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QUANTIFICATION OF UREA IN COMMONLY CONSUMED INDIAN TEA FROM DIFFERENT REGIONS USING AISE-BASED BIOSENSOR WITH UREASE-IMMOBILIZED NYLON MEMBRANE

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Abstract

Tea, is the most extensively used non-alcoholic caffeinated beverage in society today. A biosensor was created by immobilising urease enzyme on a nylon membrane and attaching it to an ammonium ion selective electrode (AISE). The enzyme electrode was characterized by Fourier transform infra-reds pectroscopy (FTIR) and Field emission scanning electron microscopy (FESEM) techniques with particle size ranging between 0.09µm-0.49µm.in diameter. The biosensor showed optimum response within 20s at pH 5.5 in 0.05mM urea conc. in sodium phosphate buffer and 40˚C. It exhibited excellent sensitivity of 38 mV/decade and lower detection limit is 0.001 mM, and wider linear range from 0.001 to 0.80 mM. Analytical recovery of added urea which were found to be 99.8%, 101.04%, 108.35%, 103.9%, 98.99%, 104.7%, 99.93%, 102.34%, 101.76%, and 103.02%. The average urea content for green tea was found to be approximately 130.57 mg/L whereas for tea it was approximately 122.93 mg/L. These findings could be beneficial for the health of the consumers.

Keywords: *Tea, AISE, Immobilization, Urease, Urea, Biosensor*

1. INTRODUCTION

The cheapest beverage that people drink is tea, second only to water. Since ancient times, drinking tea has been seen as a practise that promotes health. There is scientific support for this view provided by current medicinal research. With each new study that is published in the academic literature, the evidence for the health advantages of tea use becomes more compelling. Since ancient times, people have grown the tea plant *Camellia sinensis* and utilised the leaves for medical purposes. The chemicals in tea, a widely consumed beverage, are now being discovered to have medical benefits. There is encouraging evidence that green tea helps prevent cancer in human, animal, and cell culture research. Black tea may provide similar health benefits, according to growing evidence (Khan et al., 2013).

Tea has a long history in China, where it has been consumed as a beverage with complex flavours and used medicinally for a very long time. Due to its numerous widely recognised health benefits, green tea has become more well-liked and is now drank all over the world. 2014's (Yang et al.) Green tea is a tasty hot or cooled beverage. In order to make it, tea is commonly brewed in hot water at a temperature of 90 degrees Celsius or 194 degrees Fahrenheit, which is slightly below boiling (Castiglioni et al., 2015).

Young plant leaves are picked, withered, steamed or pan-fried, and then dried to make green tea. Many of the beneficial components included in tea leaves are preserved by this process, which also aids in preventing fermentation.

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

The health advantages of green tea are due to the high antioxidant content of the beverage. Tea contains high levels of polyphenols, which are organic substances that lower inflammation, guard against oxidative stress, and stop cell damage (Khan et al., 2013). Catechins are a family of natural phenols and antioxidants found in high concentrations in green tea. The most prevalent catechin in green tea is called epigallocatechin-3-gallate (EGCG). It has been demonstrated that ECGC promotes weight loss, boosts cognitive function, improves cardiovascular health, controls blood sugar levels, supports digestive health, and guards against several cancers (Musial et al., 2020)..

Urea is a molecule that is abundant in nature, and study of this compound is important for agricultural chemistry, food science, environmental monitoring, and medicinal treatment (Pundir et al., 2018). Urea is a small, two-atom, water-soluble byproduct of the metabolism of nitrogen and proteins with a molecular weight of 60 g/mol (Raymond et al., 2018). It is a tasteless, colourless solid that dissolves readily in water, without any acidic or alkaline properties, and is almost non-toxic (Wei et al., 2001; Dhawan et al., 2009). It has been created in the bodies of many different species as a byproduct of the urea cycle. The majority of food items also include urea, which increases the blood urea level. The objective of the current work is to utilise a biosensor to calculate the urea content of Tea.

