Research Article

An Amperometric Biosensor Based on Alanine Dehydrogenase for the Determination of Low Level of Ammonium Ion in Water

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1. Introduction

NH$_4^+$ ion is known as an essential element for plant growth at low concentration in the world’s water system. However, high concentration of this species can result from the incomplete degradation of biological waste and sewage by microorganisms, excretion by animals and plants, release of fertilizers, agricultural run-off, and industrial emissions [1–5]. Wee et al. [6] revealed that the mechanism of NH$_4^+$ toxicity in fish might be similar to that in mammals. NH$_4^+$ ion also plays an important role as a precursor for aerosol formation [7]. Thus, the development of a sensitive and selective analytical method for NH$_4^+$ ion is significant.

NH$_4^+$ ion can be a menace to public health as it can be an indicator of disorder or disease such as kidney disorder, stomach bacterial infection, or liver dysfunction by ingestion of NH$_4^+$-contaminated food [5, 8–10]. A chronically elevated concentration of NH$_4^+$ ion in blood can severely perturb brain function [11]. At the organismal level, NH$_4^+$ causes hyperventilation, hyperexcitability, coma, convulsions, and finally death. Determination of NH$_4^+$ ion in natural water is therefore of a significant interest in environmental biological studies and environmental evaluation of water since it is known to be toxic for aquatic organisms at concentration above 0.025 mg/L [4, 9, 12–14].

Due to an increasing environmental awareness and stricter regulations for pollution control, a large number of studies have been demonstrated to determine NH$_4^+$ ion in water. These include flow spectrophotometric [15], solvent extraction spectrophotometric [16], UV-VIS spectrophotometric [8], potentiometric [17, 18], flow injection analysis with fluorometric [19], amperometric methods [1, 9], molecular method with ammonia-oxidizing bacteria [20–22], continuous colorimetric assays [3, 23], and fluorometric [4, 24]. However, these methods are either time consuming or require elaborate preparation procedures. For instance, NH$_4^+$ ion determination based on spectrophotometric, fluorometric, and colorimetric methods although simple, economical, and easy for automation, they require pretreatment of the samples to avoid background interferences. The
most common technique used with optical measurement is the Berhelot’s reaction [3, 25]. But still, there are disadvantages such as slow kinetics, consumption of reagents, and the irreversibility of the reaction itself [4]. Whereas potentiometric involved selective electrode for NH$_4^+$ ion measurement, suffer interference from alkali metal ions [26, 27].

Enzymatic biosensors have been extensively applied in clinical, food, and environmental areas due to the advantages of fast detection speed, high selectivity, and sensitivity. Enzyme immobilisation onto electrode surfaces is the crucial step in fabricating electrical enzyme biosensors. The procedure used to immobilise enzymes should result in stability, allow diffusion of substrates and products, and ensure an efficient electron transfer [28]. In the present study, amperometric method involving modification of SPE by incorporating the AlaDH enzyme in photoHEMA membrane might offer rather simple, economical, reliable, rapid, sensitive, reproducible analytical procedures and control of the distribution and orientation of the immobilised enzymes. This considerably improved the sensitivity of the analysis when compared with the potentiometric-based procedures. Moreover, this method does not suffer from alkali metal ions (mainly potassium ion) interferences which affect the potentiometric nonactin-based NH$_4^+$ electrodes. Figure 1 represents the electrode design implying the mechanism of enzymatic reaction by immobilized AlaDH in photoHEMA.

In enzymology, the AlaDH enzyme catalyses the chemical reaction as shown in the following equation (see (1); [29]). AlaDH enzyme catalyses the reductive amination of pyruvate to L-alanine in the presence of cofactor NADH [30]. The enzyme has a fourfold affinity for pyruvate higher than that for L-alanine [31]. During enzymatic redox reaction, AlaDH enzyme consumes NH$_4^+$ ion for specific amination of pyruvate and NADH is oxidized to NAD$^+$. The electrochemical oxidation of NADH which occurs at working electrode produces two electrons (see (2); [32]) resulted in current raised proportional to the concentration of analyte NH$_4^+$. Thus, the signal was determined indirectly by monitoring the enzymatic consumption of NADH with amperometric method.

