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Enzymatic Determination of Urea in Milk by Sequential Injection with Spectrophotometric and Conductometric Detection

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In this work, an analytical system based on the coupling of gas diffusion separation and sequential injection analysis for urea determination in milk is presented. A versatile manifold that could simultaneously be used for either spectrophotometric or conductometric detection was constructed. The sample and urease solution are sequentially aspirated into the holding coil and sent to a thermoreactor, where urea is enzymatically hydrolyzed by urease and converted into ammonium. This stream merges an alkaline solution at a confluence point where ammonia is formed. Ammonia diffuses through a hydrophobic membrane and modifies the bromothymol blue indicator color, when spectrophotometric detection is used, or changes the conductance of a boric acid solution acceptor stream, when conductometric detection is used.This methodology was applied to the determination of urea in 18 milk samples and the results were statistically comparable with those furnished by the enzymatic recommended procedure. The detection limits were 2.6×10^{-4} and 2.8×10^{-5} mol L^{-1} for conductometric and spectrophotometric detection, respectively. Repeatability (relative standard deviation, RSD) was better than 3.7% and 2.6% for conductometric and spectrophotometric detection, respectively.

KEYWORDS: Sequential injection; milk urea determination; urease; gas-diffusion; spectrophotometry; conductimetry

INTRODUCTION

Sequential injection analysis (SIA), proposed by Ruzicka and Marshall in 1990 (*1*), has become an important alternative to other automatic methods such as flow injection analysis (FIA) (*2*). It presents some advantages such as lower reagent consumption, higher degree of automation, and the potential to carry out multideterminations without the need of manifold reconfiguration. This technique is based on the sequential aspiration of a well-defined sample and reagent zones into a holding coil with subsequent flow reversal to propel and mutually disperse the stacked zones toward the detector.

In a complex matrix like milk, the possibility of using automatic analysis becomes significant since laborious sample manipulation can be performed inside the flow tubes. The use of the sequential injection technique in milk analysis is still very recent, and only few works describe its application in this matrix. In this work we demonstrate that SIA can be a powerful tool in milk analysis by developing a system for the enzymatic determination of urea. Urea is an important compound present in blood and organic fluids, passing by simple diffusion directly to milk. This is the reason the levels of urea in milk must be periodically monitored, since they can be used to predict the state of animal health, as an indicator of the protein-feeding efficiency; besides, urea excretion may represent a significant pollutant to air and water (*3*).

Some flow injection systems were described for the determination of urea in urine (*4*) with chemiluminescence detection, with conductometric detection in serum (*5*), with colorimetric detection in soils (*6*) and wines (*7*), and with potentiometric detection in fertilizers (*8*). Concerning this determination in milk, a FIA system with potentiometric detection (*9*) and another with spectrophotometric detection (*10*) were described. Only one work described the SIA determination of urea in milk (*11*), by use of potentiometric detection.

In this work, we propose an alternative sequential injection system that can be used with either spectrophotometric or conductometric detection with the same manifold configuration. In this approach, urea is enzymatically hydrolyzed by urease and converted to ammonia. The ammonia diffuses through a hydrophobic membrane in a gas diffusion unit, modifying the characteristics of the acceptor stream. This stream is subse-

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quently directed to the detector. The inclusion of a gas diffusion unit in the system allowed urea determination without any prior sample treatment, since it allows the selective passage of ammonia through the hydrophobic membrane, thus eliminating the possible interference from proteins and colloids present in milk.

MATERIALS AND METHODS

Reagents and Solutions. All chemicals used were of analytical reagent grade, and water from a MilliQ plus system was used throughout.

A 0.01 M Tris/HCl solution (pH 6.5) was prepared by mixing 160 mL of 0.10 mol L^{-1} HCl with 200 mL of 0.10 mol L^{-1} tris-(hydroxymethyl)aminomethane (Merck, 1.08382) and the volume was then completed with water to 2.0 L.