A nickel enzyme called urease (EC 3.5.1.5) breaks down urea by converting it to ammonia (NH3), carbonic acid (H2CO3), and carbamic acid (H2NCOOH) via carbamic acid (Fig. 1).1. Bicarbonate (HCO3) and ammonium (NH4+) ions balance the amounts of carbonic acid and NH3 in aqueous solutions, respectively. Urease is produced by invertebrates, fungi, bacteria, and plants, and most species exhibit a high degree of structural and functional homology. The carbamylated lysine and hydroxyl group in the urease active site connect two Ni2+ ions (Savane et al., 2020).

Potentiometry employing polymeric membrane ion-selective electrodes (ISEs) is a well-established analytical method for evaluating the physiological status of key electrolytes. The advantages of potentiometric sensors include their small size, quick response time, ease of use, low cost, and resistance to turbid and coloured interferences. ISEs also feature a number of distinctive qualities. As opposed to other analytical methods that produce total concentration, they provide information on the concentration of free ions (ion activity). A significant decrease in sample volume has no effect on the detection limits because, at least theoretically, they are independent of sample volume. Jiawang and Wei (2020) claim that these features set ISEs apart as an indication electrode or detector.

As a critical indication for environmental and medical applications, ammonium ion concentration is of interest to academics from a wide range of sectors. As an illustration, ammonium is considered to be both a potential biomarker of an enzyme byproduct in important physiological reactions and a natural indication of water quality. Potentiometric ion-selective electrodes (ISEs), as an alternative to conventional analytical methods used to detect ammonium ions, have attracted the attention of the scientific community due to advantages like cost effectiveness, user friendliness, and miniaturisation ability, which enables simple portable measurements (Cuartero et al., 2020).

On multiple organs and tissues, the uremic toxin with the highest plasma concentration has a variety of direct and indirect effects. According to research by Colombo et al. (2023), the substance urea is harmful to the kidneys (indirectly promoting renal fibrosis), the fat cells (causing insulin resistance), other blood components (causing erythropoietin carbamylation), the circulatory system (CVS), and the digestive tract (causing epithelial barrier disintegrate and microbiome alteration).

The level of urea in cooked tea must be determined in order to assess its authenticity and quality. A variety of analytical procedures have been used to examine the presence of urea in foods and beverages. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have both been used to measure the concentrations of various compounds in tea samples (Malinowska and Szymandera, 2008). Spectroscopic methods such as Fourier-transform infrared spectroscopy (FTIR)

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

and near-infrared spectroscopy (NIRS) have shown promise as rapid and non-destructive procedures for analysis of tea. Additionally, many compounds has been identified and quantified using mass spectrometry (MS) methods such liquid chromatography-mass spectrometry (LC-MS) (Melini and Acquistucci, 2008, Köseoglu et al., 2016). But when the Academics are searched for determination of urea in drinkable tea, no such data or approach is available and the topic has been neglected so far. Though industry professionals have a variety of options for precisely detecting the urea level in Tea samples thanks to these several analytical procedures, we have developed a potentiometric biosensor using AISE with urease enzyme immobilized on nylon membrane. This methods offers advantages such as rapid response, compact size, cost-effectiveness, and resistance to interference, making them highly suitable for precise and efficient electrolyte level evaluation compared to other analytical techniques.

2. METHODOLOGY

2.1. Materials used: The reagents required for the procedures include urease, sodium phosphate buffer, tris-acetate buffer, ethanol, glutaraldehyde, cysteamine dihydrochloride, chitosan, methanol, deionized water, 0.1 M NaCl (reference filling solution), ISAB (Internal Standard Addition Buffer), 25% glutaraldehyde solution (for the preparation of 2.5% glutaraldehyde), Nessler's reagent, tri-chloroacetic acid (TCA), and absolute ethanol.

