Novelty Journals

ISSN 2394-7349

International Journal of Novel Research in Engineering and Science
Vol. 6, Issue 2, pp: (44-54), Month: September 2019 - February 2020, Available at: www.noveltyjournals.com

Inexpensive Metal Oxide Gas Sensors for Salivary Urea Quantitation

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Abstract: **A low-cost metal oxide gas sensor was used to detect ammonia and urea in saliva by constructing a test strip that contains an enzyme solution and a porous hydrophobic membrane. Test results with saliva and saliva spiked with ammonia and/or urea showed that a power law calibration along with a nano Arduino microprocessor was sufficient to distinguish and quantify these two biomarkers. The sensitivity of the metal oxide gas sensor and the simplicity of operation was well suited to construct a prototype that can provide portable and accurate information on salivary urea nitrogen (SUN) and initial dissolved ammonia in saliva. The simplified power law calibration is limited when very high (>200 ppm) dissolved ammonia is present in the saliva sample when the sample has a normal SUN concentration. However, this deviation of accuracy due to the simple algorithm deployed can be used to alert the test user that the dissolved ammonia levels in the saliva sample are well beyond normal. For SUN values equal to or greater than 40 mg/dl, high initial ammonia and high urea concentration are readily distinguished by the algorithm and sensor system.**

Keywords: **Ammonia, Dehydration, Diagnostic, Diffusion, Gas Permeable Membrane, Metal Oxide Gas Sensors, Point-of-care, Point-of-use, Saliva, Urea, Urease.**

I. INTRODUCTION

Saliva is finding increasing use as a biological fluid source for rapid, point-of-care testing [1]. The primary advantages to using saliva as a means of diagnosing conditions, such as: the relative low invasiveness of sample acquisition; the ability to access a medium with a range of biomarkers that include proteins, electrolytes, hormones, metabolites, antibodies, and DNA/RNA; and the reduced risk of infectious disease exposure to the health care provider or laboratory technologist. However, there are no established specific set of criteria for collecting a saliva sample [2]. The range of FDA approved clinical laboratory tests for saliva is considered to be relatively limited due in part to the lack of standard means for testing, relatively low concentration of biomarkers relative to blood, and the limited understanding of how biomarkers transport form capillaries to salivary glands, especially due to circadian rhythms.

One saliva biomarker that has generated interest in rapid, point-of-care testing due to its relevance for health screening is urea [3]. A colorimetric saliva urea detection test strip is marketed in some countries [4]. Ongoing research is reported in the scientific literature on developing similar test strips that are easier to use and have better capabilities for quantification due to detection of a wider variation in shades of color. Since the enzyme urease is well suited to detect salivary urea, these test strips are specific for urea and can screen for high levels of urea in saliva. Blood urea nitrogen (BUN) is a diagnostic measurement often used to detect kidney disease and track dehydration. Salivary urea nitrogen (SUN) concentration has been shown to track well with BUN [5], indicating that urea concentration in saliva can be relevant for kidney disfunction screening and a potentially less invasive means of determining an individual's degree of hydration.

Using urea as a biomarker for a specific diagnosis is challenging. Elevated urea concentrations can be due to a variety of reasons such as chronic kidney disease, dehydration, or liver dysfunction. More patient information or measurements of other biomarkers would be required to distinguish between these and potentially other underlying conditions that lead to elevated urea concentration in the blood or saliva. Also, poor oral and/or gastrological health may generate high levels of ammonia in saliva that would interfere with an accurate assessment of urea since the color change in these test strips when employing urease for detection is generally due to increased pH as urea is converted into ammonia.

In this paper, we report on a new strategy to quantify urea in saliva that can help distinguish between various causes for deviations from normal concentrations. A rapid, point-of-care method is described here where urea and other gases from saliva are quantified using inexpensive metal oxide gas sensors. The test strip component uses Korteweg stresses and viscous fingering in order to generate distinct sensor signals for urea vs. gases dissolved in saliva. A previous publication explains the use of this fluid phenomena in detail [6]. A mathematical analysis of the gas sensor response based on diffusion and Michaelis-Menten reaction kinetics as well as a practical approach to developing readouts for SUN and ppm of dissolved ammonia are provided in this paper to demonstrate how the metal oxide gas sensor [7] signal is used as a saliva analytical device. By deploying this new strategy, it is anticipated that there is potential for other biomarkers present in saliva to be detected and quantified as well.

II. MATERIALS AND METHODS

Saliva Urea Assay

The urea concentration of the saliva samples was determined using the Quantichrom Urea Assay Kit DIUR-100 (BioAssay Systems). This spectrophotometric assay can measure urea concentration up to 100 mg/dl in biological fluids without pretreatment. It uses a chromogenic reagent that forms a color complex with urea. Since this assay does not use urease and does not measure urease reaction products, the reading is unaffected by ammonia. The lack of interference from ammonia was verified by our saliva samples containing spiked amounts of concentrated ammonia.

