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Proof of Concept for a Urine-Based Method for Monitoring Blood Phenylalanine for Patients with PKU

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PROOF OF CONCEPT FOR A URINE-BASED METHOD FOR MONITORING BLOOD PHENYLALANINE FOR PATIENTS WITH PKU

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by
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Abstract

There is a great need for an at-home, simple, inexpensive, and noninvasive testing method to monitor the blood phenylalanine (Phe) levels of patients with PKU. PKU is a rare genetic disorder that affects approximately one out 15,000 people, which results in dangerously high levels of Phe in the blood. This project aims to show proof of concept for such a test, which is based upon a previously developed test method that was historically used to diagnose PKU from a urine sample. This redesigned test method represents the optimization of a little-understood reaction between Fe^{3+} ions and phenylpyruvic acid (PPA) that results in a color change. PPA is a metabolic byproduct of elevated blood Phe levels, thus enabling it to be used as an indicator of this genetic disease. The improved test increases sensitivity to the presence of PPA by two orders of magnitude over the previous method by including custom test substrates, controlled lighting conditions, and smart phone technology. The process by which the test substrates were developed is presented, as well as their performance for the quantification of PPA concentration in a urine sample. The additional accuracy of the refined method is primarily provided by the implementation of a custom-made device to control light conditions combined with the measurement of color using a smart phone to photograph test samples combined with an RGB color application for color quantification. The final test proposed is inexpensive, safe, and simple enough for a lay person to do at home. Further optimization and calibration to individuals with PKU will be needed for the test to be commercially viable.

Dedication

This thesis is dedicated to my parents who always supported my education, especially my father Michael Cholewczynski for fostering a curiosity in science and engineering from a young age. I would also like to acknowledge my late uncle, Paul Franzen Jr. for helping to financially support my graduate education.

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Chapter 1

Introduction

PKU is a rare genetic disease that causes the buildup of phenylalanine which results in severe developmental delays if not diagnosed and treated early in life[1,2,3]. Treatment is nearly entirely diet based and requires rigorous monitoring of Phe levels in the blood to avoid the effects of the disease, especially in the cases of pediatric and maternal PKU[1,4,5,6] . Current monitoring methods are time consuming, complex, and invasive[7]. This project attempts to show a proof of concept for a new method of non-invasive urine testing that could be used to monitor blood Phe levels in PKU patients. This test is based on improving the sensitivity of a previously existing colorimetric diagnostic test for PKU marketed as Phenistix[8.9] by regulating light conditions, improving the color changing substrate, and taking quantitative measurements of color by using a smart phone to digitally photograph the samples combined with a red-green-blue (RGB) reading application to obtain a quantitative color value.

Chapter 2

Background

2.1. Disease and Treatment

PKU (phenylketonuria) is a rare genetic condition that affects about 1 in 15,000 births in the US[6.10]. Patients must be homozygous recessive to show symptoms and about 1-2% of the world population is a carrier for the gene[7]. This condition is characterized by abnormally low levels of a hepatic enzyme called phenylalanine hydroxylase[7,1]. This enzyme converts phenylalanine into tyrosine and without it phenylalanine levels in the body rise and build up in the neurological system. Abnormally low levels of tyrosine are also observed[11]. This causes severe developmental impairment if not treated, as well as seizures, microcephaly, hyperactivity, and EEG abnormalities[2,12]. Less sever signs also include hypopigmentation and a musty odor[12,7].

The disease is usually managed by consuming a diet low in phenylalanine, which is supplemented by medical foods to provide the proper balance of nutrition[1,5,6]. The goal is to keep blood Phe levels between 120-360 μM. Untreated individuals may have Phe levels exceeding 1200μM, while non-PKU individuals have an average blood Phe concentration of 60 μM[1]. PKU patients control their blood Phe levels by drinking Phe-free medical formulas that contain all of the other amino acids, which are available through medical suppliers. The medical food formula is then supplemented by controlled quantities of low protein foods, such as fruit and vegetables[1,6]. Previously it was advised that the diet only be continued until age 10 when most development had been complete, but more recent research has found that continuing the

diet, and controlling Phe levels, is beneficial to PKU patients of all ages and is especially important in helping with depression, anxiety, attention deficit hyperactivity disorder, and headaches[7,6]. Weekly blood tests are generally used in infants to monitor Phe levels and become less frequent as the patient ages[1].

The high Phe levels are most detrimental in the central nervous system, so some therapies have focused on trying to prevent Phe from permeating the blood brain barrier rather than controlling systemic levels[11,13]. All large neutral amino acids use the same transport mechanism into the brain, so it is thought that simple competition with other amino acids may be able to prevent high neurological Phe levels[11,13]. Therapy with tyrosine and tryptophan has been used with some success in PKU patients[13], although it can be difficult to maintain high enough systemic levels of these other amino acids to see an effect in mental status[11].

Tetrahydrobiopterin (BH_4) has been used to control blood Phe levels and has been demonstrated to increase platelet serotonin levels in PKU patients[14]. Serotonin is an important neurotransmitter and low levels of it are correlated with high Phe levels and poor mood. BH_4 therapy may be able to improve neurological symptoms by increasing serotonin and lowering Phe levels in addition to dietary therapy[14].

PKU is a genetic disease and so gene therapy has also been evaluated as an option for treatment, specifically targeting the liver where the deficient enzyme usually resides. Limited success has been seen in the use of viral vectors in PKU mice, and it is thought that further reduction of the native viral genes is needed[15]. Some of the successes with viral vectors were gender dependent (better results in male mice), which could lead to confusion in protocols in the future[15]. The use of a non-viral vector, minicircle naked-DNA vectors have seen the most

success, with reduction of blood Phe levels and hypopigmentation for a year in PKU mice[15]. These therapies are still in animal testing stages and it is hard to predict when they may be able to help human patients.

Pharmacutical and gene therapy are promising for the treatment of PKU, but currently most patients are on a dietary monitoring program under the supervision of a doctor.

2.2. History of PKU Testing

The devastating effects of phenylketonuria (PKU) were seen long before a disease was ever identified. The disease caused severe mental impairments resulting in PKU patients who often needed to be institutionalized for the majority of their adult lives[7,12]. The first hint at an underlying mechanism occurred when a desperate mother took her two children to a Norwegian biochemist and physician, Dr. Følling, in 1934[7,12]. At first he was unwilling to help, but the mother insisted that her children were originally developing normally, and gradually started emitting a musty odor and fell behind their peers in mental development, even losing milestones in coordination, a now common narrative from parents of PKU children[12]. Eventually, he tested the children for phenols in their urine using ferric chloride ($FeCl_3$). This test should have turned red or purple in the presence of phenols, but instead turned green. Through careful chemical evaluation it was determined that the chemical causing the color change was phenyl pyruvic acid (PPA), a metabolic result of high levels of phenylalanine building up in the patients' systems[7,12,16]. The exact mechanism of this color change is still unknown, but the rate of reaction is known to be affected by the concentration of both the ferric ions and the PPA, and

is sensitive to light[2,10]. The color change is also temporary, going from a yellow-orange to a blue-green, and back to a slightly darker yellow-orange within a few minutes of time[2,10,17]. Attempts have been made to try to stabilize the color change by using different sources of Fe^{3+} ions or reducing temperature, with limited success[17]. The degree of color change is also be affected by urine pH and interference from phosphates[10,18].

All infants born in the United States have been tested for this condition since the 1960's when effective treatment for the condition was finally available at a reasonable cost. Before it was easy and cost effective to create a formula of amino acids excluding phenylalanine, low Phe diets were attempted, but usually resulted in malnutrition[7]. With no available treatment, few children were tested for this condition until major developmental delays had already occurred. When the medical-food formula became cheaper to produce, through synthetic chemistry, infant screening programs were implemented in many developed nations to detect children with this genetic condition[7]. Originally these screening programs used the ferric chloride test, or slight variations on it, such as the Phenistix test, which involved easy-to-use strips of paper impregnated with ferric ions and other chemicals to optimize the color change reaction[2,18]. The Phenistix test included an organic acid and a phosphate binding agent to get optimum color change regardless of changes in urine composition that occur with variations in diet[8]. This test was easy to use, but couldn't yield a diagnosis until about 3 weeks after birth when most infants had gone home from the hospital[18].

Similar to the ferric chloride based test was the DNPH (dinitrophenylhydrazine) based urine test. This test also worked through a reaction with PPA, but instead of a green color, it created a yellow precipitate as an indication of unusually high levels of PPA[2,9]. This reagent was

much more caustic than the ferric chloride solutions that could be used to yield similar results and was less stable for long term storage in labs, but some facilities preferred to use it[9].

Some infants invariably fell through the cracks when trying to track them down for further testing, although some areas implemented programs where cards for urine samples to be dried on were mailed to new mothers and sent back to the hospital and others even had nurses make home visits a few weeks after birth[7]. This screening process was further complicated by possible false negatives given by PPA based tests if the urine was contaminated with bacteria from feces, as could often be the case with diaper based testing[19] and mothers often complained that getting a urine sample from an infant was too difficult to carry out[20]. There has also been criticism of this screening program for the damage that can be done by treating false positives with a low Phe diet[21]. For example, there is a case study of two sisters who tested positive using the ferric chloride test for PKU, but in actuality had normal blood Phe levels, and merely excreted large amounts of Phe and its metabolic byproducts[22]. Had these sisters been screened using the ferric chloride test, they may have been subjected to unnecessary dietary restriction in childhood.

The iron chloride-based urine test was eventually replaced by the Guthrie blood test, which was invented by Dr. Robert Guthrie in 1962[18,21]. The Guthrie test is still used in hospitals today to diagnose infants with PKU and was the first leap forward from the initial urine-based infant screening method. It is a bacterial inhibition assay and relies on a heel prick to gather blood from an infant, which is then placed on a paper card for further processing[1]. It can give a reliable diagnosis within just a few days after birth; although if this first test is positive, a second test is carried out a few weeks later to ensure a correct diagnosis before treatment is started[1].

The inventor of the test, Dr. Robert Guthrie, was an American bacteriologist and physician whose daughter suffered from developmental delays[7,21]. Witnessing his daughter's struggles, and those of children like her, led to his interest in PKU and ultimately the test for which he is best known[7]. In the USA, the Guthrie test has largely been replaced with tandem mass spectroscopy; however, the original test is still favored in many places due to its ease to carry out and low cost[7].

Once a diagnosis has been made, the gold standard is HPLC testing of blood plasma for Phe levels to manage the condition with diet[23]. This test requires trained technicians and a blood sample. Often the patient can be tested at most once a week and the turnaround time is about a week for the test results to be returned to the patient's family. Many adults are tested less often than this[1]. This misses many of the highs and lows that diet causes, which can be hourly on scale[24]. A faster, layperson-administered test would make it easier for patients, and the parents of patients, to monitor the immediate effects of what they eat on their blood Phe levels, thus enabling their ability to more tightly control Phe levels on a daily basis. This could reduce the need for doctor visits and help patients self monitor with greater accuracy[25].

Current research looking into novel PKU diagnositic and monitoring tests mainly fall into two catagories; those using enzymes[26,27] and those using chromatography[28,29]. Enzyme based tests have been developed for both serum[26] and urine[27] of PKU patients. Serum based tests are unsuitable for home use, but may offer an alternative in a lab setting to the current process. An enzyme based urine test could be used at home if the enzyme was sufficiently stabilized, but this is not the case currently. At least two different novel methods of chromatography for the analysis of the urine of PKU patients have been developed since 2006[28,29]. These could possibly

be beneficial in a lab setting by quantifying multiple urine analytes relevant to PKU at the same time[28,29]. The need for a non-invasive at-home monitoring test has not yet been met.

Chapter 3

Objectives and Significance

The primary objective of this research is to create an easy to use, at-home, inexpensive, urine-based test for PKU patients to monitor their blood Phe levels. A urine test is noninvasive and likely to lead to higher patient compliance, especially for infants where blood draws are difficult and high Phe levels can do the most damage[1,6,30]. It would also provide a means for daily Phe-level monitoring while waiting for blood Phe level results from the laboratory.

A previous test for PKU diagnosis was the basis for this monitoring test. Phenistix was patented in 1962 and used to diagnose infants with PKU[8]. However, the lowest PPA value that can be detected by this method is 910 μM (15 mg/dL) which is high enough that neurological damage is already being done[7]. While other colorimetric tests had been used in the past[3,7,9], this formulation was chosen for its relatively benign reagents, quick results, and easy readability. Also, unlike colorimetric tests looking for Phe directly, this method does not rely on enzymes, which may require careful handling and storage[23,28,29]. Our test aims to modify this simple, urine-based, colorimetric test to heighten its sensitivity and accuracy through the use of controlled lighting, an RGB reading app on a smart phone, and a modified color-indicating substrate for testing.

Chapter 4

Materials

The materials used for these experiments included a smart phone, synthetic and human urine, a version of the Phenistix formula, and several test substrates. Details about each of these materials are included below to aid in replication of these experiments.

The phone used for the test was an iPhone 5, using the app ColorHelper by <codete/> to read the RGB values off of the pictures taken.

Preliminary experiments for test-method development were conducted using a synthetic urine, which was formulated from the major components of human urine as reported in the literature[31] (full composition is provided in table 5.1). Distilled water was used as the solvent.

The liquid formula used to produce Phenistix was duplicated using the directions in USPO patent number 3,048,475 (full composition is provided in table 5.2)[8].

For testing, phenylpyruvic acid (PPA) was purchases from Amresco and creatinine was purchased from TCI.

The light source for the light box were battery powered LEDs from SopoTek and were labeled as “7ft 20 LED warm white”.

The other materials used in this research included creatinine test strips, filter paper, anodisks, paper towel sheets, oriented polymer substrates, and hydrogel bandages. The creatinine test strips used were AdultaCheck6 tests produced by SCiTECK and Diagnostics, Inc. The filter paper used for the experiments were made by Whatman, grade 1, 10mm circles. The Anodisk substrates were made by Whatman and labeled as Anodisk 13, 0.2 µm membrane disk.

The white paper towels were made by Bounty™ and purchased at a local store. The oriented polymer substrate was harvested from pregnancy tests sold by Walgreens. The hydrogel bandages were manufactured for MPM medical, Inc. and went by the trade name “Cool Magic clear hydrogel sheet wound dressing.”

Chapter 5

Methods

The experiments carried out utilized synthetic urine and Phenistix formula, the creation of which is outlined in the next section to aid in replication. Experiments gradually evolved in complexity from simple vial tests, to a variety of custom substrate based tests. The way each test was carried out was based on information learned from the previous test. The methods used for each test are outlined below to add context for the results and to aid in replication.

The synthetic urine was created by combining the inorganic urine analog components listed in Table 5.1[31] in 1.0 L of water, heating to 37 °C, and stirring until completely dissolved. The magnitudes of each component added are detailed in Table 5.1. The solution was put through a sterile filter (500 mL rapid flow bottle top filter 0.2 μM aPES membrane, 75 mm diameter, 45 mm neck, Nalgene, from thermo scientific) into an autoclaved glass bottle. Human urine samples were provided by the primary researcher on an as-needed basis.

Item	Formula	Formula weight (g/mol)	mg/L	M	Source
Sodium Chloride	NaCl	58.4	8001	0.14	Sigma Aldrich
Potassium Chloride	KCl	74.6	1641	0.02	Sigma Aldrich
Potassium Sulfate	K_2SO_4	174.3	2632	0.02	Amresco
Magnesium Sulfate	$MgSO_4$	120.4	783	0.01	Sigma Aldrich
Magnesium Carbonate	$MgCO_3$	84.3	143	0.002	Fisher
Potassium Bicarbonate	$KHCO_3$	100.1	661	0.01	Amresco
Potassium Phosphate	K_3PO_4	212.3	234	0.001	Sigma Aldrich
Calcium Phosphate	$Ca_3(PO_4)_2$	310.2	62	0.0002	Sigma Aldrich
Urea	H_2NCONH_2	60.1	13400	0.22	Fisher

Table 5.1. Constituents of synthetic urine[31]. The fourth column shows the amount of chemical added to one liter of water in milligrams.

The Phenistix formula was created following one of the formulations provided in the US patent for this testing system[8]. This involved mixing a 20% ethanol solution and dissolving ferric ammonium sulfate, to provide Fe^{3+} ions; magnesium sulfate, a phosphate complexing agent; and cyclohexylsulfamic acid, to decrease the pH, into this solution while heating and stirring. Phosphates and high pH can interfere with color change, so this formulation is meant to provide optimal color change with the complexities of human urine. The resulting solution was kept covered and refrigerated until needed for use. The components of this solution are provided in Table 5.2.

Solutes	Formula	Formula weight (g/mol)	g/100mL	M	Source
Ferric Ammonium Sulfate	$NH_4Fe(SO_4)_2 \cdot 12H_2O$	482.25	5.8	1.20	Amresco
Magnesium Sulfate	MgSO4	120.37	8.9	7.39	Amresco
Cyclohexylsulfamic Acid	C6H13NO3S	179.23	6.5	3.63	TCI
Solvents	formula	Formula weight (g/mol)	ml/100mL		
Water	H_2O	18	80		Purified in Lab
Ethanol	C_2H_6O	46.07	20		Sigma Aldrich

Table 5.2. The composition of the Phenistix formula. The chart is divided into two sections by the shading. Unshaded regions show the dry solutes while the shaded regions designate the liquid solvents. The fourth column shows the amount of each chemical in grams added in a 100 mL batch.

Filter paper test substrates were prepared by soaking filter paper disks in the Phenistix formula until saturated (about 2 min.) and then laying them on a clean glass surface and drying in an oven at 100 °C for 20 min.

Hydrogel bandages were prepared as test substrates due to their ability to hold liquid and their colorless transparent appearance. These substrates were created in two ways. Both methods involved drying the bandage at room temperature until completely collapsed (4 days), then soaked in the Phenistix solution for 24 hours to rehydrate and saturate the hydrogel with the reactant. Then, half the bandages were dried in an oven for an hour at 100 °C to reduce their water content and the other half were used fully hydrated.

Anodisks are a substrate made of alumina and manufactured so that they have oriented pores that open to the flat of the disks and run perpendicular to this surface. While not absorbent, the orientated pores hold liquid so that the path length of light passing through is greater than that of filter paper of the same thickness. Anodisk-based test substrates were

prepared by laying the Anodisks on a clean glass sheet and adding 20 μL of Phenistix formula to each disk and then dried in an oven at 100 °C for 10 min. This resulted in dry disks ready for testing, which had a uniform orange color.

Ordinary white paper towels were also used as a test substrate by soaking in Phenistix formula until saturated (about 5 seconds) and drying at room temperature on a clean glass sheet. This created an absorbent substrate with color changing ability.

Test rolls were created by hand rolling 4 in. by 1 in. strips of white paper towel lengthwise and then tightly wrapping them in parafilm until securely fastened. The rolls were then cut into disks with a razor blade so that they were about 2 mm high and 6 mm in diameter. These disks were dipped in the Phenistix formula until saturated and then taken out and laid flat on a clean glass plate to dry at room temperature for 24 hours. The rolls were then stored in a beaker at room temperature.

The light box was created by taking a 12 in. x 8 in. x 8 in. cardboard box and covering it in duct tape to make it light tight, except for the bottom, which was left open. The inside was painted white to increase the reflection of light and reduce the appearance of shadows in photos taken inside the box. A 7-ft. long string of LEDs was attached to the inside top perimeter of the box to provide light. A section of wire without lights was used to attach the LED string to the power unit, which was taped to the outside of the box. The wire was threaded along the inside of the box so as not to be visible in any test photos. Baffles of cardstock were placed between the lights and the main chamber, parallel to the top of the box, to reduce glare from the LEDs, thus providing a diffuse lighting environment. An opening was cut in the top of the box to allow enough space for the camera on an iPhone 5 to have an unobstructed view of the

bottom of the box. The edges of the opening were covered in tape to add mechanical integrity and cover sharp edges. Several pictures of the testing set up are provided in Figure 5.1.

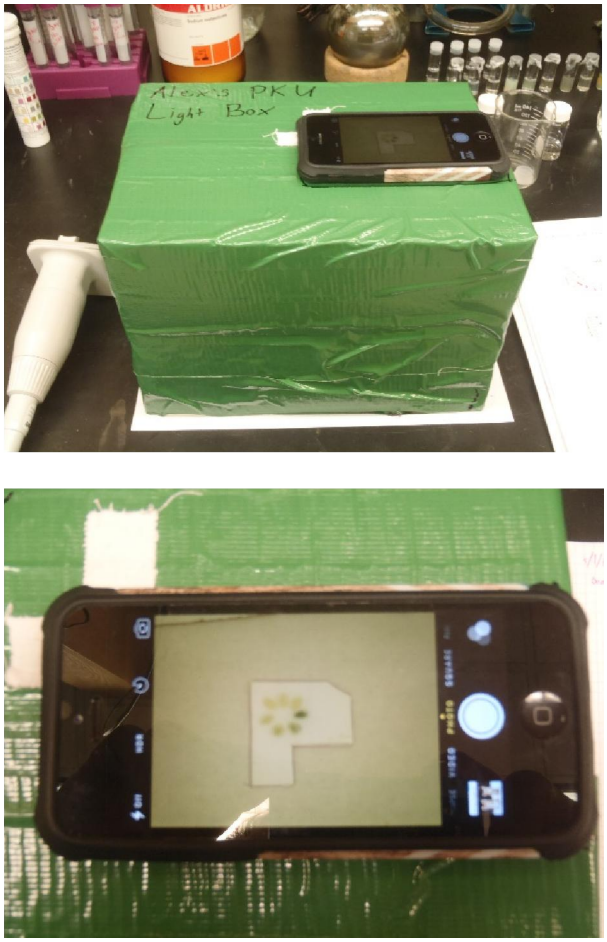

Figure 5.1. Light box in use. An oblique view (left) shows the box on a table with the iPhone in use on top. A top view (right) shows the screen of the phone viewing through the port in the top of the box to the illuminated the test samples below. The different colors of the test samples are due to their exposure to different concentrations of PPA.

