

The validation of urea determination in saliva using optical urea biosensor with *p*-dimethylaminobenzaldehyde (DMAB) reagent



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Abstract. The validation of urea determination in saliva using the biosensor method and the *p*-dimethylaminobenzaldehyde (DMAB) method has been successfully conducted. Anthocyanin compounds from *Chatarantus roseus* flowers were extracted using the maceration method with methanol as the solvent, yielding a total of 22.60% with a total concentration of 6.01 mg/L. The obtained extract was subjected to qualitative anthocyanin testing, showing a positive result was indicated by the formation of a reddish color and quantitative testing with the formation of a faded yellow color. The maximum wavelength (λ max) of anthocyanin was 664 nm with an absorbance of 0.674. The saliva samples were obtained from three different ages i.e: children, teenagers, and adults. During the saliva collection procedure, participants were instructed to abstain from consuming any food or beverages, with the exception of water, for a minimum of 1 hour prior to sample collection. All samples were stored in a freezer at 4-8°C until needed for analysis. Linearity test results were assessed using a calibration curve, yielding a coefficient of determination (R^2) of 0.978 with a sensitivity of 0.033 for the biosensor and an R^2 of 0.975 with a sensitivity of 0.685 for DMAB. The limit of detection (LOD) values were determined as 7.203×10^{-11} M for the biosensor and 6.984×10^{-6} M for DMAB, while the limit of quantification (LOQ) values were 2.182×10^{-10} M and 2.116×10^{-5} M, respectively. Statistical analysis using a t-test showed $t_{\text{calculated}} = 1.314$, compared to $t_{\text{table}} = 4.302$, indicating that $t_{\text{calculated}} < t_{\text{table}}$. This result suggests no statistically significant difference between the biosensor and DMAB methods, confirming that the biosensor method is comparable in performance to the DMAB method. Additionally, urea concentration measurements showed that adult samples exhibited the highest urea levels among the tested samples, which may indicate an association with potential health risks, including dental caries, kidney failure, and liver damage.

Keywords: biosensor, *Catharantus roseus*, DMAB, urea, saliva

INTRODUCTION

Urea is an organic compound composed of carbon, nitrogen, and hydrogen, and it is found in various samples such as milk [1], food [2], water [3], saliva [4], and blood [5]. In the human body, urea is the largest end product of nitrogen metabolism, excreted primarily through urine [6]. The normal concentration of urea in

blood ranges from 7-20 mg/dL [7]. Elevated levels of urea may indicate kidney dysfunction or other related health issues [8]. Therefore, the analysis of urea is crucial, particularly for human health monitoring. As previously mentioned, urea is also present in saliva, which is a fluid in the mouth consisting of a mixture of secretions from glands within the oral cavity. The



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properties of saliva, whether acidic or basic, are influenced by the food consumed [9].

Urea can be determined through various methods based on electrical and optical measurements [10][11][12]. Electrical methods include amperometry [13], potentiometry [14], and electrochemical impedance spectroscopy (EIS) [15]. As mentioned above, the detection of urea continues to develop such as biosensors. Numerous of studies on urea detection via biosensors have been reported. For instance, urea has been quantified using a smartphone-based pH measurement system with Ag nanoparticles[16] and Prussian blue for urea detection through impedimetric analysis also has been reported [17]. Therefore, the measurement results must be validated using an established method that has been proven to be reliable. Study on urea detection based on optical measurement has been reported using a pectin-chitosan polyelectrolyte complex (PEC) membrane [18][19]. In this study, a urea biosensor was developed based on previous work reported In this study, urea concentration in human saliva was measured using the developed biosensor, which was validated using a spectrophotometric method using *p*-dimethylaminobenzaldehyde (DMAB). Recent advancements include a urea biosensor employing iron-doped nickel oxide for potentiometric measurements.

This work demonstrated that the fabricated urea biosensor effectively determined urea levels in human saliva: however, validation through alternative analytical methods has not yet been performed. Thus, further validation of urea concentration measurement is required. In this study we used *p*-dimethylaminobenzaldehyde (DMAB) as colorimetric reagent measured by a UV-Vis spectrophotometry method. In this work, saliva urea levels has been assessed across various age groups, and the results were statistically compared using a t-test to validate any significant differences in urea concentration value between the biosensor and DMAB methods.

METHODOLOGY

Extraction of *Catharanthus roseus*

As much as 500 g of *C. roseus* flowers were first cleaned by separating the petals from the stems, then macerated using 500 mL of methanol as the solvent for 24 hours at room temperature (25°C). After maceration, the mixture was filtered to obtain the filtrate and residue. The residue was repeated by similar procedure to obtain the remind anthocyanins. This partitioning process continued until a clear *n*-hexane layer was obtained. The methanol layer was subsequently evaporated at 50°C to obtain anthocyanins [19].

