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# EVALUATION OF FLOW INJECTION ANALYSIS FOR DETERMINATION OF UREA IN SHEEP'S AND COW'S MILK

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Difficulties in measuring the urea content in sheep's milk often occur with spectral photometry due to the high protein and fat concentrations of the milk. In this study an enzymatic flow procedure (*QuickChem 8000 Ion Analyser*, Lachat Instruments, Milwaukee, USA) to determine the urea content in ovine and bovine milk was evaluated. Urea content is determined by the Berthelot reaction after splitting it enzymatically with urease. The free ammonia diffuses through a teflon membrane into a stream of reagent solutions. Detection takes place by means of a reaction between the ammonium ions with hypochlorite and salicylate producing a green colour, which is measured spectrometrically in a flow meter at 660 nm. By using a diffusion cell chemical deproteinisation of milk is not necessary and capacity is high. The assessed procedure exhibited high accuracy and precision and reached a sample capacity of 55 samples an hour. Storage of the milk samples for several days as well as chemical preservation with bronopol had no effect on the measurement procedure. Due to the complexity of the apparatus and the costs associated therewith, the device proves less suitable for routine diagnostics but rather serves as a reference method for the measurement of urea concentration in milk.

**Key words:** Urea, milk, flow injection analysis, evaluation

Rising costs and sinking profits in dairy milk production and changing nutritional habits of consumers have resulted in the growing importance of ovine milk. An economical control based on suitable parameters is thus becoming increasingly necessary in this agricultural sector. Numerous studies list milk urea content as a simple and efficient parameter for evaluating the metabolic status of cattle (Ropstad and Refsdal, 1987; Pehrson, 1996; Steen et al., 1996). The application of this parameter in assessing sheep herd health requires studies on two different levels. First of all, the relevance of milk urea should be discussed and physiological values for dairy sheep must be determined. Jelinek et al. (1996) and Bedő et al. (1997) found a significant correlation between the urea concentration in plasma

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and that in milk. In their studies, Cannas et al. (1998) confirmed a high correlation between protein content in feed and urea concentration in milk. Flöck (2000) described an increase of milk urea content at the beginning of the grazing season.

The routine use of this metabolic parameter, secondly, requires rapid and efficient analytical methods. Spectrophotometry is generally used in the analysis of cow's milk for urea. Colorimetric procedures are based on the reaction of urea with a colour reagent and the photometric measurement of the colour complex (Brown, 1959; McDowell, 1972; De Jong et al., 1992). Enzymatic analysis offers very specific measurement of the urea concentration by means of indirect ammonia detection (Wolfschoon-Pombo et al., 1981; Oltner and Sjaunja, 1982; Tschager and Jager, 1988).

Series measurements with a large number of samples place great demands on measurement procedures. Accuracy and precision aside, the processing of large sample quantities requires rapid, inexpensive and simple methods (Oltner et al., 1985). Andersson et al. (1986) assessed a flow procedure in which the urea concentration is measured by means of a concentration-dependent colour conversion of an indicator solution due to the enzymatic decomposition of urea. Another form of automation used successfully in the measurement of macronutrients in milk is infrared spectrometry. Due to the fact that other substances (e.g. lactose) absorb infrared rays in the urea absorbance spectrum, only approximate values can be measured by infrared spectrometry (Lefier, 1996). Quantitative analysis of ewe's milk with infrared systems requires special calibrations based on ovine milk (Baumgartner, 2000). In this study a method based on flow injection analysis is described.

## Materials and methods

Measurements were carried out with a QuickChem 8000 automated ion analyser from Lachat Instruments (Milwaukee, USA). As this procedure was designed for quantitative detection of ammonia in fluids, the enzymatic hydrolysis of urea to ammonia and carbon dioxide took place 30 min prior to measurement.

### *Basic principle (Switala, 1993)*

After enzymatic hydrolysis of urea the diluted sample was injected into a carrier stream of water. Ammonium ions were converted to monochloramine through a reaction with hypochlorite ions. After diffusion through a gas-permeable membrane monochloramine reacts with salicylate ions in the presence of sodium nitroprusside to form a green indophenol compound which is measured photometrically at 660 nm (Fig. 1).