$$\text{Pyruvate} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{AlaDH}} \text{L-alanine} + \text{H}^+ + \text{NAD}^+ + \text{H}_2\text{O}$$  (1)

$$\text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2\text{e}^-$$  (2)

2. Experimental Section

2.1. Reagents. All chemicals used were of analytical grade and deionised water was used throughout for solution preparation. Stock solution of 0.0032 mg/mL L-alanine dehydrogenase enzyme (AlaDH, E.C. 1.4.1.1, from Bacillus subtilis) (Sigma) was prepared by mixing an appropriate amount of AlaDH enzyme solution with 10 mM phosphate buffer pH 7 in an appendorf tube and stored at 4°C [33]. 10 mM β-nicotinamide adenine dinucleotide, reduced form (NADH, 98%) from Sigma was prepared fresh with an ice cool 10 mM phosphate buffer pH 7 and used promptly. 50 mM sodium pyruvate (C$_3$H$_2$NaO$_3$, 99%) (Sigma) stock solution was prepared by dissolving an appropriate amount of pyruvate salt in deionised water. 10 mM phosphate buffer pH 7 was prepared by adding 10 mM dipotassium hydrogen phosphate (K$_2$HPO$_4$, 98%) (Fluka) to 10 mM potassium dihydrogen phosphate (KH$_2$PO$_4$, 99.5%) (Fluka) and adjusted to the required pH value [33].

A standard stock ammonia solution was prepared by dissolving the required amount of concentrated ammonia solution (NH$_3$, 25%) (MERCK) in the deionised water. The ammonia solution used has been standardised with Nessler method using ammonium chloride (NH$_4$Cl, 99.5%) salt. A homogeneous stock solution of monomer 2-hydroxyethyl methacrylate (C(CH$_3$)$_2$COOCH$_2$CH$_2$OH, 97%) (Aldrich) was prepared by mixing appropriate amounts of monomer HEMA and initiator 2,2-dimethoxy-2-phenylacetophene (DMPP) (C$_{16}$H$_{18}$O$_3$, 98%) (Fluka) in a vial and wrapped with aluminium foil. The mixture was then stirred gently for a few minutes and stored at 4°C.

2.2. Instrumentation. Chronoamperometric measurements were performed with an AUTOLAB PG12 (AUT 71681) Potentiostat/Galvanostat. Screen-printed carbon paste electrode (SPE) designed by Universiti Kebangsaan Malaysia and manufactured by Scrint Print Co was coated with immobilised AlaDH enzyme membrane and used as the working electrode. Ag/AgCl and glassy carbon electrode were used as the reference and auxiliary electrodes, respectively. A magnetic stirrer was used to stir the electrolyte during the amperometric measurements. Measurements of pH were made with pH-meter (MeterLab PHM 210) using a combined glass electrode. AlaDH enzyme-containing pHEMA membrane was prepared by UV-initiated photopolymerisation with an UV-exposure unit (RS Components 196–5251).

2.3. Construction of Biosensor. To fabricate the AlaDH enzyme electrode, a mixture of monomer HEMA and AlaDH enzyme of 5 μL was prepared in the ratio of 1 : 1 with the enzyme concentration of 0.6 units. The mixture was then deposited onto the SPE and exposed to long-wave ultraviolet radiation for 500 seconds with an extensive nitrogen (N$_2$) gas purging.

2.4. Optimisation of Biosensor Responses. Quantitative determination of NH$_4^+$ ion was performed by chronoamperometry using AlaDH enzyme electrode. The capability of the electrochemical biosensor was characterised in terms of optimal conditions, dynamic range, detection limit, reproducibility and repeatability studies, hydrodynamic study, and shelf life study. The studies were carried out at room temperature and 5 mL of 10 mM phosphate buffer pH 7 was used as the carrier solution under constant stirring condition (100 rpm). The substrates mixture containing NH$_4^+$ ion,
pyruvate, and NADH was added into the amperometric cell after a stable baseline obtained. The measurements taken were expressed as the current difference, defined as the stable current obtained after electrochemical reaction minus that of the stable baseline current. The measurements were carried out in triplicates.

