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Flow Analysis of Lactose and Glucose in Milk with an Improved Electrochemical Biosensor

R. Pilloton

Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma,
Via Orazio Raimondo, Roma, Italy

&

M. Mascini*

Istituto di Chimica Analitica, Università di Firenze,
Via G. Capponi 9, Firenze, Italy

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ABSTRACT

A hydrogen peroxide electrochemical sensor, coupled with two enzymes (β-galactosidase and glucose oxidase) immobilized on the surface of the sensor, has been applied in flow analysis of lactose and glucose in milk samples. The developed procedures are very simple and the short response time allows the analysis of milk directly on dairy farms.

INTRODUCTION

Indirect determination of lactose through a commercial electrochemical glucose analyser has been described (Kosinski, 1981); however, direct determination of lactose in milk by electrochemical biosensors has only recently been reported (Pilloton *et al.*, 1987).

Immobilized β-D-galactosidase cleaves lactose to D-glucose and D-galactose, then immobilized glucose oxidase (GOD) forms hydrogen peroxide. A platinum electrode (polarized at +650 mV versus Ag/AgCl),

* To whom correspondence should be addressed.

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protected with a cellulose acetate dialysis membrane (100 dalton molecular weight cut off (MWCO)), measures the hydrogen peroxide locally formed. In the same samples glucose determination was realized by omitting the first enzymatic reaction.

The assemblies described in this paper were developed with the aim of obtaining a fast procedure for biosensor construction and a fast analytical procedure to be performed in a dairy farm. Therefore, they are a distinct advantage in comparison to previously published procedures with the same selectivity and precision.

Previously (Pilloton *et al.*, 1987), lactose analyses in various milk samples were performed with a biosensor where the two enzymes (β-D-galactosidase and GOD) were co-immobilized in the same step. Recently, we improved the features of the lactose biosensor with a new immobilization procedure of the two enzymes in two separate steps. A flowing apparatus was used for processing samples to obtain lower response time and a higher throughput of samples.

In some dairy farms, where enzymatic reactors convert lactose to glucose to obtain milk more edible for people with genetic diseases, a rapid glucose analysis is required in addition to the lactose analysis and therefore we developed a flow system where the two enzymatic reactions occur separately. Thus, glucose and lactose can be analyzed with the same apparatus.

MATERIALS AND METHODS

β-galactosidase (EC 3.2.1.23, from *Escherichia coli*, 580 U/mg solid), glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, type V, 1200 U/ml) were obtained from Sigma. Polyazetidine prepolymer solution (Hercules Polycup 172, 12% solids in water) was obtained from M. Delaney, NY, USA. All other reagents and buffers were AR grade.

Dialysis membrane, thickness 0·001 in, 12 000 dalton MWCO, was obtained from A. H. Thomas Co. (Philadelphia, PA, USA).

Cellulose acetate (53% acetyl) and polyvinyl acetate of high molecular weight were obtained from Farmitalia Carlo Erba (Milano, Italy). For casting the cellulose membrane a precision gauge tool (from Precision Gage and Tool Co., Dayton, OH, USA) was used. This membrane, with 100 dalton MWCO, was prepared as previously reported (Tsuchida & Yoda, 1983).

A reactor provided with polysulfonic hollow fibres (40 000 MWCO) was kindly supplied by ESACONTROL (Genova, Italy).

The hydrogen peroxide sensor was obtained from Instrumentation Laboratory SpA (Milano, Italy). Platinum wire was 0·5 mm in diameter; the

silver foil cathode was 0·5 cm^2 in area. The polarization unit was a VA detector (Metrohm 641, Herisau, Switzerland); the current was displayed on an Omniscribe recorder (Houston Instrument, USA).

Peristaltic pump, Microperpex 2132 for flow analysis was from LKB (Bromma, Sweden).

Enzyme-immobilized membranes

(1) Glucose oxidase membrane

The BSA-glutaraldehyde procedure was applied to obtain a GOD membrane. On a cellulose acetate membrane (100 dalton MWCO, 0·8 cm in diameter) we spotted 2 μl of bovine serum albumin solution (15% w/v), 2 μl of an enzyme solution (10 mg/ml in phosphate buffer pH 7·0, 0·1 mol/l) and 2 μl of an aqueous solution of glutaraldehyde (12·5% w/v), mixing uniformly. The membrane was left for half an hour at room temperature then washed with phosphate buffer (pH 7·0, 0·1 mol/litre) and stored at 4°C.

