = lm(ssao\_activity ~ `Time (min)`, data = .)) |>   
 mutate(  
 intercept = coef(model)[[1]],  
 slope = coef(model)[[2]],  
 equation = sprintf("y = %.4fx + %.4f", slope, intercept))  
  
# SSAO activity - initial velocity printed into a separate df  
ssao\_control\_Vo <- select(lm\_results, substrate\_concn, slope)  
  
# renaming the columns to S and V for easier use  
ssao\_control\_Vo <- ssao\_control\_Vo |>   
 rename(c('V' = slope, 'S' = substrate\_concn))  
  
table\_ssao\_control\_iniV <- ssao\_control\_Vo |>   
 rename(c('Initial Velocity' = "V",   
 'Substrate concentration' = "S"))  
kable(tibble(table\_ssao\_control\_iniV), format = "html", align = "c")

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Substrate concentration | Initial Velocity | | --- | --- | | 0.0000 | 0.0062583 | | 0.0625 | 0.0215376 | | 0.1250 | 0.0219468 | | 0.2500 | 0.0233723 | | 0.5000 | 0.0268804 | | 1.0000 | 0.0315220 |   Table 4.4: Initial Velocity for ssao activity for each substrate concentration calculated by linear regression. |

The initial velocity for each substrate concentration was tabulated and analysed using non-linear regression Michaelis-Menten kinetic model ([Equation 4.1](#eq-mm)) to generate the Michaelis-Menten curve [Figure 4.3](#fig-mmplot) and determine Km for benzylamine and Vmax values (A. 2005).

# Kinetic modelling to produce mm plots  
  
# Define the Michaelis-Menten equation  
mm\_equation <- function(S, Vm, Km) {  
 (Vm \* S) / (Km + S)  
}  
  
# non-linear fitting  
mm\_fit <- nls(V~ mm\_equation(S, Vm, Km),  
 data = ssao\_control\_Vo,  
 start = list(Vm = max(ssao\_control\_Vo$V), Km = median(ssao\_control\_Vo$S)))  
  
# Extracting the calculated parameters from above model  
params <- summary(mm\_fit)$parameters  
Km <- params["Km", "Estimate"]  
Vm <- params["Vm", "Estimate"]  
Vm\_se <- params["Vm", "Std. Error"]  
Km\_se <- params["Km", "Std. Error"]  
  
# Generating points for the fitted curve  
S\_curve <- seq(0, max(ssao\_control\_Vo$S), length.out = 100)  
V\_curve <- predict(mm\_fit, newdata = list(S = S\_curve))  
  
# mm\_plot generation  
ggplot(ssao\_control\_Vo, aes(x = S, y = V)) +  
 geom\_point(size = 3)+  
 geom\_line(data = data.frame(S = S\_curve, V = V\_curve), color = "blue")+  
 labs(title = "Micheales-Menten plot for SSAO kinetics",  
 x = "Substrate Concentration (mM)",  
 y = "Initial Velocity \n (µmol H2O2/min/mg protein)")+  
 theme\_classic2()+  
 annotate("text", x = max(ssao\_control\_Vo$S), y = min(ssao\_control\_Vo$V),  
 label = sprintf("Vmax = %.4f ± %.4f µmol H2O2/min  
 \nKm = %.4f ± %.4f mM",   
 Vm, Vm\_se, Km, Km\_se),  
 hjust = 1, vjust = 0)

|  |
| --- |
| Figure 4.3: Micheales Menten plot for SSAO enzyme kinetics using benzylamine as substrate |

From the nonlinear Michaelis Menten kinetic model, the Km and Vmax values were determined to be 0.03193mM and 0.02941 µmol H2O2/min, respectively.

## 4.5 **Enzyme Inhibition**

A key objective of this project is to measure the influence of caffeine and simvastatin on SSAO activity. 10mM caffeine stock solution was prepared by dissolving 19.4mg of caffeine in 10ml of dH2O. Further dilutions of 1 and 0.1 mM were prepared from the 10Mm stock solution and were used to in the kinetic experiments. Similarly, 100mM simvastatin stock was prepared by directly adding 4.78ml of DMSO to the vial containing 200mg of simvastatin powder. 10, 1, and 0.1mM dilutions of simvastatin were prepared from the 100mM stock solution.

