

Flow Injection System with Potentiometric Detection for the Determination of Urea Content in Milks

José L. F. C. Lima, Cristina Delerue-Matos,* and M. Carmo V. F. Vaz

CEQUP/Departamento de Química-Física, Faculdade de Farmácia (U.P.), Rua Anibal Cunha 164, 4050 Porto, Portugal

Two variant manifolds of a flow injection analysis (FIA) system are described for the determination of urea content in milks. This determination consists of the enzymatic reaction of urea with urease in which ammonium ion is formed. Ammonium is converted to ammonia by adding a NaOH solution and then led to a gas diffusion unit in which it diffuses to an acceptor channel (Tris/HCl, pH 7.5). Here, it is reconverted to ammonium ion and determined by a tubular configuration electrode sensitive to this ion. One of the FIA manifolds is based on the merging zones technique, whereas the other uses an immobilized enzyme. The results obtained were compared with those given by the Boehringer UV test and by the Official Method of Analysis of the Association of Official Analytical Chemists (AOAC) which can be applied to animal feed and adapted to the matrix studied. The relative deviation was less than 5%, and the precision of the developed methodologies considering RDS (%) was always less than 2%.

Keywords: *Ion-selective electrodes; milk; urea; immobilized enzyme; merging zones*

INTRODUCTION

Improper feeding of animals leads to both high and low levels of urea in milk, affecting milk production and fertility (Gustafsson and Palmquist, 1993). Therefore, urea determination has become very important in the last few years. Nevertheless, there is no reference method for the determination of this parameter in milks. The most common methods are colorimetric. Some are direct methods based on the measurement of a complex formed between urea and a reagent (AOAC, 1984; Thiemann, 1982); others are indirect methods based on the measurement of a product from urea degradation (enzymatic methods) which reacts with a reagent. The enzyme can be used in solution (Wolfschoon-Pombo et al., 1982; Rajamaki and Rauramaa, 1984; Dirks and Reimerdes, 1986) or immobilized physically (trapped in various gels) (Guibault and Nagy, 1973; Tor and Freeman, 1986; Koncki et al., 1992) or chemically (Meyerhoff, 1980; Palleschi et al., 1988; Przybyt and Sugier, 1990). The simultaneous use of chemical and physical immobilization is a promising approach for the immobilization of urease on a fiber-optic sensor (Luo and Walt, 1989). In either method, the difficulty is the laborious sample preparation steps and the time-consuming determinations.

Andersson et al. (1986) and Schwedt et al. (1994) determined the urea content in milks by enzymatic reaction with a spectrophotometric detector coupled to a FIA system, but both samples had to be treated before insertion into the system. The former paper uses a urease solution as a carrier, whereas the latter resorts to urease immobilization in VA epoxy resin.

This paper describes the development and evaluation of two variant manifolds of a FIA system for the

determination of urea content in milks, without prior treatment of samples. NH_4^+ is formed from the enzymatic reaction of urea with urease and can be potentiometrically detected by electrodes sensitive to this ion (Scholer and Simon, 1970).

One of the developed manifolds applied the merging zones technique in which the sample and enzyme solution are injected into separate carrier streams that subsequently merge in a confluence. This technique minimizes the reagent consumption as only the required amount of reagent is injected into the system instead of being continuously aspirated into the manifold (Bergamin et al., 1978). The other manifold used an enzyme immobilized in glass beads adapted to procedures previously described (Masoom and Townshend, 1984), which were afterward condensed in a reactor placed after the injection position.

MATERIALS AND METHODS

Reagents and Solutions. All reagents were pro-analysis grade, and doubly deionized water was used throughout. The 0.1 M urea stock solution was prepared from the solid. The urea standards used for calibration curves were prepared by dilution of this stock solution with a 0.01 M tris(hydroxymethyl)aminomethane hydrochloride solution (pH 7.5). The same Tris/HCl solution (pH 7.5) was used as a carrier and ionic strength adjuster simultaneously.

Aminopropyl glass beads (200–400 mesh, 75 Å mean pore diameter), urease (type IX-U 4002, 1.0 g per 80 000 units), and a 25% glutaraldehyde solution were obtained from Sigma Chemical Co.

The enzymatic UV test performed for comparative purposes was from Boehringer-Mannheim (catalog no. 542946, 1997).

The urease solution used in the merging zones system was prepared by dissolving 1.5 g of enzyme in 1 L of the Tris/HCl solution, whereas that used for the immobilization was prepared by dissolving 0.1 g of enzyme in 3 mL of 0.1 M sodium phosphate at pH 7.