2.2. Instruments used: Digital ion meter, Water bath,, Sonicator, UV spectrophotometer, Weighing balance,Magnetic stirrer, Centrifuge, Ammonium ion selective electrode:, FTIR, FESEM.

2.3. Assay of free Urease enzyme

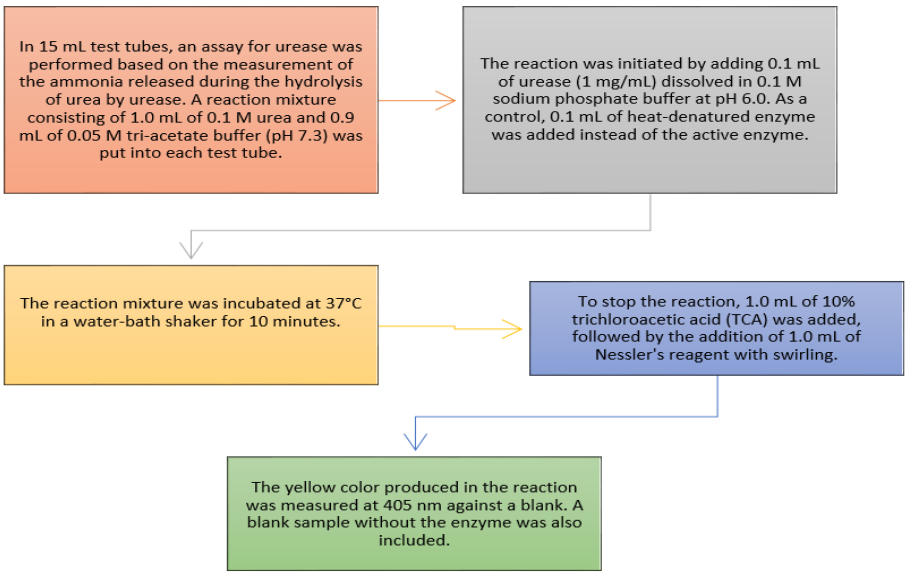

Figure 1: Stepwise representation of enzyme assay

The concentration of NH_4^+ generated throughout the experiment was extrapolated using a standard curve of NH_4^+ concentration vs. absorbance at 405 nm. One unit of enzyme activity was determined to be the amount of enzyme needed to release 1 mol of ammonia from urea hydrolysis in 1 minute under standard test conditions

2.4. Preparation of urease nanoparticles and immobilization: From SIGMA ALDRICH, the already isolated Urease enzyme was purchased. Using the technique outlined by Jakhar et al., 2019, the nano particles were created and immobilised on nylon membrane.

ISSN：1674-2974 | CN 43-1061 / N

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

2.5. Characterization of free urease nano-particles: FTIR and FESEM measurements were made to determine the size of the generated urease nanoparticles.

2.6. Characterization of urease immobilized nylon membrane: To confirm urease immobilisation on the nylon membrane, FESEM images of the nylon membrane were taken both before and after immobilisation.

2.7. Preparation of AISE electrode- The Ammonium Ion Selective Electrode (AISE) was provided by Labman. According to the electrode handbook, it was calibrated by dipping it in a 10% KCl solution for 30 minutes and then calibrating for consistent readings.

2.8. Optimization of Potentiometric Urea biosensor- The prepared urea biosensor was optimised for pH, temperature, effect of substrate concentration, response time, linear range, detection limit, analytical recovery, sensitivity, precision, reproducibility, storage stability, and interference of some metabolites in accordance with the procedure suggested by Jakhar and Pundir in their study from 2017.

2.9. Application of potentiometric urea biosensor in fermented alcoholic beverages

2.9.1. Collection of samples: as many samples of tea were collected from local market and online stores.

2.9.2. Evaluation of samples collected: A Labman Ammonium Ion Selective Electrode (AISE) was used to measure the ammonia released from the samples. The electrode was electrically connected to a Labman digital ion metre that showed the ammonia, pH, and potential readings. Each sample was subjected to a TISAB with a Labman electrode to release ammonia. Put the electrode in 20 ml of alcohol that has 1 cc of TISAB and stir to combine.