To study the effect of these inhibitors, 10µL of each inhibitor at different concentrations were added to wells containing 60µL of SSAO extract prepared by Method B (Experiment 1) in triplicate. The plates were incubated at 37℃ prior to adding the reaction mixtures containing different concentrations of the substrate.

The enzyme activity was initiated by adding the reaction mixtures the containing substrate and the fluorescence measurements were started as quickly as possible. The fluorescence values of control samples without inhibitors, and samples containing inhibitors were measured every 30 seconds for 10 minutes. This data was exported to excel and was formatted for further analysis. The fluorescence readings were converted to equivalent H2O2 concentrations using the resorufin standard curve equation. These values were plotted against time in minutes to obtain the initial velocity. The rate of hydrogen peroxide formation is provided in the appendix1 for all the kinetic experiments performed without and with different concentrations of the inhibitors. The initial velocity of each group of substrate concentrations with and without inhibitors were transferred to GraphPad to plot a nonlinear regression curve using the competitive inhibition kinetic model. This analysis was performed to calculate the Ki, Km, and Vmax of the inhibition reaction. The nonlinear regression model for competitive inhibition uses the following equation (A. 2005):

Two-way ANOVA and Ad-Hoc Tukey’s comparison was performed to compare the difference in kinetic velocity between control and samples treated with different concentrations of the inhibitor. A significant difference in the initial velocities between different concentrations of the inhibitor and the control is represented with a p-value less than 0.05.

### 4.5.1 SSAO inhibition by Simvastatin

From the competitive inhibition model [Equation 4.2](#eq-nlm-CI) , the Ki for simvastatin was calculated to be 2.145 mM and the Km and Vmax for benzylamine on SSAO were 0.01893 mM and 0.02385 µM H2O2/min respectively ( [Figure 4.5](#fig-SIM-CI) , [Table 4.6](#tbl-sim-nlm-parameters) ). The initial velocities for each simvastatin-treated SSAO sample were determined by performing linear regression on the curve of H₂O₂ formation over time, as illustrated in [Figure 4.4](#fig-ssao-sim-activity) and [Table 4.5](#tbl-sim-ini-v) .

kinetic\_data\_pivot |>   
 group\_by(test\_group) |>   
 filter(test\_group == "SIM") |>   
 mutate(substrate\_concn = factor(substrate\_concn)) |>   
 ggplot(aes(x = `Time (min)`,   
 y = ssao\_activity,   
 color = substrate\_concn,   
 group = substrate\_concn))+   
 geom\_point() +   
 geom\_smooth(method = lm, se = FALSE)+   
 scale\_x\_continuous(breaks = c(0,5,10))+  
 labs(title = "SSAO activity in the presence of 0.1, 1, and 10mM simvastatin",  
 y = "SSAO activity \n µmol H2O2/mg protein",  
 color = "Substrate Concn. (mM)")+  
 facet\_wrap(~ inhibitor\_concn)+  
 theme\_classic2()+  
 theme(legend.position = "right")+  
 theme(  
 plot.title = element\_text(size = 10, hjust = 0.5),  
 axis.title.y = element\_text(size = 10, margin = margin(t = 0, r = 15, b = 0, l = 0)),  
 axis.text.x = element\_text(size = 10),  
 axis.text.y = element\_text(size = 10),  
 legend.title = element\_text(size = 8),  
 legend.text = element\_text(size = 6),  
 plot.margin = margin(10, 10,10, 10)  
 )+  
 guides(colour = guide\_legend(ncol = 1, position = "right"))

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| Figure 4.4: SSAO activity measured over 10 minutes with benzylamine as a substrate (1, 0.5, 0.25, 0.125, 0.0625mM) and in the presence of simvastatin (SIM) at 0.1, 1, 10mM concentration. SSAO activity is presented as µM H2O2/mg protein. A – SSAO activity in presence of 0.1mM SIM, B – SSAO activity in the presence of 1mM SIM, C – SSAO activity in the presence of 10mM SIM. |

kinetic\_data\_pivot\_sim <- kinetic\_data\_pivot |> filter(test\_group == "SIM")  
  
