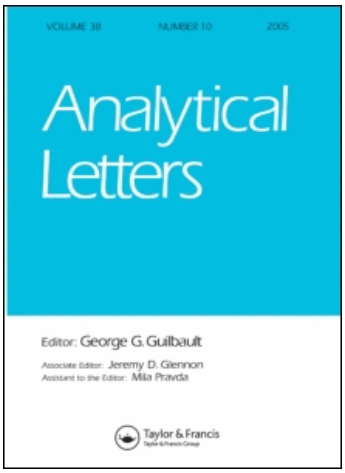

Analytical Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597227

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Online Publication Date: 01 January 2000

To cite this Article Amine, A., Moscone, D. and Palleschi, G.(2000)'Rapid Determination of Lactulose in Milk Using Seliwanoff's Reaction',Analytical Letters,33:1,125 — 135

To link to this Article: DOI: 10.1080/00032710008543041

URL: http://dx.doi.org/10.1080/00032710008543041

ANALYTICAL LETTERS, 33(1), 125-135 (2000)

RAPID DETERMINATION OF LACTULOSE IN MILK USING SELIWANOFF'S REACTION

Keywords: Spectrophotometric Method, Seliwanoff, Lactulose, milk, UHT, Sterilised.

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ABSTRACT

A rapid method based on Seliwanoff's reaction to distinguish between UHT and sterilised milk was proposed. Lactulose was determined directly in milk with no treatment. Analysis of raw milk and its carbohydrates showed that the only interference was the relatively high concentration of lactose, which imposes the lower limit of detection. Small variations of lactulose (9 mg dL^{-1}) during heat treatment could be observed when the absorbance was measured against pre-

treated milk (blank). This method showed a linear range between 17 – 170 mg dL^{-1} and a detection limit lower than that of the official HPLC method. This novel procedure was compared with a commercially available enzymatic method and the results obtained correlated well.

INTRODUCTION

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 UHT milk from sterilised milk. Several analytical methods for detection of lactulose are mainly based on gas chromatography[5], HPLC[6], enzymatic spectrophotometric[7] or amperometric[8] procedures. We also developed a method based on the use of a biosensor and microdialysis[9]. Recently a commercially available kit[10] has been proposed.

All these methods are time-consuming, require skilled operators, sample treatment before the analysis, and expensive reagents and apparatus. For example, the Kit[10] from Boehringer for lactulose determination needs 6 different enzymes, expensive reagents and about 15 hours to perform the analysis.

During the past years, colorimetric methods for sugars have been proposed for the determination of lactulose in milk[2,11-13]. These methods require separation steps, the use of chromatographic columns and/or deproteinization of the sample.

The objective of this study was the development of a rapid simple and efficient method based on Seliwanoff's reaction suitable to distinguish between UHT and sterilised milk; moreover this method could be applied for the evaluation of the efficiency of the heat treatment during industrial processing of milk.

To our best knowledge, Seliwanoff's reaction was never applied for lactulose determination in milk. This reaction is well-known[14] and occurs in

boiling aqueous hydrochloric acid (HCl) containing resorcinol. The analysis of lactulose is based on the assumption that this compound is the only source of fructose in milk. Thus, the increase in the colour intensity during Seliwanoff's reaction is proportional to the lactulose concentration in milk.

EXPERIMENTAL PROCEDURES

Reagent

Lactulose (4-O-β-D-galactopyranosyl-D-fructo-furanose) 99% purity, resorcinol and lactose were from Sigma-Aldrich (Milan, Italy). All other reagents were of analytical grade.

Seliwanoff's reagent (aqueous solution of HCl 4 mol L^{-1} containing resorcinol 0.1%) was prepared in our laboratory. This solution is stable for several months. Casein solution was prepared dissolving 2.8 g casein in 100 mL citrate-phosphate buffer 0.01 mol L^{-1}, pH 6.4.

Apparatus

Absorbance measurements were performed with an Unicam 8625 UV/VIS Spectrometer (Cambridge, U.K.), using 1-cm cuvette light path. During the tests, temperature was controlled with a Haake F3 thermostat (Berlin, Germany).

