

## A method for estimation of urea using ammonia electrode and its applicability to milk samples

Rajan Sharma<sup>1</sup>, Yudhishtir S Rajput<sup>1\*</sup>, Sumandeep Kaur<sup>1</sup> and Sudhir K Tomar<sup>2</sup>

<sup>1</sup> Department of Dairy Chemistry, National Dairy Research Institute, Karnal-132 001, India

<sup>2</sup> Department of Dairy Microbiology, National Dairy Research Institute, Karnal-132 001, India

Received 10 April 2007; accepted for publication 21 May 2008; first published online 22 July 2008

A method for the estimation of urea in milk using ammonia electrode is described. Urea is first degraded by urease enzyme into ammonium ion and carbon dioxide at neutral pH. The ammonium ion is then converted into ammonia at alkaline pH. A linear inverse relationship was observed between logarithmic concentration of ammonia or urea and electrode response. Repeatability, expressed as a coefficient of variation, was 1.77% at a level of 8.92 mM-urea in milk. The method was validated in milk samples spiked with between  $2 \times 10^{-3}$  and  $10 \times 10^{-3}$  M-urea and recovery of added urea was quantitative. Whereas, preservative sodium azide at 0.5 g/l or 2 g/l level did not affect results, lower values of urea concentration in presence of Bronopol at 0.5 g/l were observed. Urea levels in milk samples estimated by this method were comparable to standard enzymatic method. The method is simple, fast and is not prone to interference from other milk constituents.

**Keywords:** Urea, Milk, Ammonia electrode, Urease, Ammonia.

Urea is a normal constituent of milk and it forms a major part (55%) of the non-protein nitrogen of milk (Ramakrishnaiah & Bhat, 1986). Urea content of milk is affected by dietary factors, especially by the balance between the nitrogen fraction in the diet and the energy intake (Lefier, 1996; Holf et al. 1997). A high urea content in milk from unbalanced feeding of cows influences milk production and fertility (Butler et al. 1996; Larson et al. 1997). Dietary crude protein content is positively correlated with milk urea nitrogen concentrations and the latter has been proposed as a management tool for assessing dietary protein feeding (Broderick & Clayton, 1997; Godden et al. 2001; Hojman et al. 2005). Milk urea level is also linked to thermal stability of milk at higher temperature (Rajput et al. 1982, 1984). Because of clinical significance of blood urea level, a number of methods for urea estimation have been developed. The methods for estimation of urea in milk are largely derived from methods applicable to blood. These include reaction of urea with *p*-dimethyl amino-benzaldehyde (Bector et al. 1998) or diacetyl monoxime (Bhavadasan et al. 1982), measurement of change in pH (Luzzana & Giardino, 1999; International Dairy Federation, 2004) or measurement of carbon dioxide partial pressure (Jenkins et al. 1999, 2000) as a result of urea degradation

by urease, coupled reaction using urease and glutamate dehydrogenase enzymes (Wolfschoon-Pombo et al. 1981; Kampl et al. 1993), degradation of urea into ammonium ion and its measurement by ion selective electrode (Verma & Singh, 2003) and characteristic absorption bands at  $1690 \text{ cm}^{-1}$  of amide bond of urea in infrared range (Hansen, 1998). The measurement of ammonium ion by ion selective electrode is prone to interference from other cations (Verma & Singh, 2003). In the present communication, a method for estimation of urea in milk using ammonia sensitive electrode has been described.

### Materials and Methods

#### Milk samples

Pooled or individual cow raw milk samples collected from the cattle yard of the Institute in amber coloured glass containers were brought immediately to the laboratory. The samples were skimmed by centrifugation (3500 g; 10 min.; 5 °C) and unless otherwise indicated, the data presented in this article refers to skim milk.

#### Standard curve of ammonium chloride

Twenty five milliliters aqueous solutions of ammonium chloride ( $5 \times 10^{-5}$  M to  $10^{-1}$  M) were placed in 50 ml

\*For correspondence; e-mail: ys\_rajput@rediffmail.com

beaker. The ammonia electrode (Thermo Orion, USA, model: 95-12) connected to pH-meter, was allowed to dip in solution. Then, 0.5 ml high pH – ionic strength adjuster (Thermo Orion, USA) was added. High pH – ionic strength adjuster (ISA) contained sodium hydroxide, methanol and an indicator. The contents were stirred gently at a uniform rate on a magnetic stirrer. Electrode response in terms of mV was recorded when mV readings became stable (approximately 2 min). During measurement of electrode response, the temperature of all solutions was kept at 25 °C. The logarithmic of ammonium chloride concentration was plotted against mV.

