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A new enzymatic spectrophotometric assay for the determination of lactulose in milk

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Abstract

A new enzymatic spectrophotometric assay for the determination of lactulose in milk samples was developed. This method entailed the use of β-galactosidase which hydrolyses lactulose giving fructose and galactose, and fructose dehydrogenase which reacts with fructose in presence of a tetrazolium salt (MTT), giving a coloured compound which can be detected spectrophotometrically at 570 nm.

The assay showed a lactulose detection limit in milk of about 10 mg l^{-1}, a linear range of $20 \div 800$ mg l^{-1} and a relative standard deviation of 5%. The correlation with the determination of lactulose in milk using reference procedures was good. Moreover this procedure was found suitable for the quantitation of lactulose in milk after the heat treatment process, and more convenient for the rapid and sensitive estimation of lactulose if compared with previous published enzymatic methods.

Keywords: Lactulose; Milk; Enzymatic spectrophotometric method; β-D-galactosidase; FDH

1. Introduction

Recently much of the efforts devoted to the development of methods for the quantitative determination of lactulose, have been directed toward applications in the area of dairy science. Indeed lactulose (4-O-β-D-galactopyranosyl-D-fructo-furanose) which is formed during milk heat treatment [1,2] has been proposed by the International Dairy Federation (IDF) [3] and by the European Commission (EC) [4] as an analytical index to distinguish Ultra High Temperature (UHT) milk from in-container sterilised milk.

There are several analytical methods for detection of lactulose mainly based on gas chromatography [5], IDF official method based on liquid chromatography (LC) [6], also spectrophotometric [7,8] and enzymatic methods based on spectrophotometric detection [9,10] or amperometric detection [11,12]. We also developed a method based on the use of an electrochemical biosensor and microdialysis [13]. Recently a kit, commercially available from Boehringer [14] and based also on an enzymatic procedure has been proposed. One drawback of this method is the potential interference from glucose, which is quite high in milk

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where the molar ratio lactose/lactulose could be equal to 1000/1. While it is possible to minimise the effects of glucose interference by sample pre-treatment with glucose-oxidase and catalase, this approach makes the assay more difficult to automate, because it requires six different enzymes, expensive reagents and about 15 h to perform the analysis.

The present paper describes the combination of two enzymes: β-galactosidase (β-gal) from *Aspergillus orizae* and fructose dehydrogenase (FDH) from *Gluconobacter* sp. for the determination of lactulose according to the following reactions:

$$\text{lactulose} \overset{\beta\text{-galactosidase}}{\rightarrow} \text{D-fructose} + \text{galactose} \quad (1)$$

$$\text{D-fructose} + \text{MTT} \overset{\text{FAH}}{\underset{\text{PMS}}{\rightarrow}} \text{5-keto-D-fructose} + \text{MTT Formazan} \quad (2)$$

The second reaction, in presence of the electron carrier phenazine methosulfate (PMS), produces a coloured compound (MTT Formazan), which can be monitored at 570 nm using a spectrophotometric apparatus. FDH from *Gluconobacter* sp. has already been shown to be useful for the determination of fructose in biological fluids [15,16]. β-galactosidase from *Aspergillus orizae* (β-gal) shows a good thermostability and an activity for lactulose two times higher than that observed for lactose when an equimolar concentration of lactose and lactulose was used [17].

2. Materials and methods

2.1. Reagent preparation

Fructose dehydrogenase (FDH) (EC 1.1.99.11, 112 Units mg^{-1} solid), β-D-galactosidase (β-gal) from *Aspergillus orizae* (EC 3.2.1.23, 9 Units mg^{-1} solid), 5-methylphenazinium-methyl sulphate (PMS) and 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of high purity from Sigma.

Citric phosphate buffer was prepared from a mixture of 0.1 mol l^{-1} citric acid, 0.1 mol l^{-1} dibasic potassium phosphate and 0.1% of Tween 20 in distilled water. A stock solution of FDH was prepared at 125 U ml^{-1} in buffer at pH 4.9. This stock was stored at –20°C in 200 μl aliquots before use. β-galactosidase was freshly prepared in buffer at pH 4.9 at a concentration of 150 U ml^{-1}; MTT was prepared in buffer at a concentration of 0.6 mg ml^{-1} and stored at 4°C. This solution was stable for at least 1 month. PMS was prepared in buffer at a concentration of 0.6 mg ml^{-1} just prior to use.