Analysis of Anthocyanin by UV-Vis Spectrophotometry

Qualitative analysis was performed using a UV-Vis spectrophotometer to measure the visible light absorption spectrum of anthocyanins extract. The extract was placed in a cuvette, and absorbance was measured over a wavelength range of 400 nm to 700 nm, as

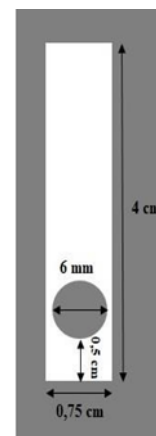


Figure 1. The construction of optical urea biosensor [20].

absorbance within the 475–550 nm range is typically exhibited by anthocyanins. The qualitative analysis was conducted to determine the maximum absorbance wavelength of the anthocyanin extract from *C. roseus* flowers. The total anthocyanin extracted from *C. roseus* flowers was determined using spectrophotometric method as previously reported [21][22] and the value obtained was being employed for subsequent concentration.

Preparation of Urea Biosensor

The urea biosensor was prepared the anthocyanin as an active compound and pectin-chitosan the PEC, which serves as the binder for the active compound. The PEC was prepared by mixing 1% pectin and 1% chitosan solutions with an optimum ratio of 3:7 followed by stirring to homogenize the mixed solution [19][20]. Next, 10 mL of is the solution was taken, and a certain amount of anthocyanin added to achieve a final concentration of 0.05 mg/L (the volume of the anthocyanin stock solution added depends on the concentration of anthocyanins obtained from the extraction process). As much as 30 μ L of the PEC anthocyanin solution was place to a circle transparent plastic, with 4 cm in diameter as presented Figure 1. The resulting solution was then dried in a refrigerator for 24 hours, after 25 μ L of urease enzyme stock solution (2.5 mg/L in 0.03 M phosphate-buffered saline (PBS) at pH 7) was added, resulting a amount of urease in each the biosensor was 0.09 mg. After the addition of the urease solution, the biosensor was stored in the refrigerator of 4 °C for 24 hours until dry.

Saliva Sample Collection and Preparation

Saliva samples were collected from three respondents who consented to participate in this study. The selection criteria for respondents were as follows: one child aged 5-11 years, one adolescent aged 12-25 years, and one adult aged 26-65 years, all residing in Banda Aceh. During saliva collection, the respondents were instructed to refrain from eating or drinking (except for water) for at least one hour before the procedure. This precaution was necessary because the oral cavity's environment becomes acidic, reaching a critical pH (≤ 5.5) within 5-30 minutes after consuming acidic food or beverages.

The oral pH gradually returns to normal due to the buffering function of saliva [23]. For saliva collection, respondents were instructed to accumulate saliva at the base of their mouth and then expel it into a collection container provided. This process was repeated until approximately 6 mL of saliva was collected from each respondent. The collected saliva samples were then stored in a freezer at 4-8°C until further analysis [23].

Determination of Urea in Saliva

Biosensor Method

The determination of urea in saliva using a biosensor was started by plotting the absorbance values vs urea concentration of 10^{-10} to 10^{-1} M. to provide a calibration curve at a maximum of anthocyanin wavelength. Absorbance measurements were recorded 5 minutes after exposing by the urea standard solutions, prepared in 0.03 M Phosphate Buffered Solution (PBS) at pH 7. This calibration curve was employed to assess the linearity, coefficient of determination (R^2), limit of detection (LOD), and limit of quantification (LOQ), with each measurement repeated three times to ensure accuracy. Saliva samples were then analyzed by applying 25 μ L of saliva onto the biosensor surface, and the absorbance was measured as similar calibration curve procedure. The absorbance values obtained were subsequently used in the equation derived from the calibration curve to determine the urea concentration in the saliva samples [24].

Biosensor DMAB Method

The determination of urea in saliva using both biosensor and DMAB reagent was started by the preparation of a calibration curve for these methods. A urea solution was prepared by dissolving 5 g of urea in deionized water. Subsequently, volumes of 2 mL; 4 mL; 6 mL; 8 mL; 10 mL; 12 mL; 14 mL; 16 mL; 18 mL; and 20 mL from the stock solution are diluted to 100 mL using 5×10^{-3} M Phosphate Buffered Solution (PBS) at pH 6.5, yielding a series of standard solutions. The ratio of the reagent volume to the standard or sample solution was maintained at 1:1 until the solution turns yellow, at which point the absorbance was measured at a wavelength of 420 nm. The calibration curve was employed to determine, coefficient of determination (R^2), limit of detection (LOD), and limit of quantification (LOQ). All experiments were three-time repetitions, saliva samples were collected from individuals across various age groups, categorized as children; adults; and the elderly. The urea determination using the p-dimethylaminobenzaldehyde (DMAB) method was performed by taking 1 mL of saliva, adding 1 mL of 5×10^{-3} M PBS at pH 6.5, and measuring the absorbance at 420 nm [25].

