Note

Milk lactose and lactulose determination by the differential pH technique

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Abstract – A differential pH technique for lactose and lactulose determination in milk samples (UHT, pasteurised, sterilised and fresh milk) is presented. With enzymatic reactions, causing a pH variation directly proportional to the lactose and lactulose contents in samples, in less than two and four minutes, respectively, the lactose and lactulose can be analysed without sample preparation procedures. The automatic methods are very simple, precise and accurate, with an excellent linearity for lactose ($R^2 = 0.9999$) and lactulose ($R^2 = 0.9997$) throughout the whole range of concentrations and good repeatability (the coefficient of variation was lower than 0.5%). With the optimised experimental conditions, a sensitivity of 0.1 mmol·L^{-1} lactulose was obtained.

Lactose / lactulose / differential pH technique / milk / enzymatic assay

Résumé – Détermination de la teneur en lactose et lactulose des échantillons de lait par la technique de pH-métrie différentielle. L'emploi de la pH-métrie différentielle pour doser le lactose et le lactulose dans des échantillons de lait (UHT, lait pasteurisé, lait stérilisé et lait cru) est proposé. Le lactose et le lactulose peuvent être dosés, en moins de deux et quatre minutes respectivement et sans traitement de l'échantillon, avec des réactions enzymatiques qui causent une variation du pH directement proportionnelle à la concentration des deux substrats dans les échantillons. Les méthodes sont automatisées et elles sont simples, précises et très exactes, avec une excellente linéarité pour le lactose ($R^2 = 0,9999$) et le lactulose ($R^2 = 0,9997$) dans toute la gamme de concentration et une très bonne répétabilité (coefficient de variation < 0,5 %). En optimisant toutes les conditions expérimentales, la méthode a obtenu une sensibilité de 0,1 mmol·L^{-1} pour le lactulose.

Lactose / lactulose / pH-métrie différentielle / lait / essai enzymatique

1. INTRODUCTION

Lactose intolerance, resulting from a shortage of enzyme lactase, which is nor-mally produced by the cells that line the small intestine, is the inability to digest significant amounts of lactose, which is the predominant sugar of milk, giving not

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dangerous but distressing results [14] (common symptoms include nausea, cramps, bloating, gas and diarrhoea, which begin about 30 min to 2 h after eating or drinking foods containing lactose [15]). Certain ethnic populations are more widely lactose-intolerant than others (75% of all African-American adults, Jewish, Native American, and Mexican-American adults, and 90% of Asian-American adults). The condition is less common among people of northern European descent [1, 10].

During heat treatment of milk in processing steps (pasteurisation, sterilisation and UHT treatment), lactose is involved both in the Maillard reaction and in isomerisation and subsequent degradation reactions.

Among the sugars derived from lactose, lactulose undoubtedly represents the most widely studied index for differentiating heated milks and for evaluating the heat load to which milk was subjected [5]. In fact, the lactulose is nowadays used as a marker of heat treatment of milk by the International Dairy Federation (IDF) [7] and by the European Commission (EC) [6] to distinguish UHT milk from in-container sterilised milk. Disaccharide (galactose + fructose) is not normally present in raw milk, but it is formed during heat treatment by isomerisation of lactose [11].

Practical and rapid methods for accurate milk lactose and lactulose determinations in foodstuffs, in order to check low lactose levels and milk product heat treatments, are not currently available.

Photometric methods, used as the reference method for lactose and lactulose determination, are very time-consuming because they require sample deproteination and fat removal, different hydrolysis and detection buffer solutions and long incubation times for each step, not practical for routine use [3].

A biosensor for lactulose determination by immobilisation of the β-galactosidase enzyme on glass beads, to have an enzyme reactor with a long lifetime, has been introduced by Compagnone et al. [4]. This procedure could provide valid support in the analysis of lactulose in milk, but a commercial reactor is not available. It needs a connection with a highly stable fructose biosensor and a long time between the sampling and result.

Chromatographic methods are in use for lactose and lactulose determination [2, 12, 13]. A dedicated and expensive detector, based on the refractive index, needs to be installed. Fat and protein in the sample must be removed. The purpose of the present work is to demonstrate that the differential pH technique [9], already in use in a great number of milk testing laboratories for urea determination [8], can also be used for lactose and lactulose determination in milk for its excellent repeatability and linearity without sample preparation procedures.

2. MATERIALS AND METHODS

2.1. Principle

The principle of the method and the instruments CL10 plus and Microlab® EFA (Diffchamb-Eurochem Lyon, France; Ardea, Rome, Italy) have already been described [9].