#### Standard curve for urea

Twenty five milliliters aqueous urea (Qualigens Fine Chemicals, India) solution (3–15 mM) were treated with 200 µl urease (EC. 3.5.1.5, Sigma Aldrich Chemicals Pvt. Ltd., USA) solution (10 mg/ml 0.05 M-Tris-HCl buffer, pH 7.0) for 15 min at 37 °C. The solution was then incubated in a water-bath maintained at 25 °C. When temperature of solution reached 25 °C, ammonia electrode was added. Then 0.5 ml high pH-ISA was added and electrode response was recorded at 25 °C when mV reading became stable. Standard curve of urea was prepared similarly to ammonium chloride standard curve.

#### Estimation of urea in milk

Twenty five milliliters skim milk was treated with 200 µl urease solution (10 mg/ml in 0.05 M-Tris-HCl buffer, pH 7.0) for 15 min at 37 °C. Milk samples were then incubated in water-bath maintained at 25 °C. Electrode response in mV was recorded at 25 °C after addition of 0.5 ml high pH-ISA. Using standard curve for urea, mV signal was converted into urea concentration ( $C_1$ ).

The urea concentration equivalent to indigenous ammonia content in milk was estimated by treating 25 ml milk similarly as mentioned above except 200 µl Tris-HCl buffer (0.05 M, pH 7.0) was added instead of urease solution. Again using standard curve of urea, mV signal was converted into urea concentration ( $C_2$ ). Urea in milk was then calculated by subtracting  $C_2$  from  $C_1$ .

#### Validation of method

Milk urea levels were raised by between 2 to 10 mM concentration by addition of calculated amounts of analytical grade urea. Urea levels were then measured as explained above. Recovery of added quantity of urea was calculated.

Urea levels in milk samples were measured by the above described method, diacetyl monoxime method (Bhavadasan et al. 1982) and an enzymatic method employing urease and glutamate dehydrogenase (kit from R-Biopharm, Germany). Paired t-test was used for comparison of results obtained by these three methods (Mendham et al. 2000).

#### Addition of preservatives

Two preservatives, Bronopol (2-bromo-2-nitropropane-1,3-diol) at 0.5 g/l and sodium azide at 0.5 and 2 g/l were added to the raw milk sample. Milk samples containing preservatives were stored up to 3 days at 4 °C. Urea levels in unpreserved and preserved samples were determined.

#### Results

Ammonium ions exist in equilibrium with ammonia gas in aqueous solutions. The equilibrium is far more favoured towards ammonia at pH values greater than 11 (pK for  $\text{NH}_3 \sim 9.0$  at 25 °C). For of this reason, ammonium chloride aqueous solution was made sufficiently alkaline to allow quantitative conversion of ammonium ion into ammonia to be finally measured by ammonia sensitive electrode. There was a linear inverse relationship between logarithmic ammonium chloride concentration and electrode response, mV (Fig. 1a). The linearity was noted at  $10^{-1}$  to  $5 \times 10^{-5}$  M-ammonium chloride. The calculated value of correlation coefficient, 0.9995, is indicative of a high degree of relationship between ammonium chloride concentration and electrode response.

