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Expression and Purification of Cytochrome 2U1 and Canine 1A2 and Metabolism of Caffeine by Cytochrome 1A2

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EXPRESSION AND PURIFICATION OF CYTOCHROME 2U1 AND CANINE 1A2 AND METABOLISM OF CAFFEINE BY CYP1A2

A Master's Thesis

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Chemistry

By

Brenda M. Wekesa

December 2021

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ABSTRACT

Cytochrome P450 enzymes are a large family of membrane heme proteins involved in eliminating drugs and the synthesis or elimination of steroids, eicosanoids, and vitamins. CYP2U1 is one of the poorly characterized P450 enzymes classified as orphan cytochrome P450 with unknown exact biological function. It is expressed in the brain and thymus as a catalyst in the hydroxylation of arachidonic acid. Unlike other cytochrome P450s, CYP2U1 is a bit unusual with forty extra amino acids. If a mutation occurs, it can lead to an early onset inherited disorder of the central nervous system called Hereditary Spastic Paraplegias (HSP). Most of the P450 enzymes in humans are known and characterized based on their structure and functions. Many of them have orthologous proteins in other species. CYP1A2 metabolizes many substrates like phenacetin, caffeine, acetaminophen, and theobromine (from chocolate) which makes it likely that there are differences between the activity of the canine and human CYP1A2 enzyme. The human CYP1A2 protein is well characterized and has a crystal structure available. This has implications in the treatment of canines in veterinary medicine and in drug metabolism studies, which are sometimes conducted in dogs. Few studies have been conducted to look at the biochemistry of the purified canine CYP1A2. Synthesis of the canine CYP1A2 gene and human CYP 2U1 followed by cloning into the pCW plasmid was done and grown in E. coli JM109 cells for the production of large amounts of proteins that were isolated by breaking the cells open through sonication, solubilized with the detergent CHAPS, and purified with Nickel affinity and ion-exchange chromatography. The purity of the protein after purification was measured by gel electrophoresis and UV-vis chromatography. The purified CYP 1A2 protein was used to characterize the metabolism of caffeine, a substance known to be metabolized by human CYP1A2.

KEYWORDS: cytochrome p450 enzymes, cyp 1a2, cyp 2u1, arachidonic acid, mutation, hereditary spastic paraplegias

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I dedicate this thesis to the Almighty God, the magnificent.

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CHAPTER 1: INTRODUCTION

1.1 Cytochrome P450 Family

Cytochrome P450 enzymes (CYP) are a large family of membrane heme proteins involved in eliminating drugs and anabolism and catabolism of steroids, fatty acids, eicosanoids, and vitamins.[1-3] There are 57 human cytochrome P450s, and surprisingly almost a quarter of them are categorized as orphan cytochrome P450s because their function, expression, and regulation are not well understood. These heme-containing proteins catalyze a large variety of oxidative reactions during metabolism involving a wide variety of endogenous and xenobiotic substrates.[4] Cytochrome P450s are named so because of their unique nature to absorb light at 450nm when reduced and complexed with carbon monoxide due to the presence of a heme-iron center that is embedded in their structure through a cysteine thiolate ligand. There are more than 50 CYP450 enzymes, but only a few are capable of metabolizing drugs expressed in the liver, small intestines, kidney, lungs, and placenta.[5] They were first discovered in 1955 in rat endoplasmic reticulum in the liver with oxidizing properties of xenobiotic substances.[6]

1.1.1 Binding Partners. The required electrons for oxidative reactions are supplied from the human cytochrome p450 reductase (CPOR), which has a molecular weight of 77kDa and is composed of 55 amino acid N-terminal transmembrane domains, which transfers electrons from Nicotinamide adenine dinucleotide phosphate (NADPH) through its cofactors Flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN) to the heme of the P450 (Figure 1). During the redox cycle, CPOR undergoes conformational changes between the closed and open forms, but only the open state can transfer the electrons to the P450s.[7]

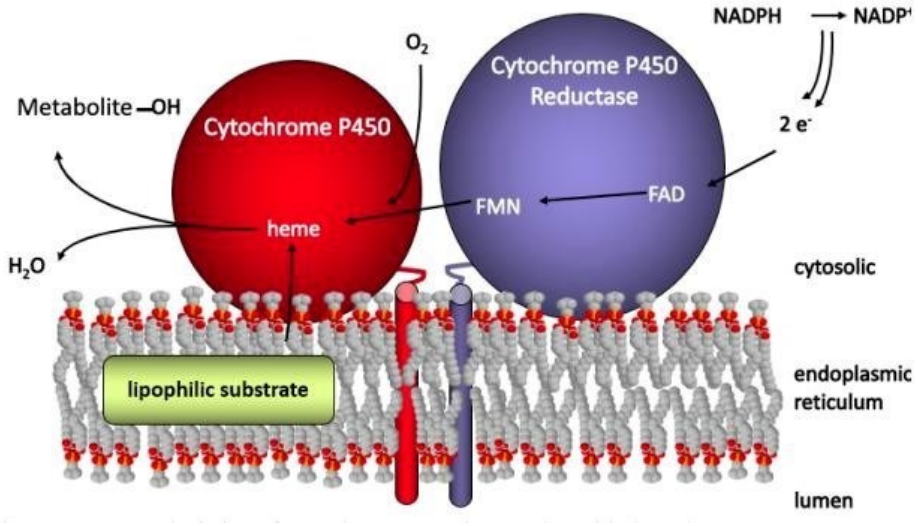

Figure 1. Cytochrome P450 in complex with the Reductase enzyme reproduced by Dr. Devore

1.1.2 The Catalytic Cycle. Figure 2 explains the catalytic cycle of P450s which are initiated when the substrate binds to the active site leading to the displacement of the water molecule normally attached to the heme (1). The binding of the substrate to the Cytochrome P450 enzyme triggers a couple of events, such as the change of spin state of the prosthetic heme and a change in the reduction potential, which is a complex process **(**2).

The substrates involved in binding are mainly hydrophobic and poorly soluble in water. This leads to a change of state of the heme iron from the low spin to the high spin state[7]. The high spin Fe^{3+} is quickly reduced to a ferrous state due to its high positive reduction potential since water is a weaker ligand for the Fe^{2+} and the difference in states tends to increase the stabilization of the ferric state in the absence of substrates. [8] Several factors play a role in the resulting reduction potential. For instance, the higher the binding affinity of the substrate to the active site of the P450, the stronger the water displacement effect resulting in a shift of the reduction potential. [9]

The presence of oxygen and carbon monoxide tends to have the same effect by binding to the ferric iron of the enzyme. Changes in temperature and pH affect the resulting equilibrium of the high-low spin of the P450s. The Oxy-P450 complex (**3**) formation through the binding of oxygen molecules which is in turn reduced to a peroxo-ferric intermediate **(4)** and later protonated to a hydroperoxo-ferric intermediate (**5**).

The effect of substrate binding reduces NADPH via cytochrome P450 reductase mostly or other associated reductases through electron transfer.[10] The second electron is transferred from either cytochrome P450 reductase, ferredoxins, or cytochrome b5 after the binding of molecular oxygen on the center of the heme at the distal axial coordination position to a peroxo-ferric intermediate. The distal oxygen atom is then protonated for the second time, yielding the first highly reactive compound, an iron (iv) Oxo species, and water through heterolysis of O-O bond (**6**) and subsequently oxygenation of the substrate to form a product complex.

1.2 Cytochrome 1A2

Cytochrome P450 1A2 is mainly located in the endoplasmic reticulum and is involved as a catalyst to many reactions in the metabolism of drugs and synthesis of steroids and lipids. The gene consists of seven exons and six introns. Human CYP1A2 consists of a 29 amino acid long transmembrane domain, a heme in its catalytic domain of multiple residues on helix F and I, and has a molecular weight of 58 kDa.[11, 12]. The core of the protein forms the heme-binding site and the proximal surface, which is the binding sites for the redox partners; NADPH cytochrome p450 oxidoreductase and cytochrome b_5 is the most conserved region compared to other known structure of the mammalian P450s.[13]

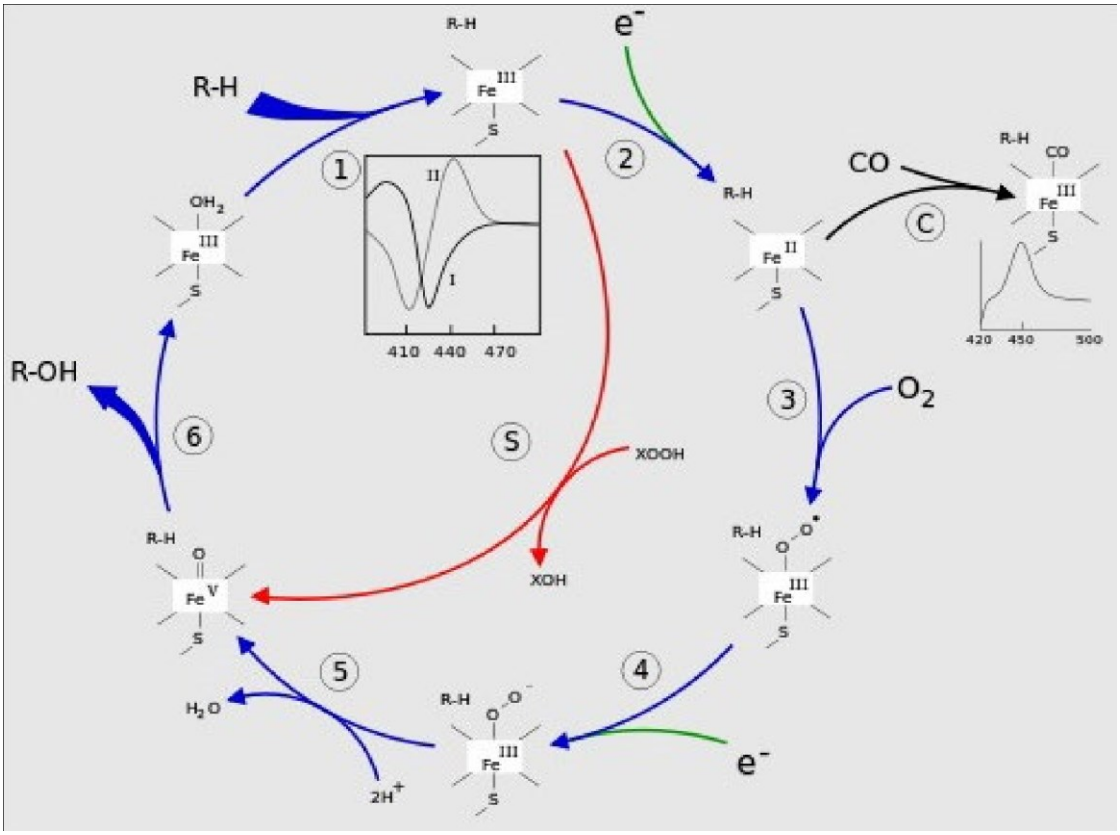

Figure 2. The Catalytic Cycle of cytochrome p450s. Used under public domain copyright.[14]

The structure is composed of 12 α-helices designated A-L and four β-sheets (1-4) respectively, with several other helices present in other P450s designated with prime or double prime designations as shown in Figure 3. Unlike the soluble prokaryotic P450s, the structure of the mammalian P450 has hydrophobic surfaces on the tip of the protein and a large insertion between helices. [15] P450s have different structural diversity and specificity that allows the binding of substrates of different sizes.[16] It has several different substrates like caffeine, phenacetin, paracetamol, theobromine, and many others, which are mostly planar polyaromatic amides and amines. [17]

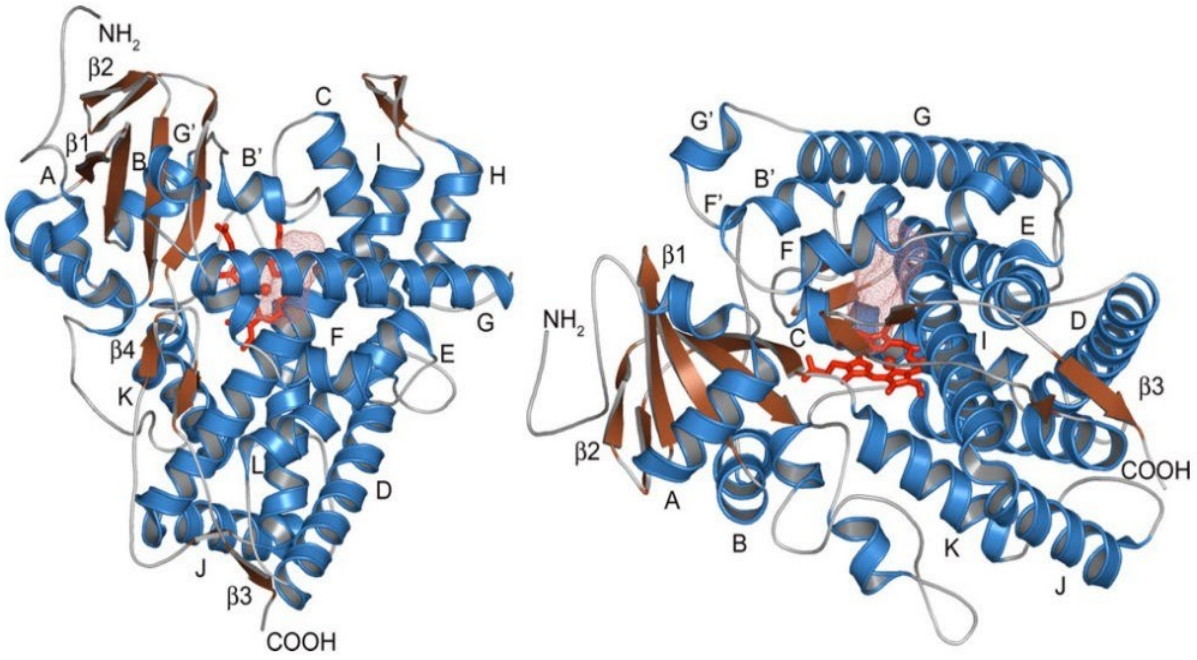

Figure 3. Human Cytochrome 1A2 Crystal Structure, adapted from reference 18

Toxicosis of Caffeine in Canines: Homologs of cytochrome P450s in different species often have similarities in the substrates that they metabolize. A lot of information is known about the human CYP1A2 enzyme, but less is known about these enzymes in cats and dogs. [19] Caffeine [(1,3,7)-trimethyl xanthine] is a natural plant alkaloid found in many plant species, beverages, foods, and some medications and is generally considered a safe compound for consumption in humans with a few mild effects.

