Chemical Assay of Urea for Automated Sensing in Milk

D. M. JENKINS,* M. J. DELWICHE,† E. J. DEPETERS,‡ and R. H. BONDURANT‡

*Department of Biological and Agricultural Engineering, †Department of Animal Science and ‡School of Veterinary Medicine, University of California at Davis, 95616

ABSTRACT

Results of a new chemical assay for urea involving enzymatic hydrolysis to ammonium and carbonate and subsequent measurement of CO₂ partial pressure are presented. The assay is simple to implement in an automated version, and the hardware used is not prone to fouling and damage by raw milk. The assay sensitivity at 24°C is about 0.367 kPa per milligram per deciliter of urea N. The assay has no dependence on milk fat in the sample, and effects of milk proteins and lactose are slight (less than 2% change in sensitivity per change in w/v percent). Observed sensitivities to urea in spiked milk samples were not significantly different from each other or from standards in distilled water or 0.1 M phosphate-buffered saline. The standard error of the assay is about 0.3 mg/dl of urea N (0.1 mM) in standard solution, and about 1 mg/dl of urea N (0.3 mM) in milk in a range of 0 to 30 mg/dl of urea N (0 to 11 mM). The assay holds promise for use in an on-line sensor to measure milk urea N in the milking parlor. (Key words: milk urea nitrogen, nutritional management, on-line biosensor)

Abbreviation key: MUN = milk urea nitrogen.

INTRODUCTION

Because feed costs constitute the largest single expense of dairy farms, recent discussion has focused on dietary efficiency. Improving N utilization is of particular interest because protein supplements are the most costly feed ingredients, and a premium is placed on the yield of milk protein (1, 3). It has been suggested (4, 14) that high concentrations of urea impair reproductive performance, and, in many localities, the environmental effect of excess N in dairy waste is a concern (9, 18). Excessive N in feed causes high systemic urea N without a corresponding increase in milk protein; values of urea and true protein in milk may be used to assess the nutritional program of lactating dairy cows (6). Various authors (4, 14) report the onset of pathologic problems when milk urea N (MUN) reaches about 20 mg/dl.

Because urea concentrations in blood and urine are important indicators of renal health, many biosensors specific to urea have been developed in the biomedical industry. These sensors hydrolyze urea with the enzyme urease (EC # 3.5.1.5),

and measure changes in solution conductivity (5, 20), pH (11, 12), or the ionic products with ion-selective electrodes, ion-sensitive field effect transistors (10, 17), or surface acoustic wave devices (15). Most of these sensors are too delicate for use with raw milk without extensive pretreatment (5, 10, 11, 15, 17, 20). Some are highly nonlinear or have a low dynamic range (10, 11, 15, 17, 20), and some are not precise enough to measure urea N to within 1 mg/dl (10, 12, 20). All of these biosensors depend on sample pH and buffering capacity.

The goal of this research was to develop a biosensor to automatically measure MUN during milking. Data from such a device could be used to manage the N balance of a herd for economic and environmental benefit. The sensor had to be able to complete one measurement cycle faster than the turnaround time for cows in the parlor (less than 10 min) and had to be capable of repeatedly measuring MUN to within 1 mg/dl (0.3 mM) in the physiological range from about 6 to 24 mg/dl (2.1 to 8.6 mM). The biosensor also had to be simple, reliable, and robust enough to withstand repeated exposure to the high lipid and protein concentrations in milk. In this paper we report on a new assay for urea that is simple, robust, and effective for measurement in milk.
MATERIALS AND METHODS

Assay Development

Device principle and theoretical sensitivity. The new assay involves the measurement of partial pressure of carbon dioxide in the sample. Under acidic conditions, the carbonate generated from the hydrolysis of urea exists primarily as dissolved CO₂. A sealed gaseous cavity in contact with the solution will pressurize to an extent proportional to the amount of urea originally in the sample as dissolved CO₂ is released into the gaseous phase.

The partial pressure, P, of a dissolved gas is given by Henry's law,

\[ P = x_g k_p, \]  

where \( k_p \) is Henry's constant and \( x_g \) is the mole fraction, or the ratio of the dissolved gas molecules in solution to the sum of all molecules in the solution. When \( x_g \) is small, the partial pressure of CO₂ can be approximated as

\[ P_{CO_2} = \frac{[CO_2]_{aq}}{[H_2O]} k_p, \]  

An empirical expression for the solubility of CO₂ in water (7, p. 242) may be used to derive

\[ k_p = E(e^{A/B} + D/T + C)^{-1}, \]  

where \( A = -159.854, B = 8741.68 \, K, C = 21.6694, D = -1.10261 \times 10^{-3} \, K^{-1}, \) and \( E = 101.325 \, kPa. \) With these values, the partial pressure of CO₂ generated by hydrolysis of urea in water is 1.03 kPa per mg/dl of urea N at 24°C.

