A CONDUCTOMETRIC METHOD FOR DETERMINATION OF ARSENIC IONS (As^{3+}) BASED ON INHIBITION OF UREASE ACTIVITY

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Abstract: This study describes a conductometric inhibition assay for the quantitative determination of arsenic ions in aqueous systems using soybean (*Glycine max*) urease. In this approach, the decrease in conductivity as a consequence of inhibition of urease activity by As (III) ions was evaluated and used to plot a calibration curve of %inhibition against arsenic concentration. The curve displayed linear relationship in the concentration range of 0.01 – 10 mg/L according to the regression equation 4.1427x + 41.0238 with an R^2 of 99.77% which shows very good correlation. Optimization of process parameters revealed pH maxima within the range of 7.0 – 7.5 and temperature within the range of 35 – 45°C. The determination of arsenic in synthetic water samples using the proposed method was satisfactory when compared with a spectrophotometric reference method and AAS analysis. The results showed no significant difference in 80% of the evaluated samples at 95% confidence level. Repeatability (precision) of the method was 4.8% RSD for 6 measurements (0.5 mg/L). The relative accuracy of the method ranged from 73.4 – 97.85%. Limit of detection (LOD) and limit of quantitation (LOQ) were 0.043 mg/L and 0.145 mg/L, respectively. Overall, the method presented in this study shows good potential for rapid determination of arsenic in water samples.

Keywords: Arsenic, assay, conductometry, inhibition, soybean, urease

Introduction

Arsenic is a toxic metalloid often classified as a heavy metal. It enters the biosphere from both geological and anthropogenic sources and is ubiquitously distributed throughout the Earth's crust, soil, sediments, water, and air (Mandal and Suzuki, 2002). Exposure of humans to arsenic occurs through the smelting industry, the use of gallium arsenide in the microelectronics industry, and the use of arsenic in common products such as wood preservatives, pesticides, herbicides, fungicides, and paints. Widespread dispersion of arsenic may also result from the combustion of fossil fuels in which it is present as a common contaminant (Howard, 2002). Arsenic is a Group 1 human carcinogen (Naujokas *et al.*, 2013) which ranks first on the Environmental Protection Agency (EPA) Priority List of Hazardous Substances (ATSDR, 2011). Acute arsenic poisoning is famous for its lethality, which stems from the destruction of the integrity of blood vessels and gastrointestinal tissue and its effect on the heart and brain. Chronic exposure to low levels of arsenic results in peripheral nerve and blood vessel damage and elevated risk for developing a number of cancers, most notably skin cancer, cancers of the liver, lung, bladder, and possibly the kidney and colon (Howard, 2002).

The ubiquity and high toxicity of arsenic calls for the development of simple and fast methods for its detection in different matrices. The current methods for analysing the metalloid involve techniques that are time consuming, expensive, requiring sophisticated instrumentation, laboratory facilities and trained staff. Such methods are not suitable for in-situ testing and monitoring. As a complement to the traditional analytical methods, there is need to develop rapid and simple methods that can be used for routine analysis of arsenic.

Bio-analytical systems have been shown to provide rapid measurements for the analysis of a wide range of toxic metals and related compounds without the complexities associated with the use of analytical equipments. Many workers have demonstrated the use of enzymes in assay systems for the inhibitive determination of heavy metals. The enzymes used include; peroxidases, xanthine oxidase, invertase, glucose oxidase, proteases, trypsin (Safar *et al.*, 2002), papain (Shukor *et al.*, 2006), acetylcholinesterase (Sabullah, 2013), molybdenum-reducing enzyme (Shukor *et al.*, 2009) glutamic dehydrogenase (Rodriguez *et al.*, 2004) and urease (Wolfbeis and Preininger, 1995; Prakash *et al*., 2008) among others. Urease (urea amidohydrolase, E.C. 3.5.1.5) is the most frequently applied enzyme for heavy metal determination. It is a nickel-containing enzyme that catalyses the hydrolysis of urea into ammonia and carbon dioxide. Studies have shown that the catalytic activity of urease is inhibited in the presence of heavy metal ions and this characteristic has been extensively employed for the determination of the metals. Arsenic ions have been shown to inactivate urease by reacting with the enzyme's sulfhydryl groups which results in the formation of arsenic sulphide (Bhattacharyya *et al.*, 2007)