  
# lm modeling to generate slope for each substrate concentration  
  
lm\_results\_sim <- kinetic\_data\_pivot\_sim |>   
 group\_by(substrate\_concn, inhibitor\_concn) |>   
 do(model = lm(ssao\_activity ~ `Time (min)`, data = .)) |>   
 mutate(  
 intercept = coef(model)[[1]],  
 slope = coef(model)[[2]],  
 equation = sprintf("y = %.4fx + %.4f", slope, intercept))  
  
lm\_results\_sim |> pivot\_wider(  
 id\_cols = substrate\_concn,  
 names\_from = inhibitor\_concn,  
 values\_from = slope  
) |> rename('Substrate Concentration (mM)' = "substrate\_concn",  
 'SIM 10 mM' = "0.1",  
 'SIM 1 mM' = "1",  
 'SIM 0.1 mM' = "10"  
 ) |> kable(align = "c")

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| | Substrate Concentration (mM) | SIM 10 mM | SIM 1 mM | SIM 0.1 mM | | --- | --- | --- | --- | | 0.0000 | 0.0091990 | 0.0133830 | 0.0115535 | | 0.0625 | 0.0125717 | 0.0185989 | 0.0161799 | | 0.1250 | 0.0139309 | 0.0180983 | 0.0169519 | | 0.2500 | 0.0159289 | 0.0181323 | 0.0190872 | | 0.5000 | 0.0147374 | 0.0191430 | 0.0217997 | | 1.0000 | 0.0200087 | 0.0232822 | 0.0254066 |   Table 4.5: Initial velocities of SSAO activity in the presence of simvastatin at 0.1, 1, and 1mM concentrations. |

library(minpack.lm)  
# Create the dataset  
data <- data.frame(  
 S = rep(c(0, 0.0625, 0.125, 0.25, 0.5, 1), each = 4),  
 I = rep(c(0, 10, 1, 0.1)),  
 V = c(0.006258, 0.01155, 0.01338, 0.009199,  
 0.02154, 0.01257, 0.0186, 0.01618,  
 0.02195, 0.01393, 0.0181, 0.01695,  
 0.02337, 0.01593, 0.01813, 0.01909,  
 0.02688, 0.01474, 0.01914, 0.0218,  
 0.03152, 0.02001, 0.02328, 0.02541))  
  
# Define the competitive inhibition model function using GraphPad's equation  
comp\_inhib <- function(S, I, Vmax, Km, Ki) {  
 Km\_obs <- Km \* (1 + I / Ki)  
 (Vmax \* S) / (Km\_obs + S)  
}  
  
# Set initial parameter values (using GraphPad's results)  
start\_vals <- list(Vmax = 0.1, Km = 0.1, Ki = 0.1)  
  
# Fit the model using nlsLM for more robust fitting  
sim\_fit <- nlsLM(V ~ comp\_inhib(S, I, Vmax, Km, Ki),   
 data = data,   
 start = start\_vals,  
 lower = c(Vmax = 0, Km = 0, Ki = 0),  
 control = nls.lm.control(maxiter = 1000, maxfev = 1000))  
  
# Extract the fitted parameters  
sim\_params <- coef(sim\_fit)  
sim\_summary\_fit <- summary(sim\_fit)  
  
  
# Calculate R-squared  
ss\_total <- sum((data$V - mean(data$V))^2)  
ss\_residual <- sum(residuals(sim\_fit)^2)  
r\_squared <- 1 - (ss\_residual / ss\_total)  
  
sim\_nlm\_values <- c(  
Km <- sprintf("%.5f", sim\_params["Km"]),  
Vmax <- sprintf("%.5f", sim\_params["Vmax"]),  
Ki <- sprintf("%.5f", sim\_params["Ki"]),  
r\_squaredv <- sprintf("%.5f", r\_squared))  
  
  
  