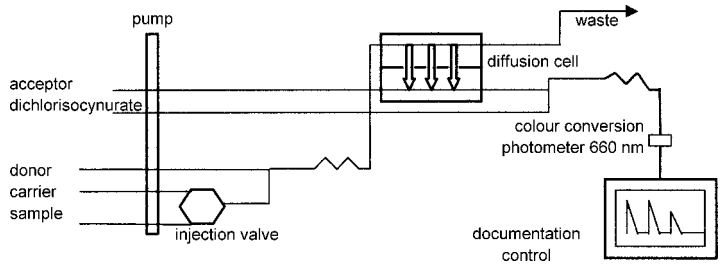


*Fig. 1.* Manifold diagram of flow injection analysis

### *Reagents*

*Buffer urease solution:* A buffer urease solution was made for the enzymatic hydrolysis of urea. For this solution, 0.5 mg of urease (U 1500, Fa. SIGMA, Vienna, Austria) and 2 ml of phosphate buffer (pH = 7.0) were used for each sample. The calculation of the required urease amount was carried out based on unit definition. One unit liberates 1.0 µmole of $NH_3$ from urea per minute at pH 7.0. For hydrolysing 1 µmol urea (60.06 µg) 2 units are needed. As declared by the manufacturer 1 mg of solids contains 16 units. As basis for the calculation, a urea content of 50 mg/dl (8.33 mmol/l) was assumed. Including excess, 0.5 mg of urease per sample was used.

*Reagents for the determination of ammonia:* The reagent solutions were composed of a sodium citrate donor, a salicylate citrate acceptor and sodium dichlorisocyanurate as a donor of hypochlorite ions. The reagent solutions were prepared according to instructions described by Lachat Instruments (Switala, 1993).

### *Preparation of the standard solution series*

From a stock solution of urea in water (1000 mg/l), standards containing 0 mmol/l, 1.665 mmol/l, 4.995 mmol/l, 8.325 mmol/l, 11.665 mmol/l and 16.65 mmol/l (correlating to 0 mg/dl, 10 mg/dl, 30 mg/dl, 50 mg/dl, 70 mg/dl and 100 mg/dl) were prepared.

### *Preparation of samples and standards*

To avoid matrix effects caused by fat deposits in the tubing, milk samples were heated to 40 °C in a water bath. Frozen milk samples were thawed overnight at refrigerator temperature and heated to 40 °C before measuring.

For enzymatic hydrolysis of urea 0.25 ml of milk (or standard solution) and 2 ml of buffer urease solution were incubated for 30 min at room temperature. The samples were arranged in the autosampler and the measurement process was carried out according to the OMNION-FIA program. A specific program for calibration was used. Calibration was accepted when a correlation coefficient of at least 0.9995 was given.

*Assessment of the flow injection analysis*

The analytic evaluation of the method was carried out according to accuracy, repeatability, linearity, influence of storage and chemical preservation, expenses and complexity of instruments. *Accuracy* was determined by adding known amounts of urea to 5 sheep's milk and 6 cow's milk samples. Considering the dilution, accuracy is expressed as the regression between measured and expected urea concentration. The FIA method was also assessed by analysing then comparing 30 samples of ewe's and 11 samples of cow's milk both by the FIA procedure and a direct colorimetric method. For the reference measurement, urea detection with 1,4-Paradimethylaminobenzaldehyde (PDAB) was used. Urea forms a yellow complex with this reagent in a low acid alcohol solution at room temperature (Lefier, 1996). Analysis was carried out following the instructions of Brown (1959) and McDowell (1970). For protein precipitation a 0.3 molar trichloroacetic acid solution was used. The clear filtrate was used for the colour reaction. The intensity of the colour was photometrically measured at 438 nm.

The *reproducibility* of the method was estimated through consecutive measurements (10 times each) of 6 ewe's and 6 cow's milk samples. It is expressed as standard deviation and coefficient of variation.

To evaluate the effects of *several days' storage and chemical preservation* of milk samples on the measurement of urea concentration, 10 sheep's milk samples were stored at +4 °C and measured after 3, 6 and 10 days. The effect of chemical conservation with bronopol (wide spectrum microtablets Mikrotabs II®, BHGmbH, Vienna, Austria) on milk urea content was examined using paired comparisons of 10 milk samples with and without bronopol. A third measurement was carried out following 10-day storage of the samples containing bronopol in a freezer at –20 °C. Due to the fact that several studies had already excluded an influence of storage and chemical preservation with bronopol on the urea content of cow's milk (Oltner et al., 1985; Carlsson and Bergström, 1994), the storage experiment was only carried out on sheep's milk. In order to evaluate the *convenience of the procedure* the following criteria were considered: complexity of instruments, capacity, expense and suitability for routine use.

## Results

Figure 2 presents the regression between measured and expected urea concentration after the addition of known amounts of urea. The recovery of added urea reached 99.3% for sheep's and 99.4% for cow's milk.