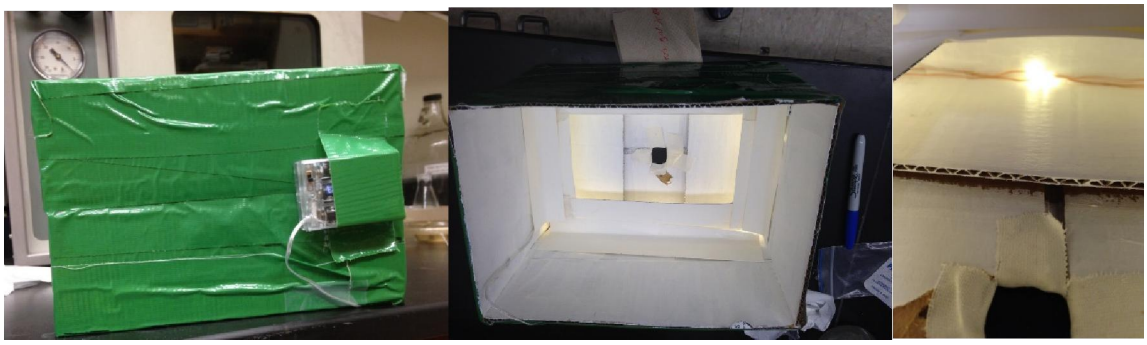

Figure 5.2. The light box when the lights are turned on. The far-left photograph shows the power unit that is attached to the outside of the box powered by 3 AA batteries with a simple on/off switch. The middle photograph shows the inside of the box from the bottom. The baffles block direct light from the LEDs and small amounts of light can be seen leaking through defining the baffles in the picture. The viewing port for the iPhone camera can be seen in the middle of the box. The far-right photograph shows a close up of the LEDs inside the box. The viewing port can be seen in the foreground for orientation. The baffles form a partial ceiling in the photo. The lights wrapped around the circumference of the box twice and the LEDs themselves tended to overlap as can be seen here.

Initial tests were done without the aid of a light box and were not analyzed as thoroughly because the goal of this initial set of preliminary studies was to find an ideal substrate for testing. Several historic tests involved the use of a liquid color change test instead of a paper substrate. An analog of these tests was created by using 5 mL glass vials, which were each filled with 1.0 mL of either water or synthetic urine spiked with various amounts of PPA. 100 L of Phenistix formula was then added to these vials (an estimated 4:1 Fe^{3+} to PPA molar ratio taken from literature to be ideal for color change) and the color change was observed at 5 min. intervals and photographed without the use of the light box.

The first substrate used to try to simplify the test for home use was filter paper. This paper was prepared as described above by soaking in Phenistix and drying in an oven. Testing was done by adding 20 μL of water or synthetic urine spiked with varying amounts of PPA to each disk of paper laying on a clean glass sheet and photographing at 5 min. intervals without the use of the light box.

The hydrogel-bandage-based substrates were prepared as described above and the testing was carried out by laying 7 of each preparation type on a clean glass surface and adding 20 μL of synthetic urine spiked with varying amounts of PPA to each piece. Photographs were taken at 5 min. intervals to monitor the color change.

The Anodisks with dried Phenistix formula were tested by adding 20 μL of synthetic urine spiked with varying amounts of PPA and photographing at 5 min. intervals to monitor the color change on a sheet of clean glass with white paper as a background without the use of a light box.

Unrolled white paper towel substrates were testing in a manner similar to the Anodisks with 20 μL of varying PPA concentration synthetic urine being added to each of seven pieces. Photographs were taken at 5 min. intervals to monitor the color change without the use of a light box.

The paper towel test rolls were first tested without the use of the light box and used a clean glass sheet on top of white paper as a background. However, this method was later further refined to include the light box and used a matte Teflon™ sheet background as outlined below. Besides these changes, the procedure was exactly the same as described below.

Testing of the paper towel rolls was done by placing seven of the test rolls on a Teflon™ sheet. Teflon™ was chosen because it is unreactive to the compounds used and is matte white in color, which was determined to be the ideal background for photographing the test samples. Tweezers were used to dip the test rolls one by one into shallow dishes of synthetic urine spiked with varying amounts of PPA, moving from lowest-to-highest to avoid contamination, until saturated (about 0.5 seconds). An example of the saturated rolls is provided in Figure 5.3.

Figure 5.3 Seven test parafilm-wrapped paper-towel rolls shortly after being dipped in synthetic urine spiked with PPA. The rolls are arranged from highest PPA concentration to lowest in clockwise order starting with the sample in the far right middle of the circle.

The light box was then placed over the test rolls and the camera placed on top of that and the LEDs inside the box turned on. A picture was taken with the preinstalled camera RGB color-app on the iPhone as quickly as possible and marked at time zero. Pictures were taken at 5 min. intervals to collect data. In between pictures, the LEDs were turned off to reduce the amount of light the samples were exposed to because the reaction is light sensitive.

The pictures were then analyzed using the ColorHelper app to obtain RGB values from the pixels near the center of each sample. These RBG values were recorded and used to evaluate the color change capacity of each substrate that was tested. The paper towel rolls with use of the light box had the most success so the data from those experiments was more thoroughly analyzed to assess accuracy. Pictures of the app in use are shown in Figure 5.4.

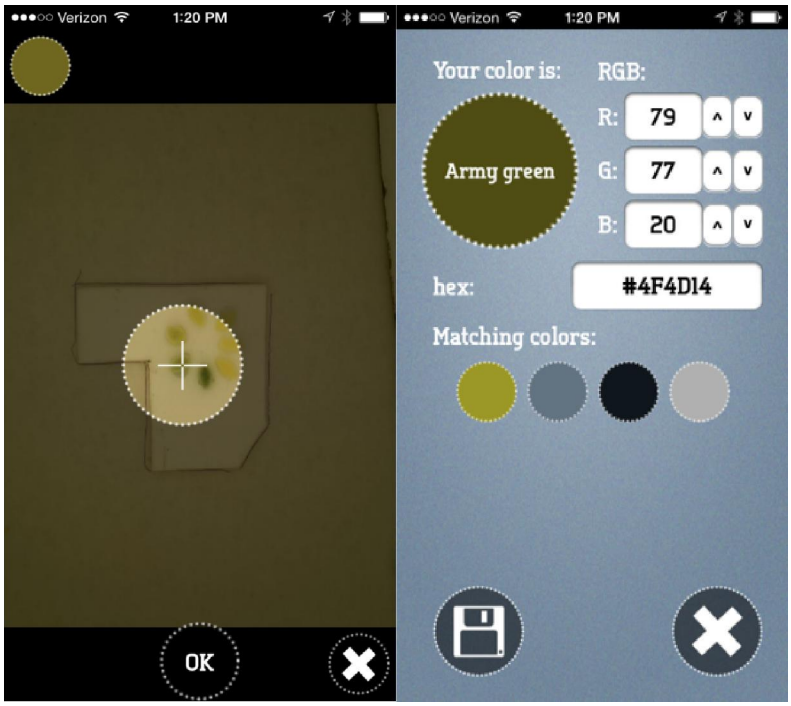

Figure 5.4. The picture to the left shows the screen of the ColorHelper app where the user selects the sample that they are interested in analyzing, which is in the center of the white crosshairs and is displayed in the top left corner of the screen. The picture on the right shows the screen after the color selection has been made and the R, G, and B values can be read, as well as the hex code for the color.

After the paper towel rolls were identified as the best testing system for synthetic urine, a similar procedure was used carry out experiments utilizing human urine spiked with PPA. Similar testing was also done with spiked synthetic urine samples that had been frozen for one week and thawed at room temperature to determine if this treatment would significantly influence the sample results if future samples needed to be frozen between acquisition and testing.

The creatinine strips were evaluated for their usefulness as a normalizing value for the hydration levels of human urine. Commercially available test strips and several concentrations of synthetic urine spiked with creatinine were used for this test. These test strips were soaked in their corresponding samples for 30 sec. before being photographed inside the light box following the same procedure as the PPA test rolls. Again, the RGB values were acquired using the ColorHelper app and processed in Microsoft excel.

Blind tests were carried out to test the ability of the testing system to identify the concentrations of unknown samples. Three random samples were made by someone other than the tester, and each tested 3 times according to the same procedure as was used to evaluate the paper towel rolls. Calibration curves were made on the same day, using the same concentrations as were used to evaluate the test substrates, and were used to analyze these samples. Same day testing helped to control for variations in test-roll age and batch, and factors like lighting and humidity.

Chapter 6

Results and Discussion

6.1. Assessment of Test Substrates

Liquid ferric ion and PPA tests have been used the most historically, and are reliable[7]. However, they require precise measurements of all reactants if the color is to be interpreted in any quantitative way, which may be difficult to carry out in a home setting by a layperson[32]. Initial tests using a liquid-containing vial rather than a solid substrate sets the bar for the color change difference (i.e., gives the greatest color change) between samples of different PPA concentration. The objective of these preliminary studies was to identify a substrate that provides the same level of color change as the liquid sample, if not more. The photo below shows the color-change results for both a vial test and a filter-paper test carried out simultaneously for easy comparison.

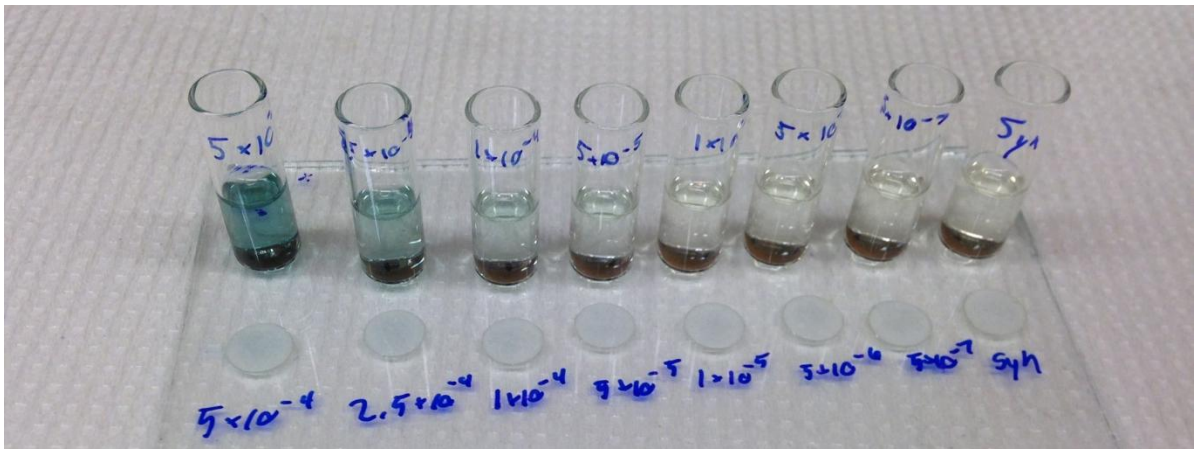

Figure 6.1.1. Results for both the liquid vial and the filter-paper-based tests utilizing the Phenistix formula and synthetic urine 5 min. after the start of the reaction. Each sample is

labeled with its PPA concentration in molarity. The vial labeled “syn” is short for synthetic urine and contains no PPA.

Water and synthetic urine were used for each type of test to understand if any of the most basic components of urine would interfere with the test. Graphs showing the red (R) RBG values for the vials for both synthetic urine and water can be seen below. The red values of the RBG values are shown because they tended to have the most reliable change in color with concentration. Green values tended to mimic the red in trend, but with less space between points. Blue values were not easy to draw trends from. Graphs of the green and blue value results can be seen in the appendix. As shown in Figures 6.1.2 and 6.1.3, despite the values in water having higher R values (indicating a lower degree of red absorbance, there is not a large difference between the changes as a function of PPA concentration for the synthetic urine and the water, thus indicating the changes in PPA concentration in the synthetic urine is qualitatively similar to that of the PPA in water.

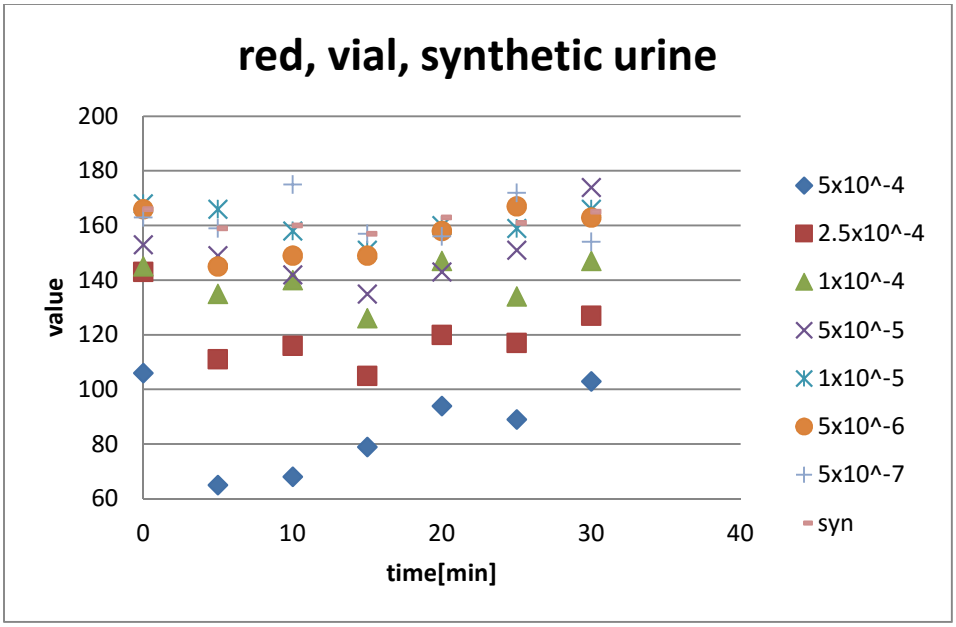

Figure 6.1.2. The red RBG values for vials of Phenistix added to seven concentrations of PPA in synthetic urine over the course of 30 minutes. The key shows which marker shape corresponds to each concentration of PPA in molarity.

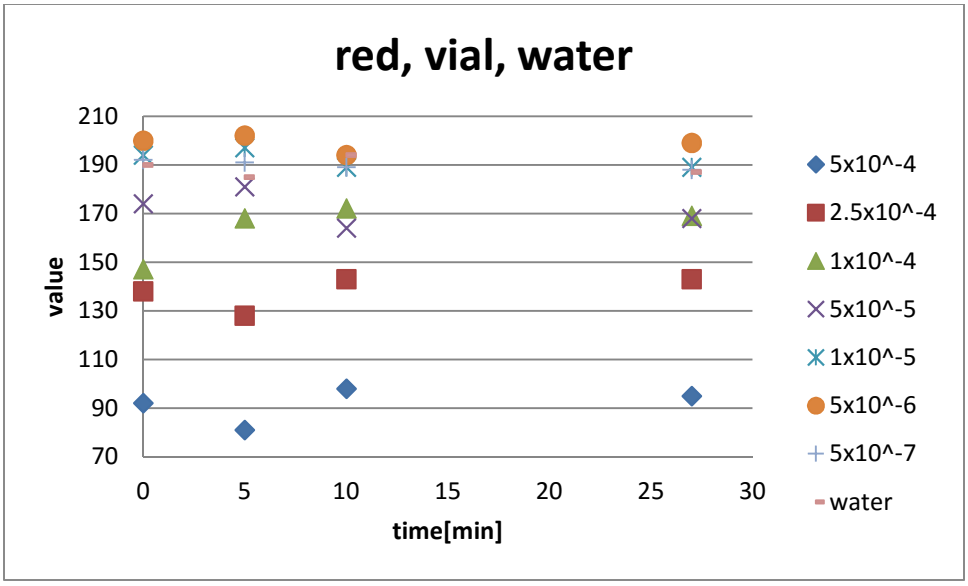

Figure 6.1.3. The red RGB values for vials of Phenistix and seven concentrations of PPA in water over the course of 27 min. The key shows which marker shape corresponds to each concentration of PPA in molarity.

These two graphs both show that the darkest color change occurred at 5 minutes, where the RGB value was the lowest (when all RGBs are high the color is white, when all are zero, the color is black). At the 5-min. time-point it can also be seen that the points are in the same order as the concentrations for the highest four concentrations (5×10^{-4} M, 2.5×10^{-4} M, 1×10^{-4} M, 5×10^{-5} M) and zero. 9.1×10^{-4} M PPA is the lowest concentration that could be detected by the Phenistix strips[8], so by using a liquid test where the path length of the light is longer, already improvements can be seen over the historic Phenistix test. The goal remains to have a solid-based coupon test (i.e., instead of a liquid-based test) to make it easier for a lay person to use at home. The results from the filter-paper test done simultaneously with the vials is shown in Figures 6.1.4 and 6.1.5. It is clear from these results that use of this substrate

provides much reduced sensitivity to changes in PPA concentration compared to the liquid vial samples.

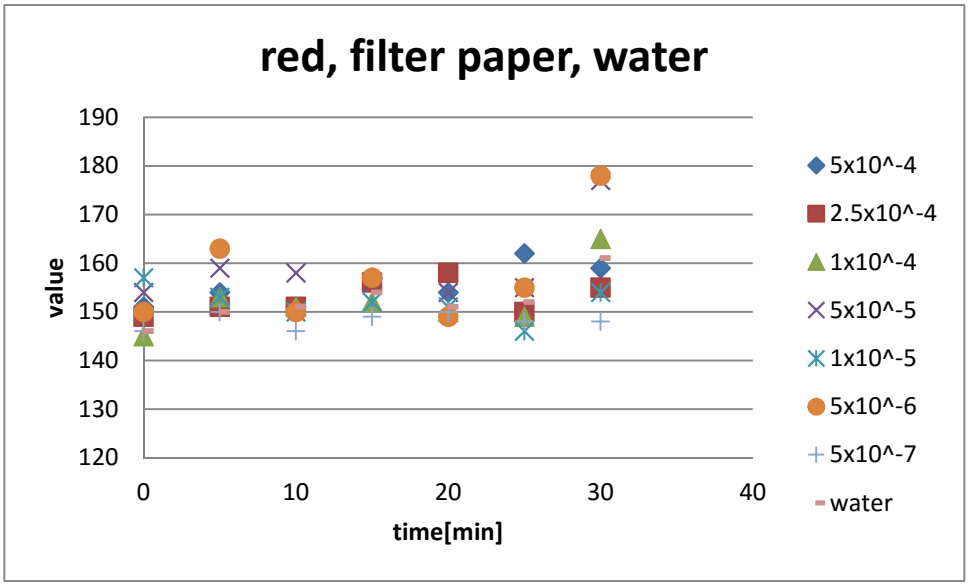

Figure 6.1.4. The red RGB values for the filter paper treated with Phenistix and 8 different concentrations of water spiked with PPA. The key shows which marker shape corresponds to each concentration of PPA in molarity.

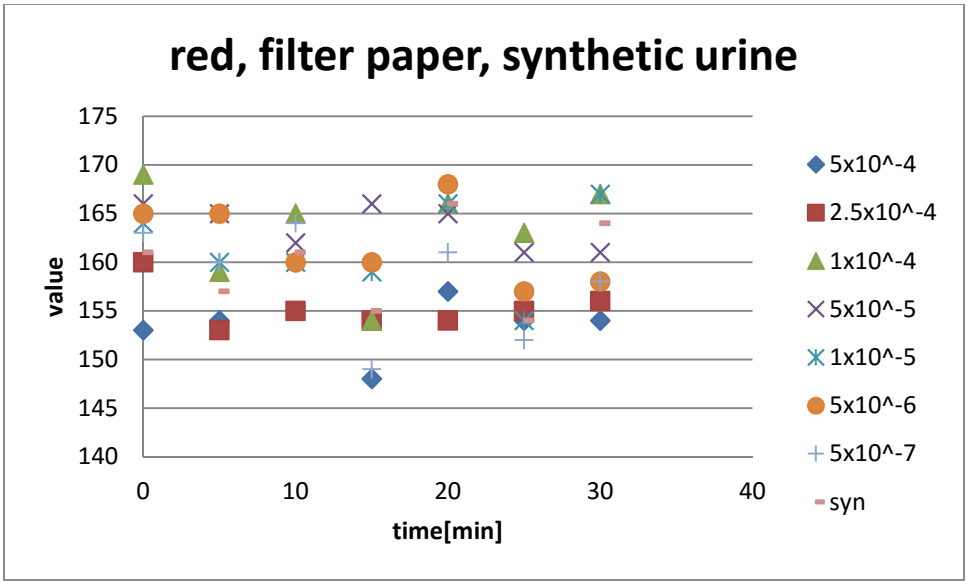

Figure 6.1.5. The red RGB values for the filter paper treated with Phenistix and 8 different concentrations of synthetic urine spiked with PPA. The key shows which marker shape corresponds to each concentration of PPA in molarity.

When compared to the vial tests it clear that the filter paper results are not very sensitive to changes in PPA concentration. It is difficult to discern any pattern at all, and the photograph (see Figure 6.1.1 above) shows that there is very little visible color change. The filter paper is designed not to hold on to liquid and so only a very thin layer of the color-changing chemical can be seen using this testing method. A substrate that holds a higher volume of liquid was investigated next to see if solid-substrate sensitivity could be improved.

Hydrogel bandages were an appealing substrate due to their availability, uniformity, and high water content. Because the bandages are mostly water by weight when fully hydrated and transparent and colorless, it was hypothesized that they would be able to showcase the color change of the PPA and Phenistix reaction. Photographs of the hydrated and dry hydrogel

bandages that were treated with Phenistix formula are shown below 5 min. after exposure to synthetic urine spiked with PPA over a range of concentrations.