The assay procedure was slightly modified from the recommended 96 well microplate procedure in order to ensure accurate and reproducible measurements with saliva. As directed in the kit assay procedure, 5 microliters of: (1) sample, (2) standard (50 mg/dl Urea supplied in the kit), and (3) blank (water) were placed in 3 separate low volume disposable cuvettes, instead of microplate wells. After equal volumes of Reagent A and B were mixed together, 200 microliters of the reagent mixture was then added to each low volume cuvette and allowed to incubate for 20 minutes at room temperature. In order to read the spectra more accurately, we used a longer integration time (1 second) and diluted each cuvette with 205 microliters of water directly prior to measurement. Absorbance from 400 nm through 800 nm was recorded rather than only using the reading at 520 nm as indicated in the assay kit. Measurements were conducted using a fiber optic spectrometer (Ocean Optics) with a water reference, at an integration time of 1 second and averaging of 5 repeat scans. This modified procedure was tested using urea standard solutions (dilutions of a PBS pH=7.4, 100 mg/ml Urea stock solution), and the accuracy was within the range shown in the assay protocol instruction sheet. Also, spiked saliva samples diluted to within the stated range of assay accuracy were measured as within the same level of accuracy as the standard urea solution.

Saliva Ammonia Assay

Hach Ammonia (Nitrogen) test strips were used to determine the concentration of dissolved ammonia in saliva. The protocol for determining ammonia in water samples was followed, as directed by the manufacturer. Briefly, saliva was collected into the analysis tube in order to have the liquid level reach the prescribed mark. The test strip was placed in the sample tube and mixed with the saliva sample for 30 seconds before removing the test strip, shaking excess liquid from the test strip, and comparing the color of the test pad to the chart provided on the test kit container. In order to verify that the ammonia assay can be accurately used in saliva, a number of tests were run. First, centrifuged and whole saliva samples were analyzed and the test kit readings were found to be the same for the centrifuged as well as the original saliva sample. Also, spiked saliva samples containing known ammonia concentration in excess to the original saliva sample gave expected readings. The Hach assay is accurate from 0 – 6 ppm of ammonia, and sample dilutions were needed in order to read higher concentrations of ammonia. Since the assay can be used in a variety of water samples, including water that can contain dissolved materials and particulates, it seems reasonable that saliva, which is essentially a clear

aqueous solution with proteins and other biochemicals, should not pose difficulties in measurement with this assay. An important feature of the Hach test strip that enables water measurements for pure water, aquarium water, and saliva samples is that the reading of the reagent color is developed due to membrane transport of the ammonia, thereby minimizing the effects of dissolved proteins and solids.

Test Strip Production

The test strip housing (Figure 1) was designed using a low-resolution, free 3-D drawing program and printed from ColorFast XT clear material. In order to keep the cost of the disposable test strip as low as possible, the materials and reagents used were adapted from commercially available materials. The test strips use standard gauze pads cut to 10 mm by 10 mm squares and a 0.22 micrometer pore diameter PTFE membrane to provide a gas permeable, but water impermeable barrier. This arrangement was demonstrated to effectively hold the enzyme reagent, collect the saliva sample, and provide a dry interface with the gas sensor. The enzyme solution was prepared from reagent grade, jack bean urease (Carolina Biologicals) using a 50% solution of water and glycerol.

Fig. 1: Top view of enzyme-loaded test strip

Saliva Sample Delivery System

The saliva sample delivery system (Figure 2) consists of a saliva funnel and connector, which were designed and printed using the same 3-D drawing program and ColorFast XT clear material used for the test strip housing. The narrow end of the funnel is sealed using an inexpensive, 9mm aluminum foil sealing sticker to allow for saliva collection directly into the funnel. The funnel is also externally marked for approximately 800 microliters from the sealed end to provide a guide for liquid saliva volume required for testing. The clear material used for funnel printing allows for visual approximation of the saliva volume inside the funnel. This delivery system helps transport saliva from the subject to the test strip using a simple saliva collection protocol with a disposable funnel, followed by movement to the test strip through the connector. The 3-D printed connector facilitates saliva movement to the test strip because placing the funnel on the connector pierces the seal of the funnel, allowing the saliva to flow through the connector and into the test strip.