Carrez I corresponds to 7.2% (w/v) $K_4Fe(CN)_6 \cdot 3H_2O$ prepared in distilled water. Carrez II corresponds to 14.4% (w/v) $ZnSO_4 \cdot 7H_2O$ prepared in distilled water.

The reference methods were the spectrophotometric enzymatic test kit 'Lactulose in Milk', (no. 139106 with further reagents) from Roche Diagnostic, (formerly Boehringer Mannheim, Germany), and the official LC method [6].

Low pasteurised and UHT milk samples were purchased from local shops.

2.2. Apparatus

Absorbance measurements were made with a Unicam 8625 UV–Vis Spectrometer (Cambridge, UK), using 1 cm cuvette light path. The effect of temperature on β-galactosidase activity was carried out with a Haake F3 Thermostat (Berlin, Germany).

2.3. Procedures

2.3.1. Sample treatment

A milk sample of 10 ml was pipetted into 50 ml conical flask and 1.75 ml of each Carrez I and II were added; the resulting solution was stirred for 2–3 min, then 6.5 ml of buffer were added. The solution was then well mixed for 2–3 min, left to rest for 30 min, then filtered through a filter paper.

Any time this procedure was carried out, the first 2–3 ml of filtrate were eliminated. The filtrate solution obtained was labelled as 'milk filtrate'.

2.3.2. Hydrolysis of standard lactulose

To each glass-stoppered test tube (7.5 × 1.0 cm) 1 ml of standard solution of lactose (35 mmol l^{-1}) and lactulose (0.44 mmol l^{-1}), a β-galactosidase solution and buffer were sequentially added, then incubated at

selected temperature and time. The total volume in the tube was 1.5 ml.

2.3.3. Hydrolysis of milk lactulose

To each glass-stoppered test tube (7.5 × 1.0 cm) 1 ml of 'milk filtrate', 0.2 ml of β-galactosidase and 0.3 ml of buffer, were sequentially added. These test tubes were placed in water bath at 65 ± 0.1°C for 12 min then cooled in tap water.

The obtained solution was labelled as 'milk hydrolysate'.

2.3.4. Absorbance measurement

100 μl of MTT, 50 μl of PMS, fructose or hydrolysate of standard lactulose or 'milk hydrolysate' and buffer were mixed in a 1 cm light path cuvette, then the reaction started with 16 μl of FDH. The resulting solution was 1.0 ml. The increase of the absorbance at 570 nm was measured every 3 min over a 12 min period at room temperature (25°C).

The volume of the 'milk hydrolysate' sample was 200 μl for sterilised and UHT milk and 500 μl for pasteurised milk. The volume of the buffer was also changed to maintain the total volume to 1.0 ml.

The acquisition of each datum was performed at least two times, then the results were averaged.

2.3.5. Blank sample

A blank sample was prepared for each milk sample. The procedure was as described above except that the buffer was added instead of FDH solution.

3. Results and discussion

3.1. Hydrolysis of standard lactose and lactulose

Previous enzymatic spectrophotometric methods [9,10,14] for lactose or lactulose are based on the hydrolysis of lactose or lactulose using β-galactosidase from *E. coli*. This enzyme has an optimum temperature between 25 and 37°C. In a recent report [17] β-galactosidase from *A. orizae* showed an activity