Method Validation

The validation of the analytical method includes the determination of the limit of detection (LOD) and the limit of quantification (LOQ) using data from the calibration curve obtained via UV-Vis spectrophotometry. The LOD and LOQ were calculated by determining the standard deviation [21]. These values

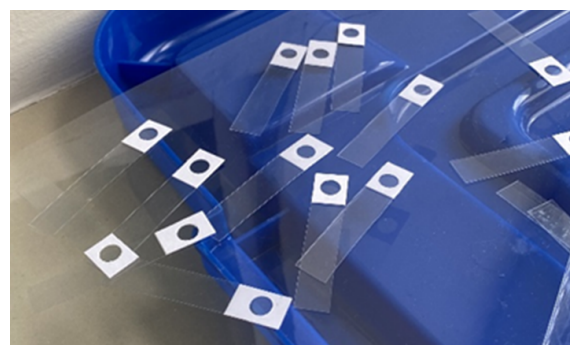


Figure 2. Urea biosensor construction

are essential for measuring the absorbance of blanks in both the biosensor and p-dimethylaminobenzaldehyde (DMAB) methods for urea determination. The LOD and LOQ values are expressed using the following formulas:

$$\text{LOD} = 3.3 \times \text{Sy}/\alpha \quad (1)$$

$$\text{LOQ} = 10 \times \text{Sy}/\alpha \quad (2)$$

Where LOD is the Limit of Detection, LOQ is the Limit of Quantification, Sy represents the standard deviation of absorbance, and α is the slope of the calibration curve.

Linearity is another crucial aspect, reflecting the linear relationship between the concentration of the sample and the corresponding analytical signal. This relationship is critical in both the biosensor and DMAB methods, ensuring that the concentration of urea correlates precisely with the absorbance values obtained [21]. Furthermore, a comparative analysis between the biosensor and DMAB methods was performed using a t-test to determine the statistical equivalence of the two methods. If the calculated t-value is less than the critical t-value from the t-table, it indicates that the biosensor method produces results comparable to those obtained by UV-Vis spectrophotometry [22]. This validation process confirms the reliability and accuracy of the biosensor method in determining urea concentrations.

RESULTS AND DISCUSSION

Performance of Urea Biosensors Based on PEC Membranes and Anthocyanins (*Catharanthus roseus*)

The urea biosensor based on a PEC membrane and anthocyanin extracted from *Catharanthus roseus* is a simple device that can be used to detect various substances, including those related to human health, such as sweat, urine, and saliva. The biosensor is assembled using transparent plastic mica and thick paper, which is affixed to form a circular section tailored to its intended size and function (Figure 2).

The successful fabrication of this optical urea biosensor, involves the use of anthocyanin as a pH indicator, which is immobilized on a pectin-chitosan polyelectrolyte complex (PEC) membrane [20]. The biosensor comprises several key components: the analyte, bioreceptor, transducer, and signal processor. The PEC membrane is created by mixing 1% solutions of pectin

Table 1. Performance of urea biosensor based on PEC and anthocyanin *Chatarantus roseus*

Parameters	Results
Linearity	10^{-10} - 10^{-1} M
Sensitivity	$0,023 \pm 1,3 \times 10^{-2}$
R ²	0,973
LoD	$5,2 \times 10^{-12}$ M
pH	7
Response Time	Minute 7 at concentration 10^{-7} M
% recovery	80,25–98,73 %
Reproducibility	10,4 %

and chitosan in a 3:7 ratio [19]. The biosensor was then dried at room temperature for 24 hours, after which a urease enzyme stock solution in Phosphate Buffered Solution (PBS) was applied. Following this treatment, the biosensor was stored in the dark to allow the surface to dry completely. The biosensor operates based on pH changes resulting from the enzymatic reaction between urease and urea. The optimal sensitivity, achieved with 0.09 mg of urease, was observed in a 0.03 M phosphate buffer solution at pH 7. The sensitivity of the biosensor over the linear concentration range of urea (10^{-10} to 10^{-1} M) was found to be 0.023, with a limit of detection (LOD) of 5.2×10^{-12} M. These optimal values will undergo further validation^[20].

Performance of the urea biosensor was shown in Table 1, highlights key parameters such as linearity, sensitivity, and reproducibility, which are essential for the accurate detection of urea in human saliva. The data indicate that urease exhibits optimal activity at neutral pH (pH 7); however, extreme increases or decreases in pH could affect the active site of urease and reduce its reactivity. The performance data of the urea biosensor suggest that pH significantly influences both sensitivity and linearity, leading to the conclusion that the observed values were optimal for its intended application [38].

Table 1 presents the performance characteristics of the urea biosensor based on a pectin-chitosan polyelectrolyte complex (PEC) membrane and anthocyanin from *C. roseus*. The biosensor exhibits a linearity range of 10^{-10} M to 10^{-1} M urea, which was suitable to detect urea in saliva sample at low to moderate concentrations. The sensitivity of the biosensor was found to be $0.023 \pm 1.3 \times 10^{-2}$, indicating a moderate response to urea concentration changes. The coefficient of determination (R²) value of 0.973 indicates a strong linear relationship between the urea concentration and absorbance, validating the reliability of the calibration curve for accurate urea detection.

The limit of detection (LoD) for the biosensor is 5.2×10^{-12} M, which indicates that the sensor is capable of detecting very low concentrations of urea, making it suitable for sensitive applications. The biosensor was also optimized at pH 7, which is consistent with the physiological pH of human saliva, ensuring that the sensor operates effectively under typical biological conditions. Further performance metrics include a

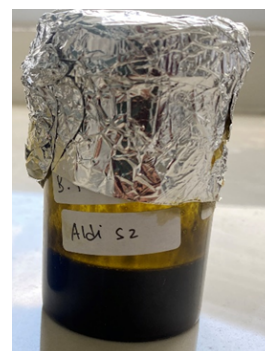


Figure 3. The crude methanolic extract of *C. roseus* flowers.

