International Dairy Journal 96 (2019) 126–131

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Accurate analysis of residual lactose in low-lactose milk: Comparing a variety of analytical techniques

Ekaterina Churakova [a], Kameshwara Peri [a], Judith Soul Vis [a], Drew Warren Smith [b], Jesse Matthew Beam [b], Marieke Petronella Vijverberg [a], Mark Cristiaan Stor [a], Remko Tsjibbe Winter [a, *]

[a] DSM Biotechnology Center, P.O. Box 1, 2600, MA, Delft, The Netherlands
[b] DSM Food Specialties USA, Inc., 3502 North Olive Road, South Bend, IN, 46628-8407, United States

ARTICLE INFO

Article history:
Received 8 October 2018
Received in revised form
9 February 2019
Accepted 10 February 2019
Available online 12 April 2019

ABSTRACT

To receive the designation "lactose-free", milk should contain <0.01% (w/w) lactose. As the analysis of such low levels of lactose is often hampered by other saccharides present or formed during milk processing, methods are required that are highly sensitive, accurate and precise. Currently, there is no international standard analysis method for the determination of lactose in low- or lactose-free milk, despite such a need from the dairy industry. We validated the analysis of residual lactose in lactase-treated UHT milk using HPAEC-PAD on a CarboPac PA100 column and compared it with a variety of commonly used analytical techniques for measuring lactose, including HPLC-RI, NMR, enzymatic kits, cryoscopy, and lactose biosensors. The results show that only one analytical technique, namely the Biomilk^{300}, an amperometric biosensor, has performance comparable with analysis by HPAEC-PAD, which remains one of the most accurate, precise and sensitive methods to assess low levels of lactose in milk.

1. Introduction

Lactose, the main carbohydrate found in milk, has experienced increased attention of late because the demand for lactose-free products by people with lactose intolerance or reduced lactose metabolism has been on the rise. Lactose is a disaccharide consisting of glucose and galactose moieties that are linked by a β-1,4 glycosidic bond. In humans, the small intestinal digestion of this sugar is promoted by lactase-phlorizin hydrolase (glycosyl hydrolase family GH1), which hydrolyses lactose into its constituent absorbable monosaccharides. Lactose intolerance is a common genetic condition connected to the deficiency of a functional lactase in adulthood (lactase non-persistence), and the percentage of the global population that exhibit this condition is estimated to be around 65% (Ingram, Mulcare, Itan, Thomas, & Swallow, 2009). Lactose intolerance may cause symptoms such as abdominal pain, flatulence and diarrhoea upon intake of lactose-containing dairy products. To satisfy the dietary requirements of the affected population for calcium and high quality protein, the global dairy industry has responded through the development of lactose-free products, commonly by pre-digestion of the lactose in milk by addition of an exogenous lactase, β-galactosidase.

Currently, there are no international agreements on the lactose concentration below which dairy products are defined as lactose-free. In some European countries, the lactose-free threshold is <0.1% (w/w), while in others the threshold is <0.01% (w/w). Chinese regulations are different again, stipulating <0.5% (w/w) lactose to allow labelling as lactose-free. In addition, terms as 'low-lactose' and 'lactose-reduced' are used in some dairy foods, whereby 'low-lactose' implies <1% (w/w) in some European countries and 'lactose-reduced' implies that lactose concentration must be lower than 2.0% (w/w) (EFSA, 2010). Clearly, the accurate and robust quantification of residual lactose in dairy products is critical for the dairy industry, both to deliver high-quality products and to meet local labelling requirements.

Traditionally, many different techniques have been used and recognised by analytical standard agencies to determine lactose in milk, such as polarimetry (AOAC, 2005a), mid-infrared detection (AOAC, 2005b), gravimetry (AOAC, 2005c), differential pH (ISO/IDF, 2010), enzymatic methods detecting either the glucose or galactose moiety of lactose (ISO/IDF, 2002a,b), and high-performance liquid

* Corresponding author. Tel.: +31 6 25638340.
E-mail address: remko.winter@dsm.com (R.T. Winter).

https://doi.org/10.1016/j.idairyj.2019.02.020

chromatography (ISO/IDF, 2007). While these methods are often simple, fast, or inexpensive to operate, their disadvantage is that they are not sensitive and/or specific enough to quantify lactose at low levels (Trani et al., 2017).

Recent literature shows the development of innovative methods for quantification of lactose at low levels in milk. Impressive limit of detections (LODs) were achieved with gas chromatography (Idda et al., 2016), ultra-high performance chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) (Garballo-Rubio, Soto-Chinchilla, Moreno, & Zafra-Gómez, 2018; Trani et al., 2017), high-performance thin-layer chromatography and fluorescence detection (HPTLC–FLD) after selective derivatisation (Morlock, Morlock, & Lemo, 2014) and nuclear magnetic resonance (NMR) (Monakhova, Kuballa, Leitz, Andlauer, & Lachenmeier, 2012). The commonly applied enzymatic methods for lactose quantification (ISO 5765-1,2/IDF 79-1,2; AOAC 984.15) (ISO/IDF, 2007) have been modified to improve their sensitivity and reduce their interference from other carbohydrates present in low-lactose milk (Gille et al., 2018; Mangan et al., 2018) (Megazymes). A novel method using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) showed excellent sensitivity and selectivity with simple sample pretreatment (van Scheppingen, van Hilten, Vijverberg, & Duchateau, 2017).