Procedure A: Determination of lactulose

To each of the glass-capped test tubes (1.7 x 15.8 cm) were sequentially added 2 mL of Seliwanoff's reagent and 0.5 mL of milk sample or standard lactulose or fructose. These test tubes were placed in a water bath at 90°C ± 0.1°C for 10 minutes and cooled in tap water. Then the resulting solution was filtered with a disposable filter (0.22 μm porosity) and the absorbance was measured at λ_{max} = 482 nm against distilled water after a few minutes. With this procedure, an increase of absorbance of 8% every 30 minutes was observed.

When a dilution step with water was carried out, (i.e. the volume in each tube adjusted to 5.0 mL with distilled water, the solution filtered and the absorbance measured), no absorbance variation was observed after one hour.

Unless otherwise stated, each value reported in this work corresponds to the mean of six measurements.

Procedure B: Heat treatment of milk

Capped tubes containing 5 mL of milk samples were placed in a water bath at 90°C ± 0.1°C, heated for selected periods of time from 0 to 100 minutes and cooled in tap water. Then the analysis of lactulose was carried out following the procedure as in A.

RESULTS AND DISCUSSION

Reaction time studies

The Seliwanoff reaction's time was studied placing the test tubes at 90°C for a period of time varying from 2.5 to 20 minutes, and then the absorbance was measured every 2.5 minutes. A constant increase of the absorbance both with standard lactulose solution (2 mmol L^{-1}) and raw milk was observed during this time. A period of time lower than 5 minutes was not sufficient to obtain a sample deproteinization and clarification, while a period of 20 minutes gave an absorbance very high and Beer's law was not valid. So 10 minutes was a good compromise between the deproteinization of the sample, colour development (in a range where the Beer's law was valid) and speed of analysis.

Figure 1 shows the calibration curves attained using the proposed method: (curve A) standard fructose, (curve B) standard lactulose, and (curve C) raw milk with added standard lactulose. The relative equations were as follows:

curve A, $y = 0.006 + 0.098\ x$, $r^2 = 0.999$

curve B, $y = 0.001 + 0.078\ x$, $r^2 = 0.999$

curve C, $y = 0.158 + 0.065\ x$, $r^2 = 0.997$

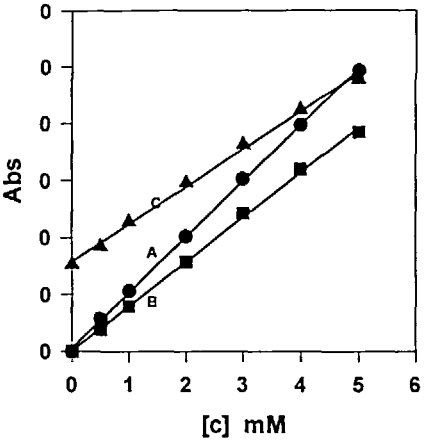

Fig. 1: Standard calibration curves of fructose (curve A), lactulose (curve B) and raw milk with added lactulose (curve C). The method used was based on Seliwanoff's reaction. Range studied (0.5 - 5 mmol/L).

(where the ordinate was the absorbance and the abscissa was the concentration in mmol/L).

From the values of the ratio of the slopes, it appears that under our experimental conditions (10 minutes at 90°C) the colour intensity obtained with lactulose is 80% of that of fructose (slope curve B/slope curve A) and the recovery of lactulose from the milk was 83% (slope curve C/slope curve B). Further studies showed that the ratio between these values did not change in the range of 2.5-20 minutes. Since the hydrolysis of lactulose and the colour development proceed at the same time, it would be difficult to attribute this low value (80%) to one of them.

Recently Schenk and Bisswanger[15] determined fructose in the presence of glucose in buffer solution using a modified Seliwanoff's reaction[16]. In this method ferric ions were added to resorcinol solution to form the red complex with fructose at 80°C for 40 minutes. Using this procedure we found that the recovery of lactulose added to milk is very low, about 30%. Similarly, in a

previous work[16] the recovery of ketoses from urine is very low. Thus, the original procedure[14] of ketose determination was adopted here.