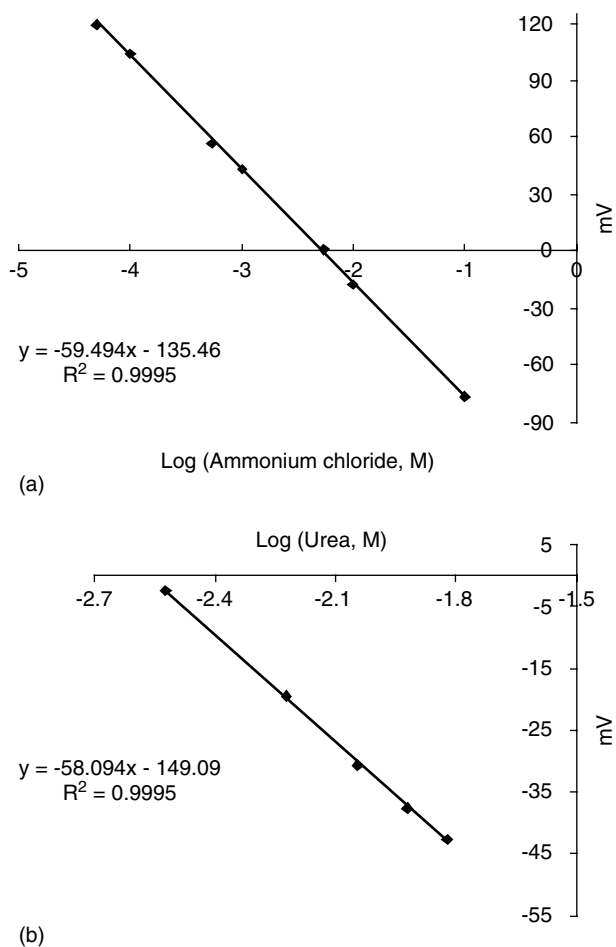
Quantitative conversion of urea into ammonium ion and ammonium ion to ammonia gas is the basis of estimation of urea by ammonia-sensitive-electrode. A linear inverse relationship between logarithmic of urea concentration and electrode response (mV) was noted (Fig. 1b). The correlation coefficient value, 0.9995, in the range of 3–15 mM-urea suggests that linear relationship exists between urea level and electrode response. Since milk urea level is in range of 3–8 mM (Kavitha et al. 2001a,b), this method is applicable to milks. Even in milk samples adulterated with so called synthetic milk (Bansal & Bansal, 1997; Paradkar et al. 2000), abnormally higher levels of urea can be measured by this method.

#### Recovery

The method was validated by spiking milk samples with known concentrations of urea (2, 4, 6, 8, 10 mM). Estimated values of urea in unspiked sampled, spiked samples and recovery calculations are shown in Table 1. The recovery of added urea to milk was in the range of 97.5 to 100%. These results validate the method for its applicability to milk.

#### Repeatability

Milk and milk with added 2.49 or 4.98 mM-urea were used for repeatability. Each milk was analysed ten times. The mean ( $\pm$ SE) urea level in these samples were  $8.92 \pm 0.05$ ,  $11.40 \pm 0.06$  and  $13.84 \pm 0.07$  mM, and the coefficients of variation were 1.77, 1.60 and 1.59%, respectively.



**Fig. 1.** Relationship between ammonium ion concentration (a) or urea concentration (b) and electrode response, mV.

#### Accuracy

The accuracy of the ammonia electrode method was evaluated by comparison with two other methods working on different principles; enzymatic method and diacetyl monoxime method.

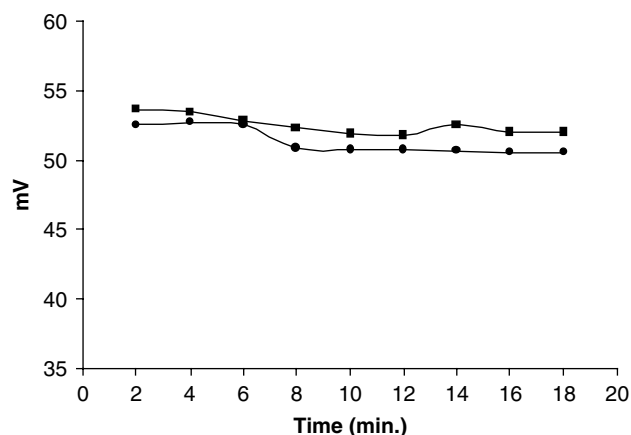
The mean value ( $\pm$ SE) of urea estimations in 10 different milk samples by ammonia electrode method, enzymatic method and diacetyl monoxime method was  $8.55 \pm 0.33$ ,  $8.51 \pm 0.31$  and  $8.83 \pm 0.33$  mM, respectively. According to the t-test, the mean results from the ammonia electrode method and enzymatic method are not significantly different ( $P < 0.05$ ) from each other. However, the difference was significant ( $P < 0.05$ ) when ammonia electrode method was compared with diacetyl monoxime method. It has been observed that the estimated values of urea in milk by diacetyl monoxime method were about 3.5% higher compared with the other two methods.