Despite these innovations focussing on increased sensitivity, the dairy industry also has a need for rapid and simple techniques that can be used by process operators to monitor lactose levels in-process. Traditionally, cryoscopy has often been used for this purpose, by which the depression of the freezing point due to the enzymatic hydrolysis of lactose is measured (Nijpels, Evers, Novak, & Ramet, 1980). More recently, biosensors have received widespread interest for this purpose due to their high specificity and ability to translate detection of the analyte to an amperometric or potentiometric signal (Eshkenazi, Maltz, Zion, & Rishpon, 2000; Kučerová, Komenská, Tomková, Skopalová, & Barták, 2017). A lactose biosensor can be engineered to detect lactose specifically or the products of lactose hydrolysis (Amamcharla & Metzger, 2011). Presently, there are several commercial lactose biosensors on the market, including Lactosens ® (DirectSens GmbH), Biomilk® (Biolan), YSI biochemistry analyser (Xylem Inc.) and SpotCheck Plus ™ (Hygiena).

To the best of our knowledge, there is currently no method available for the analysis of lactose levels in low- or lactose-free milk that has been validated and recognised as such by an international analytical standards organisation like AOAC, ISO or IDF, despite the international dairy industry having a clear need for such a method. Based on our review of the scientific literature, we believe that the HPAEC-PAD method is suitable for such validation and recognition because of its superior specificity (separation of all carbohydrates present in lactose free milk) and high accuracy, precision and sensitivity (van Scheppingen et al., 2017). The objective of our study is to compare the performance of the HPAEC-PAD method for the determination of lactose in lactase-treated UHT milk at a range of concentrations with nine commonly used analytical methods from the dairy industry to determine low lactose levels. Techniques covered in this study include enzymatic kits, commercial biosensors, NMR, HPLC according to ISO 22662/IDF 198 (ISO/IDF, 2007), HPAEC-PAD and cryoscopy (freezing point depression).

2. Material and methods

2.1. Sample preparation

Semi-skimmed (1.5% fat) UHT cows' milk was obtained from a local supermarket [Albert Hein; batch 257F27(06:15)]. Milk density was determined with a Mettler Toledo 30P Densitometer with automatic temperature control. Maxilact LGi5000 neutral lactase was obtained from DSM Food Specialties (Delft, The Netherlands). Milk was treated with lactase to prepare samples with the following (approximate) low-lactose concentrations: ~0.5% (w/w), ~0.1% (w/w) and ~0.01% (w/w). Lactose hydrolysis was performed as follows: 1.060 kg milk was incubated at 6 °C with 4816 NLU L^{-1} in a 1 L sterilised bottle and mixed by stirring at 500 rpm. Samples (~12 mL) were taken after 7.9, 14.7 and 24.1 h, pipetted into 15 mL Eppendorf conical tubes, tightly closed (to prevent evaporation) and incubated at 90 °C in a water bath for 10 min to inactivate the lactase. All samples were stored at −20 °C until further analysis, apart from the samples that were analysed directly upon cooling for freezing point depression (FPD). The unhydrolysed milk or blank milk was designated as sample A; milk hydrolysed for 7.9 h (~0.5%, w/w, lactose) as sample B; milk hydrolysed for 14.7 h (~0.1%, w/w, lactose) as sample C; milk hydrolysed for 24 h (~0.01%, w/w, lactose) as sample D. The comparison of nine methods was performed by analysing each sample with each method in independent triplicate determinations over two days, generating six analytical results for each method. Mean and relative standard deviation were calculated and reported. The samples were analysed in triplicate over one day only with HPLC with refractive index detection (RI) when it was clear that the low-lactose samples were below the LOD of this method.

2.2. Preparation of spiked samples to validate accuracy of HPAEC-PAD method

A 90 g kg^{-1} lactose solution was prepared by accurately weighing and dissolving D-lactose monohydrate (99.5% purity, Sigma Aldrich) in water. This solution was further diluted with water to 20 g kg^{-1} and 2 g kg^{-1} lactose. All three solutions were spiked to lactose-free milk (sample prepared under section 2.1 and taken after 39 h instead of 24 h to ensure complete lactose-free levels were reached). Earlier experiments (data not shown) had indicated that this sample was robustly lactose-free according to HPEAC-PAD. The final concentrations in the solutions after spiking were 0.465% (w/w), 0.094% (w/w) and 0.009% (w/w) lactose, respectively. Lactose content of all three spiked samples and the non-spiked lactose-free milk was analysed with HPAEC-PAD (Section 2.3). The lactose content of the non-spiked lactose-free milk was subtracted from the spiked samples, and the recovery was expressed as a percentage. All analyses were performed as independent triplicate measurements on two separate days, and the means and relative standard deviation (RSD%) are reported.