Bioreactor Preparation. The procedure used for the immobilization of urease was similar to that outlined by

* To whom correspondence should be addressed. Permanent address: Instituto Superior de Engenharia do Porto, Rua S. Tomé, 4200 Porto, Portugal.

Masoom and Townshend (1984). The enzyme was immobilized on readily prepared aminopropyl glass (CPG). The construction of the bioreactor involved the following steps.

(i) *Coupling.* Five milliliters of a glutaraldehyde solution (2.5% in 0.1 M phosphate buffer at pH 7.0) was added to 0.25 g of aminoalkylated glass beads in a tightly stoppered vessel through which nitrogen was bubbled to remove oxygen. The reaction was allowed to proceed for 1 h at room temperature with brief nitrogen deoxygenation every 10 min for the first 30 min. The activated glass beads were washed with distilled water.

(ii) *Immobilization.* A 0.25 g amount of activated glass beads was added to the urease solution and cooled to 4 °C for 3 h. The glass beads were then washed successively with distilled water, a 1 M KCl solution, and 0.1 M phosphate buffer at pH 7.0.

(iii) *Packing of the Reactor.* The immobilized urease was packed into a column with a 2.5 cm length and a 2 mm inside diameter (i.d.) and held in position with glass wool. When not in use, the immobilized enzyme bioreactors were stored at 4 °C in 0.1 M phosphate buffer (pH 7.0).

Apparatus. A Crison model 2002 digital voltmeter (0.1 mV sensitivity) coupled to a Kipp & Zonen recorder was used for the measurement of the potential differences between the indicating and reference electrodes.

An Orion 90-0029 double-junction electrode containing a 0.01 M Tris/HCl solution (pH 7.5) in the outer compartment was used as the reference electrode. A tubular ammonium electrode without an internal reference solution and the sensor system consisting of ionophore nonactin (Scholer and Simon, 1970) was used as the indicating electrode. As this electrode presented a low selectivity of the alkali metal ions, the ammonium cation formed was converted inside the system to ammonia gas after addition of NaOH and led to a gas diffusion unit, in which it was converted again to NH_4^+ in an acceptor channel (Canham et al., 1988). Other volatile compounds, like volatile amines, that can diffuse through the membrane do not cause interference in the electrode response (Scholer and Simon, 1970).

A Gilson minipulse 3 peristaltic pump was used for propelling the solutions which were inserted into the carrier stream by means of a homemade injector commutator (Krug et al., 1986). Omnifit PTFE tubing (0.8 mm i.d.) connected by Gilson endfittings, connectors and other Perspex devices, namely confluences, a support device for tubular and reference electrodes, and a gas diffusion unit were used in the construction of the manifolds. The support device for tubular and reference electrodes as well as the ground electrode were constructed as described by Alegret et al. (1987). The gas diffusion unit comprised two Perspex blocks forming a flow channel that was 2 mm wide, 0.5 mm deep, and 100 mm long (linear path) (Lima et al., 1994). A gas-permeable membrane, commercial PTFE tape, was placed between the two blocks.

The absorbance measurements provided by the Boehringer-Mannheim enzymatic UV test and the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) adapted to milk samples were performed with a Hitachi U-2000 spectrophotometer.

RESULTS AND DISCUSSION

FIA System Based on the Merging Zones Technique. This manifold (Figure 1) comprised a commutator for the simultaneous injection of the sample and enzyme solution into separate carrier streams, which flowed to confluence X.

The enzymatic reaction occurred in reactor L_1 , and ammonium cation was first formed and then converted in reactor L_2 to ammonia gas by the addition of a NaOH solution at confluence Y. The gas that was formed passed to a gas diffusion unit (D.U.) in which it diffused to a Tris/HCl acceptor channel (pH 7.5) and was reconverted to NH_4^+ that was detected by a tubular

