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Flow injection analysis biosensor for urea analysis in adulterated milk using enzyme thermistor

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ABSTRACT

Urea in adulterated milk is one of the major health concern, it is especially harmful to pregnant women, children, and the sick. A sophisticated and reliable detection system is needed to replace current diagnostic tools for the urea in the milk. In this work, we report a flow injection analysis-enzyme thermistor (FIA-ET) bio-sensing system for monitoring of urea in adulterated milk. This biosensor was made of the covalently immobilized enzyme urease (Jack bean) on controlled pore glass (CPG) and packed into a column inside thermistor, which selectively hydrolysed the urea present in the sample. The specific heat registered from the hydrolysis of urea was found proportional to the concentration of urea present in the milk sample. The biosensor showed a linear range 1–200 mM, with % R.S.D. 0.96 for urea in 100 mM phosphate buffer, pH 7.2. Good recoveries were obtained (97.56–108.7%) for urea up to 200 mM in the spiked milk samples with % R.S.D. 0.95. In the adulterated milk, a simple filtration strategy and matrix matching technique was used to analyse urea. The response time of the sensor was evaluated for urea, which was 2 min, and it gives satisfactory output. A good comparison was observed between the urea concentrations measured through FIA-ET and the colorimetric method. These results indicate that utilizing this system could be very effective to detect low and high level of urea in adulterated milk. The immobilized urease column exhibited a good operational stability up to 180 days when used continuously at room temperature.

1. Introduction

Adulteration of natural milk with synthetic chemicals is a serious concern for human health. Milk is an excellent source of energy, protein, minerals and vitamins (Noyhouzer et al., 2009). In India, Urea [$CO(NH_2)_2$] is commonly used as an adulterant for milk. Adulteration of milk with urea decreases the nutritive value of the milk. Urea is also a normal constituent of milk. The typical concentration of urea in milk is 18–40 mg/dL (Jonker et al., 1989). A cut-off limit for urea concentration in milk is normally accepted at 70 mg/dL. It also forms a major part (55%) of the nonprotein nitrogen of milk (Sharma et al., 2008). Urea being relatively cheap and rich in nitrogen is an economical choice to adulterate the milk. The effect of urea above cut-off limit in milk may cause indigestion, acidity, ulcers, cancers, malfunctions of kidney, etc. (Trivedi et al., 2009). Hence urea estimation in milk is of great significance.

Several conventional methods have been reported in recent years for urea analysis in milk, i.e. spectrophotometric and conductometric detection (Reis Lima et al., 2004), differential pH technique (Luzzana and Giardino, 1999). The conventional techniques as well as current wet chemistry and analytical practices are time consuming and may require highly skilled workers and expensive equipment (Loung et al., 1991). The food and dairy industries need rapid, reliable and affordable techniques for quality control. The application of thermal biosensors in food analysis is a growing field with increasing demand for reliable sensors (Bhand et al., 2010). Numerous biosensors have been reported in recent years for milk urea analysis, such as manometric biosensor (Jenkins and Delwiche, 2002; Renny et al., 2005) and potentiometric biosensor (Verma and Singh, 2003; Trivedi et al., 2009). Some of these reported biosensors have very short detection limit and low operational stability, which renders them unfit for routine analysis. There is a need for economical, reliable, robust and reproducible biosensors specifically for urea analysis in milk. Thus, thermal biosensors are good alternative devices owing to their simplicity of operation and long term stability (Pirvutoiu et al., 2002).