2.3. HPAEC-PAD

Analysis by HPAEC-PAD was performed as described by van Scheppingen et al. (2017). Briefly, for the chromatographic separation a CarboPac PA100 (250 × 4 mm) column equipped with a guard column (50 × 4 mm) of the same stationary phase was used on a Dionex ICS 5000 LC system equipped with DP dual quaternary HPLC pumps, an AS thermostated injector and a DC detector compartment with a pulsed amperometric detector (PAD). For the gradient elution, the following mobile phases were used: A, Milli-Q purified water; B, 20 mM NaOH; C, 500 mM NaOH; and D, 100 mM NaOH with 1 M sodium acetate.

During the first 37 min of the chromatographic run, isocratic conditions were employed with 8 mM NaOH (60% A + 40% B); lactose eluted after 35 min. At 37.1 min, the eluent composition was changed to 375 mM NaOH (75% C) and at 37.5 min to 80 mM NaOH and 800 mM sodium acetate (20% A + 80% D) over 1.5 min, followed by 500 mM NaOH (100% C) for 3.8 min. Before the next injection, the

system was stabilised over 15 min at the start conditions of 8 mM NaOH (60%A + 40% B). Sample preparation was performed by dispersing approximately 1 g sample in 25 mL Milli-Q purified water, vigorously mixing for 30 min on a magnetic stirrer and centrifuging for 10 min at 20,000× g. Approximately 0.5–0.6 mL of the supernatant was transferred into an ultrafiltration tube, avoiding the upper fat layer, and ultrafiltrated through a 10 kDa filter at 14,000× g, for at least 15 min. The clear filtrate was diluted at least 4 times with Milli-Q water to a lactose concentration of approximately 1 mg L^{-1}.

2.4. HPLC with refractive index detection

Analysis was performed according to method ISO 22662 on a Thermo Ultimate 3000 LC system equipped with an Aminex HPX-87P 300 × 7.8 mm column and using D-(+)-melezitoze (Sigma Aldrich) as internal standard. Biggs-Szijarto solution was used to precipitate out fat and protein. Column temperature was held at 85 °C, the refractive index (RI) detector was held at 35 °C. Twenty microlitres of sample was injected at a flow rate of 1.0 mL min^{-1} and the run time was 15 min. The mobile phase was degassed HPLC-grade water.

2.5. Freezing point depression

FPD of milk containing hydrolysed lactose was measured according to the method NEN-EN-ISO 5764:2009 using the Advanced ® cryoscope, model 4250 (Advanced Instruments Inc., Massachusetts, USA). The degree of lactose hydrolysis was determined according to manufacturer's instructions and as described previously (Nijpels et al., 1980). Samples were taken as indicated in Section 2.1 and measured in six-fold. Control experiments were performed to confirm that heat treatment of the milk at 90 °C to inactivate lactase did not influence the freezing point.

To calculate the lactose hydrolysis level (percentage of lactose hydrolysed at any time interval relative to 100% hydrolysed reference milk) a reference experiment was performed to obtain milk containing no lactose. Lactase (Maxilact LGi5000, 0.091 g) was added to 100 mL of milk in a measuring flask and mixed at 500 rpm at 37 °C for 4 h. A blank reference experiment was performed using 0.091 g of inactivated lactase (inactivated by first incubating lactase at 90 °C for 10 min). Unhydrolysed lactose level (%) was calculated according to equation (1). The concentration of residual lactose in % (w/w) then followed by multiplying the outcome of equation (1) with the concentration of lactose in the untreated milk samples as determined by HPAEC-PAD (section 2.3).

$$\text{Lactose unhydrolysed, } \% = 100\% - \frac{FP(sample,\ 6°C,\ x\ hr) - FP\ (blank,\ 6°C,\ x\ hr)}{FP(reference,\ 37°C,\ 4\ hr) - FP(reference.\ blank,\ 37°C,\ 4\ hr)} * 100\% \quad (1)$$

2.6. Enzymatic methods

Three different commercial kits were used: Lactose/D-glucose, Lactose/D-galactose (both from R-Biofarm AG, Darmstadt, Germany) and Lactose Assay Kit (K-LOLAC sequential/high sensitivity) (Megazyme, Bray, Ireland). In the lactose/D-glucose kit (R-Biofarm) lactose is hydrolysed at pH 6.6 to D-glucose and D-galactose in the presence of lactase (β-galactosidase). D-Glucose is then phosphorylated at pH 7.6 by the enzyme hexokinase (HK) and adenosine-5′-triphosphate (ATP) to D-glucose-6-phosphate (G-6-P), with the simultaneous formation of adenosine-5′-diphosphate (ADP). Subsequently, in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate, with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in the last reaction is stoichiometric to the amount of D-glucose and lactose, respectively. NADPH is measured by its light absorbance at 340 nm. The amount of lactose is calculated from the difference between the D-glucose concentrations with and without hydrolysis with lactase (β-galactosidase).