The amperometric response of the AlaDH enzyme electrode was first studied with cyclic voltammetry between −1.00–1.00 V versus Ag/AgCl at a scan rate of 0.02 V/s. Next, the hydrodynamic study was conducted by changing the applied potential in the range of 0.45–0.80 V versus Ag/AgCl. The effect of pH against biosensor response was studied by varying the pH of the carrier solution (10 mM phosphate buffer) in the range of pH 5.8–8.0. Whereas the effect of enzyme loading was carried out by changing the enzyme loading in the pHEMA membrane in the range of 0.0900–0.8250 units.

To prepare enzyme electrodes with different membrane thicknesses, different mixtures of monomer HEMA and AlaDH enzyme (0.5–6 μL) were prepared in the ratio of 1 : 1 with the constant enzyme loading of 0.6 units. The thicknesses of the membranes were then determined using digital vernier caliper with 0.01 mm precision.

To determine the influence of temperature on biosensor response, the temperature was varied from 10°C to 50°C. For the optimisation of respective NADH and pyruvate concentrations, phosphate buffer pH 7 with various concentrations of pyruvate (0.001–0.05 mM) and NADH (0.02–0.3 mM) was used. After that, the effect on biosensor performance was investigated against different NH₄⁺ ion concentration (0.03–3.41 mg/L) and the concentration of pyruvate and NADH were kept constant.

The reproducibility of the biosensor was studied by measuring the response of different SPE to the same NH₄⁺ ion concentration at 0.68 mg/L. Whereas the repeatability of the biosensor was studied using the same SPE with the same NH₄⁺ ion concentration at 0.68 mg/L and the current difference signals were taken five times for each SPE. Shelf life study of AlaDH enzyme-based electrode was studied...
measuring 1.53 mg/L NH$_4^+$ ion using the same electrode at a time interval of 2 days until a plateau response obtained.

To assess the effect of alkali (Na$^+$, K$^+$), alkaline earth (Mg$^{2+}$, Ca$^{2+}$), heavy metal (Fe$^{3+}$), amines (methylamine, ethylamine), and sulphate ion (SO$_{4}^{2-}$) interferences, two-component mixtures of NH$_4^+$ ion with interferent in different molar ratio of interferent against NH$_4^+$ ion in the range of 10–5000 were analysed by amperometric method. The current differences obtained for interferent/NH$_4^+$ ion mixtures were compared with those for pure, single component solution (0.68 mg/L NH$_4^+$ ion) using t-test.

Finally, the constructed biosensors were applied for recovery test using various standard concentrations of NH$_4^+$ ion and real sample analysis of river water. The results were validated by using established procedure of Nessler method.

3. Results and Discussions

Figure 2 shows the typical cyclic voltammograms demonstrating the electrocatalytic activity of AlaDH enzyme electrode in aqueous solutions containing 10 mM phosphate buffer pH 7 in the absence and presence of substrates 1.70 mg/L NH$_4^+$ ion. The experiment was conducted in the presence of 0.3 mM pyruvate and NADH with a scan rate of 0.02 V/s versus Ag/AgCl electrode between potential range of −1.00–1.00 V. The addition of NH$_4^+$ ion gave rise to a higher current in the potential range of 0.4–0.9 V due to the electrocatalytic oxidation of NADH during enzymatic conversion of pyruvate to L-alanine in the presence of NH$_4^+$ ion whilst the enzyme redox reaction. Hence, it seemed possible to quantify NH$_4^+$ ion concentration indirectly by measuring the increased current of NADH oxidation.

In order to choose the best potential to be applied for amperometric determination of NH$_4^+$ ion, a hydrodynamic voltammogram was recorded in the potential range of 0.45–0.80 V versus Ag/AgCl electrode (Figure 3). At applied potential of +0.6 V, the current reached plateau and therefore, this potential was adopted for further studies. Bertocchi and Compagnone [32] have reported the same result trend
substrate, product and coenzymes will also be affected by pH changes [35]. In the present work, the maximum sensitivity of the immobilised AlaDH was observed at pH 7.0 (Figure 4). Schroder et al. [31] has also reported that the pH optimum of AlaDH for both the deamination and amination reactions was ~pH 7.0.