Thermal biosensors are based on the measurement of heat, produced when a biological reaction takes place. The amount of reacted substrate is related to the heat produced through the specific enthalpy, ΔH_r, of the reaction. An advantage of this principle as compared to other analytical tools like spectrophotometric and electrochemical methods is the universal detection principle which is combined with the specificity of the biological reactions (Bjarnason et al., 1998). Thermometric detection usually employs a

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different measurement scheme of temperature across the enzyme reactor in order to increase the common mode rejection ratio. As a result, thermal biosensors typically use flow injection-based analysis. In addition, control of the carrier solution and sample is straightforward using this continuous analysis scheme (Danielsson et al., 1976a). The method combines the advantage of the enzyme selectivity and the short analysis time, low reagent consumption, particularly when the enzymes are immobilized and used in a flow injection analysis (FIA) mode (Linhares et al., 1987). A large number of enzymatic reactions have been studied by this device among which urea and glucose detection were studied extensively in serum and different food stuffs (Danielsson et al., 1976b; Bjarnason et al., 1998; Salman et al., 2008). Recently cholesterol study, heavy metal study and fructose determination is also reported by this device (Raghvan et al., 1999; Pirvutoiu et al., 2002; Bhand et al., 2010). The objective of the present work is to demonstrate the application of enzyme thermistor for selective determination of adulterated milk urea using immobilized urease enzyme.

Herein we present a simple, economical and highly stable FIA-ET biosensor for analysis of urea in adulterated milk. We have successfully demonstrated an application for urea detection in milk which is first time by this instrument. The proposed biosensor can facilitate continuous analysis of milk urea in dairy industry. Another important feature of the presented biosensor is the lower detection limit and an excellent dynamic range of detection, 1–200 mM urea with a sample throughput of 30 within an hour. The biosensor comprise of immobilized enzyme Urease (Jack bean, EC 3.5.1.5) on controlled pore glass (CPG) which selectively hydrolyses urea present in the sample. The urea hydrolysis reaction is given below

$$CO(NH_2)_2 + H_2O \xrightarrow[PB]{Urease} 2NH_3 + CO_2 + \Delta H$$

2. Experimental

2.1. Biochemical and reagents

Enzyme urease (Jack beans lyophilized 5 U mg^{-1}, EC 3.5.1.5), urea, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate monohydrate, glutaraldehyde solution 25% and tri-ethanol amine were obtained from Merck, Germany. Amino silanized CPG spherical beads (Trisoperl, size 125–140 μm, pore size 50 nm) were purchased from VitraBio (Germany). All other chemicals used were GR grade. Maxsignal® urea enzymatic assay kit was purchased from BIOO SCIENTIFIC, USA.

2.2. Solution preparation

Phosphate buffer (PB) 100 mM, pH 7.2 was prepared by mixing 100 mM of sodium dihydrogen phosphate monohydrate and 100 mM of disodium hydrogen phosphate monohydrate in double distilled water. PB was degassed before analysis. A stock solution of urea (400 mM) was prepared by dissolving 2.4024 g in 100 mL of PB (100 mM, pH 7.2). Working solution of urea was prepared daily prior to use.

2.3. Enzyme immobilization

Enzyme immobilization was achieved by glutaraldehyde cross-linking on amine functionalized CPG beads (Weetall, 1976). In brief, urease was covalently immobilized on amino propyl CPG beads according to the following procedure: 352 mg of CPG was activated with 2.5% glutaraldehyde in 100 mM PB, pH 7.2. The reaction was allowed to take place for at least 1 h inside desiccators under reduced pressure. The product which changed its colour to brick red was washed exhaustively with distilled water. Urease 200 mg (1000 U) dissolved in 1 mL PB was added to the wet activated CPG. The coupling was allowed to proceed for 30 min at room temperature and overnight at 4 °C under gentle shaking. The enzyme preparation was washed with buffer. Tri-ethanolamine (35 mg) was added to terminate all the unreacted groups on the matrix and then washed with 100 mM PB, pH 7.2. The immobilized urease was packed into a delrin column by a slurry packing method for the use in FIA mode.