In the lactose/D-galactose kit (R-Biofarm) lactose is hydrolysed to D-glucose and D -galactose at pH 6.6 in the presence of lactase (β-galactosidase). D-Galactose is oxidised at pH 8.6 by nicotinamide-adenine dinucleotide (NAD) to D-galactonic acid in the presence of β-galactose dehydrogenase (Gal-DH). The amount of NADH formed in the second reaction is stoichiometric to the amount of lactose, and D-galactose, respectively. NADH is measured by means of its light absorbance at 340 nm.

The lactose assay kit from Megazymes has the same principle as the R-Biofarm kit (measurement of NADPH absorbance at 340 nm), but has an additional enzymatic step where D-gluconate-6-phosphate is oxidised by NADP in the presence of 6-phosphogluconate dehydrogenase to D-ribulose-5-phosphate. This results in additional sensitivity, as 2 mol of NADPH are formed for every mole of glucose. Furthermore, the kit contains a glucose oxidase/catalase sample pretreatment procedure to remove residual glucose in low lactose dairy samples (Mangan et al., 2018).

All enzymatic methods were performed according to manufacturer's instructions and absorbance assayed at 340 nm on a Shimadzu CPS-240 A spectrophotometer.

2.7. Commercial biosensors: Biomilk^{300} and OneTouch

The Biomilk^{300} biosensor combines the selectivity of specific (proprietary) enzymes for each analyte with an amperometric transduction of that biological signal, which is quantifiable. This biosensor contains an electrode, with immobilised enzyme together with other electroactive components, which cause a change in the electric current when they come into contact with the analyte to be detected. Samples were analysed for lactose content with the Biomilk^{300} residual lactose biosensor according to manufacturer's instructions (Biolan, Zamudio, Spain). The biosensor was calibrated prior to analysis over the following measuring ranges, depending on the expected lactose content of the samples: 0–200 mg L^{-1}, 0–2 g L^{-1}, and 0–6 g L^{-1}. Prior to analysis, 5 mL of milk was added to the pretreatment tube containing carbon and shaken until homogeneous. Samples in the range 0–200 mg L^{-1} were measured directly; samples in the ranges of 0–2 g L^{-1} and 0–6 g L^{-1} were diluted with measuring solution an additional 10 times.

Blood glucose meters are commonly used in the dairy industry to measure residual lactose in low lactose milk (Amamcharla & Metzger, 2011) and here we tested the OneTouch Select® Plus Blood glucose monitoring system (LifeScan Inc, Milpitas, USA).

Samples were diluted to the measuring range of the glucose meter (1.1–33.3 mM) with milk before introduction on to the test strip with a 10 μL pipette. After obtaining a stable readout (in mmol l^{-1} glucose), the value was converted to lactose concentration using the stoichiometry of the lactose reaction, 1:1 (1 mol lactose hydrolysed = 1 mol glucose formed).

2.8. YSI biochemistry analyser

YSI biosensor contains exchangeable membranes with an immobilised (glucose or galactose) oxidase, which oxidises an analysed compound, producing hydrogen peroxide as a side product. Hydrogen peroxide is sequentially detected on the platinum electrode and the amperometric signal is converted to the initial concentration of reagents in the sample according to the stoichiometry of the reaction. Samples were analysed for lactose and glucose content using the YSI Model 2700 Select Biochemistry Analyzer (YSI Incorporated, Yellow Springs, Ohio) according to the manufacturer's instructions.

2.9. NMR

Milk samples (1000 μL) were treated with 40 μL HCl (4 N), mixed by vortexing for 10 min and centrifuged at 13,000× *g* for 10 min to precipitate proteins. One hundred μL of the clear supernatant (taking care to avoid the top fat layer) was accurately mixed with 100 μL of an aqueous Maleic acid standard solution (100 mM $KHCO_3$, 80 mM $NaH_2PO_4.H_2O$, 35 mM EDTA, 20 g L^{-1} maleic acid, pH 6.4). Samples were lyophilised twice until dry, dissolving in 50 μL D_2O between lyophilisation steps. After final lyophilisation samples were dissolved in 1 mL D_2O and 570 μL of the solution was transferred to an NMR tube and stabilised overnight to allow anomeric ratios of sugars to reach equilibrium. 1D 1H-NMR spectra were recorded on a Bruker Avance III HD Spectrometer operating at a proton frequency of 600 MHz equipped with a cryo probe and the following parameters: pulse program is zg with a 90° pulse, relaxation delay (d1) of 30 s, acquisition time of 2.04 s, 8 scans and probe temperature at 290 K. Half of the lactose doublet at $\delta = 4.67$ ppm, N = 0.305 H was used for quantification. The maleic acid signal (N = 2H, $\delta \pm 6.26$ ppm) was used as standard for quantification.