2.2. Reagents and standards

The concentrated buffer and the standards for the instruments CL10 plus and Microlab® EFA analyser have been described in a previous work [9].

2.2.1. Concentrated diluent

In ~60 mL of bidistilled water under continuous stirring $MgCl_2\,6\,H_2O$ (100 mmol·L^{-1}), KCl 1 mol·L^{-1}, Bronopol (0.1% w/w) and Triton X 100 (20% w/w) were dissolved, and the volume adjusted in a 100 mL volumetric flask. All the reagents were supplied by Sigma-Aldrich (Milan, Italy).

2.2.2. Working solutions

CL10

The working buffer solution was obtained by transferring with a pipette 10 mL

of concentrated buffer and 10 mL of concentrated diluent into a calibrated cylinder and diluting to 100 mL with bidistilled water. The final pH was 7.9 ± 0.1.

Microlab® EFA

The concentrated buffer was used as such. The working diluent was: 20 mL of concentrated diluent + 180 mL of bidistilled water.

2.2.3. Enzymes

The lyophilised enzymes, hexokinase (HK, EC 2.7.1.1, Hofmann La Roche, Mannheim, Germany) and glukokinase (GK, EC 2.7.1.2, Unitika, Osaka, Japan), were dissolved in glycerol 50% and used as such; the final solution activity was 200 $U \cdot mL^{-1}$ for HK and 1000 $U \cdot mL^{-1}$ for GK. The β-galactosidase from Enzeco (Enzeco® Lactase, New York, USA) was chosen from among the other enzymes tested. It was purified by gel filtration with a G-100 and concentrated on Amicon® (Lexington, USA); the solution obtained, with a final activity of ~3000 $U \cdot mL^{-1}$ at pH 7.1, was preserved in glycerol 50%.
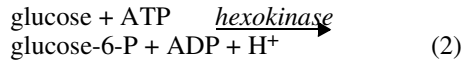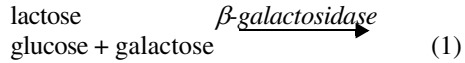
2.2.4. Calibrator

Glucose, lactose and lactulose (supplied by Sigma-Aldrich) were at least 99% pure. The glucose-lactose calibrator was prepared by dissolving the glucose 50 $mmol \cdot L^{-1}$ and lactose 100 $mmol \cdot L^{-1}$ in phosphate 5 $mmol \cdot L^{-1}$, KCl 0.1 $mol \cdot L^{-1}$ and Bronopol 0.1% solution (final pH 8.0). The lactulose calibrator was prepared by dissolving the lactulose in fresh milk sample to obtain a final concentration of 5 $mmol \cdot L^{-1}$.

2.2.5. Samples

Milk samples were collected from markets (UHT, sterilised and fresh milk), the raw milk from Cecalait (Centre d'étude et de contrôle des analyses en industrie laitière, Poligny, France). Sometimes milk samples were taken directly from the dairy plant. All the samples were used as such; no filtration, centrifugation or clarification was necessary.

2.3. Procedure for lactose determination

2.3.1. Lactose determination

$$\text{lactose} \xrightarrow{\text{β-galactosidase}} \text{glucose + galactose} \quad (1)$$

$$\text{glucose + ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P + ADP + H}^+ \quad (2)$$
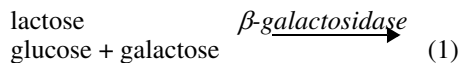
2.3.2. CL10 analyser

Twenty µL of sample (fresh and pasteurised milk or aqueous solutions) were added to the mixing chamber containing 1.2 mL of working buffer; half of the solution volume was sucked into both of the electrodes and the pH difference between the electrodes was measured. Twenty µL of hexokinase (HK) were automatically added (by peristaltic pump) to the solution remaining in the mixing chamber and then a part of the solution was sucked into only the measurement electrode. The pH change was monitored for 40 s so that the measurement of free glucose contained in the sample was obtained. Twenty µL of β-galactosidase were automatically added; after the enzyme addition, the solution was sucked into only the measurement electrode. The pH change was monitored for 90 s to observe the lactose complete hydrolysis and glucose phosphorylation.
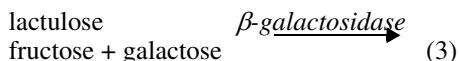
2.3.3. Microlab® EFA analyser

The analytical steps and analyte volumes used with this method were the same as those used for the CL10 instrument but automatically dispensed by the diluter/dispenser.