A 0.10 mol L^{-1} urea stock solution was prepared by dissolving 1.5 g of the solid (Sigma, U-1250) in 250 mL of the 0.01 mol L^{-1} Tris/HCl buffer solution. Working standard solutions of urea in the range of 1×10^{-3} to 5×10^{-3} mol L^{-1} were prepared by proper dilution of the stock solution in the same buffer.

The urease solution (1 g L^{-1}) was prepared by dissolving 0.010 g of lyophilized urease powder from jack beans (Fluka, type 94285, 102 units mg^{-1}) in a 10 mL volumetric flask and the volume was completed with 0.01 mol L^{-1} Tris/HCl.

For the spectrophotometric detection, the acid−base indicator was a 9×10^{-5} mol L^{-1} solution of bromothymol blue (Merck, 1.03026), obtained by dissolving 0.028 g of the solid into 500 mL of water; the pH was adjusted to 6.5 by adding the appropriate amount of sodium hydroxide (Merck, 1.06498). This solution was prepared daily.

For the conductometric detection, a 5×10^{-2} mol L^{-1} H_3BO_3 solution was prepared by dissolving 0.3 g of H_3BO_3 (Sigma, B-7660) in 100 mL of water.

The enzymatic UV test kit used for comparative purposes was purchased from Boehringer-Mannheim (catalog no. 0542946 2002).

Instrumentation. Two Gilson Minipuls 3 (Villiers-le-Bel, France) peristaltic pumps, equipped with Gilson PVC pumping tubes, were used to propel the solutions. One of the pumps was connected to the central channel of an eight-port electrically actuated selection valve (Valco VICI C15-3118E, Switzerland). The tubes connecting the different parts of the sequential injection system were made of PTFE Omnifit (Cambridge, U.K.) with 0.8 mm i.d. A 386 personal computer Samsung SD 700 (Korea) equipped with an Advantec PCL 818 L (Taipei, Taiwan) interface card, running homemade software written in Quick-Basic 4.5 (Microsoft), controlled the selection valve position and the rotation sense and speed of the peristaltic pumps.

A Julabo D-77960 (Seeldach, Germany) water bath was used to immerse the thermoreactor, maintaining a constant temperature of 37 °C for the enzymatic reaction.

The gas diffusion unit (GDU) consisted of two acrylic blocks (4 mm wide, 24 mm deep, 98 mm long), pressed against each other by four screws (*12*). A Durapore (0.22 μm pore size, Millipore) gas-permeable membrane was placed between the two acrylic blocks.

For the spectrophotometric detection, a Unicam (Cambridge, U.K.) 5625 UV−vis spectrophotometer ($\lambda = 620$ nm) equipped with a Helma (Mullheim/Baden, Germany) 178.12 QS flow-through cell with 18 μL of internal volume was used.

Regarding the conductometric detection, the conductivity was measured by use of a commercial flow-through cell with platinum electrodes, connected to a Crison 522 conductimeter (Crison Instruments, Alella, Barcelona, Spain).

The detection systems were connected to a Metrohm E586 Labograph (Herisau, Switzerland) chart recorder.

Sequential Injection Procedure. The manifold used for the urea determination in milk is shown in **Figure 1**. The protocol and time sequence for the urea determination is shown in **Table 1**.

The analytical cycle started with the aspiration (step 1) to the holding coil (HC) of the solution to be propelled (step 2) to the acceptor channel of the gas diffusion unit. This solution, depending on whether spectrophotometric or conductometric detection is concerned, was an acid−base indicator (bromothymol blue) or boric acid, respectively.