(2) β-D-galactosidase membrane

A new prepolymer, polyazetidine (PAP) recently described for the immobilization of *Escherichia coli* (Wood & Calton, 1984), was applied for enzyme immobilization. On a dialysis membrane (12 000 dalton MWCO, 0·8 cm in diameter) we spotted 5 μl of the prepolymer solution (12·5% w/v in water), 5 μl of phosphate buffer (pH 7·0, 0·1 mol/litre) and 1 mg of enzyme (β-D-galactosidase) mixing uniformly. The membrane was left for half an hour at room temperature then washed with phosphate buffer (pH 7·0, 0·1 mol/litre) and stored at 4°C. The polyazetidine reacts with various functional groups, carboxyl, hydroxyl, amine, mercaptan and free amine on another PAP chain. This reaction was also used for preparing a β-D-galactosidase reactor.

Assembling of the sensor

The membrane with immobilized GOD (100 dalton MWCO) was first placed on the platinum surface. Then a second membrane (12 000 dalton MWCO) with immobilized β-D-galactosidase was placed on. The first cellulose acetate membrane (100 dalton MWCO) eliminates interferences from electroactive chemicals such as ascorbic acid; the second one (12 000 dalton MWCO) prevents microbial attack of the enzymes and leaching of them from the sensor. The two layers were blocked with a rubber 'O'-ring.

The sensor was placed in a flow cell, with an estimated volume of 40 μl, through which, standard and sample solutions flowed by a peristaltic pump.

Assembling of the β-D-galactosidase reactor

A reactor with polysulfonic hollow fibres was used as an enzymatic reactor. The sample flowed through the hollow fibres immersed in 150 μl enzyme solution introduced as follows: β-D-galactosidase (1 mg) and polyazetidine (15 μl of a 12·5% aqueous solution) were added in 150 μl of phosphate buffer (pH 7·0, 0·1 mol/litre) and pumped, with a peristaltic pump, into the reactor. In this case the electrochemical sensor was coupled with the GOD membrane and the second dialysis membrane was used without β-D-galactosidase.

Lactose analysis

In this paper two different procedures of analysis for lactose are reported. They differ in the enzymes coupling to the electrochemical sensor.

In the first method both enzymes were immobilized on the electrode surface: GOD with BSA and β-D-galactosidase with polyazetidine procedures using as support the two cellulose acetate membranes fixed with an O-ring.

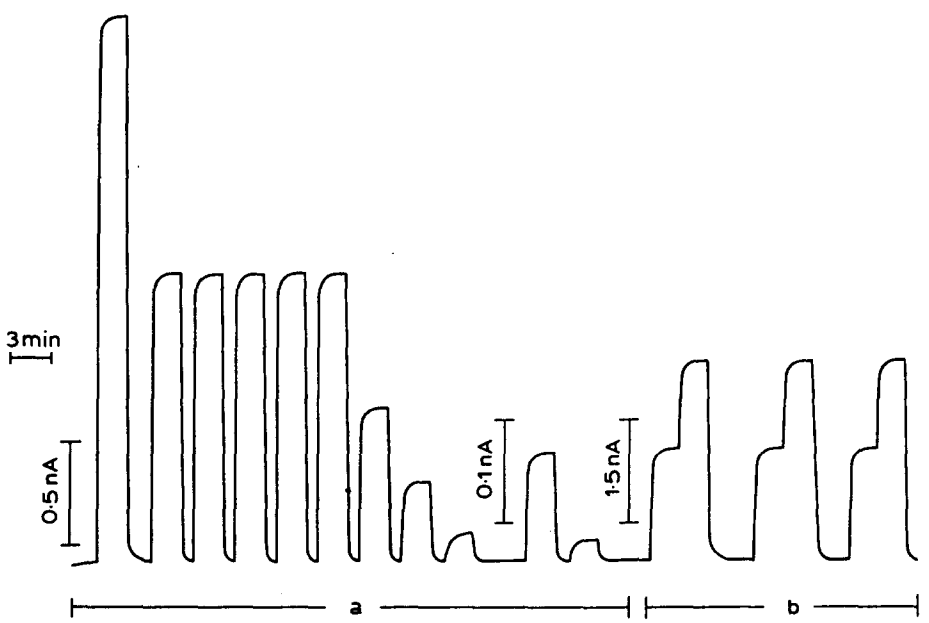

Fig. 1. (a) Recording of standard solutions (lactose 4% solution, diluted 1:50, 1:100, (5 times repeated), 1:200, 1:400, 1:800, 1:2000, 1:10000). (b) Example of the assay procedure, based on comparison of current obtained by injection of sample (diluted 1:100) and of standard added to sample.