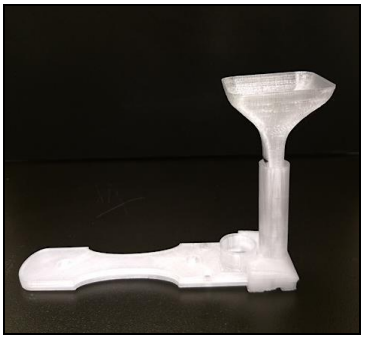

Fig. 2: Side view of the 3-D printed saliva funnel, collection device, and test strip components

Sensor System

A low-cost, SainSmart ammonia gas sensor (MQ-137) is used to measure the gases emitted by the saliva sample and gas produced from the enzymatic catalysis of urea due to urease. During saliva testing, this sensor maintains a secure connection to the test strip, allowing for gas to flow through the highly gas permeable membrane and build up in the sensor/test strip housing headspace. This sensor generates an analog voltage signal dependent on the concentration of gas present in the air space between the metal oxide film and the wire mesh protective cone. Through extensive calibration with known concentration of ammonia in water on our test strip, we have calibrated the voltage measured in the air space at equilibrium with the liquid. This calibration curve is then used to correlate salivary urea and dissolved ammonia using a timed data collection protocol. The protocol is deployed via a low-cost, Nano Arduino device and the voltage data is transmitted in real-time, analyzed and displayed on an OLED display and simultaneously via Low Energy Bluetooth (LEB) through a DSD Tech board using HM-10 LEB and a compatible smartphone app. Additionally, a SMAKN button is attached to the board, allowing for easy data collection reset. To supply power to the device, a rechargeable battery pack is attached, which can be recharged using a standard micro-USB cable. The battery pack is connected to the on/off button on the front of the device as well. The sensor system (Figure 3) is housed in a 3-D printed case, using the same 3-D printing drawing program and clear ColorFast XT material for the test strip, funnel, and connector, as described previously

Fig. 3: Top view of the sensor system in 3-D printed housing

Saliva Collection, Preparation and Testing

Each researcher provided a saliva sample and tested their own saliva either as collected or spiked with concentrated urea and/or concentrated ammonia solutions. After testing, all materials in contact with saliva were sanitized and disposed of using appropriate protocols. Highly concentrated urea solutions prepared by dissolving urea in DI water to a concentration of 100 mg/dl were used to spike the saliva samples. Also, high concentration of ammonium hydroxide solution at 10,000 ppm was prepared by dissolving ammonium hydroxide in DI water. The highly concentrated solutions required very low volumes compared to the saliva volume collected in order to adjust ammonia and/or urea levels to higher concentrations that are within the range to detect mild, moderate, and high clinically relevant levels.

To test saliva using the test strip/sensor system, steps had to be followed to prepare the saliva, sensor device, and plastic components. The sample delivery system had to first be assembled so that the test strip is uncapped and securely attached to the plastic connector. The subjects were then instructed to rinse their mouths with water and wait a few minutes before

commencing saliva collection. Saliva was then collected directly into the sealed funnel until the liquid saliva reached the black mark on the outside of the funnel (approximately 800 microliters). Once sufficient saliva had been collected, the filled funnel was gently set into the open tube of the connector without applying downward pressure to the funnel, ensuring that the funnel seal remained intact. The device was then turned on by flipping the switch on the front of the device and allowed to equilibrate by recording data until the displayed voltage no longer decreased. The button on the front of the device was then pressed to zero the data.

Once these preparatory steps had been followed, saliva data collection could begin. First, the funnel was firmly pressed down inside the connector so that the seal was broken, and saliva could flow into the connector. As soon as the seal is broken, the saliva level in the funnel starts to visibly decrease and the device button is depressed for 1-2 seconds, or until the time shown on the device display returned to zero. Immediately afterwards, the device was placed over the test strip so that the sensor was completely inside the test strip's open cylinder. The system was then allowed to record data for 10 minutes. The device sensor maintained a secure and reasonably gas-tight connection with the test strip cylinder during the duration of the data collection, which is essential for quantitative measurements of gas permeation across the PTFE membrane.

Upon completion of the saliva test, the device stops collecting data and displays the saliva ammonia amount, urea amount, and dehydration level (normal, mild dehydration, moderate dehydration, severe dehydration).

III. RESULTS AND DISCUSSION

Considering the volume of saliva that could be provided easily by a patient and the amount needed to interface with an inexpensive metal oxide gas sensor, we designed the 3-D printed test strip/sensor system to handle 800 microliters of saliva or more. The amount of time needed to collect this volume of saliva varies with the individual, but generally takes no more than 5 minutes since the funnel provides a clear visual indication when that amount is reached or exceeded. The shape of the funnel is also useful to allow the foam present when saliva is collected to collect at the top, while liquid collects at the bottom.

