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FLOW INJECTION ANALYSIS FOR THE DETERMINATION OF UREA IN COW'S MILK

By

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OLTNER, ROLAND, STAFFAN BENGTSSON and KJELL LARSSON: *Flow injection analysis for the determination of urea in cow's milk.* Acta vet. scand. 1985, *26*, 396—404. — An inexpensive and easily automated flow injection method for determination of urea in cow's milk was evaluated. Urea is hydrolysed by urease and in a gas diffusion cell the ammonia formed passes a membrane into an indicator solution. The resulting colour change of the indicator is measured at 590 nm.

The repeatability of the analysis, expressed as the coefficient of variation (C.V.), was between 0.5 and 1.2 %. Measured (y) and expected (x) milk urea concentrations after addition of known amounts of urea were related according to the equation $y = 1.00x - 0.12$ with a C.V. for the regression of 1.8 %. Recommended amounts (0.02 %) of the preservative bronopol (2-bromo-2-nitropropane-1,3-diol) added to the milk did not affect the results ($P > 0.05$).

feed monitoring; protein supply; clinical chemistry.

Inadequate feeding of cows causes lower milk production and/or impaired feed utilization and fertility. Therefore every means to achieve a better composition and control of dairy rations should be explored. In addition to chemical analysis of feedstuffs and a better control of feeding strategies, determination of various components in blood or milk has been suggested in this respect (*Rowlands* 1980, *Kaufmann* 1982, *Andersson* 1984). An example of the latter is the strong positive correlation between the protein/energy ratio in the feed and the level of urea in blood and milk (*Oltner & Wiktorsson* 1983). A high ratio impairs the utilization of feed protein, with high endogenous urea concentration and increased losses of nitrogen via the urine in consequence (*Thornton & Wilson* 1972). An additional negative factor is that the formation of urea is an energy-consuming pro-

cess. A low protein/energy ratio in the feed generally gives low urea concentrations which in turn may indicate an insufficient supply of amino acids for the production of milk and meat. Any deviation from a normal urea level in blood or milk may therefore reflect a suboptimal feeding. From a practical point of view it is advantageous to use milk for urea determinations due to the ease of obtaining milk samples from lactating cows.

Regular determinations of urea in milk from a large number of cows in a population can be helpful in several ways. Deviations from the reference range of milk urea values directly indicate an imbalance in the diet which, in most cases, will require correction. It may not be possible to detect such imbalance by conventional chemical analysis of feedstuffs. For example, several cases of heat-damaged silage were revealed by very low milk urea concentrations even though determinations of crude protein and ration checks had indicated that the cows had received an entirely adequate feeding (*Oltner*, unpublished observations).

When cows are given feedstuffs with an even quality and in the same amounts every day, day-to-day milk urea concentrations vary very little (*Oltner & Wiktorsson* 1983). However, if either the quality or the amount of feed should vary, the milk urea concentration will reflect this. Pronounced short-term variation in milk urea thus indicates the need for care regarding the quality of feedstuffs and feeding and management procedures. Large-scale milk urea determinations could also give interesting information about seasonal changes or feeding practices employed.

Recently a method for the determination of urea in milk was presented that had a relatively high capacity and yielded reliable results (*Oltner & Sjaunja* 1982). However, when used in routine work, methods for analysis of milk urea need to be inexpensive as well as rapid and accurate. The present study describes such a high capacity method based on flow injection analysis that needs a minimum of supervision and uses small amounts of low-cost reagents.

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical grade. Only glass-distilled water was used. Buffers and indicator solutions were degassed each day before use.

Indicator solution. Ammonia indicator mixture (100 mg/l, Tecator AB, Höganäs, Sweden) was dissolved in sodium dihydrogen phosphate (6 mmol/l) and pH adjusted by dropwise addition of dilute acid or base until the absorption of the solution was 0.10—0.20. An indicator stock solution can be prepared by dissolving indicator mixture (1.00 g) in sodium hydroxide (5 ml, 0.1 mol/l) and diluting to 200 ml with water.

Urease solution. Sodium dihydrogen phosphate (0.1 mol/l) and tris(hydroxymethyl)aminomethane (0.1 mol/l) are mixed to pH about 7.0. The buffer (10 ml) is used to dissolve urease (250 U, EC 3.5.1.5, Boehringer Mannheim).