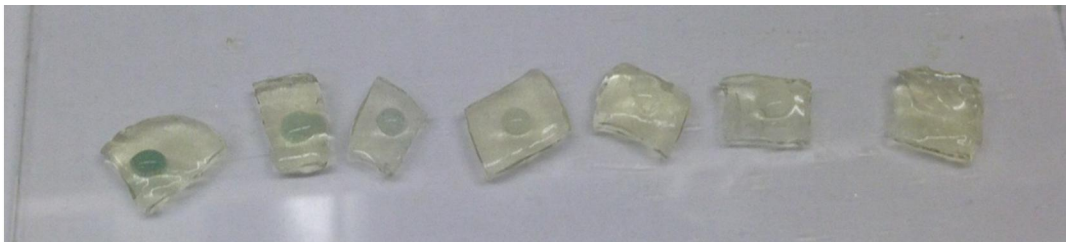

Figure 6.1.6. Fully hydrated hydrogel bandages with droplets of synthetic urine spiked with varying concentrations of PPA on their surface 5 min. after the PPA test solution was added. Concentration goes from high on the left to low on the right

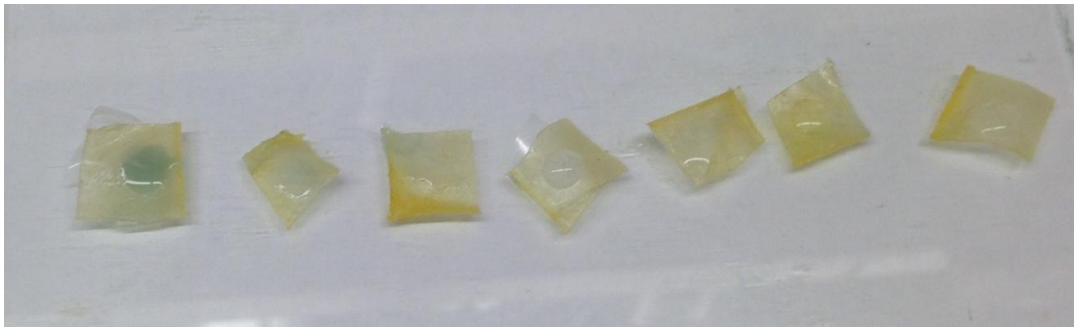

Figure 6.1.7. Dehydrated hydrogel bandages with droplets of synthetic urine spiked with varying concentrations of PPA on their surface 5 min. after the PPA test solution was added. Concentration goes from high on the left (5×10^{-4} M) to low on the right (no PPA).

As clearly shown from these photographs, the synthetic urine was not absorbed into the bandage within the 5 min. timeframe. This resulted in droplets on their surfaces with different thicknesses, leading to unreliable colorimetric readings with low sensitivity to PPA concentration compared to the liquid-vial test condition. Graphs of these readings are shown below.

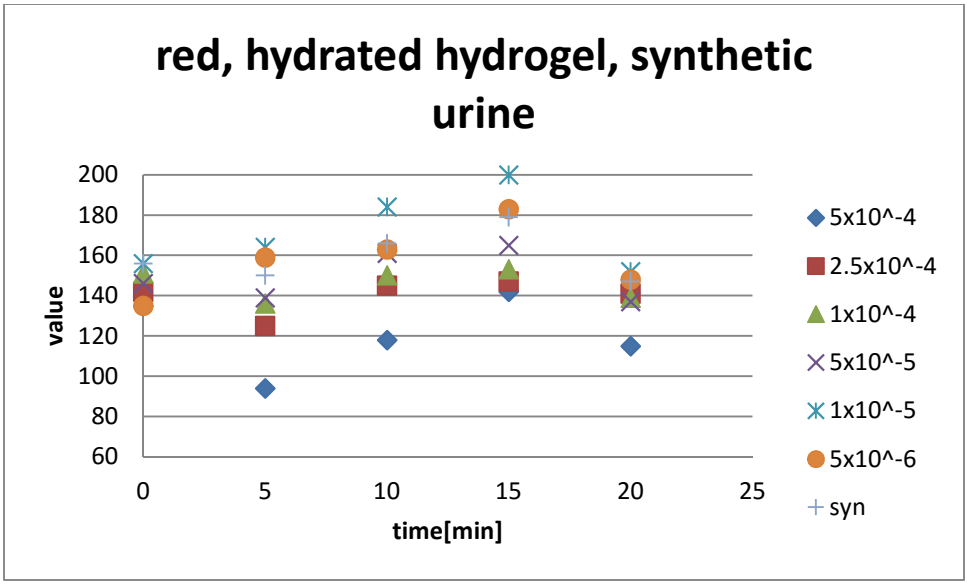

Figure 6.1.8. The red RGB values for the fully hydrated hydrogel bandage treated with Phenistix solution for seven different concentrations of PPA-spiked synthetic urine over 20 min. The key shows which marker shape corresponds to each concentration of PPA in molarity.

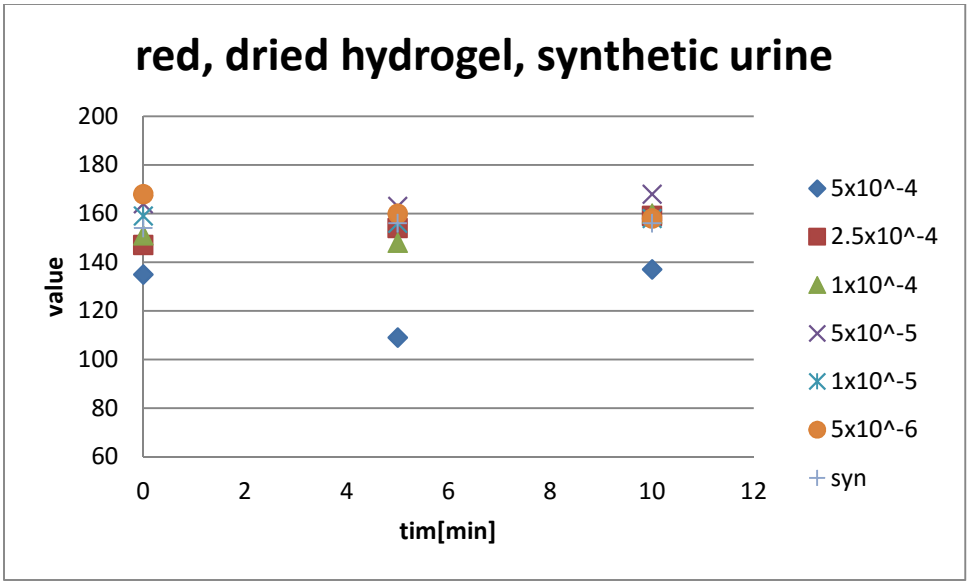

Figure 6.1.9. The red RGB values for the dried hydrogel bandage treated with Phenistix solution for seven different concentrations of PPA-spiked synthetic urine over 10 min. The key shows which marker shape corresponds to each concentration of PPA in molarity.

Although having low sensitivity, the fully hydrated hydrogel bandage showed patterns closely resembling the vial tests with respect to the lowest RGB values at 5 min. after the beginning of the test. These results indicate that the ferric ions were able to migrate out of the gel matrix to react with the droplet rich in PPA. At the end of the test the droplets were cleaned off of the gels and there was not a color change in the gel itself, suggesting the PPA did not permeate into the gel. The dried bandages adsorbed more of the synthetic urine compared to the saturated hydrogel samples, with a less-regular droplet shape, but only provided a color change pattern for the highest concentration of PPA compared to the zero value. While the fully hydrated bandage worked as well as the filter paper with synthetic urine, the results were highly dependent on the drop shape and size, which could be difficult to regulate in a home setting. Based on these results, it was hypothesized that a material with faster wicking along with higher

wicked-sample capacity (i.e., sample volume) would provide enhanced sensitivity to PPA concentration compared to the filter and hydrogel substrates.

To provide these conditions, Anodisks were evaluated next as a sample substrate that should provide faster wicking and higher sample capacity. Anodisks are a type of alumina that is formed into thin brittle disks with oriented pores. These pores allow the Anodisks to hold small volumes of fluid in vertical channels where the fluid can be observed. These disks were saturated with the liquid Phenistix formula, dried, and then small volumes of PPA spiked synthetic urine were added. A photograph of this testing set up is shown in Figure 6.1.10.

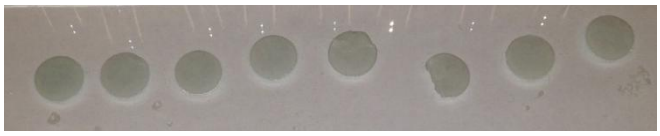

Figure 6.1.10. Anodisks pretreated with Phenistix 5 min. and then exposed to several different synthetic urine with varying concentration of PPA. PPA concentrations move from high on the left (5×10^{-4} M) to low on the right (no PPA).

As clearly observed visually from the photograph shown in Figure 6.1.10, even synthetic urine with no PPA (right-most disk) creates a color change in the Anodisks treated with Phenistix formula. This was further investigated by adding distilled water to one of the Phenistix treated disks and finding that the color change still occurred leading to the belief that this phenomenon is based on a physical color rather than a chemical reaction. The test was still analyzed to see if the Anodisks would be a suitable substrate for this test. The results of this analysis are shown in Figure 6.1.11.

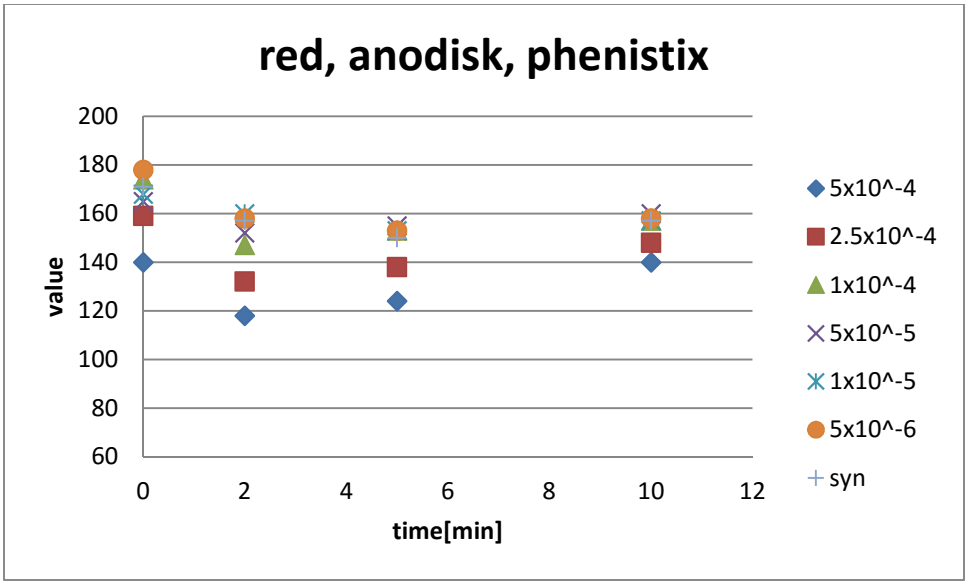

Figure 6.1.11. The red RGB values for the Anodisks pretreated with Phenistix formula at up to 10 min. after exposure to synthetic urine spiked with different amounts of PPA. The key shows which marker shape corresponds to each concentration of PPA in molarity.

Despite the small visual difference between the samples, the readings taken off of the photographs reveal that there is some difference in color change correlating to PPA concentration that reaches its peak around 2 min. after the addition of the urine analog and has almost completely faded after 10 min. While the Anodisks show some success, they still don't compare to a simple liquid test and are very brittle, making practical use difficult without breaking them. An ideal test substrate would be more mechanically robust.

White paper towels fit the ideal test substrate criteria by being fast wicking, highly absorbent, colorless, and mechanically robust. Initial tests also revealed no side reactions interfering with color change as printer paper did when coming into contact with Phenistix solution (results not shown). The paper towels were cut into 1cm squares, dipped in Phenistix

solution, dried, and had small amounts of synthetic urine with varying amounts of PPA added. A photograph of the testing set up and the resulting color changes are shown in Figure 6.1.12.

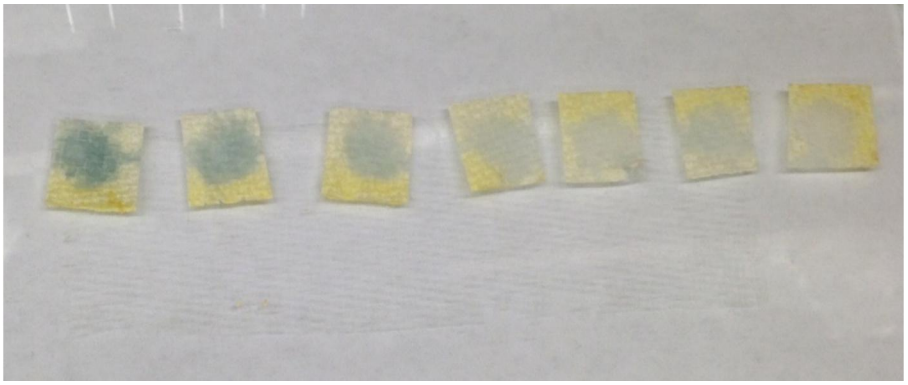

Figure 6.1.12. White paper towel sheet sections that have been treated with Phenistix formula and dried 5 minutes after synthetic urine with varying amounts of PPA had been added. Concentrations vary from high on the left ($5x10^{-4}$ M) to low on the right (no PPA). The yellow or orange color is the dry sections of the paper towel, while the white or blue sections are the wetted areas, as the amount of PPA solution added did not fully saturate the paper towel.

This testing substrate was highly absorbent and the synthetic urine spread to create a fairly even thickness of liquid giving an even color. However, the pattern on the paper towel created shadows that made measuring the color from a photograph difficult. Trying to pick a representative color patch on each sample, readings were taken and are presented in Figure 6.1.13.

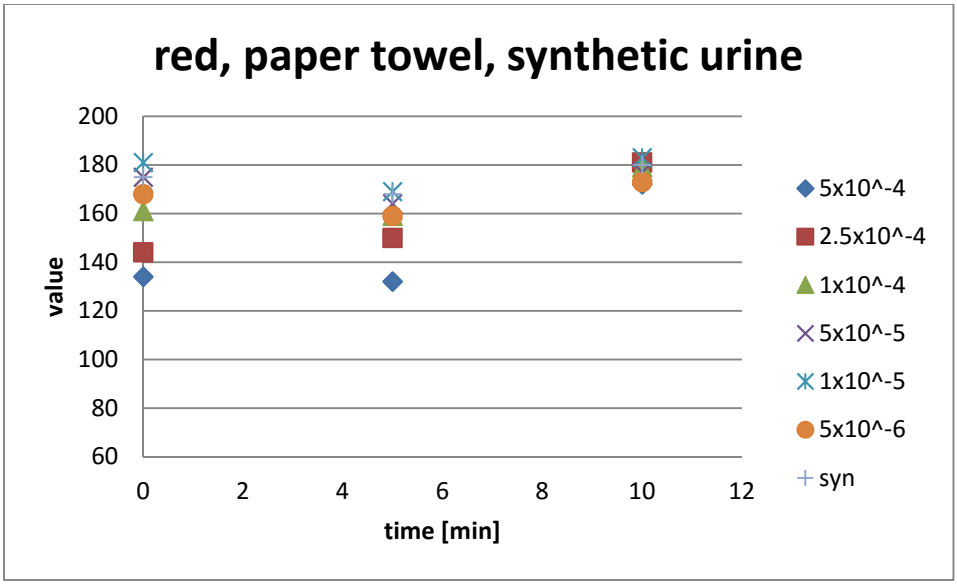

Figure 6.1.13. The red value of the RGB readout of the test section of the paper-towel substrate at three time intervals after the synthetic urine spiked with several concentrations of PPA were added. The key shows which marker shape corresponds to each concentration of PPA in molarity.

As shown in Figure 6.1.13, while the lowest red value is at 5 min., the points are the most spread out at 0 minutes. The highest 3 concentrations and the zero value are in order for the 0 and 5-minute time points. The paper towel substrate also became fairly brittle and fragile when the Phenistix was dried onto it, so some refinement was needed.

While the individual paper towel sheet substrates provided fast wicking, the sample volume that could be absorbed was still very low. Therefore it was decided to enhance the sample volume by increasing the thickness of the paper towel substrate, but in a manner to still provide very fast wicking of the synthetic urine test sample into the substrate. The final substrate that was thus tried was white paper towel rolled up, wrapped in parafilm, and sliced

to create disks, which were then dipped in Phenistix solution and dried. These will be referred to as test rolls. Several different materials were used to make test rolls, but the paper towel had the most success so it will be the only one discussed in detail here. The rolls attempted to combine all of the positive traits of other substrates by being cheap, colorless, absorbent, having vertically oriented channels, smooth texture for photography, and being mechanically robust. When one surface of the roll was dipped in liquid, the full saturation of the roll was nearly immediate. The parafilm prevented any trouble with the paper becoming brittle after Phenistix formula was dried on the substrate, although it did prevent oven drying, due to its low melting point. A photograph of testing using the rolls as substrate is shown in Figure 6.1.14. The mechanical robustness of the rolls as well as their quick wicking ability allowed the rolls to be dipped in the synthetic urine samples spiked with PPA leading to full saturation and even color.

Figure 6.1.14. The test rolls under normal lab lighting on a sheet of clean glass with white paper for a background 5 min. after being dipped in varying concentrations of synthetic urine spiked with PPA. PPA concentrations go from high on the left (5×10^{-4} M) to low on the right (no PPA).

Even with the naked eye the color gradient of the samples is clearly apparent as the concentration of PPA decreases, with the range of color intensity obviously being much greater than any of the prior substrate materials that were tested. The highest concentration is much

darker than previous solid substrate tests, and even the liquid test. The RGB readings confirm this and provide quantitative red absorbance values and are presented in Figure 6.1.15.

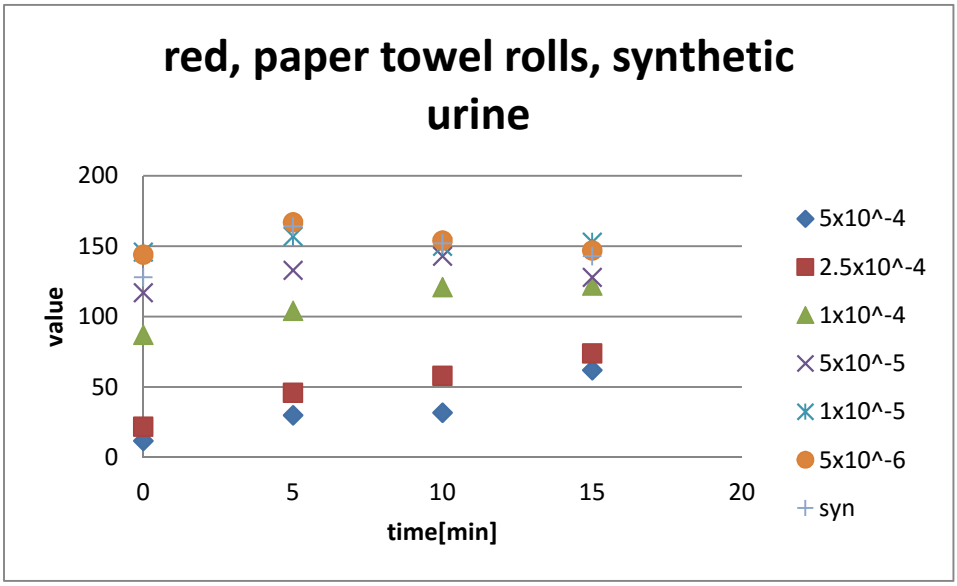

Figure 6.1.15. The red RGB values for the test rolls under normal lighting conditions at 5 min. intervals after being dipped in synthetic urine spiked with different concentrations of PPA. The key shows which marker shape corresponds to each concentration of PPA in molarity.

Figure 6.1.15 shows that the red absorbance values are very widely spaced and get much lower than with any other test substrate. As time goes on the colors of each roll start to move towards a central value, but more slowly than they did with the thinner substrates. While the darkest color is reached at 0 minutes, immediately after dipping the rolls in synthetic urine and PPA, the points are most evenly spaced at 5 min. with all but the 5 x 10^{-6} M values in the same order as their concentrations. This response is much better than any other substrate. Repetitions of this test found similar results, but variability in lighting conditions, automatic

camera focusing, and the angle of the camera relative to the samples made it difficult to compare objective RGB values from one run to the next.

The use of a light box described in the methods section made tests using these rolls much more repeatable so that tests run on different days would give similar results, allowing for statistical comparison. This was the final test set up that was evaluated for its ability to detect PPA in the urine of PKU patients in a reliable and clinically relevant manner.

6.2. Additional Substrates

In addition to the substrates discussed above, several trials were done with substrates that incorporated polyacrylic acid (PAA), which is a superabsorbent polymer sometimes used in diapers. PKU testing centers around infants because they are the patient population most at risk for adverse effects from high blood Phe levels. We therefore explored the idea of developing a substrate that could be used to collect a urine sample directly from a wet diaper. Experimenting with the idea of using a diaper directly as a test substrate led to the idea of adding PAA to some of the existing substrates to improve their absorbency. PAA was added to the hydrogels bandages, white paper towels, and test rolls. This was done by dissolving PAA into the Phenistix solution, then treating the substrates the same way their non-PAA counterparts were. This modified solution was also tried with the wicking center of a pregnancy test, which has many of the same desirable characteristics as the paper towel rolls, mainly colorlessness, oriented channels and mechanical robustness. It also had the added benefit of being commercially available so differences between samples was lessened.

Ultimately the pregnancy test polymer idea was dropped due to it being more expensive than the paper towels, performing slightly less well, and being very narrow in one dimension which made handling and photographing difficult (data not shown). The PAA also did not have the intended effect of adding absorbency to any of the substrates in any observable manner, and for the most part masked the color change taking place. When the PAA was mixed with the Phenistix formula it turned the golden-orange colored solution a deep orange-red color. This color persisted during testing which made the color change due to the ferric ion and PPA reaction less noticeable to the naked eye and when analyzed using the smart phone app and photography (data not shown).

Modifications to the Phenistix formula were also undertaken to help the filter paper substrate perform better. One test was run where the amount of ferric ions was doubled, and another was run where the Phenistix solution was added to the filter paper, dried, and then added again and dried. Both of these techniques hoped to increase the amount of color changing agent and give better color changing results. This ultimately did not help, and the results were nearly identical to the standard Phenistix formula on the filter paper and it was decided that optimizing the substrate was going to be more fruitful than optimizing the Phenistix solution.

Several substrates were tried in a rolled form, due to the success of the paper towels. These included filter paper, absorbent sheets taken from the spill kit in the lab, “amoeba” fibers of different thicknesses (fiber with irregular cross-section like the shape of an amoeba, thus providing high wicking ability), and cigarette filters. The filter paper could not be cut by the razor once rolled so disks could not be made. The sheets from the spill kit had colors that

interfered with getting accurate RGB readings, and had a quilted architecture that prevented oriented channels from forming reliably. The amoeba fibers, when made into nonwoven sheets and rolled, had difficulty holding onto the fluid and the Phenistix solution tended to form crystals with limited solubility during testing. The cigarette filters turned out to be hydrophobic and would not absorb the Phenistix solution. Each of these substrates helped to form the definition of the test substrate that would ultimately work.