Seven different raw milk samples and 31 commercial milk samples (4 sterilised, 12 UHT, 15 pasteurised) were analysed. Each sample was analysed in six replicates. The results show that the relative standard deviation (RSD) reported as the repeatability of the method for all samples was from 3 to 4%. These kinds of milk are usually subject to different conditions of heat treatments. The most commonly used method by the dairy industries is as follow: Pasteurisation 72°C for 15 sec, UHT 140°C for 3-8 sec and Sterilisation 110°C for 5-10 minutes[17].

Interference studies

The Seliwanoff reaction is specific for ketoses, so in milk, its best reactant is fructose produced from the hydrolysis of lactulose. Under our experimental conditions a concentration of fructose as low as 2 mg dL can be detected. Since lactulose is present in UHT milk[18] at concentrations of 10-51 mg dL^{-1} and in sterilised milk[2] at concentrations of 85-200 mg dL^{-1}, we decided to process these samples using Seliwanoff's reaction. Minor carbohydrates[5] as glucose and galactose present at a concentration less than 20 mg dL^{-1}, did not produce any detectable absorbance.

Other oligosaccharides and monosaccharides[19] did not interfere because they are present in milk at trace levels. Lactose concentration[19] is undoubtedly the highest present in milk (4500-5000 mg dl^{-1}). Under our conditions, high concentrations of glucose and galactose are produced from the hydrolysis of lactose, and, although aldoses, they react with Seliwanoff's reagent. In fact, 12 test tubes of standard lactose ranging from 4500 to 5000 mg dL^{-1} treated with the Seliwanoff's reagent produced an absorbance ranging from 0.159 ± 0.004 to 0.175 ± 0.004. In order to demonstrate that the reaction occurred with the aldoses and not with other products of the hydrolysis of proteins, a solution

of 2.8% casein prepared in citrate-phosphate buffer was tested. No absorbance with Seliwanoff's reaction was observed during the analysis of casein.

Analysis of milk

Seven different raw milk samples, in which lactulose was not present, were then analysed as blanks giving a mean absorbance equal to 0.152, with a standard deviation of 0.011 and RSD=7%. The values of the blanks were comparable with those obtained with lactose standards, while the value of RSD (calculated from the average of the results obtained from seven different samples, each one analysed 6 times) was higher than that obtained from the six determinations on the same milk as reported in the procedure A. This was probably due to the variability of the amount of lactose in different milk samples.

Twenty calibration curves have been performed with raw (9) and pasteurised [whole (5), semi-skimmed (3) and skimmed (3)] milk samples, where lactulose standards were added in the range (0.5-5.0 mmol L^{-1}) (17-171 mg dL^{-1}). The resulting curves gave an average value of the slope of 0.062, SD = 0.003 and a RSD = 5%. Thus we propose the following general equation for the determination of lactulose in commercial milk samples:

(1) $$Y = 0.152 \pm 0.011 + 0.062 \pm 0.003\ X$$

(where Y was the absorbance and X was the concentration of lactulose in mmol L^{-1})

In this equation the value of the intercept with its RSD of 7% includes the interference of lactose and imposes a detection limit which corresponds to the absorbance of the blank + 3 times the SD and was equal to 0.5 mmol L^{-1} (17 mg dL^{-1}).

Based on the previous equation, a sample of raw milk in which lactulose was added to give a final concentration of 60 mg dL^{-1} (a content which has been suggested by the IDF and the EC as the limit for discrimination between UHT and sterilised milk) can be rapidly detected with a RSD of about 6%.

38 different milk samples have been analysed and the values of the absorbance summarised in Table 1. From these results it appears that we can make an easy distinction between UHT and sterilised milk whereas the distinction between pasteurised and UHT milk cannot be fully appreciated.

Method of comparison

Figure 2 shows preliminary experiments on lactulose detection in sterilised and UHT milk performed both with the enzymatic Kit from Boehringer and the Seliwanoff's reaction. According to the general equation (1), the slope was = 0.92; the intercept = 9 mg dL^{-1}; $r^2 = 0.98$ and n = 8. The slope of the regression line was not significantly different from 1, nor the intercept significantly different from 0, indicating an absence of constant and proportional bias. The correlation coefficient indicated a good agreement between the two methods.