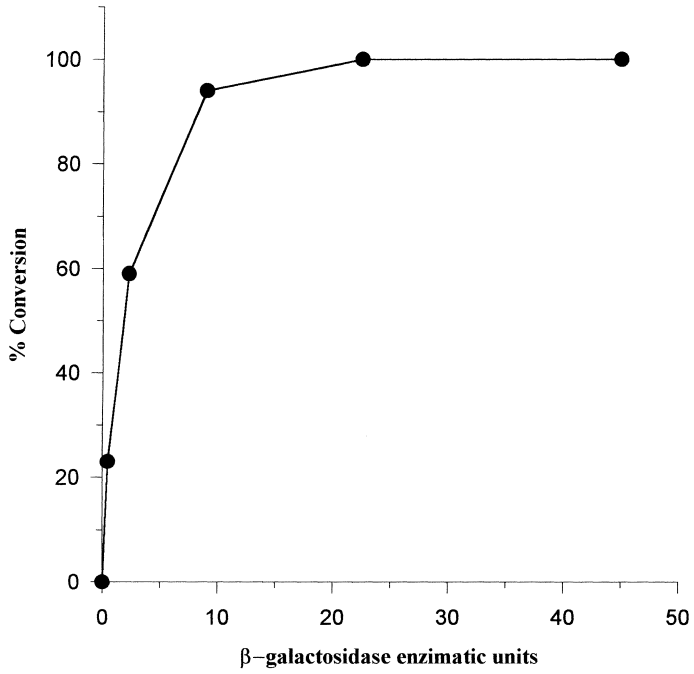

Fig. 1. Effect of β-galactosidase concentration on the conversion rate of lactulose to galactose and fructose. The fructose was measured as described in the text using the initial reaction rate. β-galactosidase from *A. orizae* was varied from 0 to 45 units. The standard was lactose 35 mmol l^{-1}/lactulose 0.44 mmol l^{-1}. The hydrolysis of lactulose was performed for 5 min at 55°C.

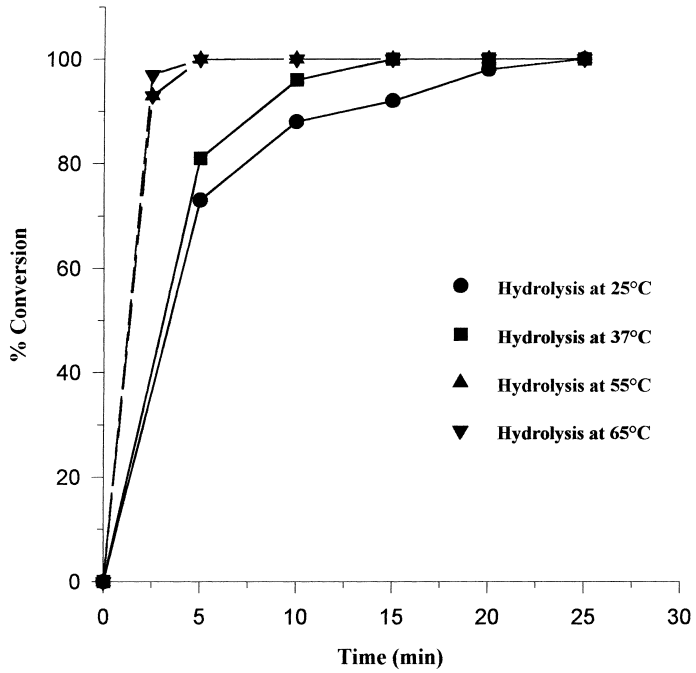

Fig. 2. Conversion rate of lactulose to galactose and fructose using standard lactose 35 mmol l^{-1}/lactulose 0.44 mmol l^{-1} with β-galactosidase from *A. orizae* (30 units) incubated at 25, 37, 55, and 65°C for a period of time variable from 2.5 to 25 min.

for lactulose two times higher than that coming from *Escherichia coli*, also showed an optimum temperature of about 55°C [17]. Thus, we studied the rate of the conversion of lactose (35 mmol l^{-1})/lactulose (0.44 mmol l^{-1}) at 55°C using different amounts of enzyme. Fig. 1 shows that the total conversion of hydrolysis (100%) was obtained during 5 min using less than 30 units of β-galactosidase. Therefore, for further experiments, 30 units of the enzyme β-galactosidase from *A. orizae* were used.

The standard solution of 35 mmol l^{-1} lactose/ 0.44 mmol l^{-1} lactulose was in the range of their natural concentration in milk after the dilution with Carrez I and II.