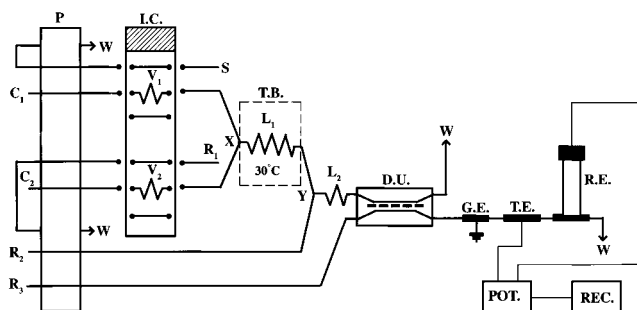


Figure 1. FIA system based on the merging zones technique: P, peristaltic pump; I. C., injector commutator; S, sample; C_1 and C_2 , carrier streams (flow rate of 0.8 mL min^{-1}); R_1 , enzyme solution; R_2 , NaOH solution (flow rate of 1.6 mL min^{-1}); R_3 , acceptor channel (flow rate of 3.2 mL min^{-1}); X and Y, confluence points; tube lengths, $L_1 = 300 \text{ cm}$ and $L_2 = 10 \text{ cm}$; V_1 , sample volume ($110 \mu\text{L}$); V_2 , enzyme volume ($250 \mu\text{L}$); W, waste; D.U., diffusion unit; G.E., ground electrode; T.E., tubular electrode; R.E., reference electrode; POT., potentiometry; T.B., thermostatic bath; REC., recorder.

selective electrode (T.E.). The electrode working characteristics were assessed in a low-dispersion FIA system, which presented a slope close to the Nernstian and a potential reproducibility of $\pm 1 \text{ mV/day}$. The electrode lifetime was >6 months.

After a former assessment for selecting approximate values of each parameter, the FIA system optimization yielded the maximum sensitivity and reproducibility without compromising the sampling rate. Equal propulsion flow rates of the sample and enzyme and equal lengths up to confluence X were used, allowing simplification of the system optimization, since the parameters to be optimized were only the sample and enzyme volumes, the concentration of the enzyme solution, the flow rate, and the reactor length. Equal flow rates at the inlet of the gas diffusion unit were used to extend the membrane lifetime (Canham et al., 1988). Under these circumstances, the average lifetime of the membranes was >1 month and their malfunctioning was detected through the decrease of the analytical signal reproducibility.

First, the enzyme concentration (0.75 g L^{-1}) was selected with regard to the maximum concentration of the selective electrode linear response in the calibration curve ($9.0 \times 10^{-1} \text{ mg/dL}$ in urea). Then, once the sample volume ($110 \mu\text{L}$) had been set, the analytical signals of different reagent volumes (from 75 up to $500 \mu\text{L}$) were recorded. It was found that for volumes higher than $375 \mu\text{L}$ there was almost no increase of sensitivity. Afterward, the effect of the analytical signals was evaluated by keeping the two parameters selected formerly and varying the third to achieve a compromise between sensitivity, reproducibility, and sampling rate. The sample volumes tested varied from 50 to $250 \mu\text{L}$, and $110 \mu\text{L}$ was the sample volume selected. Regarding the enzyme concentration, it was found that, when its concentration was doubled (1.5 g L^{-1}), sensitivity was increased by about 20%. However, when concentrations were higher, there was no significant increase. Therefore, an enzyme solution volume of $250 \mu\text{L}$ and a concentration of 1.5 g L^{-1} were the parameters selected.

As the enzymatic reaction occurs in reactor L_1 , it was necessary to select its length and flow rate so as to ensure a complete reaction. Reactor lengths in the range of 200–400 cm and different flow rates between 0.6 and 1.0 mL min^{-1} were studied, the best results

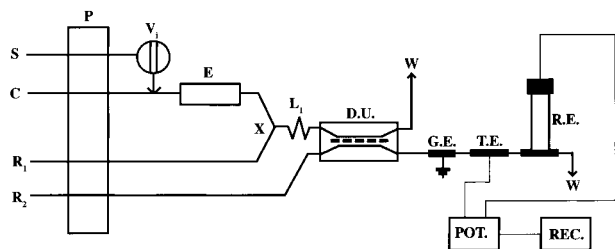


Figure 2. FIA system with an enzymatic reactor: P, peristaltic pump; V_i , injection valve ($110 \mu\text{L}$); C, carrier stream (flow rate of 0.5 mL min^{-1}); R_1 , NaOH solution (flow rate of 0.5 mL min^{-1}); R_2 , acceptor channel (flow rate of 1.0 mL min^{-1}); E, enzymatic reactor; X, confluence point; tube length, $L_1 = 10 \text{ cm}$; W, waste; D.U., diffusion unit; G.E., ground electrode; T.E., tubular electrode; R.E., reference electrode; POT., potentiometry; REC., recorder.

being obtained with a 300 cm reactor and a 0.8 mL min^{-1} flow rate.