6.3. Evaluating the Final System

A synthetic urine analog was used as the solution for a set of experiments to test the ability of the system to detect PPA. This allowed for a complete knowledge of what was in the sample so that an accurate calibration curve could be created. Six runs of the test were done and each test included 7 different concentrations of PPA. The RGB values were recorded for each sample and normalized by the background value of the photograph. In order to tell if the test was detecting a difference between concentrations a Student's t-test was run comparing each set of readings for one concentration to the nearest concentration set that was measured. This test should answer the question of whether or not the mean reading of each concentration is statistically different from readings of similar concentrations. The results are summarized in Table 6.3.1.

	Synthetic urine t-tests					
set 1[M PPA]	5x10^-4	2.5x10^-4	1x10^-4	5x10^-5	1x10^-5	5x10^-6
set 2[M PPA]	2.5x10^-4	1x10^-4	5x10^-5	1x10^-5	5x10^-6	syn
red 0 minutes	0.121	0.011	0.001	0.000	0.017	0.001
red 5 minutes	0.070	0.003	0.002	0.000	0.008	0.081
green 0 minutes	0.090	0.010	0.000	0.001	0.015	0.002
green 5 minutes	0.041	0.004	0.003	0.000	0.025	0.526
blue 0 minutes	0.153	0.135	0.006	0.056	0.621	0.375
blue 5 minutes	0.064	0.025	0.041	0.534	0.181	0.497

Table 6.3.1. Student's t-test results for synthetic urine comparisons of neighboring concentrations. Values for the rows labeled "set 1 [M PPA]" and "set 2 [M PPA]" denote which concentrations of PPA are being compared in that column. The row labels with a color and time denote whether the reading was the red, green, or blue value from the RGB code and what time the reading was taken. Values of $p < 0.05$ are highlighted and show statistical significance at the 95% confidence interval.

The results summarized in Table 6.3.1 show that a PPA concentration of as little as 5 x 10^{-6} M can be detected for red and green values at 0 minutes. Further optimization of this method should look for the greatest color difference between these two time points and use either a red or green reading. Concentrations of as little as 1 x 10^{-5} can be detected in both red and green values at 5 min. Blue values did not provide a sensitive measure of PPA concentration. The test had difficulty differentiating larger values of PPA, which is probably due to the darkness of these samples causing very low RGB values, sometimes as low as zero, giving little room for difference between raw data points. If the test is to be optimized for larger values of PPA it may require brighter lighting or a more dilute version of the Phenistix formula to

be used in creating the test rolls. The goal in creating these test rolls was to push the limit of PPA detection to its lowest level with this method, so lower concentrations were focused on when designing the test. This test successfully detected PPA at concentrations two orders of magnitude lower than the original Phenistix test.

6.3.a. Results Using Actual Human Urine

Once the test had shown the ability to detect PPA at very low concentrations in synthetic urine, human urine samples were obtained and spiked with PPA. The same procedure was then followed as in the synthetic urine to evaluate if the test could detect PPA with the added complexities of actual urine. The chart below summarizes the results of Student's t-tests carried out to see if the mean of each normalized concentration was significantly different from the concentrations closest to it.

	Real Urine t tests					
set 1 [M PPA added]	5x10^-4	2.5x10^-4	1x10^-4	5x10^-5	1x10^-5	5x10^-6
set 2 [M PPA added]	2.5x10^-4	1x10^-4	5x10^-5	1x10^-5	5x10^-6	syn
red 0 minutes	0.004	0.005	0.026	0.005	0.429	0.393
red 5 minutes	0.007	0.006	0.005	0.010	0.326	0.927
green 0 minutes	0.012	0.006	0.020	0.006	0.532	0.680
green 5 minutes	0.010	0.009	0.033	0.134	0.313	0.843
blue 0 minutes	0.149	0.008	0.533	0.835	0.763	0.316
blue 5 minutes	0.048	0.193	0.254	0.974	0.185	0.963

Table 6.3.2. Student's t-test results for human urine comparisons of neighboring concentrations. Values seen the rows labeled "set 1 [M PPA] added" and "set 2 [M PPA] added"

denote which concentrations of PPA are being compared in that column. The row labels with a color and time denote whether the reading was the red, green, or blue value from the RGB code and what time the reading was taken. Values of $p < 0.05$ are highlighted and show statistical significance at a 95% confidence interval.

Table 6.3.2 shows similar results to those of the synthetic urine. The smallest amount of PPA that could be detected was in the red and green readings at 0 minutes, although in this case the red reading at 5 minutes could also detect PPA at a concentration of 1×10^{-5}. The amount of PPA already present in the human urine before it was spiked was unknown and makes these results harder to interpret than the synthetic urine, but it seems the test has a lower detection limit with the added complexities of human urine. This could be further clarified in experiments where the PPA concentration is independently measured and compared to the test values.

6.3.b. Effect of Freezing/Thawing on PPA Measurement

A vial of PPA-spiked synthetic urine was frozen for one week and thawed to test whether freezing and thawing affects the concentration of PPA in a synthetic urine sample. The thawed sample was diluted with synthetic urine to make a standard set of concentrations that could be easily compared to measurements taken with the same sample before it was frozen. Four runs of each sample were measured and the results of the t-test comparing before and after freezing measurements is presented in Table 6.3.3.

	Student's t test--never frozen vs thawed						
PPA [M]	5x10^-4	2.5x10^-4	1x10^-4	5x10^-5	1x10^-5	5x10^-6	0
red 0	0.113	0.212	0.793	0.983	0.187	0.818	0.322
red 5	0.051	0.192	0.917	0.227	0.301	0.333	0.122
green 0	0.164	0.202	0.702	0.978	0.488	0.787	0.449
green 5	0.017	0.147	0.941	0.393	0.496	0.464	0.385
blue 0	0.328	0.299	0.610	0.297	0.651	0.476	0.422
blue 5	0.326	0.389	0.595	0.927	0.320	0.264	0.648

Table 6.3.3. Student's t-test results comparing synthetic urine samples spiked with PPA, before and after a freeze-thaw cycle. The highlighted value is the only one with a p-value of less than 0.05, meaning that all the rest of the comparisons are not indicated to be statistically different from each other.

Only one set, the one comparing pre- and post-freeze values for the green reading at 5 minutes at the concentration of 5 x 10^{-4} M PPA shows a significant difference. All 41 of the other comparisons showed no difference between the means of each set. The frozen and thawed synthetic urine samples show that the ability of color change to take place is not affected by the freezing or thawing process. Therefore, for future studies involving the collection and testing of actual human urine samples, samples should be able to be frozen for up to a week-long period of time and then thawed prior to PPA analysis without affecting the measurement of PPA that was in the fresh urine sample.

6.3.c. Creatinine Measurements

As documented in our studies, the Phenistix formula rolls can be used to measure the concentration of PPA in a urine sample over a broad range of PPA concentration. But in order to correlate these values to the blood Phe levels in a patient, they must be normalized by another

metabolite in the urine to account for differences in urine hydration. The metabolite that was chosen in this case was creatinine because it is a metabolic standard in urine testing and has a fairly constant excretion rate from the kidney independent of factors such as the amount of water consumed by the patient over a given period of time[33,] and its measurement after repeated freezing and thawing is largely unchanged[34]. Thus normalization of PPA concentration in a urine sample by the concentration of creatinine can be used to normalize the urine sample for dilution effects due to the level of patient hydration. Testing was therefore conducted to evaluate the ability to quantitatively determine creatinine concentration in urine by spiking synthetic urine with known amounts of creatinine followed by the use of color-indicating creatinine test strips. The resulting color change was then quantified using the same developed smartphone/RGB app and lightbox system that we developed for PPA concentration determination.

Commercially available colorimetric creatinine test strips were used with the light box and synthetic urine samples to first create a calibration curve. Theoretically, both the PPA and creatinine color tests could be done at the same time from the same photograph to measure each concentration using calibration curves between color and concentration. These values could be used to calculate a ratio between these two urine solutes. The creatinine strips unfortunately showed a great deal of variability as can be seen in the picture below where all three strips were exposed to the same high concentration of creatinine for 30 seconds.

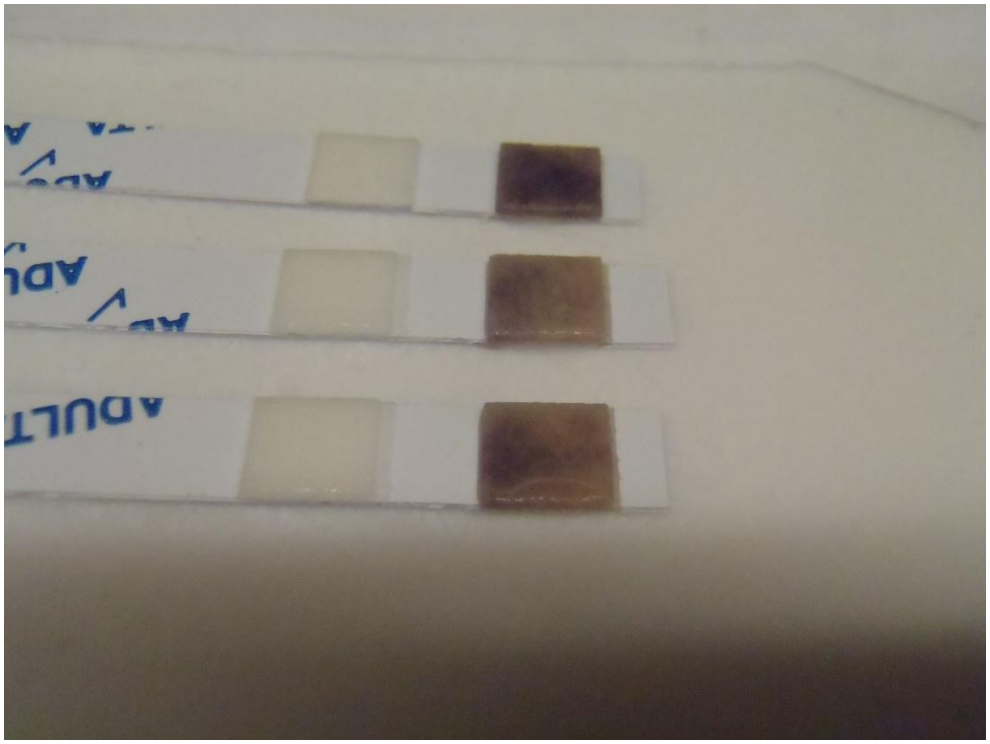

Figure 6.3.1. Photograph of three commercially available creatinine detection strips that had been exposed to 400 mg/dL of creatinine in synthetic urine for 30 seconds and let sit for another 30 seconds. Blotchy coloring can be seen on each strip as well as variation between the strips.

Even with these challenges, a calibration curve was made using the creatinine test strips, the light box, and the color identification app. This calibration curve can be seen below, along with a line of best fit.

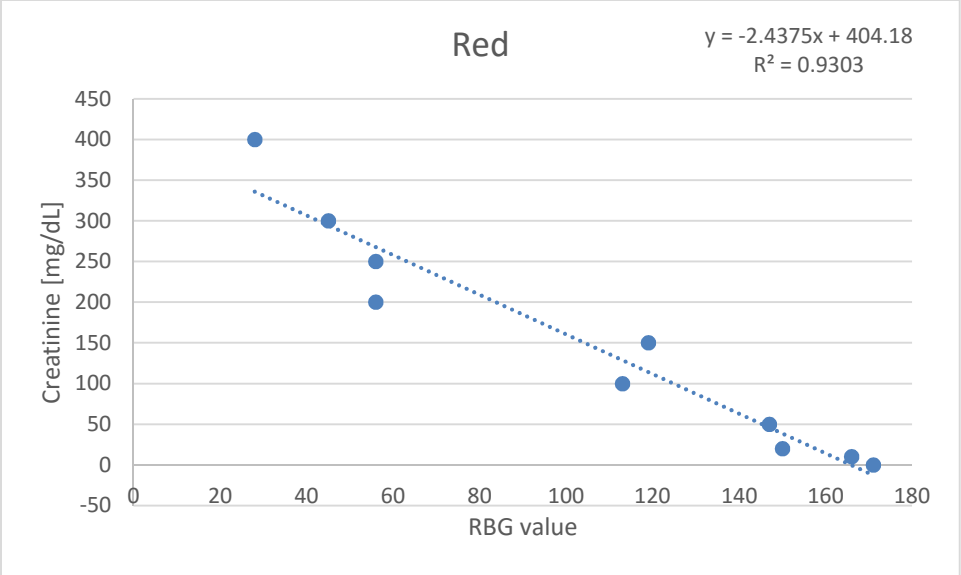

Figure 6.3.2. Calibration graph for creatinine in synthetic urine. Readings taken after 5 min.

The red value was chosen to be used for the calibration of the creatinine strips because it displayed the most linear behavior. A linear fit for the data has an r-squared value of 0.93. The strips tended not to form a uniform color across the test pad and further refinement of this part of the test may be necessary for an accurate approximation of patient blood values.

Nearly a year prior to the tests seen above, the creatinine strips were evaluated by adding 10 μL of synthetic urine with the same added creatinine concentrations as shown in Figure 6.3.2 to the test pad. A photograph of this test can be seen below.

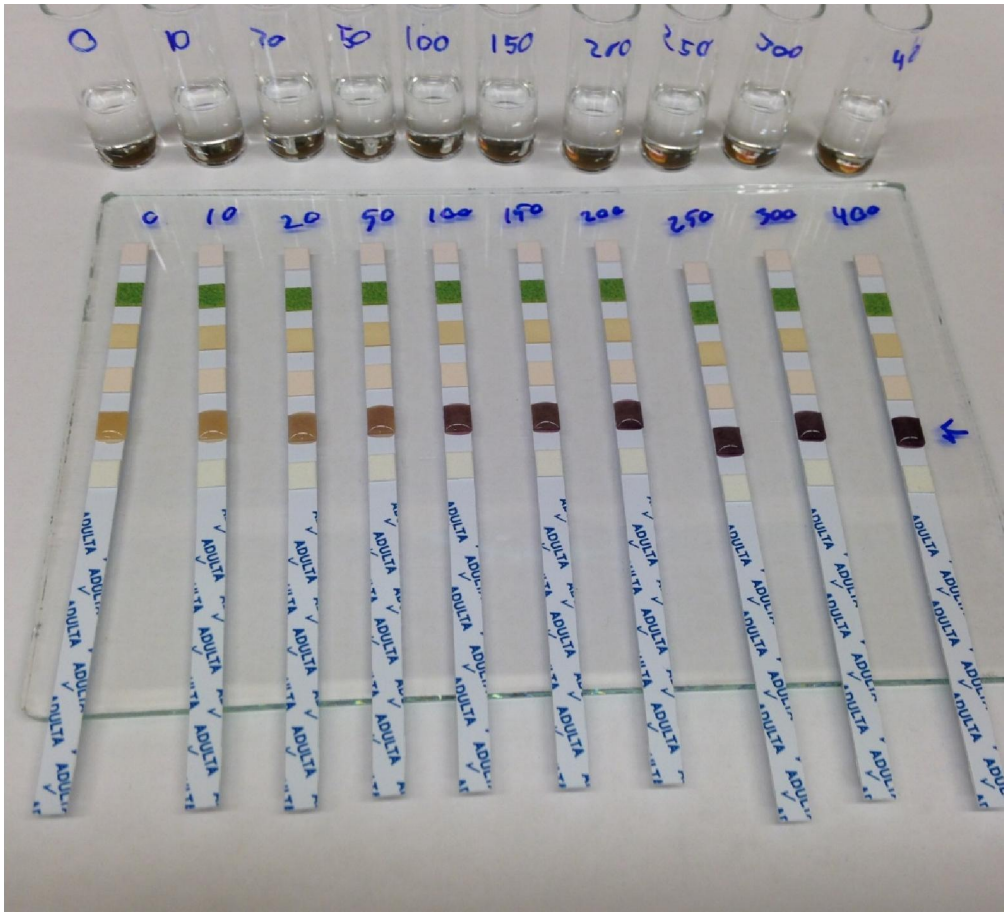

Figure 6.3.3. The creatinine test strips 60 sec. after 10 μL of each solution was added to the corresponding test pad. The arrow designates the test pad for creatine (the other test pads were for different analytes not relevent to this study). The numbers above each strip designate the concentration of creatinine and go from 0 mg/mL on the far left to 400 mg/mL on the far right.

This photograph shows that originally these test strips created a readable color that was clearly uniform and sensitive to creatinine concentration. These tests did not use the light box and were not taken by dipping the strips into the synthetic urine sample, so they were not representative of the final testing system. Unfortunately, subsequent tests with these test strips

provide inconsistent results compared to the initial tests, thus indicating that some aging phenomenon had occurred that reduced their performance. New strips were therefore ordered, hoping that newer strips would exhibit the behavior seen a year prior. However, the strips that arrived were from the same batch as the ones already possesed, and showed the same inconsistent results considered to be due to aging as with the prior set of creatinine strips, even though the expiration date for the strips was nearly a year in the future. Perhaps working with the distributor could help to ensure a higher quality control standard for this more quantitative use for the strips than was intended by the manufacturer.

6.3.d. Correlation Between Urine PPA and Blood Phe Concentration

Several studies have been done looking at the correlation between PPA and creatinine levels in the urine of PKU patinets and their blood Phe levels. One such study was used to create a calibration curve to relate these values so that the measured values from our test could be connected to patient Phe levels. A reproduction of this study's data is provided in Figure 6.3.4 along with a line of best fit[33]

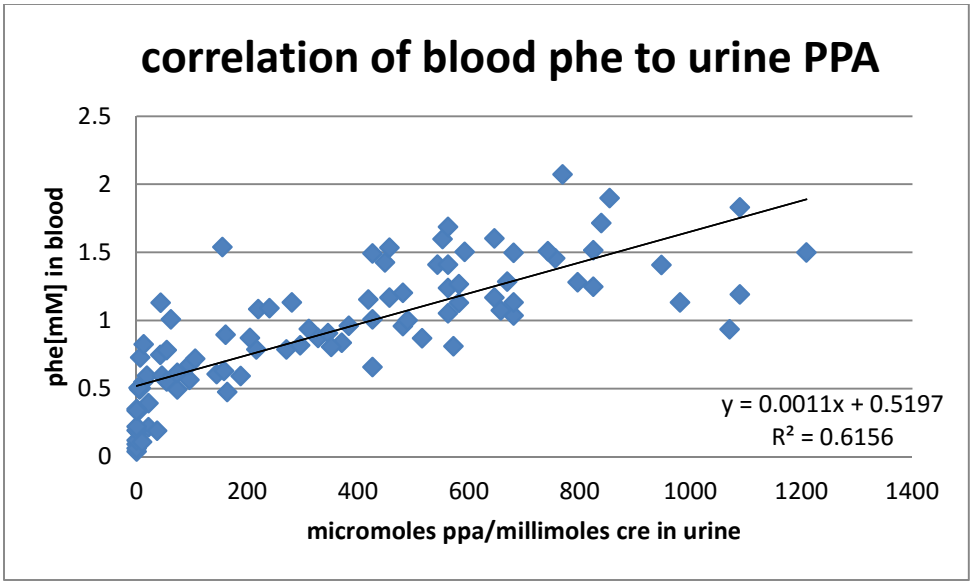

Figure 6.3.4. The correlation between PPA concentration normalized by creatinine (Cre) in the urine [μmole PPA/mmole Cre] and the concentration of Phe [mmole] in the plasma of PKU patients between the ages of 2 and 12 years[33].

Figure 6.3.4 shows that there is a large amount of scattering in the data, which is not uncommon for biological measurements. Literature shows that the relationship between these values is dependent on age and individual differences[1,7,33] . PPA stops being produced when blood Phe levels are below about 0.25 mM and renal clearance of PPA plateaus at about 1.2 mM[33], and even renal clearance of Phe is dependent on age[35]. This limits the usefulness of a generalized calibration curve for a large population with varying levels of control over their PKU diets. The data is fairly linear, and allows for a fit to be found that will be used in the blind tests to demonstrate the ability of our test to predict blood Phe levels. Future applications of this test will likely include individual calibration curves for each patient after several routine visits to their

local PKU center where blood and urine composition measurements can be made with great precision.

6.3.e Blind Tests for PPA Analysis Method

Blind tests were completed to show proof-of-concept for how the test will work in patients. Readings of creatinine and PPA unknowns were taken using the same method used to test the paper-towel-roll substrates. The calibration curves were created and used to calculate an estimated concentration for the PPA and creatinine, which were then paired and used to estimate a theoretical blood Phe value using a correlation taken from literature.

Three samples with unknown PPA concentration were created (unknown to person conducting the PPA concentration analysis), labeled A, B, and C. The test was repeated 3 times for each sample and the RGB values were averaged among the runs and normalized by the background of each run using the equation:

$$\sum \frac{value}{backgroundvalue} \cdot average\ background\ value/3$$

These normalized values are presented in Table 6.3.4.

Blind PPA samples	A	B	C
Red 0 minutes	13.56	31.85	70.11
Red 5 minutes	18.50	52.92	84.98
Green 0 minutes	15.70	36.85	69.82
Green 5 minutes	23.81	56.62	83.75
Blue 0 minutes	7.81	11.52	16.55
Blue 5 minutes	9.74	18.73	15.57

Table 6.3.4. The normalized and averaged RGB values for PPA blind samples A, B, and C at zero and five minutes after the first reading.

Both linear interpolation and a logarithmic fit were used to estimate the PPA concentration from a calibration curve of the RGB values vs. PPA concentration, resulting in the following estimates, and percent errors shown in Table 6.3.5.

A	Actual Concentration [M PPA]	linear interpolation [M PPA]	linear interpolation difference [M PPA]	linear interpolation % difference	LN fit [M PPA]	LN difference [M PPA]	LN % difference
red 0 min	3.75E-04	4.46E-04	7.08E-05	18.88	3.88E-04	1.30E-05	3.47
red 5 min	3.75E-04	4.31E-04	5.61E-05	14.96	3.89E-04	1.36E-05	3.63
green 0 min	3.75E-04	4.43E-04	6.83E-05	18.20	4.00E-04	2.46E-05	6.56
green 5 min	3.75E-04	4.12E-04	3.68E-05	9.81	3.72E-04	-2.54E-06	-0.68
blue 0 min	3.75E-04	3.58E-04	-1.70E-05	-4.53	3.62E-04	-1.28E-05	-3.42
blue 5 min	3.75E-04	4.57E-04	8.23E-05	21.94	3.92E-04	1.75E-05	4.66

Table 6.3.5. The estimated values for unknown PPA sample A, as well as percent errors and the actual concentration for 0 and 5 minute readings for red, green, and blue readings using linear interpolation and a logarithmic fit from the calibration readings.