In a closed system with no initial gaseous CO₂, the final value of the partial pressure in the enclosed cell must be adjusted for mass loading because CO₂ is depleted in the aqueous phase. If \( \alpha \) is the ratio of partial pressure with mass loading to the partial pressure with no mass loading, then \( \alpha \) can also be defined as the ratio of CO₂ in solution at equilibrium to the total CO₂ in the system,

\[ \alpha = \frac{[CO_2]_{aq} V_f}{[CO_2]_{aq} V_f + [CO_2(g)]}, \]  

where \( V_f \) is the volume of liquid in the system and \( [CO_2(g)] \) is the mole count of gaseous CO₂. We can solve for \( [CO_2(g)] \) in terms of \( [CO_2]_{aq} \) by relating the partial pressure described by the ideal gas law to that described by equation [2],

\[ CO_{2(g)} = \frac{V_g k_p}{R T [H_2O]} [CO_2]_{aq}, \]  

where \( V_g \) is the gaseous volume of the system, \( R \) is the universal gas constant, and \( T \) is absolute temperature. Substituting equation [5] into equation [4] yields

\[ \alpha = \left(1 + \frac{k_p R T [H_2O] V_f}{V_g}\right)^{-1}. \]  

We can predict the sensitivity of the device to urea by using the definition of \( \alpha \) and the fact that 1 mol of urea is hydrolyzed to 1 mol of carbonate:

\[ P_{theory} = \left(\frac{k_p R T V_f}{k_p V_g + R T [H_2O] V_f}\right) [urea]. \]

Influence of physical and chemical conditions. To operate the sensor within the given time constraints, the equilibrium between various carbonate ions and CO₂ must be considered:

\[ \text{CO}_2 \text{aq} \rightleftharpoons \text{CO}_3^{2-} + \text{H}_2\text{O} \quad \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \quad \text{H}^+ + \text{CO}_3^{2-} \rightleftharpoons 2\text{H}^+ + \text{CO}_3^{2-}. \]

The ratio of dissolved CO₂ to carbonate is dependent on pH. If the solution is alkaline, the carbonate in the system is effectively ionized in solution. Therefore, the system must be acidified in order to generate a pressure signal in real time. Based on weak acid chemistry, lowering the pH to 4.1 drives 99% of the carbonate to dissolved CO₂.

A number of different parameters have a strong effect on the system and must be corrected or controlled for. Perhaps the most important of these is temperature. The solubility constant is highly temperature dependent; \( k_p \) increases with temperature, causing the ideal sensitivity (equation [2]) and the loading correction factor (equation [6]) to change. Partial temperature compensation may be achieved by prudent design of the ratio of liquid to gaseous volumes in the cell (decreasing \( \alpha \) offsets an increase in \( k_p \)), thereby reducing the scale of errors possible from software compensation.

Temperature also has a strong influence on water vapor pressure, which may be compensated for by blanking the hydrolyzed sample against the untreated sample. This practice also corrects for the effects of background dissolved gases, ambient CO₂, and humidity levels.

The premature loss of CO₂ from the solution into the atmosphere or into the cell before it is sealed represents a significant source of sensitivity loss and possible error. Consequently, the acidified sample must be handled...
reproducibly and sealed in the cell as quickly as possible.

Another possible source of error is the variation in CO₂ solubility that is caused by other components in solution. Milk is comprised mainly of water (87.2%), lactose (4.7%), milk fat (4%), and protein (3.4%). Dissolved solids such as lactose decrease the solubility of CO₂. Therefore, the presence of lactose should make the new assay more sensitive to the presence of urea in milk. Likewise colloidal proteins in solution should increase the sensitivity of the assay by displacing water. The presence of fat in milk would be expected to lower the sensitivity of the assay because CO₂ is reported to be about twice as soluble in butterfat as in water (derived from 8, p. 367). The effect of each of these components was determined experimentally.