The enzyme urease has been isolated and characterized from various bacteria, fungi, and plants (Mobley *et al.*, 1995; Khan *et al*, 2013; Banerjee & Aggarwal, 2012; Follmer, 2008).Plant ureases hold a special place in sciencehistory because of their roles in some important landmarks of biochemistry with soybean urease being the subject of many investigations after the first findings that crude extracts of soybean (*Glycine max*) seeds contain high amounts of urease (Takeuchi, 1909). Although several studies (Kumar and Kayastha, 2010; Khan *et al.*, 2013) have characterised ureases from soybean seeds, very few have explored its applications in bioassay systems.

The use of soybean urease in bioassay systems could generate novel bio-analytical platforms with marked advantages such as cost effectiveness and stability of enzyme activity by being maintained in its natural environment. In this study, we report for the first time, the use of crude soybean urease in an inhibitive conductometric assay for the determination of arsenic ions in aqueous systems.

Materials and Methods

Materials

Sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide (BDH chemicals) ammonium sulphate, Urea (JDH chemicals), arsenic (III) standard solution, 1000 mg L^{-1}, hydrochloric acid (Merck, Germany), potassium nitrate (Burgoyne, india), Nessler's reagent was prepared from potassium iodide (Qualikems, Nigeria) and mercuric chloride (Sigma-Aldrich). Soybean seeds were purchased from the local market in Wukari, Nigeria. All chemicals are of

analytical grade and were used without further purification and all solutions were prepared with double distilled water.

Extraction of Urease

Urease was extracted from soybeans according to a slightly modified method of Khan *et al.,* (2013). Powdered soybean seeds (10 g) were soaked overnight in 100 ml extraction buffer (0.2 M sodium phosphate buffer pH 7.3) at 4ºC (Refrigeration temperature). The mixture was sieved through four layers of muslin cloth and the resulting filtrate was centrifuged at 4000 rpm for 30 min. The clear supernatant was collected and used as the crude urease extract (stored at 4ºC) while the pellets were discarded

Determination of urease activity

The catalytic activity of the extracted urease was determined by measuring the amount of ammonia released from the urea-urease reaction. The method described by Sharma *et al.,* (2009) was used with slight modifications. To 0.8 mL of assay buffer (0.2 M sodium phosphate buffer, pH 7.3), 0.2 mL of appropriately diluted urease extract was added and incubated at 37°C with 1 mL of urea solution (0.25 M in phosphate buffer). After 10 minutes, the reaction was terminated by addition of 1 mL of 0.1 M HCl. The reaction mixture was made up to 50 mL with distilled water, 2 mL of Nessler's reagent was added and the absorbance was read against a blank at 405 nm on a uv-vis spectrophotometer (APEL PD 3000).The estimation of ammonia released was carried out using an ammonium sulphate standard curve. Urease activity was estimated as µM of ammonia released. One unit of urease activity is defined as the amount of enzyme required to liberate 1 µmol of NH_3 from urea per minute under the test conditions (0.25 M urea, 0.2 M buffer, pH 7.3, 37°C).

Conductometric Inhibition Assay for Arsenic

The conductometric assay is based on measuring the decrease in conductivity that results from the inhibitory effects of As^{3+} on urease activity. Five different standard solutions of arsenic (III) ions with concentration ranging from 0.001-10 mg/L were prepared by serial dilution from a 1000 ppm stock and their inhibitory effects were tested on the extracted urease. In the procedure adopted for this study, 0.2 mL arsenic, 0.2 mL enzyme extract and 0.6 mL sodium phosphate buffer (0.2M) were pre-incubated for 15mins after which 1 mL of urea solution (0.25M) was added. The assay cocktail was left for another 10 mins and then placed immediately on ice water to stop reaction. The volume was made up to 40 mL and the conductivity of the resulting solution was taken with ORION conductivity/TDS meter (model 240). The conductivity of a blank solution was obtained and appropriate corrections were applied. Similar assays were also carried out in the absence of the inhibitor (arsenic) where distilled water was used in place of the As^{3+} solution. The level of inhibition for each tested concentration was obtained using the relationship in equation 1.

$$\% \text{ inhibition} = [Co - Ci/Co] \times 100 \quad \text{---------- (1)}$$

Where; Co is conductivity (mS/cm) obtained in the absence of the inhibitor (Arsenic)

Ci is conductivity (mS/cm) obtained after incubation with Arsenic ion

A graph of % inhibition against Arsenic concentration was plotted and used as calibration curve for estimation of unknown concentration.