It is one of the most consumed beverages believed to reduce fatigue as well as enhancing mental alertness and concentration. However, this case is not the same with canines since accidental consumption of caffeine has been reported to cause severe effects and even death [20.] Chocolate, xylitol, and grapes are some of the top ten poisons that were reported to the Pet Poisons Helpline. Toxicity in canines has implications, on the drug metabolism studies that

are sometimes conducted in dogs as well as veterinary medicine. CYP1A2 metabolizes substrates such as caffeine, phenacetine, and theobromine in coffee, etc., clearly indicating that there are differences between the activity of canine and human CYP1A2.

1.3 Cytochrome 2U1

CYP2U1 is one of the orphan P450s mainly located in the brain and thymus, although also distributed in other tissues such as the kidney, bladder, uterus, testis, and prostate.[21] The CYP 2 family is mainly composed of nine exons but, cytochrome 2U1 is highly distinct and conserved with only five exons in its sequence with an unusually long N-terminal region with eight proline residues and five arginine residues on its 20 amino acid insert along the transmembrane helix (Figure 4). [22] The human CYP2U1 shares 99, 89, 88, 87, and 83 % amino acid sequence identity with a chimpanzee, rat, dog, horse, and mouse, respectively, [23] and contains almost 50 extra amino acids located at the N-terminus of the protein. [24] Different CYPs can have the same substrates but generate different products. Drugs such as Debrisoquine and terfenadine, antihistamines, [25] serotonin, fatty acids, and N- arachidonic acid are some of the known substrates for CYP2U1. [23] Debrisoquine (Deb), a substrate to CYP 2D6 as well, has a main metabolite 4-hydroxy-Deb with CYP2D6, but with a completely different metabolite when metabolized by CYP2U1. CYP2U1 catalyzes the oxidation of Deb mainly to 1-hydroxy-Deb with smaller amounts of 4-hydroxy-Deb, 6-hydroxy-Deb, and 8-hydroxy-Deb.[26]

The sequence alignment of the 57 human CYPs showed that CYP2U1 is the longest, with at least 40 amino acids more than CYPs of families 2 and 3, and 13-33 amino acids more than the CYPs of family 4. (Figure 5).[23]

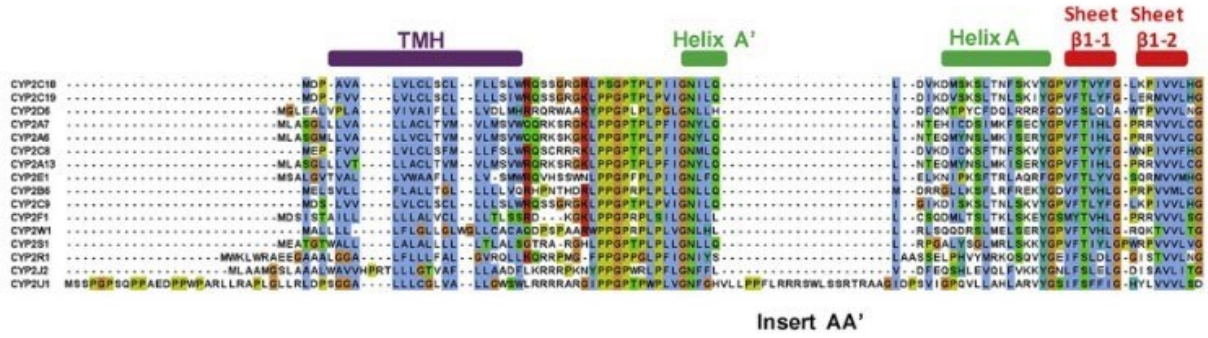

Figure 4. Amino acid alignment of the members of the human CYP2 family adapted from reference 22

Terfenadine (Terf), a derivative of terfenadine, was also analyzed with different CYPs. [26, 27] Terf and alkyl-Terf, Terf derivatives with replaced tertbutyl substituents with different alkyl chains formed different products with CYP2J2, CYP3A4, and CYP2U1 with hydroxylation in the homobenzylic position, N-dealkylation and hydroxylation in the benzylic positions, respectively. (Figure 6) [28]

CYP 2U1 plays a major role in the hydroxylation of arachidonic acid into two bioactive metabolites, 19- and 20-hydro eicosatetraenoic acid and structurally long fatty acids like docosahexaenoic acid. [21, 24] It is also suspected to play a role in iron transport and blood flow control in some tissues or organs, as well as signal transduction modulator within immune cells because of its hydroxylation properties of arachidonic acids, docosahexaenoic acids, and eicosapentaenoic acids in the thymus. [24] Studies were done on the nonalcoholic smokers, and nonsmokers as well as the alcoholic smokers and nonsmokers investigated expression levels of

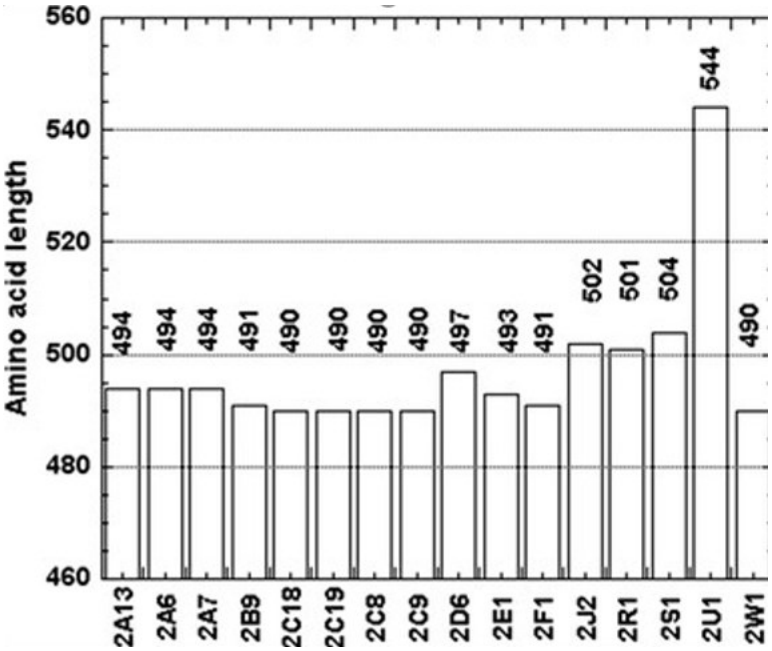

Figure 5. N-terminal alignment of CYP 2 family, adapted from reference 22

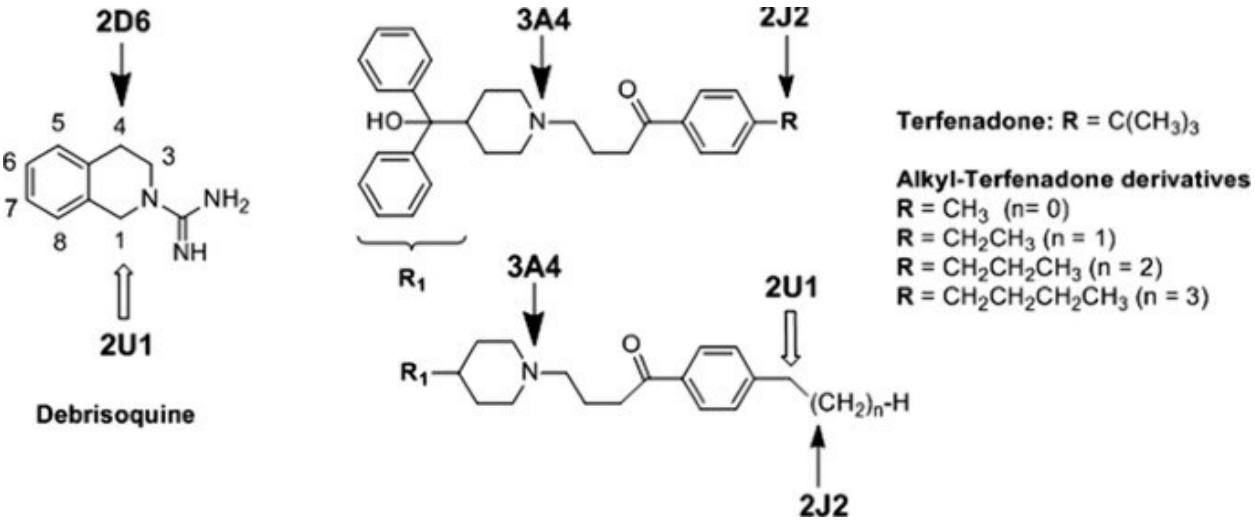

Figure 6. Oxidation sites of Debrisoquine, terfenadine, and terfenadine derivatives by P450s adapted from reference 22

CYP 2 proteins in the prefrontal cortex and amygdala and showed that CYP2U1 and CYP2E1 were expressed in all the samples and increased on alcohols and smokers. [29] These findings might have implications on the metabolism of drugs and endogenous substances.[30]

Hereditary Spastic paraplegia (HSP): Rare CYP2U1 mutations have been associated with several pathological situations and related to hereditary spastic paraplegia, a neurological disorder of the Central Nervous System that is mainly characterized by gradual spasticity and weakness of the lower limbs. [31-35] These symptoms either occur alone or with other neurological signs. Genetic studies in HSP have shown the alteration of the molecular and cellular pathway caused by changes in fatty acid, sphingolipid, and phospholipid metabolism.[35] SPG56 is a rare form of HSP due to mutations in CYP2U1. Less than 15 families have been reported so far with truncating, and missense variants in CYP2U1 observed with either homozygous or heterozygous states. [34] A study was done on the SPG56 patients, and it was observed that most of the missense variants led to inhibition of enzymatic activity due to the loss of the proper binding of the heme to the protein or modification of the protein structure. [33]

1.4 Project Objectives

The project goal is to clone the purchased gene for CYP2U1 into the pCW plasmid and use PCR for amplification. For canine CYP1A2, though, the gene was purchased pre-cloned into pCW by Twist Bioscience. The cloned gene is expressed in *E. coli* culture, sonicated to lyse the cells, solubilized with cymal-5 detergent, and separated solubilized protein by centrifugation. Purification of the protein is conducted using affinity chromatography followed by ion-exchange chromatography. The purpose of this project is to perform metabolic assays using HPLC to analyze and quantify canine CYP1A2 metabolism of caffeine in comparison to humans after purification. Protein crystals will be screened using purchased crystal kits. High-quality crystals are required to determine the crystal structure of both proteins through X-ray diffraction experiments, so crystals will likely need to be optimized before X-ray diffraction experiments.

CHAPTER 2: MOLECULAR BIOLOGY

The manufacture of well-defined and specific proteins and antibodies has been improved immensely by recombinant DNA technology with a complete solution in cloning and expression of the gene of interest. With this technology in hand, there is a possibility of designing and tailoring artificially an enzyme that can perfectly meet the requirements such as altered substrate specificity, kinetic activity, or enhanced thermal stability.

This technology depends on the ability of the restriction enzymes to cut the DNA of interest and that of the ligase in rejoining the DNA molecule in vitro into a vector to be transformed into host cell that is convenient to be manipulated experimentally through extraction and purification. Restriction endonucleases are essential in recombinant technology because they recognize a short, specific sequence of nucleotide bases that are randomly distributed throughout the DNA and cut it by catalyzing the hydrolysis of the adjacent nucleotide bonds. This ability to cut DNA at specific locations by these enzymes has led to many advancements involving the isolation of gene by researchers and their combination with other DNA molecules. [36] This chapter focuses on the theory and introduces the background of molecular cloning of cytochrome 2U1.

Polymerase Chain Reaction (PCR)-The principle of PCR requires three-step cycling; denaturation of the double DNA strand, annealing of the primers, and the extension of the primers (Figure 7). The components that are essential in this process include: (i) the DNA template to be copied, (ii) the primers, (iii) DNA nucleotide bases needed for DNA synthesis, (iv) DNA polymerase enzyme, and (v) a buffer to provide a suitable environment for the reaction. Denaturation of the double-stranded DNA duplex that is held together by hydrogen

bonds is mainly achieved through heating at 95-100˚ C. The primers, which are single-stranded nucleotide sequences complementary to one of the original DNA strands, are then attached to the dissociated ssDNA strands in the annealing process. The primers are most often in excess for the annealing to take place more effectively than the reannealing of the dissociated strands. The last step involves the use of an enzyme DNA polymerase which adds nucleotides complementary to the unpaired DNA strand on the annealed primers.

