

## Urea determination in milk by a differential pH technique

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**Abstract** — A differential pH method for the routine determination of urea in milk is presented. The measure is based upon a single enzymatic reaction, the urea hydrolysis by urease, which causes a pH variation directly proportional to the urea content in the milk sample. No sample pretreatment is required. Repeatability expressed as a coefficient of variation was 0.85 % at a level of 416 mg-urea·L<sup>-1</sup>. Recovery of added urea averaged 99.4 %. The preservatives Bronopol and sodium azide at a ten times higher level than recommended did not affect the results. Comparison with the manual enzymatic French reference method showed a very good agreement. The method is very simple, accurate and rapid. © Inra/Elsevier, Paris

**urea / differential pH / milk / enzymatic assay**

**Résumé** — **Dosage de l'urée dans le lait avec la technique de pHmétrie différentielle.** Une méthode de pHmétrie différentielle pour la détermination de l'urée dans le lait est présentée. La mesure est basée sur une seule réaction enzymatique, l'hydrolyse de l'urée par l'enzyme urease, qui provoque une variation de pH directement proportionnelle au niveau de l'urée dans l'échantillon de lait. Aucun prétraitement de l'échantillon n'est nécessaire. Le coefficient de variation de la répétabilité vaut 0,85 % au niveau de 416 mg·urée·L<sup>-1</sup>. La détermination de l'urée ajoutée était en moyenne de 99,4 %. L'addition de conservateurs tels que le Bronopol ou l'azide de sodium à une concentration dix fois supérieure à la quantité conseillée n'avait aucune influence sur les résultats. La comparaison avec la méthode manuelle enzymatique française de référence donne une très bonne corrélation. La méthode est très simple, précise et rapide. © Inra/Elsevier, Paris

**urée / pHmétrie différentielle / lait / essai enzymatique**

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## 1. INTRODUCTION

Ureic nitrogen in milk is derived essentially from the ureic nitrogen in blood; several reports have demonstrated a close correlation between urea levels in blood and in milk [3, 12]. Measuring the milk urea and the blood urea contents is approximately the same, but since milk is more easily available, it is a very suitable medium for the analysis of urea.

In mammals, urea represents the molecule used for excretion of ammonia; it is synthesized in the liver from the ammonia, which, in free form, is extremely toxic for the organism. In ruminants, the ammonia reaching the liver has two origins: exogenous, absorbed through the rumen's walls, and endogenous, originated from the amino acid of the animal organism. Its concentration in blood is highly affected by dietary factors, especially the amount of protein ingested and the protein/energy ratio [4, 8]. Consequently, determination of the urea in milk provides important information about the cows' feeding practices.

Several authors have also demonstrated a good correlation between the urea content in milk and the health problems of cows [6, 7], heat stability of milk proteins [2, 5] and cheese characteristics and yield [9–11, 13].

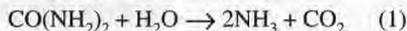
Regular determinations of urea in milk from a large number of cows can be helpful in several ways; however, for routine analysis, simple, rapid, accurate and inexpensive methods are required.

The purpose of our work has been to optimize a new method to determine the urea content in milk with an analytical apparatus which fulfils the following requirements: the instrument is small and easy to use, no sample pretreatment is necessary, the analysis rate and stability reagents are high, and it is accurate and precise.

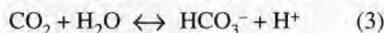
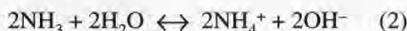
## 2. MATERIALS AND METHODS

### 2.1. Principle

The addition of urease to a urea-containing solution promotes the following reaction:



Reaction (1) is rate limited by the amount of urease added. Its half-time varies between 20 to 2 s ( $10\text{--}100 \text{ U}\cdot\text{mL}^{-1}$  of urease) and produces ammonia, which, at pH 6.7 immediately hydrolyzes, thus releasing hydroxyl ions. Carbon dioxide also hydrolyzes, with a half-time of about 30 s, thus partially neutralizing the  $\text{OH}^-$  produced by step (2):