Determination of Arsenic in Synthetic samples

The concentration of arsenic was determined in 10 synthetic water samples which were prepared by adding unknown quantities of arsenic solution to distilled water. Conductivity measurements of the levels of urease inhibition by the samples were obtained as described in above. The concentrations were then obtained from the standard calibration plot of %inhibition against concentration.

Method Validation

To validate the conductometric inhibition method, the arsenic contents of the synthetic samples were also estimated via a standard spectophotometric inhibition assay and Atomic Absorption spectroscopy (AAS).

Spectrophotometric assay

The spectrophotometric method is based on the standard assay for urease which measures the ammonia produced from the urea- urease reaction. The assay was carried out by determining the amount of ammonia released after pre-incubating the enzyme with various standard solutions of As^{3+} (0.001 – 10 obtained mg/L). Decrease in absorbance as a consequence of inhibition by As^{3+} was obtained spectrophotometrically and the percentage inhibition for each tested concentration was calculated by comparing the absorbance before and after incubation with arsenic ions using equation 2.

$$\text{Inhibition (\%)} = [Ao - Ai/Ao] \times 100 \quad \text{---------- (2)}$$

Where, Ao and Ai are the absorbances with and without incubation with arsenic ions respectively. A calibration graph of % inhibition against concentration of inhibitor (arsenic) was plotted and used to estimate the arsenic contents of the synthetic water samples which were tested in the same way.

AAS Analysis

Determination of arsenic in the synthetic samples was done using atomic absorption spectrophotometer AA-6800 (Shimadzu, Japan). A calibration curve of absorbance against concentration was prepared by running different standard solutions of arsenic and the concentrations of the samples were estimated from the standard calibration curve.

Optimization of Assay Parameters

The dependence of the conductometric assay on temperature, pH, incubation time and ionic strength was investigated and optimized; Effect of temperature was determined over a range of 20 to 55°C, pH effects were investigated over a pH range of 6.0 to 8.5 with an increment of 0.5 while keeping other parameters constant. The dependence of the system on ionic strength was evaluated in the presence of varying concentrations of KNO_3 (0.005 – 1 mM). The optimum time for the enzyme incubation with the analyte (arsenic) was evaluated at 5 minutes interval over a period of 30 min.

Results and Discussions

The quantitation of Arsenic has been achieved by measuring the decreased activity of soybean urease in the presence of Arsenic ions. Inhibition of urease activity by arsenic is ascribed to the interaction of the ion with the sulfhydryl (S-H) group of the enzyme (Bhattacharyya *et al.,* 2007). The sulfhydryl groups of many enzymes can form tight bonds with some heavy metal ions. When this happens, the enzyme cannot function well in catalysis hence there will be a marked decrease in its activity. Measurements of the level of inhibition by the metal ions offer a unique and simple tool for their quantification. Usually a graph of percentage inhibition against concentrations of the inhibitor (metal ion) is plotted and used as calibration curve for estimating the metal concentration (Rodriguez *et al,* 2004; Wang *et al*., 2009; Stepurska *et al,* 2015). Urease catalyses the hydrolysis of urea according to the reaction in equation 3

$$NH_2CONH_2 + 3H_2O \xrightarrow{Urease} HCO_3^- + 2NH_4^+ + 2OH^- \text{----- (3)}$$

The net effect of this reaction is an increase in charged products hence a rise in conductivity. Arsenic ion which is a known inhibitor of urease activity (Talat *et al.,* 2009) can cause a decrease in the resulting conductivity when introduced into the reaction medium. This forms the basis for the indirect estimation of As^{3+} content via conductometric evaluation of its inhibitory effects on urease.