2.4. Instrumentation/FIA-ET-system

The schematic set-up for FIA-ET biosensor for urea analysis is presented as Fig. 1. The set up consists of a peristaltic pump (Gilson Minipuls Evolution, France), an injection valve (type 50 from Rheodyne, Cotati, USA), sample loop (0.1 mL), enzyme thermistor, Wheatstone bridge equipped with a chopper-stabilized amplifier and data recorder. PTFE tubing (0.8 mm id) was used for connections. The carrier buffer was 100 mM PB, pH 7.2.

2.5. Procedure

For urea analysis, calibration standards were prepared by dilution of urea stock solution in 100 mM PB, pH 7.2. The immobilized urease column was mounted inside ET and was allowed to equilibrate with surrounding. Degassed carrier buffer was passed through the column initially at a higher flow rate (0.9 mL min^{-1}). Subsequently, the flow rate was brought down to the optimum value of 0.5 mL min^{-1} at which a stable baseline was achieved. Standards/spiked samples were injected and data were collected using a data acquisition system connected to the Wheatstone bridge. All measurements were carried out by injection of 0.1 mL sample at a flow rate of 0.5 mL min^{-1}. After each sample analysis, the sample loop was thoroughly rinsed with PB. The milk samples were spiked with known amount of urea after appropriate filtration and dilution and injected in to the system.

3. Results and discussion

3.1. Optimization of the FIA biosensor system

3.1.1. Ionic strength and pH of buffer

The influence of the ionic strength and pH on the activity of the enzyme urease was studied. Measurements were carried out in the flow system with varying ionic strength (10, 50, 100, 150 and 200 mM PB) and pH (6.8–7.8) for PB for 100 mM urea. The results are presented in Fig. 2a and b respectively. It is evident from Fig. 2a that with increase in the ionic strength of PB, response signal increases up to 100 mM, and then the signal starts decreasing. Thus further experiments were carried out with 100 mM PB. Fig. 2b, clearly suggests that urease exhibit maximum activity at pH 7.2 flow rate

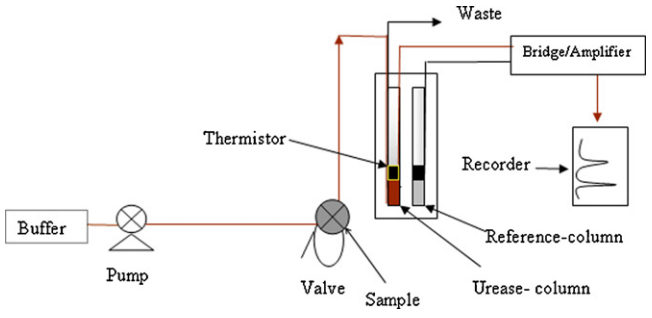


Fig. 1. Schematic set-up for flow injection analysis-enzyme thermistor (FIA-ET) biosensor for urea analysis.