The NaOH concentration used in the carrier stream was studied in order to find the minimum concentration capable of total conversion of NH_4^+ to NH_3 . The NaOH content was changed between 0.5 and 2.0 M , and the 1.0 M solution was selected. A flow rate of 1.6 mL min^{-1} and a length of reactor L_2 of 10 cm were selected to diminish the dilution of the sample plug and increase the resistance of the NaOH impulse tube.

The pH effect and the temperature over the course of the enzymatic reaction were also evaluated. Therefore, different Tris/HCl solutions with the same ionic strength and different pH levels were tested, and the highest sensitivity was obtained with pH levels close to 7.5 . The temperature effect on the enzyme activity was assessed by dipping reactor L_1 in a thermostatic bath at 25 – $41 \text{ }^\circ\text{C}$, and it was found that there was a sensitivity increase at temperatures ranging from 25 to $37 \text{ }^\circ\text{C}$. Nevertheless, when the bath temperature was greater than $30 \text{ }^\circ\text{C}$, gas bubbles were emitted; hence, $30 \text{ }^\circ\text{C}$ was the selected temperature.

Finally, the effect of the samples' prior treatment was studied by comparing the results obtained by injection of a sample with and without prior treatment (addition of 30% TCA), and equal analytical signals were obtained.

FIA System with an Enzymatic Reactor. The FIA manifold with an immobilized enzyme was plotted in Figure 2.

As the main reaction of this system occurs within the column comprising the immobilized enzyme, the dimensions and conditioning of this column were optimized.

Columns with a 2 mm i.d. and lengths varying from 1 to 6.5 cm were tested. Different injection volumes (50 – $250 \mu\text{L}$) and flow rates (0.35 – 0.65 mL min^{-1}) were also tested for each column. This study yielding column efficiency showed that up to a length of 2.5 cm there was an efficiency rise, from 2.5 to 3.5 cm there were no significant changes, and from 3.5 cm there was a slow diminishing of sensitivity due to the dispersion effect. Efficiency was assessed by comparing the analytical signals obtained from a standard with 180 mg/dL in NH_4^+ and 90 mg/dL in urea. The highest efficiency (98%) was achieved with the column with 2.5 cm , a $110 \mu\text{L}$ loop, and a 0.5 mL min^{-1} flow rate. Under these conditions, the reactor lifetime was >2 weeks when regularly used (6 h/day), and no activity loss was detected throughout this period of time. Moreover, the reactor could last longer unless systematically used. The acceptor channel flow rate was 1.0 mL min^{-1} , and the length of reactor L_1 was 10 cm . The lowest possible length was selected to avoid additional dispersion.

Determination of Urea Content in Milks. Samples were injected straight into the FIA system without prior treatment, and concentrations were evaluated by interpolation of a calibration curve. The urea standards were prepared in the carrier solution presenting concentrations ranging from 0.36 to 90 mg/dL , and a straight line with a slope close to the Nernstian was obtained. The mean values provided by each FIA system for 12

Table 1. Determinations of Urea Content in Different Kinds of Milk, Using FIA Manifolds, the Boehringer UV Test, and the Method of AOAC and the Corresponding Relative Deviation^a

sample	merging zones		Boehringer UV		method of AOAC			
	conc (mg/dL)	immobilization (conc (mg/dL))	conc (mg/dL)	RD ^d	RD ^e	conc (mg/dL)	RD ^d	RD ^e
1 ^b	25.20 ± 0.01	24.72 ± 0.01	25.92 ± 0.02	-2.8	-4.6	24.78 ± 0.05	+1.7	-0.2
2 ^b	26.22 ± 0.01	26.46 ± 0.02	25.92 ± 0.02	+1.2	+2.1	25.80 ± 0.04	+1.6	+2.6
3 ^b	27.84 ± 0.01	27.60 ± 0.01	27.48 ± 0.01	+1.3	+0.4	26.76 ± 0.04	+4.0	+3.1
4 ^c	57.30 ± 0.03	56.22 ± 0.06	56.34 ± 0.04	+1.7	-0.2	56.58 ± 0.15	+1.3	-0.6
5 ^c	40.92 ± 0.01	41.16 ± 0.05	42.48 ± 0.04	-3.7	-3.1	41.40 ± 0.12	-1.2	-0.6
6 ^c	6.42 ± 0.01	6.24 ± 0.01	6.24 ± 0.01	+2.9	+0.0	6.12 ± 0.01	+4.9	+2.0
7 ^c	7.26 ± 0.01	7.30 ± 0.01	6.96 ± 0.01	+4.3	+4.9	7.20 ± 0.03	+0.8	+1.4
8 ^c	32.88 ± 0.01	33.90 ± 0.10	33.42 ± 0.08	-1.6	+1.4	33.78 ± 0.40	-2.7	+0.4
9 ^c	16.86 ± 0.01	17.04 ± 0.08	16.74 ± 0.06	+0.7	+1.8	17.22 ± 0.08	-2.1	-1.0
10 ^c	22.44 ± 0.01	24.00 ± 0.06	23.10 ± 0.05	-2.9	+3.9	22.98 ± 0.05	-2.3	+4.4
11 ^c	12.54 ± 0.01	12.64 ± 0.04	12.36 ± 0.11	+1.5	+2.4	12.06 ± 0.14	+4.0	-4.8
12 ^c	35.94 ± 0.01	35.58 ± 0.11	36.12 ± 0.21	-0.5	-1.5	37.32 ± 0.52	-3.7	-4.7