Figure 4 provides a sketch and the dimensions of the test strip, the metal oxide gas sensor mesh interface, and the sensing surface in order to illustrate the physical basis for the mathematical models used to interpret the time dependent signals for ammonia solutions, urea solutions, and the combined ammonia and urea solutions.

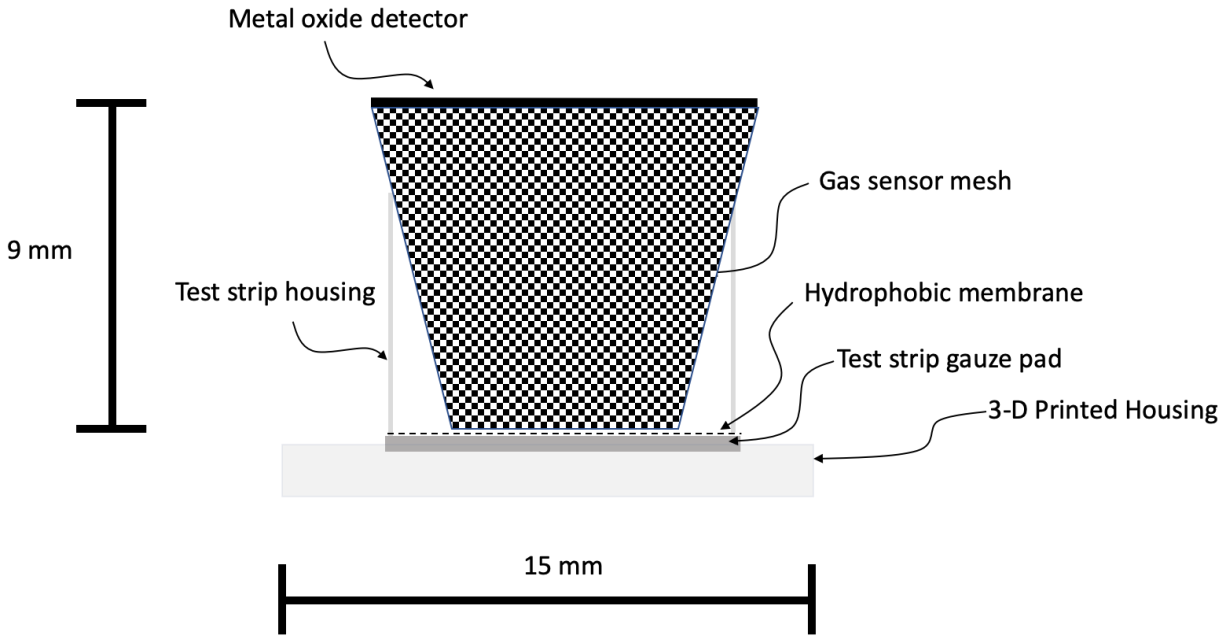

Fig. 4: Annotated side view of the test strip attached to the gas sensor

3.1 Ammonia

Ammonia in saliva can be measured in the gas phase due to unstimulated saliva having a very limited phosphate concentration (which can be as low as 2 mM) and low carbonate concentration, which yields a limited buffering action. As a result, colorimetric test strips that test for salivary urea can use pH increase as a measure of urea converted into ammonia. Ammonia in water at or above its pKa will then be in molecular form and can enter the gas phase above a liquid solution. At room temperature, the ratio of aqueous phase concentration of ammonia to its partial pressure in air is given by the Henry's law constant of 62 M/atm. It is convenient to realize that due to the molecular weight of ammonia, using this Henry's law constant results in the partial pressure of the gas in ppm being very close to the concentration in water, when using the units of ppm. It is then useful that gas metal oxide sensors specific to ammonia can have a range of detection from 5 – 500 ppm ammonia, and the production of ammonia by urease for typical values of salivary urea nitrogen can generate aqueous ammonia at concentrations well within this range.

In order to interpret the gas sensor reading, we consider that the sensor voltage reading over time can be simulated as one-dimensional diffusion through a stagnant gas film.

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \text{Equation 1}$$

There are several analytical solutions to this equation for simplified boundary conditions. One possible solution is to consider a semi-infinite media and assume that the boundary conditions are that at x = 0, C = C_0 and at x = ∞, C = 0 and initial condition that at t = 0 with x > 0, C = 0 the solution to this equation is:

$$C(x,t) = \frac{M}{\sqrt{4\pi Dt}}\exp\left(-\frac{x^2}{4Dt}\right) \qquad \text{Equation 2}$$

where M is the total quantity of analyte [ref]. Another analytical solution to Equation 1 uses the boundary conditions in the form C = C_0 at x = 0, t > 0 and the initial condition C = 0 at x > 0 and t = 0 leading to the solution obtained by Laplace Transforms as:

$$C(x,t) = C_0\,\mathrm{erfc}\left(-\frac{x}{2\sqrt{Dt}}\right) \qquad \text{Equation 3}$$