3. Results and discussion

3.1. Validating the performance of the HPAEC-PAD method for quantifying low-lactose levels in milk

Quantification of low (≤0.5%, w/w) lactose levels in milk requires a sensitive and specific method as, during processing of such dairy products, lactose and other saccharides present are converted thermally, enzymatically (β-galactosidase) or by bacterial fermentation (lactic acid bacteria) to many different derivatives (Seki & Saito, 2012). These compounds can all potentially interfere with lactose analysis. van Scheppingen et al. (2017) showed that a HPAEC-PAD method using a CarboPac PA100 column had excellent accuracy and specificity. Regarding the latter, the method did not suffer from interference from other disaccharides (e.g., allo-lactose) present in the low-lactose milk matrix (van Scheppingen et al., 2017).

Here, the performance of this method was validated by assessing its accuracy or ability to determine the 'true' lactose content in low-lactose (UHT) milk, obtained by lactase hydrolysis. Known amounts of a lactose standard were spiked to lactose-free milk and these samples were analysed using the HPAEC-PAD method to calculate the recovery (Table 1). The spiking experiments revealed that the HPAEC-PAD method had excellent recoveries across all low-lactose concentrations tested, ranging between 98 and 103%. The precision was also very good, as shown by the relative standard deviation (RSD) for triplicate measurements over two days, which was between 1.1 and 2.4%. This led us to conclude that this HPAEC-PAD method (van Scheppingen et al., 2017) for determining low-lactose concentrations in milk is suitably validated and could be assigned as standard method to compare the performance (sensitivity, accuracy and precision) of the other methods tested in this study.

3.2. Comparing performance of various methods with HPAEC-PAD to measure low lactose levels in milk

To evaluate the performance (sensitivity, accuracy and precision) of the selected methods, we prepared a single sample set with different lactose levels, by performing an enzymatic hydrolysis of lactose in UHT semi-skimmed milk using lactase (β-galactosidase). By analysing each sample in all methods in six-fold over two days, the accuracy and precision was compared. We expected to find only variation in the relative standard deviation (precision) between the analyses, but also found significant variation in the absolute value of lactose (accuracy) for the samples at different lactose levels (Table 2) when compared with HPAEC-PAD.

Milk analysed by HPLC-RI (ISO 22662; ISO/IDF, 2007) before lactase treatment (Sample A) gave a mean result of 4.77% (w/w) lactose with a RSD of 2%, which was in good agreement with the value found by HPAEC-PAD of 4.65% (w/w). Sample B (containing 0.3% (w/w) lactose according to HPAEC-PAD) could not be accurately quantified with HPLC-RI (Table 2) as lactose co-eluted with allolactose and presumably also galacto-oligosaccharides, which can be formed during the process of enzymatic lactose hydrolysis (Rodriguez-Colinas, Fernandez-Arrojo, Ballesteros, & Plou, 2014; van Scheppingen et al., 2017). The lactose concentrations in Samples C and D, 0.07 and 0.01% (w/w) by HPAEC-PAD, respectively, were too low to be detected by the HPLC-RI method (a flat baseline was observed). If a signal could be detected, however, with a more sensitive detector such as evaporative light scattering (ELSD) as the ISO 22662 method (ISO/IDF, 2007) allows, then interference by allolactose and galacto-oligosaccharides would most likely still make accurate quantification difficult. This was in line with previous results, where lactose in commercial lactose-free milk could not be detected with the HPLC-RI method (Trani et al., 2017). Clearly, the HPLC method (ISO 22662; ISO/IDF, 2007) in combination with RI detection cannot be applied for the analysis of low lactose levels (≤0.3%, w/w) in milk that has been treated with lactase or contains galacto-oligosaccharides, due to a combination

Table 1
Accuracy and precision of high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method.[a]

Lactose standard added (% w/w)	Lactose by HPAEC-PAD (% w/w)	Recovery (%)	RSD (%)
0.465	0.457	98	2.4
0.094	0.096	103	1.5
0.009	0.009	99	1.1

[a] Known amounts of lactose standard were added to lactose-free milk and analysed by HPAEC-PAD. RSD refers to relative standard deviation (n = 6).