Assay Protocols and Sample Preparations

A manual assay of the type described above was carried out in a 3.8-ml volume section of plastic tubing attached to the positive port of a 50-kPa pressure transducer (Motorola MPX 2050D, Phoenix, AZ). Standards were prepared by dissolving urea or the stoichiometrically equivalent amount of Na₂CO₃ in PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.0) or distilled water. We hydrolyzed the urea by adding 1 mg of lyophilized urease (EC # 3.5.1.5, Sigma Aldrich Chemical Corporation, St. Louis, MO) to each 10 ml of standard and incubating the samples at room temperature for 5 min. For the standard assay, a volume of 2.0 ml of the sample was added into the tubing, followed by 0.4 ml of 1.0 M citric acid to bring the pH below 4.0. The tubing was then sealed and shaken by a small DC motor. The signal from the transducer was recorded after 1 min of shaking.

We tested the effects of milk fat, casein, and lactose by assaying standards of equal urea N concentrations (30 mg/dl) and taking the regression against varying component concentrations. Stock solutions of the different components were prepared in PBS by addition of α-lactose (# L5-500, Fisher Scientific, Pittsburgh, PA), commercial heavy cream (assumed 40% milk fat), or casein (# 7078, Sigma Aldrich Chemical). Urea was added to the stock solutions to give 30 mg/dl of urea N. Samples were prepared by blending the stock solutions with solutions of 30 mg/dl of urea N in PBS. These samples were assayed by the method prescribed above.

We also tested the sensitivity of the assay in eight milk samples and compared it to the sensitivity for standards in water and PBS. Each milk sample was divided into two 10-ml volumes. One 10-ml volume from each sample was spiked with 100 μl of a stock solution of 3.03 g/dl of urea N in water. The other 10-ml volume from each sample was adulterated with 100 μl of water. These separate volumes were assayed as described above and the assay sensitivity in each milk sample was estimated based on the difference in observed pressure for the spiked and adulterated solutions. We then assayed 30 mg/dl of urea N solutions in PBS and distilled water, as well as the stoichiometrically equivalent amount of Na₂CO₃ in PBS and distilled water. Three replications were assayed for the sensitivities in each standard and sample in order to perform single-factor analysis of variance (ANOVA) and Fisher’s least significant difference (LSD) tests.

RESULTS AND DISCUSSION

The time response of an automated version of the assay is shown in Figure 1. The response can be approximated as first order with a time constant of about 3 s, reaching effective equilibrium within 20 s. The background pressure developed in the untreated sample is not negligible and must be used as a blank when samples have unknown amounts of dissolved gas or when ambient conditions are not carefully controlled.

A comparison of the standard curve for hydrolyzed urea in PBS with the pressure predicted by equation [7] is shown in Figure 2. The assay is linear over the range of interest with a standard error of 0.3 mg/dl. The predicted sensitivity is almost 37% higher than that observed, even accounting for dilution of the standard with acid and mass loading in the cell. Relating the observed sensitivity to the expression for sensitivity in equation [7], we find that the observed value for the constant kₚ is 96,700 kPa, compared to 161,000 kPa calculated from equation [3]. The reasons for this differ-

![Figure 1. Pressure recorded versus time during shaking of an automated version of the new assay: enzymatically hydrolyzed sample (●); —; Y = 6.46(1 - e⁻X/2.9); r² = 0.99, untreated sample (○).](image-url)
ence may include premature volatilization of \( \text{CO}_2 \), deviation from ideal gas behavior, and a true difference of \( \text{CO}_2 \) solubility from that described by equation [3]. The last explanation is quite plausible given observations that \( \text{CO}_2 \) solubility is inversely related to \( \text{pH} \) (13, p. 82).

We can verify the effect of mass loading in the system by showing the sensitivity of the sensor for varying values of fluid volume loaded into the cell. Standards of \( \text{Na}_2\text{CO}_3 \) in PBS equivalent to 30 mg/dl of urea N were acidified with 1 part 1 M citric acid per 5 parts of standard. Various volumes of these mixtures were assayed in the cell to determine the dependence of pressure on the ratio of liquid to gas volume. The observed data (Figure 3) correlate well with equation [7] if the value of \( k_p \) used in the equation is the same as the value fit in the standard curve (96,700 kPa). Although there is a discrepancy between the observed \( k_p \) value and that reported for pure water, the observed value is still effectively constant for a given temperature. Consequently, the system is linear and the sensor may be easily calibrated for dissolved \( \text{CO}_2 \) levels in milk.