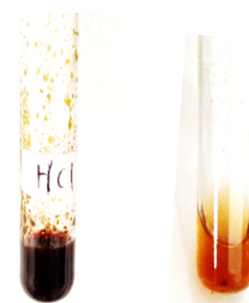


Figure 4. Anthocyanin testing with acids (left) and bases (right)

response time of 7 minutes at a concentration of 10^{-7} M, suggesting the biosensor's quick reaction to urea presence. The percentage recovery range of 80.25–98.73% highlights the biosensor's good accuracy in recovering urea from different sample matrices. Additionally, the reproducibility of 10.4% indicates relatively consistent performance across multiple measurements, which is crucial for reliable and repeatable analyses.

Extraction of Anthocyanin from *Catharanthus roseus*

The *Catharanthus roseus* were collected from Alue Naga Village, Syiah Kuala, Banda Aceh. The red flowers were air-dried indoors for 1 x 24 hour to protect them from sunlight, as anthocyanins are highly sensitive to degradation upon exposure to light. Additionally, high temperatures and mechanical agitation can contribute to the breakdown of anthocyanins and affect their color stability.

Following drying, the flowers were ground and subjected to maceration in methanol for 2 x 24 hours, with a sample-to-solvent ratio of 1:10. The mixture was filtered to separate the filtrate from the residue. The residue was then further remacerated with methanol, and the resulting methanol extract was partitioned with *n*-hexane. The partitioning process was repeated until a clear *n*-hexane layer was obtained.

The anthocyanins extracted from *C. roseus* flowers were utilized as active compounds in the fabrication of a biosensor. Other natural compounds, such as carotenoids, flavonoids, and chlorophyll, may also serve as potential candidates for use in biosensors. In addition to anthocyanins, PEC membrane consisting of pectin and

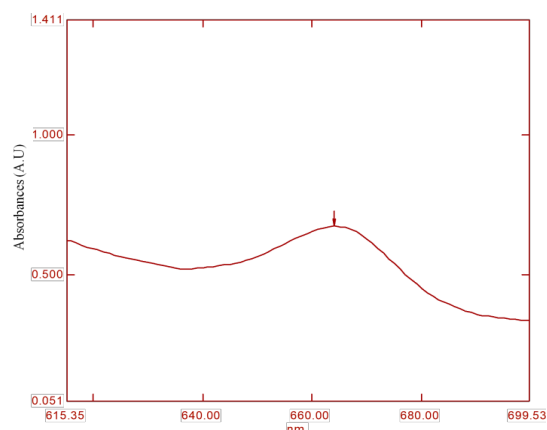


Figure 5. UV-Vis spectrum of *Catharanthus roseus* anthocyanins

chitosan was incorporated as a matrix to support and retain essential biosensor components, including enzymes or other molecular receptors. The extraction process from *C. roseus* flowers was shown in Figure 3.

Determination of Anthocyanin Colour in acid and basic

The testing of anthocyanins from *C. roseus* flowers has previously been conducted on the evaluation of flavonoids and phenols [19]. The presence of anthocyanins can also be demonstrated through a simple approach involving the addition of acidic and basic solutions to the anthocyanins extracted from the flowers. Specifically, hydrochloric acid (HCl) and sodium hydroxide (NaOH) solutions were added to sample and heated for 2 minutes, followed by observation of the resulting color changes. If the sample exhibits no change in color or reveals a reddish hue on the walls of the test tube, it indicates the presence of anthocyanins [26].

As shown in Figure 4, the reddish color observed on the heated walls of the test tube results from the addition of HCl, which creates an acidic environment (low pH) and influences the chemical structure of the anthocyanins, thereby transforming their color to reddish. Furthermore, anthocyanins undergo protonation or the addition of hydrogen ions, which can also affect their color. At a low pH, anthocyanins tend to shift to the flavilium cation form, signaling the presence of anthocyanins [27].

Testing with a base can be conducted by adding NaOH to the anthocyanins. If a gradual change in color occurs, with a transition to a yellow or faded hue from the initial appearance, it can be concluded that anthocyanins are present. This is due to the effect of increased pH upon the addition of NaOH. In this state, anthocyanins have lost hydrogen ions through deprotonation, leading to a color change to a paler or yellowish tone [27]. The acid and base testing of *Catharanthus roseus* flowers conclusively indicates the presence of anthocyanins.

Table 2. Age distribution of saliva sample respondents

Category	Age (Year)	Sex
Child	10	Female
Teenager	23	Female
Adult	56	Female

Qualitative and Quantitative Analysis of Anthocyanin

The determination of the maximum wavelength (λ_{max}) of the methanol extract of anthocyanins from *Catharanthus roseus* was performed using a UV-Vis spectrophotometer. The absorbance of the anthocyanins was measured in the visible light spectrum, specifically within the range of 600 nm to 700 nm. UV-Vis spectroscopy is utilized to measure the absorption or transmission of light by a sample at specific wavelengths. In this study, the absorption peak was found at a wavelength of 664 nm, with an absorbance of 0.674. This peak absorbance (λ_{max}) is subsequently employed as a reference point for biosensor measurements. The maximum wavelength is often used to identify and characterize materials, as it provides information about their electronic structure and aids in the identification of chemical compounds. The results regarding the maximum wavelength of anthocyanins using the UV-Vis spectrophotometer are depicted in Figure 5.