3. RESULTS AND DISCUSSION

3.1. Preparation of urease nano-particles:

At 4°C, urease nanoparticles (NPs) were produced by desolvation with ethanol. This procedure decreased the hydration layer surrounding urease molecules, boosting interactions such as Vander Waals, hydrophobic, and electrostatic forces, resulting in stable urease-NP aggregates. We cross-linked the aggregates using glutaraldehyde, interacting with -NH2 groups introduced via cysteamine dihydrochloride, to ensure their long-term stability and enzymatic activity. These urease-NP aggregates had considerably higher enzymatic activity, which was most likely due to increased active site exposure and possible structural changes during the aggregating process.

3.2. Characterization of urease nano-particles

FESEM was used to examine the the shape and dimensions of aggregates of urease NPs. The sizes of the urease NPs ranged from 90 to 100 nm, with an average diameter of 223 nm. In contrast, native monomeric urease had a diameter of 13 nm as measured by TEM in a 1992 study by Turbett et al. This finding suggests that each spherical urease NP was generated by the aggregation of 14 to 18 native urease molecules. At 4°C, urease nanoparticles (NPs) were produced using the desolvation procedure with ethanol. Lowering the moisture layer around the urease molecules caused the aggregation, allowing interactions like Vander Waals forces, hydrophobic forces, and electrostatic forces to occur. The aggregates were practically permanently cross-linked with glutaraldehyde, assisted by -NH2 groups from cysteamine dihydrochloride, while retaining their structures and enzymatic activity. The resulting urease-NP aggregates had dramatically increased enzymatic activity, which was most likely due to increased active site exposure and probable structural changes during aggregation.