The inhibition data obtained for arsenic standards is presented in Table 1. The results show a concentration dependent inhibition of urease activity by arsenic (III) ions within the

concentration range of 0.001 – 10 mg/L. The lowest tested concentration (0.001 mg/L) gave 20.95 % inhibition while the highest inhibition (observed at 10 mg/L) was 82.43%. Our projections from the obtained data suggest that 100% inhibition of urease activity would be attained at 14.24 mg/L arsenic concentration.

Table 1: Conductometric inhibition assay for arsenic

As^{3+} Concentration (mg/L)	Conductivity (mS/cm)	Inhibition (%)
0.00 (Control)	1.48 ± 0.04	0
0.001	1.17 ± 0.09	20.95
0.01	0.89 ± 0.11	39.86
0.1	0.85 ± 0.06	42.56
1	0.81 ± 0.01	45.27
10	0.26 ± 0.03	82.43

Conductivity values are presented as mean ± SD for three determinations

The calibration graph constructed by plotting percentage inhibition of urease activity against As^{3+} ion concentration is shown in Fig. 1. The plot revealed linearity for arsenic concentration in the range of 0.01 – 10 mg/L with regression equation 4.1427x + 41.0238. The slope of the calibration line reflects the sensitivity of the system. Sensitivity in this situation refers to the change in signal for a given change in concentration ($\Delta S/\Delta C$). The steeper the slope, the more sensitive the procedure i.e., the stronger the response on y-axis to a concentration change. The slope obtained in this study (4.1427) indicates that for any mg/L change in concentration, there is a 4.1427 % change in inhibition. A good correlation was established for the calibration plot as reflected by the obtained R^2 value (99.77 %). Our findings showed that the inhibition at 0.001 mg/L is not within the linear range of the calibration plot. This was noted from the large decrease in R^2 value (departure from linearity) that resulted from its inclusion in the linear model.

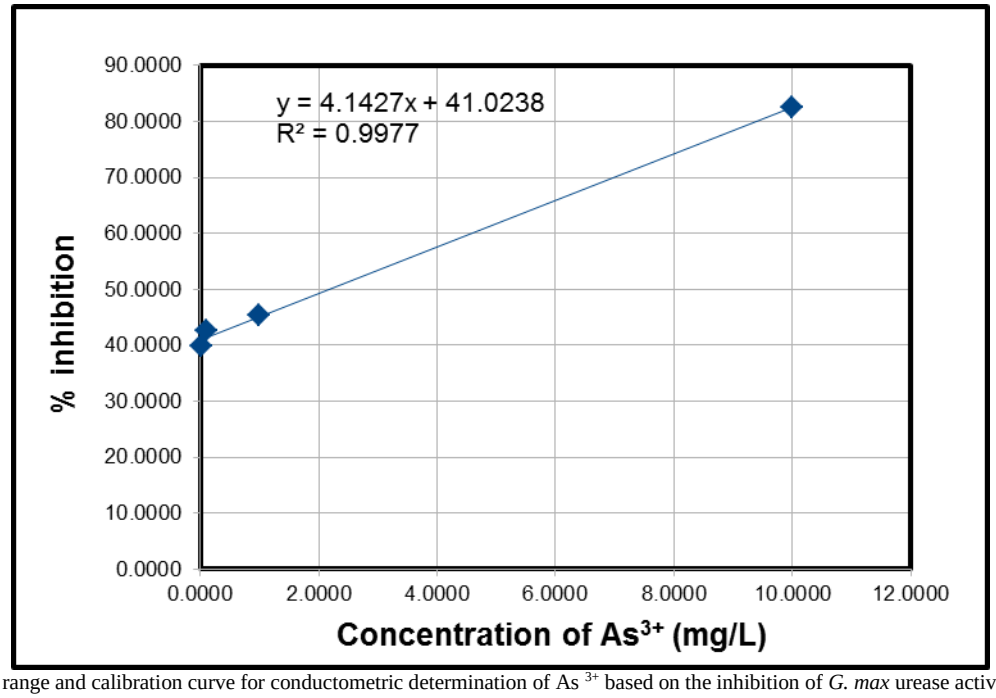

Fig. 1: Linear range and calibration curve for conductometric determination of As^{3+} based on the inhibition of *G. max* urease activity

To validate the conductometric protocol, similar inhibition assays for As^{3+} standards were carried via a standard spectrophotometric method. The procedure measures the absorbances obtained upon nesslerization of the ammonia released by the catalytic activity of urease. In the presence of As^{3+}, decreased absorbances were obtained due to decreased production of ammonia. The results of the spectrophotometric assay (Table 2) showed a % inhibition range of 24.47 – 88.59. The spectrophotometric method appears to be more sensitive than the conductometric procedure as evidenced by the higher % inhibitions recorded for all the investigated standards and the higher slope value (4.598 against 4.143). However, the data from the two methods are comparable.