The analytical procedure is simple and fast. Milk sample (100 μl) was added to 10 ml (1:100 dilution) of phosphate buffer (pH 7·0, 0·1 mol/litre) and aspirated into the system. Then standard solution (100 μl of a lactose 4% w/v in the same buffer) and milk sample (100 μl) were added to 10 ml of the same buffer. The current due to lactose for both solutions was recorded (Fig. 1) and lactose concentration in the sample was calculated with the formula:

$$\%_{sa} = 4\% \times i_{sa}/(i_{st} - i_{sa})$$

where $\%_{sa}$ is lactose content to be calculated, i_{sa} and i_{st} are the current due to the sample and standard additions, respectively.

In the second procedure the two enzymes are separate: β-D-galactosidase is covalently immobilized in a reactor with hollow fibres (as described above) and glucose oxidase is immobilized on the sensor using the BSA procedure. In Fig. 2 the general assembly of the system is shown.

With this assembly, glucose and lactose can be analyzed with the same apparatus by the switch T (Fig. 2), which eliminates the passage through the β-D-galactosidase reactor. Preliminary experiments were carried out to establish the optimum flow rate for response and recovery time (Figs 3(a)–(c) and Fig. 4). Then a flow rate of 0·7 ml/min was chosen for the limited electrical noise introduced, i.e. it gives the maximum signal/noise ratio obtained with a minimum response and recovery time obtained.

The same assay procedure reported above (1:100 sample dilution) was used and the recording is shown in Fig. 5.

This procedure has been applied for the determination of glucose in commercially available lactose free milk (Accadi from Central Dairy Milk, Milano, Italy) obtained by mixing milk with β-D-galactosidase immobilized

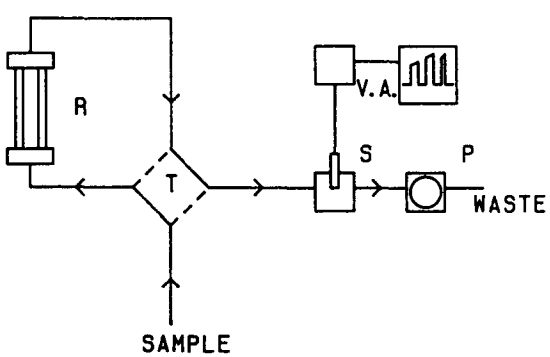

Fig. 2. Diagram representing the manifold used. R, β-D-galactosidase reactor; P, peristaltic pump; T, four way stopcock; S, sensor; VA, current detector and recorder. (The reactor was included only with the second procedure for lactose determination.)