^a Mean and standard deviation of five determinations. ^b Cow milk samples. ^c Samples of different kinds of powdered milks. ^d RD, percent relative deviation from merging zones. ^e RD, percent relative deviation from immobilization.

Table 2. Results Obtained in the Determination of Urea Content in Milk Samples ($n = 12$)

FIA manifolds		C_0^a	S^a	R^b	$t_{0.025}^c$	$t_{0.025}^d$	RSD% ^e
merging zones	Boehringer	0.995	0.375	0.998			
	method of AOAC	0.999	-0.913	0.997	+0.543	2.201	0.75 (35.94 mg/dL)
immobilization	Boehringer	0.978	5.341	0.998			
	method of AOAC	0.983	4.134	0.997	+0.175	2.201	0.8 (12.66 mg/dL)

^a Parameters of the equation $C_t = C_0 + SC_t$. ^b Correlation coefficient. ^c Calculated values for a two-tail t test. ^d Tabulated values (95% confidence level). ^e Relative standard deviation obtained from 12 consecutive injections of a milk sample. The mean concentration value obtained in the experiment is indicated in parentheses.

samples from different milks were compared with those given by the Boehringer UV test and by the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) adapted to the matrix studied, the relative deviations being less than 5% (Table 1).

Using these values, the linear relationship $C_f = C_0 + SC_r$ (Table 2) was established for both systems. In view of the regression and the relative deviation, it was found that there was good agreement between both FIA methodologies and the conventional methods.

Reproducibility was evaluated by assessing the relative standard deviation for 10 replicate injections of a milk sample with a mean concentration. The RDS value was less than 2%. The sampling rate of both systems was about 30 samples/h.

The proposed FIA systems allow determination of urea content in several milks and offer several advantages over those already described in the literature (Andersson et al., 1986; Schwedt et al., 1994). These are completely automated systems since they do not require any prior sample treatment and allow a low consumption of reagents. Even with the system that applies the merging zones technique, only the required sample volume is inserted, differing from the system developed by Andersson et al. (1986) in which the enzymatic solution was continuously aspirated. In the case of the manifold with an immobilized enzyme, the reactor lifetime was always >2 weeks, when regularly used (6 h/day), and no activity loss was found, though it could still be used after that period of time when not used systematically.

Another advantage of the developed systems is related to potentiometry which was used as a detection system, allowing us to quantify the urea content within a wider concentration range (0.36–90 mg/dL) when compared with that described by Andersson et al. (1986) and Schwedt et al. (1994) and avoiding the milk sample digestion (a tedious and boring procedure) required by colorimetric detection. Thus, the procedure can be easily implemented in laboratories for routine analyses.