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

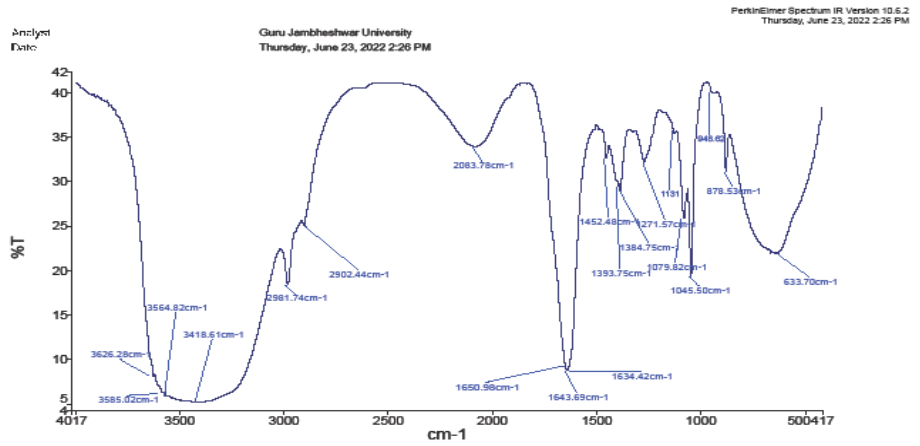

Figure 2: CURVE 1 FTIR graph of urease nanoparticles

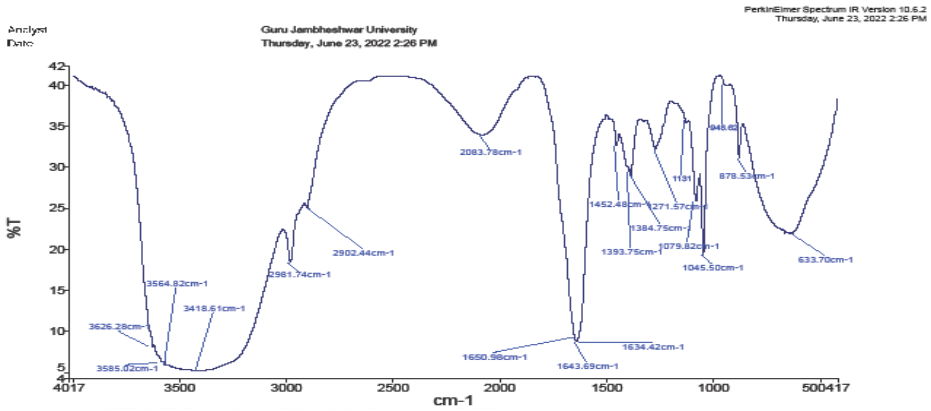

Figure 3: CURVE 2 FTIR graphs of urease nanoparticles

The curves observed in fig. 2 and 3 at different wavelengths indicated different bond stretching which are in similar trend as reported by Jakhar and Pundir in their paper in 2017.

3.3. Characterization of nylon membrane by FESEM

The untreated Nylon membrane's scanning electron microscopy (FESEM) pictures revealed a characteristic hollow beaded structure. In contrast, the Nylon membrane coated with aggregated urease nanoparticles had clusters of these nanoparticles scattered across the membrane's surface in bead-like patterns. This finding establishes the attachment and immobilisation of aggregates of urease-NPs on the Nylon membrane. The enzyme immobilised in this manner retained 86.71% of its initial activity, which was comparable to that of the native enzyme. Furthermore, the conjugation process produced a density of 1.64 mg/cm2. This result implies an increase in enzyme activity as a result of the covalent immobilisation of urease nanoparticles onto the Nylon membrane. The application of glutaraldehyde coupling, which coupled the amino groups of cysteamine-dihydrochloride made urease enzyme nps functional to the CHIT-decorated Nylon membrane, assisted immobilisation.

3.4. Construction of potentiometric urea biosensor

In order to construct a desired potentiometric biosensor for detection of urea, an ammonium ion selective electrode (AISE) was used in conjunction with a urease nanoparticle (NPs) aggregates-bound Nylon membrane. The Nylon membrane containing aggregation of urease NPs was attached to the lower, more sensitive area of the AISE in this biosensor setup. This integrated arrangement was then

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

linked to a digital ion metre. The potentiometric response was based on the AISE's properties. This method has several advantages, including a simple procedure, a reasonably quick response time, nondestructive examination, a large linear range with reasonable selectivity, and extensive use in quantifying different ions. A potential difference is formed over time in this example due to the changing concentration of NH4+ in the reaction buffer. This change in potential is caused by the urease's enzymatic hydrolysis of urea on the NC membrane, which results in the production of NH4+ and HCO3-. An ammonium ion selective electrode (NH4+ selective electrode) was used to measure this potential shift.

3.5. Optimization of urea biosensor:-

3.5.1. Optimization for Response time, temperature and pH

The biosensor's response time was evaluated at 10-second intervals from 10 seconds to 120 seconds. The biosensor based on immobilised urease NP aggregates revealed its maximal response at a pH of 5.5, which is much less than free urease, which performs best at a pH of 7.0. This shift towards lower pH for maximal activity could be due to a possible decrease in the availability of -NH2 functional groups within the enzyme structure. The optimal temperature for incubating the urease enzyme was discovered to be between 35 and 45°C, with the maximum activity recorded at 40°C. This temperature is higher than that of the native urease enzyme, which operates best at 25°C. The higher thermo-stability of the engineered nanoparticle (ENP) aggregates can be related to the increase in the optimal temperature of the urease enzyme. This improved stability is because of enzyme molecule aggregation and crosslinking with ENPs, which provides a more robust environment for the enzyme to function efficiently at elevated temperatures.

3.5.2. Effect of conc. of substrate (urea)

The biosensor's reaction and urea concentration were shown to have a hyperbolic correlation ranging from 1 to 350 M. The reaction was continuously steady above 0.025 mM, which is visible in fig.4. Notably, the present urea biosensor's working range was expanded from 0.001 to 0.08 mM. This range outperforms the capabilities of previous potentiometric urea biosensors shown in the table, demonstrating a significant improvement.