Table 2: Spectrophotometric Inhibition Assay for Arsenic Standards

As^{3+} Concentration (mg/L)	Absorbance (at 400 nm)	Inhibition (%)
0.00 (Control)	1.718 ± 0.04	0
0.001	1.301 ± 0.09	24.27
0.01	1.007 ± 0.12	41.38
0.1	0.967 ± 0.06	43.71
1	0.889 ± 0.11	48.25
10	0.196 ± 0.03	88.59

Absorbances are presented as mean ± SD for three determinations

Further, validation of the method was done with respect to its applicability for quantitative determination of arsenic. 10 synthetic samples of arsenic were analysed via the conductometric assay and the results were compared with those obtained from AAS analysis and the spectrophotometric inhibition assay (Table 3).

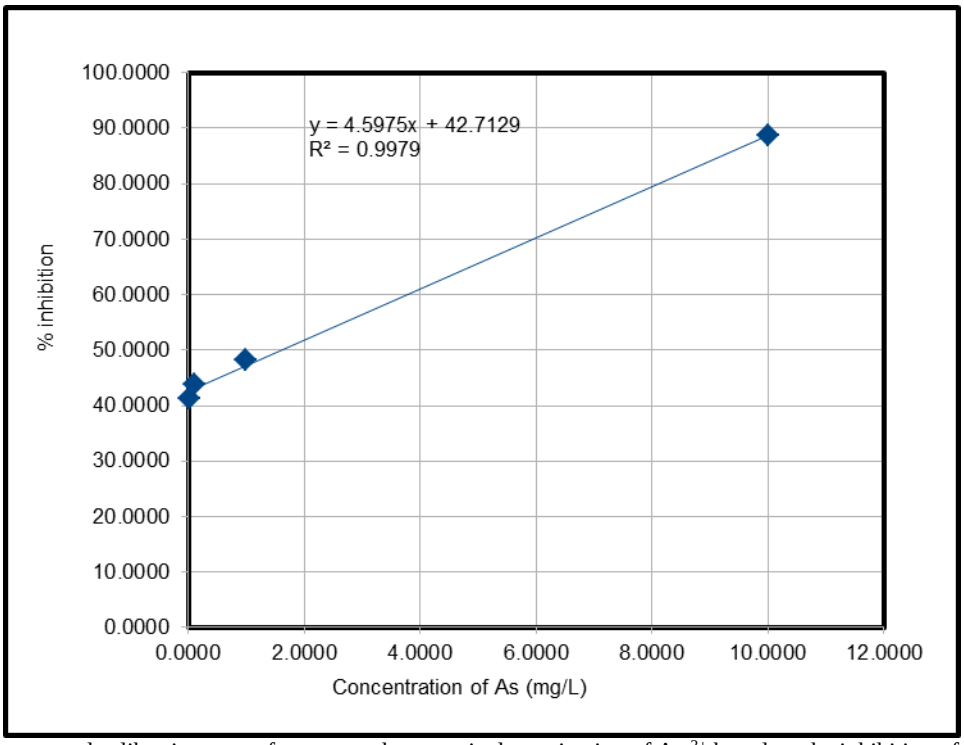

Fig. 2: Linear range and calibration curve for spectrophotometric determination of As $^{3+}$ based on the inhibition of *G. max* urease activity

Table 3: Comparative evaluation of arsenic contents of synthetic water samples measured using three methods (A, B and C)