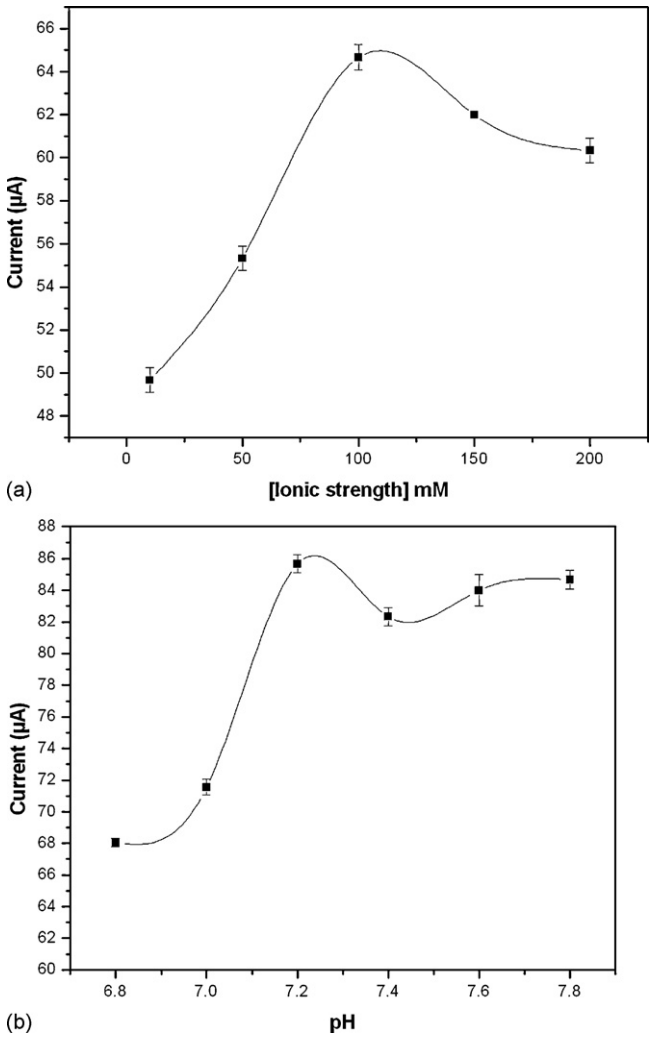


Fig. 2. Effect of ionic strength of the PB on the response of FIA-ET biosensor for 0.1 mL injection of 100 mM urea, flow rate 0.5 mL min^{-1}. Effect of pH of the phosphate buffer on the response of FIA-ET biosensor for 0.1 mL injection of 100 mM urea, flow rate 0.5 mL min^{-1}.

0.5 mL min^{-1} was found optimum to have sufficient contact time between the enzyme and the substrate.

3.1.2. Matrix matching

Commercial milk samples of different fat content (0.5%, 1.5%, and 3.5%) were purchased from local market of Goa, India. The matrix effect in the analysis of urea in milk samples was investigated. In FIA system, fat present in milk may lead to matrix related quenching and block the flow in FIA, which can decrease the device sensitivity or completely clog the column. Various sample pre-treatment techniques such as combination of centrifugation, filtration and dilution were tested to select the optimum pre-treatment procedure for milk urea analysis. Milk samples were centrifuged at 10,000 rpm for 10 min at room temperature. The centrifuged milk samples were diluted with PB to obtain different ratios of milk:PB and further spiked with known amount of urea. It was observed that 1:4 dilution of milk and PB was most responsive for urea analysis. To obtain the calibration curve for urea in spiked milk samples, a simple strategy was worked out to remove the fat and also to obtain a suitable dilution to avoid clogging of the urease column. In brief, milk samples were centrifuged, diluted and filtered through 0.45 and 0.22 μm filter (Whatman, USA). Milk analysis was carried out within the same day after sample preparation.

3.1.3. Calibration of urea biosensor in PB

The immobilized urease column was kept inside the thermistor and allowed to equilibrate overnight. The buffer solution was filtered, degassed and pumped via FIA manifold, until a constant baseline is achieved. Urea standard solutions 0.1, 1, 10, 50, 100, 150, 200, 250 mM (1 mM urea solution corresponds to 60.6 ppm) were prepared in carrier buffer and injected in to the flow stream. Response signals were recorded using a data acquisition system connected to Wheatstone bridge and plotted as current (μA) vs. urea (mM). All samples were injected in triplicates. The Michaelis constant, K_M for urea was calculated using Lineweaver–Burk plot. The K_M was found to be 100 mM. A good linear response was obtained for urea hydrolysis in the range of 1–200 mM with minimum detection limit of 0.1 mM (6.06 ppm), which is presented in Fig. 3. Linear fit of data shows a good correlation between urea and immobilized urease (R^2 = 0.99, % R.S.D. = 0.96).

3.1.4. Calibration of urea biosensor in milk

Spiked milk urea standard solutions (0.1–250 mM) of different fat content (0.5%, 1.5% and 3.5%) were injected in to the flow stream, and response signals were recorded. All samples were injected in triplicates. The FIA-ET biosensor showed an excellent dynamic range for milk urea in the range 0.1–250 mM with a good linearity (1–200 mM). The results are presented in Fig. 3. (R^2 = 0.99, % R.S.D. = 0.95). An actual sensor signal recorded for triplicate measurement of 50 and 100 mM spiked milk urea samples is presented in Fig. 3.