# MM Plot  
ggplot(data, aes(x = S, y = V, color = factor(I))) +  
 geom\_point(size = 3) +  
 geom\_line(data = data.frame(S = rep(seq(0, max(data$S), length.out = 100), 4),  
 I = rep(c(0, 0.1, 1, 10), each = 100)) |>   
 mutate(V = comp\_inhib(S, I, sim\_params["Vmax"],   
 sim\_params["Km"],   
 sim\_params["Ki"])),  
 aes(x = S, y = V, color = factor(I))) +  
 labs(title = "Competitive Inhibition of SSAO by simvastatin",  
 x = "Substrate Concentration (mM)",  
 y = "Initial Velocity \n(µmol H2O2/min/mg protein)",  
 color = "Simvastatin Concentration (mM)") +  
 theme\_classic2() +  
 scale\_color\_brewer(palette = "Set1")+  
 annotate("text",   
 x = 0.75, y = 0.01,  
 label = sprintf("Vmax = %.4f\nKm = %.4f\n Ki = %.4f",   
 sim\_params["Vmax"],   
 sim\_params["Km"],   
 sim\_params["Ki"]))+  
 theme(legend.position = "bottom")+  
 guides(colour = guide\_legend(nrow = 1))

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| Figure 4.5: SSAO inhibition by simvastatin calculated by non-linear competetive inhibition model |

sim\_ssao\_nlm\_results <- tibble(Parameters\_SIM\_SSAO = c("Km", "Vmax", "Ki", "R-squared"),   
 Values = sim\_nlm\_values)  
kable(sim\_ssao\_nlm\_results)

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Parameters\_SIM\_SSAO | Values | | --- | --- | | Km | 0.01893 | | Vmax | 0.02385 | | Ki | 2.14483 | | R-squared | 0.17029 |   Table 4.6: Non linear fit parameters for SSAO inhibition by simvastatin |

### 4.5.2 SSAO inhibition by Caffeine

Like simvastatin, caffeine also follows the competitive inhibition model ( [Equation 4.2](#eq-nlm-CI) ) and the Ki for caffeine was calculated from one independent experiment ( [Figure 4.7](#fig-CAF-CI) ). The Ki value for caffeine calculated from competitive inhibition model is 6.768mM and the Km and Vmax for benzylamine on SSAO were 0.02903mM and 0.02850 µM H2O2/min, respectively ( [Figure 4.7](#fig-CAF-CI) and [Table 4.8](#tbl-nlm-caf-fit) ).

kinetic\_data\_pivot |>   
 group\_by(test\_group) |>   
 filter(test\_group == "CAF") |>   
 mutate(substrate\_concn = factor(substrate\_concn)) |>   
 ggplot(aes(x = `Time (min)`,   
 y = ssao\_activity,   
 color = substrate\_concn,   
 group = substrate\_concn))+   
 geom\_point() +   
 labs(title = "SSAO activity in the presence of 0.1, 1, and 10mM caffeine",  
 y = "SSAO activity \n µmol H2O2/mg protein",  
 color = "Substrate Concn. (mM)")+  
 geom\_smooth(method = lm, se = FALSE)+   
 facet\_wrap(~ inhibitor\_concn)+  
 scale\_x\_continuous(breaks = c(0,5,10))+  
 theme\_classic2()+  
 theme(legend.position = "right")+  
 theme(  
 plot.title = element\_text(size = 10, hjust = 0.5),  
 axis.title.y = element\_text(size = 10, margin = margin(t = 0, r = 15, b = 0, l = 0)),  
 axis.text.x = element\_text(size = 10),  
 axis.text.y = element\_text(size = 10),  
 legend.title = element\_text(size = 8),  
 legend.text = element\_text(size = 6),  
 plot.margin = margin(8, 8, 8, 8)  
 )+  
 guides(colour = guide\_legend(ncol = 1))

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| Figure 4.6: SSAO activity measured over 10 minutes with benzylamine as a substrate (1, 0.5, 0.25, 0.125, 0.0625mM) and in the presence of caffeine (CAF) at 0.1, 1, 10mM concentration. SSAO activity is presented as µM H2O2/mg protein. A – SSAO activity in presence of 0.1mM CAF, B – SSAO activity in the presence of 1mM CAF, C – SSAO activity in the presence of 10mM CAF. |

kinetic\_data\_pivot\_caf <- kinetic\_data\_pivot |> filter(test\_group == "CAF")  
  