Table 2
Lactose concentration in milk samples of different lactose contents, according to ten methods of analysis.[a]

Method	Lactose concentration							
	Sample A		Sample B		Sample C		Sample D	
	% (w/w)	RSD (%)	% (w/w)	RSD (%)	% (w/w)	RSD (%)	% (w/w)	RSD (%)
HPAEC-PAD	4.65	2.5	0.30	0.9	0.07	1.4	0.01	2.3
HPLC-RI	4.77	2.1	n/a	–	n/a	–	n/a	–
NMR	4.67	0.4	0.32	1.1	n/a	–	n/a	–
Lactose/D-glucose (R-Biofarm)	4.57	5.1	0.58	15	0.37	25	0.15	54
Lactose/D-galactose (R-Biofarm)	4.36	3.0	0.97	24	0.41	17	0.17	21
Lactose/sequential/high sensitivity (Megazyme)	n/d	–	0.36	1.9	0.10	2.5	0.03	2.8
FPD	n/a	–	0.97	6	0.60	6	0.30	11
YSI biosensor	4.80	0.9	0.90	4.97	0.53	7.5	0.34	15
OneTouch biosensor	n/a	–	n/a	–	n/a	–	n/a	–
Biomilk^{300} biosensor	3.83	10	0.29	4.0	0.06	7.2	0.01	24

[a] Abbreviations are: HPEAC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPLC-RI, high performance liquid chromatography-refractive index; NMR, nuclear magnetic resonance; FPD, freezing point depression; RSD, relative standard deviation (n = 6); n/a, no analytical result; n/d, not determined.

of low sensitivity of the RI detector and co-elution of interfering peaks.

Initially, our results suggested that NMR could be used to measure lactose concentrations accurately in milk down to 0.07% (w/w) lactose (Sample C). Closer examination of the spectra, however, led us to conclude that, below 0.1% lactose (w/w), the proton signals related to lactose overlap with other signals from the milk matrix and thus could not be quantified reliably. Consequently, it is concluded that the NMR method employed in this study could only be used to accurately quantify lactose in samples A and B (4.65% and 0.3%, w/w, respectively). To obtain the increased sensitivity and specificity required to quantify the lactose in samples C and D (0.07% and 0.01%, w/w, respectively) with NMR, an alternative methodology like 2D selective HSQC NMR would need to be used.

In this study, three enzymatic kits were tested on the milk samples. One of these kits, the Megazyme lactose/sequential/high sensitivity kit, claims to be suitable for determining lactose concentration in lactose-free milk with a limit of detection <0.01% (w/w) (Mangan et al., 2018). In this study, however, none of the kits could accurately quantify the concentration of lactose in low-lactose milk, in line with similar work (Trani et al., 2017). For all three low-lactose milk samples (B, C, and D), the enzymatic kits overestimated the lactose concentration by at least 20% compared with HPAEC-PAD (Table 2). The lactose/D-galactose kit from Megazyme performed best out of all three enzymatic kits tested with the most precise (RSD = 2.8–1.9%) and accurate results, but compared with the HPAEC-PAD method, the lactose concentration measured in the samples was too high. In sample D, which contained the lowest amount of lactose, the lactose/D-galactose kit from Megazyme overestimated the lactose concentration by 300% compared with HPAEC-PAD. Instructions provided by the manufacturer of the lactose/D-glucose kit (R-Biofarm) mention that the accuracy is impaired when the ratio of D-glucose to lactose is more than 10:1, as is the case for low-lactose milk. In such cases, it is recommended to remove the excess D-glucose using glucose oxidase, as has been shown previously (Gille et al., 2018). Alternatively, it is recommended to use the lactose/D-galactose kit. Surprisingly, this enzymatic combination was also not satisfactory to quantify low lactose levels. It is speculated that galacto-oligosaccharides present in the samples are, besides lactose, also hydrolysed by β-galactosidases in the initial reaction of the kits, resulting in an overestimation of the amount of glucose or galactose and thus in an overestimation of the lactose concentration. This clearly illustrates the disadvantage of using an indirect method like commercially available enzymatic kits to measure low concentrations of lactose in milk.

When using the cryoscopy (FPD) method to determine residual lactose concentrations, we found that these were significantly overestimated, more than three-fold, in all samples, when compared with analysis via HPAEC-PAD (Table 2). In the cryoscopy method the depression of the freezing point of a solution in the presence of solutes (e.g., salts, sugars) is determined. During the enzymatic hydrolysis of lactose by β-galactosidases in the process to produce lactose-free milk, the concentration of glucose and galactose increases, thereby increasing the overall molar concentration of sugars in the milk, with a concomitant linear decrease in the freezing point as a result (Ramet, Novak, Evers, & Nijpels, 1979). For accurate quantification of the lactose concentration using this method, a specific calibration is used, where FPD is related to a known concentration of lactose in a milk matrix (Nijpels et al., 1980). Despite its simplicity, which makes it attractive for monitoring the degree of lactose hydrolysis in an industrial setting, our results show that this technique cannot be applied to accurate measurement of the residual lactose concentration in low-lactose and lactose-free products.