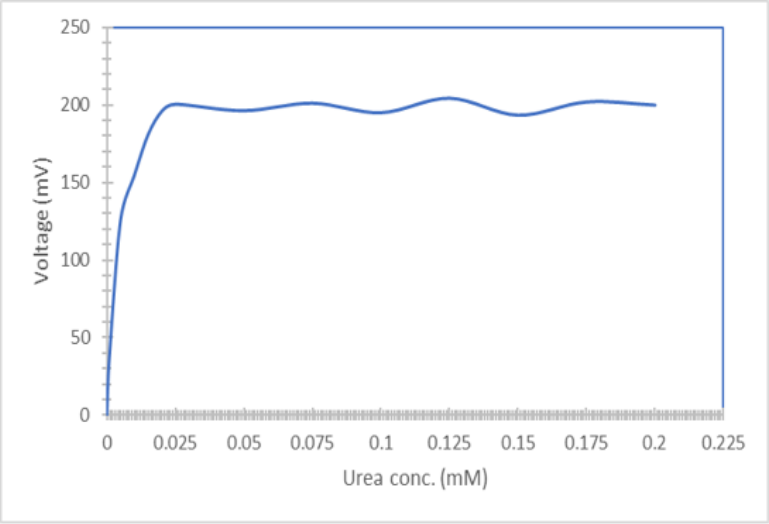

Figure 4: Voltage vs conc. Of urea graph for optimization of effect of conc. Of substrate

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DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

3.5.3. Lower detection limit

The current biosensor's detection limit was determined to be 1 mol/L, demonstrating its great sensitivity in monitoring urea contents. This detection limit is significantly lower than that of numerous previously published potentiometric urea biosensors based on different matrices, as shown in the table. It is also worth noting that the detection limit of the current biosensor beats reference methods, including the enzymic colorimetric method, which has a detection limit of 0.0005 M, which has been shown in fig 5. These findings point to the present biosensor's excellent potential for precisely monitoring urea amounts with high sensitivity and efficiency.

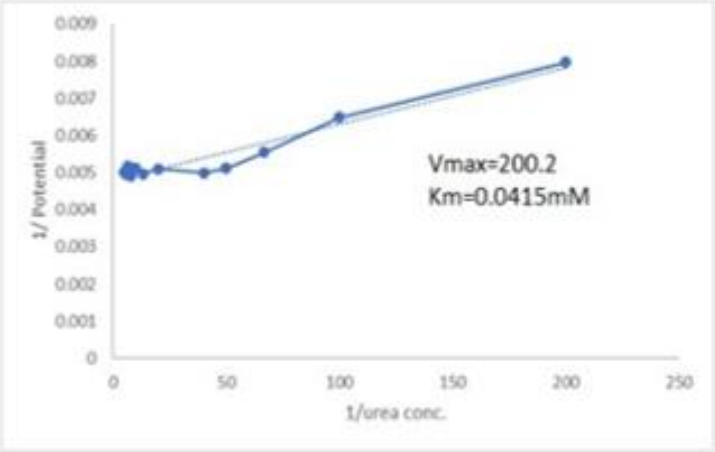

Figure 5: Graph indicating Vmax and Km of the experiment

3.5.4. Sensitivity

The current improved urea biosensor has a sensitivity of 38 mV/decade, demonstrating its higher performance when compared to previously reported potentiometric urea biosensors based on diverse materials and techniques