Analysis of milk during heat treatment

Lactulose measurement in milk could be used to optimise the milk heat processing, to check if the heating operating conditions have respected the protocols of the process, also to well understand the time-temperature profile of the milk. In this case, the lactulose content in milk collected after the heat processing was compared to the same milk sample collected before the heat processing, whose absorbance corresponds to the value of the blank. The experiments were carried out using milk plus Seliwanoff's reagent in test tubes heated at 90°C for 10 minutes, cooled in tap water, filtered and measured immediately against the blank (raw milk). No dilution steps were performed to avoid errors due to the dilution factor. A calibration curve of raw milk with added standards of lactulose in the range of 0.25-1.00 mmol L^{-1} corresponding to 8.6-34.2 mg dL^{-1} has been attained. The coefficient of correlation was r^2=0.999. The equation of the curve was Y=0.00365x (where the ordinate was the absorbance and x was the concentration in mg dL^{-1}). The value of the blank was subtracted. The detection limit was 7 mg dL^{-1}. This value was lower than that of

TABLE 1

Absorbance Measurement using Seliwanoff's Reaction of Raw Milk and of Different Types of Milk Commercially Available in Italy. Number of Samples (n) and Minimum, Maximum, Mean, Median and Relative Standard Deviation (RSD) of the Absorbance Values are Indicated.

	Raw (n = 7)	Pasteurised (n = 15)	UHT (n = 12)	Sterilised (n = 4)
Mean	0.152	0.174	0.240	0.367
RSD %	7	16	18	10
Min	0.136	0.143	0.185	0.340
Max	0.165	0.223	0.326	0.422
Median	0.154	0.158	0.239	0.350

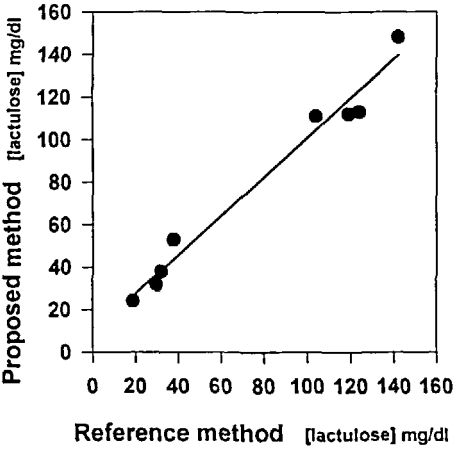

Fig. 2: Comparison between enzymatic reference method and proposed method.

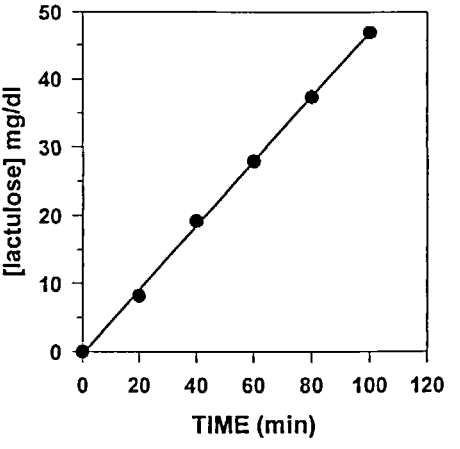

Fig. 3: Measurement of the lactulose variation in milk samples using Seliwanoff's reaction during heat treatment at 90 °C.

the official method[6] based on HPLC (20 mg dL^{-1}), however was comparable with the gas chromatographic method[5] (5-10 mg dL^{-1}), and higher than the enzymatic method[7] (1 mg dL^{-1}).

Figure 3 shows a constant increase of lactulose production in milk heated at 90°C during 100 minutes. The total variation of the absorbances during 100 minutes corresponds to a production of 47 ± 3 mg dL^{-1} of lactulose. These results were in good agreement with that reported in literature[18,20] In our experiments, an increase of 9 mg dL^{-1} each 20 minutes was measured. Thus, a variation of lactulose during heat processing as low as 9 mg dL^{-1} could be detected. This is a very important point for differentiation between high-pasteurisation and UHT processes since significant amounts of lactulose should be formed during the UHT process (10-51 mg dL^{-1})[18]

ACKNOWLEDGEMENTS

Authors wish to thank the CNR target Project Biotechnology, for financial support.

Also the Universities of "Tor Vergata" in Rome and of Mohammedia in Morocco are acknowledged for the grant received by Prof. A. Amine.

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Received: August 11, 1999
Accepted: September 1, 1999