As observed in Figure 5, the response increased with an increase in the AlaDH enzyme loading of the membrane. It shows that the enzyme loading of 0.6 units was sufficient to obtain high enough response in this system. Kwan et al. [36] has used the same amount of AlaDH enzyme to be entrapped by a poly (carbamoyl) sulfonate (PCS) hydrogel on a Teflon membrane for amperometric determination of alanine. Table 1 shows the thicknesses of pHEMA membranes containing 0.6 units of AlaDH enzyme determined using digital vernier caliper with 0.01 mm precision.

The effect of membrane thickness against AlaDH electrode response is depicted in Figure 6. The optimum thickness of AlaDH enzyme-containing photoHEMA membrane was found to be 0.23 mm from 5μL matrix due to the lowest standard deviation to make the same electrode each time and gave consistent signal against same substrates concentration. Membrane thickness of 0.25 mm was not chosen for enzyme immobilisation in the subsequent works as the thick pHEMA membrane possessed more cross-link structures, which could prevent or slow the substrates from penetrating the membrane to effect the enzymatic reaction, and thus result in lower response.

Temperature is another parameter which must be taken into consideration for biosensor performance. Figure 7 shows the profile of NH₄⁺ biosensor response against temperature. The response increases with temperature and the optimum temperature is observed at 35°C. This increased stability at higher temperature might be explained by the polymer matrix acting as a kind of scaffold thus preventing denaturation due to extensive conformational changes [37]. Anyhow, high working temperature (30°C) cannot promote the sensor performance effectively due to the reduction of the availability of dissolved oxygen in the buffer for the enzymatic reactions and the promotions of slow denaturations of enzyme [36]. When the temperature shifted to 55°C, AlaDH enzyme lost about 70% of its original activity [29]. In the present work, 25°C was selected as working temperature in amperometric NH₄⁺ ion determination using glutamate dehydrogenase (GLDH) enzyme-based probe.

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their pH environment. This will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighbourhood of the active sites. Taken together, the changes in charges with pH affect the activity, structural stability, and solubility of the enzyme. The isoelectric point of AlaDH is at pH 6.7, at which the enzyme generally has minimum solubility in aqueous solutions and the net charge on the molecule is zero [34]. In a similar manner to the effect on enzymes, the charge and charge distribution on the substrate, product and coenzymes will also be affected by pH.

![Figure 9: Determination of optimum NADH concentration in electrolyte solution containing 0.01 mM pyruvate and 1.70 mg/L NH₄⁺ ion at pH 7 (n = 3).](image)

![Figure 10: The response curve of the AlaDH electrode towards different NH₄⁺ ion concentration in the range of 0.03–3.41 mg/L at pH 7 (n = 3).](image)

**Table 1: Thicknesses of pHEMA membrane containing 0.6 units of AlaDH enzyme determined using digital vernier caliper (n = 3).**

<table>
<thead>
<tr>
<th>Total volume of AlaDH and monomer HEMA mixture in volume ratio of 1:1 (μL)</th>
<th>Thickness of AlaDH-containing pHEMA membrane (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.08 ± (0.02)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.11 ± (0.02)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.13 ± (0.02)</td>
</tr>
<tr>
<td>3.0</td>
<td>0.18 ± (0.02)</td>
</tr>
<tr>
<td>4.0</td>
<td>0.20 ± (0.03)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.23 ± (0.01)</td>
</tr>
<tr>
<td>6.0</td>
<td>0.25 ± (0.01)</td>
</tr>
</tbody>
</table>
Table 2: Data for shelf life study of AlaDH enzyme-based electrode tested in solution containing 1.53 mg/L NH$_4^+$ ion, 0.25 mM NADH, and 0.01 mM pyruvate at pH 7 over a period of 11 days ($n = 3$).