2.4. Procedure for lactulose determination

2.4.1. Lactulose determination

$$\text{lactose} \xrightarrow{\text{β-galactosidase}} \text{glucose + galactose} \quad (1)$$

$$\text{lactulose} \xrightarrow{\beta\text{-}\underline{galactosidase}} \text{fructose + galactose} \quad (3)$$

$$\text{D-glucose + ATP} \xrightarrow{\underline{glucokinase}} \text{D-glucose 6-P + ADP + H}^+ \quad (4)$$

$$\text{D-fructose + ATP} \xrightarrow{\underline{hexokinase}} \text{D-fructose-6-P + ADP + H}^+ \quad (5)$$

2.4.2. CL10 analyser

Eighty µL of sample (fresh, pasteurised, sterilised, UHT milk or aqueous solutions), 12 µL of glucokinase and 25 µL of β-galactosidase were added to the mixing chamber containing 1.2 mL of working buffer; half of the solution volume was sucked into both of the electrodes. After 180 s the pH difference between the electrodes was measured. Twenty µL of HK were automatically added to the remaining solution. After the enzyme addition, the solution was sucked into only the measurement electrode. The pH change was monitored for 240 s to observe the D-fructose-6-P formation.

2.4.3. Microlab® EFA analyser

The analytical steps and analyte volumes used with this method were the same as those used for the CL10 instrument but automatically dispensed by the diluter/dispenser.

3. RESULTS AND DISCUSSION

3.1. Optimisation of assay conditions

The factors affecting optimisation of assay conditions are the buffer pH, the buffer power, the activity of β-galactosidase at different pH and the sample volume. It would be advantageous to have the same buffer formulation for both methods. In this way, with the automatic and the semi-automatic system, both determinations could be run sequentially on the same sample. In all conditions the minimum signal which can be reliably detected by the apparatus is approximately 0.5 mpH ($1\ \text{mpH} = 10^{-3}\ \text{pH}$).

3.1.1. Lactose optimisation

For lactose determination, a sample addition of 20 µL would produce a total pH change of 0.2 pH (Δ pH = concentration × (β × dilution $)^{-1} = 140 \times (11 \times 60)^{-1}$). With a sensitivity of 0.5 mpH this would give a very good signal to noise ratio.

3.1.2. Lactulose optimisation

For lactulose determination, a larger sample volume should be added so that the signal obtained from a lactulose concentration of 1 mmol·L^{-1} a typical concentration of an indirectly-heated UHT milk, would give a signal of 7 mpH ($1 \times (11 \times 15)^{-1}$), providing a $7 \times (0.5)^{-1}$ signal to noise ratio.

The addition of a large sample volume may also improve the reaction kinetics, as demonstrated in Figure 1, where the hydrolysis of a large amount of lactose causes an acidification to pH 7.1 which favours the hydrolysis of residual lactose and lactulose for higher β-galactosidase activity.

3.2. Linearity

The regression equations and correlation coefficients obtained during the linearity test on Microlab EFA between the pH signal and lactose and lactulose concentrations in water solution samples (lactose and lactulose 0, 25, 50, 75, 100 and 150 mmol·L^{-1}) were y = 1.2574X – 0.6071 (R^2 = 0.9999) and y = 1.4717X – 3.2459 (R^2 = 0.9997), respectively.

3.3. Linearity-accuracy and lactose evaluation

A combined check for glucose and lactose determinations could be done by assaying glucose and lactose contents during the hydrolysis of a pasteurised milk sample. We added to 1 mL of milk sample 2 µL of β-galactosidase solution and 8 µL of glycerol (50%) solution for a sample final dilution of 1.00 to 1.01 and allowed the solution to be incubated at 37 °C. The hydrolysis reaction was followed by sampling every
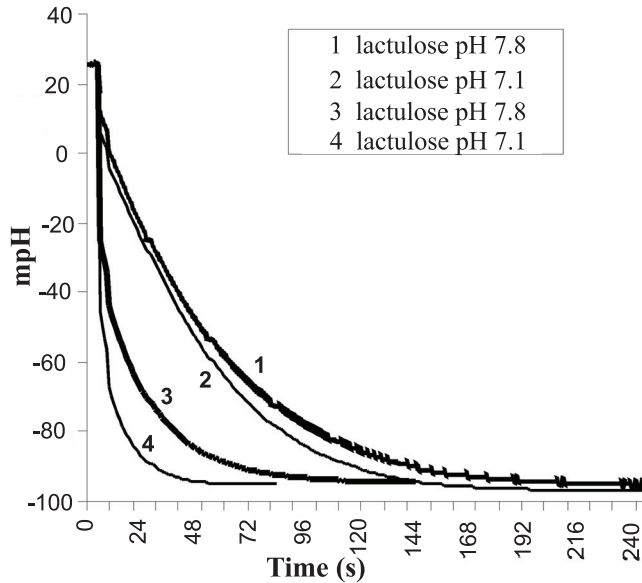
Figure 1. Lactose and lactulose hydrolyses at pH 7.8 and 7.1 with the CL10 instrument. In the reaction mixture, 10 µL of lactose or lactulose 150 mmol·L^{-1} and 40 µL of HK; 10 µL of β-galactosidase as starter for lactose and 20 µL for lactulose. The stoichiometry of the reactions is the same at the different pHs, the β-galactosidase activity on lactose increases from pH 7.8 to pH 7.1.