Fig. 2 shows the rate of conversion (hydrolysis) of standard 35 mmol l^{-1} lactose/0.44 mmol l^{-1} lactulose using 30 units of β-gal at 25, 37, 55 and 65°C. As expected, higher was the temperature, lower was the period of incubation required to reach the total conversion (hydrolysis) of lactulose (5 min at 55°C, 15 min at 37°C and 25 min at 25°C).

3.2. Analysis of sample

The proposed enzymatic assay was used to determine the lactulose concentration in milks. The milk sample was deproteinised with Carrez I and II, and then the filtrate was treated with β-galactosidase. It was observed that after the total lactulose conversion (5 min at 55°C, 15 min at 37°C and 25 min at 25°C), the enzyme β-gal was still active and when this hydrolysate was mixed with PMS/MTT and buffer, an increase in the absorbance was obtained (0.082 absorbance units/500 μl of hydrolysate sample during 12 min). When 500 μl of 'milk filtrate' were mixed with MTT/PMS and buffer without adding FDH, no change of absorbance during 12 min was observed, indicating no interferences by reducing substances eventually contained in the milk. Then we concluded that the increase of absorbance was due to the remaining activity of some impurities contained in β-gal solution. Thus the shortest time for total conversion of lactulose and the total inactivation of the enzyme activity should be selected. We observed that an incuba-

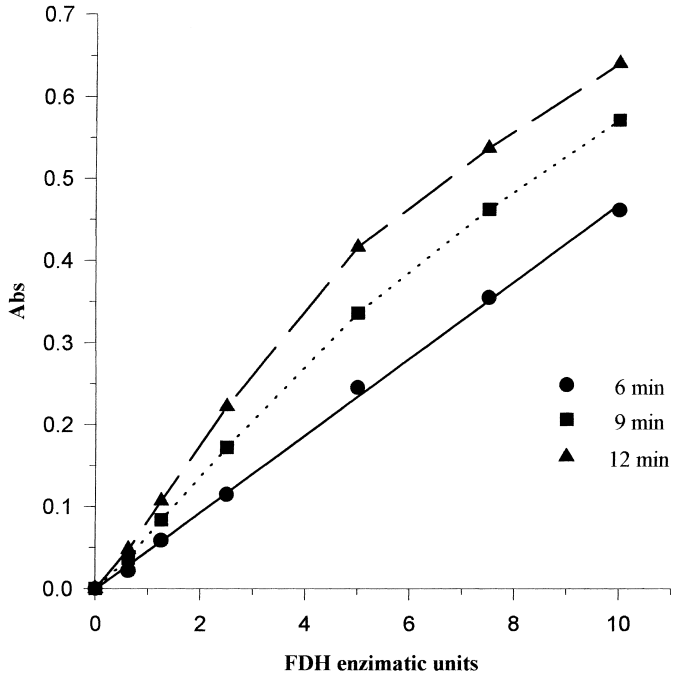

Fig. 3. Effect of FDH concentration on the reaction rate. Fructose concentration was 0.04 mmol l^{-1} and FDH was varied from 0 to 10 units ml^{-1} of reagents. The initial reaction rate was calculated from the absorbance changes over the first 6, 9 and 12 min. Buffer was citric-phosphate 0.1 mol l^{-1}, pH 4.9.

tion time higher than 10 min at 65°C was sufficient to totally inactivate the enzymatic activity of β-gal and to eliminate the absorbance increase previously observed. Thus, for further experiments we carried out the hydrolysis of milk samples at 65°C for 12 min.

3.3. Linear range and interference studies

Under the standard assay conditions, the initial rate of FDH-catalysed fructose oxidation increased with FDH concentration over the range $0 \div 10$ units ml^{-1} of reagents (Fig. 3). While these results showed that a concentration of 10 units ml^{-1} would provide higher initial rates, a concentration of 2.0 units ml^{-1} was selected for routine use, due to the relatively high cost of the enzyme.

Six consecutive analyses of standard lactulose 50 μmol l^{-1} gave a relative standard deviation of less than 3%; the same experiment repeated performing six different deproteinisations on the same milk sample gave a relative standard deviation of about 5%.