3.1.5. Urea recovery from milk samples

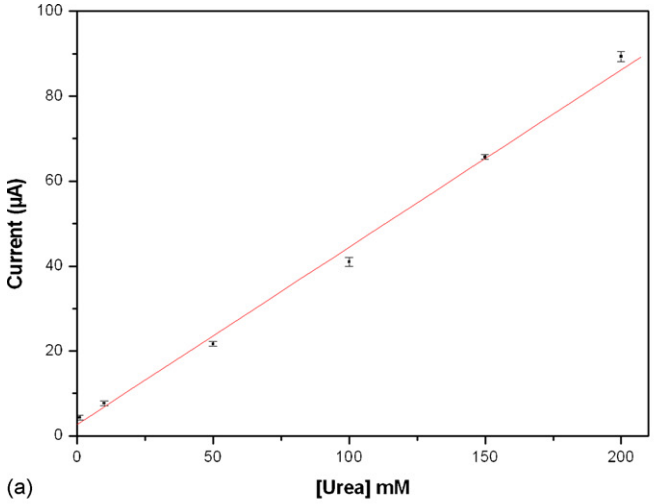
A known amount of urea standard solution was added to centrifuged, diluted and filtered milk and analysed in FIA-ET. Based on the response signal, recoveries were calculated. Table 1 shows the recovery results obtained for milk urea. Excellent recoveries in the range of 97.56–108.70% were obtained for urea in milk. This ensures the reproducibility of the presented biosensor.

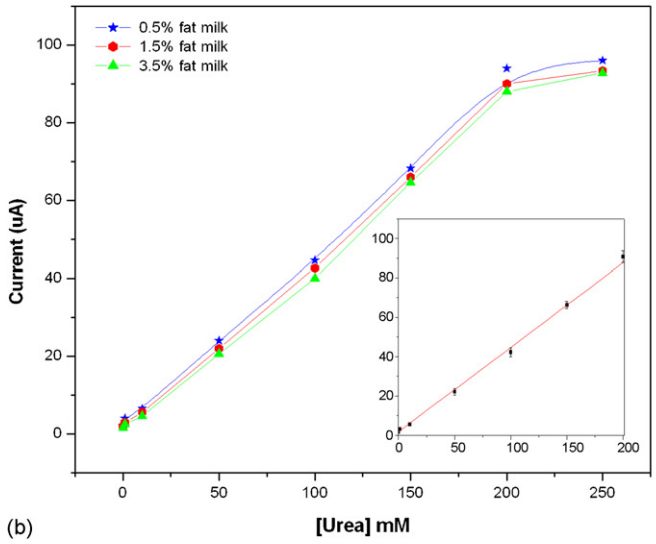
3.1.6. Sensitivity, detection limit and dynamic range

A broad linear range, 0.1–200 mM was obtained with the lower limit of detection 0.1 mM (6.06 ppm). The upper limit of detection as high as 200 mM was achieved with sensitivity of 4.17% mM^{-1} for buffer urea. For milk urea, a broad linear range, 0.1–200 mM was obtained with the upper limit of detection 250 mM and sensitivity of the signal 4.58% mM^{-1}. The reported heat of reaction for urea hydrolysis by urease is −61 kJ mol^{-1}. In our case the calculated heat of reaction in diluted milk urea samples was found to be −46.81 kJ mol^{-1}. The broad dynamic range obtained for milk urea makes the biosensor highly suitable for analysis of adulterated milk samples.

Table 1
Determination of urea recovery from commercial milk samples spiked with known amount of urea using FIA-ET biosensor.