5 min and reading the glucose and lactose with the Microlab EFA.

As a check for complete hydrolysis, 10 µL of β-galactosidase solution were added to 1 mL of the same milk sample. Figure 2 presents the obtained results; the figure presents a decrease in lactose and a simultaneous increase in glucose, thus making the sum of the two constant.

3.4. Reproducibility and repeatability

The reproducibility and repeatability of lactose and lactulose in milk samples with the EFA instrument and CL10 were tested and the results are given in the Tables I and II, with the standard deviation lower than 1.5% for the Microlab EFA and 0.5% for the CL10 analyser for lactulose, and the standard deviation lower than 0.5% for both of the instruments for lactose determination.

3.5. Specificity and interference

The specificity for lactose determination is given by the substrate characteristics of β-galactosidase. In milk, possible substrates are lactose (at high concentrations) and other β-galactosidases, but these are present at very low concentrations. In addition to glucose in the first step of the measurement, fructose and mannose can also be phosphorylated, thus increasing the glucose signal. However no effect results on lactose estimation.

The influence of preservative was evaluated by adding increased amounts of Bronopol (2-bromo-2-nitropropane-1,3-diol) to a fresh milk, UHT milk and sterilised milk. Bronopol is a non-competitive inhibitor of β-galactosidase. However, the obtained results do not show any difference within the experimental error (Tab. III) for lactose and lactulose because, with the
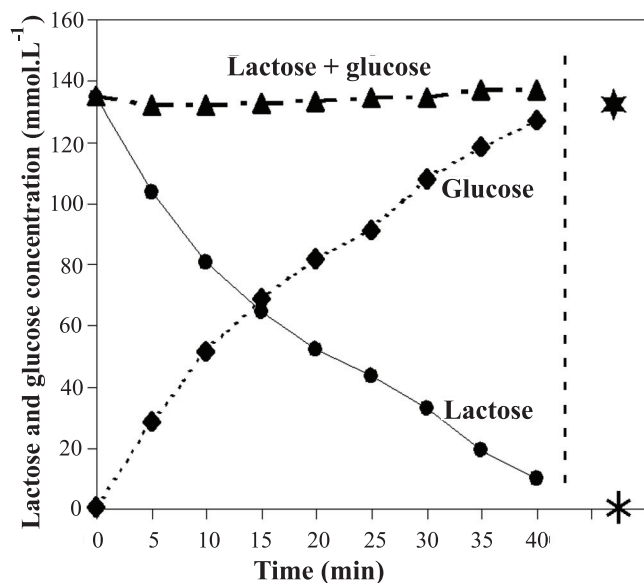
Figure 2. Lactose hydrolysis and lactose determination. 1 mL of milk sample + 10 μL of β-galactosidase solution waiting for 35 min for complete hydrolysis (stars on the graph); 1 mL of the same milk sample + 2 μL of β-galactosidase solution + 8 μL of glycerol solution sampling every 5 min (dots and squares on the graph).

Table I. Lactose and lactulose repeatability determined by the Microlab EFA and CL10 analysers; the sample volumes were 20 μL and 80 μL, respectively. The RSD obtained was lower than 0.5% for lactose and 1.5% for lactulose.