#### Influence of preservative

When milk samples were preserved with sodium azide (0.5 and 2 g/l), no interference in estimation was observed.

**Table 1.** Estimated values of urea in milk spiked with different concentrations of urea and recovery of added urea

Added Urea (mM)	Estimated urea (mM)		Recovery of spiked urea	
	Unspiked sample	Spiked samples	mM	In percent
(a)	(b)	(c)	(c-b)	$\frac{c-b}{a} \times 100$
2	3.80	5.75	1.95	97.5
4	3.80	7.76	3.96	99.0
6	3.80	9.78	5.98	99.7
8	3.80	11.80	8.00	100.0
10	3.80	13.80	10.00	100.0



**Fig. 2.** Effect of lapsed period subsequent to pH-ISA addition to ammonium chloride solution (1 mM) on electrode response at 25 °C (●) or 30 °C (■).

However, lower values (42 to 67% of initial level) of urea concentration in presence of Bronopol (0.5 g/l) were observed.

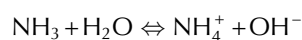
#### Urea levels in whole milk and skim milk

In four different milks, the levels of urea in whole and skim milks were measured. Mean urea levels in whole and corresponding skimmed milk were 4.86 and 5.06 mM, respectively, indicating that urea concentration in skimmed milk were about 4% higher than in whole milk.

#### Discussion

The addition of urease to milk catalyses the hydrolysis of milk urea to ammonium ion and carbon dioxide (Guilbault & Kauffmann, 1987). Ammonium ion is converted to ammonia by addition of high pH-ISA. The addition of high pH-ISA ensures the pH of the solution to be within 11 to 14 thus converting all the ammonium ions to ammonia. The ammonia electrode uses a hydrophobic gas-permeable

membrane to separate the sample solution from the electrode internal solution. Dissolved ammonia in the sample solution diffuses through the membrane until the partial pressure of ammonia is the same on both sides of the membrane. In any given sample the partial pressure of ammonia will be proportional to its concentration. Ammonia in internal filling electrode solution becomes in equilibrium with ammonium ion and generates hydroxide ion.



The relationship between ammonia, ammonium ion and hydroxide ion is given by the following equation.

$$\frac{[\text{NH}_4^+][\text{OH}^-]}{[\text{NH}_3]} = \text{constant}$$

The internal filling solution contains ammonium chloride at a sufficiently high level so that the ammonium ion concentration can be fixed. Thus

$$[\text{OH}^-] = [\text{NH}_3] \cdot \text{constant}$$

The potential of the electrode sensing element with respect to the internal reference element is described by the Nernst equation:

$$E = E_0 - S \log[\text{OH}^-]$$

where

$E$  = measured electrode potential

$E_0$  = reference potential

$\text{OH}^-$  = hydroxide concentration in solution

$S$  = electrode slope

Partial pressure of gas is dependent on temperature and therefore, standard solutions, samples and electrode must be maintained at same temperatures during analysis.

Ammonia is volatile and therefore for minimizing its escape from solution, temperature of urease treated milk (or standard urea solution) was lowered to 25 °C. At this temperature as well as at 30 °C, there was not significant drift in signal (Fig. 2). However, it is recommended to note response between 2 and 6 min. Electrode response to 1 mM and 10 mM ammonium chloride solutions between 23 to 27 °C at 1 °C interval were recorded. Electrode response was not affected by these minor changes in temperature of solution around 25 °C.

The results obtained by this study reveal that the ammonia electrode can be employed for the estimation of urea content in milk samples. The method was observed to be in close agreement with the enzymatic method of urea estimation. The method has the advantage of rapidity and a large number of samples can be handled and results are available in short span of time. Sodium azide does not interfere in assay and the method can be applied in preserved milk samples. At times, small variations in standard

curves of urea are observed and therefore, fresh standard curves needs to be prepared on each day of estimation.