Samples	Measured concentration (mg/L)			calculated t-tests		
	Atomic absorption spectroscopy (A)	Spectrometric inhibition assay (B)	Conductometric inhibition assay (C)	A&B	A&C	B&C
As-A	1.734 ± 0.04	1.711 ± 0.12	1.711 ± 0.06	0.315	0.067	0.00
As-B	1.558 ± 0.02	1.596 ± 0.04	1.433 ± 0.12	1.472	2.626	2.232
As-C	1.645 ± 0.01	1.622 ± 0.05	1.628 ± 0.04	0.781	0.714	0.162
As-D	2.292 ± 0.06	2.268 ± 0.11	2.232 ± 0.03	0.332	1.549	0.547
As-E	2.223 ± 0.16	2.205 ± 0.14	2.201 ± 0.10	0.146	0.202	0.04
As-F	2.255 ± 0.08	2.244 ± 0.06	2.034 ± 0.05	0.191	4.056*	4.657*
As-G	2.268 ± 0.03	2.166 ± 0.03	2.002 ± 0.13	4.164*	3.453*	2.129
As-H	1.897 ± 0.05	1.877 ± 0.02	1.872 ± 0.07	0.643	0.503	0.119
As-I	1.861 ± 0.02	1.850 ± 0.06	1.694 ± 0.06	0.301	4.574*	3.184*
As-J	1.862 ± 0.12	1.863 ± 0.09	1.852 ± 0.03	0.012	0.140	0.201

Values are presented as mean ± SD for three determination; applicable; *=significantly different at 95% confidence level

Our findings revealed a concentration range of 1.558 – 2.292 mg/L from AAS analysis, 1.596 – 2.268 mg/L from the spectrophotometric method and 1.433 – 2.232 mg/L from the conductometric method. The T-test evaluation of the results showed that 90% of the data from the spectrophometric method and AAS analysis are not significantly different at 95% confidence level. The Conductometric method showed 70% consistency with the AAS method and 80% with the spectrophotometric assay method. Overall, only 20% of the results were significantly different at 95% confidence level. This indicates good agreement between the three methods and demonstrates the analytical capability of the conductometric assay.

Optimization of process parameters such as temperature, pH, ionic strength and incubation time was done prior to the analysis in order to achieve maximum working capacity of the system. The effect of temperature on the urease activity assay is presented in Fig. 3. Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases with increase in temperature. However, very high temperatures can cause protein denaturation and subsequent decrease in enzyme activity. It was observed that the system exhibited optimum activity around 35 - 45°C. Further increase in temperature to 50°C, resulted in a marked decrease in enzyme activity (62% loss of activity). All determinations were carried out at 37°C.