Red *Catharanthus roseus* flowers were selected based on their higher anthocyanin content compared to white flowers. Additionally, red flowers are known for their numerous benefits, including high antioxidant properties, anticancer effects, and antibacterial and antiviral activities [28]. Quantitative testing is necessary to assess the amount of anthocyanins obtained. The concentration of anthocyanins was determined using a differential spectrophotometric method, with the samples dissolved at pH 1 and pH 4.5, and their absorbance measured in the 600-700 nm range. The differential spectrophotometry technique analyzes changes in absorbance under two or more different conditions, and it is commonly used to determine concentrations and characterize substances with specific properties. The measurements performed using UV-Vis spectrophotometry in the visible range yielded a total anthocyanin concentration of 6.01 mg/L. The total concentration of anthocyanins in *Catharanthus roseus* flowers can be ascertained based on the established equations.

Quantitative testing indicates that anthocyanins possess functional properties that exhibit greater stability in acidic conditions, whereas anthocyanins are unstable in neutral and basic solutions [29]. As such, the extraction of anthocyanins is more effectively carried out under acidic conditions. Common acidifying agents used in anthocyanin extraction include HCl and citric acid [28]. Yield is a term used to describe the outcome of a production or extraction process relative to the initial amount of material used. Yield is typically expressed as

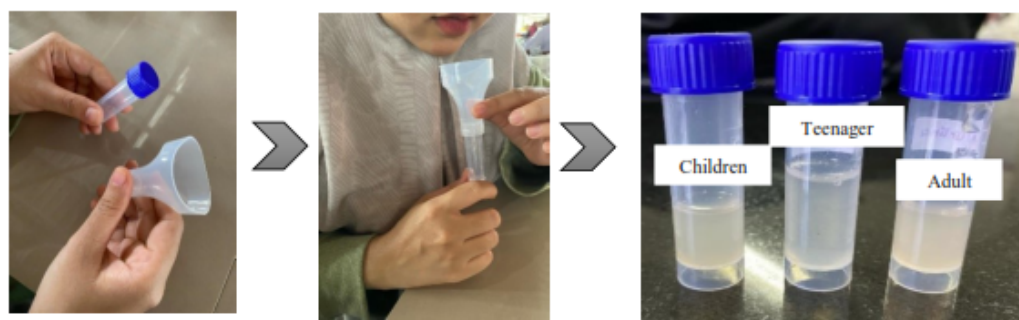


Figure 6. Saliva sample collection

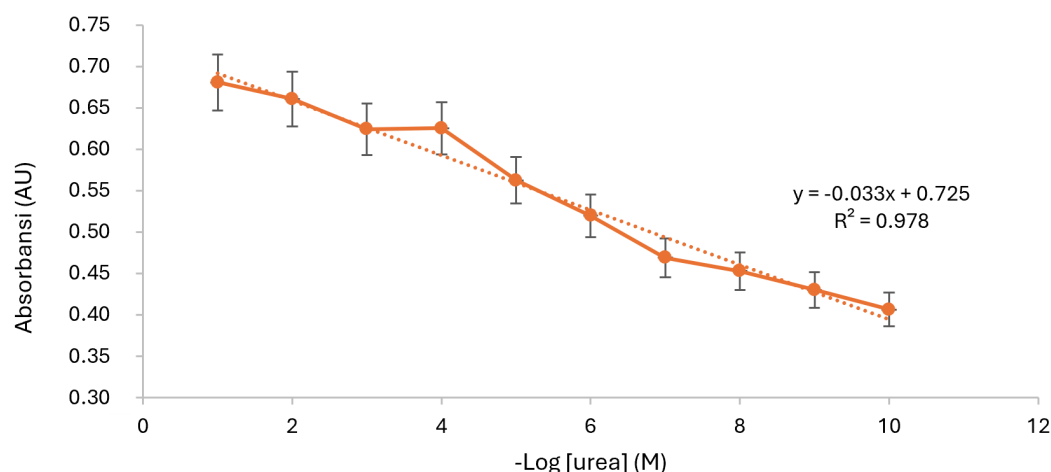


Figure 7. Biosensor calibration curve from the extract

a percentage but can also be represented as a fraction or ratio. The total yield of the methanol extract of anthocyanins obtained in this study was 22.60%. Yield refers to the amount of product generated in a process compared to the initial input material. Determining the yield of a study can assist in evaluating the efficiency of that process. A higher yield suggests that the process is more efficient in converting starting materials into products.

Saliva Sample Collection

The collection of saliva samples from these categories was based on adequate representation, as a single saliva sample is often sufficient to obtain necessary information, such as DNA analysis, hormone levels, or other biological markers. Saliva has a stable composition and can provide an accurate reflection of an individual's health status at a specific point in time [30]. Collecting a single saliva sample reduces discomfort and burden for the respondents. Saliva collection is a non-invasive procedure, though it may still be disruptive if performed repeatedly; therefore, obtaining one sample per category is more efficient and expedient than collecting multiple samples. However, there may be instances where more than one saliva sample is necessary for specific needs, such as monitoring biological changes over extended periods. The required sample size is typically determined by the specific goals of the research or medical diagnosis. The demographic details of the saliva samples collected can be found in Table 2.