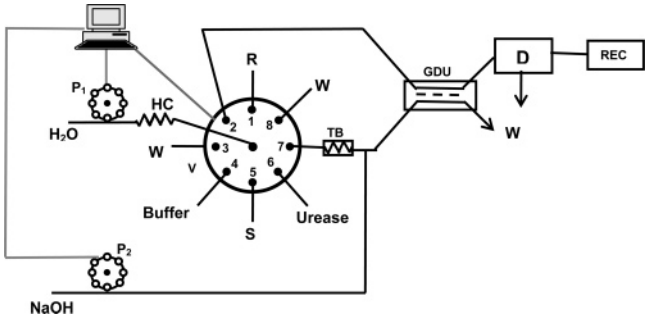

Figure 1. SIA manifold for the determination of urea in milk: $P_{1,2}$, peristaltic pumps; HC, holding coil; R, H_3BO_3 (0.05 mol L^{-1}) or bromothymol blue indicator; W, waste; S, standards or milk samples; TB, thermostatic bath (37 °C); V, eight-port selection valve; GDU, gas diffusion unit; D, detector system (UV/vis spectrophotometer or conductimeter); REC, recorder output.

To eliminate remaining solution in the HC, a washing step was included (step 3). Afterward, Tris/HCl buffer, sample, and urease solutions were sequentially aspirated to the holding coil (steps 4−6) and then sent to a thermoreactor with temperature set at 37 °C (step 7), where the enzymatic breakdown of urea occurred. To ensure maximum extent of the enzymatic reaction, the flow was then stopped for 30 s (step 8). Then the flow in the donor channel was reestablished (step 9), and pump 2 was started for propelling the sodium hydroxide solution. At the confluence point, the solution in the donor channel merged this alkaline solution, enhancing ammonia formation. When flowing through the GDU, ammonia diffused across the hydrophobic membrane, modifying the color of the acid−base indicator or the ionic composition of the boric acid acceptor stream. The solution in the acceptor channel was then propelled to the spectrophotometric or conductometric detection system (step 10), respectively. To prepare the system for the next analytical cycle, both donor and acceptor channels of the GDU were washed (steps 11 and 12).

Reference Procedure. A Boehringer-Mannheim UV test kit for urea determination in food was used for comparison purposes. Urea is hydrolyzed to ammonia and carbon dioxide in the presence of the enzyme urease. In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to form l-glutamate, whereby NADH is oxidized. The amount of NADH oxidized is stoichiometric to half of the amount of the urea present. NADH is then determined by means of its absorbance at 340 nm.

RESULTS AND DISCUSSION

The aim of this work was to devise a versatile manifold that could simultaneously be used with either spectrophotometric or conductometric detection. The manifold physical characteristics and protocol sequence were optimized regarding the spectrophotometric determination; only slight modifications were made in the alternative conductometric detection system.

Optimization of the Experimental Conditions. The study of the sequential injection system was accomplished by varying each parameter in order to optimize repeatability, sensitivity, and the gas transference across the gas-diffusion membrane.

Preliminary experiments with methylene blue dye were made to set some physical parameters (tube lengths and flow rates) in order to (i) ensure that the indicator plug would stop precisely at the top of the gas-diffusion unit (acceptor stream) and (ii) ensure that the plug buffer/sample/urease would stop in the exact point of the thermostatic bath.

The selection of the appropriate indicator, bromothymol blue (BTB), and its pH (6.5) was made according to a previous work (*13*). With the selected conditions, absorbance is directly proportional to the ammonia concentration in the donor channel. Regarding the concentration of this indicator solution, it was

Table 1. Protocol Sequence of the SIA System for the Determination of Urea in Milk Samples

step	description (spectrophotometric/conductometric)	port	time (s)	flow rate (μL s^{-1})	volume (μL)	flow direction
1	indicator/boric acid aspiration	1	5	35.3	176	reversed
2	propelling toward the gas diffusion unit	2	7	35.3	247	forward
3	dispense to waste	3	7	64.0	448	forward
4	buffer aspiration	4	4	18.4	74	reversed
5	milk sample aspiration	5	2.5	18.4	46	reversed
6	urease solution aspiration	6	5	18.4	92	reversed
7	propelling through the thermostatic bath	7	41	18.4	754	forward
8	stopped flow	7	30	0.0	0	forward
9[a]	propelling through the donor channel	7	50	18.4	920	forward
10	dispense to the detector	2	50	35.3	1765	forward
11	washing bath tube	7	80	64.0	5120	forward
12	washing the gas diffusion unit	2	10	64.0	640	forward