Analyser	Sample	Analyte	Nr.	$\overline{\mathrm{X}}$ (mmol·L^{-1})	σ_{n-1}	CV%
Microlab EFA	Pasteurised milk	Lactose	10	137.1	0.632	0.46
CL10	Pasteurised milk	Lactose	10	138.5	0.750	0.51
Microlab EFA	Bottle sterilised	Lactose	7	4.03	0.061	1.5
CL10	Bottle sterilised	Lactose	7	4.13	0.013	0.31

enzyme concentration used in this direct assay, only concentration in solution exceeding 1% may decrease the lactose signal (no effect on glucose measurement).

3.6. Ring test

An interlaboratory collaborative test for lactose determination in milk samples involving different laboratories and directed by Cecalait was carried out on 11 test sam-

ples (10 milk samples + Cecalait control aqueous solution) with two replicates. The results obtained in our laboratory (University of Milan) with two different Microlab EFA analysers compared with the results from the other laboratories (Eurochem and Macarrese (Italy), and Gembloux and Cecalait (France)), are presented in Figure 3 (according to ISO 5725) compared with the HPLC and enzymatic methods.
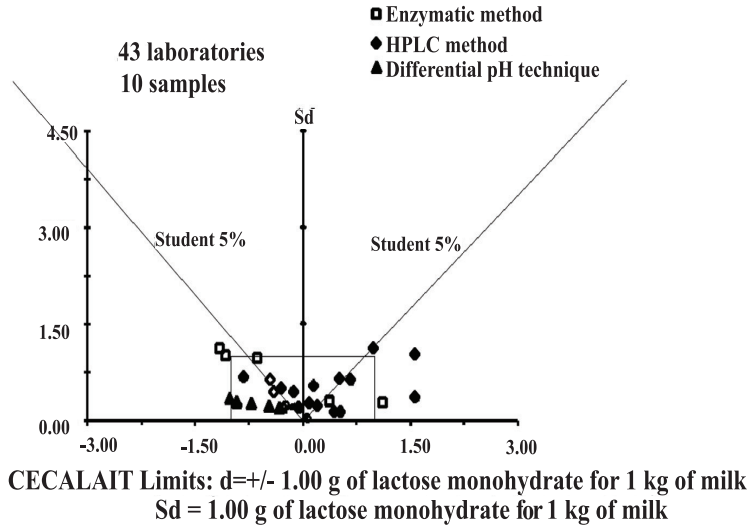
Figure 3. Ring test results. On the graph are reported the results obtained during the ring test executed on 10 milk samples, comparing three different methods of lactose determination (Enzymatic method, HPLC method and the Differential pH technique).

Table II. Lactulose reproducibility determined on different days by the CL10 analyser; the sample volume was 80 µL.

Sample	Lactulose (mmol.L^{-1})			
	09/07/02	10/07/02	08/08/02	09/08/02
Fresh milk	0	0	0.1	0
Parmalat whole UHT	0.33	0.36	0.33	0.39
Sma UHT	1.16	1.3	1.3	1.36
Stella UHT	3.44	4.4	4.31	4.13

Table III. Influence of Bronopol at two levels (0.1 and 0.5%) on three different milk samples (raw milk and UHT milk for lactose determination, sterilised milk for lactulose determination). The results are expressed in mpH.

Preservative	Lactose		Lactulose sterilised milk
	Raw milk	UHT milk	
Unpreserved	207	199	6.1
Bronopol 0.1%	208	200	6.2
Bronopol 0.5%	206	201	6.0

3.7. Lactulose determination with the CL10 analyser: recovery test

Recovery (Tab. IV) was evaluated by adding different known amounts of lactulose to aqueous solution (lactose 140 mmol·L^{-1}) and to UHT milk with endogenous lactulose concentration (1.1 mmol·L^{-1}). The average recovery referred to aqueous solution was 102%. Regression of measured lactulose

Table IV. Recovery on aqueous solution and UHT milk sample with added lactulose.

Lactulose added (mmol·L^{-1})	Aqueous solution net mpH	UHT milk net mpH	Recovery %
+ 0.5	1.6	1.5	94
+ 1.0	3	3.2	106
+ 2.0	6	6.1	102
+ 5.0	14	14.9	106

minus endogenous lactulose on lactulose content was y = 2.97X + 0.087 (R^2 = 0.9997).

4. CONCLUSION

Two important products obtained from milk, low-lactose milk and UHT or sterilised milk, need monitoring systems for on-line control. The system proposed provided the sensitivity and reproducibility required by industry with simplified operations and maintenance for operators with moderate skill and short training. The two assays also showed that by just changing the sample volume by a factor of four, it was possible to determine under optimal conditions two very similar substances which differed in concentration by a factor of 100 without any carry-over effect of lactose over lactulose determination.

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