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Current Difference ($\times 10^{-7}$ A)</th>
<th>Standard deviation</th>
<th>Relative response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.828</td>
<td>0.023</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>0.332</td>
<td>0.076</td>
<td>40.1</td>
</tr>
<tr>
<td>5</td>
<td>0.243</td>
<td>0.049</td>
<td>29.3</td>
</tr>
<tr>
<td>7</td>
<td>0.223</td>
<td>0.055</td>
<td>26.9</td>
</tr>
<tr>
<td>9</td>
<td>0.222</td>
<td>0.081</td>
<td>26.8</td>
</tr>
<tr>
<td>11</td>
<td>0.205</td>
<td>0.131</td>
<td>24.7</td>
</tr>
</tbody>
</table>

for all subsequent experiments since the sensitivity of the biosensor was sufficiently good for further studies.

To improve the performance of AlaDH electrode, various pyruvate and NADH concentrations were investigated. For the optimisation of respective pyruvate and NADH concentrations, the biosensor response to 1.70 mg/L NH$_4^+$ ion with various pyruvate and NADH concentrations was found to be 8.52 μg/L.

The response curve of NH$_4^+$ ion concentration determined using the AlaDH enzyme electrode is shown in Figure 10. A plateau region is achieved when the concentration of NH$_4^+$ ion is higher than 1.53 mg/L.

The plot of NH$_4^+$ ion concentration against current difference was linear in the NH$_4^+$ ion concentration range of 0.03–1.02 mg/L (Figure 11). The linear part of plot can be described by the regression equation $y = 0.000524x + 0.32$ with correlation coefficient, $R^2$ of 0.974. The LOD of NH$_4^+$ ion, defined here as the concentration equivalent to a signal of blank plus three times the standard deviation of the blank [38] was found to be 8.52 μg/L.

Kinetic parameters of the enzymatic reaction can be estimated by the direct linear method of the Lineweaver-Burk plot from the experimental data [39]. The apparent Michaelis-Menten constant ($K_m$) estimated from the Lineweaver-Burk plot was 0.029 mM for NH$_4^+$ ion (Figure 12).

Few methods for the amperometric determination of NH$_4^+$ ion have been developed and reported. The amperometric NH$_4^+$ electrode developed by Abass et al. [1] involved the use of SPE modified with Meldola's Blue over which is fixed a polycarbonate membrane. This sensor detect NH$_4^+$ ion indirectly by measuring the rate of decrease in current that was found to be dependent on the concentration of GLDH enzyme, 2-oxoglutarate, and NADH. The sensitivity of ammonium biosensor reported by Kwan et al. [9] using a immobilization matrix poly(carbamoyl) sulfonate hydrogel containing a biensyme system is lower when compared to the biosensor reported in this work (based on immobilised AlaDH in photoHEMA membrane) but the biosensor reported here demonstrated wider linear range of 0.029–4.26 mg/L. Another method for fluorometric NH$_4^+$ ion determination by ion chromatography (IC) using postcolumn derivatisation with o-phthaldialdehyde (OPA) under high concentrations of sodium and amino acids matrices has been developed by Kuo et al. [24] and showed good sensitivity with linear range of 0.85–85.17 μg/L and LOD of 0.85 μg/L. To remove amino acid interference, the postcolumn derivatization based on the reaction of ammonia with OPA and sulfite was applied. Thus, the ammonium biosensor developed in this work has advantage in terms of simple fabrication and application when compared to those reported so far and yet provided performance that is comparable or even better than those using other immobilization matrices.

The reproducibility and repeatability of the biosensor were evaluated at 0.68 mg/L NH$_4^+$ ion. The current difference measurement obtained using the same SPE yielded satisfactory repeatability RSD values in the range of 6.4–11.2% ($n = 5$). Whereas the measurement obtained using the different SPE gave very promising RSD values of 1.4–4.5%
solution is an important parameter a
increased the ionic strength of the medium and further
is because the presence of high concentration of ions had
[34].

will be influenced by the ionic composition of the medium
both the binding of charged substrates to enzymes and the
movement of charged molecules relative to each other. Thus
This is especially noticeable where catalysis depends on the
ratio of interferent over NH\(^+\) ion with the obtained
the biosensor response reduced significantly at high molar