The stability of the carbonate ion is of great importance to the handling of samples. Storing hydrolyzed samples too long at too low a \( \text{pH} \) leads to a loss of carbonate from solution, particularly if the samples are agitated or heated. Figure 4 shows the profiles of carbonate remaining in solution against \( \text{pH} \) for samples stored at 24\(^\circ\)C for 1 and 16 h. The \( \text{pH} \) dependence of dissolved \( \text{CO}_2 \) is also shown; the rate of carbonate loss is clearly related to dissolved \( \text{CO}_2 \). At neutral and alkaline \( \text{pH} \), the loss of carbonate after 1 h is insignificant. Samples below this \( \text{pH} \) should be adjusted to higher \( \text{pH} \) before hydrolysis and, in any case, hydrolyzed samples should be assayed within 30 min to prevent losses. Given that most milk samples range in \( \text{pH} \) from 6.5 to 6.7 (21, p. 404), no significant losses in signal should occur if samples are assayed immediately after hydrolysis.

A comparison of MUN values measured by the new method with those assayed by an autoanalyzer (Technicon Corporation Series 2 Autoanalyzer, Tarrytown, NY) demonstrates reasonable agreement between the

---

**Figure 2.** Standard curve of pressure against urea N in PBS at 24\(^\circ\)C: predicted by equation [7] using \( k_p \) value from equation 3 (—; \( k_p = 161,000 \text{ kPa}; Y = 0.501X \)), observed \( (\bullet; \cdot \cdot \cdot \cdot \cdot \cdot \cdot) \); \( k_p = 96,700 \text{ kPa}; Y = 0.367X \) (r\(^2\) = 0.999, SE = 0.3 mg/dl).**

**Figure 3.** Pressure recorded in sensor for 30 mg/dl urea N standard versus ratio of liquid to gas volume in the reaction cell at 24\(^\circ\)C: predicted by equation [7] using \( k_p \) value from equation [3] (—), observed (●), predicted by equation 7 using \( k_p = 96,700 \text{ kPa} \) (— — —).

**Figure 4.** Time and \( \text{pH} \) dependence of normalized carbonate in solution: ratio of dissolved \( \text{CO}_2 \) to total dissolved carbonate species (—), normalized total amount of carbonate remaining in solution after 1 h (●; — — —), normalized total amount of carbonate remaining in solution after 16 h (○; — — —).
Figure 5. Comparison of milk urea N (MUN) values recorded by CO₂ partial pressure method with those recorded by autoanalyzer using reaction of urea with diacetyl monoxime \( Y = 0.942 X - 1.66 \) \((r^2 = 0.964)\).

new assay and established methods. The comparison is shown in Figure 5. The milk used for these tests was preserved with bronopol and natamycin (Broad Spectrum Microtabs II, D&F Control Systems, San Ramon, CA). Because the true concentrations of urea in the milk samples are unknown, it is unclear which technique more accurately estimates MUN. The auto-analyzer screens molecules through a dialysis membrane and photometrically assays for a pink complex resulting from the reaction of urea with diacetyl monoxime (16, 19). Although this reaction is reported to give falsely high readings because of reactions with peptides, other molecules with amide bonds, substituted ureas, and some amino acids (2), we have seen that the dialyzer effectively eliminates any interferences in milk. Work is continuing to determine and better quantify the cause of the discrepancies between the two measurement systems. Preliminary data suggest that the negative intercept evident in Figure 5 occurs when the preservative is used, whereas no offset occurs when the milk is fresh and unadulterated. In light of this, on-line measurements using the new sensor may be an accurate reflection of MUN as measured using existing methods.

A summary of the regression data of pressure observed in 30 mg/dl of urea N samples against different milk component concentrations shows that the assay has little interference from the major components of milk (Table 1). The effect of lactose is significant at the 5% level. The effect, however, is slight—only a 0.6% change in assay sensitivity per change in percentage of lactose, representing an error of 0.2 mg/dl in a sample containing 30 mg/dl of urea N. The regression statistics for casein show an effect opposite to what we expected. The observed sensitivity actually decreases with an increasing concentration of casein. We speculate that the acid precipitate of casein inhibiting the diffusion of CO₂ into the gaseous phase causes this decline. Still, the observed effect is small, showing a −1.7% change in sensitivity per change in the concentration of casein. The regression statistics for milk fat show no significant effect of fat \((p\text{-value } 0.8619)\). This result is surprising given the reported difference in CO₂ solubility in fat and water and suggests that the solubility of CO₂ in the solutions tested is probably higher than that described by equation [3].

Comparisons of mean sensitivities for the eight spiked milk samples and urea and stoichiometrically equivalent solutions of Na₂CO₃ in PBS and distilled water are shown in Table 2. No differences in sensitivity were detected by single-factor ANOVA at the 5% level \((P = 0.4453)\). With the LSD test, only one milk sample was found to have a sensitivity different than the standard solutions at the 5% level. These tests indicate that the assay should be quite reliable in milk, and no correction need be made for calibrations using standard solutions in PBS or distilled water.