The advantage of using this simplified mathematical description is that it captures the observed sensor signal variation very well over the ten-minute period that data is collected (Figure 5). We rationalize that diffusion of the gas from the membrane to the surface of the sensor is the rate limiting step for the sensor response given that the manufacturer information for the sensor response is < 6 seconds, which is a significantly faster speed than the response curves shown in Figure 5. But, as can be seen in this figure, for the time zone before the maximum signal is achieved, Equation 2 yields a better fit of the data. However, since there is likely a small, but non-negligible, loss of gas at the sensor surface, presumably due to reaction on the surface and a slow leakage from the interface between the test strip and the sensor as well as a smaller amount of loss of gas from the sides of the test strip, Equation 3 provides a better fit for longer times.

Overall, it can be concluded that both solutions suggest a simple calibration. Equation 2 suggests that the maximum signal is proportional to the surface concentration. Assuming that the gas metal oxide sensor detection surface is at a distance L, the time at the maximum concentration for Equation 3 is:

$$t_{max} = \frac{L^2}{2D} \qquad \text{Equation 4}$$

and the concentration maximum will then be proportional to the total initial amount of ammonia introduced to the test strip, as follows,

$$C_{max} = 0.242\,\frac{M}{L} \qquad \text{Equation 5}$$

This mathematical model suggests that the device can be calibrated based on the initial concentration of ammonia introduced, as long as the volume applied stays constant, and that both models point to the same type of calibration.

For urea however, since it is being measured by its reaction product, a mathematical model of the gas sensor signal would need to incorporate additional mechanisms that account for reaction kinetics and diffusion to the liquid/membrane interface.

3.2 Urea

In order to measure the urea concentration, we consider its reaction in water catalyzed by the enzyme urease where 2 moles of ammonia and one mole of carbon dioxide gas are produced for each mole of urea.

$CH_4N_2O + H_2O \rightarrow 2NH_3 + CO_2$

While carbon dioxide may contribute to the metal oxide gas sensor voltage change, the sensors employed in this study are much more sensitive to ammonia than to CO_2, giving a relatively weak response when calibrated with water containing dissolved carbon dioxide.

In order to determine how best to calibrate the gas sensor for urea, we consider diffusion and reaction within the porous pad containing the test solution

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} + k_{eff}C \qquad \text{Equation 6}$$

Where the reaction rate constant k_{eff} represents a Michaelis-Menten model by a fixed concentration of urease enzyme, and it depends upon the initial and time dependent urea concentration as given by the implicit solution to the Michaelis-Menten equation.

$$t = \frac{U_o - U}{k_0 E} + \frac{K_m}{k_0 E} ln\left(\frac{U_0}{U}\right) \qquad \text{Equation 7}$$

The solution to Equation 4 needs to be done numerical in order to determine the time dependent boundary condition for the gas phase diffusion governed by Equations 2 or 3, which also must be solved numerically.