[a] The peristaltic pump 2 functions when step 9 takes place.

studied between 4×10^{-5} and 1×10^{-4} mol L^{-1}. Increasing the BTB concentration led to better sensitivity in the entire studied range. However, for the 1×10^{-4} mol L^{-1} concentration, obstruction of the flow tubes frequently occurred, because of the formation of a solid deposit due to excessive BTB concentration. Thus, the 9×10^{-5} mol L^{-1} concentration was chosen. Daily preparation of this solution was required since the pH of the BTB solution is altered by CO_2 from air and by room temperature changes.

The pH of the enzyme solution was set to 6.5 with a Tris/HCl buffer solution. The concentration of the enzyme solution was evaluated between 0.8 and 3 g L^{-1}. A 1 g L^{-1} concentration was used as it corresponded to maximum sensitivity; beyond this value, no further improvement in sensitivity was observed.

As the enzymatic reaction occurred in the coil immersed in the thermostatic bath (TB in **Figure 1**), set at 37 °C, another parameter studied was the stopped-flow period in this reactor, to allow the reaction to occur to a considerable extent. Having in mind the possibility of making this timing period as short as possible with maximum sensitivity, the stopped-flow period was studied from 20 to 40 s. From 20 to 30 s, a large (63%) increase in sensitivity was observed. Beyond this value, no further improvement was noted.

The sample volume was studied between 42 and 50 μL. Increasing sample volumes led to better sensitivity. The volume of 46 μL was chosen as a compromise between sensitivity and excessive enzyme consumption.

As the flow rate in the donor channel could have an influence on the efficiency of the diffusion process, values from 16 to 48 μL s^{-1} were tested, with a ratio of 1:1 at the confluence point. As there were no significant differences in sensitivity, it was set to 32 μL s^{-1} due to better correlation coefficient in calibration.

The NaOH concentration used in the stream merging the enzymatic reaction products was varied from 0.25 to 1 mol L^{-1}, to find the minimum concentration capable of maximum conversion of NH_4^+ to NH_3. The chosen concentration was 0.75 mol L^{-1}, as beyond this value no further improvement in sensitivity was observed.

The membrane in the gas diffusion unit was changed whenever a loss of sensitivity or linearity was observed. The average life of the membrane was approximately 3 months. No need for frequent cleaning of the tubing and membrane was observed.

As the manifold physical characteristics and protocol sequence were maintained for the conductometric detection, the only parameter to be studied was the boric acid concentration in the acceptor channel of the gas diffusion unit. It was evaluated from 0.005 to 0.1 mol L^{-1}. The best results regarding sensitivity were obtained when a 0.05 mol L^{-1} concentration was used. These results are in agreement with those of other authors (*14*).

Table 2. SIA Performance for the Urea Determination in Milk

parameter	value
concentration range	1.0×10^{-3} to 5×10^{-3} mol L^{-1}
detection limit (*a*)	spect. 2.8×10^{-5} mol L^{-1}
	cond. 2.6×10^{-4} mol L^{-1}
RSD	spect. 2.6% (4.59×10^{-3} mol L^{-1})
	cond. 3.7% (4.98×10^{-3} mol L^{-1})
sample consumption	46 μL
reagent consumption	92.5 μg urease
(per determination)	120 μg Tris/HCl
	63 mg NaOH
	8.9 μg acid–base indicator
	0.55 mg boric acid

[a] According to IUPAC recommendations.