Urea standard solutions. From a stock solution of urea in water (50.0 mmol/l) standards of 0.20, 0.40, 0.80, 1.20 and 1.60 mmol/l were prepared.

Instrumentation and performance

The apparatus used was a flow injection analyser (FIA-5020, Tecator AB, Höganäs, Sweden) with an automatic sampler (FIA-5007), a spectrophotometer (FIA-5023) and a data processing unit (FIA-5022) from the same supplier. Detailed information about the instrumentation and reagents can be found in Tecator Application Notes (note no. 50). The instrument was equipped with a gas diffusion cell (Chemifold V, Tecator AB). The set-up is described in Fig. 1. The sampler holds a total of 99 tubes. 14 positions were used for urea standards. Positions 1—5 were loaded with standards of increasing concentration to establish the standard curve and then every 10th position with a standard (0.80 mmol/l) to correct for any baseline drift.

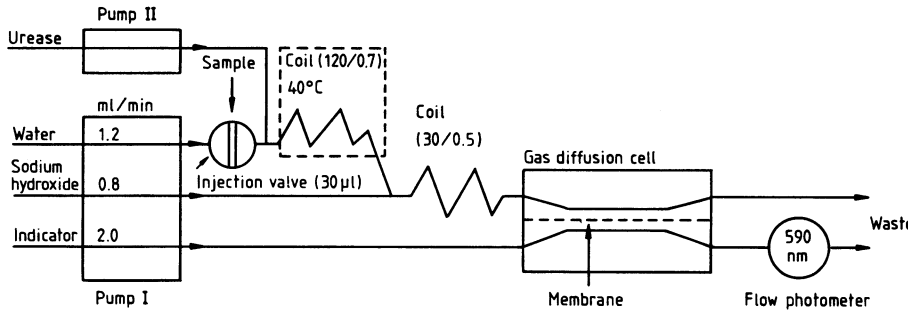

Figure 1. The flow injection manifold.

In a carrier stream of water (1.2 ml/min), samples of standard or diluted milk (30 µl) were injected automatically. Urease solution (70 µl) was added and while passing a reaction coil (120 cm, i.d. 0.7 mm, t = 14 s) kept at 40°C the urea was hydrolysed to ammonium ions and carbon dioxide by the urease. The reagent plug was then mixed with sodium hydroxide (0.2 mol/l, 0.8 ml/min) to raise the pH to above 11, at which point ammonium ions were converted to gaseous ammonia. In the gas diffusion cell, ammonia diffused through a gas-permeable membrane into the indicator which was pumped continuously (2.0 ml/min) on the other side of the membrane. The ammonia increased the pH and the concomitant colour shift of the indicator was measured by the photometer at 590 nm.

Experimental design

For the various experiments, samples of whole milk were taken from dairy cows mainly of the Swedish Red and White Breed. Before analysis, the milk was diluted with 4 parts of water (600 µl + 2,400 µl) using a dilutor (Microlab 1000, Hamilton, Switzerland).

To check the repeatability of the analysis, samples of whole milk from 5 cows were used. From each sample, 8 diluted samples were prepared and injected consecutively into the FIA apparatus.

Accuracy was determined by adding known amounts of urea to 4 samples of whole milk from different cows. Following dilution in volumetric flasks and analysis, the endogenous level of milk urea in each sample was subtracted before estimating the regression of measured on expected urea concentration. The accuracy of the analysis is expressed as the residual standard deviation (Se) of the regression. All FIA values used were means of duplicate determinations.

The reliability of the FIA method was also evaluated by comparing the results of urea determinations in milk samples from 44 cows with those obtained by the presently used routine method (*Oltner & Sjaunja* 1982). In this method an automatic instrument is used (I.L.-919, glucose/urea/creatinine analyser, Instrumentation Laboratories, Milano, Italy) where the ammonium ions formed upon urea hydrolysis participate in the reductive amination of α-ketoglutarate to glutamate. This reac-

tion is followed photometrically as the oxidation of NADH to NAD^+. The FIA values used were means of duplicate determinations.

Bronopol (2-bromo-2-nitropropane-1,3-diol), together with a little methylene blue as marker, is often used as a milk preservative in the Swedish milk recording system. Its possible effect on the milk urea determinations was examined by paired comparisons of 5 milk samples without and with bronopol at the recommended concentration (0.02 %, w/w) and at an approximately 30-fold higher concentration.