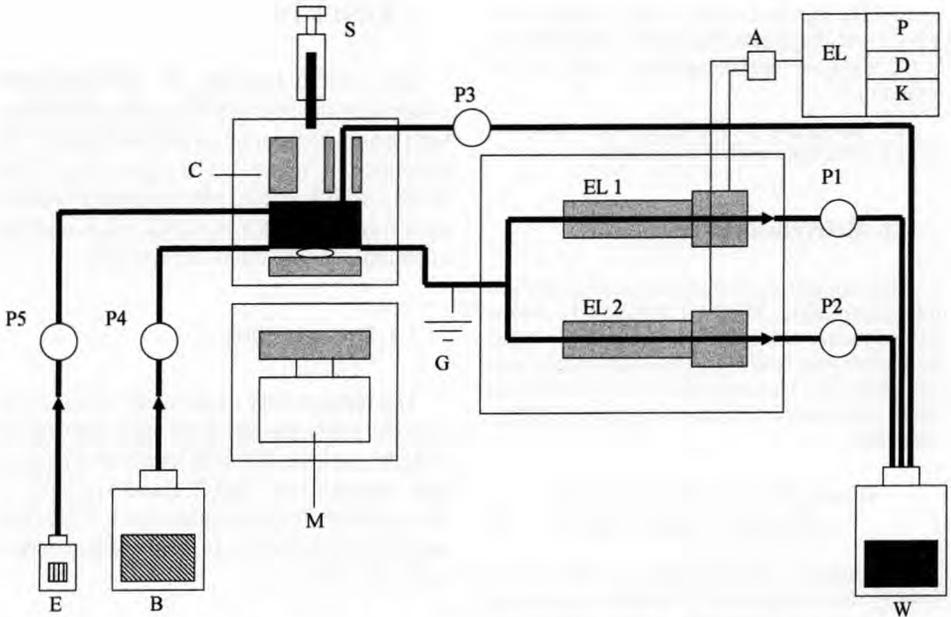
### 2.2. Instrumentation

The change in pH produced by the urea's reaction in a properly buffered system is measured by a commercially available differential pH analyzer, CL 10 Micro (kindly supplied by Eurochem, S.r.l, Ardea, Rome, Italy). The apparatus (*figure 1*) is essentially composed of a measurement block thermostatic stabilized between 30 and 37 °C, containing a mixing chamber, two capillary glass electrodes and five peristaltic pumps. The analyser is controlled by a personal computer running program 'pH 4000', with a real-time graphic display of a differential pH signal.

### 2.3. Method

Twenty  $\mu\text{L}$  of milk are added to the mixing chamber containing 970  $\mu\text{L}$  of buffer solution; a half volume of milk and buffer solution is aspirated in both the electrodes and the pH difference between the electrodes is measured. Fourteen  $\mu\text{L}$  of enzyme are then automatically added to the milk and buffer solution still remaining in the mixing chamber and the solution is aspirated in only one electrode. The new pH difference is monitored.

Endogenous ammonia in milk does not interfere with the differential pH measurement, because both electrodes contain the same diluted milk sample.



**Figure 1.** Diagram of the differential pH apparatus. A: Differential amplifier; B: buffer solution; C: mixing chamber; D: display; E1 and E2: glass capillary electrodes; EL: electronics; ES: enzyme or substrate solution; G: ground; K: keyboard; M: magnetic stirrer; P: printer; P1 to P5: peristaltic pumps; S: micropipette for injection of sample; W: waste.

**Figure 1.** Diagramme du pHmètre différentiel : A amplificateur différentiel ; B solution tampon ; C chambre de mélange ; D display ; E1 et E2 électrodes capillaires en verre ; EL électronique ; ES solution de l'enzyme ou du substrat ; G terre ; K clavier ; M agitateur magnétique ; P imprimante ; P1 à P5 pompes péristaltiques ; S micropipette pour l'injection de l'échantillon ; W déchets.