Analysis of Milk Samples. After establishment of the working conditions, the proposed system was applied to analysis of milk samples. The performance of the developed SIA systems is showed in **Table 2**. The paired results of each kind of detection with reference procedure, together with the corresponding relative deviations, are depicted in **Table 3**.

The detection limit (*15*) was 2.8×10^{-5} mol L^{-1} and 2.6×10^{-4} mol L^{-1} for the spectrophotometric and conductometric determination.

Repeatability of the system was assessed by calculating the relative standard deviations for 10 consecutive milk sample determinations and presented values of 1.2% (3.5 mmol L^{-1}) and 1.0% (4.59 mmol L^{-1}) for the spectrophotometric determination and values of 2.0% (4.55 mmol L^{-1}) and 3.7% (4.98 mmol L^{-1}) for conductometric determination. The concentration values of urea in the analyzed samples are presented in parentheses.

To evaluate the accuracy of the developed methodologies, statistical treatment of results was made by establishing a relation type of $C_{SIA} = C_0 + SC_{ref}$, being $C_0 = 0.687$ (± 0.892), $S = 0.816$ (± 0.200), and $r = 0.9793$ for the conductometric method and $C_0 = 0.453$ (± 1.710), $S = 0.873$ (± 0.411), and $r = 0.9549$ for the spectrophotometric method. In parentheses are the confidence limits for a 99% significance level (*16*). These figures show that the estimated slopes and intercepts do not differ from the values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the SIA and reference methodologies.

Concerning that urea determination becomes important in a wide range of working fields, this fully automated developed

Table 3. Results Obtained for the Determination of Urea in Milk by SIA Spectrophotometric and Conductometric Methods versus Reference Procedure

		SIA		
sample	ref procedure (mmol L^{-1})	spectrophotometric (mmol L^{-1})	conductometric (mmol L^{-1})	rel dev (%)
1	3.69	3.82		+3.5
2	3.02	3.16		+4.6
3	4.70	4.34		–7.6
4	4.60	4.32		–6.1
5	5.10	5.05		–1.0
6	3.22	3.12		–3.1
7	4.34	4.57		–5.0
8	4.16	3.90		+6.7
9	3.92		4.05	+3.3
10	4.86		4.55	–6.4
11	4.41		4.34	–1.6
12	4.22		4.15	–1.7
13	3.21		3.16	–1.6
14	4.01		4.04	+0.8
15	4.86		4.70	–3.3
16	4.73		4.43	–6.3
17	5.05		4.77	–5.5
18	4.98		4.80	–3.6

system presents the possibility of clinical, pharmaceutical, environmental, diagnostic, and food science laboratory control application.

These two simple detection alternatives were both successfully applied, showing that conductivity and spectrophotometric detection are both suitable for use in combination with sequential injection techniques, leading to rapid throughput and high reproducibility with easy manipulation.

The use of the conductometric detection, however, proved to be superior especially in one point: the acceptor solution (boric acid) showed to be very stable (and cheap) while the BTB solution used in spectrophotometric detection needed a continuous pH evaluation and correction, to avoid linearity problems.

The use of the gas diffusion technique for the urea determination in such a complex matrix like milk becomes important in this sequential injection technique, since the hydrophobic membrane avoids the passage of many possible interferents into the acceptor channel, allowing urea determination without any kind of sample pretreatment.

It should also be stressed that the enzyme consumption is about 4 times lower than in a merging zones FIA system (*9*) with the enzyme in solution for the same determination in milk.

ABBREVIATIONS USED

SIA, sequential injection analysis; FIA, flow injection analysis; GDU, gas diffusion unit; Tris/HCl, tris(hydroxymethyl)-aminomethane hydrochloride solution; PVC, poly(vinyl chloride); PTFE, poly(tetrafluorethylene); NADH, reduced nicotinamide adenine dinucleotide; GIDH, glutamate dehydrogenase; BTB, bromothymol blue; TB, thermostatic bath.

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