RESULTS

In the 5 milk samples used to estimate the repeatability of the method, mean urea concentrations varied between 3.11 and 4.87 mmol/l. The standard deviations of the distributions of results for the individual samples ranged from 0.02 to 0.04

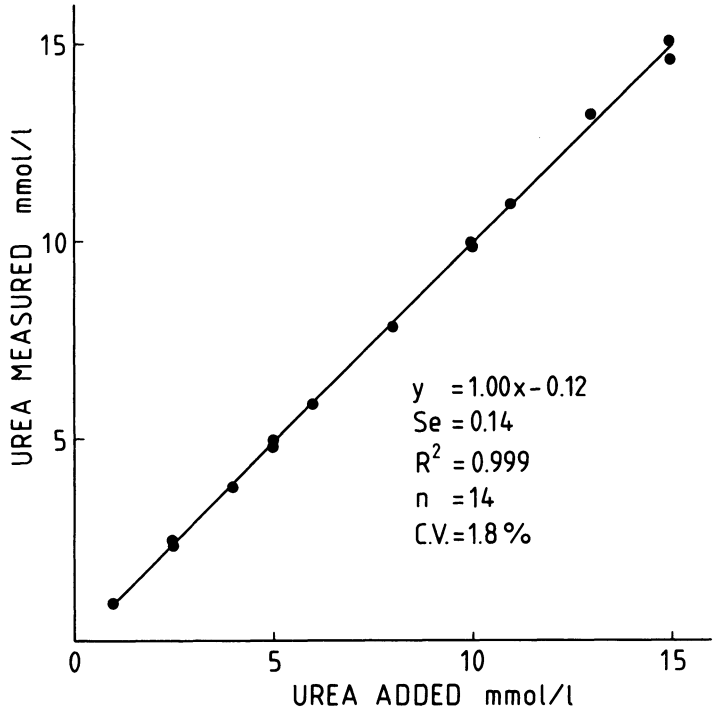

Figure 2. Accuracy of the flow injection analysis, expressed as the regression between actual (x) and measured (y) milk urea concentrations.

mmol/l, yielding coefficients of variation (C.V.) between 0.5 and 1.2 %.

The regression between measured (y) and expected (x) urea concentrations after the addition of known amounts of standard is presented in Fig. 2. They were related according to the equation $y = 1.00x - 0.12$ with a Se of 0.14 and a C.V. of 1.8 %. The recovery of added urea averaged 98 %.

Fig. 3 shows the comparison of the FIA method with the reference method. On average, the reference method gave 0.3 mmol/l higher values ($P < 0.001$). The results obtained with the FIA method (y) are related to those obtained with the reference method (x) as $y = 0.98x - 0.21$. The correlation coefficient for the regression (r) is 0.987 and the C.V. 4.7 %.

No effects on the results were found ($P > 0.05$) when bronopol was added to the milk in recommended amounts. However,