3.6. Urea conc. in tea samples

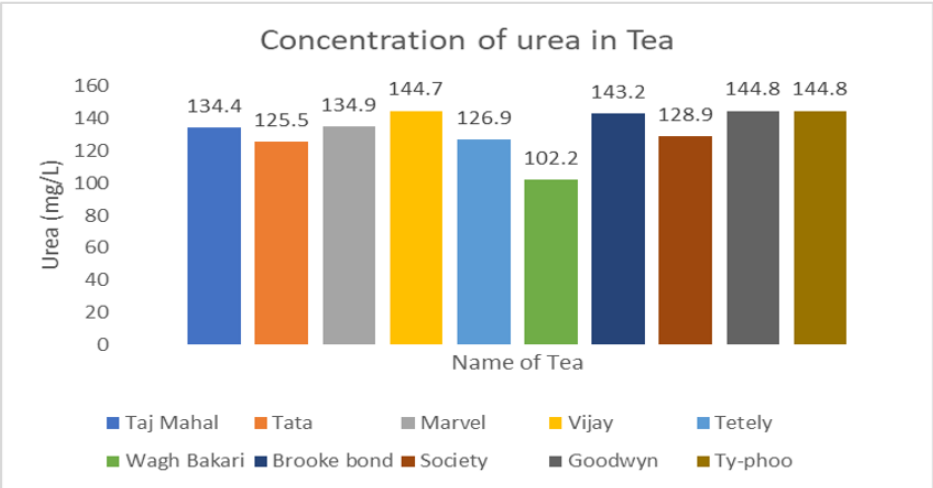

Figure 6: Graph indicating concentration of urea of different Tea.