## 2.4. Materials

The buffer solution used had the following composition: 8 mmol·L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>, 12 mmol·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 100 mmol·L<sup>-1</sup> KCl, 1 g·L<sup>-1</sup> NaN<sub>3</sub>, 10 mg·L<sup>-1</sup> acetazolamide, 2 mmol·L<sup>-1</sup> Mg<sup>++</sup>, 2 g·L<sup>-1</sup> Triton × 100, 1 g·L<sup>-1</sup> Brij 35 (supplier Fluka), 2 % v/v LM1 (supplier Kartell), pH 6.7 (at 25 °C).

Urease (E.C. 3.5.1.5.) lyophilized, supplied by Genzyme, was diluted in an aqueous solution of glycerol 50 % v/v to the final concentration of 1 800 U·mL<sup>-1</sup>.

Aqueous urea standard solutions were prepared by diluting a stock solution of 100 g·L<sup>-1</sup> urea in KCl 100 mmol·L<sup>-1</sup> and 1 g·L<sup>-1</sup> NaN<sub>3</sub>.

All of the milk samples used were raw whole milk samples or raw whole milk samples added to Bronopol (2-bromo-2-nitropropane-1,3-diol)

either individual or taken from farm tanks. The samples were stored at 4 °C and analyzed within 1 or 2 d, if not mixed with Bronopol, or within 1 week if mixed with Bronopol.

## 2.5. Reagents stability

Three preservatives, sodium azide, sodium methionate and Bronopol, were evaluated at different concentrations in relation to their influence on the urease activity.

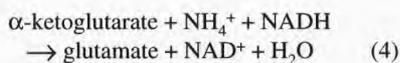
The stability assay was carried out at refrigerated and room temperature, on different lots of the reagents, added to sodium azide or Bronopol, while monitoring the following parameters: pH and buffer power for the buffer solution with sodium azide 1 g·L<sup>-1</sup> and the buffer solution with Bronopol 1 g·L<sup>-1</sup>; pH and urea con-

tent for the standard solution with sodium azide  $1 \text{ g}\cdot\text{L}^{-1}$  and the standard solution with Bronopol  $1 \text{ g}\cdot\text{L}^{-1}$ ; pH and enzyme activity for the urease solution.

All the reagents being tested were analyzed once a week for a period of 6 months.

## 2.6. Reference method

Milk samples were analyzed using the French reference method, NFV04-217-Afnor [1], a manual enzymatic determination. Urea is hydrolyzed by the enzyme urease to give ammonium ions and carbonate. The ammonium ions formed then act as substrate for the enzyme glutamate dehydrogenase:



The decrease in absorbance at 340 nm following the oxidation of NADH is monitored spectrophotometrically. Milk protein and fat have to be precipitated and filtrated before the analysis and the decrease of absorbance due to the endogenous ammonia in the milk has to be subtracted from the total absorbance variation obtained.

## 3. RESULTS

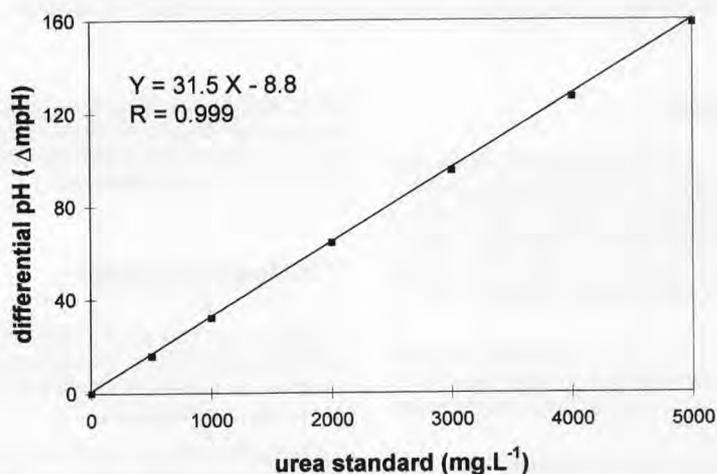
The linearity between pH signal and urea concentration, in aqueous standard solution with a urea content of up to  $5\,000 \text{ mg}\cdot\text{L}^{-1}$ , is presented in *figure 2*. The regression of the results of differential pH method ( $Y$ ) on the urea content ( $X$ ) is  $Y = 31.5 X - 8.8$ , and the correlation coefficient ( $R$ ) = 0.999.