A cycle takes 3-5 minutes repeated 20-40 times. This process requires a heat-liable polymerase enzyme to increase the yield, improve sensitivity and the length of the target DNA to be amplified. [36]

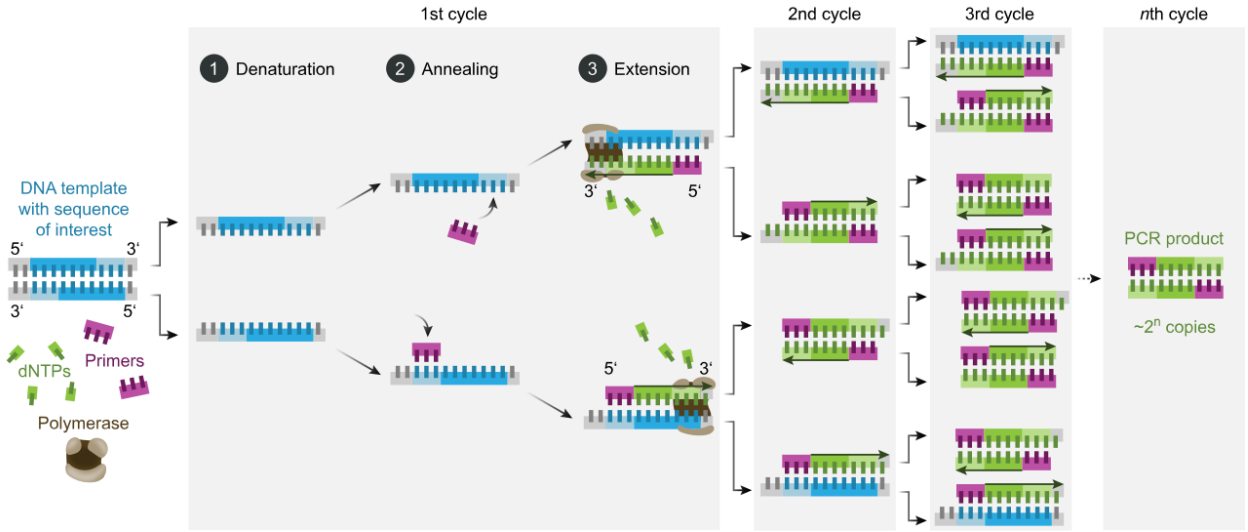

Figure 7. Theoretical amplification of PCR products. This image was made by Enzoklop, CC BY-SA 4.0 <https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons

Electrophoresis is a highly sensitive tool that is used to study, identify, and characterize proteins. [36] Biomolecules such as DNA, RNA, and proteins can be separated by the electrophoresis technique through migration on a matrix upon application of an electric current

(Figure 8).[36] Agarose, which is used to separate larger biomolecules such as nucleic acids and polyacrylamide gels for proteins, are the most used and available support matrices.[36] Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is one of the common forms of electrophoresis used to separate proteins based on size requires denaturing the activity of the protein, hence could not be used for further analysis that requires the retention of the biological activity of the protein of interest. [36] The effectiveness of the polyacrylamide gel is determined by the pore size, choice of the buffer to be used, pH, amongst other factors. [36]

The electrophoresis instrument is composed of the power supply source and a chamber for holding the running buffer and the samples. [36] A running buffer at a higher pH causes the biomolecules to be negatively charged, migrating from the cathode to the anode and vice versa at lower pH. [36] The use of dyes, fluorescent tags, or radioactive labels enables the DNA on the gel to be seen after they have separated and appears as bands on the gel. [36] A DNA marker with fragments of known lengths is run through the gel at the same time as the samples to determine the approximate number of base pairs in the sample. [36]

2.1 Methodology

Polymerase Chain Reaction. Two PCR tubes are prepared with the following contents: 35.5µL of autoclaved water, 1µL CYP 2U1, 1µL dNTPs (deoxynucleotide triphosphates), 1µL forward primer, 1µL reverse primer, 10µL 5x GC buffer, and 0.5µL Phusion polymerase. The PCR tubes are then placed on the PCR machine and run. Method of PCR2 setup is provided in Table 1. Restriction digestion was then conducted by the addition of 1µL Hind III, 1µL NdeI, and 5µL FD buffer to the PCR product.

The tubes are thereafter placed in the incubator for 1hour. DNA cleaning and concentration were done using Zymo's DNA Clean & Concentrator□-5 kit. First, the PCR products were placed in a 1.5 Eppendorf tube, and 500µL of the binding buffer was added. The contents are centrifuged at 10,000rpm for thirty seconds, and the eluent is poured into a beaker. 200µL of the wash buffer is then added and centrifuged at the same speed for thirty seconds and the eluate discarded. This procedure is done twice, and 10µL of the DNA elution buffer was added and incubated for one minute at room temperature. Centrifugation is done, and the eluate that is with the concentrated DNA is stored.

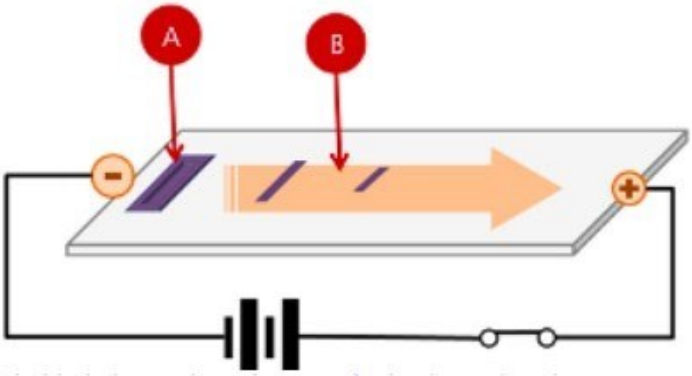

Figure 8. Electrophoresis principle. The negatively charged biomolecules move towards the cathode with applied electric current. Image by Jjw, CC BY-SA 3.0, via Wikimedia Commons, this image was not modified.

DNA Gel Electrophoresis was done to determine whether the PCR amplification procedure was successful or not. A gel is prepared by weighing out 1.5g of agarose that is put in the beaker and 100ml of 1x TAE buffer added. The contents are heated in the microwave for 30minutes carefully to dissolve the mixture. Once it was dissolved, the contents are poured on the gel runner, and the well comb was placed on one end and allowed to cool on the counter. The comb is taken out after gelling is complete, and the runner is placed on the electrophoresis

chamber. The 1X TAE buffer is added, and 10µL of the DNA ladder and 5µL of the PCR product with EZ vision one microliter of visualization dye carefully added onto the wells. The Bio-Rad Powerpack™ Basic is turned on and the voltage set at 180 V, and the gel was run.

Table 1. PCR2 method for Veriti 96 Well Thermo Cycler.

Stage	Step	Temperature° C	Time (s)
1	1	98	30
2	1	98	10
	2	53	30
	3	72	60
3	1	72	120
	2	4	Indefinitely

Cell transformation. 50 µL of JM 109 cells were put on ice for thirty minutes, added 2µL of pCW 2U1. The cells were heat-shocked in a hot water bath for thirty seconds and placed back on ice for two minutes. The mixture is pipetted onto the prepared LB-Amp plate, and glass beads are added and swirled around for a thin layer of cell formation. The plate is placed on the incubator at 37℃ overnight for colony production.

2.2 Results and Discussion

PCR was successful, as indicated by the PCR product (Figure 9). The first lane is the DNA ladder, and lanes 4-7 are the PCR products visualized with a fluorescent dye under a UV light source. The bands obtained from the PCR products were dissected, and the DNA extracted

from the gel using the Zymoclean Gel DNA Recovery Kit. The visualized PCR product and an empty plasmid backbone needed to be ligated together to make the pCW 2U1 vector with 2.5µL ligase in a 3:1 ratio. Transformation is then done after incubation and plated onto the lysogeny broth ampicillin (LB-amp) plate. The plate was incubated overnight and there were no colonies observed the next morning. Numerous attempts were made in the ligation and transformation steps with no success and a third party was contacted for cloning purposes. A full plasmid was obtained with the CYP2U1 gene, transformation done and several colonies obtained for expression and purification.

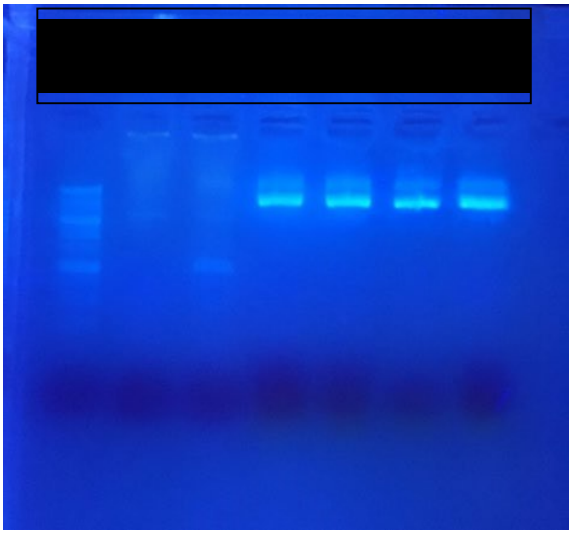

Figure 9. DNA agarose gel of CYP2U1 PCR sample. Lanes 4-7 contain PCR products. Lane 1 contains a DNA ladder.

2.3 Conclusion

Cloning was not successful in the CYP 2U1 project, and a company was contacted to produce a full vector for both the CYP 2U1 and CYP 1A2 projects. The pCW vector was suspected to be the cause of the problem as there were similar issues with one or two projects

involving the subcloning step with CYP project. The next step was the expression and purification after successful sub-cloning.

CHAPTER 3: EXPRESSION AND PURIFICATION

Protein insolubility, conformation, stability, structural stability, host cell toxicity as well as low purification yields are some of the factors that must be addressed for efficient expression and purification processes. All the cytochrome P450s investigated in this thesis utilized an *E. coli* expression system to grow large amounts of protein. After expression *in E. coli*, cells are lysed to isolate the desired protein product. The use of affinity tags has been used to address the challenges of isolating the desired protein and for the modification of protein of interest for efficient protein identification, production, and isolation from the host cells. This technique utilizes a short sequence of amino acids that are added to the proteins of interest on either the N-terminus or the C-terminus.

Polyhistidine tags are one of the commercially available affinity tags that are used mainly for the purification of poly His-tagged proteins using the immobilized metal affinity chromatography based on the interaction between the negatively charged Histidine residues and transition metals that are immobilized on the matrix, such as Nickel or Cobalt, with the unbound proteins removed during the washing step followed by the elution of the pure protein.[34] Ni (II)-nitrilotriacetic acid (Ni-NTA) has many advantages such as high affinity for histidine residues and capabilities to withstand multiple regeneration cycles and allows dissociation of the bound proteins with the use of low concentrated imidazole or changes in pH.[37] The use of imidazole, however, may result in protein aggregation or inactivity after purification, and it is highly recommended to perform dialysis for further assays, especially those involving crystallography and NMR experiments. [38-40]

3.1 Protein Expression

A resistance marker is added to the plasmid backbone to prevent the growth of plasmid free cells in the culture using an antibiotic resistance gene that is present in the bacterial host system. *E.coli* is the most widely used host system for the expression of proteins because of (i) fast growth rate, (ii) cells can be grown to high density for protein production, iii) low costs, and simple culturing conditions. [41]

3.1.1 IPTG Induction. IPTG is used to turn on the production of the desired protein since its concentration does not reduce rapidly as compared to lactose. It is similarly imported into the cell as a lactose metabolite stimulating the expression of proteins in the lac operon with a constant rate of protein expression. Presence of IPTG in the medium, inhibits Lac I from binding to the Lac O and as a result gene transcription is initiated leading to the growth of the protein of interest. [42]

3.1.2 Detergent Use for Membrane Protein Isolation. Detergents are used for the extraction of membrane-bound proteins by mimicking the physical and chemical properties of the lipid membrane and increase their solubility, which is an important factor in protein purification [39] This has impacts on the retention of the protein activity, and a special buffering system has to be improvised. Purification of these proteins uses the same chromatographic techniques as the other soluble proteins, but the main difference is always the use of the detergents in the buffers. The use of detergents, however, can affect the stability of the protein, which could interfere with binding assays and biochemical processes. [43]

Detergents are amphipathic with polar or charged head groups and an extended hydrophobic hydrocarbon chain and tend to aggregate into micelles at high concentrations interacting with both the solvent and the nonpolar surfaces of the protein. [44]

3.2 Affinity Chromatography

This is a type of liquid chromatographic technique with high-specificity interactions for separation and analysis purposes. The support matrix is usually composed of porous materials such as agarose, polyacrylamide, cellulose, and silica. A suitable ligand must be identified and placed onto the solid support of the column to interact with the biomolecule of interest selectively. The sample of interest is first injected into the column under conditions that the analyte will strongly bind to the immobilized ligand. Most of the other solutes in the sample are quickly washed from the column because they are incapable of binding to the ligand (Figure 10). An elution buffer is thereafter injected to dissociate the retained solutes in several ways, such as changing the pH or buffer composition of the mobile phase to either decrease the strength of the ligand-analyte interaction or by introducing a competing agent to displace the analyte from the ligand. The analyte is detected as it elutes and collected for later use as the column is regenerated for later use. This separation technique is highly selective and easy to perform for solute purification and quantification. [45, 46]

3.3 Ion-exchange Chromatography

Biomolecules such as proteins have exposed charged moieties at the surface that could interact with ion exchangers depending on the pI of the protein and the pH of the environment. The principle is based on the interaction between oppositely charged molecules. The ion exchanger is composed of a matrix immobilized with positively or negatively charged ligands. There are two types of ion-exchangers: cationic exchangers with carboxymethyl functional groups that are negatively charged or anionic exchangers with diethyl aminoethyl functional groups that are positively charged. A protein is negatively charged when its charge is above its pI

and could easily bind to an anionic exchanger and vice versa. Desorption of the analyte molecules is easily done by increasing the salt concentration or changing the pH of the buffer. [36] However, increased salt concentration tends to increase the surface tension of the water causing competition between the protein and the salt ions for hydration. This may in turn denature the protein by stripping off the essential layers of the water molecules from the protein surface.[37]

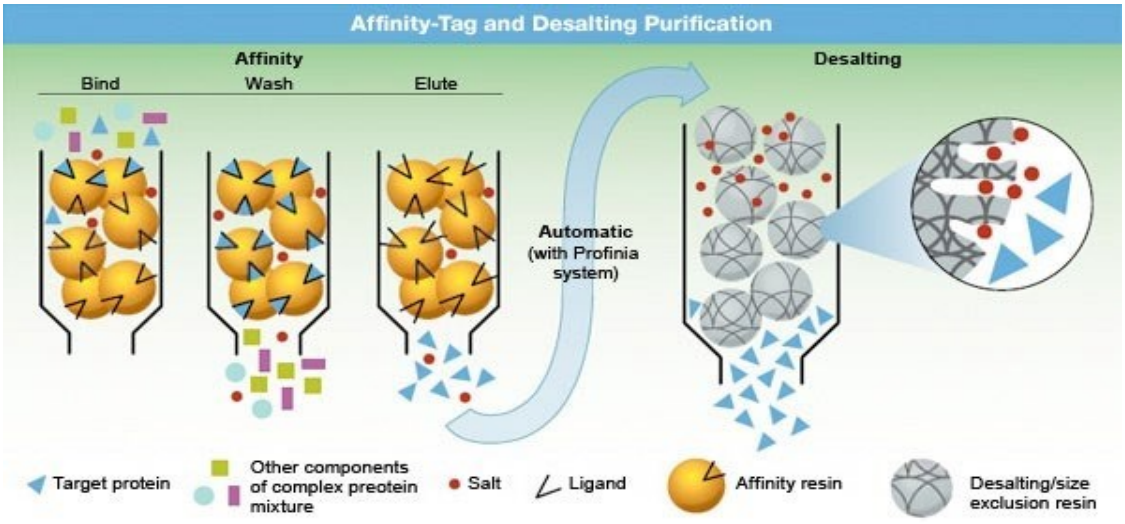

Figure 10. Principle of affinity chromatography. Image was adapted from https://www.biorad.com/webroot/web/images/lsr/solutions/technologies/lab_scale_chromatography/chromatogra phy/technology_detail/chromt11

3.4 SDS Page

A gel matrix is used in electrophoresis for the separation of proteins based on size, charge, and structure. SDS-page separates proteins based on their sizes, and this is because of the incorporated SDS detergent that denatures and binds to protein causing them to be negatively charged. These SDS-bound proteins migrate towards a positively charged electrode when current

is applied through the gel matrix. Smaller proteins migrate faster due to less resistance on the gel matrix as compared to larger ones. Once separated by electrophoresis, they can be detected using stains and their sizes determined by the protein ladder available commercially.