In the *reference measurement* comparing the flow procedure (FIA) and the colorimetric method (PDAB) on 30 ovine milk and 11 bovine milk samples, a correlation coefficient of 0.960 was estimated (Fig. 3). The mean difference between flow injection analysis and the colorimetric method was 0.47 mmol/l ±

0.58 mmol/l for ewe's milk and 0.40 mmol/l ± 0.26 mmol/l for cow's milk. Considering only the ovine milk samples the correlation coefficient was 0.961. For the cow's milk a correlation coefficient of 0.915 was determined.

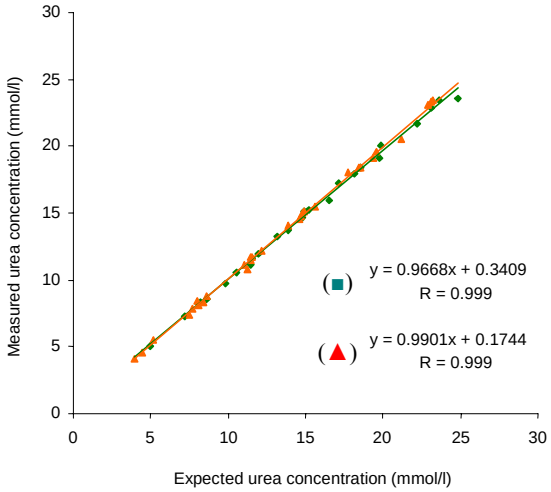


*Fig. 2.* Additional experiment: relationship between expected and actual urea concentration as measured in sheep's (■) and cow's milk (▲)

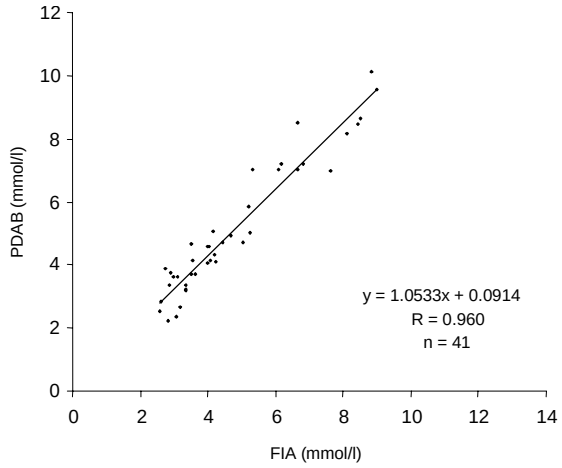


*Fig. 3.* Urea concentrations in 30 sheep's milk and 11 cow's milk samples determined by flow injection analysis (FIA) and by the colorimetric method (PDAB)

Estimation of *reproducibility* through consecutive urea measurements resulted in coefficients of variation between 0.94 and 2.72% for cow's milk and 0.93 and 1.91% for sheep's milk. Means, standard deviations and coefficients of variation for the urea concentration are shown in Table 1.

**Table 1**

Means, standard deviations (SD) and coefficients of variation (CV) in 6 sheep's and 6 cow's milk samples based on 10 consecutive determinations

| Sample no. Cow | Mean (mmol/l) | ± SD (mmol/l) | CV (%) | Sample no. Sheep | Mean (mmol/l) | ± SD (mmol/l) | CV (%) |
|---|---|---|---|---|---|---|---|
| 1 | 1.87 | 0.02 | 1.07 | 1 | 4.27 | 0.04 | 0.93 |
| 2 | 4.24 | 0.04 | 0.94 | 2 | 5.23 | 0.05 | 0.96 |
| 3 | 3.31 | 0.09 | 2.72 | 3 | 8.18 | 0.12 | 1.46 |
| 4 | 6.81 | 0.14 | 2.06 | 4 | 6.75 | 0.13 | 1.91 |
| 5 | 3.48 | 0.06 | 1.72 | 5 | 6.85 | 0.1 | 1.47 |
| 6 | 3.02 | 0.03 | 0.99 | 6 | 5.34 | 0.09 | 1.70 |

Urea levels remained unaffected by *chemical conservation* with bronopol or *freezing*. Results obtained were equal, within the experimental error, for non-preserved and preserved samples ($p > 0.05$). When non-preserved samples were stored in a refrigerator and reanalysed on day 3, 6 and 10, a significant change in the urea concentrations was first observed after six days ($p < 0.05$). The standard curve showed perfect *linearity* over the range tested, extending from 0 to 1000 mg/l (0 to 166.5 mmol/l), covering the whole physiological range in the urea content of cow's and ovine milk (Flöck, 2000).

The device reached a sample capacity of 55 samples per hour.