### TABLE 1. Summary of regression statistics: assay sensitivity to major milk components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Range tested (%)</th>
<th>Temp. (°C)</th>
<th>Baseline pressure (kPa)</th>
<th>Regression slope (kPa/%)</th>
<th>SE¹ (kPa)</th>
<th>% Error/ % Δ component²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>0–10</td>
<td>24.8</td>
<td>9.37⁴</td>
<td>0.05*</td>
<td>0.224</td>
<td>0.6%*</td>
</tr>
<tr>
<td>Casein</td>
<td>0–5</td>
<td>23.9</td>
<td>9.20⁴</td>
<td>−0.16*</td>
<td>0.245</td>
<td>−1.7%*</td>
</tr>
<tr>
<td>Fat</td>
<td>0–5</td>
<td>24.2</td>
<td>9.27⁴</td>
<td>0.007</td>
<td>0.261</td>
<td>0.07%</td>
</tr>
</tbody>
</table>

¹Standard error of observations about regression.
²Change in sensitivity per change in percent component. Calculated as ratio of regression slope to regression intercept (baseline pressure).
³Significant at the 5% level.

CONCLUSIONS

We have shown that the new assay for milk urea is simple and robust. At 24°C, the sensitivity of the assay is 0.367 kPa per mg/dl of urea N. The standard error
for buffered standards is 0.3 mg/dl in the range from 0 to 30 mg/dl. Because of the format of the assay, there are no effects of sample pH and buffering capacity except in terms of carbonate loss after urea hydrolysis. This effect should not be a problem under the short cycling times of an automated sensor. We estimate that the standard errors in milk would be within 1 mg/dl based on observed interferences by milk solids and comparison of the assay results in milk with traditional chemical methods. The presence of milk fat had no significant effect on the sensitivity of the assay, and the effects of milk proteins and lactose were slight (−1.7 and 0.6% change in sensitivity per change in w/v percent, respectively). No significant differences were detected in assay sensitivity in standards in water, buffer and milk. The assay may be adapted for use as a robust and reproducible on-line MUN sensor in the milking parlor. Automated versions of the assay have been built and tested successfully in the laboratory, and work to improve the fluid handling and the efficiency of the enzymatic hydrolysis step continues. Future work will include testing different configurations of the biosensor and engineering temperature compensation into the system.

ACKNOWLEDGMENT

This research was partially supported by USDA/BARD research project US-2638-95.

## REFERENCES


### TABLE 2. Comparisons of mean assay sensitivities in milk, PBS, and distilled water.

<table>
<thead>
<tr>
<th>Sample tested</th>
<th>Sensitivity$^1$</th>
<th>Standard Error$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea in PBS</td>
<td>0.315$^a$</td>
<td>0.005</td>
</tr>
<tr>
<td>Milk sample 7</td>
<td>0.318$^a$</td>
<td>0.011</td>
</tr>
<tr>
<td>Milk sample 8</td>
<td>0.319$^a$</td>
<td>0.005</td>
</tr>
<tr>
<td>Na$_2$CO$_3$ in PBS</td>
<td>0.320$^b$</td>
<td>0.001</td>
</tr>
<tr>
<td>Na$_2$CO$_3$ in water</td>
<td>0.320$^b$</td>
<td>0.001</td>
</tr>
<tr>
<td>Urea in water</td>
<td>0.320$^b$</td>
<td>0.001</td>
</tr>
<tr>
<td>Milk sample 2</td>
<td>0.324$^{ab}$</td>
<td>0.007</td>
</tr>
<tr>
<td>Milk sample 1</td>
<td>0.325$^{ab}$</td>
<td>0.008</td>
</tr>
<tr>
<td>Milk sample 3</td>
<td>0.326$^{ab}$</td>
<td>0.009</td>
</tr>
<tr>
<td>Milk sample 6</td>
<td>0.327$^{ab}$</td>
<td>0.007</td>
</tr>
<tr>
<td>Milk sample 4</td>
<td>0.332$^{ab}$</td>
<td>0.010</td>
</tr>
<tr>
<td>Milk sample 5</td>
<td>0.343$^{ab}$</td>
<td>0.012</td>
</tr>
</tbody>
</table>

$^a,b$Values with the same letter are not significantly different at the 5% level.

$^1$Units of kilopascals per equivalent milligram per deciliter of urea N.