For this research, subjects were instructed to refrain from eating or drinking (except for plain water) for at least one hour prior to saliva collection. This is important as the oral cavity's environment can become acidic and reach critical pH levels (≤ 5.5) within 5 to 30 minutes following the consumption of acidic foods or beverages. Subsequently, the pH of the oral cavity returns to normal due to the buffering function of saliva. The phosphate buffer solution (PBS) contained in saliva plays a crucial role in maintaining stable pH levels, particularly given the diversity of saliva from children, adolescents, and adults. The composition of saliva is significantly influenced by the foods and beverages consumed daily. Some food and drink products contain urea, which can be harmful if ingested continuously and may alter pH levels. In this study, PBS is utilized to help sustain optimal sample conditions. Additionally, PBS helps maintain isotonicity or osmotic pressure of the solution, which is essential for preserving the integrity of cells and biological components within the saliva [31]. The saliva collection process is illustrated in Figure 6.

The collected saliva samples were stored in a freezer in additional containers to maintain sample stability. If immediate analysis was not feasible, the containers were kept at low temperatures to prevent alterations in the chemical composition of the samples. Exposure to direct sunlight or extreme temperatures, which could affect the stability of the samples, was avoided until analysis was required. Care was taken to prevent contamination of the saliva samples from volatile, hazardous chemicals, and

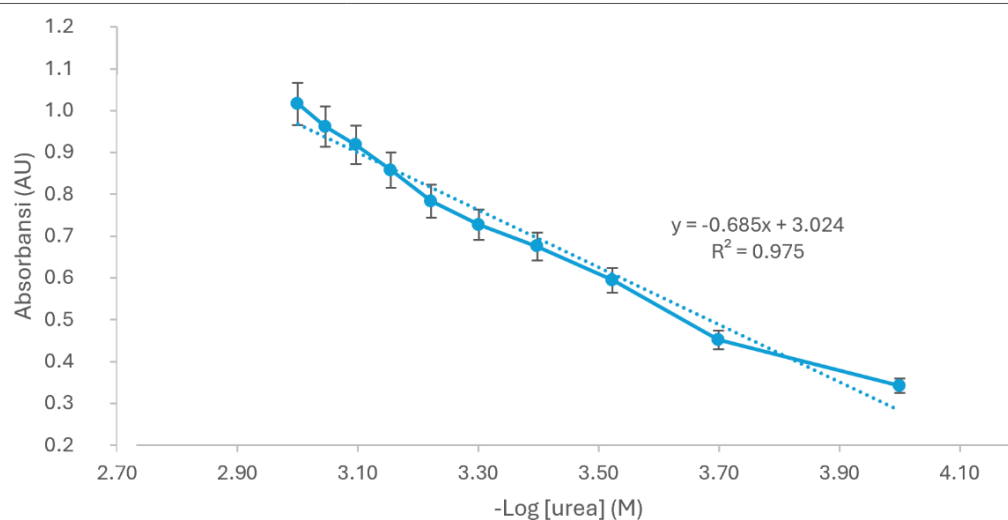


Figure 8. DMAB spectrophotometer calibration

other unwanted materials during both the collection and storage processes. Prior to analysis, the saliva samples were removed from the freezer and allowed to acclimate to room temperature.

Validation of Urea Determination Method

The calibration curve for the biosensor was obtained by measuring absorbance versus concentration using a UV-Vis spectrophotometer. This calibration curve serves as a reference to evaluate the suitability of the instrument or method employed prior to testing real samples, considering several validation parameters. The absorbance values were measured in triplicate for each concentration, and the average absorbance for each concentration was calculated. Absorbance indicates the amount of light absorbed; thus, a higher absorbance value corresponds to a greater amount of light absorption. The calibration curve concentrations for the biosensor ranged from 10^{-1} to 10^{-10} M, whereas the DMAB method utilized concentrations from 10×10^{-4} to 10^{-4} M. The obtained concentrations were then converted into $-\text{Log} [\text{urea}]$ M to facilitate measurement with low concentrations while ensuring accuracy in calculations for simplified results.

The calibration curve acquired using the UV-Vis spectrophotometer provides a basis for the method validation. The UV-Vis spectrophotometer is chosen due to its simplicity and relatively low cost, alongside its high sensitivity compared to other methods. Its high sensitivity to compounds enables the absorption of ultraviolet light or visible light, making it capable of detecting compounds at low concentrations, aligning with the objectives of this study. The results of the calibration curve for the biosensor can be observed in Figure 7.