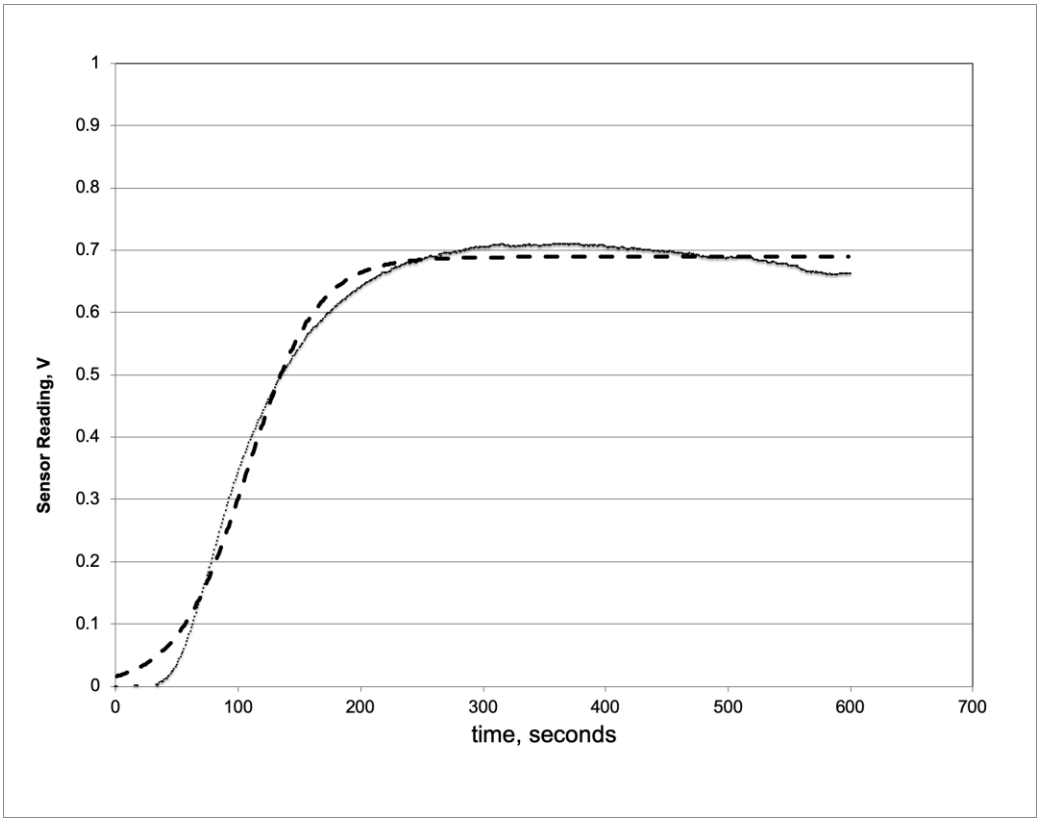

Fig. 5: Curve fitting for urea detection as a function of time

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ISSN 2394-7349

International Journal of Novel Research in Engineering and Science
Vol. 6, Issue 2, pp: (44-54), Month: September 2019 - February 2020, Available at: www.noveltyjournals.com

Based on the equations described above, it can be anticipated that the numerical solutions for the ammonia gas concentration as a function of time will have two distinct features. First, there will be a characteristic time lag before any ammonia generated by the gas can reach the sensor since it must be generated from the urease reaction which has well defined kinetics, namely on the order of micromoles of ammonia per minute. Secondly, due to diffusion within the liquid, there will be a spreading of the gas that is present at the liquid/gas membrane surface creating a broadening of the response curve. Figure 5 shows a representative kinetic curve of ammonia detection using the gas sensor for a urea solution, showing a lag time and a logistic curve shape along with a curve fit based on the equations provided above.

However, after extensive testing of our low-cost test strip/sensor system, we determined that it would be more accurate and useful to base the detection on specific endpoints rather than attempt to curve fit or obtain rate data. This became apparent due to the desire to simplify the Arduino/OLED/LEB system and lower the cost of the test strip "reader" and since the strategy is very robust in terms of reproducing data from repeat analysis of saliva samples.

Based on the analysis of kinetics and the way the gas sensor signal evolves, the simplified equation to analyze the gas sensor data is based on a power law function:

$$P_s = \left[\frac{V_c}{a(b-V_c)}\right]^{1/c} \qquad \text{Equation 8}$$

where Ps is the predicted value of salivary urea nitrogen (SUN), Vc is the corrected voltage reading after subtracting the voltage reading at an early time point due to the evolution of dissolved ammonia from saliva, and a, b, and c are experimentally fitted constants based on saliva samples with a wide range of urea and ammonia.

For ammonia, the ratio of values at key points in the detection are used, as shown in Equation 6

$$A = \left[\frac{R_{75/600}}{e(f-R_{75/600})}\right]^{1/g} \qquad \text{Equation 9}$$

where A is the initial dissolved ammonia concentration in the saliva sample in ppm, R75/600 is the ratio of voltage readings at 75 and 600 seconds respectively, and e, f, and g are experimentally fitted constants.

Figure 6 shows a parity plot for saliva samples and saliva samples spiked with urea using the spectrometer analytical kit and the test strip/sensor system. The horizontal bars represent the range of values measured using the spectrometer kit. These data indicate that the sensor system correlates well with a laboratory method that requires a spectrometer and careful measurement of the sample in order to get accurate readings. Previously [6], we demonstrated that the test strip for quantifying urea in aqueous solutions is based on Korteweg stresses and viscous fingering, thereby eliminating the need for accurate measurement of the sample being analyzed.