## Discussion

A flow injection analysis method for determining urea content in cow's and ewe's milk was evaluated. In the absence of an approved standard procedure, accuracy of the method was estimated by a standard addition experiment and through comparison with a colorimetric method (PDAB). The recovery of added urea (99.3% for ovine and 99.4% for bovine milk) correlates approximately to the values found by Oltner et al. (1985) and is also similar to that reported for the determination of urea in cow's milk by Lefier (1996). The correlation between added and recovered urea content also corresponded to the values for flow injection procedures for determination of urea in cow's milk listed in the literature. Oltner et al. (1985) found a correlation coefficient of $R = 0.999$ and Andersson et al. (1986) observed a correlation of $R = 0.998$, which confirms the high accuracy of enzymatic methods. The correlation between FIA and the colorimetric method (PDAB) was $R = 0.963$ and urea values measured by means of FIA were on average 0.47 mmol/l lower than those obtained by the colorimetric method. The photometric procedure used for reference measurement proved to cause difficulties. Although the manual method required preliminary protein precipitation, the

high fat and protein content of ovine milk led to rapid re-clouding of the filtrate, which may have affected accuracy. Flow injection analysis avoids this problem: by using a diffusion cell, deproteinisation is unnecessary (Gustafsson, 1996). The intense absorbance of the reagent blank itself was presumably also a problem of the colorimetric method. As recommended by McDowell (1972) a water blank was used which could have influenced the accuracy of the procedure. According to Brown (1959) a urea-free sample blank is required. All in all, the colorimetric method proved to be suitable for measuring the urea content of a low number of samples in the laboratory in a quick and simple manner. However, further studies concerning deproteinisation and type of blank are necessary.

Evaluation of the precision of flow injection analysis by means of repeated measurements within a 6-measurement series demonstrated a CV between 0.93 and 2.75%, proving that this method is precise and reproducible. In their assessment of an enzymatic flow injection system, Andersson et al. (1986) found a CV value of 4%. For the same procedure Carlsson and Bergström (1994) found the same value between 2 and 3%. The influence of long-term storage and chemical preservation with bronopol on the analysis procedure was tested using stored sheep's milk. Following several days' storage at +4 °C, no significant changes were found until the 6th day of storage where a significant increase was observed. Carlsson and Bergström (1994) showed that a significant change in the urea concentration in cow's milk first took place after a 16-day storage period at +4 °C. At this time the non-preserved milk was distinctly sour. The change in the urea concentration may have been due to storage-dependent production of ammonia as a result of proteolytic processes. Helaine (1977) described average ammonia concentrations of 0.26–0.34 mmol/l in milk. Pinelli (1990) reported values of 0.15 mmol/l to 0.23 mmol/l for good quality raw milk. In an experiment entailing sample storage of several days, Baumgartner (1997) found a temperature-dependent increase of ammonia in the milk. The original ammonia concentration in the milk and that arising during storage leads to a negligible falsification of measured values. Where exact measurements are needed, the actual urea concentration can be determined by measuring the blank value. Moreover, preservation with bronopol as well as the storage of samples preserved with bronopol at –18 °C showed no effect on the results as tested by means of an experimental series. These results are confirmed in the relevant literature for cow's milk. Oltner et al. (1985) first observed significantly lower results using a 30-fold excess preservation with bronopol. Carlsson and Bergström (1994) found no changes in the urea content of cow's milk following deep freezing with or without bronopol. As additional evaluation criteria, the complexity of instruments must also be considered. The procedure used here was designed to measure the ammonia concentration in fluids. For this reason, urea had to be enzymatically decomposed in the form of a preparative procedure prior to measurement (by adding buffered urease solution and incubating for a minimum of 30 min). At the

same time, the milk samples were diluted 1:8. This dilution level was also necessary in order not to exceed the linearity limit of 0.1 to 80 mg N/l (which correlates to 0.004–2.850 mmol/l of urea) as specified by the manufacturer. The incubation time was determined in an initial experiment as minimum of 30 min. According to the manufacturer, the integration of this step into the measurement system is possible, but would necessitate investigation into flow velocity, temperature regulation and incubation time. The enzymatic based flow systems found in the literature comprise the hydrolysis of urea as an integrated step in the measurement system (Oltner et al., 1985; Andersson et al., 1986; Carlsson and Bergström, 1994), which enables constant conditions concerning incubation temperature and incubation time. The sample capacity of 55 samples per hour is, however, much lower than that of infrared methods used in routine diagnostics (approx. 600 samples per hour). Furthermore, this method only analyses the urea parameter, whereas newer generations of infrared devices are capable of measuring the parameters of fat, protein, lactose, cell count and urea concentration in one cycle (Ziegmann, 1994). Due to its complexity and relatively low sample capacity as well as its high initial purchase and reagent costs, the procedure assessed in its present form is only feasible for special laboratories. Its high precision and accuracy, however, make this method suitable for assessing other methods of urea measurement and as a reference procedure for the evaluation of infrared spectrometers.

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