The results from the biosensor calibration curve shown in Figure 7 indicate that as the concentration increases, the absorbance at each concentration also increases. Each measurement point on the biosensor calibration curve includes error bars, indicating the measurement uncertainties inherent in the data. Additionally, the standard deviation of the data can be used to assess the

degree of data dispersion; a higher standard deviation signifies greater variation in the measurements. A standard deviation value that is lower than the mean is indicative of acceptable performance. The overall calibration curve for the biosensor exhibits linear behavior with a slope of 0.033 and an intercept of 0.725. Notably, there are measurement points that deviate from the linear regression line; this may be due to the pronounced sensitivity of the anthocyanin active substance utilized. Anthocyanins are known to be highly sensitive to pH and elevated temperatures, which can lead to structural degradation and the resultant loss of color. The calibration curve demonstrates linear characteristics as evidenced by the coefficient of determination (R^2). This calibration curve will subsequently be utilized to derive the regression equation for urea determination in saliva samples.

This study utilizes the p-dimethylaminobenzaldehyde (DMAB) method to conduct urea determination and to demonstrate specific colour intensity that is proportional to urea concentration with alongside favorable stability, allowing measurements to be conducted over extended periods after the reaction has occurred. The results derived plot of urea concentration and DMAB reagent as depicted in Figure 8. indicate that as concentration as we can see the absorbance increases correspondingly with the increasing urea concentration. From the figure can be observe the absorbance value exhibit a satisfactory precision as evidence by standard deviation. As low as standard deviation value indicate the data obtained is acceptable and has been proven by the error bars on the curve. The calibration curve generated using the DMAB method presents a slope value 0.685 and an intercept of 3.024. This slope value reflects the sensitivity achieved. The regression equation of DMAB calibration curve will be employed to determine urea levels in saliva.

Linearity test in addition the linearity of the curve was used to evaluate the method's responsiveness at various concentrations of analyte [32]. The linearity was determined by determination coefficient (R^2) from both calibration curve (biosensor and DMAB).

Table 3. LOD and LOQ values of biosensor and DMAB methods

Validation Method	Biosensor (M)	DMAB (M)
LOD	7.203×10^{-11}	6.984×10^{-6}
LOQ	2.182×10^{-10}	2.116×10^{-5}

Table 4. Comparison of biosensor and DMAB methods

Saliva Sample	Age (Years)	Biosensor (M)	DMAB (M)	t _{calculated}	t _{table}
Child	10	7.161×10^{-4}	5.675×10^{-4}	1.314	4.302
Teenager	23	11.66×10^{-4}	7.063×10^{-4}		
Adult	56	28.90×10^{-4}	8.649×10^{-4}		

The limit of detection (LOD) was determined to establish the lowest concentration of an analyte that produces a significant response, while the limit of quantification (LOQ) indicates the minimum concentration that can be reliably quantified (Wardani, 2022). The calculation of LOD and LOQ involves first determining the standard deviation of the blank; subsequently, LOD values are typically lower than LOQ [33]. Measurement results from the validated biosensor method demonstrated an ability to detect urea concentrations as low as 7.203×10^{-11} M. Meanwhile, the LOQ was 2.182×10^{-10} M. For the more the LOD and LOQ of DMAB method were 6.984×10^{-6} M and LOQ of 2.116×10^{-5} M as presented in Table 3.

Table 3 demonstrates that both values LOD and LOQ of these methods to be high sensitive and capable in measuring urea at very low concentrations. The exceptionally low LOD and LOQ values indicate the efficacy of this biosensor for the determination of urea in human saliva, which necessitates the measurement of urea concentrations at very low thresholds.

Comparison of Urea Concentration using the Biosensor and DMAB Methods

The t-test is a statistical technique used to compare the means of two different samples or to evaluate the significance of differences between sample means and a known value. The t-test was conducted to compare the biosensor method and the DMAB method by analyzing the calculated t-value in relation to the critical t-value, assessing whether there is a significant difference. This comparison began with formulating hypotheses regarding the methods: null hypothesis (H_0) positing that the biosensor method is equally effective as the DMAB method in UV-Vis spectrophotometry, while the alternative hypothesis (H_1) suggests that the biosensor method is less effective than the DMAB method.

Data collection involved calculating the t-value and degrees of freedom to determine whether the difference between sample means was statistically significant. The results of the t-test were interpreted based on the significance level to ascertain whether there was sufficient evidence to reject or accept the hypothesis. The t-test serves as a common tool in statistical analysis to address various research questions and make decisions based on available sample data.

The comparison of urea determination methods utilizing the biosensor and *p*-dimethylaminobenzaldehyde (DMAB) demonstrates that the biosensor exhibits good sensitivity and provides results more rapidly. Although the DMAB method is straightforward, it has a higher detection limit and requires more sample handling. The biosensor is more suited for detecting low concentrations of urea and enables fast analysis; however, it necessitates initial preparation for enzyme immobilization during device design, while being user-friendly once the biosensor is fully developed as a product. In contrast, the DMAB method can be employed for routine measurements with simpler reagent preparation requirements, albeit with several manual steps such as measuring absorbance, leading to a more time-consuming process compared to the biosensor. The comparison between the biosensor and DMAB methods based on the t-test results is presented in Table 4.