3.5 Methodology

Media preparation. LB media preparation: 2 grams of Tryptone, 1 gram of yeast extract, and 2 grams of Sodium Chloride were added in a 500ml Erlenmeyer flask, and deionized water added to 200 ml volume. The top was sealed with aluminum foil and autoclave tape and ran on the autoclave on a liquid cycle. The contents were allowed to cool once the cycle was completed and stored. TB media preparation: Three flasks was prepared for the expression of CYP 1A2 and six flasks for CYP 2U1. In each 2 L flask, 50.4 grams of prepared Terrific Broth powder was weighed, 4ml of 100% glycerol added, and the volume brought to 1000ml with deionized water. The top is sealed with aluminum foil and autoclave tape. The solution is then placed on the autoclave and run on a liquid cycle. The solution is then stored under cool temperatures after cooling for later use.

3.5.1 Buffer Preparation. All the purification buffers were prepared from 1M potassium phosphate stock at pH 7.4 prepared by measuring 800mL of deionized water into a beaker. 163g of K_2HPO_4 and 8.88g of KH_2PO_4 were added to the beaker. The pH is adjusted to 7.4 using a pH meter with sodium hydroxide and 1M hydrochloric acid solutions and the volume adjusted to 1L with deionized water. The ratios of different reagents used to prepare buffers are shown in Table 2.

3.5.2 Cell Transformation. 1 µL of the pCW 1A2 or 2U1 containing plasmid was mixed with 50µL of JM109 competent cells and placed on ice for thirty minutes.

Table 2. Reagents for purification buffers

Buffer name	1M potassium phosphate buffer(mM)	Glycerol (%v/v)	NaCl (mM)	Imidazole (mM)
Resuspension buffer	500	10	300	0
Ni-NTA wash buffer	100	10	300	10
Ni-NTA elution buffer	100	10	0	200
Ion-exchange wash buffer	50	10	0	0
Ion-exchange elution buffer	100	10	500	0

The cells were heat shocked in a 37˚ C hot water bath for thirty seconds after the incubation period and placed back on the ice for two minutes. The mixture is pipetted onto the prepared LB-Amp plate, glass beads added and swirled around for a thin layer of cell formation. The plate is placed on the incubator at 37℃ overnight for colony production

3.5.3 Expression of CYP 1A2 and CYP 2U1. A single colony of transformed cells was picked and added to the 200 ml LB media, and ampicillin was added. This starter culture is grown overnight at 37℃ on the incubator at 180 rpm. The contents are then distributed into the

three flasks of TB media the following morning, and ampicillin solution added. The cells were grown shaking for three hours, and the OD_{600} was measured. B-D-1-thiogalactopyranoside (IPTG) and aminolaevulinic acid (ALA) were added to each flask, and the temperature was reduced to 32℃, and the cells were allowed to grow for three days.

The cells are then harvested by centrifugation. The cells are equally distributed on four 400ml centrifuge tubes and centrifuged at 5000rpm for 20 minutes, and the supernatant discarded. The procedure is repeated until all the growth media has been centrifuged and the pellet resuspended with a resuspension buffer, and either stored or protein extraction done.

3.5.4 Protein Extraction. The pellets that were resuspended with the buffer are placed on a beaker and placed on ice. The tip of the sonicator probe is put near the bottom of the beaker for thirty seconds followed by a rest for another thirty seconds, alternatingly for at least three minutes. The probe is removed and a stirring rod placed into the beaker. The beaker is then placed into a dish with ice on a magnetic stirrer. While stirring, 3.2 mM Cymal-5 detergent added during the extraction step in addition to 1%v/v CHAPS with CYP1A2, but not included in the buffers which only contained 0.5%v/v. Same concentration of CHAPs detergent is used with CYP 2U1 with the exemption of Cymal- 5 detergent. The mixture is stirred for one hour on ice and then put into the centrifuge tubes. Centrifugation is done at 20000 rpm for 20 minutes, and the supernatant is stored for purification at -80 °C.

3.5.5 Protein Purification. A column with NiNTA beads is connected to the Bio-Rad Biologic LP Chromatography instrument and flushed with resuspension buffer. The lines A, C, D, and E are used and connected to the lysate, resuspension buffer, wash buffer, and elution buffer, respectively. Once everything is well set, purification is started, and the fractions are inspected and collected with the protein of interest.

The fraction with the protein of interest is placed on line C on the ion-exchange chromatography for further purification, while the ion exchange wash buffer and elution buffer are placed on lines D and E, respectively. The fractions with the purified protein are collected afterwards.

3.5.6 Absolute and Carbon Monoxide Spectra. 750µL of elution buffer with detergent is placed on the cuvette and used to auto-zero and baseline the UV-VIS. Another cuvette is used once this is done, and 750µL of the fraction is collected and scanned at a wavelength range of 750-250nm. The scan is saved, and a few crystals of sodium dithionite are added into the cuvette with the purified sample and the UV-VIS instrument auto-zeroed and baselined once more. The cuvette is bubbled with carbon monoxide and scanned at a range of 500-400nm to obtain a CO difference spectrum.

3.5.7 SDS-PAGE Analysis. Different fractions are collected including the flow-through with the lysate, the fraction with the wash buffer, as well as the elution buffer.10µL of the fractions are pipetted into an Eppendorf tube and 10µLof the loading dye with β-mercaptoethanol added to each solution. One Eppendorf with the protein ladder and the loading dye is also created with the same volume measurements. The comb and the tape on the gel are removed gently and the gel placed in the gel electrophoresis apparatus.

The SDS PAGE 10X electrophoresis buffer is prepared by dissolving 30.0g of Tris base, 144.0g of glycine, and 10.0g of SDS in 1000ml of deionized water at a pH of 8.3 and stored at room temperature. The running buffer is prepared from the 10X electrophoresis buffer by adding 50ml of the electrophoresis buffer into 450ml deionized water.

The running buffer is added to the gel runner and the samples loaded into the wells. The voltage is set to 210V, and the samples run carefully, observing the production of bubbles from the bottom of the gel. Once complete, the gel is carefully placed in water for staining. Coomassie

stain is added to the gel and placed in the microwave for 20 seconds. Water is added to the gel and microwaved again for two minutes. This process is done alternatively until the bands are easily seen.

3.6 Results and Discussion

Figure 11, briefly describes the processes conducted from the growth of the protein with the starter culture at 32°C for 24 hours which is then scaled up with the Terrific Broth media and allowed to grow further for 3 days for production of large amounts of the protein with addition of IPTG and ALA, harvested, extracted by use of sound waves and detergents and the lysate obtained after centrifugation at 5000rpm for 20minutes. The lysate is then purified by affinity chromatography with the resuspension, wash and elution buffers. Purification of CYP 1A2 and CYP 2U1 was repeated several times and optimized for better yields, such as the use of different detergents like CHAPS, Cymal-5, and Anapoe-35. The protocol with better yields was adopted for the purification of these proteins. Different conditions had to be optimized to improve the overall yields for CYP 2U1.

Cymal-5 detergent was used initially for the extraction of the CYP 2U1 with little success in binding to the cobalt column. The pH of the buffers used were not adjusted and was suspected to be the cause but there were no much improvements afterwards after fixing this issue. The concentration of IPTG and ALA were slightly increased during expression, switched the detergent to Anapoe-35, with little or no success since the overall yield was lower than 0.15µM as described in Table 1. Improved yields and binding were observed with the use of Nickel column for affinity and DEAE beads for ion-exchange chromatography with CHAPs detergent.

Nickel column was used for purification, as shown in Figure 12 followed by ion-exchange chromatography.

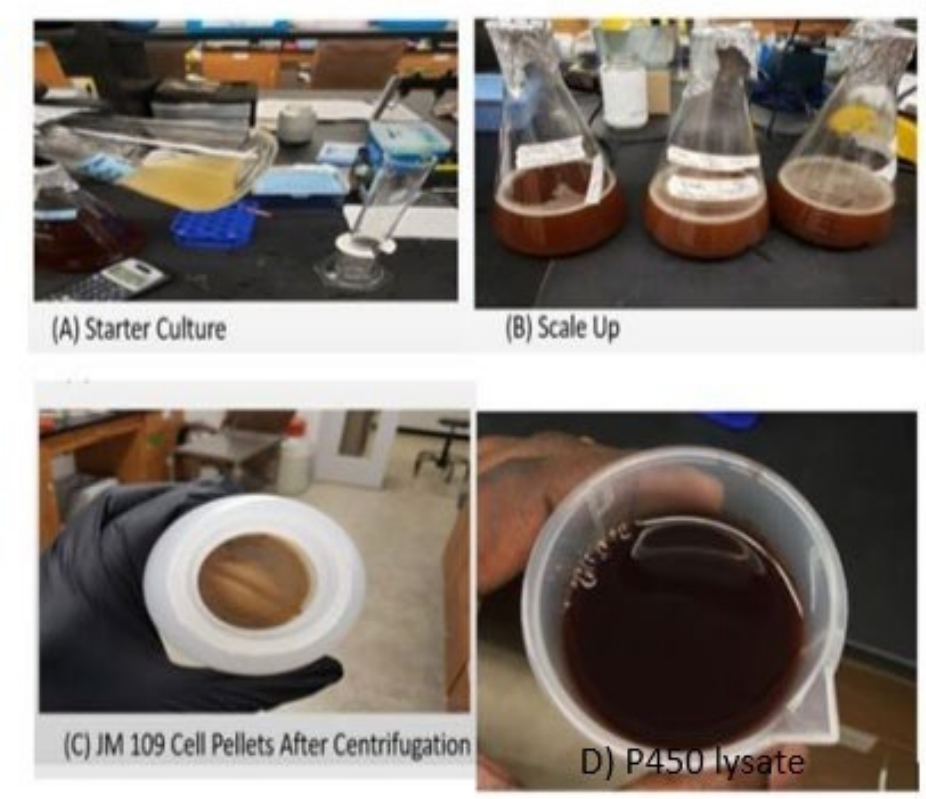

Figure 11. (A) The LB media used as the starter culture with inoculation of a single colony, B) TB media used for scale up for three days (C) Harvested protein by centrifugation (D) P450 protein to be purified after extraction.

3.6.1 Affinity Chromatography. The fractions collected with the elution buffer were selected and pooled for further analysis. The detailed assignment of this protein was made based on UV-VIS analysis. The ratio of heme to protein content, as monitored by the absorbance ratio A 420/A280, indicated that the protein of interest was present but with low yields.

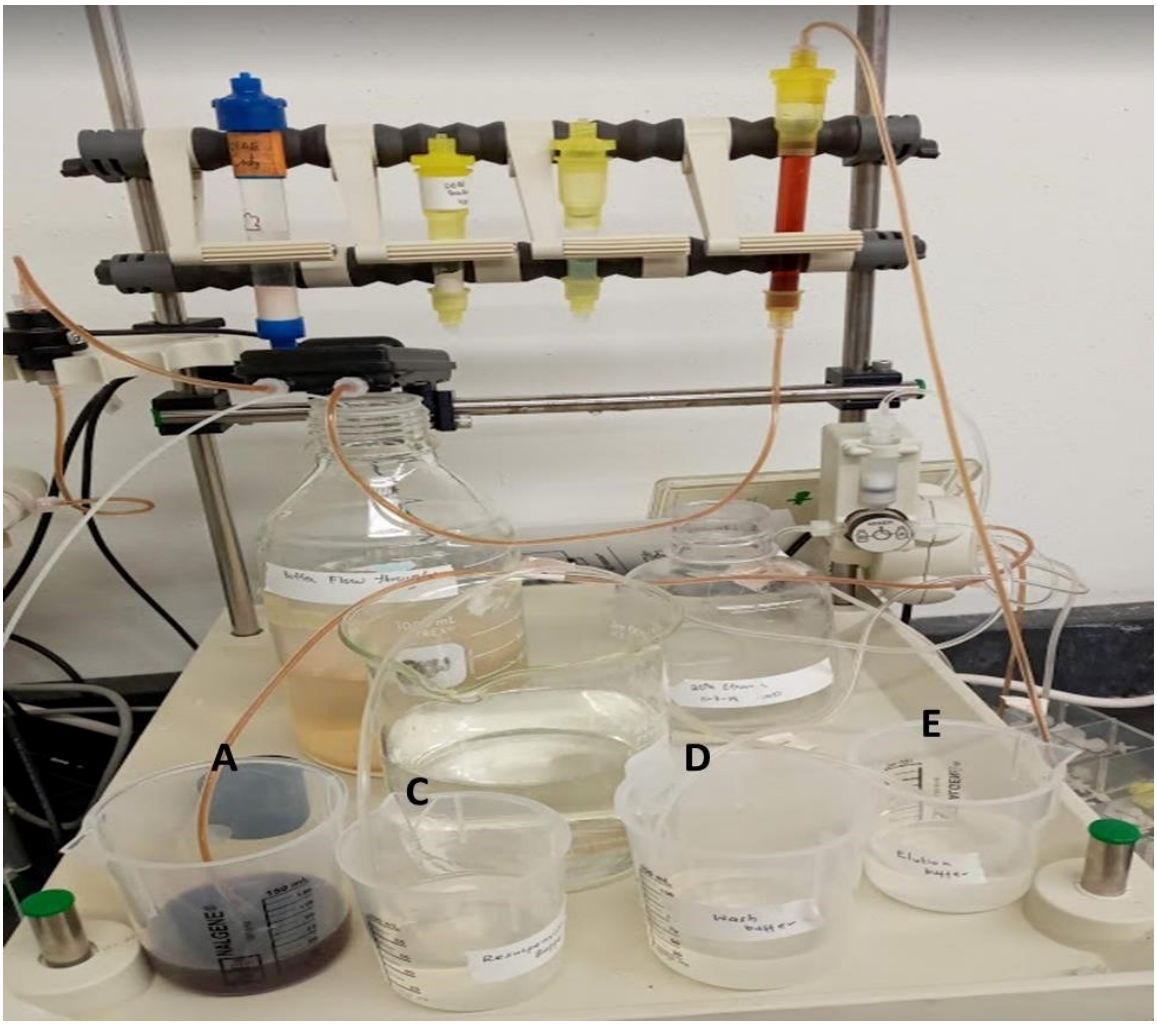

Figure 12. Nickel affinity chromatography purification technique with lines A, C, D and E representing the columns with the lysate, resuspension, wash and elution buffers respectively with the Bio-Rad Biologic LP Chromatography instrument.