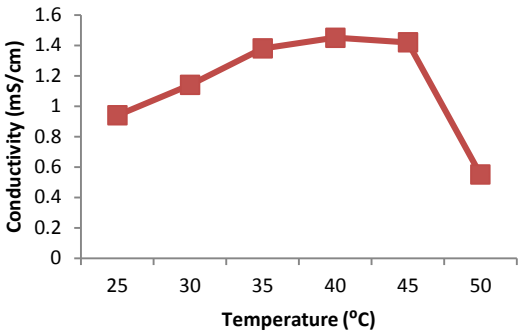

Fig. 3: Effect of temperature on conductometric urease assay

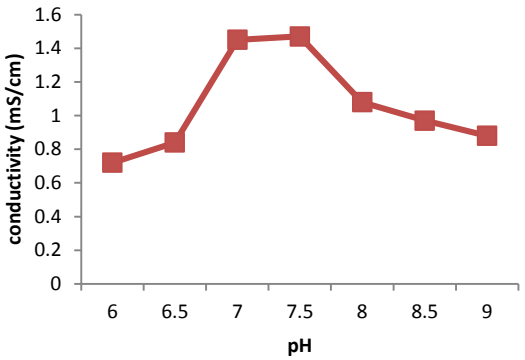

Fig. 4: Effect of pH on conductometric urease assay

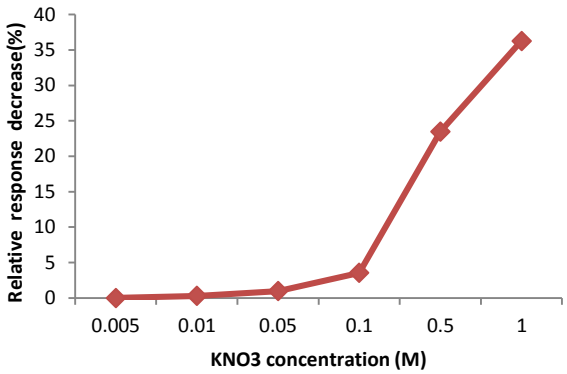

Fig. 5: Effect of ionic strength on conductometric urease assay

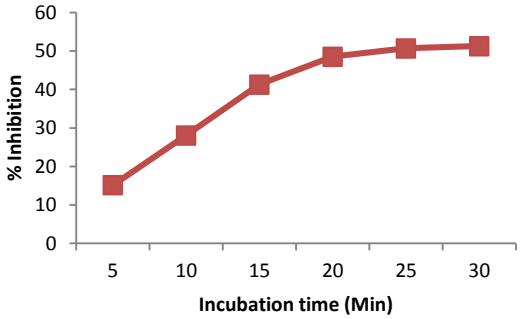

Fig. 6: Effect of incubation time on urease inhibition (evaluated for 0.1 mg/L concentration of As^{3+})

The activity profile of the urease based system over a pH range of 6.5–9.0 is presented in Fig. 4. Maximum activity was observed at pH 7.0 and it remained relatively constant up to pH 7.5 after which further increase resulted in decreased activity. About 40% decrease in activity was observed at pH 9. The relative stability of the system over a wide pH range (7.0 – 7.5) is a desirable property as it suggests that small pH related interferences would have little or no effect on the assay system. pH 7.3 was selected for this study.

Ionic strength is one of the critical factors in any conductance based assay as high concentration of ions in a sample may alter the conductance of the system and result in decreased responses. The sensitivity of the system in the presence of KNO_3 of varying ionic strength (0.005 – 1.0 M) is shown in figure 5. At 0.005 – 0.1M KNO_3 concentration, no significant decrease in assay response was observed. However between 0.5 - 1M ionic strength, a marked decrease (23.3 – 36.27%) in system response was recorded. Typical ionic strengths of natural waters have been given as 0.001 – 0.005M for surface water, 0.001 – 0.02M for portable water/ground water and up to 0.7M for seawater (Aqion, 2014). Our findings here indicate that the proposed conductometric assay would not suffer ionic strength related setbacks when used for surface and groundwater samples. However the method may not give accurate results when applied to sea water due to its very high ionic content which would cause interferences and likely give unreliable data.

The graphical representation of the results obtained due to the varied time of contact of asrenic ions (0.1 mg/L) with urease is shown in Fig. 6. The percentage inhibition increased with incubation time and maximum inhibition was reached between 25 – 30 minutes. It is evident that the longer the incubation time, the more the interaction between inhibitor and enzyme and hence the higher the inhibition. However, a longer incubation time means a longer analysis time therefore, incubation time was chosen as a compromise between the method sensitivity and time of analysis. 15 min incubation time was selected for this study, since during this period, the enzyme lost over 80% of its initial activity which is an informative indicator of sufficient inhibition.

The accuracy of the conductometric assay evaluated against the AAS results (as the true values) revealed 73.4 – 97.85 % relative accuracy. Repeatability (precision) of the method which was investigated by taking the relative standard deviation (RSD) of six replicate measurements revealed an RSD of 4.8 %. This shows that the data is tightly clustered around the mean – an indication of good accuracy. The limit of detection (LOD) for the investigated system was determined as 3 x Standard deviation (SD) of low concentration sample/slope of the calibration line while limit of quantitation (LOQ) was obtained as 10 x SD of low concentration sample/slope (Philiswa, 2011). 0.043 mg/L LOD and 0.145 mg/L LOQ were established for the method.

Conclusion

The urease – based conductometric assay described in this study seems promising for the determination of As (III) ions in aqueous systems. Evaluation of the method against standard techniques verified its suitability for quantitative analysis of arsenic. The method is very simple and does not

require the use of purified enzymes, costly reagents or sophisticated instruments therefore it can be employed in remote laboratories and for field monitoring. However, the feasibility of the method for determination of arsenic in real samples has not been evaluated. Since the proposed method is inhibition based, it may not be able to discriminate between different inhibitors of urease in a real sample matrix. Further work will therefore be geared towards ascertaining the usefulness of the method for selective detection of arsenic in samples that contain other inhibitors of the same class.

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