Biosensors are attractive analytical tools, especially for the dairy industry, due to their speed, ease of operation and low costs. Blood glucose meters are cheap, widely available, simple and robust, and for this reason, are applied frequently in the dairy industry to monitor lactose levels in milk during lactose hydrolysis by analysing the increase in glucose concentration. In this study, we tested the OneTouch Select® Plus Blood glucose monitoring system, a biosensor detecting glucose using glucose oxidase. However, we were not able to obtain quantitative results here due to elevated glucose concentration readings measured, and we speculate that this is because the test strips are very matrix-dependent and have been designed and validated for blood not milk. Similar results have been reported for various blood glucose meters, which can give an elevated glucose meter readings due to matrix interference (Amamcharla & Metzger, 2011; Schleis, 2007). Nevertheless, Amamcharla and Metzger (2011) determined the concentration of lactose in untreated milk (4.1–4.6%, w/w) by applying a calibration model at the range of 2–6% (w/w) lactose concentration and did not directly use the output of the blood glucose meter. Thus, in our opinion, blood glucose meters are not suitable for directly quantifying the lactose content in lactose-reduced milk, as they require separate lactose calibration and are matrix-sensitive.

Analysis of lactose in our milk samples with the YSI biosensor could only be performed precisely and accurately in untreated milk, sample A (Table 2), with a lactose concentration of 4.80% (w/w), compared with 4.65% (w/w) for HPAEC-PAD, and an excellent RSD of 0.9% (3.0% for HPAEC-PAD). All other samples with reduced lactose concentrations showed significant overestimation (by at

least three-fold) and worse precision in comparison with the HPAEC-PAD method (Table 2). The YSI biosensor contains exchangeable membranes with an immobilised (glucose or galactose) oxidase, which oxidises an analysed compound, producing hydrogen peroxide as a side product. Hydrogen peroxide is sequentially detected on the platinum electrode and the amperometric signal is converted to the initial concentration of reagents in the sample according to the stoichiometry of the reaction. This means that this biosensor is an indirect method for analyte quantification, and any side reaction producing even traces of hydrogen peroxide would lead to a false positive signal for lactose. Thus, this technology is not suitable for accurate quantification of low-lactose levels in milk where a β-galactosidase has been used to hydrolyse the lactose.

The Biomilk^{300} lactose biosensor was the only commercial lactose biosensor we tested that could quantify residual lactose concentrations in lactose-reduced milk with the same accuracy as the HPAEC-PAD method. While the accuracy was surprisingly good for a commercial biosensor, with lactose concentrations compared with HPAEC-PAD varying from 86 to 100% for samples B to D, respectively, the precision was relatively low, with a RSD of 24% for the lowest lactose concentration of 0.01% (Sample D, Table 2). It was, in fact, the only method we tested that could match the accuracy of the HPAEC-PAD method at the lowest lactose concentration. The accuracy of this method can be explained by the fact that the instrument is specifically designed to determine the residual lactose concentration in dairy matrices at three discrete measuring ranges, namely 0.005–0.02%, 0.02–0.2%, and 0.05–0.6%. The milk sample with the highest lactose content (Sample A; 4.65%, w/w) could not be accurately quantified with the Biomilk^{300}; the lactose concentration compared with HPAEC-PAD was 82%. This is likely due to the overall change in the sample matrix upon dilution of the untreated milk. The mode of action of the Biomilk^{300} is not disclosed but relies on a series of sequential redox reactions in the presence of lactose that result in the release of electrons at a level proportional to the lactose concentration. While the high RSD (low precision) determined at the lowest lactose concentration (Sample D, Table 2) is troubling and cannot be explained, more accurate results can be obtained by performing several replicate measurements.

4. Conclusions

This study validates the sensitivity, accuracy and precision of the HPAEC-PAD method with a CarboPac PA100 column for determining residual lactose concentrations in UHT milk at levels down to 0.01% (w/w). We compared lactose analysis by HPAEC-PAD with nine other commonly used analysis techniques and conclude that only the Biomilk^{300} lactose biosensor (Biolan) has comparable sensitivity and accuracy at all reduced lactose concentrations tested, including 0.01% (w/w) lactose. In our opinion, the other methods tested here are not suitable for measuring the lactose concentration in low-lactose or lactose-reduced milk obtained by enzymatic hydrolysis of lactose.

Acknowledgements

The authors would like to thank Erik van Leeuwen, Peter Dekker, Adriana Carvalho-de-Souza, Lucien Duchateau and Jan Metske van der Laan for providing helpful comments on this manuscript.

References

Amamcharla, J. K., & Metzger, L. E. (2011). Development of a rapid method for the measurement of lactose in milk using a blood glucose biosensor. *Journal of Dairy Science, 94*, 4800–4809.

AOAC. (2005a). Lactose in milk, Polarimetric method, method no. 896.01. In W. Horowitz (Ed.), *Official methods of analysis of AOAC International* (18th ed., p. 17). Gaithersburg, MD, USA: AOAC International.

AOAC. (2005b). Fat, lactose, protein, and solids in milk. Mid-infrared spectroscopic method, method no. 972.16. In W. Horowitz (Ed.), *Official methods of analysis of AOAC International* (18th ed., p. 23). Gaithersburg, MD, USA: AOAC International.