FDH from *Gluconobacter* is known to be selective for fructose [15,16]. However, the fructose obtained from the hydrolysis of lactulose is very low in respect to glucose and galactose obtained from the hydrolysis of lactose. The normal concentration of lactose in milk is about 140 mmol l^{-1} (49 g l^{-1}) [19]. Lactulose is absent in raw milk [2,18], however, it is present in the range of 100–510 mg l^{-1} [20] in UHT milk, 600–2000 mg l^{-1} in 'in-container' sterilised milk [2]. Fig. 4 shows the absorbance due to 40 μmol l^{-1} of fructose and the absorbance due to 8 mmol l^{-1} of glucose at different pHs. The concentration of glucose was deliberately chosen 200 times higher than the concentration of fructose. The range of pH studied was 4.0–7.0. Although pH 5.5 provided the maximum rate for FDH, pH 4.9 was chosen as the best compromise between a high response of fructose and a low response of glucose. Further studies showed that the absorbance due to the interference of glucose and galactose at concentrations higher than 5 mmol l^{-1} remained stable and was negligible (an increase of 0.035 abs units during 12 min).

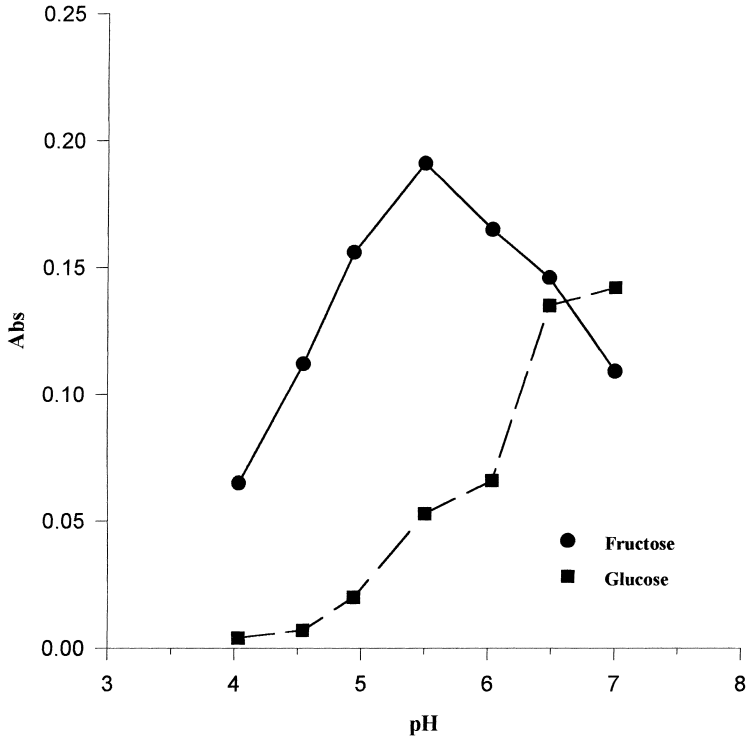

Fig. 4. Increase of the absorbance measured after 12 min using FDH (2 units ml^{-1} of reagents) and glucose 8 mmol l^{-1} or fructose 0.04 mmol l^{-1} as substrates. The pH was varied from 4.0 to 7.0.

Nine different pasteurised milks (whole, semi-skimmed and skimmed) were then analysed as blank and gave a mean absorbance equal to 0.040 ± 0.005. These values were comparable with those of lactose standards (0.035 ± 0.003).

Calibration curves were performed adding different amounts of standard lactulose to pasteurised milks in the ranges $20 \div 100$ mg l^{-1} and $200 \div 800$ mg l^{-1}. In the first case 500 μl of 'milk hydrolysate' were added and the calibration procedure gave a linear plot with equation $y = 0.0009x + 0.0002$ and correlation coefficient $r = 0.996$, y being the measured absorbance and x the lactulose concentration (mg l^{-1}). In the high range of lactulose concentration ($200 \div 800$ mg l^{-1}) only 200 μl of 'milk hydrolysate' were used; also in this case the calibration was linear over the whole range, and the regression data were $y = 0.0004x + 0.005$; $r = 0.995$. In any case 2 U ml^{-1} of FDH were used and the increase of the absorbance measured for the first 12 min. Three calibration curves gave a relative standard deviation (RSD) lower than 5%.