The data from sample A suggests that the logarithmic fit of the calibration curve data is better than the linear interpolation for estimating the PPA concentration of an unknown sample. It is difficult to tell from this unknown whether a reading at 0 or 5 minutes is the best for getting the most accurate estimate.

Unknown B had a lower concentration and its results paralleling A's are presented in Table 6.3.6.

B	Actual Concentration [M PPA]	linear interpolation [M PPA]	linear interpolation difference [M PPA]	linear interpolation % difference	LN fit [M PPA]	LN difference [M PPA]	LN % difference
red 0 min	1.50E-04	1.97E-04	4.70E-05	31.31	2.46E-04	9.60E-05	64.03
red 5 min	1.50E-04	1.47E-04	-3.16E-06	-2.11	1.93E-04	4.30E-05	28.68
green 0 min	1.50E-04	2.01E-04	5.10E-05	33.99	2.33E-04	8.31E-05	55.40
green 5 min	1.50E-04	1.52E-04	1.71E-06	1.14	1.83E-04	3.34E-05	22.26
blue 0 min	1.50E-04	2.08E-04	5.85E-05	39.00	2.55E-04	1.05E-04	70.28
blue 5 min	1.50E-04	6.67E-05	-8.33E-05	-55.53	2.02E-04	5.16E-05	34.38

Table 6.3.6. The estimated values for unknown PPA sample B, as well as percent errors and the actual concentration for 0 and 5 minute readings for red, green, and blue readings using linear interpolation and a logarithmic fit from the calibration readings.

These data from sample B show the most accurate readings for red and green values taken at 5 minutes and PPA values estimated using the linear interpolation method. From previous experiments it was determined that the blue readings were fairly unreliable and are unlikely to be used for the final method of determining patient health levels.

Unknown sample C had the lowest concentration, and the lowest overall accuracy. Its data is presented in Table 6.3.7.

C	Actual Concentration [M PPA]	linear interpolation [M PPA]	linear interpolation difference [M PPA]	linear interpolation % difference	LN fit [M PPA]	LN difference [M PPA]	LN % difference
red 0 min	6.25E-05	7.34E-05	1.09E-05	17.38	1.15E-04	5.23E-05	83.68
red 5 min	6.25E-05	7.50E-05	1.25E-05	20.02	1.05E-04	4.24E-05	67.83
green 0 min	6.25E-05	7.40E-05	1.15E-05	18.34	1.08E-04	4.59E-05	73.48
green 5 min	6.25E-05	7.51E-05	1.26E-05	20.16	9.79E-05	3.54E-05	56.68
blue 0 min	6.25E-05	1.32E-04	6.90E-05	110.46	1.56E-04	9.36E-05	149.80
blue 5 min	6.25E-05	1.09E-04	4.66E-05	74.48	2.56E-04	1.93E-04	308.96

Table 6.3.7. The estimated values for unknown PPA sample C, as well as percent errors and the actual concentration for 0 and 5 minute readings for red, green, and blue readings using linear interpolation and a logarithmic fit from the calibration readings.

For unknown sample C, the linear interpolation method for the red and green readings seem to be the most accurate, and the 0 min. readings the most accurate out of those.

Each unknown sample gives a different conclusion on the most accurate way to estimate the PPA value from the readings taken. Sample A suggests using a logarithmic fit to the data, while B and C suggest linear interpolation as the best method. Samples A and B suggest that the 5-min. reading is better than a 0 min. reading, while C shows a better accuracy for 0 min. if linear interpolation is used, but 5 min. as better if a logarithmic fit is used. All of the samples confirm previous conclusions that red and green are more useful than blue readings for estimating PPA concentration. For the remainder of the blind test trials the estimates from

linear interpolation of the 5 minute reading of the red and green samples will be used because they seem to be the most accurate for the greatest number of readings.

Further research should probably look into better standardizing the rolls so that color values are more reproducible. Experiments to optimize the region of accuracy, concerning PPA concentration, would be useful because these experiments show the greatest accuracy at high levels of PPA and ideally patients will have low levels of this product if their condition is well controlled. This could probably be done by characterizing the reaction rate as a function of each reactant concentration as well as other factors such as pH.

The blind creatinine (Cre) tests were carried out using the same commercially available test strips that were used to create the calibration curve. These strips varied from batch to batch and so accuracy was best if strips from the same tube were used each time, which limited the number of repetitions of the blind test that could be done. Therefore the analysis of these readings differs slightly from the PPA rolls in that they were normalized by a background reading, but could not be averaged over several runs. Five unknowns were created and tested using the method previously described and the RGB values divided by the corresponding RGB values of their backgrounds. A similar calibration chart had been created and fit with a line that served as the calibration equation to estimate the Cre concentration in each sample. There is only a calibration for the strips at 5 min. because of the lack of color change at 0 min. Because the fit was already linear, only one method was used to estimate the Cre concentration of each sample. The estimates, actual values, and percent error are shown in Table 6.3.8 for the five unknowns.

sample	1	2	3	4	5
red estimate	151.1158	101.004	278.9007	8.297318	90.98168
green estimate	184.9217	119.9939	274.584	8.689068	101.4431
blue estimate	123.9341	197.7033	271.4726	50.16487	13.28026
actual	200	150	400	20	100
% error red	-24.442	-32.664	-30.275	-58.513	-9.018
%error green	-7.539	-20.004	-31.354	-56.555	1.443
%error blue	-38.033	31.802	-32.132	150.824	-86.720

Table 6.3.8. The estimates for the concentration of creatinine in mg/dL and the actual concentrations, as well as the percent errors for each estimate.

From this evaluation, I conclude that the green reading leads to the best estimate of the creatinine concentration. More repetitions of this test would be necessary to make more solid conclusions and the test strips themselves had a lot of variation in color from spot to spot on the same test strip, thus making measurements difficult. Future work should improve the reliability and reproducibility of this test. Of the three RGB colors, green exhibited the lowest percent error and thus should be kept in mind when further optimizing this test.

For the final step of the proof-of-concept for the blind tests, PPA samples A, B, and C were paired with creatinine samples 4, 5, and 2, respectively, to create a ratio between the two that can be used to estimate a theoretical patient's blood Phe value using the data found in literature. A graph showing the line of best fit from data from literature correlating urine and blood values of the solutes of interest is shown above in Figure 6.3.4 and was used as an example of predicting blood Phe levels from measurements of urine PPA and Cre. The results of these calculations are provided in Table 6.3.9.

	ratio μmoles PPA/ mmoles Cre			blood Phe conc. (mM)		
	A/4	B/5	C/2	A/4	B/5	C/2
Red	58.71	1.82	0.84	0.58	0.52	0.52
Green	53.55	1.69	0.71	0.58	0.52	0.52

Table 6.3.9. Experimental estimates of the ratio of creatinine to PPA for several of the blind tests as well as their estimated blood phe levels for a pediatric PKU patient based on the correlation plot shown above in Figure 6.3.4.

While the pairs of blind samples were chosen to give the greatest range of ratios, it seems that the PPA values chosen for the majority of the tests were too low for this calibration scheme to be very effective. Most testing was done with PPA levels similar to that of a well controlled PKU patient or below so that sensitivity could be optimized; however, this means that most PKU patients do not have PPA levels this low and the calibration to blood Phe levels is not well equipped to handle these values. This exercise only is meant to demonstrate the ability of the proposed series of measurements and calculations to estimate blood Phe from PKU patients from a urine sample. However, the chosen unknown PPA values were smaller than a PKU patient who would benefit from this test, which would be more applicable for patients with blood Phe levels between 600-1200 mM.

Chapter 7

Conclusions

This research was focused on the development of an improved urine test method that could be used to estimate blood Phe concentration for patients with PKU. The developed new test method was able to demonstrate an improvement over the previously developed ferric-chloride-based urine test, Phenistix, by two orders of magnitude in sensitivity. Out of many test substrates that were tried, white paper towel rolled, bound with parafilm, sliced into disks, and treated with Phenistix formula was the most successful at creating the greatest color difference over a wide range of PPA concentrations in a urine analog. This test could detect PPA at concentrations as low as $5x10^{-6}$ M in synthetic urine and $5x10^{-5}$ M in human urine. The test was also valid on synthetic urine that had been kept frozen for one week. The improved test identified unknown concentrations of PPA and creatinine within reasonable margins of error given the large range of values. The PPA test was more accurate at higher concentrations of PPA, which are more likely to occur in patients with moderately controlled PKU[1]. Proof-of-concept was shown for estimating the blood Phe levels of PKU patients using this noninvasive testing method. Optimization of testing time, substrate composition and uniformity, and photographic conditions will likely lead to more accurate results. Improvements of the creatinine strips will also be needed for improved test accuracy. The need for a simple, inexpensive, non-invasive PKU monitoring test is great and this test is a step in the direction of meeting that need.

Chapter 8

Future work

8.1. Optimization of Testing

Several aspects of the test method can be optimized to increase its sensitivity and accuracy. The formula that the test rolls are saturated with is identical to the original Phenistix formula, which was probably optimized for flat cellulose strips. It is possible that a different concentration of each of the constituents will yield a better test for use with the paper-towel rolls, or similar substrate. It would be ideal for there to be as much spread in the color values as possible within the range of interest, which is limited by Phe metabolism to PPA (does not occur below 0.3 mM plasma Phe) and renal clearance of PPA (maximum of 40-60 micromolar PPA, corresponding to 1.1-1.2 mM plasma Phe)[33]. Making the color change as linear as possible within this range would make analysis more straightforward and improve accuracy.

This test method was developed by taking color readings at five minute intervals from 0 to 30 minutes. Five minutes or less was found to be the time best suited to give the greatest range of color values. Taking more frequent readings, or even video, to find when the darkest color change is occurring could find a better time for taking measurements to optimize accuracy.

The test strips themselves are currently made by hand and are subject to a certain level of variability in thickness and how tight they are rolled. It would be ideal to find a way that ensures consistency in creation. It also seems that how tightly the paper towel is rolled affects the color change, so this should be optimized for greatest color change in the range of interest

as well. The pregnancy test polymer with oriented channels may be another manufacturing avenue to pursue.

The light box is currently bulky and not conducive to mass-production. A 3-D printable box should be developed for greatest usability and cost effectiveness. The new design for the box should be as small and portable as possible while still maintaining controlled light conditions. Batteries and LEDs will help to keep the device portable and light. Changing the color of the LEDs from white to a specific wavelength relating to the color of the test substrates may also help to create more accurate readings.

8.2. Human Trials and Individual Calibration

Literature search has found that the correlation between PPA levels in the urine and Phe levels in the blood vary greatly by age and by individual[6]. Even when adjusted for age and creatinine level, it is difficult to create a calibration curve that would be acceptably reliable for all patients as a single population. Future work should involve getting simultaneous blood and urine samples from PKU patients to create individualized calibration curves relating PPA, creatinine, and Phe in the fluids of interest. The use of actual patients and clinicians will help to troubleshoot the test and determine its usefulness as an at-home blood Phe-level monitoring method.

8.3. App Development

Currently, an app is used to get the RGB values from photographs of the samples, but all of the calculations are done in excel. For patients to use this technology effectively it will have to be developed into an app that will do the calibration calculations for them and give them a

useful readout of an estimated blood Phe level. It would be possible to combine this app with one used for tracking meals and grams of Phe consumed to control the patient's condition as conveniently as possible. It would also be very useful for the app to enable test results to be automatically transmitted to the clinic used by the patient to provide nutritionists and physicians with this information to assist them in their efforts to help the patient control his or her blood Phe levels within a reasonable range on a daily basis.

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33 Langenbeck, U., Behbehani, A., Mench-Hoinowski, A., & Petersen, M. (1980). Absence of a significant renal threshold for two aromatic acids in phenylketonuric children over two years of age. *European Journal of Pediatrics, 134*(2), 115-118.

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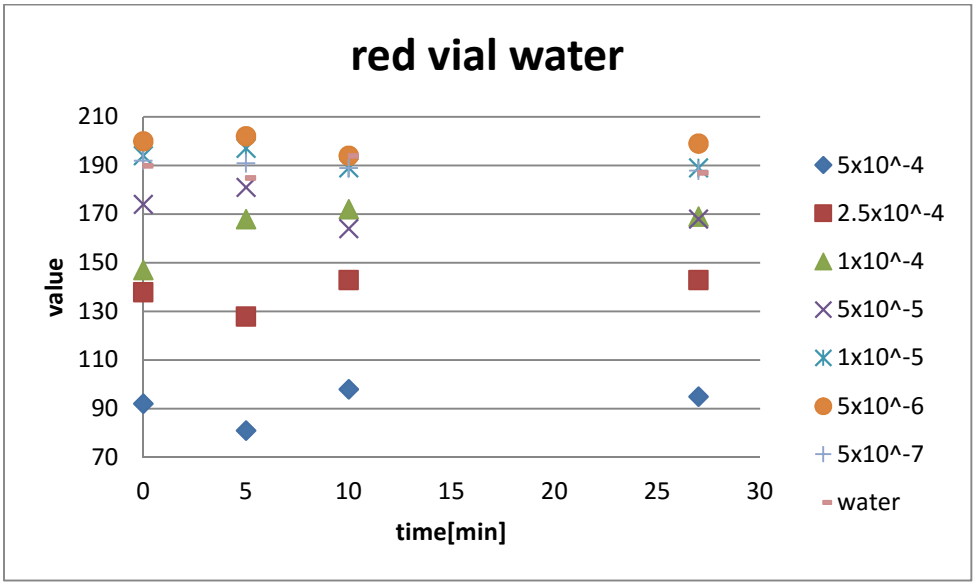
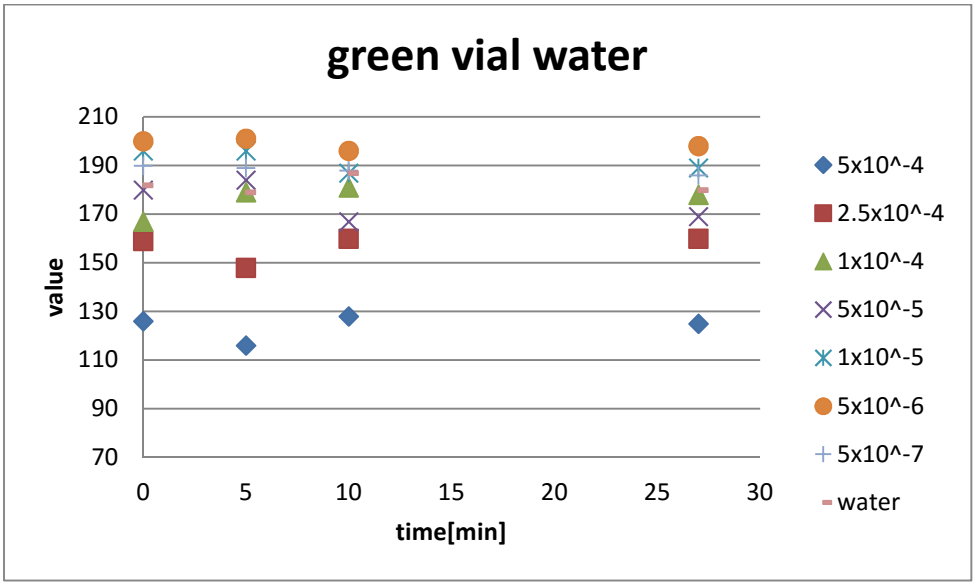
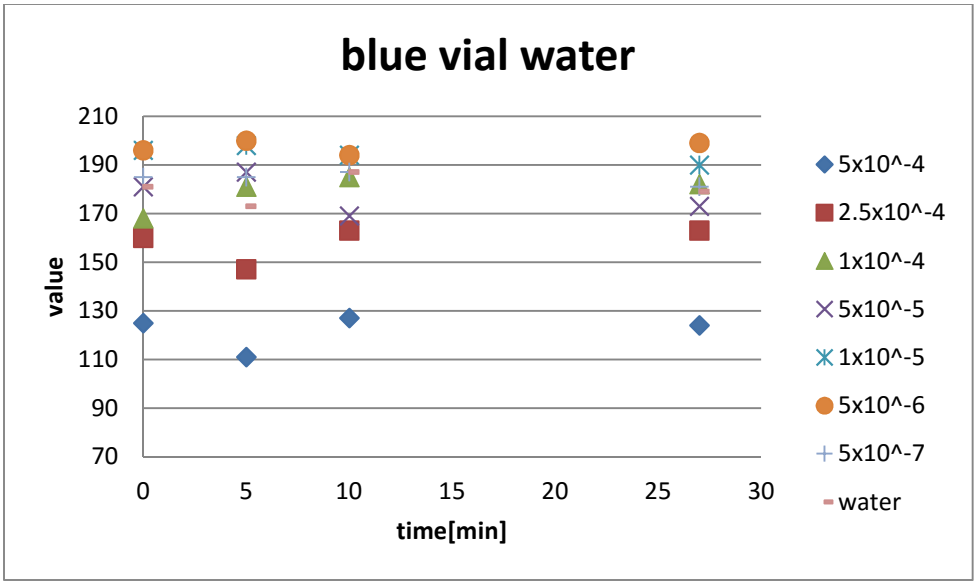
Appendix: RGB graphs of tests