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

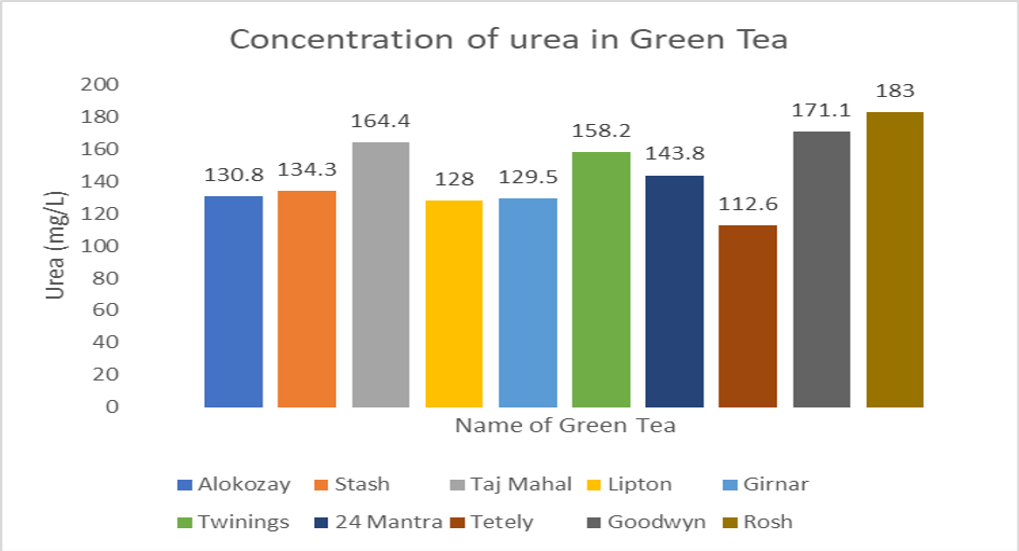

Figure 7: Graph indicating concentration of urea of different Green Tea

In the research study, the urea content (in milligrams per liter, mg/L) of various green tea brands was analyzed. Among the brands tested, Alokozay exhibited a urea content of 130.8 mg/L, while Stash contained 134.3 mg/L of urea. Taj Mahal tea was found to have a urea content of 164.4 mg/L, and Lipton tea contained 128.0 mg/L of urea. Additionally, Girnar tea was observed to contain 129.5 mg/L of urea, while Twinings had a urea content of 158.2 mg/L. The brand 24 Mantra exhibited a urea content of 143.8 mg/L, and Tetley contained 112.6 mg/L of urea. Goodwyn tea was found to have a urea content of 171.1 mg/L, and Rosh tea exhibited the highest urea content among the brands tested, with 183.0 mg/L of urea. The the urea content (in milligrams per liter, mg/L) of various tea brands was examined. Taj Mahal tea was found to contain 134.4 mg/L of urea, while Tata tea had a urea content of 125.5 mg/L. Marvel tea exhibited a urea content of 134.9 mg/L, and Vijay tea had 144.7 mg/L of urea. Tetley tea was observed to contain 126.9 mg/L of urea, while Wagh Bakari had a urea content of 102.2 mg/L. Brooke Bond tea exhibited 143.2 mg/L of urea, and Society tea contained 128.9 mg/L of urea. Goodwyn tea was found to have a urea content of 144.8 mg/L, similar to Ty-phoo tea, which also had 144.8 mg/L of urea. These results provide valuable data regarding the urea concentrations in these tea brands, which can be beneficial for consumers and the tea industry.

The results of the study demonstrated the successful development of a potentiometric urea biosensor using immobilized urease nanoparticles. The biosensor showed promising characteristics, such as a low detection limit of 1 mol/L, a wide linear range, and high sensitivity (38 mV/decade). These attributes make it a valuable tool for quantifying urea in green tea samples.

The optimization of various parameters, including pH and temperature, revealed that the biosensor exhibited optimal performance at a pH of 5.5 and a temperature of 40°C. This suggests that the immobilized urease nanoparticles had different optimal conditions compared to free urease, which typically operates at a pH of 7.0 and 25°C. The improved thermo-stability of the engineered nanoparticle (ENP) aggregates was attributed to the aggregation process, which provided a more stable environment for the enzyme to function at higher temperatures. The biosensor's response to varying urea concentrations was hyperbolic, with a working range expanded from 0.001 to 0.08 mM, surpassing the capabilities of previous potentiometric urea biosensors. The low detection limit of 1 mol/L indicates the biosensor's high sensitivity and efficiency in urea quantification.

DOI: 10.5281/zenodo.10785329
Vol: 61 | Issue: 03 | 2024

4. SUMMARY AND CONCLUSION

The study aimed to develop a potentiometric urea biosensor for the quantification of urea content in tea samples. Urease enzyme nanoparticles were prepared and immobilized on a nylon membrane, forming the basis of the biosensor. The biosensor's performance was optimized for various parameters, including pH, temperature, substrate concentration, response time, linear range, detection limit, sensitivity, precision, reproducibility, storage stability, and interference with metabolites. It was found that the biosensor exhibited excellent sensitivity, a wide linear range, and a low detection limit, making it suitable for accurate urea quantification.

In conclusion, the developed potentiometric urea biosensor based on immobilized urease nanoparticles offers a highly sensitive and efficient method for quantifying urea content in green tea samples. The biosensor exhibited excellent performance in terms of sensitivity, detection limit, and linear range. This research provides a valuable tool for monitoring urea levels in green tea, which can be significant for quality control and consumer safety in the tea industry. The optimized conditions for the biosensor's operation, such as pH and temperature, were also determined. Overall, this study contributes to the advancement of biosensor technology for urea analysis in food and beverage products. Regarding comparative studies, it is a novel work in its application part as determination of urea in tea is not paid much attention over other phenolic and non-phenolic compounds.

5. FUTURE PERSPECTIVES

Tea being one of the most widely consumed non- alcoholic caffeinated beverage after tea in society today, this study could be useful for both producers and consumers in improving and assessing the quality of this one of the most widely consumed beverages. But it also plays a big role in a number of health problems. Although Tea has been proven to be medically healthy in many aspects if consumed within the limited range, but this aspect is still untouched. Consequently, this study might help to reduce it a little bit. But this approach is not easily available and limited to laboratory only, reason being urease is a temperature sensitive enzyme and the cost of the electrode is not in reach of common man. Hence more work can be done in this field on cost effectiveness of electrode and preparation of easily available already immobilised bio-membranes.

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Vol: 61 | Issue: 03 | 2024

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