The detection limit of lactulose was dependent of the hydrolysate sample volume added and of the amount of FDH. Using our procedure, the calculated detection limit in milk sample was 10 mg l^{-1} lactulose. This value was derived from the absorbance difference of 0.010 and a maximum hydrolysate sample volume of 500 μl. This value was lower than that obtained with the official method based on LC (200 mg l^{-1}) [6], and with the gas chromatographic method (50–100 mg l^{-1}) [5] and of the same order of the Roche kit (10 mg l^{-1}) [14].

This value can be lowered if the amount of the enzyme is increased. For example, using 8 Units of FDH, the detection limit of lactulose in milk was 4 mg l^{-1} (data not shown).

3.4. Recovery study

The recovery of lactulose standards (38, 75 and 150 mg l^{-1}) was studied. Table 1 summarises the

Table 1
Recovery study (average of three deproteinisation) performed by adding standard solutions of lactulose to low pasteurised milk samples

Lactulose added ($mg\,l^{-1}$)	Lactulose found in the sample before spiking ($mg\,l^{-1}$)	Expected values ($mg\,l^{-1}$)	Found values ($mg\,l^{-1}$)	Recovery (%)	RSD (%)
150	Not Detectable	150	135	90	4
75	Not Detectable	75	73	97	5
38	Not Detectable	38	35	92	4

recovery from three deproteinisations. It should be noted that the deproteinisation of milk using Carrez I and II gave a recovery lower than 60% when lactulose standards were added to milk at a concentration less than $100\,mg\,l^{-1}$. The recovery was greatly improved (higher than 90%) if the solution was rested for approximately 30 min before the filtration (see Table 1); therefore, this resting time was applied also to the spectrophotometric reference method.

3.5. Method of comparison

To validate our procedure, two different methods of comparison have been used, one in the low and one in the high range of lactulose. In the range of lactulose below $200\,mg\,l^{-1}$ the Roche spectrophotometric kit has been applied to different UHT milk samples and the results compared with those obtained by the proposed method: the differences between the two methods are shown in Table 2 and they do not exceed ±10%. In the range higher than $200\,mg\,l^{-1}$ (detection limit of the official LC method), different amounts of lactulose standard were added to low pasteurised milk (without lactulose) and analysed by the official LC method and the proposed one. Table 3 shows that the percentages of recovery are quite similar for both methods, which are also well correlated.

Table 2
Total lactulose content of different UHT milk samples determined by the Roche spectrophotometric Kit and the proposed method

Milk samples	Spectrophotometric Kit ($mg\,l^{-1}$) A	Proposed method ($mg\,l^{-1}$) B	$(A-B)/A$ %
UHT A	131	120	8
UHT B	140	131	6
UHT C	116	128	−10
UHT D	75	82	−9
UHT E	124	134	−8
UHT F	166	173	−4

4. Conclusions

This paper has shown the good potentialities of a new method for the rapid detection of lactulose and its application in the analysis of milk.

This procedure is very simple and rapid, so it is a useful method to be adopted by the small, medium and large dairy industries for the evaluation of the efficiency of the heat treatment during milk processing.

Also this method can be easily extended to the analysis of lactulose in others fields, as in pharmaceutical products.

Table 3
Recovery of lactulose added to commercial low pasteurised milk determined by the official LC method and by the proposed method[a]

Added lactulose ($mg\,l^{-1}$)	HPLC official method ($mg\,l^{-1}$) A	Recovery (%)	Proposed method ($mg\,l^{-1}$) B	Recovery (%)
225	206	92	217	96
280	246	88	267	95
300	287	96	325	108
450	472	105	417	93
600	581	97	577	96

[a] Correlation coefficients: $A \rightarrow B$ $r = 0.980$.

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