Table 4 illustrates the comparison of the biosensor and DMAB methods across different age categories: child, teenager, and adult. The collected saliva samples were tested using both the biosensor and *p*-dimethylaminobenzaldehyde (DMAB) methods with a UV-Vis spectrophotometer. Measurements revealed varied results across the different samples. The results obtained from both methods did not show significant difference. The absorbance measurements from both the biosensor and DMAB were incorporated into the regression equation ($y = ax + b$). Based on the data, it can be concluded that absorbance increases with age, with adult samples yielding the highest measurement results among the groups studied. This finding might be attributed by dietary management and healthy lifestyle.

The t-test was performed to assess whether significant differences exist between the two methods. A two-tailed t-test was conducted with a 95% confidence interval with a critical t-value of 4.302. As shown in the table, the calculated t-value is 1.314, indicating that $t_{\text{calculated}} < t_{\text{table}}$. Therefore, it can be concluded that the biosensor and DMAB methods are comparable, with no significant differences observed, leading to the acceptance of H_0 and the rejection of H_1 .

The comparison of the biosensor and DMAB methods, as presented in Table 4, indicates that adult samples exhibit the highest values among the tested groups. This finding suggests a potential association with certain

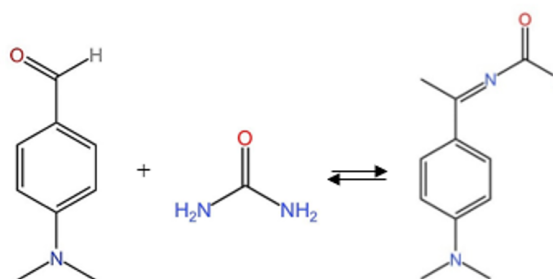
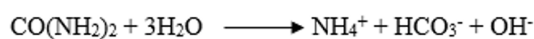


Figure 9. Urea hydrolysis reaction

diseases, such as dental caries, which may correlate with kidney failure if urea levels are excessively high. Furthermore, this indication can also be inferred from the ammonium produced during the hydrolysis of urea [34]. Elevated ammonium levels can lower pH, resulting in acidic conditions due to the production of hydrogen ions. Overall, urea serves as a vital biomarker in medical diagnostics related to health conditions, particularly concerning kidney function and protein metabolism.

The hydrolysis of urea results in the formation of ammonium and bicarbonate. This reaction, facilitated by the enzyme urease as a catalyst, accelerates the reaction rate. The urease enzyme plays a critical role in the hydrolysis of urea, as the reaction occurs at a significantly slower rate in its absence. Urease lowers the activation energy for the reaction involving urea molecules, allowing for a faster interaction with water.

In addition to accelerating reaction rates, urease exhibits high specificity for urea, typically catalyzing only the hydrolysis of urea without affecting other substrates. This specificity is essential to ensure that the desired reaction occurs efficiently without interference [37]. Optimal performance of urease occurs under certain conditions, such as near-neutral to slightly alkaline pH and at a temperature of 37°C. The hydrolysis of urea can lead to various reactions depending on the conditions and catalysts used; although ammonium is the primary product, biuret or ammonium carbamate may also form under differing conditions. The reaction that takes place during urea hydrolysis is illustrated in Figure 9.

The reaction depicted in Figure 9 illustrates that urea can be broken down into ammonium bicarbonate. Ammonium serves as a significant indicator of pH changes, potentially resulting in colorimetric shifts. In the study, a yellow color indicative of ammonium presence was observed. The reaction of urea with *p*-dimethylaminobenzaldehyde (DMAB) leads to color formation due to the generation of chromogens and functional group shifts that occur. Chromogens are compounds or molecules that can change color when reacting with specific chemicals, manifesting changes in pH or temperature. This color change serves as a fundamental indicator for urea determination analysis [35].

Urea measurement using the biosensor and DMAB methods leverages the coloration produced by each method, analyzed using a UV-Vis spectrophotometer. The resulting color is attributable to the absorption and emission of light associated with a chromophore. The atomic groups within the molecules absorb light at specific wavelengths, producing visible color. Chromophores are components of dye and pigment molecules that absorb light within ultraviolet and visible spectra, imparting color to the substance. Chromophores function by absorbing light photons, which promotes electrons to higher energy states. The perceived color results from the light reflected or emitted by the chromophore after absorption, allowing for measurable signals [36].

CONCLUSION

The study concluded that both the biosensor method and the (DMAB) method, when applied using UV-Vis spectrophotometry, demonstrated high linearity with R^2 values of 0.978 and 0.975, respectively. Sensitivity values were found to be 0.033 for the biosensor and 0.685 for DMAB. The limit of detection (LOD) was notably low for both methods, with the biosensor detecting urea down to 7.203×10^{-11} M, and DMAB detecting urea down to 6.984×10^{-6} M. The limit of quantification (LOQ) was also low, with the biosensor exhibiting an LOQ of 2.18×10^{-10} M and DMAB at 2.11×10^{-5} M. Comparison of these values demonstrates that the biosensor provides significantly lower LOD and LOQ values, indicating higher sensitivity than the DMAB method. Furthermore, a two-tailed t-test indicated no significant difference between the two methods, suggesting that the biosensor method is comparable in effectiveness to the DMAB method for urea detection. However, the biosensor method exhibited superior sensitivity, particularly in detecting lower concentrations of urea. Further research is recommended to explore additional methods for urea detection in human saliva, with a focus on enhancing the performance of the biosensor for broader applications.

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