Sample	Added amount of urea (mM)	Recovered amount of urea (mM)	Recovery, %	Found ± S.D.	R.S.D. %
Milk 1	41	44.6	108.7	44.6 ± 0.4	0.89
Milk 2	41	42.6	103.9	42.6 ± 0.4	0.93
Milk 3	41	40	97.56	40.0 ± 0.5	1.25





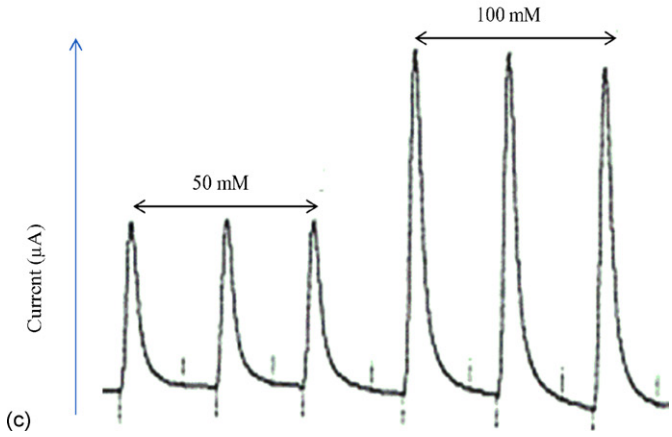


Fig. 3. Calibration plot obtained for urea using urease column in 100 mM PB, pH 7.2 for 0.1 mL of sample injection, flow rate 0.5 mL min^{-1} at 30 °C, R^2 = 0.99, % R.S.D. = 0.96. Calibration plot obtained for urea using urease column in milk with 0.5%, 1.5% and 3.5% fat. (Inset: linear range obtained for 0.5% fat milk urea. R^2 = 0.99, % R.S.D. = 0.95). Response signal recorded for two different milk urea concentrations (50 mM and 100 mM) using FIA-ET biosensor. Urea dissolved in filtered milk samples, sample volume 0.1 mL, flow rate 0.5 mL min^{-1} acquired using data acquisition system with recorder.

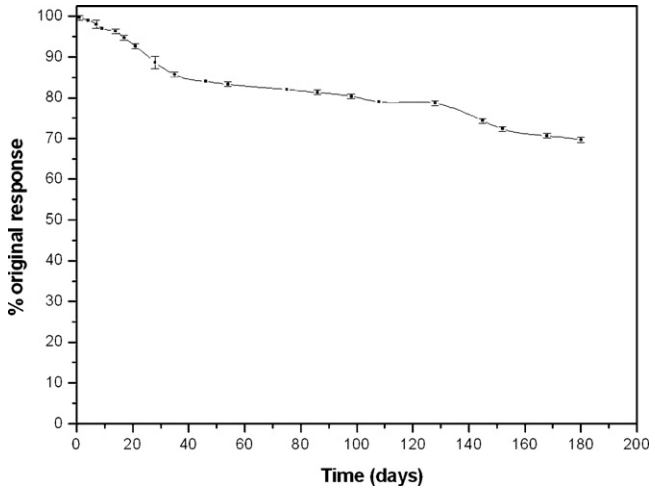


Fig. 4. Stability of urease column response to 0.1 mL, 100 mM urea using FIA-ET biosensor observed for continuous 180 days at 30 °C. Samples were injected randomly during the period.

3.1.7. Response time and sample throughput

The response of the FIA-ET biosensor was highly reproducible with a variation coefficient less than 2.5% at 30 °C and it was estimated from 20 repeated injection of 100 mM urea of 0.1 mL. Response time for urea hydrolysis by urease took about 2 min. Thus, up to 30 samples can be measured within an hour. Increased throughput can be achieved by reducing the sample injection volume down to 0.25 mL.