  
# lm modeling to generate slope for each substrate concentration  
  
lm\_results\_caf <- kinetic\_data\_pivot\_caf |>   
 group\_by(substrate\_concn, inhibitor\_concn) |>   
 do(model = lm(ssao\_activity ~ `Time (min)`, data = .)) |>   
 mutate(  
 intercept = coef(model)[[1]],  
 slope = coef(model)[[2]],  
 equation = sprintf("y = %.4fx + %.4f", slope, intercept))  
  
lm\_results\_caf |> pivot\_wider(  
 id\_cols = substrate\_concn,  
 names\_from = inhibitor\_concn,  
 values\_from = slope  
) |> rename('Substrate Concentration (mM)' = "substrate\_concn",  
 'CAF 10 mM' = "10",  
 'CAF 1 mM' = "1",  
 'CAF 0.1 mM' = "0.1"  
 ) |> kable(align = "c")

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| | Substrate Concentration (mM) | CAF 0.1 mM | CAF 1 mM | CAF 10 mM | | --- | --- | --- | --- | | 0.0000 | 0.0122429 | 0.0131826 | 0.0134182 | | 0.0625 | 0.0161367 | 0.0185402 | 0.0206733 | | 0.1250 | 0.0185621 | 0.0206289 | 0.0232647 | | 0.2500 | 0.0202061 | 0.0233087 | 0.0242922 | | 0.5000 | 0.0215960 | 0.0240157 | 0.0265001 | | 1.0000 | 0.0263826 | 0.0308273 | 0.0314966 |   Table 4.7: Initial velocities of SSAO activity in the presence of caffeine at 0.1, 1, and 1mM concentrations. |

library(minpack.lm) # For more robust nonlinear fitting  
  
# Create the dataset  
data <- data.frame(  
 S = rep(c(0, 0.0625, 0.125, 0.25, 0.5, 1), each = 4),  
 I = rep(c(0, 0.1, 1, 10)),  
 V = c(0.006258, 0.01342, 0.01318, 0.01224,  
 0.02154, 0.02067, 0.01854, 0.01614,  
 0.02195, 0.02326, 0.02063, 0.01856,  
 0.02337, 0.02429, 0.02331, 0.02021,  
 0.02688, 0.0265, 0.02402, 0.0216,  
 0.03152, 0.0315, 0.03083, 0.02638))  
  
# Define the competitive inhibition model function using GraphPad's equation  
comp\_inhib <- function(S, I, Vmax, Km, Ki) {  
 Km\_obs <- Km \* (1 + I / Ki)  
 (Vmax \* S) / (Km\_obs + S)  
}  
  
# Set initial parameter values (using GraphPad's results)  
start\_vals <- list(Vmax = 0.1, Km = 0.1, Ki = 0.1)  
  
# Fit the model using nlsLM for more robust fitting  
fit <- nlsLM(V ~ comp\_inhib(S, I, Vmax, Km, Ki),   
 data = data,   
 start = start\_vals,  
 lower = c(Vmax = 0, Km = 0, Ki = 0),  
 control = nls.lm.control(maxiter = 1000, maxfev = 1000))  
  
# Extract the fitted parameters  
params <- coef(fit)  
summary\_fit <- summary(fit)  
# R-squared  
ss\_total <- sum((data$V - mean(data$V))^2)  
ss\_residual <- sum(residuals(fit)^2)  
r\_squared <- 1 - (ss\_residual / ss\_total)  
  
caf\_nlm\_values <- c(  
Km <- sprintf("%.5f", params["Km"]),  
Vmax <- sprintf("%.5f", params["Vmax"]),  
Ki <- sprintf("%.5f", params["Ki"]),  
r\_squaredv <- sprintf("%.5f", r\_squared))  
  