In the present work, it was observed that alkali, alkaline
earth, heavy metal, and SO\(_4\)\(^-\) ions did not significantly
interfere with the quantitative determination NH\(_4\)\(^+\) ion
concentrations at low level (Table 3). The typical molar
ratio of interferent over NH\(_4\)\(^+\) ion in natural water is
low. So, the existence of interferent ions in concentration
higher than NH\(_4\)\(^+\) ion in water is not possible. However,
the biosensor response reduced significantly at high molar
ratio of interferent over NH\(_4\)\(^+\) ion with the obtained \(t\) value
higher than \(t\) critical value at 95% confident level. This
is because the presence of high concentration of ions had
increased the ionic strength of the medium and further
decreased the enzyme activity. The ionic strength of the
solution is an important parameter affecting enzyme activity.
This is especially noticeable where catalysis depends on the
movement of charged molecules relative to each other. Thus
both the binding of charged substrates to enzymes and the
movement of charged groups within the catalytic “active” site
will be influenced by the ionic composition of the medium
[34].

\(n = 5\) (Figure 13). These RSD were low enough to consider
that the membranes were reproducible using the proposed
method in the present work.

The shelf life of AlaDH enzyme-based electrode was
tested by measuring 1.53 mg/L NH\(_4\)\(^+\) ion at a time interval
of 2 days. Once measurements have been carried out, the
electrode was stored at 4°C in a refrigerator. After
2-day storage period, the biosensor sensitivity decreased
faster, reaching value of approximately 40.1% of the initial
sensitivity and continuously to 29.3% at day 5 and no
significant current differences were observed until 11-day
storage period (Figure 14). Data for shelf life study of AlaDH
enzyme-based electrode is given .5ptin Table 2.

An amperometric biosensor employing AlaDH enzyme
has been developed by Kwan et al. [36] for the determination
of alanine. This method had an operational stability of 2
days. However, the method has some problems due to other
combination salicylate hydroxylase and pyruvate oxidase
enzymes which regulate the buffer pH of the measuring
solution.

In the present work, it was observed that alkali, alkaline
earth, heavy metal, and SO\(_4\)\(^-\) ions did not significantly
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movement of charged molecules relative to each other. Thus
both the binding of charged substrates to enzymes and the
movement of charged groups within the catalytic “active” site
will be influenced by the ionic composition of the medium
[34].

![Figure 13: The reproducibility and repeatability of AlaDH enzyme
electrodes exposed to 0.68 mg/L NH\(_4\)\(^+\) ion at pH 7 \(n = 5\).](image-url)

The enzyme was found can be stabilised by K\(^+\) ion, but
the degree of stabilisation was lower than by Na\(^+\) ion. It could
be speculated that the stabilisation depends on the hydration
effect by these alkali metal ion. However, the hydration
potentials of the ions are in the order of Hofmeister’s series:
Na\(^+\) > K\(^+\) [29]. This is consistent with the order of the
interference effect observed: K\(^+\) > Na\(^+\), Fe\(^{3+}\) and Ca\(^{2+}\) ions
interfered significantly at molar ratio of interferent over
NH\(_4\)\(^+\) ion of 100 due to the formation of the insoluble
precipitation of Fe(OH)\(_3\) and Ca(OH)\(_2\) [40, 41] and has
lowered the biosensor reproducibility.

Determination of NH\(_4\)\(^+\) ion by amperometric method
can also affected by amines, particularly methylamine

Table 3: The interference effect of Na\(^+\), K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{3+}\), SO\(_4\)\(^{2-}\), CH\(_3\)NH\(_2\), and C\(_2\)H\(_5\)NH\(_2\) at different molar ratio of interferent to NH\(_4\)\(^+\) ion on the determination of 0.68 mg/L NH\(_4\)\(^+\) ion using AlaDH electrode at pH 7 \(n = 3\).