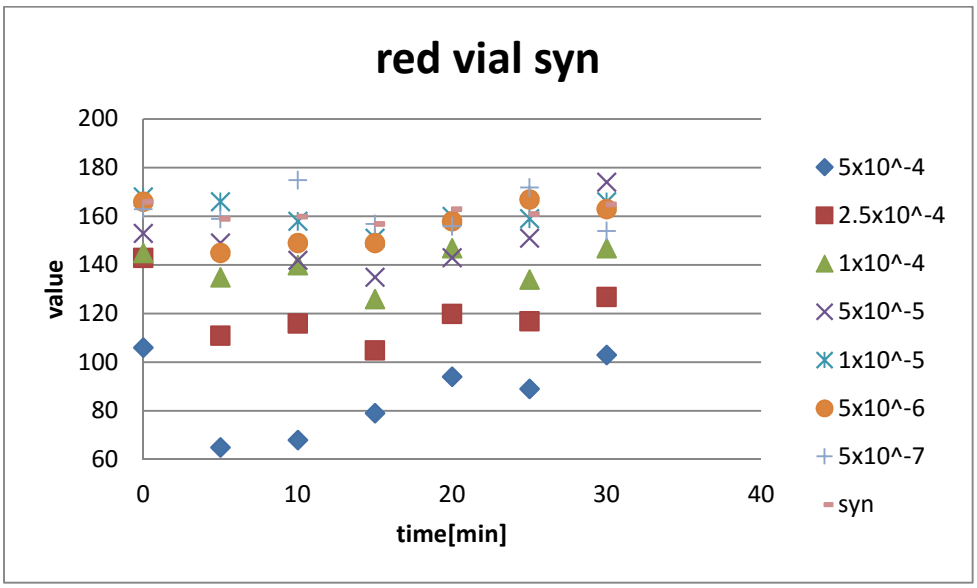
Vial tests with water

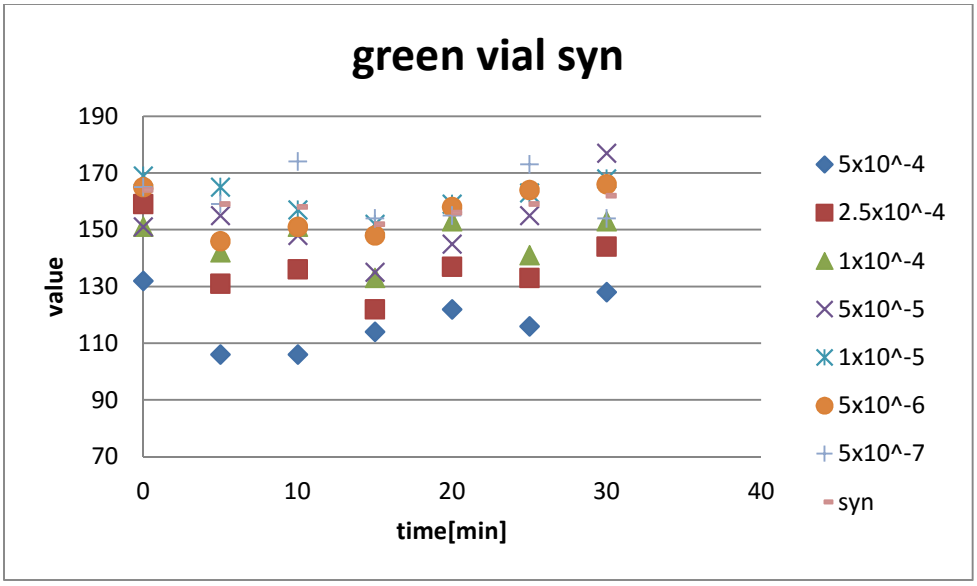


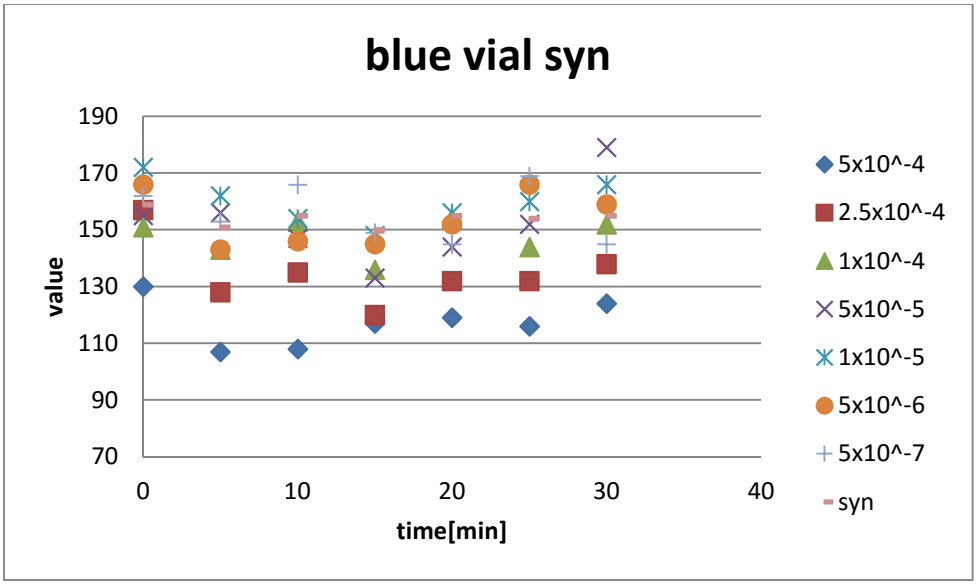




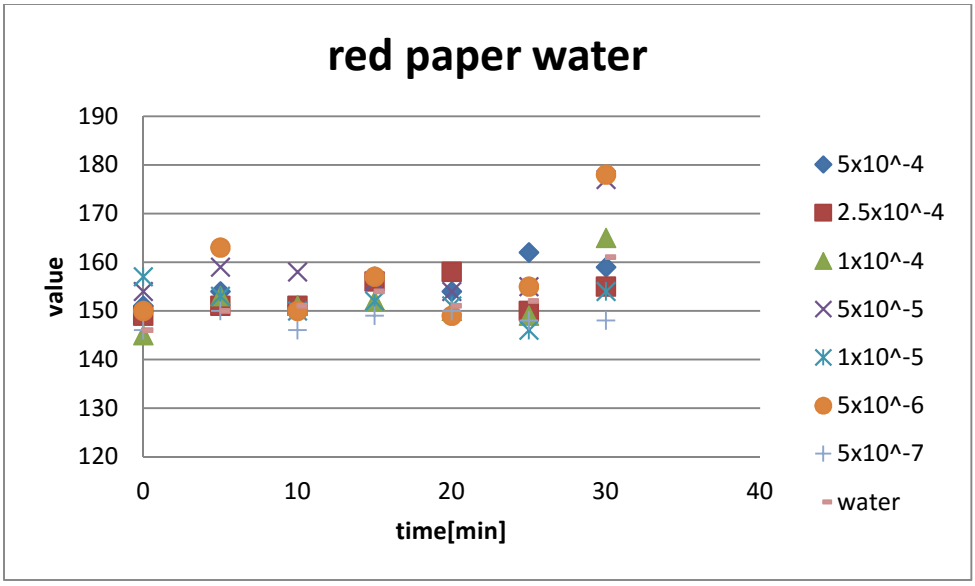
Vial tests with synthetic urine

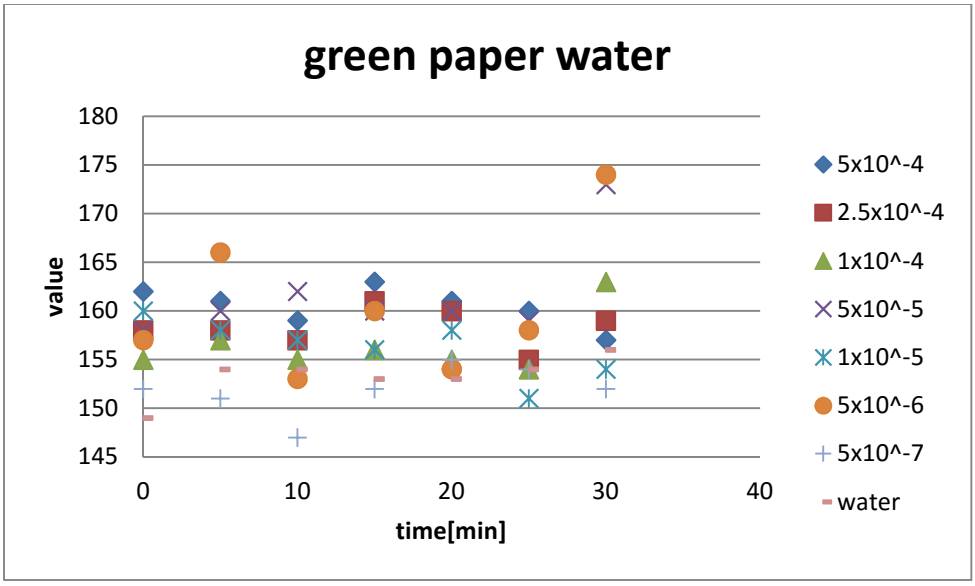


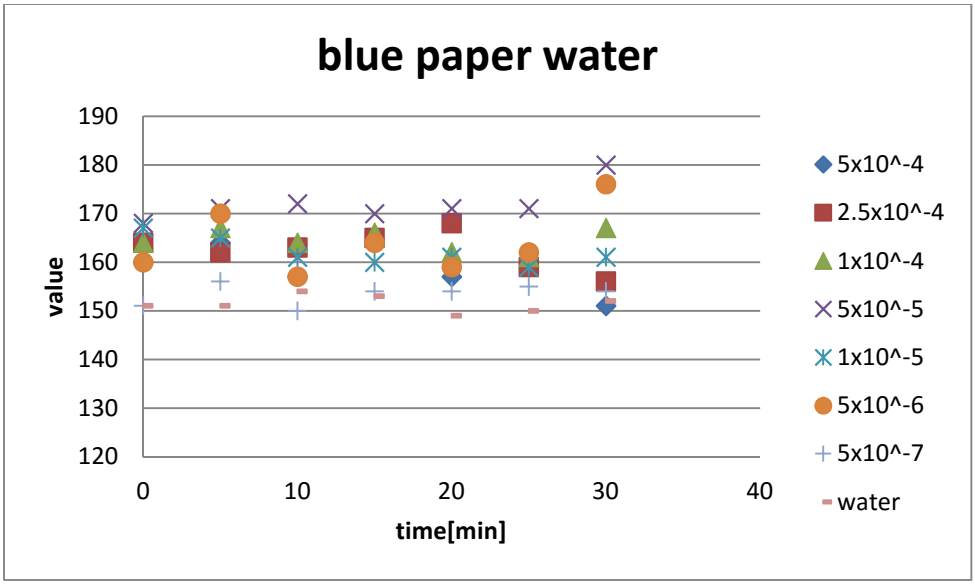




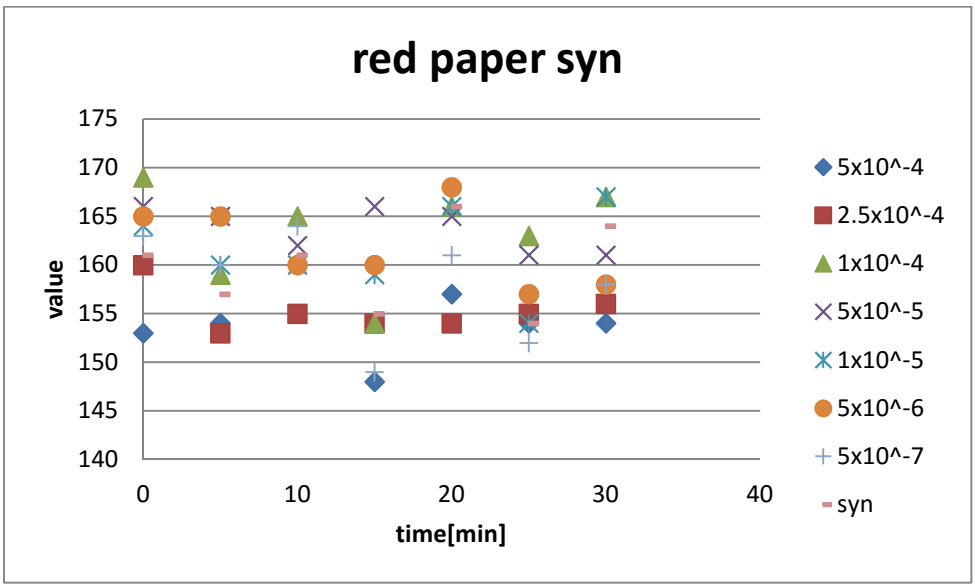
Filter paper tests with water

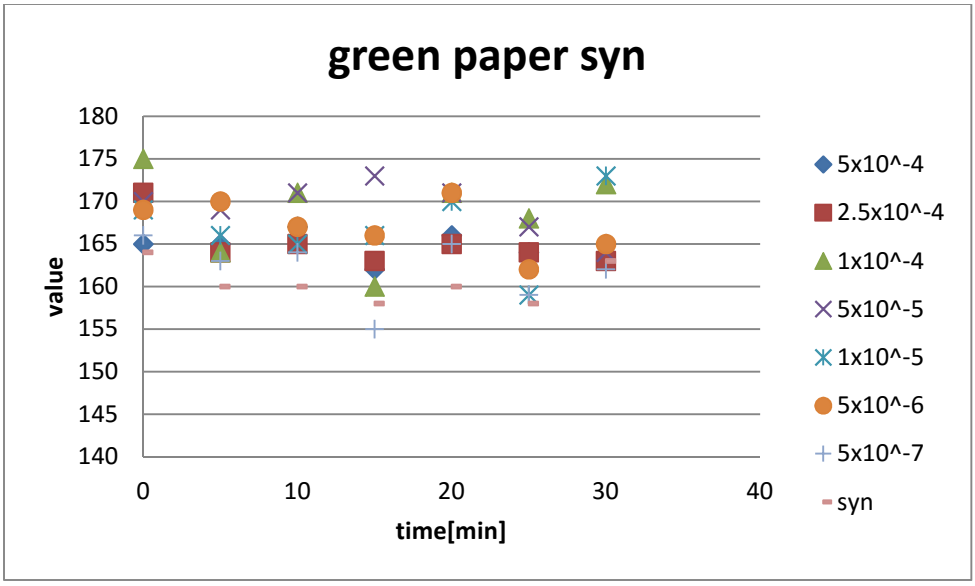

red paper water
190
180
170
160
150
140
130
120
value
0
10
20
30
40
time[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
5x10^-7
water

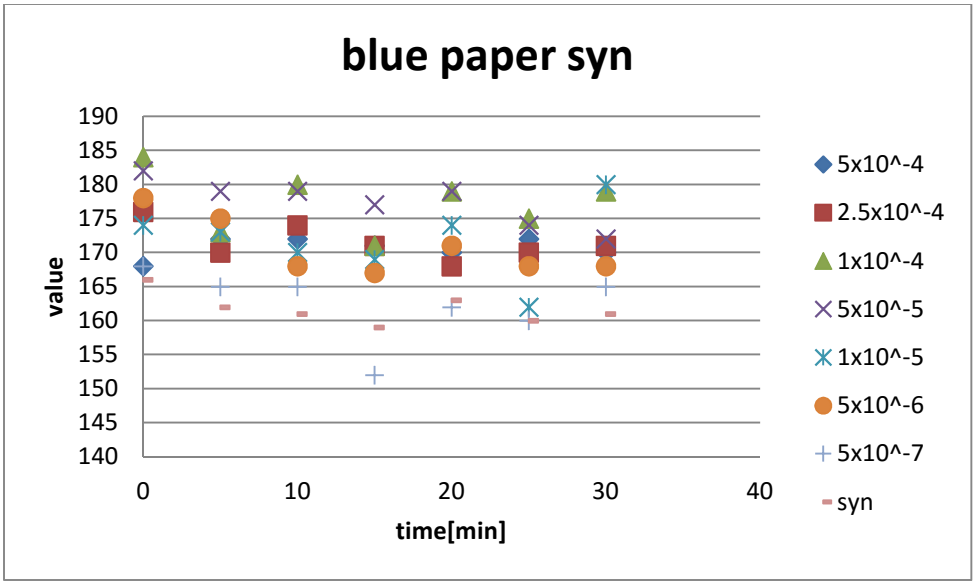

green paper water
180
175
170
165
160
155
150
145
value
0
10
20
30
40
time[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
5x10^-7
water



Filter paper tests with synthetic urine

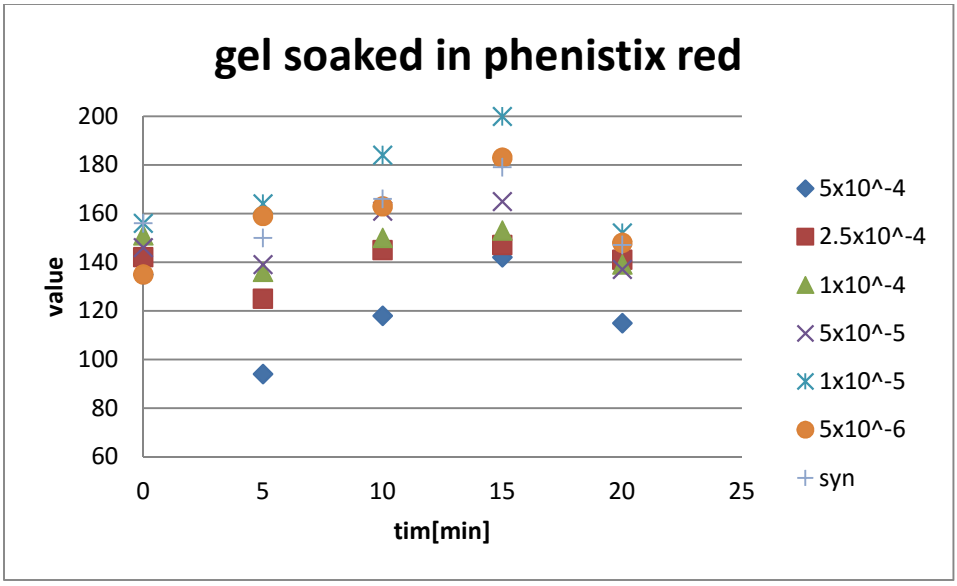


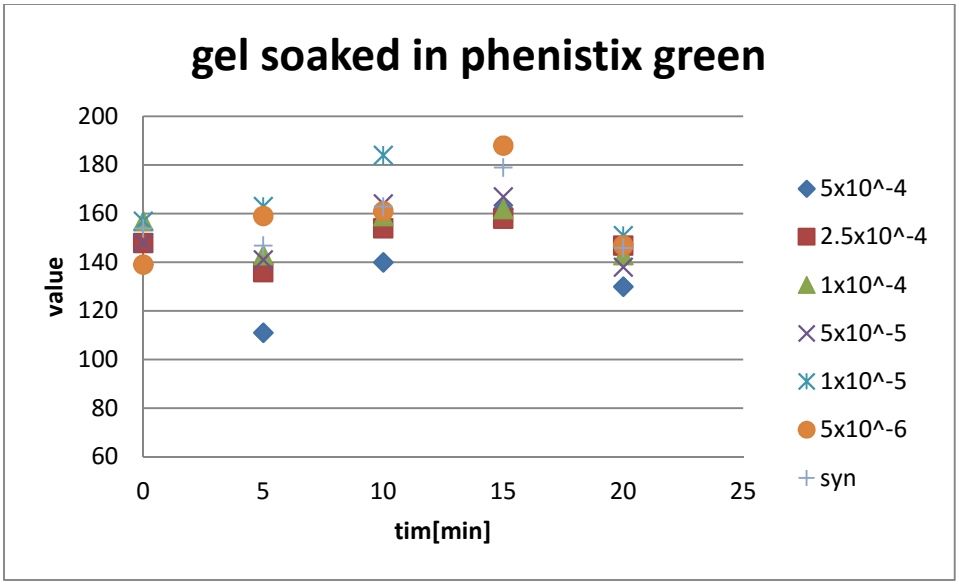


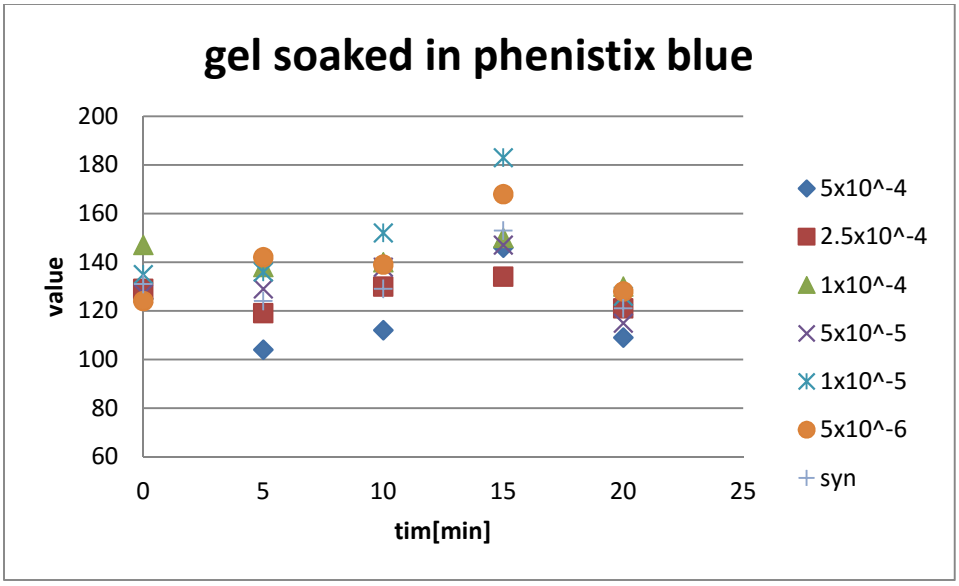


Hydrogel tests graphs

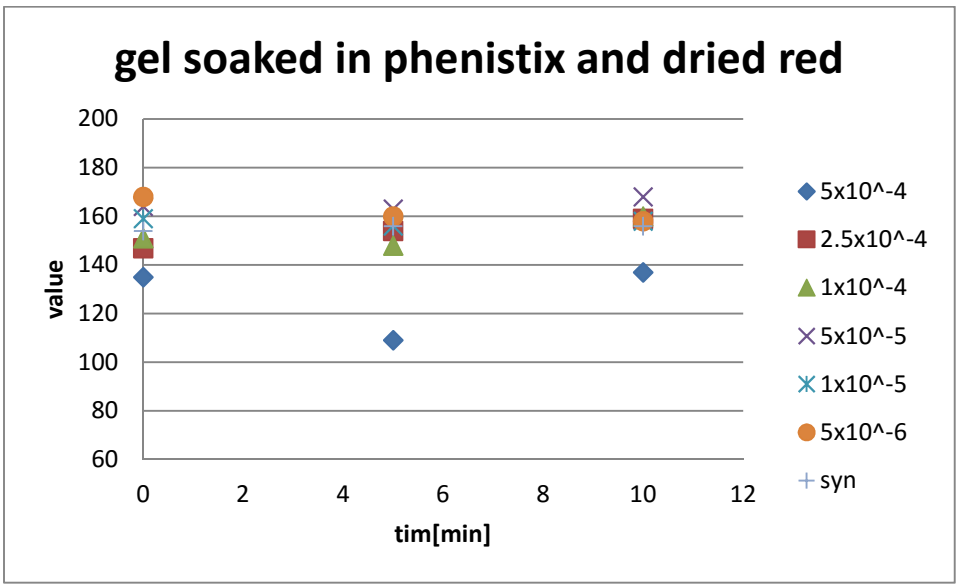
Fully hydrated

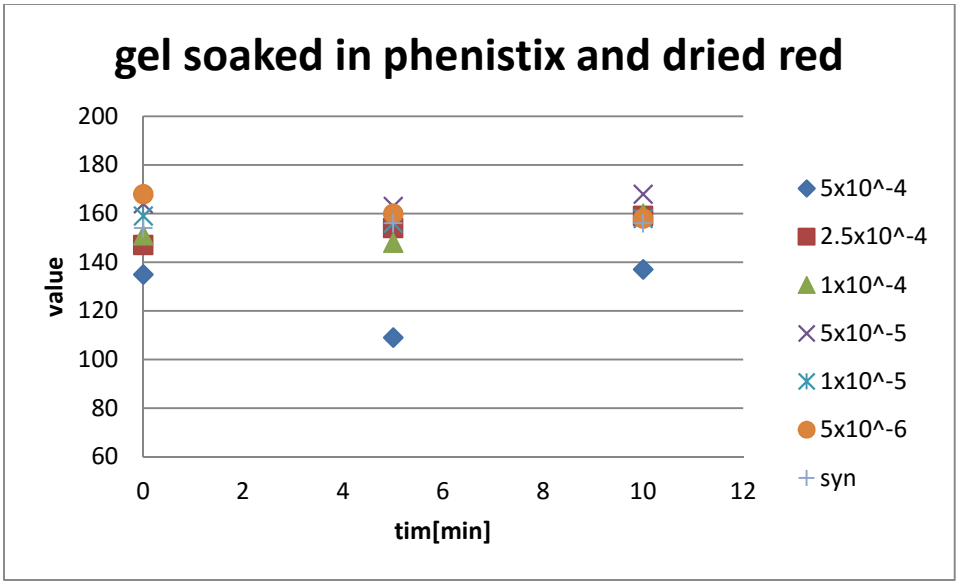

gel soaked in phenistix red
value
200
180
160
140
120
100
80
60
0
5
10
15
20
25
tim[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn

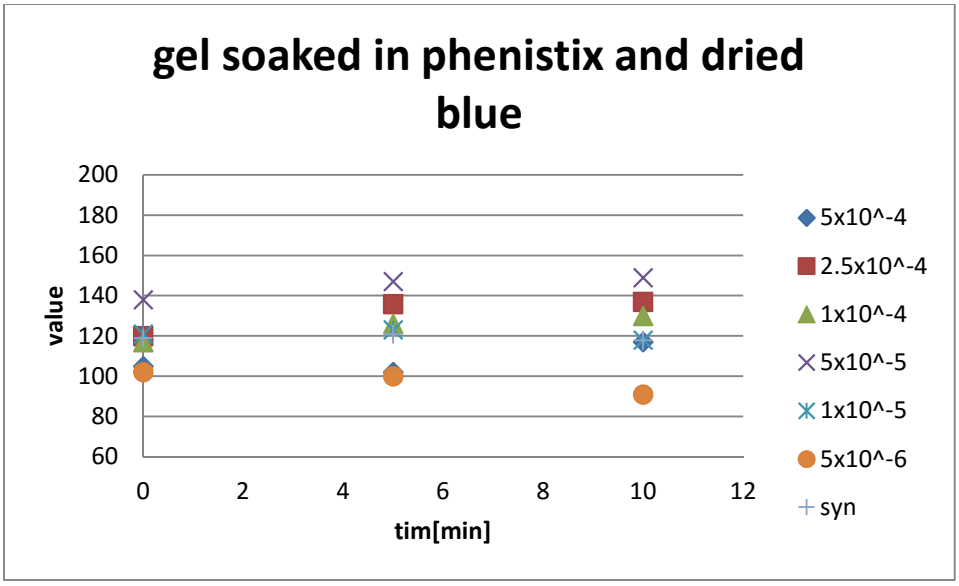

gel soaked in phenistix green
value
200
180
160
140
120
100
80
60
0
5
10
15
20
25
tim[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn