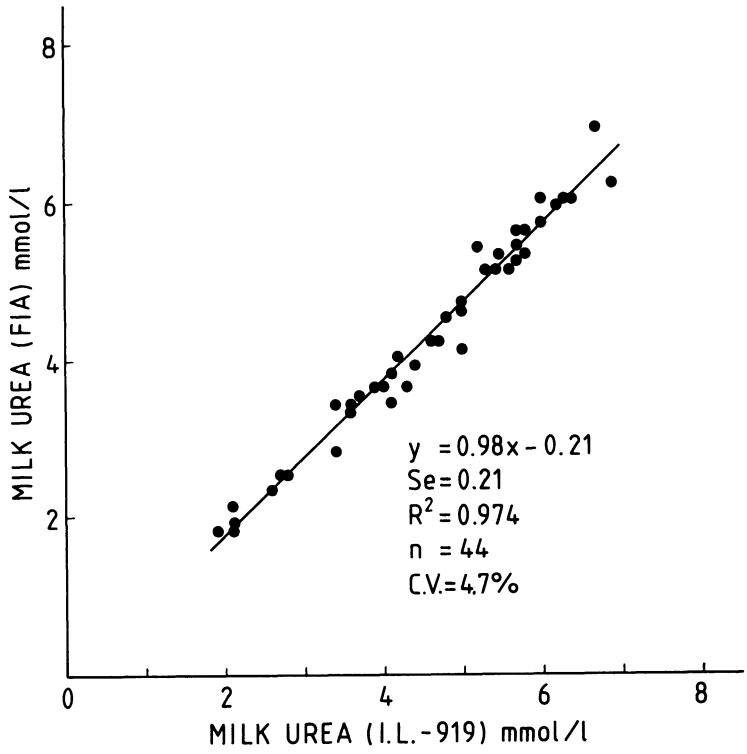

Figure 3. Comparison between results from milk urea analysis on 44 samples with a kinetic urease/glutamate dehydrogenase procedure (I.L.-919, x) and flow injection analysis (FIA, y).

bronopol at a concentration 30-fold higher than recommended gave slightly but significantly ($P < 0.05$) lower urea readings.

The cost per analysis was substantially lower with the FIA method than with the reference method, a difference mainly resulting from lower costs for chemicals and attendance.

DISCUSSION

It has been stated above that a routine method for the determination of milk urea must be rapid, accurate and inexpensive. With the FIA method presented here, 60 injections per h can be achieved. However, by merely increasing the carrier flow from 1.2 to 2.0 ml/min the capacity can be increased to 80 injections per h without obvious effects on either precision or accuracy. The loading of the sampler described is adapted for 2 injections of every sample. When single injections are to be made, recalibration is needed only after every 20th sample, thus allowing room for 90 unknown samples in the sampler.

When undiluted, unhomogenized samples are analysed, deposition of sample in the tubings and on the membrane in the gas diffusion cell will lead to carry-over errors, baseline drift and ultimately complete clogging of the tubings and the membrane. The need to dilute the samples before analysis is of course a drawback when hundreds of samples are to be analysed daily, although its effect on the precision is very small when a good dilutor is used. This is illustrated here, as the variation due to the dilution was included when calculating the precision of the method. Dilution does not completely eliminate the need for baseline corrections. There is a small and rather constant drift estimated to about 0.006 a.u. after 100 injections of milk samples.

The detector response to the change in pH in the buffer is not linear, but an ordinary titration curve. The absorbance of the indicator solution is adjusted so that the measurements are performed in the most linear range of the curve. However, although the indicator solution is buffered so as to, in a way, extend its straightest part, the non-linear relationship between pH and colour change of the indicator must be taken into consideration. This is done automatically by the data processing unit. Fig. 2 illustrates that the linear range of the method is wide enough to contain the extremes of feeding-induced variations in milk urea concentrations. The accuracy and the precision of the

method are excellent and there is no reason why duplicate analyses should be performed as a routine.

The urea determinations are disturbed by the presence of volatile amines. Ammonia is formed in stored milk, but the level of ammonia in fresh milk is low and fairly constant (*Venkatappaiah & Basu* 1952). The actual level of ammonia in a milk sample is easily determined by omitting the urease and recalibrating the instrument with an ammonium standard.

The somewhat lower values (0.3 mmol/l) obtained with the FIA method vis-à-vis the reference method are not consistent with the results of the recovery experiments for the 2 methods. No effort has been made to explain this difference, but in cases where values obtained with both methods are to be compared it must be considered.

To summarize, flow injection analysis seems to be an excellent technique for routine determinations of urea in cow's milk. It is well suited to meet the requirements of speed, accuracy, precision and price, although experience from routine use may reveal a need for modification of the method described here.

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SAMMANFATTNING

Bestämning av urea i komjölk med FIA (flödesinjektionsanalys).

Den utvärderade metoden bygger på att ammoniakgas som bildats efter hydrolys av mjölkurean diffunderar genom ett membran in i en indikatorlösning, varefter färgomslaget hos indikatorn mäts i en fotometer vid 590 nm. Reproducerbarheten, uttryckt som variationskoefficient, låg mellan 0,5 och 1,2 %. Tillsats av kända mängder ureastandard till mjölk före analys visade på en mycket god överensstämmelse mellan förväntade och erhållna värden. Normal tillsats av konserveringsmedlet bronopol (0,02 %) påverkade inte resultaten ($P > 0,05$).

FIA-metodiken bedöms vara väl lämpad för rutinmässig analys av urea i komjölk då den är snabb, säker och billig.

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