### 3.1. Repeatability

The repeatability of the method was evaluated by analyzing ten times three whole milk samples at three different levels of urea content – respectively, 140, 223 and  $416 \text{ mg}\cdot\text{L}^{-1}$ ; the coefficients of variation were 1.50, 0.86 and 0.85 %, respectively. The results are presented in *table 1*.

### 3.2. Recovery

The recovery was evaluated by adding different known amounts of an aqueous urea



**Figure 2.** Method linearity with aqueous urea standards (500, 1 000, 2 000, 3 000, 4 000 and  $5\,000 \text{ mg}\cdot\text{L}^{-1}$ ).

**Figure 2.** Linéarité de la méthode avec standards aqueux d'urée (500, 1 000, 2 000, 3 000, 4 000,  $5\,000 \text{ mg}\cdot\text{urée}\cdot\text{L}^{-1}$ ).

solution (100 g·L<sup>-1</sup>) to a whole raw milk sample with endogenous urea concentration of 247 mg·L<sup>-1</sup>.

The average recovery was 99.4 %; regression of measured urea minus endogenous urea content on urea added was:  $Y = 0.99 X + 1.2$ ,  $R = 0.999$ . The results are presented in *table II*.

### 3.3. Accuracy

The accuracy of the differential pH method was evaluated by comparison with a manual enzymatic method. The results of this method comparison, carried out in double on 47 whole milk samples, preserved with Bronopol, containing 80 to 500 mg·L<sup>-1</sup> of urea, are presented in *figure 3*.

Calculation of the regression of the results of differential pH method on the results of enzymatic manual method yields the following equation:  $Y = 0.968 X + 12.191$ ,  $R = 0.997$ .

The mean value of the 94 determinations was 300.9 mg·L<sup>-1</sup> for the manual enzymatic method and 303.6 mg·L<sup>-1</sup> for the differential pH method. The mean difference and the standard deviation of the differences between the two methods were, respectively, -2.7 and 8.5 mg·L<sup>-1</sup>.

According to the *t*-test the mean results from the two methods are not significantly different from each other. The coefficients of variation of the repeatability between the double determinations were 0.97 % for the differential pH method and 1.36 % for the manual enzymatic method.

### 3.4. Reagents stability

The activity of a urease solution 1 800 U·mL<sup>-1</sup> was measured in the buffer solution added to different concentrations of preservatives. The results are shown in *table III*. Bronopol has no inhibitory effect, sodium azide slightly inhibits the urease activity at the concentration of 2 % and sodium merthiolate

**Table I.** Repeatability on three raw whole milk samples with different urea content (results expressed in mg·L<sup>-1</sup>).

**Tableau I.** Répétabilité sur trois échantillons de lait cru avec différents niveaux d'urée (résultats en mg·L<sup>-1</sup>).