  
  
  
ggplot(data, aes(x = S, y = V, color = factor(I))) +  
 geom\_point(size = 3) +  
 geom\_line(data = data.frame(S = rep(seq(0, max(data$S), length.out = 100), 4),  
 I = rep(c(0, 0.1, 1, 10), each = 100)) %>%  
 mutate(V = comp\_inhib(S, I, params["Vmax"], params["Km"], params["Ki"])),  
 aes(x = S, y = V, color = factor(I))) +  
 labs(title = "Competitive Inhibition of SSAO by caffeine",  
 x = "Substrate Concentration (mM)",  
 y = "Initial Velocity \n(µmol H2O2/min/mg protein)",  
 color = "Caffeine Conc. (mM)") +  
 theme\_classic2() +  
 scale\_color\_brewer(palette = "Set1")+  
 annotate("text",   
 x = 0.75, y = 0.01,  
 label = sprintf("Vmax = %.2f\nKm = %.4f\n Ki = %.4f",  
 params["Vmax"],   
 params["Km"],   
 params["Ki"]))+  
 theme(legend.position = "bottom")+  
 guides(colour = guide\_legend(nrow = 1))

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| Figure 4.7: SSAO inhibition by caffeine, calculated by non-linear competetive inhibition model. |

caf\_ssao\_nlm\_results <- tibble(Parameters\_CAF\_SSAO = c("Km", "Vmax", "Ki", "R-squared"),   
 Values = caf\_nlm\_values)  
kable(caf\_ssao\_nlm\_results)

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Parameters\_CAF\_SSAO | Values | | --- | --- | | Km | 0.02903 | | Vmax | 0.02850 | | Ki | 6.76781 | | R-squared | 0.28889 |   Table 4.8: Non linear fit parameters for SSAO inhibition by caffeine |

# 5. Discussion

SSAO has been studied in various cells and tissue groups that include smooth muscle cells, intestinal tissue, heart, lungs, white adipose tissue, brown adipose tissue, kidney, retina and placenta in models such as mice, rats, and rabbits (Abella et al. 2003; BARRAND, FOX, and CALLINGHAM 1984; Barrant and Callingham 1984; María Carmen Iglesias-Osma et al. 2004; C. Li et al. 2019; Manasieva et al. 2022, 2023; Wang et al. 2018). SSAO has been detected in various tissue types in the human body as it is widely distributed in the form of serum SSAO/VAP-1 or membrane bound SSAO (Dunkel et al. 2008).

Membrane bound SSAO are found in adipose tissues, and vascular smooth muscle endothelial cells in rats, mice and humans. Brown adipose tissues were chosen to be the source of SSAO for this project to support effective use of tissues extracted from rats. A major challenge in extracting proteins and enzymes from adipose tissues is the higher concentration of lipids. Hence methods that propose to remove the fat from adipose tissues were identified and modified to fit the objectives of the experiments. The key steps used to successfully extract SSAO were, defatting the tissues with ethanol and dissolving the proteins in PBS with a pH of 8.0.

Method A involved rehydrating the tissues using a freeze-dryer. Dehydrating prior to defatting the tissues were ideal as the water present in the tissues is miscible with ethanol, this can lead to loss of proteins while defatting the tissues.

BCA and Bradford results for protein concentrations obtained by each method were compared to identify a suitable method with highest quantity of proteins. Experiment 1 of method B produced a 6.5 mg protein/ml by BCA assay. Protein concentration estimated by BCA was considered for downstream calculations as BCA is relatively more sensitive than Bradford (Noble and Bailey 2009). An advantage with BCA is that it possesses a higher tolerance for interfering species (Noble and Bailey 2009).