AOAC. (2005c). Lactose in milk. Gravimetric method Munson-walker, method no. 930.28. In W. Horowitz (Ed.), *Official methods of analysis of AOAC International* (18th ed., p. 17). Gaithersburg, MD, USA: AOAC International.

EFSA. (2010). ESFA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific opinion on lactose thresholds in lactose intolerance and galactosaemia. *EFSA Journal, 8*. Article 1777.

Eshkenazi, I., Maltz, E., Zion, B., & Rishpon, J. (2000). A three-cascaded-enzymes biosensor to determine lactose concentration in raw milk. *Journal of Dairy Science, 83*, 1939–1945.

Garballo-Rubio, A., Soto-Chinchilla, J., Moreno, A., & Zafra-Gómez, A. (2018). Determination of residual lactose in lactose-free cow milk by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry. *Journal of Food Composition and Analysis, 66*, 39–45.

Gille, D., Walther, B., Badertscher, R., Bosshart, A., Brügger, C., Brühlhart, M., et al. (2018). Detection of lactose in products with low lactose content. *International Dairy Journal, 83*, 17–19.

Idda, I., Spano, N., Ciulu, M., Nurchi, V. M., Panzanelli, A., Pilo, M. I., et al. (2016). Gas chromatography analysis of major free mono- and disaccharides in milk: Method assessment, validation, and application to real samples. *Journal of Separation Science, 39*, 4577–4584.

Ingram, C. J. E., Mulcare, C. A., Itan, Y., Thomas, M. G., & Swallow, D. M. (2009). Lactose digestion and the evolutionary genetics of lactase persistence. *Human Genetics, 124*, 579–591.

ISO/IDF. (2002a). *Lactose content, standard no. ISO 5765-1 (IDF 79-1) Dried milk, dried ice-mixes and processed cheese — Determination of lactose content — Part 1: Enzymatic method utilizing the glucose moiety of the lactose*. Geneva, Switzerland: International Organisation for Standardisation.

ISO/IDF. (2002b). *Lactose content, standard no. ISO 5765-2 (IDF 79-2) Dried milk, dried ice-mixes and processed cheese — Determination of lactose content — Part 1: Enzymatic method utilizing the galactose moiety of the lactose*. Geneva, Switzerland: International Organisation for Standardisation.

ISO/IDF. (2007). *Lactose content, standard no. ISO 22662 (IDF 198) Milk and milk products - Determination of lactose content by high-performance liquid chromatography (Reference method)*. Geneva, Switzerland: International Organisation for Standardisation.

Kučerová, P., Komenská, P., Tomková, H., Skopalová, J., & Barták, P. (2017). Determination of lactose in milk products: A comparison of three-enzyme amperometric biosensor and gas chromatography/tandem mass spectrometry. *Monatshefte Fur Chemie, 148*, 517–524.

Mangan, D., McCleary, B. V., Culleton, H., Cornaggio, C., Ivory, R., McKie, V. A., et al. (2018). A novel enzymatic method for the measurement of lactose in lactose-free products. *Journal of the Science of Food and Agriculture, 99*, 947–956.

Monakhova, Y. B., Kuballa, T., Leitz, J., Andlauer, C., & Lachenmeier, D. W. (2012). NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. *Dairy Science & Technology, 92*, 109–120.

Morlock, G. E., Morlock, L. P., & Lemo, C. (2014). Streamlined analysis of lactose-free dairy products. *Journal of Chromatography A, 1324*, 215–223.

Nijpels, H. H., Evers, P. H., Novak, G., & Ramet, J. P. (1980). Application of cryoscopy for the measurement of enzymatic hydrolysis of lactose. *Journal of Food Science, 45*, 1684–1687.

Ramet, J. P., Novak, G., Evers, P. A., & Nijpels, H. (1979). Application de la cryométrie à la mesure de l 'hydrolyse enzymatique du lactose. *Lait, 59*, 46–55.

Rodriguez-Colinas, B., Fernandez-Arrojo, L., Ballesteros, A. O., & Plou, F. J. (2014). Galactooligosaccharides formation during enzymatic hydrolysis of lactose: Towards a prebiotic-enriched milk. *Food Chemistry, 145*, 388–394.

Schleis, T. G. (2007). Interference of maltose, icodextrin, galactose, or xylose with some blood glucose monitoring systems. *Pharmacotherapy, 27*, 1313–1321.

Seki, N., & Saito, H. (2012). Lactose as a source for lactulose and other functional lactose derivatives. *International Dairy Journal, 22*, 110–115.

Trani, A., Gambacorta, G., Loizzo, P., Cassone, A., Fasciano, C., Zambrini, A. V., et al. (2017). Comparison of HPLC-RI, LC/MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. *Food Chemistry, 233*, 385–390.

van Scheppingen, W. B., van Hilten, P. H., Vijverberg, M. P., & Duchateau, A. L. L. (2017). Selective and sensitive determination of lactose in low-lactose dairy products with HPAEC-PAD. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences, 1060*, 395–399.