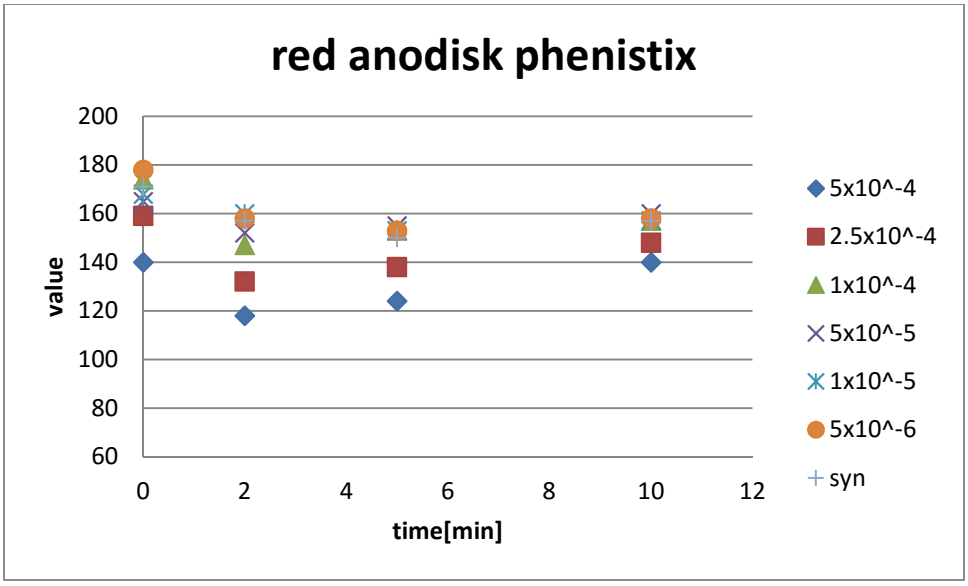
Dehydrated hydrogel tests

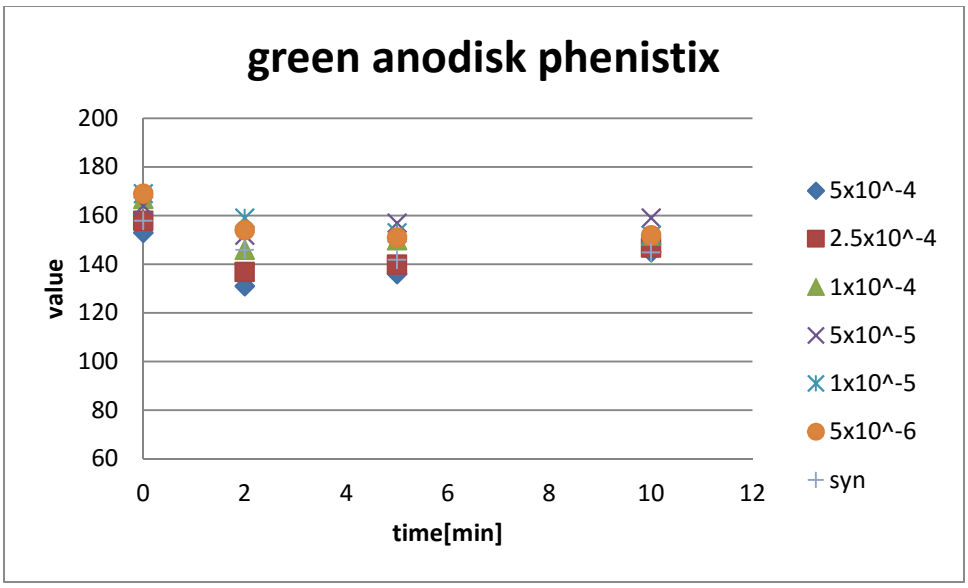


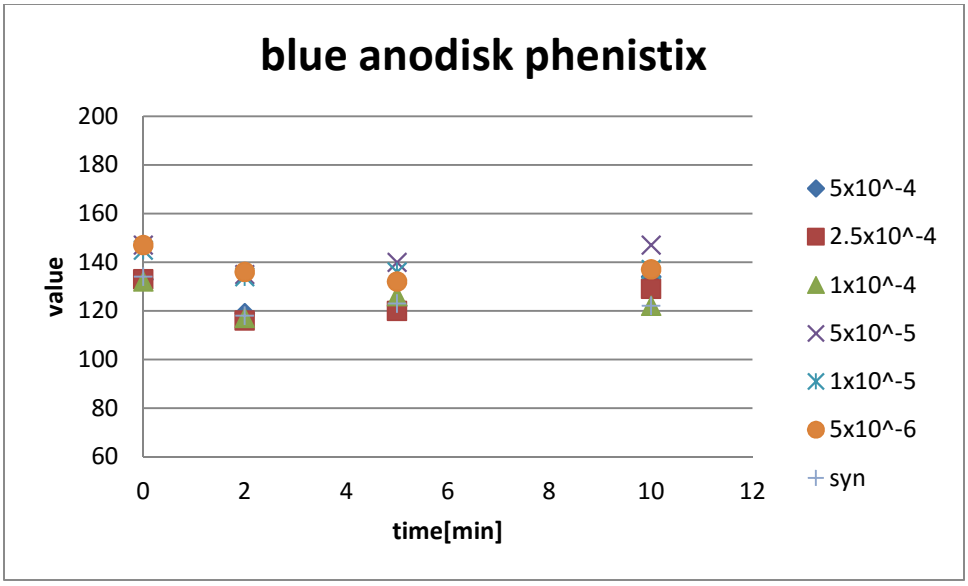




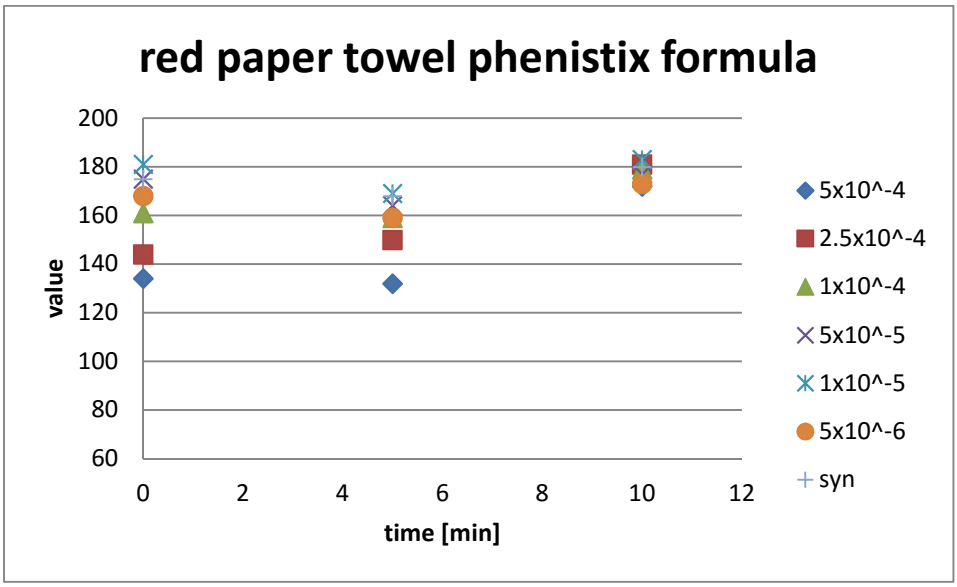
Anodisk tests

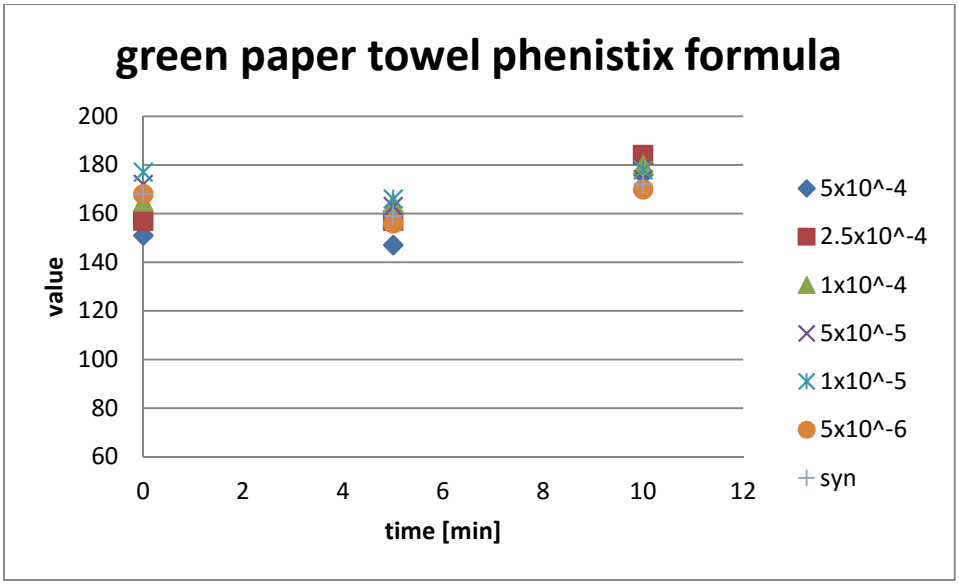

red anodisk phenistix
200
180
160
140
120
100
80
60
value
0
2
4
6
8
10
12
time[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn

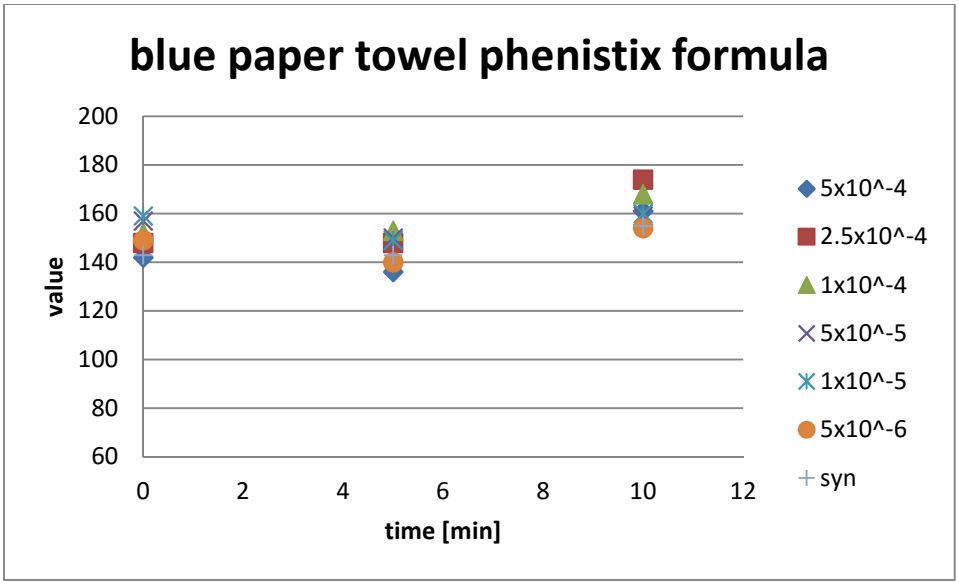

green anodisk phenistix
200
180
160
140
120
100
80
60
value
0
2
4
6
8
10
12
time[min]
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn



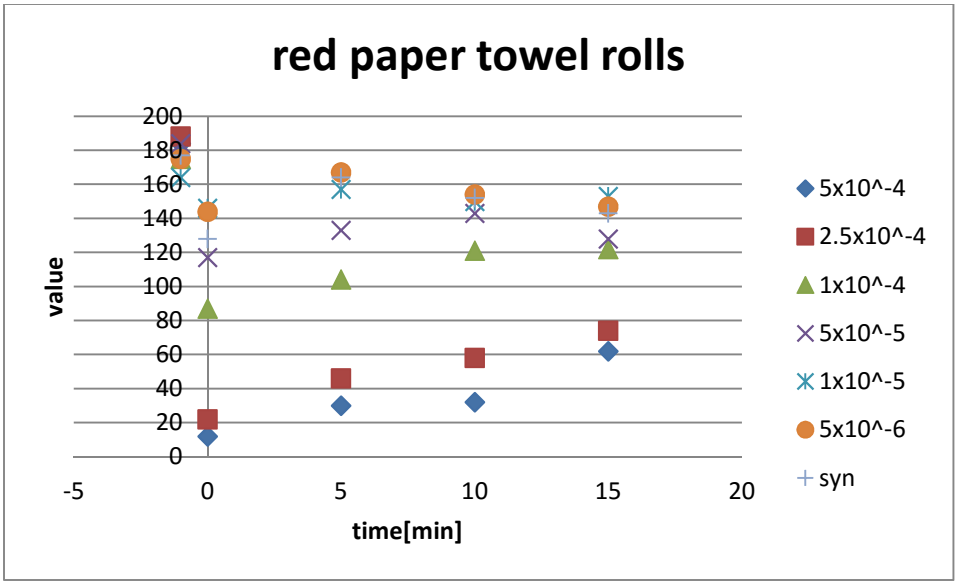
White paper towel unrolled tests

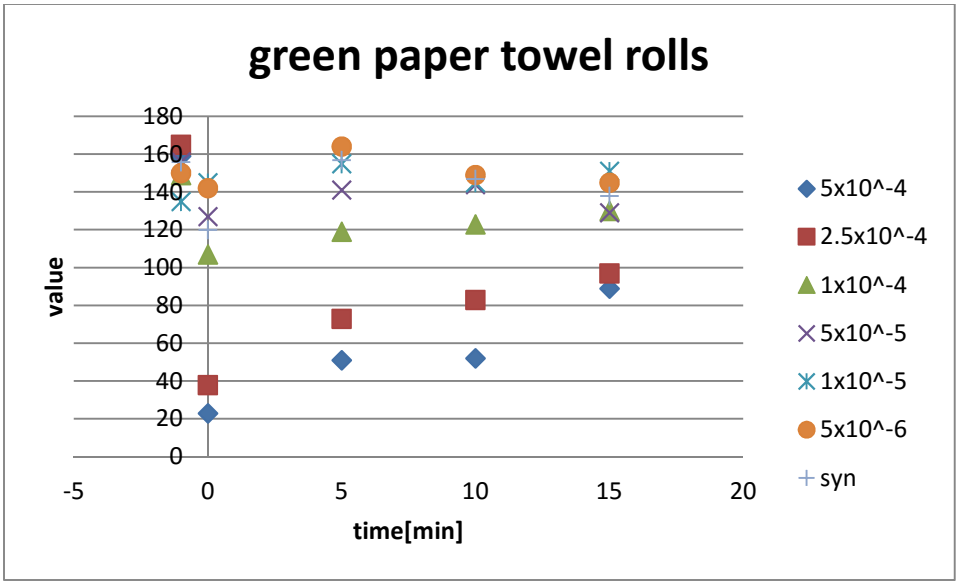


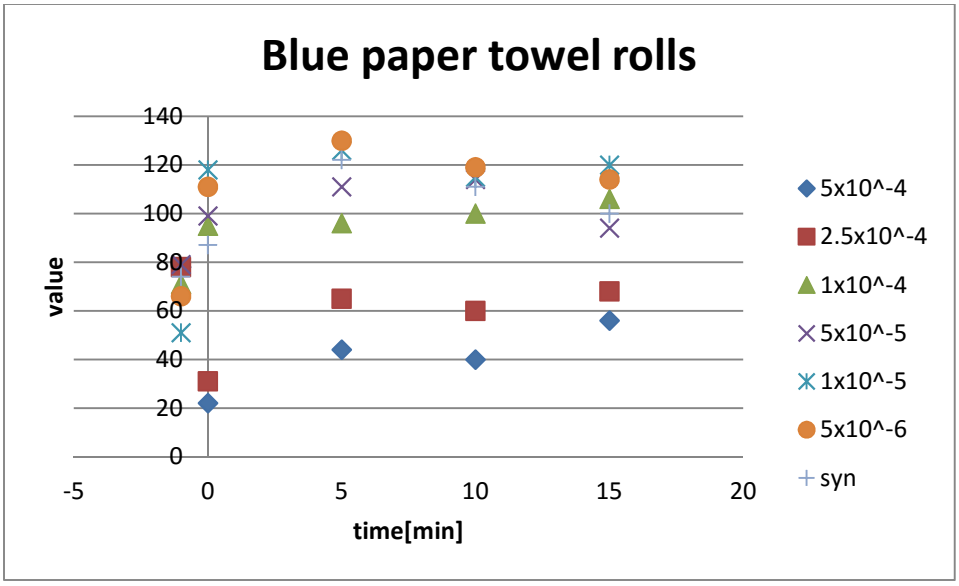




Paper towel rolls, no light box

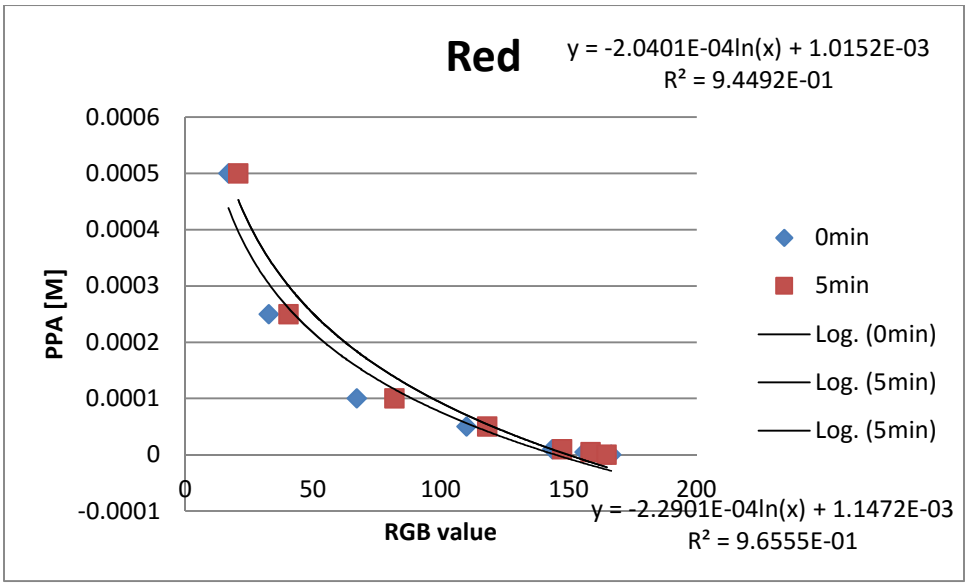

red paper towel rolls
value
time[min]
200
180
160
140
120
100
80
60
40
20
0
-5
0
5
10
15
20
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn

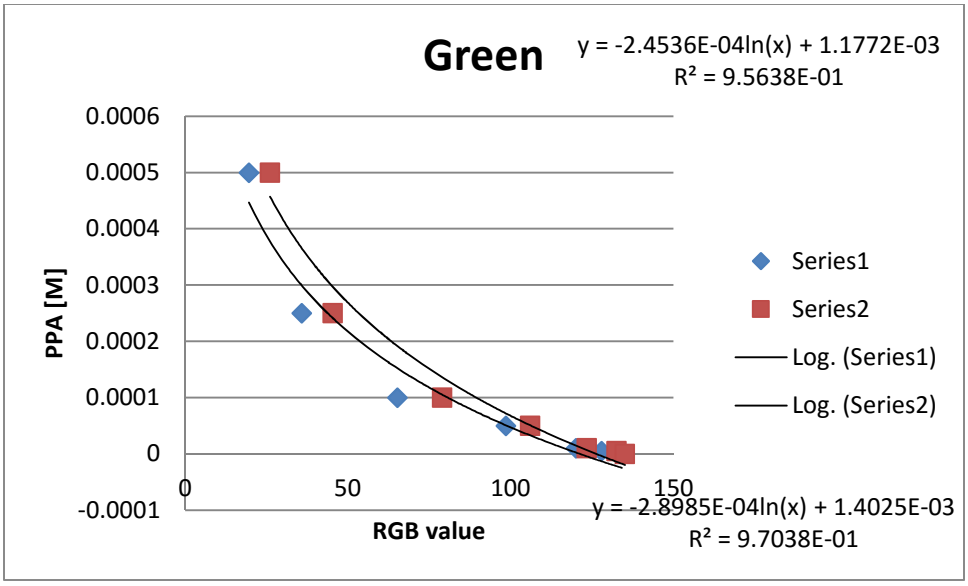

green paper towel rolls
value
time[min]
180
160
140
120
100
80
60
40
20
0
-5
0
5
10
15
20
5x10^-4
2.5x10^-4
1x10^-4
5x10^-5
1x10^-5
5x10^-6
syn

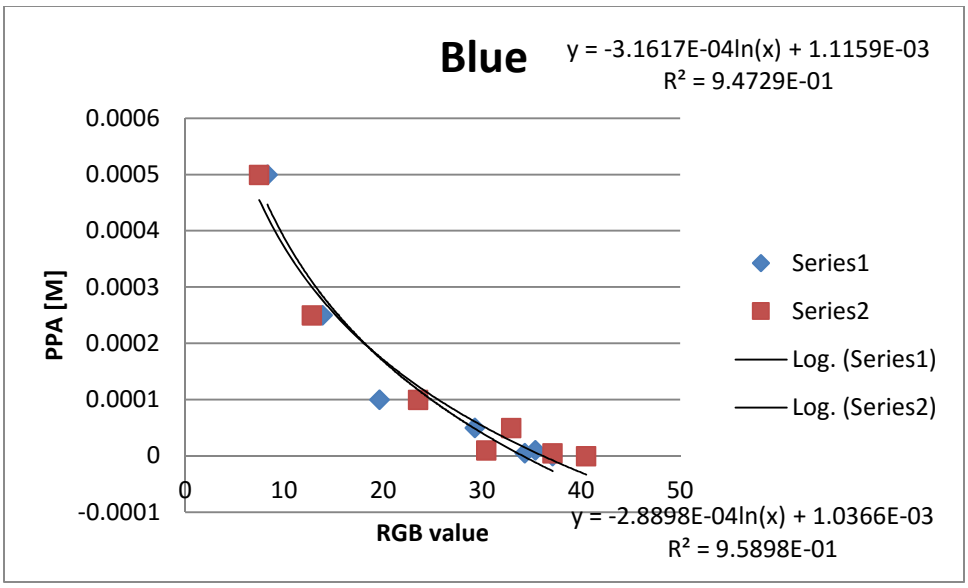


Normalized synthetic urine calibration graph (not used for blind tests). Data taken over several days.