Method A was not repeated due to freeze dryer unavailability for dehydration. Hence only one independent experiment was performed by method A. Method A also produced a more efficient fat removal with 57.3% loss in weight owing to fat and water. Method B and C were not dehydrated before defatting, which is the potential cause for a lower fat loss percentage with 38.7% and 28.7% respectively. PBS with pH 8.0 was used to dissolve the proteins as SSAO is highly stable at an optimum pH of 8.0 and the water in the buffer dissolves the proteins present in the tissue (Stevanato et al. 2011). While the samples were treated with PBS, a lower temperature was maintained to prevent loss of protein activity.  Method C involved using a tissue homogeniser, which is prone to generate heat when grinding the tissue. Hence, the homogenisation setup was modified to hold the tissues in an ice bath while homogenising at lower speeds. The tissues were homogenised with PBS instead of a lysis buffer like RIPA to keep the buffer uniform across all methods. The RIPA buffer contains higher concentrations of surfactants which can potentially hinder assays such as Bradford (Noble and Bailey 2009). The average estimated protein concentration of the extract obtained from method C was 5.43 mg/ml and 5.27 mg/ml by Bradford and BCA assays respectively. The extract obtained by method C had no significant SSAO activity with 0.016554 µmol H2O2 /mg protein. In contrast, extracts obtained in experiment 1 by methods A and B produced 2.018 and 1.641 µmol H2O2/mg protein SSAO levels respectively. Method B was chosen to perform further enzyme kinetic experiments. The SSAO activities measured by Amplex ® red are corrected to per mg protein levels calculated from BCA. After the preliminary results from experiment 1, two more independent experiments were performed by method B and method C. The protein concentration estimates by Bradford and BCA assays were closer to results from experiment 1. This was also the case for SSAO level measurements in extracts from methods B and C. Since extracts obtained by method C did not have viable SSAO activity, the extract obtained from method B was used for further analysis. In comparing the quantity of proteins extracted by other methods (Bioprotocol, CST, and RELi) with method A, method B and method C, the protocols from the literature show significantly higher quantities than the methods used in this project [Figure 5.1](#fig-protein_extraction_comparision). The protocols from the literature use Mice BAT for extracting proteins. Hence to provide a fair comparison in protocols, samples from the same animal needs to be used.

library(tidyverse)  
  
comp\_table <- tibble(Replicate = fct(c("1", "2", "3", "4")),  
 Bioprotocol = c(0.04115, 0.0431, 0.0374, 0.04),  
 CST = c(0.04892, 0.08024, 0.068, NA),  
 RELi = c(0.054944, 0.037152, 0.034784, NA),  
 Method\_A = c(0.004167, NA, NA, NA),  
 Method\_B = c(0.006743, 0.006328, 0.006432, NA),  
 Method\_C = c(0.005268, 0.004878, 0.005756, NA)  
 )  
  
comp\_table |>   
 pivot\_longer(  
 cols = !Replicate,  
 names\_to = "Protocol",  
 values\_to = "Protein\_Estimate"  
 ) |> group\_by(Protocol) |> summarise(mean = mean(Protein\_Estimate, na.rm = TRUE)) |>   
 ggplot(aes(x = fct\_reorder(Protocol, desc(mean)),   
 y = mean, fill = Protocol))+  
 geom\_bar(stat = "identity",   
 na.rm = FALSE,   
 orientation = "x",  
 show.legend = FALSE)+  
 geom\_text(aes(label = round(mean, 4)),  
 vjust = -0.5,  
 position = position\_dodge(width = 0.9),  
 size = 3)+  
 labs(  
 x = NULL,  
 y = "Average Protein Conc. \n (mg/ml/mg BAT/ml Buffer)"  
 )+  
 theme\_classic()

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| Figure 5.1: Comparison of protein extracted by different methods in literature with methods used in this study. The protein concentration measured by BCA is plotted in mg/ml/mg BAT/ml buffer used (An & Scherer, 2020; Diaz Marin et al., 2019). |

library(knitr)  
kable(comp\_table)

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| | Replicate | Bioprotocol | CST | RELi | Method\_A | Method\_B | Method\_C | | --- | --- | --- | --- | --- | --- | --- | | 1 | 0.04115 | 0.04892 | 0.054944 | 0.004167 | 0.006743 | 0.005268 | | 2 | 0.04310 | 0.08024 | 0.037152 | NA | 0.006328 | 0.004878 | | 3 | 0.03740 | 0.06800 | 0.034784 | NA | 0.006432 | 0.005756 | | 4 | 0.04000 | NA | NA | NA | NA | NA |   Table 5.1: A comparision of the amount of protein (mg/ml/mg BAT/ml) extracted from BAT by different methods in the literature and the methods used in this project |

Since there are no published methods for extracting SSAO from brown adipose tissues in rats, the results of this study cannot be compared to estimate correlations.

Comparative analysis using parametric or non-parametric tests was not performed due to the limited number of experiments. Performing statistical tests with such a small sample size would likely increase the margin of error and reduce the confidence in the results. Therefore, the findings were only tabulated and plotted to clearly present the outcomes obtained in this study.