Figures 13, and 14 show the absolute spectra of CYP 1A2 and CYP 2U1 after Nickel affinity purification with buffers at pH 7.4.

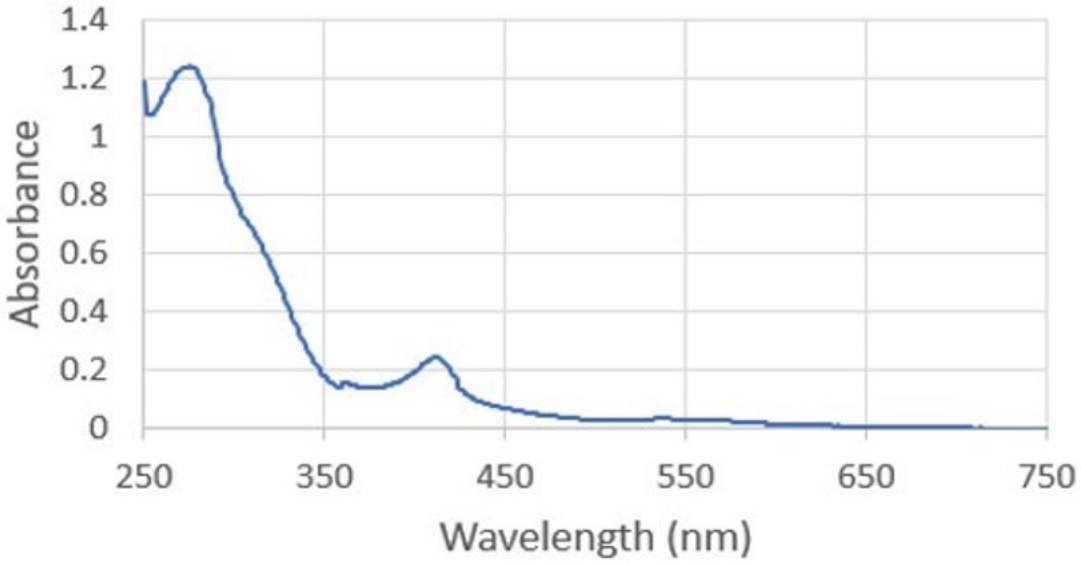

Figure 13. CYP 1A2 affinity absolute spectrum. The absorbance ratio at 420nm and 280nm wavelength used to determine the purity of the protein after affinity purification.

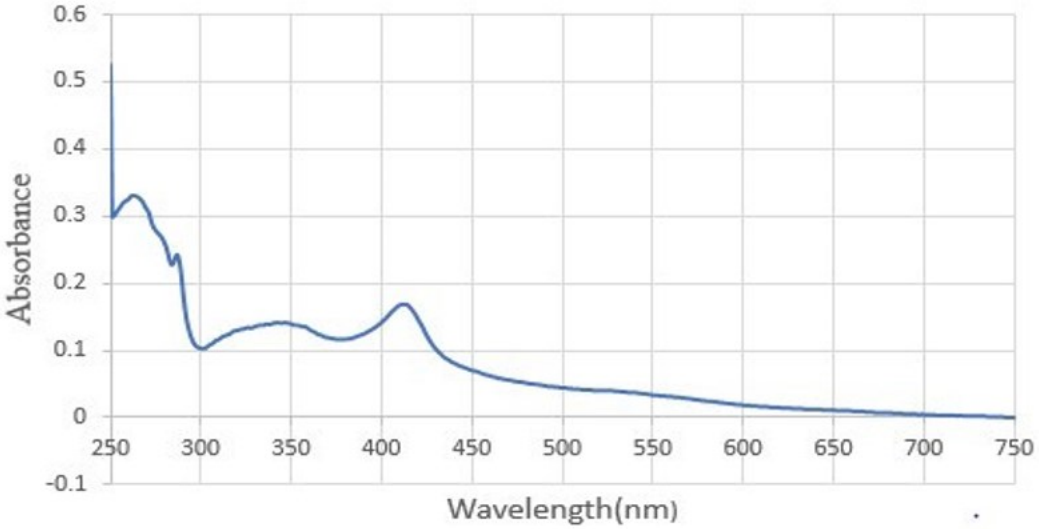

Figure 14. CYP 2U1 affinity absolute spectrum obtained to deduce the protein and heme content before ion-exchange purification.

The purified protein was confirmed by the CO difference that indicated the presence of the P450 heme with a concentration of 0.30µM for CYP 2U1 and 0.84µM for CYP1A2. The remaining fraction was saved for ion-exchange chromatography in Figures 15 and 16.

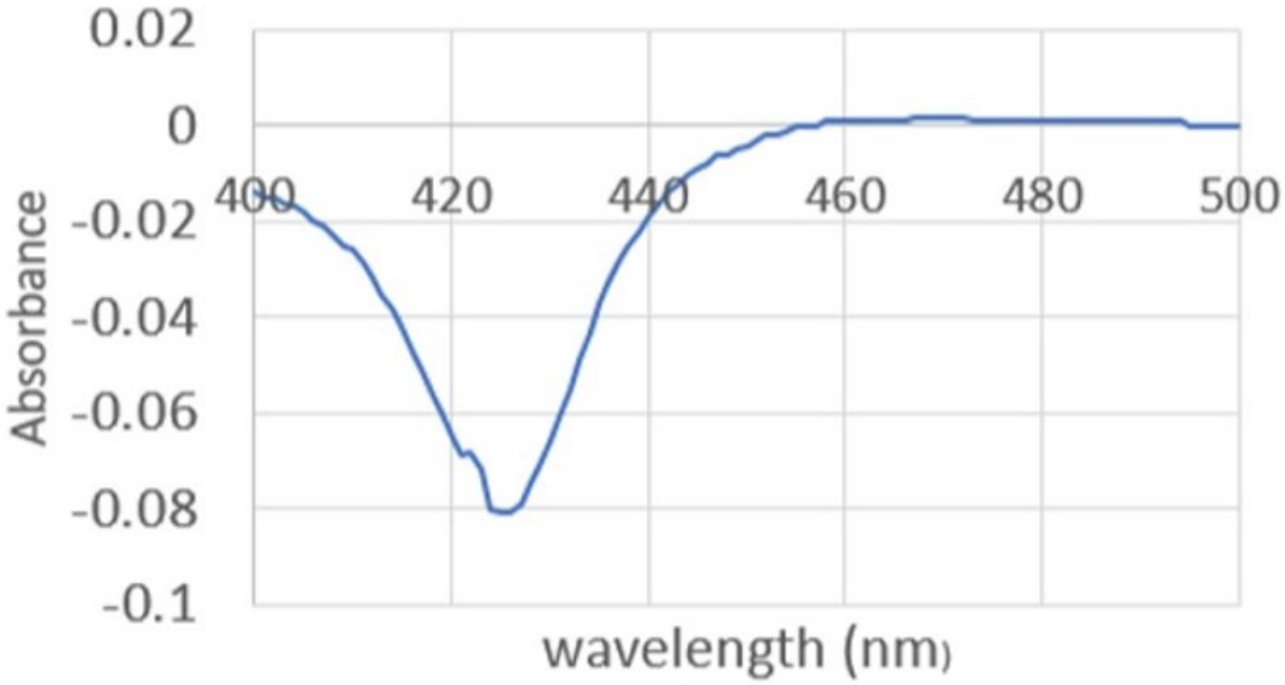

Figure 15. A Carbon monoxide spectrum confirming the presence of CYP1A2. A base line done after the addition of an excess sodium dithionite to the cuvette and the difference spectrum obtained after bubbling carbon monoxide for 45s.

3.6.2 Ion –exchange chromatography. The ratio of heme to protein improved drastically close to 1:1 after the second purification was conducted for both proteins as expected measured by absorbance A280/ A420nm.

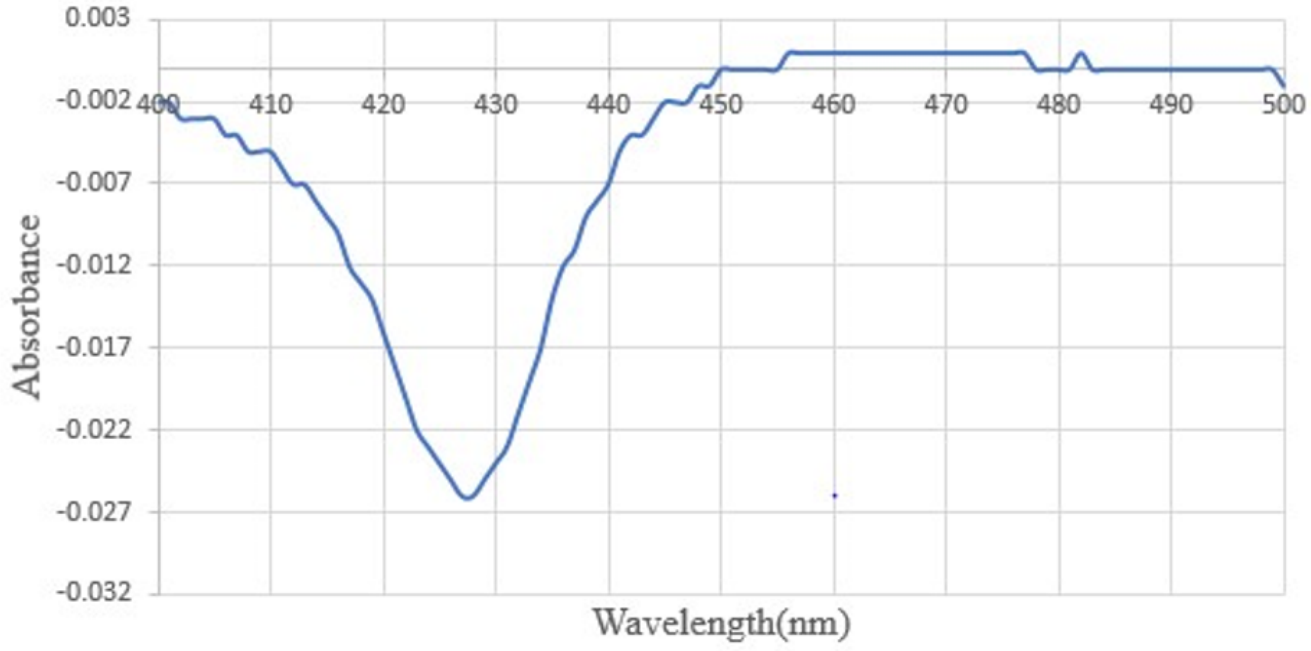

Figure 16. CYP 2U1 CO difference spectrum indicating the presence of the CYP 2U1

Figures 17 and 18 are the absolute spectra obtained after purification with ion- exchange using DEAE matrix.

3.6.3 Purification of Cytochrome b5. Cytochrome b5 was thawed and sonicated five times. It was then centrifuged for 25minutes at 19000rpm. 20ml of the resuspension buffer is then added and ground into solution. 1% of CHAPS detergent is added and stirred for one hour. It is then loaded into the Nickel column after centrifugation at 19000rpm for 30minutes. The yield produced was low, with a concentration of 2.06µM. This protein was saved at -80°C to be used for caffeine metabolism assay.