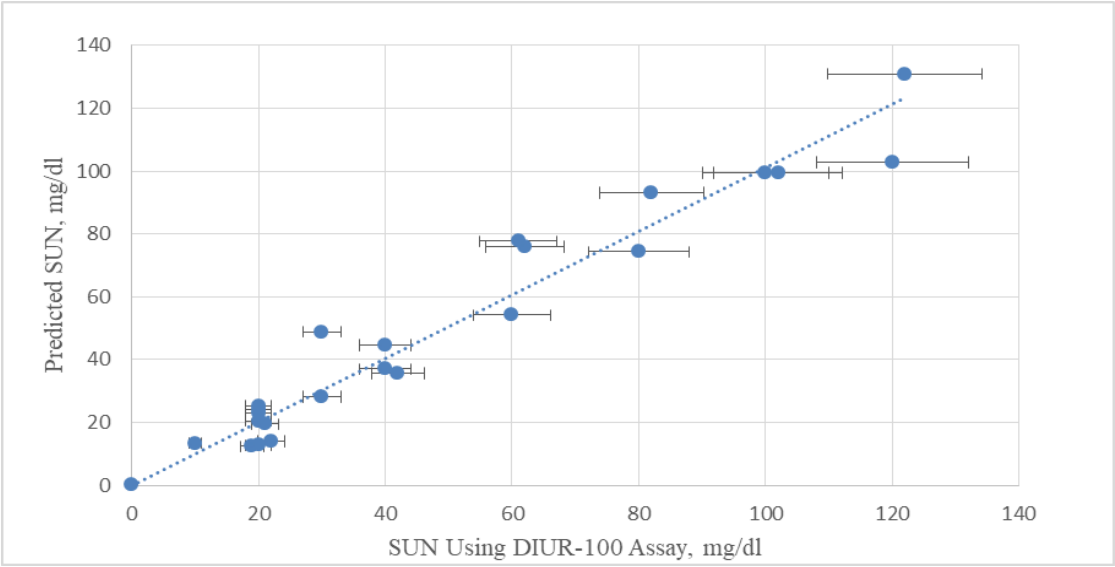

Fig. 6: Parity plot between predicted and experimentally found SUN

Novelty Journals

ISSN 2394-7349

International Journal of Novel Research in Engineering and Science
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Results from our testing of measuring initial dissolved ammonia gas in saliva are shown in Figure 7, where the analysis is more complex since the detection below 10 ppm of ammonia is not reliable due to being below the detection limit of the sensor. Above the threshold of about 20 ppm dissolved ammonia, the calibration is a power law function with the results from the Hach Ammonia Assay.

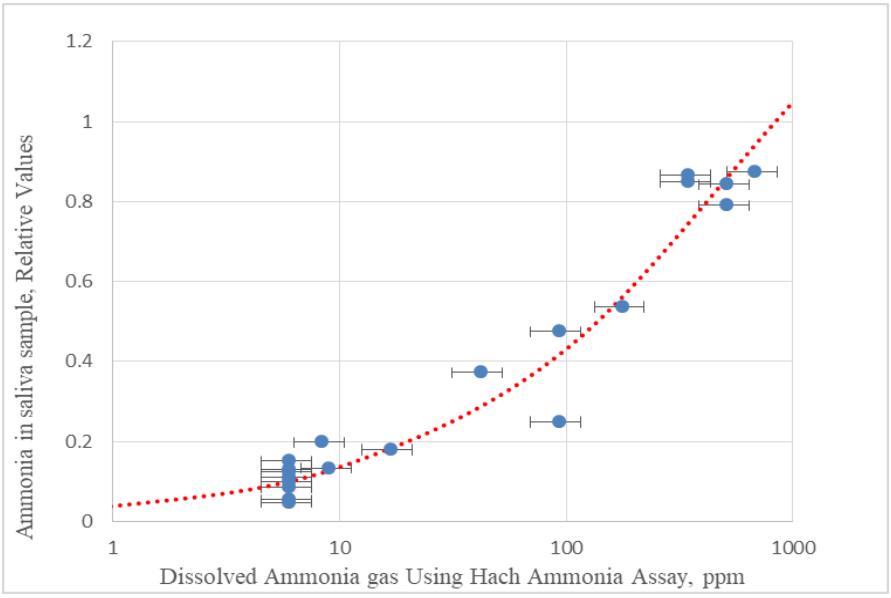
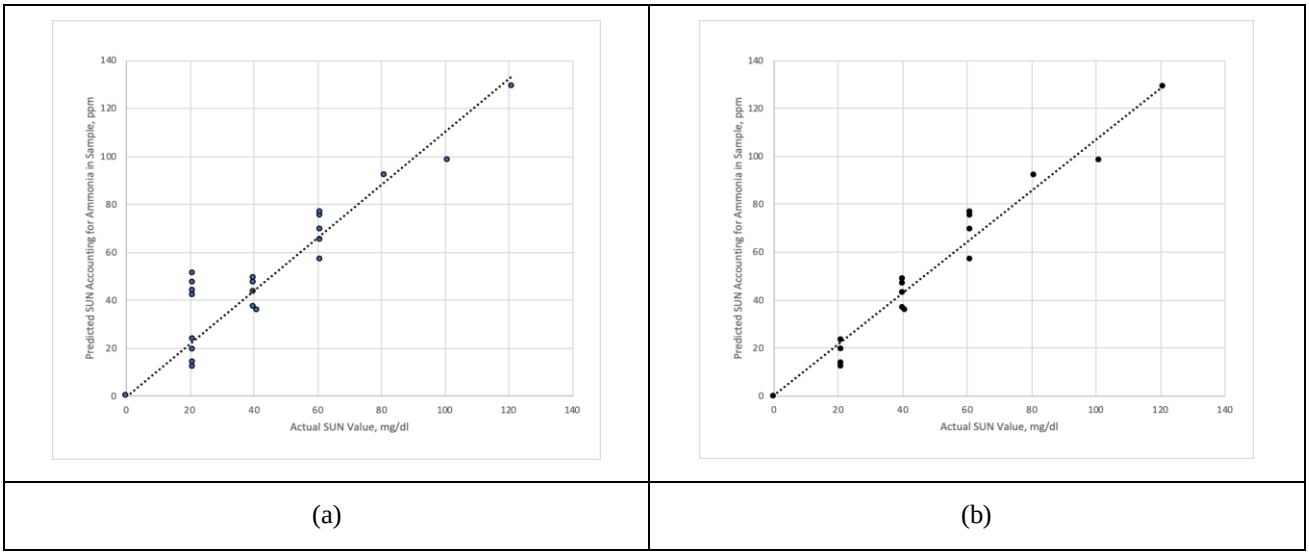