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Current difference (×10(^{-7}) A) at different molar ratios of interferent: NH(_4)(^+) ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>(0.560 \pm 0.025) (0.558 \pm 0.033) (0.589 \pm 0.027) (0.531 \pm 0.012) (0.313 \pm 0.083^*)</td>
</tr>
<tr>
<td>K(^+)</td>
<td>(0.560 \pm 0.025) (0.522 \pm 0.036) (0.164 \pm 0.047^<em>) (0.527 \pm 0.033) (0.187 \pm 0.077^</em>)</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>(0.560 \pm 0.025) (0.567 \pm 0.006) (0.535 \pm 0.017) (0.260 \pm 0.165^*) (0.291 \pm 0.066)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>(0.560 \pm 0.025) (0.556 \pm 0.043) (0.449 \pm 0.040^<em>) (0.173 \pm 0.017^</em>) (0.893 \pm 0.037)</td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>(0.560 \pm 0.025) (0.616 \pm 0.024) (0.173 \pm 0.017^<em>) (0.260 \pm 0.165^</em>) (0.291 \pm 0.066)</td>
</tr>
<tr>
<td>SO(_4)(^{2-})</td>
<td>(0.560 \pm 0.025) (0.603 \pm 0.032) (0.260 \pm 0.165^<em>) (0.260 \pm 0.165^</em>) (0.291 \pm 0.066)</td>
</tr>
<tr>
<td>CH(_3)NH(_2)</td>
<td>(0.560 \pm 0.025) (0.626 \pm 0.014^*) (0.893 \pm 0.037) (0.893 \pm 0.037) (0.893 \pm 0.037)</td>
</tr>
<tr>
<td>C(_2)H(_5)NH(_2)</td>
<td>(0.560 \pm 0.025) (0.760 \pm 0.009^*) (0.806 \pm 0.012) (0.806 \pm 0.012) (0.806 \pm 0.012)</td>
</tr>
</tbody>
</table>

\(^*\) \(t\) value > \(t\) critical value at 95% confident level with 4 degrees of freedom.
(CH₃NH₂) and ethylamine (C₂H₅NH₂) which exhibit similar physicochemical properties to NH₄⁺ ion. However, amine interferences were unexpected in natural waters due to their low content, usually at least 100 times lower than NH₄⁺ ion [23].

The performance of NH₄⁺ biosensor has been evaluated using diluted river water samples that spiked with known NH₄⁺ ion concentrations in the range of 0.17–1.02 mg/L. Table 4 shows the results of the performance of biosensor response against different river water samples spiked with known NH₄⁺ ion concentration. The average recovery values obtained were close to 100% within experimental error. Thus, NH₄⁺ biosensor developed in this study has been validated with the standard method of Nessler method. The large variations in the standard deviations observed between the spiked concentrations and the biosensor response in some samples, for example, sample 1 and 2 (Table 4), were most probably owing to the inconsistency in the spiking technique used especially when lower concentrations of ammonium ion is involved.

To ensure whether the analytical results obtained by the proposed method give reliable results when used for quantitative determination of NH₄⁺ ion, river water samples with appropriate dilution were measured by both methods. Table 5 shows the results of the comparative study of the biosensor and Nessler method. The common t-test was applied to examine whether the two methods give results that differed significantly at 95% confident level.

The results showed that the calculated t-test values were less than the t critical value of 2.776 with 4 degrees of freedom. The result shows that both methods have no statistically significant difference for NH₄⁺ ion determination. This may be due to the lack of interferences from impurities in the real sample against the developed biosensor. This indicates that the results from both methods are comparable.

### 4. Conclusion

The optimised AlaDH enzyme-based electrode was found useful for the sensitive measurement of NH₄⁺ ion. In addition, the reproducibility and repeatability of measurements of the electrode was reasonable. The preparation of AlaDH enzyme-containing membrane on SPE involved simple photolithography technique. This may be considered disposable as the fabrication method allows mass production at low cost. The electrode exhibited a high sensitivity (LOD, 8.52 μg/L) and a dynamic range between 0.03–1.02 mg/L. A good agreement between the biosensor and Nessler method demonstrated the high practically and accuracy of the biosensor in the analysis of water samples.

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