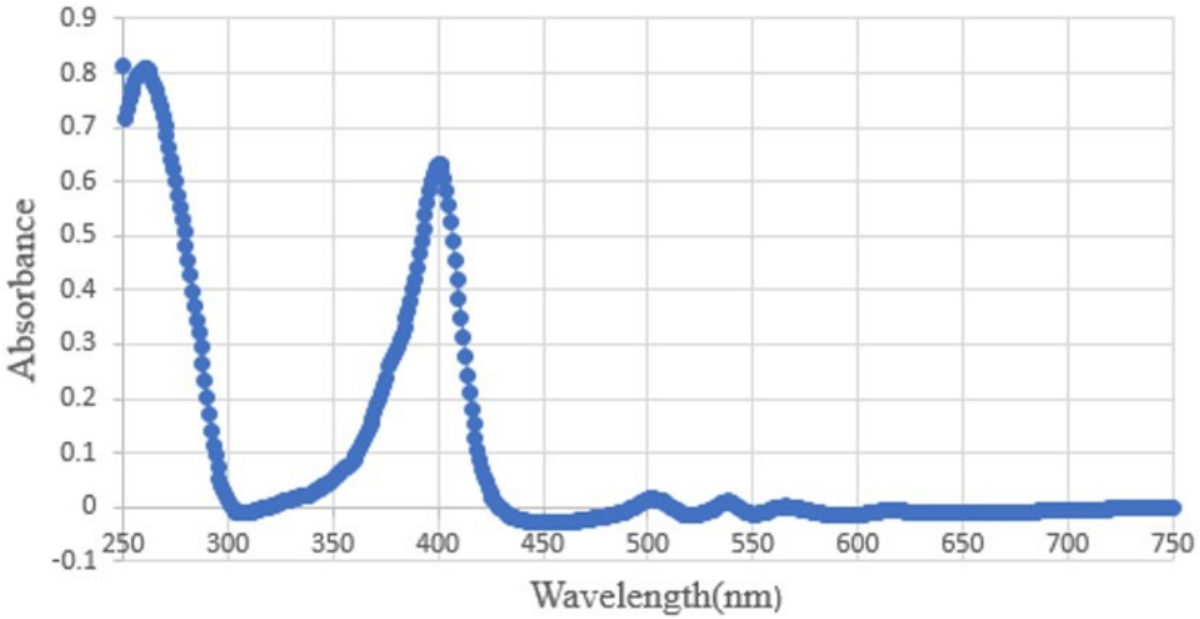

Figure 17. CYP 2U1 absolute Ion- exchange spectrum, improved

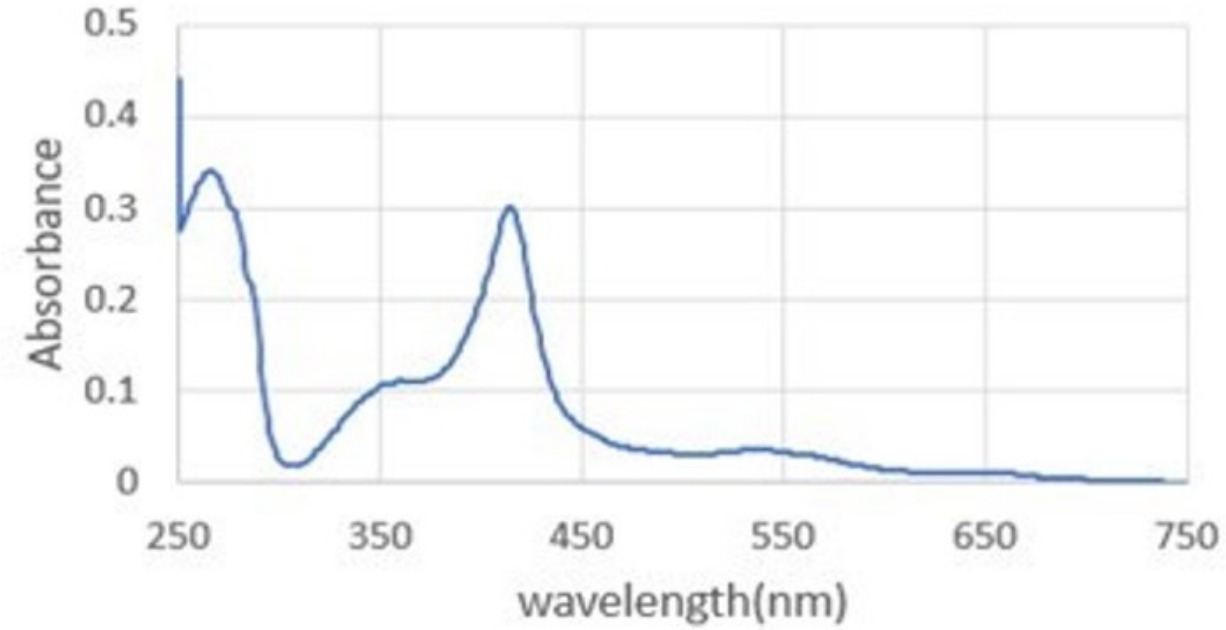

Figure 18. CYP 1A2 Absolute Ion-exchange spectrum with improved heme to protein ratio at 420nm and 280nm wavelength.

The CO difference obtained afterwards are as shown in Figures 19 and 20.

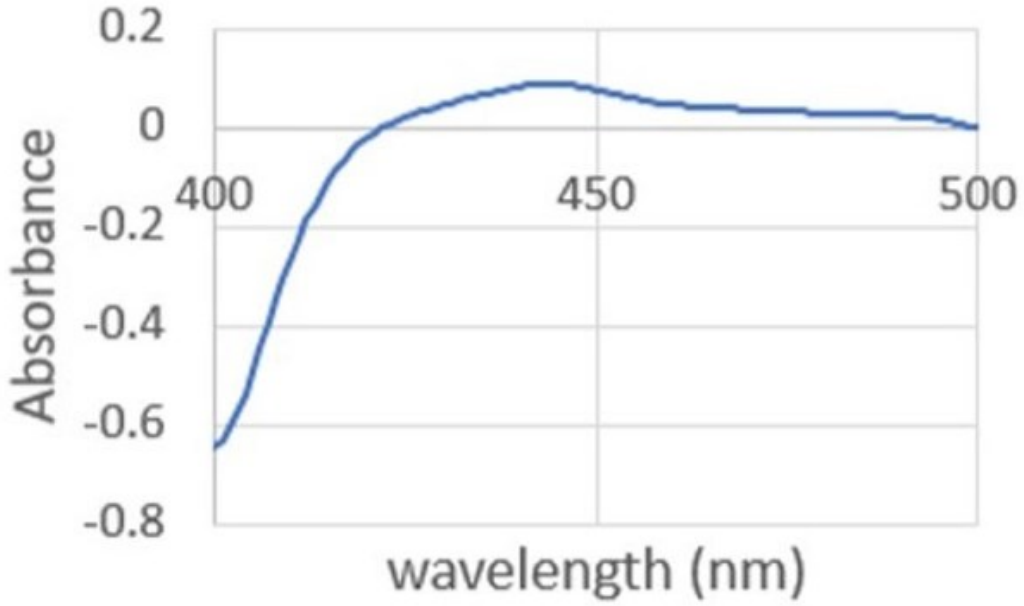

Figure 19. CYP 1A2 DEAE CO difference spectrum

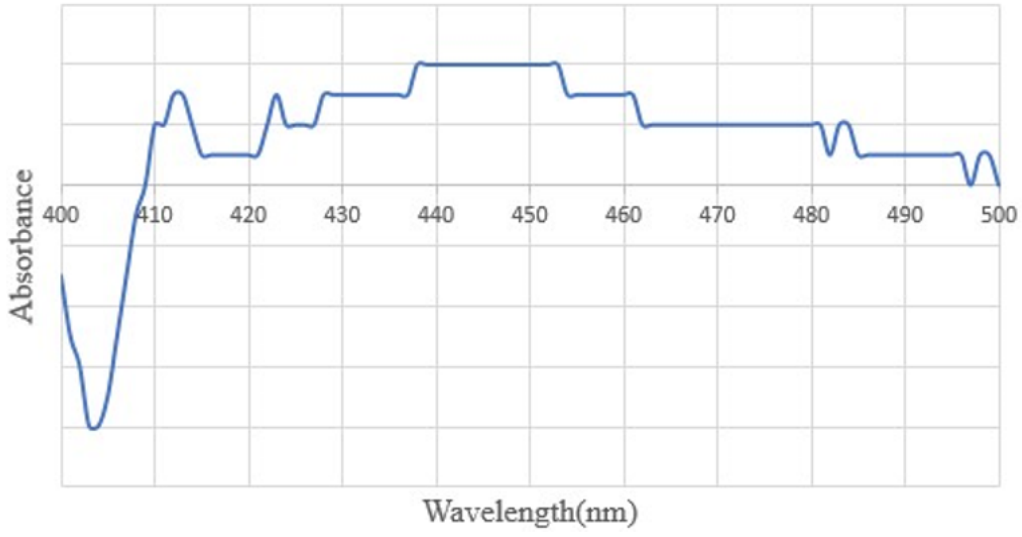

Figure 20. CYP 2U1 DEAE CO difference spectrum

3.7 Conclusion

Purification of CYP 2U1 was not an easy task as several factors had to be varied on the protocol, such as optimization of pH, detergent to be used, as well as the column to be selected for affinity chromatography. The choice of the buffer and pH to be used plays a role in purification process depending on the solubility of the protein and the potential effects on the stability of the solution which may result in denaturation or precipitation thereby affecting its purity. CHAPs detergent tremendously improved the extraction and subsequent binding of the protein to the matrix of nickel column. This was observed with an increase in intensity of the brown color of the lysate after centrifugation due to the presence of heme in P450s. Substantial amounts of expression media were used to increase the yields as listed in the Appendix. Particularly for CYP2U1, as it was discovered that a growth of 6 Liters of media was required for sufficient yields of the protein. Purification was successful, and the proteins were stored for further assays.

CHAPTER 4: X-RAY CRYSTALLOGRAPHY

Scientists can determine the structure of proteins using x-ray crystallography, a technique that provides a three-dimensional structure from a crystal. A purified and concentrated protein is required for the crystals to grow under the right conditions, and for this to occur, many factors must be put into consideration on a trial-and-error basis. The choice of precipitant, its concentration, the buffer to be used, pH, the concentration of the protein, and the temperature are among the various variables that must be put into consideration for successful crystal growth. [47, 48] Different crystal screens are available for the crystal setups with the precipitant, buffer, salt, and pH varied that can be tried with the purified protein; however, none of these conditions are guaranteed to grow crystals.[49] These can be used with different techniques (Figure 21), such as the hanging drop vapor diffusion where the drop is with an equal volume of the concentrated protein and the reservoir solution suspended over the reservoir from the glass coverslip, or the sitting drop diffusion technique with the drop placed on a special diffusion chamber, or the dialysis technique.[50, 51] The hanging drop diffusion occurs when the protein crystallizes as the vapor pressure of the solution rises.

4.1 Methodology

The purified CYP 2U1 and CYP 1A2 that were collected from ion- exchange with the elution buffer was concentrated for crystallography assay. The fractions were put into the Amicon Ultra filter tube with 25k Da cut off and centrifuged. UV-VIS was then used to determine the concentration of the protein by applying Beers Law using the coefficient factor,

absorbance at 410 nm, and the path length of the cuvette. Centrifugation of the fractions was done to achieve at least 200 µM concentration, and the sample was stored for crystallography.

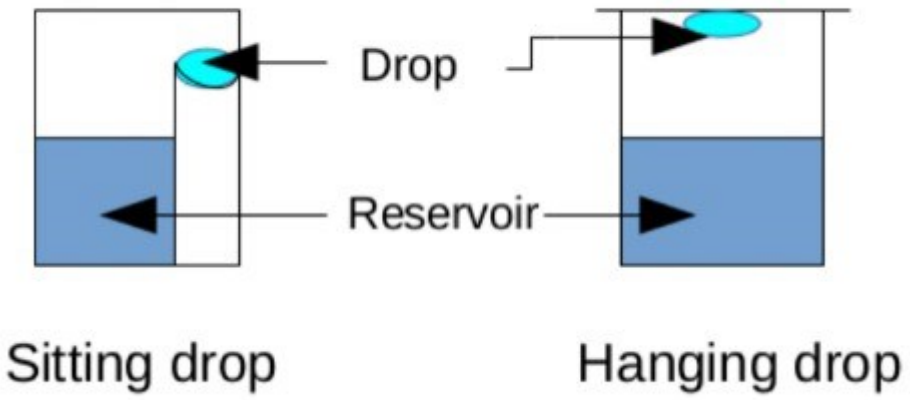

Figure 21. Crystallization techniques. https://www.pathsense.eu/wp content/uploads/2018/01/drops.png

Hampton Research crystal 1 and 2 were used for the 96-reagent setup of CYP 1A2, while Memphac was used for CYP 2U1 protein by pipetting 75µL of each reagent into the crystal screen tray. 0.5µl of the sample and the reagent were then pipetted into the sample well, and the sample tray was covered and incubated at 19°C and stored to be checked after few days. A crystal hit is optimized in a 24 well plate using the volume and concentrations that were varied based on the initial hit conditions using the crystal plate builder on Hampton Research's website into the crystal tray while 0.5 µL of the sample and reagent are carefully pipetted into the coverslip. The coverslips are then sealed with grease or immersion oil and incubated at 19°C and checked after few days as well.

4.2 Results and Discussion

The crystals that were initially observed with CYP 1A2 (Figure 22) with the crystal plate that was incubated at 19°C with the well G3 (0.01 M Zinc Sulfate heptahydrate, 0.1M MES monohydrate pH 6.5, 25%v/v Polyethene glycol monomethyl ether 5,000 and A1 (0.02 M Calcium Chloride dihydrate, 0.1M Sodium acetate trihydrate pH 4.6, 30% v/v (+-) 2- Methyl -2, 4-pentanediol). These crystals did not grow further, and the remaining purified CYP 1A2 was used for metabolism assays instead of further crystal screening.

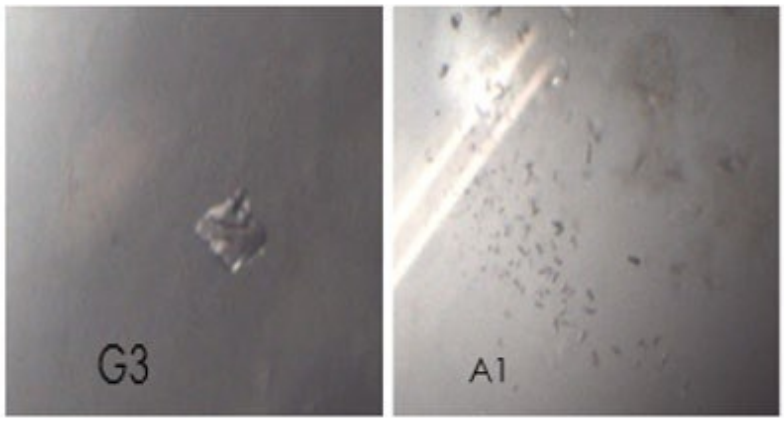

Figure 22. CYP 1A2 crystals with G3 (0.01 M Zinc Sulfate heptahydrate, 0.1M MES monohydrate pH 6.5, 25%v/v Polyethene glycol monomethyl ether 5,000 and A1 (0.02 M Calcium Chloride dihydrate, 0.1M Sodium acetate trihydrate pH 4.6, 30% v/v (+-) 2- Methyl -2, 4-pentanediol).

The CYP 2U1 crystal hit (Figure 23) was in well H9 of the well plate out of 96 wells set with conditions, (0.2M Zinc acetate dihydrate,0.1M Sodium cacodylate trihydrate pH 6.5, 18% w/v Polyethene glycol 8,000) This was further optimized, and Figure 24 shows the crystals that were observed afterward.

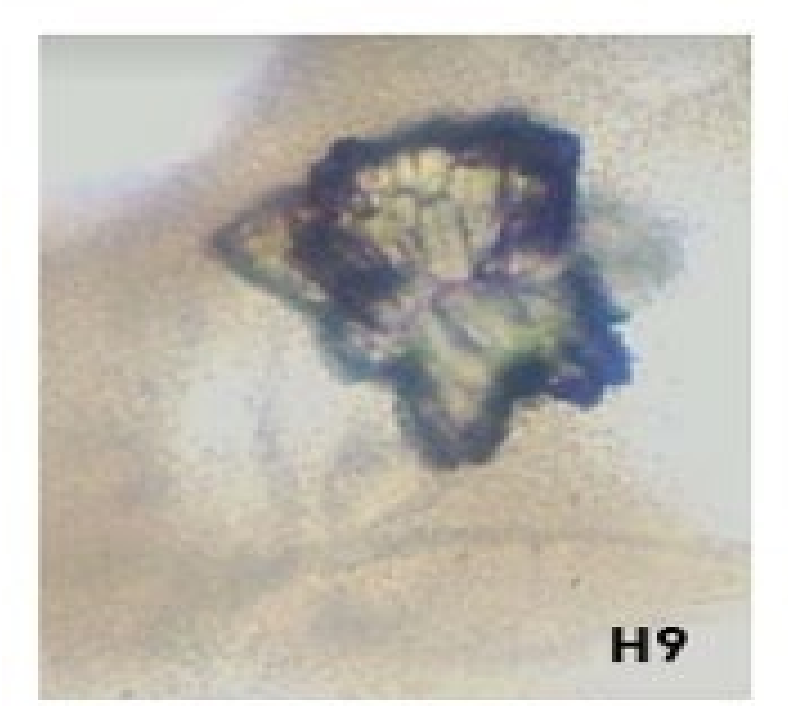

Figure 23. CYP 2U1 crystal hit with condition H9 (0.2M Zinc acetate dihydrate,0.1M Sodium cacodylate trihydrate pH 6.5, 18% w/v Polyethene glycol 8,000).