Fig. 7: Relationship between relative ammonia values and dissolved ammonia gas

Testing of saliva samples, saliva samples spiked with urea, and saliva samples spiked with urea and ammonia shows that the agreement between actual SUN values (determined by spectrometry) versus the Predicted SUN using the equations above is good except for the four data points at SUN of 21 that are above the line. Upon examining this result, those results were for very high concentrations of spiked ammonia (above 200 ppm) added to the saliva. Because such high dissolved ammonia could be due to many underlying conditions, flagging an abnormal result would be a prudent feature for this technology. Another way to address this consequence is to flag all dissolved ammonia readings > 100 ppm at short times as being unreliable if calculated SUN is < 40 mg/dl. This can be easily programmed into the nano Arduino and incorporated into the final readout.



(a) (b)

Fig. 8: (a) Plot of predicted SUN against experimentally found SUN for urea and ammonia spiked samples (b) Plot of predicted SUN against experimentally found SUN for urea and ammonia spiked samples, removing high ammonia outliers for normal SUN values

IV. CONCLUSION

A low-cost test strip/sensor system can be used to detect both dissolved ammonia and urea in a saliva sample. The saliva sample can be easily obtained in about 5 minutes and the test result can be reported in another 10 minutes. The determination of two saliva biomarkers using one disposable test strip and a portable sensor with Low Energy Bluetooth capability is a useful strategy for point of care testing, potentially providing more information about dehydration and possible underlying conditions. The inexpensive metal oxide gas sensor is deployed in this method in a way that widens the scope of these common devices to be integrated in detecting biomarkers in a liquid sample. While the more complex analysis of diffusion and reaction can be used to understand the signal, it is more prudent and relevant to use power law equations and multipoint voltage readings within an algorithm to analyze saliva samples. The trade-off in using this method is that readings are relatively accurate except when the combination of very high dissolved ammonia > 200 ppm and normal SUN are present, whereby the SUN readings would then be artificially high. Normally, high ammonia and high SUN would be present or low ammonia and high SUN could be present in someone becoming dehydrated. However, software indicating that very high ammonia is present with normal SUN should then be interpreted as having a need for further testing. In this case, a software readout that further testing is warranted would be a more prudent way to proceed.

AUTHOR CONTRIBUTIONS

Maren Eltze improved the protocol for testing saliva, built the final sensor prototype, and was the primary author of the paper. Kristin Huber discovered the need to increase the amount of saliva collected in order to improve the detection range and conducted saliva testing. Lara Eltze made the primary contribution to organizing the production of 100 test strips and conducted saliva testing. Aubrey Berger wrote the initial software for interpreting the kinetic data from the gas sensor and conducted saliva testing. Kathryn Smith developed the sensor data collection protocol and conducted saliva testing. Maria Soldevila produced the calibration solutions, determined that the Hach ammonia test strip protocol could be used for saliva and conducted saliva testing. Alan Ross refined the steps to analyze saliva and conducted saliva testing. Antonio A. Garcia directed the project, designed the 3-D printed components, wrote the final software for the sensor, and conducted saliva testing.

ACKNOWLEDGMENT

The authors were supported by a grant from Dignity Health in order to conduct this study. Dr. Abraham Lieberman of the Mohammed Ali Parkinson's Center initiated the need for an improved saliva test strip, and we are appreciative to the help and guidance he provided in order to accomplish this work.

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