**SSAO Kinetics:**

The Km values for benzylamine as a substrate for SSAO extracted from various species have been reported in the literature (Yraola et al. 2007). Since this study uses rats - *Rattus Norvegicus* as the source of SSAO, the results obtained are compared with Km values estimated from same species of sample source. A study by Yraola et al has reported a Km value of 0.0127 mM for benzylamine as a substrate for SSAO obtained from rats (Yraola et al. 2007). This correlates with the **Km value of 0.03193 mM** obtained from this study for benzylamine as a substrate of SSAO obtained from rat brown adipose tissue (Yraola et al. 2007). Moreover, the same brown adipose tissue was used by both the studies to extract the SSAO enzyme.

Ki values for Caffeine or Simvastatin have not been reported on brown adipose tissue SSAO obtained from *Rattus Norvegicus.* This study is the first to report the Ki values from caffeine and simvastatin on SSAO obtained from rats. However, a study by Sun P et al., has reported that simvastatin has reduced the release of membrane bound SSAO into circulation and thus preventing leukocyte adhesion and OGD mediated increase in vascular permeability (Sun et al. 2018). This study by Sun et al., measures the levels of SSAO by western blotting and does not provide a Ki value for simvastatin. A study by Che et al., reported that administration of caffeine to male Wistar rats caused a reduction in SSAO quantities due to accumulation of caffeine (Che et al. 2012). But no Ki values have been reported in the literature. **The Ki values obtained from this project are 2.145 and 6.768 mM for simvastatin and caffeine respectively.** The Ki values obtained are outside the inhibitor concentration range used to calculate them, indicating a need to repeat the inhibition experiments with updated concentrations that reflect the current Ki values. The fact that these Ki values fall within the millimolar range suggests that both compounds are not potent inhibitors of SSAO.

However, it’s important to note that these results are based on a single independent experiment. Additionally, this study did not use inhibitors with known Ki values as controls, which limits the ability to accurately compare the inhibition effects of different compounds. Future research should focus on addressing these limitations to improve accuracy and reduce errors, leading to more reliable results.

# 6. Limitations and future works

1. To analyse the extract from rat BAT for more specificity of substrates to distinguish the type of SSAO present. This can be achieved by comparing the reaction rates by using different substrates such as tyramine and benzylamine along with known inhibitors of other classes of deamination enzymes such as the monoamine oxidases (MAO - A and MAO - B).
2. Reanalyse the inhibition properties of simvastatin and caffeine with additional controls such as inhibitors such as the MDL72794A, PXS-4728A, methylhydrazine and semi carbazide. This could potentially increase the reliability of the inhibition kinetic results. Optimisation of inhibitor concentrations to reevaluate the Ki for simvastatin and caffeine.
3. Conduct a larger number of independent experiments to enhance the confidence interval and reduce error.
4. No subsequent assays were performed to verify complete elimination of fats by any of the methods used.
5. The extracts were stored at -20℃ instead of -80℃ (standard practice), which could have affected the stability of the enzymes present in the extracts leading to a reduced activity.

# 7. Conclusion

This study presents a comprehensive methodology for extracting SSAO from rat brown adipose tissues, demonstrating effective protein quantification through Bradford and BCA assays, even after defatting with 100% ethanol. Despite storage at -20°C, the samples retained quantifiable SSAO activity, as measured by the Amplex® Red monoamine oxidase assay. The kinetic parameters obtained for SSAO, with a Km of 0.03193 using benzylamine as a substrate, align closely with previously published results under similar conditions.

However, the Ki values for caffeine and simvastatin were derived from a single experiment, leading to a wider error margin and lower confidence in these results. Additionally, these Ki values fall within the millimolar range, suggesting that both compounds may not be potent inhibitors of SSAO. It is also worth noting that these values were outside the inhibitor concentration range used in their calculation, indicating a need for repeated experiments with adjusted concentrations that reflect the current Ki values. Moreover, the absence of inhibitors with known Ki values as controls limits the ability to accurately compare the inhibition effects of different compounds.

In conclusion, while the study successfully establishes a reliable method for SSAO extraction and activity measurement, further experiments with improved controls and optimized conditions are necessary to yield more reliable Ki values for caffeine and simvastatin. Addressing these limitations in future research will enhance the accuracy and robustness of the findings.

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