## References

- Bansal P & Bansal N** 1997 Synthetic milk – genesis, current status and options. *Current Science* **23** 904–905
- Bector BS, Moti R & Singhal OP** 1998 Rapid platform test for the detection/determination of added urea in milk. *Indian Dairyman* **50** 59–62
- Bhavadasan MK, Rajput YS & Ganguli NC** 1982 A simple colorimetric method for the determination of urea in milk. *Indian Journal of Dairy Science* **35** 263–266
- Broderick GA & Clayton MK** 1997 A statistical evaluation of animal and nutritional factors influencing concentrations of milk urea nitrogen. *Journal of Dairy Science* **80** 2964–2971
- Butler WR, Calaman JJ & Beam SW** 1996 Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cows. *Journal of Animal Science* **74** 858–865
- Godden SM, Lissimore KD, Kelton DF, Leslie KE, Walton JS & Lumsden JH** 2001 Relationship between milk urea concentrations and nutritional, management, production and economic variables in Ontario dairy herds. *Journal of Dairy Science* **84** 1128–1139
- Guilbault GG & Kauffmann, JM** 1987 Enzyme based electrodes as analytical tools. *Biotechnology and Applied Biochemistry* **9** 95–113
- Hansen PW** 1998 Urea determination in milk using Fourier transform infrared spectroscopy and multivariate calibration. *Milchwissenschaft* **53** 251–255
- Holf G, Vervoorn MD, Lenaers PJ & Tamminga S** 1997 Milk urea nitrogen as a tool to monitor the protein nutrition of dairy cows. *Journal of Dairy Science* **80** 3333–3340
- Hojman D, Gips M & Ezra E** 2005 Association between live body weights and milk urea concentration in Holstein cows. *Journal of Dairy Science* **88** 580–584
- International Dairy Federation** 2004 Milk determination of urea content. Enzymatic method using differential pH (Reference method). Standard method 195. Brussels, Belgium: International Dairy Federation
- Jenkins DM, Delwiche MJ, Depeters EJ & Bon Durant RH** 1999 Chemical Assay of Urea for automated sensing in milk. *Journal of Dairy Science* **82** 1999–2004
- Jenkins DM, Delwiche MJ, Depeters EJ & Bon Durant RH** 2000 Refinement of the pressure assay for milk urea nitrogen. *Journal of Dairy Science* **83** 2042–2048
- Kampl BK, Milas NP, Francetic & Srebocan E** 1993 Determination of the urea content in the deproteinized cow's skim milk by the urease/glutamate dehydrogenase method. *Veterinarski Arhiv* **63** 55–60
- Kavitha P, Bector BS & Sharma V** 2001a Urea content of cow milk in relation to milk yield and breed. *Indian Journal of Dairy Science* **54** 311–315
- Kavitha P, Bector BS & Sharma V** 2001b Urea content of milk of Murrah breed of buffaloes. *Buffalo Newsletter* **16** 10–12
- Larson SF, Butler WR & Currie WB** 1997 Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating dairy cows. *Journal of Dairy Science* **50** 1288–1295
- Lefier D** 1996 Analytical methods for the determination of the urea content in milk. *Bulletin of the International Dairy Federation* **315** 35–38
- Luzzana M & Giardino R** 1999 Urea determination in milk by a differential pH technique. *Lait* **79** 261–267
- Medndham J, Denney RC, Barnes JD & Thomas MJK** 2000 Vogel's text-book of quantitative chemical analysis. Sixth Edition. Pearson Education (Singapore) Pte. Ltd., New Delhi, India
- Paradkar M, Singhal RS & Kulkarni PR** 2000 An approach to the detection of synthetic milk in dairy milk: 1. Detection of urea. *International Dairy Journal* **53** 87–91
- Rajput YS, Bhavadasan MK, Singh A & Ganguli NC** 1982 Heat stability of buffalo milk as affected by the addition of urea and glyceraldehyde. *New Zealand Journal of Dairy Science & Technology* **17** 185–190

- Rajput YS, Bhavadasan MK & Ganguli NC** 1984 Effect of urea on heat-induced acidity and milk coagulation. *New Zealand Journal of Dairy Science & Technology* **19** 49–54
- Ramakrishnaiah & Bhat GS** 1986 Significance of urea level in heat stability of cow and buffalo milk. *Indian Journal of Dairy Science* **39** 60–64
- Verma N & Singh M** 2003 A disposable microbial based biosensor for quality control in milk. *Biosensors and Bioelectronics* **18** 1219–1224
- Wolfschoon-Pombo A, Klostermeyer H, Buchberger J & Graml R** 1981 Urea in the NPN-fraction of cows' milk – determination, content and influences. *Milchwissenschaft* **36** 462–466