3.1.8. Operational stability

An excellent dynamic range is achieved while the sensor is continuous in use at room temperature. The operational stability of the system was continuously monitored over the period of 180 days. About 15 measurements were made every day for 0.1 mL, 100 mM urea. The operational stability is over the studied period is presented in Fig. 4. The immobilized urease inside the thermistor presented excellent stability, over the first 30 days the enzyme activity was found 90% of the original response and there was no appreciable loss of activity. However after a period of 45 days operation, about 16% decay in response was observed. Similarly the response was monitored after 75, 150 and for 180 days and signals recorded were respectively 82%, 73% and 70% of the original response.

3.1.9. Comparison of FIA-ET and commercially available colorimetric assay

The analytical features and figures of merit of the presented milk urea biosensor were compared with Maxsignal® Urea Enzymatic Assay Kit (BIOO Scientific, USA). The kit measures the concentration of urea using the urease enzyme, which converts urea to ammonia. An alkaline hypochlorite solution is then added to the reaction mixture. The colour produced from the reaction is measured using a colorimetric multiplate reader at 620 nm. The limit of detection of urea in milk using the commercial kit is 40 ppm. The kit is designed to be used with a microplate reader. The kit is provided with reagents and a 96 well plate to perform the assay (catalogue no. #:1051 Ref.1051-01 BIOO Scientific, USA). Spiked milk urea samples corresponding to 8, 25, 50, 100 and 200 ppm were analysed simultaneously using the FIA-ET and the colorimetric assay. The results obtained are presented in Fig. 5. For colorimetric measurements (Multilabel Plate Reader Victor X4, PerkinElmer, USA was deployed with 620 nm filter). The parameters such as detection range, analysis time, sensitivity, sample throughput and cost per sample were compared. The dynamic range and the upper limit of

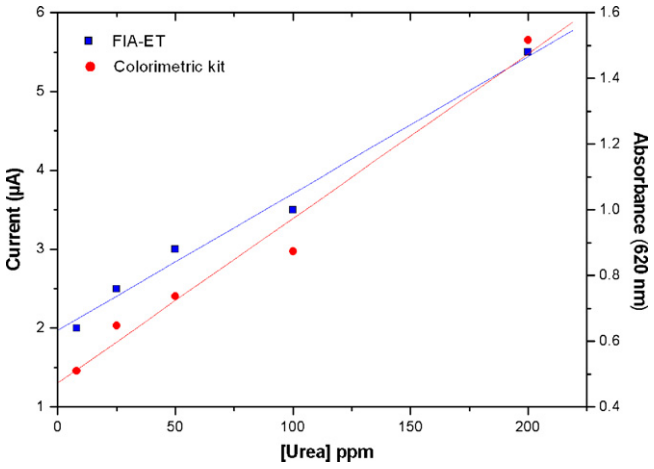


Fig. 5. Comparison of the results for milk urea concentration obtained with the FIA-ET biosensor and commercially available colorimetric plate assay (for FIA-ET $R^2 = 0.99$ and for colorimetric kit $R^2 = 0.98$).

detection for the milk urea using the kit were found much lower as against presented biosensor, which offers a manifold higher linear range 0.1–200 mM. The analysis time for the thermal biosensor is 2 min per sample with throughput of 30 h, whereas with commercial kit, up to 72 samples can be analysed within an hour. For FIA-ET biosensor, a higher sensitivity is observed. As many as 500 samples were analysed with the single urease column over the period of 180 days. The result suggests that the presented system is robust and suitable for urea determination in adulterated milk samples and can be easily adapted for routine analysis.

4. Conclusions

A flow injection analysis-enzyme thermistor biosensor for detection of urea in adulterated milk is developed and demonstrated. The method was optimized and had been successfully applied to determine urea in adulterated milk samples. The biosensor is stable at room temperature for more than 180 days and still in working condition with 30% loss of original response. Broad dynamic range (0.1–250 mM) of the sensor facilitates the urea analysis in milk. Reported method is highly reproducible, cost effective, reliable and robust. It fulfils the need of dairy processing plant for monitoring of milk urea.

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