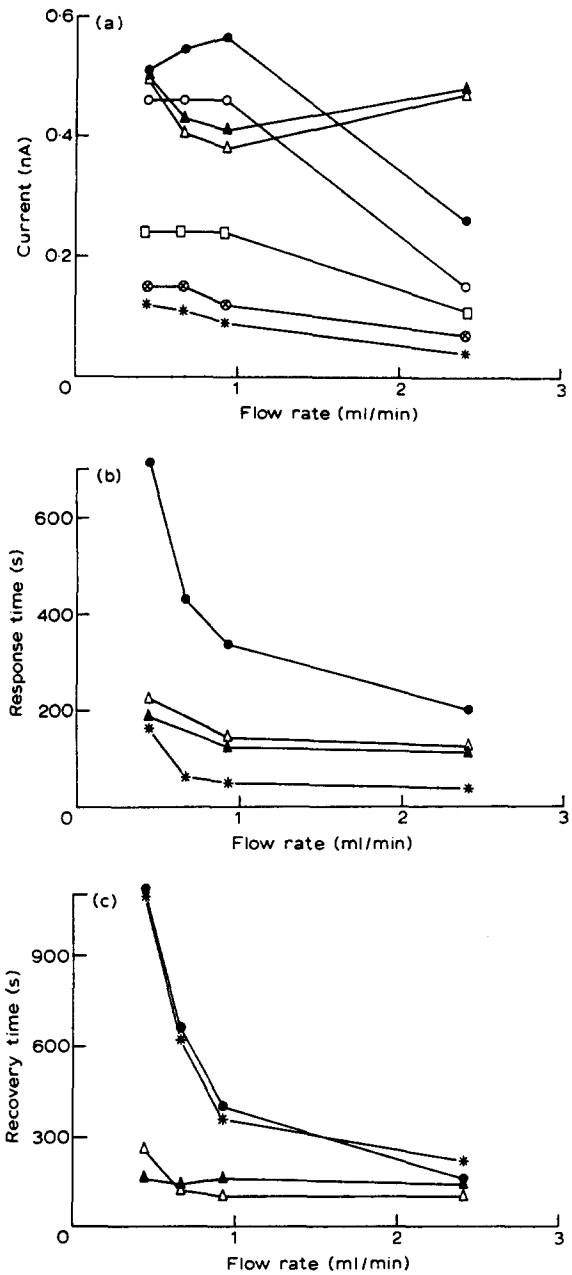

Fig. 3. (a) Current, (b) response time and (c) recovery time for lactose (using β-D-galactosidase reactor with standard solution of glucose and lactose) as function of flow rate. ▲, glucose standard (2% solution diluted 1:100) with reactor; △, glucose standard (2% diluted 1:100) without reactor; *, lactose standard (4% solution diluted 1:400); ⊗, lactose standard (4% solution diluted 1:200); □, lactose standard (4% solution diluted 1:100); ○, lactose standard (4% solution diluted 1:50); ●, lactose standard (4% solution diluted 1:40).

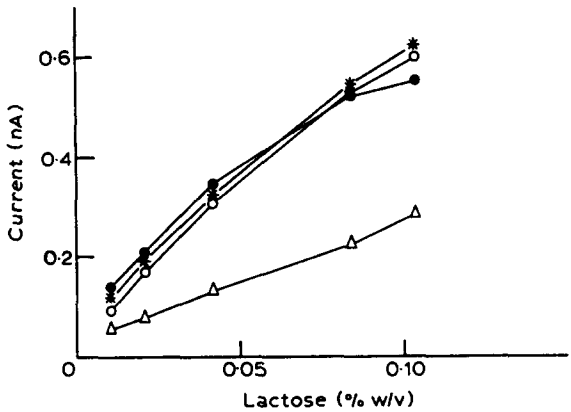

Fig. 4. Calibration curves for lactose using β-D-galactosidase reactor with standard solution at different flow rates. ●, 0·4 ml/min; ○, 0·7 ml/min; ✱, 0·9 ml/min; △, 2·3 ml/min.

on cellulose acetate fibres (SNAM Progetti patent) in a batch reactor process.

RESULTS AND DISCUSSION

With the first method described above, an improved electrochemical sensor for lactose in milk samples has been obtained using a new enzyme immobilization procedure and with a flow-through apparatus. In this case the analysis time was considerably short (4 min for determination, 95% of

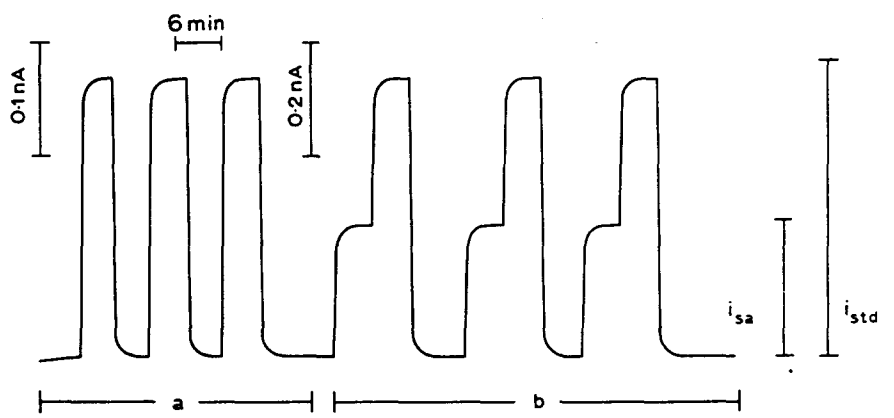

Fig. 5. Recording of flowing system using β-D-galactosidase reactor with (a) standard solution (4% lactose solution diluted 1:100) and (b) sample (fresh milk) and sample plus standard.

the response in 40 s) and reproducibility within 1% (Fig. 1). These results are a marked improvement in respect to the previously reported procedure (Pilloton *et al.*, 1987). Here the two enzymes are immobilized with two different procedures on different supports; then they are coupled in the final assembly.