LITERATURE CITED

- Alegret, S.; Alonso, J.; Bartroli, J.; Machado, A. A. S. C.; Lima, J. L. F. C.; Paulis, J. M. Construction of equipment for potentiometric determinations in flow injection analysis. *Quim. Anal.* **1987**, *6* (3), 278–292.
- Andersson, G.; Andersson, L.; Carlstrom G. Determination of milk urea by flow injection analysis. *J. Vet. Med. A* **1986**, *33*, 53–58.
- AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 14th ed.; Arlington, VA, 1984.
- Bergamin, H.; Zagatto, E. A. G.; Krug, F. J.; Reis, B. F. Merging zones in flow injection analysis. Part I. Double proportional injector and reagent consumption. *Anal. Chim. Acta* **1978**, *101*, 17–27.
- Canham, J. S.; Gordon, G.; Pacey, G. E. Optimization of parameters for gas-diffusion flow-injection systems. *Anal. Chim. Acta* **1988**, *209*, 157–163.
- Dirks, U.; Reimerdes, E. H. Eine rechnergesteuerte schnellmethode zur bestimmung von harnstoff in milcherzeugnis-sen: magermilchpulver unterschiedlicher provenienz (A rapid computer-aided method for the determination of urea in dairy products: skim milk powder from various sources). *Z. Lebensm.-Unters. -Forsch.* **1986**, *183* (2), 101–104.
- Guibault, G. G.; Nagy, G. Improved urea electrode. *Anal. Chem.* **1973**, *45* (2), 417–419.
- Gustafsson, A. H.; Palmquist, D. L. Diurnal variation of rumen ammonia, serum urea, and milk urea in dairy cows at high and low yields. *J. Dairy Sci.* **1993**, *76*, 475–484.
- Koncki, R.; Leszcynski, P.; Hulanicki, D.; Glab, S. Urea sensors based on glass pH electrodes with physically immobilized urease. *Anal. Chim. Acta* **1992**, *257*, 67–72.
- Krug, F. J.; Bergamin, F. H.; Zagatto, E. A. G. Commutation in flow-injection analysis. *Anal. Chim. Acta* **1986**, *179*, 103–118.
- Lima, J. L. F. C.; Neves, O. B. A. O.; Rangel, A. O. S. S. Flow injection determination of reducing sugar in wine in a wide concentration range using a dialysis unit and a stream splitting. In *Automatic Control of Food and Biological Processes*; Bimbene, J. J., Dumoulin, E., Trystram, G., Eds.; Elsevier: Amsterdam, 1994; pp 67–74.
- Luo, S.; Walt, D. R. Avidin-biotin coupling as a general method for preparing enzyme-based fiber-optic sensors. *Anal. Chem.* **1989**, *61*, 1069–1072.
- Masoon, M.; Townshend, A. Determination of glucose in blood by flow injection analysis and an immobilized glucose oxidase column. *Anal. Chim. Acta* **1984**, *166*, 111–118.
- Meyerhoff, M. E. Polymer membrane electrode based potentiometric ammonia gas sensor. *Anal. Chem.* **1980**, *52*, 1532–1534.
- Palleschi, G.; Mascini, M.; Martinez-Fàbregas, E.; Alegret, S. Urea determination in human sera with an ammonium-ion selective electrode made with solid inner contact and immobilised urease. *Anal. Lett.* **1988**, *21*, 1115–1129.
- Przybyl, M.; Sugier, H. Metal-metal oxide enzyme electrodes for the determination of urea. *Anal. Chim. Acta* **1990**, *237*, 399–404.
- Rajamaki, S.; Rauramaa, A. The automated determination of urea in milk. *Finn. Chem. Lett.* **1984**, 47–48.
- Schwedt, G.; Shi, R.; Stein, K. FIA-System mit einem urease-reaktor zur harnstoffanalyse in lebensmitteln (Determination of urea in foods by FIA-system with a urease reactor). *Dtsch. Lebensm.-Rundsch.* **1994**, *90* (6), 178–181.
- Sholer, R. P.; Simon, W. Antibiotika-membranelektrode zur selektiven erfassung von ammoniumionenaktivitäten (Antibiotic-membrane electrode for selective evaluation of ammonium ion activity). *Chimia* **1970**, *24*, 372–374.
- Thiemann, K. G. Eine rationelle methode zur harnstoffbestimmung in biologischem material (High-economy method for urea determination in biological material). *Mh. Vet.-Med.* **1982**, *39* (6), 186–188.
- Tor, R.; Freeman, A. New enzyme membrane for enzyme electrodes. *Anal. Chem.* **1986**, *58*, 1042–1046.
- Wolfschoon-Pombo, A.; Klostermeyer, H.; Weiss, G. Die NPN-Fraktion der kuhmilch. V. Analytik (The NPN-fraction of cow's milk. V. Analysis). *Milchwissenschaft* **1982**, *37* (2), 80–85.

Received for review September 8, 1997. Revised manuscript received January 5, 1998. Accepted January 6, 1998. We thank JNICT (PEAM SEL/516/95), and M.C.V.F.V. thanks the Ph.D. grant from PRODEP Program.

JF970767J