Top right hand logarithmic line of best fit corresponds to 0 mins, lower right corresponds to 5mins.

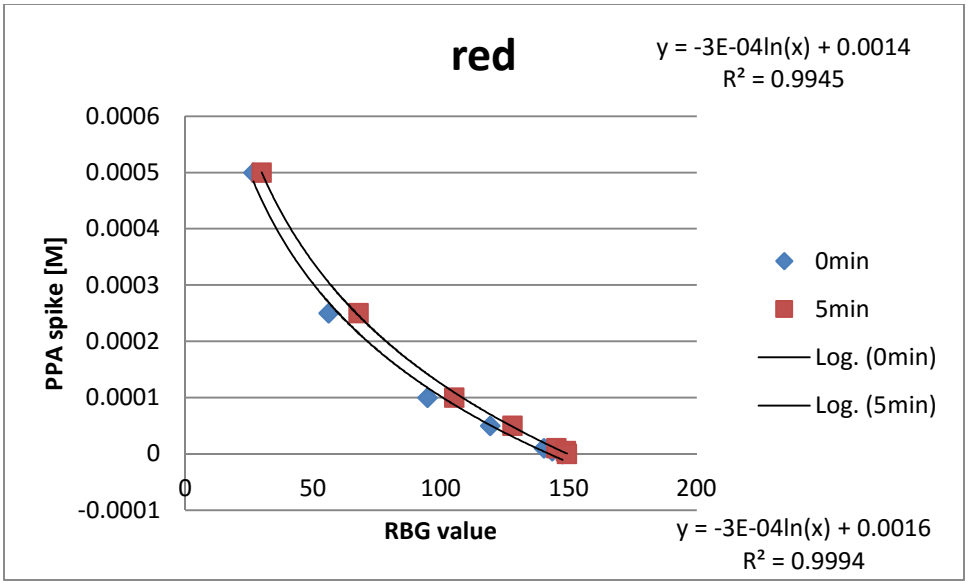


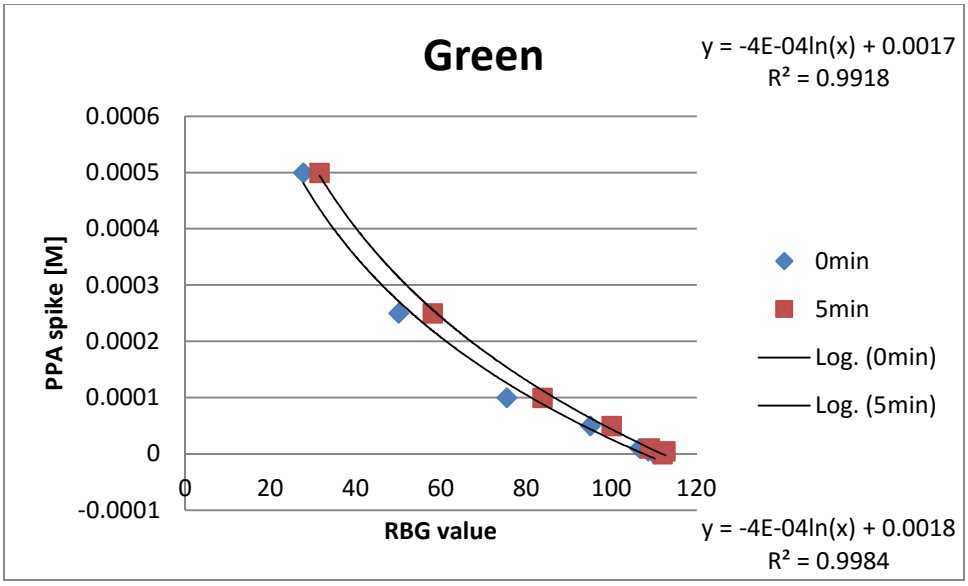


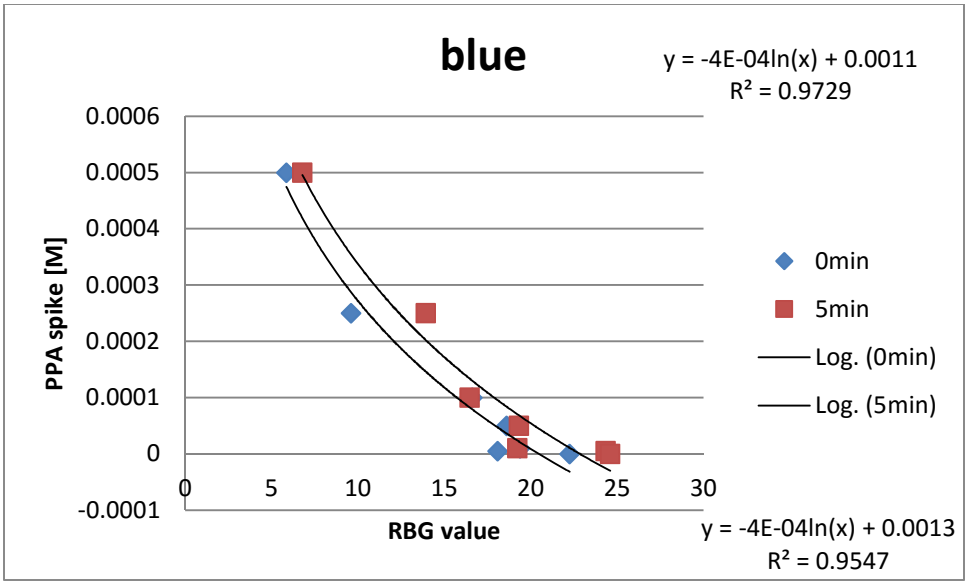


Normalized human urine calibration graph (not used for blind tests). Data taken over several days.

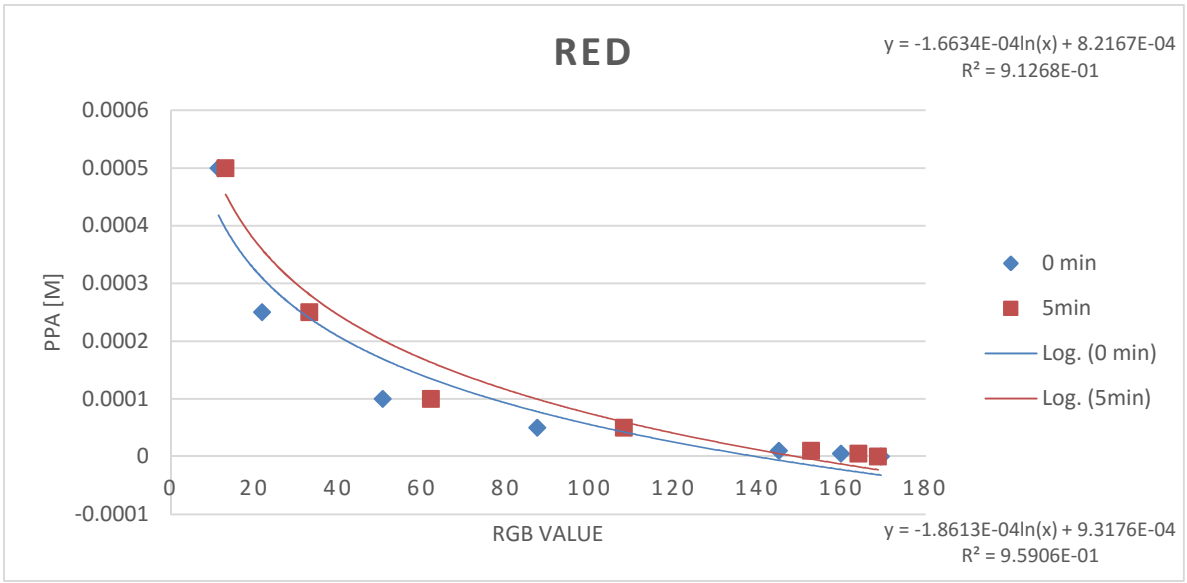
Top right hand logarithmic line of best fit corresponds to 0 mins, lower right corresponds to 5mins.

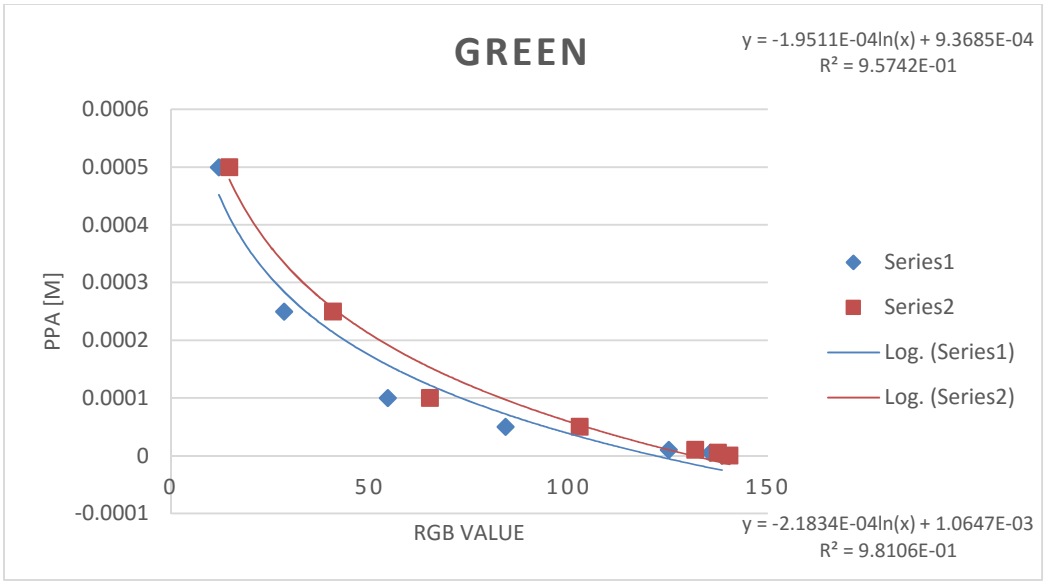

red
y = -3E-04ln(x) + 0.0014
R² = 0.9945
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
-0.0001
PPA spike [M]
0
50
100
150
200
RBG value
0min
5min
Log. (0min)
Log. (5min)
y = -3E-04ln(x) + 0.0016
R² = 0.9994

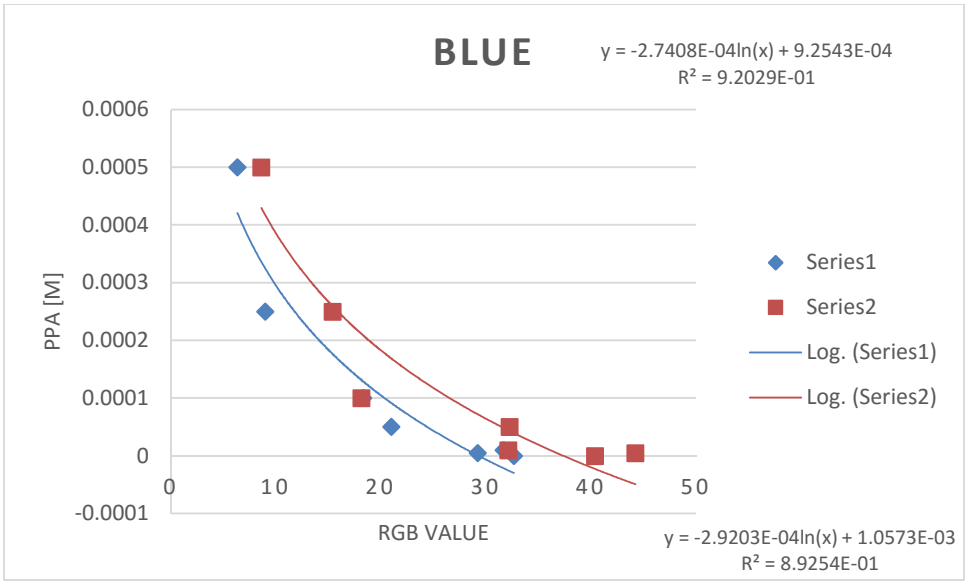

Green
y = -4E-04ln(x) + 0.0017
R² = 0.9918
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
-0.0001
PPA spike [M]
0
20
40
60
80
100
120
RBG value
0min
5min
Log. (0min)
Log. (5min)
y = -4E-04ln(x) + 0.0018
R² = 0.9984



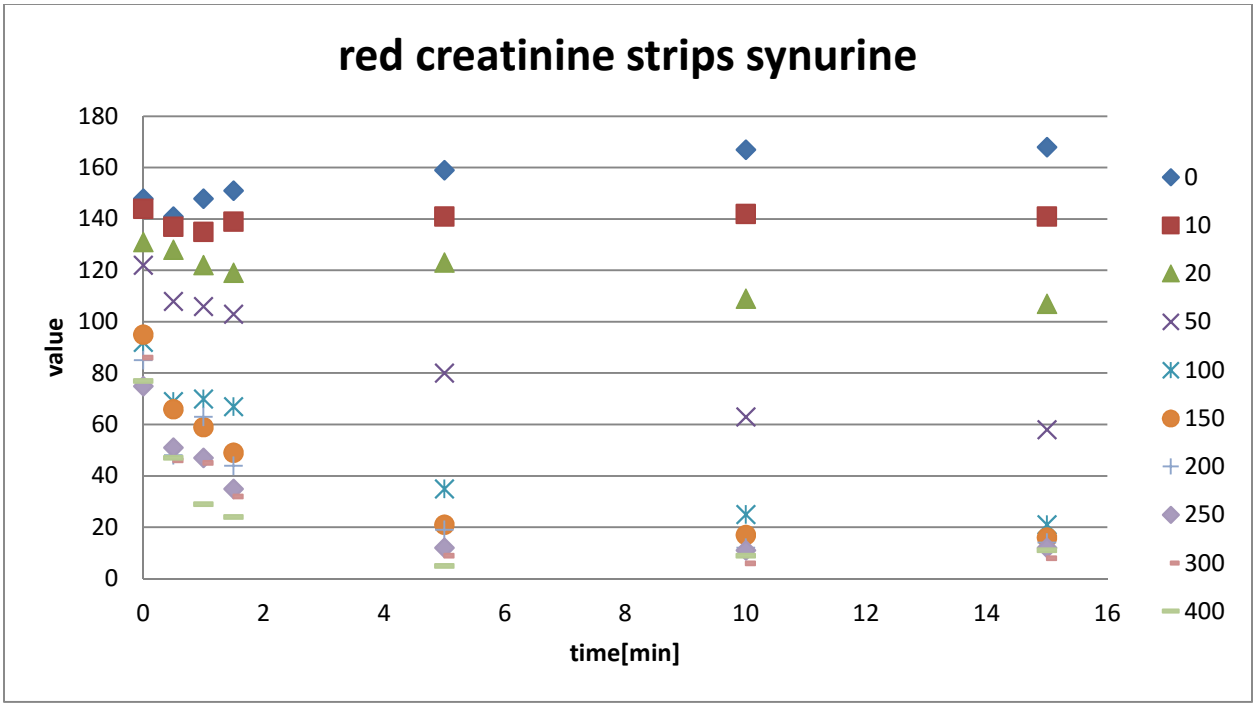
Blind test calibration graphs. Top right hand logarithmic line of best fit corresponds to 0 mins, lower right corresponds to 5mins.

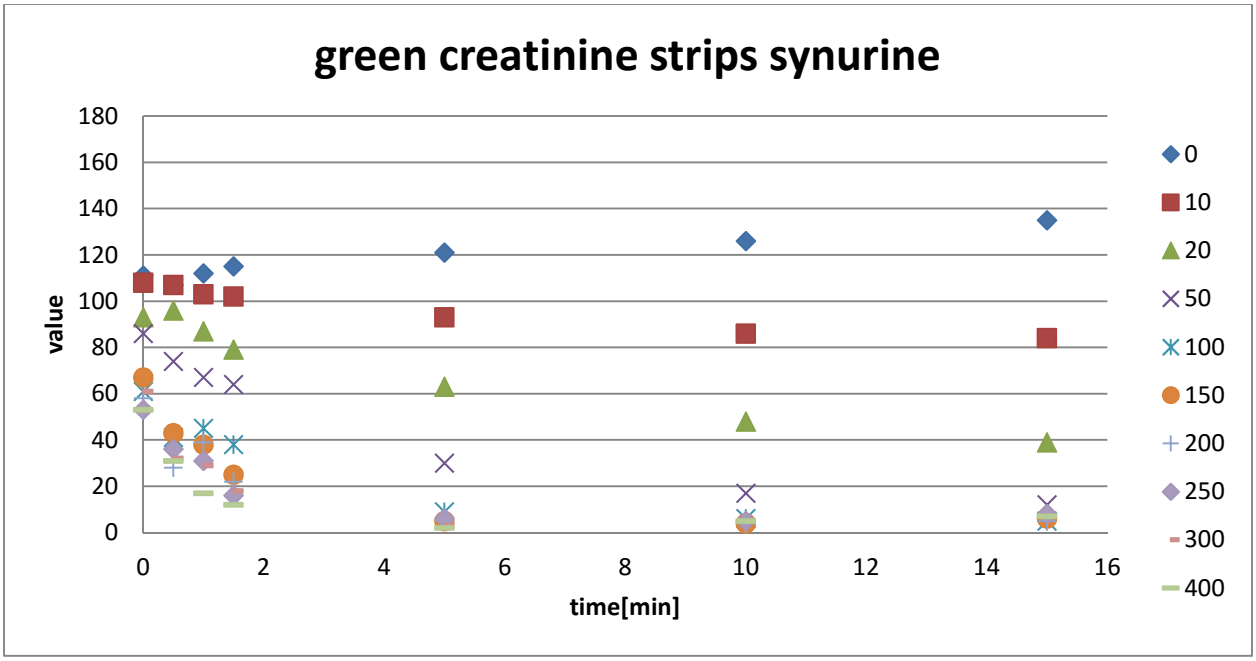


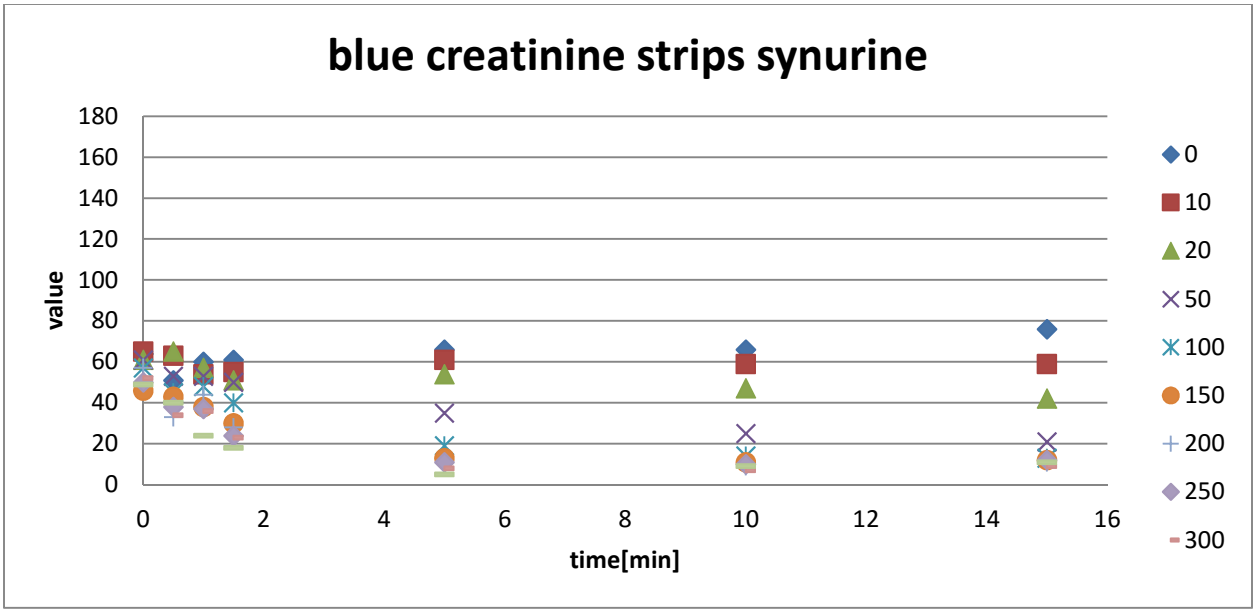


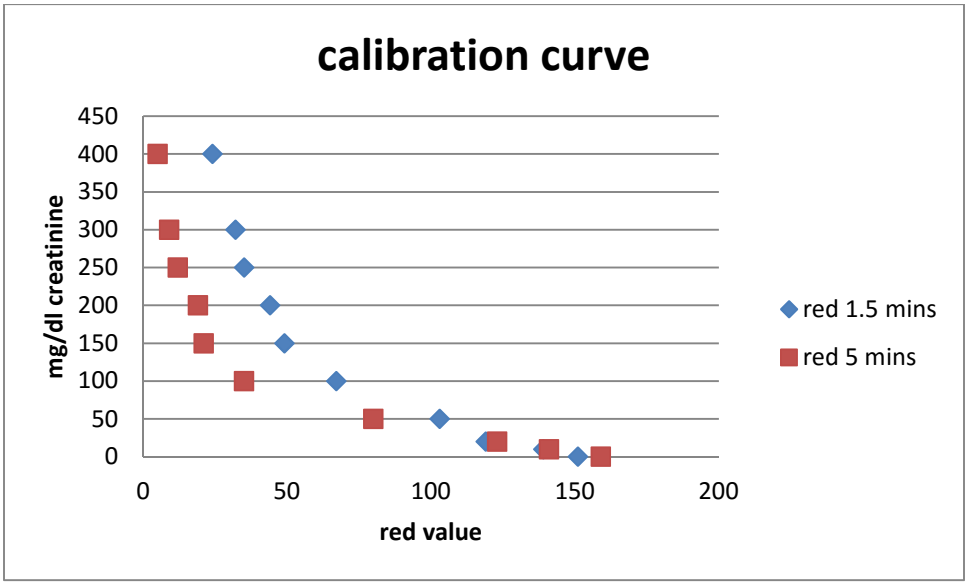


Creatinine test strips test, no light box, 1 year before deteriorated function noticed.


red creatinine strips synurine
value
time[min]
0
10
20
50
100
150
200
250
300
400


green creatinine strips synurine
value
time[min]
0
10
20
50
100
150
200
250
300
400


blue creatinine strips synurine
value
time[min]
0
10
20
50
100
150
200
250
300


calibration curve
mg/dl creatinine
red value
red 1.5 mins
red 5 mins