Optimization was done and incubated at 19°C with well plate A1 (0.1M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 1% Polyethylene glycol 8,000, B3 (0.15M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 13% Polyethene glycol 8,000) and B2 (0.15M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 9% Polyethylene glycol 8,000 as in Figure 24.

4.3 Conclusion

Initial crystal screen setups are based on a trial-and-error procedure with an aim of covering a broader range of variables as possible using commercially available crystal screen

packages. The purified protein has to be concentrated in order to increase the chances of a crystal growth. At this stage, it is common to see various combinations ranging from nothing, precipitation, or showers of microcrystals. Optimization has to be done with the crystal observed after screening with varying conditions for crystal growth. The crystal hits that were obtained disappeared after a few weeks; better crystals might have been observed with increased concentration of the purified protein.

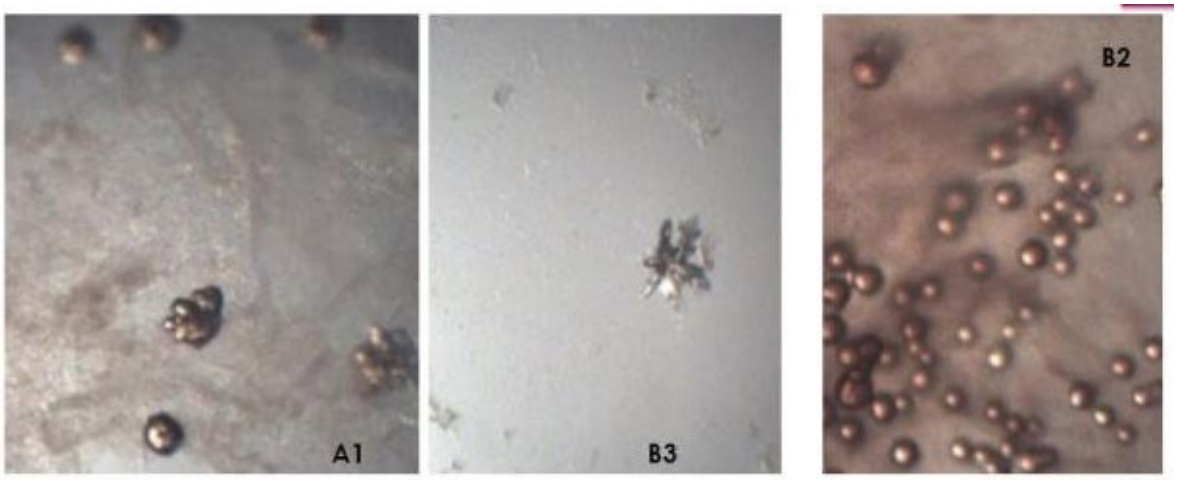

Figure 24. CYP 2U1 crystals after optimization with conditions A1(0.1M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 1% Polyethylene glycol 8,000), B3 (0.15M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 13% Polyethene glycol 8,000) and B2 (0.15M Zinc acetate dihydrate 0.1M Sodium cacodylate trihydrate pH 6.5 9% Polyethylene glycol 8,000)

CHAPTER 5: CAFFEINE METABOLISM

In humans, the metabolites of caffeine are paraxanthine (84%), theobromine (12%), theophylline (4%), and 1, 3, 7-trimethyluretic acid (1%), which are further broken down and excreted in the urine (Figure 25). Caffeine's mode of action is through antagonistic effects to adenosine and its receptors that cause drowsiness and fatigue. [52, 53] The dog enzyme is of particular interest because although the enzyme is 80% identical to the human enzyme, dogs metabolize caffeine and theobromine, known substrates of human CYP1A2, at much lower rates. Chocolate and caffeine are some of the reported toxic foods in canines that are rapidly absorbed from the gastrointestinal tract into the bloodstream after ingestion and metabolized in the liver. [19]

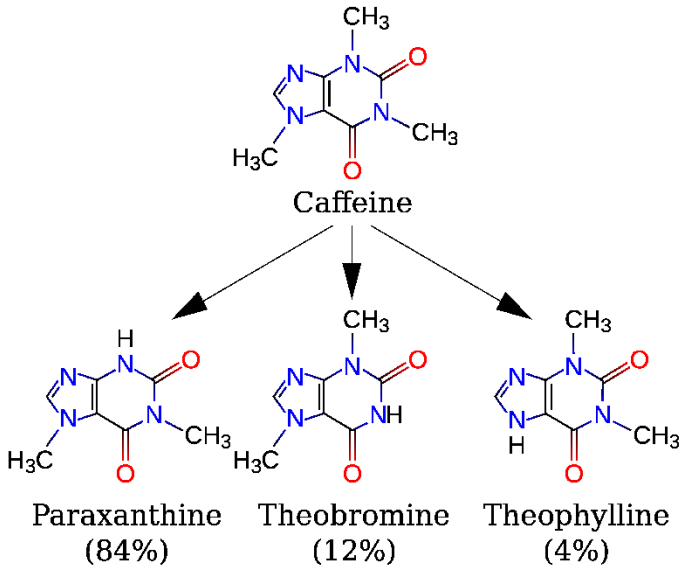

Figure 25. Caffeine metabolism.https://commons.wikimedia.org/wiki/File: Caffeine_metabolites.svg">Icey, Public domain, via Wikimedia Commons

5.1 Methodology

CYP 1A2 (30 pmol), cytochrome b5 (60 pmol), and human reductase (180 pmol) were pipetted and added into a 1.5 ml Eppendorf tube. The mixture was vortexed for 5 seconds and placed in a hot water bath for 30 minutes at 37°C. Then, 6 µL of 10 mM Caffeine, 15 µL of NADPH, and 154 µL of 1M potassium phosphate buffer were then added and incubated for 20 minutes. The reaction is further stopped by the addition of 15 µL of 6 M HCl acid. Samples were centrifuged at 12,500 rpm to remove any precipitated protein and placed in an HPLC vial for analysis.

Chromatographic conditions- HPLC analysis was performed by isocratic elution at a flow rate of 0.8 ml/min. The mobile phase composition was 15% acetonitrile, 1% acetic acid and 84% water run at a flow rate of 0.8ml/min at 275nm detector wavelength. Column used was Luna C18, 1500mm length and 46mm diameter. The injector volume used was 25µL.

5.2 Results and Discussion

The method used for HPLC analysis was developed from the Instrumental Analysis lab used for the determination of Caffeine, Theophylline, Theobromine using HPLC. A solution of 10 mM caffeine, theophylline, and theobromine were run after filtration to an auto sampler vial on HPLC for 12 minutes to indicate and determine the positions of the different compounds (Figure26).

The human reductase that was used ran out, and purification attempts by Dr. DeVore had poor yield and protein that was not active. A rat reductase was purchased instead, and we are hopeful that this protein will allow us to continue these experiments.

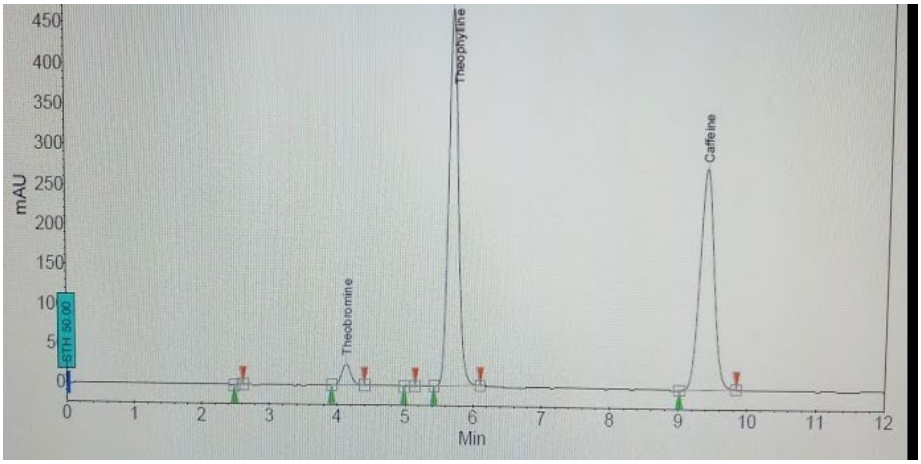

Figure 26. Caffeine, Theophylline, and Theobromine separated by HPLC analysis.

The first chromatogram (Figure 26) shows retention times for caffeine and its metabolites while the second chromatogram (Figure 27) shows the CYP 1A2 assay with the human reductase enzyme and shows the metabolite peaks at approximately 3minutes, 3.5 minutes, and 4.2 minutes showing the positions of theobromine (3.5 min), theophylline (4.2 min), and maybe paraxanthine (3.0 mins).

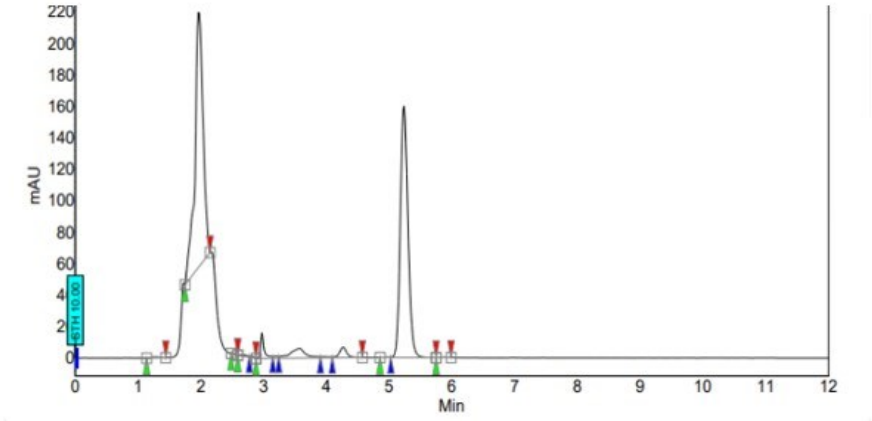

Figure 27. CYP 1A2 assay with human reductase, retention peaks at 3mins,3.5mins and 4.2 mins shows the metabolites produced.

5.3 Conclusion

Cytochrome P450s require an additional protein during oxidative reactions for the provision of electrons. The human cytochrome reductase gene was purchased, transformed in *E. coli* cells, expressed and purified using Nickel affinity for the transfer of electrons from NADPH using its flavin moieties into the heme of CYP 1A2 for an efficient metabolism of caffeine. The method used was suitable for the identification and can be used for the quantification of caffeine, theophylline, theobromine and paraxanthine.

Figure 26 shows the retention peaks of Theobromine, Theophylline and Caffeine to be at approximately 4.2mins, 5.8mins and 9.2mins respectively whereas the retention time observed with the CYP 1A2 assay as in Figure 27 indicated the retention times for NADPH, paraxanthine, theobromine, theophylline and caffeine to be 2mins, 3mins, 3.5mins,4.2mins and 5.8 mins. The changes in the peak positions could be caused by the changes in mobile phase or temperature changes.

Several purification protocols were adopted in purifying the human reductase enzyme afterwards after the depletion of the purified version and unfortunately the concentration yield was too low which affected the metabolism of caffeine assay since there were no metabolite peaks to be observed. A rat reductase gene was purchased instead and a purification protocol is to be developed to improve the yield.

We were able to observe some of the metabolites produced by CYP1A2 such as theobromine, theophylline and paraxanthine.

CHAPTER 6: FUTURE DIRECTIONS

For the future directions of these projects, further assays need to be completed to determine the optimal assay time and kinetic parameters with CYP 1A2. Also, full standard calibration samples need to be run so that the amounts of metabolites that are produced can be quantified. This can be achieved by preparation of different standards of caffeine, theobromine, theophylline and paraxanthine with different known concentration to be used in the assay. Paraxanthine was not used in this assay because it is expensive, however this has to be considered in standard preparation. These is dependent on the yield of the reductase enzyme obtained after purification and dialysis.

Different commercial screens are to be used to determine the right conditions favorable for the crystal growth of both CYP 2U1 and canine CYP 1A2 before optimization in order to determine their molecular structures. The concentration of these proteins after purification play a major role in crystal growth, a factor that has to be considered as well.

CHAPTER 7. REFERENCES

(1) Gonzalez, F. J.; Gelboin, H. V., Human cytochromes P450: evolution and cDNA-directed expression. *Environmental Health Perspectives* **1992,** *98*, 81-85.

(2) InterPro C ytochrome P450.

(3) Danielson, P. B., The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. *Current Drug Metabolism* **2002,** *3* (6), 561-597.

(4) Stark, K.; Guengerich, F. P., Characterization of Orphan Human Cytochromes P450. *Drug Metabolism Reviews* **2007,** *39* (2-3), 627-637.

(5) Slaughter, R. L.; Edwards, D. J., Recent Advances: The Cytochrome P450 Enzymes. *Annals of Pharmacotherapy* **1995,** *29* (6), 619-624.

(6) Nee, M. W.; Kim, C. A.; Garg, S.; Griffith, M. C.; Mizoue, L. S.; Rauch, P. E.; Dixon, W. J., Synthesis and chemistry of an iron (III) tetraphenylporphyrin with a covalently-attached phenolate tail. *Tetrahedron Letters* **1988,** *29* (42), 5345-5348.

(7) Montellano, O. d., *Cytochrome P450 Structure, Mechanism, and Biochemistry*. Springer International Publishing: 2015; Vol. 4.

(8) Poulos, T. L.; Finzel, B. C.; Howard, A. J., High-resolution crystal structure of cytochrome P450cam. *Journal of Molecular Biology* **1987,** *195* (3), 687-700.