	Milk 1	Milk 2	Milk 3
	141	226	412
	142	222	420
	139	222	419
	141	220	414
	140	223	416
	141	225	421
	142	224	417
	136	223	419
	143	221	411
	138	225	413
Average	140	223	416
$\sigma_{n-1}$	2.11	1.91	3.55
CV %	1.50	0.86	0.85

**Table II.** Recovery on raw milk added with 100, 200, 400, 600 and 800 mg-urea·L<sup>-1</sup>.

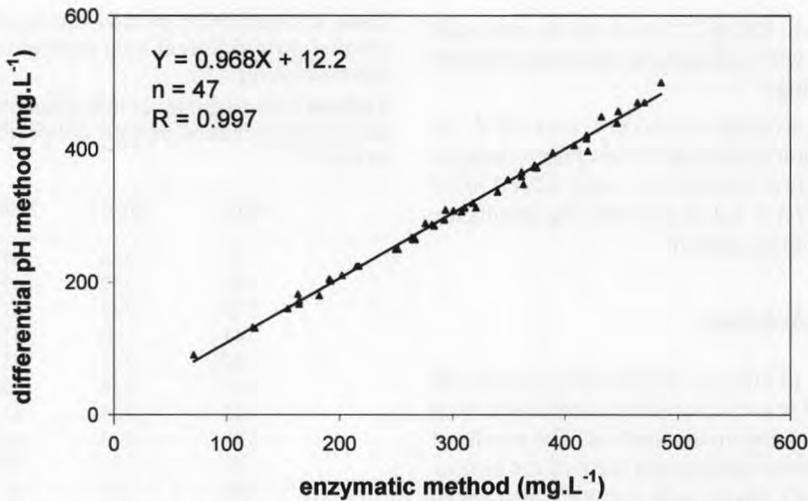
**Tableau II.** Détermination de l'urée ajoutée dans lait cru, 100, 200, 400, 600, 800 mg-urée·L<sup>-1</sup>.

	x 1	x 2	Average	Rec. %
Milk 0 (mg·L <sup>-1</sup> )	248	246	247	-
+ 100	346	346	346	99.0
+ 200	446	450	448	100.5
+ 400	643	645	644	99.3
+ 600	839	848	844	99.5
+ 800	1 041	1 032	1 037	98.8

has a strong inhibitory effect only at concentration five times higher than those normally used. However, we decided to exclude sodium merthiolate from the stability assay.

No modification of any buffer and standard solutions was observed after 6 months of storage.

The activity of the urease solutions at refrigerated temperature was unmodified after 6 months of storage, while the pH decreased of approximately 0.002 pH.



**Figure 3.** Correlation between the manual enzymatic method (standard Afnor V04-217) and the differential pH method.

**Figure 3.** Corrélation entre la méthode manuelle enzymatique (standard Afnor V04-217) et la méthode de pHmétrie différentielle.

**Table III.** Influence of different preservatives in phosphate buffer on the urease activity.

**Tableau III.** Influence de différents conservateurs en tampon phosphate sur l'activité de l'uréase.

Preservative	Concentration (%)	Activity (U·mL <sup>-1</sup> )
Bronopol	0.1	1 820
	0.5	1 760
	2.0	1 800
Sodium azide	0.1	1 790
	0.5	1 810
	2.0	1 570
Sodium merthiolate	0.1	1 800
	0.5	1 120
	2.0	490

The urease solutions at room temperature after 17 weeks of storage gradually started losing activity until the average loss of 20 % after 6 months. A gradual acidification was observed, probably caused by the protein denaturation.

### 3.5. Influence of preservatives

The influence of preservatives was evaluated by adding increasing amounts of an aqueous solution (5 %) of Bronopol (2-bromo-2-nitropropane-1,3 diol) and an aqueous solution (5 %) of sodium azide to three different raw milk samples. The results obtained (*table IV*) do not show any difference, within the experimental error, for unpreserved and preserved samples, for all the Bronopol and sodium azide concentrations tested.

## 4. DISCUSSION

The method presented here has a number of definite advantages as compared to the other existing methods for the determination of milk urea: the system uses untreated whole milk and no interference is caused by fat, fat droplets or by other suspended material.

The method derives its accuracy from the high specificity of the single enzymatic

**Table IV.** Influence of Bronopol and sodium azide at four concentration levels (0.02, 0.05, 0.1 and 0.2 %) on three different raw milk samples (results expressed in  $\text{mg}\cdot\text{L}^{-1}$ ).