We found this two step immobilization procedure more useful in terms of speed of response, lifetime and percentage of conversion of lactose in comparison to the procedure previously published; we realized that β-D-galactosidase enzyme is not compatible with the BSA procedure but, reacting with polyazetidine, the immobilization of this enzyme is mild and the procedure is easier and high enzyme activity is retained.

The second method described, based on a β-D-galactosidase reactor, is slower (10 min/analysis) but is very useful to perform analysis of glucose and lactose with a unique apparatus.

In Fig. 5 reproducibility and analysis time for lactose (10 min) can be evaluated. Using the glucose sensor and the same procedure reported above (with a 1:1000 dilution) we obtained the responses reported in Fig. 6 on some real samples.

In Fig. 7 the calibration curves of the two immobilization procedures are reported and compared with the previous procedure (enzymes coimmobilized) (Pilloton *et al.*, 1987). When the immobilization of the two enzymes occurs separately as described in this work we obtained very close curves for glucose and lactose; i.e. the hydrogen peroxide obtained from the two species is similar; this happens because the immobilized β-D-glactosidase is more active; in the procedure where the two enzymes are immobilized in the same

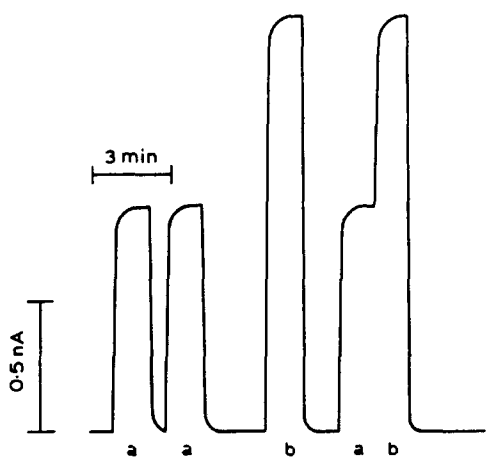

Fig. 6. Determination of glucose in a speciality milk 'lactose-free' commercially available. (a) Sample diluted (1:1000) and (b) glucose standard (2% solution diluted 1:1000) added to the sample.

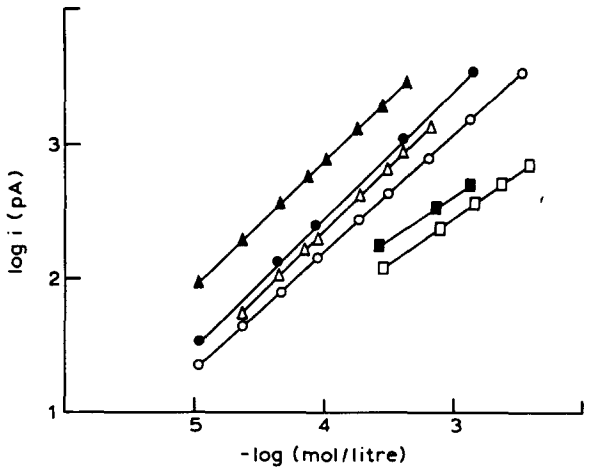

Fig. 7. Comparison of calibration curves of the methods for lactose determination. Closed symbols represent glucose calibration curves, open symbols represent lactose calibration curves. △, ▲, Measurements in open beaker, enzymes co-immobilized; □, ■, flow measurements with the β-galactosidase reactor; ○, ●, flow measurements, enzymes separately immobilized on the sensor.

step, the activity of β-D-galactosidase is lower; glucose oxidase competes and limits the β-D-galactosidase in the immobilization reaction in spite of the favourable activity ratio used (1:10) (Pilloton *et al.*, 1987).

Fast response and recovery times, simple apparatus and procedures allow the proposed method to be directly applied in dairy farms.

CONCLUSION

The proposed methods for lactose determination in milk samples do not require any preliminary sample treatment and are very simple and quick. The response time, in comparison with other methods, is very short and seems to be very useful for dairy farms where analytical data are usually requested at short notice. Moreover, these methods require very cheap instruments. Enzyme immobilization is very simple and quick and the membrane lifetime is about 1 month.

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