(9) Dubey, K. D.; Wang, B.; Shaik, S., Molecular Dynamics and QM/MM Calculations Predict the Substrate-Induced Gating of Cytochrome P450 BM3 and the Regio- and Stereoselectivity of Fatty Acid Hydroxylation. *Journal of the American Chemical Society* **2016,** *138* (3), 837-845.

(10) Sligar, S. G., *et al*, *Biochemistry*. 2007; Vol. 15.

(11) Richfield, D. Medical gallery of David Richfield 2014.

(12) Bièche, I.; Narjoz, C.; Asselah, T.; Vacher, S.; Marcellin, P.; Lidereau, R.; Beaune, P.; de Waziers, I., Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenetics and genomics* **2007,** *17* (9), 731-742.

(13) Ikeya, K.; Jaiswal, A. K.; Owens, R. A.; Jones, J. E.; Nebert, D. W.; Kimura, S., Human CYP1A2: Sequence, Gene Structure, Comparison with the Mouse and Rat Orthologous Gene, and Differences in Liver 1A2 mRNA Expression. *Molecular Endocrinology* **1989,** *3* (9), 13991408.

(14) Guengerich, F. P., *Cytochrome p450: Structure, mechanism and biochemistry*. 2005.

(15) Pylypenko, O.; Vitali, F.; Zerbe, K.; Robinson, J. A.; Schlichting, I., Crystal Structure of OxyC, a Cytochrome P450 Implicated in an Oxidative C–C Coupling Reaction during Vancomycin Biosynthesis *. *Journal of Biological Chemistry* **2003,** *278* (47), 46727-46733.

(16) Zerbe, K.; Pylypenko, O.; Vitali, F.; Zhang, W.; Rouset, S.; Heck, M.; Vrijbloed, J. W.; Bischoff, D.; Bister, B.; Süssmuth, R. D.; Pelzer, S.; Wohlleben, W.; Robinson, J. A.; Schlichting, I., Crystal Structure of OxyB, a Cytochrome P450 Implicated in an Oxidative Phenol Coupling Reaction during Vancomycin Biosynthesis *. *Journal of Biological Chemistry* **2002,** *277* (49), 47476-47485.

(17) Kawajiri, K.; Watanabe, J.; Gotoh, O.; Tagashira, Y.; Sogawa, K.; Fujii-Kuriyama, Y., Structure and drug inducibility of the human cytochrome P-450c gene. *European Journal of Biochemistry* **1986,** *159* (2), 219-225.

(18) Sansen, S.; Yano, J. K.; Reynald, R. L.; Schoch, G. A.; Griffin, K. J.; Stout, C. D.; Johnson, E. F., Adaptations for the Oxidation of Polycyclic Aromatic Hydrocarbons Exhibited by the Structure of Human P450 1A2 *[♦]. *Journal of Biological Chemistry* **2007,** *282* (19), 14348-14355.

(19) Darragh, A.; Kenny, M.; Lambe, R. F.; O'Kelly, D. A., Adverse effects of caffeine. *Irish Journal of Medical Science* **1981,** *150* (1), 47-53.

(20) Montgomery, D. L.; Lee, A. C., Brain Damage in the Epileptic Beagle Dog. *Veterinary Pathology* **1983,** *20* (2), 160-169.

(21) Karlgren, M.; Backlund, M.; Johansson, I.; Oscarson, M.; Ingelman-Sundberg, M., Characterization and tissue distribution of a novel human cytochrome P450—CYP2U1. *Biochemical and Biophysical Research Communications* **2004,** *315* (3), 679-685.

(22) Dhers, L.; Ducassou, L.; Boucher, J. L.; Mansuy, D., Cytochrome P450 2U1, a very peculiar member of the human P450s family. *Cellular and Molecular Life Sciences* **2017,** *74* (10), 1859-1869.

(23) Karlgren, M.; Miura, S.-i.; Ingelman-Sundberg, M., Novel extrahepatic cytochrome P450s. *Toxicology and Applied Pharmacology* **2005,** *207* (2, Supplement), 57-61.

(24) Siller, M.; Goyal, S.; Yoshimoto, F. K.; Xiao, Y.; Wei, S.; Guengerich, F. P., Oxidation of Endogenous N-Arachidonoylserotonin by Human Cytochrome P450 2U1 *. *Journal of Biological Chemistry* **2014,** *289* (15), 10476-10487.

(25) Abrams, W. B.; Pocelinko, R.; Klausner, M.; Hanauer, L.; Whitman, E. N., Clinical Pharmacological Studies With Debrisoquin Sulfate, a New Antihypertensive Agent. *The Journal of New Drugs* **1964,** *4* (5), 268-283.

(26) Lanz, M.; Theurillat, R.; Thormann, W., Characterization of stereoselectivity and genetic polymorphism of the debrisoquine hydroxylation in man via analysis of urinary debrisoquine and 4-hydroxydebrisoquine by capillary electrophoresis. *ELECTROPHORESIS* **1997,** *18* (10), 18751881.

(27) Lafite, P.; Dijols, S.; Zeldin, D. C.; Dansette, P. M.; Mansuy, D., Selective, competitive and mechanism-based inhibitors of human cytochrome P450 2J2. *Archives of Biochemistry and Biophysics* **2007,** *464* (2), 155-168.

(28) Lafite, P.; André, F.; Zeldin, D. C.; Dansette, P. M.; Mansuy, D., Unusual Regioselectivity and Active Site Topology of Human Cytochrome P450 2J2. *Biochemistry* **2007,** *46* (36), 1023710247.

(29) Toselli, F.; de Waziers, I.; Dutheil, M.; Vincent, M.; Wilce, P. A.; Dodd, P. R.; Beaune, P.; Loriot, M.-A.; Gillam, E. M. J., Gene expression profiling of cytochromes P450, ABC transporters and their principal transcription factors in the amygdala and prefrontal cortex of alcoholics, smokers and drug-free controls by qRT-PCR. *Xenobiotica* **2015,** *45* (12), 1129-1137.

(30) Toselli, F.; Booth Depaz, I. M.; Worrall, S.; Etheridge, N.; Dodd, P. R.; Wilce, P. A.; Gillam, E. M. J., Expression of CYP2E1 and CYP2U1 Proteins in Amygdala and Prefrontal Cortex: Influence of Alcoholism and Smoking. *Alcoholism: Clinical and Experimental Research* **2015,** *39* (5), 790-797.

(31) Tesson, C.; Nawara, M.; Salih, Mustafa A. M.; Rossignol, R.; Zaki, Maha S.; Al Balwi, M.; Schule, R.; Mignot, C.; Obre, E.; Bouhouche, A.; Santorelli, Filippo M.; Durand, Christelle M.; Oteyza, Andrés C.; El-Hachimi, Khalid H.; Al Drees, A.; Bouslam, N.; Lamari, F.; Elmalik, Salah A.; Kabiraj, Mohammad M.; Seidahmed, Mohammed Z.; Esteves, T.; Gaussen, M.; Monin, M.-L.; Gyapay, G.; Lechner, D.; Gonzalez, M.; Depienne, C.; Mochel, F.; Lavie, J.; Schols, L.; Lacombe, D.; Yahyaoui, M.; Al Abdulkareem, I.; Zuchner, S.;Yamashita, A.; Benomar, A.; Goizet, C.; Durr, A.; Gleeson, Joseph G.; Darios, F.; Brice, A.; Stevanin, G., Alteration of Fatty-Acid-Metabolizing Enzymes Affects Mitochondrial Form and Function in Hereditary Spastic Paraplegia. *The American Journal of Human Genetics* **2012,** *91* (6), 1051-1064.

(32) Citterio, A.; Arnoldi, A.; Panzeri, E.; D'Angelo, M. G.; Filosto, M.; Dilena, R.; Arrigoni, F.; Castelli, M.; Maghini, C.; Germiniasi, C.; Menni, F.; Martinuzzi, A.; Bresolin, N.; Bassi, M. T., Mutations in CYP2U1, DDHD2 and GBA2 genes are rare causes of complicated forms of hereditary spastic paraparesis. *Journal of Neurology* **2014,** *261* (2), 373381.

(33) Masciullo, M.; Tessa, A.; Perazza, S.; Santorelli, F. M.; Perna, A.; Silvestri, G., Hereditary spastic paraplegia: Novel mutations and expansion of the phenotype variability in SPG56. *European Journal of Paediatric Neurology* **2016,** *20* (3), 444-448.

(34) Lamari, F.; Mochel, F.; Saudubray, J.-M., An overview of inborn errors of complex lipid biosynthesis and remodelling. *Journal of Inherited Metabolic Disease* **2015,** *38* (1), 3-18.

(35) Durand, C. M.; Dhers, L.; Tesson, C.; Tessa, A.; Fouillen, L.; Jacqueré, S.; Raymond, L.; Coupry, I.; Benard, G.; Darios, F.; El- Hachimi, K. H.; Astrea, G.; Rivier, F.; Banneau, G.; Pujol, C.; Lacombe, D.; Durr, A.; Babin, P. J.; Santorelli, F. M.; Pietrancosta, N.; Boucher, J.-L.; Mansuy, D.; Stevanin, G.; Goizet, C., CYP2U1 activity is altered by missense mutations in hereditary spastic paraplegia 56. *Human Mutation* **2018,** *39* (1), 140-151.

(36) Sinha, R.; Khare, S. K., Protective role of salt in catalysis and maintaining structure of halophilic proteins against denaturation. *Frontiers in Microbiology* **2014,** *5*, 165.

(37) Porath, J.; Carlsson, J. A. N.; Olsson, I.; Belfrage, G., Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **1975,** *258* (5536), 598-599.

(38) Hochuli, E.; Döbeli, H.; Schacher, A., New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *Journal of Chromatography A* **1987,** *411*, 177-184.

(39) Janknecht, R.; de Martynoff, G.; Lou, J.; Hipskind, R. A.; Nordheim, A.; Stunnenberg, H. G., Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proceedings of the National Academy of Sciences* **1991,** *88* (20), 8972.

(40) Hefti, M. H.; Van Vugt-Van der Toorn, C. J. G.; Dixon, R.; Vervoort, J., A Novel Purification Method for Histidine-Tagged Proteins Containing a Thrombin Cleavage Site. *Analytical Biochemistry* **2001,** *295* (2), 180-185.

(41) Li, M.; Su, Z.-G.; Janson, J.-C., In vitro protein refolding by chromatographic procedures. *Protein Expression and Purification* **2004,** *33* (1), 1-10.

(42) Jobe, A.; Bourgeois, S., Lac represser-operator interaction: VIII. Lactose is an anti-inducer of the lac operon. *Journal of Molecular Biology* **1973,** *75* (2), 303-313.

(43) Arnold, T.; Linke, D., Phase separation in the isolation and purification of membrane proteins. *BioTechniques* **2007,** *43* (4), 427-440.

(44) Neugebauer, J. M., [18] Detergents: An overview. In *Methods in Enzymology*, Deutscher, M. P., Ed. Academic Press: 1990; Vol. 182, pp 239-253.

(45) Hage, D. S., Affinity Chromatography: A Review of Clinical Applications. *Clinical Chemistry* **1999,** *45* (5), 593-615.

(46) Pandey, A.; Shin, K.; Patterson, R. E.; Liu, X.-Q.; Rainey, J. K., Current strategies for protein production and purification enabling membrane protein structural biology. *Biochemistry and Cell Biology* **2016,** *94* (6), 507-527.

(47) Carter, C. W., Jr.; Carter, C. W., Protein crystallization using incomplete factorial experiments. *J Biol Chem* **1979,** *254* (23), 12219-23.

(48) Carter, C. W.; Baldwin, E. T.; Frick, L., Statistical design of experiments for protein crystal growth and the use of a precrystallization assay. *Journal of Crystal Growth* **1988,** *90* (1), 60-73.

(49) Jancarik, J.; Kim, S. H., Sparse matrix sampling: a screening method for crystallization of proteins. *Journal of Applied Crystallography* **1991,** *24* (4), 409-411.

(50) Hampel, A.; Labanauskas, M.; Connors, P. G.; Kirkegard, L.; RajBhandary, U. L.; Sigler, P. B.; Bock, R. M., Single crystals of transfer RNA from formylmethionine and phenylalanine transfer RNA's. *Science* **1968,** *162* (3860), 1384-7.

(51) Thomas, D. H.; Rob, A.; Rice, D. W., A novel dialysis procedure for the crystallization of proteins. *Protein Engineering, Design and Selection* **1989,** *2* (6), 489-491.

(52) Martínez-López, S.; Sarriá, B.; Baeza, G.; Mateos, R.; Bravo-Clemente, L.,Pharmacokinetics of caffeine and its metabolites in plasma and urine after consuming a soluble green/roasted coffee blend by healthy subjects. *Food Research International* **2014,** *64*, 125-133.

(53) Arnaud, M. J., *Metabolism of Caffeine and other components of coffee*. Raven Press, Ltd: Newyork, 1993.

CHAPTER 8. APPENDIX - A SUMMARY OF EXPRESSION AND PURIFICATION CONDITIONS CYP 1A2 AND 2U1 AND CORRESPONDING YIELDS

Condition	Yield	Outcome
3.2mM of Cymal-5 detergent used on both the protein and the buffers. Cobalt column was used for affinity chromatography.	Some of the protein eluted with the wash buffer and proceeded to be purified further by ion exchange using CM beads.	The protein precipitated without binding at the CM column. The pH of the buffer used was not adjusted. Might have been the cause for precipitation.
Cobalt column was used with the pH of the buffers adjusted to 7.4. Same concentration of Cymal-5 detergent used.	The same yield was obtained as before and CM beads used for ion exchange purification.	The protein did not bind to the CM column. The concentration of IPTG and ALA were adjusted.
Same conditions were used. Increased IPTG and ALA to 0.72g	Low yields obtained with the wash buffer. Ion-exchange done with CM beads.	Protein precipitation occurred. Had to use a different detergent.
10% anapole-35 detergent adopted for protein extraction.	Low yields obtained; protein eluted with the wash buffer. Ion- exchange done with CM beads.	The protein precipitated again. CHAPs detergent used in the next purification.
1% CHAPs detergent added in the protein with 0.5% in the buffer solutions.	The yield was low. Proceeded for ion exchange purification with CM beads.	Protein precipitated.
Nickel column was used for affinity and DEAE beads for ion- exchange chromatography. CHAPs detergent used.	The results were better with the protein eluting with the elution buffer in both purification steps.	The protein was saved for crystallography and the condition adopted for purification. The final yield was not enough and 6L of TB media were used instead of 3L.