**Tableau IV.** Influence de Bronopol et azide de sodium à quatre niveaux de concentration (0,02, 0,05, 0,1 et 0,2%) sur trois échantillons de lait cru.

Preservative	Milk 1	Milk 2	Milk 3
Unpreserved	267	351	217
Bronopol 0.02 %	263	352	221
Bronopol 0.05 %	270	354	219
Bronopol 0.1 %	265	352	220
Bronopol 0.2 %	269	353	218
Sodium azide 0.02 %	269	355	220
Sodium azide 0.05 %	264	351	219
Sodium azide 0.1 %	266	351	221
Sodium azide 0.2 %	265	356	220

reaction, with no interference of the most common preservatives used in milk. The pH signal obtained is directly proportional only to the urea content in the milk sample. Any spurious pH drift eventually present, due to aspecific side reactions, is automatically eliminated by the continuous blank compensation.

The apparatus is easy to use and the chemical reagents show good stability.

The method allows the analysis of 60 milk samples per hour.

The routine work flow for the method comparison study was eight samples in double per day, analyzed in parallel with both methods. With the manual enzymatic method alone, it required approximately 4 h.

## ACKNOWLEDGEMENTS

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## REFERENCES

- [1] Anonymous, Lait et produits laitiers. Détermination de la teneur en ammoniac et urée. Méthode enzymatique, norme Afnor V04-217, 1992.
- [2] Banks W., Clapperton J.L., Muir D.D., Powell A.K., Sweetsur A.W.M., The effect of dietary-induced changes in milk urea levels on the heat stability of milk, *J. Sci. Food. Agric.* 35 (1984) 165-169.
- [3] Bertoni G., Valutazione degli alimenti e dello stato metabolico dei ruminanti, Cappa V., Bertoni G. (Eds.), AIA, Roma, 1985.
- [4] Carlsson J., Pehrson B., The influence of the dietary balance between energy and protein on milk urea concentration. Experimental trials assessed by two different protein evaluation systems, *Acta Vet. Scand.* 35 (1994) 193-201.
- [5] Dalgleish D.G., Pouliot Y., Paquin P., Studies on the heat stability of milk. II. Association and dissociation of particles and effect of added urea, *J. Dairy Res.* 54 (1987) 39-45.
- [6] Doby R.T., Tritschler J.P., Prange R.W., Abdul Wahid F.T., Effect of dietary crude protein on urea in fluids of the reproductive tract of ewes and dairy cows, *J. Anim. Sci.* 59 (1986) 339 (abstr).
- [7] Ferguson J.D., Chalupa W., Impact of protein nutrition on reproduction in dairy cows, *J. Dairy Sci.* 72 (1989) 746-753.
- [8] Lewis D., Blood urea concentration in relation to protein utilisation in the ruminant, *J. Agric. Sci.* 48 (1987) 438-445.
- [9] Mariani P., Bonatti P., Sandri S., Contenuto di urea, pH, acidità titolabile e caratteristiche di coagulazione del latte di singoli allevamenti, *Ind. Latte* 28 (1992) 139-149.
- [10] Mietton B., Preparazione del latte per l'industria lattiero-casearia, *Latte* 14 (1989) 742-748.
- [11] Millet J., Incidence de la teneur en urée du lait et de la standardisation du pH du lait sur la qualité d'une pâte pressée cuite : comté, *Process* 1039 (1989) 56-62.
- [12] Oltner R., Wiktorsson H., Urea concentration in milk and blood as influenced by feeding varying amounts of protein and energy to dairy cows, *Livest. Prod. Sci.* 10 (1983) 457-462.
- [13] Pecorari M., Mariani M.S., Calzolari M.G., Tedeschi G., Il contenuto di urea nel latte: variazioni e rapporti con i parametri tecnologici, *Sci. Tec. Latt. Casearia* 44 (1993) 144-151.